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HETEROSENSORY AND HETEROCORTICAL ACTIVATION OF THE PURKINJE NEURON

by

C. Batini

Brain Research Institute
University of California, Los Angeles
Los Angeles, California

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If accepted, please send proofs to:

Dr. C. Batini
Space Biology Laboratory, Brain Research Institute
Center for the Health Sciences
University of California, Los Angeles
Los Angeles, California 90024

Heterosensory and Heterocortical Activation of the Purkinje Neuron[1]

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Summary. Convergence of heterogeneous afferents has been described in the cerebellar surface with recording of gross evoked potentials. An hypothesis is presented here that this phenomenon should appear at the single neuron level in the cerebellar cortex. Steel microelectrodes have been used for recording the unitary potential in the extracellular field of the Purkinje neuron as well as of the granular cell. The results obtained can be summarized as follows:

1. Phasic activation of peripheral receptors, visual, acoustic and somesthetic, as well as electrical stimulation of primary and extraprimary cortical areas, elicit responses in single granular cells and in single Purkinje neurons. The response consisted in both cases of a rapid burst of spikes appearing at an appropriate delay and was followed by long-lasting inhibition. This implies that the three sensory afferent pathways, as well as the cortico-ponto-cerebellar system, may converge on the single Purkinje neuron through the polysynaptic intracerebellar circuit activated by the mossy fiber afferents.

2. In the Purkinje neuron records, a second spike response shortly delayed from the mossy fiber's response was observed. It consisted usually of a single spike appearing on a slow positive wave, with a higher threshold for activation. This second response in the Purkinje neuron is attributed to the climbing fiber afferents producing a monosynaptic activation. The

convergence phenomenon of heterogeneous afferents has also been observed in this climbing fiber response.

3. Paired cortical and peripheral stimuli delivered at varying intervals have shown the existence of two different patterns of interaction at the Purkinje neurons. First, a long inhibitory interaction lasting for 100-150 msec which has been demonstrated by others, using direct stimulation of cerebellar system. This study shows that intracerebellar polysynaptic inhibitory system may be activated by more peripheral stimulations. Second, a facilitatory interaction appeared at very short interstimulus intervals, and was easily obtained at intensities which elicited no responses when unpaired. This form of facilitatory interaction (short interval) was observed only on the climbing fiber response.

The results have been interpreted and discussed in accordance with the available anatomical data. The present work has led to the hypothesis that cortical and peripheral cerebellar afferents studied here may possibly feed into precerebellar stations of convergence, which would be the sources of the mossy and climbing fibers.

Key Words: Cerebellum -- Convergence of afferents -- Mossy fiber afferent -- Climbing fiber afferent

The sensory input to the cerebellar cortex, as well as cortico-cerebellar projections, have been widely investigated by use of gross evoked potentials (see Dow and Moruzzi, 1958). Initial studies attempted a functional classification of the entire cortical surface in terms of regional localization of afferents. Later studies correlated peripheral and cortical projections to specific cerebellar regions for particular

sensory modalities (see Dow and Moruzzi, 1958).

This concept of regional specificity seems questionable, since overlap has been found between sensorial fields (Dow, 1942; Fadiga, Pupilli and Berger, 1956; Jansen, Jr., 1957; Buser and Franchel, 1960; Batini, Castellanos and Buser, 1964, 1966a) as well as interaction between heterogeneous afferents (Bremer and Bonnet, 1951; Albe-Fessard and Szabo, 1954; Levy, Loeser and Koella, 1961; Batini, 1965). More recent findings have emphasized that, over almost the whole cerebellar surface, sensory and cortical inputs appear to be largely convergent (Batini, Castellanos and Buser, 1966a).

The present study has tested the hypothesis that this "convergence" first appears at the unitary level. The problem has been investigated by recording single units from a particular area of the cerebellar cortex, while varying the applied afferent stimulus. It will be demonstrated that heterosensory as well as heterocortical afferents can influence the firing pattern of the same neuron. This "convergence" phenomenon will be shown to act on the Purkinje neuron through two parallel systems, one of which is postulated to be transmitted indirectly by the mossy fibers, the other monosynaptically by the climbing fibers. The interaction patterns between heterogeneous afferents will show functional differences of the two convergent systems.

Methods

Thirty-two adult cats were used in this experiment: 19 were deeply anesthetized with chlorolose (70 mg/Kg), 8 were decerebrated at the precollicular level under ether anesthesia, and 5 were left intact and maintained under local anesthesia. All wound edges and pressure points

were infiltrated with procaine chloride solution. All intact animals were immobilized with Flaxedil and maintained by artificial respiration. An infrared CO_2 monitor (Capnograph Godart type CG/58003) was used to sustain a constant expired CO_2 at 3 to 4%. A large anterior craniectomy was performed in order to expose the cortical surface for the electrical stimulation. A small posterior craniectomy exposed the folium and tuber vermis for microelectrode recording and was covered with agar.

Single electric shocks (0.2 msec, 1 to 5 volts) were delivered to surface electrodes 2.0 mm apart, to various cortical areas, including motor cortex, and primary visual and acoustic areas. Sensory stimulation involved short flashes (after atropinization of the pupil), clicks and subcutaneous electric shock to the forepaws. The visual system in the decerebrate animals was activated by electric stimulation of the anterior colliculi (Batini, Castellanos and Buser, 1966b).

Unit activity was recorded with stainless steel microelectrodes, prepared according to the method of Green (1958). Amplifying and display systems consisted of a P6 Grass preamplifier, a 502A Tektronix oscilloscope. Traces were photographed with a Grass camera. Spike activity was recorded on one channel of a tape recorder (2 channel Tanberg Model 6) and square waves on the second channel, for stimulus marking. A further analysis of the evoked firing was performed using a Mnemotron computer of average transients (C.A.T. Model 400B). Histograms were prepared of the spike distribution versus summed periods of time following or just preceding the stimulus presentation, the stimulus being delivered monotonously with periods between 4-10 sec. A pulse height

selector was interposed between the tape recorder and the Mnemotrom computer, in order to suppress minor spikes (discrimination level adjustable from 200 mV to 5 V), to select either positive or negative spikes, and to transform the spike into a constant square pulse (adjustable at 1-3 msec and between 250 mV and 10 V).

One hundred and twenty-seven units were recorded from the cerebellar cortex, 77 in anesthetized animals, 29 in decerebrated preparations and 21 in the intact Flaxedilized subjects.

