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THE CONTROL OF LOCOMOTION IN THE MUDPUPPY (Necturus maculatus)

BY

Blaise Matt Wheatley

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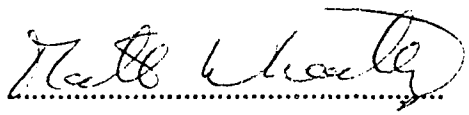

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"THE CONTROL OF LOCOMOTION IN THE MUDPUPPY (Necturus maculatus)"

SUBMITTED BY BLAISE MATT WHEATLEY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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This thesis is dedicated to my parents and to Diane, for devotion, love and support throughout.

"What does not destroy me, makes me stronger"

Friedrich Nietzsche

ABSTRACT

We have developed an in-vitro preparation for the investigation of control mechanisms of locomotion. The mudpuppy (Necturus maculatus) serves as the model system for this study. The mudpuppy is an aquatic amphibian which walks with an alternating quadrupedal gait. The in-vitro preparation consists of the first five segments of the spinal cord and the attached forelimb. N-methyl D-aspartate (NMDA) and magnesium are required for the burst pattern to emerge in the in-vitro preparation and glycine (or D-serine) potentiates the NMDA induced locomotion. The locomotor patterns in the intact animal were compared with those in the in-vitro preparation. Both patterns are similar with the exception that the Brachialis muscle in the intact animal can exhibit a double burst which is not seen in-vitro. We have also developed techniques which enabled us to record from rhythmically active interneurons during locomotion and developed criteria for identifying these interneurons. Included in these criteria are the phase of activity of the interneuron within the step cycle, the spike triggered average response of locomotor EMG, the dorso-ventral location of interneurons and the input to these interneurons from some of the afferent systems which impinge on the spinal cord. Using these criteria we have partially characterized five classes of interneurons. These include flexor (F), extensor (E), flexor→extensor (F→E), extensor→flexor (E→F), and tonic interneurons. In addition we have found that the majority of interneurons are "transitional interneurons" (F→E, E→F), whose phase of firing spans the transitional zone between swing and stance and between stance and swing respectively.

Preliminary experiments were conducted on reflex modulation in both the intact and in-vitro preparations. Electromyographic records show that the reflex responses in the intact and in-vitro preparations are modulated but that there are some differences in the control of this modulation in-vitro. In summary, the in-vitro mudpuppy preparation has

some advantages over existing in-vitro preparations used for the investigation of locomotion and should prove useful in elucidating some of the control mechanisms of locomotion in limbed vertebrates.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

As animals evolved it became increasingly important for them to develop appropriate locomotor mechanisms. Improved locomotion provided the animal with an increased range over which to gather food and allowed the animal to seek better habitats, increasing the chance of survival. In addition it provided predators with more efficient means of catching prey and prey with an increased chance of eluding predators. In order for these obvious evolutionary advantages to be realized the locomotor mechanisms of each animal had to be adapted to the animal's specific needs and environment. Vertebrates populate almost every type of habitat the earth offers. As a result, vertebrates exhibit many forms of locomotion, ranging from undulatory swimming in the phylogenetically primitive lamprey to bipedal walking in the phylogenetically advanced human. In spite of this spectrum of locomotor forms some of the strategies for neural control are proving to be similar throughout the phylum (Grillner and Dubuc, 1988).

1.2 Amphibian Locomotion

Amphibians are subdivided into three orders; apoda, urodela and anura. Apodia are limbless amphibians with snake-like bodies. Urodeles are tetrapods with long slender bodies and tails and include all families of newts, salamanders, and mudpuppies. Anura (frogs) are also tetrapods however they are characterized by having short bodies and well developed hindlimbs as adults. (For a more complete discussion of amphibian taxonomy

see Porter, 1972.) Amphibian locomotion can be subdivided into two general forms; axial swimming, characterized by lateral undulations used to initiate a propulsive wave over the length of the body (Gray, 1968) and limbed swimming/walking, characterized by the use of limbs used to propel the animal through the water or over land. The latter of these two forms of locomotion can be further subdivided into **in-phase** locomotion, when the movement of the left and right limbs are synchronized to provide the propulsive force and **out-of-phase** locomotion, when the limbs alternate to provide the propulsive force. In-phase locomotion is the common form of locomotion used by frogs when immersed in water. The forelimbs are adducted and held to the side of the body while the hindlimbs kick in a synchronized motion providing the propulsive force (Gray, 1968). Out-of-phase locomotion resembles terrestrial quadrupedal locomotion. Each pair of limbs alternate, with the forelimbs and hindlimbs moving in antiphase (the left forelimb roughly synchronized with the right hindlimb). Out-of-phase locomotion is commonly used by frogs when on land and by urodeles when on land and in the water. For the purposes of this thesis axial swimming will be referred to as "swimming" and limbed swimming/walking will be referred to as "walking." This is an over simplification of these two forms of locomotion but will help to clarify some of the concepts to follow.

1.2.1 Urodele Locomotion

Urodeles are unique among amphibia in that they exhibit two very different forms of locomotion. Urodeles are capable of walking with an alternating quadrupedal gait as well as swimming using the same lateral body undulations used by fish to locomote. The forms of locomotion used by urodeles are similar across the entire order and therefore apply to all urodeles including newts, salamanders, and mudpuppies.

Urodeles swim only when short bursts of locomotion are required, as when attempting to escape a predator or when lunging to catch food. While swimming, both forelimbs and hindlimbs are adducted and held passively against the side of the body. A travelling wave is then initiated which moves caudally over the length of the body creating the force required to propel the animal forward (Gray, 1968). Walking, as demonstrated by urodeles, resembles the alternating quadrupedal gait indicative of four legged terrestrial vertebrates with some interesting differences. First, lateral trunk movements are used to increase the stride length. These trunk movements differ from the trunk movements used during swimming (Blight, 1977). During swimming the peak of the travelling wave moves caudally over the length of the body, while in walking, the peak of the wave is stationary, midway between the forelimbs and hindlimbs. By maintaining the apex of the wave at midbody, a standing wave is created with two nodes at the points where the forelimbs and hindlimbs join the body. Contraction of the right axial musculature then moves the left forelimb to a more rostral position than would normally be attained without the axial flexion (Roos, 1964, cf Grillner, 1981). These trunk movements are absent when the animal walks slowly and increase in amplitude as the speed of the locomotion increases (Evans, 1946). A second difference between amphibian and mammalian quadrupeds is that in amphibians the limbs extend from the side of the body rather than from the ventral surface as they do in mammalian quadrupeds. The result being that the body weight is supported by the ventral surface of the body at rest and during slow locomotion. At increased speeds and when walking on land (depending on the species of urodela) more of the body weight is supported by the limbs (Evans, 1946).

1.3 Central Generation of Locomotion

In the early part of this century two hypotheses were put forth to explain how locomotion was generated. The first of these hypotheses suggested that locomotion resulted from a "chain of reflexes." One reflex initiated a response, which elicited another reflex and response, eliciting another reflex and response, the ensemble character of these reflexes being locomotion. This hypothesis, originally proposed by Philippson (Philippson, 1905; cf. Wetzel and Stuart, 1976), is often credited to Sherrington, who through extensive studies on spinal cats (Sherrington, 1910; Sherrington, 1913) emphasized the important role of reflexes during locomotion. In fact, Sherrington himself expressed reservations about the hypothesis, stating that even after deafferentation the limb still showed "traces" of reflex stepping (Sherrington, 1910). The second hypothesis of that era was purported by T.G. Brown and was based on the idea that locomotion was generated centrally and that reflexes were not needed for the production of the locomotor rhythm. T.G. Brown demonstrated that spinal animals could locomote after deafferentation and hypothesized that two "half centres" located within the central nervous system were responsible for generating the locomotor rhythm (Brown, 1914). Most of the above assessments were made after visual observation of the locomotor pattern and lacked quantitative assessments of function.

More recently, investigators have used quantitative measures to evaluate the ability of the central nervous system to generate locomotion. Székely recorded electromyographic (EMG) activity from both unilaterally and bilaterally deafferented newts and concluded that the locomotor pattern remained intact after deafferentation (Székely et al., 1969). Grillner stated that Székely's recordings after deafferentation were "identical" to those recorded in the intact animal (Grillner, 1975). It is true that much of the complex

timing required for locomotion remained after deafferentation however, Székely's records seem variable and lack quantitative gait analysis. A more complete account of locomotion after deafferentation was conducted by Grillner and Zangger (Grillner and Zangger, 1974, 1975, 1979, 1984). Grillner and Zangger demonstrated conclusively that the adult spinal cat was capable of generating rhythmic locomotor output in the absence of sensory input. This did not mean that the locomotor pattern after deafferentation was identical in all respects to that seen before deafferentation. On the contrary, the pattern after deafferentation was more variable and easier to disrupt than the locomotor pattern in the intact animal (Grillner and Zangger, 1979, 1984).

The concept of a centrally generated rhythm is now reasonably well accepted. However, how complete this locomotor rhythm is and what role peripheral input plays in shaping and generating the rhythm are two questions which have yet to be completely answered. It should also be noted however that in spite of reports demonstrating the capability of the spinal cord to generate locomotion in isolation, many believe that some afferent input is required for expression of rhythmic activity (Gray and Lissman, 1940, 1946; Shik et al., 1966; Shik and Orlovskii, 1976; Perret and Cabelguen, 1980).

1.4 Role of Afferent input in controlling locomotion

It must be recognized that the limb monitors a large number of receptor types capable of signalling an equally large number of sensory modalities. The exact consequences of input from each of the receptor types on the locomotor pattern is still unclear. Rossignol et al. (1988) summarized the role of afferent input best in saying,

> "In the open-loop mode, the system is internally calibrated from data that have been incorporated in neural elements as a result of evolution and learning. In the closed-loop mode, the system is continuously recalibrated (adapted) by feedback so that it can work with more accuracy, more stability, and with a greater range

of frequencies (bandwidth)."

Two general approaches have been used to answer functional questions on the role of afferent input during locomotion. The first approach has been to remove afferent input during locomotion and infer function from the resulting deficits. The second approach has been to stimulate afferents during locomotion and monitor the resulting perturbation to the locomotor cycle.

1.4.1 Deficits after Deafferentation

As outlined above, early deafferentation experiments were confined to visual observation of locomotor patterns, relying on the experimenter to report any deficits. Most of these reports have yielded conflicting results (reviewed by Wetzel and Stuart, 1976). Early experiments on unilateral deafferentation point to possible differences between acutely and chronically deafferented animals. If the animal was tested immediately after deafferentation then the animal did not walk (Brown, 1913). If however the animal was tested 15 months after deafferentation, locomotion was easily elicited (Brown, 1914). More recently this plasticity in the ability of the spinal cord to generate locomotion has been demonstrated in both partially deafferented (Rasmussen et al., 1986) intact cats as well as spinal cats with intact dorsal roots (Edgerton et al., 1990). In T.G. Brown's experiments he noted asymetries in interlimb coordination after chronic unilateral deafferentation. When his animals began walking they would only locomote unilaterally with the intact limb. After 20 seconds this pattern would switch to unilateral locomotion with the deafferented limb and after another short period, bilateral locomotion (Brown, 1914). A number of more recent reports have noted specific changes after

deafferentation. If unilateral deafferentation was completed while descending tracts were left intact, animals could locomote spontaneously within two weeks, although these locomotor patterns lacked accuracy and showed obvious deficits (Hnik et al., 1981; Wetzel et al., 1976; Goldberger and Murray, 1974; Goldberger, 1977). Unilateral deafferentation in both intact and spinal cats has been shown to severely disrupt interlimb coordination while having less of an effect on intralimb coordination (Wetzel et al., 1976; Giuliani and Smith, 1987). Other investigators have concluded that the transitional periods of activation may be especially dependent on afferent input (Grillner and Zangger, 1984, however, see Baev and Shimansky, 1992). These experiments suggest that afferent input, although not required, may be important in modifying and controlling the timing and coordination of the full locomotor pattern.

It should also be noted that since the demonstration of convergence from multiple neuronal systems onto interneurons (Baldissera et al., 1981), the effects of removal of afferent input must be carefully interpreted. It has been demonstrated that after deafferentation, extensor activity in the intact limb is prolonged if descending tracts are left intact (Hnik et al., 1981; Wetzel et al., 1976) while extensor activity is shortened if descending input is removed (Giuliani and Smith, 1987). This probably reflects a strong vestibulospinal input in the intact animal and demonstrates the importance of a balance between afferent and descending input (Orlovskii and Feldman, 1972).

1.4.2 Stimulation of Afferents during Locomotion

Two types of afferent stimulation have been used to investigate the role of afferent input during locomotion. Tonic stimulation involves applying a stimulus which has a

duration longer than that of the step cycle, while phasic stimulation involves applying a very short duration stimulus and monitoring its perturbation of the ongoing locomotor cycle. Sherrington showed that stepping could be elicited after tonic bilateral stimulation of superficial peroneal nerves (Sherrington, 1913). Grillner and Zangger (1979) repeated these experiments showing that the rhythm could be initiated with unilateral as well as bilateral stimulation of an afferent nerve and that increasing intensities of stimulation increased the rate of locomotion. As well, tonic afferent stimulation in conjunction with descending stimulation can initiate locomotion at stimulus intensities below those required for initiating locomotion when either input is stimulated in isolation (Shik et al., 1966; Orlovskii and Feldman, 1972). These experiments demonstrate the possible supportive and regulatory role of afferent input during locomotion.

Tonic cutaneous afferent stimulation during locomotion has a powerful effect on bifunctional muscles. A bifunctional muscle which is normally active during extension can be driven to burst during flexion after tonic afferent stimulation (Perret and Cabelguen, 1976; Perret 1986). This has been shown to be true for a number of bifunctional muscles in both the hindlimb and forelimb of the cat (Perret and Cabelguen, 1980; Cabelguen et al., 1981). Tonic afferent input may also have a stabilizing effect on the locomotor rhythm generated in the fictive cat.

Phasic input to the spinal cord during locomotion is not required for the initiation or maintenance of the basic locomotor rhythm (Grillner and Zangger, 1975, 1979, 1984). However, phasic afferent input is required for online adjustments of the motor output to external stimuli, as well as possibly supplying the spinal cord with precise timing clues throughout the step cycle. Grillner and Rossignol demonstrated that the locomotor rhythm of a single limb could be blocked by maintaining the limb in a flexed position

(Grillner and Rossignol, 1978). When the limb was slowly moved caudally into extension, a point was reached when the limb flexed, then continued stepping (Grillner and Rossignol, 1978). The mechanism responsible for the sensing of limb position could provide an important cue or trigger regulating the transition between the stance phase and swing phase of locomotion. Pearson and Duysens (1976, 1980) have also shown that the transition from stance to swing can be delayed if the ankle extensors are loaded during locomotion. Whether the signal responsible for triggering swing is hip position, ankle load or some combination of both signals an important concept emerges from these studies. First, phasic afferent input is important in regulating and controlling the step cycle. Second, there may be more than one solution to the same problem. The motor control system is full of redundancy. This fact is demonstrated every time you reach out to grasp an object. There is an infinite number of trajectories which can be taken by the arm to reach the object. Redundancy is often thought of as wasteful. In fact it may be an evolutionary advantage to be able to solve the same problem in a number ways.

1.4.2.1 Reflex modulation during locomotion

Phasic afferent stimulation has been used effectively to monitor the way in which the stretch reflex is used to assist and support the step cycle. It has been demonstrated that the monosynaptic stretch reflex and its electrical counterpart the H-reflex are modulated during locomotion (Akazawa et al., 1982; Capaday and Stein, 1986, 1987). The stretch reflex modulation observed in these experiments is functionally important to the task of walking. In the stance phase, when the soleus muscle is actively stretched, the soleus muscle reflex gain is high. Any muscle spindle input during stance would

function to assist in force production. During the swing phase when the ankle is dorsiflexed and the soleus muscle is again stretched, the reflex gain is low. Reflex activity at this point in the step cycle would be counter productive (Stein and Capaday, 1988).

Stimulation of cutaneous afferents during locomotion also results in a functionally important reflex which is modulated throughout the step cycle (Forssberg et al., 1975, 1977; Andersson et al., 1978; Prochazka, et al., 1978; Forssberg, 1979; Wand et al., 1980). This reflex has often been referred to as the "stumbling corrective reaction" and results when the dorsum of the foot contacts an object during locomotion (Forssberg, 1979). The locomotor rhythm is adjusted and the limb is lifted over the object without interrupting the animal's forward progression, but only if the stimulus occurs during the swing phase of that limb. If the same stimulus occurs during the stance phase when the limb is supporting the animal's weight the limb is not lifted over the object. This uniquely integrated reflex has been shown to occur for both the forelimb and hindlimb (Drew and Rossignol, 1987), in both intact and decerebrate animals (Forssberg et al., 1975; Prochazka et al., 1978), and in humans as well as cats (Yang and Stein, 1988, Duysens et al., 1990).

A number of neural mechanisms responsible for reflex modulation have been hypothesised. The modulation of the H-reflex during human locomotion has been hypothesised to be due to presynaptic inhibition of Ia afferent terminals onto motoneurons (Capaday and Stein, 1987; Stein and Capaday, 1988). Presynaptic inhibition allows for control of reflex gain independent of the level of activation of the motoneuron pool. In the Xenopus embryo, a stimulus applied to the animal's trunk when at rest leads to a lateral bending of the trunk away from the stimulus. When the same stimulus is applied during locomotion the response is gated so that only those stimuli applied during the

latter half of the locomotor cycle are effective. The neural mechanism responsible for sensory gating has been shown to be due to inhibition, by ipsilateral motoneurons, of an interneuron in the reflex pathway from the sensory cell (Rohon-Beard cell) to the contralateral motoneuron. As a result, when ipsilateral motoneurons are active the ipsilateral stimulus is not transmitted to the contralateral motoneurons. However when the ipsilateral motoneurons are inactive ipsilateral stimuli are free to excite contralateral motoneurons, leading to a reflex response (Sillar and Roberts, 1988). This is the only mechanism for vertebrate reflex modulation for which there is direct evidence.

1.5 In-vitro preparations for locomotion

Vertebrate in-vitro preparations were first developed over 40 years ago (Eccles, 1946; Araki et al., 1953). However, it has only been over the last 10 years that they have been used for investigating locomotion (Poon, 1980; Cohen and Wallén, 1980; Grillner et al., 1981; Roberts and Kahn, 1982). In-vitro preparations of the vertebrate brainstem and spinal cord have a number of advantages over in-vivo preparations. First, the ionic environment of the spinal cord can be easily controlled since preparations are usually superfused with a spinal cord Ringers solution. Second, since there is no blood-brain barrier all drugs and ions in the superfusate have equal access to the cells of the spinal cord and brainstem. Third, because the spinal cord is oxygenated from the superfusate, there is no need for blood and the pulse pressure associated with it, making long term intracellular recording possible. These properties make in-vitro preparations especially suited for the study of cellular mechanisms of locomotion.

In-vitro preparations can be made to locomote using a number of methods. Chemical excitants such as cholinergic agonists, dopaminergic agonists, and the

excitatory amino acids are effective in inducing locomotion when applied to the spinal cord (Poon, 1980; Cohen and Wallén, 1980; McClellan and Farel, 1985; Smith et al., 1986; Barry and O'Donovan, 1987; Smith and Feldman, 1987), while GABA antagonists, substance P and excitatory amino acids are capable of initiating locomotion when applied to the brainstem (Smith et al., 1986; Smith and Feldman, 1987). Many of the vertebrate in-vitro preparations will exhibit periods of spontaneous locomotion (Landmesser and O'Donovan, 1984; Stenhouwer and Farel, 1980, 1983; Roberts and Kahn, 1982; Smith and Feldman, 1987). Locomotion can also be induced using electrical stimulation of the midbrain or descending tracts (Cohen and Wallen, 1978; McClellan and Grillner, 1984; Atsuta et al., 1990) as well as electrical or natural stimulation of sensory pathways (Stenhouwer and Farel, 1981; Roberts et al., 1985; Smith and Feldman, 1987).

The in-vitro preparations of the lamprey and xenopus embryo, as developed by Grillner and Roberts respectively, are two preparations which have proven particularly useful in elucidating some of the circuitry and cellular mechanisms responsible for generating locomotor patterns (for review see Grillner et al., 1987, 1990, 1991; Roberts et al., 1986; Roberts and Tunstall, 1991). Dale points out the similarity in mechanisms involved in generating locomotion in both the lamprey and the Xenopus embryo despite their vastly different phylogenetic origins (Dale, 1986). We must be careful not to assume that the mechanisms responsible for generating swimming in these preparations are the same as the mechanisms responsible for generating walking in limbed vertebrates, at least until some of the circuitry and cellular mechanisms of limbed locomotion have been elucidated. One cellular mechanism which has been shown to be important in vertebrates as primitive as the lamprey and as advanced as the cat is the role of excitatory amino acid receptors (N-methyl D-aspartate, NMDA) in locomotion (Dale and Roberts, 1983;

Brodin and Grillner, 1985; Barry and O'Donovan, 1987; Douglas et al., 1990; Jordan, 1991).

In-vitro vertebrate preparations have come a long way over the last decade. What needs to be emphasized now is the search for cellular mechanisms of locomotion in higher vertebrate preparations which use limbs for locomotion.

1.6 Activity of Interneurons during locomotion

Although in-vitro preparations are well suited for investigating the activity of interneurons during locomotion, much of our knowledge of the firing patterns and properties of interneurons comes from work on the cat spinal cord. In 1972 Orlovskii and Feldman recorded extracellularly from rhythmically active interneurons in the spinal cord of the cat and found that 37% of these interneurons could be classified as mixed bursters, with their activity beginning in one phase of the step cycle and ending in another (Orlovskii and Feldman, 1972). In contrast, interneuronal recordings in the rabbit have led to the classification of rhythmic interneurons as either flexor or extensor (Viala et al., 1991). Edgerton et al. (1976) have recorded from rhythmically active interneurons during fictive locomotion in the cat and claim that the majority of interneurons had firing patterns which coincided with either the flexor or extensor phase of the step cycle. Interestingly, Grillner comments on this paper he co-authored with Edgerton and states that, "No orderly division can be made into categories related exclusively to flexor and extensor neurons" (Grillner, 1981). In a similar study Baev et al. found that the majority of rhythmically active interneurons they recorded from could be classified as bursting during flexion or extension and that only 10% could be classified as mixed (Baev et al., 1979). They suggested that the reason for their underestimate of mixed interneurons (10%) was

their use of a fictive preparation and that cyclic afferent inflow was required to convert flexor and extensor interneurons into mixed interneurons (Baev et al., 1979). The hypothesis that afferent input is capable of changing the phase of activity of a muscle during locomotion is supported by work done by Perret and co-workers (Perret and Cabelguen, 1976; Perret 1986). More recently it has been demonstrated that rhythmically active interneurons with mixed or transitional bursting characteristics are common in the forelimb of the fictive cat (Hishinuma and Yamaguchi, 1990; Terakado and Yamaguchi, 1990). However, when these interneurons are subdivided so that only last-order interneurons are considered they again can be classified as either 'flexor-like' or 'extensor-like' (Terakado and Yamaguchi, 1990; Ichikawa et al., 1991).

In simpler vertebrate systems with fewer phases of muscle activity one might expect the interneuronal firing patterns to be simpler. In the lamprey and Xenopus embryo, two species which swim rather than walk, interneuronal firing patterns are confined to the two phases of muscle activity (Grillner et al., 1991; Roberts et al., 1986). However, in the spinal dogfish interneuronal firing patterns are distributed throughout the locomotor cycle with most interneurons firing at the peak of muscle activity (Mos et al., 1990). These observations complicate the interpretation of present models of vertebrate pattern generation. Presently, models of vertebrate pattern generation have been proposed for some species: lamprey (Grillner et al., 1991), Xenopus embryo (Roberts et al., 1986), urodeles (Székely, 1965; Kling and Székely, 1968), and cats (Jankowska et al., 1967b).

A number of questions have been raised by the above studies. First, is the elusive central pattern generator (CPG) for locomotion conserved across the vertebrate phylum? Second, are transitional bursting cells part of the CPG or is the CPG more structurally and

functionally confined? Third, are there differences in the phase of activity of interneurons between the forelimb and the hindlimb in the cat? If these differences prove to be significant, do they reflect a difference in the organization of forelimb and hindlimb stepping generators? Differences between forelimb and hindlimb responses to cutaneous stimulation have been well documented (Rossignol and Drew, 1986; Drew and Rossignol, 1987). Fourth, if we assume that the last-order cells identified by Terakado and Yamaguchi (1990) and Ichikawa et al. (1991) are output cells of the CPG, are the 'flexor-like' and 'extensor-like' patterns observed in these output cells synthesized from the patterns of transitional bursting cells? Many of these questions are as yet unanswered.

In-vitro preparations of the lamprey and Xenopus embryo have been very useful in elucidating the locomotor circuitry for swimming. However, these species display relatively simple locomotor patterns when compared to walking vertebrates, and may be poor models for investigating the complex bursting indicative of limbed vertebrates. More work needs to be done to establish or sever the link between lower and higher forms of vertebrate locomotion.

1.7 Summary of Objectives

Locomotion is a complex task which in normal individuals requires relatively little attention. However, the deficits which result from disturbing the locomotor system are obvious and require great attention if they can be completed at all. Although there is little evidence for spinally generated locomotion in humans (Biraben and Roby-Brami, 1985), the hope exists that the central pattern generator, which has been demonstrated for animals from invertebrates (Getting, 1988) to cats (Grillner and Zangger, 1974, 1975, 1979, 1984) exists in humans as well. If we are to understand the cellular mechanisms

responsible for generating locomotion in limbed vertebrates we must use limbed vertebrates as models.

We have developed an in-vitro preparation of the mudpuppy (Necturus maculatus) for investigating the circuitry and control of walking in vertebrates. The mudpuppy walks with an alternating quadrupedal gait as do all urodeles and has a relatively simple spinal cord (Frankhauser, 1952). We believe that the in-vitro mudpuppy preparation has some advantages over previously identified vertebrate in-vitro preparations.

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CHAPTER 2

A COMPARISON OF INTACT AND IN-VITRO LOCOMOTION IN AN ADULT AMPHIBIAN

2.1 Introduction

In recent years much effort has been devoted to the development of in-vitro preparations for investigating the control, development and generation of locomotion. In-vitro preparations have been developed for a number of species including the lamprey (Rovainen, 1979; Cohen and Wallén, 1980; Grillner et al. 1988), larval and adult amphibians (Kahn and Roberts, 1982; Clarke and Roberts, 1984; McClellan and Farel, 1985; Stenhouwer and Farel, 1985), the chick (Landmesser and O'Donovan, 1984) and the neonatal rat (Smith and Feldman, 1987). These preparations offer many advantages over intact preparations for investigating the cellular mechanisms of locomotion. In-vitro experiments allow for strict control over the ionic and pharmacological environment, and are devoid of any pulsations related to blood pressure, allowing for stable, long term intracellular recordings (Smith and Feldman, 1987; Smith, et al., 1988). These preparations are also physically small so that oxygenation of the spinal cord can occur by diffusion from the surrounding bath. The reduced number of cells in these smaller preparations also allows for a simpler interpretation of experimental results.

A version of this chapter has been published. Wheatley, M., Edamura, M. and Stein R.B. (1992). Experimental Brain Research 88: 609-614.

In spite of the many obvious advantages of in-vitro work, each preparation has inherent limitations. The lamprey and the Xenopus embryo swim rather than walk, making extrapolation to higher forms of vertebrate locomotion difficult. Adult frogs do walk but only for short bursts of one to four steps. When walking is induced with excitatory amino acids in adult frogs, it is interspersed with other motor tasks such as wiping, jumping or swimming which makes interpretation difficult (McClellan and Farel, 1985). Some investigators have turned to neonatal and embryonic preparations which allow for a reduced preparation size (Kahn and Roberts, 1982; Clarke and Roberts, 1984; Smith and Feldman, 1987). However, neonatal and embryonic preparations are not fully developed and may have different molecular, cellular or anatomical properties than the adult animal. There is evidence that the cellular properties of motoneurons in the neonatal rat are not identical to those in the mature animal (Walton and Fulton, 1986; Fulton and Walton, 1986).

