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AN ELECTROSENSORY LATERAL LINE LOBE SLICE PREPARATION:
PYRAMIDAL CELL ELECTROPHYSIOLOGY

By

William Bruce Mathieson

A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment
of the requirements for the degree
of Doctor of Philosophy.

Department of Anatomy
University of Ottawa
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December, 1986

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'...ces choses n'étaient pas simples. Ou
peut-être alors la simple vérité était-
elle la plus difficile à démontrer.'

--Gabrielle Roy

1.

ABSTRACT

An in vitro brain slice preparation of the electrosensory lateral line lobe (ELLL) of weakly electric fish was developed and the electrophysiological characteristics of the main ELLL output neurons, the pyramidal cells, were measured. Extracellular electrode recordings demonstrated that pyramidal cells are capable of spontaneous, rhythmic spike activity. Intracellular recordings showed that intrinsic oscillations in membrane potential underlie the bursting behaviour. The majority of pyramidal cells respond to depolarizing current pulses with an initial lag in spike firing followed by a non-accommodating, higher frequency spike train.

Time and voltage-dependent properties of pyramidal cell responsiveness, as well as the effects of pharmacological blocking agents indicated that rhythmic activity and repetitive firing are dominated by a persistent, subthreshold g_{Na} which activates at depolarized levels and is the driving force behind the membrane potential oscillations and the sustained (non-accommodating) spike firing. In addition, a transient, outward potassium conductance (g_A) is responsible for the lag in spike firing by acting as a 'brake' during the initial 50-200 msec of a depolarizing stimulus.

Calcium currents and calcium-dependent potassium conductance add to the interval between spontaneous bursts but appear insufficient for spike frequency accommodation.

The in vitro behaviour of pyramidal cells differs substantially from the behaviour of the same cell type in

vivo. Possible explanations are discussed.

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ABBREVIATIONS

ACSF: artificial cerebrospinal fluid
AHP: afterhyperpolarization
4-AP: 4-aminopyridine
2-APV: 2-amino-5-phosphovaleric acid
BP: basilar pyramidal cell
DAP: depolarizing after potential
DFL: deep fiber layer
DML: dorsal molecular layer
EGTA: ethyleneglycol-bis (B-amino ethylether)-N,N'tetra
acetic acid
ELLL: electrosensory lateral line lobe
EOD: electric organ discharge
EPSP: excitatory post-synaptic potential
FPP: fast prepotential
IPSP: inhibitory post-synaptic potential
JAR: jamming avoidance response
LY: Lucifer Yellow
NBP: non-basilar pyramidal cell
NMDA: N-methyl-D-aspartic acid
pl: plexiform layer
Rin: input resistance
SP: slow potential
TEA: tetraethyl ammonium
Tm: membrane time constant
tsf: tractus stratum fibrosum
TTX: tetrodotoxin
Vm: membrane potential
VML: ventral molecular layer
VR: resting membrane potential

INTRODUCTION

Mechanisms that regulate neuronal excitability are the subject of enormous interest insofar as they ultimately govern both normal and abnormal central nervous system activity. Numerous investigations, in intact animals as well as in more recently developed in vitro preparations, indicate that neurons are equipped with a diverse set of membrane channels, many of which are modifiable under appropriate conditions. This last characteristic imparts plasticity in a neuron's response to a changing environment.

The ability to correlate biophysical properties with behavioural physiology is greatly facilitated by the use of simple model systems. With this approach, the neural substrates for isolated behaviours can be identified anatomically, manipulated physiologically or pharmacologically and the resulting behavioral effects measured. In this respect, the electrosensory system of weakly electric fish has been especially useful; anatomical, physiological and behavioural studies have been well coordinated to understand certain aspects of how central processing of electrosensory signals occurs. This thesis deals with mechanisms that regulate electrosensory function at the cellular level, which, together with the more conventional approaches to sensory physiology, should extend our current understanding of how electrosensation operates.

Electric field detection

Three orders of teleost fish have electroreceptive capabilities: siluriformes (catfish), mormyriiformes (African) and gymnotiformes (South American). While catfish are sensitive to low frequency electric signals generated from environmental sources, mormyrids and gymnotids have more highly developed electrosensory structures, and utilize self-generated electric fields for the purposes of electrolocating objects and interacting socially with conspecifics (for general reviews, see Bullock '82, Carr and Maler '86).

In the gymnotid species used in this study, a nearly sinusoidal, constant frequency electric organ discharge (EOD) is emitted from an electric organ located in the fish's tail (Knudsen '74). Electroreceptor organs are distributed about the body surface, and are highly sensitive to electric field disturbances caused by nearby objects with conductivity different from the surrounding water, or from mixing of their own EOD with those of neighboring fish. In addition to ampullary receptors sensitive to low frequency AC signals, (as found in catfish), two physiologically distinct types of tuberous receptors encode changes in EOD amplitude (P-type afferents) or timing of the zero-crossing of the EOD waveform (T-type afferents) (Scheich et al. '73). All three types of electroreceptive afferents project centrally in somatotopic

register, and synapse within the electrosensory lateral line lobe (ELLL) of the medulla (Carr et al. '82).

ELLL morphology

The continuity of the ELLL is interrupted by 3 'breaks' which partition its structure into 4 roughly equal segments: medial, centromedial, centrolateral and lateral (Carr et al. '82; Heiligenberg and Dye '82). Intracellular labelling with HRP of physiologically identified primary afferents indicated that each tuberous afferent divides to innervate the lateral, centrolateral and centromedial segments, whereas ampullary receptors innervate the medial segment alone (Heiligenberg and Dye '82). A curious feature of this organization is that each segment receives its own somatotopic representation of the body surface or 'pisciculus' which is aligned as a mirror image of those in the adjacent segments (Carr et al. '82). The functional significance of multiple maps in the ELLL is still unknown, although it reflects a general tendency for parallel processing in the electrosensory system (Carr et al. '81; Maler et al. '81a; Mathieson et al. '86).

The cytology of the ELLL has been thoroughly investigated using golgi and HRP-labelling techniques at both the light and electron microscope levels (Maler '79; Maler et al. '81a; Heiligenberg and Dye '82; Mathieson et al. '86). A simplified diagram of the cell types and their connections, based on these works, is depicted in fig.1 and described in the following paragraphs. The ELLL is a highly laminated

structure, containing at least 11 cell types and afferent input originating from 3 main sources. Primary afferent fibers from the periphery enter the ELLL rostrally and form the ventral-most lamina. As they pass in the caudal direction, terminal branches turn dorsally to ramify within the deep neuropil lamina.

In the pyramidal cell layer are located the somata of 3 cell types: basilar and non-basilar pyramidal cells which are the main output neurons of the ELLL, and one class of interneuron, the polymorphic cells. Basilar pyramidal cells (BPs) have elongated basilar dendrites that extend ventrally into the deep neuropil layer where they receive excitatory chemical synapses with P-type primary afferents. Thus, BPs respond to increases in afferent activity with an increase in firing rate (Bastian '81b). Granule cell interneurons also receive primary afferent input and then make inhibitory synapses with basilar pyramids. Measurements of the relatively large dendritic spread of the granule cell population led to the prediction that a compact excitatory receptive field for BPs would be flanked by a larger annulus of surround inhibition (Maler et al. '81a). Non-basilar pyramidal cells (NBPs) lack a basilar dendrite and therefore do not receive a direct input from the periphery. Instead, primary afferents make excitatory synaptic contact (both electrotonic and chemical) with granule cell interneurons which, in turn, make putative inhibitory synapses with NBP somata and somatic dendrites. Thus, NBPs receive electrosensory input indirectly

via a disynaptic pathway. Increases in afferent input therefore result in decreased NBP firing, the opposite of the BP response.

Granule cells also have a long ascending dendritic process (not shown in fig 1) that makes gap junction synapses only with non-basilar pyramidal cells which are offset from the parent granule cell. This special arrangement of electrotonic coupling and inhibitory synapses was predicted to endow NBPs with an off-center, on-surround receptive field organization (Maler et al. '81a). Physiological measurements of pyramidal cell receptive field properties support the neuroanatomy-based predictions (Bastian, '81b; '86a,b).

Polymorphic cells, located in the ventral aspect of the pyramidal cell layer are in a key position to regulate the receptive field properties of pyramidal neurons. Although polymorphic cells have yet to be studied physiologically, their action is presumed to be inhibitory, based on the ultrastructure of their synaptic contacts (Maler et al. '81), furthermore they appear to use GABA as a neurotransmitter (Maler and Mugnaini '86). They are driven by descending as well as P- and T-type afferent input and have an extensive axonal plexus which makes synaptic contact primarily with granule cells and, in addition, makes axo-axonic synapses with primary afferent fibers in the deep neuropil (Maler et al. '81). Thus, polymorphic cell inhibitory terminals can not only gate the incoming primary afferent input (axo-axonic), but also shape the pyramidal cell on-response via granule cell

inhibition.

Pyramidal cells are output neurons of the ELLL; their axons collect to form the plexiform lamina immediately ventral to the pyramidal cell layer. The majority of their axons project bilaterally to two midbrain structures, the nucleus praeeminalialis and layers 3,5,7,8 of the torus semicircularis (Carr et al. '81; Maler et al. '82).

Pyramidal, polymorphic and certain granule cells have extensive apical dendrites which pass dorsally into the molecular layers of the ELLL. Here the dendrites of each cell type receive a large, descending excitatory input. The only qualification to this statement is that type II granule cell apical dendrites do not reach higher than the middle of the molecular layer. The dorsal molecular layer (DML) contains vertical and parallel fibers originating in the overlying caudal lobe. These parallel fibers are oriented rostro-caudally and make multiple synapses with the distal portions of the apical dendrites of each cell type except granule type I cells. Parallel fibers in the ventral molecular layer (VML) originate in the nucleus praeeminalialis and enter the ELLL in the stratum fibrosum. In the VML they pass medio-laterally, synapsing with proximal portions of all apical dendrites. Thus, ELLL neurons are stimulated by descending inputs which differ in their origins and the geometry of their synaptic interactions.

The components of the ELLL circuitry described above evaluate amplitude-related changes in electroreceptive input

and form the neural substrate for object detection. This information is carried to the ELLL predominantly by P-type afferent fibers. The ELLL is also equipped with a separate, parallel pathway designed to relay precise timing information to higher electrosensory centers (Szabo and Fessard '74; Maler et al. '82). This pathway receives peripheral input exclusively by T-type afferents (Maler et al. '81a; Mathieson et al. '86). T-units make electrotonic synapses with spherical cells, which lie in a narrow band within the neuropil. Spherical cell axons subsequently relay the timing information to the contralateral layer 6 of the torus (Maler et al. '82). Since the spherical cells have no synaptic connections with pyramidal cells or other amplitude-sensitive neurons of the ELLL, they are not included in the wiring diagram of fig. 1. The T-unit pathway underlies social interaction among electric fish, in particular, the well documented jamming avoidance response (JAR) (Bullock et al. '72; Scheich '77; Heiligenberg '80).

Pyramidal cell physiology

Electrophysiological studies employing several species of gymnotid fish have examined the responsiveness of pyramidal cells toward a variety of electrical stimuli. The results of these investigations have identified parameters which are important to electrosensory processing in the ELLL.

Spike adaptation

Enger and Szabo ('65) first reported that ELLL pyramidal neurons are sensitive to motion and direction of objects

placed in their receptive field. Many of the units responded in a phasic manner, leading the authors to speculate on their possible role as edge detectors for moving objects. More recently, Bastian ('81b) recorded similar units, highly sensitive to the movement of small objects across a localized receptive field. Two physiological response types were identified: E-cells transiently excited by conductive objects (ie. metal sphere) and inhibited by nonconductive (ie. plastic) objects, and I-cells which respond with an opposite pattern. A subsequent report identified E and I cells by HRP-labelling, as basilar and non-basilar pyramidal cells, respectively (Saunders and Bastian '84). Since pyramidal cells evaluate electrosensory events with time-dependent components such as object movement, their electroresponsiveness must be capable of matching the timing of the incoming afferent signals. E-cells, in particular, are most sensitive to amplitude modulations with frequencies of ~64 Hz (Bastian '81b) which requires a highly phasic spike response. This characteristic was demonstrated by presenting the fish with step changes in EOD amplitude, stimuli that lack a motion component. Bastian found that both E and I cells respond abruptly to stimulation, and rapidly adapt their spike frequencies to near-baseline levels (Bastian '81b). The mean time constant for spike-frequency adaptation in E cells is 37msec (Bastian '86a).

It is important to note that this phasic firing pattern can only partially be accounted for by receptor adaptation. Electrode recording of receptor activity in response to

amplitude modulations showed that P-type afferents are moderately phasic cells (Bastian '81a; Matsubara '82). However, convergence of afferent input onto pyramidal cells results in a dramatic increase in the degree of phasic firing (Bastian '81b; '86a). The disparity in temporal coding, at receptor vs pyramidal cell level suggests that spike adaptation is mediated in part by 1) local circuitry with the ELLI, or 2) by intrinsic biophysical properties of the pyramidal cells themselves. The anatomical description of the ELLL by Maler and co-workers supports the notion that granule and polymorphic cell inhibition may add to the phasic response. Since intracellular recording of pyramidal cell electrophysiology is extremely difficult in whole animals, very little is known about intrinsic mechanisms that govern their response characteristics.

Descending input to the ELLL

The ELLL is the first-order processing station for electroreception. Output from spherical cells and pyramidal cells convey phase and amplitude-related information on to higher order electrosensory centers. The electrosensory system has also evolved an elaborate series of feedback pathways which ultimately return to the ELLL in the form of parallel fiber input as described earlier. This descending input provides the ELLL access to a number of other sensory modalities, and has recently been shown to exert considerable regulatory influence over pyramidal cell activity.

Two electrosensory nuclei occupy pivotal positions

within ELLL feedback circuitry: the nucleus praeminentialis, an isthmic structure, and the granule cell masses of the lobus caudalis, recently renamed posterior eminentia granularis. Both nuclei not only contain the parent cell bodies of the parallel fiber tracts, but in addition, integrate electrosensory input with visual (Bastian '82), proprioceptive (Bastian '74), auditory and ordinary lateral line input (Carr and Matsubara '81). In this respect descending input to the ELLL is multimodal, and provides a mechanism for non-electrosensory information to refine the way the ELLL functions during electrolocation.

Two recently published reports specify how descending input to the DML regulates ELLL output (Bastian '86a,b). This portion of the ELLL feedback loop appears to function as a gain control mechanism for pyramidal cell activity. The responses of both E and I cells to stationary or moving objects was recorded before and after interrupting input to the DML. Removal of the descending pathway altered pyramidal cell responses to stationary objects in two ways: the sensitivity of E and I cells was dramatically increased, while spike-frequency adaptation was greatly attenuated. These changes were explained by a loss of excitatory drive that parallel fibers normally provide ELLL inhibitory interneurons. Specifically, removal of excitatory input to type II granule cells, neurons of the ventral molecular layer and polymorphic cells is expected to disinhibit pyramidal cells, consistent with the recorded increases in their response to stimulation.

Descending input also controls E and I cell receptive field properties. After interruption of descending input, both cell types exhibit greatly enlarged areas of surround inhibition, a phenomenon attributed to increased granule cell activity. It is suggested that loss of DML input should reduce polymorphic cell inhibition of granule cells, thereby strengthening lateral inhibition on pyramidal cells. In addition, type I granule cell dendrites do not reach the DML, and therefore, would not lose activity by interruption of descending input (Bastian '86a).

A second consequence of descending control is that pyramidal cells are able to compensate for decreases in EOD intensity. Bastian demonstrated that E cells can increase their sensitivity to correct for decreases in the fish's electric field strength (Bastian '86b), an ability that is lost following removal of descending input to the DML (Bastian '86b).

Descending input to the VML remains to be investigated as a possible source of feedback control. The consequences of VML parallel fiber activity are likely to differ qualitatively from those in the DML, since they arrive by a different pathway, and make synaptic contacts within spatially segregated zones on the apical dendrites of their recipient neurons.

Intrinsic mechanisms

It is clear from the preceding description that the electrosensory system is equipped with several potential

mechanisms to modify ELLL function in response to a changing environment. Although the local wiring of the ELLL circuitry defines the basic properties of pyramidal neurons, and hence, output from the ELLL, qualitative changes in afferent input are sufficient to modify their excitability and cellular behaviour. In response to primary afferent input, for example, pyramidal cells are most sensitive to time-dependent modulations in EOD amplitude, a property that increases their resolution of small moving objects.

Descending input to the ELLL is an especially potent mechanism for reshaping electrosensory processing. Moreover, a yet to be explored possibility is that descending feedback to the 4 ELLL segments may differentially regulate feature extraction during electrolocation.

Even more basic to the control of neuronal behaviour are alterations of intrinsic membrane properties, phenomena that have not yet been addressed for electrosensory neurons with *in vivo* recording techniques. Unfortunately, the ability to analyze biophysical properties of ELLL neurons is extremely limited *in situ*; most electrosensitive neurons are relatively small, and hence it is difficult to obtain stable intracellular recordings. Furthermore, many features of neuronal membranes require pharmacological or electrophysiological manipulations in order to identify their presence and describe their contribution to the behaviour of the cell. Many of these procedures are not feasible in whole animal experiments, and consequently, have not been attempted in studies of

electrosensory cells.

Nonetheless, examining biophysical phenomena has been extremely fruitful in other model systems for understanding how CNS activity is regulated at the cellular level. Some examples of the more successful approaches include the biophysical substrates of conditioned learning in *Hermisenda* photoreceptor cells (Alkon '84a), ion channel properties that regulate the precise timing of motoneuron firing required in spinal pattern generators (Getting '83), or ionic conductance changes that underlie epileptogenesis in hippocampal neurons (Schwartzkroin and Prince '78).

A biophysical characterization of ELLL pyramidal cells would be equally productive. In light of the extensive anatomical and physiological information already available for electrosensory structures, some of which are outlined above, a description of the intrinsic properties of pyramidal cells is a natural continuation toward integrating cellular and sensory physiology. In a more general sense, it is increasingly evident that neurons from a broad range of CNS structures share similar membrane properties. In very few model systems, however, especially vertebrate preparations, can these new-found properties be placed in a behavioural context as well documented as is electrosensation.

Objectives:

In light of these developments, the following specific objectives were outlined for this thesis: 1) to develop a viable tissue slice preparation, allowing the recording of

electrophysiological activity of pyramidal neurons at the intracellular level. This will facilitate making a characterization of their cellular behaviour which has not previously been feasible in whole animal recording experiments. 2) Using this approach, it will be possible to measure spontaneous resting behaviour and the response properties of pyramidal cells to direct current stimulation. 3) In addition, a biophysical analysis of the ionic conductances which mediate these cellular properties will be made. 4) Wherever possible, a correlation will be made between the behaviour of cells recorded physiologically in vitro and the identity of the two types of pyramidal neurons: the basilar and non-basilar pyramidal cells. 5) The possibility of segment-specific differences in the physiology of pyramidal cells will be examined.

MATERIALS AND METHODS

I. Experimental Animals

Electrophysiological recordings were made from ELLL brain slices prepared from gymnotid fish of the species *Apteronotus leptorhynchus*. Fish were maintained at 25°C in aquarium water adjusted for conductivity to 5-10 kΩ.cm. Some experimental data using extracellular electrode recordings were taken from another species of the same order, *Eigenmannia viriscens*. The ELLL neuroanatomy of the two species is virtually identical (Maler, '79), and no significant differences were observed in their physiological behaviour.

II. Slice Preparation

Adult fish approximately 15 gms. in weight were pretreated by injecting (i.m.) 25μl of solution containing 0.25mg 2-amino-5-phosphovaleric acid (2-APV) and 1.2mg L-glutamic acid diethylester (GDEE), two acidic amino acid antagonists which interfere with N-methyl-D-aspartic acid (NMDA) and quisqualate-type receptor binding. (McLennan, '83). The injected fish were kept for 20 min in oxygenated tank water and anesthetized with ether prior to surgery (approx. 5ml ether stirred into 1L tank water). The rationale for this protocol was that the ELLL contains significant quantities of glutamate and aspartate (Nadi and Maler unpublished observations), two excitatory neurotransmitters. In vivo experiments on the role of excitatory amino acids in epileptogenesis showed that blockade of their receptor binding with 2-APV protected hyperexcitable tissue from developing

convulsive activity (Meldrum et al. '83). Furthermore, systemic application of NMDA antagonists were shown to reduce some of the damaging effects of brain anoxia (Gerhardt et al. '86). Additional studies suggested that anoxia plays a role in the development of spreading depression (Milan et al. '85), a phenomenon which is suppressed by ether anesthesia (van Herreveld and Stamm '53). Without these pretreatments, the viability of the ELL slice was greatly reduced; intracellular electrode recording indicated that a majority of neurons had undergone depolarization block and had been rendered electrically unresponsive.

The anesthetized fish were then submerged in a small dish of ice-cold artificial cerebrospinal fluid (ACSF) (Table 1) oxygenated with a 95% O₂/CO₂ mixture. Under a dissecting microscope, the skin over the head was retracted and the cranium was carefully removed with forceps to expose the full extent of the brain. Small pieces of razor blades cleaned in alcohol were used to dissect a block of brain tissue which included the ELL, corpus cerebelli and lobus caudalis. The surgical procedure itself was perhaps the most important factor in obtaining stable recordings. Many variations in the plane of section were attempted, the optimum being in the true transverse plane for the ELL (fig. 2). Tilting the slice was also beneficial; this placed the slice angle parallel to the orientation of the pyramidal cells' apical dendrites (Maler, unpublished observations) and thus minimized their amputation. A transverse orientation had

an additional benefit of sparing the majority of parallel fiber afferents originating in the lobus caudalis pars granularis, allowing orthodromic activation of the ELLL circuitry via descending input.

All cranial nerves, the ganglia of the anterior lateral line nerve (nLLA) and the spinal cord were quickly severed and the tissue block was removed from the skull. Surgery time was kept to a maximum of approximately 2 min. The tissue block was removed from the ACSF with a spatula and fine brush and excess fluid was removed with filter paper. The tissue was then fixed to a small aluminum block with a drop of cyanoacrylate glue, submerged in chilled ACSF and mounted onto an Oxford vibrating microtome. The choice of slice thickness was a compromise: thin slices promote rapid exchange of drugs and O₂ diffusion throughout the tissue, thicker slices keep a larger portion of the neuronal circuit intact. The optimum thickness was within the range of 500-700 μ m.

Each slice was gently lifted from the vibratome bath and placed onto a nylon mesh in a slice chamber apparatus (fig.3). The total time elapsed between surgical cutting of the ELLL and placement of the slice into the bath was 7-9 min.

The tissue was incubated at room temperature in oxygenated ACSF for 1-2 hours prior to electrode recording. During this recovery period, the slices were maintained using the 'fluid interface' method in which the upper surface of the slice was in contact with a humidified, oxygen-rich atmosphere through a capillary film of ACSF. This facilitated O₂

diffusion into the slice and helped reduce the length of recovery period. The ACSF flow rate was adjusted to 0.75mls/min with a Gilmont Flow Meter and was delivered to the bath by gravity from a series of bottles equipped with O₂/CO₂ bubblers. Various drugs or bath solutions could be exchanged for normal ACSF by mechanical switches to each of the bottles. The delivery tubing was kept as small as possible (i.d. 1.14mm) in order to minimize turnover time while switching solutions. The tubing plus the bath chamber volume was approximately 14ml and exchanged volumes about every 30 min. Occasionally, slices were incubated in a separate holding chamber where they were submerged in oxygenated ACSF for up to 5 hours until they were transferred to the slice chamber for electrode recording.

For the purposes of applying drugs or switching solutions during electrode recording of the slice, it was found that interface maintenance was unsatisfactory for several reasons: the low flow rates resulted in long (> 30 min) delays before drug effects were stabilized, and more importantly, even minute changes in flow rate caused the slice to move up or down enough to lose the intracellular impalement. Therefore, after the initial recovery period, slices were maintained completely submerged. Small pieces of platinum wire were cut and placed over the edges of a slice to help stabilize it and prevent it from floating away, and the flow rate was increased to approximately 4ml/min. In this way the exchange time was reduced to about 6 min and even large changes in flow rate,

such as occur during switchover of solutions, had no effect on the stability of the recording.

III. Composition of ACSF

Since the composition of gymnotid CSF had not been analyzed, ACSF was developed based on the composition of mammalian ACSF (Schwartzkroin '77; Schubert and Mitzdorf '79) and modified according to our empirical observations. The compositions of various forms of ACSF used for this study are listed in the following Table:

Table I. Composition of artificial cerebrospinal fluid (ACSF) solutions (mM).

Soln.	NaCl	NaHCO ₃	KCl	KH ₂ PO ₄	MgSO ₄	CaCl ₂	dext-rose	HEPES	TRIS	other
norm ACSF	131	20	2	1.25	2	2.5	10	--	--	--
Na-free	--	--	3.25	--	2	2.5	10	20	131	--
Mn ²⁺	129-131	--	3.25	--	--	0.2	10	20	11.4	2-10 Mn ²⁺
4-AP	131	20	2	1.25	2	2.5	10	--	--	1-2 4AP
TEA ⁺	126-116	20	2	1.35	2	2.5	10	--	--	5-15 TEA

pH set to 7.4 before bubbling with 95%O₂/CO₂

IV. Recording Apparatus

Glass microelectrodes (WPI capillary tubing) were pulled

on a Brown-Flaming puller and back-filled with either 2M NaCl for extracellular recording (10-20 M Ω resistance) or 3M KCl for intracellular recording (50-100M Ω resistance). In some experiments cells were impaled with electrodes filled with 2M potassium methyl sulfate or 3M potassium acetate. To improve the stability of intracellular recordings, electrode tips were coated with a silicon solution ('Aqua-Sil', 0.4%; Pierce Chemical Co.) and allowed to dry for about 20 min. This promoted sealing of the hydrophobic plasma membrane to the charged glass tubing following impalement of the cell.

The ELLL is a highly laminated structure (Maler '79), and the polymorphic cell layer which contains the pyramidal cells was clearly visible in the slice with the aid of a dissecting microscope and incident light source (fig 4). The recording electrode was positioned under visual guidance and lowered into the tissue with a Midgard Microstep driver. While recording from a submerged slice, glass electrodes are transparent and require application of a black coating by dipping their tips into a drop of India ink to help visualize the location of the penetration.

Electrical activity in the slice was amplified by a Dagan 8500 Preamplifier and displayed on an oscilloscope screen or paper chart recorder (Watanabe). Bipolar, silver-silver chloride electrodes were used to activate pyramidal cells orthodromically by stimulating granule cells in the lobus caudalis, or antidromically by stimulating the plexiform layer. Constant current, biphasic, square-wave pulses of 50-

100 μ A amplitude and 200-400 μ sec duration were delivered by a Grass S88 Stimulator coupled to SIU6 stimulus isolation units. For intracellular recording, pyramidal cells were stimulated directly by passing d.c. current through the recording electrode via a high impedance bridge balance circuit on the preamplifier. Following the initial impalement of the cells, hyperpolarizing current (0-0.4nA) was frequently passed through the recording electrode to help stabilize the membrane potential (V_m).

Passive electrical properties of the membrane, such as resting potential (V_R), input resistance (R_{in}), and time constant (τ_m) were routinely measured during intracellular recording. V_R was measured by recording the voltage drop after penetration of the electrode. R_{in} was measured by delivering 30 msec hyperpolarizing pulses to the cell and recording the amplitude of the voltage change across the membrane versus the intensity of the stimulus current. The slope of the plot yields the input resistance. Alternatively, R_{in} was measured directly by adjusting the bridge balance until the voltage drop just disappeared and subtracting the initial electrode impedance from the total membrane impedance. The membrane time constant was measured by delivering a square pulse of intracellular hyperpolarizing current (eg. 0.3 nA, 500 msec duration) and extrapolating the time to reach $(1-e^{-1})$ times the maximal change in V_m . (Connors and Kriegstein '86).

All intracellular recording data presented here were taken from cells which met the following criteria: the

electrode was situated within the pyramidal cell layer of the lateral, centrolateral or centromedial segments of the ELLL. All cells used in the study had a VR $\geq$ -60 mv and action potential amplitude $\geq$ 60 mv. Each unit was held at least long enough for the Vm to stabilize and measure the passive membrane properties as well as spontaneous firing behaviour.

V. EGTA injection

In some experiments, the effect of removing free intracellular Ca^{2+} was tested by injecting the specific calcium chelator, EGTA (ethyleneglycol-bis(β -aminoethyl ether)-N,N'-tetra-acetic acid) directly into the cell soma. The micropipettes were filled with a solution of 1.8M K acetate plus 0.2M K EGTA. Hyperpolarizing current (0.3-0.7nA DC) was applied across the cell membrane for up to 1 hr to allow sufficient quantities of EGTA to diffuse into the distal-most dendrites. During the injection, the changes in the electrophysiological behaviour of the cell were monitored on the strip chart recorder.

VI. Intracellular labelling

Verification of the precise localization and morphological identity of ELLL cell types recorded physiologically was accomplished by intracellular injection of the fluorescent dye, Lucifer Yellow (LY). Electrodes were filled with a 4% solution of LY in 1M lithium chloride (Relec. = 100-150M Ω). The dye was injected by passing 1 nA hyperpolarizing pulses through the recording electrode (1 Hz;

200 msec duration) for as long as possible before losing the unit (eg. 45 min.).

The electrode was pulled up and the tissue immersed in 4 % formaldehyde in 0.1M Na phosphate buffer, pH 7.4 overnight. The fixed slice was glued to a vibratome block and cut into 100µm thick sections and washed in buffer. The sections were dehydrated through a graded series of alcohol, cleared and coverslipped in methyl salicylate and sealed with nail polish. An alternative procedure (Grace and Llinas '85) recently reported to yield improved resolution and minimal shrinkage of tissue due to alcohol dehydration was also used. In this procedure, the fixed 100µm sections were washed in buffer, then dehydrated, cleared and coverslipped in dimethyl sulfoxide (DMSO). After either procedure, the resulting slides were viewed with epifluorescence microscopy through an FITC filter and photographed.

VII. Cytology

One of the criteria for assessing the viability of the slice preparation was the cytological appearance of the ELLL in vitro compared to in vivo. Since electrode recordings were made from tissue maintained in the slice chamber for periods of up to 8 hours, several experiments in which the cells were deemed 'healthy' by electrophysiological criteria were terminated by preparing the slice for light and electron microscopy.

After the recording session, fixation of the slices was achieved by immersion directly into a 2% paraformaldehyde/ 2%

glutaraldehyde solution in 0.12M Na-cacodylate buffer (pH 7.4) and stored overnight at 4 C°. The slices were then washed in buffer prior to osmication for 1 hour in an ice-cold solution of 1% osmium tetroxide and 7% dextrose in 0.12M Na-cacodylate buffer, dehydration in a graded series of alcohol, propylene oxide and finally embeddment in Araldite.

The tissue blocks were sectioned with glass knives on an LKB-III ultramicrotome to a thickness of 1-2 microns and stained with a 1% solution of toluidine blue for examination with the light microscope. The blocks were subsequently trimmed down to the tissue depth and location that included pyramidal cells recorded physiologically, and sectioned for electron microscopy. The thin sections were stained with uranyl acetate and lead citrate and viewed with a Phillips EM 300 microscope.

RESULTS

I. Viability of slices

The ultimate goal of establishing an *in vitro* brain preparation is the ability to maintain for extended periods functional neuronal circuits, including intact afferent fibers, interneurons and efferent projections, and an opportunity to experimentally manipulate the circuit in such fashion as is not possible in the intact animal. In developing a viable *in vitro* ELLL preparation, several techniques described in the Materials and Methods were critical in determining the 'health' of the slice.

a. Electrical activity:

The tissue was considered viable when, during each pass of the electrode through the slice, several cells could be punctured and recorded intracellularly. The majority of these units had resting potentials of 60 mv or greater. The V_m of healthy cells stabilized quickly (ie. <5 min) and could be recorded for several minutes to several hours. It was usual for the slice to remain 'silent' during the initial recovery period, a phenomenon common to most slice preparations (Schurr et al. '84; Bianchi et al. '86). Following 1-2 hours incubation in the chamber, it was typical of the healthiest slices to remain active for up to 10 hours.