Results

A. Evoked activity in granular and molecular layers.

When the microelectrode was inserted into the cerebellar cortex, large positive-negative or positive spikes were encountered at a depth of 0.3 to 0.4 mm corresponding to the Purkinje cell layer. This activity, which has been shown to derive from Purkinje neurons (Granit and Phillips, 1956, 1957; Andersen, Eccles and Voorhoeve, 1964), was often found at deeper levels depending upon the angle of the vertically penetrating electrode on the varying cerebellar surface. Using the method first described by Granit and Phillips (1956), the Purkinje neurons were also recognized by their antidromic response to an electrical shock applied in the subjacent cerebellar white matter. At greater cortical depths only small spikes were encountered, usually negative in polarity and of short duration. These were interpreted as the activity of the granular cells. Different afferent stimuli were applied to all units recorded from either the molecular or granular layer. The most frequently elicited response consisted of a single spike or a short burst appearing with a fixed delay after the stimulus, followed by a period of suppression of spontaneous

firing for 100-150 msec. Since all afferent fibers activated in our experiments have been shown to end in the cerebellar cortex as mossy fibers (see Discussion), we would expect that the onset of response of the units in the granular layer (first relay in the cerebellar cortex for the mossy fibers) to a given stimulus would be shorter than the delay in the response of a Purkinje neuron (which is secondarily activated by the granular cells efferents). In fact, latency of the responses elicited in the granular cells was always found to be at least 4-5 msec shorter than the latency of the responses in the Purkinje neurons to the same stimulus.

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Place Figure 1 about here

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Although it was more difficult to record from small granular cells and they generally survived for a shorter period, we were able in some instances to test all desired stimuli, and to observe evoked firing for eadh stimulus (Fig. 1).

A greater number of Purkinje neurons has been tested, almost all responding to the applied stimuli. Here again, as shown in Fig. 2, it was possible to elicit a response in the same unit by using either peripheral or cortical stimuli.

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These results were found in both the anesthetized and non-anesthetized preparations (Fig. 3). They demonstrate that a single granular and Purkinje cell can be influenced by the specific sensory systems as well

as by the cortico-ponto-cerebellar system.

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Place Figure 3 about here

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A simple hypothesis would be to attribute transmission of these heterogeneous impulses to the mossy fibers through the granular cells to the Purkinje cells. Anatomical substrates for such multiple connections between mossy fibers and granular cells have already been emphasized (Fox and Barnard, 1957; Gray, 1961). However, accurate analysis of latencies and evoked firing patterns of Purkinje neurons in this study, has shown more complex evoked activity, for which an additional afferent system must be postulated.

B. Purkinje cell evoked activity.

In these units, spontaneous firing appeared to be slightly different in the three preparations studies. The high frequency discharges first described by Brookhart, Moruzzi and Snider (1950, 1951) were more often observed in both types of non-anesthetized preparations, although to a lesser extent in the intact Flaxedilized preparation. Under chlorolose anesthesia, the most frequent pattern of discharge was a more regular low rate. However, epochs of random rapid firing, followed by periods of slow firing, or even silence appearing in a very random fashion (see also Granit and Phillips, 1956), was seen in the record of an individual cell in all three preparations. The superimposed time histogram of the spikes clearly extracts the evoked spikes from the background activity even for very high frequencies of spontaneous firing (Fig. 3).

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A simple pattern of evoked activity in a Purkinje cell is seen in Fig. 4. Each stimulus, delivered consistently every 4 seconds, elicits a burst of spikes followed by a long period of suppression of spontaneous discharges.

The delay with which different units responded to a given stimulus varied greatly. However, as shown in Fig. 5, the latencies of the first evoked spike in 25 neurons to cortical shocks (Fig. 5A) and to flashes (Fig. 5B), have two preferential latencies for either stimulus.

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Furthermore, in several records of a single unit it was possible to observe two responses following a single afferent volley (Fig. 6 and Fig. 2). These two responses were found to have the same latencies as the preferential values shown in Fig. 5. Their patterns were often different, the first consisting of a very rapid burst of spikes, the second in one or two spikes usually located in the ascending portion of a large positive wave (see also the "inactivation response" and the "D potential" described by Granit and Phillips, 1956). A further distinction between these two responses was established by using different intensities of stimulation, the "short latency" response having a lower threshold than the "long latency".

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That both the responses were generated in the same Purkinje neuron is difficult to affirm from an extracellular record. However, if they arose in two Purkinje neurons close together, they must then have independent responses to a single afferent volley. It has already been pointed out that Purkinje neurons are activated by the granular cells. The time of conduction through the granular efferents, the parallel fibers, has been calculated to be 0.3-0.5 msec (Dow, 1939), running for a maximal longitudinal length of 3-4 mm (Fox and Barnard, 1957; Gray, 1961). The observed intervals between granular and Purkinje neuron responses to the same stimulus is consistent only for the first response seen in the Purkinje neurons. Thus, another afferent system has to be considered for the second response (see Discussion).

When tested by applying various stimuli, both responses were activated, with a common latency consistent with the site of stimulation. More significantly the latency between the first and second responses were unaltered by the site of stimulation, thus lending support to the view that the anatomical convergence serves also as a converging transmission system. However, using paired stimuli at varying intervals, essential differences in the patterns of interaction between heterogeneous afferences have been found.

C. Interaction patterns in Purkinje neurons between heterogeneous afferents: inhibitory and facilitatory effects.

Paired stimuli with varying intervals were used in this experiment

to study the dynamic interaction between those cortical and peripheral projections which have been shown to converge on a single Purkinje neuron of the visuo-acoustic area in the cerebellar cortex. The paired stimuli were used in all the possible combinations provided that each of the pair activated a cerebellar response.

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As shown in Fig. 7, the spike responses elicited by a cortical test stimulus were suppressed when preceded by the flash conditioning stimulus. On reversing the order of stimulation, the cortical shocks appeared to have the same strong conditioning effect on the flash responses, suggesting a reciprocal inhibitory interaction between cortical and peripheral afferents. The mutually inhibitory effects are evident over a relatively broad time separation, with a maximum of 100-150 msec. An identical inhibitory interaction was always obtained for any pair of stimuli, if each stimulus elicited a response on the same Purkinje neuron. The long-lasting reciprocal inhibitory effect between the two heterogeneous afferent volleys has been observed to be effective either on the units responding at short latency (Fig. 7) or in those responding at long latency (Fig. 8D and 8F).

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If we now combine the two heterogeneous stimuli at short intervals a very strong facilitation is obtained. As shown in Fig. 9 the most

effective interval between the cortical and peripheral stimuli corresponded to the almost simultaneous appearance of the two expected responses. The number of spikes elicited by the double stimulation was greater than the sum of the spikes evoked by the two single stimuli. This effect was easily obtained when the intensity of both stimuli was lowered to threshold, and even more easily when the stimuli were presented at subliminal intensities. As shown in Fig. 10B and 10C, either afferent stimulus delivered separately was effective in producing a response, while their combined effect elicited a very strong response (Fig. 10D). These findings appeared to be independent of the anesthetic treatment of the animals since they have been largely confirmed on the non-anesthetized preparation (Fig. 10).

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Place Figures 9 and 10 about here

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Contrary to the inhibitory effects obtained for both long and short latency responses, facilitatory interaction was obtained only on the long latency responses of the Purkinje neurons. This difference is illustrated in Fig. 11, showing the histograms of a record in which two responses (long and short latency) were obtained for the same afferent volley. The histogram, as expected, shows a bimodal distribution of the spikes for a single stimulus (A). Combining the two stimulations did not modify the first response, but, at certain stimulation intervals, strongly enhanced the second response (B).

