AN ELECTROPHYSIOLOGICAL STUDY OF SYNAPTIC CONNECTIONS IN TISSUE CULTURES OF THE MOUSE CEREBELLUM

by

J. Martin Wojtowicz

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Department of Physiology
Faculty of Medicine
University of Ottawa
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ABBREVIATIONS

AOAA - Amino-oxyacetic acid

AP - Action potential

BS - Brain Stem

BSS - Balanced salt solution

Cb - Cerebellum

CNS - Central Nervous System

CSF - Cerebro-spinal fluid

Cx - Cerebellar Cortex

DIV - Days In Vitro

DN - Deep Cerebellar Nuclei

DRG - Dorsal Root Ganglion

E.M. - Electron Microscope

GABA - γ-aminobutyric acid

GAD - Glutamic Acid Decarboxylase

IIH - Interspike interval histogram

IPSP - Inhibitory postsynaptic potential

IS - Initial segment

Mg - Magnesium ion

P - Purkinje neurone

SCG - Superior Cervical Ganglion

SD - Somato-dendritic segment

ABSTRACT

AN ELECTROPHYSIOLOGICAL STUDY OF SYNAPTIC CONNECTIONS IN TISSUE CULTURES OF THE MOUSE CEREBELLUM

The aim of this study was to explore the feasibility of the use of tissue cultures for studying some of the mechanisms of synaptic transmission in the mammalian CNS. The cerebellar cultures are particularly suitable because their morphological features have been studied in detail, offering a possibility of correlating electrophysiological and morphological data. Another advantage of the cerebellum is that its structure and function are relatively well known in vivo so the validity of the tissue culture model can be well tested in this case.

It is well known from previous studies that functional synapses develop in the cerebellar cultures. However, the patterns of synaptic connections in cultures, in particular the interactions between cortex (Cx), deep cerebellar nuclei (DN) and brain stem (BS) have not been investigated.

Electrophysiological experiments were performed on fully developed cultures 3-4 weeks old. The cultures were placed in a perfusion chamber mounted on the stage of an inverted microscope so the electrodes could be placed near the neurones under visual control. The temperature of the perfusing solution (Earle's balanced salt solution) was maintained at 35-37^{o}C and the pH at 7.3 - 7.4.

One region of the cultures was usually activated by brief electrical pulses and synaptically evoked responses were sought in a different region. Stimulation of the DN area resulted in a synaptic activation of about 50% of the Cx neurones whereas a stimulation of the Cx area evoked 30% of the DN neurones antidromically. On the basis of known conduction velocities

and latencies of the synaptically evoked responses it has been concluded that in culture DN neurones form a synaptic projection to the Cx area, which resembles a mossy fiber pathway found in the intact cerebellum (Tolbert et al., 1976). Electron microscopic observations in culture give supporting evidence for the mossy type projection from the DN to the Cx (Hendelman et al., 1978).

Another synaptic pathway which has been well documented in culture is the monosynaptic inhibitory projection from the Cx region to the DN. Satisfactory electrophysiological and morphological evidence has been obtained to suggest that the projection is monosynaptic and that it can be routinely reproduced in culture. Electrophysiological characteristics of the pathway resemble those described in vivo by Ito et al. (1970a).

Using pharmacological blockers, such as Bicuculline, Picrotoxin and Strychnine, it has been shown that GABA is probably the neurotransmitter responsible for the inhibition. This observation is similar to that of Obata et al. (1970a) who studied an analogous synaptic connection in the lateral vestibular nucleus of cats.

In addition to the two major pathways demonstrated in cultures, evidence for recurrent axon collaterals of DN neurones and possibly Purkinje neurones has been obtained which again is in agreement with previous morphological observations in culture as well as in vivo. The presence of inhibitory interneurones in the Cx area was also evident.

Neurones from the BS area, which can be included with the cerebellar cultures, have also been studied in spite of their uncertain identity. No firm evidence could be obtained as to the terminations of these neurones in other areas of the cultures but the results suggest that these neurones are excitatory and that they are interconnected by excitatory synapses which are possibly formed by the terminals of their own recurrent axon

collaterals. A sparse inhibitory projection from the Cx to the BS area has been shown.

Such a detailed demonstration of the synaptic interconnections in tissue cultures which resemble those in vivo provides a background for further experimentation. The tissue culture model of the Cb can be used for investigation of the mechanisms of synaptic transmission and the action of various pharmacological agents on the neurones. The advantage of such a model system lies primarily in its accessibility to pharmacological manipulations which are often difficult in the intact brain.

CHAPTER I

INTRODUCTION

Part I - The Synaptic Organization of the Cerebellum

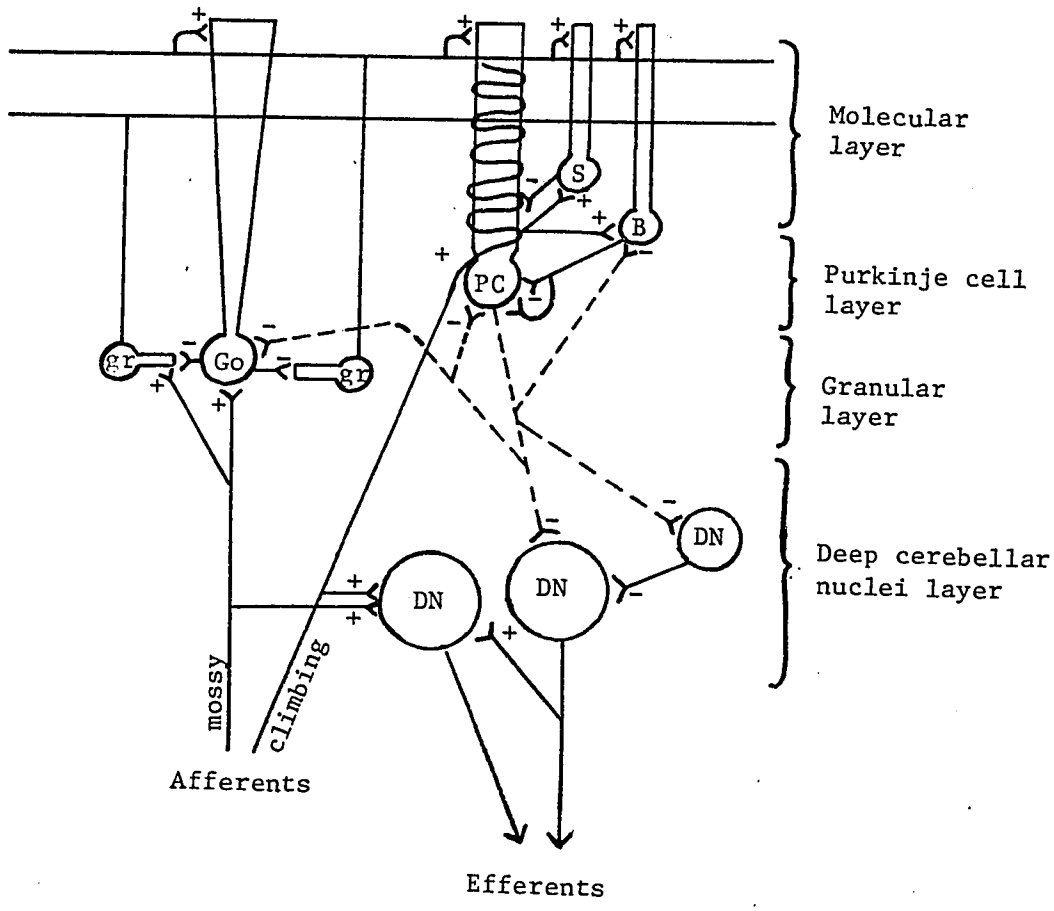
The cerebellum (Cb) is composed of two functional parts, the Cb cortex and the deep cerebellar nuclei. The cortex (Cx) can be divided into three layers; the superficial molecular layer, the central Purkinje cell layer and the internal granular layer (see diagram on page 7). Five types of neurones are found in the cortex; stellate and basket neurones in the molecular layer, Purkinje (P) neurones in the Purkinje layer and Golgi and granule neurones in the granular layer. The synaptic interconnections in the cerebellar cortex are remarkably uniform in the whole cortex and can be represented by a summary diagram shown on page 7. The deep cerebellar nuclei (DN) are of three types; lateral, interpositus and fastigial. Each nucleus is functionally related to a separate part of the cortex.

The white matter region separating the Cx from the DN contains intracerebellar fibers as well as afferent and efferent fibers of the Cb. Intracerebellar fibers are predominantly the axons of P neurones projecting to the DN and sending recurrent collaterals to the adjacent regions of the Cx. The afferents originate in the dorsal and ventral spinocerebellar tracts, the external cuneate nucleus, the vestibular nerve and nucleus, the reticular formation, the pons and the inferior olive. Most of the afferents terminate as mossy fibers and contact predominantly dendrites of granule cells. Afferents originating in the inferior olive terminate as climbing fibers and contact P neurone dendrites.

The efferent pathway from the Cb is composed of the axons of the large DN neurones which project to the thalamus, red nucleus, lateral vestibular nucleus and reticular formation.

a) Cerebellar Cortex

The characteristic feature of the mammalian Cb cortex is the location of neurones and fibers in three layers; a molecular layer, a Purkinje cell layer and a granular layer. The molecular layer contains axons of granule cells (parallel fibers), dendrites of Purkinje (P) neurones and two types of interneurones, stellate and basket. The Purkinje cell layer contains predominantly cell bodies of P neurones. The granular layer contains cell bodies and dendrites of granule cells and Golgi cells. Such a regularly layered structure is very suitable for electrophysiological experiments since various neuronal elements can be recorded from or selectively stimulated by placing the recording or stimulating electrodes at the appropriate depth beneath the cortical surface. Eccles and his associates (Eccles et al., 1966a, 1966b) extensively used stimulation of the superficial molecular layer in the analysis of intracortical synaptic connections in the cat Cb. Such a stimulation produces an excitation of the superficial parallel fibers (axons of granule cells) which in turn project to all types of neurones in the Cb cortex. Granule cells are activated antidromically while P neurones and interneurones (basket, stellate and Golgi) are activated by a synaptic input. The interneurones usually respond with multiple spike discharges lasting 10-30 msec. The number of spikes within each burst and the duration of the burst is increased by increasing the stimulus intensity. After each evoked burst there is a period of facilitation lasting up to 60 msec following the end of the burst (up to 90 msec following the first stimulus), i.e. a second parallel fiber volley applied during this period produces a stronger burst than the first one. No clear difference was found between the three types of interneurones; therefore they could be



Synaptic organization of the Cerebellum in vivo.
Summary diagram based on morphological and physiological findings.
(+) Excitatory synapse, (-) inhibitory synapse.

identified only on the basis of their location within the Cb cortex. This was usually easily done because each type of interneurone can be found at different depths under the cortical surface. No intracellular recordings could be obtained from these neurones but even on the basis of extracellular recordings the responses were identified as synaptically mediated. The progressive decrease in the latency of responses with increasing stimulus intensity was taken as an indication of a steeper rise of summated EPSPs. The long lasting facilitation of the evoked bursts was attributed mostly to the long lasting action of a neurotransmitter. The electrophysiological evidence for synaptic connections between the parallel fibers and the interneurones is in agreement with morphological studies in cats (Eccles et al., 1967, p. 58-61) and in rats (Palay & Chan-Palay, 1974).

P neurones always responded with a single synaptically mediated spike to surface cortical stimulation (Eccles et al., 1966b). This is not due to a weak or short depolarizing action of parallel fibers on these neurones, but rather is the result of a powerful inhibition which follows the onset of depolarization with a delay of a few msec and is so strong that it subsequently predominates. It was suggested by Eccles et al. (1966b) that the inhibition which prevents multiple firing of P neurones is caused by basket and stellate cells. This notion was at first supported by Llinas & Bloedel (1967) who found that in the frog Cb, which was thought not to have inhibitory interneurones, a cortical stimulation produced multiple discharges in the P neurones. However, it was later found that stellate (but not basket) cells are present in the frog Cb and subsequent studies by Rushmer & Woodward (1971) showed that an inhibition in the frog can be produced. It is possible therefore that stellate cells contribute substantially to the inhibition in mammals.

Bloedel & Roberts (1969) argued that the inhibition of P neurones following surface stimulation in cat Cb is solely an artifact, resulting from anesthesia and has nothing to do with interactions between the neurones in the intact Cb. They have shown that in decerebrate (rather than anesthetized) preparations, the P neurones are normally evoked in bursts and only after an injection of pentobarbital (15-20 mg/kg) did the P neurones respond in single spikes. They proposed that the anesthetic increased the inhibition by decreasing the rate of spontaneous firing in P neurones. This in turn would result in a disinhibition of basket cells which are normally under a tonic inhibition by P neurone recurrent collaterals. A subsequent study by Murphy & Sabah (1971b) offers some compromise in the disagreement between Eccles and Bloedel & Roberts since they have found that parallel fiber stimulation could usually produce 1 to 3 spikes in both anesthetized and decerebrate preparations.

Stimulation of the subcortical region of the Cb (white matter) can also produce synaptic excitation of Golgi, basket and stellate cells. In this case, however, multiple discharges are rare and mostly single responses are seen. P neurones also respond to such stimulation (see climbing and mossy fibers below) but in addition they can be excited antidromically. The antidromic activations are identified by a short latency (less than 1 msec), an ability to follow repetitive stimulation up to 300/sec and a characteristic inflexion on the rising phase of the spike potentials (IS-SD inflexion), which presumably signals a delay in the invasion between the axon and the large soma-dendritic expansion (Eccles et al., 1966c). The antidromic activation is a characteristic feature of P neurones and is frequently used to distinguish them from other cells in the Cb cortex.

The axons of P neurones are known to give off collaterals which feed back to the cortex and terminate on Golgi, basket and P neurones (Palay & Chan-Palay, 1974). Weak inhibition of Golgi cells has been observed after J.F. (Juxta-Fastigial, i.e. stimulation of the white matter near the fastigial nucleus) stimulation in the intact cerebellum and was attributed to P neurone collaterals (Eccles et al., 1966a). The effectiveness of collateral inhibition on P neurones has been tested on deafferented cerebellum (cut cerebellar peduncles) to avoid any possible effects produced by the activation of cerebellar afferents, and indeed an inhibition could be evoked by J.F. stimulation. This inhibition was much weaker than the inhibition produced by cortical stimulation (Eccles et al., 1966c). The inhibition of P neurones by collaterals has been further confirmed by Snider et al. (1968) who observed it in isolated folia of the cerebellar cortex. They used a transfolial stimulation in order to activate axon collaterals spreading to the adjacent folia.

b) Cerebellar Afferents

1) Climbing fibers

Stimulation of the inferior olive produces a strong synaptic activation of the P neurones which can follow a repetitive stimulation up to 100/sec. Such an activation is attributed to the climbing fibers demonstrated by a variety of histological techniques and known to originate from the inferior olive (Eccles et al., 1967, p. 36-42). The climbing fiber activation is so strong that even an inhibition sufficient to block the antidromic responses in the P neurone is unable to block the climbing fiber response. A peculiar characteristic of the

climbing fiber activation is a multiple (several spikes) response. The threshold for all spikes within a single climbing fiber burst is very similar, so the whole burst appears as an all or none response. As shown by Llinas & Volkind (1973), the bursts are produced by large all or none EPSPs. A J.F. stimulation can also produce a climbing fiber response, but in this case it is thought that the afferent axons are stimulated. Sometimes the J.F. stimulus evoked climbing fiber responses at a very long latency and it has been proposed that these are evoked indirectly via inferior olive. Recently, Llinas et al. (1974) demonstrated electrotonic coupling between neurones in the inferior olive and suggested that such coupling is responsible for these "reflex", long latency climbing fiber activations. Climbing fibers also project to the inhibitory interneurones within the cortex and presumably they are responsible for a weak activation of these interneurones following the stimulation of the inferior olive (Eccles et al., 1967).

2) Mossy fibers

A number of cerebellar afferent systems, such as spinocerebellar, pontocerebellar, vestibulocerebellar, reticulocerebellar and cuneocerebellar, terminate as mossy fibers in the granular layer of the Cb cortex and form synaptic connections with granule and Golgi cells (Eccles et al., 1967; Sasaki & Strata, 1967). The axon terminals have characteristic swellings and form complex synaptic structures (glomeruli) together with granule and Golgi cell dendrites and with Golgi cell axons. Electrical activation of the mossy fibers can be accomplished either by stimulation of afferent pathways outside the Cb or by a J.F. stimulation, since afferent fibers travel through this area. Stimulation of mossy fibers produces an excitation of granule and Golgi cells at latencies compatible with

monosynaptic transmission. Granule cells respond with bursts of spikes lasting 10-20 msec and Golgi cells with single spikes (Eccles et al., 1966e). The activation of granule cells is followed by an inhibition lasting up to 100 msec. The inhibition is thought to be produced by Golgi cells and differs slightly in time course from the inhibition caused by basket and stellate neurones.

P neurones are also excited by mossy fiber volleys but with longer latencies because they are mediated by granule cell axons. The excitation is usually in the form of single spikes and is followed by an inhibition (predominantly due to basket and stellate neurones). The evoked spikes have a variable latency and can follow stimulation up to 10/sec. The mossy fiber activation of P neurones by single stimuli is indistinguishable from that seen after the surface cortical stimulation. However, these two activations have different characteristics during repetitive stimulation, apparently because the mossy fiber path is inhibited by Golgi cells whereas the parallel fiber volley is not (Eccles et al., 1966b).

Stellate and basket cells are also excited by the mossy fibers via granule cells but unlike the case with surface cortical stimulation (see above) they usually respond to the mossy fiber stimulation with single spikes. It is believed that recurrent axon collaterals of P neurones are responsible for the suppression of bursts in these interneurones.

Afferent fibers (both climbing and mossy) give off axon collaterals to the deep Cb nuclei. Their presence has been shown electrophysiologically by Ito et al. (1970b) for vestibular and pontine afferents. The collaterals are thought to provide the excitatory drive to deep nuclear neurones which are strongly inhibited by cortical efferents.

3) Afferents from the locus coeruleus

The third type of afferent fibers which has been reasonably well established is the noradrenergic projection from locus coeruleus to the Cb cortex.

At first, Bloom et al. (1971) demonstrated the presence of thin, unmyelinated catecholamine containing fibers which terminate in the vicinity of P neurones. Subsequently, the same group of investigators (Siggins et al., 1971) demonstrated electrophysiologically that a stimulation of nucleus locus coeruleus can activate this pathway and produce long lasting inhibition of P neurones. The inhibition has a slower onset and much longer duration than the inhibition produced by intracerebellar interneurones. The most potent inhibition was seen following stimulation of locus coeruleus with a train of stimuli at a frequency of about 10/sec. These characteristics suggest a totally different mode of synaptic action from that of intracerebellar origin.

c) Cerebellar Efferents

Anatomical evidence for the projection from the Cb cortex to the cerebellar and vestibular nuclei was obtained mostly from studies using degeneration techniques. Walberg & Jansen (1961) showed the ipsilateral projection from the Cb vermis to the vestibular nuclei in cats. The majority of the fibers terminated in the lateral vestibular nucleus and only a few in the inferior and superior nuclei.

Corticofugal projections to the Cb nuclei have been shown by a number of investigators in cats (e.g. Courville et al., 1973) and in rats (Goodman et al., 1963). An especially elegant demonstration of the cortico-nuclear projection to the lateral Cb nucleus was given by

Chan-Palay (1973a) using the Golgi staining method. A general topographical organization of P neurone axons is as follows: P neurones from the cerebellar vermis (medial cortical zone) and from the medial part of the paravermis project to the vestibular nuclei and to the fastigial nucleus; P neurones from the paravermal area project mostly to the interpositus nucleus and those from the lateral cortical zone project to the lateral (dentate) nucleus. However, some overlap between these topographical zones has been observed (Courville et al., 1973).

The synaptic terminals of P neurones were identified and characterized particularly well in the dentate nucleus using the Golgi staining method (Chan-Palay, 1973a). P neurone axons are thought to terminate on both large and small neurones of the dentate (Chan-Palay, 1973b). The large neurones send axons outside of the Cb and give off numerous recurrent collaterals. The small neurones form only intra-nuclear connections (see diagram on page 7).

Electrical stimulation of the Cb cortex results in an inhibition of neurones within the deep Cb and vestibular nuclei. Intracellular recordings from giant Deiter's neurones in the lateral vestibular nucleus revealed evoked IPSPs with monosynaptic latencies (Ito & Yoshida, 1966). Concomitant extracellular recordings showed that impulses are propagated from the Cb cortex along the path followed by P neurone axons and terminate in the vestibular nuclei. It appears, therefore, that axons of P neurones are inhibitory and that they constitute the only efferent pathway from the Cb cortex. Analogous results have been obtained in deep Cb nuclei (Ito et al., 1970a). The topographical distribution of the monosynaptic projections is in a general agreement with anatomical findings.

Monosynaptic IPSPs are often followed by delayed IPSPs resulting from transsynaptic activations of P neurones from outside of the monosynaptic zones. The inhibition by P neurones is often followed by a prolonged facilitation which is the result of a disinhibition (i.e. a removal of the background inhibition). This was shown clearly by Ito et al. (1968) who found that the reversal potential for the facilitation is the same as for the evoked IPSPs. The disinhibition is believed to be the result of an inhibition of P neurones in the cortex, and in fact, a suppression of the activity in the P neurone axons was observed during the disinhibition.

Projections of the neurones in the Cb nuclei which constitute a major target for P neurone inhibition have been studied electrophysiologically by numerous investigators. It was found that the neurones from these nuclei are predominantly excitatory. Stimulation of the fastigial nucleus produces excitation of neurones in the medullary reticular formation and in the vestibular nuclei (Ito et al., 1970b), stimulation of the interpositus nucleus excites the neurones in the red nucleus and the thalamus (Tsukuhara et al., 1967; Toyama et al., 1968) and stimulation of the dentate nucleus excites the neurones in the ventrolateral nucleus of the thalamus (Uno et al., 1970).

d) Development of Neuronal Connections in the Rat Cerebellum

The development of synaptic connections has been studied electrophysiologically in the Cb of rats. Woodward et al. (1971a) found that local stimulation of the Cb cortexproduces excitation of P neurones at 4 days after birth. The delays of such responses were much longer and the variations in latency much greater than those seen in adults. These responses were attributed to immature parallel fiber connections to P

neurones. Similar responses were seen after stimulation of the peduncular region of the Cb.

Crepel (1974) found some connections between mossy fibers and P neurones in 3-4 day old rats. He observed, however, that most synapses develop between the 10th and the 30th day. In addition, he found that the inhibitory function of interneurones within the Cb cortex develops at 10-15 days. Occasional climbing fiber responses of P neurones were observed at 3 days but the mature responses did not appear until day 10. Antidromic activations of P neurones were observed in a few cases at day 3 after birth, and consistently after day 6. Similar findings are reported by Shimono et al. (1976) although they have observed presumed antidromic activations of P neurones as early as 1-2 days after birth. The typical spontaneous activity of P neurones is attained in rats at 10-15 days. Such activity is characterized by an irregular rhythm at ≈30/sec interrupted by fast bursts (Woodward et al., 1969).

Part II - Neurotransmitters in the Cerebellum

a) γ-Aminobutyric Acid (GABA)

The major candidate for an inhibitory neurotransmitter in the cerebellum (Cb) is GABA. The highest concentration of this amino acid has been detected in the Purkinje cell layer by Kuriyama et al. (1966). They also analyzed the cerebellar tissue of the rabbit for glutamic acid decarboxylase (GAD), the main synthesizing enzyme for GABA, and found that the distribution of activity of GAD paralleled that of GABA. Since the previous studies showed presence of GAD in the synaptosomal fraction of the Cb cortex the authors suggest that both GAD and GABA are present mainly in the axon terminals of the inhibitory interneurones, i.e. basket and stellate cells, and not in the somas of Purkinje (P) neurones. The second highest levels of GABA and GAD were present in the molecular layer of the Cb cortex which contains somas of basket and stellate cells. GABA-transaminase (GABA-T), an enzyme which is thought to break down GABA, was however shown to be present mainly in Purkinje and Golgi cells (histochemical assay was used). Only weak enzymatic activity was detected in the basket and stellate cells (Kuriyama et al., 1966).

Hökfelt & Ljungdahl (1972) used a different approach to study GABA neurones in the Cb. They injected small amounts of ^{3}H-GABA into the rat Cb in the presence of amino-oxyacetic acid (AOAA) (a drug which inhibits the breakdown of GABA) and studied the uptake of GABA by various neurones in the cerebral and cerebellar cortices using autoradiography. They found that radioactive GABA was taken up by stellate, basket and Golgi cells but not by P neurones. Similar results were obtained by Schon & Iversen (1972). Although GABA and GAD are not present in the somas of P cells they were found in the P neurone axon terminals of cats (Fonnum

et al., 1970; Fonnum & Walberg, 1973). Controversy still exists, however, as to the location of GABA in the synaptic vesicles found in such terminals (Davidson, 1976, p. 75).

Obata & Takeda (1969) were able to demonstrate that electrical stimulation of the cat Cb cortex in the presence of AOAA produced an increase in the release of GABA into the fourth ventricle. It is probable that during such stimulation GABA was released from the axon terminals of P neurones which are known to project to the areas near the fourth ventricle (deep Cb nuclei and vestibular nuclei).

GABA is known to have an inhibitory effect on neurones in the CNS and iontophoretic applications of GABA onto the neurones in the Cb cortex produce an inhibition of ongoing spiking activity. For example, Woodward et al. (1971b) showed that GABA mimics the effects of stellate cell inhibition in the frog Cb by producing inhibition of P neurone discharges accompanied by hyperpolarization and reduction of the membrane resistance. A similar action of GABA was found on the neurones of the lateral vestibular nucleus which receive P neurone axons (Obata et al., 1967; Bruggencate & Engberg, 1969a). However, other amino acids, for example Glycine, β-alanine and taurine also have inhibitory effects on both the cortical and vestibular neurones. No definite conclusions can be drawn concerning the differences in potency on the basis of iontophoretic application because all authors give only the values for the iontophoretic currents and these depend not only on the potency of the amino acids but also on the transport number (transport numbers were not determined). Nevertheless, GABA usually appears to be more effective than other amino acids. The estimation of actual concentrations of these amino acids at the neuronal membrane is not feasible with the use of iontophoresis.

GABA can inhibit the activity of cortical neurones when applied into the solution surrounding explanted cerebellar tissue. Okamoto & Quastel (1973) found that GABA concentrations above 0.05 mM can depress neuronal activity in explanted slices of guinea pig Cb while Glycine was 2-10 times less potent.

Gähwiler (1975a, 1976) observed that GABA in concentrations of 10^{-6} - 10^{-5} M abolished the spontaneous activity of cerebellar neurones in tissue culture while Glycine had no effect up to 10^{-3} M. In spite of large differences between these two in vitro studies they both showed that GABA is more potent than Glycine.

To show convincingly that GABA and not Glycine is the inhibitory neurotransmitter released by the neurones in the Cb, three pharmacological compounds have been frequently used; Strychnine (Glycine blocker), Picrotoxin (GABA blocker) and Bicuculline (GABA blocker). Okamoto & Quastel (1976) showed that application of Strychnine (0.005 - 0.1 mM) into the perfusing solution blocked the inhibitory effects of Glycine, β-alanine and taurine but not that of GABA. Picrotoxin (0.1 mM) on the other hand blocked the inhibitory action of GABA but not that of the other amino acids. In the intact Cb iontophoretically applied Strychnine can selectively eliminate Glycine and β-alanine depression leaving GABA effects unchanged (Curtis et al., 1971b). Similar applications of Bicuculline abolish GABA depressions but not those of Glycine. However, Bicuculline also blocks the action of β-alanine. In contrast, in lateral vestibular nucleus Bicuculline blocks only GABA depressions without blocking β-alanine.

Bruggencate & Engberg (1969a,b, 1971) extensively studied the effects of GABA, Glycine and other depressant amino acids together with the blockers Strychnine and Picrotoxin on Deiter's neurones in the lateral

vestibular nucleus. Using intracellular recordings they have observed that all of the amino acids exert rather similar depressant actions i.e. they all produce hyperpolarization associated with an increase in membrane conductance and their effects have the same reversal potential. Iontophoretic applications of Strychnine blocked the action of Glycine but not that of GABA whereas Picrotoxin had the opposite effect.

On the basis of kinetic analysis of dose-response curves for GABA and Glycine in control solutions and with respective blockers it has been proposed that the action of the blockers is competitive, i.e. they presumably compete with their respective amino acids for the receptor sites on the Cb neurones (Obata et al., 1970a; Okamoto & Quastel, 1976)

The discovery of such blocking agents as Strychnine, Picrotoxin and Bicuculline has proven useful in the investigation of the inhibitory neurotransmitters in the cerebellar pathways, although major problems have been encountered in their administration into the brain. Iontophoretic applications are often difficult especially in the case of Bicuculline and Strychnine which are poorly soluble in water. Another major problem is the uncertainty with regard to the effective concentration of a drug at the neuronal membrane and its uneven distribution around the neurone. Intravenous injections have also several disadvantages, in particular the unknown concentration of the drug at the recording site and frequent increase of the background activity of the neurones (convulsive driving) (Straughan, 1974). An alternative is offered by in vitro preparations such as brain slices and tissue cultures. In this case, the drugs can be applied in known concentrations into the perfusing solution (Gähwiler, 1975a) or locally onto the neurones by means of large pipettes (see e.g. Ransom et al., 1977).

In spite of all difficulties a large number of studies have been done on the Cb *in situ* using GABA and Glycine blockers. Woodward *et al.* (1971b) for example, found that the evoked stellate cell inhibition of P neurones in the Cb cortex of the frog was blocked by iontophoretic application of Picrotoxin but not by Strychnine. On this basis they proposed that the inhibition is mediated by GABA. Curtis & Felix (1971) reached the same conclusion after observing that the electrically evoked inhibition (presumed basket/stellate inhibition) of P neurones in the cat was blocked by Bicuculline applied iontophoretically or intravenously (0.2 - 0.6 mg/kg). In addition, Bisti *et al.* (1971) clearly demonstrated that the inhibition by Golgi cells in the Cb cortex is also blocked by 0.2 mg/kg Bicuculline or 3-5 mg/kg Picrotoxin.

Obata *et al.* (1967, 1970a) studied the evoked P neurone inhibition of vestibular neurones and found that Picrotoxin (5-10 mg/kg i.v.) blocked the inhibition whereas Strychnine (0.3 mg/kg i.v.) had no effect. The iontophoretic applications of either drug gave similar results. Ito *et al.* (1970c) also observed blocking of the evoked inhibition with Picrotoxin.

All such studies involving pharmacological blockers suggest that GABA is the inhibitory neurotransmitter in the Cb. However, other related amino acids, β-alanine, taurine, β-OH GABA and δ-aminovaleric acid cannot be excluded and even in the case of Glycine one has to be cautious because the specificity of the blocking agents has been questioned by several investigators. Davidoff *et al.* (1969) reported that in the spinal cord iontophoretically applied Strychnine does block Glycine more effectively than GABA but the difference in the degree of blocking is only quantitative and at higher doses of Strychnine, GABA was also affected. In various *in vitro* preparations of supraspinal parts

of the brain, (Gähwiler, 1976; Yamamoto, 1972; Crain, 1976) Strychnine was found to have potent convulsive effects at 10^{-8} - 10^{-5} M suggesting either unspecific effects of this drug or the presence of Glycine mediated synapses.

Godfraind et al. (1970) had consistent difficulty in observing any antagonistic action of Bicuculline on GABA inhibition in the cerebral cortex. Barnardi et al. (1976) questioned the specificity of Bicuculline in blocking the evoked inhibition in caudate neurones since they have shown by intracellular recordings that large variations in the membrane potential and membrane resistance may occur during the intravenous application of this blocker (0.3 μM/kg). Picrotoxin, however, (0.3 μM/kg) acted relatively specifically on the evoked IPSPs.

It appears therefore that the specificity of GABA and Glycine blockers can be assured only if it is tested and proven on the same type of preparation (and preferably on the same type of neurone) in which the synaptic inhibition is to be studied.

Nevertheless the evidence accumulated so far in the Cb cortex and the lateral vestibular nucleus is in favour of GABA being the neurotransmitter at inhibitory synapses. GABA fulfills most of the criteria usually specified to identify the substance as an inhibitory neurotransmitter:

1) It is present in the Cb neurones or in the axon terminals which were shown to be inhibitory by electrophysiological techniques (stellate, basket, Golgi and P neurones).

2) It is accompanied by the required synthesizing enzyme GAD.

3) It is released into the IV ventricle following P neurone stimulation.

4) It hyperpolarizes the neurones and produces a decrease in the membrane resistance suggesting opening of the ionic channels.

5) Its depressant action as well as evoked inhibition are blocked by Bicuculline and Picrotoxin.

6) Its inactivation after synaptic action is probably accomplished by a reuptake into the presynaptic terminals (Davidson, 1976, p. 76).

b) Noradrenaline (NE)

NE is another neurotransmitter which has been studied in the Cb. Noradrenergic fibers have been described in detail by Hökfelt & Fuxe (1969) and Bloom et al. (1971). Using histochemical autoradiographic and E.M. techniques they showed that noradrenergic fibers are thin, unmyelinated axons originating from the lower brainstem, probably from locus coeruleus (Olson & Fuxe, 1971). These fibers were found to project to the molecular, P neurone and granular layers of the Cb cortex. Also a sparse projection to the Cb nuclei has been found. Detailed E.M. observations showed the presence of axodendritic synapses on the P neurones which are probably formed by the noradrenergic fibers. Using permanganate fixation for E.M. it was shown that the terminals contain dense core vesicles, a characteristic of monoaminergic synapses.

Another study by Hoffer et al. (1971) showed that the NE contained within the afferent fibers may indeed play a role as an inhibitory neurotransmitter. Using iontophoresis they showed that NE depresses the spontaneous activity of "virtually all" P neurones tested. Not only the mean rate of firing was decreased but also the pattern was changed, i.e. NE increased the number of long interspike intervals without changing the short ones. A proposed β-adrenergic blocker, MJ-1999, suppressed the action of NE on P neurones. It is of interest that neurones in locus coeruleus are also inhibited by NE but their receptors

seem to be of α-type (Cederbaum & Aghajanian, 1976). Siggins et al. (1971) confirmed electrophysiologically the proposed NE projection from locus coeruleus to the Cb since they showed that electrical stimulation of locus coeruleus could produce a depression of firing of P neurones.

Recently Moises & Woodward (1976) suggested that NE may also have other effects. They were able to demonstrate that iontophoretic application of NE actually enhances the effects of Glutamate and GABA. Hoffer et al. (1975) demonstrated this phenomenon electrophysiologically by showing that evoked, synaptic responses are also enhanced during iontophoretic applications of NE.

c) Glutamate

Granule cells are the only excitatory neurones in the Cb cortex; their number, however, is very large. There have been only a few studies concerning the neurotransmitter released by these cells. It is known that some amino acids like Glutamate and Aspartate occur in large concentration in the Cb (e.g. Berl & Waelsch, 1958). However, only part of the total amino acid pool - that in the synaptic fraction - is likely to be involved in the synaptic transmission. The high affinity uptake system (Km<50 μM) for Glutamate and Aspartate is present in the cerebellar synaptosomes and it decreases in parallel with the depletion of the granule cells by a virus (Young et al., 1974). It was also found that the concentration of Glutamate but not that of Aspartate decreased by approximately 40%. These results suggest that Glutamate is a neurotransmitter present in the axon terminals of granule cells. Glutamate applied iontophoretically does have an excitatory action on P neurones thus mimicking the synaptic action of granule cell axons

(Krnjevic & Phillis, 1963). Other evidence to identify Glutamate as a neurotransmitter is missing. The major obstacle in further investigations is the lack of a selective blocker for Glutamate. There are several proposed blockers such as glutamic acid diethyl ester (GDEE), HA-966 and glutamic acid dimethyl ester (GDME). There seems to be a general agreement that these blockers reduce the excitation produced by excitatory amino acids more than that produced by acetylcholine (Davies & Watkins, 1972; Curtis et al., 1972). Only GDEE seems to have a relatively selective action against Glutamate among other excitatory amino acids (Haldeman et al., 1972; Haldeman & McLennan, 1972; McLennan, 1974). However, evidence is still contradictory (Krnjevic, 1974, p. 466).

Part III - Review of Tissue Culture Work

The first research paper on tissue cultures of the nervous system appeared in 1910; at that time Harrison was already able to culture explants of frog spinal cord for several weeks. During the next 50 years many tissues from a variety of species were cultured and several techniques were developed (Murray, 1965). It was not until 1956 that Crain published the first evidence of evoked electrical activity in neurones from chick dorsal root ganglia (DRG). Although he did not observe any intrinsic activity, the neurones had resting potentials, and action potentials could be produced by electrical stimulation. This study showed that neurones in culture develop at least some typical electrical characteristics.

The first central nervous system (CNS) tissue to be used for electrophysiological studies in culture was the cerebellum; Hild & Tasaki (1962) recorded both spontaneous and evoked electrical activity from cerebellar neurones, thus demonstrating physiological properties in cultures comparable to those *in vivo*. However, they did not detect any synaptic potentials in the neurones.

In the following years Crain & Peterson (1963, 1964) demonstrated complex discharges in fetal mouse cord explants, suggesting the presence of complex synaptic networks.

Many subsequent studies confirmed the presence of the synaptic connections between cultured neurones from a variety of tissues.

a) Dissociated Cultures

Dissociated cultures are prepared from tissue fragments which are mechanically or enzymatically dissociated prior to incubation. Cultures of DRG for example are often dissociated by gentle spreading with forceps as well as digestion with 0.25% trypsin in salt solution (see for example Scott et al., 1969; Peacock et al., 1973). Such a procedure not only separates the cells from each other but also causes substantial damage to cell processes. Nevertheless the processes can regenerate. It is probably this regenerative capability that determines the optimal age of tissues used for cultures. Nelson & Peacock (1973) used mice fetuses of different ages for dissociated cultures of cerebellum and the most successful cultures were obtained from those 11-16 days old. In fact, dissociated cultures of the nervous system are always obtained from either fetuses or animals up to a few days old, the exact age depending on the species and tissues used. Following dissociation, the cells are usually placed in Petri dishes coated with either rat-tail collagen (Bornstein, 1958) or a synthetic substance to which the tissue becomes attached. Subsequently, the cultures are incubated for several weeks in order to attain maximal growth and differentiation of the neurones. A frequently used procedure is to reduce the excessive number of non-neuronal cells by treatment with an antimitotic agent, such as cytosine arabinoside (e.g. Nelson & Peacock, 1973; Ko et al., 1976).

Dissociated cultures have been used in numerous electrophysiological experiments, the main purpose being the study of electrical properties of the neurones as well as their synaptic interactions. The experimental routine in such experiments consists of placing the cultures on the

stage of an inverted microscope and performing extracellular or intracellular recordings under visual control. During the experiments, the cultures are bathed in a physiological solution which can be changed or modified. The chemical composition of solutions which are most frequently used is presented in Table 1.

