Stability and Variability of Synapses in the Adult Molluskan CNS

Neil S. Magoski,* Andrew G. M. Bulloch**

Department of Physiology and Biophysics and Neuroscience Research Group, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada, T2N 4N1

Received 26 May 1999; accepted 22 October 1999

ABSTRACT: **Synaptic transmission was examined between identified neurons in the central nervous system (CNS) of the freshwater mollusk, *Lymnaea stagnalis*. Four identified neurons were used: Right Pedal Dorsal one (RPeD1; a dopaminergic respiratory interneuron), Visceral Dorsal two and three (VD2/3), and Visceral Dorsal four (VD4; a cardiorespiratory interneuron). Neuron RPeD1 synapses onto both VD2/3 and VD4, while VD4 makes a reciprocal synapse onto RPeD1. When compared from animal to animal, the connections were variable in sign. Previously, we demonstrated that, in a given animal, the RPeD1 → VD4 synapse could be either inhibitory, biphasic, or undetectable. The present study now expands this concept of variability by showing that the RPeD1 → VD2/3 synapse was either excitatory or undetectable from animal to animal, while the synapse from VD4 to RPeD1 was observed as inhibitory, biphasic, depolarizing, excitatory, or undetectable. Next, we used 1-day organ culture to determine if the variability observed between animals is a product of ongoing change to the sign of these identified synapses and whether or not the extent of change could be influenced by the culture conditions. Changes to the sign of transmission occurred within minutes and, more commonly, after 24-h organ culture. All three synapses were investigated before and after 1-day organ culture, in either defined medium (DM) or brain-conditioned medium (CM). Regardless of culture conditions, the RPeD1 → VD2/3 synapse showed no change of sign, i.e., it was relatively stable. However, the synapses between RPeD1 and VD4 did change sign, and when cultured in CM, the VD4 → RPeD1 synapse changed significantly more than in DM. These data indicate that variability of some synapses reflects changes at these synapses. This is the first report that specific synapses in an adult CNS can change sign, and that the sign of transmission can be modulated by environmental conditions.** © 2000 John Wiley & Sons, Inc. J Neurobiol 42: 410–423, 2000

Keywords: **synaptic transmission; freshwater mollusk; RPeD1; VD2/3; VD4**

Alterations to the sign and/or efficacy of synapses shape the configuration of neural networks (Marder, 1994; Weimann and Marder, 1994) and underlie some forms of learning (Fagnou and Tuchek, 1995; Abraham and Bear, 1996; Byrne and Kandel, 1996). Understanding these forms of synaptic plasticity is of importance for resolving the mechanisms of central pattern generation and behavioral plasticity. In a previous report (Magoski and Bulloch, 1999), we showed that the sign of synaptic transmission between identified neurons could vary between animals, e.g., monophasic versus biphasic. The present work expands on this concept by examining the variability of sign at a selection of synapses in the central nervous system (CNS) of the freshwater mollusk, *Lymnaea stagnalis*. Furthermore, we test the stability of the sign of these connections over time and in different culture conditions. The data show that the variability of some

* *Present address:* Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut 06520

** A. G. M. Bulloch is an AHFMR Senior Scientist

Correspondence to: A. G. M. Bulloch (bulloch@acs.ucalgary.ca)

Contract grant sponsor: Medical Research Council (MRC) of Canada (to AGMB)

Contract grant sponsor: MRC, Alberta Heritage Foundation for Medical Research (AHFMR) and NeuroScience Network (Canadian Centres of Excellence) Studentships (to NSM)

of the connections reflect their dynamic nature. Analogous processes are likely at work in the vertebrate nervous system, where, for example, the shape and density of dendritic spines continually change with time and experience (Cramer and Sur, 1995; Weiler et al., 1995; Wolff et al., 1995; Maletic-Savatic et al., 1999).

The molluskan CNS has been used extensively to study neuronal communication and plasticity (Kandel, 1979; Bulloch, 1989). The freshwater mollusk, *L. stagnalis* (a pond snail) offers a tractable CNS containing identified neurons with known synaptic connections and behavioral functions. Two particularly important cells are interneurons Right Pedal Dorsal one (RPeD1) and Visceral Dorsal four (VD4). These neurons are part of the respiratory central pattern generator (Syed et al., 1990; Syed and Winlow, 1991), and their synapses have been studied in some detail (Benjamin and Winlow, 1981; Benjamin, 1984; Skingsley et al., 1993; Magoski et al., 1995; Nesic et al., 1996; Magoski and Bulloch, 1999). Neuron RPeD1 also synapses onto two, essentially identical neurons known as Visceral Dorsal two and three (VD2/3). This connection has also been analyzed extensively (Winlow et al., 1981; Magoski et al., 1995; Magoski, 1996; Magoski and Bulloch, 1997).

It has been reported that RPeD1 and VD4 have mutually inhibitory connections (Syed et al., 1990; Moroz, 1991; Syed and Winlow, 1991), while RPeD1 makes an excitatory synapse onto VD2/3 (Winlow et al., 1981). However, a number of observations have suggested that the sign of these synapses can vary or that the connections can at times be undetectable. We recently examined the RPeD1 → VD4 synapse in detail (Magoski and Bulloch, 1999) and showed that it was either biphasic (depolarizing followed by hyperpolarizing), monophasic (inhibitory only), or undetectable in 48%, 39%, and 13% of preparations, respectively. Our data showed that both the biphasic and purely inhibitory synapses are monosynaptic in nature and mediated by the same transmitter, dopamine (Magoski and Bulloch, 1999). We now expand our study of synapses to include the reciprocal VD4 → RPeD1 and the RPeD1 → VD2/3 synapse. We show that the VD4 → RPeD1 connection is surprisingly variable, whereas the RPeD1 → VD2/3 connection is relatively stable. We sought to explain the animal to animal variability of all of these connections and provide evidence that, for some synapses, it reflects dynamic changes that occur within individual nervous systems. Moreover, the frequency with which a synapse changes sign can be influenced by environmental conditions.

MATERIALS AND METHODS

Animals, Dissection, and Salines

The experiments used a stock of the mollusk, *L. stagnalis*, raised and maintained at the University of Calgary. Animals had shell lengths of 15–25 mm (age ~1–4 months). Before selecting an animal, care was taken to ensure that it was not engaged in any obvious behavior, such as feeding, mating, breathing, or locomotion. All experiments required the removal of the CNS (see Magoski and Bulloch, 1997), being defined as the central ring ganglia *and* the buccal ganglia. For acute electrophysiology, the CNS was pinned to the rubber base (General Electric RTV 616) of an ~500-μl recording chamber, whereas for organ culture it was pinned to a small rubber pad. Dissection and pinning of the CNS was performed in normal *Lymnaea* saline (composition in m*M*: NaCl 51.3, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) 5.0; adjusted to pH 7.9 with 1 N NaOH). All electrophysiology was performed in high Ca^{2+}/high Mg^{2+} saline (composition in m*M*: NaCl 51.3, KCl 1.7, $CaCl_2$ 24.6, $MgCl_2$ 1.5, $MgSO_4$ 7.5, and HEPES 5.0; pH 7.9). This saline reduces the probability of polysynaptic effects by raising the action potential threshold of putative interneurons (Austin et al., 1967; Berry and Pentreath, 1976; Elliott and Benjamin, 1989). Salts were obtained from Sigma or BDH. Experiments were performed at room temperature (18–20°C).

Identification of Neurons

The neurons examined were all identifiable from preparation to preparation with a high degree of reliability. For example, RPeD1 is the only large neuron in the right pedal ganglion and is easily recognizable on the basis of size, location, color, and relatively infrequent firing pattern (Benjamin and Winlow, 1981). Neuron VD2/3 is a pair of virtually identical cells that show the same morphologic and electrophysiological characteristics, and consequently, they are treated as one neuron (Benjamin and Winlow, 1981; Magoski and Bulloch, 1997). They can be readily distinguished from the neurons around them on the basis of their large size, color, high resting potentials, and lack of spontaneous activity. For the organ culture experiments, the same neuron from the VD2/3 pair in a given preparation was investigated on both days; this was usually facilitated by drawing a map of the location of these cells. Neuron VD4 is a small, very white cell whose exact location in the visceral ganglion can differ between preparations. To ensure that the cell in question was VD4, one or more of three criteria were used: (i) VD4 always displayed a characteristic discharge of steadily broadening action potentials immediately following impalement; (ii) VD4 often displayed regenerative firing properties; (iii) VD4 always made an excitatory synapse with neurons RPeD2/3 (Syed and Winlow 1989; Nesic et al. 1996). Thus, the variability of synapses described in this study was not due to discrepancies in neuronal identification.