Evidence of tissue disruption due to surgical trauma or the effects of hypoxia was apparent from recovery periods lasting longer than the normal 2 hours, or an inability to hold stable units before premature death of the cells. Traumatized cells displayed depolarized resting potentials (less than 40 mv) which were accompanied by high levels of spontaneous activity recorded extracellularly.

Optimizing the surgical preparation of the brain and sectioning of the slices improved the tissue viability, although the success rate for obtaining a very healthy slice was still less than 25%. The greatest improvements were achieved by pre-treating the fish with 2-APV and GDEE injections, and anesthetizing the fish with ether prior to surgery. These procedures increased the success rate for obtaining healthy slices to approximately 75%, and greatly

improved the practicality of studying ELLL physiology in vitro .

b. Cytology of the slice:

Several experiments were terminated by fixation and processing for light and electron microscopy (Mat. and Meth. VII). Areas of tissue were examined which contained electrode recording sites so that a correlation could be made between the cytological appearance and the viability of the tissue.

In general, the more physiologically active the tissue, the less evidence of cytological disruption was found. For example, the outermost 50-100 μ m of all slices were exposed to the most severe shearing forces of the microtome blade. This zone was usually devoid of electrical activity and, when examined in the microscope, contained large areas of pycnotic cells and evidence of edema (not shown).

Deeper in the slice, however, a high level of electrical activity corresponded to tissue with relatively normal cytology. Electrode recordings were made in all three ELLL segments which receive tuberous electroreceptive input (Heiligenberg and Dye '82, Mathieson et al. '86). The viability and appearance of the slices were identical in each of these segments. Fig. 5 illustrates the appearance of an area of an ELLL slice which had been recorded in vitro for 8 hours. All of the ELLL laminae and major cell types described in vivo were easily recognizable and displayed minimal evidence of disruption (figs. 5-14). As expected, the greatest

damage occurred within the laminae containing fiber tracts oriented perpendicular to the plane of section. As a consequence, the deep fiber layer (DFL) tractus stratum fibrosum (tsf) and to a lesser extent the plexiform layer (pl.) contained varying amounts of extracellular edema and vacuolated axonal processes. Unraveling of the myelin sheath frequently occurred, particularly with the larger fibers. In spite of the disruption of these fiber tracts, the majority of axons displayed normal internal ultrastructure; mitochondrial swelling was not apparent and neurofilaments were evenly spaced throughout the axoplasm (fig. 10b).

In the neuropil, electrosensory afferent terminals synapsed with dendritic processes from basilar pyramidal cells, granule cells, polymorphic cells and spherical cell somata (fig. 12). Although evidence of darkened terminals and separation of synaptic junctions were found, most electrotonic and chemical synapses were qualitatively similar to those reported *in vivo* (Maler et al. '81a). The distribution of synaptic vesicles across some probability-coder terminals was somewhat dispersed compared to the normal appearance of vesicles tightly clustered around the presynaptic membrane (Maler et al. '81a). This condition does not reflect disruption of axonal structure, but in fact is a normal consequence of eliminating the high rate of electrosensory input which is characteristic of gymnotid receptor afferent fibers (Maler and Mathieson '85).

Unfixed ELLL slices present two broad cellular layers

easily recognizable with a dissecting microscope as gray bands separated by whitish fiber tracts. The most ventral of these bands is the granule cell lamina, which appeared well preserved, even following the longest recording periods in vitro. The more dorsal cell body layer is the polymorphic lamina, which contains basilar and non-basilar pyramidal cells, as well as polymorphic cells. Since the slice technique employed here was designed for recording pyramidal cell activity, the sectioning was adjusted to minimize trauma to the pyramidal cell population (eg. cutting as few dendrites as possible). Both basilar and non-basilar pyramidal cells appeared normal in light and EM sections, although occasional pycnotic cell bodies were present in even the most healthy slices (figs.8,9). It was also possible, by virtue of their characteristic ultrastructure, to identify axon terminals from polymorphic and granule cell interneurons synapsing with pyramidal somata (fig.13).

A single apical dendrite from each pyramidal cell passes dorsally through the stratum fibrosum ramifying within the ventral and dorsal molecular layers. (fig.14). The apical dendrites were studded with small spiny processes, many of which were seen receiving asymmetric, chemical synapses from descending parallel fibers (fig.14 top). In general, the soma, basilar dendrite and apical dendrites of pyramidal cells were left intact by the sectioning procedure and appeared relatively normal.

Other local interneurons, such as neurons of the

molecular layer and stellate cells, were identified in the ELLL slice and appeared normal at the light microscopic level. (fig.5). These cells are relatively scarce and were not identified in EM sections.

c. Intracellular labelling with Lucifer Yellow:

Previous studies on the intact ELLL (Maler et al. '81a), identified three neuronal cell types present within the pyramidal cell lamina: basilar and non-basilar pyramidal cells (also called E and I cells in physiological terminology of Bastian, '81b), and polymorphic cells. Since all of the recording data presented in this thesis were taken from the pyramidal cell layer, representative samples of intracellular units were injected with lucifer yellow (LY) in an attempt to correlate electrophysiological behaviour with one or all of the three possible cell types. Of ten cells filled with LY, 6 were identified as non-basilar pyramidal cells and 4 were basilar pyramidal cells. Figs. (15-16) illustrate the resulting fluorescence micrographs. These results were not unexpected, since pyramidal cells are by far the most numerous cell types in the pyramidal lamina. Moreover, polymorphic neurons are less likely to be successfully penetrated than pyramidal cells owing to their relatively smaller size (15 μ m compared to 25 μ m for a basilar pyramidal cell). Basilar and non-basilar pyramidal cell somata are approximately of equal diameter and are alternately distributed across the ELLL (Maler, '79), a pattern which reflects the nearly equal numbers of each cell type injected with LY.

Although it was possible that the electrode data presented in the following sections included occasional representation from polymorphic cells, it is reasonable to assume that the large majority of recordings were from basilar and non-basilar pyramidal cells. Correlation of cell type with electrical activity will be presented with electrophysiological data to follow.

Previous measurements of the dimensions of pyramidal cells were made with *Eigenmannia*, a smaller species with correspondingly smaller neurons (Maler '79). Another study (Saunders and Bastian, '84) used fish of comparable size and the same species as in this study. Both employed standard tissue fixation and embedding techniques which included alcohol dehydration and clearing with xylene, two treatments known to promote tissue shrinkage. As described in Mat. and Meth.VI., cells injected with LY were prepared for microscopy using DMSO dehydration and clearing, a process which reduces shrinkage (Grace and Llinas '85). Although the sample size in this study was small, the measurements made from LY-filled cells and illustrated in the micrographs in figs.15,16 give us the most accurate size estimates of basilar and non-basilar pyramidal cells to date.

Non-basilar pyramidal cells had cell bodies which measured approximately $20 \times 60 \mu\text{m}$, and a single apical dendrite which bifurcated into 15-20 fine processes ramifying into the full extent of the ventral and dorsal molecular layers (fig.15). Due to the difficulty in reconstructing the apical dendritic

tree in LY-filled cells, the full dimension of the apical dendrites was not measured. Partial reconstruction measurements indicated that they extended a minimum of 340 μ m in length by 120 μ m across. From the soma emerged numerous tiny somatic dendrites which arborized within the polymorphic lamina. The initial segment and proximal portion of the single axon was usually labelled, although the LY did not diffuse far enough along the axon to describe its course. None of the labelled pyramidal cells showed any evidence of dye-coupling with adjacent neuronal processes. Allowing for species size differences and histological shrinkage, the somatic and dendritic measurements were compatible with those previously reported for NBPs.

Fig.16 illustrates the elongated shape of a LY-filled basilar pyramidal cell. The soma measured approximately 20 μ m across and usually presented fewer somatic dendrites than the NBP, consistent with Maler's ('79) golgi data. Instead, a single highly elongated basilar dendrite descended approximately 200-400 μ m, arborizing within the deep neuropil. This basilar trunk has a diameter of approximately 10 μ m, substantially greater than the 2-3 μ m reported by Maler. The basilar dendrite terminated in a compact bush, 30-50 μ m in diameter; note that Maler ('79) estimated the basilar bush to be 50 μ m in diameter. The apical dendrite resembled that of a NBP.

The 200-400 μ m length of the basilar dendrite of LY-filled BPs was considerably greater than that reported by either

Maler (40-70 μ m) or Saunders and Bastian (Ave.137 μ m); presumably this is due to the lack of shrinkage in the slice preparation. From the profile of the BP in fig.16, it is possible that primary afferent input, which contacts the basilar dendrite at its distal bush in the neuropil (Maler et al. '81a), might be electrotonically distant from the soma and the spike initiating initial segment. Possible functional significance of these measurements will be elaborated upon in the Discussion.

d. Synaptic connectivity:

An important consideration in the viability of an in vitro brain preparation is the preservation of functional synaptic connections within the neuronal circuit to be studied. In the ELL slice, synaptic input to pyramidal cells was tested by orthodromic stimulation of either primary afferent fibers in the deep fiber lamina, or descending parallel fibers from the lobus caudalis. Fig. 17 illustrates synaptic potentials recorded from pyramidal cells following stimulation of the descending input. Suprathreshold stimuli were followed by a 3-6 msec latency EPSP or an all-or-none action potential or burst of action potentials. DC hyperpolarization of the soma prevented Vm from reaching spike threshold, revealing a complex EPSP with a rapid initial time-course lasting approximately 6 msec, and a second, much slower phase lasting about 100 msec. Hyperpolarizing the cell increased the EPSP amplitude, as expected for classic EPSPs

(Eccles '64).

Activation of primary afferent fibers was less successful at evoking synaptic potentials. Primary afferents reach the ELLL by coursing longitudinally in the caudal direction. Therefore, in the transverse plane, many of their fibers are perpendicular to the orientation of the slice and are sectioned as they enter the ELLL, rendering them unresponsive. If it had been desirable, however, to study the synaptic interactions of primary afferent fibers with pyramidal cell properties, orientation of the ELLL slice in the longitudinal plane would preserve the majority of afferents in the deep fiber layer.

Synaptic activity generated from the large numbers of interneurons was occasionally evident by the presence of spontaneous, hyperpolarizing IPSPs, recorded from pyramidal cells using acetate electrodes (fig.18). IPSPs were rarely encountered when using chloride electrodes, presumably due to reversal of their direction by intracellular chloride injection (Eccles, '64).

e. Passive membrane characteristics:

Intracellular electrode recordings were obtained from more than 140 pyramidal neurons; passive membrane parameters such as input resistance for the membrane (R_{in}), membrane time constant (τ_m), and resting membrane potential (V_m) were measured. In addition, spike amplitude was routinely measured during brief depolarizing current pulses as a measure of the stability of the recording and overall health of the tissue.

Fig.19 illustrates time constant measurement, and the data, in Table II summarizes the calculated membrane parameters.

Table II.
Electrophysiological properties of ELLL pyramidal cells in vitro.

	input resistance	resting potential	spike amplitude	time constant, τ_m
# sampled	132	96	141	8
.....				
AVE	27.1	73.9	68.2	6.75 ⁰
$\pm$ S.E.	± 1.16 Mohm	± 0.96 mv	± 0.79 mv	± 1.44 msec

II. Spontaneous activity

a. Extracellular recording:

Extracellular, single unit recordings from the polymorphic lamina revealed spontaneous activity which followed three characteristic patterns: 1) low frequency tonic firing of action potentials; 2) short bursts of action potentials of 2-20 spikes per burst, and variable interburst intervals of <1 sec; 3) pacemaker-like activity with very large bursts discharging several hundred spikes per burst and regular burst intervals, usually >10 sec (Mathieson and Maler, 1983). The records in fig.20 illustrate the typical discharge patterns of the bursting neurons.

The most frequently encountered patterns of spike firing

were by far the tonic and lower-intensity bursting. As judged by the criteria for tissue viability, both of these spiking behaviours predominated in healthy slices. The 'giant bursters' were, by contrast, most often recorded during the initial experiments, when many of the optimum slice preparation techniques had yet to be developed. Their presence was frequently accompanied by a preponderance of neurons in 'depolarization block', which had resting membrane potentials of $<40\text{mv}$ (measured by intracellular penetration) and were unresponsive to stimulation. In subsequent experiments, fish were pre-treated with 2-APV, GDEE and ether anesthesia to reduce the harmful effects of anoxia following surgery (see Mat. & Methods). With the addition of these steps to the slice preparation, the occurrence of giant bursters was eliminated.

The strongest evidence that 'giant bursters' were firing abnormally was that whenever they were encountered, groups of cells were recorded which exhibited nearly identical, complex patterns of burst firing. In one experiment, extracellular recording within the deep fiber layer directly ventral to a pacemaker-like neuron revealed a pattern of axon spiking almost identical to that of the neuron above. This indirect evidence that the more intense spontaneous bursts were being driven by injured primary afferent axons was supported by the observations that tonic firing or small bursts of spikes were never accompanied by spontaneous discharge in the underlying deep fiber layer. Presumably it is this intense abnormal activity which drove the pyramidal cells into depolarization

block; the efficacy of APV and GDEE in improving slice viability may thus be related to their ability to antagonize the response of pyramidal cells to the primary afferent transmitter. The following sections employed intrasomatic electrode recording techniques to show that, in viable slices, rhythmic activity in ELL neurons in vitro results from intrinsic membrane properties and is not driven by injured afferents.

b. Intracellular recording:

Fig. 21 illustrates a spontaneous action potential recorded intrasomatically from a pyramidal cell. Their spikes typically were preceded by a 3-5mv 'hump', and displayed a conspicuous inflection point where V_m reached spike threshold (arrow). Action potentials rapidly repolarized so that the spike duration at 50% maximum amplitude was approximately 0.5-1.0 msec. A hyperpolarizing after-potential (AHP) of 5-10 mv followed the spikes. In most respects, pyramidal cell neurons' action potentials resemble fast-Na spikes reported in other vertebrate neurons (Hille, '70).

1. Oscillations in V_m :

The values reported in Table II indicated that for cells used in this study, the resting membrane potential averaged about 74 mv, with a range of 60-90 mv. However, many units displayed an unstable V_m which oscillated above and below the resting potential in a stereotyped fashion. The trajectories

of several examples of these voltage excursions are described in fig.22. More robust oscillations consisted of a slow depolarizing ramp, followed by a steep repolarization (fig.22a-c). Rhythmicity did not depend on the electrolyte used in the recording electrode; identical patterns of slow potentials were observed while recording with potassium chloride, acetate or methyl sulfate electrodes.

In a stable recording, the frequency and amplitude of the SPs was remarkably constant, although it varied from cell to cell within a range of 0.5- 1Hz and 2-15 mv. In more depolarized neurons, the SPs traversed the subthreshold range for spike firing, and evoked a burst of action potentials which resembled the extracellularly recorded activity.

The SPs were found to be strongly voltage-dependent; current-clamping the V_m to various subthreshold values altered both the frequency and amplitude of the voltage excursions. Hyperpolarizing the neuron reduced the strength of the SPs, eliminating them at more hyperpolarized levels. Conversely, depolarizing current injection increased frequency and amplitude. In the extreme, a non-rhythmic cell (tonic firing or quiescent) could sometimes be induced to bursting behaviour by turning on this voltage-dependent oscillation. The degree of voltage-dependence, that is, how large of a voltage-step was required to elicit a substantial change in resting behaviour, varied greatly from cell to cell. In many cells, however, step-changes in V_m by as little as 5 mv above and below resting V_m were sufficient to switch the cells firing

behaviour from tonic to bursting (fig.22d).

In order to appreciate the importance of this intrinsic rhythm to ELLL pyramidal cells in vitro, wherever possible, intracellular records included a description of the cells' resting membrane properties and presence of voltage-dependent slow potentials. From a total of 131 cells, pyramidal cells were placed into 3 arbitrary classes of spontaneous behaviour; a) approximately 1/3 of those sampled were non-rhythmic at any potential, b) 1/3 were 'weakly' rhythmic eg. they displayed oscillations in V_m during subthreshold, depolarizing current injection c) 1/3 were 'strongly' rhythmic at the resting membrane potential. The data for these measurements are shown in the following Table:

Table III.

Classification of ELLL pyramidal cells by strength of membrane potential oscillation.

type of behaviour -----	% of total cells (n=131) -----
I. Strongly rhythmic	39.7
II. Weakly rhythmic	24.4
III. Non-rhythmic	35.9

While recording from rhythmic cells, measurements of R_{in} were made throughout the course of several consecutive membrane oscillations. This was carried out by delivering constant-current hyperpolarizing pulses through the recording pipette and measuring the size of the resulting voltage

deflections on the pen recorder. Surprisingly, even during the more robust SPs, no detectable changes in R_{in} were measured at different points in the rhythm. This indicates that either the number of channels involved in generating the different phases of the SPs are quite small, or that activation-inactivation of inward currents are offset by inactivation-activation of outward currents to produce no net change in R_{in} . Another possibility is that the channel openings generating SPs occur far enough out on the dendrites that fluctuations in R_{in} are too small to measure at the soma. This appears unlikely, however, considering the voltage-sensitivity of SPs to current applied at the soma.

11. Correlation of cell type with rhythmic potentials:

An interesting possibility emerged that slow oscillations in membrane potential might exist in either basilar or non-basilar pyramidal cells, but not both. In order to test this, cells were characterized according to their rhythmic membrane properties, then injected with Lucifer Yellow. In a sample of ten cells, no correlation was found between cell type and auto-rhythmicity; nearly equal numbers of NBP and BP⁺ neurons exhibited membrane oscillations. Conversely, non-rhythmic cells were equally likely to be BP or NBP cells.

11.1. Fast pre-potentials:

Superimposed upon the SP were small, depolarizing spikelets which superficially resembled so-called fast pre-potentials (FPPs) described in hippocampal neurons (Spencer and Kandel '61). Their amplitude varied from 2-5 mv in a

voltage-dependent manner; as the V_m oscillated across a slow potential, FPPs increased in frequency and amplitude reaching a maximum at more depolarized voltages (fig.22a,e). Consecutive sweeps of the oscilloscope taken at different voltages within a SP and at a faster sweep speed, illustrate the voltage-dependent behaviour of the FPPs (fig.22e). The degree of voltage-sensitivity with respect to V_m suggests that FPPs originate on, or electrotonically near the cell soma.

III. Repetitive firing properties

a. F/I data:

One approach often used to investigate how active conductances can shape neuronal behaviour is to study the input-output relationships of a cell. To address this area for pyramidal neurons, intracellularly recorded cells were delivered 80 msec square pulses of depolarizing current and the resultant spike potential frequencies were measured across the duration of the stimulus. Various protocols are often employed to generate spike frequency data; for this study, measurements made of the 1st, 2nd, and 3rd interspike intervals were used to calculate the instantaneous, intermediate and steady-state firing frequencies during repetitive firing.

More than 30 pyramidal cells were tested for their stimulus-response characteristics, and the calculated spike frequencies were plotted against total current injected to give a series of F/I curves for each neuron. Although the

responses varied greatly in their slopes and x-intercept, certain generalities were apparent from the data. A graph illustrating the F/I curves from an exemplary neuron is shown in fig.23. Most cells responded to increasingly intense stimuli by raising their spike frequencies in a roughly-sigmoidal fashion. The slope of the curve was shallow just above rheobase, and steepest at intermediate stimulus intensities. The spike frequency usually plateaued at higher current levels. In addition, the steady-state frequencies were consistently higher than the instantaneous values (fig.24), indicating that the cells were more capable of high-frequency firing during longer stimulation periods.

b. Spike accommodation:

A further series of experiments was employed to investigate the presence of slowly developing patterns of repetitive firing not visualized during short duration stimuli. Depolarizing DC injections, 400msec-1sec in duration were delivered through the electrode micropipette. Two characteristic patterns of spike potentials were recorded. First, approximately 2/3 of pyramidal cells responded with a slowly developing train of spikes which increased in frequency to the point of tonic firing (fig.25d), or rapid bursts of action potentials (fig.25c). These neurons showed no indication of spike accommodation.

In contrast to the non-accommodating neurons, roughly 1/3 of the cells tested responded in a more phasic manner (fig.26), by exhibiting partial to near total spike

accommodation. It should be noted that the occurrence of these two response types was complicated by one or more voltage-dependent processes. An accommodative cell could be made to fire non-accommodatively by manipulating the stimulus intensity and current-clamping V_m to different values.

c. Stimulus-response lag:

One of the more conspicuous features of pyramidal cell firing in response to direct stimulation was a lag period at the start of a depolarizing pulse which lasted up to 250 msec. As illustrated by the spike trains in fig.26, a pronounced lag preceded both tonic or bursting-type repetitive firing. In some cells, (eg.fig.26c) the lag period so dominated the initial 200 msec that the spike frequency was held below 25 Hz, then abruptly increased to nearly 100 Hz. The nature of the mechanisms involved in regulating spike accommodation and lag will be examined further in the following section.

It is clear from the above data that the repetitive firing properties of pyramidal cells in vitro would have a major influence on the output behaviour of cells receiving excitatory input. The ionic mechanisms responsible for the observed lag period would act as a 'brake' for spike firing, and tend to prevent high frequency responses to brief, excitatory input. Conversely, the non-accommodative nature of most pyramidal neurons would render their response to longer duration stimuli non-phasic.

The response characteristics of in vitro pyramidal cells was strikingly different from their in vivo

counterparts. Bastian's laboratory has recorded from pyramidal cells (E and I cells) *in situ* while the fish received behaviourally relevant, excitatory stimuli. As shown by the response of an E cell in fig.25a (top row)(compliments of Dr. Bastian, personal communication), pyramidal cells *in vivo* respond to excitatory input with a fast, high frequency burst of action potentials, followed by rapid spike adaptation. Spike frequencies of 450-600 Hz are not uncommon during the initial 50 msec of the burst (compared to 25-100 Hz *in vitro*). Moreover, the response is extremely abrupt, following a brief latency (10-15msec) for conduction of the afferent volley and a single synaptic delay (Bastian, '81a). The functional significance of these discrepancies will be addressed in the Discussion.

IV. Ionic conductances

a. Fast sodium conductance (g_{Na}):

From the many examples of spontaneous and stimulus-evoked spike-potentials presented in these results, it is evident that ELLL pyramidal neurons display a fast sodium current typical of most invertebrate and vertebrate neurons. The rapid rise-time of the depolarizing phase and short duration of the spike (as in fig.21), indicates rapid activation and inactivation kinetics of sodium channels, and the threshold for spike generation was in the range of 10-15 mv depolarized from rest. In addition, 0.2 μ M TTX was sufficient to block g_{Na} , and thereby eliminate fast somatic spikes.

b. Subthreshold sodium conductance:

In addition to classic fast-Na spikes, several lines of evidence suggested that, in the voltage range subthreshold for spike potentials, the excitability of pyramidal cells is to a large extent determined by the presence of a persistent Na conductance (g_{NaP}).

1. TTX sensitivity:

At resting membrane potentials, fast Na channels are for the most part closed. However, in cells which display slow oscillations, another, persistent g_{Na} provided the driving force for the SPs. Addition of $0.2\mu M$ TTX to the bath ACSF completely and reversibly eliminated pyramidal cell rhythmic behaviour ($N=10$). The strip chart recording in fig.27 illustrates at a slow speed the time course of TTX-blockade of membrane oscillations. In this experiment, the slice was maintained in the interface condition, which was reflected by the long delay before the drug had an effect. Also note throughout the records the upwardly directed pen strokes which correspond to intermittent, depolarizing current pulses that also monitored TTX block of spike potentials; fast and persistent Na channels were equally sensitive to TTX. During TTX-block, the interval between SPs increased, and the amplitude of the depolarizing phase of the voltage shift diminished until the V_m was essentially flat. Although the hyperpolarizing phase appeared unaffected by TTX, an increase in SP interval resulted from a reduction in the slope of the depolarizing ramp.

Lidocaine, a topical anesthetic also known to block sodium channels (Sugimori and Llinas '80), had a similar effect on SP generation when added to the ACSF (fig.28a).

11. Sodium sensitivity:

The sodium-dependence of subthreshold potentials was further demonstrated by substitution of normal ACSF for Na⁺ free ACSF. fig.28b illustrates a rhythmic pyramidal cell recorded intracellularly during incubation in zero-Na. As with TTX block, sodium substitution eliminated both slow and fast spike potentials.

Not shown in figs.27,28 is the observation that fast-prepotentials were also Na-dependent. Both TTX application and Na substitution abolished the brief depolarizing spikelets which frequently were superimposed upon the crest of SPs.

It could be argued that both slow and fast-prepotentials represent synaptic currents transmitted electrotonically from dendritic loci to the soma. Blocking Na channels would abolish such activity by eliminating tonic synaptic input. The voltage parameters of the subthreshold behaviour, however, suggest that these events are intrinsic to pyramidal cell membranes. This is clearly indicated by current-clamping manipulations of V_m. Manually setting V_m above or below resting values with DC injections strongly affected the amplitude and frequency of SPs. Unlike EPSPs, which decrease in amplitude or reverse direction upon depolarization of V_m, SPs and FPPs increased in strength in the positive direction.

Another possible explanation for the voltage sensitivity

of the SPs is the existence of an 'unusual' outward current that is synaptically modulated, such as the M-current. In this situation, neurotransmitter binding closes a voltage-dependent, outward K-channel to affect a slow depolarization of the membrane. This possibility appears unlikely, however (see Results IVf.).

Activation of a subthreshold inward current was particularly obvious during depolarizing current pulses. Following an initial lag, a conspicuous depolarizing ramp developed in the voltage range preceding spike firing in approximately 50% of the cells tested (fig.29). The slope of the ramp varied from cell to cell, so that cells which displayed a strong oscillation in V_m also displayed a large depolarizing ramp during injection of current pulses, often developing oscillations reminiscent of SPs. In 4 separate experiments, addition of 0.2 μ M TTX abolished the ramp providing additional evidence for the activation of a subthreshold sodium current (g_{NaP}).

iii. Rectification: Further evidence for a subthreshold, inward sodium conductance in pyramidal cells was obtained from I/V relationships when brief positive and negative current pulses of equal amplitude were delivered intracellularly. Approximately 1/3 of the cells tested ($N=30$) displayed a variable degree of apparent anomalous rectification. As shown in the top row of fig.30, the voltage deflection was larger in response to depolarization, often forming the characteristic trajectory of a stimulus-induced SP. The middle line in each stimulus series marks the resting potential and provides a reference for the current-evoked responses. Positive

dependent g_{NaP} and not a true anomalous rectification per se.

Conversely, another 1/3 of the cells displayed rectification of opposite sign. The records in fig.30, bottom row, illustrate two cells responding with negative rectification. This pattern of I/V response may correspond to cells in which depolarization activates a relatively weak g_{NaP} compared to larger, voltage-dependent outward conductances. Opening K-channels would thereby attenuate the positive voltage deflection. Similarly, a cell with a large resting g_K might also respond with negative rectification; a pulse of hyperpolarization would inactivate both g_{NaP} and g_K , increasing R_{in} and exaggerating the hyperpolarizing voltage deflection.

The remaining cells responded ohmically, with symmetrical I/V deflections. In reality, it was evident that electrical properties of these cells were regulated by a very delicate balance between inward, g_{NaP} and outward conductances (eg. g_A or $g_{K(ca)}$). This balance could be altered in favour of one direction or the other, simply by manipulating the V_m and stimulus amplitude. However, the net conductance significantly influenced the firing behaviour of the cells; strongly non-accommodating pyramidal cells generally displayed a large bias toward positive rectification, whereas accommodating cells (cf fig 30d) typically showed negative rectification.

c. Calcium conductance (g_{Ca}):

1. g_{Ca} blockade:

Data obtained from extracellular, single-unit recordings

provided indirect evidence that calcium was important in regulating pyramidal cell excitability. Burst-firing was found to be a Ca-dependent process, since both removal of Ca^{2+} , or addition of the calcium channel blocker, verapamil, to the bath reversibly abolished bursting activity (fig.31).

Electrode recording at the intracellular level reinforced the above findings. Cells which displayed regular oscillations in V_m ($N=3$), were incubated in low calcium ACSF containing 2mM Mn^{2+} to block calcium currents (Bagust and Kerkut '80). As shown in fig.32, calcium blockade altered the trajectories of the SPs by progressively increasing their frequency and at the same time diminishing their amplitude. With time the rhythm had degraded into very irregular and rapid fluctuations in V_m 2-3 mv in amplitude, which, since calcium influx was blocked, probably was a reflection of the persistent Na-current and I_A (see below). Washout with ~~normal~~ ACSF completely recovered the pre-test rhythm.

Without the aid of voltage-clamping techniques, it is impossible to judge which specific conductances were affected by the Mn^{2+} block of calcium channels. The shortening of the interval between successive oscillations strongly suggested that a Ca-dependent outward current, probably $gK(\text{ca})$, adds to the hyperpolarizing phase of the SPs, thereby regulating the frequency of rhythmic firing. The decrease in amplitude of the voltage shifts also suggested that calcium influx either contributes directly to the depolarizing phase of the SP, or indirectly by activating a calcium-dependent inward (Na)

current. In either case, since Mn^{2+} -block also interrupts synaptic transmission (Bagust and Kerkut '80), the residual, irregular potentials following calcium channel blockade must be intrinsic to the pyramidal cell.

11. Calcium spikes:

A more direct approach to demonstrate calcium currents is to record calcium spike-potentials. In order to isolate g_{Ca} from other inward and outward currents and successfully evoke clean Ca-spikes, TTX was added to the bath to block $g_{Na}(\text{fast})$ and g_{NaP} , as well as 4-AP and TEA to block g_A and g_K (delayed rectifier), respectively. This massive block of membrane conductance should, in theory, unmask Ca-spikes during depolarizing stimulation. In approximately 1/2 of the 20 pyramidal cells so treated, however, no evidence of Ca-spikes was found, even during intense depolarization.

In a small fraction of the cells ($N=4$), small amplitude Ca-spikes were evoked. These potentials had high thresholds for activation, requiring current pulses greater than 1.5 nA in amplitude (fig.33b). The remaining cells displayed low threshold (eg. 0.5nA) spike potentials with amplitudes in the range of 5-20 mv (fig.33a). The most responsive cell is shown in fig.33c at fast and slow sweep speeds; a low-threshold, short latency Ca-spike was evoked which was immediately followed by an unusual 'reverberation' in V_m . At higher stimulus intensities, a slowly developing hump in the membrane potential dominated the stimulus-response and continued for an

additional 50-100 msec after terminating the stimulus pulse (fig.33d,e). Finally, Ca^{2+} was substituted with Ba^{2+} , which is known to pass through calcium channels more readily than Ca^{2+} (Hagiwara et al. '74). At this point, stimulation was no longer necessary to generate Ca-potentials. As illustrated in fig.33f, slow, long duration calcium currents were recorded spontaneously. Superimposed on the crest of the slow calcium wave can be seen small spikelets which appeared similar to small amplitude 'early' Ca-spikes. The time course and threshold values for these calcium potentials suggest that the early evoked spikes are generated fairly near to the soma, while the high threshold, later potential probably represents spikes originating further out in the dendrites.

It is clear from these results that pyramidal cells vary considerably in the magnitude of g_{Ca} that is expressed during direct current stimulation. The range of responses included a large group of neurons that displayed no measurable calcium current, to neurons that show both low and high threshold spikes of considerable magnitude.

d. Calcium-dependent potassium conductance, $g_{\text{K}(\text{Ca})}$:

Numerous examples of vertebrate CNS neurons have been described whereby the cells' electroresponsiveness is in part determined by a prominent calcium-dependent potassium conductance (Alger and Nicol '80, Barrett and Barrett '76, Hotson and Prince '80, Krnjevic and Lisiewicz '72, Llinas and Jahnsen '82, Llinas and Sugimori '80b, Llinas & Yarom '81). Hippocampal neurons, for example, display rapid, spike

frequency accommodation which is mediated by outwardly-directed $gK(ca)$ (Madison and Nicoll '82). The fact that a majority of ELLL pyramidal cells in vitro fire repetitively with little or no accommodation, in addition to the observed difficulty in evoking calcium spike potentials, leads to the speculation that $gK(ca)$ is not an important component of their membrane conductance. The following experiments were conducted to assess the contribution of $gK(ca)$ to spike firing in pyramidal cells.

