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A CELLULAR ANALOGUE OF OPERANT CONDITIONING

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Using the hippocampal-slice preparation, we attempted to model operant conditioning in vitro by reinforcing pyramidal cell bursting responses with local micropressure applications of transmitters and drugs. The same injections were administered independently of bursting to provide a "noncontingent" control for direct pharmacological stimulation or facilitation of firing. The results suggested that the bursting responses of individual CA1 pyramidal neurons may be reinforced in a dose-related manner by response-contingent (but not noncontingent) injections of dopamine and the selective dopamine D_2 agonist, N-0923. N-0924, a stereoisomer of N-0923 that is largely devoid of D_2-agonist activity, failed to reinforce CA1 bursting. Burst-contingent injections of the excitatory neurotransmitter glutamate also failed to reinforce CA1 bursting; indeed, the glutamate applications (whether contingent or random) reduced the likelihood of bursts while increasing the frequency of solitary spikes. Reinforcement delays exceeding 200 ms largely eliminated the reinforcing efficacy of the D_2 agonist N-0437 in CA1 operant conditioning. The results are consistent with the suggestion that the behaviorally reinforcing effects of dopaminergic agents can be modeled in vitro in the hippocampal-slice preparation.

Key words: in vitro operant conditioning, reinforcement mechanisms, dopamine, N-0923, hippocampus, glutamate, delay of reinforcement, response elements, atoms of behavior, brain slice

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> A more useful way of putting it is to say that the *elements* are strengthened wherever they occur. This leads us to identify the element rather than the response as the unit of behavior. It is a sort of behavioral atom, which may never appear by itself upon any single occasion but is the essential ingredient or component of all observed instances. (Skinner, 1953, p. 94)

In studying a complex system such as the brain, it is useful to distinguish between its intrinsic properties (those primarily associated with the elements or building blocks) and its emergent properties (those primarily associated with the system as a whole or with major organizational subunits). For example, in Marie Curie's early investigations of the radioactivity of uranium compounds, a key discovery established that the radiation was an intrinsic (atomic) property of the element uranium and did not depend on the emergent (molecular) properties of the different uranium salts or on other facts related to chemistry, such as light or temperature. Establishing as a fact that radioactivity is an atomic property led Curie to important new facts, including the discovery of two new elements, radium and polonium. In the case of nervous systems, it is convenient to assume that brain cells or small neural circuits represent the elements or building blocks, and to ask which mental processes may depend primarily on cellular behavior and which may depend primarily on the organization and behavior of the system.

The cells versus systems question is important, not only intrinsically, but because it bears on the relevance of nonhuman (particularly invertebrate) models for understanding human brain functioning. Suppose that some human mental process (e.g., associative memory) depended primarily on the behavior or biochemistry of individual cells. Because there are few, if any, fundamental differences in the physiological and biochemical properties of human and invertebrate brain cells, it would be reasonable in such cases to expect that the mechanisms of human and invertebrate associative memory would exhibit many similarities. On the other hand, differences are obviously more impressive than similarities when brain circuitry and brain complexity are compared in human and snail. Hence, for those human mental functions that depend heavily on complex brain circuitry, it would be unreasonable to expect much enlightenment from invertebrate models. Twenty-five years ago, the hypothesis that learning and memory depended heavily on cellular or even subcellular events

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was highly controversial. Today, this hypothesis is strongly supported by the detailed explanatory power of current cellular models of invertebrate habituation and classical conditioning (Byrne, 1987; Kandel, 1984), and it is reasonable to expect that closely related cellular mechanisms will be found to underlie at least some of these behavioral activities in higher animals. Here, we suggest in a similar vein that the reinforcement of operant behavior also depends heavily on individual cellular behavior. As a first approach, we have attempted to show that a cellular conditioning process analogous to behavioral operant conditioning can be demonstrated in a highly reduced brain-slice preparation and in the absence of most of the brain.

Until now, little consideration has been given to the identification of the brain substrate that is modified by the reinforcement process. Because it is behavior that is reinforced, it is plausible to assume that behavioral substrates are major targets of brain reinforcement systems. A behavioral response obviously reflects the activity of many neurons. Is it the integrated activity of these neurons that is reinforced? That is, is positive reinforcement exerted at the level of complex neuronal systems? Or is it the individual activities of the relevant neurons that are reinforced? That is, is positive reinforcement exerted at the cellular level?

It is commonly believed that positive reinforcement is exerted at the systems level and probably involves the strengthening or reorganization of the neural substrate of the whole response. The sheer number and virtually infinite variation of reinforceable operant behaviors, however, make it unlikely that the substrate of the whole response is the functional unit of reinforcement. Furthermore, whether or not particular behavioral variations are treated as the same response or as different responses depends on the reinforcement contingencies. Thus, for example, if lever presses of 5-g and 10-g force are reinforced indiscriminately, both are counted as the same "correct" response; however, if they are selectively reinforced, the behavioral variations clearly are regarded as different responses. The fact that closely similar behavioral variations may be reinforced either indiscriminately or selectively is presumptive evidence that the unit of reinforcement cannot be the whole response itself. Rather, as Skinner (1953) has proposed, the whole response must be a composite made up of distinct, largely interchangeable components, and it is these response elements or "behavioral atoms" that are the more likely functional units for reinforcement. Furthermore, to account for the high behavioral resolution that can be achieved by differential reinforcement, the response elements must have a fine grain and a correspondingly fine neural substrate. These considerations, in conjunction with the theoretical work of Klopf (1982), have led us to consider seriously the possibility that individual cellular behavior may be modifiable by reinforcing stimuli (Stein & Belluzzi, 1982, 1988).

Olds (1965) was the first to report apparent evidence for the operant conditioning of single neurons. In these experiments, freely moving rats with implanted microelectrodes received food or rewarding brain stimulation contingent on appropriate spurts of high-rate single-unit activity. Firing rates were increased in a number of cases, suggesting reinforcement of the single-unit response. Unfortunately, it is not clear whether it was the behavior of the individual neuron that was being reinforced or whether some more complex response or movement, of which the neuron's activity was a part, was actually being reinforced. In some of Olds's tests, a restriction system was used to limit movement; electronic detectors were discharged by most movements, and these precluded reinforcement. Although operant conditioning was still obtained under these conditions, one cannot rule out the possible reinforcement of behavior involving undetected movements, such as postural adjustments or attentional responses. Like other investigators who have attempted to demonstrate operant conditioning of single-unit activity, Olds recognized that, if a reinforcing stimulus is delivered to a behaving animal, it is impossible to separate the reinforcement of single units from the reinforcement of more complex responses.

