PROCESSING OF MECHANORECEPTIVE INPUT IN CRAYFISH: AN *IN VIVO* AND *IN VITRO* STUDY OF THE ROLE OF THE CUTICULAR STRESS DETECTORS IN MOTOR CONTROL.

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CORNELIA SIEGLINDE LEIBROCK
26th April 1993

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Der Taucher

"Wer wagt es, Rittersmann oder Knapp,
zu tauchen in diesen Schlund?
...
Wer ist der Beherzte, ich frage wieder,
zu tauchen in diese Tiefe nieder? ..."

Friedrich Schiller (1798)

SUMMARY

Cuticular stress detectors (CSD_1 and CSD_2) are mechanoreceptors positioned in the proximal parts of the legs of decapod crustaceans. Using a reduced preparation consisting of the isolated thoracic central nervous system and a walking leg of either of the two crayfish species, *Procambarus clarkii* or *Pacifastacus leniusculus*, it has been possible to study by means of extracellular and intracellular techniques the physiological properties of the sensory fibres and the reflex responses in the four proximal muscle groups to mechanical and electrical stimulation of the receptor organs.

The location of the CSD terminals within the thoracic ganglion was determined by anterograde fillings of the afferent fibres with biocytin. The anterograde fills also showed the absence of any cell bodies within the thoracic ganglion. Thus one can be certain that the CSD nerves contain no motorneurone axons and that all fibres within the CSD nerves belong to sensory cells. These have their cell bodies in the periphery; their orthodromic direction of conduction is from the periphery to the thoracic central nervous system. Most CSD fibres terminate, and branch extensively within the ganglion. Nevertheless, some CSD1 fibres were found to pass through the ganglion and continue rostrally within the ventral nerve cord.

Intracellular recordings of impulses arriving in the central ganglion made it possible to identify the physiological properties of single receptor cells when stimulating the receptor organs mechanically. The recordings from CSD2 terminals were found to be in consensus with extracellular recordings from previous studies. CSD1 intracellular recordings revealed a class of sensory fibres not previously described. They were identified as 'high threshold' fibres, for large amplitude, high frequency stimuli were necessary for their activation. These CSD1 fibres appear to be responsible for the occurrence of a reflex response reversal in anterior levator motorneurones. This is discussed with reference to a possible role of the CSD1 receptor organ in the control of autotomy.

Intracellular recordings from sensory terminals also revealed the presence of centrally coupled sensory afferents. Coupled units often had different conduction velocities, and 'on' units were sometimes coupled with 'off' units. Lucifer Yellow usually filled two or more fibres when injected into a single cell. This is in support of central coupling, for the dye is known to cross gap junctions.

Some sensory units showed antidromic spike conduction, i.e. from the ganglion to the sense organ, being always coupled to fibres firing orthodromically. It is

suggested that the coupling provides a mechanism for amplification of the sensory signals, the antidromic spikes induced by impulses coming from coupled units. This is supported by the finding that stronger mechanical or electrical stimulation of the sensory fibres leads to an increased occurrence of antidromic spikes.

Using a number of electrophysiological and pharmacological tests, it has been demonstrated that at least 32% of all the connections recorded between the CSDs and the motorneurones of the four proximal muscle groups are monosynaptic. The reflex responses within one motorneurone group varied.

The importance of sensory feedback in shaping the final motor output was studied in preparations showing rhythmic, reciprocal activity in antagonistically operating muscle groups. Rhythmic CSD activity was shown to be able to entrain such fictive locomotor activity.

CSD1 activity during normal walking behaviour was studied in an *in vivo* preparation. Thus, reflex responses could be discussed with knowledge of the natural activation of the CSDs and related to their functional significance in locomotion.

Chapter 1

GENERAL INTRODUCTION

1.1 The neuronal basis of behaviour

The study of animal behaviour traces its origins to two different sources: field studies of animal behaviour by ethologists such as Konrad Lorenz and Niko Tinbergen, and network or cellular studies by neurobiologists such as Charles Sherrington. Indeed, it was Sherrington who, at the beginning of this century, introduced the term 'synapse' and the concept of the motor unit as the basic unit of motor activity (Sherrington, 1939). A combination of both approaches is needed to understand behaviour in terms of the nervous system. This aim has been tackled in many different ways and is now known as the field of neuroethology (Camhi, 1984).

Some of the earliest attempts involved trying to map every single component of the nervous system. For instance, Albrecht Bethe, working at the turn of the century, made a detailed study of the central nervous system of the crab, *Carcinus*. He systematically analysed its behaviour and reflexes, and examined the central nervous system histologically. He became very skilful at carrying out minute surgical operations on parts of the central nervous system. By destroying parts of a neuronal network and comparing the behaviour of the animal before and after such operations in response to localized stimulations, he was able to assess the functions of numerous nervous pathways (Florey, 1990). However, as an attempt at mapping the entire nervous system of an animal in relation to its behaviour, Bethe's work was doomed to failure. Florey (1990) translates the last passage of Bethe's third *Carcinus* paper (Bethe, 1898) as follows: "As we now overlook my whole work, I reach the sad conclusion that nothing has been gained from it for our factual knowledge. Were there not satisfaction in the search for knowledge, one would have to say in resignation: it is too difficult for us humans."

It was not until much later, well into the second half of this century when much improved equipment and techniques were available, that neurophysiologists rediscovered this approach and began to establish the neuronal basis of behaviour by identifying the cellular and synaptic components constituting networks underlying behavioural acts. This time it was not the entire nervous system under investigation, but smaller parts which were involved in the execution of well defined, stereotyped or rhythmic behaviours such as breathing, walking, swimming or courtship. Ambitious as these aims were, they were thought to be realisable in invertebrate nervous systems. For the attraction of invertebrate nervous systems is based on the fact that they contain relatively few neurones, many of which are comparatively large and can be identified as individuals. Microelectrode techniques can often be employed and allow easy experimental investigations at the single cell level. As a result of all these features,

invertebrate preparations were coined "simple" nervous systems (Usherwood and Newth, 1975).

1.2. "Simple" nervous systems

Work on a wide variety of invertebrate phyla has contributed to our understanding of how nervous systems are organised. Molluscs, in particular the sea hare, *Aplysia*, have been used in learning studies which have been at the forefront of research into molecular mechanisms that underlie long-term synaptic plasticity (Kupfermann *et al.*, 1971; Kandel, 1979). Another mollusc which has been extensively studied is *Navanax*. Its buccal ganglion contains about 200 neurones, 30 of which have been identified as being involved in feeding behaviour (London *et al.*, 1987; Woollacott, 1974). The neural basis of escape responses has been successfully analysed in a number of species, notably earthworms (Bullock and Horridge, 1965), crayfish (Wine and Krasne, 1982) and cockroaches (Ritzmann, 1984; Camhi, 1985), since the neurones involved are large ('giant') and the circuits relatively simple. Another successful approach has been to take a small autonomous ganglion and study its neural networks in detail. The lobster stomatogastric ganglion is the best studied example here (Selverston and Moulins, 1987). Of all such preparations studied within the last 40 years, three examples were chosen to give an impression of the kind of studies that have been carried out. These examples cover work on leeches, locusts and decapod crustaceans.

1.2.1. Leeches

The ventral nerve cord of the medicinal leech, *Hirudo medicinalis*, an annelid, consists of 21 discrete ganglia with long somata-free connectives between them. The cell bodies in the living ganglia are clearly visible with normal light microscopic techniques and are all accessible to microelectrode penetration. Each of the 21 ganglia contains about 400 cell bodies and each resembles every other ganglion in the chain (Macagno, 1980). It is possible to identify individual neurones reliably on the basis of their shapes, sizes and position within the ganglion. The identification can be confirmed by intracellular recording. Thus repeated recordings from one and the same neurone can be made and individual cells can be analysed for their specific characteristics, their electrical properties, their branching pattern and their synaptic connections to other cells. Thus, sensory input and motor output can be clearly identified. This is of advantage when analysing synaptic interactions within the central nervous system which has been done, for example, in three types of mechanosensory neurones which respond to various forms of cutaneous stimuli (Blackshaw, 1985) or

when studying the neural basis of behaviour. For example, the leech nervous system has been used to study the neural circuits that generate rhythmic movements of the animal such as swimming, which is produced by alternating contractions of dorsal and ventral longitudinal muscles along the length of the leech (Kristan and Weeks, 1983), and the pumping action of the heart tubes which is co-ordinated intersegmentally and bilaterally (Calabrese and Peterson, 1983).

1.2.2. Locusts

Another animal which has been extensively used in neuroethological studies is the locust. As in annelids and molluscs, only minimal dissection of the animal is necessary in order to gain access to the central nervous system. This allows intracellular electrophysiological techniques to be employed in preparations still capable of performing a limited repertoire of behaviours. Behavioural activity in such semi-dissected preparations can be compared with activity recorded extracellularly from muscles and nerves in intact animals. Since neurones penetrated during microelectrode studies can be visualised by dye-filling with, for example, horseradish peroxidase or Lucifer Yellow, this combination of techniques makes it possible to identify the neuronal components involved in the production of specific behaviours.

One of the best studied behaviours in locusts is the corrective steering mechanism that they must perform in order to maintain a stable flight course in the face of perturbations such as wind gusts or faulty motor performance. Course deviations are detected by three sensory systems, the compound eyes, the three ocelli and wind-sensitive hairs on the forehead. Information from these organs converges onto a small number (6-10 pairs) of interneurones in the brain which convey it to the thoracic ganglion where the flight motor pattern is organized. Each of these interneurones encodes a very specific type of course deviation, such as a dive or banked turn. The integration of the error message into the centrally generated flight rhythm is achieved by a population of interneurones located in the three segmental ganglia of the thorax. These thoracic interneurones (there are more than 30 pairs) are postsynaptic to specific descending deviation detectors and presynaptic to the flight motorneurones. In addition, they receive inputs from the oscillator circuit. This ensures that the descending information is gated at wing-beat frequency and that corrective signals only reach the flight motorneurones at the appropriate time in the wing-beat cycle. Thus deviations from a straight course are detected and corrected (Reichert and Rowell, 1986).

The hindleg of a locust contains a variety of proprio- and exteroceptors which give rise to some 10,000 sensory axons. Most of these make synapses with

motor- and interneurones in the ipsilateral half of the metathoracic ganglion, while some project to the mesothoracic ganglion. There are some 1000 interneurones in each half of the metathoracic ganglion and less than 100 motorneurones which are responsible for controlling the movements of a hindleg (Burrows, 1989). Extensive studies during the last 15 years have provided a clear picture of the synaptic pathways between the sensory and motorneurones. They involve at least four identified populations of spiking and non-spiking local interneurones. Together with a fifth group of interneurones that conveys sensory information between ganglia, they are part of the circuits responsible for sensori-motor integration of mechanosensory information in the locust leg (Burrows, 1989; 1992; Laurent, 1991). These circuits combine with intrinsic properties of the neurones themselves to ensure that leg reflexes are appropriately co-ordinated for a wide variety of different circumstances faced by the animal.

1.2.3. Decapod crustaceans

The central nervous system of decapod crustaceans is very similar to that of insects in that it has the same ladderlike arrangement of the ventral nerve cord with pairs of hemiganglia connected by somata-free connectives. The overall number of motorneurones present in a typical decapod crustacean is about 3000. Each walking leg is innervated by about 100 motor fibres (Laverack, 1988), this number being independent of growth or age. In contrast, the number of sensory fibres depends on the size of the animal. Sensory structures and thus sensory fibres are added at each moult in animals which grow continuously throughout their lives (Laverack, 1976). Counts of axon profiles in nerve cross sections gave numbers in excess of 100,000 axon profiles in the leg nerve of a small lobster (Govind and Pearce, 1985). Thus the number of sensory axons is a factor of 10 larger in a lobster than in a locust leg, whereas the motorneurone number is about the same. Much less is known about the number of interneurones present in the central nervous system of crustaceans. Laverack (1988) came to the conclusion that the numbers of interganglionic neurones is a factor of 10 less than the numbers of sensory fibres. This would be equivalent to the relation of 10:1 of sensory to interneurones in the locust metathoracic ganglion. Nevertheless, he explicitly stated that this ignored the numbers of intraganglionic interneurones that do not project along the connectives. Thus the number might be much higher.

The use of crustacean preparations for the study of escape reactions and of pattern generating circuitry within a small semi-autonomous ganglion have already been mentioned. The former has provided neurophysiological explanations for such

ethological concepts as fixed action patterns and habituation to a constant stimulus. Indeed, the crayfish escape circuit uses presynaptic inhibition to prevent habituation occurring as a result of the animal's own movements (Kirk, 1985). The lobster stomatogastric ganglion has not only been an excellent model for central pattern generators (see below), but has shown us that neural circuits are not hard-wired, but can be altered under the influence of a variety of neuromodulators to produce a number of different motor outputs (Katz and Harris-Warrick, 1990). Additionally, crustacean preparations have been extensively used for the study of mechanisms of motor control.

When comparing the overall numbers stated in these three examples of simple nervous systems, neurone numbers are relatively small in comparison to humans where some 10_{10}-10_{11} neurones are present in the brain alone. Apart from smaller numbers of neurones, the main attraction of using invertebrate preparations is the possibility of reliably and repeatedly recording from the same neurones in different preparations. For both these reasons, neural circuits controlling different aspects of behaviour should be more easily worked out than in the more complex nervous systems of vertebrates in general and mammals in particular. Since there is every reason to believe that nervous systems are organised in a similar manner across the Animal Kingdom, invertebrate nervous system preparations therefore provide useful models for elucidating general principles of neuronal organisation in addition to their intrinsic scientific interest. One such general principle of operation is the concept of central pattern generators (Delcomyn, 1980). Their discovery has proved to be one of the major stepping stones in the understanding of motor control in animals.

1.3. Motor control

The elements of any central motor system must perform at least three basic tasks apart from the direct control of muscle contraction (by innervation) in order to produce effective behavioural acts. First, initiation, maintenance and termination of movement (command), second, generation of the spatio-temporal impulse pattern that is translated by muscles into meaningful movements (pattern generation) and third, integration of sensory information during the ongoing movement (sensory feedback). Each of the three will be dealt with separately.

1.3.1. Command neurones

The term "command neurone" was introduced into the literature dealing with motor control by Wiersma and Ikeda (1964). They used it to describe neurones in the nerve cord of crayfish that, when stimulated, elicited co-ordinated behavioural

acts, that is they elicited rhythmic movements of the swimmerets in the abdomen of crayfish. Since then the concept of command neurones has received much controversial attention which culminated in a review article by Kupfermann and Weiss (1978). This article was published together with critical comments of 33 scientists.

Kupfermann and Weiss defined a command neurone as a neurone that is both necessary and sufficient for the initiation of a given behaviour. Sufficiency is demonstrated by stimulating a putative command neurone and showing that its stimulation elicits a defined behavioural act. For example, stimulation of specific neurones in *Tritonia* leads to withdrawal of the branchial plumes, to turning movements or swimming (Willows, 1967). Necessity is demonstrated by showing that silencing the putative command neurone eliminates or at least alters the behaviour or its corresponding motor programme. Kupfermann and Weiss were already aware of the fact that the concept could not be applied rigorously to single cells only, but that in some cases, groups of neurones when treated as a whole might satisfy the necessity and sufficiency criteria for a given behaviour, even though individual neurones of the group would fail to meet the criteria. They termed such a group a "command system". However, the whole concept implies that the organisation of motor control is a hierarchical one, and that the command precedes the operating and movement generating systems. A lot of experimental data have shown that command neurones/systems can themselves be part of the pattern generating system or be under direct influence of it. Thus identification of command neurones/systems might be more difficult than by just applying the above stated criteria. As an alternative to the hierarchical, the concept of consensus was introduced by Davis (1976). He suggested that the command function may be distributed among many neurones acting in consensus to drive a behaviour and that different neurones may perform a command function at different times in a behavioural sequence. Hence the concept of command neurones, in so far as it describes the triggering function of particular neurones in initiating the production of a motor output, can still be assumed to be a valid one.

1.3.2. Central pattern generators

Central pattern generators (CPGs) are assemblies of neurones which are capable of generating and controlling a wide variety of periodic behavioural patterns. They constitute the neuronal basis for rhythmic behaviours, where all or part of an animal's body moves in a cyclic, repetitive way, such as occurs in walking or breathing. The existence of such networks of neurones has been strongly disputed on the grounds that rhythmic, alternating activity could be achieved by reciprocal feedback from the active part to the inactive part, thus causing the inactive part to

become active. This theory negates the existence of a central pattern generator, but states that sensory feedback is responsible for the generation of rhythmic motor pattern. This hypothesis is based on studies in which reflexes were shown to produce rhythmic movements and where subsequent elimination of these reflexes by deafferentation or isolation of the nervous system abolished the rhythmic behaviour or rhythmic motor output. Leeches and earthworm, for example, were reported to exhibit a number of responses during swimming, crawling, or "walking" that seemed to form a chain of reflexes in which each movement provided the trigger for the next throughout the entire duration of the behaviour (Gray *et al.*, 1938; Gray and Lissmann, 1938). Experiments on toads showed that animals whose spinal cord had been severed just behind the head could be made to step by reflex action simply by being held against a slowly rotating drum. The stimulus eliciting stepping was stretch of a leg. In later experiments, toads were completely deafferented along the spinal cord. These experiments seemed to reveal a complete lack of co-ordinated walking and as such were thought to confirm the hypothesis that rhythmic motor output is not generated in the central nervous system, but is based on reflex activity (Gray and Lissmann, 1940; 1946).

However, since the observation that the isolated central nervous system of a locust can produce the motor pattern for flight in the absence of sensory feedback (Wilson, 1961), it has become more and more accepted that complex rhythmic behaviours can be generated by pattern generators contained within the central nervous system, a concept originally put forward by Graham Brown (1911). Nowadays, the evidence in favour of the concept of CPGs is so overwhelming (for review see Delcomyn, 1980) that it has been recognized as a general principle of operation.

For reasons as given above (section 1.2) CPGs have been particularly well studied in invertebrate systems. The networks of neurones producing the rhythmic pattern for the escape swimming behaviour in *Tritonia* (Getting, 1983; Getting *et al.*, 1980), for the heartbeat in leeches (Calabrese and Peterson, 1983) and for the activity in the stomatogastric nervous system of the lobster (Selverston *et al.*, 1976) are well known. It had been hoped that, by analysing the CPGs for different rhythmic systems, a relatively small number of mechanisms for pattern generation would emerge (Getting, 1988). Despite the relative small numbers of neurones involved in the above mentioned networks - they contain between 12 to 14 neurones each - it appears that no two pattern generators are alike (for a short summary of each of the three systems see Selverston and Moulins, 1985). Nevertheless, a more reductionist viewpoint has led to the identification of the actual properties making a network of neurones into a central

pattern generator. For such neurones not only sum synaptic inputs but have also different intrinsic properties that allow them to generate complex activity pattern and synapses are not just excitatory or inhibitory but possess an equally diverse set of properties. Thus, the existence and activity of CPGs seem to depend on the complex interactions between network, synaptic and cellular properties which were termed the "building blocks" of pattern generators (Getting, 1989).

1.3.3. Sensory feedback

Just as it is nowadays accepted that rhythmic behaviours can be generated by central networks of neurones, it is also accepted that the pattern is influenced to a greater or lesser extent - depending on the system - by sensory feedback. For example, such stereotyped behaviour as song production in Orthoptera mainly represents the output of the CPG and sensory feedback hardly plays a role in shaping the final motor pattern (Elsner, 1975). In contrast, sensory feedback is, for example, very important in the flight system of locusts, for the animal might easily encounter unexpected events or obstacles in its flight path, thus making minor or profound adjustments of the final motor output necessary. Locomotion in animals is a behavioural act based on the rhythmic motor output of antagonistically operating muscles. The modes of operation involve muscles which move either appendages and limbs in different directions (e.g. walking in arthopods) or whole body segments in undulating movements (e.g. swimming in soft bodied animals). As an animal walks over the complicated terrain of its natural habitat, the demands on the limb will vary markedly from cycle to cycle and the peripheral control will act to optimize the motor output at any one moment (Clarac, 1985). Thus, such plasticity as seen in a crab walking on a smooth surface or climbing up a gradient show the importance of sensory feedback in order to adapt the central programme to the external situation. Sensory input in general is considered to modulate the activity of the CPG, e.g. cycle duration or intensity of single bursts, but not to function in any important way to establish the basic rhythm of the motor output. Nevertheless, there are examples where rhythmic movements are generated to a large extent by reflex actions. In stick insects, the ability of the animal to walk in a variety of environments, to walk with different gaits and in different directions is achieved by co-operation of sensory feedback and endogenous pattern generators. Initiation and half of the stepping cycle is under control of a pattern generator, whereas the other half is controlled by sensory feedback from proprioceptors within the leg (Bässler, 1992). The principle of a continuous chain of reflex acts producing rhythmic movements has been described in a bivalve mollusc. The scallop swims by alternately opening and closing the valves. Sensory feedback from proprioceptors occurs

whenever the valves are opened and that input excites motorneurones to the closer muscle (Mellon, 1969). However, a study on another swimming bivalve has shown that the isolated visceral ganglion can produce bursts of efferent impulses in swimming nerves (Salanki and Varomka, 1972).

The concept of sensory feedback has been utilized in attempts to build walking machines. Sensors are built into the legs of such machines and supply information about the terrain and any obstacles in their path. The information is channelled to directly influence the movement of the legs without passing through higher order computational centres. Examples of such machines are the Adaptive Suspension Vehicle developed by K.J. Waldron and T. McGear, or Odex-III built by the American company Odetics (von Randow, 1992).

1.4. Locomotion in decapod crustaceans

Decapod crustaceans provide good models for the study of locomotor co-ordination and their underlying neuronal structures, for they show a variety of behavioural pattern that can not only be identified in whole animal preparations, but can be correlated to activity in single cells. Three different approaches have been taken in order to elucidate walking behaviour in decapods.