Our approach to understanding vertebrate locomotion has been to develop an in-vitro brainstem - spinal cord - forelimb preparation with some advantages of both reduced and intact preparations. We chose the adult mudpuppy (Necturus maculatus) as a suitable model system. The mudpuppy is an aquatic quadruped and can locomote in one of two ways. It can swim much like a fish, and can walk with an alternating quadrupedal gait. The mudpuppy, is a poikilotherm and functions well over a large range of temperatures (5 - 30°C; Scott, 1981). In spite of its primitive phylogenetic origin, it maintains many of the reflexes seen in higher vertebrate species (Wheatley and Stein, 1989; Wheatley et al., 1990). The fact that the animal is a poikilotherm allows the experimenter to reduce the temperature of the in-vitro recording chamber while still maintaining the viability of the preparation. The lowered temperature slows metabolic

processes enough to reduce the oxygen requirements of the preparation.

The aim of this study was threefold: first, to see if the in-vitro mudpuppy could be made to locomote reliably; second, to determine the optimal conditions for in-vitro locomotion; and third, to compare the intact and in-vitro locomotor patterns. Some of the preliminary results have been presented elsewhere (Wheatley and Stein, 1989; Wheatley et al., 1990).

2.2 Methods

2.2.1 Animals

Mudpuppies were obtained from a local supplier and maintained in an aquarium at 10 - 15°C. Twenty nine adult mudpuppies (body length 30-40 cm) were used during the present experiments. Intact animals were kept in a solution of tap water pretreated with sodium thiosulphate to reduce chlorine levels to those tolerated by the animal. Before dissection, animals were anaesthetized with a bath application of 3-aminobenzoic acid ethyl ester (Sigma, 1g - 5g/l).

2.2.2 Intact preparation

Bipolar electromyographic (EMG) recordings were made from 10 intact animals while walking on an aquatic treadmill. EMG electrodes were made from 75μm teflon coated silver or stainless steel wire and inserted into six forelimb and shoulder muscles: latissimus dorsi (LD), dorsalis scapulae (DS), procoracohumeralis (PRO), pectoralis (PECT), brachialis (BR), and external ulnae (EU) (Fig.1, and Gilbert, 1973). Two EMG wires were inserted in each muscle after an incision in the skin overlying the muscle. The wires were then fed under the skin to the dorsal surface of the animal where they exited and either attached to a "back pack" sutured to the animals skin or lead directly to the preamplifiers. After recovery from anaesthesia the animals walked freely on the treadmill. EMG signals were differentially preamplified and stored on an FM recorder for later analysis.

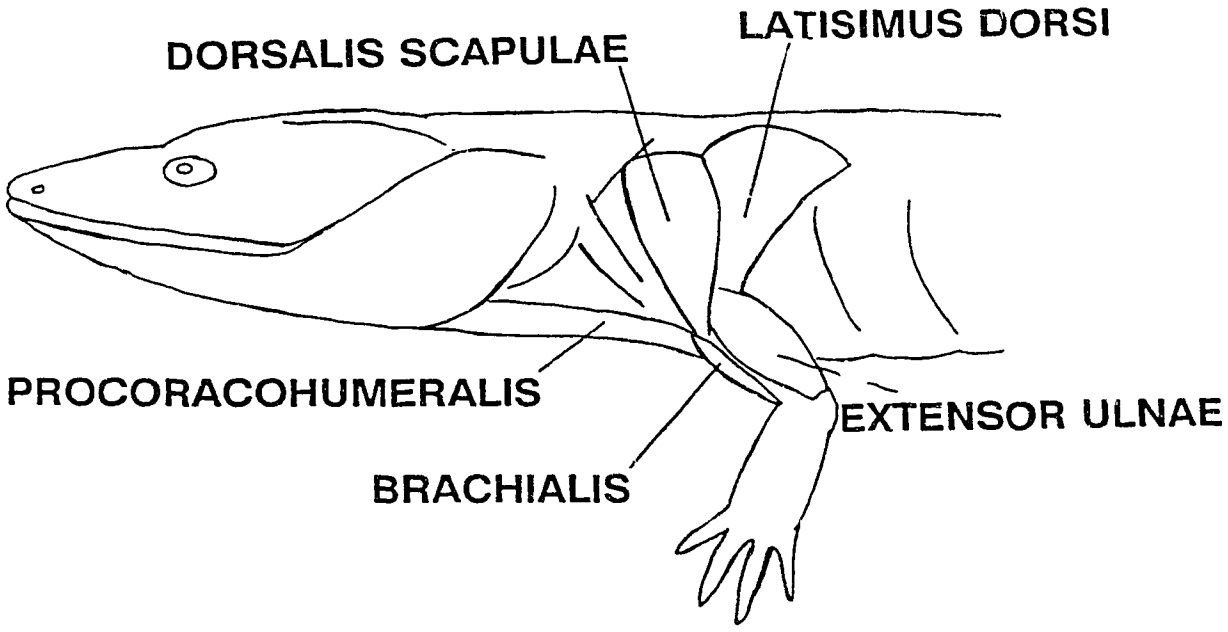

Fig.1 Diagram of mudpuppy with anatomical positions of muscles used during recording. Latissimus dorsi (LD), Dorsalis scapulae (DS), Procoracohumeralis (PRO), Extensor ulnae (EU), Brachialis (BR). The Pectoralis muscle (PECT) is on the ventral surface and is not shown.

2.2.3 In-vitro preparation

Brainstem-spinal cord-forelimb dissection

In vitro experiments were performed on 19 mudpuppies. Data was collected from four animals to construct the concentration response curves to NMDA with and without magnesium. Eight animals were used for the in-vitro gait analysis. Twelve animals were used in the experiments involving glycine (five of these animals overlaped with the in-vitro gait analysis group).

The in-vitro dissection was performed as follows. A longitudinal incision was made along the dorsal surface of the animal and epiaxial muscles were removed from the caudal border of the cranium to the sixth cervical vertebrae. The right suprascapular cartilage was removed to expose the brachial plexus and the skin of the upper forelimb was removed. A laminectomy was completed from the first through fifth cervical vertebrae to expose the first five segments of the spinal cord. This was long enough to include all the innervation of the forelimb (Székely and Czéh, 1967). A craniotomy exposed the hindbrain, and the decerebration was performed at the caudal diencephalon. (The animal can also be spinalized by sectioning the spinal cord at the caudal border of the medulla). The vertebral column and right forelimb were then removed from the rest of the body and placed in a petri dish containing 100% oxygenated spinal cord Ringers (NaCl, 115mM; $CaCl_2$, 2mM; KCl, 2mM; $MgCl_2$, 1.8mM; Hepes, 5mM, pH 7.3; glucose, 1g/l). While in the petri dish the brachial plexus was exposed, the paraspinal muscles were removed and the dura mater covering the spinal cord was opened. The total time for dissection takes less than 45 minutes.

Once the dissection was complete, the preparation was transferred to the recording chamber and perfused with cooled and oxygenated spinal cord Ringers at a

flow rate of about 5 ml/min. The recording chamber was kept at 15°C. In the recording chamber the spinal cord was fixed by pinning the vertebral column to the Sylgard Resin (Dow Corning) which coated the base of the bath. The forelimb was held by clamping the procoracoid cartilage to the wall dividing the limb bath from the spinal cord bath.

After a one hour recovery period the animals showed a withdrawal reflex to pinching of the limb. Locomotion was then induced by bath application of 100μM N-methyl DL-Aspartate (NMA, Sigma). When the effects of glycine were being investigated the bath application of 100μM NMA included 1μM glycine (Sigma). In-vitro EMG recordings were made in much the same way as when the mudpuppies were intact except that only two muscles (BR and EU) were available to record from. Intact and in-vitro walking patterns were compared to determine whether the two locomotor patterns were the same. The four parameters used were: cycle duration, flexor burst duration, extensor burst duration, and interval from the onset of flexion to onset of extension (flex - ext interval). The means were compared using a two-sided t test at the $p<0.01$ level (Zar, 1974).

2.3 Results

2.3.1 Intact animal recording

EMG's were recorded from the muscles of the shoulder and elbow while the animal walked on an underwater treadmill (Fig. 1 and 2). Walking in these animals was episodic. When the animal began to walk, the speed of the treadmill was adjusted to match the speed of the animal. During locomotion the muscles of the forelimb showed EMG burst patterns which were indicative of the function of the limb. The external ulnae (EU) functions to extend the elbow and therefore it's EMG burst was limited to the extensor phase when elbow extension helps to propel the animal forward. The brachialis (BR) flexes the elbow and shows a variable burst pattern, ranging from a single burst during the flexor phase to a double burst pattern with bursts both in flexion and extension. The variable activation of BR is shown in Figure 3. This figure is taken from an intact animal during a two minute stretch of walking. Each trace starts at the beginning of the flexor phase and ends with the beginning of the subsequent step cycle. The implications of this burst pattern will be addressed in the Discussion. The pectoralis muscle (PECT) supports the animal and exhibits a single burst throughout the extensor phase of locomotion. The latissimus dorsi (LD) also shows a single burst during extension and functions to draw the limb posteriorly (retraction). Dorsalis scapulae (DS) raises the limb, while procoracohumeralis (PR) functions to draw the limb forward (protraction). DS and PR also demonstrated variable muscle activation patterns, although the variability in BR was most pronounced.

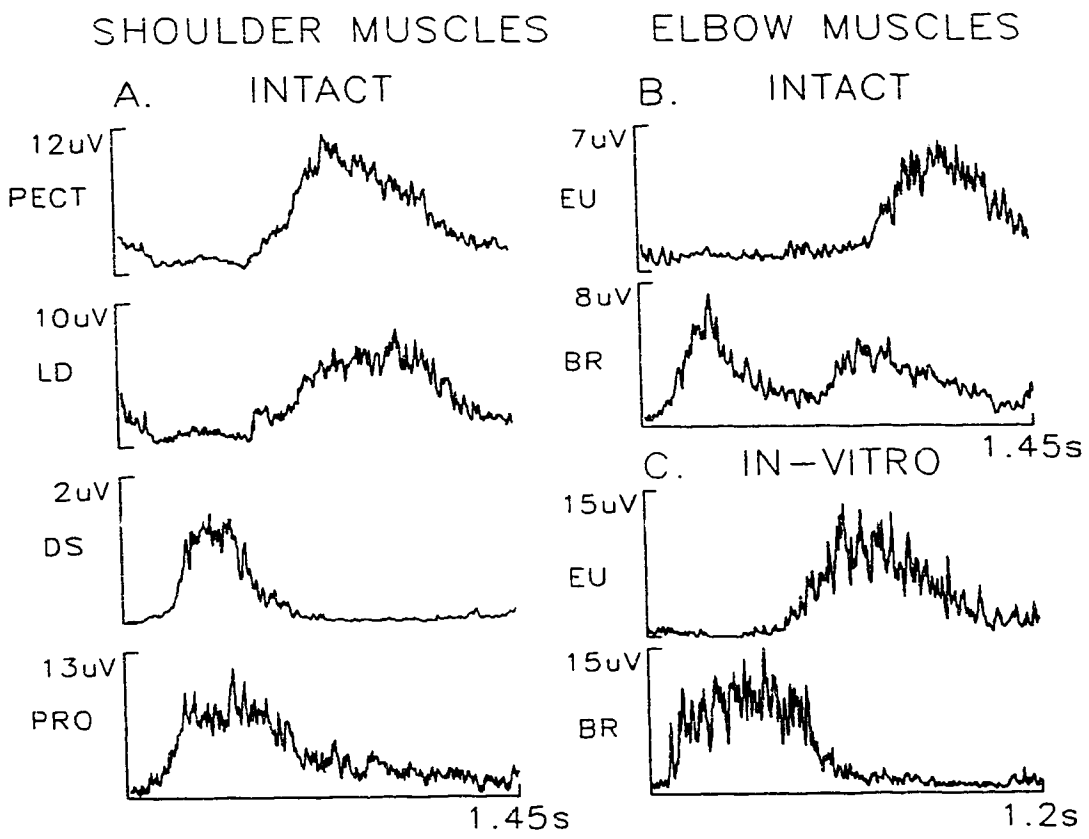

Fig. 2 Patterns of electromyographic (EMG) activity recorded from the mudpuppy while walking. A) Shoulder EMG's recorded from an intact mudpuppy. B) Elbow EMG's recorded from the same animal as in A. C) Elbow EMG's recorded from an in-vitro preparation after bath application of 100μM NMA. All traces are one complete step cycle beginning with flexion.

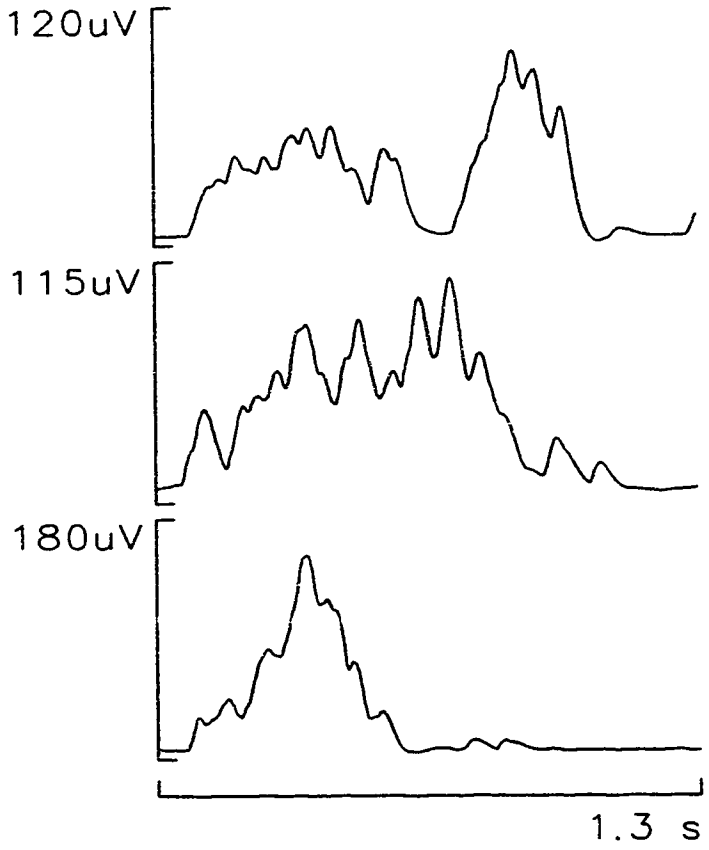

Fig. 3 Electromyograms recorded from the Brachialis (BR) of an intact mudpuppy walking on the treadmill. All traces were selected from a single mudpuppy during a 2 minute sequence of walking.

2.3.2 In-vitro preparation recording

The in-vitro preparation began to walk within a couple of minutes of superfusion with NMA. The pattern was weak at first but increased in speed and amplitude over the next few minutes. Once the intensity of walking was maximal it maintained this level for 30 - 90 min. At this point the rhythm began to break down, becoming less regular as both flexor and extensor bursts decrease in intensity and frequency. The preparation was then refractory to further additions of NMA. After a washout period of 20 - 60 min, the preparation could be made to walk again with superfusion of NMA. This sequence could be repeated many times. When the experiment was complete the preparation could be stored unoxygenated at 2°C for periods of up to 5 days. Any time during this period the preparation could be made to walk by repeating the steps outlined above.

In an initial series of experiments step cycle frequency was plotted against the concentration of NMA added to the bath. A saturating dose response curve was obtained from 4 mudpuppies (Fig 4). Locomotion was never initiated at doses below 50 μM, and reached a maximum of 1.5 Hz at about 200 μM. The preparation also showed desensitization to NMA after long periods of exposure to the drug. In our experience this desensitization was quite variable. Nonetheless, a single dose usually initiated and maintained locomotion for more than one hour. This desensitization is a common feature of responses mediated by excitatory amino acids (Nowak et al., 1984; MacDonald, et al., 1987). To minimize the desensitization we kept the concentration of NMA at the minimum dose required for near maximal step cycle frequencies (100 μM).

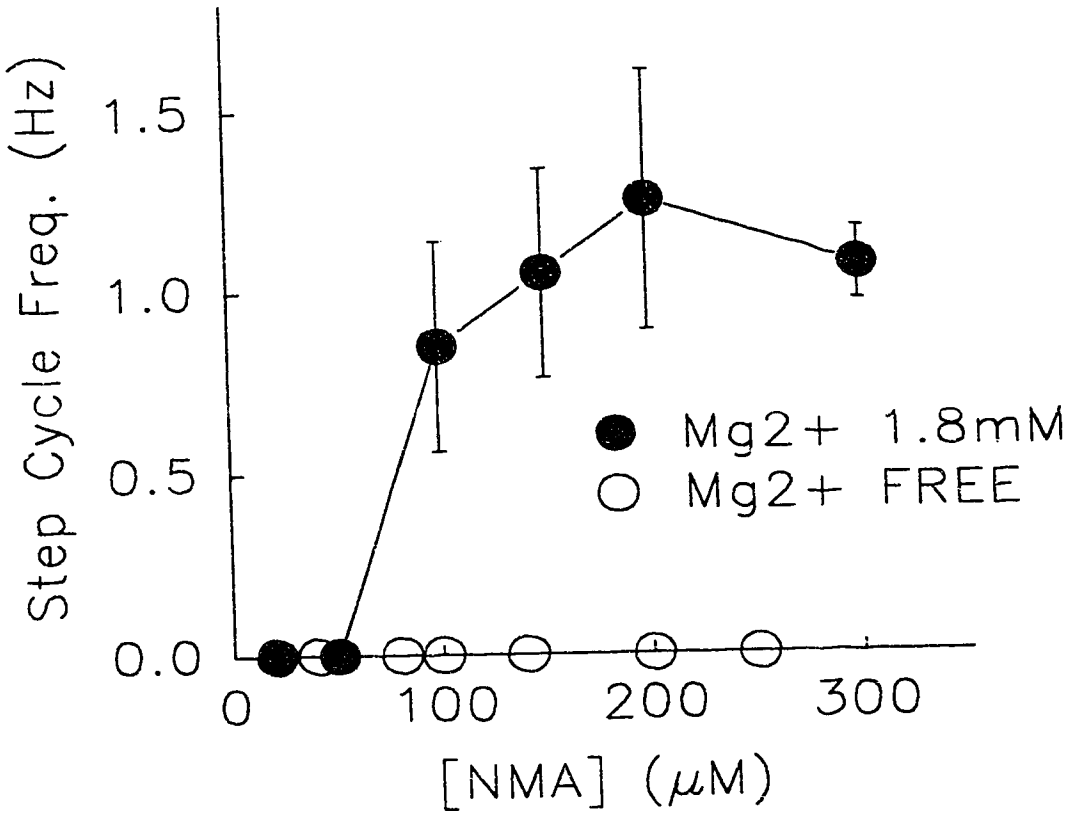

Fig. 4 Dose response curves for bath application of NMA to <u>in-vitro</u> preparation. Open circles () are in magnesium free bath and closed circles (●) are in 1.8 mM magnesium (mean ±SE).

The in-vitro preparation required the presence of Mg^{2+} in the bathing solution. Without magnesium the muscles were tonically active, but showed no phasic burst pattern (Figure 4). This is consistent with a response mediated by NMDA receptors. On a separate population of mudpuppies the effects of glycine were investigated. Glycine has been shown to be a modulator of the NMDA receptor (Johnson and Ascher, 1987). A comparison of the step cycle frequency in-vitro after 100μM NMA and after 100μM NMA and 1μM glycine showed that the frequency was significantly increased with glycine present (Fig 5).

2.3.3 Locomotor patterns

A comparison of the activity patterns of the BR and EU muscles in 8 in-vitro and 10 intact preparations can be seen in figure 6. Four indices were used: the step cycle duration, the duration of the first flexor burst, the duration of the extensor burst and the duration between the onset of the flexor burst and the onset of the extensor burst (flex - ext interval) (Fig.6A). Parameters were averaged from 8 -10 consecutive cycles in each animal. The step cycle durations from the intact and in-vitro preparations were statistically different ($p<0.01$). Possible explanations for this difference include: i) The dose (100μM) of NMA used may have been submaximal in these in-vitro experiments and therefore the discrepancy did not reflect a difference in the pattern of walking. ii) Glycine may have been required for maximal activation of the NMDA receptor. (Glycine was not added to the bath in any of the in-vitro experiments in figure 6.) The pattern of locomotion can best be analyzed by plotting the duration of each gait parameter as a percentage of the step cycle duration. This normalizes for the variation in cycle duration between the two preparations and allows for a comparison between intact and in-vitro preparations. After

normalization there is no statistical difference in flexor burst duration, extensor burst duration, or flex - ext interval between the intact and in-vitro conditions.

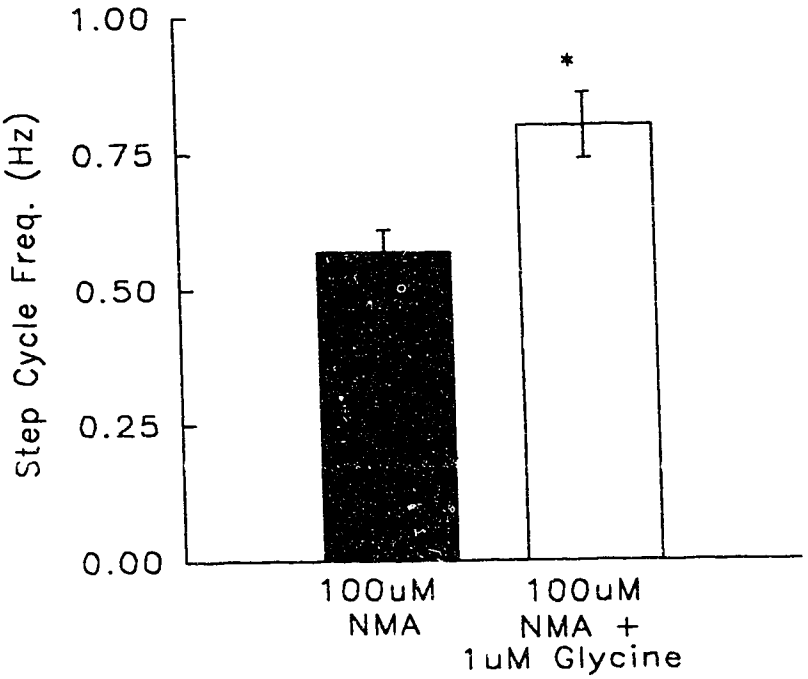

Fig. 5 Effect of glycine on step cycle frequency. Filled box (■ n=9) is mean step cycle frequency (mean ±SE) with bath applied 100μM NMA. Open box (□ n=3) is mean step cycle frequency with bath applied 100μM NMA and 1μM glycine (*two-sided t test $p<0.01$).

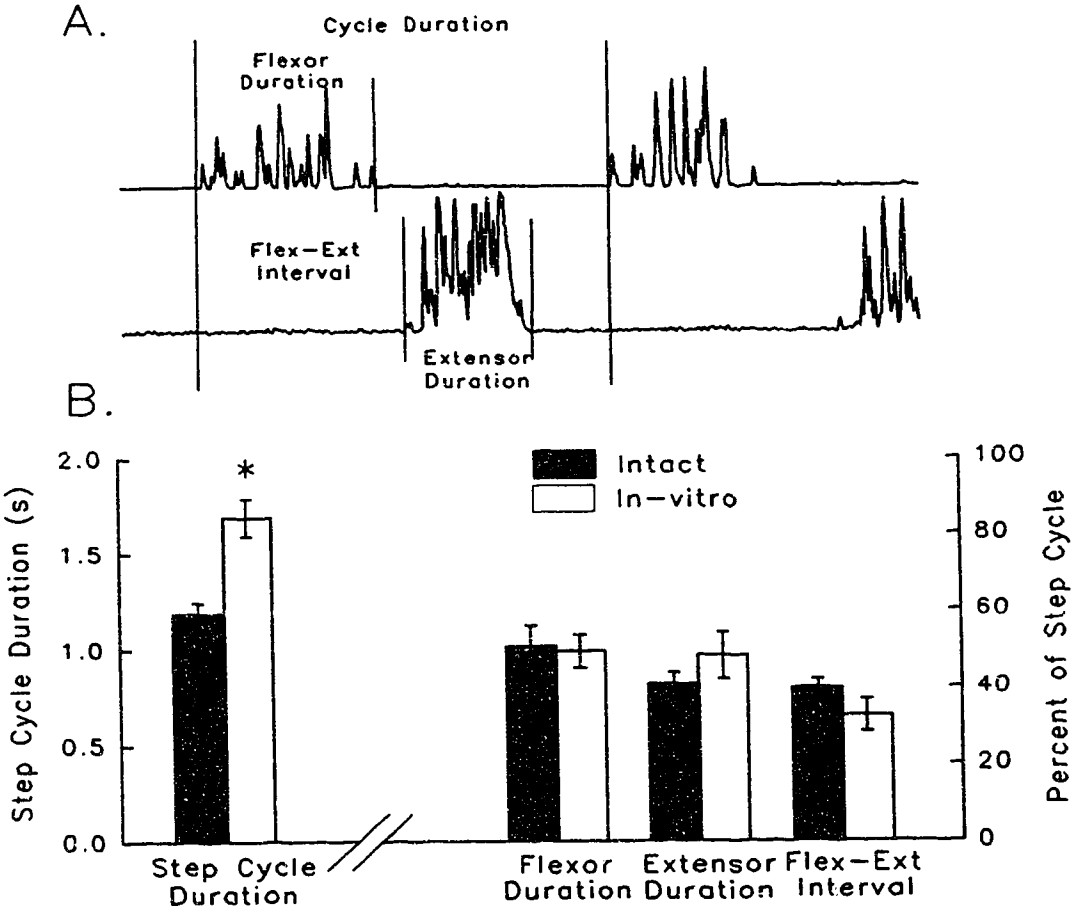

Fig. 6 Locomotor patterns. A) EMG traces showing the points where gait analysis parameters are measure. B) Gait analysis parameters (mean ±SE) are plotted for intact and in-vitro preparations. Flexor duration, extensor duration, and flex-ext interval are mormalized to the step cycle duration (*two-sided t test $p<0.01$).

2.4 Discussion

The EMG's recorded in the intact animal were similar in some respects to those recorded by Székely et al. in newts (Triturus cristatus) but differed in other respects (Székely et al. 1969). Particular differences were noted in the activation of LD and EU muscles during the step cycle. They noted that LD and EU muscles showed bursts of activity during both flexion and extension. In contrast we have never seen LD nor EU active during the flexor phase. Székely et al. reported that all muscles showed variability in their duration and pattern of activation and this variability depended upon the position of electrodes in the muscle. Although electrode placement affects the pattern of activation, it can not account for all changes in activity patterns seen during locomotion. The output patterns seen during locomotion show cycle to cycle variations. We have seen the BR activation change from a single burst per step cycle to a double burst from one step cycle to the next (fig 3). This variability is only present in the flexor muscles, while extensors always show a single burst during the extensor phase of the step cycle.

The variability in flexor activation is similar to that seen in the knee flexor group of cat, and human (for review see Grillner, 1981). Perret and Cabelguen (1980) demonstrated the plasticity of this pattern of activation in a bifunctional muscle, semitendinosis, during locomotion in the cat. In their figure 1C, semitendinosis changes from a double bursting muscle to a single bursting muscle from one step to the next. This same change in activity is seen in the BR muscle of the mudpuppy (Fig. 3), although the BR in the mudpuppy is a monoarticular muscle. It is interesting to note that the BR of the in-vitro preparation rarely shows the second burst seen in the intact animal, although it can overlap the EU burst as seen in figure 2C.

A number of possibilities exist for explaining the dual burst in the intact animal and

the lack of it in-vitro. Perhaps some afferent feedback is absent in the in-vitro preparation. In the hindlimb of the cat, the hip has been shown to provide important afferent input during locomotion (Grillner and Rossignol, 1978). In the in-vitro preparation the shoulder musculature and associated afferents have been removed resulting in some loss of afferent input from the moving limb. This explanation seems unlikely in light of Székely et al. (1969) experiments with deafferented newts. Alternatively, descending input from higher centres to the central pattern generator may be important in eliciting the double burst pattern. In the in-vitro preparation the brainstem is left intact while descending input from structures rostral to the diencephalon have been removed. Finally, one could argue that NMA induced locomotion is not 'real' locomotion but is rather a component of real locomotion. Although direct evidence supporting this suggestion is lacking, simplified patterns have been seen in other in-vitro preparations. Smith et al. (1988) recorded EMG's from the neonatal rat in-vitro and showed alternating flexor and extensor patterns from sartorius and semimembranosus respectively. Sartorius in the adult cat usually displays a complex pattern of activation during walking (Grillner, 1981), which is not seen in any of the records shown by Smith et al. More work needs to be done to discern what factors contribute to the second burst in the flexor muscles during the extensor phase and why it is absent in the in-vitro preparation.

