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Modulation of transmitter release during early stages of synapse formation between identified *Lymnaea* neurons

By

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Abstract

Physical contacts between synaptic partners bring about specific changes in the secretory machinery of their target cells, however the precise identity of signaling molecules and the underlying mechanisms remain elusive. Utilizing soma-soma synapses between identified *Lymnaea* neurons, the present study investigated changes in transmitter secretory machinery during early synaptogenesis. Specifically, the dopaminergic neuron, right pedal dorsal 1 (RPeD1), and the peptidergic neuron, visceral dorsal 4 (VD4), which make mutual inhibitory synapses *in vivo*, were individually isolated from the intact brain and paired in cell culture. Close juxtaposition of the isolated somata resulted in chemical synapse formation between the cell bodies and these synapses were electrophysiologically similar to those seen *in vivo*. In this configuration, VD4 was first to establish its inhibitory synapse with RPeD1 (12 hours), whereas the reciprocal synapse developed later (18-24 hours). To investigate evoked secretory capabilities of RPeD1 during synaptogenesis, VD4 somata were used as an electrophysiological bioassay. I found that a single unpaired RPeD1 was able to release dopamine, however, this capability was transiently suppressed after soma-soma pairing with VD4. Transmitter suppression from RPeD1 was VD4 cell specific and required *de novo* protein synthesis. Moreover, VD4-induced suppression of transmitter release from RPeD1 was independent of membrane contact and did not involve receptor tyrosine kinase mediated signaling.

Since VD4 initiated synaptic transmission with RPeD1, I hypothesized that VD4-induced suppression of transmitter release from this cell involved FMRFamide release from VD4. To test this, RPeD1 was cultured in the presence of FMRFamide and

transmitter release was examined with an assay cell. Consistent with the hypothesis, transmitter release from RPeD1 was suppressed by FMRFamide.

To define the intracellular mechanism mediating VD4/FMRFamidergic responses in RPeD1, various pharmacological agents were used. I found that both VD4 and FMRFamide-induced effects in the giant dopamine cell involved lipoxygenase metabolites of the arachidonic acid (AA) signal cascade. Moreover, the inhibitors of AA signaling also blocked inhibitory synapse formation between VD4 and RPeD1.

In summary, this study demonstrates that FMRFamide release from VD4 during early stages of synaptogenesis modulates the secretory machinery of RPeD1 through an AA pathway. These data underscore the importance of transmitter/receptor interactions during synapse formation.

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For keeping my spirits up outside the lab, I am grateful to the many great friends that I have made during graduate school. I am particularly indebted to my girlfriend, Royale Underhill, for all her love, support, patience, and tolerance.

As a parting note, I would like to finish with a short passage from Hunter S. Thompson, an author with an uncanny knack of cutting straight to the bone:

"Look outside," I said.

"Why?"

"There's a big...machine in the sky...some kind of electric snake...coming straight at us."

"Shoot it," said my attorney.

"Not yet," I said. "I want to study its habits."

Dedication

I dedicate this dissertation to the memory of my friend and mentor, Dr. Nikita Grigoriev. I will never forget your scientific brilliance, your sense of humor, and your willingness to always help others. This dissertation would not have been possible without you.

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List of Abbreviations

4-BPB	4-bromophenacyl bromide
5-HT	5-hydroxytryptamine
AA	arachidonic acid
ABS	antibiotic saline
ACh	acetylcholine
AChR	acetylcholine receptor
ARIA	acetylcholine receptor inducing activity
B5	buccal neuron 5
B19	buccal neuron 19
BDNF	brain derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CGC	cerebral giant cell
CM	conditioned media
CNS	central nervous system
CO	carbon monoxide
DA	dopamine
DM	defined media
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
F.E. test	Fisher's exact test
FLRFamide	phe-leu-arg-phe-amide
FMRFamide	phe-met-arg-phe-amide
GDPFLRFamide	gly-asp-pro-phe-leu-arg-phe-amide
HPLC	high-performance liquid chromatography
HVA	high-voltage activated
Ig	immunoglobulin
LTD	long term depression

LTP	long term potentiation
LVA	low-voltage activated
MASC	muscle-specific co-receptor
MuSK	muscle-specific kinase
NCAM	neural cell adhesion molecule
NDGA	nordihydroguaiaretic acid
NGF	nerve growth factor
NO	nitric oxide
NSF	*N*-ethylmaleimide-sensitive fusion protein
NT-3	neurotrophin 3
PFPA	pentafluoropropionic acid
PKC	protein kinase C
PSA	polysialic acid
RPD1	right parietal dorsal 1
RPeD1	right pedal dorsal 1
SD	standard deviation
SLT	supralateral radular tensor
SNARE	soluble NSF attachment protein receptors
SNAP	soluble NSF attachment protein
SNAP-25	synaptosomal-associated protein-25
TrkA	tyrosine receptor kinase A
VD2/3	visceral dorsal 2/3
VD4	visceral dorsal 4
VDCC	voltage dependent calcium channels
VJ	visceral J

Chapter 1: Introduction

1.1. General introduction

During development, individual neurons within the central nervous system are faced with the daunting task of locating and forming synaptic connections with a small set of select target cells. This synaptic connectivity is initially determined by migration of the post-mitotic cell, genesis of the growth cone, directed axon extension towards potential target cells, and the selection of specific postsynaptic neurons (Haydon and Drapeau, 1995). The newly formed synapses are refined further during later stages of development by competition between neurons themselves for a limited set of target cells (Goodman and Shatz, 1993; Haydon and Drapeau, 1995). These stereotypical patterns of neural connectivity established during development provide the framework for proper functioning of the entire nervous system in adult animals, yet the precise cellular and molecular mechanisms orchestrating this specificity of synaptic connections are poorly understood. It is generally accepted, however, that target cell selection and specific synapse formation are both contingent upon cell-cell signaling between the potential pre and postsynaptic neurons. Moreover, contacts between select synaptic partners are also known to bring about specific changes in the synaptic machinery of their companion cell (Fitzsimonds and Poo, 1998; Zoran et al., 1990). These changes are critical for future synaptic transmission between the neurons and their target cells. Nevertheless, neither the precise identity of the cell-cell signaling molecules, nor the nature and timing of such changes have been fully deduced.

This gap in our knowledge in the area of neurodevelopment and synapse formation in both vertebrates and invertebrates is largely due to the anatomical

complexity of the intact brain as cell-cell interactions between defined sets of pre and postsynaptic neurons are often difficult to investigate directly. One of the main objectives of this study, therefore, was to utilize a simple *in vitro* model system, compromised of individually identifiable pre and postsynaptic neurons, to investigate cellular changes that occur during the early stages of synaptogenesis.

This introductory chapter will provide an overview of the literature that pertains to the completed research. This review will first cover the basics of synapse formation from early stages of target cell selection to consolidation and competition dependent remodeling of the synapse. Emphasis will be placed on the nature of signals that are exchanged reciprocally between the synaptic partners and on the mechanisms by which various signaling molecules bring about specific changes in their target cells. Since it has been shown that neurotransmitter secretory machinery is regulated immediately upon contact with specific postsynaptic partners (Chow and Poo, 1985; Xie and Poo, 1986), the involvement of neurotransmitters in target cell selection and synapse formation will be analyzed. Moreover, biochemical and ion channel mechanisms underlying vesicular release of neurotransmitters will also be examined. Special emphasis will be placed on dopamine and FMRFamide, as these are the two transmitters utilized by neurons that were used in this study. The chapter will then conclude by giving a rationale for choosing *Lymnaea* as a model system for studies on synaptogenesis.

1.2. Synapse Formation

The formation of discrete chemical synapses in the central nervous system is dependent on a number of sequential steps, each of which determines the specificity, the type, and the strength of the final connection. Prior to synapse formation, neurons must

extend their axons toward appropriate target sites. Axons often navigate towards a general target area in large bundles and defasciculate at specific points to invade potential postsynaptic sites (Holt and Harris, 1998). This site-specific defasciculation may be controlled by relative changes in cell adhesion molecules such as fasciclin II in *Drosophilia* (Lin et al., 1994) or NCAM (neural cell adhesion molecule) in vertebrates (Rutishauser, 1985). After target invasion, advancing growth cones are directed towards discrete topographic locations, and finally into particular cell layers containing the target neurons (Holt and Harris, 1998). This targeting is under the control of chemoattractive and chemorepulsive molecules that can form gradients of either surface bound or diffusible guidance cues (for reviews see Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Following the identification of a prospective target neuron, the advancing growth cone stops and undergoes a number of reciprocal inductive interactions with the postsynaptic target that culminate in the precise juxtaposition of presynaptic neurotransmitter release sites with postsynaptic receptor clusters (for review see Glass and Yancopoulos, 1997; Ruegg, 1996). The newly established functional chemical synapse is further modified by activity dependent mechanisms that can either strengthen, weaken, or remove the synapse entirely if inappropriate (Budnik, 1996; Davis and Goodman, 1998; Goodman and Shatz, 1993; Katz and Shatz, 1996). Together, these steps ensure the formation of precise patterns of synaptic connections that are required for the proper functioning of the adult central nervous system.

1.3. Growth cone guidance and target cell selection

As growth cones advance towards their targets, they appear to be guided by at least four different mechanisms. These include contact-mediated attraction,

chemoattraction, contact-mediated repulsion, and chemorepulsion (Goodman, 1996). *In vivo*, these four mechanisms are thought to act in concert to repel extending axons away from non-target sites, thus preventing path finding errors, while simultaneously attracting them to appropriate targets. Representative molecules from each of the four categories are discussed below. Although examples of each mechanism will be dealt with individually, it should be noted that the distinction between these mechanisms is at best arbitrary and blurred (Goodman, 1996). For example, in some instances a secreted molecule may become immobilized either on the extracellular matrix or bound to a cell surface, thus giving the impression that the observed response may have involved "contact-mediated" signaling. Furthermore, a given guidance molecule which acts as an attractant for one cell type may be a repellent for another (Holt and Harris, 1998; Tessier-Lavigne and Goodman, 1996), consequently, it is often difficult to assign a particular trait to any given molecule.

1.3.1. Diffusible growth cone guidance molecules

The hypothesis that growth cones may be guided by attractive gradients of diffusible factors released by their targets was first proposed by Ramón y Cajal at the turn of the century. Since then, a multitude of factors have been identified that may serve this function. These include: nerve growth factor (NGF; Gunderson and Barrett, 1980), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3; Song et al., 1997); secreted proteins in the netrin family (Kennedy et al., 1994; Mitchell et al., 1996), and neurotransmitters such as acetylcholine (McCobb et al., 1988; Zheng et al., 1994), serotonin (Goldberg and Kater, 1989; Goldberg et al., 1990; Haydon et al., 1984), and dopamine (McCobb et al., 1988; Spencer et al., 1998). It is highly probable that these

diffusible molecules contribute to cell-cell communications that can occur from some distance during early development.

Netrins are secreted proteins of ~ 600 amino acids that are thought to form a gradient in the extracellular environment which regulates early stages of growth cone guidance (reviewed by Goodman, 1996). In both vertebrates (Kennedy et al., 1994) and *Drosophilia* (Mitchell et al., 1996), netrins released by cells in the ventral midline, attract growth cones that are extending towards the midline. This attraction occurs simultaneously with the active repulsion of other growth cones that are developmentally destined for targets away from the midline. In this fashion, netrins serve as both long-range diffusible chemoattractive and chemorepulsive molecules that guide extending axons either towards or away from target areas (Goodman, 1996; Kennedy et al., 1994; Mitchell et al., 1996).

Elegant experiments conducted by MM Poo's lab (eg: Song et al., 1997; Zheng et al., 1994) at the level of individual growth cones have demonstrated the roles of NT-3, BDNF, and ACh in axon guidance. This work also examined levels of the intracellular signaling molecule cAMP in the growing neurons and together these studies led to an interesting hypothesis; intracellular signals interact with external diffusible molecules to regulate growth cone guidance and target cell selection (Song et al., 1997). Focal application of NT-3, BDNF, or ACh *in vitro* onto the growth cones of extending *Xenopus* motor neurons caused the extending axon to turn towards the source of the signal molecule (Song et al., 1997; Zheng et al., 1994). This clearly demonstrates that the above compounds can act as diffusible molecules and are thus capable of regulating growth cone guidance. With the inclusion of the non-hydrolysable analogue competitor of

cAMP, Rp-cAMP, in the bath media, it was found that the extending growth cones turned away from the BDNF or ACh source. Although the ACh-induced turning behavior is thought to be transduced through Ca^{2+}-calmodulin dependent protein kinase II in these neurons (Zheng et al., 1994), the switch in polarity of the turning behavior caused by the elevated levels of cAMP demonstrate a plasticity mediated by the interaction between external and internal signals. In other words, intracellular levels of cAMP in the growth cone can act as a switch, which reverses the polarity of its response to diffusible cues present in the external environment. Such a mechanism may explain why extending neurites abruptly change their direction of growth upon contact with specific cells, termed "guidepost" cells, during development. Song et al. (1997) hypothesized that cells may be initially attracted to guidepost or target cell areas originally due to the gradient of diffusible factors released by the these cells. Upon arrival at the guidepost cells, changes in intracellular cAMP in the growth cone, perhaps triggered by contact with the guidepost cells, may switch the growth cone responsiveness from a positive (attraction) to negative (repulsion). This would prevent the extending axon from being "stalled" prematurely at the guidepost cell and cause extension in a new direction (Song et al., 1997).

In parallel with the chemoattractive molecules described above, chemorepulsion is also thought to guide growth cones during development of the nervous system. The primary chemorepulsive molecules identified to date are members of the semaphorin protein family (Goodman 1996) and a variety of classical neurotransmitters (Zheng et al., 1994). Semaphorins are a family of both secreted and cell surface molecules that appear to be evolutionarily conserved from insects to humans (Kolodkin et al., 1993). This

family of proteins is defined by a conserved 500 amino acid sequence that contains 14-16 cysteines, many blocks of conserved residues, and no obvious repeats (Kolodkin et al., 1993). *In vivo*, expression of semaphorin II in *Drosophilia* muscle cells that do not normally secrete this protein, inhibits certain identified motor neuron growth cones from forming normal synaptic terminal arborizations on these muscles (Matthews et al., 1995). This effect was discrete in that it did not prevent another class of motor neurons from forming synapses on their correct targets. Thus, semaphorin II can act *in vivo* as a selective target-derived signal that inhibits the formation of specific synapses. *In vitro* expression of human semaphorin III in COS cells has been shown to cause chemorepulsion of growth cones from the rat dorsal root ganglion (DRG) that are advancing in the presence of NGF (Messersmith et al., 1995). This secretion from COS cells did not repel a different subset of DRG neurons growing in the presence of NT-3. Since rat DRG neurons either terminate dorsally (NGF responsive) or ventrally (NT-3 responsive) in the spinal cord, this data may suggest that semaphorin III functions in the patterning of sensory projections by selectively repelling axons that normally terminate dorsally (Goodman, 1996; Messersmith et al., 1995).

Following the demonstration that neurotransmitters could be released from advancing growth cones in cell culture prior to contact with any prospective target cells (Hume et al., 1983), this class of molecules became the focus of a number of studies aimed at elucidating their role in neurite outgrowth and guidance. Acetylcholine (Lipton et al., 1988), the amines serotonin (Haydon et al., 1984; McCobb et al., 1988) and dopamine (Lankford et al., 1987; McCobb et al., 1988; Spencer et al., 1996), glutamate (Mattson et al., 1988), and the gaseous neurotransmitter nitric oxide (Hess et al., 1993)

have all been shown to inhibit neurite outgrowth, growth cone motility; and in the case of serotonin, specific synapse formation (Haydon et al., 1984). These diffusely released molecules are good candidates for distant cell-cell signaling between growth cones. For instance, by regulating the transmembrane fluxes of many ions, neurotransmitters can not only influence neuronal excitability, but they also activate various cascades of second messengers which either directly or indirectly influence the integrity of the growth cone cytoskeleton (McCobb et al., 1988). Indeed, the neurotransmitter activated voltage-gated calcium channels have been shown to change the internal homeostasis of growth cone calcium concentration, thus affecting growth cone motility and neurite extension (Anglister et al., 1982; Cohan et al., 1987). In keeping with the diverse roles of other chemoattractants and repellents, the neurotransmitter-induced effects on growth cone motility are cell type and often growth cone specific. For example Haydon et al. (1984) and Spencer et al. (1996) have clearly demonstrated that different neurons respond in dissimilar ways to the same transmitter. In other words, specific transmitters may inhibit growth cone motility of one cell type while having no effect on another neuron. Together, these data support the idea that neurotransmitter release from elongating growth cones may help to regulate the pattern of neural architecture during early development.

Overall, these studies clearly show that diffusible molecules are able to guide an extending growth cone *in vitro* in both an attractive and a repulsive manner. Whether appropriate levels of these compounds exist in the developing embryo, and in appropriate spatial and temporal patterns relative to the extending growth cones, remains to be demonstrated.

1.3.2. Contact mediated target cell selection molecules

After axonal invasion of appropriate target regions, their growth cones transiently probe the surface of many possible targets in search for contact mediated attractive cues. At this stage of target cell selection, surface bound molecules that regulate the adhesiveness between the advancing growth cone and the prospective target cell often act as attractive cues (Goodman 1996; Rutishauser et al., 1988). Although a number of molecules fall into this category (eg: cadherins: Fannon and Colman, 1996), most work in this area has remained focused on the immunoglobulin (Ig) superfamily of cell adhesion molecules including the neural cell adhesion molecule (NCAM) in vertebrates (Rutishauser et al., 1988) and the related Fasciclin molecules in *Drosophilia* (Chiba et al., 1995; Davis et al., 1997). NCAM is a cell surface bound molecule with five Ig and two fibronectin domains in its extracellular region (Cunningham et al., 1987). Upon contact, NCAMs expressed on opposed cell membranes bind homophilically and adhere the membranes together (Rutishauser et al., 1988). This binding may not be directly involved in synapse formation *per se* (Bixby and Reichardt, 1987), however the increased extent and/or duration of membrane contact is thought to enhance the efficacy of interactions between other cell surface molecules. In this manner, NCAM may serve a role in a permissive hierarchy in which increased cell-cell contact allows for the initiation of further interactions between the cells regulated by a second molecule(s) (Bixby et al., 1987; Rutishauser et al., 1988). Interestingly, the interaction between NCAMs on opposed cell membranes may be regulated by binding of the large carbohydrate moiety of polysialic acid (PSA) (Goodman, 1996; Rutishauser et al., 1988; Rutishauser 1991). PSA

binding to NCAM, prevents the physical interaction between opposed cell membranes, thus lowering the relative adhesiveness and probability of interaction between two cells. By increasing or decreasing the relative amount of bound PSA, the adhesiveness of an advancing growth cone can be regulated such that it is lower when traversing through an area of inappropriate targets and high in the vicinity of correct targets (Goodman, 1996; Rutishauser et al., 1988; Rutishauser 1991). In this manner, NCAM provides a flexible mechanism whereby advancing growth cones can be targeted to select group of potential postsynaptic cells.

The Fasciclins are a set of invertebrate cell adhesion molecules from the immunoglobulin superfamily that are related to NCAMs (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Their role in target cell selection and specific synapse formation has been well established at the *Drosophilia* neuromuscular junction, a model system in which synapses between individual, identified motor neurons and their discrete target muscles can be studied directly (Chiba et al., 1995; Davis et al., 1997). Additionally, the *Drosophilia* model system has the advantage of being amendable to genetic manipulation. In two separate studies, ectopic expression of either Fasciclin II or III on muscles that normally do not express these molecules resulted in the formation of inappropriate synapses (Chiba et al., 1995; Davis et al., 1997). Thus, the mere presence of expressed Fasciclin molecules on the muscle surface was often sufficient for growth cones to recognize them as specific synaptic partners. From these studies, it was concluded that Fasciclin II and Fasciclin III are indeed target recognition molecules that function during early development as contact-mediated attractive cues to ensure that appropriate synapses are formed between motor neurons and their specific muscle targets.

When a growth cone probes the surface of prospective target cells, it not only comes in contact with attractive cues on appropriate targets but also repulsive signals on inappropriate targets. These cell surface molecules will prevent the formation of improper synaptic connections and, together with attractive cues, guide growth cones in the right direction (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Two molecules have recently been identified as possible contact-mediated repulsive cues: collapsin in the avian nervous system (Luo et al., 1993) and connectin at the neuromuscular junction in *Drosophilia* (Nose et al., 1994). Collapsin is a 100 kD glycoprotein from the chick brain that contains a single immunoglobulin-like domain in its C-terminal region (Luo et al., 1993). Although it appears that this molecule may be secreted as a diffusible repulsive signal, a basic region near its C-terminal likely binds to negative charges displayed on cell surfaces or in the extracellular matrix (Luo et al., 1993). Collapsin was found to induce the collapse of advancing dorsal root ganglion growth cones *in vitro* (Luo et al., 1993), and this was thought to have involved the depolymerization of actin at the leading edge of the growth cone (Fan et al., 1993). Collapsin is homologous to semaphorin III (see section 1.3.1), a family of chemorepulsive molecules which are composed of both membrane bound and diffusible members (Goodman, 1996). Connectin is a cell surface protein, from the leucine-rich repeat family in *Drosophilia,* that is expressed on the surface of a subset of embryonic muscles and the motor neuron growth cones that innervate them (Nose et al., 1992; Nose et al., 1994). Ectopic expression of connectin on muscles that normally do not display this molecule caused a change in the topographic pattern of innervation of these muscles (Nose et al., 1994). Motor neurons that normally innervate these muscles were repelled

away from their correct targets and thus failed to make appropriate synaptic connections. Together, these studies on collapsin and connectin show that molecules attached to the surface of potential targets can regulate target cell selection by preventing the formation of inappropriate synaptic connections. These repulsive molecules signal to the advancing growth cone that the contacted cell is an inappropriate target and that it should continue its search for specific target cells elsewhere.

From the contact-mediated and diffusible guidance molecules discussed above, it becomes apparent that growth cone guidance and specific target cell selection rely upon a combination of both growth repulsive and permissive molecules that are expressed *en route*. This notion has led Goodman (1996) to propose a model in which, multiple, bi-functional, targeting signals which are aligned along common boundaries serve partially overlapping functions, yet work in concert to guide growth cones either away from inappropriate cells or toward specific targets. This model thus predicts that any one, cell-specific molecule does not determine target cell identity, rather this is determined by a combination of various molecules that are expressed by both the target and its neighboring cells.

A specific synaptic partner selection mechanism working along these lines would be highly reliable and robust. Furthermore, removal of any one molecule by genetic knockout would have very little disruptive effect on the targeting of the neurons. On the other hand, however, the introduction of this same molecule into a novel location may disrupt the normal pattern of signaling molecules, thus resulting in targeting abnormalities (Goodman, 1996). This trend is readily apparent in the *Drosophilia* model system in which genetic knockout phenotypes are much less different from wild type than

the phenotypes created by ectopic expression of guidance molecules on inappropriate targets (eg: Chiba et al., 1995; Nose et al., 1994; Mitchell et al., 1996).

1.4. Cell-cell interactions during early synapse formation

1.4.1. Target cell induced changes during synaptogenesis: Presynaptic changes

Following contact with a correct target cell, the presynaptic growth cone undergoes a rapid morphological reorganization in order to form a functional synapse. The lamellepodia of the growth cone, which had already begun to shrink on entering the vicinity of the target cell, disappear as do the extended filopodia. Neurotransmitter vesicles from the core of the neurite are advanced along microtubules to the tip in contact with the target cell (Levitan and Kaczmarek, 1997). These specializations are, at least in part, orchestrated by the postsynaptic cell and enable the presynaptic neuron to release larger amounts of transmitter into the developing synaptic cleft. This rapid but discrete and functional reorganization has been shown *in vitro* using *Xenopus* motor neurons and their associated target muscles (Chow and Poo, 1985; Evers et al., 1989). In these studies, acetycholine release from the growth cone was enhanced immediately after contact with the target muscle cell. This enhanced release was accompanied by a redistribution of both transmitter vesicles and the secretory proteins at the contact site.

In the intact brain, anatomical constraints deter an examination of early stages of synapse formation, therefore the neuromuscular junction, where both pre and postsynaptic elements can be examined simultaneously, has remained the focus of many studies on synapse formation. For example, motor neuron B19, from the buccal ganglion of the snail *Helisoma trivolvis*, forms appropriate chemical synapses in culture only with its normal *in vivo* synaptic partner, the supralateral radular tensor (SLT) muscle (Zoran et

al., 1993). This identified neuron does not form chemical synapses with inappropriate/non target muscles. Following cell-cell contact between B19 and the target SLT muscle, the resting cytosolic calcium levels have been shown to rise rapidly in the presynaptic cell at the contact site (Zoran et al., 1993). This rise in calcium does not occur when B19 contacts an inappropriate muscle cell. Similarly, a rise in presynaptic calcium is seen in *Xenopus* motor neurons following contact with a "myoball" in culture (Dai and Peng, 1993). The functional significance of this rise in presynaptic calcium levels in both *Helisoma* and *Xenopus* has not yet been determined, however, it may permit local reorganization of the cytoskeleton (Haydon and Zoran, 1994; Neher and Zucker, 1993) and a priming of the secretory machinery (Funte and Haydon, 1993; Haydon and Zoran, 1994; Zoran et al., 1990) such that synaptic vesicles are mobilized to the plasma membrane for evoked release into the developing synaptic cleft. Calcium can regulate the association of the secretory protein synapsin with vesicles and the actin cytoskeleton through calcium/calmodulin-dependent protein kinase II (Haydon and Zoran, 1994), thus providing a signal pathway from target cell contact to vesicle accumulation at the contact site. The rise in the presynaptic calcium level may play a further role in synapse formation by regulating gene expression in the neuron, leading to a subsequent phase of transcription and translation necessary for consolidation of the synapse (Haydon and Zoran, 1994). Changes in internal calcium levels have been shown to alter gene expression in a number of models (Finkbeiner and Greenberg, 1998), and at least in the case of *Xenopus* (Dai and Peng, 1993) the contact induced increase in calcium concentration has been found to reach the nucleus.

Upon contact with its specific partner cells, the postsynaptic cell induces further changes in the presynaptic cell to assure efficient synaptic transmission. In the frog (Robitaille et al., 1990), the leech (Fernandez-de-Miguel et al., 1992), and rat cerebellar granule cells (D'Angelo et al., 1994), Ca^{2+} channels have been shown to be up-regulated and redistributed in the cell following contact with a synaptic partner. A similar phenomenon has been seen in cultured hippocampal neurons where Ca^{2+} channels, along with other synaptic proteins (synapsin, synaptophysin, synaptotagmin, etc.) redistribute at synaptic sites (Fletcher et al., 1991; Basarsky et al., 1994). Together, the placement of voltage-gated calcium channels and neurotransmitter secretory machinery at the synaptic site, along with the removal of these components from extrasynaptic regions, facilitates the formation of discrete transmitter release sites only at the synapse, while suppressing release elsewhere. Not only does contact with the postsynaptic cell determine the location of transmitter release sites, in some instances it may even determine the type of transmitter that is to be released into the synaptic cleft. Schotzinger et al. (1994) demonstrated that when sweat glands from the footpad of rats are innervated by sympathetic neurons which express a noradrenergic phenotype, signals from the gland cells reduced the expression of noradrenergic traits in the neurons, in turn changing them to a cholinergic phenotype. Although the identity of the retrograde signal that causes these many changes in the presynaptic cell are unknown, these effects are aimed at localizing the correct transmitter filled vesicles and the appropriate secretory machinery, at the developing synaptic site. In the adult nervous system, this will form the basis of chemical synaptic communication between synaptic partners.

1.4.2. Target cell induced changes during synaptogenesis: Postsynaptic changes

Contact between the prospective synaptic partners also brings about changes in the postsynaptic cell. These changes are either induced by physical contacts (membrane-receptors) between the potential partner cells or via the release of diffusible substances from the presynaptic neuron (anterograde signals). Activation of specific postsynaptic receptors in turn initiate a cascade of events that involve the development of postsynaptic specializations, transmitter receptor clustering, and ion channel modulation (for recent reviews see Colledge and Froehner, 1998; Glass and Yancopoulos, 1997; Ruegg 1996). These changes in the postsynaptic cell are crucial for the future synaptic transmission.

Since synapse formation between the CNS neurons is difficult to study directly, most research aimed at synaptogenesis has remained focused on changes that occur on the postsynaptic target muscle cell during innervation by a motor neuron. At a developing neuromuscular junction, a newly arrived motor axon triggers numerous modifications in the distribution of acetylcholine receptors (AChR) in the muscle membrane (Hall and Sanes, 1993; Salpeter, 1987). It is likely that the postsynaptic receptor clustering results from both a redistribution of pre-existing AChRs and from synthesis of new receptors (Anderson and Cohen, 1977; Colledge and Froehner, 1998; Sanes, 1997). In both instances however, acetylcholine receptors are clustered under the motor neuron contact site thus facilitating postsynaptic responsiveness to the released transmitter. Voltage gated sodium channels become co-clustered with the acetylcholine receptors on the muscle membrane under the motor neuron contact site (Boudier et al., 1992; Flucher and Daniels, 1989) ensuring efficient transduction of the chemical signal into electrical action potentials.

In contrast to the lack of knowledge concerning retrograde signals from post- to presynaptic cell, a wealth of molecular evidence exists regarding the identity of anterograde signals that lead to synaptic differentiation at the postsynaptic membrane (for recent review see Colledge and Froehner, 1998). Two molecules that are released as anterograde signals from motor neurons are ARIA (acetylcholine receptor inducing activity: Jessell et al., 1979; Loeb and Fischbach, 1995) and agrin (Nitkin et al., 1987; Meier et al., 1995; Wallace et al., 1991). These molecules can independently cluster pre-existing acetylcholine receptors at the synaptic contact site and induce the transcription of new nicotinic acetylcholine receptors at subsynaptic nuclei (Ruegg, 1996).

Agrin was first isolated and characterized from basal lamina extracts of *Torpedo californica* where it was found to induce aggregation of acetylcholine receptors in cultured myotubes (Nitkin et al., 1987). Since then, agrin isoforms have been found in many populations of neurons in the vertebrate CNS (O'Conner et al., 1994), with highest expression levels being found during developmental time periods coinciding with synapse formation (Li et al., 1997). At the developing neuromuscular junction, this 200 kDa protein is synthesized and secreted by motor neurons where it becomes incorporated into the synaptic basal lamina (Denzer et al., 1995; Nitkin et al., 1987; Ruegg et al., 1992). From the basal lamina, agrin activates a receptor tyrosine kinase, MuSK (muscle-specific kinase), on the surface of the muscle through an unidentified muscle-specific co-receptor, MASC (Glass et al., 1996). Activation of MuSK leads to tyrosine phosphorylation of the acetylcholine receptor • -subunit through the intracellular peripheral membrane protein rapsyn (Apel et al., 1997; Ferns et al., 1996; Wallace et al., 1991). Although the precise reason for the phosphorylation of the • -subunit by rapsyn

remains unclear (Glass et al., 1997), it may help to recruit the receptor, along with other postsynaptic proteins, to the primitive MuSK based cytoskeletal scaffold (Apel et al., 1997; Colledge and Froehner, 1998; Glass and Yancopoulos, 1997). Overall, the function of agrin at the developing neuromuscular junction is relatively clear and well characterized: once released from the motor neuron, it binds to its specific receptor on the muscle where it induces acetylcholine receptor clustering at the contact site. In the CNS however, agrin does not seem to be involved in synaptogenesis; neurons cultured from mice carrying a mutation in the agrin gene (which disrupts neuromuscular junction assembly - see Li et al., 1999; Gautam et al., 1999) were shown to reliably form synapses. Its presence in the CNS does nevertheless suggest that this molecule may serve in functions other than synapse formation (Hilgenberg et al., 1999).

At the developing neuromuscular junction, transcription of synapse-specific genes in the postsynaptic muscle cell is, at least in part, regulated by trophic molecules released from the presynaptic motor neuron. ARIA, originally identified as a factor extracted from chick brain and spinal cord tissue, has been shown to increase acetylcholine receptor synthesis in cultured myotubes (Jessell et al., 1979; Ruegg, 1996). This protein is initially synthesized in the presynaptic neuron as a transmembrane precursor, the extracellular portion of which is released onto the basal lamina by proteolytic cleavage (Falls et al., 1993; Loeb and Fischbach, 1995). Further cleavage of the molecule in the basal lamina is thought to release an EGF-like region, which then acts on the target muscle (Loeb and Fischbach, 1995). In the target muscle, ARIA activates the transcription of transgenes in the subsynaptic nuclei that carry the regulatory elements for • - and • -subunits of acetylcholine receptor genes (Chu et al., 1995; Jo et al., 1995). The

end result of ARIA release from the presynaptic motor neuron is an increased synthesis of acetylcholine receptor subunits in the region directly under the neuron-muscle contact site (Ruegg, 1996).

In addition to the recruitment of specific neurotransmitter receptors, functional exclusion of specific receptor-subtypes from developing synapses may also occur following contact with a target cell. For example, in leech, a pressure-sensitive (P) neuron receives a 5-hydroxytryptamine (5-HT) mediated inhibitory synaptic connection from the Retzius (R) neuron. This inhibitory effect is via the activation of Cl^- channels present in the P neuron (Fuchs et al., 1982). Prior to contact with the R cell, the P neuron expresses PKC-dependent excitatory cation channels on its surface membrane that are opened by 5-HT. Contact with the R neuron activates a tyrosine kinase in the P cell which, in turn, prevents PKC-dependent activation of the 5-HT modulated cation channel (Catarsi and Drapeau, 1992; Drapeau, 1990). In other words, 5-HT causes excitation of the P neuron prior to contact with the R cell but inhibition after contact. This switch involves a suppression of 5-HT activated PKC-dependent excitatory cation channels on the postsynaptic membrane. Although it appears that the loss of channel activation by PKC is mediated by membrane contact (Drapeau et al., 1989), the precise cellular and molecular mechanisms involved and the timing of these events are not well understood.

1.5. Competition dependent synaptic remodeling

In addition to particular pre and postsynaptic cellular changes, synapse specificity also relies upon activity dependent competition between neurons. This competition in the developing nervous system is most likely for the limited amounts of trophic factors released by any select target cell (Kandel et al., 1991; Purves and Lichtman, 1985).

Neurons must receive a certain level of trophic support in order to remain alive and will thus compete with any other neuron that innervates a common target cell. Differing presynaptic inputs are thought to be identified by the target cell via their differing patterns of activity, and it is the extent of this activity that determines the level of trophic support that it might receive (Levitan and Kaczmarek, 1997). Hebb (1949) postulated that synaptic efficiency increases at synapses where electrical activity is coincident between pre and postsynaptic targets. Conversely, synaptic efficiency decreases at those synapses where presynaptic activity is not coincident with postsynaptic activity. This hypothesis has been investigated at neuromuscular junctions and excitatory synapses where depolarization of the postsynaptic target cell following activity in the presynaptic cell strengthens the synapse (Constantine-Paton, 1990; Lo and Poo, 1991). At these two types of synapses, at least, Hebb's hypothesis does seem to hold true.

A number of studies have begun to establish the possible mechanisms that may account for "Hebbian synapses". Using nerve-muscle co-cultures from *Xenopus* embryos, Lo and Poo (1991) demonstrated that when one of two neurons innervating a single muscle cell is repeatedly stimulated, its synapse is strengthened while the synapse made by the non-stimulated neuron is suppressed. If both neurons were stimulated synchronously, synaptic suppression seldom occurred. Asynchronous stimulation, on the other hand, led to the suppression of one or both of the synapses. Direct focal application of acetylcholine to the nerve-muscle synapse resulted in a depression of the synapse. This ACh-induced depression was prevented by synchronous electrical stimulation of the presynaptic motorneuron with the ACh pulse (Dan and Poo, 1992). Depolarization of the muscle by either ACh or neuronal stimulation, increases intracellular calcium levels in

the muscle that may in turn trigger the release of a retrograde messenger molecule (Dan and Poo, 1992; Goodman and Shatz, 1993; Levitan and Kaczmarek, 1997). Presynaptic neurons that are active during the release of this retrograde messenger are protected from elimination, whereas connections made by electrically inactive neurons are removed (Dan and Poo, 1992; Levitan and Kaczmarek, 1997). The identity of the retrograde signal, and the mechanism by which activity prevents elimination at the developing neuromuscular synapse, however, are presently unknown.

The activity dependent mechanisms alone cannot however account for competition between neurons that form inhibitory synapses. Specifically, Hebbian competition relies on coincident depolarization of the pre- and postsynaptic cells, as can be found at excitatory synapses and neuromuscular junctions; it does not take into account inhibitory synapses at which the presynaptic activity inhibits the firing patterns of the postsynaptic cell. It therefore seems reasonable to postulate that the mechanisms other than electrical activity may serve to determine the specificity of synapse formation at inhibitory synapses. Unfortunately, as compared with their excitatory counterparts, even less is known about mechanisms that determine the specificity of inhibitory synapses. This information is of key importance for our understanding of neurodevelopment since a vast majority of synapses between neurons in the CNS are inhibitory in nature.

1.6. Neurotransmitter release

The regulated release of diffusible chemical transmitter signals is the primary means of communication between neurons, and from neurons to muscles at the neuromuscular junction (Bajjalieh, 1999). Also, since action potential evoked release of

neurotransmitter in some models exists prior to target cell contact, it may therefore serve in functions other than the transynaptic communication (see section 1.3.1; Goldberg and Kater, 1989; Lauder 1993; Voronezhskaya et al., 1992). Following synapse formation, discrete transmitter release into the synaptic cleft is a tightly regulated mechanism of transducing electrical activity in the presynaptic cell into a chemical signal that is detectable by the postsynaptic cell. It is this chemical signaling between pre- and postsynaptic cells that is the basis for the functioning of the entire nervous system, thus, this review will now focus in detail on the mechanics of neurotransmitter release.

Katz and his colleagues first demonstrated at the neuromuscular junction that neurotransmitter release from the nerve terminal occurs in the form of multi-molecular packets. With the discovery that nerve terminals contain vesicles (Palade, 1954; Robertson, 1956), it was proposed that these synaptic vesicles form the structural basis for quantal liberation of neurotransmitter (del Castillo and Katz, 1957; Katz, 1969). This "vesicular hypothesis" of transmitter release states that the fusion of a transmitter filled vesicle with the surface membrane, and the resulting exocytotic discharge of the vesicle contents into the synaptic cleft, is responsible for the generation of the electrophysiologically detectable quanta. Strong evidence supporting this hypothesis was established by measuring increases in cell membrane capacitance during exocytotic release (Fernandez et al., 1984; Gillespie, 1979; Lindau and Neher, 1988; Neher and Marty, 1982). The capacitance of a cell is directly proportional to its surface area. Stepwise, as opposed to smooth, increases in cell membrane capacitance measured during bouts of exocytotic discharge indicated the fusion of small, discrete membrane bound vesicles with the surface membrane. More recently, using a combination of patch-clamp

measurement of capacitance and high resolution amperometry to detect secretory products, the vesicular hypothesis has been directly confirmed in at least 2 cell types: mast and chromaffin cells (Alvarez de Toledo et al., 1993; Robinson et al., 1995; Oberhauser et al., 1996). The process leading to the vesicular release of transmitter has multiple steps. These can roughly be broken down into 3 stages each of which will be discussed separately: 1) calcium influx through voltage dependent calcium channels (VDCC), 2) interaction between calcium and docked vesicles 3) release of vesicle contents to the external medium via fusion with the plasma membrane.

1.6.1. Ca^{2+} Influx is mediated through voltage dependent calcium channels

Transmitter release from the presynaptic terminal is triggered by an increase in intracellular calcium concentration that occurs via influx through voltage dependent calcium channels located in the plasma membrane. These channels, of which there are various types, open in response to action potential induced membrane depolarization (Goda and Sudhof, 1997). Calcium entry through VDCCs leads to an increased, but non-uniform, distribution of calcium beneath the cell membrane (Chad and Eckert, 1994; Fogelson and Zucker, 1985). Localized domains of high calcium arise just inside the pore of the channels since calcium entry through the pore occurs faster than calcium diffusion away into the cytoplasm (Matthews, 1996). To determine if the localized increases in calcium concentration would reach sufficient levels necessary for exocytosis, Llinàs et al. (1992) used a calcium-dependent photoprotein to optically measure internal calcium concentrations. These studies estimated that the Ca^{2+} levels might exceed 100 • M in the presumed presynaptic zone at the squid giant synapse.

Based primarily on pharmacological and biophysical criteria, VDCCs can be broadly bisected into two main groups: low-voltage activated channels (LVA; T-type channels) and high-voltage activated channels (HVA; N-, L-, P-, and Q-type channels) (Lnenicka and Hong, 1997). The HVA channels have higher activation thresholds while the LVA channels show a rapid voltage-dependent inactivation (Carbone and Lux, 1984). Also, HVA channels are more permeable to Ba^{2+} than Ca^{2+} with the reciprocal being true for LVA channels (Fox et al., 1987a; Carbone and Lux, 1987). Furthermore, LVA channels are more sensitive to Ni^{2+} than Cd^{2+} while the opposite view is held for HVA channels (Nowycky et al., 1985; Fox et al., 1987a,b).

