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Electrophysiological studies of mouse cerebellum neurons in culture

by

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Submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science.

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LIST OF ABBREVIATIONS

Ach - Acetylcholine.
ACH - Autocorrelation histogram.
ADT - Analog data tape.
AF - Alternate firing.
AHP - Afterhyperpolarization.
ap - Action potential.
APN - Absolute pair number.
ATI - Analog tape interface.
bic - Bicuculline.
BS - Brain stem.
BSS - Basic salt solution.
BW - Bin width (BX in CCH figure legends).
CA - Catecholamine.
CB - Common bursting.
CCH - Cross correlation histogram.
CF - Climbing fiber (in introduction). (Common firing in results).
CFR - Climbing fiber response.
CI - Common inhibition.
CTALOG - Label of disk file storing address and other frequently used data for disk data records.
Cx - Cerebellar cortex.
DE - Directional excitation.
DN - Deep cerebellar nuclei.
DRG - Dorsal root ganglion.
EDT - Encoded data tape.
EI - Enigmatic interactionns.
Em - Membrane potential.
EM - Electron microscope.
EPSP- Excitatory postsynaptic potential.
GABA- Gamma amino butyric acid.
glu - L - glutamic acid.
GTF - Gigantocellular tegmental field.
HCA - dl - homocysteic acid.
HRP - Horseradish peroxidase.
IIH - Interspike interval histogram.
IO - Inferior olive.
IPSP- Inhibitory post synaptic potential.
LC - Locus ceruleus.
LM - Light microscope.
LVN - Lateral vestibular nucleus.
MAM - Methyl azoxy methanol acetate.
MEM - Minimal essential medium.
mes V - Mesencephalic nucleus of trigeminal nerve.
MF - Mossy fiber.
mmf - Milli - milli farad (= microfarad).
mmV - Microvolt.
MMV - Monostable multivibrator.
NE - Norepinephrine.
NPM - Nucleus parabrachialis medialis.
O.D.- Outside diameter.
PCG - Pontine central gray.
PDS - Paroxysmal depolarizing shift.

PN - Purkinje neuron.
PSP - Post synaptic potential.
RF - Reticular formation.
SC - Spinal cord (except in section 4.4.0.2 of discussion where it refers to nucleus subceruleus).
SG - Spike generation site.
ST - Spike train.
STDISP - Spike Train DISPlay subroutine (p 70)
SVN - Superior vestibular nucleus.
TTL - Transistor transistor logic.
TXIH- Triggered cross interval histogram.
VN - Vestibular nuclei.
XIH - Cross interval histogram.

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ABSTRACT

Investigation of synaptic connections in a group of neurons in culture from the peduncular region of the pontine tegmentum (hereinafter referred to as brain stem (BS) neurons) was carried out by means of two simultaneous single unit recordings. Primarily extracellular records were obtained, and correlations between the spike trains were determined by cross correlation methods (Perkel et al, 1967b) as well as various modifications of time series analysis adapted to the BS culture system.

Correlations obtained betwen the spike trains were then reformulated in terms of possible synaptic mechanisms which could give the observed correlation. This resulted in a multiplicity of possible explanations, and thus a smaller number of simultaneous extracellular/intracellular recordings of two different neurons were made so that synaptic activity could be directly observed rather than inferred as from the purely extracellular records.

Because of the low rates of spontaneous activity present in the BS neurons, the majority of cell pairs were recorded using double barreled electrodes from which iontophoretically applied glutamate (glu) could be utilized to increase the rate of activity of the neurons. Most BS neurons were consistently excited by glu, although some anomalous responses to glu were observed.

A large degree of correlated activity was observed in the recorded neurones. Of 92 extracellular cell pairs, 64% exhibited a definite

correlation, 12% could possibly have had an interaction, but were ambiguous, and only 24% were definitely non-correlated. Of the 59 correlated extracellular cell pairs, three principal interaction categories could be distinguished: correlated bursting, directional excitation, and a small number of miscellaneous interactions. Correlated bursting was the numerically greatest interaction with 46 (78%) of the correlated pairs exhibiting some form of correlated bursting. Directional excitation was observed in 5 (8%) of the cell pairs, and may represent a direct excitatory synaptic connection from one neuron to the other, although conclusive evidence of this was not obtained in any of the extracellular data.

15 good cell pairs comprised the intracellular data, and here again correlated bursting was the largest interaction type (4 cases). Intracellular records revealed that common bursting was due to the occurrence of a large depolarization of variable size and duration. The variability from one intracellular recorded burst to another implied that the large depolarization was the sum of numerous nearly synchronous EPSP's. In one extra/intra cell pair, a large consistently evoked EPSP (from the extracellular cell) was observed. When other intracellular records were analyzed by the techniques of extracellular spike triggered intracellular averaging, an additional 2 EPSP's were observed as well as 3 nondirectional interactions which consisted of a depolarizing potential extending backwards and forwards from the time of the extracellular spike.

The intracellular records provided evidence for predominantly excitatory interactions among the BS neurons, although two cases of common inhibition

were also found in the intracellular data. Intracellular recordings revealed a more extensive interaction among the neurons than shown in the extracellular data.

With the excitatory nature of the BS to BS connections established, the data were examined in order to see if the type of culture had any influence on the interactions. Two types of BS cultures may be distinguished; BS - Cx cultures in which cerebellar cortex and deep cerebellar nuclei are included, and pure BS cultures in which almost all of the cerebellar elements have been eliminated. No difference could be found between the frequency of occurrence of the various interaction types and the type of culture. Only when the cell pairs were grouped by the electrode configuration used; one glu and one single barrelled, or two glu electrodes, was a difference apparent. The interactions in the cultures recorded with 2 glu electrodes were almost exclusively (92%) correlated bursting, whereas this category made up only 57% of interactions in cell pairs recorded using one glu electrode.

A model which has been proposed to explain this data is that the BS neurons consist of a network of interconnected neurons with excitatory interactions in which most synapses are weak. When glu is applied at one site, a portion of the network is activated, but the activity rate is not sufficiently high to obscure interactions (non-bursting type) which may be occurring with the cell on the other electrode. With two glu electrodes, a greater number of neurons are activated, and when proper synchrony of spikes occurs, unlimited positive feedback causes synchronous firing in all cells in the network until accumulated refractoriness ends the cycle. Such an event would correspond to a correlated burst.

Chapter I

INTRODUCTION

1.1 GENERAL OVERVIEW OF PROJECT

1.1.1 Statement of problem

The initial aim of this investigation was to use electrophysiological recording techniques to attempt to determine the nature of synaptic interactions among a group of neurons found in cultures of newborn mouse cerebellum and cerebellar peduncle. Also it was desired to utilize various techniques of spike train analysis to asess their utility in revealing synaptic connections in a neural culture system. At the time the project was initiated, primarily electrophysiological data was available on these neurons, and anatomically all that could be said about them is that they formed a distinct cellular configuration at the light microscopic (LM) level, and appeared to originate from the peduncular region of the brain and were thus called Brain Stem (BS) neurons.

The projections of the neurons to various regions of the culture were identified on the basis of antidromic activation of the extracellularly recorded neurons by focal electrical stimulation (Wojtowicz, 1978). It was found that the BS neurons could be antidromically activated from both cerebellar cortical (Cx) and Deep nuclear (DN) areas of the cultures. Also, BS

neurons could be excited, apparently synaptically, after Cx stimulation, but no decision was possible on whether this was mediated by excitatory cortical projections or collaterals of antidromically activated BS or DN neurons.

On the basis of the available data (as well as the assumption that the BS neurons were a homogenous cell group) it was decided to investigate the synaptic interactions among the neurons by a method using some technique other than focal electrical stimulation. The use of two unit recordings was chosen as a method of determining the connections of the BS neurons with each other. Initially it was proposed to investigate the synaptic connections of the BS neurons first with each other, and then with the other areas of the culture, although to date it has only been carried out among the BS neurons.

1.1.2 Electrophysiological approach

The nature of the approach used to look for synaptic interactions among the neurons was determined to a large extent by the properties of the neurons and technical constraints. Ideally, intracellular recordings from two neurons would give the least ambiguous results. This method was deemed impractical because even though the BS neurons are fairly large (by mammalian standards) they are still small enough that a great deal of effort could be expected in obtaining sufficiently stable intracellular records for the records to be of any use. The mechanical parameters of the micromanipulators and chamber

at the beginning rendered this approach (and still do) impractical.

Accordingly it was decided that simultaneous extracellular recordings would be employed. The ability to see the neurons in the culture was of immense value in knowing approximately where to go to find a given type of neuron, but the optimal recording location required that a neuron be active as the electrode is moved inward to allow for very fine adjustments of the electrode to be made once a spike is picked up to prevent mechanical injury. Earlier single unit recordings on the BS neurons revealed that they were either silent, slowly firing, or phasically active but at rather long intervals (Hendelman et al, 1977). This required some means of increasing their rate of activity. Electrical stimulation was a possibility, but the closely packed nature of the cells and their abundant axon collaterals as well as DN axons passing through made selective focal stimulation a virtual impossibility. It was for this reason that the use of iontophoretically applied excitatory amino acids (glutamic (glu) or homocysteic (HCA)) using double barrelled microelectrodes was decided upon. Two glutamate containing electrodes were employed in the majority of experiments due to the flexibility in modulating the firing of each neuron that this configuration gave. When this approach was adopted, it was assumed that the action of glutamate was exerted primarily on the neuron being recorded from, and that glu was excitatory to all neurons in the CNS.

1.2 ANATOMICAL CONSIDERATIONS

Two major culture methods of nervous tissue may be distinguished, dissociated cultures and explant cultures. The latter may be further subdivided into roller tube and Maximow types. Dissociated cultures bear the least resemblance to the living brain, as they are made by taking pieces of brain tissue and using either mechanical or enzymatic techniques to dissociate the tissue into a homogenate of single cells. These are then plated on a supportive surface and allowed to grow. A primary advantage of this technique lies in the detailed visualization of neuronal processes and their growth.

Roller tube cultures consist of small brain pieces placed on collagen coated coverslips, and then subjected to continuous rotary motion in the incubator. This causes considerable flattening of the explant (much more so than occurs in the Maximow cultures) and results in transformation of original tissue piece to a sheet of cells. This essentially two dimensional culture is thin enough so that neuronal somata and sometimes processes may be visualized under a microscope. The effect of the change from a 3D to 2D organization must be considered in the reorganization of connections in the tissue piece.

Maximow cultures are made by taking a relatively large piece of tissue, (but not more than about 500 microns thick), placing it on a collagen covered cover slip and incubating it in a Maximow chamber. The medium is changed twice weekly. The progress of the explant may be observed microscopically after a

few days in vitro. The course of neural differentiation in these cultures most closely resembles that occurring in the living state, including maintenance of 3D relations among the neurons. Visibility of neuronal locations is quite adequate for electrophysiological recordings, although the thickness restricts unambiguous identification to the nuclear outlines of the cells.

After explantation, an outgrowth of glial and other proliferating cellular elements occurs, as well as a thinning of the explant. There is likely some degree of translocation of neuronal somata. Depending on the thickness of the explant, there may be a necrotic zone in the central region in which no details may be seen due to its thickness. This zone consists of an area of dead tissue in its lowermost portion, adjacent to the cover slip, but the upper layer is still living. Presumably this will include some neurons and other cells. In this area, it is very difficult to see anything because of its thickness (W. J. Hendelman, personal communication).

The topological transformations occurring in the tissue slice in culture from the original organization have been studied by Seil,(1972). In cultures of cerebellum it was found that neurons which had already matured retained their positions relative to one another (approximately) and only granule cells, which were produced postnatally in the external granular layer underwent migration. The production of laminated structures within explants of cerebral cortex also (Calvet, 1974) suggests that it is reasonable to assume that the geometric relations of

neural masses in explant cultures will be preserved. (Of course this does not take into account changes which may be produced by neuritic growth in vitro).

Within the cultures used in this investigation, usually 3 areas (Cx, DN and BS) could be demarcated quite readily. The Cx was at the periphery of the explant and myelinated axons were sent inward to the DN area, which was close to the ciliated ependymal tissue. The BS area was always located adjacent to the ependymal tissue, and could be in continuity with the DN area. The distinctiveness of the areas was variable between cultures.

1.2.1 Anatomy of region of interest

Two levels of complexity will be revealed by a consideration of the cultures and actual brain systems used in this study. The cerebellar cultures are the simpler ones (compared to the brainstem) due to a number of features of the cerebellar cortex: its homogenous constitution, anatomical isolation and well characterized cytoarchitecture. The deep nuclear cell groups represent a problem in that the exact identification of which group is present is difficult, but they do have a relationship with the cortex which, as a first approximation, may be assumed to be constant.

Anatomically the BS explants seem to present a simpler organization than their cerebellar counterparts, but examination of the corresponding region in the animal reveals a great variety of intermingled nuclei and fiber tracts. Also, the

distance scale for variation is much smaller in this region than in the cerebellum, and when one considers the limits of variation possible when cutting the explants manually, the possibilities for the origin of the BS neurons become very numerous.

1.2.1.1 Gross anatomy

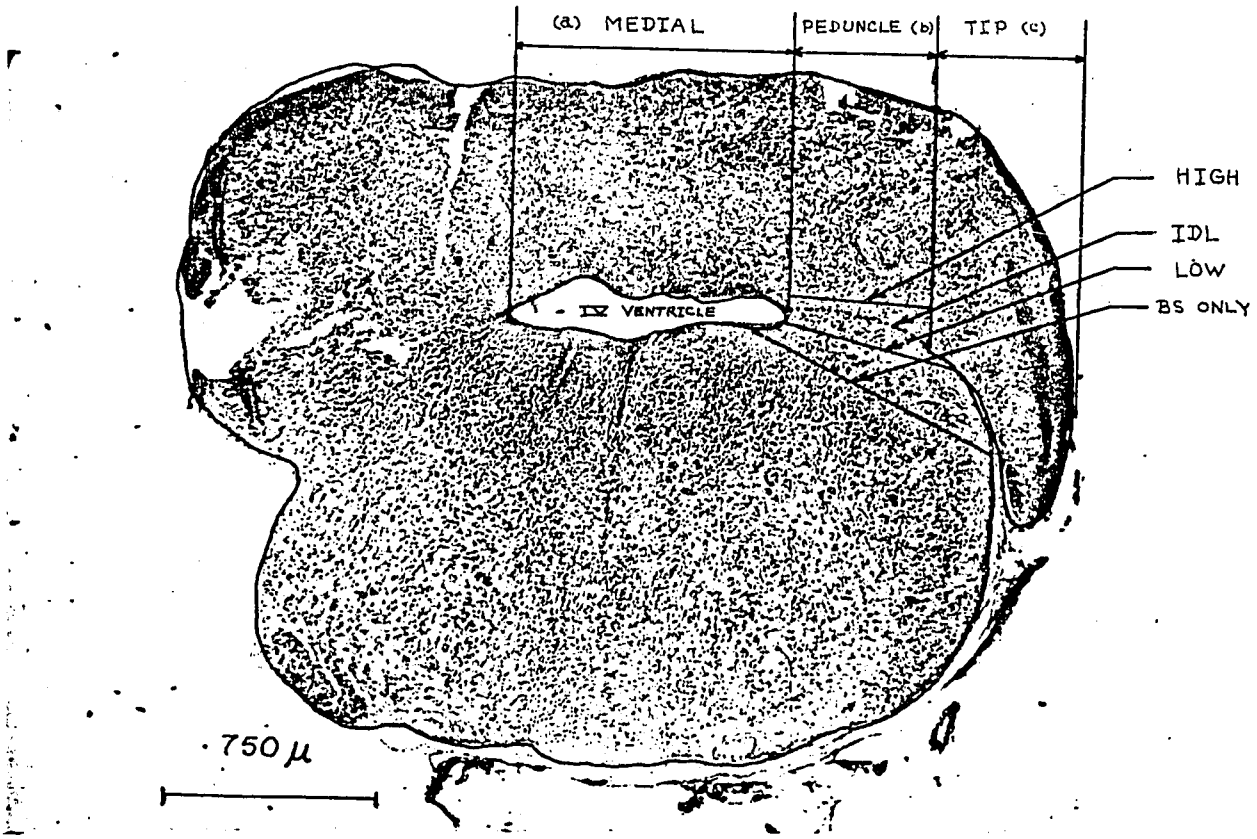
The region of interest may be broken down into three basic areas: the cerebellum, the cerebellar peduncles and the area of brainstem lying immedialy ventral to the cerebelar peduncles. The cerebellum is the largest of the above mentioned structures in the newborn mouse, and only the peduncles are in continuity with the underlying brainstem. This anatomical isolation makes it possible to prepare pure cultures of cerebellar cortex and DN. In Fig (IF1) the further anatomical subdivisions of the cerebellum into a lateral piece and a medial piece are illustrated. Separation of the cerebellum from the brainstem by severance of the peduncles gives rise to three possible culture types: (a) those derived from the lateral tip region, designated as tip cultures, (b) those medial to the tip and including variable amounts of peduncular tissue, and (c) sections taken from the medial portions of the cerebellum. All explants are made by cutting saggital sections through the cerebellum. Types (a) and (c) give essentially pure cortex-DN cultures and are not considered further.

It is in the cultures of cerebellum and peduncle where the existence of a distinct cell group, the BS neurons, is observed.

Cultures may be prepared which either include the BS neurons with the cerebellum or as 'pure' BS cultures in which the bulk of cerebellar tissue is removed before explantation (although some cortical remnants are found in even the most pure of explants). The dividing line between the cerebellum and peduncles is shown by (IDL). in Fig (IF1). The origin of the BS neurons is shown to be in the medial portion of the peduncular region by the observation that if the piece of peduncular tissue to be explanted is divided into medial and lateral halves, the appearance of cells with BS anatomical features is most often restricted to the medial piece (W. J. Hendelman, unpublished results).

At birth the dimensions of the mouse brainstem are about 1.5 mm dorsal to ventral and 2 mm across. The rostro caudal distance encompassed by the cerebellar peduncles is about 350 microns which represents almost 3/4's of the dorsal surface of the dorsal pons. The segment of tissue representing the peduncular explant consists of the cerebellar peduncles in addition to a variable portion of the dorsolateral pons whose boundaries are shown in fig (IF1). The dorsal boundaries of this area may be rather completely described and correspond roughly to the medullary-pontine junction caudally, and the termination of the cerebellar peduncle rostrally (almost at the pontine-midbrain junction). Laterally the boundary is the lateral exterior surface of the pons, and medially the limit is the lateralmost extent of the fourth ventricle. The ventral boundary of the piece is less precise, but may be taken as a

Figure IF1



Newborn mouse brainstem-cerebellum cross section showing where various sections taken for different culture types.

High- Represents section made to yield only Cx-DN culture.

Low - Is approximate ventral boundary of explant when both Cx and BS included.

IDL - Represents dividing line between peduncle and cerebellum.

BS only explants are made by taking piece of tissue which is bounded dorsally by IDL and ventrally by BS only line.

(Note: all boundaries approximate).

line running from the lateral tip of the fourth ventricle in a ventro-lateral direction to the exterior pontine surface. As manual excision is performed, it is likely that the ventral boundary will be the most variable in view of the small size of the pontine region.

1.2.1.2 Anatomy of mouse BS neurons in culture

Before moving on to a consideration of the possible precursors of the BS neurons, their anatomy in culture will be considered. In culture BS neurons may be identified as a closely packed group of large cells (25-30 diameter), always adjacent to ciliated ependymal tissue of the fourth ventricle, usually as a well circumscribed collection of cells. The appearance of the living BS neurons is illustrated in Fig IF1. Occasionally a more diffuse arrangement is possible when multiple areas of ependymal tissue occur. The cells are distinguished by a large eccentric nucleus, prominent nucleoli, and large, distinctive refractile granules located on the periphery of the cytoplasm (Hendelman and Marshall, in preparation). The granule abundance is variable from culture to culture, and in rare cases the granules may appear and then disappear, (over several weeks), although this may be an effect of the culture medium. Myelinated axons are occasionally observed to traverse the BS region. It is not possible to see projecting axons from the BS area in living cultures. It must be noted that not all of the BS neurons have granules, and often neurons are seen in the BS region which look identical to the

granulated neurons (in terms of nuclear and somatic profiles), but do not have any granules. Whether they represent BS neurons which have lost their granules, or another neuronal type, is not clear. There is no way of determining what the significance of these non-granulated cells is on the basis of the small amount of anatomical data which was gathered during the majority of the electrophysiological experiments. All that can be said about them is that they appear to be 'belong' in the BS region, as there are other neurons which may be distinguished quite readily from the non-granular cells.

Of the distinct neuronal types found in and around the BS region the most common one has a larger nucleus than BS cells, and a clearly visible spherical somatic outline which is also larger. Also, no granules are visible within the cytoplasm. When present, these cells are solitary. On morphological criteria these neurons may be reasonably considered as cells of the mesencephalic nucleus of the trigeminal nerve (Allerand, 1971; Wolf, 1964). Within the BS area itself it is often possible to see regional differences among the neurons either in distribution of granules or of neuronal size. The relevance of these variations is at present unknown. The extent and thickness of the BS region are also observed to vary among cultures, but this is not accompanied by very obvious changes in the morphology of the neurons as seen in the living cultures.

At the light microscopic level several staining techniques have been employed to visualize the processes of the BS neurons. The Holmes stain gives primarily impregnations of axons, but

rarely allows for visualization of the BS cell bodies. The Golgi technique is the standard method which is employed for the staining of both cell bodies and their processes, but attempts to apply this procedure to BS neurons by Aggerwal and Hendelman led to completely negative results. A more recent technique, the intracellular injection of horseradish peroxidase, has been employed with considerable success by Hendelman and Marshall (1978 and in preparation) in the staining of BS neurons, as well as other cell types within cerebellar cultures.

The basis of the HRP technique is that considerable intracellular protein transport is carried out bidirectionally between the soma, and the processes of the neuron. When a neuron is impaled by a micropipette filled with a solution of HRP, and the enzyme is injected iontophoretically into the neuron, within several hours distribution of the HRP to even the most distal processes will have occurred. In order to visualize the spatial location of the HRP, the culture is treated with a chemical mixture which contains hydrogen peroxide, and a dye precursor which gives a colored product when oxidized by the oxygen evolved from the reaction of the peroxide with the HRP. Aside from the great advantage of being able to see the detailed projections of a single chosen neuron, HRP also has the advantage that it is electron dense, and after examination at the LM level, if the culture is prepared for EM, parts of the injected neuron may be identified by their very dark appearance.

The HRP stained BS neurons were seen to have 2 or 3 dendrites originating from the soma which were initially stout

and gradually tapered. Usually one dendrite was larger than the rest. The dendrites generally had variable numbers of spinelike or irregular projections. In more peripherally sited neurons the dendrites were observed to extend beyond the boundaries of the BS region. Multiple axon-like processes were usually found for each BS neuron. One of the axons originated from either the soma, or the proximal portion of a large dendrite, while others were seen to result from the distal continuations of dendrites. No direct evidence for the unmyelinated nature of the axons is available, but their small calibre and failure to observe myelin in the living culture BS region make it very likely. The axons were observed to form a dense plexus in the BS region, as well as sending numerous projections to the outgrowth region. A few axons were detectable in the cortical and DN areas. The axons possessed small varicosities.

Investigation of the monoamine content of the cultures has been carried out by Hendelman and Ferguson (in preparation) using the technique of glyoxylic acid induced fluorescence. So far their studies have revealed that fluorescent axons with varicosities, similar to those of the BS neurons stained by HRP are present in the outgrowth area of the explant. These are not present when the BS region is absent. Unfortunately the autofluorescence and monoamine fluorescence in the area of BS cell bodies is such that visualization of fluorescent somata is not possible. The evidence for the presence of monoamine, most likely NE, containing neurons in BS cultures is thus quite clear, but what fraction of the neurons are of this nature is

not known. The necessity of such evidence in BS neuron identification will be discussed later.

1.2.2 In vivo anatomy of mouse brainstem region

When work on the BS neurons was initiated, the opinion was that the region represented a homogeneous group of cells. Consequently this bias permeated all early experiments and little thought was given to detailed anatomical descriptions of the cultures. The argument for BS homogeneity is essentially anatomical, and for that group of neurons which possess all of the BS morphological criteria (observable at the LM level) is likely to be correct. There are reasons, however, for believing that other neuronal types are present with the 'classic' BS neurons. A number of lines of evidence lead to the conclusion that the question is not which cell group the BS neurons represent, but rather of how many cell groups do the BS neurons represent. (Physiological evidence for this is considered in the discussion).

Rather than present a detailed case incorporating all the pieces of evidence, just the following statements will be made. In view of the diverse number of neuronal groupings known to be present in the area explanted, it is unreasonable to expect only one to survive. The use of explants with small or large pieces of brainstem tissue gave groups of BS neurons which were also smaller or larger, but at the LM level no gross differences in cell types or morphological change was apparent.

1.2.2.1 Overview of cell groups

Within the area of the peduncular region explant may be found portions of the following cell groups: vestibular nuclear complex, cochlear nuclei, trigeminal nuclear complex, at least two NF containing nuclei, portions of the midbrain central gray, parts of the dorsal pontine reticular formation as well as portions of other poorly delimited nuclei in the area (Ramon-Moliner and Nansereau, 1974). Each one of the regions mentioned may be subdivided further, in some cases quite extensively, to give a rather large list of possibilities.

A detailed examination of the possible group of origin of the BS neurons will not be made here as this is dealt with at length in the discussion. Rather, the more simplistic view prevailing at the initiation of the project will be presented, and this should be contrasted with the more detailed view currently existing. Initially, the possibilities for the origin of the BS neurons were thought to be rather limited primarily due to what then appeared to be a very restricted area of the brain stem being used for the cultures. It was thought that the primary groups in the area would be the vestibular nuclei, the locus ceruleus and Mesencephalic V.

Another factor which makes the identification of the origin of neurons in a given piece of brain tissue from a newborn animal difficult is that the cytoarchitecture of the area is in many cases not the same as that of the corresponding area in the adult. In the newborn, the brain is still undergoing considerable development, and this may take the form of

increases in the size of neurons in some areas, while in other areas there is still division of neuroblasts and differentiation of these cells into neurons as well as cell migrations. Detailed information on what is occurring postnatally is only available for a few select areas of the brain (such as cerebellum).

1.2.3 Cerebellum

1.2.3.1 Anatomy

Anatomically, the adult mouse cerebellum consists of a thin convoluted layer of cortex underlain by a much thicker layer of white matter in which are imbedded the deep cerebellar nuclei. Two large groups of fibers, the cerebellar peduncles, on either side of the cerebellum attach the cerebellum physically to the brainstem and are the routes for all cerebellar afferents and efferents.

The cerebellar cortex can be divided into three distinct layers; the molecular layer, Purkinje cell layer, and the internal granule cell layer. Of these three, the molecular layer is the most superficial, and is composed of numerous thin granule cell axons (parallel fibers) making extensive contact with Purkinje cell dendrites, and the dendrites of inhibitory interneurons. The dendrites of the Purkinje cells are arranged in a multibranched planar fashion perpendicular to the course of the parallel fibers. Their dendrites are the most numerous of cells sending dendrites to the molecular layer. Also in the molecular layer are two of the inhibitory interneurons of the

cerebellum: the basket and stellate cells which are postsynaptic to the parallel fibers and inhibit Purkinje cells. The third inhibitory interneuron of the cortex, the Golgi cell has its soma located within the internal granular layer and sends dendrites locally and to the molecular layer. Below the molecular layer the cell bodies of the Purkinje cells are arranged in a regular monolayer with a single large dendrite directed to the molecular layer where it arborizes extensively. An axon which emerges from the other end of the cell is directed towards the deep cerebellar nuclei. Collaterals are given off by axons of Purkinje neurons (PN) which make contacts on the dendrites of other PN, basket and Golgi cells (Eccles et al, 1967) in the molecular layer. The granule cell layer is innermost and consists of very numerous tiny granule cells, the terminations of the mossy fibers (the cerebellar glomeruli) and Golgi cell somata. Each glomerulus is a complicated structure consisting of a central mossy fiber axon contacting a number of granule cell dendrite endings and a Golgi cell dendrite. The granule cell dendrites are also contacted by a Golgi cell axon. The whole complex is ensheathed by a layer of glia (Eccles et al, 1967). The axons of the granule cells pass outwards into the molecular layer where they bifurcate and run considerable distances bidirectionally parallel to the surface, and perpendicular to the PN dendrites, as the parallel fibers.

The cortex is underlain by a rather thick area of white matter which is composed primarily of a network of myelinated axons in which are imbedded the subcortical cerebellar nuclei.

Four distinct pairs of nuclei may be distinguished within the cerebellum proper; these are the fastigial, globose, embolliform and dentate nuclei which are generally designated as the deep cerebellar nuclei. However, there is abundant electrophysiological and anatomical evidence for including the lateral and parts of the superior vestibular nuclei with the other cerebellar nuclei (Eccles et al, 1967).

Anatomically the subcortical cerebellar nuclei are less well characterized than the cerebellar cortex. The four classical nuclei are irregularly shaped masses or sheets of cells surrounded by axons. The neurons comprising them are medium sized multipolar cells. All of the fibers projecting to the cortex give off collaterals to the deep nuclei. A massive projection is also received from the PN's of the cortex. Only the vestibular nuclei that are classed as subcortical nuclei receive fibers from the PN, and this is primarily from the vermis (or midline region) of the cerebellum. The axons of the DN cells project mainly to areas outside the cerebellum. Collaterals are given off to other DN cells (Sotelo and Anguat, 1973), and a small projection is sent to the cerebellar cortex where presumably the axons terminate as mossy fibers (Gould and Graybiel, 1976). The main number of DN axons course outward through the cerebellar peduncles and synapse on numerous brainstem nuclei, as well as projecting to the thalamus. The fastigial nucleus in particular makes extensive connections with the vestibular nuclei.

Two major cerebellar afferent systems may be distinguished: the mossy fibers (MF) and the climbing fibers (CF). The MF's are numerically the largest input, and as a mossy fiber ascends the peduncle to the cortex, it gives off collaterals to the DN, and once it reaches the cortex, it branches many times and forms synapses with granule cell dendrites in the glomeruli. The MF's originate from almost all parts of the neuraxis. The CF's are much less numerous, and arise mainly from the inferior olivary nuclei (IO). Their importance lies in the unique connection formed between a CF and a Purkinje cell with a single CF forming numerous synapses on the primary and secondary dendrites of the PN. The CF's also give off collaterals to Golgi cells. Also a CF axon may invest more than one PN, but only one CF is found on each PN. A third input to the cerebellum, whose functional significance is not yet fully understood, is the projection from the locus ceruleus (LC). This consists of thin unmyelinated axons which project primarily to the cortex where the axons divide and terminate in the vicinity of the PN cell bodies, although a small fraction of the axons penetrate to the granular layer (Olson and Fuxe, 1971; Bloom et al, 1971). No mention of a projection to the DN was made in the just mentioned papers, although it is quite likely on the basis of labelled NE uptake investigations of the cerebellum (Ishii and Friede, 1967).

1.2.3.2 Cerebellar development in vivo.

Development of the cerebellum begins with the formation of DN and PN neurons from a common precursor. (Miale and Sideman,

1961) The Purkinje cells migrate towards the periphery where they organize themselves in the form of a monolayer. No inner granule cell layer is present at this time, and the precursors of the granule cells are in the form of an external granule cell layer on the outer surface of the cerebellum. These cells divide to produce granule cell precursors which migrate inwards. They are also thought to be the origin of the basket and stellate cells (Mugnaini, 1969). At the level of the molecular layer the inward migrating granule cells give off two cytoplasmic protuberences which proceed to elongate in opposite directions and form the parallel fibers (Mugnaini, 1969). This is the stage of cerebellar maturation attained when the mouse is born. Subsequent events involve the production of a full complement of granule cells, their migration, and the development of the Purkinje cell dendrites and synapse formation with the parallel fibers. Also mossy fibers grow in to synapse with the granule cell dendrites.

1.2.3.3 Cerebellar development in vitro.

The explant develops in culture roughly according to the way it would have continued its maturation in vivo. In the first several days following explantation, the explant becomes thinner, and a profuse growth of glial and mesodermal cells occurs at its margins. However no proliferation of neurons (aside from the continuing division of neuroblasts giving rise to the granule cells and inhibitory interneurons of the molecular layer) or large scale translocations of their somata

occur. Granule cell precursors are produced in the external granular layer and attempt to migrate inwards. For unknown reasons granule cells do very poorly in cultures, and vast numbers die off giving a much smaller (although still considerable when compared to other cell type numbers) population of internal granule cells (Wolf, 1964). This granule cell death has been attributed to toxicity of the culture medium (Allerand, 1971). Also in the cultures granule cells in the external granule cell layer may begin to develop synaptic connections with other neurons to give an external granule cell layer which has no couterpart in vivo. The DN's do not assume their in vivo oriented arrangement, but are oriented in a seemingly random fashion, although mainly facing out, and may form layers 2-3 cells thick. (Allerand, 1971; Seil, 1972; Wolf, 1964; Hild, 1966).

Despite these variations a reasonably consistent culture anatomy is attained. Areas which can be readily distinguished are cortex, subcortical nuclei and ependyma. The geometry of the explant is more or less a linear transformation of the explant with the cortical area lying above the DN cell groups, and ependymal tissue (from the ependymal lining of the fourth ventricle). Below, or beside the ependymal tissue are found groups of neurons derived from brainstem nuclei (BS). Myelinated fibers link cortical and DN areas, and also are seen going between BS and DN areas.

When the non-cortical cell types are considered, three basic varieties may be distinguished on the basis of morphology

and silver staining with a modified Holmes technique (Allerand, 1971). The classification employed here is the same as used by Allerand (1971). Type I cells are medium to large multipolar cells with long dendrites and are intensely stained by the silver stain (Allerand, 1971). These correspond to cells of the deep cerebellar nuclei and vestibular nuclei. Type II cells are large multipolar closely packed cells with large nuclei, prominent nucleoli, and possess refractile granules whose number increases with age. They do not stain with the silver stain and are generally located below the ependyma (in relation to the cortex). These are the cells which are thought to correspond to the BS neurons on which the work to be described was done. Type III cells are very large, adendritic neurons which occur singly, and in small numbers. They are stained, by the silver stain, and correspond most closely with the mesencephalic nucleus of the trigeminal nerve (Mes V).

The cell types present in a culture depend on the boundaries of the explant, and on chance. Explants including peduncular tissue give rise to BS and vestibular neurons, and relatively pure BS cultures can be obtained by using only peduncular tissue although a few PN are invariably present. The BS neurons send unmyelinated axons throughout the explant and even beyond in many cases. Usually in the cultures there is a band of fibers running on the outside edge of the cortical area which is composed primarily of DN axons (Seil, 1972). This structure has no homologue in vivo.

1.2.3.4 Physiology of cerebellum in vivo

Within the cerebellar cortex the granule cells are the only excitatory neurons and all others are inhibitory. Both major afferent systems to the cerebellum (CF + MF) are excitatory. Perhaps the most distinctive feature of the cerebellar circuitry is that the preponderance of inhibitory connections tends to prevent the occurrence of reverberatory activity within the cortex following an input (Eccles et al, 1967).

The two afferent systems have rather different effects. The climbing fibers form very strong excitatory synapses on PN's and activation of a CF causes a burst of action potentials to be generated by the PN. This synapse is one of the most powerful excitatory synapses known in the brain as it is capable of eliciting a response from a PN even when the same cell is incapable of being antidromically activated (Llinas, 1974). Usually 1 or 2 full size spikes are produced with a number of abortive spikes at a frequency of about 500/sec (Eccles et al, 1967). This burst of ap's, or climbing fiber response (CFR) is sufficiently distinctive that it allows the separation of activity in an extracellularly recorded PN into simple spikes and complex spikes which are the CFR's. In this manner the relationship between the two afferent systems may be investigated. Mossy fibers, on the other hand, branch profusely in the cerebellum and activation of a single MF activates as many as 600 granule cells (Eccles et al, 1967). A very general description of the effects of a mossy fiber input is that numerous granule cells upon which the MF terminates are

activated, as well as the Golgi cells whose dendrites are within the activated glomeruli. Action potentials spread along the parallel fibers of the activated granule cells eliciting EPSP's in dendrites of PN's and also in the inhibitory interneurons. Initially activation of PN's occurs, but the subsequent activation of the inhibitory interneurons can either inhibit the PN directly (basket and stellate cells), or inhibit granule cells (Golgi cells). Also the recurrent collaterals of the PN's inhibit other PN's. Thus the net effect of a MF input is to produce a structured burst (with the spacing of the spikes and duration being proportional to the excitation impinging on the PN), and a subsequent depression of the excited cells and of neighboring neurons.

The outputs of the DN cells are excitatory. Basically there are two excitatory inputs to the DN; collaterals of CF's and MF's, and one inhibitory input, the PN projection. The intranuclear circuitry is more complex than that of the cortex (or at least looks to be so). Sotelo and Angaut (1973) have described the fine structure of the cerebellar nuclei and lateral vestibular nucleus as being quite similar.

1.2.3.5 Physiology of cerebellum in vitro

Cultures of mouse cerebellum form numerous synaptic contacts, many of which are equivalent to those in vivo, and others which may be described as abberant. (Although this may be because the cells in vivo have not been exposed to the proper conditions to make them exhibit the same "abberant"

connectivity). Presently the group of BS neurons is the most difficult to categorize in terms of how closely its connections duplicate those in vivo.

There is good evidence of synaptic interactions occurring between PN's and other cortical neurons. The activity of single PN's in culture is generally in the form of groups of ap's alternating with silent periods (Leiman and Seil, 1973; Calvet, 1974). The activity is dependent on the cell density with low density cultures, and seemingly isolated cells displaying a regular firing rate (Schlapfer et al, 1972). Also culturing explants in the presence of an antimitotic agent (MAM) results in the almost total absence of granule cells and is reported to cause a regular pattern in most of the PN (Calvet et al, 1974). Electrical stimulation in the cortical region gives burst response in PN's at low rates of stimulation, but high rates give single spikes or no response (Leiman and Seil, 1973). The presence of inhibitory interactions in cortical areas has been inferred from the response of cultures to convulsants. Bicuculline which is an inhibitor of GABA action (thought to be the inhibitory transmitter of the cerebellum) causes an increase in firing rate of PN at low concentrations (Gahwiler, 1975). As the concentration of bicuculline is raised, the average firing rate decreases, but peak firing rate remains the same, and the cells fire in long bursts separated by long pauses (Gahwiler, 1975). Introduction of GABA into the bathing medium with the bicuculline reverses the effect.

Several investigators have recorded activity from two or more PN's to see whether correlations exist, and a number of different findings have been reported (which is not surprising in view of the number of different culture techniques utilized). In explant cultures, Calvet et al, (1974) report lack of correlated activity among PN's in cortex-DN cultures. They claim to have found, however, that in cerebellar cultures grown with a piece of brainstem, synchronous burst discharge occurred regularly in the PN's (Calvet and Lepault, 1975). These were correlated with similar coincident burst discharges and slow wave potential shifts occurring within the BS explant. Their data for cerebellum alone, however, do not support their contention that the PN's exhibited uncorrelated activity because of the crude method of data analysis that they employed (involving the use of bursts in one neuron to construct post burst histograms in the other neuron).

Work on roller tube cultures by Schlapfer et al (1972) revealed a high degree of correlation among PN's. They found a large amount of correlated bursing, and in the case of nonburst correlated cells a high degree of correlation using first order recurrence time histograms. 75% of all cell pairs they examined possessed correlated activity.

In the DN region slow regular activity has been described (Handelman et al, 1977) as well as simultaneous bursts in two cells (Calvet et al, 1974; Wojtowicz, 1978). DN neurons can be antidromically activated from the cortex, and inhibited by cortical electrical stimulation. In culture, reciprocal

connections have been found to occur between cortex and DN with the cortex to DN projection inhibitory and the DN to cortex connection excitatory (Wojtowicz et al, 1978). Whether the DN to cortex connection is mono or poly synaptic is not known at present.

Inhibitory axons go from the cortex to the BS region (Hendelman et al, 1977) and while it is known that BS axons pass through the cortical region (Hendelman et al, 1977) and may influence the activity of cortical explants (Wojtowicz, 1978) the exact termination of BS axons in cortex is not known. Among BS neurons excitatory synaptic actions have been observed (Marshall et al, 1977). The possibility of a pathway between BS and DN has not yet been investigated, although axons from each area traverse the other (Hendelman and Marshall, in preparation), and antidromic activation of DN may be achieved from BS (Wojtowicz, 1978).

The connections between PN and DN neurons match the pathways found in vivo (Wojtowicz et al, 1977). Within the cortex both excitatory and inhibitory pathways exist although it has thus far not been possible to investigate the intracortical connections any further. Reciprocal connections may also exist between cortex and BS (Wojtowicz et al, 1978), but the lack of knowledge of the identity of the BS neurons makes it difficult to say whether or not this pathway occurs in vivo.

In the cerebellar culture system being investigated in this laboratory, study of the projections from PN to DN using both electrophysiology and pharmacology (Wojtowicz, 1978; Wojtowicz

et al, 1978) has given strong evidence that the projection from PN to DN is replicated in culture, and that the transmitter is very likely GABA. This work has also demonstrated that projections from DN to PN also occur, and it is possible that there may be excitatory connections between the DN and BS neurons. In the studies by Wojtowicz (1978), BS neurons were studied both extracellularly and intracellularly, but with quite a different approach being used (all were single unit records) than in this study. Cortical stimulation was found to elicit IPSP's in a small fraction of BS neurons tested (although this number may be larger in some cultures; K. C. Marshall, unpublished results), and these were found to be relatively short (less than 50 ms duration). Excitatory effects on BS neurons were also observed with the stimulation in cortex, but it was not possible to verify whether these were due to activation of BS collaterals, or the activation of DN axons in cortex which send collaterals to synapse on BS neurons.

Degeneration studies involving the BS region have failed to show any sign of BS projections to the cortex (Aggerwal and Hendelman, unpublished results), but they and Privat and Drian (1975) have observed mossy fiber type terminals in the cortical regions of the explants. Privat and Drian claim that these were absent when no BS area was included in the explant, but Aggerwal and Hendelman have seen mossy fiber type terminals in pure cerebellar explants (Aggerwal, 1977).

The cerebellar culture system is a useful model in which the effects of culturing on the development of synaptic

connections may be compared to those of in vivo development. A different approach which has been adopted by Llinas et al (1973) and (Puro and Woodward, 1978) is the use of either ferret cerebellum in which the granule cell layer has been destroyed by infection with a virus, or the use of X-irradiation to kill granule cell precursors in newborn animals. Essentially, this gives a preparation in which the effect of granule cell elimination may be examined for its effect on cerebellar physiology occurring in vivo. Some of the changes observed in this system which parallel those occurring in culture are the formation of mossy fiber synapses directly on PN cell bodies and dendrites, and more disorganized nature of the PN dendritic tree.

1.2.4 Other culture systems.

At present quite a large number of areas from the CNS have been grown in culture, and only select portions of the field will be dealt with briefly. One system which has been dealt with in detail is the projections from dorsal root ganglia (DRG) to spinal cord (SC) in culture. This system is of interest because it demonstrates that projection and transmitter sensitivities are retained largely intact. Crain (1977) indicated that DRG neurons in culture form synaptic connections with the dorsal SC, and that the cells are depolarized by GABA rather than hyperpolarized as are most neurons. This is consistent with the in vivo findings that the neurotransmitter at primary afferent terminals is GABA. Crain (1977) stated that

occasionally synaptic interactions were present between the DRG neurons, but these were very few in number. Ransom et al (1977a,b,c) have used a dissociated SC system in which they have been able to demonstrate the continuing nature of this specificity even under such extreme conditions. They used simultaneous intracellular recordings from two neurons, and were able to identify DRG neurons, and other spinal cord neurons. This was done on the basis of an elegant technique involving the intracellular injection of current pulses into the recorded cell, and on the basis of the observed time constants of the transients, were able to decide whether the neuron had an extensive dendritic tree (SC neurons) or none (DRG) cells. When they separated the cells in this manner, only excitatory connections were observed from DRG to SC, and no connections from DRG to DRG or SC to DRG.

Other work by Crain that is of relevance to work done on the BS cultures, is his observation (Crain 1977) that synchronous activity may be induced in any culture system tested by the application of convulsants (or it may occur spontaneously). This activity could be induced by either strychnine, or bicuculline, and most of Crain's data demonstrates synchronous slow wave potentials, although it is quite likely that a more detailed investigation would have demonstrated that these were due to correlated spike discharges. Such events have been observed in cultures of spinal cord (Nelson et al, 1977), brainstem (Crain, 1977), hippocampus (Zipser et al, 1973; Peacock et al, 1978), cerebral cortex

(Calvet, 1974), cerebellar cortex (Nelson and Peacock, 1973) and hypothalamus (Gahwiler et al, 1978). This phenomenon is considered in more detail within the discussion.

1.3 SPIKE TRAIN ANALYSIS.

For the purposes of analyzing interactions among extracellularly recorded spike trains a number of methods may be employed. The primary differences among these methods lie in the depth of analysis performed. Since the interactions occurring among RS neurons were not known, it was decided to employ the technique of cross correlation analysis since it appeared to be a powerful technique for demonstrating synaptic interactions. The approach taken was essentially that of Perkel, Gerstein and Moore (1967a, 1967b).

Rather than investigate the mathematical basis for the spike train analysis methods here, the bulk of this material has been briefly stated when required in rest of thesis and the account which follows is essentially a description of the technique of the cross correlation method as this was the primary test employed. It should be noted that the underlying theory is essentially identical for all the analysis methods considered in this study.

The basic premise on which Perkel et al (1967a) based their analysis of extracellularly recorded data is that there is a great deal of information encoded within a train of spikes which may be revealed by the use of the appropriate analysis techniques. For the basis of mathematical study the idealized

spike train is considered to be composed of two components; the spikes themselves, and the intervals between them. Only the intervals are of importance, for as the authors observe; in a train where spikes are indistinguishable from one another, only their spacing carries any information.

Because the record of neural activity is obtained by extracellular recording, no information is available about the process that is producing this train of intervals. It may be, however, characterized, and a hierarchy of classification levels are available. On the lowest level lie the order independent measures, and above these lie various levels of order dependent measures. The mean rate is an order independent measure which expresses all of the information in a spike train as one number. The distribution of interspike intervals as presented by the interspike interval histogram (IIH) gives a more detailed description of the spike train than the mean rate, but it too is an order independent measure.

Order dependent measures are more difficult to quantify because many possible ways of ordering the spikes exist. The relations among adjacent spikes may be shown in the cross-interval histogram (XIH) (Rodieck et al, 1962), and the autocorrelation histogram (ACH) demonstrates recurring patterns within the spike train (Marczynski and Sherry, 1971; Perkel et al, 1967a; Gerstein and Kiang, 1960). The sign test developed by Brudno and Marczynski (1977) may be used to investigate the non-recurrent patterns in a ST, and other pattern tests are devisable.

For the purposes of cross-correlation analysis, the ACH is the primary measure of order employed (Perkel et al, 1967a). This leads to the concept of stationarity (and how this connection is made should shortly become clear). In the mathematical analysis of time series data such as a set of spikes from a neuron, the mechanism responsible for the observed output is unknown, but nevertheless several distinct categories of mechanisms may be distinguished. The primary division is between a stationary, and a non-stationary process. A stationary process is one in which the stochastic process which produces the output of the system under consideration is invariant throughout the period of observation. A non-stationary process is one in which the events observed are functions of different processes in some unknown manner.

As this is a crucial distinction in the theory of spike train analysis, it will be further clarified. Assume for now that the system under consideration is a stationary process, and assume that it has a number of internal states for which there exist a constant set of transition probabilities from a given state to any other state. This is known as a Markov chain, or Markov process. If the transition probabilities are unknown, but an external observer is capable of recording the state changes in the process, then by observing the process over a sufficiently long period of time, the state transition probabilities may be calculated.