The in-vitro preparation is made to locomote with a bath application of the excitatory amino acid N-methyl aspartate (NMA). Magnesium is required during the bath application of NMA, consistent with an NMDA mediated response. The NMDA receptor/channel complex is blocked at resting membrane potential by Mg^{2+} (Nowak et.al, 1984), and this reversible block endows the NMDA receptor/channel complex with its unique current voltage curve which can promote oscillation of the membrane potential

and bursting (for review see Watkins and Collingridge, 1989). Without magnesium both flexor and extensor muscles are tonically active but show no bursting.

The dose response curve for NMA (fig 4) is similar in some respects to those obtained by Brodin and coworkers (Brodin et al., 1985) in the lamprey. It has a characteristic plateau at concentrations above 200μM in the presence of magnesium, and at this plateau the frequency of bursting rarely goes above about 2Hz. However the curve we obtained in magnesium-free medium does differ from that of Brodin and Grillner (1986). With no magnesium in the bath they found a steep linear relationship between burst frequency and NMA concentration. In the mudpuppy the characteristic response in magnesium free medium was tonic firing of both flexors and extensors but no bursting was ever seen. Magnesium is also not required for bursting in the Xenopus embryo after application of NMDA (Dale and Roberts, 1984). Recently, Atsuta et al. (1990) have induced locomotion in the neonatal rat preparation with brainstem stimulation and found that in a magnesium free bath the preparation could still be made to locomote but at lower stimulus intesities. Finally, Smith et al. (1988) have plotted dose response curves for NMDA application in the neonatal rat preparation. No plateau occurs in the dose response curve, but their doses may be below those needed for a maximal response.

Since the discovery of the potentiating effect of glycine on the NMDA response in cultured mouse neurons (Johnson and Ascher, 1987), the role of glycine in the central nervous system has expanded. It is now believed that glycine receptor activation is an absolute requirement for activation at the NMDA receptor/channel complex (Kleckner and Dingledine, 1988; Thomson, 1990). This implies that some basal level of glycine is present in-vivo. Recent evidence suggests that in mammals, glycine levels in the cerebrospinal fluid in-vivo are 8 - 13 μM (Ferraro and Hare, 1985; Skilling et al., 1988).

However, in in-vitro experiments the glycine mediated potentiation of the NMDA response is variable (Ascher and Johnson, 1989). This is not the case in our in-vitro preparation. We observed a consistent increase in step cycle frequency after the co-administration of glycine and NMA (Fig 5). The step cycle frequency (1/step cycle duration) for the intact animal was 0.84 ± 0.04 Hz (Figure 6), for the in-vitro with glycine and NMA 0.80 ± 0.06 Hz (Figure 5), and for the in-vitro preparation with NMA only 0.59 ± 0.04 Hz (Figure 5 and 6). This potentiation of step cycle frequency was large enough to bring the step cycle frequency in-vitro into the range seen in the intact animals. There was no statistical difference ($p<0.01$) in step cycle frequency between the intact animals and the in-vitro preparations with NMA and glycine.

The burst patterns were generally similar in the intact and in-vitro preparations preparations. The step cycle duration was significantly less in the intact animal than in the in-vitro preparation without glycine but, the duration was substantially reduced by the addition of glycine in-vitro. After normalization, the durations of the flexor and extensor bursts were not statistically different, with the flexor occupying approximately 50% of the step cycle in both conditions. The duration of the interval from the onset of flexion to the onset of extension was also not statistically different.

In conclusion, the in-vitro mudpuppy offers some interesting advantages over some of the current in-vitro preparations used to study the cellular mechanisms of locomotion. We are currently working on developing this preparation for use in intracellular studies to elucidate mechanisms used in the control of walking in adult vertebrates.

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CHAPTER 3

AN IN VITRO PREPARATION OF THE MUDPUPPY FOR SIMULTANEOUS INTRACELLULAR AND ELECTROMYOGRAPHIC RECORDING DURING LOCOMOTION

3.1 Introduction

In vitro preparations of the vertebrate spinal cord were first developed over 40 years ago (Eccles, 1946; Araki et al., 1953). Since that time the advantages of in vitro over in vivo preparations have been well documented (Kudo, 1978; Bagust and Kerkut, 1981). However, vertebrate in vitro preparations only began to be used for the investigation of locomotor mechanisms in the early 1980's (Poon, 1980; Cohen and Wallén, 1980; Grillner et al., 1981; Roberts and Kahn, 1982). Many of the early preparations were based on lower vertebrate models with relatively simple nervous systems. The reduced cell number of the lower vertebrate spinal cord made circuit analysis easier, while the spinal cord's small size and short diffusion distance made these in vitro preparations especially robust. However, these lower vertebrates swim like a fish rather than walk, raising the question of how similar these locomotor mechanisms are to those in higher vertebrates. Mammalian in vitro spinal cord preparations have also been developed (Bagust and Kerkut, 1981; Shapovalov et al., 1981), but only in the last few

A version of this chapter has been published. Wheatley, M. and Stein, R.B. (1992). Journal of Neuroscience Methods 42: 129-137.

years have mammalian preparations been adapted for investigating locomotor mechanisms (Kudo and Yamada, 1987; Smith and Feldman, 1987; Smith et al., 1988). These mammalian preparations walk rather than swim and do have a spinal cord architecture characteristic of higher vertebrates. However, to maintain viability in vitro, neonatal animals (less than 7 days old) must be used, whose locomotor system may still be undergoing developmental changes. As well, the ability to record from interneurons during locomotion in these preparations has yet to be demonstrated. We have developed an in vitro preparation of the mudpuppy (Necturus maculatus) with the long term viability and simplicity (Fankhauser, 1952) of lower vertebrate preparations as well as the morphological characteristics and locomotor capabilities of higher vertebrate preparations. The mudpuppy is an aquatic amphibian that normally walks with an alternating quadrupedal gait. It has the advantage of being poikilothermic and can therefore be maintained in vitro for long periods of time at reduced temperatures. With application of excitatory amino acids to the bath the animal can be made to locomote in vitro (Wheatley et al., 1991).

This report demonstrates that it is possible to record intracellularly from interneurons while recording electromyographic (EMG) activity from forelimb flexor and extensor muscles during locomotion. In most in vitro preparations the spinal cord is either physically separated from the musculature (Bagust and Kerkut, 1981) or all movement is blocked with neuromuscular blocking agents (Roberts and Kahn, 1982). These precautions are taken to ensure that there is no movement of the preparation while recording intracellularly. However, when all locomotor movements are blocked, all feedback related to the movement is also blocked. Although this afferent feedback is not required to generate the basic locomotor rhythm, it may play an important role in

modifying the locomotor pattern (eg., Grillner and Zangger, 1984). The in vitro mudpuppy preparation allows for simultaneous intracellular and electromyographic recordings, while maintaining some movement related afferent feedback.

3.2 Methods

3.2.1 In vitro preparation

Adult mudpuppies (Necturus maculatus, body length 30-40 cm) were obtained from a local supplier and maintained in an aquarium at 20 - 22°C. Before surgery, animals were anaesthetized with application of 3-aminobenzoic acid ethyl ester (Sigma, 1g - 5g/l) to the water in which the mudpuppy is placed. The dissection was performed as follows: a longitudinal incision was made along the dorsal surface of the animal and epiaxial muscles were removed from the caudal border of the cranium to the sixth cervical vertebrae. The right suprascapular cartilage was removed to expose the brachial plexus and the skin of the upper forelimb was removed. A laminectomy was completed from the first through fifth cervical vertebrae to expose the first five segments of the spinal cord. This was long enough to include all the innervation of the forelimb musculature (Székely and Czéh, 1967). The animals were spinalized by sectioning the spinal cord at the caudal border of the medulla. The vertebral column and right forelimb were then removed from the rest of the body and placed in a petri dish containing 100% oxygenated spinal cord Ringers (NaCl, 115mM; $CaCl_2$, 2mM; KCl, 2mM; $MgCl_2$, 1.0mM; Hepes, 5mM, pH 7.3; glucose, 1g/l). While in the petri dish the brachial plexus was exposed, the paraspinal muscles were removed and the dura mater covering the spinal cord was opened. The dissection takes about 45 minutes to complete.

Once the dissection was complete, the preparation was transferred to a recording chamber (120 ml) and perfused with cooled (15°C) and oxygenated spinal cord Ringers at a flow rate of 3 - 10 ml/min. In the recording chamber the spinal cord was stabilized by pinning the vertebral column to the Sylgard Resin (Dow Corning) which coated the

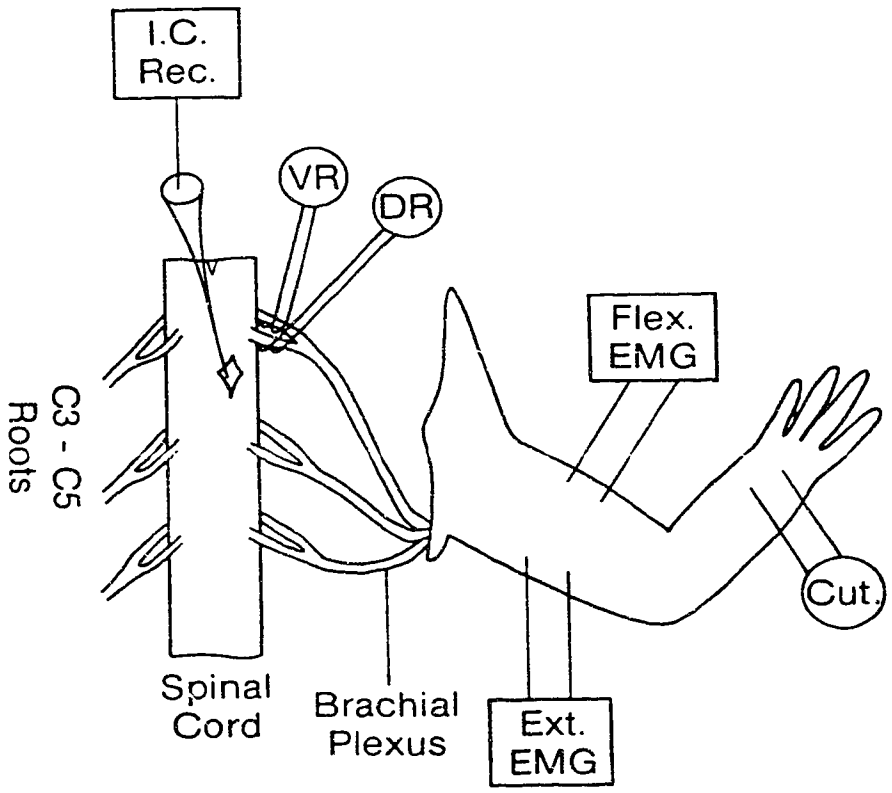

Fig. 1 In vitro preparation from the mudpuppy, consisting of isolated spinal cord and forelimb attached by the brachial plexus. VR (ventral root), DR (dorsal root), IC (intracellular recording), Flex EMG (electromyographic activity from brachialis muscle), Ext EMG (electromyographic activity of the extensor ulnae muscle), Cut (cutaneous stimulating electrodes).

base of the bath. The forelimb was stabilized by pinning the procoracoid cartilage to the base which was coated with Sylgard (Fig. 1).

After a recovery period of one hour the preparation shows a withdrawal reflex to pinching of the limb. At this point animals may display some spontaneous locomotion, but the steps are infrequent and sequences are rarely longer than a few steps. Locomotion is chemically induced by application of 30μM N-methyl D-aspartate and facilitated with 5μM D-serine (NMDA and D-serine, Sigma) to the bath (Wheatley et al., 1992).

3.2.2 Stimulation and recording

EMG recordings were made using bipolar stainless steel wires (50μm) inserted in forelimb flexor and extensor muscles (Brachialis and Extensor ulnae respectively). Bipolar ventral and dorsal root stimulating electrodes are made from teflon coated silver wire (75μm), bared and bent so that the tip has a 100 - 200μm hook. Cutaneous stimulating electrodes are also made from teflon coated silver wire and are inserted percutaneously into the dorsum of the foot using a 30 gauge hypodermic needle to test for cutaneous input from this region to interneurons and motoneurons during locomotion. Intracellular recordings from interneurons were made using glass microelectrodes (borosilicate glass, Sutter Instruments; resistance $\approx$ 100Mohms), filled with 2M potassium acetate. Only cells with membrane potentials greater than -45mV were used.

3.2.3 Data analysis

Intracellular records were filtered (DC to 5kHz) while EMG records were filtered (10 - 300Hz) and rectified before being digitized and stored on a computer for later analysis.

Spike-triggered averages and crosscorrelations of electromyographic (EMG) records were triggered from the intracellular action potentials of interneurons during locomotion (see below). Interneurons were classified using three criteria: their response to afferent stimulation, their spike-triggered average to ongoing EMG, and their phase of firing during the step cycle. The phase of firing of a rhythmic interneuron was computed by triggering an average of the filtered (10 - 300Hz) and rectified EMG of the forelimb flexor and extensor muscles for 2 seconds before the spike and 2 seconds after. A minimum of 300 sweeps were required in order to assess the crosscorrelation.

The digitally smoothed crosscorrelations of both the flexor and extensor EMG tend to approximate damped sinusoids out of phase with one another (see Fig. 6). If the interneuron always fired action potentials at the midpoint of the flexor phase of the step cycle, its spike would coincide with the positive peak of the flexor correlation. The positive peak of the flexor correlation would approximate the midpoint of the flexor phase of the step cycle, while the positive peak of the extensor correlation would approximate the midpoint of the extensor phase of the step cycle. In an idealized step cycle the midpoint of the flexor phase of the step cycle corresponds to a phase value of 0.25, while the midpoint of the extensor phase corresponds to a phase value of 0.75 (Fig. 2). With two perfect sinusoids in antiphase the positive peak of the flexor correlation would coincide with the negative peak of the extensor correlation. However, there was often some asymmetry between the flexor and extensor correlations. If the flexor and extensor crosscorrelations were asymmetric, the phase values for interneurons were computed separately for the flexor and extensor correlation and the values were averaged. The maximum difference between the two computed values was 0.16 with a mean difference of 0.06 and a standard error of 0.011. These criteria may not be valid for interneurons

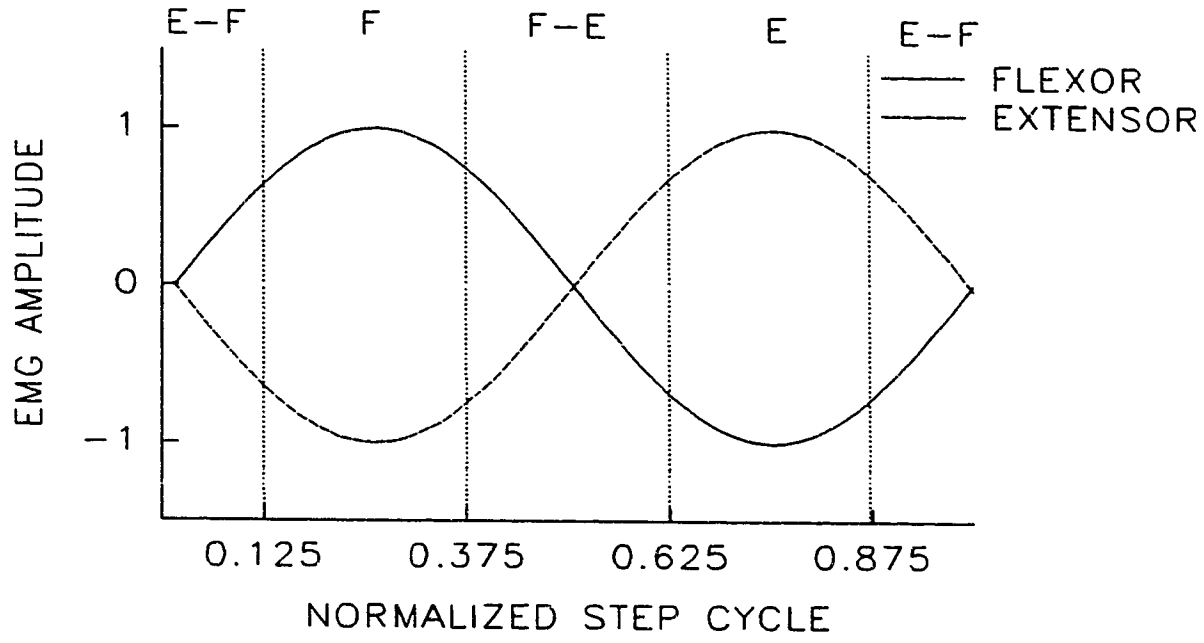

Fig. 2 Schematic diagram of crosscorrelation used for classifying the phase of an interneuron during locomotion. Interneurons are classified as either F (flexor, 0.125 - 0.375), F→E (flexor → extensor, 0.375 - 0.625), E (extensor, 0.625 - 0.875), or E→F (extensor → flexor, 0.875 - 1.0 and 0 - 0.125).

which burst twice during one step cycle as has been demonstrated in the cat (Orlovskii and Feldman, 1972; Baev et al., 1979; Ichikawa et al., 1991), however we have not found any double bursting interneurons. Once the phase value for the interneuron was calculated, interneurons were classified as either flexor (F, phase = 0.125 - 0.375), flexor → extensor (F→E, phase = 0.375 - 0.625), extensor (E, phase = 0.625 - 0.875), or extensor → flexor (E→F, phase = 0.875 - 1.0 and 0 - 0.125).

To determine if the interneuron being recorded was connected to the motoneuron pools of the brachialis and extensor ulnae muscles or receiving common input from some source, the spikes were used to trigger an average of EMG on a short time scale. These spike-triggered averages (STA) of ongoing rectified and filtered (10-300Hz) EMG require a minimum of 500 spikes for proper assessment. The EMG is averaged for 100ms prior to the intracellular action potential and 100ms after. A facilitation or inhibition was said to be significant if the peak of the correlation was more than 2 standard deviations greater than the distribution of the EMG for the 100ms period prior to the trigger.

The latency of the STA was computed as follows. The average locomotor EMG 200ms prior to the stimulus was subtracted from the STA so that the STA was centred around zero. Using a least squares estimator, a simple linear regression was fitted to the data beginning at the rising edge (for excitation) or falling edge (for inhibition) of the correlation (Zar, 1974). The equation of the regression line is computed using the time coinciding with the rising edge of the action potential as the origin (t=0). The time was then evaluated for v=0 which gives a measure of the latency of the facilitation or inhibition (see Fig. 3).

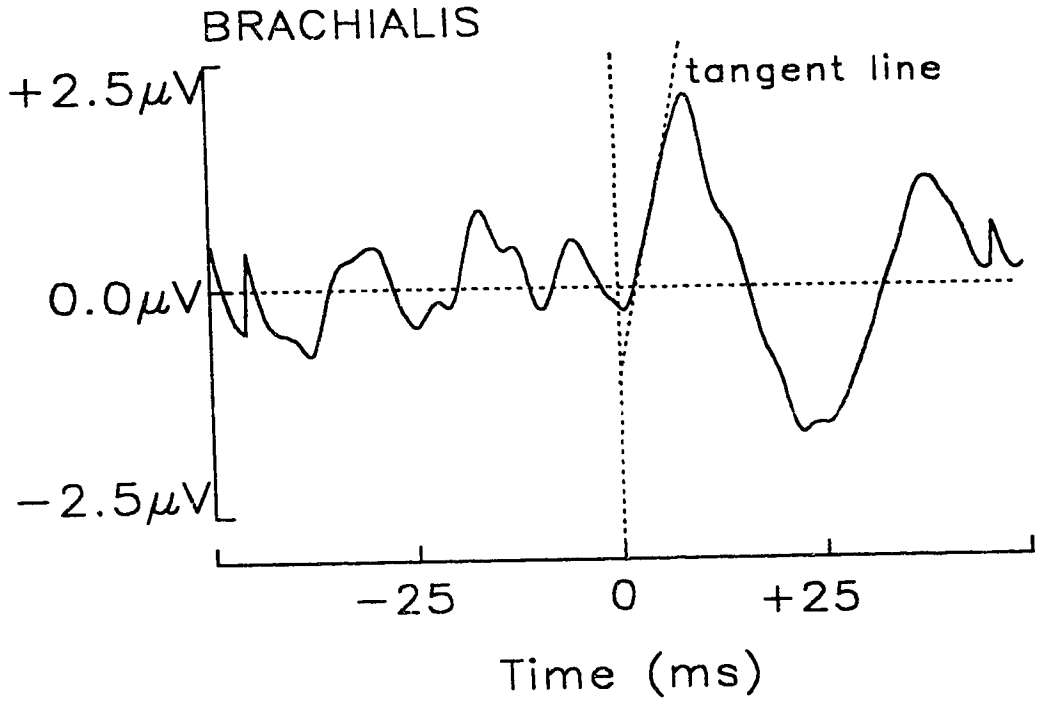

Fig. 3 Method used for determining the latency of response in a spike-triggered average (STA). The tangent line is a linear regression fitted to the data beginning at the rising or falling edge of the correlation so as to minimize the mean square errors. The intercept of the fitted line (v=0) estimates the latency to the response.

3.3 Results

3.3.1 Locomotor patterns

Locomotion was induced with a bath application of 30μM NMDA and 5μM D-serine. D-serine is a co-agonist at the NMDA receptor channel complex and potentiates the NMDA response as well as reduces the desensitization of the receptor to NMDA (Johnson and Ascher, 1987; Vyklický et al., 1990; Benveniste et al., 1990). After the application of both NMDA and D-serine the locomotor pattern builds over the first minute reaching a steady state within 3-5 minutes. At this point the preparation would continue to produce rhythmic activity for 60 - 120 minutes (variable) at which time the locomotor pattern would become less rhythmic. The preparation was then washed with spinal cord Ringers for 30 - 60 minutes. After the onset of the wash locomotor bursting would decrease in amplitude, stopping completely within 1 - 2 minutes. When the wash was complete, the NMDA application could be repeated. The preparation could be made to walk repeatedly over the 36 - 48 hour period.

It was important to cool the preparation during the experiment. If the preparation was maintained at 25°C it rarely lasted beyond 8 - 10 hours. However, if the preparation was cooled and maintained at 15°C, the preparation would often show locomotor activity 36 - 48 hours after the start of the experiment (figure 4). Interestingly, cutaneous and dorsal root evoked reflexes could often be maintained for over 100 hours with no apparent change in threshold or amplitude. For reasons which are not yet clear the flow rate of the superfusate should be kept below 10ml/min. At high flow rates the preparation did not last beyond 1 - 2 hours.

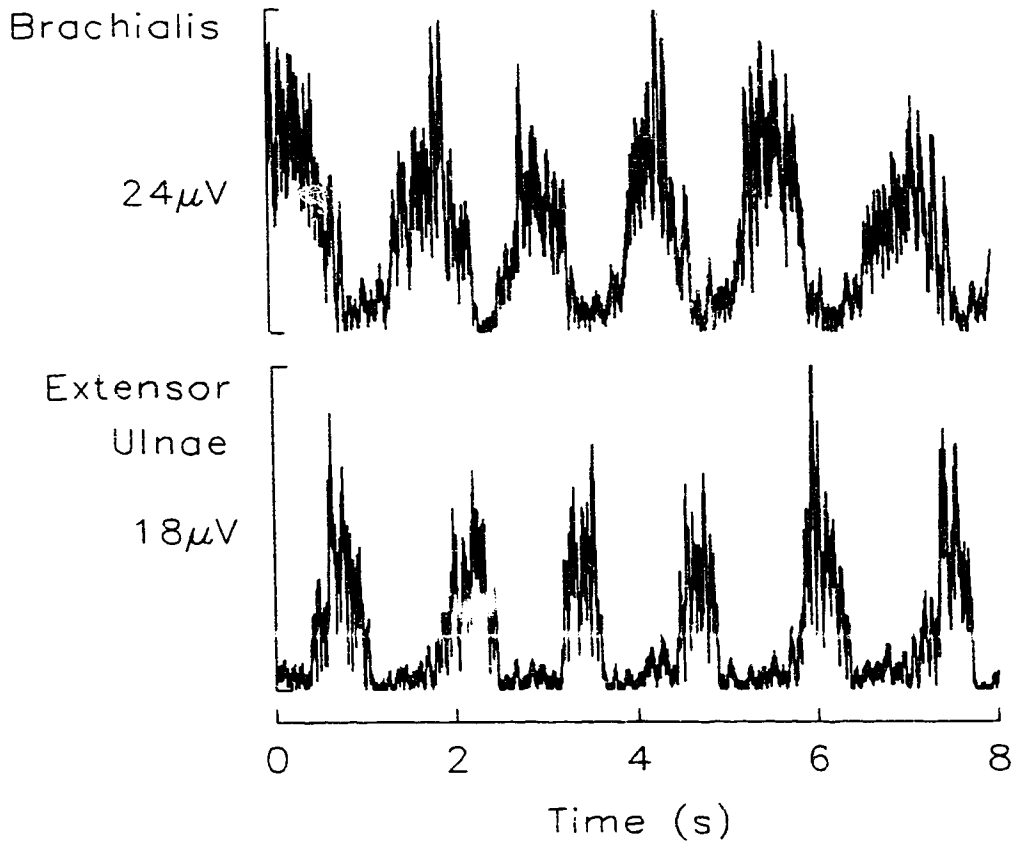

Fig. 4 Locomotor EMG recorded 36 hours after the start of the experiment. NMDA (25μM) was used to initiate locomotion.

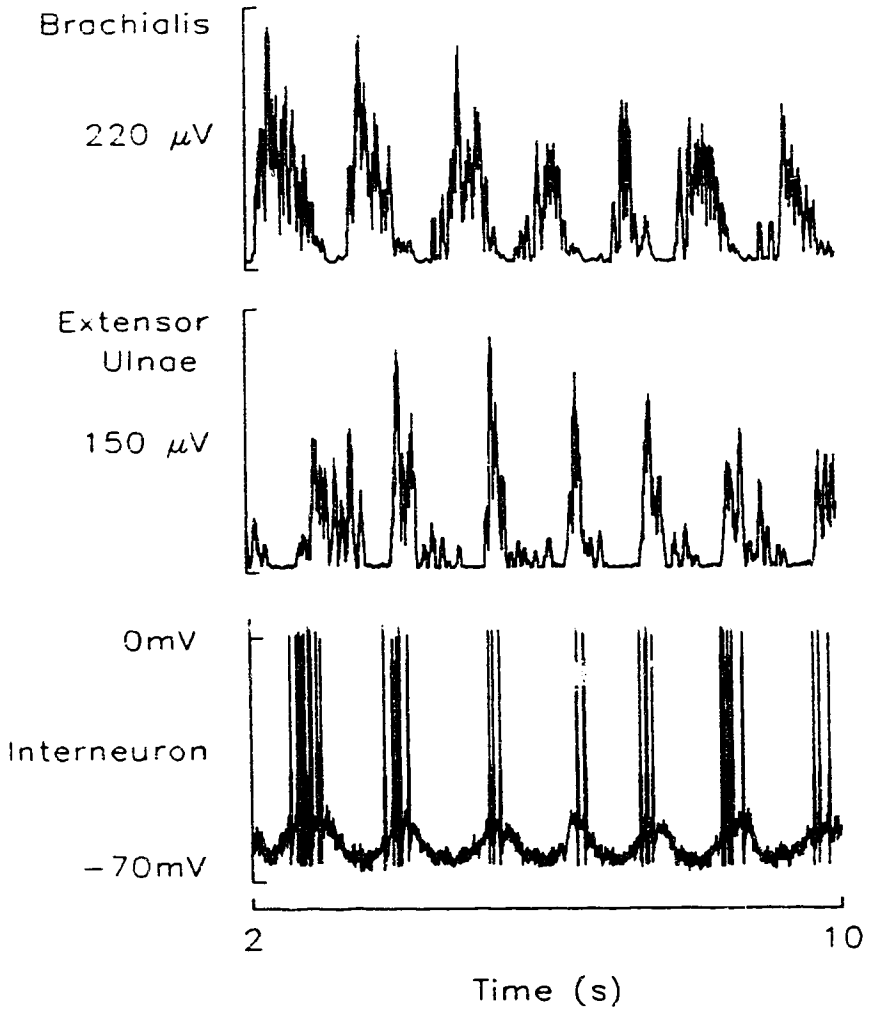

Fig. 5 Simultaneous intracellular and EMG recordings during locomotion. Brachialis (elbow flexor), Extensor ulnae (elbow extensor).

3.3.2 Intracellular recording during locomotion

One of the advantages of this preparation is the ability to record intracellularly from interneurons while the animal is moving. This is accomplished by mechanically isolating the forelimb from the spinal cord. During the dissection, shoulder and paraspinal muscles are removed and the vertebral column is pinned to the base of the recording chamber. The only connection between the spinal cord and the moving forelimb is the brachial plexus. Once these precautions are taken, long term (30 - 60min) intracellular recordings from both motoneurons and interneurons can be made (see Fig. 5).

