Circuits constructed from identified *Aplysia* neurons exhibit multiple patterns of persistent activity

D. Kleinfeld,* F. Raccuia-Behling,* and H. J. Chiel‡
*Solid State and Quantum Physics Research Department, AT&T Bell Laboratories, Murray Hill, New Jersey 07974; and
‡Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT We have used identified neurons from the abdominal ganglion of the mollusc *Aplysia* to construct and analyze two circuits in vitro. Each of these circuits was capable of producing two patterns of persistent activity; that is, they had bistable output states. The output could be switched between the stable states by a brief, external input. One circuit consisted of co-cultured L10 and left upper quadrant (LUQ) neurons that formed reciprocal, inhibitory connections. In one stable state L10 was active and the LUQ was quiescent, whereas in the other stable state L10 was quiescent and the LUQ was active. A second circuit consisted of co-cultured L7 and L12 neurons that formed reciprocal, excitatory connections. In this circuit, both cells were quiescent in one stable state and both cells fired continuously in the other state. Bistable output in both circuits resulted from the nonlinear firing characteristics of each neuron and the feedback between the two neurons.

We explored how the stability of the neuronal output could be controlled by the background currents injected into each neuron. We observed a relatively well-defined range of currents for which bistability occurred, consistent with the values expected from the measured strengths of the connections and a simple model. Outside of ths range, the output was stable in only a single state. These results suggest how stable patterns of output are produced by some in vivo circuits and how command neurons from higher neural centers may control the activity of these circuits.

The criteria that guided us in forming our circuits in culture were derived from theoretical studies on the properties of certain neuronal network models (e.g., Hopfield, J.J. 1984. *Proc. Natl. Acad. Sci. USA.* 81:3088–3092). Our results show that circuits consisting of only two co-cultured neurons can exhibit bistable output states of the form hypothesized to occur in populations of neurons.

INTRODUCTION

A variety of neural circuits controlling behavior exhibit multiple stable patterns, or states, of electrical activity. In these states each of the cells in the circuit show a relatively constant level of activity for a time that is long compared to the period between action potentials. Examples of stable output patterns are seen in some circuits that control motor outputs. In such circuits the same group of motor neurons and interneurons are capable of firing in one of several relatively long-lived, fixed patterns of activity (for review see Delcomyn, 1980; Kristan, 1980; Selverston, 1980; Roberts and Roberts, 1983; Cohen et al., 1988). The multiplicity of stable output patterns in neural circuits could result from some combination of intrinsic cellular properties, the connections between neurons, and external inputs. The precise interplay among these features has been difficult to establish with in vivo preparations.

An alternative approach to studying the mechanisms that give rise to emergent behavior in the nervous system is to construct circuits in cell culture. This approach allows simplified circuits to be examined so that certain cellular and synaptic properties can be held constant while others are varied. It also provides a means to test theoretical ideas that suggest how the proper choice of neuronal connectivity and neural bias currents, i.e., constant current inputs to each cell, can lead to multiple stable output states (Little, 1974; Hopfield, 1982). For example, circuits consisting of two neurons that interact via reciprocal connections of the same sign are expected to produce stable outputs in either of two states, i.e., they have bistable outputs. The output can be driven between the two states by a brief perturbation. We emphasize that while bistability is an *intrinsic* property of such circuits, the transitions between states are caused by *extrinsic* inputs.

We report the construction and analysis of two circuits that exhibit multiple stable patterns of output. One circuit consisted of neurons that interacted via reciprocal, inhibitory connections. A second circuit consisted of neurons that interacted via reciprocal, excitatory connections. The criteria used to design the circuits were motivated by the theoretical studies of Hopfield (1982, 1984)

Please address correspondence to David Kleinfeld, Room 1G-233, AT&T Bell Laboratories, 600 Mountain Ave., Murray Hill, NJ 07974.

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on the collective behavior of populations of neurons. We used our circuits to analyze how bias currents can control whether the neuronal outputs were stable in a single state, or bistable in one of two states. The control of the in vitro circuits by external inputs has analogies to the modulation of neuronal excitability in in vivo circuits by command neurons (Kupfermann and Weiss, 1978).

We constructed our circuits using identified neurons from the mollusc *Aplysia*. The neurons were co-cultured using the procedures developed by Schacher and Proshansky (1983) (see also Kaczmarek et al., 1979; Dagan and Levitan, 1981). These techniques allow the spatial location and growth conditions of the neurons to be controlled in a relatively precise manner. The extensive literature on the neurophysiology of *Aplysia* (Frazier et al., 1967; Kandel et al., 1967; Koester and Kandel, 1977) guided us in selecting neurons that formed the desired connections in vitro. However, three of the four neuronal connections that we observed in vitro are not reported to occur in vivo. Further, our circuits do not correspond to any that have been described in *Aplysia* or in other in vivo preparations.

The paper is organized as follows: The theory of bistability in neuronal circuits is briefly reviewed, with emphasis on the criteria that should be satisfied for a circuit to produce two stable outputs. Experimental evidence is presented that the circuits we constructed satisfied the criteria suggested by the model. The basic bistable output properties of the circuits and the effect of different bias currents on the stability of the outputs were probed.

Preliminary aspects of this work have been presented (Kleinfeld et al., 1988*a* and *b*).

THEORETICAL BACKGROUND

The work of Hopfield (1982; 1984) describes how idealized neurons can be used to form networks that are capable of exhibiting multiple, stable output states. In each stable state some of the neurons are active while the others are quiescent. The output of the network will converge from an arbitrary initial state to a particular stable state. This convergence property has been proposed as a prototype for associative memory.

The functioning of Hopfield's network requires that the neurons have nonlinear firing characteristics and that there is synaptic feedback between many of the neurons. The equations describing the dynamics of this and related models are reviewed elsewhere (Amit, 1989; Kleinfeld and Sompolinsky, 1989). Here we focus on a version of Hopfield's model that contains only two neurons and two stable states (Hopfield, 1984). This circuit preserves some of the features of the original network, such as the relation between the stability of the output and the magnitude of external inputs to each neuron. The output of the two neurons is determined by a set of circuit equations in which there is a continuous relation between the input to each neuron and its output. We take this relation to be strongly nonlinear, i.e., the 'high-gain' limit, so that the stable states are clearly discernible (Fig. 3 in Hopfield, 1984). Bistability within populations of neurons has been described by Harth et al. (1970), Wilson and Cowan (1972), and Amari (1972) using similar equations.

Bistable output activity is expected to occur when the neurons, and the interactions between these neurons, approximately fulfill the following criteria (Fig. 1 *A*):

(*a*) To clearly identify the output states, the activity of the neurons have discernible quiescent and active levels. We refer to these levels as "OFF" and "ON," respectively. Thus the rate of firing of the neurons must change in an abrupt, nonlinear manner as a function of the input current (Fig. 1 *B*; the width of the transition is defined by an effective current I_W).

(*b*) To provide a feedback pathway to stabilize the two output states, the neurons are connected by reciprocal connections of the *same* sign (Fig. 1 *A*). The strength of each connection, J, is taken as the value of the current that enters the postsynaptic cell when the presynaptic neuron is firing at its steady-state rate.[1]

(*c*) To provide the temporal integration that allows each neuron to maintain a constant level of activity, the duration of the postsynaptic response is long compared with the period between action potentials in the presynaptic cell.[2] Alternately, the postsynaptic cell can have a suitably long membrane time constant.

The input to each neuron contains contributions from both its presynaptic partner and external sources, such as a stimulating electrode. The synaptic inputs should be capable of driving the postsynaptic neuron between its ON and OFF levels if the connections are sufficiently strong (i.e., $|J| > I_W$) and the bias current, I_0, is properly set relative to the threshold current, I_T. When both of these conditions are satisfied, a brief stimulus, ΔI, should

[1]We express the synaptic strength in terms of an effective synaptic current to simplify the comparison between J and value of the bias currents. In a related work, it was expedient to express the observed synaptic strength as the value of the temporally integrated postsynaptic potential (Kleinfeld and Sompolinsky, 1988). The two expressions are equivalent for relatively small changes in the postsynaptic potential.

[2]In a circuit with many neurons the activity of the postsynaptic cell is maintained by integrating the input from a large population of presynaptic cells; there is no restriction on the synaptic time-constant. In two-cell circuits, the average over a population must be replaced by a time average over the activity of the single presynaptic neuron.

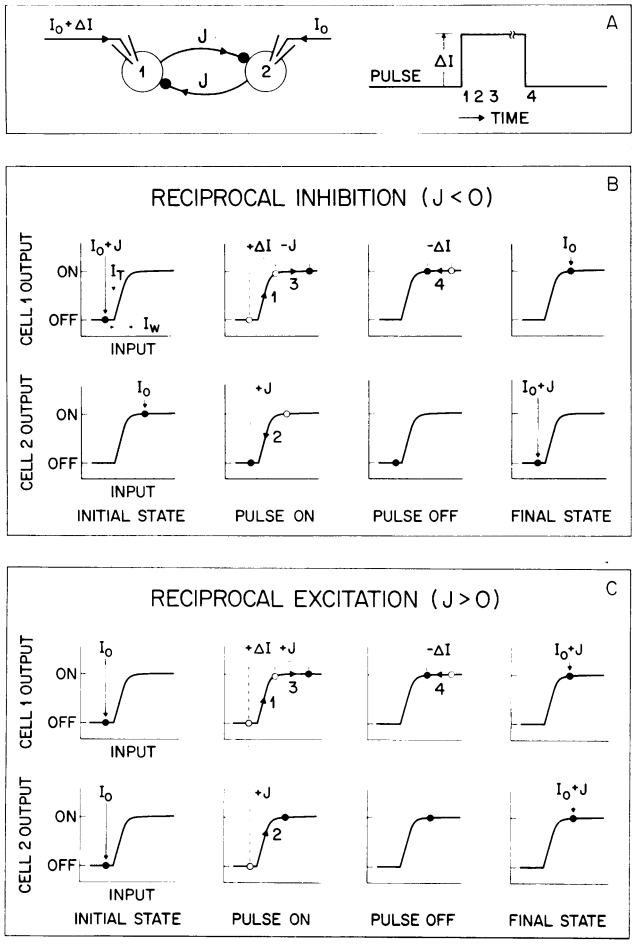

FIGURE 1 Idealized bistable behavior for circuits of two neurons connected by slow, reciprocal connections. (*A*) Schematic of the circuit. The connections have strength J and a decay-time τ_s. The current injected into each cell consists of two components, a constant bias current I_0 and a transient component ΔI. (*B*) The dynamic properties of a circuit with reciprocal, inhibitory connections. The input-output relation for each neuron is taken to be a saturating nonlinear function characterized by a threshold I_T and an effective width I_W. This circuit exhibits bistable output when the bias currents satisfy $I_T + I_W \leq I_0 \leq I_T - J$. A transition between states is elicited by a current-pulse $\Delta I \geq I_T + I_W - (I_0 + J)$; see text for details. (*C*) The dynamic properties of a circuit with reciprocal, excitatory connections. This circuit exhibits bistable output when the bias currents satisfy $I_T + I_W - J \leq I_0 \leq I_T$. A transition from the quiescent state to the active state is elicited by a current pulse, $\Delta I \geq I_T + I_W - I_0$, into either cell; see text for details.

be capable of inducing a transition between the two stable states.

It is assumed for simplicity that the strength of the connections are relatively constant in time. In practice, these strengths may change over time as a result of intrinsic mechanisms, such as facilitation and fatigue, or as a result of a change in the firing rate of the presynaptic cell. We suspect that bistable outputs will still occur if the strength is sufficiently large. Other aspects of the behavior of the circuit, such as the absolute firing rate of each neuron, require a more detailed analysis than the one considered here.

Reciprocal inhibition ($J < 0$)

Inhibitory connections tend to maintain the neurons at opposing levels of activity. In one stable state cell 1 is active and cell 2 is quiescent (ON/OFF) and in the other state cell 1 is quiescent and cell 2 is active (OFF/ON). These states are observable when the bias currents are adjusted so that all cells would be active if their synaptic inputs were removed (Fig. 1 *B*). A transition between the stable states is initiated by injecting a pulse of current into the quiescent neuron (cell 1 in Fig. 1 *B*).

