1985

Cutaneous and Supraspinal Control of the Axial Muscles in the Rat: Implications for Behavior

Mark Steven Cohen

Cutaneous and Supraspinal Control of the Axial Muscles in the Rat: Implications for Behavior

A thesis submitted to the faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Mark Steven Cohen

April 1985
The Rockefeller University
New York, NY

Table of Contents

Abstract

The axial muscles of the rat are active in a wide variety of behaviors; of special interest is their involvement in sexual behavior. In this research their responses to cutaneous inputs and supraspinal influences were studied.

The pudendal nerve (PN), which innervates the skin regions contacted by males during sexual behavior, was stimulated electrically and the responses in the dorsal roots, the lateral longissimus muscle (LL) and its muscle nerves (MN's) were recorded. Conduction velocities in the PN were 54 m/s for the largest A-β fibers and averaged 10 m/s for A-δ fibers. Stimulation (stim) of the PN afferents at currents below A-δ threshold potentiated LL motoneuron firing and evoked activity in otherwise silent nerves. The first responses in females occured 8.8 ms after the last shock in a 3 shock stim; in males the onset was 11.4 ms. MN firing peaks occurred at latencies of 14, 24 and 105 ms in both sexes; the105 ms peak was larger in males. Firing was usually depressed from 37 to 53 ms. Comparable responses were seen with ipsi- or contralateral PN stim; these were facilitated by bilateral stim. In EMG recordings, unit activity was consistent with that seen in the MN's.

After total spinal cord transections in the thoracic region, the PN-evoked response in the LL nerves (PNER) consisted of a single activity peak at 22.8 ms: the earliest responses are at least partly segmental. To help identify supraspinal inputs to the PNER various partial transections of the spinal cord were made. When the ventrolateral (VL) columns were spared the PNER was like that in intact animals. If the VL columns were cut the PNER was like that in fully transected rats.

Bilateral convergence of PN inputs was seen in the totally transected animals; thus, such convergence occurs segmentally. The lesions which substantially altered the PNER were similar to those which eliminate lordosis in behaving rats.

Electrical stim within the medullary reticular formation evoked activity in the LL MN's at latencies of 2.6 to as much as 70 ms from the effective shock in a stim train. Responses were evoked by contra- as well as ipsilateral stim. Combined stim of the brainstem and PN's demonstrated facilitatory effects upon the excitability of LL MN's and evoked responses not seen with stim to either site alone; the response latency was shorter than that to either stim applied alone.

Chapter 1

Supraspinal Control of the Lumbar Axial Muscles and Their Involvement in Behavior

The muscles of the lower back, specifically the lumbar axial muscles are active in an enormous variety of animal behaviors, including running (Muybridge 1887), standing (Slijper 1946), burrowing and exploratory behaviors (Schwartz-Giblin, Femano and Pfaff 1984) and vestibular-induced postural adjustment (Suzuki and Cohen 1964), yet, as compared to the limb muscles, for example, their control has not been studied widely.

Anatomy of the Lumbar Axial Muscles

Three major epaxial muscle groups, present in reptiles, birds and mammals, were distinguished by Vallois (1922). These included **i**) the transversospinalis system which is a group of vertebral muscles located between the spinous and articular processes of the vertebrae, **ii**) the ilio-costalis system, a group of muscles located more laterally, overlying the proximal portions of the ribs and **iii**) the longissimus system. The latter is well-developed in the rat (Brink 1978, Brink and Pfaff 1980a), taking origin from the iliac crest and from the lumbar and sacral vertebrae by way of the lumbosacral aponeurosis, which completely covers the transversospinalis musculature. Fibers of the longissimus muscles attach to the proximal portions of the last three or four ribs and to the diapophyses and lateral edges of the lumbar vertebrae. In the rat, the transversospinalis system is anatomically distinct, except at the level of the metapophyseal tendons, whereas the iliocostalis system is fused with one component of the lumbar longissimus system forming the lateral longissimus (Brink 1978, Brink and Pfaff 1980a); the lumbar longissimus sytem is present also as the medial longissimus and as a short fiber system. As it extends rostrally the fibers of the lateral longissimus become mingled with those of the longissimus dorsi (Brink and Pfaff 1980a). Distinct innervation exists for the transversospinalis, lateral longissimus and medial longissimus systems: the transversospinalis is innervated by medial branches of the

dorsal rami and the lateral longissimus by lateral branches. Innervation of medial longissimus derives from branches of the L6, S1 and S2 dorsal rami which merge, typically, to form two muscle nerves (Brink and Pfaff 1980a).

Unilateral stimulation of the lateral longissimus muscle group in the rat results in lateral flexion of the spine and bilateral activation of the muscle induces local dorsiflexion. Bilateral stimulation of the caudal portion of the lateral longissimus was seen also seen to produce rump elevation (Brink 1978, Brink and Pfaff 1980a).

Certain cutaneous reflexes of the axial muscles have been described, for example, the abdominal and erector spinae skin reflexes (Kugelberg and Hagbarth 1958). Carlson and Lindquist (1976) reported that stimulation of the nerves supplying the skin of the lumbar back elicits EMG activity in the longissimus dorsi and mutifidus spinae at minimal latencies of 6 ms in the spinal cat. Monosynaptic reflexes of the axial muscles in the cat were infrequently seen by Carlson (1978b) and such reflexes are weak in lumbar axial muscles of the rat (Brink & Pfaff 1981).

Central Control of the Axial Muscles: the Reticulospinal System

In the rat, the lateral longissimus muscle is innervated by the axons of motoneurons located in the lumbar and thoracic regions of the spinal cord (Brink, Morrell and Pfaff 1979). These neurons are located in the medial column of the ventral horn as in other animals (Elliott 1944).

The motoneurons of the axial muscles receive inputs from a variety of supraspinal centers. In the cat, monosynaptic excitation and inhibition of motoneurons of the longissimus dorsi were seen in response to electrical stimulation in the medial medullary brainstem, in the region of the MLF, as well as in sites in Deiter's nucleus (Wilson et al. 1970). Monosynaptic connections from the

pontomedullary reticular formation to the axial muscle nerves of longissimus dorsi, interspinales and spinalis dorsi were reported by Peterson et al. (1979). Peterson et al. also reported a regional organization of the reticular formation in the cat in which neurons projecting to the medial reticulospinal tract (RST) are found dorsorostrally in the pontine reticular formation and gigantocellular nucleus and the units projecting caudally via either the ipsilateral or contralateral RST are found more caudally and ventrally. This topographic organization is consistent with the anatomical results of Nyberg-Hansen (1966). In the rat, as studied by Zemlan et al. (1979), labelled cells were found throughout the medullary gigantocellular nucleus following HRP applications restricted to the ventrolateral columns of the spinal cord at low thoracic levels. Thus, in the rat, the topographic organization of the reticular formation would appear to be somewhat different. Reticulospinal neurons were reported in the rat by Fox (1970); monosynaptic connections from reticular formation units to lumbar motoneurons were demonstrated by Shapovolov and Gurevitch (1970), who unfortunately do not report clearly which muscles are activated by these motoneurons. In the rat, short-latency excitation can be seen in the lateral longissimus muscle (Femano et al. 1984a and b) and in the lateral longissimus muscle nerves (Brink & Pfaff 1981). In the latter case, minimal latencies of 3 ms were reported from the effective shock in a multiple shock stimulus train. As described by these authors, these responses are consistent with monosynaptic activation in the rat.

The reticulospinal system is described as a primarily *medial motor sytem* by Kuypers (1964): motor fibers originating from the reticular formation terminate primarily within the ventromedial columns of the ventral horn (a result noted also by Nyberg-Hansen 1965), which are associated with motor nuclei that project to the axial muscles and the muscles of the proximal limbs. Other medial systems described by Kuypers include the fibers of the vestibulospinal, tectospinal

and intersitiospinal tracts. Peterson and Abzug (1975) have demonstrated an extensive pattern of interconnection between the vestibular nuclei and medial reticular formation that may be involved in the coordination of these descending systems

The fibers of the RST branch extensively at various spinal levels; Peterson and his collaborators (1979) reported that 22/23 reticulospinal neurons projecting to the cervical spinal cord also sent branches to lumbar levels. As mentioned by Peterson (1979), the reticulospinal system is well situated to mediate those behaviors which rely preferentially on the proximal musculature. The system of branching of fibers of the RST also makes them ideal candidates for the control of behaviors which require the coordinated control of muscles at several segmental levels.

One of the most revealing ways in which to study the control of motor systems is in the context of well-defined behaviors. One extensively studied behavior involving the epaxial muscles is lordosis, the sexual posture of the female rat.

Lordosis Behavior

Lordosis, the mating posture of the female rat, is a necessary behavior for successful procreation. As described from film analyses by Pfaff and Lewis (1974) and by Pfaff (1980) the naturally-occuring mating sequence takes place as follows: Following a series of proceptive behaviors by the female including ear-wiggling and 'hopping and darting' the male approaches the female from the rear and makes contact with her flanks with his forepaws and often presses his head and upper body against her lower back. This is followed by his rapidly stroking her ventral flanks. He then grasps her and moves forward pressing his lower body against her tailbase area. After initial contact by the male, the female

extends both front and rear legs. She then begins to elevate her perineal region in a manner that facilitates genital contact by the male. It is after she has attained a highly stereotyped pose with her tailbase elevated, tail deflected, head raised and back dorsiflexed that the animal is said to have adopted the lordosis posture.

Somatosensory Control of Lordosis

The stimulation provided by the male rat can be mimicked crudely by the experimenter using manual stimulation. Sufficient stimulation to elicit lordosis in this case requires stroking of the anterolateral flanks followed by light pressure to the lower back and perineum (Pfaff et al. 1977). The latter stimulation method demonstrates that visual, olfactory, thermal, auditory, and gustatory stimulation are not required. Experiments have also been performed with rats in natural encounters, after the female has been blinded, deafened and rendered anosmic (Kow and Pfaff 1976); the male rat is still reliably able to elicit lordosis in these cases. Thus, the necessary components of the stimulus are purely somatosensory.

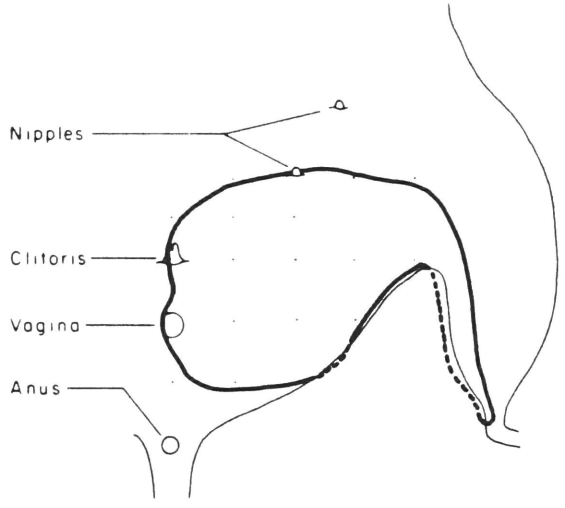
Stimulation with air puffs designed to activate only hair receptors is insufficient to evoke lordosis even in well primed animals. Likewise, cutaneous stimuli such as brushing, which can activate all hair unit types in the dorsal root ganglion never evoked lordosis (Kow and Pfaff 1979). Type I hair-skin units respond to brushing, and while they respond to pressure, their pressure threshold is lower than the minimal pressure required to initiate lordosis in the majority of rats (Kow et al. 1979), they therefore can not be sufficient to evoke lordosis. When the male presses against the female's perineum a relatively broad area of her skin is stimulated. Such pressure does not activate punctate skin deformation units (Kow and Pfaff 1979), therefore these units cannot be essential contributors to the somatosensory control of lordosis behavior. Non-cutaneous receptors, such as those from muscles and joints cannot be essential for eliciting

lordosis; non-mechanoreceptive units are also irrelevant (Pfaff 1980). Slowly increasing pressure on the perineal skin (Kow et al. 1979) however, can induce the behavior, thus, information transmitted via slowly-adapting pressure receptors, the only remaining receptor type excited by cutaneous stimulation of the perineum is required. This does not of course rule out the importance of the other receptor types which may be contibutory to the natural behavior. The slowly adapting pressure unit is probably the same as the type II unit described by Iggo and coworkers (1966) in the cat.

Tactile information from the perineal region is transmitted centrally via the pudendal nerves (Kow and Pfaff 1973) and it has been demonstrated that destruction of these nerves is followed by significant reduction in the lordosis performance of female rats in response to standard stimuli (Kow and Pfaff 1976). Recordings from the pudendal nerve have shown its receptive field (figure 1-1) to include much of the perineum, extending to the midline, laterally to the posteroventral flanks, caudally to about halfway between the anus and vagina and rostrally to the approximate level of the lowest nipple (Kow and Pfaff 1973). Interestingly, the receptive field size of the nerves is increased as much as 22% by the same hormonal pretreatments which condition the animals for lordosis behavior, further emphasizing its importance in the behavioral pathway.

Involvement of the Axial Muscles in Lordosis

The postural adjustments needed to perform lordosis have been described in detail following analysis of x-ray films taken during copulatory behavior (Pfaff et al. 1978); this method allows close examination of the changes in orientation of the skeleton and joints. Elevation of the rump area is accomplished primarily by dorsiflexion of the vertebral column, hindlimb extension and to some extent pelvic rotation. Several other postural adjustments are seen in lordosis, including forelimb extension and head elevation, the latter as a result of the spinal



Location of receptive field of the perineofemoral branch of the pudendal nerve in a representative estrogen-treated female rat. *Thick line:* the receptive field, determined by recording nerve responses during stimulation of the skin with a small camel hair brush. *Broken line:* the field extending slightly beyond the ventral surface.

from: Kow and Pfaff, Neuroendocrinology 13:299-313 (1973)

Figure 1-1

The cutaneous receptive field of the perineofemoral branch of the pudendal. The receptive field is outlined in heavy black lines and extends beyond the ventral surface of the hind limbs as indicated by the broken lines. (From Kow and Pfaff 1973).

dorsiflexion.

The functions of the various epaxial muscle groups, including lateral and medial longissimus and transversospinalis in lordosis behavior were defined in a series of ablation experiments by Brink (1978; also Brink and Pfaff 1980b). Destruction of medial longissimus caused little change in the quality of lordosis responses seen in the experimental animals. Complete removal of lateral longissimus or transversospinalis individually, however, while it left the animal capable of locomotion and unimpaired on balance beam tests, resulted in significant decreases in lordosis performance. Ablation of the latter two muscles in combination was highly effective in reducing lordosis performance scores and destruction of all three muscle groups caused large decreases in performance. From these data, Brink concluded that the most important muscles involved in lordosis are lateral longissimus and transversospinalis, but since removal of these muscles did not completely eliminate the rump elevation component of lordosis, significant contributions must be made by other musculature; likely candidates include the intermammilares, mammilosylodei and short fiber components of the sacral musculature.

Estrogenic Control of Lordosis

Lordosis behavior, is subject to strong control by steroid hormones (Beach 1948). There is a dose-dependent increase in the behavior with estrogen injections (Kow and Pfaff 1975a) and a reciprocal relationship between hormone dose and intensity of somatosensory stimulation (Kow, Montgomery and Pfaff 1979). Treatment with progesterone further potentiates the behavior and its combined effects with estrogen are most visible in the females which respond best to estrogen. (Pfaff 1970). While there exist a number of cells in the dorsal horn of the spinal cord which bind the steroid (Morrell et al. 1982), estrogen receptors are located predominately in the forebrain regions in the rat (Pfaff 1968) and many

other species (Morrell and Pfaff 1978). Specifically, the septum, amygdala, preoptic area, tuberal hypothalamic nuclei, pituitary and several mesencephalic subtectal nuclei bind estrogen with high affinity, not only in rat but in all species studied thus far, including several varieties of fish, lizards, amphibians, birds, rodents, primates and others.

Although the majority of the estrogen receptors are located in the hypothalamus and electrical stimulation of the ventromedial nucleus of the hypothalamus causes a facilitation of lordosis in response to either manual stimulation or male mounting (Sakuma & Pfaff 1979a) and lesions in that nucleus cause decreased lordotic responsiveness (Sakuma & Pfaff 1979b), it is evident from the lack of responsiveness by hypothalamic cells to cutaneous stimuli (Bueno and Pfaff 1976) that the primary pathways of lordosis do not require trans-hypothalamic transmission of sensory information on a lordosis-by-lordosis basis. Furthermore, the electrical stimulation effects in the VMN require at least 15 min, and usually about 1 hour, of stimulation to appear (Sakuma & Pfaff 1979a). Female rats with spinal transections, however, do not display lordosis when exposed to somatosensory stimuli which would otherwise be adequate to evoked the behavior in the intact animal (Kow, Montgomery and Pfaff 1977). It is thus of interest to understand the mechanism through which supraspinal influences are capable of exerting their control over distal spinal circuits and to examine the role of intrinsic spinal pathways in the pudendal nerve-evoked response.

Neurons projecting to the medullary reticular formation from the mesencephalic central gray have been characterized by Sakuma and Pfaff (1980a) and the excitability of such cells, as measured by the somatic invasion of antidromic spikes was shown to be estrogen dependent (Sakuma and Pfaff 1980b). Units in the central gray, in turn receive inputs from the VMN; although no differences in

latency were seen in estrogen-treated vs untreated females, antidromic invasion of cells in the VMN occurred after a mean latency of 13.3 ms in females and at 16.9 ms in males (Sakuma and Pfaff 1981). The threshold for stimulation within the central gray for antidromic invasion of the somata of such cells was lower in estrogen treated females than in untreated females (Sakuma and Pfaff 1981). A key question in developing an understanding of the role of estrogen in controlling behavior is to define the manner in which the hormonally-modulated influences of higher centers act upon spinal cord mechanisms controlling sensori-motor integration.

The experiments described in this thesis utilize our knowledge of the neural inputs and outputs of lordosis behavior to establish a stimulation and recording protocol which excites pathways that must be involved in lordosis behavior. Using this paradigm, responses in the muscles of the lower back were evoked successfully by stimulation of a cutaneous nerve supplying the perineal region. It is shown that these electrical responses are subject to supraspinal influences and that activity in the medullary reticular formation may influence the evoked responses. The use of the paradigm thus provides a novel perspective from which to study the neural control of the lumbar axial muscles. Many parallels exist between these results and results obtained from the study of lordosis behavior. There are also aspects of the electrical evoked responses which differ from behavioral data. These parallels and differences are noted in the discussion sections following the presentation of the experimental results.

Chapter 2

The Pudendal Nerve-Evoked Response In Axial Muscles

Summary

In Urethane-anesthetized rats recordings were made of the afferent volley in the dorsal roots and of the electrical activity of the lateral longissimus muscle and motor nerves during electrical stimulation of a cutaneous branch of the pudendal nerve. Male and female rats were used; the females were ovariectomized and either pretreated with estradiol or left without hormonal treatment. Conduction velocities in the pudendal nerve were 54 m/s for the largest A-β fibers and averaged 10 m/s for A-δ fibers. Excitation of pudendal nerve afferents at stimulus currents below threshold for A-δ fibers strongly potentiated the firing of axial motoneurons and evoked activity in nerves which were otherwise silent. Trains of three shocks to the pudendal nerve were considerably more effective than double or single shock trains. Repetition rates as low as 1/s had an excitatory effect on the lateral longissimus muscle. Recordings from the axons of the epaxial motoneurons of female rats showed a strong activation of neuronal firing with an onset latency of 8.8 ms from the first shock of a three ms, three shock train; the response onset in male rats, at 11.4 ms, differed significantly from that in the females. Peak spike activity occurred at mean latencies of 14, 24 and 105 ms in both sexes. A period of depressed firing was usually present from 37 to 53 ms. Males differed in having a larger peak in activity at 105 ms, but the overall profile of the responses was similar in males and females. Responses of comparable magnitude were seen with ipsilateral or contralateral pudendal nerve stimulation; these were facilitated by bilateral stimulation. In electromyographic (EMG) recordings, both unit and field potentials were seen in response to pudendal nerve stimulation. This unit activity was consistent with the firing pattern seen in the muscle nerves.

Introduction

The axial muscles are active in a wide variety of behaviors in the rat and other animal species. Their contribution to locomotion was recognized by Muybridge (1887) and has been analyzed in depth by Slijper (1946). EMG recordings made from the lateral longissimus (LL) and transversospinalis (TS) muscles in the rat (Schwartz-Giblin, Femano and Pfaff 1984) have demonstrated their activation during burrowing and exploration. The axial muscles also receive reflex activation in vestibular-induced postural movements (Suzuki and Cohen 1964). As first studied in the cat by Carlson and Lindquist (1976) and in the rat by Schwartz-Giblin, Halpern and Pfaff (1984) back muscle activity can be induced by electrical stimulation of the cutaneous nerves supplying the overlying skin. Among the best studied behaviors involving the muscles of the epaxial region is lordosis, the female rat sexual posture. To understand better the control of these muscles under behaviorally-relevant circumstances the stimulation and recording protocol used in these experiments was patterned after that occuring during lordosis, recognizing that although this cannot be expected to mimic accurately the complex behavioral pattern associated with rat sexual behavior (Pfaff and Lewis 1974), such stimulation will nevertheless activate neuronal circuitry that must be utilized as a substrate for lordosis.

Naturally occurring lordosis behavior is induced by cutaneous stimulation of the flanks followed by stimulation of the perineum by the male rat (Pfaff and Lewis 1974) and involves activation of the LL and TS muscles, which are necessary for the female to adopt the lordosis posture (Brink and Pfaff 1980). Adequate manual stimulation of the skin of the flank and perineum in unrestrained, awake rats induced electrical activation of LL and TS (Schwartz-Giblin, Femano and Pfaff 1984); from these studies it is known that cutaneous stimulation evokes responses in the axial muscle groups at latencies as brief as 10 ms from flank

stimulation and within 50 ms from the perineal stimulation in many instances.

Responses to perineal stimuli are transmitted centrally by the pudendal nerve, the sole cutaneous innervation for much of the region (Kow and Pfaff 1975). Transection of the pudendal nerve substantially decreases the efficacy of standard stimuli in eliciting lordosis (Kow and Pfaff 1975). In the cat, the pudendal nerve has an efferent component in at least some of its branches (Ueyama et al 1984); it is shown here, that the branches of the pudendal nerve used in the present experiment do not apparently innervate the muscles of the perineum. The fibers of the pudendal nerve enter the CNS primarily via the L5-S1 dorsal roots (Kow and Pfaff 1975; see also Thor et al. 1982). Brink et al. (1979) showed that the LL and TS muscles are innervated predominately from the lateral branches of the dorsal rami of the L3-L5 spinal nerves, with cell bodies in the lower thoracic and upper lumbar spinal cord. Kow, Zemlan and Pfaff (1980) determined that many spinal neurons innervated by perineal stimulation are found in the L5-S1 level. Thus, any pudendal nerve information must ascend several segments via either second order cells or axon collaterals before exciting the epaxial motoneurons.

Elevated levels of estrogen are permissive for lordosis behavior and the receptive field size of the pudendal nerve is itself modulated by estrogen levels (Komisaruk, Adler and Hutchison 1972). While the majority of estrogen-concentrating cells are located in higher neural centers (Pfaff and Keiner 1973), and intact pathways between the brain and spinal cord are required for lordosis to occur (Zemlan, Kow and Pfaff 1983), the regions of the brain known to be involved in lordosis behavior, including the ventromedial nucleus of the hypothalamus and midbrain central gray areas show only weak electrical responsiveness to somatosensory stimulation of the perineum (Sakuma and Pfaff 1980b & 1980c, Bueno and Pfaff 1976) suggesting that much of the moment-to-moment

control of lordosis takes place at spinal levels. It was thus of particular interest to characterize the shortest latency responses of epaxial muscle motoneurons to lordosis-relevant stimuli as these are likely to result from activation of intrinsic spinal pathways. This conduction path from pudendal afferents to axial motoneurons is a probable locus of convergence of hormonal and sensory information.

Given the above considerations of the naturally occurring behavior a study was undertaken to explore the responses evoked in the axial musculature by electrical stimulation of the pudendal nerve.

Experiment 1

In order to understand fully the time course of the responses evoked by pudendal nerve stimulation it is necessary to ascertain whether or not the pudendal nerve contains a significant complement of motor fibers in order to rule in or out the possibility that stimulation of the pudendal nerve evokes muscle reflexes of non-cutaneous origin. Since it is well known that muscle efferents and afferents usually travel together in a given nerve the branch of the pudendal nerves used in these studies was tested for the presence of muscle efferents by electrically stimulating the cut distal segments.

Materials and Methods

Surgical Preparation

Prior to the experiment the animals were injected intraperitoneally with 140 mg/kg of Urethane (Sigma Chemical Co.) in 40% solution with distilled water. Occasionally small supplementary doses, usually 0.2 ml, of anesthetic were administered to maintain constant anesthetic depth. An injection of 0.2 cc of Atropine was administered routinely to prevent mucus accumulation. Before beginning any surgery, the skin in the dorsal midline area and in the dorsal pos-

terior area of the hindlimbs was infiltrated with 2% Lidocaine HCl (Xylocaine, Astra Pharmaceutical Products).

The pudendal nerve was exposed through a dorsal approach; after an incision of the dorsal skin about 5 mm lateral to the tailbase, the gluteus maximus was cut at its medial attachment and reflected together with the overlying skin. The pudendal nerve was desheathed and dissected bluntly in order to remove as much connective tissue as possible. The nerve was then ligated with 9-0 nylon monofilament near its proximal end and cut preserving its distal portion.

Following the surgery the animal was moved to a stereotaxic apparatus and its head was lightly clamped in place. A clamp was attached to one of several exposed spinal processes. Rectal temperature was maintained at 37 degrees centigrade through the use of a servo-controlled DC heating pad and a heat lamp. The experiments were terminated by administration of a lethal dose of Chloropent (Fort Dodge Laboratories).

Stimulation

The distal portions of the pudendal nerves on both sides of the animal were placed onto stimulating hook electrodes (as shown diagramatically in figure 2-1) made of Teflon-coated .010" stainless steel, with exposed tips having an interpolar distance of approximately 1.5 mm. The stimulators on each side were Grass SIU5 stimulus isolation units, driven by a combination of Tektronix oscillators and pulse generators and by a Grass S4 unit. Stimulus current was monitored differentially on an oscilloscope as the voltage developed across a 10K ohm resistor in series with the stimulating electrode. Stimulation consisted of single shocks and trains of various magnitudes as described below.

Results

Stimulation of the distal segment of the pudendal nerve with currents as high as 1 mA produced no detectable movement of the rat. The perineal region

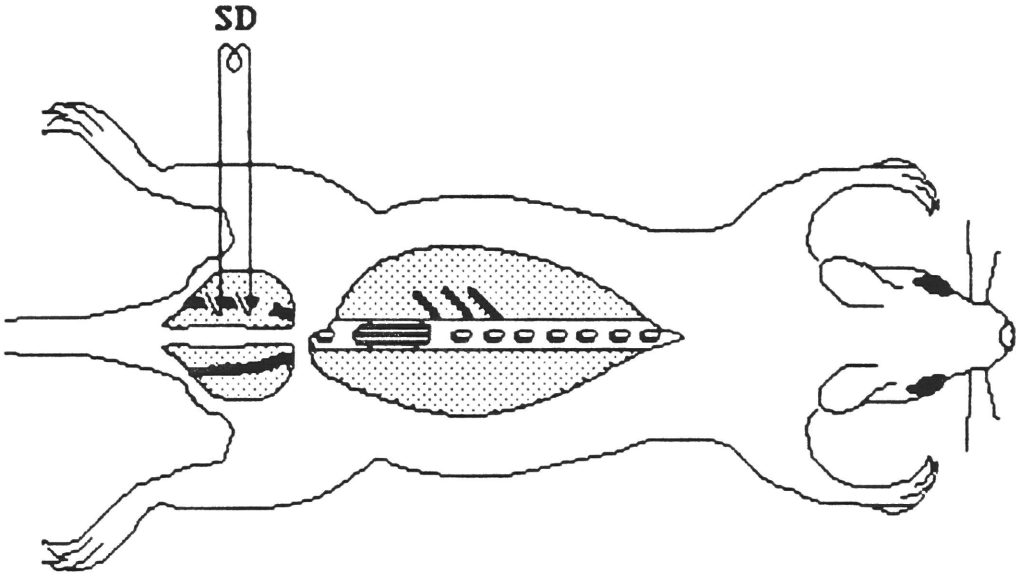


Figure 2-1

Diagram of the stimulation site used in Experiment 1. A hook electrode was used at the site labelled SD to stimulate the distal portion of the pudendal nerve. Movements (or the lack thereof) were observed with a dissecting microscope in the perineal region and elsewhere.

particular, including the muscle walls of the pelvis, was viewed through a dissecting microscope and no movement could be seen in that region.

Discussion

Ueyama et al. (1984) have shown that the pudendal nerve, which branches profusely, contains muscle efferents in at least some of its branches. The pudendal nerve is known, for example, to innervate the bulbocavernosus and external urinary sphincter. In the rat, too, a number of branches of the nerve were visible during the dissection, proximal to the branch used for stimulation in this experimental series. It is not possible to rule out the possibility that the branch of the pudendal nerve used in our experiments contains a small number of muscle efferents innervating muscles whose movements are too small to be seen in this manner described above, however, any such muscle efferents are likely to account for only a small fraction of the fibers seen in the nerve. On the basis of these experiments the branch of the pudendal nerve used here is thought to be primarily cutaneous.

Experiment 2

Dorsal Root Recordings

To interpret the total latency from pudendal nerve stimulation to the occurrence of responses in the axial muscles or muscle nerves it is necessary to know the time spent in peripheral conduction within the pudendal nerve. It is of interest, as well, to determine the threshold of the various fiber components of the nerve in order to design test stimuli which activate primarily the fibers relevant to lordosis behavior (cutaneous pressure fibers) without activating other fiber groups, such as C-fiber nociceptors, or A-delta fibers, which do not contribute to the behavior (Pfaff et al. 1977). Therefore, the response of the dorsal roots to stimulation of the pudendal nerve were studied.

Materials and Methods

Studies of the dorsal root responses were carried out in 8 Sprague-Dawley rats of both sexes.

Surgical Preparation

The pudendal nerves on both sides of the animal were exposed as before. The nerves were then either left intact or ligated with 9-0 nylon monofilament and cut near their distal end, preserving the proximal portion. A laminectomy of L1, L2 and sometimes T13 was performed to expose the dorsal surface of the spinal cord. Immediately prior to dorsal root recording the dura was opened with iridectomy scissors and the dorsal roots were visualized. The roots themselves were always handled with glass hooks and were covered with a vaseline and mineral oil mixture to prevent drying.

Stimulation

The proximal portion of the pudendal nerves on both sides of the animal were placed onto the stainless steel stimulating electrodes described previously. For some experiments involving antidromic invasion of the pudendal nerve one of the L5, L6 or S1 dorsal roots was stimulated via .005" platinum-iridium hook electrodes, insulated, except at the tips, with Teflon. The stimulus apparatus was as described above.

Recording

In order to circumvent persistent noise problems, bipolar recordings from the dorsal roots were made through the platinum hook electrodes or from the pudendal nerves via the stainless steel electrodes. In these recordings, the interpolar distance was usually widened to about 4 mm. The recording electrodes were connected to a locally placed differential amplifier with a gain of 10,000. Electrical responses were attenuated below 30 Hz and above 3 KHz to improve the signal to

noise ratio. The raw incoming signal was monitored on an oscilloscope. The recording and stimulation sites are shown diagramatically in figure 2-2 in which SP represents the proximal stimulation site for the pudendal nerve and RD represents the recording site for the dorsal roots.

Data Analysis

An Apple IIe computer was used for on-line (and in several cases off-line) analysis of the data. Dorsal root activity was either photographed from the oscilloscope screen or observed using the RC Electronics APPLESCOPE digital oscilloscope system, and in most cases the average of twenty-five or fifty stimulus trials was derived in order to improve the signal to noise ratio. Response latencies to the various components of the dorsal root responses were measured from the photographic records or from printouts of the averaged responses.

Following dorsal root recording experiments the entire course of the pudendal nerve was exposed by removing portions of the ischium and lateral musculature. Measurements of the nerve length were made by laying a thread along the course of the nerve and measuring the length of that thread. The average of three independent measurements was used.

Results

The mean threshold intensity (T) of pudendal nerve stimulation to evoke responses in the S1 through L5 dorsal roots was 21 μA and ranged from 1.5 to 50 μA when 200 μsec square pulse shocks were used. The first electrical responses in these roots were recorded 1.6 ms after a single shock to the pudendal nerve (see figure 2-3). The length of the pudendal nerve from stimulation site to recording site averaged 8.5 cm, yielding a conduction velocity of 54 m/s for the fastest fibers in the rat pudendal nerve. They can thus be classified in the A-β range. The evoked response was a complex wave with a number of irregular peaks showing a broad spectrum of conduction velocities of afferents within the

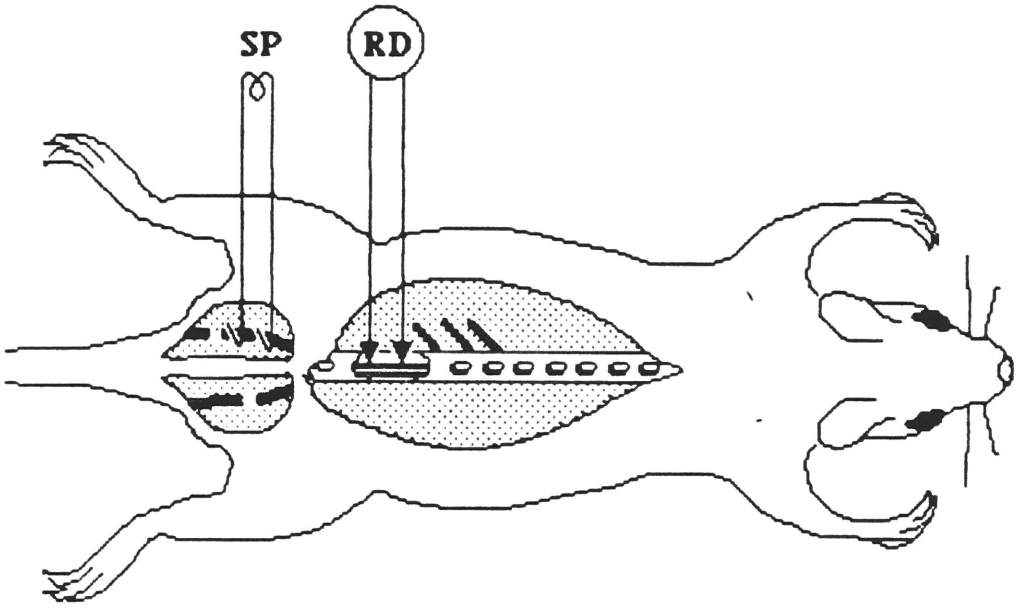


Figure 2-2

Diagram of the stimulation and recording sites used in Experiment 2. At the SP site, the proximal portion of the pudendal nerves on either side of the animals were stimulated through stainless steel hook electrodes. Recordings from the dorsal roots were made through a laminectomy at the RD site. The dorsal root ac tivity was recorded through platinum hook electrodes.

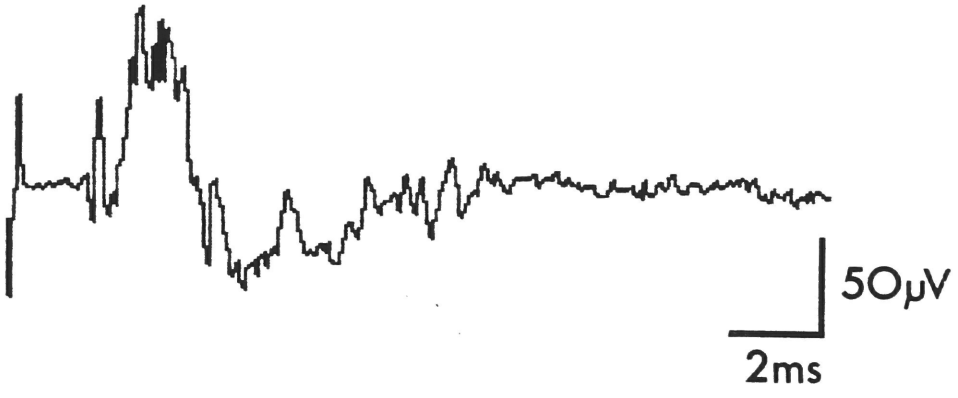
Figure 2-3a

The averaged responses to 20 consecutive 50 μA single shocks to the pudendal nerve recorded from the ipsilateral L6 dorsal root at the dorsal root entry zone. Latency from stimulation to arrival of the afferent volley is 1.6 ms. Activity is visible as late as 10 ms indicating the activation of A-δ fibers.

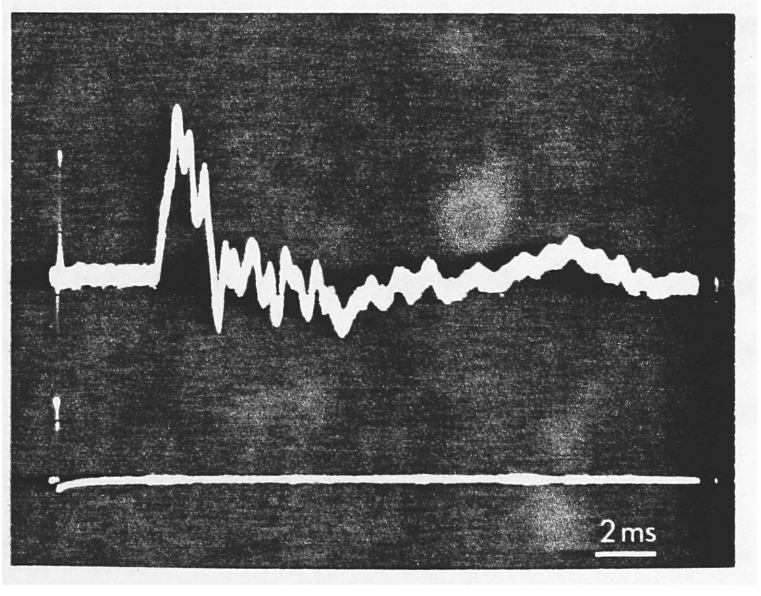
Figure 2-3b

Antidromic volley recorded in the pudendal nerve to stimulation of the S1 dorsal root without signal averaging. Stimulation rate was 200/sec with 75 μA ipsilateral stimulation.

A



B



nerve. As stimulus current was elevated from threshold the magnitude of the first peaks increased, but ceased to do so with stimulation at more than about 5 x T. At stimulus intensities of 10 times T or more, a late (8.2 ms latency to peak) broad wave occasionally appeared. With a conduction velocity of 10.4 m/s the later wave is probably conducted via A-δ fibers. The late wave in particular was more distinct when the antidromic volley initiated by dorsal root stimulation was recorded from the cut end of the pudendal nerve.

During stimulation of the pudendal nerve, contractions of the abdomen were visible in response to stimulus trains with amplitudes of 270 μA (mean) or more. In the males, small movements were visible in the scrotum at similar or slightly lower stimulus intensities. Such contractions, of both the abdomenal area and scrotum, possibly the results of the cremasteric reflex, were present following sectioning of the portion of the pudendal nerve distal to the stimulation site.

Discussion

Due to the apparent absence of muscle nerve fibers in the branch of the pudendal nerve stimulated, the activity seen in the dorsal roots was assumed to originate primarily from cutaneous afferents. It is not possible, however, to rule out the possibility that this portion of the nerve contains some deep receptor or proprioceptive afferents, although the origin of such afferents is not obvious from the anatomy of this branch of the nerve, which does not appear to approach any skeletal or visceral tissue. Any muscle afferents would probably have to be from group II or group III, as Hnik (1978) has shown that the conduction velocity of group I fibers in the rat sciatic nerve were from 60-80 m/sec.

On the basis of the results of these experiments, it was determined that stimulation of the pudendal nerve with currents less than 200 μA could be expected to activate primarily A-β fibers without activation of A-δ fibers in most cases.

The contractile activity in the perineum must itself have been mediated synaptically by an intraspinal route, because of its continued occurrence following sectioning of the distal portion of the pudendal nerve.

Experiment 3

Epaxial Muscle Nerve Recordings

As part of the study of the neural circuitry interposed between the pudendal nerve and the axial muscles it is of value to determine the minimal latency from stimulation of the pudendal nerve to muscle activation. Determinations of this latency from the responses of units within the lateral longissiumus muscle are hampered by several factors. The conduction velocity of muscle fibers within lateral longissimus is approximately 7 m/s (Schwartz-Giblin et al. 1984) and may introduce significant delays into the recording. The synaptic delay at the neuromuscular junction in these muscles has not been determined and it too introduces an unknown quantity to the response latencies.

Even under optimal conditions an electrode located within the muscle mass would only be able to record the activity of a small number of muscle units, a fact of particular interest because the lateral longissimus muscle, though composed primarily of fast-twitch glycolytic fibers, is heterogeneous (Schwartz-Giblin, Rosello and Pfaff 1983, Carlson 1978b), containing both fast-twitch oxidative glycolytic and slow-twitch oxidative fibers. The latter comprise as much as 62% of the fibers in the medial deep region of the muscle in the rat. There is no *a priori* reason to expect that these muscle fiber types would be recruited at similar latency by stimulation of the pudendal nerves, and in fact there is data to suggest that cutaneous afferents selectively evoke activity in subpopulations of muscle units (Kanda, Burke and Walmsley 1977). Recordings of units within the muscle might therefore be subject to significant variability due to small variations in placement of the revording electrodes.

Recordings made directly from the muscle nerves, however, are not subject to these difficulties. The conduction time from the spinal cord to the muscle nerves in the rat has been measured, and is reported by Brink (1981) to be 1.1 ms. The nerve contains a large number of fibers, presumably a mixture of axons of various motor unit types, and therefore it should be possible to detect the first units activated by recording from the whole nerve. Stability of the recording site is also favorable under these circumstances.

Materials and Methods

In order to test for differences in the responses of animals in differing hormonal states, forty-six Sprague Dawley rats of both sexes, females weighing from 226 to 440 grams and males with weights between 240 and 580 grams, were used in this study. All of the females were ovariectomized and were divided among three hormonal pretreatment groups: untreated, implanted with a 5 mm silastic implant containing crystalline estradiol (Smith 1977), or pretreated with a 10 microgram subcutaneous injection of estradiol benzoate in corn oil on at least three separate days. The latter two treatments produced similar behavioral changes in regard to the priming of lordosis behavior and were thus considered as a single 'estrogen-primed' treatment group.

Behavioral Testing

Each animal was tested for lordosis performance before beginning the acute phase of the experiment. The scoring procedure is similar to that described by Harlan et al. 1983. In this assay, the rats are rated for lordosis responsiveness to manual stimulation, consisting of repetitive stroking of the anterior flanks followed by light pressure applied to the perineum and dorsal tailbase, after which the animal is lifted off of the ground. Lordosis responses were rated on a scale from 0 to 3 as follows (see figure 2-4):

0.....No lordosis

1.....Slight lordosis; back is elevated, but not horizontal

2.....Moderate lordosis; back is approximately level

3.....Strong Lordosis; head and rump are elevated above the thorax

Except for those experiments using non-treated females or males, the female in the colony with the highest lordosis score was routinely selected for experimentation. In no case did any male or untreated female rate a lordosis score other than zero.

Surgical Preparation

The pudendal nerves on both sides of the animals and the L3, L4 and L5 lateral longissimus muscle nerves were prepared as described above. At least a 3 mm length of the muscle nerve was usually available for recording.

Stimulation

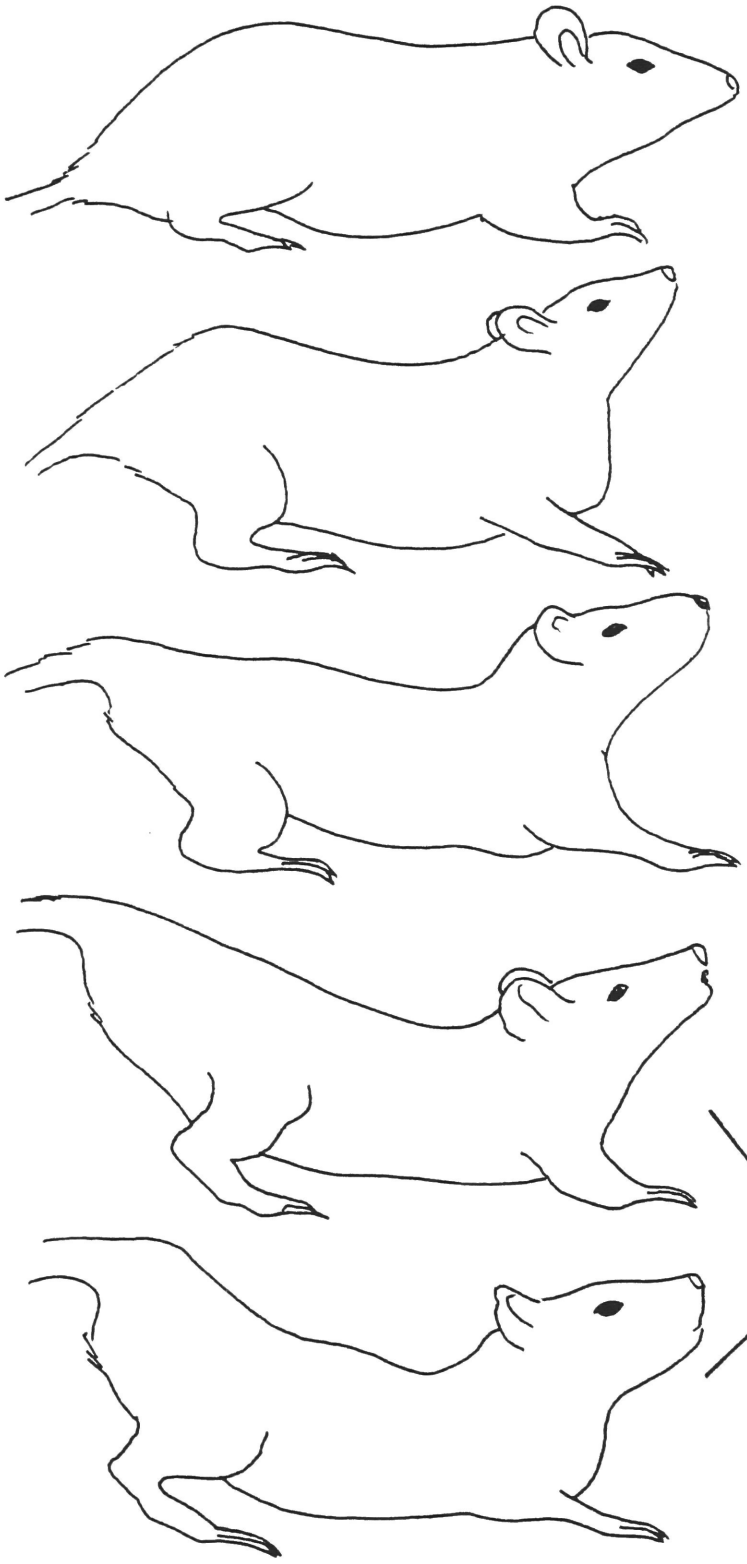
The stimulus apparatus was as described above for Experiment 2. Specific stimulus parameters were a variable in these experiments and are described in detail below. The standard stimulus consisted of a train of three 200 μsec square pulse shocks separated by 1.5 ms and repeated at a rate of 2/s. This choice of stimulus parameters was based on the results of initial experiments described below.

