Squires' study (170 μsec for wave V). It is clear, therefore, that investigators should control, or at least monitor, body temperature in studies of ABR latency (*23, 24*).

The mechanisms underlying the correlation of body temperature and ABR latency are unknown. Temperature might affect ABR latency by retarding receptor activity, neuronal conduction, or synaptic transmission at any point up to and including the inferior colliculus (*18, 19*). By varying stimulus and organismic variables, and observing corresponding changes in the rhythm, it may be possible to determine the locus of the effects. The ABR's may thus be useful for investigating the effects of body temperature on neural activity in the human.

NOEL K. MARSHALL
EMANUEL DONCHIN*
Cognitive Psychophysiology Laboratory, Department of Psychology, University of Illinois, Champaign 61820

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4. Wave I probably reflects eight nerve action potentials; waves II to V are presumed to be generated by the cochlear nucleus, superior olive, lateral lemnicus, and inferior colliculus, respectively (*3*).
5. The mean (± standard deviations) latency (in milliseconds) of waves I to V observed in our laboratory were 1.79 ± 0.11, 3.00 ± 0.14, 3.97 ± 0.14, 5.17 ± 0.21, and 6.00 ± 0.16, respectively, at 64-dB hearing level based on 80 observations (eight subjects, ten recordings). The interpeak latencies for waves I to III and III to V were 2.18 ± 0.11 and 2.02 ± 0.29, respectively. All components of the ABR can be observed at the appropriate latencies.
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11. Because of equipment malfunction, data from the tenth recording session of subject 2 were unusable. A session at 1600 hours was therefore added for a total of 17 recordings.
12. Sessions lasted 30 to 45 minutes. The subjects were instructed to relax and sleep if they could during the recording period. M. Amadeo and C. Shagass [*Psychophysiology* **10**, 244 (1973)] reported that there is little or no change in the amplitude and latency of the ABR's from waking to sleep.
13. Hearing level threshold was established on the basis of the mean level of intensity at which three staff members could no longer detect the presence of continuous white noise. The hearing level for click stimuli delivered at 11 per second was established in a similar manner.
14. The latency determinations were made by a trained observer, who did not know the purposes of the experiment. Peaks were identified in a computer display of the ABR's by positioning a cursor at the peaks. The latency value at the cursor was identified by the computer. Latency was determined with 0.02-msec resolution.
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* Address reprint requests to E.D.

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GABA Analogues Activate Channels of Different Duration on Cultured Mouse Spinal Neurons

Abstract. *Voltage-clamp recordings from mouse spinal neurons grown in culture were used to study the membrane current fluctuations induced by 12 substances structurally similar to γ-aminobutyric acid (GABA). Fluctuation analysis provided estimates of the electrical properties of the elementary events underlying these responses. Estimates of the mean conductance of channels activated by all of the substances except glycine did not differ significantly from that estimated for GABA, whereas mean durations of agonist-activated channels all differed significantly from that found for GABA. The results indicate that all of the substances tested except glycine activate channels of similar conductance but of different durations.*

γ-Aminobutyric acid (GABA) is an amino acid endogenous to a variety of nervous systems, where it is thought to function as a neurotransmitter, inhibiting excitability through an increase in Cl^- ion conductance (*1*). This inhibitory action, shared by a number of naturally occurring amino acids, can also be mimicked by a variety of synthetic substances thought to resemble the GABA molecule stabilized in different configurations (*1*). The advent of fluctuation analysis and single-channel recording techniques has shown that the elementary events associated with the macroscopic effects of neurotransmitters can be described quantitatively in terms of the electrical dimensions of ion channel events (*2*). We have applied fluctuation analysis to the membrane responses induced by GABA, glycine, taurine, and a number of synthetic substances in cultured mouse spinal neurons and report that all of the agonists except glycine activate ion channels of similar conductance but variable duration.

