Synaptic Precedence During Synapse Formation Between Reciprocally Connected Neurons Involves Transmitter-Receptor Interactions and AA Metabolites

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INTRODUCTION

The stereotypic patterns of neural connectivity established during development provide the framework for proper functioning of the entire nervous system in adult animals. However, the precise cellular and molecular mechanisms orchestrating this specificity of synaptic connections remain poorly understood (see Albright et al. 2000). It is generally accepted that target cell selection and specific synapse formation is contingent on cell-cell signaling between potential pre- and postsynaptic neurons. Moreover, contacts between appropriate pre- and postsynaptic partners are also known to bring about specific changes in the synaptic machinery of their target cells during early synapse formation (Fitzsimonds and Poo 1998; Zoran et al. 1990). These changes range from an upregulation of the secretory capabilities (Chow and Poo 1985; Zoran et al. 1996) to a complete switch in the transmitter phenotypic characteristics of presynaptic neurons (Asmus et al. 2000; Schotzinger and Landis 1988; Schotzinger et al. 1994). However, neither the precise identity of any given cell-cell signaling molecule nor the underlying mechanisms has yet been fully defined (see Haydon and Drapeau 1995; Jessell and Sanes 2000; Sanes and Lichtman 1999).

In addition to the above-described cell-cell signaling during early stages of synaptogenesis, the final patterns of neuronal connectivity are refined further by activity dependent competition between neurons (Albright et al. 2000; Kandel et al. 1995; Constantine-Paton 1990). Together, these activity patterns are out of sync. The support for this hypothesis stems from a number of studies at the neuromuscular junction (Dan and Poo 1992; Lo and Poo 1991) and in the nervous system (Constantine-Paton 1990). Together, these studies demonstrate that in most instances of excitatory synaptic transmission at peripheral and central synapses, the synaptic competition may follow the Hebbian postulate. Whether a similar activity dependent interaction is also functional at inhibitory synapses, where presynaptic activity suppresses postsynaptic excitability, remains to be determined.

In our laboratory, we have developed synapses between the somata of identified Lymnaea respiratory neurons right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4). These neurons establish reciprocal inhibitory connections in a soma-soma configuration, in the absence of neurite outgrowth. These soma-soma synapses are both morphologically and electro-
physiologically similar to those seen in vivo (Feng et al. 1997, 2000; Hamakawa et al. 1999; Syed et al. 1991; Woodin et al. 1999). Specifically, RPeD1 and VD4 form reciprocal inhibitory synaptic contacts in which RPeD1 releases dopamine and VD4 secretes FMRFamide-like peptide. Using soma-soma synapses, we report here on a novel form of synaptic interaction between VD4 and RPeD1, which are two of the mutually connected neurons from the central respiratory rhythm-generating network in Lymnaea (Syed et al. 1990). We demonstrate that although a reciprocal inhibitory synapse develops between VD4 and RPeD1 after 24–36 h of cell pairing, VD4 is the first cell to establish synaptic transmission with RPeD1 (within 12–18 h). We show that during early synapse formation, VD4 “captures” RPeD1 as a postsynaptic partner by rendering its transmitter releasing capabilities incapable of secretion. This transmitter suppression of RPeD1 was VD4 cell specific, mimicked by its transmitter (FMRFamide-like peptides), and required transcription and de novo protein synthesis in VD4 but not in RPeD1. Both VD4 and FMRFamide-induced transmitter suppression in RPeD1 involved an arachidonic acid (AA)-mediated cascade. The perturbation of AA pathway in RPeD1 not only prevented both the VD4 and FMRFamide-induced suppression of transmitter release but also enabled RPeD1 to initiate synaptic transmission with VD4 at a much earlier time point. Moreover, a single RPeD1 that had developed transmitter secretory capabilities overnight, prior to its pairing with VD4, was immune to VD4-induced transmitter release. Under these experimental conditions, reciprocal inhibitory synapses developed concurrently between the cells after only 12–18 h of pairing.

Taken together, this study suggests a novel mechanism by which synaptic interaction might regulate the timing of synapse formation between reciprocally connected (inhibitory) neurons. Moreover, our data underscore the importance of transmitter/receptor interactions in regulating the secretory machinery of synaptic partners during early synapse formation.

**METHODS**

**Animals**

All experiments were performed on neurons isolated from the fresh water snail Lymnaea stagnalis maintained in aquaculture at the animal care facility of the University of Calgary. Animals were raised at room temperature (20–22 °C), in well-aerated and de-chlorinated tap water. Snails were exposed to a 12/12-h light/dark cycle and fed lettuce (1 time per week) and Purina Trout Chow (5 times per week). Animals with shell lengths of 15–20 mm (approximately 2–4 mo old) were used in all experiments.