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Discussion

The unit potentials recorded from the cerebellar cortex were classified as granular cell and Purkinje cell activity according to the layer in which they were found. The identification of the Purkinje neurons as established in the present work has been discussed in previous studies (Granit and Phillips, 1956; Andersen, Eccles and Voorhoeve, 1964; Suda and Amand, 1964) and needs no further comment. But the identification of the granular cell is not supported by direct evidence. The possibility exists for recording from one of the few stellate neurons within the granular layer. However, the few direct afferent fibers to the stellate cells (Jontov, 1958) have been described as arriving from the medulla (Jontov, 1959). Since the results of our experiments showed units responding with very short delay to cortical as well as visuo-acoustic afferences it would appear that they were from the granular cells. In the present investigations, the afferent cerebellar pathways activated by either cortical stimulation or peripheral visual and acoustic, have been demonstrated to run through the brachia pontis (Jansen and Brodal, 1954) and end in the granular layer as mossy fibers (Jelgersma, 1917; Lorente de Nó, 1935; Snider, 1936). Similarly, the spino-cerebellar afferents activated by somesthetic stimulation, end in the granular layer as mossy fibers (Miskolczy, 1931; Brodal and Grant, 1962). Thus in each case the activated granular cells transmitted the impulses monosynaptically to the Purkinje neurons. However, the results have shown that a single afferent volley elicited two responses in the Purkinje neurons. The following reasons demonstrate that these two responses are completely independent: (i) a single modality afferent

volley can elicit the "first" or the "second" separately; (ii) when recorded simultaneously they may show different sensitivities to the intensity of stimulation; (iii) the facilitatory interaction between heterogeneous afferents occured only on the "second response". According to the conduction time of the efferent fibers from the granular cells, only the first response can be attributed to the arrival of the impulses through the mossy fibers.

An explanation for the "second response" requires the existence of another afferent system. In fact the Purkinje cell dendrites receive monosynaptically independent afferences over the climbing fibers. Lorente de Nó (1924) and Mettler and Lubin (1942) claimed that all cerebellar afferents end as mossy fibers, the climbing fibers belonging only to the intracerebellar systems (Carrea, Reissig and Mettler, 1947; Scheibel and Scheibel, 1954). However, Szentágothai and Raikovits (1959) using the Nauta-Gyga method, recently demonstrated degenerating climbing fibers after various extracerebellar lesions. The majority of these fibers seem to originate in the inferior olive passing through the inferior peduncle, but some degeneration was also found after lesion of the brachium pontis and brachium conjunctivum. Therefore, the possibility of a direct activation of the Purkinje neurons by afferent stimulations through the climbing fibers cannot be excluded. This point of view is also supported in experiments by Jansen and Fangel (1961), where two different components of evoked activity were seen in the Purkinje layer after cortical stimulation: the first was attributed to the arrival of impulses through the mossy fibers, the second to those through the climbing fibers.

One of the main results from this study is the suggestion that afferent volleys coming from the activation of different pathways may converge at the level of a single neuron in the cerebellar cortex. Other investigators (Batini, Castellanos and Buser, 1966a), using the method of gross evoked potentials, were able to demonstrate with macro-electrodes, for a given point on the cerebellar surface, the coexistence of three kinds of convergence: hetero-cortical convergence (Dow, 1939; Jansen, Jr., 1957), hetero-sensory convergence (Bremer and Bonnet, 1951), and convergence between cortical and peripheral volleys (Albe-Fessard and Szabo, 1954; Green, 1958). These results are here largely confirmed and extended to the level of single unit recordings in the visuo-acoustic region where independent systems seem able to converge on the Purkinje neurons from each source studied.

The problem thus arises to determine, for each of the two afferent systems, the site of convergence of such heterogeneous sources, and inversely, for each source, the locus of divergence of the two afferent cerebellar systems. Part of this complex hodological problem has been already discussed in previous work to which the reader is referred (Batini, Castellanos and Buser, 1966a, 1966b). The hypotheses suggesting that the pontine nuclei possess the anatomical substrate for a pre-cerebellar common relay, serving both peripheral and cortical afferences, can be maintained as long as no other evidence is added. This view is a result of the fact that pontine fibers have been demonstrated to end in the cerebellar cortex exclusively as mossy fibers. However, this system can be readily postulated only for the convergence of part of the heterogeneous impulses arriving via granular cells.

An analogous center for pre-cerebellar convergence for the direct afferents to Purkinje neurons is suggested to lie in the inferior olive. It has been shown that the main source of degenerating climbing fibers in cerebellar cortex arises from olivar lesions (Szentagothai und Rajkovits, 1959). Spinal afferents to the inferior olive have been described (Brodal, Walberg and Blackstad, 1959) as well as cortical connections arising from both primary and extraprimary areas (Mettler, 1935a, 1935b; Snider and Barnard, 1949; Walberg, 1956). The localization of the afferent fibers within the inferior olive is possibly compensated by the diffuse distribution of the olivo-cerebellar connections (Brodal, 1940a, 1940b). Such a system receiving afferents from specific pathways and having multiple afferents from each subnucleus to Purkinje neurons, is strongly suggestive of a converging relay.

The functional significance of the heterogeneous convergence at the cerebellar cortex has already been pointed out (Batini, Castellanos and Buser, 1966a, 1966b). Here the functional significance of the double independent afferents via Purkinje neurons can be discussed in the frame of results obtained with paired stimuli.

The long-lasting inhibitory effect described in our experiments appears to be a fundamental property of all afferents arriving in cerebellar cortex and acting at the level of the Purkinje neurons. A polysynaptic inhibitory system on the Purkinje neurons and the granular cells has been recently described (Andersen, Eccles and Voorhoeve, 1964; Eccles, Llinás and Sasaki, 1966a, 1966b) to be found in the basket and Golgi cells which are interposed between granular and Purkinje neurons.

Inhibitory effects in this system are assumed to be activated by mossy fibers. However, evidence exists for a second mechanism for the activation of this system through the collaterals of the climbing fibers synapsing on Golgi cells of the granular layer (Scheibel and Scheibel, 1954).

Results of the present study have shown that a facilitatory response may be obtained at the Purkinje neuron if the paired stimuli occur within a very short period of time. The upper limit for this temporal relation is probably determined by the transmission time of the polysynaptic inhibitory system from the Golgi cell to the Purkinje neuron. Since a collateral pathway is shown between the climbing fiber and the Golgi cell, it is clear that a second ascending volley on the climbing fiber may find the Purkinje neuron inhibited as a result of the first volley. This assumption is strengthened also by our earlier results obtained by using the gross evoked responses: paired cortical and peripheral stimuli delivered at short intervals and at low intensity elicited a very strong facilitation of the late component of a complex cerebellar evoked potential (Levy, Loeser and Koella, 1961; Batini, 1965). The same strong facilitatory effect on the early component of the evoked potential is elicited by a double shock delivered at very short intervals on the inferior olive (Batini, in preparation).

FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Responses of a granular cell to various stimuli in an anesthetized animal. A. Single electric shock to the sigmoid gyrus; B. to the lateral gyrus; C. to the ectosylvian gyrus; D. flash; E. click. The stimuli are marked by a dot.

Fig. 2. Responses to various stimuli obtained in a Purkinje neuron of an anesthetized animal. A. Flash; B. click; C. subcutaneous electric shock in the left forepaw; D. cortical stimulus of the visual cortex; E. of the acoustic cortex; F. of the motor cortex. Note that the "first" and the "second response" are clearly obtained in all records.

Fig. 3. Superimposed time histograms of the spike responses of a Purkinje neuron in an intact Flaxedilized animal. In this and following histograms number of spikes is shown on the ordinate, time in seconds on the abscissa, and N equals the number of stimuli presented. A. Acoustic stimulation; B. somesthetic stimulation of the right forepaw; C. electric shock of the sigmoid gyrus. The stimuli are delivered just before 0 time. Note that the "first" and the "second response" are more clearly evident in C.

Fig. 4. Record of a Purkinje neuron in an anesthetized preparation. A. Responses to flashes; B. to electric shock of the sigmoid gyrus; C. clicks. The stimuli are marked by a dot. The unit responds to each stimulus with a burst of spikes at short latency ("first response"), followed by a long period of inhibition.

Fig. 5. Latency distribution of 25 Purkinje neuron responses after

stimulation of the sigmoid gyrus (A) and flashes (B). Number of units is shown on the ordinate, time in 5 msec increments is plotted on the abscissa. The latency of each unit has been calculated as a mean of ten presentations of the stimulus. See text for further explanation.

Fig. 6. Double response in a Purkinje neuron to flash stimulation (the stimulus artifact is marked by a dot) in a decerebrated animal. Note the difference between the "first response", which consists of a rapid burst of spikes, and the "second response", which consists of two spikes appearing on the ascending portion of a slow wave which has been shortened by the time constant. See "D" potential in the text for further description of the slow potential. The double response is followed by a long period of inhbiition.

Fig. 7. Inhibitory interaction by paired stimuli in a Purkinje neuron of an anesthetized animal. A. Control of the response to a single flash stimulus (left side), and an electric shock to the acoustic cortex (right side). These elicit the "first response" only. Note the difference in the artifact. B. Left column, the response to the test stimulus (cortical shock) is suppressed by the conditioning (flash) stimulus presented at increasing intervals. In the right column an identical effect is obtained when the flash is the test stimulus and the cortical shock is the conditioning stimulus. C. Control of the response to a single stimulus as in A, after the double stimulation period.

Fig. 8. Superimposed time histograms showing inhibitory interaction by paired stimuli. On the "second response", flashes (1) and sigmoid gyrus stimulation (2) in an anesthetized animal. A. Control for spontaneous activity; B. responses to flashes; C. response to cortical shocks; D. inhibition of the flash response. The test flash stimulation is applied before the conditioning stimulus but the response to the test stimulus is expected after the conditioning stimulus response. E. Summation of the two responses which are expected to appear simultaneously. F. Inhibition of the cortical test response.

Fig. 9. Superimposed time histograms of the facilitatory interaction by paired stimuli in Purkinje neuron of an anesthetized animal. 1. Flashes; 2. motor cortex stimulation. Note the long latency of the responses. Number of spikes on the ordinate and time in msec on the abscissa.

Fig. 10. Superimposed time histograms of the facilitatory interaction by paired stimuli of a Purkinje neuron in a decerebrated preparation. The stimuli are applied shortly before 0 time. A. Spontaneous activity; B. subliminal somesthetic stimulation to the right forepaw; C. subliminal clicks; D. paired stimuli at short intervals at the same intensities as in record C.

Fig. 11. Superimposed time histograms of the facilitatory interaction by paired stimuli on the "second response" of a Purkinje neuron in an anesthetized animal. 1. Flashes; 2. electric shock to the sigmoid gyrus. A. Control before applying the double stimulation. Note the bimodal distribution of the

spike response due to the appearance of the "first" and the "second response" on the unit record. B. Paired stimuli at varying short intervals. The second response only is strongly facilitated at certain intervals (see second line in B). C. Control after applying the double stimulation.

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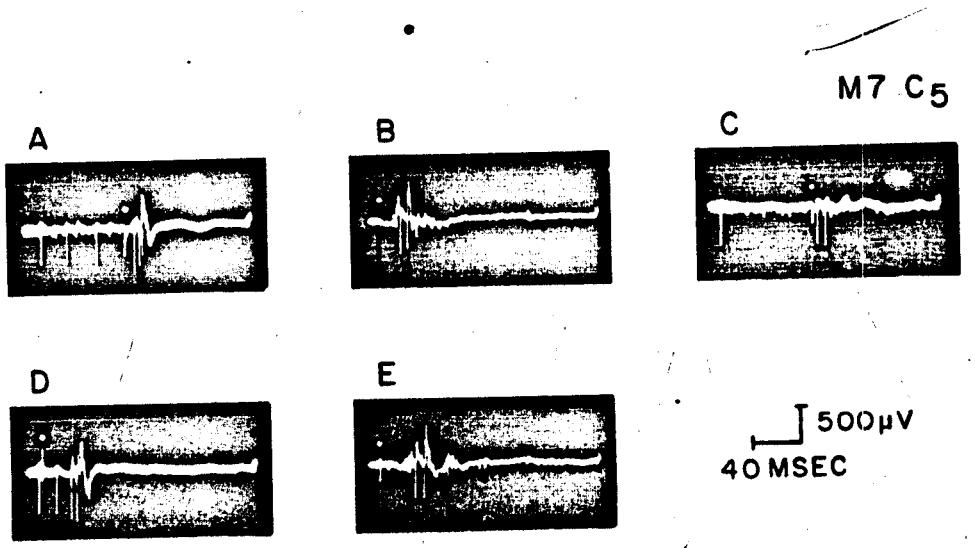