Our solutions to this problem are (a) to use a greatly reduced experimental preparation—the brain slice—and (b) to deliver the reinforcing stimulus—a microinjection of dopamine or cocaine—locally to the neuron being conditioned. For our initial experiments, the hippocampal brain slice had many advantages. First, due to a fortuitous anatomy, the hippocampus can be cut into slices that preserve

the viability and activity of the neurons in the intact structure (Andersen, Bliss, & Skrede, 1971). Neurophysiological studies show that the electrical activity recorded from slices is comparable to that obtained from an intact preparation (Schwartzkroin, 1981). Second, the hippocampus is the target of putative dopamine and endorphin reinforcing systems. Dopamine projections to hippocampus have been described with the presubiculum-CA1 field as the main target area (Bischoff, 1986; Verney et al., 1985). The hippocampus also is well innervated by enkephalin and dynorphin projections and is rich in mu, delta, and kappa opiate receptors (McLean, Rothman, Jacobson, Rice, & Herkenham, 1987). Finally, hippocampal units in the CA1 and CA3 fields occasionally fire in characteristic bursting patterns. Hippocampal bursting has attractive possibilities as a potentially reinforceable cellular response, because such bursting is associated with sharp increases in intracellular calcium (Krnjevik, Morris, & Rupert, 1986). Kandel (1984) has suggested that calcium influx may serve as the ionic marker of recent activity for activity-dependent presynaptic facilitation in cellular classical conditioning, and we have speculated along similar lines that calcium influx may prime the bursting hippocampal cell for chemical reinforcement (Stein & Belluzzi, 1988).

Consistent with the above-described anatomy, we have been able to demonstrate apparent "operant conditioning" of hippocampal CA1 single-unit activity with local administrations of dopamine, cocaine, or the dopamine D_2 agonist N-0437 as reinforcement, and similar operant conditioning of CA3 single-unit activity with local administrations of dynorphin A as reinforcement (Belluzzi & Stein, 1983, 1987; Stein & Belluzzi, 1988, 1989). Application of the reinforcing substances had opposite effects on subsequent firing rates, depending on the activity pattern of the neuron at the time of drug administration. If the neuron had been bursting just before the injections, the rate of bursting was increased; if the neuron had been firing in a nonbursting mode or was silent at the time of the injection, the bursting rate was unaffected or decreased. These observations, therefore, are consistent with the possibility that the bursting of hippocampal cells may be operantly conditioned in vitro by contingent microinjections of reinforcing transmitters or drugs. In support of this suggestion, we report here the results of new experiments in which we evaluated the cellular reinforcing efficacies of (a) different concentrations of dopamine, (b) two stereoisomers of N-0437 that differ 100-fold in their D_2 affinity, (c) the excitatory neurotransmitter glutamate, and (d) immediate versus delayed dopaminergic reinforcement.

METHOD

The experiments were performed on transverse hippocampal slices prepared from male Sprague-Dawley rats (200 to 270 g). The rats were lightly anesthetized with Metofane® and decapitated. The brain was removed rapidly (45 to 60 s) from the skull and chilled to 4 °C in oxygenated artificial cerebrospinal fluid (ACSF) containing NaCl (124 mM), KCl (5 mM), $CaCl_2$ (2.4 mM), $MgSO_4$ (2 mM), KH_2PO_4 (1.25 mM), $NaHCO_3$ (26 mM), and glucose (10 mM). Using plastic tools, the hippocampus was dissected and rinsed repeatedly with cold ACSF to minimize cell damage. The hippocampus was positioned on a McIlwain tissue chopper and sectioned into eight to ten 400-μm slices. Using an eyedropper, six to eight slices were individually transferred to a static chamber where they were supported on nylon mesh at the surface of the ACSF solution in an oxygenated atmosphere (95% O_2, 5% CO_2, 500 mL/min) at 35 °C. The slices were preincubated for at least 2 hr, during which time the volume (1.25 mL) of ACSF in the static chamber was changed every 30 to 40 min.

Single-barreled micropipette blanks (Omega dot borosilicate, 1 mm outside diameter, 0.5 mm inside diameter) were pulled on a vertical puller (Kopf 700B) and backfilled with vehicle (165 mM saline) or test drug in vehicle. The micropipette was connected to a pressure injector (Med Sys PPM-2), and the tip was broken back under microscopic control to permit pressure ejection of a droplet approximately 10 μm in diameter (0.5 pL) following a 50-ms application of nitrogen at 15 psi.

Following incubation, the micropipette was aimed at spontaneously active hippocampal cells in the pyramidal cell layer of CA1 (Figure 1). Action potentials were amplified (Dagan 2400 preamplifier), displayed on a digital storage oscilloscope (Tektronix 5223), and pro-