In general, only HVA channels appear to play a role in evoked neurotransmitter release (Lnenicka and Hong, 1997). Pharmacological blockage of the N-type channel with • -CgTX GVIA, a peptide from the venom of the marine cone snail *Conus geographus*, has been shown to block evoked transmitter release from synaptic terminals in the mammalian CNS (Kamiya et al., 1988; Luebke et al., 1993), sympathetic nervous system (Hirning et al., 1988), and from frog motor terminals (Kerr and Yoshikami, 1984). Similarly, P-type channel blockers such as the peptide • -agatoxin from the venom of the funnel web spider *Agelenopsis aperta,* have been shown to block transmitter release in the mammalian CNS (Luebke et al., 1984; Regehr and Mintz, 1994; Castillo et al., 1994). In invertebrates, the P-type channel has been shown to be involved in transmitter release at the squid giant synapse (Llinàs et al., 1989) and motor terminals in the crayfish (Araque et al., 1994). In some models, it appears that both P- and N-type channels are involved in triggering transmitter release at the same synaptic terminal (Luebke et al., 1993). This has been demonstrated in *Aplysia* where the activity of both N- and P- type

channels is necessary for evoked transmitter release from synaptic terminals in the central ring ganglia (Fossier et al., 1994).

In order to minimize the time delay between calcium influx and exocytotic discharge of the synaptic vesicle contents, the site of calcium influx, the VDCCs, must be physically located within close proximity to the vesicle secretory machinery (Bennett, 1997). Any significant increase in the spatial separation between these two sites will increase the time required by the calcium signal to reach the putative calcium sensor site (see section 1.6.2) thus jeopardizing the efficacy of transmitter release at the synapse. Furthermore, an increased diffusion distance may result in the maximal calcium concentration reaching the secretory machinery to be potentially lower than the threshold concentration required for exocytosis (Bennett, 1997). To overcome this spatial problem, the membrane bound protein syntaxin has been proposed to serve as a physical linkage that holds the VDCCs in close proximity with the transmitter vesicle secretory machinery (Bennett et al., 1992; Bennett, 1997). Binding sites for syntaxin on the cytoplasmic loop of N-type VDCCs have been identified (Sheng et al., 1994,1996) and may serve to localize these channels to the synaptic release site.

1.6.2. Calcium influx triggers primed and docked vesicles

In neurons, synaptic vesicles cluster at synaptic sites and those closest to the presynaptic membrane are "docked" to the membrane (Heuser and Reese, 1981; O'Conner et al., 1994). Rothman (1994) first proposed the SNARE (soluble NSF attachment protein receptors) hypothesis in an attempt to explain the molecular chain of events that leads to the targeting and docking of these vesicles to specific sites on the plasma membrane. At the core of this hypothesis is the direct interaction between

identified proteins on the surface of the transmitter filled vesicles and proteins on the plasma membrane at the synaptic release site. This interaction in turn targets the vesicles to distinct release sites and provides a mechanism whereby exocytosis can be regulated by other cellular processes (Hanson et al., 1997; Rothman 1994). The SNARE molecules include syntaxin and synaptosomal-associated protein (SNAP-25) on the interior surface of the plasma membrane, and synaptobrevin on the surface of the vesicle (Zheng and Bobich, 1998). The physical interaction between synaptobrevin on the vesicle and the 2 plasma membrane bound SNAREs results in the formation of a stable 7S complex, the docking complex that tethers the vesicle to discrete sites on the presynaptic membrane (Rothman, 1994).

Following the formation of the tethered docking complex, vesicles must be primed for their sensitivity to the arrival of a calcium trigger that catalyzes exocytosis (Zheng and Bobich, 1998). The 7S docking complex provides a high affinity binding site for soluble *N*-ethylmaleimide-sensitive fusion protein attachment proteins (SNAPs) (McMahon and Sudhof, 1995). The binding of • -SNAP is thought to displace synaptotagmin, an integral membrane protein on the vesicle, and recruit soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) to form a 20S complex called the fusion complex (Zheng and Bobich, 1998). Binding to SNAP stimulates the ATPase activity in the NSF, which then hydrolyzes ATP to prime the vesicle. At this stage, the vesicles are closely attached to the plasma membrane by the 'activated' SNARE complex and are ready to interact with other lipids or proteins in the plasma membrane and undergo fusion in response to a Ca^{2+} trigger (Bennett, 1997).

Calcium influx through VDCCs (see section 1.6.1) raises the calcium concentration around the primed vesicles and acts as a trigger for the opposed vesicle to fuse with plasma membrane thereby releasing the vesicular contents to the external environment. Electrophysiological analysis has shown that for each vesicle to be released, 4 independent calcium binding sites must be filled (Bennett, 1997; Bertram et al., 1996; Stanley 1997). The synaptic vesicle associated membrane protein synaptotagmin contains two C2 domains, motifs that can bind calcium and phospholipids, and has thus been proposed as the putative calcium sensor in the SNARE complex (Brose et al., 1992; Chapman and Jahn, 1994; Li et al., 1995). This molecule has been reported to bind the N-type calcium channel (Leveque et al., 1994); and treatment with antibodies against synaptotagmin reduces exocytosis (Elferink et al., 1993). Together, this data strongly implies that synaptotagmin acts as a molecular calcium sensor for the docked and primed vesicles. A rise in intracellular calcium signals through synaptotagmin to activate the primed SNARE complex. In turn, this activation causes the vesicle to undergo fusion with the plasma membrane and subsequent transmitter exocytosis.

1.6.3. Vesicle fusion and exocytosis of the neurotransmitter

Calcium influx and, binding with synaptotagmin, causes a rapid electrostatic and/or conformational change in the fusion complex. This restarts the process of fusion that was arrested in a primed state prior to calcium influx, and allows the vesicle to fuse with the plasma membrane (Goda and Sudhof, 1997). Presently, there are several proposed models that can account for the creation of a fusion pore through which the transmitter would pass. In the hemifusion model, the role of the synaptic complex

proteins is to bring the lipid bilayers so close that they virtually fuse on their own. The energy released by the stable SNARE complex may be used to overcome the energy barrier between the two membranes and thus contribute directly to membrane fusion (Zheng and Bobich, 1998). In an alternate model, the fusion pore model, a protein channel-like structure is formed between the vesicle and the plasma membrane (Monck and Fernandez, 1994). The channel-like pore is thought to expand when its subunits separate and the lipid molecules invade the space between the proteins (Zheng and Bobich, 1998). Synaptophysin, a protein integrated into the vesicle membrane, has 4 membrane spanning domains, a topology that is very similar to gap junction proteins (Johnston et al., 1989; Johnston and Sudhof, 1990). Due to this fact, and because it can form ion channels in lipid bilayers (Thomas et al., 1988), synaptophysin has been implicated in playing the role of the initial channel-like structure.

1.7. FMRFamide

FMRFamide (Phe-Met-Arg-Phe-amide) was first discovered in the clam *Macrocallista nimbosa* (Price and Greenberg, 1977). Since then, it has also been found in a number of molluscs (Price 1986; Price et al., 1987), including *Lymnaea stagnalis* (Ebberink et al., 1987), and a variety of other phyla and species (Boer et al., 1980; Chronwall et al., 1984; Grimmelikhuiijzen and Spencer, 1984; Weiss et al., 1984). Although much of the early work with FMRFamide focused on its direct excitatory effect on heart musculature in various molluscs (Greenberg and Price, 1979; Painter et al., 1982), in *Lymnaea*, this peptide has been shown to act in several different central neural networks which regulate heart beat (Buckett et al., 1990), egg laying (Brussaard et al.,

1989) and respiration (McKenney 1992). Furthermore, FMRFamide also modulates the sensory-motorneuron network that controls gill withdrawal in *Aplysia* where it mediates either short or long term synaptic depression (Montarolo et al., 1988; Schacher et al., 1993).

In *Lymnaea*, the FMRFamide locus spans >20 kb and compromises at least 5 exons (I-V), punctuated by 4 introns (Santama et al., 1995). With neuron specific post transcriptional regulation at the level of differential RNA splicing, this gene has the potential to produce at least 13 distinct peptides (see figure 1.1). Exon II encodes mainly for the tetrapeptides FMRFamide/FLRFamide while exon III encodes for the heptapeptides GDP/SDPFLRFamide (Saunders et al., 1992,). In the *Lymnaea* CNS, alternate RNA splicing generates at least two different mRNA moieties, one compromising exons I and II and another including exons I and III but excluding II (Saunders et al., 1992). These two moieties are expressed in a differential and mutually exclusive manner in the CNS (Bright et al., 1993; Santama et al., 1995; Saunders et al., 1992).

1.7.1. FMRFamide: receptors

FMRFamide receptors in molluscs come in 2 distinct types: a family of G-protein coupled receptors that activate K^+ channels and/or inhibit Ca^{2+} channels, and a ligand-gated Na^+ channel (Brezina et al., 1987 a,b; Bahls et al., 1992; Cottrell 1997; van Tol-Steye 1999). The effects of these channels are predominantly inhibitory for the G-protein coupled receptor and excitatory for the ligand-gated Na^+ channel. The most extensively studied G-protein coupled receptor is linked to the opening of the S-channel in *Aplysia*

sensory neurons (Belardetti and Siegelbaum, 1988). Activation of this receptor increases the open probability of the S-channel (Belardetti et al., 1987) in a PTX-sensitive manner (Volterra and Siegelbaum, 1988). This channel may also signal via the lipoxygenase metabolites of arachidonic acid (Piomelli et al., 1987a, 1987b: see section 1.7.2). A similar G-protein/arachidonic acid pathway activated by FMRFamide has been described in other abdominal ganglion neurons from *Aplysia* (Brezina et al., 1987), in neuron B5 from the buccal ganglion of *Helisoma trivolvis* (Bahls et al., 1992), and from caudo-dorsal cells of *Lymnaea* (Kits et al., 1997). In *Lymnaea*, FMRFamide can also have an inhibitory effect on neurons, involving suppression of a Ca^{2+} current (Dreijer et al., 1995; van Tol-Steye, 1999). Thus, taken together, the activation of the FMRFamide G-protein coupled receptor inhibits electrical activity in molluscan neurons by acting on a number of ion channels in the plasma membrane. Interestingly, along with inhibition of electrical activity, exogenous application of FMRFamide has also been shown to prevent evoked transmitter release in cultured molluscan neurons (Bahls et al., 1992; Man-Son-Hing et al., 1989).

A fast excitatory response and an increase in membrane conductance in response to exogenous FMRFamide application were first found in *Helix aspersa* (Cottrell et al., 1984). Strong evidence that this current was carried by a peptide gated ion channel was obtained using outside-out membrane patches from *Helix* C2 neurons (Cottrell et al., 1990). Inward currents were observed following external FMRFamide application even with 5'-*O*-(2-thiodiphosphate), a G-protein blocker, in the recording pipette (Green et al., 1994). This suggested a tight receptor/channel coupling with no involvement of a G-protein (Cottrell, 1997). The channel was cloned from *Helix* (Lingueglia et al., 1995) and

subsequently expressed in *Xenopus* ooyctes (Lingueglia et al., 1995; Zhainazarov and Cottrell, 1998) and human embryonic kidney (HEK-293) cells (Coscoy et al., 1998). The expressed channel formed a tetramer in the oocyte plasma membrane, had a higher affinity for FMRFamide than for FLRFamide, and could be pharmacologically blocked by amiloride or benzamil (Coscoy et al., 1998; Lingueglia et al., 1995; Zhainazarov and Cottrell, 1998). Although the FMRFamide gated Na^+ channel is the first peptide gated channel to be described, it has only been activated by exogenous application of the ligand; whether this channel exists at synapses remains to be demonstrated.

1.7.2. FMRFamide: signal transduction pathway

Although little is known about the actual receptor structure, the signal transduction pathway for the G-protein linked FMRFamide receptor has been fairly well characterized in molluscs. Working with the *Aplysia* S-channel, Piomelli et al. (1987) found that the FMRFamide induced increase in open probability of this channel was mediated via lipoxygenase metabolites of arachidonic acid. This notion has since been supported further in *Aplysia* where blockers of the lipoxygenase pathway of arachidonic acid prevent long-term heterosynaptic inhibition caused by FMRFamide (Wu and Schacher, 1994). Similar results have been obtained in other molluscan species: FMRFamide activates the lipoxygenase pathway of the arachidonic acid signaling cascade which in turn acts on K^+ channels in both *Lymnaea* (Lopes et al., 1998; Kits et al., 1997; van Tol-Steye, 1999) and *Helisoma* (Bahls et al., 1992). Along with the activation of a K^+ channel, the FMRFamide/arachidonic acid pathway also suppresses a Ca^{2+} channel in the plasma membrane (van Tol-Steye, 1999). This inhibition of inward

Ca^{2+} current tends to hyperpolarize and, in concert with the increase in outward K^+ current, will inhibit the cell.

1.8. Dopamine

The role of dopamine as a neurotransmitter in both vertebrates and invertebrates is well established. In *Lymnaea*, dopamine was first found in an identified neuron, Right Pedal Dorsal 1 (RPeD1) by Cottrell et al. (1979). This cell is part of the respiratory central pattern generator in *Lymnaea* (Syed et al., 1990; Syed and Winlow, 1991) and it makes a variety of monosynaptic connections in the CNS (Berry and Cottrell, 1975; Winlow et al, 1981). Synaptic transmission from RPeD1 to a number of target cells has been shown to be dopaminergic and it most likely works through a D_2-like G-protein coupled receptor (Magoski et al., 1995, Spencer et al, 1998).

Along with its function as a neurotransmitter in the CNS, DA has also been hypothesized to play a role in neural development (Haydon et al., 1984; Lauder 1993) in both molluscs and vertebrates. Specific DA receptors are expressed transiently during development (Lankford et al., 1987) and exogenous DA application has been shown to regulate growth cone activity *in vitro* in both vertebrate (Lankford et al., 1988) and invertebrate (McCobb et al., 1988; Spencer et al., 1998) model systems. Furthermore, DA release from growth cones at the tips of extending neurites has been shown to occur prior to target cell contact (Spencer et al., 1998) and perturbation of embryonic DA levels causes developmental abnormalities in *Lymnaea* (Voronzhskaya, 1990; Voronzhskaya et al., 1992). Together, these data support the postulate that release of this transmitter prior to the formation of synapses may play a role in axonal pathfinding, target cell selection, and specific synapse formation.

1.9. Summary

From the above review, it is clear that synapse formation proceeds through a number of complex stages; beginning with an initial growth cone guidance and target cell recognition through to activity dependent competition and remodeling of the forming connections. Furthermore, it is clear that interactions between the prospective pre and postsynaptic cells bring about specific and necessary changes in their partners to ensure efficient release and detection of chemical transmitter substances. At all stages of synaptogenesis, precise signaling between the partner cells is required for the synaptic program to proceed normally. In most of the preparations studied to date, neither the precise timing or identity of these signals, nor the cellular and molecular mechanisms through which these signals regulate synaptic programs, are fully understood. The answers to these questions are difficult to discover because specific synapses between defined sets of pre and postsynaptic neurons are difficult, if not impossible, to investigate in the intact brain. Ideally, both pre and postsynaptic neurons must therefore be studied in a preparation where not only the somata but also their synapses are directly accessible to both cellular and molecular analysis.

1.10. Rational for using *Lymnaea* soma-soma synapses as a model system

The freshwater pond snail, *Lymnaea stagnalis*, has a relatively simple CNS consisting of ~20,000 neurons distributed in 11 ring ganglia. This snail has been used extensively for neurophysiological studies on the neural control of circulation (Bekius, 1972; Hubendick, 1978; Syed and Winlow, 1991c), feeding (Yeoman et al, 1996), respiration (Jones, 1961; Hubendick, 1978; Syed and Winlow, 1991a, 1991b; Inoue et al., 1996), locomotion (Haydon and Winlow, 1982, Syed and Winlow, 1989, 1991c),

synaptic transmission (Berry and Cottrell, 1975; Winlow et al., 1981), synapse formation (Feng et al., 1997; Syed et al., 1990; 1992b; Syed and Spencer, 1994), neurohormonal actions and peptidergic mechanisms (Schot et al., 1981; Roubos and Smeets, 1989; Leung et al., 1990), neurite outgrowth (Kits et al., 1990; Syed et al., 1992a), and identification of trophic molecules (Fainzilber et al., 1996).

The rationale for choosing *Lymnaea* is straightforward. These snails have a simple and easily accessible nervous system with large and individually identifiable neurons. These neurons have large somata that facilitate direct electrophysiological access thus simultaneous recordings can easily be made from both pre and postsynaptic neurons. Moreover, the adult neurons can be extracted from the intact brain and cultured *in vitro*. In cell culture, identified neurons not only regenerate their axonal processes but they also recapitulate their patterns of specific synapse formation. Thus, networks of neurons can be reconstructed *in vitro* where they generate rhythmic activity similar to that generated by the intact brain.

Taking advantage of identified *Lymnaea* neurons, one of the main objectives of this study was to determine how secretory machinery of synaptic partners is modulated during early synapse formation. In this study, individually identified neurons right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4) were chosen because these cells form mutual inhibitory connection *in vivo* (Syed and Winlow 1991b) which can be reconstructed under appropriate cell culture conditions (Feng et al., 1997; Syed et al., 1990). RPeD1 and VD4 are part of the respiratory central pattern generator in *Lymnaea*; a 3 cell circuit of which these 2 neurons are an important component (Syed et al., 1991; Syed and Winlow, 1991b). This CPG has been reconstructed in culture where these 3

neurons were shown to be sufficient to generate respiratory rhythm similar to that seen *in vivo* (Syed et al., 1990).

Although *Lymnaea* neurons are able to re-establish their *in vivo* synaptic connections in cell culture, the reconstructed synapses that form between the neurites are located at a distance from the cell body. This configuration precludes direct electrophysiological and/or molecular access to the synaptic site. To circumvent this problem, Feng et al. (1997) built upon previous work on other invertebrate neurons (Fuchs et al., 1982; Haydon, 1988; Nicholls et al., 1990; Cooper et al., 1992; Klein, 1994) and established chemical synapses between directly opposed somata. With this synaptic model, Feng et al. (1997) demonstrated that *Lymnaea* neurons reform their specific synapses in a soma-soma configuration. Furthermore, Feng et al. (1997) showed that these synapses are target cell specific and similar to those seen either *in vivo* (Syed and Winlow, 1991) or those that form between their neurites *in vitro* (Syed et al., 1990). Using this simple model system, the specificity of synapse formation and the precise timing of events underlying synaptogenesis can be examined directly at a resolution not possible elsewhere.

1.11. Specific aims and hypotheses

One of the main objectives of this research was to utilize an *in vitro* soma-soma preparation consisting of identifiable pre- and postsynaptic neurons to study modulation of transmitter secretory machinery during early stages of synapse formation. This model presents the unique advantages of being able to control the timing and location of target cell contact. The ultimate goal was to exploit this model to understand both intercellular and intracellular signaling mechanisms that regulate synapse formation between

interneurons from the central nervous system. The specific aims of this research are as follows:

Specific Aim #1: To determine the sequence of soma-soma synapse formation between RPeD1 and VD4.

Since this pair of cells form a mutually inhibitory synapse *in vivo*, I examined the sequential development of this synapse *in vitro*. I asked the question whether this cell pair directly proceeds to a mature, mutually inhibitory synapse, or if there are characteristic early synaptic configurations that precede the final connection. Also, due to the fact that RPeD1 only appears to utilize one transmitter (dopamine: Magoski et al., 1995), I examined the regulation of this cell's secretory machinery during soma-soma synapse formation with VD4. Specifically, I tested whether interactions between the two cells during early phases of synapse formation caused changes in RPeD1's secretory capabilities.

Specific Aim #2: To determine the intercellular signal responsible for the modulation of transmitter release from RPeD1 during synaptogenesis.

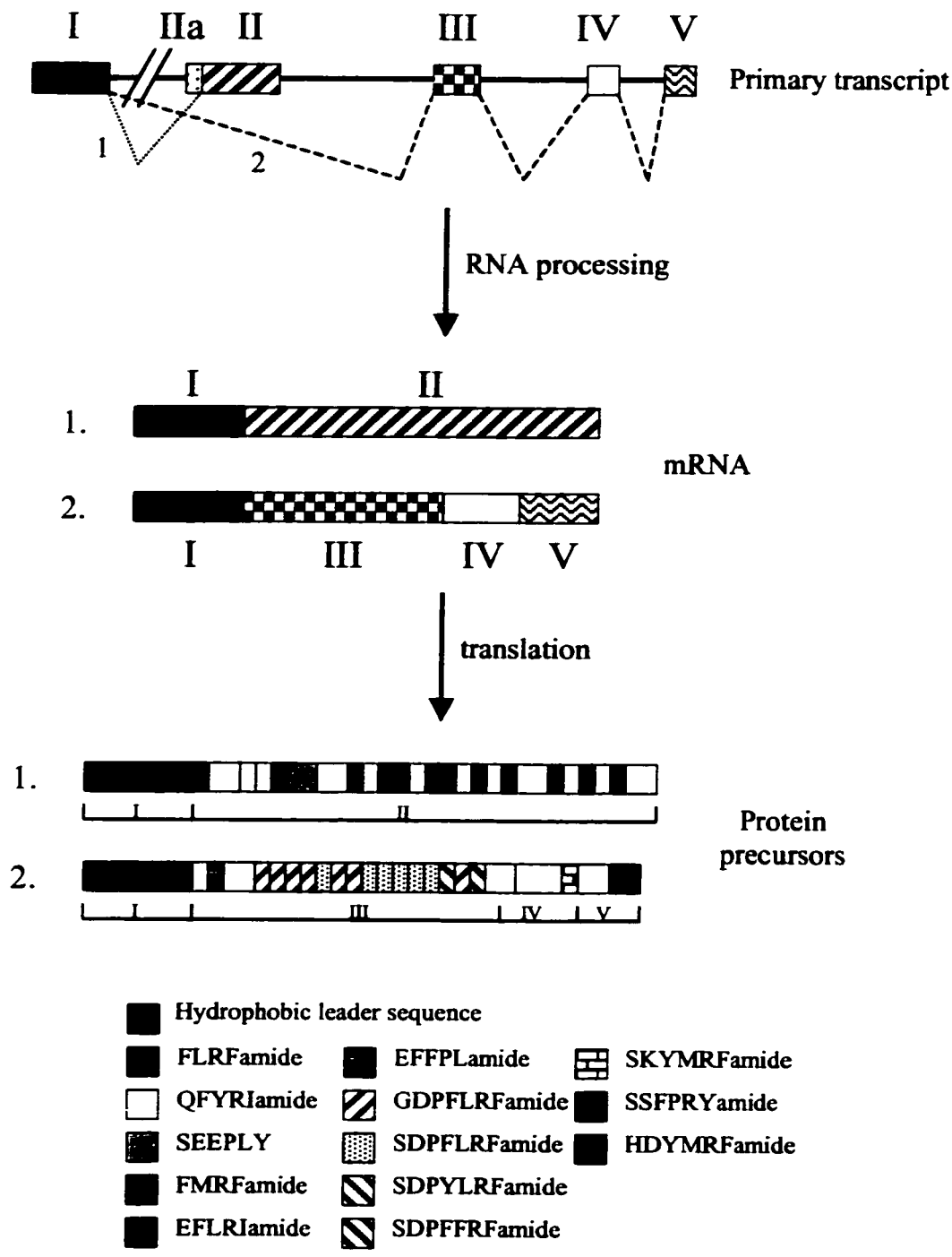
Following from the first part of the study in which transmitter release from RPeD1 was found to be transiently suppressed during synapse formation with VD4, I examined the nature of the intercellular signal(s) responsible for causing this. Specifically, I set out to determine whether the suppression was target cell specific, if it required an active synthesis of new protein, and if it was due to membrane bound or

diffusible signal molecules. In particular, since I found that VD4 acquired the ability to synaptically release transmitter first, I investigated the possibility that transmitter release from VD4 regulated the secretory machinery of RPeD1.

Specific Aim #3: To determine intracellular signal cascade in RPeD1 that mediates transient transmitter suppression during specific synapse formation.

To validate the findings that release of a FMRFamide-like peptide from VD4 is responsible for the transient suppression of dopamine release from RPeD1, I investigated the second messenger cascade in RPeD1 that transduced both the VD4- and peptide-induced effects. I hypothesized that a common intracellular signaling cascade in RPeD1 mediates both the target cell contact and FMRFamide effects.

Figure 1.1. Model depicting differential splicing and expression of the FMRFamide gene in *Lymnaea stagnalis*. The primary RNA transcript of the locus (top of the panel) contains at least five exons and is alternatively spliced in two modes. Differential splicing results in the generation of at least two distinct mRNA types; type 1 (1: consisting of exons I and the part of exon II shown) and type 2 (2: comprising exons I, III, IV, and V). These are expressed in the CNS in a mutually exclusive manner. Each mRNA encodes a distinct protein precursor containing multiple, non-overlapping peptides (shown at the bottom of the panel). The two protein precursors share a common hydrophobic leader sequence, which is also expressed in the CNS in a mutually exclusive fashion. (Modified from Santama et al., 1995).


I
IIa
II
III
IV
V
Primary transcript
1
2
RNA processing
I
II
1.
mRNA
2.
I
III
IV
V
translation
1.
2.
Protein
precursors
Hydrophobic leader sequence
FLRFamide
EFFPLamide
SKYMRFamide
QFYRIamide
GDPFLRFamide
SSFPRYamide
SEEPLY
SDPFLRFamide
HDYMRFamide
FMRFamide
SDPYLRFamide
EFLRIamide
SDPFFRFamide

Chapter 2: Methods and Materials

2.1. Animals

All experiments were performed on neurons from the fresh water pond snail, *Lymnaea stagnalis* (Gastropoda, Pulmonata, Basommatophora, Lymnaeidae: see fig. 2.1A). The snails used in this study were derived from an inbred population raised at the Free University Amsterdam and were maintained in aqua-culture at the animal care facility at the University of Calgary. Animals were raised at room temperature (20°C to 22°C) in well aerated, de-chlorinated tap water. Snails were exposed to a 12/12 hour light/dark cycle and fed lettuce (1x/week) and Purina Trout Chow (5x/week). Individual animals with shell lengths of 15-20 mm (approximately 2-4 months old) were used in all experiments.

2.2. Dissection

Animals were dissected in a standard *Lymnaea* saline containing (in mM): NaCl 40.0, KCl 1.7, $CaCl_2$ 4.1, and $MgCl_2$ 1.5. The saline was buffered with 10.0 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid: Sigma) and the pH was adjusted to 7.9 with 1N NaOH (Syed and Winlow, 1991). Antibiotic saline (ABS) was prepared by the addition of Gentamycin (Sigma: 150 μg/mL) to autoclaved standard saline.

Animals with an approximated shell length of 15-20 mm were deshelled with scissors and anesthetized in 10% (v/v) Listerine in standard saline for 10 minutes. Anesthetized snails were pinned down on a silicone rubber based (General electric RTV 616) dissection dish containing ABS. All instruments were pre-sterilized with 75% ethanol and subsequent cell isolation/culture procedures were performed in a sterile tissue

culture laminar flow hood (Ridgway et al., 1991; Syed et al., 1990; Syed et al., 1999). The central nervous system (CNS) was exposed by making a dorsal midline incision through the body wall and removed as described previously (Ridgway et al., 1991; Syed et al., 1999). Briefly, the body wall and the penis were pinned back and the esophagus, which is encircled by the central ring ganglia, was cut near the stomach. The buccal mass was then rolled anteriorly, pulling the attached stump of the esophagus through the ring ganglia, and pinned down. The cerebral commisure was cut and both central ring and buccal ganglia were removed using fine dissection scissors (Fine Science Tools, #5).

2.3. Cell culture

2.3.1. Media and dish preparation

All cell culture experiments were performed by placing isolated *Lymnaea* neurons in cell culture dishes containing defined media (DM) for a specific time period (2-24 hours). DM consisted of serum-free 50% (v/v) Liebovitz's L-15 media (formula #82-5154 EL, GIBCO) with additional salts (in mM: NaCl 40.0, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, HEPES 10.0), 10mM glucose, 1.0 mM L-glutamine, and 20 μg/mL gentamycin. The pH was adjusted to 7.9 with 1N NaOH and the solution was filtered (Millipore Sterivex-GV, 0.22 μM filter unit) and stored in autoclaved bottles.

Two types of cell culture dishes were used in this study. For most experiments, uncoated plastic culture dishes were used (25mm, Falcon-3001). In a minority of experiments that required better cell adhesion and better optical viewing, Poly L-Lysine coated glass-coverslip attached dishes were used. These dishes were prepared by drilling a 15 mm diameter hole in the bottom of recycled plastic dishes and attaching glass coverslips (22x22 mm, Baxter) across the hole using non-toxic 100% silicone glue (Dow

Corning). These dishes were sterilized with ethanol and overnight exposure to UV light in a laminar flow hood to prepare for cell culture. Poly L-Lysine (Hydrobromide, FW: 50,000–110,000; Sigma P2636) was dissolved in 0.15 M tris[hydroxymethyl]aminomethane (TRIS) buffer (pH 8.4) to reach a concentration of 1 mg/mL. This solution was syringe filtered (0.22 μM Millipore filter) and added to the glass-coverslip attached dishes (2 mL/dish) which were then left overnight in a sterile laminar flow hood. The following day, the Poly L-Lysine solution was removed and the dishes were washed with sterile water (3 x 30 min; see Wong et al., 1983) and stored at 4°C until used.

2.3.2 Single cell isolation

Following removal from the whole animal (section 2.2), the isolated central ring ganglia were washed serially in ABS (3 x 15 minutes) and treated with trypsin (Type III, 2 mg/mL) dissolved in DM for 24 minutes at room temperature. This enzymatic activity was subsequently stopped by placing the ganglia in trypsin inhibitor (2 mg/mL), also dissolved in DM for 15 minutes. The ganglia were then pinned to the bottom of a black silicone rubber (General Electric, RTV616) dissection dish containing high osmolarity DM (40 mM glucose; Sigma G7021). Under a stereo dissection microscope (Zeiss), both the outer and inner connective tissue sheaths were removed mechanically using fine forceps (DuMont #5). At this point, the somata of individual neurons could easily be identified based on their position, size, and colour (Benjamin and Winlow, 1981; Berry and Cottrell, 1975; Syed et al., 1990; Ridgway et al., 1991: see fig. 2.1B). Identified cells were selectively isolated by applying gentle suction via a microsyringe (Gilmont, GS 110) attached to a fire polished glass pipette (see fig. 2.2). To prepare pipettes for

single cell extraction, glass capillary tubes (WPI, 2mm, 1B200F) were coated with Sigmacote (Sigma, SL-2) and pulled on a vertical pipette puller (DKI, 700C). The tips were broken and fire polished on a microforge to create a tip diameter slightly larger than the soma of the desired cell. Isolated cells were subsequently transferred to pre-prepared culture dishes (see section 2.3.1) and left undisturbed in the tissue culture hood at room temperature.

2.3.3. Soma-soma pairing

To obtain soma-soma synapses, somata of identified cells were juxtaposed as described previously (Feng et al., 1997). Briefly, careful isolation of identified neurons resulted in individual somata with long axon stumps attached. Somata were gently placed in the culture dishes such that their axon stumps overlapped close to where they exit from the cell body. Within a few hours in culture, the cells retracted the axon stumps bringing the somata into direct contact with one another. The soma-soma paired cells were left undisturbed overnight, allowing the formation of chemical synapses.

2.4. Electrophysiology

2.4.1. Intracellular recordings: isolated cells

To study the synaptic connections formed between neurons in the soma-soma cell culture configuration, simultaneous intracellular recordings were made from the opposed somata. Conventional sharp intracellular glass microelectrodes (1.5 mm internal diameter, TW 150F-6, WPI: resistance 30-60 MΩ) were prepared on a vertical microelectrode puller (Kopf, 700C) and filled with a saturated K_2SO_4 solution. These electrodes were connected to the amplifier headstages and the electrode tips were positioned near the cells using Narishige micromanipulators (MM 202 and MM 204,

Tokyo, Japan). A ground electrode created from chloride coated silver wire was placed in the bath and, prior to cell impalement, the resistance of the microelectrodes was balanced using a Grass stimulator (S88). A Zeiss (Axiovert 135) inverted microscope was used to view the cells. Neurons were impaled with the electrodes and the intracellular signals were amplified via a dual channel preamplifier (NeuroData, IR-283), viewed using a digital oscilliscope (Fluka 2000), and recorded on a chart recorder (Gould 2400S).

2.4.2. Detection of transmitter release

Neurotransmitter release from the soma of RPeD1 was measured electrophysiologically using an assay (sniffer) cell. In most cases, a VD4 soma was freshly isolated (section 2.3.2) and placed in the dish containing cells that had been cultured overnight. In specific experiments, either a VD2/3 or a VJ soma was used as assay cells. In experiments where the culture medium contained a drug or other substance not present in normal DM, the solution in the culture dish was replaced with saline or DM prior to addition of the assay cell. After 1 hour recovery time, the culture dish was relocated to the electrophysiology rig and the assay cell was impaled with an intracellular microelectrode (section 2.4.1). Careful manipulation of the electrode allowed the assay cell to be lifted from the surface of the dish while maintaining intracellular electrophysiological access to the cell. The assay cell was then maneuvered within close proximity of the RPeD1 somata and its ability to release transmitter was determined electrophysiologically by examining responses in the assay cell to RPeD1 stimulation. *In vivo*, RPeD1 inhibits VD4 and VJ but excites VD2/3, effects that are mimicked by exogenous dopamine application (Magoski et al., 1995).

2.5. High-performance liquid chromatography

To quantify intracellular dopamine contents of lone or soma-soma paired cultured RPeD1 somata, high-performance liquid chromatography (HPLC) protocols were used similar to those described in Magoski et al., (1995). Briefly, RPeD1 somata were cultured alone or in soma-soma pairs with VD4 for18 or 24 hours in DM (see section 2.3.2, 2.3.3). These somata were subsequently isolated with a sigmacoted glass capillary tube (section 2.3.2), placed individually in ~10 µl of pentafluoropropionic acid (PFPA: Pierce), and frozen at -70°C until analysis. Following cell culture and isolation, all ensuing HPLC protocols were conducted by L.G. Bauce. Samples were taken through 2 freeze-thaw cycles and diluted with PFPA to 100 µl in an autosampler vial. The chromatographic system consisted of a Waters HPLC pump, an ESA Coulochem guard cell at +0.75 V, a Waters intelligent sample processor autosampler, a Beckman ultrasphere 5 µm octadecyl ion-pairing column (4.6 x 45 mm) thermostated at 50°C, a graphite/cyanoacrylate electrode, and a Bio analytical systems amperometric detector set for 0.65 V. Separation in the column utilized a mobile phase consisting of (in mM) 30 trisodium citrate, 10 citric acid, 1 EDTA, 100 sodium perchlorate, and 10 sodium dodecyl sulphate at a flow rate of 2 ml/min.

2.6. Specific compounds and their application

Three different methods of drug application were used in this study. First, to test the chronic effects of specific compounds, drugs were added into the DM before cells were placed in the culture dish and left for the full length of the culture time. Second , drugs were bath perfused using a Minipuls 2 (Gilson) peristaltic pump during intracellular recording for acute effects. Finally, some drugs were applied in small puffs

directly to the soma of cultured cells using a glass pipette (tip diameter: 10-20 μm) attached to an Eppendorf microinjector (5242). To avoid physical disturbances resulting from the pressure application, the glass pipette was positioned at a distance (~200-300 μm) and angle to the cell soma.

The protein synthesis inhibitors anisomycin (Sigma A1899) and actinomycin D (Sigma A4262) were dissolved in saline to make stock solutions and added to the DM in the culture dishes to reach the desired final concentrations (12.5 μg/ml and 5.0 μg/ml respectively).

Arachidonic acid (AA: Sigma A9673), 4 bromophenacyl bromide (4-BPB: Sigma B2006), nordihydroguaiaretic acid (NDGA: Sigma N5023), and the receptor tyrosine kinase blocker lavendustin A (Sigma L2400) were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions and frozen as aliquots. Individual aliquots were thawed, diluted with DM so that the final DMSO concentration was less than 0.1% v/v, and added to the culture dishes. In a separate series of experiments, 4-BPB and NDGA were bath applied using the peristaltic pump.

The synthetic peptide phe-met-arg-phe-amide (FMRFamide: Sigma 6910) was dissolved in 50 mM acetic acid to reach a concentration of 10 mM and frozen in 50 μl aliquots. These were thawed, diluted with DM, and either added to the culture dishes or puffed directly onto the cultured cell somata in the desired concentration ranges.

The dopamine receptor blocker (±)-sulperide (RBI S-112) and the sodium channel blocker benzamil (RBI B-116) were dissolved in saline and bath applied to the cells during eletrophysiological recording.

Statistics

The statistical software program "SigmaStat for Windows" (v. 4.0, Jandel Scientific, San Rafael, CA.) was run on a personal computer and used to calculate mean±SD throughout. Differences between mean values from each experimental group were tested using either a paired or unpaired Student t-test, or a repeated measures analysis of variance (ANOVA). Data that quantified the presence or absence (incidence) of certain phenomenon were analyzed using Fisher's exact test (F.E. test) while distribution in different categories was tested by Chi-Square (χ^2) analysis. Differences were considered significant if $p < 0.05$.

Figure 2.1. The freshwater pulmonate mollusc, *Lymnaea stagnalis*, as a model system.

A) Ventral view of the intact animal.

B) Isolated central ring ganglia.

The central ring ganglia (excluding buccal ganglia) was isolated from the intact animal and pinned down on a dissection dish (dorsal view). The commissure between the right and left cerebral ganglia was cut to facilitate access to the pedal ganglia. Individual neuronal somata located within the various ganglia are discernable including those used in the present study. These include: RPeD1 = right pedal dorsal 1, VD4 = visceral dorsal 4, VD2/3 = visceral dorsal 2/3, RPD1 = right parietal dorsal1.

A

B

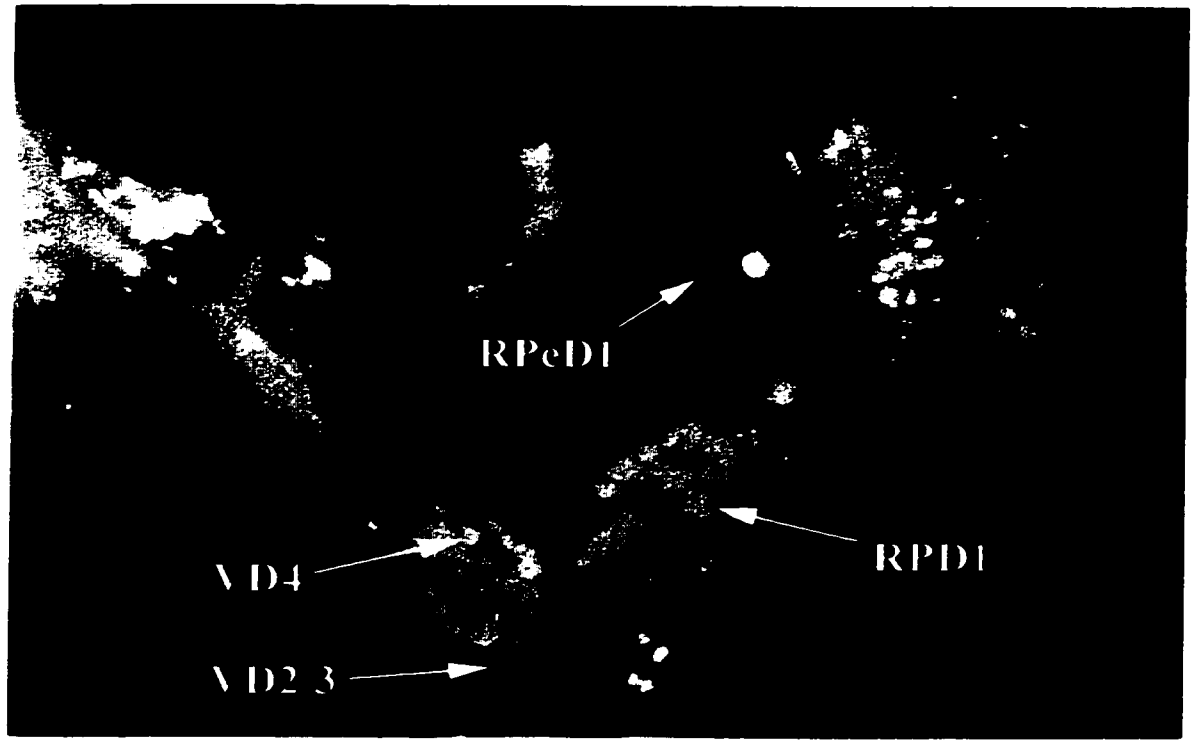
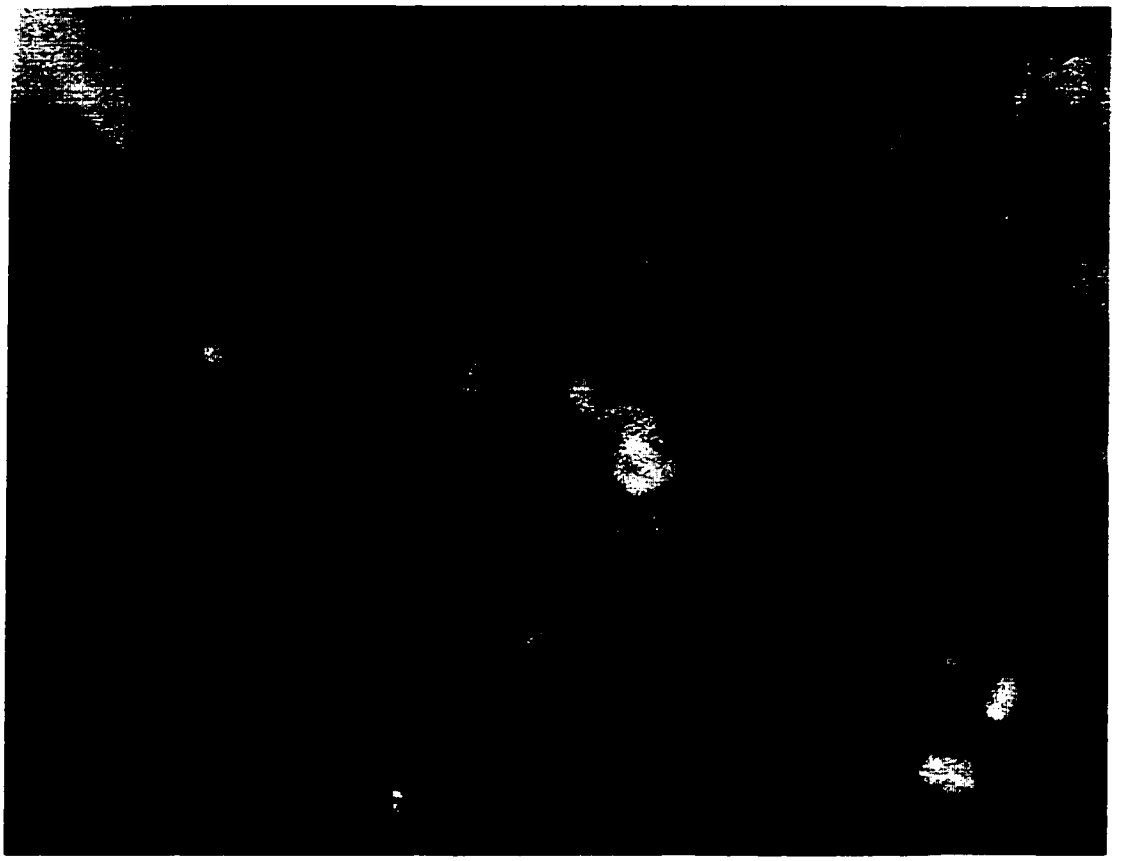


Figure 2.2. *In vitro* isolation of identified neurons.