1.4.1. Behavioural studies

In an aquarium, the rock lobster *Jasus lalandii* can use all of its legs, but most of the time only the three back pairs are actively employed while the two front pairs remain immobile (Clarac, 1984). Such studies on freely walking animals have, for example, provided insight in the pathways involved in interleg co-ordination (Cruse and Müller, 1986; Müller and Cruse, 1991). They have also led to the elucidation of the stepping pattern used by the animal facing different situations such as forward or backward walking, walking at different speeds or having to adapt their walking pattern after amputation of a leg (Barnes, 1975).

1.4.2. *In vivo* studies

Experiments, where animals walk on a treadmill, have demonstrated that the behaviour exhibited here is similar to that in freely moving animals (Ayers and Davis, 1977; Chasserat and Clarac, 1983). Therefore, data from electrophysiological recordings obtained under such experimental conditions will represent the actual activity in normally behaving animals. Such *in vivo* studies have led to detailed knowledge of the involvement of different motorneurone pools in locomotion.

Walking is effected by the co-ordinated simultaneous movements of 6-7

joints each operating in either of two planes: dorso-ventrally or anterior-posteriorly. The two most important pairs of muscles for locomotion are the promotor and remotor and the levator and depressor. Movements at the thoraco-coxal joint, the most proximal of all joints, are performed by the promotor and remotor muscles which are largely responsible for the forward and backward movements of the entire leg. Contraction of the promotor muscle leads to protraction of the leg, that of the remotor muscle to retraction. The levator and depressor muscle at the coxo-basipodite joint move the leg in the vertical plane, thus playing a major role in determining the duration of the swing and stance phase of each step and the amount of load one leg has to bear. Levator contraction raises the leg and depressor activity lowers it. The flexor and extensor muscles in the meropodite move the mero-carpodite joint in a complimentary plane to that of levator and depressor in the basipodite. They are of more importance in sideways walking than in forward and backward locomotion. All the other muscles and joints are of less importance and are involved in locomotion in varying degrees depending on species and mode of locomotion (Ayers and Davis, 1977; Barnes, 1977).

In vivo studies are also extremely useful when studying activity and stimulation of receptor organs during normal behaviour.

1.4.3. *In vitro* preparation

The third approach taken when studying motor control in crustacean legs is isolation of the parts of the nervous system one is interested in. The isolated preparation of the thoracic cord of crayfish as developed in Bristol by Sillar and Skorupski (1986) and further refined by Chrachri and Clarac (1987) is a good device for studying the interactions between sensory and motor systems. The preparation offers several advantages: the stimulation and recording from sense organs is very selective, and one sense organ can be singled out from all the organs present in a decapod's leg. Intracellular recordings are made possible by easy access to the ganglion. Motorneurones, interneurones and terminals are easily identified by correlating intracellular and extracellular recordings. In recent years, this preparation has been of particular interest, because it allows integrative mechanisms of motor control to be examined in single cells.

Nevertheless, it should be kept in mind that any study at the single cell level - which aspires to elucidate behaviour in animals - only has value, if recordings can be correlated with and explained in terms of the normal activity in animals. Crayfish are excellent study objects to meet this demand, for both *in vivo* and *in vitro* preparations are known and are used in the study of motor control.

1.5. Reflexes

1.5.1. The concept

A reflex may be defined as a relatively simple action that is elicited by particular types of sensory stimuli, independently of volition. The three classical components of a reflex arc are therefore the sensory inflow pathway, the central relay site and the motor outflow pathway. Reflexes are not easily distinguished from fixed-action patterns (for discussion see Kandel, 1976), but the most widely used differentiating feature is that the strength of the motor action is graded with the intensity of the stimulus in a reflex, whereas the stimulus acts as a trigger, causing release of the motor act in a all-or-none form in a fixed-action pattern.

Reflexes can be generated by a variety of stimuli such as slipping on a wet surface, itching or touching a hot plate and may involve movements of a single muscle, as the stretch reflex, or rather complex behavioural acts such as it occurs in the crayfish escape response (tail flip) (Mill, 1982). In locomotor systems, reflexes confined to single joints of the same leg are called intrasegmental and have been extensively studied in crustacean systems like the thoraco-coxal muscle receptor organ (e.g. Skorupski, 1992) or the coxo-basal chordotonal organ (e.g. ElManira *et al.*, 1991a). Intersegmental reflexes are reflexes where the afferent sensor and the target motor action are located in different joints of the same leg. Intersegmental reflexes originating from chordotonal organs are often found to be weaker in their effect than intrasegmental reflexes; the influence of the coxo-basal chordotonal organ is more effective - expressed in the number of sensory connections onto the motorneurones - in the muscles moving the coxo-basipodite joint than in the muscles moving the thoraco-coxopodite joint (ElManira *et al.*, 1991b). Other reflexes are found to have their origin in one leg and effect the motor output in a neighbouring leg, or are even found to influence complicated actions such as corrective righting behaviour involving the whole body (Hisada and Neil, 1985).

Since Sherrington (1906) identified reflexes as "the unit reaction in nervous integration" reflexes were accepted to play an important role in the co-ordination of motor control. They were first studied in reduced vertebrate (mainly dog and cat) preparations. In crustacean preparations, mechanisms of integration and processing of sensory information became of particular interest since the early 1960s. Bush (1962) showed that chordotonal organs were responsible for reflexes in crustacean limbs which tended to resist the imposed movement. He termed these reflexes 'resistance reflexes'. They are analogous to the myotactic reflex of vertebrates, and depend upon sensory activation of chordotonal organs to produce facilitation of motor neurone discharge of passively stretched muscles. Subsequent work showed that

these resistance reflexes in crustaceans are 'automatically' suppressed when the animal is walking normally, and only become manifest when a leg joint is prevented from assuming its 'correct' position (i.e. as determined by the normal motor output) in the locomotor cycle (Barnes *et al.*, 1972; Spirito *et al.*, 1972). However, this is not the whole story. It has also been observed that resistance reflexes actually can reverse in sign and become assistance reflexes (DiCaprio and Clarac, 1981).

1.5.2. Reflex modulation

Reflexes not only modify the final motor output (see 1.3.3.), but sensory feedback and consequently reflexes themselves are subject to extensive modification, particularly in locomotor systems, in order to contribute effectively to the production of a meaningful behaviour. In cats, for example, a tactile stimulus to the dorsum of the paw evokes a reflex response that depends, during walking, on the phase of the stepping cycle. During the swing phase, the reflex results in flexor activation, whereas during the stance phase extensors are excited. This is functionally useful in that a stimulus to the dorsum of the paw during the swing phase is very likely to originate from a fixed object being in the path of the moving leg. Such an obstacle is best overcome by an additional flexion of the leg. During the stance phase, on the other hand, a stimulus to the dorsum of the paw is most likely to stem from a moving object. The most effective way for the animal to avoid such an object would be to perform an increased rapid extension followed by flexion (Forssberg *et al.*, 1975).

In recent years, work done mainly on isolated preparations has shown that there are a number of ways in which reflex modulation can take place. One mechanism is parallel processing of sensory information. Parallel pathways leading to the same motorneurones with opposite reflex signs exist. The state of the central nervous system 'decides' which pathway will produce a reflex response in the motorneurones. Thus, the sensory information is gated by the central activity. This phenomenon is, for example, well known for the crustacean thoraco-coxal muscle receptor organ (Bush and Skorupski, 1990). Secondly, presynaptic effects can have a profound effect on the transmission of sensory information. Presynaptic inhibition is a process in which synaptic efficacy is reduced by preventing the release of transmitter from presynaptic endings. The idea that presynaptic inhibition in sensory afferents could account for the depression of monosynaptic excitatory postsynaptic potentials (epsps) mediated by Group Ia fibres, when a conditioning stimulus was delivered to Group I afferent flexor muscle fibres in the mammalian spinal cord was first put forward by Frank and Fuortes (1957). Indeed, in 1988, Jimenez and co-workers were able to attribute the presence of primary afferent depolarizations (PADs) in Ia

afferents to the stimulation of Group I afferent fibres (Jimenez *et al.*, 1988). Such depolarizations can shunt incoming sensory impulses by increasing the conductance, thus effectively reducing the size of an incoming spike and thereby decreasing the amount of transmitter released (Cattaert *et al.*, 1992b; Kennedy *et al.*, 1974). PADs have not only been found in vertebrate systems such as the above stated example or the cutaneous primary afferents of cats (Gossard *et al.*, 1989), but also in invertebrate nervous systems. They have been reported in crayfish tactile sensory afferents (Kennedy *et al.*, 1974; Kirk, 1985), and in crayfish chordotonal sensory afferents (Cattaert *et al.*, 1990). Thirdly, the occurrence and effectiveness of reflexes also depends on postsynaptic effects. For example, as has been described above (section 1.2.2), the shaping of the motorneurone response might entirely depend on the channelling of the sensory information by different groups of interneurones or, in case the connection is a direct one, upon the excitability of the postsynaptic motorneurone. Motorneurone excitability is expressed by the value of its membrane potential which oscillates during rhythmic activity accordingly to the phase of movement. Thus, the flow of information from sense organ to effector can be modified and depends both on the behavioural state of the animal and the phase in the cycle of the ongoing rhythm.

1.6. Receptor organs

In order to understand the mechanisms which underlie sensory feedback in motor control, one has firstly to have knowledge of the sensory organs themselves. An initial integrative process occurs in the periphery, in that different receptor cells encode different sensory information. There is abundant sensory information available in crustacean legs as there are a multitude of different receptor organs present; such as the chordotonal organs spanning all the joints, the muscle receptor organs, of which the thoraco-coxal muscle receptor has been particularly well studied, or the funnel canal organs, which are believed to be the equivalent to the insect campaniform sensilla (Gnatzy *et al.*; 1984; Schmidt and Gnatzy; 1984). Sense organs are the interpreters of the internal and external world.

1.7. Cuticular stress detectors (CSDs) - the scope of this study

This thesis is concerned with the role that two mechanoreceptors, situated in the proximal part of the crustacean leg, play in the control and co-ordination of motor output.

The next chapter (Chapter 2) introduces CSD_1 and CSD_2 and deals with particular aspects of their physiological, anatomical, morphological and behavioural properties. In particular, attention will be given to CSD_1, for less is known about its

role in locomotion. For this purpose, *in vivo* experiments were performed which supplied the information necessary to be able to interpret data obtained from *in vitro* experiments in relation to their functional significance. Most of the methods and techniques used will be described in this chapter.

The third chapter deals with the functional influence that the cuticular stress detectors exert on static and rhythmic motor activity in the proximal leg muscles. Their synaptic connections to motorneurones in the central nervous system and the reflex responses of motorneurones to their activity are investigated. It will also be shown that sensory input from the cuticular stress detectors can entrain fictive locomotor rhythms in motorneurones.

The fourth chapter is concerned with the observation that the reflex loops between cuticular stress detector one and anterior levator motorneurones can have a positive as well as a negative sign. This response reversal is discussed with regard to a possible involvement of the receptor organ in the control of autotomy.

Most data available to date about processing of sensory information show that peripheral mechanisms in the sense organs themselves play an important role in producing the plasticity needed for efficient motor output under different conditions. Chordotonal organs in crustaceans, for example, are known to possess two to four types of sensory cells for the detection of position and rate of movement of the joint (Clarac, 1977). Also, neuromodulators, such as proctolin, serotonin and octopamine, have been shown to influence the discharge frequency in sensory afferents (ElManira *et al.*, 1991c; Pasztor and Bush, 1987). In recent years, more and more studies have examined mechanisms occurring in the central parts of the sense organs, namely in the afferent terminals. The picture emerging shows that such phenomena as primary afferent depolarizations (see 2nd paragraph of section 1.5.2.) or electrical coupling of sensory terminals as found in the touch cells of leeches (Baylor and Nicholls, 1969) or in chordotonal receptor cells in crayfish (ElManira *et al.*, 1993) are also very important in the processing of sensory feedback. The fifth chapter presents evidence for electrotonic coupling in mechanoreceptor afferent terminals within the central nervous system of crayfish. The occurrence of antidromic spikes is linked to this phenomenon and will be discussed in terms of its functional role.

Altogether, this study deals with particular aspects of motor control in decapod crustacean and will show that despite their common name, the two cuticular stress detectors play quite different roles in shaping of the final motor output in crayfish legs.

Chapter 2

ANATOMY, PHYSIOLOGY AND *IN VIVO* ACTIVITY OF CUTICULAR STRESS DETECTORS IN CRAYFISH.

2.1. INTRODUCTION

Cuticular stress detectors (CSD_1 and CSD_2) are mechanoreceptors in the legs of decapod Crustacea (for review see Clarac, 1976). They are positioned in the proximal part of the leg and each consists of an elastic tissue strand joined to an external area of soft, uncalcified cuticle. The strand, which is innervated by bipolar neurones, does not cross a joint, nor is it attached to a muscle. The name of these organs is derived from the fact that they respond to any stimulus that results in the deformation of the pliable cuticle, mainly pressure applied or transmitted to the basi-ischiopodite region of the exoskeleton. In both CSD_1 and CSD_2, 'on' and 'off' units can be identified, with the 'on' units in the majority. 'On' units are defined as responding when mechanical stress is applied to the cuticle, leading to indentation of the soft cuticle and shortening of the receptor strand. 'Off' units are defined as being active when the stress is removed from the cuticle resulting in release of the soft cuticle and lengthening of the receptor strand. There are phasic, phaso-tonic and tonic units. As tension in muscles also puts strain onto the cuticle, the receptors additionally respond to tension in the muscles of the coxo-basipodite, mero-carpopodite and propo-dactylopodite, i.e. all the joints which move the leg dorso-ventrally (Clarac, 1977). They also respond to movement of the basio-ischiopodite and ischio-meropodite joint, but to a smaller extent. It should be noted that all these responses to joint movement are smaller than those observed in the chordotonal joint receptors. (Clarac *et al.*, 1971).

CSD_1 is found dorsally in the basipodite or, in the case of a fused basi-ischiopodite region, proximal to the breakage plane. Wales *et al.* (1970) first described CSD_1 in *Homarus gammarus* as a sensory organ with a strand spanning an anterior area of soft cuticle and a posterior cuticular projection. The strand lies in a plane at right angles to the long axis of the limb. It is innervated by 20-30 bipolar cells. Later, the same organ was reported in *Carcinus maenas* (Wales *et al.*, 1971). Here, the strand is posteriorly attached to a peg and is innervated by more than 40 bipolar cells. The nerve arises from the main leg nerve in the coxa and not close to the sense organ as in *Homarus*. The soft cuticle associated with CSD_1 is largest in the Macrura, smaller in the Anomura and smallest in the more heavily calcified Brachyura (Clarac, 1976).

CSD_2 is situated ventrally in the ischiopodite or, in the case of a fused basi-ischiopodite region, distal to the breakage plane. CSD_2 was first mentioned by Clarac and Masson (1969) in *Astacus* and *Maia* and later described by Wales *et al.* (1971). CSD_2 in *Carcinus* is very small, comprises about 30 sensory cells and consists

of two strands. As with CSD_1, the main strand is orientated in a plane in parallel with the diameter of the leg. The receptor nerve is short and joins the main leg nerve just proximal to the breakage plane (=area of former ischiopodite). The soft cuticle associated with CSD_2 in *Astacus* has an area many times larger than that in *Carcinus* and the receptor organ is a larger and seemingly more important structure. It also consists of two strands, and is innervated by more than 50 bipolar cells (Vedel *et al.*, 1975). The nerve leaves the main leg nerve close to the basi-ischiopodite joint in the basipodite (Moulins and Clarac, 1972). Similarly to CSD_1, the thin cuticle associated with CSD_2 decreases in size from the Astacidea, through the Palinuridae and Anomura to the Brachyura.

CSD_2 has been studied by electron microscopy by Moulins and Clarac (1972) who found that the distal processes of the sensory, bipolar cells are enclosed in scolopidial structures. Most of those are embedded in a strand of elastic tissue, but some are directly attached to the cuticle. The scolopidia are similar to the ones found in crustacean chordotonal organs (Whitear, 1962). Thus, the CSD receptors were classified together with the chordotonal organs of decapod legs. Nevertheless, the connective tissue between the scolopale cells and the matrix of the strand in the CSD_2 organ is different to that found for most chordotonal scolopidia. In the propodactylopodite chordotonal organ (PDCO), Mill and Lowe (1971) suggested that the type of connective tissue can have a direct influence on the sensitivity of the transduction process of sensory information. Also, chordotonal proprioceptors span joints and monitor position of joints, unlike the CSDs which monitor cuticular stress. Therefore, it has been concluded that the CSD receptors can best be compared to the campaniform sensilla of insects (Clarac, 1977). No electron microscopic study has been undertaken for the CSD_1 organ.

The fact that the most effective stimulus for the CSDs is pressure on the cuticle led Clarac in 1976 to propose that these receptor organs are used by the animal to detect the force generated when the leg makes contact with the ground. Ten years later in 1986, Klärner and Barnes demonstrated that the highest frequencies of CSD_2 discharge occurred when the leg was placed on the ground at the onset of the power stroke, and that CSD_2 discharge frequencies positively correlated with the rate of force generated by the leg during the power stroke. Therefore, CSD_2 receptor organ activity encodes information about the first contact that the leg makes with the ground at the beginning of the power stroke and the load borne by each leg.

Several studies have examined the role of CSD_1 in autotomy (see Chapter 4), but comparatively little information is available concerning the role of CSD_1 in locomotion. The following experiments were undertaken to remedy this situation, and

also to further our understanding of the cuticular stress detectors in general. Only with a fuller knowledge of their anatomical and physiological properties will it be possible to understand the role they play in the control of motor activity.

2.2. MATERIAL AND METHODS

2.2.1. Animals

Both male and female crayfish of the species *Pacifastacus leniusculus* and *Procambarus clarkii* were used in the experiments. They were obtained from a commercial supplier and maintained in aerated aquaria at 14-18°C.

2.2.2. **Methylene blue** (35 preparations)

In preliminary experiments, methylene blue was used to locate and identify the neuronal structures of the cuticular stress detectors within a walking leg. After the removal of the cuticle around the receptor region and some of the connective tissue which would have prevented the stain from reaching the nervous tissue, the preparation was either exposed to a very weak solution of methylene blue (1% made up in distilled water and diluted with saline) for more than 5 hours or to a more concentrated solution (dark blue colour) for 20-30 min (Wales *et al.*, 1970) .

After successful staining, the preparation was fixed in 10% ammonium molybdate for up to 24h, after which it was rinsed, dehydrated and cleared. Preparations could then be kept in methyl salicylate in the dark.

2.2.3. *In vitro* **preparation** (120 preparations)

The *in vitro* preparation consisted of the 3rd, 4th and 5th thoracic ganglion, the 5th leg motor nerves and one of the cuticular stress detectors (Fig.2.1A and B). In some preparations, all 5 thoracic ganglia were present. The initial dissection is as described by ElManira *et al.* (1991a): Animals were decapitated. The abdomen, thoracic carapace and viscera were removed and the thorax was pinned dorsal side up in a large petri dish. Thoracic muscles were removed and the sternal artery was cannulated and perfused for 5 min. The endoskeleton that covers the proximal neuromuscular system was excised. The thoracic appendages of ganglia 3 and 4 were removed, and the whole ventral nerve cord, with the 5th leg still attached, was pinned out in a petri dish lined with a transparent, electrically neutral resin (Sylgard). The preparation was bathed continuously with oxygenated saline (adapted from van Harreveld (1936) concentrations in mM: 195 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.6 $MgCl_2$, 10 Tris; pH adjusted to 7.5) and cooled by a Peltier effect cooler. Promotor, remotor, anterior and posterior levator, and depressor nerves were isolated.

The two large leg nerves, anterior and posterior distal roots (adr and pdr), that innervate the distal limb were cut in most experiments. The nerve leading to one

of the cuticular stress detectors was carefully dissected so as to keep the organ intact. Hence, it was possible to mechanically stimulate the receptor organ by placing a probe with a blunt end on the area of uncalcified cuticle near the attachment point of the strand (Fig.2.1C).

By forward and backward sinusoidal movements of the probe, which was driven by an electromechanical transducer, the soft cuticle of the CSD was indented and released, thereby shortening and lengthening the receptor strand and stimulating 'on' and 'off' units respectively. The forward and backward movements of the electromechanical transducer were transmitted to the probe - which was attached to the end of a soft spring - as isometric forces. There was not much space around the CSD sensory organs, when recording *in vitro* from different sensory and motor units, therefore it was not possible to attach a force transducer (Pixie) to the tip of the stimulating probe and to measure the forces applied to the CSD organs directly. Hence, the forces were measured indirectly by correlating the movement at the tip of the mechanical probe with the force needed to produce such a movement. This movement could be directly related to the movement of the electromechanical transducer arm and hence could be correlated to the voltage output of the transducer. Thus, the amplitude of the stimulus monitor was used to determine the forces applied to the soft window of the CSD organs (Fig.2.2). A phase lag between the driving voltage and the movement of the mechanical transducer arm or the movement at the tip of the probe was not observed. These comparisons were done by visual inspection, therefore the occurrence of minute phase lags cannot be excluded, but was thought to be negligible.

The stimulating frequency (of around 0.6Hz) of the sinusoidal movement was chosen, because it corresponded to the setting of 1 on the scale of the movement transducer and was thought to be adequately slow, in order to show 'on' and 'off' units stimulated at different phases. In most experiments, sine stimuli were used in order to smoothly indent and relax the soft cuticle and to avoid any sudden changes in the stimulating parameters. Nevertheless, ramp stimuli were sometimes used in order to allow better identification of the properties of the sensory units.

When using sinusoidal stimuli, both the frequency and amplitude can be changed. In some experiments higher frequencies were used, but care was taken to be always below the intrinsic oscillation frequency of the stimulating probe. At frequencies where the probe started to oscillate itself, these oscillations added to the amplitude set by the electromechanical transducer and resulted in a larger force acting on the soft window than indicated by the monitor trace. In addition, such larger amplitudes resulted in having a period out of contact between probe and cuticle, for

the probe was generally placed in such a way on the cuticle that at maximum elongation of the strand the probe was just in contact with the soft cuticle.

