fered to the same hares in a similar feeding trial. The lack of response to β-sitosterol (Table 3) demonstrates that hares do not avoid all secondary metabolites.

Thus a single identifiable secondary plant metabolite can function as a deterrent to snowshoe hare browsing. The concentrations of PME found in catkins and foliar buds of winter-dormant green alder are sufficient to deter feeding by snowshoe hares. However, the low levels of PME found in internodes suggest that hare preferences for mature over juvenile internodes are controlled by factors other than those leading to avoidance of buds and catkins. Thus green alder may have at least a two-level defense system during winter—defense of growth stages (mature and juvenile internodes) and parts within growth stages (buds and catkins compared to internodes).

JOHN P. BRYANT
GREGORY D. WIELAND
Institute of Arctic Biology, University of Alaska, Fairbanks 99701
PAUL B. REICHARDT
VINCE E. LEWIS
MICHAEL C. MCCARTHY
Department of Chemistry, University of Alaska

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3. In a representative experiment free-ranging and captive hares rejected all of the catkin and bud biomass offered while rejecting 40 percent and none, respectively, of internodes offered.
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8. PME was quantified by exhaustive ether extraction of plant part followed by concentration of the extract to a known volume. A portion of the concentrate was filtered through a Waters Silica Sep-Pak cartridge and spiked with a measured quantity of pinosylvin dimethyl ether as an internal standard. Quantitative analysis was carried out by gas chromatography (OV-101).
9. Supported by NSF grants DEB-8207170 and DEB-7823919.

30 December 1982; revised 11 April 1983

Identification of Presynaptic Neurons by Laser Photostimulation

Abstract. *An optical method involving the use of a laser and a novel fluorescent dye as a photostimulation probe has been developed to identify presynaptic neurons in a large ensemble of cells. Illumination of an extracellularly stained neuron by the laser microbeam evokes action potentials. With this technique an interneuron connecting identified leech neurons was quickly located. The method speeds up the elucidation of neuronal networks, especially when small cells are involved.*

Understanding the cellular basis of any central nervous system function requires mapping the neuronal networks controlling that function. Much progress has been made toward this end, especially in the evaluation of simple networks in some invertebrate ganglia that control behavioral reflexes or more complex behavioral acts such as swimming, feeding, defensive withdrawal, and simple learning (*1*, *2*).

