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FROG SPINAL MOTONEURONS: THEIR SUPRASPINAL
SYNAPTIC INPUT AND ANATOMICAL LOCALIZATION

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

by

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Approved for publication
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"...the next minute you'd see that frog whirling
in the air like a doughnut--see him turn one summerset,
or maybe a couple, if he got a good start, and come
down flat-footed and all right, like a cat."

--from The Notorious Jumping Frog
of Calaveras County by Mark
Twain (Samuel Clemens), 1865

PREFACE

For more than fifty years the central nervous system of the cat has been the subject of extensive anatomical and physiological study which has proven very fruitful in elucidating the principles of mammalian nervous function. The anuran has been in the repertoire of the neurophysiologist and the neuroanatomist longer than the cat but in this century the study of its central nervous system has languished in comparison. Considering the unique place in phylogeny of the anuran spinal cord and the wealth of accessory information available concerning the laboratory use of anurans, now is an opportune time to apply the tools of modern neuroanatomy and neurophysiology to the study of the anuran spinal cord.

The subject of this thesis is the motoneurons which innervate the hindlimb of the frog. The work is divided into two sets of experiments, each with its own introduction, methods, results and discussion sections. The physiological experiments are concerned with descending fibers in the lateral funiculus, known to have powerful excitatory synaptic input to lumbar motoneurons, and with whether these fibers are propriospinal or supraspinal. The anatomical experiments are concerned with the localization of lumbar motoneurons and with the organization of their groupings.

Motoneuron locations were determined both by physiological and by anatomical means but the anatomical means provided more data. The maps of motoneuron locations provided by this data will be useful for future physiological study of the frog spinal cord.

In addition to the two main parts of the thesis there is a general introduction to the anuran spinal cord and an appendix of supplementary information.

An essential part of this work was the development of a technique for making intracellular recordings from the anuran spinal cord in situ. This technique allows the exploration of problems not

possible with the old technique of studying the excised spinal cord in vitro, e.g. identification of separate peripheral inputs, and it enables the cord to be kept in better physiological condition.

At the time when this work was begun only one other investigator (Fukami, 1961, 1962) had used an in situ anuran cord preparation and he had published little information on his methods, therefore it was necessary to develop this preparation anew. The preparation as it was finally used required techniques as sophisticated as those used to study mammalian spinal physiology. Considerable time and effort was expended to develop these techniques; it was painfully learned that one cannot rip open a frog spinal column and begin immediate recording with any hope of the preparation surviving for long. The considerable time required to develop the preparation necessarily limited the time remaining for experiments; this accounts for the relatively small number of units which are reported.

ACKNOWLEDGMENTS

I wish to thank Bernice Grafstein for her guidance and criticism during the course of this work. I am deeply indebted to Victor Wilson for his aid and encouragement. I am especially grateful to my wife, Judy, for her continuous advice, support, and patience.

Adele Edison, Steve Green, Alex Mauro, Marion Murray, and Dick Wylie have been especially helpful and encouraging at critical moments throughout this project.

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I extend humble thanks to Detlev Bronk and to numerous other friends and colleagues who have made my graduate education a rewarding and satisfying experience.

Finally, I would like to thank Pyramus for keeping me company through the long hours of writing.

ABSTRACT

Techniques were developed for maintaining a bullfrog spinal cord in situ and for recording intracellularly from lumbar motoneurons in this preparation. Fibers in the contralateral and ipsilateral lateral funiculi of the spinal cord which originate at or above the calamus scriptorius were found to produce EPSPs in flexor and extensor motoneurons innervating the hindlimb. These EPSPs appeared to be generated monosynaptically on the somas and proximal dendrites of the motoneurons.

Chromatolysis was used to map the location of motoneurons innervating hindlimb muscles in the bullfrog, Rana catesbiana. These motoneurons were located in the dorsolateral cell column of the ventral horn, but not in the ventromedial column. A somatotopic organization was found, with motoneurons innervating proximal muscles located ventrally and rostrally to those innervating distal muscles. A functional organization was found, in which motoneurons controlling the flexor muscles around any joint were located rostrally to those controlling the extensors. Electrophysiological mapping gave results consistent with this arrangement.

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GENERAL INTRODUCTION

Preview

Anuran amphibians (frogs and toads) occupy a unique place in vertebrate phylogeny making their central nervous system especially interesting as a subject of comparative investigation: they are the oldest living relative of the first tetrapod to evolve from a fish.

The transition from fish to tetrapod was probably one of the most important steps in the evolution of the vertebrates. Consider simply the changes in the nervous system required for tetrapodal movement on the land. The development of spinal mechanisms for the movement of the limbs, for example in walking and scratching, is associated with the appearance in anurans and all higher tetrapods of clearly defined motor neurons organized into linear groups and of a distinct gray matter organized into dorsal and ventral horns. Prominent swellings in the spinal cord, caused by expansion of the gray matter and of the motoneuron population, are associated with the brachial and lumbar regions which innervate the limbs.

The development of supraspinal mechanisms for limb movement, for example in posture, balance, predation, or escape, obviously required the development in anurans and all higher tetrapods of more extensive long motor and sensory pathways. Amphibians are the first vertebrates known to have direct sensory fibers to supraspinal levels and anuran amphibians show better development of spino-cerebellar and spino-vestibular tracts than do urodele amphibians (salamanders). Tegmentospinal, vestibulospinal, and reticulospinal fibers are believed to be present in all vertebrates, but their first prominent development probably occurs in the anurans.

The work that follows is concerned with the motoneurons that innervate the frog hindlimb, including both the organization of their groupings and the synaptic actions of lateral funiculus supraspinal fibers upon them. In addition, certain aspects of their function in spinal reflexes will be discussed. The relationship of the central

nervous system in anurans to that in higher vertebrates will be considered.

The Anuran Spinal Cord

A brief review of the anatomy of the spinal cord of anuran amphibians (frogs and toads) will facilitate presentation of the experimental results. Detailed descriptions of the gross and microscopic anatomy are available in works by Gaupp (1899), Haslam (1889), Ariens Kappers et al. (1936), Silver (1942), Kennard (1959), and Nieuwenhuys (1964).

The spinal cord of the frog (Figure 1) exhibits brachial and lumbar enlargements separated by a thoracic region and ending in a long filum terminale. From the cord emerge ten pairs of roots, numbered from two to eleven, the first having disappeared during development (see discussion of nomenclature in Appendix). The largest branch of root 2 is the hypoglossal nerve, innervating the tongue. A smaller branch of root 2 combines with roots 3 and 4 to form the brachial plexus, innervating the forelimbs. Roots 5, 6, and 7 innervate the thoraco-abdominal body wall while roots 8, 9, 10, and 11 form the lumbar plexus innervating the hindlimbs.

Toad spinal cords are usually more compressed in length relative to the spinal canal than are frog spinal cords. In other respects their cord structure is assumed to be the same.

Dorsal Horn Anurans are the first group in phylogeny with clearly defined dorsal horns appearing in the gray matter of the spinal cord. The gray matter of the fish spinal cord is shaped like an inverted "Y" whereas in the anuran it looks more like the "H" shape seen in all higher vertebrates (Ariens Kappers et al., 1936). This separation of the dorsal gray into two distinct horns is probably caused by the development of the dorsal funiculi in the midline between the two dorsal horns.

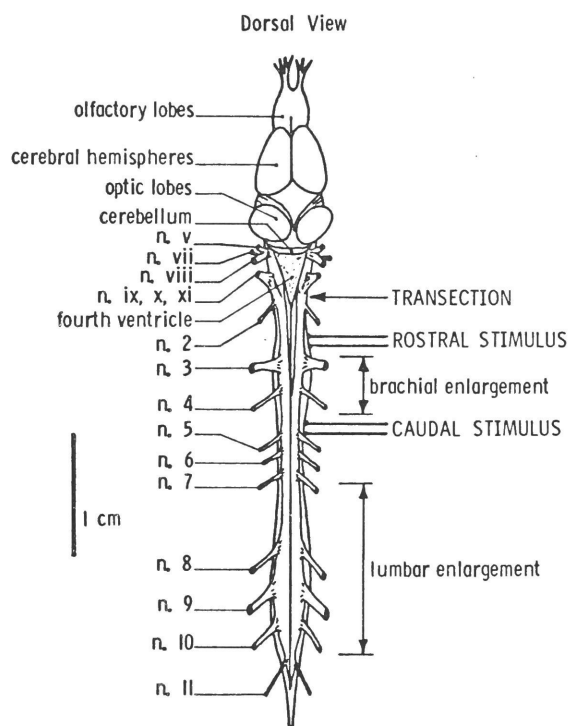


Figure 1. Dorsal view of the central nervous system of the bullfrog (*Rana Catesbiana*). The nomenclature of Gaupp (1899, see Appendix for discussion) is used. Spinal root one exists in the embryo but disappears by adulthood. Motoneurons supplying the muscles of the hind-limb lie in the lumbar enlargement served by roots 8, 9, 10, and 11.

The anuran spinal cord is the first one in phylogeny in which the dorsal roots separate into medial and lateral divisions on entering the cord. The lateral division, consisting of fine medullated and unmedullated fibers, enters the cord as a bundle comparable to the tract of Lissauer found in higher vertebrates (Nieuwenhuys, 1964) and it terminates in a dense meshwork, the dorsal neuropil of Silver (1942). The larger medial division, consisting of heavily medullated fibers, enters the dorsal funiculus and splits into ascending and descending branches which have collaterals going into the gray matter (Nieuwenhuys, 1964). It is these collaterals which are thought to provide direct synaptic connections to the motoneurons.

Dorsal Root Collaterals Anuran motoneurons have prominent dendrites which end with extensive branches in the dorsal horn near the entry of the dorsal root (Silver, 1942; Kennard, 1959). Dorsal roots have short collaterals which terminate in the dorsal horn region near the distal dendrites of the motoneurons (Liu and Chambers, 1957; Joseph and Whitlock, 1968b; Liu, 1969). In the phylogenetic progression anuran, alligator, lizard, cat, and primate the dorsal root collaterals become longer and terminate in progressively more ventral positions; the terminals move from the dorsal horn in anurans to the ventral horn in the cat and primate (Joseph and Whitlock, 1968a). Joseph and Whitlock (1968a) have suggested that this demonstrates a phylogenetic movement of the dorsal root collateral synapses from distal dendrites in anurans to proximal dendrites and somas in cats and primates.

Brookhart and his coworkers (Brookhart and Fadiga, 1960; Fadiga and Brookhart, 1960) have corroborated by physiological means that anuran dorsal root collaterals synapse on the distal dendrites in the dorsal horn. However, recent physiological studies on the cat (Burke, 1966; Burke, 1967; Nelson and Frank, 1967; Smith et al., 1967; Rall et al., 1967) have demonstrated that the situation is not radically different from that in the anuran; dorsal root collateral synapses are widely distributed over the soma-dendritic surface of the

motoneuron with most synapses situated on the dendrites, many quite distally. Cat dorsal root collaterals probably terminate in the ventral horns on motoneuronal dendrites which run between the somas in a longitudinal direction (Sprague and Ha, 1964; Sterling and Kuypers, 1967a, 1967b).

Whether the difference between anurans and cats in the post-synaptic location of dorsal root terminals is sufficient to account for the weak excitation these terminals cause in anurans (Brookhart and Kubota, 1963) compared to the strong excitation they cause in cats (e.g. Brock et al., 1952; Coombs, et al., 1957b; Eccles et al., 1957) is a question which will be taken up further in the Physiological Discussion section.

Ventral Horn Unlike urodele amphibians (those with tails, e.g. salamanders) and fishes, anuran amphibians have distinct ventral horns in which clearly defined motoneurons are organized in linear columns. The anuran thus has the beginning of a columnar organization of motoneurons which is found in all higher tetrapods; there are two columns of motoneurons in the anuran but this increases to as many as seven in the cat (Romanes, 1953). The details of anuran motoneuron organization will be discussed further in the Anatomical section.

Ascending Pathways Amphibians are the first vertebrates known to have direct sensory fibers ascending to supraspinal regions. Some fibers project from the lumbar dorsal roots through a fasciculus gracilis onto a primordial nucleus gracilis located in the medulla (Woodburne, 1939; Joseph and Whitlock, 1968b). Anurans are thought (Ariens Kappers, 1936) to have a more developed spino-cerebellar tract than urodeles because of the greater development of the limbs and proprioceptive sense in anurans. Recent anatomical (Joseph and Whitlock, 1968b; Ebbesson, 1969) and physiological (Rushmer, 1970) evidence indicates that lumbar dorsal root fibers ascend in the dorsal funiculus directly to the cerebellum. Direct spino-vestibular fibers ascend in the lateral funiculus (Ebbesson, 1969).

Additional evidence on ascending pathways in anurans is not available; not until recently have degenerating fiber staining techniques become available which allowed the tracing of fiber pathways in nonmammalian species (Ebbesson, 1970). Previous work based on the tracing of fibers in normal material is now considered unreliable.

Descending Pathways Investigators using normal material have described tectospinal, cerebellospinal, tegmentospinal, vestibulospinal, and reticulospinal fibers (see reviews by Papez, 1929; Noble, 1931; Ariens Kappers et al., 1936; Nieuwenhuys, 1964) but the descriptions are vague as to the origin, course, and fine termination of these fibers. Vestibulospinal, reticulospinal, and possibly tegmentospinal (precursors to rubrospinal) fibers are the only ones generally thought to exist in primitive vertebrates like the anurans (Nieuwenhuys, 1964).

Using the experimental techniques of staining degenerating fibers, Robinson (1968) has recently demonstrated a tectospinal pathway but only a few fibers descend into the spinal cord and those do not descend any further than the brachial enlargement. Additional evidence on descending pathways, based on experimental anatomy, is even more scanty than that available on ascending pathways (see above).

There has been a paucity of physiological work on descending pathways in the anuran, perhaps due to the lack of anatomical information. The details of descending pathways in anurans will be discussed further in the Physiological section.

PHYSIOLOGICAL INTRODUCTION

Liu and Chambers (1957; Liu, 1969) first drew attention to the differences in terminal areas of pathways in the anuran spinal cord. Hemisection of the spinal cord 2 or 3 segments above the lumbar region, which interrupted all descending fibers including propriospinal fibers, resulted in terminal degeneration near the somas and short lateral dendrites of ventral horn motoneurons; section of the dorsal roots produced terminal degeneration in the dorsal horn where the only motor processes are the distal portions of the motoneurons' dorsal dendrites.

Brookhart and his coworkers, using an in vitro preparation of the anuran spinal cord (Brookhart et al., 1959; Machne et al., 1959) confirmed by potential field recording (Brookhart and Fadiga, 1960) that lateral funiculus fibers terminate in the ventral horn near the somas and proximal dendrites of the motoneurons and that dorsal root collaterals terminate in the dorsal horn near the distal dendrites of the motoneurons. They demonstrated by intracellular recording (Fadiga and Brookhart, 1960) that both of these pathways make monosynaptic contact with spatially distinct regions of the motoneuron membrane: lateral funiculus fibers with the somas and proximal dendrites and dorsal root collaterals with the distal dendrites.

Brookhart's studies of the physiology of anuran lateral funiculus fiber connections to lumbar motoneurons left several questions unanswered: (1) The spinal cord was stimulated in the caudal thoracic region, only 2 or 3 segments above the level of recording, so it was impossible to know whether the fibers were short propriospinal ones or long fibers descending from supraspinal regions. The ventrolateral funiculus of the cat contains both long and short fibers with direct connections to lumbar motoneurons (Lloyd, 1941) so it would be of interest to know whether the same situation exists in the anuran. (2) The spinal cord was stimulated on the ipsilateral side but the contralateral side might also have input to the motoneurons. Fibers in the contralateral ventrolateral funiculus of the cat make

monosynaptic connections to lumbar motoneurons (Willis et al., 1967); do they in the anuran? (3) The spinal cord was excised, the in vitro preparation, thus severed from the leg nerves so it was impossible to identify the kinds of motoneurons being studied. Ventrolateral funiculus fibers in the cat have excitatory synapses on about equal fractions of the extensor and flexor motoneuron pools (Willis et al., 1967) but the separate vestibulomotoneuronal and reticulomotoneuronal fibers, which pass through the ventrolateral funiculus, have excitatory synapses on different functional groups of motoneurons and they never make monosynaptic contact with the same lumbar motoneurons (Wilson and Yoshida, 1969; Grillner et al., 1970). Is there a similarity in the distribution of lateral funiculus fibers in the anuran or does its motor specialization for jumping require an unusually large input to one functional group of motoneurons, e.g. extensors? (Although the cat also jumps, the frog is more specialized for this one activity.)

In this part of my thesis lateral funiculus fibers descending from supraspinal regions on both ipsilateral and contralateral sides of the anuran spinal cord were studied with respect to their region of termination on the soma-dendritic membrane of lumbar motoneurons and with respect to their distribution to functionally different motoneurons.

In order to conduct these experiments I had to develop techniques for use of the in situ preparation of the anuran spinal cord. This was important for two reasons. One is that various small peripheral nerves were made available for the identification of flexor and extensor motoneurons. Another is that since the animal's circulatory system was kept functioning, the spinal cord was maintained in a more natural state, especially in regard to oxygenation and ionic environment.

Since the major portion of my time and effort was devoted to developing and perfecting an in situ preparation of the anuran spinal cord, a large portion of the results are presented as confirmation of

the viability of that preparation. In addition the results indicate that the lateral funiculus fibers which were investigated by Brookhart in the lumbar region originate bilaterally at or above the base of the medulla and synapse on equal fractions of lumbar extensor and flexor motoneurons.

PHYSIOLOGICAL METHODS

In Situ Preparation

The in situ anuran cord preparation has not been widely used for intracellular recording, but if physiological study of the anuran central nervous system is to be as fruitful as has been that of the cat, the use of this preparation will be necessary. Fukami (1961, 1962) first made intracellular recordings from an in situ anuran spinal cord, using the toad, but he reported few technical details about the preparation. Aside from recording EPSPs from muscle afferents (Fukami, 1961) and studying the anodal break response in the motoneurons (Fukami, 1962) he did nothing further using the preparation.

While my work was in progress, Simpson (1969) completed his doctoral research using an in situ preparation of the spinal cord of the small frog, Rana pipiens. Simpson extended Fukami's work by investigating the different patterns of EPSPs from various hindlimb afferents, both muscular and cutaneous. In some respects the techniques which we independently developed are similar. However, I have used the large frog, Rana catesbiana, because it has more accessible nervous structures. In addition I have used a lower temperature than did Simpson (9°C vs. 21°C-24°C) because this was essential for keeping the large frog preparation viable for several days.

Many preparations of anuran nervous tissue are surprisingly rugged and require little in the way of sophisticated technique. However, the in situ spinal cord is unusually delicate. For example, it is especially sensitive to blood circulation; oxygenated Ringer's cannot be so readily circulated around the cord as it can be in the in vitro preparation, therefore the normal circulation of blood must be maintained.

Since the preparation is more difficult than might be expected for an anuran and since this fact was learned after considerable trial-and-error, the method for preparing and recording from the in situ

spinal cord will be described in detail. Hopefully future investigations of anuran spinal physiology will be aided by the detailed description of the preparation which is to follow.

Animals

The animals used in all physiological experiments were female (because they are larger than males) bullfrogs, Rana catesbiana, with body weights in the range of 500-650 grams.

Preoperative care of the animals is described in the Appendix. Two weeks before they were to be used in an experiment, frogs were removed from the 15-20°C animal room and placed in an icebox kept at 9°C, the temperature at which all physiological experiments were conducted.

Ringer's Solution

The usual composition of the Ringer's solution was (mM): NaCl, 75; NaHCO₃, 25; Na₂HPO₄, 5; KCl, 4; CaCl₂, 1; Tris, 5; Glucose, 20. This was bubbled with 95% O₂ + 5% CO₂. (CaCl₂ was not added until the rest of the mixture had equilibrated with the gas for one hour.) This composition closely matched the measured composition of frog plasma at 9°C (courtesy of D. Elliot and M. Rizack) and such a Ringer's improved the viability of the preparation. The pH of the solution at 9°C when equilibrated with the gas mixture was 7.2.