1. EGTA injection:

A total of eight neurons were injected with potassium EGTA, a chelator of intracellular calcium, by passing hyperpolarizing current directly through the micropipette. Membrane properties such as slow potential oscillations and spike frequency accommodation were compared at the onset and throughout the EGTA injection. If $gK(ca)$ is involved in either of these processes, chelating intracellular calcium would alter the firing properties of the cell.

Injection of EGTA had a rapid and consistent effect on all 8 cells tested. First, chelation of free intracellular calcium caused a steady depolarization in V_m which plateaued up to 10 mV positive to rest. This suggested that EGTA removed a tonic, outward conductance shifting V_m in the depolarizing direction. Second, EGTA effectively eliminated spike accommodation. Fig. 34a,b demonstrates how cells that initially fired with little spike accommodation were minimally effected by EGTA. In contrast, cells which were strongly accommodating

(fig.34c,d) were dramatically effected by the drug, becoming completely tonic.

In general, cells tested with EGTA became more highly responsive to depolarizing pulses. This was evident even in cells that were initially only weakly accommodating. The implication is that interspike interval, as well, is determined in part by calcium-dependent processes.

The observed changes in firing after injection of EGTA were not due simply to the depolarizing effect of EGTA on the V_m . This possibility was eliminated by manually clamping V_m to the resting value and maintaining the stimulus at a constant intensity. The results suggest that the wide range in observed accommodation levels was due to the presence of a corresponding range of $gK(ca)$ levels.

The significance of internal Ca^{2+} or $gK(ca)$ on rhythmic membrane potentials was more difficult to decipher. Except for the already mentioned EGTA-induced depolarization, cells which were non-rhythmic were unaffected in resting membrane potential. Cells which displayed SPs, however, responded to EGTA in an apparent contradiction to the earlier, zero-calcium experiments. As shown in fig.35, membrane oscillations became progressively more intense with increasingly long interburst intervals. The increased level of excitability is consistent with a mechanism whereby EGTA had removed the braking action of $gK(ca)$ during burst firing. However, the same current should also be expected to contribute to the interburst interval. Note in fig.35 that even after lengthy

EGTA injection, burst episodes terminated with a prominent afterhyperpolarization. This suggests that the hyperpolarizing phase of SPs or bursts of spikes is at least partially mediated by a different outward current(s) than simply $I_{K(Ca)}$. Alternatively, although EGTA was injected for up to 1 Hr, the possibility is recognized that $g_{K(Ca)}$ channels out on the most distal dendrites were not affected by injecting the soma, and these distal sites may be important in regulating rhythmicity.

11. Apamin:

The bee venom toxin, apamin, has been reported to specifically block Ca-activated potassium channels in several vertebrate cell preparations (cf. Hugues et al. 1982). Addition of 100nM apamin to the bath had no measurable effect upon pyramidal cell responsiveness. However, increasing the dose to 400nM mimicked several of the EGTA-induced changes in firing properties (N=3). The cell recorded in fig.36 best illustrates the apamin-effect because it displayed a strong spike accommodation, presumably mediated by $g_{K(Ca)}$ (panel a). Treatment with apamin completely abolished the accommodating response (panel b), which was partially returned during washout with normal ACSF (panel c).

Apamin also reduced the amplitude of the post-spike hyperpolarization. Compare the AHP before and after apamin in fig.36d,e. The amplitude of the undershoot was reduced by >50% and the duration of the AHP was curtailed. Note that the duration of the action potential itself was unaffected by

apamin.

The remaining two cells tested with apamin showed similar patterns of change, the magnitude of which corresponded with the presence of spike-frequency accommodation. These results, together with the EGTA data, support the interpretation that non-accommodating pyramidal cells have little $gK(ca)$ while the repetitive firing of accommodating cells is strongly influenced by this outward current.

e. Voltage-dependent Potassium currents:

Two voltage-dependent outward currents were identified in pyramidal cells which could be differentiated by their pharmacological sensitivities. The fast transient potassium current (I_A), and the classic delayed rectifier (I_K) were both found to play a large role in determining the excitability of these neurons.

1. gK :

The potassium conductance identified as the delayed rectifier (gK) by Hodgkin and Huxley (1952) is perhaps the most difficult to study in isolation from other outward currents. Although TEA applied externally in 1-10mM concentrations has been shown to block delayed K-currents without interfering with I_A or the slow-AHP associated with $gK(ca)$ (Barrett and Barrett '76; Thompson '77; Segal et al. '84), other studies have found a TEA-sensitive component of fast, Ca-dependent K-currents (Adams et al. '82; Zbicz and Weight '85). Therefore, when ELLL slices were treated with 5-

10mM TEA, the most definitive statement to be made was that a population of K-channels, including gK, was blocked. Nonetheless, the consequences of blocking K-channels with TEA was immediately obvious. The cells became hyperexcitable, firing paroxysmal trains of spike potentials. Unfortunately, this inevitably led to the cells entering depolarization block (not shown).

In a separate series of experiments, CsCl electrodes were used to block potassium channels by injecting Cs⁺ directly into the cell soma. This treatment non-specifically blocks K-channels, with the exception of the M-channel (Bezanilla and Armstrong '72; Halliwell and Adams '82). The result from one such cell is shown in fig.37 top row). Depolarizing pulses were delivered to the cell to inject the Cs⁺ and monitor changes in the spike trajectories. At T=0 (not shown) a normal train of spikes with typical undershoot of the AHP was recorded. After 30 min of Cs⁺ injection, the AHP had been reduced from 8mv to approximately 1mv (panel a). By T=40 min, a newly formed depolarizing after-potential (DAP) was apparent during the interspike intervals (panel B). This inward-directed DAP was interpreted as being a component of the persistent, subthreshold gNa normally masked by the powerful outward K⁺ currents. Support for this notion was obtained in the next panel (c) where 0.2 μ M TTX had been added to the bath. The photograph was made just before TTX block of spike potentials was achieved, when the drug had reduced gNaP sufficiently to eliminate the DAPs.

Cs⁺-blockade of potassium channels also increased the duration of the action potential, presumably by impairing spike repolarization. These results, together with the strong TEA effect on spontaneous activity, suggests that K-currents are important in maintaining low levels of background activity in pyramidal cells.

ii. IA:

First described in molluscan neurons (Connors and Stevens, 1971b, Neher, 1971) and more recently in vertebrate neurons (Gustafsson et al. '82; Stansfeld et al. '86, see also review by Rogawski in TINS '85), IA is a transient, outward potassium current with time and voltage-dependent properties differing from other outward conductances. It activates rapidly in the voltage range subthreshold for spike formation and inactivates with a double-exponential time constant over a 200-400 msec period (Connor and Stevens '71b; Neher '71). Although IA is partially activated at rest potentials, a cell must receive a pre-conditioning hyperpolarization in order to fully remove inactivation (Neher '71).

Several lines of evidence suggested that ELLL pyramidal cells have a large IA which regulates the input-output relationships as recorded intracellularly. Firstly, repetitive firing in response to depolarizing pulses was typically preceded by a variable delay and subsequent low spike-frequency period; the duration of this lag roughly corresponds to the inactivation times for IA (cf. fig.26).

Since IA inactivates with time at positive membrane potentials, current-clamping V_m at different values should affect the lag in spike reactivity, if indeed it is mediated by IA. The left and center columns in fig.38 illustrate the voltage-dependence of the lag period. At each membrane potential tested, the magnitude of the depolarizing pulse was adjusted so that the same final stimulus voltage was reached. At depolarized V_m s, the response lag was reduced, whereas hyperpolarization increased lag time indicating de-inactivation of an outward conductance.

The presence of an IA-like current during repetitive firing was also demonstrated pharmacologically, with the addition of 1mM 4-AP to the bath solution. At this dose, 4-AP has been shown to selectively block A-channels (Thompson '77; Zbicz and Weight '85). As indicated in fig.38c, 4-AP abolished the response lag to direct stimulation, and allowed the cell to fire an immediate, high-frequency burst of spikes.

In addition to regulating stimulus-response patterns, IA also determined the trajectory for individual spikes and interspike intervals. Blocking IA with 4-AP effectively eliminated the early phase of the AHP, as well as broadening the spike duration by as much as 300% (fig.37d,e). Thus, the contribution of IA toward spike repolarization is significant, and this current also adds to the hyperpolarizing action of g_K and $g_K(ca)$ in forming the interspike interval.

f. M-current:

Previous studies provided evidence for a cholinergic input to the ELL, possibly from vertically descending fibers of eurydendroid cells in the lobus caudalis (Maler et al. '81a) and involving only muscarinic receptors (Phan and Maler '83). In other CNS structures, activation of muscarinic receptors has been associated with the closure of the so-called M-channel and its corresponding conductance (Adams and Brown '82). Pyramidal neurons in the ELL slice were therefore tested for the presence of an M-current and responsiveness to cholinomimetic drugs.

First, atropine (1 μ M) was added to the bath to block the action of any endogenous Ach. Next, the cell was current-clamped to depolarized Vm to activate gM, and given step hyperpolarizing pulses. Closing and subsequent opening of M-channels should be evidenced by a hyperpolarizing 'sag' and depolarizing 'hump', respectively (Halliwell and Adams '82). As illustrated by the record in Fig.39, no indication of M-current was obtained in any of the cells tested.

This negative finding was given support by the addition of 1 μ M carbachol, a potent Ach agonist (Halliwell and Adams '82). In three separate experiments, application of the drug had no measurable effect on the firing pattern, membrane oscillations or Rin. These results might have a number of explanations: First, pyramidal cells may not receive a cholinergic input. Second, Ach receptors might not be coupled to M-channels in pyramidal cells. In other cell preparations,

Ach has been shown to modulate such diverse channels as Ca-dependent K-channels (Bernardo and Prince '82b; North and Tokimasa '82; Cole and Nicoll '84; Tokimasa '85; Madison and Nicoll '86) or the A-channel (Wadman et al. '86). In addition, the density of Ach receptors in the ELLL is low, even in the dorsal molecular layer where the highest level of QNB-binding was measured (Phan and Maler '83). This lamina is where the distal-most dendrites of the pyramidal cells are found. Small currents associated with the M-channel at these distal origins might not be resolvable at the soma. Alternatively, the in vitro preparation may somehow alter M-channel properties making them unresponsive to voltage manipulations.

V. Other effects of 4-aminopyridine

The previous section presented data to show that 4-AP treatment of ELLL slices had direct effects on the electrophysiology of pyramidal cells, probably by blocking an IA-like outward current. In addition, 4-AP acted indirectly on pyramidal cells through the actions of other, unidentified elements in the ELLL circuit. These actions initiated several rather striking changes in the electrical behaviour of pyramidal cells.

While recording from pyramidal cells in slices superfused with 4-AP, spontaneous spike activity gradually increased in spite of only minor decreases in VR. Frequently, DC hyperpolarization was required to maintain reasonable firing rates. During this period of hyperexcitability, a very regular, large amplitude oscillation (approx. 2 Hz) in

membrane potential developed which is illustrated at two time scales in fig.40. This pattern occurred in all of the cells treated with 4-AP ($N > 15$) and so dominated their electrical behaviour as to induce a pacemaker-like burst firing. Frequently, the large oscillations were superimposed upon a second, extremely slow wave (approx. 0.2 Hz; fig.41). It should be noted that these 4-AP potentials were different from the slow potentials that characterized untreated cells. The drug-induced oscillations were larger amplitude waves, often 20mv peak-to-peak, and lacked the typical slow inward ramp and subsequent steep hyperpolarizing phase of the SPs. Furthermore, in contrast to SPs of untreated cells, the large waves (both 0.2 and 2 Hz forms) increased in amplitude when constant-current hyperpolarization was applied, and decreased during depolarization. The membrane voltage had no effect on the frequency of the potentials. The chart records in fig.41a,b demonstrate this rectification phenomenon.

Several experiments were performed in an attempt to determine if 4-AP waves resulted from turning on intrinsic conductances or from activating an intense, synchronous synaptic input. Bath application of 0.2 μ M TTX completely abolished all spiking and wave potentials (fig.42). This result was inconclusive, however, since TTX blocks not only g_{Na} , but nerve activity as well.

In a separate experiment, calcium influx was blocked by addition of Mn^{2+} to the bath, which resulted in the elimination of 4-AP waves (fig.41c). As the calcium block

occurred, the depolarizing part of the wave was eliminated, leaving the resting potential at its baseline level. This result indicates that the waves are Ca-dependent events, driven either by 1) large Ca-currents involved in the depolarizing phase of the wave, 2) synaptically mediated depolarization followed by an unidentified repolarizing current, possibly $gK(Ca)$. The data seems to rule out the first possibility since large Ca-currents were difficult or impossible to evoke in a majority of pyramidal neurons. Also, cells treated with 4-AP plus Na- and K-channel blockers to unmask Ca^{2+} -currents never exhibited these large amplitude oscillations suggesting that they are not mediated by calcium.

The drug-induced potentials appeared operationally similar to the giant bursters encountered in unhealthy slices. This form of bursting was found to have the same voltage-dependence that 4-AP waveforms had. These data are consistent with observations on other cell preparations that afferent nerve terminals are also sensitive to 4-AP (Llinas et al. '76; Shimahara '81). 4-AP treatment therefore probably evokes hyperexcitability in ELLL afferent fibers, generating synchronous, giant EPSPs in pyramidal cells.

DISCUSSION

Recent advances in the technology of in vitro CNS preparations have stimulated an increase in our understanding of the cellular mechanisms that regulate neuronal excitability. Unfortunately, however, apart from studies of the comparatively simple nervous systems of invertebrate animals, very little data pertaining to intrinsic membrane physiology can be applied to gaining an understanding of the regulation of specific behavioural patterns or sensory capabilities.

The electrosensory system of weakly electric fish is one area of vertebrate neurobiology where cellular mechanisms could be examined in a more behavioural context. The ELLL, in particular, has been thoroughly described anatomically with respect to its intrinsic circuitry (Maler, '79; Maler et al. '81a; Mathieson and Maler '86) as well as its connections with higher order electrosensory areas (reviewed by Carr and Maler '86). The ELLL has a relatively simple, laminated structure which facilitates study of its cellular components at the physiological level. In addition, this first-order sensory area subserves two well documented electrosensory behaviours: electrolocation of objects (Bastian '81a,b), and the jamming avoidance response (JAR) which is used during social interactions with conspecific fish (Bullock et al. '72; Heiligenberg '77;'80;'83). From these studies is emerging a detailed analysis of the function of the electrosensory system correlated with the structure of the various

electroreceptive centers.

To date, the electrophysiology of the ELLL has been explored primarily with extracellular electrode recordings of pyramidal neurons in response to various behavioural stimuli (Enger and Szabo '65; Scheich '77; Bastian '81b, '86a,b; Partridge et al. '81; Matsubara '82). Although pyramidal cell activity under these conditions correlates well with predictions made from neuroanatomical diagrams (Bastian '86a), none of the interpretations take into account the cellular properties of pyramidal cells themselves. The results presented in this thesis are the first attempt to study pyramidal cell physiology at the intracellular level, and to identify the cellular properties which have functional consequences for the overall output of the ELLL.

The ability to characterize the biophysical properties of pyramidal cells demonstrates the usefulness of an in vitro preparation of the electrosensory lateral line lobe. By incorporating into the slice technique several key steps which minimize the damaging effects of surgical hypoxia and preserve the structural integrity of most of the ELLL circuit, it was possible to maintain viable tissue for more than 10 Hr, and record from stable pyramidal cell units on a routine basis.

It is important that the interpretation of physiological recordings from neural tissue is based on a solid understanding of the surrounding circuitry. This is especially true of in vitro preparations such as the ELLL slice. As shown in fig 1, the main neuronal components of the ELLL

circuit diagram include the pyramidal cells, and two types of inhibitory interneurons, the granule and polymorphic cells. The major inputs to the lobe are from primary afferent fibers in the deep fiber layer, and parallel fibers descending from the lobus caudalis and nucleus praeeminentialis.

In the transverse, 500-700 μm thick slices used in this study, the following structures and connections should remain virtually intact: First, the dendritic trees of pyramidal, granule and polymorphic cells are oriented parallel to the plane of the slice. In each of these cell types, the maximum longitudinal spread of the dendritic processes, as measured by golgi impregnation, is less than 250 μm (Maler '79). Therefore, excluding those neurons located in the most superficial 125 μm of the slice, these main cell types of the slice should be undamaged by the vibratome knife.

Since pyramidal cells are output neurons of the ELLL, their axons are cut as they pass through the plexiform lamina toward the lateral limniscus. It was possible, however, to activate pyramidal cells antidromically by stimulating the proximal portion of their axons within the plexiform layer. In contrast, the local collaterals of granule cells and probably polymorphic cells form an intrinsic plexus of axon terminals which contact nearby pyramidal cells (Maler et al. '81a) and, as such, will probably remain intact in the slice.

Although slice preparation interrupts the normally high tonic input to the ELLL, it is possible to activate orthodromically not only the pyramidal cell population, but

the inhibitory interneuronal pool as well. By stimulating the granule cell mass in the overlying caudal lobe to activate descending parallel fiber input, or primary afferents in the deep fiber lamina, it is feasible to examine the interaction of synaptic currents with the intrinsic currents described in this study.

Pyramidal cell electrophysiology in vitro

The ELLS slice preparation used in this thesis was designed to investigate the electrophysiological characteristics of pyramidal cells in as close to their natural state as possible. The laminar organization of the ELLS, which segregates the cellular and fiber tracts into discrete layers facilitated electrode recording of a pure population of basilar and non-basilar pyramidal cells. Intracellular labelling with Lucifer Yellow confirmed this important assumption; all of the physiological data reported here are representative of electrical activity of BP and NBP neurons.

The results of these data demonstrate that pyramidal cells in vitro exhibit several characteristic patterns of spontaneous activity. While nearly 1/3 of the cells have a stable resting membrane potential, the remaining cells are capable of undergoing rhythmic oscillations in Vm. These slow potentials are voltage-dependent so that direct depolarization of the cell soma increases their amplitude and frequency. The presence or absence of slow potentials appear to correlate

with spontaneous bursting or tonic spike activity recorded extracellularly.

Repetitive firing:

When pyramidal cells are injected with depolarizing current pulses, they respond with a characteristic pattern of repetitive spike firing. Although the response patterns vary from cell to cell, two striking generalizations can be made from their input/output relationships. During long duration stimulation (eg. up to 1sec), the early response of pyramidal cells can be characterized as a period of low frequency spike firing. This pattern is clearly illustrated by an initial delay lasting up to 30 msec prior to the 1st action potential volley (cf fig.25). The early response, described as a 'lag' in spike frequency, continues for an additional period of 50-250msec during which spike frequencies rarely exceed 100Hz (eg. fig26). Following the lag period, the majority of cells enter a period of higher frequency spike firing which persists throughout the duration of the stimulus pulse; they display little or no spike frequency accommodation and as such are tonic neurons (fig.25).

Ionic conductances:

Characterization of the membrane conductance of pyramidal cells indicates that spontaneous rhythmic activity and the repetitive firing properties are mediated by the actions of at least 6 different ionic currents. Two currents, in particular, dominate the physiological behaviour of these cells: a persistent, subthreshold sodium current (I_{NaP}) and a transient

outward current similar to the A-current.

Several lines of evidence support the conclusion that gNaP contributes substantially to pyramidal cell activity. First, the spontaneous, slow potentials are driven by voltage-dependent processes which activate in the voltage range subthreshold to spike initiation. Altering V_m by as little as 5 mv can transform a non-rhythmic cell into one that displays robust SPs. Furthermore, SPs are sodium dependent; blockade of Na influx eliminates rhythmic activity. The progressive flattening of the SP rising phase which accompanies TTX block suggests that gNaP drives the depolarizing component of membrane oscillations.

Additional evidence for gNaP activation occurs during the response to positive current injection. A subthreshold current pulse elicits a slowly growing depolarizing ramp in the voltage trace, often triggering the appearance of SPs. This inward ramp is sensitive to TTX block. When compared to voltage deflections evoked by equal amplitude current pulses in the hyperpolarizing direction, many cells display a form of inward rectification interpreted, in part, as a consequence of activating gNaP. It is reasonable to assume that the persistent, depolarizing action of gNaP is largely responsible for the lack of spike frequency accommodation when challenged with long duration stimuli.

The effect of IA on pyramidal cell activity is equally dramatic. Due to the time and voltage properties of IA, the action of this current is especially obvious during the early

response to stimulation. The kinetics of IA are such that activation occurs rapidly (within msec) and inactivation proceeds with a time constant of 200-300 msec, depending on the membrane potential (Neher '71). This outward current counteracts the depolarizing effect of positive current injection, resulting in the prominent lag period in spike train formation clearly visible in the stimulus-response profiles (cf fig.26). The importance of IA to electroresponsiveness is most obvious when the current is blocked with 4-AP or inactivated by steady-state depolarization; under these conditions pyramidal cells become highly excitable and react rapidly to depolarizing stimuli by firing a high frequency burst of spikes (fig38).

IA may also influence spontaneous oscillations in membrane potential. Current clamping V_m to depolarized levels results in SPs of increased frequency and amplitude. Depolarizing the cell not only activates g_{NaP} , but in addition inactivates (with time) IA, which would further unmask the voltage excursion driven by g_{NaP} . Thus, some of the variability in the strength of pyramidal cells' rhythmic behaviour could be accounted for by differences in the strength of IA. Unfortunately, it was impossible to test this idea pharmacologically, since treatment with 4-AP also induced the formation of the giant EPSP-like waves which overwhelmed the more subtle, voltage-dependent properties of the intrinsic SPs.

In addition to the classic sodium and delayed rectifier

currents, calcium and calcium-dependent potassium currents influence pyramidal cell activity. The contribution of these last two currents is far less demonstrable than that of either I_A or g_{NaP} .

By virtue of the inability to demonstrate calcium spikes in at least 1/2 of the cells tested, I_{Ca} is probably not a major contributor to the total membrane conductance. Even in the remaining fraction of cells that Ca-spikes could be evoked, a massive blockade of Na and K channels was required in order to sufficiently unmask their presence. In light of the relatively weak calcium influx, it is possible that calcium entry via voltage activated channels does not play a major direct role in pyramidal cell excitability.

Calcium currents, however small, indirectly regulate rhythmic activity and repetitive firing by activating $g_{K(Ca)}$. This is evident from the effects of eliminating calcium from the bath or blocking Ca channels with manganese. In spontaneously rhythmic cells these treatments substantially reduced the interval between SPs, indicating that a calcium-dependent outward current ($g_{K(Ca)}$) regulates the frequency of membrane oscillations. Spike frequency accommodation, as well, depends on $g_{K(Ca)}$. In those cells which displayed accommodation, blockade of $g_{K(Ca)}$ with apamin or chelating calcium with EGTA injection eliminated the accommodation whereas non-accommodating cells were unaffected by these drugs. The insensitivity of non-accommodating cells to $g_{K(Ca)}$ block provides further support to the notion that calcium and

calcium-dependent potassium channels play minor roles in pyramidal cell physiology *in vitro*. The accommodating cells appear to belong to a different functional class; it will be important for future studies to determine whether this functional distinction corresponds to a morphologically unique population of pyramidal cells.

Fast pre-potentials (FPPs):

An intriguing aspect of pyramidal cell electrophysiology is the presence of spontaneous spikelets, referred to as fast pre-potentials. Their appearance does not seem to correspond to injury potentials, since many neurons deemed healthy by physiological criteria displayed stable patterns of FPPs throughout long duration recording sessions. In general, they were sensitive to voltage manipulation and TTX treatment, similar to that of the larger amplitude slow potentials. The consequence of FPPs for spike activity is apparent during periods of membrane depolarization, either spontaneously at the crest of an SP, during the EPSP which follows orthodromic stimulation, or during direct injection of positive current. Each form of depolarizing event is sufficient to increase FPP amplitude above the threshold for spike initiation.

The duration and amplitude of FPPs observed in ELLL pyramidal cells resemble similar potentials recorded in hippocampal pyramidal neurons *in vivo* (Kandel and Spencer '61) and *in vitro* (Swartzkroin '77). The precise nature of hippocampal FPPs has yet to be defined, but has been proposed to originate from localized patches of dendritic membrane

referred to as 'hot spots' (Kandel and Spencer '61). Computer modelling of CA1 electroresponsiveness (Traub and Llinas '79) predicted that FPPs recorded at the soma were, indeed, resulting from sodium dendritic spikes. Alternatively, simultaneous recordings from separate electrodes and intracellular labelling of hippocampal cells indicates that some FPPs are electrotonic coupling potentials transmitted via soma-somatic and dendro-dendritic gap junctions (MacVicar and Dudek '80; 81).

The ultrastructure of the ELLL supports the possibility of pyramidal cell FPPs arising from electrotonic synapses. Non-basilar pyramidal cells, in particular, make gap junction contacts with ascending processes from granule cell dendrites, whereas basilar pyramidal cells appear incapable of forming gap junctions (Maler et al. '81a; Mathieson et al. '86). This arrangement predicts that only non-basilar pyramids should have FPPs. The intracellular recordings presented here, however, argue against FPPs being electrotonic coupling potentials; FPP sensitivity to current injection or oscillation in V_m suggests that they arise on or near the cell soma. Furthermore, the granule cell dendritic processes are long and thin (eg 50-100 μm from the pyramidal cells, 2-5 μm diameter, Maler '79). It seems likely that the granule cells are electrotonically too remote from the NBPs to be influenced by such small amplitude fluctuations in V_m .

It has been proposed that FPPs in hippocampal cells may function as a booster mechanism to amplify weak synaptic

signals in distal dendritic loci sufficiently to invade the soma (Kandel and Spencer '61). In light of the size measurements taken from LY-filled ELLL pyramidal cells (as in fig. 16), it seems feasible that FPPs might fulfill a similar requirement for amplifying electrosensory input from the periphery. Since primary afferents synapse onto the distal bush of the basilar dendrite, EPSPs must be conveyed up to 400 μ m before reaching the soma. Localized patches of membrane with active sodium conductance, situated part way along the basilar dendritic trunk could act as FPP generating sites and provide a mechanism to increase the gain of ascending EPSPs. Localizing sodium 'hot spots' on the dendritic trunk would also render them voltage- and TTX-sensitive, consistent with the recording data. This hypothesis predicts that only basilar pyramids should have FPPs. Further combined morphological and physiological studies will be required to resolve this issue.

Intersegmental Comparison

An intriguing aspect of electrosensory organization is the existence of multiple somatotopic maps in the ELLL and higher-order centers. Tuberous electroreceptors form identical, mirror image maps of the body surface in the centrolateral, centromedial and medial segments (Heiligenberg and Dye '82; Carr et al. '82). The possibility has been raised that intersegmental differences in synaptic organization of ascending and descending inputs to the ELLL may allow for independent analysis of selected features in the fish's environment (Heiligenberg and Dye '82). However, previous

studies on the afferent projections to the ELLL and their synaptic connectivity have failed to detect any such differences (Maler et al. '81a; Mathieson et al. '86). The results presented here addressed the issue by comparing the electrophysiological characteristics of pyramidal cells in all three segments that receive tuberous receptor input. In each parameter measured, pyramidal cells behaved similarly regardless of the segment they occupied. If there are segmental differences in electrosensory processing, they do not appear dependent on such pyramidal cell properties as input resistance, rhythmic membrane activity or repetitive firing patterns. It is possible that differences in the ionic conductance of pyramidal cells are present at the segmental level, but could not be resolved with the limited sample tested in this study. The functional significance of this interesting phenomenon awaits further study.

Comparison with other cell types

Perhaps the most exciting revelation to emerge from the membrane biophysical data presented here and in other recent studies, is an invalidation of the long-held view that a vertebrate neuron's soma and dendrites function strictly as passive cables. By exploring the ionic currents that underlie excitability at rest and at subthreshold membrane potentials, it is clear that neurons are capable of a diverse range of active conductances. As with ELLL pyramidal neurons, many other cell types in both invertebrate and vertebrate CNS structures utilize a characteristic subset of currents which

dominates their electroresponsiveness.

Non-inactivating sodium conductances:

El11 pyramidal cells exhibit a sodium current (INaP) which activates in the voltage range 10-20mv negative to that of fast sodium currents. INaP also differs from the fast sodium current due to its slow inactivation, which is manifested by long duration, spontaneous membrane oscillations and the slow depolarizing ramp evoked by current injection. This current is labelled 'persistent', because without the aid of voltage clamping techniques it is impossible to determine whether INaP is non-inactivating or slowly inactivating.

Sodium conductances with properties similar to gNaP have been identified in several other cell preparations. In squid axons, so-called 'sleepy sodium channels' were described as non-inactivating, but were opened at relatively high threshold voltages (Matteson and Armstrong '82). More recently, slowly inactivating sodium channels which also open at subthreshold potentials were described in giant axons of squid and cockroach, as well as in leech and barnacle neurons (Gilly and Armstrong '84; Yawo et al. '85; Kleinhaus and Johansen '86; Davis and Stuart '86). In the squid giant axon, they have been called 'threshold channels' because they are a major inward current carrier at the threshold voltage for spike initiation (Gilly and Armstrong '84).

GNaP is present in certain mammalian neurons, as well. In hippocampal CA1 cells *in vitro*, depolarizing current injection evokes a train of slow prepotentials which are

similar in voltage-sensitivity and trajectory to ELLL slow potentials (Langthorn et al. '84). CA1 cells also display a TTX-sensitive, inward-directed rectification when given subthreshold current pulses (Hotson et al. '79). Later experiments identified the driving force underlying the slow potentials to be a persistent, subthreshold sodium current (MacVicar '85). Thus, in both CA1 and ELLL pyramidal cells, subthreshold excitability levels are regulated largely by similar gNaPs.

In cerebellar slices, stimulation of purkinje cell somata evokes all or none plateau potentials which inactivate slowly and are sodium-dependent. These potentials are normally masked by large calcium and calcium-dependent currents, but the Na currents probably enable the cell to respond to stimulation with sustained, repetitive firing (Llinas and Sugimori '80a) in much the same way that ELLL pyramidal cells respond non-accommodatively to prolonged stimulation.

The most thoroughly investigated form of gNaP occurs in neocortical neurons in vitro. In tissue slices and in culture, neocortical cells possess a slowly inactivating sodium conductance which activates between resting potential and spike threshold (Connors et al. '82; Stafstrom et al. '82; '84a,b; '85; Dichter et al. '86). This inward current is responsible for the inward rectification characteristic of neocortical neurons (Stafstrom et al. '82; '84a) and regulates repetitive firing properties by dominating the membrane conductance between spikes (Stafstrom et al. '82).

It is probable that gNaP determines to a large extent the F/I relationships of pyramidal neurons in both ELLL and neocortex. The roughly sigmoidal shape of ELLL F/I curves (fig 23) appears to correspond to the primary, secondary and tertiary frequency ranges described in other neurons (Schwindt and Calvin '73; Langthorn et al '84). The increase in slope which occurs at the transition from primary to secondary range firing is a reflection of turning on gNaP. Neocortical neurons in vitro appear to lack a primary frequency range; the slope of the F/I curve raises abruptly into a steep linear portion corresponding to a secondary firing range. Stafstrom et al. ('84b) attribute this to gNaP activating at relatively negative potentials.