Recording

The exposed nerve branches were very short, seldom exceeding about 7 mm, thus, killed end recordings were impractical. Monopolar recrdings were precluded by problems with noise contamination, thus bipolar recordings were made from the muscle nerves via platinum-iridium hook electrodes with an interpolar distance of 1 to 2 mm. The recording electrodes were connected to a locally placed differential amplifier with a gain of 10,000. Electrical responses were attenuated

Figure 2-4

Scoring procedure for lordosis performance of the female rat in response to manual "fork" stimulation. In the stimulation tests the experimenter rubs the animal's flanks with his or her thumb and ring finger, then lifts up the animal, applying slight pressure to the rat's tailbase, back and perieneum. Lordosis is observed as the degree to which the back is arched upward and is scored on an ordinal scale from 0, indicating no lordosis, to 3, for strong lordosis performance. Figure adapted from Brink (1978).



0 (No Lordosis)

1 (Slight Lordosis)

2 (Moderate Lordosis)

3 (Strong Lordosis)

below 30 Hz and above 3 KHz to improve the signal to noise ratio. The output of the differential amplifier was fed to a window discriminator used in either a threshold detection or windowing mode. When the spikes were of small amplitude the discriminator threshold was set conservatively, resulting in occasional missed spikes rather than in false triggering on noise. The raw incoming signal was monitored on an oscilloscope. An example of the raw spike data is shown in figure 2-6a. The recording and stimulation sites are shown diagramatically in figure 2-5 in which SP represents the proximal stimulation site for the pudendal nerve. RN is the recording site for muscle nerve recordings.

Data Analysis

An Apple IIe computer was used for on-line (and in several cases off-line) analysis of the data. The muscle nerve data was displayed as PST or ISI histograms through the use of a data acquisition program developed in the laboratory (Cohen and Pfaff 1984, see Appendix I).

Peaks in the PST histograms were quantified by the following off-line analysis (figure 2-6): A baseline noise level was computed as the mean and standard deviation of the number of spikes per bin in the 250 ms prior to each stimulus. The histograms were then smoothed using a boxcar averaging technique in which the number of spikes per bin was set equal to the average of the bins in its neighborhood. With binwidths of 0.5 ms this neighborhood was set to include the current bin and its four nearest neighbors. For 1 ms bins the average of the bin and its two nearest neighbors was used and for 5 ms bins the average of the bin and its right hand nearest neighbor was used. These boxcar widths were chosen empirically in trial runs to minimize distortion of the data while removing unwanted high frequency noise. After smoothing, the bins containing a number of spikes outside the range of the previously-derived mean, plus or minus 2.57 standard deviations were considered 'significant' peaks. The range of +/-

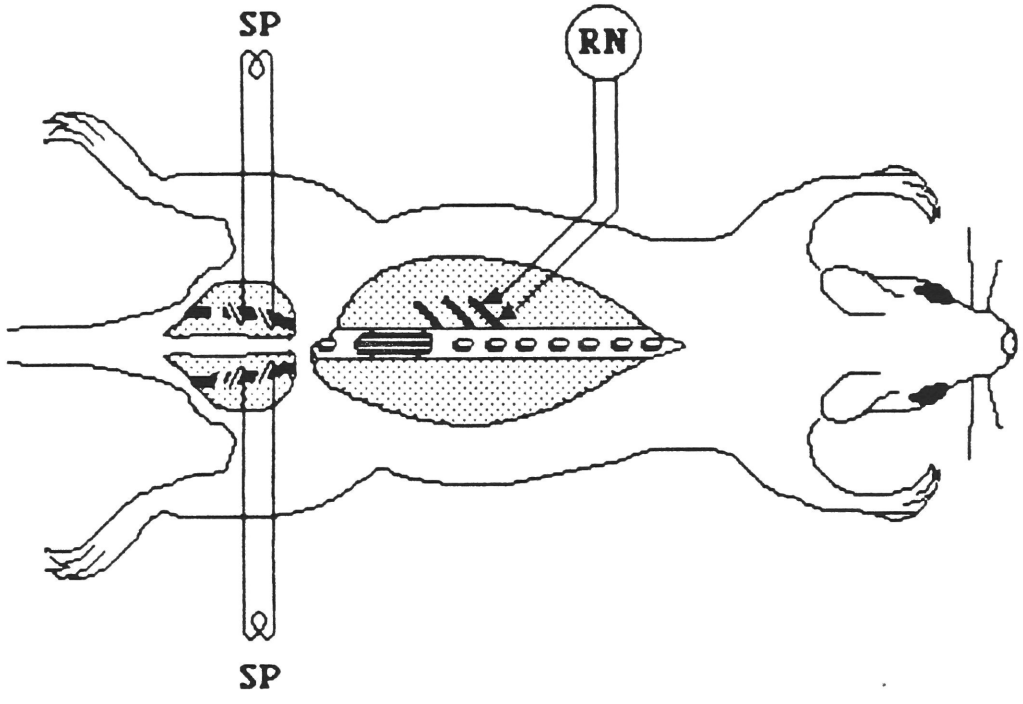
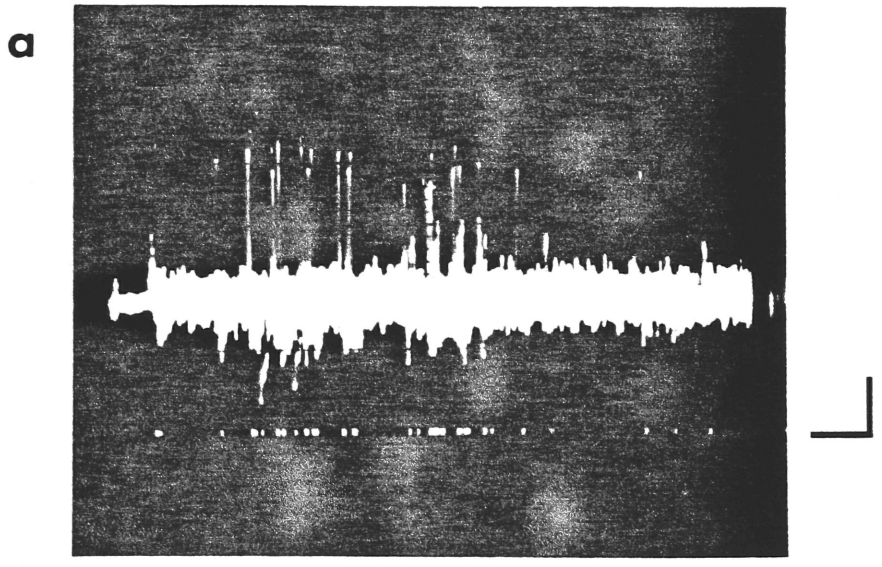


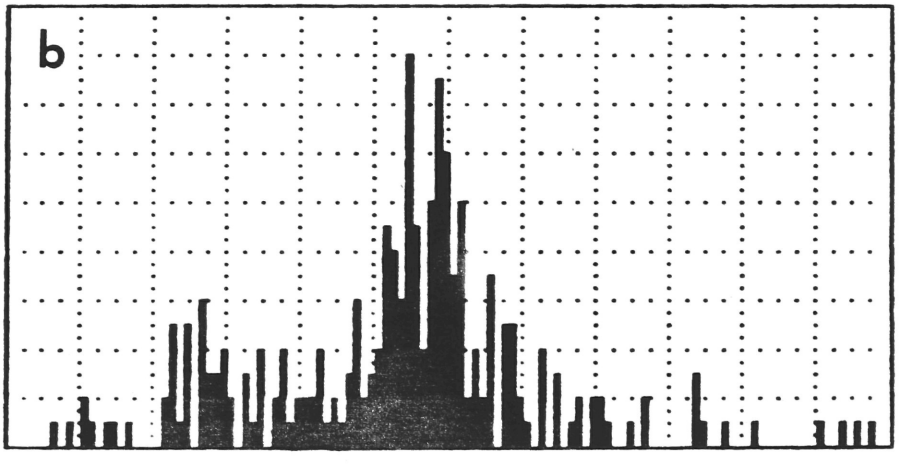
Figure 2-5

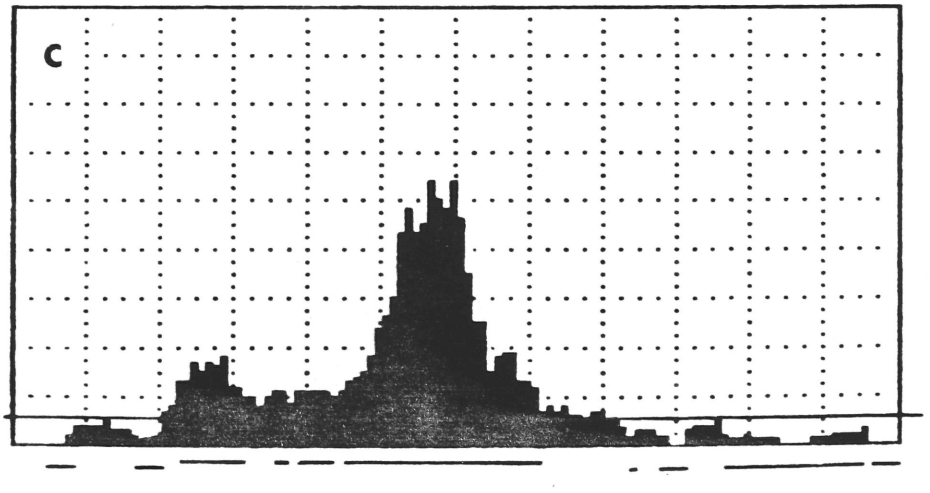
Diagram of recording and stimulation sites used in Experiment 3. SP is the site for stimulation of the proximal portion of the pudendal nerves. Recordings of the activity in the branches of the L3, L4 and L5 dorsal rami which innervate the lateral longissimus muscle were made through bipolar hook electrodes. RN shows schematically the site for muscle nerve recording. For clarity, only the L3 muscle nerve recording site is shown.

Figure 2-6

a) Electrical spike activity in the L5 muscle nerve during 100 μA bilateral stimulation of the pudendal nerve with triple shock trains at a rate of two per second (ten superimposed sweeps). Horizontal calibration bar: 5 ms, vertical calibration: 20 μV. **b and c)** Post-stimulus time (PST) histograms of the spike activity in the L4 epaxial muscle nerve following 1/s bilateral stimulation of the pudendal nerves with trains of three shocks at a current of 50 μA; 78 accumulated sweeps. Horizontal divisions are 5 ms, vertical divisions are 2 spikes. Mean and standard deviations of the baseline firing rate are calculated from the 250 ms period immediately preceding the stimulus. The raw histogram **b** is first smoothed using a boxcar averaging technique in which the number of spikes per bin was set equal to the average of the bin and its four nearest neighbors. After smoothing, **c**, the mean is plotted (horizontal line on that figure) and the difference of each bin from the mean is calculated. The horizontal lines below the histogram indicate bins which differ from the mean by more than 2.57 standard deviations. Peak heights are recorded in multiples of the mean baseline firing rate (not shown).


a


b


c

2.57 standard deviation units was chosen to correspond roughly to a 99% confidence interval. The amplitude of the peaks was quantified in multiples of the mean baseline firing rate (see figure 2-6). This method of analysis, while essentially arbitrary, was designed to produce a repeatable and objective peak detection system producing normalized measurements of peak heights. See *Appendix II* for a description of the theoretical justification for this method of quantification.

Results

Usually, after placing the muscle nerves onto the hook electrodes, there was spontaneous activity present for several seconds. However, 25% of the nerves tested were silent with or without stimulation; we postulate that these were damaged in surgery. Occasionally (4/55 tested nerves) there were spontaneously firing periodic units present in some nerves for the duration of an experiment. These units fired at a mean modal frequency of 21/s and their firing rate was independent of pudendal nerve stimulation. In many of the remaining cases a number of units were spontaneously active in these nerves but their separate firing rates could not be assessed using the available window discriminator. The amplitude of the muscle nerve spikes was usually from 20 to 100 μV.

During trains of stimulation with the standard stimulus, consisting of repeated triple shock trains (see methods), there was usually a marked increase in the overall firing rate of the muscle nerves (figure 2-7). Usually the spike activity continued to increase for the first 10 to 20 seconds of stimulation and declined gradually following its cessation. PST histograms of the nerve spikes following each stimulus train demonstrate that latencies from the stimulus to muscle nerve activity cluster in discrete ranges (figure 2-8).

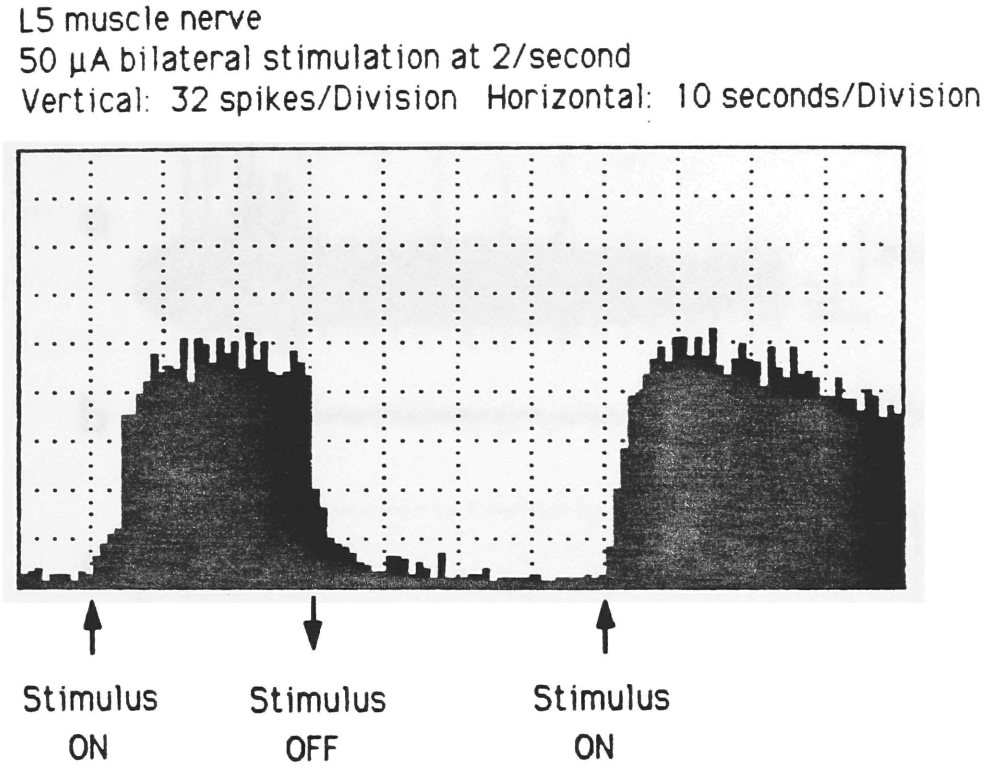


Figure 2-7

Firing rate histogram of L5 muscle nerve activity before, during and after stimulus bouts, each horizontal division is 10 seconds and each vertical division is 32 spikes. The first ten seconds record the spontaneous activity in the muscle nerve. Bilateral stimulation of the pudendal nerve starts at ten seconds (first upward arrow) and continues for 30 seconds at a rate of two triple shock, 50 μA trains per second. Stimulation is discontinued at the downward arrow. Finally, stimulation is reapplied at 90 seconds (second upward arrow) and continues for the duration of the recording. The activity continues to increase for approximately 6 seconds after starting the stimulation (12 trains). In this example the firing rate of the L5 nerve diminished gradually over a period of approximately 10 seconds following the cessation of the stimulus. The second stimulus bout shows evidence of some fatigue after about 30 seconds of stimulation (60 trains).

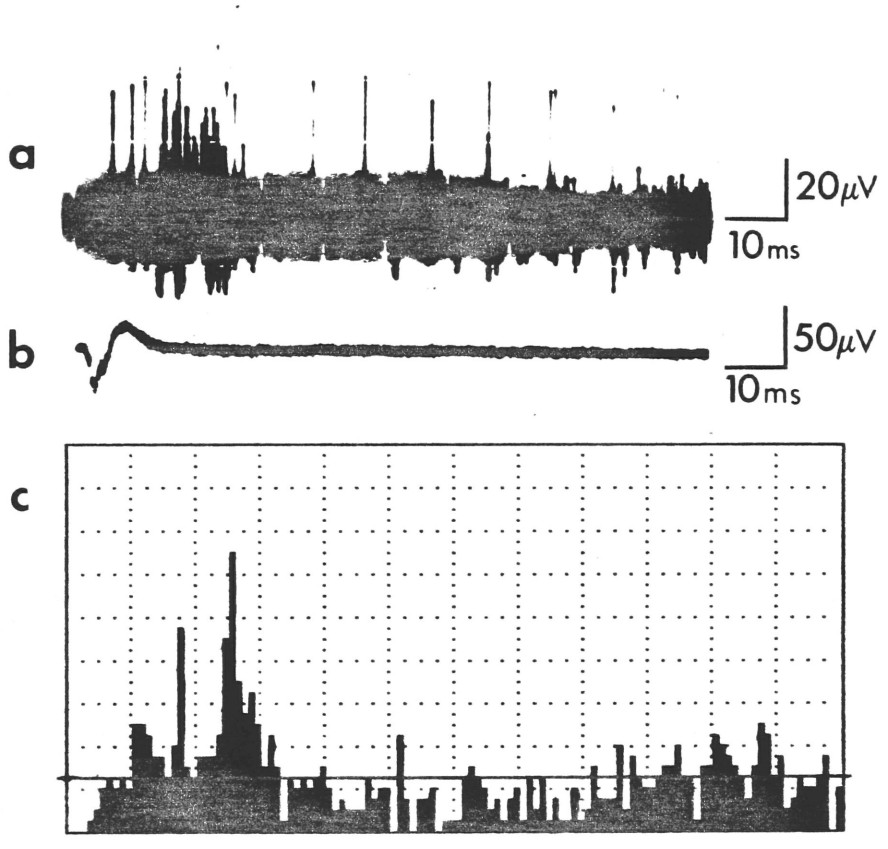


Figure 2-8

Spike activity in the L3 muscle nerve during 50 μA bilateral stimulation of the pudendal nerve with triple shock trains at a rate of two per second (ten superimposed sweeps). A clustering of nerve spike activity is evident at about ten to thirty ms post-stimulus. **b**) Simultaneously recorded afferent volley in the L6 dorsal root, negativity down. **c**) PST histogram generated simultaneously with **a** and **b** above, but for 74 stimulus iterations. In this example early peaks are evident between 10 and 30 ms follwoing the stimulus onset. There was no significant depressed period. A third significant peak is seen at 108 ms. The first three milliseconds of the PST recording are suppressed to avoid contamination by the stimulus artifact. Calibration bar for **a**: 10 ms x 20 microvolts; for **b**: 10 ms x 50 microvolts; in **c**, vertical divisions are 4 spikes, horizontal divisions are10 ms.

Stimulus Parameters

Several experiments were performed to optimize the stimulus parameters used to evoke the epaxial responses described above. Figure 2-9 shows that while single and double shocks were in some cases effective in evoking reactions from the epaxial muscle nerves, in all cases they were substantially less effective than triple shocks. Stimulation with four or more shocks failed to potentiate the muscle nerve activity further. A standard stimulus configuration of a train of three 200 μsec shocks with an intershock interval of 1.5 ms was thus adopted, but the latencies were nevertheless quantified conservatively from the first shock of the train, since stimulation with single shocks had a small, but by the criteria described above, significant effect in eliciting the axial muscle nerve responses. Thresholds for significant nerve responses were not routinely determined. Stimulus currents producing obvious axial muscle nerve responses ranged from 4 to 240 μA with a mean of 58 μA.

Electrical response patterns as described above were evoked by unilateral stimulation of the pudendal nerve on either side of the animal, with a tendency for stimulation contralateral to the recording site to be more effective. In all nine experiments where it was examined, bilateral stimulation was effective in eliciting nerve responses at intensities where unilateral stimulation to either side was not (figure 2-10), or when unilateral stimulation was successful, bilateral stimulation was much more so. The facilitation was more obvious in the early (8-35 ms) than in the later (105 ms) response peaks. These facilitatory effects demonstrate that spinal afferents responding to cutaneous inputs converge upon interneuronal elements.

Repetition rates of one train per second elicited nerve responses but the response magnitude usually increased with stimulus repetition rates up to 10/s; this was particularly visible when stimulus currents were near threshold. Stimu-

Figure 2-9

Responses of the L5 muscle nerve to 1/sec bilateral stimulation of the pudendal nerves. The left-hand column shows the raw histogram and the right-hand column presents the histograms after smoothing and peak detection. The horizontal lines in the figures on the right represent the mean baseline level of spike activity in the nerve. Below these smoothed histograms are lines indicating the presence of bins containing a number of events significantly greater or less than the mean. Stimulation with single shocks, shown in **a** and **b**, produces significant activity in the nerve at 19 ms. Two shock stimulation, **c** and **d**, is more effective than single shocks and stimulation with three shocks, as shown in **e** and **f**, is even more so. Four shock stimulus trains, **g** and **h**, do not further potentiate the responses. The magnitude of the first peak in **a** and **b** is 2.4 times the mean baseline level. The first peak in **c** and **d** is 3.5, in **e** and **f** it is 4.2 and in **g** and **h** it is 4.3. The horizontal scale is 10 ms/division in all figures and the vertical scale is marked on the raw histograms. Top row: 98 sweeps; second row: 106 sweeps; third row: 144 sweeps; bottom row: 109 sweeps.

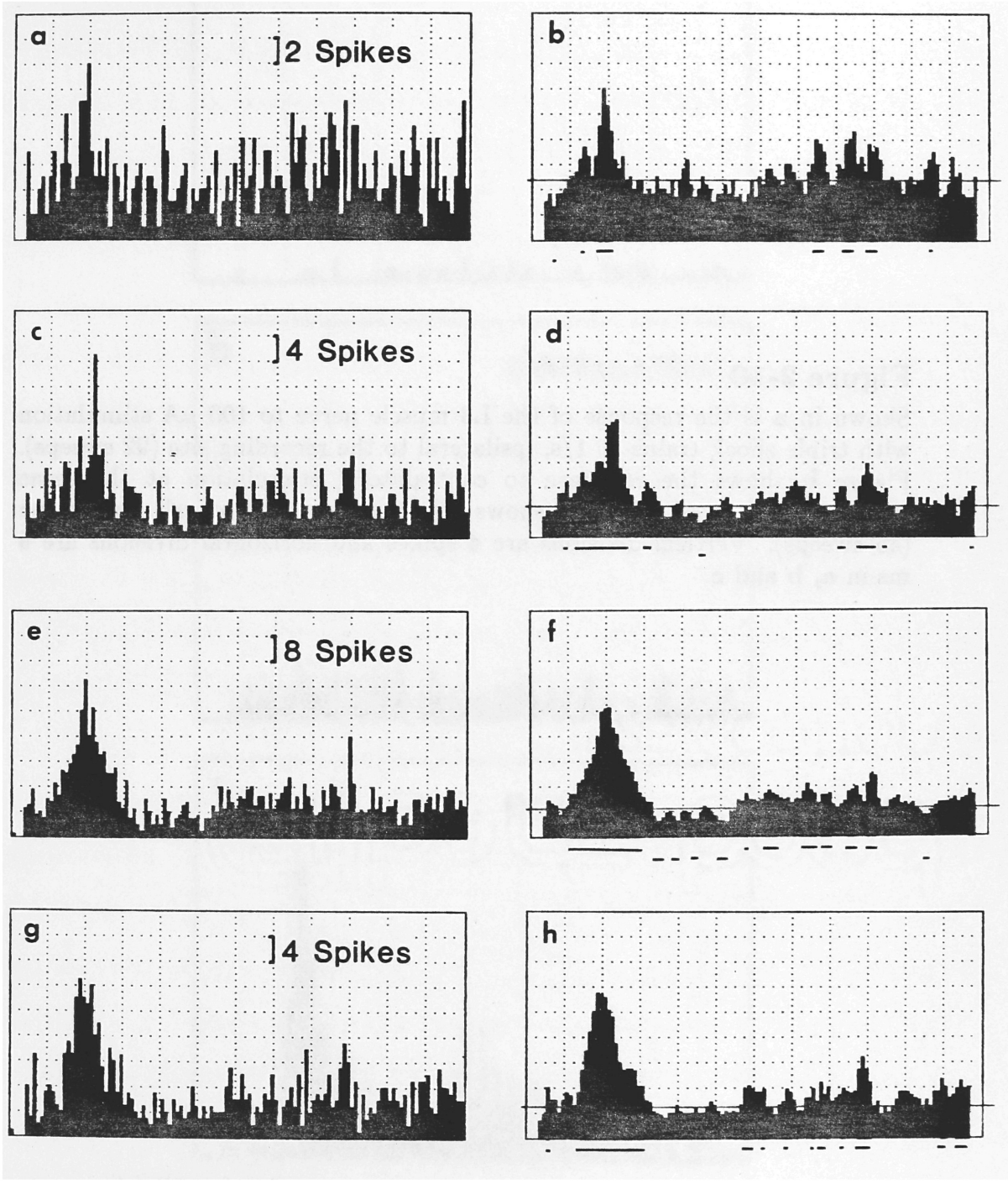
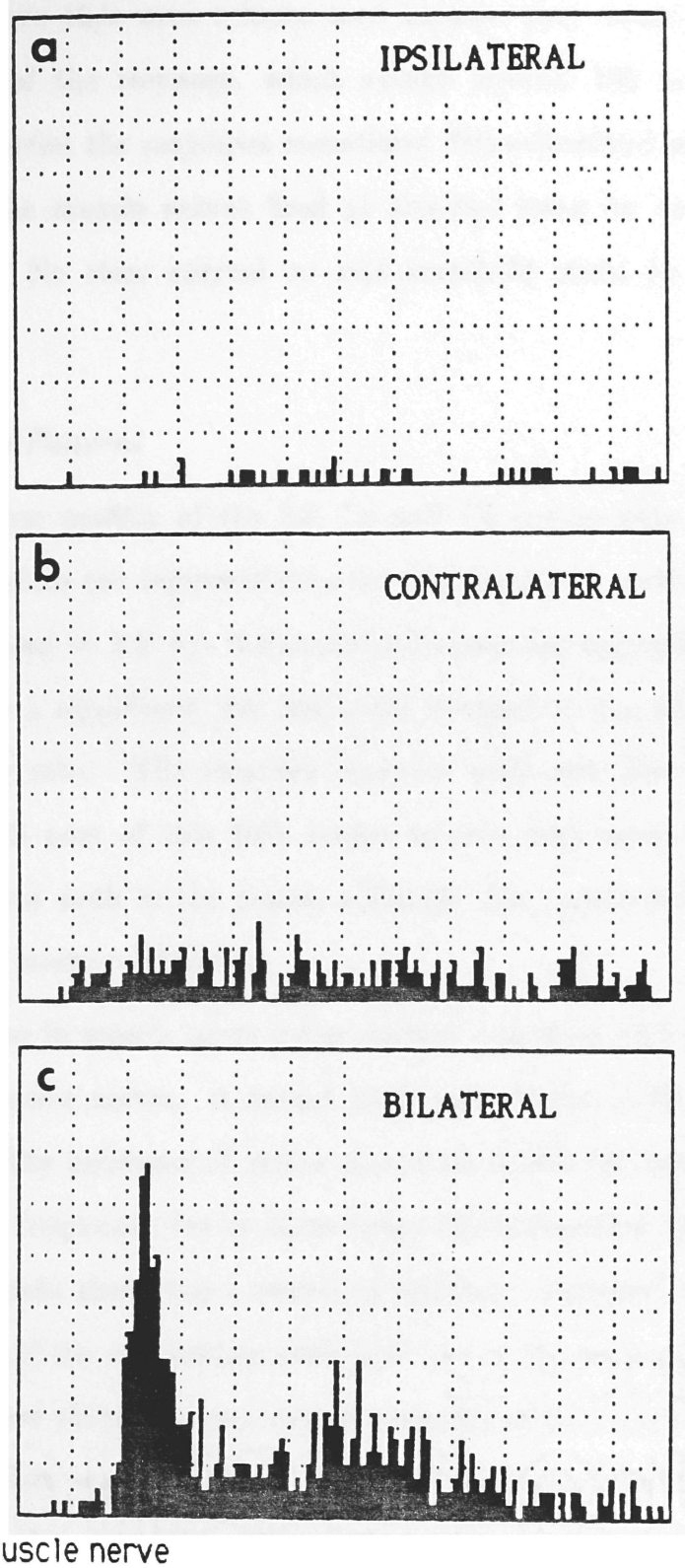

a
]2 Spikes
b
c
]4 Spikes
d
e
]8 Spikes
f
g
]4 Spikes
h

Figure 2-10

Shown in **a** is the response of the L3 muscle nerve to 100 μA stimulation with triple shock trains at 1/s, ipsilateral to the recording site (92 sweeps). Figure **b** shows the response to contralateral stimulation at the same intensity (105 sweeps) and **c** shows the response to bilateral stimulation (45 sweeps). Vertical divisions are 4 spikes and horizontal divisions are 5 ms in **a, b** and **c.**



L3 muscle nerve
100 µA stimulation at 1/second
Vertical: 4 spikes/Division Horizontal: 5 ms/Division

lation rates above 10/s were seldom used because they would obscure the temporal features of the response, which extend beyond 100 ms. At the higher stimulus frequencies the responses sometimes desynchronized with respect to the stimulus and the muscle nerves fired at elevated rates for the duration of the stimulus bout. No clear current or rate threshold could be detected for this phenomenon.

Nerve Response Patterns

The response profiles of the L3, L4 and L5 nerves were indistinguishable. Table 1 summarizes the temporal data for the responsive nerves. Within 5 to 13 milliseconds (mean = 8.8 +/- 0.4 s.e.m.) following the beginning of the stimulus train there was a significant (see Methods) increase in the probability of nerve firing in female rats. The response onset in male rats was 11.4 ms (+/- 1.7 s.e.m.). A small part of this 30% longer latency may result from the slightly longer conduction path in the males, although this cannot possibly account for the over 2.5 ms latency difference.

The increase in muscle nerve firing reached a peak at 14.2 (+/- 0.3) ms in 53 of the 55 responsive nerves. A second peak was present at 25.1 (+/- 0.6) ms in 48/55 nerves. The incidence of action potentials within the latency interval from 8 to 35 ms was frequently ten or more times the background firing rate. Following the early peaks there was a period of relatively depressed activity beginning at 37.1 (+/- 1.3) ms and lasting until 52.8 (+/- 1.8) ms in 45/55 nerves tested. In all cases where there was any change in spike activity during stimulation the predominant effect was excitatory. It cannot be determined from these experiments whether the relatively quiescent period is the result of active inhibition, synchronization of the motor units during the first peak in activity at 14 ms, or both. Figure 2-11 shows an axial muscle nerve recording made during pudendal nerve stimulation in which there is a striking damped periodicity to the evoked

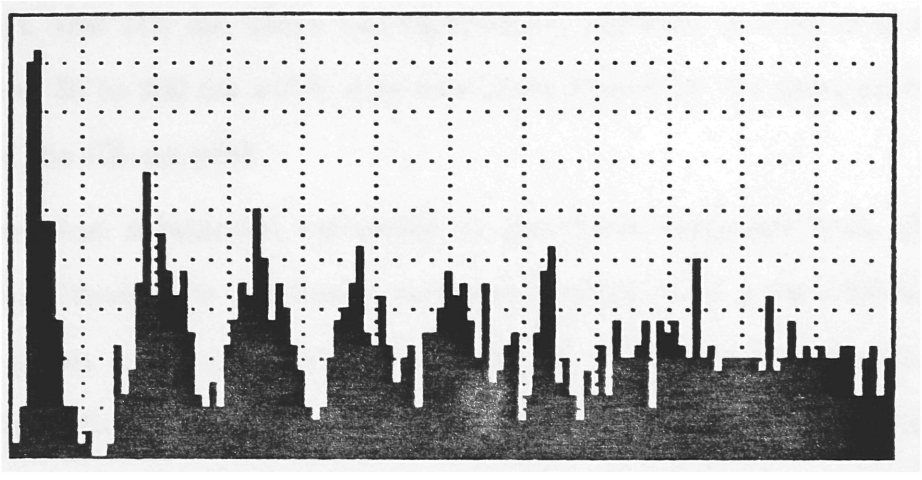


Figure 2-11

PST histogram of spike activity in the L5 epaxial muscle nerve during 1/s, 100 μA bilateral stimulation of the pudendal nerve with triple shock trains, 59 accumulated sweeps. Horizontal divisions are 50 ms and the vertical scale is 4 spikes/division. Note the damped periodicity to the response.

response. Records such as these may result from entrainment of individual motoneurons firing periodically at similar rates.

A third major histogram peak (designated in Table 1 as 'late response') was often (51/55 cases) present at 105.5 (+/- 1.8) ms after which the activity returned gradually to baseline (figure 2-7). In some cases, while there was no clear peak near 100 ms, there was significantly elevated activity over the broad range from 60 to 200 ms which may have been caused by the same process which produced the 105 ms peak.

There was substantial variability in the nerve responses both within and across individuals. As previously mentioned, there were a variable number of activity peaks. At times a nerve that was not initially responsive to pudendal nerve stimulation became so several hours later or vice versa. It was not possible to correlate such changes in the responses with changes in either temperature or depth of anesthesia.

Effects of Sex and Lordotic Responsiveness

When the data were grouped by sex the overall electrical response patterns were similar in three treatment groups. Table 1 presents the data separated by sex and lordosis score. The magnitude of the late (100 ms) response was significantly larger in the males than in either the lordosis-responsive or unresponsive females and there was a trend ($p < .05$) for the onset in elevated nerve activity to occur earlier in the females than in the males.

Ruling Out a Dorsal Root Reflex

Based on several lines of evidence, the early responses shown here are unlikely to represent dorsal root reflexes (DRR's). The DRR is known to occur following stimulation of cutaneous nerves under conditions of depressed spinal cord temperature and low stimulation rates (Toennies 1938). The typical DRR

Table 1

Temporal profiles of the responses of the axial muscle nerves to electrical stimulation of the pudendal nerve with trains consisting of three shocks, 200 μsec in duration. All latencies are measured with respect to the first shock in the train. The responses of the L3, L4 and L5 nerves did not differ from one another and are combined in the table. In the first row, **L** is the lordosis score in reponse to manual stimulation. In quantifying the nerve responses, each nerve was considered to be an independent trial. If multiple recordings were made from the same nerve those responses were averaged.

(**a**) Mean latency, in ms from the beginning of the stimulus train. (**b**) Standard error of the mean.

*Indicates pairs which are significantly different (by one-way analysis of variance) at the .05 level.

**Indicates significance at the .01 level.

Table 1
Summary of the features of the
Pudendal Nerve-Evoked Response

		Total	Female	L<2	L≥2	Male
Onset	Frequency	55/55	44/44	25/25	17/17	11/11
	Mean [a]	9.3	8.8*	7.9	10.0	11.4*
	s.e.m. [b]	±0.4	±0.4	±0.5	±0.5	±1.7
First Peak (8<T<20ms)	Frequency	53/55	44/44	25/25	17/17	9/11
	Mean	14.2	14.1	14.2	14.3	14.6
	s.e.m.	±0.3	±0.4	±0.5	±0.6	±0.9
Second Peak (15<T≤35)	Frequency	48/55	39/44	21/25	17/17	9/11
	Mean	25.1	24.3	24.9	23.3	28.8
	s.e.m.	±0.6	±0.6	±0.9	±1.0	±1.2
Quiet Period Begin	Frequency	45/55	39/44	24/25	14/17	6/11
	Mean	37.1	36.7	34.9	40.4	39.7
	s.e.m.	±1.3	±1.4	±1.7	±2.2	±4.9
End	Mean	52.8	52.7	50.2	57.8	53.1
	s.e.m.	±1.8	±2.0	±2.4	±3.4	±4.2
Late Response	Frequency	51/55	40/44	22/25	17/17	11/11
	Mean	105.5	105.8	105.9	105.1	104.3
	s.e.m.	±1.8	±2.0	±2.8	±3.1	±4.0
	Magnitude	3.55	2.46**	2.49	2.40	7.26**
	s.e.m.	±0.71	±0.60	±0.79	±0.31	±2.39

consists of a wave of activity several milliseconds in duration which is recorded in the afferent fibers 4 to 10 ms following stimulation of the cutaneous fibers or dorsal roots. At stimulation rates greater than 1/s the DRR drops out rapidly. By contrast, the early responses in these experiments were undiminished, and often appeared potentiated with stimulation at rates up to 10/s (the maximum rate used, see above). To conclusively test the hypothesis that the responses described here are due to dorsal root reflexes the activity of lateral longissimus muscle units in response to pudendal nerve stimulation was assessed.

Experiment 4

Materials and Methods

Six ovariectimized, estrogen-treated, female rats, all showing lordosis scores greater than 2 in response to manual stimulation were used in these experiments. The pudendal nerves were prepared as in Experiments 2 and 3.

Surgical Preparation

A midline incision was made to expose the superficial back musculature. A 5 to 10 cm long cut in the aponeurosis on one or both sides of the animal was made approximately 7 mm lateral to the midline. The aponeurosis was then sutured to the skin and reflected, exposing the medial and lateral longissimus muscles. In most cases the medial longissimus muscle was removed. The muscles were covered with a layer of vaseline and mineral oil mixture to prevent drying of the tissue.

Recording

EMG unit recordings were made differentially either from a pair of Teflon-coated .005" tungsten wires, whose insulation was notched several times along their length, or from a pair of braided stainless steel wires insulated with Teflon except at the tips which were formed into hooks. These were inserted directly into the

LL muscle mass. The output from these electrodes was fed to a locally placed differential amplifier as above.

Data Analysis

Unit data from the lateral longissimus muscle was analyzed using the PST histogram generating system described above and was subjected to the identical quantitative analysis.

Results

Recordings of units through electrodes in the lateral longissimus muscle showed a pattern of activation substantially similar to that of the muscle nerves (figure 2-12). A prominent peak in activity was visible near 15 ms (15.1 +/- 2.4) and a second peak was apparent near 30 ms (29.7 +/- 3.2). A late peak near 100 ms (102 +/- 7) was present in 3 out of the 6 animals. Table 2 summarizes the responses of the muscle units and muscle nerves.

Discussion

The activation of LL muscle units at latencies corresponding to those seen for action potentials within the muscle nerves, and presumably caused by them, demonstrates that the pudendal nerve-evoked responses are not merely reflections of a dorsal root reflex.

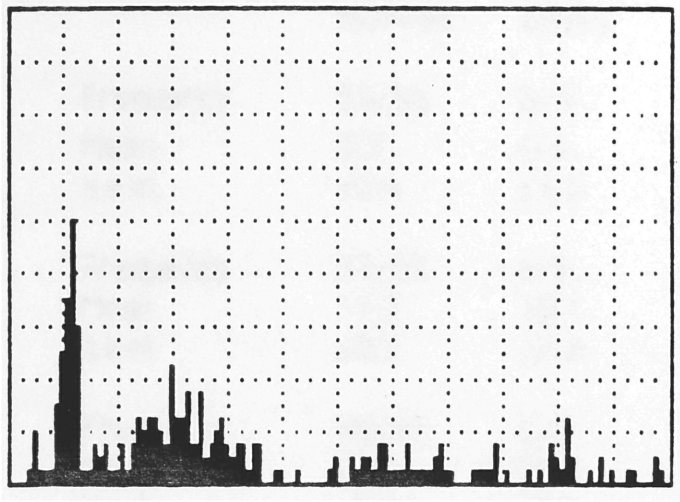


Figure 2-12

Responses of lateral longissimus muscle units to bilateral stimulation of the pudendal nerves with three shock 200 μA trains at a rate of 2/s. A pronounced activity peak is evident between ten and fifteen ms and elevated activity is present from 25 to 35 ms. The vertical scale is 8 spikes/division, the horizontal scale is10 ms/division.

Table 2 comparison of muscle nerve and muscle unit responses

		Muscle Nerves	**Muscle Units**
Onset	Frequency	55/55	6/6
	Mean	9.3	9.4
	s.e.m.	±0.4	±1.3
First Peak (8<T<20)	Frequency	53/55	6/6
	Mean	14.2	15.1
	s.e.m.	±0.3	±2.4
Second Peak	Frequency	48/55	5/6
	Mean	25.1	29.7
	s.e.m.	±0.6	±3.2
Quiet Period Begin	Frequency	45/55	3/6
	Mean	37.1	37.0
	s.e.m.	±1.3	±4.9
End	Mean	52.8	53.3
	s.e.m.	±1.8	±5.2
Late Response	Frequency	51/55	3/6
	Mean	105.5	102
	s.e.m.	±1.8	±7

General Discussion

Considerations of Afferent Fiber Types Relevant to Lordosis

As described in the introduction, the stimulus protocol used in these experiments was intended to resemble, to the extent possible, the inputs relevant to lordosis behavior. Kow and Pfaff (1979) have shown that the receptor type most likely to be involved in lordosis is the type II pressure unit as described in the cat by Iggo (1966). Burgess et al. (1968) and Hunt and McIntyre (1960, using a different classification scheme) have shown that centripetal conduction from these receptors is mediated by A-β fibers. Nociceptors, known to project via C-fibers are not involved. The extent of the contribution made by other receptor types, including, for example hair/skin units is not known. C-fiber mechanoreceptors, responsive to gentle mechnical stimulation of the skin, as first described by Zotterman (1939) and demonstrated by Douglas and Ritchie (1957) to make up the bulk of the first C deflection elcited by electrical stimulation of cutaneous nerve, lack the ability to respond to rapidly changing stimuli and require 150-200 msec of stimulation at six times threshold to produce a consistent response (Bessou et al. 1971). Since lordosis can be elicited within 160 msec of stimulation by the male rat (Pfaff and Lewis 1974), it would seem unlikely that these receptors contribute significantly to the behavior. Thus, for these experiments, stimulus currents were usually kept below 150 μA. Stimulation at this intensity could be expected to activate Aβ fibers without activation of A-δ or C-fibers. Thus, we are confident that the stimuli which were used in these experiments activated the fibers most relevant for lordosis while minimizing the activation of higher threshold A-δ and C-fibers.

Probable Central Route of the Pudendal Nerve-Evoked Response

To determine whether or not a supraspinal pathway is involved in the pudendal nerve-evoked response the response onset time was compared with the

minimal conduction time, including synaptic delays, required for a spino-bulbo-spinal (SBS) loop, given the known short-latency, monosynaptic connections from the medullary reticular formation to the axial muscles in the cat (Wilson, Yoshida and Schor 1970) and the short-latency connections in the rat (Femano et al. 1984). The mean response latency of 8.8 ms in females includes 1.6 ms of conduction time to the dorsal roots. Brink and Pfaff (1981) demonstrated that LL muscle nerve units could be excited at latencies of 3-5 ms from stimulation of the medullary reticular formation; of this, 1.5 ms is accounted for by synaptic delay and conduction time from the motoneurons to the muscle nerve. The minimal spino-bulbo-spinal pathway would contain at least two more synapses, accounting for 1 ms of the central delay, leaving 3.2 ms (8.8 - 1.6 - 3 - 1) for conduction along the ascending spinal pathway. Shimamura and Livingston (1963) calculated that in the cat, the conduction velocity of spinoreticular fibers, approximately 60 m/sec in their experiments, was as much as twice that of the reticulospinal fibers. If similar relative conduction velocities exist in the rat it is not possible to rule out a supraspinal route on the basis of latency alone. However, experiments with rats one day following complete spinal cord transection (discussed in the following chapter), demonstrate the presence of vigorous short latency responses in the LL muscle nerves to pudendal nerve stimulation, indicating that the short latency responses described here are at least partially segmental.

Since the early responses are subject to bilateral convergence and are apparently mediated by segmental pathways, this convergence must take place within the spinal cord. In recordings made from units located predominately in the dorsal and intermediate gray of the rat spinal cord that were responsive to cutaneous stimulation within the receptive field of the pudendal nerve, Kow and Pfaff (1980) found no units which responded to contralateral inputs. However, in

horseradish peroxidase studies of the terminal fields of the pudendal nerves in the cat, both Thor and coworkers (1982) and Ueyama et al. (1984) have reported contralateral spinal projections, described by the latter group as crossing in the dorsal commissural gray and terminating in the ventromedial portions of the dorsal horn; no direct projections to the ventral horn were seen. The variance between these anatomical studies and the physiological work of Kow and Pfaff (1980) may be attributable to species differences or to physiological specificity of the crossed fibers. The site of bilateral convergence of the pudendal nerve-evoked response within the spinal cord remains to be demonstrated.

The lack of a robust hormone effect on the latency or peak amplitudes of the pudendal nerve-evoked response was unexpected, since our initial hypothesis, based on the effects of hormone levels on lordosis behavior, was that the two female groups (strong vs. weak or absent lordosis) would differ from one another and that they would both be different from the males. Specifically, it was supposed that the enhanced responsiveness to appropriate somatosensory inputs apparent in receptive females would be manifest in increased excitability of the axial muscle nerves to pudendal nerve stimulation in the acute preparation. There are several explanations for the present data. First, evoking the response ordinarily requires a fairly complex sequence of stimuli including flank stimulation preceding perineal pressure (Pfaff and Lewis 1974). This stimulus paradigm, although it activates fibers that are part of the behavior's neural substrate, would be unlikely by itself to evoke the full behavior in the intact female. In addition, these experiments were always done on deeply anesthetized animals, and hormone-sensitive pathways, possibly involving many synapses higher in the neuraxis, may be severely depressed under these conditions. It is also possible that in the awake state the pathways evoked by pudendal nerve stimulation are actively inhibited in the unprimed rat. Lordosis behavior is a highly coordinated

series of movements undoubtedly involving polysynaptic pathways which may themselves be subject to anesthetic depression and which thus did not exert endocrine-dependent influences on the reflexes recorded here. Therefore, although the pudendal nerve-evoked response in axial muscles is undoubtedly a *substrate* for lordosis it cannot be expected to explain the behavior fully.