Identified neuronal somata were extracted individually from enzyme pre-treated ganglia by applying gentle pressure through a fire polished pipette. In this photomicrograph, a glass pipette is positioned on top of a visceral dorsal 4 (VD4) soma and the arrow represents the direction of force applied during the cell extraction process. (figure modified from Syed et al., 1999).



Chapter 3: Formation of Soma-Soma Synapses Between RPeD1 and VD4

3.1 Introduction

All nervous system functions in an adult animal rely upon the precise patterns of synaptic connections that are established during early development (Goodman, 1996; Goodman and Shatz, 1993; Hall and Sanes, 1993). The precise cellular and molecular mechanisms that determine the specificity of synapse formation between potential synaptic partner cells, however, remain largely unknown. This lack of fundamental knowledge regarding synaptogenesis is due, at least in part, to the anatomical complexity of the mammalian brain. For instance, the identity of individual pre and postsynaptic partner neurons in the intact mammalian central nervous system (CNS) is virtually impossible to deduce at a single cell level. Thus, the cell-cell interactions between defined sets of synaptic partners cannot be studied reliably. Furthermore, synaptogenesis in the intact nervous system occurs in a highly complex environment, which often contains a multitude of extrinsic signaling molecules, whose precise identity and involvement in specific synapse formation is also yet to be elucidated.

In contrast to the situation in the central nervous system, a great deal is known about both intrinsic and extrinsic cell-cell signaling molecules that determine the specificity of synapse formation at the neuromuscular junction (Broadie and Bate, 1993; Ding et al., 1983; Landmesser et al., 1990; Schotzinger et al., 1994). Since synapse formation between an individual motor neuron and its corresponding muscle cell can be investigated directly both *in vivo* (Schotzinger et al., 1994) and *in vitro* (Chow and Poo, 1985; Poyer and Zoran, 1996; Stoop and Poo, 1995; Zoran et al., 1990, 1991) this model has become the standard for studies on synapse formation. Utilizing nerve-muscle

preparations from both invertebrate and vertebrate species, a variety of cell-cell signaling molecules have not only been identified, but their involvement in synapse formation has also been determined (Fitzsimonds and Poo, 1998). Since less is known about synapse formation in the intact brain, it is tempting to extrapolate studies at peripheral synapses to synaptogenesis in the nervous system. However, intricacies of synaptic connections in the intact brain warrant that the mechanisms underlying synapse formation in the nervous system may be different from those at the neuromuscular junction.

The acquisition and regulation of appropriate neurotransmitter secretory capabilities is arguably one of the most important steps that each neuron must undergo during development. This ability is not only necessary for the functioning of mature synapses, but it may also play a role in regulating many other aspects of neuronal development such as neurite outgrowth (Bulloch and Hauser, 1990; Goldberg and Kater, 1989; Goldberg et al., 1990; Hess et al., 1993; Lankford et al., 1987,1988; Lauder 1993; Lipton et al., 1988; Mattson et al., 1988; McCobb et al., 1988; Spencer et al., 1996) and specific target cell selection (Haydon et al., 1985; Spencer et al., 1998; Zheng et al., 1994). Various steps underlying the development of transmitter secretory capabilities during synapse formation between identified pre and postsynaptic partners have been investigated *in vitro* in both vertebrate (Chow and Poo, 1985; Evers et al., 1989; Stoop and Poo, 1995; Tabti and Poo, 1994; Xie and Poo, 1986) and invertebrate (Davis et al., 1998; Haydon 1988; Kuffler et al., 1987; Poyer and Zoran, 1996; Zoran et al., 1990; Zoran et al., 1996) preparations. Using a nerve-muscle preparation from the frog *Xenopus*, Mu-Ming Poo's laboratory has demonstrated a rapid up-regulation of ACh

release from motor neurons immediately after contact with appropriate (Chow and Poo, 1985; Evers et al., 1989), but not inappropriate, target muscle cells (Xie and Poo, 1986). This increase in ACh release occurred within seconds following neuron-muscle contact (Chow and Poo, 1985; Evers et al., 1989; Sun and Poo, 1987), suggesting that although neurons are endowed with release capabilities prior to muscle contacts, its modulation is target cell contact dependent. Similar results have been reported for discrete neurons from the snail *Helisoma trivolvis*; buccal neuron 5 (B5) forms chemical synapses within seconds of contact with a partner cell (Haydon and Man-Son-Hing, 1988; Haydon and Zoran, 1989; Zoran et al., 1990). The fast time course of this synaptogenesis indicates that the transmitter secretory machinery in B5 is functional prior to target cell contact (Haydon and Zoran, 1994). In contrast, another buccal neuron (B19) from the same snail requires several hours of contact by a specific target cell prior to coupling its action potentials with neurotransmitter secretion (Zoran et al., 1990, 1991). This coupling requires cell-cell signaling between the synaptic partners, though the precise identity of various molecules involved has not yet been deduced (Haydon and Zoran, 1994). Together, these studies demonstrate that both modulation and/or acquisition of presynaptic transmitter capabilities are regulated during initial stages of synapse formation, however neither the precise timing of these changes nor the signaling molecules have yet been identified.

Although transmitter secretion capabilities of neurons at identified synapses have been studied during early stages of neuromuscular development (Chow and Poo, 1985; Evers et al., 1989; Poyer and Zoran, 1996; Xie and Poo, 1986; Zoran et al., 1990, 1991; Zoran et al., 1996) and at a novel (synapse does not exist *in vivo*) neuron-neuron synapse

in *Helisoma* (Haydon 1988), relatively little is known about the cellular mechanisms by which neurotransmitter release is organized at central synapses. A notable exception are studies in the leech where synapse formation between pressure sensitive and Reitzus neurons has been investigated *in vitro* (Catarsi and Drapeau, 1993; Drapeau, 1990; Drapeau and Sanchez-Armass, 1988). These studies, however, were primarily focused on the postsynaptic cell and presynaptic changes during synapse formation were not closely investigated. To date, no studies have specifically examined the mechanisms by which secretory machinery of mutually connected neurons is regulated during synapse formation.

One of the main objectives of this study, therefore, was to examine how transmitter release capabilities of an identified *Lymnaea* neuron are regulated during synaptogenesis. Specifically, I took advantage of identified neurons right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4) which are both synaptically and functionally well characterized, and comprise the neural network that underlies the respiratory central pattern generator (CPG) (Syed and Winlow, 1991). In culture, these cells reliably reform appropriate inhibitory connections with each other (Syed et al., 1990) that are electrophysiologically similar to those seen *in vivo* (Syed and Winlow, 1991). Since synapses between the neurites of *in vitro* sprouted neurons are located at some distance from the soma, and thus not amenable for direct cellular analysis, a soma-soma synapse preparation for these neurons has also been developed (Feng et al., 1997). This preparation enables direct electrophysiological access to the synaptic site and provides experimental control over the timing of cell-cell contact; two factors that are crucial for analysis of synapse formation but cannot be controlled in neurite-neurite synaptic

cultures. This study investigated sequential changes that occurred during the formation of an appropriate, mutually inhibitory synapse between two identified neurons from the central ring ganglia. Moreover, as RPeD1 is known to contain and release dopamine as its sole transmitter, the present study focused on testing the transmitter release capabilities of this cell to determine the mechanisms by which neurosecretion is modulated during early synaptogenesis.

3.2 Results

3.2.1 RPeD1 and VD4 formed a mutually inhibitory synapse in the soma-soma configuration

To establish that RPeD1 and VD4 can form appropriate chemical synapses *in vitro*, these neurons were individually extracted from the intact ganglia and juxtaposed in a culture dish containing defined media (DM). After 24 hours in culture, simultaneous intracellular recordings were made from the paired cells and chemical synapses were tested electrophysiologically. Out of 43 soma-soma paired RPeD1/VD4 cells, 21 established a mutually inhibitory synapse. Specifically, electrically induced action potentials in RPeD1 not only inhibited the spiking activity in VD4 (fig. 3.1B) but they also produced 1:1 inhibitory postsynaptic potentials (IPSPs) (fig. 3.1D). Similarly, induced action potentials in VD4 prevented spontaneous spiking activity in RPeD1 and also produced 1:1 IPSPs (fig. 3.1C,E). This reciprocal inhibitory synapse formed between the somata after 24 hours of pairing was similar to that reported earlier by Feng et al. (1997) and established further the usefulness of soma-soma model for future studies on chemical synapse formation.

3.2.2 VD4 was first to establish inhibitory synaptic communication in the soma-soma configuration

To determine the time course of synaptogenesis between soma-soma paired VD4 and RPeD1 cells, neurons were paired *in vitro* and simultaneous intracellular recordings were made after 18 to 24 hours of cell culture. After 18 hours, 73% (n=30) of soma-soma paired cells exhibited a one-way inhibitory synapse between VD4 and RPeD1 (fig. 3.2A,B); whereas only 17% displayed a mutually inhibitory synapse. In other words, evoked action potentials in VD4 produced 1:1 IPSPs in RPeD1 but not vice-versa. In comparison, when left for 24 hours in cell culture, 21 out of 37 pairs formed a mutually inhibitory synapse while 11 pairs remained one way, i.e., VD4• RPeD1. Between 18 and 24 hours, the incidence of mutual synaptic inhibition increased significantly (• $^{2}(2)$ = 16.286, $P < 0.001$) with inhibitory connections from RPeD1 to VD4 formed in 57% of the 24 hour pairs (see figure 3.2C). It is important to note that a one-way inhibitory synapse was observed exclusively from VD4 to RPeD1. Synaptic communication from RPeD1 to VD4 appeared only in those instances where a mutually inhibitory synapse had formed between the paired cells. Together, this data demonstrates that VD4 is first to establish synaptic communication while RPeD1 only becomes competent for synaptic transmission at a later time window.

An additional trend noted in the progression from 18 to 24 hours was an increased incidence of electrical coupling between the soma-soma paired cells. In 6 pairs left for 24 hours, hyperpolarizing current injected into either of the cells elicited a similar hyperpolarization in the corresponding partner cell. A similar degree of electrical coupling was not seen at 18 hours. Soma-soma paired cells display an increased level of

somatic contact over time (Syed, pers. comm.) and this likely accounts for the increase in detectable electrical coupling between the partner cells.

3.2.3 Transmitter release from RPeD1 was suppressed by VD4 during early synaptogenesis

The absence of an electrophysiological response in VD4 to induced action potentials in RPeD1 after 18 hours in the soma-soma configuration is likely due either to one or both of the following possibilities: 1) VD4 does not express appropriate dopamine receptors on its soma surface membrane at early time points during synapse formation or 2) RPeD1 is unable to release neurotransmitter during this time window. The presence of appropriate receptors on the surface of the VD4 soma at 18 hours was demonstrated electrophysiologically using a biological source to apply exogenous transmitter. Isolated somata of RPeD1 cultured alone for 24 hours were impaled with an intracellular electrode and maneuvered in close proximity to the VD4 soma which had established a one-way inhibitory synapse with its paired partner RPeD1 (fig. 3.3A). Care was taken to ensure that no physical contact occurred between the lone RPeD1 and the paired VD4 soma. In all instances, depolarization induced action potentials in the lone RPeD1 caused a delayed inhibition of spiking activity in VD4 (n=6: fig. 3.3B). This data demonstrates that: 1) the isolated somata of a lone RPeD1 cell is competent to release transmitter in culture prior to contact with a prospective target cell and 2) a paired VD4 soma, in a one way synapse with RPeD1, posseses functional receptors for the transmitter released by a lone RPeD1.

To test whether the absence of synaptic transmission from RPeD1 to VD4 (after 18 hours in culture) could be attributed to the failure of paired RPeD1 somata to release transmitter, an *in vitro* assay system was first developed to detect dopamine release from these cells. RPeD1 neurons were cultured individually overnight and freshly isolated VD4 somata were used to detect evoked transmitter release. Specifically, these freshly isolated VD4 somata were placed in the culture dish containing the cultured giant dopamine cells, impaled with sharp intracellular electrodes, and maneuvered within close proximity to the cultured RPeD1 somata. Simultaneous intracellular recordings from both the assay cell and the RPeD1 soma, and subsequent RPeD1 stimulation consistently generated a delayed inhibitory response in the assay VD4 (fig. 3.4A: n=29). This positive detection demonstrates that the utilization of assay cells in this manner provided a reliable method to detect evoked transmitter release from RPeD1 somata and thus justifies their usage in further experiments. To examine the time course of acquisition of transmitter release capabilities *in vitro*, RPeD1 were isolated and assayed for this ability at various time points using the above mentioned method. Following 2-3 hours in culture, only 20% (n=1 of 5) of the RPeD1s were capable of evoked transmitter release. The incidence of transmitter release from RPeD1 after 5-6 hours in culture was also low (14%: n=1 of 7: P=1.000 F.E. test). After 12-13 hours in these conditions, the incidence of evoked transmitter release rose to 71% (n=5 of 7), however, this increase in the incidence of transmitter release was not significantly different from cells plated for 2-3 hours (P=0.242 F.E. test). The incidence of evoked transmitter release from cells that were left either for 18-19 hours or 23-24 hours were essentially identical (92%: n=12 of 13, and 89%: n=17 of 19 respectively). These values however, differed significantly

from those cells that were tested at 2-3 hours (P=0.008 and P=0.006 respectively, see figure 3.4B). These data demonstrate that RPeD1 cultured alone for greater than 12 hours are capable of evoked transmitter release that can be detected reliably by using a freshly isolated assay cell.

To test the hypothesis that RPeD1 somata in an 18 hour, 1 way synapse with VD4 are unable to release transmitter, a similar "sniffer" cell strategy was used. Specifically, RPeD1 was soma-soma paired with VD4 and left in culture for 18 hours. Subsequent electrophysiological analysis revealed a one-way (VD4• RPeD1) inhibitory synapse between the paired cells. A freshly isolated VD4 assay cell was then maneuvered in close proximity to the paired RPeD1 (fig. 3.5A) and the intracellular activity from both cells was recorded via sharp electrodes. After 18 hrs of soma-soma pairing with VD4, RPeD1 failed to release detectable amounts of transmitter following electrical stimulation (n=0 of 10: fig. 3.5B). This data is consistent with the hypothesis that soma-soma pairing with VD4 suppresses transmitter release from RPeD1 during early synapse formation.

Since mutual inhibitory synapses between RPeD1 and VD4 were detected after 24 hours of cell pairing, these data suggested that the suppression of transmitter release from RPeD1 was transient. To test this possibility, freshly isolated assay cells were positioned in close proximity to RPeD1 somata in 24 hour, mutually inhibitory synapses with VD4. In all soma-soma pairs that exhibited mutually inhibitory synapses (n=7), non-synaptic transmitter release from RPeD1 was detected by the assay cell. The incidence of detectable transmitter release from lone and paired dopamine cells during the 18 and 24 hour time windows is represented graphically in figure 3.6. The incidence of transmitter

release from lone RPeD1 somata at both 18 and 24 hours were identical (P=1.000 F.E. test). Soma-soma paired RPeD1 somata, however, display a significant increase in the incidence of evoked transmitter release when monitored at 18 hour and 24 hour (P<0.001). Together, these data demonstrate that the target cell induced suppression of transmitter release from the RPeD1 somata is indeed transient.

3.2.4 RPeD1 contains and releases dopamine

To rule out the possibility that the absence of transmitter release from a paired (18 hr) RPeD1 involved suppression of transmitter (dopamine) synthesis, high-performance liquid chromatography (HPLC), pharmacology, and electrophysiological assay techniques were used. Specifically, RPeD1 has previously been shown to contain dopamine (Ausdirk 1985; Cottrell et al., 1979; Elekes et al., 1991; Werkman et al., 1991) and its synaptic transmission with a large number of follower cells is dopaminergic (Magoski et al., 1995). Both single and paired RPeD1 cells were removed from the culture dish and their dopamine contents were analyzed by HPLC. Lone RPeD1 somata cultured for 18 hours were found to contain 0.70 ± 0.09 pmol dopamine /cell (n=4). It is important to note that neither serotonin, octopamine, nor norepinephrin were detected in appreciable quantities in this cell. Similar levels of dopamine were detected in RPeD1 somata that were paired with VD4 for 18 hr or 24 hrs (Table 3.1). No significant difference between any of these groups was revealed using an unpaired student's t-test (P>0.05). This data demonstrates that dopamine levels in lone RPeD1 somata are identical to that of the paired cells; hence RPeD1's inability to release transmitter at 24 hr (at present) cannot be attributed to the lack of dopamine contents.

Molluscan neuropharmacology in general suffers from the lack of appropriate tools that are available in their vertebrate counterparts. However, in *Lymnaea*, one of the most effective pharmacological blockers of DA receptors is the D-2 DA receptor antagonist (±)-sulperide (Magoski et al., 1995; Werkman et al., 1987). This compound has been shown to block the effect of both acutely applied DA and postsynaptic responses in RPeD1's target cells *in vivo* and *in vitro* (Magoski et al., 1995). In this study, (±)-sulperide was tested for its ability to block non-synaptic responses in assay cells to RPeD1 stimulation. As shown in the representative electrophysiological traces (figure 3.7), prior to (±)-sulperide application, induced action potentials in a lone RPeD1 caused a slightly delayed inhibition of spiking activity (fig. 3.7A) and membrane hyperpolarization (fig. 3.7B) in a freshly isolated assay cell. These RPeD1-induced non-synaptic responses in the sniffer cell were blocked in the presence of 50 μM (±)-sulperide (bath applied: fig. 3.7C,D). This data demonstrates that RPeD1-induced non-synaptic responses in the assay cell most likely involved dopamine release from RPeD1.

To further rule out the possibility that VD4 contact may have altered the transmitter phenotype of a paired RPeD1, various other target cells were used as transmitter release assay cells. Specifically, three different neurons, each of which responds in a characteristic way to both RPeD1 stimulation *in vivo* and exogenous DA, were used to detect transmitter release from the giant dopamine cell. VD4, the assay cell used for the majority of the experiments in this thesis, and visceral J (VJ) are inhibited *in vivo* by RPeD1, an effect that can be mimicked by exogenous dopamine application (Magoski et al., 1995). Visceral dorsal 2 (VD2), on the other hand, is excited by RPeD1 and exogenous dopamine application both *in vivo* and in cell culture. Moreover,

dopamine-induced effects on VD2 are also blocked by the dopamine receptor antagonist (Magoski et al., 1995). A representative example of electrophysiological responses recorded from these cells in response to the stimulation of a lone RPeD1 is shown in figure 3.8. Induced action potentials in RPeD1 inhibited spiking activity in VD4 (n=29: fig. 3.8A) and VJ (n=5: fig. 3.8B), whereas VD2 was excited following RPeD1 stimulation (n=5: fig. 3.8C). Taken together, these data demonstrate that both excitatory and inhibitory effects of RPeD1 stimulation on its target cells involve a single transmitter and suggests that VD4 -induced suppression of transmitter release from RPeD1 does not involve a switch in transmitter phenotype.

3.3 Discussion

Large, individually identified neurons in an assortment of invertebrate species offer unique advantages as compared with their vertebrate counterparts. One of the most striking feature that makes various invertebrate preparations better suited for studies on synapse formation is the ability of their adult neurons to regenerate axonal processes and to re-establish specific synapses both *in vivo* (Syed et al., 1992) and *in vitro* (Bodmer et al., 1984; Bulloch and Syed, 1992; Camardo et al., 1983; Fuchs et al., 1981; Hadley et al., 1983; Haydon 1988; Henderson 1983). Most pertinent to this study is the soma-soma synapse preparation, originally developed for leech neurons (Fuchs et al., 1982) and subsequently adopted in *Helisoma* (Haydon 1988), *Aplysia* (Klein 1994) and *Lymnaea* (Feng et al., 1997). In previous preparations, however, isolated neurons were maintained apart in culture for extended periods of time, then placed together in non-adhesive culture conditions, and subsequently plated in their final soma-soma configuration on adhesive culture dishes (Haydon, 1988). This complex protocol prohibits electrophysiological

analysis of cell-cell signaling mechanisms and temporal sequencing of events that occur during early stages of synapse formation. The *Lymnaea* soma-soma preparation utilized in the present study is the only one in which neurons are paired immediately following isolation from the CNS, thus, various time sensitive cellular events underlying synapse formation could be investigated directly. For example, if the *Helisoma* protocol (Haydon, 1988) was used in the present study, the interaction between the two cells which occurred between 12 and 24 hours of membrane contact could not have been examined electrophysiologically, thus, the VD4-induced suppression of transmitter release phenomenon would have been missed entirely.

A variety of developing/regenerating neurons have been shown to release transmitter prior to target cell contact and synapse formation (Spencer et al., 1998; Young and Poo, 1983). Coupled with the evidence that some transmitters and their receptors are expressed transiently during development (Daval et al., 1987; Lankford et al., 1987), these studies led to the idea that in addition to their conventional involvement in trans-synaptic communication, neurotransmitters may also participate in neurodevelopment (Haydon et al., 1984; Lauder 1988; Lauder, 1993). Further studies indicated that this may indeed by the case since perturbations of endogenous neurotransmitters during embryonic development cause both morphological (Shuey et al., 1992; Tennyson et al., 1983; Voronezhskaya, 1990; Voronezhskaya et al., 1992) and synaptic abnormalities (Goldberg and Kater, 1989). Data from a number of laboratories now support the proposition that transmitter release prior to target cell contact is involved in axonal pathfinding and target cell selection (Haydon et al., 1984, Lauder, 1993; Song et al., 1997; Spencer et al., 1998; Zheng et al., 1994). In Lymnaea, RPeD1 growth cones were

shown to contain (Magoski et al., 1995) and release (Spencer et al., 1998) dopamine prior to contact with target cells, suggesting that the transmitter-receptor interactions may help determine target cell selection. The data presented in the present study showed that RPeD1 soma are also capable of transmitter release. This somatic release was detected only after several hours of neuronal culture, possibly suggesting that transmitter secretion from RPeD1 *in vivo* occurs solely at specific synaptic sites and that the cell body becomes competent to release transmitter only after its synaptic contacts are severed. This notion does not conform to a general conclusion drawn from studies on the mollusc, *Planorbis corneus*, where transmitter release was reliably detected *in vivo* from the giant dopamine cell soma (Chen et al., 1995). RPeD1's inability to release transmitter within a few hours of its isolation could also be attributed to physical damage that occurred during CNS removal from the intact animals. Previous studies examining non-synaptic transmitter release prior to target cell contact *in vitro* (Chow and Poo, 1985; Spencer et al., 1998) utilized neurons which were maintained for a number of days in culture. Further experiments are required to test whether RPeD1 soma in the intact *Lymnaea* CNS are indeed capable of transmitter release or if this phenomenon occurs only after cells are removed from the central ring ganglia.

From the above studies, the importance of non-synaptic neurotransmitter release in neurite outgrowth and synapse formation is apparent, however its direct involvement in synapse formation has not yet been demonstrated. Additionally, it is interesting that non-synaptic release from the VD4somata prior to contact with the dopamine cell was never detected. VD4 is a peptidergic neuron that is thought to release FMRFamide-like peptides onto postsynaptic targets (McKenney, 1992; Santama et al., 1995). Although

somatic release of peptides has been reported *in vivo* for the molluscs (*Helix*: Darbon et al., 1996), exocytotic release of peptides from growth cones prior to target cell contact has not been described. This may be due to the relative increase in energy expenditure necessary to synthesize peptides, as compared to classical neurotransmitters, and thus preclude non-synaptic release and involvement of these molecules in developmental roles that have been ascribed to their classical counterparts. Moreover, the absence of transmitter release from VD4 prior to target cell contact is reminiscent of the synaptic strategy seen in *Helisoma* neurons B5 and B19. Neuron B5 is capable of transmitter release prior to cell contact and forms indiscriminate connections with a variety of cells that are refined at a later time point. On the other hand, B19 releases transmitter only after contact with a discrete set of target neurons (Haydon and Zoran, 1994; Zoran et al., 1990, 1991). This represents an underlying difference in developmental synaptic programming, a situation that is likely occurring between the two *Lymnaea* neurons used in the present study. Furthermore, the difference in somatic transmitter release capabilities *in vitro* between RPeD1 and VD4 suggests that synaptic and non-synaptic release mechanisms may be fundamentally different.

Since transmitter release from a single RPeD1 neuron was detected in all instances, it can be argued that the so-called "synapses" between soma-soma paired cells may be artificial. Although Feng et al. (1997) have provided conclusive morphological and electrophysiological evidence validating that synapses between somata are identical to those seen *in vivo*, this issue still requires additional clarification. For the purpose of this discussion, I will refer to transmitter release as "non-synaptic" if it occurred from the soma prior to target cell contact or from areas of the soma other than the target cell

contact site, whereas "synaptic" release will imply exocytotic discharge that occurs directly at the target cell contact site. Prior to target cell contact, RPeD1 was found to release transmitter non-synaptically from all area of its soma. A similar non-synaptic, somatic release of transmitter has also been well documented in both vertebrate (Chow and Poo, 1985; Huang and Neher, 1996; Jaffe et al., 1998; Johnson and Pilar, 1980; Zaidi and Matthews, 1997) and invertebrate (Anderson et al., 1998; Chen et al., 1996; Darbon et al., 1996) preparations. Non-synaptic, somatic release is characterized by slower release kinetics that exhibit a greater sensitivity to Ca^{2+} (Huang and Neher, 1996) than synaptic release, possibly signifying that the somatic secretory machinery is not physically linked in close proximity to the site of Ca^{2+} influx (Huang and Neher, 1996). Further, Zaidi and Matthews (1997) have demonstrated that non-synaptic, somatic release may be differently regulated than that of its synaptic counterpart. This differential regulation of synaptic versus non-synaptic transmitter release can also be inferred from previous work with the *Lymnaea* soma-soma model. Feng et al. (1997) showed that when placed in a 3 cell soma-soma-soma configuration with both a target and non-target neuron, RPeD1 released transmitter only at the contact site with the appropriate target and not from any other areas on the soma. In other words, contact with the incorrect target cell suppressed non-synaptic somatic release without preventing discrete synaptic release at the correct target cell contact site. For an earlier example, Chow and Poo (1985) detected non-synaptic release from both the soma and the tip of an extending *Xenopus* motor neuron neurite *in vitro*. After the growth cone had contacted an appropriate target muscle however, a reduction in the amount of non-synaptic transmitter released was detected at the soma (Chow and Poo, 1985). This reduction in non-synaptic

release was postulated to represent either a redistribution of transmitter synthesis/packaging/secretory machinery within the neuronal cytoplasm and/or a differentiation of the neuronal plasma membrane (Chow and Poo, 1985). A similar mechanism can account for the transient suppression of dopamine release observed in the present study. For instance, VD4 contact may induce a switch in transmitter secretory programs in RPeD1, resulting in a transient down-regulation of non-synaptic release in favor of specific synaptic release. During this phase, evoked action potentials would become uncoupled from the secretory machinery, thus rendering the neuron incapable of transmitter release. Following the formation of a mature, mutually inhibitory synapse, the soma may regain its ability to release transmitter non-synaptically, similar to that recorded *in vivo* from the giant dopamine cell in the snail, *Planorbis corneus* (Anderson et al., 1998; Chen et al., 1996). These possibilities however remain to be tested.

An additional attribute of the *Lymnaea* model is that since synapses between RPeD1 and VD4 are mutually inhibitory, the cellular and molecular mechanisms underlying synapse formation between reciprocally connected neurons can be investigated directly. For instance, by studying the RPeD1 and VD4 pair, one may gain insight into the cellular and molecular mechanisms by which synaptic machinery of reciprocally interconnected neurons is organized during synapse formation. In the context of this cell pair, both neurons must not only arrange their own transmitter secretory machinery, as a developing presynaptic partner, but they must also be concurrently engaged in the organization of their postsynaptic sites. This fundamental difference between *Lymnaea* model system and neuromuscular junction adds additional complexity to the developing synapse, nevertheless, it may be more representative of synaptogenesis

that goes on in a developing brain. Examples of such mutual inhibitory synapses include central pattern generators, such as those described for swimming in the mollusc *Clione* (Norekian and Satterlie, 1993), for controlling stomach muscles in crustaceans (Dickinson and Marder, 1989), or for aerial respiration in *Lymnaea* (Syed and Winlow, 1991). Furthermore, recent evidence suggests that certain axo-dendritic synapses in the mammalian CNS have a degree of "bi-directionality": GABA released exocytotically from the dendrite signals through receptors on the terminal bouton of the presynaptic cell (Zilberter et al., 1999). Studies on *Lymnaea* neurons will thus provide further insights into mechanisms by which mutual inhibitory synapses are put together during development of the central nervous system.

Studies on both vertebrate and invertebrate preparations have shown that cell-cell contact between pre and postsynaptic neurons bring about specific changes in the secretory machinery of their corresponding partner cells. Most information on transmitter releasing capabilities of a presynaptic neuron before and after its synaptic contact with a postsynaptic cell comes from studies on motor neuron connections with their target muscle cells. In both vertebrate (Chow and Poo, 1985; Evers et al., 1989; Tabti and Poo, 1994; Xie and Poo, 1986) and invertebrate (Poyer and Zoran, 1996; Zoran et al., 1990; Zoran et al., 1996) preparations, transmitter release capabilities of the presynaptic neuron have been shown to be immediately up-regulated following contact with a target muscle cell. Similarly, Haydon (1988) demonstrated that a postsynaptic neuronal contact enhances the neurotransmitter release capabilities of a presynaptic *Helisoma* neuron. Taken together, these data from various preparations show a general trend: target cell contact enhances transmitter release properties of its presynaptic neuron.

In the present study, however the transmitter release properties of RPeD1 were found to be transiently suppressed during early synaptogenesis. This data is thus in complete contrast to those obtained in other species. Although the reason for this apparent discrepancy between *Lymnaea* and other models is unclear, it is tempting to speculate that synapse formation between reciprocally connected neurons may follow a trend that is different for those of uni-directional synapses. In our RPeD1 and VD4 model, each neuron would have to acquire not only its pre but also a postsynaptic status, therefore, it is likely that these events will progress through sequentially discrete and more regulated phases than that of unidirectional synapses. Furthermore, it is interesting to note that all the investigations concerning regulation of secretory machinery mentioned above dealt with neuromuscular/excitatory synapses and often display a degree of reliance on electrical activity (Poyer and Zoran, 1996). In the current study, all cells were cultured in a defined media that lacks trophic factors. Under these conditions both RPeD1 and VD4 show little spontaneous activity (Bjorgum, pers. comm.) and it is thus unlikely that activity-dependent mechanisms play any role suppression of DA release from the giant dopamine cell.

For proper wiring of mutually interconnected networks, the precise timing of synapse formation between synaptic partners must be tightly regulated either through extrinsic or intrinsic cellular signaling. For instance, rather then having all synaptic connections form simultaneously upon contact, the synaptic partners must influence their corresponding target such that each pair will first acquire either its pre or postsynaptic status. Having completed one half of their synaptic program, the neurons must then change their role in this synaptic partnership. From data presented in this chapter I

propose that VD4-induced suppression of transmitter release from RPeD1 may be a mechanism that allows synaptogenesis between these partners to follow such a regulated differential time course. Since this study was carried out *in vitro*, in a relatively simple culture media containing no extrinsic trophic molecules, the VD4-induced transmitter suppression in RPeD1 can only be attributed to intercellular signaling between the two cells.

Previous studies have demonstrated that the postsynaptic target cell may change the transmitter phenotype of its corresponding presynaptic neuron (Schotzinger et al., 1994). A possible mechanism to account for the loss of detectable transmitter release from RPeD1 may therefore be VD4-induced switch in its transmitter phenotype. Three lines of evidence presented in this study do not support this postulate. First, HPLC measurement detected no appreciable changes in dopamine levels at various stages of synapse formation between RPeD1 and VD4. Second, a selective dopamine receptor blocker, (±) -sulperide, always blocked RPeD1-induced responses in the assay cells. Finally, three different cell types each responded to the transmitter released from a lone RPeD1 in a manner reminiscent of their characteristic responses to exogenous dopamine.

Taken together, the data in this chapter demonstrate that synapse formation between RPeD1 and VD4 follows a well-organized pattern of events that regulate mutual inhibitory synapses between the soma-soma paired cells. The developing synapse is initially dominated by VD4 which consistently forms an inhibitory connection onto RPeD1 prior to the commencement of reciprocal transmission. During this transient, 1-way phase, RPeD1 is unable to release transmitter. Since these experiments were

performed in the absence of extrinsic trophic/signaling molecules, all necessary signals coordinating these steps must therefore be inherent to the cells themselves.

Figure 3.1. RPeD1 and VD4 formed a mutually inhibitory synapse in the soma-soma configuration.

RPeD1 and VD4 were cultured in the soma-soma configuration (**A**: white scale bar = 100 μm) in DM for 24 hours. Simultaneous intracellular recording revealed a mutually inhibitory synapse between the somata. Specifically, electrical stimulation (black bars under traces) of either RPeD1 (**B,D**) or VD4 (**C,E**) inhibited spontaneous action potentials in the partner cell (**B,C**) or produced unitary 1:1 inhibitory postsynaptic potentials(**D,E**).

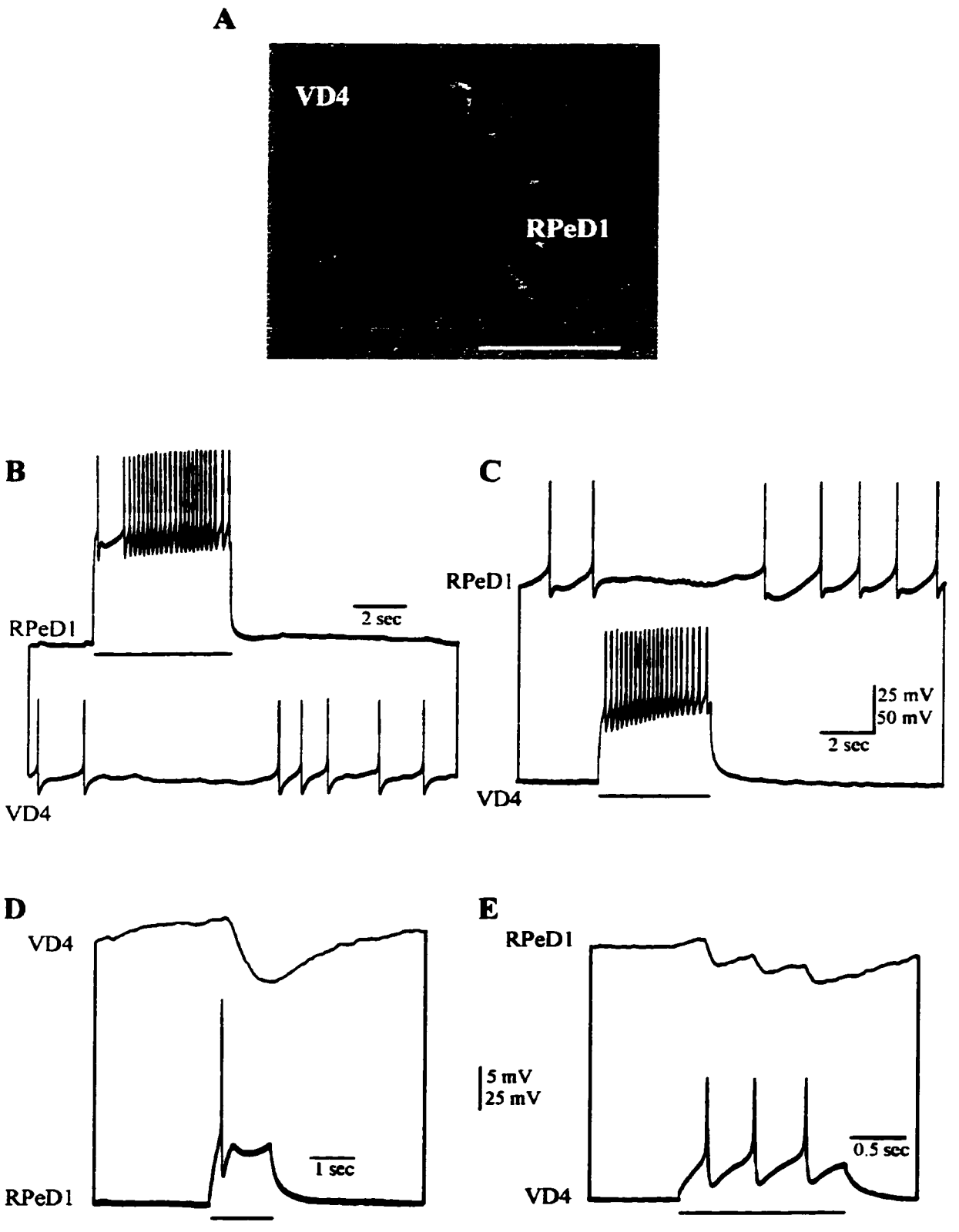
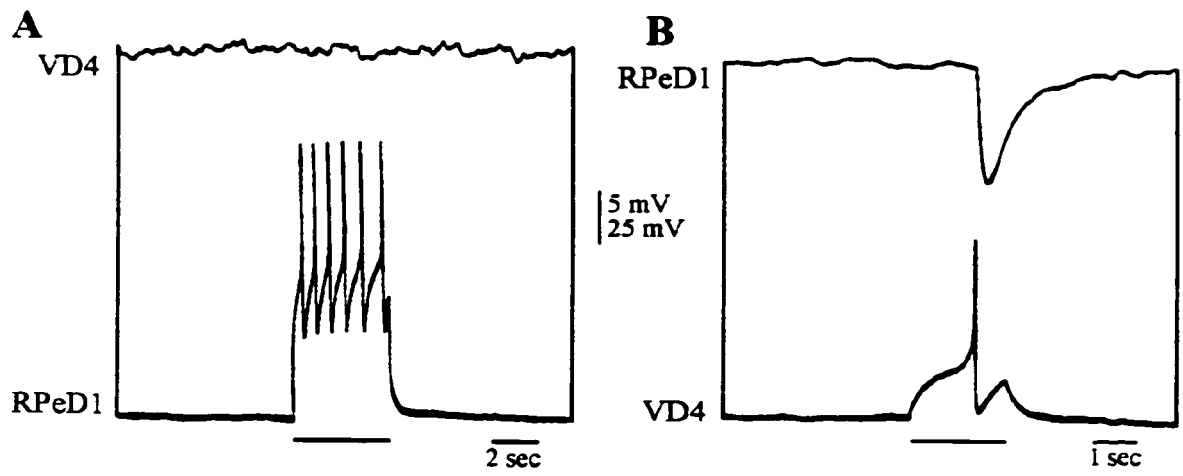

A
VD4
RPeD1
B
RPeD1
2 sec
VD4
C
RPeD1
25 mV
50 mV
2 sec
VD4
D
VD4
1 sec
RPeD1
E
RPeD1
5 mV
25 mV
0.5 sec
VD4

Figure 3.2. VD4 was first to establish a synaptic contact with RPeD1.

RPeD1 and VD4 were placed in the soma-soma configuration in DM and left for 18 hours. Simultaneous intracellular recordings were made from both cells and these revealed an inhibitory synaptic connection from VD4 to RPeD1 but not vice versa. Specifically, evoked action potentials in RPeD1 did not induce an electrophysiologically detectable response in VD4 (**A**), whereas action potentials in VD4 produced 1:1 inhibitory postsynaptic potentials in the RPeD1 soma (**B**). **C.** The percentage of cell pairs that either formed 1 way (VD4• RPeD1), mutually inhibitory, or no chemical synapse after 18 or 24 hours. It is important to note that in no case was a 1-way synapse detected between RPeD1 and VD4 (RPeD1• VD4) at 18 or 24 hours.