Reciprocal excitation ($J > 0$).

Excitatory connections tend to maintain both neurons at the same level of activity. In one stable state both neurons are quiescent (OFF/OFF) and in the other state both neurons fire (ON/ON). These states can be observed when the bias currents are set so that the neurons are initially off (Fig. 1 *C*). The bistability can be probed by injecting a pulse of current into either cell and observing the transition from the state OFF/OFF to the state ON/ON (Fig. 1 *C*). In principle the circuit can remain in the state ON/ON indefinitely, although in practice physiological mechanisms such as postsynaptic desensitization and spike adaptation will gradually decrease the stability of this state.

Stability diagrams

We illustrate how bias currents can be used to control whether the output of a circuit is stable in a single state or in one of two states, i.e., bistable. We consider the possibility of differences in connection strength and cellular properties between the two neurons. The strength J_{12} is defined as the steady-state input from cell 2 to cell 1, I_{T1} is defined as the threshold current of cell 1 and I_{01} is the bias current of cell 1; a similar convention holds for J_{21}, I_{T2}, and I_{02}. Note that we can neglect I_{W1} and I_{W2} compared with $|J_{12}|$ and $|J_{21}|$, respectively (see Results).

When there is no interaction between two neurons, or only a unidirectional connection, there can be no bistable output behavior. The output will be stable in one of four states; they are ON/ON, ON/OFF, OFF/ON, and OFF/OFF (Fig. 2 *A*). The choice of stable output states

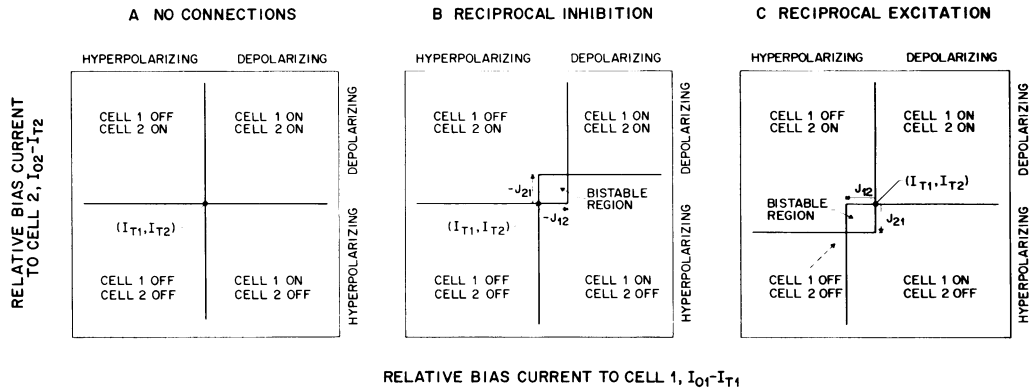

FIGURE 2 Stability analysis of idealized circuits of two neurons. The stable output states are shown as a function of the relative bias current into each cell, $I_{01} - I_{T1}$ and $I_{02} - I_{T2}$, respectively. (*A*) The stable output states for two cells that have no connections or only a single connection. There are four monostable output states: ON/ON, OFF/ON, OFF/OFF, and ON/OFF. (*B*) The stable output behavior for a circuit with reciprocal inhibitory connections, where J_{12} is the synaptic input from cell 2 to cell 1 and J_{21} is the input from cell 1 to cell 2. When the bias currents lie within the bistable region the output of the circuit is stable in both the state ON/OFF and the state OFF/ON. A transient, external perturbation to the circuit can cause a transition between these states. (*C*) The stable output behavior for a circuit with reciprocal excitatory connections. When the bias currents lie within the bistable region the output of the circuit is stable in both the states OFF/OFF and ON/ON.

depends on the value of the bias currents into the two cells relative to that of the threshold currents.

In circuits with reciprocal inhibitory connections the output can be bistable when the synaptic input to each neuron can drive it between quiescence and firing. The ranges of bias currents that permit bistability is illustrated in Fig. 2 *B*. Outside of these ranges the circuit is expected to remain in one of the four monostable states. Aspects of this behavior may be tested experimentally. In particular:

(*a*) There should be a range of bias currents for which the output is stable in both the state ON/OFF and the state OFF/ON. The upper bounds to this range are given by the magnitude of the steady-state synaptic input into cell 1 and cell 2, i.e., $-J_{12}$ and $-J_{21}$, respectively (Fig. 2 *B*). In practice these bounds may be only approximate, as it is difficult to measure the precise value of the connection strengths.

(*b*) The ratio of the maximum relative bias currents for which bistable output occurs, $(I_{01} - I_{T1})/(I_{02} - I_{T2})$, should nominally equal the ratio of connection strengths, J_{12}/J_{21}. The ratio of the strengths may be measured with greater accuracy than the individual strengths. Thus we expect that the shape of the bistable region should reflect differences in the strength of the two connections (Fig. 2 *B*).

(*c*) The relative bias currents that separate the bistable region from monostable regions, and separate different monostable regions, should coincide with the expected values (Fig. 2 *B*).

A similar analysis holds for the case of reciprocal excitation (Fig. 2 *C*). A comparison of the behavior of our in vitro circuits with the predictions of the model serve to highlight the extent to which the observed behavior can be accounted for in terms of idealized neuronal properties.

METHODS

Culture techniques

Juvenile *Aplysia californica* (~5g), raised in mariculture, were either a gift of the Howard Hughes Medical Institute (Woods Hole, MA), or purchased from Sea Life Supply (Sand City, CA). All procedures made use of an isotonic saline (460 mM NaCl, 10 mM KCl, 10 mM Hepes, 10 mM glucose, 27 mM $MgCl_2$, 27 mM $MgSO_4$, and 10 mM $CaCl_2$; pH 7.6), a modified Liebovitz's L-15 medium (400 mM NaCl, 10 mM KCl, 0.20 mM $NaHCO_3$, 0.45 mM KH_2PO_4, 1.35 mM Na_2HPO_4, 30 mM glucose, 2.3 mM glutamine, 27 mM $MgSO_4$, 27 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.54% [wt/vol] of an L-15 powder mix without inorganic salts and glutamine [No. 82-5154EA; Gibco, Grand Island, NY]; pH 7.6), and a medium for plating (5% [vol/vol] fetal calf serum [Gibco] and 1% [vol/vol] of a penicillin/streptomycin/fungizone solution [No. 600-5240; Gibco] in modified L-15 medium). *Aplysia* hemolymph was extracted from ~500 g animals (Sea Life Supply), filtered in two stages (5.0-μm filter [No. 4264; Gelman Sciences, Inc., Ann Arbor, MI] and 0.45-μm filter [No. 4262; Gelman Sciences, Inc.]) and stored in separate lots at −15°C.

Identified neurons were harvested from the abdominal ganglion as described (Schacher and Proshansky, 1983). In brief, ganglia were isolated, digested for 2.6 h at 36°C in 1.0% (wt/vol) protease (type IX; Sigma Chemical Co., St. Louis, MO) prepared in modified L-15 medium, washed three times in isotonic saline, and stored until use (<3h) in plating medium at 4°C. Ganglia were pinned and desheathed in a dish coated with Sylgard (Dow Corning Corp., Midland, MI) that was refreshed hourly with cold (4°C) plating media. Neurons were identified by their position, size, and the branching pattern of their axons (Frazier et al., 1967; Kandel et al., 1967). Cells were isolated by gently inserting a micropipette at the junction between the initial

segment and the soma and slowly teasing the cell out of the ganglion. Isolated neurons were transferred and plated onto 50-mm dishes (Falcon Labware, Oxnard, CA) coated with poly-*D*-lysine and containing 4 ml of 50% (vol/vol) hemolymph in modified L-15 medium. We sketched the morphology and relative position of the cells at the time of each plating. The cells exhibited extensive outgrowth after 2–3 d in vitro, as shown for co-cultured LUQ and L10 neurons in Fig. 3. Cultures were maintained at 18°C and the medium was exchanged at 2–3 d intervals.

Electrophysiology

Standard current-clamp methods were used. Neurons were penetrated with single-barrel microelectrodes filled at their tips with 4 M K-acetate ($R_E \simeq 20$ MΩ). A single electrode was used for measurements of the circuit properties of the co-cultures while a two-electrode current clamp was used for most measurements of synaptic connections. Electrotonic coupling between neurons was measured as described previously (Bennett, 1977). The cultures were perfused with an equal mixture of isotonic saline and modified L-15 medium, T ≃ 22°C, during the recording sessions. For some measurements charybdotoxin (CTX), isolated from *Leirus quinquestriatus* venom (Latoxan, Rosans, France) as described previously (Miller et al., 1985), was added to the recording media. CTX blocks calcium-activated potassium channels (Miller et al., 1985; Hermann, 1986). All measurements were performed on cultures 3–7 d after plating.

RESULTS

Synaptic connections in vitro

Reciprocal inhibition

The neurons that we selected as candidates for the inhibitory system were L10 and selected left upper quadrant (LUQ) neurons, i.e., L2–L6 ($n = 87$). Multiphasic connections from L10 to the LUQs, but no connections from LUQ to L10, occur in vivo (Kandel et al., 1967) and were studied in vitro by Camardo et al. (1983). We observed a slow, inhibitory connection from L10 to LUQ in 60% of the preparations and an inhibitory connection from LUQ to L10 in 35% of the preparations. Reciprocal inhibitory connections occurred in 25% of the LUQ/L10 pairs (Fig. 4 *A*). Neurons that formed reciprocal connections generally had ~500-μm axon stumps that were crossed near the soma. The inhibitory response decayed with a complex time dependence; the decay constant, defined as the time for the postsynaptic response to decay to half of its maximum value, was $\tau_S = 5$–10 s for LUQ/L10 pairs ($n = 22$) (Fig. 4 *A*).

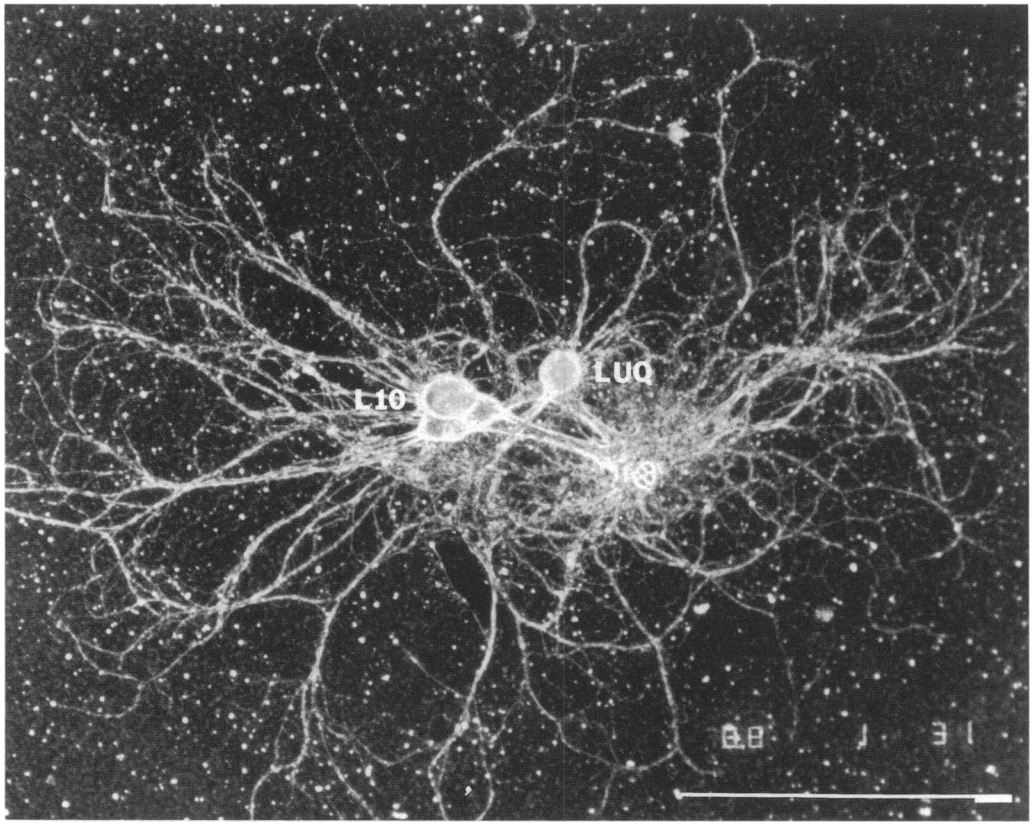

FIGURE 3 A co-culture of L10 and LUQ neurons; the LUQ is putatively L3. These neurons were connected by reciprocal inhibitory connections. Photographed under dark field illumination 3 d after the initial plating. The scale bar corresponds to 1 mm.