Chapter 3

Spinal Cord Transections and the Pudendal Nerve-Evoked Response

Summary

In order to examine the supraspinal control of the pudendal nerve-evoked response (PNER) the pudendal nerve evoked activity in the axial muscles following spinal transections was studied. After total transections of the spinal cord in the thoracic region, the evoked responses consisted only of a single peak in activity 22.8 ms (+/- 1.4 ms) following the pudendal nerve stimulus, demonstrating that the earliest responses previously described for the PNER are at least partly segmental in origin. To help identify supraspinal inputs to the PNER a series of partial transections of the spinal cord were made to cut selectively different columns of tracts within the cord. Four different surgeries were performed: transections of the entire dorsal half of the cord; ablations of all of the medial columns; combined lesions of the dorsal half and medial columns, sparing only the tracts within the ventrolateral columns and bilateral transections of the lateral column. Following all transections except those of the lateral columns, the PNER was similar to that seen in intact animals. After lateral column lesions however, the responses were like those seen in the totally transected animals. The combined results suggest that supraspinal influences upon the PNER are conveyed primarily via the ventrolateral columns of the spinal cord.

Bilateral convergence of afferent information was evident in the totally transected animals, indicating that such convergence occurs at segmental levels. The lesions which substantially altered the PNER were analogous to lesions which eliminated lordotic responsiveness in behaving female rats (Kow and Pfaff 1977)

Introduction

In the previous chapter the responses of the axial muscle nerves to electrical stimulation of the pudendal nerves in the rat were described, and have been referred to as the pudendal nerve-evoked response (PNER). On the basis of an 8.8 ms minimal latency from pudendal nerve stimulation to the onset of the axial muscle nerve activation we hypothesized that this evoked response resulted at least in part from segmental circuitry. Arguments on the basis of latency alone are not definitive however, since measured from the first shock in our stimulus trains there is just sufficient time for a spino-bulbo-spinal (SBS) loop not, however, with the 4-6 ms brainstem delay seen by Shimamura and Livingston in the SBS pathway in the cat. While the minimal latency suggests a solely intrinsic spinal pathway for the earliest elements of the PNER, the relatively long duration of the neural activation, which lasts over 100 ms, suggests considerable processing of the afferent information, perhaps including brainstem mechanisms. Purely segmental conditioning effects by cutaneous nerves have been demonstrated, however, which have a time course in the same range as that seen in the PNER. Crenna, Schieppati and deCurtis have demonstrated facilitation of the triceps surae monosynaptic reflex at latencies in excess of 100 ms in animals made spinal with transections at the T12 level, while Rudomin has shown primary afferent hyperpolarization with a time course of 80 ms or more.

Electrical stimulation within the medullary reticular formation (Femano, Schwartz-Giblin and Pfaff 1984a and 1984b) can evoke responses in the axial muscles. Brink et al. (1981) demonstrated activation of ML and LL muscle nerves in the rat following electrical stimulation of the brainstem at latencies as short as 3 ms from the effective shock in multiple shock trains. In the cat, monosynaptic connections from Deiter's nucleus, and from units activated by stimulation within the medial longitudinal fasciculus, have been demonstrated for

the longissimus dorsi muscle (Wilson, Yoshida and Schor 1970), which is homologous to the anterior continuation of the longissimus portion of the lateral longissumus in the rat (Brink 1978).

It was with these considerations in mind that several experiments were performed on the effects of spinal cord transections upon the pudendal nerve-evoked response.

Experiment 1

Materials and Methods

Seventeen female Sprague-Dawley rats with weights from 260 to 354 grams were used in these experiments.

Surgical Preparation

Complete spinal cord transections were made in seventeen rats, of which three died post-operatively. All of the animals used were ovariectomized females treated with at least three subcutaneous 10 μg injections of estradiol benzoate in corn oil solution, or implanted with 5 mm silastic tubes containing estradiol (Smith 1977). Pretreatment with steroids in this way eliminated differences in hormonal state as a source of variablility in the results. Before beginning the experiments the animals were tested manually for lordosis performance according to the method of Harlan et al. (1983; see chapter 2). In these experiments the lordosis scores ranged from 0 to 3.

For spinal cord transections the rats were anesthetized with 25 mg/kg chloropent and the skin overlying the surgical field was infiltrated with 2% Lidocaine HCl (Xylocaine, Astra Pharmaceutical Products). The spinal cord was exposed through the intervertebral space between the T2 and T3 spinous processes, usually without removing any tissue or performing a laminectomy. Some of the total transections were made through a laminectomy of the T8 verte-

bra. In making the transections the cord was cut with a dull spatula and enough neural tissue was aspirated posterior to the cut for the entire cross-section of the cord to be seen under the dissecting microscope. This ensured the completeness of the transections. After sectioning, a ball of cotton was placed into the resultant gap in the spinal cord and the muscles overlying the vertebral column were sutured together; the skin was then pulled together with wound clips. Following the cord surgery the animals were allowed to recover for one or two days and their behavior was observed. Responses to manual stimulation previously adequate to elicit lordosis were noted, as were responses to tail and foot pinches. The completeness of the total transections was verified *post mortem* by gross dissection.

Recording and Stimulation

Details of the recording and stimulation methods are similar to those described in the previous chapter and will thus be summarized briefly here.

For the acute phase of the experiments the rat was anesthetized with 140 mg/kg of Urethane injected intraperitoneally. After removal of the medial longissimus muscle the L3, L4 and L5 spinal nerves were exposed by blunt dissection and the motor branches were separated, then ligated and cut at their distal ends. The proximal ends were then placed onto a bipolar platinum hook electrode for recording. The pudendal nerves on both sides of the animal were exposed surgically and placed onto bipolar stainless steel hook electrodes. The recording and stimulation setup was as shown in figure 2-5.

Our standard stimulus consisted of a train of three 200 μsec shocks separated from each other by 1.5 ms and repeated at a rate of twice per second, although other stimulus repetition rates were occasionally used. Further details of the surgical, recording and stimulation procedures can be found in the previous chapter. Following the surgery the animals were placed in a stereotaxic

apparatus and their heads were clamped lightly in place. Rectal temperature was maintained at 37 degrees Centigrade with a servo-controlled DC heating pad and a heat lamp.

Recording

The outputs of the recording electrodes from the muscle nerves were amplified locally at a gain of 10,000 and bandpass filtered at 100 Hz and 3 kHz. These were then fed to a window discriminator which was used to trigger an Apple IIe computer programmed to produce Post-Stimulus Time (PST) and firing rate histograms of the data on-line (Cohen and Pfaff 1984). Raw data was also monitored on an oscilloscope. The window discriminator was set to trigger only on fairly large spikes. This resulted occasionally in missed spikes but was resistant to false triggering.

Data Analysis

After the recording session the rats were given an intracardiac injection of 0.2 cc of Heparin (1000 U/ml). They were then perfused with saline followed by 10% formalin for 10 minutes or more. The vertebral columns were removed from approximately the C2 through the L3 level and placed in 30% sucrose formalin mixture. The completeness of the total transections was verified *post mortem* by gross dissection.

Where possible, the firing patterns of the axial muscle nerves were analyzed statistically using the method described by in the previous chapter and in Appendix II wherein the peaks were detected by comparison to the prestimulation firing rate. In other cases, in which the prestimulus firing rate was minimal (or the nerve was silent) the latency of the major peaks was noted and no attempt was made to quantify the magnitudes. Latencies were measured from the first shock of the three shock stimulus trains, because it has been shown previously that in the intact animals, single shock stimuli were effective in eliciting activation of the

epaxial muscle nerves.

Results

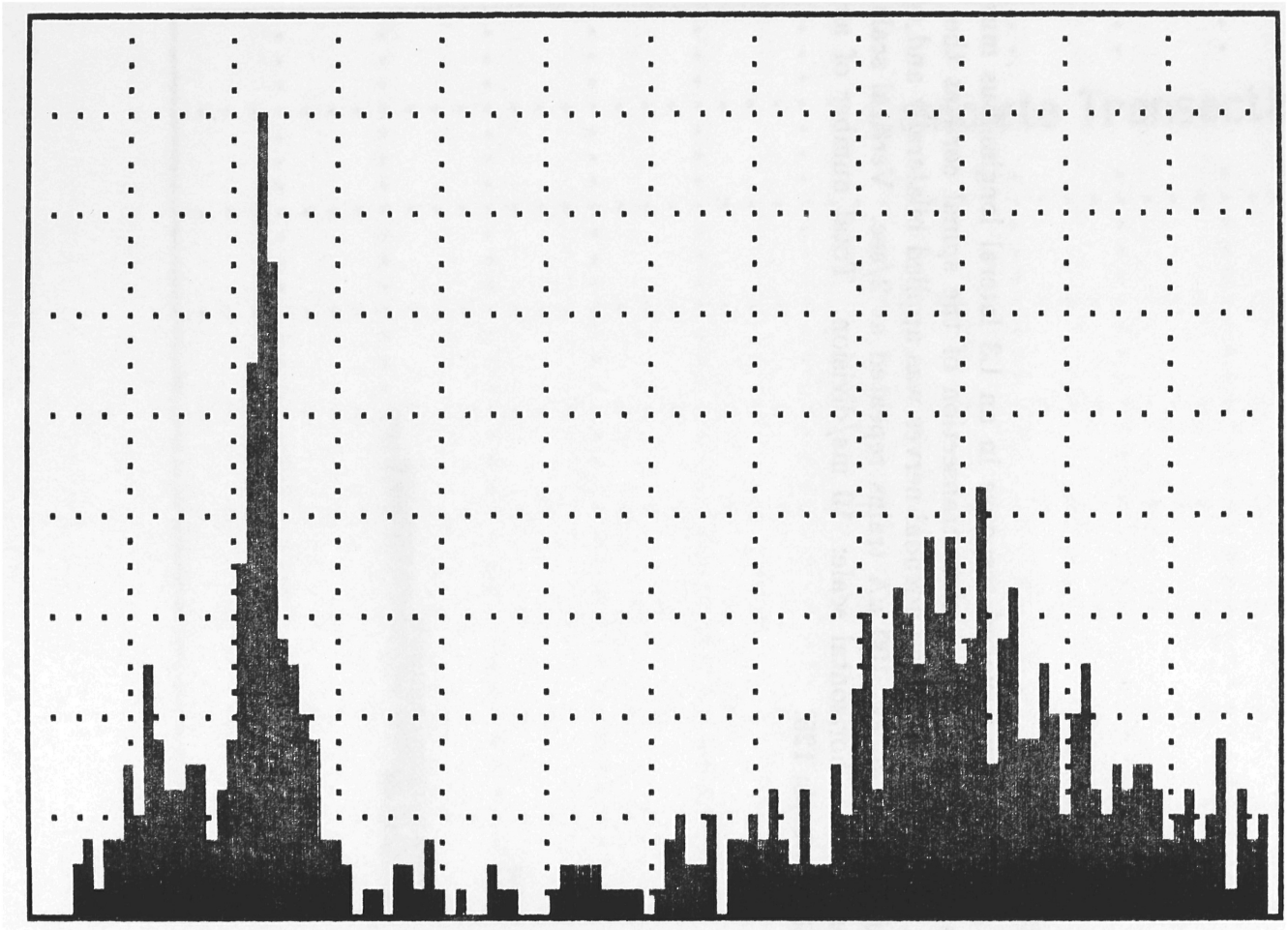
Figure 3-1 shows the temporal profile of the pudendal nerve-evoked response, in an intact animal, to bilateral stimulation of the pudendal nerve with our standard stimulus of triple shock trains repeated at a rate of twice per second. Several distinctive features are noted. In particular there are two early peaks in nerve firing at approximately 15 and 25 ms following the stimulus. After this, there is usually a period of relatively depressed activity from 35 to 50 ms followed by a third late peak in activity near 100 ms. Figure 3-2 shows the response to similar stimulation following a complete transection of the spinal cord. This response differs from that shown in figure 3-1 in a number of its features. The total activity level in the transected animals was markedly reduced as compared to the intact animal; frequently (5 out of 14 animals) all of the nerves were completely silent and showed no firing at all in response to pudendal nerve stimulation. When they were responsive there was never the large activation seen in intact animals. The first signs of evoked firings occur at a latency of approximately 18.5 ms, with a peak at about 20 ms. Among the completely transected animals the mean latency of this isolated activity peak was 22.8 (+/-1.4 s.e.m.) ms. The nerve activity had returned to its low baseline level by 35 ms following the stimulus onset.

In all cases there was a complete absence of activity synchronized to the stimulus at post stimulus latencies greater than 35 ms in the animals which received complete spinal cord transections in marked contradistinction to the intact animals.

In the transected animal, unlike the intact rat, *unilateral* stimulation was *ineffective* in eliciting activation of the axial nerves; bilateral stimulation was

Figure 3-1

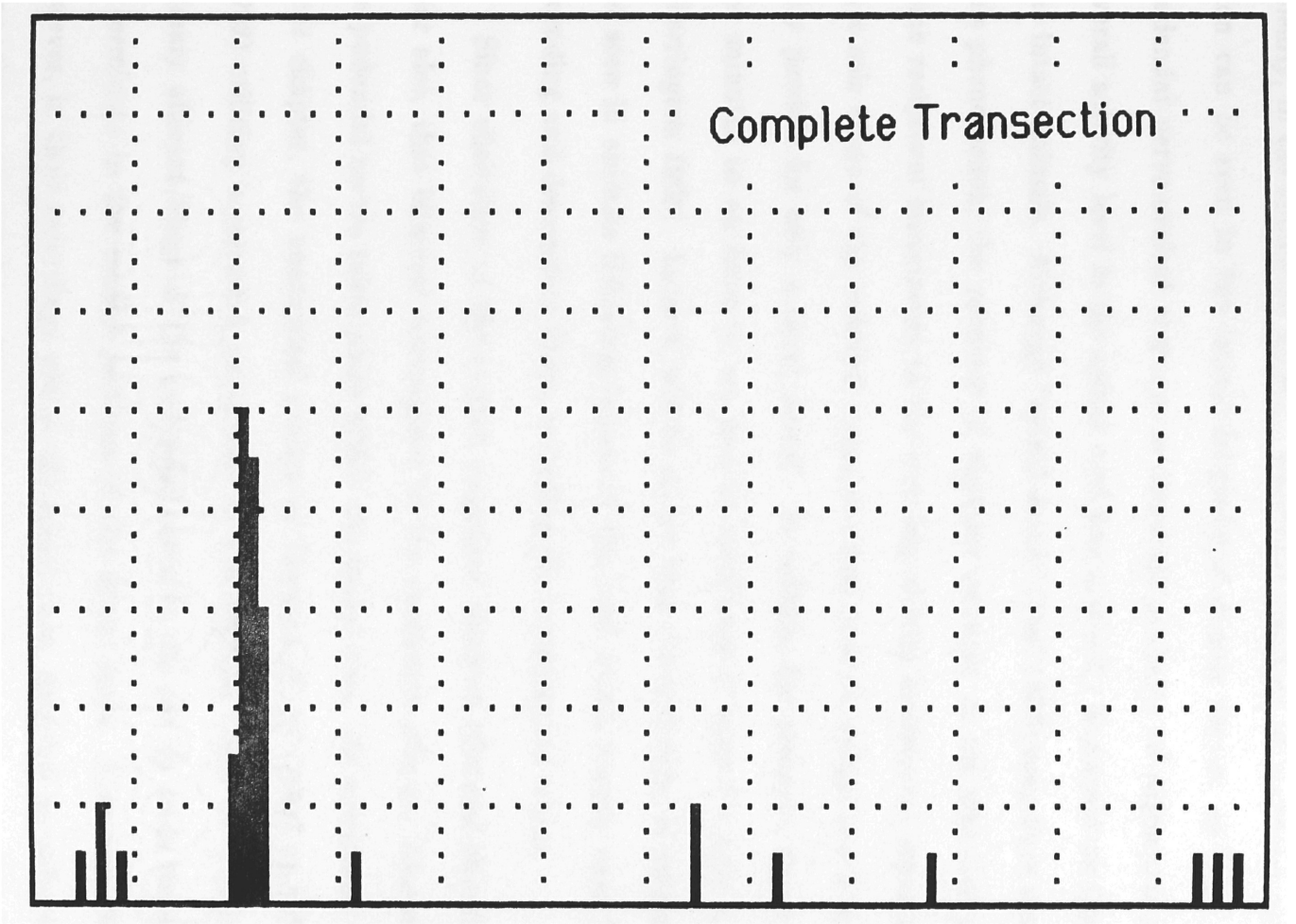
The response evoked in the L4 lateral longissimus muscle nerve by bilateral stimulation of the pudendal nerves with triple shock trains at 50 μA repeated twice per second. Eighty-seven accumulated sweeps. Vertical scale: 4 spikes/division, horizontal scale: 10 ms/division.



L4 muscle nerve
50 µA Bilateral Stimulation at 2/sec
Vertical: 4 spikes/division Horizontal: 10 ms/division
Total number of sweeps: 87

Figure 3-2

The pudendal nerve-evoked response in an L3 lateral longissimus muscle nerve 1 day following complete transection of the spinal cord at the T4 level. Stimulation to the pudendal nerves was applied bilaterally and consisted of triple shock, 100 μA trains repeated at 2/sec. Vertical scale: 2 spikes/division, horizontal scale: 10 ms/division. Total number of accumulated sweeps: 125.



L3 muscle nerve
100 µA Bilateral Stimulation at 1/sec
Vertical: 2 spikes/division Horizontal: 10 ms/division
Total number of sweeps: 125

always necessary.

Discussion

Clearly, in the spinalized animal, significant response to pudendal nerve stimulation can be seen in the lateral longissimus muscle nerves: at least part of the pudendal nerve-evoked response is therefore probably of segmental origin. The overall activity level in the spinal cord was markedly depressed as compared with the intact animals. Although "spinal shock" (Hall 1850) may have contributed to this phenomenon, the presence of vigorous response to tail and foot pinches and some reciprocal movements in the rear legs during locomotion argue against this as a sole cause of the reduced responsiveness, indeed, spinal shock in rats probably persists for only a short period. In rabbits, for example, shock lasts for a few minutes to an hour or so, and in carnivores it lasts for only a few hours (Sherrington 1906). Later it will be shown that the reduction in responsiveness is not seen in animals following lesions of the cord which destroy over 75% of the ascending and descending fibers, including the corticospinal tracts.

Since elicitation of the evoked responses required bilateral stimulation, it is clear also, that bilateral convergence of the cutaneous afferent information from the pudendal nerves takes place within the spinal cord. As mentioned in the previous chapter, the anatomical results of Ueyama et al. (1984) and Thor et al. (1982) utilizing horseradish peroxidase as a retrograde tracer have indicated that primary afferent fibers of the pudendal nerve in the cat do cross the spinal cord to terminate in the medial portions of the dorsal horn. Kow and Pfaff (1980), however, in their recordings within the spinal cord, detected no units which were responsive to stimulation within the receptive field of the contralateral pudendal nerve. The variations in results utilizing these two different methods may result in part from species differences. The data provided by these transection experiments, however, do indicate the presence of some crossed fibers in the spinal cord

responsive to pudendal nerve stimulation in the rat.

Experiment 2

Responses of animals with partial transections of the spinal cord. Total spinal transections have a dramatic impact on the later components of the pudendal nerve-evoked response, while leaving an early component intact. These results are interpreted as clear evidence of the involvement of supraspinal centers in the pudendal nerve-evoked response. Having seen these changes in the activation of the epaxial muscle nerves it was of interest to gather more information as to which spinal cord tracts were involved. To answer this question, we made partial transections of the spinal cord, in order to destroy transmission in a subset of the ascending and descending pathways. The intended lesions included: dorsal half lesions, lesions of the medial columns, lesions of the lateral columns and combined ablations of the dorsal half of the cord and medial columns (sparing the ventrolateral columns). The results of such tractotomies point strongly to the reticulospinal and vestibulospinal systems as candidates for the transmission of supraspinal information. These transection results parallel closely the results of Kow, Montgomery and Pfaff (1977) as well, in that transection of similar spinal cord tracts was effective in depressing both lordosis behavior and the pudendal nerve-evoked response.

Materials and Methods

Experiments were performed in fourteen ovariectomized and estrogen-pretreated female Sprague-Dawley rats with weights from 250 to 325 grams.

Surgical Preparation

Surgical and anesthetic procedures to expose the spinal cord were identical to those described for Experiment 1 above. All spinal cord exposures were made at the T2-3 level, without the need for a laminectomy. For partial transections the cord was cut with a microknife. The surgical exposure was then sutured closed

as described for Experiment 1. All animals were allowed at least one day of recovery following these partial transections. After this period, withdrawal reflexes to tail and foot pinches were active.

The recording and stimulation procedures were exactly as described for Experiment 1.

Data Analysis

Analysis of the electrical responses was as described above.

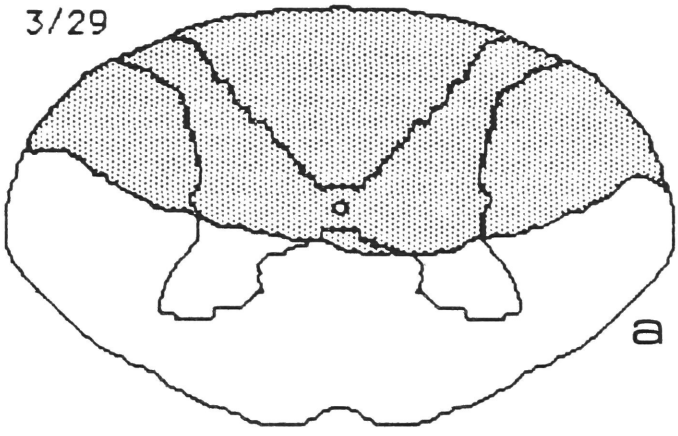
After perfusion with saline followed by formalin a section of vertebral column containing the lesion was removed and cryoprotected in sucrose/formalin. A section of the spinal cord containing the lesion and intact tissue for several segments rostral and caudal to the lesion was removed from the vertebral column and embedded in gelatin/albumin mixture and allowed to set in fixative for 3-7 days. Cords with dorsal half lesions and combined dorsal and medial lesions were sliced coronally using a freezing microtome into 50 or 75 μm sections. Spinal cords with medial and lateral column lesions were sliced parasagitally and horizontally respectively. The tissue was then stained conventionally with cresyl violet and luxol fast blue, and the extent of the surgical lesions was assessed under the micrscope. Drawings of the sections were made under a projection microscope. As verified histologically, the actual lesions were generally similar to the intended lesions.

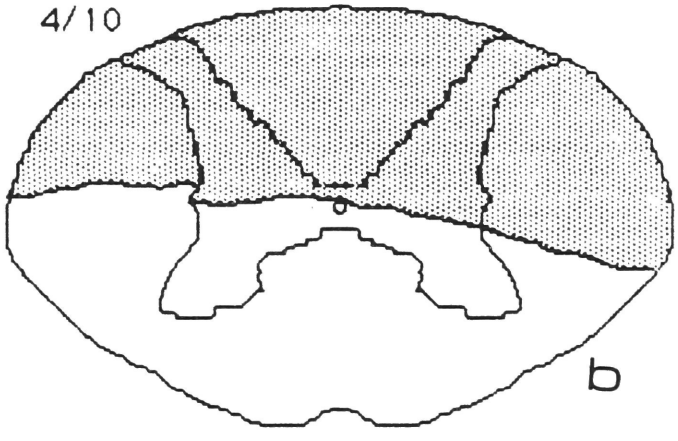
Transections of the Dorsal Half of the Spinal Cord. Three animals were lesioned in the dorsal half of the spinal cord; figure 3-3 shows the actual lesion sites.

A representative PNER from one of these animals is shown in figure 3-4. The PNER in all of the animals with dorsal half lesions was substantially similar. The figure shows a broad peak in activity in the time interval from 10 to 30 ms following the stimulus onset. This is followed by a period of relatively reduced

Figure 3-3

Locations of lesions of the dorsal columns of the spinal cord made in 3 animals. Lesioned areas are shaded, intact areas are indicated in white. The locations of the lesions was reconstructed from cresyl violet and luxol fast blue stained sections.


3/29
a


4/10
b

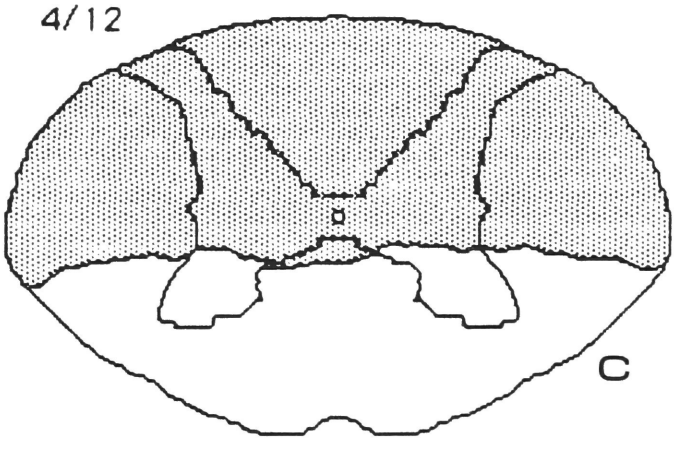
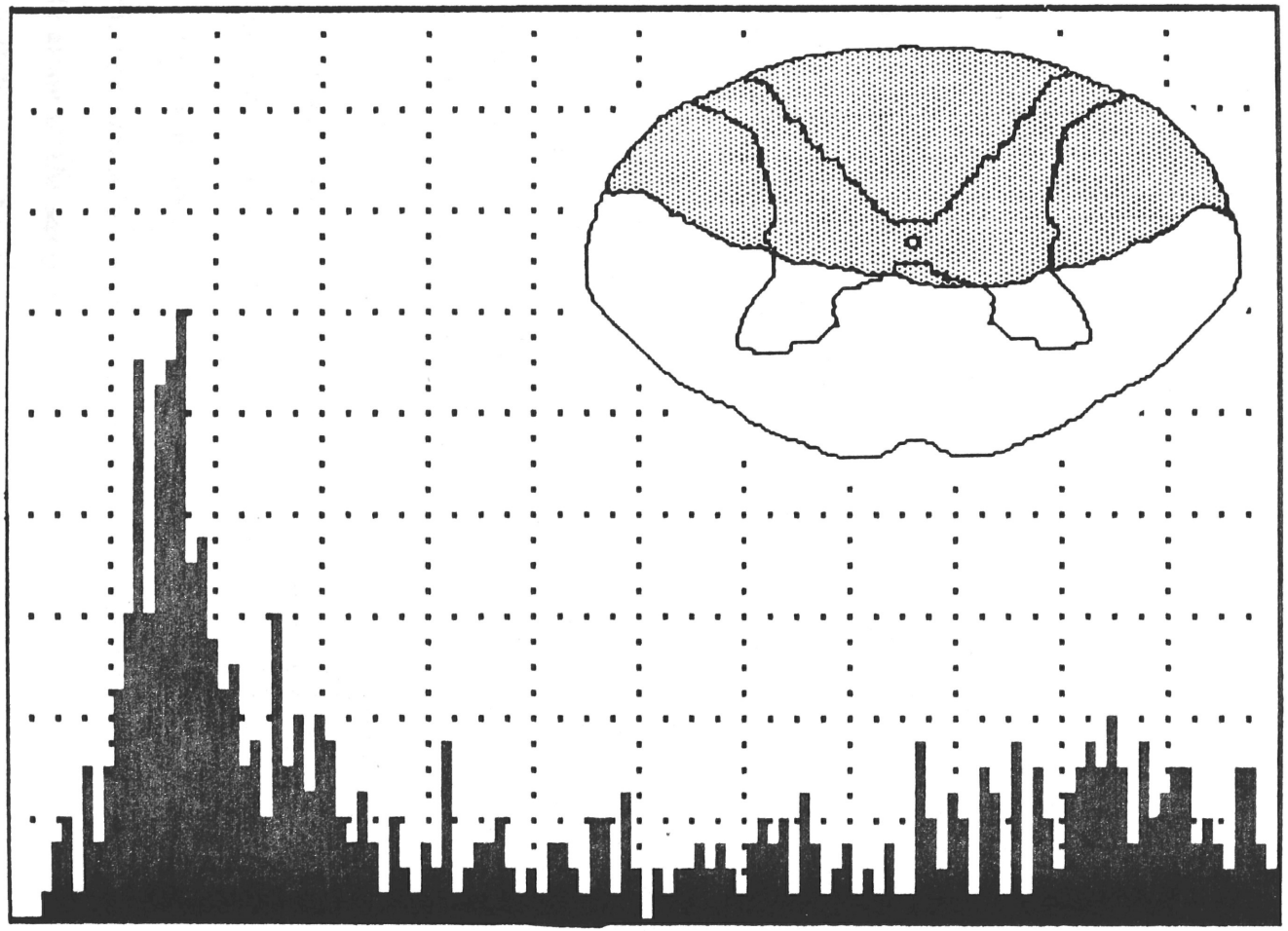

4/12
c

Figure 3-4

The pudendal nerve-evoked response recorded in the L3 lateral longissimus muscle nerve of a rat one day following transection of the dorsal columns of the spinal cord as shown in the inset. Stimulation applied to the pudendal nerves consisted of triple shock bilateral 20 μA trains repeated at 2/sec. The PST histogram shows the accumulated responses to 82 stimulus iterations. Vertical scale: 4 spikes/division, horizontal scale: 10 ms/division.



L3 Muscle Nerve
20 µA Bilateral Stimulation at 1/sec
Vertical: 4 spikes/division Horizontal: 10 ms/division
Total number of sweeps: 82

spike activity lasting from approximately 30 to 60 ms. Elevated activity is apparent between 100 and 150 ms after the stimulus train. In overall character and specific latencies for the response onset, early peaks, onset and duration of the quiescent period and location of the peak near 100 ms, these responses were similar to those seen in the intact animals. The second early peak near 25 ms was not seen in one of the animals.

Stimulation to either the ipsilateral or contralateral pudendal nerve alone evoked responses in the lateral longissimus muscle nerves. Bilateral summation of the activity was readily demonstrated. The overall activity level was high as compared to the totally transected animals and was similar to that seen in intact rats.

Transections of the Medial Columns. Figure 3-5 shows the actual lesion sites for five animals which received ablations intended to destroy the medial portions of the spinal cord. The extent of the lesions, especially near the most ventral part of the cord varied somewhat. Several of the lesions did not completely destroy the ventromedial columns bilaterally.

The pudendal nerve-evoked responses of these animals were comparable to each another; a typical example is shown in figure 3-6. All of the features seen in the intact animals were seen in the lesioned animals and at similar latencies: again the PNER's of the partially transected animals were similar to those seen in the intact animals. In four of the five animals the 100 ms peak was especially prominent, exceeding the magnitude of the earlier activity peaks.

In these animals also, while responses were still seen to unilateral stimulation, bilateral stimulation produced greater responses than stimulation to either side alone at the same stimulus intensity. The apparently very high general level of activity seen in the accompanying figure 3-6 does not represent a consistent

Figure 3-5

Locations of lesions of the medial columns of the spinal cord made in 5 animals. Lesioned areas are shaded, intact areas are indicated in white. The locations of the lesions was reconstructed from cresyl violet and luxol fast blue stained sections.

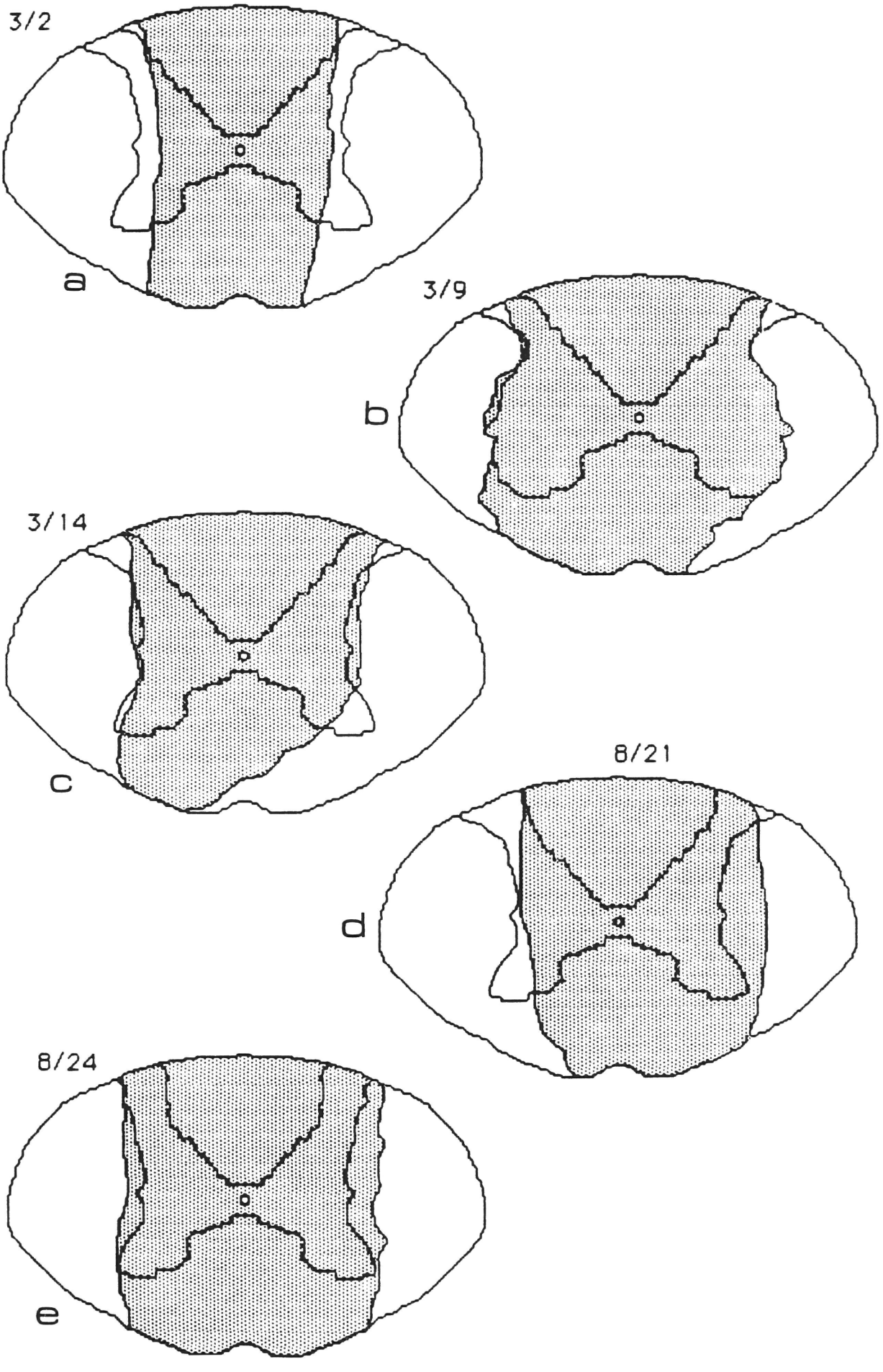
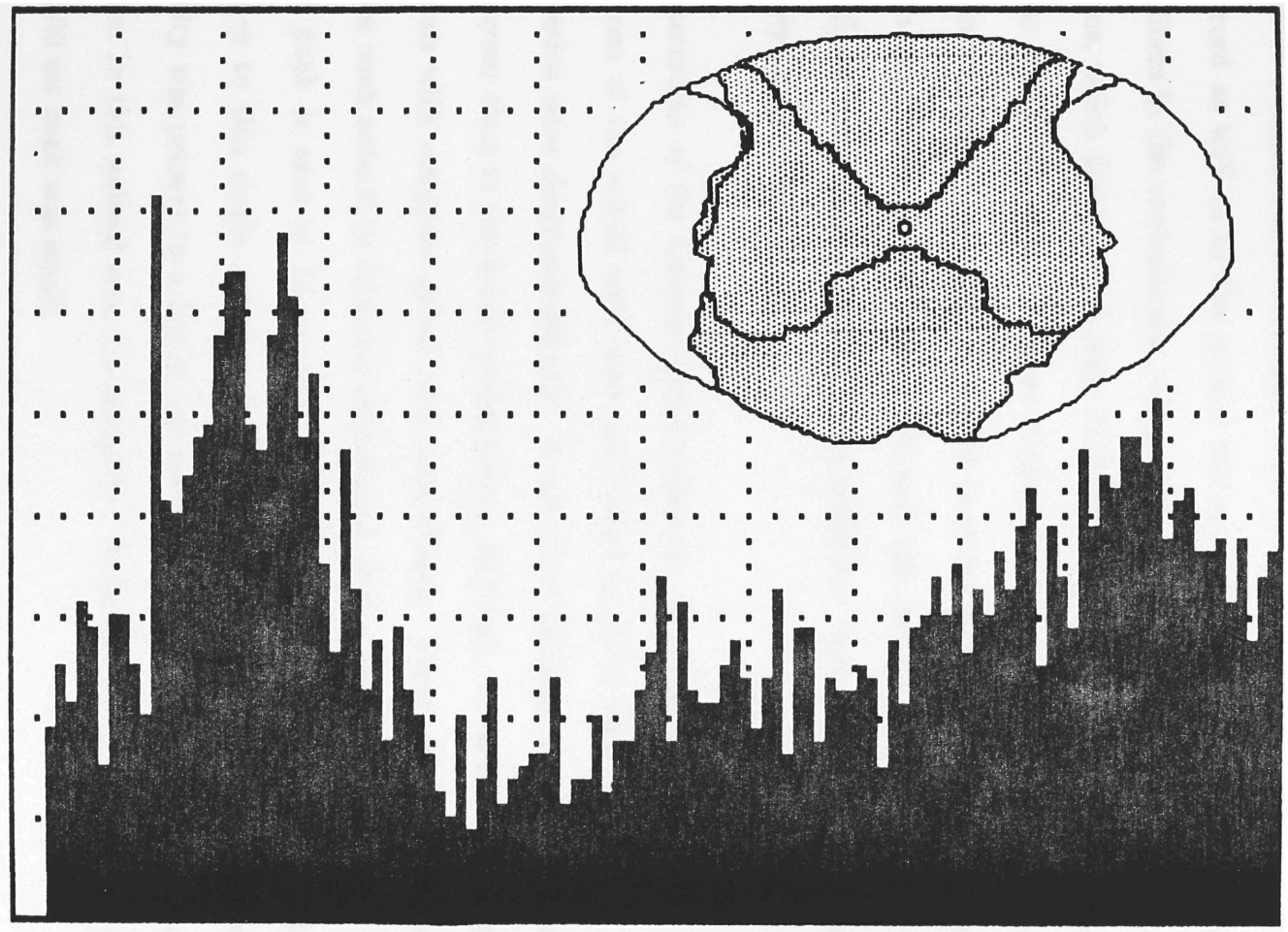

3/2
a
3/9
b
3/14
c
8/21
d
8/24
e

Figure 3-6

The pudendal nerve-evoked response recorded in the L4 lateral longissimus muscle nerve of a rat one day following transection of the medial columns of the spinal cord as shown in the inset. Stimulation applied to the pudendal nerves consisted of triple shock bilateral 200 μA trains repeated at 2/sec. The PST histogram shows the accumulated responses to 170 stimulus iterations. Vertical scale: 8 spikes/division, horizontal scale: 10 ms/division.



L4 Muscle Nerve
200 µA Bilateral Stimulation at 2/sec
Vertical: 8 Spikes/division Horizontal: 10 ms/division
Total Number of Sweeps: 170

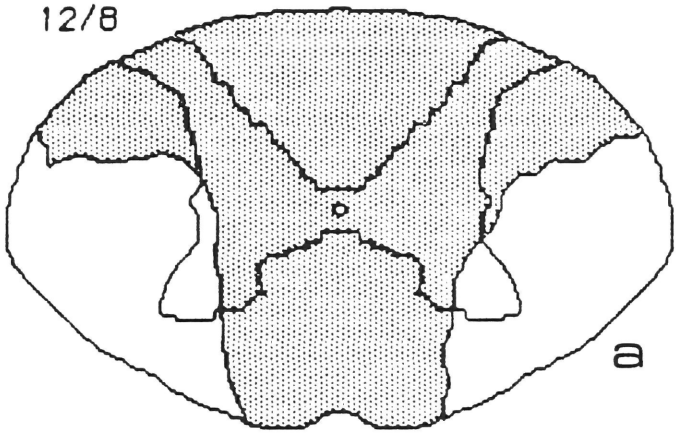
difference in overall activity between these animals and those which received other partial spinal transections.

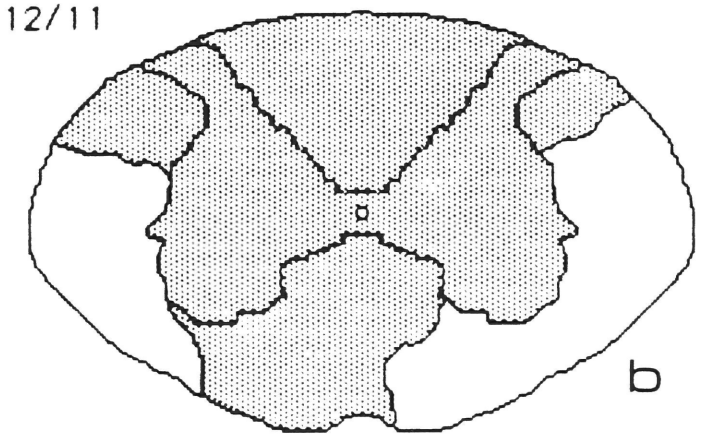
Combined Lesions of Dorsal and Medial Columns. Figure 3-7 shows lesions made in three animals which destroyed most of the fibers in the dorsal half of the spinal cord as well as in the medial columns. These lesions leave intact primarily the fibers in the ventrolateral columns of the spinal cord. Remarkably, after such lesions, which destroyed at least 75% of the white matter in the cord at the level of the lesion, the pudendal nerve-evoked response did not differ from that seen in the intact animal. A typical example is shown in figure 3-8. Prominent response peaks are visible at latencies of 15 and 100 ms and (not visible in the figure) significant periods of depressed firing incidence were often present from approximately thirty to 50 ms.

Transections of the Lateral Spinal Columns. Bilateral transection of the lateral portions of the spinal cord were performed in three animals. Figure 3-9 shows the lesion sites diagrammatically. In all three animals, the overall activity level was lower than in the intact preparations, although not as low as that seen in the animals with complete spinal cord transections. Figure 3-10 shows the response of one such animal to bilateral stimulation of the pudendal nerves. A single distinct peak is seen at 14 ms with no further significant activity. The average latency to this single activity peak was 15.3 (+/-3.2) ms. In one animal, late activity was present in a few of the recordings. As shown in figure 3-9c the transection in this animal was not complete. Even in this animal the magnitude of the 100 ms peak was small.

Figure 3-7

Locations of combined lesions of the medial and dorsal columns of the spinal cord made in 3 animals. Lesioned areas are shaded, intact areas are indicated in white. The locations of the lesions was reconstructed from cresyl violet and luxol fast blue stained sections.


12/8
a


12/11
b

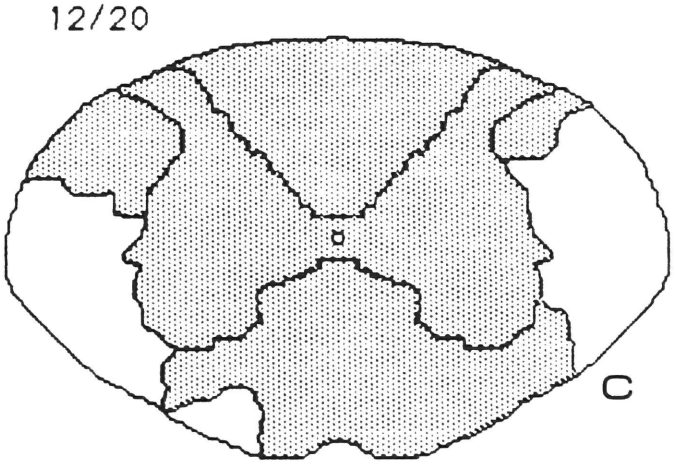
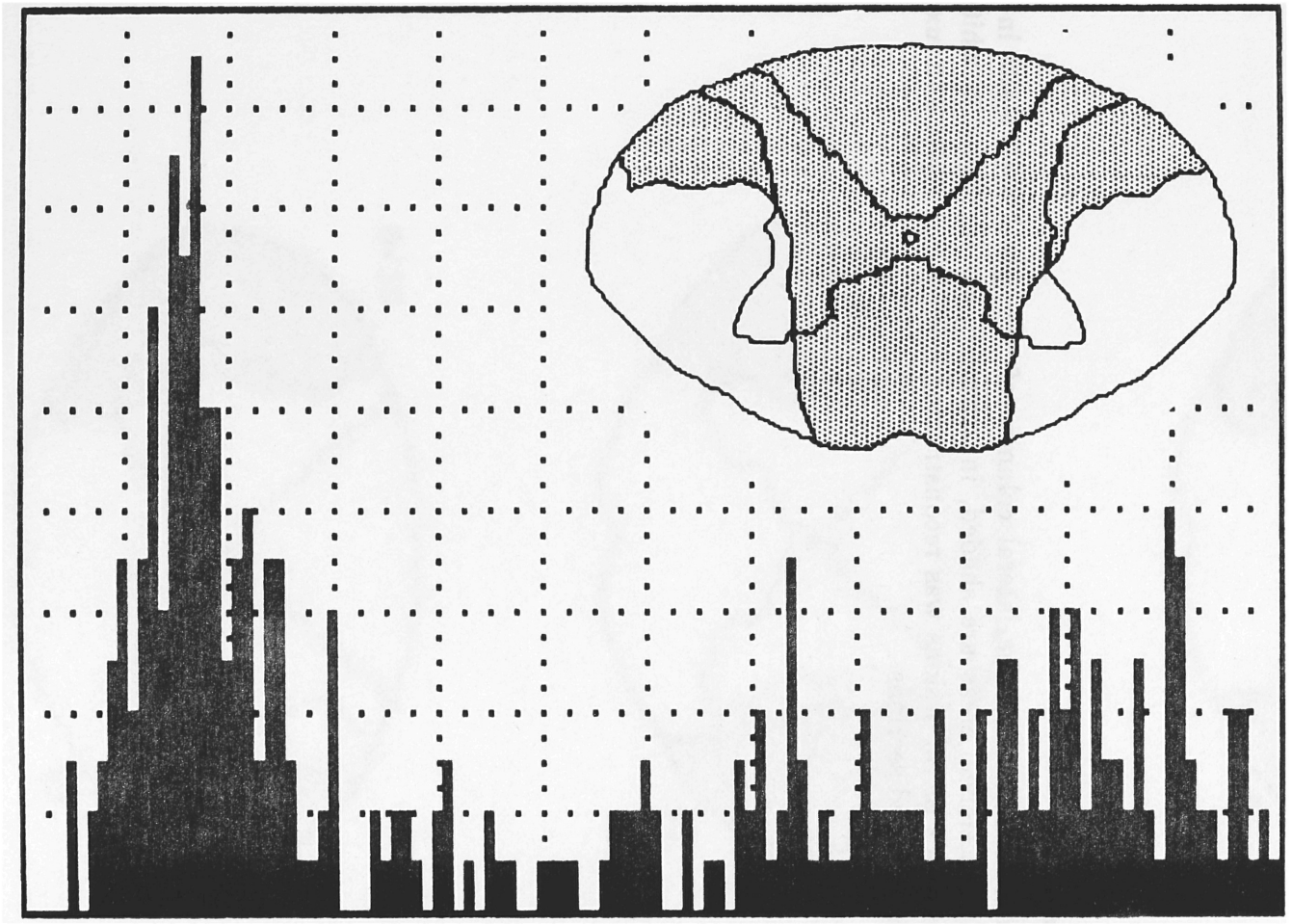

12/20
c

Figure 3-8

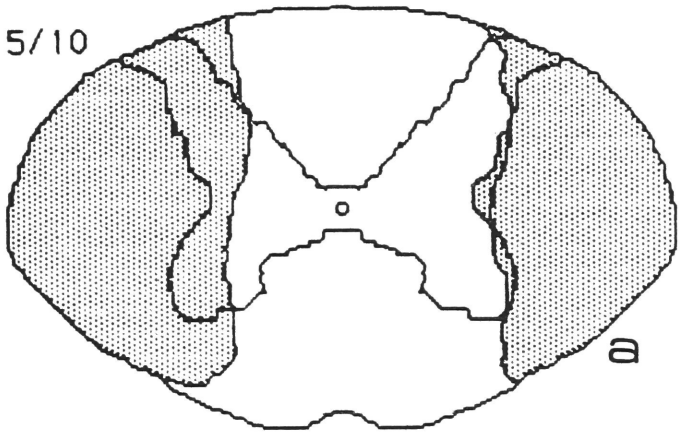
The pudendal nerve-evoked response recorded in the L4 lateral longissimus muscle nerve of a rat one day following transection of the medial and dorsal columns of the spinal cord as shown in the inset. Stimulation applied to the pudendal nerves consisted of triple shock bilateral 75 μA trains repeated at 2/sec. The PST histogram shows the accumulated responses to 80 stimulus iterations. Vertical scale: 2 spikes/division, horizontal scale: 10 ms/division.