Anesthesia

The best anesthetic was tricaine methanesulphonate (Finquel, Ayerst; previously MS-222, Sandoz; see Graham-Jones, 1964) because its rapid action and short time course made possible the accurate control of the degree of anesthesia. Sodium pentobarbital (Diabital, Diamond) was also used but the response to this anesthetic at 9°C was slow and variable; it frequently caused death.

All drugs were prepared in Ringer's solution at a concentration appropriate to administer the initial dose in a volume of 2-3 cc. This was injected into a subcutaneous lymph sac. Subsequent injections, in volumes of 0.5-1.0 cc, were made via a cannula in the sciatic vein of the hindleg.

The usual dose of tricaine methanesulphonate (200 mg/kg) required 5-15 minutes to take effect and lasted 60-90 minutes. A lower dose (100 mg/kg) would cause the same degree of immobility for 30-45 minutes and a higher dose (300 mg/kg) would last for 2-3 hours.

The usual dose of sodium pentobarbital (40 mg/kg) required 2-3 hours to take effect and lasted 12-24 hours. In some animals this dose was not effective but larger doses (50-60 mg/kg) which were likely to immobilize all animals were sometimes lethal.

Sometimes heavy anesthesia was used throughout an experiment and the spinal cord was left attached to the brain. More frequently, the spinal cord was severed from the brain and kept under little or no anesthesia since this preparation had healthier motoneurons and was longer lasting. Other effects of the presence or absence of anesthetic will be presented with the results.

Muscular activity was blocked by succinylcholine (Succostrin, Squibb) which did not lower the blood pressure as d-tubocurarine (Tubocurarine Chloride, Eli Lilly) does (Wright and Taylor, 1949, Fukami, 1961). Its action was rapid and short in duration so there was little danger of accidentally administering a lethal dose.

In an animal at 9°C, the usual dose of succinylcholine (2.5 mg/kg) would take effect in 5-10 minutes and last for 2-3 hours. Subsequent to the initial administration, doses of 1 mg/kg were given as required.

Mounting

The animal was mounted in a rigid frame by fixing clamps to the left iliac bone of the pelvis, to the transverse processes of the vertebrae at about the level of the fifth vertebra (thoracic), and to the upper jaw and skull. Mounted in this way the animal was about three-quarters submerged in a water bath which was refrigerated to keep the rectal temperature at 9°C (Plate 1).

The water bath and atmosphere above it were saturated with oxygen by constant bubbling. The portions of the frog's skin which were not submerged were covered with moist gauze strips. After the completion of surgery a tent of thin plastic sheet was draped over the frame holding the frog's body so that the moist oxygen atmosphere above the bath was kept in contact with the skin.

Respiration

Mechanical respiration of the lungs was not used because it was a source of vibration which would have caused instability in intracellular recording. The low metabolism at 9°C allowed the adequate exchange of oxygen and carbon dioxide through the skin. (At normal temperatures a significant amount of respiratory exchange takes place between frog skin and water (Krogh, 1904) and at lower temperatures hibernating frogs may stay under water for months (Dickerson, 1906).) The preparation could be kept in good physiological condition for at least 3 days under these conditions.

Surgery

A dorsal laminectomy was performed along the length of the spinal canal from the first to the ninth vertebrae (Plate 2). After skin and muscle were removed from the dorsal surface of the vertebrae, a high-speed (air-turbine) dental drill was used to remove all but a thin layer of bone above the spinal cord. The remaining bone was chipped away with a pair of Rongeur bone cutters which were specially

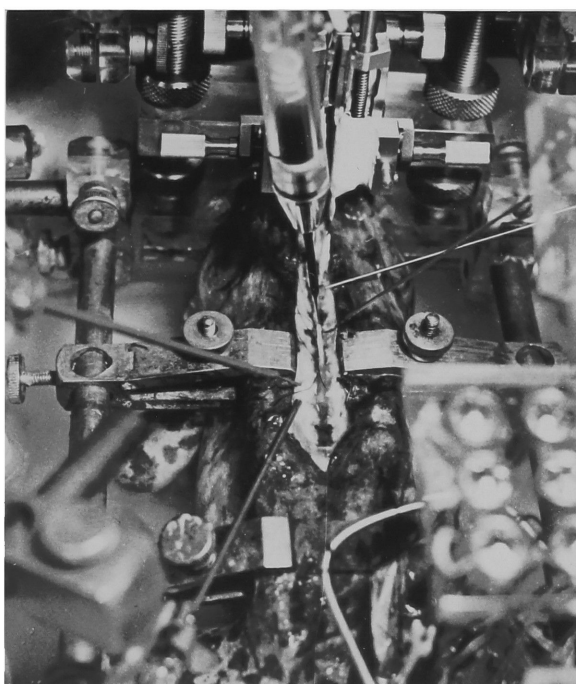


Plate 1. Overview of in situ frog spinal cord preparation. The animal was rigidly attached to a rectangular frame by clamps on the head, on the transverse processes of thoracic vertebrae, and on the left iliac bone of the pelvis.

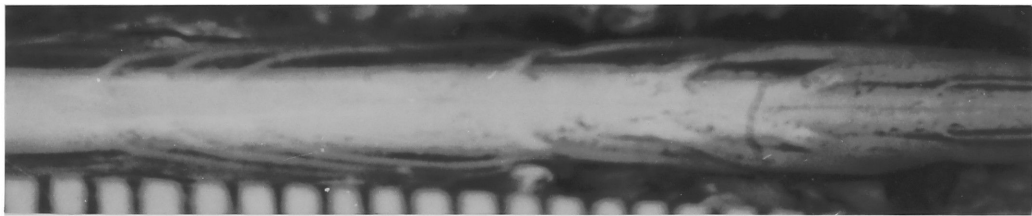


Plate 2. Dorsal view of in situ frog spinal cord with dura, arachnoid, and dorsal vein removed. From the rostral to the caudal direction (left to right) one can see the thoracic dorsal roots (5, 6, and 7) and the lumbar dorsal roots (8, 9, 10, and 11). Scale in millimeters.

machined to have a small tip. Powdered thrombin (Topical, Bovine Origin, Parke Davis) was applied to exposed bone marrow to stop bleeding. Care was taken during bone cutting to avoid damage to the vertebral arteries lying on each side of the vertebrae and to avoid damage to the dorsal vein (*V. vertebralis interna dorsalis*, Gaupp, 1899; *V. spinalis posterior*, Ecker, 1864, and Haslam, 1889). The dorsal vein drains the spinal cord as well as the dura in the bullfrog whereas in smaller species it may only drain the dura (Tregear, 1958; Bremer, personal communication). If the vein is removed in the bullfrog, the spinal cord will stay in good physiological condition only as long as blood is kept flowing from the small veins leading from the dorsal surface of the cord. There is one of these small veins (which normally drain into the dorsal vein) for each cord segment.

During the laminectomy frequent rinses with cold, oxygenated Ringer's solution kept the spinal cord moist and washed away blood and bone chips. After the laminectomy, a steady flow of Ringer's solution at 9°C and in equilibrium with a 95% O₂ + 5% CO₂ gas mixture was run over the spinal cord to supplement the oxygen and glucose supply of the vascular system and to prevent the drying of the tissue.

Under a dissecting microscope, the dura and some lateral branches of the dorsal vein (which do not drain the spinal cord) were cut, allowing the dura and dorsal vein to be gently retracted in the lumbar region of the spinal cord. Bleeding was arrested by momentarily pinching small, nonessential, veins with number 6 watchmaker's forceps. Care was taken not to damage the veins draining the dorsal surface of the cord into the dorsal vein.

Just prior to the insertion of a recording micropipette into the spinal cord, a small section of pia overlying the dorsal funiculus was carefully dissected away with fine hooks and knives. Sometimes the application of a digestive enzyme was found useful to soften the pia for dissection (Hafeman and Miller, 1967). The enzyme mixture (collagenase and elastase, Calbiochem, 40 mg/ml of each) was soaked up

in a $\frac{1}{2} \times 1$ mm rectangle of filter paper and applied to the appropriate region of the pia for 10-30 minutes.

When transection of the spinal cord was desired, it was made at the level of the calamus scriptorius (Figure 1) by means of a suction pipette. A suction pipette had several advantages over a small knife: (1) it allowed the removal of nerve tissue without damaging the dorsal vein or ventral artery, (2) it allowed the removal of a section of the medulla thus visibly isolating the cord from the brain, (3) it resulted in instant removal of blood, thus keeping the surgical field clear, and (4) it resulted in a more rapid arrest of bleeding from the exposed brain and spinal cord stumps.

Various hindleg nerves were surgically exposed and mounted on stimulating electrodes. Skin of the leg was drawn up to form a pool which was filled with oxygenated mineral oil to prevent drying of the nerves and to insulate the electrodes from contact with surrounding tissue.

Stimulation

All stimuli were electrical pulses, rectangular in shape and 0.05-0.10 msec in duration, generated by standard circuitry and isolated from ground. Pulses were delivered singly at a rate of 2 per second unless otherwise noted.

Stimulation was delivered to hindleg nerves by placing the appropriate nerve across a bipolar electrode constructed of a pair of fine platinum wires mounted 1 mm apart on a plastic support. Oil in the pool over the leg insulated the electrodes from surrounding tissue and reduced stray currents by reducing the volume of saline adhering to each nerve. Current spread at the branches of fine peripheral nerve twigs sometimes caused difficulty in ascertaining which twig was being stimulated but this was overcome by putting a stimulating electrode on both twigs in question and picking the twig that had a lower threshold.

Stimulation was delivered to the lateral funiculi of the spinal cord through bipolar electrodes constructed of a pair of stainless steel wires (diameter 0.015 in) mounted 1 mm apart on a small micro-manipulator. The tips of the wires were electrolytically tapered and then the entire wire was coated with several layers of varnish. An oblique area was exposed at the tip by grinding the wire on an Arkansas stone under a dissecting microscope. One pair of electrodes was mounted on the left side of the cord and the other pair on the right. They were lowered into place by separate micromanipulators from the dorsal aspect and perpendicular to the cord. The exposed oblique surfaces of the wires came to rest against the sides of the cord, one pair on each side. The electrodes were located in one of two longitudinal positions: either caudally, just above dorsal root 5 or rostrally, just below dorsal root 2.

The arrangement of the different stimulating electrodes allowed input to be directed to a motoneuron over 3 final pathways: ventral root, dorsal root, and lateral funiculus.

Recording

Recording circuits were conventional ones employing a bridge and a neutralized input capacity amplifier (Bioelectric Instruments). All recording was made in the D.C. mode unless otherwise stated and positive potentials were displayed upward on the oscilloscope. Negative current (5 μ a for 3 minutes) could be passed through the recording micropipette to cause deposition of methyl blue dye into the motoneuron being studied (Thomas and Wilson, 1966). Later this motoneuron could be located in histological sections of the spinal cord.

Intracellular recordings were made through a glass micropipette filled with 2 M KAc which had been saturated with methyl blue dye. To aid intracellular penetration, the tip of the micropipette was gently broken before filling it with electrolyte (the jagged edge apparently aids membrane puncture). Micropipettes used for recording had a resistance of 3 to 7 megohms. The micropipettes had inside tip

diameters of 0.4-0.8 μ and outside tip diameters of 0.6-1.2 μ as measured from electronmicrographs (Forman and Cruce, 1971).

A micropipette was inserted into the cord from the dorsal aspect in the region of the lumbar enlargement by means of a micromanipulator. Insertion was made at an angle of 10-15° from the vertical (passing ventrally and laterally from a point near the midline on the dorsal surface of the cord) because this caused the micropipette to pass through the densest part of the motoneuron pool.

Motoneurons were located by following antidromic focal potentials. After being impaled by a micropipette, most motoneurons with a resting potential greater than 50 mv maintained that potential steadily for 2 to 20 minutes, which is not uncommon for anuran motoneurons (Katz and Miledi, 1963). Three motoneurons lasted as long as 90 minutes before decay of the membrane potential set in. Since motoneurons with less than 50 mv resting potential generally showed erratic responses and rapid decay of the resting potential, they were routinely discarded. Although over 70 initially healthy motoneurons (RP > 50 mv) were impaled, the short recording time available in most limited the sample size for quantitative measurements (see Figure 10 for numbers). The antidromic spike, when it either fully or partially invaded the motoneuron soma (Coombs et al., 1957a; Machne et al., 1959) was used as a means of identification of motoneurons (Eccles et al., 1957).

Histology

At the end of an experiment an animal was killed by perfusion with fixative. The cord was removed, embedded in paraffin, and sectioned at 25 μ . Before staining, the sections were scanned under a microscope to locate the blue stain deposited inside motoneurons by marking with the micropipette. The sections were then lightly stained with cresyl violet or toluidine blue. The details of the histological procedure are given in the Appendix.

Mapping

Not all motoneurons studied in a given experiment were marked with intracellular dye. There were two reasons for this: (1) the process of moving dye out of a micropipette by passing current often raised the resistance of the micropipette, making it unsuitable for further recording; if a micropipette was giving good recordings, it would not be casually sacrificed for marking a cell, (2) marked motoneurons had to be spaced about 2 mm apart along the cord so that they could be identified separately from each other in sections of the cord; the small length of spinal cord under study only allowed space for about 3 cells to be marked in one experiment.

The small number of marked cells in one experiment served as reference marks for locating the other recorded cells on a map according to readings taken from the micromanipulator. The results from mapping the locations of motoneurons in the physiological experiments are discussed in the section on the anatomical experiments.

PHYSIOLOGICAL RESULTS

The Motoneurons

The motoneurons had an average resting potential of 60 mv (range 50-90 mv) and their intracellular action potentials (spikes, Figure 2) had an average amplitude of 75 mv (range 50-100 mv). Conduction velocity in the motor axons, calculated from the latency of the antidromic spike, ranged from 8 to 22 m/sec.

Segmental Synaptic Input

The effect of hindlimb muscle afferent volleys upon lumbar motoneurons was studied as a means of monitoring the health of the preparation and for comparison with the effect of lateral funiculus volleys.

Upon stimulation of a peripheral muscle nerve, the lowest threshold response evoked in a motoneuron was usually an excitatory post-synaptic potential with a short latency, the M-EPSP (Figure 3). If the motor axon of the impaled cell was contained in the nerve being stimulated, a greater intensity of stimulation (about 1.5 times threshold for the M-EPSP) would evoke an antidromic spike (Figure 2a).

An M-EPSP was always evoked in a motoneuron by stimulation of the nerve containing the motoneuron's axon; usually M-EPSPs were evoked by stimulation of other peripheral motor nerves. An M-EPSP of maximum size, even from an homonymous source, was never large enough to fire an orthodromic spike.

Spatial summation of M-EPSPs from different peripheral nerves was never large enough to exceed spike threshold, either. However, spatial summation of an M-EPSP and an EPSP evoked by a volley in supraspinal pathways (see below) would exceed spike threshold.

M-EPSPs (Figure 3) had a rapid rising phase, a steady latency in response to a single shock, and a time-to-peak which was constant

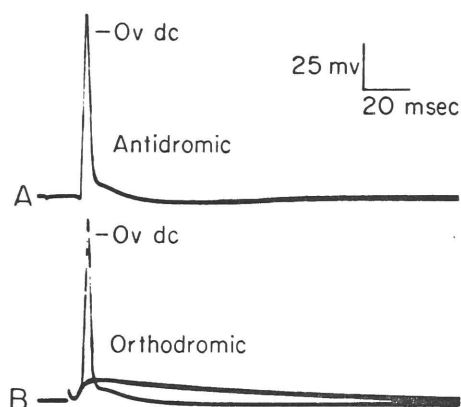


Figure 2. Intracellular action potentials (spikes). A) Segmental antidromic spike evoked in a peroneal nerve motoneuron by stimulation of the peroneal nerve. B) Suprasegmental orthodromic spike evoked in the same motoneuron by stimulation of the rostral ipsilateral lateral funiculus of the spinal cord. The LF-EPSP exceeded the spike threshold in about half the traces. Resting potential of the cell was 90 mv. Spikes were retouched to improve visibility. In this and all succeeding intracellular recordings, positive potentials are displayed in an upward direction.

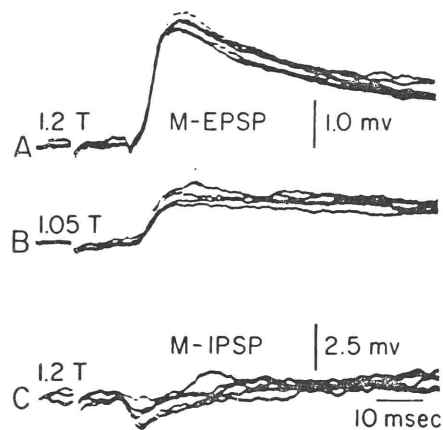


Figure 3. Segmental post-synaptic potentials. A, B) M-EPSP evoked in a peroneal nerve motoneuron (RP > 50 mv) by stimulation of the nerve ramus adductorius at 1.05 (B) and 1.2 (A) times threshold. D.C. recording. C) M-IPSP evoked in a ramus adductorius motoneuron (RP < 50 mv) by stimulation of the mixed nerve to the gracilis muscles and the ventro-medial thigh skin at 1.2 times threshold. Recording time constant: 405 msec. The different total latencies in A and B compared to C are due to different peripheral conduction times. Segmental latencies, measured from the arrival time of the fastest dorsal root volley, were 2.5 msec for the M-EPSP and 5.8 msec for the M-IPSP. The notch preceding the EPSP in A is caused by the extracellular field potential of antidromic spikes in adjacent motoneurons. The extracellular field potential for B and C was negligible.

with increasing stimulus intensity, making it probable that they were evoked through a monosynaptic connection. M-EPSPs did not show a decrease in amplitude with increasing low frequency stimulation (2-6 per second) nor with heavy pentobarbital anesthesia but both of these conditions did depress long-latency, variable EPSPs (for example, from cutaneous nerves) which were clearly polysynaptic (see also Kubota and Brookhart, 1963). The segmental delay of M-EPSPs, calculated as the time between the earliest arrival of a volley in the cord dorsum and the foot of the M-EPSP, ranged from 2.6 to 3.7 msec.

Hyperpolarizing potentials were evoked by stimulation of some peripheral muscle nerves (Figure 3c) in two naturally depolarized cells (resting potential < 50 mv), but no routine search was made for IPSPs in other cells by artificially depolarizing them (passing current through the micropipette). The amplitude of these M-IPSPs was increased by depolarization of the cell and decreased by hyperpolarization, therefore they were true inhibitory post-synaptic potentials, not disfacilitation (Toyama et al., 1968) and they were generated near the recording electrode, that is on the soma and proximal dendrites (Kubota and Brookhart, 1963; Smith et al., 1967).

Suprasegmental Synaptic Input

The effect upon lumbar motoneurons of lateral funiculus volleys from supra-lumbar regions was studied to determine whether lateral funiculus fibers having monosynaptic contact with the motoneurons were part of a short or long descending system and whether they descended ipsilaterally, contralaterally, or bilaterally. Since it is well established (Fadiga and Brookhart, 1960) that lateral funiculus fibers from the ipsilateral side in the lower thoracic region (caudal level) make monosynaptic contact with lumbar motoneurons, most attention was directed to the effects of stimulation of the lateral funiculi, both ipsilaterally and contralaterally, in the region of the calamus scriptorius (rostral level).

Upon stimulation of the lateral funiculus of the spinal cord, the lowest threshold response evoked in a motoneuron was usually an EPSP, the LF-EPSP, which was either simple (Figure 4) or complex (Figure 5) in appearance. Hyperpolarizing post-synaptic potentials, LF-IPSPs, were also observed, though not routinely searched for (see above) and one is shown in Figure 4c.

Complex LF-EPSPs (Figure 5) were composed of two parts, a short-latency or "early-simple" wave resembling the short-latency, simple LF-EPSP (Figure 4) and a "late-complex" wave. The "late-complex" wave had a long and variable rising phase, a variable latency, a variable time-to-peak, and was reduced in amplitude by increasing low-frequency stimulation or by administering heavy pentobarbital anesthesia, making it likely that this portion of the LF-EPSP was generated by polysynaptic connections to the motoneuron. The "late-complex" wave was generally more prominent in contralateral LF-EPSPs than in ipsilateral ones (Figure 5). All further use of the term LF-EPSP will implicitly refer to the "simple" or "early-simple" waves of the LF-EPSP.