For a non-stationary process, assume the same set of states as for the stationary process considered previously, but now

assume that several Markov processes are in operation sequentially, each with its own set of state transition probabilities. The way in which this system would now work is, for example, with some initial state (S1), process 1 would give another state, say S3 for an output. Then the control shifts to another process, say process 3 which gives another state starting from S3, and then another process takes over from there etc. If the same external observer were to view this system and to calculate transition probabilities from the observed succession of states, any resemblance between this, and any of the various processes collectively responsible for the output would be purely fortuitous due to the multiplicity of processes involved in producing the states.

If one replaces the word "states" above with observed interspike interval from a neuron, then the requirement for stationarity is quite clear, as only a stationary record can be meaningfully interpreted. Mathematically, testing for stationarity is quite complex (Cox and Lewis pp 37, 1966), although there are several simpler practical tests which may be used (Landolte and Correia, 1978; Bryant et al, 1973).

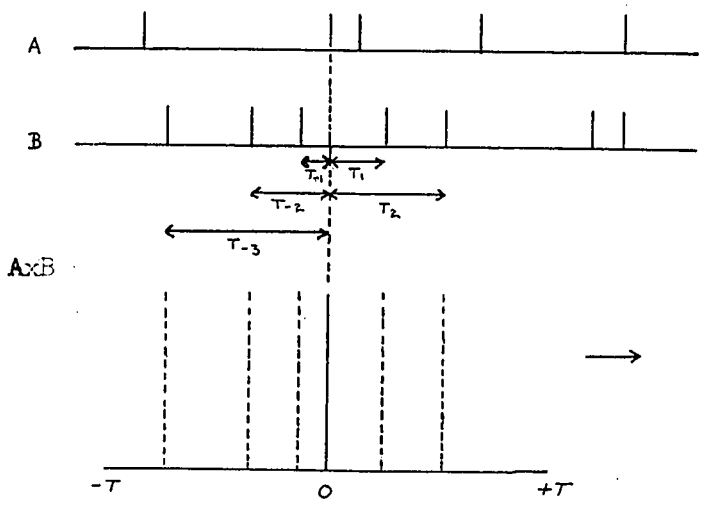
Stationary processes may be further subdivided into renewal and non- renewal processes. The distinction here is that in the renewal process the lengths of successive intervals are statistically independent whereas in a non-renewal process some type of dependence exists. Most neural processes can be expected to be non-renewal processes (Perkel et al, 1967a) and the tests for this are essentially equivalent to the order dependent measures mentioned earlier.

Quantification of the interactions among two sets of point processes is accomplished by computation of the cross correlation histogram (CCH) between the two trains. The mechanism of the computation of the CCH from spikes in Train A to spikes in Train B is illustrated in Fig (I1). Essentially the technique involves measuring the times from a spike in one ST to all other spikes following and preceding the reference spike in the other spike train. These times are known as forward and backward recurrence times. When the recurrence times are computed for every spike in Train A, and histograms of the forward and backward times made, the CCH is the result. The only significant property of the CCH that should be noted here is that computation of the CCH for B to A yields a CCH whose shape (but not numerical values in each bin) is simply the mirror image of the CCH from A to B. Also, the ACH may be viewed as the cross correlation of the spike train with itself, and since the resulting CCH is symmetric, only the positive half is used for display purposes.

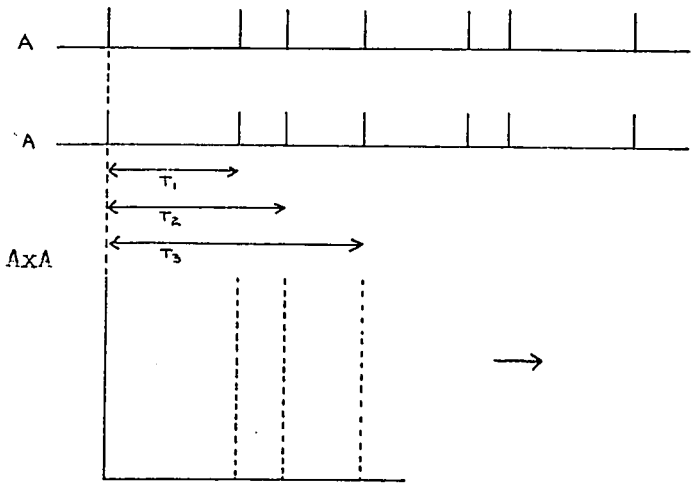
Interpretation of the CCH is dependent on the complexity of the features encountered, and is also semi-quantitative. The null hypothesis for the relationship among the two trains assumes no interaction. As a consequence of this the anticipated CCH is flat (as a first approximation) and the expected number of events per bin may be calculated from the first order statistics of both trains (for more details see Fig 20 or page 156).

Figure 11

a). Mechanism of CCH computation



b). ACH as CCH of spike train with itself



Aside from a few simple cases, there is no rigidly specified algorithmic method for assignment of synaptic relationships to observed cross correlations, but with refinements to the method, ways of distingushing among possible circuits in pairs of neurons may be devised. The use of the CCH interpretation method is illustrated in detail for one specific connection type; distinguishing the result of a strong excitatory synapse directly from one neuron to another from the result of a third neuron forming excitatory synapses on the two neurons from which the ST's are being recorded.

Consider a directionally coupled pair of neurons (Fig (I2)) in which the presynaptic neuron (pre) forms a strong synapse on the postsynaptic cell (post) which is insufficiently strong for a single spike from the presynaptic neuron to elicit a spike in post every time. Furthermore assume that both neurons receive a continuous synaptic barrage which is not correlated between the two cells. The resultant spike trains, when analyzed (computation done from pre to post) will yield a CCH whose most prominent feature is a peak to the right of the origin. The exact shape of this peak is a property of the PSP induced by pre in post (Perkel et al (1967b)), and the distance of the peak from the origin is approximately the conduction time from pre to post.

The mapping of various ACH's of the two cells for a direct connection is illustrated in figure (I2). (Here A represents pre, and B represents post). Whenever cell A fires, the probability of firing in cell B is increased after a time T1

Figure I2

CCH of directly coupled cell pair (both aperiodic cells)

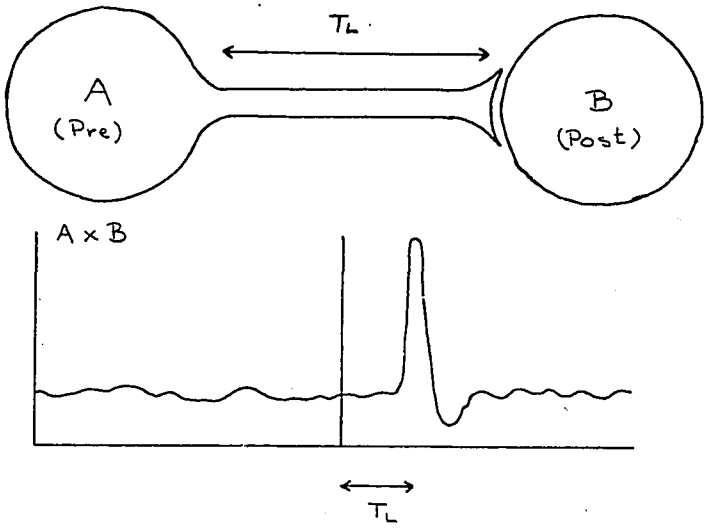


Figure I3

CCH of directly coupled cell pair (periodic postsynaptic cell)

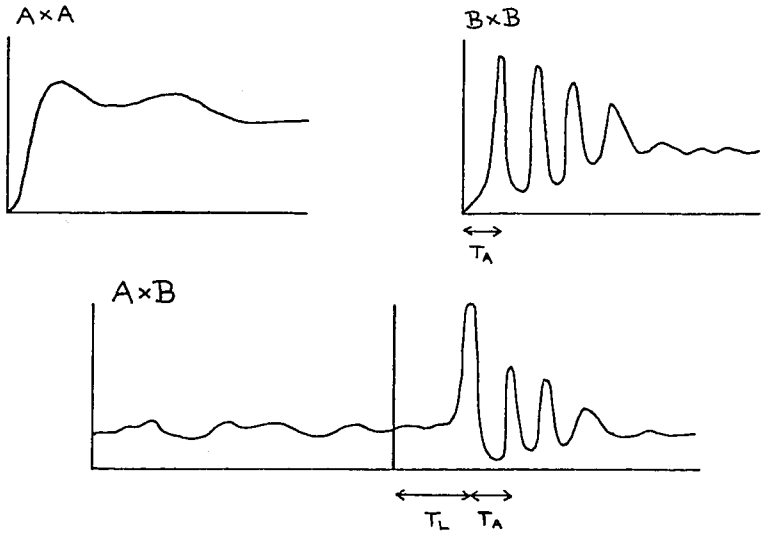
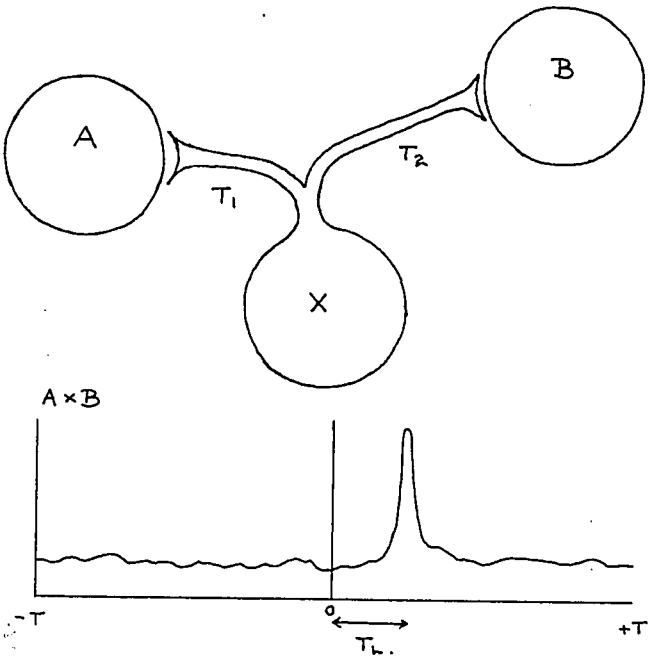
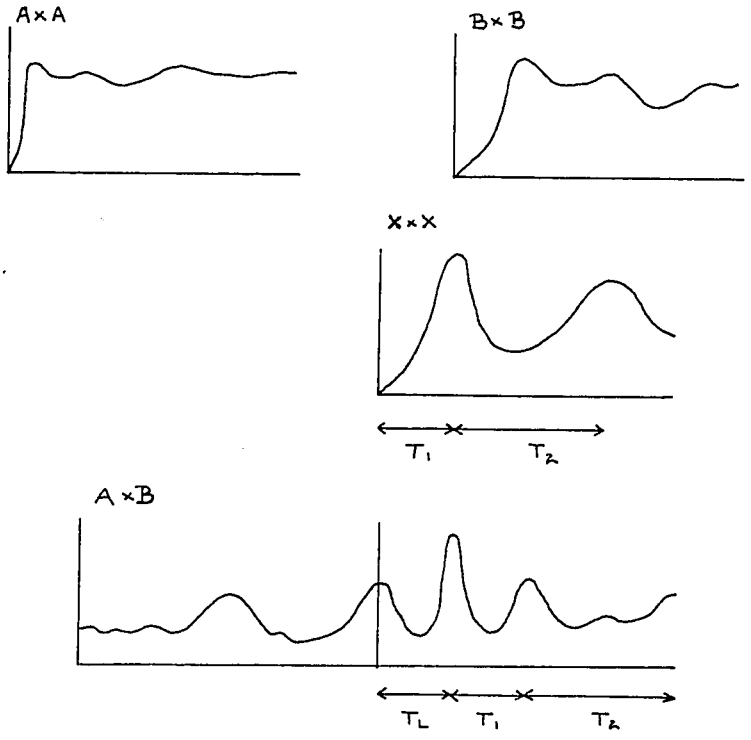


Figure I4
Common excitation to A and B

a). Aperiodic driver (X)



b). A and B aperiodic, X periodic



The ability to eliminate possible interactions by consideration of ACH mappings in the CCH was one of the factors responsible for using two glutamate electrodes, as this allowed the activities of both neurons to be modulated. This did not work out as expected due to lack of specificity of the glu modulation just to the cells being recorded from. While the initial approach was rather naive, the most glaring deficiency was the assumption that pairs of neurons could be viewed as being 'isolated' unless they had a strong synaptic connection between them, in which case only a few possible types of interactions were thought to be possible. The major assumptions from the early approach utilized in the analysis of data from the earliest experiments can be summarized in the list below:

1. Correlations between neuronal spikes are either the result of:

   a) Excitatory or inhibitory synaptic interactions from one neuron directly to the other.

   b) An excitatory or inhibitory synaptic connection directly from a third neuron to both neurons being recorded.

2. The bulk of background synaptic activity in neurons is uncorrelated.

3. In the analysis of data, every spike may be assumed to be equivalent. A corollary of this statement is that spike patterning should not affect the interaction.

(This is not the case, although it is a reasonable approximation in slow firing cells).

Chapter II

METHODS

2.1 CULTURE TECHNIQUES.

All cultures were prepared and nurtured in the laboratory of W. J. Hendelman. Explantation of the cultures was performed under aseptic conditions in a positive pressure hood, and all operations were done manually. Newborn mice were anesthetized with ether, killed by cardiac puncture, the tip of the cranium cut away, and the brains quickly removed. The cerebellum and brainstem were separated from the remainder of the brain, and the resulting piece was trimmed to just include the cerebellum and dorsal portion of the pons. The meninges were carefully removed from the surface of the brain, and then manual saggital sections of the cerebellum were cut with a razor blade. The locations of the sectioning for the medial, central and lateral pieces are shown in diagram (IF1) and have been considered previously in Introduction.

For the BS cultures, the piece which includes the cerebellar peduncle is used, and if a combined cerebellar BS culture is desired, the whole peduncular piece is laid onto a collagen coated cover slip. For the preparation of the BS only cultures, the cerebellum is removed as much as possible manually. It may be seen in figure (IF1) that there appears to be a natural dividing line between the cerebellum and the

brainstem. This may correspond to the foramen of Lushka (W. J. Hendelman, personal communication). The remaining piece of peduncular tissue may be transferred to the cover slip as is, or the slice may be transsected to give medial and lateral halves of which the lateral half has been observed not to give neurons with the cytoarchitectonic characteristics of the BS neurons in most cases (W. J. Hendelman, unpublished results). This distinction is fairly recent, and while the last groups of pure BS cultures were based on this division, it is not known if it was done in the earliest "pure" BS cultures.

Since manual manipulation is carried out for all of the procedures, it is especially likely in the case of the BS explant, because of its small size, that its boundaries will be the most variable. At present this has not been investigated in great detail, although paraffin sections of the explanted region have been prepared and examined.

The explants are then incubated in Maximow chambers (at 35.5 °C (to within 1 C°) utilizing standard techniques (Crain, 1977). No antibiotics are used within the culture medium, and the medium is changed twice weekly under aseptic conditions equivalent to those maintained during the explantation procedure. The cultures are monitored weekly, and details begin to become apparent several days after the explantation. The cultures are not worked on for physiology until after three weeks in vitro. The incubator temperature is maintained

The culture medium is only a partially defined medium, and consists of:

50% Eagle Minimal essential medium (MEM) which is a completely

defined medium. (Gibco, ctalog #12-127)

25% human cord serum (Gibco).

25% chick embryo extract (Gibco).

The glucose concentration of the total medium is 1100 mg%.

Occasionally other media have been used, and for these no analysis is available. The most relevant analysis would be the amino acid composition of the media as both glycine and GABA in the medium have been reported to cause variations in the exciteability of the cultures (Nelson et al, 1977).

Cultures to be worked on are selected by W. J. Hendelman according to morphological criteria, and the exigencies of the experimental situation. The cultures are viewed in his laboratory to establish mutual anatomical frames of reference before they are used for experimentation. Selection criteria have not been uniform during the course of the study due to progressively greater knowledge of the culture system.

2.2 EXPERIMENTAL METHODS

2.2.1 Equipment

2.2.1.1 Chamber

All experiments are carried out within a plexiglass chamber mounted atop of the stage of an inverted microscope. The chamber has a central well in which the cover slip containing the culture is placed and adheres to the bottom by surface tension. BSS aerated with 95% air and 5% CO2 enters the well on the right and leaves on the left. The usual perfusion rate is about 2 ml/min. Temperature of the fluid within the chamber is

monitored by means of a thermocouple in the side of the well. The fluid is heated by means of a heating coil enclosing the BSS containing glass tubing just outside the chamber. The temperature within the chamber is kept to within ±1 C° by means of occasional visual monitoring of the chamber temperature and manual adjustment of heating current via a potentiometer. Also within the well are a reference electrode for the Mentor preamp and a Platinum wire which serves as the ground lead for the iontophoresis units and voltage follower.

The microscope is an inverted type, and the culture is viewed through the bottom of the chamber. During experiments a magnification of 640X is used with oil immersion objective in direct contact with the chamber. Illumination is provided via a lamp and condenser system mounted above the chamber. The microscope objective remains stationary while the chamber (mounted on the microscope stage) is moveable.

2.2.1.2 Micromanipulators

The electrodes are inserted into the culture through the open top of the chamber. One micromanipulator is mounted on the chamber base, whereas the other is mounted on the steel plate on which the microscope is also mounted.

A hydraulic microdrive (Narashige model MO-10) carrying a probe for a voltage follower (model VF-1, WP Instruments) is mounted on the chamber. A rather coarse micromanipulator is attached by a screw directly to the chamber (see MF1). To this manipulator is fastened another micromanipulator which carries

the microdrive. A plexiglass probe holder is attached to the end of the microdrive and holds the electrode at the end of the probe.

This microdrive has sufficient precision for extracellular recording but is not stable enough for intracellular work. As it is attached to the chamber, it moves with it and thus cells recorded from this electrode may be recorded from while the chamber is moved about to locate another cell with the second electrode.

The other microdrive is a stepping type (made by AB-Transvertex) which advances in 4 micra steps. As it is mounted external to the chamber, the electrode retains a constant relationship to the field of view, but not to the culture. Generally this manipulator is set up so that the electrode intersects the culture in the center of the field of view. (Manual controls are available to move the electrode in 3 mutually perpendicular directions in a continuous manner). Because this micromanipulator has the position of the electrode fixed with respect to the chamber, the electrode must be withdrawn before moving the stage.

2.2.1.3 Electrical apparatus

Both electrodes are mounted on probes which consist of a voltage follower close to the electrode. Henceforth the designations E1 and E2 will be applied to the probes associated with the stepping and hydraulic drives respectively (as well as to the neurons recorded with these probes).

E1 has a voltage follower mounted directly on the drive, and has bidirectional connections with the Mentor amplifier (Intracellular Probe System (model N-950). In addition to recording the Mentor amp may also be used for passing currents (pulses or steady) through the recording electrode. The currents may be set using controls on the amplifiers or by applying control voltages to the stimulus input jacks. Also on this drive are plug terminals for the outputs of 4 iontophoresis units and a ground connection for the units. Attachments are made to these terminals using gold plugs attached to fine platinum wires which are then directly inserted into the bath solution or the iontophoresis solutions. It is possible to measure the impedance of the electrode on the probe at any time using a built in measuring circuit.

E2 contains the voltage follower circuitry within the probe also, but the power and output leads are located in an external box. The output of this unit is fed directly into a Grass DC amp (model P18), and its output goes to the oscilloscope and then to the tape recorder(s). This system is only capable of recording. The ground wire for this system consists of a platinum wire immersed in the bath. This has the effect of forcing the Mentor reference electrode to ground also. The effects of this seem to be limited to introducing slightly more noise into the Mentor output as well as rendering the 10 mV calibration on the Mentor inoperative. No other functions on the Mentor are affected.

The primary problem associated with the E2 setup is the AC noise level which has occasionally been as high as 1 mV. Generally, however, it ranges from about 500 V and up. Much of this was previously eliminated by setting the Grass amp high pass filter cutoff at 300 Hz. This made the AC tolerable, but information on slow potentials was totally lost. In the Mentor amplifier 60 Hz interference is less of a problem because a built in notch filter can be used on the output if desired.

This high level of AC interference existing on E2 has been one of the major factors responsible for lack of definite analysis of many early spike records as often it was not possible to adequately separate the spike from the 60 HZ interference. While much thought was initially given to ways to reduce this interference by searching for ground loops etc, the final elimination of the AC on E2 appeared insoluble, and its presence was finally accepted. Near the end of the experiments, a purely fortuitous observation revealed that touching the battery wires and ground simultaneously reduced the amplitude of the 60 Hz component to less than half of its previous value. This may perhaps be accounted for by assuming that the battery, heating coil, and associated metal act as an antenna, and inductively couple the induced AC to the BES flowing through the heating coil with the coil acting like a transformer winding. Another factor which cut down the noise was the successful construction of a 60 Hz notch filter (Using circuit described by Ferris (1974)).

This rather detailed consideration of the noise level problem was done to illustrate the large effect that seemingly trivial problems can have on the results obtained: it seems reasonable to say that the high noise level on E2 led to the introduction of an unavoidable sampling bias towards larger amplitude ap's on one electrode. (While this would not have drastically changed the results, it would have led to a much more complete automated analysis of the data instead of having to rely on visual analysis of the high noise records.)

Two oscilloscopes are employed for data display during experiments. One is a Tektronix (model 5103N-D13) storage scope which has been modified to make outputs available from the amplifiers, and also it has a gate output which gives a + going pulse at the beginning of each sweep which remains high for the duration of the sweep. The other scope is a Tektronix (model 5103N-D12) which is utilized primarily for photography as it has an oscilloscope camera (model PC-24, Nikon Kohden Kogyo Co.) semipermanantly mounted on its face. Both are dual trace scopes, and depending on the amplifier modules used, can display up to 4 signals. The most common arrangement is to use one differential amplifier module which has adjustable high and low pass filters and a dual trace amplifier module with no filtering capacity in the storage scope.

E2 and E1 are fed into the amplifiers of the storage scope, and the amplifier outputs are used as inputs to the tape recorders. The inputs of the other scope are generally taken from the output amplifiers of the tape recorders to allow

monitoring of the recorded signals, and adjustment of the gain whenever overloading occurs.

A variety of tape recorders has been used for data recording. For intracellular recording all data is recorded on an FM recorder, while for extracellular data either the FM recorders or a standard cassette recorder (Sony CF-550A is used). Most of the extracellular records reside on audio cassettes either recorded later from the Phillips FM recorder, or real time.

Two FM recorders are used for data recording. Initially only a Phillips (model ANA-LOG-7) recorder was employed. In the model used only 3 data tracks and one voice track were available. At the most commonly used recording speed of 3 3/4 ips the frequency response using FM recording is 0 - 1250 Hz. The primary disadvantage of this machine is the high cost of the tape cassettes (about $80). The other FM recorder used is a Hewlett Packard (model 3960) which records on conventional 1/4" reel to reel audio tapes. The entire width of the tape is used to record 4 data channels or 3 data and one voice channel. Since a speed of 3 3/4 ips is not available on this machine, all data is recorded at 7 1/2 ips. Only FM recordings are possible and at this speed the frequency response is 0 - 2500 Hz. This machine has the very useful feature of being able to play/record both forwards and backwards, but it also has the disadvantages of only FM recording ability and no erase function.

For extracellular data recording two cassette recorders have been employed. While convenient to use, both machines have

the rather undesirable features (at least for data recording) of having automatic gain control used on the input signals. Thus a large transient spike will decrease the recorded amplitude of the input signal immediately following with the gain then slowly increasing back to its original level with a long time constant (in the range of 30 sec). These features could be tolerated for most extracellular records as no distortions occurred in the interspike intervals, but the amplitude of the spike was distorted in such a manner that it is impractical to attempt to reconstruct the actual size of the spike from cassette data. The frequency response of the Sony recorder model CF-550A was found to be 30 - 18000 Hz which is more than adequate for the extracellular records.

Iontophoresis units (model 160, WP Instruments) are used to deliver eject and retain currents to the iontophoretic barrels of the electrodes. Although they have a range of up to 1000 na, the maximum currents used were generally in the range of 20 na or less. The units may be manually operated, or an external pulse may be used to apply a preset current to the electrode for the duration of the pulse. When this mode of operation was employed, an unacceptable amount of noise was generated by the input pulse edges. This was reduced to a tolerable level by placing a 20 mmf and a 0.1 mmf capacitor in parallel with the pulse input, and a 0.1 mmf capacitor between the output of the iontophoresis unit and ground.

A Grass (model S88) stimulator is employed for the generation of square wave pulses either for intracellular

current injection or to repetatively activate iontophoresis units. For both applications the output of the stimulator is directly connected to the input of the unit being driven, and the voltage set on the stimulator voltage control. As the accuracy of this dial is not very great, the actual value of the voltage is made more precise by monitoring the waveform on an oscilloscope.

For auditory monitoring of the amplified spikes, the outputs of the oscilloscope amplifiers are led to either an audio amplifier/speaker unit (if FM recorder being used), or if cassette recorders are being employed, the pause switch is depressed in the record mode so that the input signals may be listened to. Auditory detection of spikes is very efficient when small signal to noise ratios exist (as when approaching a neuron with the electrode).

A signal averager (Northern model NS-575) is employed either to calculate various frequency histograms (using NS-590 module) or to do averaging of signals (using NS-588 correlation module). Generally the unit is used offline.

2.2.1.4 Microelectrodes

Single barrelled microelectrodes are made by heating a piece of 1.5mm O.D. glass tubing in a microelectrode puller to give two single barrelled electrodes. Tip size and taper are controlled by varying the pull strength and heating coil current. Trial and error is used to find optimal heating and pull parameters, and for a given heating coil shape the values

are reproducible. All microelectrodes are examined under a microscope, and selected microelectrodes are filled either by boiling them in EtOH at reduced pressure, or by direct injection of the filling solution. For extracellular recording the electrodes are filled with 3M NaCl solution while for intracellular recordings 2 M K citrate is used.

Double barrelled electrodes are pulled from blanks consisting of two fused pieces of 3 mm O.D. glass tubing. Filling is accomplished by boiling in water, and then introduction of the required filling solutions by first withdrawing all of the water in the electrode except that quantity which is in the thin part, and then refilling with the required solution. The electrodes are left overnight to allow for diffusion of the ions throughout the water in the tip area. In the electrodes employed 3M NaCl was used for the recording barrel, and generally 0.2 M Na glutamate solution was used in the other barrel. In several experiments 0.2 M homocysteic acid (HCA) was used in place of glutamate.

Electrodes are chosen for use by first inspecting the tip under a microscope and measurement of the impedance. If the impedance is too high, the electrode tips are broken by gently touching the electrode tip to a glass surface under microscopic control. Generally electrodes used for extracellular recording had impedances of 10 - 30 M while the intracellular electrodes, due to their smaller tip diameter had impedances of 20 - 40 M . Double barrelled electrodes were given the additional test of observing the noise increase produced by iontophoretic currents

in the range of 0 - 20 na. This would separate out the blocked electrodes from the good ones before they were inserted into a culture.

2.2.2 Experimental types

2.2.2.1 Extracellular experiments

Four possible electrode combinations may be used for extracellular recording, although the majority of extracellular records were made with two double barrelled glutamate electrodes.

Spontaneous activity was recorded using two single barrelled electrodes, but was very infrequently employed due to the low level of spontaneous activity present in BS neurons, and also the difficulty of locating cells without first damaging them or killing them. Some of the extracellular records were obtained using one single barrelled electrode and one double barrelled iontophoresis electrode. This configuration was utilized in the first experiments, and additional records of this nature were obtained during intracellular experiments when spontaneously active neurons were encountered on the intracellular electrode during a penetration of the culture.

The majority of extracellular records were obtained using two double barrelled electrodes. Several advantages result from the use of this method; cells are easily located, and the nature of possible synaptic connections between a pair of cells can be clarified by the use of different glu curents to see if the expected firing rate changes occur when the permutations of cell excitation are applied.

In order to locate a pair of neurons the BS area of the explant is first located, and examined to determine if the orientation is favorable for unencumbered electrode penetrations. Then one electrode (always E2 initially) is used to locate a neuron. An attempt is made to sample the culture randomly, subject to the constraint that the location chosen not interfere with the entry of the other electrode into the explant.

The electrode is moved slowly into the selected area of the culture with a few na of eject current being passed on the glu barrel. The loudspeaker is closely monitored for the presence of spikes, and when the firing of a neuron is heard the motion of the electrode is terminated. Then attention is shifted to visual monitoring of the spikes, and first it is ascertained if the spikes are of an adequate size for unambiguous discrimination. If the spikes are too small, the electrode is moved backwards and forwards very slowly in an attempt to increase the spike size. Should this procedure fail, then usually another cell is sought unless interesting activity is observed in the cell. If this is the case, then a photographic record would allow for spike separation, or the electrode could be withdrawn, and another track made after displacing the electrode slightly in another direction relative to the track. Theoretically this should allow for the finding of a more suitable recording location for the cell, but only in a very thin culture could one be reasonably sure that the same cell was being recorded from.

Once a spike having a satisfactory signal to noise ratio is obtained, a decision on whether or not to use it is made. If the cell appears to be damaged (excessively long duration spike), or if two or more spikes of similar amplitude are present on the electrode, the record is of little use. Also it is necessary to insure that the activity of the cell does not result from mechanical stimulation of the neuronal membrane by the electrode. This may usually be ascertained initially by shutting off the eject current, and if the cell is seen to quickly cease to fire, or at least slow down then it is most likely that the electrode is not pressing on the cell. It is easy to rule out the effects of gross physical effects on the cell since they are so obvious, and the cells usually do not survive long after such electrode contacts. It is also possible for the mechanical effects to be more subtle, and only manifest themselves when vibratory motions of sufficient magnitude to change briefly the firing pattern of the neuron are imparted to the electrode by external shock waves. This problem is not likely to be significant in the majority of the experiments because of the measures taken to reduce transmission of vibrations through the microscope-chamber-electrode assembly.

Once a satisfactory cell is found on E2, essentially the same procedure is repeated with E1, except that the continuing presence of E2 is checked periodically. When a satisfactory cell is also obtained on E1, then portions of the activity are recorded, and various combinations of iontophoresis currents in the two electrodes are tested to reveal if any interaction is

present, or if one was initially seen to be present, to see the effect of the different iontophoresis conditions on the interaction. Not all activity is recorded; usually iontophoresis conditions are adjusted until a stable mode of activity seems to result, and then a recording is made. The length of the tape record is a function of how much data would be required to show up any interaction, the stability of the cell pair and how much tape is left. For each pair of cells an attempt is made to record activity with the four possible permutations of iontophoresis conditions. The levels of current used are kept low as high currents give excessively regular firing and cause very fast decrease in spike size of many of the cells. Low currents are used when it is desired to see what type of synaptic input is occurring to the cell. So many variables are involved in making this type of a decision that it was not studied quantitatively.

The length of a recording made for a pair of cells depends on the type of activity exhibited by them. If both fire in an irregular manner, then this is taken as an indication of abundant synaptic input, and long recordings under a variety of iontophoresis conditions are made. If an interaction is obvious while recording from the pair, manipulation of the glu current levels on both cells is carried out in such a manner that the resulting records may allow a decision to be made of whether the interaction is consistent with a direct anatomical connection, or something else. In the case of two regularly firing neurons, very long records would be required to show up any relationship

(to allow for the detection of temporary periods of phase locking which would occur with two uncorrelated cells firing at different but regular rates). Such cases are usually played with by manipulation of the iontophoresis conditions until a change in firing patterns is observed.

It is obvious from the description of the method employed that a sampling bias is present. First, the neurons which are recorded from are all glutamate sensitive, have spikes larger than the noise level of the electrode on which they are recorded, and fire tonically upon glutamate application. Thus if there are any neurons in the culture which are glu insensitive, glu inhibited, are very small, or are phasically firing they will not be recorded from. If they are recorded they would be assumed to be damaged cells, cells which have been lost, or some explanation which is consistent with the current mental model of the cultures. The proportion of such neurons in the cultures is likely not large, but there is no way of estimating their number using the neuron finding method which was employed. In some of the last experiments a number of neurons with phasic glu responses, or glu inhibitions which could be repeatedly persuaded to repeat their "anomalous" response were encountered. No systematic study was undertaken because they refused to fire fast enough to obtain sufficient data for a rigorous study of interactions with other neurons.

2.2.2.2 Intracellular experiments

All intracellular experiments were done with one electrode (E1) intracellular and E2 extracellular. The procedures for finding the extracellular cell are essentially the same as described previously, although the interest criterion of the E2 spike train is relaxed, and cells which can be depended on to fire when an intracellular penetration occurs are selected even if they fire regularly.

Once a cell is located with E2, the intracellular electrode is inserted into a selected cell. This is accomplished by choosing the cell, and adjusting the position of the stage so that the electrode tip will enter the selected cell roughly in the center of the nucleus (the nucleolus is a convenient aiming point). This method was found to produce the most stable intracellular records presumably because the tip was in the soma.

Because of the short length of most of the intracellular records, recording is begun as soon as a large potential shift is observed during the advance of the intracellular electrode. Depending on the decay of the membrane potential immediatly after penetration, the electrode may be advanced or backed off a step or two in an effort to stabilize the potential.

If the impaled neuron is firing rapidly, it is hyperpolarized to a level sufficient to stop spike generation by current injection through the electrode. This is done so that the extracellular spike may be used as a trigger for the averaging of synaptic noise in the intracellular record giving a

record uncontaminated by ap's. When this operation is done, after withdrawal of the intracellular electrode to a point just outside the cell a control record is made with E2 firing to eliminate the possibility that any averaged events encountered are field potentials from the E2 cell. It would have been interesting to study correlations between the intracellular and extracellular spike trains, but the short length of the records required a choice to be quickly made of whether spike to spike interactions were to be studied, or E2 spike/E1 synaptic activity correlations. The latter was almost always the case.

During intracellular recording experiments as many visible cells as possible were impaled until the electrode became blocked. The extracellular electrode was moved every few pairs, or when the cell on E2 was lost.

Intracellular recordings are restricted to more superficial cells, and thus thinner cultures are selected for these experiments. Because of the large degree of visual control possible in selection of cells, it is easy to restrict the cells impaled to those readily identifiable as typical BS neurons with granules.

During most of the intracellular experiments the membrane potentials of the cells were monitored on a chart recorder at a low speed. This was done so that an independent calculation could be made for the membrane potential of a neuron aside from the one made from the tape. Although calibration signals were recorded on at least one tape for a given intracellular experiment, they often yielded anomalous results due to changes

in tip potential which often occurred close to penetration. The estimates of initial membrane potential were computed from the voltage difference on leaving the cell from the membrane potential present just on entry to the cell. In some cases it was obvious that there were changes in the tip potential between these two times as unreasonable values of Em were computed. A system to give more accurate results would be to record each attempt at penetration so that records would be obtained of the electrode tip potential changes as it approached the cell.

2.3 DATA ANALYSIS METHODS

2.3.1 Extracellular data

2.3.1.1 General overview

The primary aim of the data analysis was to determine if activity of a pair of neurons was correlated or not and thus most of the data consisted of tape or film records of neuron pairs. Not all cell pairs recorded from were filmed or taped, and of those which were only the ones that seemed correlated had a note made about them. Also single cell records were occasionally made of interesting activity. Of these only those from one culture were systematically analyzed to look at the effects of bicuculline on spike patterns. The data for correlated cell pairs with no actual ST records, single cell records etc was not systematically analyzed, but was used mainly for the purposes of settling ambiguous results for cell pairs in the same culture. In theory it should be possible to sort cell pairs neatly into correlated and non-correlated categories, but

it was discovered that a large ambiguous category resulted due to various deficiencies in the data or analysis.

To obtain quantitative data on the numbers of correlated pairs vs the number of noncorrelated pairs, it was necessary to set up categories to select the subset of records which could in theory be analyzed with sufficient thoroughness to classify them as interacting/noninteracting. Since a variety of analysis methods was utilized, it was also necessary to quantify the confidence in the result. Because of changes in the concept of what constitutes an exhaustive analysis, data between early and late experiments may not be directly comparable in a few cases. The method detailed below was applied uniformly to all cell pairs to have a uniform basis for comparison of the correlations.

Tape records are sorted into good and bad categories. The records put in the bad categories are those in which there is excessive noise on the tape, excessive amount of electrical artifacts or clear indication of mechanical stimulation on one of the neurons. These are not considered further for the purposes of assessing interactions.

The good quality records are next sorted by the amount of data they contain. This decision is a rather rough one, but it is based on the principle that the amount of data required to make a decision about the correlation of activity in two trains of spikes is a function of the nature of the activity type in each ST (Perkel et al, 1967b). The records are sorted into categories of insufficient data and sufficient data to make a

decision. This puts many of the very short records, or those pairs in which one or both cells are very slowly firing, two pacemakers etc. in the insufficient data category. In a number of cases the quantity of data from a pair considering only the film or tape record may be inadequate, but other observations made on one or both cells during the experiment may result in the interaction being considered reasonably likely. These pairs are counted among the interactions, and the remaining pairs constitute the insufficient data category.

Every record has been visually inspected at least once (some of the cell pairs that just vaguely seem to have an interaction have been looked at many times), and a number of interactions are evident at this level. However visual analysis is quite qualitative and is most efficient for certain types of interaction detection such as correlated bursting. With adequate feedback from those records on which a CCH analysis was done, more types of interactions can be recognized. In the aspect of finding correlated patterns in the data, visual and auditory monitoring is the most efficient of any of the methods used. However a detailed calculation of the degree of certainty associated with the postulated interaction is of great assistance in decreasing the degree of scepticism in the conclusion among those who are less intimately acquainted with the raw data. A mathematical rationalization of visual burst analysis has been performed and may be located in the mathematical appendix. Similar reasoning could be used for other types of pattern correlation.

Many of the records were limited to a purely visual analysis as a consequence of the inability of the spike discrimination hardware to identify spikes from one or both neurons with the requisite degree of fidelity. Of the records that can be computer analyzed, not all have been done so either because of the very likely possibility that there was no correlation in the data or the presence of 60 Hz interference too large to allow one spike to be adequately discriminated at the time the major EDT preparation for computer analysis was done. (No 60 Hz notch filter).

In an attempt to make the results of visual analysis more valid, all records having presumed interactions were first ranked by degree of confidence in the interaction. Then all of the less likely pairs were examined again on a different day with a very critical frame of mind to determine whether the initial impression was subjectively replicable. If it was not, then these records were assigned to the no interaction group. Since it is likely that some of the records visually analyzed may be loosely correlated, the records from the visual analysis which still remained borderline were placed in the interaction category in the hope that the number of misclassified records would be very small, and that maybe they would balance. (Actually, there are probably more records misclassified in the no interaction group as those cells which exhibited correlated bursting were identifiable virtually every time, but if no bursting was exhibited and if a subtle interaction was occuring it may have been missed visually).

Some of the records were entirely on film, and initially manual measurement of the intervals was attempted. The cumulative errors in the trains quickly became excessive, and the job of data entry to the computer via cards and the data checking were most laborious. Some of the records have had their CCH's computed by using a piece of graph paper and aligning the center mark with the first spike in the train being used, and then marking in the squares below when spikes occur in the other train. This method is also labor intensive, and requires extreme concentration to maintain accuracy, but allowed for a number of the pairs to be examined. Depending on the rate of firing of the cells, the CCH from the film may be accumulated at a rate of anywhere between 30 to 100 spikes/hour.

Of the records which were not run through the whole spectrum of computer tests, it has been possible in several cases to do CCH estimates using the averager and some simple external logic. The time involved in analyzing each pair is far too large for the method to be applied to any but borderline cases which are found by the visual analysis as it is also required to compute IIH's for each of the cells used, and all of the data has to be manually retrieved from the averager memory.

Of the records which were computer analyzed, a large number of possible methods were available to test for interaction. Not all were used for a given cell pair if the CCH showed unmistakable evidence of an interaction. For those CCH's which were almost flat, or flat but elevated or depressed below the expected level, a detailed investigation of individual spike to

spike interactions making up the CCH as well as pattern triggered CCH approximations was done. Also the raw data were again consulted to insure that the patterns appearing at this detailed level of analysis were not artifacts. If no interaction were found as a result of this, or if insufficient data were present to justify running some of the tests, the cells were placed into the no interaction category.

The total number of interactions was computed by summing all of the numbers in the various categories of analysis. Cell pairs with no interaction were also combined. The category of insufficient data was left as is. All of the data was then sorted by culture type, and was the basis on which the type of activity was contrasted with the culture type. Because only good data was used, and descriptions of interactions in the experimental notes were used only if they survived a critical reapraisal, some cultures were completely eliminated.

2.3.1.2 Tape documentation

Raw data tapes are referred to as Analog Data Tapes (ADT). Following an experiment, tape documentation is carried out in which cell pair record parameters, experimental conditions, and readily visible changes in neural activity are recorded. At this time each cell pair is assigned an Absolute Pair Number (APN) which is of the form (XX-YY-ZZ) where: XX represents the number of the culture, YY is number of cell on E1, and ZZ is sequential number of cell on E2.

2.3.1.3 Computer data analysis

(i). EDT Preparation.

Selected ADT records are first formed into Encoded Data Tapes (EDT) prior to computer analysis. The tapes used for the EDT's are 60 minute Maxell cassette tapes on which two channels of uniform amplitude pulses (representing the encoded spikes from the two electrodes), as well as record separational information is encoded for input to the terminal. The use of Maxell tapes has given the best results with the tape recorder used as their frequency response and output are superior to a number of other brands which were tested.

To make the EDT, the ADT output of the selected record is passed through two amplitude discriminators whose output is fed into the Analog Tape Interface (ATI). This circuit produces the appropriate (+) going pulses which are recorded on the EDT for each input spike as well as simultaneous pulses on each channel at the beginning and end of each record and the (-) going pulses of the tonebursts recorded on the EDT for aural identification of records. Voice data giving EDT number and side number is placed at the beginning of each EDT. Full circuit details may be found in the terminal appendix.

Before an EDT is made of a record, it is thoroughly inspected on a storage scope. If different iontophoresis conditions, or noise are present in the ADT record, then the record is divided into sections so that each segment represents the same applied glu currents and is free of noise. When the divisions have been decided upon, the segment of ADT record is

again carefully inspected on a storage scope to determine the minimum trigger level to get all spikes free of noise should be and the optimum degree of filtering (if it is required). Outputs of the discriminators (for both neurons) are used to trigger fast sweeps of the scope to insure that all spikes are triggering pulses and that only one cell ap is being selected. The average duration of the spike as obtained by this method is used to set the ATI monostable multivibrator (MMV) periods so that multiple triggerings of the discriminator within a spike will not result in multiple pulses being recorded on the EDT.

Once it has been satisfactorally established that all spikes in both cells will be faithfully discriminated, the EDT record is made. The ADT is first rewound to a point slightly before the starting point of the desired tape segment, and the tape recorder making the EDT is started recording. At this time the ATI data inputs are disabled. A short tone burst of (-) going pulses is recorded on both channels of the EDT and the ADT recorder is then started. When the desired segment starts coming through on the ADT, the ATI enable switch is pressed and a stopwatch is started. The enable signal first causes simultaneous (+) going spikes to be written on the two channels of the EDT, and then allows the pulses from the discriminators to pass through the unit and be written as (+) spikes on the tape. When the endpoint of the ADT data segment is reached, (actually very close to the end of the segment) the disable switch on the ATI is activated concurrently with the stopping of the stopwatch. This operation interrupts the flow of data from

the discriminators, and puts two simultaneous pulses at the end of the record. After about 5 sec the ATI tone switch is again briefly activated to signal the end of a record. The stopwatch time is recorded and is one of the crude checks used in the examination of the data once it is on disk in the 360.

The records which are made for the EDT's have the tape counter location of the ADT segment used, absolute pair number of the cell pair, spike shape and amplitude, filtering used on the ADT output, trigger levels on discriminators, ATI MMV periods, and the total record time (stopwatch time) noted. Each EDT record is assigned its own unique identification number which is of the form; (EDT tape #, serial location of record on tape). Also a note is kept of which ADT channel goes into which EDT channel.

(ii). Data Transmission.

The data recorded on the EDT's is an exact replica of the interspike intervals in both spike trains, and when fed into the playback section of the ATI will yield a 1 - 2 microsec (+) TTL level pulse for every (+) going pulse on the tape. This is put directly into the input of the terminal.

Data entry to the 360 is a complicated affair which begins with the physical translocation of the terminal to the third floor of the physics building (loc of the computing center) as carefully as possible due to the extremely temperamental nature of this device. Once the terminal and its power supplies have been successfully relocated within the cramped confines of the room containing the Microdata computer and its associated

peripherals, a quick check of the status LED's on the terminal is carried out. If everything appears to be in order, the serial output lead of the terminal is connected to a plug from the Microdata via a pair of alligator leads, and secured by tape. A small amount of data is read into the Microdata from the terminal to see if transmission is in order.

When the condition of the terminal has been discovered to be satisfactory, the terminal is placed in a non transmit mode, and the Microdata program to receive the data is started. While in this mode, the Microdata receives all input data from the terminal and stores it on disc until the flipping of a switch on its control panel lets it know that it can stop. The EDT playback is then started and when the first toneburst is heard, the terminal timers are enabled by pressing a switch. This operation also sends a special code (called interrupt code) before any of the interval data is transmitted. This code is interpreted by the Microdata as the start of a record. Within this code is sent the binary number encoded in the settings of the 8 data switches of the terminal control panel. This code is used to send the number of the record. Data transmission rate from the terminal to Microdata is at 2400 baud.

Timing of the interspike intervals continues until a second tone burst is heard, at which time the terminal timers are manually stopped. Then it is necessary to wait for the next tone burst, and start the timers again for the next record. A stopwatch is also used to recheck the record times during data transmission.

When the last record has been played through the terminal, the data receive portion of the Microdata program is terminated. Then the amount of data inputted from the terminal can be ascertained, and selected portions of the data, or all of it in sequence can be viewed on a TV monitor or a hard copy made on a nearby high speed printer. Illegal codes within the data are flagged by asterisks in the printout of the data, but it is far easier to check the data once it is on disk on the 360. (All Microdata programs written by Mark Pepin, U of O computing center).

Before transmission to the 360 is done, the data in the Microdata disk is sorted into two long files (one for each SP) and reconverted to numerical form. The interrupt codes are changed to negative numbers and remain in the data as record delimiters. The two files are then sent to the 360 from the Microdata via a high speed data link between the two machines. An assembler program running concurrently in the 360 (this program also written by Marc Pepin) receives the data and writes it into two disk files, and prints how much data it has received when transmission stops. This data is now in a form which may be accessed by standard Fortran programs, and the appropriate analysis carried out.

(iii). Computer Data Analysis.

Once the interspike interval data is on the 360 disk, it is manipulated by a variety of program packages, but the format of the data remains identical. Only a cursory description of each program package is given here.

The first package goes through the data in each file seeking out the negative numbers to find the record boundaries. This information is then used to create a new disk file (CTALOG) which contains the address information for each record as well as its sum. After the program is finished, it prints out the CTALOG file as well as a numeric listing of each record. To test for fidelity of transmission, first the sums of each record in CTALOG listing are compared, and if the discrepancy is very large, the numerical listing of the cell pair is examined to see if the source of error can be found. The first and last intervals in each ST record should be equal and simultaneous, and serve as a rough check of fidelity of data transmission. In practice this is used as the primary check because although theoretically there should be an equal number of clock periods in both of the ST's, there may be a difference of several hundred, and the record may be verified as being accurately transmitted. Careful examination of the ST's in several cases where this has occurred revealed that the trains had not been shifted by the magnitude of the discrepancy, and that part of it may be accounted for by short intervals from the tone burst causing unequal times to be transmitted in the end of the record. (This is the part of the record which contains no spike interval data, so the errors are irrelevant if they occur here). When the terminal was first being tested, extensive manual comparison of the interspike intervals obtained using the terminal, and by playing the EDT into the averager were done. These tests show that the terminal can be counted on to either

transmit data perfectly or when it malfunctions, it is always in such a bizarre manner that its lack of proper operation is immediatly obvious.