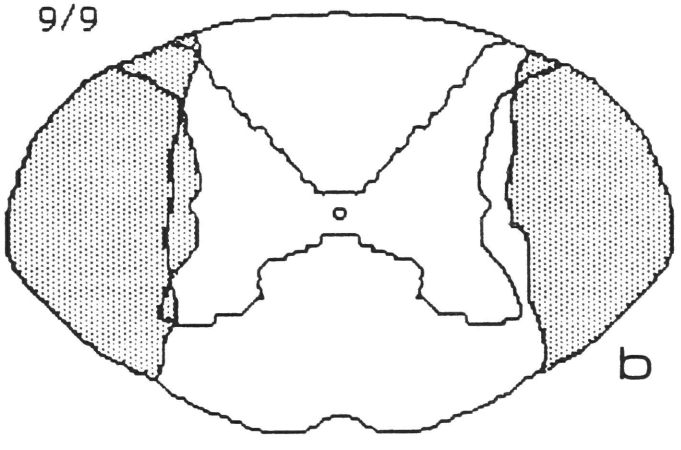


L4 Muscle Nerve
75 µA Bilateral Stimulation at 2/sec
Vertical: 2 spikes/division Horizontal: 10 ms/division
Total Number of Sweeps: 80

Figure 3-9

Locations of lesions of the lateral columns of the spinal cord made in 3 animals. Lesioned areas are shaded, intact areas are indicated in white. The locations of the lesions was reconstructed from cresyl violet and luxol fast blue stained sections.


5/10
a


9/9
b

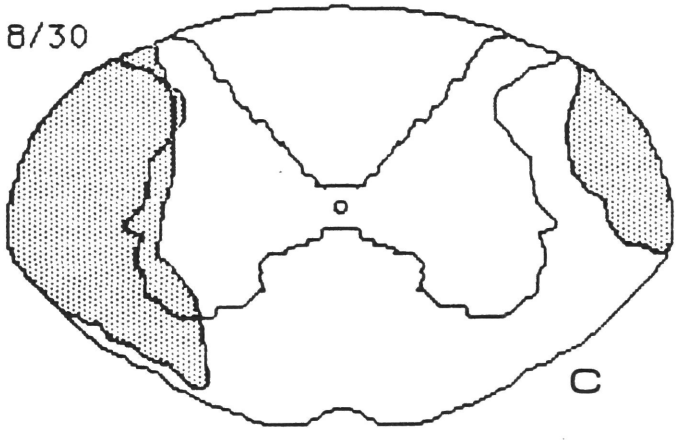
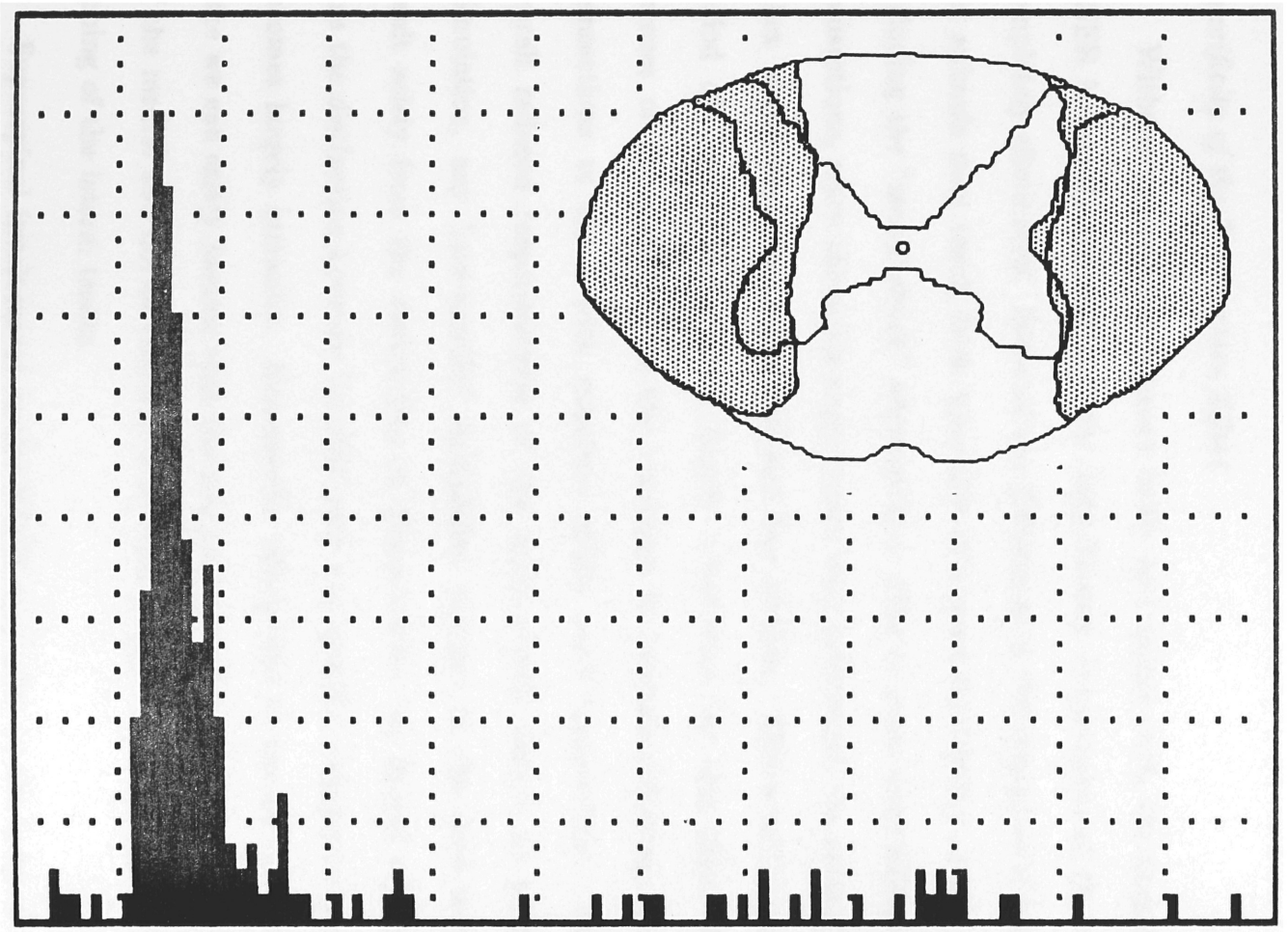

8/30
c

Figure 3-10

The pudendal nerve-evoked response recorded in the L4 lateral longissimus muscle nerve of a rat one day following transection of the lateral columns of the spinal cord as shown in the inset. Stimulation applied to the pudendal nerves consisted of triple shock bilateral 75 μA trains repeated at 2/sec. The PST histogram shows the accumulated responses to 39 stimulus iterations. Vertical scale: 2 spikes/division, horizontal scale: 10 ms/division.



L4 Muscle Nerve
75 µA Bilateral Stimulation at 2/sec
Vertical: 2 spikes/divison Horizontal: 10 ms/division
Total number of sweeps: 39

General Discussion

The results of the experiments described in this chapter display strong evidence for a component to the PNER which is purely segmental: following complete transections a short latency component is still present. The experiments show also that there is bilateral convergence, intrinsic to the spinal cord, of cutaneous information upon the motor pathways of the axial muscles.

Specificity of the Transection Effect

Without connections between brain and spinal cord, the magnitude of the PNER is greatly reduced and the long latency components of the response are completely eliminated. Some of the differences in the responses of intact and spinal animals may result from generally decreased excitability of the spinal cord, including the "spinal shock" phenomenon. One or more days following complete transections, when the acute experiments were performed, the animals had strong reflex withdrawal response to tail and foot pinches. This would suggest that the period of "spinal shock" had largely terminated by this time. In addition, because of the similarity of the responses in animals following lateral column transections to those with complete spinal cord transections - including the overall reduced responsiveness of the axial muscle nerves to pudendal nerve stimulation, any 'non-specific' excitability changes in the cord would have to result solely from the destruction of fibers within the lateral columns. In this case the distinction between 'specific' and 'non-specific' changes in the spinal cord becomes largely semantic. Non-specific effects due to trauma are controlled for since we can safely assume that the trauma induced by the combined transections of the medial and dorsal columns is at least as great as that involved in the sectioning of the lateral tracts.

Supraspinal involvement in the PNER may exist in the form of a reciprocal

pathway between the brain and spinal cord, or as a descending facilitatory influence from the brain upon segmental circuitry, or both. Shimamura and Livingston (1963) have described a spino-bulbo-spinal (SBS) reflex in the cat, whose involvement extends to the spinal nerves in the thoracic region as well as to the other domains of the spinal cord. Presumably, nerves of the axial muscles make a major contribution in this region, since limb muscles are not involved. The response latencies seen in the cat SBS reflex, would translate to approximately 9.7 ms in the rat, assuming comparable conduction velocities in the latter species: 1.6 ms from pudendal nerve to cord dorsum (Cohen et al. 1984), 1.0 ms in the ascending and 2.1 ms in the descending spinal pathways (based on Shimamura and Livingston's estimated conduction velocities of 65 and 33 m/s respectively), 4 ms of central delay in the brainstem and at least 1 ms of synaptic delay in the spinal cord. Thus, the known SBS reflex could contribute to the early activity in the PNER. The early activity remains, however, following complete transection of the spinal cord and thus certainly does not require the activation of an SBS pathway, although SBS responses may contribute to the PNER intact animals. The activity at 100 ms may still be the result of a supraspinal loop, and in fact allows time enough for a transcortical route. Cortical evoked responses to pudendal nerve stimulation have been seen in humans (Haldeman et al. 1982) and single units in the pericruciate cortex of cats have been shown to respond to pudendal nerve stimulation (Slimp and Towe 1980). The decrease in the magnitude of the early activity in the PNER following total spinal cord transections, relative to intact animals is likely to result from the removal of facilitatory input from supraspinal centers, since that response is at least partially segmental, but experiments based on transections alone cannot fully distinguish these possibilities.

Identity of the Pathways Involved in the Pudendal Nerve-evoked Response

Each of the partial spinal cord transections destroyed a subset of the fibers within the spinal cord, yet only lesions involving destruction of the lateral columns produced gross disruption of the pudendal nerve-evoked responses similar to those seen following complete spinal cord transections.

Transections of the dorsal portion of the spinal cord in the rat destroy the descending fibers of the corticospinal system (Brown 1971, Elger et al. 1977). Fibers of the rubrospinal system run in the dorsal part of the dorsolateral columns in the rat (Brown 1974), as do fibers which take origin in the raphe magnus. Dorsal half lesions could also be expected to destroy ascending fibers of the cuneate and gracile fasciculi as well as the fibers of the dorsal spinocerebellar tract. Thus, none of these fiber tracts can be necessary for pudendal nerve-evoked activity similar to that in intact animals.

Successful lesions of the medial columns, like those in 3-5a and 3-5b can be expected to destroy the corticospinal system and the cuneate and gracile fasciculi as discussed above in reference to the dorsal half sections. Fibers of the tectospinal tract descend in the ventromedial columns but only as far as the upper cervical regions (Waldron et al. 1969) in the rat. In the cat, the medial vestibulospinal tract descends in this region (Nyberg-Hansen 1966). The fibers of the midbrain reticular formation descend in the ventromedial columns of the rat, but only as far as the midthoracic levels. Thus these fibers of theses pathways are unlikely to affect directly the responses we have studied.

The transections of the dorsal half of the cord and medial columns were intended to spare only the ventrolateral columns of the spinal cord. The descending fiber systems in the rat in this spared region include a major component originating, predominately ipsilaterally, in the nucleus gigantocellularis of the medullary reticular formation; vestibulospinal fibers of the ipsilateral lateral

vestibular nucleus; axons from the locus ceruleus and from pars caudalis of the pontine reticular formation. The nucleus raphe magnus and contralateral red nucleus also contribute fibers to the ventrolateral columns in the rat (Zemlan et al. 1979). Ascending fibers of the ventrolateral columns include a large spinoreticular component terminating ipsilaterally through much of the pontine and medullary reticular formation. Other significant contributors to the white matter of the ventrolateral cord in the rat include the spinothalamic tract, spinovestibular tract (terminating estensively in the lateral vestibular nucleus), the ventral spinocerebellar tract, spinotectal fibers and fibers projecting to the mesencephalic central gray (Zemlan et al. 1978). Any or all of these fibers may be necessary for the production of teh pudendal nerve-evoked response in the the intact animal.

Possible Sources and Mechanisms of Descending Control

In the cat, electrical stimulation of the medullary reticular formation (Peterson, Pitts and Fukushima 1979) and of the vestibular nucleus (Wilson, Yoshida and Schor 1970) have been shown effective in exciting the motoneurons of the epaxial muscles of the neck and thoracic back. Brink and Pfaff (1981) have demonstrated that in the rat stimulation of these brainstem regions, and also of the midbrain, potentiates the reflex activation of the lateral longissimus and medial longissimus muscle nerves evoked by stimulation of the dorsal roots and in some cases may excite these muscle nerves directly. Such observations lead us to suspect that descending influences from the reticular formation, lateral vestibular nuclei and central gray may each be significant contributors to the long latency components of the PNER.

Supraspinal influences upon the excitability of segmental pathways are well documented in the cat, especially in reflexes of hindlimbs (Hongo et al. 1969, Holmqvist and Lundberg 1961, Rudomin et al. 1983, Amassian et al. 1982, Martin, Haber and Willis 1979, Lundberg, Malmgren and Schomburg 1977).

Considerable evidence exists to indicate that suprapinal nuclei (Rudomin et al.1983) and cutaneous inputs (Engberg, Lundberg and Ryall 1968) can modulate the transmission of Ia and Ib reflex pathways either through changes in the excitability of interneurons or by alterations in the level of primary afferent depolarization and there is evidence that the sign of the effects from cutaneous afferents and brainstem is the same (Rudomin 1983). The primary afferent depolarization of cutaneous fibers in the A-α, A-β and A-δ ranges of conduction velocity is subject to modulatory control from brainstem structures including the raphe magnus and reticular formation (Martin, Haber and Willis 1979). It is possible that an analogous system, acting upon interneuronal elements in the pathway mediating the pudendal nerve-evoked response, conveys modulatory influences from supraspinal centers. The loss of such influences may be responsible for the reduction in the magnitude of the shortest latency components of the PNER following spinal cord transection.

In a series of experiments on the effects of partial transections of the spinal cord on lordosis behavior, Kow and his collaborators (1977) showed that intact fibers in the ventrolateral columns of the spinal cord were both necessary and sufficient for lordotic responsiveness to appropriate somatosensory stimuli. The transections described in this chapter closely parallel those made by Kow et al. The parallel between their results and those described here is striking: lesions which disrupt lordotic responsiveness also drastically alter the pudendal nerve-evoked response, other lesions, destroying the majority of the long spinal conduction pathways leave both the pudendal nerve-evoked response and lordosis performance intact.

Electrical stimulation of the lateral vestibular nucleus (Modianos and Pfaff 1977) and of the central gray (Sakuma and Pfaff 1979a) have each been shown to facilitate lordosis behavior in freely moving female rats, and lesions of the central

gray (Sakuma and Pfaff 1979b), medullary reticular formation (Modianos and Pfaff 1979) and lateral vestibular nucleus (Modianos and Pfaff 1976) produce decrements in lordosis performance, even when such lesions produced no impairments of posture or movement. Interruption of the fiber pathways conveying descending information from these brainstem structures also markedly reduce the magnitude and time course of the PNER and thus provide evidence that the mechanisms underlying the PNER parallel those which provide supraspinal control of lordosis behavior.

Chapter 4

Brainstem Stimulation Effects on the Pudendal Nerve-Evoked Response

Summary

Stimulation with bipolar electrodes located within the medullary reticular formation including the gigantocellular and parvocellular regions evoked activity in the lateral longissimus muscle nerves at latencies of 2.6 to as much as 70 ms from the effective shock in a multi-shock train. In all cases, trains of stimulation were required to elicit such activity in the nerves. Response latencies varied with location of the brainstem stimulating electrode and with stimulus intensity. Although most of the stimulating sites were ipsilateral to the recordings, when contralateral stimulation was used, evoked responses were sometimes seen. Combined stimulation of the brainstem and pudendal nerves demonstrated facilitatory effects upon the excitability of motoneurons of the lateral longissimus muscle. Combined stimulation to the brainstem and pudendal nerves evoked responses not visibile with stimulation to either site alone. The response latency to combined stimulation was shorter than that to either stimulus applied individually.

Introduction

Following transections of the spinal cord which include the ventrolateral columns the pudendal nerve-evoked response is reduced to a single prominent peak in activity. This activity peak occurs approximately 23 ms after complete spinal cord transections and at 15 ms after destruction of the lateral columns alone. Following lesions which spare the ventrolateral columns the evoked responses are similar to those seen in intact animals, and consist of multipeak responses with significant activity extending to well over 100 ms.

Located in the ventrolateral portion of the spinal cord of the rat are pathways to and from the reticular formation and vestibular nuclei (Zemlan et al. 1978, 1979), as well as descending fibers originating in the nucleus subceruleus and raphe magnus regions of the brain (Zemlan et al. 1979). Ascending tracts include also spinotectal and spinothalamic fibers (Zemlan et al. 1978).

The pathways connecting the spinal cord and the reticular and vestibular nuclei are of particular interest because of their reciprocal nature, their known involvement in axial muscle control and their pattern of innervating spinal nerves at several segmental levels. This pattern of connectivity makes these spinobulbospinal systems a likely candidate for the mediation of axial muscle responses to cutaneous stimulation. Direct electrical stimulation of the medullary reticular formation in the region of the MLF has been shown to activate the motoneurons of the longissimus dorsi system in the cat via monosynaptic connections (Wilson, Yoshida and Schor 1970). Brink (1981) demonstrated the presence of short, 3-5 ms, latency pathways from the reticular formation to the lateral longissimus muscle nerves in the rat and Femano et al. (1984a and 1984b) have shown that EMG activity in the rat lateral longissimus muscle can be evoked at latencies of 4-7 ms by electrical stimulation of sites within the medullary reticular formation.

It is known from the work of Peterson et al (1975) that the majority (86%) of the reticulospinal neurons having axon terminals within the cervical enlargement also have branches which extend to lower spinal levels. This pattern of branching makes such reticulospinal units ideally suited to the coordinated activation of the axial musculature in a behavior such as lordosis which requires the simultaneous control of axial muscle groups occurring at several spinal levels.

Recordings made within the reticular formation of freely moving, unanesthetized rats demonstrate that cells in that region are responsive to somatosensory stimulation of the perineum. Manual stimulation of the type effective in eliciting lordosis in estrogen-primed animals (see methods, chapter 2, Experiment 3), evoked both excitatory and inhibitory responses in units identified by antidromic invasion from the spinal cord as reticulospinal (Kow and Pfaff 1982). Such units may well be involved in the brainstem control of lordosis. The majority of these units responded "promptly" to the cutaneous stimulation. Others responded only after longer latencies, and may be involved in producing a tonic descending drive to spinal units.

On the basis of these results, and those reported in the previous chapter with partial spinal cord transections, it was of interest to examine in detail the effects of brainstem activation upon the PNER.

Materials and Methods

Brainstem stimulation experiments were performed in a total of seventeen female Sprague-Dawley rats. The animals were ovariectomized females with weights from 214 to 278 grams all of which were pretreated with 10 μg subcutaneous injections of estradiol benzoate in corn oil or subcutaneous implants with 5 mm silastic tubes cointaining crystalline estradiol. Each animal was tested for lordosis performance in response to manual stimulation on the day of the experiment.

The animals were anesthetized with 140 mg/kg intraperitoneal injections of Urethane (Sigma Chemical Co.) in 40% solution with distilled water, supplemented as needed to maintain constant anesthetic depth (N=8 animals) or with 50 mg/kg Sodium Pentobarbitol in propylene glycol and aqueous solution (Nembutal, Abbott Laboratories), supplemented as needed (N=9 animals).

The pudendal nerves on both sides of the animal were prepared for stimulation and the L3, L4 and L5 muscle nerves were prepared for recording as discussed in chapter 2. Following the nerve surgery the animals were moved to a stereotaxic apparatus. For these experiments, earbars were inserted and the head of the animals was clamped in place to an incisor bar adjusted to 5 mm below earbar zero. Lidocaine HCl in 2% solution (Xylocaine, Astra Pharmaceutical Products) was injected into the skin overlying the cranium and neck. A midline incision was made over the dorsal surface of the cranium and the fascia overlying the parietal bone was scraped away with a spatula. The neck muscles and their tendons were bluntly dissected over the midline and cut away from the occipital crest with scissors. The occipital bone was then scraped clear of tissue and the neck muscles, together with the overlying skin, were deflected, using suture tied to the stereotaxic frame. In most cases the dorsal surface of the brainstem, at the level of the obex, could be exposed at this point by removal of the dura and pia with no removal of bone. Occasionally it was necessary to remove small amounts of the occipital bone with rongeurs to obtain the desired exposure. In two experiments (those using a vertical electrode approach, as described below), the head of the animals was adjusted in the sterotaxic apparatus such that the head was levelled between the lambda and bregma points. The occipital bone was removed along the midline, from the level of the foramen magnum up to the

level of the sagittal sinus, approximately 1 mm caudal to the lambdoid suture.

Stimulation

Stimulation was applied to the brainstem via a twisted pair of 75 μm Teflon-covered stainless steel electrodes or by a similar twisted pair of tungsten electrodes. The wire pairs were coated with cyanoacrylate glue and the tips of the electrodes were cut obliquely at an approximately 45 degree angle to yield a sharp end for penetration into the brain. Stimulation was applied to the pudendal nerve with stainless steel hook electrodes as described in previous chapters. Brainstem stimulation was produced by a combination of Tektronix function generators and a Grass S4 unit. The brainstem electrode was driven through an SIU5 stimulus isolation unit.

Two types of penetration were used for the stimulating electrodes. Most of the electrodes were inserted at a 30 degree rostro-ventral angle measured with respect to the vertical. In these preparations the initial electrode tracks were placed at the rostro-caudal level of the obex 0.7 mm lateral to the midline. This penetration was calculated to reach the gigantocellular area of the medullary reticular formation without the need for removal or penetration of cerebellar material. Some penetrations were also made 1.7 mm lateral to the midline. Occasionally penetrations were made up to 1 mm rostral or caudal to the obex.

In two of the experiments the brainstem electrode was inserted vertically, through the cerebellum, 5 mm caudal to the lambda suture, and either 1.7 or 0.7 lateral to the midline.

Initially, 50 to 250 ms trains of shocks at an internal frequency of 200 Hz and with a magnitude of 30 μA repeated once every two seconds were used as search stimuli to detect sites which activated the axial musculature. Once such an effective site was found the responses in the axial muscle nerves was assessed. The optimal stimulus configuration for the brainstem stimulus was not

extensively studied, because for the purposes of these experiments, it was desirable to use the shortest duration stimulus trains possible.

Experiments, the results of which are described in detail below, were performed to study the effect of varying the interval between brainstem and pudendal nerve stimulation. The interval could be continuously varied such that the brainstem stimulation preceded or followed that to the pudendal nerves.

Histological Verification of the Stimulus Sites

Following the acute experiments the rats were administered an intracardiac injection of 0.2 ml Heparin (1000 U/ml) and perfused for 5 minutes with isotonic saline followed by 10 minutes of perfusion with 10% Formalin mixture. The heads were removed and placed in 30% Sucrose-Formalin mixture for cryoprotection and allowed to harden for several days. The brains were removed and left in the Sucrose-Formalin mixture until they were sliced, usually after several more days. The brains were blocked at the same angle, with respect to the vertical, at which the electrodes had been inserted and 50 μm frozen sections were prepared of the region containing the electrode tracks. These were stained with Cresyl-Violet according to standard methods. The location of the electrode tracks was compared with a standard series of sections prepared at the same angle to the vertical as the specimen slices.

Results

Direct Stimulation Effects

Effective sites were found at all of the rostro-caudal and medio-lateral sites described in the methods section. At the sites used, movement of the thoracic and/or lumbar axial muscles was grossly visible with trains of stimulation.

Once activity was detected grossly in the muscle, the muscle nerves were

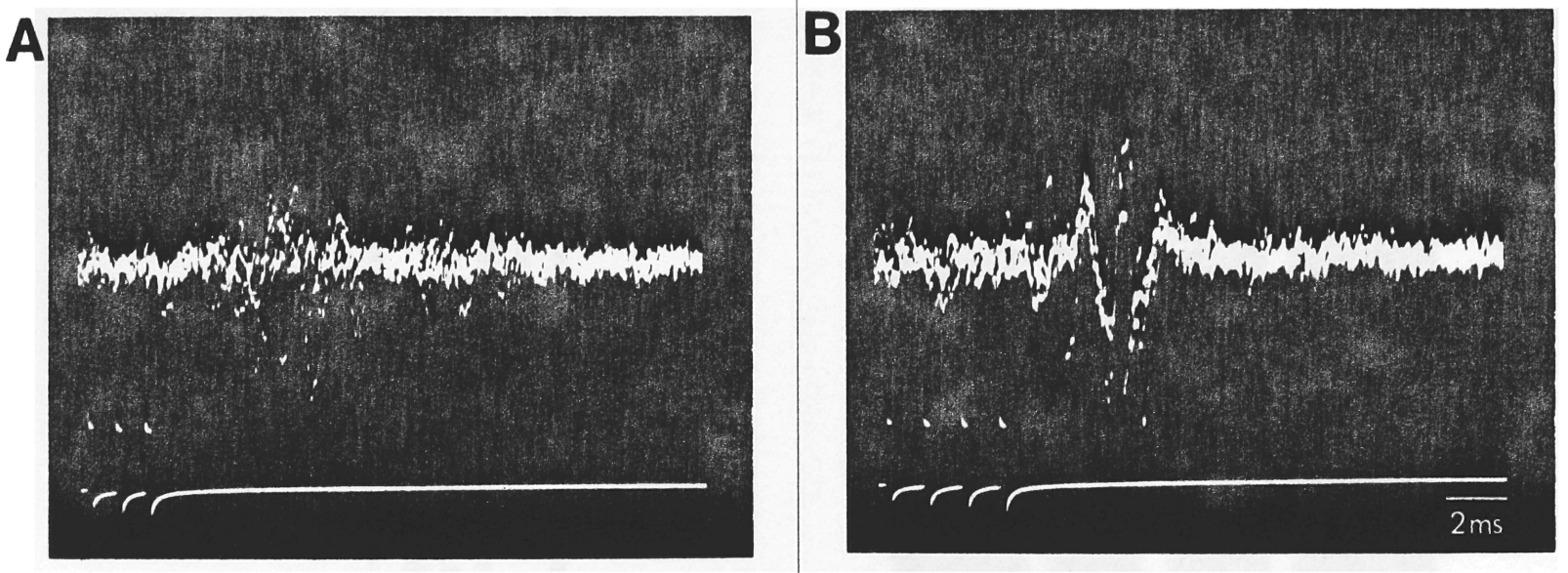
placed onto the recording electrodes. In some cases, sites which were effective at evoking visible abdomenal muscle activity were also effective in eliciting action potentials in lateral longissimus muscle nerves. The length of the stimulus train to the brainstem was shortened until activity could no longer be detected in the nerves. The minimal train length required varied with the different stimulation sites. Stimulation with fewer than three shocks in the stimulus train was never seen to elicit responses in the lateral longissimus muscle nerves (figures 4-1, 4-2, 4-5). Higher frequency trains were also tested. These were often more effective than lower frequency trains at evoking muscle nerve responses.

In order to test the effects of brainstem conditioning stimuli on the pudendal nerve-evoked responses, it was desirable to use short stimulus trains for the brainstem stimuli. Thus, the intershock interval in the stimulus trains was frequently set as high as 1 kHz and currents as high as 200 μA were sometimes used. The average current effective in producing either facilitation of the pudendal nerve responses or direct activation of the muscle nerves was 45.0 (+/-7.5) μA.

Response latencies varied considerably with stimulation to different sites. In most cases, the response did not occur at a fixed latency with respect to the stimulus train, suggesting a polysynaptic path from brainstem to spinal motoneurons. At times the latencies were as long as 70 ms. Each stimulation site appeared to have a characteristic latency associated with it. At 4/24 effective sites, direct activation of the muscle nerves was seen at latencies as short as 2.6 ms. With stimulation at these locations the response latency was more tightly fixed to the stimulus. These short latency responses are consistent with a monosynaptic connection from the brainstem to the axial motoneurons (Brink 1981), although this could not, of course, be tested with this stimulation and recording paradigm. At one stimulus site, increasing the stimulation current

Figure 4-1

Responses recorded in the L5 lateral longissimus muscle nerve to electrical stimulation of the brainstem in the region of the medullary reticular formation. The bottom trace in both figures shows the stimulus as recorded differentially across a resistor interposed into the stimulus path. Both figures are the superimposed resonse records to 5 stimulus repetitions. Trace **A** on the left shows the responses triple shock trains at a stimulus intensity of 150 μA repeated at 2/sec., trace **B** on the right shows the response evoked by stimulation with four shock trains. The 2 ms calibration bar refers to both figures.



L5 muscle nerve
150 µA Brainstem Stimulation at 2/sec
5 repetitions

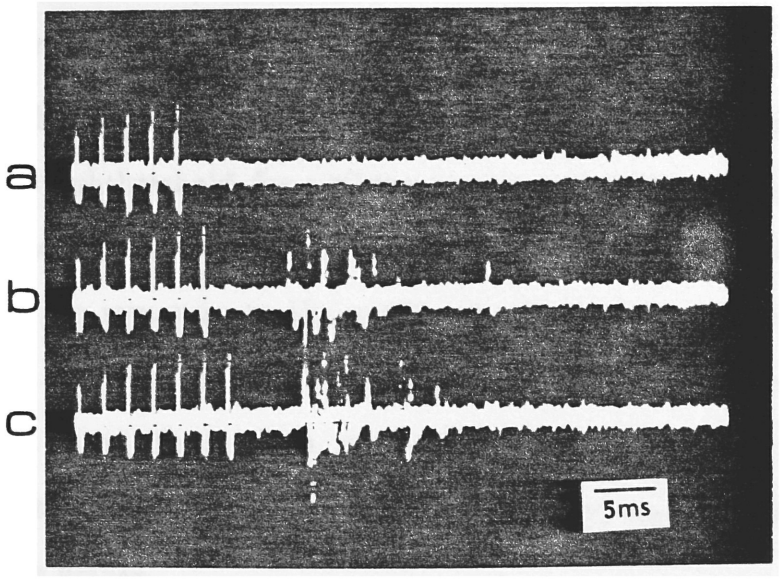


Figure 4-2

Responses recorded in the L3 lateral longissimus muscle nerve to electrical stimulation within the reticular formation of the medulla with a current of 80 μA at a repetition rate of 1/sec. Each trace, A, B and C is the superimposition of the responses to 5 repetitions of the stimulus. In A the stimulus consisted of a 5 shock train, in B it was a 6 shock train, and in C a 7 shock stimulus train was used. The stimulus artifacts are clearly visible at the left of all three traces. The 5 ms calibration refers to all three figures.

from 30 μA to 200 μA decreased the latency to the earliest responses from 30 to 3 ms, possibly as a result of current spread.

Convergence of Pudendal Nerve and Brainstem Inputs

Combined stimulation of the pudendal nerves and brainstem at stimulus intensities subthreshold for evoking responses to either stimulus alone revealed mutual facilitation of the axial muscle nerve responses to the two inputs (figure 4-3, 4-4): the responses to the combined stimulation were greatly augmented as compared to stimulation of either the brainstem or pudendal nerves alone. Brainstem sites from which such facilitation could be produced were often effective in facilitating the pudendal nerve-evoked responses in more than one of the muscle nerves.

When the effects of combined stimulation were examined in greater temporal detail it was apparent that the response latency with respect to either stimulus alone could be decreased by the combined stimus as shown in figure 4-5. Figure 4-5 also demonstrates that combined stimulation of the brainstem and pudendal nerve could induce the firing of additional units.

The effect of shortened latency is demonstrated more quantitatively in figure 4-6 which presents a histogram of the response latency to the brainstem and pudendal nerve stimuli alone and to the combined stimulation of both sites. Whereas the brainstem stimulus was subthreshold and the pudendal nerve stimulus evoked a response at a minimal latency of 27 ms, the combined stimulus elicited a substantially larger response at a 15 ms minimal latency.

In 2 out of 22 effective sites combined stimulation of the brainstem and pudendal nerves was capable of revealing short latency responses which were not otherwise visible. Figure 4-7 shows the responses of an L3 muscle nerve to separate stimulation of the pudendal nerves and brainstem and to the combined

Figure 4-3

Firing rate histogram of the responses recorded in the L5 muscle nerve to stimulation of the pudendal nerves and brainstem reticular formation. The horizontal bars at the bottom indicate periods of stimulation; the upper gray bars indicate pudendal nerve stimulation and the lower black bars indicate periods of brainstem stimulation. Brainstem stimulation consisted of 7 shock trains at a current of 30 μA repeated at 2/sec. Stimulation to the pudendal nerves with triple shock trains was applied bilaterally at a current of 50 μA and was also repeated at 2/sec. Horizontal divisions in the figure are 20 seconds and vertical divisions are 32 spikes.

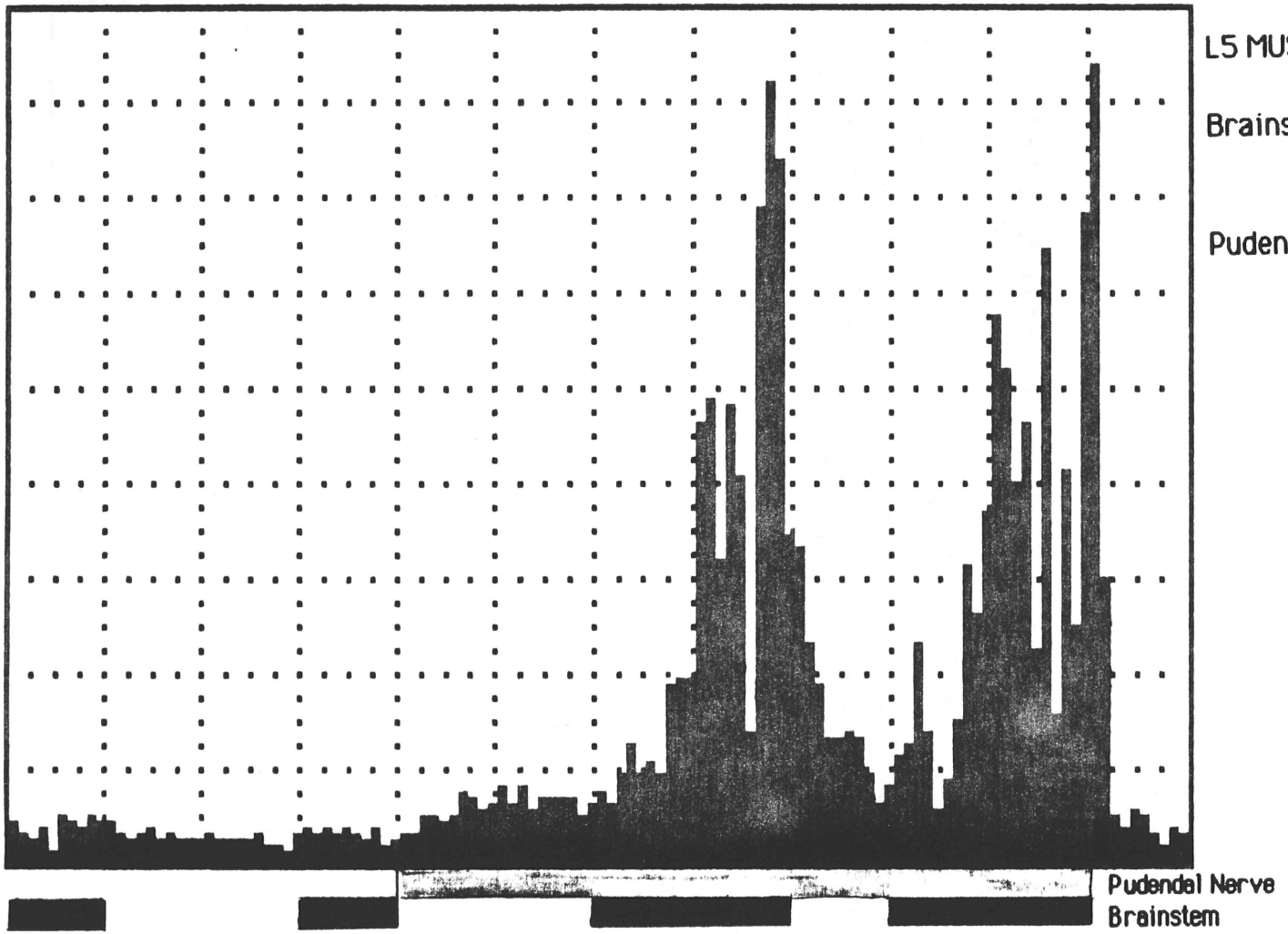
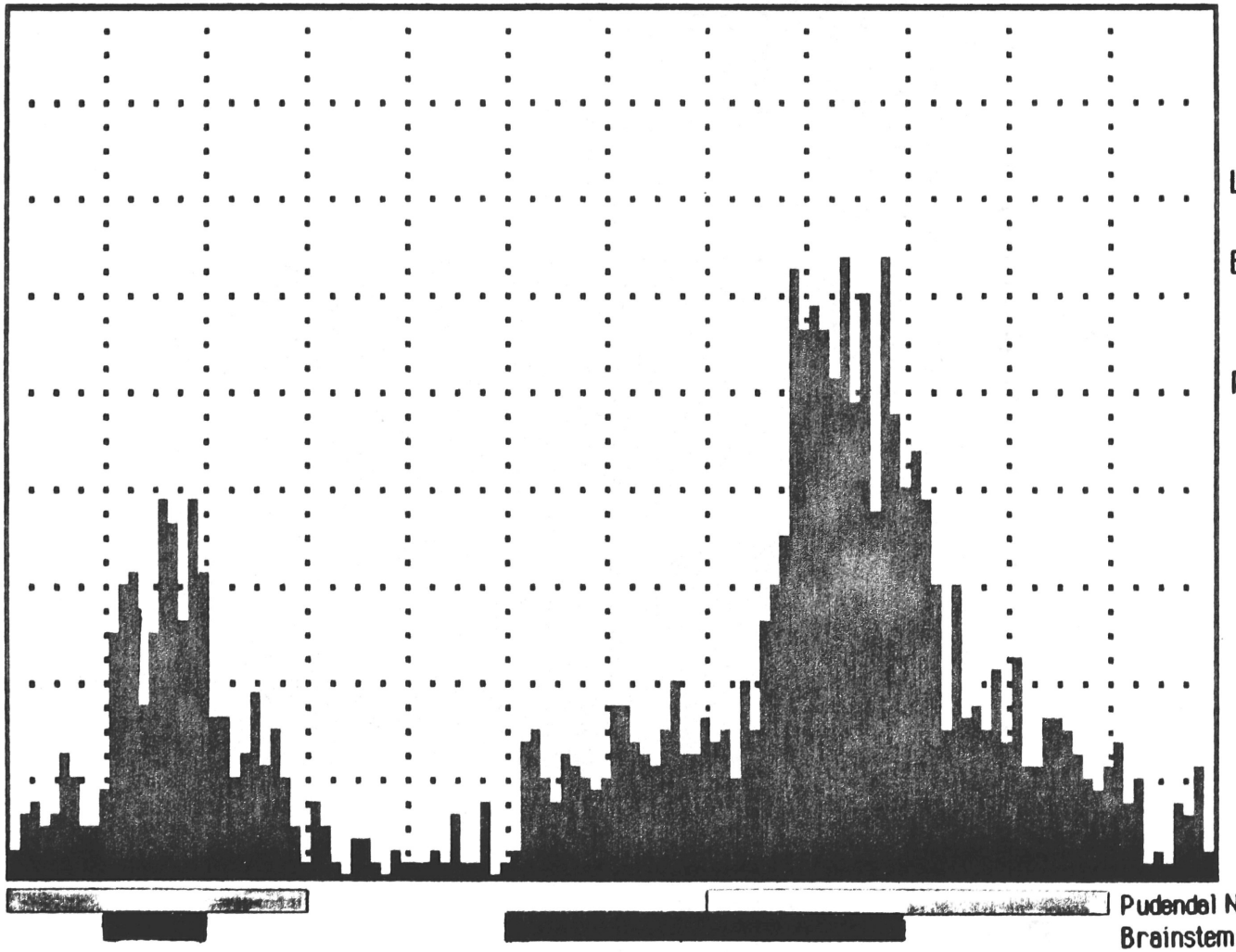

L5 MUSCLE NERVE
Brainstem: 30 µA x 7 shocks
repeated at 2/sec
Pudendal Nerves: 50 µA Bilateral
2/sec
Pudendal Nerve
Brainstem
Vertical: 32 spikes/division
Horizontal: 20 sec/division

Figure 4-4

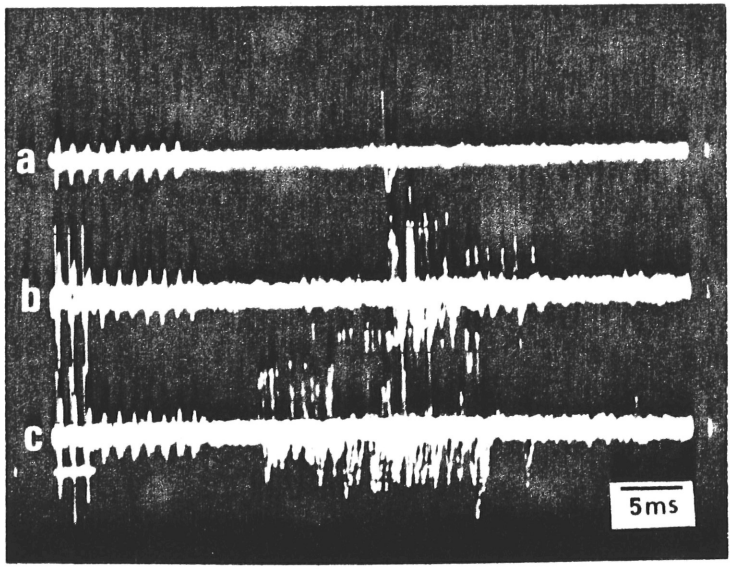
Spike activity records of the responses recorded in the L4 lateral longissimus muscle nerve to stimulation of the pudendal nerves and brainstem reticular formation. The horizontal bars at the bottom indicate periods of stimulation; the upper gray bars indicate pudendal nerve stimulation and the lower black bars indicate periods of brainstem stimulation. Brainstem stimulation consisted of 7 shock trains at a current of 15 μA repeated at 2.sec. Stimulation to the pudendal nerves, consisting of triple shock trains was applied bilaterally at a current of 100 μA and was also repeated at 2/sec. Horizontal divisions in this figure are 10 seconds and vertical divisions are 8 spikes.



Vertical: 8 spikes/division
Horizontal: 10 sec/division

Figure 4-5

Responses recorded in the L3 lateral longissimus muscle nerve to stimulation of the brainstem within the region of the medullary reticular formation and to stimulation of the pudendal nerves. Stimulus artifacts are clearly visible in all three traces. In **a**, stimulation consisting of 11 ms trains at 1kHz with a current of 30 μA was applied to the brainstem at a repetition rate of 2/sec. Bilateral pudendal nerve stimulation with triple shock, 50 μA trains was applied during the period recorded in **b** (the stimulus artifact from the brainstem stimulator continues to contaminate this trace, even in the absence of brainstem stimulation during this period). The repetition rate for the pudendal nerve stimulus was also 2/sec. Combined stimulation to the brainstem and pudendal nerves sites evoked the responses shown in **c**. The small white bar below trace **c** indicates the period during which the pudendal nerves were stimulated in the second and third traces. The 5 ms calibration refers to **a**, **b** and **c**.