**Dissection**

Snails were dissected in a standard Lymnaea saline containing (in mM) 51.3 NaCl, 1.7 KCl, 4.0 CaCl2, and 1.5 MgCl2. All chemicals were purchased from Sigma unless stated otherwise. The saline was buffered with 10.0 mM HEPES, and the pH was adjusted to 7.9 with 1 N NaOH (Syed and Winlow 1991). Antibiotic saline (ABS) was prepared by adding Gentamycin (150 μg/ml) to the sterile saline.

Animals with shell length of 15–20 mm were deshelled with scissors and anesthetized in 10% (vol/vol) Listerine in standard saline for 10 min. Anesthetized snails were pinned down on a silicone-rubber-based (General electric RTV 616) dissection dish containing ABS. All instruments were presterilized with 70% 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, 1999).

**Cell culture**

All cell culture experiments were performed on isolated Lymnaea neurons plated in Falcon 3001 dishes containing defined media (DM). DM consisted of serum-free 50% (vol/vol) Liebowitz’s L-15 media (formula 82-5154 EL, GIBCO) with additional salts (in mM) 40.0 NaCl, 1.7 KCl, 4.1 CaCl2, 1.5 MgCl2, 10.0 HEPES, 10 mM glucose, 1.0 mM L-glutamine, and 20 μg/ml gentamycin. The pH was adjusted to 7.9 with 1 N NaOH, and the solution was filtered (Milipore Sterivex-GV, 0.22-μm filter unit) and stored in autoclave bottles. Following their removal from the intact animals, the isolated central ring ganglia were washed in ABS (3 × 15 min) and treated with trypsin (Type III, 2 mg/ml dissolved in DM) for 20–25 min at room temperature. The enzymatic activity was subsequently neutralized by placing the ganglia in trypsin inhibitor (2 mg/ml), also dissolved in DM for 15 min. The ganglia were pinned to the bottom of a black silicone-rubber (General Electric, RTV616) dissection dish containing high-osmolarity DM (40 mM glucose). Under a stereo dissection microscope (Zeiss), both the outer and inner connective tissue sheaths were removed mechanically using fine forceps. Identified cells were selectively isolated by applying gentle suction via a microsyringe (Gilmont, GS 110) attached to a fire polished glass pipette (see Syed et al. 1999 for details).

To obtain soma-soma synapses, identified neurons were juxtaposed on either poly-l-lysine-coated or untreated plastic dishes (3001 Falcon) containing DM (see Feng et al. 1997 for details).

**Electrophysiology**

To test for synaptic connections, simultaneous intracellular recordings were made from the juxtaposed somata. Conventional, intracellular glass microelectrodes (1.5 mm ID, TW 150F-6, WPI, resistance, 30–60 MΩ) were prepared on a vertical microelectrode puller (Kopf, 700C) and filled with a saturated solution of K2SO4. The electrodes were connected to the amplifier headstages and were used to impale neurons by Narishige micromanipulators (MM 202 and MM 204, Tokyo). A chloride-coated silver wire was used as ground electrode, and the microelectrode resistance was balanced using a Grass stimulator (S88). A Zeiss (Axiovert 135) inverted microscope was used to view the cells, and these were subsequently impaled with sharp electrodes and intracellular signals were amplified via a dual-channel preamplifier (NeuroData, IR-283), viewed using a digital oscilloscope (Fluka 2000), and recorded on a chart recorder (Gould 2400S).

**Detection of transmitter release**

Neurotransmitter release from RPeD1 somata was detected electrophysiologically by using a postsynaptic, dopamine sensitive somata (VD2, VD4, VJ) as an assay (the “sniffer cell”) cell. In experiments where the culture medium contained a drug or other substances not normally present in DM, the medium was replaced with saline or DM prior to the addition of a sniffer cell. Transmitter release from RPeD1 was detected with an isolated sniffer neuron that was maneuvered into close proximity of RPeD1. It is important to note that 100% of the cells (n = 28) (also see Spencer et al. 2000) used as a sniffer neuron were viable and responsive to exogenously applied dopamine. Sniffer cells were able to detect DA release regardless of their position relative to soma of RPeD1, suggesting that secretion occurred from all of the soma of an isolated RPeD1.

**Chemicals**

All chemicals used in this study were purchased from Sigma Chemicals with the exception of (-)-sulpiride, which was acquired from Research Biochemicals (S-112).
The protein synthesis inhibitor anisomycin (A1899) and the transcription blocker actinomycin D (A4262) were dissolved either in saline or DM and applied at a final concentration of 12.5 and 5.0 μg/ml, respectively. 5-hydroxytryptamine (5-HT; H9523) was dissolved in saline to a final concentration of 10⁻⁶ M. AA (A9673), 4-bromophenacyl bromide (4-BPB: B2006), and nordihydroguaiaretic acid (NDGA: N5023) were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and frozen as aliquots. Individual aliquots were thawed, diluted with DM (the final DMSO concentration was less than 0.1% vol/vol), and added to the culture dishes. Regarding irreversible AA inhibitor 4-BPB, isolated RPeD1 neurons were treated briefly (15 min) in a culture dish and subsequently plated on 3001 dishes either as single cells or paired with VD4. NDGA, on the other hand, was added to the culture dish after immediate pairing and the drug solution was replaced only with normal DM prior to intracellular recordings.