The question has been raised whether the ability to demonstrate two sodium conductances with different kinetic parameters reveals the existence of two separate populations of sodium channels. It is possible that INaP and the fast INa associated with action potentials could flow through the same channel, provided that there is a substantial overlap in the activation/ inactivation parameters (m_{∞} , h_{∞} curves, Hodgkin and Huxley '52); at membrane potentials where m/h overlap occur, a non-inactivating inward current would flow. This possibility seems unlikely, however, since INaP (measured with voltage clamp in neocortical cells) activates along a far broader range of voltages than predicted by overlap of m_{∞} / h_{∞} curves (Stafstrom et al. '85). Further evidence that two separate sodium channel populations exist was presented in

abstract form (Sugimori and Llinas '80): lidocaine differentially blocks the fast and slow forms of gNa. This report was never substantiated in manuscript form, however, and similar treatment of ELLL pyramidal neurons fails to discriminate between gNa and gNaP (fig.28).

Recent advances in characterizing the structure of sodium channels (Noda et al. '84) has led to speculation that fast and slow sodium channels differ in a circumscribed segment of the channel which is enriched in arginine and lysine. Binding of the neurotoxins batrachotoxin and grayanotoxin to fast Na-channels reduces inactivation and lowers the threshold for channel opening, possibly by modifying the acidic residues (Khodorov and Revenko '79; Seyama and Narahashi '81; Yawo et al. '85).

Calcium conductances:

Numerous CNS cell types have been shown to have large calcium currents which vary widely in their spatial distribution and kinetic requirements. In certain cells their activity is the critical determinant for regulating bursting behaviour and repetitive firing in response to synaptic input.

In gastropods, many central neurons are autorhythmic, discharging spikes in a bursting pacemaker pattern (Smith, '80). The essential biophysical characteristic of bursting cells is the presence of a negative slope resistance (NSR) region in the current-voltage relationship (Wilson and Wachtel '74) which is invariably accompanied by a non-inactivating

inward current (Smith '80). The charge carrier of this 'pacemaker' current is controversial; It has been identified by some authors as a TTX-insensitive sodium current in *Aplysia* and *Otala* (Smith et al. '75), a calcium current in *Helix* (Eckert and Lux '76) and *Tritonia* (Thompson '76) or a mixture of I_{Ca} / I_{Na} (Johnston '76; Kramer and Zucker '85). The intracellular Ca^{2+} concentration was also measured by Arsenazo III absorbance (a Ca-sensitive dye) and found to oscillate in proportion to the pacemaker cycle, providing additional support to a role for calcium in pacemaker activity (Gorman and Thomas '78).

Calcium influx indirectly regulates the frequency of bursting by activating $gK(Ca)$ (Meech and Standen '75; Eckert and Lux '76; Carnevale and Wachtel '80). The effect is mimicked by direct intracellular injection of Ca^{2+} which results in the post-burst repolarization of the membrane (Meech '72; Gorman and Thomas '78).

The contribution of calcium conductances to neuronal excitability in vertebrates has been investigated in a variety of tissue preparations. In cat spinal motoneurons, voltage clamp analysis *in situ* revealed that repetitive firing is regulated by a persistent inward current which activates within the voltage range traversed by the interspike interval (Schwindt and Calvin '73). The current (I_I), which is carried by Ca^{2+} (Schwindt and Crill '80a;b), is partially activated during the primary F-I firing range, and strongly activated during the transition to secondary range firing (Schwindt and

Crill '82). Furthermore, it is responsible for the observed region of negative slope conductance in motoneurons (Schwindt and Crill '77) which permits them to fire non-accommodatively to sustained depolarization (Schwindt and Crill '82).

Several forms of g_{Ca} have been described which influence bursting behaviour in mammalian neurons. Synaptic activation of Purkinje cell dendrites evokes the formation of a calcium-dependent plateau potential and calcium spikes which invade the soma and result in burst of fast somatic action potentials (Llinas and Sugimori '80b). In inferior olivary neurons, two spatially distinct calcium currents mediate a low frequency oscillation in membrane potential and rhythmic firing; a high threshold calcium spike originates at dendritic locations while a low threshold Ca -spike is somatic in origin. The somatic g_{Ca} requires a conditioning pre-pulse of hyperpolarization, eg. as in a post-burst AHP. Differential activation of these two conductances, simply by changing the intensity of background synaptic activity, may be sufficient to regulate the two naturally occurring oscillating frequencies observed in these cells (Llinas and Yarom '80a;b).

Similarly, thalamic neurons display two functional states of excitability, shown with *in vivo* recordings to be regulated by a persistent inward conductance (Deschenes and Steriade '82) and identified as g_{Ca} *in vitro* (Llinas and Jahnsen '82). At negative potentials, a somatic g_{Ca} is de-inactivated and cells respond to stimulation with a burst of spikes. At depolarized V_m , g_{Ca} is inactivated and the cells

fire tonically (Jahnsen and Llinas '84a;b). Thus, manipulation of calcium conductance can dramatically alter the input-output response of a cell.

In hippocampal CA3 neurons, spontaneous bursting is generated by an intrinsic, TTX-insensitive conductance (Wong and Prince '78), identified by voltage clamping techniques as a persistent, g_{Ca} that underlies a negative slope conductance region in the I-V curve (Johnston et al. '80). Subsequent work reported that in both CA1 and CA2 neurons, burst firing is regulated by a persistent calcium current (Brown and Griffith '83b).

The presence of calcium-dependent potassium currents appears to be a universal property of vertebrate neurons. They contribute to the so-called fast AHP, implicated in the repolarization of action potentials in bullfrog sympathetic neurons (MacDermott and Weight '80; Adams et al. '82) and CA3 cells of hippocampus (Zbicz and Weight '85). Intracellular calcium also activates a slower form of hyperpolarization (slow AHP) (Krnjevic and Lisiewicz '72; Krnjevic et al. '78) which is essential for the low frequency discharge observed in frog motoneurons (Barrett and Barrett '76) and spike frequency accommodation in CA1 hippocampal cells and supraoptic neurons (Madison and Nicoll '84; Bourque et al. '85). Post-burst hyperpolarization, as well, depends on calcium influx during spike discharge which activates a long duration, AHP (Alger and Nicoll '80; Hotson and Prince '80; Schwartzkroin and Stafstrom '80; Gustafsson and Wigstrom '81; Hablitz '81;

Aghajanian et al. '83; Brown and Griffith '83a).

In light of the abundance of evidence that calcium and calcium-dependent currents regulate excitability in such a wide spectrum of central neurons, it is somewhat surprising that in ELLL pyramidal cells, calcium-sensitivity is difficult to demonstrate for most repetitive firing properties. In several respects, pyramidal cells most closely resemble neocortical neurons in vitro : the responsiveness of both cell types is dominated by a persistent sodium current, neither cells have prominent calcium currents and consequently, $gK(ca)$ plays a less prominent role in regulating spike frequency. The last feature is manifested by the relative lack of spike accommodation which is characteristic of their response to current injection.

IA:

A rapidly activating, transient K current was reported almost simultaneously in neurons of two species of gastropod (Connor and Stevens '71b; Neher '71). Currents with pharmacological, time and voltage sensitivities similar to the so called A-current have since been reported in a variety of molluscan and vertebrate nerve cells (Alkon '84a Gustafsson et al '82; Stansfield et al. '86). IA appears to be a universal component of neuronal membrane currents and is a major determinant in spike firing patterns (Rogawski '85). During repetitive firing, the inactivation kinetics of IA prevent rapid membrane depolarization in the interspike interval and thereby allow firing with very low spike frequencies (Connor

and Stevens '71c). At presynaptic sites, IA is also believed to regulate neurotransmitter release by rapidly repolarizing the nerve terminal and restricting Ca^{2+} influx (Llinas et al. '76; Shimahara '81).

The sensitivity of the A channel and its ability to alter cellular behaviour has been documented in photoreceptor cells of the marine snail, *Hermisenda* (reviewed by Alkon, '84a,b). During associative learning in these animals, light-induced depolarization of the receptor membrane becomes progressively augmented which, in turn, causes a time-dependent inactivation of IA. This increases the cell's responsiveness to subsequent stimuli for as long as the membrane remains in the depolarized state. Interestingly, this behavioural change outlasts the direct effect of depolarization on IA inactivation. Since IA participates in spike repolarization, its blockade in *Hermisenda* neurons prolongs spike duration and allows enhanced calcium influx to accompany action potentials. This cascade of effects is hypothesized to indirectly modify IA via Ca^{2+} -calmodulin dependent protein kinase phosphorylation of the A channel gating mechanism. Hence, long after the conditioning-induced depolarization disappears, IA modification continues to evoke the behavioural change.

In ELLL slices, IA appears to influence pyramidal cell activity both as an intrinsic current and indirectly via pre-synaptic mechanisms. Acting as a 'brake' during the initial phase of a depolarizing response, IA counteracts the developing inward gNaP to maintain low spike frequencies. The

appearance of large EPSP-like potentials following 4-AP block of IA suggests that synaptic input to pyramidal cells may also be regulated by IA-like currents.

ELLL pyramidal cells in vitro vs in vivo

Accommodation:

Although pyramidal cell physiology has been investigated in intact fish almost exclusively at the extracellular level, it is possible to draw comparisons between their response properties in vivo vs in vitro. Bastian recorded E and I cell activities from immobilized fish while manipulating the intensity of their electroreceptive input (Bastian '81b, '86b). By examining the contrasting patterns of spike responses evoked in vitro vs in vivo, it is clear that removal of the ELLL from its normal ascending and descending connections dramatically alters pyramidal cell excitability and electroresponsiveness.

Several possible explanations could account for the discrepancies in firing behaviour under the two conditions. As shown by Bastian's extracellular recordings (and illustrated in fig.25), pyramidal cells in situ (E cells) respond to excitatory electrosensory input with two distinct phases: an early, very brief high frequency burst of spikes (up to 600Hz for a period of 20-50 msec), followed by a second phase of low frequency firing (spike adaptation) In vivo, therefore, pyramidal cells are both highly excitable and phasic in their response to peripheral input.

Both of these properties are, to a large extent, lost after slice preparation. Pyramidal cells *in vitro* have an early phase of low responsiveness (100-200Hz), lasting for about 100-250 msec; this phase appears to result from a strong IA-like conductance. The second phase of response is usually non-accommodative, due to the combined influences of gNaP activation and a weak gK(ca).

At least some of these characteristics can be explained strictly in terms of neurocircuitry. In the intact brain, basilar pyramidal cells receive monosynaptic excitation from P-type primary afferent terminals. In addition to this direct afferent input, a disynaptic, inhibitory pathway via granule cell interneurons is also activated (Maler et al. 81). Therefore, during an increase in afferent stimulation, BPs experience a rapid excitation (monosynaptic pathway) followed by a delayed inhibition (disynaptic pathway). In the slice preparation, however, pyramidal cells were stimulated by direct current injection, which bypassed the synaptically mediated excitation-inhibition sequence probably present in *in vivo* processing. Thus the adaptation of ELLL basilar pyramids to sustained input is probably due to disynaptic inhibition rather than to accommodation based on outward K currents such as IK(ca). This conclusion is made intuitively plausible by the temporal response characteristics of the E-cells (basilar pyramids). Bastian ('81b) has shown that E-cells respond optimally to amplitude modulations (AMs) of about 60 Hz. A long lasting outward current such as IK(ca) would presumably

act as a low pass filter and block the response to such high frequency modulations. $INaP$, by summing with synaptic depolarization, would increase the slope of an EPSP and therefore allow an E-cell to follow higher frequency AMs. Thus, it may be that the presence of a strong, persistent INa and a weak $IK(ca)$ is a cellular specialization which optimizes E-cells for responding to rapidly modulated primary afferent input. In nature these modulations might be produced by rapid movement of objects with respect to the electroreceptors.

A certain number of cells (approx. 1/3) did show spike frequency accommodation which was primarily due to $IK(ca)$. It will be important to determine if these accommodating cells are a different morphological cell type, or whether pyramidal cells can, under certain circumstances, display a more powerful $IK(ca)$.

I-cells (non-basilar pyramids) also adapt in vivo to sustained electroreceptive input (Bastian '81b). The input to non-basilar pyramids is quite complex (Maler et al. '81a) and it is not evident how the circuitry could account for the adaptive properties of these cells. Bastian ('81b) reported that I-cells respond best to AMs with lower frequency (about 16 Hz) and it will be interesting to determine whether this is a circuitry property or due to a greater $IK(ca)$ in these cells.

Spike-frequency lag:

It is not yet possible to understand why pyramidal cells in vivo can produce a rapid, high frequency response to

primary afferent input while *in vitro* the same cell type will respond to intense depolarization with only a low frequency firing (lag phase). There are at least three possible explanations to this discrepancy:

1) IA inactivation: The normal input to pyramidal cells *in vivo* (from primary afferents and descending input) may keep these neurons in a state of tonic depolarization. Since IA inactivates when the cell is depolarized (Connor and Stevens '71b; Neher '71) this would mean that *in vivo*, IA would not normally counteract the depolarizing effect of primary afferent input; with all extrinsic input severed *in vitro*, pyramidal cells would be hyperpolarized and IA inactivation would be removed. Subsequent depolarization would therefore be strongly counteracted by the activation of IA.

There are two serious difficulties with this scenario:

a) When Bastian ('86a,b) eliminated a descending projection to the ELLL pyramidal cells, he recorded a decrease in their rate of spontaneous activity; this is presumably because the pyramidal cells hyperpolarize after this tonic excitatory input is removed. The hypothesis presented above would predict that, under these circumstances, IA should be unmasked and cause a lag in the cells' response to increased input. In fact, the opposite was observed since the cells then respond even more vigorously to increased primary afferent input (Bastian '86a). b). When descending input is blocked, ELLL pyramidal cells display an enhanced 'surround' inhibition (Bastian '86a) presumably associated with a hyperpolarization

of the cells. It would therefore be expected that an object moving across the receptive field of a pyramidal cell will first hyperpolarize it (due to surround inhibition) and then depolarize it (center response). The inactivation of IA will be removed while the cell is hyperpolarized and IA should then be strongly activated during the subsequent stimulation of the center of the receptive field. The hypothesis above predicts that the center response should be weaker due to the braking action of IA. Again Bastian's results ('86a) demonstrate that even when there is a potent inhibitory surround, the center response is rapid and vigorous.

Therefore, this first hypothesis-that IA is inactivated by tonic depolarization in vivo- appears to be inconsistent with the data from Bastian's ('81b; '86a) experiments.

2). The activation kinetics for IA may also explain why ELL cells in vivo respond more quickly to afferent input. As suggested by the occurrence of fast prepotentials recorded at the soma, EPSPs in the basilar dendrites of basilar pyramids may be capable of initiating all or none, dendritic spikes at localized 'hot spots'. If these spikes depolarize the soma membrane faster than IA activation takes place, they would evoke an intense burst of spikes immediately followed by IA repolarization; the fast prepotentials do, in fact, often trigger action potentials in vitro. In this hypothesis, IA would add to the inhibition of pyramidal neurons by granule cells, and hence, increase the phasic response to peripheral stimulation. This possibility is weakened, however, because

IA activation is rapid ($<10\text{msec}$, Zbicz and Weight '85) whereas a typical burst response from Bastian's recordings may last up to 20msec (personal communication).

It should also be considered that the fast prepotentials would have to come from different sources for basilar and non-basilar pyramidal cells. For a basilar pyramid, the basilar dendrite is an attractive candidate, because dendritic spikes at this location would boost primary afferent input. For non-basilar pyramids, excitatory drive is derived from the ascending dendrites of granular interneurons (via electrotonic synapses, Maler et al. '81a). In non-basilar pyramids, therefore, fast prepotentials presumably originate from granule cell-NBP gap junction synapses. Clearly, this interesting hypothesis will require a great deal of further study.

3) IA modulation: A third hypothesis is that synaptic input, present *in vivo* but lost *in vitro*, is capable of suppressing IA. There are, in fact, several examples in the literature of IA being modulated by synaptic activity. Recent studies in Hermisenda giant neurons indicate that 5-HT can suppress IA by increasing its inactivation rate constant. Furthermore, this neurotransmitter effect is mediated indirectly by a second messenger system; forskolin, a cAMP substrate, mimics the effect of serotonin treatment (Acosta-Urquidí '86). In other studies, IA was shown to be inhibited by cAMP-dependent phosphorylation in Bag cells of *Aplysia* (Strong '84), but activated by the neuropeptide, FMRF-amide in bursting neurons

L4 and L6 (Ruben et al. '86). In vertebrate Raphe neurons, noradrenaline acts via $\alpha 1$ receptors to inhibit IA and cause burst firing (Aghajanian '85). More recently, in cultured hippocampal neurons, IA was reported to be blocked by acetylcholine, even in conditions where an M-current was not detectable (Nakajima et al. '86). This latest finding is of particular interest for ELLL functioning, since pyramidal neurons probably receive a descending, cholinergic input from Eurydendroid cells of the caudal lobe (Maler et al. '81b; Phan and Maler '83). This input could account for the intense, early response of pyramidal cells in vivo, by tonically suppressing A-channel activity. Although this hypothesis is elegantly simple, it lacks experimental support. The ELLL slice preparation is, however, well suited to testing its validity since it is possible to stimulate descending fiber-systems in the slice, or to apply putative neurotransmitters or modulators. It will be especially important to test whether the descending cholinergic input can, in fact, modify IA.

gK(ca) modulation:

By analogy with membrane conductances in other cell types, the calcium-dependent potassium conductance in ELLL pyramidal cells is also a strong potential target for neuromodulation. Numerous studies indicate that gK(ca) is modified in a variety of tissues. In sensory neurons of Aplysia, one type of facilitation is mediated by serotonin, which acts presynaptically via cAMP to close the so-called S-channels (Siegelbaum et al. '82). Noradrenaline induces

gK(ca) in guinea pig hepatocytes (Burgess et al. '79), but inhibits the same conductance in rat sympathetic neurons (Horn and McAfee '80), and hippocampal CA1 cells (Madison and Nicoll '82; Haas and Konnerth '83; Adams and Lancaster '85). Dopamine induces gK(ca) in CA1 cells (Bernardo and Prince '82c), while histamine has the opposite effect (Haas and Konnerth '83). FMRF-amide reportedly has a dual action, capable of both potentiating and inhibiting gK(ca) in *Helix* neurons (Cotrell '82).

Muscarinic agonists, in addition to blocking conductance of the M-channel, are reported to block gK(ca) in hippocampal CA1 cells (Bernardo and Prince '82a,b; Cole and Nicoll '83, '84), myenteric neurons (North and Tokimasa '83) and Bullfrog sympathetic neurons (Tokimasa '85). Interestingly, block of gK(ca), either by the action of Ach (Cole and Nicoll '83), NA (Madison and Nicoll '82) or calcium sequestration (Madison and Nicoll '84), is accompanied by a reduction in spike frequency accommodation during repetitive firing similar to apamin and EGTA blockade of accommodation recorded in ELLL pyramidal cells (RESULTS, IVd.).

The potential for neuromodulation of IA or gK(ca) may be an important feature of electrosensory processing in nature. Modifying these channel properties may extend the range of responses of ELLL neurons as required during a variety of behavioural situations and seasonal fluctuations in the life of the fish. For example, during the night/ day cycle, as the behaviour of gymnotid fish varies, so too, are the

requirements placed upon the electrosensory system. In daylight hours, fish are generally stationary and gregarious; their electrosensory activity may be primarily involved in social interactions (ie. the JAR). At night, they actively engage in solitary hunting excursions (ie. electrolocation of objects) (Bastian '86a). While hunting for food, fish can often be seen swimming rapidly back and forth alongside an object. This moves the object in and out of the receptive fields of pyramidal cells, a manoeuvre hypothesized to sharpen electrosensory acuity (Bastian '81b). The temporal responsiveness of pyramidal cells during this process must somehow be matched to the frequency of the amplitude modulations it 'sees' as the object passes across its receptive field. Stated another way, the faster the object appears to move, the more phasic the pyramidal cells' response must be. Clearly, under these circumstances, the ionic currents (and surround inhibition) which mediate spike frequency accommodation need to be maximally activated in order to provide the appropriate response.

In contrast, social behaviour requires far less temporal and spatial acuity from the P-system of afferents. When in a group, fish are highly responsive to the low frequency amplitude modulations resulting from the summation of one or more fishs' EOD signal (Matsubara and Heiligenberg '78). In this setting, the requirements for pyramidal neurons may include large receptive fields, with little need for spike adaptation.

South American knife fish normally live in large tropical rivers which experience huge seasonal fluctuations in water resistivity. Since the electroreceptors in the fishs' skin detect changes in electrical conductivity, their sensitivity also varies with the seasons (Knudsen '74). It is probable that pyramidal neurons must somehow adjust their own excitability to compensate for the changing electroreceptive input.

Two recent reports by Bastian (1986a,b) describe one mechanism which could be used for this purpose in ELL processing of electrosensory signals. Descending fibers originating in the nucleus praeminentialis of the midbrain are a part of a feedback loop which acts as a gain control for pyramidal cell responsiveness. Normally, descending input activates polymorphic cells which, in turn, inhibit the granule cell interneurons. This lessens the surround inhibitory response of E cells to increased receptor firing. In addition, polymorphic cells make axo-axonic, inhibitory synapses with primary afferent axons (Maler et al. '81), and hence, increased polymorphic cell activity will partially shunt out receptor input. Therefore, this descending influence maintains a low level of responsiveness and a weak surround inhibition (eg. less phasic). When Bastian interrupted these descending fiber tracts, pyramidal cell excitability and surround inhibition increased.

Descending input could also impose long-lasting control on pyramidal cell excitability by modulating one or more of its ionic conductances. For example, modifying the gating

properties of IA would significantly change the early response for pyramidal cells, while modifying $gK(ca)$ or $gNaP$ would alter the late response.

There is growing evidence that muscarinic receptors are coupled to $gK(ca)$ channels, and possibly to M-channels, through the breakdown of phosphatidylinositol and activation of C kinase (so called PI effect) (Nishizuka '84). This view is supported by the observations that muscarinic agonists induce PI degradation (Labarca et al. '84), and phorbol esters, which activate C kinase, mimic Ach block of accommodation as well as the PI effect (Malenka et al. '86).

These findings may help explain some of the more puzzling results presented here regarding the role of calcium-dependent conductances in pyramidal cell rhythmic oscillations. As indicated by the experiments depicted in fig 35 and 36, injection of EGTA blocked spike frequency accommodation (fig 35), as expected with reduced $gK(ca)$ activity, yet had no effect on the post-burst repolarization phase of the slow potentials (fig 36). This suggests that a different conductance besides $gK(ca)$ is responsible for SP repolarization. One such possibility is the voltage-dependent activation of an M-current. This idea seemed attractive in light of the histochemical evidence for a muscarinic input to the ELLL (Maler et al. '81b), Phan and Maler '83.). However, although application of muscarinic antagonists to ELLL slices had variable, and sometimes dramatic

effects on pyramidal cell rhythmicity (not illustrated), no evidence of M-current was measured electrophysiologically. The possibility still exists, however, that in vitro, tonic influence of Ach (or other undefined transmitters) inhibit both $gK(ca)$ and gM . If Ach transduction is via calcium-dependent C kinase and the PI effect in ELLL pyramidal cells, (as occurs in hippocampus), EGTA removal of calcium may unmask the M-current and increase its contribution to membrane hyperpolarization. This would explain why burst repolarization is so strong following EGTA injection. Unfortunately, the inability to measure M-current, even in the presence of atropine, is difficult to reconcile with this hypothesis.

General implications for in vitro model systems

In vitro model systems such as the ELLL slice preparation are powerful research tools, making possible investigation of many regulatory mechanisms which are too difficult to approach with conventional methods in intact animals. The ability to achieve stable electrode recordings for relatively long periods, while controlling the composition of the bathing medium, has been instrumental in characterizing the growing inventory of ion channels and how they function within the membrane to regulate the behaviour of a given cell.

In spite of the many advantages to the in vitro approach to electrophysiology, it is clear from the data presented in this study that the interpretation of such work must be made with great care. Many reports on the in vitro properties of central neurons are taken as an accurate representation of

their *in vivo* counterparts, in spite of not having a solid basis for comparison. As with ELLL pyramidal cells, interruption of the normal synaptic drive may be sufficient to radically alter such basic physiologic parameters as the input-output relationships of a cell, or the functional state of the ion channels that make up the composite membrane conductance. Technical advances in tissue maintenance will continue to extend the life of neural preparations, and these concerns will become even greater as long-term changes in membrane properties express themselves.

The data to emerge from this and future studies of ELLL neurons are unique in these respects. Unlike similar data reported in more complex model systems, the cellular properties of ELLL neurons examined *in vitro* can be interpreted within a growing framework of neurocircuitry and behavioural physiology. In this way, the physiological consequences of tissue preparation and maintenance can be recognized, and even utilized, to formulate and test predictions about the mechanisms that govern electrosensory processing.



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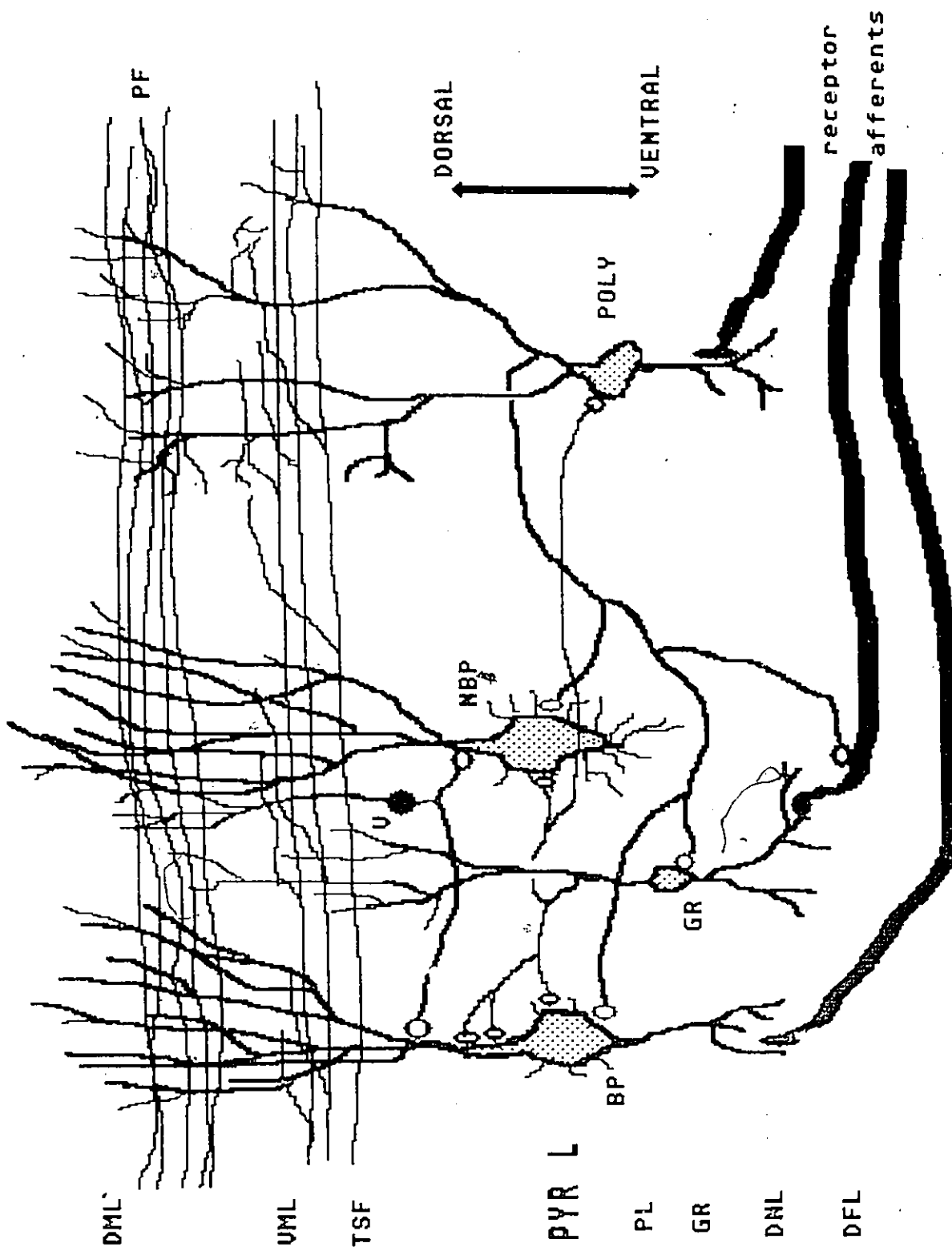
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Figure 1. A simplified schematic diagram of the ELLL circuitry. Primary afferent input from tuberous and ampullary receptors at the fish's body surface form the deep fiber layer (DFL) on the ventral surface of the ELLL. The pyramidal cell layer (PYR L) contains cell bodies of 3 different classes of neurons: basilar pyramidals (BP), non-basilar pyramidals (NBP) and polymorphic cells (POL). Basilar pyramidals possess a long basilar dendrite which extends ventrally into the deep neuropil (DNL) where it receives excitatory synaptic input (filled circles) from the primary afferent terminals. Non-basilar pyramidals lack a basilar dendrite and therefore do not receive direct primary afferent input. Pyramidal cell axons (not shown) leave the ELLL via the plexiform layer (PL).

Three classes of interneurons make inhibitory synaptic contact (open circles) with basilar and non-basilar pyramidals: neurons of the ventral molecular layer (V), granule cells, which occupy the granule cell layer (GR), and polymorphic cells. BPs, NBPs, polymorphic cells, neurons of the ventral molecular layer, and a subset of granule cells have large apical dendrites which extend dorsally into the molecular layers. Here they receive descending, excitatory contact: parallel fibers (PF) from neurons in the nucleus praeeminentialis enter the ELLL via the tractus stratus fibrosum (TSF) and ramify in the ventral molecular layer (VML). PFs originating in the lobus caudalis of the cerebellum ramify in the dorsal molecular layer (DML).

FIGURE 1.



62

Figure 2. Blocking diagram for ELLL slice preparation.

The orientation of a typical 600 μ m slice as cut from the brain of *A. leptorhynchus* is shown from dorsal (left panel) and caudal (right panel) views. The slice is transverse to the ELLL (ie 45° from the brain stem axis). The knife cut is tilted 10° as indicated to preserve the apical dendrites of pyramidal cells which bend rostrally as they course toward the dorsal molecular layer of the ELLL. SP= cut end of spinal cord; LC= lobus caudalis of the cerebellum; Cb= corpus cerebelli; TeO= optic tectum; FB= forebrain.

2.