L3 muscle nerve

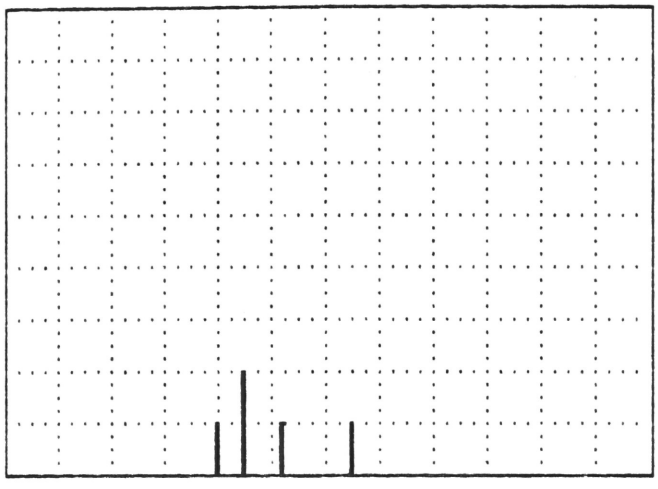
←Brainstem : 30 µA x 11 ms x 1 kHz

←Pudendal nerves: 50 µA bilateral

←Combined brainstem and Pudendal Nerve Stimulation

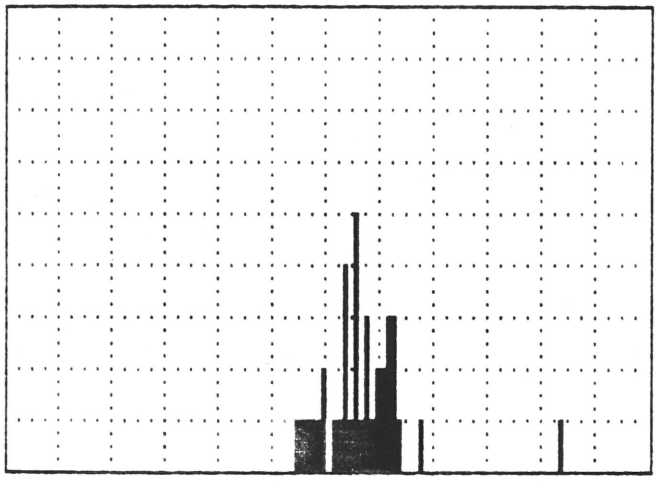
Figure 4-6

Post stimulus time histograms of the responses of the L4 lateral longissimus muscle nerve to stimulation of the pudendal nerves and to stimulation within the medullary reticular formation. Brainstem stimulation consisting of 5 shock, 30 μA trains repeated at 2/sec evoked the minimal response shown in the upper figure. The response to 10 μA bilateral stimulation of the pudendal nerves with 3 shock trains is shown in the middle figure. The effects of combined stimulation of the brainstem and pudendal nerves with the stimuli described above produced the response shown in the bottom figure. Vertical divisions for all three figures are 1 spike, horizontal divisions are 5 ms. The top figure is the accumulated response to 31 repeated stimulus iterations, the middle trace is the response to 30 iterations and the bottom trace is the response to 28 iterations of the combined stimulus.

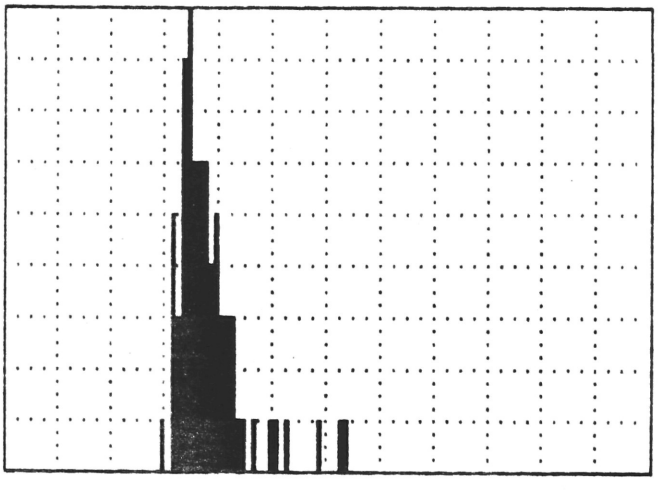


L4 muscle nerve

Brainstem Stimulation:
30 µA x 5 shocks
Repeated at 2/sec
Total Number of Sweeps: 31



Pudendal Nerve Stimulation:
10 µA Bilateral at 2/sec
Total Number of Sweeps: 30

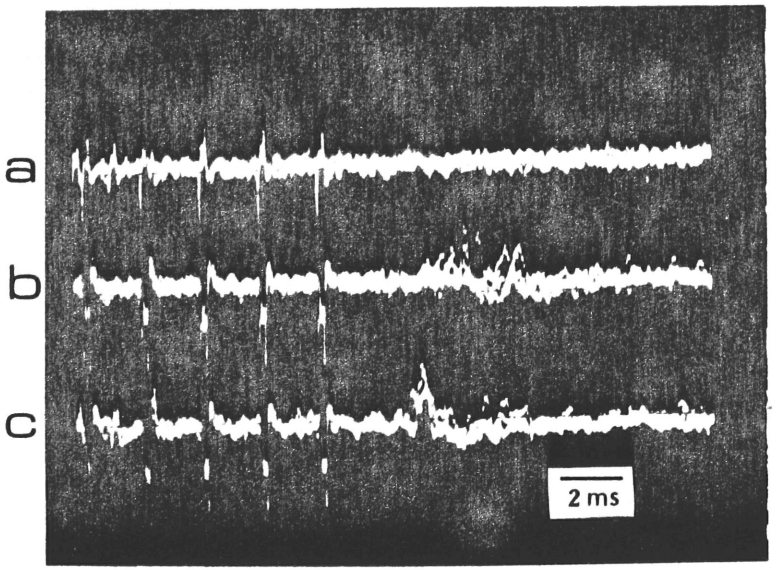


Combined Brainstem
& Pudendal Nerve Stimulation
Total Number of Sweeps: 28

Vertical: 1 spike/division Horizontal: 5 ms/division

Figure 4-7

L3 lateral longissimus muscle nerve responses to stimulation of the brainstem and pudendal nerves. Top trace (**a**): bilateral 10 μA stimulation of the pudendal nerves with triple shock trains repeated twice per second. Middle (**b**): stimulation within the medullary reticular formation with 20 μA five shock trains repeated twice per second. Bottom (**c**): combined stimulation of the brainstem and pudendal nerves. All traces show superimposed responses to 10 stimulus iterations. The 2 ms calibration bar applies to all traces. The horizontal bar, labelled pn in the figure indicates the period of pudendal nerve stimulation. The stimulus artifacts from the brainstem stimulus are clearly visible.



L3 muscle nerve

Pudendal Nerves: 10 µA bilateral

Brainstem: 20 µA x 5 x 500 Hz

Combined Brainstem and Pudendal Nerve Stimulation

10 repetitions

stimuli. Stimulation with a five shock train within the brainstem did not evoke fixed latency responses. When the brainstem stimulus was conditioned by a three shock bilateral train to the pudendal nerves, a fixed latency response was seen in the muscle nerve 2.6 ms following the last shock in the stimulus train.

Condition-Test Intervals

In figure 4-8 the evoked response, not present with stimulation of the pudendal nerve or brainstem alone, was facilitated by simultaneous stimulation of the brainstem and pudendal nerves (figure 4-8a), and occurred at a latency of 10 ms with respect to the first shock in the stimulus train (the effective shock could not be determined with this experimental design. When the pudendal nerve stimulus was delayed by as little as 2.5 ms (4-8b), the response was much attenuated. Shifting the pudendal nerve stimulus later still, until it in effect occured simultaneously with the response relative to the brainstem, showed little or no facilitation of the evoked responses. Facilitation of the pudendal nerve-evoked response by brainstem stimulation was present with condition-test (C-T) intervals of up to 80 ms in some combinations of brainstem stimulation and nerve recording sites.

In figure 4-9 is shown a series of experiments on C-T intervals in which the response to combined stimulation of the brainstem and spinal cord occurred at a latency of approximately 18 ms from the first shock in the combined sitmulus; no response was present to stimulation of the brainstem or pudendal nerve alone. When the pudendal nerve stimulus was shifted 5 ms later, so that it was preceded by 5 ms of brainstem stimulation the response occurred at a latency of 13 ms with respect to onset of the pudendal nerve stimulus, at an essentially constant latency with respect to the brainstem stimulation. Delaying the pudendal nerve stimulus still further, so that it was preceded by 10 ms of brainstem stimulation produced an evoked response at a latency of approximately 9 ms from the onset of the pudendal nerve stimulation.

Figure 4-8

Responses recorded in the L3 lateral longissimus muscle nerve to stimulation of the brainstem within the region of the medullary reticular formation and to stimulation of the pudendal nerves. Stimulus artifacts are clearly visible in all three traces. The period of pudendal nerve stimulation is indicated by the horizontal bars below each trace. In trace A stimulation to the pudendal nerves and brainstem were applied simultaneously, in B the pudendal nerve stimulus was delayed by 2.5 ms and in trace C it was delayed to four ms following the end of the brainstem stimulus. Pudendal nerve stimulation consisted of bilateral triple shock trains at 20 μA. Stimulation to the brainstem was with 1 kHz, five shock trains at a current of 20 μA. The 2 ms calibration bar refers to all three traces.

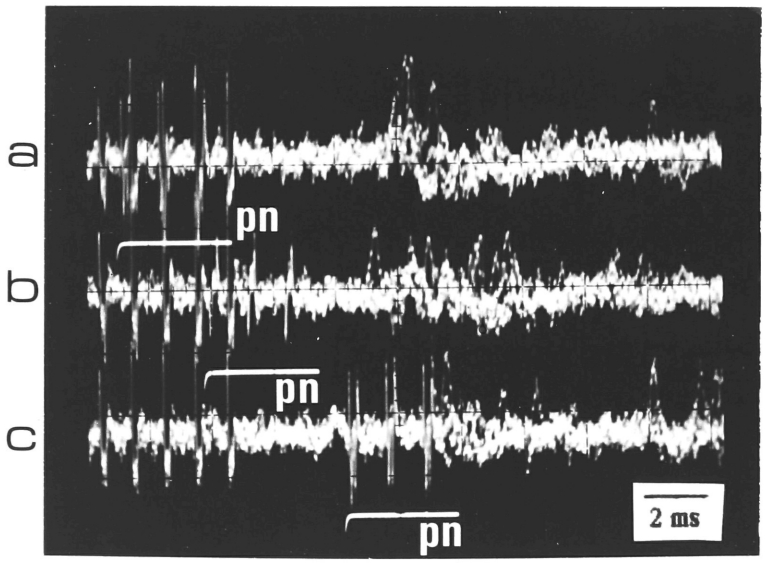
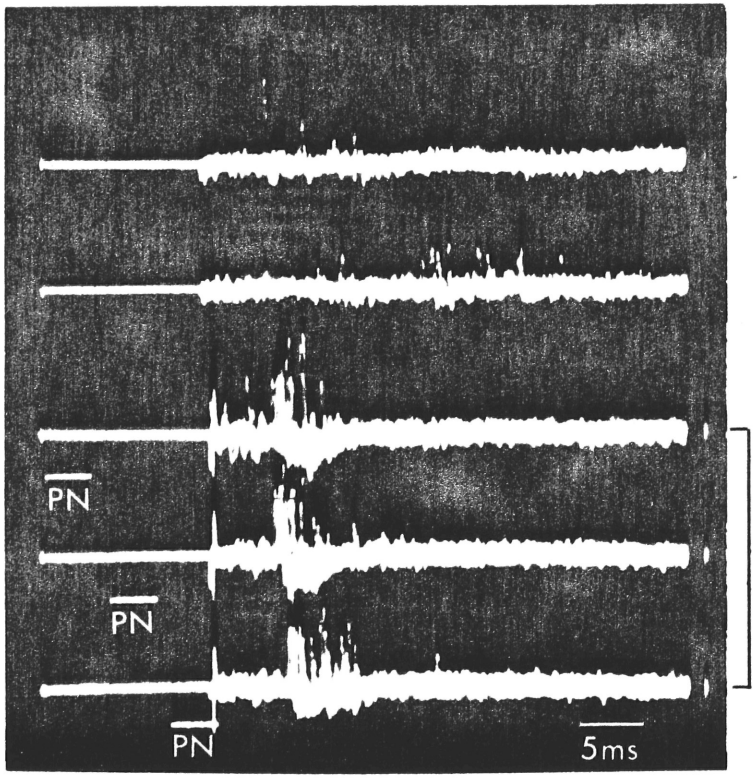

a
b
c
pn
pn
pn
2 ms

Figure 4-9

L3 lateral longissimus muscle nerve responses to stimulation of the medullary reticular formation and pudendal nerves. Artifact suppression is responsible for the flatness of the traces for approximately the first 15 ms in all traces. The apparent spike at the end of the blanking period in the bottom three traces is a secondary effect of the artifact suppresssion cricuitry. The top trace shows the response to stimulation within the medullary reticular formation of the brainstem with 30 μA, 250 Hz trains with a duration of 12.5 ms, repeated at 1/sec. The seond trace shows the response to bilateral stimulation of the pudendal nerves with 10 μA bilateral triple shock trains. In the bottom three traces are shown the responses to combined stimulation of the brainstem and pudendal nerves. The pudendal nerves were stimulated during the period indicated by the horizontal bars labelled PN. The top such bar applies to the second and third traces. The other two markers apply to the traces immediately above them. In the middle trace, the pudendal nerve stimulus occurred at the beginning of the brainstem train. In the trace below that, the pudendal nerve stimulus was preceded by 5 ms of brainstem stimulation. In the bottom trace, the pudendal nerve stimulus was delayed by 10 ms with respect to the onset of the brainstem stimulus. The 5 ms calibration refers to all 5 traces.



bs

pn

bs + pn

Brainstem: 30μA x 250 Hz x 12.5 ms
Repeated at 1/sec

Pudendal Nerves: 10 μA Bilateral at 1/sec

10 Repetitions

Effective Sites

At 22 different electrode locations, stimulation at depths ranging from 1.7 to 3.4 mm, at sites 0.7 mm lateral to the obex, were effective in facilitating activity evoked in the axial muscle nerves by pudendal nerve stimulation. In one animal stimulation 1.0 mm rostral and 0.7 mm lateral to the obex at a depth of 4.1 mm was also effective. From one location, 0.7 mm lateral to the obex and at a depth of 2.5 mm, inhibition of the pudendal nerve-evoked activity was seen. At 15 of the 22 sites which produced facilitatory effects, stimulation to the reticular formation alone also evoked activity in the muscle nerves. Figure 4-10 shows a map of the sites from which brainstem stimulation was effective in eliciting muscle nerve activity and/or in modulating the pudendal nerve-evoked responses.

Discussion

Estimates of Current Spread

Using the data of Bagshaw and Evans (1976) we estimate the current spread from our highest stimulation strengths of 200 μA to be 1.4 mm. The average stimulus current used was 45 μA and has an expected current spread, by their method, of 0.7 mm.

Convergence of Brainstem and Pudendal Nerve Inputs

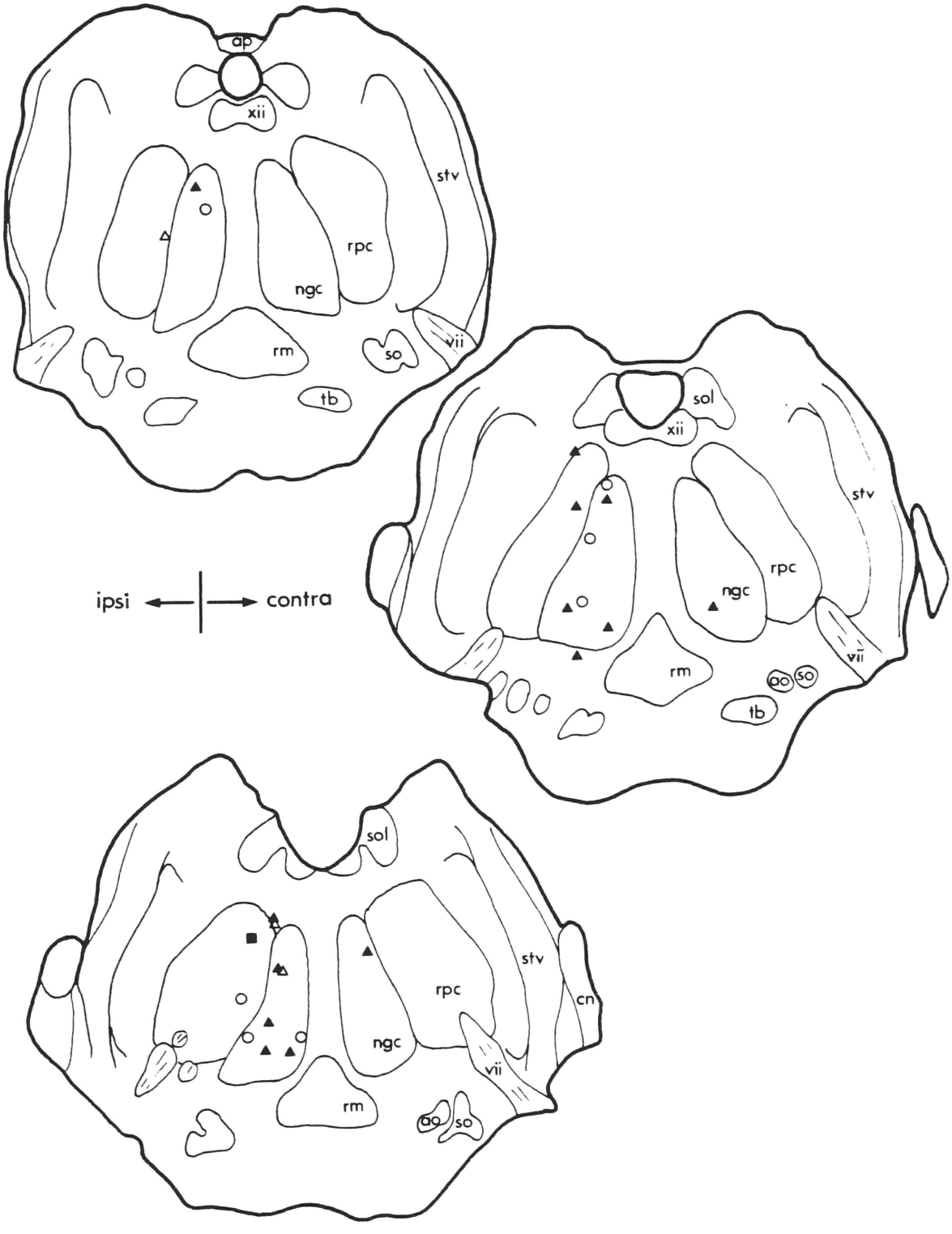
These results demonstrate that the input pathways from the pudendal nerves and the brainstem converge at or before the final common path of the motoneurons innervating the axial longissimus muscles. The exact locus of convergence remains obscure however; these experiments do not provide information which would distinguish between a brainstem or spinal cord locus.

The large shifts in latency, on the order of 10 to 15 ms as seen in figures 4-5 and 4-6, which occur when brainstem and pudendal nerve stimulation are combined, suggest the presence of two alternative pathways to the motoneuron pool,

Figure 4-10

Diagram of brainstem stimulation sites. After histological processing of the brainstem, 50 sections were prepared at the same angle as the stimuating electrode penetration (30 degrees from the horizontal). Electrode locations were derived from the stained tissue sections and the locations of effective stimulation sites were note onto standard drawings as follows: Open triangles (△) indicate sites from which electrical stimulation within the brainstem evoked activity in the muscle nerves, but from which no facilitation of the pudendal nerve-evoked response was produced. Open circles (○) indicate sites from which brainstem stimulation facilitated the pudendal nerve-evoked response, but did not produce muscle nerve activity in the absence of pudendal nerve stimulation. Sites from which brainstem stimulation both facilitated the pudendal nerve-evoked responses and evoked muscle nerve activation are indicated by filled triangles (▲). From one site, indicated by a filled square (■), stimulation within the brainstem inhibited the pudendal nerve-evoked responses. The sections differ by 150 in rostrocaudal position, the uppermost section being most caudal.

Abbreviations: *ap* - area postrema; *ao* - accessory olive; *cn* - ventral cochlear nucleus; *ngc* - gigantocellular reticular nucleus; *rm* - raphe magnus; *rpc* - parvocellular reticular nucleus; *so* - superior olivary complex; *sol* - solitary nucleus and tract; *stv* - spinal tract of the trigeminal nerve; *tb* - trapezoid body; *vii* - facial nerve; *xii* - hypoglossal nucleus.


ap
xii
stv
rpc
ngc
rm
so
vii
tb
sol
xii
stv
rpc
ngc
vii
rm
oo
so
tb
ipsi
contra
sol
stv
rpc
cn
ngc
vii
rm
oo
so

perhaps one via a supraspinal loop, and one by a more direct segmental route, which is subject to facilitatory descending drive. When the pudendal nerve input is allowed sufficient time to activate the motoneuron pool as implied from our earliest nerve onset latencies, the brainstem responses would be facilitated as in figure 4-8b, in contrast to figure 4-8c.

Chapter 5

General Discussion and Speculations

These experiments have shown that responses in the lateral longissimus muscle and muscle nerves can be evoked by stimulation of the pudendal nerves with electrical current. The responses occur at latencies as short as 8.8 ms and persist for over 100 ms. The overall pattern seems to be one of short latency excitation, relative quiescence , then renewed activity.

The presence of short latency responses has led us to experimentation in animals which have been subject to complete transections of the spinal cord, in order to expose any purely segmental components to the responses. In these animals, short latency responses are still present and are still subject to bilateral convergence of of pudendal nerve inputs. The longer latency responses apparently require some input from supraspinal centers. To indicate which supraspinal regions are involved a series of tractotomies was performed, which indicated the sufficiency of tracts located in the ventrolateral spinal cord in producing responses similar to those seen in the intact animal. Because of the known connectivity of pathways in that part of the spinal cord the study of brainstem inputs to the pudendal nerve-evoked response was begun with electrical stimulation. Such stimulation was effective in potentiating the responses to pudendal nerve stimulation. The locus of such onvergence has not yet been determined.

Much of the work described here was motivated by an interest in the physiological mechanisms underlying lordosis behavior. We are struck by several general observations on the parallels, and differences, between the pudendal nerve-evoked response and lordosis behavior:

Stimulation of the appropriate inputs to lordosis behavior, elicits responses from the muscles and muscle nerves essential for the behavioral output. These neural inputs and outputs must form a substrate for lordosis behavior. The slowly-adapting pressure receptors, Ruffini endings, are known to project to the

spinal cord via A-β fibers (cf. Burgess and Perl 1973, review). Based upon measurements of the actual stimuli present in lordosis (Kow and Pfaff 1976), it is this receptor type which is thought to be of primary importance in the somatosensory stimulation of lordosis behavior. Electrical stimulation of the pudendal nerve probably results in highly synchronous activation of many units in the nerve, and the pattern of excitation, for example the specific fibers activated, would not be expected to mimic accurately the responses to complex stimuli such as those which might result from natural mating encounters. Other receptor types, not thought to be involved in lordosis, including C-fiber nociceptor and thermoreceptors were probably not activated by the stimuli used in these experiments.

The patterns seen in the pudendal nerve-evoked response are not, in general altered by hormonal pretreatment Some significant differences are seen in the male and female rats. The initial hypothesis on beginning the experimental series, was that some response changes would be seen as a result of estrogen treatment. Specifically, it was hypothesized that female rats which are behaviorally receptive, i.e. display strong lordotic responsiveness to manual stimulation should show relatively enhanced excitability to pudendal nerve stimulation. Several lines of explanation may help us in interpreting this result:

Firstly, these animals were always systemically anesthetized during these experiments. Anesthetics are well known to disrupt the transmission of signals over multisynaptic pathways, of the type likely to mediate the hormonal influences on lordosis. Thus, the anesthesia may have rendered the hormonal effects invisible.

Secondly, lordosis as a complete behavior requires an intricate pattern of sensory inputs including cutaneous stimulation of the flanks followed by perineal pressure. To the limited extent that our stimulus paradigm mimics the sensory inputs to lordosis it does so only for the perineal pressure com-

ponent, whereas the other components of the behavior may constitute the hormonally sensitive parts. It is known, for example that response to stimulation of cutaneous nerves whose receptive field includes the flank region are altered by estrogen treatment (Schwartz-Giblin, Halpern and Pfaff 1984).

Finally, the possibility remains that under some as yet undeveloped, more sensitive transformation of the multi-unit data, significant differences in the responses of the various treatment groups would be revealed. The analysis methods presented here allowed comparison only of latency and peak magnitude relative to baseline activity. For example, hormonally mediated changes in segmental excitability may be manifest as changes in the mean activity level of the muscle nerves during stimulation. However, because the actual mean activity level is exquisitely sensitive to small changes in settings, for example, of the window discriminator or to overall health of the nerves, it was not possible use this as a basis for inter-individual comparisons.

Supraspinal influences upon the pudendal nerve-evoked response are transmitted via fibers located in ventrolateral spinal cord. Partial spinal cord transections, similar to those described here, have indicated that intact connections in this region of the cord are necessary and sufficient to allow functional lordosis in behaving animals (Kow, Montgomery and Pfaff 1977). The results on the effects of spinal cord transections on the pudendal nerve-evoked responses closely parallel the behavioral result, in that transections of virtually all of the long ascending and descending fibers of the spinal cord except those in the ventrolateral columns leaves the pudendal nerve-evoked response essentially unchanged, whereas destruction of the lateral columns drastically reduces the magnitude and time course of the evoked muscle nerve activity.

These considerations of the parallels between the pudendal nerve-evoked response and lordosis behavior are consistent with the hypothesis that the

hypothalamus and other brain regions modulate the excitability of intrinsic spinal pathways, possibly by way of elements within the reticular formation, to potentiate the transmission of cutaneous information to axial motoneurons, which in turn contribute to the lordosis posture.

Among the supraspinal influences upon the pudendal nerve-evoked response are those stemming from elements within the reticular formation - possibly including fiber bundles passing through that region. While electrical stimulation within the reticular formation does not enhance lordosis performance, this brainstem region is thought to be an important way station in transmitting influences from the ventromedial nucleus of the hypothalamus and midbrain central gray regions of the brain.

Supraspinal influences upon the excitability of segmental pathways are well known (cf. Lundberg 1967, review). In many cases these effects appear to be mediated by interneurons in the reflex pathways. For example, Hongo et al. (1969) showed evidence suggesting that axons within the rubrospinal tract project monosynaptically upon interneurons mediating Ia inhibition as well as those mediating Ib excitation and inhibition. Similar effects were seen in the inhibitory and excitatory reflex responses to low threshold joint receptors and to cutaneous inputs, again apparently via the interneurons in those pathways. Facilitation of the Ia inhibitory pathway from extensors to flexors evoked by stimulation of the vestibulospinal tract is also mediated by monosynaptic projections to Ia inhibitory interneurons (Grillner, Hongo and Lund 1967).

Classically, the effects of reticular inputs upon the interneurons of the spinal cord have been thought to be largely inhibitory (Holmqvist and Lundberg 1959, Lundberg 1967). Much recent work has focused on the brainstem control of primary afferent depolarization and its interneuronal mediation. Considerable evidence exists already to indicate that suprapinal nuclei (Rudomin et al.1983) and

cutaneous inputs (Engberg, Lundberg and Ryall 1968) can modulate the transmission of Ia and Ib reflex pathways and there is evidence that the sign of the effects from cutaneous afferents and brainstem is the same (Rudomin 1983). The primary afferent depolarization of cutaneous fibers in the A-α, A-β and A-δ ranges of conduction velocity is subject to modulatory control from brainstem structures including the raphe magnus and reticular formation (Martin, Haber and Willis 1979).

Interneurons within the spinal cord represent a likely locus of convergence of brainstem and cutaneous, pudendal inputs. As described by Jankowska and McCrea (1983, page 107), "*Use of the same interneurones by several fibre systems is by no means infrequent; on the contrary, it appears to be a rule in all types of interneurones investigated*". Such convergence of pudendal and brainstem inputs upon spinal circuitry is perhaps the simplest hypothesis consistent with the present data, since it does not presuppose the necessity of a supraspinal loop. In this model of the system, brainstem influences on the pudendal nerve-evoked response would be present in the form of tonic descending excitatory drive. Sullivan et al. (1985) have shown that pudendal nerve stimuli effective in eliciting activation of the lateral longissimus muscle, as measured by EMG unit activity, are associated with desynchronization of the cortical encephalogram, which in turn are thought to reflect state changes in the reticular formation.

Although the results of the experiments described in this thesis do not demonstrate any evidence of hormonal effects upon the pudendal nerve-evoked response, it is still reasonable to speculate that the circuitry activated by electrical stimulation of the pudendal nerve in the manner described evokes activity in the same pathways which mediate lordosis behavior. It is obvious that the behavior involves the coordinated activation of muscle systems (e.g. those of the back, neck and legs) which are themselves involved in countless other behaviors.

Intuitively, and on the basis of the essential conservatism of evolution, it would seem highly unlikely that a separate hormonally controlled set of physiological connections would have evolved to subserve lordosis behavior. Instead, it seems much more likely that hormonal influences are manifest as subtle changes in the strengths of interconnections of extant neural elements, a little higher excitability here, a little stronger inhibition there. Viewed in this context it does not appear surprising that the relatively non-physiological system of electrical nerve stimulation followed by measurements of resulting neural activity should not reveal the subtleties of these hormonally induced changes. The pudendal nerve-evoked response instead reveals a pattern of functional connectivity from a cutaneous receptive field in the perineum to motoneurons in the back, well situated to subserve lordosis behavior.

Introduction

In the past several years the microcomputer has come to represent an attractive alternative to the use of mini and mainframe computing machines in the scientific laboratory. Since the release of the Apple computer in 1976 the microcomputer has been an approachable and inexpensive tool for the handling and organization of large amounts of data. Such machines have in addition become increasingly powerful as the cost of the electronic subcomponents has dropped and as increasing volumes of software have become available. The *Apple* computer is particularly well suited to scientific use owing to its flexible and well-documented input/output (I/O) structure, low cost, powerful built-in graphics capabilities and widespread acceptance in scientific laboratories.

My research necessitated the use of a system for the analysis of time-interval distributions of neural and electromyographic spike data. It was important to acquire and analyze data on-line, forming histogram representations of the time-interval distributions. While various dedicated hardware packages exist already for this purpose, I developed my own microcomputer-based system for reasons of both cost and future flexibility in altering the parameters of my analyses. Although it is possible to use a software timing loop to do the actual timing of spike arrivals (Femano and Pfaff 1983) that procedure is not compatible with on-line graphing and data analysis; I chose instead to use a hardware and software based system.

The system described here will allow the computer to accept spike data and to analyze the raw data stream into post-stimulus time (PST) or interspike interval (ISI) histograms which are plotted as the data arrives. The vertical scale of the histogram is automatically adjusted as the total number of events per bin increases. Artifact suppression during stimulation is built in. A novel feature of the acquisition system is its ability to replot the data with different binwidths,

i.e. with different temporal magnification for more fine-grained examination of any portion of the histogram *without* the need to reacquire the data. This feature is particularly valuable in the study of phenomena which are subject to fatigue or are not stationary in time (Cohen et al. 1983). Binwidths from 100 microseconds to 6.4 seconds can be selected, although there is a trade-off between total acquisition time and temporal resolution (see below). In addition to its functions in data acquisition, the system also includes the capability to store and retrieve data on disks, to send it to a graphics plotter, to produce graphs with arbitrary titles and to get numerical readings of the number of events stored in each histogram bin (the latter with some limitations, see below).

The program is designed for ease of use, even by the computer novice. All of the interactions with the user are made as "friendly" as possible and in most cases the user is advised of incorrect data entries and given an opportunity to review all important parameters before commencing a data acquisition period.

This appendix, based in large part on material published in Brain Research Bulletin (Cohen and Pfaff 1984) describes the complete implementation of the analysis system, including the design plans and source code for the computer programs. Detailed study of the programs, circuits or indeed of the computer itself should not be necessary either to grasp the functional logic of the system or to implement the ISI and PST histogram generator on an Apple II. These more 'primitive' details then are intended for a reader with a specific interest in the computer algorithms used.

While I developed the acquisition system with the analysis of neural signals in mind it may also be used with other data types. In the ISI mode, for example it may be used to estimate the mean frequency of almost any periodic event (within the limits of its temporal resolution and maximum acquisition time). Although a great deal of attention was devoted to the development of a system

capable of on-line analysis, the present program is equally suited to the analysis of taped data (Schwartz-Giblin, Femano and Pfaff 1984). Also, the temporal resolution and accuracy are high enough that I have been able to use it to calibrate several types of electrical equipment, for instance stimulators, interval timers and so on.

System Overview

The acquisition system consists of an integrated package of both electronic components (hardware) and computer programs (software). In the interest of clarity I will describe the hardware and software segments separately.

Hardware

The basic computer setup required for the acquisition system is an Apple II+ -type computer (Apple II+ or Apple IIe) with at least 48K of memory, one or preferably two disk drives, a video monitor and a graphics printer and printer interface card. I used an Apple IIe computer with two disk drives, an Epson FX80 printer and a Microbuffer printer interface. The addition of a so-called 80-column card, while not strictly necessary, makes the video displays considerably easier and in the program reprinted in figure I-3a its use is assumed. Several other computers are available which are sufficiently similar to the Apple to be compatible in this application. In addition to the primary computer hardware the system requires the use of a circuit card containing an integrated circuit known as a *6522 versatile interface adaptor,* (Rockwell Doc. Number 29000, 1981) and its necessary support components as well as a signal conditioning circuit which converts the raw data stream into signals compatible with the electrical requirements of the computer system. A functional equivalent of the circuit card containing the 6522 may be purchased commercially or, as in my case, easily constructed using wirewrap techniques (De Jong 1982). The signal conditioning circuit, though not commercially available, is rather straightforward in design

and quite easy to assemble.

The 6522 counts and times events and sends and receives signals on the Apple's internal data lines. It is also used to interrupt the functioning of the Apple computer's microprocessor and thereby redirect program execution.

Software

The software is written in Applesoft BASIC and in 6502 assembly language. While BASIC is easy to use it runs rather slowly, so all critical timing functions are written in the much faster-running assembly language. The graphic display functions are also written in assembly language so that display update is as rapid as possible.

The software is arranged heirarchically in three levels. Initial setup parameters are entered using a highly interactive program written in BASIC. When the user requests initiation of an acquisition run, the next level, a machine language subroutine, is called which initializes the computer for data acquisition and then immediately starts to plot whatever data is presently stored in a designated region of computer memory. As the data comes in, the plotting program is interrupted and control is sent to the final data acquisition level to enter the new data into memory, after which plotting is reinitiated. When the user issues a stop command the input lines are disabled and a final version of the histogram is plotted. Subsequently control is returned to the BASIC section of the program.

The software includes its own memory management in which the computer memory is 'temporally mapped'; event times are serially arranged in the physical memory. The Temporally-Mapped Memory concept allows a considerable increase in acquisition speed and is of great convenience in the plotting and analysis of the temporal data.

Construction Details

Hardware

Figure I-1 shows the schematic of the signal conditioning system. Two identical channels were required, one which accepts signals on the data line and a second which monitors stimulus events. The circuit is powered directly from the computer power supply and requires only a few milliamps to operate. IC1 is an optional inverter which will allow triggering on positive or negative going waveforms. Its output is fed to a comparator, IC2, whose output is normally held at 5 volts (referred to as 'high'). When the signal level exceeds a voltage determined by VR1, the threshold control, the comparator output drops to near 0 volts ('low') which in turn triggers IC3, a 'one-shot'. The resistors and capacitor surrounding IC3 are adjusted to yield a trigger pulse of 60 microsecond duration - long enough to be reliably sensed by the computer and short enough to maintain high temporal resolution. In normal use the spike input is driven by a window discriminator for greater precision in triggering.

Figure I-2 is a wiring diagram for the 6522 circuit card. This was mounted on a Vector (tm) 4609 which inserted into the Apple computer expansion slot number 5. Please note that in this application the PB6 and PB7 pins are be connected together. This represents a small alteration of the native configurations of the commercially available board which can be implemented by simply placing a jumper wire across the appropriate pins (Port 2 pins 7 and 8 on the John Bell board and DL15 and DL16 on the Interactive Structures product). The description of this circuit board is necessarily sketchy, but for further information the reader is referred to the excellent text by De Jong (1982) which also has an in-depth description of the functions of the 6522 integrated circuit. In my implementation the signal conditioning circuit and the 6522 card was connected via a ribbon cable which exits through a slot in the back of the computer. Power sup-

Figure I-1

Interface circuit required to condition incoming spike and trigger pulses. Threshold crossings result in the output of a 60 microsecond pulse. Two such circuits are required, one for the trigger line and one for the spike line. The respective outputs of these circuits are connected to the CA1 and CA2 inputs of the 6522 card shown in figure I-2. Power is also supplied via the 6522 card (see figure 2b). After constructing the circuit, the PULSE WIDTH potentiometer must be adjusted to yield a 60 microsecond pulse with each threshold crossing. The switch labelled '+' and '-' allows inversion of the incoming signal enabling detection of either positive or negative going transitions. A supply bypass capacitor should be placed as near as possible to the 311 to avoid unwanted oscillations. All capacitance values are in microfarads except as noted and all resistances are in Ohms.

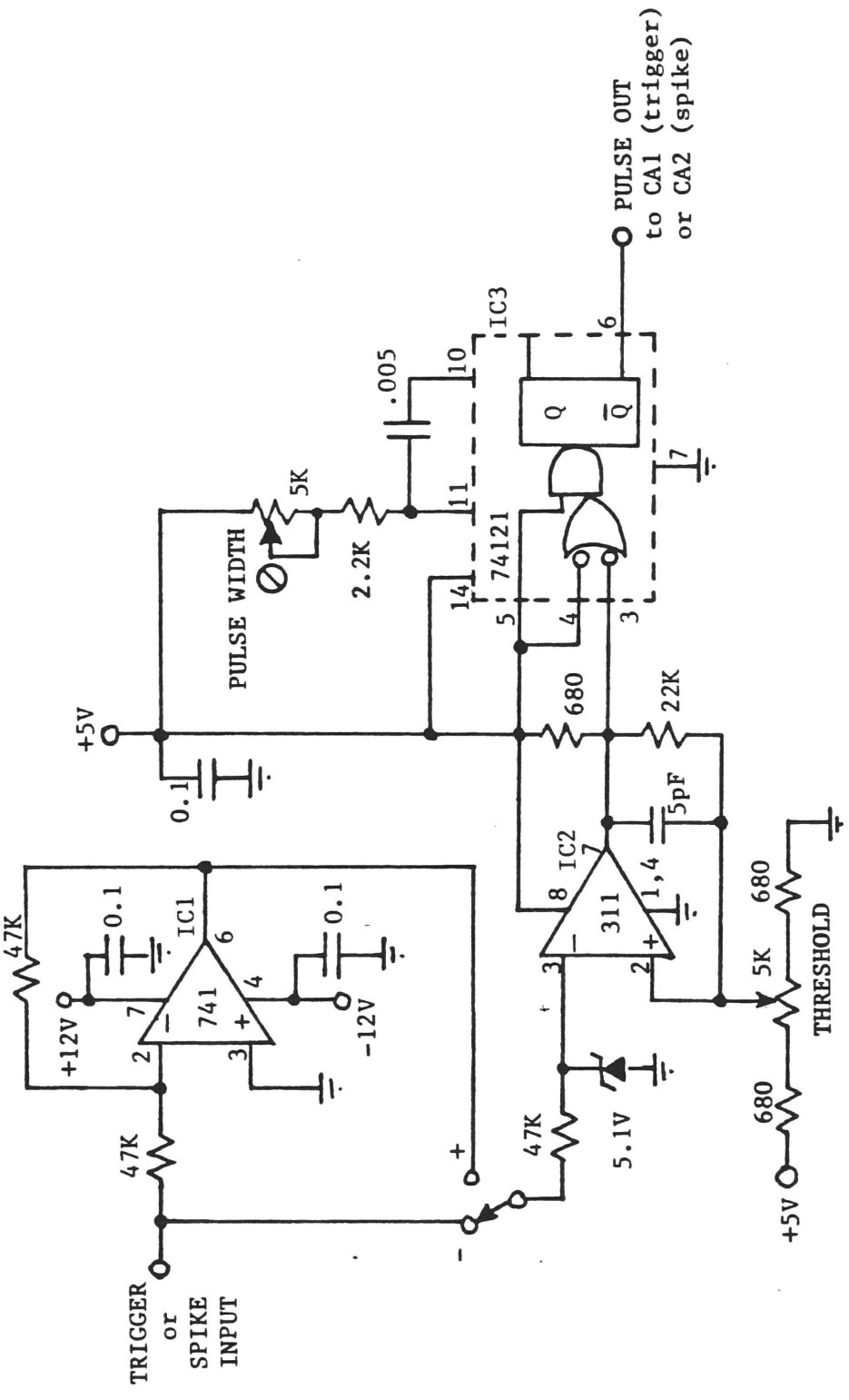

TRIGGER or SPIKE INPUT
47K
+12V
0.1
IC1
741
-12V
0.1
47K
+
-
47K
5.1V
IC2
311
1,4
5pF
680
22K
+5V
0.1
PULSE WIDTH
5K
2.2K
.005
IC3
74121
Q
Q̄
PULSE OUT
to CA1 (trigger)
or CA2 (spike)
5K
680
680
THRESHOLD
+5V

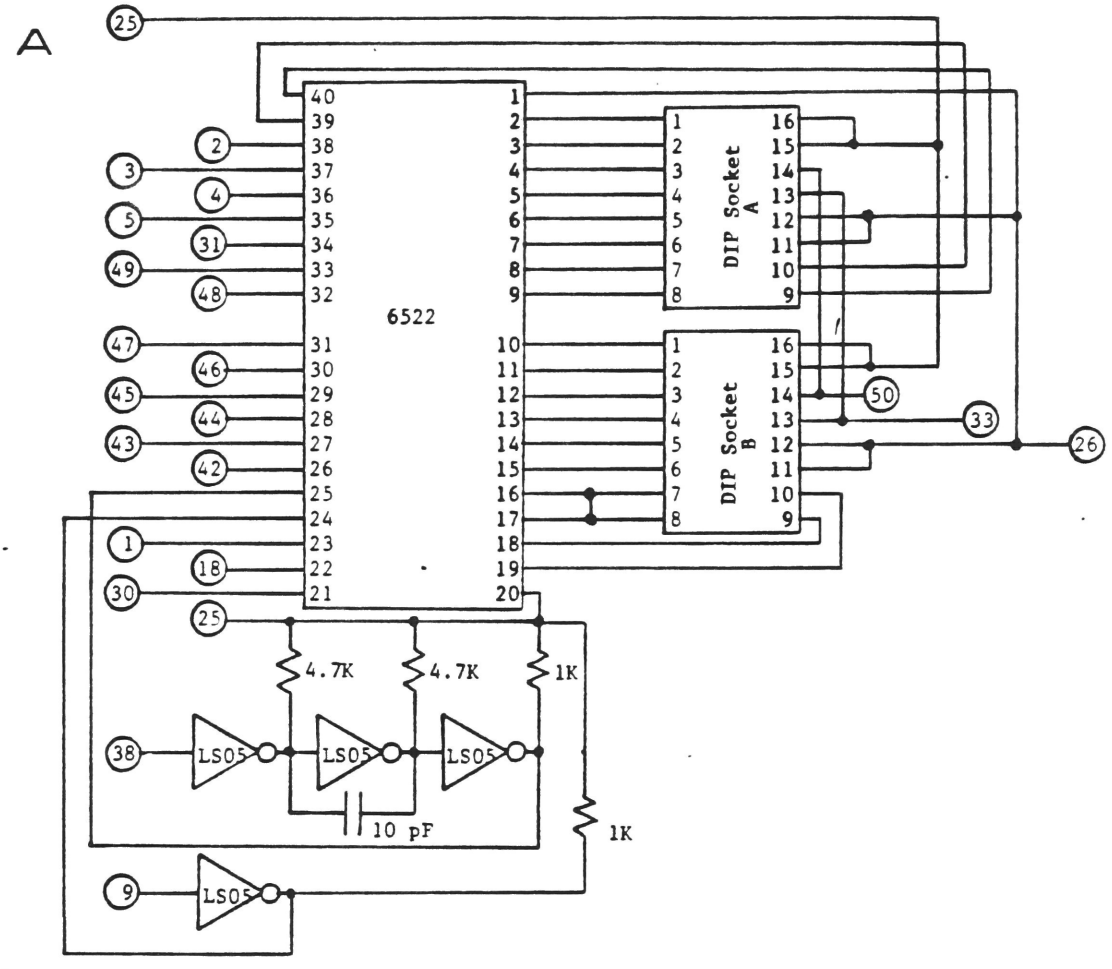
Figure I-2a

Wiring diagram for the 6522 card. Numbers in circles refer to the pin numbers used in the Apple expansion card slots. Uncircled numbers refer to pin numbers for the integrated circuit and the DIP sockets. Note the jumper connecting PB6 and PB7.

Figure I-2b

Diagram of the lines available from DIP sockets A and B. Although the circuit described in the present article utilizes only the CA1, CA2 and power supply pins, the remaining input/output lines are made available for future expansions. Please note that the power supply pins differ from those supplied by John Bell. In particular, the +12V and -12V lines aren't connected on that product and the user will have to connect a jumper wire to those connectors. The DI09 circuit board manufactured by Interactive Structures does not have DIP sockets on board. Labels in parentheses correspond to the labelling system used by Interactive Structures.

A



B

(DL1) PA0	1	DIP Socket A	16	NC	(DL9) PB0	1	DIP Socket B	16	NC
(DL2) PA1	2		15	+5 Volts	(DL10) PB1	2		15	+5 Volts
(DL3) PA2	3		14	+12 Volts	(DL11) PB2	3		14	+12 Volts
(DL4) PA3	4		13	-12 Volts	(DL12) PB3	4		13	-12 Volts
(DL5) PA4	5		12	GND	(DL13) PB4	5		12	GND
(DL6) PA5	6		11	GND	(DL14) PB5	6		11	GND
(DL7) PA6	7		10	CA2 (CL0)	(DL15) PB6	7		10	CB2
(DL8) PA7	8		9	CA1 (CL1)	(DL16) PB7	8		9	CB1

ply connections are were brought out through this cable.

Software

A complete listing of the software for the acquisition system is reprinted in figure I-3. Part A is the BASIC language portion and part B is the machine language section. Flow charts for the machine language portion are shown in figures I-4 through I-9. These follow very closely the logic contained within the actual program. Line 835 of the BASIC program uses a command specific to the microbuffer printer interface which required alteration when the program was used with other interface cards. The BASIC portion was confined into a small region of memory by using the HIMEM command. This was necessary in order to leave the largest possible memory range available for data storage. In order to fit the program into that space it was necessary to 'pack' the program, removing all REM statements and combining multiple statements on each program line.