Many interneurons show membrane potential oscillations locked in time to the locomotor cycle, indicating a strong drive from the central pattern generator (Jordan, 1981). The peak to peak amplitude of observed locomotor drive potentials (LDP) ranged from less than 1mV to 15mV. Not all rhythmically active cells show membrane potential oscillations. However, most of the cells showing LDP's have action potentials superimposed on the LDP. Those that do not have spikes superimposed on the LDP presumably have LDP's which are subthreshold or alternatively the spike generating mechanism of the cell may have been damaged by the intracellular penetration.

A typical crosscorrelation is shown in figure 6. This is the crosscorrelation for the interneuron shown firing in figure 5. This interneuron had a mean resting membrane potential of -62mV with a large locomotor drive potential. This interneuron was classified as an F→E interneuron with a phase of activity of 0.51. After averaging 3100 sweeps the

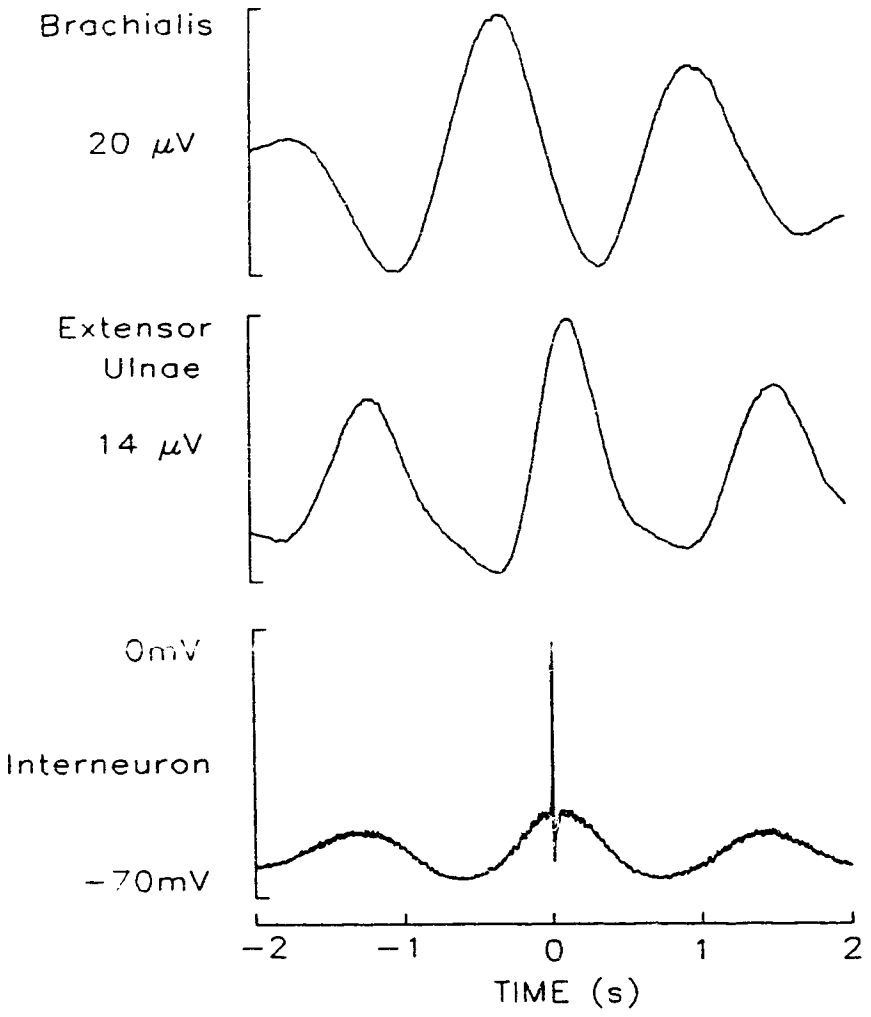

Fig. 6 Crosscorrelation of the locomotor EMG with the firing of an interneuron (same interneuron displayed in figure 5). Crosscorrelation is completed using 400 spikes. This cell is classified as an F→E interneuron with a phase of activity of 0.51.

locomotion.

The identification of rhythmic cells as interneurons is made using several criteria. First, motoneurons are identified by antidromic activation from the ventral root, while interneurons are identified by the absence of antidromic activation. While it is possible that ventral root stimulation may not antidromically activate all motoneurons, it is less likely when intracellular depolarization is coupled with supramaximal ventral root stimulation. Second, if an interneuron responds to dorsal root stimulation the cell must show a graded excitatory postsynaptic potential (epsp) or inhibitory postsynaptic potential (ipsp) and must not follow high frequency (300Hz) stimulation of the dorsal root. This second step is used to ensure that the recording is not from the axon of a dorsal root ganglion cell.

The ability to record intracellularly from interneurons while recording EMG activity allows for the use of intracellular action potentials of the interneuron as a trigger for averaging the EMG. This can result in large post-spike facilitations and inhibitions of ongoing EMG activity. Figure 7 shows the action potential from an interneuron used as the trigger for a STA of EMG from the elbow flexor (Brachialis) and extensor (Extensor ulnae) muscles during locomotion. This particular interneuron was classified as an extensor (E) interneuron with a phase of activity of 0.63. The action potentials in this interneuron are correlated with a clear postspike facilitation of flexor EMG and an inhibition of extensor EMG. The shortest latency post spike facilitation or inhibition we have found has been 5ms.

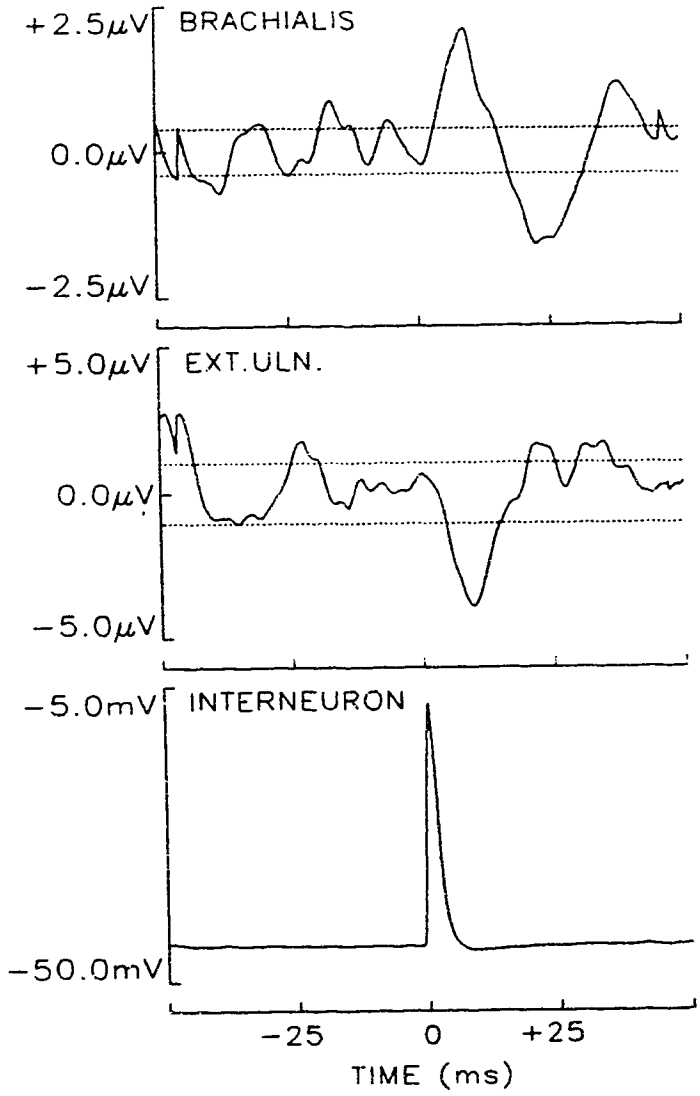

Fig. 7 Spike triggered average response of an E (extensor) interneuron with a phase of 0.63. This interneuron shows a facilitation of flexor EMG (Brachialis) and an inhibition of the extensor EMG (Extensor ulnae). Average of 520 sweeps. Dotted lines indicate ± 1 SD of the prespike EMG.

3.4 Discussion

This preparation offers some advantages over previously described in vitro locomotor preparations. The preparation is viable for relatively long periods of time in vitro. For comparison, the neonatal rat preparation survives for 7 - 8 hours (Smith and Feldman, 1987), while the hemisected mouse spinal cord survives for 8 - 10 hours (Bagust and Kerkut, 1981). The mudpuppy when cooled to 15°C remains viable for more than 36 hours. Interestingly, the threshold and amplitude of the cutaneous and dorsal root evoked reflexes may be unchanged after 72 hours while the locomotor pattern generating mechanism does not survive much longer than 36 - 48 hours. If NMDA is applied after 48 hours, tonic co-contraction of forelimb muscles results. Small inhibitory interneurons in the central nervous system are especially susceptible to anoxia (Davidoff et al., 1967; Davidoff et al., 1967; Francis and Pulsinelli, 1982; Arregui and Barer, 1980). Perhaps such interneurons are important in the CPG, and these small locomotor interneurons may be selectively lesioned by anoxia after 36 - 48 hours in vitro while interneurons subserving reflexes are spared. Alternatively interneurons controlling locomotion may be located deeper in the spinal cord than interneurons subserving reflex pathways, making them the first to be lesioned by a progressive anoxia. This could reflect an important difference between interneurons controlling reflexes and interneurons controlling locomotion.

The flow rate in the bath is an important parameter to regulate during the experiment. We found that at flow rates above 10 - 20 ml/min the preparation would survive only 1 - 2 hours. This observation has also been noted by those working with the neonatal rat preparation (John Greer, unpublished observations). Presumably something

is washed out or not allowed to equilibrate at the higher flow rates. Bagust and Kerkut state that their neonatal mouse spinal cord preparation survives best at higher flow rates (Bagust and Kerkut, 1981). However, their "high" flow rates are only 10ml/min, which is in agreement with our results.

Another advantage of the in vitro mudpuppy preparation is the fact that the animal normally walks with an alternating quadrupedal gait as do higher vertebrate quadrupeds. In contrast the lamprey and Xenopus embryo swim rather than walk (Cohen and Wallén, 1980; Grillner et al., 1981; Roberts and Kahn, 1982). It should be noted that some of the afferent input from the "intact" forelimb is removed when the shoulder musculature is removed. This means that the complement of afferent information coming from the forelimb may not be complete. However, deafferentation in these animals has little effect on the timing and complexity of the motor output (Székely et al. 1969; and unpublished observations).

One of the more significant advantages this preparation offers is the ability to make simultaneous intracellular and EMG recordings while the animal is moving. This can be accomplished because the spinal cord is pinned down and mechanically isolated from the moving forelimb. The ability to record intracellularly from an interneuron, using its action potential to trigger an average of the ongoing EMG, provides the experimenter with a method for identifying the interneuron's output. It should be mentioned however that not all postspike facilitations and inhibitions are indications of linkage between the CPG and the motoneuron pools. Alternatively the facilitation or inhibition may result from common input to the rhythmic interneuron and the motoneuron pool (Kirkwood, 1979). For example, an ascending tract cell could receive corollary input from the CPG during locomotion (Arshavsky and Orlovsky, 1976), in which case it may show a post spike

facilitation to one of the motoneuron pools when in fact they are not functionally connected. Some of these complications may be resolved by intracellular dye injection to identify tract cells from segmental interneurons.

The in vitro mudpuppy preparation offers some advantages over other in vitro preparations for the investigation of locomotor mechanisms. The preparation's long term viability, its relatively simple nervous system, its partially intact afferent system, and the ability to record intracellularly while recording EMG could provide a method for answering some of the pharmacological and physiological questions which can not be answered with other preparations.

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CHAPTER 4

THE ACTIVITY OF INTERNEURONS DURING LOCOMOTION IN THE IN-VITRO NECTURUS SPINAL CORD

4.1 Introduction

The spinal cord is capable of generating rhythmic locomotor output in the absence of phasic afferent input (for review see Rossignol et al., 1988). Yet, despite numerous demonstrations of this ability, relatively little is known about the activity of interneurons responsible for generating the locomotor rhythm in walking vertebrates. The most complete description of the activity of interneurons, their interconnections and input-output properties during locomotion has come from work on lower vertebrate preparations that swim rather than walk (Grillner et al., 1991; Roberts et al., 1986). Do these results apply to walking vertebrates or are modifications and added complexity needed to sustain legged locomotion? The answers are largely unknown.

When constructing models of central pattern generation the appropriate output of the circuit should be generated using known firing patterns and connections of interneurons within the circuit. A number of individuals have recorded from rhythmically active interneurons during locomotion in the cat in an attempt to classify interneurons according to their phase of firing within the step cycle (Orlovskii and Feldman, 1972;

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Edgerton et al., 1976; Baev et al., 1980; Hishinuma and Yamaguchi, 1990; Terakado and Yamaguchi, 1990; Yamaguchi 1991; Ichikawa et al., 1991; Viala et al., 1991). The range of phase values for these interneurons varies greatly. Most authors group interneurons as either flexor or extensor after correlating their firing patterns with the neurogram of the step cycle (Orlovskii and Feldman, 1972; Edgerton et al., 1976). However, estimates of the proportion of interneurons which can be classified as mixed or transitional, with a phase of firing that correlates with the transitional period between flexion and extension or extension and flexion, range from 10% (Baev et al., 1980) to 55% (Gelfand et al., 1988). These transitional interneurons may be essential when constructing the circuit for locomotion (Gelfand et al., 1988).

Input to the central pattern generator must also be described from descending, afferent, and propriospinal fibres that initiate, adapt and modulate the output of the circuit. The effects of afferent input onto interneurons of the CPG are often inferred from intracellular recordings of motoneurons or electromyographic records of muscles activity. However, motoneurons are not considered part of the CPG (Grillner et al., 1988). Therefore, afferent input can modify the output via direct effects on motoneurons independent of their effect on the interneurons of the CPG. There are few direct recordings of the effects of afferent input on rhythmically active interneurons of higher vertebrates during locomotion (Hishinuma and Yamaguchi, 1990; Shefchyk et al., 1990; Yamaguchi, 1991).

To study the effects of afferent input onto interneurons during locomotion it is important to monitor the afferent input while the animal is locomoting and not while the animal is at rest. Reflexes which result in flexion at rest may result in flexion or extension

function of afferent input onto interneurons during locomotion in walking vertebrates. Recently, a class of interneurons that have direct projections to motoneurons and that receive Group I and II input have been identified in laminae VI and VII of the L4 segment (Edgley and Jankowska, 1987; Cavallari et al., 1987). These interneurons have been shown to respond to stimulation of the mesencephalic locomotor region (Edgley et al., 1988) and at least some of them are rhythmically active during locomotion (Shefchyk et al., 1990). These neurons are functionally separate from a population of interneurons located in laminae IV and V which also respond to group II afferent stimulation. This latter population of group II interneurons do not have group I input nor do they project monosynaptically to the motor nuclei in lamina IX (Edgley and Jankowska, 1987) Functionally, subdividing interneurons according to their projections, input and activity during locomotion is an important technique for classifying interneurons.

Smith et al. (1991) have successfully localized the area generating the respiratory rhythm in the brainstem of the neonatal rat by serially sectioning the brainstem and monitoring the integrity of the respiratory motor output. We have attempted to localize the central pattern generator for forelimb locomotion in Necturus maculatus (mudpuppy) by making serial sections of the spinal cord while monitoring the electromyographic records of active flexor and extensor muscles. The mudpuppy is an aquatic amphibian which walks with an alternating quadrupedal gait (Wheatley et al., 1992). By reducing the area required for intracellular recording we can effectively increase our yield of functionally important locomotor related cells. We have therefore made intracellular recordings from rhythmically active interneurons within this localized area of spinal cord (Wheatley and Stein, 1992), and attempted to characterize these cells during locomotion according to their phase of activity during the step cycle and their response to afferent stimulation.

4.2 Methods

4.2.1 In-vitro preparation

Thirty one adult mudpuppies (greater than 30cm length) were used for the current experiments. Before surgery, animals were anaesthetized by applying 3-aminobenzoic acid ethyl ester (Sigma, 1g - 5g/l) to their water. Only a brief description of the methods will be given since a detailed outline has been provided in a previous paper (Wheatley and Stein, 1992). After the animal was anaesthetized the first five segments of the spinal cord and attached forelimb were removed and placed in a petri dish which was superfused with cooled (15°C) and oxygenated spinal cord Ringers (NaCl, 115mM; $CaCl_2$, 2mM; KCl, 2mM; $MgCl_2$, 1.0mM; Hepes, 5mM, pH 7.3; glucose, 1g/l). While in the petri dish the brachial plexus was exposed, the paraspinal muscles were removed and bipolar teflon coated silver wires (75μm) were inserted into the elbow flexor (Brachialis) and extensor (Extensor ulnae) muscles for electromyographic (EMG) recording of muscle activity during locomotion. Bipolar teflon coated silver wires (75μm) were also inserted percutaneously into the dorsum of the foot to stimulate cutaneous input from this region. When the dissection was complete the preparation was transferred to an in vitro recording chamber where bipolar ventral and dorsal root stimulating electrodes were hooked under the ventral roots leaving the 3rd and 4th segments (C3, C4) and the dorsal root entering the 3rd segment (C3). Ventral and dorsal root electrodes were made from teflon coated silver wire (75μm) bared for 100μm at the tip.

4.2.2 Localizing the central pattern generator

An attempt was made to localize the central pattern generator (CPG) for

locomotion. In ten animals locomotion was induced using a bath application of NMDA (25μM) and D-serine (5μM) while the EMGs from the elbow flexor and extensor muscles were monitored. Using fine dissecting scissors, serial sections of the spinal cord were made at 5mm intervals starting from either C1, proceeding caudally (n=6) or from C5, proceeding rostrally (n=4). The purpose of the serial sectioning was to localize the minimal area required for generating the locomotor pattern. Locomotion was judged to be "normal" if, the EMG of the flexor (when cutting rostrally) or the extensor (when cutting caudally) was rhythmic, the step cycle duration did not exceed 3 seconds and the peak amplitude of the EMG was at least 50% of the amplitude prior to sectioning. No attempt was made to quantify any deficits which resulted from the sectioning. Using the flexor when cutting rostrally and the extensor when cutting caudally ensures that interrupting the innervation of the muscle is not misinterpreted as a disruption of the locomotor rhythm. As the spinal cord area was reduced, the preparation required longer periods of time to recover from each serial section. Serial sections were made every 5 - 15 minutes until the locomotor pattern was no longer intact. However, when the locomotor pattern recovered, the recovery never took longer than 40 seconds.

4.2.3 Intracellular recording

Locomotion was induced with a bath application of N-methyl D-aspartate (25μM NMDA) and D-serine (5μM). Intracellular recordings from interneurons were made using standard glass (borosilicate) microelectrodes with a resistance of about 50-100 Mohms. We recorded from 50 rhythmically active interneurons. Interneurons were identified by the absence of antidromic activation from the ventral roots and an inability to follow high frequency (300Hz) stimulation of the dorsal root.

A cell classified as an interneuron was further classified according to its phase of activity within the step cycle. The phase of activity of an interneuron was calculated by crosscorrelating the intracellular action potentials with the ongoing filtered (10-300Hz) and rectified locomotor EMG. Details of this technique and the rationale for subdividing the step cycle are given in Wheatley and Stein (1992) and only a brief description will be included below. The step cycle was assumed to begin and end with the onset of the flexor EMG burst; the peak flexion occurred at a phase of approximately 0.25 and peak extension at approximately 0.75 as indicated schematically in Fig. 3. On this basis, interneurons were classified as either flexor (F, phase = 0.125 - 0.375), flexor → extensor (F→E, phase = 0.375 - 0.625), extensor (E, phase = 0.625 - 0.875), or extensor → flexor (E→F, phase = 0.875 - 1.0 and 0 - 0.125) depending upon their phase of activity within the step cycle (Wheatley and Stein, 1992).

Interneurons were further classified according to their input from specific afferent systems. Afferent input from the dorsal root of segment C3 was monitored after graded electrical stimulation of the dorsal root to segment C3. Cutaneous afferents from the dorsum of the foot were stimulated by applying a short train of electrical impulses through electrodes in this area. The threshold for activation of afferents was chosen as the stimulus intensity required to elicit a electromyographic response in either the Brachialis or Extensor ulnae.

4.3 Results

4.3.1 Localizing the central pattern generator

Sectioning the spinal cord and monitoring motor output was at best a crude method for localizing the CPG. However, it did reduce the volume for intracellular recording. Each segment of the spinal cord was sectioned three times: first, at the rostral border of the preceding dorsal root; second, midway between the dorsal root and the preceding dorsal root; and third, at the caudal border of the dorsal root. Fig. 1 illustrates some of the sections made to the spinal cord during two experiments. When the sections were made rostrally, from C5 to C2, sections up to and including mid C3-C4 did not affect the rhythm. The section rostral to C4 compromised the motor output to the Extensor ulnae (EU), an elbow extensor, by removing some of the innervation to that muscle. In spite of removing innervation to EU, the locomotor rhythm in Brachialis (BR), an elbow flexor, remained unaffected. The next section at C3 abolished the locomotor rhythm; the extensor muscle was silent while the flexor fired tonically. The locomotor pattern did not recover within 15 minutes and the recording was stopped. Following all previous sections the locomotor pattern recovered within 40 seconds.

In a second set of experiments serial sections were made caudally from C1 to C4. In these experiments the rhythm was severely compromised after serial sections caudal to C2. This would indicate that sections caudal to C3 and rostral to C2 are not required to generate the basic locomotor rhythm. This demonstrates that a 10 - 15 mm length of spinal cord in an adult animal (body length 300 mm) is required to generate locomotion in the forelimb. As a result of these experiments, all intracellular recordings were made from a 10 - 15 mm section of spinal cord spanning the first dorsal root at segment C3

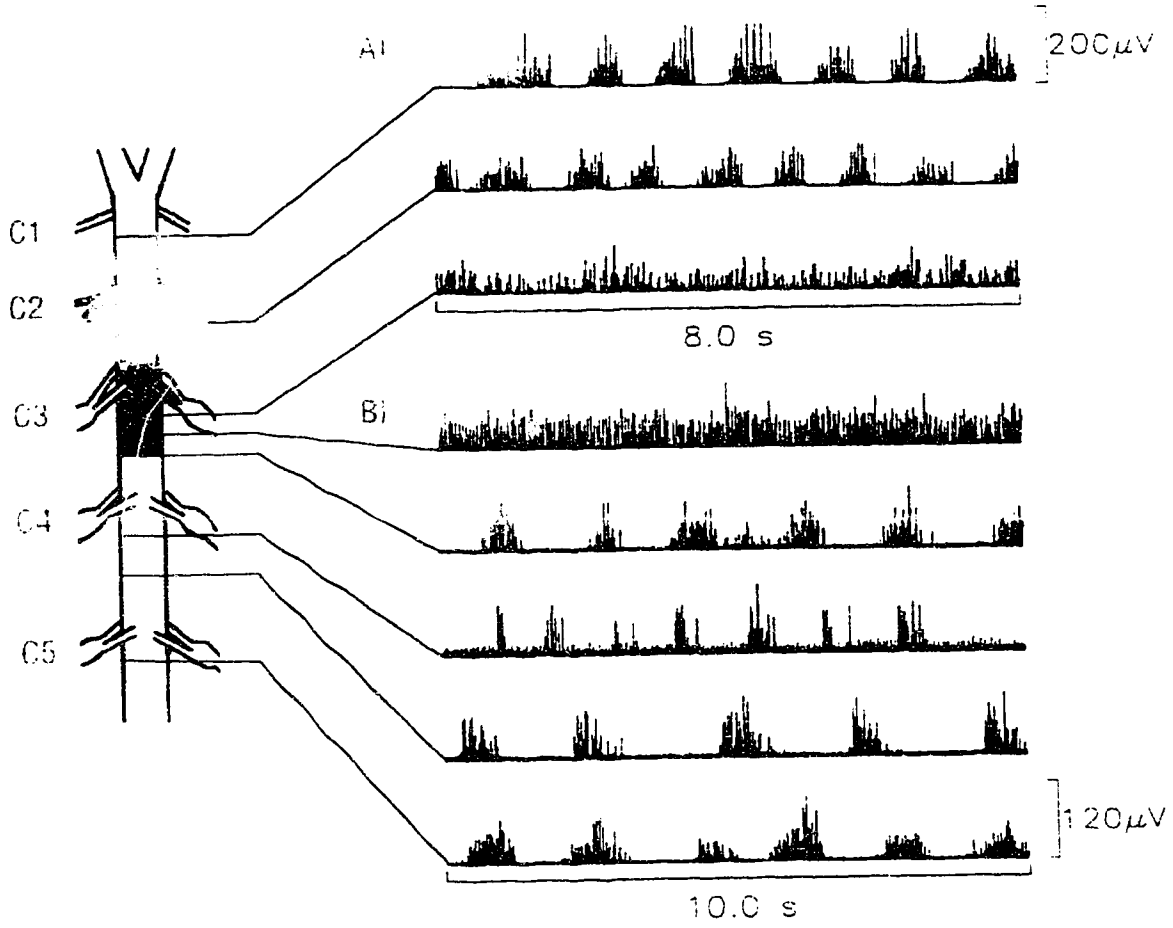

Fig. 1 The CPG is localized by recording EMG activity from Brachialis (BR) and Extensor ulnae (EU) muscles after serial sections of the spinal cord. A) EMG recorded from EU after serial sections of the spinal cord from C1 to C3. B) EMG recorded from BR after serial sections of the spinal cord from C5 to C3. Hatched area indicates the minimal area required for the generation of locomotion in-vitro.

(shaded area in Fig. 1).

4.3.2 Intracellular activity of interneurons

We recorded from 50 rhythmically active interneurons spanning segment C3 during NMDA induced locomotion. Fig. 2A depicts an intracellular interneuronal recording with simultaneous recording of elbow flexor and extensor EMG. Fig. 2B is the crosscorrelation of the action potentials from the interneuron shown in Fig. 2A above with the ongoing locomotor EMG from the elbow flexor and extensor muscles.

This interneuron was classified as an F interneuron (phase = 0.23) and was located 200μm below the surface of the cord. This interneuron showed no STA to either the flexor or extensor EMG when 3100 sweeps were averaged (Wheatley and Stein, 1992) and showed no response to electrical stimulation of the dorsum of the foot. However, this interneuron did respond to low level dorsal root stimulation (Fig. 2C) with an inhibitory postsynaptic potential (IPSP). The threshold for evoking the IPSP was 1.2 times threshold. This particular interneuron had a large locomotor drive potential (LDP) although not all rhythmically active interneurons had LDP's.