Neurones from DRG of a chick were studied by Scott et al. (1969) who found that resting potentials and action potentials were similar to those found in vivo. However, no evidence of spontaneous activity could be obtained. Peacock et al. (1973) performed more advanced studies on mouse cultures including both spinal cord and DRG. These workers were able to demonstrate spontaneous activity in the neurones from spinal cord and DRG and they also showed that the DRG neurones had retained the characteristic shape of their action potentials. In addition, several pairs of neurones; spinal cord-spinal cord, and DRG-spinal cord were recorded which showed synaptic coupling. Similarly, Fischbach & Dichter (1974) recorded from pairs of neurones in chick spinal cord and showed that IPSPs, EPSPs and action potentials could be generated in some of these cells by stimulation of other distant cells. The postsynaptic potentials were easily reversed by passing current through the recording electrodes. They also measured the conduction velocity along unmyelinated neurites and some current-voltage relationships of cells. Nelson & Peacock (1973) performed similar studies on cerebellar neurones of mouse and also found evidence for both spontaneous and evoked postsynaptic potentials.

The major problem in most of such studies is to identify the type of neurones from which one is recording. For example, it is very difficult to distinguish motor neurones from other types of neurones found in tissue cultures of the spinal cord. The main abnormality found in all but a few of these studies is the lack of myelinated fibers.

Further studies performed on dissociated cultures involved the determination of pharmacological characteristics of synapses. Obata (1974) found that neurones from rat DRG and superior cervical ganglion (SCG) in cultures are depolarized by GABA applied either iontophoretically or into the bath. Such an effect by GABA was expected from *in vivo* experiments (e.g. Krnjevic, 1974). Acetylcholine depolarized SCG but not DRG cells (a finding also in agreement with *in vivo* experiments). The action of acetylcholine on the SCG cells was blocked by d-tubocurarine but not by atropine.

The extension of these studies by O'Lague and coworkers (O'Lague *et al.*, 1974) gave rather unexpected results. They showed that excitatory, cholinergic-nicotinic synapses are present on neurones in cultures of isolated SCG, i.e. they are formed by cells within the ganglion. They also showed that when skeletal muscle fibers are incubated together with SCG, cholinergic neuromuscular synapses are formed with similar pharmacological properties. No evidence of noradrenergic synapses could be found (Nurse & O'Lague, 1975). On the other hand a study by Ko *et al.* (1976) who utilized combined explant cultures of spinal cord and dissociated neurones from SCG, found normal cholinergic-nicotinic synaptic connections formed by axons of spinal cord neurones on SCG neurones.

It has been established therefore that neurones in dissociated cultures develop normal electrical characteristics and can form functional synapses. However, the chemical nature of the neurotransmitters and the sensitivity of synaptic receptors to pharmacological agents may differ from those found *in vivo*.

Recently Nelson and his associates published a series of articles

which illustrate how the dissociated cultures can be successfully used in studying the electrophysiological and pharmacological properties of neurones. One particularly interesting paper is the last one of the series, which deals with the sensitivity of spinal cord neurones to two putative neurotransmitters: Glycine and GABA (Nelson et al., 1977). They have demonstrated that a Glycine (0.4 mM) or GABA (0.4 mM) containing medium desensitized the spinal cord cells to Glycine and GABA respectively but there was no cross desensitization indicating that there are separate receptors for these two amino acids. GABA produced a depression of all spontaneous synaptic activity in neurones whereas Glycine decreased the frequency of IPSPs only. On the basis of these and other observations the authors propose that Glycine is the major inhibitory substance in the spinal cord while GABA may have a presynaptic action. Such proposals are in agreement with in vivo studies (e.g.Krnjevic, 1974).

In some of the dissociated cultures, cells are capable of aggregating into groups, and fibers may form fascicles. The synaptic connections in such reaggregated cultures are quite complex (Crain & Bornstein, 1972). These cultures may bridge the gap between dissociated cultures and explant cultures which will be described next.

b) Explant Cultures

A typical feature of explant cultures is the retention of the topographical distribution of neurones characteristic for a given tissue. Two different techniques are available for growing explant cultures; the roller tube method and the Maximow method.

The roller tube method was used by Hild & Tasaki (1962) for

cultures of cerebellum. They explanted fragments of tissue, placed them in a drop of plasma on a coverslip and subsequently added chick embryo extract to form the clot which kept the tissue in place. The coverslip was then placed in a test tube containing a nutrient solution. The test tube was constantly rotated in a drum, so that the fluid could gently spread the tissue down to a monolayer of cells.

The Maximow method was adapted to nervous tissue by Murray (see Murray, 1965). Explants are placed on a collagen coated coverslip, covered with a drop of feed and sealed in a Maximow chamber (see methods). During incubation for several weeks, the cells attach to the collagen base and spread to form a layer a few hundred micra thick. The living cultures of both kinds can be examined under a microscope. The experimental setup for electrophysiological recordings is usually quite similar to that described for dissociated cultures. However, the great majority of workers perform extracellular rather than intracellular recordings in these cultures. Salt solutions most frequently used for maintaining the cultures during the experiments are listed in Table 1.

Explant cultures have been used for studying the synaptic interactions between groups of neurons. For example, recordings by Crain & Peterson (1964) revealed that in cultures containing explants of both DRG and spinal cord single electrical stimuli in DRG area evoked long lasting (up to 500 msec) field responses in the spinal cord. Using standard electrophysiological criteria, such as potentiation by a second stimulus, the authors established that these responses were synaptically mediated. In addition, a Glycine blocker, Strychnine (1-10 µg/ml), was found to potentiate the responses presumably by removing a tonic (Glycine) inhibition. Other studies by Crain & Bornstein (1964, 1974) showed that

not only can synaptic connections be found in explant cultures but also that they are actually formed in culture. By taking explants from very young fetal mouse neo-cortex and hippocampus, these authors were assured that the synapses found in these cultures must have been formed in culture. In fact, in younger explants (1-2 DIV), only short latency, short duration responses were seen - these could have been recorded from neurites. Parallel E.M. studies demonstrated only a few immature synapses. Older explants showed long lasting spontaneous and evoked discharges and numerous synaptic profiles under E.M.

One particularly interesting type of response seen in explants of neo-cortex is a short latency positive wave followed by several repetitive discharges at a frequency of about 10/sec (Crain & Bornstein, 1964). The response occurs synchronously throughout a wide area of the explant and probably represents the synaptic activity in a complex reverberating network of neurones. Similar repetitive, synchronous discharges of neurones were also reported by Calvet (1974). It is suggested by Crain (1976) that the response patterns are reminiscent of those found in isolated pieces of neonatal cortex in situ. It appears possible therefore that the neuronal circuitry in both cases is similar.

Somewhat similar responses were obtained in hippocampal explants using both extracellular (Crain, 1976) and intracellular (Zipser et al., 1973) recordings. These intracellular records are particularly valuable as they provide evidence for some of the mechanisms responsible for the generation of long lasting neuronal discharges. Most notably long lasting (1-2 sec) depolarizing potentials were observed which could be increased in time and amplitude even more by Strychnine or Bicuculline (10^{-6} M).

Crain and coworkers performed numerous studies on other parts of the brain grown in cultures and consistently demonstrated "organotypic" synaptic networks (review by Crain, 1976).

In many cases the evoked and spontaneous neuronal discharges could be modified by application of various pharmacological agents such as Bicuculline, Picrotoxin, Strychnine, d-tubocurarine, caffeine and more recently (Crain et al., 1977) opiates. These experiments illustrate the advantages of explant tissue cultures in pharmacological studies over in vivo preparations. Particularly important is the opportunity of adding the drugs to the bathing solution in known concentrations without the interference of blood-brain barrier or vascular side effects.

One agent which has been extensively used by Crain (review in Crain, 1976) and others (e.g. Gähwiler et al., 1973) is the magnesium ion (Mg). 5-15 mM Mg has been used in tissue culture and other in vitro preparations (e.g. Richards & Sercombe, 1970) to identify electrical signals which cross chemical synapses on the premise that such signals are more sensitive to Mg interference than ones which do not. Although such an action of Mg is supported by studies on peripheral synapses (del Castillo & Engbaek , 1954; Jenkinson, 1957) it has also been known that Mg has a direct depressant effect on the neuronal membrane (Frankenhauser & Meves, 1958). The actions of Mg on transmitter release are antagonized by calcium ions while membrane stabilizing effects of these divalent cations are additive. In addition it appears that in the CNS this direct effect may be even more pronounced than in the periphery (e.g. Kato et al., 1968; Erulkar et al., 1974; Hackett & Llinas, 1973; Marshall et al., 1975). Crain (1976, p. 168) has acknowledged this evidence and used Mg with caution in order to avoid any direct depressant effect. This was

apparently possible if the concentration of Mg was adjusted individually for each experiment so the simple spike responses (presumably direct responses of neurites) were not changed and yet more complex responses (presumably synaptically mediated) were blocked.

The accumulated evidence obtained from intracellular and extracellular recordings as well as from the effects of pharmacological agents proves that explant cultures contain complex networks of synaptically interconnected neurones and that some of these connections are similar to their counterparts in vivo. No evidence of abnormal connections has been found as yet; therefore, these cultures have frequently been called organotypic.

The two culture systems, dissociated cultures and explant cultures, are both useful and complementary in the study of neural tissue. Dissociated cultures offer more accessibility to individual neurones, thus better opportunity to study membrane properties, mechanisms of synaptic transmission, etc. A particularly useful aspect of these cultures is the easy visualization of neural processes under the microscope due to the lack of neuroglia. The main problem is the difficulty in identifying the types of neurones, which completely lose their topographical orientation during preparation of the tissue. Another problem is the lack of myelination. It is probable that the lack of myelin is the price paid for good visibility because the glia cells which normally surround neuronal processes are also responsible for their myelinization (see for example review by Lumsden, 1968).

Organotypic explant cultures on the other hand are suitable preparations for studying the synaptic relationships between neurones within relatively complex networks. It seems that an ideal culture

system which would combine the advantages of both dissociated and explant cultures is yet to be developed.

c) Tissue Cultures of the Cerebellum

In the previous section of this introduction different culture systems have been described together with their applications in the investigation of CNS. In this section all relevant work on tissue cultures of cerebellum will be described. Investigators in this field are using primarily two types of cultures: roller tube type and Maximow type.

Morphological features of the cerebellar roller tube cultures were described by Hild (1966) with the use of Holmes' silver stain. He has found that these cultures contained well developed neurones which apparently had only a few synaptic contacts. However, this could have been the result of a limitation of the staining technique. Electrophysiological experiments by Hild and Tasaki (1962) who were the first to record from Cb cultures, failed to detect any synaptic interactions, although they have demonstrated that the neurones were excitable and fired spontaneous impulses. These negative findings (with respect to the presence of synapses) in both morphological and physiological studies must have been caused by some unfavorable tissue culture conditions because they are not in agreement with more recent physiological findings.

According to Schlapfer et al. (1972) the living roller tube cultures of rat cerebellum contain large neurones ("presumably Purkinje neurones") which can be clearly seen in the living state under the light microscope. Occasionally, dendrites and myelinated axons emerging from these cells can also be visualized. In the central part of the cultures the neurones

belonging to cerebellar nuclei could be observed. Because of the relatively good visibility of neurones in these roller tube cultures, they were subsequently used for electrophysiological and pharmacological studies. Schlapfer et al. (1972) studied different patterns of spontaneous activity of Purkinje (P) neurones in these cultures as recorded by two different electrodes. 77% of the neuronal pairs showed statistically correlated spike activity. Such a correlation, indicating more or less simultaneous firing suggests strongly the presence of synapses on these P neurones. The results can be interpreted as evidence of a common, excitatory synaptic input on P neurones supplied by the granule cells (or perhaps by deep nuclear neurones).

Another study by Gähwiler et al. (1973) supplied further evidence for the synaptic interactions in similar cultures; they argued that P neurones located in the periphery of the culture fire action potentials regularly (presumably endogenous activity) whereas others have a rather irregular rhythm which is determined partly by their endogenous rhythm and partly by the synaptic input. After addition of 8 mM Mg into the bathing solution the activity of centrally located neurones was either stopped or significantly modified whereas the activity of peripheral ones was slowed down by only about 20%. The authors concluded that since Mg is generally assumed to block synaptic transmission selectively, the relatively weak effect on peripheral neurones may be explained by the absence of active synaptic connections on these neurones. This appears to be a reasonable conclusion, but the strong effects of Mg on centrally located neurones must be interpreted with caution in view of the recent evidence for a direct depressant effect of Mg (see results).

Pentobarbital (20 µg/ml) had similar effects to those of Mg but d-Tubocurarine (10 µg/ml) or Atropine (20 µg/ml), which are cholinergic blocking agents, had no significant effect on the spontaneous activity.

In the subsequent study, Gähwiler (1975a) used roller tube cultures for investigations of the effects of GABA and its blockers Bicuculline and Picrotoxin on P neurones. As reviewed in section II of this introduction there is considerable evidence for GABA being the inhibitory neurotransmitter released by P neurones as well as by stellate, basket and Golgi cells in the cerebellum. In culture, Gähwiler found that 10^{-6} M GABA (in the perfusing solution) usually slowed the rate of firing and 10^{-5} M stopped it completely. However, it is not clear if GABA acted directly on the P neurones or on other neurones in the culture (e.g. granule cells) which in turn caused a disfacilitation of P neurones. Bicuculline (10^{-8} M) and Picrotoxin (10^{-5} M) reversed the inhibitory effects of GABA showing that these two blockers are effective in culture. In contrast, Strychnine (10^{-7} - 10^{-5} M) had no clear antagonistic action on GABA. Bicuculline and Picrotoxin also changed the pattern of the spontaneous firing in P neurones. Picrotoxin at 10^{-7} - 10^{-4} M had excitatory effect but above 10^{-4} M it depressed the activity. The excitatory effect of Picrotoxin was associated with the change of pattern of firing as shown by the interspike-interval histograms. A main feature of the changes produced by Picrotoxin was the reduction of the number of long interspike-intervals and the increase of the number of short intervals. The effects of Bicuculline were similar except that much lower concentrations were needed. 10^{-10} - 10^{-6} M produced excitation and above 10^{-6} M it caused inhibition.

Gähwiler suggests that the reduction of the number of long interspike

intervals means the reduction of the inhibitory influence upon P neurones. Clearly, however, other interpretations are possible. It is also difficult to account for the low concentration of Bicuculline used in this study. Other tissue culture experiments required much higher concentration in the range of 10^{-7} - 10^{-5}M (review by Crain, 1976).

In another study by Gähwiler (1975b) effects of noradrenaline (NE) and related drugs were shown. It was demonstrated that NE (applied into the perfusing solution) had a depressant action on P neurones in agreement with the findings in vivo. However, the effects were only partly reversible and probably require confirmation by other workers in order to be accepted.

Numerous morphological studies on cerebellar explant cultures grown in Maximow chambers have appeared (Wolf, 1964; Bornstein & Murray, 1958; Seil & Herndon, 1970, Allerand, 1971; Seil, 1972). The degree of differentiation of individual neurones and topographically separated regions in these cultures has been described best by Seil in 1972. In mouse cultures Seil could clearly differentiate between cerebellar cortex (Cx), deep nuclei (DN) and peduncular areas of the Cb interconnected by nerve fibers. He extended his observations in a recent paper (Seil & Leiman, 1977) to show that three different cerebellar nuclei (middle, interpositus and lateral) can be found in cultures depending on the part of Cb from which the explants were taken and he confirmed the morphological characteristics of a deep nuclear region reported by Hendelman & Aggerwal (1974). These very well organized cultures grown in Seil's laboratory have been used in electrophysiological studies by Leiman & Seil (1973) which demonstrated that an electrical stimulation

of one region of the culture could produce synaptically mediated responses in another part. Both excitatory and inhibitory synaptic connections were observed. Although the cultures were too thick to permit the identification of individual neurones during recording, it is probable that they stimulated and recorded in the cortical region. Some of the responses resembled roughly the excitation-inhibition sequences observed in *in vivo* P neurones after stimulation of the cortical surface (Murphy & Sabah, 1971b). It appears therefore that granule cell-P neurone synaptic connections are functional in cultures and that inhibitory interneurones are also present. This electrophysiological evidence confirmed earlier ultrastructural studies which reported the presence of the synaptic profiles in similar cultures (Seil & Herndon, 1970; Wolf & Dubois-Dalq, 1970).

In their latest paper Seil & Leiman (1977) focused their attention on the activity of DN neurones. In contrast to cortical neurones no spontaneously active cells were seen in DN. Action potentials could be induced only by mechanical pressure exerted by the electrode tip. It was proposed that the DN neurones were unable to generate intrinsic, spontaneous activity but the finding differs from observations by Calvet (1974) and Wojtowicz *et al.* (1975). Seil and Leiman found that stimulation of the DN region by a nearby electrode produced trains of spikes from the DN neurones. In addition, these neurones could be inhibited by stimulation of the cortical region. During pharmacological tests Bicuculline (10^{-6} - 10^{-4} M) was added to the bathing solution and it increased the rate of activity of cortical neurones but did not activate DN neurones.

Another group of researchers used explant cultures of Cb which have

been described morphologically by Privat & Drian (1975). In 1974, Calvet published her observations on the pattern of firing of P neurones and DN cells in Cb cultures of rats and cats. Although large variations were seen from culture to culture, P neurones were usually firing in bursts whereas DN cells fired single spikes at long intervals (several seconds). Simultaneous recordings with two electrodes were used to study the correlation of firing in pairs of neurones. No such correlation was seen in P neurones whereas spikes in DN cells often occurred simultaneously. In cultures modified by addition of an antimitotic agent (MAM) into the growing medium Calvet et al. (1974) did find correlation between bursts of P neurones. The correlated bursts were usually followed by periods of inhibition which were synchronous with positive slow waves. The major morphological change in these cultures grown with MAM is a depletion of granule cells. Additional ultrastructural evidence suggested that the number of synapses usually attributed to P neurone collaterals was increased in these cultures and it is proposed that the recurrent collaterals are responsible for the correlation of bursts and for the positive slow wave potentials.

In cultures which include peduncular regions Calvet & Lepault (1975) also observed correlated bursting activity of P neurones but without associated slow waves. The peduncular region of Cb cultures often contains neurones of brain stem origin and Calvet proposed that in this case they are responsible for the correlation of bursting between P neurones. However, no direct evidence was presented to support this suggestion.

One more study on explant cultures of the Cb appeared by Geller & Woodward (1974). They recorded spontaneous activity of P neurones with multibarrelled electrodes and tested the sensitivity of the neurones to

various putative neurotransmitters such as GABA, Glycine, Glutamate, Acetylcholine as well as some pharmacological agents - Picrotoxin and Bicuculline. They conclude that P neurones in cultures respond to all these substances in a similar way as P neurones *in vivo*.

Cb cultures used in the experiments to be described in this project are grown in the laboratory of Dr. W. Hendelman. The morphological features of these cultures compare favourably with cultures grown in other laboratories. Although their topographical organization is somewhat inferior to the cultures of Seil, the visibility of individual neurones in the living state seems to be better due to a lesser thickness of the tissue. Cx, DN and brain stem regions can be clearly distinguished in unstained cultures. The detailed morphological features of these cultures will be reviewed in part IV and are in general agreement with the findings of Seil (1972) although some differences in the development of P neurones and in the number of granule cells are apparent. These differences may be due to the different characteristics of the incubation media used in each case. Because of the differentiation of different topographical regions, these cultures are suitable for studies of synaptic transmission. In the experiments to be described an effort was made to combine most of the techniques used by other investigators in order to characterize the synaptic connections between three different regions in these cultures. Single unit recordings of spontaneous as well as evoked activity have been used to detect synaptic interactions. Iontophoresis of putative neurotransmitters and bath applications of various test drugs were used for pharmacological studies. Such a combination of techniques allowed more detailed investigations of neuronal connections in the Cb cultures than in the previous studies.

Part IV - Morphological Characteristics of Tissue Cultures Used in the Present Study

Two types of cultures were used in this project. One type (I) contains only cerebellar tissue and is obtained by dissecting the Cb as shown in Fig. 5 (see methods). In cultures of type II a small piece of brain stem (BS) tissue is included by making a cut slightly lower than that shown in Fig. 5.

A schematic topographical map of type I cultures is shown in Fig. 1. The cortical area is characterized by a spotted appearance due to the presence of small granules in the neuropil. The region of cerebellar nuclei has no granules and is characterized by a network of myelinated axons. Individual neurones in both areas can be distinguished but often only the nucleus and nucleolus are clearly seen. The morphological features of cortical and DN neurones have been studied using the Golgi-Cox method (Hendelman et al., 1978). The most typical neurones in the cortical area are P neurones, about 20 μ in size, with numerous spines on soma and dendrites (Fig. 2). The dendritic tree of P neurones in culture differs from that in vivo and generally instead of a single apical dendrite there are several dendrites emerging from the soma. As described by Aggerwal (1977) this is a normal developmental stage in the ontogeny of the P neurone occuring in vivo at 4-6 days after birth. In culture however, these perisomatic dendrites are retained during the subsequent growth and are usually not reorganized into a mature dendritic tree. Another characteristic feature of these neurones in culture is that they remain dispersed in the cortical area, i.e. they do not form a single layer. It has been proposed that this condition is the result of the

FIGURE 1: Schematic drawing showing the topography of the cerebellar cultures, and micrographs of Cx and DN neurones in living culture. The large circles in the cortical area represent Purkinje cells and the smaller ones, granule cells. Myelinated axons connecting the cortical and deep nuclear regions are shown.

The upper photograph on the right shows Purkinje neurones (arrows). The middle photograph shows myelinated fibers (arrows) which are probably axons of the Purkinje neurones. The lower photograph shows the neurones (arrows) of the deep cerebellar nuclei.

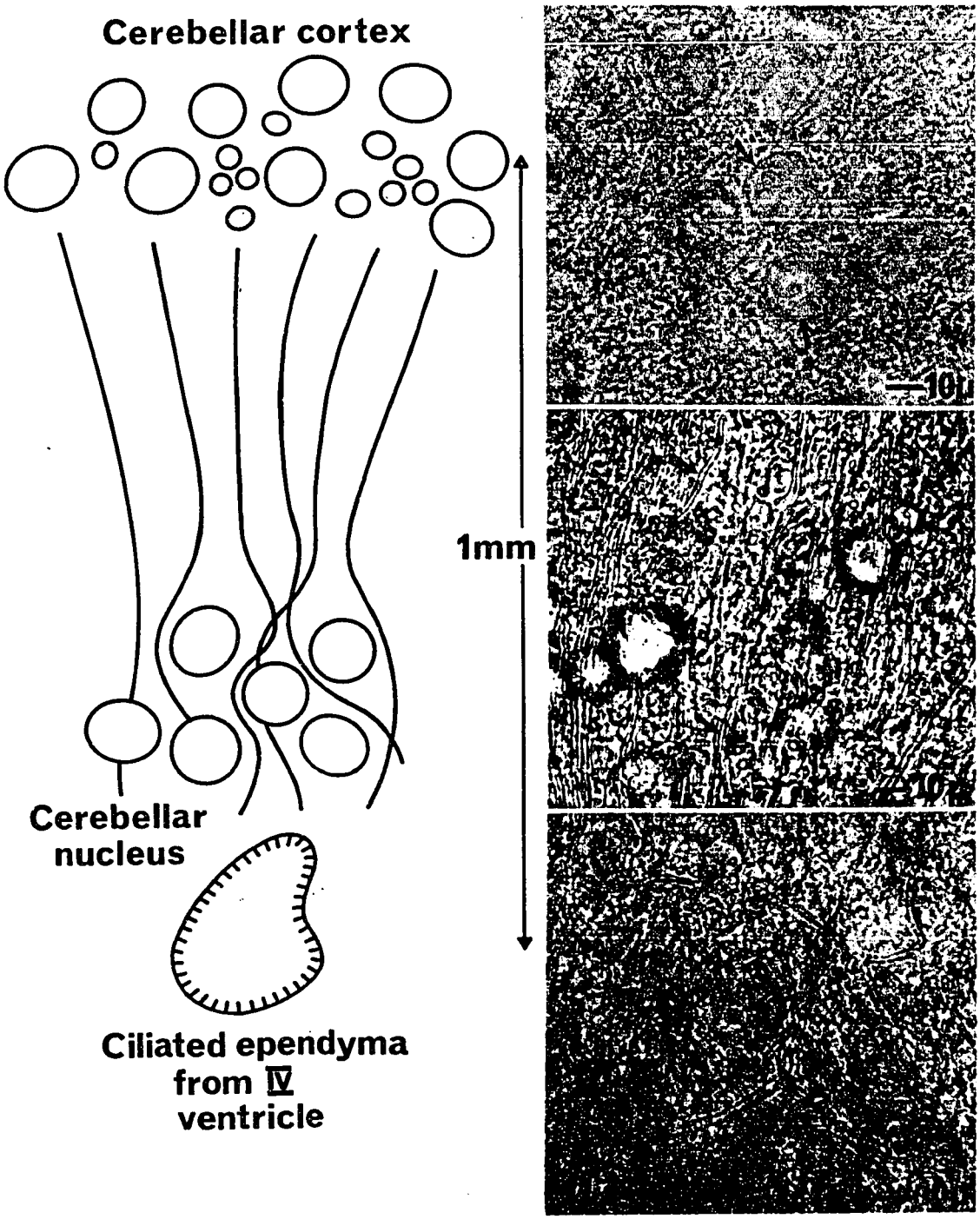

Cerebellar cortex
1mm
Cerebellar nucleus
Ciliated ependyma from IV ventricle

FIGURE 1

reduced number of granule cells in culture (Aggerwal, 1977). Other neuronal types, granule, stellate and Golgi, have also been described in the cortical area of the cultures and are similar to in vivo cerebellum (Fig. 2). In addition, detailed electron microscopy studies of the cortical region of the cultures have been carried out by Drs. A. Aggerwal (1977) and W.J. Hendelman (Hendelman et al., 1978) and they have reported the following observations. Both symmetric (inhibitory) and asymmetric (excitatory) synaptic contacts are present in cultures. Among the profiles forming symmetric synapses on P neurones two types can be distinguished; one resembling terminals of recurrent axon collaterals of P neurones and the other resembling terminals of stellate (or basket) neurones. Many of the profiles forming asymmetric synapses on P neurones can be attributed to the axons of granule cells. Some of these synaptic contacts resemble parallel fiber-P neurone synapses from in vivo Cb, others however are much larger and contact more dendritic spines than usually observed in vivo. This finding is interpreted as evidence for hypertrophy of parallel fiber terminals. In addition, a distinctly different type of terminals can frequently be observed resembling mossy fiber terminals of in vivo Cb. These structures form asymmetric synapses on granule cell dendrites and occasionally also on P neurones. Since these terminals are seen in cultures lacking extracerebellar afferents, they are presumably originating from DN neurones. A mossy fiber projection from the DN to the Cx has recently been described in vivo (Gould & Graybiel, 1976).

The DN neurones are often difficult to visualize in living cultures but can be distinguished from other neurones by their topographical location (Fig. 1). When impregnated with the Golgi-Cox technique, the DN neurones are seen as bipolar or multipolar and are usually found in

FIGURE 2: Golgi preparations of cerebellar cultures (from Hendelman et al., 1978).

4 - Cx-DN

5 - Granule cell

6 - Golgi cell

7,8,9 - Purkinje neurones

10 - DN neurone

Bar in all except #4 is 20 μ

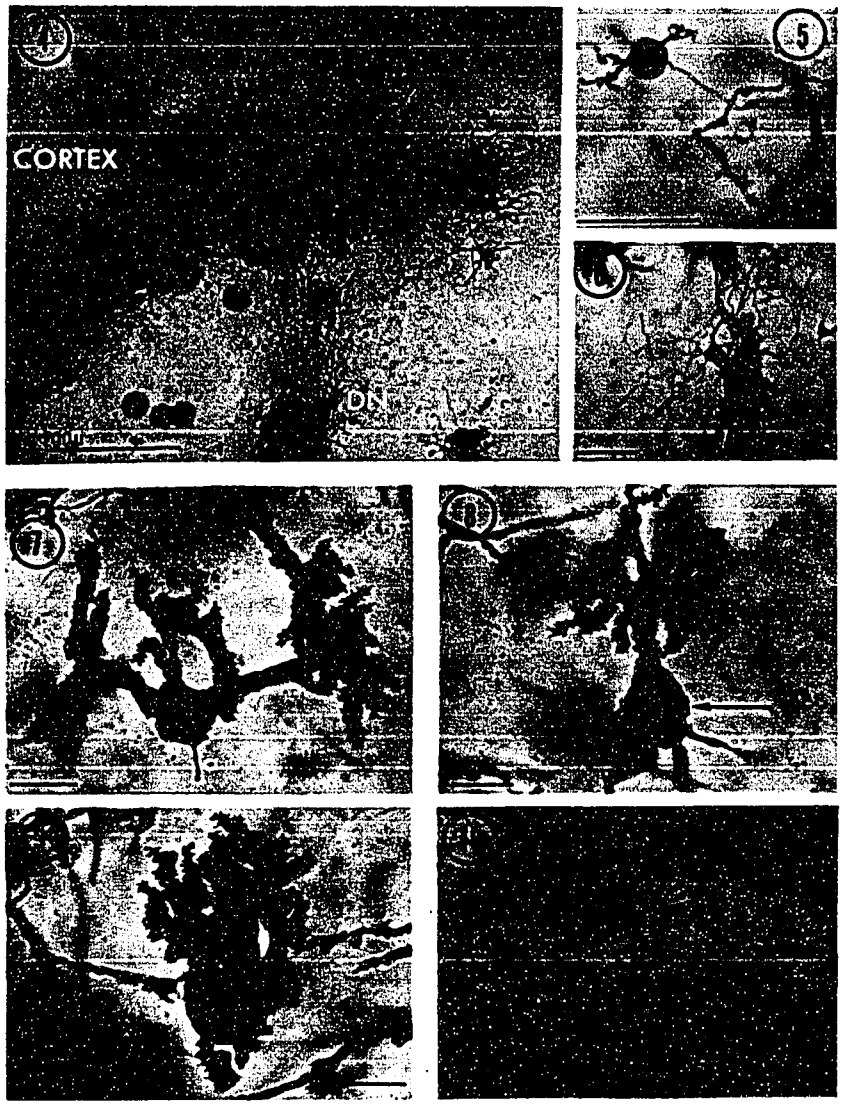


FIGURE 2

clusters of up to ten cells (Fig. 2). The soma measures 15-25 μ and has 2-6 dendrites with a few branches measuring up to 200 μ. There are only a few spines on soma and dendrites (Hendelman & Aggerwal, 1974).

Myelinated axons (1-2 μ in diameter) can be seen projecting between the two areas of the cultures (Fig. 1). Some of these are probably emerging from DN neurones but most are presumed to be axons of P neurones and their collaterals. The internodal distance in these axons is uncertain. On a few occasions clear interruptions of the myelin were observed which could represent the nodes of Ranvier, however, in most cases no nodes could be seen even when the axons were traced for relatively long distances (e.g. 400 μ). It seems likely therefore that most of the axons have a continuous myelin sheath along their whole length, as has been reported by Hild (1957) and Bornstein & Murray (1958).

Cultures of type II include BS neurones positioned near the IV ventricle. These neurones contain characteristic, large granules at the periphery of the cytoplasm (Fig. 3A) and are somewhat larger than other neurones in culture. Additional information on morphological features of the BS neurones has been obtained in this laboratory using intracellular staining with horseradish peroxidase (Marshall & Hendelman, unpublished observations). The neurones stained by this method usually had a few stout dendrites and long axons which did not appear to be myelinated. Axonal processes emerged either from the soma or from dendrites and gave off numerous branches throughout the cultures. Fine axonal branches formed characteristic plexuses in the BS area (Fig. 3B, C).

A few P neurones have also been stained with horseradish peroxidase showing myelinated axons projecting to the DN area. Proximal segments of these axons gave off recurrent collaterals while distal segments formed numerous branches which terminated as large swellings (presumed synaptic

FIGURE 3: BS neurones (Marshall & Hendelman, unpublished observations).

A - living culture

B - a single neurone stained with horseradish peroxidase

C - axonal branches of a single neurone (cell body not shown)

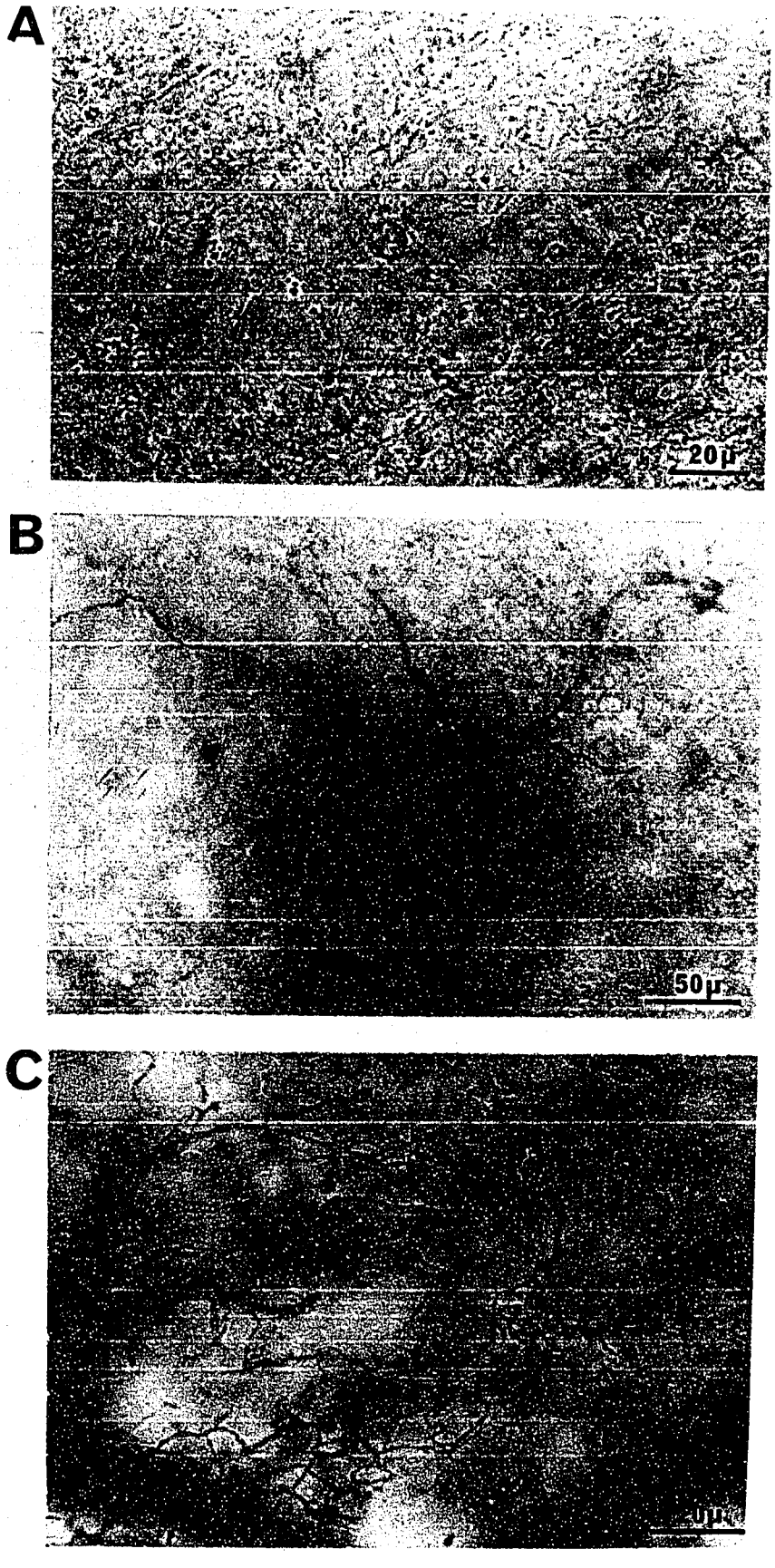


FIGURE 3

boutons).

I have used a modified Holme's silver stain (see methods) on many cultures, following electrophysiological experiments in order to confirm the topographical separation of the cortical and the DN areas and the proper placement of the electrodes (Fig. 4A). The axonal processes stain well with this method and frequently the axons of the P neurones could be identified and traced from the cortical to the DN region (Fig. 4B). The axons of DN neurones were difficult to identify in the dense network of fibers characteristic of the DN area, but at least in one case the axon of a DN neurone could be followed (Fig. 4C). According to Sèil & Leiman (1977) these axons project to the cortex and give off recurrent collaterals to adjacent DN neurones. Neuronal cell bodies stained in only a few cultures and two examples are shown in Fig. 4C.

FIGURE 4: Holme's preparations of cerebellar cultures

A - The whole explant showing Cx, DN and BS areas. Arrow indicates the site of placement of a stimulating electrode used during an electrophysiological experiment.

B - Cx region showing axons of P neurones projecting towards DN area. Short arrow indicates the nucleus of one P neurone, axon of this neurone is giving off a recurrent collateral (long arrow) about 60 μ away from the soma.

C - DN region showing two neurones (short arrows). A dashed line follows a process which appeared to originate from one of the neurones (curved arrow). This may be the axon making a sharp turn in the DN area and projecting towards cortex (thin arrow).

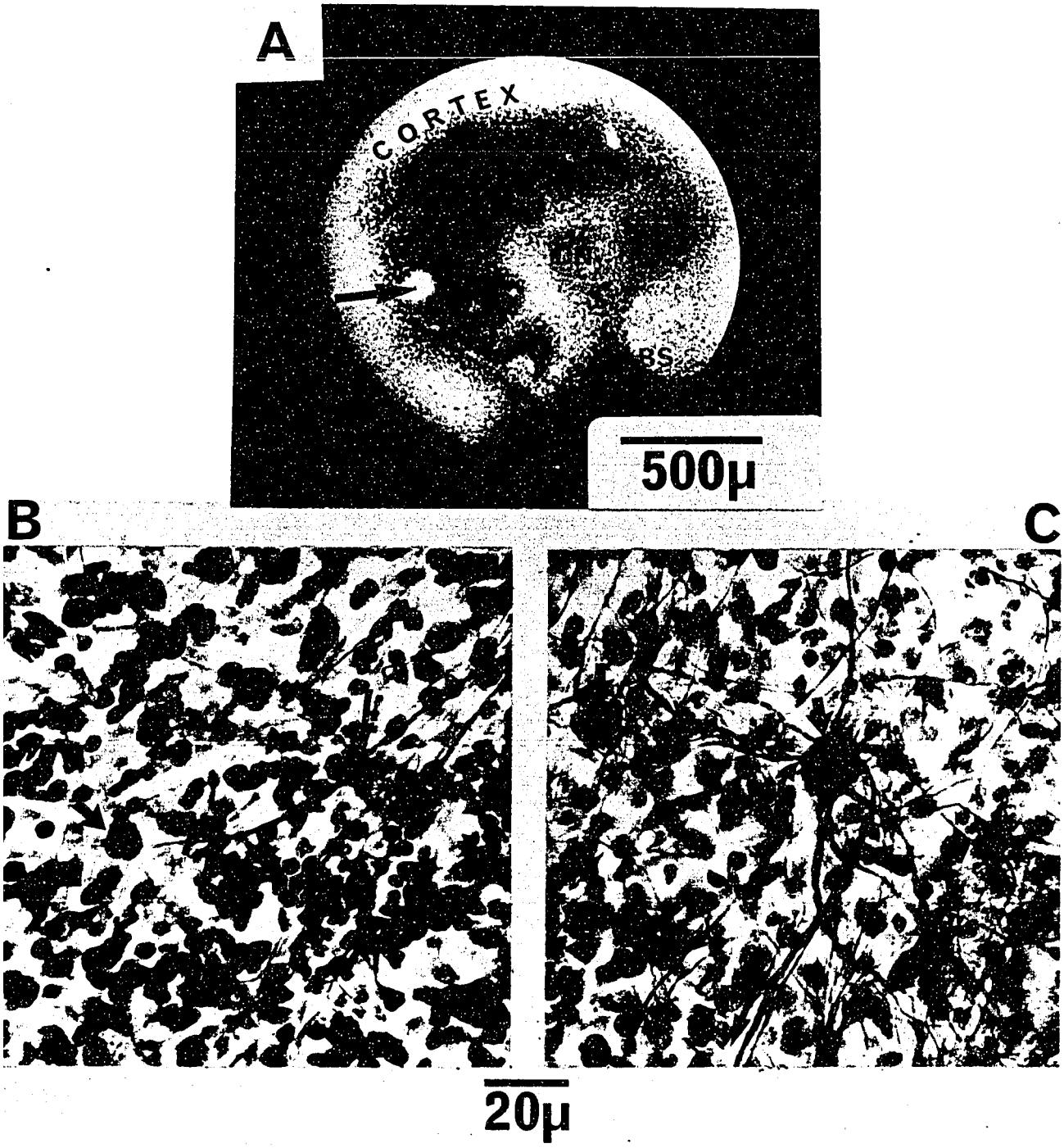


FIGURE 4

CHAPTER 2

METHODS AND MATERIALS

a) Tissue Cultures and Experimental Setup

Cultures were obtained by explanting 1 mm thick, parasaggital sections of newborn mice cerebella (Cb) (Fig. 5). Most cultures (about 2/3) originated from explants taken from the part of the Cb situated directly above the peduncles and the remaining 1/3 originated from the lateral parts of the Cb. The medial sections were rarely used because of poor myelination. The explants were placed on the collagen-coated coverslips and subsequently incubated in the standard Maximow chambers at 35.5°C. Routine techniques for the maintenance of cultures were followed (Wolf, 1964; Allerand, 1971). The nutrient medium, which was changed twice a week, consisted of 50% Eagle's minimal essential medium, 25% human cord serum, 25% chick embryo extract and 11 g/l dextrose Cultures could also be successfully grown using adult (author's) serum instead of the cord serum. The mature cultures (3-4 weeks *in vitro*) were examined in their living state (40x oil immersion objective) and those containing clearly visible neurones and well myelinated fibers were selected for experiments.