2.2.4. Recording

Platinum wires, placed in contact with the nerves and insulated with petroleum jelly (Vaseline), were used to record extracellularly from or to stimulate the nerves. The ganglion was desheathed to make intracellular recordings with glass microelectrodes possible. The microelectrodes were filled with 3M KCl (resistance 9-30MOhm). Intracellular recordings were made from the neurites, where it is possible to record action potentials and postsynaptic potentials. A bridge circuit was used for recording and passing depolarizing or hyperpolarizing current pulses. The electrical recordings were amplified, displayed on an oscilloscope and stored using either a digital or FM tape recorder.

Motorneurones were identified by the following criteria:

(1) one-to-one correlation of intracellularly recorded action potentials and spikes recorded in one of the motor roots.

(2) depolarization of motorneurones leading to action potentials in the cells, these centrally produced action potentials being recorded in the distal motor root in a one-to-one relationship.

(3) electrical stimulation of the muscle root producing an antidromic spike which travels from the periphery to the ganglion.

(4) collision of spikes when combining method (2) and (3).

CSD terminals were identified by their response to mechanical and electrical stimulation of the receptor organs using comparable criteria to those used for the recognition of motorneurones.

Neurones not identifiable by the above criteria were classed as interneurones.

2.2.5. Anatomy (37 preparations)

Biocytin anterograde fillings of the CSDs were performed in order to localize the terminals of the CSD receptor cells within the 5th ganglion. The distal end of either CSD_1 (11 preparations) or CSD_2 (8 preparations) nerve, cut immediately proximal to the elastic strand, was submersed in a biocytin solution (3% in 0.05M Tris, pH 7.6), separated from the normal saline bath by a small Vaseline well. After 24-48h at 4°C the preparation was fixed overnight in a 1% cacodylate buffer (pH 7.6) containing 2.15% paraformaldehyde and 0.25% gluteraldehyde. After being rinsed three times in a solution of 0.05M Tris containing 0.9% NaCl and 0.1% saponine, it

was left for three days in a solution of the Vectastain Elite ABC kit (Vector Laboratories). The preparation was then washed in a 0.05M Tris and 0.9% NaCl buffer, continuously for three days using a slow drip. The stain was precipitated in a Diaminobenzine solution (2.5mg DAB in 10ml 0.05M Tris, 0.9% NaCl, and 0.01% H_2O_2). Cobalt (0.0225% Cobaltchloride) and Nickel (0.0187% Nickel(II)-sulfamathydrate) were used to intensify the staining (Izzo, 1991). After thorough washing the preparation was dehydrated and mounted in DPX. The stained terminals were drawn using a Camera lucida.

Fixation and precipitation procedures were carried out by Marie-Christine Auriac.

Lucifer Yellow (5% in 1M LiCl, microelectrode resistance 40-70MOhm) (Stewart, 1978) was used to stain individual CSD terminals (9 CSD_1 and 9 CSD_2). It was injected intracellularly using 2-8nA hyperpolarizing current for 30-60min. After a short rest, the preparation was fixed for 12 hours in 1% Paraformaldehyde, dehydrated in a series of alcohols, cleared in methyl salicylate and photographed in wholemount preparation under a fluorescence microscope.

2.2.6. Nerve sections (10 preparations)

Transverse sections of the CSD nerves (5 CSD_1 and 5 CSD_2) were made as close as possible to the bipolar cell bodies. The nerves were dissected and all connective tissue removed. The nerves were then fixed in a 1% cacodylate buffer containing 2.15% paraformaldehyde, rinsed four times in 0.1M cacodaylate buffer and postfixed by buffered 2% osmium tetroxide (OsO_4). The pH of all solutions was adjusted to 7.6. After repeated rinsing, the preparations were dehydrated in a series of alcohols and in a propylene oxide solution. They were then embedded in Epon resin. Sections of one micrometer thickness were cut using an LKB microtome with glass blades. The sections were mounted on glass slides and stained using a mixture of methylene and azure blue in equal concentrations.

Fixation, cutting and staining was performed by Marie-Christine Auriac.

2.2.7. *In vivo* preparation (35 preparations)

Only animals of the species *Pacifastacus leniusculus* which weighed more than 70g were used.

The animals were held above a treadmill which was submersed in an aquarium filled with aerated freshwater. The attachment was counterbalanced so that the animal was at its normal weight in water. A coiled spring allowed some movement in all directions (Barnes, 1977). This was important, for it made it possible to

recognize when the crayfish was trying to turn. In general, the animals walked forwards, but occasionally backwards. The treadmill had a diameter of 38cm, was 14cm wide and turned freely under the walking crayfish (Fig.2.3).

Pairs of silver wire insulated except for their tips were used to record CSD and motor activity. They were inserted through small holes in the exoskeleton made with an insect pin and were glued in place with cyanoacrylate glue. Myograms were obtained from the levator and depressor muscle groups in the coxopodite, CSD_1 recordings in the basipodite and CSD_2 recordings in the ischiopodite. CSD recordings were considered to be satisfactory when pressure applied to the soft cuticle of the receptor organ elicited a larger response than that produced by any other stimulus (Fig.2.4).

The position of the leg under investigation was monitored following the method of Cruse and Müller (1984). An insulated silver wire whose tip had been briefly heated to produce a small ball of bare silver was attached to the tip of the leg. This wire served as the third electrode of a potentiometer which records a voltage proportional to the position of the recording electrode relative to two plate electrodes. These two plate electrodes (16cm x 20.5cm) were positioned under water at the front and back end of the treadmill and were connected to a sine wave oscillator producing alternating current of 90-300mV at 10kHz. Alternating current was used in order to avoid electrolysis. Thus an electrical field was produced parallel to the longitudinal axis of the animal. The signal from the recording electrode was rectified and amplified to give a DC signal proportional to the forward and backward movements of the leg tip.

2.2.8. Data analysis

Electrophysiological data were either displayed using a Gould electrostatic paper recorder (ES 1000) or selectively collected via a CED 1401 interface into a computer. CED software (SIGAVG and SPIKE2) was then used for further analysis and results were displayed using an XY-plotter.

With the SIGAVG programme it is possible to capture analogue data before and after a set point (peri-trigger mode) in several traces simultaneously. These single frames in their entirety can be superimposed, moved around the x- and y-axes, amplified, averaged and any number of sweeps from different files can be combined to create a new file.

The SPIKE2 programme was used to capture very long stretches of analogue data (up to 3000s) simultaneously in several traces and to transform it into digital pulses. A modified version of a programme written by Jean-Yves Barthe in the

SPIKE2 language was used to automate the process. The digital data were then used for further analysis, e.g. drawing phase histograms and making cross correlations between two different channels.

The following graphical packages were used in the production of graphs and histograms: CA-Cricket Graph; SigmaPlot; Minitab.

2.3. RESULTS

2.3.1. Receptor morphology

Methylene blue stains of CSD_1 in the walking legs of *Procambarus clarkii* revealed the same structural organisation as Wales *et al.* (1970, 1971) found for the receptor organ within the walking legs in *Homarus gammarus* and *Astacus leptodactylus*. Only one difference is worth mentioning: In *Procambarus clarkii*, the receptor nerve splits into two parts towards the strand. However, the main features of the receptor organ are the same: The receptor strand does not cross a joint, nor is it attached to a muscle, but is connected to an area of uncalcified, soft cuticle. The bipolar sensory cells, numbering up to 40, are of reasonably uniform size (about 20μm across the width of the cell body) with the exception of about 5-8 fairly large somata (35-60μm). The nerve joins the anterior distal root (adr) of the main leg nerve close to the organ in the basipodite.

The structure of CSD_2 in *Pacifastacus leniusculus* is very similar to the description given by Wales *et al.* (1971) for the walking legs in *Astacus leptodactylus*. As in *Astacus* two distinct strands can be identified. Their posterior insertion points are very different. The main strand originates from the soft cuticle of the ventro-posterior wall, whereas the accessory strand, which is smaller, has its posterior insertion onto harder cuticle more distally than that of the main strand. Both their anterior insertions end at the ventral part of the soft cuticle. Here, an island of slightly denser calcification is externally visible. Referred to as the 'tongue' (see Fig.2.1C, to the right of the arrow tip), this region represents the best place for mechanical stimulation of CSD_2 as it is most sensitive to any applied force (Klärner and Barth, 1986). Contrary to the description of CSD_2 given by Wales *et al.* (1971), the main strand in *Pacifastacus* does not form a half turn of a spiral, but lies across the diameter of the leg as a flat, broad sheet. There are about 60 sensory cells whose axons join the posterior distal root (pdr) close to the ischio-basipodite joint (Fig.2.5).

2.3.2. Cross sections

The transverse nerve sections of CSD_1 and CSD_2 show that there are two clearly distinct groups of fibres in CSD_2 consisting of a lot of very small (diameter: ≤7μm) and a few very large units (diameter: 10-23μm). In contrast, the CSD_1 nerve root contains very large, intermediate and small axons. The cell number seems to be inversely correlated with the axon diameter. There is a large number of small cells (≤7μm), some fibres of intermediate size (diameter: 8-10μm) and very few large axons

(see photos, Fig.2.6, and histograms, Fig.2.7). As expected from the number of cell bodies being stained with methylene blue, there are more CSD_2 than CSD_1 axons within the sensory nerves.

2.3.3. Branching pattern of CSD afferents within the ganglion

Biocytin anterograde fills of the CSD terminals have revealed three major differences between CSD_1 and CSD_2 afferents (Fig.2.8). First, there are more CSD_2 fibres (in agreement with the findings from methylene blue stained whole mounts and transverse sections of sensory nerves). Second, the CSD_2 fibres branch mainly in two regions of the ganglion. Very long processes occupy the rostral part of the ganglion following the contours of the neuropile. This is where the levator motorneurones are located (ElManira *et al.*, 1991a). Shorter terminals end caudally close to their point of entry into the ganglion, in the region where depressor motorneurone dendrites branch (Fig.2.8D). In the latter area, CSD_1 fibres also branch extensively, but fewer CSD_1 afferent endings can be found in the rostral region. Third, some CSD_1 fibres run straight through the ipsilateral half of the ganglion towards the midline where they undergo a 90° turn and continue rostrally. The stain can be seen beyond the fourth thoracic ganglion (Fig.2.8A).

When using Lucifer Yellow to stain single terminals, two different types of afferent CSD_1 fibres were found. Some fibres would enter the ganglion and branch within the neuropile. As seen with Biocytin, the more extensive branching occurs in the caudal region of the neuropile (Fig.2.8C). A second class of fibres were found to have rather few branches terminating within the neuropile. The main branch (=axon) passes through the ganglion and after a turn continues rostrally (Fig.2.8B). In all Lucifer Yellow fills more extensive staining of small branches within the ganglion was found than for the preparations with biocytin (compare Fig.2.8A with 2.8B and C). Both dyes consist of small molecules and should thus spread equally well within neurones. Hence, the difference is attributed to the fact that biocytin is applied in the periphery and has to travel all the way to the ganglion, whereas Lucifer Yellow is injected intracellularly within the ganglion.

Physiological support was provided for the existence of CSD_1 afferents that extended rostrally from the 5th thoracic ganglion. In two experiments, two additional extracellular electrodes were placed in contact with the ventral nerve cord (VNC), one rostrally of the 4th thoracic ganglion and one rostrally of the 1st thoracic ganglion. During one of these experiments, the impulses of a CSD_1 terminal, recorded intracellularly within the 5th ganglion, could not only be correlated with their extracellular spikes in the CSD_1 nerve root, but were also seen to occur in the

VNC in front of the 4th ganglion (Fig.2.9A and B). The impulses were first seen in the sensory nerve root, then in the 5th ganglion and later in the ventral nerve cord in front of the 4th ganglion, thus confirming them to be of a sensory nature. Recordings of the electrode rostrally to the 1st ganglion were too noisy to allow identification of the CSD_1 unit to be made here. However, electrical stimulation of the VNC in front of the 1st ganglion gave rise to a spike, at constant latency, recorded intracellularly in the sensory terminal under investigation. This suggests that these particular CSD_1 sensory axons extend at least this far anteriorly in the ventral nerve cord. The conduction velocity between the VNC electrode in front of the 1st thoracic ganglion and the intracellular electrode in the 5th thoracic ganglion was $1.3ms^{-1}$. A conduction velocity of $1.2ms^{-1}$ was calculated for the distance between the intracellular electrode and the recording electrode in front of the 4th thoracic ganglion. These values are sufficiently similar for the presence of an added synaptic delay to be discounted. The conduction velocity between the CSD_1 receptor organ in the basipodite of the leg and the intracellular electrode in the 5th thoracic ganglion was $4.0ms^{-1}$ (Fig.2.9C). This supports the impression gained from the Lucifer Yellow fills that the axons of these particular CSD_1 fibres become smaller in diameter when extending into the VNC.

2.3.4. Physiological properties of CSD afferents

2.3.4.1 General

Intracellular recording techniques made it possible to characterize and classify single afferent fibres accordingly to their response to mechanical stimulation of the soft windows associated with the organs. Since previous workers have already established that there are 'on' and 'off' units, with 'on' units being in the majority and established their coding properties (Clarac *et al.*, 1971; Klärner and Barth, 1986; Wales *et al.*, 1971), no detailed survey of sensory properties was undertaken in this study. (For a more detailed analysis of all the units recorded see Marchand *et al.*, in preparation; the data were not available to the author of this study, because all these data are kept in Marseille, France).

Overall, 16 'on' units and 10 'off' units were identified for the CSD_2 receptor organ, two of which are shown in Figure 2.10. In addition, 3 fibres were found which showed no response to pressure applied to the soft window.

In CSD_1, 13 'on' units and 1 'off' unit were recorded when the stimulating frequency of the mechanical transducer was set to its usual value of 0.6Hz. In addition, 14 CSD_1 units were recorded which only responded when the mechanical stimulator, indenting and releasing the CSD_1 receptor strand, oscillated at a much higher frequency than 0.6Hz.

2.3.4.2. High threshold fibres

This phenomenon of particular CSD_1 fibres not responding to the normal stimulation of 0.6Hz was first observed in extracellular recordings where frequencies in excess of 8-15Hz resulted in the activation of large amplitude CSD_1 units (Fig.2.11A). One was therefore tempted to conclude that these particular CSD_1 fibres responded only at higher frequencies. Nevertheless, due to the intrinsic properties of the stimulating probe, it often occurred that the probe itself started to vibrate at higher frequencies. Thus the amplitude of oscillation was also increased and hence the force applied to the soft window. At intermediate frequencies (about 8Hz), these large fibres were not activated when care was taken to ensure that the stimulator probe did not vibrate and was hence oscillating at a small amplitude. Only when the amplitude was larger than a particular threshold level did these particular CSD_1 fibres respond to mechanical stimulation of the soft window (Fig.2.11B). Increasing the amplitude at low frequencies did not result in the stimulation of the large CSD_1 fibres, even when very large amplitudes were applied. A combination of high frequency and large amplitude is equivalent to stronger forces pushing and releasing the soft window of the receptor organ. These particular CSD_1 fibres therefore only seem to be stimulated when particularly large forces acted on the cuticle.

Since spike sizes recorded extracellularly are positively correlated to the size of the axon, calling these high threshold fibres 'large' does not only describe the amplitude of their extracellular, electrophysiological recordings, but also their true axon size. Correlating the intracellular recorded impulses of high threshold fibres with extracellularly recorded spikes, it was possible to identify these units as the large CSD_1 fibres in the extracellular trace.

No attempt was made to ascertain the exact values of frequency and amplitude necessary to stimulate the high threshold CSD_1 receptor cells. Firstly, preparations varied, and secondly, both parameters were linked to each other so that the threshold was either reached at a fairly high frequency with a smaller amplitude, or at a lower frequency with a larger amplitude. Insufficient data were available to draw a frequency-amplitude correlation curve.

2.3.5. Depression of CSD reflexes

The reflexes elicited by either CSD_1 or CSD_2 stimulation in the proximal muscle groups were different within as well as between groups. This will be described in detail in the next chapter. In addition, CSD_1 and CSD_2 reflex responses showed different degrees of decrement during long term stimulation. When mechanically stimulating the CSD_2 receptor organ, the intensity of the reflex responses remained

approximately constant for the whole duration of the stimulation (Figs 2.12 and 2.13), which in some experiments exceeded five minutes. In contrast, CSD_1 mechanical stimulation over time periods of longer than 20s was accompanied by a conspicuous reflex decrement (Figs 2.14 and 2.15). The reflex excitation of the depressor motorneurones declined over a period of 24 cycles to 3% of its initial value in terms of the average number of spikes per stimulus cycle, while the effectiveness of the reflex inhibition of the levator motorneurones declined by an approximately equivalent amount (to 16%) over the same time period. When comparing the average number of sensory spikes per stimulus cycle elicited in the CSD_1 receptor cells at the beginning of the stimulation and later on during the stimulation, it could be seen that there was no equivalent decline (Fig. 2.15). Thus the reflex decrement was mainly a central phenomenon rather than being due to sensory adaptation. As such, this phenomenon could be called reflex depression. The reflex decrement was reversible. Even after periods of rest as short as 9s, the reflex spontaneously recovered to its original strength when stimulation re-commenced (Fig. 2.16). Depression was not only observed in the extracellular motor root recordings, as shown in Figs 2.14 and 2.16, but also in intracellular recordings of motorneurones in the fifth ganglion (data not shown here). Additionally, the effect was observed in interneurones (Fig.2.17). A possible site for this depression is thus the synapses made by the sensory afferents themselves, as has been suggested by the work of Marchand & Barnes (1992), who studied habituation - a form of reflex depression - of dactyl sensory afferent reflexes in crayfish.

2.3.6. *In vivo* experiments

In order to elucidate the behavioural significance of CSD_1 it is not sufficient to have a knowledge of its anatomy and of the physiological properties of the afferent cells, but it is also necessary to study its activity during normal behaviour. Hence, CSD_1 recordings were made during normal locomotor activity in the crayfish. Satisfactory recordings of CSD_1 activity in either the 4th or 5th walking leg were achieved in 12 experiments, the two legs showing no difference in the timing of CSD_1 activity.

The CSD_1 nerve is relatively short before it joins the main leg nerve. Inserting electrodes through the cuticle in order to record the electrical signals of its sensory cells is a procedure prone to crosstalk from the main leg nerves. Therefore, only large impulses in the CSD_1 recordings were selectively identified as being CSD_1 units and used in the analysis. Any small CSD_1 spikes which might have been present, but which could not be distinguished from the background noise, were discarded.

Hence, all phase histograms are biased towards the large units. The term 'large' is used here to describe the CSD_1 units which had an amplitude larger than the background noise and should not be confused with the high threshold units in part 2.3.4.2.

The first question addressed was concerned with the timing of CSD_1 activity within the step cycle. The step cycle is divided into two parts: the return and the power stroke. In forward locomotion, the return stroke comprises the time when the leg, at its posterior extreme position (PEP), leaves the ground and moves forward until it touches the ground at its anterior extreme position (AEP). There the power stroke begins and lasts as long as the leg is in contact with the ground. During the power stroke, the leg moves backwards, thus propelling the animal forwards. In order to correlate sensory and motor activity to their occurrence in each step cycle, phase histograms were drawn (see Figs 2.18 and 2.19). All phase histograms represent the neuronal activity during a number of steps. Phases of 0 and 1 correspond to the AEP. Thus the histograms show the distribution of sensory or motor activity within the step cycle, starting and finishing at the AEP. Stride lengths vary from step to step. Hence, each of the 20 bins in each histogram represents 1/20th of the step cycle and not a fixed time length. The same argument holds true for the duration of return and power strokes. Thus there is not a fixed point for the PEP within the histograms. Instead the relative occurrences of the PEPs are shown in an additional histogram drawn under the main one. Large variations in the relative position of the PEP were an indication of irregular walking patterns (compare Fig. 2.19 with Fig. 2.18).

The onset of CSD_1 activity occurred sometime during the return stroke and very clearly did not coincide with the switch from return to power stroke at the AEP (Fig.2.18). In most recordings, it could be divided into two sub-bursts, the first beginning sometime during the return stroke and the second at approximately the AEP, i.e. simultaneously with the onset of the power stroke. CSD_1 activity terminated before the leg was lifted off the ground at the end of the power stroke.

In addition to monitoring occurrences of CSD_1 spikes within the stepping cycle, CSD_2, levator and depressor activity were recorded. Sensory cells of CSD_1 are stimulated before CSD_2 activity occurs, but activity in both receptor organs declines over approximately the same time course during the power stroke (Fig.2.19). The start of CSD_2 activity occurs at around the AEP when the leg touches the ground, as previously reported by Klärner and Barnes (1986) for *Astacus leptodacylus*. They were able to correlate CSD_2 activity with the rate of increase in load borne by the leg when it is in contact with the ground. Correlation of CSD_1 activity with motor activity of coxopodite muscles showed that CSD_1 starts bursting before the depressor burst occurs (Fig.2.20B) and around or shortly after the peak of activity in the levators is

seen (Fig.2.20A). No distinction between anterior and posterior levators was made.

2.4. DISCUSSION

2.4.1. Morphology and anatomy

The morphology of the CSD receptor organs, using methylene blue staining techniques, has been extensively studied in different species of Decapod Crustacea, mainly in crabs, lobsters and crayfish (see Introduction 2.1.). Nevertheless, repeating these experiments was useful for two reasons. First, extensive application of this technique yielded the necessary knowledge and expertise in recognizing the surroundings of the receptor organ. Thus, the dissection performed in subsequent experiments leading to the *in vitro* preparation could be carried out without damaging the sensory organs themselves. This allowed direct electrophysiological recordings from the CSD nerve roots to be made, while at the same time stimulating the receptor cells mechanically. Secondly, neither species of freshwater crayfish used in this study had been used in CSD studies before. The results from the methylene blue staining therefore allowed a comparison of the morphology of these sense organs among several crayfish species. As it turned out, the CSD receptor organs in both *Pacifastacus* and *Procambarus* have morphological properties that fit the descriptions given by Wales *et al.* (1970; 1971) for *Astacus leptodactylus*. Minor differences are mentioned in the Results.