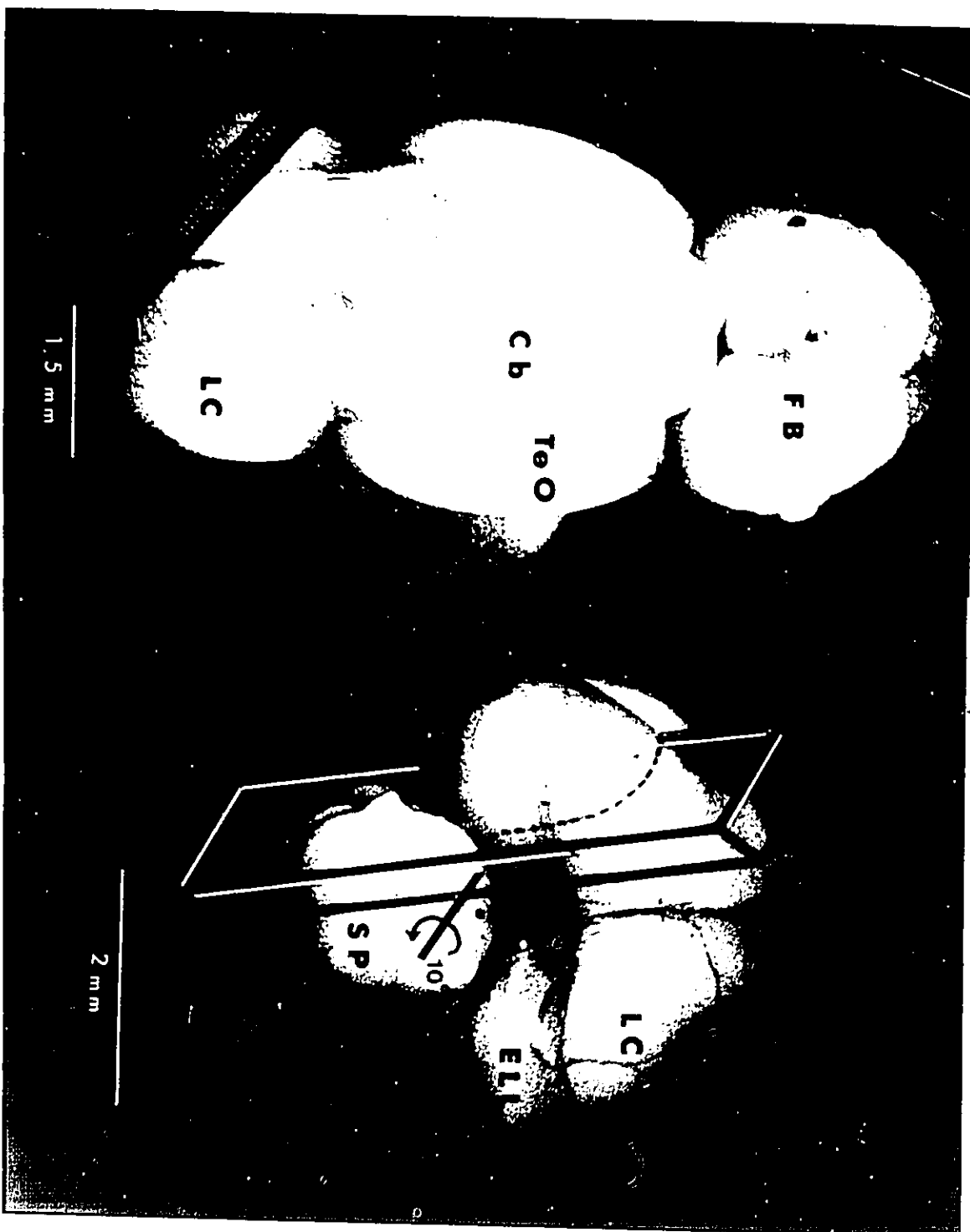


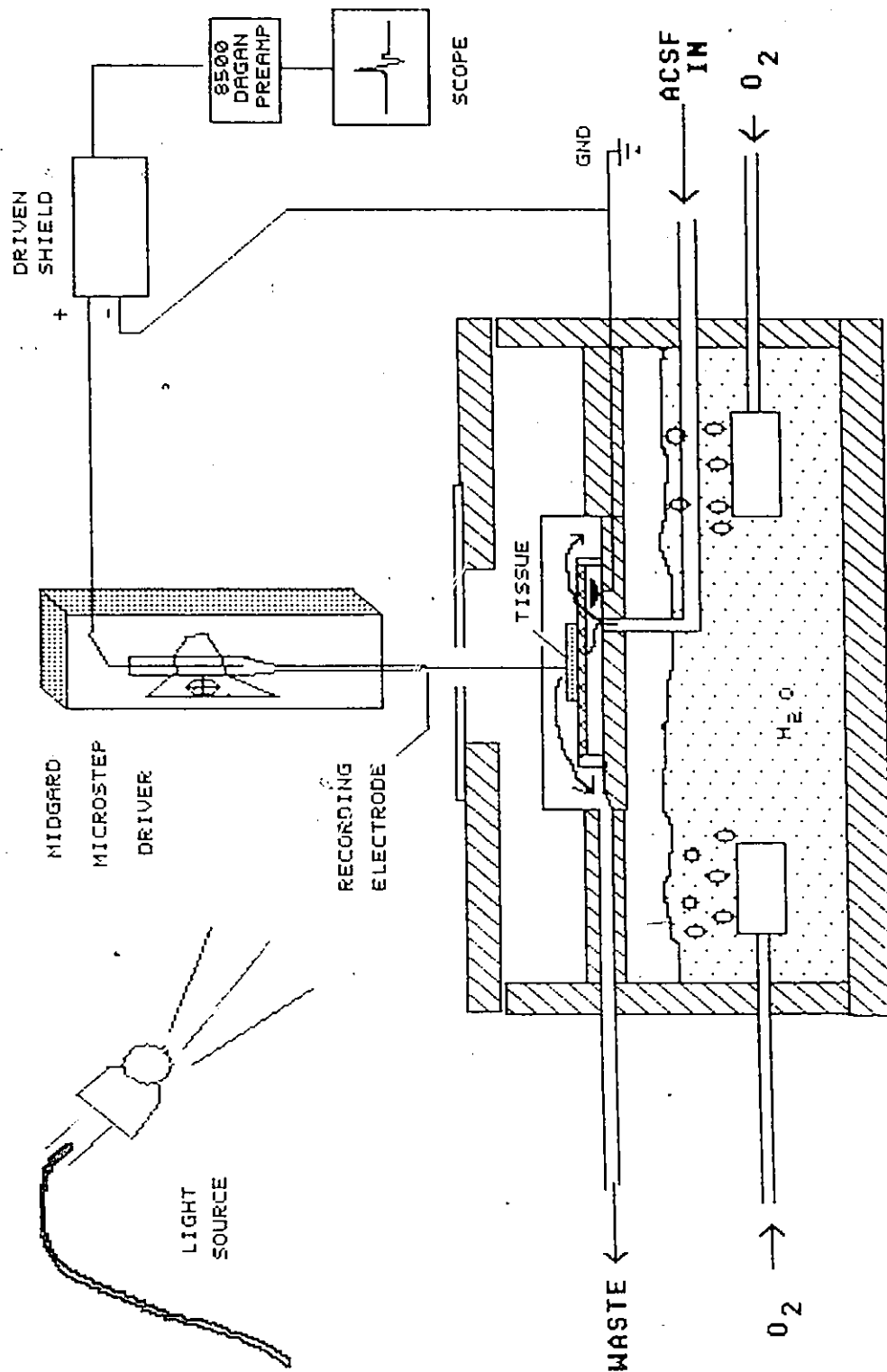
Figure 3. Slice chamber apparatus.

The slice chamber consists of 2 plexiglass baths: the smaller upper well contains the tissue slice which rests on a nylon mesh. Oxygenated ACSF enters the well from below and flows over and around the tissue and is withdrawn by suction to a waste bottle. The depth of the ACSF in the well is regulated by paper wicks that draw the fluid into the waste outflow port.

In the 'interface' technique, the ACSF layer flowing over the tissue is restricted to a capillary film which acts as the interface between the tissue and a humidified, oxygen-rich atmosphere created by bubbling O₂ into the water-filled outer chamber.



FIGURE 3.



SLICE CHAMBER SETUP

Figure 4. Transverse ELLL slices photographed in place in the slice chamber apparatus.

These two consecutive slices were resting on a nylon mesh stretched across the slice chamber well. Small pieces of platinum wire were placed across the tissue to anchor the slices while recording with the 'submerged slice technique'. The ELLL lies between the brain stem (BS) ventrally and the lobus caudalis (LC) dorsally. The alternating gray and white bands of the ELLL are clearly visible through the dissecting microscope. P= pyramidal cell layer; df= deep fiber layer.

4.

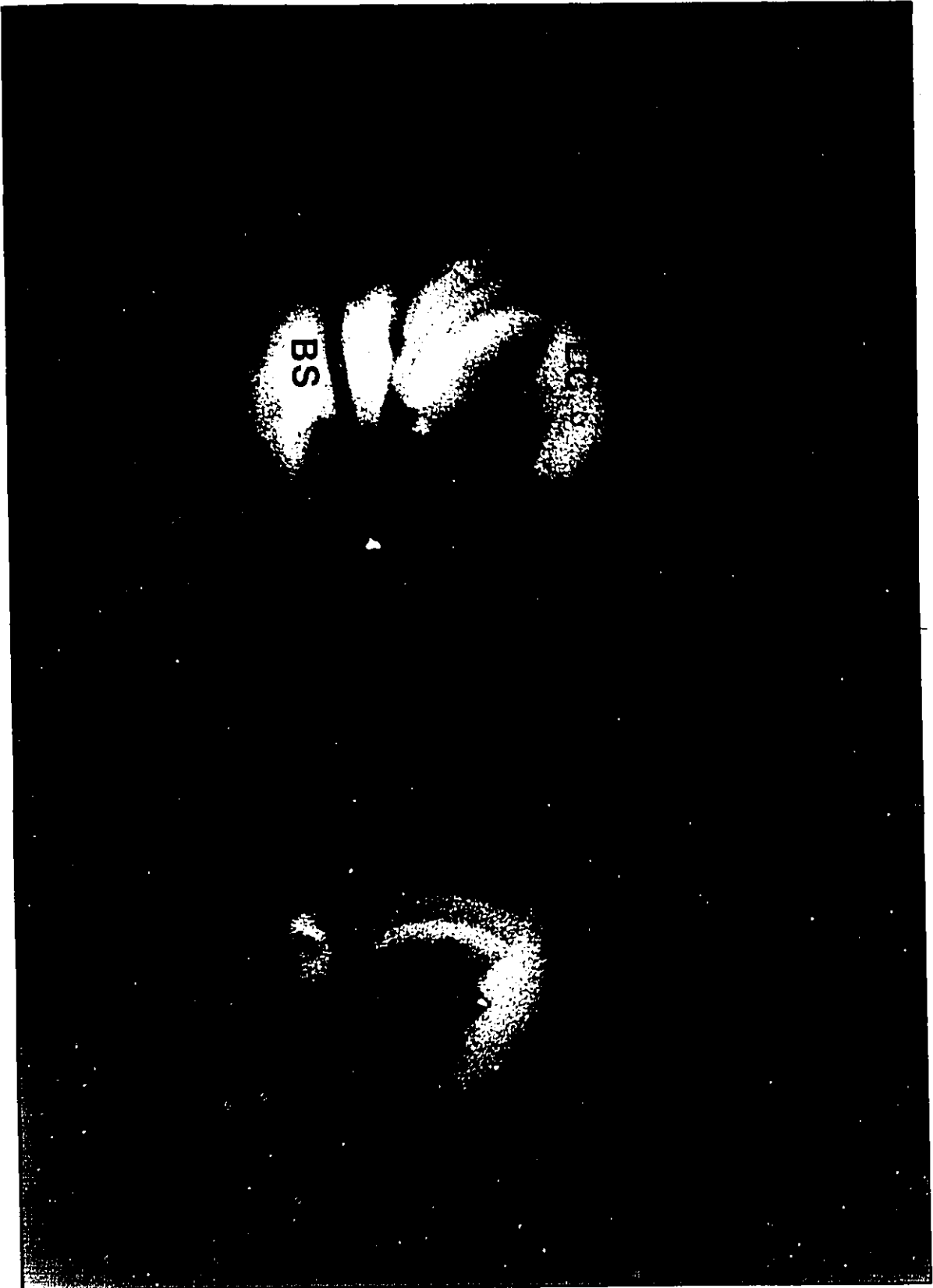
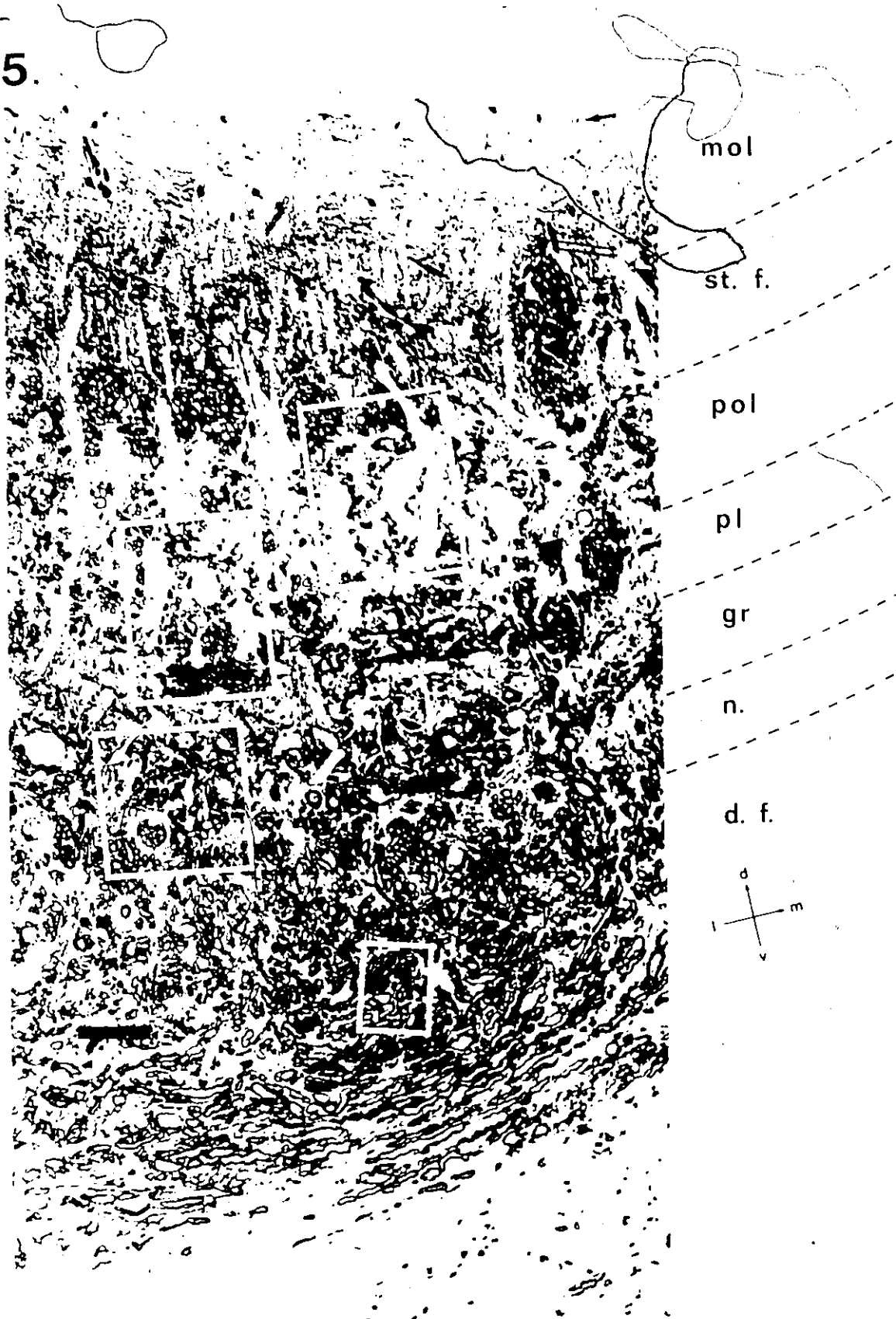


Figure 5. Light micrograph of ELLL slice following 8 Hr recording period.

The slice was fixed and embedded, and sectioned to the depth of tissue that was recorded electrophysiologically. All ELLL laminae, including their major neuronal constituents are intact and show minimal evidence of cytological damage. Boxed- in areas are shown at higher magnification in figures 6-8. For all light micrographs, sections were stained with toluidine blue. Large arrow indicates a polymorphic neuron typically located at the ventral edge of the pyramidal cell layer. Single small arrow in the molecular layer indicates a stellate cell; small double arrows indicate a neuron of the ventral molecular layer. Bar marker= 40 μ m.

Dotted lines at right of figure indicate approximate boundaries of the various laminae: mol= molecular ; pol= pyramidal layer; st.f.= stratum fibrosum; pl= plexiform layer; gr= granule cell layer; n= neuropil; d.f.= deep fiber layer. Directional markers are drawn relative to the longitudinal axis of the ELLL.

5.



mol

st. f.

pol

pl

gr

n.

d. f.

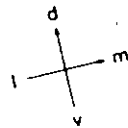


Figure 6. Light micrographs of the deep fiber and neuropil laminae.

a. The deep fiber layer contains primary afferent axons and very rare multipolar cells (not shown in this photograph). In healthy slices, the majority of primary afferents appeared undamaged, although some tissue swelling was evident in the extracellular compartment, especially near the pial surface of the ELLL.

b. The deep neuropil (DN) lies between the deep fiber and granule cell (Gr) laminae. A large spherical cell (Sph) lies in the neuropil and is surrounded by a characteristic 'halo' of glial processes. Within the neuropil, numerous primary afferent terminals make synaptic contact with dendrites of basilar pyramidal, granule and polymorphic cells, as well as spherical cell bodies (see electron micrograph in Fig.12). Closely packed granule cells form a dense cellular band dorsal to the neuropil.

Bar marker = 8 μ m.

6.

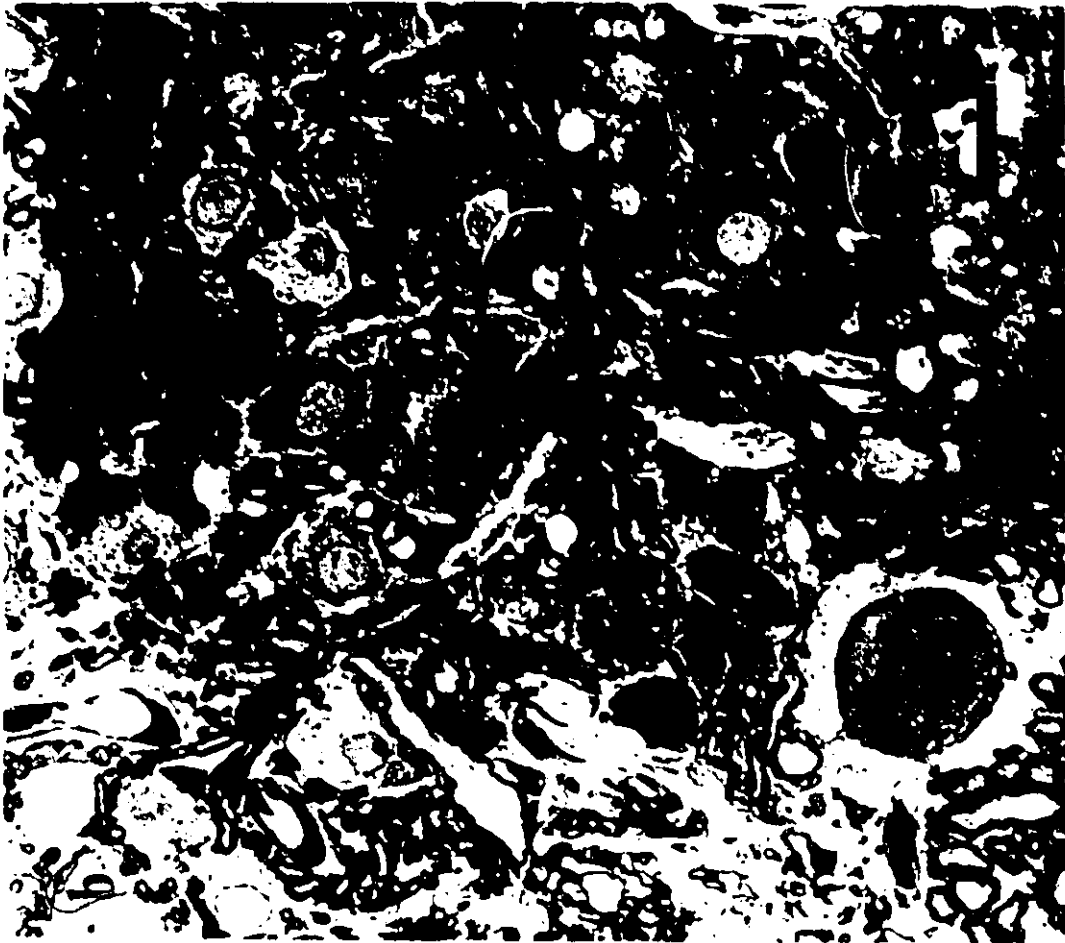


Figure 7. Light micrographs of the pyramidal cell layer.

a. A myelinated axon (probably a granule cell axon) appears to make an en passant terminal (arrow) before contacting the soma of a pyramidal cell (pyr). Axons from the pyramidal neurons collect into an efferent fiber tract, the plexiform layer (PL) which forms the ventral border of the pyramidal cell layer. Marker= 8 μ m.

b. A large pyramidal cell (pyr), possibly an NBP is visible with its large apical dendrite by-passing a blood vessel to extend dorsally through the stratum fibrosum (ST) into the molecular layer. Glial cells (arrows) often crowd the pyramidal cell somata. A polymorphic neuron occupies a typical location at the ventral aspect of the pyramidal cell layer. Note the more densely-staining cytoplasm of the polymorphic neuron.

Bar marker= 8 μ m.

7.

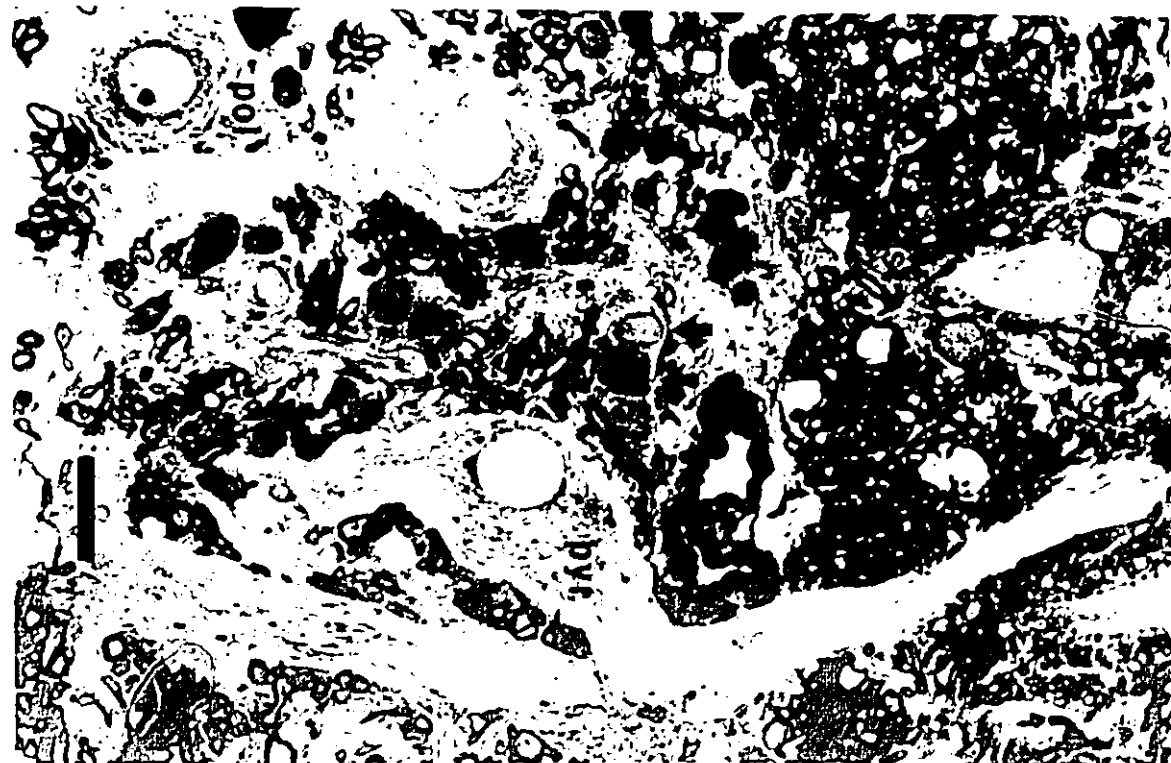
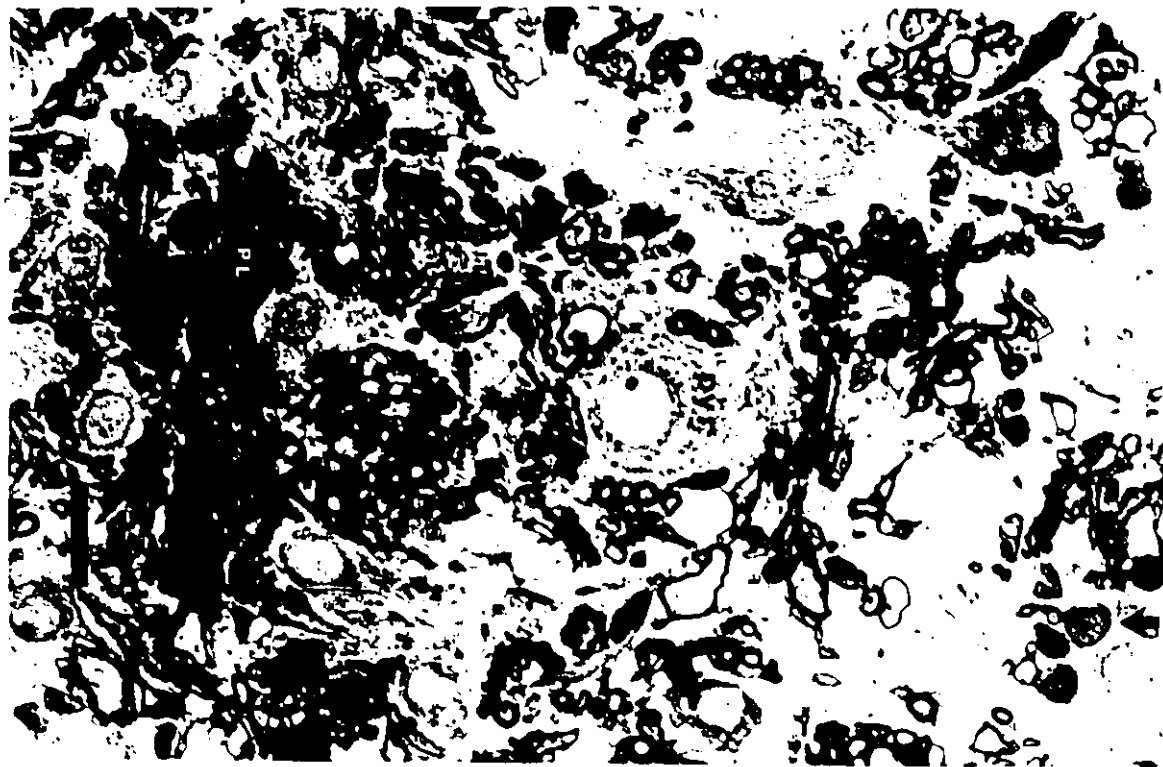


Figure 8. Left: A non-basilar pyramidal cell (NBP) with its large apical dendrite (arrow) appears completely normal in its cytological preservation. Gr=granule cell. Right: A basilar pyramidal cell (BP) is visible with the proximal portion of its basilar dendrite (open arrow). The darkened profile of a dead neuron is indicated at the top by a filled arrow. Note that the BP is also somewhat pycnotic, and was probably damaged partially by the slice procedure.

Bar markers= 8 μ m.

8.

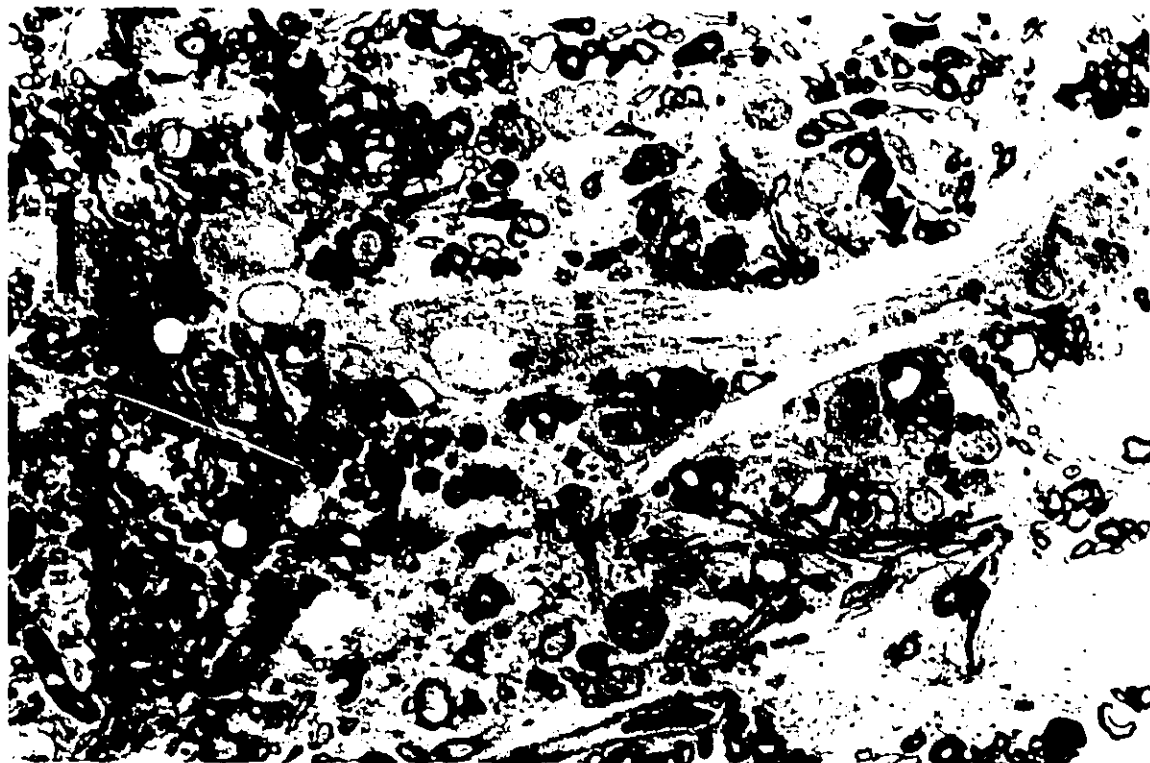


Figure 9. Light micrograph of ELLL in vitro showing moderate tissue damage. In comparison with the condition of the slice photographed in Fig.5, this slice contains several degenerating cells associated with loss of tissue viability. In the pyramidal cell layer (P) viable pyramidal cells (open arrow) are intermingled with darkened pyramidal cells in the early stages of cell death (closed arrow). Numerous examples of highly vacuolated axon 'ghosts' are visible in the stratum fibrosum (ST), granule cell (Gr) and plexiform layers. This slice had been maintained for approximately 7 Hr prior to fixation and was exhibiting physiological characteristics of a 'tired slice'.

DF= deep fiber layer

MOL= molecular layer

V= neuron of the ventral molecular layer

Bar marker= 40 μ m.

9.



Figure 10. EM micrographs of deep fiber and neuropil layers.

a. Several axon terminals are making chemical synapses with dendrites from granule cells (gr), basilar pyramidal cells (pyr) and polymorphic cells (not present in this photograph) in the deep neuropil.

b. Primary afferent axons in the deep fiber layer show some disruption of myelin lamellae and swelling in the extracellular space. The axoplasm, mitochondria and neurofilaments of the majority of the fibers were preserved.