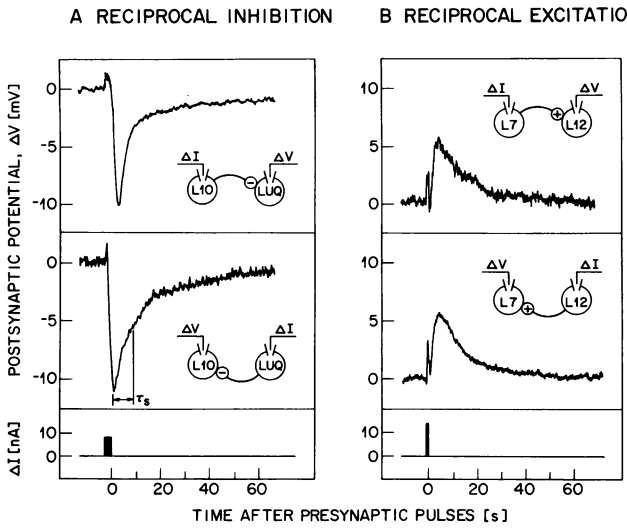

FIGURE 4 The reciprocal interactions between the pairs of neurons used to construct each circuit. (*A*) The interactions between L10 and an LUQ, putatively L4. These cells formed reciprocal inhibitory connections. The observed hyperpolarization was measured in response to a train of action potentials, generated by a set of current pulses, ΔI (50-ms pulses at 200-ms intervals for 2 s), in the presynaptic cell. The membrane potential of either cell was −50 mV and the electrotonic coupling was $\kappa = 0.1$ (*B*) The interactions between neurons L7 and L12. These cells formed reciprocal excitatory connections. The observed depolarization was measured in response to a train of action potentials, generated by a set of current pulses, ΔI (50-ms pulses at 200-ms intervals for 1 s), in the presynaptic cell. The membrane potential of the postsynaptic cells were adjusted to ~−55 mV before the train to prevent the cells from firing. The initial spike in the records results from electrotonic coupling between the cells ($\kappa = 0.2$).

Many of the co-cultured LUQ/L10 pairs formed electrotonic connections ($\kappa \equiv \Delta V_{post}/\Delta V_{pre} = 0.1$–$0.3$). Pairs that contained appreciable electrotonic coupling in parallel with reciprocal inhibitory connections allowed excitation as well as inhibition between cells. These pairs were rejected for further study.

Reciprocal excitation

The neurons selected as candidates for constructing the excitatory system were L7 co-cultured with L12 ($n = 43$). A slow, excitatory connection from L7 to L12 was present in 37% of the preparations. A slow, excitatory connection from L12 to L7 was present in 40% of the preparations. Reciprocal excitatory connections were observed in 23% of the preparations (Fig. 4 *B*). These co-cultures also contained weak electrotonic coupling, $\kappa = 0.1$–0.2. The rise time of either depolarization was ~2 s and the decay time was $\tau_S \simeq 10$–15 s ($n = 10$). On occasion, a fast inhibitory response was observed ($n = 3$) (Fig. 4 *B, top*); this component had no effect on the properties of our circuits.

The connection strength from L12 to L7 decreased with use. The amplitude of the depolarization in L7 was repetitively probed by inducing short trains of action potentials in L12 at 30-s intervals. An ~15% decrease in the peak amplitude of the response occurred after each train. No change was observed in the connection strength from L7 to L12 under similar testing.

Neuronal input-output relations

To obtain circuits with the desired output properties, we required neurons with continuous, nonbursting behavior. Neuron L10 is known to exhibit bursting in vivo (Frazier et al., 1967; Koester et al., 1974) as well as in vitro (Obaid et al., 1989). This precluded the use of L10s that exhibited bursting in culture. Fortunately, in about half of the instances L10 did not burst or burst rather irregularly. In all other respects L10 behaved normally. Continuous firing behavior was obtained when the irregularly bursting cells were bathed in ~100 nM CTX ($n = 5$).

We consider the input-output relation for nonbursting L10s. The response to a succession of increasingly strong steps of depolarizing current is shown in Fig. 5. The crucial feature is that an increase in the amplitude of the current by 0.01 nA could change the output of L10 from quiescence to one of continuous firing. The firing rate increased relatively little when the amplitude of the

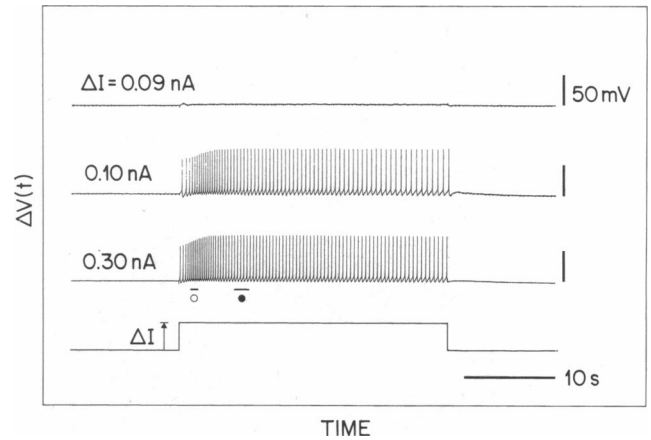

FIGURE 5 The firing behavior observed in a nonbursting L10 with current pulses, ΔI, of increasing amplitude. Note the change from quiescence to firing that occurs when the current is increased by only 0.01 nA, i.e., from 0.09 to 0.10 nA. Regions marked by ○ and ● nominally correspond to those used to calculate the initial and steady-state firing rates, respectively; see Fig. 6 *A*.

current steps was further increased; compare the traces for 0.10 and 0.30 nA in Fig. 5.

The complete input-output relation for L10 was measured under quasistatic conditions, allowing the neuron to rest 300 s between 30-s pulses of current (Fig. 6 *A*; open symbols refer to the initial firing rate and solid symbols refer to the steady-state rate). The width of the transition from quiescence to firing was $I_W \lesssim 0.01$ nA. Although the firing of L10 showed adaptation, there was an abrupt change from quiescence to firing at all times (Fig. 6 *A*).

The input-output relations for LUQ, L7, and L12 neurons are shown in Fig. 6, *B–D*, respectively. Each datum represents the average firing rate measured under quasistatic conditions. Note that whereas LUQ neurons are reported to exhibit bursting outputs in vivo (Frazier et al., 1967; Kramer and Zucker, 1985; Scholz et al., 1988), we did not observe bursting under our culture conditions. The essential feature of the data is that the activity of all of the neurons changed in an abrupt manner from quiescence to firing at a rate of the order of 1 spike/s when the input current was increased by ~0.01 nA. Thus, the neurons exhibited a sharp transition from the OFF to an ON level for input currents on the scale of 0.1 nA. This scale corresponds to the synaptic and bias currents appropriate for our experiments (see next section). We note for completeness that all of the neurons were capable of firing repetitively over a much broader range of currents, as illustrated for L12 (Fig. 6 *D, inset*).

The input-output relation for L12 was significantly altered by continuous activity. The width of the input-output curve increased from $I_W \lesssim 0.01$ nA to $I_W \simeq 0.1$ nA when the injection current was increased in a linear ramp (~1 pA/s), as opposed to being increased in steps. When the current was subsequently decreased the threshold

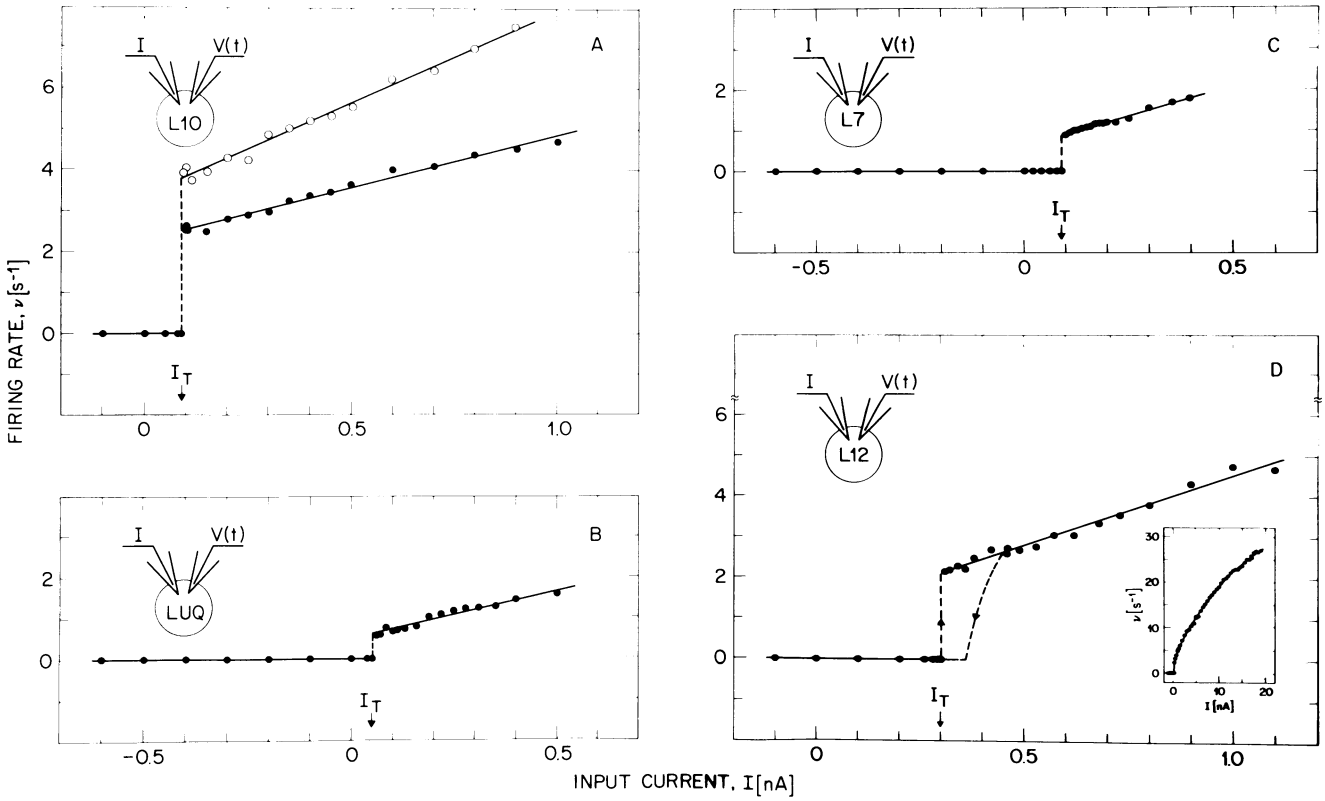

FIGURE 6 The input-output relations for the neurons used in both circuits. The firing rate was measured as a function of the input current under quasistatic conditions (see text). The threshold current, I_T, corresponded to the minimum current for which the neuron fired continuously. (*A*) The input-output relation for L10. Open circles (○) correspond to an average over the second through sixth interspike interval, i.e., the initial rate. Filled circles (●) correspond to an average over the 15th through 20th interspike interval, i.e., the steady-state rate; see Fig. 5. (*B*) The input-output relation for an LUQ, putatively L3. The firing rate was relatively constant in time; each datum corresonds to the rate averaged over the initial 30 action potentials. (*C*) The input-output relation for L7. The firing rate was relatively constant in time; each datum corresponds to the rate averaged over the initial 30 action potentials. (*D*) The input-output relation for L12. Each point is an average over the 2nd through 12th interspike interval. Inset shows the firing rate values over an extended range of input currents. The dashed line (- - -) indicates the approximate output behavior of L12 under conditions in which the current was continuously increased, then smoothly decreased.

current was observed to increase by 0.1–0.2 nA (Fig. 6 D, *dashed line*). The input-output relations of LUQ, L7, and L10 remained essentially constant under similar testing.