The synthetic peptide phe-met-arg-phe-amide (FMRFamide: P6910) was dissolved in 50 mM acetic acid to obtain a concentration of 10 mM and subsequently frozen in 50-μl aliquots. These were thawed, diluted with DM, and either added to the culture dishes or puffed directly onto the somata in the desired concentration ranges.

Statistics

All data were analyzed using the statistical software program SigmaStat for Windows (v. 4.0, Jandel Scientific, San Rafael, CA.). Data that quantified the incidence of certain phenomenon were analyzed using Fisher’s exact test (F. E. test) while distribution in different categories was tested by χ² analysis. Differences were considered significant if P < 0.05.

RESULTS

VD4 is first to establish inhibitory synapse with RPeD1 in a soma-soma configuration

In a soma-soma configuration, both RPeD1 and VD4 establish mutually inhibitory synapses in cell culture within 24–48 h (Feng et al. 1997). To determine which of these two neurons established synaptic transmission first, cells were paired in vitro and simultaneous intracellular recordings were made either on day 1 (12–24 h) or day 2 (36–48 h). We found that in the majority of cases (73%), VD4 was the first cell to establish an inhibitory synapse with RPeD1 (Fig. 1). Specifically, after 12–18 h of pairing, intracellular recordings revealed that induced action potentials in VD4 produce 1:1 inhibitory postsynaptic potentials (IPSPs) in RPeD1 (n = 30 Fig. 1A), whereas action potentials in RPeD1 failed to generate a postsynaptic response in VD4 (Fig. 1B). Although 10% soma-soma paired cells did not establish synapses at 12–18 h, 17% cells exhibited reciprocal inhibitory connections. In the case of mutually connected pairs, we do not, however, know which direction of synapse developed first. A larger population of paired cells developed reciprocal, inhibitory synapses after 24–36 h of cell pairing (Fig. 1, C and D). These data (summarized in Fig. 1E) indicate that 21 of 37 pairs exhibited reciprocal connections on the second day, and thus the incidence of bi-directional synaptic transmission increased significantly [χ²(2) = 16.286, P < 0.001] on day 2, at which time a larger proportion (47%) as compared with 17% on day 1; see Fig. 1E) of RPeD1 neurons was found to be synaptically connected with VD4. It is important to note that a one-way inhibitory synapse was observed only between VD4 and RPeD1 and not vice versa. Together, these data demonstrate that VD4 is the first cell to establish synaptic connection with RPeD1 (within 12–18 h) and that a vast majority of giant dopamine cells become capable of synaptic transmission only at a later time period (24–36 h).

Transmitter release from RPeD1 is suppressed by VD4 during early synaptogenesis

To test whether a soma-soma paired RPeD1 was capable of transmitter release, and if a paired VD4 did indeed possess functional dopamine receptors, the transmitter secretory (RPeD1) and dopamine response (VD4) properties of both cells were examined. Specifically, in a first series of experiments, the freshly isolated somata of either RPeD1 or VD4 were manipulated in close proximity to soma-soma paired cells.

![Image](http://jn.physiology.org/https://jn.physiology.org/DownloadedFrom)
RPeD1 and VD4 (Fig. 2A). Simultaneous intracellular recordings were made from paired cells to confirm that the synaptic transmission between them was indeed in one direction, i.e., from VD4 to RPeD1 (not shown). A freshly isolated RPeD1 somata which was capable of dopamine release (see Fig. 3D) was introduced to the culture dish, impaled with a sharp electrode and maneuvered in close proximity (but not contacting) to a paired VD4 cell (Fig. 2A). Dopamine release from RPeD1 was induced via current injection, and a nonsynaptic electrophysiological response was tested in VD4 (Fig. 2B). Induced action potentials in RPeD1 consistently generated a nonsynaptic inhibitory response in the paired VD4 (6 of 6 cells), demonstrating not only that a paired VD4 possessed functional transmitter receptors for the released transmitter but that an unpaired RPeD1 was indeed capable of transmitter release.

To test further for the consistency and reliability of transmitter secretion from a lone RPeD1 and to demonstrate that the released transmitter was indeed dopamine, RPeD1 was plated as a single cell, and various different postsynaptic somata were used as sniffer cells. We found that when manipulated to within close proximity of a single RPeD1 cell, various somata of its in vivo target neurons responded as they do in vivo to the induced release of transmitter (Fig. 3). That is, the intracellular stimulation of a single RPeD1 at 12–18 h, produced nonsynaptic, inhibitory (VD4, n = 29; VJ cell, n = 5) and excitatory responses (VD2, n = 5) in the sniffer cells (Fig. 3, A–C). To confirm that the nonsynaptic responses observed in the sniffer cells were the result of dopamine release from RPeD1, the dopamine receptor antagonist sulphuride (10^{-4} M) was perfused into the culture dish. In all instances (n = 17), sulphuride reversibly blocked nonsynaptic dopaminergic responses in the sniffer cell (data not shown) (but see Spencer et al. 2000).