For electrophysiological experiments, the cultures (on their coverslips) were transferred from the Maximow chambers to a chamber on the stage of an inverted microscope so that the electrodes could be placed near the neurones under visual control (Fig. 6A). The chamber had a volume of about 2 ml and was constantly perfused with a slightly modified Earle's balanced salt solution (BSS) (see materials) which was equilibrated with 95% air and 5% CO_2 giving a pH of 7.3 - 7.4 as measured in the chamber. The pH depended on the rate of flow of the solution

Figure 5: Plan of dissection of the newborn mouse cerebellum. Coronal view. (Courtesy of Dr. A. Aggerwal)

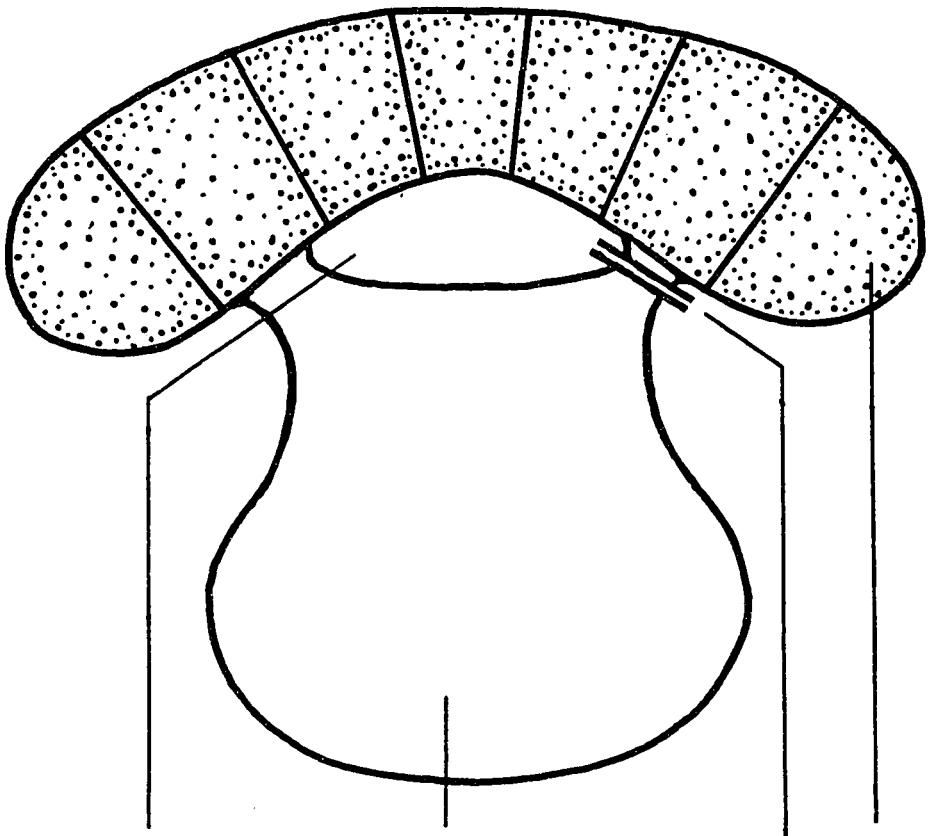

IV VENTRICLE
BRAIN STEM
CEREBELLUM
CEREBELLUM IS SEPARATED HERE FROM THE PEDUNCLES AND BRAIN STEM

FIGURE 5

through the chamber and its temperature, so these parameters had to be kept relatively constant in order to achieve a steady pH throughout an experiment. Since it was not practical to keep a pH probe in the chamber at all times the pH measurements were made only occasionally.

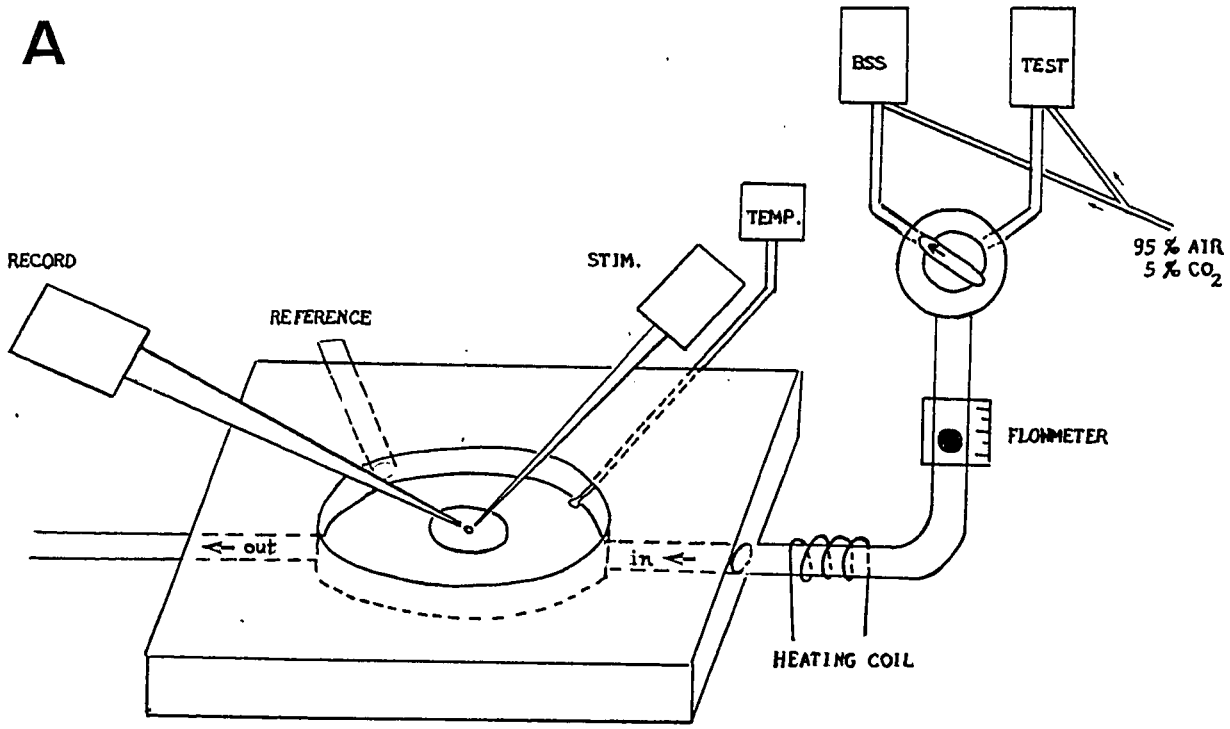
The flow rate was regulated by a valve placed on the inlet tubing and it was set at 5-7 ml/min. The temperature of the bathing solution was kept at 35-37°C by means of a heating coil placed around the inlet tubing and it was continuously monitored with a thermistor probe placed in the chamber. Various test substances (e.g. pharmacological agents) were dissolved in BSS in separate reservoirs, which could be switched into the perfusing system. Depending on the rate of perfusion, a complete exchange of solutions in the chamber took 3 to 5 minutes. This time was accounted for by the time necessary to replace the control solution in the inlet tubing and in the chamber itself. The exchange time has been determined using coloured solutions and agrees well (±1 min) with the time course of the action of easily diffusible substances (e.g. magnesium ions) on the neurones.

b) Stimulation

For monopolar electrical stimulation single platinum, palladium or tungsten needles were used. These were etched electrolytically to a fine tip and insulated with "Insulex" except for about 40 micra from the tip. Negative current pulses of 0.1 msec duration were delivered through the electrode via a constant current stimulus isolation unit (Grass). The effective current spread (stimulation field) was determined individually for every electrode by recording direct excitations from nearby neurones. Such excitations were recognized by their short latency (less than 0.5 msec) and steady following of high frequency stimulation

Figure 6A: Experimental setup. The chamber was placed on the stage of an inverted microscope so the objective (40x oil) could be positioned directly underneath the culture whereas a condenser of the microscope was above the chamber. The bottom of the chamber was made of thin transparent glass. The recording and stimulating electrodes were placed in the culture using micromanipulators.

Figure 6B: Diagram showing the electronic equipment used during experiments. Data was usually photographed directly from the oscilloscope screen. In some experiments, data was collected on tape and analyzed later.


A
BSS
TEST
TEMP.
RECORD
STIM.
REFERENCE
95 % AIR
5 % CO2
FLOWMETER
out
in
HEATING COIL

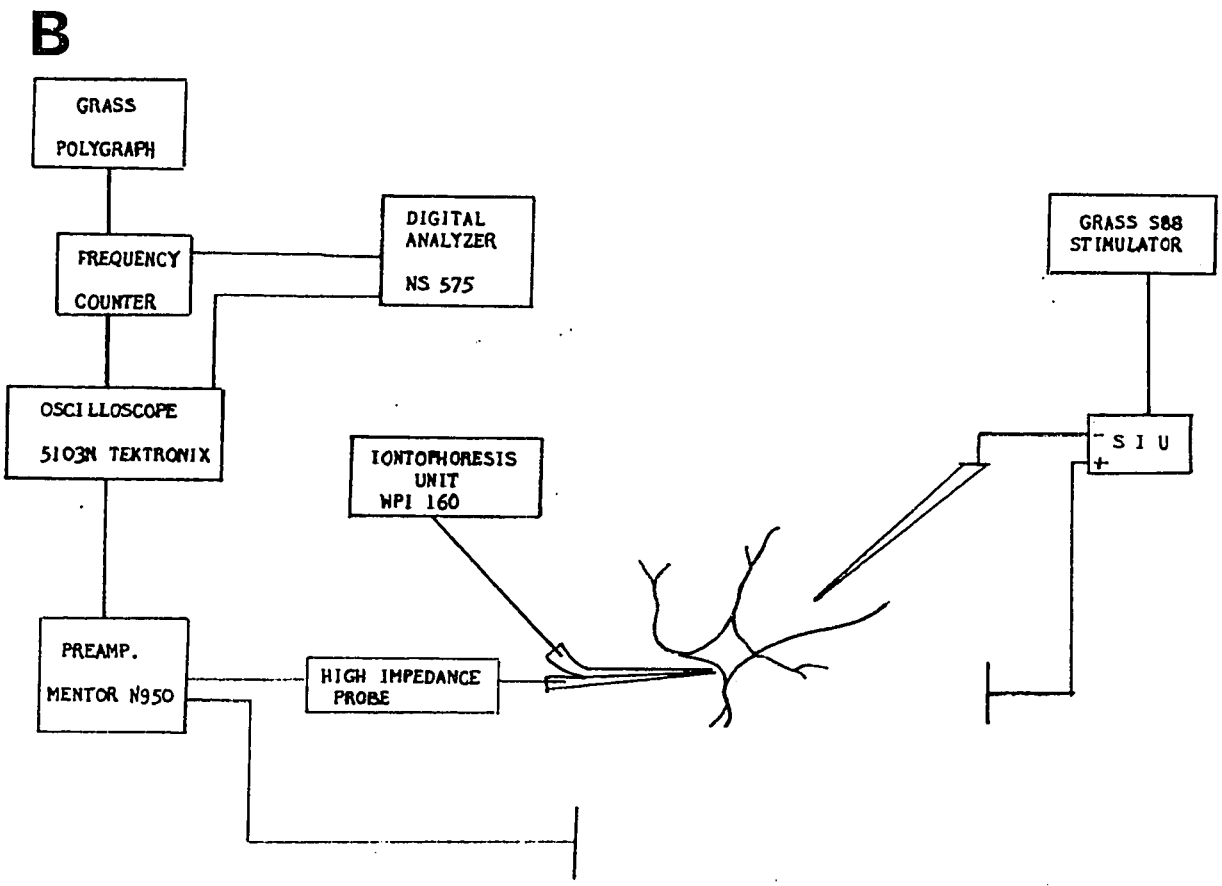

B
GRASS
POLYGRAPH
DIGITAL
ANALYZER
NS 575
FREQUENCY
COUNTER
GRASS S88
STIMULATOR
OSCILLOSCOPE
5103N TEKTRONIX
S I U
IONTOPHORESIS
UNIT
WPI 160
PREAMP.
MENTOR N950
HIGH IMPEDANCE
PROBE

FIGURE 6

(above 100/sec). According to these observations a minimal stimulus intensity required to produce a direct excitation of neurones situated very close (about 30 micra) to the electrode was about 1 µA. As the distance increased, higher intensity was necessary. Stimuli of up to 15 µA were tested and these resulted in a direct excitation of neurones within a radius of up to 100 micra. These findings are in good agreement with the results obtained by other experimenters who used similar electrodes (review by Ranck, 1975). The effective spread of a stimulating current for axons would be expected to be only slightly larger than for neurones (Begshaw & Evans, 1976).

In the initial phase of experimentation twin bipolar electrodes made of two platinum needles set apart 100 µ were used. However, there were several problems with such an arrangement:

- the stimulation field varied considerably with different electrodes.
- relatively high currents (50-1,000 µA) had to be passed to achieve a reasonable current spread.
- such electrodes caused serious damage to the surrounding tissue due to the high current density as well as mechanical vibrations.

Hence, the use of the bipolar electrodes was abandoned in favour of monopolar ones.

c) Recording

For extracellular single cell recordings Ag/AgCl electrodes were used, connected to the preparation via fine micropipettes filled with 3 M NaCl (resistance 5-10 MΩ, tip diameter 1-3 µ). The recording pipette was sometimes fused with four other pipettes forming five barrelled iontophoretic electrodes, the other barrels being filled with 0.2 M sodium glutamate,

pH 8.0; 0.5 M GABA, pH 6.0; 0.5 M Glycine, pH 5.0 and 1.0 M sodium acetate, pH 7.0. Negative current was used to expel glutamate and positive currents for both GABA and Glycine. Sodium acetate was used for current controls.

The multibarrelled electrodes were often broken off in a special way so the recording pipette protruded a few micra beyond the iontophoretic pipettes. This procedure facilitated subsequent recordings considerably and it minimized the damage of tissue surrounding the neurones. The diameter of the whole complex at the tip was about 5 μ.

The electrodes were mounted on a high impedance probe placed close to the preparation and connected to standard recording equipment (Fig. 6B). A polygraph was usually used to display the rate of firing of neurones, while the individual action potentials (APs) were photographed from the screen of an oscilloscope. Histograms were constructed using a digital analyzer and subsequently photographed.

Successful recordings from single neurones were obtained when the tip of the electrode was placed within a few micra from a neuronal membrane. If such visual identification was not possible the activity was assumed to originate from a single neurone when the amplitude of the AP was uniform. The amplitude of APs was usually 0.4 - 1.0 mV and it clearly depended on the distance of the electrode from a neurone. The polarity of APs also varied with the distance; it was mostly negative when the electrode was relatively far from the neurone and gradually became bipolar (positive-negative) as the electrode was moved closer.

For intracellular recording, pipettes were filled with 2 M potassium citrate (resistance 10-30 MΩ). Penetration of the neuronal membrane usually coincided with an injury discharge and a 20-60 mV drop of the potential at the electrode tip.

d) Materials

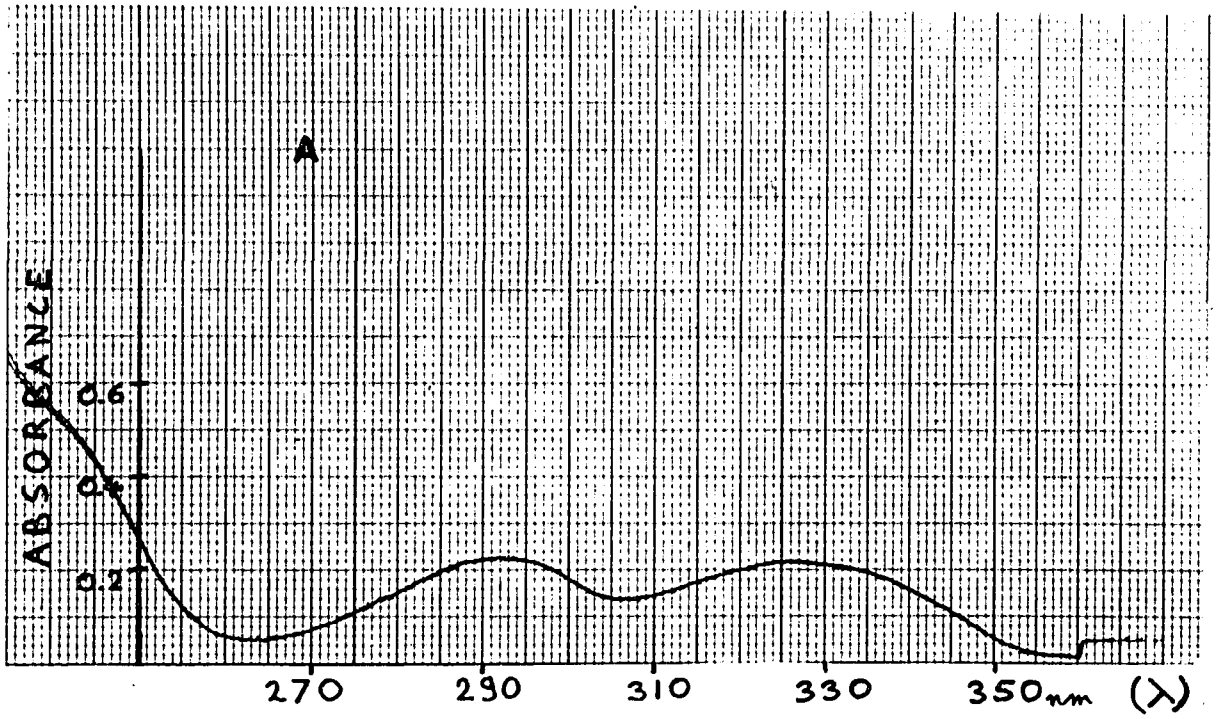
Several BSSs which are commonly used for tissue culture experiments are listed in Table 1. Concentrations of some solutes in cerebro-spinal fluid (CSF) of the rabbit are also included for reference, because similar data for the mouse are not available. It is apparent that Earle's BSS resembles most closely the CSF (note especially the concentrations of calcium and bicarbonate) so this solution was chosen as a basic perfusion medium. The solution was slightly modified, i.e. phenol red was removed to avoid any possible toxic effects of this substance and the concentration of bicarbonate was lowered to 24 mM in order to maintain a proper pH during the recording. In addition, 5 g/l extra dextrose was added, a procedure used by many investigators in order to supply an extra energy source for the neurones. It should be noted that most BSSs (including Earle's) contain a rather high concentration of potassium (5.4 mM) in comparison to CSF (2.9 mM) which can produce a depolarization of neurones; however, this effect is probably small (e.g. Hösli et al., 1972; Ransom et al., 1977).

For pharmacological tests Bicuculline (Pierce), Strychnine (Sigma), Picrotoxin (Sigma) and Bicuculline-methiodide (Pierce) were obtained as free compounds. These were then added to the BSS a few minutes before it was switched into the perfusing solution.

The absorbance spectra of Strychnine and Bicuculline in ultraviolet light have been measured in order to check the purity of these substances. The spectrum for Strychnine agreed with the expected values (Beckstead & French, 1971). The spectrum obtained for Bicuculline gave two characteristic peaks at 292 nm and 327 nm representing Bicucine and Bicuculline proper (Fig. 7A). However, the calculated absorption

Figure 7A: Ultraviolet spectrum of Bicuculline solution.
50 µM in Earle's BSS, pH 7.
The spectrum of Bicuculline-methiodide was identical.

Figure 7B: #1, control sample from the fresh solution 100 µM.
#2, 3, 4, successive samples taken during incubation of the solution at 37°C for 7, 14 and 21 minutes respectively.
Note the increase of the first peak (Bicucine) and the decrease of the second peak (Bicuculline).
Bicuculline-methiodide was stable under similar conditions.


A
ABSORBANCE
0.6
0.4
0.2
270
290
310
330
350nm (λ)

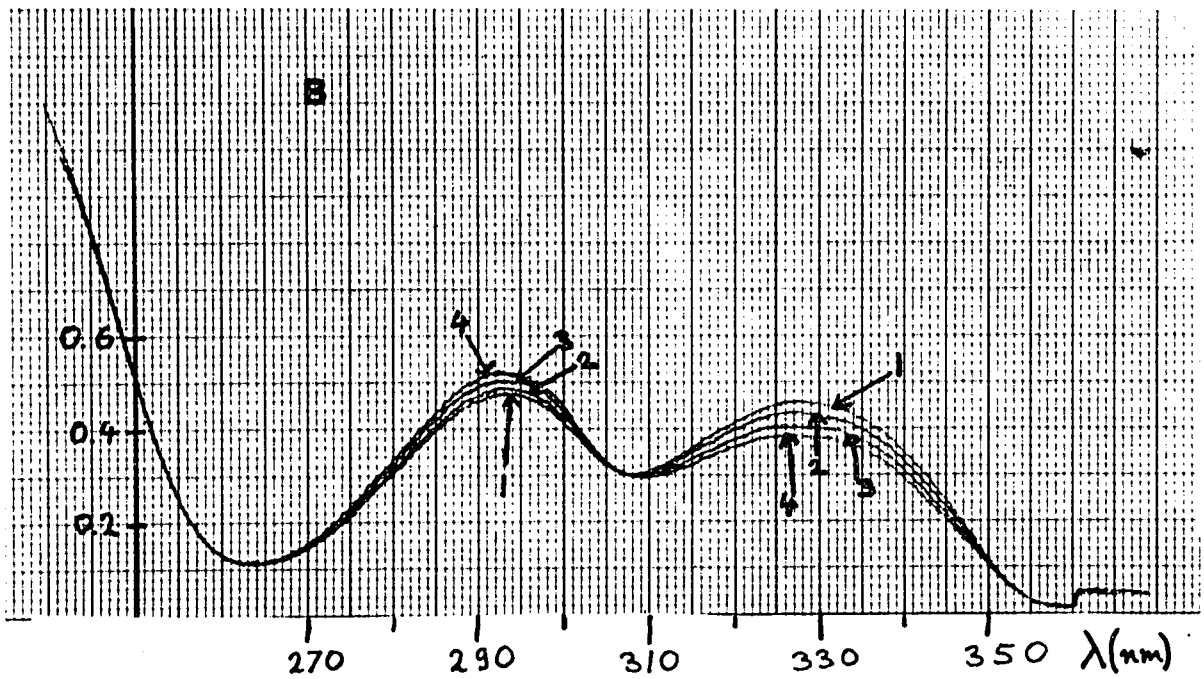

B
0.6
0.4
0.2
4
3
2
1
270
290
310
330
350 λ(nm)

FIGURE 7

coefficient ($\log \varepsilon = 3.6$ at 327 nm) was substantially lower than the value evident from Fig. 2 of Olsen et al. (1975) ($\log \varepsilon = 4.3$). This result was consistently obtained when Bicuculline was dissolved in either Earle's BSS or distilled water and using fresh samples of the drug from two companies (Sigma, Pierce). We have expressed concern to Dr. Olsen about this discrepancy in a personal letter and as a result it has been confirmed that the value given in their paper is incorrect. The error made by these authors was simply a mechanical one and they do give the correct value for $\log \varepsilon = 3.66$ in their later paper (Olsen et al., 1976).

Olsen et al. (1975) also reported that Bicuculline is quickly hydrolyzed to inactive Bicucine when dissolved in distilled water (pH 7.6 adjusted with Tris), especially at high temperatures (37°C). Control experiments were performed to determine how much Bicuculline was hydrolyzed during the experiments, using spectrophotometric assay. It has been found that at 37°C Bicuculline in Earle's BSS (pH ≃ 7) was quickly hydrolyzed (about 20% decrease in 20 minutes) while Bicuculline-methiodide was stable (Fig. 7B). This effect would not be significant because during the electrophysiological experiments the solution in the chamber was being exchanged every 3-5 minutes. However, samples taken from the outlet of the chamber had a concentration of Bicuculline decreased on the average by about 25% from the initial values (2 tests, range 24-26%). The additional decrease was undoubtedly due to an increase in the temperature of the solution during its passage through the heating coil as well as due to the rise in the pH of the solution during mixing in the chamber. As a result of these control experiments all concentrations of Bicuculline given in the results are corrected (i.e. reduced by 25%) to account for the hydrolysis.

e) Holme's Staining Procedure (C.D. Allerand, personal communication to Dr. W.J. Hendelman)

(1) Do not wash culture or drain drop before fixing.

(2) Fix: 10% Formalin in Boric Acid Buffer

12.4 gm/L Boric Acid (27.5 ml) Na Borate

19.0 gm/L Na Borate (22.5 ml)

Distilled H_2O (197 ml)

Fix 5 days in refrigerator.

(3) Rinse with 80% ETOH and store in 80% ETOH in refrigerator for 1 month - change ETOH twice weekly.

(4) PLASTIC FORCEPS

50% ETOH 5'

25% ETOH 5'

Distilled H_2O 15'

(5) 20% Ag NO3 room temperature 1½ hours

(6) Rinse distilled H_2O x 3 for 10'

(7) Impregnate in the following solution 1 CS/staining dish.

Add in the following order:

12.4 gm/L Boric acid (27.5 ml)

19.0 gm/L Na Borate (22.5 ml)

Distilled H_2O (197 ml)

Mix well

1% Ag NO_3 (0.5 ml)

Mix well

10% Pyridine in H_2O (2.5 ml)

Mix well

Make freshly each time before use and warm before adding to dish.

Impregnate for ± 25 hours at 40°C.

(8) Remove CS from impregnating solution. Drain well - do not wash - keep in the dark. Place in reducing solution 6'.

Reducing solution - make freshly and warm to 32^{o} before using.

Hydroquinone 1 gm

Na Sulfite 10 gm

Distilled H_2O 100 ml

(9) Running H_2O 10'

(10) Rinse distilled H_2O

(11) Gold chloride 0.2% 1-6' in the dark

(12) Rinse distilled H_2O

(13) Oxalic Acid 2% 15'

(14) Rinse distilled H_2O

(15) Na Thiosulfate 5% 5'

(16) Running water 10'

(17) Distilled H_2O rinse

(18) Dehydrate in successive ETOH

(19) Clear Xylene

(20) Mount Permount

N.B. Items 5 through 15 should be carried out in the dark or dim light; it will improve the whiteness of the background. For these items I recommend plastic forceps.

CHAPTER 3

RESULTS

Part I - Electrophysiological Identification of Axonal Projections in Cultures

In living, unstained cultures the individual unmyelinated neuronal processes cannot be distinguished. Myelinated axons can be seen but it is not possible to trace them to their cells of origin. During the course of electrophysiological studies it was possible to activate neurones antidromically and in this fashion determine the projections of their axons. The results presented below will be discussed in view of morphological data available on the Cb cultures. In addition, antidromic activations have been used to give estimates of the axonal conduction velocities.

Reliable identification of the axonal projection can be made only if the stimulus is applied sufficiently far from the cell soma as to avoid a direct stimulation of the cell body or dendrites. Since the maximal spread of the stimulating current did not exceed 100 μ (see methods) and the maximal length of the dendrites in cultured neurones is less than 300 μ it was decided that a minimum 400-500 μ separation between the stimulating and recording sites is sufficient for the experiments.

Four criteria have been used for identification of antidromic activations of neurones:

1. Constant latency of evoked action potentials

Prepotentials of the antidromic activation (axonal spike and initial segment spike) have a relatively short duration (0.5 - 1.0 msec) so that the activation of the cell body cannot be delayed by more than about 0.5 msec. In addition, the safety factor for the antidromic activation is often relatively high, so such an activation

is usually less dependent on another (synaptic) input to the neurone than a synaptic activation.

2. Following of high frequency stimulation (above 100/sec) by an action potential

This criterion is somewhat arbitrary because it does not distinguish between antidromic and strong synaptic input. It is, however, a usual characteristic of antidromic activations and an unusual characteristic of synaptic activations.

3. Collision between a spontaneous action potential (generated in the region of the cell soma) and an evoked action potential (generated by a stimulus in the region of the axon terminal).

If the evoked action potential is antidromic, then collision will occur because both action potentials will be conducted along the same axon in opposite directions (Tasaki, 1949). Theoretically the minimal expected time period between the spontaneous and the antidromically evoked action potentials should be approximately equal to the refractory period of the axon plus the doubled conduction time along this axon.

4. The separation between the axonal spike, the initial segment (IS) spike and the somato-dendritic (SD) spike (Eccles, 1966, p. 47).

The axonal spike usually invades the IS of the axon with a relatively high safety factor which is also the case for invasion of the SD region by the IS spike, so there is little difference in time between the activation of IS and SD during antidromic invasion. However, if a neurone is hyperpolarized either by passing a current through the cell membrane or by afterhyperpolarization (e.g. during repetitive activation) or by an

IPSP, a delay between axonal, IS and SD spikes can be observed because the excitation time is prolonged by the polarization. Usually the delay between IS and SD spikes is more pronounced than that between axonal and IS spikes. In the case of synaptic activation, EPSPs are also thought to excite the IS before they produce the SD spike, however, a clear delay between IS and SD spikes usually does not occur, probably because the hyperpolarization of the SD is eliminated by the incoming EPSP, so the time difference between excitation of IS and SD is kept small.

Since none of the described criteria is completely flawless, at least 3 out of 4 criteria had to be fulfilled before the activation was classified as antidromic. The activations of BS, DN and P neurones were attempted and the results are reported below.

a) Brain Stem Neurones

Approximately 20% of nearly 100 BS neurones studied appeared to be evoked antidromically by stimulation of either DN or Cx areas. The data from 11 neurones which were studied in detail is presented in Table 2. A projection to the DN area could be demonstrated only in a few cases because in the majority of the cultures, the DN and BS regions were placed too close to each other to allow selective stimulation. One example of antidromic activation of a BS neurone is shown in Fig. 8. Criteria 1, 2 and 4 are well illustrated in this case. An AP evoked in this neurone followed stimulation at up to 100/sec with constant latency. At 200/sec the delay between IS and SD spikes was observed and although the latency of the SD spike varied at this frequency, that of the IS spike remained fairly constant. Occasionally a complete block of the SD spike was evident and only the IS spike remained.

FIGURE 8. Antidromic activation of a BS neurone (superimposed traces, extracellular recording). This neurone was activated by stimulation of the DN area 400 μ distant. Continuous suprathreshold (10 μA) stimulation evoked APs at a constant latency with a frequency of 1/sec (top) and 50/sec (middle). At 200/sec (bottom) the latency of the IS spike was fairly constant but that of the SD spike varied. Thick arrow indicates the IS-SD separation. It can also be seen that at times the large SD spike failed and only the smaller IS spike (upper thin arrow) or even a smaller (possibly axonal) spike (lower thin arrow) was evoked.

Note - this neurone is also shown in Table 2 (cell #11).

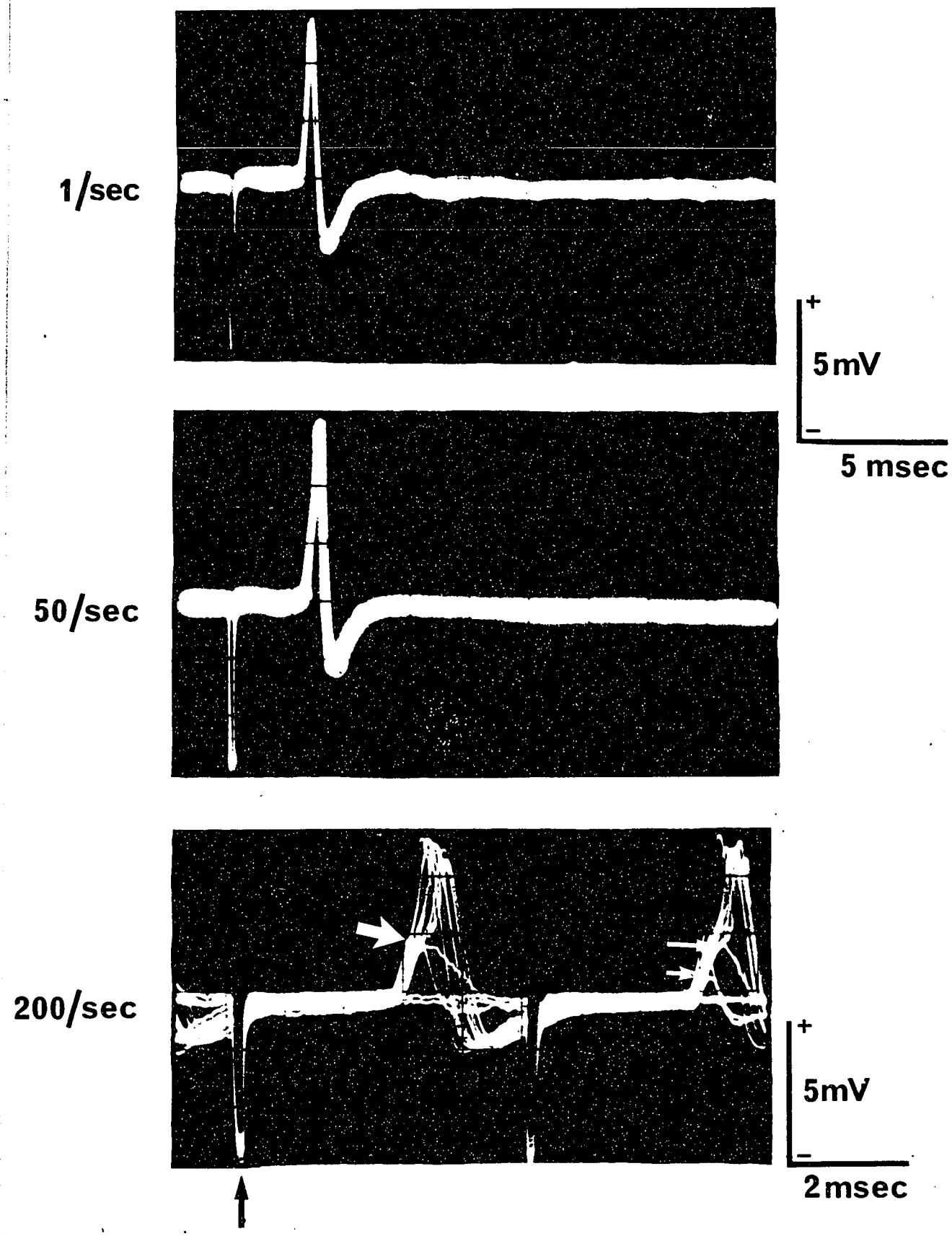


FIGURE 8

The phenomenon of collision is illustrated in another neurone which was evoked by cortical stimulation (Fig. 9). The test for collision has been carried out in the following manner. First the intensity of stimulation was set to 50% above the threshold value and the latency of the response was measured as the time period between the beginning of the stimulus artifact to the beginning of the evoked AP (A in Fig. 9). Secondly, the ability of the evoked response to follow two successive stimuli ("refractoriness") was determined (B, C, D in Fig. 9). The minimal separation between two APs was assumed when the second response was occurring in only about 50% of trials (C in Fig. 9). Thirdly, the least interval between a spontaneous spike (originating in the cell soma) and the evoked spike was measured (F in Fig. 9); this latter time was called the collision interval.

The collision test was judged positive if the collision interval was equal to the refractoriness of the neurone plus the doubled latency of the potential. This reasoning was based on the assumption that the refractoriness was a measure of the refractory period of an axon and that the latency was an indication of the conduction time along the axon in either antidromic or orthodromic direction (see discussion).

For all BS neurones shown in Table 2, including the neurone from Fig. 9, the measured collision intervals were approximately equal to the predicted values. All intervals were measured from the photographed records so the accuracy of these measurements was usually ± 0.1 - 0.2 msec (5-10% maximum error).

Other neurones (about 20% of all BS neurones studied) also appeared to be antidromically activated, i.e. they fulfilled criteria #1 and 2, or #1 and 4 or #2 and 4. But they could not be tested with the collision test because they were not spontaneously active. These activations could

FIGURE 9: Superimposed traces of antidromically evoked responses in a BS neurone activated by the stimulation of a cortical region 800 μ distant.

A - Evoked spike at a frequency of 1/sec.

B,C,D - Pair of stimuli to cortical region with increasing interstimulus interval.

E,F,G - Pulses generated by a positive phase of spontaneous APs, are used to initiate the oscilloscope sweep and a delayed stimulus to cortical region with increasing delays. Stimulus artifacts are indicated by arrows.

Results of the collision test in this neurone were as follows: latency - 2.4 msec (A), refractoriness - 3.7 msec (C), collision interval - 8.2 msec (F).

The predicted collision interval ($2 \times 2.4 + 3.7 = 8.5$) is resonably close to the observed value of 8.2 msec.

Note - time intervals between the spikes were measured at their negative peaks.

Inset - relation of recording and stimulating electrodes with respect to the neurone.