Electrophysiology

Current clamp was performed using electrodes of 20–30 MΩ resistance when filled with 2 *M* potassium acetate. Data were collected with a dual-channel intracellular amplifier equipped with a bridge balance [see Magoski and Bulloch (1997) for details]. On some occasions microelectrode penetration of neurons was aided by exposing the surrounding connective tissue sheath to a small pronase crystal (Sigma type XIV), held by forceps, for 1–3 s. The CNS was then rinsed five times in cold (~4°C) normal saline to remove excess enzyme. During electrophysiology, the chamber was perfused at a rate of ~3 mL/min using a peristaltic pump.

Organ Culture

The CNS was cultured for ~24 h to examine synapses over time. A CNS was pinned to a small rubber pad, and the synapses investigated. It was then taken to an aseptic laminar flow hood, rinsed (3 × 5 min) in antibiotic saline (ABS; normal saline with 150 μg/mL gentamycin, Sigma G3632), and placed in a sterile glass vial (Kimble 08360) containing 6 mL of chemically defined medium (DM) or brain-conditioned medium (CM). The DM was serum-free, 50% (v/v) Liebowitz L-15 medium (Gibco, special order), with added inorganic salts (concentration in m*M*: NaCl 40.0, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, and HEPES 5.0; pH 7.9) and 20 μg/mL of gentamycin. CM was made by incubating DM with 2 *Lymnaea* CNS/mL for 72 h (see Ridgway et al., 1991). The vial was kept in a humidified incubator at room temperature (18–20°C). The next day, the preparations were removed and investigated in the same order that they were originally examined. Note that although the electrophysiology was performed in high Ca^{2+}/high Mg^{2+} saline, the organ cultured preparations spent most of their time in either DM or CM.

Statistical Analysis

The statistical software program, Instat 2.01 (GraphPad Software) was used to calculate the mean and standard error of the mean (S.E.M.) of each data point and to perform Fisher's exact test. This test examines the association between two variables and was used to test hypotheses about differences in frequency. Data were considered significantly different if the two-tailed *p* value was less than 0.05.

RESULTS

Acutely Isolated Preparations

We first examined whether the sign of the RPeD1 → VD2/3 synapse, the RPeD1 → VD4 synapse, and the VD4 → RPeD1 synapse varied between acutely isolated CNS preparations. The synaptic sign refers to the effect of presynaptic stimulation on both the membrane potential and excitability of the postsynaptic neuron, and, for the purpose of the present study, is as follows: (i) *excitatory*, where a depolarizing excitatory postsynaptic potential (EPSP) elicited postsynaptic action potentials; (ii) *inhibitory*, where an inhibitory postsynaptic potential (IPSP) hyperpolarized the postsynaptic neuron, prevented postsynaptic action potentials, and reversed close to −90 mV; (iii) *biphasic*, where a biphasic postsynaptic potential (BPSP) displayed a depolarizing phase followed by a hyperpolarizing phase (despite the fact that the biphasic synapse had a depolarizing phase, it was not excitatory because the depolarization was likely mediated by a Cl^- conductance that clamped the membrane below spike threshold; Magoski and Bulloch, 1999); (iv) *depolarizing but not excitatory*, where a depolarizing postsynaptic potential (DPSP) inhibited postsynaptic action potentials (like the depolarizing phase of the BPSP, the DPSP had a reversal potential below threshold and again likely involved a Cl^- conductance); and (v) *undetectable*, where no physiological synapse could be detected. Although we recognize that "undetectable" is not a synaptic sign *per se*, we included it here because, while it is important to determine the sign of a detectable synapse, it is equally important to note when the neurons are not connected.

Different Signs of Transmission at RPeD1 → VD2/3 and RPeD1 → VD4 Synapses

Both of the synapses made by RPeD1 proved variable, although the connection onto VD4 displayed more forms than the connection onto VD2/3. Each PSP was investigated over a range of postsynaptic voltages, typically between −40 and −90 mV. The average resting membrane potentials of the neurons was: −56.0 ± 0.7 mV for RPeD1 (n = 537), −74.4 ± 0.7 mV for VD2/3 (n = 381), and −53.0 ± 0.8 mV for VD4 (n = 267). For the RPeD1 → VD2/3 synapse, RPeD1 excited VD2/3 in 80% of the cases (n = 305), whereas the synapse was undetectable in the remaining 20% of preparations (n = 76; Fig. 1). For the RPeD1 → VD4 synapse, we recently reported that RPeD1 inhibited VD4 in 39% of the preparations (n = 104), in 48% of the cases made a biphasic synapse onto VD4 (n = 129), and in the remaining 13% of preparations the synapse was undetectable (n = 34; numerical data taken from Magoski and Bulloch, 1999; Fig. 2). The biphasic synapse was likely not a product of a polysynaptic pathway, inasmuch as the connection was maintained in high divalent saline and showed a constant and rapid (more rapid than the inhibitory form itself) latency (Magoski and Bulloch, 1999). The biphasic and inhibitory synapses were also distinctly different. If a biphasic synapse was present, it would be detected, particularly at more negative