4.3.3 Phase of activity of interneurons

Rhythmically active interneurons with a wide range of firing characteristics have been found in the mudpuppy spinal cord. Although the range of firing frequencies demonstrated by rhythmically active interneurons covered a wide range (1 - 300Hz), all bursts of action potentials were phase locked to the locomotor cycle. Interneurons whose firing was not correlated to locomotion, were not included in the present study. Interestingly, no interneurons with double bursts were found during the experiments. The

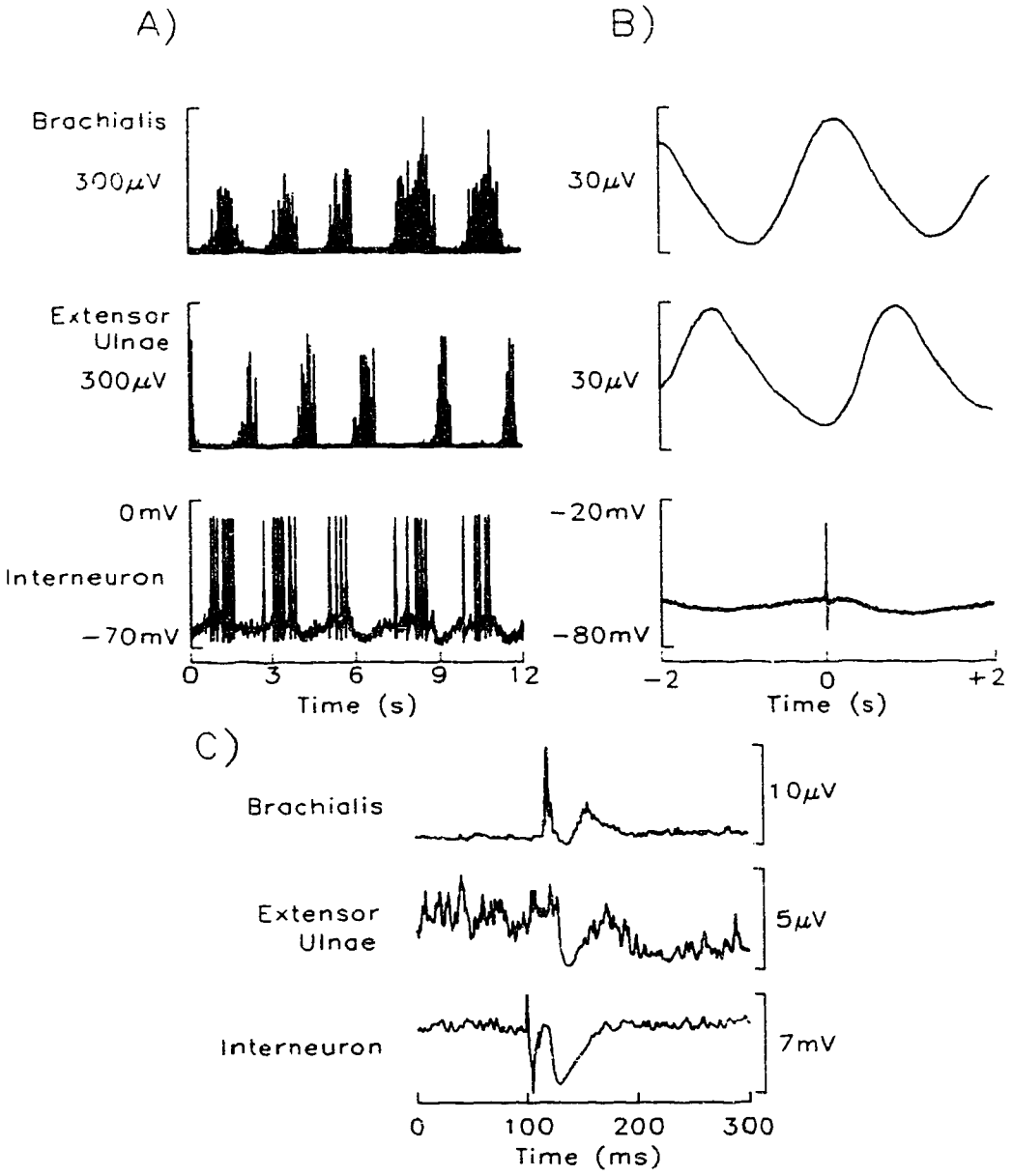

Fig. 2 A) Intracellular recording from a flexor (F) interneuron (phase = 0.23) with the simultaneous EMG recorded from Brachialis and Extensor ulnae muscles. B) The corresponding crosscorrelation of the action potentials from the flexor interneuron in A to the Brachialis and Extensor ulnae muscles. C) Interneuron responds to dorsal root (C3) stimulation with an ipsp during locomotion. The EMG response to stimulation is also shown. The negative deflection preceeding the stimulus in the interneuronal recording is an intracellular current injection used to monitor the input resistance of the interneuron.

duration for the burst of action potentials characteristic of each interneuron varied greatly. Some cells fired only one action potential every locomotor cycle while others fired continuously, modulating their firing frequency in phase with the locomotor cycle. The mean percentage of the step cycle occupied by interneuronal action potentials was 56% ± 35%. Burst patterns were of two general forms. A minority of interneurons were active throughout the step cycle with an increase in firing frequency during one part of the cycle (28%, 14/50). Most interneurons fired a single burst of action potentials during one part of the step cycle and were silent for the rest of the step cycle (72%, 36/50). There were no significant differences in the percentage of the step cycle occupied by the burst between categories of interneurons (F, E, E→F, F→E). Some interneurons showed rhythmic locomotor drive potentials without superimposed action potentials. Clearly, the absence of action potentials superimposed upon a rhythmic LDP could mean that the LDP was subthreshold for spike generation or the spike generating mechanism of the cell was damaged during the intracellular penetration.

A histogram showing the phase of activity for all interneurons is plotted in Fig. 3. The phase histogram for interneuronal firing is bimodal with peaks in the F→E and E→F transitional periods. In fact 68% (34/50) of all rhythmically active interneurons are classified as transitional bursting cells with the largest number classified as F→E interneurons (44%, 21/50). For comparison, included in Fig. 3 is a histogram of the phase of firing of motoneurons during locomotion. The majority (91%, 10/11) of motoneurons burst in either the flexor (F) or extensor (E) periods of locomotion.

4.3.4 "Tonic" cells

We have recorded from a group of rhythmically active interneurons (n=5) with

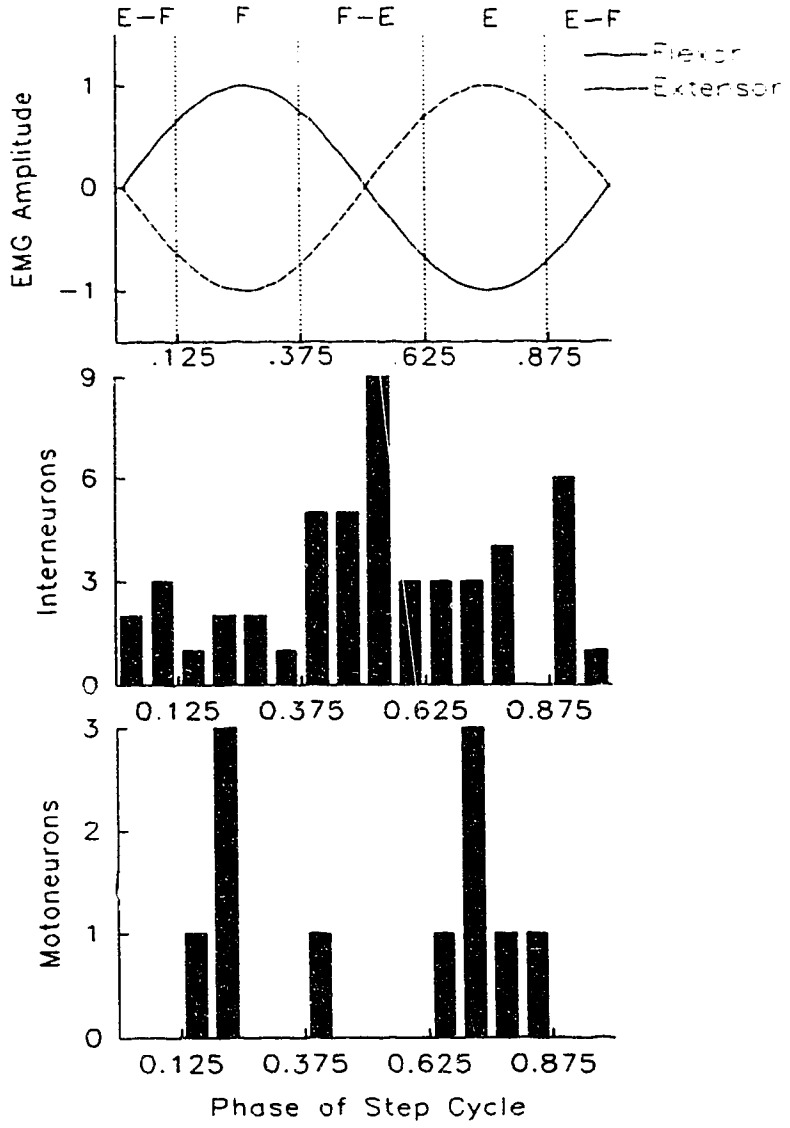

Fig. 3 Histogram of the phase of firing of interneurons and motoneurons. (Top) A schematic representation of the crosscorrelation of flexor and extensor muscles during locomotion (Wheatley and Stein, 1992). (Middle) Histogram of the phase of firing for interneurons during locomotion (n=50). (Bottom) Histogram of the phase of firing for motoneurons during locomotion (n=11).

a long burst duration which varied but usually lasted about 10 seconds. The action potentials were superimposed on a large (10mV) membrane depolarization. These cells did not receive any afferent input and were located at about the same depth as the rhythmically active interneurons that were phase locked to the locomotor cycle. The periods of action potentials from these tonic cells were highly correlated with periods of reciprocal flexor and extensor bursts of the muscles active during locomotion (Fig. 4). In fact, when the tonic cells fired, locomotor rhythmicity was strong and when they did not, rhythmicity was reduced or absent.

4.3.5 Location of interneurons

The location of interneurons, measured as the depth from the dorsal surface of the spinal cord, was recorded in some experiments. The dorso-ventral diameter of the cervical spinal cord of the mudpuppy ranges from 800 - 1200μm. Rhythmically active interneurons were found 200 - 700μm below the surface with a mean depth 395 $\pm$ 160μm. For comparison, rhythmically active motoneurons were found 550 - 750μm below the dorsal surface of the spinal cord with a mean depth of 650 $\pm$ 83μm. The population of interneurons whose location was accurately recorded (n=9) was too small to assess the significance of differences in location between specific categories of interneurons (F, E, E$\rightarrow$F, F$\rightarrow$E).

4.3.6 Afferent input to interneurons during locomotion

Afferent input to interneurons was tested after stimulating the dorsal root of the 3rd spinal segment or the skin covering the dorsum of the foot. 29% (10/35 tested) of all interneurons tested responded with a graded excitatory or inhibitory postsynaptic

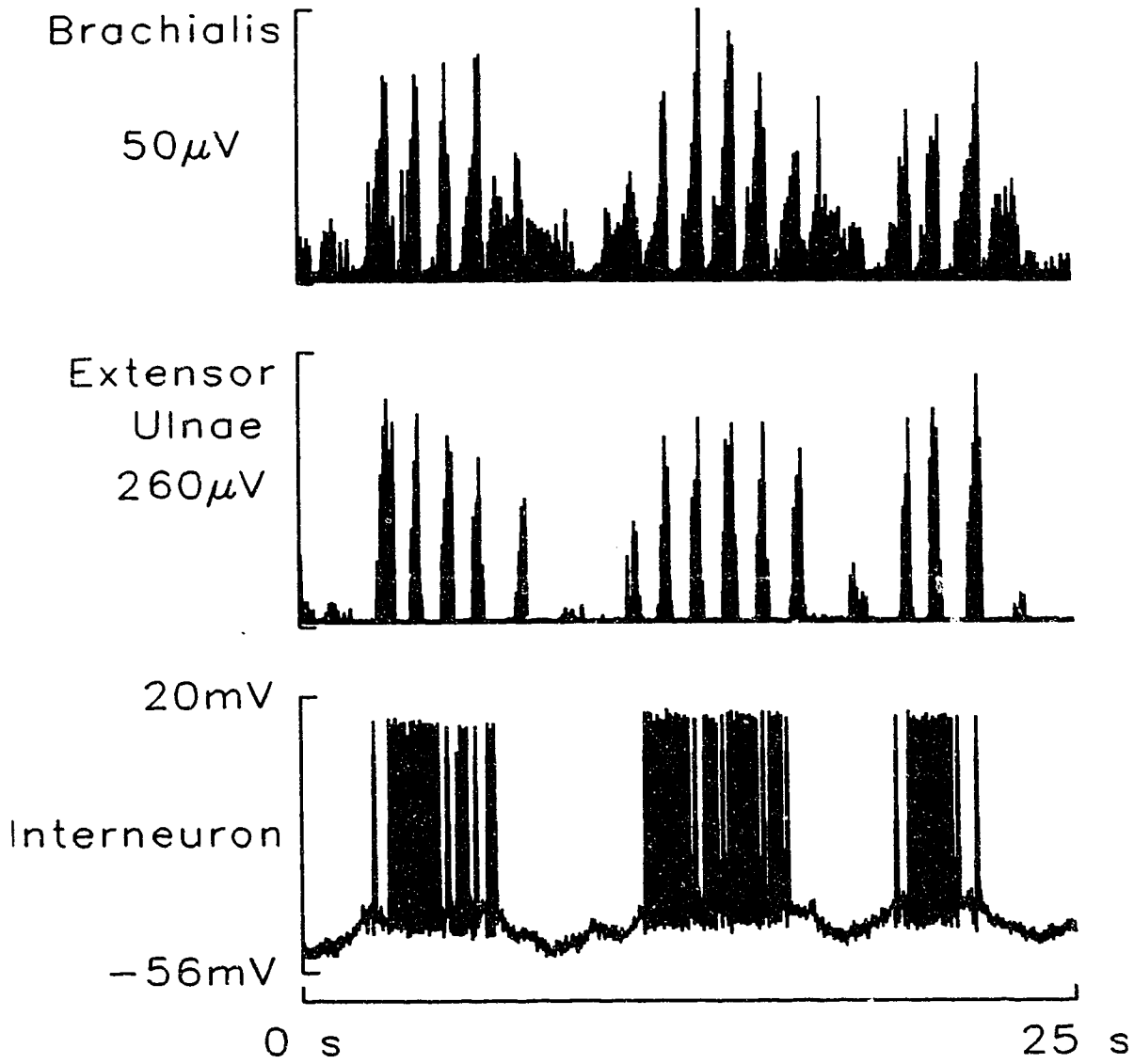

Fig. 4 "Tonic" cells. Intracellular recording from an interneuron that fires continually during locomotion and is silent when locomotion ceases. Top traces are EMG recordings from Brachialis and Extensor ulnae muscles during locomotion.

	FLEXOR	FLEX→EXT	EXTENSOR	EXT→FLEX
NUMBER	6	22	10	12
MEAN DEPTH	450μm (n=4)	380μm (n=4)	250μm (n=1)	? (n=0)
STA (flexor)	0	3(+ve)	3(+ve)	1(+ve), 3(-ve)
STA (extensor)	0	3(+ve)	1(-ve)	0
DR input (excite)	0/4	3/14	0/7	0/10
DR input (inhibit)	4/4	0/14	0/7	1/10
Cutaneous (excite)	0/4	3/14	0/7	0/10
Cutaneous (inhibit)	1/4	0/14	0/7	0/10

Table 1 Summary of interneurons.

potential to stimulation of the skin or the dorsal root.

When interneurons were subdivided into categories according to their phase of activity, significant differences in afferent input were noticed. All flexor interneurons tested responded to afferent stimulation and all of these responses were inhibitory (100%, 4/4 tested). In contrast all flexor → extensor interneurons which responded to afferent stimulation responded with excitatory postsynaptic potentials (36%, 5/14 tested). Extensor interneurons never showed any response to afferent stimulation while only one extensor → flexor interneuron responded to afferent stimulation. Remember that only afferent input from the 3rd segment dorsal root and the skin was tested. This leaves open the possibility of afferent input from the 4th or 5th dorsal roots ipsilaterally or the 3rd, 4th or 5th roots contralaterally. In some experiments both ipsilateral and contralateral input from the 3rd dorsal root was tested and interneurons were found which responded only to contralateral dorsal root stimulation (EPSP) supporting the possibility that some rhythmically active interneurons may be sensitive only to contralateral input.

4.4 Discussion

4.4.1 Localizing the central pattern generator

In the present study, a 10 - 15 mm section of spinal cord spanning segment C3 was found to be essential for the generation of the locomotor rhythm. This corresponds to an area consisting of less than 2 segments of the spinal cord. This distribution of the mudpuppy pattern generator overlaps the rostral portion of the forelimb motoneuron pools. Motoneuron pools supplying forelimb muscles are distributed from the rostral half of C3 to the caudal half of C5 (Székely and Czéh, 1967; Stephens and Holder, 1985). In the lumbosacral enlargement of the cat a "leading" area located at L3-L5 has been postulated as the site of the "generator mechanism" responsible for "determining the rhythm of oscillations in the whole spinal hindlimb center" (Deliagina et al. 1983; Gelfand et al., 1988). This "leading" area also overlaps the rostral portion of the motoneuron pools innervating the hindlimb.

Deliagina et al. (1983) were careful not to suggest that only L3-L5 segments are capable of rhythm generation. On the contrary, they found that more caudal segments could generate the rhythm but that these segments were less capable of rhythm generation. They suggest that rhythm generation is distributed throughout the lumbosacral spinal cord of the cat. This distributed pattern generator or network of pattern generators has also been proposed for lower vertebrates such as the lamprey (Grillner et al., 1991). In contrast, the pattern generator to the forelimb of the mudpuppy is not widely distributed, and can be localized to an area covering parts of two spinal cord segments. These results are consistent with transplantation experiments in Urodela conducted by Székely (1963). By transplanting segments from the brachial level of the

spinal cord to the thoracic level, Székely showed that only certain segments of the spinal cord were capable of generating motor output (Székely, 1963).

4.4.2 Classification of interneurons during locomotion

Tonic interneurons.

Many models of pattern generation require some tonic excitation to enable them to burst rhythmically. The "tonic" cells outlined above could assume this role. We have very little information on these tonic cells other than they do not respond to any afferent input that we tested. In addition we used their action potentials to spike trigger average the EMG from brachialis and extensor ulnae and have found no post spike facilitations or inhibitions from these cells to the muscles investigated (Wheatley and Stein, 1992). This suggests that they are removed from the motoneuron pools of the Brachialis and Extensor ulnae muscles by more than one synapse as would be expected of cells which could drive the locomotor rhythm.

Transitional interneurons.

In the present study a large proportion of rhythmically active interneurons have been classified as "transitional interneurons" with their peak of activity spanning the region between the flexor and extensor phases of locomotion. In contrast, the activity of motoneurons closely parallels the flexor or extensor phases of the locomotor cycle. As might be expected, the pattern of motoneuronal activity corresponds to the pattern of muscle activity recorded from the forelimb of the mudpuppy during locomotion (Wheatley et al., 1992). A number of questions need to be addressed. First, are the interneurons

that we recorded part of the CPG? Second, are transitional interneurons common in other locomotor preparations? Third, what role do transitional interneurons play in pattern generation?

There are certain caveats that must be considered in relation to the first question. Although some of the interneurons recorded may constitute part of the CPG, others may be driven rhythmically by it or by afferent input. For example, tract cells and propriospinal interneurons may also discharge rhythmically in time with the locomotor cycle and yet are not considered part of the CPG. We must also be careful not to label interneurons as receiving no afferent input. We have only tested the afferent input from the dorsum of the foot and the 3rd dorsal root. We can not exclude the possibility that these interneurons receive input from other dorsal roots. Finally, we must be careful when interpreting the activity patterns of rhythmically active interneurons when under the influence of NMDA. The NMDA receptor/channel complex conducts calcium and displays voltage dependent behaviour. The combination of these two properties can lead to pacemaker activity in neurons devoid of any synaptic input (Grillner and Wallén, 1985).

Comparison with other preparations.

In the lamprey and Xenopus embryo the phase of activity of rhythmically active interneurons has been shown to coincide with one of two phases of activity indicative of rhythmically active motoneurons during locomotion (Buchanan and Grillner, 1987; Buchanan et al., 1989; Roberts et al., 1986). This may not seem surprising considering that undulatory swimming in these species is reciprocal with only two phases of muscle activity. However, in the dogfish which also has only two phases of muscle activity, the phase of activity of rhythmically active interneurons does not always coincide with the two

phases of activity of motoneurons. A minority of rhythmically active interneurons in the dogfish could be classified as transitional bursting cells with activity patterns which span the two phases of locomotion (Mos et al., 1990a, 1990b).

In the cat, the number of transitional interneurons may depend on the presence of cyclic afferent input. It has been hypothesized that afferent input may convert flexor and extensor interneurons into transitional interneurons (Baev et al., 1980). Orlovskii and Feldman (1972) recorded extracellularly from rhythmically active interneurons in the decerebrate walking cat and found that 37% of all interneurons could be classified as mixed or transitional bursters, with their activity beginning in one phase of the step cycle and ending in another. More recently Gelfand et al. (1988) repeated these experiments and classified 55% of the interneurons as transitional interneurons. In contrast, Edgerton et al. (1976) recorded from rhythmically active interneurons during fictive locomotion and reported that the majority of interneurons had firing patterns which coincided with either the flexor or extensor bursts during locomotion. In a similar study Baev et al. (1980) also found that the majority of rhythmically active interneurons could be classified as either flexor or extensor and that only 10% could be classified as mixed or transitional bursting interneurons. Baev et al. (1980) suggest that the underestimate of transitional or mixed interneurons (10%) in their study could have been due to the lack of cyclic afferent input in the fictive preparation and that cyclic afferent inflow may be required to convert flexor and extensor interneurons into mixed interneurons. In the in-vitro mudpuppy the forelimb is moving, supplying some cyclic afferent input. It is possible that the preponderance of transitional interneurons in the in-vitro mudpuppy results from their being driven into that phase by cyclic afferent inflow.

Models of pattern generation.

A role for transitional interneurons has recently been proposed by Baev and Shimansky (1992). They suggest that afferent inflow from the moving limb is greatest at the transition periods between swing and stance and between stance and swing (Baev and Shimansky, 1992) and yet these are the points in the step cycle when afferent stimulation has little or no effect on the step cycle duration (Duysens, 1977; Lennard, 1985; Baev and Shimansky, 1992). They suggest that a "model" of afferent inflow is produced by the CPG, reducing afferent transmission at these points. In this way the "real" afferent inflow can be compared to the "model" afferent inflow and used as an error signal to correct the aberrant step cycle when the two signals are temporally dissociated. According to this hypothesis the large number of transitional interneurons are required to control timing in the step cycle.

An alternative role for transitional interneurons has been proposed by Shik and Orlovsky (1976) after modifications of the "ring" hypothesis (Székely, 1965; Kling and Székely, 1968). They suggest that spatial and temporal summation from a relatively large number of rhythmically active interneurons in the transition areas of the step cycle account for the rapid phase transition between swing and stance and between stance and swing.

Finally, Gelfand et al. (1988) have included transitional interneurons in their modification of the half center model of pattern generation (Jankowska et al., 1967a,b). The transitional interneurons in their model are responsible for supplying delayed excitation to the antagonist half center. In doing so, these interneurons have replaced the need for "fatigue" within the interneuronal circuits.

It is too early to define the exact function of transitional interneurons in the CPG

of the mudpuppy or other vertebrate spinal cords. More experiments are needed to characterize the transitional, flexor, extensor, and tonic interneurons morphologically and to determine the physiological connections between them. The in-vitro mudpuppy preparation promises to be the most amenable preparation for doing the necessary experiments to decide between possible models for vertebrate walking.

4.5 References

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CHAPTER 5

REFLEX MODULATION DURING LOCOMOTION IN THE MUDPUPPY (Necturus maculatus)

5.1 Introduction

It is generally agreed that the basic locomotor rhythm is generated within the central nervous system (Delcomyn, 1980), but afferent input plays a vital role in adapting the basic locomotor rhythm to suit environmental conditions (reviewed by Rossignol, et al., 1988). When an obstacle touches a cat's paw during the swing phase of locomotion, the limb flexes to lift the paw over the obstacle. When the same obstacle is encountered during the stance phase of locomotion the animal does not flex the limb but rather extends the limb. Thus, the reflex response to a cutaneous stimulus varies in sign and amplitude at different times in the step cycle (Forssberg, et al., 1975; Prochazka, et al., 1978; Andersson, et al., 1978). This modulated response has been referred to as the "stumbling corrective reaction" (Forssberg, 1979).

Phase dependent cutaneous reflexes have been demonstrated in animals throughout the vertebrate phylum. Species as phylogenetically diverse as the Xenopus embryo and the human have been shown to modulate cutaneous input during locomotion (Sillar and Roberts, 1988; Duysens et al., 1990; Yang and Stein, 1990). Cutaneous

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reflexes from the limb of the cat have been thoroughly investigated. Cutaneous stimulation of the dorsum of the paw results in a variety of electromyographic (EMG) responses. During the swing phase of locomotion, when the limb is lifted off the ground, the stimulus is followed by short latency (10ms) and long latency (25ms) excitatory responses in the knee flexor muscles as well as a relatively small amplitude, short latency (10ms) excitatory response in the knee extensor muscles. During the stance phase of locomotion, when the limb is supporting the animal, the same stimulus results in a short latency (10ms) inhibition of the knee extensor muscles followed by a longer latency (25-50ms) excitation; the knee flexor muscles are silent (Forssberg, 1981). Similar responses are seen in spinal, mesencephalic and intact cats with notable quantitative differences (Forssberg, 1979). Drew and Rossignol (1987) have also monitored phase dependent cutaneous responses in the forelimb of the cat and found that the responses were similar to those recorded from the hindlimb.

The source and mechanism of the reflex modulation have been a source of debate. The first clue as to the source of the modulation came after it was shown that the modulated response was intact in a 'fictive' cat (Anderson et al., 1978). In the absence of movement related feedback the source of reflex modulation had to be of central origin. Central modulation of afferent input has been postulated to occur at four distinct but not mutually exclusive sites (reviewed by Sillar, 1991). The proposed first site of sensory gating is at the level of the incoming afferent terminals (Gossard and Rossignol, 1990). Primary afferent depolarization (PAD) via interposed GABAergic interneurons is believed to result in a decrease in transmitter release during certain phases of the step cycle (reviewed by Rudomin 1990). A second possible level of sensory gating is believed to occur at the level of the interneurons interposed between

the afferent terminals and the motoneurons. Sillar (1991) further classifies these cells as non-modulated interneurons and modulated premotor interneurons (Shefchyk et al., 1990; Sillar and Roberts, 1992). The central pattern generator (CPG) is believed to provide not only the drive to the motoneuron pools but also to modulate the efficacy of afferent transmission via its effects on specific interneurons (Moschovakis et al., 1991). The final proposed site of reflex modulation is at the level of the motoneuron. Motoneurons have a higher probability of firing action potentials when excitatory postsynaptic potentials (EPSP) are superimposed on the depolarized phase rather than the hyperpolarized phase of the locomotor drive potential.

We have developed an in-vitro preparation of the mudpuppy (Necturus maculatus) for investigating some of the mechanisms controlling reflex modulation in walking vertebrates (Wheatley et al., 1992). With this preparation we can compare the response to cutaneous stimulation of the dorsum of the foot in the intact animal and in-vitro preparation. The bath application of NMDA to the in-vitro preparation, produces walking which has many of the characteristics shown by the intact animal walking freely on an aquatic treadmill (Wheatley et al., 1992).

5.2 Methods

A total of thirty-four adult mudpuppies were used for both the intact and in-vitro experiments. Mudpuppies were obtained from a local supplier and maintained in an aquarium at room temperature. Sixteen animals were used for the intact animal experiments while eighteen animals were used for the in-vitro experiments. All animals were anaesthetized with a bath application of 3-aminobenzoic acid ethyl ester (Sigma, 1g - 5g/l) prior to any invasive procedures. Some of the methods have been described in previous papers and will only be outlined below (Wheatley et. al., 1992; Wheatley and Stein, 1992; Wheatley et. al., 1993;).

5.2.1 Intact animal

Sixteen adult mudpuppies (body length 25-40 cm) were used during the intact animal experiments. During the experiments animals were kept in a solution of tap water pretreated with sodium thiosulphate to reduce chlorine levels to those tolerated by the animal. Before dissection, animals were anaesthetized with a bath application of 3-aminobenzoic acid ethyl ester (Sigma, 1g - 5g/l).

Bipolar electromyographic (EMG) recordings were made from the intact animals while walking on an aquatic treadmill. EMG electrodes were made from 75μm teflon coated silver or stainless steel wire and inserted in two forelimb muscles: brachialis (BR), and extensor ulnae (EU) (Gilbert, 1973). Two EMG wires were inserted into each muscle after an incision in the skin overlying the muscle. The wires were then fed under the skin to the dorsal surface of the animal where they exited and were either attached to a "back pack" sutured to the animal's skin or led directly to the preamplifiers. After recovery from

anaesthesia the animals walked freely on the treadmill. Bipolar teflon coated silver wires (75μm) were inserted percutaneously into the dorsum of the foot so that cutaneous stimuli could be applied during locomotion. The cutaneous stimulus was applied as a train of three pulses with an interpulse interval of 5ms (200Hz) at an intensity of less than 2 times threshold. Threshold was measured as the stimulus intensity required to elicit a reflex response in the elbow flexor (brachialis) at rest. Stimuli were delivered pseudorandomly at a minimum interstimulus interval of 2 seconds and a maximum interstimulus interval of 7 seconds. At stimulation frequencies greater than 1/second, the intracellularly recorded postsynaptic potential (PSP) is significantly attenuated (Simpson, 1976). Each trial was conducted for a minimum of 15 minutes to ensure that at least 200 stimuli were delivered.