By increasing the stimulus intensity, LF EPSPs could usually be made large enough to generate a spike on the rising phase (Figure 2b). This is quite unlike M-EPSPs which, as previously mentioned, were never large enough to fire spikes.

LF-EPSPs had a rapid rising phase, a steady latency in response to a single shock, and a time-to-peak which was constant with increasing stimulus intensity, making it probable that they were evoked through a monosynaptic connection. LF-EPSPs did not show a decrease in amplitude with increasing low-frequency stimulation nor with heavy pentobarbital anesthesia but both of these conditions did depress long-latency, variable EPSPs (for example, in the "late-complex" wave) which were clearly polysynaptic.

LF-EPSP latencies from the caudal level had a range of 2.5 to 4.0 msec; from the rostral level, 4.0 to 8.0 msec (Figure 6). Segmental delay, determined by measuring the difference between the

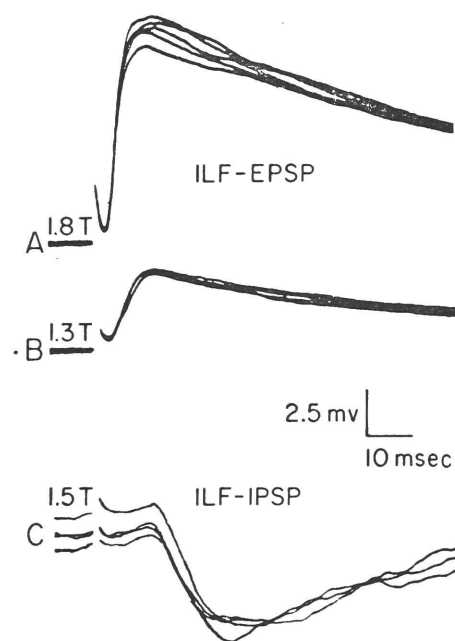


Figure 4. Suprasegmental post-synaptic potentials of the "simple" type. A, B) ILF-EPSP evoked in a peroneal nerve motoneuron by stimulation of the rostral ipsilateral lateral funiculus at 1.3 (B) and 1.8 (A) times threshold. C) ILF-IPSP evoked in a tibial nerve motoneuron by stimulation of the rostral ipsilateral lateral funiculus at 1.5 times threshold. The extracellular field potential for B or C was negligible but for A caused some distortion of the rising phase.

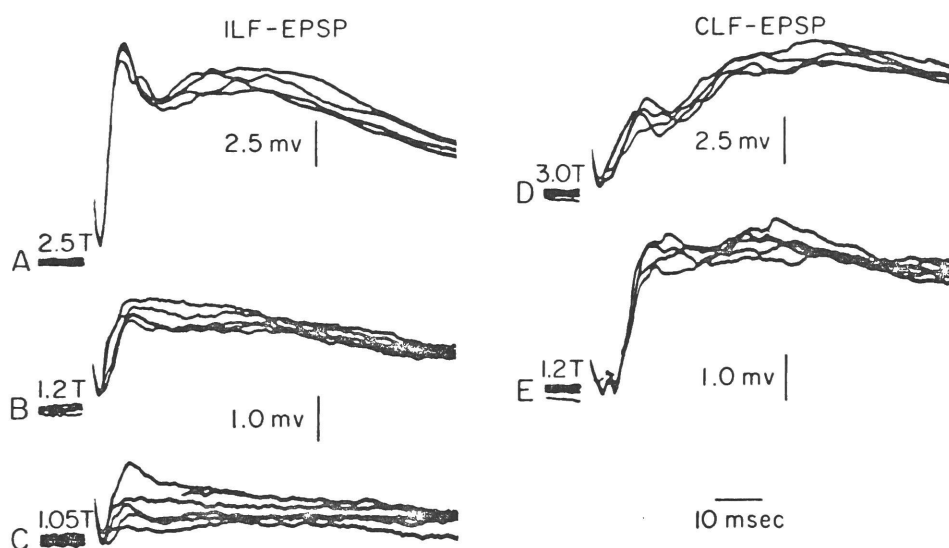


Figure 5. Suprasegmental post-synaptic potentials of the "complex" type. A, B, C) ILF-EPSP evoked in a tibial nerve motoneuron by stimulation of the rostral ipsilateral lateral funiculus at 1.05 (C), 1.2 (B), and 2.5 (A) times threshold. D, E) CLF-EPSP evoked in the same motoneuron by stimulation of the rostral contralateral lateral funiculus at 1.2 (E) and 3.0 (D) times threshold. The notch which appears just past the peak of the "early-simple" wave of the EPSP in A and D is an artifact caused by an intense extra-cellular field potential. For B, C, and E the extracellular field potential was less distorting. Note the "late-complex" wave which prolongs the decay phase in A, D, and E and which is most prominent in the CLF-EPSPs (D and E).

latency of the leading edge of the volley recorded on the cord surface and the latency of the LF-EPSP, had a range of 2.5 to 3.4 msec. This is similar to the range of segmental delays I measured for the monosynaptic M-EPSP (2.6-3.7 msec).

The latencies of rostral LF-EPSPs form two groups in the histogram (Figure 6) but this is probably an artifact of the spread of latencies since the latencies of the caudal LF-EPSPs form only one group (Figure 6) and since two distinct volleys were never detected in recordings from the cord surface. There is the possibility that the rostral stimulating electrodes were located over a synapse in the descending pathway and were stimulating in some cases the pre- and in other cases the post-synaptic sides; there is insufficient data to eliminate that possibility completely. However, that would still mean that the descending fiber population was the same one in the region of the rostral electrodes as in the region of the caudal electrodes. Therefore the descending fiber population will be treated as one throughout this study.

The latencies of the rostral and caudal LF-EPSPs were plotted against their respective conduction distances and a linear regression (see Wilson *et al.*, 1970 for a discussion of this analytical method) was calculated through the points: the mean conduction velocity was 8 m/sec and the mean segmental delay was 2.7 msec. The mean conduction velocity is appropriate for A fibers and the mean segmental delay is appropriate for a monosynaptic delay.

Synaptic Location

The rise-time or time-to-peak is an important parameter for characterizing the site of generation of an EPSP on the soma-dendritic membrane (e.g. Rall, 1967; Fadiga and Brookhart, 1960). If two EPSPs are compared, and assumed to have the same synaptic mechanism and equal time courses for transmitter release, the one with the greater time-to-peak is electrotonically further away from the recording electrode.

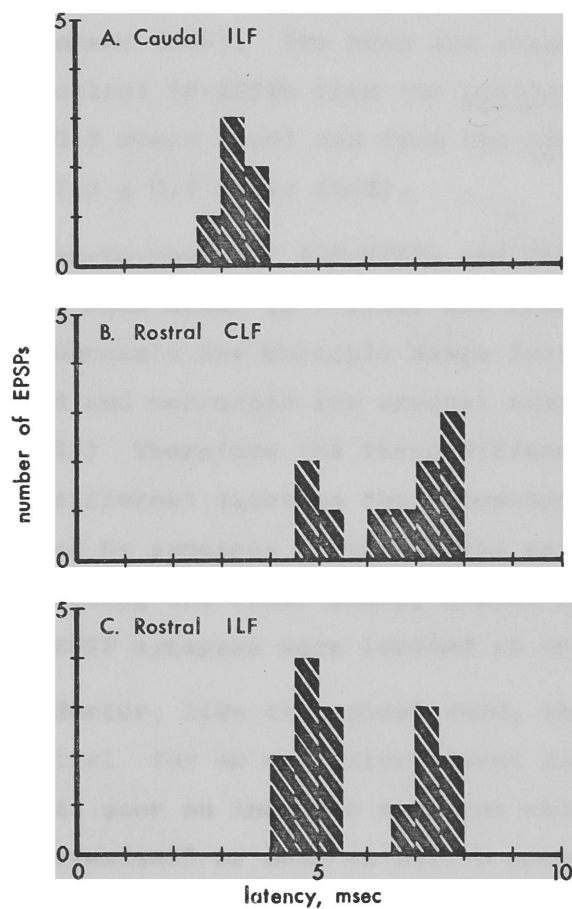


Figure 6. Latency of LF-EPSPs in lumbar motoneurons. A) Caudal ILF-EPSP evoked by stimulation of the caudal ipsilateral lateral funiculus. (The caudal CLF-EPSP had a similar histogram.) B) Rostral CLF-EPSP evoked by stimulation of the rostral contralateral lateral funiculus. C) Rostral ILF-EPSP evoked by stimulation of the rostral ipsilateral lateral funiculus. Note the apparent separation of the rostral latencies into two groups. This is probably an artifact (see text).

Time-to-peak was measured (Figure 7) after correcting the EPSP shape for distortion caused by extracellular field potentials (as recorded after withdrawing the micropipette to a position just outside the cell). The mean and standard deviation of the time-to-peak for M-EPSPs was 8.2 ± 0.5 msec (n=6). The mean and standard deviation of the time-to-peak for rostral LF-EPSPs from the ipsilateral funiculus (ILF-EPSPs) was 6.3 ± 0.3 msec (n=8) and from the contralateral funiculus (CLF-EPSPs) was 7.3 ± 0.7 msec (n=8).

The means of time-to-peak for ILF-EPSPs and CLF-EPSPs were significantly different from each other ($p < 0.01$) and from the mean for M-EPSPs ($p < 0.05$). (Duncan's New Multiple Range Test, p. 136 of Edwards, 1960, was used and corrected for unequal sample size according to p. 96 of Winer, 1962.) Therefore the three different EPSPs were probably generated at different sites on the motoneuronal membrane. ILF-EPSPs were generated by synapses closer to the recording electrode than the synapses generating the other EPSPs; M-EPSP synapses were the farthest away and CLF-EPSP synapses were located at an intermediate site.

In a volume conductor, like the spinal cord, the direction of an extracellular potential for an excitatory event indicates whether a recording electrode is near an inactive membrane which is a source of current (positive potential) or near an active membrane which is a sink of current (negative potential) (Lorente de No', 1953). When recordings are made extracellular to motoneuron somas, a positive potential associated with an EPSP is usually interpreted as indicating that the EPSP is generated at an active site distant from the soma, that is on the distal dendrites (Brookhart and Fadiga, 1960; Terzuolo and Araki, 1961). Thus the extracellular field potential for M-EPSPs, which was always in a positive direction, (Figure 7) indicates that these EPSPs were generated on distal dendrites. By similar reasoning, an extracellular potential near a motoneuron soma which is in a negative direction, such as that recorded during LF-EPSPs (Figure 7), indicates that the active membrane, in this case the site of lateral

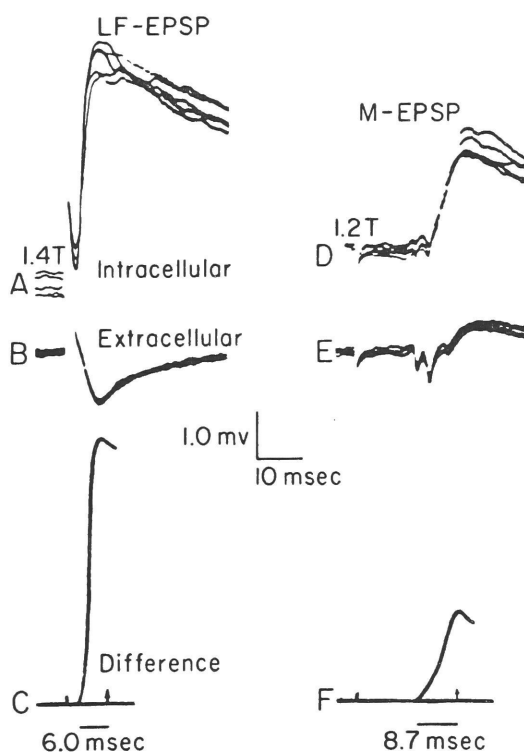


Figure 7. Measurement of EPSP time-to-peak. A, D) Intracellular recordings in a peroneal nerve motoneuron of EPSPs evoked by stimulation of (A) the rostral ipsilateral lateral funiculus at 1.4 times threshold and (D) the peroneal nerve at 1.2 times threshold. B, E) Extracellular recordings made adjacent to the motoneuron during the similar stimuli after withdrawing the electrode from the cell. C, F) Reconstructions of the actual EPSP time course made by subtracting the extracellular potential from the intracellular potential. Time-to-peak is indicated by bar beneath each drawing. Note that the extracellular potential of the LF-EPSP is negative and of the M-EPSP is positive, indicating different synaptic sites (see text).

funiculus synapses, is near the recording site, i.e. the soma and proximal dendrites of the motoneuron.

Both the time-to-peak measurements and the direction of the extracellular field potentials are in agreement that the ILF-EPSP was generated on the soma and proximal dendrites and that the M-EPSP was generated on the distal dendrites. The CLF-EPSP was similar to the ILF-EPSP in the direction of its extracellular field potential which indicates that both EPSPs were generated near the cell body. The greater time-to-peak of the CLF-EPSP might suggest that it was generated more on the proximal dendrites and less on the soma than the ILF-EPSP. An alternative explanation for the differences in rise time of the CLF- and ILF-EPSP might be that the contralateral volley had a slightly less synchronous arrival time than the ipsilateral volley (this could be caused by marked reduction in fiber diameter in the terminal region, say due to branching; a change in volley synchrony in this region would likely escape detection in recordings from the cord surface).

Interaction of EPSPs

The interaction of two or more homonymous EPSPs evoked by muscle afferents or lateral funiculus stimulation was studied to confirm whether or not these EPSPs were evoked via the same synapses which have previously been investigated in anurans: Fadiga and Brookhart (1962) established that dorsal root EPSPs and Caudal LF-EPSPs interact differently.

When pulse pairs were delivered to the rostral lateral funiculus (Figure 8), a second LF-EPSP was either the same size as the first one, causing a doubling in the amount of membrane depolarization, or was facilitated. However, under the same conditions a second M-EPSP was not the same size as the first one, but was depressed in amplitude; it did not cause much additional depolarization of the membrane.

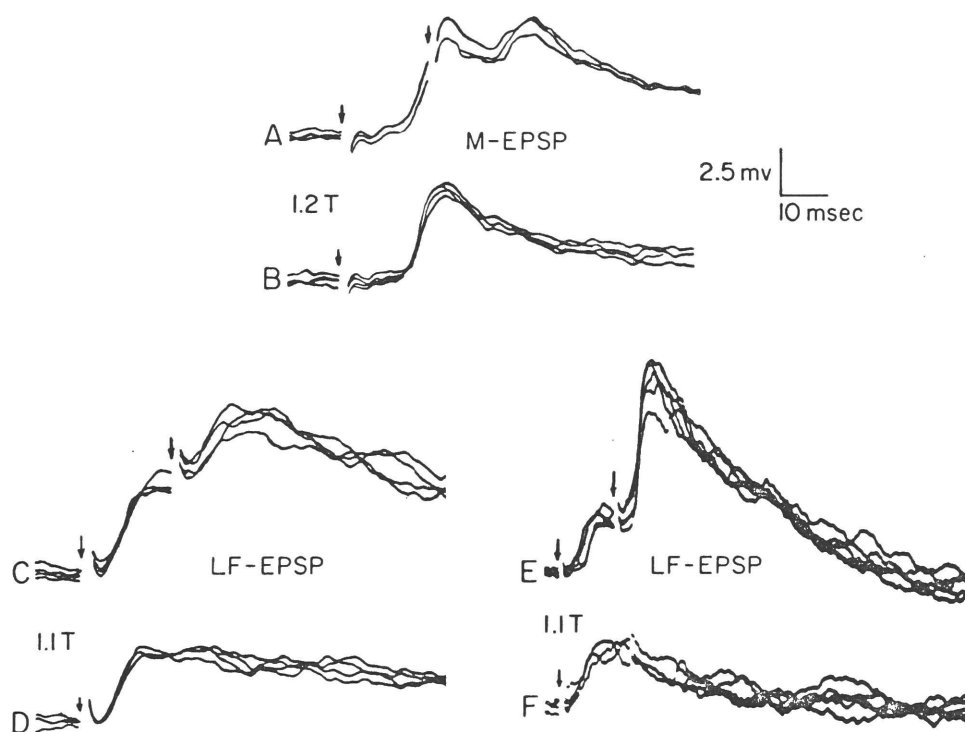


Figure 8. Interaction of pairs of EPSPs. A, B) M-EPSP evoked in an unidentified lumbar motoneuron by stimulation of the crural nerve at 1.2 times threshold where indicated by the arrows. Single pulse in B and double pulse in A. C, D) LF-EPSP evoked in the same motoneuron by stimulation of the rostral ipsilateral lateral funiculus at 1.1 times threshold where indicated by the arrows. Single pulse in D and double pulse in C. E, F) LF-EPSP evoked in a ramus adductor motoneuron by stimulation of the rostral ipsilateral lateral funiculus at 1.1 times threshold where indicated by the arrows. Single pulse in F and double pulse in E. Recording time constant in E, F: 405 msec. Note that a second LF-EPSP is either the same amplitude as the first one (C) or facilitated (E) but a second M-EPSP is depressed in amplitude compared with the first one (A).

A similar difference was observed in the homonymous interaction of the two kinds of EPSPs when they were evoked in rapid succession (Figure 9). Individual LF-EPSPs were of approximately the same amplitude and showed a consequent increase in the mean level of membrane depolarization. Individual M-EPSPs following the first one were generally of smaller amplitude and the resultant membrane depolarization was not greatly increased above the level caused by a single M-EPSP. Possible explanations for these differences will be taken up in the discussion.

Interaction of EPSPs from heteronymous sources revealed that a single M-EPSP and a single LF-EPSP were additive without reduction in either EPSP, regardless of temporal sequence. Subthreshold EPSPs from the two sources could add up to exceed threshold for the cell and fire a spike. Single M-EPSPs from two different peripheral nerves were also additive without reduction, but they were never large enough to exceed spike threshold.

Distribution of Suprasegmental Synaptic Input

LF-EPSPs were evoked in most lumbar motoneurons which were sampled, regardless of whether the motoneurons were flexors or extensors, whether the lateral funiculus was stimulated at the rostral or caudal level, or whether the lateral funiculus was stimulated on the ipsilateral or contralateral side of the spinal cord (Figure 10 and Table I).

Section of the lateral funiculus at the level of dorsal root 5 abolished rostral ILF-EPSPs in all cells tested and abolished CLF-EPSPs in half the cells tested. This suggests that fibers stimulated in the lateral funiculus at the level of the calamus scriptorius (rostral stimulation) continue to descend in the lateral funiculus on the ipsilateral side; on the contralateral side some fibers cross above the level of dorsal root 5 and descend in the ipsilateral lateral funiculus while some descend on the contralateral side and cross below the level of dorsal root 5.

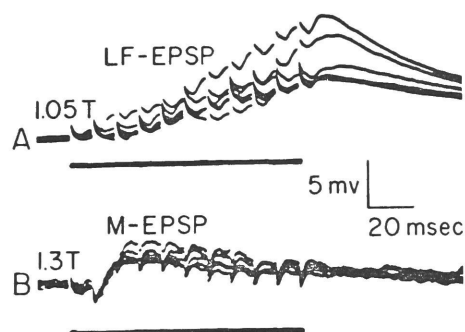


Figure 9. Interaction of trains of EPSPs. A train of pulses was delivered at 10 msec intervals for 100 msec (indicated by solid bar beneath recording). The pulse train was repeated twice each second. A) LF-EPSP evoked in a peroneal motoneuron by stimulation of the rostral ipsilateral lateral funiculus at 1.05 times threshold. B) M-EPSP evoked in the same motoneuron by stimulation of the nerve ramus adductorius at 1.3 times threshold (submaximal). The effects were the same at other stimulus intensities and at other pulse intervals in the range 10 to 100 msec (frequency 100 to 10 per second). At pulse intervals greater than 100 msec the M-EPSPs did not overlap and behaved as separate EPSPs, relatively unaffected by each other.