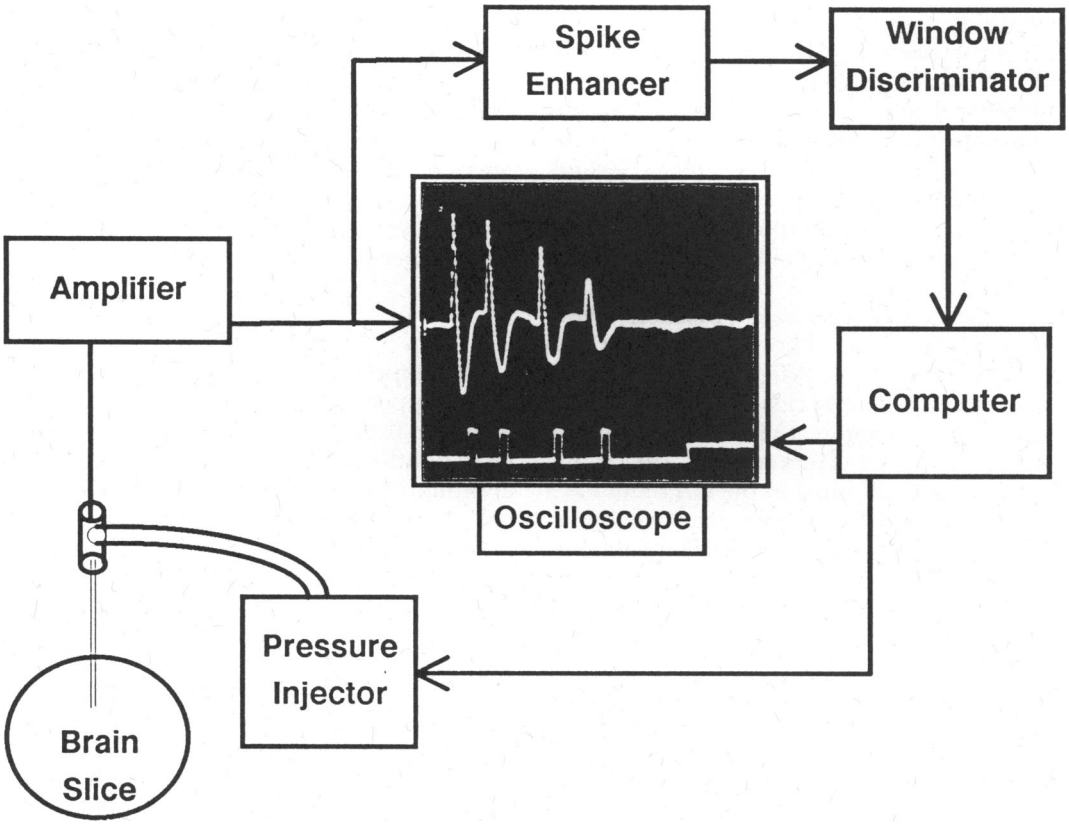

Fig. 1. Schematic diagram of cellular operant conditioning experiment. A single-barreled glass micropipette for simultaneous recording and pressure injection is filled with dopamine (1 mM in 165 mM saline) or other drugs and aimed at spontaneously active hippocampal cells in the CA1 layer. Amplified action potentials are processed by a spike enhancer and window discriminator to increase the signal-to-noise ratio and to isolate signals when multiple-unit activity is encountered. When the computer recognizes a reinforceable burst of activity (based on criteria established individually for each test neuron before operant conditioning), the pressure-injection pump is activated for 50 ms to deliver an approximately 10 μm-diameter droplet of drug close to the cell. Drug-induced increases in bursting are necessary, but not sufficient, evidence of cellular operant conditioning, because the chemical treatments may directly stimulate or facilitate cellular firing. As a mandatory control for such pharmacological stimulation, the same drug injections are also administered independently of bursting on a noncontingent or "free" basis. Cellular-reinforcing effects may be inferred only if the noncontingent injections are relatively ineffective. Inset, upper trace: Burst of firing recorded extracellularly from a CA1 cell exhibiting typical decrescendo pattern with progressively shorter and broader spikes occurring later in the burst. Lower trace: 1-ms logic pulses triggered by each spike. Spikes that satisfy the preset amplitude criteria of the discriminator are converted to logic pulses for counting by the computer. The onset of the 50-ms reinforcing injection is shown as a smaller displacement from baseline.

cessed through a spike enhancer and window discriminator to increase the signal-to-noise ratio and to isolate signals when multiple-unit activity was encountered. Settings of the discriminator were continuously monitored on a second oscilloscope (Tektronix 5113) to ensure that proper settings were maintained throughout the experiment. Important criteria for the selection of suitable cells included a signal-to-noise ratio of at least 4:1 and relatively stable baseline activity. Digitized action potentials from the discriminator were counted by a computer (Data General Eclipse 120); when a reinforceable burst of activity (see criteria below) occurred, the computer operated the pressure-injection pump to eject the solution from the micropipette tip close to the cell.

During operant conditioning, micropressure injections of drug were applied through the recording pipette directly to the cell for 50 ms following bursts of activity that met the criteria. Drug-induced increases in bursting

are a necessary, but not sufficient, indication of cellular operant conditioning, because the drug treatments might directly stimulate or facilitate cellular firing. As a mandatory control for such pharmacological stimulation, the same drug injections were administered independently of bursting on a noncontingent basis (see Matched Injections below). Cellular reinforcing effects were inferred only if the noncontingent injections were relatively ineffective. A burst was defined as a train of neuronal action potentials containing a minimum of n spikes with a maximum interspike interval (ISI) of 10 ms (see Figure 1). A spike-counting computer program accumulated successive spikes occurring within 10 ms of each other and recognized a burst if the total count equaled n or more. The value of n was set individually for each test neuron so that bursts occurred at a baseline rate of approximately five per minute; for most units, $n = 3$ or 4. During the reinforcement phase, injections were made following each burst at the end of the first ISI that did not contain a spike.

A complete neuronal operant conditioning experiment involved six stages. (a) Baseline: The rate of bursting prior to operant conditioning was determined in a baseline period of approximately 5 to 10 min. (b) Reinforcement: Each burst was then followed by an injection of the test solution. To minimize injection artifacts, neuronal activity during and for 3 s after each injection was excluded from the analysis and had no programmed consequences. (c) Extinction: Reinforcement was terminated and recording continued until the baseline burst rate was recovered. (d) Matched (free) injections: Burst-independent injections of the test solution were given at regular intervals to determine the direct pharmacological effects of the micropressure injections on neuronal activity. The number of injections per minute was matched to the three to five highest injection rates obtained during the prior reinforcement period. Again, neuronal activity during and for 3 s after each injection was excluded from the analysis. Occasionally, a burst would occur within 500 ms of a programmed free injection; on these occasions, in order to minimize adventitious reinforcement, the programmed injection was delayed 500 ms. (e) Washout: A second baseline period allowed residual effects of drug administration to dissipate and baseline burst rates to return. (f) Reacquisition: A second period of reinforcement was scheduled, whenever possible, in order to compare rates of original acquisition and reacquisition and to ascertain the viability of the preparation following noncontingent injections. For similar reasons, the scheduling of contingent and noncontingent injections was occasionally reversed so that a period of free injections would precede the first reinforcement period.

RESULTS

Dopaminergic Reinforcement of CA1 Units

Results from a representative experiment in which dopamine was used as the reinforcing agent are shown for a hippocampal CA1 unit in Figure 2. In two separate periods of operant conditioning (REINF), the frequency of bursts was rapidly increased above the baseline rate after several burst-contingent dopamine micropressure injections. The same dopamine injections administered noncontingently (MATCH) failed to increase the frequency of bursts and even tended to suppress bursting (although in this case they did not suppress overall spike activity). Note also in Figure 2 that the bursting rate turned down at the end of both reinforcement periods. This effect is consistently observed when high rates of bursting have been generated by the reinforcement procedure, and may be explained by the direct inhibitory (hyperpolarizing) effect of high dopamine concentrations. To protect units from excessive concentrations of dopamine (or other reinforcing drugs), we typically terminate the reinforcement period at the point at which the acquisition curve turns down. In this regard, it may be noted that when compared with more conventional neurophysiological experiments, a rather high pipette concentration of dopamine (1 mM) was required in these studies. However, because drug injections had to be delivered to individual cells in close temporal relation to bursts of activity, it was necessary to use an exceedingly short injection duration (50 ms) and a small volume (0.5 pL). After diffusion to action sites, these minute droplets of drug presumably are diluted to concentrations comparable to those produced in more conventional neuropharmacological studies, in which lower initial concentrations are usually