A
VD4
RPeD1
2 sec
B
RPeD1
5 mV
25 mV
VD4
1 sec

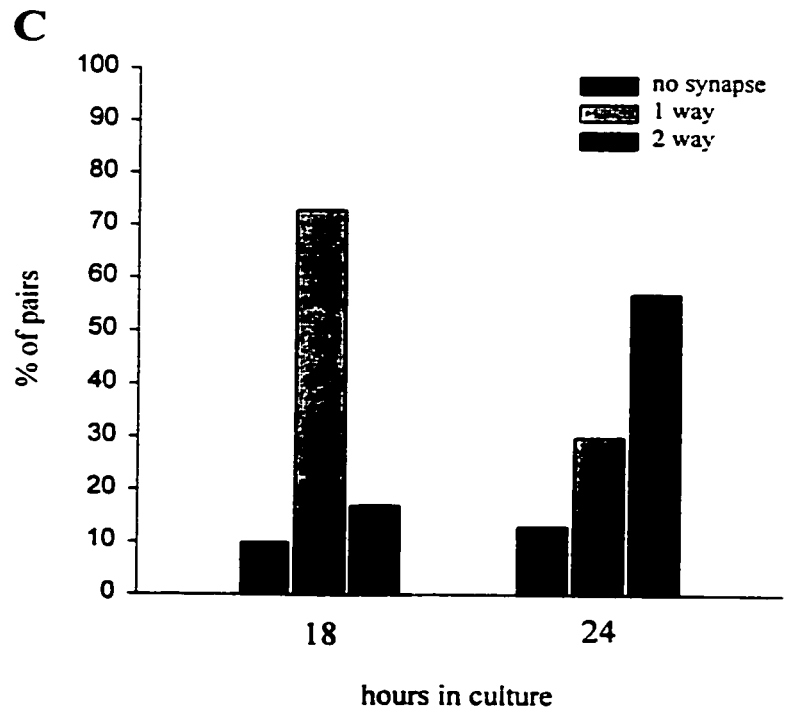
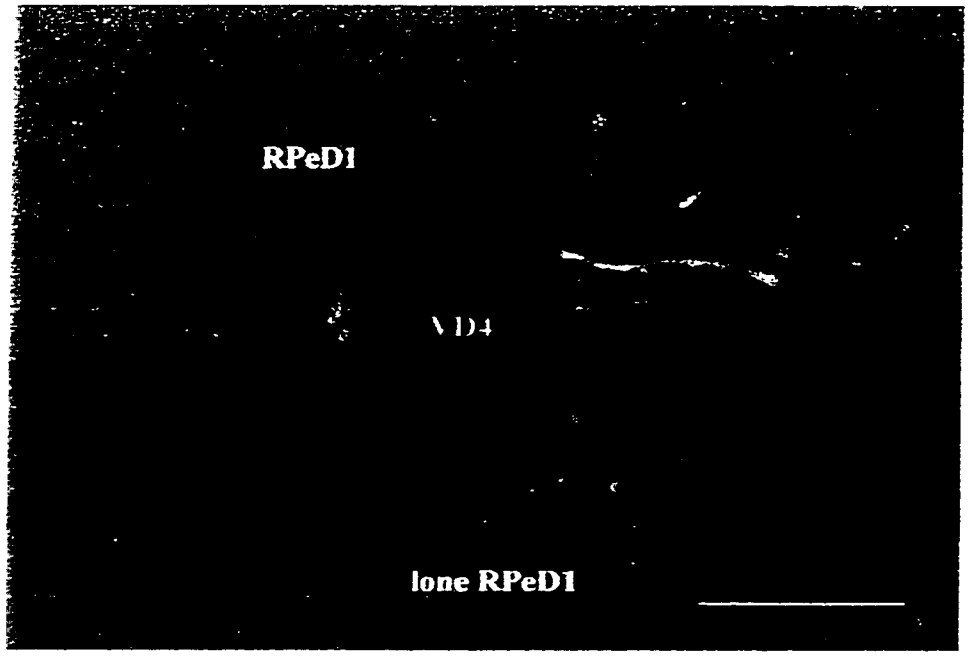

C
100
90
80
70
60
50
40
30
20
10
0
% of pairs
no synapse
1 way
2 way
18
24
hours in culture

Figure 3.3. VD4 in a 1 way inhibitory synapse with RPeD1 exhibited functional dopamine receptors.

Isolated soma of RPeD1 and VD4 were plated in a soma-soma configuration for 18 hours. Following electrophysiological characterization of a 1-way synapse between (VD4• RPeD1), a RPeD1 soma cultured alone in the same dish (> 500 μm away) was impaled and maneuvered within a close proximity (~ 10-20 μm) of VD4 soma (**A**: scale bar = 100 μm). Electrical stimulation of the lone RPeD1 soma (black bar) consistently inhibited the firing of spontaneous action potentials in the paired VD4 (**B**).

A



B

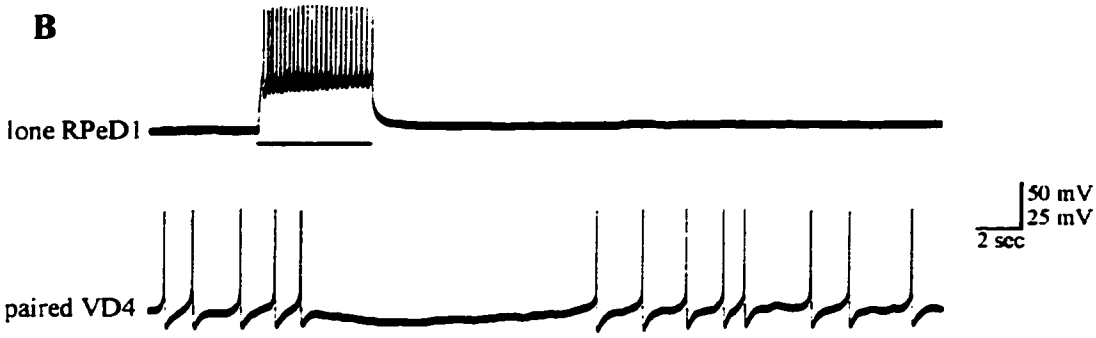
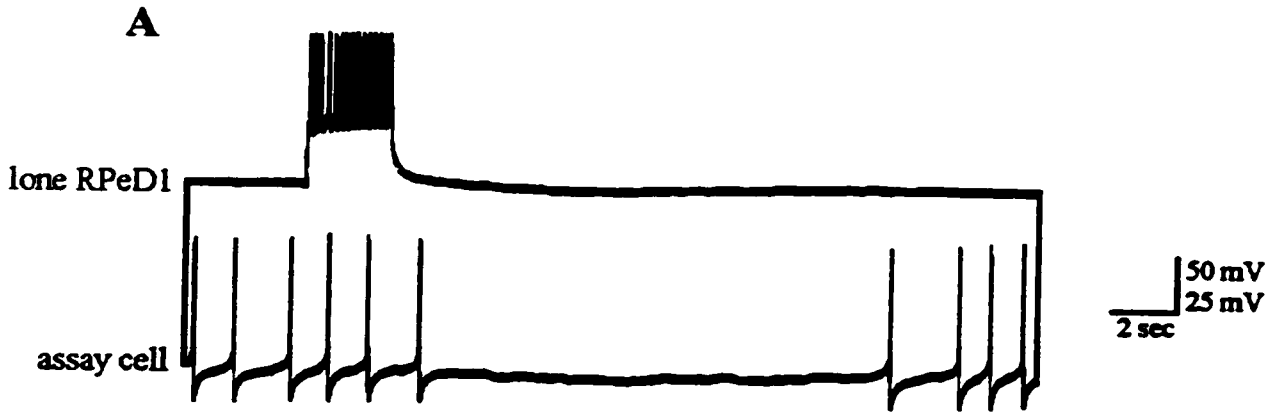


Figure 3.4. Freshly isolated VD4 somata (assay cell) reliably detected evoked transmitter release from a lone RPeD1.

Dopamine responsive, assay cell (VD4) was freshly isolated and placed in a cell culture dish containing lone RPeD1 soma. The assay cell was impaled with intracellular electrodes and maneuvered within close proximity, but not touching, the cultured RPeD1 somata. **A.** Evoked action potentials in RPeD1 (24 hours) inhibited the firing of spontaneous action potentials in the assay cell. The incidence of detectable transmitter release from a lone RPeD1 was initially low but increased dramatically after 12 hours in culture(**B**).


A
lone RPeD1
assay cell
50 mV
25 mV
2 sec

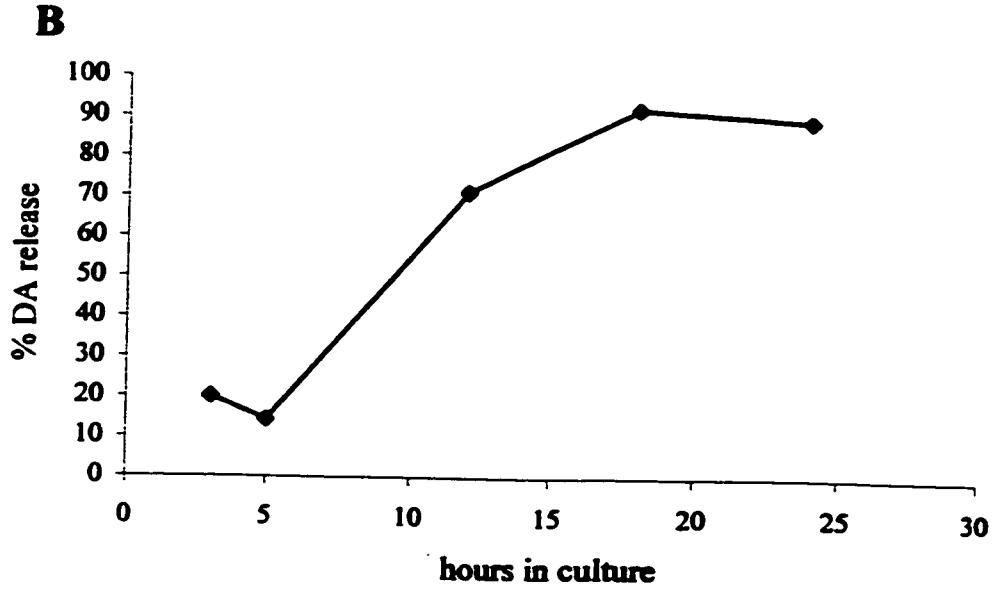
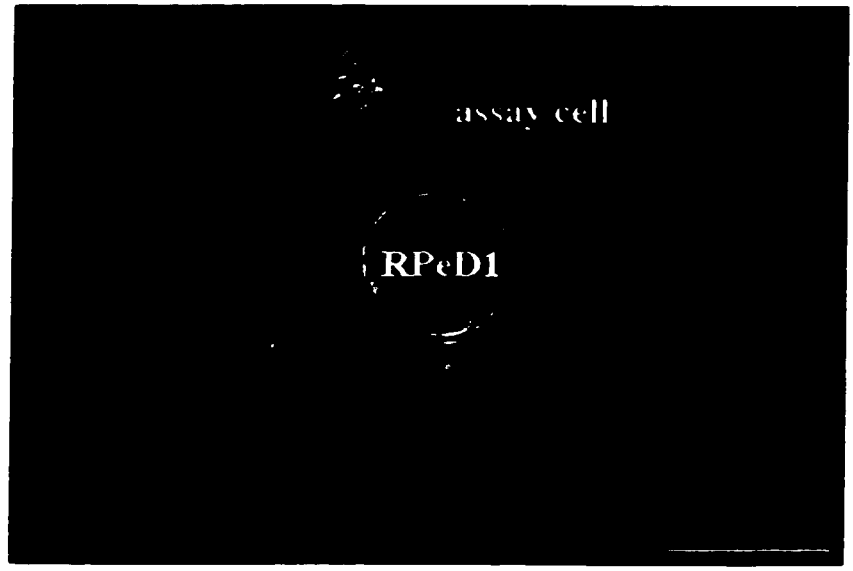

B
% DA release
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
hours in culture

Figure 3.5. RPeD1 somata in a 1-way synapse with VD4 did not exhibit dopamine release.

A freshly isolated assay cell was impaled with an intracellular electrode and positioned close to RPeD1 somata which had already established a 1 way synapse with VD4 (VD4• RPeD1) (**A**: scale bar = 100 μm). In this configuration, transmitter release from RPeD1 could not be detected by the assay cell. Specifically, evoked action potentials in the soma-soma paired RPeD1 did not inhibit the spontaneous firing of action potentials in the assay cell (**B**).

A



B

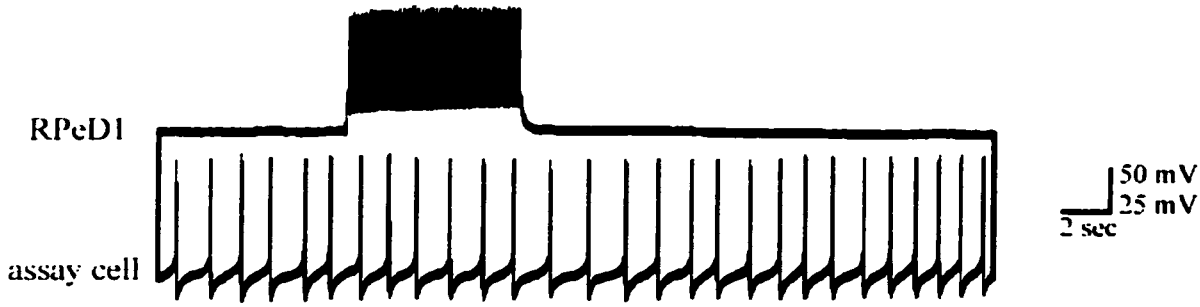


Figure 3.6. Summary data comparing the incidence of detectable transmitter release from lone or paired RPeD1 somata after 18 or 24 hours in culture.

At these time points, transmitter release from lone RPeD1 somata was consistently detected by the assay cell. Soma-soma paring between RPeD1 and VD4 however, transiently suppressed RPeD1's ability to secrete detectable amounts of transmitter.

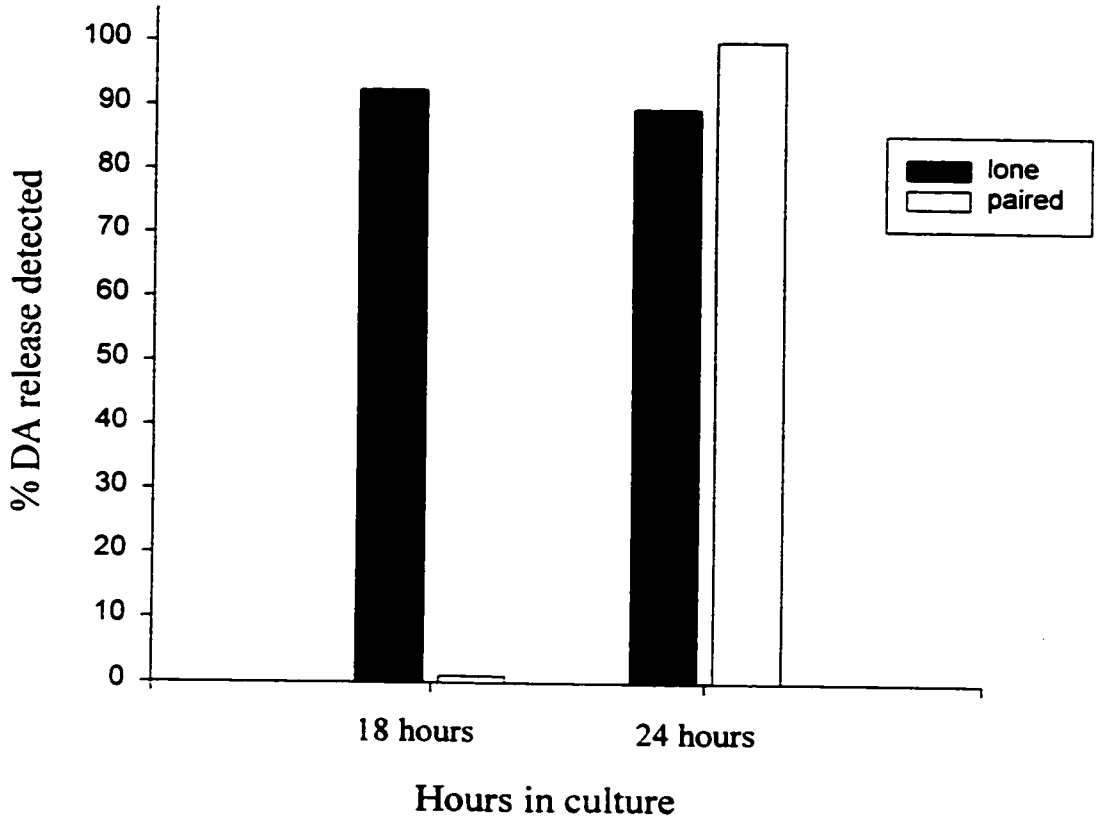

100
90
80
70
60
50
40
30
20
10
0
% DA release detected
lone
paired
18 hours
24 hours
Hours in culture

Table 3.1. Isolated RPeD1 somata cultured in DM contain dopamine.

RPeD1 somata were cultured either alone or paired with VD4 in a soma-soma configuration for 18 or 24 hours. The dopamine contents of each individual soma were analyzed by HPLC. Comparable amounts of dopamine were detected in all RPeD1s, irrespective of their time or configuration in culture.

Sample	[Dopamine] mean ± std
18 hour lone RPeD1	0.70 ± 0.09 pmol/cell
18 hour paired RPeD1	1.10 ± 0.40 pmol/cell
24 hour paired RPeD1	1.00 ± 0.20 pmol/cell

Figure 3.7. Isolated somata of RPeD1 in culture release dopamine

To test whether the transmitter released from a lone RPeD1 is indeed dopamine, assay cells were freshly isolated and positioned within close proximity to RPeD1. Electrically induced action potentials in the lone RPeD1 either inhibited spontaneous active action potentials in the assay cell (**A**), or produced a compound hyperpolarizing response (**B**). These dopaminergic responses were blocked by a dopamine D_2-receptor antagonist, (±)-sulperide (**C, D**).

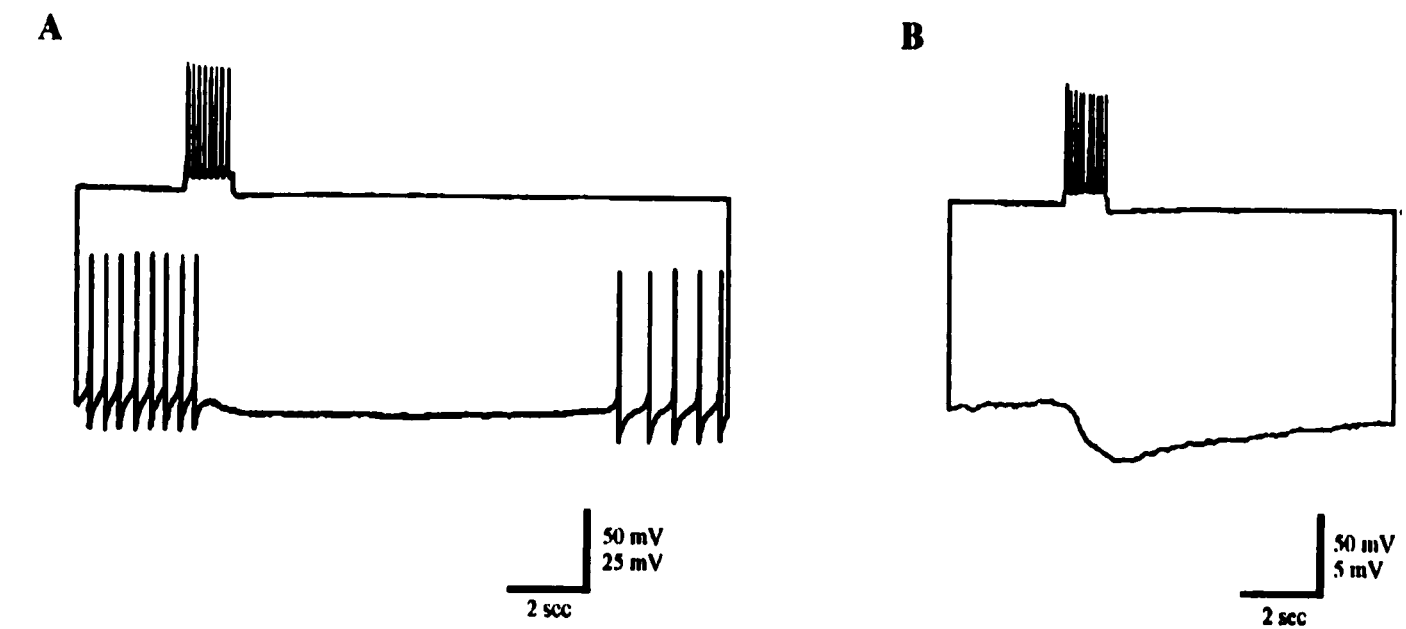
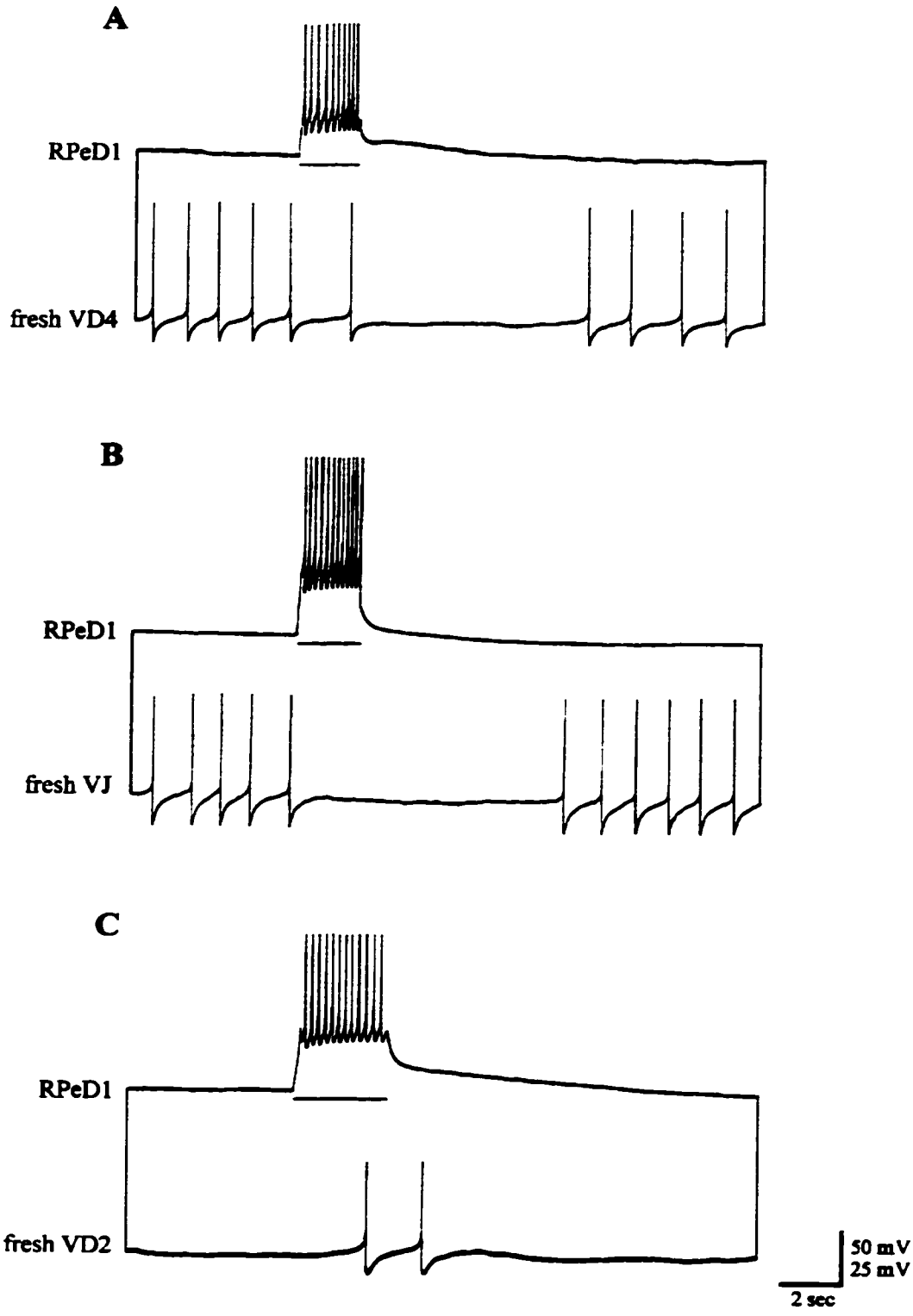

A
50 mV
25 mV
2 sec
B
50 mV
5 mV
2 sec
C
D

Figure 3.8. RPeD1-induced effects on various neurons were target cell type specific.

Three different cells from the visceral ganglion; namely VD4, VJ, and VD2 were freshly isolated and used to assay transmitter release from lone RPeD1 (18 hrs in culture) in DM. Both VD4 (**A**) and VJ (**B**) showed inhibitory responses to evoked action potentials in the lone RPeD1 somata, whereas VD2 exhibited an excitatory response to RPeD1 stimulation (**C**).


A
RPeD1
fresh VD4
B
RPeD1
fresh VJ
C
RPeD1
fresh VD2
50 mV
25 mV
2 sec

Chapter 4: Transmitter Release is Modulated via a Retrograde Signal from VD4

4.1. Introduction

Discrete synapse formation between appropriate target cells is generally thought to, at least in part, depend on coincident signaling between the prospective pre and postsynaptic partners (Fitzsimonds and Poo, 1998; Haydon and Zoran, 1994). Anterograde signaling molecules released from the presynaptic cell cause the clustering of suitable transmitter receptors (Hall and Sanes, 1993; Salpeter, 1987), and their associated ion channels (Boudier et al., 1992; Flucher and Daniels, 1989) in the postsynaptic membrane directly under the developing synaptic site (Colledge and Froehner, 1998; Glass and Yancopoulos, 1997; Ruegg, 1996). Similarly, retrograde messengers released from the postsynaptic target can also induce changes in the presynaptic cell, which in turn ensure that the appropriate transmitter is released in sufficient quantities at the developing synaptic site (Dan and Poo, 1994; Fitzsimonds and Poo, 1998; Grinnel, 1995; Hall and Sanes, 1993). This bi-directional signaling continues after initial stages of synaptogenesis and serves an important role in both maintenance and plasticity of synaptic connections in the adult animals (Fitzsimonds and Poo, 1998; Hall and Sanes, 1993).

A number of molecules have been identified as putative retrograde messengers during early stages of synapse formation. These can generally be classified into 3 separate categories: membrane-permeant molecules, secreted factors, and membrane-bound factors (Fitzsimonds and Poo, 1998). Among the membrane-permeant molecules are arachidonic acid (Bolshakov and Siegelbaum, 1995; Lynch et al., 1989), and the gases nitric oxide (NO: Bohme et al., 1991; O'Dell et al., 1991) and carbon monoxide

(CO: Zhuo et al., 1993). These molecules are produced in the postsynaptic cell, often in response to a Ca^{2+} trigger (Dumuis et al., 1988; East and Garthwaite, 1991), and diffuse freely across the synaptic cleft to affect the presynaptic cell (Fitzsimonds and Poo, 1998). The gases NO and CO are very attractive candidates for retrograde signaling since they diffuse rather easily in both aqueous and lipid environments, allowing rapid trans-synaptic signaling. Nevertheless, these gaseous messengers have a limited range of action due to a short half-life (Meulemans, 1994). In contrast to the diffusible release of membrane-permeant factors, secreted retrograde molecules are actively released by exocytosis from the postsynaptic cell (Fitzsimonds and Poo, 1998). This category of retrograde signaling molecules includes classical transmitters (Nirenberg et al., 1996; Zilberter et al., 1999), and neurotrophic factors such as brain derived neurotrophic factor (BDNF: Figurov et al., 1996; Lohof et al., 1993) and nerve growth factor (NGF: Blochl and Thoenen, 1995, 1996). The last class of retrograde signaling molecules is membrane bound and function via direct interaction between molecules expressed on the opposed cell membranes (Dai and Peng, 1993; Drapeau et al., 1989; Zhu et al., 1994, 1995; Xie and Poo, 1986). Examples of membrane-bound retrograde molecules are, neural cell adhesion molecule (NCAM: Zhu et al., 1994, 1995) and S-laminin (Hunter et al., 1991).

Much of what is known regarding retrograde signaling in the nervous system comes from studies on synaptic plasticity at fully developed synapses (for review see Goda, 1994; Hawkins et al., 1994). Specifically, long term potentiation (LTP) and long term depression (LTD) have been extensively studied in attempts to identify modulatory signals that are released by postsynaptic cells to alter the responsiveness of the presynaptic neuron to subsequent stimuli (Bliss et al., 1986; Dolphin et al., 1982; Lynch

et al., 1989; Mulkey et al., 1993; Mulkey et al., 1994). It has been postulated that most changes observed during synaptic plasticity at an adult synapse might be reminiscent of various developmental stages, thus, it is tempting to speculate that the underlying mechanisms might also be similar. However, as compared with LTP and LTD, relatively fewer studies have investigated retrograde signaling between defined pre and postsynaptic neurons during synaptogenesis in the central nervous system (Drapeau et al., 1989). At this time, therefore, it is difficult to ascertain whether the signaling molecules and the mechanisms underlying synaptic plasticity in the nervous system are indeed related to those observed during early synapse formation.

The soma-soma synapse model described in chapter 3 provides a tractable system for investigating signaling molecules that are involved in cell-cell interactions between prospective synaptic partners during early stages of synapse formation. In the previous chapter, I have demonstrated that VD4 is first to establish trans-synaptic communication with RPeD1 and that soma-soma pairing of this cell with RPeD1 results in the suppression of transmitter release from the giant dopamine cell. In this study, to identify cell-cell signaling molecules, I tested a general hypothesis that VD4-induced suppression of transmitter release in RPeD1 is cell type specific, involves new protein synthesis, and is mediated via FMRFamide release from VD4.

4.2 Results

4.2.1 VD4- induced suppression of transmitter release from RPeD1 was target cell contact specific

To test whether the suppression of transmitter release from RPeD1 was VD4 cell contact specific, the giant dopamine cell was soma-soma paired with two other identified

neurons, right parietal dorsal 1 (RPD1) and the cerebral giant cell (CGC). Both of these cells are considered non-target cells of RPeD1 since *in vivo*, they do not make either physical or synaptic contacts with the giant dopamine cell (Syed, pers. comm.). In the first series of experiments, RPeD1 and RPD1 were soma-soma paired on plain plastic dishes containing DM and left undisturbed for 18 hours. Simultaneous intracellular recordings were made after 18 hours in culture and these revealed the presence of a novel inhibitory connection from RPD1 to RPeD1 in 82% of pairs tested (n=17: fig. 4.1B). Specifically, electrically induced action potentials in RPD1 prevented the spontaneous firing of action potentials in RPeD1. In none of the cell pairs tested, however, was there any evidence for the reciprocal synaptic connection (fig. 4.1A). In other words, evoked action potentials in RPeD1 had no effect on the resting membrane potential of RPD1. Manipulation of a freshly isolated assay cell near the RPeD1somata revealed that this cell was still capable of evoked transmitter release (n=5: fig. 4.1C).

To examine further the target cell specificity of transmitter suppression from a soma-soma paired RPeD1, a cerebral giant cell (CGC) was paired with the dopamine cell. After 18 hours of soma-soma contact, simultaneous intracellular recordings revealed an inhibitory chemical connection from CGC to RPeD1 but not vice versa (n=9: fig. 4.2A,B). Induced action potentials in CGC hyperpolarized RPeD1 from its resting potential in 66% of the paired cells (fig. 4.2B). Conversely, action potentials in the giant dopamine cell had no effect on the resting membrane potential of CGC (fig. 4.2A) in all 9 soma-soma pairs. An assay cell approach demonstrated that 8 of these 9 RPeD1 cells were indeed capable of evoked non-synaptic, transmitter release (fig. 4.2C).

Since both RPD1 and CGC failed to suppress transmitter release from RPeD1, I conclude the following. 1) The suppression of transmitter release from soma-soma paired RPeD1 cells observed in chapter 3 was not due to physical disturbances that might have resulted from soma-soma pairing. 2) VD-4 induced suppression of transmitter release from RPeD1 was cell type specific. 3) Notwithstanding the fact that both CGC and RPD1 had established a novel synapse with RPeD1, this inappropriate inhibitory synapse alone was not sufficient to induce transmitter suppression from RPeD1. 4) Finally, even though RPeD1 cells paired with RPD1 and CGC were competent to release transmitter non-synaptically, they did not establish inappropriate synapse with these cells. From these studies, therefore, it seems safe to conclude that cell contact-induced suppression of transmitter release from RPeD1 is target cell type specific.

4.2.2 VD4-induced suppression of transmitter release from RPeD1 required transcription and *de novo* protein synthesis.

Due to the fact that a single RPeD1 was competent to release dopamine, I asked the question whether VD4-induced suppression of transmitter release from RPeD1 involved an active process, requiring both transcription and *de novo* protein synthesis. To address this issue directly, cells were soma-soma paired in the presence of either a transcription blocker (actinomycin D: 5 μg/ml) or a protein synthesis inhibitor (anisomycin: 12.5 μg/ml). Simultaneous intracellular recordings were made following 18 hours in cell culture and evidence for synaptic communication was sought electrophysiologically. From 12 soma-soma paired cells maintained in anisomycin, direct intracellular stimulation of VD4 failed to induce any electrophysiologically detectable signal in 92% of the paired RPeD1 cells (fig. 4.3B). These data confirmed

earlier studies (Feng et al., 1997) where synapse formation between soma-soma paired RPeD1 and VD4 was completely blocked by this protein synthesis inhibitor. RPeD1 stimulation under these experimental conditions produced a compound, non-synaptic response in 58% of the VD4 cells (fig. 4.3A). Similarly, in 86% of the soma-soma pairs maintained in the presence of actinomycin D, RPeD1 stimulation produced a compound inhibitory response in VD4 (fig. 4.3C), whereas a reciprocal VD4 to RPeD1 synapse was observed in only 17% of the paired cells (fig. 4.3D: n=14). Freshly isolated assay cells manipulated within close proximity to the paired RPeD1 somata were able to detect evoked, non-synaptic transmitter release from 80% of the dopamine cells cultured in the presence of anisomycin (n=5: fig. 4.4A) and from 86% of the cells in actinomycin D (n=14: fig. 4.4B). The above data are summarized in figure 5.

As compared with soma-soma pairs maintained in normal DM, RPeD1/VD4 pairs cultured in the presence of both anisomysin and actinomycin D had significantly lower incidences of inhibitory synaptic transmission from VD4 to RPeD1 with a corresponding increase in the reciprocal (RPeD1• VD4) connection (fig. 4.5A: (• $^{2}(6) = 46.854$, $P < 0.001$). Similarly, the incidence of detectable, evoked, non-synaptic DA release from a paired RPeD1 increased significantly compared to that of controls, in both anisomycin ($P = 0.022$ F.E. test) and actinomycin D ($P = 0.002$ F.E. test: fig. 4.5B). Taken together, these data demonstrate that transcription and *de novo* protein synthesis are necessary for both the formation of VD4 to RPeD1 synapses and for VD4-induced suppression of transmitter release from the soma of the giant dopamine cell.

4.2.3 VD4 induced suppression of DA release from RPeD1 does not involve cell-surface bound molecules.

Since transmitter suppression from a paired RPeD1 was VD4 contact specific, I asked the question whether this phenomenon required cell-cell signaling via membrane contacts between the cells. In an initial series of experiments, single RPeD1 neurons were soma-soma paired with paraformaldehyde fixed VD4 somata. Following 18 hours in culture, membrane contact between the dopamine cell and the fixed VD4 was confirmed visually (fig. 4.6A). For cell pairs that had maintained their contact for the 18 hour time interval, a freshly isolated assay cell was added to the culture dish and the paired RPeD1 was tested for its ability to release transmitter following electrical stimulation. In 86% of the RPeD1/fixed VD4 pairs tested, the assay cell detected evoked, non-synaptic DA release from the RPeD1 soma (n=6: fig. 4.6B). These data show that sustained membrane contact with a fixed VD4 did not affect the viability of RPeD1. Furthermore, it demonstrates that membrane contact with VD4 alone was insufficient to suppress transmitter release from the giant dopamine cell.

In view of the fact that neurotrophic factors released from target cells have been shown to alter neurotransmitter secretory machinery of the innervating presynaptic neuron, I hypothesized that if such a molecule were to be involved in VD4-induced suppression of transmitter release from RPeD1, it would function via a receptor tyrosine kinase (Trk). Lavendustin A, a potent and selective inhibitor of TrkA receptors, has previously been shown to block trophic factor induced signaling between soma-soma paired *Lymnaea* neurons (Hamakawa et al., 1999; Woodin et al., 1999), therefore, cells were paired in the presence of lavendustin A (10 μM) and transmitter secretory capabilities were tested electrophysiologically. Synapses formed in the presence of this compound were electrophysiologically similar to those observed in DM alone.

Specifically, simultaneous intracellular recordings revealed that RPeD1 and VD4 paired in DM containing lavendustin A, formed a mutually inhibitory synapse after 24 hours in culture (fig. 4.7A,B). The paired (synapses) RPeD1 in these cultures were able to release transmitter non-synaptically, as shown by the electrophysiological responses exhibited by a freshly isolated assay cell (fig. 4.7C). Similarly, cells paired in lavendustin A for only 18 hours formed a one-way (VD4• RPeD1) inhibitory synapse, analogous to that observed in control pairs (fig. 4.7D,E). When tested for its release capabilities, RPeD1 somata in these one-way synapses were unable to release DA following electrical stimulation (fig. 4.7F). Taken together, these data demonstrate that inhibitory synapse formation between soma-soma paired cells does not involve trophic factor release from the paired cells and that VD4-induced suppression of transmitter release from RPeD1 is independent of intrinsic diffusible trophic factors.

4.2.4 Exogenous FMRFamide mimicked VD4-induced suppression of transmitter release from RPeD1

VD4 is known to contain and release FMRFamide-like peptides (McKenney, 1992; Santama et al., 1995), and its postsynaptic effects are mimicked, in almost all instances, by either FMRFamide or its related peptides (McKenney, 1992: also see fig. 5.1). Since VD4 was first to initiate synaptic transmission with RPeD1 (see chapter 3), I hypothesized that VD4-induced suppression of transmitter release from RPeD1 may involve signaling via the release of a FMRFamide-like peptide from VD4. To test this hypothesis directly, RPeD1 somata were cultured alone in the presence of exogenous (10^{-6}M) FMRFamide. After 18 hours, the media was replaced with normal DM and freshly isolated assay cells were introduced to the culture dish. The lone RPeD1 cells were

stimulated electrically to induce DA release. Despite repeated stimulation, however, assay cells failed to detect transmitter release from 79% of the FMRFamide treated RPeD1 somata (n=14: fig. 4.8A). Similar treatment with a related peptide, GDPFLRFamide (10^{-6}M), also was found to suppress transmitter release in 77% of cells tested (n=9). Compared to control values, the incidence of detectable release dropped significantly in the presence of either of these related peptides ($P<0.001$ F.E.test). Heat inactivation of FMRFamide prior to addition to the culture media rendered this peptide incapable of blocking the transmitter release from a lone RPeD1 (n=4: fig. 4.8D: $P=1.000$ F.E. test compared to control).

Since exogenously applied FMRFamide and its related peptide exert inhibitory (hyperpolarizing) effects on the giant dopamine cell (McKenney, 1992: also see fig. 5.1A), I asked whether any other transmitter capable of inhibiting activity in RPeD1 could also mimic FMRFamide-induced effects on RPeD1. To test the specificity of FMRFamide's response, therefore, a second inhibitory transmitter was tested. Pressure application of 5-hydroxytryptamine (5-HT, 10^{-5}M) caused a similar, inhibitory response in cultured giant dopamine cells as acute FMRFamide application (data not shown), therefore, this amine was chosen to test whether application of an inhibitory transmitter would be sufficient to suppress RPeD1's secretory capabilities. RPeD1 was isolated and cultured for 18 hours in DM containing 10^{-6}M 5-HT. Prior to electrophysiological analysis of the cells, the DM containing 5-HT was replaced for normal saline, and a freshly isolated assay cell was placed in the dish to detect transmitter release from RPeD1. In all RPeD1 cells tested (n=7), the assay cells were able to detect induced, non-synaptic transmitter release from the soma of the giant dopamine cell (fig. 4.8B). These

data demonstrate that the inclusion of an inhibitory neurotransmitter in the culture media is not sufficient to suppress transmitter release from RPeD1 and that this phenomenon is specific to FMRFamide and its related peptides. These results also demonstrate that both VD4 and FMRFamide-induced membrane hyperpolarization alone may not be sufficient for transmitter suppression from RPeD1.

I next sought to determine, whether FMRFamide-induced suppression of transmitter release involved protein synthesis, similar to that with VD4-induced suppression. Lone RPeD1 somata were cultured in DM containing a mixture of FMRFamide (10^{-6}M) and anisomycin (12.5 μg/ml). The culture medium containing both of the above compounds was subsequently replaced with normal saline after 18 hours and dopamine release from the RPeD1s was tested with a freshly isolated assay cell. Interestingly, even in the presence of the protein synthesis inhibitor, FMRFamide still suppressed induced transmitter release from RPeD1 (n=5: fig. 4.8C: $P<0.001$ F.E. test). These data thus provide direct evidence that the steps requiring *de novo* protein synthesis during VD4-induced suppression of transmitter release do not involve the giant dopamine cell and therefore most likely reside in VD4. The above data, comparing the effects of various treatments on RPeD1's secretory capabilities, are summarized in figure 4.9.

4.3 Discussion

In this chapter, I have demonstrated that VD4-induced transmitter suppression from RPeD1 is target cell specific and requires *de novo* protein synthesis. This suppression does not involve cell surface signaling between the paired cells, nor receptor tyrosine kinase mediated signaling. I do, however, provide direct evidence that either FMRFamide or a related peptide, released from VD4, is sufficient to suppress transmitter

release from RPeD1. Accordingly, this study underscores the importance of neurotransmitter release in the maturation of developing synapses in the nervous system.