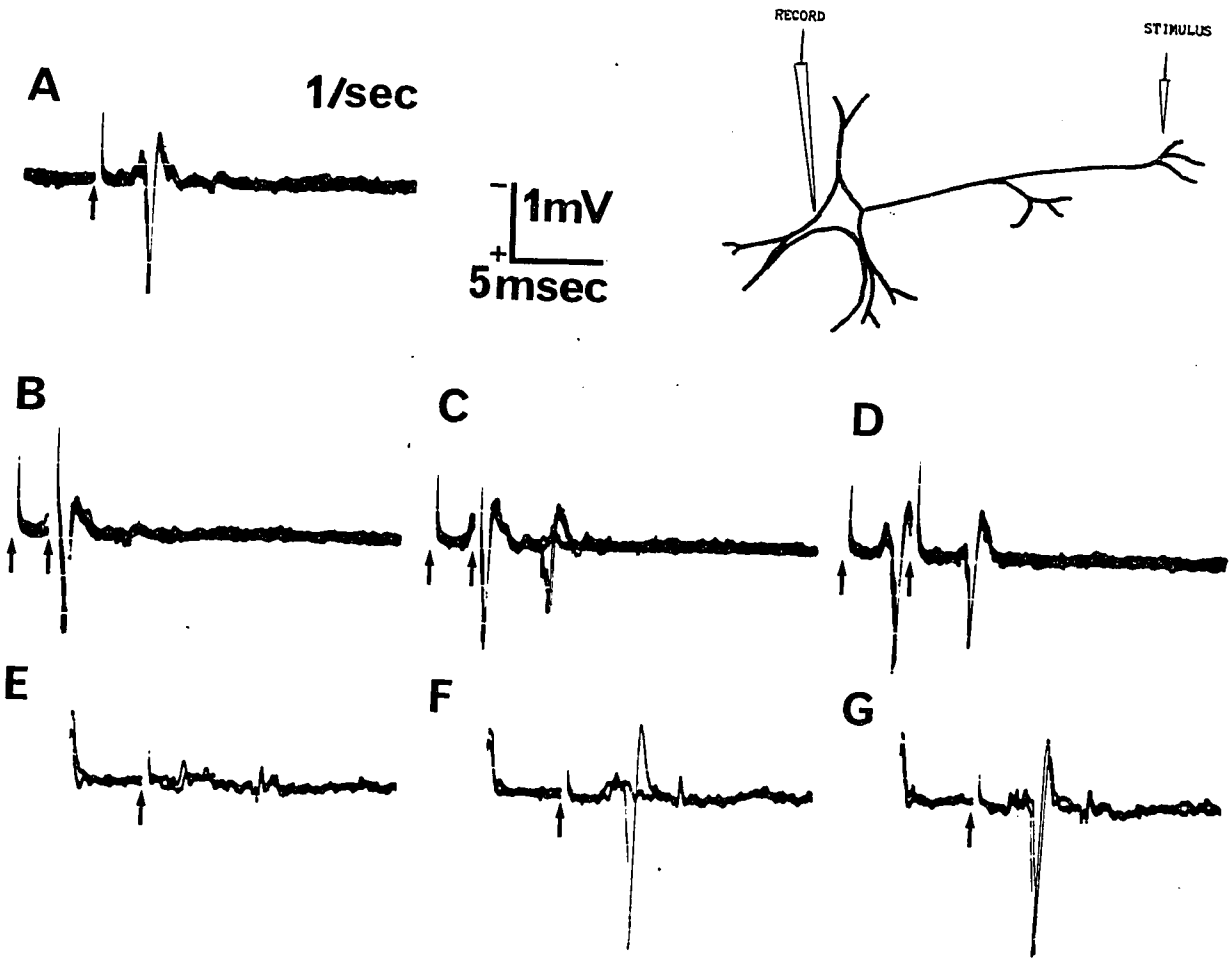


FIGURE 9

have been antidromic but they are not included in Table 2 because the fulfilment of only two criteria was considered inadequate, especially in view of very strong synaptic activations seen in these neurones (see part IV, e) which could be mistaken for the antidromic ones.

Conduction velocities were calculated for the antidromic activations on the basis of latencies and distances separating stimulating and recording electrodes (Table 2). The estimated conduction velocities have a range of 0.14 - 0.48 m/sec (average 0.24 m/sec; standard deviation 0.1). Such calculations do not take into consideration the time utilized by a stimulus to generate an AP in the fiber. However, since the duration of the stimulating pulse was 0.1 msec it is expected that the excitation occurred during this time, which is small (less than 5%) in comparison to the total latencies.

b) Deep Nuclear Neurones

About 30% of DN neurones appeared to be activated antidromically by a cortical stimulation. Data from 14 units which were studied in detail are presented in Table 3. The same four criteria were used for distinguishing antidromic activation as in the case of BS neurones. An example is shown in Fig. 10. The neurone was evoked with constant latency after every stimulus even at frequencies greater than 100/sec (A, B in Fig. 10). The refractoriness was determined (C, D, E) and the time corresponding to the minimal spike separation (D) was compared with the minimal separation between the spontaneous and evoked APs (G). The difference in time was found to be equal to the latency determined in (A). Since the predicted time difference should be equal to the doubled latency, the collision test is apparently not completely conclusive in this case. Such discrepancy between theoretical and experimental

FIGURE 10. Superimposed traces of antidromically evoked responses from a neurone in the cerebellar nucleus activated by stimulation of a cortical region.

A, B - Evoked spikes

C, D, E - Pair of stimuli to cortical region with reducing interstimulus interval.

F, G, H - Pulses, generated by rising phase of spontaneous action potentials, are used to initiate the oscilloscope sweep and a delayed stimulus to cortical region with reducing delays.

Stimulus artifacts are indicated by arrows.

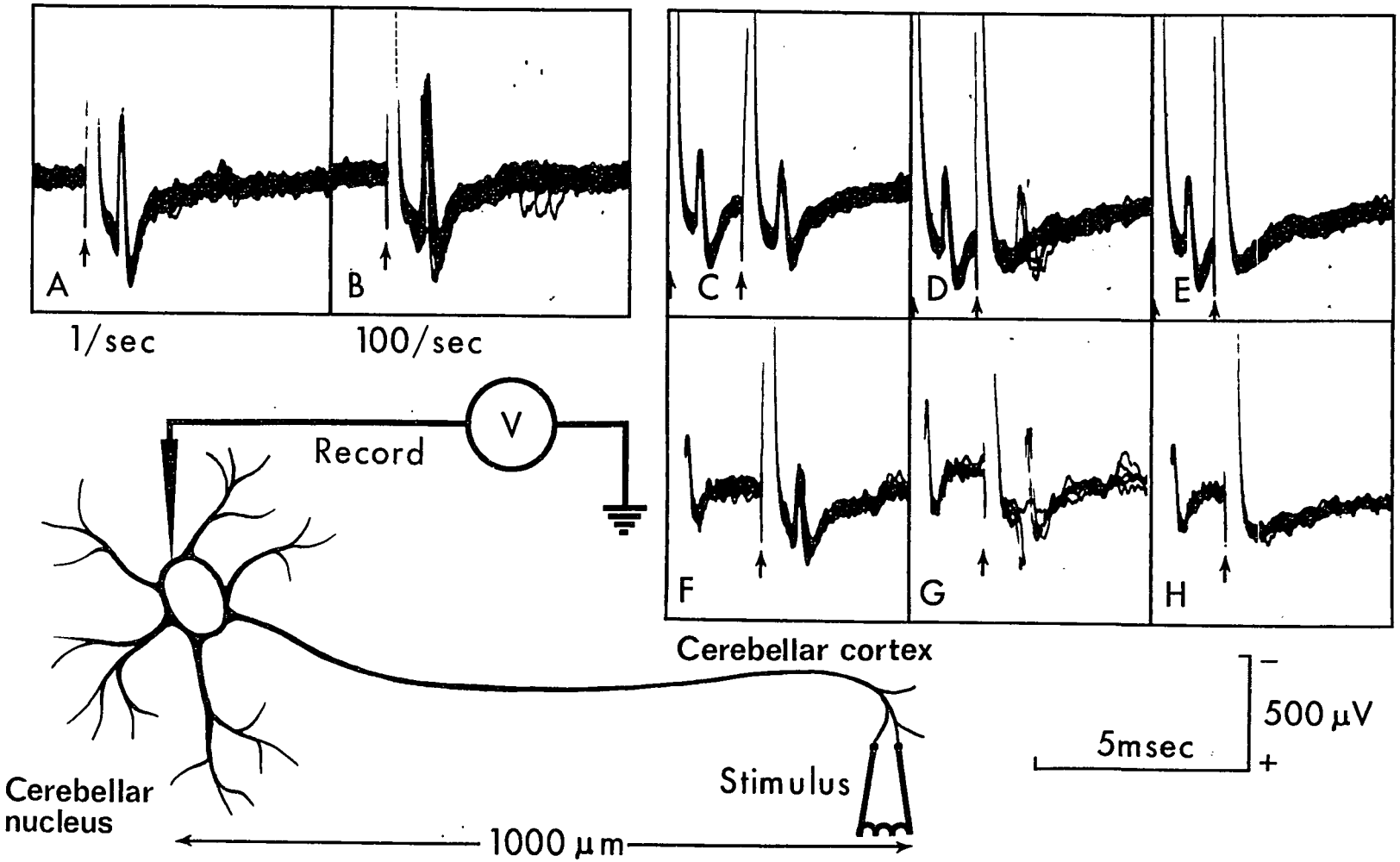

A
B
1/sec
100/sec
C
D
E
F
G
H
V
Record
Cerebellar cortex
Cerebellar nucleus
Stimulus
1000 µm
500 µV
5msec
–
+

FIGURE 10

FIGURE 11. Antidromic activation of a deep cerebellar neurone following the stimulation of a cortical region 500 μ distant.

A- Superimposed sweeps showing the evoked responses at threshold stimulus intensity (6 μA).

B, C - Same at higher intensity (50% above threshold and higher frequency of stimulation). Note that in C the first spike in the train was evoked at much shorter latency than the remaining ones. This was consistently observed in other neurones at high frequency stimulation (e.g. Fig. 8) but usually the first spike was not shown in the records (i.e. photographs were taken after the train of stimuli began).

D, E, F, G, H, I - Collision test

D, E, F - Pair of stimuli to cortical region with increasing interstimulus interval.

G, H, I - Pulses, generated by a rising phase of spontaneous action potentials, are used to initiate the oscilloscope sweep and a delayed stimulus to cortical region with increasing delays.

Stimulus artifacts are indicated by arrows.

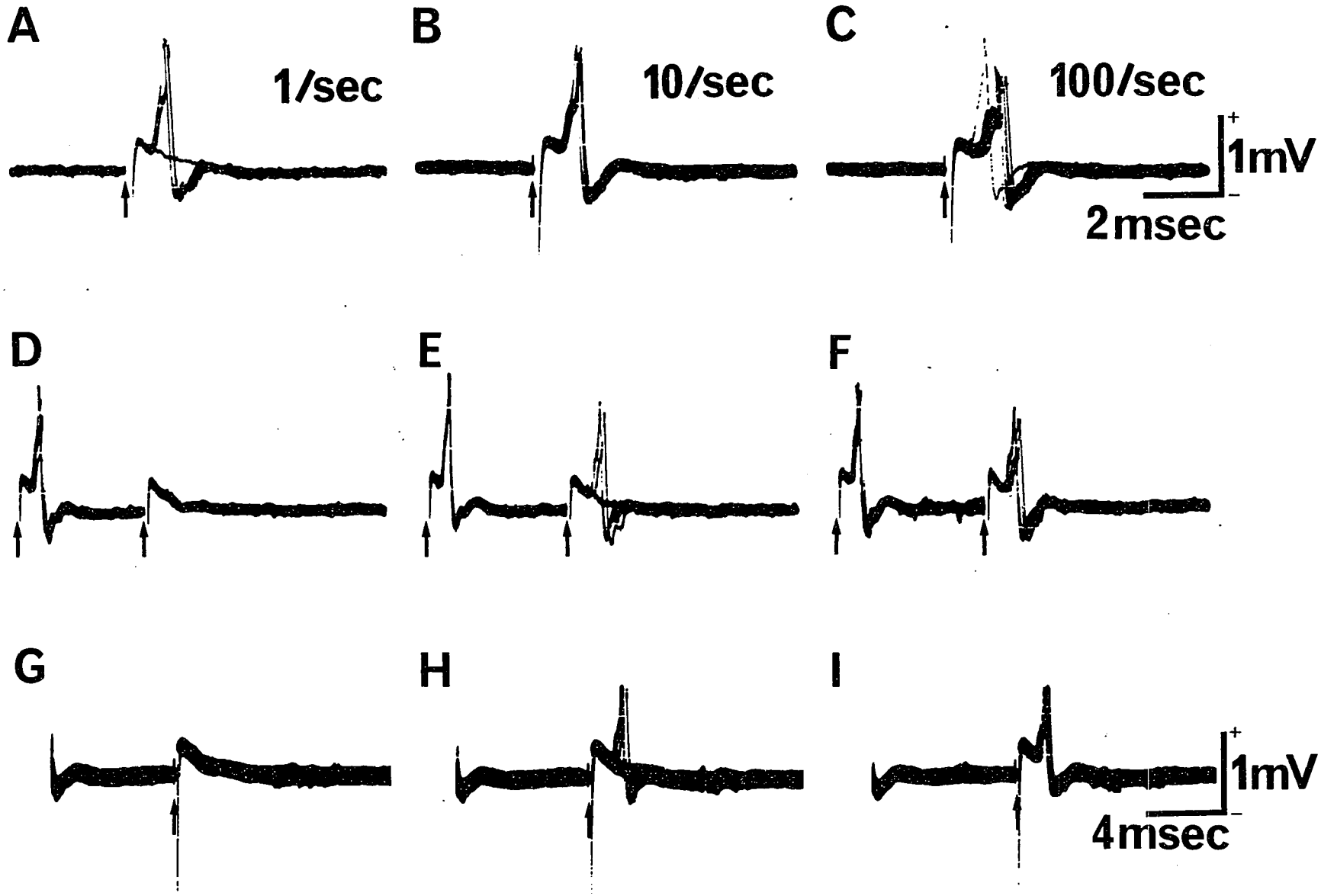


FIGURE 11

values was a consistent finding in all but one DN neurone. Another example is shown in Fig. 11. This neurone fulfilled criteria 1, 2 and 4 (A, B, C in Fig. 11) so there is little doubt that the potential was antidromically evoked but the refractoriness was unusually long (7.6 msec in D, E, F) and the minimal time between spontaneous and evoked spikes was 8.4 msec (G, H, I) instead of the expected 9.0 msec (0.7 + 7.6 + 0.7 = 9.0). Clearly then, the formula to predict a collision time is not appropriate for DN neurones. Most factors which can influence the collision test have been discussed by Fuller and Schlag (1976). Tissue culture conditions present still more problems in the interpretation of the collision and these will be discussed later.

From the latencies of the antidromic APs given in Table 3 the conduction velocities were calculated. It was found that for these neurones the velocities vary between 0.16 and 1.7 m/sec giving an average value of 0.71 m/sec (standard deviation 0.48). In some experiments (not illustrated) two stimulating electrodes were used positioned at different distances from a neurone so that the conduction time along the axon could be measured in two ways:

1 - as a difference in latencies of the APs evoked by each electrode.

2 - as a time period between the beginning of the stimulus artifact and the beginning of an evoked AP.

Both measurements gave almost identical conduction times indicating that a one point antidromic activation is a valid way of estimating the conduction velocity.

35 DN neurones besides those shown in Table 3 appeared to be antidromically activated, i.e. they fulfilled criteria #1 and 2. The collision test (criterion #3) was not performed because in these cases

other aspects of neuronal function were under investigation. Also, frequently the DN neurones were not spontaneously active and for this reason the collision test could not be performed. The IS-SD separation (criterion #4) was not seen in these neurones but this does not preclude the possibility that they were antidromically activated because visualization of the IS spike probably depends on a favourable position of the electrode (i.e. close to the IS).

In 25 of these neurones sufficient records were obtained to measure latencies of the responses and to calculate conduction velocities. These results are presented in Fig. 12 together with those from Table 3. The calculated conduction velocities had a range of 0.17 - 2.0 m/sec and the average value was 0.60 m/sec (standard deviation - 0.42) which is not significantly different from the average value of 0.71 m/sec obtained in Table 3. Thus, it can probably be accepted that most DN neurones which fulfilled only criteria #1 and 2 were antidromically activated.

c) Purkinje Neurones

It was not possible to activate cortical neurones antidromically except for a few isolated cases. This is an unusual finding since *in vivo* the antidromic activation of P neurones is commonly observed and in fact used as one of the criteria to distinguish P neurones from other neurones in the Cb cortex (Eccles *et al.*, 1967, p. 71). *In vivo*, the antidromic activations can be achieved by placing the stimulating electrode in the white matter of the Cb near the deep nuclei. A similar procedure in culture often produced a synaptic excitation but not an antidromic one. The general lack of such activation in cultures is particularly unexpected in view of the ample morphological evidence showing that axons of P neurones project to the region of DN. In order

FIGURE 12. Axonal conduction velocities of DN neurones.

Shaded bars - neurones which satisfied at least 3 out of 4 criteria for antidromic activation (values from Table 3).

Open bars - neurones which satisfied only criteria 1 and 2 (see text).

Note that conduction velocities for both groups of neurones have a similar range.

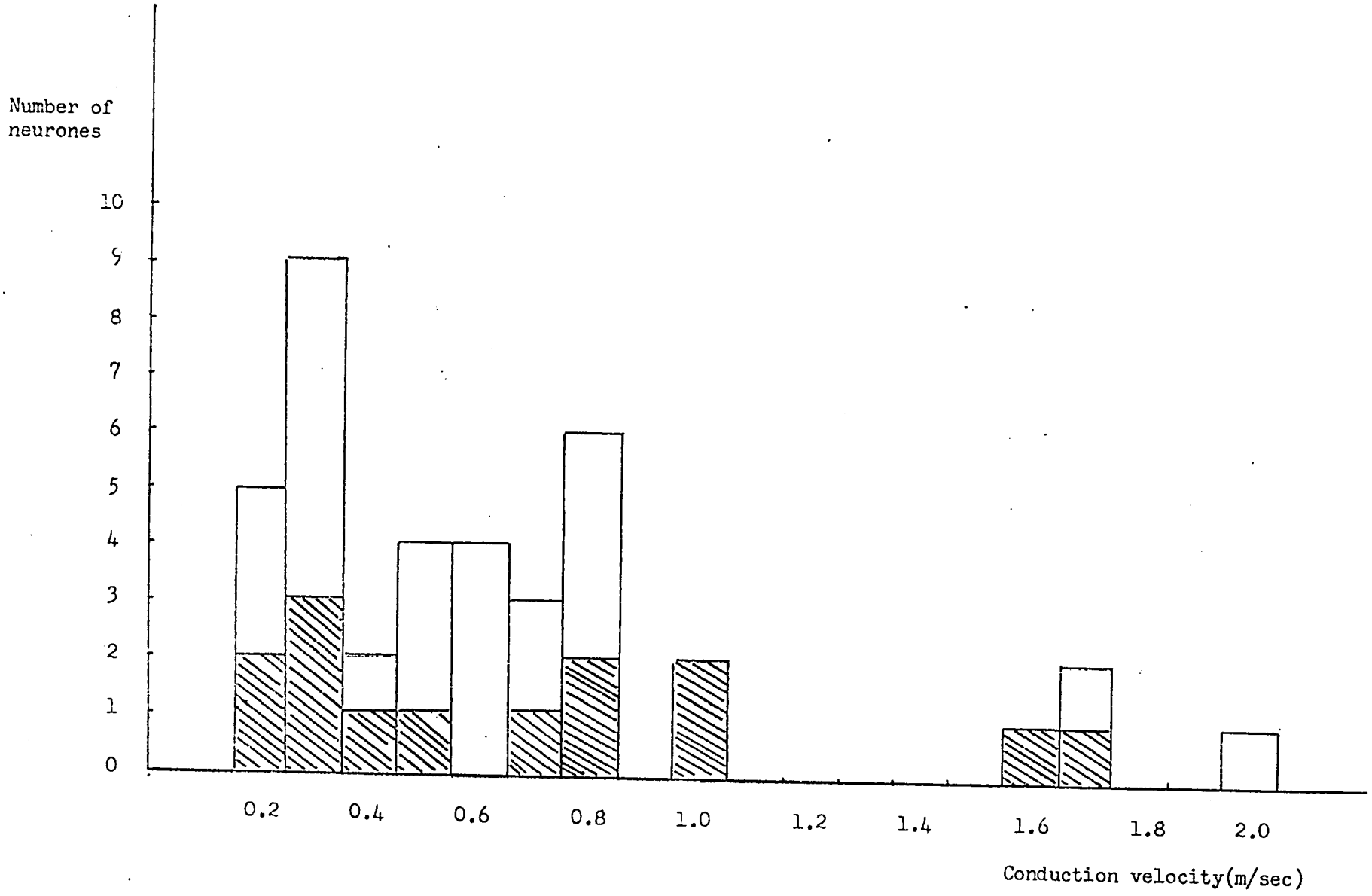

Number of neurones
10
9
8
7
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Conduction velocity(m/sec)

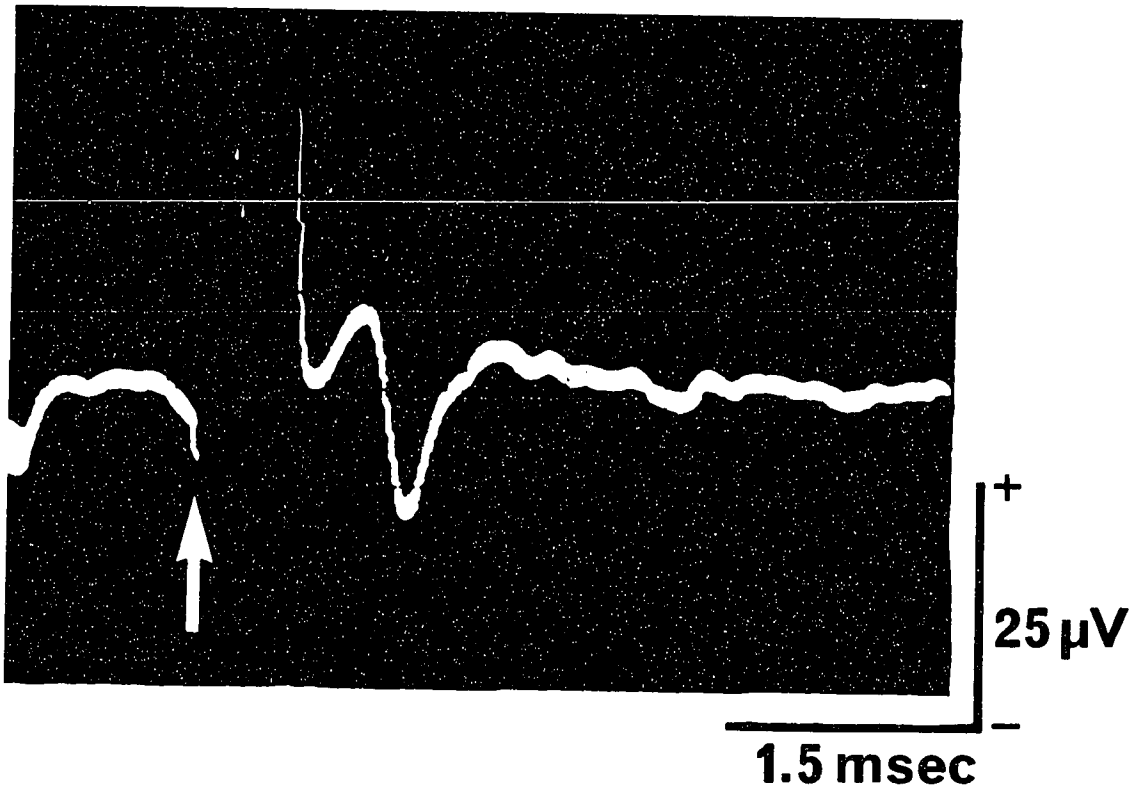
FIGURE 12

to investigate this problem recordings were made from individual myelinated fibers (presumed axons of P neurones). Stimulation of the DN region produced evoked responses in some of these fibers. However, in many cases no responses could be observed. It is probable that the large number of failures was simply the result of inappropriate placement of the electrode (insufficiently close to the fiber), because such a placement was very difficult to achieve. Therefore, the significance of these negative findings is not clear.

It is not certain if the obtained potentials were all or none in nature because they were too small to be seen in the noisy baseline and could be recorded only with the aid of an averaging computer. However, they had a short duration and a diphasic configuration resembling propagated APs rather than subthreshold depolarizations. They could also follow stimulation at 100/sec without any change in latency or any reduction in amplitude suggesting a direct excitation (e.g. Fig. 13, bottom trace). The recorded potentials resembled those seen in the nodes of Ranvier in frog's myelinated nerve fibers (Hodgkin, 1964, p. 51) but no nodes were observed in culture.

Thus it is likely that at least some of the P neurone axons can be excited antidromically. The cell bodies are also excitable since they can easily be activated by synaptic inputs or by a direct stimulation with electrical pulses. On the basis of these observations it can be proposed that the reason for the lack of antidromic activations of P neurones is the inability of the axonal spike to invade the soma. The recordings made from eight individual fibers (Table 4) allow for an approximate estimate of the conduction velocities giving the mean value of 0.26 m/sec (standard deviation 0.09).

FIGURE 13. Top - recording from a myelinated fiber. A bipolar stimulating pulse (negative-positive) was used in order to minimize the stimulus artifact. Distance from the stimulating electrode was 200 μ and the stimulus intensity 5 μA. Record was averaged 1000 X at the frequency of 1/sec.
Bottom - another myelinated fiber. Stimulus was at 3 μA and the distance 400 μ. Record was averaged 2000 X at the frequency of 100/sec. Arrow indicates the beginning of stimulus artifacts.



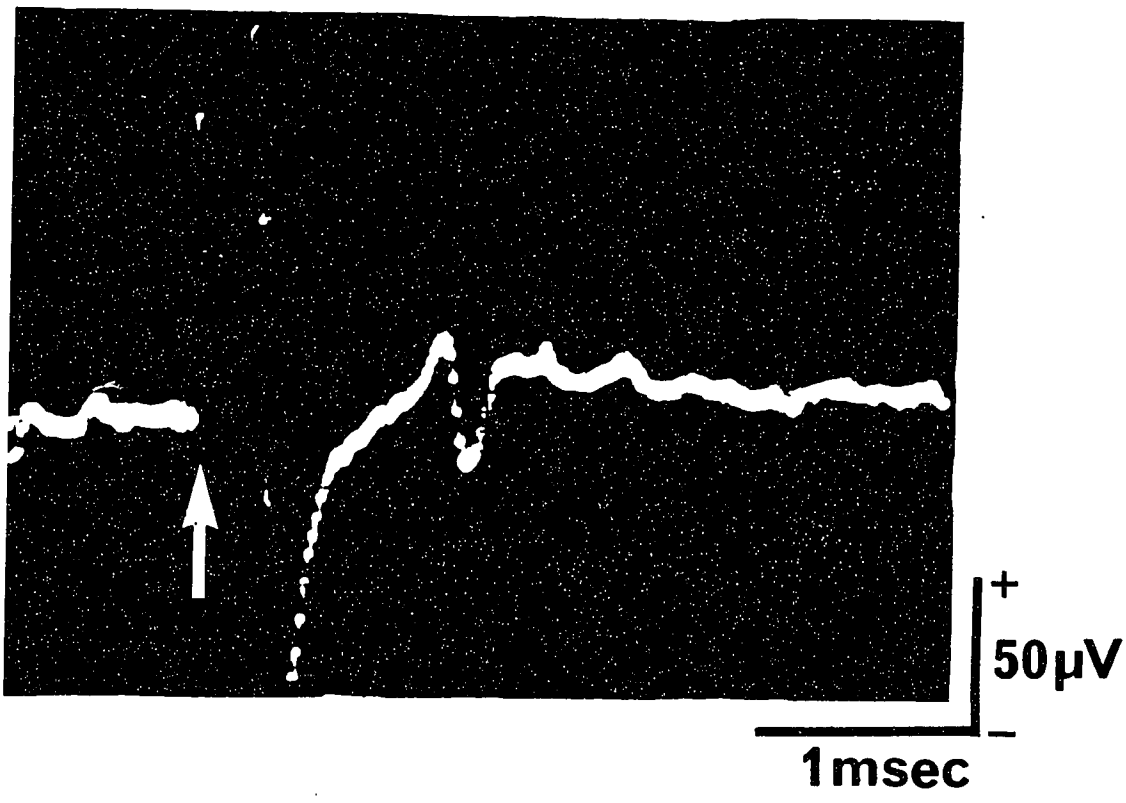


FIGURE 13

Part II - Spontaneous Activity

a) Extracellular Recordings

Preliminary experiments showed that spontaneous activity could be recorded in cultures by simply inserting a recording electrode into the tissue near neurones. Initially this activity was studied in order to determine if the characteristic patterns of firing could be associated with various types of neurones found in our Cb cultures. It was hoped that once such patterns were found they would help to identify the neurones whenever visual identification was not possible.

Of course it is difficult to establish if the activity of a neurone is truly spontaneous or if it is generated by some external factor such as a slight vibration of the recording electrode. In fact it was sometimes observed that the mechanical pressure exerted by the electrode on the neurone could increase the rate of its firing. In most cases, however, the recording electrode could be moved slightly towards or away from the soma without changing the rate of firing. Thus in these cases it is reasonable to assume that the APs were spontaneously generated by the nerve cells within the culture.

Among the population of 154 neurones which could be visually or electrophysiologically identified (see below) 70% of the cortical neurones, 67% of the DN neurones and 44% of the BS neurones were spontaneously active (Table 5). However, these ratios varied considerably from culture to culture. The most characteristic pattern of the spontaneous activity in cortical neurones was irregular firing with occasional short bursts while the average rate of activity varied from 1 to 50 spikes/sec (usually 5-20/sec) (Fig. 14). The firing of DN neurones was also irregular in most cases but usually without clear

FIGURE 14. Ratemeter recordings of the characteristic patterns of spontaneous activity in cultured neurones. Records were taken from four different cultures which all had a brainstem region.

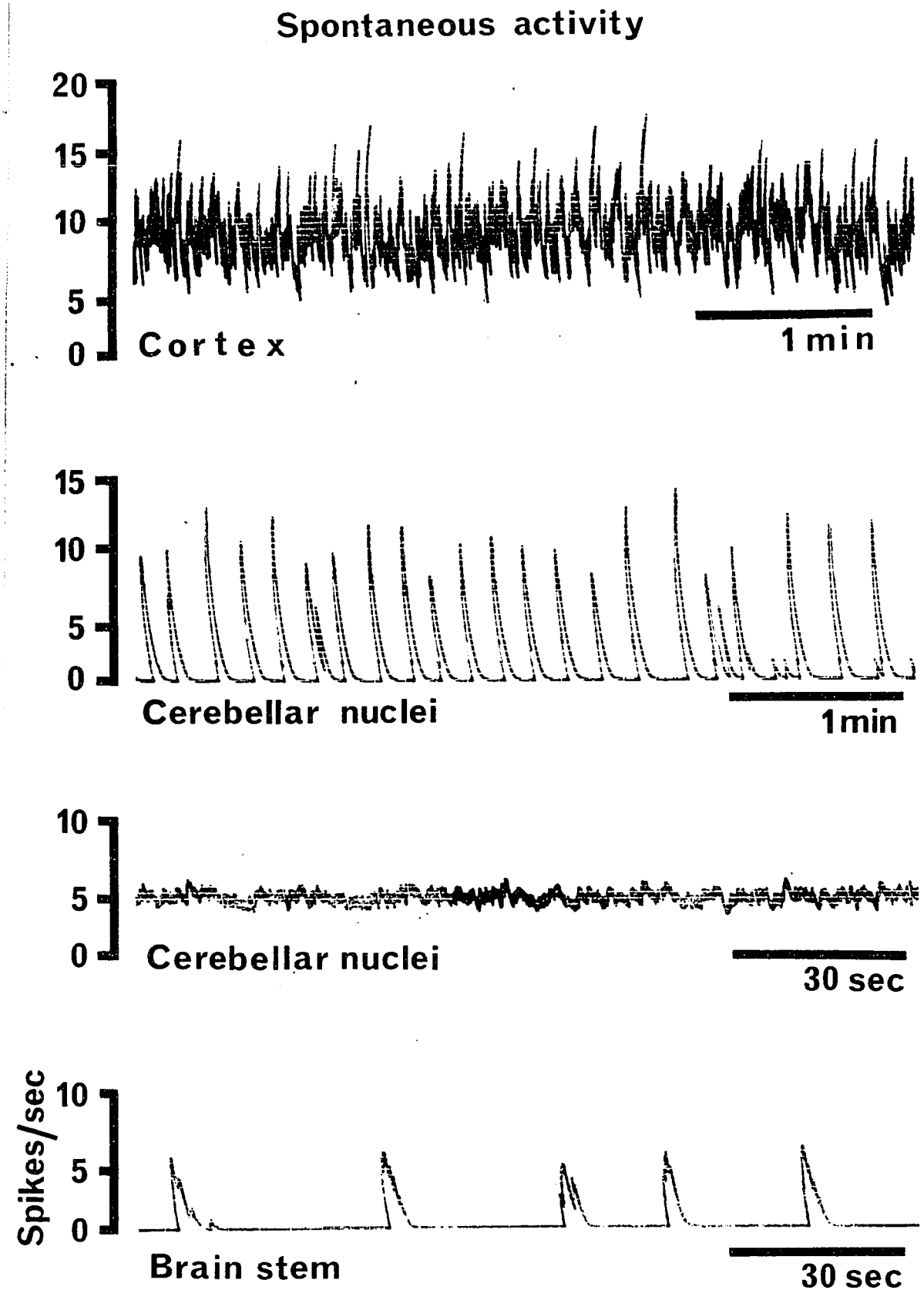

Spontaneous activity
20
15
10
5
0
Cortex
1 min
15
10
5
0
Cerebellar nuclei
1min
10
5
0
Cerebellar nuclei
30 sec
Spikes/sec
10
5
0
Brain stem
30 sec

FIGURE 14

bursting, and the average rate was usually slower (1-5/sec). Other units fired regular single spikes or regular bursts of spikes (Fig. 14). Bursts in DN neurones were usually longer than bursts in cortical neurones. BS neurones usually fired with irregular single spikes at a slow rate (less than 5/sec) and in some cases they fired in bursts of spikes separated by silent periods (Fig. 14). The rate and pattern of the spontaneous activity in each region varied widely from culture to culture and also in different neurones within the same culture. The differences in the characteristics of spontaneous activity in the three regions of the culture were only quantitative and it was not possible to ascribe any unique pattern to just one neuronal type.

It is likely that the number of neurones in each region which were not spontaneously active has been underestimated in this series of experiments, because they were often difficult to identify. These silent cells were noticed only if they could be activated by stimulation with another electrode or if it was observed under the microscope that the recording electrode was clearly touching the neuronal membrane and yet no activity was detected. In other cases, the silent neurones could easily remain unnoticed. This problem could be minimized by using iontophoretic electrodes containing an excitatory substance such as glutamate, which excites silent neurones and therefore makes it possible to detect their presence. It must be realized, however, that even this technique is not flawless because the leakage of glutamate cannot be completely eliminated even when the retaining iontophoretic current is used so the results obtained may still give too high a proportion of active neurones (Obata et al., 1970b). In addition, it should not be assumed that glutamate excites all neurones. Although this is a common assumption, recent findings by Yamamoto (1976) show

that some Cb interneurones can be inhibited by glutamate.

With the use of glutamate electrodes it was determined in a separate series of experiments that only 43% of DN neurones were spontaneously active in contrast to 67% shown in Table 5. Unfortunately, no additional systematic studies were done on other types of neurones.

The characteristics of the spontaneous activity were not consistently different in cultures derived from lateral or medial parts of the Cb and no clear differences could be observed between cultures of Cb only, and those which included BS neurones.

The spontaneous activity of neurones could be modified by various chemical agents such as magnesium ions and the convulsants Bicuculline, Picrotoxin and Strychnine which are known to affect synaptic transmission. Their effects will be dealt with in subsequent chapters. It was also observed that the temperature of the perfusing solution had an effect on the spontaneous activity. The rate of firing in cortical neurones was usually decreased by lowering the temperature from 35 to 25^{o}C, and increased by raising it, whereas in DN neurones opposite effects were sometimes seen.

b) Intracellular Recordings

During intracellular recordings, it was possible to detect spontaneous synaptic activity, which would not be apparent with extracellular technique. Typical examples of spontaneous EPSPs and IPSPs are shown in Fig. 15. Although these recordings have been obtained from only a few neurones, some apparent differences have been observed between cortical and DN areas. Thus, while in the cortical neurones both EPSPs and IPSPs were seen, in the DN neurones IPSPs were predominant.

It can be postulated that the IPSPs in the DN neurones were produced by P neurone axons which project to this region (see part IV).

FIGURE 15. Intracellular recordings of spontaneous synaptic activity.

Top trace - EPSPs and IPSPs in a cortical neurone.

Bottom trace - IPSPs in a DN neurone. Membrane potential in this neurone was 30 mV.

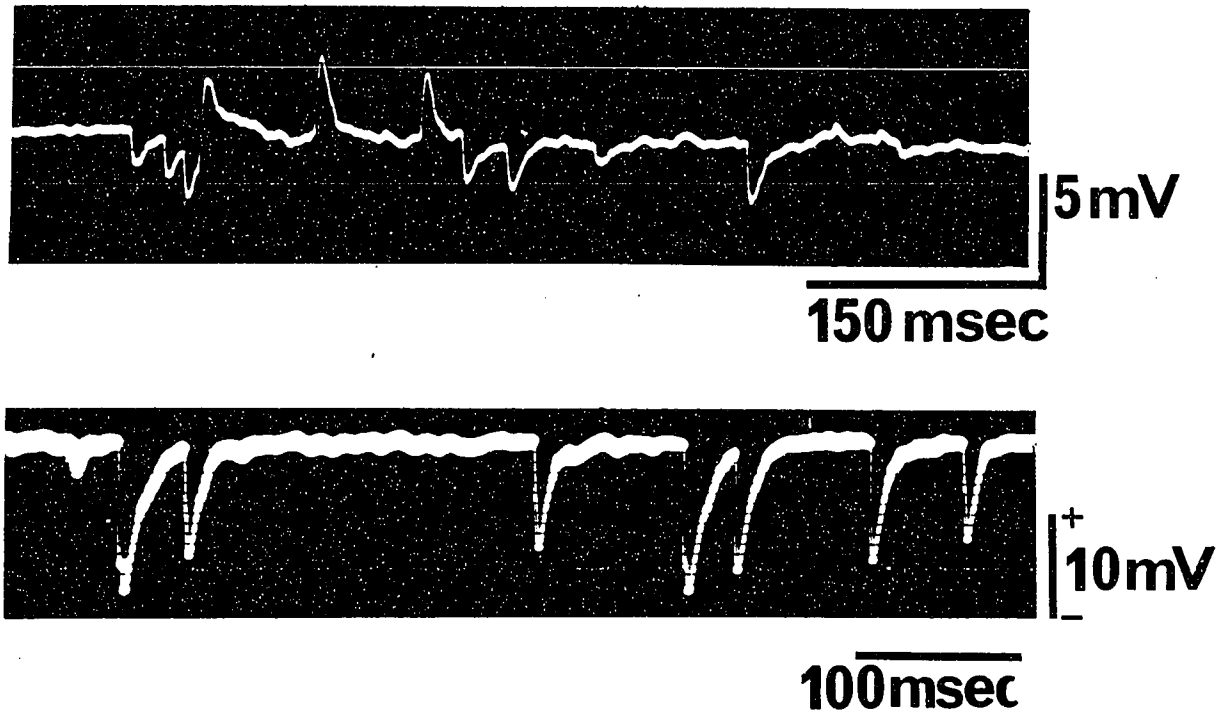


FIGURE 15

Part III - Effects of Magnesium Ions (Mg) on Membrane Excitability and Synaptic Transmission

Mg has been used by other investigators as a selective blocking agent of synaptic transmission in tissue cultures. In the initial experiments, I have used 8-10 mM magnesium chloride to study the effects of this cation on spontaneous activity. The purpose of these experiments was to distinguish between pacemaker neurones and the neurones which are driven by a synaptic input. In all but a few of about 100 neurones tested in all regions of cultures, the spontaneous activity was completely stopped by magnesium (Fig. 16) while in the rest the activity was greatly depressed. This observation differs from that of Gähwiler et al. (1973) who observed cessation of activity in about 50% of the neurones. It must be noted, however, that these authors used roller tube cultures which tend to be more dispersed. This in turn could result in reduced synaptic density on neurones.