The anatomy of the afferent fibres was studied in biocytin backfills and with Lucifer Yellow dye injection. Both methods failed to reveal any cell bodies, thus proving all axons within the CSD nerves to be afferent fibres. They also show the extensive branching of the terminals within the ganglion. It is interesting to note that the areas in the neuropile where the primary afferents end largely overlap with the areas where the dendritic trees of the motorneurones innervating the proximal muscle groups are located, particularly the ones of the anterior levator, depressor and promotor motorneurones (ElManira, 1991a; 1991b; Skorupski and Sillar, 1988). Thus, monosynaptic connections between sensory afferents and motorneurones are possible from a pure morphological point of view (further information in Chapter 3). The main areas occupied by the arborizations of CSD_1 and CSD_2 primary afferents are strikingly different. Most CSD_1 primary afferent branching occurs caudally in the ganglion in the region where the depressor motorneurone dendrites are located, whereas more of the CSD_2 terminals end in the rostral region of the neuropile where the anterior levator dendrites are found. This fact could suggest that the anterior levator muscle receives preferential input from CSD_2, while the depressor muscle receives more input from CSD_1. Experiments in Chapter 3 show that the pathways underlying reflex responses

indeed support this hypothesis. An average number of 4.6 CSD_1 units connect onto each depressor motorneurone, whereas only an average of 3.8 CSD_1 fibres connect with each anterior levator motorneurone. In accordance with the morphological locations of CSD_2 terminals within the ganglion, more CSD_2 fibres produce a response in anterior levator than in depressor motorneurones, on average 5.8 and 4.2 fibres per motorneurone respectively (see Chapter 3, Fig.3.14).

Both, biocytin fills and methylene blue stains showed very clearly that there are far more CSD_2 than CSD_1 sensory units. The same difference was observed when counting the axons in the sensory nerves of CSD_1 and CSD_2. However, absolute numbers of axons were much greater than expected. Between 67 and 80 axons were present in the CSD_1 nerve whereas only about 40 cell bodies were visible in the strand. Figures for CSD_2 were 166 to 172 axons, but only 60 cell bodies were seen with methylene blue. There are two possible reasons for this discrepancy. Firstly, staining of sensory cells with methylene blue is often capricious, especially when the cells are small in size. The presence of large amounts of connective tissue in the basi-ischiopodite region of the leg did not facilitate the staining procedure and particularly small cells might have easily remained unstained or unnoticed. Secondly, in sensory nerves such as the CSD nerve roots, the possibility that some of the axons originate outwith the CSDs from other sensory structures cannot be excluded. Indeed, Wales *et al.* (1971) made the statement that the sensory nerve bundles to CSD_1 and CSD_2, like the nerves to other chordotonal organs, also carry fibres which innervate the surrounding hypodermis. Together, these two reasons could account for the fact that axon counts in sectioned sensory nerves give higher numbers than the methylene blue staining of the CSD receptors. Interestingly, axon counts in motor roots also yield higher numbers than nerve backfills do (Cattaert *et al.*, 1992b; Moffett *et al.*, 1987; Parson, 1982). The two possible explanations given for this are comparable to those given above. Firstly, that backfills fail to demonstrate cell bodies belonging to cells that have very small axons and, secondly, that it is possible (or even probable) that motor roots contain afferent axons in addition to the motorneurones (Tse *et al.*, 1983).

Counts of axon numbers within the nerve cross sections show that an overwhelming majority of the cells are small. Thus the ratio of small to large fibres is heavily biased towards the small cells. The same relationship between small and large fibres has been observed by Cooper and Govind (1991) in their study of the propodactylopodite chordotonal organ (PDCO) in the lobster claw. Following the classification of small and large sensory cells in chordotonal organs as either position or movement sensitive, respectively (Mill and Lowe, 1973), they concluded that the position sensitive, tonic fibres are in the majority. A lot of similarities exist between

the cuticular stress detectors and the chordotonal organs in the legs of crustaceans. Hence, it is reasonable to apply the same classification criteria for CSD fibres and think of the small CSD fibres as being tonic and large fibres as being phasic cells. However, no analysis of electrophysiological data was undertaken to prove or disprove the point.

The finding that particular CSD_1 fibres do not terminate within the 5th ganglion, but join the ventral nerve cord and project into the anterior region of the central nervous system beyond the thoracic ganglia is interesting in that it indicates the possible direct influence of a leg mechanoreceptor onto neurones in other ganglia. The number of CSD_1 units involved is not known. The effect of mechanoreceptor activity on the motor output in neighbouring legs is well documented. Stimulation of dactyl sensory afferents (DSAs) (Müller and Clarac, 1990), CSD_2 (Klärner and Barnes, 1986) and TCMRO (Sillar *et al.*, 1987) is known to influence the timing of the CPG in ipsilateral legs. In addition, reflex connections from the CBCO in the 5th walking leg of crayfish to motorneurones in the swimmeret system have been demonstrated (Cattaert *et al.*, 1992a). The mechanisms underlying all these effects are thought to involve interneurones which provide the coupling elements between CPGs in neighbouring legs or even between thoracic and abdominal ganglia. In the case of these CSD_1 fibres, no branches were observed entering more anterior thoracic ganglia, so that a direct role for these anteriorly-going afferent axons in, say, interleg co-ordination is unlikely. Since spike transmission in these CSD_1 afferents was recorded in the connective in front of the 1st thoracic ganglion, it seems more likely that the destination of these axons is in higher brain centres, e.g. the brain or sub-oesophageal ganglion.

2.4.2. Receptor stimulation

Normal stimuli are considered to be any forces or loads acting on the cuticle and resulting in its deformation. Such strains will be transmitted along the hard cuticle to the area of soft cuticle. The soft cuticle associated with the receptors most probably serves as a transducer by being buckled under the stresses present and producing a change in length of the strand. The location of the organs in the legs is such that the soft areas are unlikely to make contact with hard surfaces in the environment. Therefore pressure applied directly to the soft cuticle is not considered to be a "normal" stimulus, but it will mimic the receptor organ stimulation as it results in shortening and lengthening of the strand (Clarac *et al.*, 1971). Klärner and Barth (1986) described the stimulation of CSD_2 as following: "When force is exerted on the basipodite in the direction of the long leg axis, compressing the leg longitudinally, the

tongue of stiff cuticle which projects into the compliant cuticle of CSD_2 moves inwards. Possibly the leg is also compressed and the compliant cuticle deformed in turn in a similar way while the leg is pressing against the ground during walking." Therefore natural stimuli such as leg loading, movement of joints and tension in muscles close to the receptor region (Clarac *et al.*, 1971; Klärner and Barnes, 1986), which all result in deformation of the cuticle, were mimicked by indenting and releasing the soft cuticle and shortening and lengthening the receptor strand directly.

2.4.3. Physiology

Although, as described above, CSD_1 and CSD_2 afferents have different branching patterns within the ganglion, the physiological properties of CSD_1 and CSD_2 as described by previous authors (review Clarac, 1976; Klärner and Barth, 1986) appear very similar. Phasic, tonic and intermediate phaso-tonic fibres are known for both receptor organs. In this study, evidence for the presence of a fourth type of sensory fibre is presented. This fibre type has only been identified in CSD_1 receptor cells. It is different in that it does not respond to the normal stimulation parameters which were sufficient to stimulate all other fibres. It is possible to alter mechanical stimulation parameters in a number of ways. When using sine wave stimuli, both the frequency and amplitude of the oscillations can, in theory, be altered independently of each other, but non-linear properties of the probe used in these experiments meant that care had to be taken to ensure that this was the case. The amplitude is positively correlated to the force with which the stimulating probe pushes against the window. Although changes in frequency do not alter the amplitude of the applied forces, they do alter the rate at which these forces are applied. Four different extreme modes of stimulation are thus possible:

low frequency - small amplitude
low frequency - large amplitude
high frequency - small amplitude
high frequency - large amplitude.

The first mode is classed as "normal" stimulation parameters, because most of the CSD receptor cells are stimulated at around a frequency of 0.6Hz and at small amplitude. In contrast, only the last two modes would stimulate the 4th class of CSD_1 units. Presumably, they are phasic fibres where a combination of high amplitude and high frequency is necessary to provide a sufficiently high rate of force production to excite them. Unfortunately, there is an unlimited number of intermediate stimulation modes possible and there were insufficient data to assess their specific stimulation parameters. Chapter 4 will deal with their functional

significance.

2.4.4. Identification of sensory units

All sensory units were identified by correlation of extracellular spikes with intracellularly recorded impulses. These correlations were done both during mechanical and electrical stimulation of the receptor cells. The possibility that the correlation is a consequence of a volley of co-incident afferent discharges in different units has to be considered, but is thought to be unimportant for two reasons. First most cells (particularly the earlier ones) were also identified by a third method. Depolarizing current pulses injected into the afferent terminals resulted in an antidromic spike which was recorded in the extracellular trace (Fig.2.21), thus dispelling any doubts as to the identification of the sensory unit. Secondly the correlations were all done in order to identify a unit as a sensory unit from either CSD_1 or CSD_2. This aim was achieved, for it would not matter which extracellular spike exactly corresponded to the intracellularly recorded one.

2.4.5. Reflex depression = habituation?

Klärner and Barth (1986) found that CSD_2 fibres show no adaptation during sinusoidal mechanical stimulation of the soft cuticle of CSD_2. Their finding has been extended in this study where it is shown that the reflexes that occur in response to CSD_2 stimulation do not decline. In contrast, reflex responses to CSD_1 stimulation show a remarkable reflex decrement within the first half minute of sinusoidal stimulation. This reflex decrement can be termed depression, as it is not due to sensory units adapting to the stimulation. The decline in reflex responsiveness was also shown to disappear after a rest as short as 9s. Such recovery after a rest is one of the criteria for the identification of habituation (Thompson and Spencer, 1966) and supports the idea that the reflex decline could be termed habituation. Nevertheless, no experiments were performed to show the occurrence of dishabituation, thus it is thought to be more appropriate to refer to the observed phenomenon as reflex depression and not as habituation. The mechanism(s) underlying reflex depression in the motorneurone and interneurone response to CSD_1 stimulation is not known, but its existence suggests that CSD_1 sensory feedback is of rather less importance in the control of long lasting, tonic motor output than in the control of fast phasic activity (see Chapter 3).

2.4.6. Activation pattern of CSD_1 in normal locomotion

When trying to understand the functional role played by the cuticular

stress detectors in the control of motor output, it is useful to have some knowledge of the anatomical and physiological properties of their sensory cells. This has been covered in the first sections of the Results. However, only with the knowledge of their activation patterns during normal behaviour is it possible to link this information to any functional influence they may have.

For a long time it has been known that CSD_2 provides information about the onset of the power stroke and the load being borne by a leg (Klärner and Barnes, 1986). Recordings of CSD_1 in freely moving animals were made for the first time in this study. The results obtained suggest that CSD_1 provides slightly different information than CSD_2. Generally, CSD_1 receptor cells were active much earlier than CSD_2 units in each step cycle. Nevertheless, part of its activity in normal locomotion (the second sub-burst) occurs simultaneously with activity in CSD_2 and thus is likely to encode the same information. However, the first phase of CSD_1 activity occurs much earlier within the step cycle and must therefore encode different information. CSD_1 cells start firing before the leg touches the ground, when it is still in mid-air during the return stroke. It was not possible to link CSD_1 activity to either of the two extreme positions of the leg (AEP or PEP), or to the depressor muscle activity as has been done for CSD_2. The only correlation possible is with activity in the levator muscles. CSD_1 sensory fibres always started firing at around the time when the levator myogram recordings showed their highest firing frequencies. In crabs, it is known that contractions of the anterior levator muscle powerfully stimulate CSD_1 in restrained animals (Clarac, 1976). In crayfish, as in crabs, the soft window is extremely close to the insertion of the anterior levator apodeme on the cuticle of the basipodite. Thus, the contraction of the levator muscle fibres pulling at the apodeme will certainly put some stress on the cuticle very close to the receptor organ. This has not only been shown in experiments in crabs (see fig.6A, Clarac *et al.*, 1971), but was also investigated in the present study on crayfish (data not shown). Twice in an *in vitro* preparation, a hair was attached to the anterior levator apodeme which for that purpose had not been removed during the dissection, thus making it possible to stimulate CSD_1 sensory units by pulling on the apodeme. The possible function of these two sub-bursts of CSD_1 activity in walking will be discussed in the next chapter when the reflexes produced by the CSDs have been described.

FIGURE 2.1:

A: Posterior view of a walking leg of a crayfish.

Cuticular stress detector 2 (CSD_2) lies in full view (shown by the asterisk). Cuticular stress detector 1 (CSD_1) is positioned dorso-anteriorly in the basipodite, and is therefore not visible in this view (its position is indicated by the arrow).

Other abbreviations: basip., basipodite; carpop., carpopodite; coxop., coxopodite; ischiop., ischiopodite; merop., meropodite; prop., propodite.

B: *In vitro* preparation showing the thoracic ganglia 3, 4 and 5 and the 5th left leg.

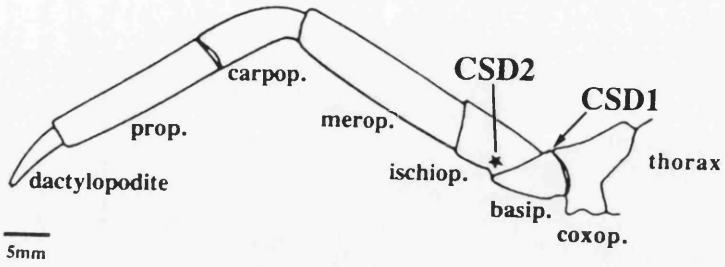
Abbreviations: adr, anterior distal root; a.lev, anterior levator motor root; dep, depressor motor root; ME, intracellular microelectrode; mech.stimulation, mechanical stimulation; pdr, posterior distal root; p.lev, posterior levator motor root; pro, promotor motor root; rem, remotor motor root.

C: Magnified drawings of the basi-ischiopodite region of the leg. The arrows indicate the location of the blunt probe on the CSD windows and direction of its movement ('on' and 'off') when stimulating the receptor organs.

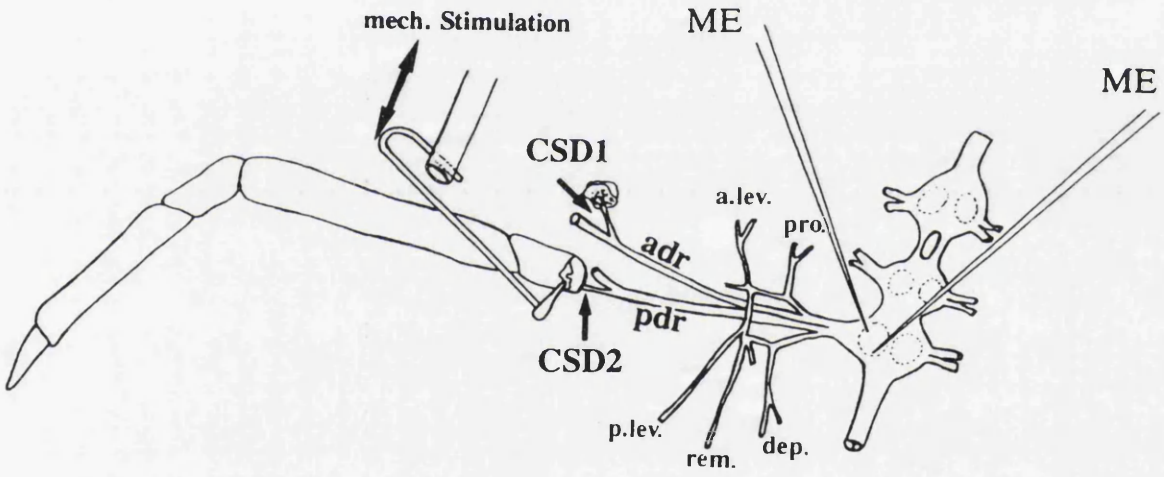
CSD_1: Dorsal-anterior view of basi- and ischiopodite showing the almost circular shape of the soft cuticle associated with the CSD_1 receptor organ.

CSD_2: Ventral-posterior view of the ischiopodite displaying the large, elongated area of soft cuticle. The arrow tip points to the 'tongue', so called because of its shape. It represents the most sensitive location for mechanical stimulation.

A


carpop.
CSD2
CSD1
prop.
merop.
thorax
ischiop.
dactylopodite
basip.
coxop.
5mm

B


mech. Stimulation
ME
ME
CSD1
a.lev.
pro.
adr
pdr
CSD2
p.lev.
rem.
dep.

C

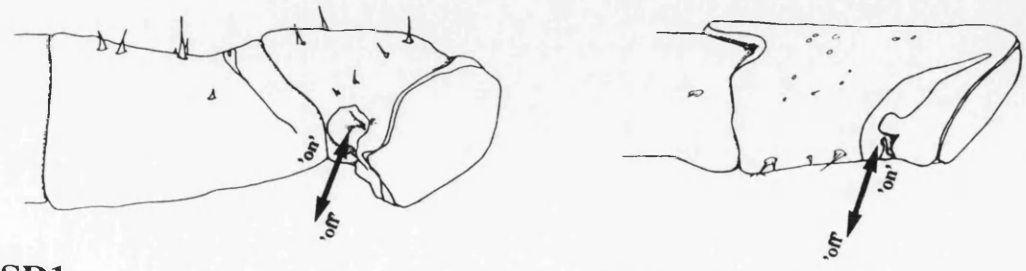

CSD1
CSD2

FIGURE 2.2:

Calibration curve of mechanical stimulator probe showing the relationship between the voltage settings of the electromechanical driver unit and the force being applied to the pliable window of the CSD receptor organ.

The voltage output on the stimulus monitor is linearly correlated to the amplitude of the forward and backward movements of the stimulating probe (not shown). By measuring the forces necessary to move the stimulating probe to different degrees, it was possible to correlate displacement of the probe with the force such a displacement would yield. Thus it is possible to correlate the amplitude of the oscillating stimulator probe with the force applied to the window and thus to the receptor strand

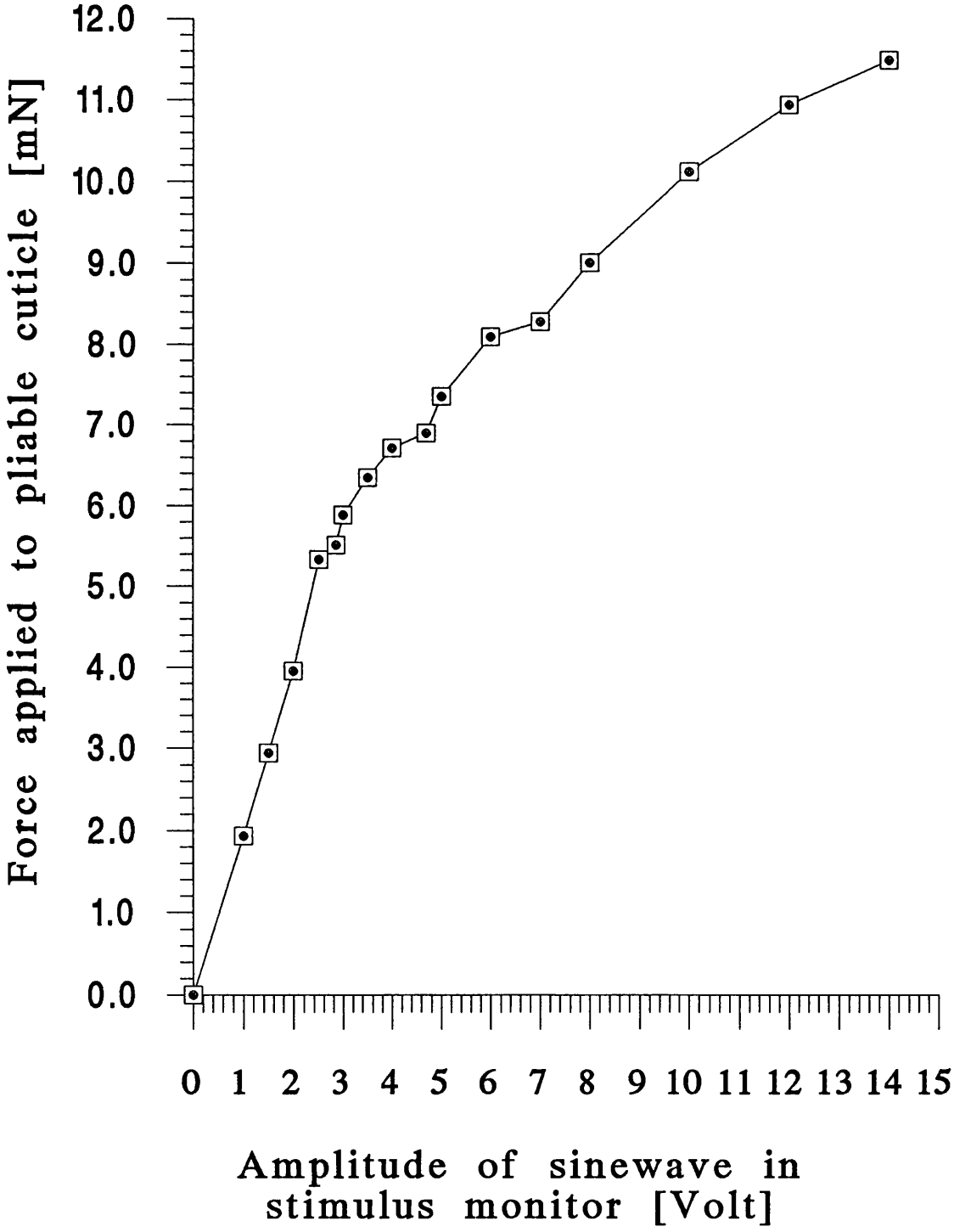

Force applied to pliable cuticle [mN]
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Amplitude of sinewave in stimulus monitor [Volt]

FIGURE 2.3:

Treadmill arrangement (from Barnes, 1977). See text for explanation.