Comparison of observed neuronal properties with theoretical criteria

The stability of the output activity will depend on there being a sufficiently strong synaptic input under steady-state conditions. The synaptic input must be capable of driving the postsynaptic cell between quiescence and firing in order to supply the feedback necessary to stabilize the two output states. This implies that the input must be large compared to the width of the input-output curves, i.e., $|J| \gg I_W$ with $I_W \lesssim 0.01$ nA (Fig. 6, *A* and *B*).

Consider first the connections between LUQ and L10. The L10 fires at ~2 spikes/s when it is biased just above its threshold value (Fig. 6 *A*). A measure of the postsynaptic response in an LUQ biased near its threshold value, with L10 firing at ~2 spikes/s, sets a lower bound on the magnitude of the connection strength. A similar argument holds for the postsynaptic response in L10 with LUQ firing near its minimum rate, ~0.5 spikes/s (Fig. 6 *B*).

A particularly strong hyperpolarization observed in LUQ in response to a sustained train of action potentials in L10 is shown in Fig. 7 *A*. Here, as in general, the magnitude of the hyperpolarization remained essentially constant over the ~100-s time course of the measurements. Its specific value depended on firing rate of the presynaptic L10. For rates in the range ν = 2–3 spikes/s, the average steady-state connection strength for the L10 to LUQ connection was $J = \Delta V/R_{IN} \simeq -0.15$ nA (±30% SDM; $n = 5$),[3] where R_{IN} is the measured input resistance of the neuron. The largest hyperpolarization observed in L10 in response to a sustained train in LUQ is shown in Fig. 7 *B*. The magnitude of the hyperpolarization for this connection was fairly independent of the firing rate of LUQ over the range ν = 0.3–1 spike/s (Fig. 7 *B*). The steady-state value of the connection strength for the LUQ to L10 connection was $J \simeq -0.10$ nA (±50% SDM; n = 4) (see footnote 3). The stability condition $|J| \gg I_W$ was fulfilled for both inhibitory connections.

A similar result was found for the L7 to L12 to L7 connections in the excitatory circuit, for which $J \sim -0.05$ nA (±50% SDM; n = 5 reciprocally connected pairs). (See footnote 3). However, after sustained activity in these cells the strength of the L12 to L7 connection

[3]The value of J is only an estimate that sets the order of magnitude of the synaptic current. At this level of approximation, we did not account for differences in potential between the soma and cell processes of the postsynaptic neuron (Spira et al., 1988) and for changes in the resistance of the neuron caused by the synaptic input.

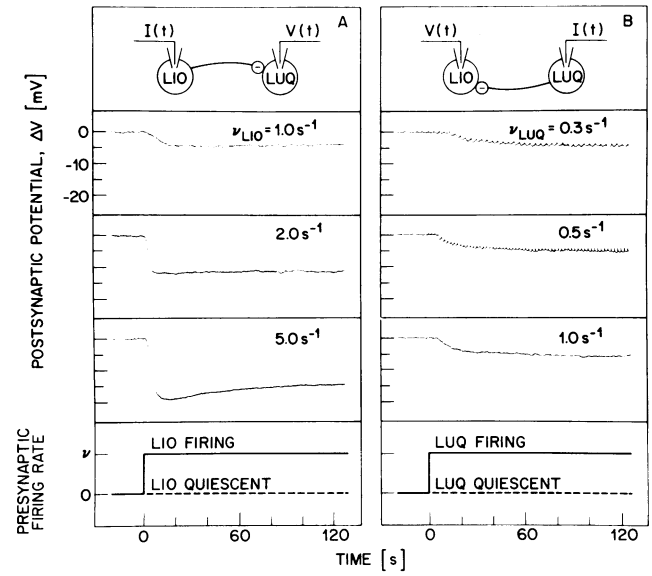

FIGURE 7 The steady-state interaction between L10 and LUQ neurons that were biased near their threshold values. (*A*) The inhibitory response measured in an LUQ, putatively L4, for different rates of firing in L10 ($V_{rest} \simeq -45$ mV). Action potentials were elicited in L10 by 50 ms, 7 nA, pulses of current. The firing rate of L10 in our circuits was typically ν = 2 spikes/s to ν = 3 spikes/s. For ν = 2 spikes/s, the hyperpolarization was $\Delta V \simeq -12$ mV, corresponding to $J \simeq -0.25$ nA ($R_{IN} \simeq 50$ MΩ). (*B*) The inhibitory response measured in L10 in response to different rates of firing in an LUQ, putatively L4 ($V_{rest} \simeq -50$ mV). Action potentials were elicited in the LUQ by 50 ms, 8 nA, pulses of current. The firing rate of the LUQs in our circuits were typically ν = 0.3 spikes/s to ν = 0.5 spikes/s. The hyperpolarization for these rates was $\Delta V \simeq -5$ mV, corresponding to $J \simeq -0.15$ nA ($R_{IN} \simeq 35$ MΩ). The spikes in the data record were caused by electrotonic coupling ($\kappa \lesssim 0.2$).

decreased and the transition width and threshold current of L12 increased (Fig. 6 *D*).

The stability of the output states also depends upon temporal integration of the synaptic input. This implies that the duration of the postsynaptic response must be long compared with the time between action potentials in the presynaptic cell. The decay time of the synaptic input for all connections was $\tau_s \sim 10$ s (Fig 4, *A* and *B*). This time was greater than the longest period between action potentials, $\nu^{-1} < 2$ s (Fig. 6, *A–D*), fulfilling this condition. Variations in firing rates on the time scale of τ_s should not affect the stability of the circuits.

To summarize, we identified three criteria as guides for the construction of circuits that should exhibit bistable output. These were (*i*) neurons with a nonlinear input-output relation, (*ii*) reciprocal connections of the same sign, and (*iii*) integration over past activity by the relatively long decay time of the interactions. All three criteria could be satisfied by co-cultures of L10 and LUQ

neurons. Co-cultures of L7 to L12 neurons satisfied these criteria at the onset of activity but not with extended use.

Circuit dynamics

Reciprocal inhibition: basic response

The dynamic behavior of the circuits with reciprocal inhibition was probed in co-cultures of L10 and LUQ ($n = 8$). To construct an appreciable number of ciruits with reciprocal chemical connections, negligible electrotonic coupling ($\kappa \lesssim 0.1$) in parallel with the chemical connections, and negligible spike adaptation, we often plated L10 with two or more LUQs. The pairwise connection strengths in these circuits were determined by hyperpolarizing all neurons, except the cells of interest, to suppress the inhibitory response (e.g., Camarado et al., 1983). This allowed us to unravel the connectivity when two or more neurons were strongly coupled.

We first discuss the dynamic behavior of a circuit consisting of L10 and two LUQs; similar data were obtained with co-cultures of L10 and a single LUQ. The L10 formed reciprocal inhibitory connections with each of the LUQs, and there was essentially no electrotonic coupling in parallel with these connections ($\kappa < 0.05$). The two LUQs were coupled by a strong electrotonic connection ($\kappa = 0.5$); this coupling synchronized the firing of these cells. According to the model (Fig. 1 *B*) this circuit should have two stable states, one with L10 active and the LUQs quiescent, and vice versa.

The dynamic response of the above circuit is shown in Fig. 8. The proper bias currents were set by (*a*) adjusting the currents to maintain the three neurons at their quiescent level, (*b*) increasing the current to L10 to a value $\lesssim 0.1$ nA above its threshold current, (*c*) increasing the currents to the LUQs to values $\lesssim 0.1$ nA below the value at which the neurons began to fire. The circuit remained in the state with L10 active and the LUQs quiescent until current pulses were simultaneously injected into the LUQs (time 1, Fig. 8). We used relatively long current pulses to accentuate the inhibitory

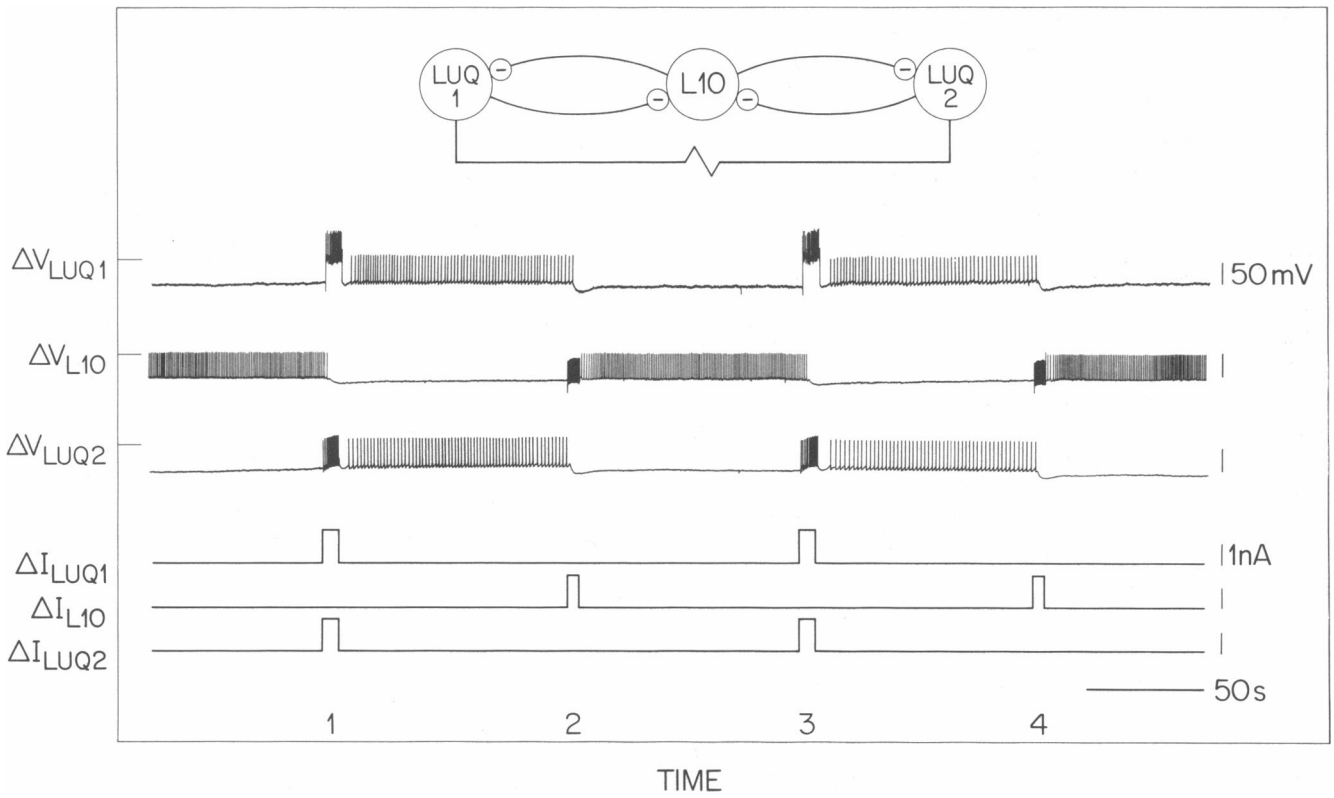

FIGURE 8 The basic dynamic behavior of the inhibitory circuit. This particular circuit consisted of L10 co-cultured with two LUQs. The L10 formed reciprocal connections with each LUQ; the LUQs were coupled by a strong electrotonic connection and thus functioned essentially as a single cell. The bias currents were $I_0 = 0.15$, 0.55, and 0.15 nA for LUQ1, L10, and LUQ2, respectively. The circuit was initially in the state OFF/ON, with the LUQs quiescent and L10 active. At time 1 a brief pulse of current was injected into the LUQs, causing a transition to the state ON/OFF. Subsequent transitions were induced at times 2, 3, and 4.

response. The pulse caused the LUQs to fire persistently and L10 to become quiescent. The circuit remained in the new state until a pulse was injected into L10 (time 2, Fig. 8). This pulse caused the output to return to the original state. The alternation between states was elicited repeatedly (e.g., times 3 and 4, Fig. 8), at least 20 times, without obvious fatigue of the output.

