to commissural stimulation was radically changed (Fig. 1c) (*9*). Figure 2 shows that animals with neonatal lesions generate short-latency, large negative responses to commissural stimulation throughout the entire extent of the molecular layer. The maximal response was obtained 200 to 250 μm above the granule cell layer. In terms of latency and wave form, the responses at all levels of the molecular layer were identical to those recorded in the inner molecular layer of normal rats (Fig. 2). These results were replicated in six rats with verified complete entorhinal lesions.

To summarize, the commissural response is found in both the inner and outer molecular layers of the dentate gyrus in adult rats that received a lesion of the entorhinal cortex at an early age (11 days). In normal rats the maximum response to commissural stimulation is found only in the inner molecular layer (Fig. 1, b and c, and Fig. 2).

The most plausible interpretation of these data is that the commissural axons that migrate into the outer molecular layer after neonatal entorhinal lesions do in fact form permanent functional synaptic connections in that region. The similarity of latency and wave form of the potentials in normal animals and those with lesions argues against the abnormally located responses being caused by postlesion pathology. Also relevant to this argument are our studies on changes in the entorhinal system after neonatal commissural lesions. Anatomical experiments have shown that the entorhinal projections are not changed by these lesions (*10*) and we have data showing that the laminar profile of the response to entorhinal stimulation is also unaffected.

We have provided data indicating that the acetylcholinesterase-containing septal projections to the dentate sprout after entorhinal lesions (*11*). The significance of these different forms of postlesion growth in the hippocampus with respect to the behavioral consequences of entorhinal lesions are not clear, but the results reported here, combined with earlier behavioral work (*1*), suggest that abnormal connections that form after brain damage may play a critical role in phenomena such as recovery from brain damage.

GARY LYNCH
SAM DEADWYLER
CARL COTMAN

Department of Psychobiology,
University of California,
Irvine 92664

References and Notes

1. G. E. Schneider, *Brain Behav. Evol.* **3**, 295 (1970).
2. G. Lynch, S. Mosko, T. Parks, C. Cotman, *Brain Res.* **50**, 174 (1973); G. Lynch, B. Stanfield, C. Cotman, *ibid.*, in press.
3. D. C. Goodman and J. A. Horel, *J. Comp. Neurol.* **127**, 71 (1966); G. Raisman, *Brain Res.* **14**, 25 (1969); R. D. Lund and J. S. Lund, *Science* **171**, 804 (1971); R. Y. Moore, A. Bjorklund, U. Steveni, *Brain Res.* **33**, 13 (1971).
4. L. R. Marotte and R. F. Mark, *Brain Res.* **19**, 41 (1970).
5. P. Anderson, T. W. Blackstad, T. Lomo, *Exp. Brain Res.* **1**, 236 (1966).
6. T. Lomo, *ibid.* **12**, 18 (1971).
7. Others (*5, 6*) have shown that as an electrode is moved through the molecular layer, the maximum negative extracellular current produced by stimulation of a given afferent system coincides exactly with the point of maximum innervation by that afferent. Thus, in precisely laminated structures, such negativity is the extracellular reflection of many dendritic excitatory postsynaptic potentials (EPSP's). A plot of the magnitude and polarity of these extracellular potentials versus the vertical position of the electrode is called a laminar profile and can be used to establish the locus of activated synaptic endings. In the present situation the recorded slow-wave potential met the following criteria for an EPSP: (i) It followed high-frequency (100-hz) stimulation. (ii) Fiber potentials corresponding to the "axonal" spikes described by Lomo (*6*) were commonly recorded before the onset of the negative slow potential. (iii) It is unlikely that a volley of incoming presynaptic potentials could generate the source-sink relations of the slow depth responses recorded by our microelectrodes; the extracellular current flow from axons would be perpendicular to that occurring within the dendrites postsynaptically (*5, 6*).
8. Glass micropipettes with a tip diameter of 1 μm and impedance values of 1 to 10 megohms were lowered into the hippocampal formation until the granule cell layer of the dentate gyrus was reached. Potentials were led through a preamplifier to an oscilloscope and averaging computer. Unit recordings were obtained with the use of a high-frequency (0.5 to 10 khz) filter. Stimulation of the CA3 field of the contralateral hippocampus or of the ipsilateral entorhinal cortex was accomplished with glass-coated tungsten microelectrodes (tip diameter of 10 μm). These stimulation electrodes were manipulated until the maximal cell discharges and associated evoked field potentials were obtained within the granule cell layer. The recording electrode was then raised dorsally in 50-μm steps, and eight responses at each successive step were averaged and recorded until the pyramidal cells of the hippocampus (CA1) were encountered by the electrode tip. (Comparable results were obtained when the profile was constructed by lowering the electrode in 50-μm steps from CA1 to the dentate.) The recording procedure was repeated three or four times per animal (all in the same anterior-posterior plane of the dorsal hippocampus). In earlier experiments the recording electrode was moved slightly in a mediolateral plane to aid in locating the ventral extent of the recording tract. At the conclusion of later experiments in this series, a small quantity of fast green dye was ejected from the tip of the microelectrode using negative current of 10 to 15 μa. This provided precise localization of a particular electrode trace. After these procedures animals were killed and the brains were removed and stained with cresyl violet. The position of stimulating and recording electrodes as well as lesion placements were carefully checked and plotted on reconstructions of relevant sections.
9. Recording immediately after surgery was vastly complicated in these experiments by the fact that the rat brains were far from mature in the immediate postlesion period, that is, at 11 days of age.
10. G. Lynch, T. Parks, B. Stanfield, C. Cotman, in preparation.
11. G. Lynch, D. Matthews, S. Mosko, T. Parks, C. Cotman, *Brain Res.* **42**, 508 (1972); C. Cotman, D. Taylor, D. Matthews, G. Lynch, in preparation.
12. Supported by NSF grant GB35315X and NIMH grants R-MH19793-01 and MH19691.