Mouse spinal neurons were grown in tissue culture according to methods previously described (*3, 4*). At the time of the experiment, normal maintenance medium was replaced by Hanks balanced salt solution containing 1 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, and 1 μ*M* tetrodotoxin to eliminate all evoked synaptic activity and allow clearer study of the pharmacologic responses. The recording medium and the drug solutions were all buffered to *p*H 7.4 with 25 m*M* Hepes. Intracellular recordings under voltage clamp were made at room temperature (23° ± 1°C) on the modified stage of an inverted phase microscope through the use of two

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electrodes filled with 3*M* KCl. The use of these electrodes changed the inversion potential of the amino acid responses from about −60 mV to between −20 and −10 mV (*3*), and this value remained stable throughout the experiment. GABA, glycine, and taurine (Sigma) were applied to individual cells by iontophoresis from pipettes containing 1*M* solutions or by pressure from pipettes containing 50 μ*M* amino acid dissolved in the same medium used for recording. The synthetic analogues of GABA used were γ-amino-β-hydroxybutyric acid (GABOB), imidazole acetic acid (IAA), δ-amino-valeric acid (DAV), muscimol, β-guanidinopropionic acid (BGP) (all from Sigma); 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP), piperidine-4-sulfonic acid (P4S), and isoguvacine (provided by P. Krogsgaard-Larsen, Copenhagen, Denmark); 3-aminopropanesulfonic acid (3APS) and *trans*-4-aminocrotonic acid (TAC) (provided by G. A. R. Johnston, Canberra, Australian Capital Territory). All of the analogues were applied by pressure from pipettes containing 20 μ*M* to 1 m*M* agonist. On each cell, GABA responses were compared with those of one or more other agonists through the use of concentrations sufficient to permit fluctuation analysis and indicate the driving force underlying the membrane response. Fluctuation analysis was carried out on responses obtained with the membrane potential clamped to −60 or −70 mV, since this holding potential provided a driving force of 40 to 50 mV.

Results were obtained from 61 spinal cord neurons. All of the substances examined produced membrane responses that inverted at the same potential as GABA (*3*, *5*), suggesting that, like GABA (*3*), the analogues increase the Cl^- ion conductance of these neuronal membranes. The membrane current responses to all of the substances were accompanied by the appearance of additional membrane current variance (Fig. 1), which was directly proportional to the increase in membrane current (not shown), suggesting that similar elementary mechanisms underlie responses of different amplitude. The variable decline in amplitude seen during some of the current responses probably reflects desensitization, since the inversion potential, and thus the driving force measured during the declining phase of these responses, did not change (*5*). The agonist-induced variance was analyzed on the assumption that it reflects the statistical variation in the number of agonist-activated open ion channels about a mean level of open channels and, further, that there is a low probability of a channel's being open (*6*). The average conductance, γ, of a single channel can then be estimated from the relation $\gamma = \sigma^2/[\Delta I \times (V_c - E_r)]$, where σ^2 is the variance associated with the agonist-induced increase in membrane current (ΔI), E_r is the reversal potential of the agonist response, and V_c is the holding potential under voltage clamp (*2*). Estimates of γ for all of the substances studied except glycine were not significantly different from the value obtained for GABA (Table 1). Glycine, as reported previously (*5*), activated channels whose conductance was approximately twice that of GABA.

Estimates of the average duration of the open channels were obtained from power spectral density plots of current fluctuations. For holding potentials in the −60- to −70-mV range, all the agonist spectra included in this study were approximated by a single Lorentzian term, $S(f)/S(0) = 1/[1 + (f/f_c)^2]$, where $S(f)$ denotes the spectral density as a function of frequency f, and f_c is the half-power frequency of the spectrum (Fig. 2) (*2*). If we assume a relatively simple model of channel operation, the single Lorentzian character of the spectra suggests that the agonists each open a single population of channels whose durations are exponentially distributed about an average duration, given by the equation $\tau = 1/2\pi f_c$ (*2*). The average duration of channels activated by the various substances ranged from 2.3 msec for taurine to 76.3 msec for muscimol. Although absolute values for calculated channel duration varied from cell to cell, each estimated duration differed significantly from that of channels activated by GABA (Table 1). For all analogues tested, channel durations were, as for GABA (*7*), insensitive to changes in membrane potential over the −50- to −90-mV range.