A microelectrode search for the neu-

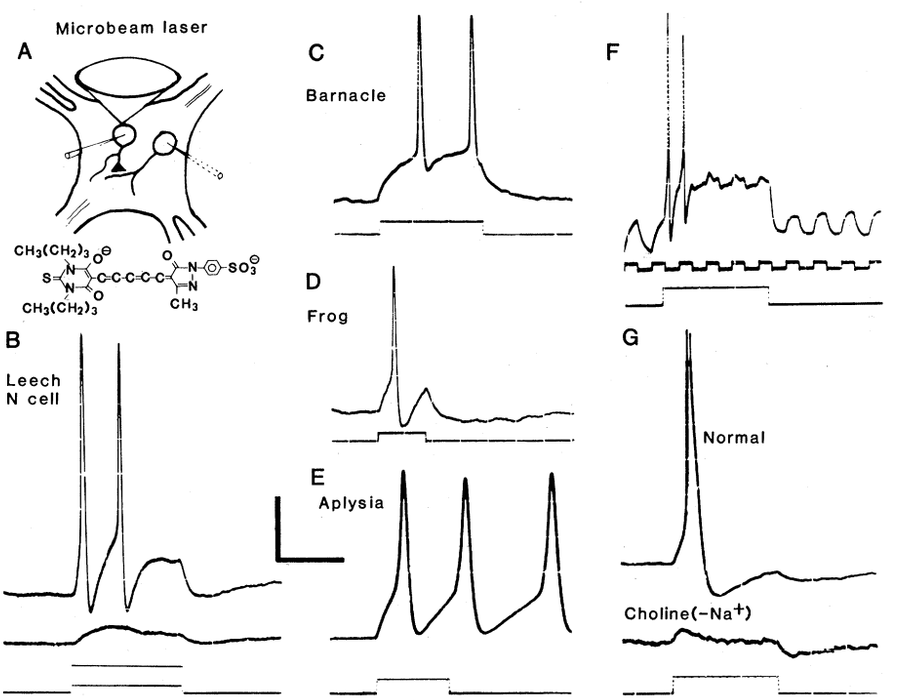

Fig. 1. Photostimulation of nerve cells. (A) Simplified scheme of the experimental arrangement for detecting presynaptic neurons. For more details on the microbeam optics, see Grinvald and Farber (*13*). The structure of RGA-30 is also shown. (B) Subthreshold and suprathreshold stimulation of the leech N sensory cell. Square pulses denote duration and relative amplitude of laser light. Subthreshold stimulation required 75 percent light attenuation. Scale is 16 mV (vertical) and 40 msec (horizontal). (C to E) Photostimulated action potentials from barnacle (C), frog (D), and *Aplysia* (E) neurons. Scale is 40 mV and 40 msec. (F and G) Biophysics of photostimulation. (F) Measurement of membrane resistance during photostimulation of the leech P cell. Injected current (3 nA) is indicated by the small, periodic square pulses. (The time constant for the resulting change in membrane potential is approximately proportional to the membrane resistance.) Note the recovery immediately after illumination. Scale bar is 20 mV and 100 msec. (G) Two photostimulations of the same leech N cell. The first stimulation was in choline-substituted, Na^+-free saline (scale, 4 mV and 40 msec); the second, in normal saline (scale, 15 mV and 40 msec).

rons presynaptic to a given cell may be difficult even in a simple nervous system, for many, often small, neurons must be impaled in pairs. Frequently, even a diligent search for presynaptic neurons leaves the elucidation of networks incomplete (*3*). Therefore there is need for a technique enabling rapid and noninvasive stimulation of specific cells. A solution we have developed is to photostimulate neurons with laser light; rapid scanning of individual neurons with a laser microbeam may be used to find the cells that synaptically affect an electrically monitored neuron.

Arvanitaki and Chalazonitis (*4*) detected light-induced changes in membrane potentials in *Aplysia* neurons, which are often intrinsically photoexcitable, and *Sepia* axons vitally stained with neutral red, methylene blue, or similar compounds. Fork (*5*) demonstrated that some pigmented *Aplysia* neurons can be photostimulated with a high-power laser. However, most neurons, especially in the ganglia of other invertebrates, cannot be stimulated under these conditions.

Our approach has been to design molecular probes that, in the presence of light, depolarize the membrane by forming transient channels (*6*). A photostimulation probe, designated RGA-30, was discovered and an improved analog, designated RH-500, was then synthesized (*7*). The probe binds to the neuronal membrane and acts as a molecular transducer. Illumination of a stained neuron by a laser microbeam 1 to 2 μm in diameter induces an abrupt, reversible depolarization and subsequent action potentials.

Most of our experiments were performed on segmental ganglia of the leech *Hirudo medicinalis*. We also used frog sympathetic ganglia and neurons from the mollusks *Balanus nubilus*, *Aeolidia papillosa*, *Aplysia californica*, and *Limax*. A 50-mW He-Ne laser was used (Spectra Physics model 125A); the power of the laser microbeam on the cell was 0.011 W. In all preparations even long laser light pulses (5 seconds in duration) had no observable effect on the electrical activity or resting potential of the neurons before their incubation with the photostimulation compound. Ganglia were incubated in a normal Ringer solution containing 10 to 100 μ*M* RGA-30 or RH-500. The solution was changed back to normal Ringer after 20 minutes. Staining the neurons did not alter their resting potential or the shape of their action potentials (*8*).

Figure 1, A to E, shows the results of photostimulating nerve cells from four preparations. Stained ganglia required a short light pulse (5 to 60 msec) for suprathreshold stimulation, and the light-induced depolarization frequently appeared identical to the response to a square current pulse. The cells could be restimulated by the laser pulse 1 to 20 times. (When neurons could no longer be photostimulated, they could usually still fire action potentials upon intracellular current injection.) Identified sensory neurons, interneurons, and motoneurons could all be stimulated.

Aeolidia and *Balanus* neurons required 100 μ*M* RGA-30 for photostimulation, whereas 20 μ*M* was sufficient for the leech. *Aplysia* ganglia were even more difficult to stain. In all preparations it was clear that the effect of the illumination is limited to the illuminated area in three dimensions. When the miniature light spot was positioned on a neighboring neuron, the monitored neuron did not fire (*9*).

In an attempt to clarify the mechanism underlying photostimulation, we measured membrane resistance in leech sensory cells during the photostimulation procedure. Figure 1F shows that membrane resistance decreases with the light pulse. When the cell repolarizes, its membrane resistance often returns to 50 to 100 percent of the prestimulus level within 1 minute. To determine the reversal potential, we used two electrodes to adjust the membrane potential and simultaneously measure the laser-induced voltage changes (*10*). The reversal potential was found to be −10 to −20 mV (20 to 30 mV above the resting membrane potential). Such a reversal potential in leech sensory cells indicates that more than one ion is involved in photostimulation. To test the involvement of Na^+ and native sodium channels, experiments were performed with Na^+-free solution and tetrodotoxin (TTX) (Sigma). When choline was substituted for Na^+ in Ringer solution for leech and barnacle preparations, the light-induced initial depolarization was one-fifth to one-tenth that seen with normal saline (Fig. 1G). In experiments in which barnacle ganglia were incubated in