FIGURE 2.4:

Checking the accuracy of CSD_1 *in vivo* recordings. The animal is immobilized on a wooden board out of the water.

A: Recording taken before the animal was placed on the treadmill.

● ● ●: Gentle indentation of the soft window of CSD_1.

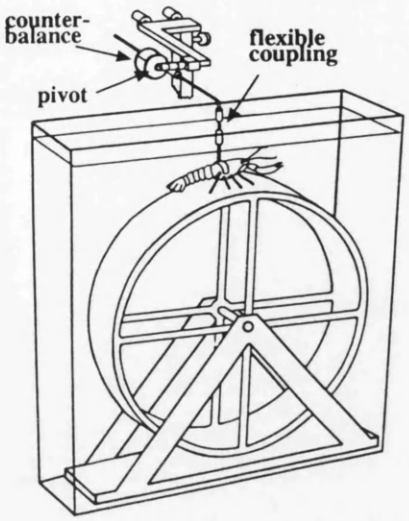
▲ ▲ ▲: Brushing along the dactyl, thus stimulating the dactyl sensory afferents (DSAs).

B: Recording after the animal had been on the treadmill for about an hour.

● ● ●: Stimulating CSD_1 soft window

◆◆◆: Pushing on the anterior levator apodeme

Notice the different reflexes in levator and depressor muscles.


counter-
balance
flexible
coupling
pivot

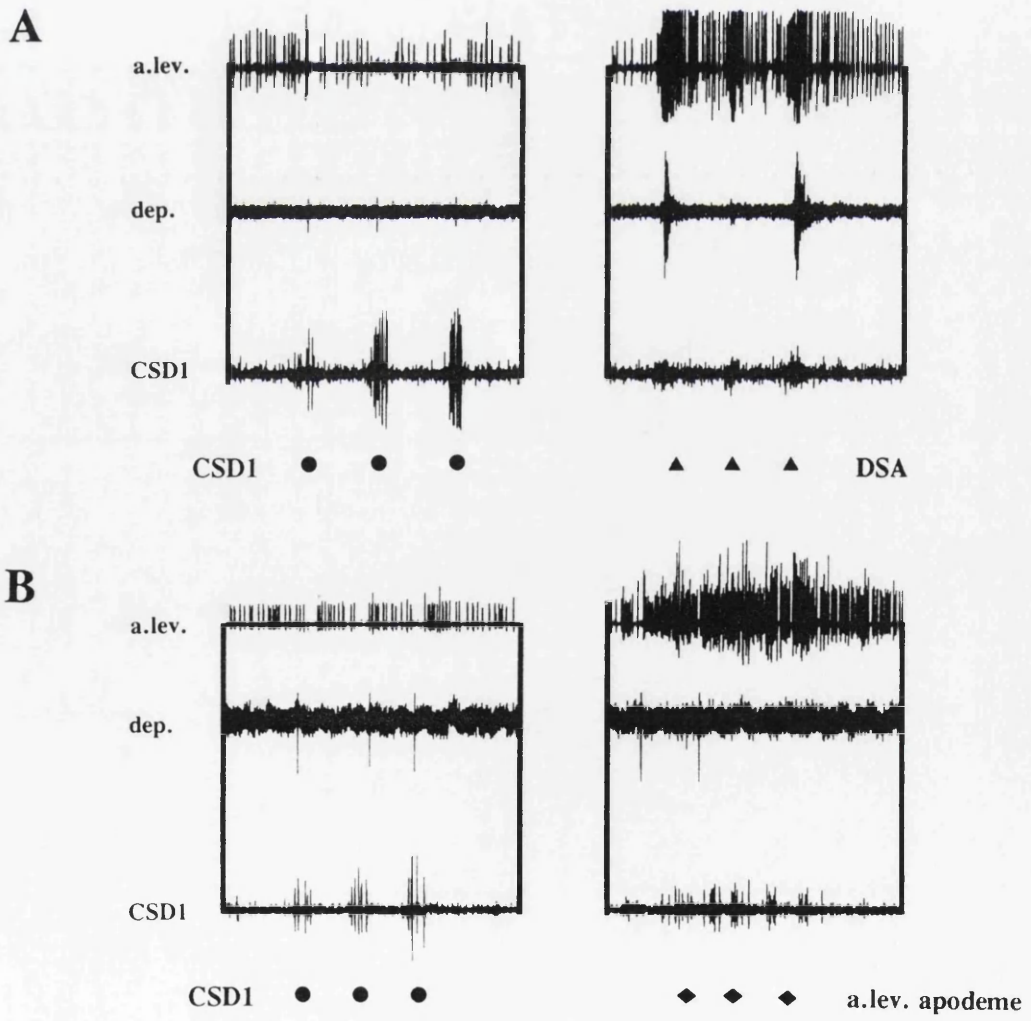

A
a.lev.
dep.
CSD1
CSD1
DSA
B
a.lev.
dep.
CSD1
CSD1
a.lev. apodeme
4s

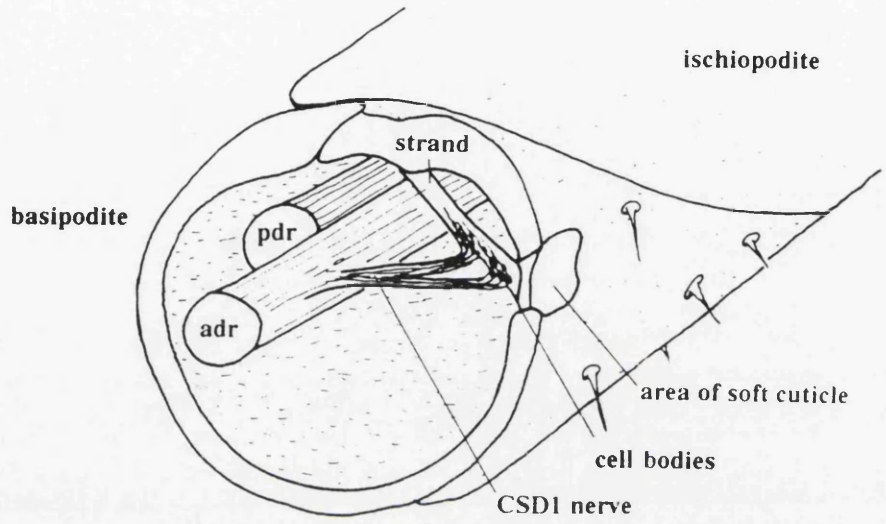
FIGURE 2.5:

Stereograms showing the relative position of the CSD receptor organ, strand, bipolar cell bodies, area of soft cuticle and nerve, within a walking leg.

A: The nerve root of CSD_1 joins the anterior distal root (adr) fairly close to the elastic strand within the basipodite. The position of the receptor organ is shown from a plane just distal to the coxo-basipodite joint.

B: The CSD_2 nerve root is much longer than the CSD_1 nerve before joining one of the two main leg nerves, the posterior distal root in case of CSD_2 . The CSD_2 accessory and main strands and the cell bodies are hidden in this view, which shows the arrangement of the nerve roots within the leg from a plane through the basi-ischiopodite joint. The strands are indicated by dashed lines, see Wales *et al.* (1971) for a view from the other side.

A


ischiopodite
strand
basipodite
pdr
adr
area of soft cuticle
cell bodies
CSD1 nerve

B

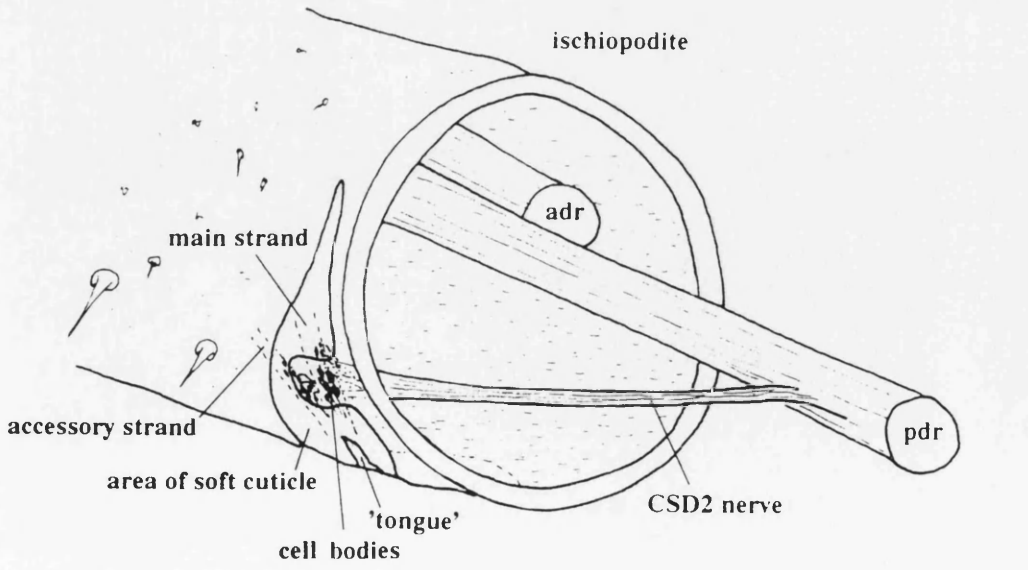
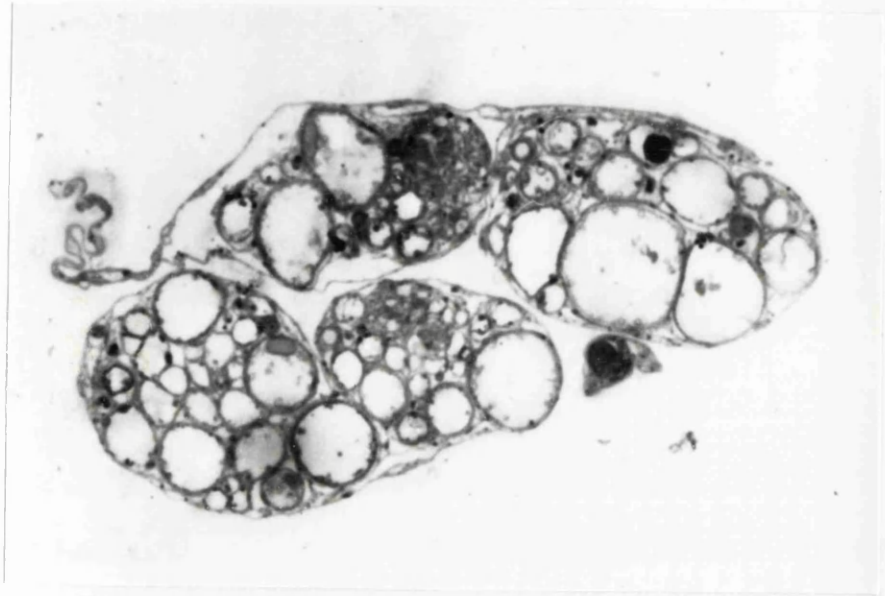

ischiopodite
adr
main strand
accessory strand
area of soft cuticle
'tongue'
cell bodies
CSD2 nerve
pdr
1mm

FIGURE 2.6:

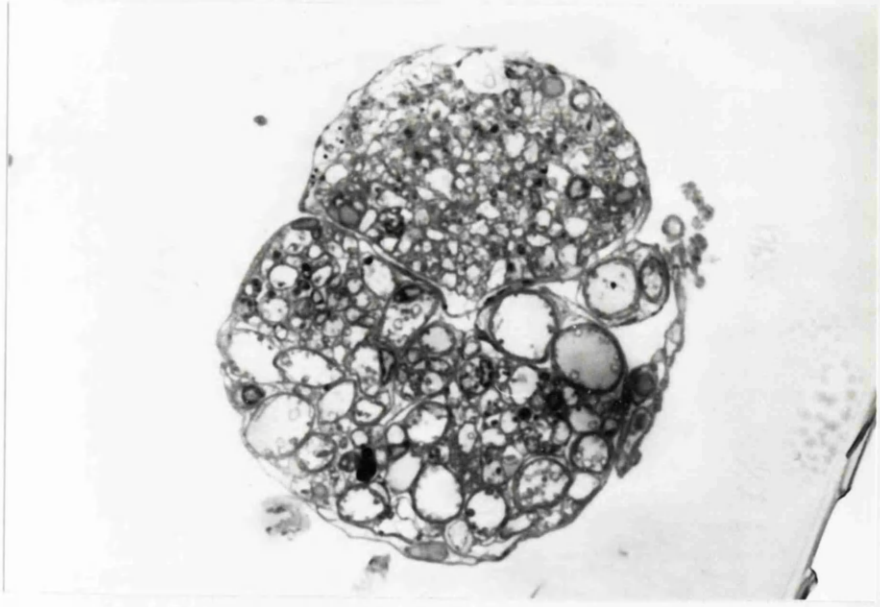
Cross sections through the nerve roots of CSD_1 (A) and CSD_2 (B). Notice the large number of small cells in the nerve root of CSD_2.

CSD1



25μm

CSD2



25μm

FIGURE 2.7:

Histograms showing the size distribution of axons in cross sections of CSD_1 and CSD_2 nerve roots.

A,B: CSD_1
C,D: CSD_2
Abbreviation: n, number of axons.

In the cross sections shown in Fig.2.6, axons are not perfectly circular in shape, probably due to fixation and cutting procedures. Therefore, the axon cross sectional area is the most accurate measurement of axon size, and was used to draw the histograms. Nevertheless, most authors talk about axon diameters. Therefore, the values for axon diameters (shown in the upper x-axes) were calculated from the cross sectional area measurements (shown in the lower x-axes). The calculation assumes axons were circular in cross section, which as discussed above was by no means always the case.

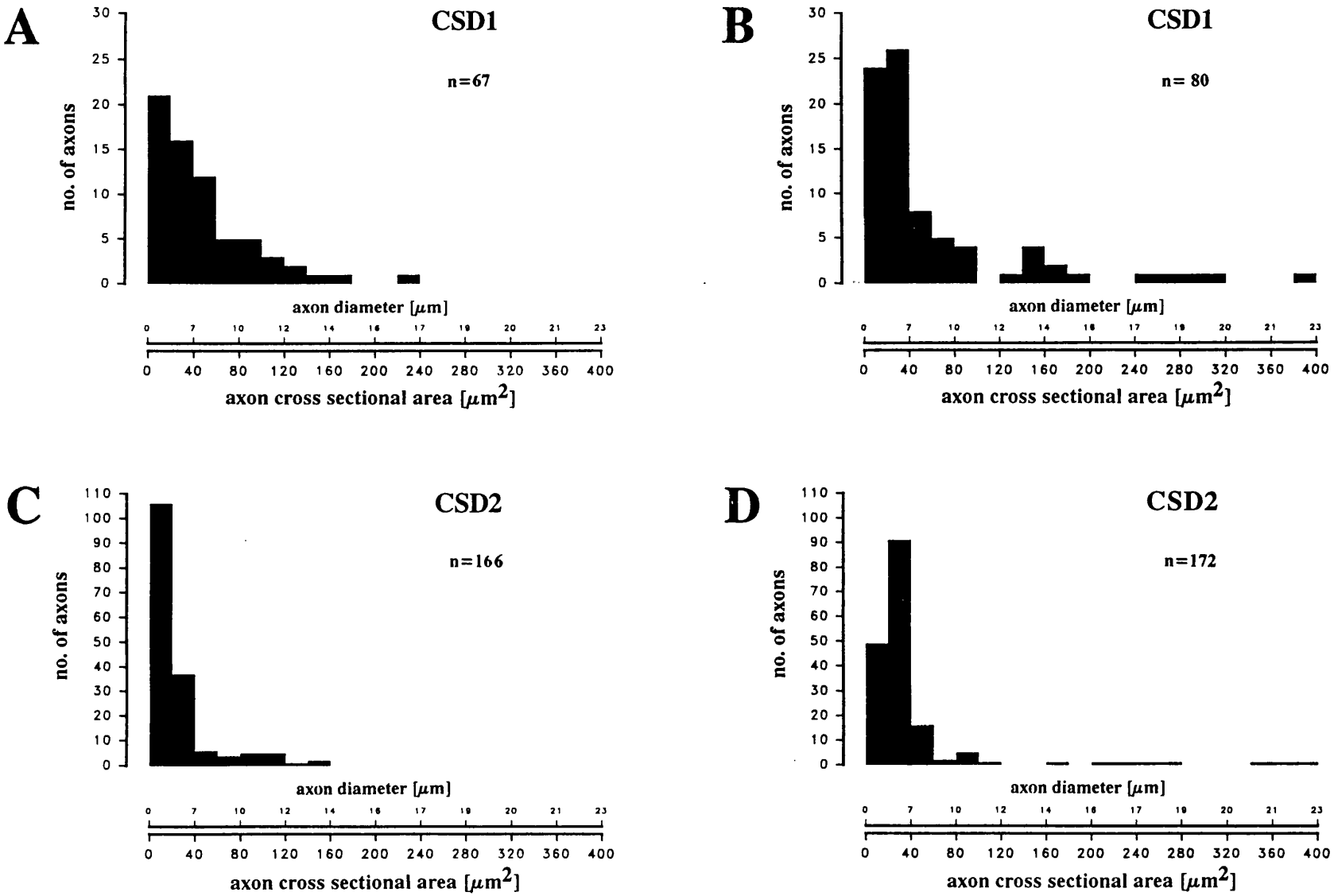

A
CSD1
n=67
no. of axons
axon diameter [μm]
axon cross sectional area [μm²]
B
CSD1
n= 80
no. of axons
axon diameter [μm]
axon cross sectional area [μm²]
C
CSD2
n=166
no. of axons
axon diameter [μm]
axon cross sectional area [μm²]
D
CSD2
n=172
no. of axons
axon diameter [μm]
axon cross sectional area [μm²]

FIGURE 2.8:

A and D:

Biocytin anterograde fills of the CSD terminals. The dye was applied distally close to the receptor organs and allowed to travel towards the ganglion into the afferent terminals. The axons branch in a similar manner to the dendritic trees of motorneurones or interneurones. Thus the afferents are able to make multiple synapses with postsynaptic cells. Both drawings are from fills of CSD afferent terminals in the 5th left hemiganglion.

B and C:

Two Lucifer Yellow fills of single CSD_1 fibres.

Only the 5th left hemiganglion is shown. Drawings were made from a number of photographs, all focusing at different planes.

The dashed line in all drawings indicates the extent of the neuropile. The straight dashed line in A and D is drawn to indicate the location of the midline between the left and right halves of the 5th thoracic ganglion.

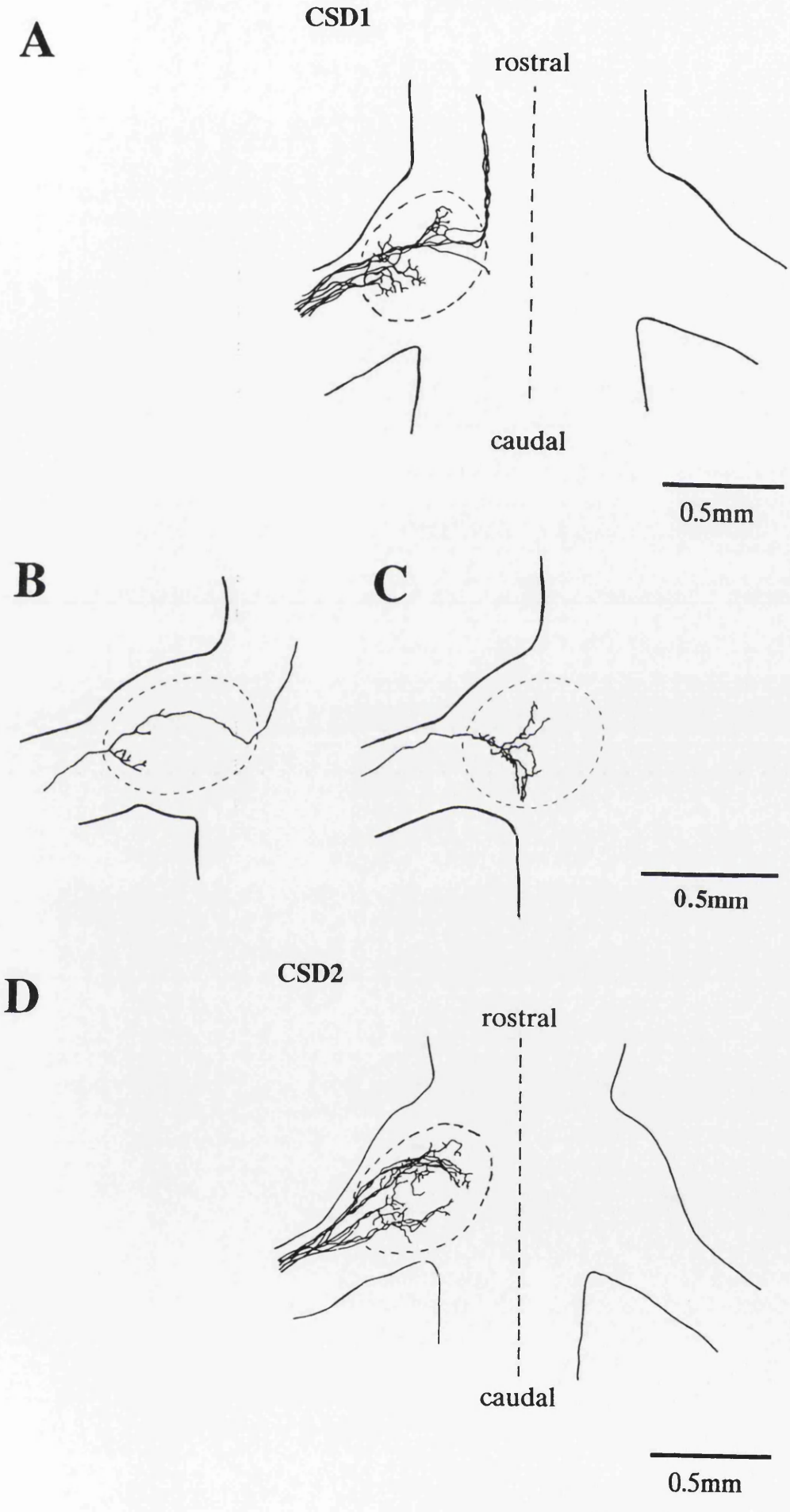

A
CSD1
rostral
caudal
0.5mm
B
C
0.5mm
D
CSD2
rostral
caudal
0.5mm

FIGURE 2.9:

Electrophysiological recordings of one CSD_1 fibre whose intracellular spike was identified in the extracellular recording of the CSD_1 receptor nerve and in the extracellular recording of the ventral nerve cord in front of the 4th thoracic ganglion (for questions on the appropriateness of identification see Discussion, part 2.4.4.).