13 November 1972; revised 23 March 1973

Methionine Sulfoximine–Resistant Mutants of Tobacco

Abstract. *Selecting mutants from populations of haploid plant cells cultured in vitro may provide a rapid method for recovering agriculturally useful variants. Mutants of* Nicotiana tabacum *were recovered which were resistant to methionine sulfoximine, an analog structurally similar to methionine. Induction of chlorosis was prevented or less evident in mutant plants that were inoculated with* Pseudomonas tabaci, *a bacterial pathogen which produces a toxin that is a structural analog of methionine. Several mutants show a specific increase in the level of free methionine.*

Recent advances in the somatic cell genetics of higher plants have demonstrated that it is possible to utilize selective techniques to recover mutant individuals from populations of single haploid cells cultured in vitro (*1*). The experiments reported here were designed to pose two questions: (i) Is it possible to select mutants of a higher plant which have an altered response to a pathogen by recovering cells which are resistant to the toxin produced by that pathogen? (ii) Is it possible to increase selectively the level of a nutritionally important component in a plant by selecting mutants resistant to a toxic structural analog of that component? Both of these questions can be resolved by recovering and analyzing mutants of *Nicotiana tabacum* which are resistant to the methionine analog, methionine sulfoximine (MSO). It has been demonstrated by Braun (*2*) that toxin produced by *Pseudomonas tabaci*, the bacterial pathogen which causes the wildfire disease of tobacco, is a structural analog of methionine. Methionine sulfoximine, although not the true bacterial toxin, will elicit an identical response from tobacco leaves, and mutants of *Chlorella vulgaris* resistant to MSO are also resistant to the toxin. Methionine sulfoximine produces a chlorotic halo on tobacco leaves which is similar to the halo induced by the pathogen.

Haploid plants of *Nicotiana tabacum* cv. Havana Wisconsin 38 were generated by anther culture, and the haploid composition of the tissue was confirmed cytologically. Populations of single haploid cells from in vitro cultures of *N. tabacum* and haploid protoplasts from leaf mesophyll cells were obtained and plated as previously described (*1, 3*). Cell density when protoplasts were plated and cells were cultured was always greater than 2×10^3 cells per milliliter.

After isolation, protoplasts or cells were treated with 0.25 percent ethyl methanesulfonate for 1 hour and plated. The cultures were permitted to grow for 2 weeks, overlaid with an equal volume of medium containing 10 m*M* MSO, and incubated for 3 months. Surviving calluses were recovered and placed on medium containing no MSO. Each callus was grown for several months, divided, and retested for resistance to 10 m*M* MSO. Those calluses which retained resistance were diploidized by further culture as calluses and regenerated into whole plants for further genetic and physiological analysis.