We have applied fluctuation analysis to membrane responses elicited in cultured mouse spinal neurons by natural

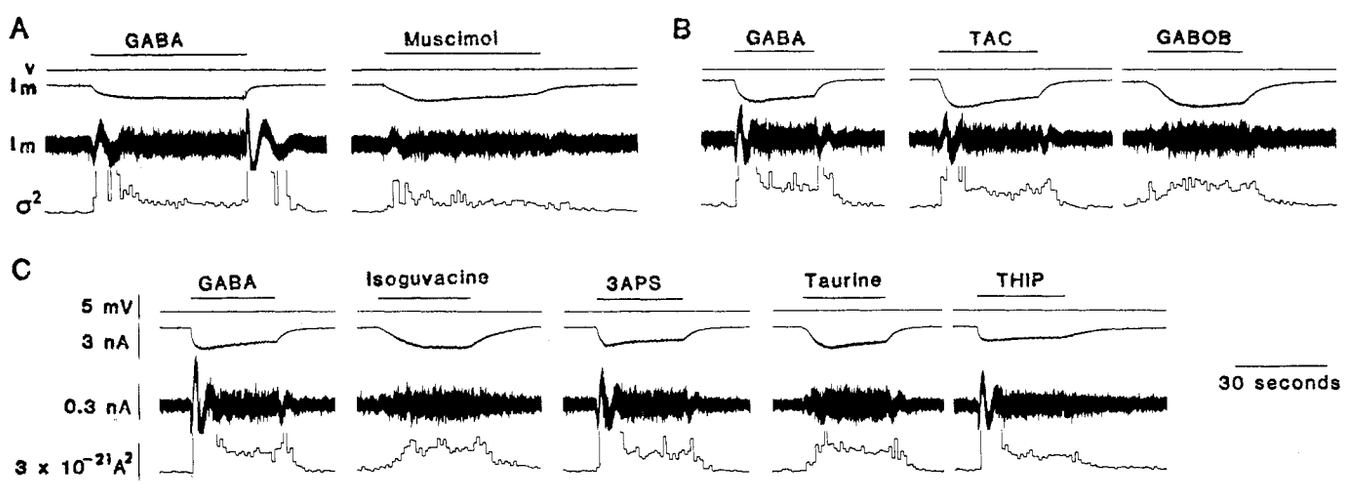

Fig. 1. Membrane response to GABA analogues on cultured mouse spinal neurons. The figure contains three sets of pen-recorder traces showing intracellular recordings from three different cells made through the use of KCl microelectrodes. The cells in (A) and (B) were voltage-clamped to −60 mV, and that in (C) was held at −70 mV. Analogues were applied by pressure from closely positioned micropipettes containing 20 μ*M* muscimol, 50 μ*M* TAC, 200 μ*M* GABOB, 50 μ*M* GABA, 1 m*M* isoguvacine, 200 μ*M* 3APS, 1 m*M* taurine, and 200 μ*M* THIP. The GABA response in (A) was evoked by iontophoretically applied GABA. Application periods are marked by bars. The traces show membrane voltage (V); low gain, d-c–coupled membrane current with inward current downward (I_m, upper trace); high gain, a-c–coupled membrane current (I_m, lower trace); and membrane current variance (σ^2). Traces in (A) were recorded with a bandwidth of 0.1 to 100 Hz, while those in (B) and (C) were recorded with a bandwidth of 0.2 to 500 Hz. All of the analogues reversibly increased membrane current and variance. The multiphasic waves apparent in a number of the a-c–coupled traces are a result of the settling of the filter in response to the relatively rapid change in membrane current.

and synthetic substances structurally related to GABA and have estimated the properties of the Cl^- ion channel events underlying the responses. The principal finding is that for all of the substances tested except glycine, channel conductance varies much less than channel duration. As previously reported (*5*), γ for glycine-activated channels was approximately twice that for GABA. Although the naturally occurring amino acid taurine evoked an elementary event whose conductance was similar to GABA's, it was about 1/20 as potent as GABA on a molar basis in producing responses comparable to GABA.