A Technical Description of the System

After the user has chosen the type of histogram (ISI or PST) and specified the trade-off between temporal resolution and total acquisition time, he or she issues a start command. This enables interrupts from the spike and trigger lines and initiates the plotting routine. The latter scans through the data storage region of memory and forms a histogram display of whatever data is stored there. In the present implementation the plotting routine forms a histogram having 120 bins, but 16K of computer memory is available for data storage. Thus a histogram made up of 16K "microbins" is formed in memory and several of these microbins are combined to form a single bin in the finished histogram.

As data arrives on the spike or trigger lines the plotting program is interrupted and the arrival time of the incoming spike or trigger data is noted. Each distinguishable arrival time is mapped to a different place in computer memory and the contents of that memory location is incremented.

Figure I-3a

BASIC language section of the histogram program. This portion is responsible for most of the user interaction. As shown it is set up to work with an Apple IIe computer with an Apple 80 column card and a Microbuffer printer interface card. Minor changes would be required for its use with other hardware configurations. In typing in this program, all comments (REM statements) must be removed in order to condense the program sufficiently for it to fit into the available memory space.

```
 10  REM   ********************************
 20  REM   *    HISTOGRAM GENERATING      *
 30  REM   *      DATA ACQUISITION        *
 40  REM   *           SYSTEM             *
 50  REM   *                              *
 60  REM   *  COPYRIGHT OCTOBER, 1983     *
 70  REM   *       MARK S. COHEN          *
 80  REM   ********************************
100  REM HIMEM:8192
110  LET PS = 16718:
     LET HI = 17297:
     LET IS = 16640:
     LET LINES = 17792:
     LET FA = 17937:
     LET SWITCH = 18041:
     LET CI = 16695:
     REM    PS    IS PST INIT   HI    IS GRAPH   FA    IS FASTP
     LOT   LINES IS GRID   CI    ENABLES INTERRUPTS
120  HOME :
     TEXT :
     LET D$ =  CHR$ (4)
129  REM TITLE PAGE
130  VTAB 10:
     PRINT "ISI AND PST HISTOGRAM GENERATOR"
140  VTAB 20:
     PRINT  TAB( 18)"MARK COHEN":
     PRINT  TAB( 18)"APRIL, 1983"
150  PRINT D$;"BLOAD ISI AND PST":
     REM  MACHINE LANGUAGE SUBROUTINES
160  LET D$ =  CHR$ (13) +  CHR$ (4):
     TEXT :
     ONERR  GOTO 1290
170  PRINT D$;"PR#3":
     REM  TURN ON 80 COLUMNS
179  REM MAIN MENU
180  VTAB 3:
     PRINT "SELECT:":
     VTAB 5:
     PRINT  TAB( 10)"A" TAB( 10)"Generate PST Histogram"
190  VTAB 8:
     PRINT  TAB( 10)"B" TAB( 10)"Generate ISI Histogram"
200  VTAB 11:
     PRINT  TAB( 10)"C" TAB( 10)"Examine data stored on disk"
210  VTAB 14:
     PRINT  TAB( 10)"D" TAB( 10)"Catalog Disk 2"
220  VTAB 17:
     PRINT  TAB( 10)"Q" TAB( 10)"QUIT"
230  VTAB 22:
     PRINT "PRESS A,B,C,D or Q":
     GET A$
240  ON A$ = "A" OR A$ = "B" GOTO 260:
     ON A$ = "C" GOTO 530:
     ON A$ = "Q" GOTO 700:
     IF A$ = "D" THEN
          PRINT D$;"CATALOG D2":
         GET A$:
         GOTO 160
```

```
250  INVERSE :
     PRINT "PLEASE... PRESS A,B,C,D or Q ":
     NORMAL :
     GOTO 230
259  REM HISTOGRAM ACQUISITION SET-UP
260  HOME :
     VTAB 5:
     PRINT "Please enter the binwidth, in msec, you wish to see
      displayed.":
     PRINT "This should be a multiple of 0.1 msec. (e.g. 4.1)
270  PRINT :
     INPUT "BINWIDTH (followed by 'RETURN'):";BW
280  LET BW = ( INT ((BW + .0001) * 10)) / 10:
     LET CLOCK = 1
289  REM SET CLOCK RATE TO CORRESPOND TO BINWIDTH
290  IF BW > 12.8 THEN
          LET CLOCK = 2:
         IF BW > 25.6 THEN
              LET CLOCK = 5:
             IF BW > 64.2 THEN
                  LET CLOCK = 10:
                 IF BW > 128 THEN
                      LET CLOCK = 20:
                     IF BW > 256 THEN
                          LET CLOCK = 50:
                         IF BW > 645 THEN
                              LET CLOCK = 100:
                             IF BW > 1280 THEN
                                  LET CLOCK = 500
300  IF BW < .1 THEN
          LET BW = .1
310  IF BW > 6400 THEN
          PRINT "I'm sorry, the maximum binwidth is 6400 msec."
         :
         GOTO 270
320  PRINT :
     PRINT "Please enter your desired artifact suppression (in
     msec.)":
     PRINT "This should be an integer multiple of ";.1 * CLOCK"
      msec."
330  PRINT :
     INPUT "ARTIFACT SUPPRESSION:";AS
340  LET M = 25.5 * CLOCK:
     IF AS > M THEN
          PRINT "I'm sorry, the maximum duration is "M" msec.":
         GOTO 330
350  LET AS = ( INT ((AS + .0001) * 10 / CLOCK)) * CL / 10
360  HOME :
     VTAB 10:
     PRINT  TAB( 10)"ARTIFACT SUPPRESSION";:
     POKE 36,50:
     PRINT AS;:
     POKE 36,57:
     PRINT "milliseconds"
370  PRINT  TAB( 10)"BINWIDTH";:
     POKE 36,50:
     PRINT ;BW;:
     POKE 36,57:
```

```
     PRINT "milliseconds"
380  PRINT  TAB( 10)"SAMPLING RATE";:
     POKE 36,50:
     PRINT CLOCK * 100;:
     POKE 36,57:
     PRINT "microseconds"
390  LET DW = BW * 120:
     LET TB$ = "milliseconds":
     IF BW > 8.3 THEN
          LET TB$ = "seconds":
         LET DW = BW * .120
400  PRINT  TAB( 10)"DISPLAY WIDTH";:
     POKE 36,50:
     PRINT DW;:
     POKE 36,57:
     PRINT TB$
410  PRINT :
     PRINT "IS THIS CORRECT (Y/N)?":
     GET B$:
     ON B$ <  > "Y" GOTO 260
420  POKE 898,(BW * 10 / CLOCK):
     POKE 899,16:
     REM PASS BW, VG TO LOCATIONS ACCESSSED BY MACHINE LANGUAGE
      ROUTINESPOKE IN CLOCK RATE AND ARTIFACT SUPPRESSION
430  LET BTIMER = CLOCK * 50:
     POKE 897,( INT (BTIMER / 256)):
     POKE 896,(BTIMER -  PEEK (897) * 256)
440  POKE 16395,(255 - AS * 10 / CLOCK)
449  REM INITIALIZE OFFSET TO 0
450  POKE 904,0:
     POKE 905,150:

460  POKE 50440,0:
     POKE 50441,0:
     REM  STOP TIMERS
470  POKE 16407,66:
     POKE 16406,190:
     HOME :
     VTAB 21:
     PRINT  TAB( 5)"PRESS 'G' TO START, 'S' TO STOP";:
     IF A$ = "A" THEN
          PRINT ", '1' FOR SINGLE SWEEP"
480  GET B$:
     IF B$ = "1" THEN
          POKE 16406,46:
         REM  END FLAG
490  ON B$ = "S" GOTO 160:
     ON B$ <  > "G" AND B$ <  > "1" GOTO 470:
     ON A$ = "B" GOTO 520:
     CALL PS
499  REM PLOTTING ROUTINE
500  CALL HI
510  GOSUB 720:
     GOTO 850
519  REM ISI INITIALIZATION
520  CALL IS:
     GOTO 500
529  REM STORED DATA HANDLER
```

```
530  HOME :
     VTAB 3:
     PRINT "Is the data stored as:"
540  VTAB 8:
     PRINT  TAB( 20)"A" TAB( 5)"Graph"
550  VTAB 11:
     PRINT  TAB( 26)"or"
560  VTAB 14:
     PRINT  TAB( 20)"B" TAB( 5)"Raw Data"
570  VTAB 22:
     PRINT  TAB( 5)"TYPE A or B":
     GET B$:
     ON B$ = "B" GOTO 610:
     IF B$ <  > "A" THEN
          INVERSE :
          PRINT "PLEASE... ENTER A or B ":
          NORMAL :
          GOTO 570
579  REM GRID SET UP
580  GOSUB 1260:
     HGR :
     HCOLOR= 3:
     CALL LINES
590  CALL FA:
     REM  FAST PLOT FOR DATA STORED FROM 768 TO 888
600  GOTO 640
610  GOSUB 1260
620  POKE 896, PEEK (22018):
     POKE 897, PEEK (22019):
     POKE 902, PEEK (22016):
     POKE 903, PEEK (22017):
     REM MAKE A COPY OF VARIABLESIN LOWER VARIABLE SPACE
630  LET CLOCK = ( PEEK (22019) * 256 +  PEEK (22018)) / 50:
     GOTO 1110
639  REM   TITLE LINE
640  HOME :
     VTAB 21:
     PRINT  TAB( 25)TI$:
     GOSUB 730
650  HOME :
     VTAB 21:
     PRINT "P to Print Graph, H to read Histogram, Return to ex
     it":
     GET B$:
     ON B$ = "P" GOTO 670:
     ON B$ = "H" GOSUB 1350:
     IF B$ <  >  CHR$ (13) THEN
          INVERSE :
          PRINT "PLEASE ENTER P or H ":
          NORMAL :
          GOTO 650
660  GOTO 160
669  REM SET UP FOR PRINTING
670  PRINT "Would you like a new title? (default title is ";TI$
     ;")":
     GET B$:
     IF B$ = "Y" THEN
          GOSUB 800:
```

```
        GOTO 160
680  IF B$ <  > "N" THEN
         INVERSE :
        PRINT "PLEASE ENTER Y or N ":
        NORMAL :
        GOTO 670
690  GOSUB 820:
     GOTO 160:
     REM PRINT USING DEFAULT TITLE
699  REM EXIT
700  TEXT :
     HOME :
     PRINT "TO RESTART, TYPE 'GOTO 140'":
     PRINT "TO RESTART USING CURRENT DATA TYPE 'GOTO 1090'"
710  PRINT :
     PRINT "ERROR "; PEEK (222);" ON LINE "; PEEK (219) * 256
      +  PEEK (218):
     END
719  REM LABEL HISTOGRAM
720  HOME :
     VTAB 21
730  IF  PEEK (901) = 0 THEN
         LET GAIN = 16 /  PEEK (899):
        GOTO 750
740  LET GAIN = 2 ^  PEEK (900) * 16
750  LET TS =  PEEK (902) + 256 *  PEEK (903)
760  LET BW =  PEEK (898) * ( PEEK (897) * 256 +  PEEK (896))
      / 500:
     LET TB$ =  STR$ (BW / 100) + " sec/Div":
     IF BW < 100 THEN
         LET TB$ =  STR$ (BW * 10) + " ms/Div"
770  LET OS = (38400 - 256 *  PEEK (905) -  PEEK (904)) / 10:
     LET CLOCK = ( PEEK (897) * 256 +  PEEK (896)) / 50
780  PRINT  TAB( 3)"Vertical: ";GAIN;" Spike(s)/Div" TAB( 20)"H
     orizontal: ";TB$
790  PRINT  TAB( 3)"Total number of sweeps: ";TS; TAB( 20)"Offs
     et: ";OS * CL;" msec.":
     POKE 36,60:
     PRINT "press any key";:
     GET B$:
     PRINT :
     RETURN
799  REM PRINTING HANDLER
800  HOME :
     VTAB 21:
     PRINT "FIRST LINE OF TITLE:":
     INPUT TA$
810  HOME :
     VTAB 21:
     PRINT "SECOND LINE OF TITLE:":
     INPUT TI$:
     PRINT D$;"PR#1":
     PRINT  CHR$ (9)"G":
     HOME :
     VTAB 21:
     PRINT TA$:
     GOSUB 845:
     GOTO 840
```

```
820  HOME :
     VTAB 21
830  PRINT D$"PR#1":
     PRINT  CHR$ (9)"G":
     GOSUB 845:
     REM PRINT GRAPH THEN TITLE
840  PRINT D$;"PR#3":
     RETURN
845  PRINT TI$:
     PRINT  TAB( 10)"VERTICAL: ";GA;" SPIKE(S) / DIV    HORIZON
     TAL: ";TB$:
     PRINT  TAB( 10)"TOTAL # OF SWEEPS: ";TS;"     OFFSET: ";OS
      * CL;" MS.";:
     RETURN
849  REM OPTIONS MENU
850  TEXT :
     HOME :
     PRINT "SELECT:"
860  VTAB 4:
     PRINT  TAB( 10)"C" TAB( 8)"Continue data collection"
870  VTAB 6:
     PRINT  TAB( 10)"D" TAB( 8)"Save raw Data to disk (uses lot
     s of disk space)"
880  VTAB 8:
     PRINT  TAB( 10)"G" TAB( 8)"Save Graph to disk"
890  VTAB 10:
     PRINT  TAB( 10)"P" TAB( 8)"Print current histogram"
900  VTAB 12:
     PRINT  TAB( 10)"N" TAB( 8)"Plot New data"
910  VTAB 14:
     PRINT  TAB( 10)"R" TAB( 8)"Replot current data"
920  VTAB 16:
     PRINT  TAB( 10)"H" TAB( 8)"Read Histogram
930  VTAB 18:
     PRINT  TAB( 10)"Q" TAB( 8)"QUIT"
940  LET Q =  FRE (0)
950  GET B$:
     PRINT  CHR$ (13):
     REM CARRIAGE RETURN
959  REM CONTINUE ACQUISITION
960  IF B$ = "C" THEN
          HOME :
         VTAB 21:
         PRINT  TAB( 10)"press 'S' to stop":
         CALL CI:
         GOTO 500
970  IF B$ = "D" OR B$ = "G" THEN
          GOTO 1050:
         REM  DISK HANDLER
980  IF B$ <  > "N" THEN
          GOTO 1010
990  HOME :
     VTAB 10:
     PRINT "ERASES DATA IN MEMORY, OK?":
     GET B$:
     IF B$ <  > "Y" AND B$ <  > "y" THEN
          GOTO 850
1000 PRINT "Do you wish to use the same settings of binwidth, h
```

```
      istogram type etc (Y/N)?":
      GET B$:
      GOTO 1100
1010  IF B$ = "P" THEN
           GOSUB 800:
          GOTO 850
1020  IF B$ = "H" THEN
           GOSUB 1350:
          GOTO 850
1030  ON B$ = "Q" GOTO 700:
      ON B$ = "R" GOTO 1110
1040  INVERSE :
      PRINT "PLEASE ENTER C,D,G,P,N,R or Q ":
      NORMAL :
      GOTO 860
1049  REM DISK STORAGE HANDLER
1050  INPUT "What is the title? ";TI$:
      IF  LEN (TI$) > 30 THEN
           PRINT "Title is too long":
          GOTO 1050
1060  PRINT "Which disk should I save to (1 or 2)?":
      GET DN$:
      LET DN =  VAL (DN$):
      IF DN <  > 1 AND DN <  > 2 THEN
           INVERSE :
          PRINT "PLEASE ENTER  1 or 2 ":
          NORMAL :
          GOTO 1060
1070  HOME :
      VTAB 21:
      PRINT  TAB( 10)"...saving ";TI$;" to disk number ";DN
1080  IF B$ = "D" THEN
           PRINT D$;"BSAVE ";TI$;", A$5600, L$4000, D";DN:
          GOTO 850
1090  PRINT D$;"BSAVE ";TI$;", A$0300, L$EF, D";DN:
      GOTO 850
1100  ON B$ = "Y" GOTO 420:
      ON B$ = "N" GOTO 160:
      INVERSE :
      PRINT "PLEASE ENTER Y or N ":
      NORMAL :
      GET B$:
      GOTO 1100
1110  PRINT :
      TEXT :
      PRINT D$;"PR#3":
      PRINT
1119  REM REPLOT HANDLER
1120  PRINT "Enter a binwidth between "0.1 * CLOCK" and "12.8 *
      CLOCK" milliseconds":
      PRINT "The current binwidth is "BW" milliseconds"
1130  PRINT :
      INPUT "WHAT IS YOUR NEW BINWIDTH? ";BW:
      LET BW = ( INT ((BW + .0001) * 10 / CLOCK)) * CLOCK / 10
1140  IF BW > 12.8 * CLOCK THEN
           LET BW = 12.8 * CLOCK
1150  IF BW < .1 * CLOCK THEN
           LET BW = .1 * CLOCK
```

```
1160  PRINT "BINWIDTH IS ";BW
1170  PRINT :
      PRINT "Please enter the offset (in msec.) you wish to see
      displayed.":
      PRINT "Offset is the amount that the origin is shifted to
      the right.
1180  PRINT "This should be a multiple of ";CL / 10;" msec. or 0
      ."
1190  PRINT :
      INPUT "OFFSET:";OS$
1200  IF OS$ = "" THEN
           LET OS$ = "0"
1210  LET OS =  VAL (OS$)
1220  LET OS = ( INT ((OS + .0001) * 10 / CLOCK)) * CL / 10:
      REM  CONVERT TO MULTIPLE OF CLOCK
1230  PRINT "OFFSET IS:";OS:
      LET OB = 38400 - OS * 10 / CL
1240  POKE 905, INT (OB / 256):
      POKE 904,(OB -  PEEK (905) * 256)
1250  PRINT "Total time displayed will be ".120 * BW" seconds, O
      K? ":
      GET B$:
      ON B$ <  > "Y" GOTO 1110:
      HOME :
      POKE 898,(BW * 10 / CLOCK):
      POKE 899,16:
      POKE 16392,211:
      CALL HI + 5:
      GOTO 510
1259  REM RECALL STORED DATA
1260  INPUT "What is the title? ";TI$
1270  PRINT "Which disk is the graph or data on (1 or 2)?":
      GET DN$:
      LET DN =  VAL (DN$):
      IF DN <  > 1 AND DN <  > 2 THEN
           INVERSE :
          PRINT "PLEASE ENTER 1 OR 2":
          NORMAL :
          GOTO 1270
1280  PRINT D$;"BLOAD "TI$", D"DN:
      RETURN
1289  REM ERROR HANDLER
1290  POKE 216,0:
      LET EC =  PEEK (222)
1300  IF EC <  > 6 THEN
           GOTO 1320
1310  HOME :
      PRINT TI$;" was not found on disk ";DN:
      INPUT "Please try a new title ";TI$:
      RESUME
1320  IF EC = 254 THEN
           PRINT "PLEASE REENTER THIS VALUE (first press return)
          ":
          GET B$:
          RESUME
1330  IF EC = 9 THEN
           HOME :
          PRINT " DISK FULL ":
```

```
          GET B$
1340  GOTO 700
1349  REM READ HISTOGRAM
1350  HOME :
      CALL SWITCH:
      LET I = 1:
      LET P = I:
      VTAB 23:
      PRINT "USE M , . / to move cursor,  RETURN to stop.":
      REM  SWITCH TURNS ON HIRES1
1360  GET B$:
      IF B$ =  CHR$ (13) THEN
           RETURN
1369  REM CURSOR MOVEMENT
1370  IF B$ =  CHR$ (44) THEN
           LET I = I - 1
1380  IF B$ = "M" THEN
           LET I = I - 10
1390  IF B$ =  CHR$ (46) THEN
           LET I = I + 1
1400  IF B$ = "/" THEN
           LET I = I + 10
1410  IF I < 1 THEN
           LET I = 1
1420  IF I > 120 THEN
           LET I = 120
1430  HCOLOR= 0:
      HPLOT P,155 TO P + 1,155:
      HPLOT P,7 TO P + 1,7:
      LET P = I * 2 + 2:
      HCOLOR= 3:
      HPLOT P,155 TO P + 1,155:
      HPLOT P,7 TO P + 1,7
1440  VTAB 21:
      PRINT  TAB( 10)"TIME=";I * BW + OS * CL;" msec      ";:
      POKE 36,40:
      PRINT "N=";GA *  PEEK (768 + I) / 16;"     ":
      GOTO 1360:
      REM  READ HEIGHT DIRECTLY FROM GRAPH
```

Figure I-3b

6502 machine language subroutines for the acquisition and real time plotting of ISI and PST histograms. This section requires the presence of 6522 versatile interface adaptor card in slot 5 of the Apple computer. If the card is to reside in another slot, lines 13 through 22 will need to be changed and the program re-assembled. The second digit of the hexadecimal address in these lines is the slot number. Thus, line 13 would be altered to read, "T1CL EQU $C401" if the 6522 card is placed in slot 4.

```
2   ********************************************
3   *      ISI AND PST HISTOGRAM GENERATOR       *
4   *             By Mark S. Cohen               *
5   *              COPYRIGHT 1983                *
6   *                                            *
7   ********************************************
8            ORG  $4100
9   HIPAGE   EQU  $95        ;SET MEMORY RANGE FOR
10  LOPAGE   EQU  $56        ;DATA STORAGE.
11  ISIL     EQU  $A1        ;INTERRUPT VECTOR FOR ISI (LO-BYTE)
12  ISIH     EQU  $41        ;REQUIRES SECOND ASSEMBLY.
13  PAD      EQU  $C501      ;PORT A OUTPUT REGISTER.
14  DDRA     EQU  $C503      ;DATA DIRECTION REGISTER A.
15  T1CL     EQU  $C504      ;TIMER 1 HIGH AND LOW COUNTERS
16  T1CH     EQU  $C505      ;ARE SET BY USER (PRIMARY CLOCK RATE).
17  T2CL     EQU  $C508      ;TIMER 2 COUNTS TIMER 1 PULSES AND IS
18  T2CH     EQU  $C509      ;USED TO INDEX MEMORY.
19  ACR      EQU  $C50B      ;AUX. CONTROL REG.
20  PCR      EQU  $C50C      ;PERIPHERAL CONTROL REG.
21  IFR      EQU  $C50D      ;INTERRUPT FLAG REGISTER.
22  IER      EQU  $C50E      ;INTERRUPT ENABLE REGISTER.
23  MPOINTL  EQU  $08        ;LOW BYTE OF CURRENT MEMORY LOCATION.
24  MPOINTH  EQU  $09        ;HIGH BYTE OF CURRENT MEMORY LOCATION.
25  VGNS     EQU  $FC        ;PRODUCT OF VERTGAIN AND NS.
26  NS2      EQU  $FD        ;FOR DATA SHUFFLING.
27  IAL      EQU  $FE        ;BASE ADDRESS LOW.
28  TIMERL   EQU  $0380      ;LOW BYTE OF BIN TIMER.
29  TIMERH   EQU  $0381      ;HIGH BYTE OF BIN TIMER.
30  BWIDTH   EQU  $0382      ;NUMBER OF MINI-BINS PER PLOTTED BIN.
31  VERTGAIN EQU  $0383      ;VERTICAL GAIN (POINTS/SPIKE).
32  GAINRED  EQU  $0384      ;AMOUNT OF GAIN REDUCTION (IF VERTGAIN<1).
33  REDUCE   EQU  $0385      ;REDUCE FLAG; DECREASE GAIN IF SET.
34  TOTALL   EQU  $0386      ;SWEEP COUNTER, LOW
35  TOTALH   EQU  $0387      ;AND HIGH BYTE. COPIES IN $5600 AND $5601.
36  ORGL     EQU  $388       ;ORIGIN OF HISTOGRAM
37  ORGH     EQU  $389
38  DONE     EQU  $4008      ;SET BY USER.
39  LASTFLAG EQU  $400A      ;SET DURING FINAL PLOT OF DATA.
40  SUPPRESS EQU  $400B      ;ARTIFACT SUPPRESSION VALUE.
41  BINNUM   EQU  $400C      ;CURRENT BIN NUMBER
42  NSL      EQU  $400F      ;NUMBER OF SPIKES/BIN (LO BYTE).
43  NSH      EQU  $4010      ;NUMBER OF SPIKES/BIN (HI BYTE).
44  Y_POS    EQU  $400D      ;COPY OF BAR Y POSITION.
45  X        EQU  $4011      ;X FOR PLOTTING GRID LINES
46  Y        EQU  $4012      ;Y FOR SAME
47  I        EQU  $4012
48  ERASEFL  EQU  $4013      ;ERASE FLAG, SET ON BIN OVERFLOW.
49  SHOT     EQU  $4014      ;FLAG FOR SINGLE SWEEP MODE.
50  T_OUT    EQU  $4015      ;T2 TIME OUT FLAG.
51  INTRPTL  EQU  $4016      ;INTERRUPT VECTOR
52  INTRPTH  EQU  $4017
53  HPOSN    EQU  $F411      ;PLOTTING CALLS TO APPLE MONITOR.
54  HPLOT    EQU  $F457
55  PLOT     EQU  $F45A
56  INCRY    EQU  $F504
57  HLIN     EQU  $F53A      ;DRAWS A LINE FROM INTERNAL POSITION
58  *POINT SPECIFIED BY INPUT CONTAINED IN 6502 REGISTRS.
```

```
                    59  *ISI HISTOGRAM INITIALIZATION
4100: 78            60  ISI     SEI             ;DISABLE INTERRUPTS DURING INITIALIZATION.
4101: A9 56         61          LDA  #LOPAGE
4103: A0 04         62          LDY  #04
4105: A2 95         63          LDX  #HIPAGE    ;CLEAR DATA STORAGE RANGE FROM LOPAGE+4
4107: 20 64 45      64          JSR  CLEAR      ;TO HIPAGE.
410A: A9 00         65          LDA  #$00       ;CONFIGURE PORT A
410C: 8D 03 C5      66          STA  DDRA       ;FOR INPUT.
410F: 8C 15 40      67          STY  T_OUT      ;SET THE TIME OUT FLAG.
4112: 8D 0C C5      68          STA  PCR        ;TRIGGER ON NEGATIVE EDGES.
4115: 8D 86 03      69          STA  TOTALL     ;INITIALIZE TOTAL SPIKES
4118: 8D 87 03      70          STA  TOTALH     ;TO ZERO.
411B: A9 E0         71          LDA  #$E0       ;INITIALIZE T1 TO TOGGLE PB7 IN ITS
411D: 8D 0B C5      72          STA  ACR        ;FREE RUN MODE.
4120: A9 A1         73          LDA  #ISIL      ;GET INTERRUPT VECTOR LOW BYTE.
4122: 8D FE 03      74          STA  $3FE       ;STORE IT IN $03FE.
4125: A9 41         75          LDA  #ISIH      ;STORE INTERRUPT HIGH BYTE
4127: 8D FF 03      76          STA  $3FF       ;IN $03FF.
412A: A9 7E         77          LDA  #$7E       ;DISABLE OTHER INTERRUPTS.
412C: 8D 0E C5      78          STA  IER
412F: A9 81         79          LDA  #$81       ;ENABLE INTERRUPTS
4131: 8D 0E C5      80          STA  IER        ;FROM CA2 ONLY.
4134: AD 01 C5      81          LDA  PAD        ;CLEAR IFR0 AND IFR1
4137: 58            82          CLI             ;ENABLE FUTURE INTERRUPTS.
4138: 60            83          RTS


                   105  *PST INITIALIZATION SEQUENCE
414E: 78           106  PSTINIT  SEI             ;DISABLE INTERRUPTS DURING INITIALIZATION.
414F: A9 56        107          LDA  #LOPAGE
4151: A0 04        108          LDY  #04
4153: A2 95        109          LDX  #HIPAGE
4155: 20 64 45     110          JSR  CLEAR      ;CLEAR THE MEMORY RANGE USED FOR DATA STORAGE.
4158: A9 00        111          LDA  #$00       ;CONFIGURE PORT A FOR INPUT.
415A: 8D 14 40     112          STA  SHOT       ;CLEAR ONE-SHOT FLAG.
415D: 8D 0C C5     113          STA  PCR        ;TRIGGER CA1 AND CA2 ON NEGATIVE EDGES.
4160: 8D 86 03     114          STA  TOTALL     ;INITIALIZE TOTALL AND TOTALH
4163: 8D 87 03     115          STA  TOTALH     ;TO ZERO.
4166: 8C 15 40     116          STY  T_OUT      ;SET THE TIME OUT FLAG.
4169: 8D 03 C5     117          STA  DDRA       ;FOR INPUT.
416C: A9 E0        118          LDA  #$E0       ;INITIALIZE T1 TO TOGGLE PB7 IN ITS
416E: 8D 0B C5     119          STA  ACR        ;FREE RUN MODE.
4171: AD 16 40     120          LDA  INTRPTL    ;GET INTERRUPT VECTOR LOW BYTE.
4174: 8D FE 03     121          STA  $3FE       ;STORE IT IN $03FE.
4177: AD 17 40     122          LDA  INTRPTH    ;SO THAT INTERRUPTS CALL THE APPROPRIATE
417A: 8D FF 03     123          STA  $3FF       ;INTERRUPT SEQUENCE FOR PST.
417D: A9 7C        124          LDA  #$7C       ;DISABLE OTHER INTERRUPTS.
417F: 8D 0E C5     125          STA  IER
4182: A9 83        126          LDA  #$83       ;ENABLE INTERRUPTS
4184: 8D 0E C5     127          STA  IER        ;FROM CA1 AND CA2.
4187: AD 01 C5     128          LDA  PAD        ;CLEAR THE IFR
418A: 58           129          CLI             ;ENABLE FUTURE INTERRUPTS.
418B: 60           130          RTS
```

```
                    152  *ISI INTERRUPT ROUTINE
41A1: 78            153  ISIINT   SEI             ;DISABLE INTERRUPTS DURING PROCESSING.
41A2: 98            154           TYA             ;SAVE THE CONTENTS OF THE 6502.
41A3: 48            155           PHA
41A4: 8A            156           TXA
41A5: 48            157           PHA
41A6: A9 20         158           LDA  #$20       ;SET UP MASK FOR BIT 5.
41A8: 2C 0D C5      159           BIT  IFR        ;CHECK IFR5 (TIME OUT FOR T2).
41AB: F0 03         160           BEQ  GOOD       ;IF NOT SET, GO ON.
41AD: EE 15 40      161           INC  T_OUT      ;SET TIME OUT FLAG.
41B0: AC 08 C5      162  GOOD     LDY  T2CL       ;GET TIMER LOW BYTE (CLEAR IFR5).
41B3: AD 09 C5      163           LDA  T2CH       ;GET TIMER HIGH BYTE.
41B6: 85 07         164           STA  $07        ;HIGH BYTE IN $07.
41B8: 84 06         165           STY  $06        ;LO BYTE IN $06.
41BA: AD 80 03      166           LDA  TIMERL     ;GET LOW BYTE FOR TIMER 1.
41BD: 8D 04 C5      167           STA  T1CL       ;PERIOD = 2(TIMERL-TIMERH+2)CLOCK CYCLES.
41C0: AD 81 03      168           LDA  TIMERH     ;TIMER 1 HIGH BYTE.
41C3: 8D 05 C5      169           STA  T1CH       ;THIS COMMAND STARTS THE COUNT.
41C6: A9 FF         170           LDA  #$FF       ;INITIALIZE T2 TO COUNT DOWN
41C8: 8D 08 C5      171           STA  T2CL       ;FROM TOP OF HIPAGE
41CB: A9 95         172           LDA  #HIPAGE    ;AT A RATE SET BY TIMER 1.
41CD: 8D 09 C5      173           STA  T2CH       ;START T2.
41D0: AD 15 40      174           LDA  T_OUT      ;CHECK THE IFR FLAG.
41D3: D0 2B         175           BNE  CHECK      ;IF SET, GET OUT.
41D5: A5 07         176           LDA  $07        ;GET T2CH
41D7: C9 56         177           CMP  #LOPAGE    ;T2<=LOPAGE, GET OUT.
41D9: 90 25         178           BCC  CHECK
41DB: C9 95         179           CMP  #HIPAGE
41DD: D0 05         180           BNE  GO2        ;NOT ON HIPAGE, SO GO ON.
41DF: CC 0B 40      181           CPY  SUPPRESS   ;IF T2CL<SUPPRESS,
41E2: B0 1C         182           BCS  CHECK      ;SUPPRESS THIS POINT.
41E4: A0 00         183  GO2      LDY  #$00       ;CLEAR THE Y-REGISTER.
41E6: 18            184           CLC             ;CLEAR THE CARRY
41E7: A9 01         185           LDA  #$01       ;USING INDIRECT ADDRESSING,
41E9: 71 06         186           ADC  ($06),Y    ;INCREMENT M (M IS LOCATION [$07]I[$06]).
41EB: F0 25         187           BEQ  OVI        ;IF ZEROED DO OVERFLOW.
41ED: 91 06         188           STA  ($06),Y    ;PUT THE INCREMENTED NUMBER BACK.
41EF: A9 01         189           LDA  #01        ;ADD ONE TO THE SPIKE COUNTER
41F1: 18            190           CLC
41F2: 6D 86 03      191           ADC  TOTALL
41F5: 8D 86 03      192           STA  TOTALL     ;LOW BYTE
41F8: A9 00         193           LDA  #00
41FA: 6D 87 03      194           ADC  TOTALH     ;CARRY INTO HIGH BYTE
41FD: 8D 87 03      195           STA  TOTALH
4200: AD 00 C0      196  CHECK    LDA  $C000      ;READ THE KEYBOARD
4203: 10 0A         197           BPL  RETURN1    ;IF NOT PRESSED, GET OUT.
4205: 8D 10 C0      198           STA  $C010      ;CLEAR THE KEYBOARD STROBE.
4208: C9 D3         199           CMP  #$D3       ;HAS "S" BEEN PRESSED?
420A: D0 03         200           BNE  RETURN1    ;IF NOT, KEEP ACQUIRING.
420C: 4C 74 43      201  FINISH1  JMP  FINDONE   ;LEAVE WITH INTERRUPTS DISABLED.
420F: 4C 64 43      202  RETURN1  JMP  FINMORE   ;LEAVE WITH INTERRUPTS ENABLED.
4212: 78            203  OVI      SEI             ;DISABLE INTERRUPTS
4213: 20 DD FB      204           JSR  $FBDD      ;BEEP,
4216: A9 AA         205           LDA  #$AA       ;THEN PUT ASTERISK
4218: 8D 50 07      206           STA  $750       ;ON LOWER LEFT OF SCREEN.
421B: D0 EF         207           BNE  FINISH1    ;ABORT THE RUN.
```

```
                    225  *PST INTERRUPT ROUTINE - SINGLE SWEEP
422E: 78            226  INTRPTS  SEI             ;DISABLE FURTHER INTERRUPTS DURING PROCESSING.
422F: 98            227           TYA             ;SAVE THE CONTENTS OF THE 6502.
4230: 48            228           PHA
4231: 8A            229           TXA
4232: 48            230           PHA
4233: A9 02         231           LDA  #$02       ;CHECK TO SEE WHICH LINE REQUESTED INTERRUPT.
4235: 2C 0D C5      232           BIT  IFR        ;CA1 (TRIG) SETS IFR1 (BIT 2)
4238: D0 3D         233           BNE  RESETS     ;IF SET, DO RESET SEQUENCE.
423A: A9 20         234           LDA  #$20       ;SET MASK FOR BIT 5.
423C: 2C 0D C5      235           BIT  IFR        ;CHECK IFR5 (TIME OUT FOR T2).
423F: D0 22         236           BNE  OUTS       ;IF SET THEN IGNORE THIS POINT
4241: AC 08 C5      237           LDY  T2CL       ;GET TIMER LOW BYTE (CLEARS IFR5).
4244: AD 09 C5      238           LDA  T2CH       ;GET THE TIMER HIGH BYTE.
4247: 85 07         239           STA  $07        ;HIGH BYTE IN $07.
4249: 84 06         240           STY  $06        ;STORE IT IN $06.
424B: C9 56         241           CMP  #LOPAGE    ;T2<=LOPAGE, GET OUT.
424D: 90 14         242           BCC  OUTS
424F: C9 95         243           CMP  #HIPAGE
4251: D0 05         244           BNE  GOS        ;NOT ON HIPAGE, SO GO ON.
4253: CC 0B 40      245           CPY  SUPPRESS   ;IF T2CL<SUPPRESS,
4256: B0 0B         246           BCS  OUTS       ;SUPPRESS THIS POINT.
4258: A0 00         247  GOS      LDY  #$00       ;CLEAR THE Y-REGISTER.
425A: 18            248           CLC             ;CLEAR THE CARRY
425B: A9 01         249           LDA  #$01       ;USING INDIRECT ADDRESSING,
425D: 71 06         250           ADC  ($06),Y    ;INCREMENT M (M IS LOCATION [$07][$06]).
425F: F0 0B         251           BEQ  OVS        ;IF ZEROED, DO OVERFLOW.
4261: 91 06         252           STA  ($06),Y    ;PUT THE INCREMENTED NUMBER BACK.
4263: A9 02         253  OUTS     LDA  #$02       ;CHECK FOR STIM.
4265: 2C 0D C5      254           BIT  IFR
4268: D0 0D         255           BNE  RESETS     ;IF PRESENT DO RESETS.
426A: F0 2C         256           BEQ  CHECKS     ;IF NOT, THEN LEAVE.
426C: 78            257  OVS      SEI             ;DISABLE INTERRUPTS
426D: 20 DD FB      258           JSR  $FBDD
4270: A9 AA         259           LDA  #$AA
4272: 8D 50 07      260           STA  $750
4275: D0 2D         261           BNE  FINISHS    ;ABORT THE RUN.
                    262  *PST RESET SUBROUTINE - SINGLE SWEEP
4277: AD 14 40      263  RESETS   LDA  SHOT       ;IS THIS THE FIRST TRIGGER?
427A: D0 1C         264           BNE  CHECKS     ;IF NOT, IGNORE.
427C: AD B0 03      265           LDA  TIMERL     ;GET LOW BYTE FOR TIMER 1.
427F: 8D 04 C5      266           STA  T1CL       ;PERIOD = 2(TIMERL-TIMERH+2)CLOCK CYCLES.
4282: AD B1 03      267           LDA  TIMERH     ;TIMER 1 HIGH BYTE.
4285: 8D 05 C5      268           STA  T1CH       ;THIS COMMAND STARTS THE COUNT.
4288: A9 FF         269           LDA  #$FF       ;INITIALIZE T2 TO COUNT DOWN
428A: 8D 08 C5      270           STA  T2CL       ;FROM TOP OF HIGH PAGE
428D: A9 95         271           LDA  #HIPAGE    ;AT A RATE SET BY TIMER 1.
428F: 8D 09 C5      272           STA  T2CH       ;START T2.
4292: EE 86 03      273           INC  TOTALL     ;SET TOTALL TO 1.
4295: EE 14 40      274           INC  SHOT       ;SET SHOT FLAG.
4298: AD 00 C0      275  CHECKS   LDA  $C000      ;READ THE KEYBOARD
429B: 10 0A         276           BPL  RETURNS    ;IF NOT PRESSED, GET OUT.
429D: 8D 10 C0      277           STA  $C010      ;CLEAR THE KEYBOARD STROBE.
42A0: C9 D3         278           CMP  #$D3       ;HAS "S" BEEN PRESSED?
42A2: D0 03         279           BNE  RETURNS    ;IF NOT, KEEP ACQUIRING.
42A4: 4C 74 43      280  FINISHS  JMP  FINDONE    ;LEAVE WITH INTERRUPTS DISABLED.
42A7: 4C 64 43      281  RETURNS  JMP  FINMORE    ;LEAVE WITH INTERRUPTS ENABLED.
```

```
                302  *PST INTERRUPT ROUTINE - MULTIPLE SWEEP
42BE: 78        303  INTRPTM  SEI             ;DISABLE FURTHER INTERRUPTS DURING PROCESSING.
42BF: 98        304          TYA             ;SAVE THE CONTENTS OF THE 6502.
42C0: 48        305          PHA
42C1: 8A        306          TXA
42C2: 48        307          PHA
42C3: A9 20     308          LDA  #$20       ;SET MASK FOR BIT 5.
42C5: 2C 0D C5  309          BIT  IFR        ;CHECK IFR5 (TIME OUT FOR T2).
42C8: F0 03     310          BEQ  TIMEOK     ;IF NOT SET THEN GO ON.
42CA: EE 15 40  311          INC  T_OUT      ;IF TIME OUT SET T_OUT.
42CD: AC 08 C5  312  TIMEOK  LDY  T2CL       ;GET TIMER LOW BYTE.
42D0: AD 09 C5  313          LDA  T2CH       ;GET THE TIMER HIGH BYTE.
42D3: 85 07     314          STA  $07        ;HIGH BYTE IN $07.
42D5: 84 06     315          STY  $06        ;STORE LOW BYTE IN $06.
42D7: A9 02     316          LDA  #$02       ;CHECK TO SEE WHICH LINE REQUESTED INTERRUPT.
42D9: 2C 0D C5  317          BIT  IFR        ;CA1 (TRIG) SETS IFR1 (BIT 2)
42DC: D0 36     318          BNE  RESETM     ;IF SET, DO RESET SEQUENCE.
42DE: AD 15 40  319          LDA  T_OUT      ;CHECK TIME OUT FLAG.
42E1: D0 1A     320          BNE  OUTM       ;IF SET GET OUT
42E3: A5 07     321          LDA  $07        ;CHECK T2CH
42E5: C9 56     322          CMP  #LOPAGE    ;T2<=LOPAGE, GET OUT.
42E7: 90 14     323          BCC  OUTM
42E9: C9 95     324          CMP  #HIPAGE
42EB: D0 05     325          BNE  GOM        ;NOT ON HIPAGE, SO GO ON.
42ED: CC 0B 40  326          CPY  SUPPRESS   ;IF T2CL<SUPPRESS,
42F0: B0 0B     327          BCS  OUTM       ;SUPPRESS THIS POINT.
42F2: A0 00     328  GOM     LDY  #$00       ;CLEAR THE Y-REGISTER.
42F4: 18        329          CLC             ;CLEAR THE CARRY
42F5: A9 01     330          LDA  #$01       ;USING INDIRECT ADDRESSING,
42F7: 71 06     331          ADC  ($06),Y    ;INCREMENT M (M IS LOCATION [$07][$06]).
42F9: F0 0B     332          BEQ  OVM        ;SEE IF BIN IS FULL.
42FB: 91 06     333          STA  ($06),Y    ;PUT THE INCREMENTED NUMBER BACK.
42FD: A9 02     334  OUTM    LDA  #$02       ;CHECK FOR STIM.
42FF: 2C 0D C5  335          BIT  IFR
4302: D0 10     336          BNE  RESETM     ;IF PRESENT DO RESETS.
4304: F0 4A     337          BEQ  CHECKM     ;IF NOT, THEN LEAVE.
4306: 78        338  OVM     SEI             ;DISABLE INTERRRUPTS
4307: 20 DD FB  339          JSR  $FBDD      ;OVERFLOW ROUTINE. BEEP
430A: A9 AA     340          LDA  #$AA       ;THEN PLACE ASTERISK ON SCREEN.
430C: 8D 50 07  341          STA  $750
430F: D0 4B     342          BNE  FINISHM    ;ABORT THE RUN.
4311: EA        343          NOP
```

```
                346  *PST RESET SUBROUTINE - MULTI SWEEP
4314: A5 07     347  RESETM   LDA  $07        ;IF NOT IN HIPAGE
4316: C9 95     348           CMP  #HIPAGE
4318: D0 0C     349           BNE  STARTM     ;THEN RESTART.
431A: CC 0B 40  350           CPY  SUPPRESS   ;IS LOW BYTE IN SUPPRESS RANGE?
431D: 90 07     351           BCC  STARTM     ;IF NOT, START TIMERS.
431F: F0 05     352           BEQ  STARTM     ;IF LOW BYTE=SUPRESS, START TIMERS.
4321: AD 15 40  353           LDA  T_OUT      ;CHECK FOR TIME OUT.
4324: F0 39     354           BEQ  RETURNM    ;IF NOT TIME OUT THEN IGNORE.
4326: AD 80 03  355  STARTM   LDA  TIMERL     ;GET LOW BYTE FOR TIMER 1.
4329: 8D 04 C5  356           STA  T1CL       ;PERIOD = 2(TIMERL-TIMERH+2)CLOCK CYCLES.
432C: AD 81 03  357           LDA  TIMERH     ;TIMER 1 HIGH BYTE.
432F: 8D 05 C5  358           STA  T1CH       ;THIS COMMAND STARTS THE COUNT.
4332: A9 FF     359           LDA  #$FF       ;INITIALIZE T2 TO COUNT DOWN
4334: 8D 08 C5  360           STA  T2CL       ;FROM TOP OF HIGH PAGE
4337: A9 95     361           LDA  #HIPAGE    ;AT A RATE SET BY TIMER 1.
4339: 8D 09 C5  362           STA  T2CH       ;START T2.
433C: A9 01     363           LDA  #01        ;THIS SECTION ADDS ONE TO THE
433E: 18        364           CLC             ;TOTAL NUMBER OF STIMULI COUNTED.
433F: 6D 86 03  365           ADC  TOTALL     ;LOW BYTE FIRST.
4342: 8D 86 03  366           STA  TOTALL
4345: A9 00     367           LDA  #00
4347: 8D 15 40  368           STA  T_OUT      ;CLEAR THE TIME OUT FLAG.
434A: 6D 87 03  369           ADC  TOTALH     ;THEN CARRY INTO HIGH BYTE
434D: 8D 87 03  370           STA  TOTALH
4350: AD 00 C0  371  CHECKM   LDA  $C000      ;READ THE KEYBOARD
4353: 10 0A     372           BPL  RETURNM    ;IF NOT PRESSED, GET OUT.
4355: 8D 10 C0  373           STA  $C010      ;CLEAR THE KEYBOARD STROBE.
4358: C9 D3     374           CMP  #$D3       ;HAS "S" BEEN PRESSED?
435A: D0 03     375           BNE  RETURNM    ;IF NOT, KEEP ACQUIRING.
435C: 4C 74 43  376  FINISHM  JMP  FINDONE    ;LEAVE WITH INTERRUPTS DISABLED.
435F: 4C 64 43  377  RETURNM  JMP  FINMORE    ;LEAVE WITH INTERRUPTS ENABLED.
4362: EA        378           NOP
4363: EA        379           NOP
4364: A9 00     380  FINMORE  LDA  #00        ;CLEAR THE TIME OUT FLAG.
4366: 8D 15 40  381           STA  T_OUT
4369: AD 01 C5  382           LDA  PAD        ;CLEAR THE INTERRUPT FLAG REGISTER.
436C: 68        383           PLA
436D: AA        384           TAX             ;RESTORE THE 6502 REGISTERS.
436E: 68        385           PLA
436F: A8        386           TAY
4370: A5 45     387           LDA  $45
4372: 58        388           CLI             ;ENABLE INTERRUPTS
4373: 40        389           RTI
4374: 78        390  FINDONE  SEI             ;DISABLE INTERRUPTS (AGAIN).
4375: A9 7F     391           LDA  #$7F       ;DISABLE ALL INTERRUPTS
4377: 8D 0E C5  392           STA  IER
437A: A9 00     393           LDA  #00        ;CLEAR THE TIME OUT FLAG.
437C: 8D 15 40  394           STA  T_OUT
437F: A9 D3     395           LDA  #$D3       ;SET DONE FLAG = 'S'.
4381: 8D 08 40  396           STA  DONE
4384: 68        397           PLA             ;RESTORE THE 6502 REGISTERS.
4385: AA        398           TAX
4386: 68        399           PLA
4387: A8        400           TAY
4388: A5 45     401           LDA  $45
438A: 40        402           RTI
```

```
                409  *PLOTTING ROUTINES
                410  *THIS SECTION PLOTS THE DATA THAT HAS
                411  *BEEN COLLECTED ABOVE, GENERATING HISTOGRAMS.
                412  *COORDINATES AND SEVERAL VARIABLES MUST BE
                413  *SET UP BY A BASIC PROGRAM WHICH CALLS THIS ROUTINE.
4391: A9 00     414  GRAPH    LDA  #$00
4393: 8D 08 40  415           STA  DONE       ;INITIALIZE DONE.
4396: A9 7F     416           LDA  #$7F       ;SET COLOR = WHITE
4398: 85 E4     417           STA  $E4
439A: A9 01     418           LDA  #$01       ;INITIALIZE GAINRED TO ONE.
439C: 8D 84 03  419           STA  GAINRED
439F: A9 00     420           LDA  #$00       ;INITIALIZE LASTFLAG
43A1: 8D 0A 40  421           STA  LASTFLAG
43A4: 8D B5 03  422           STA  REDUCE     ;AND REDUCE FLAG.
43A7: A9 20     423           LDA  #$20       ;SET $E6 (PAGE INDICATOR) TO #$20
43A9: 85 E6     424           STA  $E6
43AB: 2C 54 C0  425           BIT  $C054      ;SET HGR SOFT SWITCH.
43AE: 2C 53 C0  426           BIT  $C053      ;DISPLAY  HI-RES SCREEN 1
43B1: AD 57 C0  427           LDA  $C057      ;
43B4: AD 50 C0  428           LDA  $C050      ;SET TO MIXED MODE.
43B7: A0 00     429  ERASE    LDY  #00        ;INITIALIZE Y TO 00.
43B9: 8C 13 40  430           STY  ERASEFL    ;CLEAR THE ERASE FLAG.
43BC: A9 20     431           LDA  #$20       ;SET LOWER LIMIT OF CLEAR.
43BE: A2 3F     432           LDX  #$3F       ;SET ENDING PAGE NUMBER.
43C0: 20 64 45  433           JSR  CLEAR
                434  *USE IAL AND IAH TO INDEX DATA STORAGE FOR GRAPH
43C3: A9 00     435           LDA  #$00
43C5: 85 FE     436           STA  IAL
43C7: A9 03     437           LDA  #$03       ;STORAGE IS FROM $0300 UP.
43C9: 85 FF     438           STA  IAL+1
43CB: 20 80 45  439           JSR  GRID
43CE: A9 00     440  ALOOP    LDA  #$00       ;INITIALIZE BINNUM
43D0: 8D 0C 40  441           STA  BINNUM     ;TO ZERO.
43D3: AD 88 03  442           LDA  ORGL       ;INITIALIZE MPOINTH-MPOINTL TO ORGH-ORGL
43D6: 85 08     443           STA  MPOINTL
43D8: AD 89 03  444           LDA  ORGH
43DB: 85 09     445           STA  MPOINTH
43DD: EE 0C 40  446  NEXTBIN  INC  BINNUM     ;NEXT BIN
43E0: AE 82 03  447           LDX  BWIDTH     ;X COUNTS NUMBER OF MINIBINS TO SUM IN NS.
43E3: A9 00     448           LDA  #$00       ;INITIALIZE NSL TO ZERO.
43E5: 8D 0F 40  449           STA  NSL        ;ZERO THE SUM LOCATIONS
43E8: 8D 10 40  450           STA  NSH        ;NSL AND NSH.
43EB: C6 08     451  BLOOP    DEC  MPOINTL    ;LOOK AT NEXT BIN.
43ED: A9 FF     452           LDA  #$FF       ;IF MPOINTL IS
43EF: C5 08     453           CMP  MPOINTL    ;EQUAL TO ZERO
43F1: D0 02     454           BNE  SUM        ;DO SUMMATION.
43F3: C6 09     455           DEC  MPOINTH    ;OTHERWISE GO TO THE NEXT PAGE.
43F5: 18        456  SUM      CLC             ;CARRY WILL BE A FLAG.
43F6: AD 0F 40  457           LDA  NSL        ;ADD [MPOINTH-MPOINTL] TO NSL.
43F9: A0 00     458           LDY  #$00       ;USING INDIRECT ADDRESSING.
43FB: 71 08     459           ADC  (MPOINTL),Y
43FD: 8D 0F 40  460           STA  NSL
4400: A9 00     461           LDA  #00        ;CLEAR THE ACCUMULATOR
```

```
4402: 6D 10 40  462          ADC  NSH        ;AND ADD THE CARRY TO NSH.
4405: 8D 10 40  463          STA  NSH
4408: CA        464          DEX             ;DECREMENT THE COUNTER
4409: D0 E0     465          BNE  BLOOP      ;IF COUNTER=0 PLOT THE BIN
440B: AD 85 03  466          LDA  REDUCE     ;IF REDUCE FLAG SET, CONTRACT GRAPH.
440E: F0 06     467          BEQ  TEST       ;OTHERWISE TEST NSL-NSH.
4410: 20 31 45  468          JSR  CONTRACT
4413: 38        469          SEC             ;FORCE A BRANCH TO PLOTBAR.
4414: B0 1B     470          BCS  PLOTBAR
4416: AD 10 40  471  TEST    LDA  NSH        ;IF NSH IS ZERO
4419: F0 0C     472          BEQ  TESTNSL    ;TEST NSL.
441B: EE 13 40  473  SET     INC  ERASEFL    ;SET ERASE AND REDUCE FLAGS.
441E: EE 85 03  474          INC  REDUCE
4421: 20 31 45  475          JSR  CONTRACT   ;CONTRACT THE GRAPH.
4424: 38        476          SEC             ;FORCE A BRANCH TO PLOTBAR.
4425: B0 0A     477          BCS  PLOTBAR
4427: AD 0F 40  478  TESTNSL LDA  NSL
442A: C9 91     479          CMP  #$91       ;IS NSL > 145?
442C: B0 ED     480          BCS  SET        ;SET FLAGS AND CONTRACT.
442E: 20 FD 44  481          JSR  HEIGHT     ;IF NSL<145 FIND BAR HEIGHT.
4431: AC 0C 40  482  PLOTBAR LDY  BINNUM     ;STORE VGNS IN $0300+BINNUM.
4434: 91 FE     483          STA  (IAL),Y
4436: A5 FC     484          LDA  VGNS
4438: F0 5A     485          BEQ  H          ;IF VGNS=ZERO, GET NEXT BIN.
443A: 85 FD     486          STA  NS2        ;SAVE A COPY OF VGNS.
443C: 18        487          CLC             ;COMPUTE X POSITION
443D: A0 00     488          LDY  #$00
443F: AD 0C 40  489          LDA  BINNUM     ;GET THE BIN NUMBER
4442: 2A        490          ROL             ;DOUBLE IT
4443: 69 02     491          ADC  #$02       ;AND ADD THE OFFSET.
4445: 90 02     492          BCC  C          ;IF THIS SETS THE CARRY
4447: A0 01     493          LDY  #$01       ;SET Y=1.
4449: 8D 11 40  494  C       STA  X          ;SAVE X POSITION IN X & Y.
444C: 8C 12 40  495          STY  Y
444F: AA        496          TAX             ;XPOS INTO X-REGISTER.
4450: 18        497          CLC             ;CLEAR THE CARRY FOR SUBTRACTION.
4451: A9 9A     498          LDA  #$9A       ;FIND YPOS=154-VGNS
4453: E5 FC     499          SBC  VGNS
4455: 8D 0D 40  500          STA  Y_POS      ;SAVE A COPY IN Y_POS
4458: 20 11 F4  501          JSR  HPOSN      ;FIND THE COORDINATES, USING A MONITOR CALL.
445B: A5 1C     502  DLOOP   LDA  $1C        ;PLOT THE POINT, USING STANDARD APPLE
445D: 51 26     503          EOR  ($26),Y    ;TECHNIQUES.
445F: 25 30     504          AND  $30
4461: 51 26     505          EOR  ($26),Y
4463: 91 26     506          STA  ($26),Y
4465: C6 FD     507          DEC  NS2        ;DECREMENT NS2 (i.e. GET NEXT Y-COORDINATE).
4467: F0 06     508          BEQ  F          ;IF VGNS =0 THEN MOVE ON.
4469: 20 04 F5  509          JSR  INCRY      ;OTHERWISE GET NEXT Y VALUE
446C: 4C 5B 44  510          JMP  DLOOP      ;AND PLOT IT
446F: AE 11 40  511  F       LDX  X          ;RELOAD PROCESSOR REGISTERS.
4472: AC 12 40  512          LDY  Y
```

```
4475: E8        513           INX             ;WITH NEW X POSITION (DOUBLE WIDTH)
4476: A5 FC     514           LDA  VGNS       ;RESET THE COUNTER (NS2).
4478: 85 FD     515           STA  NS2
447A: AD 0D 40  516           LDA  Y_POS
447D: 20 11 F4  517           JSR  HPOSN      ;FIND THE COORDINATES, USING A MONITOR CALL.
4480: A5 1C     518 ELOOP     LDA  $1C        ;PLOT THE POINT.
4482: 51 26     519           EOR  ($26),Y
4484: 25 30     520           AND  $30
4486: 51 26     521           EOR  ($26),Y
4488: 91 26     522           STA  ($26),Y
448A: C6 FD     523           DEC  NS2        ;DECREMENT NS2 (i.e. GET NEXT Y-COORDINATE).
448C: F0 06     524           BEQ  H          ;IF VGNS =0 THEN MOVE ON.
448E: 20 04 F5  525           JSR  INCRY      ;OTHERWISE GET NEXT Y VALUE
4491: 4C 80 44  526           JMP  ELOOP      ;AND PLOT IT
4494: A9 78     527 H         LDA  #$78       ;IF BINNUM IS EQUAL TO 120
4496: CD 0C 40  528           CMP  BINNUM
4499: F0 03     529           BEQ  HIBIN      ;THEN STOP THEN LOOK AT FLAGS.
449B: 4C DD 43  530           JMP  NEXTBIN    ;OTHERWISE PLOT THE NEXT BINNUM.
449E: AD 00 C0  531 HIBIN     LDA  $C000      ;READ THE KEYBOARD PORT.
44A1: 30 03     532           BMI  IDENTIFY   ;IF NOT CLEAR, BRANCH TO COMPARE.
44A3: AD 08 40  533           LDA  DONE       ;IF CLEAR, READ DONE
44A6: C9 D3     534 IDENTIFY  CMP  #$D3       ;IF DONE NOR KYBD EQUAL "S"
44AB: 8D 10 C0  535           STA  $C010      ;CLEAR THE KEYBOARD STROBE.
44AB: F0 0B     536           BEQ  LAST?      ;CHECK LAST FLAG, IF "S".
44AD: AD 13 40  537           LDA  ERASEFL    ;CHECK THE ERASE FLAG.
44B0: F0 03     538           BEQ  GOA        ;IF CLEAR THEN START AT BINNUM=0 AGAIN.
44B2: 4C B7 43  539           JMP  ERASE      ;IF SET ERASE AND REPLOT THE HISTOGRAM.
44B5: 4C CE 43  540 GOA       JMP  ALOOP
44B8: 78        541 LAST?     SEI             ;DISABLE INTERRUPTS FOR FINAL GO-THROUGHS.
44B9: A9 D3     542           LDA  #$D3       ;SET DONE FLAG
44BB: 8D 08 40  543           STA  DONE       ;TO "S"
44BE: AD 0A 40  544           LDA  LASTFLAG   ;SEE IF LASTFLAG IS SET.
44C1: F0 21     545           BEQ  SETFLAG    ;IF NOT, SET IT.
44C3: AD 13 40  546           LDA  ERASEFL    ;CHECK THE ERASE FLAG.
44C6: F0 03     547           BEQ  GETOUT     ;IF CLEAR THEN DO EXIT SEQUENCE.
44C8: 4C B7 43  548           JMP  ERASE      ;OTHERWISE ERASE.
44CB: AD 86 03  549 GETOUT    LDA  TOTALL     ;MAKE A COPY OF TOTAL  COUNT
44CE: 8D 00 56  550           STA  $5600      ;IN BOTTOM OF DATA RANGE
44D1: AD 87 03  551           LDA  TOTALH
44D4: 8D 01 56  552           STA  $5601
44D7: AD 80 03  553           LDA  TIMERL     ;COPY THE TIMER SETTINGS
44DA: 8D 02 56  554           STA  $5602
44DD: AD 81 03  555           LDA  TIMERH
44E0: 8D 03 56  556           STA  $5603      ;TO A CONVENIENT PLACE FOR STORAGE.
44E3: 60        557           RTS             ;GO TO CALLING PROGRAM.
44E4: EE 0A 40  558 SETFLAG   INC  LASTFLAG   ;SET LASTFLAG
44E7: 4C B7 43  559           JMP  ERASE      ;GO THROUGH MAIN LOOP ONCE MORE.
```

```
44FD: 85 FD     579  HEIGHT   STA  NS2        ;MAKE A COPY OF NSL.
44FF: A2 08     580          LDX  #$08       ;THIS SECTION MULTIPLIES NSL BY VERTGAIN.
4501: A9 00     581          LDA  #00        ;CLEAR A.
4503: 46 FD     582  BR1      LSR  NS2        ;SHIFT NS INTO THE CARRY.
4505: 90 04     583          BCC  BR2        ;IF THE CARRY=0 THEN SKIP ADDITION.
4507: 18        584          CLC             ;CLEAR THE CARRY FOR ADDITION.
4508: 6D 83 03  585          ADC  VERTGAIN   ;COLLECT THE SUM OF THE PRODUCTS
450B: 6A        586  BR2      ROR             ;IN THE ACCUMULATOR. ROTATE IT
450C: 66 FC     587          ROR  VGNS       ;INTO THE PRODUCT LOCATION
450E: CA        588          DEX             ;DECREMENT THE BIT COUNTER
450F: D0 F2     589          BNE  BR1        ;UNTIL EIGHT BITS HAVE BEEN COUNTED.
4511: C9 00     590          CMP  #$00       ;IF A IS NOT ZERO
4513: D0 07     591          BNE  NEWVG      ;THEN GET A NEW VERTGAIN.
4515: A5 FC     592          LDA  VGNS       ;IF VGNS<145 PLOT IT.
4517: C9 91     593          CMP  #$91       ;OTHERWISE...
4519: B0 01     594          BCS  NEWVG      ;REDUCE VERTGAIN.
451B: 60        595          RTS
451C: 4E 83 03  596  NEWVG    LSR  VERTGAIN   ;DIVIDE THE GAIN BY TWO
451F: AD 0F 40  597          LDA  NSL
4522: EE 13 40  598          INC  ERASEFL    ;SET THE ERASE FLAG TO REPLOT DATA.
4525: D0 D6     599          BNE  HEIGHT     ;AND TRY AGAIN.


4531: AE 84 03  610  CONTRACT LDX  GAINRED    ;REDUCE GAIN BY SHIFTING NSH-NSL TO THE
4534: 18        611  ILOOP    CLC             ;RIGHT (i.e. DIVIDING BY TWO) USE THE X
4535: 4E 10 40  612          LSR  NSH        ;REGISTER AS A COUNTER.
4538: 6E 0F 40  613          ROR  NSL
453B: CA        614          DEX             ;DECREMENT THE COUNTER.
453C: D0 F6     615          BNE  ILOOP      ;LOOP UNTIL X=0.
453E: AD 10 40  616          LDA  NSH        ;TEST NSH FOR ZERO.
4541: D0 07     617          BNE  ER_RED     ;IF NOT ZERO THEN ERASE AND REDUCE.
4543: AD 0F 40  618          LDA  NSL        ;IS NSL STILL TOO BIG?
4546: C9 91     619          CMP  #$91
4548: 90 0A     620          BCC  K          ;IF NOT THEN GET OUT.
454A: A2 01     621  ER_RED   LDX  #$01       ;OTHERWISE RESET THE COUNTER.
454C: 8E 13 40  622          STX  ERASEFL    ;SET THE ERASE FLAG.
454F: EE 84 03  623          INC  GAINRED    ;INCREASE REDUCTION
4552: D0 E0     624          BNE  ILOOP      ;FORCED BRANCH TO ILOOP
4554: AD 0F 40  625  K        LDA  NSL        ;RETURN WITH NSL IN ACCUM.
4557: 85 FC     626          STA  VGNS       ;AND IN VGNS
4559: 60        627          RTS


                638  * SUBROUTINE CLEAR. WRITES ZEROS INTO RANGE SPECIFIED BY A,X &Y*
                639  *REGISTERS ON ENTRY (I.E. FROM A-Y TO X-00).
4564: 85 FF     640  CLEAR    STA  IAL+1      ;SET UP BASE ADDRESS HIGH.
4566: A9 00     641          LDA  #00        ;SET UP BASE ADDRESS LOW.
4568: 85 FE     642          STA  IAL
456A: 91 FE     643  A        STA  (IAL),Y    ;CLEAR A LOCATION.
456C: C8        644          INY             ;INCREMENT Y FOR THE NEXT LOCATION.
456D: D0 FB     645          BNE  A          ;LOOP UNTIL A PAGE IS CLEARED.
456F: E6 FF     646          INC  IAL+1      ;GO TO THE NEXT PAGE.
4571: E4 FF     647          CPX  IAL+1      ;STOP NOW?
4573: B0 F5     648          BCS  A          ;NO, CLEAR ANOTHER PAGE.
4575: 60        649          RTS
```