It was a greater surprise that antidromic activations of neurones were also strongly affected by Mg. The DN neurone shown in Fig. 17A for example had been excited antidromically by a cortical stimulation and yet 8 mM Mg completely abolished its response even at maximal possible stimulus intensity. 12 other neurones from BS and DN areas were tested in a similar way using 5-10 mM Mg. The antidromic activations were reversibly blocked in six neurones; in six others the threshold for activation was reversibly increased by 10-200% and in one neurone there was no change in the threshold (Table 6). It can be seen from the table that considerable differences in sensitivity to Mg were observed between the neurones, however, the reason for this is not known. It is perhaps of interest that such differences occurred even between neurones within the same culture. An example is shown in Fig. 17B.

FIGURE 16. Ratemeter recordings of spontaneous activity in a DN neurone (top trace) and a Cx neurone (bottom trace). 8 mM $MgCl_2$ produced a complete block of the activity. Arrow up indicates the time when $MgCl_2$ was switched into the perfusing solution and arrow down when it was switched off.

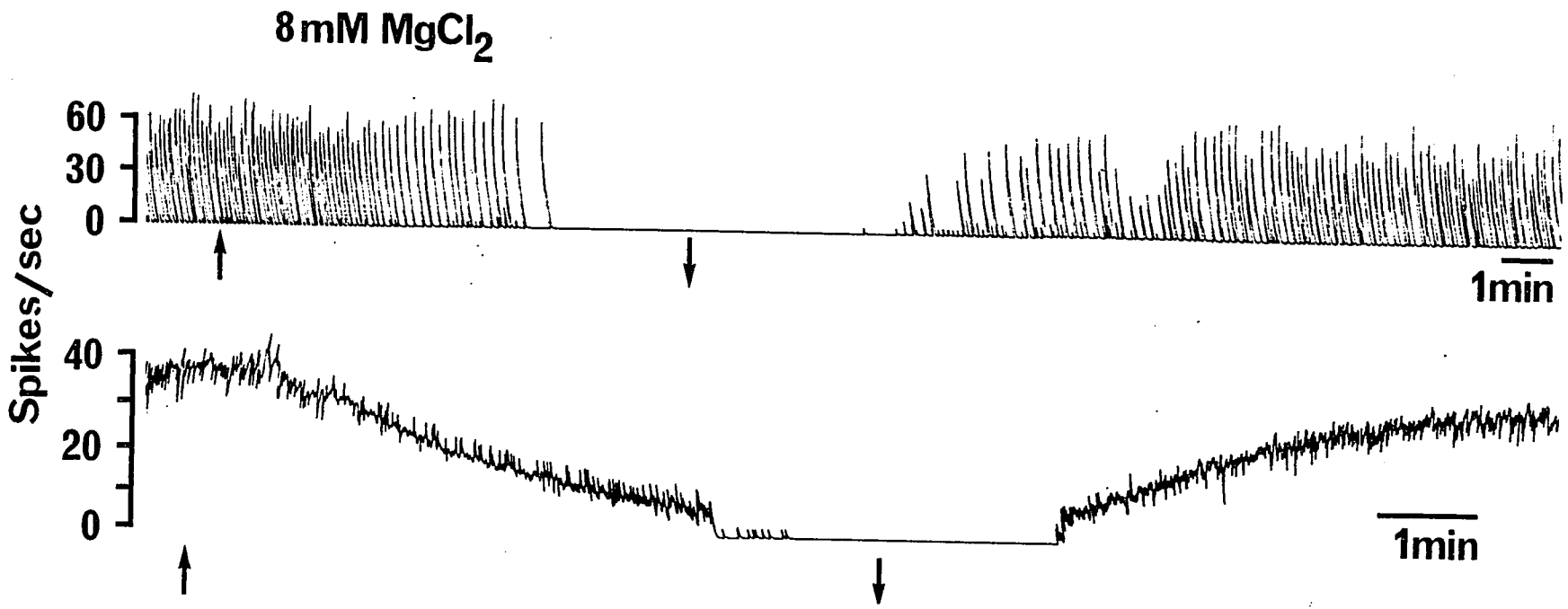


FIGURE 16

FIGURE 17. A - Effect of Mg (8mM) on the antidromic activation of a DN neurone. Note, this neurone is also shown in fig. 10.
B - Effect of Mg (10mM) on the antidromic action potential, field response and a synaptic action potential in a BS neurone.
The antidromic AP is superimposed on the negative field potential and has the latency of 2.2 msec. The synaptic activation (possibly due to recurrent axon collateral of BS neurone, see section IVe) has the latency of about 8 msec. Mg was switched into the perfusing solution after the control record was taken. During the continuous perfusion, the Mg concentration in the chamber gradually increased and reached a sufficient concentration to block the synaptic potential first (Mg 4 min) followed by the antidromic one (Mg 6 min). The field potential was reduced in amplitude to about 30% of its initial value. Recovery occurred in a reversed order.
First record - single sweep, all others - superimposed sweeps.

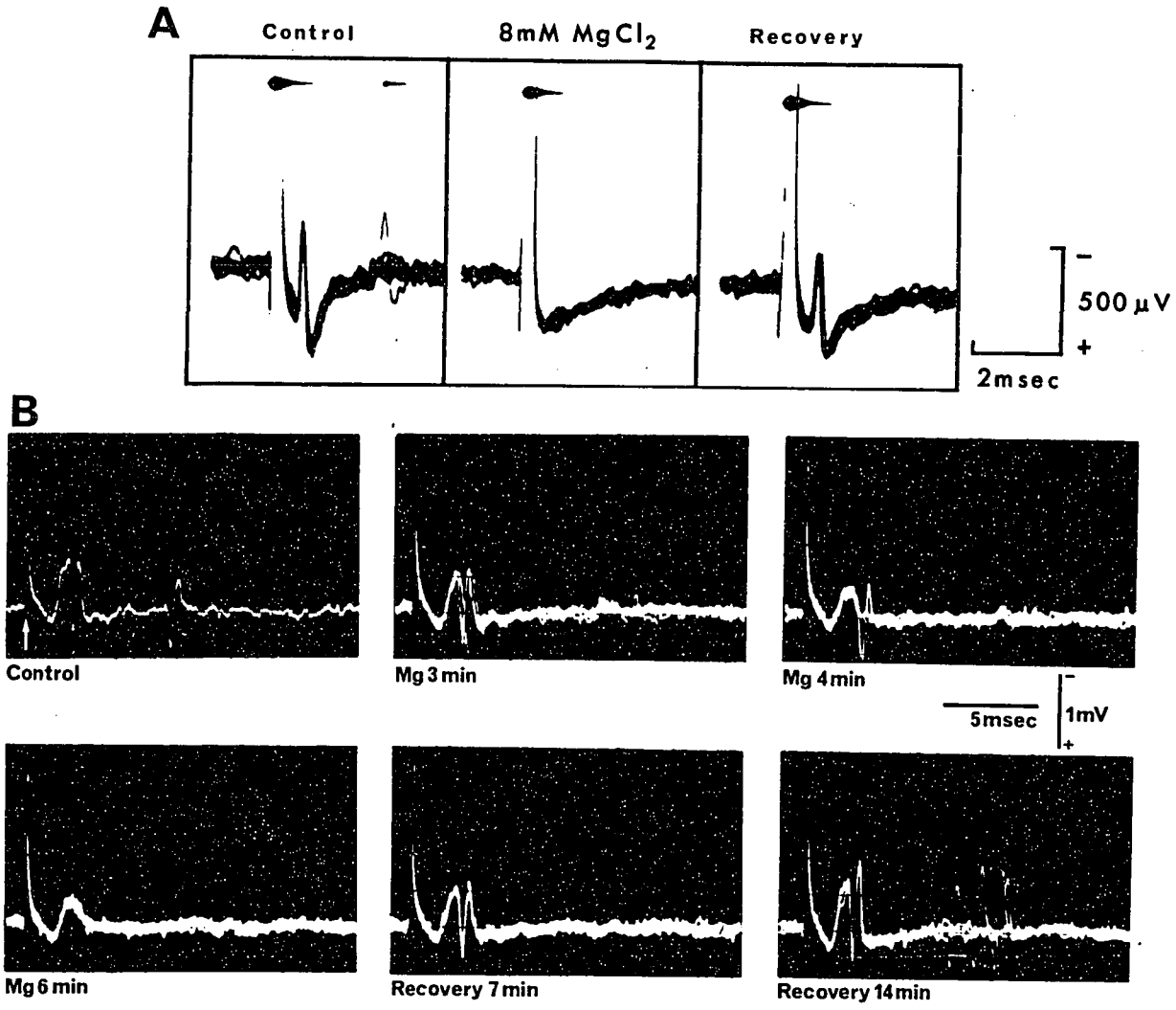

A
Control
8mM $MgCl_2$
Recovery
500 µV
2msec
B
Control
Mg 3 min
Mg 4 min
5msec
1mV
Mg 6 min
Recovery 7 min
Recovery 14 min

FIGURE 17

In this case an antidromic spike from a single neurone is superimposed on a field potential which is likely to represent antidromic activations of other neurones in the area because it had a similar latency as all antidromically evoked spikes in this culture. 10 mM Mg completely blocked the antidromic activation of the neurone shown, whereas in another neurone tested (not illustrated) the threshold was raised by only about 10% of its control value.

Figure 17B also shows that the action of Mg is not necessarily unselective because in this and many other cases studied it has been observed that the synaptic excitation was more readily blocked by Mg than the antidromic one. It appears, however, that to achieve a selective block of the synaptic transmission careful adjustment of the concentration of Mg is necessary in order to avoid a direct depression of the membrane.

In other experiments responses to iontophoretically applied glutamate were used as a test of neuronal excitability. It was found in tests on 19 Purkinje and BS neurones that even strong excitations caused by 7 to 10 second pulses of glutamate were markedly reduced by 10 mM Mg (Fig. 18) and a considerable depression was seen by 5 mM Mg. To test whether this depression could be explained by the removal of ongoing excitatory synaptic activity, the effects of 5 mM Mg with 3.6 mM Ca were tested, as it has been found that the Mg depression of transmitter release at the neuromuscular junction is reversed by this concentration of Ca (del Castillo & Engbaeck, 1954; Jenkinson, 1957). It can be seen from Fig. 19A that 3.6 mM Ca or 5 mM Mg depressed neuronal excitability to about the same degree, and that they were <u>additive</u> in this action. Figure 19B illustrates the composite data from four neurones tested in a similar manner, in which complete data were obtained. Glutamate pulses

FIGURE 18. Continuous ratemeter record showing the effect of 10 mM Mg on activity evoked by 10-nA iontophoretic pulses of glutamate. Iontophoretic pulses are signalled by indicator record at top. Considerable latency for the effects of Mg is largely explained by lag time for perfusion system and mixing in the bath (about 5 min). An arrow up indicates when test Mg solution was switched into the perfusion system and arrow down when it was switched off.

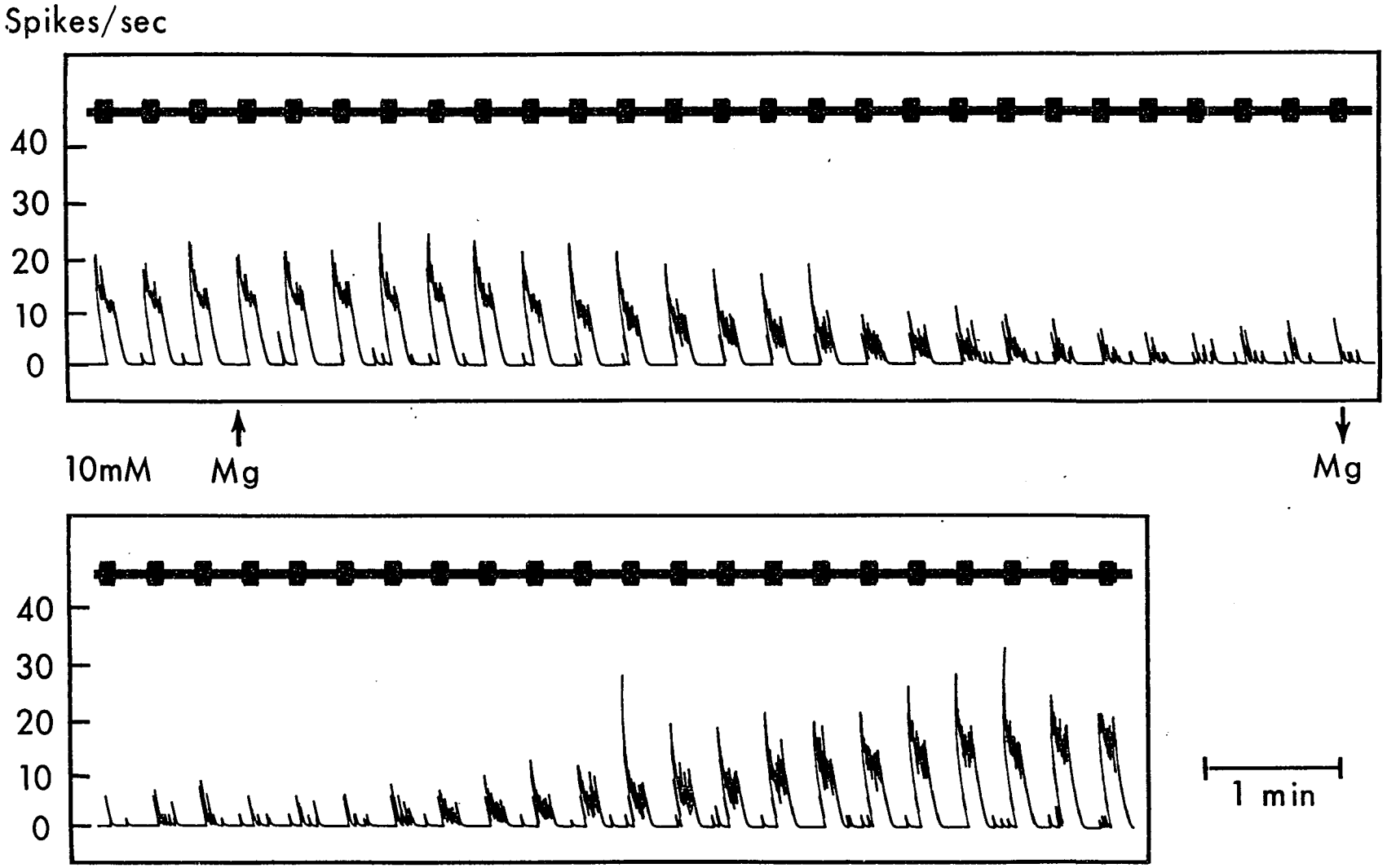


FIGURE 18

FIGURE 19 A - Partial ratemeter records of a neuronal firing during glutamate application (20 nA). Records were taken during perfusion by solutions containing different concentrations of divalent cations.

B - Composite data from four neurones tested as in A, using 10 and 20nA iontophoretic pulses of glutamate. Standard errors indicated.

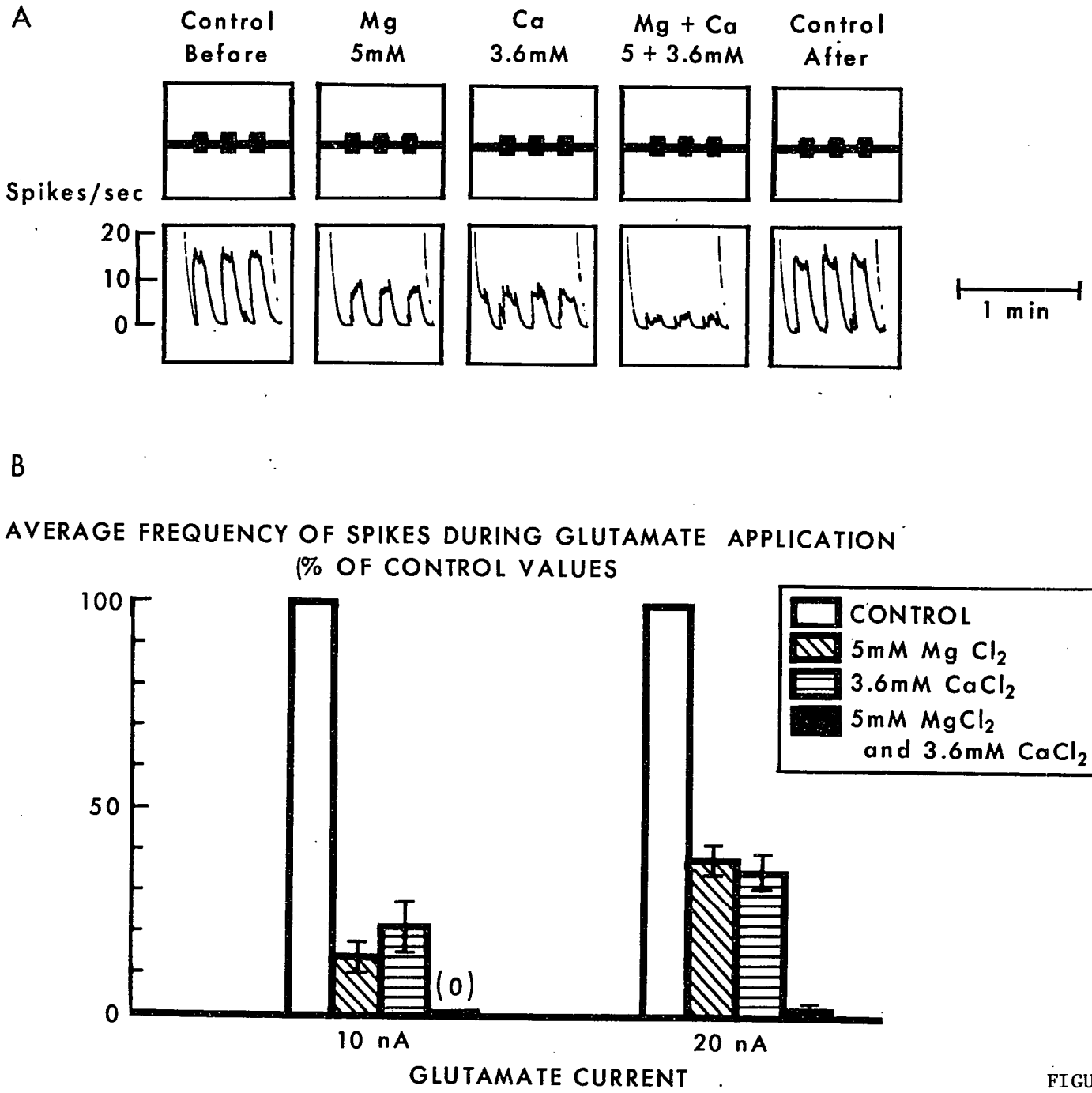

A
Control
Before
Mg
5mM
Ca
3.6mM
Mg + Ca
5 + 3.6mM
Control
After
Spikes/sec
20
10
0
1 min
B
AVERAGE FREQUENCY OF SPIKES DURING GLUTAMATE APPLICATION
(% OF CONTROL VALUES
100
50
0
(0)
CONTROL
5mM Mg Cl2
3.6mM CaCl2
5mM MgCl2
and 3.6mM CaCl2
10 nA
20 nA
GLUTAMATE CURRENT

FIGURE 19

of two current intensities were used. Statistical analysis of the number of action potentials evoked by the glutamate pulses show that Mg at 5 mM and Ca at 3.6 mM depress the excitability and that the combination of both gives a significantly greater depression than either one alone (Student t test, $p<0.05$). Control experiments using solutions having increased osmolarity (equivalent to the test solutions) showed that osmolarity changes within this range have no detectable effect on spontaneous activity or on membrane excitability as tested by glutamate pulses.

Part IV - Synaptic Connections

a) Deep Nuclei (DN) to Cerebellar Cortex

A stimulating electrode was placed in the DN area with the intention of activating directly most DN neurones. This was feasible since these neurones are usually found in small clusters. In several experiments APs were recorded from DN neurones adjacent to the stimulating electrode in order to determine the stimulation field (see methods). It is of interest to point out here that the latency of direct activations varied considerably in different units even when the distance from the stimulating electrode was the same. Thus in some neurones the latency was within 0.1 msec whereas in others it was as long as 0.4 msec even with suprathreshold stimuli (e.g. Fig. 28A, part IVe). No obvious explanation is available for this finding but it should be noted since it is important in the interpretation of the following experiments.

Only larger neurones (above 15 μ in diameter) of the cortical area were studied. This includes both Purkinje (P) and large Golgi neurones since in the living state they cannot be differentiated on morphological grounds. In this section they will be referred to jointly as cortical (Cx) neurones. It is probable, however, that a majority of these neurones are P neurones simply because they outnumber Golgi neurones by 3 to 1 (Palay & Chan-Palay, 1974, p. 102, see discussion).

In most cultures studied, stimulation of DN evoked APs in Cx neurones. Examples are shown in Fig. 20. The latency was variable near threshold and as the intensity of stimulus was increased the latency became progressively shorter and more constant (A, B, C in Fig. 20). This was probably due to the gradual recruitment of EPSPs.

FIGURE 20. A - Superimposed sweeps of the evoked action potential in a Cx neurone after 1/sec stimulation of the DN area 500 μ distant. Stimulus (arrow) intensity was progressively increased from just above threshold (5 μA) to its maximal value (15 μA), resulting in a more constant and shorter latency of the evoked response. At higher frequency of stimulation the latency was variable even at 15 μA (not illustrated).

B - Superimposed sweeps of the evoked action potential in another Cx neurone. The stimulus intensity was set at 8 μA (≈3x threshold) and the frequency of stimulation was gradually increased from 1/sec to 20/sec. Note that the latency was more variable and often shorter at higher frequency. Both neurones were recorded from the same culture.

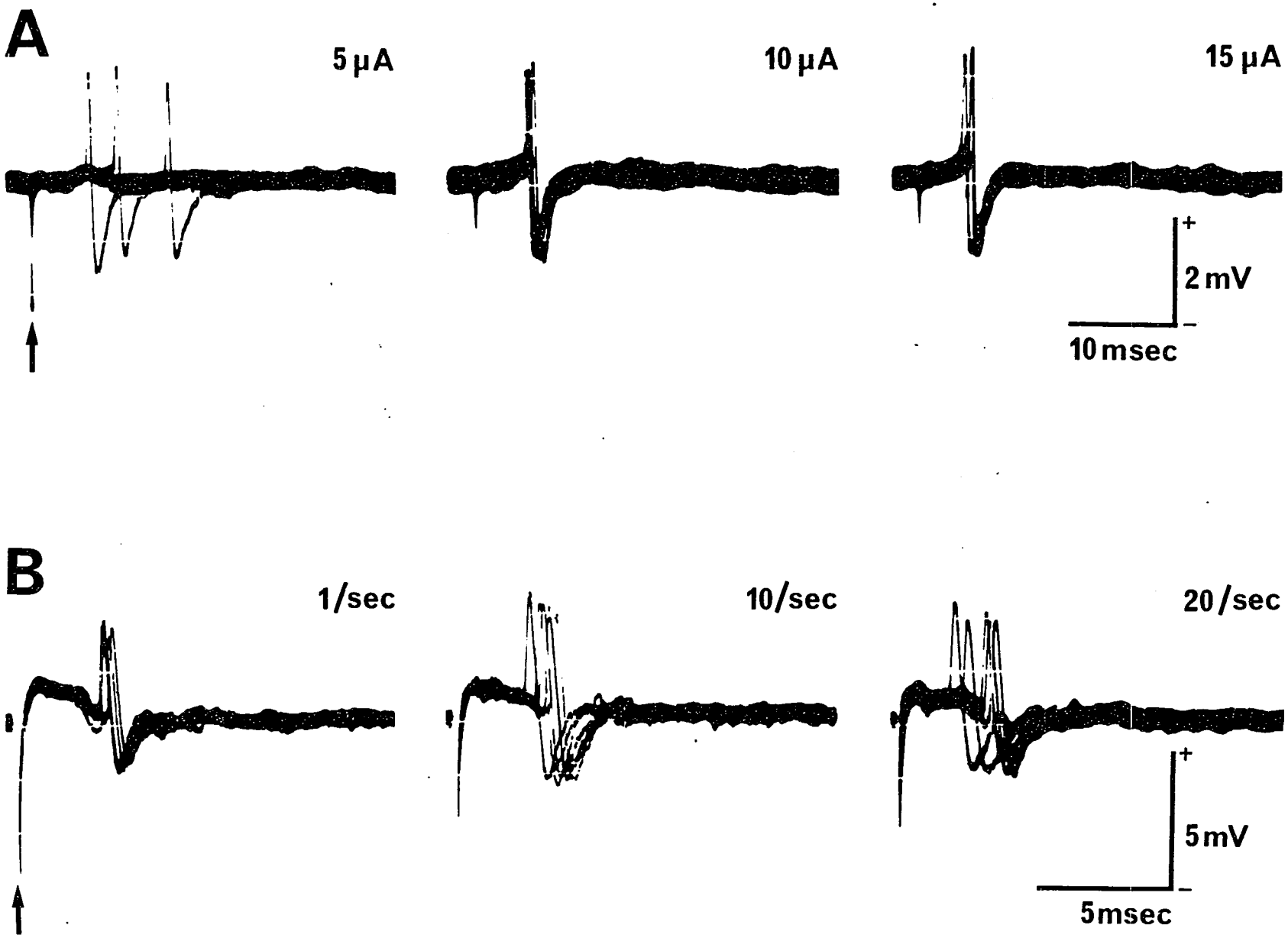
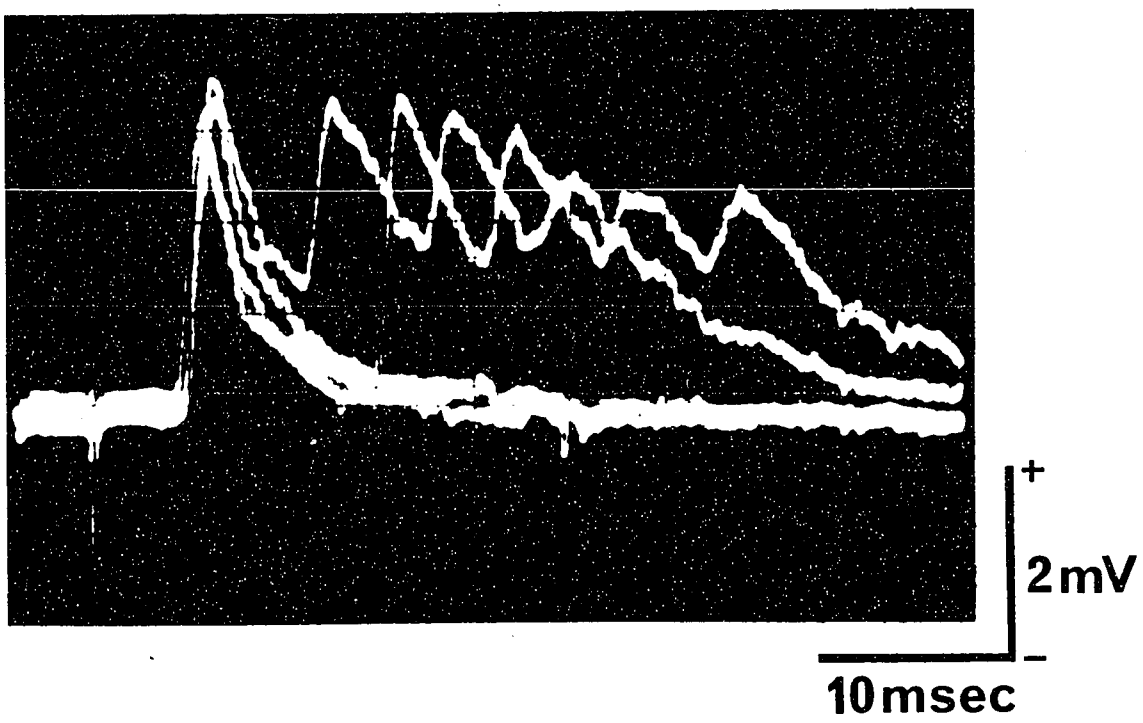


FIGURE 20

However, even at suprathreshold intensity the latency varied by 1-3 msec, especially when the frequency of stimulation exceeded 1/sec (D, E, F). Characteristically the latency sometimes became shorter as the frequency of repetitive stimulation was increased to 20-50/sec, possibly due to the summation of the successive EPSPs, but the action potentials could not follow the stimulation at frequencies above 50/sec. The evoked potentials in other neurones had similar characteristics but they often failed to follow the stimulation at frequencies above 10/sec and the latencies were more variable even at a low frequency (1/sec) and suprathreshold intensity (see Table 7 for summary). The evoked potentials were seen in both spontaneously active as well as in inactive neurones. All the properties of the evoked APs suggest that there is a synaptic, excitatory connection between the DN and the Cx. In addition, in about 10% of the Cx neurones a suppression of the spontaneous activity has been observed, indicating an inhibitory connection. These findings are supported by a preliminary series of intracellular recordings performed on 10 Cx neurones. In 9 of these neurones evoked synaptic potentials were obtained. Clear, graded EPSPs were evoked in 2 neurones and one of them is shown in Fig. 21 (top trace). In this particular case the initial simple EPSP was sometimes followed by a complex EPSP which varied in amplitude and duration with successive stimuli. An increase in the stimulus intensity produced an increase in the amplitude of the initial EPSP but not in that of the complex EPSP. APs could not be evoked, presumably because of partial deterioration of the neurone. In 5 other neurones, short depolarizing potentials were immediately followed by IPSPs (Fig. 22, top trace). It can be proposed that these potentials represent a rising phase of EPSPs which are partly superimposed on the

FIGURE 21. Top - Evoked EPSPs in a Cx neurone. Distance 800 μ (4 superimposed sweeps, DC-intracellular recording). Bottom - Evoked bursts in another Cx neurone. Distance 400 μ (5 superimposed sweeps, extracellular recording).



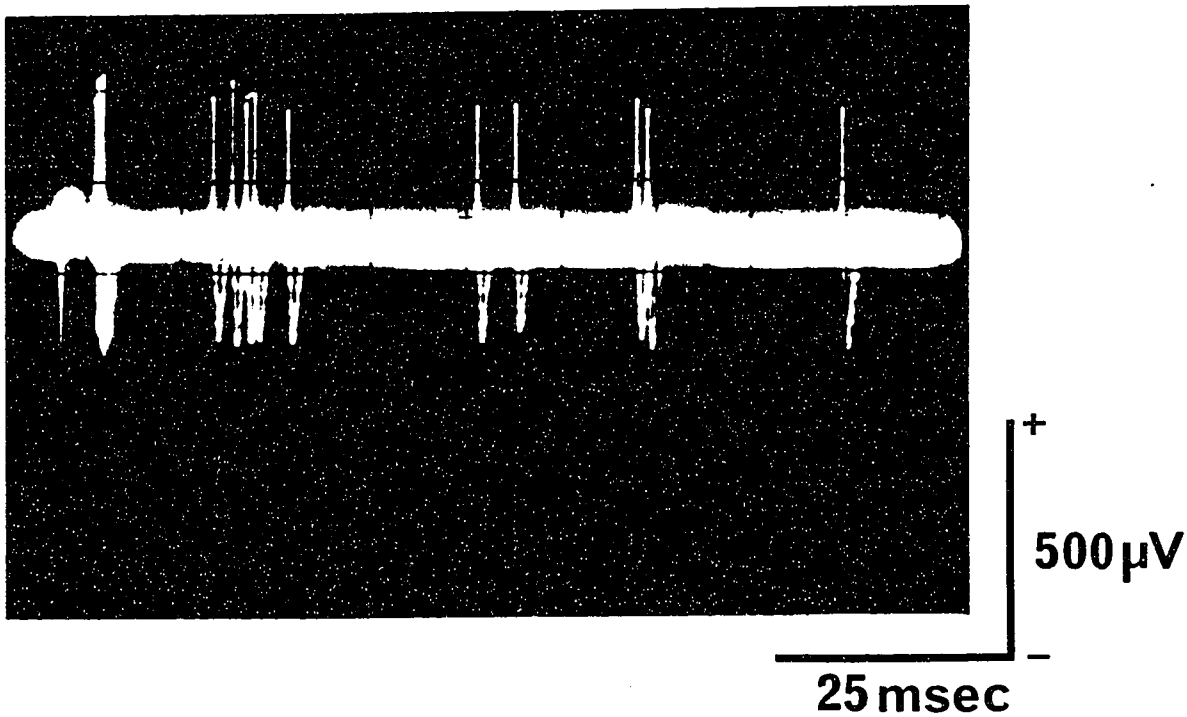


FIGURE 21

FIGURE 22. Top - Evoked responses (rising phase of EPSP followed by IPSP) in a Cx neurone. DN stimulation. Distance 500 μ. AC-intracellular recording.
Bottom - Evoked IPSPs in another Cx neurone in the same culture. DC-intracellular recording.

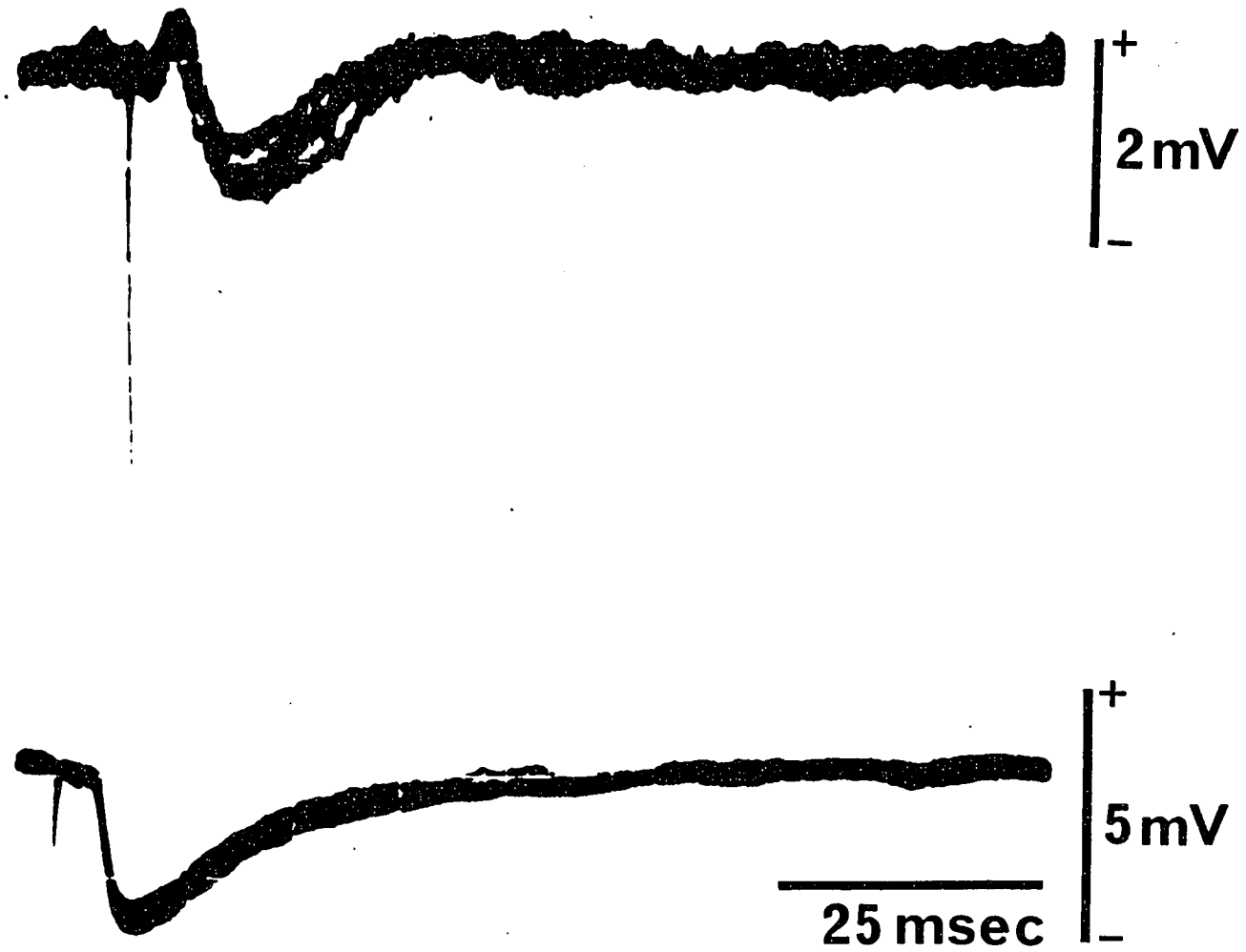


FIGURE 22

IPSPs. These responses are very similar to those observed by Eccles et al. (1967, p. 83) during the stimulation of parallel fibers in the cat Cb. They were able to separate EPSPs from IPSPs by a gradual hyperpolarization of the neurones, but in culture the intracellular recordings were not sufficiently stable to confirm this finding.

In the remaining neurones only evoked IPSPs were observed (Fig. 22, bottom trace). The latencies of two evoked EPSPs, IPSPs as well as presumed EPSP-IPSP sequencies were in the same range as the latencies of evoked extracellular spikes (see Table 7).

The proportion of evoked neurones from a total of about 90 studied (extracellular and intracellular) was about 50% but wide variations in this ratio have been noticed from culture to culture.

In order to determine if the synaptic projection from DN to Cx is monosynaptic the results of all extracellular studies have been summarized in Table 7. Shown in this table are the latencies of single evoked spikes similar to those of Fig. 20 as well as those of EPSPs and IPSPs. All latencies were measured from the beginning of the stimulus artifacts to the beginning of the potentials. From each latency a hypothetical synaptic delay of 0.5 msec was subtracted and subsequently the distances separating the stimulating and recording electrodes in each case were divided by these corrected latencies. Such calculations would give the values for the conduction velocities of presynaptic axons if the connections were monosynaptic. The calculated values from 32 neurones gave the mean value of 0.20 m/sec (standard deviation - 0.09) and the range between 0.04 and 0.47 m/sec. Such a slow conduction is inconsistent with a mean value for DN axonal velocities of 0.71 m/sec

(see part I of the results), and therefore the DN-Cx projection is unlikely to be uniformly monosynaptic. Nevertheless many of the individual values shown in Table 7 could be consistent with a monosynaptic projection because they are within the range of DN conduction velocities (refer to Fig. 12). Axons giving rise to such projection would have to be slowly conducting axons of DN neurones (0.2 - 0.5 m/sec).

In five neurones (≃ 10% of all evoked neurones) the evoked responses occurred in form of bursts; usually 2-5 spikes at a frequency of 50-200/sec (Fig. 21, bottom trace). The number of spikes within the bursts was usually dependent on the stimulus intensity. In two cases it was also observed that the number of spikes evoked by each stimulus was increased by moving the recording electrode closer to the neurone. The latencies of the first spikes within the bursts were in the same range as those of singly evoked spikes and thus they were suggestive of polysynaptic rather than monosynaptic transmission.