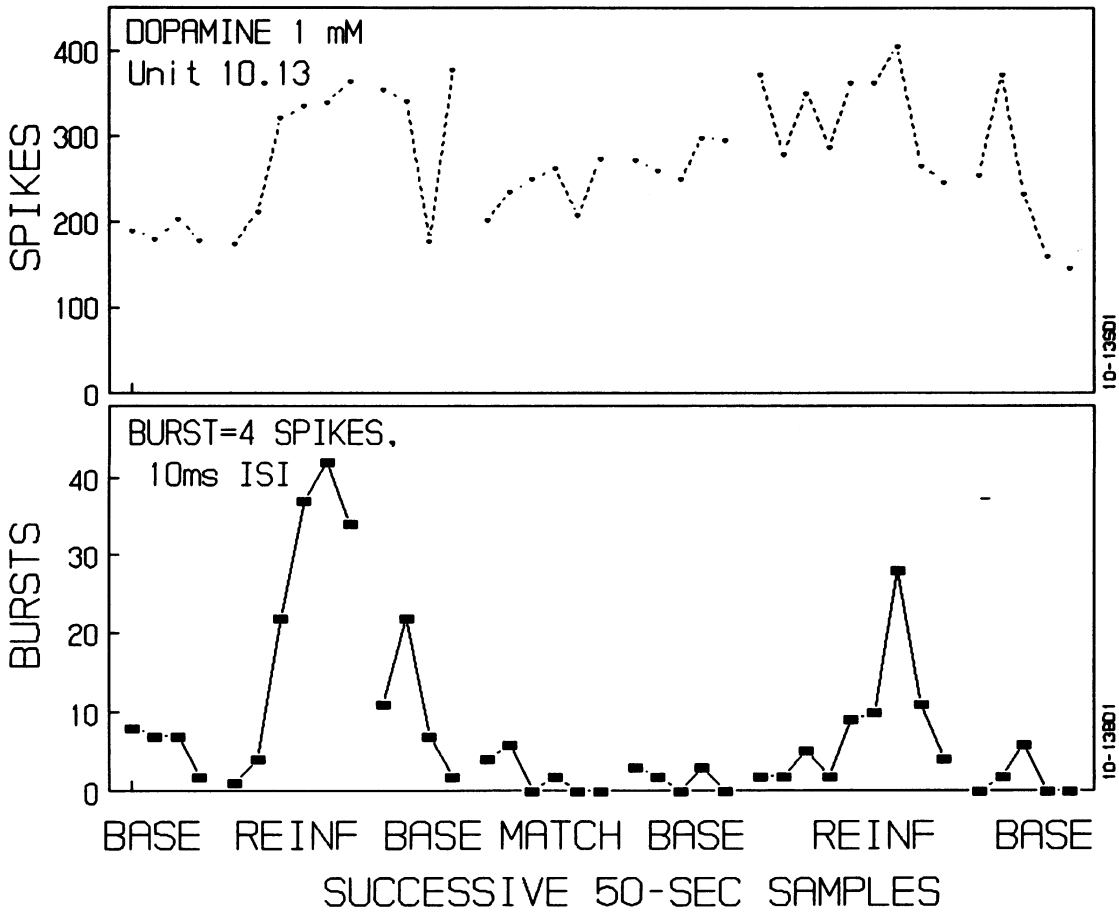

Fig. 2. Operant conditioning of the activity of a CA1 pyramidal cell in a slice of dorsal hippocampus with local injections of dopamine used as reinforcement. The activity of the unit throughout seven phases of a complete experiment is shown. Each point shows the number of bursts (lower graph) or the total number of spikes (upper graph) in successive 50-s samples. Prior to the first baseline phase, a burst criterion of four or more spikes was selected. This criterion gave a burst rate for this unit that never exceeded eight per 50 s in the initial baseline period (BASE). In the reinforcement period (REINF), dopamine HCl (1 mM in 165 mM saline) was applied for 50 ms immediately after each burst; the burst rate increased to a maximum of 42 per 50 s. Following a second baseline period, the same number of dopamine injections was delivered independently of the unit's behavior as a control for possible general stimulant effects (MATCH). The number of injections was matched to that earned during the last six 50-s periods of the reinforcement phase. Bursting rates were increased by contingent dopamine injections during the reinforcement periods but were not increased when the same injections were administered noncontingently in the matched-injection period.

applied in greater volume and for much longer durations.

Dopamine dose-response data are summarized in Figure 3. Plotted here are the mean peak bursting rates obtained in the predrug baseline, reinforcement, and noncontingent phases from experiments in which different groups of cells were given operant conditioning training at various concentrations of dopamine. The dopamine reinforcement function exhibited a sharp peak at 1 mM and fell off abruptly when this optimal concentration was either halved or doubled. These results are consistent with earlier dose-finding pilot work and suggest that the range of effective dopamine doses in cellular operant conditioning is highly constrained. Twenty-four of 37, or 65%, of the cells tested at 1 mM exhibited a pattern of reinforcement-related changes in responding consistent with an operant conditioning interpretation (like that depicted in Figure 2); of the 13 negative cases, 9 cells failed to show