To define the precise time course over which RPeD1 neurons become capable of transmitter release, dopamine secretion was analyzed over a series of time points. We found that only 20\% (n = 1 of 5), and 14\% (n = 1 of 7) of the freshly isolated RPeD1 somata were able to release transmitter at 2–3 and 5–6 h,
respectively. Dopamine release was, however, detected consistently from RPeD1 neurons at 10–12 h (71%, n = 5 of 7) and 18 h (92%, n = 12 of 13), respectively. No further differences were observed at 24 h where 17 of 19 (89%) RPeD1 were found capable of dopamine release (Fig. 3D). The incidence of detectable transmitter release was significantly different at 18 and 24 h compared with cells plated for only 2–3 h (P < 0.008 and P < 0.006, respectively, FE test). Taken together, the preceding data demonstrate that although a single RPeD1 is capable of dopamine release within a few hours of its isolation, its ability to release transmitter, however, increases/improves with time.

Because 92% of single RPeD1 reliably could release transmitter at 12–18 h, the absence of postsynaptic response in VD4 following stimulation of the paired RPeD1 may thus be due to the lack of transmitter release from its partner cell RPeD1. To test whether a paired RPeD1 could release transmitter, a freshly isolated VD4 somata was thus used as a “sniffer” cell (Fig. 4A), and RPeD1’s ability to release transmitter was tested. Despite repetitive stimulation, we failed to detect nonsynaptic transmitter release from a paired RPeD1 (n = 10; Fig. 4B). These data show that a paired RPeD1 is incapable of transmitter release (12–18 h) and raise the possibility that this suppression may involve contact with VD4 neuron.

VD4-induced suppression of transmitter release from RPeD1 is target cell contact specific and requires transcription and de novo protein synthesis

Because transmitter release from a paired RPeD1 was suppressed only transiently (from 12 to 24 h), we next asked whether VD4-induced suppression of transmitter release from RPeD1 involved transcription and/or new protein synthesis. To address this possibility, cells were soma-soma paired either in the presence of a transcription blocker (actinomycin D, 5 μg/ml) or a protein synthesis inhibitor (anisomycin, 12.5 μg/ml). Simultaneous intracellular recordings were made on day 1 and evidence for synaptic communication was sought electrophysiologically. As shown previously (Feng et al. 1997), both protein synthesis (n = 12) and transcription (n = 14) inhibitors blocked synapse formation (electrophysiological evidence only) between the paired cells. Under these experimental conditions, however, RPeD1 pairing with VD4 failed to suppress transmitter release from RPeD1, and in most instances diffused dopamine secretion was reliably detected by the sniffer cell (Fig. 5).

To test whether transmitter suppression from a paired RPeD1 was specifically due to contact with VD4, RPeD1 cell was soma-soma paired with another identified cell, termed the cerebral giant dopamine cell (CGC). Because, in vivo, RPeD1 does not make physical or synaptic contacts with CGC neuron, we hypothesized that pairing this cell with RPeD1 in a soma-soma configuration would not suppress transmitter release from the giant dopamine cell. After 12–18 h of cell pairing, a sniffer cell (VD4) was introduced to the culture dish and manipulated in close proximity of RPeD1 somata. Transmitter release from RPeD1 was induced via direct intracellular stimulation, and a nonsynaptic response was detected in 8 of 9 VD4 somata. These data demonstrate that a nontarget cell (CGC) contact does not suppress transmitter release from RPeD1 (Fig. 5).
VD4-induced suppression of transmitter release from RPeD1 was mimicked by exogenous FMRFamide