Once the CTALOG file has been created, it is used by all program packages to find the required records in the data files. The first program to use it is a simultaneous spike train display routine (STDISP) which prints out each pair of records in an analog form (time to length conversion) with the two trains one above the other. Each line is divided into 100 intervals each representing an equal interval of time which is specified in the input to the program. The number of spikes in each train during that particular interval is printed. Depending on the interval time chosen, the display either looks like the two trains viewed on a storage scope, or a simultaneous moving average of the trains. For a first look at the data generally a line time of 2.5 sec is used. This is adequate to show how each ST firing pattern changes with time and how the two ST's interact. This may serve as a crude test of stationarity although it is more suited to the visualization of interactions.

At some time during the analysis procedure, the CTALOG file is modified so that it contains additional information about each pair of ST's. This data is not essential to the analysis, but it was noticed that it was being entered so often that it could be a part of the CTALOG file and be printed out with every analysis done.

Once data has been cataloged and checked it is analyzed. A standard program package allows for the calculation of CCH's

both of a whole record or any given time segment of the record. While it is not mathematically correct to analyse the whole record, (as there may be nonstationarities in the data), it does serve to separate those records in which the same interaction occurs throughout the record from those in which the interaction may vary.

To test for stationarity, a qualitative estimate may be obtained from STDISP output, or a moving average technique is applied to the data using a stationarity test program. This program goes through both ST's and calculates the number of spikes which occur in an interval (user specified) and prints two histograms concurrently for the whole duration of the trains. Thus, one has a visual record of the stability of the average of each ST, and it may also be determined if gross rate changes are shared by the two cells. From an inspection of this output, the times for segmentation of the trains are determined.

The standard analysis program computes both single ST and dual ST parameters. Segmentation data may be inputted for each cell pair, but, once the segmentation times have been established, they are added to CTALOG and automatically entered to save on keypunching. For each ST segment, the interspike interval histogram (IIH), autocorrelation histogram (ACH) and cross interval histogram (XIH) are computed and displayed as well as the mean and standard deviation for each ST. The CCH is also computed and displayed as well as a count of the actual number of events in the forward and backward portions of the CCH and the expected number of events calculated from the means of

the two ST's and the bin width. In order to facilitate comparisons of CCH's and ACH's for different segments of the same pair, all are computed at the same bin width.

Interpretation of the CCH is carried out visually, and the CCH may be immediatly classified as showing significant features, maybe showing something, or essentially flat. The bin width used depends on the expected latency of any possible interaction, and in the case of peaks confined to a single bin, a shorter bin width is used to recalculate the CCH to see if the feature persists in several bins and insure that it is not an artifact (Bryant et al, 1973). Stability of an interaction may be tested by segmentation of a record, and comparison of the resulting CCH's to see what features, if any change from one CCH to another.

The greatest problems of interpretation arise when the observed CCH does not have a simple form, but nevertheless has features which are definitely not flat. With these particular cells it is wished only to attempt to show that there is an interaction or not, rather than determine what the interaction is. There are a variety of ways to go about this, and several of them are used.

One way is to shuffle both of the ST's and to recompute the CCH for the shuffled case. Then by subtracting the shuffled CCH from the actual CCH one may obtain a difference CCH which may be compared to the original. No quantitative conclusions are possible, but what is sought is the persistence of features from the original CCH in the difference CCH. Also significant is the

degree which the expected number of events/bin differs from the actual number/bin in the control CCH as compared to the original.

Another method which was utilized to check the data was to calculate the recurrence times for each spike of one of the ST's (generally this was done for the ST with the least number of spikes). Then, this was converted into a display of the positions of the spikes in the other ST around each spike for the ST in which the recurrence times were calculated. By examining this printout it was possible to determine if the interaction among the two ST's changed, and also if there were points where correlation occurred.

The last method which was employed was the construction of what was referred to as a Triggered Cross Interval Histogram (TXIH). This was a two dimensional decomposition of a CCH (as explained more fully in Fig 20), and consisted of the normal XIH for one cell of the pair and another XIH of the other cell in which the number of spikes occurring within a specified time limit of each triggering spike pattern in the other cell was recorded.

The above methods were concerned with determining the presence and nature of interactions among two ST's, but it is also possible to determine something of the synaptic input to a neuron by examing its ST alone. The ACH provides an indication of whether or not any recurring pattern exists within the intervals. Since no method of quantitating ACH's was known, they were classified into several categories with the categories

being determined by the type of ACH's encountered up to that time: ie if a completely new form came up that would not easily fit, then it was put into a category by itself. The XIH was also used to see what the distribution of interval pairs was, especially in the same cell when recorded with other cells in a different record, or between various segments of a record.

The exact sequence of the tests carried out on a given cell pair would really be a function of its interaction, and the order dependence properties of its constituent ST's. For some of the records it was necessary to write new analysis programs in order to demonstrate what was occurring. This was particularly true of those records in which a correlation could be clearly seen in the raw data, but was of a phasic nature, and was lost in the noise created by the remainder of the activity which was non correlated.

2.3.2 Intracellular data

All analysis of intracellular data was performed manually with the exception of averaging for presumed PSP's. Documentaion was carried out as for extracellular data with the same system of APN's being used. The only differences were that intracellular events checked for were periods of spike generation, and periods of non- spike firing where the records were suitable for averaging.

2.3.2.1 Common data analysis

For each cell recorded intracellularly the amplitude of the membrane potential and spike amplitude were used to classify the data. Cells having an Em of greater or equal to 40 Mv or an ap 40 mv were put into the category of "good" intracellular recordings. Cells which did not meet these criteria were placed in a second catergory, but not totally rejected. In order for a cell to be classified as good it also had to produce an action potential, either spontaneously on impalement, or with depolarizing current injection. Action potential amplitudes were measured, and the result expressed in the form of an amplitude above resting Em, and the amplitude of the afterhyperpolarization was also measured if possible. Also for each ap the duration of the ap, and the approximate duration of the AHP were also measured. This was done only for a few of the initial ap's in the record and did not take into account changes in ap parameters with deterioration.

Intracellular recordings in which low membrane potentials existed were analyzed visually, and if correlations were seen they were noted, and treated as extracellular data obtained from damaged cells if the correlation appeared to be between the spike like potentials observed. All of these recordings are labelled as "quasi intracellular" due to the ambiguities which could arise from the interpretation of some of the data. Other cells in which the Em was not as badly deteriorated were treated like intracellular data from the good cells if interactions were observed, but the potentials were subjected to close scrutiny to insure that they were what they really appeared to be.

The properties of the extracellular cell which was present with each intracellular cell were qualitatively studied, and its glutamate response as well as firing pattern with each intracellular cell noted.

2.3.2.2 Total data analysis

(I). Spike to spike correlations.

Since the purpose of the intracellular experiments was not primarily to obtain more dual spike train data, automated analysis of the two ST's was not done. (in any case most of them would have been too short for analysis). Since there was a possibility of interaction, each intra/extra pair was visually analyzed, first on a storage scope at about 0.5 sec/div to see if there were any long time scale interactions among the spikes, and then on a short time scale triggering on each spike in turn to look for short latency interactions.

(II). PSP Analysis.

This was done in the most detail for each cell pair. Almost all of the intracellular cells recorded were hyperpolarized soon after impalement to stop ap generation, and it was this interval which was examined in detail. Only a visual analysis was used, and this was done on a storage scope using 0.1 or 0.2 sec/div sweep speed to look at the high gain AC for short synaptic potentials and the DC for longer potentials. A rough estimate of the PSP types and durations was obtained in this manner, and correlations were sought of PSP input and E2 cell activity. When correlations were observed, that portion of

the record was filmed, or more often the required section of the record was retaped on another FM recorder at 30 ips, and a chart record of the E2 cell with either the DC or AC intra (whichever showed the correlation) was made with a playback speed of 1 7/8 or 15/16 ips to give a maximum slowdown of 16/1 over the original. This was usually adequate to demonstrate the interactions with minimal distortion of the potentials. (Not as good as a film record, but much easier to store and handle).

To look for evoked PSP's from the E2 cell, averaging of the AC coupled intracellular record triggered by the E2 spikes was used. Only those sections of the intracellular record having no intracellular spikes, and a relatively stable DC baseline were used for averaging. Obviously for averaging it is desirable to employ as large a number of sweeps as possible, but constraints are imposed by the length of the record, the firing rate of the E2 cell, and the length of the extracellular control period. To facilitate comparisons of the averages, first the maximum number of averages possible in the control period was determined and then as many such averages as possible were computed during the intracellular records where this was possible. Each average was generally computed in 256 bins using a bin width of 50 to 100 micro sec and finally recorded on a chart recorder by operating the averager output at slow sweep. Thus each average and the control were directly comparable, and a serial record of the averaged potential with respect to time since penetration existed for the cell.

Although not all records had extracellular controls, averages were computed for all cells wherever it was possible. The assumption underlying this was that since not one control had shown the existence of a field potential from the extracellular spike, there was a low probability of the other averaged data being simply a field potential. Also, the averaged intracellular data is a form of cross correlation, (Kirkwood and Sears, 1978) and could be informative. In order to carry out this average both a foward and backward average triggering on the spike were done by playing the tape forward and backwards.

Chapter III

RESULTS

3.1 EXTRACELLULAR RESULTS

3.1.1 General observations

3.1.1.1 Spike parameters

Most of the extracellular recordings attained were of a single cell, although occasionally two (and very rarely three) cells were recorded on the same electrode. The most commonly observed spike waveforms were biphasic initially positive going spikes and a triphasic (initial and final small positive peaks) with a large negative peak. Other more complex forms were less often observed. Amplitudes of the spikes ranged from 100 - 200 mmV to as much as 5-10 mv, and were generally constant in each cell except when cell deterioration or electrode movement occurred.

Spike duration was quite variable ranging from less that 1 ms to as much as 20 ms. Generally it was observed that undamaged cells had relatively short duration spikes with fast rise and fall times. Damaged cells had long duration spikes which were often seen to get progressively smaller and longer as the cell deteriorated.

The exact nature of the spike parameters was not recorded in detail for each cell, except the duration which was measured to establish the minimum allowable interspike interval in the

computer analyzed data. Also the spike parameters were more closely checked when there were possibilities of more than one spike being present. While some information regarding the possible synaptic input to the cell may be obtained from an analysis of the spike amplitude and duration, this was very seldom done as the spike train (ST) of a cell was considered as a series of point processes except when decremental bursts occurred.

3.1.1.2 Spontaneous activity

Spontaneous activity of the cells was not studied systematically because of its complete absence or very low occurrence in most cells. Occasionally, faster rates of spontaneous activity were seen, but they tended to be phasic.

Activity observed on glutamate (glu) electrodes with retain current on is not herein classified as spontaneous because there is still a possibility of glu leakage, or of current effects. Thus the only occasions during which spontaneous activity was seen was in early experiments where single barrelled electrodes were used, and during intracellular experiments where spontaneous extracellular activity was sometimes recorded on the advancing intracellular electrode.

While a more detailed knowledge of the spontaneous activity would be useful in seeing what the normal interactions were in the cultures, its excessively low rate, and the extreme difficulty of finding cells without causing initial mechanical damage by the electrode made it impractical to study except when

it arose in a coincidental manner. Even this activity could not really be characterized as spontaneous if the other electrode in the culture was releasing glu.

3.1.1.3 Glutamate response

In all experiments done, at least one iontophoretic electrode was employed to increase the rate of activity of the neurons. A large majority of BS neurons were found to have their firing rate increased by glu, and this was an efficient way of locating neurons without damaging them, and then activating them and modulating their activity. Initially it was believed that the glu response was localized to the region of the neuron being activated, but phenomena observed when two glu electrodes were employed made it apparent that this is not always the case. Thus the effects of glu will first be considered on the cell from which the recording is being made, and then the effects on a distant cell which are possibly a direct effect of the glu will be considered.

A. Effect on cell at glu electrode.

While the response to glu is rather variable, most cells in the BS region are activated by glu. There are a small number of neurons which respond to glu in an atypical manner, but their small number and difficulty of study makes it difficult to decide exactly what type of phenomenon their activity represents.

The sensitivity of glu responsive cells is quite variable using the same iontophoretic electrode. This is to be expected

as the distance from the glu electrode tip to the glu senstive membrane of of the cell being recorded may be quite variable. When two glu electrodes were employed, it was occasionally possible to activate one neuron with both electrodes when the electrodes were separated by the whole width of the BS area. However, about as often it was not possible to activate a neuron on one electrode from the other iontophoretic electrode even when the two electrodes appeared to be very close together.

There is generally a threshold glutamate current which must be exceeded before the cell begins to fire. This is most obvious in cells which are completely silent when the glu is off. The i glu required to exceed the threshold is quite variable, with the most sensitive cells beginning to fire when the retain current is decreased to 0 while the less sensitive ones require high values of eject current. Most often fairly low values of eject currents were used (0-4 na) with up to 10 na for some cells.

The glu induced activity was proportional to the i glu, but the exact nature of the activity was dependent on the cell. It has been demonstrated (Denavit-Saubie and Champagnat, 1978) that the effect of glu on a cell is dependent on the type of synaptic inputs being received by a cell at the time the glu is applied. Thus the observed activity of a cell would be a function of the glu induced depolarization and the input to the cell. Figure 1 shows the IIH of a neuron at three different glu current levels, and demonstrates an increase in rate and regularity with the higher currents. This can be taken as

fairly typical of most BS neuron glu responses, except that this cell was somewhat more glu sensitive than most.

Another phenomenon which was often seen was that of decreased spike size in a glu stimulated cell. It was possible to induce this effect in all cells with high glu currents, but the maximum firing frequency and rate of decrease of spike size were functions of the cell involved. This process presumably reflects excess depolarization of the neuron by the glu. In the cells in which this occurred readily it was difficult to obtain sufficiently long records with the spike large enough to be discriminable, and hence these may be classified as non correlated due to insufficient data.

An adaptation to glu is seen in some cells with the firing frequency decreasing with time for a given current. Wide variations in the rate of adaptation are seen with some cells exhibiting no observable adaptation while cells such as the one in Fig 2 have a reproducible adaptation response (at least over a short term). This effect was considered when records were analyzed because it represents a nonstationarity. There were some records of neurons which responded atypically to glu application. These have been divided here into categories of phasic and glu inhibited cells. It is possible that the cells classified as phasic glu responders may simply represent an extreme case of glu adaptation, but the examples described are sufficiently different from the glu adaptation commonly observed that a separate category was felt to be warranted.

Figure 1
Influence of glu current on neuron rate

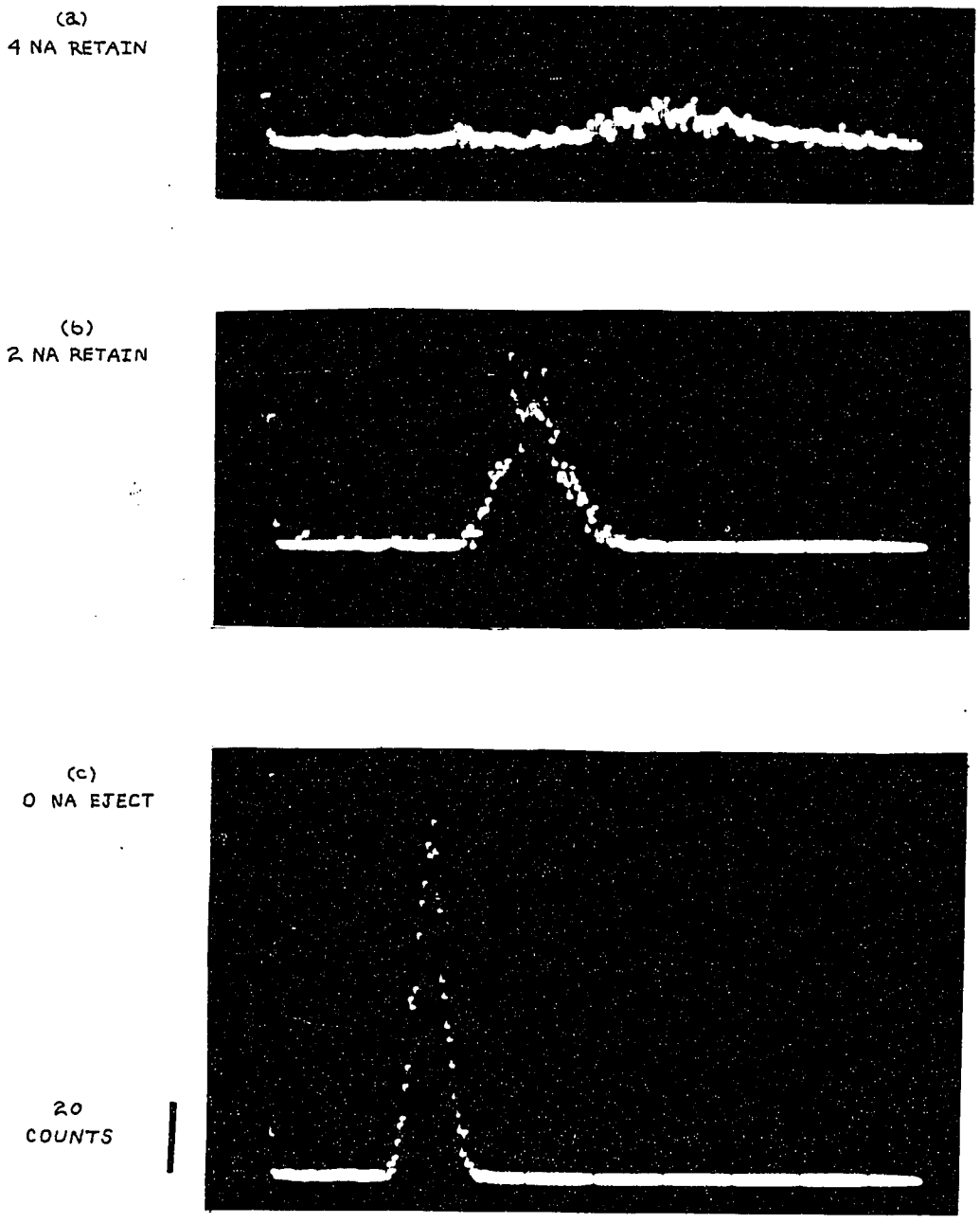
Interspike interval histograms for three different values of glu current on a neuron are illustrated. Vertical bar represents 20 counts, and horizontal line represents 250 ms. Bin width of 2 ms used for all histograms.

a). 4 na retain current. Activity primarily slow and irregular, although small proportion of shorter intervals present as indicated by small peak around 180 ms.

b). 2 na retain current. Rate of activity faster and also more regular than in (a).

c). 0 na eject current. Fast firing and very regular.

Figure 1



Neurons responding phasically to glu are the most common of the atypical glu responders and also the most frustrating to study. These neurons respond to glu application with a brief interval of firing immediatly following, or a variable time after glu application. Then they cease to fire and do not fire again until the glu is switched to retain for a while, and then turned on once again. Often they can be induced to fire no more than one burst every few minutes and thus periods of few hours would be required to gather sufficient data to check for signs of interaction with other neurons.

The proportion of these neurons in the culture cannot be estimated because the sampling method employed tends to miss them, and when they were first seen the lack of maintained activity was interpreted as loss of the cell and another cell was sought. Three of these cells have been investigated in detail. Two of these were located in culture 19, and should not strictly be classified as phasic responders since they were not recorded on the glu electrode. They did, however, consistently fire a one to two second long burst of spikes when the glu on the other electrode was turned on. One of these was investigated by applying glu from various parts of the culture, and applications in several widely separated areas were found to give activation of this neuron. In each case there was an interaction of some type with the cell on the T2 electrode, but it was not readily classifiable. This interaction is illustrated in Fig. 17. Of the more conventional phasic glu responders (from culture 20), one was consistently activated

Figure 2
Glutamate adaptation response

All 4 records represent the response of a single cell on E1 to glu iontophoretically applied from its electrode. In all cases illustrated here an eject current of 2 na was used. Counts represent number of spikes occurring in E1 cell in successive 1 second intervals.

Glu was switched from retain to eject on E1 at the initiation of each record (arrows), and the value of the glu current on E2 was constant for the whole record. Different cells were present on the E2 electrode for each adaptation response illustrated, as well as different eject currents on these cells.

Not shown were cases where no apparent adaptation response occurred on E1, but whenever this was seen, the rate of E1 was very low, and thus adaptation may not have been readily percieved.

a). Pair (17-1-2) with E2 on retain.

b). Pair (17-1-4) with E2 on 9 na eject.

c). Pair (17-1-5) with E2 on 4 na eject.

d). Pair (17-1-6) with E2 on 4 na eject.

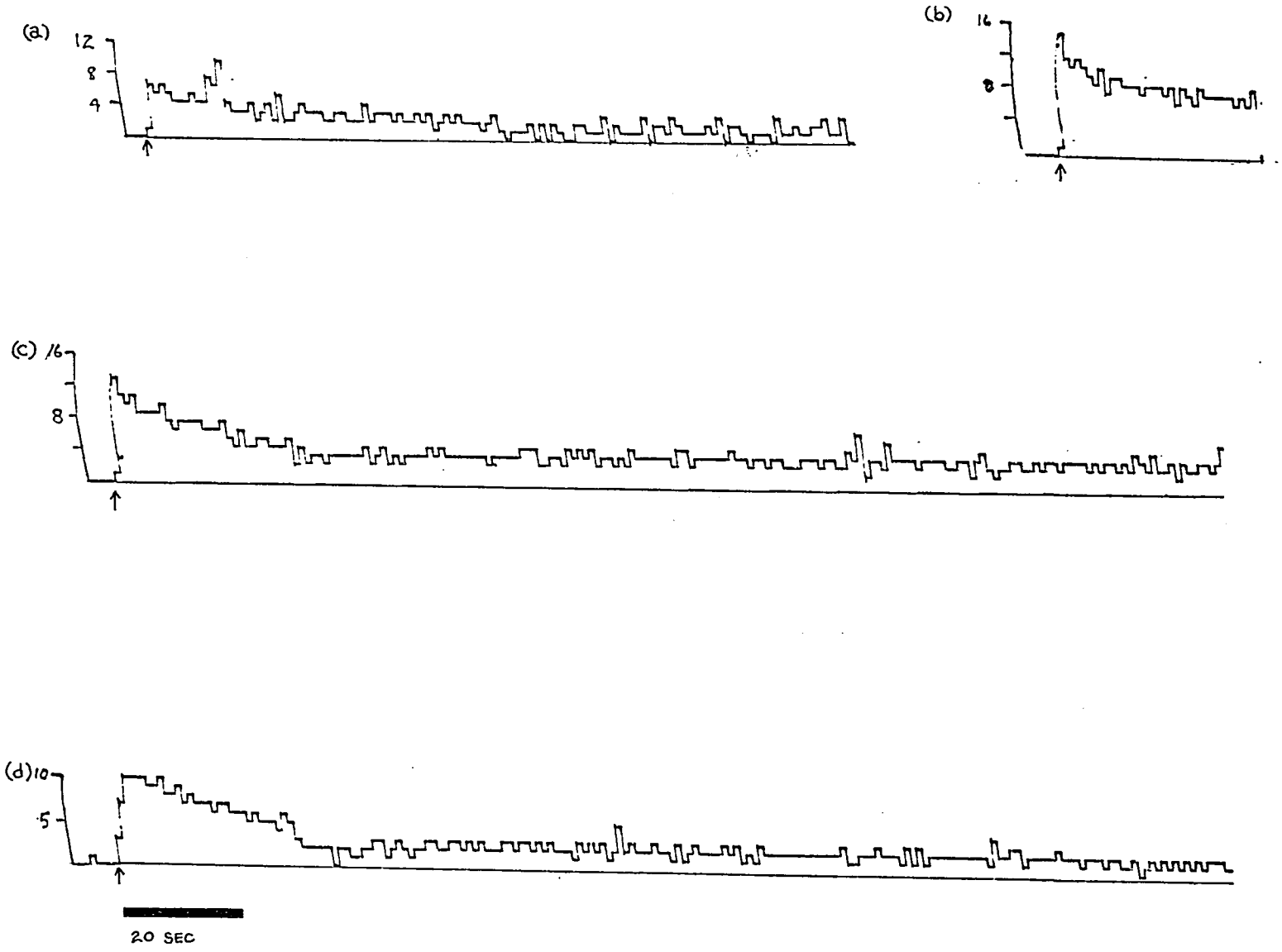


Figure 2

with glu pulses for which it would fire four spikes every time. In contrast to the two neurons in culture 19, no correlation was observed with the other cell being recorded. Another phasically responding neuron in culture 20 was the E2 cell for intracellular pair (20-7-4) (shown in Fig. 24) Bursts were consistently observed in this cell concommitant with large EPSP's in E1. Because at least some of these resulted from glu application, it seems likely that the phasic glu responses may be synaptically mediated.

Cells inhibited by glu were the most rare of the atypical glu responders, and only one was studied in such a manner that the glu inhibition could be clearly seen. Fig 3 demonstrates the firing rate of this neuron in response to putting glu on eject or retain. There is a consistent decrease in firing rate when glu is initially applied, but there does appear to be a gradual return to the original firing rate. Maximum activity occurs with 4na retain current. One complicating factor in the interpretation of this effect is that this neuron is one of the type which exhibits large spontaneous rate changes, and the points marked ? in the figure indicate where the firing rate rather abruptly goes from 0 to about 20/sec with no change in iontophoresis conditions. However, sometime later, turning up the glu current again stopped the firing.

II. Longer Range Glu Effects.

In many cell pairs it has been observed that variations in the glu current at one electrode produce effects on the firing rate of the other cell. This may be the result of synaptic

interaction, but some cases may be due to a direct effect of the glu from both electrodes on one cell. This type of interaction is to be expected on anatomical grounds as HRP results have shown that BS neurons have a wide dendritic field which may often extend to distant parts of the culture. Thus a fortuitous positioning of the electrodes could result in two parts of a neuron being stimulated from distant locations. An interaction observed under such conditions could be direct effect of the glu stimulation.

There is evidence to indicate that this type of interaction occurs, but it would be difficult to exclude the possibility that an increase in the rate of one neuron being produced by glu ejection on both iontophoretic electrodes was not instead due to activation of a population of excitatory neurons being activated by the distant electrode and causing the rate increase. Two pairs of neurons in culture 11 exhibited this type of interaction. Because of the inability to decide among the possibilities, they are classified as enigmatic interactions.

A synergistic effect is also occasionally seen with two glu electrodes. Application of glu to one neuron at a time produces no activity in the other neuron, and moderately regular activity in the stimulated neuron. Essentially the same, but opposite effect occurs when glu is applied only to the other neuron. When identical glu currents are applied to both neurons simultaneously, a very different pattern of activity results in the two cells. This takes the form of correlated bursting or of shared periods of firing and non firing in the cell pair. This

Figure 3
Glutamate inhibition

Record represents continuous count of number of spikes occurring in cell in successive 1 second bins.

1). Start record (4 na retain i on).

2). 4 na glu switched on. Note immediate rate reduction.

3). I glu reduced to 0 na eject.

4). Glu switched to 4 na retain - immediate rate increase.

5). Continuation of record from one above.

6). 0 na eject current put on.

7). These spikes represent storage scope screen erase electrical artifacts, and occur throughout the record as two closely occurring spikes approximately every 2 seconds. May also be seen later in record when cell ceases to fire again.

8). Glu eject current increased to 4 na.

9). Glu on retain.

10) Glu put on 4 na eject.

?) Spontaneous rate change.

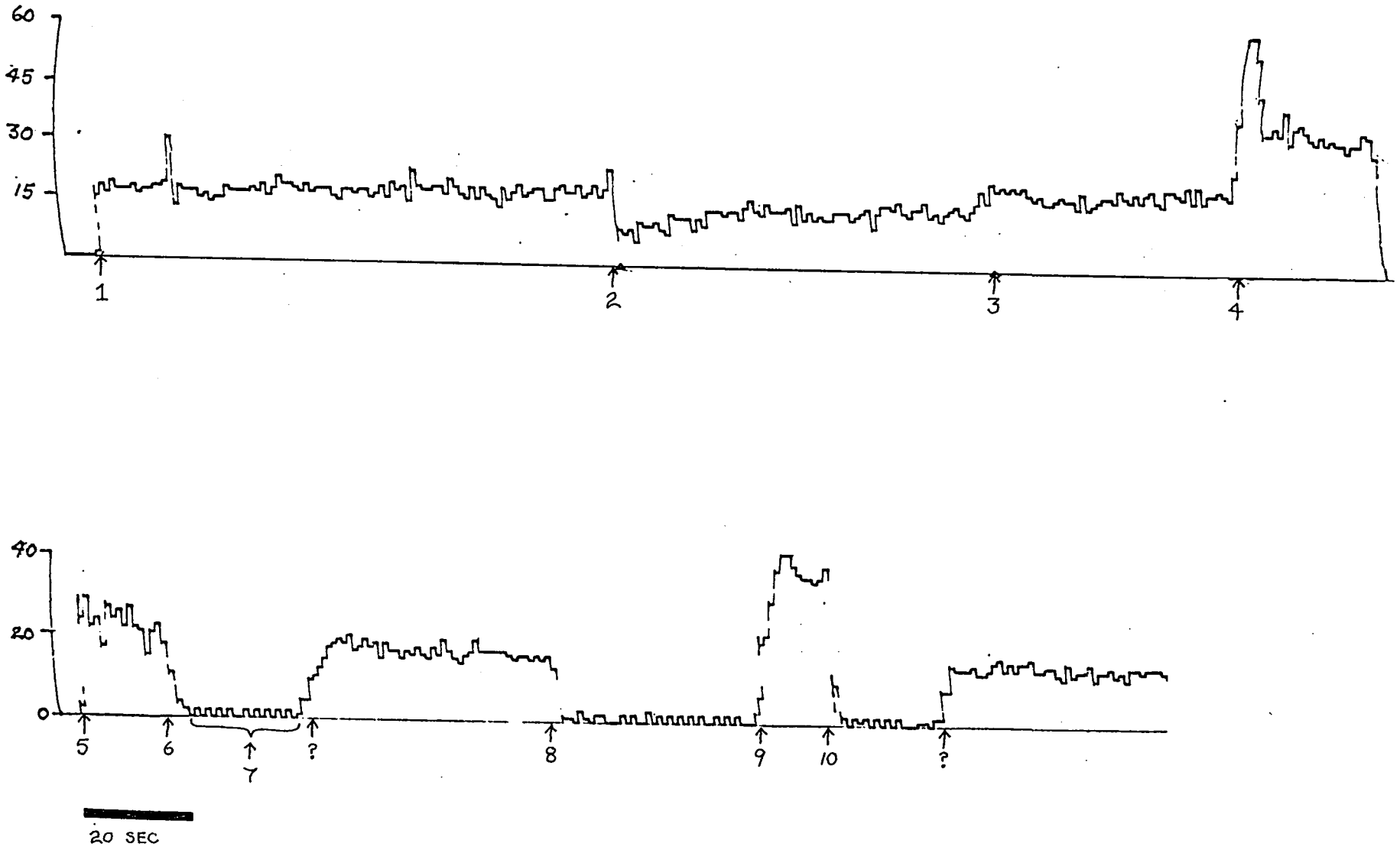


Figure 3

is especially obvious in some cell pairs, and in many other cases fine adjustment of iontophoresis currents can produce synchronized activity.

3.1.2 Interactions

For the purposes of data classification, the following categories of major interactions have been designated:

1. Directional excitation (spike to spike) (DE)
2. Common bursting (CB).
    a) Frequent or very obvious CB.
    b) Occasional or less obvious CB.
    c) Common firing
3. Miscellaneous interactions.
    a) Alternate firing (AF).
    b) Common inhibition (CI).
    c) Enigmatic interactions (EI).

The category of enigmatic interactions was created as there still remained a few pairs of neurons with definite but weird correlations at the termination of data classification.

Decision of what is a major interaction in a pair of neurons is qualitative, and if other interactions are present, they are not generally mentioned. Evidence is present for a

significant fraction of cell pairs with multiple interactions, but only in cultures in which intracellular records were also obtained has it been possible to be reasonably certain about the probable existence of a presumed multiple interaction. Thus they are described individually later. Within the extracellular data multiple explanations were usually possible for the observed activity. Without correlative intracellular evidence about mode of intracellular genesis of various spike patterns, only the most clear interaction was considered in the tabulation of interactions . Also the lability of the interaction is generally not given as it proved to be too difficult to quantitate this variable. If it is given, it is only to illustrate specific cases.

3.1.2.1 Ordering of spikes in spike trains.

Many useful inferences may be made about the synaptic input a neuron is receiving by examining the ST for signs of order. On the basis of extracellular data alone such results must be interpreted with caution as a number of different explanations may be formulated to account for the observed activity. However the occurrence of a common pattern in many cells could be taken as evidence of a similar synaptic input and not many intracellular recordings would be required to see what was causing that pattern.

Bursts were the most widely observed pattern in all of the cultures. Their predilection to occur simultaneously in pairs of cells made them very easy to see or hear. A variety of forms

could be distinguished of which the decremental burst is the most stereotyped and easiest to see. This burst form consists of closely spaced spikes with the amplitude of each spike decreasing as a function of the serial position of the spike within the burst. During the decremental burst, the firing rate of the neuron was maximal for that cell. (See Fig. 8 for examples of bursts). Also, while bursts varied in duration, rate and number of spikes, for a given neuron the burst configuration was generally stable, often to the point of the bursts being superimposable.

There is a strong similarity between the decremental burst observed in the BS neurons and climbing fiber responses (CFR) occurring in PN's of the cerebellum. In the cerebellum it is a very strong depolarization which is the underlying factor producing the complex spike. In BS neurons it is likely that a very strong depolarization also is the factor responsible for the burst, although it seems to be due to the activation of a number of synapses simultaneously, but not necessarily as the result of a single axon termination.

In the analysis of simultaneously recorded Purkinje cells from cerebellum, (Bell and Grimm, 1969, Bell and Kawasaki, 1972) the stereotyped nature of the CFR allowed each ST to be decomposed into two sets of point events; the CFR and the simple spikes. This allows for a less ambiguous analysis of the interactions among these point events. The same rationale should apply in the analysis of BS culture data, but its validity is contingent on the demonstration of the burst to be

an identifiable event which is most likely in the case of the decremental burst.

The strongly decremental burst seems to be one extreme of a continuum of steadily increasing excitation impingent on a neuron (which happpens to be correlated among many cells in the BS cultures). With other patterns the increase in firing is not as pronounced as in the decremental burst, and the spike size may not decrease at all. These are more difficult to pick out visually in the presence of other activity.

Thus there are two components making up the spike train of the majority of BS neurons; the individually occurring spikes, and the bursts (those that are reasonably decremental so that they can be seen). Both of these can be treated as point processes in the analysis of correlation between two trains. One unfortunate quality of the most strongly decremental bursts is that although their rapid decrease in spike size makes for excellent visual identification, often the smaller spikes are below the threshold of the discriminator, and it has not been possible to develop an algorithm which will unambiguosly identify them in the computer data except in a select few cases.

Simple spikes may also be patterned. The simplest pattern is that of a pacemaker cell in which the successive intervals are approximately the same. A cell exhibiting this pattern of firing is either receiving no synaptic input (if it is completely glutamate driven), or if it spontaneously firing it could be receiving very many non correlated synaptic inputs which would have the same effect as a steady depolarization

(Segundo and Perkel, 1967). Since spontaneously active pacemakers were never seen in BS regions, pacemaker cells were considered to be neurons which were driven primarily by the glutamate.

The description of other spike patterns is less easy, due to differences in terminology among various workers. For example, the pattern of a group of spikes with short, irregular spike separations within the group and delimited from another group by a long interspike interval has been referred to simply as a burst by Calvet and Lepault (1975), and Schlapfer et al (1972). These two groups of investigators made no distinction of whether the patterns were regular or irregular, and it is easy to see differences among their published data. Similar burst patterns to those observed by Calvet and Lepault were seen in several BS neurons (correlated in pair (23-1-1) as shown in Fig 7 Since such a pattern could be the result of either a periodic common excitation or inhibition, more detailed burst description is necessary for the purposes of deciding among the possibilities.

3.1.2.2 Computer analyzed data

A standard analysis was performed on all computer analyzed records, and it was then determined whether the resulting CCH represented an interaction. Where satisfactory evidence existed that the interaction was real, then an attempt was made to determine the cause of the interaction.

3.1.2.3 CCH classification

A first look at the CCH's will see them fall into 3 categories.

1. Those in which an interaction is visually quite obvious, and at least two bins wide in the CCH. Every such case was the result of a real correlation.

2. CCH's in which either there appears to be a complex pattern of some type, or a uniformly elevated or depressed baseline (with respect to the computed control CCH for that pair).

3. CCH's which are essentially flat. Not considered further.

The CCH's of the first category can be divided into primary features which are directly caused by the interaction and secondary features which are the result of periodicities in neurons (not necessarily those recorded from; Perkel et al, 1967b) and these may sometimes completely obscure the primary features (Bryant et al, 1973). The secondary features are used to infer the presence of other neurons in the interaction by comparing them with the ACH's of both of the neurons used in the construction of the CCH. The primary effect is estimated from a display of the raw data (computer generated, and if this is insufficient, the original data is looked at again).

Correlated cell pairs could be divided into frequent interactions, in which the interaction occurred often throughout the record, and occasional interactions, in which only certain

parts of the record had a correlation. These could not be shown in the conventional CCH and in order to illustrate the interaction it was necessary to use the pattern triggered cross interval histogram (TXIH) or selection of only certain patterns from the trains to use for calculation of the cross correlation. These will be dealt with later. The obvious cross correlations could be the result of an interaction which was either spike to spike or spike group to spike group.

All CCH figures are displayed in a standard format. The direction of the CCH computation is from A to B and is symbolized by AxB. The autocorrelations of the two cells are displayed below the CCH with the train A ACH (AxA) on the left and train B ACH (BxB) on the right. Unless indicated in text legend, all time calibrations are in ms.

For the CCH of each pair of neurons a set of statistical data is provided. The explanation of the parameters is as follows:

NA = number of intervals in train A.

NB = number of intervals in train B.

AMEAN, BMEAN indicate, respectively, the mean interval duration in each of the trains in seconds.

PA, PB are mean firing rates for cells A and B in spikes/sec.

TIME = total duration of record in sec.

BX = bin width used in calculation of CCH and ACH.

EVAL = expected value of mean number of counts/bin, and is computed by:

$$EVAL = NA*BX*PB$$

NF, NB represent the total number of events in forwards and

backwards sections of CCH.

MEANF, MEANB are the mean number of events/bin in forward and backward directions.

No such statistical data compilation was performed on the ACH's of the cells.

3.1.2.4 Directional interactions

The pattern in all of the directional interactions was that of a spike in one train being followed by a spike in another train with a certain latency. Fig 4 illustrates a case of such an interaction where the latency from B to A is about 35 ms. Neither ST is periodic as is visible from the ACH's and no periodicities are apparent in the CCH. Another 2 cases of such directional spike to spike interactions were encountered in the computer analyzed data and 2 more were discovered by manual or averager analysis. The basic data for all these cells is summarized in table 1

In three pairs of cells (pair (2-3-3) analyzed twice) the data proved amenable to CCH computation using the signal averager. All of these cases showed a directional correlation from one cell to the other. It must be noted that the histogram obtained is only an approximation to the CCH as not all spikes are used in the computation. This approximation was felt to be reasonable when the interaction latency was short, and the spike separation in the triggering train was of the same order as the total duration of the computed histogram. Also it was necessary

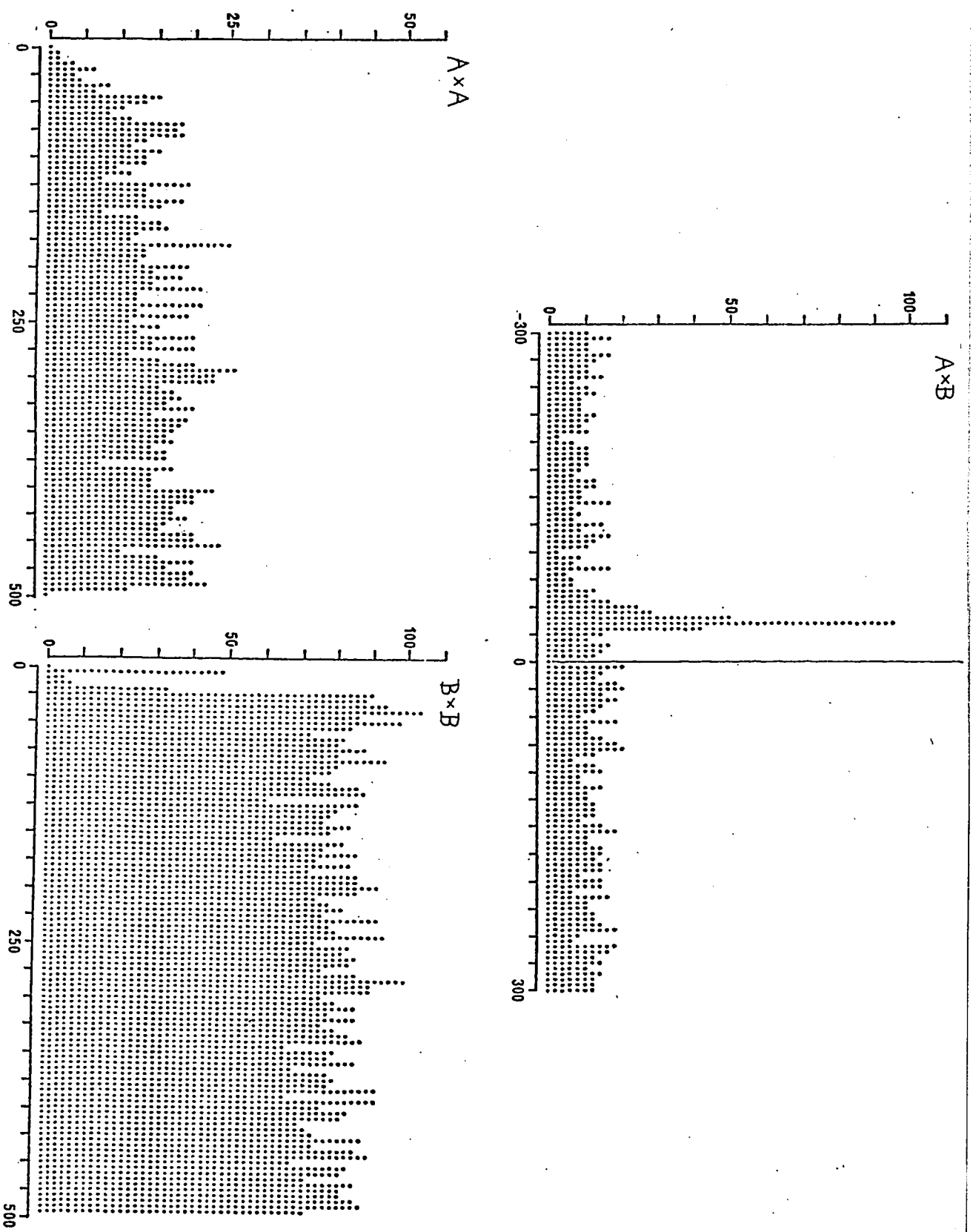
Figure 4
Computer analyzed directional excitation

This histogram computed on whole computer analyzed record for pair (3-2-1). Within this record, E1 rate increased greatly twice (spontaneouly) and interaction was not present, or very weak during these times. Strength of modulation given in Table 1 is based on the highly correlated segments.

NA = 845 NB = 1223

AMEAN=	1.2773	PA=	0.7829	TIME=	88.1324
BMEAN=	0.3799	PB=	2.6324	BX=	0.0050
EVAL=	0.9081				
NF=	1269	NB=	1250		
MEANF=	12.6900	MEANB=	12.5000		
SDF=	3.5908	SDB=	10.5825		

Figure 4



that not many spikes with very short time separations occurred within the train triggering the averager sweep. All these conditions were satisfied in the pairs of cells used.

Figures 5 -7 show the CCH approximations obtained from the three pairs of cells used. In all of them the relationship appears to be reasonably directional. The expected number of events per bin was computed from the IIH and mean rate of each of the ST's using the methods described on page 96.

In order to see how many of the cell pairs could have been due to a direct anatomical connection each will be considered in some detail, and the effects of the glutamate pointed out.

Pair (2-3-3), which is illustrated in Fig. 5 was the most tightly coupled of all the DR pairs encountered. There was virtually no activity in the two neurons except for single spikes on E1 which were always followed by a spike on E2. When the glutamate was turned up slightly on E2, occasional spikes occurred not associated with the prior occurrence of an E1 spike, but the activity of E1 also increased. At one point in the record where both cells were firing faster, a burst occurred in E2 (the first of the record) which was followed by one in E1 with no spikes in E2. Insufficient data was available to assess the significance of this.

Pair (3-2-1), which is shown in Fig 4, was unusual in that the interaction between the two neurons was strongly dependant upon the rate of E1. When E1 was firing slowly, a fairly strong modulation of the activity of E2 was observed with an E2 spike

occurring within 30 ms of the B1 spike. The activity on B1 was independent of the glu current on B2 and the interaction was maximal for low glu currents. With higher values of glu current, correlated bursting occurred on B2 and here the burst in B2 seemed to precede the one in B1 (This portion of the record was not computer analyzed because the higher glu current caused the spike size of B2 to decrease, and also evoked activity in a second neuron on the electrode which could not be distinguished from the decreased size original B2 spike by simple amplitude discrimination. CB's occurred in all three cells).

At the constant level of glu current where the interaction was maximal, the B1 cell was phasic. Generally the cell would fire slowly, and the interaction among the two neurons occurred. Then the rate of B1 would abruptly increase with little change in the rate of B2, but the interaction was no longer present. This is not what would be expected of a direct excitatory synapse from B1 to B2. Visual observation, as well as segmentation of the record and computation of the CCH's confirmed the reversability of this change in interaction. One interesting aspect of the interaction is that at one point where the glu was turned down so that B2 was not firing, when B1 was slowly firing, no interaction was observed between the cells (B2 didn't fire at all) until the glu was turned up slightly, and then the interaction was again present.

Of the other interactions, both pairs (3-4-1) and (23-2-1) had records which dealt with only one set of glu currents, and

Figure 5
Very tightly coupled directional excitation

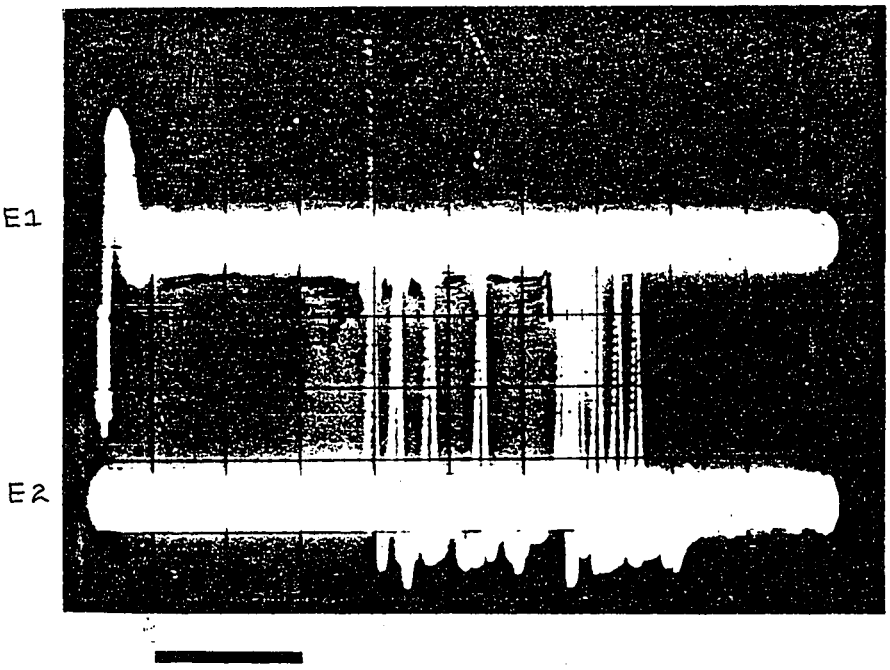
This data from pair (2-3-3). F1 top and F2 bottom.

a). Superimposed sweeps with F1 triggering. F1 spikes + down and 500 microvolt per division. F2 + up and same voltage scale. This record made with 2 na of glu current on F2, and from initial part of record. Latency from F1 to F2 decreases in later part of record. Calibration bar represents 10 ms.

b). Histogram of latencies from F1 spike to F2 spike. For section of record compiled to give this histogram, every F1 spike was followed by a spike in F2, although latency became shorter near end of record. This histogram is equivalent to a CCH from F1 to F2 as F1 fired at most once/second. To the left of the origin there are no events in the range 0 - 500 ms. Occasionally a second spike occurred on F2 following a spike on F1, but its minimum latency was 100 ms, and it would thus have no effect on the shape of the illustrated histogram. Glu current was kept constant.