In many instances evoked negative field potentials were recorded which had a duration of 2-4 msec and were composed of several small spikes (≃ 200 μV). These potentials were usually ignored since it was not possible to determine precisely if they were directly or synaptically evoked. The latencies of six such responses are given in Table 7. The average conduction velocity (0.36 m/sec) for these field potentials is higher than for extracellular single spikes and the latencies were always more constant. These field potentials could represent monosynaptic activations of cortical interneurones (e.g. granule cells).

b) Brain Stem (BS) to Cerebellar Cortex

A series of experiments was specifically designed to demonstrate a

projection from the BS area to the cortex. However, electrical stimulation by single or repetitive (10/sec) pulses usually failed to produce any response in the Cx neurones. In the same cultures a stimulation of the DN region readily evoked spikes in the cortex. Only in a few cases did the BS stimulation produce a long latency (above 5 msec) excitation of the Cx neurones, which, in accordance with the conduction velocity of BS neurone axons, was probably polysynaptic.

The interpretation of these occasionally positive results is difficult because of the possibility of stimulating axon collaterals of DN neurones which might spread to the BS region. Alternatively BS neurones could excite the DN neurones which in turn project to the cortex. However, the results were not sufficiently reproducible to encourage further investigation.

c) Cerebellar Cortex to Deep Nuclei

The presence of an inhibitory pathway from the Cb cortex to the DN is well documented in vivo. The purpose of this series of experiments was to verify if an analogous pathway can be reproduced in cultures.

Useful data were obtained from a population of 129 DN neurones from 60 cultures. These neurones were selected on the basis of a favourable signal to noise ratio (at least 2:1) and stable recording for periods of at least 20 minutes (and often for several hours). 76% of these neurones did not have any spontaneous activity or were active at a rate of less than 5 spikes/sec and were therefore continuously activated by iontophoretic application of glutamate. In 84% of the neurones this activity could be inhibited as a result of electrical pulses applied through stimulating electrodes in the cortical region (Fig. 23A).

FIGURE 23. Examples of evoked inhibitory responses in DN neurones.
A - Top - 20 superimposed traces showing the evoked inhibition of the activity of a DN neurone.
Bottom - The same in the form of a peristimulus histogram. Note also a slight facilitation following the inhibition. The peristimulus histogram is the result of 250 sweeps, the bin width is 2 msec. Vertical calibration in this and all other histograms is given as number of spikes per bin. Arrows show the time of the application of the stimulus. Time calibration is equal for both traces.
B - Top - 5 superimposed sweeps of evoked unitary IPSPs (stimulus intensity was just at threshold).
Bottom - Several superimposed sweeps of the evoked summated IPSPs (stimulus intensity was at 2x threshold).
Note - Irregularities of the baseline were caused by spontaneous IPSPs. Intracellular, DC recording. Calibration is equal for both traces.

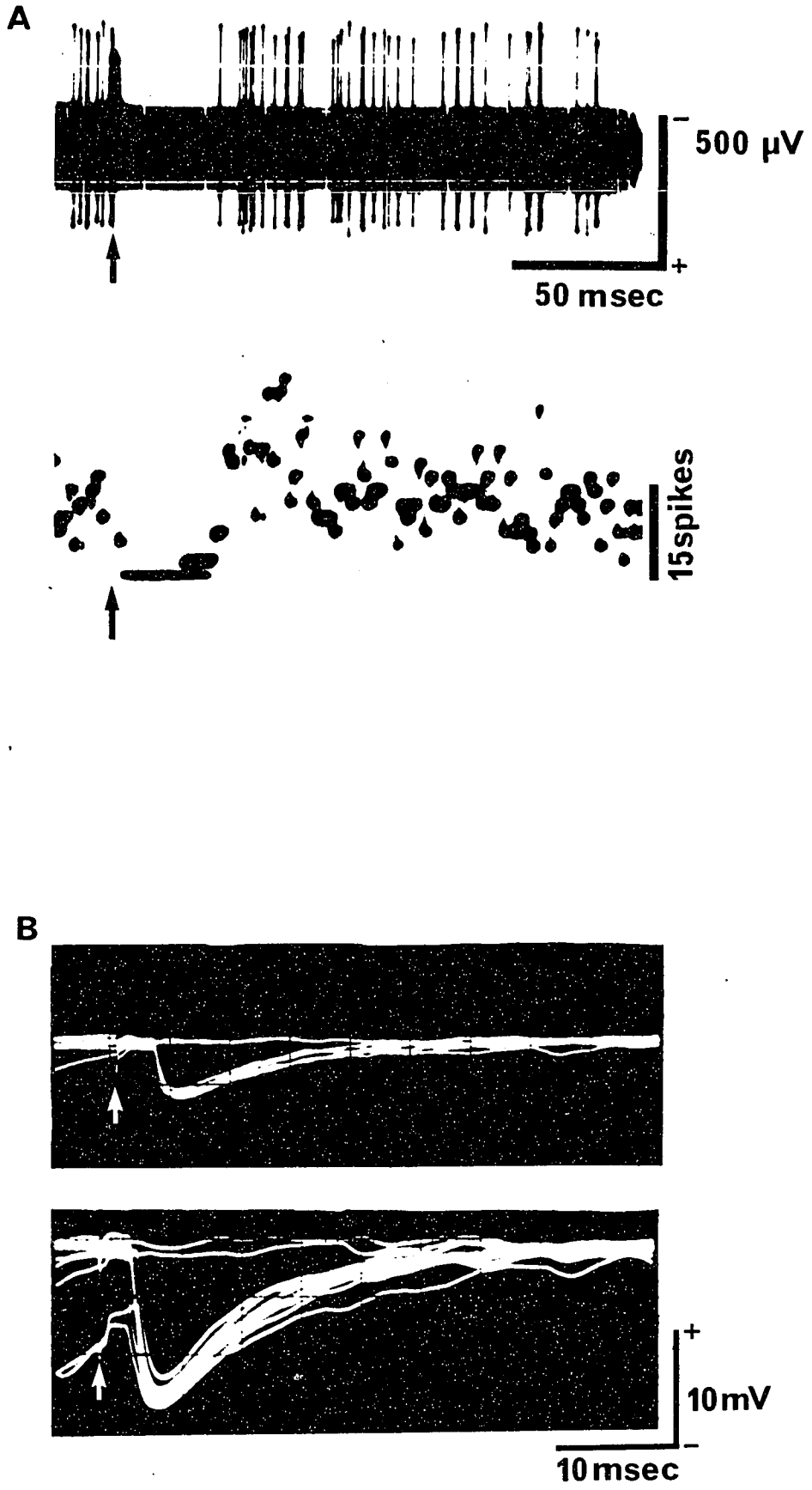


FIGURE 23

However, it is unlikely that this number represents the actual proportion of neurones which can be inhibited, because the stimulating electrode was designed to activate neurones in only a small part of the cortex.

In several experiments two independent stimulating electrodes were placed in the cortical area. In these cases all DN neurones seemed inhibited by one of the electrodes and frequently by both. The maximal duration of the inhibitory period varied from 10-300 msec in different neurones depending on the intensity of stimulus as well as on the rate of background firing of the neurone (Fig. 24). Under our experimental conditions the usual inhibition lasted 20-60 msec with the stimulus intensity at 5-10 μA and the background firing at 10-20/sec. The latency of the onset of inhibition varied from 1 to 6 msec, the usual value being less than 4 msec. In addition, with intracellular recording IPSPs were observed in 9 DN neurones. These IPSPs had a duration of 10 to 70 msec and were the result of summated unitary IPSPs such as the one shown in Fig. 23B. The latencies of the evoked IPSPs ranged between 2.4 - 5.0 msec (mean ≈ 4.0 msec) (see Table 8). Even those IPSPs with a latency greater than 4 msec steadily followed high frequency stimulation (up to 40/sec) suggesting monosynaptic transmission.

Assuming a monosynaptic pathway, conduction velocities were calculated from the latencies of the evoked IPSPs (Table 8) and gave a range of 0.11 - 0.26 m/sec (mean 0.15 m/sec; standard deviation 0.05). The average value is lower than the average conduction velocity of P neurone axons (0.26 m/sec, refer to Table 4) but, considering large standard deviations in both experiments and a likely systematic error resulting in an overestimation of the values presented in Table 4 (see discussion), the latencies of the evoked IPSPs may still be consistent with monosynaptic transmission.

FIGURE 24. The effect of increasing stimulus intensity on the duration of evoked inhibition. The threshold was about 3 μA. The background activity was maintained at 10-15/sec throughout the experiment. 64 sweeps, 2 msec/bin. Note that the bin counting the stimulus artifact was off the screen in these and all other peristimulus histograms.

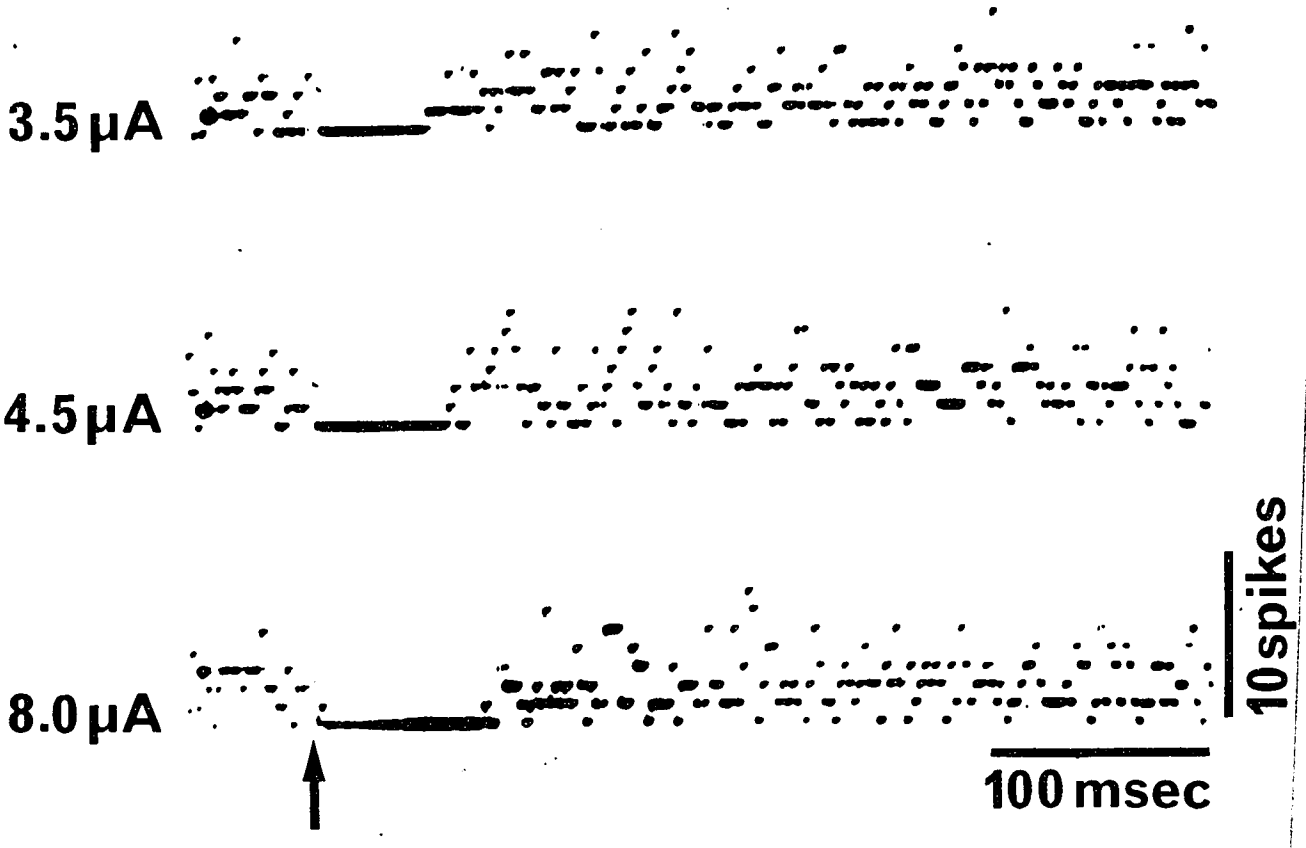


FIGURE 24

In about 50% of DN neurones the inhibition was preceeded by either antidromic or synaptic excitation. These cases presented a problem in interpretation because it was not clear how much of the inhibition was the result of synaptically evoked IPSPs and how much of it was simply the manifestation of a depression of firing following evoked spikes (e.g. due to afterhyperpolarization). The neurone shown in Fig. 25 is an example. The background activity in this neurone was quite regular as shown by the interspike-interval histogram (IIH) (Fig. 25C). A cortical stimulus produced an antidromic spike at a very short latency (less than 2 msec) which was followed by a silent period and subsequent oscillatory discharges (Fig. 25A). The initial silent period is equal to the time intervals between the discharges and also exactly equal to the interspike time interval. Clearly in this case the initial silent interval can be explained purely on the basis of the afterhyperpolarization although some short synaptic inhibition cannot be ruled out. In addition to the usual form of displaying the pattern of spontaneous activity (IIH in Fig. 25C) another form is shown in Fig. 25B. This histogram was constructed by triggering the sweep of a digital analyzer with spontaneous spikes instead of the trigger from the stimulator. Since the histogram is the result of many successive sweeps, it is displaying the pattern of firing in the neurone. It can be seen that this pattern is very similar to the pattern observed following an antidromic spike (Fig. 25A). It appears therefore that antidromic excitation can create an apparent "inhibition" by resetting of the background firing of a neurone. However, in most neurones which were excited at a short latency a genuine inhibition was observed. This could be demonstrated by adjusting the intensity of the stimulus and showing that the onset of

FIGURE 25. A - Peristimulus histogram showing the effects of cortical stimulation (arrow) on the activity of a DN neurone. In this neurone the stimulus produced a short latency antidromic activation (off the screen) which was followed by a depression of firing and oxcillatory rebounds. 128 sweeps, 2 msec/bin.
B - "Autohistogram" (see text) showing the pattern of spontaneous activity in the same neurone. Sweeps were triggered by spontaneous spikes. 64 sweeps, 2 msec/bin.
C - Interspike interval histogram. The peak of the histogram corresponds to 41 msec, the maximal count of the histogram is 70 spikes and the bin width is 1 msec.

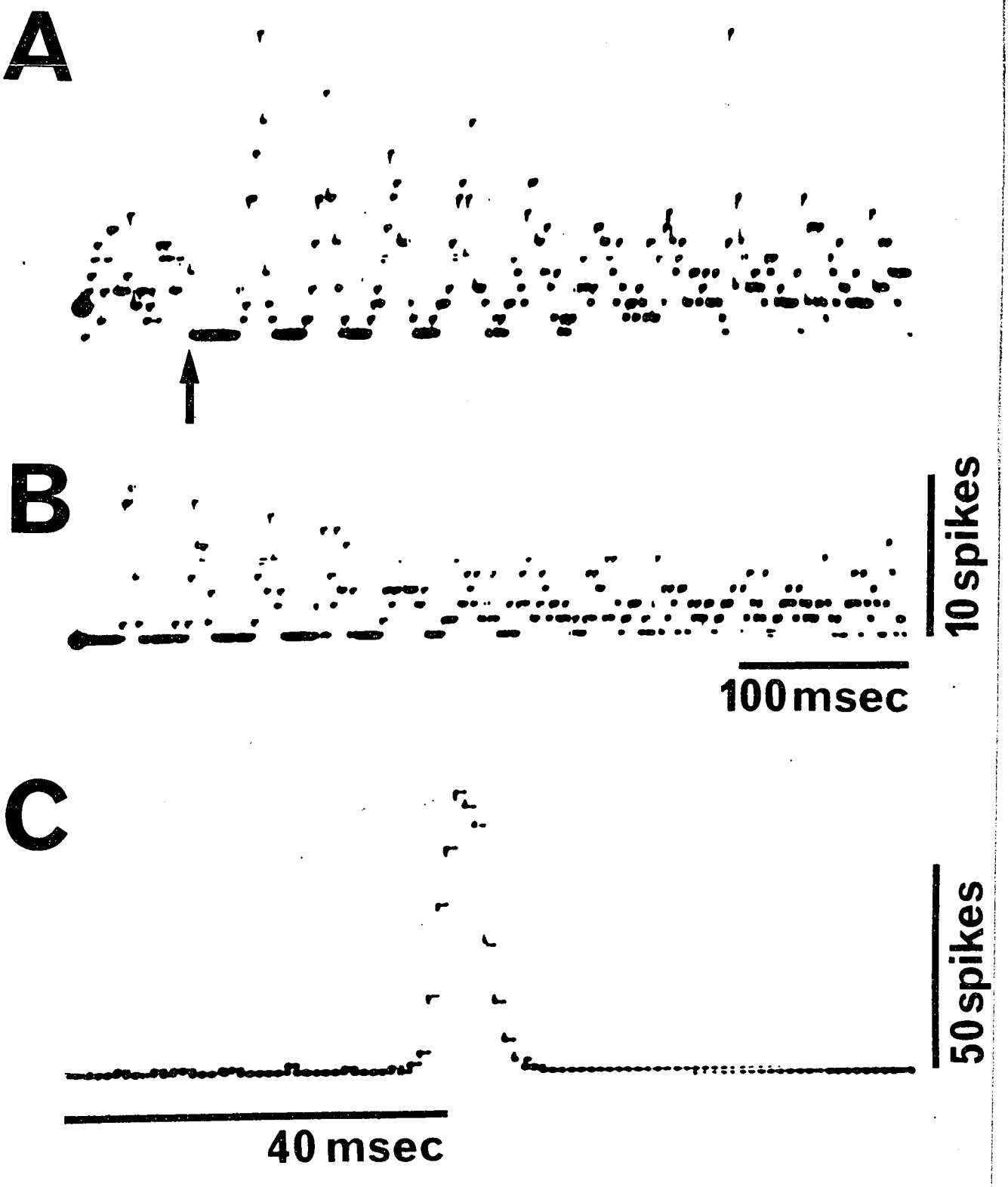


FIGURE 25

the inhibition is not concomitant with the threshold for an evoked spike, or by observing that an increase in the stimulus intensity (beyond the threshold for excitation) produces an increase in the duration of the inhibition.

In 44% of the synaptically inhibited neurones the initial inhibition was followed by a series of oscillatory discharges (usually 3-4) similar to those shown in Fig. 25 but not triggered by the evoked spikes (Fig. 26A). The time interval between these discharges was approximately equal to the interspike time interval of the background firing. This was indicated particularly in some tests where the levels of the glutamate application were adjusted to modify background firing rates. In these cases the repetition rate of the postinhibitory discharges was found to change in accordance with the change in the rate of background firing (Fig. 27).

In 21% of the inhibited neurones a facilitation was observed following the evoked inhibition (Fig. 23, 26B). The facilitation usually lasted 50-100 msec and is thought to be the result of disinhibition (see discussion).

d) Cerebellar Cortex to Brain Stem

Seven cultures have been used in a separate series of experiments in order to determine if electrical stimulation can produce an inhibition of the BS neurones similar to that seen in DN. In five cultures cortical stimulation produced a depression of neuronal firing lasting 20-100 msec. However, only 10 neurones out of some 40 tested in these cultures showed this response. The inhibition was in all respects similar to that of DN neurones but occurred at longer latency. This was more accurately determined during intracellular recordings

FIGURE 26. A - Oscillatory postinhibitory discharges in a DN neurone.

Top - 5 superimposed sweeps showing the evoked inhibition and oscillatory discharges.

Bottom - The same in the form of a peristimulus histogram. 200 sweeps, 2 msec/bin.

B - Facilitation of firing in a DN neurone.

Left - 10 superimposed sweeps showing the evoked inhibition and subsequent facilitation.

Right - The same in the form of a peristimulus histogram. It should be noted that the threshold for the facilitation is lower than that for the inhibition. 200 sweeps, 2 msec/bin.

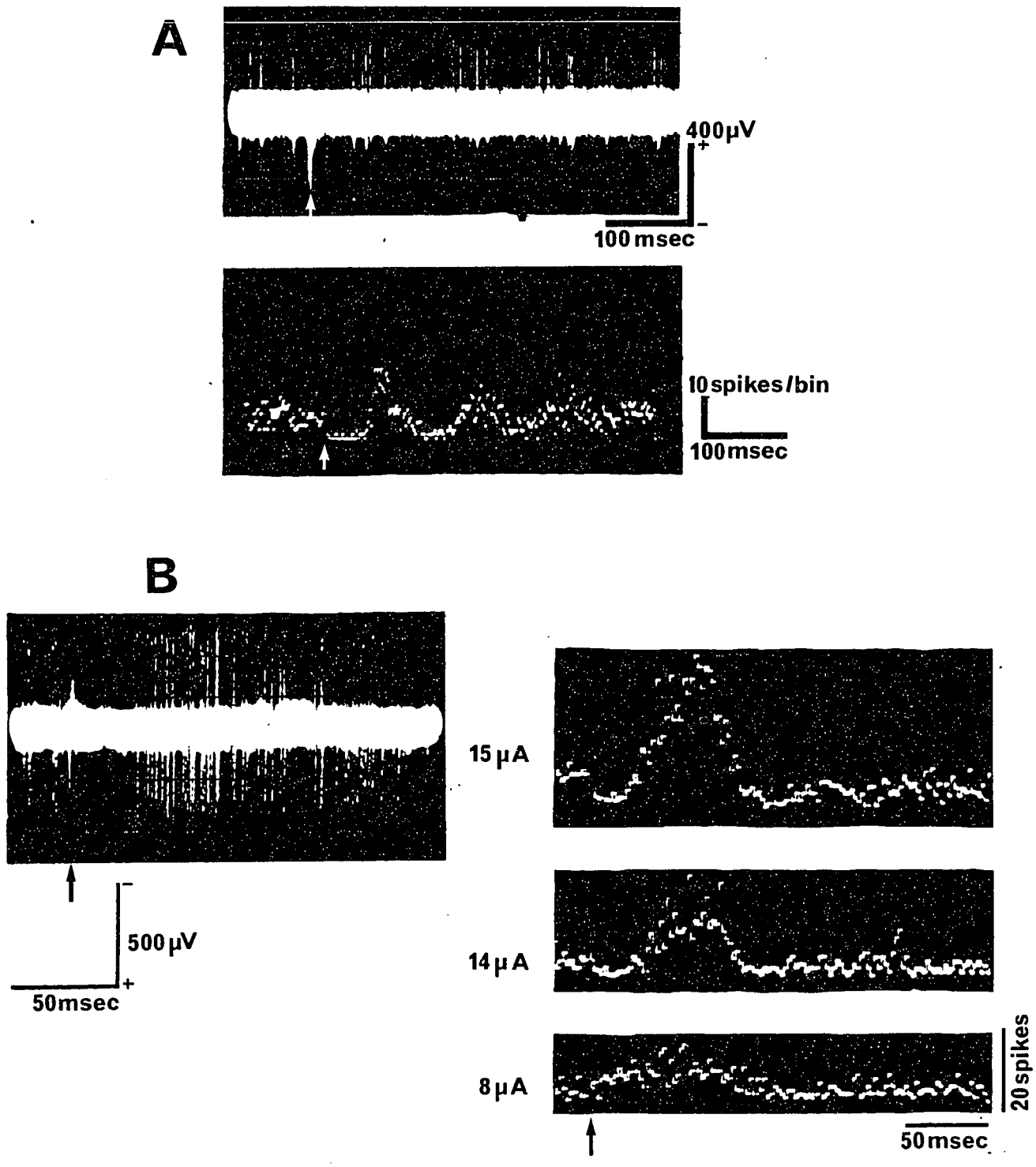

A
400μV
+
−
100 msec
10 spikes/bin
100msec
B
500μV
−
+
50msec
15 μA
14 μA
8 μA
20spikes
50msec

FIGURE 26

FIGURE 27 The effects of changing the rate of background firing in a DN neurone on the oscillatory postinhibitory discharges.

Top row - Peristimulus histogram showing the evoked inhibition and postinhibitory discharges. Interspike interval histogram (IIH) showing the background activity. Autohistogram showing the background activity.

Middle row - The same sequence at a higher rate of background activity.(Stimulus intensity was increased to 11 μA in order to maintain the duration of the inhibition approximately constant).

Bottom row - The same sequence at still higher rate of background activity.

Interpretation - The peristimulus histograms demonstrate that the postinhibitory discharges are more pronounced when the rate of background activity is increased from 15/sec (top) to 23/sec (middle) and 31/sec (bottom) and that the rate of repetition of these discharges follows the rate of the firing. To the right of each peristimulus histogram is a corresponding IIH giving the rate and pattern of the background firing. In the right column the background firing is presented in the form of autohistograms (see text page 98) which show that as the rate of the background activity is increased the rhythmicity of the firing increases, mimicking the postinhibitory discharges.

Calibrations - Horizontal and vertical calibrations in the bottom right corner apply to all peristimulus histograms and autohistograms, 2msec/bin for all. Horizontal calibrations for each IIH are given in the figure. Bin size was 1msec.

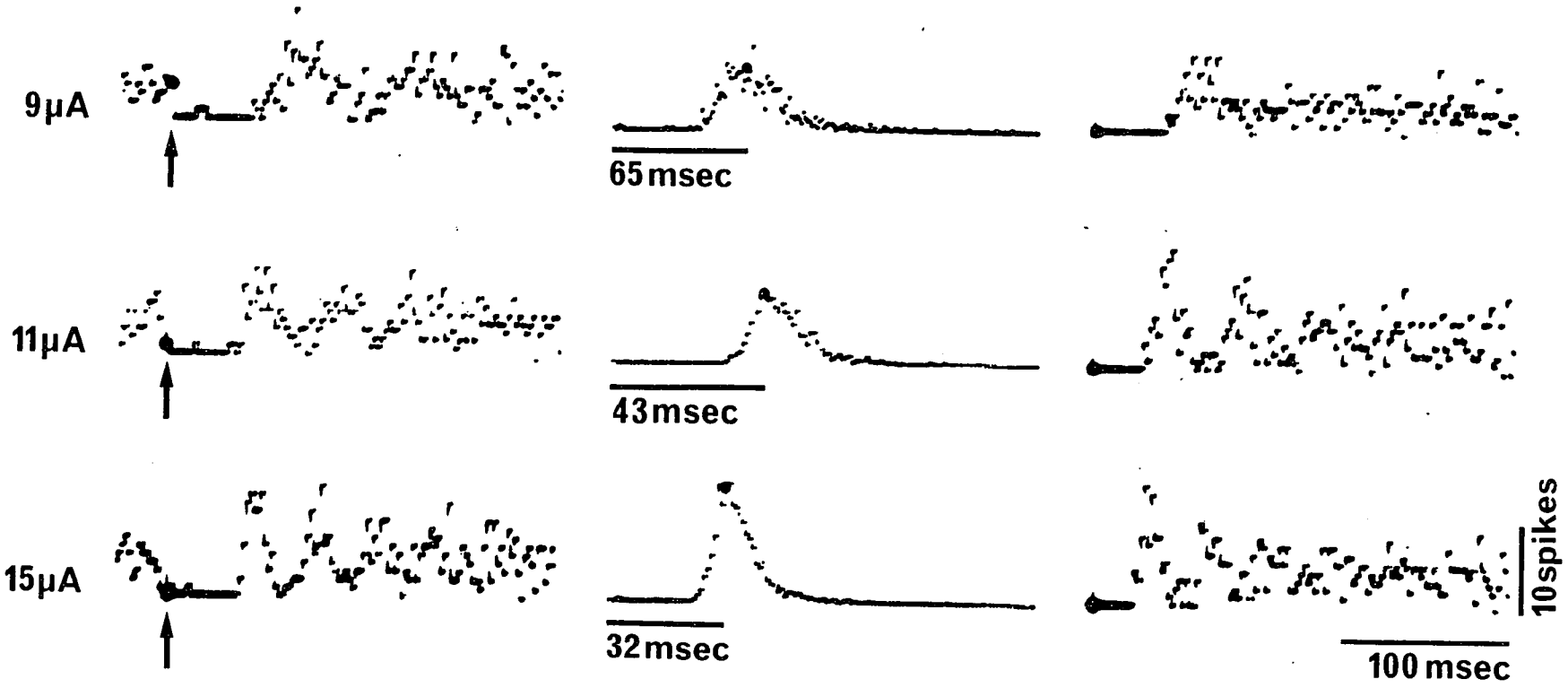


FIGURE 27

obtained in 4 neurones. The evoked IPSPs (Fig. 28B, bottom trace) had latencies ranging between 8-15 msec which is about three times longer than the latencies seen in DN neurones at comparable distances. Such long latencies suggest that the IPSPs in BS neurones are not monosynaptic. In 3 BS neurones, the inhibition was followed by a late excitation (e.g. Fig. 28B, bottom trace).

e) Electrophysiological Evidence for Recurrent Axon Collaterals in DN and BS neurones

In 18% of the DN neurones tested, a cortical stimulation produced single spike responses with a 2-7 msec latency and which were clearly synaptically mediated (Fig. 28A). This was established on the basis of a negative collision test and variable latency. The evoked APs could not follow repetitive stimulation at frequencies above 10/sec. In several cases these synaptic responses were observed together with antidromic ones in the same neurones (see for example Fig. 17A).

During experiments in which both stimulating and recording electrodes were placed in the DN, electrical stimulus sometimes produced a direct activation of a neurone followed by multiple synaptic activation (Fig. 28A). Such responses could be produced by DN neurone recurrent axon collaterals forming excitatory connections in the DN area.

About 100 BS neurones were studied in separate experiments and it was observed that in most cases cortical stimulation produced an excitation. In about 10% of the neurones, the activation was certainly antidromic and in another 10% it appeared to be antidromic but these latter neurones were not rigorously tested (see part I). The majority of the BS neurones were probably synaptically activated as suggested by a variable latency of these responses during repetitive stimulation

FIGURE 28 A - Evoked responses in DN neurones.

Top - Several sweeps showing synaptically mediated extracellular responses in a DN neurone (spikes retouched). Stimulus (arrow) was applied to the cortical area.

Bottom - Several sweeps showing a short latency, direct activation and subsequent synaptic activations in another DN neurone. Stimulus was applied to the DN area. Time calibration is equal in both cases.

B - Evoked responses in BS neurones.

Top - Several sweeps of the evoked EPSPs and action potentials in a BS neurone. Stimulus was applied to the cortical area 600 μ distant. The intensity of stimulation was set at just threshold for the generation of action potentials.

Bottom - The evoked IPSPs in another BS neurone in the same culture. Note that the IPSPs were sometimes followed by a late excitation. Intracellular AC recordings. The peaks of action potentials were cut off during the transfer of the data onto the magnetic tape.

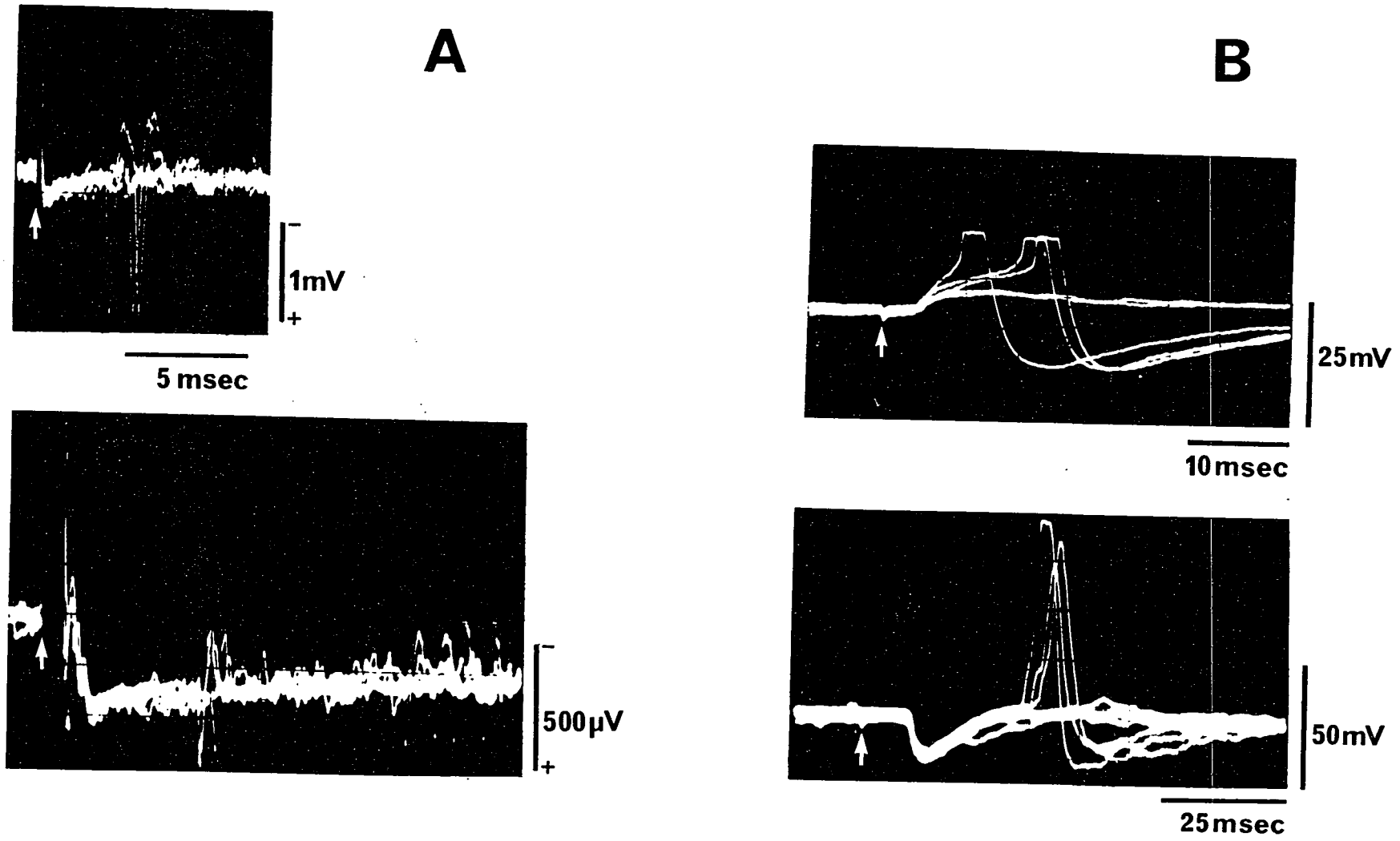


FIGURE 28

FIGURE 29 Evoked responses in a BS neurone following stimulation of a cortical area.

A, B - Superimposed sweeps at 1/sec and 50/sec respectively.

C,D,E,F,G - Collision test.

C,D,E - 2 stimuli to the Cx region with progressively increasing interstimulus interval.

F,G - Spontaneous APs triggered the sweeps of the oscilloscope and the delayed stimuli to the Cx region. Note that the minimal time separating the spontaneous and evoked APs (F) is not longer than the minimal time separating two evoked spikes (D).

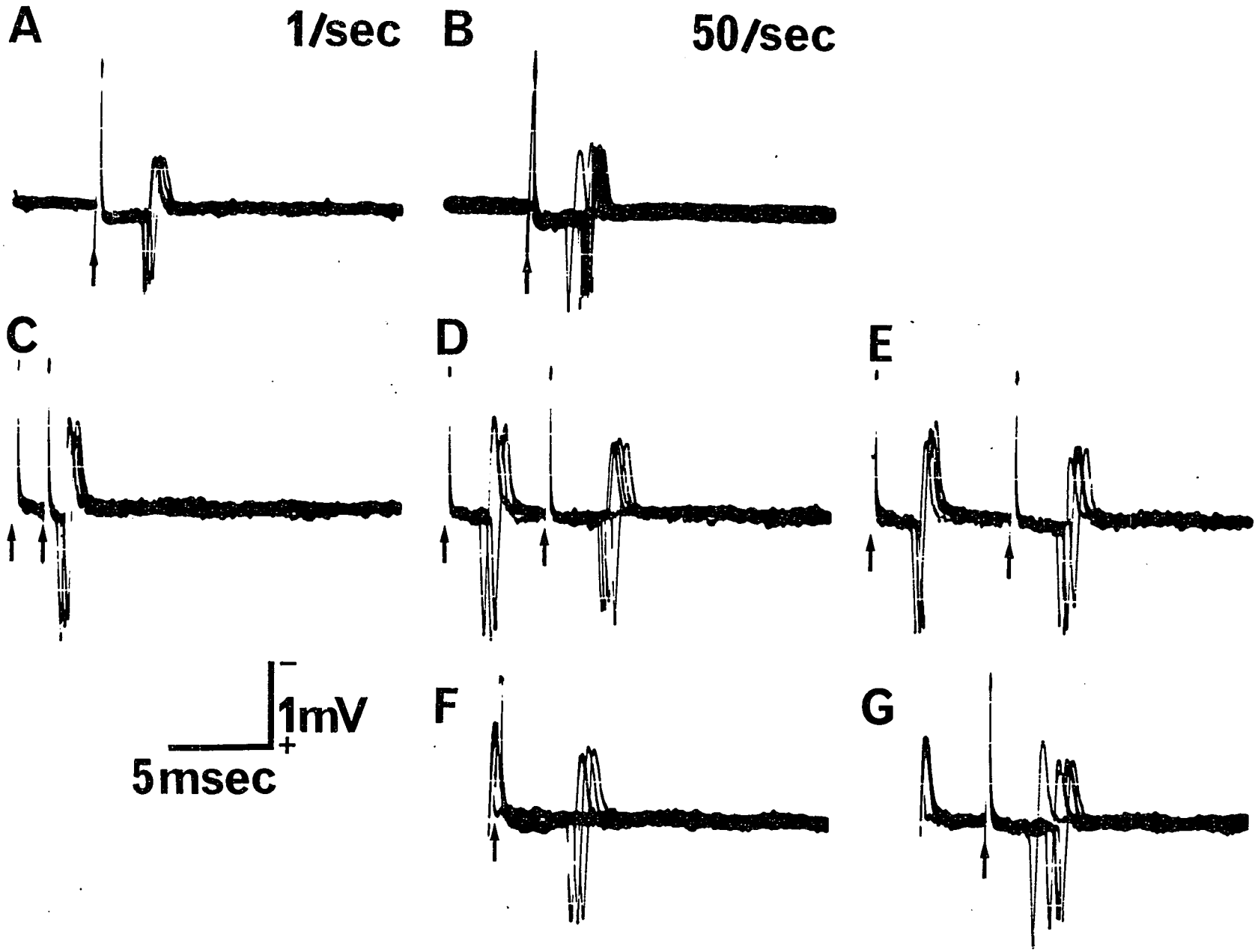


FIGURE 29

at high frequencies. Results of collision tests confirmed that the responses were synaptically mediated (Fig. 29).

The latency and strength of these synaptic activations varied in different neurones. In some, the activation was quite secure even during stimulation at 50/sec (e.g. Fig. 29) and occurred at latencies of 2-4 msec, but in others the activation was weak and occurred at relatively long latencies (6-10 msec, see for example Fig. 17B). Several neurones could be evoked at two or more distinctly different latencies depending on the stimulus intensity.

Intracellular recordings in 5 BS neurones demonstrated evoked EPSPs capable of producing a strong activation. Figure 28B (top trace) shows the evoked EPSPs which were just at threshold for generation of APs. An increase in the stimulus intensity resulted in a summation of more EPSPs and consequently in a more constant and shorter latency of the evoked APs. It is thought that these excitatory responses are mediated by recurrent axon collaterals of either DN or BS neurones but at the present time this issue still remains unresolved.

Part V - Pharmacological Studies

a) DN Neurones

Pharmacological studies were conducted on 111 DN neurones for the purpose of investigating the identity of the neurotransmitter responsible for the inhibition in the Cx-DN pathway. During these experiments pharmacological blocking agents were added into the perfusing solution. The action of these blockers was determined by studying their effects on the evoked inhibition and on depressions produced by iontophoretic applications of the putative neurotransmitters GABA and Glycine.