We explored two issues concerning the long-term stability of the output states. These studies utilized circuits of L10 co-cultured with a single LUQ ($n = 3$). The bias levels were adjusted as described above.

The first issue we examined was whether each state was stable for a time that was long compared with the decay time of the interactions after cessation of a pulse ($\tau_s \sim 10$ s). We observed that each of the two output states were stable for at least 500 s. The data in Fig. 9 *A* emphasizes the long-term stability of the state ON/OFF, with LUQ active and L10 quiescent. Initially the output was in the state OFF/ON, with LUQ quiescent and L10 active. We then injected a pulse of current into LUQ, causing a transition to the state ON/OFF. The brief delay in the onset of firing of LUQ corresponds to the decay of the inhibitory input from L10. The circuit remained in the ON/OFF state for 500 s (Fig. 9 *A*). After this time we injected a pulse of current into L10 and observed a transition back to the state OFF/ON. Similar results were observed for the long-term stability of the state OFF/ON. These results show that the bistable output persisted for a period at least 50 times the decay constant of the synaptic input. This implies that the stability of the output depends on continuous feedback between the neurons.

After relatively long periods of sustained activity the synaptic input often weakened and the connections in the LUQ/L10 circuits became incapable of maintaining one cell quiescent while the other cell fired. The onset of this fatigue is illustrated in Fig. 9 *B*. The output of the circuit was initially in the state OFF/ON. With sustained activity the connection from L10 to LUQ fatigued and both cells began to fire. Thus the output was no longer in a

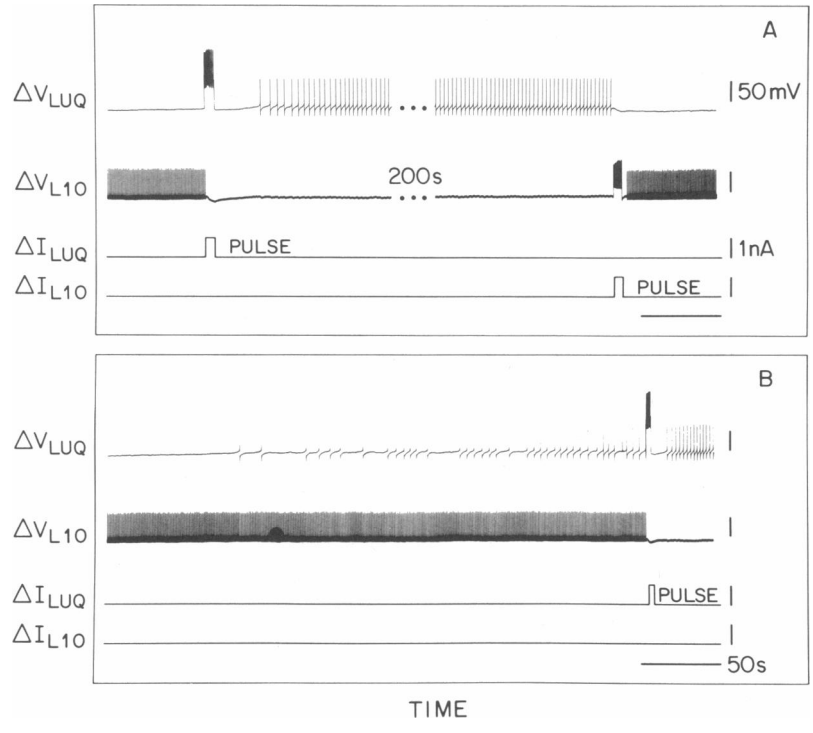

FIGURE 9 Bistable behavior in an inhibitory circuit consisting of a LUQ/L10 co-culture. (*A*) Illustration of the long-term stability of the output. A current pulse to LUQ elicited a transition from the state OFF/ON to the state ON/OFF. The outputs remained in this state for 400 s, ~25 times the decay constant τ_s of the interaction. Note, however, the slight weaking of the inhibition in L10 over this period. A transition from the state ON/OFF to the state OFF/ON was subsequently elicited by a pulse to L10. (*B*) Illustration of the effect of gradual fatigue of the connection from L10 to LUQ on the stability of the output. The data shown was taken after the circuit had been in the state OFF/ON for nearly 1,000 s. The connection began to weaken, allowing LUQ to gradually start firing. Thus the output state of the circuit became ambiguous. A transition to the state ON/OFF was subsequently elicited by a current pulse to LUQ. The LUQ to L10 connection did not have an opportunity to fatigue and this output is unambiguous.

well-defined state. However, a subsequent current pulse to LUQ, whose connection to L10 had not fatigued, caused a transition to the well-defined state ON/OFF.

A second issue was the stability of the output states when one of the cells fired erratically. This was addressed by examining the output of circuits in which the LUQ fired irregularly ($n = 2$). The basic bistable output behavior of one such circuit is shown in Fig. 10 *A*. Note that we used relatively weak current pulses to induce transitions in this circuit. The long-term stability of the output state ON/OFF, with LUQ active and L10 quiescent, is shown in Fig. 10 *B*. The long-term stability of the

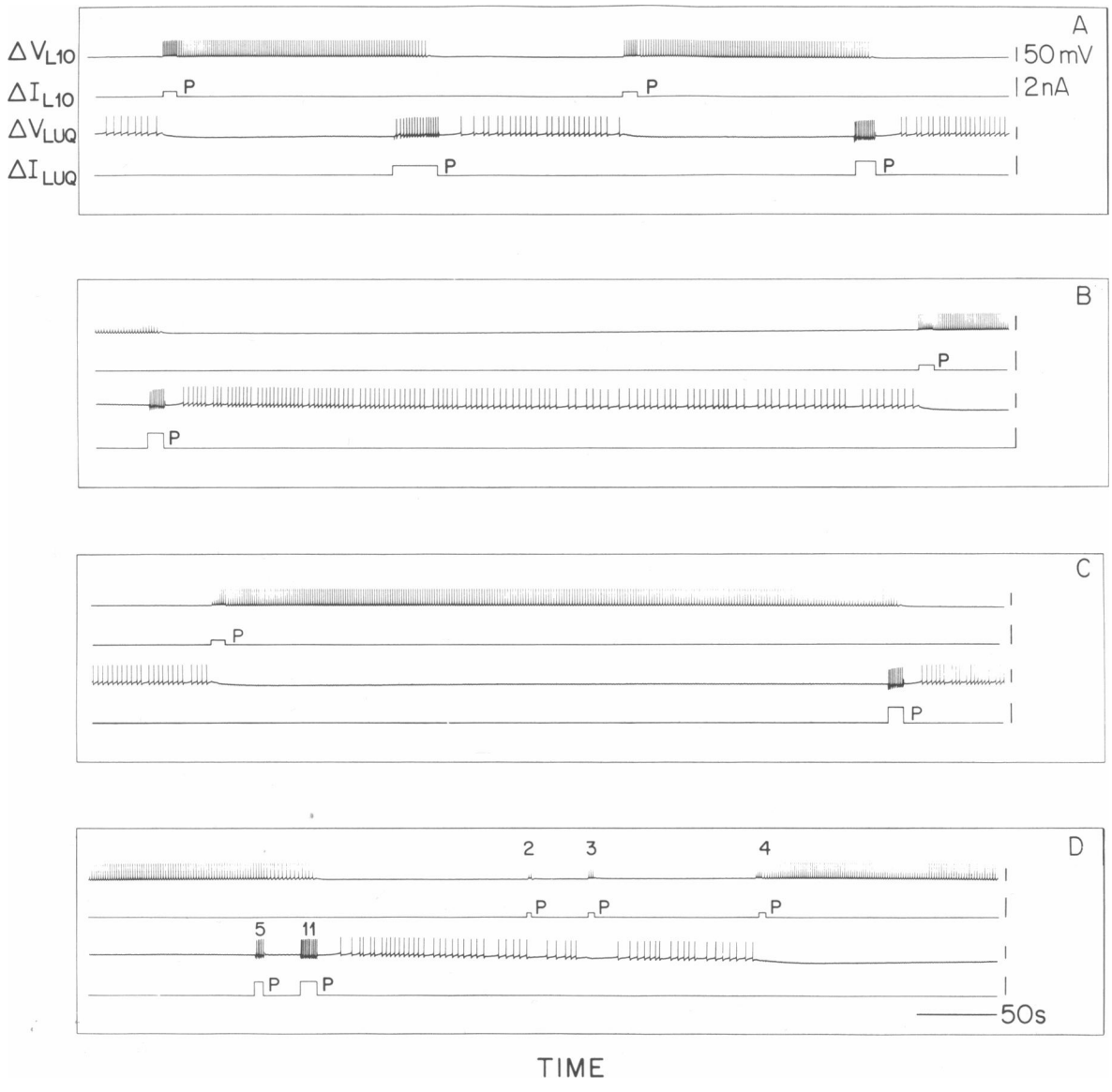

FIGURE 10 Bistable behavior in an inhibitory circuit consisting of co-cultured LUQ and L10 neurons in which the LUQ, putatively L3, had a slightly erratic firing rate. Injection of a current pulse is indicated by a '*P*'. (*A*) The basic bistable response of the circuit. Note that relatively weak current pulses were used to cause transitions between the states ON/OFF and OFF/ON. (*B*) Demonstration of the long-term stability, ~500 s, of the state OFF/ON. Note the relatively erratic firing of LUQ. The fluctuations are faster than the integration period characterized by the decay time, τ_s, of the synaptic response. (*C*) Demonstration of the long-term stability, ~500 s, of the state ON/OFF. (*D*) The effectiveness of the duration of the current pulse in initiating a transition between states. A pulse to LUQ that elicited 11 action potentials, but not one that elicited five action potentials, caused a transition from the state ON/OFF to the state OFF/ON. A pulse to L10 that elicited four action potentials, but not pulses that elicited either two or three action potentials, caused a transition from the state OFF/ON to the state ON/OFF.

companion state OFF/ON is shown in Fig. 10 *C*. These data show that the output of the circuits were relatively insensitive to fluctuations in the firing rate that were fast compared to the time scale of the synaptic integration, i.e., τ_s.

A final issue we examined was the number of action potentials that a brief current pulse had to cause in the presynaptic cell to trigger a transition. This number should depend on the detailed strengths of the connections and the bias levels and input resistances of the neurons. In practice relatively few action potentials were required. Two examples are shown in Fig. 10 *D*. Initially the output was in the state ON/OFF. The first pulse of current injected into LUQ caused it to fire five action potentials, only partially inhibiting L10. A second, longer pulse caused LUQ to fire 11 action potentials and induced a transition to the state OFF/ON (Fig. 10 *D*). We next attempted to cause a transition back to the state ON/OFF. Injection of pulses into LUQ that were sufficient to cause either two or three action potentials transiently inhibited L10. With four action potentials a complete transition occurred (Fig. 10 *D*; note the gradual increase in amplitude of the inhibition in LUQ). These results show that transitions can be induced by relatively low firing rates.

Reciprocal inhibition: stability analysis

We discussed above the basic phenomenology and long-term stability of the bistable output. In those studies the bias currents were adjusted so that the output of each neuron was near its optimal sensitivity to changes in its synaptic inputs. We now present the results of measurements on the stability of the output as a function of the bias levels of each cell. As discussed in the theory section (Fig. 2 *B*) there are four possible output states: ON/ON, ON/OFF, OFF/ON, and OFF/OFF. The output of the circuit may be stable in only one of these states, i.e., monostable, or it may be stable in both the states ON/OFF and OFF/ON, i.e., bistable.

We judged the stability of the output for each set of currents as follows: If both neurons remained quiescent for a time $\gtrsim$200 s, the output of the circuit was judged to be monostable in the state OFF/OFF. Similarly, if both neurons fired for a time $\gtrsim$200 s, the circuit was judged to be monostable in the state ON/ON. If one neuron remained quiescent while the other was active, the output was potentially bistable. We checked this possibility by exciting the quiescent cell with a current pulse. Two outcomes were observed: (*i*) The active cell was momentarily inhibited but soon returned to its previous level of activity. The cell that we injected with a current pulse returned to quiescence. The original state is thus the only stable state and the circuit is monostable. (*ii*) The cell that we injected with a current-pulse became active and inhibited its postsynaptic partner from further firing. The circuit thus appears to be bistable. This was confirmed by pulsing the now quiescent cell to return the output of the circuit to its original state.