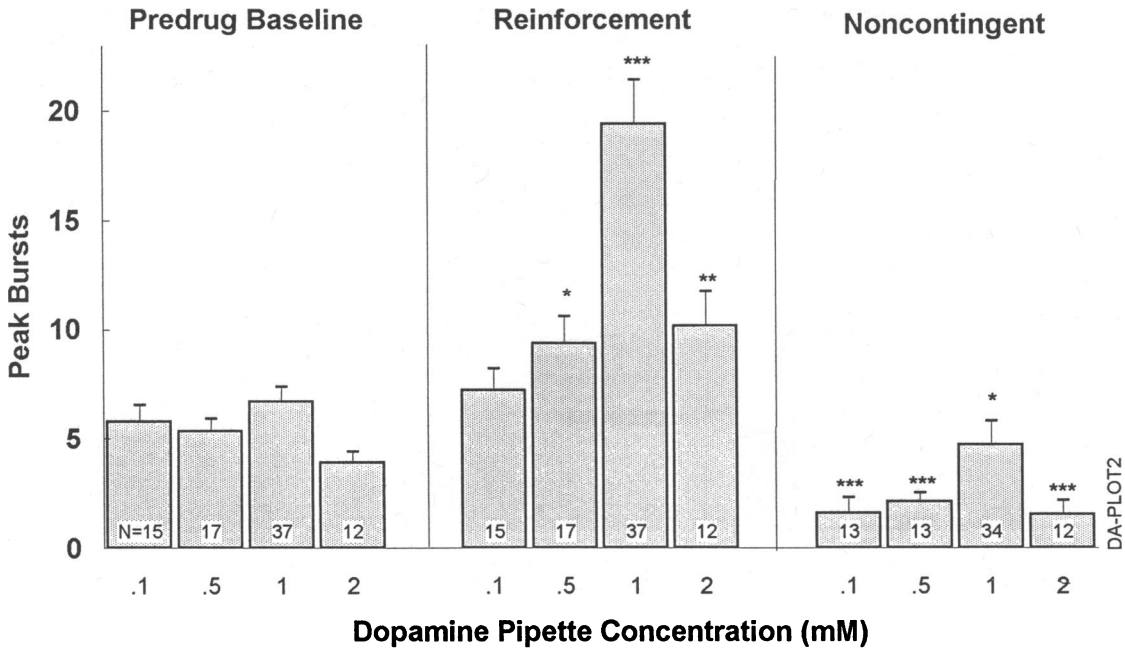

Fig. 3. In vitro operant conditioning of CA1 bursting as a function of dopamine dose. Eighty-one cells received operant conditioning training at various concentrations of dopamine. Bars show mean peak rates of bursting ± *SEM* in the predrug baseline, reinforcement, and matched free injections (NONCONTINGENT) phases of the seven-phase experiment and are calculated by averaging the two highest 50-s bursting scores for each unit in each phase and then averaging over treatment groups. See Figure 2 for a representative experiment obtained from a unit in the 1 mM group. N = number of cells. Differs statistically from predrug baseline (within-groups comparison), $*p < .05$, $**p < .01$, $***p < .001$.

reinforcement-induced increases in bursting above baseline rates, and 4 cells had nonselective increases in bursting after both contingent and noncontingent dopamine applications. However, in the majority of cases, noncontingent dopamine applications at any test concentration failed to facilitate—and often suppressed—CA1 bursting; this observation suggests that nonspecific stimulation is an unlikely explanation of the facilitating action of contingently administered dopamine.

D_2 Reinforcement of CA1 Units

In previous work (Black, 1987; Stein & Belluzzi, 1989), we showed that the D_2-preferring agonist N-0437 was an effective reinforcer of CA1 bursting activity, whereas the D_1 agonist SKF 38393 was ineffective. To establish the specificity of N-0437's action at D_2 receptors, we compared the cellular reinforcing efficacies of its stereoisomers, N-0923 and N-0924, which differ 100-fold in their affinity for D_2 receptors. In the dose range of 1 to 6 mM, only the D_2-active isomer N-0923 was effective as a reinforcer of CA1 bursting; even at the highest concentration of 6 mM, N-0924 was inactive (Figure 4). The reinforcing action of N-0923 (6 mM) was completely blocked by coadministration of the selective D_2 antagonist sulpiride (10 mM) but not by coadministration of the D_1 antagonist SCH 23390 (1 mM) (see Figure 5).

Failure of Glutamate to Reinforce CA1 Bursting

To examine further the role of nonspecific stimulation, we attempted to reinforce CA1 bursting with micropressure injections of the excitatory transmitter glutamate. Unlike dopamine, glutamate is not associated with the reinforcing properties of drugs in the intact organism (Self & Stein, 1992). In contrast to dopamine and N-0923, glutamate (0.05 to 0.5 mM) failed to facilitate CA1 bursting; indeed, both contingent and noncontingent glutamate applications reduced the likelihood of bursts while at the same time increasing the frequency of individual spikes (Figures 6 and 7).

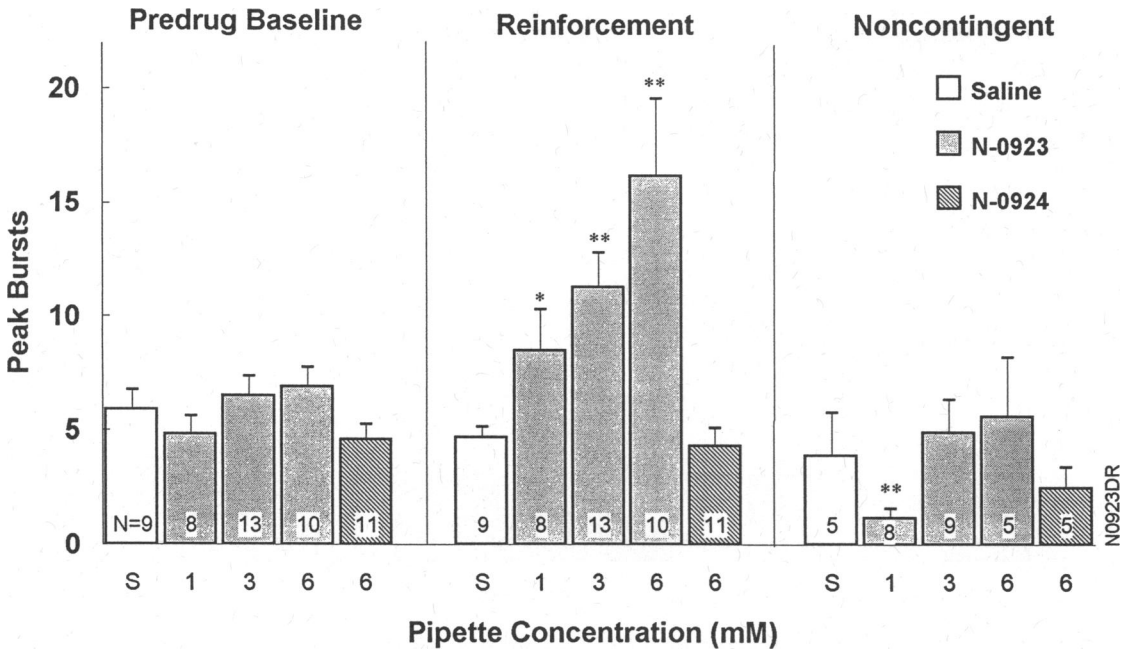

Fig. 4. In vitro operant conditioning of CA1 bursting with the selective D_2 agonist N-0923 and its D_2 inactive isomer N-0924 (see text and Figure 2 for procedure). Bars show mean peak rates of bursting ± *SEM* in the three experimental phases indicated, calculated as described in Figure 3. N = number of cells. Differs statistically from predrug baseline (within-groups comparison), $*p < .05$, $**p < .01$.