We cannot be certain whether the substances we used are acting at the same or different receptor sites. GABA responses in these cells can be modulated by drugs that do not alter glycine responses (*8*), which suggests that these two amino acids do not act at the same site. Furthermore, glycine does not compete with GABA in binding assays carried out with frozen rat synaptic membranes, whereas all of the other substances we used do (*9, 10*). Thus, the synthetic analogues and taurine appear capable of interacting with some GABA receptor sites. We have found that the mean duration of membrane channels opened by an agonist is highly correlated with the concentration of that agonist required to displace 50 percent of bound GABA from frozen rat synaptic membranes (*11*). The high correlation suggests that the biochemical and electrophysiological assays are measuring a common variable that reflects the interaction of the agonists with receptors for GABA. If we assume that taurine and the synthetic substances are indeed all acting at GABA receptors on cultured neurons, the results demonstrate that the kinetics of GABA-receptor-coupled anionic channels, and apparently not their conductance, depend on the structure of the agonist. A similar conclusion has been reached for cationic channels activated by agonists at cholinergic (*12*) and glutamate-sensitive synapses (*13*). Our results together with further analysis of other structures interacting with GABA receptors coupled to Cl^- ion channels should provide insights into the structural requirements for Cl^- ion channel activation and the relationship between agonist binding and channel kinetics. Furthermore, these data should allow assessment of how well the binding and the channel-activating characteristics of GABA analogues can account for the relative potencies of the substances when applied in vivo.

Table 1. Properties of chloride-permeable channels activated by naturally occurring and synthetic substances structurally related to GABA in cultured mouse spinal neurons (shown as means ± standard deviations). Channel conductance was estimated in picosiemens.

Agonist	Channel conductance (pS)	Channel duration (msec)	Observations (*N*)	Cells (*N*)
GABA	15.4 ± 3.5	30.4 ± 2.2	380	61
Glycine	32.3 ± 8.0*	6.2 ± 1.3*	71	3
Taurine	15.5 ± 3.0	2.3 ± 0.3*	17	3
THIP	16.9 ± 5.5	13.0 ± 4.0*	28	6
Isoguvacine	17.5 ± 1.4	16.5 ± 2.5*	13	3
DAV	18.6 ± 6.0	9.0 ± 1.4*	11	3
IAA	16.0 ± 4.5	5.5 ± 1.0*	32	3
TAC	16.6 ± 3.3	24.8 ± 5.5*	34	4
BGP	13.4 ± 2.3	8.9 ± 1.4*	29	3
GABOB	16.3 ± 4.2	15.3 ± 3.7*	26	4
Muscimol	17.9 ± 3.3	76.3 ± 14.9*	79	14
3APS	17.5 ± 3.4	9.0 ± 1.8*	48	7
P4S	16.2 ± 1.4	8.3 ± 0.5*	26	4

*Significantly different from GABA, *t*-test ($P < .001$).