Figure 1

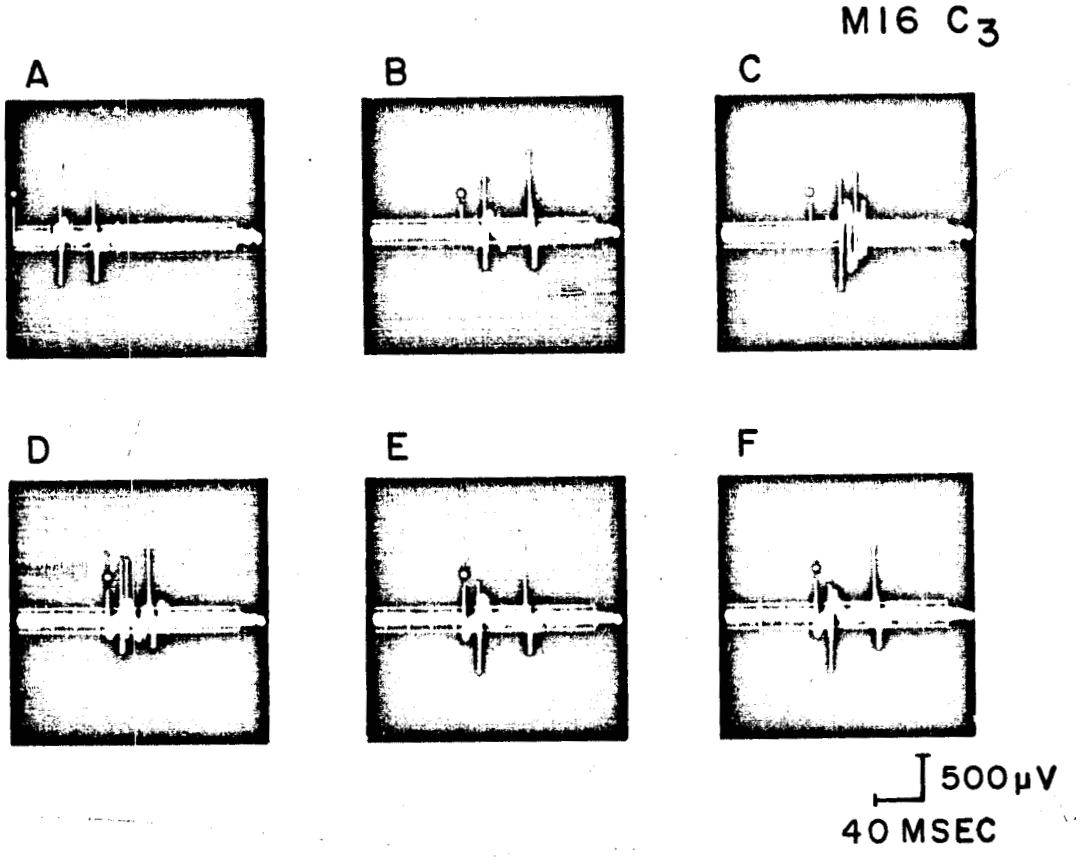


Figure 2

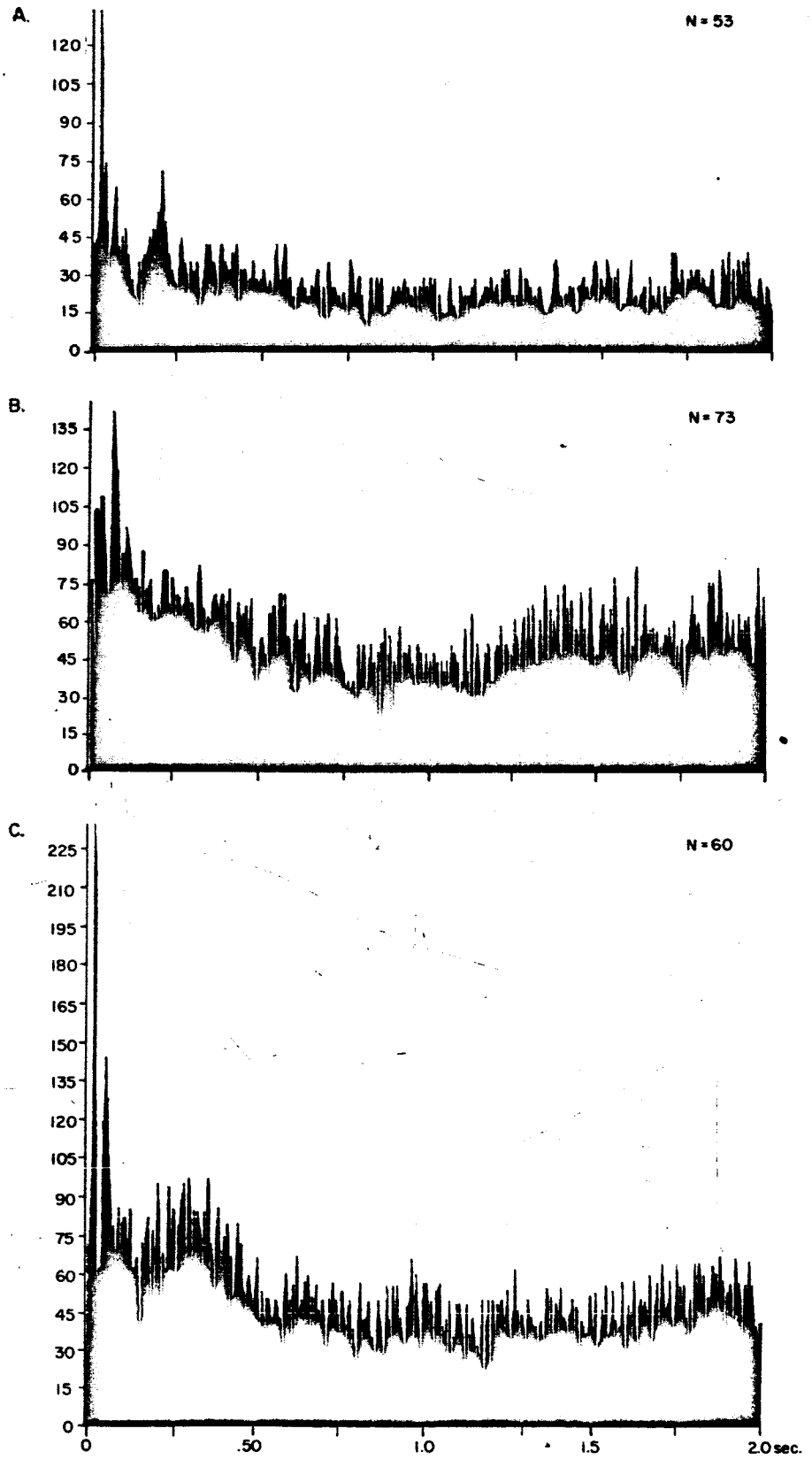
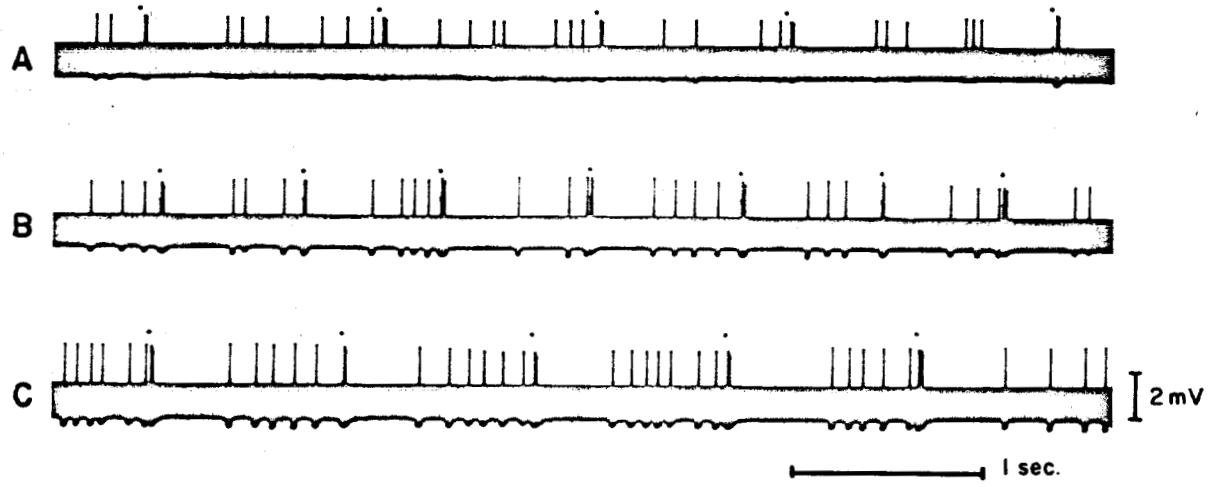