An example of data from the stability analysis is given in Fig. 11. Initially the bias currents were adjusted so that LUQ was active and L10 was quiescent. We probed the stability by injecting a strong current pulse into L10. The LUQ was briefly inhibited but resumed firing. The output was judged monostable in the state ON/OFF for this set of bias currents. We next increased the level of the bias current to L10 only and again probed the output. Injection of a pulse into L10 now caused a transition to the state OFF/ON. We observed that the output returned to its original state when we subsequently injected a current pulse into LUQ. The output was judged bistable for this set of bias currents. This data shows how a change in the bias current to a single cell changed the output behavior of the circuit from monostability to bistability.

We reemphasize that the focus of our analysis was on the steady-state behavior of the output. Thus the output of the network was assigned only after a neuron remained active for a period that was substantially longer than τ_s (~10 s; Fig. 4 *A*).

The compiled results from the stability analysis with a particular L10/LUQ co-culture are shown in Fig. 12. We used sets of currents that were separated in value by 0.05 nA in acquiring this data. There are three noteworthy

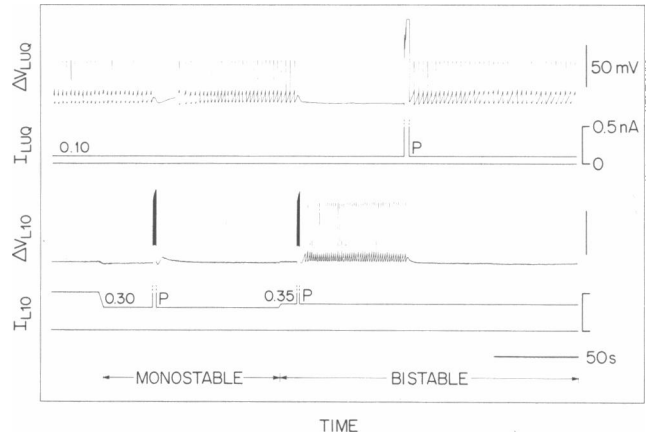

FIGURE 11 Illustrative example of monostable output and bistable outputs from the stability analysis. The bias currents were first adjusted to I_{01} = 0.10 nA and I_{02} = 0.30 nA, for which LUQ was active and L10 quiescent. A strong current pulse to L10 momentarily inhibited LUQ, but the original activity soon recovered. Thus the output was monostable in the state ON/OFF. The bias current to L10 only was changed to I_{02} = 0.35 nA. Pulsing L10 now elicited a transition to the state OFF/ON. Subsequently pulsing LUQ caused a transition back to the state ON/OFF. The output was thus bistable.

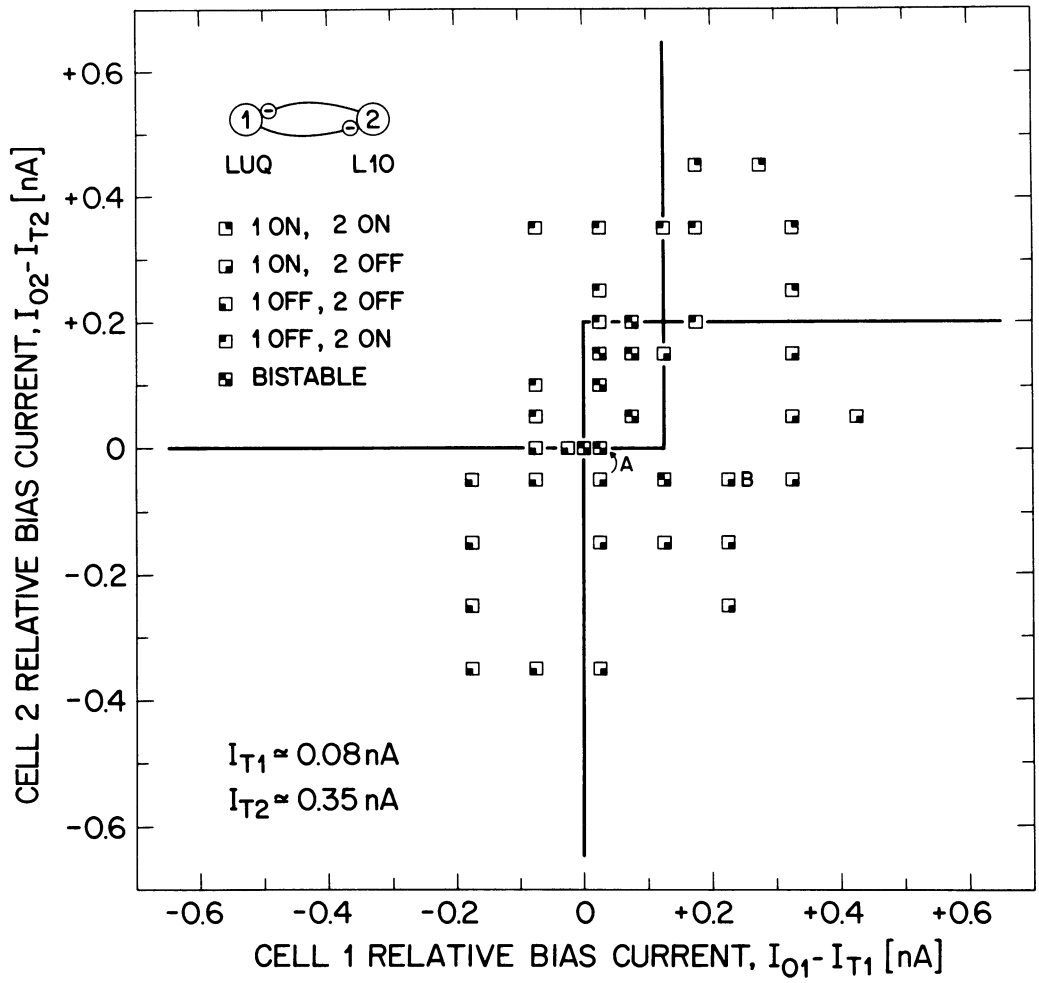

FIGURE 12 Compilation of the stability analysis on the LUQ/L10 inhibitory circuit. The data labeled *A* corresponds to the data shown in Fig. 11. The essential features of the data were: (*i*) There was a small range of bias currents for which the output was bistable in the states ON/OFF and OFF/ON. (*ii*) The shape of the bistable region was nearly twice as large for currents injected into L10 as for currents injected into LUQ. (*iii*) The relative bias currents that separated the bistable region from monostable regions, and separated different monostable regions, agreed with theory (cf. Fig. 2 *B*).

features:

(*a*) There was a range of bias currents for which the ouput was stable in both the state ON/OFF and the state OFF/ON, i.e., bistable (■; Fig. 12). The order of magnitude of this range was ~0.1 nA. This corresponds to the magnitude of the steady-state value of the synaptic inputs ($J_{12} \sim J_{21} \sim 0.1$ nA).

(*b*) The shape of the bistable region was about twice as large for currents injected into L10 as for currents injected into LUQ. According to our theoretical arguments (Fig. 2 *B*) the connection from LUQ to L10 should consequently be roughly twice as strong as that from L10 to LUQ. This is consistent with the connections strengths observed for these cells (Fig. 13; a similar ratio of strengths was observed under steady-state conditions).

(*c*) The relative bias currents that separated the region of bistable output from the monostable regions, and separated different monostable regions, were in good agreement with the predictions of a simple model (cf. Figs. 2 *B* and 12).

The number of consecutive measurements that could be performed on a given preparation was limited by changes in the excitability of the cells. These changes occurred over a period of a few hours and reflected, in part, drift caused by gradual clogging of the electrodes. The compiled results in Fig. 12 are from our most robust preparation. Similar findings, over a smaller range of bias currents, were observed with other preparations ($n = 4$).

Reciprocal excitation: basic response

We now consider the output properties of co-cultures of L7 and L12 ($n = 6$). These neurons form slow, reciprocal

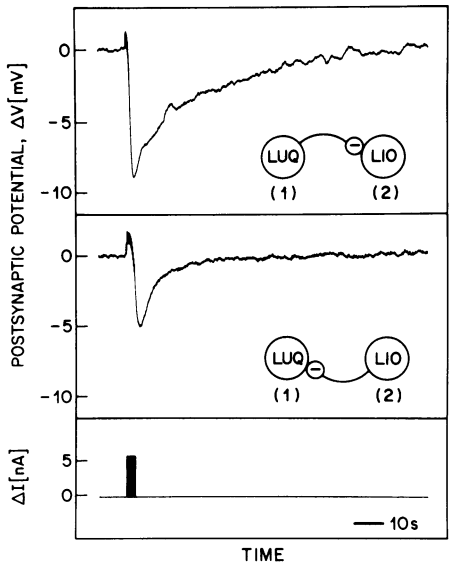

FIGURE 13 The relative connection strengths between the co-cultured L10 and LUQ neurons used to examine the stability of the output state as a function of the individual bias currents (Figs. 11 and 12). The inhibitory responses were elicited by a train of action potentials, generated by a set of current pulses, ΔI (50-ms pulses at 200-ms intervals for 3 s), in the presynaptic cell. The input resistance of either cell was $R_{IN} \simeq 25$ MΩ. Thus the connection from LUQ to L10 is roughly twice the strength of the connection from L10 to LUQ.

excitatory interactions (Fig. 4 *B*) and are expected to produce a distinct, bistable output (Figs. 1 *C* and 2 *C*). Thus the behavior of the excitatory circuits provides information that is complementary to that obtained with the inhibitory circuits.

The basic response of the excitatory system is shown in Fig. 14. We first set the bias currents just below the threshold values for the cells. The neuronal output remained quiescent. The subsequent injection of a current pulse into L7 caused L7 to fire immediately and L12 to fire ~1.5 s later. The latter delay coincides with the rise time of the depolarization (Fig. 4 *B*). Sustained activity in both cells was observed after cessation of the pulse. The brief inactivity of L7 after termination of the pulse was caused by a hyperpolarizing afterpotential. The change in the output of the circuit from the quiescent to the active state is in agreement with the model (Fig. 1 *C*). A similar transition was observed when the pulse was injected into L12, rather than L7, in accord with the approximate symmetry of the connections (see below; trial 6 in Fig. 15).

The duration of the active state was limited by at least two mechanisms. First, the strength of the L12 to L7 connection decreased by ~30% over the time course of the active output (see earlier section). Secondly, the value of the threshold current increased over the same period (Fig. 6 *D*). These effects can cause a circuit in its active state to relax to its quiescent state. They can also lead to a decrease in the duration of the output after successive trials, as observed (see below; cf. trials 5 and 6 in Fig. 15).

A circuit in the active state could be returned to the quiescent state by injecting a hyperpolarizing current pulse into one or both neurons. The duration of this pulse needed to be 20–30 s. This time coincides with the nearly complete decay of the excitatory response observed after a brief train of activity in the presynaptic cell (Fig. 4 *B*) but was substantially less than the duration of the output in the state ON/ON.

Reciprocal excitation: stability analysis

The limited duration of the state ON/ON in a bistable L7/L12 circuit prevented us from performing a complete stability analysis, such as the one discussed for the inhibitory circuit. However we could perform a partial analysis in which the response of the circuit was probed in a series of measurements using increasing values of bias current ($n = 3$). In particular, the bias currents were increased in steps from a set of values where both cells were quiescent, in the state OFF/OFF, to a set where the output was potentially bistable (broken arrow, Fig. 2 *C*).

We focus on the results obtained with a particular circuit (Fig. 15). A relatively long current pulse was injected into L12 to insure that the postsynaptic response in L7 consistently reached its maximum amplitude. At low values of bias current the circuit remained in the quiescent state after a pulse (trial 1, Fig. 15). As the bias currents were increased, in steps of 0.1 nA, we observed the beginnings of sustained activity after a pulse. For the response in trial 3 (Fig. 15) the model suggests that the bias current of L7 is properly set but that of L12 is too low. We thus increased the bias level of L12. The circuit exhibited sustained firing in response to a pulse (trial 5; Fig. 15), in agreement with the model. The duration of the active output was long compared with the decay time of the connections but decreased with successive trials (cf. trials 5 and 6, Fig. 15). The full activity recovered after a rest period of ~30 min.