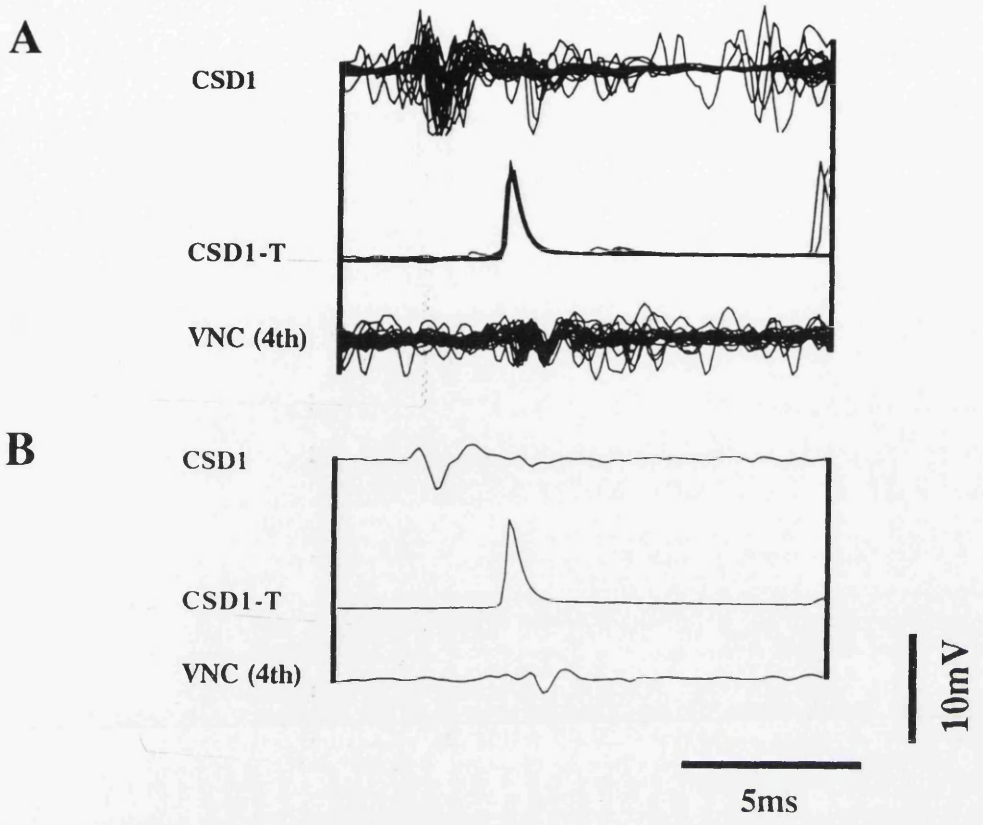
A: 25 sweeps, all triggered on the intracellular spike, are superimposed in one drawing. The recording was made during mechanical stimulation of the CSD_1 receptor organ.

B: Average of all 25 sweeps in A.

C: Electrical stimulation of, from top to bottom, the CSD_1 nerve root, the ventral nerve cord rostrally to the 4th thoracic ganglion, and the ventral nerve cord (VNC) in front of the 1st thoracic ganglion. The latencies reflect the distances between the recording and stimulating electrodes. Hence, the latency is longest when electrically stimulating the VNC in front of the 1st thoracic ganglion, and shortest when stimulating the VNC in front of the 4th thoracic ganglion.

Each of the three drawings shows three superimposed sweeps.

Abbreviations: CSD1-T, CSD_1 afferent terminal; el.st., electrical stimulation; VNC(1st), ventral nerve cord rostrally to the 1st ganglion; VNC(4th), ventral nerve cord rostrally to the 4th ganglion.


A
CSD1
CSD1-T
VNC (4th)
B
CSD1
CSD1-T
VNC (4th)
10mV
5ms

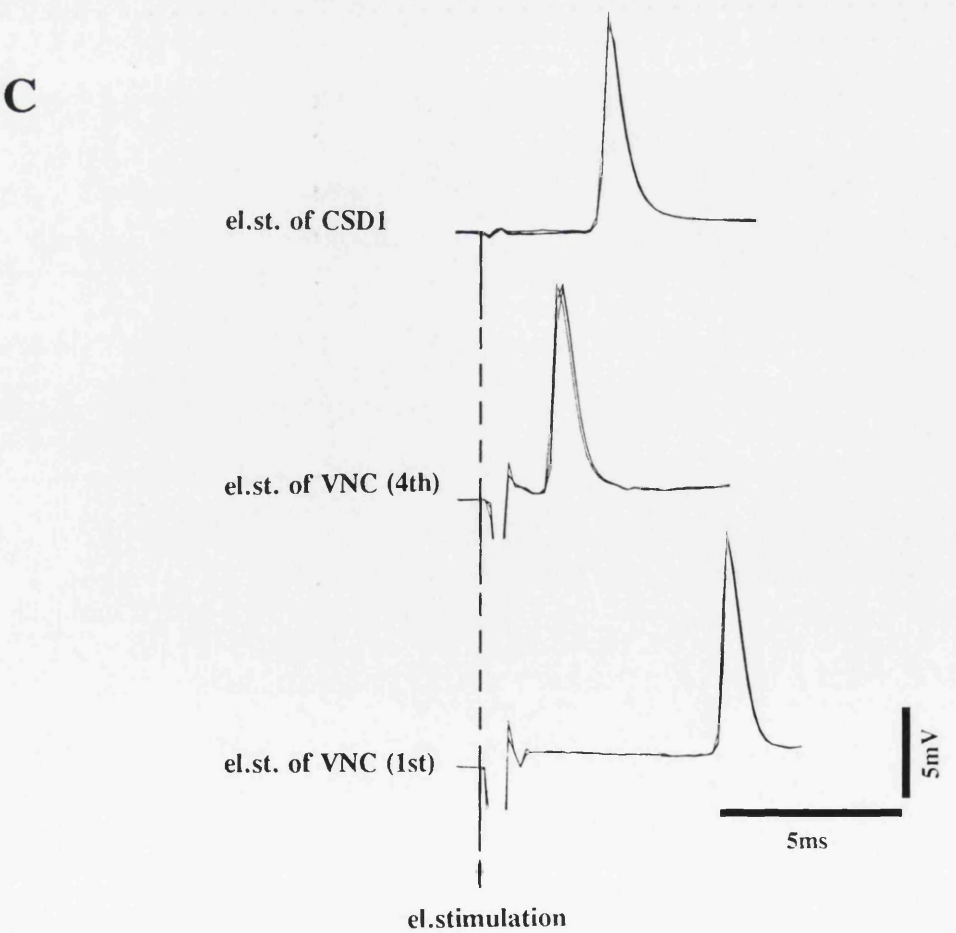

C
el.st. of CSD1
el.st. of VNC (4th)
el.st. of VNC (1st)
5mV
5ms
el.stimulation

FIGURE 2.10:

Intracellular recording from two CSD_2 afferent terminals simultaneously with the entire activity in the CSD_2 nerve. The use of ramp rather than sinusoidal mechanical stimulation of the CSD_2 receptor organ allowed tonic and phasic units to be distinguished from each other. CSD_2 unit (a) is a phasic 'off' unit, while CSD_2 unit (b) is a phasotonic 'on' unit.

Sensory afferent spikes are propagated by electrotonic spread within the ganglion (Cattaert *et al.*, 1992b; Chapter 5). The spike size, therefore, is dependent on the location of the recording electrode, i.e. the further into the ganglion the intracellular recording is made, the smaller the spike is.

Abbreviation: mech.st.monitor, mechanical stimulus monitor.

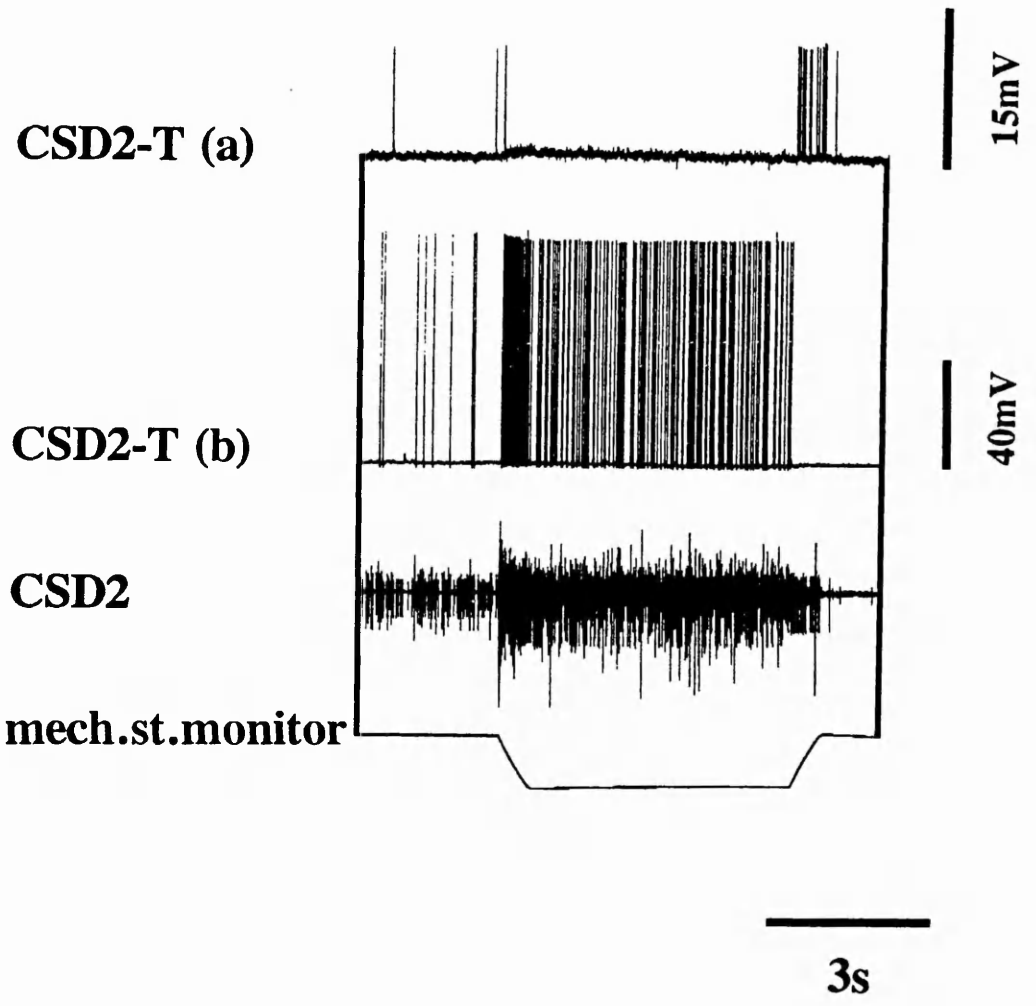

CSD2-T (a)
CSD2-T (b)
CSD2
mech.st.monitor
15mV
40mV
3s

FIGURE 2.11:

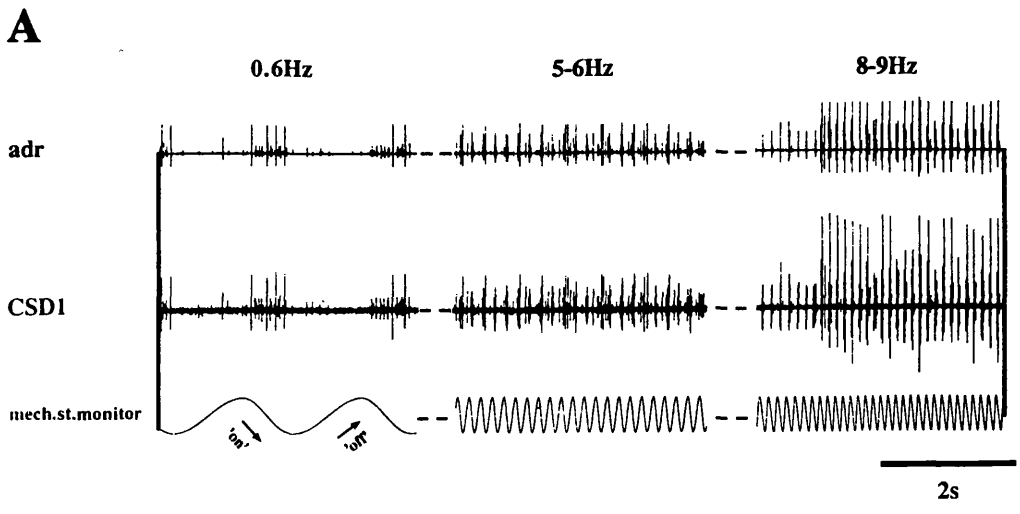
Effect of frequency and amplitude changes on the responses of high threshold CSD_1 fibres.

A: Effect of frequency

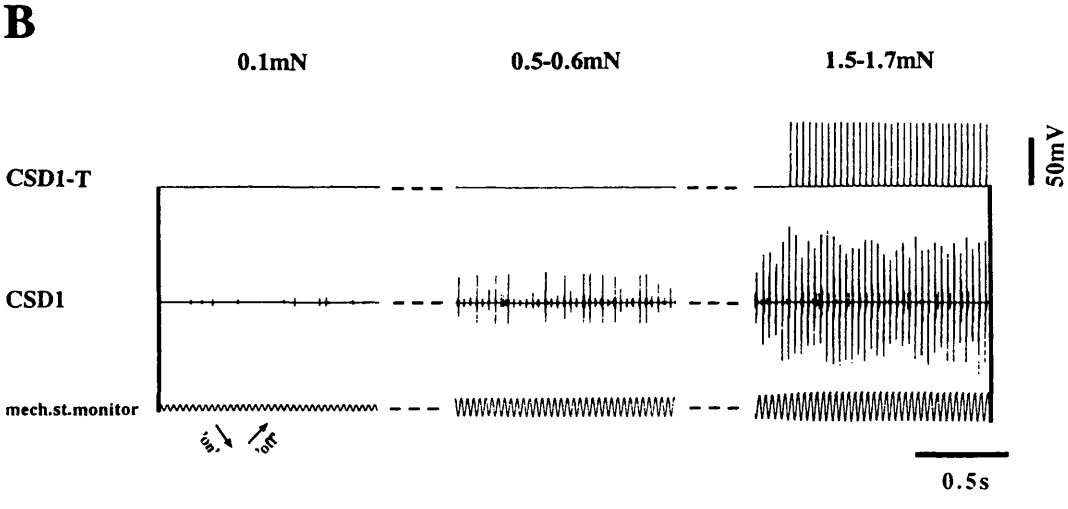
Three "snapshots" from a continuous record. The frequency of the sinusoidal signal which was used to drive the mechanical probe was steadily increased, while the amplitude was kept constant. At around 8Hz to 9Hz, very large units in the extracellular CSD_1 trace occurred in addition to the smaller units which had already been active at lower frequencies (0.6Hz and 4-6Hz). NB: 8-9Hz was below the intrinsic oscillation frequency of the stimulating probe, which in this case was around 20Hz.

B: Effect of amplitude

Three "snapshots" from a continuous record of extra- and intracellular CSD_1 activity. The frequency of the mechanical stimulation was constant at 30Hz, while the amplitude was continuously increased. The CSD_1 terminal only responded at large amplitudes, when large amplitude extracellular spikes were visible in the extracellular trace. Smaller CSD_1 units, identified by their smaller spikes in the CSD_1 extracellular trace, were active at smaller stimulus amplitudes.



amplitude constant: 1.8mN



frequency constant: 30Hz

FIGURE 2.12:

Parts of a continuous record showing the reflex responses of an interneurone, recorded intracellularly in the 5th thoracic ganglion, and anterior levator, posterior levator and depressor motorneurones, recorded extracellularly in their respective motor roots, to mechanical stimulation of CSD_2. The record starts immediately after stimulation was begun (to the left of the dashed lines). 30s later (to the right of the dashed lines), the response intensity seems to have decreased marginally (see also next figure).

Abbreviation: IN., interneurone.

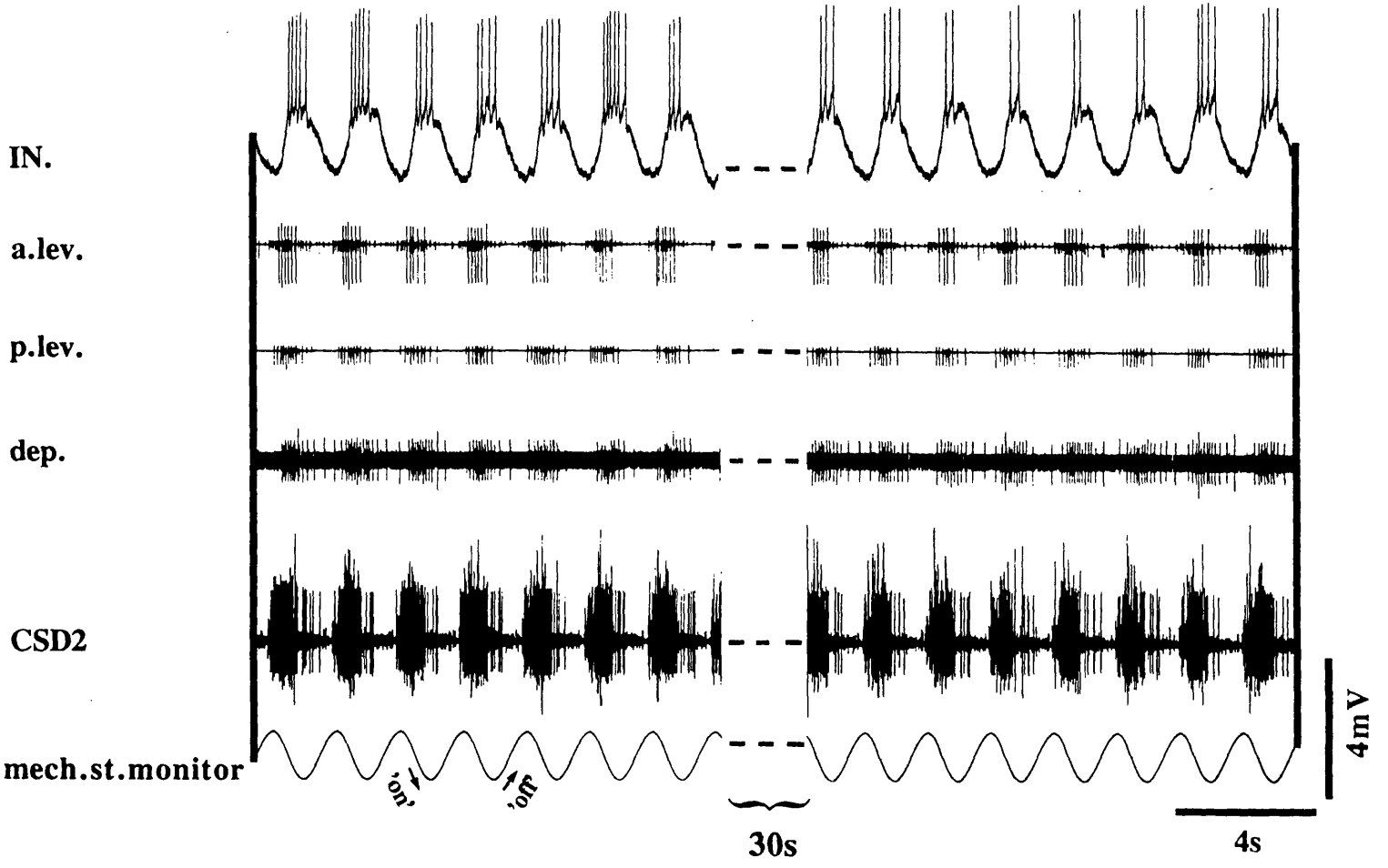

IN.
a.lev.
p.lev.
dep.
CSD2
mech.st.monitor
'on'
'off'
30s
4s
4mV

FIGURE 2.13:

Histograms showing the pooled activity of units in CSD_2, anterior levator and depressor nerve roots at two different points in the recording illustrated in Fig.2.12. Each histogram represents the average number of spikes occurring per stimulus cycle. These were calculated from the pooled activity in 10 consecutive stimulation cycles. The left hand histograms were compiled from the first 10 cycles of mechanical stimulation of the CSD_2 receptor organ, the right hand histograms from 10 cycles of activity 34s later. Both anterior levator and depressor motorneurones are excited by CSD_2 'on' fibres. No adaptation occurs in the CSD afferent fibres, and only a small decline can be seen in the number of spikes in the motor roots.