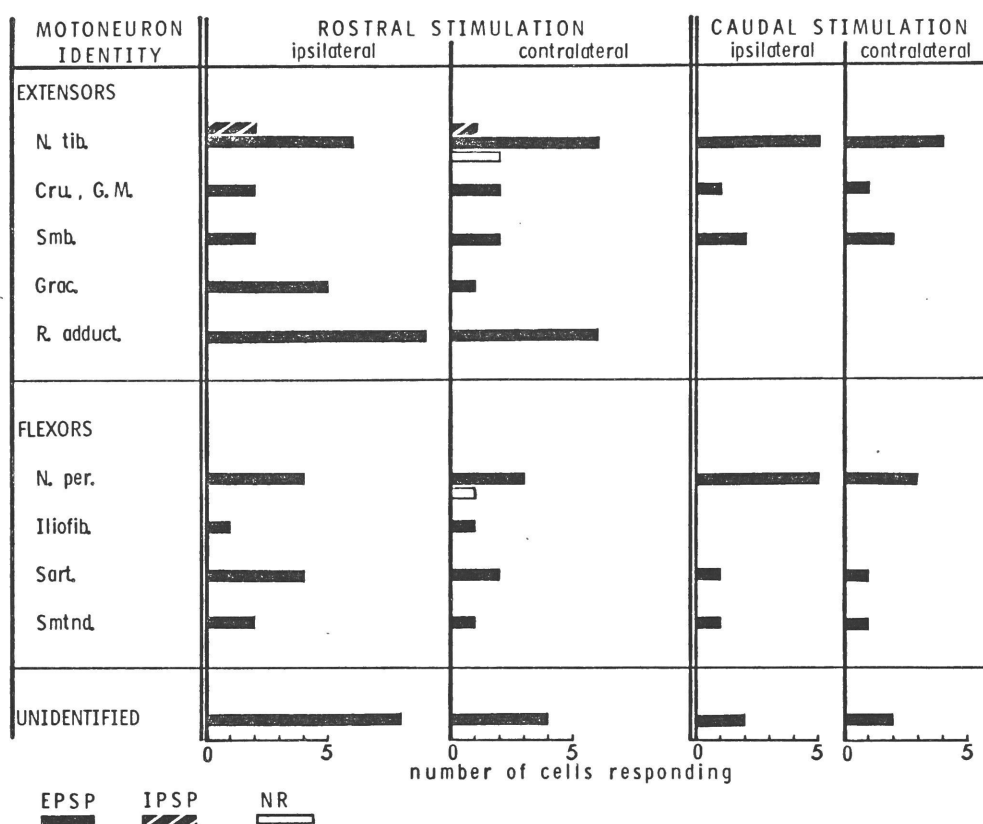


Figure 10. Distribution of lateral funiculus post-synaptic potentials. Bar length indicates the number of cells (scale at bottom) in any particular category. Bar shading indicates whether there was no response (open), IPSP (striped), or monosynaptic EPSP (solid). At the top of the figure is given the location of the stimulating electrodes. A cell was tested with either rostral or caudal stimulation but not with both. Many cells were tested with both ipsilateral and contralateral stimulation. Results from twelve animals. Abbreviations used: N. Tib. - tibial nerve; Cru., G.M. - cruralis and gluteus magnus; Smb. - semimembranosus; Grac. - gracilis major and gracilis minor; R. adduct. - nerve ramus adductorius which innervates adductor magnus, quadratus femoris, and obturator externus; N. per. - peroneal nerve; Iliofib. - iliofibularis; Sart. - sartorius; Smtnd. - semitendinosus. Tibial and peroneal nerves are classified according to the function of the ankle muscles they innervate. (See the Appendix for further comments on muscle nomenclature and function.)

Table I
Distribution of Monosynaptic LF-EPSPs

	Rostral		Caudal	
	Ipsi	Contra	Ipsi	Contra
Extensor	92% (26)	85% (20)	100% (8)	100% (7)
Flexor	100% (11)	88% (8)	100% (7)	100% (5)
Unident	100% (8)	100% (4)	100% (2)	100% (2)

PHYSIOLOGICAL DISCUSSION

Several features of anuran spinal physiology, particularly on the segmental level, which are not entirely unique to my study, will be discussed to demonstrate the healthy condition of the preparation and to provide the reader with a general orientation to anuran spinal physiology. This will be followed by a discussion of synaptic input from suprasegmental levels with particular emphasis on the findings of my study concerning supraspinal fibers in the lateral funiculus. Finally, there will be some speculation as to the possible origin and function of these fibers.

Anuran Spinal Physiology

The Motoneurons The frog motoneurons which I studied had greater resting potentials (a mean of 60 mv) than many investigators have found. Brookhart, for example, found mean resting potentials ranging from 44 to 49 mv in several different studies (Fadiga and Brookhart, 1960; Kubota and Brookhart, 1963). Katz and Miledi (1963) noted that variability in responses, particularly in hyperpolarizing potentials, became evident below 60 mv and became pronounced below 40 mv. While the large resting potentials of my motoneurons attested to a healthy condition, they also may have been responsible for the paucity of IPSPs (see discussion below).

The conduction velocity in the motor axons (8-22 m/sec) identifies them as A fibers (Erlanger and Gasser, 1937) or axons of the twitch motor system rather than of the small-nerve motor system (Kuffler and Gerard, 1947) if the slowing effect of the low temperature (Gasser, 1931; Katz and Miledi, 1965), i.e. 9°C, is taken into account.

Since small-nerve motoneurons are also located in the ventral horn (Ito and Watanabe, 1962) and since only large motoneurons are likely to survive puncture with the size of micropipette used (Katz and Miledi, 1963) it is likely that small-nerve motoneurons were not included in the sample.

Segmental Delay Time The segmental delay times which I observed for M-EPSPs (2.6-3.7 msec) and for LF-EPSPs (2.5-3.4 msec) may seem large for a monosynaptic delay, even in anurans. For example, Fadiga and Brookhart (1960) found that segmental monosynaptic delay for either pathway was 1.5-2.0 msec. However, their measurements were made at a higher temperature (15-17°C) than were mine (9°C) and synaptic delay is known to lengthen with decrease in temperature (Katz and Miledi, 1965). My measurements of synaptic delay were comparable to the values (3-4 msec) found at similar low temperatures by other investigators (Katz and Miledi, 1963; Richens, 1969).

Time-to-Peak The time-to-peak values (Figure 7) which I obtained for M-EPSPs (8.2 msec), CLF-EPSPs (7.3 msec), and ILF-EPSPs (6.3 msec) may seem large, even for anurans. For example, Fadiga and Brookhart (1960) found values of 3.2 msec for DR-EPSPs and 2.3 msec for LF-EPSPs. However, the lower temperature of my experiments would be expected to increase the time course of transmitter release (Katz and Miledi, 1965) and thereby increase the time-to-peak (Rall, 1967) to approximately the values I have obtained. Other investigators working in anuran motoneurons at similar low temperatures have found that miniature EPSPs had average time-to-peak values of about 10 msec (Colomo and Erulkar, 1968) and a range of 2-20 msec (Katz and Miledi, 1963).

Interaction of EPSPs The interaction of two or more homonymous M-EPSPs in succession resulted in a membrane depolarization which was not much greater than that caused by the first EPSP; the individual M-EPSPs following the first EPSP were depressed in amplitude (Figures 8 and 9). This is the same effect which was seen by previous investigators (Fadiga and Brookhart, 1962) when studying the interaction of DR-EPSPs; it is further evidence that the M-EPSP and the DR-EPSP were evoked by the same synapses.

The interaction of homonymous LF-EPSPs is different: whether the individual EPSPs are facilitated or unaltered they summate to

produce increasing membrane depolarization (Figures 8 and 9). This is true of LF-EPSPs evoked from the caudal level as well (Fadiga and Brookhart, 1962).

The frequency dependence of the amplitude of individual moto-neuronal EPSPs in a train has not been well studied in frogs but the effect on the mean level of membrane depolarization is striking at all frequencies (Fadiga and Brookhart, 1962): LF-EPSPs summate to increase depolarization, DR-EPSPs do not. The mechanisms responsible for these effects are not known but several possibilities will be mentioned:

- (1) Presynaptic differences in transmitter mobilization and storage (Curtis and Eccles, 1960; Mallart and Martin, 1967) could account for EPSPs at the lateral funiculus synapse being facilitated or unaltered when repetitively evoked (Figure 9A) while those at the segmental synapse on the same post-synaptic membrane were depressed (Figure 9B).
- (2) Presynaptic inhibition (Eccles, 1964; Bergmans and Colle, 1964; Kostyuk and Timchenko, 1965) could be present only in segmental pathways, thus accounting for depression of M-EPSPs following the first in a train (Figure 9B).
- (3) Postsynaptic addition of polysynaptic EPSPs (Willis et al., 1967) might create an apparent maintenance or facilitation of LF-EPSP amplitude (Figure 9A) since polysynaptic activity is more apparent in supraspinal pathways in the anuran than in segmental pathways.
- (4) Postsynaptic location of the segmental synapses on the distal motoneuron membrane could theoretically (Brookhart and Kubota, 1963; Burke, 1967) account for the reduction in amplitude of M-EPSPs following the first one in a train (Figure 9B). However, Porter (1970) has recently found that monkey cortico-motoneuronal EPSPs show facilitation although they are apparently generated by synapses on the distal dendrites; postsynaptic location of synapses may be of less importance to repetitive interactions than has been supposed.

Segmental Synaptic Input

The monosynaptic EPSP which can be evoked in lumbar motoneurons by stimulation of the frog dorsal root, the monosynaptic DR-EPSP (Fadiga and Brookhart, 1960) has been shown by Simpson (1969) to be similar in several ways to the monosynaptic EPSP which can be evoked by stimulation of muscle afferents, the M-EPSP. Indeed, Fukami (1961) earlier found monosynaptic M-EPSPs in toad brachial motoneurons but it is not certain that they were generated only on the dendrites.

I confirmed, by measurement of synaptic delay, time-to-peak, extracellular field potential, and interaction of multiple EPSPs that the M-EPSP and the DR-EPSP are identical; the monosynaptic DR-EPSP of Brookhart was evoked by stimulation of muscle afferents in the dorsal root.

The monosynaptic DR-EPSP rarely, if ever, evoked spike discharge (Brookhart and Kubota, 1963); the M-EPSP, which I observed (Figure 3), never evoked spike discharge. This is in agreement with Simpson's (1969) findings concerning muscle afferent input to frog lumbar motoneurons but is quite different from the situation found in the cat where muscle afferent volleys can produce large enough EPSPs to evoke motoneuronal spikes (Brock et al., 1952; Coombs et al., 1957b; Eccles et al., 1957).

In the cat, muscle afferent collaterals to the motoneurons are responsible for the monosynaptic ventral root spike evoked by dorsal root stimulation (Eccles and Pritchard, 1937; Renshaw, 1940; Lloyd, 1943a) or by muscle stretch (Lloyd, 1943c); they mediate the pre-synaptic side of the myotatic reflex (Lloyd, 1943b). However, in the anuran there is no monosynaptic ventral root spike evoked by dorsal root stimulation (Eccles, 1946; Bremer, 1953; Lloyd, 1959; Brookhart et al., 1959) nor by muscle stretch (Marx, 1950); a myotatic reflex has usually not been found (Kato, 1934; Bremer et al., 1942) although Sassa (1921) reported eliciting one in semitendinosus muscle. The absence of these effects in the anuran is now accounted for by the

small, sub-threshold EPSPs produced in their motoneurons by muscle afferent volleys.

The weak influence of anuran muscle afferents on motoneuron discharge might be due to the dendritic location of their synapses: a dendritic EPSP would decay in amplitude as it is electrotonically conducted to the soma (Fadiga and Brookhart, 1963; Rall, 1967). However, recent studies of cat motoneurons have demonstrated that muscle afferent synapses, although distributed on all regions of the motoneuron membrane, are found predominantly on the dendrites, both proximal and distal (Burke, 1966; Burke et al., 1967; Smith et al., 1967; Rall et al., 1967) yet cat muscle afferent volleys can evoke large enough monosynaptic EPSPs to fire the motoneurons. Thus the difference between cats and anurans in the location of muscle afferent synapses doesn't appear striking enough to completely account for the differences in muscle reflexes of the two species.

I suggest that an additional factor accounting for the weak influence of anuran muscle afferents on motoneuron discharge could be that only a small number of muscle afferents converge onto each motoneuron. In cats, each afferent fiber from a given muscle makes synaptic contact with virtually all of the motoneurons innervating that muscle (Mendell and Henneman, 1971). Nothing is currently known about the number of muscle afferents converging onto anuran motoneurons. A study of the distribution of muscle afferents among anuran motoneurons would be a valuable contribution towards understanding the relative importance of dendritic synapses in the control of motoneuron excitation. Such an investigation is now made possible by the in situ frog spinal cord preparation where individual muscle nerves can be selectively stimulated and by the localization of anuran motoneuron pools (see Anatomical section).

Previous investigators have noted the scarcity of segmental IPSPs in anuran motoneurons (Brookhart and Kubota, 1963; Simpson, 1969). However, by using no anesthesia and by stimulating the 8th

dorsal root while recording from motoneurons of the 9th or 10th ventral roots, Kubota and Brookhart (1963) were able to elicit DR-IPSPs.

Many of my experiments were conducted on spinal animals in the absence of anesthesia so anesthesia cannot account for the scarcity of M-IPSPs which I observed. Since the 8th dorsal root enters the region of the lumbar spinal cord populated predominantly by flexor motoneurons and the 9th and especially the 10th ventral roots leave in the extensor regions (see Anatomical section), it is likely that Brookhart was observing the effects of afferents from antagonistic muscles; stimulation of anuran muscle nerves is well known to cause inhibition of cutaneously evoked twitch reflexes (Kato, 1934) and antagonist muscles are the most effective in causing this inhibition (Bremer and Bonnet, 1942). However, I frequently stimulated antagonist afferents without observing obvious M-IPSPs.

A more likely explanation remains for the scarcity of M-IPSPs in my experiments. Katz and Miledi (1963) noted that anuran IPSPs didn't occur in motoneurons with resting potentials exceeding 60 mv but became progressively prominent as cells were depolarized from that level. My motoneurons had resting potentials exceeding 50 mv whereas most of Brookhart's (Kubota and Brookhart, 1963) had resting potentials under 50 mv. The large resting potentials attested to the healthy conditions of my motoneurons but these large resting potentials also prevented the discrimination between excitatory and inhibitory action on the basis of potential changes. In the future the routine testing of motoneurons by the passage of depolarizing current through the micropipette might circumvent this.

Suprasegmental Synaptic Input

Previous investigators established that fibers in the lateral funiculus had a powerful excitatory influence on frog lumbar motoneurons (Brookhart et al., 1959; Brookhart and Kubota, 1963), but it was not known whether these fibers originated in spinal or in supraspinal regions

since they stimulated the lateral funiculus in the lower thoracic segments. My experiments demonstrated that lateral funiculus fibers from the rostral end of the spinal cord had a similar excitatory influence on frog lumbar motoneurons (Figures 4 and 5).

The physiology of rostral LF-EPSPs was similar to that previously demonstrated for caudal LF-EPSPs (Fadiga and Brookhart, 1960; 1962). Fibers in the rostral lateral funiculus produced LF-EPSPs with monosynaptic segmental latency; maximal LF-EPSPs were frequently large enough to fire motoneuronal spikes (Figure 2); the short time-to-peak of the LF-EPSPs and the negative extracellular potential associated with them (Figure 7) were evidence that they were evoked on the somaproximal dendritic membrane; two or more LF-EPSPs in succession were capable of temporal summation to produce increased membrane depolarization (Figures 8 and 9).

Fibers in the contralateral pathway, never before investigated, were also shown to synapse monosynaptically on ipsilateral lumbar motoneurons (Figure 10 and Table I) and to produce LF-EPSPs (Figure 5) with physiological properties similar to those produced from the ipsilateral side. The negative extracellular potential associated with CLF-EPSPs indicates that they are generated in the region of the motoneuron soma, like ILF-EPSPs; their greater time-to-peak suggests that they are generated somewhat further away from the recording electrode, perhaps on the proximal dendrites.

Some polysynaptic excitatory activity (Figure 5) was evoked by lateral funiculus stimulation, especially from the contralateral side. This phenomenon was not seen in previous investigations of the lateral funiculus (Fadiga and Brookhart, 1960), but it can probably be attributed to the lighter anesthesia as well as to the more rostral level of stimulation.

LF-IPSPs (Figure 4) have not previously been reported (cf. Fadiga and Brookhart, 1960; Kubota and Brookhart, 1963). Their paucity in my experiments was unexpected considering the supraspinal

inhibitory effects which exist in anurans (Kato, 1934) but these effects may not be mediated by fibers in the lateral funiculus. More likely, as discussed above, the large resting potentials of the motoneurons precluded the appearance of IPSPs.

Supraspinal Pathways

Fibers in the lateral funiculus of the anuran spinal cord, known to have powerful excitatory effects on lumbar motoneurons (Fadiga and Brookhart, 1960), have now been shown to originate bilaterally at or above the level of the calamus scriptorius (caudal end of the fourth ventricle and beginning of the spinal cord). In the following pages I will speculate on the possible origin and function of these fibers. Since so little is known about the anatomy and function of supraspinal tracts in the anuran, this speculation will draw heavily on information about other vertebrate species, where the anatomy and function is better known.

Origin Kato (1934) described, in an anuran, an excitatory supraspinal pathway. When the caudal, dorsal region of one cerebral hemisphere was stimulated, twitches were evoked in muscles of the contralateral hindlimb. By making hemisections at various levels of the brain and cord, he found that the pathway decussated in the caudal medulla and rostral spinal cord. It is not known whether Kato was observing monosynaptic or polysynaptic effects, but his effects might have been mediated by the lateral funicular supraspinal pathway.

I may have been stimulating portions of the same fiber tract both before and after decussation; that is my ipsilateral effects were mediated by the part of the contralateral tract which had crossed in the medulla and my contralateral effects were mediated by the part of the contralateral tract which crossed more caudally, in the spinal cord. The fact that contralateral fibers may have had a different synaptic termination and more polysynaptic connections than ipsilateral fibers would not prevent them from being part of one tract but these differences would certainly raise some doubts about this hypothesis.

In view of Kato's findings, it is curious that ablation of one hemisphere of an anuran (Loesser, 1905) causes nothing more dramatic than a "muscular weakness" on the crossed side for a period of 3 to 4 days (see also review by Aronson and Noble, 1945). More caudal regions of the anuran brain cause more dramatic motor deficits when they are ablated.

Ablation and stimulation experiments on the anuran brain have demonstrated that there are powerful motor effects emanating from the tectum (Loesser, 1905; Abbie and Adey, 1950; Goodman, 1958), the cerebellum (Loesser, 1905; Goodman, 1958), the lateral vestibular nucleus (Abbie and Adey, 1950; Barale et al., 1968) and the medulla or rhombencephalic reticular formation (Loesser, 1905; Abbie and Adey, 1950).

Whether the pathways from these regions to the spinal cord are mono- or poly-synaptic is not known. Because stimulation in the brain regions produces slow and phasic reactions, it has been proposed that the pathways to the motoneurons are polysynaptic with relays in the reticular formation and propriospinal pools (Noble, 1931; Ariens Kappers et al., 1936; Abbie and Adey, 1950; Goodman, 1958).

Classical anatomical studies, using normal material described the presence in anurans of tectospinal, cerebellospinal, tegmentospinal, vestibulospinal, and reticulospinal tracts (see reviews by: Papez, 1929; Noble, 1931; Ariens Kappers, 1936; Ariens Kappers et al., 1936; Nieuwenhuys, 1964) but the descriptions are vague as to the origin, course and termination of the fibers, due to the inherent limitations of studies using normal material.

Experimentally, it has been shown (Jankowski and Afelt, 1964) that transection of the anuran spinal cord at its rostral end does not result in massive degeneration of fiber tracts, such as occurs in mammals. However, if there were long descending fibers scattered throughout the white matter of the spinal cord amongst the many

shorter fibers, they could only be detected by the recently developed techniques for staining degenerating fibers (Nauta, 1957; Heimer, 1970; Ebbesson, 1970).