5.2.2 In-vitro preparation

Eighteen adult mudpuppies (25 - 40cm length) were used for the in-vitro experiments. After the animal was anaesthetized, as for the intact preparation, the first five segments of the spinal cord and attached forelimb were removed and placed in a petri dish which was superfused with cooled (15°C) and oxygenated spinal cord Ringers (NaCl, 115mM; $CaCl_2$, 2mM; KCl, 2mM; $MgCl_2$, 1.0mM; Hepes, 5mM, pH 7.3; glucose, 1g/l). While in the petri dish the brachial plexus was exposed, the paraspinal muscles were removed and bipolar wires were inserted for electromyographic (EMG) recording of cutaneous stimulation, as described above. When the dissection was complete the preparation was transferred to an in-vitro recording chamber where bipolar ventral and dorsal root stimulating electrodes were hooked under the ventral roots leaving the 3rd and 4th segment (C3, C4) and the dorsal root entering the 3rd segment (C3). Ventral and dorsal root electrodes were made from teflon coated silver wire (75μm) bared for 100μm

at the tip.

Locomotion was induced in-vitro with a bath application of N-methyl D-aspartate (25μM NMDA) and D-serine (5μM). Afferent input from the dorsal root was monitored after graded electrical stimulation of the dorsal root entering spinal cord segment C3. Cutaneous input from the dorsum of the foot was monitored by applying a short train of electrical impulses through the bipolar electrodes in the dorsum of the foot as described for the intact animal above. The dorsal root stimulation was applied as a single 0.5ms pulse and was maintained at less than 2 times threshold. Threshold was measured as the stimulus intensity required to elicit a reflex response in the elbow flexor (brachialis) and at least 200 stimuli were required for each trial as in the intact animal experiments.

The bath application of all drugs was maintained by a continuous gravity perfusion at a flow rate of 3 - 5 ml/min. It is important to keep the perfusion flow rate below 10ml/min to maintain optimum viability of the preparation (Wheatley et al., 1992). In another set of experiments (n=3) NMA was added to the bath while the reflex response to cutaneous stimulation was monitored. In these in-vitro preparations the EMG response to cutaneous stimulation of the dorsum of the foot (2XT) was monitored for a short period prior to administration of the drug. The drug was added for a period of 5 minutes before the preparation was washed with spinal cord Ringers. The reflex was continually monitored throughout the drug addition and for periods up to one hour following the administration. The amplitude of the reflex response was then plotted as a percent of the reflex response prior to administration. Four separate combinations were tested: NMA (100μM), NMA (100μM) + bicuculline (20μM), NMA (100μM) + picrotoxin (20μM), NMA (100μM) + strychnine (5μM).

5.2.3 Data analysis

EMG records were differentially preamplified, rectified and filtered (10 - 300 Hz) and stored on a computer's hard disk using an A/D disk storage program (Axotape, Axon Instruments). All channels of data were digitally sampled at 5 or 7 kHz and latter analyzed using a customized software package written by one of the authors (Wheatley, 1992).

Cutaneous and dorsal root stimuli were delivered pseudo-randomly to the dorsum of the foot and to the dorsal root entering segment C3. The step cycle was arbitrarily chosen to begin with the onset of the flexor burst and to end with the onset of the next flexor burst. Each step cycle could be divided into 2 - 10 segments while the stimuli in any given segment could be averaged together and compared to the average response in any other segment. The undisturbed step cycles (step cycles without stimuli) were also averaged so that the mean level of EMG at different phases of the step cycle could be subtracted from the averaged response to stimuli at those phases. This removed the background EMG leaving the response to the stimuli centred at 0mV.

5.3 Results

5.3.1 Cutaneous reflex in the intact animal

The EMG response to cutaneous stimulation of the dorsum of the foot in the intact animal was quite variable. Generally, two reflex responses could be discerned in each of the flexor and extensor muscles. The usual response in the flexor (BR) was a short latency (40-70ms) inhibition at all points in the step cycle, followed by a variable long latency (70-100ms) excitatory response (Fig. 1A). The reflex responses recorded in EU were more consistent than those recorded in BR. A consistent short latency (40-70ms) inhibition could be seen in EU during stance. This was usually followed by a longer latency (70-100ms) excitatory response which was only seen during the stance phase of locomotion (Fig. 1B).

The average data for the intact animals (n=5) are displayed in figure 2. Reflex responses were measured after subtracting the background EMG for a period 100ms before the stimulus. In this way the reflex response could be investigated with the locomotor trend removed. After subtraction, excitatory responses are displayed as values above zero while inhibitory responses are displayed as values below zero. Each point on the graph was plotted as the mean ± the standard error of the mean and a line was drawn through the mean of each point. At the bottom of figures 1 and 2 are the average step cycles for each of the muscles recorded from during the experiments. The step cycle plots were created by taking the average step cycle from the individual experiments, normalizing the cycle duration to 1 and averaging those step cycles together. Step cycles are displayed from the onset of the flexor EMG burst (beginning of swing phase) until the onset of the next flexor burst (end of stance phase).

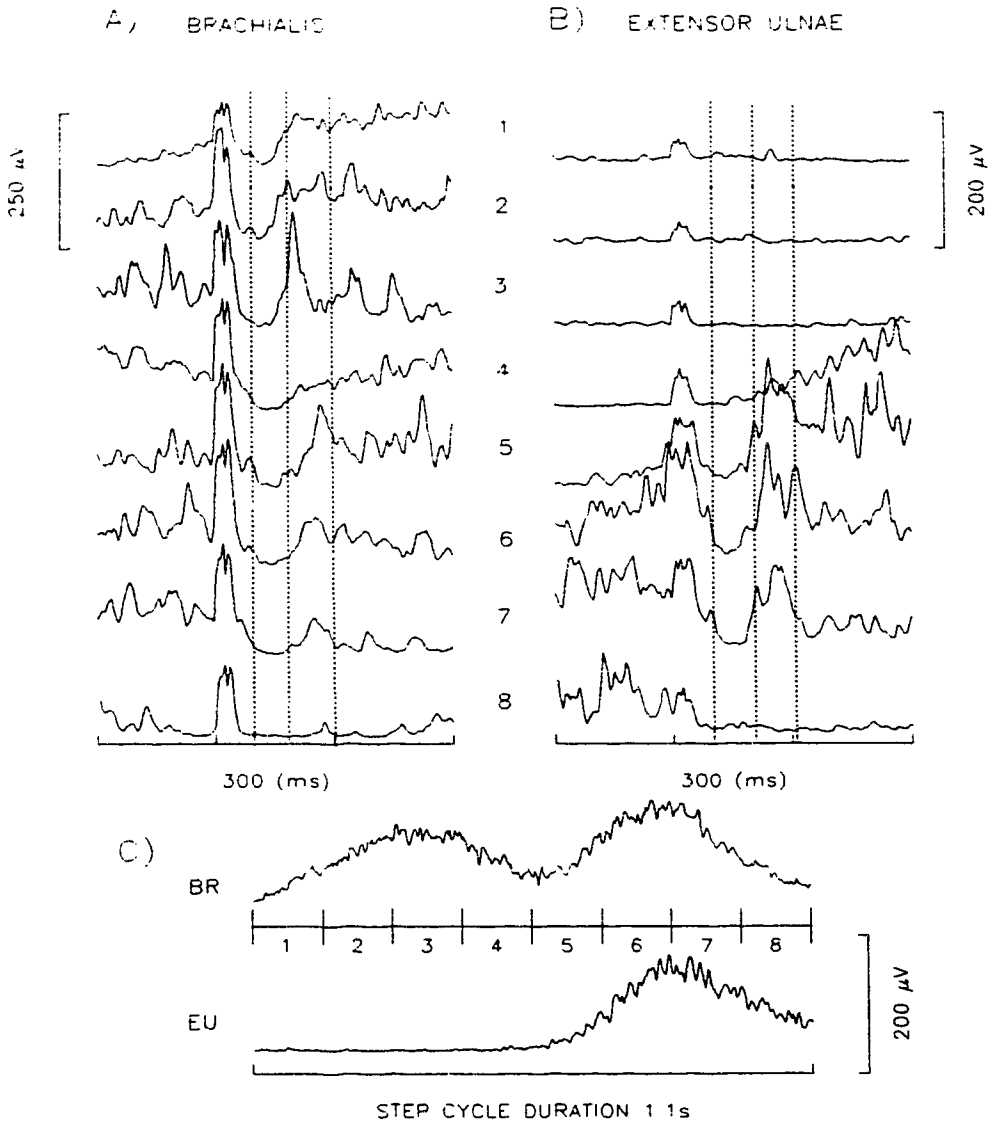

Fig. 1 Cutaneous reflex response in forelimb muscles of the intact mudpuppy during locomotion. A) Average EMG response in the elbow flexor, Brachialis (BR) to cutaneous stimulation of the dorsum of the foot at 8 points in the step cycle (183 stimuli). Stimulus artifact preceeds the first dotted line. Dotted lines indicate the beginning and end of the short latency (40-70ms) and long latency (70-100ms) responses. B) Average EMG response in the elbow extensor, Extensor ulnae (EU). C) Average step cycle recorded from BR and EU during locomotion. The 8 averaging windows appear below the BR trace.

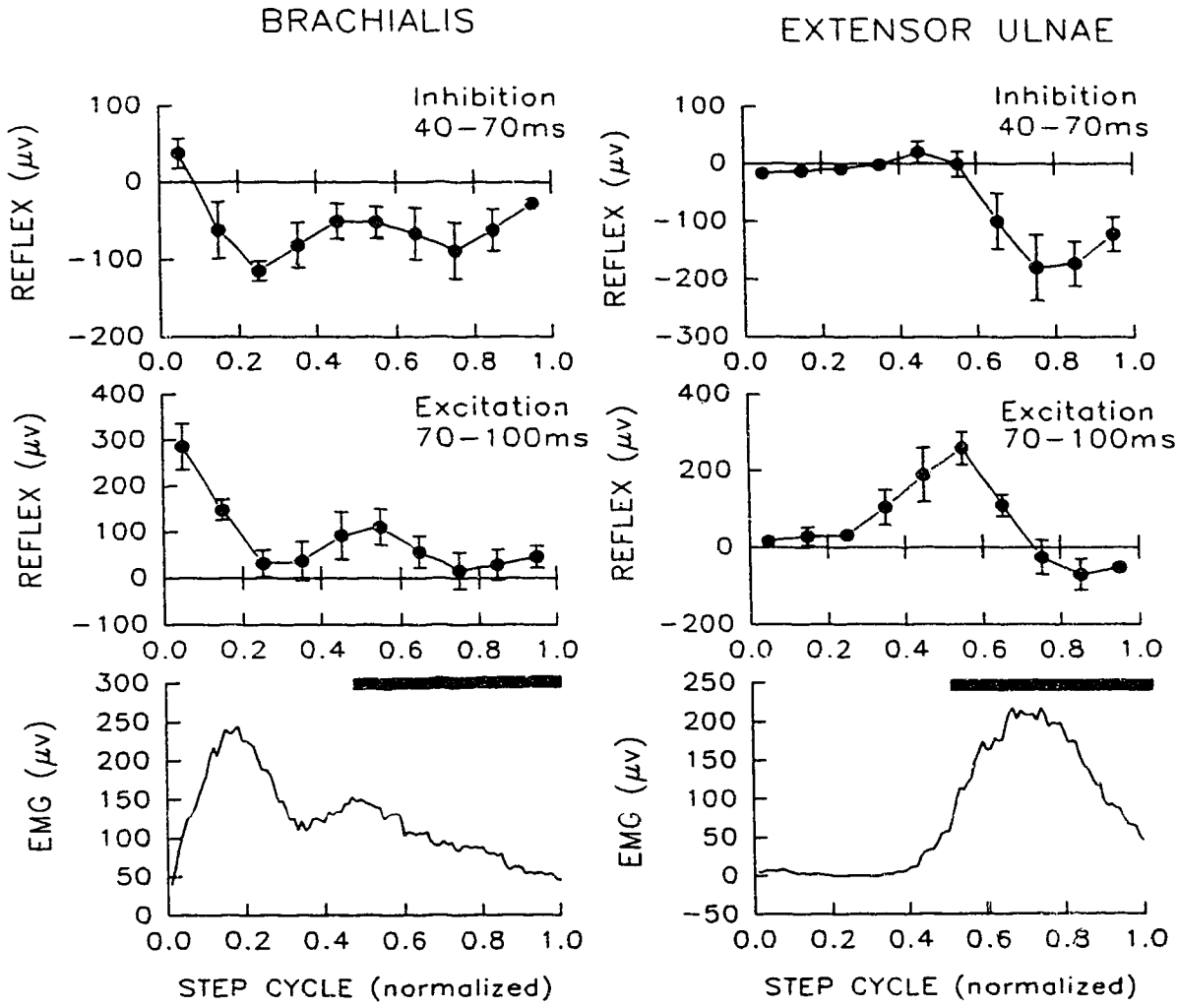

Fig. 2 Average reflex responses in Brachialis and Extensor ulnae in the intact animal during locomotion. The mean (± SE) reflex amplitude is plotted after subtrating the locomotor EMG for a period 100ms prior to the stimulus. The short latency (40-70ms) and long latency (70-100ms) responses are plotted above the average normalized step cycle for each muscle. Thick lines at the top of each step cycle indicate the stance phase of locomotion. (n=5)

From the average plots it can be seen that the short latency inhibition of BR was present throughout the step cycle with the possible exception of early swing phase (Fig. 2). The lack of inhibition at this point in the step cycle could result from a lack of background EMG. Unlike excitatory responses, inhibitory responses must be superimposed on a background level of EMG in order to be seen. The long latency excitatory response in BR was largest in early swing phase with a small second peak during the second burst in BR.

The short latency inhibitory response in EU was inversely related to the amplitude profile of the step cycle EMG for EU, with the peak inhibition occurring in mid stance (Fig. 2). The inhibition of EU only occurs during the stance phase, since even small amounts of background EMG are not inhibited during the swing phase (Fig. 1B). In contrast, the long latency excitatory response is phase advanced to the step cycle EMG, reaching a peak in early stance phase, when the locomotor EMG is still rising (Fig. 2).

5.3.2 Cutaneous reflex in the in-vitro preparation

Some of the reflex responses in the in-vitro preparation differ qualitatively and quantitatively from those in the intact animal. The reflex responses in-vitro were also more consistent than those in the intact animal. As in the intact animal, cutaneous stimulation was followed by two responses in the flexor muscle (Fig. 3A). However, unlike the intact animal the short latency response (40-70ms) was excitatory and highly modulated. Following the short latency excitation was a long latency (70-100ms) excitation which was largest in early swing phase, similar to that seen in the intact animal. Unlike the intact animal this long latency response remained throughout the step cycle (Fig. 4).

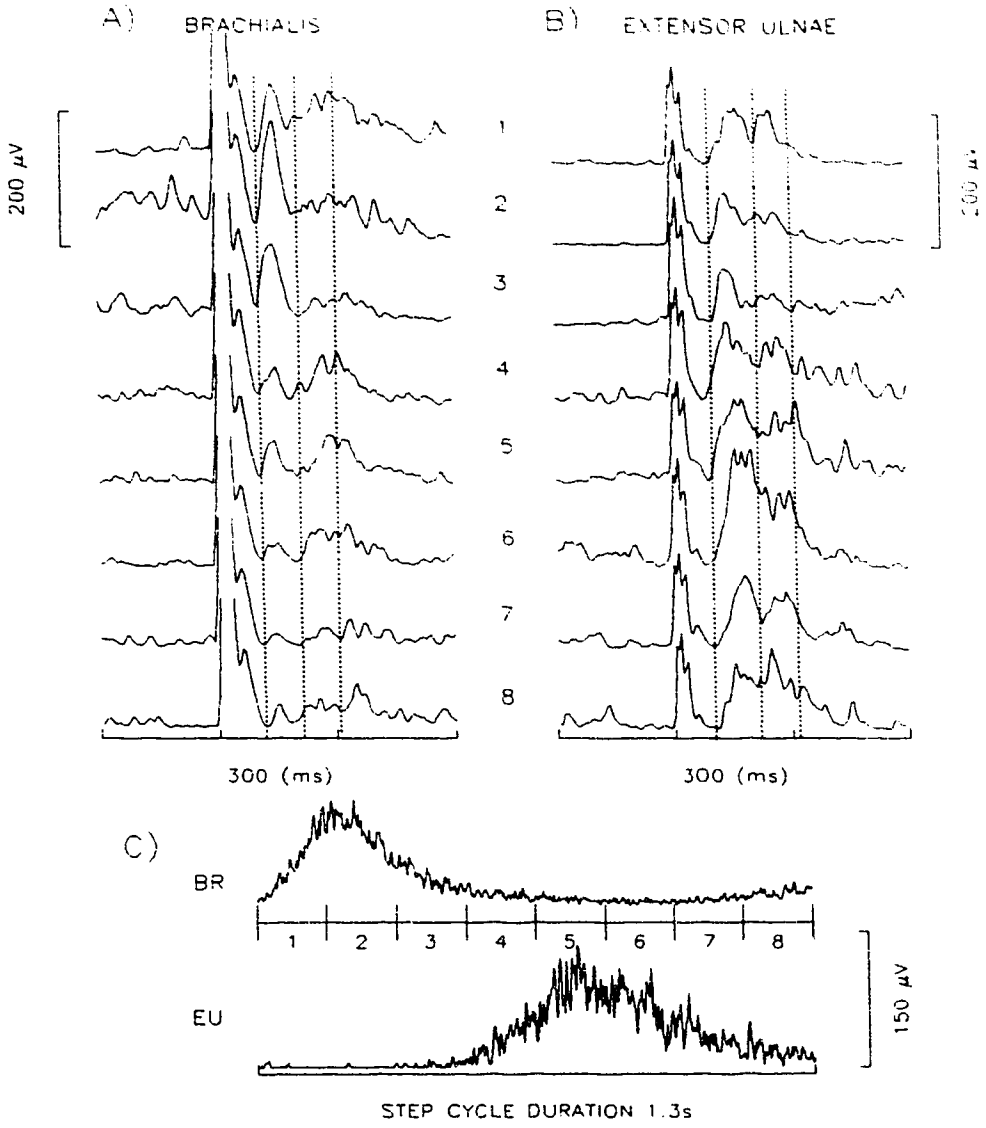

Fig. 3 Cutaneous reflex response in forelimb muscles of the <u>in-vitro</u> mudpuppy during locomotion. A) Average EMG response in the elbow flexor, Brachialis (BR) to cutaneous stimulation of the dorsum of the foot at 8 points in the step cycle (308 stimuli). Stimulus artifact preceeds the first dotted line. Dotted lines indicate the beginning and end of the short latency (40-70ms) and long latency (70-100ms) responses. B) Average EMG response in the elbow extensor, Extensor ulnae (EU). C) Average step cycle recorded from BR and EU during locomotion. The 8 averaging windows appear below the BR trace.

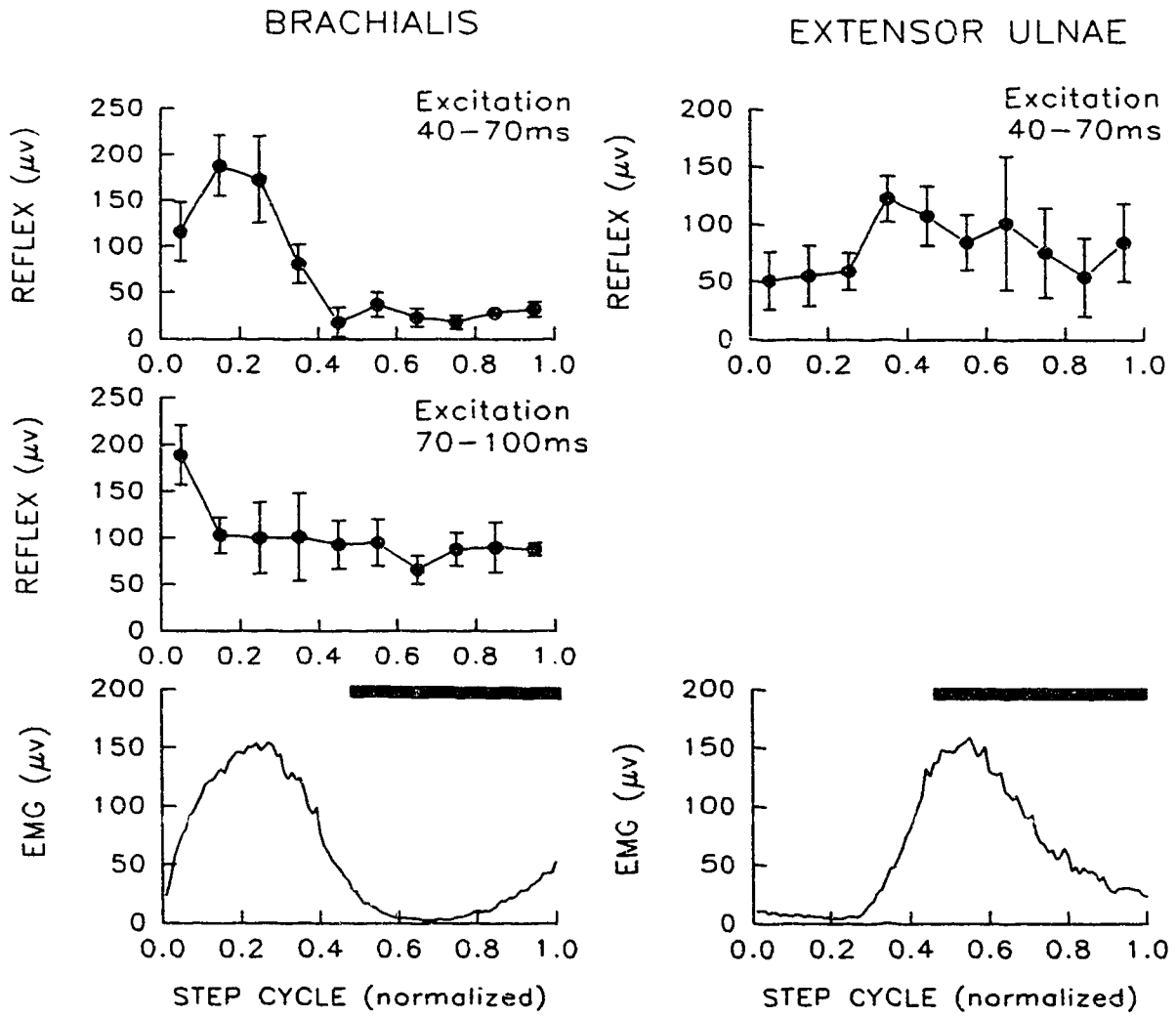

Fig. 4 Average reflex responses in Brachialis and Extensor ulnae in the in-vitro preparation during locomotion. The mean (± SE) reflex amplitude is plotted after subtrating the locomotor EMG for a period 100ms prior to the stimulus. The short latency (40-70ms) and long latency (70-100ms) responses are plotted above the average normalized step cycle for each muscle. Only the short latency response is plotted for EU. Thick lines at the top of each step cycle indicate the stance phase of locomotion. (n=7)

The reflex response seen in EU after cutaneous stimulation in-vitro differed markedly from that seen in the intact animal (Fig. 3B). The short latency inhibition seen in the intact animal was reduced or absent in the in-vitro preparation while the latency of the excitatory response was reduced, enveloping most of the area originally occupied by the inhibition. As a result, the excitatory response in EU shown in Fig. 4, occurs during the short latency period (40-70ms). We believe the short latency excitatory response seen in the in-vitro preparation is the same as the long latency excitatory response seen in the intact animal. Notice that the peak of the excitatory response in-vitro, although more variable, leads the EU locomotor EMG as did the long latency excitatory response in the intact animal.

5.3.3 Cutaneous reflex depression with NMA

When NMA or NMDA is applied to the spinal cord to initiate locomotion there is a period during which the reflex response to cutaneous stimulation is reduced or abolished. When NMA is applied at concentrations required to induce locomotion (100μM) the attenuated reflex response lasts until the animal begins to walk (Fig. 5). It has been suggested that the CPG is responsible for modulating or gating the reflex response to cutaneous stimulation (Sillar, 1991). It has also been suggested that GABAergic interneurons are important in controlling reflex transmission (Eccles et al., 1963; Davidoff, 1972). The cutaneous reflex depression caused by NMA was effectively blocked by adding the GABA antagonist bicuculline to the bath (Fig. 5). Picrotoxin also effectively reduced the reflex depression induced by NMA but was not as effective as bicuculline (Fig. 5). In addition, when NMDA and strychnine, a glycine antagonist, were applied to the bath, a 4 fold increase in reflex amplitude was observed (Fig. 5). The

effects of bicuculline, picrotoxin, NMDA and strychnine were all reversed after washing the preparation with spinal cord Ringers.

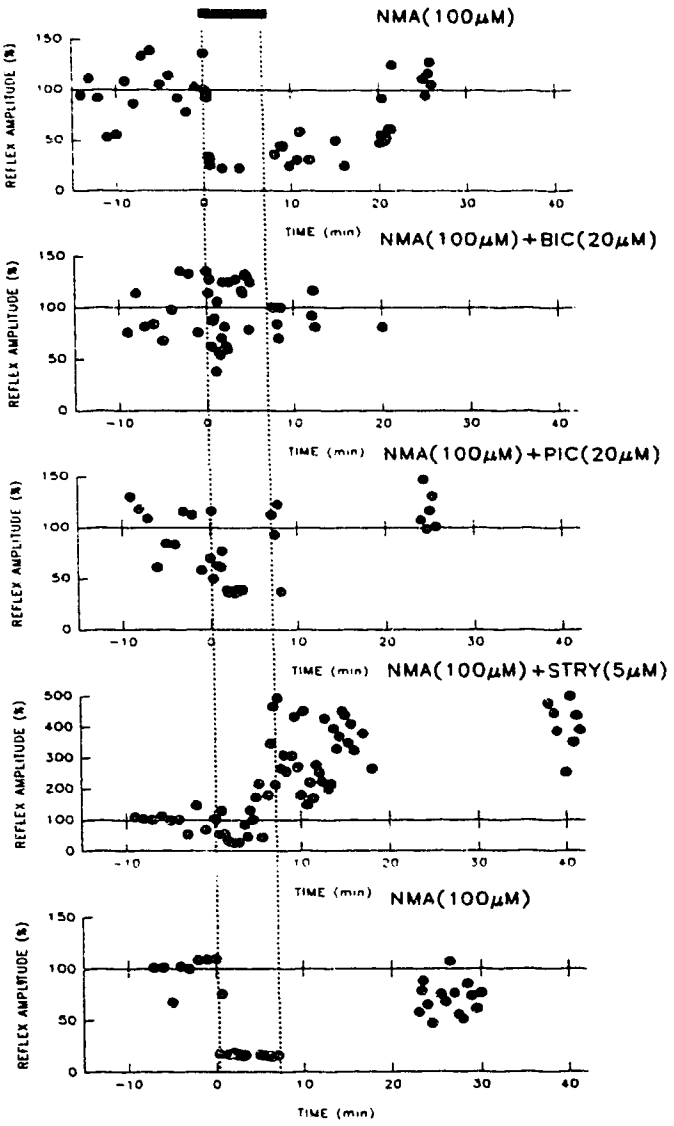

Fig. 5 Cutaneous reflex depression with N-methyl DL-aspartate (NMA). The reflex response is plotted before during and after the addition of drugs to the in-vitro bath. Drug additions are marked by the dotted lines. NMA, NMA + bicuculline (BIC), NMA + picrotoxin (PIC), NMA + strychnine (STRY) were added in separate trials.

5.4 Discussion

5.4.1 Cutaneous reflex responses

Cutaneous reflex modulation has been thoroughly investigated in the forelimb and hindlimb of the cat (for review, Rossignol et al., 1988). However, relatively little is known about the reflex response in lower walking vertebrates (Lennard, 1985; Schomburg and Steffens, 1988). We have found that many of the reflexes found in higher vertebrates have correlates in lower vertebrates, such as the mudpuppy. For example, during the swing phase of locomotion, cutaneous stimulation of the dorsum of the foot resulted in inhibition followed by excitation in the forelimb flexor (BR) of the intact mudpuppy, while in the forelimb flexor (BR) of the cat the inhibition and longer latency excitation were preceded by a short latency excitation (Drew and Rossignol, 1987). During the stance phase of locomotion, the same cutaneous stimulus results in a short latency inhibition in BR of the mudpuppy while there is no response seen in BR of the cat (Drew and Rossignol, 1987). We must remember that BR in the intact mudpuppy bursts twice during each step cycle (Wheatley et al., 1992) while in the cat it bursts only once during each step cycle. If there was an inhibition of BR during stance in the cat it would not be evident in the EMG recordings since BR is not active during the stance phase. Consistent with this suggestion is the finding that intracellular recordings from hindlimb flexor motoneurons in the cat have revealed IPSP's during the stance phase (Schomburg and Behrends, 1978).