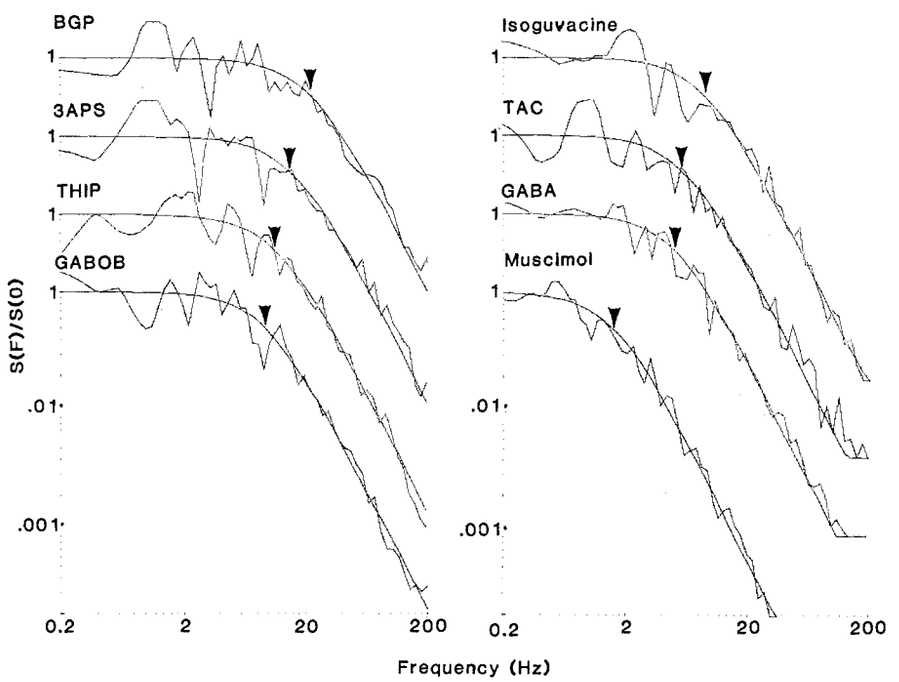

Fig. 2. Power spectral density plots of membrane current fluctuations evoked by GABA and its analogues on cultured mouse spinal neurons. The spectra were obtained as the difference between spectra derived from baseline fluctuations and those calculated during agonist applications. The spectra have been normalized by dividing each spectral density point, $S(f)$, by the zero frequency asymptote of the spectrum, $S(0)$, and fit by a least-squares analysis. Each spectrum is closely approximated by a single Lorentzian equation (solid lines). The cutoff frequency, f_c (downward arrowhead), is related to mean channel duration, τ ($\tau = 1/2\pi f_c$). Values of f_c and the corresponding τ for the agonists are as follows: GABOB ($f_c = 9.4, \tau = 17$ msec), 3APS (15.4, 10.3), THIP (11.7, 13.6), BGP (21.4, 7.4), isoguvacine (8.6, 18.4), TAC (6, 26.6), GABA (5.1, 31.2), and muscimol (1.8, 86.8). The spectra for GABA, THIP, isoguvacine, and 3APS were all obtained from the same cell.

JEFFERY L. BARKER
DAVID A. MATHERS
Laboratory of Neurophysiology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

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Genetic Evidence That Protein Synthesis Is Required for the Circadian Clock of *Neurospora*

Abstract. *Small doses of cycloheximide given at intervals (pulses) cause phase shifts of the circadian clock of* Neurospora. *The effects of this drug on the clock are mediated through its inhibition of protein synthesis, since two cycloheximide-resistant mutants whose 80S ribosomes are resistant to cycloheximide showed no phase shift after exposure to the drug.*

Metabolic inhibitors have been used to analyze molecular mechanisms underlying circadian rhythmicity in order to identify pathways of cellular biochemistry necessary for clock function. The rationale of this approach is as follows. If continuous application of the inhibitor alters the period length of the rhythm or if a pulse alters its phase, then the cellular target of the inhibitor is presumed to be important for the clock.

However, unequivocal identification of the target of clock alteration is difficult because most drugs affect multiple sites in the cell. For example, cycloheximide (CHX) inhibits cytosolic (80*S*) protein synthesis in eukaryotes; continuous application of this drug alters the period length of the clock in *Euglena* (*1*), and pulses cause phase shifts in *Acetabularia* (*2*), *Aplysia* (*3*), and *Gonyaulax* (*4, 5*). In addition, pulses of other inhibitors of protein synthesis, such as puromycin and anisomycin, cause phase shifts in *Acetabularia* (*6*) and *Aplysia* (*3, 7*). These results seem to suggest that protein synthesis in 80*S* ribosomes is necessary for clock function. However, under some conditions CHX inhibits mitochondrial function (*8*) and transport of amino acids and sugars (*9*), and it has been suggested (*4, 10*) that the effects of CHX on circadian rhythmicity are mediated through some mechanism other than inhibition of protein synthesis.