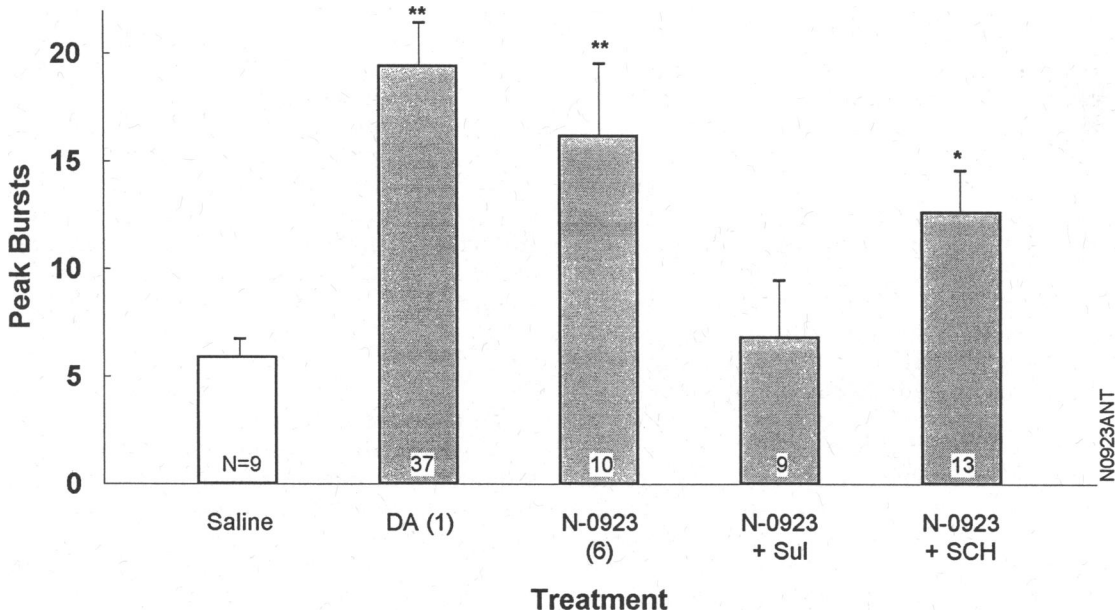

Fig. 5. The selective D_2 antagonist sulpiride, but not the selective D_1 antagonist SCH 23390, blocks CA1 in vitro operant conditioning with the D_2 agonist N-0923 used as reinforcement (see text and Figure 2 for procedure). Cells reinforced with 6 mM N-0923 or 1 mM dopamine (DA) exhibited significantly more bursts than controls reinforced with saline (SALINE). When 10 mM sulpiride was added to the N-0923 solution (N-0923 + Sul), the reinforcing action of N-0923 was abolished. On the other hand, when 1 mM SCH 23390 was added to the N-0923 solution (N-0923 + SCH), the reinforcing action of N-0923 was only slightly diminished. Bars show mean peak bursting scores in reinforcement periods ± *SEM*. N = number of cells. Differs from saline (between-groups comparison), $*p < .05$, $**p < .01$.

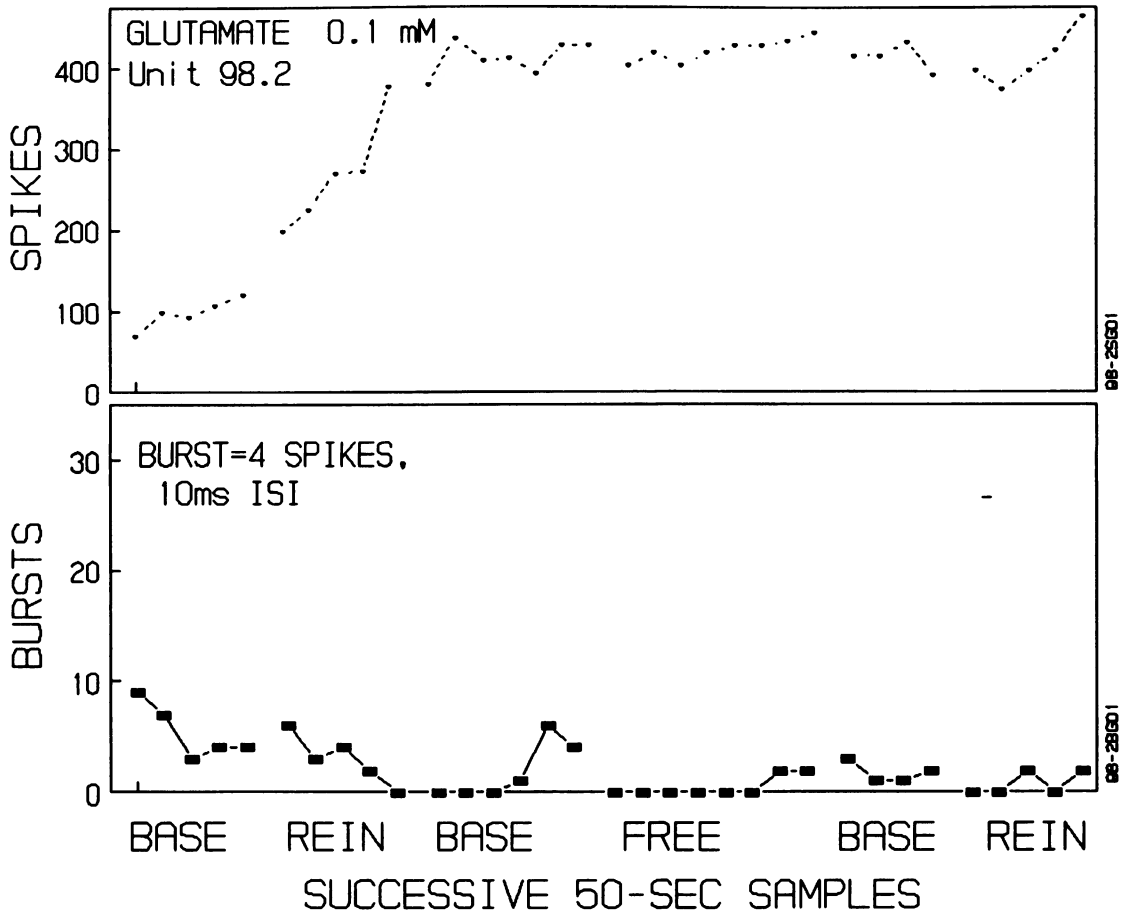

Fig. 6. Unsuccessful operant conditioning of CA1 bursting in a dorsal hippocampal slice with local injections of the excitatory neurotransmitter glutamate (0.1 mM) used as reinforcement. Burst frequencies were suppressed both by contingently applied (REINF) and noncontingently applied (FREE) glutamate, but in both cases total spike counts were quadrupled. For details, see text and Figure 2.

Again, these glutamate data are inconsistent with the interpretation that the dopaminergic agents facilitated hippocampal bursting by nonspecific stimulation. The burst-suppressant action of glutamate was unexpected, but this observation opens the interesting possibility that glutamate might activate mechanisms that are designed to counteract or oppose the dopaminergic reinforcement mechanism (Carlsson, 1988).

Delayed Reinforcement in CA1 Operant Conditioning

In behavioral operant conditioning, it is well established that the effectiveness of a reinforcing stimulus is sharply reduced when its presentation following the operant response is delayed (Renner, 1964). Reinforcement with electrical brain stimulation, by eliminating the necessity for consummatory responses, permits precise temporal control of the interval between the operant response and primary reinforcement. By using this method to deliver primary reinforcement and by taking care to minimize possible sources of conditioned or secondary reinforcement, we found that delays as short as 1 s markedly impeded the acquisition of self-stimulation behavior (Figure 8). Demonstration of a similar delay-of-reinforcement decrement in CA1 operant conditioning would lend support to the hypothesis that cellular and behavioral reinforcement processes are interrelated.