Specific postsynaptic cell contact has previously been shown to alter presynaptic transmitter release capabilities during early stages of synaptogenesis. For example, in both molluscan (Zoran et al., 1990) and amphibian (Chow and Poo, 1985; Xie and Poo, 1986) *in vitro* neuromuscular preparations, contact with discrete target cells causes a rapid increase in transmitter release. These effects were target cell specific since contact between two motor neurons themselves, or with other inappropriate muscle targets, failed to modulate the secretory machinery of the presynaptic cell (Xie and Poo, 1986; Zoran et al., 1990; Zoran et al., 1996). These data indicate that specific intercellular signals required for presynaptic changes are generated only in particular postsynaptic target cells. In the present study, RPeD1 did not form inappropriate synaptic connections with two non-target neurons, nor did the contact with these neurons suppress its transmitter secretory capabilities. Together with a previous study on RPeD1 soma-soma synapses (Feng et al., 1997), this demonstrates that RPeD1 is specific in its target cell selection and that this selectivity is maintained in the soma-soma configuration. Furthermore, this data argues for the presence of one or more specific retrograde signals, generated only in particular target cells, which are necessary to induce transient suppression of transmitter release from the RPeD1 soma and subsequent synapse formation.

De novo protein synthesis tends to be a requirement for synapse formation in most, but not all, models studied to date. Utilizing soma-soma synapses, Feng et al. (1997) have previously demonstrated that synapse formation between RPeD1 and VD4 is blocked by anisomycin. The concept that protein synthesis is required for synaptogenesis

was further established by the demonstration that soma-soma pairing resulted in gene induction in RPeD1 and VD4. Specifically, van Kesteren et al (1997) demonstrated that, compared with their single counterparts, several genes were up-regulated in paired RPeD1 and VD4. The precise identity, function and the underlying mechanisms for the majority of these newly synthesized proteins, however, remain unknown. In the present study, protein synthesis was found to be necessary for synapse formation, however, RPeD1's ability to release transmitter from its somata did not require *de novo* protein synthesis. In other words, it seems reasonable to assume that the ability to release transmitter somatically was either pre-assembled in the intact brain or was activated in the brief time period between isolation of the central ring ganglia from the intact animal and placement of the individual cells in culture. In either case blocking protein synthesis in culture did not impinge upon RPeD1's ability to secrete transmitter non-synaptically.

The most common intercellular signals known to regulate transmitter release at developing and mature synapses involve cell surface molecules and/or neurotrophins (Fitzsimonds and Poo, 1998; Haydon and Zoran, 1994). In snail (Zoran et al., 1990; Zoran et al., 1996) and amphibian (Xie and Poo, 1986) neuromuscular models, contact induced signaling molecules have been shown to regulate the acquisition of presynaptic transmitter secretory capabilities, however, the exact identity of such membrane bound molecule(s) remains elusive. Since soma-soma membrane contact with a paraformaldehyde fixed VD4 did not suppress transmitter release from RPeD1, the data presented in this chapter rules out the possibility that membrane bound molecules are likely involved in the contact mediated suppression of transmitter release from RPeD1. It is feasible that the fixation treatment may have either removed or altered membranous

components or the structural integrity of the membrane bound molecule/s in VD4, however, our data are nonetheless consistent with early studies on leech neurons (Drapeau et al., 1989). Specifically, a fixation procedure on leech neurons, identical to the one used in the present study, did not alter membrane bound molecules such that a fixed cell was still able to activate cell-cell signaling in a manner similar to that of healthy cells.

Neurotrophins have long been considered as potential candidates for regulating synaptic development and for modulating the strength of existing synapses in the adult nervous system (for review see Fitzsimonds and Poo, 1998; McKay et al., 1999). Furthermore, in both vertebrates (Vicario-Abejon et al., 1998) and invertebrates (Hamakawa et al., 1999; Woodin et al., 1999) neurotrophic factors have been shown to regulate excitatory synapse formation *in vitro*. This trophic factor -induced synapse specificity required *de novo* protein synthesis and was mediated via receptor tyrosine kinases (Hamakawa et al., 1999; Woodin et al., 1999). These recent studies from our laboratory have also shown that the unidentified trophic factors released from isolated central ring ganglia appear necessary for the formation of particular excitatory synapses (Hamakawa et al., 1999; Woodin et al., 1999) but not for inhibitory synapses (Feng et al., 1997). Although data presented by Feng et al. (1997) excluded extrinsic trophic factors for their role in inhibitory synapse formation, this study did not rule out the involvement of trophic factors that might be released from the paired cells themselves. In the present study, I have demonstrated that a potent inhibitor of the receptor tyrosine kinase did not affect the ability of soma-soma paired cells to form appropriate inhibitory synapses. Thus, this study adds to the general conclusion drawn by Feng et al (1997) that neither

extrinsic nor intrinsic trophic factors are required for the formation of inhibitory synapses between soma-soma paired cells. In addition, the present study also demonstrated that VD4-induced transmitter suppression from RPeD1 did not involve intrinsically derived, trophic factors. The possibility of other neurotrophins and/or their specific receptors cannot, however, be ruled out.

In the present study, chronic application of FMRFamide and its related peptide GDPFLRFamide, suppressed transmitter release from cultured RPeD1 somata. Acute application of FMRFamide has previously been shown to reduce evoked transmitter release from both *Helisoma* (Man-Son-Hing et al., 1989) and *Aplysia* neurons (Abrams et al., 1984; Piomelli et al., 1987). In these molluscan species, FMRFamide reduced both the Ca^{2+} influx through voltage gated channels and, independently, reduced the sensitivity of the transmitter secretory machinery to the incoming Ca^{2+} trigger. Although these studies clearly demonstrated a mechanism by which exogenous FMRFamide can reduce transmitter release, a natural synaptic source of this peptide in *Helisoma* remains un-identified. In other words, although possible FMRFamidergic innervation has been reported in *Aplysia* (Small et al., 1992), it is unknown whether the cells used in *Helisoma* receive any FMRFamide mediated synaptic input in the intact animals. Furthermore, the action of FMRFamide in these studies was investigated in the context of synaptic modulation at existing synapses, thus, a possible role in transmitter release during synaptogenesis remained unexplored.

In the *Lymnaea* respiratory central pattern generator, VD4 is thought to release a FMRFamide-like peptide as a neurotransmitter. Evidence for this is twofold: exogenous application of FMRFamide to identified postsynaptic targets mimics VD4's inhibitory

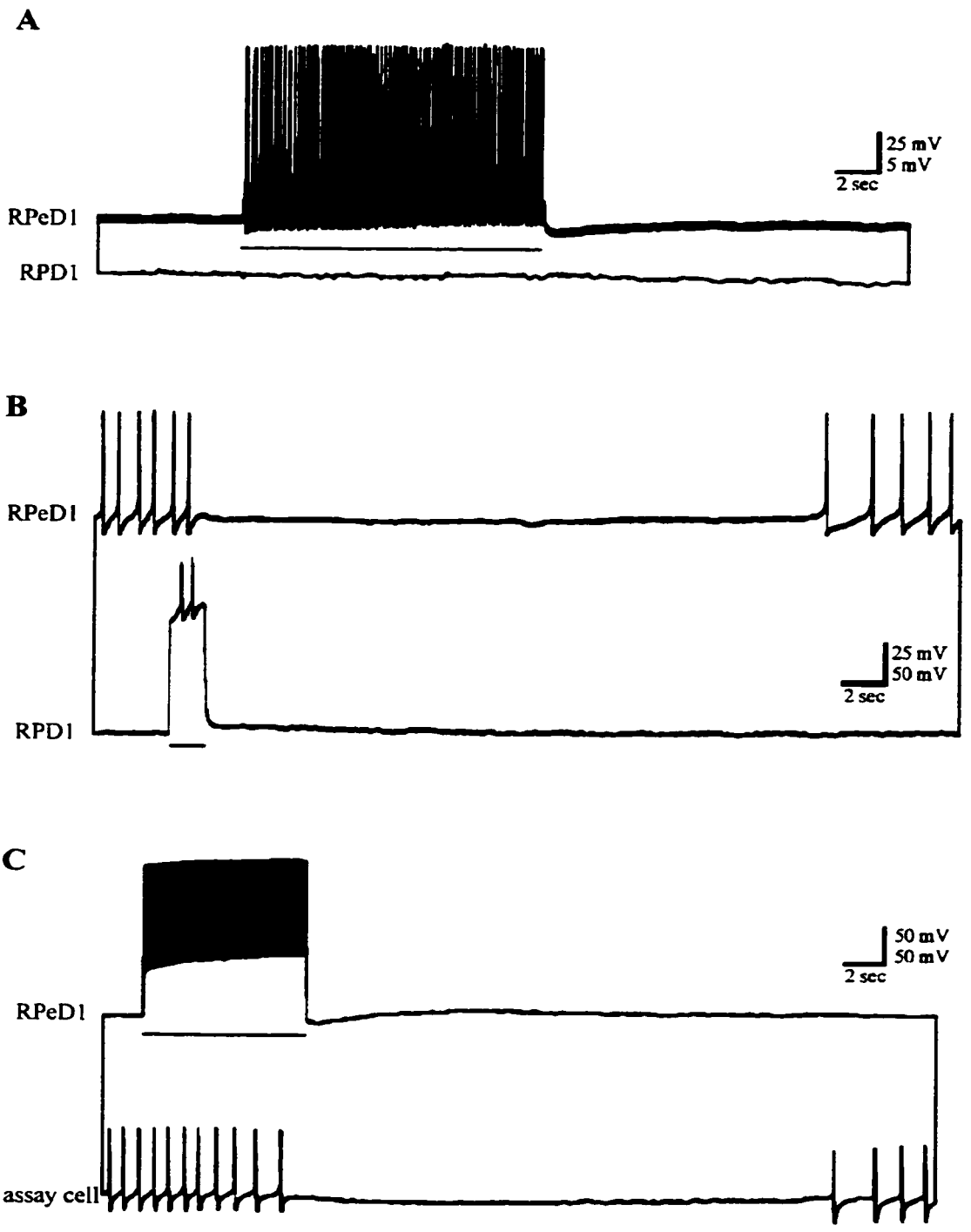
connections (McKenney, 1992) and VD4 has been shown by both *in situ* hybridization (Santama et al., 1995; Saunders et al., 1992) and immunohistochemistry to contain FMRFamide-like peptides (McKenney, 1992). Electrophysiological and pharmacological data obtained from *in vitro* studies (see chapter 5) supports the hypothesis that synaptic transmission between soma-soma paired VD4 and RPeD1 is also mediated via FMRFamide-like peptides.

In combination with previously published studies, the data obtained here suggest the following: prior to interactions between the two cells, RPeD1 is able to release transmitter non-synaptically while VD4 cannot. During initial stages of synapse formation, an early, unknown signal between the two cells enables VD4 to release a FMRFamide-like peptide. Release of this peptide from VD4 at synaptic contact sites with RPeD1 serves as a retrograde signal, which transiently suppresses dopamine release from this cell. Following this transient period, possibly representing a redistribution/reorganization of transmitter secretory and/or packaging machinery, RPeD1 gains the ability to release dopamine synaptically at the contact site, as well as non-synaptically over other areas of the soma. Since RPeD1 is able to release transmitter independent of protein synthesis, and a cocktail of FMRFamide and anisomycin was still able to suppress dopamine release, the protein synthesis dependent step is most likely inherent to VD4. This protein synthesis dependence may represent either the actual synthesis of the FMRFamide-like peptide, or synthesis of secretory machinery components necessary for the acquisition of functional exocytotic capabilities. Although the suppression of transmitter release occurs independent of protein synthesis in RPeD1,

it is still unclear whether the subsequent recovery from this suppression also involves de *novo* protein synthesis.

Figure 4.1. Transmitter suppression from RPD1 was VD4 cell contact specific.

To test whether transmitter suppression from RPeD1 was VD4 cell contact specific, the giant dopamine cell was paired in the soma-soma configuration for 18 with a non-target cell right parietal dorsal 1 (RPD1). Simultaneous intracellular recordings (18-24 hrs) showed the absence of synapse between RPeD1 and RPD1 (**A**). However, a novel inhibitory synapse was detected between RPD1 and RPeD1. Specifically, induced action potentials in RPD1 inhibited the firing of spontaneous action potentials in RPeD1 (**B**). To test whether a RPeD1 somata paired with RPD1 was indeed capable of transmitter release, a freshly isolated assay cell (VD4) was maneuvered in close proximity to the giant dopamine cell and transmitter release was induced via current injections. Under these experimental conditions transmitter release from RPeD1 was detected reliably. (**C**).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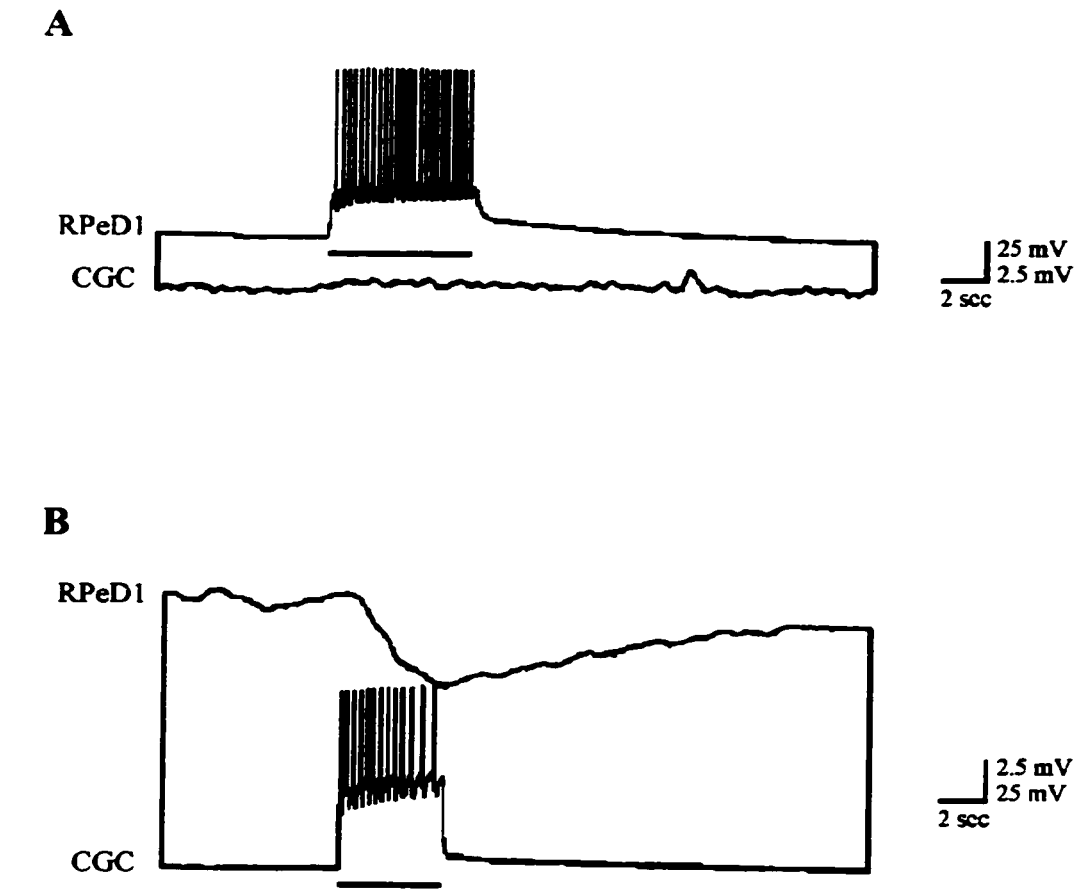
25 mV
5 mV
2 sec
RPeD1
RPD1
B
RPeD1
25 mV
50 mV
2 sec
RPD1
C
50 mV
50 mV
2 sec
RPeD1
assay cell

Figure 4.2. Soma-soma contact with the non-target cell CGC did not suppress evoked transmitter release from RPeD1.

RPeD1 and the cerebral giant cell (CGC) were isolated and cultured in the soma-soma configuration for 18 hours. Simultaneous intracellular recordings were made from the paired cells and no synapse was detected between RPeD1 and CGC (**A**). An inappropriate inhibitory connection was however detected from CGC to RPeD1. Specifically, induced action potentials in CGC produced a compound postsynaptic hyperpolarizating response in RPeD1 (**A**). Induced transmitter release from RPeD1 soma was reliably detected by a freshly isolated assay cell (**C**).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RPeD1
CGC
25 mV
2.5 mV
2 sec
B
RPeD1
CGC
2.5 mV
25 mV
2 sec
C
RPeD1
assay cell
25 mV
5 mV
2 sec

Figure 4.3. Synapse formation between VD4 and RPeD1 somata requires *de novo* protein synthesis.

RPeD1 and VD4 soma were isolated and cultured in the soma-soma configuration for 18 hours in DM containing either the protein synthesis blocker anisomycin (12.5 μg/ml) or the transcription blocker actinomycin D (5 μg/ml). In the presence of anisomycin, electrical stimulation of RPeD1 (black bar) prevented the firing of action potentials in VD4 (**A**), whereas VD4 stimulation produced no electrophysiologically detectable response in the RPeD1 soma (**B**). Similarly, when cultured in actinomycin D, induced action potentials in RPeD1 inhibited spontaneous firing in VD4 (**C**) in the absence of a reciprocal connection (**D**).

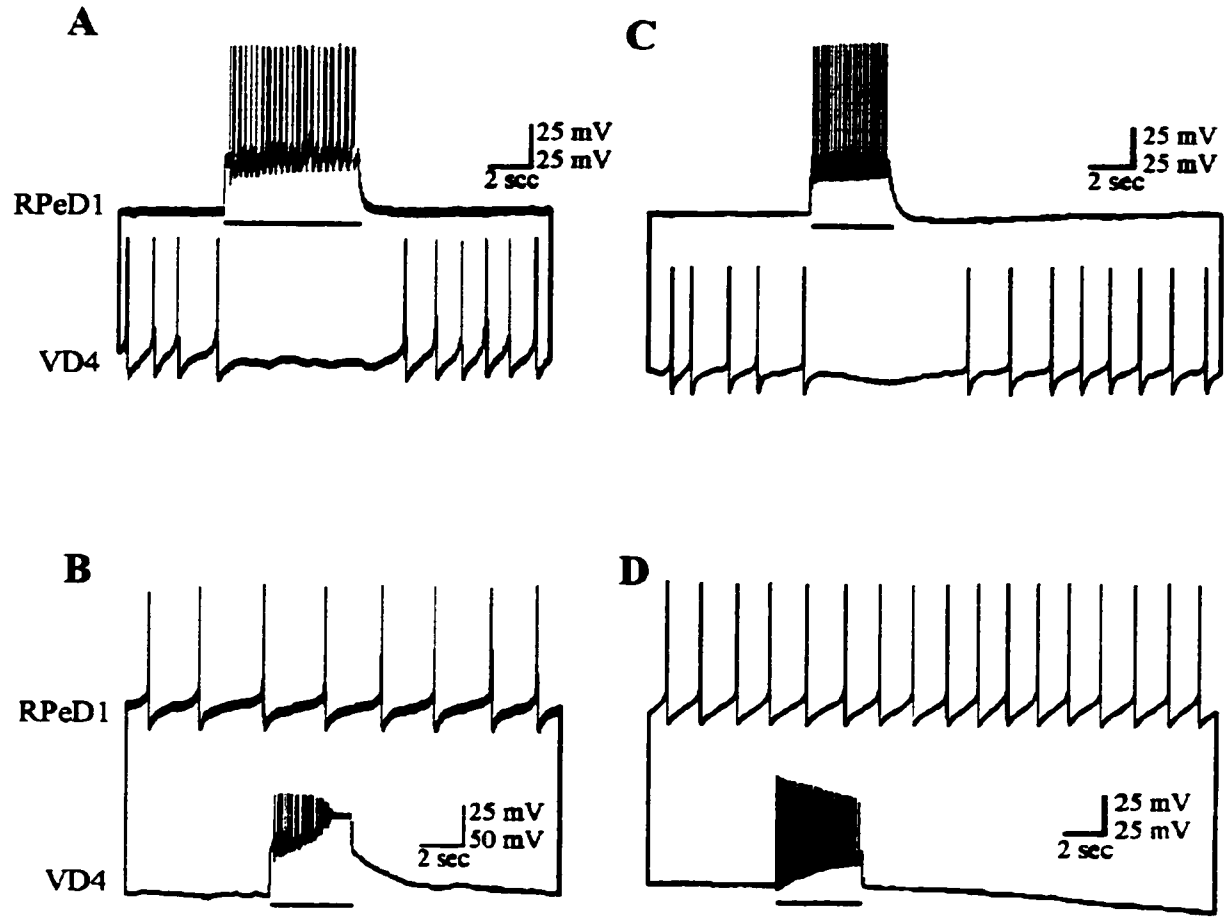

A
25 mV
25 mV
2 sec
RPeD1
VD4
C
25 mV
25 mV
2 sec
B
RPeD1
25 mV
50 mV
2 sec
VD4
D
25 mV
25 mV
2 sec

Figure 4.4. VD4-induced suppression of DA release from RPeD1 requires *de novo* protein synthesis.

RPeD1 and VD4 soma were isolated and cultured in the soma-soma configuration for 18 hours in DM containing either anisomycin (12.5 μg.ml) or actinomycin D (5 μg/ml). A freshly isolated assay cell was subsequently added to the culture dish, impaled with an intracellular recording electrode and manipulated in close proximity to the paired RPeD1 somata. Induced action potentials (black bars) in the soma-soma paired RPeD1 maintained either in anisomycin (**A**) or actinomycin D (**B**) inhibited the firing of spontaneous action potentials in an assay cell.

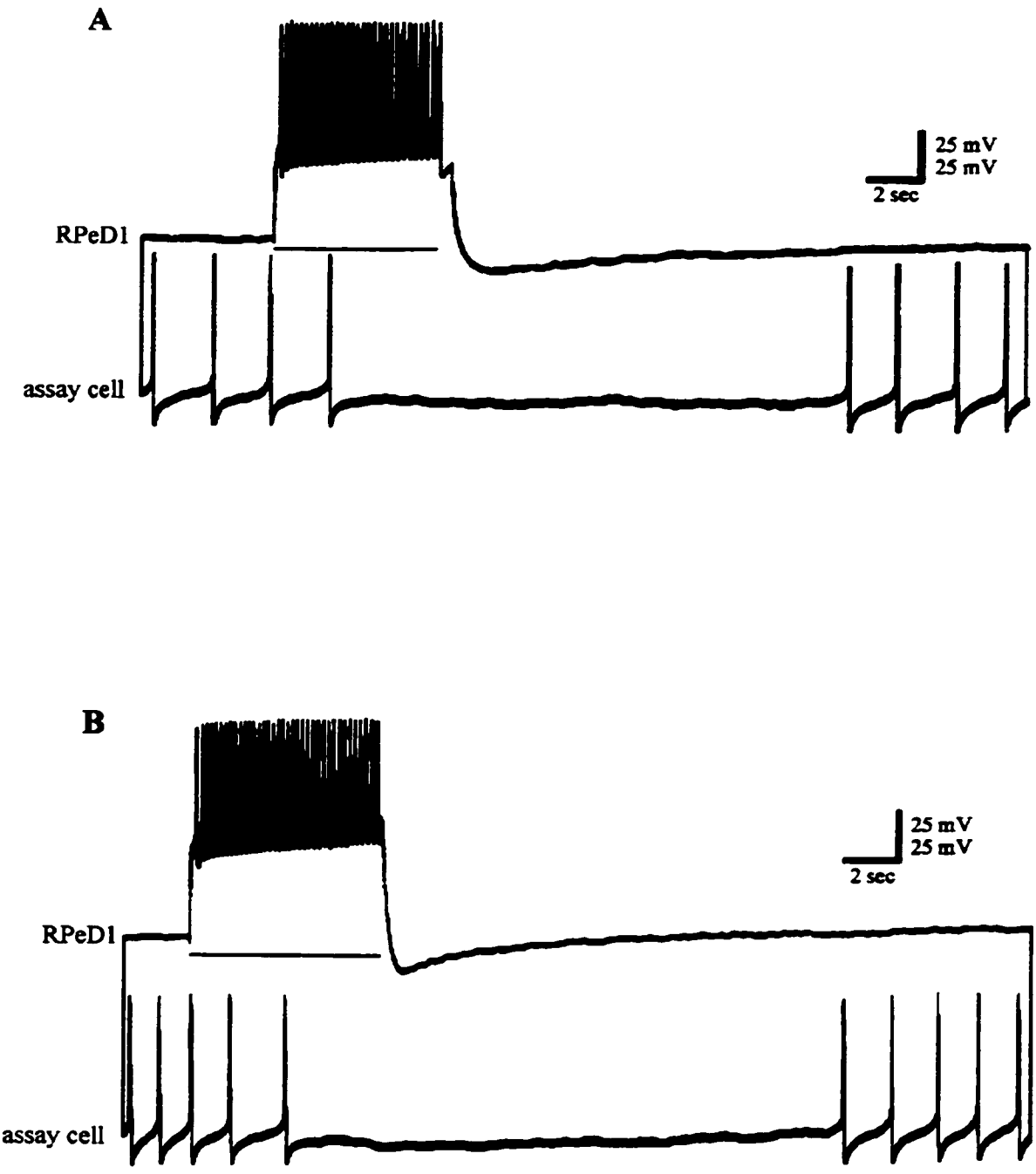
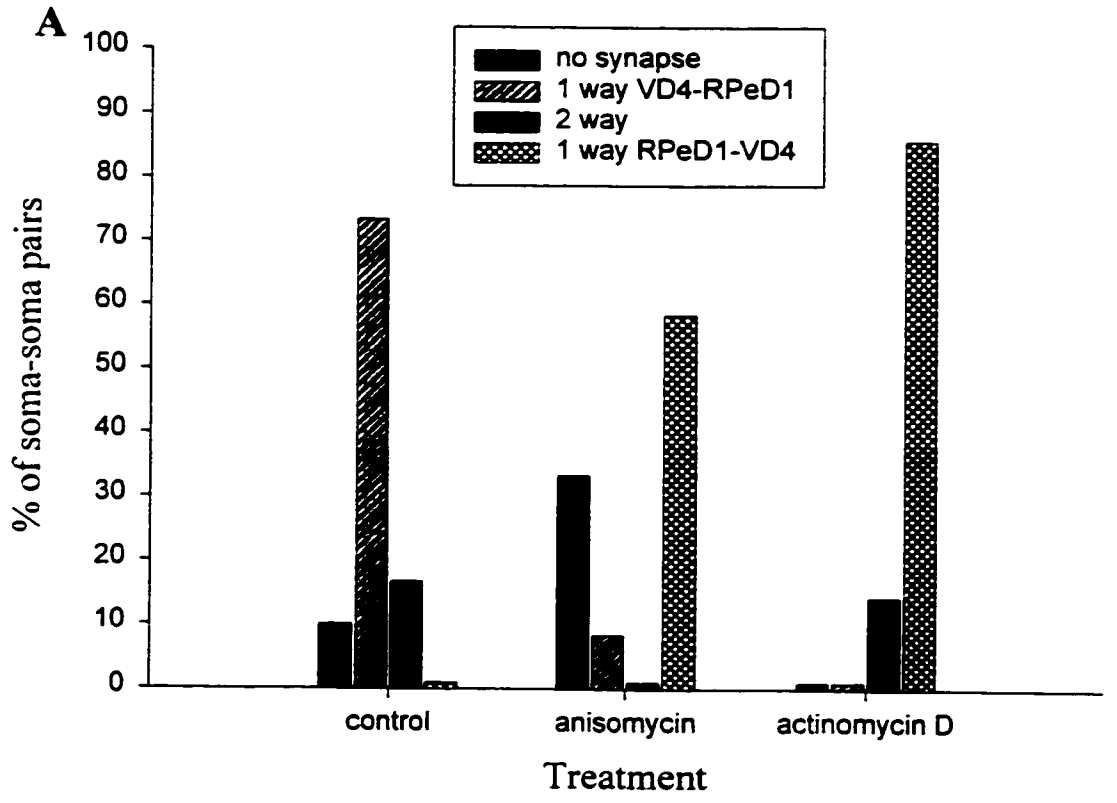

A
25 mV
25 mV
2 sec
RPeD1
assay cell
B
25 mV
25 mV
2 sec
RPeD1
assay cell

Figure 4.5. Summary data showing the incidence of synapse formation between VD4 and RPeD1 and the dependence of VD4-induced suppression of transmitter release from RPeD1 on protein synthesis.

A. The incidence of 1 way (VD4• RPeD1) inhibitory synaptic communication between the paired cells decreased significantly in the presence of either a protein synthesis blocker (anisomycin), or a transcription blocker (actinomycin D), with a concurrent increase in the incidence of 1 way (RPeD1• VD4) inhibitory communication, as compared to control levels ($P<0.001$).

B. The incidence of detectable evoked transmitter release from RPeD1 somata paired with VD4 (for 18 hour) increased significantly with the inclusion of anisomycin ($P=0.022$) or actinomycin D ($P=0.002$) in the culture media.


A
100
90
80
70
60
50
40
30
20
10
0
% of soma-soma pairs
no synapse
1 way VD4-RPeD1
2 way
1 way RPeD1-VD4
control
anisomycin
actinomycin D
Treatment

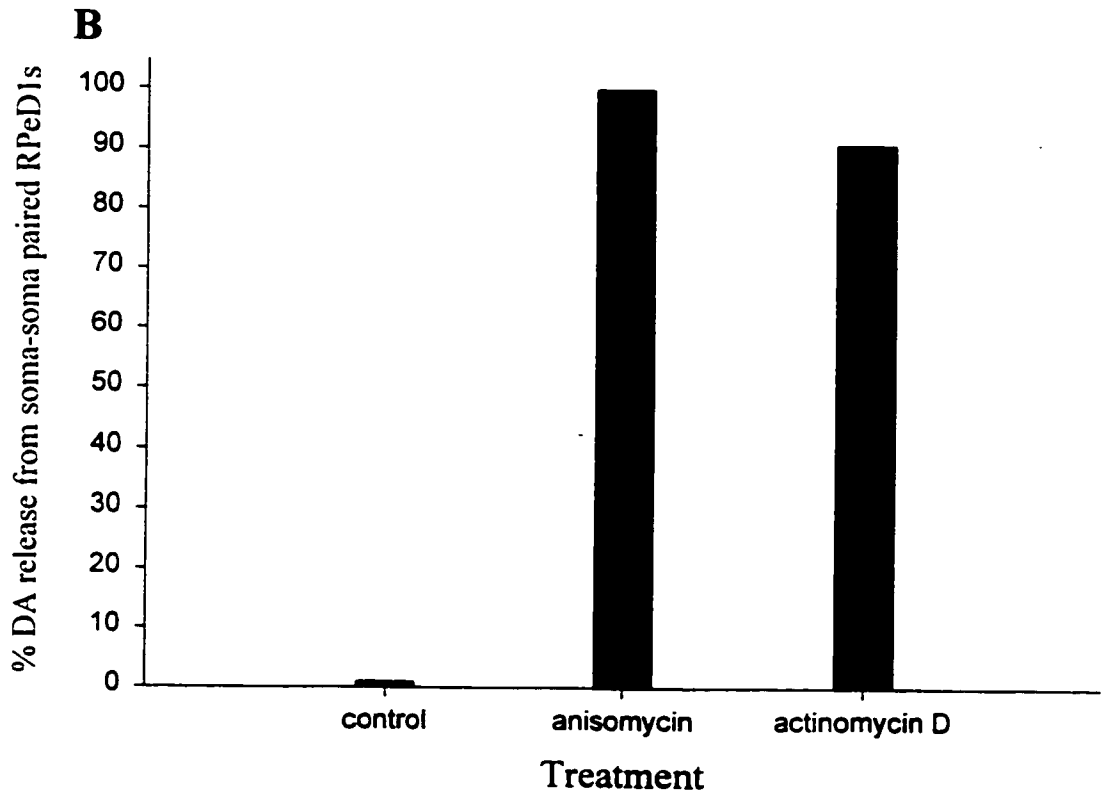
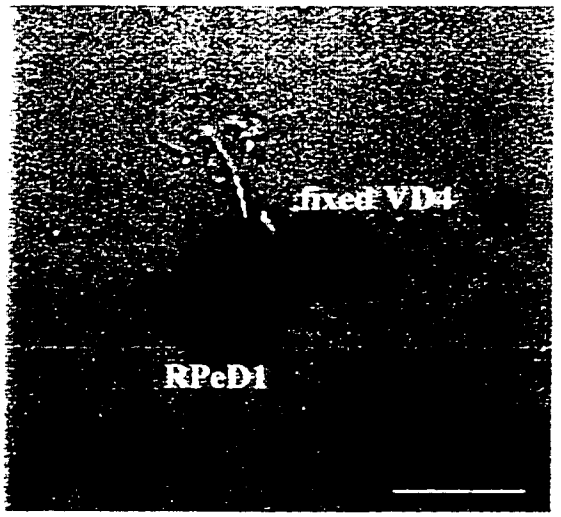

B
100
90
80
70
60
50
40
30
20
10
0
% DA release from soma-soma paired RPeD1s
control
anisomycin
actinomycin D
Treatment

Figure 4.6. VD4 membrane contact with RPeD1 alone was insufficient to suppress transmitter release from the giant dopamine cell.

Isolated RPeD1 somata were soma-soma paired with a paraformaldehyde pre-fixed VD4 and both cells were maintained in contact with each other for 18 hrs. Following visual inspection, the cells that remained in contact over the 18 hrs period were further analyzed (**A**: white scale bar = 100 μm). Specifically, a freshly isolated assay cell was impaled with an intracellular recording electrode and positioned in close proximity to the RPeD1 somata. Action potentials in the RPeD1 soma (in contact with the fixed cell) consistently inhibited the spontaneous firing of action potentials in the assay cell (**B**).

A



B

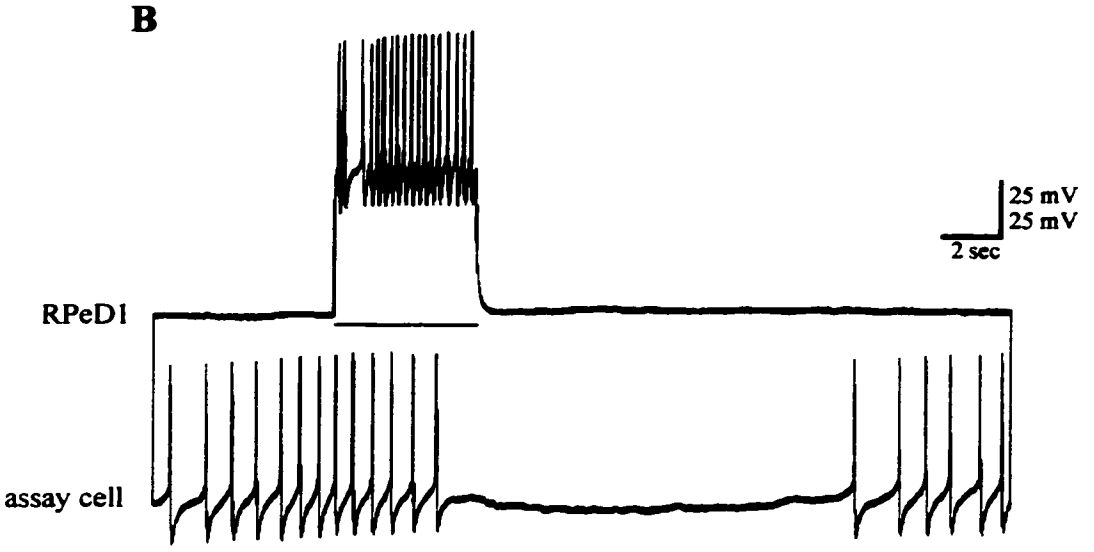


Figure 4.7. Inhibitory synapse formation between RPeD1 and VD4, and VD4-induced suppression of transmitter release from the giant dopamine cell, do not require receptor tyrosine kinases.

Soma-soma synapses between RPeD1 and VD4 were constructed in DM containing the receptor tyrosine kinase inhibitor, lavendustin A. Simultaneous intracellular recordings from RPeD1 and VD4 after 24 hours of pairing revealed a mutually inhibitory synapse between the paired cells (**A,B**). Freshly isolated assay cells consistently detected evoked transmitter release from RPeD1 somata in these pairs (**C**). Soma-soma pairs examined after only 18 hours in cell culture revealed a transient 1-way inhibitory synapse between VD4 and RPeD1 as was observed in control pairs (See Figure 3.2). Specifically, induced action potentials in VD4 inhibited spontaneous spiking in RPeD1 (**E**), whereas the synaptic connections between RPeD1 and VD4 was absent (**D**). Assay cells failed to detect evoked DA release from RPeD1 somata in this 1 way synaptic configuration (**E**).

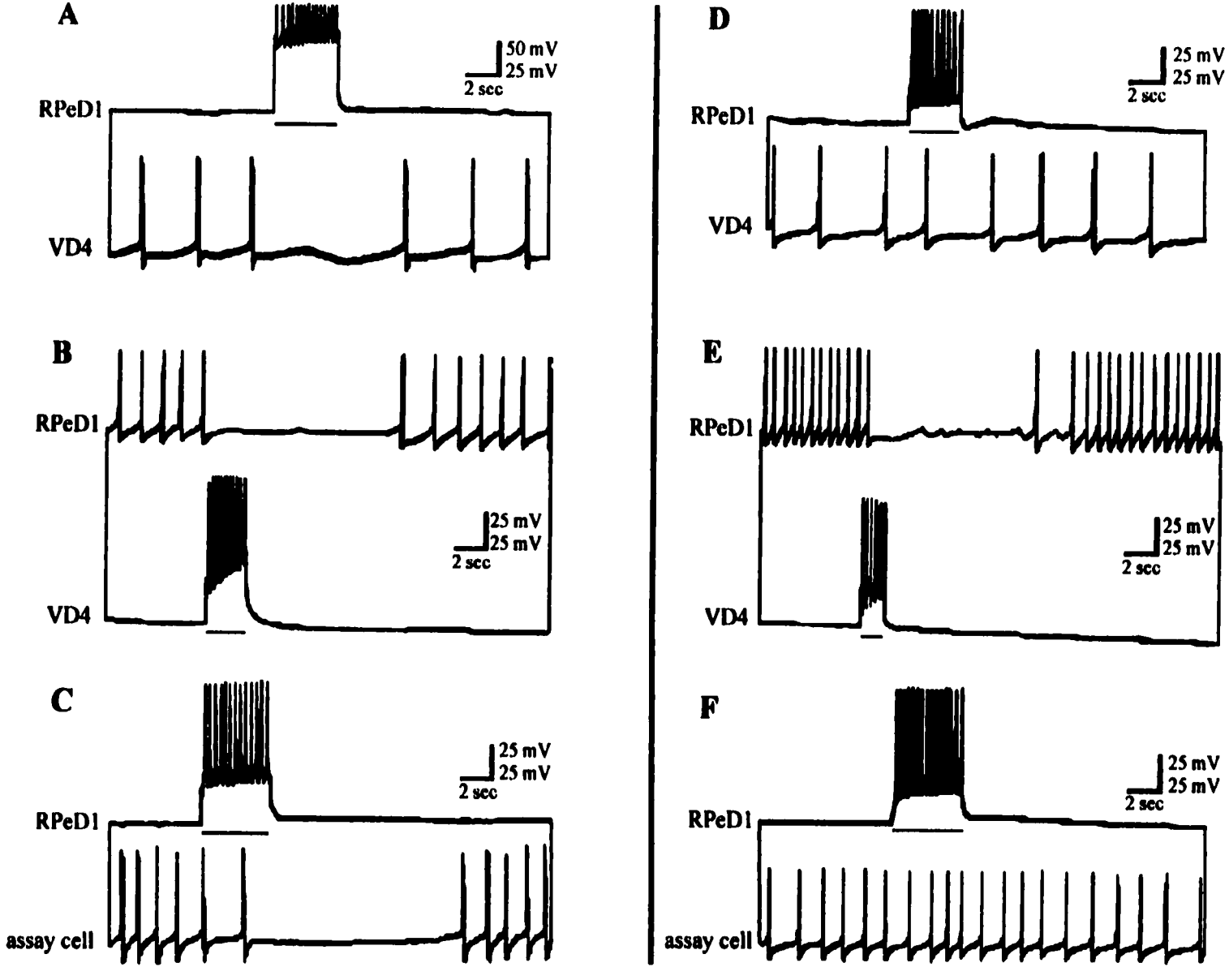

A
50 mV
25 mV
2 sec
RPeD1
VD4
B
RPeD1
25 mV
25 mV
2 sec
VD4
C
25 mV
25 mV
2 sec
RPeD1
assay cell
D
25 mV
25 mV
2 sec
RPeD1
VD4
E
RPeD1
25 mV
25 mV
2 sec
VD4
F
25 mV
25 mV
2 sec
RPeD1
assay cell

Figure 4.8. Exogenous FMRFamide suppressed evoked transmitter release from lone RPeD1 somata.

RPeD1 somata were isolated and cultured alone in DM containing various additional compounds. After 18hrs, the media was substituted with normal DM and the RPeD1 soma was assayed for its ability to release transmitter. **A.** An assay cell failed to detect transmitter release from the RPeD1 soma that was maintained in the presence of FMRFamide (10^{-6}M). **B.** In contrast, RPeD1 somata cultured in the presence of another inhibitory transmitter, 5-HT (10^{-6}M), released detectable levels of transmitter. **C.** RPeD1 cells cultured in the presence of both FMRFamide (10^{-6}M) and the protein synthesis blocker anisomycin (12.5 μg/ml), failed to release detectable amounts of transmitter. **D.** Similarly, heat-inactivated FMRFamide (10^{-6}M - 30min. in boiling water) also failed to suppress transmitter release from a lone RPeD1 cell.