Using the new techniques, Robinson (1968) has shown that there are anuran tectospinal fibers but they project only as far caudally as the brachial region of the spinal cord. Reticulospinal fibers (Robinson, personal communication) do project to all regions of the spinal cord but their connection to the motoneurons is still uncertain. As yet there is no experimental evidence on other anuran supraspinal fibers.

Recently Robinson (1969) has demonstrated in the lizard (a reptile) by means of degenerating fiber stains and chromatolysis that scattered rubrospinal, vestibulospinal, and reticulospinal fibers descend as far as the lumbar enlargement but he did not study the relationship of the degenerating terminals to the motoneurons.

It is generally accepted that vestibulospinal and reticulospinal pathways are phylogenetically old and present in all vertebrate classes (Ariens Kappers et al., 1936; Ariens Kappers, 1936; Nieuwenhuys, 1964) and that these pathways always have fibers making direct connections to the motoneurons (Shapovalov, 1970) but there is less known about rubrospinal (segmentospinal) pathways. Even in cats, which clearly have a rubrospinal pathway (Nyberg-Hansen and Brodal, 1964; Petras, 1967), the monosynaptic connections to lumbar motoneurons are sparse (Shapovalov, 1970). Thus it is reasonable that vestibulospinal and reticulospinal pathways might exist in anurans, perhaps with monosynaptic connections to lumbar motoneurons, but any other direct pathways are highly unlikely.

Path Having established that reticulomotoneuronal and vestibulomotoneuronal fibers might exist in anurans there is still the question, would they be found in the lateral funiculus? At present this can only be answered by observing the location of these pathways in other vertebrates.

In the lizard (Robinson, 1969) the lateral funiculi contain primarily reticulospinal fibers, some ipsilateral or decussating above the spinal cord and some contralateral or decussating within the spinal cord; vestibulospinal (from Deiter's nucleus) fibers decussate above the cord and lie entirely in the medial portion of the ventral funiculus.

In cats vestibulospinal fibers (from Deiter's nucleus) are found in the lateral regions of the ventral funiculus (Pompeiano and Brodal, 1957; Nyberg-Hansen and Mascitti, 1964) and they lie especially lateral in the caudal medulla (Busch, 1964). Hence, it is possible that stimulation of the anuran lateral funiculus at the calamus scriptorius would evoke volleys in vestibulomotoneuronal fibers as well as reticulomotoneuronal ones.

Distribution Supraspinal fibers in the anuran lateral funiculi make monosynaptic excitatory connections with over 85% of both flexor and extensor motoneurons in the lumbar spinal cord (Figure 10 and Table I). This is similar to the distribution of fibers in the ventral and lateral funiculi of the cat spinal cord (Willis et al., 1967).

Furthermore it has been shown in the cat that there are two complementary and mutually exclusive supraspinal systems in the ventral and lateral funiculi: (1) the lateral vestibular nucleus (Deiter's nucleus) makes monosynaptic excitatory connections to some hindlimb extensors; (2) the pontine reticular formation makes monosynaptic excitatory connections to many hindlimb flexors and some hindlimb extensors not served by Deiter's nucleus (Grillner et al., 1968; Lund and Pompeiano, 1968; Wilson and Yoshida, 1969; Grillner et al., 1970). No lumbar motoneuron has excitatory connections from both systems.

Function While vestibulospinal and reticulospinal systems are probably phylogenetically old and present in all vertebrate classes, there are modifications of these systems for the peculiar functional

needs of particular species. For example, several classes of fish (Graham and O'Leary, 1941; Rovainen, 1967; Shapovalov, 1970) as well as urodele amphibians (Herrick, 1948) have large reticular cells (Mauthner and Müller cells) which send large, fast conducting motor fibers into the spinal cord; their function is probably to cause rapid synchronous contraction of axial muscles in a whiplike escape response.

Supraspinal fibers in the anuran lateral funiculi are also specialized in comparison to their mammalian counterparts. The anuran excitatory fibers synapse on the somas and proximal dendrites of motoneurons and probably because of this (Brookhart and Kubota, 1963) the EPSPs which can be evoked by volleys in these fibers are sufficiently large to fire spikes in the motoneurons. The analogous mammalian excitatory supraspinal fibers synapse mostly on the dendrites (Shapovalov, 1970) and probably because of this the EPSPs which can be evoked by volleys in these fibers are too small to fire spikes in the motoneurons.

Why should anurans have powerfully excitatory supraspinal fibers? Jumping is an activity for which anurans are specialized and an activity which would require powerful, rapid supraspinal excitation of both flexor and extensor motoneurons. The explosive bullfrog jump begins with an extensor thrust of the hindlimbs which lasts about 25 milliseconds; there is a period of ballistic coasting, lasting about 300 milliseconds, followed by a rapid flexion of the hindlimbs back into squatting position, a phase which lasts about 25 milliseconds (Gray, 1968). The extensor and flexor phases of jumping, because of their rapid, synchronous onset and massive involvement of hindlimb muscles are just the sort of actions for which the lateral funiculus fibers, with their intense monosynaptic input to the motoneuron somas, appear suited.

Little is known about the motor centers controlling jumping in anurans, but the medulla, especially the caudal regions, appears to be essential (Sherrington, 1906; Afelt, 1965). The cells of the reticular

formation, already suspected of being major contributors to the lateral funiculus, are likely candidates for the control of jumping since they are prominent in the lower medulla. With its various inputs, e.g. from vestibular, cerebellar, and tectal regions, the reticular formation seems ideally situated for the integration of visual and positional information necessary for a frog to accurately jump at a moving target and catch it in mid-air (see Davidson, 1963, for dramatic photographs of this activity).

ANATOMICAL INTRODUCTION

The problem of the central origin of motor axons innervating individual muscles of the vertebrate limb has long been of interest and has been well reviewed by Sherrington (1892), Strauss (1946), and Romanes (1964). The localization of motoneurons within the frog spinal cord is of special interest to comparative neuroanatomy because anuran amphibians (frogs and toads) are, among the living vertebrates, perhaps the most closely related to the first vertebrate to evolve limbs (Noble, 1931; Romer, 1966, 1968; Hecht, 1969; Colbert, 1969) and because they are the most primitive vertebrate in which clearly defined motoneurons are organized into linear columns in a distinct ventral horn (Nieuwenhuys, 1964).

The localization of frog spinal motoneurons is of special concern here because during the physiological investigations for this thesis it was necessary to locate particular types of motoneurons for intracellular recording. The information available in the literature concerning the localization of frog spinal motoneurons (Silver, 1942; Stussi, 1960) was inadequate for this task.

Silver (1942) used stimulating microelectrodes within the frog lumbar spinal cord to investigate the general regions of localization of motoneuron function. Stussi (1960), using the same method, confirmed Silver's results and mapped the locations of regions of the lumbar spinal cord which excited specific hindlimb muscles.

Unfortunately the electrical stimulation technique has two major weaknesses. (1) There is uncertainty about whether the electrical current stimulates motoneuron cell bodies or other spinal structures. (2) The results are based on visual observation of muscle twitches but the deep leg muscles cannot be seen, so their contraction must be inferred from leg movement. Furthermore, neither Silver nor Stussi appear to have taken into account the effect which variation in the lumbar plexus has on the location of motoneurons in different frogs (Sherrington, 1892, Romanes, 1951).

Since the location of specific motoneurons in the frog spinal cord is uncertain from Silver's and from Stussi's results, the usefulness of these works for detailed comparison with mammalian work (e.g. Romanes, 1951) is impaired. I have made a more precise investigation of the localization of motoneurons in the frog spinal cord, in part because of its value to comparative neuroanatomy.

Functional and somatotopic maps of motoneuron locations in the cat spinal cord (Romanes, 1951) paved the way for more advanced exploration of mammalian spinal physiology by means of the intracellular recording technique (e.g. Eccles et al., 1957). I have prepared similar maps for the frog spinal cord as an aid to my study of frog spinal physiology.

I investigated the location of spinal motoneurons in the frog using the technique of retrograde chromatolysis which has proven to be an accurate means to locate mammalian spinal motoneurons (Romanes, 1951). I took into account the effects of plexus variation in preparing maps of the locations of motoneurons innervating individual muscles of the hindlimb.

ANATOMICAL METHODS

Retrograde Reaction

Retrograde reaction of the perikaryon was used to map the location of motoneurons within the bullfrog spinal cord. The phenomenon of retrograde reaction, first observed by Nissl (1892), most thoroughly described by Bodian and Mellors (1945), and recently reviewed by several authors (Beresford, 1965; Cole, 1968; Cragg, 1970), consists of changes seen in the perikaryon when the axonal process of a nerve cell is injured by a cut, a crush, or a tear. A decrease in basophilic stainable material (Nissl substance), or chromatolysis, is most commonly observed and is sometimes accompanied by swelling of the perikaryon and displacement of the nucleus to the cell border.

Retrograde reaction reaches a peak (measured by extent of cell swelling or extent of Nissl clearing) in a few days to a few weeks after axonal injury. Then the reaction subsides and the cells either atrophy and die or return to normal. The time course is fairly uniform for a given species and temperature (Ebbesson, 1970). Among the closely related species in the genus Rana, a wide variety of times have been reported for the peak of the retrograde reaction, but the variation may have been due to the different temperatures used (lower temperatures prolong the time course). For example in Rana pipiens (21-24° C), the maximum reaction of motoneurons occurs 14-21 days after nerve injury (Porter et al., 1963), while in Rana temporaria (20° C) it occurs in 20-50 days and maximum perikaryal swelling is at 31 days (Edstrom, 1959).

In Rana catesbiana (22-24° C), the species used in the present experiments, it has been reported that retrograde reaction of motoneurons is detectable after 8-11 days (Cerf and Chacko, 1958), but the time of maximum reaction has never been determined. In a pilot study I found that the maximum retrograde reaction of motoneurons in response to transection of the spinal roots occurred in Rana catesbiana

(20° C) at 28-35 days.

Animals

Female bullfrogs, Rana catesbiana, weighing 350-550 grams, were obtained and cared for, preoperatively, in the manner described in the Appendix. After surgery the animals were returned to a 20° C animal room where they were fed small leopard frogs, Rana pipiens, once a week.

Surgery

Sodium pentobarbital (Diabutal, Diamond, 40 mg/kg) or tricaine methanesulphonate (Finquel, Ayerst, previously MS-222, Sandoz, 200 mg/kg) diluted in saline to a volume of 2-3 cc and injected subcutaneously was used to induce anesthesia. One hindlimb was opened and a single nerve was injured, care being taken not to injure adjacent nerves. In early experiments, the contralateral hindlimb was left intact as a control, but, in later experiments, as the appearance of the retrograde reaction became more familiar, a different nerve was injured on the contralateral side as well.

Retrograde reaction is generally less pronounced when nerves are severed distally, for example in the leg region, than when nerves are severed proximally, for example near their exit from the spinal cord (e.g. Romanes, 1941). Romanes (1951) overcame this problem in the cat by making use of the observation of Howe and Bodian (1941) that repeated section of a peripheral nerve led to a more uniform degree of chromatolysis in the motoneurons of the spinal cord.

In the present experiments, the double-injury technique of Romanes was found to produce more pronounced and uniform chromatolysis than either a single transection or a single crush to peripheral nerve in the bullfrog. However, no technique of injuring a peripheral nerve produced a response as intense as that seen when a single injury was administered to a spinal nerve.

The primary injury was always a crush, since this allowed a loose loop of thread to be placed around the nerve, marking the nerve for easy location when the second operation was performed. It did not matter much whether the secondary injury was a transection or a crush, so either type was used. The secondary injury was made 7-14 days after the primary one, the exact day not being critical.

The function of the muscles whose nerves were experimentally injured was determined by the procedures given in the Appendix. There is also in the Appendix a discussion of the nomenclature used for muscles and nerves (Gaupp, 1896-1904).

Histology

Between 28 and 35 days after the primary injury, the animal was anesthetized then perfused with amphibian ringer followed by fixative. The spinal cord with roots intact was removed immediately through a dorsal laminectomy and stored in fixative for 24 hours. The pelvis and hindlimbs were dissected until the lumbar plexus was exposed bilaterally allowing a drawing of the plexuses to be made.

After the roots on one side were cut short, to make a left-right marker, the spinal cord was transected just rostral to the seventh dorsal root and the remaining caudal portion, or lumbar enlargement, was embedded in paraffin, sectioned at 25 μ , and stained with cresyl violet or toluidine blue. Further details of the histological procedure are given in the Appendix.

Anatomical Analysis

Reconstructions of the cell columns in the ventral horn were prepared by Elliot's (1942) projection method, with particular attention being paid to the longitudinal changes in these columns as well as their arrangement in a given transverse plane.

In experimental spinal cords, a count was made of reacting cells, i.e. those showing chromatolysis, counting only those with a nucleolus present. The nucleolus is so small that it appears in only one 25 μ section, while the perikaryon of a cell may extend through several sections.

A map of reacting cell locations in the transverse plane was prepared for each experimental spinal cord by noting the dorso-ventral position of the cells within a cell column.

A map of reacting cell locations on the longitudinal axis was prepared in the following manner. The number of reacting cells in each section was counted and the counts from each ten successive sections (250 μ) were summed. The resulting count was displayed as a histogram of cell density along the longitudinal axis.

Romanes (1951) noted, in the cat, that a motoneuron pool is displaced in a rostral or a caudal direction with reference to segmental markers according to whether the plexus for that cord is prefixed or postfixed. A prefixed plexus has a greater contribution from more rostral roots than a postfixed plexus (Sherrington, 1892) and consequently a motoneuron pool is displaced more rostrally.

In the present experiments, the frog was found to have pre- and post-fixed plexuses with consequent displacements of motoneuron pools. As will be shown in the results section, the entire dorso-lateral cell column, which exists only in the lumbar region, is displaced in a rostral or a caudal direction according to the kind of plexus it serves, but a given motoneuron pool retains always its same longitudinal position within the dorso-lateral cell column.

The caudal edge of the dorsal root entry was used as a reference marker to delineate segments along the longitudinal axis because the caudal edge has a distinct point of entry to the spinal cord and the dorsal roots are fairly uniform in their arrangement from cord to cord.

A "standard" longitudinal map was prepared for each spinal cord, regardless of plexus form, by adjusting the rostral or caudal location of the dorso-lateral cell column to match the location of that column in a spinal cord with a "standard" plexus. This was done by projection of the experimental column onto the "standard" column. This procedure had the added advantage that differences in the length of the column in different animals could be adjusted by altering the magnification of the projector until the length of the projected cell column exactly matched that of the "standard" cell column.

ANATOMICAL RESULTS

Lumbar Plexus

The contributions of spinal roots 8, 9, 10, and 11 to the lumbar plexus vary in different individual frogs. Spinal root 8 is usually of moderate size and frequently contributes to the lumbar plexus. Spinal roots 9 and 10 vary a great deal in size but always contribute to the plexus. Spinal root 11 is usually small and frequently does not contribute to the plexus.

Previous descriptions of the variation in the lumbar plexus of the frog were inadequate for the purposes of this investigation. Sherrington (1892) divided frog plexuses into two groups and Gaupp (1899) illustrated four forms, but neither author described the complete range of variants nor their frequency. Since accurate location of motoneurons requires consideration of the plexus variation (Romanes, 1951) it is necessary, as a preliminary to reporting the experimental results, to describe plexus variation in the frog.

A survey of 82 cases revealed seven distinct forms of the lumbar plexus in the frog, Rana catesbiana, based on the size of the contributing spinal roots (Figure 11). The different plexus forms shown in Figure 11 are positioned from left to right according to whether they receive greater contributions from rostral or from caudal roots. The "D" type plexus is taken to be the normal or "standard" form of the plexus because it occurred with the greatest frequency, i.e. in about one-third of the cases. Forms "C", "B", and "A" are progressively more prefixed, that is having larger contributions from the more rostral spinal roots. Forms "E", "F", and "G" are progressively more postfixed, that is having larger contributions from the more caudal spinal roots.

Forms "C" and "D", although having the same apparent contribution from the spinal roots, were separated on the basis of the difference in the bridge between roots 8 and 9. The bridge in form

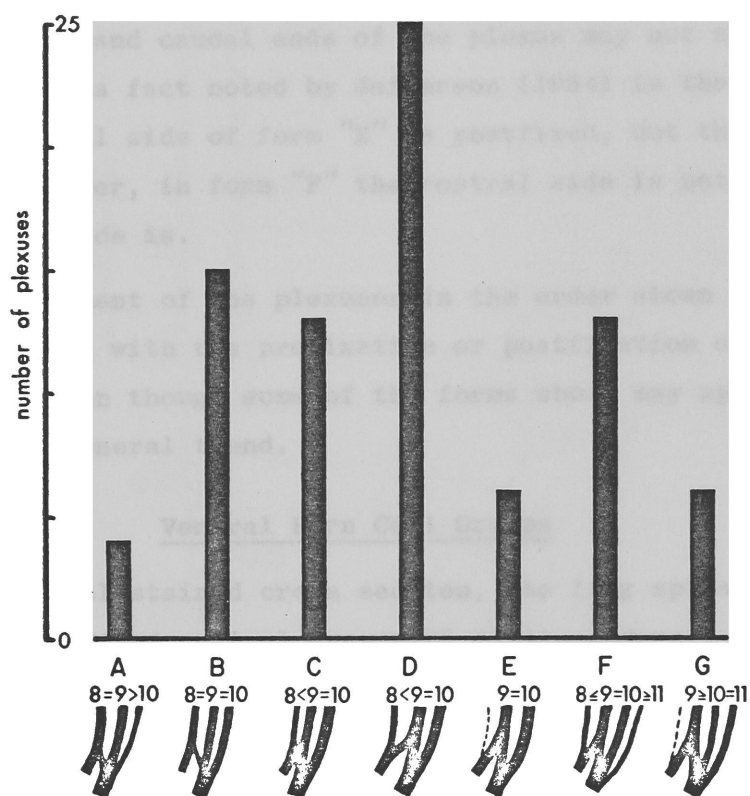


Figure 11. Lumbar plexus forms. The frequency of appearance of different forms of the lumbar plexus of the frog, *Rana catesbiana*, are displayed from left to right in approximate order of the shift from domination of the more rostral roots, 8 and 9, to domination of the more caudal roots, 10 and 11, or from more prefixed to more postfixed form.

"C" appears to be a transitional stage between that in form "B" where root 8 contributes to the sciatic nerve and that in form "D" where root 9 contributes to the crural nerve.

Form "E" is an aberrant form in which the plexus is shortened, having only a thin filament contributed by root 8, but none by root 11. It is assumed to represent a transition between forms "D" and "F".

The rostral and caudal ends of the plexus may not shift in fixation together, a fact noted by Jefferson (1954) in the cat. For example, the rostral side of form "E" is postfixed, but the caudal side is not. However, in form "F" the rostral side is not postfixed while the caudal side is.

The arrangement of the plexuses in the order shown in Figure 11 has correlated well with the prefixation or postfixation of the motoneuron columns, even though some of the forms shown may appear to deviate from the general trend.

Ventral Horn Cell Groups

Seen in Nissl stained cross section, the frog spinal cord exhibits a well-defined central gray with distinct dorsal horns and prominent ventral horns (Figure 12). The large ventral horn cells, or motoneurons, are found in two distinct columnar groups within the frog spinal cord. A small column, in a ventro-medial position exists throughout the length of the cord except in the lumbar swelling, while a larger column, in a dorso-lateral position, exists only in the region of the brachial and lumbar swellings.