Reflex responses in extensor muscles of the forelimb during locomotion are similar in the cat and mudpuppy. The extensor ulnae (EU) in the mudpuppy is an elbow extensor, similar in function to the triceps group in the forelimb of the cat. In both the

mudpuppy and the cat the reflex response to cutaneous stimulation in the elbow extensor is inhibition followed by excitation. During the swing phase of locomotion the reflex response differs in these two species. In the cat there is a short latency excitation while in the mudpuppy there is no reflex response (Drew and Rossignol, 1987).

The ultimate functional consequence of stimulation of the dorsum of the foot may be similar in the cat and mudpuppy, although the strategies used to achieve the objective may be different. During early stance phase the long latency excitatory response in EU functions to propel the animal forward and the limb posteriorly, away from the stimulus. During the swing phase of locomotion the long latency excitation of the flexor may function to increase the amplitude of the swing phase effectively moving the limb forward, over the stimulus.

There are some important considerations to take into account when evaluating functional responses in the mudpuppy. First, the amplitudes of the reflex responses in the mudpuppy were not as large as those in the cat. In the cat, reflex responses to cutaneous stimulation may be 2 to 5 times the amplitude of the locomotor EMG while in the mudpuppy reflex responses are rarely more than 1 - 2 times the amplitude of the locomotor EMG. Second, the animal is aquatic and does not have to maintain its centre of gravity over its limbs in order to maintain balance. Third, when needed the animal can swim using lateral body undulations and therefore does not depend solely on its limbs for propulsion. For these reasons it may not be imperative to the survival of the animal to control the cutaneous reflex as precisely as it is controlled in land-dwelling quadrupeds.

The cutaneous reflex responses in-vitro differ markedly from those in the intact animal. The short latency inhibition seen in BR in the intact animal is replaced by a short

latency excitation in the in-vitro preparation. This excitatory response may be suppressed by the inhibitory response in the intact animal. In addition, the excitatory response seen in EU appears throughout the step cycle while the inhibitory response is reduced. The reduced inhibitory response in the extensor muscles of spinal animals is not unusual and has been shown to occur in the cat (Forssberg, 1979).

There are two possible explanations for the discrepancy between the results obtained in the intact and in-vitro preparations. First, the short latency excitatory response may be suppressed by descending input in the intact animal which has been removed in the in-vitro preparation. Many supraspinal centers have inhibitory actions on segmental reflex actions (Baldissera et al., 1981). In particular, the reticulospinal system has strong inhibitory actions on reflex transmission (Engberg et al., 1968). Second, some of the afferent input has been removed in the in-vitro preparation. It is possible that input from these afferent sources normally regulates transmission in the intact animal (Rudomin, 1990). Alternatively, the difference in the reflex response noted in-vitro could be the result of the way in which locomotion is initiated. NMDA may alter the 'state' of spinal reflexes. In the lamprey spinal cord edge cells transmit sensory information to ipsilateral and contralateral interneurons (Viana Di Prisco et al., 1990). Alford et al. (1990) have shown that NMA induced locomotion leads to rhythmic activity in edge cells that is not seen during sensory induced locomotor activity. The implication is that NMA may alter the processing of afferent input in a way that is not seen during spontaneous episodes of locomotion. This may explain the difference in reflex responses seen in the intact and in-vitro preparations. We have recorded from rhythmically active interneurons during NMDA induced locomotion and have attempted to monitor the modulation of PSP's during locomotion (Wheatley and Stein, 1992). We have recorded from only a small population

of interneurons with the appropriate afferent input but have found little evidence of modulation of PSP's in spite of a modulated EMG response (unpublished observations). This aspect of the study requires further investigation.

5.4.2 Neural Mechanisms

There have been a number of recent reviews of possible mechanisms and sites of cutaneous reflex modulation (Sillar, 1989; 1991). Sillar (1991) has defined four sites where reflex modulation is postulated to occur. One of the proposed sites is at the level of the primary afferent (Gossard and Rossignol, 1990). The CPG is believed to modulate transmitter release from afferent terminals by depolarizing primary afferents during locomotion (PAD) (Dubuc et al., 1988). In addition, peripheral afferent stimulation has been shown to result in PAD via GABAergic interneurons (Rudomin, 1990). These GABAergic interneurons are believed to mediate inhibition of afferent activity both presynaptically and postsynaptically (Peng and Frank, 1989a; Peng and Frank, 1989b). Evans and Long (1989) have shown that peripherally generated PAD is the result of non-NMDA mediated synaptic transmission. In fact, NMDA antagonists have no effect on the DR evoked PAD while the same PAD is effectively blocked by the non-NMDA antagonist AP5 (Evans and Long, 1989; Hackman and Davidoff, 1991). We have shown that the cutaneous reflex can be abolished with the addition of NMA to the in-vitro bath and that this blockade can be reversed with the addition of bicuculline and reduced with the addition of picrotoxin (Fig. 5). Picrotoxin seems less effective than bicuculline in blocking the hypothesized GABA mediated reflex depression. The reason for this reduced efficacy may be because of the different pharmacological blockade induced by the two drugs. Bicuculline is a competitive antagonist at the GABA recognition site. Picrotoxin is

reported to be a noncompetitive antagonist acting at a site separate from the GABA recognition site. In addition it does not alter the kinetics of single chloride channels but instead acts to decrease the probability of opening the channel (Simmonds, 1980). This evidence suggests that NMDA mediated synaptic transmission from interneurons of the CPG via last order GABAergic interneurons is responsible for the depression of the cutaneous reflex and in light of Evans and Long's (1989) results, that this pathway is separate from the pathway activated by peripheral afferent stimulation. Recently, GABA immunoreactive cells have been found that contact not only primary afferent fibers but also spinal interneurons (Maxwell et al., 1990; Christenson et al., 1991).

The cutaneous reflex response in the in-vitro mudpuppy is reduced by NMA but greatly facilitated by the addition of NMA and strychnine (Fig. 5). Eccles et al. (1963) have shown that strychnine, a glycine antagonist, reduces postsynaptic inhibition while having little effect on presynaptic inhibition. This postsynaptic inhibition is believed to be due to glycinergic recurrent inhibition of motoneurons by Renshaw cells (reviewed by Baldissera et al., 1981). Davies and Watkins (1983) report that polysynaptic excitation of Renshaw cells by segmental afferents is selectively depressed by NMDA antagonists. In light of this evidence, our results might suggest that NMA drives glycinergic interneurons which postsynaptically inhibit motoneurons. If this were the only explanation then we would not expect the addition of bicuculline to abolish the reflex depression (Fig. 5). More likely, glycinergic interneurons play a role in limiting the depolarization of motoneurons either via recurrent inhibitory pathways from the axon collaterals of motoneurons and/or via specific glycinergic interneurons which regulate the gain of polysynaptic reflex transmission at the neurons interposed between afferents and motoneurons.

5.5 References

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The purpose of these investigations was to develop an in-vitro preparation to be used to study the control of limbed locomotion in vertebrates. We did not begin the investigations with the mudpuppy as a model. In fact we tried a number of other preparations including the frog, toad and rat before deciding on the mudpuppy. The mudpuppy proved to be an excellent model system for many of the reasons outlined in this thesis.

Getting (1986) defined eight steps in studying central pattern generators:

1. Description of behaviour.
2. Characterization of motor pattern.
3. Identification of motor neurons and interneurons.
4. Localization of pattern generating neurons.
5. Mapping of synaptic connectivity.
6. Characterization of cellular properties.
7. Manipulation of network, synaptic or cellular properties.
8. Reconstruction of pattern generator, motor output and behaviour.

Although we have addressed some of the points outlined above, I would like to take this time to discuss some of the problems and future directions which may lead to a more thorough understanding of central pattern generation in the mudpuppy.

Double burst in Brachialis

One of the more interesting results to emerge from the comparison of the intact and in-vitro locomotor patterns in chapter 2 was the finding that Brachialis (BR) exhibited a double burst in the intact animal and only a single burst in the in-vitro animal. We offered three explanations for this finding: one hypothesis dealt with the lack of afferent feedback, a second hypothesis dealt with the lack of descending input, a third hypothesis alluded to the effects of NMDA on the locomotor pattern.

We have conducted a series of separate experiments which may shed some light on the source of the second burst in BR in the intact animal. When the intact animal is allowed to locomote freely in the aquarium, he will both walk along the bottom as well as swim in open water using his limbs. We have recorded EMG from forelimb muscles in these animals and have found that the double burst in BR is present both while swimming, when there is no foot contact, and while walking, when there is foot contact (unpublished observations). In a separate set of experiments the intact animal was anaesthetized and decerebrated. In addition, a laminectomy was performed, exposing the first four cervical segments. In these animals all afferent and propriospinal input remained intact. The animals were then made to locomote by applying NMDA (25μM) to the spinal cord. In these animals EMG recordings from BR always only show the single burst indicative of the in-vitro animal (unpublished observations). These two experiments suggest that the single burst is not a function of lost propriospinal or afferent input. Two explanations remain. Descending input to the central pattern generator may be responsible for the second burst in BR. Alternatively, NMDA may be responsible for initiating a locomotor pattern which differs in some respects from that in the intact animal. The finding that a pharmacologically induced locomotor pattern differs somewhat from

the locomotor pattern in the intact animal is not new. Pearson and Rossignol (1991) have shown that in spinal cat, the patterns induced with clonidine differ in some respects from those induced with DOPA/nialamide which in turn differ from those recorded in the intact cat.

In light of these findings it is important to ensure that alternative methods of initiating locomotion are investigated in the mudpuppy. We have already stimulated an area in the brainstem (McClellan and Grillner, 1984), which is similar to the 'MLR' (mesencephalic locomotor region) of the cat, and found that we could initiate and maintain locomotion in-vitro (unpublished observations). It may be important to compare results obtained in the 'NMDA induced' preparation with those obtained in the preparation after brainstem stimulation. Alternative methods of initiating locomotion have been demonstrated for other preparations (Atsuta et al., 1990; Atsuta et al., 1991; Greer et al., 1992). It would be of interest to see if any of these methods initiated the double burst in BR.

Identification of Interneurons

In this thesis we have used a number of physiological criteria for identifying interneurons during locomotion. Included in these criteria are their phase of firing during the step cycle, their response to afferent stimulation and their spike-triggered average of EMG during locomotion. We have recently begun to include morphological criteria for identification of interneurons as well. We have injected interneurons with procion yellow, an intracellular fluorescent dye, and studied interneuron morphology in whole mounts of the spinal cord. With this technique we hope to add morphological criteria to the present physiological criteria for identifying interneurons within the central pattern generator.

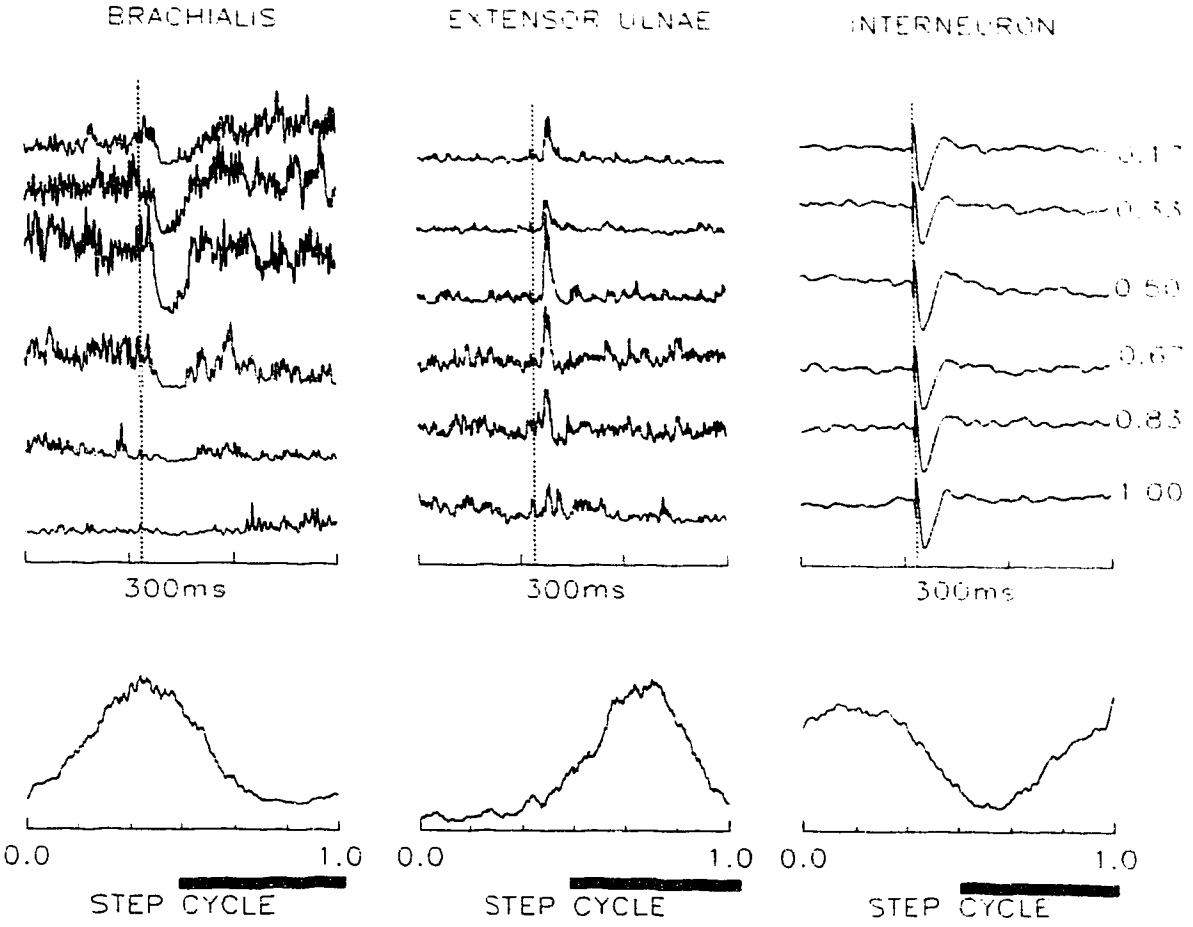

Fig. 1 Intracellular and electromyographic response to dorsal root stimulation (DR) during locomotion. Dotted line indicates the onset of the stimulus. Each trace indicates the average response to DR stimulation in one of 8 averaging windows during the step cycle. Below each series of traces is the average step cycle for that muscle (brachialis and extensor ulnae) or cell (interneuron). Notice that the reflex response in the extensor is modulated while the intracellular response is not.

Reflex modulation during locomotion

In chapter 5 we have described the modulation of cutaneous and dorsal root reflexes during locomotion. In addition to the electromyographic data, we have also recorded from a number of rhythmically active interneurons during locomotion. While recording from these interneurons we have activated afferent pathways in an attempt to monitor intracellularly the modulation of PSP's during locomotion. Our population of cells with adequate PSP's is small as of yet, however one interesting result has emerged. In some of the cells the EMG response to stimulation is modulated while the interneuronal PSP is not (Fig. 1). If the mechanism of modulation was due to presynaptic inhibition of the afferent terminals, one would expect the intracellularly recorded response in the second order interneurons to be modulated as well. In fact, in many cases it is not modulated. These findings suggest that either the site of reflex modulation is not at the level of the primary afferent or that the presynaptic inhibition of afferent input is not only afferent specific but also specific to particular branches of the afferent.

Conclusion

In this thesis we have demonstrated that the in-vitro preparation of the mudpuppy (Necturus maculatus) serves as a good model for investigating the central pattern generator for locomotion. In addition it serves as a model for the study of central and peripheral interactions during locomotion. We have demonstrated that the locomotor patterns in-vitro are similar to those recorded from the intact animal. We have also shown that it is possible to record routinely from interneurons and motoneurons while recording simultaneously from the muscles. In this manner the intracellular and electromyographic responses can be compared to responses recorded from "fictive" animals. We have also

shown that a majority of rhythmically active interneurons are active within the transition periods of the step cycle, suggesting that some of the current models of pattern generation may need to be altered in order to accommodate these new findings. In addition we have shown that reflex modulation occurs in both the intact and in-vitro preparation of the mudpuppy but that some of the control strategies may have been altered in the in-vitro preparation. Further characterization of the physiological and morphological properties of specific interneuronal classes would be useful in understanding the organization of the central pattern generator.

I believe it would be useful to investigate the source of the second burst in BR. The second burst seems to be controlled independent of the first. How is the second burst generated? How do reflexes affect the first and the second burst? Are afferent pathways to each burst controlled separately? Can this second burst be generated using one of the alternative methods for initiating locomotion in-vitro? Are the interneurons which excite BR during the first burst the same interneurons responsible for the second burst? There are many interesting questions which surround this second burst in BR.

The mudpuppy also exhibits two forms of locomotion as outlined in chapter 2. It swims like a fish and walks like a quadruped. These forms of locomotion are smoothly integrated in an escape response. It would be of useful to investigate the coordination of these behaviours during an escape. It would also be interesting to initiate this escape response in-vitro and to investigate how the central pattern generator controls the two behaviours.

As with most new preparations the ground work needs to be laid before any complex investigations can be undertaken. I hope that I have laid some of this ground work and that future investigators will find this thesis useful in their quest to understand

the control of locomotion in the mudpuppy.

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APPENDIX

MANUAL: MATT AVERAGING PROGRAM

1) SETUP

This averaging package was written by Matt Wheatley while a graduate student in the Division of Neuroscience in 1991. The source code has not been listed but is available from Matt Wheatley or Dr. R.B. Stein in the Division of Neuroscience at the University of Alberta. The program allows for the display and hardcopy printout of data recorded with the Axotape program (Axon Instruments). It also allows the user to average (straight average or phase specific average), smooth, fit, subtract, and measure the data.

The executable program is run by typing MATTAVE on the command line (the executable program is MATTAVE.EXE).

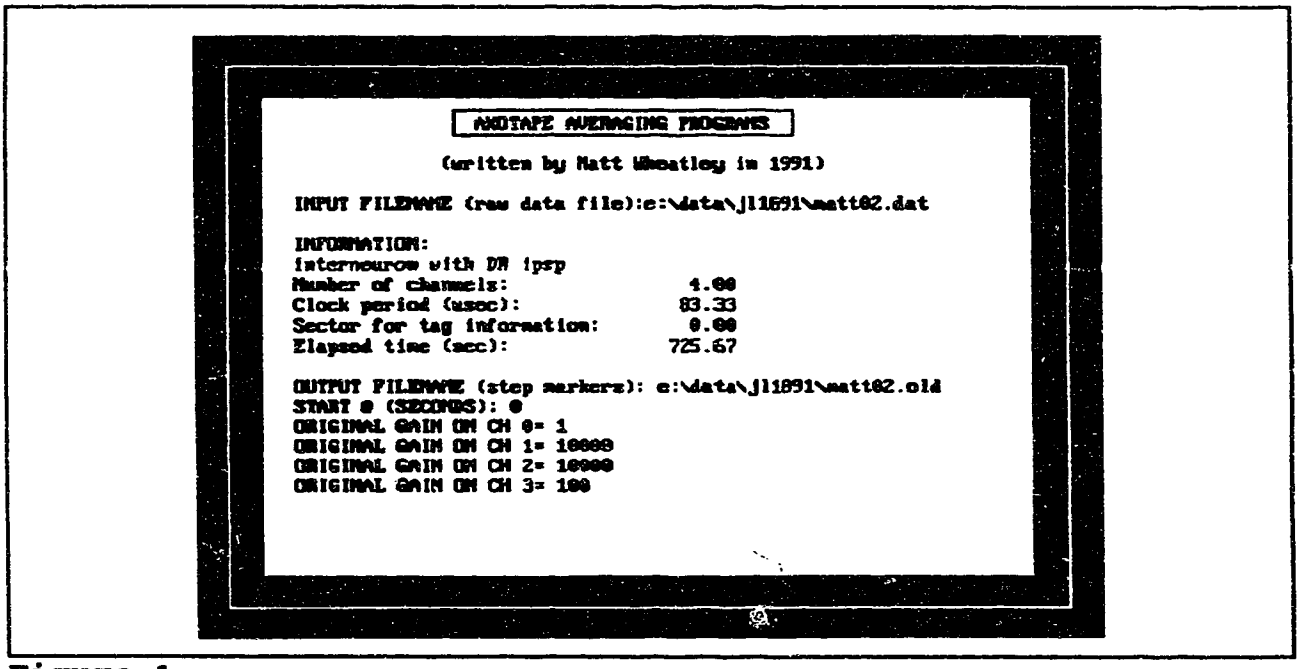

Figure 1

1) The program asks for a number of items as input (Fig. 1).

First, the program requires an input **filename**. This will be the data file stored by Axotape and will have a *.DAT extension. The format of the file can be obtained by reading the manual for the Axotape program. Remember that if the file is not in the current directory you will have to supply a path.

Note: The display portion of the program will handle 16 channels. However the averaging and phase averaging subroutines will only handle 4 channels at the present time. This is due to the limitation placed on the system by DOS's 640K barrier. The program interrogates the computer and allocates the maximum available RAM (below 640K) to the averaging arrays.

2) When the input file is read, the information attached to the file will be displayed on the screen.

* The first line lists a title or header the experimentor supplied when recording the data originally. This usually contains some description of the data and any other information deemed important to the specific file.

* The second line lists the number of channels (up to 16) on the recorded file.

* The third line lists the clock period or sample time in μsec. This is the clock period used by the A/D for sampling <u>all</u> the channels. It must be multiplied by the number of channels and converted to frequency to obtain the per channel sample rate.

eg. clock period = 83.33μs

4 channels * 83.33μs = 333.33μs/channel

1/333.33μs = 3kHz sample rate / channel

* The fourth line gives the sector for tag information. This option is used by Axotape but is <u>not</u> used by the MATTAVE.EXE program.

* The last line lists the total elapsed time of the file in seconds.

3) The program will then ask for an output filename. This output file stores the step markers or cursor values selected by you in the main program (see cursor measurements below). These values are latter used for phase averaging the data.

4) The program asks "where in the file would you like to start." This allows the user to jump forward to any part of the file. The same command is used in the main program and is called **JUMP**.

5) The program also asks for the recorded gain on each channel. This will calibrate all y scale bars in all menus appropriately. All y bars in the program are displayed in mV. Remember that Axotape requires full scale input to be ± 10volts.

eg. Axotape full scale voltage = ± 10 V

gain = 100

± 10 V / gain 100 = ± 100mV full scale voltage

2) MAIN MENU

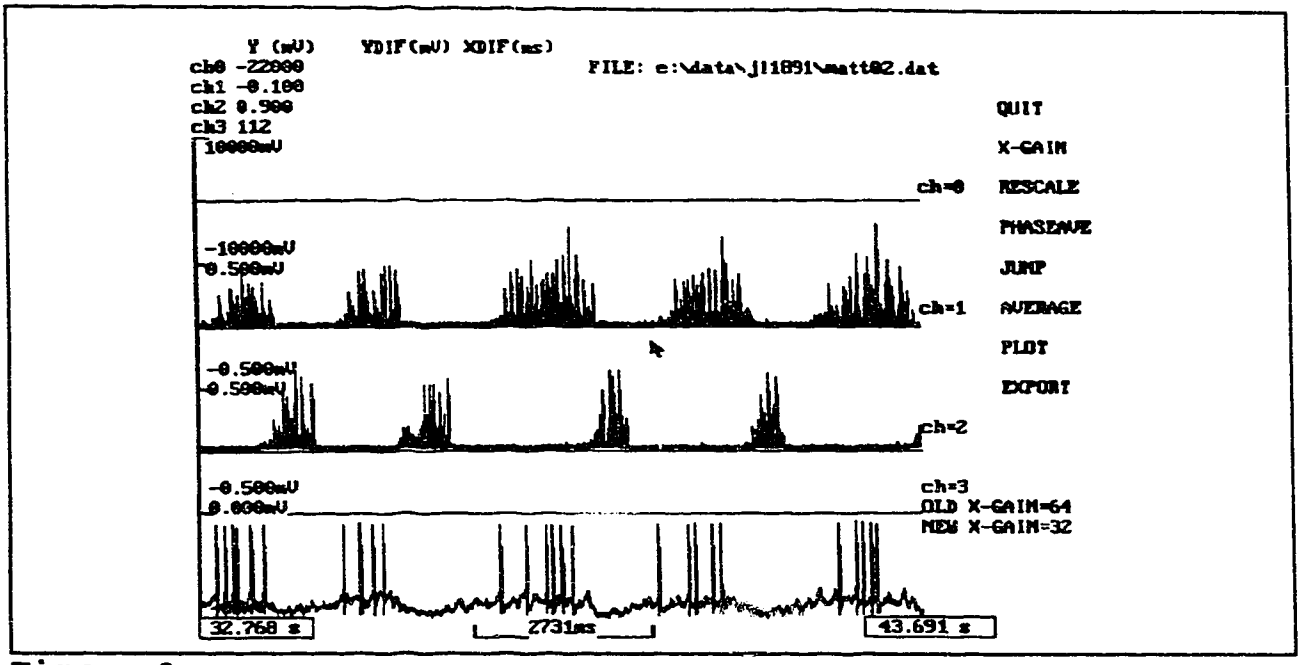

Figure 2

The main menu displays all channels on the screen (Fig. 2). Starting with channel 0 at the top.

Remember, Axotape has a strange convention for numbering channels. On the A/D board channel 15 is the first channel sampled. Therefore in two channel mode the input must be put into channel 15 and 14. This has to do with the count down timer on the A/D board. Don't ask questions just accept it. (This only occurs on the 16 channel TL-125 model not on the 40kHz 8 channel TL-40).

I have reversed the convention for simplicity.

channel 15 on the A/D = ch 0 on the display
channel 14 = ch 1
channel 13 = ch 2 etc.

The y scale bars are located to the left of each trace. The full scale voltage (± 10 V) has been divided by the gain for each channel so that the y bars are in mV.

eg. ch 2 gain = 20000

ch 2 = ± 0.5mV

Each trace also has a magenta zero line indicating 0 mV. The channel marker (eg. ch=1) is attached to the zero line and moves with it.

At the bottom of the screen are two boxes at the left and right limits of the traces. These indicate where in the file the screen starts and ends (seconds).

Also at the bottom of the screen is an X bar indicating in ms what the scale is for the present display.

THE CURSOR (Fig. 3)

In the top left of the screen is a real time readout of the cursor value (arrow) for any given channel. As the cursor moves around the screen the values for each channel change appropriately. In this way the voltage value for any displayed point can be found simply by placing the point of the arrow over the data point and reading out the voltage from the display.

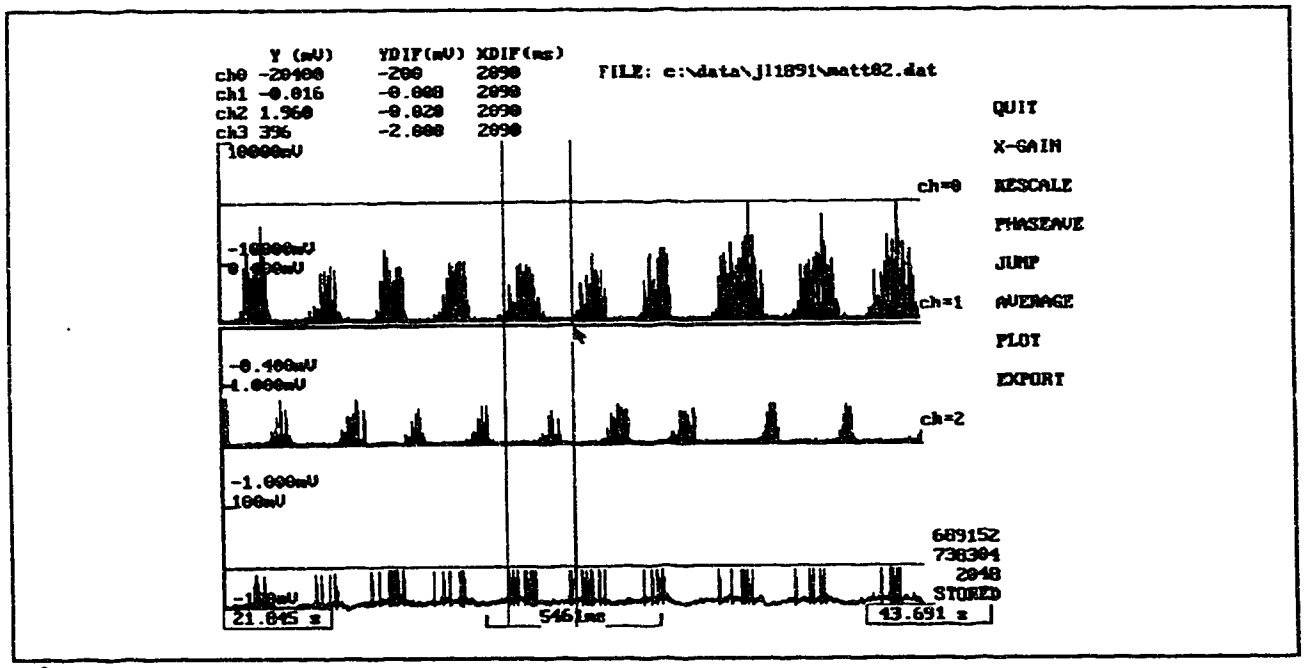

Figure 3

Included in the upper left display is a value calculated from the difference in the cross hairs for selected cursor readings. To anchor the cross hair to any point on the screen you must point to the appropriate point and press the left button on the mouse. To anchor the second cross hair you must move the cursor to a different point and press the left button again. At this point an x and y difference (YDIF and XDIF) will be calculated for all channels relative to the area demarcated by the cross hairs. At this point you can erase the cursors by pressing the middle button on the mouse or you can store the cursor values in the output file selected during the setup by pressing the right button on the mouse. When the values have been stored, a readout of the file location values, the x difference and the word STORED will appear in the lower right

corner of the screen. The cursor marks will remain on the screen until a new screen is displayed. At this point new cursor values can be selected, displayed or stored.