Figure 5

(a)



(b)

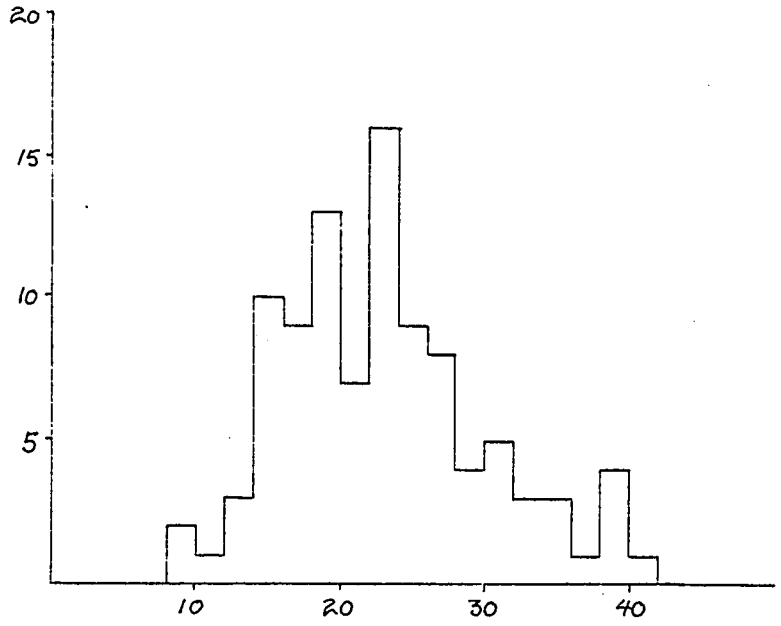


Figure 6
Directional excitation CCH's for pairs (23-1-1) and (23-2-1)

a). Pair (23-1-1) CCH approximation. Since interaction was so prominent, no expected value was calculated. Direction is E2 to E1. Statistical data:

Forward sweeps = 335 Backwards sweeps = 334

Forward events = 195 Backward events = 79

Time scale for both (a) and (b) is in ms.

b). Pair (23-2-1) CCH approximation. Direction is E2 to E1. Since interaction was weaker, EVAL was calculated. For estimates of PA and PB, interspike interval histograms of E1 (A) and E2 (B) whole records were used.

(i). Calculation of PA, PB

NA = 367 NB = 500

TA = 55.5sec TB = 54.5 sec

PA = 6.67/sec PB = 9.17/sec

(ii). Calculation of expected value/bin.

Forward sweeps = 231 Backward sweeps = 230

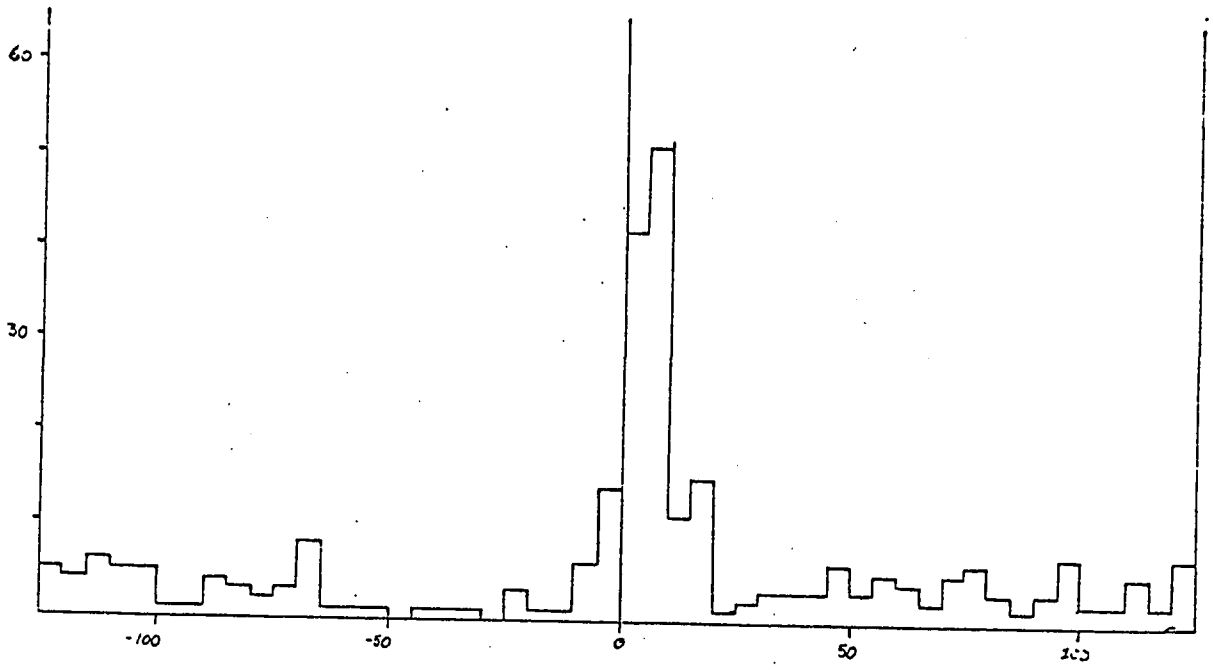
Forward events = 254 Backward events = 274

for a bin width of 5 ms, EVAL = 7.7

height of major peak (at 32 counts) is well in excess of EVAL.

Figure 6

(a)



(b)

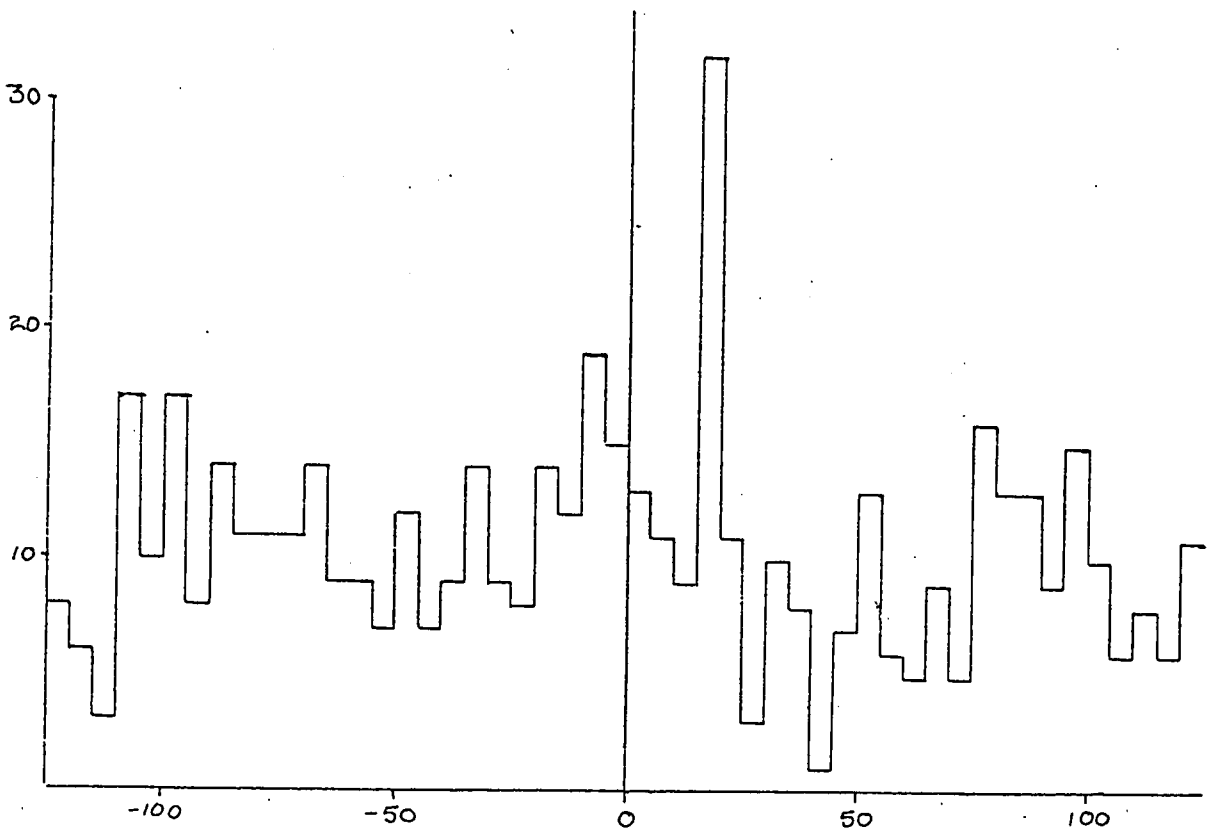


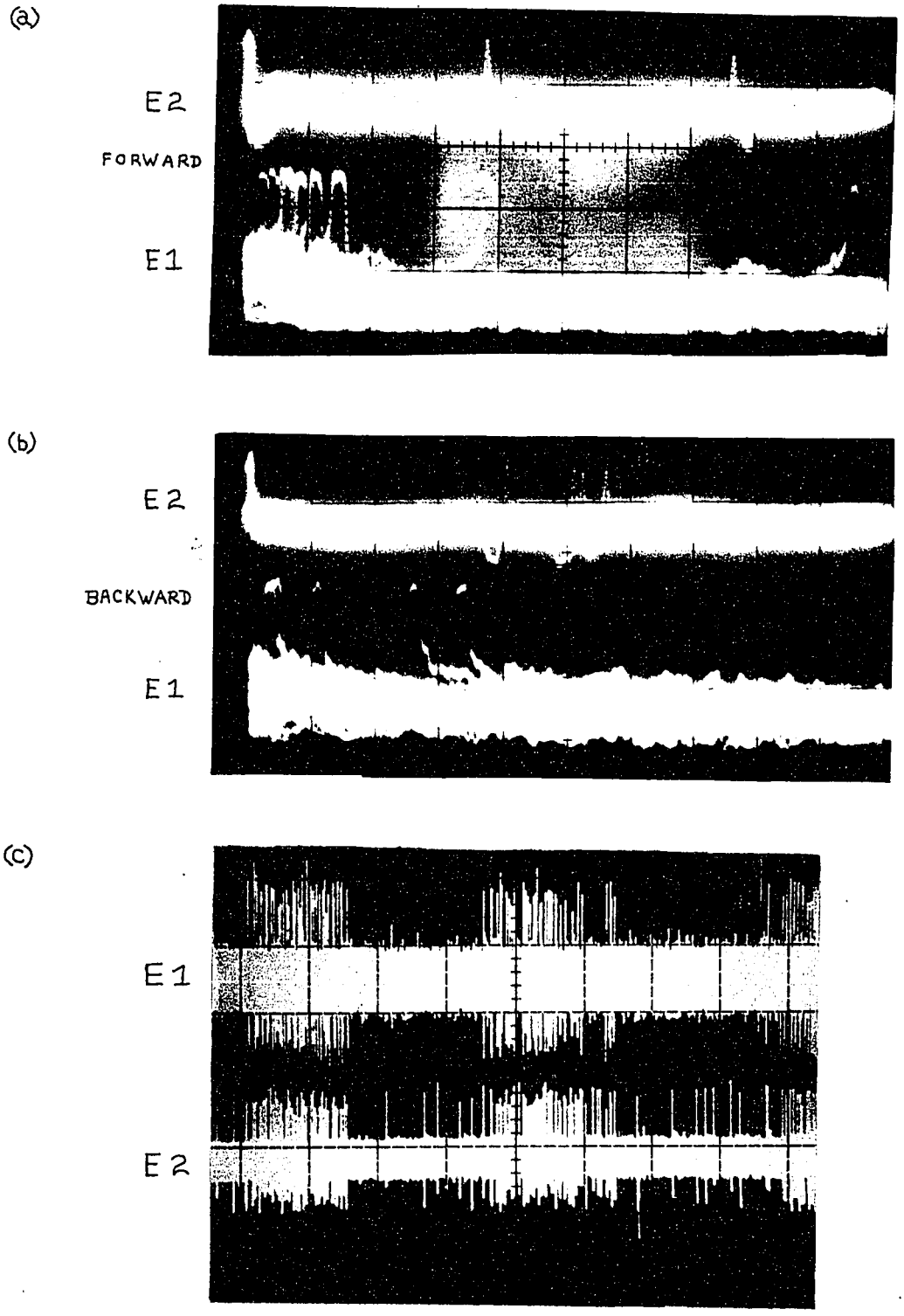
Figure 7
Raw data for pair (23-1-1)

a). Superimposed sweeps triggering on E2 spike (top) in forward direction. E1 spike follows with short latency. Time scale is 2 ms/div for (a) and (b).

b). Superimposed sweeps triggering on E2 spike in backwards direction. Occasional E1 spikes precede E2 spike with short latency but far fewer than follow E2 spike.

c). Slow sweep of cells showing additional interaction. Here time scale is 5 sec/division. Both cells fire grouped spikes separated by long silent periods. E1 does not fire at all during interval between groups, but E2 begins to fire slowly near end of silent period, and directional excitation occurs once E1 begins to fire. E2 glu current constant during this activity pattern.

Figure 7



thus no tests could be done on the interaction. Only in Pair (23-1-1) was the interaction noticed immediately, and the direction was from P2 to P1. Turning up the glu current, however, decreased the rate of P2 drastically, and increased the P1 rate. One possible explanation for this is that glu activated inhibitory neurons projected to P2 and thus caused the rate decrease. (Evidence for this is that the IPSP's seen in pair (23-3-1) only began when the glu was turned on P2). The CCH's for pairs (23-1-1) and pair (23-2-1) are shown in Fig 6 . Raw data for pair (23-1-1) shown in Fig 7 .

3.1.2.5 Common bursting

Common bursting (CB) was the most commonly encountered interaction among BP neurons. Its frequency of occurrence, as well as the nature of the bursts were very variable. All of the burst to burst relational parameters were very sensitive to the level of applied glu current. Figure 8 illustrates four cases of correlated bursts encountered in the experiments.

First, data on computer analyzed CB cells will be presented to illustrate the different forms of CCH's resulting. No distinction between burst types is made during the analysis, but visual rechecking of the raw data is used to make inferences on possible synaptic mechanisms of the interaction in conjunction with the CCH. Spike group to spike group interactions were primarily the result of common burst firing in the cells. (No distirction is made here between the types of burst which were involved). To translate the CCH into a

mechanism accounting for the interaction it is necessary to consider the raw data for each case to determine whether it represents common excitation or inhibition.

The many forms which these CCH's exhibit are a consequence of doing a correlation spike by spike on an interaction phenomenon which operates on the level of spike groups. The fundamental relationship is a peak centered on the origin, or displaced to one side depending on the exact nature of the interaction between the two bursts and the temporal sequence of the spikes within each burst.

In order to illustrate the relationship between the original ST and the CCH, representative interactions have been selected from the computer data and included as text figures. When single spikes occur in a given interval, they are represented by +'s and when multiple spikes occur, the number of spikes in the interval is listed. Each pair of lines is from a different segment of the record, and was chosen on the basis of most closely fitting the CCH.

Fig (9) shows a case of two neurons which fire essentially all bursts. There are some minor periodicities in the CCH, but the primary feature is the broad peak assymetrically placed on the origin. Examination of the ACH's reveals cell A to be an aperiodic cell, and cell B to fire most of its spikes in bursts as is exemplified by the initial peak, valley and second peak. In order to elucidate the exact interaction in this case it is necessary to go back to the original data, representative portions of which are shown in Fig 10

Figure 8
Raw data of correlated bursting.

a). Bursts from pair (3-2-1). E1 top, E2 bot. E2 fires moderately decremental burst (only last spike is smaller) and second cell on E2 electrode (very small spikes following big E2 spikes) fires only when both E1 and E2 burst together. E1 cell fires 1 to 3 spikes with E2 burst. Time scale = 0.2 sec. No voltage scale given because all these records taken from cassette data tape.

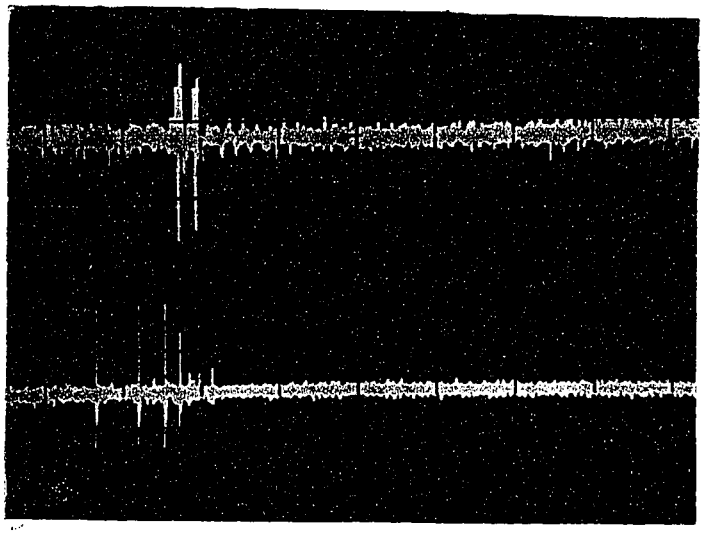
b). Fast sweep of bursts from pair (14-5-6). E1 top, E2 bot. E1 rate in illustrated burst is about 190/sec, whereas single spike firing rate is only 5 - 10 spikes/sec. E2 fires decremental bursts of which only one spike is seen in the photograph. Time calibration = 20 ms.

c) Correlated decremental bursts in pair (14-4-6). E2 fires only decremental burst with very few spikes, whereas E1 fires both spikes and bursts. E1 is one of burst firing cells which consistantly have a long interval following the burst. Time calibration = 400 ms.

d). Pair (24-5-2) CB's. E2 fires rarely and usually in doublets. This is associated with long burst in E1 (rate in photo is increased for about 4 sec). E2 rate little affected by glu. Same E2 cell fires very closely correlated spikes with new E1 cell in pair (24-6-2).
Time calibration = 1 sec.

Figure 8

(a)



(b)

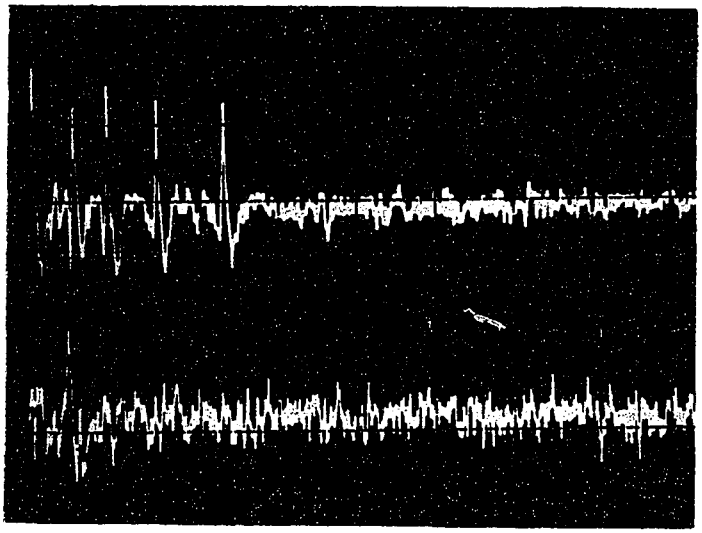
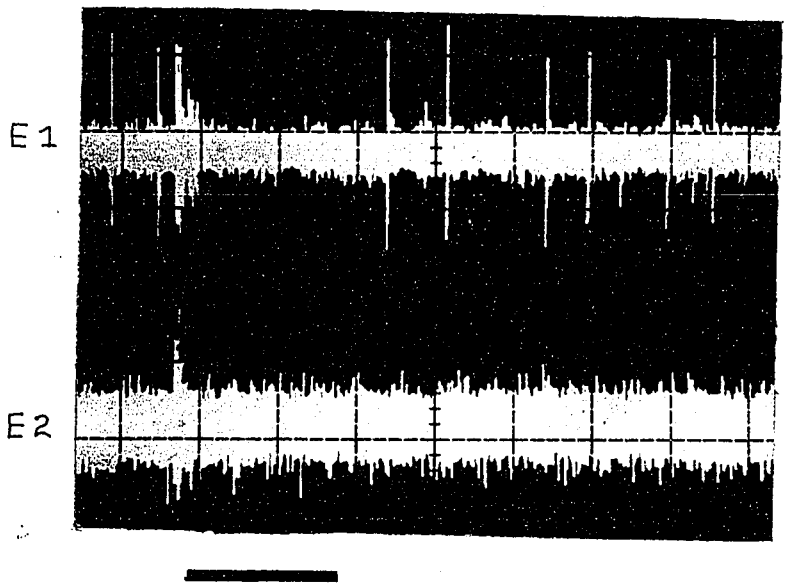
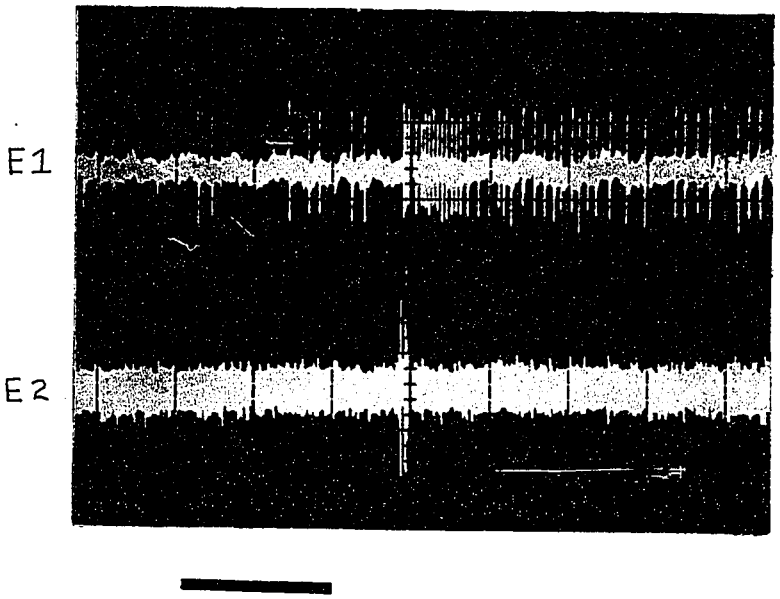


Figure 8

(c)



(d)



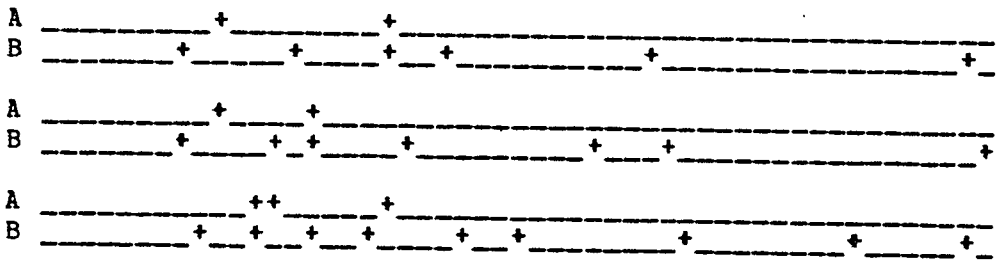


Figure 10: Pair (4-5-2) BW = 10ms

Cell B fires before A which usually fires two spikes per burst. Most of the spikes in the B burst occur after A has fired and thus the peak is broader on the right.

Fig (11) illustrates a case of two neurons which fire many common bursts but also fire spikes which are generally not correlated. Train A is shown to have a minor complex periodicity (in ACH) which may appear in very weak form to the right of the origin. (This may indicate a minor spike to spike interaction from A to B). ACH of cell B show it to be primarily a bursting cell with an interburst interval of about 1.2 sec. The spike data is shown in Fig 12

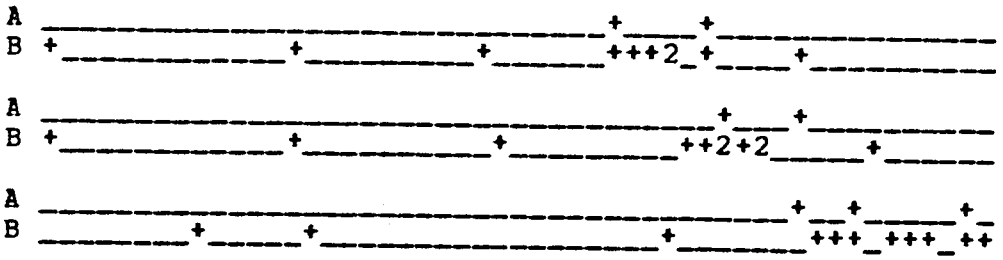


Figure 12: Pair (11-6-3) BW = 10ms

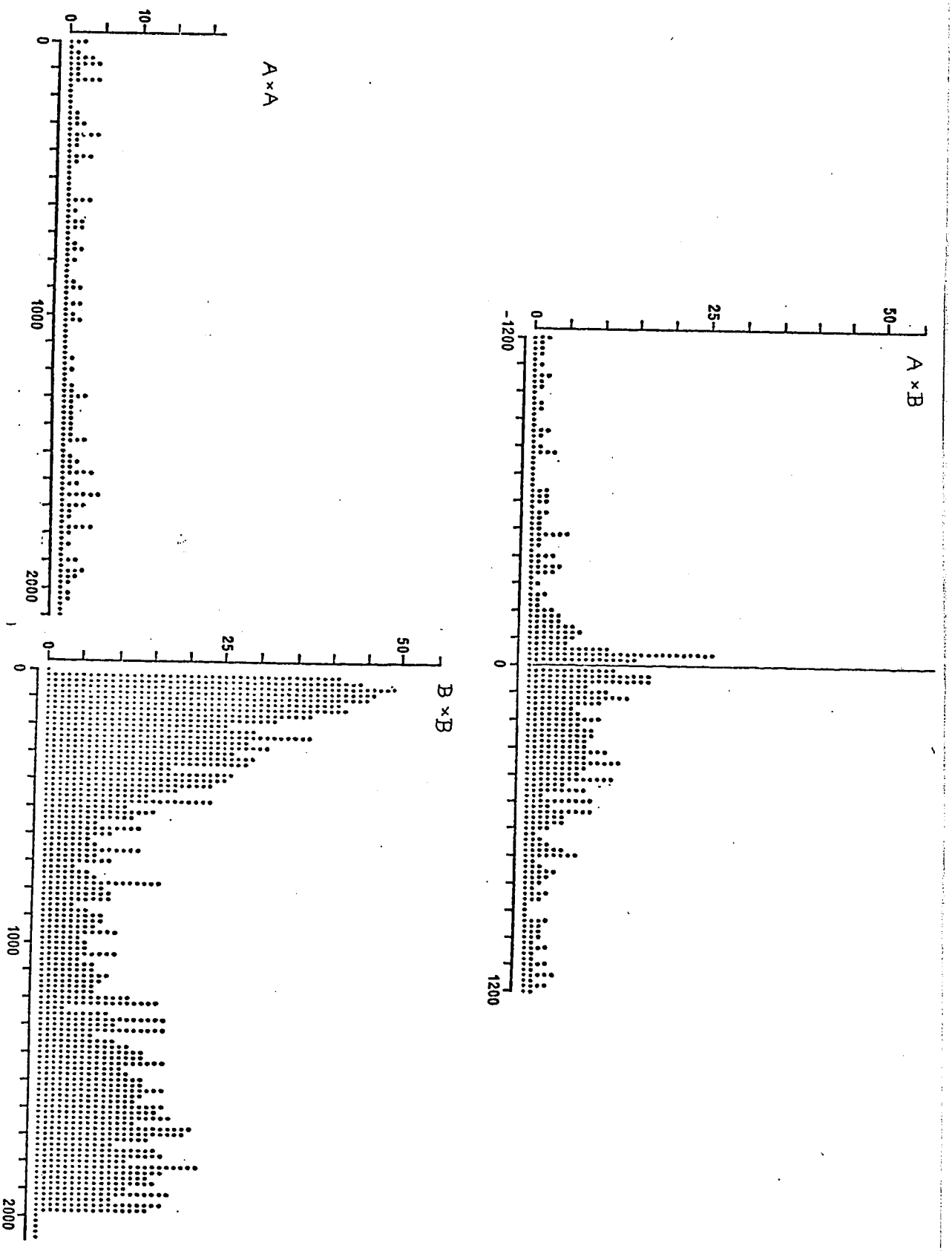
Figure 9
Pure common bursting.

This is data for pair (4-5-2). Statistics for this CCH are:

NA = 69 NB = 232

AMEAN=	1.2773	PA=	0.7829	TIME=	88.1324
BMEAN=	0.3799	PB=	2.6324	BX=	0.0200
EVAL=	3.6326				
NF=	491	NB=	274		
MEANF=	4.9100	MEANB=	2.7400		
SDF=	3.7606	SDB=	3.3841		

Figure 4



Cell B fires first, and cell A soon after. The burst in cell A is over before that in cell B and thus the peak is shifted to the right. A long silent period follows the cell B burst which is not apparent on the CCH at this time scale. (On a broader time scale a peak is seen at about 1.2 to 1.3 sec which is where the cell begins to fire again). The dip at about -220 ms is a consequence of a longer than usual interval preceding the burst in cell B. It is not included as a primary feature because it is less often present than the burst.

Fig (13) illustrates a situation where cell B is firing both bursts and spikes while cell A fires spikes or bursts only with the B bursts. Examination of the ACH's reveals B to be aperiodic and A to be a typical bursting cell. The interaction is shown in Fig 14.

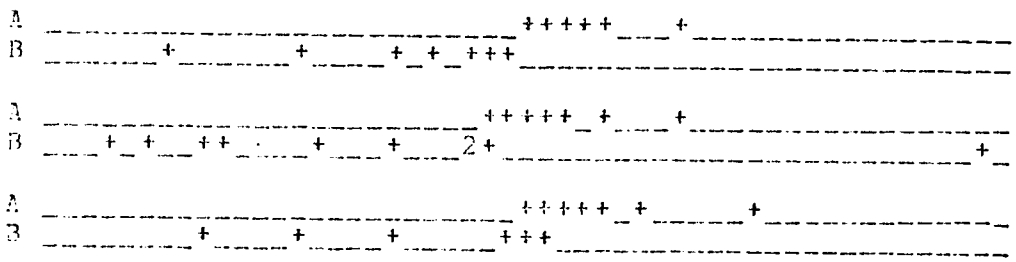


Figure 14: Pair (14-1-2) BW = 10ms

B fires before A and thus the peak is to the left of the origin. A silent period of about 400 ms follows the B burst. For this particular cell pair, computer records for two other sets of iontophoresis conditions were available, and the only feature remaining constant was the peak on the left and the dip on the right of the origin on the CCH.

All of the previous records illustrated cell pairs in which at least one of the bursts was decremental. The CCH of Fig 15 shows a pair of cells which periodically fired short groups of spikes, but no decremental bursts. Both ACH's are very periodic with miror variatiors and fit the CCH very well. The primary feature of the interaction is shown in Fig 16. The peak at the origin results from the common firing during the spike groups (SG). The times t1 and t2 are very similar, and are approximately equal to the inter spike-group interval. This, and other periodicities in the ST's gives the recurring pattern of peaks on either side of the origin. This

Figure 11

Correlated bursting: Pair (11-6-3)

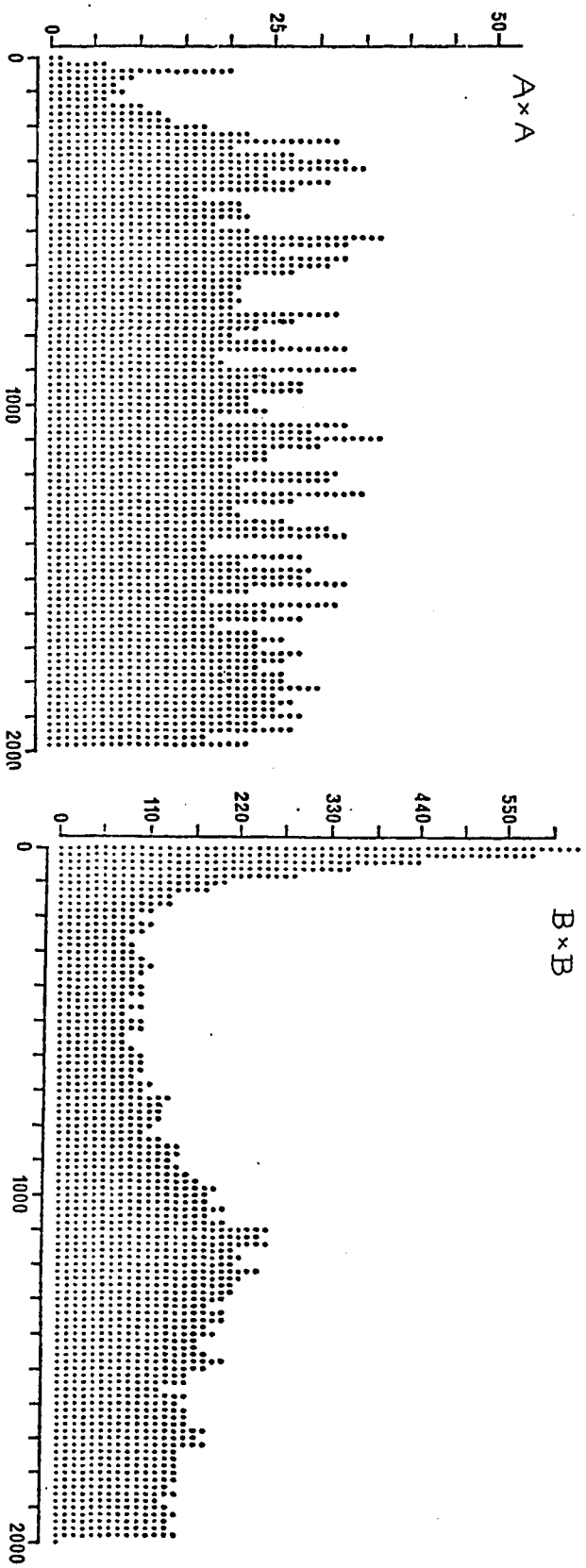
Cell B fires primarily in bursts as is evident from the shape of its ACH. Cell A has variable firing pattern depending on applied glu current, but correlated bursting interaction occurs only when the glu current on cell A exceeds a threshold value sufficient to cause cell A firing.

Statistics for this pair are:

NA = 432 Nb = 1112

AMEAN=	0.4671	PA=	2.1409	TIME=	201.8616
BMEAN=	0.1815	PB=	5.5087	BX=	0.0200
EVAL=	47.6139				
NF=	5475	NB=	4972		
MEANF=	54.7500	MEANB=	49.7200		
SDF=	19.8421	SDB=	11.3605		

Figure 11


0
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50
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0
1000
2000
0
110
220
330
440
550
B×B
0
1000
2000

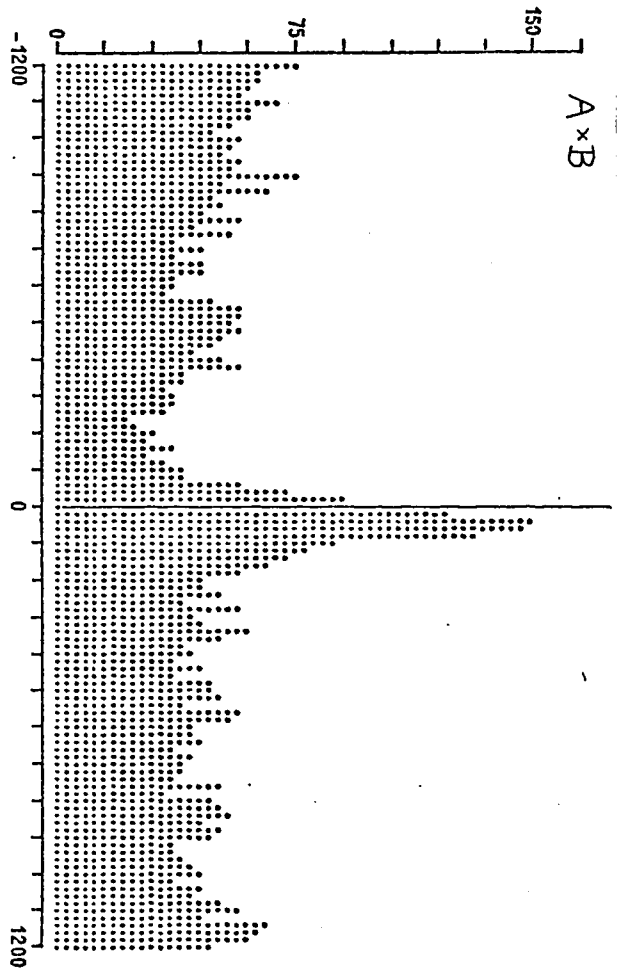

0
75
150
A×B
-1200
0
1200

Figure 13
Correlated burst followed by silent period

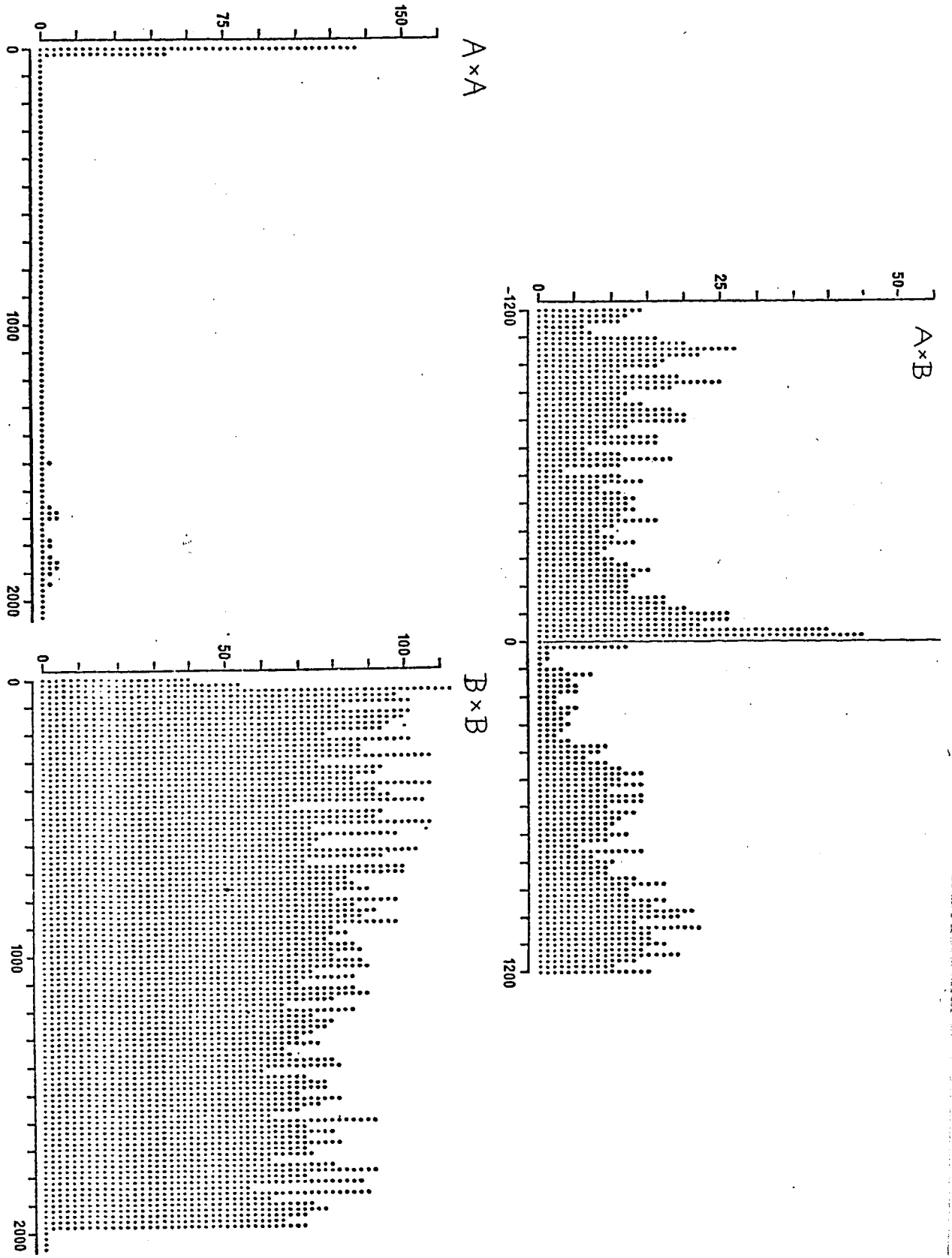
Pair (14-1-2) under conditions where silent period following burst most prominent.

Statistics for this pair:

NA = 137 NB = 589

AMEAN=	1.1380	PA=	0.8787	TIME=	155.9208
BMEAN=	0.2647	PB=	3.7776	BX=	0.0200
EVAL=	10.3515				
NF=	1099	NB=	1453		
MEANF=	10.9900	MEANB=	14.5300		
SDF=	5.3056	SDB=	6.1522		

Figure 13


A×A
0
75
150
0
1000
2000
A×B
50
25
0
-1200
0
1200
B×B
0
50
100
0
1000
2000

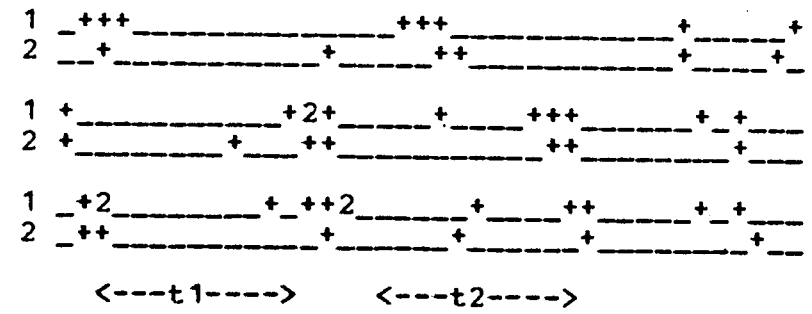


Figure 16: Pair (15-3-3) at 40 ms BW

case was also studied under different iontophoresis conditions, and in the least periodic case only the central peak flanked by two valleys remained. Thus the primary feature of the interaction is seen to remain constant, but just the periodicity was absent.

3.1.2.6 Summary of computer analyzed correlated bursting

Table 2 summarizes the results of the computer data analysis. It should be noted that there are two categories of interactions which are accounted in this table; those cell pairs in which the CCH was sufficient to see that there was an interaction (corresponding primarily to tonic interactions), and those in which it was necessary to do a visual analysis (or a more sophisticated computer analysis) of the data to see the interaction. The latter category consists of two distinct types of cases. The most numerous were interactions in which correlated bursting occurred only infrequently amid much uncorrelated spike activity in the pair of cells and thus the

Figure 15
Rhythmic correlated bursting

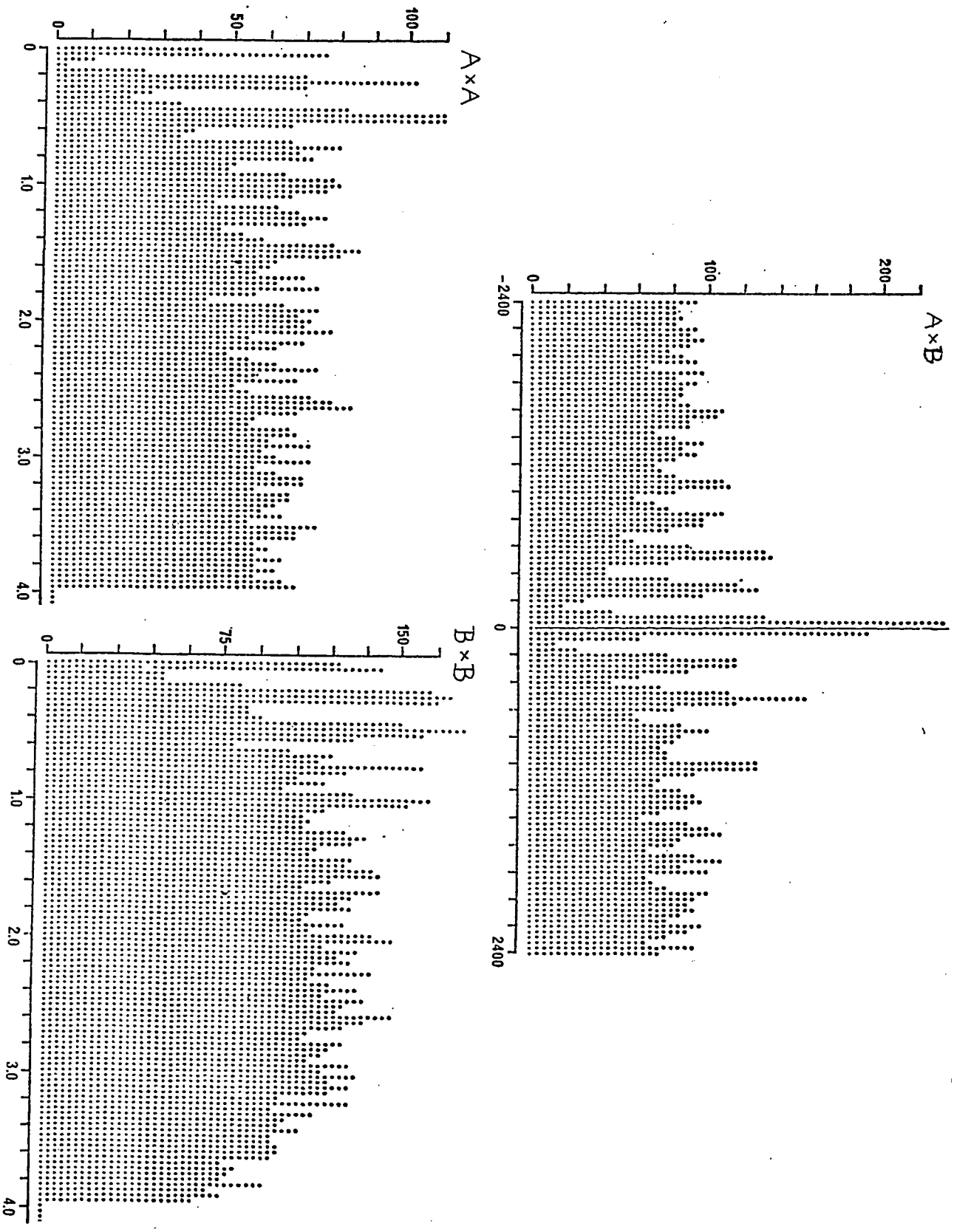
Pair (15-3-3) rhythmic CB data under most rhythmic set of iontophoresis conditions. For this diagram, the times in the CCH are given in ms and the ACH times are given in seconds.

Statistics for this pair:

NA = 460 NB = 575

AMEAN=	0.3461	PA=	2.8891	TIME=	159.2278
BMEAN=	0.2769	PB=	3.6112	BX=	0.0400
EVAL=	66.4490				
NF=	8624	NB=	8691		
MEANF=	86.2400	MEANB=	86.9100		
SDF=	21.6587	SDB=	24.1640		

Figure 15


A×A
0
50
100
0
1.0
2.0
3.0
4.0
A×B
-2400
0
100
200
0
2400
B×B
0
75
150
0
1.0
2.0
3.0
4.0

correlated bursting was not visible in the CCH (phasic interactions). Only one case of another type of correlation existed, and this was the interaction of a cell with a phasic glu response with the neuron on E2 (pair (19-3-2)). Several of the complex bursts in the phasically responding cell were put on an EDT, but only about 60 or so spikes were present. Visually, it was possible to see a change in the firing pattern of the cell on the other electrode when the bursting in the phasically active cell began, but no significant correlation in the CCH was seen.

In the computer analyzed data, a total of 14 pairs of neurons were found to exhibit correlated bursting which was fairly obvious (this number includes 2 category 2 CCH's which had a small central peak, and were found to exhibit CB using visual confirmational analysis).

When these are grouped into categories corresponding to the examples of CB CCH's given in figures 9 to 16, the following distribution is seen:

3 pairs with CB only.

6 pairs with single peak at origin.