Because N-0437 produced reliable baselines of operant conditioning, this agent was used as the reinforcing substance to study the effects of delayed reinforcement on CA1 operant conditioning. The efficacy of conditioning asso-

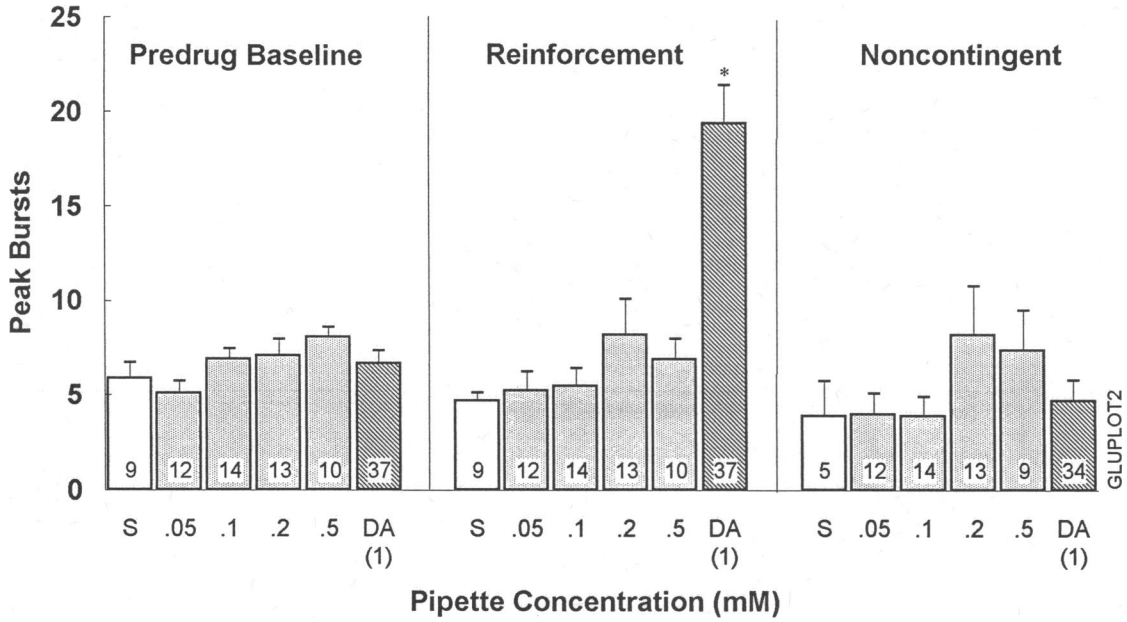

Fig. 7. Summary of glutamate experiments. Bars show peak rates of bursting (calculated as described in Figure 3) of cells given operant conditioning training at various concentrations of glutamate, 1 mM dopamine (DA), or saline (S). N = number of cells. Differs from saline (between-groups comparison), $p < .05$.

ciated with reinforcement delays of 0, 100, 200, or 500 ms was determined in an experiment involving 32 CA1 cells; each cell received operant conditioning at a single reinforcement delay. A delay-of-reinforcement gradient was generated by averaging the peak bursting rates of the test cells in each delay group (Figures 9 and 10). The curve indicates that reinforcements delays exceeding 200 ms largely elim-

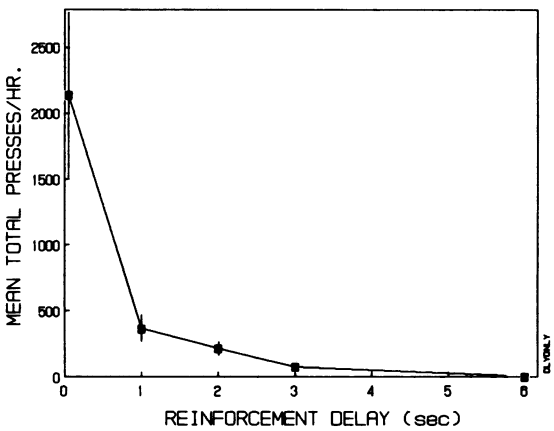

Fig. 8. Acquisition of operant behavior (medial forebrain bundle self-stimulation) as a function of reinforcement delay. Total lever-press responses on Day 1 of training are shown for different groups of rats whose responses were reinforced after the indicated delay. Note that a delay of only 1 s produced a rate decrease of approximately 80%. Vertical lines represent ± *SEM* (after Black, Belluzzi, & Stein, 1985).

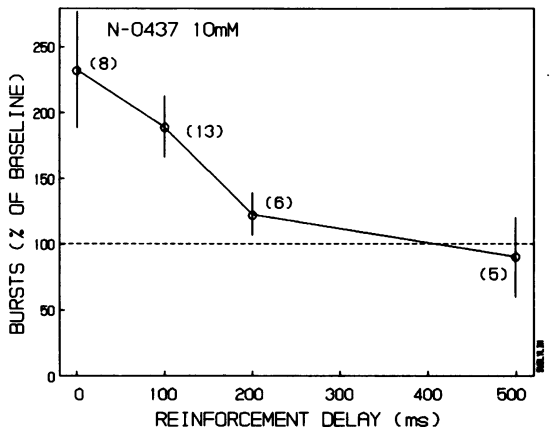

Fig. 9. Delay of reinforcement gradient in cellular operant conditioning with N-0437 (10 mM) as reinforcement. Compare with the behavioral delay-of-reinforcement curve shown in Figure 8. Number of neurons tested at each reinforcement delay is indicated in parentheses. Vertical lines represent ± *SEM* (after Belluzzi & Stein, 1987).