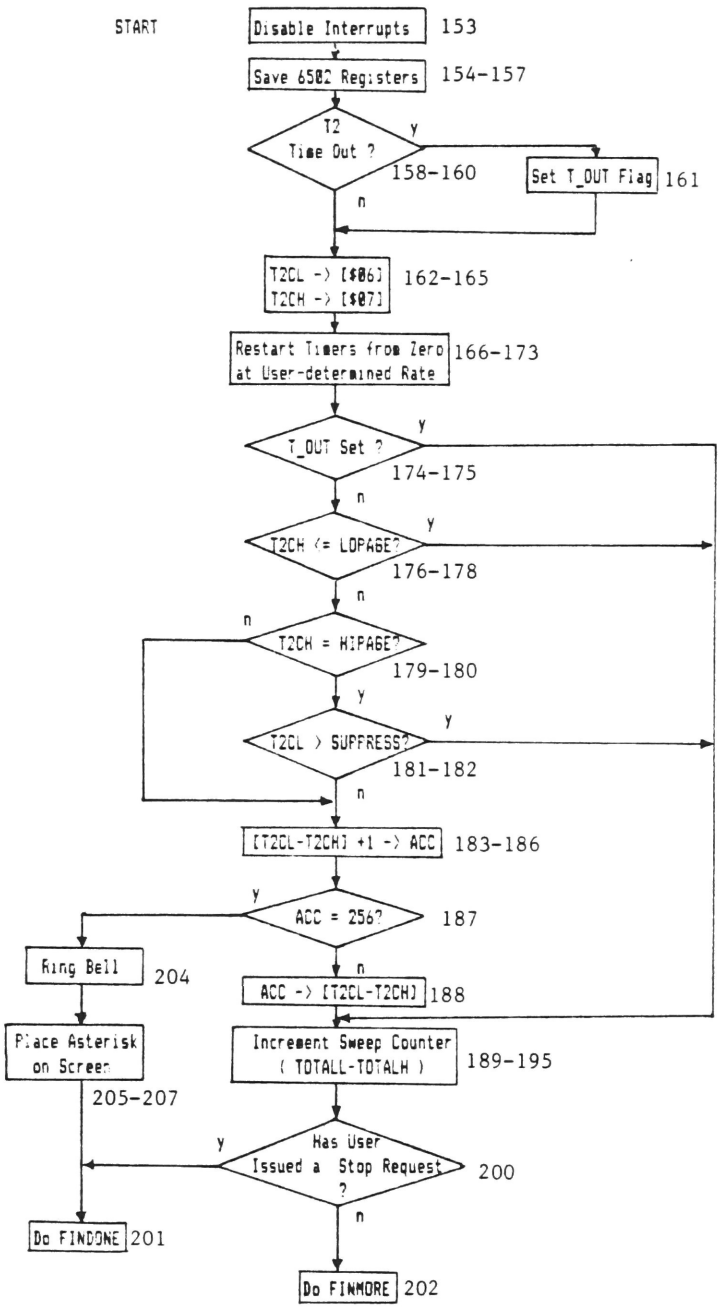
```
                660 *PLOT A GRID OF POINTS ON HIRES PAGE.
4580: A2 03     661 GRID    LDX  #3        ;PLOT 3,9
4582: A0 00     662         LDY  #0
4584: A9 09     663         LDA  #9
4586: 20 57 F4  664         JSR  $F457     ;PLOT THE POINT
4589: A9 03     665         LDA  #3
458B: A2 00     666         LDX  #0
458D: A0 99     667         LDY  #153
458F: 20 3A F5  668         JSR  $F53A     ;HLINE TO 3,153
4592: A9 F4     669         LDA  #244
4594: A2 00     670         LDX  #0
4596: A0 99     671         LDY  #153
4598: 20 3A F5  672         JSR  $F53A     ;HLINE TO 244,153
459B: A9 F4     673         LDA  #244
459D: A2 00     674         LDX  #0
459F: A0 09     675         LDY  #9
45A1: 20 3A F5  676         JSR  $F53A     ;HLINE TO 244, 9
45A4: A9 03     677         LDA  #3
45A6: A2 00     678         LDX  #0
45A8: A0 09     679         LDY  #9
45AA: 20 3A F5  680         JSR  $F53A     ;HLINE TO 3,9
45AD: A0 00     681         LDY  #0        ;MAKE SURE THAT Y=0
45AF: A9 19     682         LDA  #25       ;PLOT X,Y WHERE Y=25
45B1: 8D 12 40  683 AA      STA  Y         ;TO 238, STEP 16
45B4: A9 08     684         LDA  #8        ;AND X=8 TO 153 STEP 5
45B6: 8D 11 40  685 GRID1   STA  X         ;LOOP FOR X-COORDINATES.
45B9: AA        686         TAX            ;PUT ACC. IN X REGISTER.
45BA: AD 12 40  687         LDA  Y         ;RESTORE Y-COORDINATE.
45BD: 20 57 F4  688         JSR  $F457     ;HPLOT
45C0: A0 00     689         LDY  #0        ;REZERO THE Y REGISTER.
45C2: 18        690         CLC
45C3: AD 11 40  691         LDA  X
45C6: 69 05     692         ADC  #5        ;INCREMENT X BY 5
45C8: C9 EF     693         CMP  #239      ;IF X>238 GET NEXT Y
45CA: 90 EA     694         BCC  GRID1     ;OTHERWISE LOOP.
45CC: AD 12 40  695         LDA  Y         ;
45CF: 18        696         CLC
45D0: 69 10     697         ADC  #16       ;INCREMENT Y BY 16
45D2: C9 BA     698         CMP  #138      ;IF Y>137 DO VERTICAL GRID.
45D4: 90 DB     699         BCC  AA
45D6: A9 17     700         LDA  #23       ;PLOT X=23 TO 223 BY 20
45D8: 8D 11 40  701 BB      STA  X
45DB: A9 0D     702         LDA  #13       ;AND Y=13 TO 149 BY 4
45DD: 8D 12 40  703 GRID2   STA  Y         ;SAVE Y
45E0: AE 11 40  704         LDX  X         ;GET X-COORDINATE.
45E3: 20 57 F4  705         JSR  $F457     ;HPLOT
45E6: A0 00     706         LDY  #0        ;CLEAR THE Y-REGISTER.
45E8: 18        707         CLC
45E9: AD 12 40  708         LDA  Y
45EC: 69 04     709         ADC  #4        ;INCREMENT Y BY 4
45EE: C9 96     710         CMP  #150      ;IF Y<150 PLOT NEXT Y
45F0: 90 EB     711         BCC  GRID2
45F2: AD 11 40  712         LDA  X         ;OTHERWISE GET NEXT X
45F5: 18        713         CLC
45F6: 69 14     714         ADC  #20       ;WHICH IS X+20
45F8: C9 E0     715         CMP  #224      ;IF LESS THAN 223, DO IT AGAIN.
45FA: 90 DC     716         BCC  BB
45FC: 60        717         RTS
```

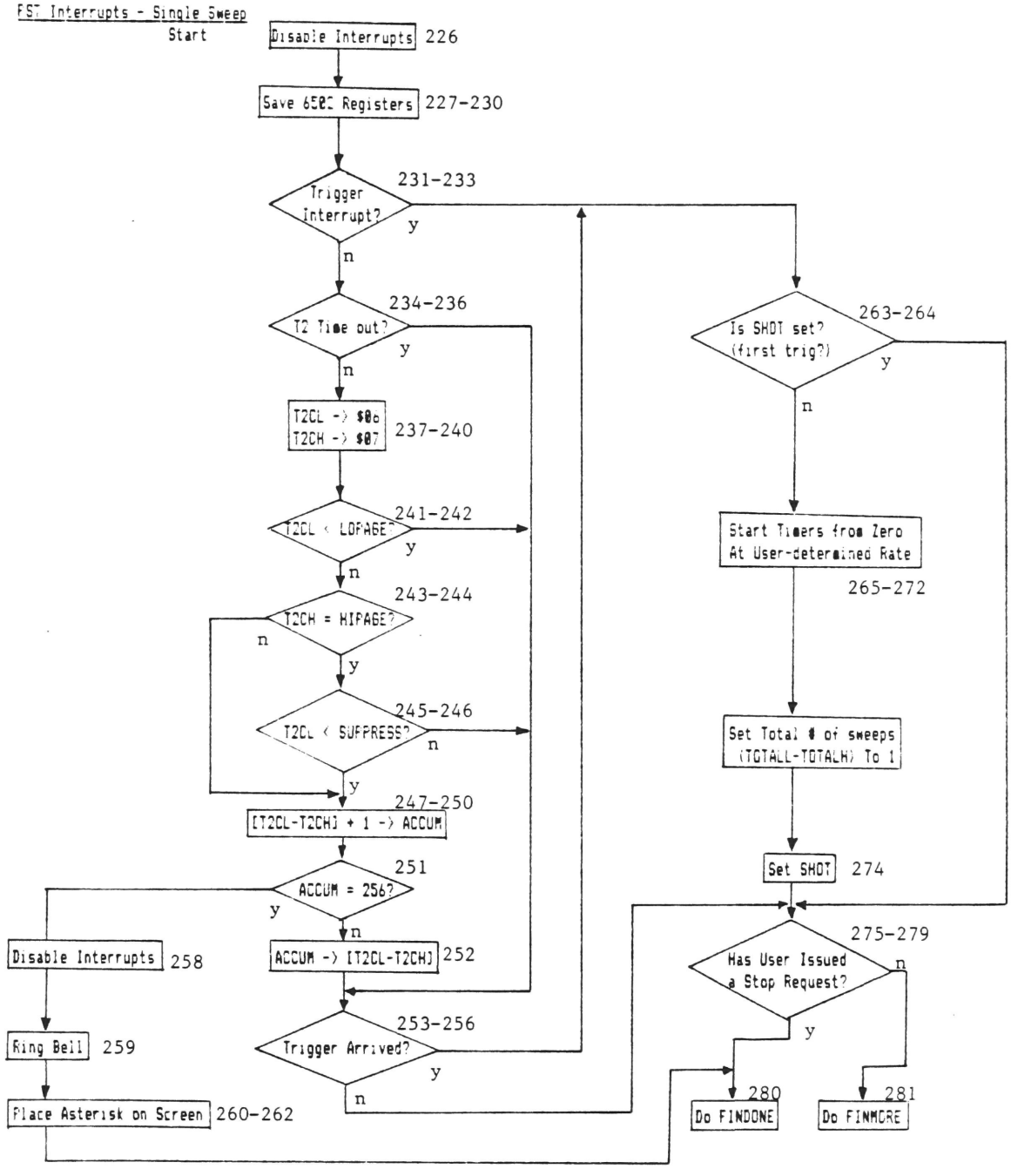
```
                738 *HI RES GRAPHICS ROUTINE FOR PLOTTING HISTOGRAM DATA QUICKLY.
4611: A9 00     739 FASTPLOT LDA  #0
4613: 85 FE     740          STA  IAL        ;INITIALIZE IAL TO $0300
4615: A9 03     741          LDA  #$03
4617: 85 FF     742          STA  IAL+1
4619: A9 00     743          LDA  #0
461B: 8D 12 40  744          STA  I          ;SET I(COUNTER) TO ZERO
461E: 8D 11 40  745          STA  X          ;SAME FOR X
4621: EE 12 40  746 NLOOP    INC  I
4624: AD 12 40  747          LDA  I
4627: 0A        748          ASL             ;PUT 2*I INTO ACCUMULATOR
4628: 18        749          CLC             ;ADD 3
4629: 69 02     750          ADC  #2
462B: 8D 11 40  751          STA  X          ; THIS IS X POSITION
462E: AE 11 40  752          LDX  X
4631: A9 99     753          LDA  #153       ;PREPARE TO PLOT X,153
4633: A0 00     754          LDY  #0
4635: 20 57 F4  755          JSR  HPLOT
4638: A2 00     756          LDX  #0         ;NOW HLINE TO 153-[$0300+I]
463A: AC 12 40  757          LDY  I
463D: 38        758          SEC
463E: A9 99     759          LDA  #153
4640: F1 FE     760          SBC  (IAL),Y
4642: A8        761          TAY
4643: AD 11 40  762          LDA  X
4646: 20 3A F5  763          JSR  HLIN
4649: EE 11 40  764          INC  X          ;DO THIS FOR 2*I+3
464C: A9 99     765          LDA  #153
464E: A0 00     766          LDY  #0
4650: AE 11 40  767          LDX  X
4653: 20 57 F4  768          JSR  HPLOT
4656: A2 00     769          LDX  #0
4658: AC 12 40  770          LDY  I
465B: 38        771          SEC
465C: A9 99     772          LDA  #153
465E: F1 FE     773          SBC  (IAL),Y
4660: A8        774          TAY
4661: AD 11 40  775          LDA  X
4664: 20 3A F5  776          JSR  HLIN
4667: AD 12 40  777          LDA  I
466A: C9 78     778          CMP  #$78       ;IS I=120?
466C: 90 B3     779          BCC  NLOOP
466E: 60        780          RTS

                791 *TURN ON HI-RES 2 WITHOUT ERASING IT.*
4679: A9 20     792 SWITCH   LDA  #$20       ;SET $E6 (PAGE INDICATOR) TO #$20
467B: 85 E6     793          STA  $E6
467D: 2C 54 C0  794          BIT  $C054      ;SET HGR SOFT SWITCH.
4680: 2C 53 C0  795          BIT  $C053      ;DISPLAY  HI-RES SCREEN 1
4683: AD 57 C0  796          LDA  $C057      ;
4686: AD 50 C0  797          LDA  $C050      ;SET TO MIXED MODE.
4689: 60        798          RTS
```

Figure I-4 through I-9:

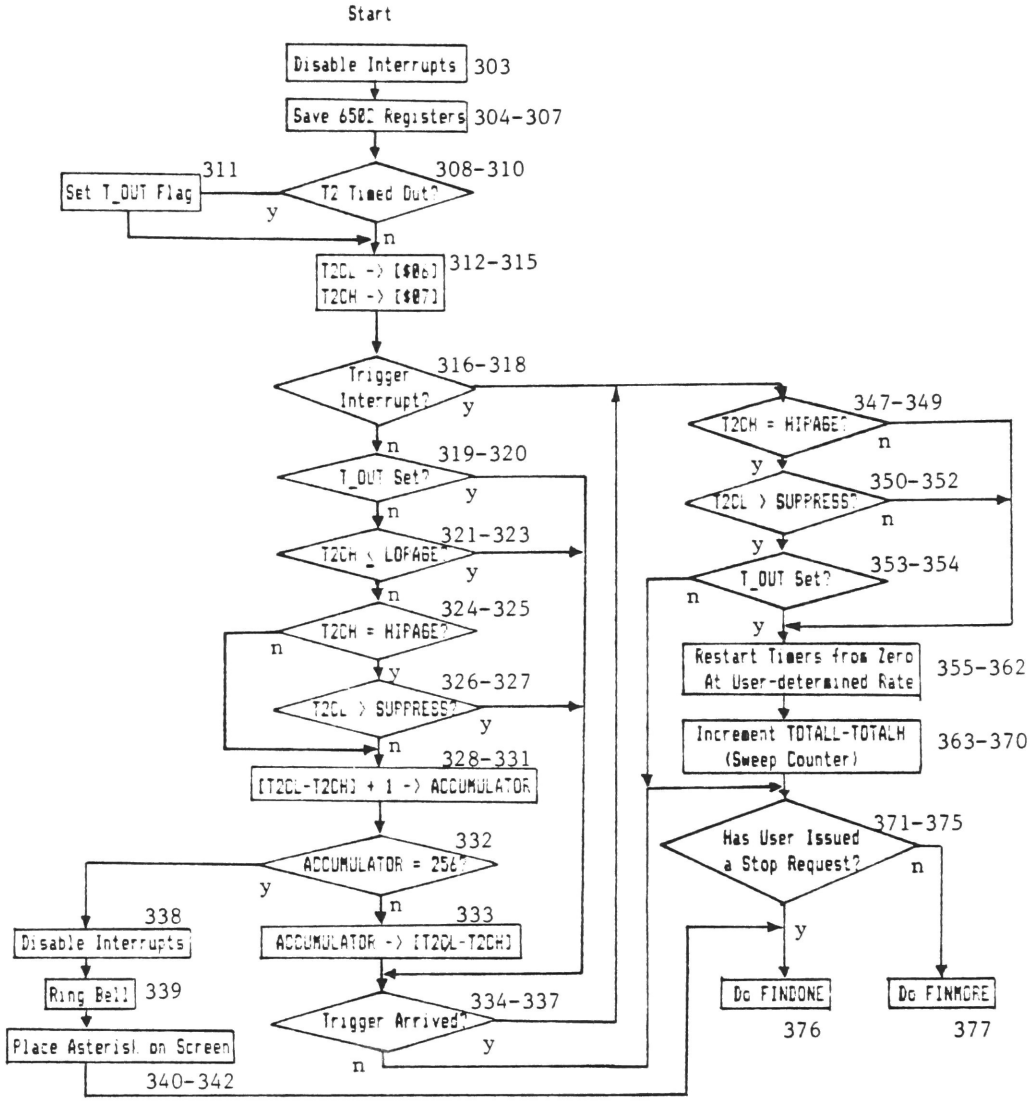
Flow charts detailing the procedures in figure I-3b. Numbers preceded by a "$" sign are in standard hexadecimal notation. The numbers to the right of the boxes refer to the line numbers used in I-3b for cross-reference. The notation [$06] means, "the contents of memory location $06" and the notation T2CL -> [$06] means, "transfer the contents of T2CL to memory location $06". When the '=' is used in boxes not including a question mark it is being used as an assignment statement, thus "MPOINTL = ORGL" means that the variable, MPOINTL is being assigned the value of variable ORGL. Variable names are shown in capital letters and are the same names used in figure I-3B.

ISI INTERRUPTS

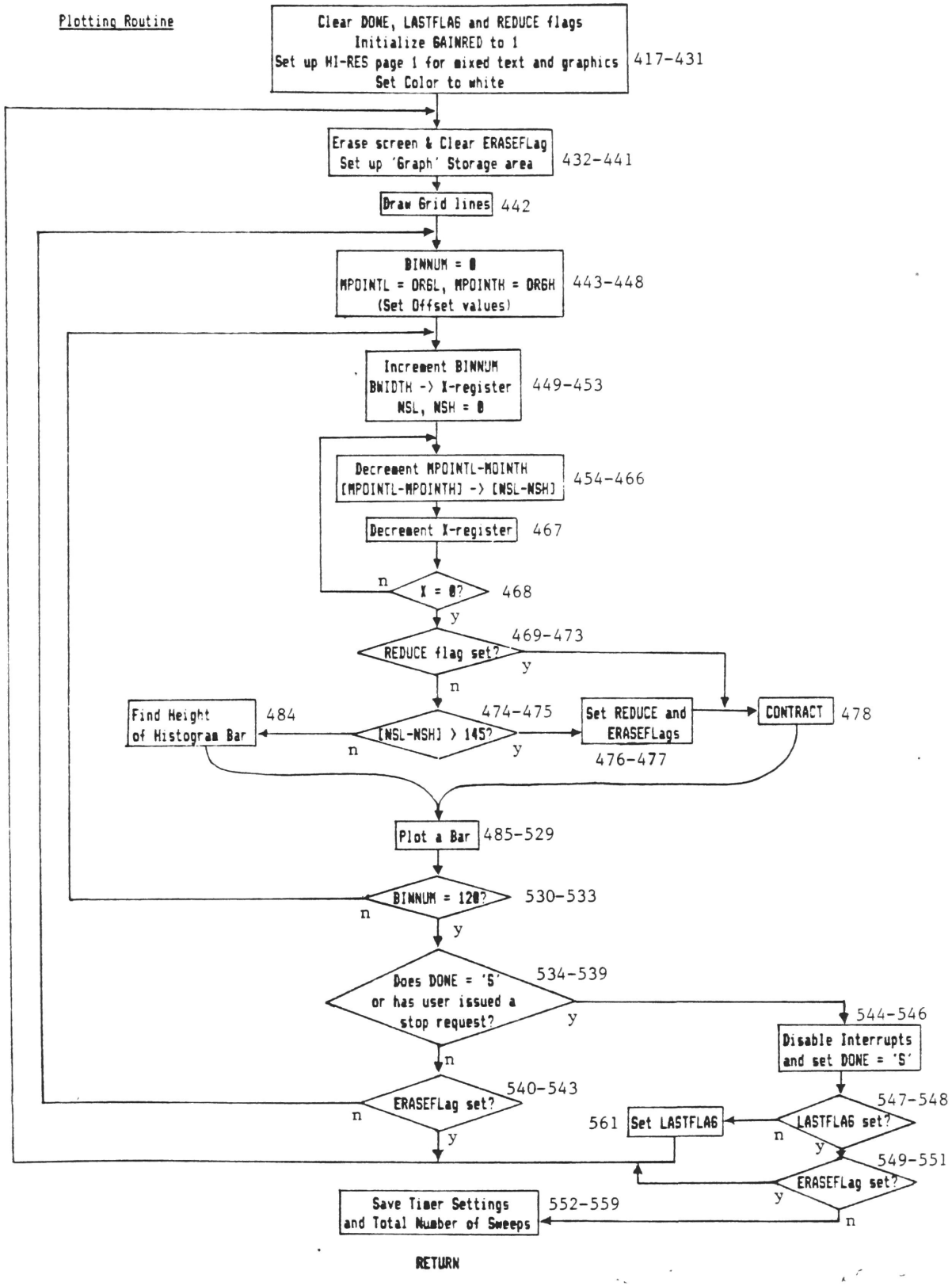



FST Interrupts - Single Sweep
Start
Disable Interrupts 226
Save 65C2 Registers 227-230
Trigger Interrupt? 231-233
y
n
T2 Time out? 234-236
y
n
T2CL -> $0b
T2CH -> $07 237-240
T2CL < LOPAGE? 241-242
y
n
T2CH = HIPAGE? 243-244
n
y
T2CL < SUPPRESS? 245-246
n
y
[T2CL-T2CH] + 1 -> ACCUM 247-250
ACCUM = 256? 251
y
n
Disable Interrupts 258
ACCUM -> [T2CL-T2CH] 252
Ring Bell 259
Trigger Arrived? 253-256
y
n
Place Asterisk on Screen 260-262
Is SHOT set? (first trig?) 263-264
y
n
Start Timers from Zero At User-determined Rate
265-272
Set Total # of sweeps (TOTALL-TOTALH) To 1
Set SHOT 274
Has User Issued a Stop Request? 275-279
n
y
Do FINDONE 280
Do FINMORE 281

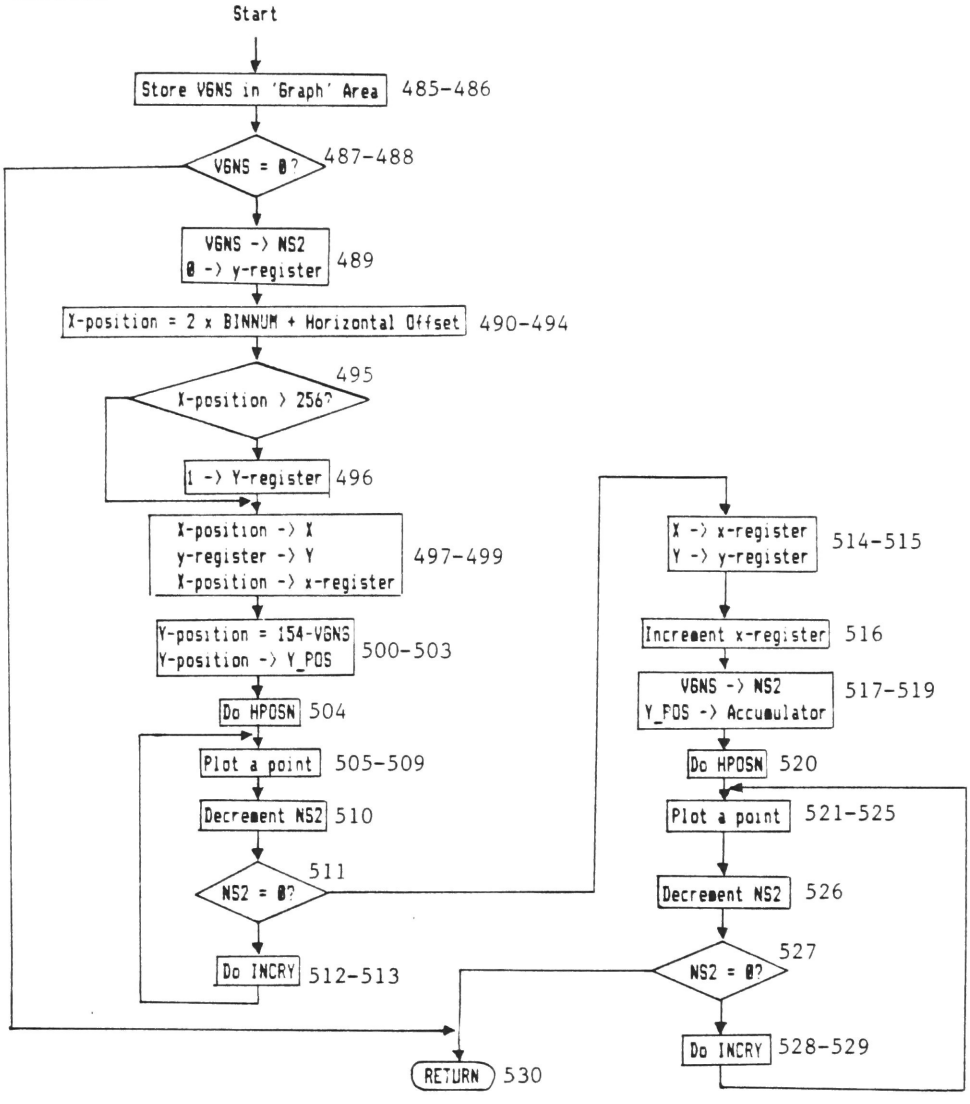
PST Interrupts - Multiple Sweep



Plotting Routine

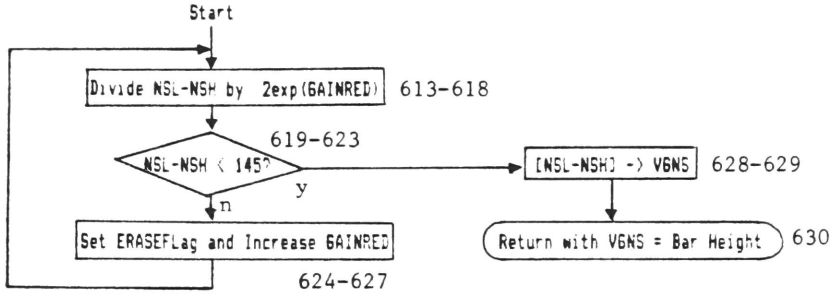


Plot a Bar


Start
Store VGNS in 'Graph' Area
485-486
VGNS = 0?
487-488
VGNS -> NS2
0 -> y-register
489
X-position = 2 x BINNUM + Horizontal Offset
490-494
X-position > 256?
495
1 -> Y-register
496
X-position -> X
y-register -> Y
X-position -> x-register
497-499
Y-position = 154-VGNS
Y-position -> Y_POS
500-503
Do HPOSN
504
Plot a point
505-509
Decrement NS2
510
NS2 = 0?
511
Do INCRY
512-513
RETURN
530
X -> x-register
Y -> y-register
514-515
Increment x-register
516
VGNS -> NS2
Y_POS -> Accumulator
517-519
Do HPOSN
520
Plot a point
521-525
Decrement NS2
526
NS2 = 0?
527
Do INCRY
528-529

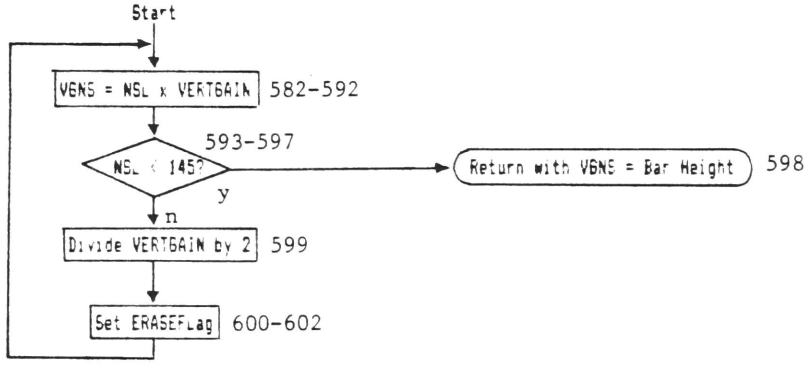
A

Contract



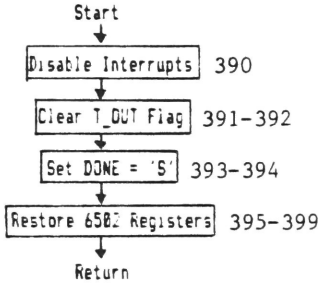
B

Height

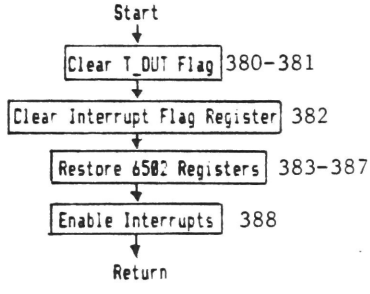


C

Findone



Findmore



As noted above, the 6522 card is used for two major functions: to count the number of cycles on the Apple system clock (and therefore to act as the timer for the acquisition run) and to generate interrupt requests as data arrives on either the spike or trigger input lines. The timer function is performed by using two resettable count-down timers built into the chip. Timer T1 consists of two 8 bit latches and a 16 bit counter. The latches are used to store the data which is to be loaded into the counter which, once loaded, counts down at the system clock rate. In my program T1 is configured to alternate between high and low states after each countdown cycle. Timer T2 is also a presettable countdown timer which is set to count the number of cycles of T1. T2 is used to time the arrival of spike data, while the rate at which T1 counts out determines the temporal resolution of the program (see below).