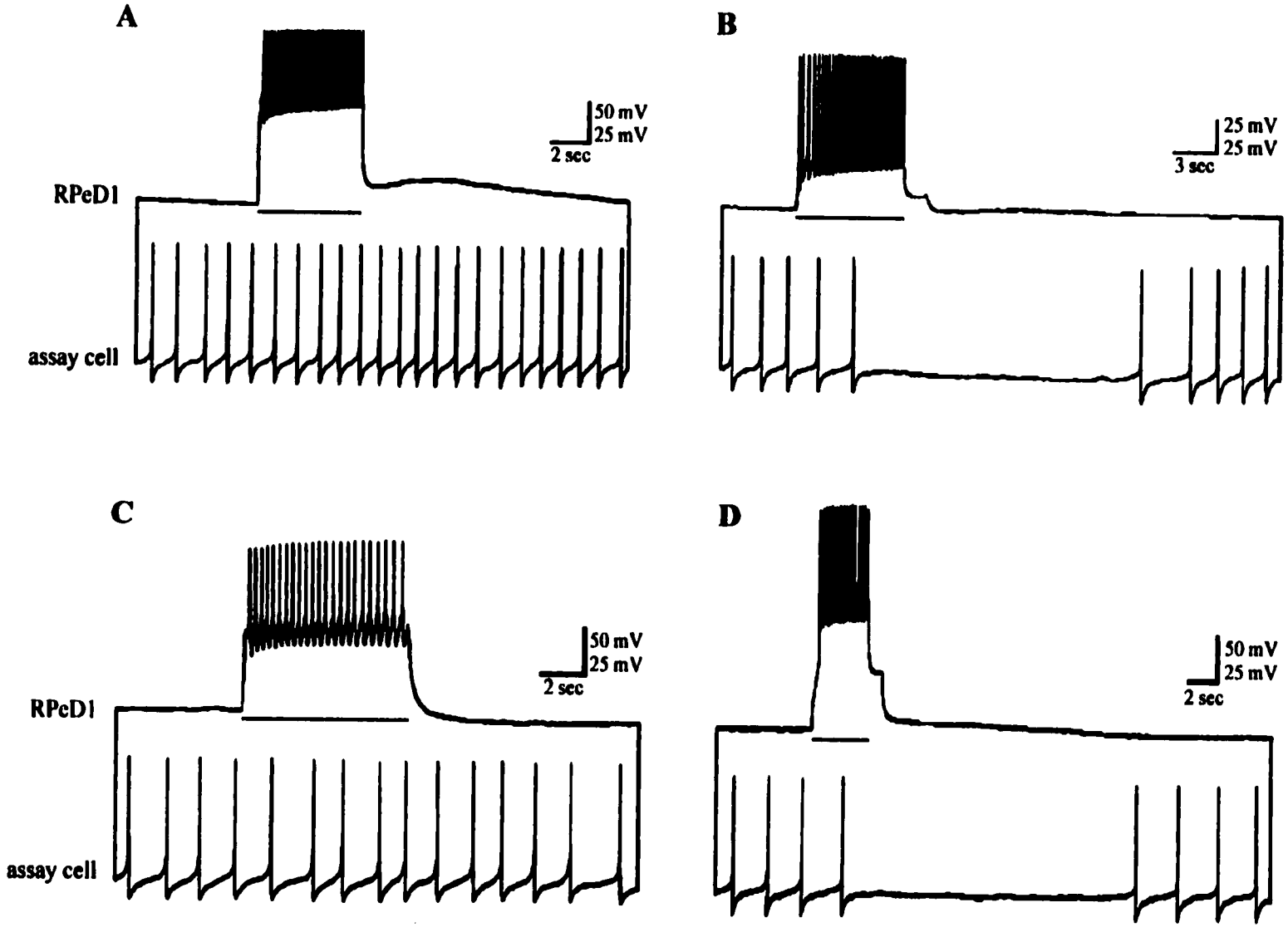
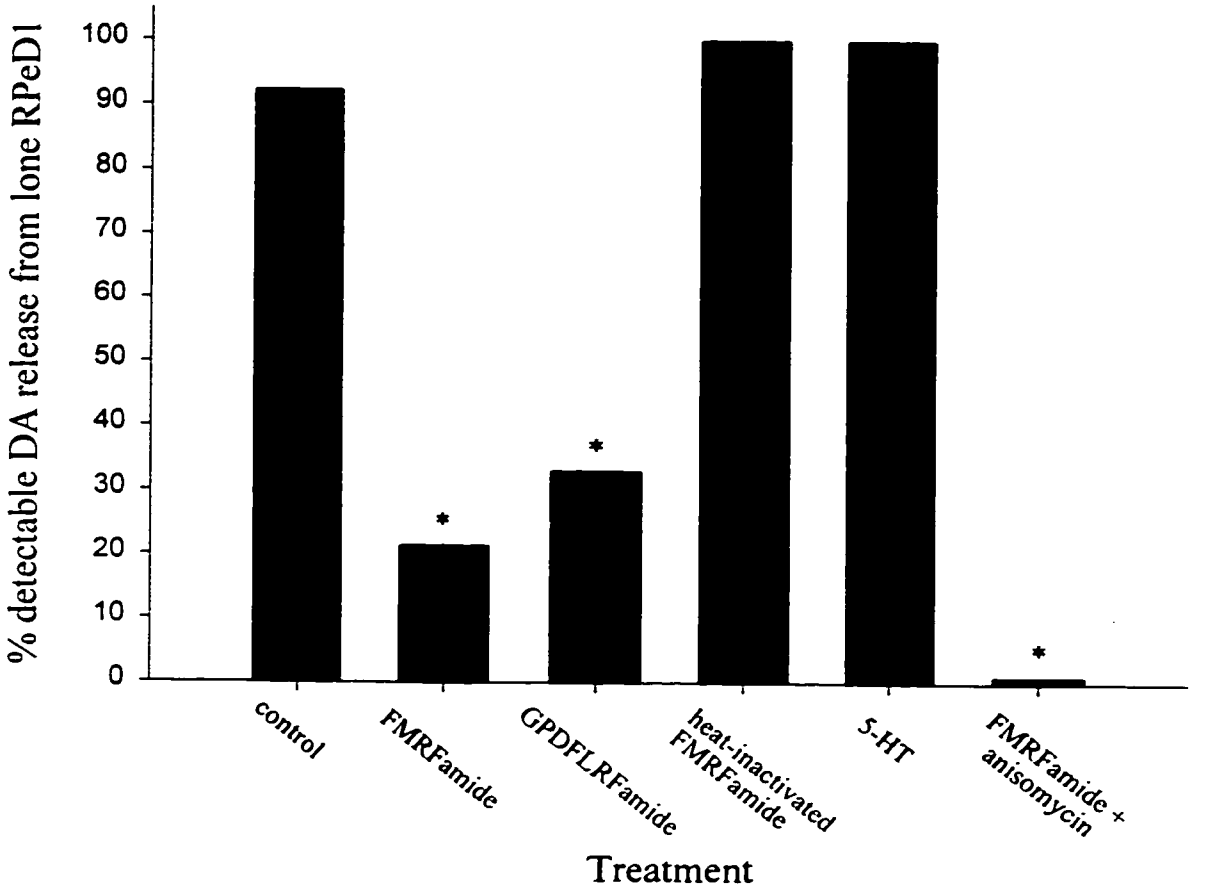

A
50 mV
25 mV
2 sec
RPeD1
assay cell
B
25 mV
25 mV
3 sec
C
50 mV
25 mV
2 sec
RPeD1
assay cell
D
50 mV
25 mV
2 sec

Figure 4.9. Summary data showing the incidence of evoked transmitter release from RPeD1 somata cultured alone under various experimental conditions.

As compared to control cells cultured in DM alone, the incidence of detectable transmitter release was significantly lower for RPeD1 maintained in FMRFamide ($P<0.001$), GDPFLRFamide ($P<0.001$), or a cocktail of FMRFamide and anisomycin ($P<0.001$). On the other hand, neither the inhibitory transmitter 5-HT, nor the heat-inactivated FMRFamide, had any effect on the incidence of detectable transmitter release ($P=1.000$ for both).


% detectable DA release from lone RPeD1
100
90
80
70
60
50
40
30
20
10
0
*
*
*
control
FMRFamide
GPDFLRFamide
heat-inactivated FMRFamide
5-HT
FMRFamide + anisomycin
Treatment

Chapter 5: Transmitter Release is Modulated by an Arachidonic Acid Signal Cascade in RPeD1

5.1 Introduction

The neuropeptide FMRFamide is a well-known modulator of synaptic transmission in various molluscan species (Bahls et al., 1992; Mackey et al., 1987; Man-Son-Hing et al., 1989; Montarolo et al., 1988; Santarelli et al., 1996; Schacher et al., 1993; Schacher and Montarolo, 1991; Small et al., 1992; Xu et al., 1995). For instance, by working on both pre and postsynaptic neurons, FMRFamide evokes short and long-term synaptic depression at the sensory-to-motor neuron synapse of the gill withdrawal reflex circuit in *Aplysia* (Abrams et al., 1984; Belardettie et al., 1987; Mackey et al., 1987; Montarolo et al., 1988; Piomelli et al., 1987). Belkin and Abrams (1993) have since demonstrated that FMRFamide activates at least two K^+ currents in the postsynaptic motor neurons which in turn decrease the responsiveness of these cells to subsequent excitatory synaptic inputs. A similar FMRFamide- induced activation of K^+ currents in the presynaptic cell was shown to result in the reduction of evoked transmitter release from the sensory neuron (Belardetti et al., 1987; Piomelli et al., 1987). Thus, in the *Aplysia* gill withdrawal circuit, FMRFamide acts on both pre and postsynaptic cells to bring about an overall reduction in the motor output.

In *Helisoma*, Man-Son-Hing et al., (1989) provided the first direct evidence that FMRFamide reduces evoked neurotransmitter release from *in vitro* cultured neurons by at least 2 separate mechanisms. Specifically, utilizing soma-soma synapses between *Helisoma* buccal neurons 5 and 19, they demonstrated that evoked postsynaptic currents were significantly reduced in the presence of FMRFamide. This FMRFamide-induced

reduction in synaptic transmission involved a decrease in the macroscopic, voltage-activated calcium current in presynaptic neuron B5, and a reduction in the sensitivity of its secretory machinery to intracellular calcium levels (Man-Son-Hing et al., 1989).

Although the receptor(s) mediating inhibitory FMRFamidergic responses have not yet been well characterized, the signal transduction pathway downstream of these receptors is known to involve the arachidonic acid (AA) cascade. In a variety of molluscan preparations (*Aplysia*: Belardetti et al., 1989; Buttner et al., 1989; Critz et al., 1991; Piomelli et al., 1987; *Helisoma*: Bahls et al., 1992; *Lymnaea*: Kits et al., 1997; Lopes et al., 1998), pharmacological blockers of the AA pathway attenuate the FMRFamide-induced increase in outward (K^+) current. Furthermore, FMRFamide-induced effects are mimicked by exogenous AA (Bahls et al., 1992; Critz et al., 1991; Piomelli et al., 1987). Downstream of AA, the signal cascade splits into at least 3 separate pathways mediated by lipoxygenase, cyclooxygenase, or cytochrome p50 metabolites respectively (Piomelli et al., 1987). In the majority of studies, the lipoxygenase branch of this cascade appears to be the primary downstream signaling pathway underling FMRFamide–induced activation of K^+ channels (Bahls et al., 1992; Kits et al., 1997; Lopes et al., 1998; Piomelli et al., 1987).

In most of the studies described above, FMRFamide was shown to act as a short term modulator of synaptic strength at fully mature synapses. Whether developing or newly formed synapses are also susceptible to FMRFamide-induced modulation of synaptic efficacy has not been previously demonstrated. Earlier in this thesis (chapter 4), I established that chronically applied FMRFamide and related peptides mimic VD4's effect on long term suppression of evoked transmitter release from RPeD1 during

synapse formation. The current study is a logical extension of this work and was designed to elucidate mechanisms by which both VD4 and FMRFamide-induced suppression of transmitter release was brought about in the giant dopamine cell. Specifically, I set out to test the hypothesis that VD4/FMRFamide-induced effects on RPeD1 are mediated through the metabolites of the AA pathway and that the perturbation of this second messenger cascade would render both VD4 and exogenous FMRFamide incapable of suppressing transmitter release from RPeD1.

5.2 Results

5.2.1. Exogenous FMRFamide induced varied responses in RPeD1

Previous studies on isolated ganglionic preparations have shown that RPeD1 exhibits a biphasic response to exogenously applied FMRFamide (Skingsley et al., 1993). To test the responsiveness of an *in vitro* isolated RPeD1 to FMRFamide, the giant dopamine cell was maintained in cell culture. After 12-18 hrs, intracellular recordings were made from a lone RPeD1 and FMRFamide was applied via pressure ejection. At a concentration of 10^{-6}M, FMRFamide consistently produced an inhibitory response in RPeD1 (n=12: fig. 5.1A). Specifically, a 0.5 second pressure pulse of FMRFamide (10^{-6}M) temporarily prevented the spontaneous firing of action potentials in RPeD1. Increasing the FMRFamide concentration (10^{-5}M) generated a biphasic response in most RPeD1 somata tested (73%: n=15: fig. 5.1B) which was characterized by an increase in the firing frequency followed by a period of reduced spontaneous activity. Interestingly, increasing the FMRFamide concentration further (10^{-4}M), produced a pure excitatory response in RPeD1 which lead to spike generation in 12 of 17 cells tested (fig. 5.1C), whereas the remaining 5 cells exhibited a biphasic response which was similar to that

produced by 10^{-5}M FMRFamide. These data, summarized graphically in figure 5.2, demonstrate that the incidence of inhibitory, biphasic, and excitatory responses changed significantly with increasing concentrations of applied FMRFamide ($\bullet\ ^2(4)=63.912$, $P<0.001$). The percentage of giant dopamine cells responding in a solely inhibitory manner decreased from 100%, at the lowest concentration, to 27% and 0% at higher concentrations of FMRFamide. Conversely, pure excitatory responses were seen only at the highest concentration of peptide (70% at 10^{-4}M FMRFamide). The incidence of biphasic responses to FMRFamide was highest at the intermediate peptide concentration (73% at 10^{-5}M) and lower (29% at 10^{-4}M) or non-existent at the other levels tested. Taken together, these data demonstrate that FMRFamide-induced effects on cultured RPeD1 neurons are concentration dependent and that a change in its responsiveness to higher concentrations is not merely quantitative; rather, it involves a complete switch from being purely inhibitory (10^{-6}M) to excitatory (10^{-4}M). The biphasic nature of FMRFamide response in the majority of RPeD1 cells led us to hypothesize that the giant dopamine cell most likely expresses two different types of FMRFamide receptors which exhibit varied sensitivities to exogenously applied peptide.

5.2.2. Excitatory FMRFamidergic response in RPeD1 was mediated by peptide-gated Na^+ channels

Since a FMRFamide-gated Na^+ channel has previously been identified and cloned from C2 neurons of *Helix aspersa* (Coscoy et al., 1998; Cottrell, 1997; Cottrell et al., 1990; Lingueglia et al., 1995) and was shown to mediate fast, excitatory responses in this snail, I asked whether the FMRFamidergic (10^{-4}M) excitatory response observed in RPeD1 also involved this peptide-gated ion channel. To test this hypothesis, individual

RPeD1 neurons were isolated and cultured overnight in DM. The culture media was replaced with normal saline prior to intracellular recordings and FMRFamide applied exogenously. FMRFamide (10^{-4}M) produced a pronounced depolarization in RPeD1 (mean = 13.3 ± 1.6 mV) from a holding membrane potential of –80 mV in all cells tested (n=6: fig. 5.3A). The normal saline was subsequently replaced with Na^+-free saline and FMRFamide was reapplied using the same application pipette. In the Na^+-free saline, FMRFamide (10^{-4}M) induced effects in RPeD1 were significantly reduced (mean = 2.0 ± 0.7 mV) from the holding potential of –80 mV ($P<0.001$ student's t-test; n=6: fig. 5.3A and B).

After demonstrating the dependence of the depolarizing response on external Na^+, benzamil, a pharmacological tool that has been shown previously to block the excitatory FMRFamidergic response in *Helix* (Lingueglia et al., 1995) was utilized. Acutely applied FMRFamide (10^{-4}M) depolarized all 6 cells from their holding membrane potential of –80mV (fig. 5.3C). These depolarizing effects in RPeD1were significantly (75% reduction) reduced in the presence of 10^{-5}M benzamil. (fig. 5.3D). Analysis of the mean amplitude of FMRFamide-induced depolarizing responses before, during, and after the benzamil treatment revealed a statistically significant reduction (fig. 5.4: ANOVA $P<0.001$). Following a 15minute wash with normal saline, the mean magnitude of FMRFamide-induced depolarizing response in RPeD1 returned to its baseline level (fig. 5.4). Since the inhibitory component of the biphasic response remained unperturbed in the presence of benzamil (unpubl. obs.), and excitatory responses returned to near baseline levels following washout, these data show that this drug is indeed specific (non-

toxic) and effective. Together, these data thus demonstrate that FMRFamide-induced excitatory response in RPeD1 likely involves a peptide gated Na^+ channel.

5.2.3. FMRFamide-induced inhibitory response in RPeD1 was mediated via the AA pathway

FMRFamide-induced inhibitory responses in various molluscan neurons (Volterra and Siegelbaum, 1988), including *Lymnaea* (van Tol-Steye, 1999) are mediated via G-protein coupled receptors that signal through AA metabolites. To test for the involvement of AA in the mediation of FMRFamide induced inhibitory effects in RPeD1, two well characterized (Bahls et al., 1992; Belkin and Abrams, 1993; Critz et al., 1991; Lopes et al., 1998; Piomelli et al., 1987a, 1987b) pharmacological blockers (NDGA and 4-BPB) of the AA pathway were used. Individual RPeD1 were isolated and cultured overnight in DM which was subsequently replaced with normal saline prior to intracellular recordings. Cells were impaled with intracellular electrodes and their responses to FMRFamide (10^{-6}M) were tested either in the absence or presence of the AA pathway blockers. FMRFamide-induced inhibitory effects in RPeD1 were consistently blocked by both NDGA (10^{-5}M) (n=7: fig. 5.5A,B) and 4-BPB (10^{-5}M) (n=6: fig. 5.5C,D). In the presence of the latter compound, the inhibitory response to 10^{-6}M FMRFamide was blocked in all 6 cells tested however in 4 cells, a slight increase in spontaneous firing was observed in RPeD1 following peptide application.

If the above drugs were indeed specific, then I reasoned that they should block only the inhibitory and not the excitatory component of the FMRFamide-induced (10^{-5}M) biphasic response in RPeD1. To test this possibility, RPeD1 was isolated and maintained in cell culture for 12-18 hrs and FMRFamide (10^{-5} M) was pressure applied either in the

absence or presence of NDGA and 4-BPB. In all instances (n=3) FMRFamide-induced inhibitory but not the excitatory effects were completely blocked by 10^{-5}M NDGA (fig. 5.6A,B) and 10^{-5}M 4-BPB (n=6: fig. 5.6C,D). These data thus confirm that inhibitory but not the excitatory component of the FMRFamide-induced biphasic response is mediated by the metabolites of AA pathway.

5.2.4. Inhibitors of Arachidonic acid pathway prevented VD4-induced suppression of dopamine release from RPeD1

To test the hypothesis that VD4-induced suppression of transmitter release from RPeD1 involved FMRFamide activated metabolites of the AA pathway, the giant dopamine cell was first cultured alone in DM containing both FMRFamide (10^{-6}M) and NDGA (5 x 10^{-6}M). Alternately, isolated RPeD1 somata were pre-incubated for 15 minutes in 4-BPB (10^{-5}M), an irreversible phospholipase A_2 blocker, and then cultured alone in FMRFamide (10^{-6}M). Following 18 hours in either experimental condition, the media was replaced with normal saline, and a freshly isolated (untreated) VD4 assay cell was added to the culture dish. RPeD1 was tested for its transmitter release capabilities by injecting depolarizing current pulses. The incidence of transmitter release from RPeD1 somata maintained for 18 hrs in the presence of various compounds is shown graphically in figure 5.7. Neither 4-BPB (n=6: 10^{-5}M in 0.1% DMSO) nor NDGA (n=10: 5 x 10^{-6}M in 0.1% DMSO) alone affected RPeD1's ability to release transmitter in DM (P=1.000 F.E. test for both). However, FMRFamide (10^{-6}M)-induced suppression of transmitter release from RPeD1 was prevented by both 4-BPB (n=7) and NDGA (n=10). Specifically, as compared with cells maintained for 18 hours in the presence of FMRFamide alone, both 4-BPB and NDGA prevented the peptide-induced suppression

of transmitter release from RPeD1 ($P<0.002$ F.E. test, FMRFamide vs. 4-BPB/FMRFamide: $P<0.003$ F.E. test, FMRFamide vs. NDGA/FMRFamide). These data strongly support the hypothesis that FMRFamide-induced suppression of transmitter release from RPeD1 involves AA and its metabolites. To test this possibility further, lone RPeD1 somata were cultured in the presence of AA for 18 hrs and evoked transmitter release capabilities were detected by an assay cell. In the presence of AA (5×10^{-6}M in 0.1% DMSO), only 33% (n=6) of the cultured RPeD1 somata were able to release a detectable amount of transmitter. This incidence of transmitter release was significantly lower than that found in control conditions ($P<0.017$: F.E. test). These values were nevertheless comparable to the incidence of transmitter release detected from FMRFamide treated cells (21%). Taken together, the above data strongly support our claim that FMRFamide-induced suppression of transmitter release from RPeD1 indeed involves an AA pathway.

5.2.5. Inhibitory synaptic transmission between VD4 and RPeD1 involves AA pathway

Although the above data demonstrate that FMRFamide-induced suppression of transmitter release from RPeD1 involves AA, they do not directly test the possibility that the synaptic transmission between VD4 and RPeD1 is also mediated via AA pathway. To test this possibility, VD4 and RPeD1 were individually isolated and paired for 24 hours in a soma-soma configuration. Simultaneous intracellular recordings were made from both cells and neurons exhibiting mutually inhibitory synapse were selected for further experimentation. Specifically, pairs in which RPeD1 inhibited the firing of action potentials in VD4 (fig. 5.8A) and vice versa (fig. 5.8B) were bath perfused with the

inhibitors of AA metabolites. In 75% of the tested pairs, perfusion of NDGA (10^{-5}M) did not affect synaptic transmission from RPeD1 to VD4 (fig. 5.8C), whereas the synaptic transmission from VD4 to RPeD1 was completely blocked (n=4: fig. 5.8D). Similarly, in 5 out of 6 pairs perfused with 4-BPB (10^{-5}M), the inhibitory transmission between VD4 and RPeD1, but not from RPeD1 to VD4, was completely blocked.

Interestingly, in the presence of 4-BPB but not NDGA, an excitatory component of the VD4-RPeD1 transmission become evident, even though the cells were consistently held at similar membrane potentials (fig. 5.9). Specifically, when RPeD1 was maintained at membrane potentials of –70 to 80 mV, compound action potentials in VD4 induced a brief but fast depolarization phase (fig. 5.9B,C) followed by a slower, longer lasting hyperpolarization. The depolarizing potentials observed here and in many other instances of recording from normal cells (not shown) did not lead to the firing of action potentials in RPeD1. However, bath perfusion with 4-BPB (10^{-5}M), which completely blocked the inhibitory component, greatly facilitated the VD4-induced depolarizing responses in RPeD1 (fig. 5.9D), often to a magnitude that generated action potentials RPeD1 (fig. 5.9E).

To test whether the excitatory component of VD4-induced depolarization in RPeD1 involved a second transmitter, this synaptic transmission was also tested in the presence of benzamil (Na^+ channel blocker). Soma-soma paired VD4/RPeD1 neurons were simultaneously impaled with sharp electrodes and synapses tested electrophysiologically. (fig. 5.10A, 5.11A). The paired cells were first bath perfused with 10^{-5}M 4-BPB which blocked the inhibitory component and uncovered the excitatory phase of the biphasic response in RPeD1 (fig. 5.10B, 5.11B). To test whether the

excitatory component was mediated by the FMRFamide-gated Na^+ channel, the preparation was bathed with benzamil (10^{-5}M) and synapses were re-tested. In the presence of benzamil, the excitatory component of the synaptic transmission between VD4 and RPeD1 was completely abolished (n=4: fig. 5.10C, 5.11C).

Taken together, the above pharmacological manipulation of the VD4 to RPeD1 synapse *in vitro* revealed that the synaptic transmission between VD4 and RPeD1 is indeed mediated by a FMRFamide-related peptide, which activates an AA mediated pathway in the giant dopamine cell. The above data also raise the possibility that synaptic communication from VD4 to RPeD1 may include a weak excitatory component, which underlies a dominant inhibitory phase. Furthermore, this experiment indicates that these responses are mediated by a FMRFamide-like peptide, which acts on two different receptor sub-types.

5.2.6. Synapse formation between VD4 and RPeD1, and VD4-induced suppression of transmitter release from RPeD1 were both blocked by inhibitors of the AA pathway

In chapter 3, I demonstrated that VD4 is first to establish synaptic communication with RPeD1 in the soma-soma configuration. Subsequently, I showed that VD4-induced suppression of transmitter release from RPeD1 involves FMRFamide release from VD4 and the activation of an AA mediated pathway in the giant dopamine cell. I next sought to determine whether synapse formation between VD4 and RPeD1 and the VD4-induced suppression of transmitter release from RPeD1 could also be blocked by inhibitors of the AA pathway. To test this hypothesis, cells were soma-soma paired either in the presence of NDGA (5×10^{-6}M), or RPeD1 was pre-incubated for 15 minutes in 4-BPB (10^{-5}M)

prior to pairing with VD4. After 18 hours in culture, the synapses were tested electrophysiologically by simultaneous recording from both cells. Under control conditions (DM only), an inhibitory connection between VD4 to RPeD1 was found in 73% of the pairs while mutually inhibitory synapses were seen in only 17% of the soma-soma pairs (n=30: fig. 5.12). None of the soma-soma pairs in control conditions had an inhibitory connection from RPeD1 to VD4 without the presence of reciprocal communication. Cell pairs in which the RPeD1 was pretreated for 15 minutes with 0.1% DMSO showed a similar ratio of synapse formation: in 4 out of 5 pairs tested, 1 way inhibitory synapse formation was found from VD4 to RPeD1. Likewise, when DMSO was included in the culture media (0.1%), 3 out of 4 cell pairs tested exhibited a 1 way inhibitory synapse from VD4 to RPeD1. In contrast, treatment with 4-BPB or NDGA caused significant changes in the incidence of inhibitory synapse formation between these two cells (• $^{2}(6)=49.467$, $P<0.001$). Intracellular recordings from soma-soma pairs exposed to 4-BPB revealed an inhibitory connection from RPeD1 to VD4 in 7 out of 9 pairs. The reciprocal inhibitory connection from VD4 to RPeD1 was present in only 1 of these 9 cell pairs. Furthermore, under these conditions, VD4 to RPeD1 inhibitory connections were never seen alone. Chronic treatment with NDGA, on the other hand, reduced the formation of inhibitory synaptic connections in both directions. When cultured in NDGA, 78% cells (n=9) failed to establish any synaptic connection, while 22% formed a 1-way inhibitory connection (VD4• RPeD1) - an incidence greatly reduced from control levels. These data demonstrate that AA pathway inhibitors specifically suppress the incidence of inhibitory synapse formation between VD4 and RPeD1, however, the effects of NDGA are less specific than that of 4-BPB. Since in the

absence of synaptic activity between VD4 and RPeD1, the giant dopamine cell was able to establish its inhibitory synapse with VD4, these data also demonstrate that the transmitter release from the giant dopamine cell was no longer suppressed by VD4.

Interestingly, although the inhibitory synaptic connection between VD4 and 4-BPB pre-treated RPeD1 paired in DM was rarely observed (n=1 of 9), in 3 soma-soma pairs strong excitatory synaptic transmission was detected between VD4 and RPeD1. Specifically, invoked action potentials in VD4 increased the frequency of action potentials in an RPeD1 that was spontaneously active (fig. 5.13A), caused the firing of action potentials in an electrically silent RPeD1 (fig. 5.13B), or induced 1:1 EPSPs in RPeD1 (fig. 5.13C). Since a hyperpolarizing current pulse applied to VD4 had no effect on the membrane potential of a paired RPeD1 (fig. 5.13A), and the rising phase of VD4-induced EPSP in RPeD1 (fig. 5.13C) occurred only after the presynaptic action potentials, I believe that the above excitatory synaptic transmission was mediated chemically.

Moreover, it was observed that in instances of excitatory synaptic communication between VD4 and RPeD1, dopamine release was successfully detected from all RPeD1 cells. These data thus demonstrate that VD4 -induced suppression of transmitter release from RPeD1 is contingent upon the formation of appropriate inhibitory but not the inappropriate excitatory synapse between the soma-soma paired cells.

5.3 Discussion

In this chapter, I provided the first direct evidence that FMRFamide induced effects in RPeD1 are mediated by two distinct receptors: one, a peptide-gated Na^+ channel and the other linked to AA metabolite mediated signaling. Blocking the AA pathway by

specific inhibitors not only prevented FMRFamide-induced suppression of transmitter release from the giant dopamine cell, but it also blocked specific inhibitory synapse formation between VD4 and RPeD1. These data thus underscore the possibility that neuropeptides such as FMRFamide may play a novel developmental role in synapse formation in addition to their previously established role as modulators of synaptic efficacy at mature synapses.

FMRFamide-like peptides have long been implicated as transmitters utilized by VD4 at synaptic contacts with a number of its target cells. This assumption, however, is largely based on immunohistochemical (McKenney, 1992) and *in situ* hybridization (Santama et al., 1995; Saunders et al., 1992; Skingsley et al., 1993) data, in addition to the fact that VD4-induced postsynaptic effects (*in vivo*) in most instances are mimicked by exogenous FMRFamide (McKenney, 1992; Skingsley et al., 1993). Since in a number of other studies (McKenney, 1992; Nesic et al., 1996), FMRFamide, or related peptide, application alone failed to mimic VD4-induced effects on particular target cells, this neuron was hypothesized to contain more than one transmitter. Consistent with this notion were data from the intact ganglia, which showed that when stimulated electrically, VD4 produced a biphasic (fast excitation followed by slower depolarization; Benjamin, 1984; Magoski, 1996; Park and Winlow, 1993) response in RPeD1 and that only the inhibitory component of this dual response was mimicked by FMRFamide-like peptides (Nesic et al., 1996). However, Skingsley et al., (1993) noted that exogenous FMRFamide could evoke a biphasic response (fast depolarization followed by a slower hyperpolarization) in intact RPeD1 somata. The depolarizing phase of this biphasic response was seen only at higher concentrations of the applied peptide ($\bullet\ 10^{-4}$M).

Although in most studies VD4-induced depolarizing response did not lead to spiking activity in RPeD1, Park and Winlow (1993) did nevertheless found a small percentage of intact ganglionic preparations in which action potentials in VD4 produced spikes in the giant dopamine cell. In the present study, the VD4 to RPeD1 soma-soma synapse occasionally exhibited a biphasic component (for example see fig. 5.10A), however the depolarizing phase never lead to spiking in RPeD1 under control conditions. Since in the presence of 4-BPB, induced action potentials in VD4 generated strong excitatory responses in RPeD1 that were attenuated by the peptide-gated Na^+ channel blocker benzamil, our data support a general conclusion that both components of the biphasic response are produced by a FMRFamide-like peptide. The possibility that VD4 may also release more than one transmitter, however, can still not be excluded.

In all studies described above, neither the receptor coupling nor the second messenger pathways were examined in detail. The present study is thus the first to demonstrate that FMRFamide-induced effects on isolated RPeD1 somata are not only FMRFamide specific but they are also dose-dependent. Specifically, I have demonstrated that at lower concentrations (10^{-6}M), FMRFamide produced a prolonged inhibitory response in RPeD1. At a concentration of 10^{-5}M, however, FMRFamide generated a biphasic (excitation followed by inhibition) response in RPeD1 which switched to being completely excitatory at a concentration of 10^{-4}M. These data thus demonstrate that FMRFamide alone is sufficient to mimic VD4's biphasic postsynaptic effects on the giant dopamine cell.

Little is known about various types or subtypes of peptidergic receptors in invertebrates. However, if I assume that molluscs such as *Lymnaea*, *Aplysia*, and

Helisoma possess at least a limited diversity of various subtypes of peptide receptors, each of which affects different ion channels at particular optimal peptide concentrations, then it is perhaps reasonable to propose that a dynamic range of postsynaptic responsiveness could be achieved by varying the concentration of any given peptide at a specific synapse. For instance, as shown in figure 5.1, if VD4 were to release FMRFamide in the range of 10^{-6}M then the postsynaptic response in RPeD1 would be expected to be purely inhibitory, whereas this peptide released at a concentration of 10^{-5}M and 10^{-4}M will likely induce a biphasic and/or pure excitatory response respectively. Thus, the sign of the postsynaptic response, and even the role this synapse plays in the neural circuit, could possibly altered merely by changing the relative concentration of peptide released from the presynaptic cell.

The data presented in this chapter demonstrate that FMRFamide-induced excitatory and inhibitory effects in RPeD1 involved two different receptors: one that gates ion channel (excitatory) and the other coupled through an AA signal cascade (inhibitory). The time course of these postsynaptic responses (fast depolarizing - direct ion channel gating and slow prolonged - G protein coupled pathway) is consistent with this respective pathway coupling. Although the precise concentration of FMRFamide released at VD4 to RPeD1 synapse is at present difficult to deduce, I can speculate from electrophysiological data that it would be in the range of 10^{-7} to 10^{-6}M since postsynaptic reponses in RPeD1 to VD4 stimulation are predominantly inhibitory. However, in instances where a biphasic response is observed I suggest that the FMRFamide release may range from 10^{-5} to 10^{-4}M.

The inhibitory component of the biphasic response in RPeD1 appears to be mediated by a receptor similar to that demonstrated in other molluscan species (Bahls et al., 1992; Belkin and Abrams, 1993; Critz et al., 1991; Piomelli et al., 1987; Sasaki et al., 1997; Yanaura et al., 1993), namely, a G-protein coupled receptor that signals through an arachidonic acid signal cascade. In other preparations, this receptor is characterized by a relative high sensitivity to external FMRFamide and a long time delay between ligand binding and ion channel opening/closing (Belkin and Abrams, 1993). The excitatory component of the FMRFamidergic response in RPeD1, on the other hand, is reminiscent of a peptide-gated Na^+ channel that has been described in other molluscs (Cottrell et al., 1990). This channel is shown to be highly selective to Na^+ ions, exhibits relatively low sensitivity to external FMRFamide, and it can be blocked by benzamil (Coscoy et al., 1998; Cottrell 1997; Lingueglia et al., 1995; Zhainazarov and Cottrell, 1998). In the present study, the kinetics of the biphasic response (fast excitation followed by delayed inhibition), coupled with its dependence on FMRFamide concentration (see fig. 5.1), the differing pharmacological responses of excitatory (see fig. 5.3) and inhibitory responses (see fig. 5.6), and the dependence of the excitatory response on external Na^+, all support the conclusion that these two distinct responses are mediated by separate receptors.

The involvement of arachidonic acid pathway in FMRFamidergic synaptic transmission in molluscs is well-established. For example, in *Helisoma* (Bahls et al., 1992), *Aplysia* (Belkin and Abrams, 1993; Critz et al., 1991; Mackey et al., 1987; Piomelli et al., 1987; Schacher et al., 1993), and *Lymnaea* (Lopes et al., 1998; van Tol-Steye, 1999), FMRFamide activates phospholipase A_2 which subsequently synthesizes of AA. Arachidonic acid is subsequently broken down into a variety of metabolites

(Piomelli et al., 1987) each of which in turn can activate further signaling cascades in the cell (Lopes et al., 1998; Piomelli et al., 1987). In molluscs, FMRFamide/AA-mediated effects have consistently been found to signal through lipoxygenase pathway, downstream of AA (Bahls et al., 1992; Lopes et al., 1998; Piomelli et al., 1987). In the present study, I demonstrated that both phospholipase A_2 (4-BPB) and a lipoxygenase inhibitor (NDGA) attenuated FMRFamide-induced inhibitory effects on cultured RPeD1 somata. Furthermore, AA was also found sufficient to mimic both the FMRFamide and the VD4-induced suppression of transmitter release from RPeD1. The data presented in this study is consistent with previous findings in *Aplysia*, *Lymnaea*, and *Helisoma*. (Bahls et al., 1992; Lopes et al., 1998; Piomelli et al., 1987) and together, these studies suggest that FMRFamide receptor and its signaling pathway is conserved in various molluscan species. Moreover, taken together with data presented in chapter 3 and 4, this study demonstrates that VD4-induced suppression of transmitter release from RPeD1 involves FMRFamide release from VD4, which in turn activates an AA mediated pathway in RPeD1.

FMRFamide-induced, acute suppression of transmitter release has previously been reported in both *Aplysia* (Abrams et al., 1984; Piomelli et al., 1987) and *Helisoma* (Haydon et al., 1991; Man-Son-Hing et al., 1989). In both of these species, the FMRFamide-induced suppression of transmitter release was shown to be mediated via lipoxygenase metabolites of AA (Bahls et al., 1992; Piomelli et al., 1987). These lipoxygenase metabolites activate K^+ channels, which in turn hyperpolarize the neurons (Belkin and Abrams, 1993, 1998; Critz et al., 1991; Lopes et al., 1998). Lipoxygenase metabolites have also been shown to decrease voltage activated Ca^{2+} currents which, in

parallel with the activated outward K^+ current, serves to hyperpolarize the cell. This reduction in voltage activated Ca^{2+} current may also result in reduced Ca^{2+} influx in response to action potentials, thus reducing the total amount of transmitter that is released at the synaptic sites. In *Aplysia*, a potential source of FMRFamide in the tail shock/siphon withdrawal circuit are modulatory interneurons located in the left pleural ganglion (LPL16: Mackey et al., 1987; Small et al., 1992). Peptide release from these neurons is thought to modulate the siphon withdrawal reflex by reducing transmitter release from the sensory neurons (Mackey et al., 1987). A similar physiological role for FMRFamide in *Helisoma* has yet to be described. The present study is the first to show that a similar mechanism of FMRFamide-induced suppression of transmitter release occurs during synaptogenesis between two well-characterized respiratory CPG neurons.

Since AA itself can also act as a retrograde messenger (for review, see Fitzsimonds and Poo, 1998), it is therefore feasible that VD4 induced effects on RPeD1 may be mediated by AA release from VD4. This postulate is however inconsistent with data presented in this study for the following reasons. 1) 4-BPB is an irreversible blocker of phospholipase A_2 (Belkin and Abrams, 1993; Blackwell and Flower, 1983) -an enzyme which is required for the synthesis of AA. 2) RPeD1, but not the VD4 somata, were pretreated with this drug prior to soma-soma pairing, therefore, the formation of AA was prevented only in RPeD1, not in VD4. 3) If VD4 were to produce AA as a retrograde signal, this molecule would have still been available to suppress transmitter release from RPeD1 by diffusing between the closely opposed somata and activating downstream metabolites in the giant dopamine cell. In other words, AA production in VD4 would have suppressed transmitter release from RPeD1 by acting downstream of the

pharmacological block. This was not however the case, since the transmitter secretory capabilities of giant dopamine cells remained unperturbed following treatment with the phospholipase A_2 inhibitor. Thus, the data in this study rule out the possibility that VD4-induced suppression of transmitter release from RPeD1 involves AA release from VD4.

An important finding of the present study is the apparent presence of FMRFamide-gated Na^+ channels at the synaptic contact site between VD4 and RPeD1. Following perfusion with 4-BPB, which removed inhibitory responses, evoked action potentials in VD4 consistently produced excitatory postsynaptic potentials in RPeD1 (see fig. 5.9). These excitatory potentials were often sufficiently large to cause spiking in RPeD1 (see fig. 5.9,5.11), an effect that was never seen in control soma-soma pairs. Furthermore, these EPSPs could be completely abolished by subsequent perfusion with benzamil (fig. 5.11). Although the precise mechanism by which 4-BPB "uncovered" these excitatory responses remains to be investigated, one possibility is that under normal conditions the inhibitory component dominates and thus prevents the depolarizing potential from reaching the spike threshold. Removal of this inhibitory component by 4-BPB may thus allow the giant dopamine cell to generate spikes. A similar switch from hyperpolarizing to depolarizing response to applied FMRFamide has also been reported in *Aplysia* sensory neurons after 4-BPB treatment (Belkin and Abrams, 1993). Since this switch in the neuronal response to applied FMRFamide occurred within minutes of 4-BPB perfusion, the involvement of newly synthesized receptors is highly unlikely. The electrophysiological and pharmacological data presented here leads us to conclude that a FMRFamide-gated Na^+ channel and a FMRFamide receptor G-protein coupled with AA

signal cascade are co-expressed postsynaptically on the RPeD1 somatic membrane. Although further experiments are required to substantiate these claims, our data are nevertheless the first to support such a possibility.