DISCUSSION

Basis for the bistable output

The firing properties of the neurons in our circuits and the connections between the neurons met theoretical criteria for bistable systems: (*a*) The output of individual neurons changed abruptly from quiescence to firing as their input current was increased (Fig. 6). This allowed us to distinguish between two distinct levels of output activity. (*b*)

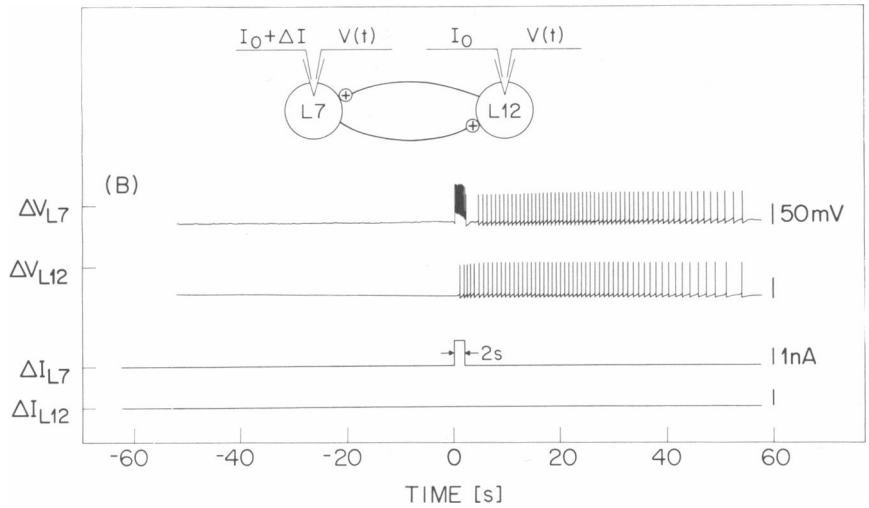

FIGURE 14 The basic dynamic behavior of an excitatory circuit consisting of co-cultured L7 and L12 neurons. The bias currents, I_0 = 0.10 nA and 1.05 nA for L7 and L12, respectively, were set ~ 0.05 nA below their threshold values. The transition from the quiescent to the active state was induced by injecting a brief current pulse into L7. The electrotonic coupling was κ = 0.1.

Pairs of identified neurons were connected by reciprocal chemical connections of the same sign. The magnitude of the synaptic inputs were sufficiently large to allow activity in the presynaptic cell to switch the activity in the postsynaptic cell between quiescence and firing at a nearly constant rate. (*c*) The characteristic decay time of each interaction after a pulse (Fig. 4) was long compared to the period between action potentials in the presynaptic cell (Fig. 6), providing a means of temporal integration.

The circuits we constructed were capable of producing either of two stable patterns of output activity when appropriate, constant bias currents were injected into the neurons. A first circuit consisted of co-cultured L10 and LUQ neurons. These neurons formed reciprocal, inhibitory connections. The output could be reliably and repeatedly switched between the states ON/OFF and OFF/ON by a brief, perturbing current pulse (Δt ~1 s) (Figs. 8 and 10). The stability of each state persisted for a time t ~ 1,000 s, even when one of the neurons fired erratically (Fig. 10). A second circuit consisted of co-cultured L7 and L12 neurons. These neurons formed reciprocal, excitatory connections. The output could be switched between the states OFF/OFF and ON/ON (Figs. 14 and 15). The properties of both circuits were consistent with the behavior predicted from theoretical arguments for bistability (Fig. 1).

The results support the hypothesis that the long-term stability of output resulted from the interactions between the neurons, as opposed to intrinsic cellular properties. (*a*) The stable outputs of both circuits persisted for times that were long compared to the observed decay constant of the interactions. The output states in the inhibitory circuit were stable for >500 s, about 50 times the decay constant (Figs. 9 and 10), and the active state in the excitatory circuit was stable for ~100 s, four to five times the decay constant (Figs. 14 and 15). The persistent activity resulted from the feedback between the two neurons. This feedback continuously regenerated the synaptic input that stabilized the output. (*b*) Changing the bias current to one cell could change the persistent output properties of both cells (Figs. 11 and 15), illustrating the necessity of interactions. None of the neurons showed persistent activity in response to a brief depolarizing current pulse when the neighboring cell in the circuit was inhibited (Figs. 11 and 14). The same pulse, however, could initiate a transition to a stable ouput state when both cells were properly biased (Figs. 11 and 15); compare trials 3 and 5 in Fig. 15. The bistable outputs of both circuits occurred only over a restricted range of bias currents (Figs. 12 and 15) whose magnitude was consistent with the observed values of the connection strengths (Figs. 12 and 13). Outside this range the output was monostable in one of four possible states. Lastly, neurons cultured in isolation did not fire after the termination of a current pulse (e.g., Fig. 5).

The stability of the output states was insured if the interactions were of sufficient strength and duration. These minimum criteria could be met in a variety of ways. For example, the strength of the connection from L10 to LUQ was roughly proportional to the firing rate of L10, whereas the connection from LUQ to L10 was roughly independent of the rate of firing of LUQ (Fig. 7). Both connections provided sufficient feedback to allow circuits to achieve stable outputs. Furthermore, while the firing

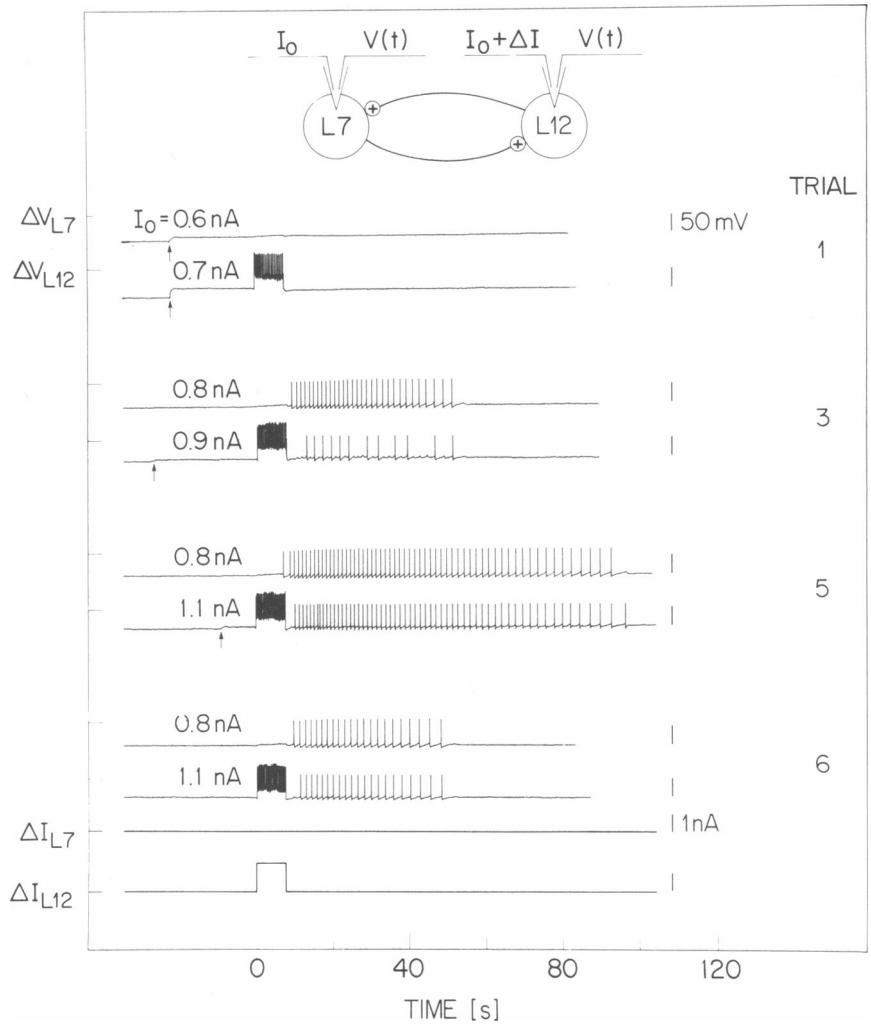

FIGURE 15 The dynamic behavior of an L7/L12 circuit as a function of the bias current to each neuron. We show four data records taken from a set of consecutive trials. The value of the bias current was increased in 0.1-nA steps between trials; the arrows indicate the time of the increase. Current pulses were injected into L12 at 120-s intervals. The partial synchrony in the firing of the neurons results from the electrotonic coupling (κ = 0.2).

rates of the individual neurons differed (Fig. 7), they were essentially constant over the range of inputs, either synaptic currents or bias currents, used with these circuits. Thus the neurons were characterized as either OFF or ON.

Relevance to in vivo circuits

Many circuits involved in the generation of motor activity produce well-defined sequences of output activity. These circuits are referred to as central pattern generators (CPGs). The individual output states of CPGs may be stable for a time that is long compared with the time for a transition between the states. Reciprocal connections of the same sign, such as those studied in our in vitro circuits, provide a mechanism for stabilization. Neurons that control synergistic motor outputs are connected by excitatory chemical connections or strong electrotonic connections while neurons that control antagonistic outputs are connected by inhibitory connections (Selverston and Moulins, 1987; Getting, 1988). This form of connectivity stabilizes the individual output states in the CPG controlling swimming in the mollusc *Tritonia* (Getting, 1981, 1983, 1989; Kleinfeld and Sompolinsky, 1988). The output states in other CPGs, such as the circuit controlling the pyloric rhythm in the crustacean stomatogastric ganglion (Miller and Selverston, 1982) and the CPG controlling heartbeat in the leech (Thomspon and Stent, 1976; Peterson and Calabrese, 1982), appear to be stabilized by intrinsic cellular properties as well as reciprocal connections.

The circuits we constructed function as "memories."

Introduction of a brief, ~1-s pulse of activity changed the output state of the circuit for up to 1,000 s. Persistent electrical activity can be evoked in regions of association cortex in monkeys during short-term memory tasks ($\Delta t \sim$ 10–100 s) (Fuster and Jervey, 1982; Miyashita and Chang, 1988). It is an open question as to whether this persistent activity represents a stable state that is maintained by recurrent synaptic interactions (Sompolinsky, 1988; Toulouse, 1989).

Our circuits functioned only when the synaptic inputs were capable of driving the neurons between their on and off levels. A consequence of this constraint is that the operating level of each neuron must be set so that the synaptic inputs can drive the neuron through its transition region. The basal value of these levels are an intrinsic property of the neurons and are presumably matched to the average value of the synaptic input in vivo. We controlled these levels in our in vitro circuits by injecting a constant bias current into the neurons. These levels could also be controlled by a constant, external synaptic input.

Our work suggests how external inputs or modulation of neural threshold levels may control the output of circuits in vivo. This may be relevant to the control of certain motor circuits by command neurons (Kupfermann and Weiss, 1978). A single command neuron can activate a relatively large circuit (Kennedy et al., 1966). One mechanism for this activation is a tonic, synaptic input that changes the bias level of one or more neurons in the circuit. This mechanism appears to play a dominant role in turning on and off the CPG controlling the swim rhythm in the mollusc *Tritonia* (Getting and Dekin, 1985). It may also play a role in changing the output state of a circuit controlling navigation in the silkworm moth (Olberg, 1983). A second mechanism is the release of a neurohormone that changes the threshold properties of one or more neurons in a circuit. This mechanism may be relevant, in part, to the control of the pyloric rhythm in the stomatograstric ganglion (Nagy and Dickinson, 1983; Marder and Hooper, 1985; Harris-Warrick, 1986).

Relevance to model neural networks

Recent theoretical work on associative neural networks suggests how highly interconnected circuits of many neurons can produce such multiple, temporally stable output states (Little, 1974; Hopfield, 1982, 1984; Amit et al., 1985*a* and *b*; Gardner, 1988). These model networks may be relevant to aspects of associative memory and pattern recognition (Hopfield, 1982). Extensions of the model proposed by Hopfield (1982) suggest how networks with multiphasic connections (Sompolinsky and Kanter, 1986; Kleinfeld, 1986), time-dependent connections (e.g., synaptic fatigue) (Peretto and Niez, 1986) and disynaptic interactions (e.g., presynaptic facilitation or inhibition) (Dehaene et al., 1987) may produce a sequence of stable output states. The application of these models to the production of rhythmic motor output by CPGs is discussed by Kleinfeld and Sompolinsky (1988).