Silver's (1942) otherwise excellent account of ventral horn cell groups in the frog spinal cord did not describe the relationship of these groups to the emergence of the spinal roots. Since accurate location of motoneurons requires a consideration of their topographical organization into groups (Romanes, 1951), it is necessary, as a preliminary to reporting the experimental results, to describe in detail the columnar arrangement of motoneurons in the lumbar ventral

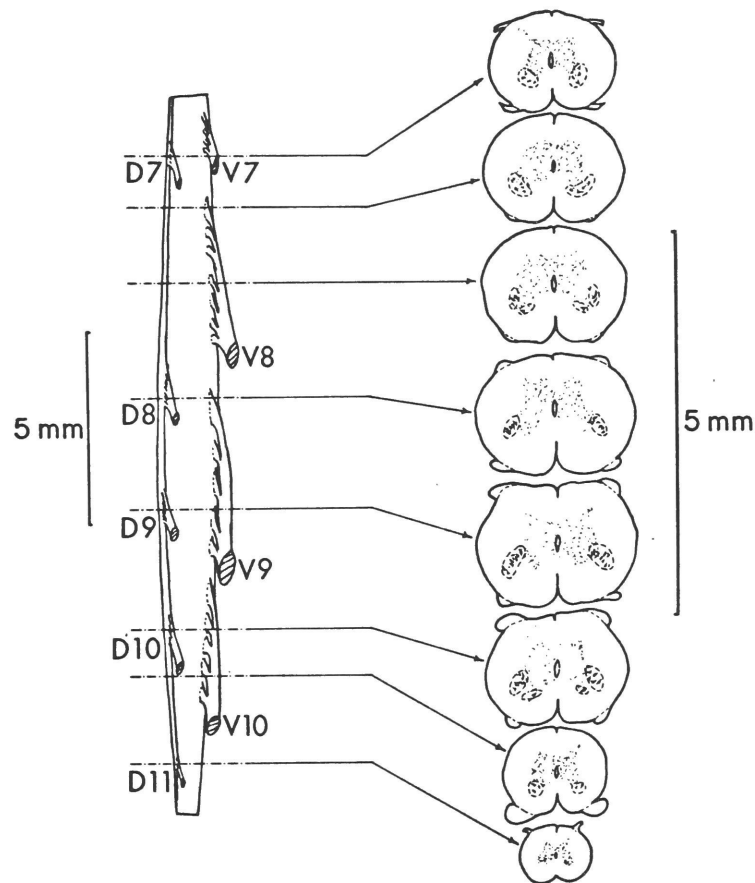


Figure 12. The lumbar enlargement of the frog spinal cord. From an animal with a "standard" or type "D" plexus. Calibration: 5 mm. The lateral view of the spinal cord, on the left, is one half the scale of the transverse sections, on the right. The transverse sections are composite tracings from several adjacent 25 μ thick sections made at the levels indicated by the arrows (ventral edge of a section is towards the bottom of the page). Motoneurons are the large cells appearing at the ventral borders of the ventral horns. The density of motoneurons in a given transverse drawing is not an accurate representation of motoneuron density on the rostro-caudal axis (see Figure 13 for this) but the location of motoneurons in a given transverse section is an accurate representation of the extent of the motoneuron column at that level of the cord. Separate motoneuron columns are indicated by dashed lines encircling the motoneurons.

horn of the frog spinal cord.

Beginning at the rostral end of the lumbar enlargement and proceeding caudalward (Figure 12), there is a small ventro-medial column of motoneurons. This column expands in a dorsal and lateral direction and then separates into two distinct columns in the middle of segment 8, a ventro-medial one and a dorso-lateral one. At the caudal edge of segment 8, the ventro-medial column has disappeared while the dorso-lateral one has continued to expand in a ventral direction. Between the middle of segment 9 and the caudal edge of segment 10, the dorso-lateral column expands markedly in a dorsal direction while its ventral portion recedes. This dorsal expansion never clearly separates into another column, even though the cells in it are somatotopically distinct from others in the dorso-lateral column. In the caudal portion of segment 10, a separate ventro-medial column reappears and runs on for a short distance as the dorso-lateral column tapers off.

When spinal roots 8, 9, and 10 serving the hindlimb were injured, a retrograde reaction was seen (Plate 3, Figure 13) in almost all cells of the dorso-lateral motoneuron column, but only a few reacting cells were seen in the ventro-medial cell column (Figure 13). Reacting cells in the ventro-medial column were probably due to the small nerve branches to the muscles of the abdomen and lower back which branch off the spinal roots distal to the level of the injury. When individual nerves to hindlimb muscles were sectioned, no reacting cells were ever seen in the ventro-medial column.

The fact that all cells of the dorso-lateral cell column did not react may suggest that some of these cells do not supply axons to the lumbar roots, but a more likely explanation is that the chromatolytic reaction is never complete in a given population of cells (Romanes, 1951).

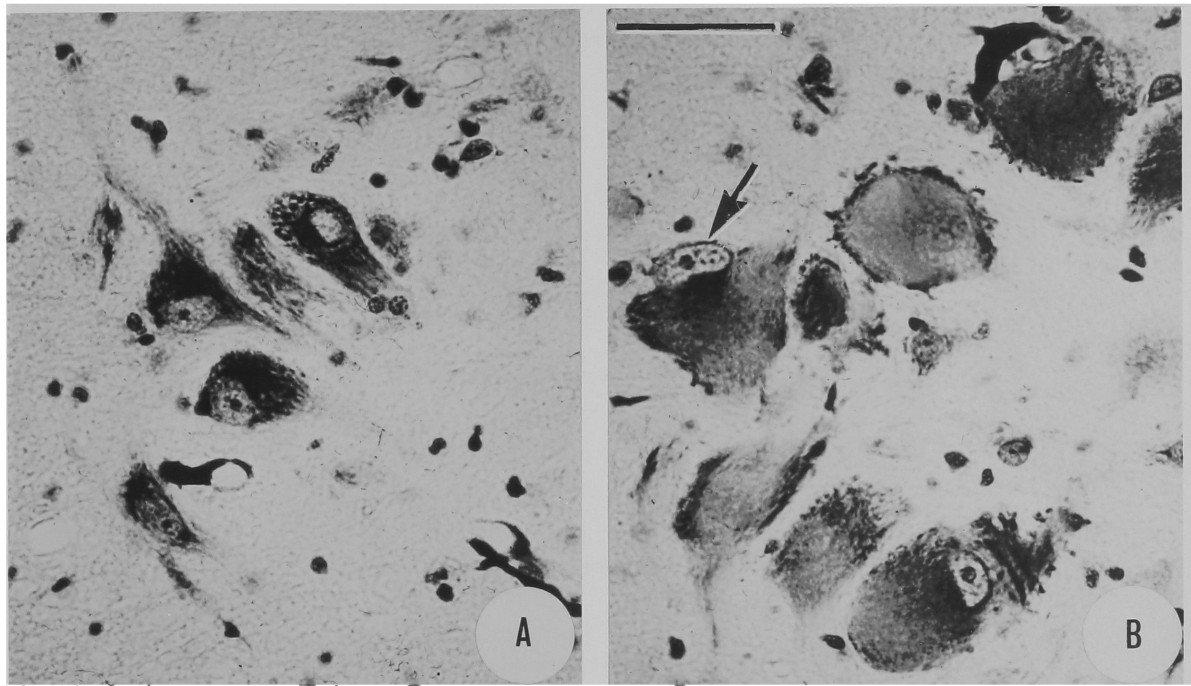


Plate 3. Maximum chromatolytic reaction. Motoneurons in lumbar spinal cord of frog. (a) Control side of spinal cord, cells are normal in appearance. (b) Experimental side of spinal cord 34 days after injury of spinal roots 8, 9, and 10, cells show retrograde reaction characterized by swelling of the soma, clearing of the Nissl substance (chromatolysis) and eccentricity of the nucleus (arrow). Calibration: 50 μ .

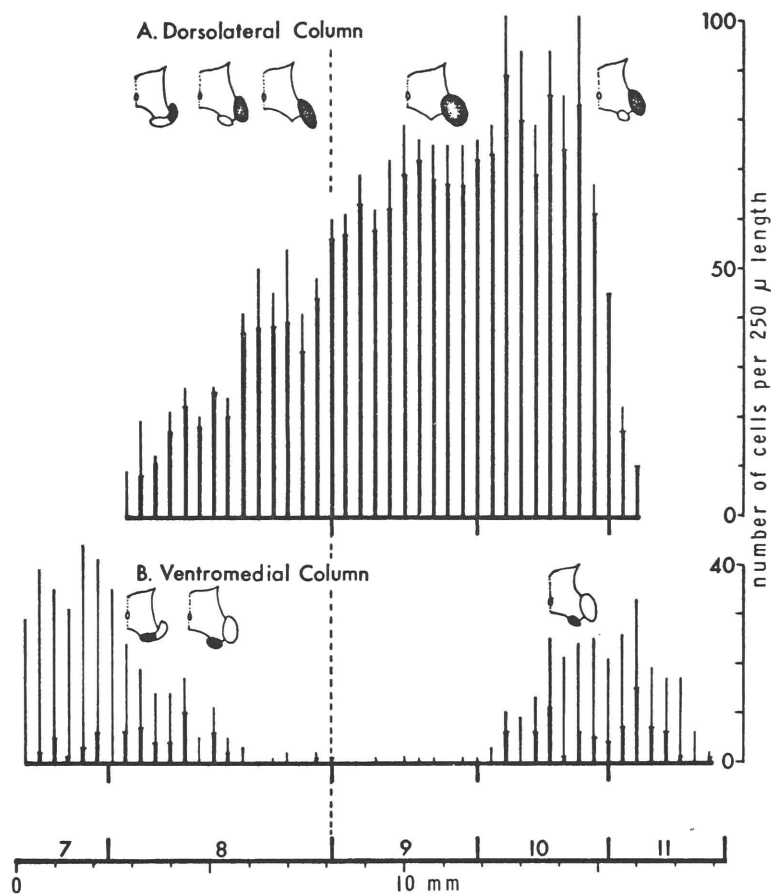


Figure 13. Lumbar motoneurons. The location of chromatolytic (heavy vertical lines) and normal (thin vertical lines) motoneurons on the experimental side of the spinal cord 34 days after injury of the lumbar spinal roots 8, 9, and 10 distal to the sympathetic ganglia. The frog had a "standard" or "D" type of plexus. Numbers on the longitudinal scale indicate segments of the spinal cord as delineated by the caudal edges of the dorsal roots. The location of the two motoneuron columns are shown on the small inset diagrams of transverse sections; the location of chromatolytic motoneurons is indicated in solid black. The transverse sections are positioned at their approximate longitudinal location.

Correction for Plexus Variation

The longitudinal location of a given motoneuron pool, as determined by chromatolysis, varies among different individual frogs, but this variation is well correlated with the variation in plexus forms. In a frog with a postfixed plexus, a given motoneuron pool is located in a more caudal position while in a frog with a prefixed plexus, a motoneuron pool is located more rostrally (Figure 14). Not only is a given motoneuron pool shifted longitudinally, but the entire dorso-lateral cell column is shifted in position according to the type of plexus (heavy baselines in Figure 14). The dorso-lateral cell column was displaced as much as one-half segment up or down in the case of prefixed or postfixed plexuses, respectively.

After plexus variation was taken into account in mapping, there was still some variation in the number of chromatolytic motoneurons resulting from the same nerve injury in different frogs (Figure 15) and in some cases there was no distinct reaction at all. The causes of this variation are not truly known, but it also occurs in the chromatolytic reaction in cats (Romanes, 1951). When there was a distinct reaction, regardless of the exact number of motoneurons which reacted, the pool of motoneurons always had its densest portion in the same location (Figure 15).

Maps of chromatolytic motoneurons from different frogs were converted to a standard size and position by adjusting the dorso-lateral cell column to match the column in a frog with a "standard" or "D" type of plexus (see Methods). This method resulted in reproducible maps of motoneuron locations, regardless of plexus variation. The uncorrected maps of ankle motoneurons shown in Figure 14 can be compared with the corrected maps in Figure 15.

As was found with the plexuses themselves, sometimes one end of a column had a greater shift in fixation than the other end. For example in Figure 14, case 1509R has a plexus which is postfixed on the caudal side, but not on the rostral side. Its dorso-lateral column

shows a similar shift compared to the column from the "standard" or "D" type plexus in case 1332 pictured just above, that is the caudal end is shifted further caudally, or is postfixed, but the rostral end is about normal. The extension of one end of the column in case 1509R resulted in the whole column being longer than normal, but the projection method, as well as compensating for longitudinal shifts in the whole column, compensated for different column lengths. Thus the projection method of transforming all maps onto a "standard" map was able to compensate for plexuses with aberrant fixation such as type "F" as well as for plexuses with simple pre- or post-fixation.

Motoneuron Location

The locations of motoneurons innervating various hindlimb muscles, as determined by chromatolysis and corrected for plexus variation, are shown in Figures 15 - 18. Motoneuron locations along the longitudinal axis of the spinal cord are mapped as histograms of the density of chromatolytic cells per 250 μ . Their locations in the transverse plane are mapped on drawings of the ventral horn cell columns as blackened areas. There is considerable intermingling of the motoneurons to different muscles; motoneurons for a single muscle are not organized into a compact nucleus.

Even though the motoneurons are not organized into discrete nuclei, it is still possible to detect organization in their arrangement. The motoneuron maps in Figures 15-18 are presented according to a functional scheme, that is the joint which the innervated muscle acts upon (pelvis, knee, or ankle-toes) and the specific action on that joint (flexion or extension). The means of determining the function of a specific muscle are given in the Appendix.

The organization of the motoneurons can also be considered according to a somatotopic scheme, that is according to the location of the muscle innervated in the limb. Both functional and somatotopic analyses will be presented in the discussion.

Confirmation by Physiological Mapping

During the course of the physiological experiments, the locations of motoneurons studied by intracellular recording were mapped by a combination of stereotaxic and dye marking techniques (see Physiological Methods) and identified by antidromic stimulation. Although a much smaller number of motoneurons was mapped by this means, the results were in complete agreement with the results obtained by the retrograde reaction method (Figure 19). A given motoneuron in a physiological experiment was usually encountered in the dense portion of its pool as mapped by the chromatolytic method. This suggests the potential usefulness of these motoneuron maps for future physiological experiments.

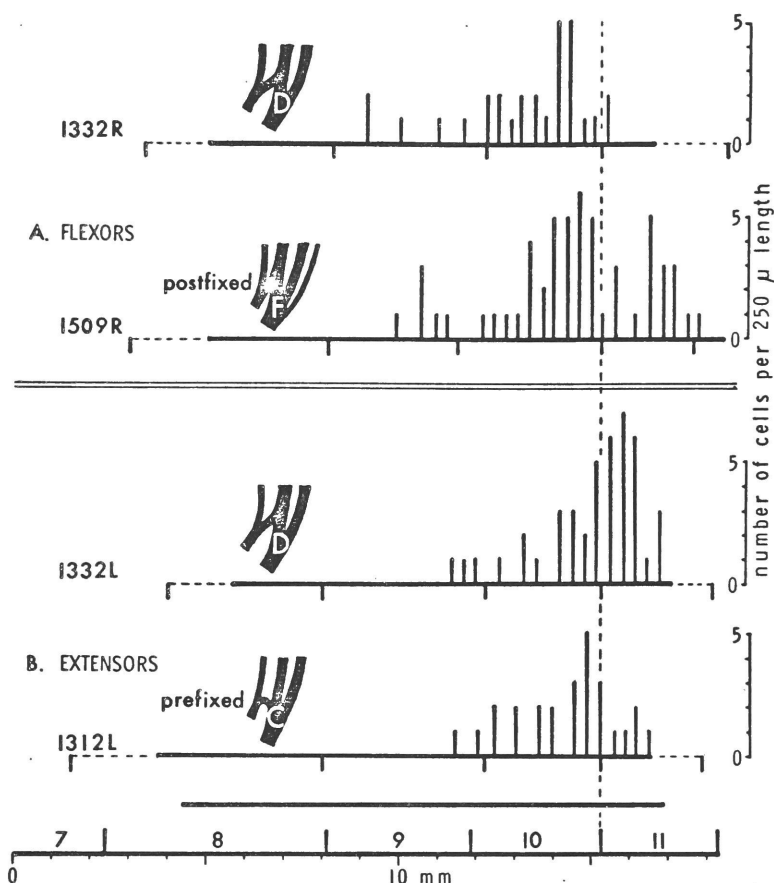


Figure 14. Ankle and toe motoneurons, uncorrected. The location of chromatolytic motoneurons resulting from injury to the nerves innervating ankle and toe muscles. Results uncorrected for plexus variation or variation in length of the dorso-lateral column. The heavy line underneath each motoneuron pool indicates the longitudinal extent of the dorso-lateral column in that frog. The heavy line at the bottom of the page indicates the length and location of the dorso-lateral column in a "standard" frog with a type "D" plexus. Frogs with different plexuses are compared to show the longitudinal shift of motoneuron pools and of the dorso-lateral column. (Motoneuron pool 1332R is from the right side of the same frog where 1332L is from the left side.)

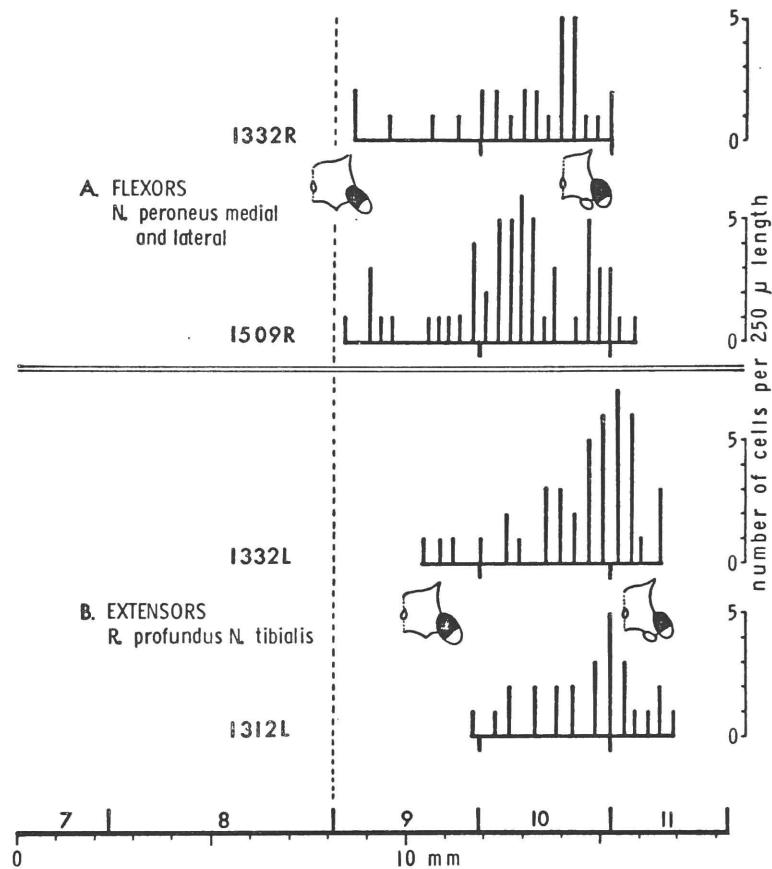


Figure 15. Ankle and toe motoneurons, corrected. The location of chromatolytic motoneurons resulting from injury to the nerves innervating ankle and toe muscles. The results are the same ones shown in Figure 14, but corrected for plexus variation and variation in length of the dorso-lateral column. Notice the reproducibility of the location of motoneuron density in different frogs. At A two unusual cases show extreme differences in the shape of the dense population of motoneurons, but in both cases the location of the density is the same, i.e. in segment 10. At B are shown two more typical cases in which the shape of the dense population as well as the location is reproduced.