One of the greatest advantages of working with identified and functionally well defined neurons is that the information obtained at a single cell level can be extrapolated to deduce the network configuration of the entire circuit. Since VD4 and RPeD1 are well-characterized respiratory central pattern generator (CPG) neurons, the data obtained in the present study help us to understand the cellular, synaptic and pharmacological basis of FMRFamidergic synaptic transmission between VD4 and RPeD1. Specifically, *in vivo*, VD4 and RPeD1 form a mutually inhibitory synapse as part of the respiratory CPG (Syed and Winlow, 1991). Although, the cellular and synaptic basis of dopaminergic neurotransmission at the RPeD1 to VD4 synapse is well defined (Magoski et al., 1995; Barnes et al., 1994), less is known about the peptidergic synaptic transmission between VD4 and RPeD1. The present study demonstrates that the synaptic transmission between VD4 and RPeD1 is FMRFamidergic, involves two different receptors, and is partially mediated via the AA pathway. The co-localization of excitatory and inhibitory FMRFamide receptors in RPeD1 may provide a degree of functional plasticity within the respiratory CPG. For example, since the two receptor types are activated by different external peptide concentrations, the efficacy of synaptic form and strength could thus be directly regulated by VD4 releasing differing amounts of transmitter at any time given time point. This scenario may fit behavioral repertoire following long periods of respiratory inactivity during which peptide filled vesicles likely accumulate in VD4 at its contact sites with the giant dopamine cell. I therefore propose that under these

conditions, VD4 may directly initiate the respiratory activity by exciting the giant dopamine cell. Once activated, RPeD1 then takes over and VD4 may fall back to serve its conventional modulatory role. During early phases of respiratory rhythm generation, enhanced FMRFamide release may continue to prime the CPG by raising the level of excitability in the giant dopamine cell (Syed et al., 1990; Syed and Winlow, 1991). This possibility however remains to be examined experimentally.

A further exciting finding of this study relates to the complete switching of synaptic sign (from inhibitory to excitatory) between VD4 to RPeD1 in the presence of phospholipase A_2 inhibitor, 4-BPB. Specifically, I demonstrated that if RPeD1 were pretreated with 4-BPB prior to pairing with VD4, 30% of the soma-soma pairs established excitatory synaptic communication from VD4 to RPeD1. These data are important and significant since they demonstrate that neurotransmitter (such as FMRFamide)-induced activation of the AA pathway is required for the formation of appropriate inhibitory synapses between VD4 and RPeD1. On the other hand, these data provide first direct evidence that in the absence of AA metabolites, these neurons can establish excitatory synapses. Although it appears that this excitatory synapse is novel (ie: rarely or never appears in the intact animal), I feel that it is an appropriate synapse in that its presence *in vivo* likely contributes to the early depolarization phase of the commonly seen biphasic VD4 to RPeD1 synapse. Thus, instead of the formation of an inappropriate synapse, I postulate that by blocking the inhibitory component of the biphasic transmission I have "unmasked" a component of synaptic communication common between these two cells. Since these excitatory synapses formed in DM (does not contain extrinsic trophic factors), this data is in sharp contrast to other studies from

our laboratory which showed growth factors are required for excitatory, but not inhibitory, synapse formation (Feng et al., 1997; Hamakawa et al., 1999; Woodin et al., 1999). Although the expression of appropriate postsynaptic receptor(s)/signal pathway in RPeD1 and release of an excitatory transmitter from the presynaptic cell (VD4) are capable of occurring independently from extrinsic trophic support in the current study, the possibility of intrinsic trophic support (ie: partner cell derived factors) could not be ruled out. Moreover, since the precise mechanisms underlying trophic factor-induced inhibitory/excitatory synaptic switching are at present unknown (Hamakawa et al., 1999 Woodin et al., 1999), the data presented in this study are an early indication of possible intracellular signaling pathways involved.

In summary, the data presented in this chapter demonstrate that both VD4 and FMRFamide exert diverse effects on RPeD1, which involve two distinct receptors and an AA mediated pathway. Moreover perturbation of AA metabolites not only blocks appropriate inhibitory synapse formation between VD4 and RPeD1 but, consistent with our hypothesis, also blocks VD4-induced suppression of transmitter release from the giant dopamine cell. Finally, perturbation of the FMRFamide activated AA pathway results in inappropriate excitatory synapse formation between VD4 and RPeD1. Together, these data thus underscore the importance of neurotransmitter - especially neuropeptides – receptor interactions during early synapse formation between the CNS neurons.

Figure 5.1. FMRFamide exerts diverse effects on isolated somata of RPeD1.

Individual RPeD1 somata were cultured overnight in DM and subsequently impaled with sharp electrodes. Various concentrations of FMRFamide were tested for their ability to modulate RPeD1 activity. **A.** At a concentration of 10^{-6}M, a brief pulse of FMRFamide consistently produced an inhibitory response in RPeD1, which transiently prevented the spontaneous firing of action potentials in this cell. **B.** A brief pulse of FMRFamide applied at a concentration of 10^{-5}M, however, induced a biphasic (excitation followed by inhibition) response in RPeD1, whereas at a concentration of 10^{-4}M, it produced a purely excitatory response in RPeD1 (**C**). (Peptide applied at arrows).

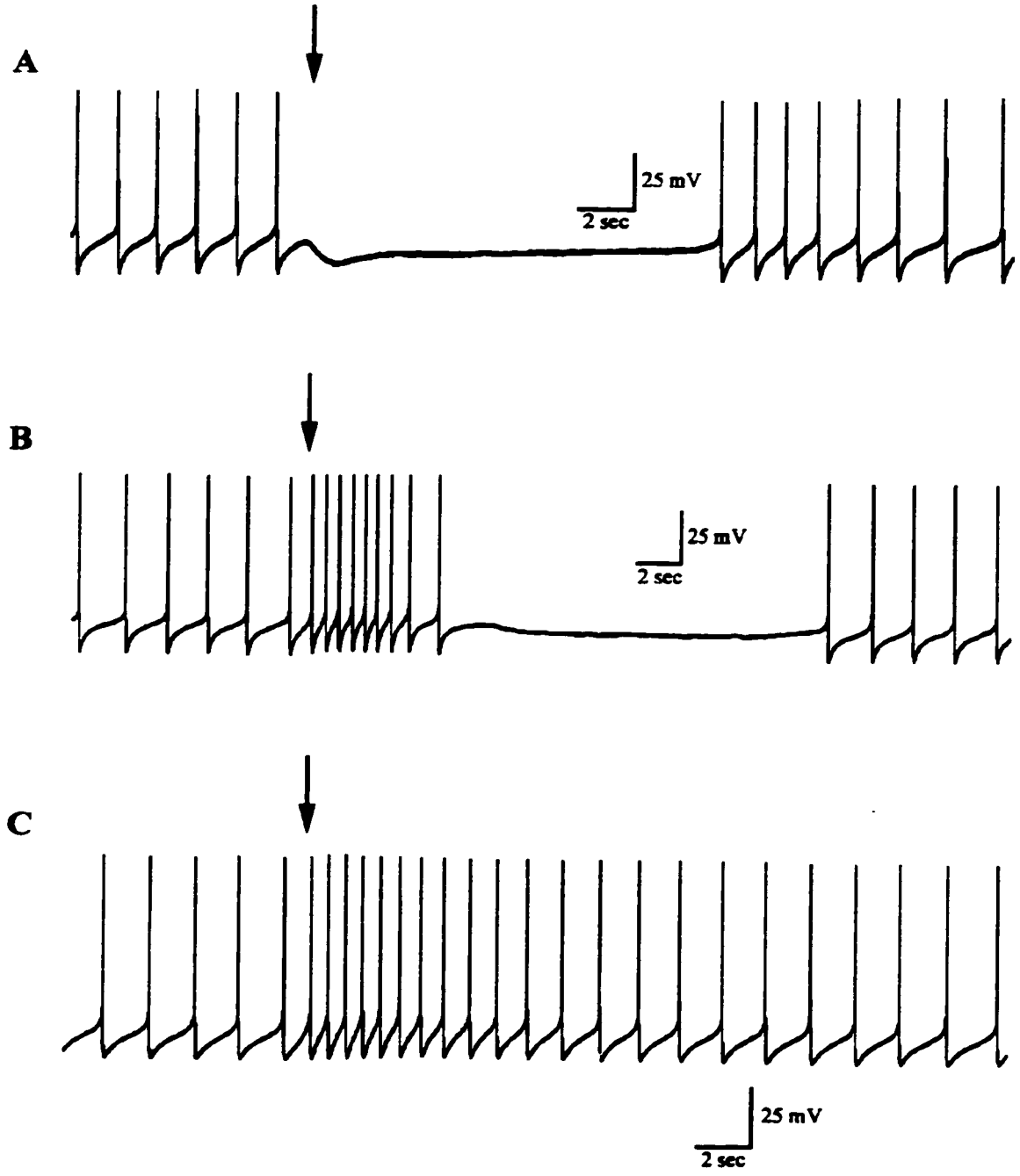

A
25 mV
2 sec
B
25 mV
2 sec
C
25 mV
2 sec

Figure 5.2. Summary data showing the effects of various FMRFamide concentrations on RPeD1 activity.

At lower concentrations (10^{-6}M), RPeD1 consistently responded in an inhibitory manner to exogenously applied FMRFamide, whereas, 10^{-5}M and 10^{-4}M FMRFamide produced biphasic and excitatory effects respectively.

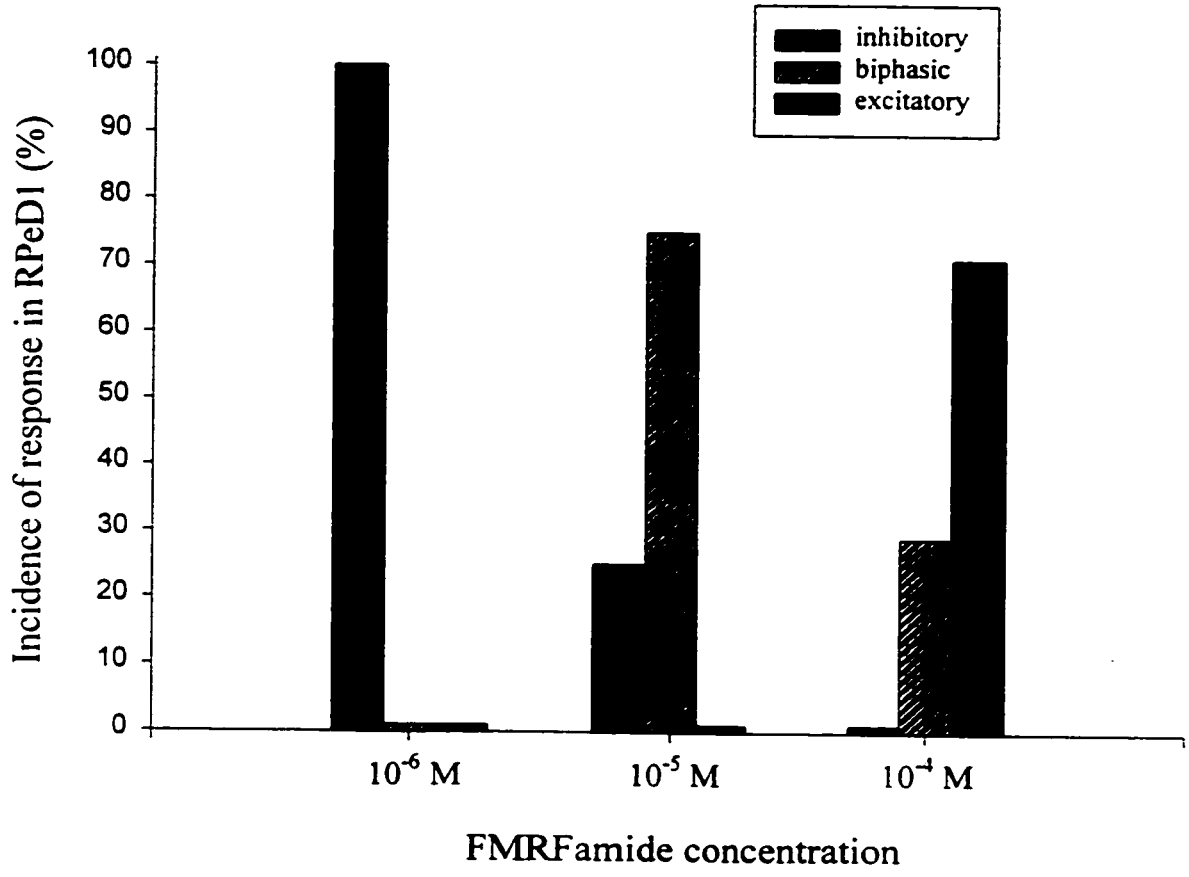

inhibitory
biphasic
excitatory
Incidence of response in RPeD1 (%)
100
90
80
70
60
50
40
30
20
10
0
10-6 M
10-5 M
10-4 M
FMRFamide concentration

Figure 5.3. FMRFamide-induced excitatory response in RPeD1 was sensitive to external Na^+ and benzamil.

Individual RPeD1 somata were isolated and cultured overnight in DM. The bath solution was subsequently replaced with normal saline and the RPeD1 somata were impaled with intracellular microelectrodes. A brief pulse of FMRFamide (10^{-4}M) from a closely positioned pipette consistently caused a large depolarization in RPeD1 somata held at a membrane potential of –80 mV (**A**). In the presence of Na^+-free saline, FMRFamide (10^{-4}M also dissolved in Na^+-free saline) application caused only a minimal depolarization (perhaps an artifact of pressure pulses) in the RPeD1 (membrane potential = –80 mV: **B**. Similarly, FMRFamide (10^{-4}M) induced a large depolarizing response from –80 mV (**C**), that was blocked by benzamil (10^{-5}M: **D**). (Peptide applied at arrows).

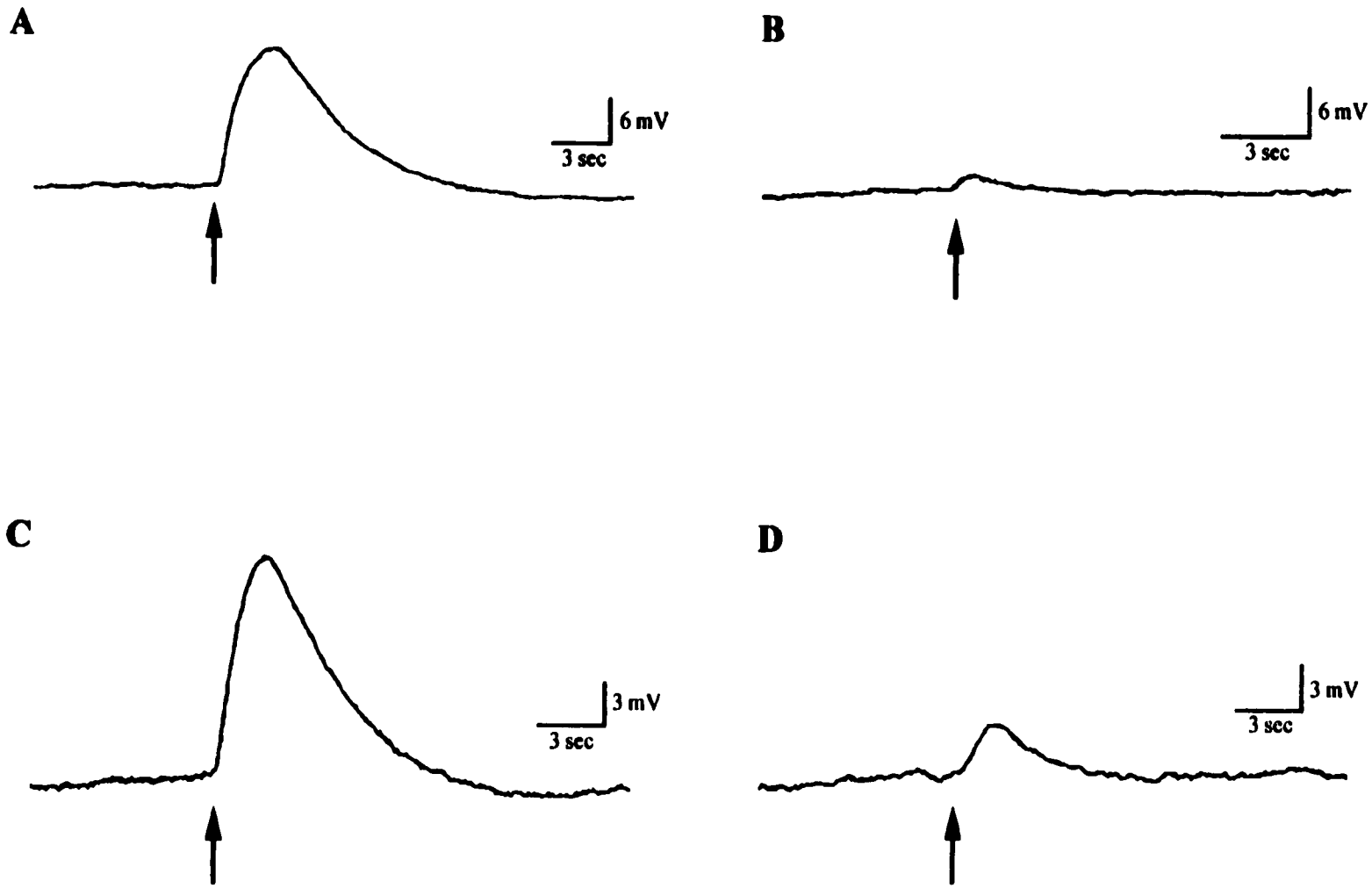

A
6 mV
3 sec
B
6 mV
3 sec
C
3 mV
3 sec
D
3 mV
3 sec

Figure 5.4. Summary data showing the effects of benzamil treatment on FMRFamidergic-induced excitatory responses in RPeD1.

A group of 6 RPeD1 somata were sequentially tested for their response to pressure applied FMRFamide (10^{-4}M) before, during, and after bath perfusion with the Na^+ channel blocker, benzamil. During the initial control period in saline, normalized depolarizing responses from a holding potential of –80 mV were relatively steady. These were however significantly reduced (~ 30% of the normalized control values) after bath perfusion of benzamil. The depolarizing response to applied FMRFamide were partially restored following wash out with normal saline (normalized values on graph generated by dividing each measured depolarization by the mean depolarization elicited in that particular cell for each treatment: points on graph = mean ± SEM, * denotes significant difference from control).

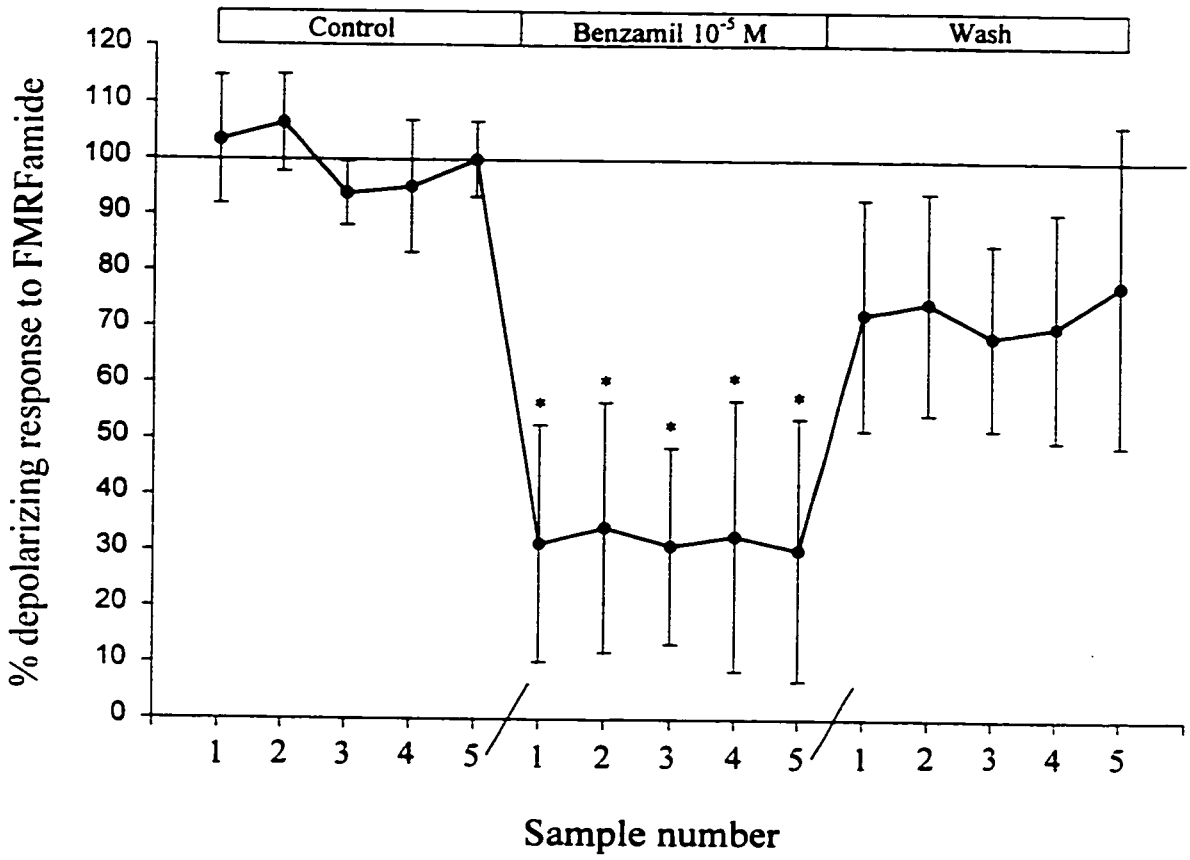

Control
Benzamil 10^{-5} M
Wash
% depolarizing response to FMRFamide
120
110
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Sample number

Figure 5.5. Inhibitors of AA metabolites blocked FMRFamide-induced inhibitory responses in RPeD1.

RPeD1 somata were individually isolated and cultured overnight in DM. Following replacement of the bath media with normal saline, intracellular electrophysiological recordings revealed consistent inhibitory responses to low concentrations (10^{-6}M) of pressure applied FMRFamide (**A,C**). **B**. These inhibitory responses were completely blocked in the presence of NDGA (10^{-5}M: an inhibitor of the lipoxygenase branch of the arachidonic acid signaling pathway). Bath perfusion with 4-BPB (10^{-5}M: an inhibitor of phospholipase A_2) on the other hand, switched the RPeD1's responsiveness to FMRFamide from inhibitory to excitatory (**D**). Specifically, following 4-BPB perfusion, a brief pulse of FMRFamide (10^{-6}M) increased the rate of spontaneous action potentials in RPeD1 (**D**) and completely removed the inhibition seen in control cells (**C**). (Peptide applied at arrows).

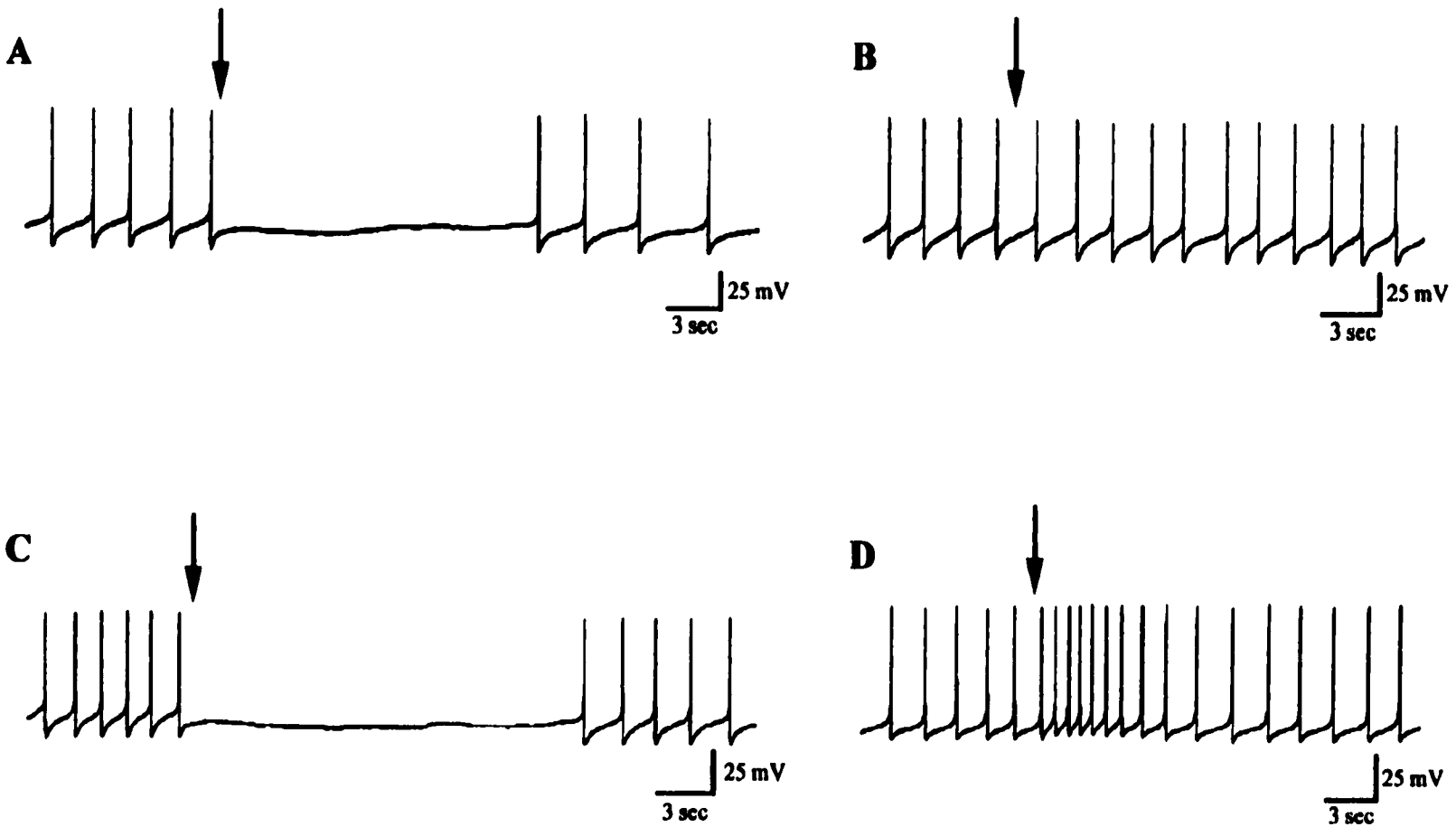

A
25 mV
3 sec
B
25 mV
3 sec
C
25 mV
3 sec
D
25 mV
3 sec

Figure 5.6. FMRFamide induced inhibitory, but not the excitatory, responses in RPeD1 were blocked by the inhibitors of the AA pathway.

Intracellular recordings were made from individually cultured RPeD1 somata and FMRFamide (10^{-5} M) was applied exogenously. In normal saline, FMRFamide (10^{-5}) consistently produced a biphasic response, which was characterized by fast depolarizing phase followed by a slower hyperpolarizing component (**A**). In the presence of NDGA (10^{-5}M), however FMRFamide caused a purely excitatory response (**B**). Similarly, in the presence of 4-BPB, the biphasic response in RPeD1 (**C**), switched completely to a pure excitatory (**D**). (Peptide applied at arrows).

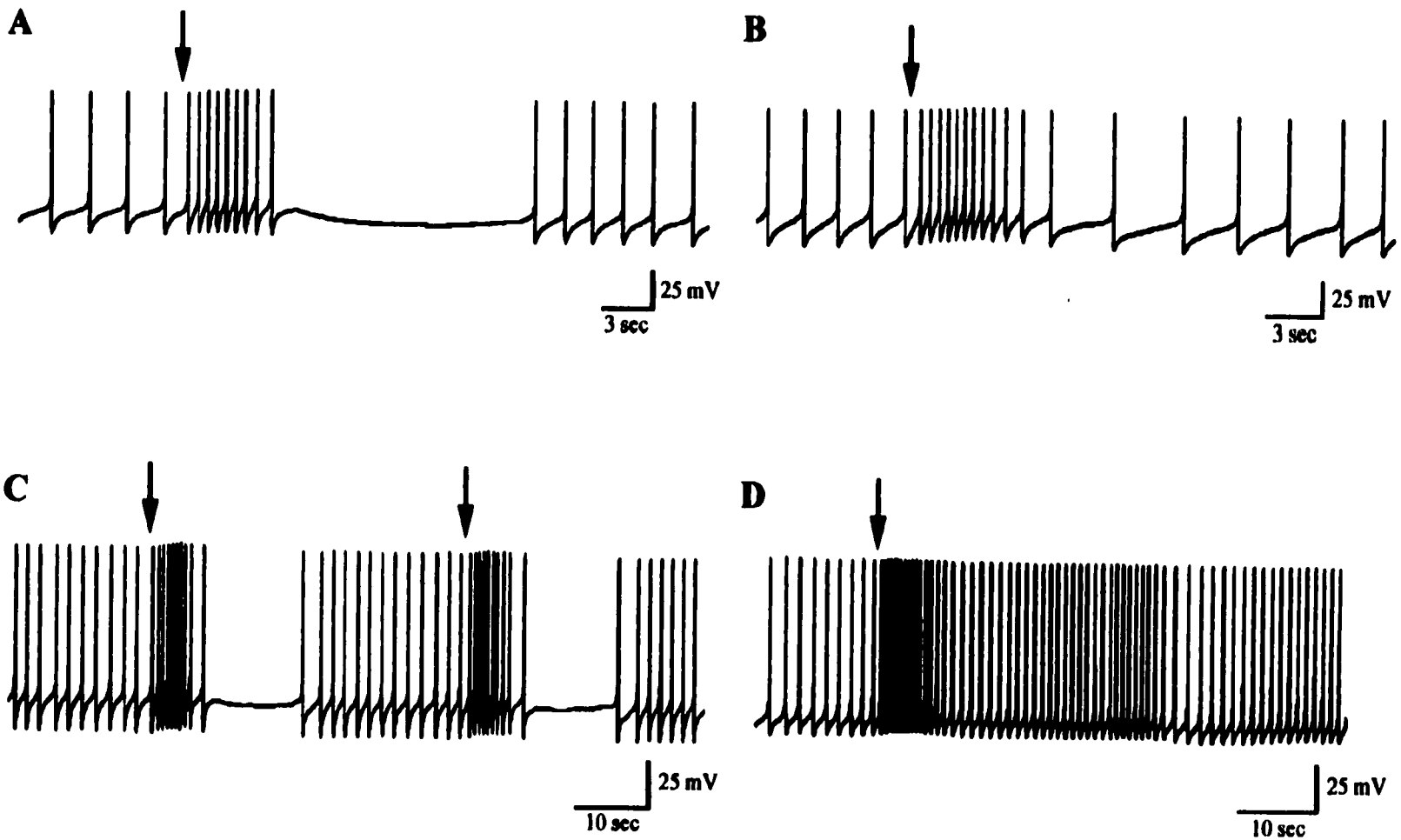

A
25 mV
3 sec
B
25 mV
3 sec
C
25 mV
10 sec
D
25 mV
10 sec

Figure 5.7. FMRFamide-induced suppression of transmitter release from RPeD1 involved the AA pathway.

Individually isolated RPeD1 somata were cultured alone for 18 hours in DM containing various inhibitors of the AA pathway. The experimental solution was subsequently exchanged with normal saline and a freshly isolated assay cell was added to the culture dish to test for evoked transmitter release from RPeD1. As shown previously (fig 4.10), evoked transmitter release was consistently detected from RPeD1 that was cultured in DM alone (control), but not from cells that were chronically treated with FMRFamide (10^{-6}M). FMRFamide-induced suppression of transmitter release from RPeD1 was however prevented by both 4-BPB (phospholipase A_2 blocker 10^{5}M) and NDGA (lipoxygenase blocker 5 x 10^{-6}M). FMRFamide-induced effects on transmitter suppression from RPeD1 were also mimicked by arachidonic acid (5 x 10^{-6}M) (* indicate significant difference from control).

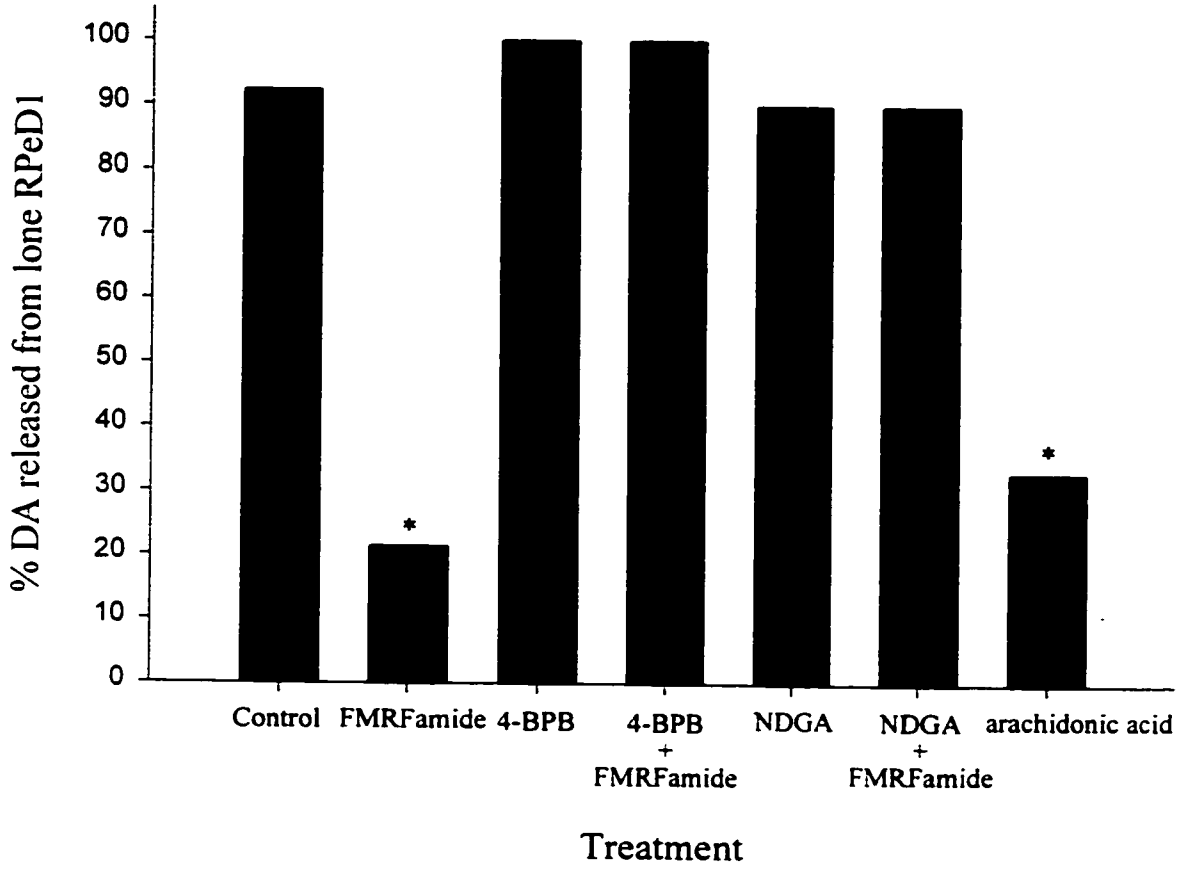

% DA released from lone RPeD1
100
90
80
70
60
50
40
30
20
10
0
*
*
Control
FMRFamide
4-BPB
4-BPB + FMRFamide
NDGA
NDGA + FMRFamide
arachidonic acid
Treatment

Figure 5.8. Inhibitory synaptic transmission between VD4 and RPeD1 was blocked by NDGA.

VD4 and RPeD1 were cultured in a soma-soma configuration for 24 hours. Simultaneous intracellular recordings indicated the presence of a mutually inhibitory connection. Specifically, induced action potentials in RPeD1 inhibited spontaneous action potentials in VD4 (**A**) and vice versa (**B**). Following bath perfusion with NDGA (10^{-5}M), the soma-soma connection was re-tested. RPeD1 stimulation under these experimental conditions continued to inhibit spontaneous action potentials in VD4 (**C**), however, the VD4-induced inhibitory response in RPeD1 were completely blocked by NDGA (**D**).

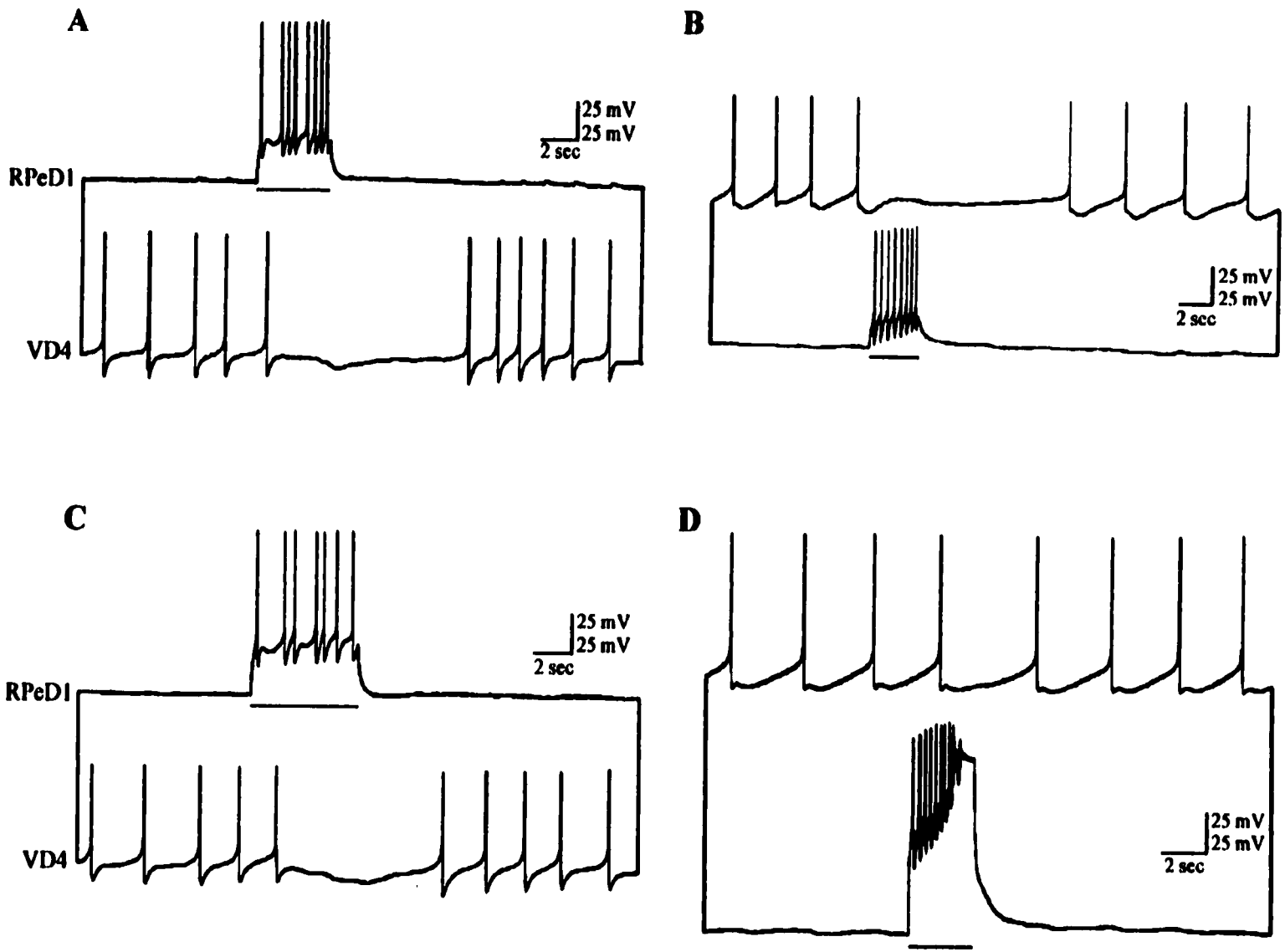

A
RPeD1
VD4
25 mV
25 mV
2 sec
B
25 mV
25 mV
2 sec
C
RPeD1
VD4
25 mV
25 mV
2 sec
D
25 mV
25 mV
2 sec

Figure 5.9. 4-BPB altered the VD4–induced inhibitory response in RPeD1 to an excitatory response.

VD4 and RPeD1 were paired in normal DM and synaptic connections were analyzed electrophysiologically. **A.** Induced action potentials in VD4 produced a large, compound, postsynaptic potential in the giant dopamine cell held at –60mV. **B.** At a membrane potential of –70 mV, induced action potentials in VD4 produced a biphasic response in RPeD1. This was characterized by small, fast, 1:1 depolarizations followed by a slower, compound, hyperpolarization. The fast depolarizating response did not involve electrical coupling since hyperpolarizing current in VD4 (dotted line) had no effect on the membrane potential of RPeD1. **C.** Further hyperpolarization of RPeD1 to –80 mV resulted in larger 1:1 depolarizations in response to action potentials in VD4. **D.** Bath perfusion with 4-BPB (10^{-5}M) resulted in a large increase in the amplitude of depolarizing postsynaptic potentials in RPeD1 that was held at the same membrane potential (-80 mV). **E.** These large, excitatory, postsynaptic potentials observed in 4-BPB were often of sufficient amplitude to induce action potentials in RPeD1, even if this cell was maintained at –80 mV.