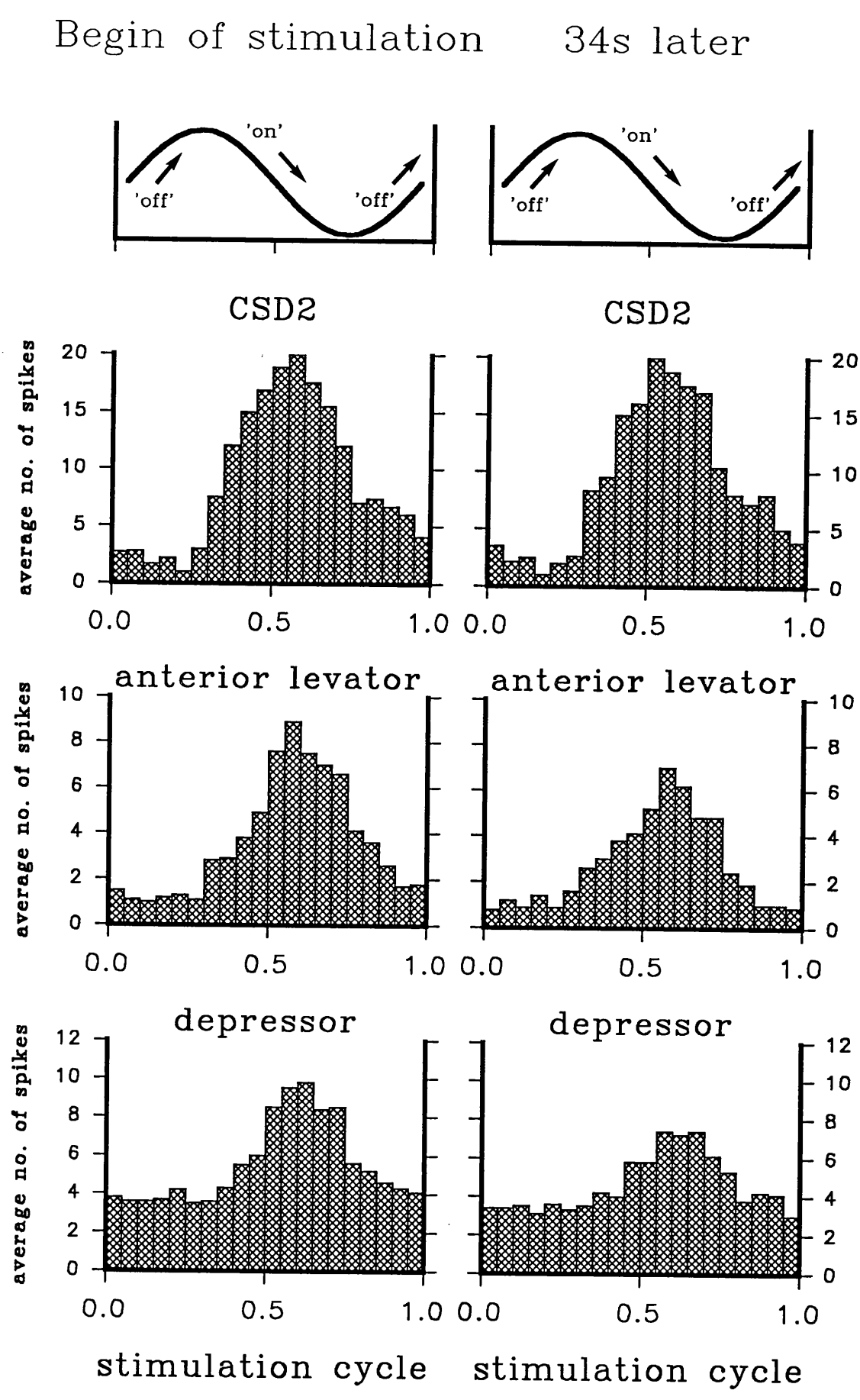

Begin of stimulation
34s later
'on'
'off'
'off'
'on'
'off'
'off'
CSD2
CSD2
average no. of spikes
20
15
10
5
0
0.0
0.5
1.0
anterior levator
anterior levator
10
8
6
4
2
depressor
depressor
12
stimulation cycle
stimulation cycle

FIGURE 2.14:

Parts of a continuous record showing the reflex responses occurring in four proximal nerve roots to mechanical stimulation of CSD_1. A significant decrement in reflex response is noticeable when comparing the record at the beginning of the stimulation (to the left of the dashed horizontal lines) to a later stage (to the right of the dashed lines). At the start of stimulation, anterior and posterior levators are completely inhibited (with the exception of one large unit in the anterior levator), while the depressor shows strong excitation in several units. Later, the activity in anterior and posterior levator units is only intermittently inhibited and the depressor units are only weakly excited. There is no obvious adaptation in CSD_1 activity.

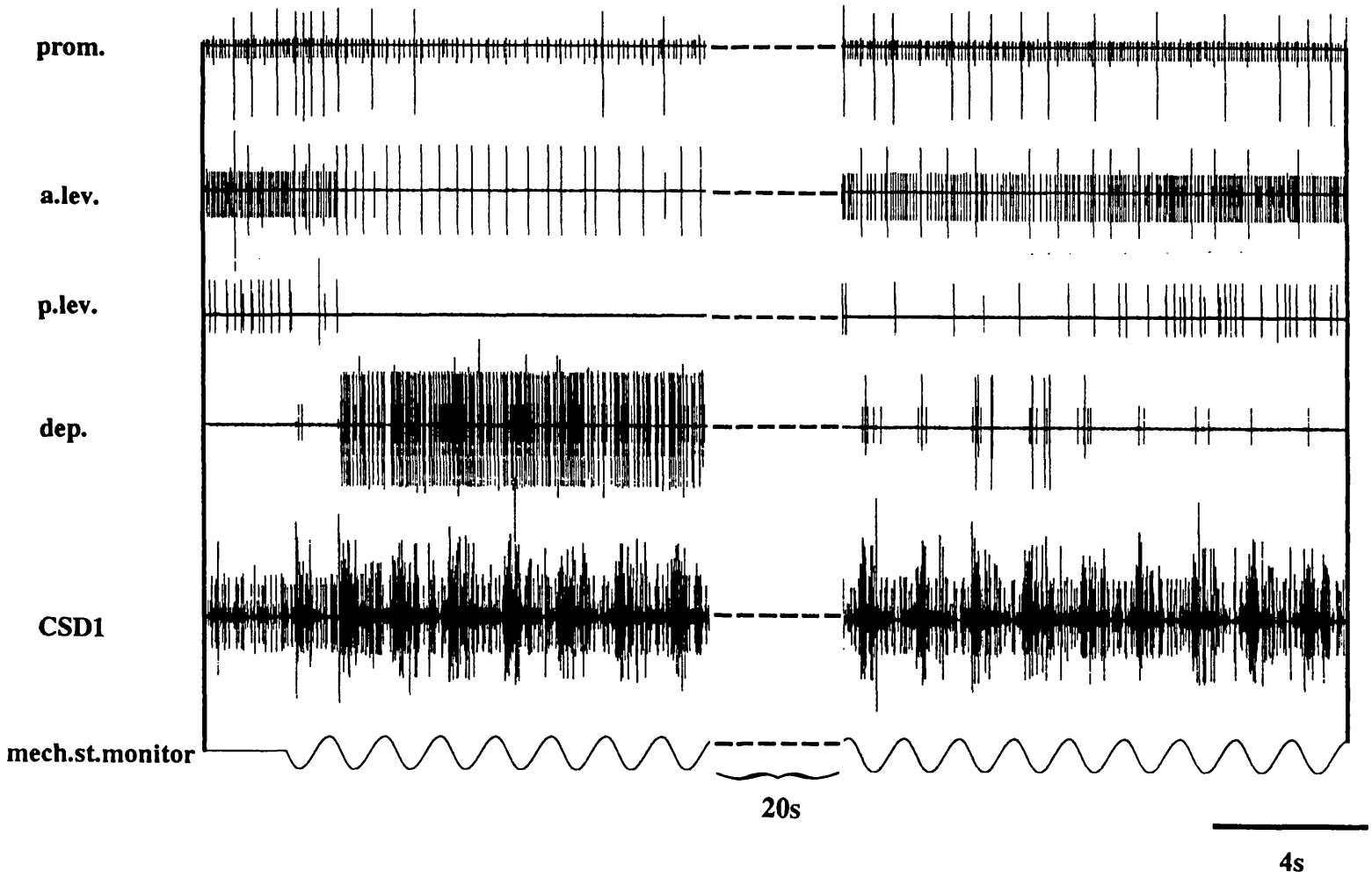

prom.
a.lev.
p.lev.
dep.
CSD1
mech.st.monitor
20s
4s

FIGURE 2.15:

Histograms showing the pooled activity of units in CSD_1, anterior levator and depressor nerve roots at two different points in the recording illustrated in Fig.2.14. Each histogram represents the average number of spikes occurring per stimulus cycle. These were calculated from the pooled activity in consecutive stimulation cycles. The left hand histograms were compiled from the first 7 to 10 cycles of mechanical stimulation of the CSD_1 receptor organ, the right hand histograms from 8-10 cycles of activity 20s later. At the beginning of CSD_1 stimulation the spontaneous firing in anterior levator motorneurones is almost completely abolished, whereas the depressor shows excitation with a peak of activity during the 'on' phase of the stimulation. 20s later (right hand diagrams) the activity level in the anterior levator has increased to an extent that hardly any inhibition in anterior levator motorneurones is noticeable, whereas most of the activity in the depressor units has died down to a few spikes occurring during the 'on' phase of the stimulation. There is a slight decrease in CSD_1 activity.

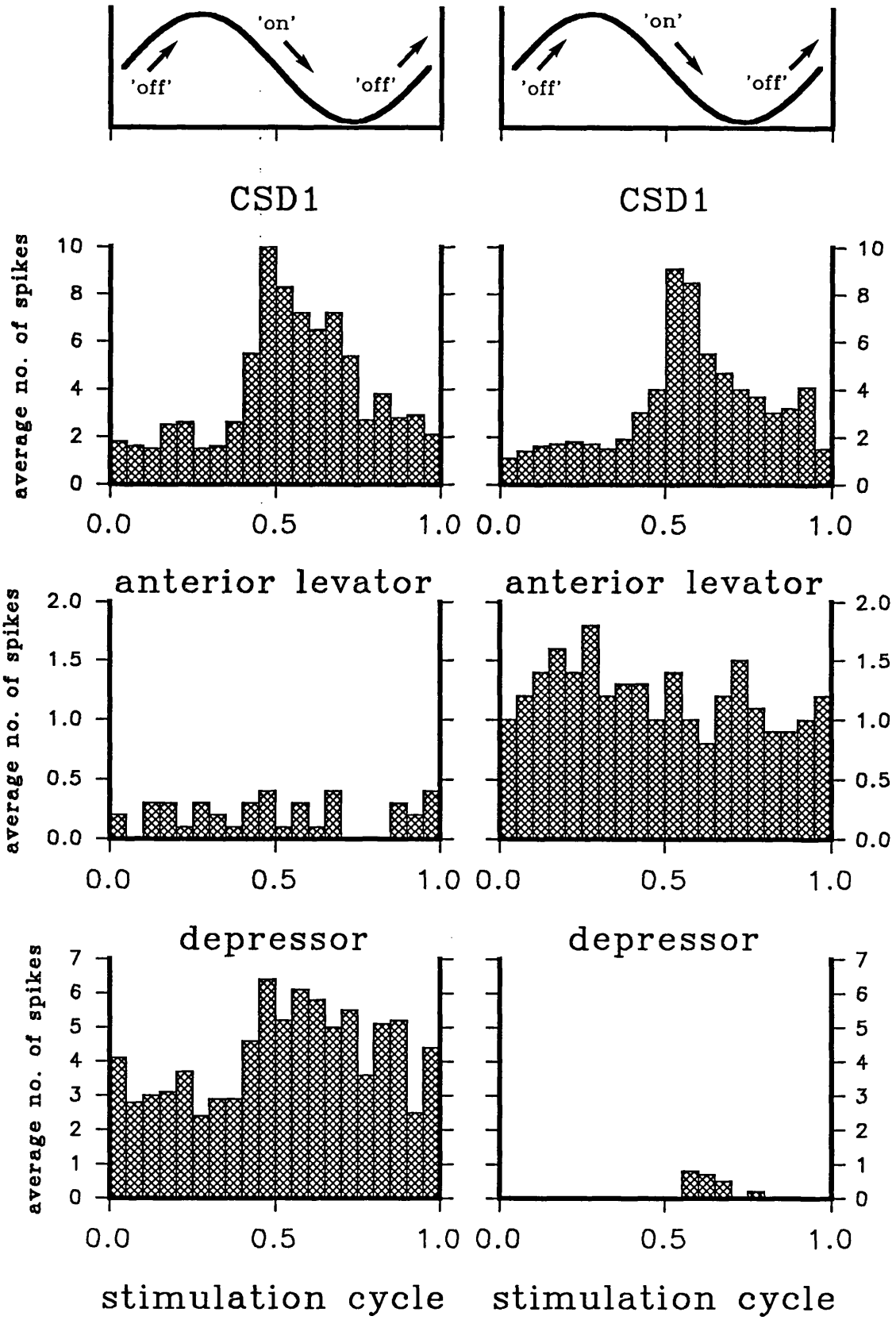

Begin of stimulation
20s later
'on'
'off'
'off'
'on'
'off'
'off'
CSD1
CSD1
average no. of spikes
10
8
6
4
2
0
0.0
0.5
1.0
anterior levator
anterior levator
2.0
1.5
1.0
0.5
0.0
depressor
depressor
7
6
5
4
3
2
1
0
stimulation cycle
stimulation cycle

FIGURE 2.16:

Continuous record of five motor roots when stimulating CSD_1 intermittently. When a reflex decrement was obvious, i.e. when anterior levator spikes were again visible and the firing frequency of the depressor units had diminished (to the left of all lines of larger dots), the stimulation was stopped. When stimulation was resumed after a short rest (indicated by the lines of smaller dots), the reflex response had recovered its original strength, thus demonstrating spontaneous recovery after a rest following reflex depression.

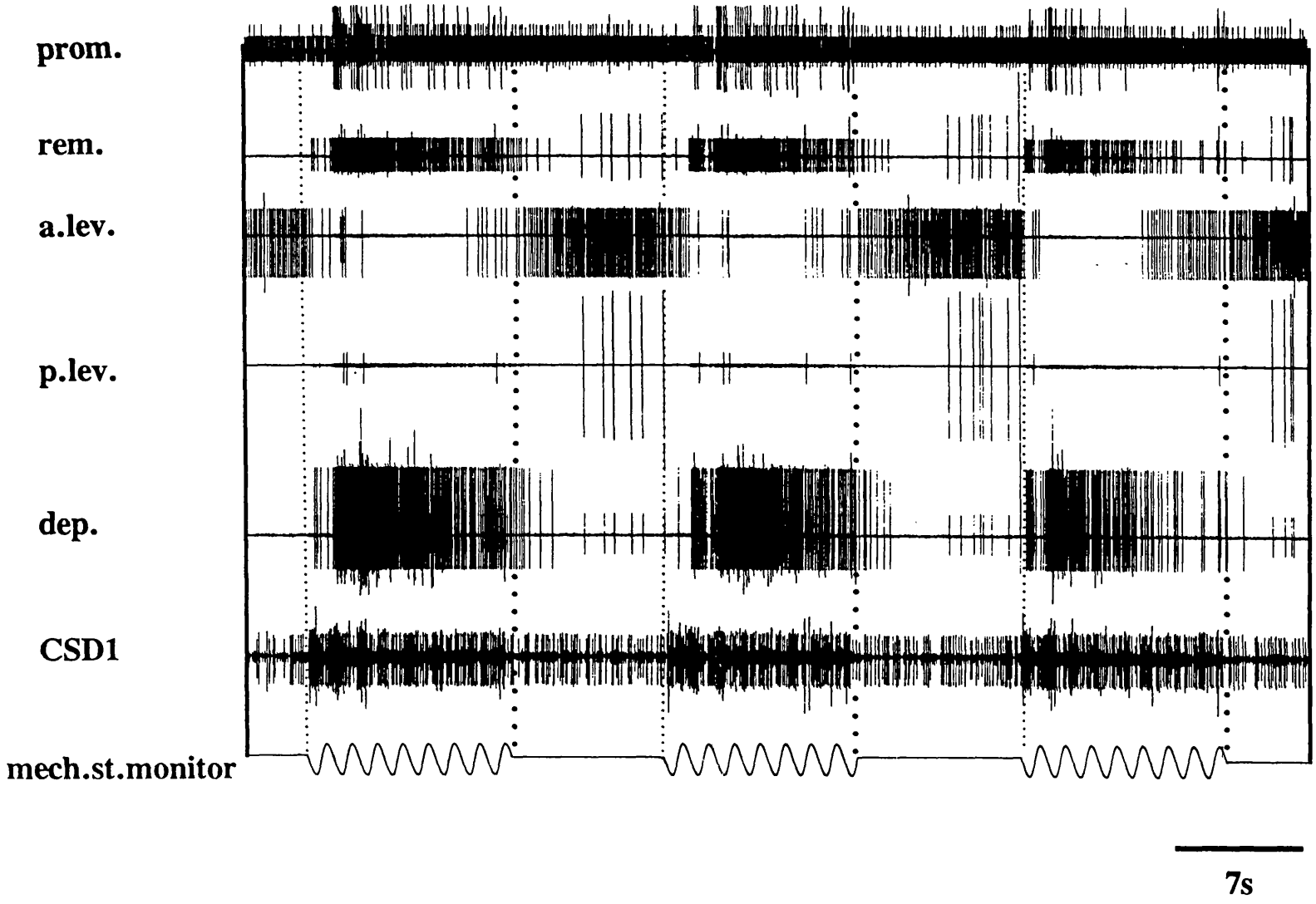

prom.
rem.
a.lev.
p.lev.
dep.
CSD1
mech.st.monitor
7s

FIGURE 2.17:

Reflexes resulting from CSD_1 mechanical stimulation in promotor, anterior levator and depressor motor roots, and in one interneurone are shown. The initial strong reflex response declines rapidly after the first 12s of stimulation. As can be seen from the intracellular recording, the reflex response does not disappear altogether, but becomes subthreshold.

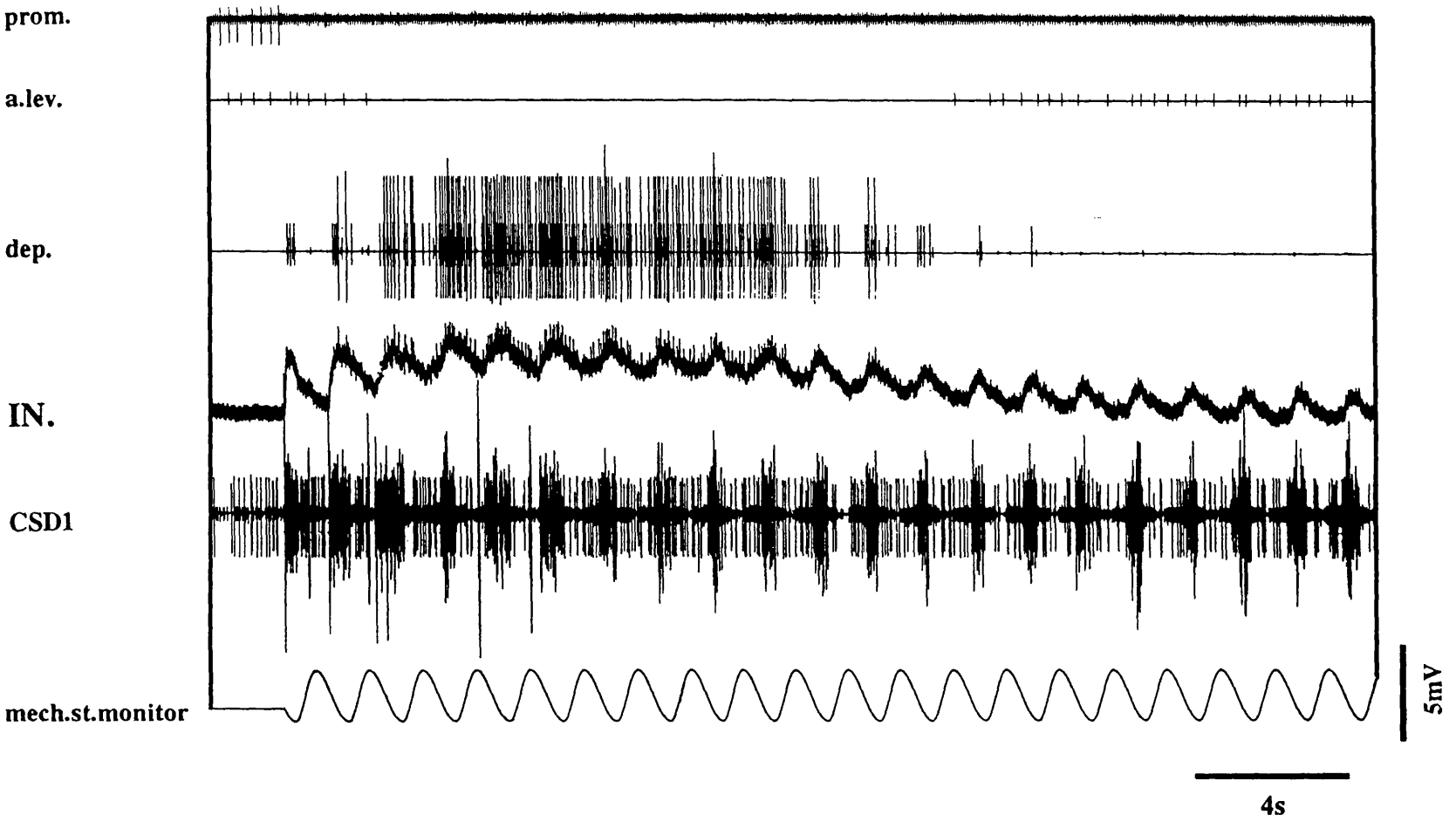

prom.
a.lev.
dep.
IN.
CSD1
mech.st.monitor
5mV
4s

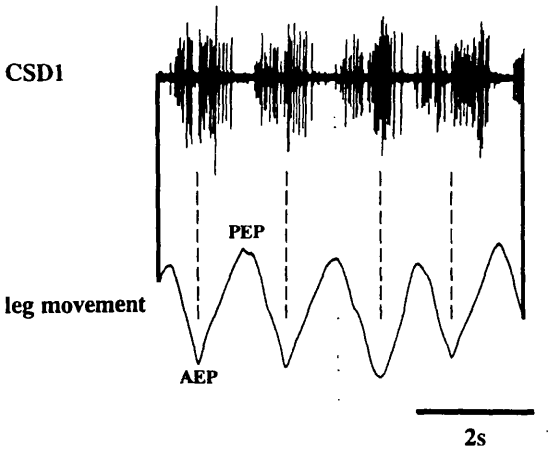
FIGURE 2.18:

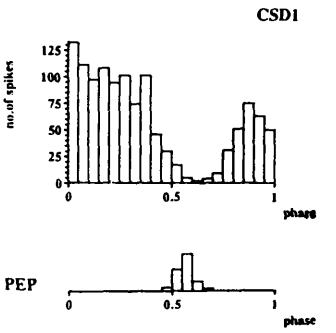
Sensory activity in CSD_1 recorded extracellularly in the 4th walking leg of a crayfish walking on a treadmill, along with the forwards-backwards components of the movement of the tip of the leg. The histogram represents the relative occurrence of the sensory activity during 62 steps. The lower histogram shows the relative times of occurrence of the posterior extreme position (PEP) during the same 62 steps. Phases of 0 and 1 represent the anterior extreme position (AEP); i.e the beginning of the power stroke. The PEP represents the beginning of the return stroke.