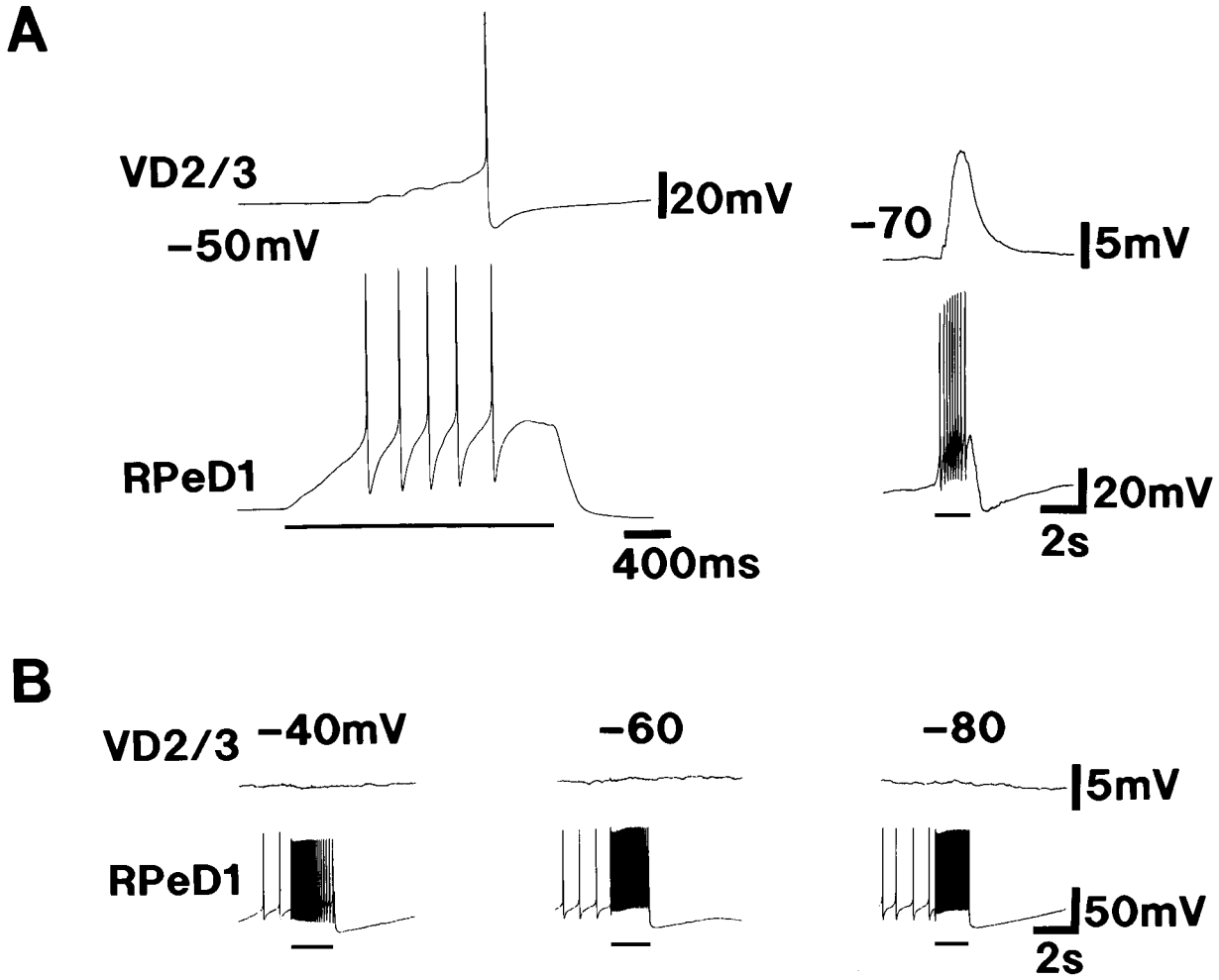

Figure 1 Neuron RPeD1 either excited or was not connected to VD2/3. (A) In the majority of preparations, RPeD1 excited VD2/3. When VD2/3 was held at −50 mV, a short burst of action potentials in RPeD1 caused VD2/3 to fire a spike. At a more negative membrane potential of −70 mV, action potentials in RPeD1 caused VD2/3 to depolarize. RPeD1 membrane potential = −55 mV. (B) In another preparation, the synapse from RPeD1 to VD2/3 was undetectable. The synapse did not elude detection, because a PSP could not be elicited at a range of postsynaptic membrane potentials (given across the top). RPeD1 membrane potential = −55 mV. Bars indicate the duration of depolarizing current injection into RPeD1.

postsynaptic voltages. Thus, a biphasic synapse would not be categorized as an inhibitory synapse just because the depolarizing phase was overlooked.

The possibility that seasonal or environmental changes were related to the differences seen in the sign of transmission was investigated. No obvious correlation between the month, day of the week, time of day, or feeding schedule and the sign of the RPeD1 → VD2/3 or RPeD1 → VD4 synapse was observed (data not shown).

A question arising from the preparations with undetectable synapses is whether RPeD1 tends to be disconnected from multiple targets within a preparation. To determine to what extent RPeD1 was connected to both VD2/3 and VD4, both synapses were investigated in the same preparation. For this purpose, the synapses were considered only as connected (i.e., excitatory, inhibitory, or biphasic) or not connected (i.e., undetectable). When RPeD1 was connected to VD2/3 it was connected to VD4 86% of the time (n = 96). In a markedly lower number of preparations, 9%, RPeD1 had no detectable synapse onto either VD2/3 or VD4 (n = 10). Finally, in even fewer preparations RPeD1 was connected to VD2/3 but not VD4 3% of the time (n = 3) and in 2% of the cases RPeD1 was connected to VD4 but not VD2/3 (n = 2).

There were three intriguing cases where the sign of the RPeD1 → VD4 synapse changed *during* the

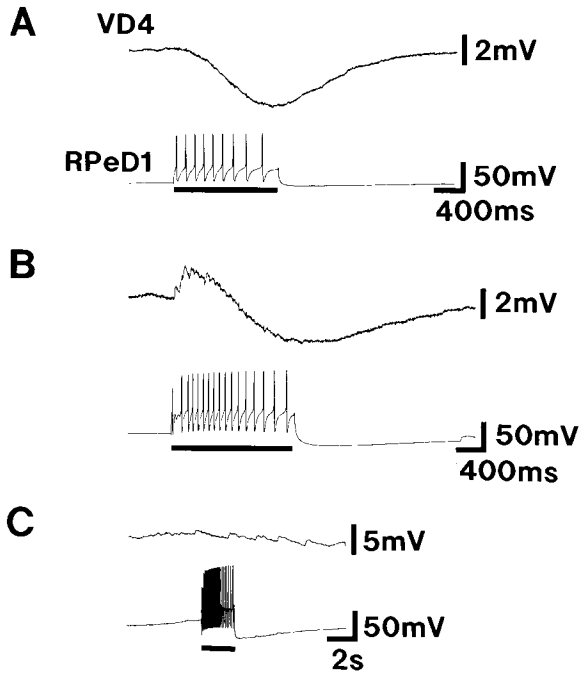

Figure 2 Signs of synaptic transmission at the RPeD1 to VD4 connection. (A) Stimulation of RPeD1 inhibited VD4. Membrane potentials: RPeD1 = −56 mV; VD4 = −70 mV. (B) Neuron RPeD1 biphasically depolarized then hyperpolarized VD4. Membrane potentials: RPeD1 = −58 mV; VD4 = −70. (C) The synapse from RPeD1 to VD4 was undetectable. Membrane potentials: RPeD1 = −50 mV; VD4 = −70. Bars indicate the duration of depolarizing current injection into RPeD1. In all cases, the synapse was tested with the membrane potential of VD4 held at various voltages, typically between −40 and −90 mV. For simplicity, only the postsynaptic voltage of −70 mV was presented here.

course of an experiment. Two synapses spontaneously changed from inhibitory to biphasic and another from biphasic to inhibitory. These changes were unambiguous and persisted for the remainder of the experiment (total time 40–90 min). Figure 3 shows a biphasic synapse that changed to an inhibitory synapse over a matter of minutes. These data suggest that the difference of synaptic sign between acutely isolated preparations reflects changes occurring within individual preparations.

Different Signs of Transmission at VD4 → RPeD1 Synapses

The sign of the reciprocal synapse from VD4 to RPeD1 in acutely isolated CNS preparations was also investigated. The sign of the VD4 → RPeD1 synapse was particularly diverse (Fig. 4). Neuron VD4 inhibited RPeD1 16% of the time (n = 40), made a biphasic connection to RPeD1 in 31% of cases (n = 78), and in 26% of the preparations VD4 depolarized but did not excite RPeD1 (n = 65). Neuron VD4 excited RPeD1 in 10% of the preparations (n = 25) and the connection was undetectable 17% of the time (n = 44). No correlation between the month, day of the week, time of day, or feeding schedule and the sign of the VD4 → RPeD1 synapse was observed (data not shown).

Organ Culture Preparations

Given the above data, we sought to examine whether synaptic transmission can change over time within a preparation. This was suggested by the observation that a few of the RPeD1 → VD4 synapses spontaneously changed sign during the recording period. Two hypotheses were tested: first, that the sign of transmission can change within a preparation, and second, that the frequency of changes can be modulated by neurotrophic factors. To this end, synapses were examined before and after 1-day organ culture in either

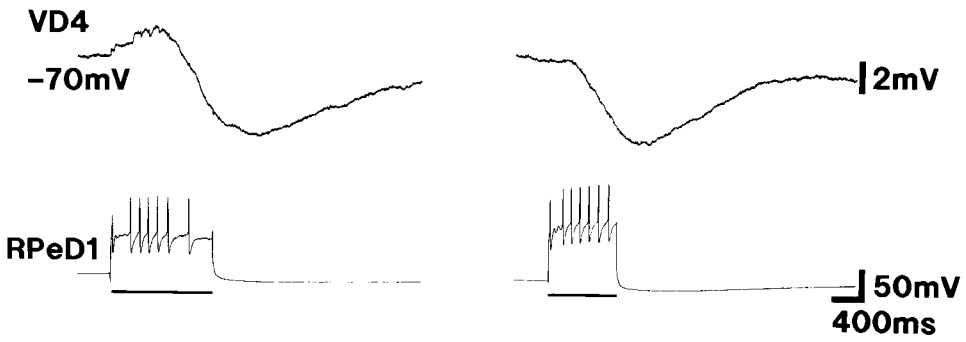

Figure 3 Conversion of the sign of transmission at a synapse from RPeD1 to VD4. Initially, stimulation of RPeD1 produced a BPSP in VD4. Approximately 6 min later the synapse was tested again, and RPeD1 elicited a monophasic IPSP in VD4. The sign did not change again throughout the remainder of the experiment. No connection from VD4 to RPeD1 was detected at any time during the experiment. RPeD1 membrane potential = −55 mV. Bars indicate the duration of current injection into RPeD1.