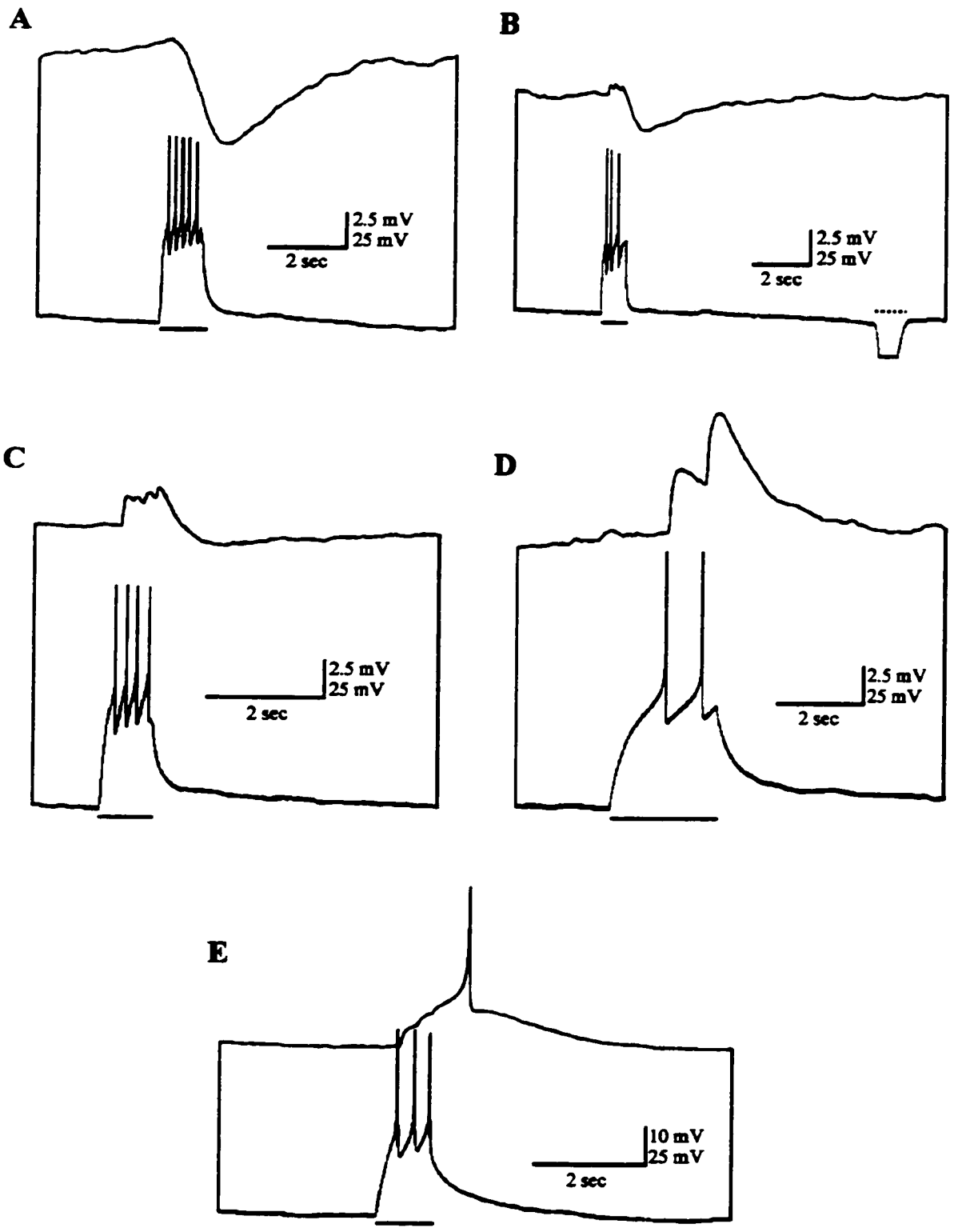

A
2.5 mV
25 mV
2 sec
B
2.5 mV
25 mV
2 sec
C
2.5 mV
25 mV
2 sec
D
2.5 mV
25 mV
2 sec
E
10 mV
25 mV
2 sec

Figure 5.10. VD4-induced excitatory responses in RPeD1 were blocked by benzamil.

Soma-soma synapses were reconstructed between VD4 and RPeD1 in DM. **A.** Simultaneous intracellular recordings after 24 hours in culture revealed the presence of an inhibitory synapse between VD4 and RPeD1. Specifically, induced action potentials in VD4 caused small, 1:1 postsynaptic potentials in RPeD1 and prevented spontaneous firing. **B.** Following bath perfusion with 4-BPB (10^{-5}M), induced action potentials in VD4 resulted in an increase in RPeD1's spontaneous firing rate. **C.** This excitatory response was blocked by subsequent bath perfusion with benzamil (10^{-5}).

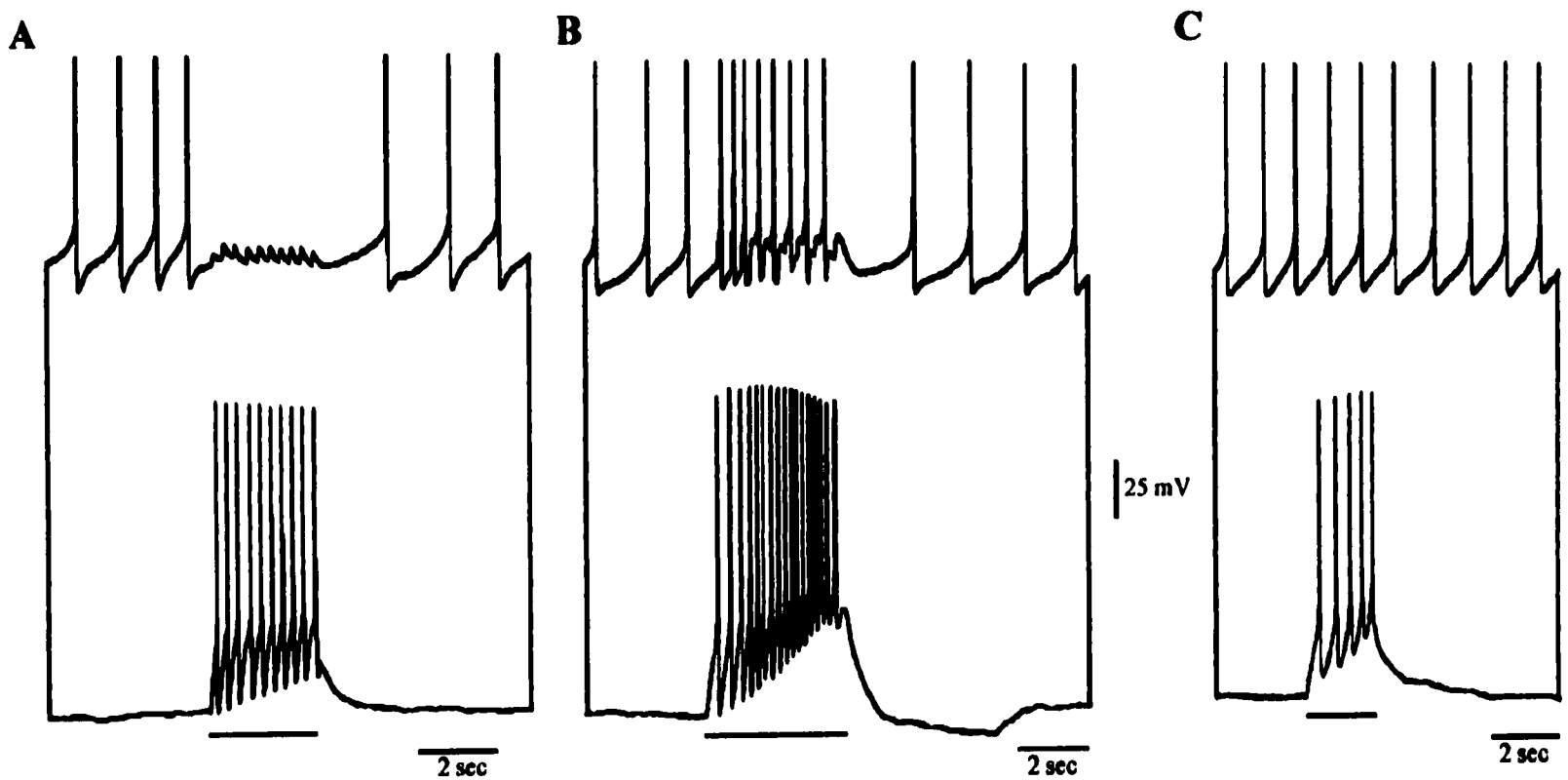

A
B
C
25 mV
2 sec
2 sec
2 sec

Figure 5.11. Benzamil blocked VD4-induced depolarizing responses in RPeD1.

A. When held at a membrane potential of –80 mV in DM, an induced action potential in VD4 elicited a fast, depolarizing response in a soma-soma paired RPeD1. This VD4-induced EPSP did not lead to action potentials in RPeD1. **B.** Following perfusion with 4-BPB (10^{-5}M), however an evoked action potential in VD4 caused a larger postsynaptic response in RPeD1 which lead to action potential generation in the giant dopamine cell. **C.** VD4-induced depolarizing responses in RPeD1 were completely blocked by bath perfusion with benzamil (10^{-5}M).

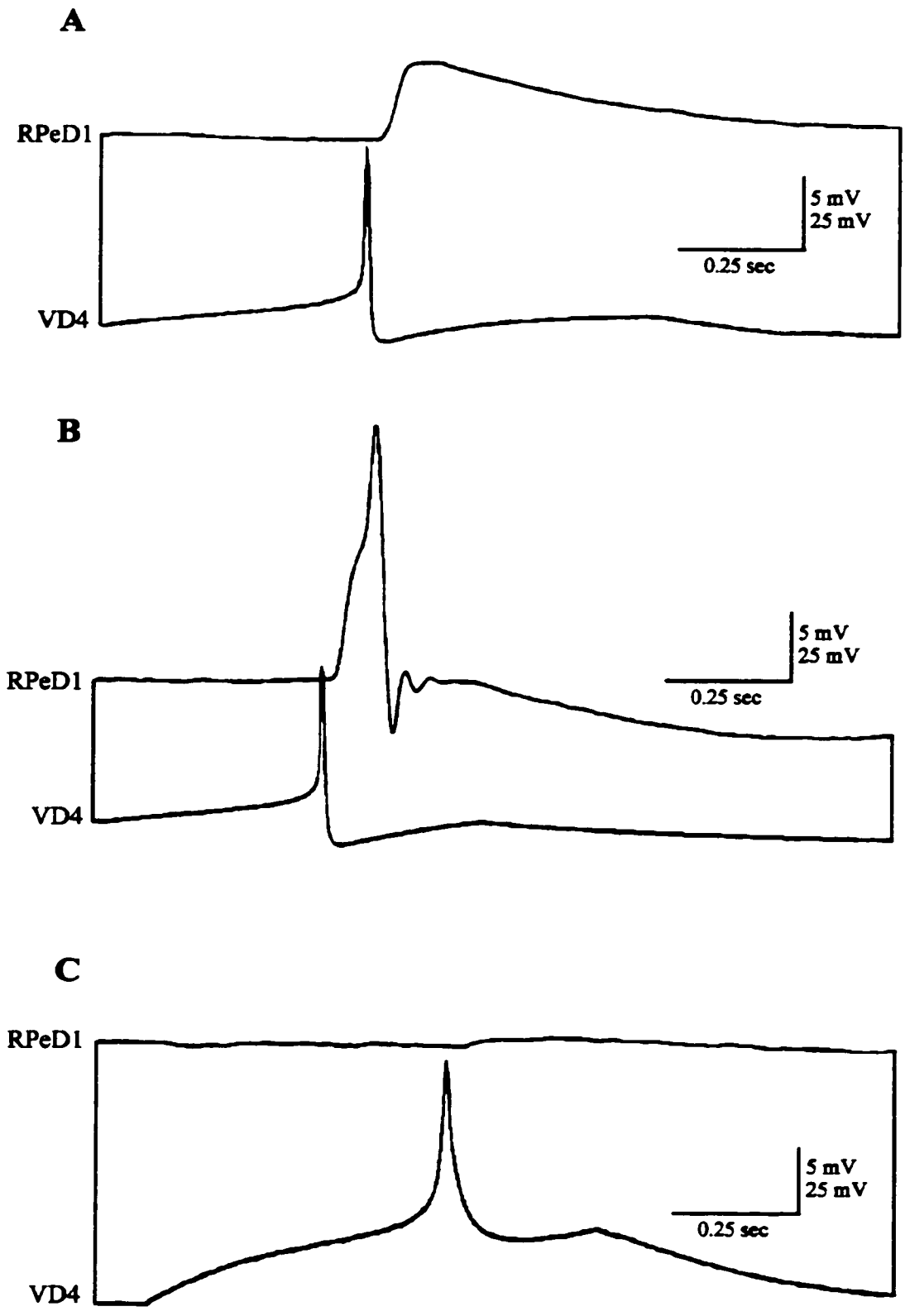

A
RPeD1
VD4
5 mV
25 mV
0.25 sec
B
RPeD1
VD4
5 mV
25 mV
0.25 sec
C
RPeD1
VD4
5 mV
25 mV
0.25 sec

Figure 5.12. Synapse formation between VD4 and RPeD1, and the VD4-induced suppression of transmitter release from RPeD1, were blocked by NDGA and 4-BPB.

To determine the significance of AA in synapse formation between VD4 and RPeD1, and the VD4-induced suppression of transmitter release from RPeD1, the giant dopamine cell was paired with VD4 either in the presence of NDGA or following pretreatment with 4-BPB. Specifically, in the later protocal, the dopamine cell was pretreated with the irreversible phospholipase A_2 blocker, 4-BPB, and subsequently paired with an untreated VD4 in DM. After 18 hours of pairing, the soma-soma synapse was characterized electrophysiologically. Compared to control (DM) conditions, the incidence of 1 way inhibitory synapse between VD4 and RPeD1 was significantly reduced in both experimental conditions. Conversly, the incidence of 1 way inhibitory (RPeD1• VD4) communication was greater in the 4-BPB pretreated experimental group than control pairs. A freshly isolated assay cell detected evoked transmitter release from the RPeD1 soma in all pairs exposed to either 4-BPB or NDGA.

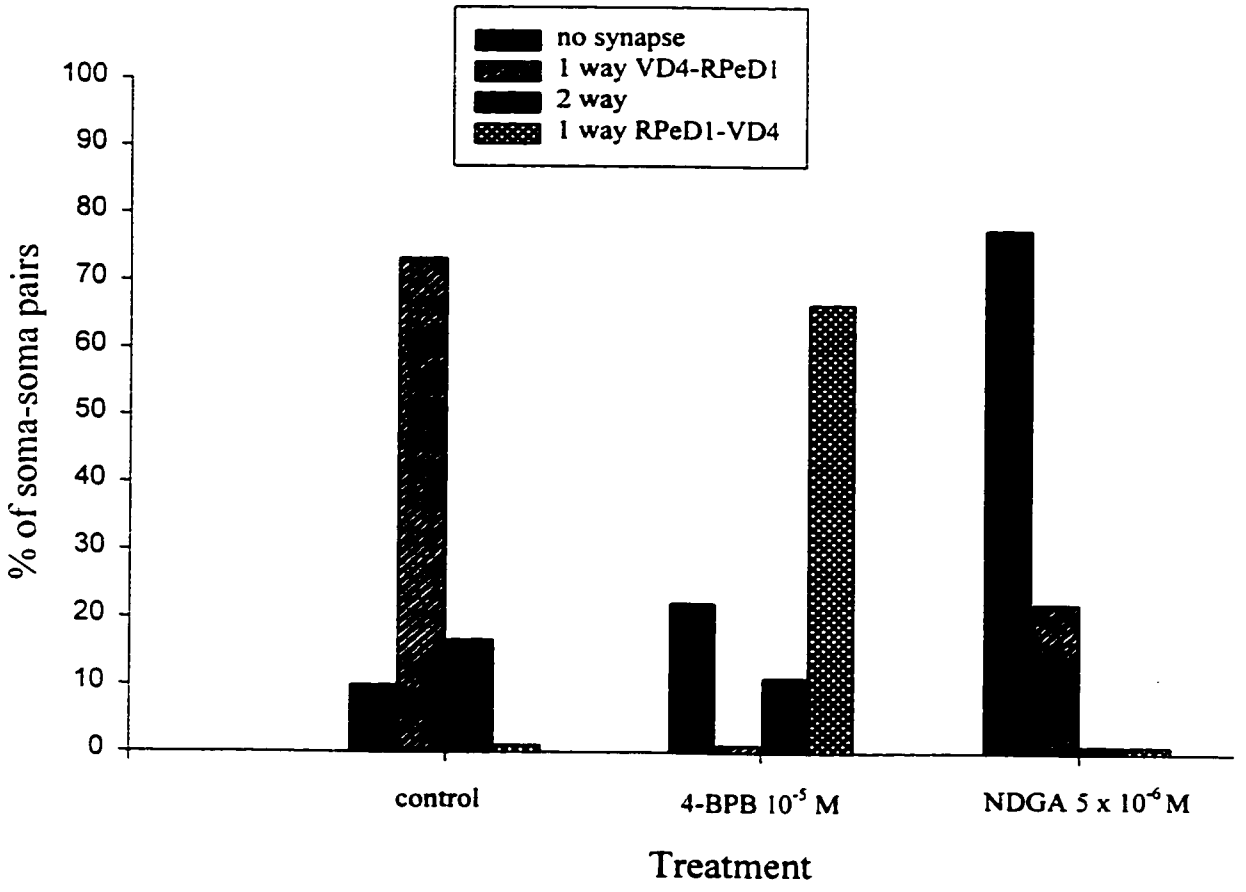
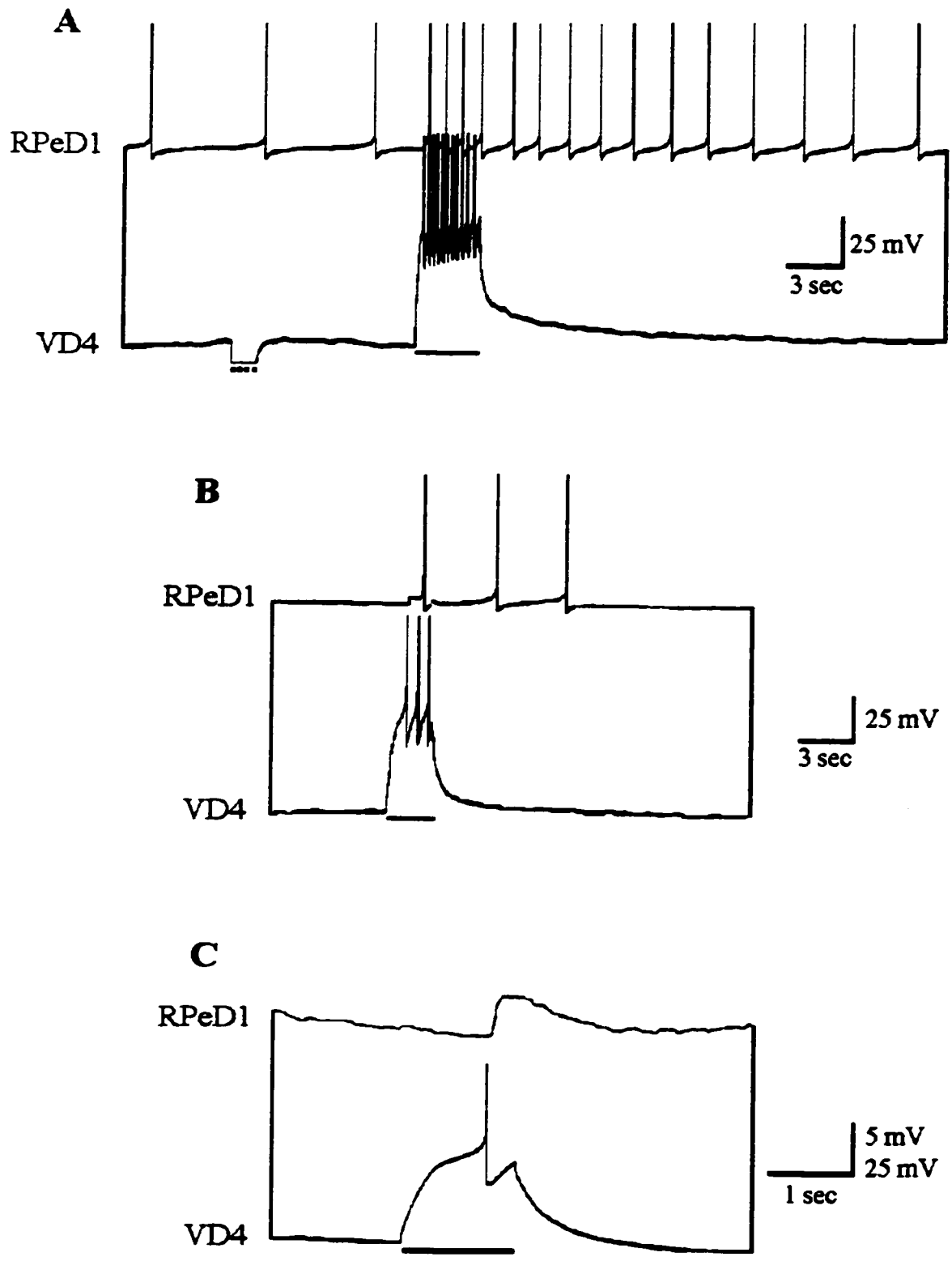

no synapse
1 way VD4-RPeD1
2 way
1 way RPeD1-VD4
% of soma-soma pairs
100
90
80
70
60
50
40
30
20
10
0
control
4-BPB 10-5 M
NDGA 5 x 10-6 M
Treatment

Figure 5.13. An inappropriate excitatory synapse developed between VD4 and RPeD1 in the presence of a phospholipase A_2 inhibitor, 4-BPB.

An isolated somata of RPeD1 were pretreated with 4-BPB (10^{-5}M) and subsequently soma-soma paired with VD4 in DM. Following 18 hours in DM, synapses were tested electrophysiologically. In 3 of 9 pairs tested, VD4 was found to form an excitatory chemical connection with the RPeD1 soma. Specifically, evoked action potentials in VD4 increased the frequency of spiking activity in RPeD1 (**A**), induced a quiescent cell to fire action potentials (**B**), and caused 1:1 depolarizing postsynaptic potentials in an RPeD1 somata held at –60 mV (**C**). The depolarizing potentials did not involve electrical coupling between the cells since the injection of hyperpolarizing current in VD4 (dotted line in **A**) had no effect on RPeD1's membrane potential.


A
RPeD1
VD4
25 mV
3 sec
B
RPeD1
VD4
25 mV
3 sec
C
RPeD1
VD4
5 mV
25 mV
1 sec

Chapter 6: General Discussion and Future Directions

All nervous system functions in adult animals are contingent upon precise connectivity patterns (synapse formation) that are established during early development. Therefore, understanding cellular and molecular mechanisms underlying synapse formation is fundamental to our comprehension of not only how the nervous system is put together during early development, but also how it functions in the adult animal. The anatomical complexity of the mammalian brain, and the intricate nature of synaptic connectivity between its neurons, presents formidable challenges, sending neurobiologists in search of appropriate *in vitro* model systems. Therefore, as an alternative to the complex *in vivo* environment, various simple *in vitro* model systems have been developed (Bulloch and Syed, 1992; Chow and Poo, 1985; Haydon 1988; Feng et al., 1997; Fuchs et al., 1982; Klein, 1994; Syed et al., 1990; Xie and Poo, 1986; Zoran et al., 1993). These models have since provided significant and fundamental information about cellular and molecular mechanisms by which specificity of synapse formation might be generated in the intact brain. Notwithstanding the fact that mechanisms underlying synapse formation between cultured CNS neurons resemble those that are functional at the NMJ, at present it is difficult to assert whether these data would also be applicable to the intact brain.

In the present study, I took advantage of a soma-soma synapse model recently developed for *Lymnaea* neurons (Feng et al., 1997). It can be argued that since synapses in general do not form between the cell bodies of neurons in the intact brain, this preparation is rather artificial and perhaps irrelevant to studies on synapse formation. Nevertheless, previous studies from others (Fuchs et al., 1981; Haydon 1988; Klein,

1994; Man-Son-Hing et al., 1989) and our laboratory (Hamakawa et al., 1999; Feng et al., 1997; Woodin et al., 1999), have provided both morphological and electrophysiological evidence demonstrating that synapses developed between the somata of identified neurons are similar to both those seen *in vivo* and those that form *in vitro* between neurites. Moreover, the data presented in the present study provides further support in favor of the argument that synapse formation between soma-soma paired neurons RPeD1 and VD4 does indeed follow a well organized synaptic program which is regulated in a highly coordinated manner. In light of this evidence, I would strongly argue that this preparation is indeed appropriate and relevant to similar studies that are being conducted in *Lymnaea* either *in vitro* or *in vivo*.

A central theme in developmental neurobiology is that synapse formation is a highly regulated process and involves dynamic interactions between extending growth cones of both pre and postsynaptic neurons. *En route* towards its target, a growth cone encounters (physical contacts) various potential partners, however, it does not establish indiscriminate synaptic connections with these non-target cells. The nature of signals that instruct the growth cone to terminate its advance and establish a synaptic connection following contact with an appropriate target cell are fundamental to our understanding of synapse formation in the nervous system; their precise identity and the underlying mechanisms, however, have not yet been deduced. This particularly holds true with respect to retrograde signaling molecules that are released from the postsynaptic cells to modulate the secretory machinery of their presynaptic partners.

One of the main objectives of this research was to search for signaling molecules and underlying mechanisms that regulate the secretory machinery of an identified neuron

during early synapse formation. To this end, a soma-soma cell culture preparation for *Lymnaea* neurons (Feng et al., 1997) was used in combination with an assay technique; together, this model enabled me to detect evoked transmitter release from RPeD1 at various different time points during the course of synaptogenesis with VD4. Utilizing this model system, I have demonstrated that synapse formation between the somata of two molluscan neurons follows a sequential program composed of well-coordinated and discrete steps. Specifically, the dopaminergic neuron (RPeD1) was found to be capable of evoked transmitter release from all areas of its soma prior to target cell contact; conversely, the peptidergic neuron (VD4) released transmitter only at specific synaptic sites. Upon somatic contact, VD4 rapidly acquired the ability to release transmitter (as evidenced by its ability to form a synapse), whereas RPeD1's ability to release transmitter was transiently suppressed. Following a time delay of approximately 6 hours, RPeD1 regained its transmitter secretory capabilities both at the contact site with VD4, and at all other areas over its somatic surface.

Since in most other model systems presynaptic secretory machinery was found to be up-regulated immediately after contact with a postsynaptic cell (Chow and Poo, 1985; Haydon and Zoran, 1989; Zoran et al., 1990; Zoran et al., 1991; Zoran et al., 1996), I found VD4-induced suppression of transmitter release from RPeD1 to be highly intriguing. Moreover, I discovered that VD4-induced suppression of transmitter release from RPeD1 was contingent upon *de novo* protein synthesis and transcription. Together, these studies suggested that the target cell contact-induced suppression of transmitter release from RPeD1 is an active process that involves gene induction. Consistent with this notion are recent molecular studies on *Lymnaea* soma-soma paired cells that show

several genes and their products are indeed up-regulated during synapse formation between RPeD1 and VD4. Specifically, utilizing differential display polymerase chain reaction techniques, van Kesteren et al., (1996, 1997) have provided direct evidence showing that, compared with their individually cultured partners, soma-soma pairing between RPeD1 and VD4 results in the up-regulation of several genes. The identity of some of these genes has now been revealed (van Kesteren personal communication) and their functional significance in synapse formation is being deduced. This approach may enable us in the future to identify genes that are involved in VD4-induced suppression of transmitter release from RPeD1. Subsequently, mRNAs of interest can be injected into individual neurons prior to soma-soma synapse formation and the effects of this manipulation on subsequent synapse formation can be studied electrophysiologically. Alternately, these mRNAs could be introduced into axotomized neurons *in vivo* and their involvement in synapse formation can be tested after regeneration of synaptic connections. Such a protocol would not only help define molecular mechanisms underlying synapse formation, but this approach would also help us to determine whether the data obtained from *in vitro* studies also applies *in vivo*. Such experiments are highly feasible in *Lymnaea* since, not only do its neurons regenerate *in vivo* following axotomy and single cell transplantation (Syed et al., 1992), but also foreign mRNA can be expressed both in neurons and their isolated axons (van Minnen et al., 1997).

As mentioned above, cell-cell contacts between pre and postsynaptic partners can bring about specific changes in the synaptic machinery of their target cells. This cellular signaling may involve either membrane bound molecules (Dai and Peng, 1993; Drapeau et al., 1989; Xie and Poo, 1986) or retrograde diffusible messengers released by the

synaptic partners (Schacher et al., 1993). Since growth cones of developing neurons have been shown to release neurotransmitter and are themselves sensitive to exogenously applied transmitter, it was therefore hypothesized that these diffusible molecules may also serve to determine the synapse specificity (Goldberg and Kater, 1989; Lipton and Kater, 1989). A direct involvement of neurotransmitters in synapse formation, however, has not yet been determined. Furthermore, even less is known about the cellular signaling molecules that function in a developing nervous system down stream from the transmitter receptor. The present study is the first to demonstrate that FMRFamide receptor activation, and the underlying second messenger pathway, are essential for synapse formation between VD4 and RPeD1. Although the involvement of other neurotransmitters such as 5HT, DA, or ACh and their receptors in the *Lymnaea* model is yet to be determined, the present study does nevertheless underscore the importance of transmitter/receptor interactions in synapse formation between the identified neurons.

Earlier studies in both vertebrates and invertebrates have shown that transmitter release from presynaptic neurons is up-regulated immediately after contact with its postsynaptic target (Chow and Poo, 1985; Haydon and Zoran, 1989; Zoran et al., 1990; Zoran et al., 1991; Zoran et al., 1996). These studies lead Haydon and Drapeau (1995) to propose the idea that neuronal secretory machinery in most instances is "ready, set, and go" for synapse formation immediately upon contact with their target cells. Furthermore, the ability of neurons to alter transmitter release sites during synapse formation is also documented. For instance, somatic release from frog motor neurons was shown to be down regulated in favor of discrete release at a growth cone contacting a target cell (Chow and Poo, 1985). It seems reasonable to postulate that the suppression of somatic

release may involve the redistribution of secretory machinery, ion channels, and synaptic vesicles from the cell body to specific synaptic sites. Consistent with this idea are data from our laboratory which show that Ca^{2+} hot spots corresponding to enhanced Ca^{2+} channel activity are indeed clustered at specific contact sites between soma-soma paired cells (Feng, 1998). These data demonstrate that Ca^{2+} channels may cluster at specific contact sites between pre and postsynaptic neurons, thus regulating the transmitter release to occur only at discrete synaptic sites. It is therefore reasonable to assume that cell-cell signaling that triggers Ca^{2+} channel clustering, or its redistribution at specific synaptic sites, may render RPeD1 incapable of transmitter release. This notion, however, is inconsistent with experimental data which showed that after an initial transient period of dormancy, RPeD1 begin to secrete transmitter, albeit indiscriminately, from all somatic surface areas. This release from all areas of the soma at 24 hours was perplexing; it casts doubt as to whether the neurotransmission between these cells was indeed synaptic and did not involve diffused release. Although further experiments would be required to determine fully whether the transmitter release from RPeD1 is indeed synaptic, at this juncture, I speculate that having established its synapse with VD4 (24hours), the giant dopamine cell may begin to function as a large varicosity - thus releasing transmitter from all around its cell body. This hypothesis can be tested by pairing RPeD1 simultaneously with two other neurons; one target cell, such as VD4, and the other a non-target neuron like VD1, and then testing RPeD1's ability to release transmitter both synaptically and non-synaptically. Indeed Feng et al. (1997) have demonstrated that under such an experimental condition, RPeD1 releases transmitter at specific synaptic sites with VD4, whereas non-synaptic release from other areas of its cell

body is completely suppressed. Further support for the notion that transmitter secretory machinery in a three-cell configuration is discretely localized was obtained by Ca^{2+} imaging techniques. Specifically, Feng (1998) demonstrated that Ca^{2+} hot spots in RPeD1 develop only at its contact site with VD4 and not VD1. Based on these studies, and the data presented here, I speculate that under experimental conditions where RPeD1 is in contact with only one of its target cell, its somata becomes non-selective and releases dopamine indiscriminately from all areas. Pairing with a non-target cell, however, precludes RPeD1 from non-selective release and under these conditions it becomes selective in releasing transmitter only at specific sites with VD4. Measuring capacitance changes in RPeD1 during various stages of synapse formation may enable this hypothesis to be tested further. For instance, measuring changes in capacitance during exocytotic discharge, Huang and Neher (1996) found that somatic, non-synaptic exocytosis has slower release kinetics than synaptic. A similar method could thus be used to test whether a switch in secretory program occurs in RPeD1 during synaptogenesis. Any change in the time delay between cell depolarization and the increase in capacitance (due to the fusion of secretory vesicles with the surface membrane) over the course of synaptogenesis would indicate a switch from non-synaptic to synaptic transmitter release strategies.

In the present study, the contrasting excitatory and inhibitory effects of FMRFamide on RPeD1 were concluded to be due to the peptide binding to two different receptor types. In *Aplysia*, Belkin and Abrams (1998) have recently demonstrated a similar biphasic response to applied FMRFamide in which the excitatory component could be magnified by application of AA signaling blockers. These authors hypothesized

that the biphasic response could be due to activation of different receptor subtypes or may involve different second messenger signal cascades. Similarly, in *Lymnaea*, FMRFamide binding to one receptor in RPeD1 may activate two separate ion channels (one leads to excitation and other inhibition), which might be responsible for a biphasic response in this cell. Although the ionic dependence and pharmacological data obtained in the present study point toward the expression of a FMRFamide-gated Na^+ channel in RPeD1, this issue still needs to be resolved fully. For example, experiments utilizing voltage clamp techniques would be required to carefully analyze response kinetics and thus enable differentiation between the presence of an ionotropic (ligand gated channel) or metabotropic (second messenger coupled) FMRFamide receptor in RPeD1. Alternately, in view of the fact that a FMRFamide-gated Na^+ channel has been cloned from another mollusc (*Helix*: Lingueglia et al., 1995), evidence for the presence of a similar channel on RPeD1 could be sought using molecular techniques.

An important issue not discussed in the main body of this thesis is whether the transient suppression of transmitter release from RPeD1 is an essential component of its synaptic program and is inherent to the giant dopamine cell itself. This possibility can be tested in the future by pairing RPeD1 with target cells that are only postsynaptic to the giant dopamine cell. For example, RPeD1 should be paired with VD2/3, a pair of identified neurons which receive excitatory input from the giant dopamine cell but do not form a reciprocal connection back onto RPeD1. Soma-soma pairing and subsequent assay cell techniques can be employed to determine transmitter release capabilities of the giant dopamine cell under these conditions. RPeD1's inability to release transmitter at 18 hours would indicate that a transient suppression of dopamine release is indeed an

intrinsic component of its synaptic program, however, these data might undermine the importance of VD4/FMRFamide-induced transmitter suppression from RPeD1 during early synapse formation.

Although the functional significance of VD4-induced suppression of transmitter release from RPeD1 remains unclear, it is tempting to speculate that this process may involve competition between these cells for common synaptic partners. Specifically, since both RPeD1 and VD4 innervate some common postsynaptic partners, it is feasible that by suppressing RPeD1's secretory machinery, VD4 may actively outcompete this cell in its attempts to innervate shared postsynaptic targets. Following VD4's formation and consolidation of synaptic communication with a target, RPeD1 may be allowed (by removing transmitter suppression) to innervate this common target cell. This possibility needs to be tested experimentally, however, if the data were consistent with the hypothesis, then I would dare to propose further that, in addition to neuronal hierarchy at the network level (command neuron concept – see Xin et al., 1996), there also exists a synaptic hierarchy. This model would predict that neurons such as VD4 which serve higher order functions (cardiovascular control) may take precedence in target selection over other lower order (functionally) neurons such as RPeD1.

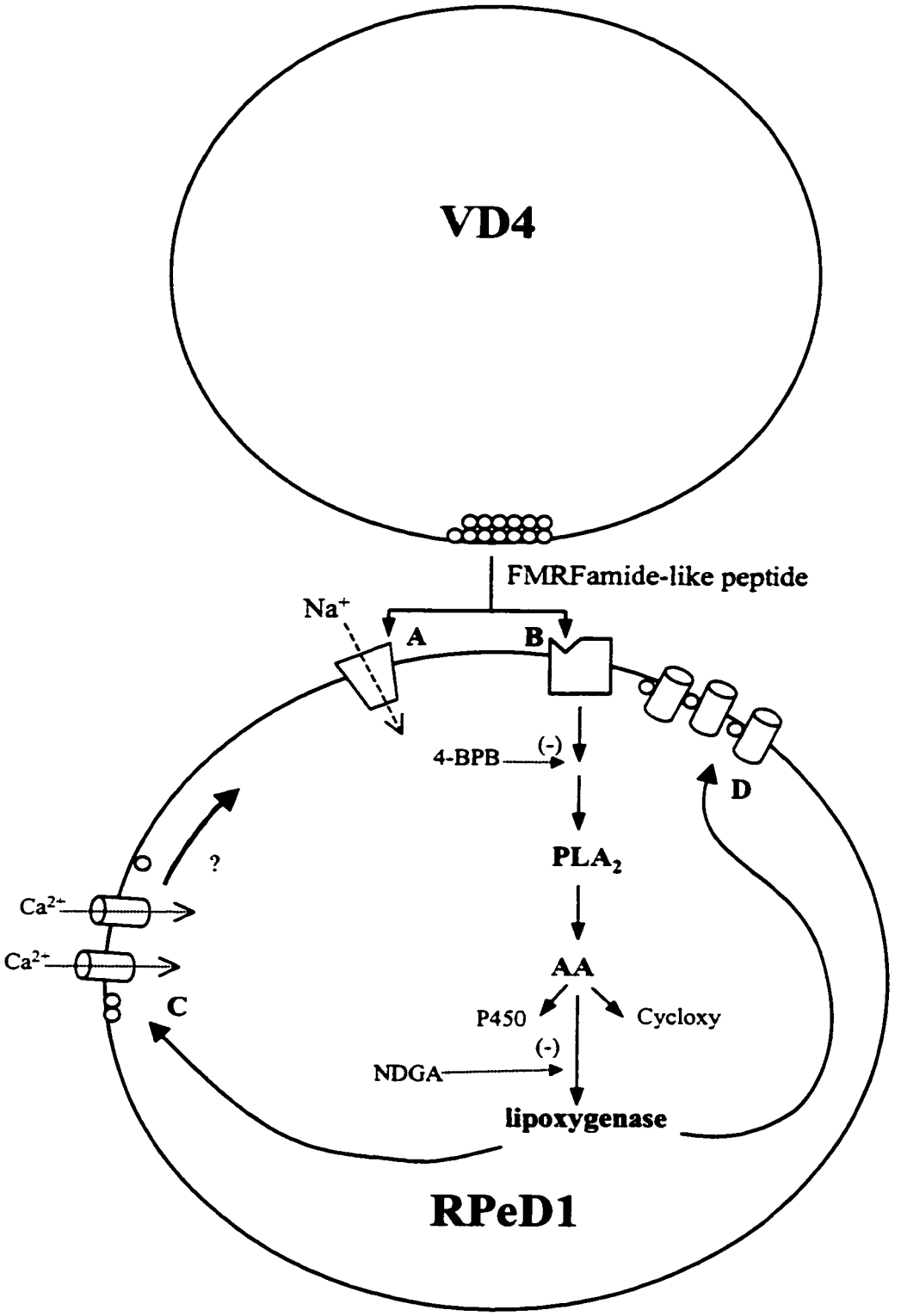
The model system approach is superficial if the data obtained cannot be related back to the functioning of neuronal networks in the intact nervous system. One of the greatest strengths of the *Lymnaea* model is that information obtained *in vitro* on the synaptic physiology of RPeD1 and VD4 connections is directly applicable to the cellular and synaptic basis of rhythm generation that underlies respiratory behavior in the intact animal. For instance, in addition to providing direct insights into the involvement of

VD4 and its transmitter FMRFamide in synapse formation, the present study also demonstrated that the synaptic transmission between VD4 and RPeD1 is FMRFamidergic and involves two different peptide receptors: one that directly gates a Na^+-channel and one that signals via AA metabolites.

In summary, I utilized a soma-soma preparation to characterize cell-cell interactions during early stages of synapse formation. Electrophysiological techniques revealed a novel event during synaptogenesis; namely, a transient suppression of non-synaptic transmitter release, which preceded the formation of discrete synaptic communication between the paired cells. This suppression required protein synthesis and was mediated by synaptic release of the neurotransmitter FMRFamide from VD4 that signaled via the AA second messenger in RPeD1. This study underscores the importance of transmitter/receptor interactions during early stages of discrete synapse formation between identified *Lymnaea* neurons. Furthermore, the data presented strengthens the validity of soma-soma synapse model for further studies on the cellular and molecular mechanisms that underlie specific synapse formation in the nervous system.

Figure 6.1. Overall summary.

During early stages of soma-soma synapse formation between VD4 and RPeD1, VD4 releases a FMRFamide-like peptide as a transmitter which suppresses DA release from the giant dopamine cell. This peptide acts on two different receptor types on RPeD1: an excitatory peptide-gated Na^+ channel (**A**) and an inhibitory receptor likely coupled through a g-protein mediated pathway (**B**). Activation of this second type of receptor, in turn, activates phospholipase A_2 (PLA_2) and subsequently causes the formation of arachidonic acid (AA). Lipoxygenase metabolites downstream of AA suppress RPeD1's secretory capabilities. This may work through one or a combination of two mechanisms: **C**) a redistribution and association of voltage gated Ca^{2+} channels and secretory machinery to the target cell contact site or **D**) a transient suppression of both the Ca^{2+} channels and secretory machinery already located at the developing synaptic site. Both 4-BPB, which prevents the activation of PLA_2, and NDGA, which prevents the formation of lipoxygenase metabolites, are sufficient to block both VD4- and FMRFamide induced suppression of transmitter release from RPeD1.


VD4
FMRFamide-like peptide
Na+
A
B
4-BPB
(-)
D
PLA2
?
Ca2+
Ca2+
AA
C
P450
Cycloxy
(-)
NDGA
lipoxygenase
RPeD1

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