VD4 expresses the FMRFamide gene and both contains and releases FMRFamide-like peptides (Santama et al. 1995; Saunders et al. 1992; Skingsley et al. 1993). Moreover, its postsynaptic effects in almost all instances are mimicked by either FMRFamide or its related peptides (McKenney 1992). Because VD4 was the first cell to initiate synaptic transmission with RPeD1, we hypothesized that VD4-induced suppression of transmitter release from RPeD1 was likely mediated via the release of a FMRFamide-like peptide from VD4. To test this hypothesis directly, a single RPeD1 was cultured for 12–18 h in the presence of exogenous (10⁻⁶M) FMRFamide. The DM containing FMRFamide was subsequently replaced with normal DM, and a freshly isolated VD4 soma (sniffer cell) was introduced to the culture dish. RPeD1 was stimulated electrically to cause dopamine release. Despite repeated stimulation, however, we failed to detect dopamine release from 79% of the FMRFamide pretreated somata of RPeD1 (n = 14; P < 0.001 compared with control, FE test: Fig. 6). Heat-inactivated FMRFamide, on the other hand, was ineffective in suppressing transmitter release from RPeD1. To test whether FMRFamide-induced suppression of dopamine release from RPeD1 also involved new protein synthesis in this cell, RPeD1 was cultured for 12–18 h in the presence of anisomycin (12.5 μg/ml). The culture medium containing the preceding compounds was subsequently replaced with normal DM, and RPeD1’s ability to release dopamine was tested at 12–18 h of post culture. We discovered that even though RPeD1 was cultured in anisomycin, FMRFamide was still able to suppress dopamine release from RPeD1 (n = 5; P < 0.001 compared with control, FE test: Fig. 6). These data suggest that FMRFamide-induced suppression of transmitter release is independent of new protein synthesis in RPeD1. FMRFamide-induced effects were also mimicked by a related peptide, GDPFLR-99amide (10⁻⁷M). Under these conditions as well, a freshly isolated sniffer cell did not detect evoked transmitter release from 70% of the cultured RPeD1 somata (n = 9; P < 0.001 compared with control, FE test, Fig. 6).

To determine whether the peptide-induced effects on dopamine release from RPeD1 were FMRFamide specific, 5-HT (10⁻⁶M), which also inhibits RPeD1 activity (not shown), was tested for its ability to suppress transmitter release from RPeD1. Specifically, RPeD1 somata were isolated and cultured in the presence of 5-HT. After 12–18 h, the culture media was replaced with normal DM, and freshly isolated sniffer cell was used to detect evoked dopamine release from RPeD1. Chronic 5-HT treatment (n = 7) failed to suppress transmitter release from a lone RPeD1 cell (Fig. 6). Taken together, these data show that the VD4/FMRFamide-induced suppression of transmitter release from RPeD1 cell transmitter specific and is contingent on transcription and protein synthesis in VD4, or alternatively, relies on synapse formation.

FMRFamide-induced suppression of transmitter release from RPeD1 is mediated via arachidonic acid pathway

FMRFamide-induced postsynaptic effects on a number of molluscan neurons (Lymnaea, Kits et al. 1997; van Tol-Steye et al. 1999; Helisoma, Bahls et al. 1992; Aplysia, Piomelli et al. 1987) are generally mediated via the metabolites of AA. To test whether FMRFamide-induced effects on dopamine suppression from RPeD1 also involved AA, RPeD1 was cultured in the presence of 5 μM AA, and RPeD1’s ability to release dopamine was tested at 12–18 h. We found that in most instances (67%; n = 6, Fig. 7), the sniffer cell failed to detect dopamine release from an AA-pretreated RPeD1. These data demonstrate that both VD4- and FMRFamide-induced effects on transmitter suppression from RPeD1 may also involve the activation of AA cascade. To test this possibility further, a single RPeD1 was cultured in DM containing FMRFamide and various inhibitors of the AA metabolites. It is important to note that in the case of 4-BPB (irreversible blocker), neurons were treated with this drug for only 15 min and subsequently plated on the culture dishes containing normal DM + FMRFamide. NDGA, on the other hand, was added to the culture dishes at the time of plating and washed with normal DM prior to intracellular recordings. A sniffer cell was used to detect dopamine release from RPeD1 after 12–18 h of cell culture. In the presence of the inhibitors of the AA pathway such as 4-BPB (n = 6) and NDGA (n = 10), FMRFamide failed to suppress dopamine release from RPeD1 (Fig. 7). Similarly, neither 4-BPB nor NDGA alone affected the dopamine release capabilities of single RPeD1. Taken together, these data demonstrate that FMRFamide-induced transmitter suppression in RPeD1 involves AA pathway and/or its metabolites.

VD4-induced suppression of dopamine release from RPeD1 is prevented by AA pathway inhibitors

To determine whether various inhibitors of the AA pathway could also prevent VD4-induced suppression of transmitter release from RPeD1, a single RPeD1 cell was pretreated with 4-BPB for 15 min, and after drug washout with normal DM, it
was soma-soma paired with an untreated VD4 for 12–18 h. We found that after pretreatment with 10 μM 4-BPB (n = 9), the incidence of one way inhibitory synaptic transmission from VD4 to RPeD1 was significantly reduced from 75% in the control pairs to 0% in the presence of AA pathway inhibitor $\chi^2(6) = 49.467, P < 0.001$: Fig. 8]. In contrast, however, the incidence of RPeD1 ↔ VD4 synapse was significantly enhanced in the presence of 4-PBP (from 18% to 78%; Fig. 8). These data demonstrate that VD4-induced suppression of transmitter release by RPeD1 is mediated by an AA signaling cascade and that blocking this synaptic transmission permits RPeD1 to establish its inhibitory synapse with VD4 (Fig. 8).