On the right portion of the screen are a number of options which can be selected either with the mouse or by using the highlighted "hot key" for each selection.

MOVING THROUGH THE FILE

cursor

The user can move through the file to display successive windows in one of two ways. First, moving the cursor off the screen will shift the display forward or backward depending upon if the movement is to the right or the left respectively.

ctrl + arrow

An easier method of shifting the display is to use the arrow key. Using the left and right arrow keys will shift the screen by ⅛ of a full screen forward and back respectively. Using ctrl + arrow will shift the screen one full screen.

jump

If one needs to move a larger distance through the file the JUMP key is used. JUMP will allow the user to jump to any point in the file by inputting the value in seconds in the lower right corner when prompted.

ADJUSTING THE DISPLAY PARAMETERS

The display gains can be manipulated in either the x direction or the y direction. If a larger window is required in the x direction, select **X-GAIN**. In the lower right corner a prompt is displayed showing the old x-gain and asks for a new x-gain. The program creates a screen buffer which retrieves from the file a section of data 512 points * number of channels long. The x-gain is a value which corresponds to the number of these screen buffers you would like to display. This means that the amount of data displayed will depend on the sample rate chosen.

eg. 4 ch, sampled at 5kHz / channel, x-gain = 10

512/5000 = 0.102 seconds * x-gain 10 = 1.02 seconds

the amount of data in bytes displayed corresponds to a segment ...
512 * 2 * 4 * 10

512 = number of points / channel in screen buffer
2 = the data points are stored as 2 byte words
4 = the number of channels recorded
10 = x-gain

bytes displayed = 40960 bytes (40kb)

The program includes a screen interpolation (min-max) routine which displays the full resolution of the data but allows for rapid display. It does this by finding the minimum and maximum values over a variable number of points (x-gain) and displays the min and the max value at the same x value. In this manner for an x-gain of 256 only two points are plotted instead of 256 points. This is done because the bottle neck of all IBM compatible computers is the graphics functions.

The display amplitude can be adjusted by selecting **RESCALE**. After selecting rescale the program will prompt (again in the bottom right) for the channel you would like to rescale. After selecting the channel the program will prompt for an upper and lower limit. This corresponds to the upper and lower limit (in mV) you would like displayed in the window provided for that channel.

eg. If you have an intracellular recording of a neuron with a membrane potential of -60mV recorded at a gain of 100. This neuron will be displayed in a window with an upper limit of 100mV and a lower limit of -100mV. If you want to look at post synaptic potentials superimposed on the membrane potential, you could rescale with an upper limit of -50mV and a lower limit of -70mV. This would centre the trace in the window and multiply the display gain by 10.

HARDCOPY AND ASCII OUTPUT

Hardcopy output can be obtained by either selecting **PLOT** on the right menu or pressing shift + print screen on the keyboard. The PLOT function plots the screen to an HPGL compatible plotter in HPGL mode. The shift + print screen function is a very useful function which requires the inclusion of a small program which comes with the Axotape program you purchased. The program is called PPRINTSC.EXE and prints the contents of the screen to a laserjet or laserjet compatible printer.

ASCII output of any trace can be obtained by selecting **EXPORT** from the menu on the right. EXPORT will prompt for a filename in which to store the ASCII file. Along with the data will be included the gains of each channel, the filename, the sample time / channel, and the x-gain used to display the sample. Using these values you can easily compute the duration of the sample. You are required to compute the duration of the sample because the interpolated results are what are dumped to the ASCII file. This means that regardless of the sample time or x-gain there will always be 1024 points dumped to the file.

Remember, the ASCII file will be stored in the current directory if you do not supply a path.

3) AVERAGE (Fig. 4)

AVERAGE allows the user to use one of the channels as a trigger and to trigger off a threshold level on that channel. Averages of all channels will be made time locked to the threshold crossing on the trigger channel.

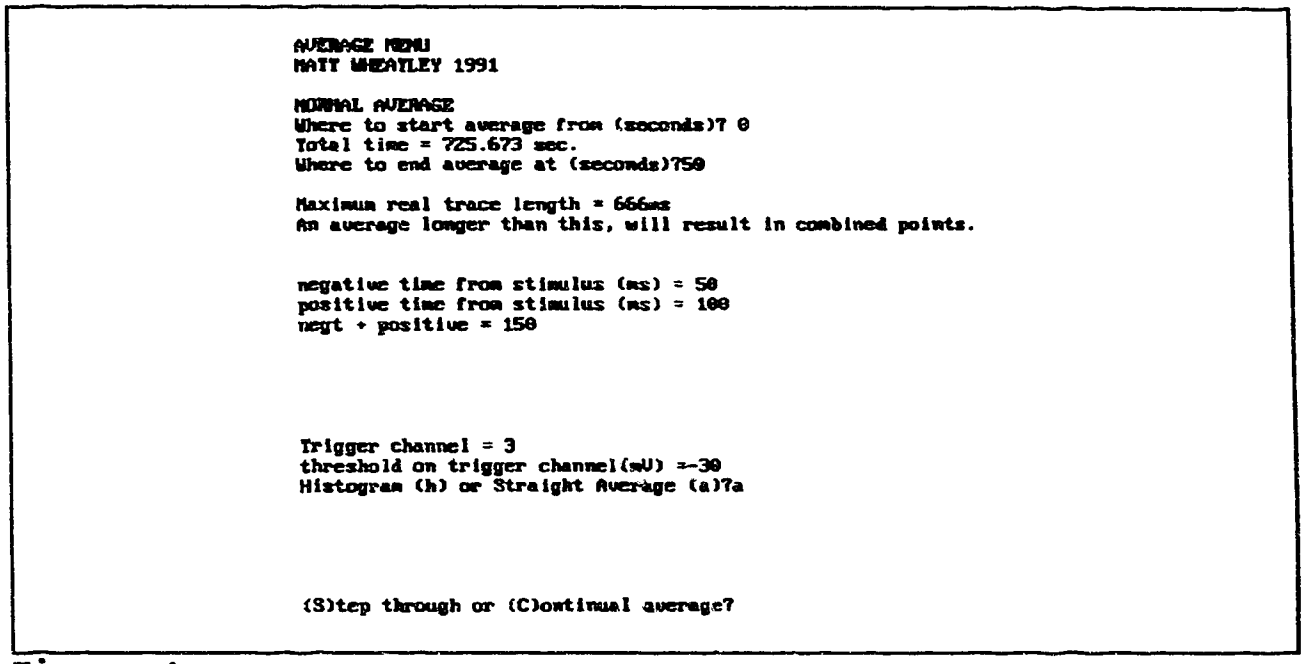

Figure 4

After selecting AVERAGE a menu is displayed which prompts the user for a number of input items (Fig. 4).

It first asks where you want to start the average from (seconds). Next it will display the total length of the file and ask where you want to end the average (seconds). The program will only look for trigger points within the segment of the file designated by start and end.

The program will then display two lines which state...

> **"Maximum real trace length = 666ms. An average longer than this will result in combined points."**

Due to the limitation placed on the program by DOS the maximum average will be limited to 666ms. For an average longer than this the program will combine points, effectively lowpass filtering the data. It will prompt you for the number of points to combine and will tell you the cut off frequency which will result when those points are combined.

The program can do negative time averaging. The program can find a trigger and average data which occurred before the trigger. It will ask you the length of time you need displayed before the stimulus occurs. It will also ask for the length of time you want displayed

after the stimulus occurs. These will then be combined by the program and a total trace length will be displayed.

The next prompt will be for the channel you would like to use as the stimulus channel. It will only search on this channel for threshold crossings. Once the channel has been selected it will prompt for the threshold in mV for the positive going trigger. One of the pitfalls of the program the way it is currently set up is that threshold value must be decided upon before entering the AVERAGE menu. The threshold function acts like a "schmidt trigger" and will not retrigger until the trace goes below the threshold.

HISTOGRAM OR AVERAGE

The program prompts the user to select "**histogram**" or "**straight average.**" In histogram mode an average as well as a histogram will be displayed on the screen. The histogram subroutine divides the trace into 100 bins and displays the histogram under the average. The histogram function requires threshold values for all channels, and will prompt the user for these values. The trigger channel remains the same while the threshold crossings on other channels contribute to the histogram.

The final input required by the AVERAGE routine is whether the user wants to "**step**" through the average or to "**continue**" through the average accepting all threshold crossings as valid. If "continual average" is chosen, the average will build from the first to last threshold crossing, overlaying each trace over the previous trace. If "step" is chosen, the current trace will be drawn in magenta and will wait for the user to "**reject**" it (pressing R) or to "**accept**" it (any other key). If the trace is accepted, the magenta trace is over written in yellow, included in the average and a new magenta trace is displayed. If the trace is rejected, the trace is over written in grey, not included in the average and a new trace is displayed in magenta. The number of accepted and rejected traces is displayed at the bottom of the screen and is updated with each new trace.

AVERAGE OR CLEAR AND AVERAGE (Fig. 5)

Once the traces are overlaid the average can be displayed by choosing AVERAGE (which will overlay the average) or CLR&AVE (which will clear the overlay and display the average alone.

SMOOTH

A data smoothing routine was written which will low pass filter the data. The SMOOTH option will prompt the user for the number of points in the window used by the moving average. If 20 points are chosen the data point is averaged with 10 points preceding it and 10 points following it. In this way no phase shift occurs. The original average is saved and the data smooth is only on the displayed data. Therefore the original average can be retrieved at any point by selecting SMOOTH and then RESTORE. Remember that the smooth allows functions on the original data. Therefore if you smooth once with a window of 10 points and smooth again

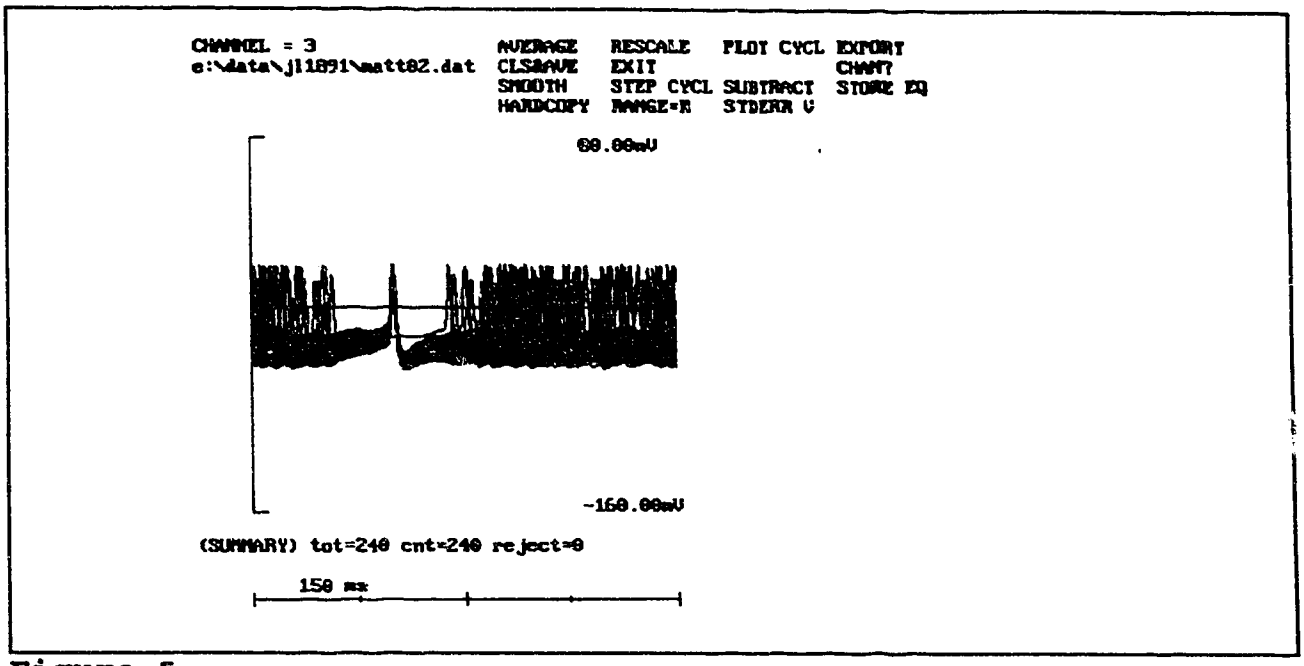

Figure 5

with a window of 20 points you do not have a smooth of 30 points. The smooth is a 20 point smooth.

HARDCOPY AND ASCII OUTPUT

Hardcopy output can be obtained by either selecting **HARDCOPY** or pressing shift + print screen on the keyboard. The HARDCOPY function plots the screen to an HPGL compatible plotter in HPGL mode. The shift + print screen function uses the Axotape program PPRINTSC.EXE and prints the contents of the screen to a laserjet or laserjet compatible printer.

ASCII output of the trace can be obtained by selecting **EXPORT** from the menu on the right. EXPORT will prompt for a filename in which to store the ASCII file. Along with the data will be included the gains, the filename, the sample time / channel, and the x-gain used to display the sample. Using these values you can easily compute the duration of the sample. You are required to compute the duration of the sample because the interpolated results are what are dumped to the ASCII file. This means that regardless of the sample time or x-gain there will always be 1024 points dumped to the file.

Remember, the ASCII file will be stored in the current directory if you do not supply a path.

RESCALE

RESCALE functions the same way it did in the main menu (see main menu above).

EXIT

EXIT will return you to the main menu.

STEP CYCLE

STEP CYCLE is only available in the PHASEAVE subroutine.

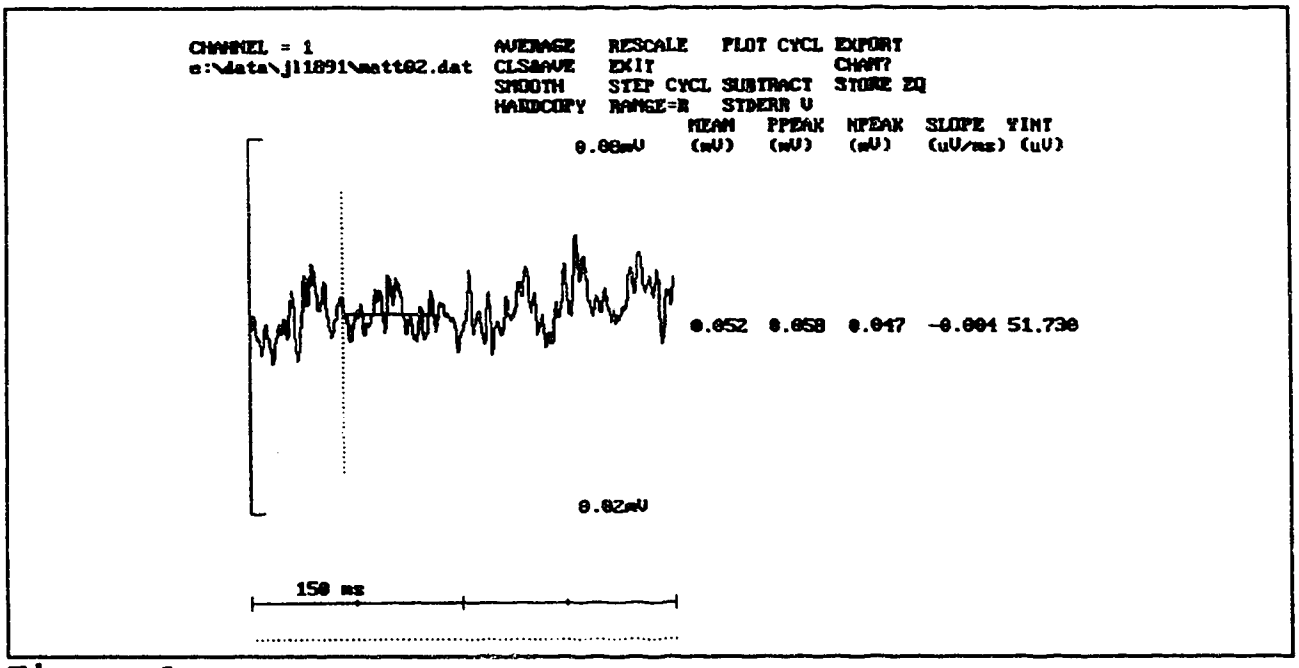

Figure 6

RANGE (Fig. 6)

RANGE is a subroutine which requires as input a starting and ending point in the average trace and then uses that "range" to find a number of values for the user. The total number of points in the trace are displayed in the bottom right corner of the screen. The start and end of the range are chosen relative to the total number of points in the trace. The range is then displayed in magenta while the rest of the average is displayed in white. To the right of the trace are five values: the mean of the range, the positive peak of the range in mV, the negative peak of the range in mV, the slope of a best fit line through the range and the y intercept of that best fit line. The data is fit using a least squares estimation of the line. The x and y axis for the linear fit are drawn on the screen as a yellow dotted line while the best fit line itself is drawn in green through the data points. The equation of the best fit line can be stored by selecting STORE EQ. This equation of the line can then be subtracted from the trace using SUBTRACT to remove any trend in the data.

STANDARD DEVIATION (Fig. 7)

STDERR V is a subroutine which takes as input a range similar tc the RANGE function and computes the standard deviation over that range of data points. The range is displayed in magenta while the standard deviation is displayed as dotted error bars at + 1SD and -1SD of the mean of the range displayed.

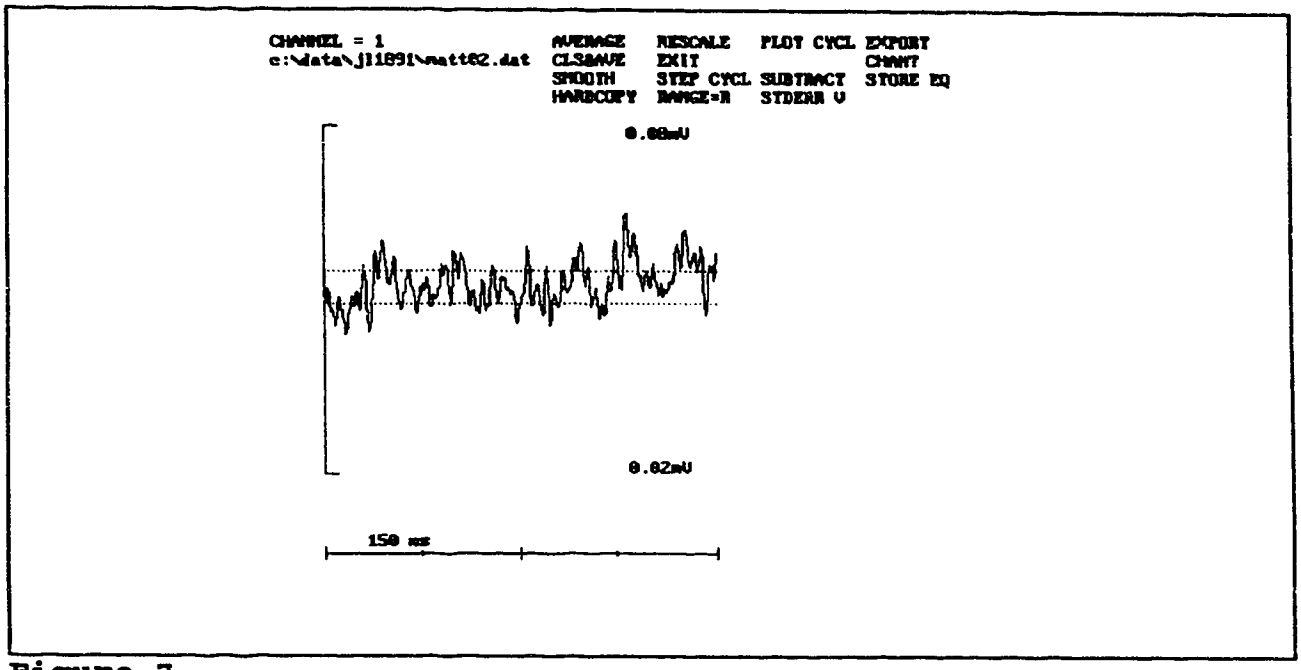

Figure 7

4) PHASEAVE (Fig. 8 and 9)

The **PHASEAVE** subroutine has the same options as the AVERAGE subroutine but requires different input. Only those differences will be documented here. PHASEAVE requires as input file markers stored in the output file designated during the setup. File markers are chosen as outlined in CURSOR in the main menu above (Fig. 3). The subroutine searches for threshold crossings as outline in AVERAGE above only in areas bounded by the cursor markings. Any threshold crossing is then analysed according to the phase of the bound area (step cycle) in which it is found.

The subroutine first prompts the user to decide on the number of divisions he would like the cursor bound area to be broken up into. The area or step cycle can be broken into 1 - 10 divisions.

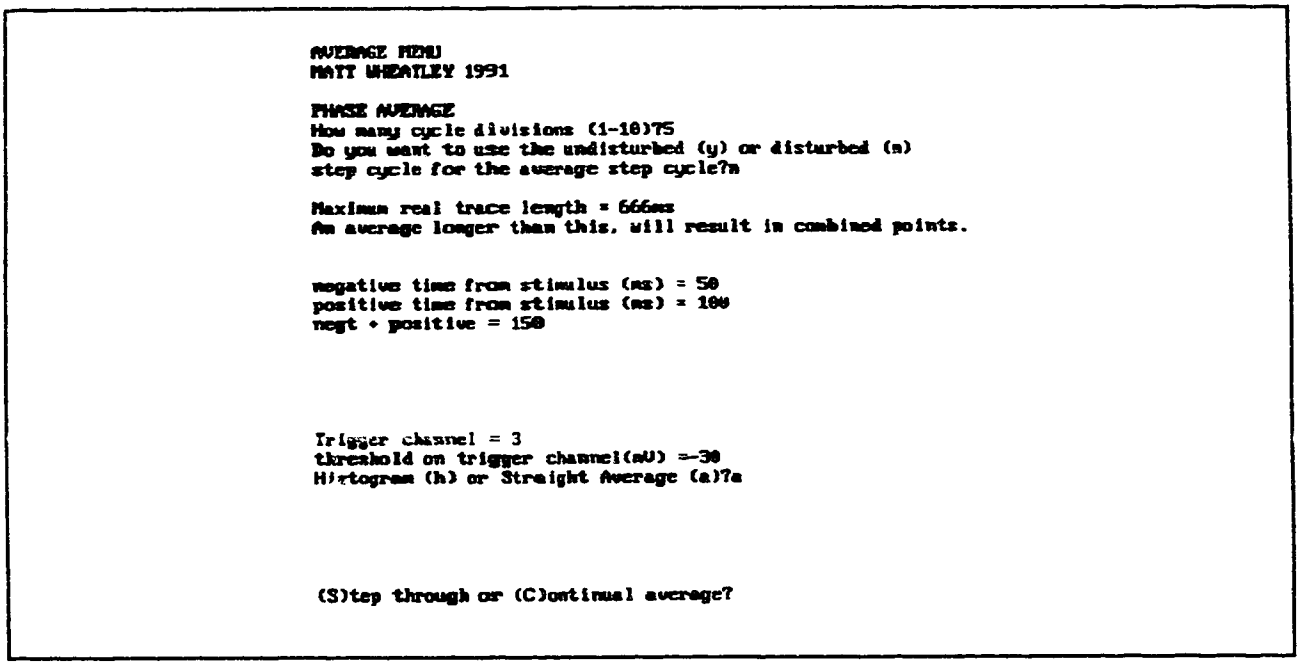

Figure 8

The subroutine then asks if you would like the **disturbed** or **undisturbed** step cycle for the average. This requires some explanation. PHASEAVE computes two averages. The averages of the stimuli at different phases of the step cycle and the average of the step cycle that has been used to determine the phases. If the step cycle average is computed from all step cycles with stimuli in them then the step cycle average is from the "disturbed" step cycle. If the step cycle average is computed from all step cycles without any stimuli then the step cycle is said to be "undisturbed." In this way changes in step cycle duration and amplitude can be compared between the disturbed and undisturbed step cycle.

All other inputs are the same as those used in the AVERAGE menu.

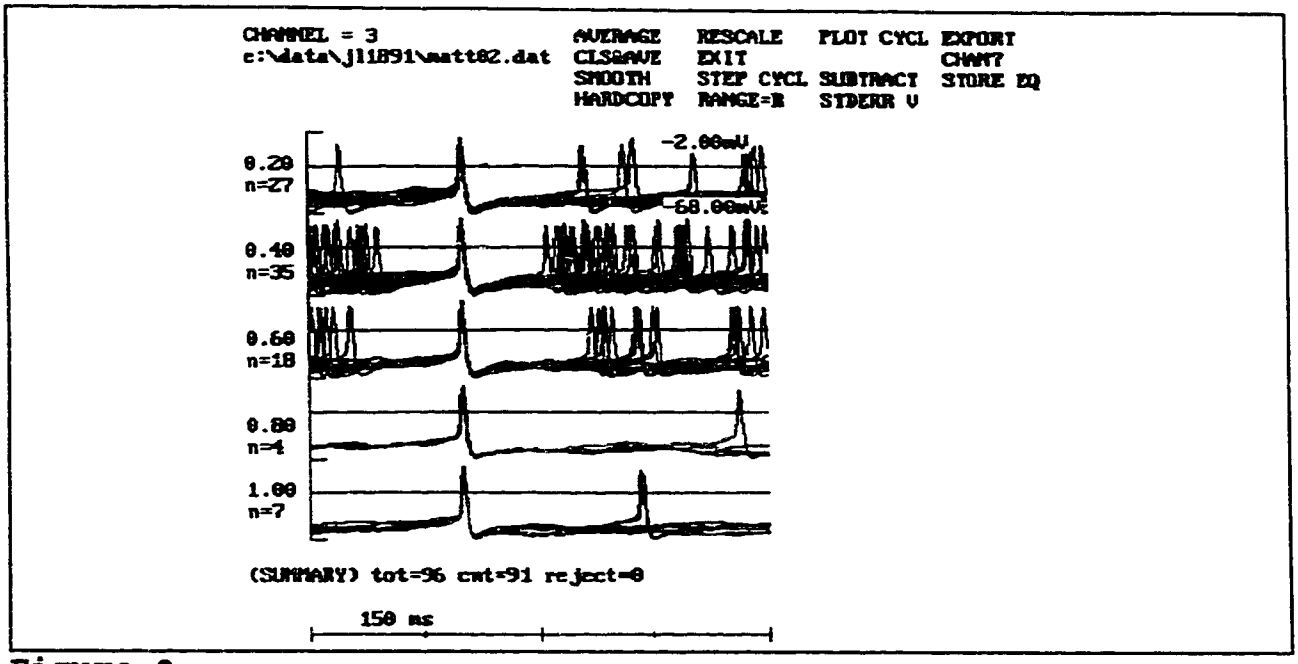
CHANNEL = 3
c:\data\j11891\matt02.dat
AVERAGE
CLS&AVE
SMOOTH
HARDCOPY
RESCALE
EXIT
STEP CYCL
RANGE=R
PLOT CYCL
SUBTRACT
STDERR V
EXPORT
CHAN?
STORE EQ
-2.00mV
-68.00mV
0.20
n=27
0.40
n=35
0.60
n=18
0.80
n=4
1.00
n=7
(SUMMARY) tot=96 cwt=91 reject=0
150 ms

Figure 9