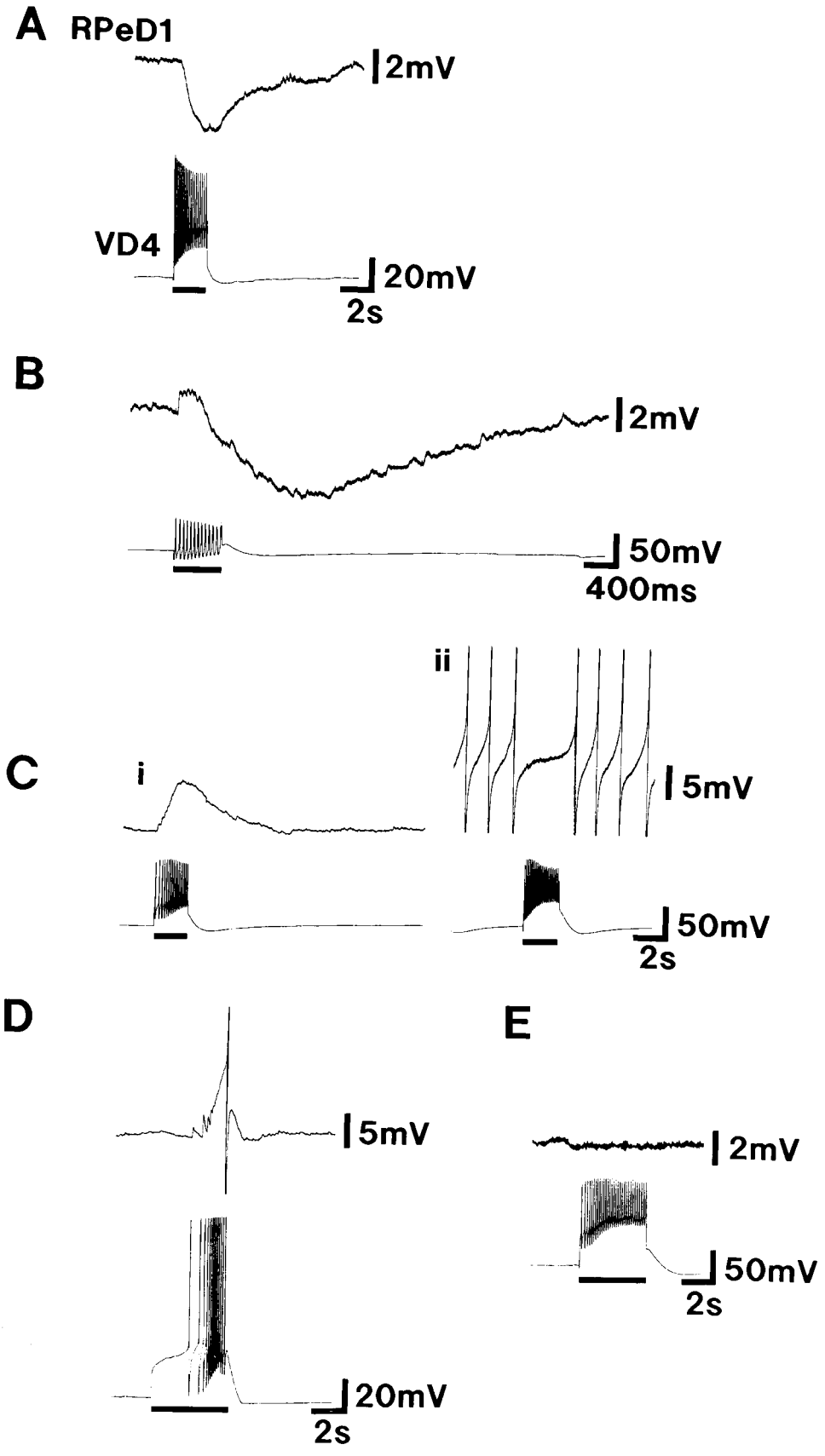
A
RPeD1
2mV
VD4
20mV
2s
B
2mV
50mV
400ms
C
i
ii
5mV
50mV
2s
D
5mV
20mV
2s
E
2mV
50mV
2s

Table 1 Sign of the RPeD1 → VD2/3 Synapse before and after Organ Culture

	DM	CM
Unchanged		
E → E	13	11
U → U	2	1
Total	14	12
Changed		
E → U	0	1
(*n*)[a]	(15)	(13)

E, excited; U, undetectable.

[a] Number of preparations in which synapses changed or were unchanged in DM or CM following 1 day of organ culture.

Table 2 Sign of the RPeD1 → VD4 Synapse before and after Organ Culture

	DM	CM
Unchanged		
B → B	5	2
I → I	3	5
U → U	3	1
Total	11	8
Changed		
B → I	1	5
I → B	1	1
B → D	1	1
B → U	1	0
Total	4	7
(*n*)[a]	(15)	(15)

B, biphasic; D, depolarized but not excited; I, inhibited; U, undetectable.

[a] Number of preparations in which synapses changed or were unchanged in DM or CM following 1 day of organ culture.

defined medium (DM) or conditioned medium (CM; which contains neurotrophic molecules; Ridgway et al., 1993). DM and CM experiments were performed in parallel; DM can be considered a control or neutral culture condition.

The RPeD1 → VD2/3 Synapse Was Stable in Organ Culture

For the RPeD1 → VD2/3 synapse, in DM 0% (0 of 15) of the RPeD1 → VD2/3 synapses changed synaptic sign, while in CM, only 8% (1 of 13) of the synapses changed ($p > 0.05$, Fisher's exact test; Table 1). Thus, the RPeD1 → VD2/3 synapse appears to be rather stable, inasmuch as neither of the two hypotheses hold true for this connection. An important consideration was whether or not the synapse was deteriorating in organ culture. This did not seem to be the case, as only one RPeD1 → VD2/3 synapse, in this case cultured in CM, changed from excitatory to undetectable (Table 1).

The RPeD1 → VD4 Synapse Changed in Organ Culture

The RPeD1 → VD4 synapse, a more variable connection, was examined before and after 1-day organ culture in DM or CM. Some of these organ cultures were the same preparations in which the RPeD1 → VD2/3 synapse was examined. The only practical manner in which to quantify change to synaptic sign was to record the fact that a change occurred, as too many types of changes were possible to analyze separately. In DM, 27% (4 of 15) of the RPeD1 → VD4 synapses changed synaptic sign, while in CM, 47% (7 of 15) of the synapses changed sign. Despite this trend, the effect of CM on the frequency of change was not significant ($p > 0.05$; Table 2). As shown in Figure 5, the most common change in sign for the RPeD1 → VD4 synapse following organ culture, particularly in CM, was that of biphasic to inhibitory. Overall, this data satisfies the first hypothesis, in that the synapse does indeed change sign over time, although the second hypothesis was not satisfied, as CM did not enhance the frequency of change.

Figure 4 Signs of synaptic transmission at the VD4 to RPeD1 connection. (A) Neuron VD4 inhibited RPeD1. Membrane potentials: RPeD1 = −70 mV; VD4 = −60 mV. (B) Stimulation of VD4 biphasically depolarized then hyperpolarized RPeD1. Membrane potentials: RPeD1 = −70 mV; VD4 = −52 mV. (Ci) Neuron VD4 depolarized RPeD1. Membrane potentials: RPeD1 = −70 mV; VD4 = −55 mV. (Cii) When RPeD1 was driven to fire action potentials (membrane potential −40 mV), and VD4 was again stimulated, it resulted in inhibition of RPeD1, not excitation. (D) Neuron VD4 excited RPeD1, producing discrete EPSPs and a single action potential. Membrane potentials: RPeD1 = −50 mV; VD4 = −60 mV. (E) The synapse from VD4 to RPeD1 was undetectable. Membrane potentials: RPeD1 = −70 mV; VD4 = −58 mV. Bars indicate the duration of depolarizing current injection into VD4. The membrane potential of RPeD1 was held at various voltages, typically between −40 and −90 mV, and the synapse was tested. For simplicity only specific postsynaptic voltages were presented here.