Bar Markers: a= 0.5 μ m

b= 0.7 μ m

10

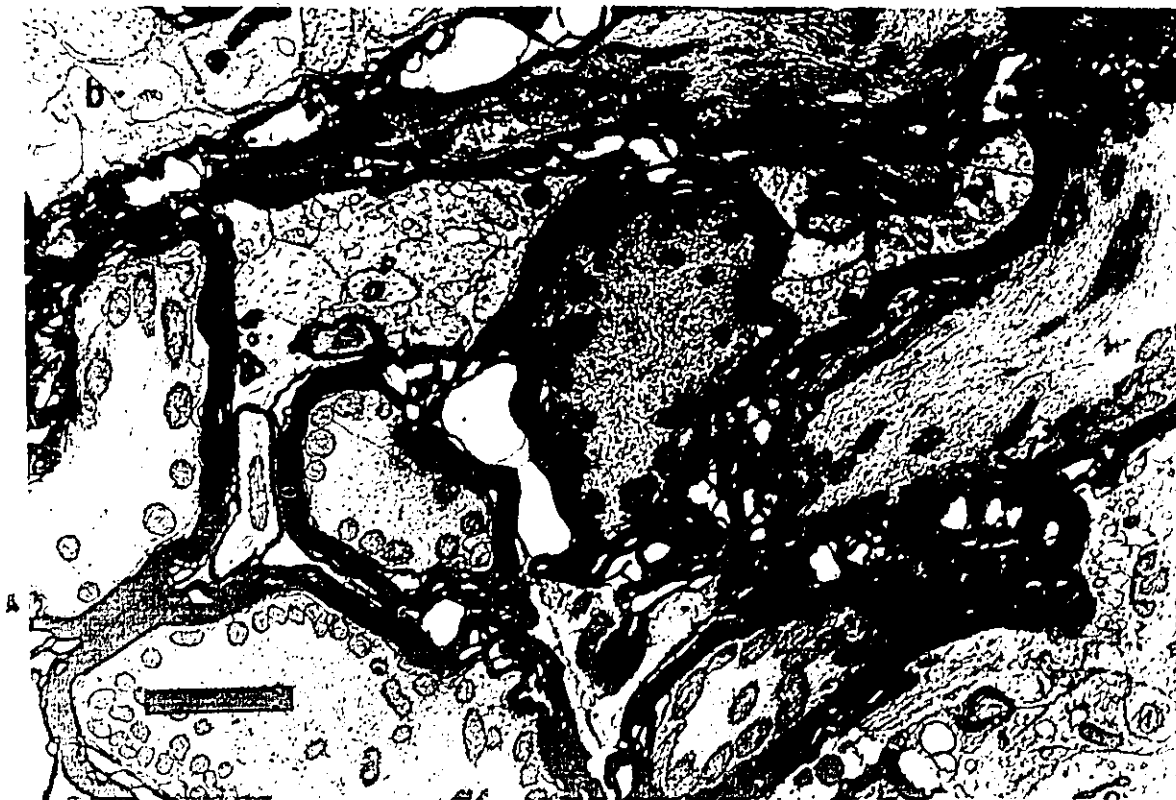
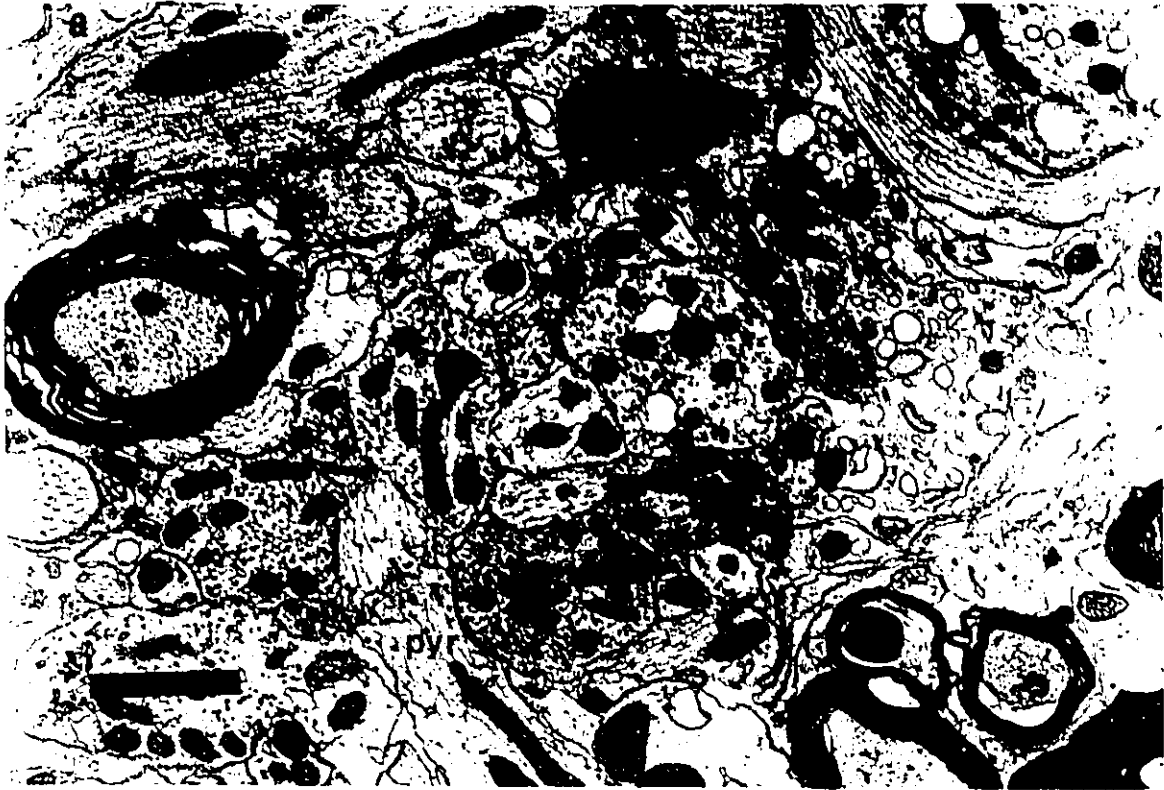
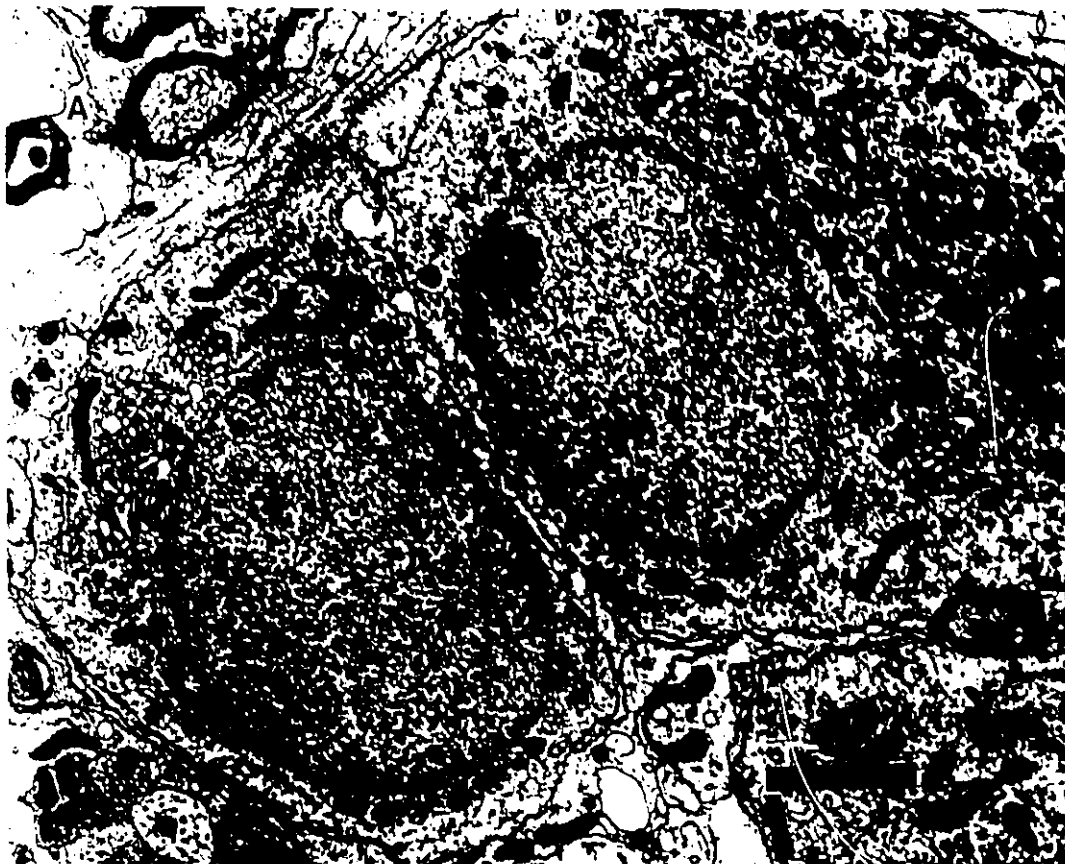


Figure 11. EM micrograph of granule cells. Examples of 3 granule cells are shown. Granule cells are closely packed, with dense cytoplasm. Their somata receive occasional chemical and electrotonic synapses with primary afferent and polymorphic axon terminals (not visible in this photograph). The majority of these contacts were intact in the slice preparation although degenerating terminals were sometimes apparent (arrow in B).

Bar markers: A= 1.1 μ m.

B= 1.6 μ m

11



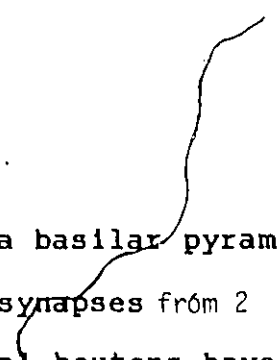


Figure 12. EM micrograph of a basilar pyramidal dendrite (PYR) in the deep neuropil with synapses from 2 primary afferent axon terminals. The terminal boutons have rounded synaptic vesicles clustered around asymmetric chemical synapses (arrows), typical of primary afferent-pyramidal cell junctions. Pyramidal cell dendrites are differentiated from granule cell dendrites by their relatively large size and electron lucent dendroplasm.

Bar marker= 0.5 μ m.



12



Figure 13. EM micrographs of a pyramidal cell soma (b) and initial segment (a). Axon terminals from granule and polymorphic cell interneurons form numerous symmetric chemical synapses (arrows) with pyramidal cell somata. The concentration of these inhibitory synapses is especially high around the pyramidal cell axon's initial segment, which is identified in part by neurofilament bundles (*).

Bar markers= 1 μ m.

13



Figure 14. EM micrographs of the stratum fibrosum and molecular layers.

Top. An apical dendrite, probably from a pyramidal neuron, can be seen making numerous asymmetric chemical synapses with parallel fibers in the molecular layer. The synapses usually occur on dendritic spines (arrows), and are very well preserved after several Hr in vitro. Bottom. The fibers of the stratum fibrosum are nearly all cut transversely in the slice orientation used in this study. Consequently, many axons (particularly the larger ones) die during the course of the experiment.

Bar markers: Top= 0.5 μ m.

Bottom= 0.9 μ m

14

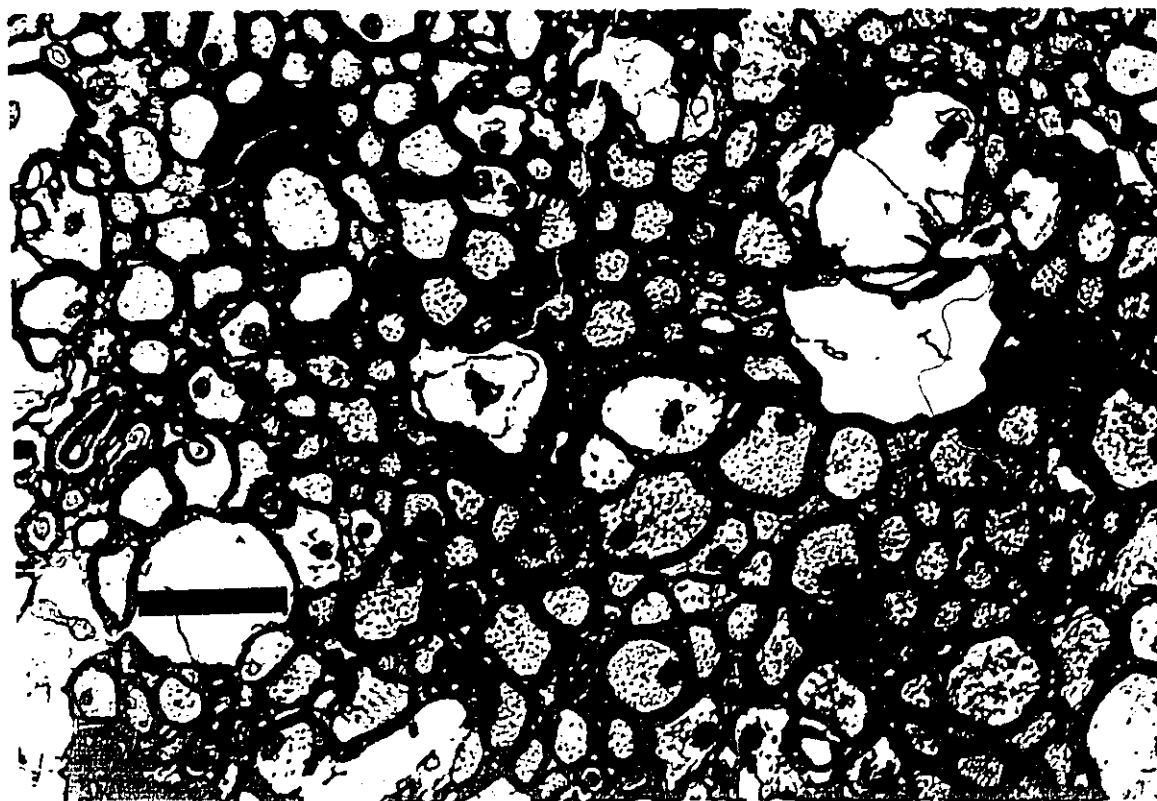
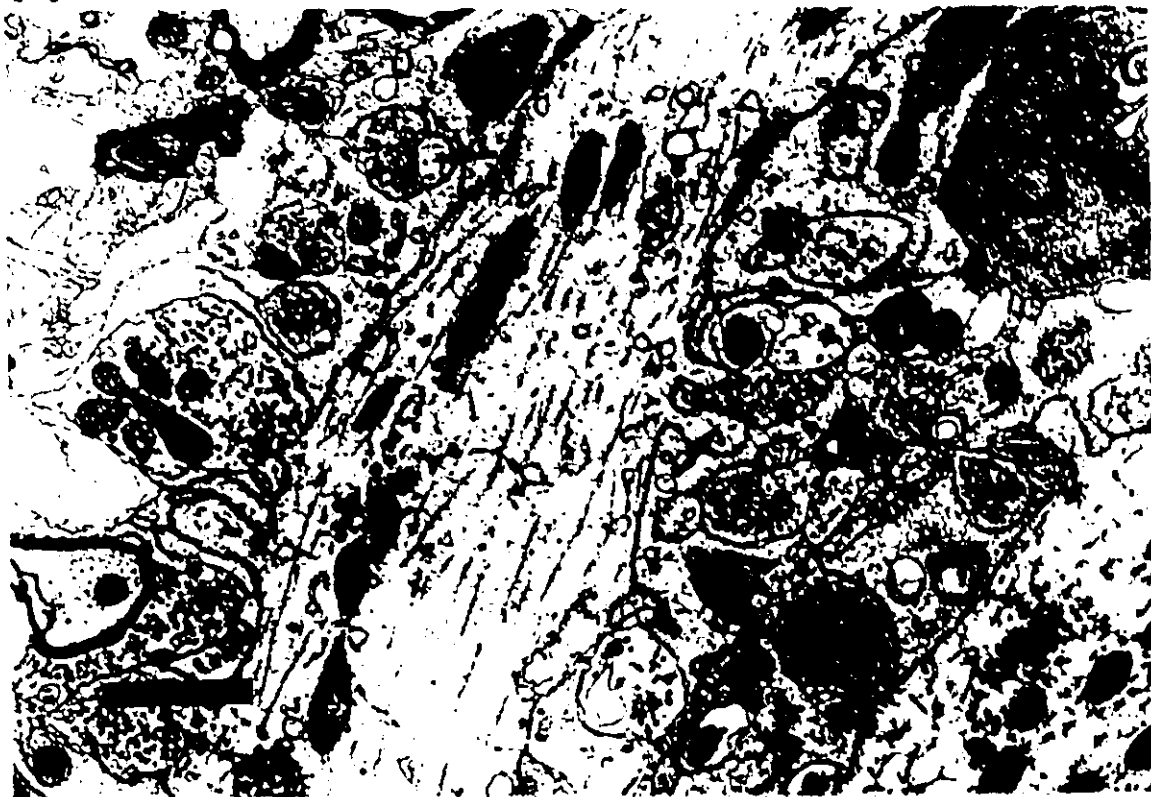


Figure 15. Lucifer Yellow-injected non-basilar pyramidal cell. Intracellular recordings were made with pipettes filled with LY to characterize their spontaneous and stimulus-evoked spike activity. After identifying the cells physiologically, they were injected with LY by passing hyperpolarizing current pulses (1 Hz, -2nA) for up to 1 Hr prior to fixation and visualization of the injected cell with fluorescence microscopy. This cell was identified as a non-basilar pyramidal cell by its morphology and position in the ELL. A large apical dendrite extends dorsally into the ventral and dorsal molecular layers where it bifurcates and divides into an extensive apical dendritic tree. Numerous somatic dendrites are visible extending a short distance into the surrounding pyramidal cell layer. Conspicuously absent is a basilar dendrite, present only in basilar pyramidal cells.

Bar marker= 70 μ m.

15

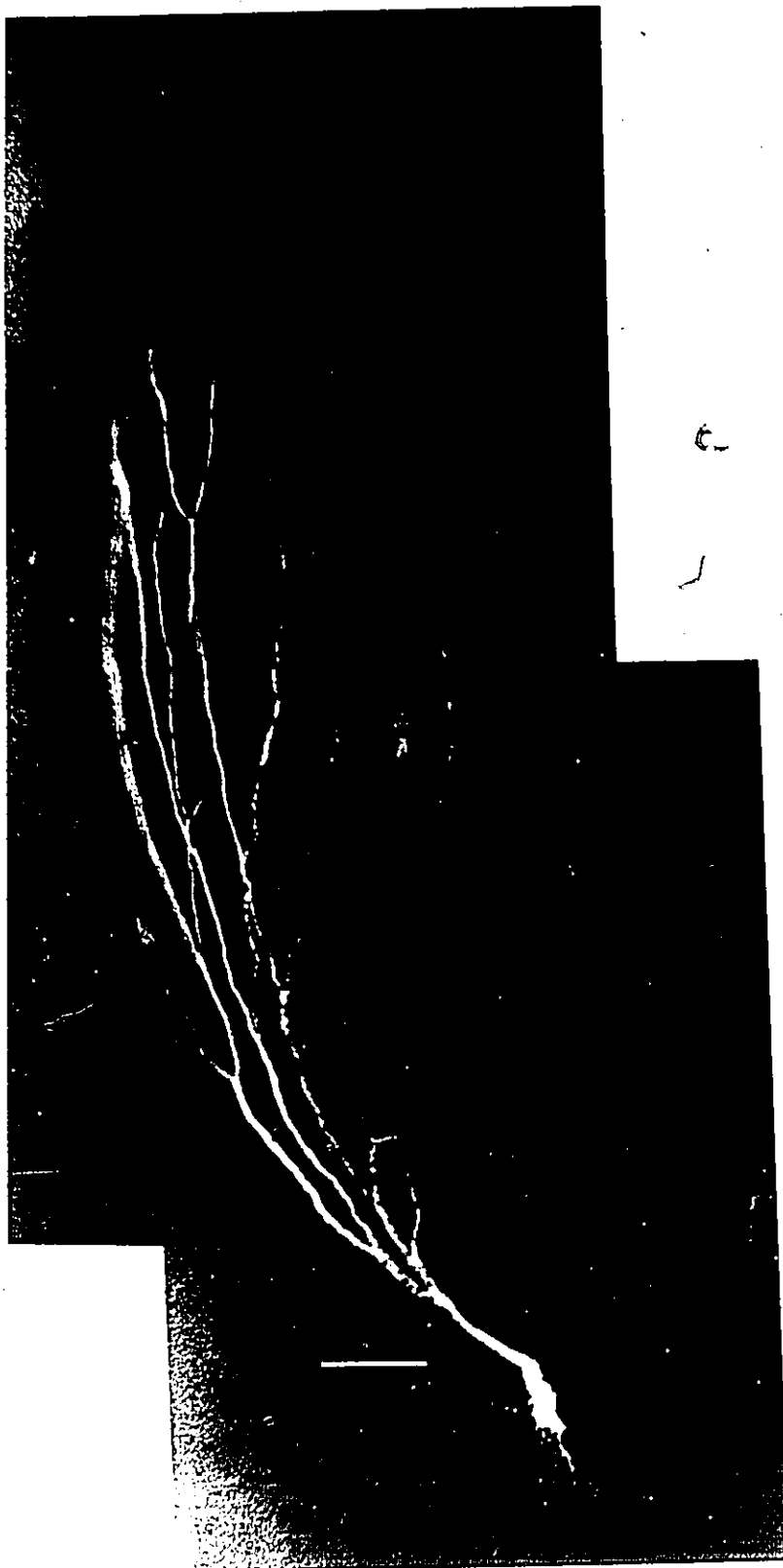


Figure 16. Lucifer Yellow-injected basilar pyramidal neuron. Similar to the cell in Fig. 15, this neuron was injected with LY and processed for fluorescence microscopy. It is identified as a basilar pyramidal cell due to the presence of its long, basilar dendrite, which extends ventrally into the deep neuropil layer where it receives direct primary afferent input. The basilar dendrite is smooth and unbranched as it descends through the plexiform and granule cell layers, then divides into an extensive basilar bush within the neuropil. Note that the BP has fewer somatic dendrites than the NBP, but possesses the long apical dendrite. —

Bar marker= 100 μ m.

16

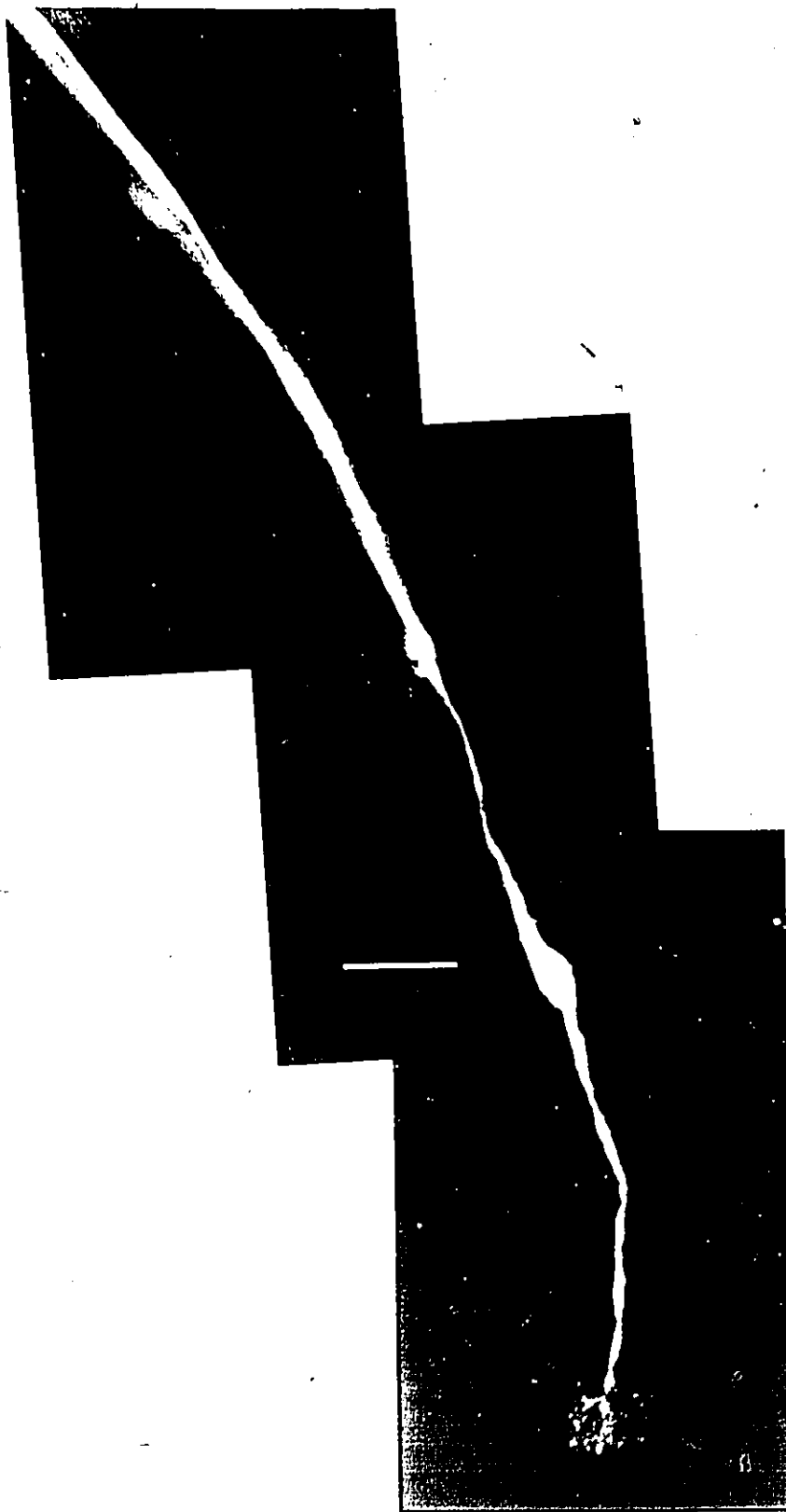


Fig. 17

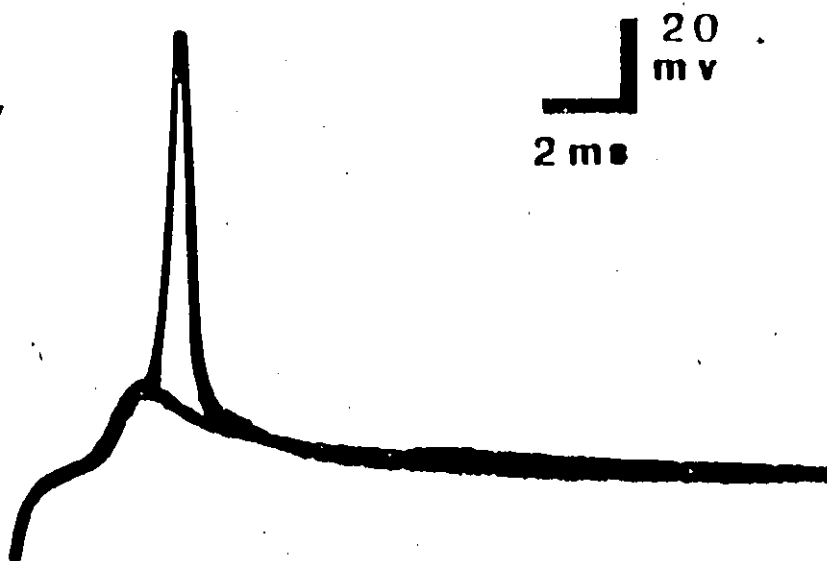


Fig. 18



Figure 17. Excitatory postsynaptic potentials. Stimulation of granule cells in the lobus caudalis activates descending input from parallel fibers which, in turn, contact pyramidal cell apical dendrites. Following a 3-6 msec delay, suprathreshold stimulation evoked all-or-nothing spikes or bursts of spikes. Hyperpolarizing the cell with steady-state current injection prevented the cell from reaching threshold for spike initiation, revealing an EPSP.

Figure 18. Spontaneous, hyperpolarizing IPSPs were occasionally encountered. In this recording, a depolarizing slow potential with a burst of spikes is visible at the start of the trace, followed by rhythmic, hyperpolarizing IPSPs.

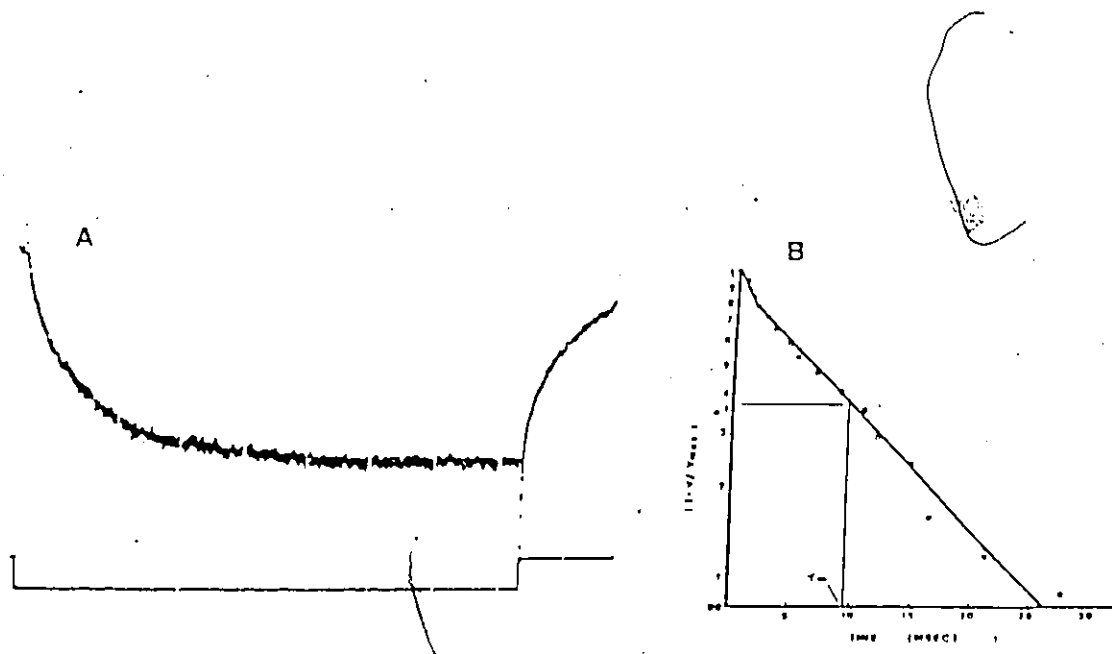


Figure 19. Calculation of membrane time constant, T_m .

A. A 60 msec, hyperpolarizing current pulse (current monitor, lower trace) was applied and the resulting voltage deflection was photographed. The charging of the membrane with respect to time was calculated as the fraction of maximum voltage change (expressed as $1 - V/V_{max}$) and plotted against time on semilog. paper (B). Interpolating $1/e$ yields a T_m of 9.6 msec for this neuron.

Figure 20. Extracellular recording of spontaneous, rhythmic activity in pyramidal cells. Two forms of rhythmic bursting are shown: in a) small bursts of spikes, typically 5-20 spikes/burst fire with a frequency of approximately 1-4 Hz. A second, less frequent form of 'giant' bursting is shown in b): large bursts of several hundred spikes/burst occurred at very regular intervals (eg. 20 sec burst cycle).

Figure 21. Spontaneous, intracellularly recorded action potential. The spike is preceded by a 3-5 mv hump, and shows a fast rising phase, repolarizing phase and after-hyperpolarization typical of most neurons. Arrow marks the inflection point at threshold voltage for spike initiation.

FIGURE 20.

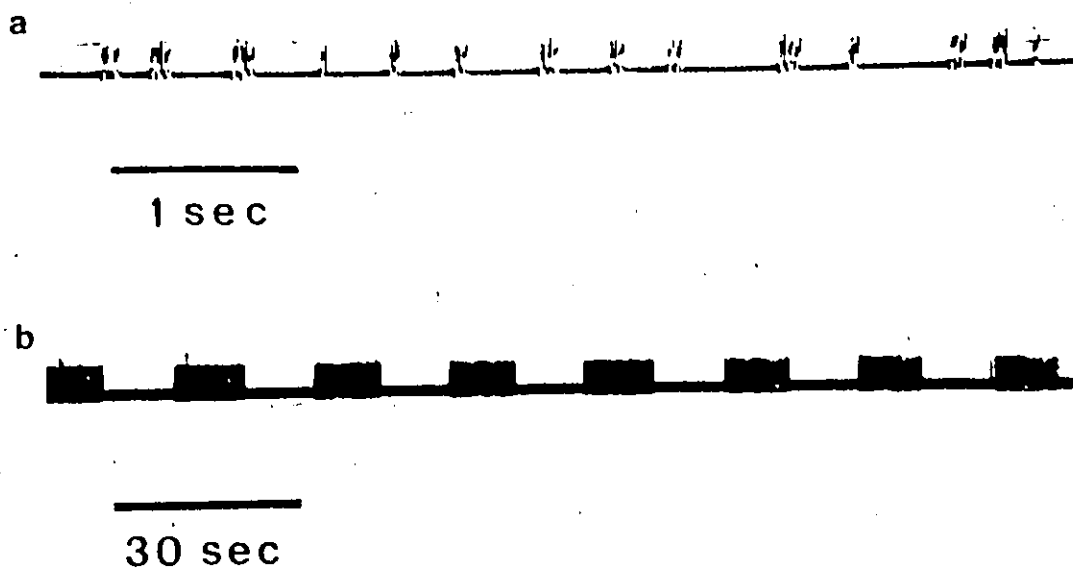


FIGURE 21.