2 pairs with peak at origin followed by dip.

3 pairs with rhythmic CCH.

It is interesting that, although a silent period often followed a burst, only in culture 14 were CCH's encountered where a prominent dip was present on one side of the peak. Similarly, all three pairs of rhythmic bursting were from culture 15.

Leaving aside the post burst firing depression and silent period for now, all of the CB CCH's have as their most prominent feature the large broad peak straddling the origin. During a given set of iontophoresis conditions, the burst latencies are generally constant, but varying the glu current on one of the electrodes can change the time relationship of the burst overlap.

Examination of the widths of the peaks revealed an extreme range of 80 - 320 ms for peak duration, but a mean value for the peak duration was calculated to be 160 ms (for this computation all records, including serial records of the identical cell pair under different iontophoresis conditions were used). Since all peak duration values except one were in the range of 80 to 200 ms, it is likely that this particular time value may reflect some aspect of the mechanism causing correlated bursting in the cultures. The peak which occurs in the CB CCH's corresponds roughly to the time of overlap of the bursts in the two cells, and perhaps the measured value of burst overlap is required to maintain culture correlated bursting. (This concept discussed later using Calvins ideas on recruitment of neurons into burst firing patterns).

3.1.2.7 Manual burst analysis

(I). Visual burst analysis.

All cases of correlated bursting with the exception of those which were computer analyzed and had obvious CCH's were found by visual burst analysis. In addition, a visual check was

done on all of the computer analyzed data to see how the bursts looked in relation to the CCH. Fig 8 shows four examples of correlated burst raw data to illustrate the ease with with such correlations may be detected, and to demonstrate the variability in burst parameters encountered.

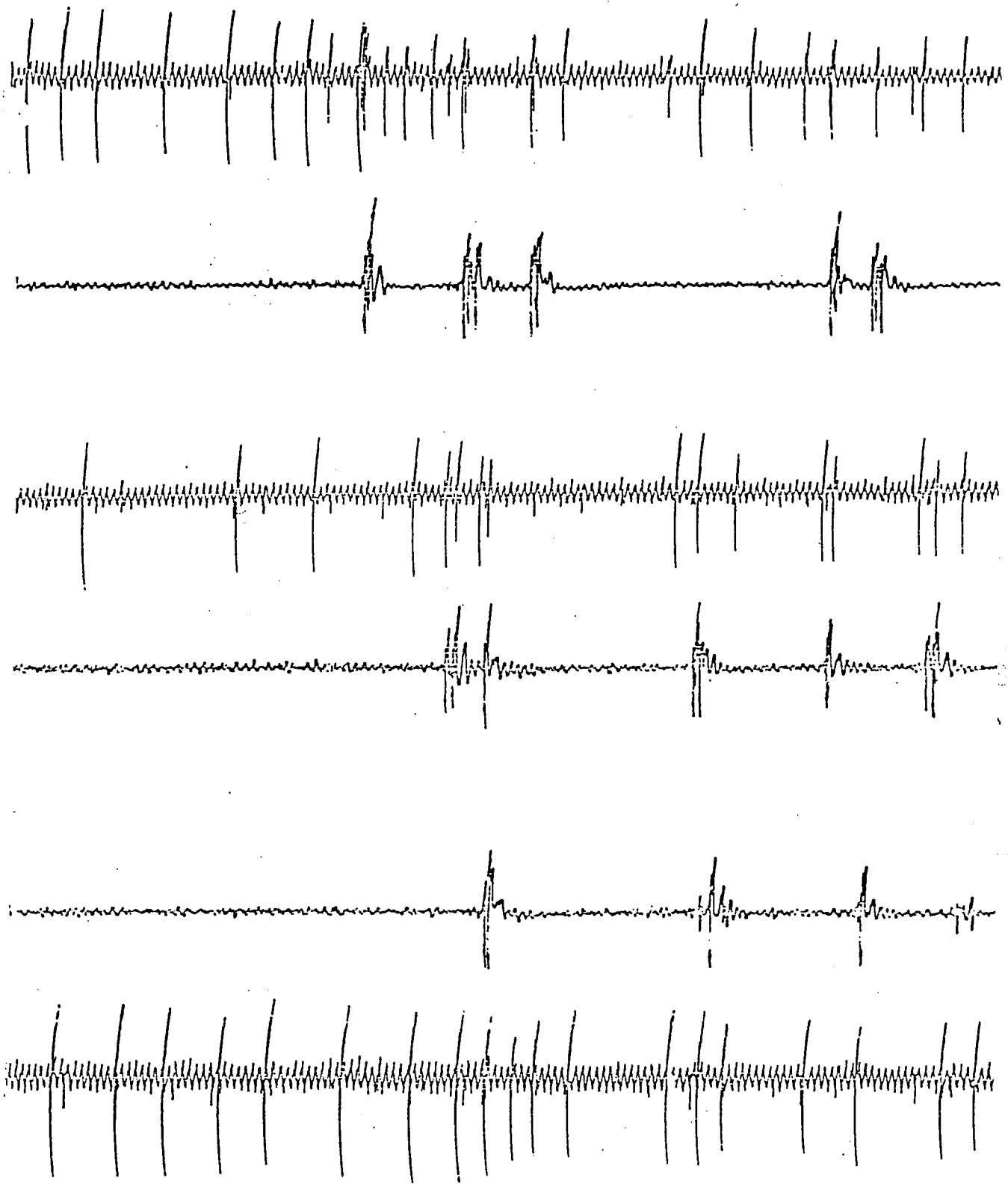
The major disadvantage of this method was that it could show that an interaction was present, but much other data was not obtained due to the excessive effort involved. One such piece of information which would have been interesting was the time relationship between the bursts, and its consistency. In the case of purely burst firing cells it was possible to measure this parameter simply by triggering on the bursts and watching the scope, but when non bursting activity was present, numerous passes through the data were required. Also, based on visual analysis, only a crude guess could be made about whether the interaction was 'frequent' or 'occasional' in terms of its probable CCH shape.

Aside from the total number of neurons in which CB was seen, it is not worthwhile to make finer distinctions on the basis of visual analysis. Thus the treatment of neurons analyzed in this manner will be more descriptive and only interesting bursts will be discussed in this section. In almost all of the cultures whenever bursts occurred in one neuron, bursts or spikes occurred in the other cell also. There was one major exception to this: culture 21 which was a pure BS culture. Here bursts were observed to occur in two neurons, but no correlation between their onset or ending was seen. Many

Figure 17
Enigmatic interaction

Three examples of bursts in a cell with a phasic glu response are illustrated to show the definite presence of an interaction, but its elusive nature. The phasic cell was on E1 (bot cell in diagram) and was recorded on a single barrelled electrode. It fired a long burst following the turning on of glu eject current on E2. The time from initiation of glu application to burst firing in E1 was rather variable, as was the exact structure of the E1 burst. This data from pair (19-6-3).

Figure 17



unusual burst features were seen in this particular culture including very long bursts, and what looked like decremental bursts, but the minimum spike size occurred within the center of the burst after which the spike size increased again. This is not to say that the cell pairs were non-correlated, but the correlation was not of the nature encountered up to that time. On a visual level it was impossible to decide what was going on between these neurons.

Culture 14 was the most interesting in terms of bursting. Here every pair of neurons recorded from exhibited correlated bursting. As several parameters were modified in this particular experiment (culture feed and use of HCA instead of glu electrodes) this may be an atypical culture. In some of the pairs of neurons the bursts occurred with very similar time onsets, and in one case, (pair (14-1-1)), the first spike of the burst occurred simultaneously in both neurons, while later spikes in the burst were not seen to have such a close correlation. (The simultaneous spikes were also seen to occur when no bursts were occurring in the cells). If this cell pair did not represent two spike generating sites on the same neuron, then it indicates that within the BS cultures, such close spike correlations can result from the same mechanism responsible for the correlated bursts.

(II). Common firing. (CF)

Four cell pairs were encountered in which the interaction was a nondirectional correlation of spikes in the two neurons. The data on these cells is given in Table 3 . These cells are

urusual in that 3 of the 4 are from culture 24. As well as firing correlated spikes very close together, the cells were also quite slow firing and were hard to drive with glutamate. An illustration of the raw data from one of these cell pairs is given in Figure 18 Here one spike is triggering the scope sweep in every case, but the photographs are taken in both the forward and reverse directions. Pair (14-1-1) which also exhibited this behavior, is not included in the tally for the CF interactions as the CB which occurred was also very prominent. Within the bursts in this pair there was no close spike correlation, but generally before a burst, spikes would occur within 1 ms of each other in both cells.

Most likely the category of CF is a subset of CB as 3 of the 4 cells which exhibited CF also participated in CB's with other neurons. The CF cells fired only spikes when they were participating in a CB interaction with another neuron. Thus it is reasonable to assume that the CF cells represent two simultaneously recorded neurons of the type which fire primarily single spikes in response to the correlated culture activity (which results in bursts in most other neurons).

3.1.2.8 Effect of Bicuculline on BS neurons

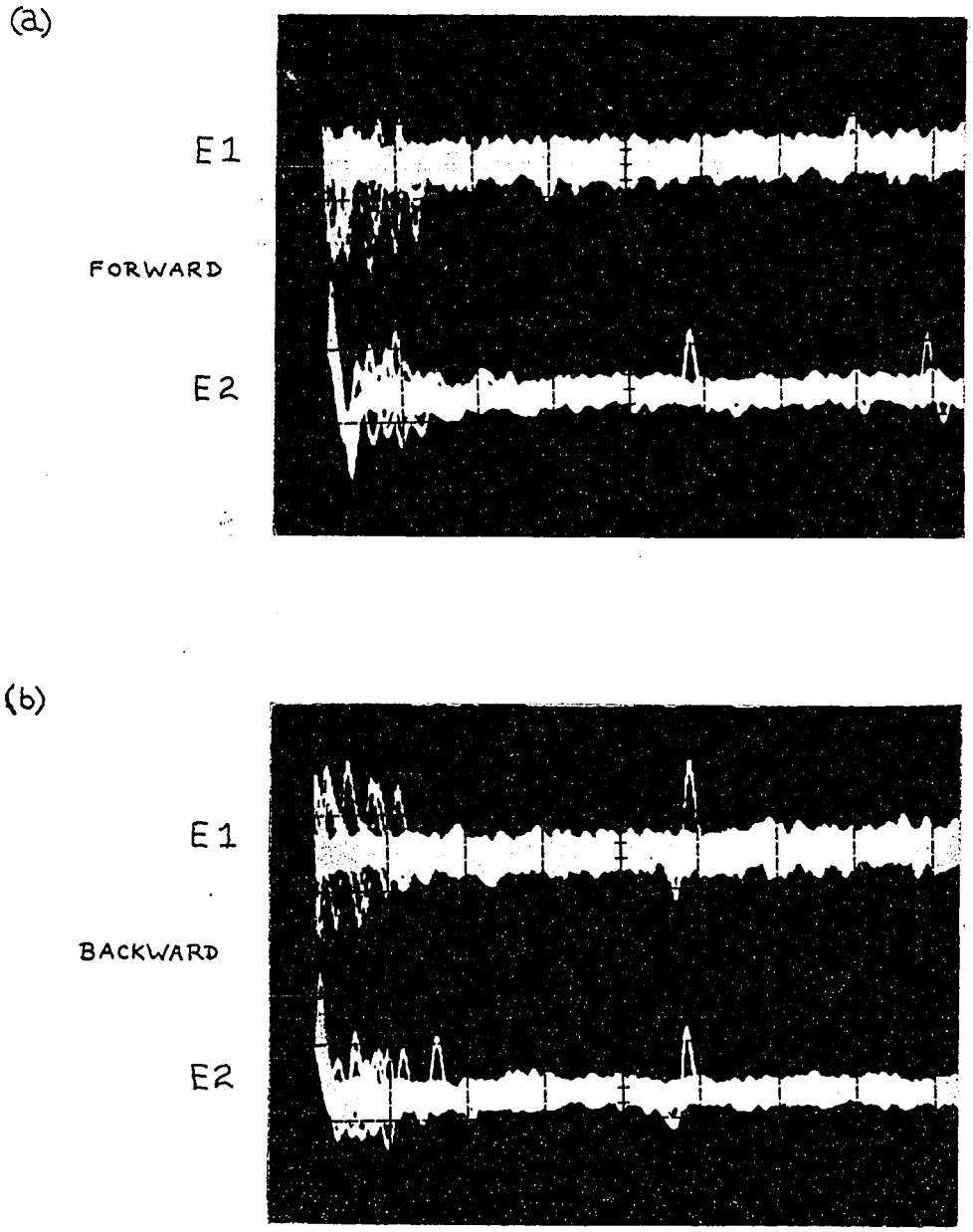
In two experiments, bicuculline (bic) was added to the BSS in an order to increase the rate of activity in the BS neurons (1 experiment) and to see if there was an increase in correlated activity caused by the bic (1 experiment). The rationale behind the use of bic in the initial experiment was that perhaps there

Figure 18
Common firing

This pair, (24-6-2), consisted of two slowly firing cells in which very closely correlated single or double spikes occurred. The lack of directionality may be observed by comparing the forward triggered E2 traces (part (a)), with the backward E2 triggered traces (part (b)). E2 is the bottom spike in each case. One interesting feature of this cell pair is that the cells were separated by virtually the whole extent of the culture BS region.

Time calibration = 5 ms.

Figure 18



was a tonic inhibition from PN's to the BS neurons, and this would be reversed by the bic. It was observed that a greater amount of correlated bursting was observed when the bic was employed, but this was a non-quantitative finding. In experiment 17 a rigorous study of the effects of bic was carried out with the aim of discovering whether an increase in the correlation of a single pair of neurons occurred from no drug to drug states. A standard technique was used for all of the pairs of cells recorded. First, in standard BSS, the response to two fixed glu currents on both neurons was recorded. Then bic infusion was initiated, and after four minutes, the glu on both cells was turned on again at the same currents as the control period, and for the same length of time. Then normal BSS was switched back on, and after an additional four minutes the glu was again turned on to obtain the post drug control.

In total, 7 pairs of neurons were recorded under these conditions of which 5 pairs exhibited correlated bursting predominantly during the period of bic infusion. However, it proved difficult to hold most of the neurons for the entire period of time required to check for the effects of the bic. (on one electrode the same neuron was present for all 7 pairs, but on the other electrode the stability of the neurons proved to be less).

Six of 7 pairs of neurons had EDT's prepared, and when the CCH's were computed, only one pair showed an interaction, and this was during the presence of bic. For the one cell that was present during all of the records good single cell records were

available of its response to bic, but this was seen to be quite variable. The basic effect was an increase in bursting while the bic was present, but the rate of firing could be either increased or decreased. Visual and auditory examination disclosed an increase in the irregularity of firing in the neuron, but no quantitative measure of this change was computable by any of the existing analysis programs, and it was not considered worthwhile to write one just for this cell.

In five cases, with different cells on the other electrode, bic was observed to cause correlated bursting. Of these cases, only one pair had both a pre and post drug control with both neurons firing at a high rate. This pair (17-1-7) will be considered in detail, and while extrapolation from one pair of cells is generally risky, in this case the results and conclusions can be taken as quite typical of the response of the BS neurons to bic.

When CCH's were computed on this pair, it was surprising that no interaction between the cells was observed. Several bin widths were tried, as well as various segmentations and still no interaction seemed to be present; the CCH was not flat, but the shape for each segment was not the same. When the raw data was visually examined, the occurrence of marked correlated bursts in the latter half of the bic record was observed. These bursts were rather long and were followed by a long silent period in each of the cells. They were not present in the control record, or the post drug control

Examples of STDISP format displays of the activity of the two cells involved in the computation of the TXIH are shown in the figure 19 The bin width for this figure is 30 ms, and A represents E1 and B is E2. The first part of the activity is from the initial portion of the bic record where the cells were firing mainly single spikes, and some irregularity is already apparent. The second part of the record illustrates the bursts.

Part 1. Uncorrelated activity at start of bic infusion.

```
A 1..1...1..1...1..1...1...1....1..1..1...1..1...1
B ....1......1.......1..........1.........1.....

A ..1....1...1.....1...1......1...1....1...1...1..
B ......1.......1.......1........1......1......1..

A ..1....1..1......1...1....1....1....1..1......1.
B ....1........1.......1.......1......1.......1...
```

Part 2. Bursts with Bic.

```
A ..1.....1..1..1..1.1..1......1...1..1.211....111
B ......1......1.....1......1...1....1.11211......

A ...1...1..1...1........1.111221.111.............
B .......1.....1.........1..11221.................

A ..1.1.1..1.11.11..1.1...........1.....1..1..1..
B .....1...1.1....1..1........1..........1......1
```

Figure 19: Pair (17-1-7) (Bic) at 30ms BW

In several cases it was not possible to demonstrate a phasic interaction such as that seen in pair (17-1-7). Thus, the method by which this cell pair was shown to be correlated will be discussed in detail, and it may also be applied to the other cell pairs in which this was the case. One favorable

feature of this cell pair was the existence of a large amount of data for each of the cells. The minimum number of spikes in any of the three records was 907 in the E2 pre bic control, and all together there were 6988 E1 spikes and 5397 E2 spikes.

When the pair was first picked up, the iontophoresis of glu caused regular firing in both neurons. Absolutely no sign of bursting was seen, and it is likely that no correlation among the cells exists on the basis of the CCH. Once the infusion of bic containing BSS was begun, and the glu turned on again, little change from the initial pattern was seen in the first part of the drug record. Within a minute or so of the bic being on, it was possible to notice a greater irregularity in the firing of both of the neurons (as well as an increased rate of both), but no interaction seemed to be occurring. Finally, in the last portion of the bic record the bursts began to occur, and continued until the end of the record, although they were separated by many single spikes. In the post bic control period, the reverse sequence of firing was encountered with the firing becoming more and more regular in the bic free period. Bursting was seen in one of the cells during this time, but no corresponding bursts were seen in the other cell.

Computation of TXIH's for each of the cases yields sufficient data to test the effect of the bic on the pattern statistically by using the chi square test to compare the pattern of short intervals occurring within each TXIH to the expected number assuming independence of the spike trains. For the pre and post drug controls no difference between the

theoretical and actual TXIH was observed. Only when bic was present was there a significant difference seen, and this allowed rejection of the null hypothesis at a greater than 99.5% confidence level. Full details of the test are given in Fig 20

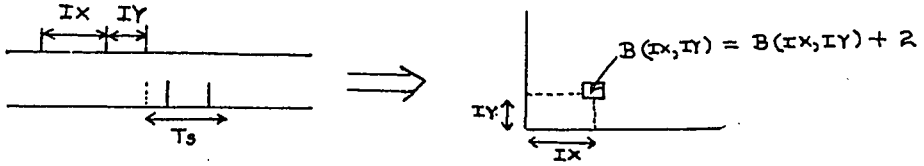
Application of the TXIH to other cell pairs was done, but only in this case was the data sufficient to allow a high degree of confidence to be attached to the result. Many of the cases which were present in the computer entered data could have likely been resolved using this method of analysis as well as other methods, but the data quantity was insufficient.

3.1.2.9 Miscellaneous interactions

This category includes all of the interactions which were observed on only several occasions and thus their significance is not clear. On the basis of extracellular data, only the category of alternate firing (AF) may be readily distinguished. Common inhibition most likely occurs in some of the extracellular records, but the inability to distinguish common inhibition superimposed upon tonic excitation from rhythmic common excitation on the basis of the extracellular data makes it unprofitable to pursue this topic until it is considered in the intracellular data. One of the enigmatic correlations has been illustrated in Fig 17 Only the category of alternate firing (AF) will be considered in detail and it has been observed in two pairs of neurons. In both cases it did not persist for long after it was noticed. Fig (21) is the CCH of two neurons which exhibit alternate firing with some overlap.

Figure 20
Statistical analysis of Pair (17-1-7) TXIH

Within CB's of this pair, 'direction' of correlation was found to be strongest from E2 to E1 TXIH is constructed by counting number of spikes occurring in E1 within interval Ts following every pair of E2 intervals as shown below:



Null hypothesis assumes that no correlation exists between the two trains of spikes. Thus the expected number of P1 spikes occurring within a time Ts from a random starting point is:

$$NE1 = Ts*P1 \qquad \text{where } P1 = \text{firing rate of P1.} \qquad (1)$$

Applying this to the TXIH, where in triggering train (E2) there are A(i,j) events in bin (i,j), the expected number of events in corresponding bin for follower XIH (B(i,j)) is:

$$BE(i,j) = A(i,j)*Ts*P1 \qquad (2)$$

In this pair of cells, only short intervals were of interest. A bin width of 90 ms was employed, and the range of (0-270) ms on both axes of the TXIH was searched for correlations (total of 9 bins). Statistical quantitation of the result was achieved by using the chi square test with the value of chi squared (X2) being computed from:

$$\chi 2 = \sum_{i=1}^{3} \sum_{j=1}^{3} \left(\frac{B(i,j) - BE(i,j)}{BE(i,j)} \right)^2$$

In order to make the results mathematically correct, the various bins in the contingency table were combined to achieve a total of 5 or greater (Freund, 1971). This is indicated in the tabular data by the '*' present in a bin indicating that it was combined with the totals of the other *'ed bins. In this table of the number of events within the bins, the XIH of the triggering cell is on the left with the number of interval pairs entered within each bin. Follower (E1) table is on the right. Topmost entry is number of E1 spikes within 20 ms of E2 interval. Lower numerical entry is expected number of E1 spikes based on equation (2) above. For each calculation the value of P1 is taken as the reciprocal of the mean E1 rate for the given set of experimental conditions being investigated.

Figure 20 continued

Statistical parameters of data under various conditions.

Condition	N2	N1	EN1	P1	P2	X2	*X2	rHo
Pre bic control	908	79	83	3.34	4.59	2.21	3.84	<95
Bic infusion	1936	360	289	4.51	7.47	45.06	12.84	>99.5 **
Post bic control	1558	232	239	4.67	7.62	2.92	7.82	<95

The only condition where a statistically significant difference exists between the expected number of E1 spikes (EN1) and the actual number of E1 spikes (N1) is during the period of Bic infusion. The X2 value for this condition required for the null hypothesis to be rejected at probability level rHo is given by *X2. The difference during bic infusion allows rejection of the null hypothesis at a greater than 99.5 % confidence level. It may also be noted that even though both neurons did not return to their baseline firing rates (rates are given by P1 and P2 and are in spikes/sec), the values in the TXIH reverted to those expected by chance.

Figure 20 (continued)

(a). Control

Number of E2 events

2 *	3 *	105
1 *	1 *	2
6 *	1 *	2 *

Number of E1 spikes (within 20ms)

0 0.184 *	1 0.276 *	13 ← # of E1 spikes 9.77 ← expected # E1 spikes
0 0.092 *	0 0.092 *	0 0.184 *
1 0.552 *	0 0.092 *	1 0.184 *

(b). With bicuculline

8 *	239	902
12 *	55	225
19 *	6 *	9 *

4 1.20 *	62 35.85	161 135.3
2 1.80 *	13 8.25	29 33.75
13 2.85 *	0 0.90 *	1 1.35 *

(c). Post bic control

3	265	377
7	226	266
5	5	2

1 0.46 *	42 40.40	49 57.47
3 1.06 *	39 34.45	42 40.55
0 0.76 *	1 0.76 *	0 0.30 *

As both have a long cycle, a large time scale is required to show the interaction. The only significant feature is the broad dip with its minimum in the vicinity of the origin. The interaction for this pair is given in Fig 22.

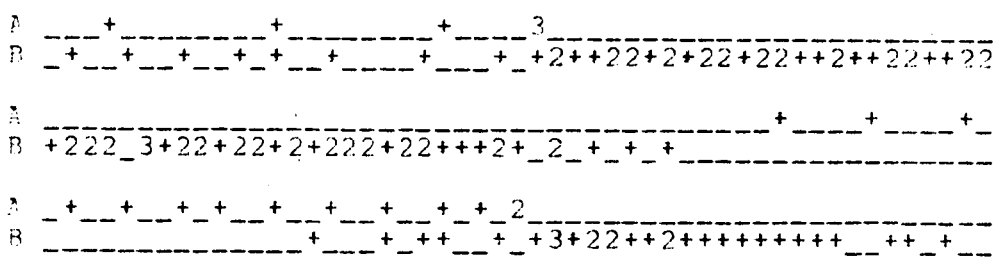


Figure 22: Pair (20-5-1) BW = 50ms

One unusual feature of this pair is that it appears to be a case of correlated bursting. Initially the cell on F1 (cell A in Fig 22) was firing while the F2 cell was silent. Then the F2 cell would begin to slowly fire while the F1 cell still fired. When F2 began to fire quickly, it was quite abruptly, as may be seen in Fig 22 and Fig 23 part (a). What is not very obvious in the raw data presented in Fig 23, is the apparent occurrence of simultaneous decremental bursts in both neurons when F2 begins its rapid firing. (This is not at all obvious in F2 at the time scale employed, and may be faintly seen as two very small spikes in F1 just after the big spike ceases). When F2 begins to slow down, which it also does abruptly, the small spike on F1 is occasionally seen to slowly become larger, and then to fire while F2 is silent. Also this relationship does not hold for long; a second computer record made about 10 minutes into the

record no longer reveals the alternate firing, and gives a relatively flat CCH. Also, in this portion of the record, the small fast E1 spikes are seen to be correlated with long silent periods in E2 (fig 23, part (b)).

Figure 21
Alternate firing CCH

Data used to construct CCH is from first part of record from pair (20-5-1) where pronounced alternate firing (AF) occured. Time scale for CCH is given in ms, and for the ACH's in sec

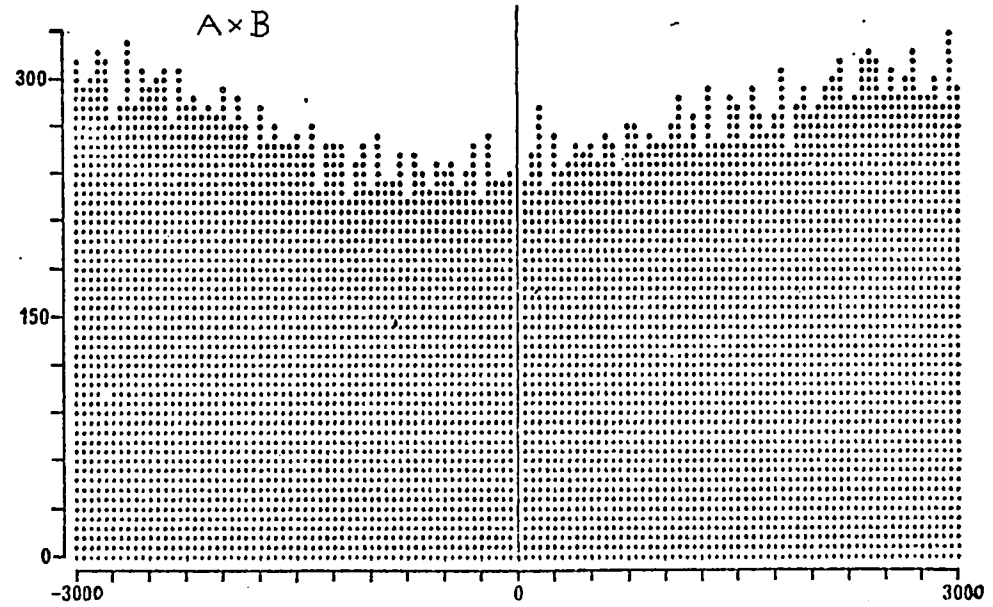
NA = 963 NB = 2374

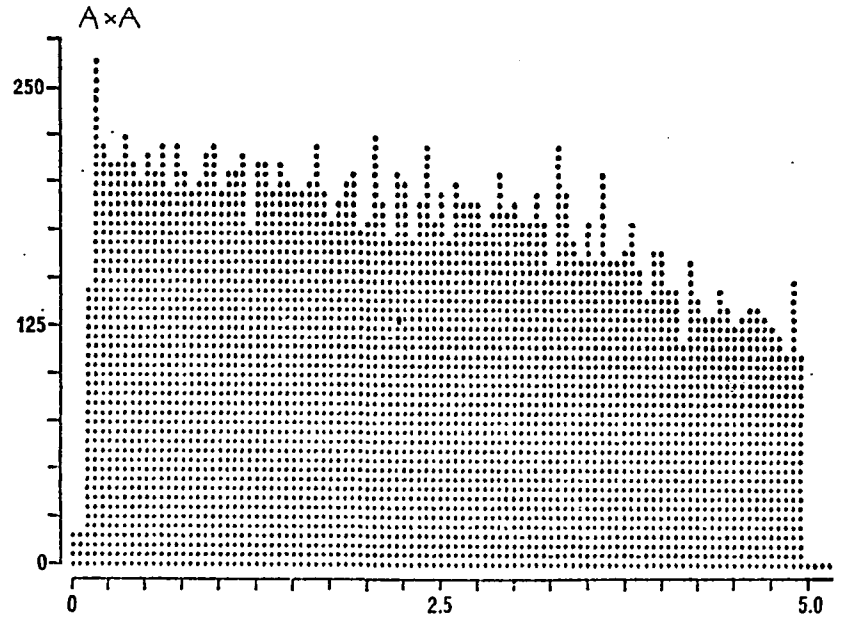
AMEAN=	0.3505	PA=	2.8533	TIME=	337.5994
BMEAN=	0.1422	PB=	7.0320	BX=	0.0500
EVAL=	338.6902				
NF=29201		NB=29379			
MEANF=	292.0098	MEANB=	293.7898		
SDF=	26.0109	SDB=	39.4593		

Figure 23
Raw data from alternate firing cell pair

a). Prominent AF in initial part of data record. E1 top and E2 bottom. No glu electrode present on E1. When E1 ceases to fire, it is with a decremental burst. This burst may be faintly seen in the form of two very small spikes following the last large E1 spike. Time calibration is 1 sec/div.

b). Section of later part of pair (20-5-1) record where alternate firing no longer present. Instead get curious correllation of increase in E! rate with much decreased spike size accompanied by long silent interval in E2. Time calibration = 0.5 sec/div.


A×B
300
150
0
-3000
0
3000


A×A
250
125
0
0
2.5
5.0

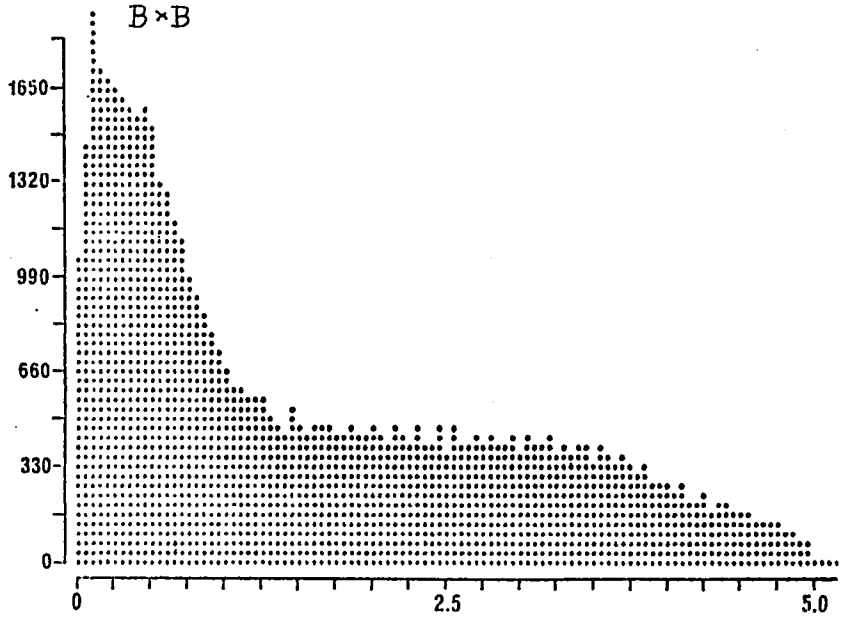
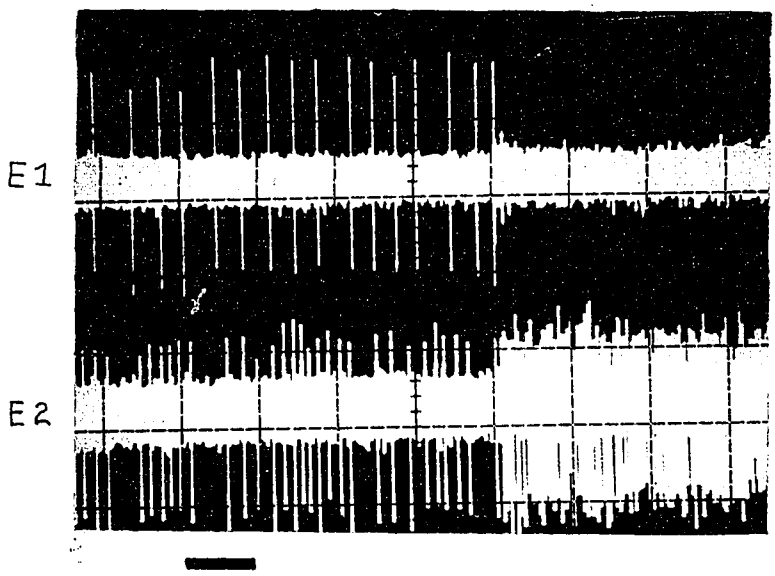

B×B
1650
1320
990
660
330
0
0
2.5
5.0

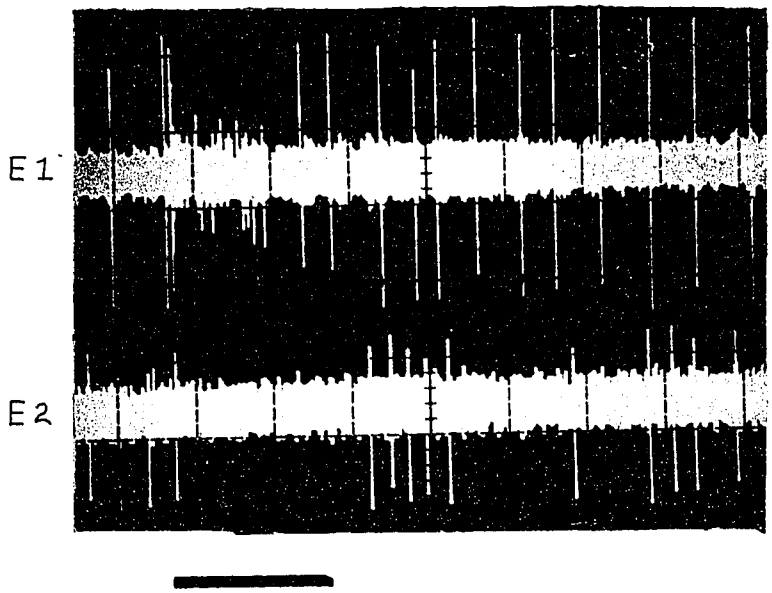
Figure 21

Figure 23

(a)



(b)



3.2 INTRACELLULAR RESULTS

While intracellular recording furnished a greater quantity of information for each stable cell pair, it was more difficult to obtain the intracellular data. Numerous cells were penetrated, but of these few were usable. A rough indication of the greater difficulty of intracellular recordings is that of 52 penetrations made in 7 experiments only 12 had membrane or action potentials greater than 40 mV, and even some of these deteriorated rapidly. The length of the record was not used as one of the criteria as it was difficult to obtain very stable recordings with most neurons exhibiting a constant Em for longer than 5 minutes. Only in a few cases were recordings stable for 10 or more minutes

Other records were of poorer quality in terms of membrane potential and spike size, but did exhibit a definite correlation with the activity of the extracellular cell. These were also analyzed as correlated pairs, but in some cases it may not be possible to ascertain the exact nature of the interactions due to deterioration of the cell. If small spike like potentials were present, and were stable, these were analyzed in the same manner as extracellular data and given the designation 'quasi intracellular'.

3.2.1 Basic electrical parameters

In determining the basic electrical parameters of the BS neurons, only the cells in the good category

were used. For this group the mean membrane potential was 48 mV and the mean ap size was 64 mV. Table 4 summarizes the characteristics of all these cells.

All except one of the neurons exhibited spike activity on impalement, but there was a great variability in the subsequent nature of the spike train (discussed later). All of the ap's exhibited a large afterhyperpolarization (AHP) following the ap. These AHP's were observed to summate if the ap's occurred sufficiently close together. Most of the spikes were uniform with no inflections except when deterioration set in, or if a burst occurred.

3.2.1.1 Spike generation

Spike generation activity in the impaled cells could be either regular, patterned, phasic or absent. (since only 12 cells are being considered general categories will be used). Most of the cells were observed to fire fairly regularly upon impalement. This is very likely due to depolarization resulting from electrode penetration as examination of the spike trains on a short time scale revealed a sequence of continuous depolarization finally followed by a spike and repolarization after which the process began anew. Occasionally this process could be interrupted by a sufficiently strong EPSP, but otherwise no evidence of synaptic input could be seen.

These cells either would continue to fire like this until the spike began to decay, or they repolarized and

slowed down. The former case was the most common, and usually a hyperpolarizing current was passed to terminate the firing.

Only one cell was encountered where no spikes were seen shortly after penetration. Only after passage of depolarizing currents of up to 3 na did the cell finally concede to fire, and in every case the pattern consisted of two spikes for every large decrease in membrane potential followed by silence until further depolarization or repolarization - depolarization occurred.

3.2.1.2 Synaptic activity

The synaptic activity occurring in the neuron was of primary interest, and in most cells shortly after impalement the membrane was hyperpolarized to a level sufficient to stop spike generation so that synaptic activity could be more readily observed. The currents required varied with each neuron. Very strong excitatory synaptic input could make the neuron fire while very hyperpolarized. This was observed in two neurons in which bursts occurred.

Total synaptic activity of a neuron was very variable and thus difficult to quantitate. Both EPSP's and IPSP's were present, but with the EPSP's generally being more numerous. Unitary synaptic potentials were infrequently observed. Most potentials large enough to be readily discernable visually proved to have a number of components on closer examination.

Spontaneously occurring EPSP's were usually 50 ms or less in duration and came in a wide variety of shapes, but most had slow rise times. Often they were grouped and the summated potentials were of a rather varied nature.

One distinct category of EPSP is the one associated with a burst. This was usually seen to have an abrupt onset, and slow decay which could extend over as long as 10 seconds. These will be referred to as Paroxysmal Depolarizing Shifts (PDS) using the terminology of (Matsumoto and Ajmone Marsan, 1964). On closer inspection these are observed to not be a unitary event, but rather the result of numerous summated EPSP's. In every case where a PDS has been observed, a burst or an acceleration of firing in the extracellular cell has been observed concomitant with the PDS.

Of the neurons in the good category, 4 were found to exhibit either a PDS or bursting. Three of these fired spikes during the burst and the other exhibited huge EPSP's. Each case will be considered separately because of the small number of cells and the importance of the synaptic correlate of the bursts.

The single neuron which did not fire ap's exhibited large (~10 mV) abrupt depolarizations of variable duration (0.5 to 10 sec). Their large size is possibly due to the spike generating mechanism of the neuron being inactivated and thus not having the mechanism of afterhyperpolarization to restore the membrane potential towards its resting level. The variable duration, and jagged appearance of the

depolarizing potential suggested a summation of many EPSP's which had a nearly simultaneous onset. An illustration of three such potentials is shown in Fig 24

Of the three neurons which fired spikes during the burst, two fired only a single long burst during the entire record, while the other fired occasional short bursts. In all these cases the depolarization associated with the burst was not of the same magnitude as that observed in pair (20-7-4) possibly as a result of the spikes being present. An increase in EPSP frequency was observed, and also the occurrence of EPSP-like potentials which were followed by hyperpolarizations. These could possibly represent abortive spikes or dendritic spikes.

In all of these neurons a decrease in spike size was seen when spikes followed one another with short temporal separations. Depending on the length of the interval between the two spikes, the second spike could either be normal in appearance, but just smaller if the interval between the spikes was in the range of the spike duration, or with complex spiky potentials occurring if the spike occurred with a shorter separation.

The one cell which exhibited the short bursts fired 6 in the period of observation. Of these 5 were preceded by a depolarization of variable latency whereas the other appeared to be immediatly preceded by an IPSP. The IPSP may be coincidental, but it is possible for an IPSP to initiate a burst also. (Doesn't seem to be important in BS neurons). Three of these bursts are shown in figure 30

Figure 24
Intracellular/extracellular record of correlated bursting

Three of the shorter EPSP's recorded from pair (20-7-4) in conjunction with E2 bursts are illustrated. E2 top, E1 bot. Note variable shape and amplitude of E1 EPSP associated with E2 burst. In top 2 bursts close temporal correlation between EPSP onset and E2 firing may be noted, but this is less pronounced in bottom burst. Time calibration = 0.5 sec. Voltage calibration is 10 mv (applies to E1 only).

Figure 24

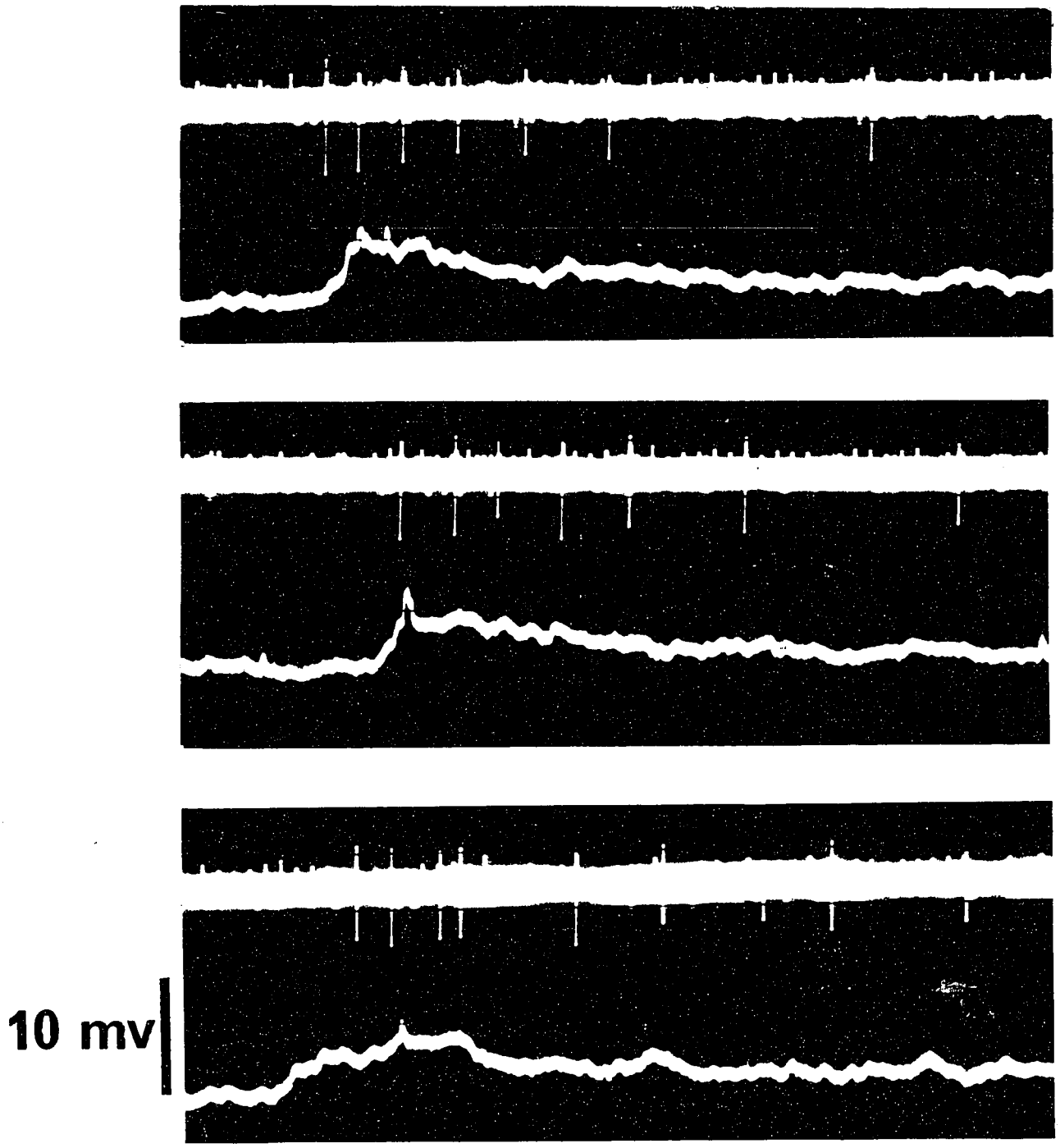
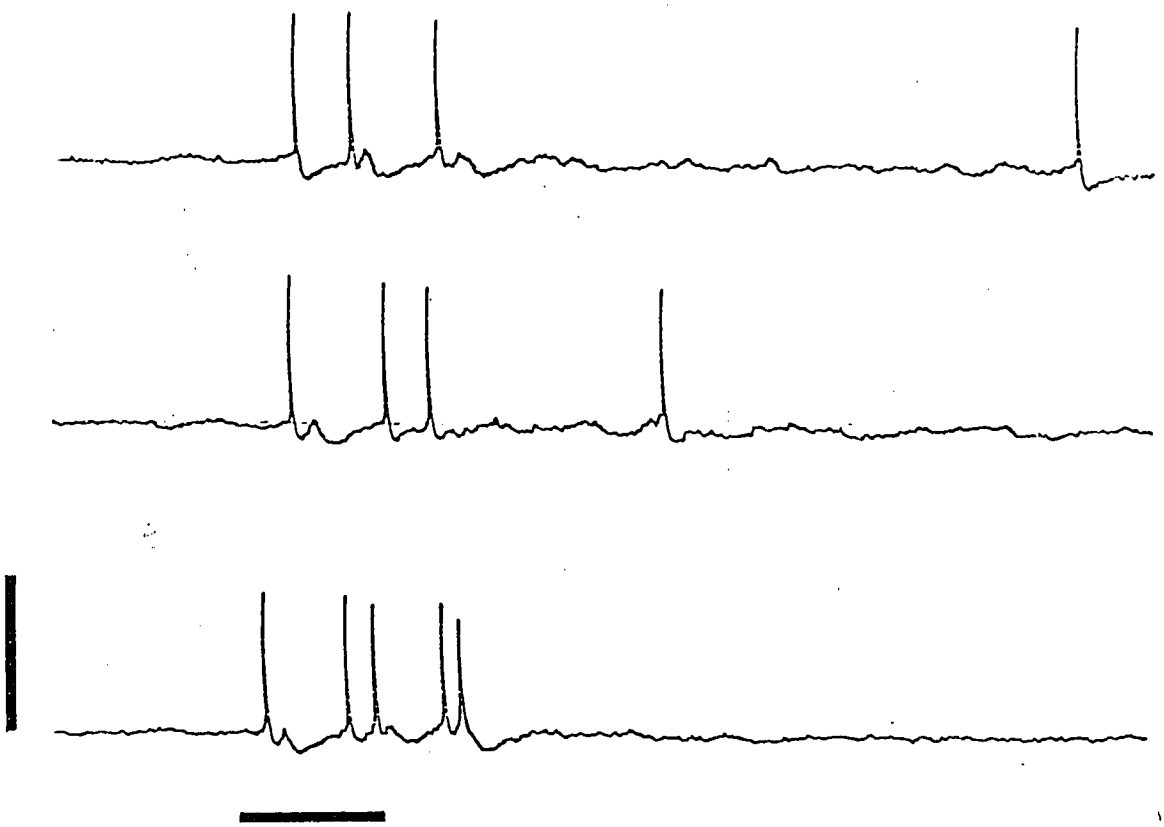


Figure 25

Intracellularly recorded short bursts



Three bursts from pair (24-2-1) are illustrated. Note that single spikes were not followed by depolarizing afterpotentials whereas spikes in burst were.

Time calibration: 140 ms.

Voltage calibration: 66 mv.

Fast components of spike distorted as this record from chart recorder. For more faithful reproduction of spike at faster sweep see Fig. 30 .

The variability and small number of cells exhibiting bursting is not unusual when it is noted that this is from a total of 12 relatively short records. However all of the data is consistent in showing the underlying event to be a large, composite depolarization. This is considered further in the interactions section.