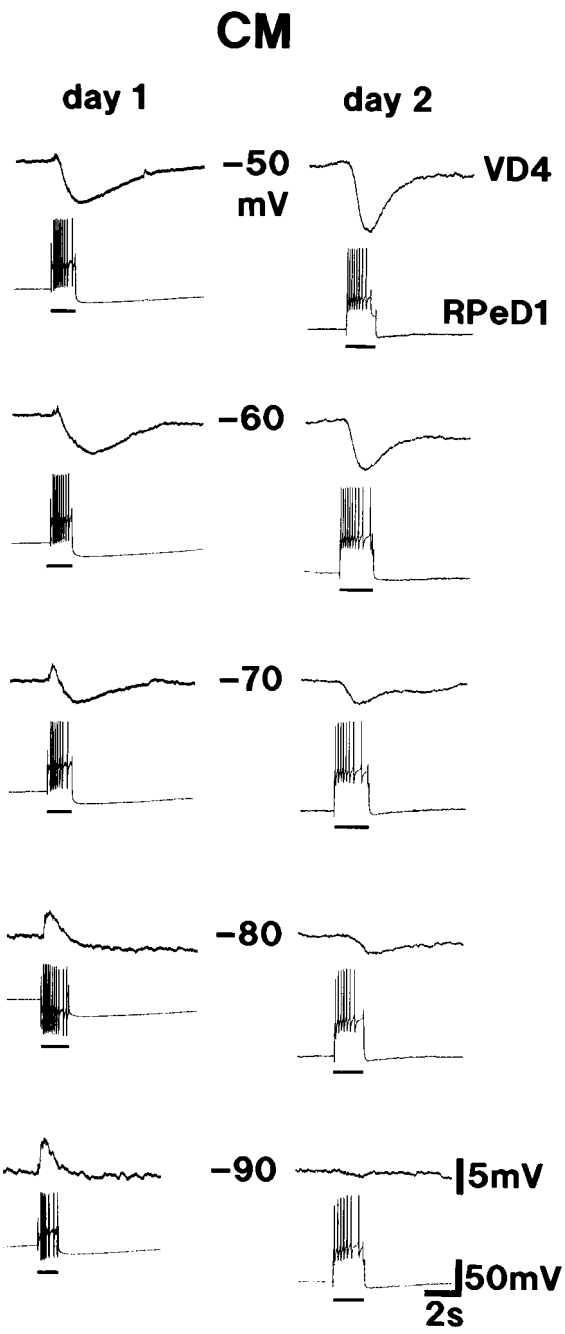

Figure 5 The RPeD1 → VD4 synapse changed from biphasic to inhibitory following organ culture. In this case the culture medium was CM. On both days, VD4 was held at the same range of designated membrane potentials and a PSP was elicited. On day 1, a BPSP was elicited in VD4 by RPeD1 stimulation, while on day 2 RPeD1 stimulation elicited an IPSP in VD4. The reduced frequency of firing in RPeD1 on day 2 was intentional. The biphasic response could become somewhat obscured during high-frequency input from RPeD1, and to demonstrate with certainty that the synapse on day 2 was in fact only inhibitory, a lower frequency of stimulation was chosen. RPeD1 membrane potential on both days = −70 mV. Bars indicate the duration of depolarizing current injection into RPeD1.

As with the RPeD1 → VD2/3 connection, the RPeD1 → VD4 synapse did not deteriorate in organ culture. One synapse, in this case cultured in DM, changed from connected to undetectable (Table 2).

Because both the RPeD1 → VD2/3 and the RPeD1 → VD4 synapses were investigated in many of the same preparations, this allowed comparison of two different synapses with a common presynaptic origin. Data were pooled from DM and CM because neither of these conditions promoted more change for these two synapses. Only 4% (1 of 28) of the RPeD1 → VD2/3 synapses changed following organ culture, while in the same preparations, a significantly larger proportion, 37% (11 of 30), of the RPeD1 → VD4 synapses changed ($p < 0.003$). This comparison emphasizes the relative stability of RPeD1's connection to VD2/3 compared with that to VD4.

The VD4 → RPeD1 Synapse Changed in Organ Culture and Change Was Enhanced by CM

The reciprocal VD4 → RPeD1 synapse was also investigated in organ culture. In DM, 40% (6 of 15) of the VD4 → RPeD1 synapses changed sign, compared to 87% (13 of 15) in CM. This result is significant, indicating that the synaptic sign changed more following organ culture in CM than in DM ($p < 0.03$; Table 3). For the VD4 → RPeD1 synapse, the most frequent change to synaptic sign was that of depolarizing to inhibitory synapse (Fig. 6). The behavior of the VD4 → RPeD1 synapse following organ culture satisfies both of the hypotheses; the connection changes over time, and the extent of change is enhanced by neurotrophic factors.

As with the RPeD1 synapses, the VD4 → RPeD1 synapse did not deteriorate in organ culture. Three synapses in DM (20%) and three in CM (20%) changed from connected to undetectable, whereas one in DM (7%) and two in CM (13%) changed from undetectable to connected (Table 3). Thus, while there was loss of function for some synapses, there was also gain of function for others.

DISCUSSION

Both the network properties of neurons and the ability to store information depend on the sign and/or efficacy of chemical transmission (Marder, 1994; Weimann and Marder, 1994; Fagnou and Tuchek, 1995; Abraham and Bear, 1996; Byrne and Kandel, 1996). Thus, it is important to examine both the efficacy *and* the sign of synapses. We have reported on the variability of connections between identified *Lymnaea* neurons. As well, we have shown that some of these connections are stable, whereas others show

Table 3 Sign of the VD4 → RPeD1 Synapse before and after Organ Culture

	DM	CM
Unchanged		
B → B	2	0
D → D	3	1
U → U	3	0
I → I	1	1
Total	9	2
Changed		
D → I	1	3
B → D	0	1
D → B	1	0
D → U	0	2
I → U	3	0
E → B	0	2
I → B	0	1
B → U	0	1
E → D	0	1
U → B	0	1
U → D	0	1
U → I	1	0
Total	6	13
(*n*)[a]	(15)	(15)

B, biphasic; D, depolarized but not excited; E, excited; I, inhibited; U, undetectable.

[a] Number of preparations in which synapses changed or were unchanged in DM or CM following 1 day of organ culture.

change in the sign of transmission over time, which can be influenced by culture conditions.

Variability in Synaptic Sign

Previously, we demonstrated that the sign of the RPeD1 → VD4 synapse was variable between animals, with inhibitory, biphasic, or undetectable connections (Magoski and Bulloch, 1999). This lead us to examine the RPeD1 → VD2/3 and the VD4 → RPeD1 synapses in the present study, and we also found differences in the sign of transmission for these connections. The VD4 → RPeD1 synapse displayed the most physiological and functional differences in sign, with inhibitory, biphasic, depolarizing, excitatory, or undetectable connections. In contrast, the RPeD1 → VD2/3 synapse was more predictable, usually manifesting itself as a simple EPSP, or less frequently it was undetectable. When both of RPeD1's synapses were examined in the same preparations, their presence appeared to be interdependent, i.e., in general, RPeD1 was either connected to both VD2/3 and VD4, or was not connected to either postsynaptic neuron.

Neuron RPeD1 uses dopamine as its neurotransmitter (Magoski et al., 1995); thus, the undetectable RPeD1 → VD2/3 or RPeD1 → VD4 synapses could be due to lack of dopamine release or absence of postsynaptic dopamine receptors. Alternatively, the synapse may not have been formed during development. The enigma of two different chemical connections at the RPeD1 → VD4 synapse can be explained by our prior work. Magoski and Bulloch (1999) showed that the biphasic synapse is mediated by dopamine acting on two dopamine receptors: one that is sulpiride-insensitive and likely activates a Cl^- conductance, and a second that is sulpiride-sensitive and probably activates a K^+ conductance. The inhibitory RPeD1 → VD4 synapse is mediated solely by the sulpiride-sensitive, K^+ conductance-coupled receptor.

The VD4 → RPeD1 synapse has been reported as inhibitory (Syed et al., 1990; Moroz, 1991; Syed and Winlow, 1991), biphasic (Benjamin, 1984; Skingsley et al., 1993), and in a preliminary report ($n = 2$), as excitatory (Park and Winlow, 1993). Skingsley et al. (1993) indicated that the inhibitory effects of VD4 on RPeD1 are mediated by SDPFLRFamide or GDPFLRFamide. Furthermore, the depolarizing, but not excitatory, effects of VD4 on RPeD1 might be glutamatergic (Nesic et al., 1996). Thus, differences in synaptic sign may reflect a divergence in the types of transmitters released by VD4. However, none of the transmitters thought to be present in VD4 produce true excitation in RPeD1 (Nesic et al., 1996). Also, preliminary attempts on our part to correlate the sign of the VD4 → RPeD1 synapse with a response to glutamate or GDPFLRFamide were inconclusive (data not shown). Consequently, differences in postsynaptic receptors may also contribute to variability at the VD4 → RPeD1 synapse.