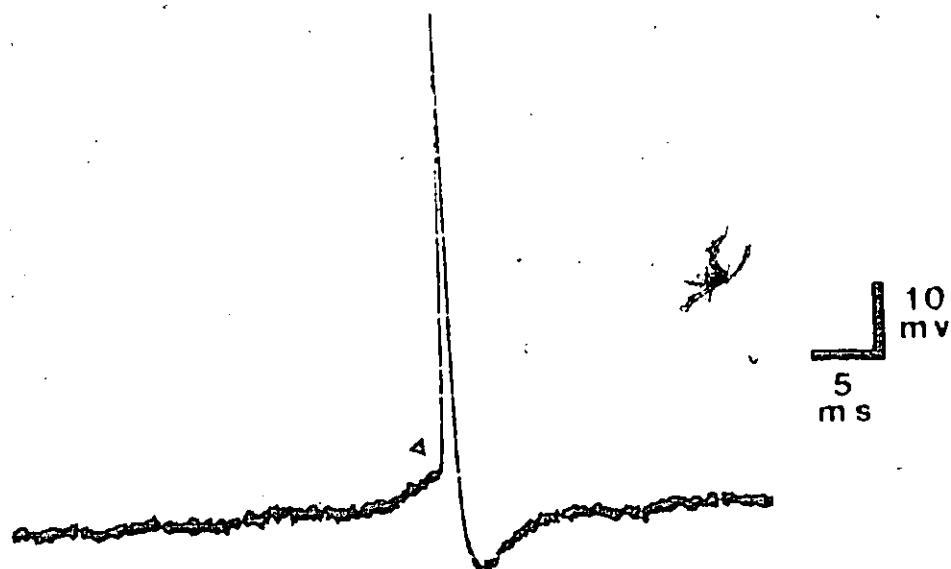
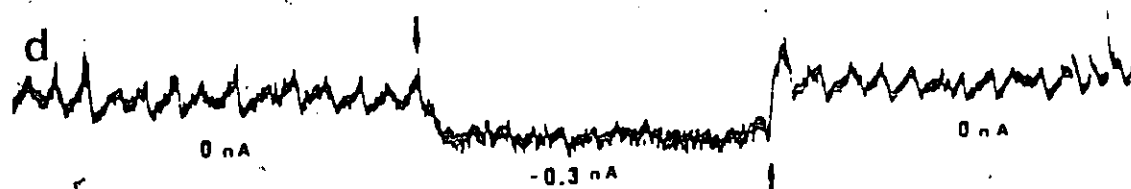
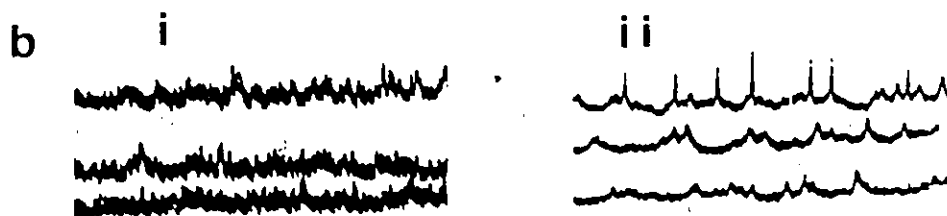
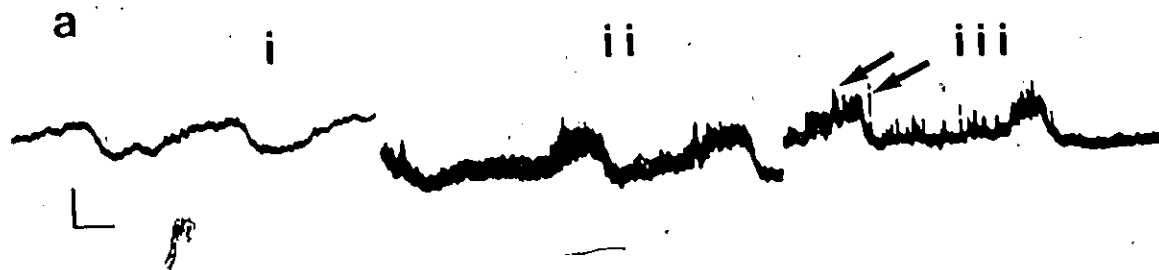


Figure 22. Spontaneous slow and fast pre-potentials.

a.i-iii. Oscilloscope traces at slow speed from 3 cells with large SPs at resting membrane potential. The rhythm typically consisted of a slow rising phase followed by a steeper repolarization. Fast pre-potentials (arrows) frequently were superimposed on the depolarized portion of the SPs. b). Two weakly rhythmic cells; depolarizing V_m between resting and rheobase levels increases the frequency and amplitude of SPs. c). A non-rhythmic cell transformed into a rhythmic cell when V_m was depolarized to rheobase. d). A strongly rhythmic cell becomes nearly quiescent when hyperpolarized by negative D.C. injection. e). FPPs recorded spontaneously from the cell in all at different points along the SP. As V_m depolarizes, the FPPs increase in frequency and amplitude.

Bar markers=	(ai,aiii)	1 sec,	5 mv
	(aii)	0.5 sec,	5 mv
	(bi)	0.5 sec,	10 mv
	(bii)	0.5 sec,	5 mv
	(c)	0.5 sec,	5 mv
	(d)	0.5 sec,	3 mv
	(e)	5 msec,	2 mv

FIGURE 22.



e.

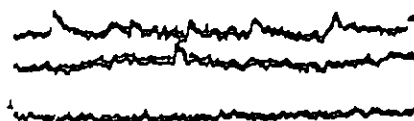
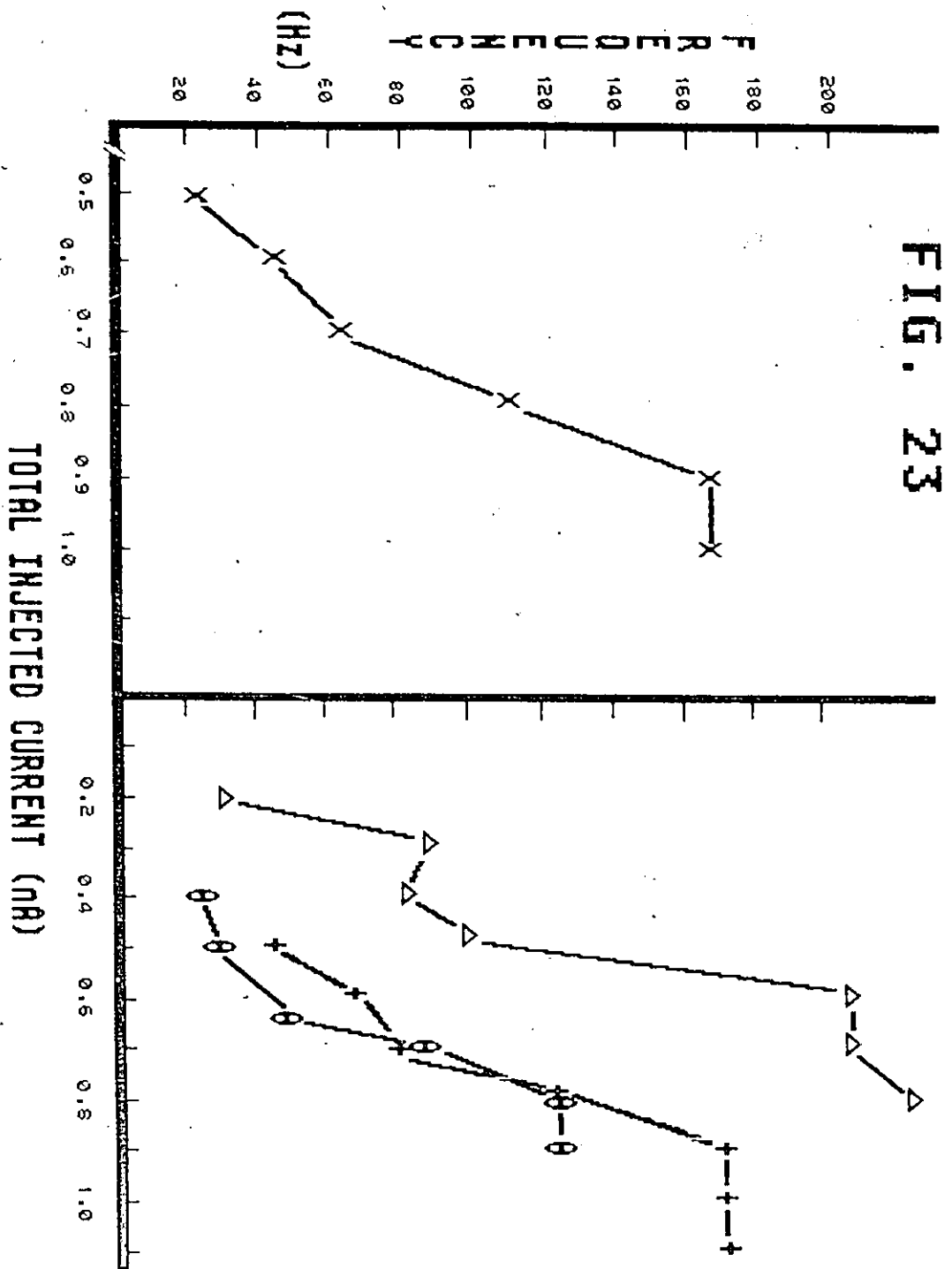


Figure 23. Spike frequency/ current stimulus curves. The spike frequency, calculated from the first interspike interval was plotted against the magnitude of 80 msec, depolarizing current pulses. Since the measurements were made while the cells were current-clamped at various V_m s, the abscissa was calculated as total current by adding the stimulus pulse to the steady-state current values.

The left panel shows an exemplary neuron's response: at low stimulus intensities, spike frequency increases gradually. The slope of the F/I curve is maximal at intermediate stimulus intensity, and reaches a plateau at higher levels.

The right panel shows F/I curves for 3 other neurons to illustrate the range in electroresponsiveness.



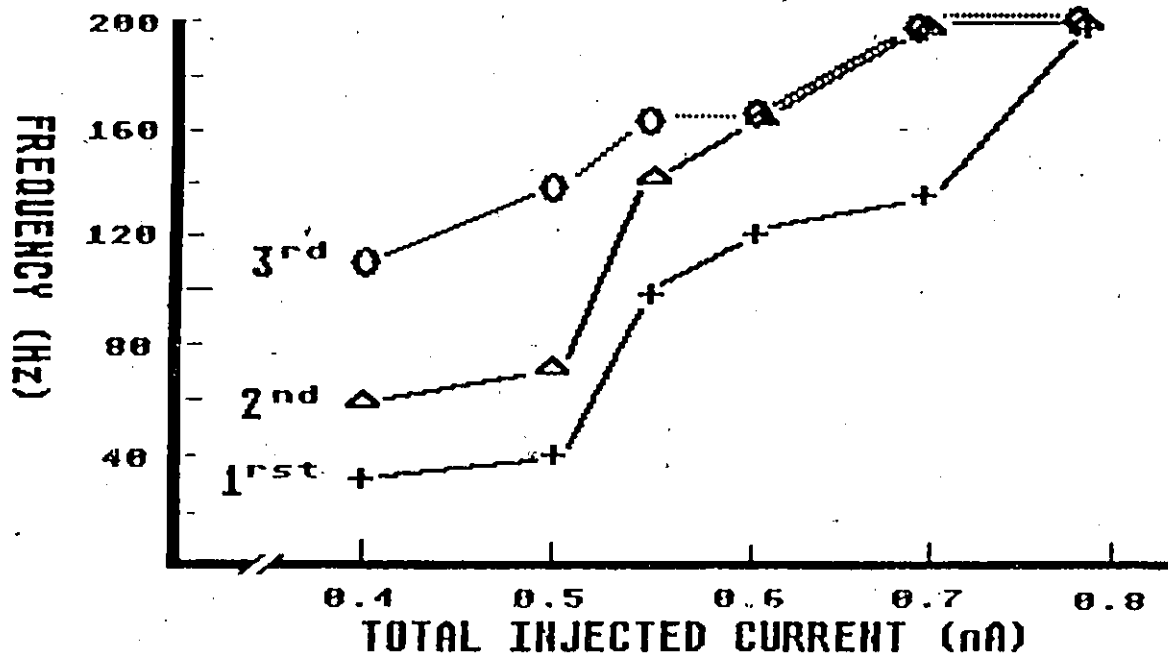


Figure 24. F/I curves from a neuron calculated from the 1st, 2nd, and 3rd interspike intervals. The sigmoidal shape of spike frequency increases is obvious in all 3 curves. The first interval, representing 'instantaneous' frequency values was consistently lower than both 2nd (intermediate) or 3rd (steady-state) interspike interval values.

Figure 25. Repetitive firing characteristics of pyramidal cells in vivo and in vitro.

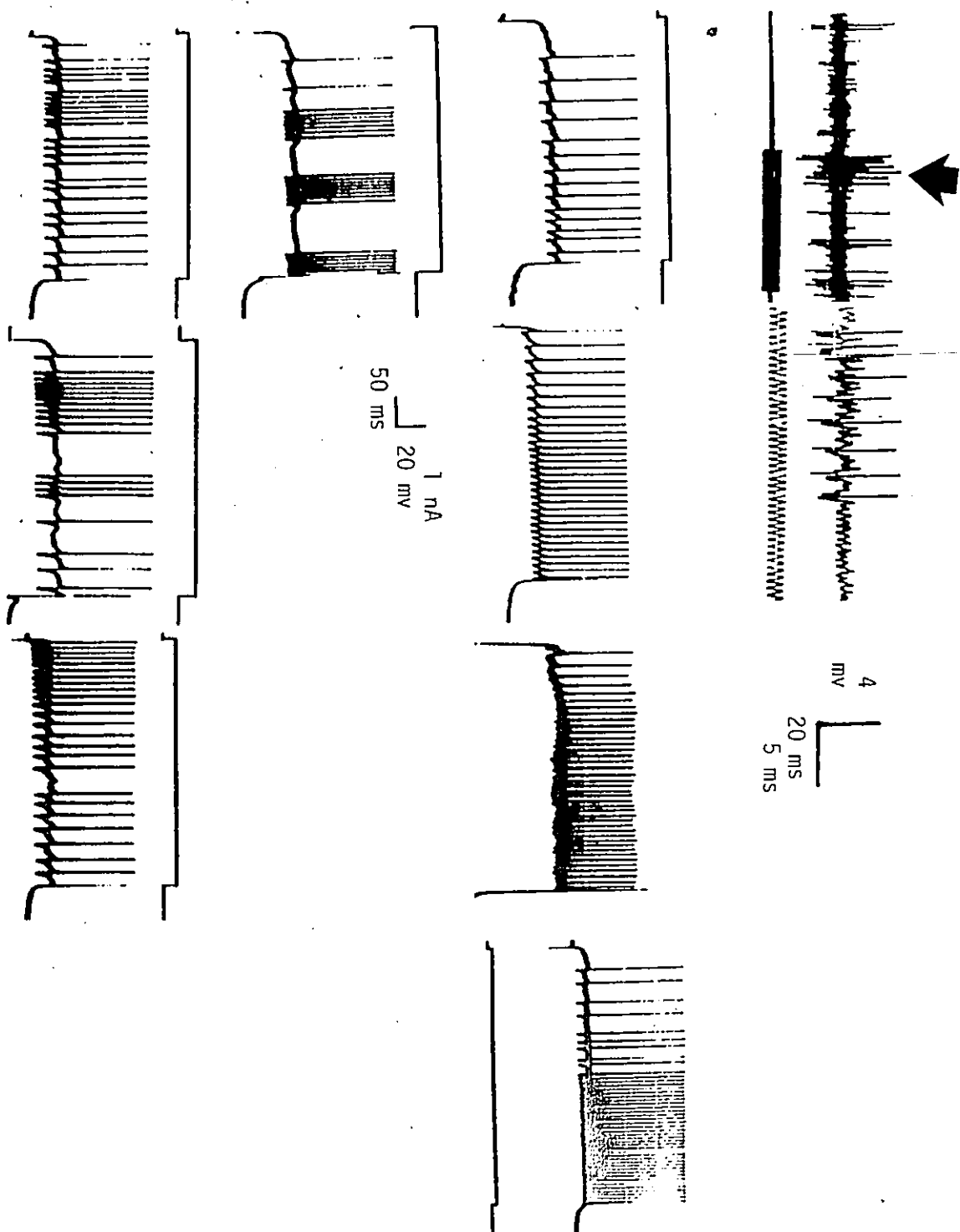
TOP ROW: Cells recorded in vivo (compliments of J. Bastian). While a fish was presented with a simulated EOD modulation (lower trace), pyramidal cell response was measured extracellularly (upper trace). Left panel: at the onset of increased electrical stimulation, the cell responds with an abrupt burst of spikes (600 Hz maximum, arrow) which rapidly adapts. Right panel: expanded view of initial burst response. Marker= 20msec (left panel); 5msec (right panel).

SECOND ROW: Pyramidal cells recorded in vitro stimulated directly by intracellular current injection. The range of stimulus responses are shown from 4 different cells. The cells responded to the stimulation with a slowly developing train of spike potentials (250 Hz maximum) which displayed no evidence of spike frequency accommodation. The 4 responses illustrate the range in magnitude of the 'lag' in spike frequency. Markers= 50 msec, 20mv.

THIRD ROW: Another non-accommodating cell which responded with a lag in spike firing followed by repetitive bursts of spikes.

BOTTOM ROW: Accommodating cells' response. Approximately 1/3 of all cells tested responded with varying degrees of spike frequency accommodation. Marker= 50msec, 20mv.

FIGURE 25.



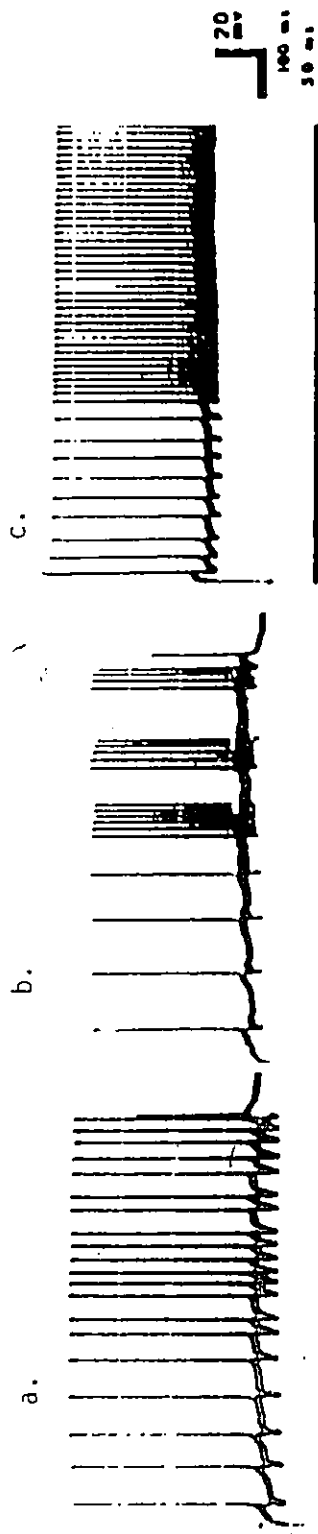


Figure 26. Spike frequency 'lag'.

The stimulus-response of 3 different cells demonstrates the range in repetitive firing types. Each of these cells responded to depolarizing current pulses with a lag in spike firing which kept the spike frequency low during the initial 50-250 msec of stimulation. In the extreme case shown at the right, the prominent lag abruptly ends after 200 msec allowing the cell to fire a high frequency spike train.

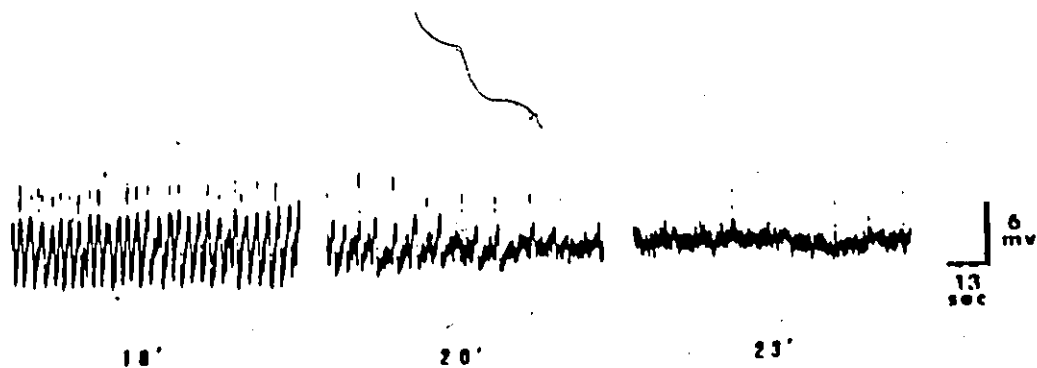


Figure 27. TTX block of Slow Potentials.

The strip chart record shows at slow speed a strongly rhythmic cell with regular, 0.4 Hz SPs. TTX ($0.2\mu\text{M}$) added to the bath abolished the voltage oscillations, demonstrating a Na-dependent component of the rhythm. During the early stages of the TTX block, the slope of the depolarizing ramp of the SP gradually diminished until the SP was eliminated.

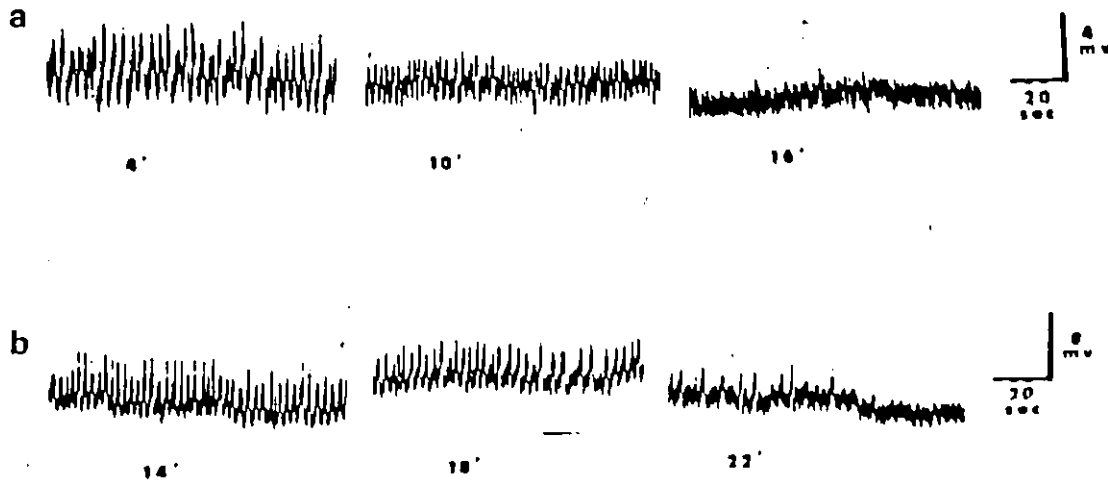


Figure 28. Sodium-sensitivity of spontaneous slow potentials.

Spontaneously rhythmic neurons were treated with 1.4mM lidocaine (a) or sodium-free ACSF (b) to test the effect of blocking Na influx on the propagation of membrane oscillations. Both treatments reduced the amplitude of SPs, finally abolishing all rhythmic activity.

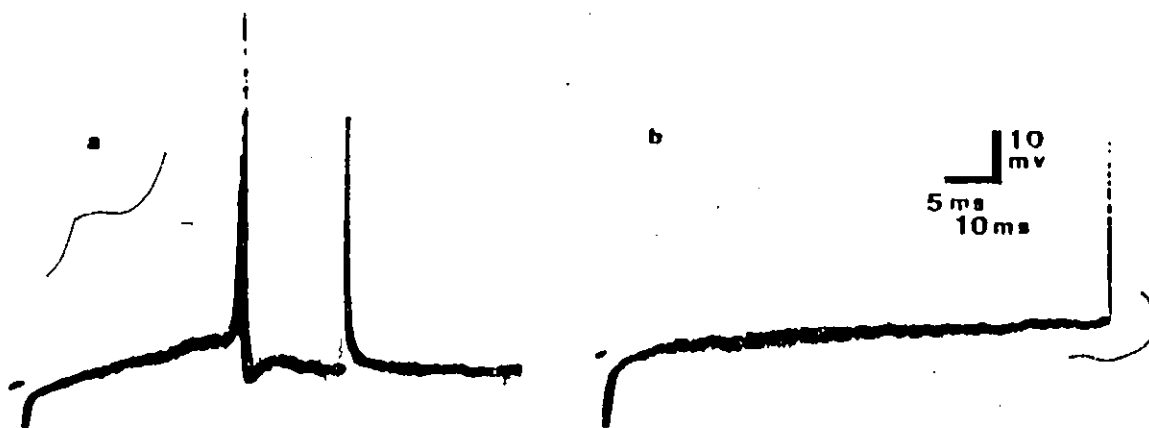


Figure 29. TTX sensitivity of $g_{Na(P)}$.

When pyramidal cells were given brief, depolarizing current pulses, they responded with a subthreshold depolarizing ramp in V_m . As the ramp traversed the voltage threshold for spike initiation, a single spike or burst of spikes resulted (a). The depolarizing ramp was abolished by TTX-treatment (b), providing additional evidence for the presence of a persistent, subthreshold sodium conductance.

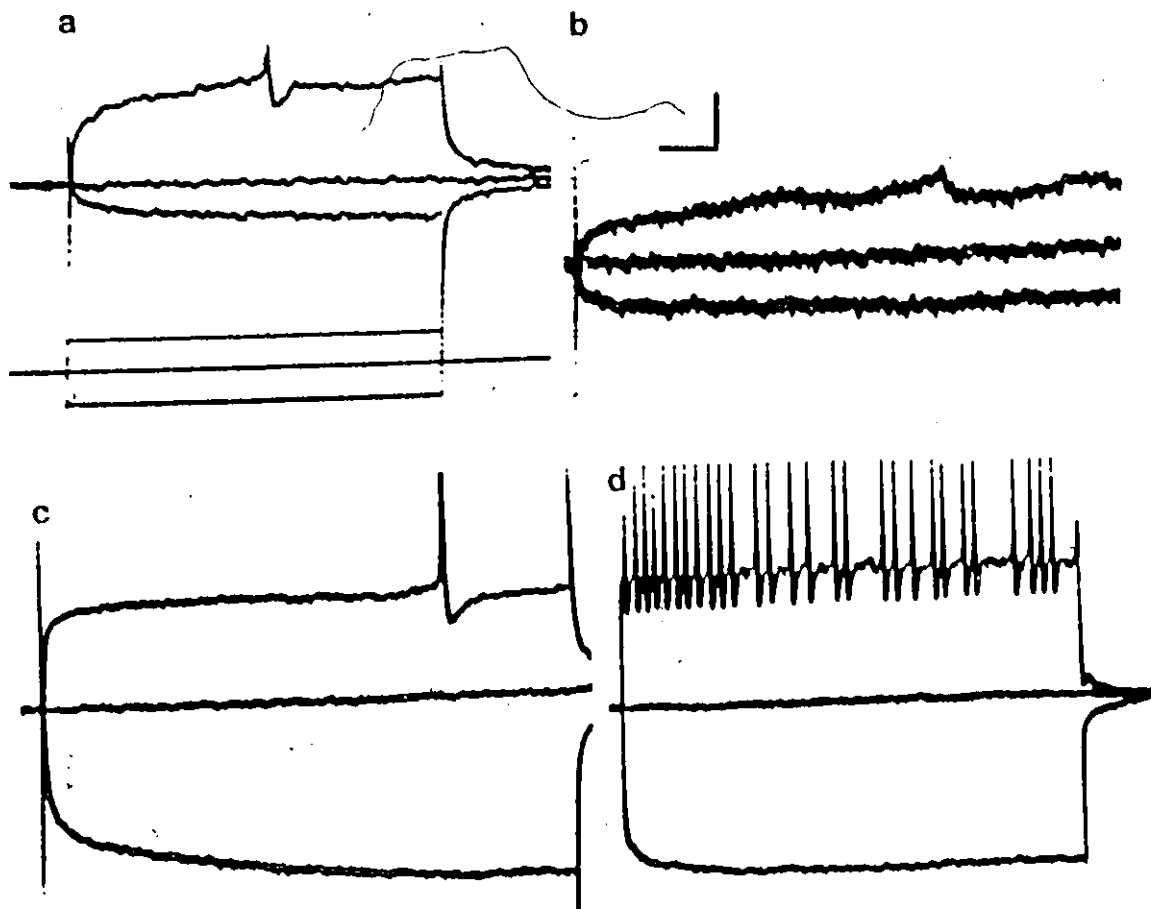


Figure 30. Rectification patterns in 4 different pyramidal cells. Each cell was stimulated with equal-amplitude, positive and negative current pulses as indicated in the lower current monitor traces in a). The resulting voltage deflections indicate non-linear I/V relationships. The cells in a) and b) display an apparent anomalous rectification; their depolarizing response is larger than the hyperpolarizing response. The cells in c) and d) show rectification in the negative direction.

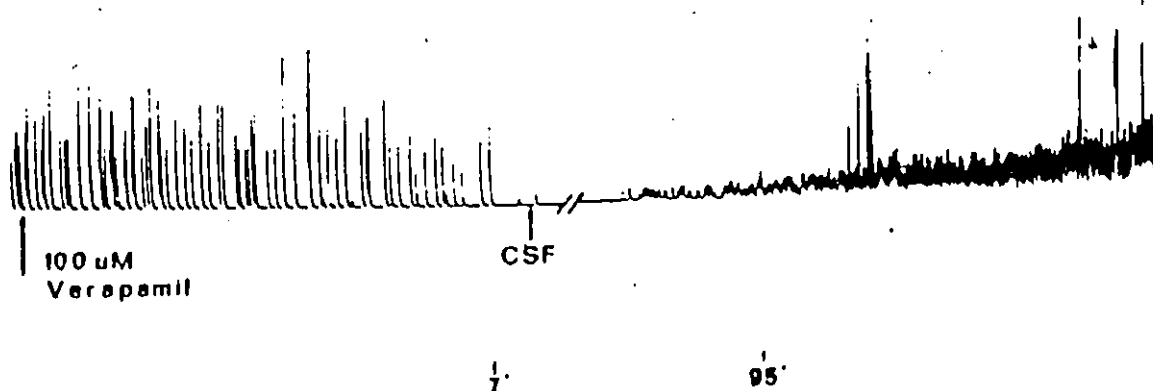
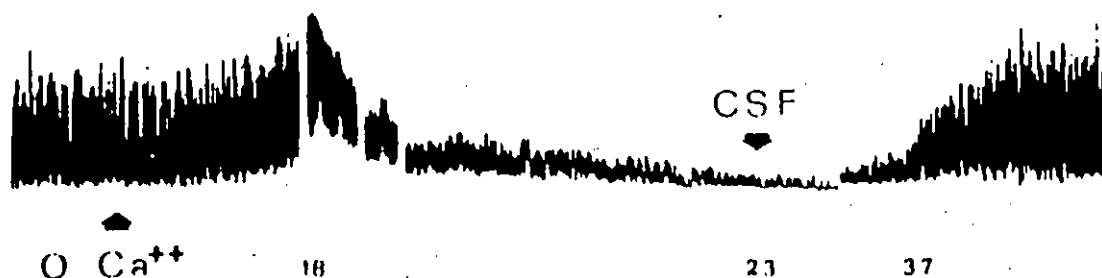


Figure 31. Effect of blocking calcium influx on spontaneous, extracellular burst-firing.

Top trace: A cell firing spontaneous, bursts of spikes was recorded on a rate meter before and after removal of calcium from the ACSF solution. At T=18 min, the cell passed through a brief period of increased excitability as the Ca was washed from the tissue, followed by a gradual decrease in burst firing. Return of normal ACSF at T=23 min restored the spontaneous bursting activity.