Two of the input lines to the 6522, namely CA1 and CA2 can be used to generate interrupts, and the so-called 'Interrupt Flag Register' on the 6522 can be polled to determine which of these lines was the source of the interrupt request. In the application described here both the spike and data lines can generate interrupts. For further information on the 6522 the reader is referred to De Jong 1982 and the Rockwell 6522 reference manual.

An unusual feature of this program is the way in which the spike arrival time is used to create a memory map of the incoming data. As mentioned above, data arrival initiates an interrupt request. Figure I-4 is a flow chart describing the servicing of that interrupt for ISI data. After performing housekeeping functions related to interrupts in general (Emerson 1983) the first procedure is to read the current setting of timer T2, then reset T2 to count down from a number corresponding to the top of the data storage range. In my case this value is $95FF (note: numbers preceded by a "$" sign are in standard two-byte hexadecimal notation). After verifying the validity of the time value and checking to

see that it's not within the artifact suppression region, the arrival time, in hexadecimal, is used directly to point to a location in computer memory. Thus if T2 reads $91C2, memory location $91C2 is incremented to mark the arrival of a spike at that time. This scheme has the advantage of relative ease of programming and high speed in servicing data interrupts. Obviously the interrupt must be ignored if the timer value points to a location outside of the data storage range available in RAM. Failure to protect against this problem can lead (and has led) to many interesting forms of computer crashes. As can be seen from the memory map in figure I-10 my program uses a 16K space in RAM between $5600 and $95FF. This region was selected as the largest free memory area in the computer which does not impinge on the Hi-Res graphic screen area and which still leaves adequate memory available for the BASIC program. Since each memory location can store only one byte of data, the maximum number of spikes which can be stored in any one time microbin is 255. Therefore a program which uses this memory mapping scheme *must* check for bin overflow and warn the user of its occurrence. My overflow handling routine halts acquisition before bin overflow and alerts the user by means of the Apple BELL routine. Two interrupt exit paths are provided (figure I-9c and 9d). FINMORE simply returns to the plotting program with interrupts still enabled while FINDONE disables all future interrupts and sets various flags which signal termination of the acquisition period.

PST interrupt handling is analogous to the above ISI handling routine (see figures I-5 and I-6). The key difference is that interrupts may be requested from either a trigger or data line. In practice the trigger line is set up to record the occurrence of stimuli to the experimental preparation. The identity of the signal generating the interrupt is determined by querying the Interrupt Flag Register of the 6522. If the interrupt was generated by the presence of a data pulse, the

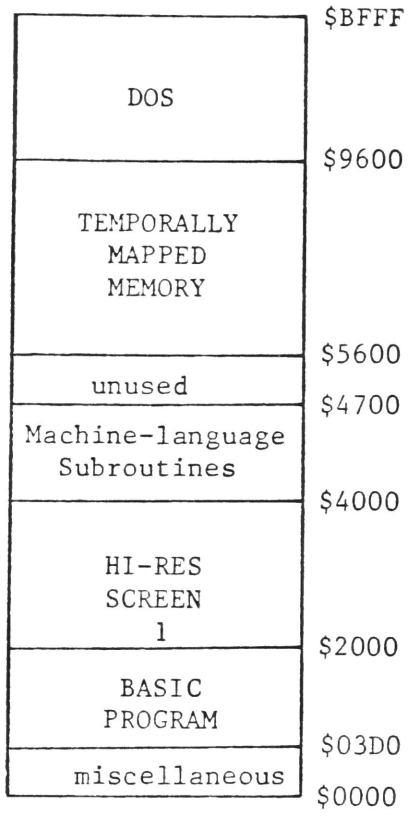


Figure I-10

Memory map of the uses of computer memory by the histogram program. In addition to these locations, zero page locations $06 through $09 and $FC through $FF are used.

arrival time is noted, but the clock is not reset. If the interrupt is generated from the trigger line however, the clock *is* reset and a counter for the total number of stimuli is incremented. Two forms of PST interrupts are available: single or multiple sweep. The former accepts only a single interrupt from the trigger line then acquires data until the user issues a stop request, while the latter forms a cumulative histogram of responses following repeated triggers. Artifact suppression is handled by simply ignoring data arriving within a user-determined time period. Both the trigger and spike lines are tested, the former so that the clock does not get reset (PST mode) with the arrival of successive stimuli in a train.

Interrupt handling occurs as a background task in this application. The foreground task is the graphing of the data currently in RAM. A flow chart for the plotting segment of the program is displayed in figures I-6 and I-7. Much of the code is devoted to an automatic rescaling feature (figure I-9a and I-9b) which is necessary to display histogram data whose magnitude may vary widely. In practice the vertical scale is expanded when the amount of data is relatively small and contracted for very large amounts of data. Extensive is made of the graphics routines available in the Apple ROM (Mesztenyi 1982) but the programmer should be warned that some of these routines are rather slow and their use may compromise the real-time plotting features of the analysis system.

Because the amount of data stored following each acquisition is large, the expense of saving raw data onto floppy disks was unrealistically high. Fortunately, the finished histogram can be represented in under two hundred bytes of data (only the number of events per bin need be saved together with identifying information). To do this, a second 'temporally-mapped memory' area is established in page $03 of memory and is referred to as the 'graph' area in the flow charts and program. Using this condensed representation I was able to save up

to 105 histograms on a single floppy disk under DOS 3.3 and with a simple DOS modification I was able to expand this to 210 histograms on each side of a floppy disk.

This program was designed to be useable by other members of our laboratory and to be made available to scientists elsewhere. Thus I extended considerable effort towards making the system 'friendly' and intuitively clear. This was facilitated by extensive use of menus and error checking. I feel very strongly that this style is of great importance in the development of programs for use by the biological scientist, whose background and interests may not include computers *per se.*

Specifications and Limitations

The overall resolution of the acquisition system is limited by the amount of time required to process the incoming data and by the total amount of free memory available for data storage. I have chosen a 100 microsecond minimum clock cycle (which represents the maximum event counting rate) as a compromise which is significantly faster than most neurophysiological processes yet allows adequate time for processing. Even at this rate it is not possible for the computer to service both trigger and stimulus interrupts in time to plot data in the first 100 microsecond bin. Fortunately in our application this period was usually artifact suppressed anyway (see below). With 16K of free memory in the computer it is possible to record 1.6384 seconds of data at this temporal resolution. The total acquisition time divided by the clock rate is a constant, equal to the amount of free memory available for data storage (in this case 16384 bytes). By slowing the clock it is possible to record a longer data stream at lower resolution. Because the Apple computer can store only one byte of data at each memory location, this program can only store up to 255 events in any given microbin. While this should not pose a problem in most physiological studies, especially

when one considers the large number of microbins, the user should recognize it as a limitation of the program. The program warns the user of microbin overflow by producing an audible 'beep' and halting data acquisition. All of the acquired data remains valid however.

The *Read Histogram* function reads the total number of events per bin by reading the condensed histogram in page $03 of RAM. This in effect means that its resolution is limited to that of the graphic presentation. In practice this means that the number presented as the total number of spikes per bin is accurate to within +0 to -0.67%.

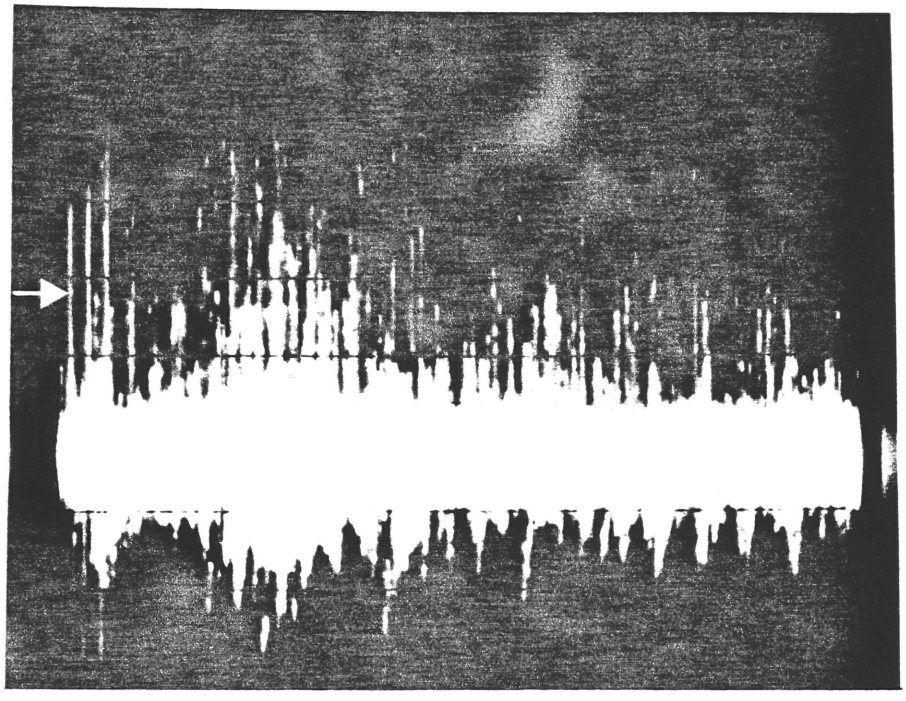
Figure I-11 shows records of the PST histogram program in actual use. The top trace represents the raw incoming spike signals, the bottom trace is a histogram derived from the same acquisition run. As can be readily seen, the histogram provides a considerable clarification of the data.

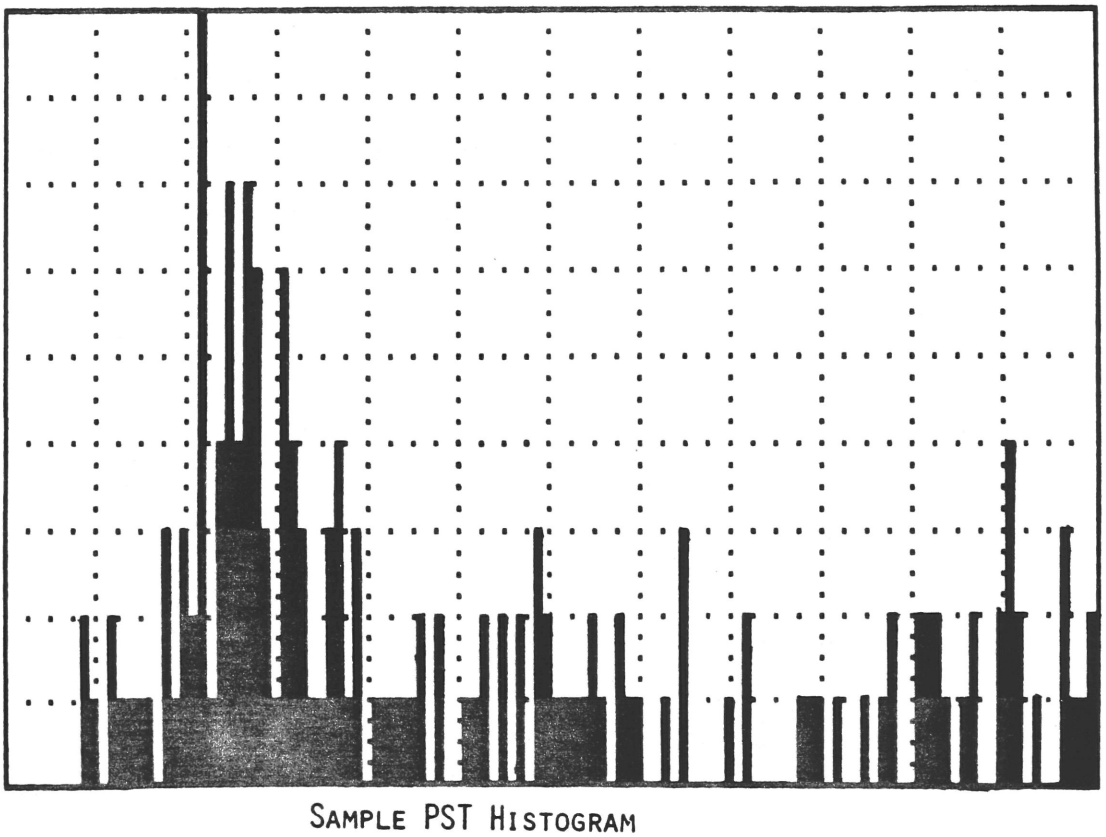
Availability

Functional equivalents of the 6522 circuit card I built are available commercially from John Bell Engineering as the 6522 Apple II parallel interface and from Interactive Structures as the DI09 board. These can be adapted with minor modifications as described in figure I-2. The 6522 chip itself is manufactured by several companies, notably Rockwell Engineering and Mostek, and can be easily acquired. I have also written a version of this program for use on Apple II+ and compatible computers without 80-column cards. This version is now in use in our laboratory as well.

Figure I-11

Data from a pudendal nerve-evoked response of a epaxial muscle nerve. The top record represents the raw data as displayed by repetitive sweeps on an oscilloscope. Approximate trigger level for the window discriminator is indicated by arrow at left. The bottom record is the resulting PST histogram with three milliseconds of artifact suppression. Time scales are both 5 msec/Div. The histogram, as displayed on the computer monitor, contains the text shown. The top line is a title which can be chosen by the user.





SAMPLE PST HISTOGRAM

VERTICAL: 1 SPIKE(S)/DIV HORIZONTAL: 5 MS/DIV

TOTAL NUMBER OF SWEEPS: 11 OFFSET: 0 MSEC.

FIG. 11.

Summary

I have designed a data acquisition system for the Apple computer ideally suited to the production of PST and ISI histograms of neural or electromyographic spike data. The overall cost of the system is substantially lower than that of most commercially available systems and the performance, particularly the rebinning feature, exceeds that of many of the commercial products. Inasmuch as the program was designed and written by a physiologist, I feel that it is well matched to their specific needs of others. Since the program described above produces hard-copy printouts of the acquired data it has also saved significant amounts of money which would otherwise have been spent on graphic arts. Many extensions of this program possible, for including on-line and off-line statistical analyses (Li and Chan 1981; also see appendix 2).

Appendix II

Quantitative Analysis of Histogram Data

Introduction

The presentation of data in histogram form carries with it some unique problems in analysis. The results described here are much concerned with response latencies, referred to locations of peak unit activity. It was therefore necessary to develop an objective analysis sytem, which was capable of detecting the features apparent in the data by visual examination. The method described here, while essentially arbitrary is both highly repeatable and insensitive to experimenter bias.

Figures II-1A and II-1B show examples of data which illustrate the problem. In the first figure, one can see unambiguous peaks centered at about 12, 23 and 92 ms. There is also a suggestion of a trough or relatively quieter period from 30 to 60 ms. The next figure illustrates data in which peak detection by eye is tricky: is there a period of increased activity center at 20 ms or not? In order to increase our objectivity in analyzing such data, we felt that it was important to have a method of detecting peaks that could not be biased by our hypotheses. Such a method is outlined below.

The Analysis Method (an example)

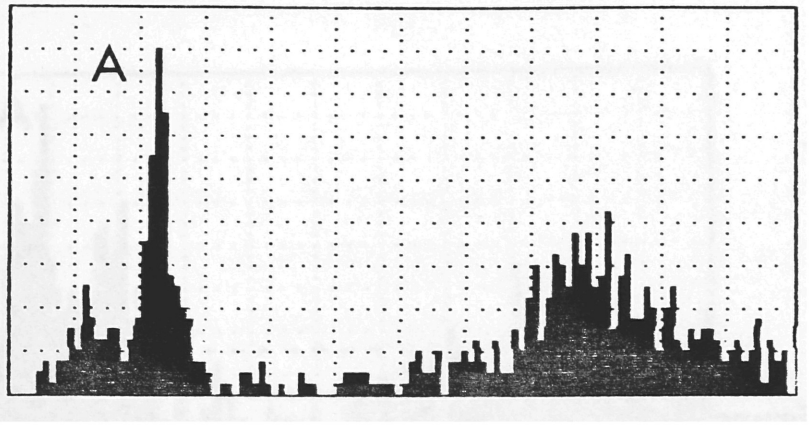
Figure II-2A shows a PST histogram of the type used in much of the data presentation. The horizontal line superimposed upon it shows the mean firing rate during the 50 msec period immediately preceding the stimulus. Note that this is the period at the right end of the PST histogram in II-2B. After finding the mean level the standard deviation from the mean level during the baseline interval is then computed according to standard methods. The dotted lines in the figure bracket the region within 2.57 standard deviations from the mean baseline firing rate.

The final stage of the analysis is to locate the bins which differ from the

Figure II-1

[A] PST Histogram of the response recorded in the L4 muscle nerve to bilateral stimulation of the pudendal nerve with repeated 50 μA triple shock trains. Vertical scale: 4 spikes/division, horizontal scale: 10 ms/division. Clear peaks are visible at 12, 23 and 92 ms.

[B] PST Histogram of the response of the L5 muscle nerve to 1/s 40 μA bilateral stimulation of the pudendal nerve with repeated single shocks. Vertical scale: 2 spikes/division, horizontal scale: 10 ms/division.


A

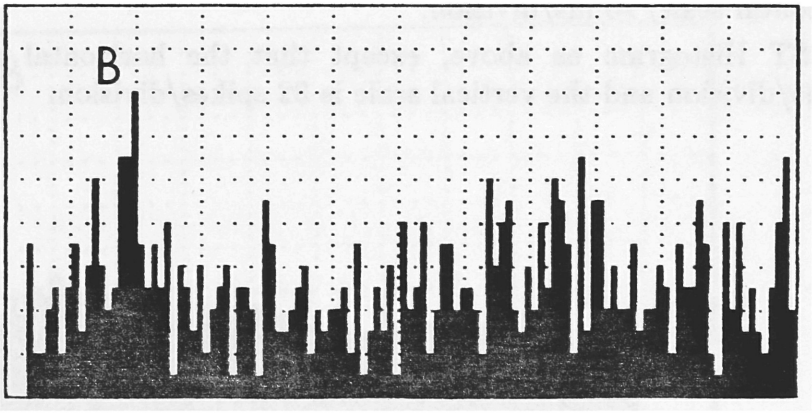
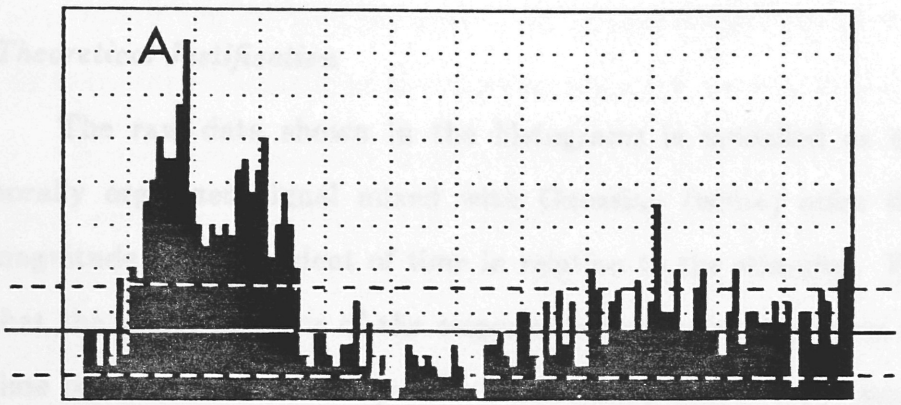
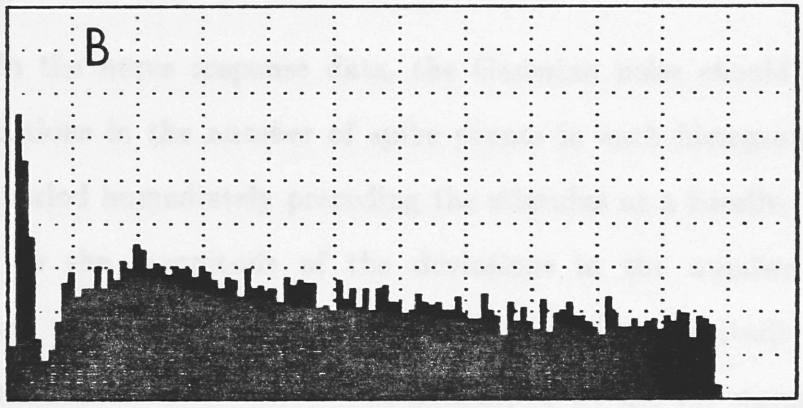

B

Figure II-2

[A] PST Histogram of the response recorded in the L4 muscle nerve to bilateral stimulation of the pudendal nerve with repeated triple shock trains. The solid horizontal line indicates the mean firing rate in the nerve during the 60 ms period immediately preceding the stimulus. The broken horizontal lines indicated the the range of +/-2.57 standard deviations from the mean. Vertical scale: 4 spikes/division, horizontal scale: 10 ms/division.

[B] PST Histogram as above, except that the horizontal scale is 100 ms/division and the vertical scale is 32 spikes/division.


A


B

baseline mean by more than 2.57 standard deviations, and to note the time, and the magnitude of those bins (again, in standard deviations). Bins which differ from the mean by more than 2.57 standard deviations are taken to indicate significant peaks and troughs in the data.

Theoretical Justification

The raw data shown in the histograms is modelled as consisting of temporally organized signal mixed with Gaussian (white) noise fluctuations whose magnitude is independent of time in relation to the stimulus. We assume further that the signal portions of the responses are of essentially zero magnitude by the time of the next stimulus pulse. This assumption is made somewhat more tenable by our selection of stimulus rates. Stimulus repetition rates were in general selected such that the temporally organized response to each stimulus appeared, at least by eye, to be complete before the onset of the next stimulus (see figure II-2b).

In the nerve response data, the Gaussian noise should show up as random fluctuations in the number of spike events in each histogram bin. By using the time period immediately preceding the stimulus as a baseline we can quantify the noise as the magnitude of the deviations in the number of events per bin. Because we assume the noise to be Gaussian, its magnitude can be conveniently measured in units of standard deviations, so we use the standard deviation of the number of spikes per histogram bin in the time period immediately preceding the stimulus as a normalized measure of noise amplitude. If the noise is normally distributed, 99% of the bins in the baseline period will contain a number of events that is within +/-2.57 standard deviations from the mean. We can thus say with 99% confidence that a bin differing from the mean by more than 2.57 standard deviations does not do so due to noise alone.

The production of binned histograms acts on the responses as a low pass filter. This can be seen intuitively. Summming the number of events falling within a 5 msec time interval results in a loss of the information on variations over a time course shorter than 5 msec. In other words it filters out the information above 200 Hz = (1/5 msec). It is thus important that the baseline noise determination be made at the smallest binwidth used in peak detection.

In the early phases of data collection the peak detection method had not yet been devised and consequently baseline information was not saved at the finest binwidths. It was thus necessary to low pass filter any data which was collected at finer binwidths before comparing it to the baseline period which was acquired with coarser bins. To accomplish this boxcar averaging was used to smooth the data, removing the high frequency fluctuations. Filtering by boxcar averaging however, is not equivalent to the filtering introduced by binning into PST histograms. In more concrete terms, the filtering introduced by summing into bins introduces a sharp low pass cutoff at the binning frequency, while boxcar averaging produces filtering with a more gentle rollof at frequencies above the reciprocal of the boxcar width. For these reasons it would be inaccurate to speak of a 99% confidence interval in data analyzed in this fashion, although a criterion level of 2.57 standard deviations was used in peak detection.

While noise amplitude can be conveniently assessed in standard deviation units, peak response amplitude cannot. Summing repetitive stimulus trials into PST histograms has an averaging effect that reduces the amplitude of noise with each acquired sweep. Thus, the standard deviation measured in a histogram composed only of the responses to a few stimulus trials would be greater than that derived from a larger number of responses. Peaks measured in the smaller sample would be smaller, in terms of standard deviation units than peaks detected in the larger sample. Instead, peak response heights were measured in

multiples of the mean firing rate, in which case peak amplitudes are essentially independent of the number of trials.

The assumption that the noise is Gaussian is fundamentally an *a priori* one of course and there is in fact data to suggest that the spectral density of neural noise has significant 1/f components (Verveen and Derksen 1968). Although a spectral analysis of the baseline noise has not been made of the nerve data reported here, modelling the noise as 'white' probably introduces at worst only a small error which is more than outweighed by its computational convenience.

References

Abzug C, Maeda M, Peterson BW and Wilson VJ (1974) Cervical branching of lumbar vestibulo-spinal axons. Journal of Physiology 243:499-522.

Amassian VE, Batson DE and Eberle L (1982) Does the red nucleus contribute dynamically to contact placing in cats? Journal of Physiology 327:62p-63p.

Amassian VE (1979) The use of contact placing in analytical and synthetic studies of the higher sensorimotor system. In: Asanuma and Wilson (Eds). *Integration in the Nervous System.* Igaku-Shoin, Tokyo.

Bagshaw EV and Evans MH (1976) Measurement of current spread from microelectrodes when stimulating within the nervous system. Experimental Brain Research 25:391-400.

Ball J (1937). Sex activity of castrated male rats increased by estrin administration. Journal of Comparative Psychology 28:273-283.

Ball J (1937) A test for measuring sexual excitability in the female rat. Comparative Psychology Monographs 14:1-37.

Barfield RJ and Chen JJ (1977) Activation of estrous behavior in ovariectomized rats by intracerebral implants of estradiol benzoate. Endocrinology 101: 1716-1732.

Beach FA (1948). *Hormones and Behavior.* New York: Hoeber.

Bessou P, Burgess PR, Perl ER and Taylor CB (1974) Dynamic properties of mechanoreceptors with unmyelinated (C) fibers. Journal of Physiology 34:116-131.

Brink EE (1978) Muscles, nerves and motoneurons involved in a lordotic reflex, or in tail movements, of the rat. Doctoral thesis, the Rockefeller University.

Brink EE, Morrell JI and Pfaff DW (1979) Localization of lumbar epaxial motoneurons in the rat. Brain Research 170:23-41.

Brink EE and Pfaff DW (1980a) Vertebral muscles of the back and tail of the albino rat (*rattus norvegicus albinus*). Brain, Behavior and Evolution 17:1-47.

Brink EE and Pfaff DW (1980b) Ablations of lumbar epaxial musculature: effects on lordosis behavior of female rats. Brain, Behavior and Evolution 17:67-88.

Brink EE and Pfaff DW (1981) Supraspinal and segmental input to lumbar motoneurons in the rat. Brain Research 226:43-60.

Brown LT (1971) Projections and temination of the corticospinal tract in rodents. Experimental Brain Research 13:432-450.

Brown LT (1974) Rubrospinal projections in the rat. Journal of Comparative Neurology 154:169-188.

Bueno J and Pfaff DW (1976) Single unit recording in hypothalamus and preoptic area of estrogen-treated and untreated ovariectomized female rats. Brain Research 101:67-78.

Burgess PR and Perl ER (1973) Cutaneous mechanoreceptors and nociceptors. In *Handbook of Sensory Physiology, Somatosensory System* A. Iggo (Ed.) New York: Springer-Verlag.

Burgess PR, Petit D and Warren RM (1968) Receptor types in cat hairy skin supplied by myelinated fibers. Journal of Neurophysiology 31:833-848.

Carlson H and Lindquist C (1976) Exteroceptive influences on the lumbar back muscle tone and reflexes in the cat. Acta Physiologica Scandinavica 97:332-342.

Carlson H (1978a) Morphology and contraction properties of cat lumbar back muscles. Acta Physiologica Scandinavica 103:180-197.

Carlson H (1978b) Histochemical fiber composition of lumbar back muscles in the cat. Acta Physiologica Scandinavica 103:198-209.

Carlson H (1978c) Observations on stretch reflexes in lumbar back muscles of the cat. Acta Physiologica Scandinavica 103:437-445.

Cohen MS and Pfaff DW (1984) On-line data acquisition system using an Apple (tm) computer. Brain Research Bulletin 13:205-223.

Cohen MS, Schwartz-Giblin S and Pfaff DW (1983) Responses of epaxial muscles and motor nerves to electrical stimulation of the pudendal nerves in the rat. Society for Neurosciences Abstracts (9) 316.15.

De Jong, ML (1982) *Apple II Assembly Language* Indianapolis: Howard W. Sams & Co.

Douglas WW and Ritchie JM (1957) Non-medullated fibres in the saphenous nerve which signal touch. Journal of Physiology 135:385-399.

Duysens J and Loeb GE (1980) Modulation of ipsi- and contralateral reflex responses in unrestrained walking cats. Journal of Neurophysiology 44(5):1024-1037.

Eccles JC, Kozak W and Magni F (1961) Dorsal root reflexes of muscle group I afferent fibres. Journal of Physiology 159:128-146.

Elger CE, Speckmann, E-J, Caspers DH and Janzen RWC (1977) Cortico-spinal connections in the rat. I. Monosynaptic and polysynaptic responses of cervical motoneurons to epicortical stimulation. Experimental Brain Research 28: 385-404.

Elliott HC (1944) Studies on the motor cells of the spinal cord. IV. Distribution in experimental animals. Journal of Comparative Neurology 81:97-103.

Emerson, RL (1983) Interrupts and Apples. Call-A.P.P.L.E. pp. 35-39 February 1983.

Engberg I, Lundberg A and Ryall RW (1968) Reticulospinal inhibition of transmission in reflex pathways. J. Physiol. 194:201-223.

Femano, P.A. and Pfaff D.W. (1983) Time-interval acquisition on a 6502-based microcomputer. Behavior Research Methods & Instrumentation 15:521 .

Femano PA, Schwartz-Giblin S and Pfaff DW (1984a) Brain stem reticular influences on lumbar axial muscle activity. I. Effective sites. American Journal of Physiology 246(15):R389-R395.

Femano PA, Schwartz-Giblin S and Pfaff DW (1984b) Brain stem reticular influences on lumbar axial muscle activity. II. Temporal Aspects. American Journal of Physiology 246(15):R396-R401.

Fox JE. Reticulospinal neurones in the rat. Brain Research 23:35-40 (1970)

Grillner S, Hongo T and Lund S (1967) Interactin between the inhibitory pathways from Deiter's nucleus and Ia afferents to flexor motoneurones. Acta Physiologica Scandinavica

Hagbarth K-E and Naess K (1950) Reflex effects of tetanic stimulation of different fibre systems in the hind limb of the cat. Acta Physiologica Scandinavica 21:336-361.

Hagbarth K-E (1952) Excitatory and inhibitory skin areas for flexor and extensor motoneurones. Acta Physiologica Scandinavica 26 suppl. 94:1-58.

Haldeman S, Bradley WE, Bhatia NN and Johnson BK (1982) Pudendal evoked responses. Archives of Neurology 39:280-283.

Hall M (1850) *Synopsis of the diastaltic nervous sytem; or the system of the spinal marrow; and its reflex arcs.* London, J. Mallett.

Harlan RE, Shivers BD, Kow L-M and Pfaff DW (1983) Estrogenic maintenance of lordotic responsiveness: requirement for hypothalamic action potentials. Brain Research 168:67-78.

Holmqvist B and Lundberg A (1961) Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alpha motoneurones. Acta Physiologica Scandinavica 54, suppl. 186:1-51.

Hongo T, Jankowska E and Lundberg A (1969) The rubrospinal tract II. Facilitation of interneuronal transmission in reflex paths to motoneurones. Experimental Brain Research 7:365-391.

Hnik P (1978) The increased response of chronically de-efferented rat muscle spindles to stretch. Brain Research 21:448-451.

Hunt CC and McIntyre AK (1960) An analysis of fibre diameter and receptor characteristics of myelinated cutaneous afferent fibres in cat. Journal of Physiology 153:99-112.

Jankowska E and McCrea (1983) Shared reflex pathways from Ib tendon organ afferents and Ia muscle spindle afferents in the cat. Journal of Physiology 338:99-111.

Kanda K, Burke RE and Walmsley B (1977) Differential control of fast and slow twitch motor units in the decerebrate cat. Experimental Brain Research 29:57-74.

Komisaruk BR, Adler NT and Hutchison J (1972) Genital sensory field: enlargement by estrogen treatment in female rats. Science 178:1295-1298.

Kow L-M and Pfaff DW (1973) Effects of estrogen treatment on the size of receptive field and response threshold of pudendal nerve in the female rat. Neuroendocrinology 13:299-313.

Kow L-M and Pfaff DW (1975a). Induction of lordosis in female rats: two modes of estrogen action and the effect of adrenalectomy. Hormones and Behavior 6:259-276.

Kow L-M and Pfaff DW (1975b) Dorsal root recording relevant for mating reflexes in female rats: identification of receptive fields and effects of peripheral denervation. Journal of Neurobiology 6(1):23-37.

Kow L-M and Pfaff DW (1976) Sensory requirements for the lordosis reflex in female rats. Brain Research 101:47-66.

Kow L-M, Montgomery MO and Pfaff DW (1977) Effects of spinal cord transections on lordosis reflex in female rats. Brain Research 123:75-88.

Kow L-M and Pfaff DW (1977) Sensory control of reproductive behavior in female rodents. Annals of the New York Academy of Sciences 290:72-97.

Kow L-M, Montgomery MO and Pfaff DW (1979) Triggering of lordosis reflex in female rats with somatosensory stimulation: quantitative determination of stimulus parameters. Journal of Neurophysiology 42(1):195-202.

Kow L-M and Pfaff DW (1979) Responses of single units in sixth lumbar dorsal root ganglion to mechanostimulation relevant for lordosis reflex. Journal of Neurophysiology 42(1):203-213

Kow L-M, Zemlan FP and Pfaff DW (1980) Responses of lumbosacral spinal units to mechanical stimuli related to analysis of lordosis reflex in female rats. Journal of Neurophysiology 43(1):27-45.

Kow L-M and Pfaff DW (1982) Responses of medullary reticulospinal and other reticular neurons to somatosensory and brainstem stimulation in anesthetized or freely-moving ovariectomized rats with or without estrogen treatment. Experimental Brain Research 46:191-202.

Kugelberg E and Hagbarth KE (1958) Spinal mechanism of the abdominal and erector spinae skin reflexes. Brain 81:290-304.

Kuypers H.G.J.M. (1964) The descending pathways to the spinal cord, their anatomy and function. In *Organization of the Spinal Cord,* J.C. Eccles, J. Schade (Eds.) New York: Elsevier.

Li, H.F. and Chan, F.H.Y. (1981) Microcomputer-based spike train analysis. Computer Programs in Biomedicine 13:61-72.

Lundberg A (1967) The supraspinal control of transmission in spinal reflex pathways. In *Recent Advances in Clinical Neurophysiology,* L Widen (ed.) Amsterdam: Elsevier.

Lundberg A (1979) Multisensory control of spinal reflex pathways. Progress in Brain Research 50:11-28.

Lundberg A, Malmgren K and Schomburg ED (1977) Cutaneous facilitation of transmission in reflex pathways from Ib afferents to motoneurones. Journal of Physiology 265:763-780.

Magni F and Willis WD (1964) Subcortical and peripheral control of brain stem reticular neurons. Archives of Italian Biology 102:434-448.

Malsbury CW and Pfaff DW (1974) Neural and hormonal determinants of mating behavior in adult male rats. A review. In L.V. diCara (Ed.), *Limbic and Autonomic Nervous System Research.* New York: Plenum.

Martin RF, Haber LH and Willis WD (1979) Primary afferent depolarization of identified cutaneous fibers following stimulation in medial brainstem. Journal of Neurophysiology 42(3):779-790.

Mesztenyi, .K (1981) Notes on hi-res graphics routines in Applesoft in VJ Golding (Ed.) *All About Applesoft* . Seattle: Apple Puget Sound Program Library Exchange.

Modianos DT and Pfaff DW (1977) Facilitation of the lordosis reflex in female rats by electrical stimulation of the lateral vestibular nucleus. Brain Research 134:333-345.

Morrell JI and Pfaff DW (1978) A neuroendocrine approach to brain function. Localization of sex steroid concentrating cells in the vertebrate brains. American Zoologist 18:447-460.

Morrell JI, Wolinsky TD, Krieger MS and Pfaff DW (1982) Autoradiographic identification of estradiol-concentrating cells in the spinal cord of the female rat. Experimental Brain Research 45:144-150.

Muybridge EA (1887) *Animals in Motion: an electro photographic investigation of consecutive phases of animal movement.* Philadelphia: University of Pennsylvania.

Nyberg-Hansen R (1965) Sites and mode of termination of reticulospinal fibers in the cat. An experimental study with silver impregnation methods. Journal of Comparative Neurology 124:71-100.

Nyberg-Hansen R (1966) Functional organization of descending supraspinal fiber systems to the spinal cord. Anatomical observations and physiological correlations. Ergebnische Anatomie Entwickl.-Gesch. 39:1-48.

Parsons B, MacLusky NJ, Krieger MS, McEwen BS and Pfaff DW (1979) The effects of long-term estrogen exposure on the induction of sexual behavior and measurements of brain estrogen and progestin receptors in the female rat. Hormones and Behavior 13:301-313.

Peterson BW and Abzug C (1975) Properties of projections from vestibular nuclei to medial reticular formation in the cat. Journal of Neurophysiology 38:1421-1435.

Peterson BW, Maunz RA, Pitts NG and Mackel RG (1975) Patterns of projection and branching of reticulospinal neurons. Experimental Brain Research 22:333-351.

Peterson BW (1979) Reticulospinal projections to spinal motor nuclei. Annual Review of Physiology 41:127-40.

Petras JM (1967) Cortical, tectal and tegmental fiber connections in the spinal cord of the cat. Brain Research 6:275-324.

Pfaff DW (1968) Autoradiographic localization of radioactivity in the rat brain after injection of tritiated sex hormones. Science 161:1355-1356.

Pfaff DW (1970) Nature of sex hormone effects on rat sex behavior: specificity of effects and individual patterns of response. Journal of Comparative and Physiological Psychology 72: 45-50

Pfaff DW and Keiner M (1973) Atlas of estradiol-concentrating cells in the central nervous system of the female rat. Journal of Comparative Neurology 151:121-158.

Pfaff DW and Lewis C (1974) Film analyses of lordosis in female rats. Hormones and Behavior 5:317-335.

Pfaff DW (1970b) Nature of sex hormone effects on rat sex behavior: specificity of effects and individual patterns of response. Journal of Comparative and Physiological Psychology 72: 45-50

Pfaff DW, Montgomery MO and Lewis C (1977) Somatosensory determinants of lordosis in female rats: behavioral definition of the estrogen effect. Journal of Comparative and Physiological Psychology 91(1):134-145.

Pfaff DW, Diakow C and Montgomery MO (1978) X-ray cinematographic analysis of lordosis in female rats. Journal of Comparative and Physiological Psychology 92(5):937-941.

Pfaff DW (1980) *Estrogens and Brain Function,* New York: Springer Verlag.

Rockwell International Inc. Versatile Interface Adaptor Part Number R6522. Doc. Number 29000 D47 Rev. 4 (1981).

Rudomin P, Nunez R and Madrid J (1975) Modulation of synaptic effectiveness of Ia and descending fibers in spinal cord. Journal of Neurophysiology 38:1181-1195.

Rudomin P, Jimenez I, Solodkin M and Duenas S (1983) Sites of action of segmental and descending control of transmission on pathways mediating PAD of Ia- and Ib-afferent fibres in cat spinal cord. Journal of Neurophysiology 50(4):743-769.

Sakuma Y and Pfaff DW (1979a) Facilitation of the lordosis reflex of female rats from the ventromedial nucleus of the hypothalamus. Journal of Physiology 288:189-202.

Sakuma Y and Pfaff DW (1979b) Deficit in the lordosis reflex of female rats caused by lesions in the ventromedial nucleus of the hypothalamus. Journal of Physiology 288:203-210.

Sakuma Y and Pfaff DW (1979c) Facilitation of female reproductive behavior from mesencephalic central gray in the rat. American Journal of Physiology 237:R278-284.

Sakuma Y and Pfaff DW (1979d) Mesencephalic mechanisms for integration of female reproductive behavior in the rat. American Journal of Physiology 237:R285-290.

Sakuma Y and Pfaff DW (1980a) Cells of origin of medullary projections in the central gray in the rat mesencephalon. Journal of Neurophysiology 44(5):1002-1011.

Sakuma Y and Pfaff DW (1980b) Excitability of female rat central gray cells with medullary projections; changes produced by hypothalamic stimulation and estrogen treatment. Journal of Neurophysiology 44(5):1012-1023.

Sakuma Y and Pfaff DW (1980c) Covergent effects of lordosis-relevant somatosensory and hypothalamic influences on central gray cells in the rat mesencephalon. Experimental Neurology 70:269-281.

Sakuma Y and Pfaff DW (1981) Electrophysiologic determination of projections from ventromedial hypothalamus to midgrain central gray: differences between female and male rats. Brain Research 225:184-188.

Sakuma Y and Pfaff DW (1982) Properties of ventromedial hypothalamic neurons with axons to midbrain central gray. Experimental Brain Research 46:292-300.

Schieppati M and Crenna P (1984) Natural cutaneous stimulation induces late and long-lasting facilitation of extensor motoneurons in the cat. Brain Research 293:259-267.

Schwartz-Giblin S, Rosello L and Pfaff DW (1983) A histochemical study of lateral longissimus muscle in rat. Experimental Neurology 79:497-518.

Schwartz-Giblin S, Halpern M and Pfaff DW (1984) Segmental organization of rat lateral longissimus, a muscle involved in lordosis behavior: EMG and muscle nerve recordings. Brain Research 299:247-257.

Schwartz-Giblin S, Femano PA and Pfaff DW (1984) Axial electromyogram and intervertebral length guage responses during lordosis behavior in rats. Experimental Neurology 85:297-315.

Shapovalov AI and Gurevitch NR (1970) Monosynaptic and disynaptic reticulospinal actions on lumbar motoneurons of the rat. Brain Research 21:249-263.

Sherrington CS (1897) Decerebrate rigidity and reflex coordination of movements. Journal of Physiology 22:319-332.

Sherrington CS (1906) *The Integrative Action of the Nervous System.* New Haven: Yale University Press.

Shimamura M and Livingston RB (1963) Longitudinal conduction systems serving spinal and brain stem coordination. Journal of Neurophysiology 26:258-272.

Slijper EJ (1946) Comparative biologicanatomical investigations on the vertebral column and spinal musculature of mammals. Kon. Ned. Akad. Wet. Verh. (Tweedie Sectie), 42(5):1-128.

Slimp JC and Towe AL (1980) Effects of pudendal nerve stimulation on neurons in pericruciate cortex of male domestic cats. Experimental Neurology 67:181-204.

Smith ER, Damassa DA and Davidson JM (1977) Hormone administration: peripheral and intracranial implants. In: *Methods in psychobiology.* Vol. 3 New York: Academic Press.

Suzuki J -I and Cohen B (1964) Head, eye body and limb movements from semicircular canal nerves. Experimental Neurology 10:393-405.

Thor KB, Kuo DC, deGroat WC, Blais D and Backes M (1982) Alterations of HRP-labelled pudendal nerve afferent projections in the sacral spinal cord of the cat during neonatal development and after spinal cord transection: correlation with physiological plasticity of a spinal somatovesical reflex. Society for Neuroscience Abstracts (8) 84.8.

Toennies JF (1938) Reflex discharge from the spinal cod over the dorsal roots. Journal of Neurophysiology 1:378-390.

Ueyama T, Mizuno N, Nomura S, Konishi A, Itoh K and Arakawa H (1984) Central distribution of afferent and efferent components of the pudendal nerve in cat. Journal of Comparative Neurology 222:38-46.

Vallois, H (1922) Les transformations de la musculature de l'espisome chez les vertebres. Archives de Morphologie Generale et Experimentale 13:1-538

Verveen AA and Derksen HE (1968) Fluctuation Phenomena in Nerve Membrane. Proceedings of the IEEE 56(6):906-16

Walmsley B, Hodgson JA and Burke RE (1978) forces produced by medial gastrocnemius and soleus muscles during locomotion in freely moving cats. Journal of Neurophysiology 41(5):1203-1216

Waldron HA and Gwyn DG (1969) Descending nerve tracts in the spinal cord of the rat. I. Fibers from the midbrain. Journal of Comparative Neurology. 137:143-154.

Wilson VJ, Yoshida M and Schor RH (1970) Supraspinal monosynaptic excitation and inhibition of thoracic back motoneurons. Experimental Brain Research 11:282-295.

Whalen RE and Nakayama K (1965) Induction of estrous behavior: facilitation by repeated hormone treatment. Journal of Endocrinology 33:525-526.

Young WC (1961) The hormones and mating behavior. In WC Young (Ed.), *Sex and Internal Secretions* Vol.2, third edition. Baltimore: Williams & Wilkins.

Zemlan FP, Leonard CM, Kow L-M and Pfaff DW (1978) Ascending tracts of the lateral columns of the rat spinal cord: a study using the silver impregnation and horseradish peroxidase techniques. Experimental Neurology 62:298-334.

Zemlan FP, Low L-M, Morrell JI and Pfaff DW (1979) Descending tracts of the lateral columns of the rat spinal cord: a study using the silver impregnation and horseradish peroxidase techniques. Journal of Anatomy 128(3):489-512.

Zemlan FP, Kow L-M and Pfaff DW (1983) Effects of interruption of bulbospinal pathways on lordosis, posture and locomotion. Experimental Neurology 81:177-194.

Zotterman (1939) Touch, pain and tickling: an electrophysiological investigation on cutaneous sensory nerves. Journal of Physiology 95:1-28.

End