The model neural networks can produce stable firing patterns if the neurons exhibit nonlinear firing characteristics and there are feedback pathways between the neurons. In model networks with a small number of neurons, the period of synaptic integration must be long compared to the period between action potentials. These constraints also allowed our in vitro circuits to produce bistable outputs. It is interesting that some features of the model networks were realized in co-cultures that consisted of only two cells. This suggests that our circuits may serve as building blocks for the construction and analysis of more complex in vitro networks.

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Note added in proof: Subsequent to the submission of this work, an interesting study by Koester and Alevizos (1989) showed that LUQ neurons form a limited number of inhibitory connections back onto L10 in vivo. These occur in addition to the previously described connections from L10 to the LUQs (Kandel et al., 1967).

REFERENCES

Amari, S. I. 1972. Characteristics of random nets of analog neuron-like elements. *IEEE (Inst. Electr. Electron. Eng.) Trans. Sys. Man Cybern.* 2:643–657.

Amit, D. J. 1989. Modeling Brain Function: The World of Attractor Neural Networks. Cambridge University Press, New York.

Amit, D. J., H. Gutfreund, and H. Sompolinsky. 1985*a*. Spin-glass models of neural networks. *Phys. Rev. A.* 32:1007–1018.

Amit, D. J., H. Gutfreund, and H. Sompolinsky. 1985*b*. Storing infinite numbers of patterns in a spin-glass model of neural networks. *Phys. Rev. Lett.* 55:1530–1533.

Bennett, M. V. L. 1977. Electrical transmission: a functional analysis and comparison to chemical transmission. *In* Handbook of Physiology. Sec. 1. The Nervous System. Vol. 1. E. R. Kandel, editor., American Physiology Society, Bethesda, MD. 357–416.

Camardo, J., E. Proshansky, and S. Schacher. 1983. Identified *Aplysia* neurons form specific chemical synapses in culture. *J. Neurosci.* 3:2814–2820.

Cohen, A. H., S. Rossignol, and S. Grillner, editors. 1988. Neural Control of Rhythmic Movements. John Wiley & Sons, New York.

Dagan, D., and I. B. Levitan. 1981. Isolated identified *Aplysia* neurons in cell culture. *J. Neurosci.* 1:736–740.

Dehaene, S., J.-P. Changeux, and J.-P. Nadal. 1987. Neural networks that learn temporal sequences by selection. *Proc. Natl. Acad. Sci. USA.* 84:2727–2731.

Delcomyn, F. 1980. Neural basis of rhythmic behavior in animals. *Science (Wash. DC).* 210:429–498.

Frazier, W. T., E. R. Kandel, I. Kupfermann, R. Waziri, and R. E. Coggeshall. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica. J. Neurophys.* 30:1288–1351.

Fuster, J. M., and J. P. Jervey. 1982. Neuronal firing in the inferotemporal cortex of the monkey in a visual memory task. *J. Neurosci.* 2:361–375.

Gardner, E. 1988. The space of interactions in neural network models. *J. Phys. A.* 21:257–270.

Getting, P. A. 1981. Mechanism of pattern generation underlying swimming in *Tritonia.* I. Neuronal network formed by monostable connections. *J. Neurophysiol. (Bethesda).* 46:65–79.

Getting, P. A. 1983. Mechanism of pattern generation underlying swimming in *Tritonia.* III. Intrinsic and synaptic mechanisms for delayed excitation. *J. Neurophysiol. (Bethesda).* 49:1036–1050.

Getting, P. A. 1988. Comparative analysis of invertebrate central pattern generators. *In* Neural Control of Rhythmic Movements in Vertebrates. A. H. Cohen, S. Rossignol, and S. Grillner, editors. John Wiley & Sons, New York. 101–127.

Getting, P. A. 1989. Reconstruction of small neural networks. *In* Methods in Neuronal Modeling: From Synapses to Networks. C. Koch and I. Segev, editors. MIT Press, Cambridge, MA. 171–194.

Getting, P. A., and M. S. Dekin. 1985. Mechanism of pattern generation underlying swimming in *Tritonia.* IV. Gating of central pattern generator. *J. Neurophysiol. (Bethesda).* 53:466–480.

Harris-Warrick, R. M. 1986. Chemical modulation of central pattern generators. *In* Neural Control of Rhythmic Movements. A. H. Cohen, S. Rossignol, and S. Grillner, editors. John Wiley & Sons, New York. 285–331.

Harth, E. M., T. J. Csermely, B. Beek, and R. D. Lindsay. 1970. Brain functions and neural dynamics. *J. Theor. Biol.* 26:93–120.

Hermann, A. 1986. Charybdotoxin specifically blocks Ca-activated K conductance of *Aplysia* neurons. *Soc. Neurosci. Abstr.* 12:789.

Hopfield, J. J. 1982. Neural networks and physical systems with emergent collective computational properties. *Proc. Natl. Acad. Sci. USA.*79:2554–2558.

Hopfield, J. J. 1984. Neural with graded response have collective computational properties like those of two-state neurons. *Proc. Natl. Acad. Sci. USA.* 81:3088–3092.

Kandel, E. R., W. T. Frazier, R. Waziri, and R. E. Coggeshall. 1967. Direct and common connections among identified neurons in *Aplysia. J. Neurophysiol. (Bethesda).* 30:1352–1376.

Kaczmarek, L. K., M. Finbow, J. P. Revel, and F. Strumwasser. 1979. The morphology and coupling of *Aplysia* bag cells within the abdominal ganglion and in cell culture. *J. Neurobiol.* 10:535–550.

Kennedy, D., W. H. Evoy, and J. T. Hanawalt. 1966. Release of coordinated behavior in crayfish by single central neurons. *Science (Wash. DC).* 254:153–155.

Kleinfeld, D. 1986. Sequential state generation by model neural networks. *Proc. Natl. Acad. Sci. USA.* 83:9469–9473.

Kleinfeld, D., G. F. Raccuia, and H. J. Chiel. 1988*a*. Reciprocal inhibition between identified *Aplysia* neurons maintained in culture. *Biophys. J.* 53:427a. (Abstr.)

Kleinfeld, D., G. F. Raccuia, and H. J. Chiel. 1988*b*. Circuits with bistable outputs constructed from identified neurons. *Soc. Neurosci. Abstr.* 14:841.

Kleinfeld, D., and H. Sompolinsky. 1988. Associative neural network model for the generation of temporal patterns: theory and application to central pattern generators. *Biophys. J.* 54:1039–1051.

Kleinfeld, D., and H. Sompolinsky. 1989. Associative neural network models for central pattern generators. *In* Methods in Neuronal Modeling: From Synapses to Networks. C. Koch and I. Segev, editors. MIT Press, Cambridge, MA. 195–246.

Koester, J., E. Mayeri, G. Liebeswar, and E. R. Kandel. 1974. Neural control of circulation in *Aplysia.* I. Interneurons. *J. Neurophys.*37:476–496.

Koester, J., and E. R. Kandel. 1977. Further identification of neurons in the abdominal ganglion of *Aplysia* using behavior criteria. *Brain Res.* 121:1–20.

Koester, J., and A. Alevizos. 1989. Innervation of the kidney of *Aplysia* by L10, the LUQ cells, and an identified peripheral motoneuron. *J. Neurosci.* 9:4078–4088.

Kramer, R. H., and R. S. Zucker. 1985. Calcium-induced inactivation of calcium currents causes the interburst hyperpolarization of *Aplysia* bursting pacemaker neurons. *J. Physiol. (Lond.)* 352:131–160.

Kristan, W. B., Jr. 1980. Generation of rhythmic motor patterns. *In* Information Processing in the Nervous System. H. M. Pinsker and W. D. Willis, Jr., editors. Raven Press, New York. 241–261.

Kupfermann, I., and K. R. Weiss. 1978. The command neuron concept. *Behav. Brain Sci.* 1:3–39.

Little, W. A. 1974. The existence of persistent states in the brain. *Math. Biosci.* 19:101–120.

Marder, E., and S. L. Hooper. 1985. Neurotransmitter modulation of the stomatogastric ganglion of decapod crustacean. *In* Model Neural Networks and Behavior. A. I. Selverston, editor. Plenum Publishing Corp., New York. 313–337.

Miller, C., E. Moczydlowski, R. Latorre, and M. Phillips. 1985. Charybdotoxin, a protein inhibitor of single Ca^{+2}-activated K^+ channels from mammalian skeletal muscle. *Nature (Lond.)* 313:316–318.

Miller, J. P., and A. I. Selverston. 1982. Mechanisms underlying pattern generation in lobster stomatogastric ganglion as determined by selective inactivation of identified neurons. IV. Network properties of pyloric system. *J. Neurophys.* 48:1416–1431.

Miyashita, Y., and H. S. Chang. 1988. Neuronal correlate of pictoral short-term memory in the primate temporal cortex. *Nature (Lond.).* 331:68–71.

Nagy, F., and P. S. Diokinson. 1983. Control of a central pattern generator by an identified modulatory interneurone in crustacea: modulation of the pyloric motor output. *J. Exp. Biol.* 105:33–58.

Obaid, A. L., D. Kleinfeld, T. D. Parsons, G. F. Raccuia, and B. M. Salzberg. 1989. R15 and other rhythmically active *Aplysia* neurons exhibit endogenous bursting in culture: possible activators of in vitro neuronal assembles. *Biophys. J.* 55:175a. (Abstr.)

Olberg, R. M. 1983. Peromone-triggered flip-flopping interneurons in the ventral nerve cord of the silkworm moth, *Bombyx mori. J. Comp. Physiol. A.* 152:297–307.

Peterson, E. L., and R. L. Calabrese. 1982. Dynamic analysis of a rhythmic neural circuit in the leech *Hirudo medicinalis. J. Neurophys.* 2:256–271.

Peretto, P., and J. J. Niez. 1986. Collective properties of neural networks. *In* Disordered Systems and Biological Organization. E.

Bienenstock, F. Fogelman, and G. Weibusch, editors. Springer-Verlag, Berlin. 171–185.

Roberts, A., and B. L. Roberts. 1983. Neural Origin of Rhythmic Movements. Cambridge University Press, Cambridge, UK.

Schacker, S., and E. Proshansky. 1983. Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* 3:2403–2413.

Scholz, K. P., L. J. Cleary, and J. H. Byrne. 1988. Inositol 1,4,5-triphosphate alters bursting pacemaker activity in *Aplysia* neurons: voltage-clamp analysis of effects on calcium currents. *J. Neurophys.* 60:86–104.

Selverston, A. I. 1980. Are central pattern generators understandable? *Behav. Brain Sci.* 3:535–571.

Selverston, A. I., and M. Moulins. 1987. Comparison with other systems. *In* The Crustacean Stomatogastric Ganglion: A Model for the Study of Central Nervous Systems. A. I. Selverston and M. Moulins, editors. Springer-Verlag, New York. 307–313.

Sompolinsky, H. 1988. Statistical mechanics of neuronal networks. *Physics Today.* 41:70–80.

Sompolinsky, H., and I. Kanter. 1986. Temporal association in asymmetric neural networks. *Phys. Rev. Lett.* 57:2861–2864.

Spira, M., I. Segev, R. Werman, and Y. Yarom. 1988. Modulation of the electrotonic structure of *Aplysia* neurons by subthreshold voltage shifts and serotonin. *Soc. Neurosci. Abstr.* 14:384.

Thompson, W. J., and G. S. Stent. 1976. Neural control of heartbeat in the medicinal leech. III. Synaptic relations of the heart interneurons. *J. Comp. Physiol.* 111:309–333.

Toulouse, J. 1989. Perspectives on neural networks and their relevance to neurobiology. *J. Phys. A.* 22:1959–1968.

Wilson, H. R., and J. D. Cowan. 1972. Excitatory and inhibitory interactions in localized populations of model neurons. *Biophys. J.* 12:1–24.