It is important to note that the incidence of electrical coupling between the paired neurons increases with time in culture, and because electronic coupling renders the data analysis difficult, the cells exhibiting electrical connections are not therefore included in the data presented in Fig. 8. Thus a larger percentage of pairs (not included in Fig. 8) were the cells that were found to be electrically coupled. We wish to point out that our failure to detect electrophysiological signals for functional synaptic transmission from VD4 to RPeD1, under conditions where 4-BPB disrupted the AA pathway, does not rule out the possibility that synaptic morphology may be present between the two cells.

**FIG. 7.** FMRFamide-induced suppression of transmitter release from RPeD1 involved the AA pathway. Individually isolated RPeD1 somata were cultured alone for 12–18 h in DM containing various inhibitors of the AA pathway. The experimental solution was subsequently exchanged with normal saline, and a freshly isolated sniffer cell was added to the culture dish to test for percent of cells that were capable of evoked transmitter release. The 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 effects on single RPeD1 were mimicked by exogenous AA (5 μM, n = 6). The FMRFamide-induced suppression of transmitter release from RPeD1 was, however, prevented by both 4-bromophenacyl bromide (4-BPB; phospholipase A2 blocker, 10 $-7$ M, n = 6) and nordihydroguaiaretic acid (NDGA; lipooxygenase blocker 5 $\times$ 10 $-6$ M, n = 10), (*, significant difference from control), whereas these compounds alone had no effect on the percent of cells from which dopamine release was detected. Note that the control data used in Figs. 6 and 7 are identical and are represented here for comparative purposes.

**FIG. 8.** Synapse formation between VD4 and RPeD1, and the VD4-induced suppression of transmitter release from RPeD1 were blocked by 4-BPB. To determine the significance of AA pathway in synapse formation between VD4 and RPeD1 and the VD4-induced suppression of transmitter release from RPeD1, the giant dopamine cell was pretreated with 4-BPB. Specifically, the dopamine cell was pretreated with the irreversible phospholipase A2-blocker, 4-BPB and subsequently paired with an untreated VD4 in DM. After 12–18 h 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 cells pretreated with 4-BPB $\chi^2(6) = 49.467, P < 0.001, n = 9$. Conversely, the incidence of 2-way inhibitory (RPeD1 ↔ VD4) communication was greater in the 4-BPB pretreated experimental group than the control pair. A freshly isolated sniffer cell detected evoked transmitter release from the RPeD1 soma in all pairs exposed to 4-BPB. Note: the control data presented here are identical to that of Fig. 1 and are used here for comparative purposes.

VD4 fails to suppress transmitter release from a dopamine secreting RPeD1

To test whether VD4-induced effects on transmitter suppression in RPeD1 are time dependent, RPeD1 was first allowed to gain its dopamine secretion capability (day 1 in culture) and was subsequently paired with VD4. Specifically, RPeD1 was isolated and cultured alone overnight. Dopamine release was detected by the sniffer VD4 on day 2 (Fig. 9 A), and RPeD1 was subsequently paired with VD4. Simultaneous intracellular recordings were made from RPeD1 and VD4 after 12–18 h of pairing. In 17 of 17 pairs (100%), a bidirectional synapse was detected between RPeD1 and VD4 and action potentials in either cell generated corresponding 1:1 IPSPs in the postsynaptic neuron (Fig. 9, B and C). These data show that if RPeD1 is given a “head start,” VD4 fails to suppress its transmitter release, and under these experimental conditions, a reciprocal inhibitory synapse always develops concurrently.

**DISCUSSION**

In this study, we have demonstrated that, in addition to their involvement in synaptic transmission in the adult brain, transmitter/receptor interactions between neurons can also regulate the efficacy of secretory machinery during early synapse formation. Specifically, although a single dopaminergic neuron
VD4 and RPeD1, allowing RPeD1 to become the soma-soma pairing blocked synaptic transmission between in RPeD1. Perturbation of AA metabolites in RPeD1 during the dopamine cell were mediated via an AA-signaling cascade tide- and VD4-induced suppression of transmitter release from FMRFamide and another FMRFamide-like peptide. Both pep-
fects on RPeD1 were mimicked by exogenously applied only. Moreover, the VD4-induced, transmitter suppressive ef-
recision and de novo protein synthesis dependent step in VD4 transcription and de novo protein synthesis dependent step in VD4 only. Moreover, the VD4-induced, transmitter suppressive ef-
cts on RPeD1 were mimicked by exogenously applied FMRFamide and another FMRFamide-like peptide. Both peptide- and VD4-induced suppression of transmitter release from the dopamine cell were mediated via an AA-signaling cascade in RPeD1. Perturbation of AA metabolites in RPeD1 during soma-soma pairing blocked synaptic transmission between VD4 and RPeD1, allowing RPeD1 to become the first cell to establish synaptic transmission with VD4. It is important to note that acutely applied FMRFamide or its related peptides do not suppress synaptic transmission between RPeD1 and VD4 pairs under control experimental conditions (not shown). Taken together, our data demonstrate that transmitter-receptor interactions between neurons during early synapse formation may serve as a mechanism by which the precise timing of synapse formation between mutually interconnected neurons can be regulated.