IPSP's were less frequent than EPSP's, although results from electrical stimulation in the cortical region of the explant make it likely that most BS neurons receive inhibitory input. Two types of IPSP's have been observed. One type has a rapid rise time and short duration. Two neurons have been observed in which these were the primary synaptic events occurring, but the recordings were not of the best quality. The other type of IPSP has a slower onset and a time course of 100 - 200 ms These seem to be the complement of the PDS since in every case where one was observed, a diminution of the other cell's extracellular activity was observed for the duration of the IPSP.

3.2.2 Synaptic interactions

3.2.2.1 Spike to spike interactions

Because of the regular nature of most of the intracellularly recorded spike data, and its short length, it was not judged worthwhile to do CCH's of it with the extracellular spike train. Visual analysis of the ST's

revealed possible interactions, but in each case the amount of data was insufficient to convincingly illustrate the correlation.

3.2.2.2 Evoked PSPs

In every feasible case PSP's evoked from E2 were sought in E1. If large enough these could be visually detectable, but if not then extracellular spikes were used to trigger averaging of the intracellular synaptic noise. In order for a correlated voltage event to be considered an evoked PSP, it had to satisfy the following set of criteria: non zero latency, relatively constant duration and shape, as well as following a wide range of E2 firing rates. For a voltage event obtained by averaging the following additional criteria were required: no spikes present in the interval used for averaging, presence of a satisfactory extracellular control record made with the intracellular electrode just after leaving the cell, and the event appearing in several sequential averages.

All cell pair records were visually inspected for evoked PSP's by watching an oscilloscope sweep triggered by E2. Only one neuron was found in which there was an EPSP large enough to be readily seen above background intracellular activity. The neuron in which this EPSP occurred had a membrane potential of only 33 mV on impalement, and deteriorated rapidly. Figure 26 shows sequential EPSP's being triggered by the E2 spike. Initial EPSP's were

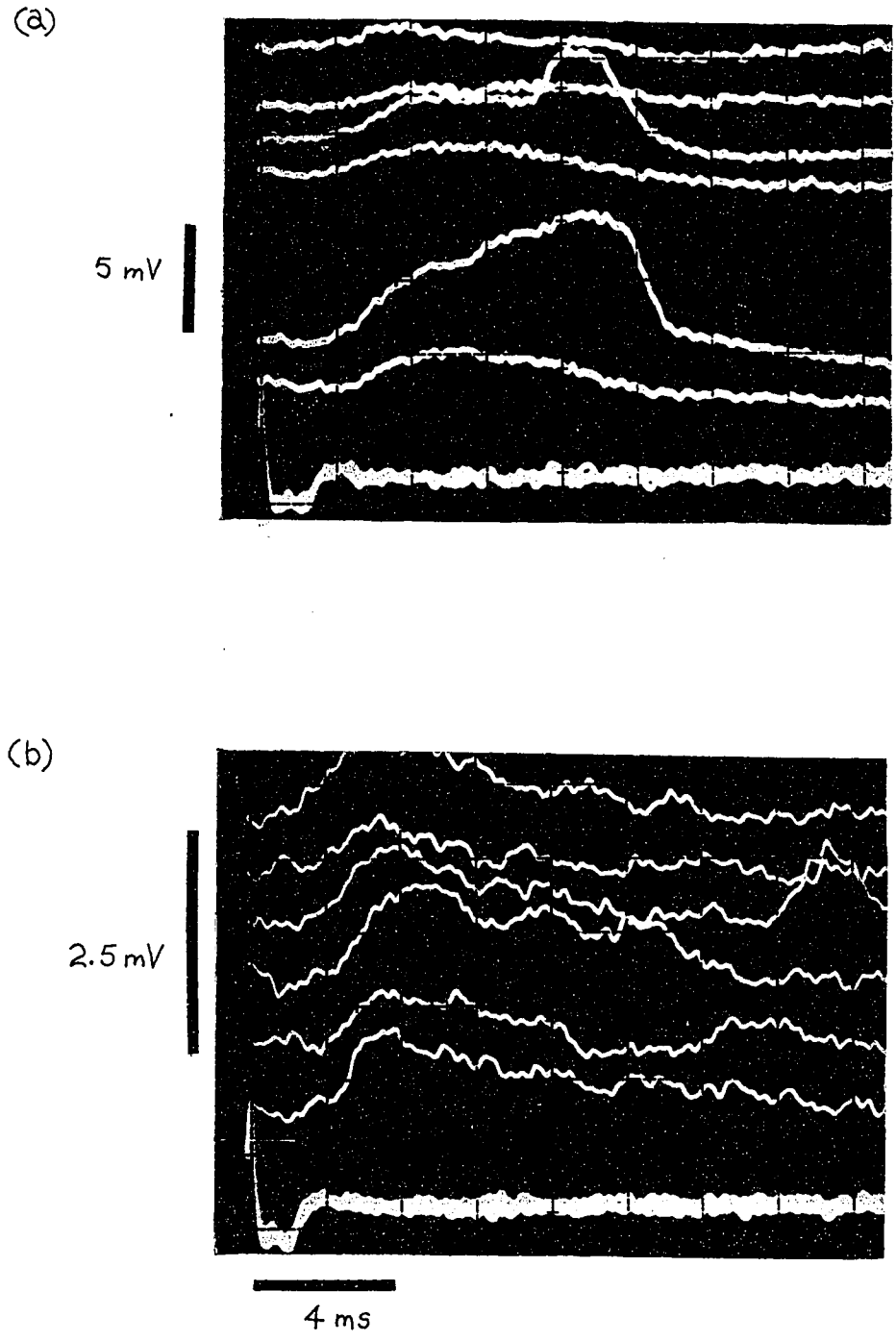
Figure 26
Evoked EPSP

This data from pair (23-27-6) with F1 intracellular, F2 extracellular. Time scale identical for both photos. Both photos consist of AC coupled intracellular potential with F2 spike (extreme bottom of picture) triggering sweeps. Successive F1 traces were manually vertically displaced. In (a), unaltered F1 data is displayed, but in (b) F1 has been low pass filtered (range 0 - 1 KHz) to better illustrate the EPSP's.

a). Evoked depolarizing potentials visible immediately after F1 penetration. Several other potentials such as large F1 potential (second from bottom) were seen in initial 15 sec of record, but not always associated with an F2 spike. These may represent abortive spikes, as they were no longer present once membrane potential deteriorated slightly.

b). F2 evoked EPSP's present in period after (a) taken. Size of evoked EPSP's in this photo comparable to small EPSP's visible in (a) despite deterioration of membrane potential. Much variability in size of EPSP's may be observed. Following this part of record EPSP size declined progressively, but EPSP's could still be consistently evoked by F2 spikes at rates of up to 12/sec.

Figure 26



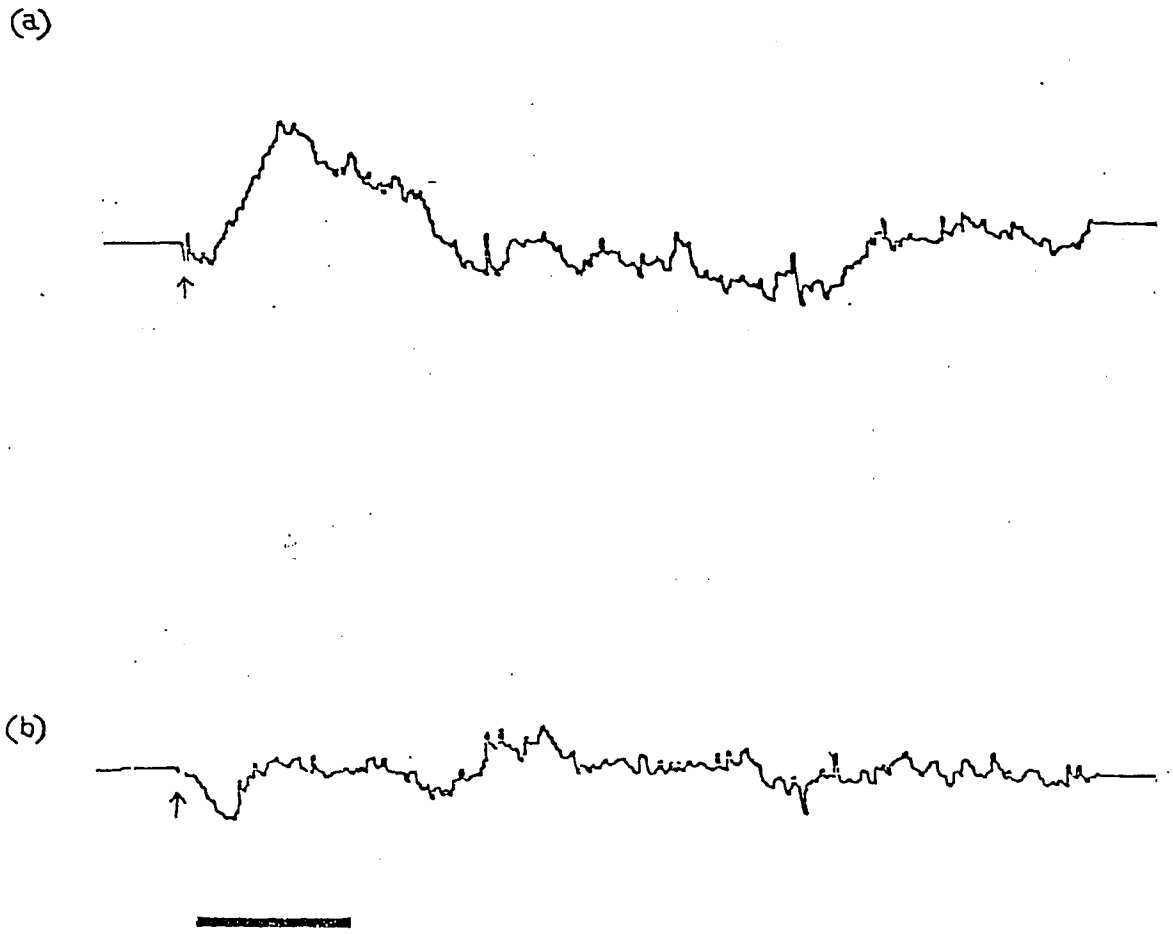
occasionally 5 mv or more in amplitude, and may represent abortive spikes. The EPSP latency was 1 - 2 ms, and considerable size variations occured. There is little doubt that this potential represents a true synaptic potential, but a more detailed study of the connection was precluded due to only about a minute of activity being recorded before severe P1 deterioration occurred.

This example illustrates well the problems caused by low quality recordings: this cell exhibited what looked like an EPSP, constant latency, and ability to follow at high P2 rates. The most puzzling aspect is the large size of the potential: in pair (20-7-4) the amplitude of the intracellular depolarization occurring with the extracellular burst is about 10 mV. Thus such a large potential would be expected to trigger at least one spike, if not a burst. It is possible that the large potential represents an abortive spike (this would also explain the occasionally anomalously fast fall time of the potential) and that further depolarization completely inactivated the spike generating mechanism thus allowing the pure EPSP to be seen.

All other correlated EPSP's were seen as a result of averaging. Two types of events were encountered: those which exhibited a definite latency, and others which appeared to have no latency at all. Only one cell satisfied all the criteria necessary to classify it as an evoked EPSP. (ie non zero latency and flat extracellular control). It is shown in Fig 27. Another case with a latency of 1.8 ms was seen, but no extracellular control was obtained.

Figure 27

Directional averaged evoked EPSP



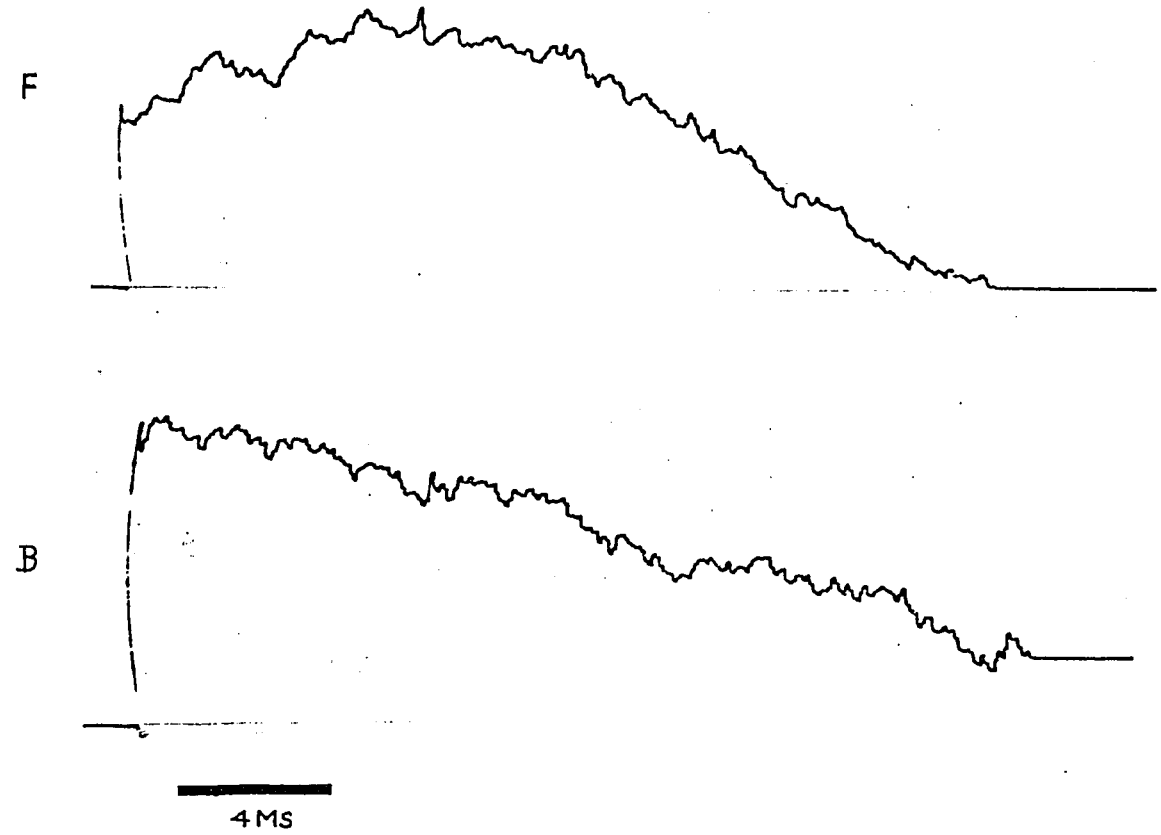
(a). EPSP evoked from E2 to E1 in pair (18-5-3). Arrow indicates where average starts. 32 E2 spikes used to do average shown.

(b). Extracellular control on E1 for this pair. 32 spikes also used in construction of this average.

Time calibration for both is 4 ms.

Figure 28

Bidirectional extracellular triggered intracellular potential



F represents averaged E1 potential triggering on E2 spikes in forward direction and B represents average triggering in backwards direction. 128 E2 spikes used in both forward and backward averages.

Extracellular controls on E1 not shown as they were not to scale as only about 55 E2 spikes present in control period. Both controls essentially flat. In order to make estimate of significance of illustrated intracellular averages, difference between max and min values (in the form of counts) in all averages and their controls were computed. Then by extrapolation (linear) from the control maximum differences, the significance of the events displayed is apparent.

Forward: Max - Min is 8579. Fwd control dif is 729 for 52 sweeps. Extrapolated dif for control is 1752.

Backward: Max - Min is 7663. Bwd control dif is 762 for 50 sweeps. Extrapolated difference for control is 1828.

The other class of EPSP like events consisted of a zero latency peak which could also be seen to extend backwards if reverse averaging was carried out. An example of such an event is shown in Fig 28. Interpretation of such events is difficult as they could represent common firing of other neurons in the culture synapsing on E1, or it could be the result of E1 and E2 receiving a common inhibition (Taylor et al,1978).

Table 5 contains a summary of all neurons in which averaging was carried out and the results. Rigorous criteria for identifying the event were satisfied in 3 cells, but the number of cells in which evoked EPSP's exist is likely larger.

3.2.2.3 Correlated bursting

In every case of an intracellular burst, a corresponding extracellular burst was seen. In Fig 24 the rate of firing in the extracellular cell is seen to roughly parallel the level of E1 depolarization. In the two longer bursts, no such relationship is visible, and it is merely possible to conclude that both neurons exhibited a shared increase in firing rate.

Fig 29 is a CCH manually constructed from E2 spikes to E1 spikes in pair (23-8-2) for the duration of the burst. The lack of any definite pattern shows that the relationship during a burst is not spike to spike. This lack of a

clear correlation in the CCH demonstrates the non-stationary nature of the burst, and supports the validity of rejecting the verdict of the CCH in favor of one from visual burst analysis when bursts are seen in the original ST.

The case of pair (24-2-1) is rather interesting because it seems to provide evidence for multiple spike generation (SG) sites in the BS neurons. (This is electrophysiological evidence; anatomical studies have shown multiple axon-like processes leaving the neurons). The spike seen on E2 during this record is rather small, and it was not noticed until the larger spike also present on the electrode temporarily stopped. In the portion of the record under consideration this cell did not fire once. This small spike was seen to faithfully follow each single E1 spike with a latency of less than a millisecond. Possible phase shifts occurring as a result of filtering make it meaningless to further refine the latency measurement. This interaction broke down during the bursts, for here E2 was seen to fire more spikes than E1, and to fire them at times when E1 was not firing. This is shown in Fig 30. Some of the E2 spikes were observed to occur during what appeared to be abortive spikes on E1 during the burst

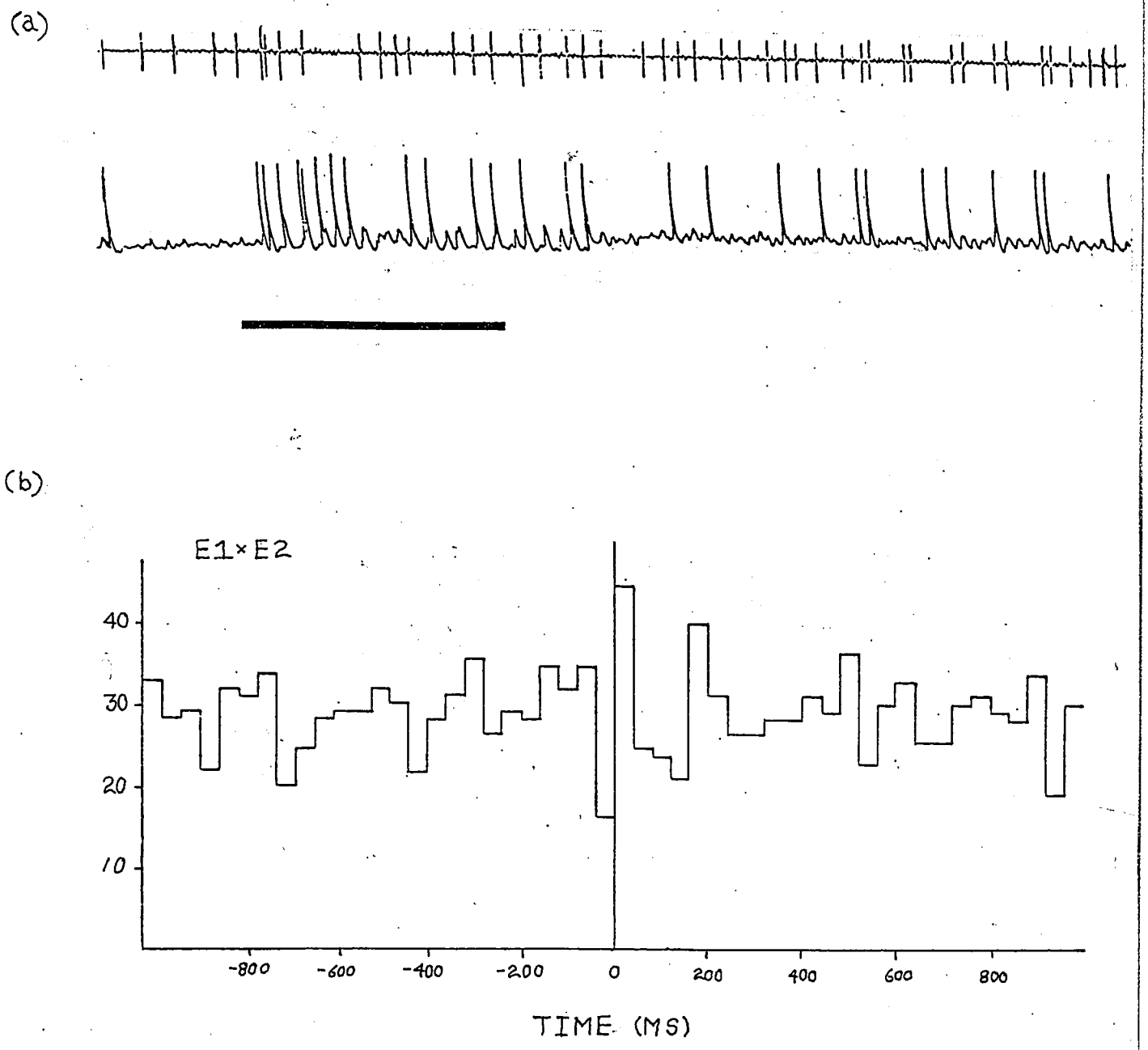
A number of possible hypotheses for this phenomenon were generated, and of these the most plausible were that the E2 spike was either from a second spike generation site or E1, or some type of field potential being generated at the E2 location by the spike generation site being recorded

Figure 29
Intracellular/extracellular long correlated burst

a). Chart record of both neurons as burst begins. Only increase in firing rate is evident in both cells. E2 top, E1 bottom. Time calibration is 1.0 sec.

b). Manually constructed (from film data) CCH from E1 spikes to E2 spikes during burst. Time calibration in ms.

Figure 29



by E1. Both of these hypotheses are equally plausible for single spikes, but it is difficult to account for the potentials seen during a burst via the field potential type of mechanism, especially as the amplitude of the E2 spike does not vary to the degree the E1 potentials during the burst are observed to vary.

3.2.2.4 Shared inhibition

Two cell pairs were encountered in which large IPSP's on E1 were associated with long pauses in the extracellular cell. Fig 31 illustrates a sample of the raw data from one such pair (23-3-1), and a manually constructed CCH from E1 IPSP onset to E2 spikes. The membrane potential of the intracellular cell in this record was 32 mv when the IPSP's were occurring and at this time it was too depolarized to fire full size spikes and thus the sharp positive going excursions probably represent a mixture of abortive spikes and EPSP's.

Even though this phenomenon was clearly seen in only two cells, it does appear significant that in both these cases the IPSP's occurred only when both cells were firing rapidly. Pair (23-3-1) showed no spontaneous IPSP's while the E2 cell was silent, but when the glu was put on E2 the spiky potentials appeared on E1 and E2 began to fire spikes. The onset of the IPSP's coincided with the start of the activity in E2. In the other case (pair (23-8-2)) the common inhibition was seen only after the cells had fired a long burst together.

Figure 30
Two spike generating sites in same neuron ?

In all photo's, intracellular DC trace on top and filtered extracellular spike on bottom. For (a) and (b), time calibration is 20 ms, and for (c) it is 40 ms.

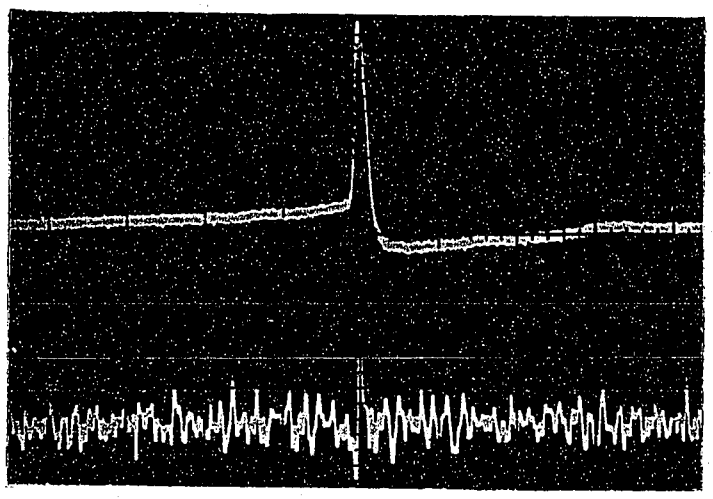
a). Typical correlation between single E1 spike and E2 spike.

b). Intracellular spike during burst. Depolarizing potential following E1 spike is associated with prominent E2 spike, and smaller E2 spikes appear to be present between first E1 spike and the depolarizing potential.

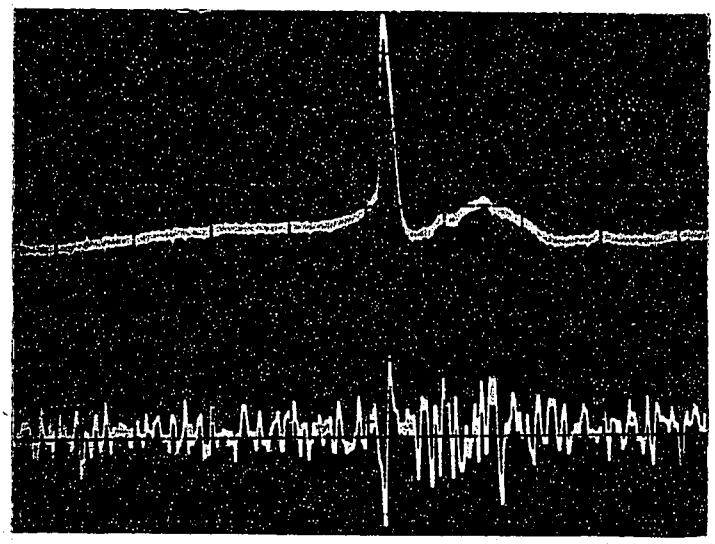
c). Slower sweep of another burst. Again, multiple potentials are associated with closely grouped E1 spikes.

Figure 30

(a)



(b)



(c)

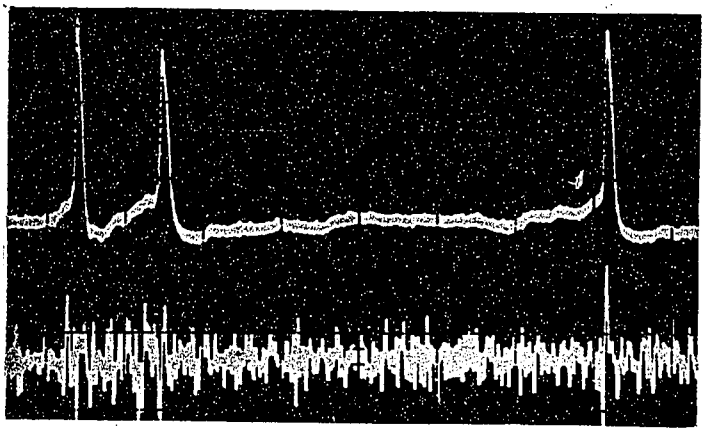
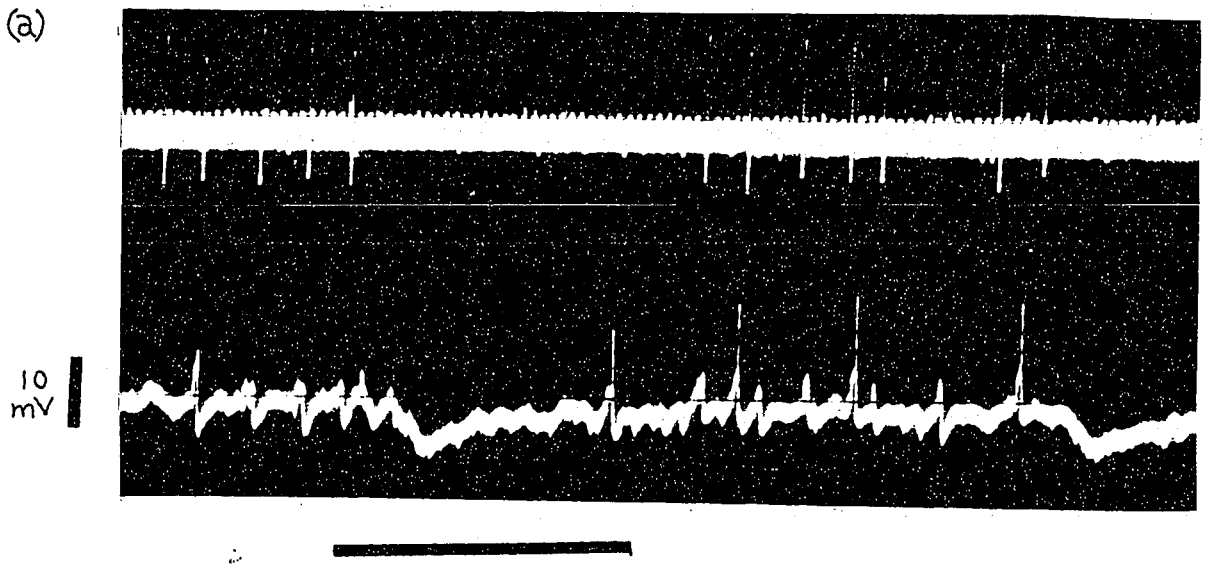


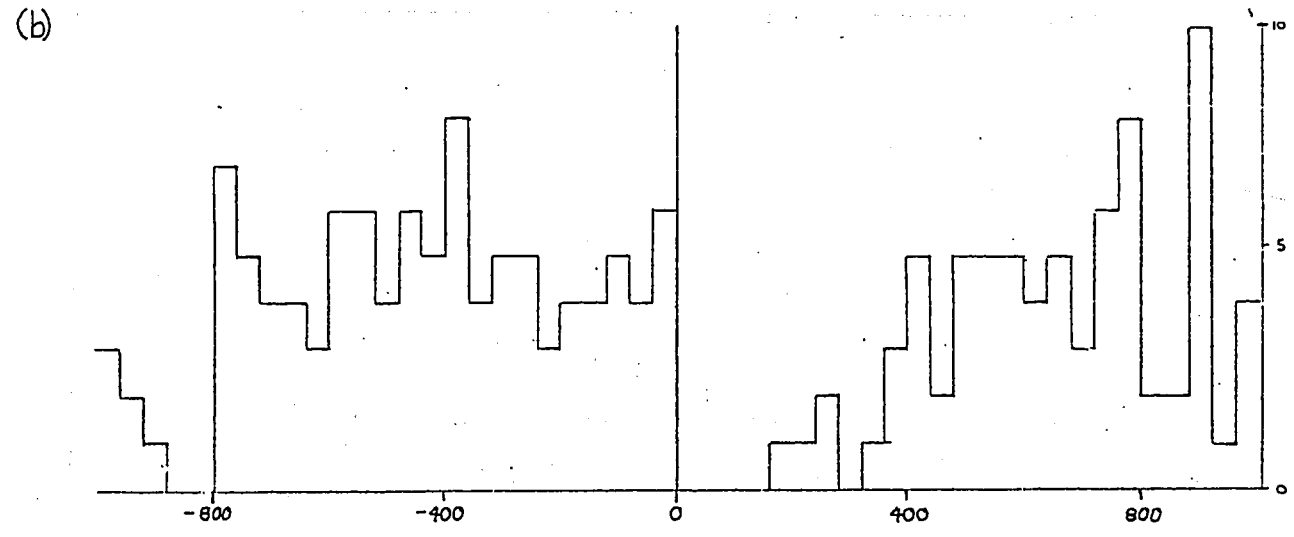
Figure 31
Common inhibition

a). Raw data recorded from pair (23-3-1). E2 extracellular (top) and E1 intracellular. Voltage calibration applies to E2 only. Time scale represents 0.5 sec.

b). CCH manually constructed (from film data) from onset of E1 IPSP to E2 spikes. 11 IPSP's were used in the construction of this CCH. Time units are ms.

Figure 31





3.2.2.5 Miscellaneous synaptic interactions

This category includes events which are presumeably due to synaptic interactions, but not in an easily classifieable way, and are thus lumped together.

In many cases the effect of iontophoresis of glutamate at a distant location was observed to increase the synaptic input visible intracellularly. This did not require that the E2 cell fire, and in most cases there was no correlation between the E2 spike and a distinct EPSP in E1. This demonstrates that the iontophoresis of glu is not specific to a single neuron, and most likely activates several neurons simultaneously. Only the neuron closest to the iontophoretic electrode is likely recorded from.

Another event indicative of some nature of synaptic interactions between the E1 and E2 cells was the observation in several cases of pronounced changes in E2 firing pattern following impalement of E1. This took the form of the silencing of E2 (most common) or the induction of a purely burst firing pattern in E2 (one case). All of these pairs when subsequently analyzed for spike to spike interactions did not yield any clearcut evidence of an interaction. Thus it is seen that the effects of the two neurons upon one another may be a result of their activity in determining the activity of the network. Thus the large increase in E1 activity generated by its impalement is sufficient to change the activity of the neurons synapsing on E2 to such an extent that an obvious

perturbation of E2 firing pattern is visible. (This method has been used by Schlapfer et al (1972) to demonstrate synaptic interactions among neurons by puncturing one neuron of a pair in such a manner as to kill it and noting the response of the other member of the pair in response to this).

3.2.2.6 Multiple interactions

Because of the greater information available as a result of intracellular recordings, it was anticipated that more multiple interactions would be encountered. This proved to be the case and revealed that a multiplicity of possible interactions may exist among all BS neurons. Because of the diversity of such combinations, two of the clearest examples will be used to illustrate the correlations encountered.

Pair (23-8-2) was found to have the following interactions: an evoked EPSP from E2 with 1.8 ms latency, a long period of mutually increased firing which was followed by the appearance of IPSP's in E1 associated with long interspike intervals in E2. As this pair was used in the construction of a CCH from E2 to E1 during the burst, and no strong evidence of an interaction obtained, it may be concluded that the evoked EPSP from E2 to E1 is ineffective in the modulation of the E1 ST.

For this pair it was also observed that the E2 activity pattern became one of more irregular spike firing

concomitant with an increased number of E1 EPSP's visible after the burst. For this pair of neurons it appears as if the interactions observed are due to strong common excitatory and inhibitory inputs, and thus the nature of the observed interaction will depend upon the correlation of the synaptic inputs to the two cells.

The pattern of common excitation and inhibition may also be seen in pair (23-3-1). In this pair the activity of both cells was nonexistent in the absence of iontophoresis of glu on E2. When this occurred the onset of E2 spikes and E1 EPSP's and IPSP's was rapid. The distribution of this common inhibition within the culture may be seen to be extensive as another cell pair recorded extracellularly on E1 with the same E2 cell experienced shared long interspike intervals. (Pair (23-1-1) which is shown in Fig 7 part (c)).

3.2.3 Influence of culture type on activity

In order to put together all of the intracellular and extracellular results in a form where the effects of the types of cultures and experimental conditions could be investigated on the interaction types, the intracellular results were first converted to 'extracellular results'. This was done by grouping all of the strong interactions observed intracellularly into their corresponding extracellular categories. When this was done, only one of the cell pairs with an evoked EPSP had an EPSP deemed sufficiently strong to significantly influence the firing

of the postsynaptic cell. Of the total 15 intracellular pairs considered, 9 were felt to have correlations of such a nature that they could have been perceived as being correlated had they been recorded extracellularly.

Table 6 is a total summary of the extracellular results, and Table 8 is a summary of the intracellular results. In the extracellular table, the 'quasi intracellular' cell pairs have been treated as extracellular cell pairs. All pairs are classified by their primary interaction in this table. Tables 7 and 9 are partitionings of this table on the basis of the type of culture, or the type of electrode configuration employed. To keep the numbers for comparison reasonably large, the use of 2 glu or 2 HCA electrodes has been taken as identical. No statistical analysis has been performed on the data as far too much variability is present in the data to give statistically meaningful results.

Two basic culture types could be distinguished in the experiments; BS and cerebellar cultures (BS-ctx) and pure BS cultures. This grouping is not as distinct as it once appeared as there are likely other variable cell types present in the BS area. Table 7 summarizes the interactions grouped by culture. While differences are evident, they are minor, and in view of the variability between the cultures, most likely not significant. This point will not be pursued further here as it is discussed later.

A far greater influence on the results was evident from the type of electrode configuration which was used as may be seen in

table 9 . Again, as in the case of the culture comparison, no statistical evaluation was performed, but it is clear that almost exclusively correlated bursting interactions are present in the configuration with two glu electrodes. Roughly equal numbers of cell pairs were obtained from either pure BS or BS-ctx cultures using the two electrode configurations, and the results were the same here when the breakdown was made in this manner. The effect is most likely a direct interaction of the glutamate with the culture, and how this may be achieved is discussed in discussion.

TABLE 1

Directional spike to spike correlations

PAIR #	DIRECTION	LATENCY (MS)	STRENGTH	TEST
(2-3-3)	E1 to E2	20	1.00	no
(3-2-1)	E1 to E2	35	0.69	no
(3-4-1)	E1 to E2	<40	0.45	no
(23-1-1)	E2 to E1	<15	0.53	yes
(23-2-1)	E2 to E1	20	0.13	no

DIRECTION - of correlation.
LATENCY - Approximate latency of maximal response in follower cell. When < before latency value response is constant over that time period.
STRENGTH - Given by ratio:
(#spikes follower cell)/(# spikes leader) with the spikes occurring in the relevant time period
TEST - Indicates whether or not test for monosynaptic excitation made during record.

TABLE 2

Summary of computer analyzed cell pairs

CULTURE	TOTAL	CCH1	CCH2	CCH3	VCOR	TCOR
2	1	1				1
3	1	1				1
4	3	1	1	1	1	2
6	1	1				1
9	5		1	4	1	1
10	1	1				1
11	2	1	1		1	2
14	4	3	1		1	4
15	4	4				4
17	6	1	2	3	4	5
18	1		1			0
19	2		2		1	1
20	2	1	1		1	2
TOTALS	33	15	10	8	10	25

TOTAL - number of pairs from culture analyzed by computer.

CCH1 - Obvious correlations.

CCH2 - Unable to decide on interaction by CCH alone.

CCH3 - No correlation apparent from CCH.

VCOR - Number of pairs in groups CCH2 and CCH3 in which CB detected visually.

TCOR - Total number of pairs showing correlation (CCH1 + VCOR).

TABLE 3

Common firing cell pairs

PAIR #	SEPARATION	RATE
(14-1-1)	<1	F
(24-3-1)	<4	S
(24-4-1)	16	VS
(24-6-2)	1	VS

SEPARATION - temporal separation between spikes in ms.

RATE - of firing within cell pair:
F - fast
S - slow
VS- very slow

TABLE 4

Good intracellular recordings parameters

PAIR #	Em	TOT	SPIKE	AHP
(18-3-3)	50	90	75	15
(18-4-3)	84	66		
(18-5-3)	56	60		
(18-6-3)	44	45		
(18-7-3)	46	52		
(20-7-4)	40+	82	65	17
(23-4-2)	40	60	47	13
(23-8-2)	45	62	51	11
(23-13-3)	40	86	73	13
(23-14-3)	43	64	52	12
(23-22-5)	40	39	30	9
(24-2-1)	46	79	66	13

Em Initial membrane potential (mv).

TOT SPIKE + AHP (also in mv)

SPIKE Magnitude of spike (mv).

AHP Magnitude of afterhyperpolarization in mv.

TABLE 5

Results of averaging for PSP

PAIR #	PSP	CNTRL	LAT
(18-3-3)	DEPSP	1	0.8
(18-4-3)	x	2	-
(18-5-3)	NEPSP	2	0
(18-6-3)	NEPSP	2	0
(18-7-3)	x	2	-
(20-7-4)	x	2	-
(23-4-2)	x	1	-
(23-8-2)	DEPSP	2	1.8
(23-12-3)	x	1	-
(23-14-3)	NEPSP	1	0
(23-27-6)	DEPSP	1	1.8

PSP Type of PSP

x - none

DEPSP - directional EPSP

NEPSP - nondirectional EPSP.

CNTRL Presence or absence of control on E2.

1 - Control present.

2 - No control present

LAT Latency of PSP in ms.

TABLE 6

Total extracellular correlations

CULTURE NC	CULTURE NP	PDEL TS	PDEL TN	PDEL MS	INTERACT DEF	INTERACT ?	INTERACT NI	INT TYPE DE	INT TYPE CF	INT TYPE CB	INT TYPE AF	INT TYPE EI	EXPDA CUL	EXPDA EL
2	6	3	-	-	3	-	-	1	-	2	-	-	1	1
3	6	3	-	-	3	-	-	2	-	1	-	-	2	1
4	10	3	-	-	3	-	4	-	-	3	-	-	1	1
5	3	-	-	-	1	-	2	-	-	1	-	-	1	1
6	3	-	-	-	3	-	-	-	-	3	-	-	2	1
9	7	2	-	-	1	-	4	-	-	1	-	-	1	2
10	4	2	-	-	2	-	-	-	-	1	-	1	1	2
11	11	1	-	1	5	1	3	-	-	3	-	2	1	2
12	3	2	-	-	1	-	-	-	-	1	-	-	1	2
14	7	-	-	-	7	-	-	-	-	7	-	-	3	3
15	11	1	1	-	7	2	-	-	-	7	-	-	3	3
17	8	-	-	-	5	1	2	-	-	5	-	-	2	2
18	2	-	-	-	1	1	-	-	-	1	-	-	1	1
19	6	1	-	1	2	1	-	-	-	-	-	2	2	1
20	6	2	-	-	3	-	1	-	-	2	1	-	3	1
21	14	3	-	5	1	2	3	-	-	1	-	-	2	1
23	12	-	-	1	6	3	2	2	-	2	1	1	3	1
24	5	-	-	-	5	-	-	-	3	2	-	-	1	1
TOTALS	124	23	1	8	59	11	21	5	3	43	2	6		

Legend for Table 6

CULTURE

NC - Culture number.
NP - Number of pairs in that culture.

PDEL - Breakdown of pairs deleted from analysis.

TS - Records too short.
TN - Records too noisy.
MS - Mechanical stimulation of neuron occurs during record

INTERACT - Breakdown of interaction classifications.

DEF - Number of definate interactions that occur.
? - Number of pairs for which no decision is possible.
NI - Number of pairs with no interaction.

INT TYPE - Breakdown of interactions into classes.

DE - Directional excitation (spike to spike)
CF - Common firing (spike to spike)
CB - Common bursting (all types)
AF - Alternate firing.
EI - Enigmatic interactions.

EXPDA - Experimental data parameters.

CULA - Anatomical type of culture.
1 - BS + cortex.
2 - BS only (pure).
3 - BS only with other unknown cells.

EL - Electrode configuration employed.
1 - One glu electrode and one single barrel electrode.
2 - Two glutamate electrodes.
3 - Two homocysteic acid electrodes.

TABLE 8

Summary of intracellular results by culture

CULTURE	TOT	NC	NNC	AESP	CB	CI
18	5	2	3	2		
20	1	1			1	
23	8	5	3	3	2	2
24	1	1			1	
Totals	15	9	6	5	4	2

TOT - Total intracellular in good or correlated categories.

NC - Number of pairs with correlation.

NNC - Number of pairs with no correlation.

AESP - Number with averaged EPSP.

CB - Number with correlated bursting.

CI - Number with common inhibition.

Intracellular results were converted to 'extracellular' results by consideration of how evident the interaction would likely have appeared had the same cell pair been recorded extracellularly only. Of the 15 intracellular pairs, 5 were judged to be 'correlated' on this basis, and the other 10 were considered to be non-correlated. The 5 correlated cells had the following correlations:

DE = 1
CB = 4

There were also two intracellular pairs in which common inhibition was seen, but these were not included in the total tally of interactions due to the unpredictability of verifying such interactions in the purely extracellular data.

TABLE 7

Interactions grouped by culture type.

(a). Total numbers of interactions.

TYPE	TOT	NP	NC	N?	NNC	DE	CF	CB	AF	EI
BS	82	18	43	9	13	5	0	32	2	4
BS-CTX	55	14	21	2	18	1	3	15	0	2
TOTALS	137	32	64	11	31	6	3	47	2	6

(b). Percentages of each category in table as fraction of total number of pairs in category.

TYPE	NP	NC	N?	NNC	DE	CF	CB	AF	EI
BS	22	51	11	16	6	0	38	2	5
BS-CTX	25	38	4	33	2	5	27	0	4

(c). Percentages of correlated cell pairs in each group within each type of correlation category.

TYPE	DE	CF	CB	AF	EI
BS	12	0	75	4	9
BS-CTX	5	13	71	0	11

TABLE 9

Interactions grouped by electrode configuration

(a). Total numbers of interactions.

TYPE	TOT	NR	NC	N?	NNC	DE	CF	CB	AF	EI
1+2	87	22	37	6	22	6	3	22	2	4
2+2	51	10	27	5	9			25		2
TOTALS	137	32	64	11	31	6	3	47	2	6

(b). Percentages of each category in table as fraction of total number of pairs in category.

TYPE	NR	NC	N?	NNC	DE	CF	CB	AF	EI
1+2	23	47	8	23	7	4	24	2	5
2+2	20	53	10	18			49		4

(c). Percentages of correlated cell pairs in each group within each type of correlation category.

TYPE	DE	CF	CB	AF	EI
1+2	17	10	57	5	12
2+2			92		8

Chapter IV

DISCUSSION

4.1 SYNAPTIC INTERACTIONS.

4.1.1 Effect of Glutamate on BS neurons.

4.1.1.1 Standard effects.

From the experimental data, it can be seen that most BS neurons are excited by glu, in keeping with the findings of other workers on neurons of the CNS. What is interesting is the responsiveness variation that is encountered among the BS neurons. The most relevant possible explanations for this are the dendritic geometry of the neuron, the diffusion geometry of the glu around the tip, and the possible presence of especially glu sensitive areas on the dendrites of the recorded neuron (hot spots in the terminology of Ransom et al, 1977c). The consideration of these possibilities which follows is almost entirely theoretical due to the obvious difficulty of measuring the relevant data. When the analysis of iontophoresis of glu is considered from a theoretical point of view (Gradisek et al, 1978), solution of the diffusion equations shows that the concentration of glu around the tip is spherically symmetrical. Depending on the orientation of the tip to the target membrane, and the distance of the tip, quite different depolarizations can be produced. In culture it may be expected that the presence of cellular elements will distort the diffusion pattern to a

variable extent. In the model of glu diffusion considered, the fall off in glu concentration may be approximated by an exponential function, and in the steady state condition, a greater glu current would have a greater distance of action.

No consideration of any of the factors which could enter into the determining of the glu diffusion pattern was done on a detailed level due to the complexity of the problem, and the virtual impossibility of performing such measurements in the cultures. However, consideration of the theoretical situation easily leads to the conclusion that once a steady state is attained, the effects of a given current should be reproducible. One factor which was not considered (because of its theoretically more complicated nature) was the possibility that it was changes in the glu current (and subsequent change in glu release rate) that were responsible for some of the observed effects in the culture. Presumably this would be a possibility in some of the phasic glu dependent interactions, but since many of the interactions were tonic, then in long records with low to moderate glu currents, the assumption of a steady state glu diffusion pattern seems reasonable.

The effect of glu can be considered to be primarily on the dendrites of the BS neurons. This has shown to be the case in other systems; in cat spinal motorneurons Zieglgansberger and Champagnat, (1978) demonstrated that the latency of intracellulary recorded depolarization increased with increasing distance of the glu electrode from the neuron. Also Barker and Ransom (1978) working in cultures of dissociated spinal cord

found that excitatory responses were obtained from all the dendrites of neurons on which they iontophoresed glu. Both they and Ransom et al, (1977c) found areas of the dendrites where far larger depolarizing responses were obtained (hot spots). The implication from this is that all dendrites of neurons which pass through the area of glu diffusion will be depolarized to some extent.

4.1.1.2 Glu adaptation.

One factor which makes the data here difficult to interpret is that on the basis of extracellular recordings it is not possible to determine whether the observed adaptation response is due to a direct effect of the glu on the neuron, or to a synaptically mediated effect caused by the activation of nearby neurons. The glu adaptation response for a given neuron is quite variable over time, and in the neuron used for the adaptation example, there were times when no adaptation response occurred. The contribution of the various factors in the production of the response could be determined either by intracellular records of the glu response (not done) or by study of adaptation in the presence of agents (such as manganese) which block synaptic transmission.