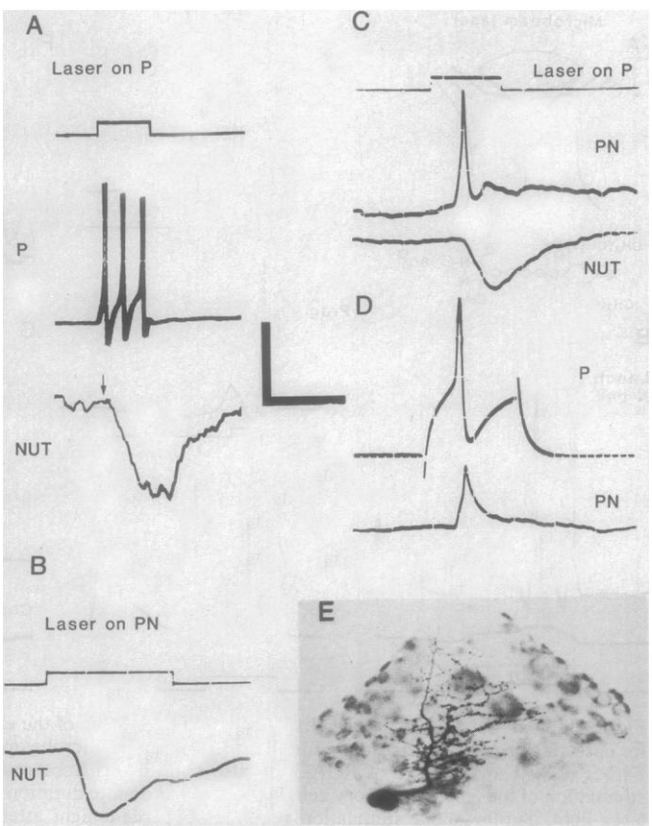

Fig. 2. Detection of the interneuron by scanning of the laser microbeam. (A) Controlled two-microelectrode experiment: the microbeam was positioned over the P cell. Photostimulation resulted in its firing (middle trace) and the subsequent IPSP in the NUT (bottom trace). Photostimulation of the same cell was successfully repeated seven times. The top trace shows duration of the laser pulse. Scale is 20 mV and 100 msec for the P cell and 4 mV and 100 msec for the NUT. The arrow shows the timing of the peak of the first action potential in the P cell (latency, 5 to 6 msec). (B) Discovery of a neuron presynaptic to the NUT, the PN interneuron. Scale is 10 mV and 40 msec. (C) Two-electrode experiment in which the PN interneuron and NUT were impaled. Photostimulation of the medial P cell resulted in firing of the PN neuron, which evoked an IPSP in the NUT after 1 msec (scale, 10 mV and 40 msec for both recordings). In five other experiments, direct stimulation of the PN neuron with a microelectrode evoked an IPSP in the NUT. (D) Direct electrical stimulation of the P cell (scale, 40 mV and 40 msec) evoked an excitatory postsynaptic potential and a subsequent action potential in the PN interneuron (scale, 20 mV and 40 msec). In five experiments identical to those in (C) and (D), the PN interneuron was injected with Lucifer yellow. Its typical morphology is shown in (E) (the small soma is out of focus).

20 μ*M* TTX in order to block sodium channels completely, the depolarization was larger than in Ringer with choline, but half to one-quarter of that in normal Ringer solution. Hence, photostimulation may be attributed to the formation of transient, light-induced, and probably nonspecific channels. However, these channels are small because they exclude choline.

The light pulse in the presence of these photostimulation probes often causes some irreversible photochemical damage. Only a limited number of repetitions (1 to 20, usually more than 3) of the photostimulation were possible. However, the fast recovery of the membrane resistance (Fig. 1F) indicates that the effect of light and the photostimulation probe on the membrane is different from the well-known photodynamic damage (from which recovery is slow or absent) observed with other dyes (*7*). The experiments described below indicate that the photochemical damage does not limit the usefulness of the technique, because even one or two photostimulations are sufficient to discover unknown synaptic connections.

Established neuronal pathways in the leech ganglion were confirmed by photostimulation. Figure 2A shows that a photostimulated medial sensory P cell was indeed synaptically connected to the NUT (cell 251). The same experiment was repeated 25 times, and in 22 experiments a microbeam pulse on the P cell evoked the inhibitory response. More than ten other established synaptic connections, both chemical and electrical, were each confirmed three to ten times by photostimulation.