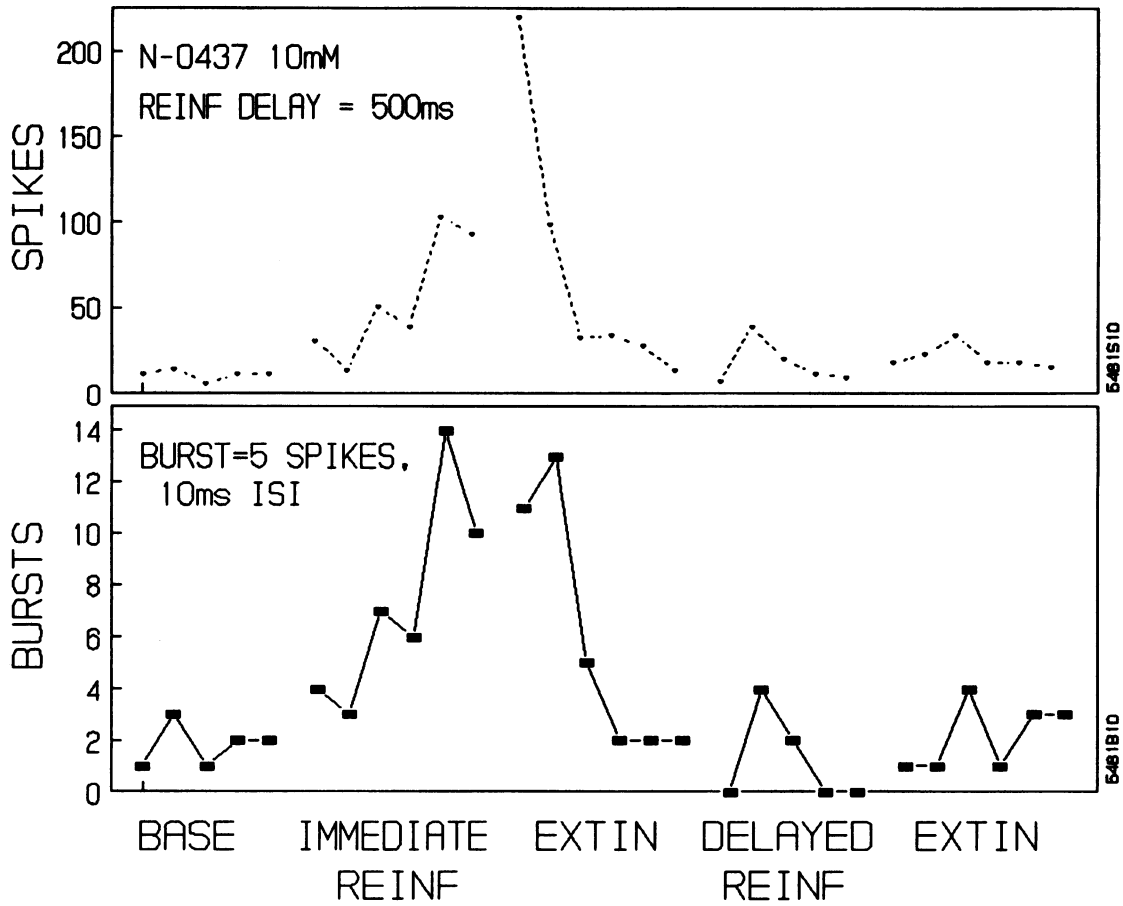

Fig. 10. In vitro operant conditioning experiment for a representative CA1 unit in which immediate and 500-ms delayed applications of the D_2 agonist N-0437 were used as reinforcement. A period of nonreinforced responding (EXTIN) followed each reinforcement (REINF) period. For details, see text and Figure 2.

inate the effectiveness of N-0437 reinforcement in CA1 operant conditioning. The steep gradient of reduced effectiveness of delayed reinforcement again makes it unlikely that pharmacological stimulation or some artifact of the injection procedure accounts for the increase in neuronal bursting when reinforcement is immediate. Rather, the stringent requirement for contingency supports the interpretation that a cellular reinforcement process, which resembles behavioral operant conditioning in many of its properties, has been identified in these experiments.

DISCUSSION

The results described above are consistent with previous work (Stein & Belluzzi, 1988, 1989) that suggested that hippocampal CA1 bursting may be reinforced in vitro by dopaminergic agents such as dopamine itself, cocaine, and certain dopamine D_2 receptor agonists. Here we showed that the reinforcing effects of the dopaminergic agents are dose dependent, are selectively antagonized by D_2 antagonists, and are abolished if the reinforcing injections are delayed for more than 200 ms after the occurrence of the hippocampal bursting response. As noted, a major concern is that these observations may merely reflect the pharmacological excitation or facilitation of neuronal activity rather than the operation of a novel cellular process analogous to operant conditioning. Accordingly, as mandatory evidence of cellular operant conditioning, we have always required that the noncontingent presentation of positive agents will be relatively ineffective; indeed, noncontingent applications

of dopamine, cocaine, and D_2 agonists failed to facilitate and even tended to suppress the bursting of hippocampal CA1 cells.

Nevertheless, it may be argued that solutions of neurotransmitters or drugs applied immediately after a neuronal burst may prevent complete repolarization of the neuron, thereby reducing the transmembrane potential and increasing the likelihood of action potentials occurring in the future. One approach is to monitor the transmembrane potential with an intracellular electrode during cellular conditioning experiments. However, this approach is technically difficult, and, in any case, intracellular recording by other investigators (e.g., Gribkoff & Ashe, 1984; Malenka & Nicoll, 1986) has already shown that the direct effects of dopamine on CA1 membrane potential are generally small and inconsistent. Indeed, in cells with extremely stable membrane potentials, Malenka and Nicoll reported that dopamine usually produced a small *hyper*polarization (consistent with our own findings that noncontingent dopamine applications reduce CA1 bursting). An alternative approach is to test nonspecific depolarizing agents, such as glutamate, for reinforcing activity in the cellular operant conditioning test. In the present experiments, we found that burst-contingent injections of glutamate in a range of doses failed to reinforce CA1 bursting; indeed, this excitatory neurotransmitter reduced the likelihood of bursts while increasing the frequency of firing in a nonbursting mode. These negative findings with glutamate clearly contradict the nonspecific-stimulation hypothesis. And because glutamate is not associated with the reinforcing properties of drugs in the intact organism, these negative results provide further support for the cellular reinforcement hypothesis.

Finally, it may be useful to consider briefly a biochemical signaling mechanism for cellular reinforcement that is suggested by this work. What is required is a plausible mechanism that will elevate the bursting probability of a hippocampal CA1 cell if—and only if—that cell's dopaminergic reinforcement receptors are activated shortly after the occurrence of a burst response. In other words, only recently active cells can be eligible for reinforcement. Three possible ionic markers of recent activity, and hence reinforcement eligibility, are Na^+ influx, K^+ efflux, and Ca^{++} influx. Because calcium influx is a universal signal for activating intracellular biochemistry, it is proposed that calcium influx is the ionic signal that primes the cell for the reinforcement message. If so, the next step is to identify the intracellular event or second messenger that is activated by the dopaminergic reinforcement signal. It is well established that dopamine D_2 receptors (as well as mu-opioid receptors that mediate heroin's reinforcing action and cannabinoid receptors that mediate marijuana reinforcement) all share the ability to couple inhibitory G proteins that inhibit adenylate cyclase activity and stimulate potassium conductance (for review, see Self & Stein, 1992). Hence, if a recently active CA1 cell is exposed to dopamine, inside that cell a brief temporal conjunction of two biochemical signals will be produced—a sudden rise in Ca^{++} and a sudden drop in cyclic AMP and K^+. This conjunction of signals would constitute a biochemical AND function and could reinforce bursting by causing the modification of membrane proteins that control cellular excitability.

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