The mechanism of the glu adaptation could be considered as that of firing rate adaptation which is observed to occur in most neurons in response to a constant current applied intracellularly. Zieglgansberger and Puil (1973) have presented evidence that this may not be the case, for they found that in

cat spinal motorneurons, iontophoretic glu did not produce sustained firing even when the intracellularly recorded depolarization was more than adequate to produce repetitive firing in the cells had it been produced by by an intracellularly injected current. They hypothesized that this was somehow related to the membrane resistance of the neuron, since motorneurons, being large cells, had low membrane resistances, whereas other cells which they found could be repetitively driven by glu all had high membrane resistances, and would represent smaller cells. Two conclusions can be drawn from this observation. If the response of the motorneurons is not peculiar to them, then by the type of glu response seen, it should be possible to determine roughly the size of the neuron being recorded from. Secondly, since high glu currents cause an increase in membrane conductance, and at these current levels the spike is generally observed to fail, and also adaptation is faster, it may be that the adaptation is dependent on the increase in membrane conductance caused by the glu. Dostrovsky and Pomeranz (1977) have also studied the effects of glu on spinal cord neurons (recorded extracellularly) and encountered both lack of adaptation and adaptation to glu iontophoresis. Because the firing rate adaptation to glu was not affected by prior activation by aspartate, they interpreted the results as due to desensetization of specific glu receptors.

In the BS cultures, the adaptation of many neurons was minimal, and this could be related to the low glu currents generally used. In the papers discussed, the currents used were

much higher, on the order of 200 na and up, and while it is risky to compare iontophoretic current data from system to system, here the difference seems to be of a sufficient magnitude to infer that the low currents employed in most experiments with the BS neurons did not cause appreciable adaptation. This was essential for doing meaningful ST analysis on the data, as shared rate changes in neurons can give spurious correlations (Perkel et al, 1967b) Evidence for faster adaptation at high glu concentrations is provided by the work of Gahwiler (1976) who applied glu in the bathing medium of cerebellar cultures, and noted that low concentrations of glu caused a sustained increase in firing frequency of the neurons, while higher glu levels caused only transient frequency increases, and overall were depressant. Neurons with phasic responses to glu in the BS cultures have been insufficiently characterized to make any decision on the reason for their glu response.

4.1.1.3 Glu inhibition.

Despite the almost universal excitatory action of glu on various neural systems, direct glu inhibition has been reported by Yamamoto (1978), Yamamoto et al, (1977). Here the most critical fact to be proven is that the inhibition is in fact direct, and not the result of the glu activating a nearby projection of inhibitory neurons which project to the neuron being recorded. In the work done by Yamamoto (on cerebellar slices), the glu inhibited cells were located in the granular

layer of the cerebellum, and the inhibition persisted even when bic perfusion was employed (on the assumption that all cerebellar inhibition is GABA mediated) or when magnesium was used to stop synaptic transmission. Evidence was also available for a dual effect of the glu with the inhibitory response being inactivated relatively quickly, and the neuron being excited later.

The cases of apparent glu inhibition encountered among the BS neurons are not decisive as the appropriate control experiments have not been done. Three possible explanations may be offered for the observed inhibitions in BS:

1. The inhibition is a direct effect.

2. The inhibition is the result of inhibitory neurons being activated which inhibit the neuron being recorded.

3. The inhibition is due to a current effect from the iontophoretic electrode to the neuron, and if this is so many anomalous types of responses can be expected (Zieglgansberger and Puil, 1973).

The adaptive and inhibitory responses described herin could reflect unusual reponses of the BS neurons to glu. This possibility has not been pursued as part of this study, but should be further investigated, using intracellular recording.

4.1.1.4 Modulation of neuronal activity by glu.

The investigation of the interaction of iontophoretically applied glu with the synaptic input received by a neuron has been investigated by Zieglgansberger and Puil, (1973) and Denavit-Saubie and Champagnat, (1978). The possibilities for interaction are quite large, and so only a general treatment of the topic will be performed. One effect of glu application is the transformation of subthreshold EPSP's to spikes. This is presumably due to the summated depolarizations of the EPSP and glu being sufficient to exceed the firing level of the cell. In motorneurons, Zieglgansberger and Puil, (1973) found that the effect was not a simple summation since EPSP's with fast rise times were apparently more effective in eliciting spikes with glu application than slower rising EPSP's. This they interpreted as an effect of the glu increased conductance on the membrane time constant which selectively reduced the size of slower rising EPSP's. They also observed a reduction in the sizes of both EPSP's and IPSP's which was a function of the iontophoretic current. This was much more pronounced for IPSP's and occasionally an IPSP could be reversed. It is possible that glu may in some cases produce bursts in the neuron by an intrinsic mechanism, for (Zieglgansberger and Puil, 1973) reported occasional conversion of the AHP following a spike into an afterdepolarization. In hippocampal cultures, Zipser et al (1973) have demonstrated that the mechanism of burst generation in these neurons is an afterdepolarization.

It is possible to use the effect of applied glu to reveal the synaptic events underlying a pattern of activity in a neuron as has been done by Denavit-Saubie and Demontigny, (1978) in respiratory neurons of the cat. Respiratory neurons studied were either inspiratory or expiratory neurons, and fire bursts during the corresponding phases of the respiratory cycle. If this pattern were due to alternating excitation, identical (except for phase shift) effects of iontophoresed glu would be expected on the cells. Since different effects of glu on the two classes of neurons were obtained, the implication is that two different sources of synaptic input are responsible for the pattern of firing in respiratory neurons.

Within the BS cultures, low glu currents were employed with most neurons in an attempt to see what the subthreshold synaptic input to the cell was. This data could not be quantitatively incorporated, but was efficiently used to get interactions occurring among cells. (This may be an artifact of the changing glu current modifying whole culture activity rather than elevation of EPSP's correlated with the other cells firing past threshold as was generally assumed). The glu modulatory effect was utilized extensively in experiments, but aside from saying that the effects of a given glu current on an interaction were often reproducible, no inferences regarding the mechanism of the interaction can be made.

One factor which seems to be reasonably certain is that cells which fire regularly over the entire range of glu currents studied are not receiving any significant synaptic input during

the time of glu application (this requires that they be completely inactive when no glu is being applied). Regular firing has been used by some investigators as evidence of no synaptic input to a cell. In PN's in culture, many of which are spontaneously active, Schlapfer et al (1972) considered regularly firing cells to be isolated, and Gahwiler et al (1973), also working in cultured cerebellum found that addition of magnesium to the bathing medium would lead to either regular firing in a cell, or complete cessation of firing. Another possibility which must be considered is that a regularly firing neuron is receiving a large number of uncorrelated EPSP'S which would have the same effect as a constant depolarization (Segundo and Perkel, 1967). If this is the case in the regular glu driven neurons in BS cultures, then this non-correlated synaptic activity must be of low amplitude to account for the lack of spontaneous activity, and must also remain uncorrelated when the glu is applied. On the basis of intracellular recordings it appears as if the culture is very inactive in the absence of glu application.

In two experiments HCA was employed instead of glu. For the purposes of this study the effects of these two compounds will be considered as identical although a number of differences do exist among them. HCA has been reported to not cause an increase in the membrane conductance, or even to cause a decrease (Engberg et al, 1975). If it is in fact true that there is less of an adaptation with the HCA, this could account for the qualitative observations that HCA seemed to be better at

exciting neurons tonically without causing large spike size decreases. The duration of action of HCA is also reported to be longer than that of glu (Watkins, 1978) since HCA is not readily taken up by neurons. For the purposes of this study these differences can be assumed to be minimal, as they do not substantially affect the model to be presented for the effects of excitant amino acids on the BS cultures.

4.1.2 Direct evidence for synaptic interactions

Only the combined intracellular/extracellular recordings gave direct evidence of synaptic interactions, and these were most definite in the direction E2 to E1. Common activity could not be as rigorously proved, although the assumptions made on the basis of combined intra/extra recordings allowed the distinguishing of many more interactions unambiguously than did the totally extracellular records.

4.1.2.1 General intracellular synaptic activity

Several features of the intracellular activity were apparent which may be related to the activity observed when low glu currents were used in the extracellular recordings. One feature which appears significant is the very low rate, or total absence of synaptic activity observed when intracellular experiments were performed in the absence of a glu electrode in the culture. This suggests that much of the synaptic activity observed in the intracellular recordings which were carried out with the extracellular electrode also in the culture was artifactual in

the sense that it would not likely be present had only intracellular recordings been done. It appears that activity in some of the neurons is induced no matter where in the culture iontophoresis of glu occurs. This matter will be dealt with in more detail when the effect of glu on interactions is considered.

Another feature which appeared to be significant was the almost exclusive observance of EPSP's. This is unusual as this phenomenon was as marked in the BS-ctx cultures as it was in the pure BS cultures. Electrophysiological stimulus-response studies done by K. C. Marshall (unpublished results) indicate that neurons in the BS region may receive inhibitory synaptic input from PN's.

A possible explanation for this phenomenon utilizes the projection from PN to Deiters neurons in vivo as an analogy. In this projection, the size of the unitary evoked IPSP is on the order of 200 microvolts, and the combined effects of the total projections to a given neuron result in a continuous hyperpolarization of the neuron due to the tonic bombardment from the PN's (Eccles et al, 1967) This could also be occurring within the BS cultures, although this would require that the activity of the PN's in the cortical area be non-correlated.

The EPSP's observed exhibit no remarkable features except for their apparent tendency to occur in groups. This feature has been noted in several intracellular records when little synaptic input was visible. In two of these neurons which were not firing rapidly following electrode penetration, spikes could

be seen to be evoked by these grouped EPSP's. This could explain the phasic activity which was usually visible in most spontaneously active BS neurons, but how the activity begins, and what terminates it cannot be answered. This phenomenon, involving shared input which is temporally patterned in two neurons may be responsible for some of the directional spike to spike interactions which were observed.

4.1.2.2 Correlated Intracellular extracellular activity

I. Direct excitatory.

Of the interactions observed, most were what appeared to be direct synaptic interactions from E2 to E1. All except one of these had very small EPSP's and thus required averaging for their demonstration. The latencies of the evoked EPSP's were quite short and were in the range of about 1 to 2 ms. The preponderance of the shorter latency EPSP's suggests that some of them could have represented a common excitatory input to the two cells.

The averaged forms of the EPSP's looked as if they were unitary, but careful examination of the intracellular record on the scope triggering on E2 spikes seemed to show a number of components in all of the records which were examined. This does not necessarily imply that the averaged EPSP is the result of several EPSP's which are consistently evoked following the E2 spike, as the different shapes could also represent the modified EPSP (unitary) with the modifications arising from the interaction of the EPSP with the other synaptic potentials

impinging on the neuron. It is also possible that the EPSP is the result of a distributed synapse on the postsynaptic neuron. To give the large variations sometimes seen in the size of the synaptic potential, a high failure rate at axon branch points would be required.

Averaged PSP's that had zero latency, and also extended backward in time could be the result of either common excitation or common inhibition (Taylor et al, 1978). The predominance of common excitation among the BS neurons, as well as the failure to detect evoked inhibition from one BS neuron to another make it very likely that these interactions do in fact represent common excitation received by the two neurons.

It is interesting, but perhaps not surprising in view of the small number of intracellular pairs, that only one case of directly evoked excitation with an EPSP large enough to be visible occurred. If the ambiguities mentioned in the results section in regard to this cell pair (23-27-6) are disregarded for now, then two explanations are possible.

One explanation is that this phenomenon represents nothing unusual, and that the BS neurons consist of a randomly connected network of excitatory neurons. On this basis one could expect the majority of synapses to be weak, but there would occur at one extreme, heavy projections from one neuron to another. This is attractive from a modelling viewpoint, but there is no evidence that the BS - BS interconnections are random. The presence of highly organized projection patterns in explant culture systems DRG to spinal cord (Ransom et al 1977b),

Cerebellar cortex to DN (Wojtowicz et al, 1978), retina to tectum (Smalheiser and Crain, 1978), makes it likely that the connections which are occurring within the BS cultures are also organized in some fashion, and the fact that they look random may only reflect the inability to fathom the organizing principles involved in the specification of the connections.

The other possibility is that there exist different neuron types, some of which form stronger connections than the majority of BS neurons. On the basis of the extracellular results, it would appear that their proportion in the culture would be low, but this is based on data which mainly involve the use of two glu electrodes which could obscure this interaction through the induction of correlated culture activity.

II. Common Excitatory Interactions.

With regard to common excitation in intracellular extracellular pairs, there also seemed to be a continuum of strength of the common input. The lower orders of correlation were not analyzed in a quantitative manner, but several common categories were noted:

1. Shared excitation from glu induced activity. This pattern consisted of no activity in either cell until the glu was put on eject, and then synchronous onset of spikes in the glu stimulated cell, and EPSP's/spike occurred in the intracellular cell. This activity generally had no spike to spike correlation or E2 spike EPSP correlation.

2. Grouped EPSP's and greater firing rate briefly in the E2 cell. This was seen on several occasions, and would likely correspond to shared non-decremental bursts observed in the extracellular pairs.

3. Correlated bursting. As this will be considered further later, only the following points will be made now.

   a) Synaptic correlate of the correlated burst is a large irregular depolarization whose onset is roughly synchronous with the extracellular burst, and whose time course parallels the extracellular bursting.

   b) The burst is almost certainly a composite event made up of numerous summated EPSP's as both its shape and duration are highly variable from burst to burst.

III. Common inhibition

Two cases of common inhibition were encountered in which the relationship was very clear. One extracellular pair (23-1-1) also can be said to most certainly experience common inhibition as the E2 cell exhibited long pauses correlated with IPSP's in the intracellular cell in pair (23-3-1). The consecutive set of three pairs recorded with E2 in this culture demonstrates well the value of intracellular records in the elucidation of multiple interactions. What is not clear is the

extent of such strong inhibition in the cultures as no other inhibitions of this nature were recorded in the other cultures. This particular culture was a BS only culture which was contaminated with other pontine neurons. Thus the inhibition could be either from an unknown neuronal type in the explant, or from PN's some of which are almost always explanted along with the tissue fragment used for the BS only cultures.

The large amplitude (6 - 10 mv) and duration of about 200 ms (or more) are much larger and longer than the IPSP's resulting from cortical stimulation (Wojtowicz, 1978). It is possible that correlated PN firing could be responsible, but this is speculative. An interesting property of the IPSP's in this culture was their association with both bursting, and other activity in the culture. In pair (23-3-1) no IPSP's (or for that matter any synaptic activity) were observed until the glutamate on E2 was put on, and then both spike-like potentials as well as the IPSP's began in E1, along with the activity in E2. In pair (23-8-2) no IPSP's were observed until the neurons fired a long correlated burst, and then a similar pattern of correlated IPSP's with E2 silent periods occurred. This suggests that either reciprocal connections exist between the excitatory and inhibitory cells, or that the glutamate is activating both excitatory and inhibitory cell populations. No cases of direct inhibition from one BS neuron to another have been observed.

4.1.2.3 Indirect evidence from extracellular data

While abundant evidence for the excitatory nature of the BS interactions exists, the small fraction of cell pairs which exhibited what could be reasonably termed evidence of direct synaptic excitatory interactions is puzzling. When these cell pairs are considered in more detail, evidence can be found for other possible circuits being responsible for the observed interaction.

One of the advantages of being able to modulate the activity of each of the neurons over a wide range was thought to be decision of whether presumed directional excitatory interactions were actually that or the result of shared excitation. All cases of directional excitation occurred when only one glutamate electrode was employed. It is highly unlikely that this is coincidental in view of the electrode configuration being more highly correlated with the type of interactions observed than the culture type (table 9). This suggests that the observation of direct synaptic interactions may not be possible with the use of two glu electrodes, because the activity patterns induced within the culture overwhelm the effects of the synaptic connection.

A number of possible explanations may be offered for the directional interactions observed of which the simplest is that the correlations may represent direct synaptic connections between the two neurons, but that the effect of the glu on modulating other neurons in the culture which project to both is sufficient to obscure the interaction. This is supported by the

observation that all of the interactions occurred when the rate of activity was fairly low, and that rate increases in either of the cells decreased the intensity of the interaction. (The failure of the presumed postsynaptic cell in pair (3-2-1) (whose CCH shown in Fig 4 to follow at high E2 rates could be explained by a synapse whose effect is rate dependent, or the change in culture state indicated by the abrupt E1 activity increase causing the effectiveness of the E1 induced EPSP in E2 being somehow nullified: the correlation could be masked by increased input, though the EPSP is still present).

It would thus appear that the interactions observed through the use of glu for activating neurons are in a sense quite "artificial". It may be hypothesized that the glu released at an electrode has a rather wide field of influence within the culture, though at a particular electrode location only one spike is recorded. The glu effect would not have to be one of induction of spikes in the cells it influences; it would be sufficient to cause a depolarization and change in membrane resistance of the dendrites which would affect the shape and size of PSP's induced distal to the main area affected on the dendrite.

For a given glutamate current, it appears that a stable mode of activity exists within the culture in which either a synaptic interaction between two cells may be observed, or some recurrent spatial activity pattern existing in the culture is modulating the activity of the two neurons to give the observed temporal spike separations. For a change in glu current, all of

the parameters influenced by glu in the cells being influenced by the glu would be changed, and the mode of activity which existed at the previous current level would no longer be stable, and a new pattern would result. Depending on the nature of the synaptic connections received by the cells being recorded from, just about any type of pattern could result. The most common pattern was that of correlated bursting. This was observed less frequently in the cultures in which only one glu electrode was used, but it seemed to occur more at the higher glu currents. (Insufficient data to decide this fully). This idea is explored in greater depth in the section on correlated bursting.

If the interactions designated by DE are in fact direct excitation, then it is highly likely that the EPSP's generated are all subthreshold as in each case glutamate iontophoresis on the presumed postsynaptic cell was required for the interaction to be expressed.

4.2 CORRELATED BURSTING

Correlated bursting (CB) was the most common pattern observed in the BS cultures, although there were large variations in the degree of correlated bursting occurring within a culture. CB could occur under every type of electrode configuration, but was seen most often with two glu electrodes.

Spontaneous CB was observed on a number of occasions, but it was rather rare. The fact that it does occur has to be taken into account in any theory which tries to explain the mechanism of generation of the correlation. With only one glu electrode

CB was seen often, but generally higher glu currents were required to induce it, and also the bursts were not as often fully decremental as in the cultures where two glu electrodes were used. Nevertheless, CB was the most common interaction in this electrode configuration also. Using two glu electrodes revealed that CB could be observed very frequently and for many pairs it was also possible to induce bursts by carefully adjusting the glu currents. It was this latter phenomenon which gave the first hint that iontophoretic glu was doing more than simply modulating the activity of only the recorded neurons.

It was also possible to increase the frequency of CB with the use of Bicuculline (bic). When this was done using a configuration with only one glu electrode, there was a greater quantity of bursting observed. The bursts induced by bic are similar to the ones seen with glu, but may be slightly longer. When two glu electrodes were employed with bic, it was discovered that for a pair of neurons in which no CB is present at one set of glu currents, the addition of bic to the BSS can cause the same glu currents to yield CB which is not present once the bic is washed out. Possibly this reflects some role for bic in blocking the effect of GABA from inhibitory synapses, but may also reflect a non-specific excitatory effect of the bic. Zipser et al (1973) found that bic caused enhancement of the PDS's observed in hippocampal cultures. This may be a direct effect on the neuron, since in the hippocampus this PDS seems to represent an afterdepolarization following a spike. Barker and Ransom (1978) reported that in dissociated spinal

cord cultures, bic would convert biphasic GABA responses (initial short depolarization followed by longer hyperpolarization) to purely excitatory events.

Induction of correlated bursts within a culture with two iontophoretic electrodes can be quite dramatic. (The observations to be described are applicable to both glu and HCA induced CB's). Several pairs of neurons have been studied where the response of each to the glu eject current on its associated electrode alone was regular firing. In most of these cases the other neuron did not respond at all to this eject current. When both electrodes were put on eject together, the initial few seconds of activity were usually regular, but a progressive grouping of spikes could be noticed which eventually turned into decremental bursts.

4.2.0.4 Possible mechanisms for CB

Several possible mechanisms which cause burst type firing in neurons can be rejected fairly quickly. One mechanism which has been observed to cause bursts in neurons which receive common inhibition is the excitatory rebound which follows the IPSP. If the mechanism for this involved the rebound from PN inhibition among the BS neurons, this would be eliminated by bicucculine. Inhibition may play a role in the generation of CB's, but it is not necessary.

One possible method which was envisaged as the cause of the CB's was the existence of a discrete neuronal type (designated as a master neuron) which formed strong excitatory connections

on numerous neurons within the culture. It is possible that neurons which form such connections do exist within the cultures, but their numbers are not large. Such a mechanism could operate to some extent in the triggering of bursts, but it also seems not necessary.

To test the latter theory experimentally would be rather difficult in the BS cultures as the exact nature of the interaction would not be easily predictable. If the synapse was sufficiently strong to cause a burst every time a spike occurred, then the time relationship between the burst onsets should be more constant. Also, at low rates of activity, one would expect greater stereotypy to occur among the bursts. If it is assumed that the interaction is one of the master neuron spike causing a spike rather than a burst in the postsynaptic cell whenever it fires, then, when the master neuron was firing a much more frequent occurrence of short latency spike to spike interactions would be expected. Such an entity as the master neuron may exist within the cultures, but it may function only to initiate the burst, and the resulting activity may be independent of its influence from this point on with other factors being responsible for the maintenance and termination of the bursting.

One avenue which seems to say something about the genesis of CB's in the neurons is the variety of models designed to account for epilepsy. Many points of similarity can be found between the state of correlated bursting in the culture and in epilepsy. The same convulsants which produce epileptiform

activity in vivo also have a similar effect on cultures of the brain. Crain (1976) has used strychnine and bicuculline in a number of culture systems to produce synchronized activity. Within the cortex topical application of acetylcholine (Ach) (Ferguson, 1974) or the introduction of penicillin topically onto the cortex (Andersen et al, 1969) produces correlated activity. Basically the pattern of activity recorded from cortical neurons during epileptiform activity consists of bursts of high frequency spikes (decremental by the terminology used in this work) separated by silent periods. When the bursts are recorded from a number of neurons simultaneously they are seen to have a large amount of overlap of the times they are firing. This has been seen in 4 neurons simultaneously by Harner and Sgro (1978) from the cerebral cortex.

A general mechanism proposed to account for this state is an excess of excitation over inhibition; either as the result of increased activity or numbers of excitatory terminals, or from a decrease in the level of inhibition as caused by convulsant agents. With an adequate level of excitation, the inhibition may be disregarded, and the system may be approximated by a network of neurons with purely excitatory connections. Such systems have only positive feedback, and when this becomes sufficiently large, the system fires at its maximal rate, and burst termination is achieved either by synchronous occurrence of refractory periods (MacGregor and McMullen, 1977) or the excess depolarization causing failure of the spike generating mechanism. In computer

models of randomly connected networks of neurons with purely excitatory connections, rhythmic synchronized activity has been found to occur, and also, when a random input is applied to the neurons, the presence of temporally synchronous spike groups in the input causes firing in many of the neurons. These are referred to by MacGregor and McMullen (1977) as synchronized clusters, and if they are fed back into the system essentially unlimited positive feedback occurs to give synchronous firing in all the cells.

A productive way to look at such phenomena is to consider the possible states which can exist within a neural network. This approach has been applied by Ferguson, (1974) to Ach induced synchronous burst discharges observed within the cerebral cortex. The basic assumption is that the neural network may be viewed as having a dynamic pattern which is a description of the electrical activity which is occurring. For example, with the cortex the normal pattern is one where there is not a very large degree of correlation among the neurons which is thought to be maintained by a tonic desynchronized inhibition. The state has "resiliency" in that an excess of excitation may cause a brief change, but is not maintained. When the amount of inhibition is decreased, or the amount of excitation greatly increased, a new dynamic pattern is created which is self sustaining, and in the case of epileptic activity, consists primarily of a highly synchronous discharge with the neurons firing at high rates. Epilepsy is referred to as a pathologic dynamic pattern by Ferguson, (1974).

The relationship of this type of activity to epilepsy is rather general, as the exact nature of the activity in any system would be influenced to a great extent by the idiosyncratic synaptic connections of the system, and also by the ratios of inhibition to excitation. Support for the state with synchronous burst discharges being a state with predominant excitation has been provided by the work of Nelson et al. (1977) in cultures of dissociated mouse spinal cord. They saw correlated bursting occurring synchronously within two neurons, and as they were recording intracellularly from both neurons, they could see that the event was a simultaneous large amplitude depolarization. They grew a number of cultures in medium which had a high level of glycine present, and when they worked on these cultures, they found that most of the neurons were discharging in these sychronous burst patterns. This effect was reversible in about 18 hours when the cultures were transferred to normal culture medium. By the use of pharmacological testing they established that the effect of the glycine was to cause a decrease in the magnitude of the response to inhibitory amino acids. This was interpreted as a desensitization of the receptors. This data illustrates quite elegantly that this type of gross activity variation may be induced relatively easily by a variety of means. Crain (1976) has discussed this concept also regarding the induction of correlated activity in various types of CNS cultures with convulsants.

An interesting theory by Calvin (1972) addresses itself to the role of burst type discharges in the generation of

correlated bursting. He refers to this as a recruitment model, which illustrates a plausible mechanism for the propagation of bursting activity throughout a network once it begins. He first considers the effect of temporal summation of EPSP's on the size of the resultant EPSP. By a variety of simulations he could show that a high frequency burst was capable of producing quite large EPSP's when the intervals between the spikes were in the range of the subsynaptic membrane time constant. Then based on a consideration of a typical cortical neuron Calvin went on to calculate the number of neurons which would be required to exhibit burst discharges in order for the depolarization to be sufficient to cause the neuron they synapse on to also begin discharging in bursts. For the cortical neuron this was calculated to be only 1% of the inputs. Thus, the mechanism implied by the model is that once a certain number of neurons begin to fire bursts (another requirement is that the bursts must overlap), other neurons are "recruited" into burst firing patterns also. Usually this is prevented by inhibition not allowing such a buildup, but in the absence of inhibition, or in the presence of certain chance grouping of spikes in neurons, the firing pattern may become established and stable.

Presently it is premature to definitely apply any one of these theories to the system of BS neurons. It is likely that the use of glu electrodes is the source of the excitation that is driving the system into a bursting state What is not clear at the moment is how the CB state is initiated. If it is assumed that once frequent CB's occur in synchrony, that this may

maintain the state as long as is desired, then a number of triggers may be visualized for the initiation. It could be a unique neuron type as was suggested earlier, or it could be a chance pattern of activity which sets the system into the correlated bursting state from which it cannot emerge once it enters. On the basis of this model, a set of coupled relaxation oscillators should be able to achieve the correlated state by phase locking at a certain point and making the irreversible transition. (This analogy is not that far removed from reality as the effect of glu on a neuron in the absence of synaptic activity is closely represented by a relaxation oscillator (personal neuronal modelling studies)).

One matter that is purely speculative is a consideration of whether or not it would be possible for an individual multiaxoned BS neuron to intrinsically generate bursts. This mechanism has been suggested by Nelson and Peacock (1973) for the bursts (correlated) in dissociated cultures of cerebellum. If a neuron with multiple spike generating sites (SG's) is capable of producing such activity, then there must be some mechanism of preventing the neuron from bursting when it is firing only single spikes. The most likely mechanism would be some kind of modulation of the degree of coupling between the interconnected SG's, and one possible way to do this would be to use strategically situated inhibitory synapses to shunt most of the current from a distal site before it reaches the soma. Such synapses would be most effective if located on the proximal dendrites of the neuron.

4.2.0.5 Effect of glutamate on interactions

With the great influence on the activity of the BS neurons which has been demonstrated for glu it remains to tackle the question of how much information on the synaptic interactions can be obtained from the data. In one sense the glu induced activity can be treated as artifactual and it does cause a certain amount of blurring of the synaptic interactions as it is the state of the whole culture which is being recorded rather than just the interaction between the two cells being held on the electrodes. In the present system it would be difficult to get around this problem as some source of excitation appears to be needed in the BS area. Of the electrode configurations employed, the combination of one glu electrode with a non glu electrode seems optimum in terms of encountering the largest number of correlation types. However, the system is certainly not comparable to the use of Aplysia preparations (Bryant et al, (1973)) in which the activity of each neuron may be monitored intracellularly for long periods, and the cells specifically activated by means of intracellular current pulse injection according to any desired firing pattern. Such a system is more suited to the detailed analysis of synaptic connections where the properties of a specific synapse are to be studied in detail. Presently the BS culture system does not have this type of specificity as there exist no anatomical criteria to separate distinct regions as may be done with the cortical and DN areas in the cerebellar explants.

While the general effects of the glutamate on the culture make electrophysiological to anatomical correlations on the basis of extracellular data more ambiguous, it does provide an interesting system for the study of the interactions as a whole of a small group of neurons. On the basis of the extracellular data obtained it was seen that a great variety of activity modes could occur within a culture. Interactions were not constant for long in most cases, and on the basis of a short record from a cell it was not possible to decide if any interaction existed or not because in many cells which were initially non-correlated, waiting would reveal correlated states between the two cells or a correlation could be induced by variation of the glu currents. It is thus necessary to speak of observed interactions and potential interactions. It is likely, within the BS cultures, that most if not all randomly selected cell pairs will exhibit some correlation under a specific set of conditions. As all this occurs with essentially the same set of anatomical connections, it is clear that the determining influence on a neuron is not the specific synapses it receives from a given neuron, but rather the activity of the other neurons which project upon both of the neurons. If this activity is small compared to the strength of the synapse, then a cross correlation will give a result which corresponds to the synaptic interaction, but if the input activity is much larger, then almost any desired pattern may be created.

Methods presently available do not allow the study of these detailed modes of activity, but several extreme ranges of

activity could yield interpretable data. The lower extreme is the state of very slow firing where strong synaptic connections could be more prominent, and the other extreme is the correlated bursting state. It would be possible to obtain some data about the nature of the synaptic connections when CB is occurring by the quantitative analysis of the burst interactions. When compared with other systems in epileptic states (in which the anatomical connections are known) it would be possible to make more informed guesses of what the circuitry organization among the BS neurons is. This would be analogous to the work by Llinas and Volkind, (1973) and DeMontigny and Lammare, (1973), using harmaline induced tremor for studying the connections between the cerebellum and its subcortical nuclei.

4.3 APPLICABILITY OF SPIKE TRAIN ANALYSIS TO BS CULTURE SYSTEM.

The use of spike train analysis in the study of the BS culture system has proven to be useful in the quantitation of the observed interactions, but in a strict sense, it was not as useful as it was initially expected to be. Most of the interactions were detectable visibly, and thus it may be argued that the elaborate procedure for analysis of spike train data was not necessary as the interactions could be seen. It is also possible to argue, however, that this simple manual approach may be useful as a first approximation, but that automated analysis is necessary to more fully characterize the interaction and because a quantitative measure of the correlation is available, it is far easier to compare different parameters of the correlation recorded under different conditions.

4.3.1 Limitations of ST analysis

Many of the limitations which were found for the ST analysis method were due to unsuitability of just cross correlation methods to deal with the interactions occurring within the BS culture system. One of the primary requirements for this method to yield meaningful results is that the spike trains be stationary. This requirement was seldom satisfied. Previously demonstrated data of adaptation of neuron firing rate, or of large seemingly spontaneous rate changes within some neurons make it very difficult to justify accepting the verdict of a CCH as representing the correlation. Also, the interactions which occurred were sometimes seen to be multiple, and rate dependent (examples would be the seeming simultaneous common inhibition and direct excitation in pair (23-1-1) and seeming direct excitation occurring only at low E1 rates in pair (3-2-1)). For cases such as these, if one interaction occurs sufficiently often, then it will appear in the CCH, but a clearly visible set of multiple interactions could quite concievably result in a flat CCH. Such anomalous, nonstationary interactions are not limited to the BS culture system, and have also been described within Aplysia by Bryant et al (1973).

Another feature of the BS cell pair records which complicated analysis was the effect of glu to regularize neuronal discharge in many instances. As has been pointed out by Perkel et al (1967b), finding whether or not a correlation exists among two pacemaker neurons is very difficult because of the possibility of phase locking among uncorrelated regular

spike trains. This difficulty is inherent within the BS system, as the use of glu electrodes has proved to be optimal in the activation of the neurons. Low glu currents were often used to obtain more irregular activity, but often this resulted in quite low rates which necessitated very long records to obtain sufficient data for meaningful analysis. Also, within the BS cultures, it appeared that correlations from slowly firing neurons occured less often than in pairs of neurons which were faster firing. As an extreme example, culture 9 (which was not mentioned in the results because only one of the CCH's computed showed even mild evidence of correlation), had very long records made for the neurons under minimal glu currents (all electrodes were blocked, so glu application was by diffusion from the tips), and although activity was very irregular and very slow minimal correlation occurred. This may reflect activation of insufficient numbers of neurons to raise culture activity to a level to give a correlation.

One very great advantage which simultaneous intracellular recordings have (at least in invertebrate preparations where the recording stability is far greater than in mammalian neurons), is the ability to obtain any desired activity pattern in the spike train of a neuron by driving it with depolarizing pulses corresponding to the generated spike train. In this manner, the problems associated with regular ACH's are avoided, and CCH's exhibiting non-random features are most meaningful if both neurons have flat ACH's (or essentially Poisson process type spike trains as used by Bryant et al, 1973). Such selectivity was not possible within the culture system.

All of the above statements have been concerned with the CCH as the primary measure of interaction, and the limitations are the most severe in the utilization of only CCH analysis. There are many other correlation measures which may be applied to the system, and these were utilized to a lesser extent due to the late time of their introduction. For example, the TXIH seems to show greater ability to detect correlations than the CCH, but requires much larger amounts of data than the CCH. Also, the decomposition of the ST's into various types of point events corresponding to meaningful spike patterns within the train, and calculation of CCH's between these point events may give a more meaningful correlation than assuming that every spike is equivalent. This could be done in the BS system for bursts and spikes in an analogous manner to the analysis of cerebellar ST data by Bell and Grimm (1969) as was discussed earlier. The use of spike patterns rather than spikes is quite mathematically correct (although more complex to analyze in detail) and represents a more relevant approach to use in the analysis of simultaneously recorded ST's (W. Tatton, personal communication).

In conclusion, the limitations which exist within the cultures are severe only if "classic" ST analysis methods as developed by Perkel et al (1967a, 1967b) and Moore et al (1970) are utilized. It is important to note, however, that modifications will be required to deal with the idiosyncracies of every system under study, and that by not limiting oneself to a particular method such as only CCH analysis, delving into the

more generalized mathematical background of the correlation methods which is essentially time series analysis (rigorously expounded by Cox and Lewis, 1966) may yield measures more relevent to the system under study. The primary utility of all of the statistical methods is that they provide quantitative data, and this often may highlight subtleties which would pass unnoticed using a more elementary analysis, although the generalized correlation is usually visible at both levels.

4.3.2 Advantages of ST analysis

One of the primary advantages of entry of spike interval times into a large computer is the great flexibility which results from this approach, even though many of the computations which were performed on the data could have been done utilizing existing equipment (eg calculation of CCH approximation using the signal averager and external logic). In terms of amount of time consumed in data preparation for analysis, the two methods are equivalent, but the major difference lies in the flexibility of analysis possible once the data is in a computer accessible form. For example, with certain sets of data, it would be possible to use the averager and computer to compute identical CCH's, but whereas with the computer data it would be no extra trouble to compute the IIH's ACH's and other measures on the ST's, using the averager, computation of each of these measures would require the replay of the data every time it was wished to compute a given function of the data. Aside from this drawback, the difficulty of obtaining the data within a numeric format

makes further calculations on various parameters of the ST's almost prohibitive using the averager. The only reason that some CCH's were computed by this method was due to the development of conceptually elegant (but physically laborious) methods to examine several pairs of cells for which the semi-automated method of analysis proved to be faster solely because only two cell pairs were being analyzed.

Another advantage of computer analysis of the data, is that in the event that other tests are discovered which may be applied to the data, all of the previously entered data is present in a form such that the test may be applied with minimal effort, whereas using the averager, use of a new test would require construction of additional logic and playing back all the data through it. If only a very specific correlation is being sought, this problem would never arise, but in the case of the BS to BS synaptic interactions, what was relevant in the data was not known, and maximal use of the data was made. (This can be overdone also; and a rough maxim given by Perkel et al (1967a) suggests that analysis overkill is occurring when the amount of numeric output from analysis program begins to exceed that originally present in the raw data).

Another advantage of the use of automated analysis is that feedback from the computer analysis of the data, when combined with the sensory experience of the raw data gives a deeper understanding of the relationship of the two ways of looking at the interactions. This will not be discussed further due to its vague and qualitative nature, but it does have the seemingly

paradoxical effect of rendering the automated analysis redundant by attuning the senses to relevant patterns in the data by a process of association. Such a process is most useful if the feedback is rapid as in the case of short delays between experiments and computer analysis of the data. The difficulties involved in direct access to the 360 computer at Ottawa U during the major part of the project made this feedback suboptimal, but present expansion of time sharing facilities could give far shorter time delays between experiments and seeing the results.

4.4 POSSIBLE ORIGIN OF BS NEURONS

The general area of origin of the BS neurons can be specified in some detail now, but a definite correlation is still not possible. It is likely that some of the BS neurons are from the LC based on monoamine fluorescence and HRP injection. (Hendelman and Ferguson, in preperation). However, the results do not agree with the physiological data now available from the LC in vivo, and more work would be required to find the discrepancy. It is likely, though, that on electrophysiological criteria, the BS neurons consist of a number of cell types in variable proportions of which one unique cell type is the predominant one. A description of the region explanted is given before discussion of the possibilities for the BS neurons in terms of the various regions to be described. The various nuclei which were mentioned briefly in the introduction are now described in more detail.

4.4.0.1 Vestibular nuclei

The vestibular nuclei (VN) are a rather heterogeneous group of nuclei lying in the dorsolateral brainstem, and extend roughly from the caudal pons to the medulla. Four principal nuclei are commonly distinguished, although a more detailed examination reveals 7 other minor cell groups which belong in the vestibular complex (Brodal et al, 1962). While there is a heavy projection from the vestibular nerve to the nuclei, Brodal et al (1962) states that only about half of the vestibular neurons receive primary vestibular afferents while the remaining neurons receive numerous other projections, both from other vestibular nuclei as well as from varied parts of the brain.

There is an especially intimate relationship between the VN and the cerebellum, particularly the vermis. Both the lateral vestibular nucleus (LVN) and the superior vestibular nucleus (SVN) continue dorsally within the cerebellar peduncles to fuse with the lateral deep cerebellar nuclei (Korneliussen, 1968). The LVN is grouped functionally with the cerebellar nuclei (Sotelo and Angaut, 1973) and receives an especially heavy projection from the Purkinje neurons of the vermis. A projection back to the cortex has not been described for the LVN, but it is likely (if LVN is analagous to DN) that bidirectional connections exist between it and the cerebellar fastigial nucleus. The giant cells of Deiters are the best known cells of the LVN, but both medium and small cells also exist within it. All sizes of neurons receive cerebellar

afferents as well as collaterals from other neurons within the nucleus and from outside.

Within the explants utilized for the BS only cultures, the LVN and SVN will be included in every case. It is less certain of whether the medial and descending vestibular nuclei will be incorporated. With these two nuclei reciprocal connections between the cortex and DN would exist (Alley et al, 1975). It is unlikely that individual nuclei could be distinguished in culture as there is overlap between all of the nuclei of the vestibular complex in vivo. Such nuclei are referred to as "open" by Mannen (1965). The neurons in the VN are classified as isodendritic by Ramon-Moliner and Nauta (1966) as are neurons of the reticular formation and the Locus ceruleus (LC). Thus anatomical criteria alone would be insufficient to decide if a neuron was part of the vestibular complex in culture.

Aside from connections with the cerebellum, the VN also have extensive long ascending and descending connections, and locally, numerous reciprocal connections with the reticular formation, occulomotor nuclei, and other cranial nerve nuclei. No true internucial neurons are described within the VN complex, but Brodal et al (1962) note that some neurons give off a rich plexus of thin axons within the nucleus as well as projecting outside the nucleus. These could function in a similar manner to interneurons. VN to VN interactions have been described as predominantly excitatory (Shimazu, 1972).

The only factor which can be seen to argue against BS being VN is the virtual absence of myelin in the BS cultures. This is

not a serious obstacle, for the degree of myelination may be heavily influenced by culture conditions. For example, PN's in pure cerebellar cortical cultures myelinate poorly whereas cortical/DN cultures have PN's with well myelinated axons (W. J. Hendelman, personal communication). Also, not all cells in the VN have myelinated axons, and given the complex relations of the VN to the rest of the brain, it would not be possible to a priori predict their behavior in culture.

4.4.0.2 Monoaminergic nuclei

Within the explanted area may be included several NE containing cell groups of which the LC (group A6 of Dahlstrom and Fuxe, 1964) is the largest. Of the other NE containing nuclei, group A4 would be most likely to be included. Within the pontine NE nuclei, there are no clear divisions; the nuclei described by Dahlstrom and Fuxe are areas where aggregations of NE containing cells are found, but scattered neurons with NE fluorescence are found throughout the pons, linking all of the nuclei (Amaral and Sinammon, 1977). The pontine NE neurons share a common origin from a location in the lateral wall of the fourth ventricle (Seiger and Olson, 1973) and migrate from this area to their final locations. The area of origin is essentially the area occupied by the LC in the adult. Within the fetal rat, Seiger and Olson, (1973) also describe many fluorescent cells scattered throughout the cerebellar peduncle, roof of the fourth ventricle, and even within the developing cerebellum.

If Seiger and Olson's (1973) view of the common origin of the pontine NE neurons is correct, then it would be likely that neurons which were destined to migrate to other areas would be explanted along with the LC. If there are functional differences among the pontine NE neurons, then this could perhaps explain the variance between the predominance of excitatory interactions (in view of the expected inhibitory action of NE; this is discussed later) observed among BS neurons in this study, and the catecholamine fluorescence results from the BS cultures obtained by Hendelman and Ferguson (in preparation). This does not imply that the BS neurons are another pontine NE cell group; the properties of the LC neurons could also change in the culture system.

The electrophysiological literature dealing with pontine NE cell groups (aside from the LC) is virtually nonexistent. This could well be a consequence of these cell groups being small and poorly delimited, and lack of certainty of whether units encountered are actually NE neurons or not. Thus in view of the common origin of the pontine NE neurons, and their extensive interconnections (Amaral and Sinnamon, 1977), the LC will be taken as the archetype of the pontine NE nuclei.

Most of the literature data on the LC is based on the rat, but should be quite applicable to the mouse as the gross anatomy of the LC varies little among various species of rodents. In rodents, the LC is a compact cell group lying adjacent to the fourth ventricle in the rostral pons and midbrain. Caudally, the LC is bounded medially by the fourth ventricle and laterally

(and dorsally and ventrally) by the medial and superior vestibular nuclei. Moving rostrally, the LC is less closely apposed to the ventricle, and is now medially bounded by the pontine central gray (PCG) and laterally by the MES V which is intimately in contact with the lateral border. The ventral boundary at this point consists of the gigantocellular tegmental field (GTF) of the pontine RF.

Cytoarchitectural criteria may be used to divide the LC into a larger dorsal portion and a smaller ventral portion (subceruleus). The dorsal LC consists primarily of medium sized bipolar or fusiform neurons with a smaller number of large multipolar cells scattered throughout the region. Within the subceruleus there is a higher proportion of multipolar cells and less dense packing of the neurons. Swanson (1976) states that most if not all neurons in the LC contain NE, although nonfluorescent cells have been described (Amaral and Sinnamon, 1977).

Examination of Golgi stained dorsal LC in rat (Swanson, 1976) revealed that the multipolar cells had 3 to 5 large dendrites which branched only once or twice, and in the periphery of the nucleus extended long distances into the surrounding neuropil. Short spines were seen irregularily along the dendrites. Some of the neurons were seen to have richly branching local axon plexuses and multiple axons emerging from one neuron were occasionally seen.

Widespread projections to the whole of the CNS occur from the LC, and a single neuron may send collaterals to both the

cerebral and cerebellar cortices (Nakamura and Iwana, 1975). There is some evidence for the formation of different synapse types between the remote and local projection areas; synapses with cerebellum and cerebral cortex are described as lacking presynaptic membrane specializations and being not in direct contact with neurons (Amaral and Sinnamon, 1977) whereas synapses in the nucleus tractus solitarius are formed on dendritic spines and are reported to be classical synapses (Chiba and Doba,1975). The physiological significance of this is as yet unknown. Reciprocal connections exist from the LC to the raphe nuclei, RF and other NE cell groups (Amaral and Sinnamon, 1977).

Afferents to the LC originate in widespread areas of the CNS, and if it receives a projection from every area it innervates, then it would have afferents from the whole of the brain. The areas of interest in the peduncular region sending axons to the LC are the vestibular nuclei and fastigial nucleus (Cederbaum et al., 1978), fastigial neurons (Snider et al, 1976), as well as trigeminal complex neurons.

The effects of LC stimulation on the nuclei it innervates have generally been described as inhibitory (Amaral and Sinammon, 1977). This has been shown to be not strictly correct in the cerebellum by (Woodward et al, 1977), and that the effect is more precisely one of decreasing neuron spontaneous activity and increasing the sensitivity of the neuron to both excitatory and inhibitory input. Data in the literature at this time is inadequate to determine the effect of LC connections with

brainstem nuclei as well as the expected effects of LC to LC connections (in view of the modulatory function of NE described just previously). IF 5-HT is used as an analog, then both inhibitory or excitatory effects should be possible as both effects have been described for 5-HT projections from the raphe (Couch, 1970)

4.4.0.3 Trigeminal nuclear complex

Classically four main nuclei are recognized within the trigeminal complex and are: spinal nucleus of V, motor nucleus of V, principal nucleus of V, and mesencephalic nucleus of V (mes V). The total extent of the complex is from the cervical levels of the spinal cord to the midbrain. These nuclei have been further subdivided into a variety of other nuclei by various workers. For the purposes of this summary the classification of Astrom (1953) has been adopted because his categories were based on the brain of the mouse.

Basically the nuclei of V may be grouped into sensory nuclei, association nuclei and motor nuclei. Most of the nuclei are located in the ventrolateral portion of the brainstem, and only mes V and supratrigeminal nuclei (N V s) (both sensory nuclei) are likely to be included in the area explanted for peduncular cultures.

The mes V nucleus is situated in the dorsal brainstem, medial to the fourth ventricle just lateral to the LC. The extent of the nucleus is from the rostral pons into the midbrain. Mes V neurons are primary sensory neurons which may

be viewed as dorsal root ganglion cells moved to a central location. They receive primarily afferents from muscle spindles of jaw muscles and tooth receptors (Jerge, 1963a). The cells are large spherical neurons which are primarily unipolar, and have projections to the supratrigeminal nucleus, sensory nuclei of V as well as cerebellum and likely LC (Torvik, 1956). In addition terminals have been observed ending near other mes V cells (Pearson, 1949), but their functional importance is not known. Not all cells in mes V are unipolar, and in fetal and newborn brains of several species variable number of bipolar and multipolar cells exist (Pearson, 1949), and these are believed to undergo a transition to the unipolar form with age. In human mes V a few multipolar cells are found even in the adult (Pearson, 1949) but in rat the cells are said to be predominantly unipolar in the newborn and no data are available for the mouse. Cells having unipolar shape have been identified in explants of cerebellar peduncle from newborn mouse by Allerand (1971), but whether all mes V assume this form in culture in mouse is unknown as Hild (1966) has demonstrated the persistance of multipolar mes V neurons in cultures from newborn kitten. The intimate association of the LC with mes V has been commented upon by numerous workers, although the nature of their interaction does not appear to have been investigated electrophysiologically.

Of the remaining trigeminal nuclei, the nucleus supratrigeminalis is the most dorsally located. This nucleus has been described as a dorsomedial extension of the principal

sensory nuclei (Torvik, 1956) from which it is distinguished by a looser arrangement of cells, and larger size of the cells than found in the sensory nucleus. The nucleus is poorly delimited from its surroundings and consists of medium sized oval or triangular cell bodies which emit a number of fairly short dendrites (Astrom, 1953). A similarity to neurons of the reticular formation has been suggested by Torvik (1956) because the cells have fairly long processes (presumably axons).