There are other potential sources of differences in synaptic sign. Different receptors may be localized on different postsynaptic axon collaterals and activated in various combinations, depending on the extent of transmitter release from presynaptic terminals. This is in essence an issue of efficacy, in which different degrees of efficacy at specific sites of release combine to produce variability and a distribution of synapses. Such a possibility notwithstanding, we would argue that the fundamental issue of changes in the effects of presynaptic stimulation on both postsynaptic membrane potential and excitability, i.e., sign, can be separated from changes to efficacy. These events may reflect a level of complexity beyond that of changes in efficacy, where the manner in which the presynaptic neuron signals the postsynaptic neuron is different both functionally and likely mechanistically. Another potential source of variability could be animal-to-

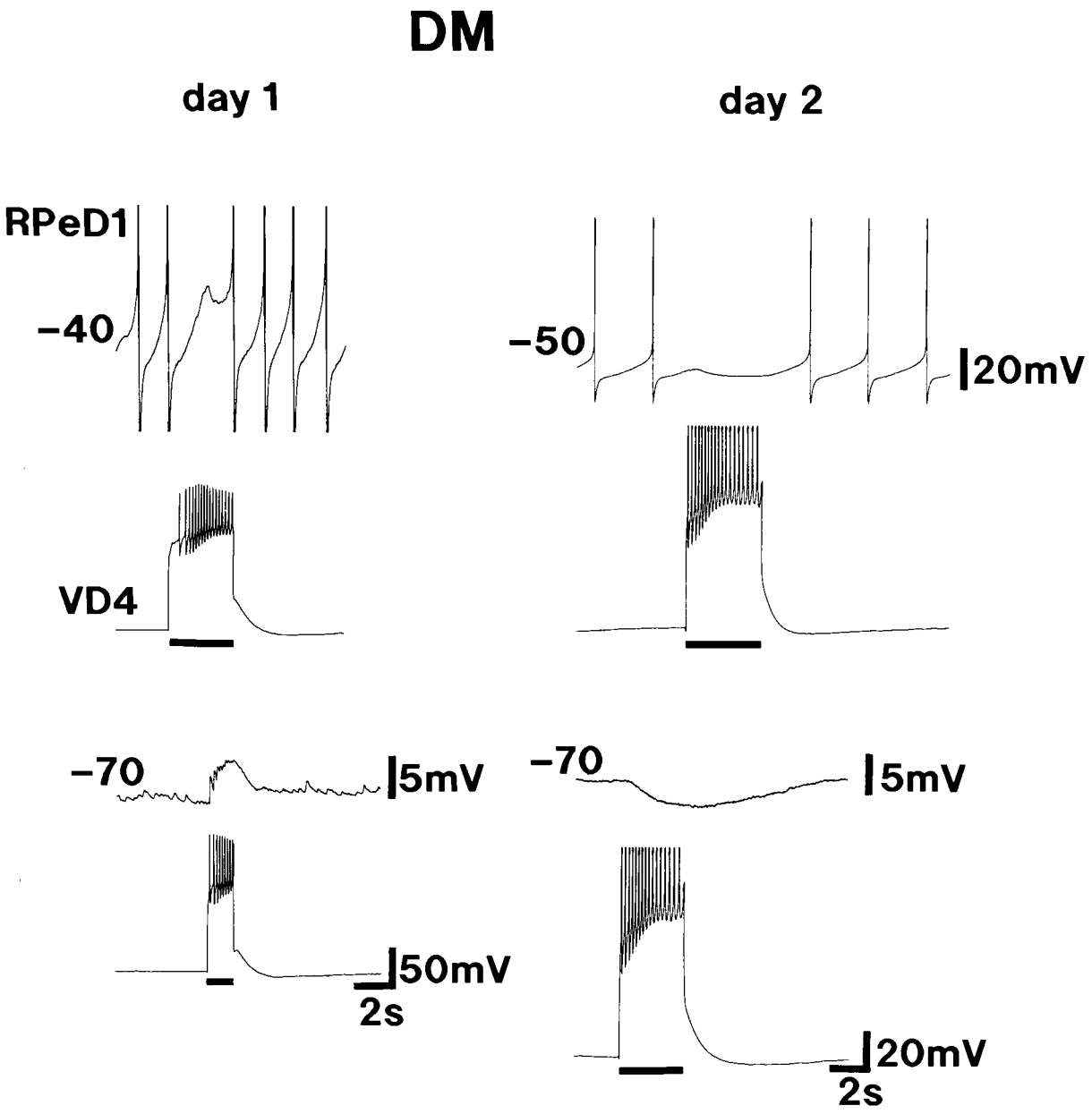

Figure 6 The VD4 → RPeD1 synapse changed from depolarizing to inhibitory following organ culture. In this case the culture medium was DM. The VD4 → RPeD1 synapse was investigated in the same preparation before and after 1-day organ culture. For each day, RPeD1 was held at the designated membrane potentials, and a PSP was elicited. On both days, when RPeD1 was held at a potential above threshold, the synaptic input inhibited spiking; however, the underlying mechanism was different, because on day 1 the synapse was depolarizing at −70 mV (likely Cl^--dependent), while on day 2 the synapse was hyperpolarizing at −70 mV (likely K^+-dependent). VD4 membrane potential: day 1 = −60 mV; day 2 = −62 mV. Bars indicate the duration of depolarizing current injection into VD4.

animal alterations in ionic gradients. For example, a specific K^+/Cl^- co-transporter is developmentally regulated such that its expression mediates a change in GABA responses from depolarizing to hyperpolarizing upon hippocampal maturation (Rivera et al., 1999). In the present work, varying the degree of expression or expressing different transporters could account for changes to some of the synapses, especially those activating Cl^--permeable channels. Alternatively, shunting conductance(s) could cause a

synaptic input to decay and not consistently reach the soma (the recording site), which would make it appear as if a synapse was absent. The latter is unlikely because of the robust nature of the preparation and the use of high Ca^{2+}/high Mg^{2+} saline. Finally, there is a small possibility that variability is the product of polysynaptic activation of putative interneurons, although this is unlikely because of prior evidence for monosynapticity of connections (Skingsley et al., 1993; Magoski and Bulloch, 1997, 1999) and the presence of high divalent ions.

Differences in synaptic sign imply functional differences, such as connected versus not connected or inhibitory versus excitatory. However, the difference between depolarizing, biphasic, and inhibitory synapses is less straightforward. Although all of these synapses are functionally inhibitory, the time course of inhibition is different. For example, the depolarizing form of inhibition causes a decrease in excitability without a large change in voltage, particularly at resting potential, and recovery is quite rapid. Conversely, the hyperpolarizing, K^+-dependent form of inhibition causes a significant change in postsynaptic membrane potential, and recovery is prolonged. Additionally, depolarization could regulate voltage-dependent phenomena, such as voltage-sensitive adenylate cyclase (Reddy et al., 1995). Furthermore, the changes that likely occur to postsynaptic Cl^- concentration, during biphasic and depolarizing transmission, could modulate G-protein–dependent processes, as suggested by Lenz et al. (1997).

Variations in synaptic sign may be linked to the behavioral state of the animal (Lukowiak, 1980; Lukowiak and Freedman, 1983). In crayfish, exogenous serotonin modulation (facilitation or inhibition) of synaptic input to an identified interneuron depends on the social dominance status of the animal (Yeh et al., 1997). Variations in synaptic connections may also depend on the release of neuromodulators (Selverston, 1992; Marder, 1994). In the crab stomatogastric nervous system, presynaptic inhibition selectively reduces chemical excitation, without effecting electrical transmission at mixed chemical/electrical synapses (Coleman et al., 1995).