All neurones tested could be inhibited by iontophoretic ejections of GABA at 1-20 nA; frequently even the diffusion of GABA from the electrode was sufficient to cause a strong depression of the spontaneous or the glutamate generated activity (e.g. Fig. 30). On the other hand, Glycine was usually less potent and in 24% of the neurones it did not have any apparent effect except for that attributable to an iontophoretic current. In many neurones a rebound excitation was observed after the termination of the application of these substances (Fig. 30). During the pharmacological studies the ejecting currents of GABA and Glycine were adjusted so that they produced approximately equal effects. Usually these currents were set at a level which was sufficient for an almost complete depression of the activity during the period of their application (e.g. control record in Fig. 30). In several experiments, GABA (or Glycine) applications were made very short in order to mimic as closely as possible the evoked synaptic inhibition (Fig. 34). The effects of currents alone were determined by passing current through the barrel filled with sodium acetate. This was done either by passing

positive current pulses equal in amplitude to those of GABA or Glycine (e.g. Na^+ pulses in Fig. 30) or by applying negative (balancing) pulses simultaneously with GABA or Glycine (e.g. balancing current in Fig. 34).

GABA Blockers - Bicuculline, Bicuculline-Methiodide and Picrotoxin

The specific action of Bicuculline was demonstrated by comparing its effects on evoked inhibition and on iontophoretic applications of GABA and Glycine. An example is shown in Fig. 30. This neurone was inhibited by pulses of GABA and Glycine as well as by the electrical stimuli applied in the cortical region of the culture. As the concentration of Bicuculline in the perfusing solution was increased from 1 to 9 μM the GABA effect and the evoked inhibition were progressively blocked without changing the effect by Glycine. Bicuculline produced strong spontaneous bursting in the neurone at 3 and 9 μM but the specific action of Bicuculline towards GABA was still apparent. It was found in most neurones tested that a 1-5 μM solution of Bicuculline was sufficient to block significantly the evoked and GABA inhibitions but not those of Glycine (Table 9). Concentrations below 1 μM had no effect. The block of the inhibition was judged to be significant when the duration of inhibition was decreased by at least 25% or if the average rate of spikes during the period of inhibition increased by about 25% of that seen in a prestimulus control period.

Bicuculline as well as other GABA blockers increased the average rate of firing and produced spontaneous bursting (Fig. 31). Using relatively large electrodes sometimes made it possible to record from several cells simultaneously and it was observed that the bursts occurred in all cells synchronously (Fig. 32). Such bursts usually occurred in the neurones which were spontaneously active or which were continuously activated by

FIG. 30.

Effects of Bicuculline on inhibitory responses in a DN neurone. Left column - polygraph records showing the effects of Bicuculline (1, 3, 9μM) on the iontophoretic applications of GABA and Glycine. Black bars above each record show 10 second periods when retaining current (10 nA) was switched off and the indicated, positive, ejecting currents were switched on (GABA 0 nA, Glycine 30 nA and Na^+ 30 nA). Note that in this neurone GABA diffusion alone was sufficient for a complete depression of activity. Right column - peristimulus histograms showing the effects of Bicuculline (1, 3, 9μM) on the evoked inhibition of the same neurone (bin width 2 msec, 70 sweeps).
Note - recoveries of all responses were obtained after each concentration of Bicuculline but only the final recovery after 9μM Bicuculline is shown. Arrows show the time of stimulation.

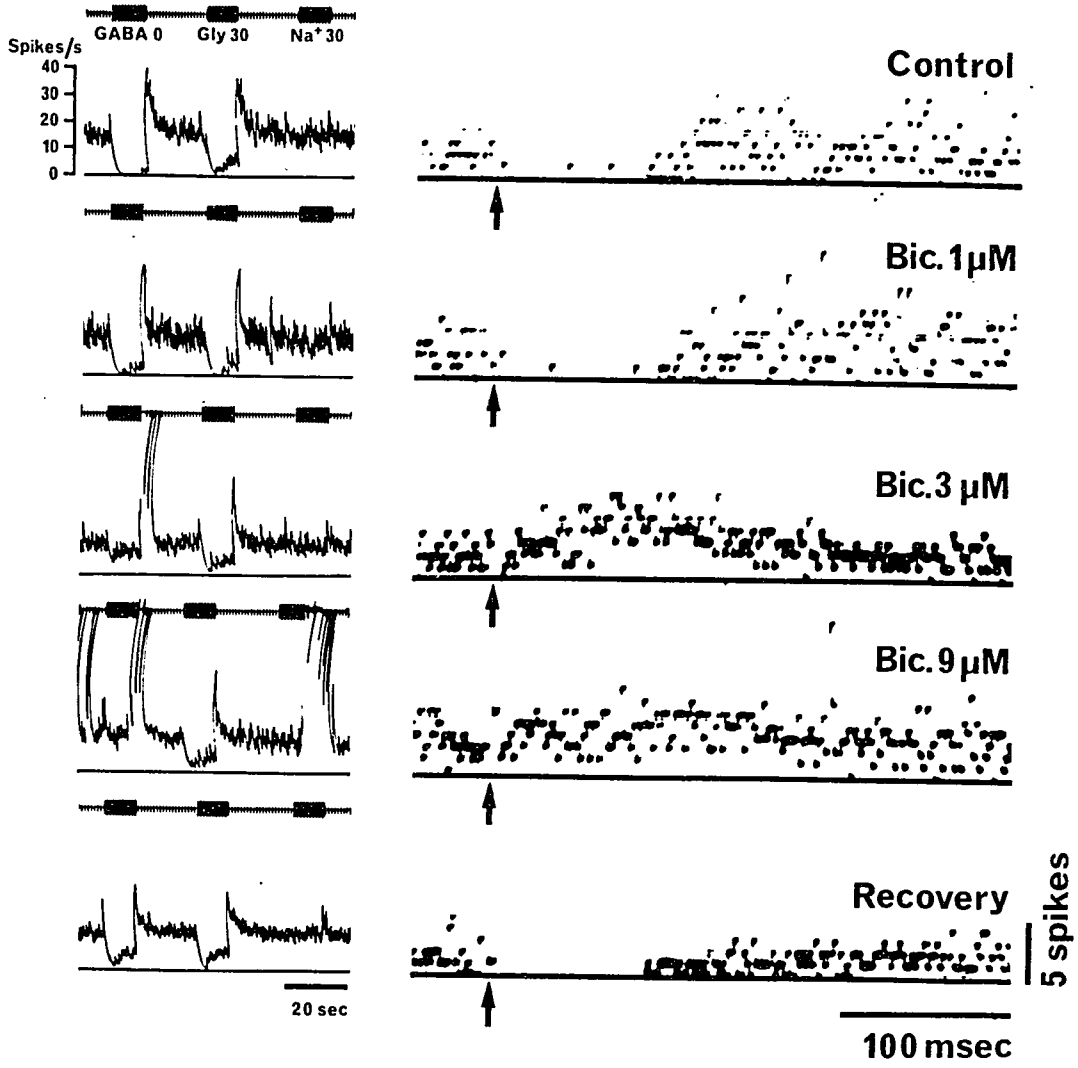

GABA 0
Gly 30
Na+ 30
Spikes/s
40
30
20
10
0
20 sec
Control
Bic. 1 µM
Bic. 3 µM
Bic. 9 µM
Recovery
5 spikes
100 msec

FIGURE 30

FIGURE 31. Effects of Bicuculline (6 μM) on the activity of a DN neurone. This neurone was not spontaneously active but it was continuously activated by an iontophoretic application of glutamate. Note that the average rate of firing was increased and that Bicuculline produced strong bursting.

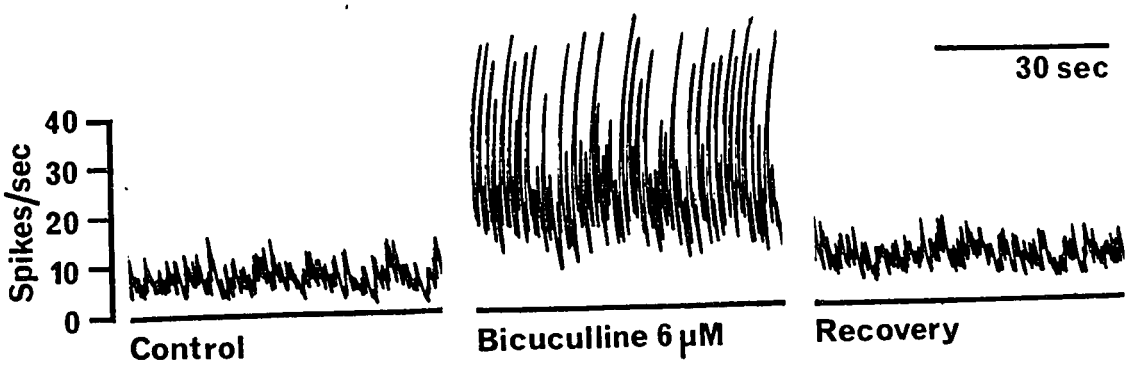

30 sec
40
30
20
10
0
Spikes/sec
Control
Bicuculline 6 µM
Recovery

FIGURE 31

FIGURE 32. Continuous recording from the DN area during perfusion with Bicuculline (4.5 μM). Note a gradual increase in the activity of several units resulting in a strong burst followed by a period of depression. This sequence was repeated several times during a 15 minute period of perfusion with Bicuculline.

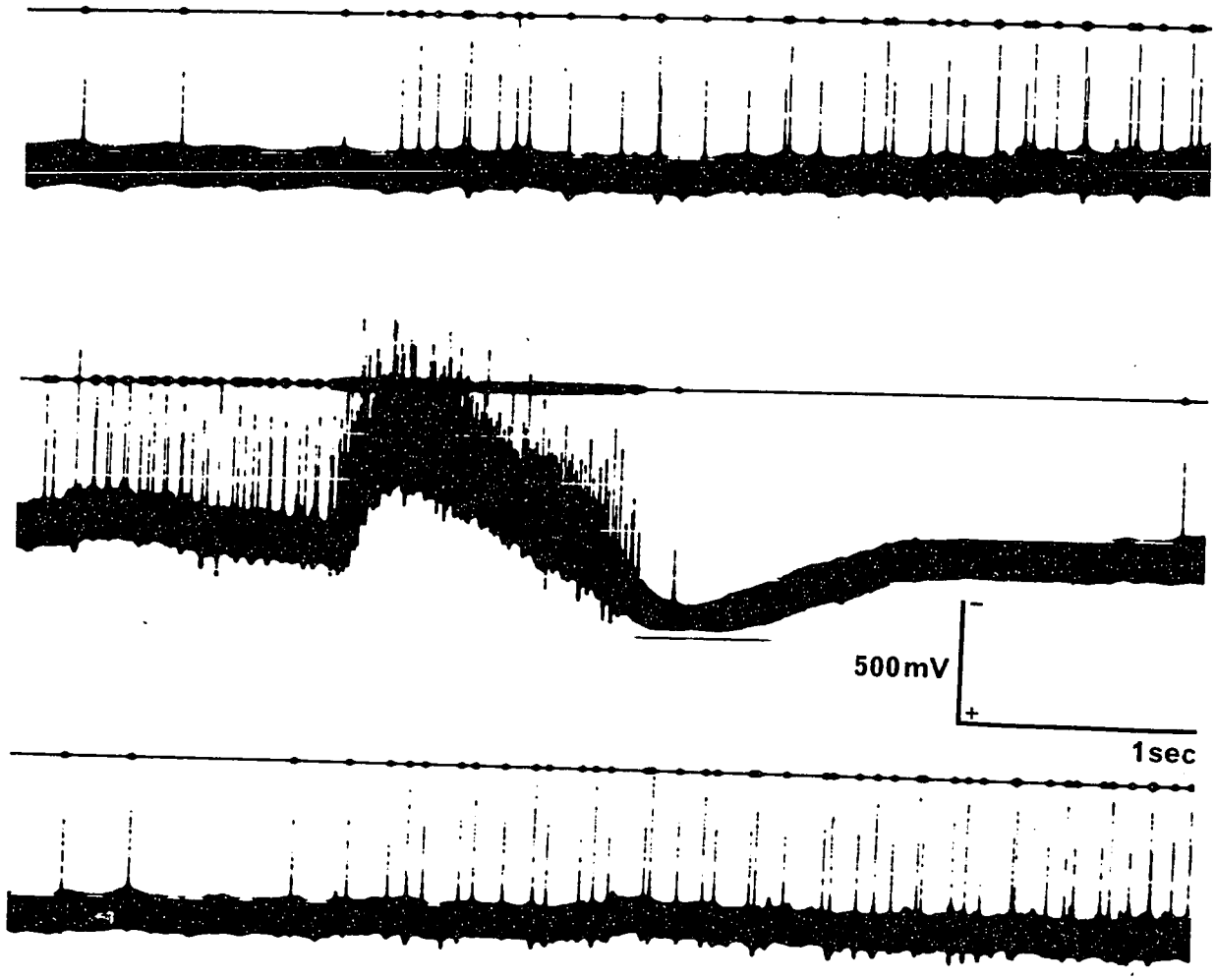


FIGURE 32

glutamate. Without glutamate application, the silent neurones usually didn't burst. The bursting activity could and occasionally did mask the inhibition, but is was clearly observed that the evoked inhibition was blocked exclusive of the influence of bursts. In order to ensure that an apparent blocking of inhibition was not the result of an increase in the rate of background activity, this activity was maintained at approximately control levels or lower by adjusting the iontophoretic current of glutamate with which the cells were driven.

Bicuculline abolished the late facilitation (refer to part IVc) in DN neurones suggesting that this response was due to a disinhibition rather than to a synaptic excitation (Fig. 33A). On the other hand, the short latency excitation of DN neurones (refer to part IVe) was enhanced by Bicuculline while the inhibition was blocked. In peristimulus histograms this appeared as an increase in the probability of firing during the time of the original excitation and inhibition (Fig. 33D). In addition, in 53% of the neurones tested, cortical stimulation evoked long lasting excitation (up to 500 msec) even though most of these neurones did not appear to be activated synaptically prior to the application of Bicuculline (Fig. 33B). The postinhibitory discharges (refer to part IVc) were abolished by Bicuculline in all neurones tested (Fig. 33C).

Bicuculline-methiodide had similar effects as Bicuculline but appeared to be less potent on a molar basis. A direct comparison made on two neurones showed that 10-15 μM Bicuculline-methiodide had the same effect as 5 μM Bicuculline. Results from other neurones tested with only one of the blockers seem to confirm the somewhat higher effectiveness of Bicuculline (refer to Table 9 for summary). All effects of Bicuculline and Bicuculline-methiodide were

FIGURE 33. Effects of Bicuculline on evoked responses in DN neurones.

A - Upper trace: Peristimulus histogram showing the inhibition of a DN neurone followed by a facilitation (control solution). Lower trace: The same neurone in Bicuculline 5 μM. Note that the inhibition is greatly reduced and that the facilitation is completely abolished.

B - Upper trace: Inhibition of a DN neurone after cortical stimulation (control solution). This neurone was also evoked antidromically at short latency. Lower trace: The same cell in Bicuculline 3 μM. The inhibition and discharges are replaced with a long excitation.

C - Upper trace: Inhibition of a DN neurone followed by regular discharges at 10/sec (control solution). This neurone was spontaneously active at the rate of 10/sec. Lower trace: The same cell in Bicuculline methiodide 10 μM. The inhibition and the discharges were blocked.

D - Upper trace: Short latency synaptic activation, followed by the inhibition of a DN neurone (control solution). The excitation of this neurone is also shown in Fig. 28A (top trace). Lower trace: The same cell in Bicuculline 3 μM. The excitation is enhanced (first two bins following the stimulation) while the inhibition is blocked. Also occasional bursts were evoked. Arrows indicate the time of stimulation. 128 sweeps, 2 msec/bin for all records except that in C which has 250 sweeps.

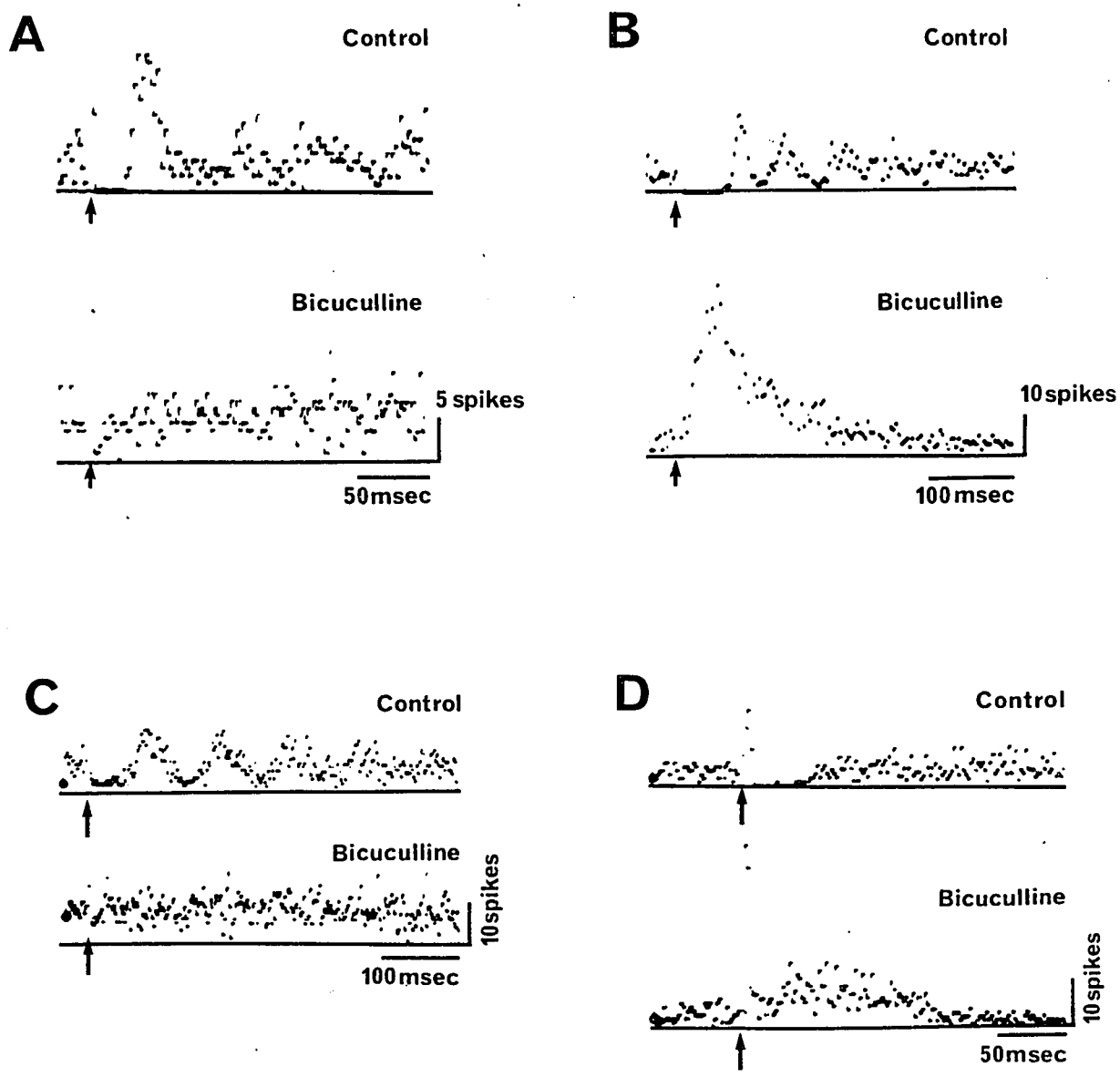

A
Control
Bicuculline
5 spikes
50msec
B
Control
Bicuculline
10spikes
100msec
C
Control
Bicuculline
10spikes
100msec
D
Control
Bicuculline
10spikes
50msec

FIGURE 33

FIGURE 34. Left column: Electrical stimulation with single pulses in the cortical region results in inhibition of a DN neurone (top photograph, 10 superimposed sweeps). The lower three records are peristimulus histograms constructed from 200 superimposed sweeps (bin width 2 msec). The inhibition was blocked by Bicuculline-methiodide (11.0 μM) (middle record) and recovery was complete (lower record).

Right column: GABA iontophoresis (0 nA) to the neurone inhibits activity. Arrow up indicates the time when the negative retaining current (10 nA) was switched off and arrow down when it was switched back on. Simultaneously, a balancing current of opposite polarity was passed through the adjacent barrel filled with sodium acetate. The peristimulus histograms (200 sweeps, bin width 5 msec) show the effect of Bicuculline-methiodide on GABA depressions.

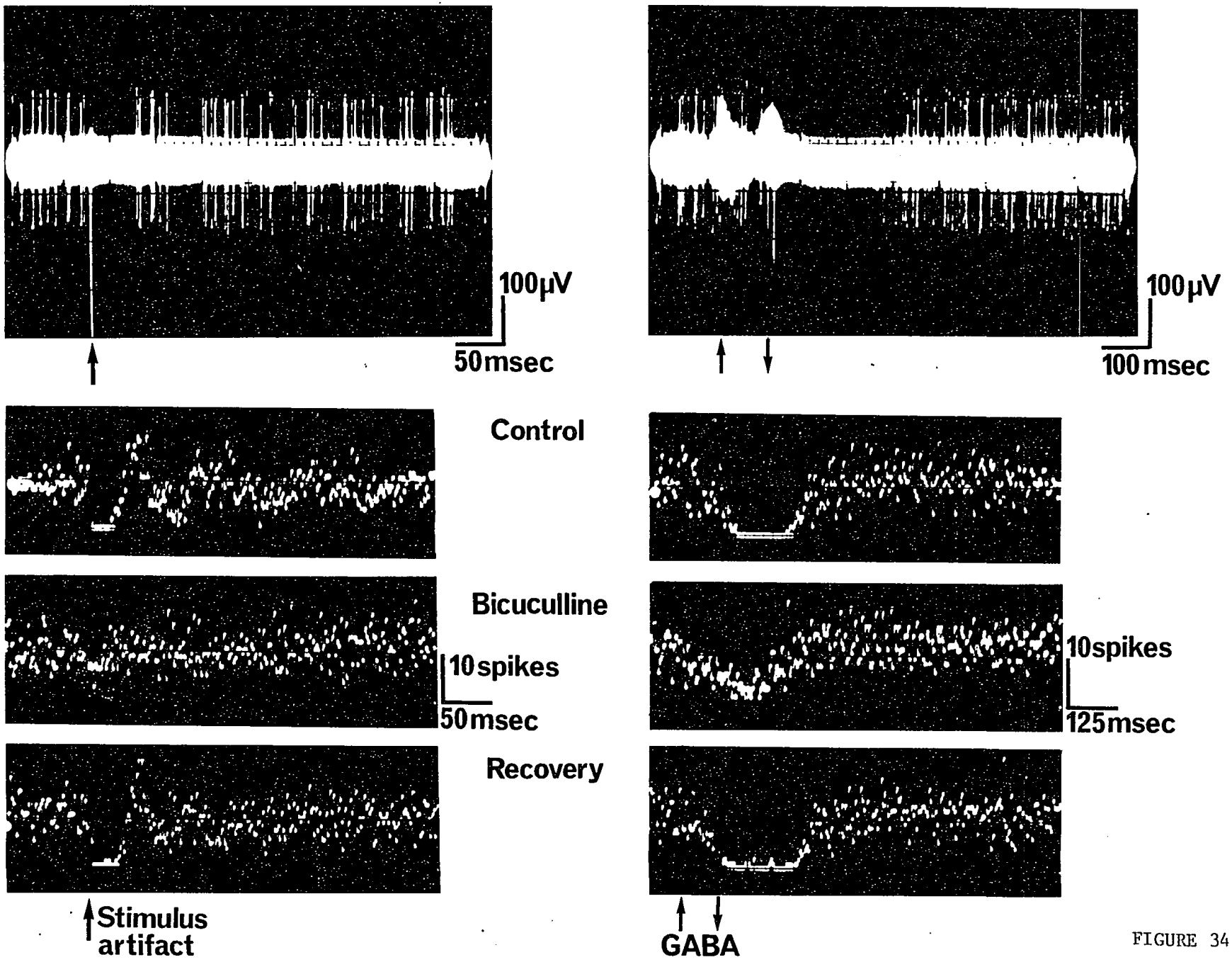

100µV
50msec
100µV
100msec
Control
Bicuculline
10spikes
50msec
10spikes
125msec
Recovery
Stimulus
artifact
GABA

FIGURE 34

reversible immediately following the washing of cultures with control BSS (see e.g. Fig. 34).

The effects of another GABA blocker, Picrotoxin, were very similar to those of Bicuculline but occurred at higher concentrations (Table 9). In Fig. 35 for example Picrotoxin was tested on the same neurone as that shown in Fig. 30. It can be seen that at 5 μM Picrotoxin almost completely blocked the evoked inhibition and the effect of GABA but not that of Glycine. A complete block of evoked inhibition was seen in this cell at 10 μM as compared to 3 μM in the case of Bicuculline. The effects of Picrotoxin were reversible but recovery occurred more slowly (usually 10-20 minutes) than for Bicuculline.

Glycine Blocker - Strychnine

Strychnine acted as a specific Glycine blocker when applied into the perfusing solution at concentrations up to 10 μM (Table 9, Fig. 36). The neurone shown in Fig. 36 was inhibited by cortical stimulation as well as by iontophoretic pulses of GABA and Glycine. As the concentration of Strychnine in the solution was increased from 1 to 10 μM the evoked inhibition and the effect of Glycine were reduced whereas the effect of GABA remained unchanged. In the same neurone Bicuculline at 7.5 μM blocked the inhibition and the action of GABA but not that of Glycine. The same results were found in 2 other neurones but using Bicuculline-methiodide and Picrotoxin as GABA blockers.

Above 10 μM the action of Strychnine was relatively unspecific, i.e. it often blocked the action of GABA as well as that of Glycine (Table 9). Evoked inhibition in most DN neurones tested was reduced or completely blocked by 10-20 μM Strychnine (Table 9). Strychnine also modified the pattern of glutamate-induced firing of neurones.

FIGURE 35. Effects of Picrotoxin on inhibitory responses in a DN neurone.

Left column - Polygraph records showing the effects of a 5 μM solution of Picrotoxin on the iontophoretic applications of GABA and Glycine. Black bars above each record show the 10 sec. periods when retaining current (10 nA) was switched off and the indicated positive, ejecting currents were switched on (GABA 0 nA, Glycine 30 nA and Na+ 30 nA).

Right column - Peristimulus histograms showing the effects of Picrotoxin on the evoked inhibition of the same neurone (bin width 2 msec, 70 sweeps).

Note - The same neurone is also shown in Fig. 30.

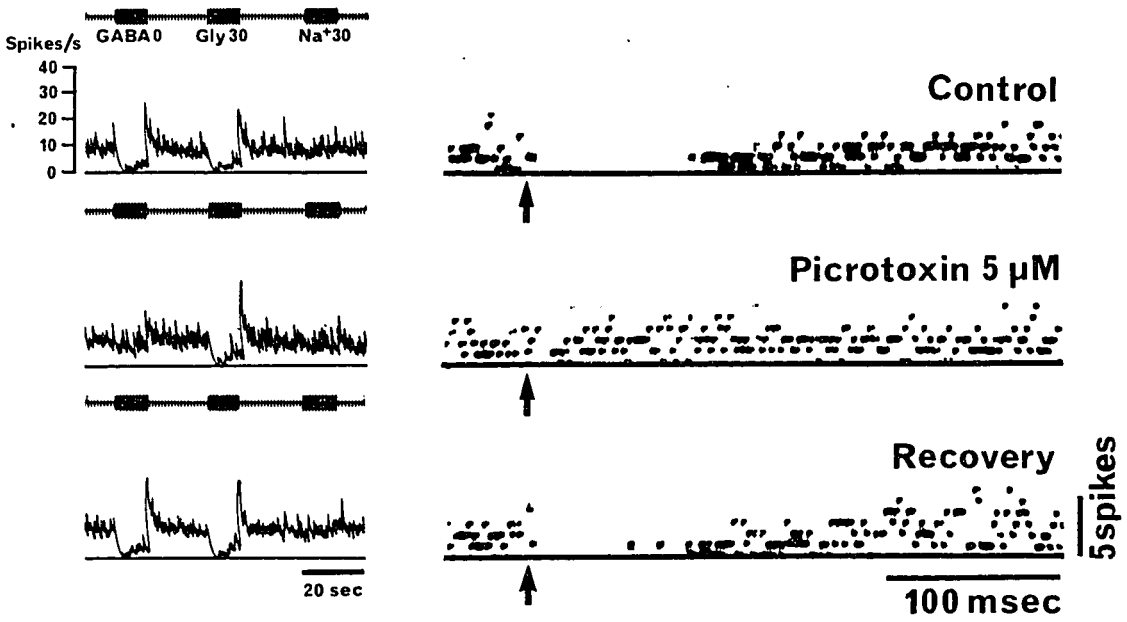

Spikes/s
GABA 0
Gly 30
Na+30
40
30
20
10
0
20 sec
Control
Picrotoxin 5 µM
Recovery
5spikes
100 msec

FIGURE 35

FIGURE 36. Effects of Strychnine on inhibitory responses in a DN neurone.

Left column - Polygraph records showing the effects of Strychnine (1, 5, 10 μM) on the iontophoretic applications of Glycine (20 nA) and GABA (10 nA). Black bars above each record show the 10 sec. periods when retaining current (10 nA) was switched off and the indicated, positive, ejecting currents were switched on. Na+ at 20 nA did not have any apparent effect on this neurone.

Right column - Peristimulus histograms showing the effects of Strychnine (1, 5, 10 μM) on the evoked inhibition of the same neurone (bin width 2 msec, 128 sweeps).

Note - Recoveries were obtained after each concentration of Strychnine but only the final recovery after 10 μM is shown.

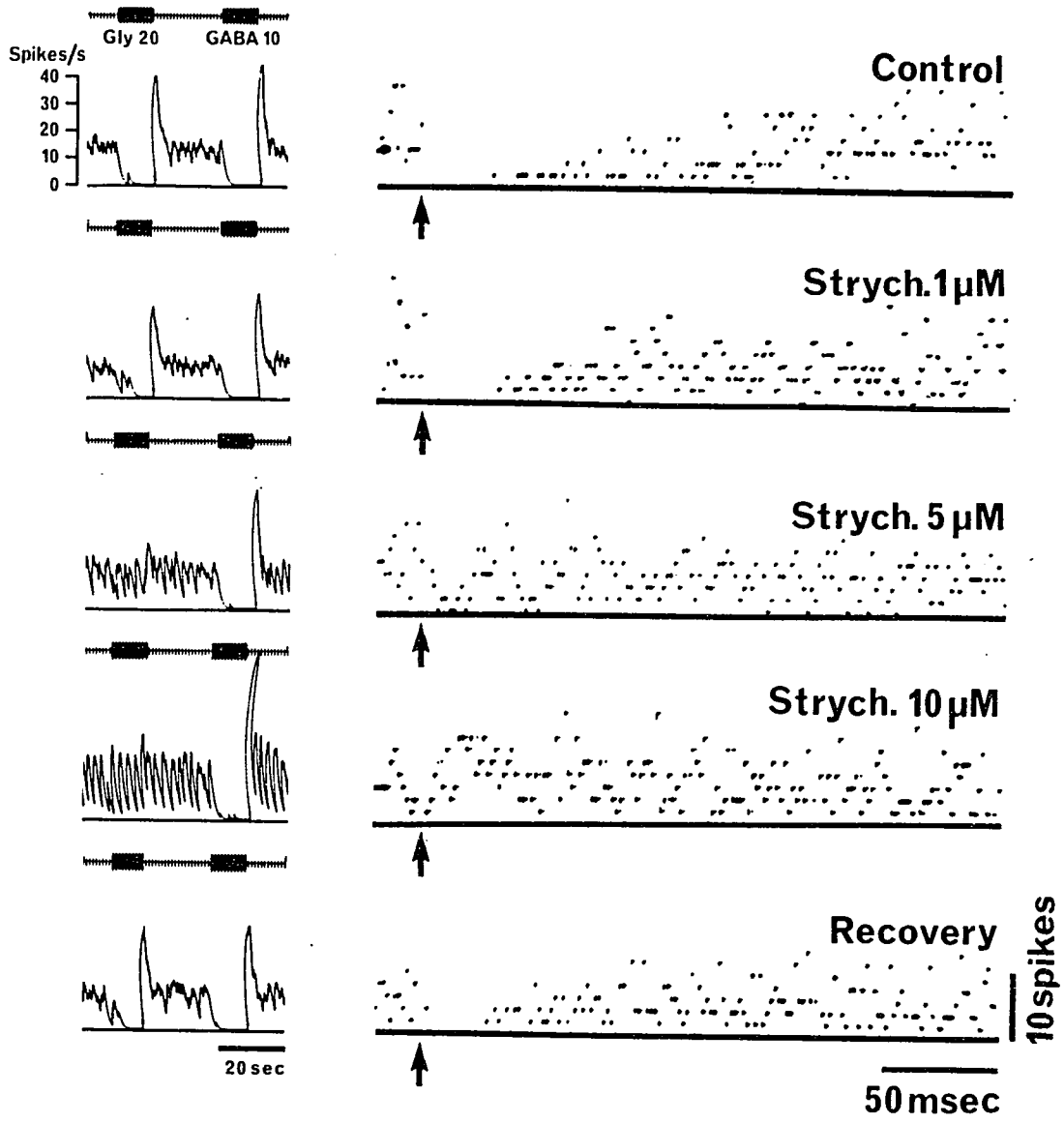

Gly 20
GABA 10
Spikes/s
40
30
20
10
0
Control
Strych.1µM
Strych. 5µM
Strych. 10µM
Recovery
10spikes
20 sec
50msec

FIGURE 36

The most characteristic change was the production of repetitive bursting as shown for example in Fig. 36. In addition Strychnine usually increased the average rate of firing, however, in some neurones the rate was decreased or unchanged. The effects of Strychnine were reversible but it usually took 15-20 minutes for full recovery to occur.

Table 9 represents the summary of all pharmacological tests. The evoked and GABA inhibitions were blocked at similar concentrations by GABA-blockers whereas Glycine was unchanged. Strychnine below 10 μM blocked the evoked inhibition only in a small proportion of the tests and it showed clear preferential blocking of Glycine over GABA. The concentration of 10 μM seemed to be on the border line between specific and unspecific actions of Strychnine. Thus, in some neurones Glycine was blocked while GABA remained unchanged whereas in others GABA was also blocked. This reduction of the specific action of Strychnine was clear at higher concentrations (15-20 μM), although Glycine was usually more strongly blocked than GABA. Strychnine blocked the evoked inhibition even in these neurones which were not sensitive to iontophoretic applications of Glycine.

b) Cx Neurones

Electrical stimulation in the Cx area often produced an excitation of the Cx neurones which was sometimes followed by an inhibition. These responses were very similar to those obtained in the Cx as a result of DN stimulation (refer to part IV a). In order to test a possibility that GABA is the neurotransmitter responsible for the inhibition, the effects of GABA and the blockers Bicuculline and Strychnine were studied in a few preliminary experiments. In 3 neurones, the evoked and GABA inhibitions were blocked by 4,4 and 20 μM respectively suggesting that GABA may be the

neurotransmitter in the Cx region. Strychnine was tested on 3 other neurones and blocked the evoked inhibition at 5,10 and 12 μM respectively but it was probably acting unspecifically. The unspecific action was clearly demonstrated in one of these neurones which was not sensitive to Glycine and yet GABA depression and the evoked inhibition were blocked. Both blockers had potent convulsant effects at concentrations as low as 1-5 μM.

A proposed glutamate blocker, glutamic acid diethyl ester (GDEE), was tested on 14 Cx neurones in order to determine if it can be used as a selective blocker. Such a blocking agent would be very useful in further studies of excitatory synaptic connections in culture. In these experiments neurones were activated by brief iontophoretic pulses of either glutamate or another excitatory amino acid homocysteate. GDEE was applied into the perfusing solution at progressively increasing concentrations. In most neurones GDEE (1-5 mM) drastically reduced the spike size without any apparent change in the rate of the spontaneous firing and often this effect was irreversible. Only in 3 neurones the spikes size remained sufficiently large to complete the tests and only in one case did GDEE block glutamate selectively without blocking of homocysteate. These results suggest rather unspecific action of GDEE. Other proposed glutamate blockers have not been tested.

c) BS Neurones

It has been shown electrophysiologically that there is a Cx-BS inhibitory projection which resembles the Cx-DN pathway although in case of BS neurones it is uncertain if the projection is monosynaptic. A GABA blocker Bicuculline (5-10 μM) readily blocked the evoked inhibition in 8 neurones tested and produced bursts similar to those observed in DN but shorter. Effects of Strychnine on BS neurones were not studied.

Part VI - Tables

Table 1: Chemical formulations of various BSSs as compared to cerebrospinal fluid and plasma of the rabbit.

References: Earle's, Hanks, Gey's, DMEM (BSS), and L-15 (BSS) from a catalog of Microbiological Associates; Simm's from Paul (1960); Crain's from Crain & Pollack (1973).

Values for CSF and plasma from Davson (1967).

Table 1

Compound	Earle's	Hank's	Gey's	Simm's	DMEM(BSS)	L-15(BSS)	Crain's	Plasma	CSF
NaCl	116 *	136	136	137.5	110	138	137	-	-
KCl	5.4	5.4	5.0	2.7	5.4	5.4	2.7	-	-
$CaCl_2$	1.8	1.3	1.5	1.3	1.8	1.3	1.0	5.6	2.5
$MgSO_4$	0.8	0.8	0.3	-	0.8	0.8	1.0	-	-
$MgCl_2$	-	-	1.0	1.0	-	1.0	-	2.0	1.7
NaH_2PO_4	1.0	-	-	-	0.9	-	-	-	-
Na_2HPO_4	-	0.3	0.8	1.5	-	1.3	1.4	-	-
KH_2PO_4	-	0.4	0.2	-	-	0.4	0.15	-	-
$NaHCO_3$	26	4.2	2.7	11.9	44.0	-	6.0	25	22
Dextrose	5.5	5.5	5.5	5.5	5.5	5.0	5.5	8.3	5.3
Total Na	143.0	140	140	152	155	141	146	148	149
K	5.4	5.8	5.2	2.7	5.4	5.8	2.85	4.3	2.9
Cl	125	143	146	145	119.0	148	142	106	130
Osmolality	309	304	302	321	333	305	306	298	305

*Concentrations in mequiv/liter

TABLE 2. Antidromic activations of brainstem neurones and the calculated conduction velocities. Conduction velocities were calculated from latencies and distances given in the table. Criteria 1, 2, 3, 4 which were used to identify the antidromic responses are explained in the text. Note that each neurone fulfilled at least 3 out of 4 criteria.

* This neurone is shown in Fig. 9.

** This neurone is shown in Fig. 8.

TABLE 2

Cell#	Distance (μ)	Latency (msec)	Criteria 1 2 3 4	Cond. veloc. (m/sec)
1	800±50	2.4 (±0.2)	x x x x	0.33*
2	700	5.0	x x x x	0.14
3	800	2.5	x x x x	0.32
4	500	2.0	x x x x	0.25
5	600	2.6	x x x x	0.23
6	400	2.2	x x x -	0.18
7	400	2.0	x x x -	0.20
8	800	5.0	x x x -	0.16
9	1000	2.1	x x - x	0.48
10	500	2.5	x x - x	0.20
11	400	2.2	x x - x	0.18**

TABLE 3. Antidromic activations of DN neurones and the conduction velocities. Distances separating the stimulating and recording electrodes and the latencies of antidromic spikes are indicated. Criteria 1, 2, 3 and 4 are described in the text.