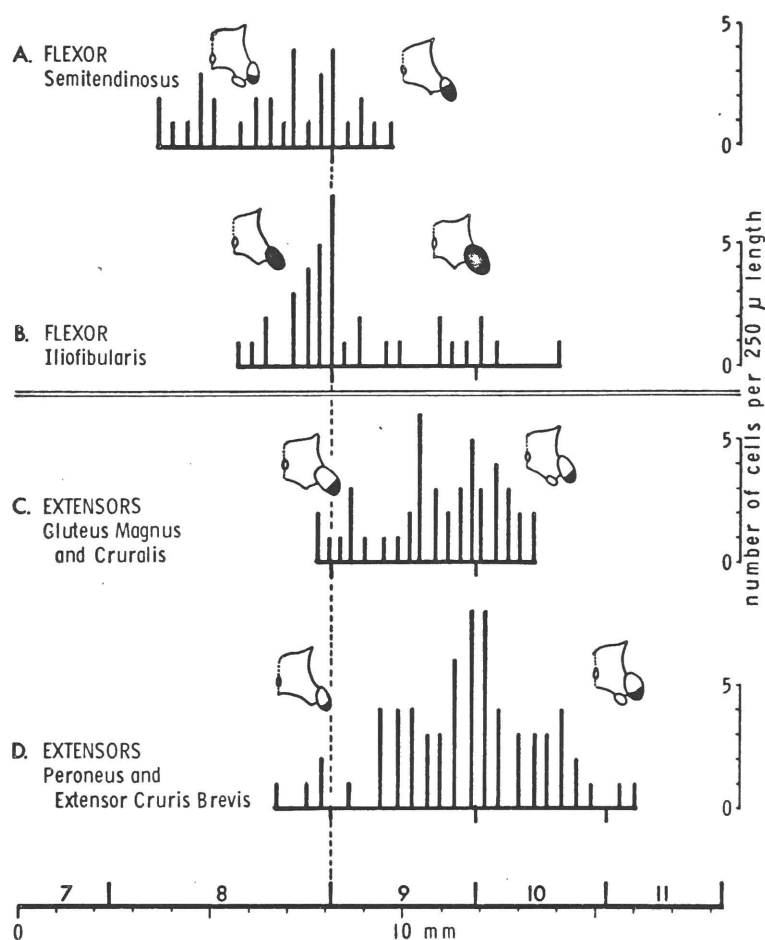


Figure 16. Knee motoneurons. The location of chromatolytic motoneurons resulting from injury to the nerves innervating muscles which act on the knee. Two of the muscles also have actions on other joints: M. semitendinosus is a weak pelvic extensor, M. iliofibularis is a weak pelvic flexor. The results are corrected for plexus variation and variation in length of the dorso-lateral column. The frogs had the following plexus forms: (a) frog #1505-R, type "C", (b) frog #1508-R, type "C", (c) frog #1319-R, type "B", (d) frog #1321-R, type "A".

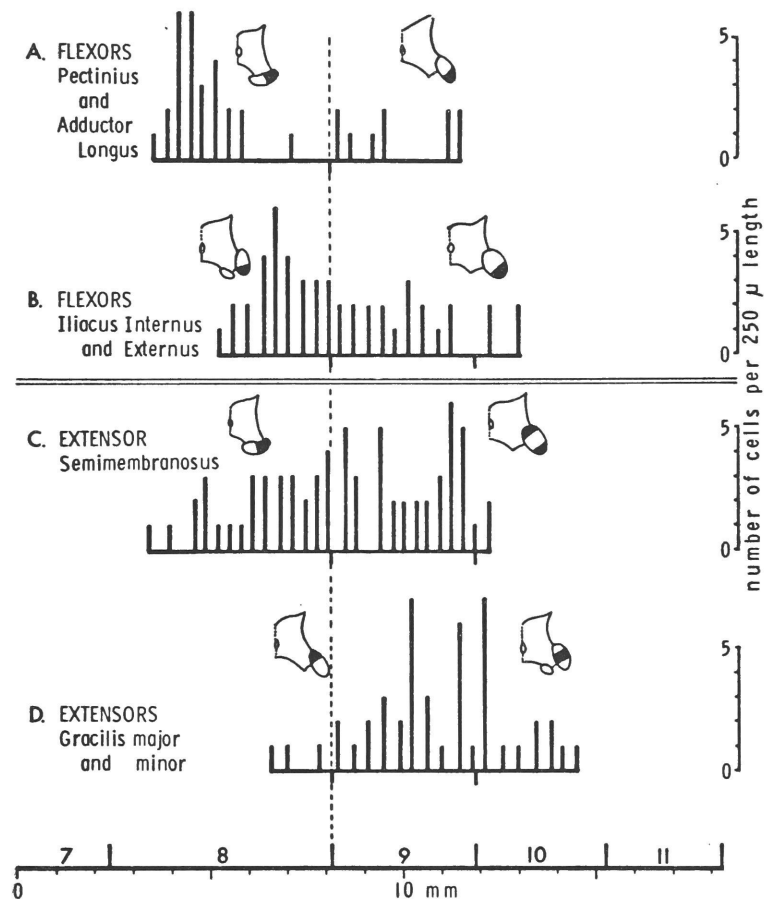


Figure 17. Pelvic motoneurons. The location of chromatolytic motoneurons resulting from injury to the nerves innervating muscles which act on the pelvis. The results are corrected for plexus variation and variation in length of the dorso-lateral column. The frogs had the following plexus forms: (a) frog #1312-R, type "C", (b) frog #1501-R, type "D", (c) frog #1507-R, type "F", (d) frog #1504-R, type "F".

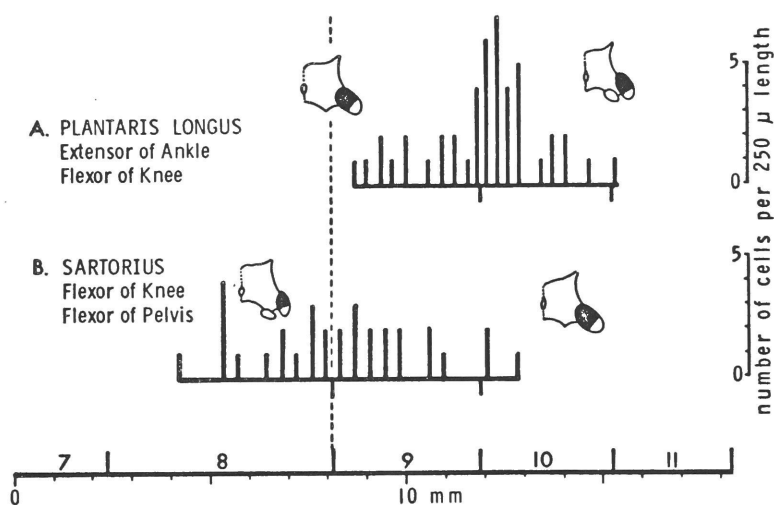


Figure 18. Dual function motoneurons. The location of chromatolytic motoneurons resulting from injury to the nerves innervating plantaris longus and sartorius, muscles with actions on two joints. Plantaris longus is a strong ankle extensor and weak knee flexor; sartorius is a strong pelvic flexor and a weak knee flexor. The results are corrected for plexus variation and variation in length of the dorso-lateral column. The frogs had the following plexus forms: (a) frog #1509-L, type "F", (b) frog #Ch-127-L, type "C".

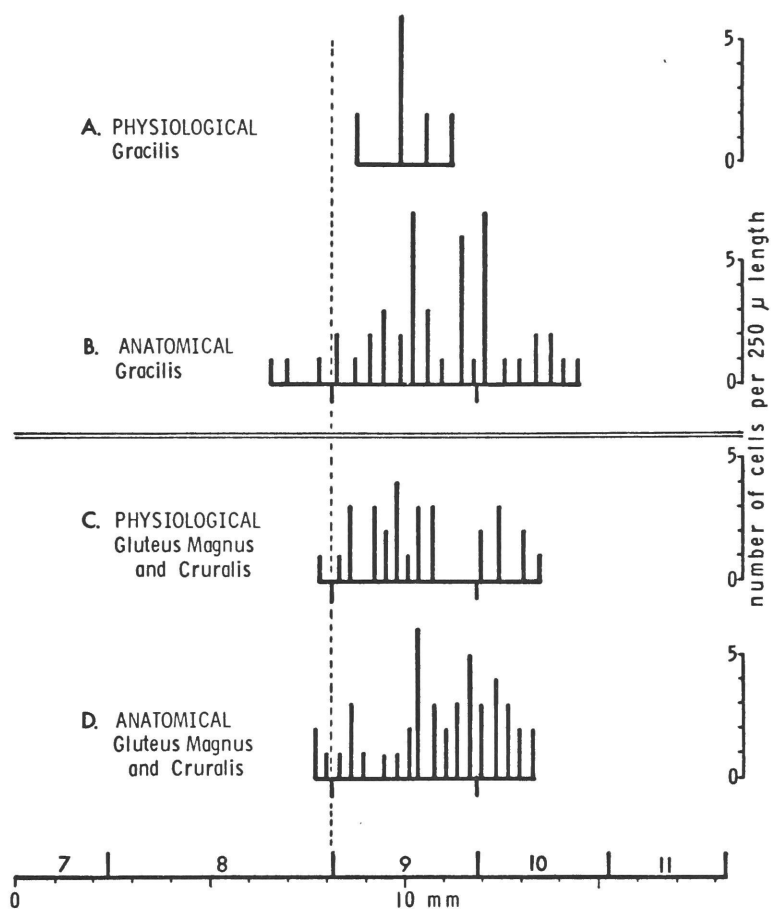


Figure 19. Physiological vs. anatomical mapping. A comparison of the locations of motoneurons for the same muscles obtained by the intracellular staining technique and by the chromatolytic method.

ANATOMICAL DISCUSSION

The association of prominent lateral cell groups in the ventral horn with the spinal roots serving the brachial and lumbar plexuses is a feature common to all limbed vertebrates (Elliott, 1942, 1944; Romanes, 1953, 1964) except urodele amphibians (salamanders), which have no prominent lateral cell groups (Strauss, 1946; Hughes, 1968). Kaiser (1891) first postulated that the lateral cell groups of the ventral horn innervate the limb musculature. Strauss (1946) and Sprague (1948) have exhaustively reviewed the early development of this concept. More recently, Romanes (1951) has demonstrated that the lateral cell groups in the cat innervate the hindlimb by observing the localization of chromatolysis following peripheral axonal injury.

The situation in anuran amphibians (frogs and toads) was first described by Brissaud and Bauer (1903) who observed chromatolysis in the lateral cell column following leg amputation. Silver (1942) and Stussi (1960) reported that only stimulation in the lateral regions of the ventral horn caused muscle twitches in anuran hindlimbs. My experiments have corroborated these findings by demonstrating that motoneurons innervating individual hindlimb muscles are localized to the lateral cell column.

There have been numerous theories concerning the organization of motoneuron location (see Strauss, 1946 and Sprague, 1948 for review) including the following: that they are organized relative to individual muscles, to individual peripheral nerves, to articular limb segments, or to functional and morphological muscle groups. Each of these theories can be supported in some degree by the experimental data which various investigators have presented, however, none of them has been satisfactorily proved.

Two theories of motoneuron organization have been predominant amongst the investigators of motoneuron location. The first is that there is a functional organization according to synergistic and

antagonistic muscles or according to muscles causing movement around one joint. The second is that there is a somatotopic organization according to the morphological location of the various muscles in the limb. I believe that there is some merit to both of these theories, since function and morphological location of a muscle are considerably interdependent.

Both the functional and somatotopic theories of motoneuron organization will be presented separately as they apply to frog motoneurons and their areas of agreement and disagreement will be discussed.

The events occurring during development which might be responsible for the functional and somatotopic organization of spinal motoneurons will be discussed. This will lead into a discussion of vertebrate motoneuron organization in general.

Functional Analysis

General Organization Motoneurons in the lumbar spinal cord of the frog have a location on the longitudinal axis which is correlated with the proximo-distal location of the joint whose movement they control. Motoneurons innervating muscles which act on the most distal joint are located most caudally in the cord; those innervating muscles which act on the most proximal joint are located most rostrally in the cord. For example, motoneurons innervating ankle and toe muscles are located primarily in segments 10 and 11 (Figure 15); motoneurons innervating the knee muscles are located primarily in segments 8, 9, and 10 (Figure 16); motoneurons innervating the pelvic muscles are located primarily in segments 8 and 9 (Figure 17). Thus there are regions of the ventral horn which are primarily involved with movement around one joint, even though overlap of adjacent regions is extensive. Within each joint control region of the spinal cord (e.g. pelvis, Figure 17) there is a finer degree of functional organization: flexor motoneurons are located in a more rostral part of the spinal cord than extensor motoneurons.

Different segments of the lumbar spinal cord also have another kind of functional significance due to overlap of adjacent joint control regions. Segment 8 contains most of the flexor motoneurons for pelvic and knee action and segment 9 contains most of the extensor motoneurons for these same joints (Figures 16 and 17). Segment 10 contains most of the motoneurons having both flexor and extensor action on the ankle and toes (Figure 15).

In the transverse plane there is also a functional separation of motoneurons. In segment 8, which contains predominantly flexors, knee flexors tend to be more dorsal in position than pelvic flexors. In segment 9, which contains predominantly extensors, pelvic extensors tend to be more dorsal than knee extensors (compare Figures 16 and 17).

Exceptions Some muscles exert action on two joints rather than one (dual-function muscles) and therefore are difficult to classify according to the general scheme. One action is usually prominent and independent of joint position while the other is usually weak and position dependent (see Appendix). For example, the knee flexors, semitendinosus and iliofibularis (Figure 16), also act on the pelvic joint where semitendinosus is a weak extensor and iliofibularis is a weak flexor. The motoneurons for these two muscles are located in the region of the spinal cord where their most prominent actions are controlled, that is the knee flexor region. They also overlap with the region controlling their weak pelvic functions. However, contrary to the general rule, the weak extensor motoneurons, semitendinosus, are rostral to the weak flexor motoneurons, iliofibularis.

Sartorius and plantaris longus illustrate the extremes of displacement which the motoneurons of dual-function muscles can have (Figure 18). Sartorius, whose prominent action is pelvic flexion and whose weak action is knee flexion, has its motoneurons located in the region where the motoneurons for both of these functions are controlled, i.e. segment 8. The location of plantaris longus motoneurons, however, is less satisfactory from a functional point of view. Their prominent

action is ankle extension, but their location is slightly rostral to (although overlapping with) the other ankle extensors (Figure 15). Plantaris longus motoneurons do not overlap with the region controlling their weaker function, knee flexion (although they do run for some distance into the knee control region (Figure 16)). This dislocation of plantaris longus motoneurons from the knee flexor region is one of the outstanding exceptions to the general functional organization of motoneurons. As will be seen below, however, it is an excellent example of somatotopic organization, since the plantaris longus motoneurons are located in the region of the cord where somatotopically related muscles, in the shank, also have their motoneurons.

Somatotopic Analysis

Longitudinal Organization The longitudinal arrangement of frog lumbar motoneurons has been discussed functionally, according to motoneuronal control of joint movement, but the motoneurons also have a somatotopic organization along this axis (Table II). Most muscles which act on the pelvis or on the knee are somatotopically close to each other in the thigh; the motoneurons for these thigh muscles are located predominantly in segments 8 and 9. (Motoneurons for shank muscles are located in segments 9 and 10; motoneurons for foot and toe muscles are located in segments 10 and 11.)

Special Examples The muscles peroneus and extensor cruris brevis (Figure 16, Table II) are exceptions functionally but not somatotopically. Although their action is on the knee, they are located in the shank; their motoneurons are located in segments 9 and 10, the somatotopic shank region.

Another shank muscle, plantaris longus (Figure 18, Table II) which has a primary action on a different joint, the ankle, has its motoneurons in almost exactly the same location as do peroneus and extensor cruris brevis. Its somatotopic location in the leg is matched by an appropriate location of its motoneurons in the somatotopic shank region of the spinal cord, segments 9 and 10.

Table II
Motoneuron Organization

Muscle Innervated	Muscle Function	Muscle Location	Motoneuron Location	
			Column	Segment
Iliac. I. & E.	Pelvic	Deep Proximal	V	8
Pect. & A.L.	Flex.	Thigh	V	8
GM. & Cru.	Knee	Lateral Thigh	V	9
Per. & ECB.	Ext.	& Shank	V	9/10
Smtnd.	Knee	Midline-Distal	V-M	8
Iliofib.	Flex.	Thigh	VMD	8
Sartor.			D	8/9
Smb.	Pelvic	Medial	V-D	9
Grac.	Ext.	Thigh	D-M	9
Plant. Long.	Ankle Ext.	Medial Shank	D	9/10
N. Pero. M. & L.	Ankle, Toe Flex.	Lateral Shank & Dorsal Foot	D	10
N. Tib. R. Sup.	Ankle, Toe Ext.	Medial Shank & Plantar Foot	D	10/11

Transverse Organization In the transverse plane of the spinal cord there is also a somatotopic arrangement of motoneurons. The single dorso-lateral group of motoneurons can be subdivided, on somatotopic grounds into two groups, a ventral one innervating proximal and medial muscles and a dorsal one innervating lateral and distal muscles. In segments 10 and 11 it is possible to describe a third more dorsal and medial group containing the motoneurons which innervate the distal muscles of the foot (Plates 4 and 5).

Three Dimensional Organization If the transverse and longitudinal somatotopic arrangement of motoneurons are combined, a picture emerges of three columns of cells (ventral, dorsal, and caudo-dorsal) which innervate somatotopically different groups of muscles. Each different somatotopic group of muscles also forms a functional unit. The somatotopic columnar organization of motoneurons is summarized in Table II.

In segment 8, motoneurons in the ventral column innervate the deep proximal muscles of the thigh (pelvic flexors). In segments 9 and 10, motoneurons in the ventral column innervate the muscles on the lateral surface of the thigh and on the lateral surface of the shank (knee extensors).

In segment 8, motoneurons which lie in the medio-dorsal column innervate muscles in the midline and distal thigh (knee flexors). In segment 9, motoneurons which lie in the dorsal column innervate muscles on the medial surface of the thigh (pelvic extensors). In segments 9 and 10, motoneurons in the dorsal column innervate the muscle on the medial surface of the shank (ankle extensor).

In segment 10, motoneurons in the most dorsal column innervate the lateral shank and dorsal foot muscles (ankle and toe flexors). In segments 10 and 11 the motoneurons in the most dorsal column innervate the medial shank and plantar foot muscles (ankle and toe extensors).

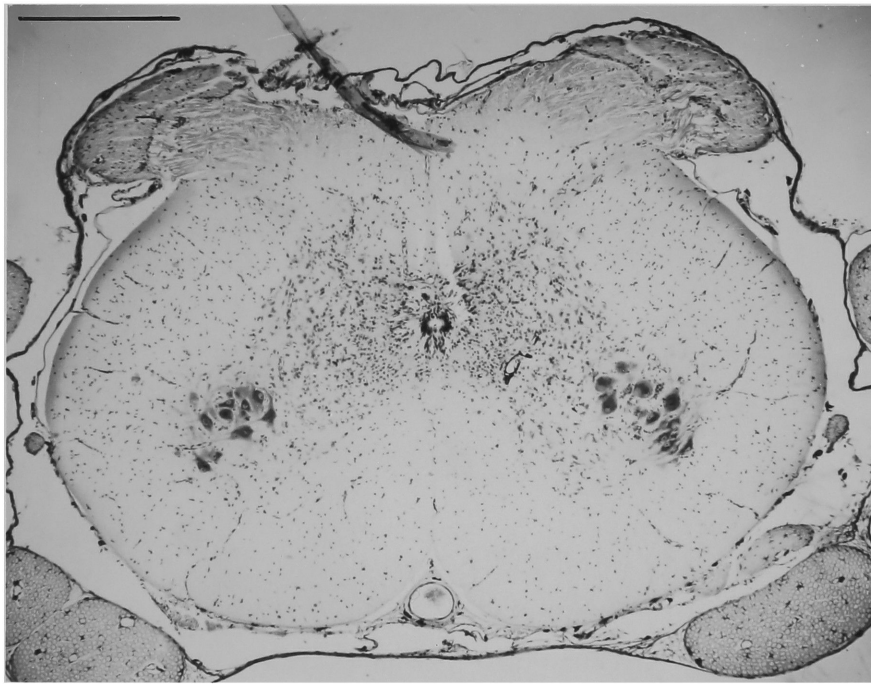


Plate 4. Ankle and toe motoneurons, low-power. On right side of spinal cord, nerves to ankle and toe motoneurons were injured 34 days before fixation. Notice reacting cells in dorsal part of lateral column. Calibration: 500 μ .