Figure 3

M26 C_{10}



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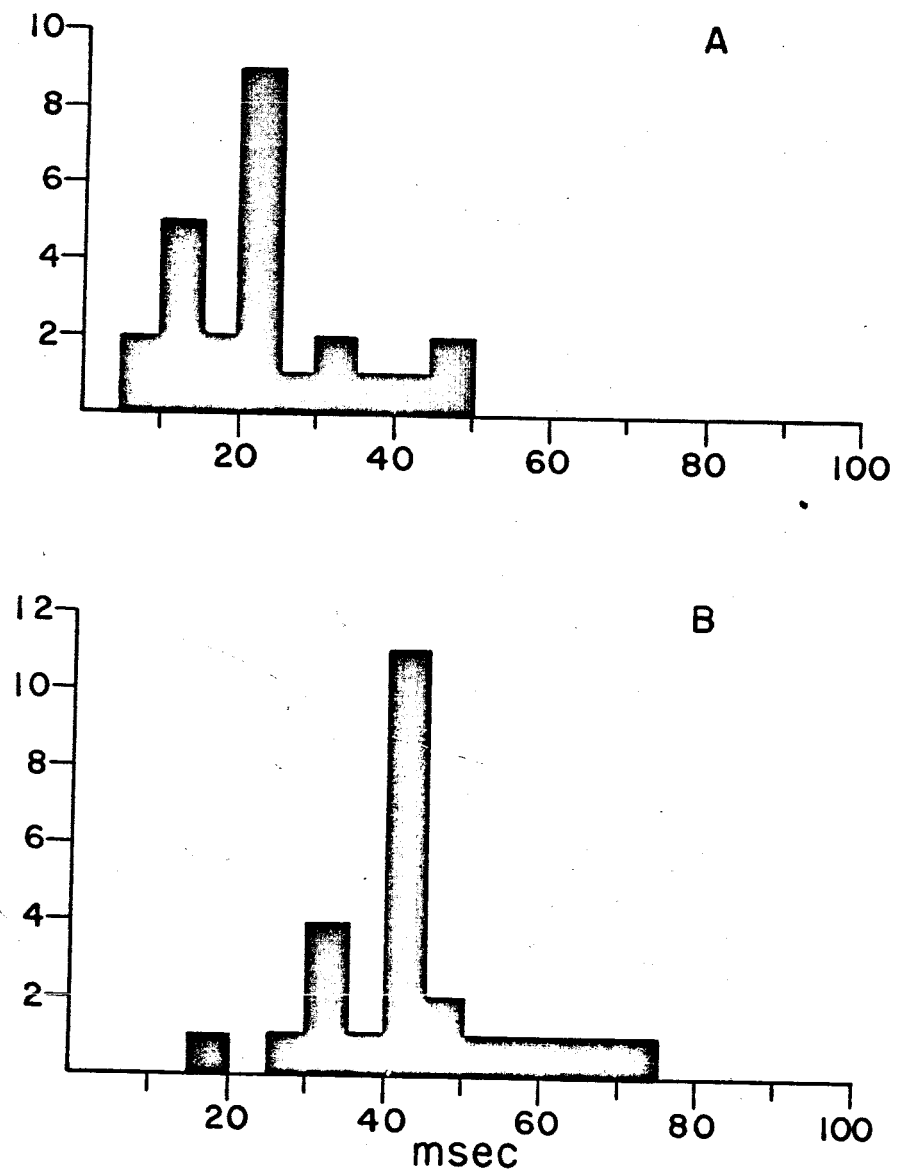

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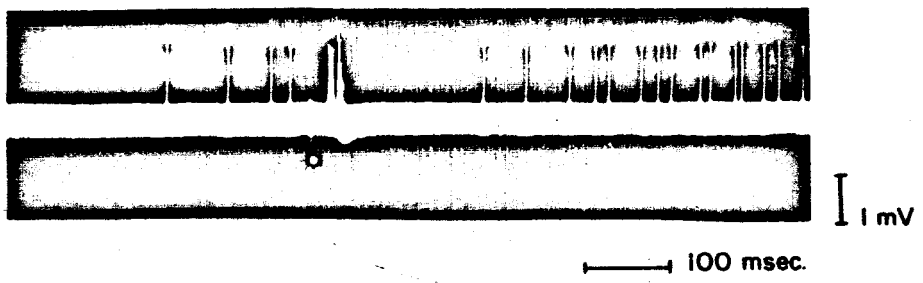

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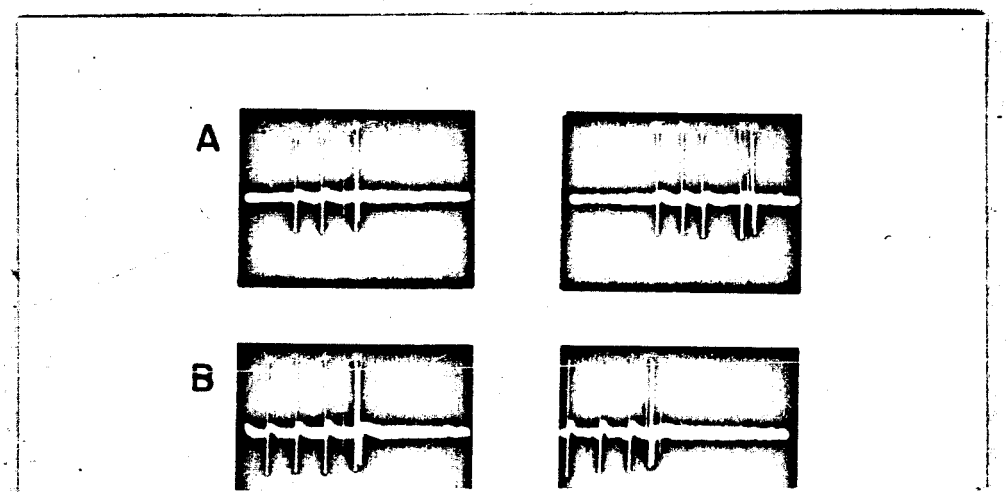