Neurons RPeD1, VD4, and another cell known as the input three interneuron constitute the respiratory central pattern generator (CPG) in *Lymnaea* (Syed et al., 1990; Syed and Winlow, 1991). Mutual inhibition between RPeD1 and VD4 is thought to be essential for rhythm generation (Syed et al., 1990; Moroz, 1991; Syed and Winlow, 1991). However, RPeD1 and VD4 are also part of circuits involved in locomotion and cardiovascular function (Buckett et al., 1990) and these networks may be configured in more than one state. Spencer et al. (1999) recently reported that the frequency of both spiking in RPeD1 and the spontaneous bursting of Input three were reduced in animals whose respiratory behavior had been operantly conditioned. Whether similar variability in synaptic connectivity can be correlated with different behavioral states remains to be seen. In another example, the swallowing CPG in lobsters is temporarily configured by borrowing neurons from other CPGs (Meyrand et al., 1991). By eliminating a synapse, or changing its sign, neurons may be brought into or out of a circuit, or their role within a circuit altered (the "polymorphic network" concept; Getting, 1989).

An unusual aspect of our study is that all of the synapses showed, to one extent or another, cases where no connection could be detected. Such "undetectable" synapses could be the result of transmitter release or receptor levels being so low that PSPs are not large enough to be monitored. Hence, we use the term "undetectable" as opposed to "absent" synapses, as the synapses could be physiologically present, but the neurons are clearly not *functionally* connected.

Changes to Synaptic Sign

The rapid conversion of sign at a RPeD1 → VD4 synapse suggested that dynamic changes might account for the differences in synapses displayed by acutely isolated preparations. This was tested by examining the synapses before and after 1-day organ culture. For the synapses between RPeD1 and VD4, the sign did change, indicating that synapses change sign over time within the animal. However, time is likely not the most important variable in producing change; rather, the conversion of sign is probably driven by processes discussed above (both *in vivo* and *in situ*), as attested by the sudden sign switch at three of the RPeD1 → VD4 synapses. Magoski and Bulloch (1999) showed that the biphasic and inhibitory synapses from RPeD1 to VD4 are likely produced by the activation of two dopamine receptors. Thus, a possible mechanism for changing the RPeD1 → VD4 synapse would be to alter the activation or expression of these receptors. Furthermore, the various mechanisms contributing to variability (receptor localization, differential transmitter release, changes in efficacy, changes in ionic gradients, shunting conductances, polysynaptic pathways) hypothesized in the previous section of the Discussion could function in the organ culture and result in the synapses changing over time within a preparation.

Although the RPeD1 → VD4 synapse changed following organ culture, CM did not affect the frequency of change. Thus, change at this synapse is likely due to a variable other than a diffusible molecule. A dichotomy was apparent between the RPeD1

→ VD2/3 and RPeD1 → VD4 synapses. The RPeD1 → VD4 synapse changed significantly more often following organ culture than the RPeD1 → VD2/3 synapse. Thus, the RPeD1 → VD4 synapse is malleable, whereas the RPeD1 → VD2/3 synapse is relatively stable. Neurons RPeD1 and VD4 may have more diverse physiological roles than VD2/3 (whose function remains to be determined), this being reflected in the plasticity of their synapses. This also demonstrates that organ culture does not result in widespread change or deterioration, as specific synapses respond differently. That said, the fact that some synapses changed from detectable to undectable, particularly the VD4 → RPeD1 synapse (see below), raises the possibility that recording quality or cell health may contribute to connections becoming undetectable. There is an inherent limitation to the inclusion of undetectable synapses, as one can never be absolutely sure that these connections are absent for physiological rather than pathological reasons. While pathology is conceivable, we feel it is unlikely, as we consistently observed negative membrane potentials and little variability in, or obviously low values for, input resistance, suggesting favorable recording conditions as well as healthy cells.

For the VD4 → RPeD1 synapse, the frequency of change in sign was remarkable. Of particular note were the overall number of changes, and that some synapses were fundamentally different following organ culture, such as excitatory versus biphasic. This is in keeping with the concept that organ culture mimics events *in vivo*, where the VD4 → RPeD1 connection had a diverse distribution of synapses, which remained equally diverse following organ culture. The ability of a synapse in the adult brain to change the very nature of its signaling is striking. Our observations suggest that a significant degree of "plasticity" exists at this synapse, but its behavioral significance remains to be determined.

The VD4 → RPeD1 synapse also showed more changes to the sign of transmission following organ culture in CM than in DM. This suggests that there is a component(s) of CM that promotes this form of synaptic plasticity. The change itself in CM was quite random, although given that this synapse was variable to begin with, exposure to the neurotrophic factors in CM simply enhances the frequency of synaptic changes. Organ culture in murine NGF, a potential component of CM (Ridgway et al., 1991), did not promote more change to any of the three synapses (data not shown). Neurotrophic molecules can modulate synaptic transmission (Lo, 1995; Berninger and Poo, 1996). For example, organ culture of the *Aplysia* CNS in transforming growth factor-α enhances sensory-to-motor neuron transmission (Zhang et al., 1997). The results of the present study suggest that a component of CM can regulate synapses in the *Lymnaea* CNS. This is the first report that synaptic sign, as opposed to efficacy in its strictest sense, can be modulated by the neurotrophic environment. Collectively, these studies support a role for neurotrophic factors as neuromodulators in the CNS.

The authors thank Garry Hauser for technical assistance, Nadine Magoski, Olivera Nesic, Steven Barnes, Richard Hawkes, and Quentin Pittman for comments on earlier drafts of the manuscript.

REFERENCES

Abraham WC, Bear MF. (1996). Metaplasticity: the plasticity of synaptic plasticity. *Trends Neurosci* 19:136-130.

Austin G, Yai H, Sato M. (1967). Calcium ion effects on *Aplysia* membrane potentials. In: Invertebrate nervous systems, Wiersma CAG, ed. (Chicago, Illinois: University of Chicago press), pp. 39-53.

Benjamin PR. (1984). Interneuronal network acting on snail neurosecretory neurones (yellow cells and yellow-green cells of *Lymnaea*). *J Exp Biol* 113:165-185.

Benjamin PR, Winlow W. (1981). The distribution of three wide-acting synaptic inputs to identified neurons in the isolated brain of *Lymnaea stagnalis* (L.). *Comp Biochem Physiol* 70A:293-307.

Berninger B, Poo M-M. (1996). Fast actions of neurotrophic factors. *Curr Opin Neurobiol* 6:324-330.

Berry MS, Pentreath VW. (1976). Criteria for distinguishing between monosynaptic and polysynaptic transmission. *Brain Res* 105:1-20.

Buckett KJ, Peters M, Benjamin PR. (1990). Excitation and inhibition of the heart of the snail, *Lymnaea*, by non-FMRFamidergic motoneurons. *J Neurophysiol* 63:1436-1447.

Bulloch AGM. (1989). The cellular basis of neuronal plasticity. (New York, New York: Manchester University Press).

Bulloch AGM, Hauser GC. (1990). Sprouting by isolated *Helisoma* neurons: enhancement by glutamate. *Int J Dev Neurosci* 8:391-398.

Bulloch AGM, Ridgway RL, Harrington KC, Tetzlaff W. (1991). Glutamate modulates a NGF-like protein and a NGF-like mRNA in the *Lymnaea* CNS. *Soc Neurosci Abstr* 17:598.6.

Bulloch AGM, Syed NI, Ridgway RL. (1994). Neurite outgrowth and synapse formation by *Lymnaea* neurons: towards a characterization of molluscan neurotrophic factors. *Neth J Zool* 44:317-326.

Bulloch AGM, van Kesteren R, Hauser G, Fainzilbe M, Ibanez, C, van Minn J, Smit G, Geraerts W, Vreudgenhil E. (1996). Trk related tyrosine kinase receptors cloned from the *Lymnaea* CNS. *Soc Neurosci Abstr* 22:301.25.

Byrne JH, Kandel ER. (1996). Presynaptic facilitation re-

visited: state and time dependence. *J Neurosci* 16:425-435.

Coleman MJ, Meyrand P, Nusbaum MP. (1995). A switch between two modes of synaptic transmission mediated by presynaptic inhibition. *Science* 378:502-505.

Cramer KS, Sur M. (1995). Activity-dependent remodeling of connections in the mammalian visual system. *Curr Opin Neurobiol* 5:106-111.