Bottom trace: A different cell was treated with 100 μM verapamil to block Ca-channels. The drug treatment caused a reversible loss of spontaneous burst firing.

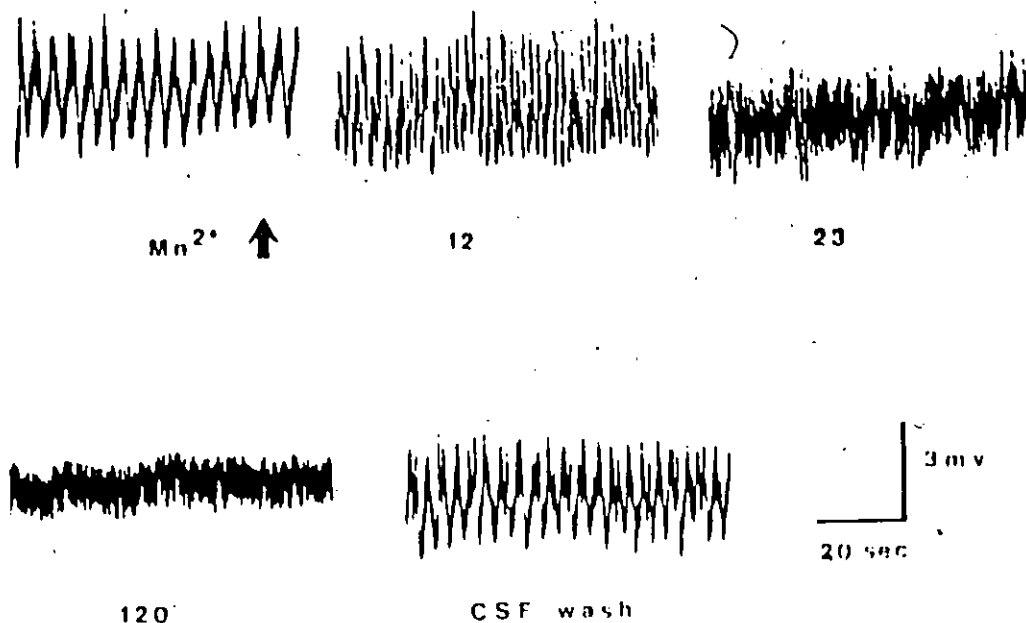
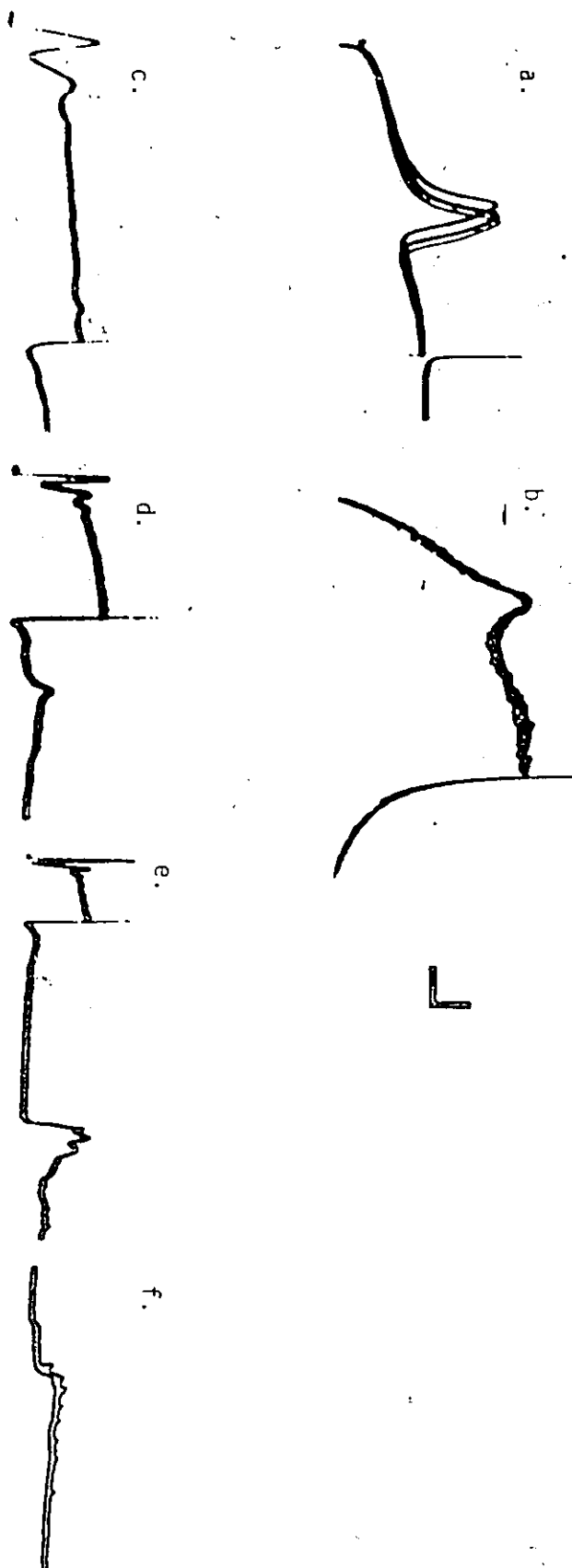


Figure 32. Effect of manganese block of calcium influx on spontaneous slow potentials.

A stip chart recording of a strongly rhythmic cell before and after addition of 2mM Mn^{2+} / 0mM Ca^{2+} to the bath solution. During the block of Ca-channels, the interburst interval gradually decreased (eg at 12' and 23') until the rhythm had degraded into very rapid, small amplitude oscillations in membrane potential (eg 120'). Washout with normal ACSF completely restored the pre-manganese rhythm..

Figure 33. Ca^{2+} -spikes. Slices were perfused with $0.2 \mu\text{M}$ TTX to block Na-channels, 2mM 4-AP to block IA, and $10\text{--}15\text{mM}$ TEA to block remaining K-channels. In the TOP ROW, two types of Ca-spikes are illustrated when these cells were stimulated with current pulses of varying amplitude. (a): low threshold Ca-spikes. Several spikes are superimposed to show their characteristic, broad shape. Stimulus current = 0.7nA , marker = 10msec , 20mv . (b): high threshold Ca-spikes superimposed illustrate an early spike followed by a broad, slow ramp occasionally outlasting the duration of the stimulus current. Stimulus amplitude = 6nA , marker = 5msec , 10mv . (c): In a different experiment (starting at the left) TTX, 4-AP treated cell shows low and high threshold Ca-spikes (Stim. amplitude = 1.6nA). (d) is at slower sweep speed to show the late Ca-spike outlasting the stimulus. (e): TEA (10mM) was added to the bath, which unmasked more of the Ca-current, especially the late components. (f): Ba^{2+} was substituted for calcium, resulting in the appearance of spontaneous Ca-potentials resembling the late, stimulus-evoked Ca-spikes. Markers = 20msec , 10mv (1st panel); 10msec , 10mv (2nd); 50msec , 10mv (3rd); 500 msec , 20mv (4th panel).

FIGURE 33.



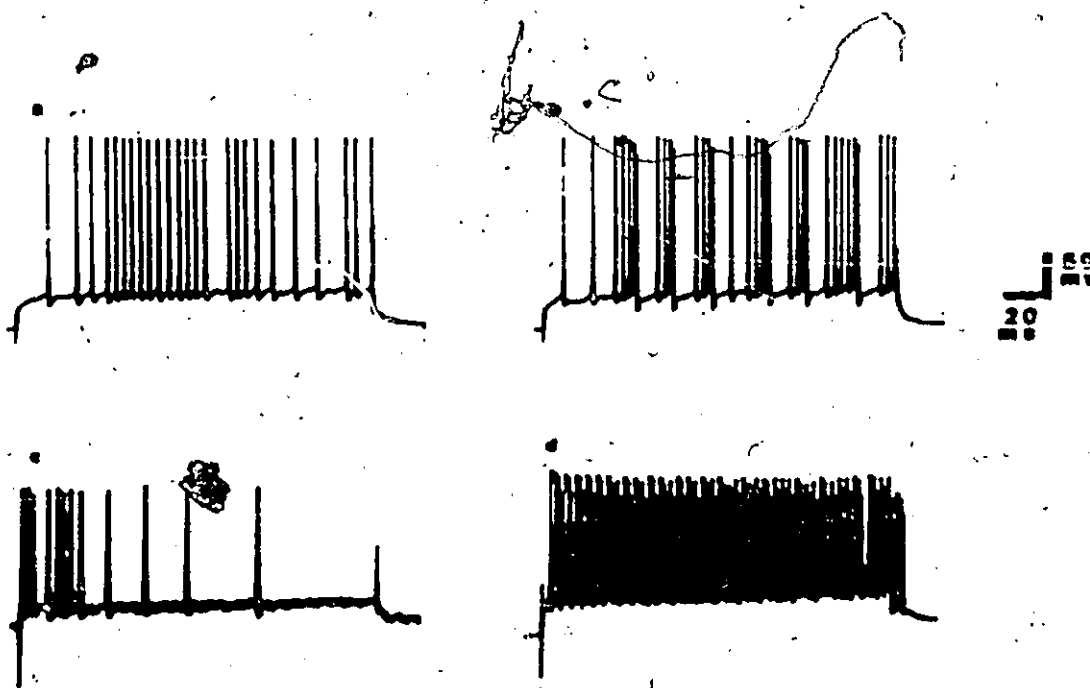


Figure 34. Effect of EGTA on accommodation. Spike frequency accommodation was recorded in cells before (left column) and after (right column) intracellular injection of K^+ EGTA. The micropipette was filled with 0.2M EGTA and K acetate and injected into the soma by delivering 1 Hz, -2nA current pulses for up to 1 Hr. Two experiments are depicted in the top and bottom rows, respectively. In both cases accommodation, which was evident in the pre-injection photographs (a and c) was abolished within 10 min of EGTA injection (b and d). In each experiment V_m was manually clamped to a constant value and the stimulation amplitude was kept constant. While EGTA eliminated accommodation, it had no effect on the 'lag' in spike frequency.

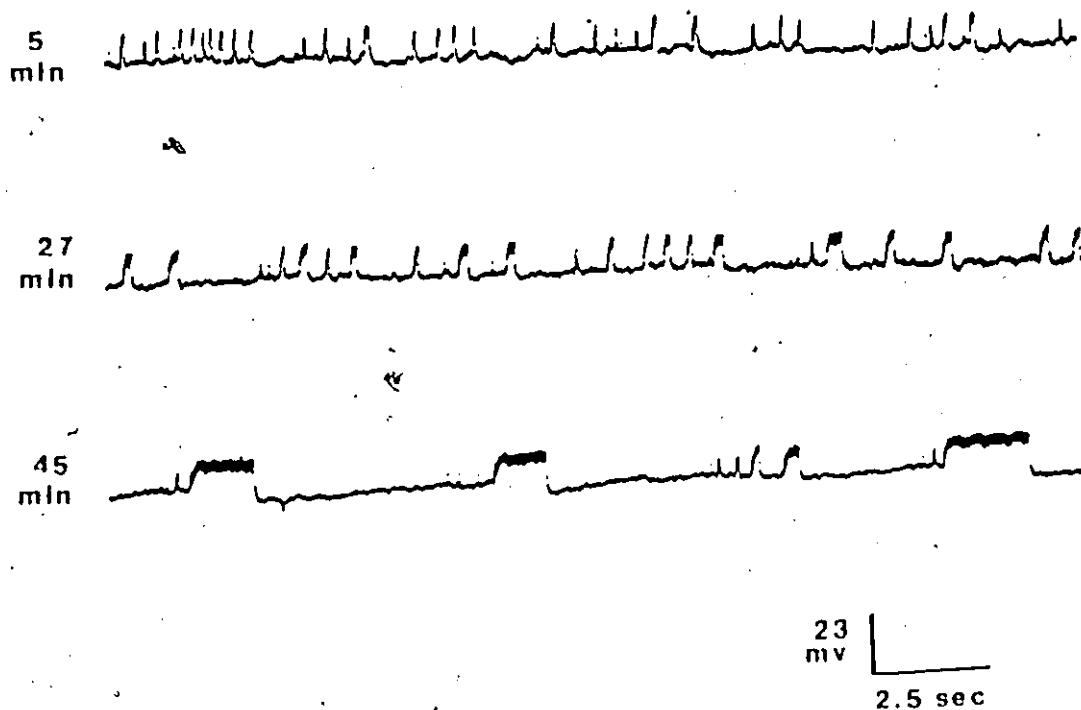


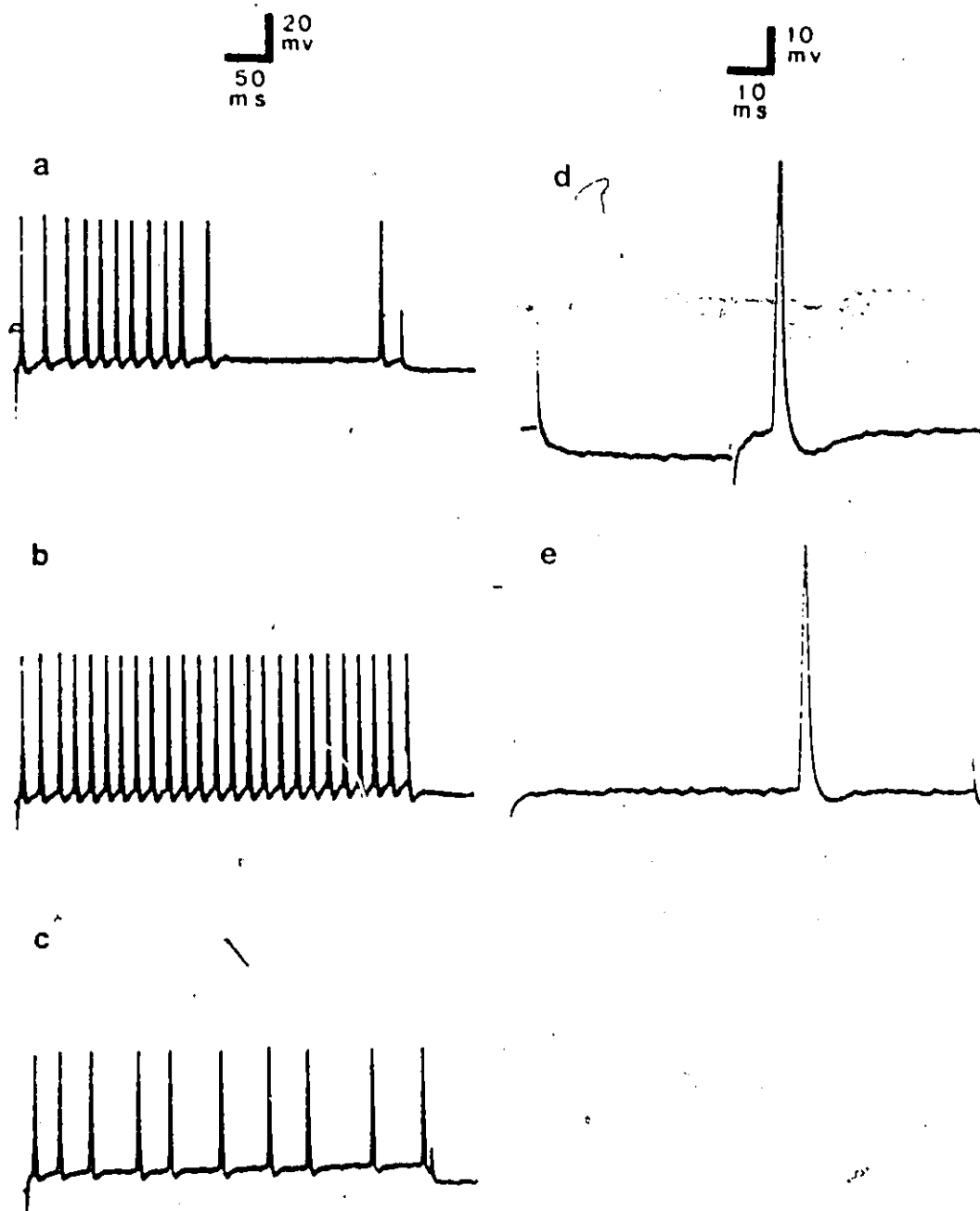
Figure 35. Effect of EGTA on spontaneous rhythmic activity. The strip chart record shows a bursting cell at various times during the injection of EGTA to chelate free intracellular calcium. At T=5 min EGTA injection, rapid bursts (~5 Hz) of 3-10 spikes/burst began to increase in duration and interburst interval. The pattern developed until by T=45 min the rhythm consisted of large bursts lasting 1-2 sec with very long interburst intervals. Note that at T=45 min the bursts terminated abruptly and were followed by a prominent, slow-AHP.

Figure 36. Effect of apamin on spike frequency accommodation and AHP amplitude.

A cell which displays accommodation (a) was superfused with 400nM apamin. 10 min after bath application of the drug, accommodation was eliminated (b). Washout of the drug with normal ACSF partially restored the accommodating response (c).

Apamin also affected the duration of the AHP. In d), an untreated cell has a typical 8-10 mv AHP following an anode-break evoked spike potential. Application of 400nM apamin reduced the AHP amplitude to approximately 50% of the pre-drug value (e).

FIGURE 36.



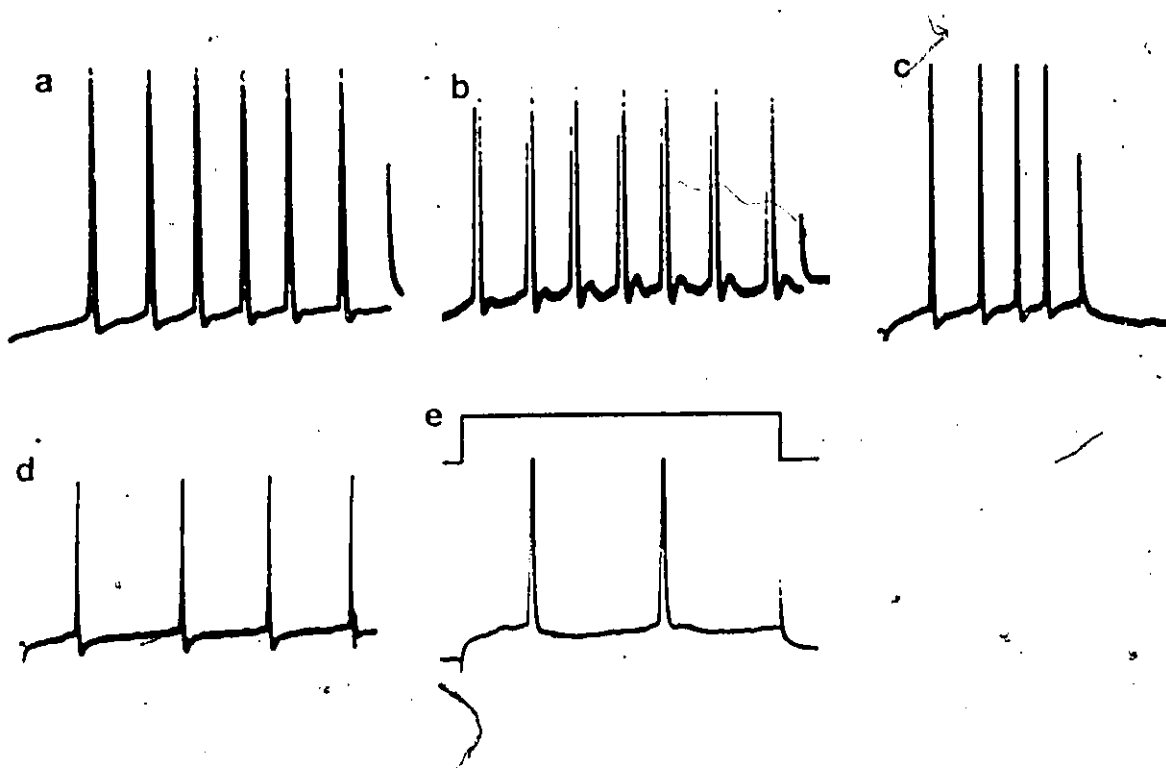


Figure 37. Effect of Cs^+ injection and 4-AP treatment on electroresponsiveness. a. Stimulus-response following $+0.4\text{nA}$ stimulus pulse, after 30 min injection of Cs^+ (1 Hz, $+0.4\text{nA}$ depolarizing pulses) AHP amplitude is reduced from pre-injection levels of 8 mv to approximately 1-2 mv. The remaining AHP corresponds to an early component of spike repolarization not blocked by Cs^+ -injection. b). After 40' Cs^+ , spike duration increased and a depolarizing afterpotential (DAP) appeared in the interspike interval. c). DAP was abolished by addition of $0.2\mu\text{M}$ TTX. d). Normal stimulus response. e). Response following 4-AP treatment; early AHP is blocked, leaving a slow, later component of AHP intact. Note increase in spike duration.

Figure 38. Effect of I_A -like current on stimulus-response 'lag'.

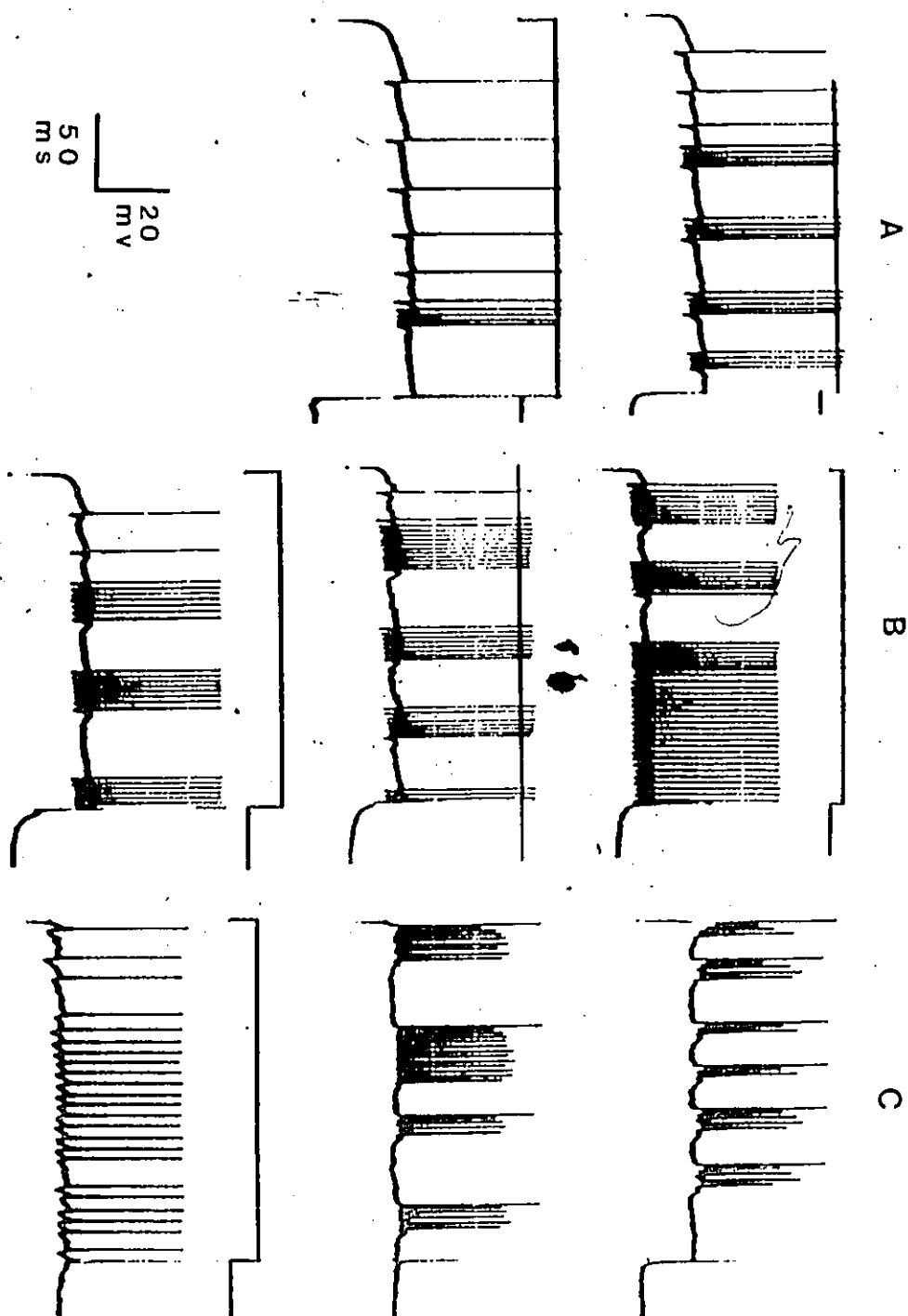
A. The cell was stimulated with depolarizing current pulses (400msec duration) at depolarized V_m to inactivate I_A , and hyperpolarized V_m to remove I_A inactivation. In lower panel, V_m was hyperpolarized by steady-state current of -0.2nA ; a prominent lag in spike firing is evident. In top panel V_m is depolarized by $+0.3\text{nA}$ D.C. injection; the stimulus pulse (adjusted to give the same total current injected) evoked a much smaller lag.

B. Another cell stimulated as in A; during hyperpolarization (lower panel, -0.3nA D.C.), at rest (middle panel), and depolarization (top panel, $+0.4\text{nA}$ D.C.).

C. Lower panel: during -0.3nA D.C. hyperpolarization, the stimulus evokes a spike train with a moderate lag. Middle panel: at the same V_m and stimulus pulse, 58 min after the addition of 1mM 4-AP; lag is greatly reduced and spike frequency increased.

Top panel: 80 min after 4-AP addition; lag is eliminated.

FIGURE 38.



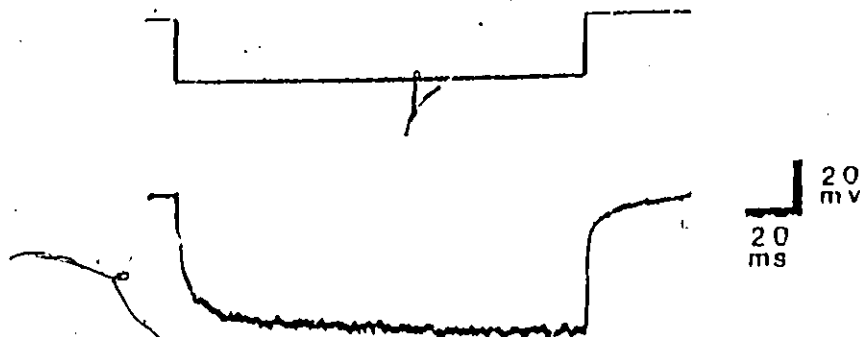


Figure 39. Pyramidal cells were tested for the presence of an M-current. Atropine (1mM) was added to the bath to block the action of endogenous Ach and the cell was depolarized with steady current injection to activate M-channels, if present. The cell was given brief hyperpolarizing pulses (current monitor, upper trace) that would inactivate M-current. No indication of M-current relaxation was evident in the form of either a 'sag' in the hyperpolarizing voltage deflection, or a 'hump' following the termination of the current pulse.

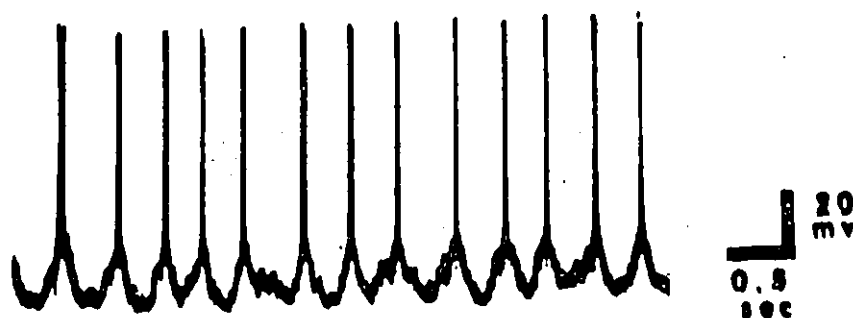


Figure 40. 4-AP induced membrane oscillations are illustrated at fast and slow sweep speed of the oscilloscope.

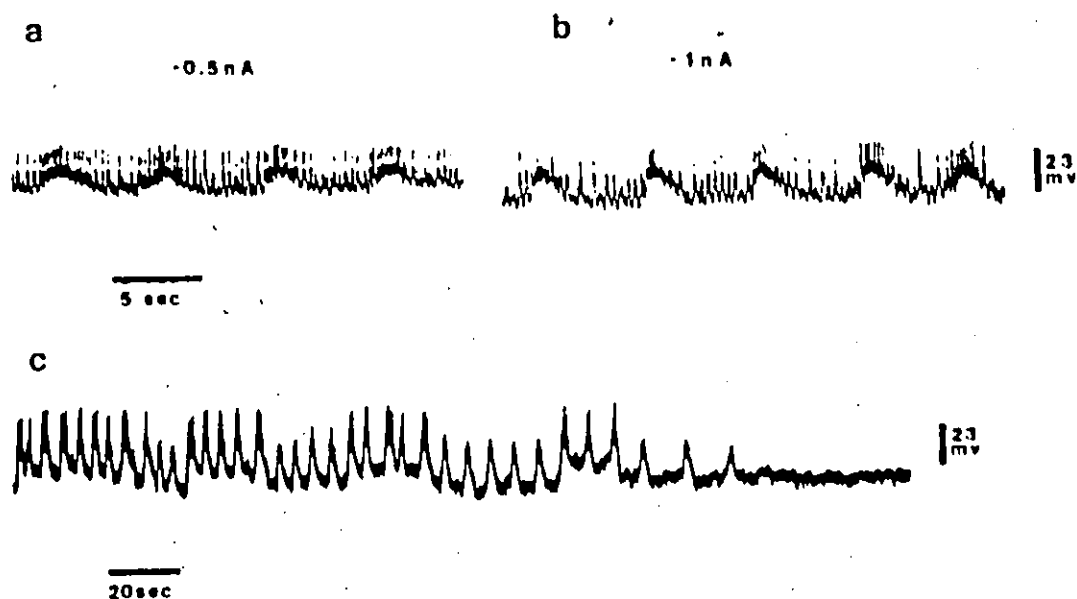


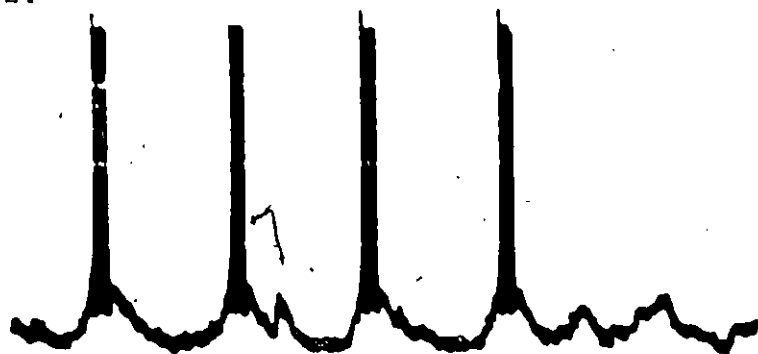
Figure 41. Voltage and calcium dependency of 4-AP induced waves.

The strip chart recordings in a) and b) illustrate the voltage-sensitivity of the waves. Two forms of oscillation are visible: slow waves (0.2 Hz) with higher frequency waves (2 Hz) superimposed on them. Hyperpolarization of the V_m increased the magnitude of both slow and fast wave forms. In trace c) a different 4-AP treated cell was superfused with 2mM manganese to block calcium influx. Both slow and fast wave forms were abolished by the block in Ca-channels

Figure 42. TTX block of 4-AP induced bursting.

Bath application of 1mM 4-AP induced the formation of large membrane oscillations and burst firing (top trace). This form of rhythmicity was blocked by addition of 0.2 μ M TTX; 16 min after drug application, the interburst interval is considerably lengthened (middle trace), and the rhythm is eliminated at T=22 min (bottom trace).

FIGURE 42.



20
mv
200
ms