An analysis of approximately 2.7×10^7 viable protoplasts yielded 33 presumptive MSO-resistant calluses, and 1.9×10^7 viable cells from in vitro cultures yielded 19 presumptive calluses. Most of these presumed mutants were unstable in expression of the MSO resistance. After further growth of the calluses on medium lacking MSO, 49 of the 52 presumed mutants were found to segregate tissue which was no longer MSO resistant. The phenotype of the unstable tissue exhibits many characteristics common to presumed mutants recovered from animal cell cultures (*4*). Further work focused on the three remaining calluses. Callus 1 was recovered from in vitro cultured cells, while calluses 2 and 3 were recovered from protoplasts. Diploid plants were regenerated from each of the three calluses. Each mutant plant was crossed to a nonmutant plant to give F_1 progeny. The F_1 progeny were self-fertilized to yield an F_2 generation. One hundred F_2 seedlings from each of the three crosses were germinated under sterile conditions on White's medium and tested for resistance to MSO. The results are presented in Table 1. The pattern of transmission of mutant 1 is complex, perhaps best described by the ratio expected from two recessive loci with additive effects, and segregating independently, yielding a 9 : 3 : 3 : 1 ratio. The patterns of transmission of mutants 2 and 3 appear simpler and best explained by single semidominant loci yielding a 1 : 2 : 1 ratio. Since the mutants were derived from mutagenic treatment and there is the possibility of extensive genetic damage, the ratios must be considered preliminary. Crosses between mutants 2 and 3 indicate that they are possibly allelic. Since these two mutants were derived from protoplasts isolated in two different experimental series, they are different mutational events.

Table 1. Growth of 100 F_2 seedlings on medium containing 10 m*M* methionine sulfoximine.

Mutant number	No growth	Slight growth	Normal growth
1	59	37	4
2	31	51	18
3	36	42	22

Figure 1 demonstrates the reaction of the leaves upon inoculation by *P. tabaci* or application of MSO. After infection with *P. tabaci* the susceptible plant forms chlorotic halos that surround a brown necrotic spot at the site of inoculation. Methionine sulfoximine causes a similar reaction except that the necrotic spot is not as pronounced. Inoculation of all three homozygous mutant plants and a variety of *N. tabacum* carrying a naturally occurring genetic resistance (Burley 21) (*5*) does not lead to production of the characteristic chlorotic halo. Leaves of mutant 1 do show a definite necrotic spot after infection. Mutants 2 and 3 show small dark areas at the site of infection with *P. tabaci*. Burley 21 is unaffected by infection. The small dark areas on mutants 2 and 3 are similar to the blackfire disease caused by *P. angulata*. This observation is quite striking since *P. angulata* is considered by many plant pathologists (*6*) to be a variety of *P. tabaci* which does not produce toxin. All three mutant types are susceptible to infection by *P. angulata*, while Burley 21 is resistant. Under the experimental conditions used in this work, mutants 2 and 3 are distinctly more resistant to the effects of infection by *P. tabaci* than is the