Elegant studies from the Landis laboratory have demonstrated that interactions between sweat gland (Schotzinger and Landis 1988) and sympathetic neurons and between perios-
teum and sympathetic neurons (Asmus et al. 2000) during development induce a complete change in neuronal transmitter phenotype from noradrenergic to cholinergic properties. These effects involve nonsynaptic release of acetylcholine (ACh) from sympathetic neurons and a subsequent release of trophic molecule(s) from the sweat gland (Guidry and Landis 1998; Schotzinger and Landis 1988). A similar switch from cholin-
ergic to glutamatergic transmitter has also been documented in the developing visual system (Wong et al. 2000); however, the precise identity of signaling molecule(s) in this instance remains to be resolved.

The preceding studies suggest that the lack of detectable postsynaptic response from a soma-soma paired RPeD1 might involve a switch in its transmitter phenotypic properties from dopaminergic to some unknown transmitter. Contrary to this assumption, the HPLC analysis of either single or paired RPeD1 neurons did not reveal a significant difference in the dopamine contents (Lovell 2000). Moreover, both synaptic (paired RPeD1) and nonsynaptic (single RPeD1) responses in VD4 were blocked by dopamine receptor antagonist sulpiride (not shown) (but see Spencer et al. 2000). Because a paired VD4 cell was responsive to dopamine release from a single RPeD1, these data demonstrate that during early synapse formation the functional dopamine receptors are indeed present in VD4. Therefore our data show that VD4 cell contact induces a complete, albeit transient suppression of transmitter release from RPeD1.

Target-cell-induced changes in the secretory machinery of presynaptic neurons have previously been well documented. For instance, in Helisoma the motor neuron B19 becomes competent of transmitter release only after its contact with an appropriate muscle target cell (Zoran et al. 1990). Here we demonstrated that although a single unpaired RPeD1 was indeed competent of transmitter release, its secretory capabilities were acutely suppressed only after its contact with VD4. We have also shown that if a single RPeD1 was allowed to develop its transmitter secretion capabilities overnight, prior to its pairing with VD4, not only did VD4 fail to suppress dopamine release but also a reciprocal inhibitory synapse developed concurrently between the paired cell (Fig. 9). Taken together the data presented in this study suggest that transmitter suppression from RPeD1 is not only VD4 cell contact specific but that it is also time dependent. That is, if RPeD1 cell was given a head start to develop its transmitter secretory capabilities (12–18 h), its subsequent pairing with VD4 would fail to induce transmitter suppression. Therefore the transient suppression of transmitter release from RPeD1 suggests a new mechanism by which the timing of synapse formation might be regulated between mutually connected neurons. Because both RPeD1 and VD4 have several common postsynaptic targets in vivo (Syed and Winlow 1991), we speculate that VD4-induced suppression of transmitter release may enable this cell to “out-compete” RPeD1 for a synaptic target. Once VD4’s connectivity patterns are fully established, it may permit dopamine secretion from RPeD1, thus allowing it to innervate appropriate targets. The VD4-induced suppression of transmitter release from RPeD1 may thus define the temporal pattern of connectivity in this model. This hypothesis although needs to be tested experimentally, the data provided in Fig. 9 are, however, consistent with this postulate.

Regarding mechanisms of VD4-induced transmitter suppression in RPeD1, we observed that a freshly isolated VD4 fires more spontaneous action potentials (and/or often burst of spikes) as compared with its in vivo counterpart, which is generally quiescent. After 12–18 h of culture, VD4’s sponta-
neous activity patterns, however, decline to the levels seen in vivo (Syed et al. 1990). Because in a soma-soma configuration, VD4 was always the first cell to establish synaptic contact with RPeD1, we propose that this initial activity pattern and hence
the release of a FMRFamide-like peptide(s) at VD4-RPeD1 synapse would suppress transmitter release from RPeD1. Because after 12–18 h, the spontaneous activity levels in VD4 decline to its in vivo level, this would allow RPeD1 to recover from peptide-mediated synaptic depression, thus restoring the bi-directional synaptic transmission at 24–36 h. Consistent with this observation are our data, which showed that in instances where synaptic transmission between VD4 and RPeD1 was blocked by the inhibitors of AA pathway, the VD4-induced suppression of transmitter release from RPeD1 was prevented. Moreover, this perturbation also allowed RPeD1 to establish synaptic transmission with VD4 at a much earlier time point (12–18 h). Taken together, these data elude toward the possibility that as seen at the excitatory synapses, synaptic activity-dependent interaction may also be functional at the inhibitory synapses, albeit in the opposite direction (i.e., synaptic depression).