Further studies of the medial P to NUT connection suggested that it was polysynaptic (*11*). We therefore searched for the unknown interneuron in the medial P to NUT synaptic pathway by using photostimulation. After scanning about 50 cells on the ventral side (*12*) of the ganglion, we discovered a 10-μm-diameter cell that forms an inhibitory synapse with the NUT (Fig. 2B). We have named this cell the PN neuron. It is found in the cluster of small cells posterior to the P cell corresponding in position to cells 51 to 54 (*2*).

While the use of a single electrode and the microbeam was far easier than the use of a pair of microelectrodes, photostimulation is still equivalent only to a stimulating electrode that cannot record. Because precise latencies are unknown, it is more difficult to determine whether monosynaptic or polysynaptic connections are detected. Nevertheless, after fast optical detection of connectivity (as in locating the cell body of a presynaptic neuron), the synaptic connections can be studied in detail with conventional electrophysiological techniques.

We confirmed that the PN neuron is indeed an interneuron in the P to NUT pathway by using pairs of microelectrodes to record synaptic activity between P to PN and PN to NUT (Fig. 2, C and D). In these experiments the PN interneuron was also injected with Lucifer yellow in order to unambiguously identify it by its characteristic morphology (Fig. 2E). Furthermore, when the PN neuron was killed with a 30-second laser pulse the P cell could no longer evoke a synaptic potential in the NUT, but the P to AE motoneuron connection remained functional.

IRA C. FARBER
AMIRAM GRINVALD
Department of Neurobiology, Weizmann Institute of Science, Rehovot, 76100 Israel

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8. With some leech neurons we observed an increased rate of spontaneous firing by T cells but not P or N sensory cells. This phenomenon has been eliminated with the new probe, RH-500 (I. C. Farber *et al.*, unpublished results).
9. Theoretically, the laser microbeam offers excellent spatial resolution in three dimensions: practically, the in-plane resolution is about 2 μm and the out-of-plane resolution is 4 to 8 μm. Thus small neurons can be selectively stimulated without difficulty. However, when very small cells are stimulated, one should control for the possibility of stimulation of neuropil processes or cells in deeper layers. (Excessive staining may lead to a deterioration of the resolution by a factor of ~ 2.)
10. Laser light can modulate the electrical characteristics of the microelectrode tip. To eliminate artifacts it was necessary to minimize the gate leakage current of the recording electrometer.
11. The following observations suggest a polysynaptic pathway: (i) occasional variability in a relatively long (5- to 6-msec) latency of onset, (ii) occasional failure of single action potentials to elicit an inhibitory postsynaptic potential (IPSP), (iii) reversible blocking of the IPSP solution containing 15 m*M* Ca^{2+} and 20 m*M* Mg^{2+}, (iv) occasional stimulation of the P-evoked excitatory response (1 to 2 mV) in the NUT, and (v) failure of the injection of 3 *M* tetraethylammonium ion into the P cell to affect the IPSP recorded from the NUT. Some of these observations differ from those for *Macrobdella decora* [A. L. Kleinhaus and S. Brand, *Comp. Biochem. Physiol.* **70A**, 37 (1981)].
12. An inverted fluorescence microscope can be used to search the dorsal surface for neurons presynaptic to a ventral postsynaptic cell.
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4 April 1983

Nestmate and Kin Recognition in Interspecific Mixed Colonies of Ants

Abstract. *Recognition of nestmates and discrimination against aliens is the rule in the social insects. The principal mechanism of nestmate recognition in carpenter ants (*Camponotus*) appears to be odor labels or "discriminators" that originate from the queen and are distributed among, and learned by, all adult colony members. The acquired odor labels are sufficiently powerful to produce indiscriminate acceptance among workers of different species raised together in artificially mixed colonies and rejection of genetic sisters reared by different heterospecific queens.*

A fundamental proposition of sociobiology, that altruistic behavior can evolve by natural selection only if the beneficiaries of the behavior are related to the donors, requires that helpers somehow direct their aid preferentially toward kin (*1*). Insect societies exhibit an extreme form of altruism in which most colony members do not reproduce at all but instead help others in their reproductive effort. The means by which social insects attempt to distinguish kin from nonkin have recently been subjected to intensive experimental and theoretical analysis (*2–4*). Two principal recognition mechanisms seem likely to occur in highly eusocial ants and bees: each colony member may produce genetically determined odor labels or "discriminators" that are distributed among all nestmates to form a collective colony odor; or the queen may produce discriminators that are distributed to all of her workers (*5*). We here report experimental evidence suggesting that queen discriminators serve as recognition cues in monogynous ants of the genus *Camponotus*. These acquired odor labels are sufficiently