Few primary afferents are received by the nucleus, and projections have been described from rostral levels of the neuraxis as well as mes V (Astrom, 1953). The latter projection is of the form of a single collateral of mes V branching extensively among the cells of the supratrigeminal nucleus. The function of the supratrigeminal nucleus has been described as that of an association nucleus, presumable mediating trigeminal reflexes (Jerge, 1963b). Electrophysiological investigations by Jerge (1963b) revealed that neurons within supratrigeminal nucleus could be activated by jaw movements or pressure stimuli applied to intraoral receptive fields. Convergence of sensory modalities was observed onto the neurons leading to the conclusion that they were homologous to spinal interneurons. This would lead to the expectation (carrying the spinal analogy further) that it would project to the motor nucleus of V, but other projections within the brainstem are also a possibility.

4.4.0.4 Reticular formation

Most of the cells classified as belonging to the pontine reticular formation lie ventral to the area under consideration, although it is almost certain that scattered cells will be found all the way to the dorsal surface. About these nothing can be done or said. Of the various 'nuclei' of the RF, the gigantocellular tegmental field is the most relevant.

This cell group was mentioned earlier in connection with the LC A number of cell groupings are included in the GTF including the nuclei reticularis pontis oralis and reticularis pontis caudalis. Many giant cells are scattered through the GTF, mainly medially and these send long axons both rostrally and caudally as well as giving off numerous collaterals within the brainstem (Schiebel and Schiebel, 1958). The LC receives a heavy investation from the GTF (Steriade and Hobson, 1976) and also sends axons back to this area. The most fascinating thing about the LC-GTF connections is that this particular system has been implicated in the periodic generation of desynchronized sleep (Steriade and Hobson, 1976). In the explanted area the expected number of giant cells would not be large, but if hypertrophic GTF-LC synapses occurred in culture (as a consequence of the GTF neurons not having most of their projection area around), then it would be reasonable to expect that cells found electrophysiologically to form strong, widespread synapses with other cells could likely represent this particular connection. It is also possible that they are present within the LC itself as Olzewski and Baxter (1954).

described large, unpigmented cells within the human LC which they considered to be displaced neurons from the nucleus reticularis pontis oralis.

4.4.0.5 Other cell groups

Other cell groups are present in the area explanted, but these may be considered to be irrelevant because only small portions would be included, and the group would not necessarily be consistently present in the BS cultures. One group which is definitely known to be excluded is the dorsal cochlear nucleus which is present on the external lateral surface of the cerebellar peduncle.

4.4.1 Comparison of BS culture results with literature data

4.4.1.1 Anatomy

Systematic anatomical studies of cerebellar cultures have been carried out by Wolf (1964), Allerand (1971) and Seil (1972). Only the studies by Allerand and Seil have dealt with additional non-cerebellar neuronal types present in the peduncular region. Of these two workers, the results of Allerand (1971) are most directly applicable to the culture system used in this laboratory.

As was mentioned in the introduction, Allerand distinguished 3 primary groups of non-cerebellar neurons; types I, II and III. Leaving aside the type I neurons for the moment, her types II and III may be seen to correspond respectively to the BS neurons worked on here and Mes V. The correlations

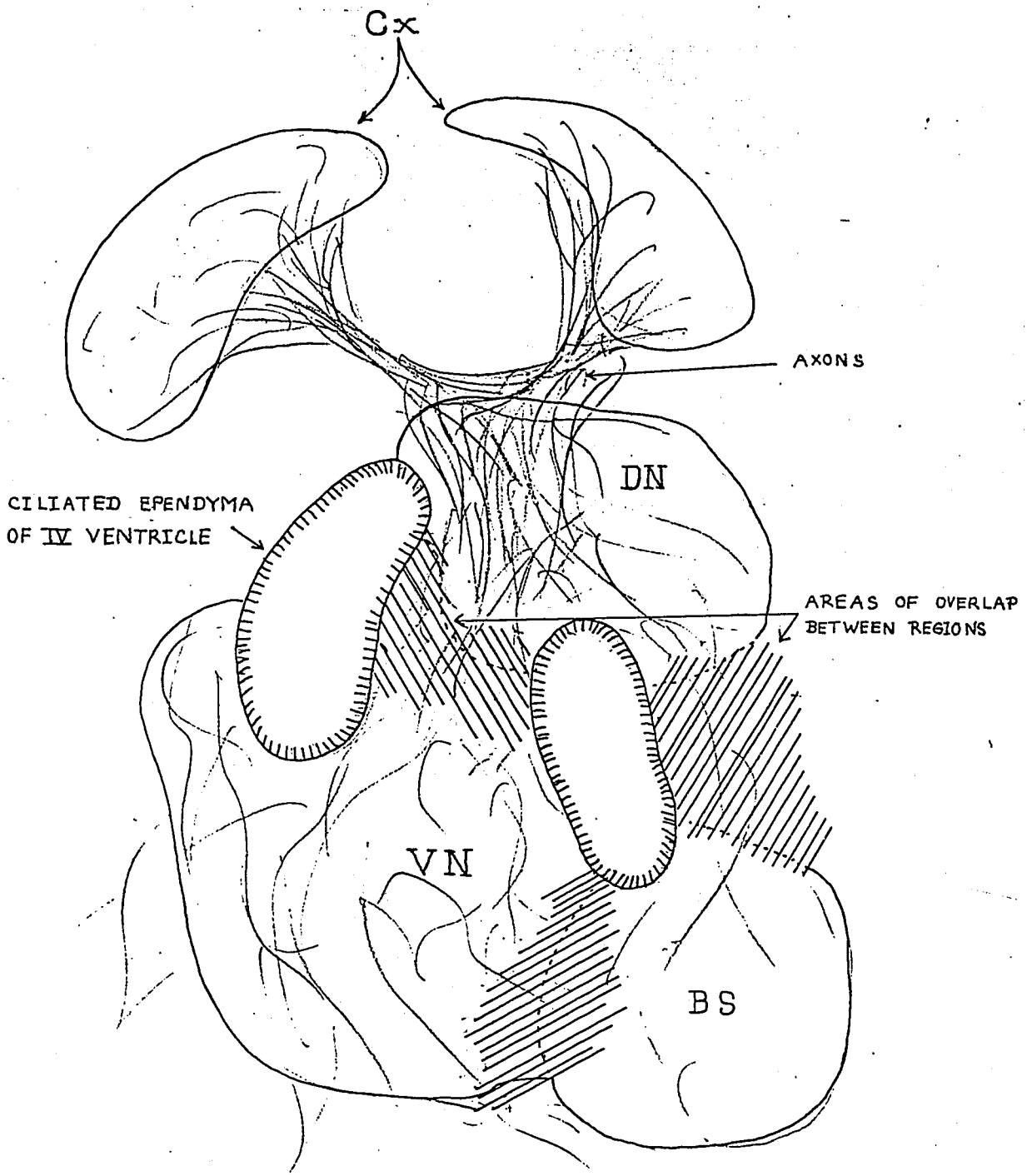
between type II and BS are very strong at the LM level in living cultures. A notable point she mentions is the lack of staining of BS neurons using the Holmes method. (This has also been observed by Hendelman). This could explain The conspicuous absence of a similar neuronal category from the work of Seil (1972), as his results were based entirely on Holmes stained cultures.

The type I neurons of Allerand (1971) and vestibular neurons of Seil (1972) seem identical on the basis of the Holmes stain. These neurons are described as having myelinated axons by Allerand, and to be at the level of, or ventral to the ependymal cysts by Seil (1972). Undoubtedly, many of these neurons are from the dorsally located vestibular nuclei, but aside from giant cells, which are likely from the LVN, no further distinctions may be made.

Comparison of the anatomical data of Allerand and Seil with that from the BS cultures from Hendelmans laboratory is complicated by incomplete information to compare all data equally. Thus, informed extrapolations were made to fill in the gaps, and resulted in the following picture which is shown in Fig D1.

The only difference between this and the previous view of the cultures is that the non-cerebellar neurons have been divided into a VN group and a BS group with the BS neurons being defined as previously (introduction). The VN neurons may be described as those non-cerebellar neurons which are not located in the BS area and are stained by the Holmes technique and may

FIGURE D1


Cx
AXONS
DN
CILIATED EPENDYMA
OF IV VENTRICLE
AREAS OF OVERLAP
BETWEEN REGIONS
VN
BS

have myelinated axons. These would not necessarily be a clearly defined group as they could be physically distinct (as shown by Seil, 1972) or could merge with the DN or BS neurons. They are morphologically similar to the DN neurons (as a consequence of similarity between DN and LVN, SVN).

It is now necessary to look at the BS cultures herein employed to find the counterpart of VN. Such a study has not been systematically done. However, since BS neurons are likely present in Seils cultures, (just invisible by his criteria), it seems likely that the same selective perception occurred in the cultures used here. The material to be presented as evidence consists primarily of incidental observations and one Holmes stained culture. It does, however, put previously disconnected pieces of data together in an aesthetically pleasing way. Further experiments to obtain quantitative anatomical data are required to determine the validity of the theory given.

Most BS cultures which were worked on were not examined in detail outside the BS area, except when other neuron types appeared to be present, so that recordings could be restricted mainly to those cells fitting the BS description. One class of cultures existed in which myelinated neurons could be seen next to the BS region. These were called 'contaminated' BS in the results. It is very likely that most of these cells are not DN (W. J. Hendelman, personal communication), and in one such 'contaminated' culture the successful Holmes impregnation occurred, three large impregnated neurons similar in appearence to the 'vestibular neurons' described by Allerand and Seil were

observed in an area of the culture by the ependyma which was neither DN or BS. Thus it is quite possible that the pontine unidentified neurons are equivalent to the VN neurons described by Allerand and Seil.

One neuronal type which is described identically by Allerand, Seil, Wolf and Hendelman is the cells of Mes V which are distinctive by their large size and lack of dendrites. Such cells have been described as rare by all authors, although in the cultures used here, they were very rarely seen, and when found, usually only one Mes V cell was present in the culture. It thus seems certain that BS are not Mes V.

Should the division of peduncular neurons into BS and VN areas prove correct, identification of the origin of BS neurons is not very greatly simplified. Both groupings are quite arbitrary and based on purely morphological criteria. One significant difference may be a paucity of neurofibrils in the BS neurons compared to the VN neurons (which is the explanation offered by Allerand (1971) to explain differences in Holmes impregnation of the two types). It is not at all clear, though, how these anatomical differences relate to the physiology of the neurons.

One simplifying factor in identification of some of the BS neurons may be the existence of CA containing neurons, presumably localized to the BS area of the explants (Hendelman and Ferguson, in preperation). Axonal profiles in the outgrowth zone of the explants very similar to the fluorescent axons have been observed in HRP injected BS neurons (Hendelman

Marshall, in preperation). Victorov et al (1978) have shown very similar fluorescent axons growing out of peduncular culture of mouse. The peduncular area giving rise to the BS neurons is included in their cultures, and they attribute the origin of these fibers to the LC. The tendency of CA containing BS neuron axons to grow away from the explant may possibly be used to identify the CA containing somata within the cultures by electrical stimulation in the outgrowth zone. The majority of neurons in the cerebellar explants have axons which remain within the original confines of the culture, or if they do enter the outgrowth, they usually turn back after going a short distance (Allerand, 1971; Seil 1972).

4.4.1.2 Physiology

No work, aside from that described herein, (or by Wojtowitz, 1978) has been previously done on the physiology of the BS neurons. Some workers have used cultures which would most certainly include the BS neurons, but lack of precise description of the neurons recorded from the brainstem portion of the explant make comparison difficult.

Calvet and Lepault (1975) studied the effects of culturing cerebellar cortex with or without an underlying section of brainstem, and reported that more synchronous bursting activity in the cortical explant occurred with the brainstem present. This was apparently initiated by cells from the brainstem driving the PN's, but no mention was made of the size of the brainstem piece used. It is likely much larger than the one

employed in this study, as Privat and Drian (1975), (who work with Calvet on cerebellar cultures) report using a section comprising half of the dorso-ventral thickness of the pons as their brainstem piece. Such a large piece could either contain an excitatory neural network normally projection to the cerebellum which undergoes synchronous firing, or portions of the inferior olive (IO) may be included. When the IO is explanted with cerebellum, Gahwiler (1978) reports that complex spikes occur in PN's following electrical stimulation in the expected location of the IO. This suggests that the strength and specificity of the IO to PN climbing fiber synapse is retained in culture. The results of Calvet and Lepault (1975) could thus result from PN's in Cx-brainstem cultures being strongly driven by synchronously firing olivary neurons as in harmaline induced rhthmic cerebelaary activity (Llinas and Volkind, 1973).

4.4.2 What are the BS neurons?

A number of possibilities have been presented for the possible identity of the BS neurons as well as evidence for what they are likely not. One complicating factor is that it is unlikely that the BS neurons are a homogenous group. Morphologically distinct cells have been described within the BS group (eg Mes V) and thus other neurons morphologically similar to the BS neurons can be expected. This is a large problem in electrophysiological experiments as it is not possible to tell whether or not one is recording from a

not. Electrophysiological data obtained during HRP experiments, just after a neuron is impaled would give direct electrophysiological and anatomical data for the same neuron. Thus far only single unit recordings have been obtained in HRP experiments, and electrodes used in HRP experiments have larger tip diameters than citrate electrodes normally used in intracellular recordings. This gives poorer quality and shorter duration records for cells impaled with HRP electrodes.

On the assumption that the majority of neurons identifiable as BS in the cultures are identical, it may be argued that a sufficiently large sample of cell pairs will illustrate the BS to BS interaction by virtue of it being numerically the largest. When this type of reasoning is applied to the results, the incidence of correlated bursting dwarfs all other interactions, but a number of possibilities are available to explain its presence. It is evident that excitation from one neuron to another appears to be the predominent interaction, but it cannot be decided whether the CB is caused by recurrent excitatory connections within the BS group, or by synchronous firing in another excitatory neuron population driving the BS neurons.

4.4.2.1 Some general considerations.

Most of the systems discussed can be expected to exhibit oscillatory activity. For example, the VN with their excitatory interconnections could easily give synchronous activity, whether through a coupled relaxation oscillator type mechanism, or by being driven in synchrony (Demontigny and Lamarre, 1973) as

occurs in harmaline induced tremor. In cultured VN neurons synchronous burst discharges are very likely, for Wojtowicz (1978) has described correlated bursting in cultured DN neurons in the presence of bicucculine. If connections between DN and VN in culture approximate those in vivo, then they should be synaptically coupled, and when bursts occur in DN they would be also expected in VN, especially the LVN, as it may be viewed as merely a displaced portion of the deep nuclei. Steriade and Hobson (1976) implicate excitatory connections from the GTF to the vestibular nuclei as the source of eye movements during REM sleep. The VN form reciprocal connections with the RF, and thus very likely the GTF, and thus this would represent another purely excitatory system capable of exhibiting correlated bursting. In view of the anticipated excitatory networks among the trigeminal and other nuclei, clearly the presence of evidence indicating recurrent excitatory interactions is of no help in narrowing down the choices for the BS neurons.

On the basis of fluorescence histochemistry of the BS cultures, Hendelman and Ferguson (in preparation) have proposed that the BS neurons correspond to the LC. If the physiological data on the LC currently available in the literature is accepted at face value, then the implication is that LC to LC connections should be inhibitory. This is clearly at variance with the electrophysiological findings. The "inhibition" produced by LC stimulation in its various target areas has been shown to be a more complex phenomenon than a simple IPSP, and is not really inhibition at all. In cerebellum (Moises et al, 1978; Hoffer et

al, 1973) the actions of LC stimulation have been described as being of slow onset and long duration. In hippocampus both inhibition and excitation have been reported following LC stimulation with inhibition predominating (Finch et al, 1978; Segal and Bloom, 1976). This suggests that the role of NE is modulatory rather than classical inhibition or excitation. In light of these findings, it is not possible to predict what action an LC to LC synaptic connection would have as no comparable studies (simultaneous two unit recordings in LC) have not been carried out.

Another complicating factor in the consideration of LC as BS is the greater potentialities shown by monoaminergic neurons. Evidence has been compiled by Amaral and Simammon (1977) that the LC may be considered as a central homologue of the sympathetic ganglia; more generally, there are grounds for considering all central monoamine containing neurons as being related to the sympathetic ganglia as they have similar developmental patterns (Seiger and Olson, 1973) and all central monoaminergic nuclei can innervate target organs normally innervated by sympathetic nerves; eg. iris (Olson and Seiger, 1972; Seiger and Olson, 1978)). Pursuing this analogy further it may be seen that sympathetic neurons do not appear to be as rigidly programmed as other central neurons, as the transmitter type of cultured sypathetic neurons may be changed from NE to Ach, or various mixtures of the two (in the same neuron) by appropriate experimental manipulations (Patterson et al, 1978). What is especially significant here is the extent to which a

target tissue in culture can shape the innervation pattern it receives from a monoaminergic neuron, as well as influencing its choice of transmitter (Patterson et al, 1978). Should the same process occur normally in the development of central monoamine systems, but with the various target groups of neurons influencing the specification of the monoaminergic neuron connections and transmitter, then surprises are likely.

There is no fundamental obstacle from preventing the existence of a synapse which utilizes NE as a transmitter, but induces an EPSP in the postsynaptic cell. The effect of a synapse depends mainly on the associated subsynaptic receptor molecules rather than the type of transmitter involved as is evidenced by the multifunctional effects of each of the various central neurotransmitter ccandidates on different neuronal populations.

4.4.1.2 Possible system developing into BS neurons.

On indirect evidence, one possible candidate for a NE containing cell group which may possibly have excitatory interconnections will be considered: the nucleus parabrachialis medialis (NPM). Fluorescent cell bodies have been reported in this nucleus by (Pin et al, 1968) and Chu and Bloom (1974). The NPM is shown by conventional anatomical techniques to be lateral to the LC and surround the branchium conjuctivum by Taber (1961), and to be medially continuous with the LC in cat (Jones and Moore, 1974). In the rat, the nucleus has also been described lateral to the superior cerebellar peduncle by

Palkovits and Jacobowitz (1974) as well as by Swanson and Hartman (1975). Both of these groups of authors have also described fluorescent axons and terminals within the nucleus. (This nucleus is also sometimes referred to as the marginal nucleus of the branchium conjunctivum). Taber (1961) describes the majority of the cells in the NPM of the cat as being small, although a few large multipolar cells are described by Taber as being similar to the cells of the SC. In rodents, only the smaller neurons are found within the NPM (B. E. Jones, personal communication). In cat Chu and Bloom (1974) report that the NE cells of the NPM project to the cerebellum along with those of the LC.

One apparently severe drawback which must be overcome is that within the rat, there are no fluorescent cells within the NPM, and the fluorescent neurons reported in the cat NPM are those of the LC (B. E. Jones, personal communication), which in the cat is more dispersed than in rodents. However, the fluorescent neurons in the cat NPM are large and multipolar and would thus correspond most closely to cells in the subceruleus (SC) rather than the dorsal LC. This may reflect a functional difference between the SC and dorsal LC, as Bertrand et al (1973), in a detailed mapping of neurons firing in synchrony with portions of the respiratory cycle, found the highest density of such cells in the NPM, SC and Kolliker - Fuse nucleus. Very few units exhibiting respiratory phase locking were encountered by them in the dorsal LC.

The gap which must be bridged to account for BS as NPM is between the anatomy and physiology. Bertrand and Hegelin (1971) postulate a respiratory synchronizing function for the NPM and associate it with the apneustic center. To account for respiratory rhythm generation by the NPM, and also to explain the results of their experiments involving resetting of the rhythm by electrical stimulation, they postulate at least two systems of self re-exciting neuronal populations within the nucleus. There seems to be sufficient evidence to accept their findings as correct. The problem arises because all literature examined showed mutually exclusive studies; either fluorescence and no physiology or physiology with no consideration of monoamine transmitters. It does appear likely, in view of respiratory units in SC and CA neurons in NPM, that CA neurons are participating in some manner in the generation of the respiratory discharge. If the physiological role of CA neurons in apneusis is identical in mouse as in cat, then the correlation pointed out would remain valid, despite the lack of fluorescent cells in the NPM of rat.

The BS cultures may thus be analogous to the apneustic center. Consideration of the effects of the NE neurons in the generation of rhythmic activity in self - reexciting networks of neurons leads to a number of possibilities about their role. A direct role may be postulated if the NE cells form recurrent excitatory synapses. Also it is possible for the NE cells to have a modulatory role, but the effects of this are more difficult to predict. The possibilities:

1. The BS neurons actually represent a NE containing network (involved in respiratory discharge generation) which form recurrent excitatory NE synapses.

2. The activity of BS neurons is accounted for by a self re-exciting neuronal population from the NPM, but the NE cells are not the ones generating the correlated firing; this is due to another population of interconnected excitatory neurons, and the role of the NE cells is to modulate the exciteability level of these cells.

3. BS neurons are LC, but in culture they have formed either:

   a) Recurrent excitatory synapses (analagous to point 1 above).

   b) Under the culture conditions, LC will form connections with excitatory populations to give bursting type activity. (Analagous to point 2 above).

If these are the only possibilities of relevance, then it is clear that these possibilities may not be decided between on the basis of existing data since the necessary experiments were not carried out. However, it would seem likely that relatively simple experiments could be used to distinguish among the first three possibilities. Possibility 1 may be most simply tested by observing the response of BS neurons to iontophoretically

released NE; if a large number of excitatory responses are observed then it is possible that NE is an excitatory transmitter between the BS neurons. In order to prove this, however, would involve rather detailed experiments involving adrenergic blocking agents and intracellular recording in order to distinguish this possibility from the the one of NE increasing the excitation in a purely excitatory group of neurons. The only possibility which would discredit this line of reasoning would be if predominantly inhibitory effects of NE were observed.

One major question which can be asked about the role of the NE is whether it is acting as a modulator or as a classic transmitter. It is were to simply increase the effectiveness of PSP's as it has been postulated to act in cerebellum (Moises et al, 1978), then the existence of a second population of excitatory neurons with recurrent collaterals would have to exist in order to produce the correlated firing. On the other hand, if it exerted a direct inhibitory effect, the circuitry of the BS cultures may be too complex to be useful.

What can be said now is that the BS culture system has been characterized to a great extent, but not sufficiently to allow for the nature of the synaptic connections to be worked out in detail. However, as there is clear evidence of monoaminergic (presumably NE) neurons, the system could be very useful in the investigation of monoaminergic neurons with other cell groups in the brainstem or the well characterized cerebellar portions of the cultures. In order for this to be the case, the experiments

briefly outlined above would be required to characterize in greater detail the nature of the monoaminergic responsiveness and synaptology within the BS cultures.

4.5 MODEL WHEREBY GLU CAN PRODUCE SYNCHRONIZED ACTIVITY.

Leaving aside the question of identity of the BS neurons, one may ask of what use are the BS cultures? As just another ill defined neural network, not very much. One field where the system could be useful is in the investigation of a small network of mammalian neurons under various experimental conditions (pharmacological electrical etc) in order to see how the network behavior is affected.

One model which may be directly tested using the BS cultures is that of a neuron population with purely excitatory connections. To a large extent this appears to be what the BS system represents. The manner in which this has been studied, using two iontophoretic electrodes, has not been reported previously, and the disadvantage arising from this system producing whole culture correlated activity when only limited projections are being looked for are advantages if it is wished to study the change from a desynchronized activity mode to a synchronized activity mode in the cultures. Since it has proven possible to induce correlated bursting in virtually all pairs of neurons in BS cultures using two glu electrodes, the system would be ideal in determination of the significant parameters involved in the state transition. This could possibly be a model system for epilepsy, but would also be of substantial

theoretical interest in the modelling of excitatory networks by computer in which the component of glu excitation is included. This, to the best of my knowledge, has not been done either.

Footnotes

1 p41

Glucose concentration which is mentioned is that which is utilized within the culture medium, and is kept this high so that better myelination of the axons will occur (W. H. Hendelman, personal communication). Within the BSS solution used during the experiments, the glucose concentration is 6 gm/l (33 mM), and the total ionic strength of the BSS has been calculated as 309 milliosmoles. This is not significantly different from the osmolality of human CSF which is about 306 mosml.

2 p56

Hyperpolarizing currents were utilized in essentially all cells recorded intracellularly, but often the current was not applied when an interaction was seen. Roughly speaking, all of the cell pairs in which prominent interactions occurred were not maintained by hyperpolarizing currents, while the cells in which evoked averaged PSP's were sought were.

In the case of the cells which exhibited correlated bursting, one had no hyperpolarizing current put on it during the bursts (pair (20-7-3)) and the two other cells which fired one long burst each were hyperpolarized at the time. Pairs (23-3-1) and (23-27-6) had no hyperpolarization applied when the interaction was most prominent. All cells for which averaging was carried out were hyperpolarized to various levels during this time as only one cell did not exhibit spike activity following penetration.

3 p 138

The criteria which were used to determine whether or not a voltage event obtained by averaging was actually a PSP were more rigid than necessary, and discriminated against the smaller PSP's. This was due to the requirement that the event appear in sequential averages and be comparable in shape and latency. This requirement was established so that the same number of sweeps could be used in both the average, and the control average to make the two directly comparable, but was not essential as the control could be scaled up if need be.

The most relevant factor to be remembered is that the presence of an evoked PSP is established by a comparison of sequential averages, and the event being present in these.

TERMINAL APPENDIX

1.1 TERMINAL DESCRIPTION

1.1.1 Output format

Interspike interval data within the terminal is stored as 16 bit binary coded decimal (BCD) numbers in two seperate stacks. Before looking in detail at the manner in which this is accomplished, the output of the terminal as seen by the computer will be described.

All of the timer data as well as control signals are serially transmitted on an output line (asynchronously) at a maximum data rate of 2400 bits/sec (2400 baud). Since the transmission is asynchronous, a start and stop bit are a part of each byte transmitted, and so the rate is 240 bytes/sec.

The smallest unit of transmission is a byte, and each byte is divided into two 4 bit sections (nibbles). The first nibble contains information about data origin, etc and is called the control nibble (Nc). The last four bits contain one digit of data and are called the data nibble (Nd).

A word is defined as one complete data number, and may be from 1 to 4 bytes in length (as only one data digit/byte is

transmitted). Leading zeroes from data are not transmitted. An overflow condition in a timer (representing a count of 10000) is coded as a single byte. Control words are always 4 bytes long.

The data transmitted contains all the information necessary to recreate the original spike train, and to keep records separate. (data words are transmitted in the same order they are recieved by using a first in first out (FIFO) stack as data memory). A program on the Microdata seperates data into spike trains, reassembles the numerical data, and inserts control data at the appropriate places before transmission to 360 disk.

The functions of each bit within Nc are as follows: (Bit 0 is first one and Bit 3 is last one).

Bit 0 continuation flag (CF). For first byte of a new word CF=0, and for every subsequent byte it is 1. Its function is to indicate the start of a new word.

Bit 1 Control word flag (CWF) For data words CWF=1, but if the word is a control word, CWF=0 only in the first byte of the control word (and also in any subsequent bytes containing the data number 'E').

Bit 2 Overflow flag (OF). When OF=0 no overflow is present, but when the byte is an overflow byte, OF=1.

Bit 3 Spike train number (ST)

ST=0 indicates timer1 as data source.

ST=1 indicates timer 2 as data source.

Data words may be of two types; numbers <=9999 or overflows. Numerical data is transmitted without leading zeroes, and thus may be from 1 to 4 bytes in length. The number 0000, however, is transmitted as a single 0 in order to prevent errors in the possibility of an overflow occuring, and then a spike occuring very shortly therafter with the result that the timer interval following the overflow would be 0. Timer overflows are represented internally within the terminal as the number F000. The digit F in the leading position is the code used by the output logic to signal an overflow, and a single overflow byte is transmitted.

Control words are used for indicating the beginning and end of records. The terminal logic places a control word at the same point in each spike train when a start command is recieved by the terminal. Each control word is 4 bytes in length, and, although there is a possibility of transmitting information within a control word, this feature has not been extensively used. The structure of the control word is as follows:

Byte 1 = 000X1110 where

X=ST and digit E (1110) in Nd represents control word code.

Byte 2 = 110XZZ00 where:

X=ST, and ZZ are two bits which are used in internal data transmission for control word generation.

Byte 3 = CCCCNNNN

Byte 4 = DDDDNNNN where:

NNNN can be any number in the range of 0 to E (if NNNN = E, then CWF=0)

Only minimal error detection logic is used within the Microdata program during data conversion; it simply looks at the Nd of data words and checks whether they are in the range of 0 to 9. Errors during data transmission are very low (no quantitative data available) as the distance from terminal to computer during data transmission is about 3 meters.

The primary error check utilized is the time of the record. Using a stopwatch, the time of each record transmitted through the terminal is obtained every time a new record is transmitted. Also, on every EDT simultaneous spikes are present at the beginning and end of each record. These intervals can later be located in the data, and equivalence of times checked for.

1.2 TERMINAL LOGIC

1.2.1 Overview

Basically the terminal consists of two timers to obtain interspike intervals, a buffer memory to store them, output logic necessary to encode the data for serial transmission to an external computer, and control logic to keep everything running smoothly. In addition, the unit is also obsolete (thanks to advances in micro-computer technology), and anyone who now uses

discrete logic to build such a unit (or a circuit of equivalent complexity), (for any other reason other than as a breadboard before putting it all on a chip) is either crazy, or a masochist.

1.2.2 Analog Tape Interface (ATI)

1.2.2.1 Function

The function of the ATI is both to make the encoded data tapes (EDT) and to read them into the terminal. EDT's are cassett tapes recorded on a stereo cassette recorder at 1 7/8 ips and consist of two channels of spike train data as well as record separation information.

An EDT record is a portion of an extracellular record in which spikes may be separated by simple amplitude discrimination, and is recorded without interruptions. At the beginning of the record, following the recorder turn on artifact, is a tone burst consisting of negative going spikes. This is the signal for enabling the timers on the terminal when the record is being read. Then, after a variable time, two simultaneous positive spikes signify that the initial part of the data record is being read. Each ap is transformed into a + spike of about 0.75 V and 400 microsec duration. At the conclusion of the record, two simultaneous positive spikes occur, and then another tone burst of negative spikes about 5 seconds later.

The output portion of the ATI accepts 2 discriminator outputs (TTL levels) and transforms them into the short positive spikes which are written on tape. In addition, manual switches are sued to initial writing of data (and creation of simultaneous initial spikes) and to stop writing of data and generate the trailing spikes. The function of these spikes is to check the validity of data transmission, as the times from the first to last spike on each channel should be equal if data was correctly transmitted. Also a switch is used to put negative going tone bursts on the tape to give an aural indication of end of records.

The input portion of the ATI accepts the tape recorder output while it is playing the EDT, and outputs a short TTL level pulse (for each channel) every time a positive spike is detected. Negative spikes from the tone bursts do not give any output as they are used as an aural cue to know when to enable or disable terminal timer. The input portion output is connected directly to the terminal inputs.

1.2.2.2 Logic

In the interests of brevity and sanity the detailed description of the ATI logic has been omitted. Anyone wishing to see it may do so by coming to room 348 in Medicine.

1.3 TERMINAL OPERATION

1.3.1 Design philosophy

The terminal is an anachronistic device remaining from the days before microprocessors when people still built such things. It is constructed almost entirely of small and medium scale integration TTL logic IC's. Many of the units in the terminal which are constructed of multiple chip assemblies may now be purchased oin a single IC package. Thus a detailed description of the logic of the device would not serve much purpose (except from the point of view of future historians) as it is unlikely that anyone will ever chose to utilize this method to do something of this nature again.

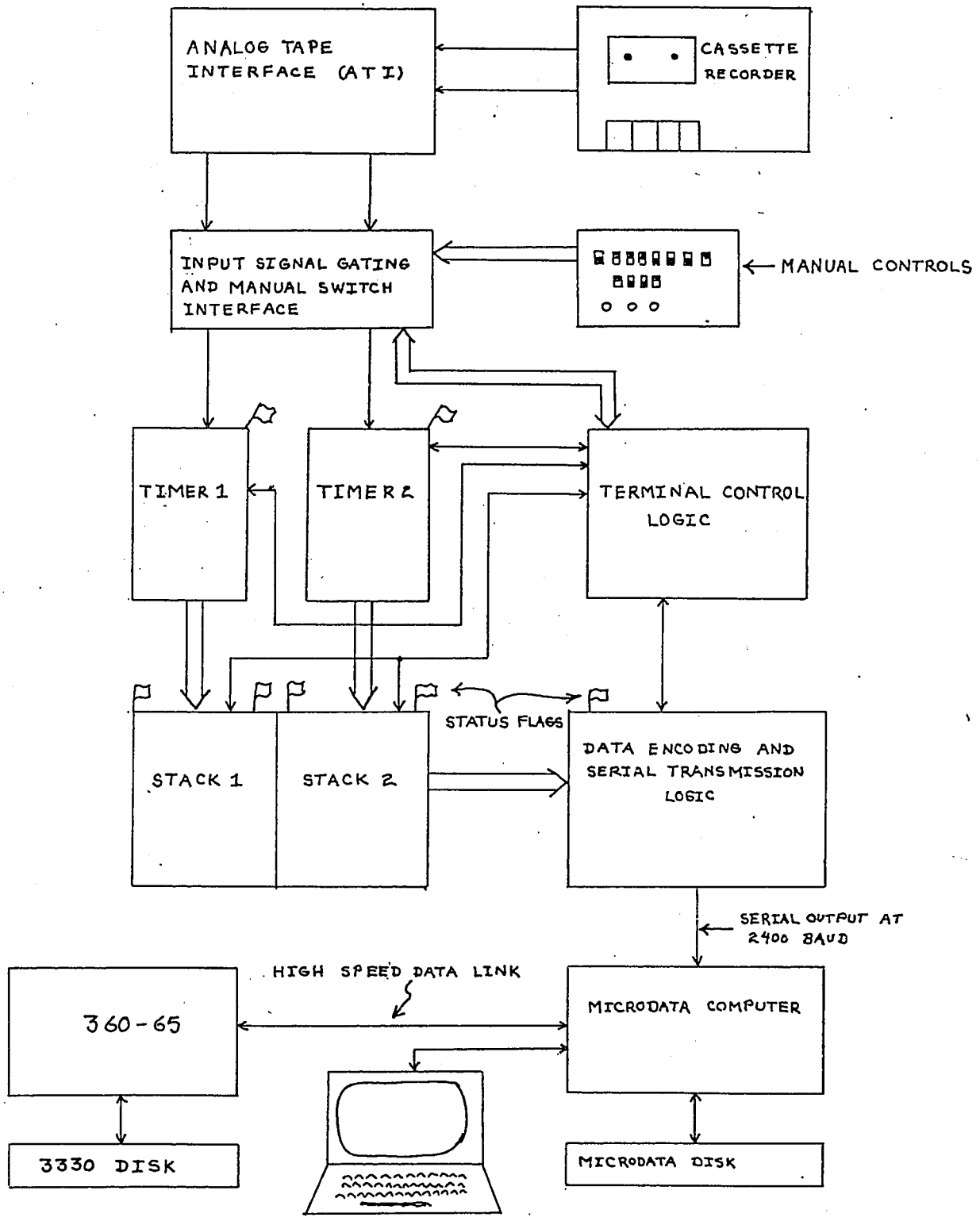
The design philosophy employed was essentially a method of translating flowcharts into digital circuits using a varient of the state machine concept as described by Clare (1973). Basically, a state machine is a unit which has a number of internal states and possible link paths between these states. A clock is used to make a transition from one state to another every clock cycle, and this transition is dependant on the value of logical variables, or may be direct. Essentially it is the decomposition of a flowchart of a process into a number of states, and their connections so that it may realize a given algorithm.

Hierarchies of state machines may be put together, with the high level state machines giving commands to the lower level ones. The terminal which was built is essentially a two level state machine with the highest level machine controlling the data flow from the timers to stacks, and from stacks to output

units. The second level state machines are the stacks, and output unit. The timers do not fit the definition of a state machine, as they are totally controlled.

Figure T1 shows the basic units making up the terminal, and their relationship with the data input and the computer. The terminal control logic is the high level state machine, and is controlled by a switch panel. Data from the EDT goes through the ATI where the signals on the tape are converted to narrow TTL level pulses. These go into the input signal gating logic which is under manual control. When it is disabled, no data from the ATI makes it to the timers of the terminal, and when enabled, the pulses go straight through. Two timers are employed (one for each ST), and they may use a clock frequency of any desired value, although the standard clock period for timing was 200 microsec. The timers may, under manual control, be enabled or disabled. Each timer has a flag (TF) which is used to inform the terminal control logic about its status. When the timers are enabled, they begin to count clock pulses until an input pulse from the ATI comes in. This stops the count, and sets the TF. The control logic is constantly scanning the timer flags, and when it detects that one is set, it loads the count from the timer into its associated stack, then resets the flag, and restarts the timer. The other condition which sets the timer flag is when an overflow occurs. Each timer has a maximum count of 9999, and when this is exceeded, the TF is set, and this results in the number F000 being stored in the stack. In the absence of input pulses the timers give a series of overflows at a rate dependant on the clock frequency.

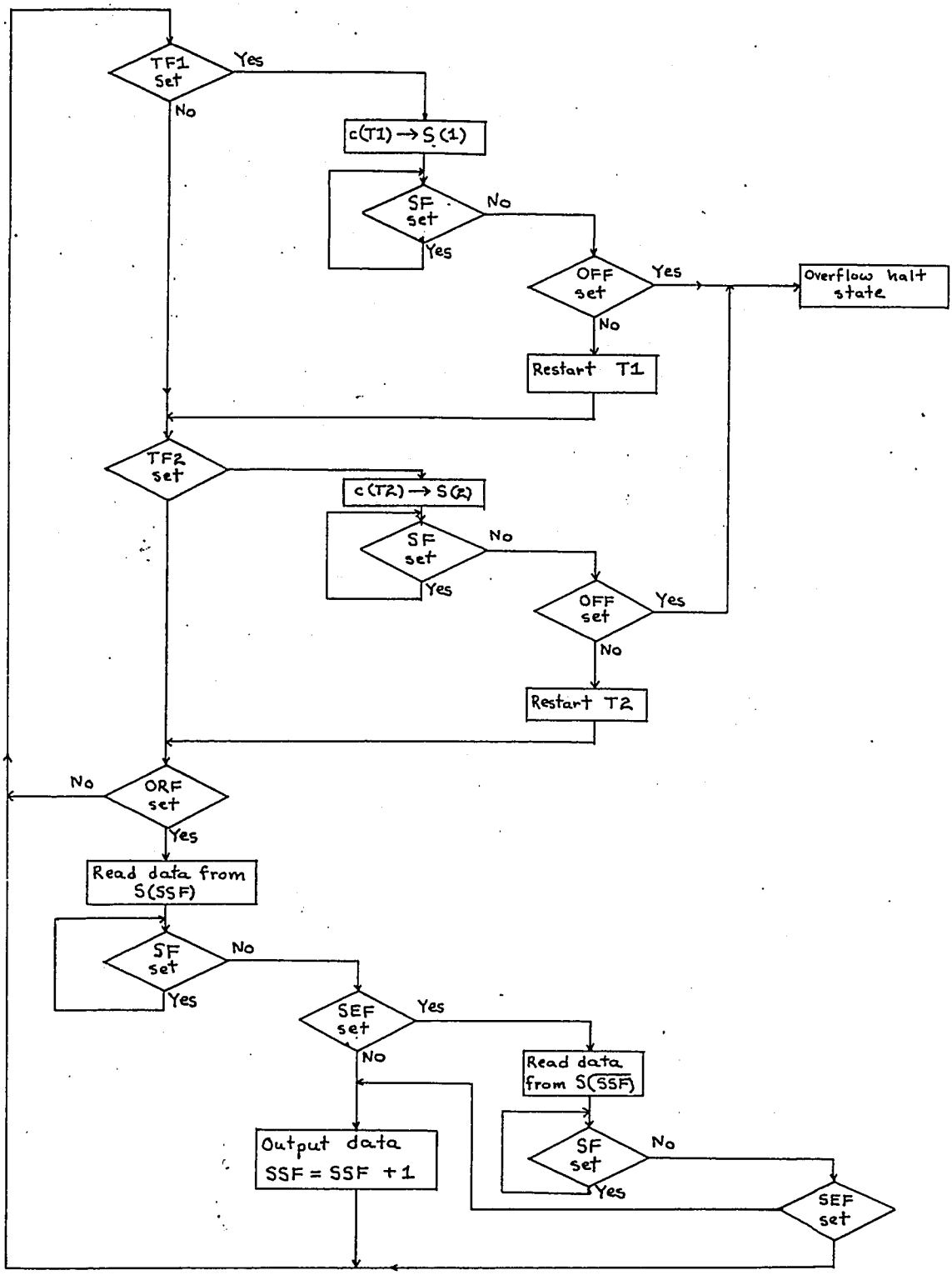
Figure T1
Data flow in terminal/computer



Two stacks exist only in theory, but actually the logic is common, and they simply use different areas of a 1024 word (16 bit words) memory. Each stack occupies 255 words for data storage, and the 256'th word is used to store the pointers for the first free data address, and the first data location. The stack is A first in first out stack (FIFO) and will not be described further.

In order to see the sequence of operations which are being executed by the control unit, diagram T2 should be consulted. This flowchart shows the cycle which this unit goes through. Each step takes 1 microsec to execute, and thus the longest a timer would have to wait for servicing would be about 18 microsec. The logic checks the state of each of the timer flags in turn, and if the flag is set, it goes through a routine which loads the contents of the timer into its appropriate stack, and then cycles through a loop until the stack has finished its store cycle. Once the stack has finished, it checks to see if an overflow has occured in the stack (> than 255 numbers) and if this is the case the terminal stops. If everything is in order, the timer is restarted and the next timer flag is checked and the same sequence of storage operations performed if necessary. Once the timers have been serviced, the logic next looks at the state of the output flag. The output unit accepts a data word from a stack, and information from which timer it is from, and then proceeds to cut up the data word into up to 4 bytes with their associated control information to be transmitted to the computer. While it is engaged in this process, it clears its

Figure T2
Operation sequence of terminal control logic



output ready flag (ORF) and does not set it until it has finished transmitting the data. The state of the ORF is checked, and if the output unit is engaged in transmitting data, the control unit goes back to checking the timer flags to see if any spikes have occured in the meantime. If the ORF is set, then the control unit checks to see if there is any data in the stacks to be transmitted. In order to balance transmission between the stacks, a flip flop (SSF) is used to alternate stack selection, and once data has been read from one stack during a cycle, it is read from the other stack first during the next cycle. The control logic asks the stack designated by SSF if it has any data, and if the response is negative (as indicated by the setting of the stack empty flag (SEF)), it asks the next stack if it has any data. Once it finds data in a stack this data is loaded into the output unit, and it begins to transmit it.

The control logic may be taken as the general case of a state machine, and both the stack, and output board go through similar such circular sequences of state transitions, although they wait for orders from the control logic. The output of the terminal goes into a Microdata computer as was explained earlier within the Methods section.

MATHEMATICAL APPENDIX

1.1 PRELIMINARY COMMENTS

This section was originally envisaged as a major part of the thesis dealing in detail with the mathematics involved in spike train analysis. Subsequent rethinking of the thesis organization resulted in substantial amounts of material being moved out of this appendix into the body of the thesis. Other material was still thought of being included, but the present length of this treatise makes further elaboration on mathematical matters questionable. It would have been most likely more prudent to dispose of this appendix at an earlier stage, but the concept refused to go away, and like its anatomical counterpart in the body, this vestigial remnant remains.

After the final format of the major sections of the thesis was achieved, a mixture of not clealy related ideas were left for inclusion in this appendix. If the bridging text were to be inclded, much duplication between earlier sections of the thesis would occur: eg with the simple exposition of ST analysis to be found in the introduction. Presenting vaious aspects of the statistics in a more rigorous maner as well as the assumptions involved would be more mathematically correct than the explanations now employed. Also certain assumptions made about

the neurons should have been examined in more detail to make necessary corrections for the BS culture system. This had the danger of taking things rather far afield from the original research proposal.

Finally, after considerable deliberation, a decision was made to include the mathermatical rationale behind visual burst analysis This was to have been a component of the math appendix, but now constitutes essentially the whole of the appendix. One section which was of great interest, but was omitted, was that of modelling of neural networks. A program to simulate networks using an adaptation of the 4 state variable neuromime model (MacGregor and Lewis, 1977; Macgregor and McMullen, 1978) was written, and run with the inclusion of constant depolarizing currents in a self re-exciting neuronal network to simulate the effects of glu. In the networks tested recurrent patterns were seen to occur, and most often a direct "synapse" between two neurons was not implied from the CCH computed for that pair. Undoubtedly the BS neuron system could have been simulated, but the vast number of perturbations posible (due to so many parameters in a single neuron which can be varied, not even considering the interconnections among the neurons) would have required far more detailed intracellular data on various electrical parameters of the BS neurons to get meaningful simulations Without this data, it remains an interesting mathematical curiosity, but nevertheless a useful one (but not useful enough to be inserted into the thesis).

1.2 RATIONALE BEHIND VISUAL BURST ANALYSIS

One problem which was observed with many records is that when correlated burst activity is present at a very low rate within a background of seemingly non-correlated activity, then the CCH may not demonstrate the interaction. Since the occurence of near simultaneous complex spikes in two cells appears significant, data were visually checked for the presence of simultaneous bursts.

This phenomenon was found to occur very frequently, although not all cells had the same rate of simultaneous bursting. As many cell pairs were classified by this method, a mathematical analysis was made of its validity.

A numerical measue of the pobability of obtaining the measured number of burst coincidences is required. One primary requesite for the method to be described to work is that the bursts be unambigously distinguishable. This criterion is satisfied in the case of decremental bursts, and is discussed within the results section. Let both ST's be replaced by only the bursts which occur, and let Na be the number of bursts in train A and Nb the number in train B. It is next assumed that one can unambiguosly divide the bursts into coincident and non-coincident bursts. (This is possible in virtually all of the cell pairs being considered). Let Td be the maximal duration of the burst encountered, and assume that bursts will occur only at integral multiples of Td. Then the number of intervals possible in the record considered is:

Nt = T/Td where T is total time of record (1)

Thus the model is:

Let Nc equal the number of coincident bursts. It is next assumed that the bursts are independant, and may occur in any pattern in either ST. (This is not strictly correct, but it makes the mathematics much easier). Thus, the total number of combinations of the two burst patterns is N* where:

$$N^* = \binom{Nt}{Na} \cdot \binom{Nt}{Nb}$$

It is known that Nc bursts are coincident. Thus they must stay together, and can be arranged in:

$$N^*c = \binom{Nt}{Nc}$$

ways. The remaining uncorrelated bursts must be distributed at random, but have only (Nt - Nc) intervals remaning for then to go in. Furthermore, no more coincidental pairing are allowable for these remaining bursts. This leaves N*a = Na - Nc bursts in ST A and N*b = Nb - Nc bursts in ST B. The number of ways of arranging these bursts in N*t = Nt - Nc intervals is:

$$\binom{N^*t}{N^*a} \cdot \binom{N^*t - N^*a}{N^*b}$$

Thus the probability of obtaining the observed pattern is given by:

$$Pc = \frac{\binom{Nt}{Nc} \cdot \binom{Nt - Nc}{Na - Nc} \cdot \binom{Nt - Na}{Nb - Nc}}{\binom{Nt}{Na} \cdot \binom{Nt}{Nb}}$$

As an example, consider one cell pair where 3 correlated bursts were seen in each ST in 438 seconds. No uncorrelated butsts were seen, and the longest burst was 500 ms. Thus Nt = 876 and Na = 3, and Nb - Nc = 0. Substituting these numbers into the above formula gives a value of $Pc = 8.95 \times 10^{-9}$. This probability is sufficiently low to assume that the observed arrangement of bursts did not occur by chance. Indeed, it is sufficiently low, that even if the bursts cannot occur at random with respect to one another, there is ample room for addition of modification factors which will still leave the same result: the presence of correlated bursts is definately significant.

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