In one study in *Neurospora*, CHX did not alter the period length of the circadian conidiation rhythm in cultures growing on agar medium (*11*). However, CHX inhibited conidiation itself at such low concentrations that the clock could not be assayed under conditions where significant inhibition of protein synthesis occurred. For assaying the *Neurospora* clock, we have developed a liquid culture system that allows us to administer the drug in short pulses (*12, 13*) and have demonstrated that pulses of CHX cause phase shifts of the *Neurospora* clock (*14*).

In order to determine whether the phase-shifting effects of CHX are mediated through inhibition of protein synthesis, we examined the effects of the drug on two CHX-resistant mutants (*15*) in which the site of resistance is protein synthesis on 80*S* ribosomes (*16*). These mutants allow a direct test of whether CHX affects the *Neurospora* clock through its inhibition of protein synthesis or through some other unknown mechanism. If CHX acts through protein synthesis, then in these two CHX-resistant mutants the clock should also be resistant to CHX. If CHX acts through some other mechanism not affected by the mutations, then the clock of the mutants should show the same sensitivity to CHX as the wild-type strains. We now show that CHX alters the clock through its effects on protein synthesis.

The following strains of *Neurospora crassa* were used. The double mutant *bd, pan-2* (*17*) was crossed (*18*) to the cycloheximide-resistant mutants *cyh-1* and *cyh-2*. From these crosses, we isolated the triple mutants *bd, pan-2, cyh-1* and *bd, pan-2, cyh-2*. These two strains (referred to as the CHX-resistant strains) and *bd, pan-2* (referred to as the CHX-sensitive strain) were used for all experiments.

The strains were grown as described (*13*). Conidial inocula of the three strains were added to liquid glucose medium [0.3 percent glucose, Vogel's salts (*18*), 0.001 percent pantothenate]. All experiments were carried out at 25°C. The cultures were grown in constant light for 33 hours and formed mycelial mats. Disks (11 mm in diameter) were cut from the mats with a cork borer and washed in, and transferred to, liquid medium without pantothenate. At this time the disks were placed in constant darkness for the duration of the experiment. This light-to-dark (LD) transition sets the clock to a unique phase point from which it begins to "free run" (*12, 13*).

Beginning 15 hours after the onset of darkness, sets of six disks were treated with CHX (0.1 μg/ml) every 4 hours for the next 24 hours. At the end of each of these pulses, the disks were rinsed in drug-free medium and transferred to pantothenate-supplemented solid medium in race tubes, where they grew and formed conidial bands with circadian periodicity. Control disks to which CHX had not been added were also washed and transferred to race tubes at this time. The phase of the rhythm on the race tubes directly reflects the phase of the rhythm in liquid culture (*13*) and was calculated by linear regression analysis (*19*). The effect of the CHX pulse was determined by calculating the phase difference between a CHX-treated culture and a control culture transferred to race tubes at the same time.

The effects of CHX on protein synthesis were examined in the three strains as described (*14*). At 15 hours after the LD transition, circadian time 5 (CT 5), CHX was added to sets of six disks of each of the three strains at concentrations of 0, 0.1, or 0.5 μg/ml. After a 30-minute incubation period in CHX, L-[^{35}S]methionine (7.3 μCi; 1195 Ci/mmole; Amersham) was added, and the disks were incubated for 1 hour. The protein was precipitated with 10 percent trichloroacetic acid, and incorporation was measured by liquid scintillation counting. Protein was determined by the Lowry (*20*) method.

Figure 1 shows phase response curves for CHX pulses for the CHX-sensitive and CHX-resistant strains, in which the differences between control and experimental phases are plotted as a function of circadian time of the beginning of the CHX pulse (CT 12 is defined as the time of the LD transition). The wild-type

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