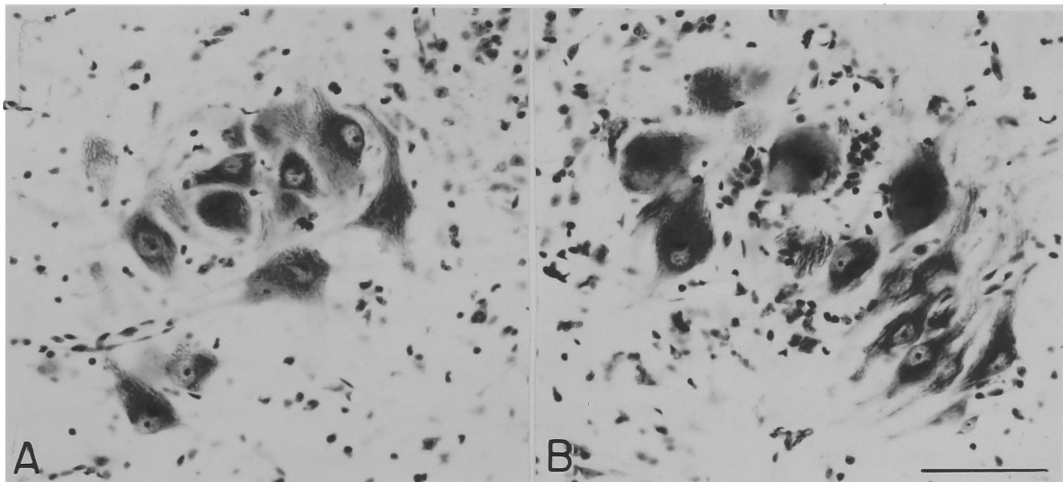


Plate 5. Ankle and toe motoneurons, high-power. High-power view of the spinal cord in Plate 4, showing (A) left side or control and (B) right side or experimental at 34 days. Calibration: 100 μ .

Motoneuron Development

Certain events which occur during development are probably responsible for the orderly somatotopic and functional arrangement of motoneuron location.

Differentiating Phase Motoneurons differentiate and take up anatomical positions in a sequence which parallels the orderly growth of motor fibers into the developing limb bud (for a general review of this concept see Strauss, 1946). Cells which develop from the lateral and the ventrolateral sectors of the early neural tube migrate into position in the lateral and medial regions of the ventral horn, respectively, where they differentiate into motoneurons (Watterson, 1965). This differentiation occurs first in the more rostral segments of the spinal cord and proceeds caudally; it also occurs earlier in the most medial and ventral regions of the ventral horn and proceeds laterally, then dorsally (Angulo y Gonzales, 1940). Motor nerve fibers from the spinal cord first grow into regions of axial musculature, then into proximal limb regions and finally into distal limb regions; shortly after the limb is completely innervated, motor activity appears in the same proximo-distal sequence (Romanes, 1941).

The parallel order of motoneuron differentiation and muscle innervation in development is thought to account for the somatotopic arrangement of motoneurons in adult vertebrates (Hughes, 1968). According to this theory, motoneurons in the medial ventral horn differentiate first and their fibers innervate the first region of the body reached by outgrowing nerve fibers, the axial muscles. (In the adult the medial column of motoneurons innervates the axial muscles.) Lateral motoneurons differentiate next and their fibers innervate the limb muscles. Lateral motoneurons in the rostro-ventral region differentiate earlier and their fibers reach proximal limb muscles; in the caudo-dorsal region they differentiate later as their nerve fibers are innervating the distal muscles. (In the

adult, the rostro-ventral motoneurons innervate the proximal limb muscles and the caudo-dorsal motoneurons innervate the distal limb muscles.)

In the frog, the hindlimb nerve growth sequence has been partly described (Taylor, 1943; Hughes, 1965) and may be compared with the motoneuron organization which I found in the adult. The first nerve branches to appear in the hindlimb are those of the crural nerve innervating the deep and proximal thigh muscles. This is followed by the appearance of the ramus profundus anterior of the sciatic nerve innervating the muscles on the lateral surface of the thigh. I found that (Table II), in the adult frog, motoneurons innervating the deep-proximal thigh muscles lie in the ventral cell column of the 8th segment; lateral thigh motoneurons lie in the ventral cell column of the 9th segment. Thus, as will be further explained below, in the adult, the motoneurons are in an anatomical order in the spinal cord which matches the developmental order of motoneuron differentiation and of nerve growth into the hindlimb.

It is not yet known whether nerves form synapses with the muscles in the same sequence as they grow into the hindlimb. However, if this is assumed along with certain assumptions about the sequence of motoneuron development (that ventral cells differentiate first in a rostro-caudal sequence, followed by more dorsal cells; that these cells send fibers into the hindlimb in the order in which the cells differentiate) then, based on the adult motoneuron organization, it may be predicted what sequence of hindlimb nerve growth would be observed if the work of Taylor and Hughes were to be extended to cover the entire hindlimb.

The muscles in Table II are arranged in such a hypothetical order, the lower the number the earlier the innervation. It would be interesting to know if the muscles are actually innervated in this order, but no one has yet studied the exact order of innervation of individual hindlimb muscles.

Degenerating Phase Hughes (1965, 1968) reported that as the innervation of the limb bud in anuran embryos was being completed there occurred simultaneously a decrease in the number of fibers entering the limb and a decrease in the number of motoneurons in the ventral horn of the spinal cord. Little is known about what goes on during this phase, but Hughes suggested that after an initial over-growth during differentiation there followed this phase of degeneration of cells and fibers which had not made the appropriate functional connections.

During this degenerating phase many alterations in the pattern of motoneuron organization could take place. For instance, the basic developmental sequence of connections, which would be reflected in a somatotopic organization of motoneurons, could be altered in favor of a functional organization, grouping together those motoneurons with similar actions.

The present experiments suggest that most of the functional organization seen in the location of frog motoneurons is a consequence of the somatotopic organization and therefore of the initial pattern laid down in development. However, the very complex functional organization seen in the mammalian spinal cord, for example of the cat (Romanes, 1951), is more difficult to explain simply on somatotopic and developmental grounds. Perhaps in the cat there is, during development, a more extensive rearrangement of hindlimb innervation patterns along functional lines during the degenerating phase of motoneuron and fiber development. A comparison between the frog and the cat of the spinal motoneuron organization, to be discussed in the next section, indicates that this might be the case.

Vertebrate Motoneuron Organization

Longitudinal In the present experiments it was found that frog lumbar motoneurons have a longitudinal functional arrangement where flexor motoneurons are rostral to extensors and motoneurons controlling proximal joint function are rostral to those controlling distal joints. These results are in agreement with Sherrington's (1892) findings from stimulation of frog spinal roots and with Silver's (1942) and Stussi's (1960) findings from stimulation of the frog spinal cord. I have suggested that the apparent functional organization of frog motoneurons is a consequence of somatotopic patterns laid down during development.

In the cat lumbo-sacral cord, some flexor motoneurons are rostral to extensors (Romanes, 1951) just as in the frog, but cat knee motoneurons are organized quite the reverse of frog knee motoneurons, that is extensors are rostral to flexors. Furthermore, the arrangement of cat knee motoneurons does not fit the longitudinal somatotopic developmental scheme where proximal limb muscles are innervated by rostral motoneurons and distal limb muscles are innervated by caudal motoneurons. Rather a functional organization seems to have intruded. This functional arrangement could have been superimposed on the somatotopic arrangement during the degenerating phase of motoneuron development.

Transverse In the transverse plane in the frog spinal cord there is a tendency for knee and pelvic motoneurons to lie in separate parts of the lateral column, but not for flexor and extensor motoneurons to be separated. For example in segment 8, knee flexor motoneurons are somewhat dorsal to pelvic flexor motoneurons, but in segment 9, the extensor region, it is the pelvic motoneurons which are dorsal of the knee ones. I have suggested that this transverse organization in the frog is, like the longitudinal organization, a consequence of somatotopic patterns laid down during development.

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In the transverse plane of the cat spinal cord there is a quite different and more complex arrangement of motoneurons, both somatotopically and functionally. Motoneurons for proximal muscles are located ventrally; those for distal muscles, dorsally. Motoneurons for flexors are located medially; those for extensors, laterally. Once again, in the cat functional and somatotopic arrangements do not coincide, possibly due to extensive reorganization along functional lines during the degenerating phase of motoneuron development.

Three Dimensional In the cat, somatotopically or functionally related motoneurons are grouped together in long columns, whereas in the frog they are grouped together in short columns within a segment. This difference in organization may be related to the fact that the dendrites of cat motoneurons are quite extensive in a longitudinal direction within the columns of motoneurons (Sterling and Kuypers, 1967b) whereas frog motoneurons have very extensive dendrites in a radial direction within the segment (Silver, 1942; Kennard, 1959, Nieuwenhuys, 1964). The dendrites of frog motoneurons have not been specifically studied with regard to their longitudinal extent, but they are known to extend radially to a much greater degree than do those of cat motoneurons.

Dendritic Frog motoneurons send dendrites into the lateral margin of the spinal cord where they form an extensive plexus (Silver, 1942). Silver (1942) found that by stimulation of this plexus he could elicit, at very low threshold, contractions of discrete muscles in the hindlimb. This suggests that there may be a functional organization within the dendrites of the lateral plexus. It is not yet known to what extent the radial organization of frog motoneuron dendrites is restricted to segmental functional groups, but in the cat the longitudinally oriented dendrites appear to course within a column among motoneurons which are functionally related (Sterling and Kuypers, 1967b).

Little is truly known about the importance of dendrites in motoneuron function except that they form a much larger postsynaptic surface than do the cell bodies (Romanes, 1964). Romanes (1964) has suggested that it may be of more importance functionally to consider the location and relationship of the dendrites than of the cell bodies of motoneurons. More detailed comparisons between frog and cat motoneuron dendrites might provide some clue as to the functional importance of dendrites.

Phylogenetic It is tempting to postulate that there is a phylogenetic shift of motoneuron dendrites from a radial orientation in the most primitive tetrapods, frogs, to a longitudinal orientation in higher tetrapods, like cats. Such a shift might account for the fact that a frog has only one lateral column of motoneurons, whereas a cat has seven. The organization of motor function, and of dendrites, over several segments may have caused the break-up of the lateral motoneuron group into a greater number of columns in the higher vertebrates.

Most tetrapod vertebrates have their motoneurons organized into columns in the ventral horn and the number of columns present increases progressively from anuran amphibians (frogs and toads) to reptiles to birds to mammals (Romanes, 1953). However, one tetrapod group, the urodele amphibians (salamanders) have no distinct columnar organization of their motoneurons nor even a prominent ventral horn (Strauss, 1946; Hughes, 1968) and they, therefore, more closely resemble fishes than they do anuran amphibians. Urodeles are phylogenetically more recent than anurans but there are several characteristics of their nervous systems which are more primitive, probably as the result of specialized development (Strauss, 1946).

Even the functional organization of urodele motoneurons seems more primitive than that of anurans. Szekely and Czeh (1967) used stimulating electrodes in the brachial spinal cord of the Axolotl to map the functional location of motoneurons innervating the forelimbs.

They found that motoneurons with functional similarity were grouped in small "islands" scattered over several segments. This is quite different from the situation in the frog where motoneurons with functional similarity tend to be grouped in one segment and to some extent even in one part of the column. Thus frogs appear to be the most primitive tetrapod showing some of the columnar organization of spinal motoneurons which is characteristic of higher vertebrates.

It is tempting to speculate that the columnar motoneuron organization found in the anuran spinal cord accounts for the general faster and more precise movements which anurans can make as compared to urodeles. This in turn gives anurans the motor apparatus for more aggressive food seeking behavior than urodeles. And it may account for the fact that some anurans have adapted to an essentially aerial habitat; climbing trees and gliding from them to the earth requires considerably more muscular dexterity than simply crawling across the land.

The columnar organization of motoneurons in the frog may represent the beginning of an evolutionary trend which has allowed each class of vertebrates to develop greater degrees of muscular coordination and control. There are of course many parts of the nervous system which have evolved in parallel with the motoneuron organization and which have an important part in motor activity, e.g. supraspinal fiber pathways and dendritic orientation.

Much more needs to be known about the organization of motor systems in vertebrates intermediate between amphibians and mammals in order to test this hypothesis about columnar organization. Furthermore some quantitative method of analyzing the complexity of muscular control should be applied to studies of different vertebrate classes. In addition it might be useful to study motor organization and control in different species within one vertebrate class which has shown extremes of adaptive radiation for motor activity, e.g. the amphibians or reptiles. Such a study might provide information as to the limits and capabilities of a particular kind of motor system.

APPENDIX

Nomenclature

Confusion has arisen in the literature concerning nomenclature for frog anatomy. Ecker's original Anatomie des Frosches (1864) was published in German and translated into English by Haslam (1889). Ecker and Weidersheim (1881) revised and expanded the earlier (1864) work and this edition was then further revised by Gaupp (1896-1904). The two editions most often available are the Haslam translation of the early Ecker and the Gaupp revision of Ecker and Weidersheim. These two editions of Anatomie des Frosches use different nomenclature in several places and are the source of the confusion.

In this thesis the nomenclature of Gaupp (1896-1904) is used because that is the most recent and the most comprehensive anatomy of the frog. There are only a few items where this makes any difference. Haslam's Ecker numbered the spinal roots in the adult from 1 to 10. Gaupp's Ecker and Weidersheim observed that the first embryonic spinal root disappeared in the adult but the embryonic numbering system was retained. This resulted in numbering the adult roots from 2 to 11. Hindlimb muscles which are of interest in this thesis and which have conflicting nomenclatures are listed in Table III. For further information the reader is referred to Dunn (1900, 1902).

Muscle Function

For this thesis it was desired to focus attention on the two most important movements of the bullfrog hindleg--the squatting posture and the explosive jump (or swim-kick). This limitation of the analysis of muscle function made it much easier to compare various hindlimb muscles according to function and also seemed justified in view of the normal activity of the limb. Gaupp's (1899) descriptions of frog muscle function do not provide sufficient information to relate the muscle functions to kicking or squatting. Therefore it was necessary to obtain new information on the function of frog hindlimb muscles.

Table III

Differences in hindlimb muscle nomenclature of
Haslam (1889) and Gaupp (1896)

Names of Muscles Haslam (1889) translation of Ecker (1864)	Names of Muscles Gaupp (1896) revision of Ecker & Weidersheim (1881)
Triceps femoris	Triceps femoris
Vastus internus	Caput anticum seu cruralis
Rectus femoris anticus	Caput medium s. Tensor fasciae latae
Vastus externus	Caput posticum s. Gluteus Magnus
Rectus internus major	Gracilis major
Rectus internus minor	Gracilis minor
Biceps	Ileo-fibularis
Ileo-psoas	Iliacus internus
Gluteus	Iliacus externus
Gastrocnemius	Plantaris longus

There are three recognized methods of determining muscle function (Beever, 1904). The first is to mechanically pluck the muscle in a dead animal and observe the movement caused. The second is to electrically stimulate a muscle in a live animal and observe the effect of the contraction. The third is to observe (visually or by electrical recording) the contraction of the muscle during an actual movement.

The third, or physiological, method is more accurate than the first two, or anatomical, methods because it reports the actual function of a muscle in concert with other muscles rather than its potential function as determined in isolation. The physiological method is ideal for the analysis of a complex movement such as made by a newt forelimb in walking (Szekely et al., 1969). However, in the case of the bullfrog, fine adjustments of posture on the ground or accurate movements using the hindleg are not normally important (Dickerson, 1906). Even the celebrated bullfrog jumping accuracy is largely dependent on the orientation given by the forelimbs (Gray, 1968). Thus an anatomical analysis appeared justified.

Experiments were conducted using a modified type "two" anatomical method to determine the most important function of each of the major muscles in the hindlimb of the frog. Only movements in the horizontal plane were studied (with the frog lying on its belly). Flexion was defined as movement around a joint which resulted in that joint assuming the same folded position which it assumed when the frog's hindleg was in its normal squatting posture--that is folded up against the ventrolateral surface of the frog's body. Extension was defined as movement around a joint which resulted in that joint assuming the same position which it assumed when the frog's hindleg was employed in jumping or swimming--that is fully opened in a straight line pointing backwards from the frog's body.

A frog was pithed, its hindleg skinned, and the muscle nerves exposed. A small bipolar electrode was used to apply electrical stimuli to the nerves. After threshold for muscle twitch was determined for each nerve using a 0.1 msec pulse, the pulses were applied at a rate of 50 per second (creating a tetanus) and the resulting joint movement was observed. In cases where there was the possibility of current spread to more than one nerve appropriate sections of surrounding nerves were made to eliminate this possibility.

Many muscles had relatively simple action:

1) Flexion of thigh at pelvic joint: pectini^{us}, adductor longus, ili^{us} internus, ili^{us} externus, tensor fascia latae.

2) Extension of thigh at pelvic joint: semimembranosus, gracilis major, gracilis minor, adductor magnus, quadratus femorus, obturator externus.

3) Extension of shank at knee joint: gluteus magnus, cruralis, peroneus, extensor cruris brevis.

4) Flexion of foot on ankle joint: tibialis anticus longus, tibialis anticus brevis (all muscles served by the common peroneal nerve, medial and lateral divisions).

5) Extension of foot on ankle joint: tibialis posticus (served by R. profundus of tibial nerve).

Toe movements are complex but may be simply described according to whether these movements appear when the rest of the leg is flexing or extending:

1) Plantarflexion (curling of toes towards ventral surface of foot and relaxation of web between toes): muscles served by Ramus superficialis and R. profundus of tibial nerve below branches to the muscle tibialis posticus.

2) Dorsiflexion (curling of toes towards dorsal surface of foot and spread of web between toes): muscles served by medial and lateral branches of common peroneal nerve below branches to the shank muscles.

Some muscles displayed actions on two joints, but in each case the action on one joint was simple and "strong." By the term "strong" is meant that under tetanus of the muscle it was difficult to move that joint in opposition to the muscle:

1) Semitendinosus: strong flexion of shank on knee. Also weak extension of thigh to 90° angle on pelvis when thigh was initially in fully flexed position.

2) Iliofibularis: strong flexion of shank on knee. Also weak flexion of thigh to 90° angle on pelvis when thigh was initially in fully extended position.

3) Sartorius: strong flexion of thigh on pelvis. Also weak flexion of shank to 90° angle on knee when it was fully extended.

4) Plantaris longus (Gaupp, 1899, also called Gastrocnemius by Haslam, 1889): strong extension of foot on ankle. Also weak movement of shank to 90° angle on knee whether from initial position of full flexion or full extension.

Preoperative Animal Care

Female bullfrogs, Rana catesbiana, were shipped by air from Jacques Weil in Rayne, Louisiana. Since frogs were obtained in all seasons of the year, uniform metabolic activity was insured by special storage procedures. Newly arrived frogs were kept for two weeks to six months in tanks of fresh tap water in a room where the air temperature was maintained at $15-20^{\circ}$ C. Since bullfrogs are cannibals both in the wild and in captivity (Dickerson, 1906), they were easily fed by putting small leopard frogs, Rana pipiens, into the tanks once a week.

The period of adaptation in the $15-20^{\circ}$ C animal room also served as a period for treating or removing unhealthy animals. Soon after arrival, bullfrogs were rinsed in a dilute cleansing solution (PhisoHex, Winthrop) and this rinse was repeated once on each of the next 3 or 4 days. This prevented the development or spread of "red-leg," a skin disease in amphibians, and sometimes cured individuals afflicted with

a mild case of the disease. Animals which still showed red-leg symptoms after several days of phiso hex rinse as well as any animals which displayed sluggish activity were separated from the pool of experimental animals and destroyed.

Histological Procedure

The animal was perfused through the heart with Ringer's solution until all the blood was washed out. This was followed by perfusion with fixative until all of the skin was perfused and the legs became stiff. The cord was then removed from the animal and stored in fixative for 24 hours. At first Bouin's fixative was used but in later experiments it was found that buffered glutaraldehyde gave better fixation:

Bouins

85 cc picric acid saturated
12 cc formalin
3 cc acetic acid

Glutaraldehyde

dilute to 2% with 0.05 M cacodylate buffer (pH 7.2)
use at 4°C

Cacodylate Buffer (0.05 M) at pH 7.2

25 cc sodium cacodylate (0.02 M)
2.1 cc hydrochloric acid (0.02 M)
72.9 cc water

Spinal cords from electrophysiological experiments were dehydrated and rehydrated with acetone rather than the usual ethyl alcohol because the methyl blue dye used for intracellular staining was very soluble in ethyl alcohol.

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