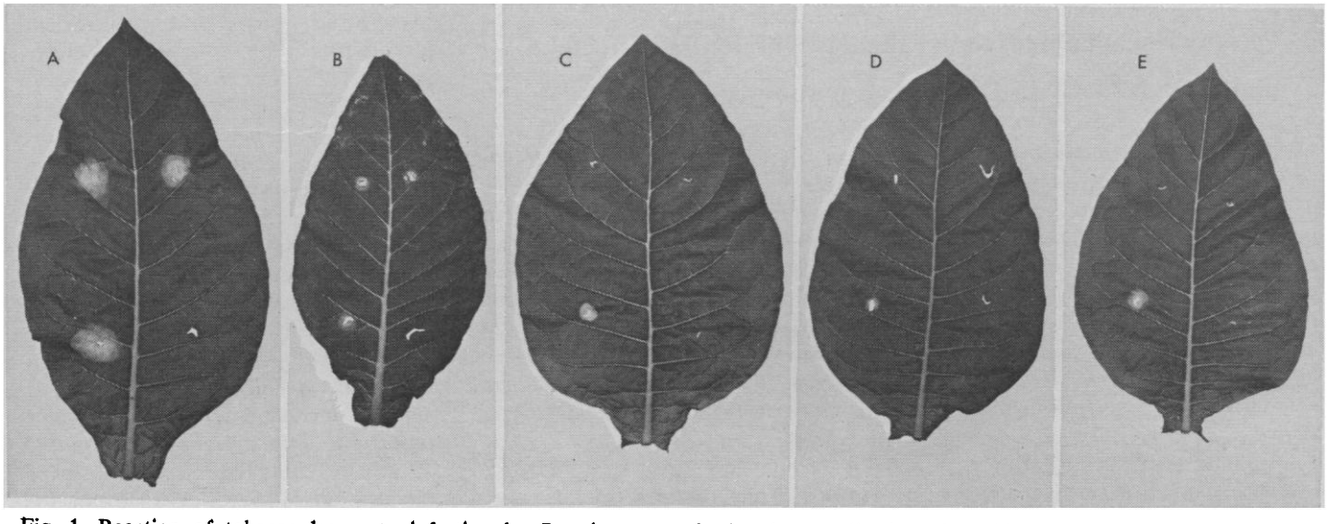

Fig. 1. Reaction of tobacco leaves to infection by *Pseudomonas tabaci* and to application of methionine sulfoximine (MSO). (A) Control leaf from a wild-type plant; (B) leaf from mutant 1; (C) leaf from mutant 2; (D) leaf from mutant 3; (E) leaf from cv. Burley 21 which carried a naturally occurring resistance to *P. tabaci*. The leaves were inoculated at two points in the apical region with 0.1 ml of a 3- to 4-day-old culture of *P. tabaci* in nutrient broth. One-tenth milliliter of a 0.1 m*M* solution of MSO was applied to the left side of the basal portion of the leaf. Uninoculated nutrient broth was applied to the right basal region. Leaves were examined for their reaction after 6 days.

Table 2. Concentrations of certain free amino acids in tobacco leaves. Concentrations are in nanomoles per gram, fresh weight. Fully expanded young leaves with veins removed were homogenized at room temperature. An equal volume of 10 percent trichloroacetic acid was added to the homogenate and then centrifuged. The acid-soluble supernatant was run on an amino acid analyzer. Each value was calculated from three replicates. Level of glycine, alanine, and proline are included to demonstrate that the increases in methionine in mutants 2 and 3 are specific to that amino acid.

Tobacco	Methionine	Glycine	Alanine	Proline
Havana Wisconsin 38	0.4 ± 0.2	1.3 ± 0.3	1.8 ± 0.3	0.3 ± 0.1
Mutant 1	.3 ± .2	1.4 ± .3	1.7 ± .5	.4 ± .2
Mutant 2	1.9 ± .5	1.7 ± .5	2.0 ± .4	.5 ± .2
Mutant 3	2.4 ± .6	1.2 ± .2	1.5 ± .3	.4 ± .2

wild-type variety from which they were selected. The naturally occurring resistance of Burley 21 is superior to that of the mutants. The failure to observe chlorosis in the mutant plants is due primarily to resistance to the action of the toxin, for neither bacterial multiplication nor toxin appearance is inhibited in the mutants.

The level of free amino acids in young, fully expanded leaves of wild-type and mutant tobacco is presented in Table 2. Mutants 2 and 3 both show significant specific increases in the level of free methionine. Free methionine levels are also increased in callus cultures and stem and root tissue in these mutants. The methionine content of the total proteins of these tissues is not significantly increased.

Even though MSO and the toxin produced by *P. tabaci* resemble methionine structurally, current evidence suggests that they may interfere with the enzyme glutamine synthetase rather than directly with methionine metabolism (*7*). However, levels of glutamine are not significantly altered in the leaf tissue of the mutant plants. Elevated intracellular levels of methionine may possibly protect against MSO uptake (*8*).