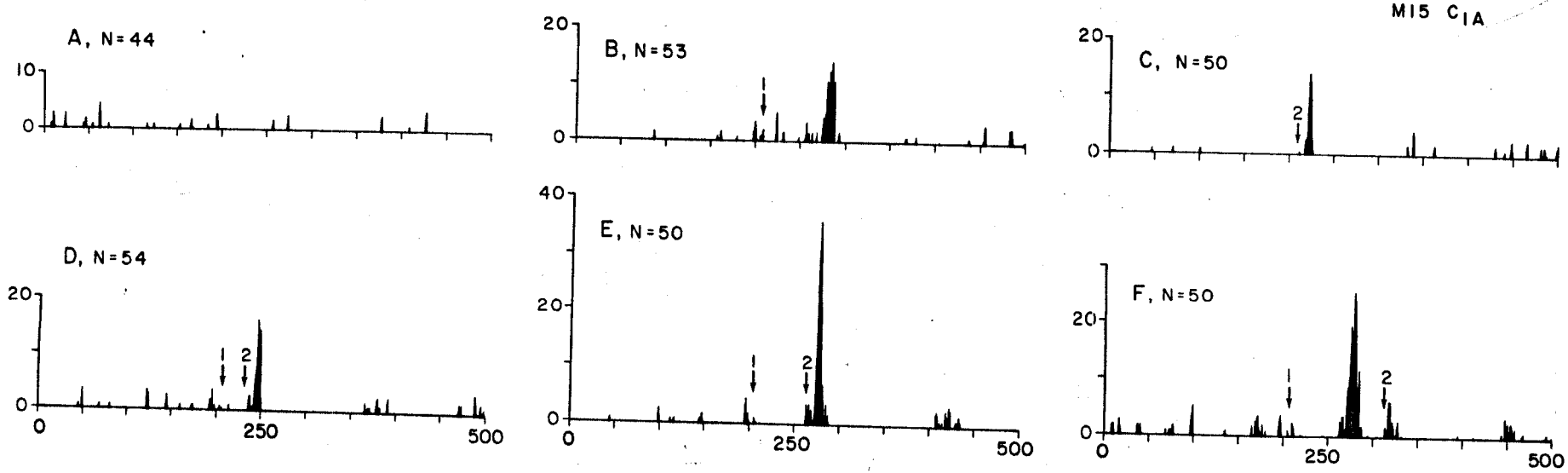
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Figure 8