A number of studies have begun to establish the possible mechanisms that may account for “Hebbian synapses,” which are thought to involve, i.e., activity-dependent suppression of synaptic transmission. Using nerve-muscle co-cultures from *Xenopus* embryos, Lo and Poo (1991) demonstrated that when one of the two neurons innervating a single muscle cell was repeatedly stimulated, its synapse was strengthened while the synapse made by the nonstimulated neuron was suppressed. However, if both neurons were stimulated synchronously, synaptic suppression seldom occurred. The identity of the retrograde signal and the mechanism by which activity prevents synapse elimination at the developing neuromuscular junction are, however, presently unknown. In this study, we have demonstrated that the release of FMRFamide-like peptide from VD4 during early stages of synapse formation and the activation of AA pathway in RPeD1 are sufficient to suppress transmitter release from RPeD1. Because treatment of VD4, but not RPeD1, with actinomyces D and anisomycin prevented VD4-induced suppression of transmitter release from RPeD1, we suggest that both transcription and translation dependent steps involved in VD4-induced transmitter suppression in RPeD1 were most likely required for peptide synthesis and its secretory machinery in VD4.

The involvement of an AA signal pathway in FMRFamide-gic synaptic transmission in mollusks 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 et al. 1999), FMRFamide activates phospholipase A₂, which is required for AA synthesis. This molecule is subsequently broken down into a variety of metabolites (Piomelli et al. 1987), each of which can activate further signaling cascades in the cell (Lopes et al. 1998; Piomelli et al. 1987). In mollusks, FMRFamide/AA-mediated affects have consistently been found to signal through lipoxygenase pathway downstream of AA (Bahls et al. 1992; Lopes et al. 1998; Piomelli et al. 1987). We have demonstrated that both phospholipase A₂ (4-BPB) and a lipoxygenase inhibitor (NDGA) attenuated FMRFamide-induced inhibitory effects on cultured RPeD1 somata and blocked synaptic transmission between VD4 and RPeD1 (Fig. 7) (for NDGA, see Lovell 2000). 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 are 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 receptors and its signaling pathway are conserved in various molluscan species. Moreover, this study demonstrates that VD4-induced suppression of transmitter secretion from RPeD1 involves release of FMRFamide-like peptide(s) from VD4, which in turn activate 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), and these affects were also shown to be mediated via lipoxygenase metabolites of AA (Bahls et al. 1992; Piomelli et al. 1987). Specifically, AA metabolites were shown to activate K⁺ channels, which in turn hyperpolarized 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 Ca²⁺ currents, which in parallel with the activated outward K⁺ currents, hyperpolarize the cell further. This reduction in voltage activated Ca²⁺ current may also result in reduced Ca²⁺ influx in response to action potentials, thus reducing the total amount of transmitter at the synaptic sites. AA has recently been shown to inhibit not only Ca²⁺ channels but also the dopamine transporter in human cells (Ingram and Amara 2000). The preceding studies offer a number of possible mechanisms, which may account for both VD4- and FMRFamide-induced suppression of dopamine secretory machinery in RPeD1; however, the precise nature of cell-cell signaling remains to be defined. Because synaptic transmission between RPeD1 and VD4 neurons paired in a soma-soma configuration (24–36 h) remains unperturbed in the presence of FMRFamide (data not shown), we suggest that neither FMRFamide-induced suppression of Ca²⁺ current nor an enhancement of K⁺ current in RPeD1 is likely sufficient to produce long-term depression of dopamine release from this cell. We thus propose that FMRFamide-activated AA pathway may exert direct effect on synaptic machinery, although the precise nature of such mechanism remains unknown.

FMRFamide has not only been shown to suppress transmitter release at mature *Helisoma* synapses in culture (Man-Son-Hing et al. 1989), but it also induces synaptic depression and a decrease in the number of presynaptic varicosities at newly formed sensory to motor neuron synapse in *Aplysia* (Mackey et al. 1987; Montarolo et al. 1988; Schacher et al. 1993). These studies suggest that in addition to modulating neuronal excitability, FMRFamide may also depress the efficacy of transmitter release at both mature and newly formed synapse. In addition to the above-described acute affects, our data demonstrate that FMRFamide can also chronically suppress transmitter release from RPeD1 for many hours. Moreover, we showed that this transmitter suppression would occur only if both cells were paired immediately after their isolation. Specifically, when RPeD1 was given a head start, its subsequent paring with VD4 would fail to induce transmitter suppression. Taken together, our data suggest that unlike the above-mentioned conventional role in synaptic modulation, FMRFamide-like peptide released at synaptic site may also regulate the secretory machinery during early stages of synapse formation. These data thus underscore the importance of peptide neurotransmit-
ters in regulating the timing of synapse formation between mutually connected neurons.

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