Elekes K, Kemenes G, Hirip L, Geffard M, Benjamin PR. (1991). Dopamine- immunoreactive neurones in the central nervous system of the pond snail *Lymnaea stagnalis*. *J Comp Neurol* 307:214-224.

Elliott CJH, Benjamin PR. (1989). Esophageal mechanoreceptors in the feeding system of the pond snail *Lymnaea stagnalis. J Neurophysiol* 61:727-736.

Fagnou DD, Tuchek JM. (1995). The biochemistry of learning and memory. *Mol Cell Biochem* 149:279-286.

Fainzilber M, Smit AB, Syed NI, Wildering WC, Hermann PM, van der Schors RC, Jimmenez CR, Li KW, van Minnen J, Bulloch AGM, Ibanez CF, Geraerts W. (1996). CNRF, a molluscan neurotrophic factor that interacts with the p75 Neurotrophin receptor. *Science* 274:1540-1543.

Getting PA. (1989). Emerging principles governing the operation of neural networks. *Annu Rev Neurosci* 12:185-204.

Haydon PG, Winlow W. (1981). Morphology of the giant dopamine-containing neuron, R.Pe.D.1, in *Lymnaea stagnalis* revealed by Lucifer Yellow CH. *J Exp Biol* 94:149-157.

Kandel ER. (1979). Behavioral biology of *Aplysia*. (San Francisco, California: Freeman Press).

Lenz RA, Pitler TA, Alger BE. (1997). High intracellular Cl^- concentrations depress G-protein-modulated ionic conductances. *J Neurosci* 17:6133-6141.

Lo, DC. (1995). Neurotrophic factors and synaptic plasticity. *Neuron* 15:979-981.

Lukowiak K. (1980). CNS control over gill withdrawal behaviors in *Aplysia*: satiation causes an increase in the suppressive control in older but not in younger animals. *J Neurobiol* 11:591-611.

Lukowiak K, Freedman L. (1983). The gill withdrawal reflex is suppressed in sexually active *Aplysia*. *Can J Physiol Pharm* 61:743-748.

Magoski NS, Bauce LG, Syed NI, Bulloch AGM. (1995). Dopaminergic transmission between identified neurons from the mollusc *Lymnaea stagnalis. J Neurophysiol* 74:1287-1300.

Magoski NS (1996) Dopaminergic transmission in *Lymnaea*: physiology, pharmacology, and plasticity. Ph.D. thesis, University of Calgary, Calgary, Alberta, Canada.

Magoski NS, Bulloch AGM. (1996). Nerve growth factor (NGF) enhances transmission at a dopaminergic synapse in the mollusk, *Lymnaea stagnalis. Soc Neurosci Abstr* 22:301.23.

Magoski NS, Bulloch AGM. (1997). Localization, physiology, and modulation of a molluskan dopaminergic synapse. *J Neurobiol* 33:247-264.

Magoski NS, Bulloch AGM. (1999). Differential activation of dopamine receptors produces variability in sign at an identified synapse. *J Neurophysiol* 81:1330-1340.

Maletic-Savatic M, Malinow R, Svoboda K. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* 283:1923-1927.

Marder E. (1994). Polymorphic neural networks. *CurRev Biol* 4:752-754.

Meyrand P, Simmers J, Moulins M. (1991). Construction of a pattern-generating circuit with neurons of different networks. *Nature* 351:60-63.

Moroz LL. (1991). Monoaminergic control of respiratory behaviour in the freshwater pulmonate snail, *Lymnaea stagnalis* (L.). In Signal Molecules and Behavior, Winlow W, Vinogradova OS, Sakharov DA, eds. (Manchester, U.K.: Manchester University Press), pp. 101-123.

Nesic O, Magoski NS, McKenny K, Syed NI, Lukowiak K, Bulloch AGM. (1996). Glutamate as a putative neurotransmitter in the mollusc, *Lymnaea stagnalis*. *Neuroscience* 79:1255-1269.

Park J-H, Winlow W. (1993). Variant synaptic connections in the respiratory central pattern generator in semi-intact preparations of *Lymnaea*. *J Physiol* 473:198P.

Reddy R, Smith D, Wayman G, Wu Z, Villacres EC, Storm RR. (1995) Voltage-sensitive adenylyl cyclase in cultured neurons. A calcium-independent phenomenon. *J Biol Chem* 270:14340-14346.

Ridgway RL, Syed NI, Lukowiak, K, Bulloch AGM. (1991). Nerve growth factor (NGF) induces sprouting of specific neurons of the snail, *Lymnaea stagnalis*. *J Neurobiol* 22:377-390.

Ridgway RL, Syed NI, Bulloch AGM. (1993). *In vitro* evidence for multiple neurotigenic factors in the central nervous system of pulmonate molluscs. *Acta Biol Hung* 44:109-115.

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. (1999). The K^+/Ca^{2+} co-transporter KCC2 renders GABA hyperpolarization during neuronal maturation. *Nature* 397:251-255.

Selverston, AI. (1992). Pattern generation. *Curr Opin Neurobiol* 2:776-780.

Skingsley DR, Bright K, Santama N, Van Minnen J, Brierley MJ, Burke JF, Benjamin PR. (1993). A molecularly defined cardiorespiratory interneuron expressing SDPFLRFamide/GDPFLRFamide in the snail *Lymnaea*: monosynaptic connections and pharmacology. *J Neurophysiol* 69:915-927.

Spencer GE, Winlow W. (1994). Variant synaptic connections of an identified peptidergic neurone of *Lymnaea stagnalis. J Physiol* 475:150P.

Spencer GE, Syed NI, Lukowiak, K. (1999). Neural changes after operant conditioning of the aerial respiratory behavior in *Lymnaen stagnalis. J. Neurosci.* 19:1836–1843.

Syed NI. (1988). The neural control of locomotion in *Lymnaea*. Ph.D. thesis, University of Leeds, Leeds, United Kingdom.

Syed NI, Winlow W. (1989). Morphology and electrophysiology of neurons innervating the ciliated locomotor epithelium in *Lymnaea stagnalis* (L.). *Comp Biochem Physiol* 93A:633-644.

Syed NI, Lukowiak K, Bulloch AGM. (1990). In vitro reconstruction of the respiratory central pattern generator of the mollusk *Lymnaea*. *Science* 250:282-285.

Syed NI, Winlow W. (1991). Respiratory behavior in the pond snail *Lymnaea stagnalis* II. neural elements of the central pattern generator (CPG). *J Comp Physiol* 169A: 557-568.

Syed NI, Richardson P, Bulloch AGM. (1996). Ciliary neurotrophic factor, unlike nerve growth factor, supports neurite outgrowth but not synapse formation by adult *Lymnaea* neurons. *J Neurobiol* 29:293-303.

Weiler IJ, Greenough WT, Hawrylak N. (1995). Morphogenesis in memory formation: synaptic and cellular mechanisms. *Behav Brain Res* 66:1-6.

Weimann JM, Marder E. (1994). Switching neurons are integral members of multiple oscillatory networks. *CurR Biol* 4:896-902.

Werkman TC, Van Minnen J, Voorn P, Steinbusch HWM, Westerink BHC, De Vlieger TA, Stoof JC. (1991). Localization of dopamine and its relation to the growth hormone producing cells in the central nervous system of the snail *Lymnaea stagnalis*. *Exp Brain Res* 85:1-9.

Wildering WC, Lodder JC, Kits KS, Bulloch AGM. (1995). Nerve growth factor (NGF) acutely enhances high-voltage-activated calcium currents in molluskan neurons. *J Neurophysiol* 74:2778-2781.

Winlow W, Haydon PG, Benjamin PR. (1981). Multiple postsynaptic actions of the giant dopamine-containing neurone R.Pe.D.1 of *Lymnaea stagnalis*. *J Exp Biol* 94: 137-148.

Wolff JR, Spatz WB, Laskawi R, Missler M. (1995). Structural dynamics of synapses and synaptic components. *Behav Brain Res* 66:13-20.

Yeh S-R, Musolf BE, Edwards DH. (1997). Neuronal adaptations to changes in the social dominance status of crayfish. *J Neurosci* 17:697-708.

Zhang F, Endo S, Cleary LJ, Eskin A, Byrne JH. (1997). Role of transforming growth factor-α in long-term synaptic facilitation in *Aplysia*. *Science* 275:1318-1320.