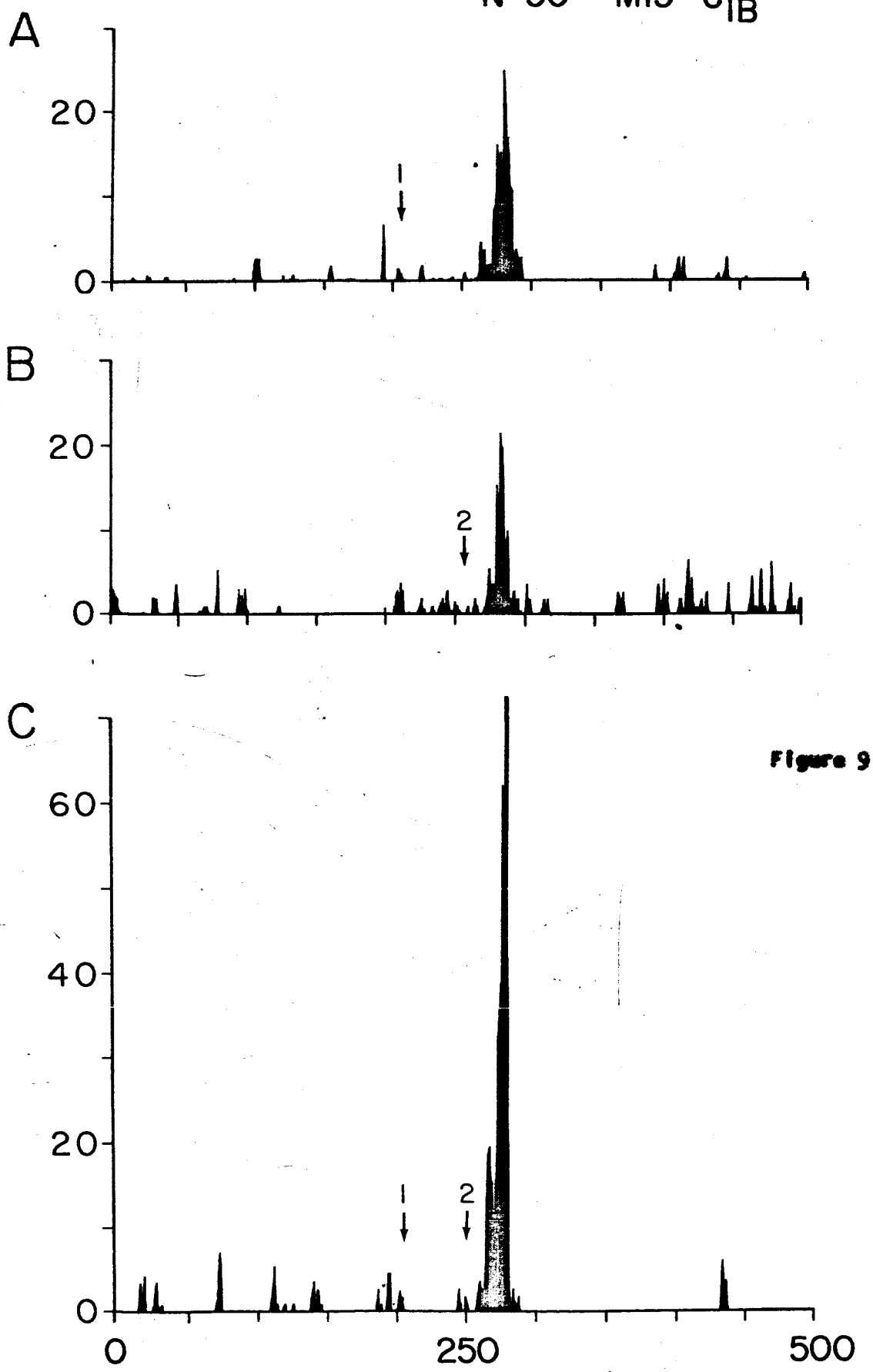

Figure 9

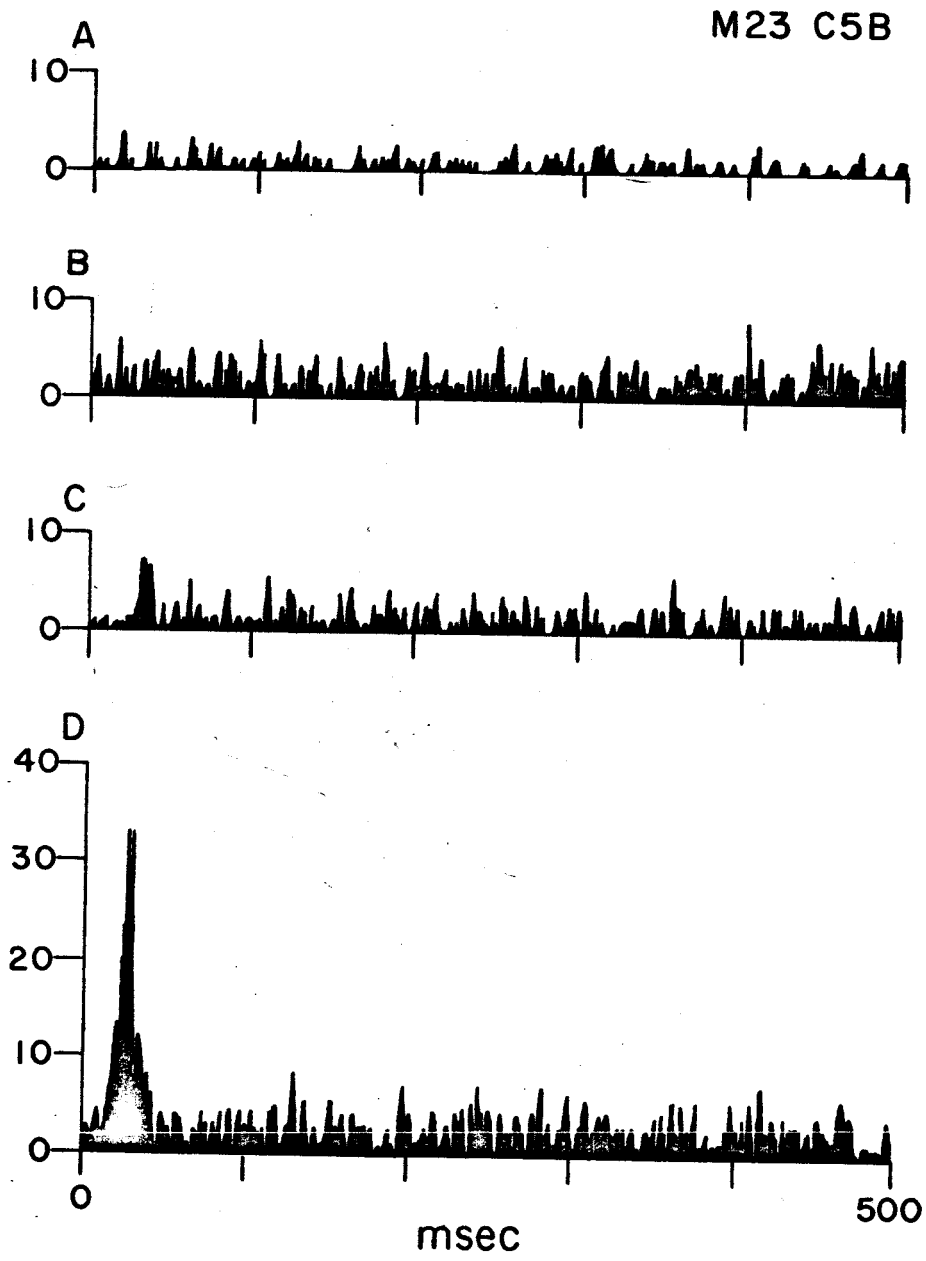

Figure 10

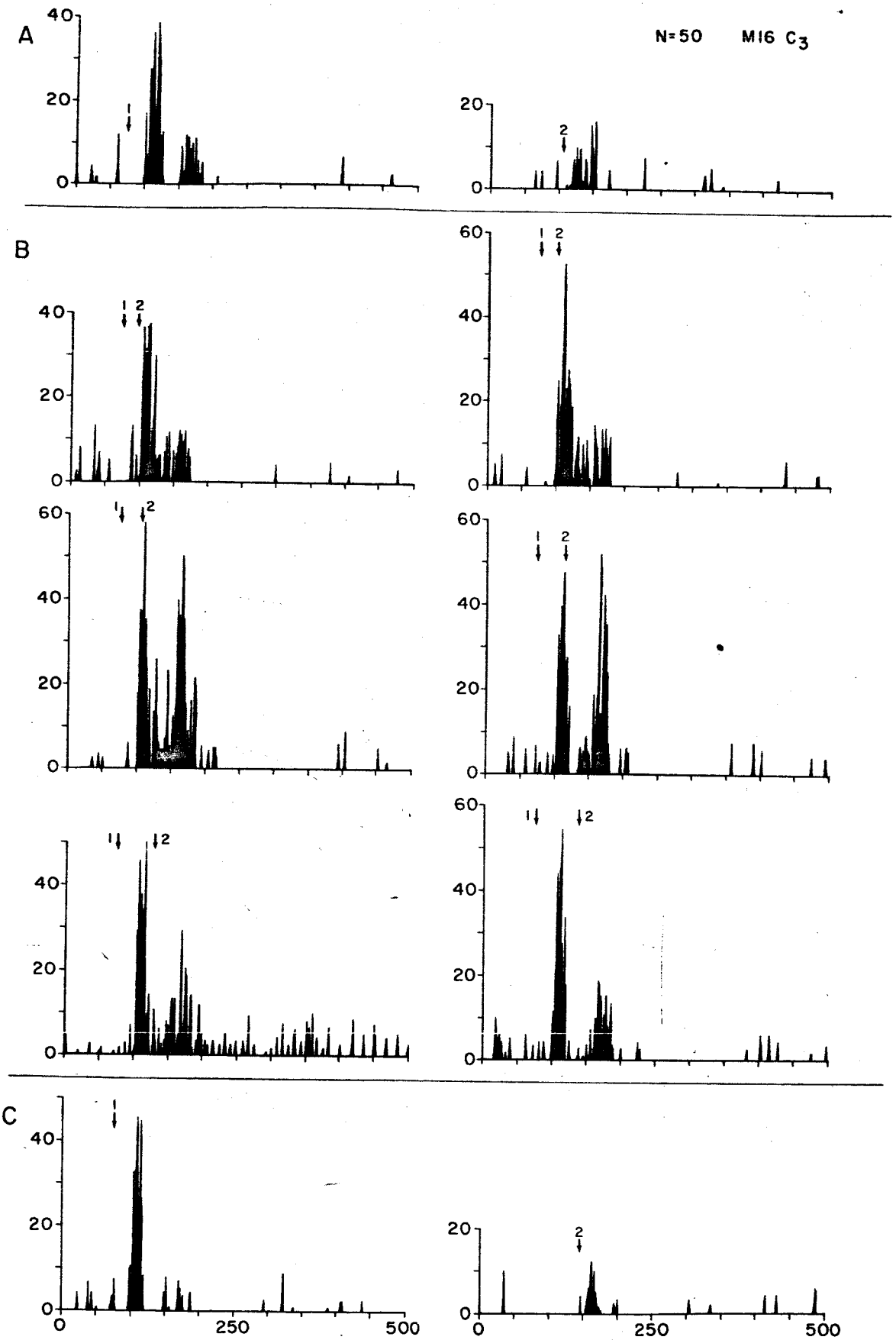

Figure 11