PETER S. CARLSON

Department of Biology,
Brookhaven National Laboratory,
Upton, New York 11973

References and Notes

1. P. S. Carlson, *Science* **168**, 487 (1970).
2. A. C. Braun, *Phytopathology* **45**, 659 (1955).
3. P. S. Carlson, H. H. Smith, R. D. Dearing, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 2292 (1972).
4. M. Harris, *J. Cell. Physiol.* **78**, 177 (1971).
5. E. E. Clayton, *J. Hered.* **38**, 35 (1947).
6. G. B. Lucas, *Diseases of Tobacco* (Scarecrow Press, New York, ed. 2, 1965).
7. S. L. Sinden and R. D. Durbin, *Nature* **219**, 379 (1968).
8. F. Meins and M. L. Abrams, *Biochim. Biophys. Acta* **266**, 307 (1972).
9. This research was partially supported by the Atomic Energy Commission and partially by PHS grant GM 18537. I thank Rosemarie D. Dearing for excellent technical assistance, Drs. C. C. Litton and H. A. Skoog for providing cultures of *Pseudomonas tabaci* and *P. angulata*, and my colleagues at Brookhaven for many stimulating discussions.

9 February 1973

Δ^9-Tetrahydrocannabinol and Ethanol: Differential Effects on Sympathetic Activity in Differing Environmental Setting

Abstract. *Serum dopamine β-hydroxylase activity, a useful biochemical index of peripheral sympathetic nervous activity, was measured in rats treated with Δ^9-tetrahydrocannabinol or ethanol or both substances. After 7 days of treatment with either substance, serum dopamine β-hydroxylase activity decreased significantly. Combined treatment with both agents enhanced the effects of each given alone. In rats subjected to immobilization stress, treatment with Δ^9-tetrahydrocannabinol appeared to potentiate the stress-induced increase in serum enzyme activity. Treatment with ethanol, with or without Δ^9-tetrahydrocannabinol, effectively blocked this increase in enzyme activity. These results show that both substances have significant effects on the sympathetic nervous system which are critically influenced by environmental setting.*

The social use of marijuana has been guided more by folklore than by scientific knowledge. Street lore suggests that the effects of marijuana can be enhanced by the simultaneous use of alcohol (especially in the form of sweet wines). While this practice may seem well based from what we know of the pharmacological effects of these agents, little biochemical evidence for their interaction is available. The synthesis of Δ^9-tetrahydrocannabinol (Δ^9THC), believed to be the major active substance in marijuana, has permitted a more systematic study of the interactions of Δ^9THC with other drugs. We report the results of one such study. Using a simple biochemical marker, dopamine β-hydroxylase (DBH) in serum, we found that Δ^9THC and ethanol have profound effects on the sympathetic nervous system and that these effects (and their interactions) are critically influenced by the environmental setting in which the drugs are given.

Dopamine β-hydroxylase catalyzes the formation of the neurotransmitter noradrenaline from dopamine. The enzyme is present in the serum of man and other mammalian species (*1*). Serum DBH arises mainly from sympathetic nerve terminals (*2*) and is elevated by stress of various types (*3*). The activity of this enzyme is therefore a useful biochemical index of the activity of the sympathetic nervous system and provides a convenient means to study the effects of drugs and their interactions on sympathetic nervous activity. We investigated the effects of Δ^9THC and ethanol on sympathetic nervous activity of normal rats and rats subjected to repeated immobilization, a procedure used as an experimental model of stress in animals (*4*).

Sprague-Dawley rats weighing about 200 g were divided into four groups. One group (12 rats) received Δ^9THC (20 mg per kilogram of body weight) in combination with ethanol (400 mg/kg); another group (16 rats) received Δ^9THC (20 mg/kg) in a mixture of homologous serum and polyethylene glycol (3 : 1, by volume); and two groups (12 rats each) received either ethanol (400 mg/kg) or serum–polyethylene glycol mixture alone. Half the rats in each group were immobilized with a specially designed restraining device (*4*) for 2 hours daily for seven consecutive days. The remaining rats in each group served as nonimmobilized controls. The Δ^9THC was either dissolved in ethanol or suspended in serum–polyethylene glycol. All animals were injected daily (100 μl of drug or vehicle subcutaneously) at the same time, and immobilization was started 45 minutes after the injection. Baseline blood samples were drawn from the tail vein in all animals before the experiment was begun, and samples were also taken 20 hours after the fourth and seventh immobilization periods. Immobilized and nonimmobil-