* This neurone is shown in Fig. 11.

** This neurone is shown in Fig. 10.

TABLE 3

Cell #	Distance (μ)	Latency (msec)	Criteria 1 2 3 4	Cond. veloc. (m/sec)
1	800±100	5.0±0.2	x x x x	0.16
2	500	0.7	x x x x	0.71*
3	1000	0.6	x x x -	1.67**
4	800	0.5	x x x -	1.6
5	1000	1.6	x x x -	0.77
6	500	0.9	x x x -	0.55
7	500	0.5	x x x -	1.0
8	500	0.6	x x x -	0.83
9	400	1.2	x x - x	0.33
10	400	1.2	x x - x	0.33
11	500	0.5	x x - x	1.0
12	500	2.0	x x - x	0.25
13	700	2.5	x x - x	0.28
14	800	1.8	x x - x	0.44

TABLE 4. Conduction velocities of myelinated fibers. Extracellular potentials were recorded from single myelinated fibers (diameter 1-2 μ) in response to the stimulation in a DN area. The distances between the stimulating and recording electrodes are indicated. Latencies were measured from the beginning of the stimulus artifact to the beginning of the potential.

TABLE 4

Fiber #	Distance (μ)	Latency (msec)	Cond. veloc. (m/sec)
1	300±25	2.2	0.14
2	300	1.4	0.21
3	300	1.4	0.21
4	400	1.7	0.23
5	400	1.7	0.23
6	200	0.7	0.28
7	400	1.0	0.40
8	400	1.0	0.40

TABLE 5. Spontaneous activity in neurones. This table shows the proportion of spontaneously active neurones in each region of the cultures. Data was collected from 25 cultures using extracellular recordings with NaCl electrodes.

TABLE 5

	# of neurones studied	# of neurones active (% of total)
Cortex	66	46 (70%)
Deep nuclei	30	20 (67%)
Brainstem	55	24 (44%)

TABLE 6. Summary of the results showing the effect of Mg on antidromically evoked potentials in cultured neurones. Note: Criteria 1, 2, 3 and 4 are listed and explained in the text (see part 1 of Chapter 3).

TABLE 6

Cell #	Concn. of Mg mM	Criteria for antidromic activation				Effect on stimulus threshold for activation
		1	2	3	4	
1	10	x	x	x	x	↑ 100%
2	5	x	x	x	x	Block
3	10	x	x	x	x	↑ 200%
4	8	x	x	x		Block
5	8	x	x	x		Block
6	10	x	x	x		Block
7	10	x	x	x		↑ 10%
8	10	x	x	x		Block
9	10	x	x	x		↑ 100%
10	10	x	x	x		↑ 70%
11	8	x	x		x	Block
12	10	x	x		x	↑ 50%
13	10	x	x		x	No effect

TABLE 7. Summary of Cx neurones activated by the stimulation of DN area. Latencies of evoked potentials were measured to the initiation of earliest spike and hypothetical synaptic delays of 0.5 msec were subtracted from each value. Distances separating stimulating and recording electrodes were divided by the corrected latencies. The obtained conduction velocities would represent the conduction velocities of DN neurone axons if the DN-Cx projection was monosynaptic.

TABLE 7

Cell #	Latency (msec)	Distance (mm)	Calculated conduction velocities (assuming mono-synaptic transmission)
1	10±0.5	0.4±0.1	0.04
2	5	0.5	0.11
3	4	0.4	0.11
4	4.5-10	0.5	0.12
5	4	0.5	0.14
6	4-7	0.5	0.14
7	4-5	0.5	0.14
8	5-10	0.5	0.16
9	3.5	0.5	0.17
10	3-6	0.5	0.20
11	2	0.4	0.20
12	4-6	0.7	0.20
13	4-6	0.7	0.20
14	4-6	0.7	0.20
15	3-4	0.5	0.20
16	3-4	0.5	0.20
17	3-4	0.5	0.20
18	2.5-6	0.5	0.25
19	2.5	0.5	0.25
20	6	1.4	0.27
21	4	1.1	0.31
22	2-4	0.5	0.35
23	2	0.7	0.47
			$\bar{x}$=0.20, S.D.=0.09
EPSP 1	3.5	0.5	0.17
EPSP 2	5.0	0.8	0.18
IPSP 1	3	0.4	0.16
IPSP 2	4-5	0.4	0.11
Field potential #			
1	3	0.8	0.32
2	3	0.8	0.32
3	3	0.8	0.32
4	3	0.8	0.32
5	2.5	0.8	0.4
6	1.5	0.5	0.5
			$\bar{x}$=0.35, S.D.=0.07

TABLE 8. Summary of the evoked IPSPs in the DN neurones. The latencies of the IPSPs are given together with the distance separating stimulating and recording electrodes which was 500 μ in all cases. A hypothetical synaptic delay of 0.5 msec was subtracted from each latency and the distance was divided by these corrected latencies. The obtained values should give the conduction velocities of presynaptic axons if the transmission was monosynaptic.

TABLE 8

Cell #	Latency (msec)	Distance (mm)	Calculated conduction velocities (assuming mono-synaptic transmission) (m/sec)
1	2.4	0.5±0.1	0.26
2	3.0	"	0.20
3	4.0	"	0.14
4	4.0	"	0.14
5	4.2	"	0.13
6	4.2	"	0.13
7	4.5	"	0.12
8	4.5	"	0.12
9	5.0	"	0.11
			$\bar{x}$=0.15, S.D.=0.05

TABLE 9. The effects of four pharmacological blockers on inhibition of neuronal activity in the cerebellar nuclei. The inhibition was produced by cortical stimulation or by iontophoretic applications of GABA or Glycine. Percentages show the proportion of neurones in which the inhibition was significantly reduced (see text) by a blocker at a given concentration. Numbers in parentheses indicate the number of neurones tested.

*Concentrations of the blockers in the perfusing solution.

TABLE 9

Blocker	Inhibition	1-4 μM*	5-9 μM*	10-20 μM*
Bicuculline	Evoked	94% (17)	100% (12)	100% (3)
	GABA	71% (7)	100% (10)	100% (3)
	Glycine	0% (2)	20% (5)	50% (2)
Bicuculline methiodide	Evoked	67% (3)	67% (6)	100% (9)
	GABA	67% (3)	71% (7)	93% (15)
	Glycine	0% (2)	0% (3)	33% (6)
Picrotoxin	Evoked	0% (2)	67% (3)	100% (10)
	GABA	0% (2)	50% (4)	100% (8)
	Glycine	0% (2)	0% (2)	14% (7)
Strychnine	Evoked	0% (4)	30% (10)	69% (45)
	GABA	20% (5)	0% (5)	70% (20)
	Glycine	75% (4)	75% (4)	89% (18)

CHAPTER 4

DISCUSSION

Part I - Antidromic Responses of DN, BS and P neurones

As described in the results the neurones in both DN and BS areas were activated antidromically by electrical stimuli in the Cb cortex. The 4 criteria used for identification of such antidromic excitations are listed in Part I of the results. Probably the most reliable criterion is the collision test between spontaneous and evoked APs. However, there are several problems in the evaluation of this test. Theoretically, the minimal time between a spontaneous AP (generated in the area of cell soma) and an evoked AP (generated in the axon) should be equal to the time necessary to propagate an AP from the soma to the site of electrical stimulation plus the refractory period of the axon plus the time necessary for the evoked AP to propagate back towards the cell soma. In practice the determination of these three time intervals is not feasible, therefore, two simplifications have been made in the results. First it was assumed that the conduction velocities for spontaneous (orthodromic) and evoked (antidromic) APs are equal. This assumption may not be correct because an antidromic AP can be produced in the fiber during the relative refractory period following the spontaneous orthodromic AP so the antidromic AP will be conducted at least partly in the refractory fiber. It may be expected that the conduction velocity in the refractory fiber is slower than in the recovered fiber and this was confirmed in the present study. For example, inspection of Figures 8-11 in Part I of the results reveals that at higher frequency of stimulation the latency of antidromically evoked APs was longer than at low

frequency, presumably because of the slowed conduction velocity.

The second assumption is that the refractory period of the axon can be estimated by measuring the minimal time interval at which two APs can be evoked in the cell soma by a pair of stimuli of equal intensity applied to the axon. This assumption again is not entirely justified because such time interval will include not only the refractory period of the axon but also the additional delay produced by a slower conduction of the second AP (because it is conducted in the relative refractory period of the first one).

One should note that the first assumption will tend to underestimate the predicted collision time whereas the second assumption will tend to overestimate it by approximately the same amount. As a result, the two errors cancel each other and in fact the experimental collision time interval is in reasonable agreement with the predicted one (see Fig. 9). Fuller & Schlag (1976) suggested that it is better to consider the collision time as an interval between a spontaneous spike and the beginning of the stimulus which will evoke a spike in 50% of trials. In this case the predicted collision time should be equal to the latency of the evoked spike plus the refractory period as determined by twin stimuli. This method includes the same two assumptions as described above and indeed the measured collision times obtained by Fuller & Schlag were different (usually shorter) from the predicted values. This has also been seen in the present study but the method could not be used on all neurones because often the initial phase of a spontaneous AP could not be visualized, a condition which is necessary to measure the collision interval according to Fuller & Schlag. Instead, the collision was measured as a time interval between corresponding peaks of spontaneous and evoked APs.

In DN neurones, the measured collision times were consistently shorter than the predicted ones by an amount approximately equal to the latencies of the evoked APs. It is considered unlikely that these APs were synaptically activated because in the case of conventional synapses no collision at all would be expected, and besides, the phenomenon was observed in neurones which fulfilled three other criteria for antidromic activation (Fig. 11). The discrepancy could have been the result of an underestimation of the conduction time in the orthodromic direction or of an overestimation of the refractory period. It is not certain what the actual reason was but it was clearly a characteristic property of the DN neurones which distinguished them from BS neurones.

The latencies of the antidromic activations of BS and DN neurones were used to calculate the conduction velocities of their axons assuming straight trajectory between recording and stimulating sites. For BS, the average conduction velocity was 0.24 m/sec (range 0.14 - 0.48 m/sec). This is not an unreasonable figure since the axons of the BS neurones are unmyelinated and very thin (less than 1 μ). The velocity of parallel fibers in an immature rat Cb is about 0.2 m/sec (Shimono et al., 1976) and these fibers are probably of similar size. Fishbach & Dichter (1974) give a value of 0.15 m/sec for the conduction velocity of unmyelinated neurites of similar diameter in culture. It is, however, surprising that only 10-20% of the neurones were activated antidromically, because virtually all neurones stained with horseradish peroxidase (see introduction Part IV) had very extensive axonal branches projecting throughout the culture and some neurones appeared to have more than one axon.

Unfortunately the exact identity of the BS neurones in our cultures is not known so a direct comparison to the in vivo counterparts cannot be

made. One possibility is that these are the neurones of the nucleus locus coeruleus, since this nucleus is located in the brain stem near the cerebellar peduncles (W.J. Hendelman, personal communication). Antidromic activation of neurones in locus coeruleus of the adult rat have been studied and conduction velocities of 0.4 - 1.3 m/sec were found (Nakamura & Iwama, 1975; Foiers & Mogenson, 1976). Considering that fibers in mouse culture may be slightly smaller and immature in comparison to adult animals, the values for the conduction velocity seem to support the possibility that a group of BS neurones in culture is in fact originating from the locus coeruleus. Unfortunately, other electrophysiological properties of these neurones are in disagreement with this hypothesis. First, electrical stimulation of the BS area does not produce an inhibition of cerebellar P neurones with any consistency as is observed in vivo (Siggins et al., 1971) after stimulation of the locus coeruleus. On the contrary, a few effects which have been obtained in cultures were usually excitatory. In addition, these neurones appear to form excitatory synaptic connections with each other as was indicated by the results in Part IVe. This was shown particularly well in cultures containing only BS neurones (Marshall et al., 1977) so the possibility of excitatory collaterals spreading from the DN region was eliminated.

Another possible source of BS neurones in culture is the lateral or superior vestibular nucleus (Allerand, 1971; Seil, 1972). The neurones of the lateral nucleus are myelinated and fast conducting (Ito et al., 1964) so they are unlikely candidates, but the superior nucleus remains as a possibility. The relatively sparse inhibitory projection from the Cx (results, Part IVd) would favour correlation with the superior rather than the lateral nucleus. The lack of synaptic connections between the BS neurones and the Cx region points in the same direction, since only

medial and descending nuclei project to the cortex (Palay & Chan-Palay, 1974).

Calvet & Lepault (1975) proposed that BS neurones in similar cultures connect to the cortical area. This may well be the case but it must be recognized that in their cultures the BS area may include all vestibular nuclei as well as the pons (Privat & Drian, 1975).

For DN neurones the conduction velocities have also been determined on the basis of antidromic excitations, giving the range of 0.16 - 1.7 m/sec (average 0.71 m/sec). Such a wide range is rather unexpected, because if these fibers are myelinated a 10-fold range in conduction velocities would suggest a 10-fold range in the diameter (Waxman & Bennett, 1972). At the most, a 3-fold range in diameter has been observed suggesting that the group of high values around 1.6 m/sec is artifactual and may be the result of an excessive spread of the stimulating current. The majority of values have a much lower range, 0.16 - 1.0 m/sec (Fig. 12, Table 3) giving the average value of 0.55 m/sec. Alternatively, the high values may reflect the conduction velocities of the fibers which reached a higher developmental stage than the remaining fibers.

The DN neurones had usual diameters of 15-25 μ and those from which antidromic potentials have been recorded (30% of total) did not have any electrophysiological or morphological characteristics which could distinguish them from the remaining neurones in this area suggesting that they were representative of the whole population. The axons of these neurones in culture are assumed to be myelinated as is the case *in vivo*, but the conduction velocities are much lower than 5-30 m/sec reported by Ito *et al.* (1970a). In cultures, the diameter of myelinated axons does not exceed 2 μ, so clearly their slow conduction could be at least partly related to the difference in size since in cats these fibers

measure 4-7 μ (personal observation). A further reduction of the conduction velocity may be due to immature electrical characteristics of the axonal membrane or of the myelin.

Hopkins & Lambert (1973) have found that the conduction velocity of unmyelinated fibers in the cervical sympathetic trunk of rats increases with age. But the increase was only 2-3-fold during the period between the 20th and 100th day after birth. A more dramatic increase (≈10-fold) was observed by Huttenlocher (1970) in the pyramidal tract neurones of kittens during the first few weeks after birth. The large increase in the conduction velocity was concomitant with myelinization of the axons.

None of the Cx neurones in culture were activated antidromically by stimulation of the subcortical regions. The activation was expected in the P neurones since their axons project to the DN area. In the cat Cb, the antidromic activation is commonly observed and even in immature rats it has been studied by several researchers (Shimono et al., 1976; Crepel, 1974). In all previous studies, the antidromic activation of P neurones has been a consistent finding although Crepel (1974) has shown that in very young rats (3-10 days) the refractory period of P neurone axons is longer than in adults, so consequently they cannot conduct impulses at a frequency higher than about 50/sec. The latencies of antidromic activations were much longer than in adults but the actual conduction velocities were not measured. Only on day 20 the P neurone axons attained their adult characteristics.

In culture, the P neurone axons appear to transmit both spontaneous and evoked impulses in the orthodromic direction (results, Part IIb, IVc), so clearly they are excitable. Cell somas are also excitable since they can be readily activated by synaptic inputs. Thus the most likely reason

for the lack of antidromic excitation is an inability of the axonal spike to invade the soma. Such a block of antidromic invasion could be caused by insufficient current density generated by the axonal spike at the soma or by a powerful short latency inhibition. Both possibilities require further investigation.

Estimations of the conduction velocity of P neurone axons could not be accomplished on the basis of antidromic potentials, so in a series of experiments recordings were made directly from the axons. Only in a few cases was it possible to obtain evoked potentials which without doubt originated from myelinated axons. These potentials resembled closely the potentials recorded from the nodes of Ranvier in frog's nerve (Hodgkin, 1964) but in culture no nodes could be seen at the site of the recording or anywhere else along these fibers. The nature of the recorded potentials is not understood, nevertheless it is felt that their latencies do represent the conduction times along the axons. The values for the conduction velocities given in the results (Part Ic) were calculated on the basis of latencies measured from the beginning of the stimulus artifact to the initiation of the potential in each case, giving an average value of 0.26 m/sec. An alternative procedure is to measure the latencies to the negative peak (refer to Fig. 13) because this peak may indicate the time of activation of the fiber at the site near the recording electrode. Such a correction is most significant for the potentials evoked at short latencies and the conduction velocities calculated in this fashion give an average value of 0.21 m/sec. Another correction is probably required to account for the time utilized by the stimulus to initiate the impulse in the fiber but this time could not be measured.

The measured conduction velocity is of the same order of magnitude

as the values obtained for the DN neurones on the basis of antidromic excitation but much slower than conduction velocities of P neurones *in vivo*. In cats, P neurones conduct at 15-20 m/sec (Ito & Yoshida, 1966). Some reduction in the velocity could be the result of the smaller size of the fibers in culture (1-2 μ) as compared to cats (3-5 μ, Eccles *et al.*, 1967, p. 242). But according to Waxman & Bennett (1972) such an approximately three-fold reduction in diameter may account only for a three-fold reduction in velocity which is much less than the difference observed. A further decrease in the conduction velocity might be explained by some immature electrical characteristics of either the myelin sheath or the axonal membrane.

As it has been stated in Part IV of the introduction, the myelinated fibers in culture generally lack the nodes of Ranvier. The authors of two previous studies noted the lack of nodes in cerebellar cultures, but they did not comment on the possible significance of this finding (Hild, 1957; Bornstein & Murray, 1958). In a later review of tissue culture work, Murray (1958) presented the photographs of nodes in both living and stained fibers but did not report how often such nodes could be seen. In our cultures, I have seen the nodes only a few times in spite of extensive searching. According to Bodian (1951) (see also a review by Waxman, 1972), the ratio of internodal distance to fiber diameter in the white matter of mammalian CNS is around 200/1 so the expected internodal distance of P neurone axons in culture should be 200-400 μ. Obviously this is not the case in our cultures.

It is considered likely that the lack of nodes may be responsible for the slow conduction velocity observed in culture but the mechanism of impulse conduction in such continuously myelinated axons is not known.

Summary of Antidromic Activations

Antidromic activations demonstrated that the axons of BS and DN neurones project to the Cx area in culture. The axons of the BS neurones conduct at 0.24 m/sec. Such a slow conduction is consistent with a small diameter of these umyelinated processes. Axons of the DN neurones conduct at 0.71 m/sec. The observed value is much slower than the usual conduction velocities of DN neurone axons in vivo. It is proposed that the slow conduction is the result of some immature or abnormal electrical characteristics of these axons in culture. The Purkinje neurones could not be activated antidromically. It is thought that the lack of such an activation is due to inability of the axonal spike to invade the soma.

Part II - Synaptic Connections

a) Purkinje Neurones

P neurones were distinguished from other neurones in the cortical area on the basis of their relatively large size (15-20 μ). Some of the Golgi cells may be of similar size but their number cannot be estimated because the only quantitative study available included all Golgi cells (large and small) in the analysis (Palkovits et al., 1971). The ratio for the total number of P neurones to Golgi cells is 3:1 according to this study.

Using a Golgi staining procedure, the Golgi cells have only infrequently been seen in culture and all of them were small (see Fig. 2). Therefore, it is assumed that a great majority of the recordings obtained from large neurones in the cortical area of the cultures were from P neurones.

Electrical stimulation of distant regions in culture often produced evoked responses in P neurones. During the initial phase of experimentation 5-10 mM Mg was used to identify the responses which are synaptically mediated on the assumption that Mg interferes with the synaptic transmission selectively. This approach has been used extensively in tissue culture (e.g. GÄhwiler et al., 1973) and other in vitro preparations (e.g. Richards & Sercombe, 1970). It was soon realized, however, that in our cultures Mg could also produce a strong depression of the membrane excitability (results, Part III). Other authors have also reported similar observations. Thus, Yamamoto (1972) reported that in isolated sections of the guinea pig hippocampus neuronal excitability was depressed by 6.5 mM Mg. Hackett & Llinas (1973) observed a depression of parallel fibers in the isolated cerebellum of the frog by 5-20 mM Mg and Erulkar et al. (1974) observed a

depression of motor neurones in the isolated spinal cord of the frog by 10 mM Mg. Other authors reporting depression of neuronal excitability in the CNS by Mg have used iontophoretic applications (Kato et al., 1968; Kato & Somjen, 1970; Kelly et al., 1970; Somjen & Kato, 1968) so that the concentrations of Mg at the neuronal membrane have been uncertain.

In our preparation, a selective block of the synaptic transmission would have been possible only if the membrane excitability was continuously monitored. However, since such a procedure was not practical, I have used in later experiments only electrophysiological criteria to distinguish the synaptically mediated responses from direct activations.

Electrical stimulation of the DN region evoked APs in about 50% of the P neurones. These were all synaptic responses characterized by variable latencies and inability to follow high frequency stimulation (results, Part IVa).

In the preliminary analysis of the data (see Table 7), the evoked potentials were judged to be polysynaptic because the calculated conduction velocities for the DN neurone axons were slower then the actual velocities measured on the basis of antidromic activations. It was assumed in these calculations that the only delay involved in the synaptic transmission is 0.5 msec. More detailed considerations will be presented below.

The synaptic delay has been assumed to be 0.5 msec throughout the present study on the basis of the usual values 0.3 - 0.5 msec reported in the mammalian CNS (e.g. Ito & Yoshida, 1966). However, an additional time should be allowed for the generation of APs by the EPSPs. This time may differ considerably in different neurones depending on the synaptic input so it is difficult to estimate accurately. Intracellular recordings obtained in P neurones so far were not sufficiently stable to

measure the rise time of EPSPs directly, but the usual value for spike generation by the EPSP is 0.5 msec (Eccles, 1964; review by Berry & Pentreath, 1976), so it is tentatively used in this discussion. Another delay of up to 0.4 msec may be caused by a delayed activation of the DN neurones in response to the electrical pulse (results, Part IVa). Assuming the distance of 500 μ and considering all possible delays, the maximal latency of a monosynaptically evoked AP in a P neurone may be as long as 2.1 msec, see below;

0.4 msec - activation of a DN neurone

0.7 msec - conduction time along the DN neurone axon

0.5 msec - synaptic delay

0.5 msec - generation of the AP by an EPSP

2.1 msec - total latency

The calculated latency is considerably shorter than most latencies of single, evoked spikes suggesting a polysynaptic rather than monosynaptic pathway (refer to Table 7). But the latencies of the field potentials appear to be close to a monosynaptic range. These field potentials usually had several small components so they may have originated from the groups of granule cells present in the Cx area (see drawing in Fig. 1). The granule cells could in turn relay the APs to the P neurones.

Preliminary intracellular recordings from P neurones suggest a disynaptic rather than monosynaptic pathway because the latencies of the EPSPs were in the same range as those of extracellular spikes, whereas if they were monosynaptic the maximal delay of 1.6 msec would be expected (assuming a distance of 500 μ).

During intracellular recordings prominent IPSPs were evoked in the P neurones and a similar inhibition was sometimes observed with extracellular recordings. The inhibition could have been generated by either

inhibitory interneurones in the Cx region or by recurrent axon collaterals of P neurones. In most neurones, the latencies of the IPSPs were in a monosynaptic range for slowly conducting P neurone axons so they could have resulted from antidromic activation of P neurone axon collaterals (Fig. 22, bottom trace). In some cases, however, the latencies of the evoked IPSPs were so long that they could not be explained on the basis of monosynaptic transmission and therefore must have been generated indirectly via cortical interneurones.

In about 10% of the P neurones, DN stimulation produced bursts of spikes. Such responses were usually observed in interneurones and not in P neurones in the cat Cb, but as reviewed in the introduction, the lack of bursting *in vivo* may have resulted from the anesthesia. The bursts seen in cultures may reflect multiple activation of DN neurones such as shown in Fig. 28A.

The electrophysiological findings reported in this thesis are in agreement with morphological findings by Aggerwal (1977) and Hendelman *et al*. (1978). They have found that mossy fiber terminals contacting granule cell dendrites are present in cultures even though the extra-cerebellar afferents are absent. These mossy fibers could originate from DN neurones and thus provide the morphological substrate for the demonstrated functional connection between DN and P neurones.

At first the DN-Cx projection was thought to represent an aberrant synaptic connection in tissue culture because it was not previously reported *in vivo* (Wojtowicz *et al*., 1975). Only recently Tolbert *et al*. (1976) published their evidence for a similar projection in the intact cat Cb. Using retrograde labelling with horseradish peroxidase and anterograde labelling with tritiated leucine they have shown that neurones

from the DN project to the granular layer of the Cb cortex. They were able to confirm the morphological findings by antidromic activations of DN neurone axons from the cortex. Independently, Gould & Graybiel (1976) have demonstrated the DN-Cx projection using retrograde staining with horseradish peroxidase.

A monosynaptic connection between mossy fibers and P neurones is not normally found in vivo. But a study by Llinas et al. (1973) demonstrated that in an agranular Cb of the ferret, mossy fibers can contact P neurones directly, presumably because of insufficient number of granule cells. In culture, the number of granule cells is reduced in comparison to in vivo Cb, apparently due to toxicity of the chick embryo extract to these neurones (Allerand, 1971). It is conceivable that under such circumstances mossy fibers of DN neurones can contact P neurones directly in culture. Electron microscopic studies by Aggerwal (1977) revealed the presence of a few direct synapses between mossy fiber terminals and the P neurones in culture.

b) Deep Cerebellar Neurones

Stimulation of the cortical region produced an inhibition in a great majority of DN neurones. The latency of the onset of inhibition was usually 2-4 msec. The latencies were most accurately measured during intracellular recordings and these gave values between 2.5 and 5 msec. The evoked IPSPs were almost certainly monosynaptic because they could follow repetitive stimulation at high frequency with constant latency. In no case were synaptically evoked APs in either Cx or DN region observed to follow such stimulation sufficiently well to mediate the transmission. The monosynaptic projection is also supported by morphological evidence

showing the P neurone axons projecting directly to the DN area. A monosynaptic projection would be expected in parasaggittal sections of the Cb since Ito et al. (1970a) have observed that monosynaptic zones in the cortex are situated more or less directly above each DN region. The calculated conduction velocities for P neurone axons, assuming monosynaptic transmission, appeared slower than the directly measured conduction velocities of myelinated axons, 0.26 m/sec, but these axonal recordings are difficult to interpret and as discussed in the first part of this chapter they are likely to overestimate the conduction velocities.

My electrophysiological experiments suggest that virtually every DN neurone receives an inhibitory input from a large area of the Cx because most DN neurones could be inhibited no matter where the stimulating electrode was placed within the Cx. Since the cortical neurones were often spontaneously active it would be expected that they constantly inhibit the DN neurones. This was indeed confirmed during intracellular recordings which showed frequent spontaneous IPSPs in the DN neurones (Fig. 15). The spontaneous IPSPs were similar in duration and amplitude to the evoked ones. However, the IPSPs did not overwhelm the excitatory activity completely and occasionally EPSPs were also seen. During extracellular recordings from the DN neurones some spontaneous activity has been detected, a finding which differs from that of Seil & Leiman (1977) who did not see any spontaneous activity in the DN area except for that which presumably originated from P neurone axons. I feel, however, that their criteria for distinguishing fiber responses are not sufficiently well defined to accept this interpretation. Especially the criterion of "the variations in amplitude which fiber potentials demonstrate

in response to electrical stimuli of varying intensity" is difficult to comprehend since it is generally accepted that fibers can produce all or none spikes as do the neuronal somata. Thus the failure of Seil & Leiman to detect any spontaneous activity in the DN may simply be the result of a misidentification of neuronal activity for fibers. The spikes which I recorded in the DN are unlikely to have originated from fibers because even during specific attempts such fiber recordings were very difficult. However, one should also notice that the BSS used as bathing solution in the experiments by Seil & Leiman is different from the Earle's BSS used in the present study. They have used Simm's BSS (refer to Table 1) which has a 2.7 mM concentration of K^+ as compared to about 5.4 mM in Earle's BSS. According to work by Hösli et al. (1972) and Ransom et al. (1977) such a difference in the K^+ concentration should cause only a few mV drop in the resting potential of the neurones, nevertheless it is possible that the DN neurones in my experiments were slightly more depolarized and thus generated activity more easily than the neurones of Seil & Leiman (1977).

It is considered unlikely that the spontaneous discharges were due to the contamination of the DN region by the cortical neurones because the cultures were selected for the experiments on the basis of a clear separation between the DN and Cx regions (see chapter 1, part IV and chapter 2 for details).

Electrically evoked inhibition in some DN neurones was followed by a facilitation. This response resembles the disinhibition studied by Ito et al. (1968) in vestibular nuclei. GABA-blockers abolished the facilitation in our experiments, confirming that it was the result of disinhibition in DN neurones. A possible mechanism is the activation of inhibitory interneurones by the cortical stimulation, resulting in inhibition of Purkinje cells, with a consequent removal of their tonic inhibitory influence on the DN neurones. A similar facilitation could in

principle be the result of a rebound excitation such as that observed after iontophoretic applications of the inhibitory amino acids (see Fig. 30). I consider this possibility less likely, however, since in some of the neurones which were carefully studied, the facilitation occurred at a stimulus intensity lower than that necessary to produce the inhibition (see Fig. 26B).

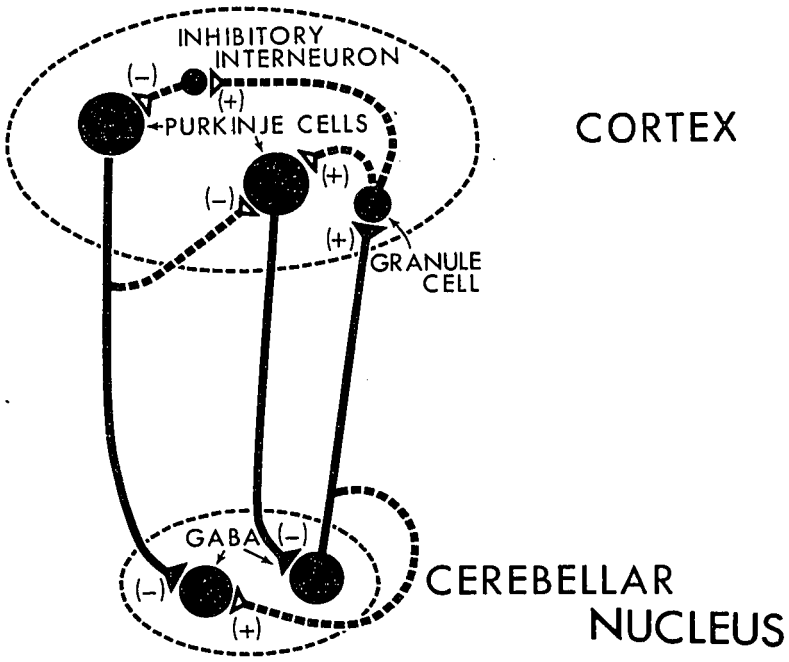
Another characteristic response in DN neurones was the presence of rhythmic discharges following the inhibition (Fig. 26A). In the preliminary report, it was suggested that these discharges may be the result of successive periods of excitatory and inhibitory synaptic activity which would presumably be mediated by a chain of neurones (Wojtowicz et al., 1976). It could be speculated for example that once the DN neurones are inhibited by a cortical volley they respond with a rebound excitation which in turn will activate the DN-Cx pathway and thus produce the next inhibitory volley via the Cx-DN pathway. Alternatively, the cortical stimulation could produce an antidromic activation of the recurrent axon collaterals of the DN neurones which may contact inhibitory interneurones (recurrent inhibition) and these in turn could cause successive cycles of inhibition and rebound excitation in the DN neurones (a similar mechanism has been proposed in the thalamus by Andersen & Eccles, 1962). However, on the basis of the correspondence of the steady rate of neuronal firing and the periodicity of the postinhibitory discharges, I now feel that these responses are simply the result of a resetting of the regular firing of a neurone. That is, an evoked inhibition delays the occurrence of a spike by a certain period of time after which the neurone resumes its background firing. A similar idea was suggested by Murphy & Sabah (1971a) to explain a functional role of the climbing fibers in the Cb.

In some DN neurones a cortical stimulation evoked synaptically mediated responses at a short latency (results, Part IVe). These responses were enhanced by GABA blockers presumably because the blockers removed the concomitant inhibition. It is proposed that these are the responses of recurrent axon collaterals of the DN neurones.

c) Summary of Synaptic Connections

The cortical and DN regions in cultures are interconnected. The axons of DN neurones project to the Cx and they contact synaptically the Cx neurones. On the basis of the latencies of the evoked responses in the P neurones and the conduction velocities of DN neurone axons the DN-Cx projection was judged to be predominantly disynaptic (see diagram on page 148). The inhibitory responses in the P neurones were attributed to P neurone axon recurrent collaterals which are known to be inhibitory in vivo and to the inhibitory interneurones in the Cb cortex. The axons of P neurones project from the Cx to the DN area and contact the DN neurones monosynaptically. As in vivo, the projection is inhibitory. The excitatory responses in DN neurones were attributed to antidromic activation of the recurrent axon collaterals of other DN neurones.

5-10 mM Mg was initially used as a selective blocker of synaptic transmission. However, subsequent experiments demonstrated that a significant depression of the membrane excitability could result even at such low concentrations of Mg. This was shown using the threshold for antidromic activation and iontophoretically applied glutamate as tests of the excitability. The additive depressant effects of increased calcium concentrations provided further evidence that the effects were largely on the membrane excitability and not on the transmitter release mechanisms.



Summary of synaptic connections in tissue cultures.

Part III - Pharmacological Studies

The main objective of my pharmacological studies was to identify a neurotransmitter substance responsible for the inhibition of DN neurones. GABA blockers blocked the inhibition in parallel with blocking of iontophoretic GABA pulses, indicating that GABA is likely to be the neurotransmitter in the pathway studied. Such a finding is in general agreement with other studies performed *in vivo* although these studies were not dealing specifically with DN neurones. In the lateral vestibular nucleus, Bruggencate & Engberg (1971) demonstrated a selective action of iontophoretically applied Strychnine and Picrotoxin on the depressions produced by iontophoretic applications of Glycine and GABA respectively. They didn't study, however, the effects of these blockers on the synaptic inhibition. Obata *et al*. (1970a) used Picrotoxin and Strychnine for the investigation of evoked inhibition produced by P neurones in the lateral vestibular nucleus. Intravenous injections of Picrotoxin (8-16 micromoles/kg) blocked the synaptic inhibition while Strychnine was ineffective even at up to 2.1 micromoles/kg (≈2 x convulsive dose). These results were not entirely conclusive because the selectivity of the blockers was shown only with iontophoretic applications of these drugs and not with intravenous injections. It appears therefore that this thesis is the most complete pharmacological study of the inhibition produced by the P neurones.

Besides blocking the inhibition of DN neurones, GABA blockers frequently produced a long excitation which replaced the inhibition (Fig.33). It is possible that the excitation was the result of an activation of axon collaterals of antidromically excited DN neurones. In the control BSS such an excitation was likely to be mostly suppressed by a powerful inhibition and in fact only occasionally the evoked responses were seen.

It was consistently observed that GABA blockers produced an increase in the background activity in DN neurones which in most cases was followed by a generation of bursts (Figs. 31 and 32). The increase in activity can be explained by a removal of the tonic inhibitory input to the neurones by the blocker. Bursts in turn could be generated by the mutual, spontaneous excitation of neurones via their axon collaterals. Nelson et al. (1977) observed similar bursts in cultured spinal cord neurones after incubation with a high concentration of Glycine (0.4 mM). They have demonstrated with intracellular recordings that under such conditions the frequency of occurence of spontaneous IPSPs was decreased. Gähwiler (1975) has found that 10^{-10} - 10^{-6} M Bicuculline increased the rate of firing of cortical neurones in the roller tube cerebellar cultures. Although my present study is mainly concerned with DN neurones, I have made several tests on cortical neurones and found that higher concentrations of Bicuculline, i.e. 1-5 µM, are necessary to influence both cortical and DN neurones.

The derivative Bicuculline-methiodide was also tested because it is more soluble and more stable in physiological solutions (Olsen et al., 1975, 1976). It was found that Bicuculline-methiodide was somewhat less effective as a blocker than Bicuculline, but since the difference was small, Bicuculline-methiodide is probably the compound which should be used in future experiments of this kind.

Picrotoxin was also found to be selective for GABA, but less active than Bicuculline on a molar basis. The effective concentration of Picrotoxin (5-15 µM) is within the range reported by Gähwiler (1975) who found that 10^{-7} - 10^{-4} M is necessary to increase the rate of activity of neurones in roller tube cerebellar cultures.

Unexpectedly, Strychnine was also found to block the evoked inhibition. This occurred in most cases at concentrations above 10 μM when Strychnine also often blocked iontophoretic pulses of GABA. Therefore, the effects of Strychnine did not necessarily contradict the results obtained with GABA blockers. It should be noted that the concentration of 10 μM is considerably higher than the usual convulsant dose of 0.3 mg/kg (≈1 micromole/kg) reported *in vivo* (e.g. Curtis *et al.*, 1971a).

However, in three neurones from three different cultures, Strychnine blocked the evoked inhibition and the effects of Glycine but not those of GABA. All those neurones were also tested with GABA-blockers and it was found that the evoked and GABA inhibitions were abolished whereas Glycine depressions were unchanged. In view of this evidence, it seems possible that a certain number of P neurones release a neurotransmitter other than GABA; one which is blocked by both Strychnine and GABA blockers. Taurine and β-alanine depressions have been found to be blocked by both Glycine and GABA blockers (e.g. Curtis *et al.*, 1971b) and would be possible mediators of this effect.

The effectiveness of Strychnine in blocking the evoked inhibition has been demonstrated in other parts of the brain (besides the spinal cord) by several workers. Yamamoto (1972) demonstrated that 3-8 μM Strychnine can block the evoked IPSPs in slices of the hippocampus. Similar effects have been observed in tissue cultures of the hippocampus and neocortex at 3-10 μM (Zipser *et al.*, 1973; Crain, 1976) and it has been suggested that taurine may be the neurotransmitter blocked by Strychnine. On the other hand, Gähwiler (1976) proposed that Strychnine acts unspecifically even at low concentrations (10^{-8} - 10^{-6} M), presumably

by changing the effectiveness of both excitatory and inhibitory synapses. This proposal requires further investigation.

Summary of Pharmacological Studies

Pharmacological blocking agents were used to study the nature of a neurotransmitter at the P neurone - DN neurone synapse. Two putative transmitters, GABA and Glycine, were applied onto the DN neurones iontophoretically and they both depressed the activity in these neurones. In most cases, Bicuculline and Picrotoxin blocked the synaptic inhibition and the GABA depressions in parallel without affecting Glycine suggesting that GABA is the transmitter responsible for the synaptic inhibition. GABA blockers also increased a spontaneous activity in neurones and enhanced a synaptic excitation, both effects can be explained by the removal of the inhibition.

These studies demonstrate the feasibility of the tissue culture model for investigation of pharmacological properties of synapses in the CNS. The usefulness of the model lies primarily in its accessibility to various pharmacological manipulations which are often difficult in vivo.

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