FIGURE 2.19:

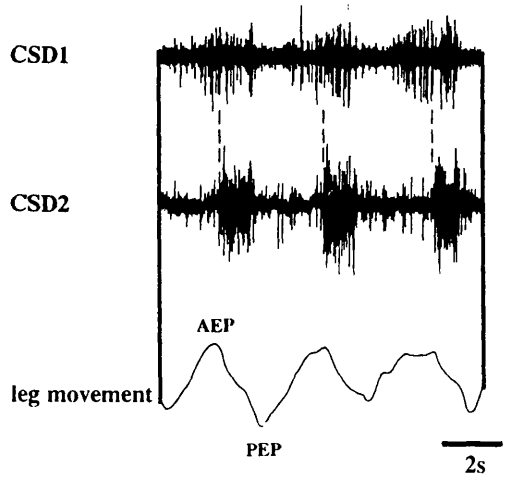
Sensory activity in CSD_1 and CSD_2 recorded simultaneously in the 4th walking leg of a crayfish walking on a treadmill, along with the forwards-backwards components of the movement of the tip of the leg. The histograms represent the relative occurrence of the sensory activity during 53 steps. The bottom histogram shows the relative times of occurrence of the posterior extreme position (PEP) during the same 53 steps. Phase of 0 and 1 represent the anterior extreme position (AEP).

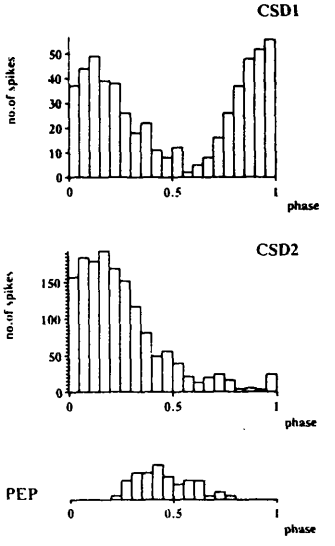
CSD_1 activity starts before the sensory cells in CSD_2 are stimulated (see dashed lines). Notice the fairly slow walking speed (about 0.4Hz), the irregularities in the movement trace and the fairly large variations of power and return stroke durations (PEP histogram), all indicating that the walking pattern was rather slow and hesitant.


CSD1
PEP
leg movement
AEP
2s


CSD1
no.of spikes
125
100
75
50
25
0
0
0.5
1
phase
PEP
0
0.5
1
phase

n=62


CSD1
CSD2
AEP
leg movement
PEP
2s


CSD1
no.of spikes
50
40
30
20
10
0
0
0.5
1
phase
CSD2
no.of spikes
150
100
50
0
0
0.5
1
phase
PEP
0
0.5
1
phase

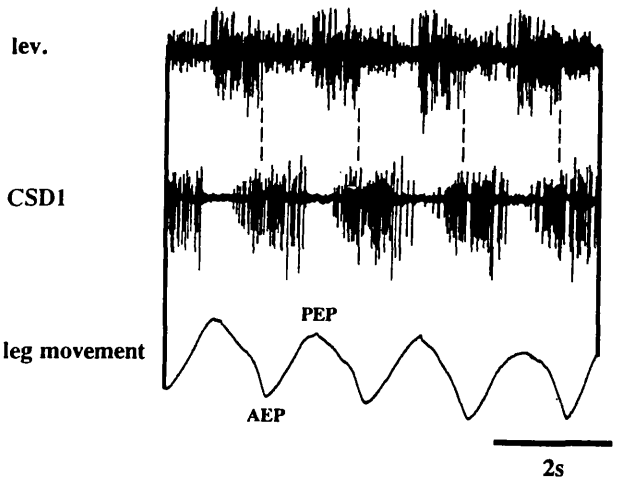
n=53

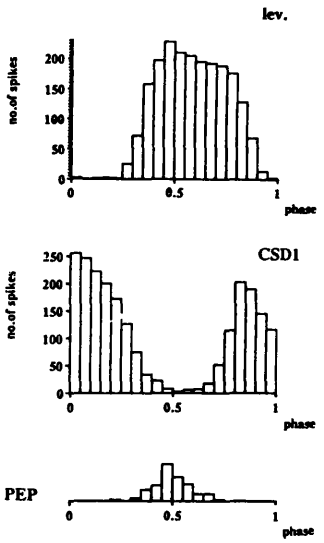
FIGURE 2.20:

Muscle activity (myogram) recorded from levators (A) and depressors (B) simultaneously with the CSD_1 neurogram and the forwards-backwards components of the movement of the tip of the leg of a crayfish during walking on a treadmill. The phase histograms (see Fig.2.18 for details) were compiled from 88 steps (A) and 42 steps (B).

Dashed lines in A indicate the end of the levator bursts, while dashed lines in B represent the beginning of the depressor bursts.

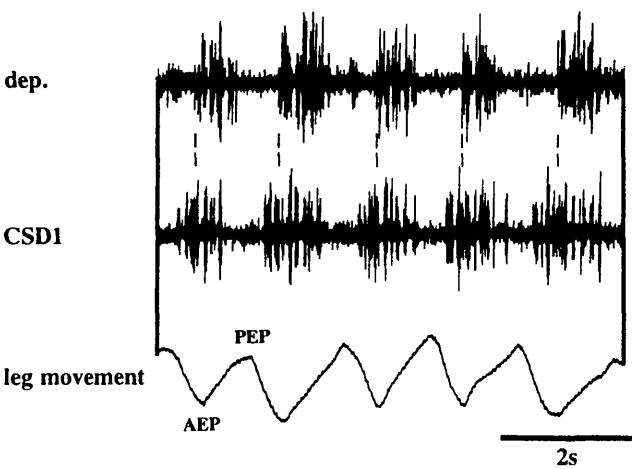
A

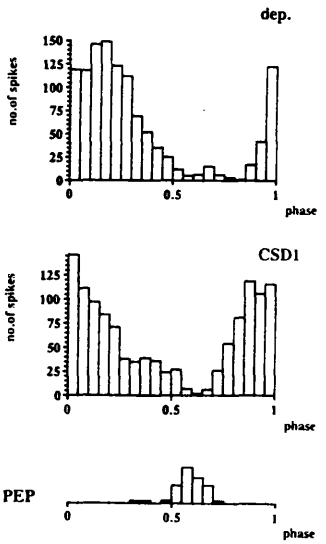

lev.
CSD1
leg movement
PEP
AEP
2s


lev.
no.of spikes
phase
CSD1
PEP

n=88

B

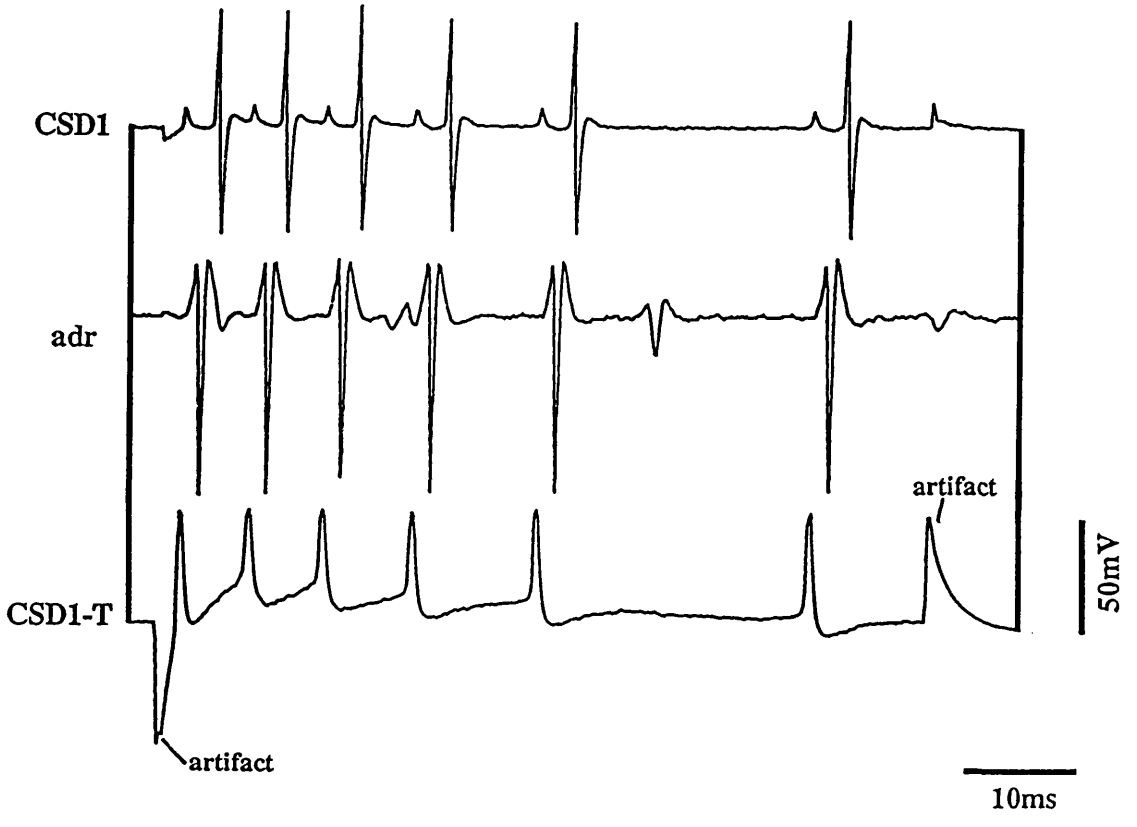

dep.
CSD1
leg movement
PEP
AEP
2s


dep.
no.of spikes
phase
CSD1
PEP

n=42

FIGURE 2.21:

Injecting intracellularly positive current pulses (+16nA) results in spike generation in the sensory terminal. These impulses travel antidromically within the axon to the sensory organ, thus allowing identification of the extracellular spike in the adr and CSD_1 nerve root recording.


CSD1
adr
CSD1-T
artifact
artifact
50mV
10ms

Chapter 3

SYNAPTIC CONNECTIONS OF THE CUTICULAR STRESS DETECTORS IN CRAYFISH: MONO- AND POLYSYNAPTIC REFLEXES AND THE ENTRAINMENT OF FICTIVE LOCOMOTION IN AN *IN VITRO* PREPARATION.

3.1. INTRODUCTION

The co-ordination of muscles, joints and limbs necessary to produce an effective motor act is achieved by the interaction of centrally located pattern generators and integration of sensory feedback from the periphery (Bush and Clarac, 1985). In Crustacea, sensory information from the legs stems from a multitude of different sense organs. An important role in the control of movement is played by proprioceptors, such as the chordotonal joint receptors or the muscle receptor organs, and by exteroceptors such as the dactyl sensory afferents (Barnes, 1977; Bush, 1977; Clarac, 1977; Clarac, 1990; Clarac and Barnes, 1985; Libersat *et al.*, 1987b).

The cuticular stress detectors are also thought to play a role in postural adjustment and locomotion (Clarac, 1976). They are mechanoreceptors situated in the proximal part of the leg. The physiological properties of these receptors have been studied in detail (for review see previous chapter) and their afferent responses during natural stimulation have been investigated in whole animal preparations (Klärner and Barnes, 1986; previous chapter). Insight into the behavioural function of CSD activity has been gained by studying the reflexes elicited in leg muscles by CSD stimulation. Clarac and Wales (1970) showed that CSD_1 stimulation elicits a clear response in the posterior levator and depressor muscles and also seems to influence the activity of the anterior levator. These reflex responses are easily obtained with *Carcinus maenas, Palinurus vulgaris* and *Homarus gammarus*, but are much more labile in *Astacus leptodactylus* (Clarac *et al.*, 1971). Moffett (1975) demonstrated excitatory reflex connections to both anterior and posterior levator muscles in the land crab, *Cardisoma guanhumi*. In contrast, Findlay (1978) found that the anterior levator is inhibited by CSD_1 stimulation in *Carcinus maenas*. Vedel and Clarac (1979) showed that the stimulation of CSD_1 receptors in the rock lobsters, *Panilurus vulgaris* and *Jasus lalandii*, increases activity in all the mero-carpopodite and coxo-basipodite muscles, although to different degrees.

Electrophysiological studies of CSD_2 have mainly been carried out in the Astacidea and show clear reflex connections between CSD_2 and the flexor muscles (flexor and accessory flexor) (Vedel *et al.*, 1975) and between CSD_2 and the anterior levator. The anterior levator and flexor reflexes were also described by Klärner and Barnes (1986), who stated that CSD_2 stimulation modulates spontaneous muscle activity rather than elicits more muscle spikes.

All these studies demonstrate that CSD activity has an effect on motor activity in the legs of decapod crustaceans, and led Clarac (1976) to propose that these

receptor organs are used by the animal to detect the force generated when the leg makes contact with the ground, like the funnel canal organs of the dactyl which are sensitive to cuticle distortion and record contact of the leg with the ground (Libersat *et al.*, 1987a; Shelton and Laverack, 1968). This hypothesis was tested by Klärner and Barnes (1986) who demonstrated that the highest frequencies of CSD_2 discharge occurred when the leg was placed on the ground at the onset of the power stroke. They also demonstrated a CSD_2 influence on the co-ordination of neighbouring ipsilateral legs. Tying a wire round the ischiopodite of the fourth walking leg where the CSD_2 receptor organ is located compressed the soft window constantly. They observed that the timing of the stepping pattern in the third walking leg was less precise in animals with chronically stimulated CSD_2 than in control animals. Therefore, it seems that the CSD_2 receptor organ not only modulates the posture of the entire leg depending on the weight supported by the animal, but also plays a role in the co-ordination of all legs. In previous studies, interpretation of the functional significance of CSD_1 has been more or less hypothetical due to the lack of information on CSD_1 activity during locomotion. This situation has been remedied by the *in vivo* experiments described in the previous chapter. In locomotion, CSD_1 sensory fibres are first stimulated when the anterior levator motorneurones have reached their highest discharge rate during the return stroke in each step cycle. They stop firing at about the same time as the CSD_2 units during the power stroke. CSD_1 is also thought to play a role in regulating the motor output in autotomy either by preventing accidental autotomy or by promoting limb fracture upon injury (see Chapter 4).

Rhythmic co-ordinated activity similar to the muscle activity in normally moving animals is known to exist in isolated nervous preparations of animals such as the cockroach (Pearson and Iles, 1970), crayfish (Sillar and Skorupski, 1986), dogfish (Grillner *et al.*, 1976), lamprey (Wallén and Williams, 1984), rat (Cazalets *et al.*, 1990) and cat (Grillner, 1985; Grillner and Zangger, 1975). This rhythmic motor activity occurring without the aid of any phasic sensory feedback is named 'fictive locomotion' (Grillner, 1981). In the crayfish isolated thoracic preparation, fictive locomotion was first described by Sillar and Skorupski (1986) in the proximal muscle groups of the walking legs. They demonstrated the occurrence of spontaneous, reciprocal activity of promotor and remotor motorneurones, with levator motorneurones firing in phase with promotor bursts. This resembles the muscle activity in intact animals during forward locomotion. Chrachri and Clarac (1990) later elaborated on the system and showed that the isolated preparation could display activity patterns mimicking backward as well as forward locomotion.

The existence of such fictive locomotion in the crayfish *in vitro*

preparation is utilized in this project to study the functional role of the cuticular stress detectors in locomotion. Two approaches are taken to elucidate the extent to which the two receptor organs are involved in shaping the motor output generated by the central nervous system. Firstly, the synaptic connections between the sensory afferents and the motorneurones controlling the proximal muscle groups of the leg are studied in detail using both extracellular and intracellular techniques. They demonstrate the presence of both mono- and polysynaptic pathways. Secondly, it has been shown that reflex inputs to motorneurones are not the only way by which proprioceptive feedback influences the motor output, but that phasic afferent signals can influence the timing of the central pattern generator (Andersson and Grillner, 1983; Andersson *et al.*, 1978a; 1978b; Bässler, 1985; Clarac and Chasserat, 1983; Grillner *et al.*, 1981; Elson *et al.*, 1986; Sillar *et al.*, 1986). The ability of phasic CSD stimulation to entrain the pattern of fictive locomotion is thus studied in rhythmically active *in vitro* preparations.

This study focuses on the proximal muscle groups which play a dominant role in generating leg movements during locomotion, concentrating on the levator and depressor muscles which lift and lower the leg, but not ignoring the promotor and remotor muscles which move the leg forwards and backwards.

3.2. MATERIAL AND METHODS

3.2.1. Animals

All reflex experiments were performed on male and female crayfish, *Procambarus clarkii*, which measured between 8 and 15cm in length. Crayfish of the species *Pacifastacus leniusculus* were used in the entrainment experiments.

Crayfish of both species were obtained from a commercial supplier and maintained in aerated aquaria.

3.2.2. *In vitro* preparation

An *in vitro* preparation consisting of the isolated thoracic central nervous system and a fifth walking leg was used as described in Chapter 2.

3.2.3. Stimulation of sensory fibres

CSD sensory fibres were stimulated either electrically or mechanically as described in Chapter 2.

Mechanical stimulation was used to study the motorneurone responses to CSD stimulation. Care was taken to ensure that, during stimulation, the blunt end of the stimulator probe was permanently in contact with the soft cuticle of the receptor organs. Thus, it was possible to link the motorneurone responses to either the 'on' stage (corresponding to indentation of the soft cuticle and shortening of the receptor strand) or the 'off' stage (corresponding to release of the soft cuticle and lengthening of the receptor strand) of the stimulation.

When studying the influence of CSD stimulation in rhythmic preparations (entrainment experiments), mechanical stimulation of the CSD organs was arranged so that it comprised not two, but three stages. By allowing the blunt probe to come off the soft cuticle during the sinusoidal movement, the stimulation consisted of an 'on', an 'off' and a third state, when no stimulation at all occurred. It is believed that this resembled most closely the CSD activity that would occur naturally during walking. It also made it possible, for purely technical reasons, to use a wider range of cycle durations.

Electrical stimulation activates simultaneously a number of sensory units irrespective of their physiological properties. Hence motorneurone responses to electrical stimulation of the CSDs cannot be identified as either 'on' or 'off' response. However, electrical stimulation does allow one to control precisely the duration and timing of the stimulus and was useful when trying to ascertain whether the responses

were direct (monosynaptic) or transmitted via interneurones (polysynaptic). Electrical stimulation was also sometimes used in the entrainment experiments.

3.2.4. Saline

Tests for monosynaptic or polysynaptic pathways included the use of salines with ionic concentrations differing from the concentration used normally.

	normal saline	high Ca^{++}/high Mg^{++}	zero Ca^{++}/high Mg^{++}
NaCl	195	158.4 *	195
KCl	5.4	5.4	5.4
$CaCl_2$	13.5	34	0
$MgCl_2$	2.6	6.5	16.1
TRIS	10	10	10

* adjusted to maintain the same osmolarity as in normal saline.

All values are in mM, pH was adjusted to 7.5.

The application of high Ca^{++}/high Mg^{++} and zero Ca^{++}/high Mg^{++} was localized by placing a ring of Vaseline with separate in- and outflows around the fifth ganglion, thus restricting the effect of the changed ionic concentrations. The rest of the preparation was perfused with normal saline.

3.2.5. Fictive locomotion

Rhythmic preparations exhibiting fictive locomotion were used to study entrainment. The muscarinic agonist, oxotremorine, which is known to induce and enhance rhythmic motor activity in the isolated crayfish preparation (Chrachri and Clarac, 1987) was added to the saline to obtain and/or maintain rhythmicity in preparations. Bath concentrations of between 10^{-6}M and 10^{-5}M were used. In a few experiments, 5-HT (serotonin) at concentrations between 2 x 10^{-7}M and 5 x 10^{-7}M was used in addition to oxotremorine. It was thought to enhance further the rhythmicity of the preparations.

3.2.6. Recording and data analysis

Methods used for extracellular and intracellular recordings were identical to those in Chapter 2. Data capture and analysis of intracellular recordings using CED software were also carried out as described in Chapter 2.

Extracellular spike data from entrainment experiments was analysed by first converting the analogue data, written to the hard disc via the CED 1401 interface, into standard pulses using a manually controllable discriminator within the CED SPIKE2 programme. Then bursts were identified by applying two criteria:

The beginning of a burst was said to occur when two consecutive action potentials first occurred at less than a minimum interpulse interval (set at between 10 and 100ms in different experiments). Similarly the end of a burst was defined as occurring when action potentials first occurred at intervals longer than a predetermined value. This maximum interpulse interval determined when a spike was too far away from the last one for it to belong to the burst (set at between 100 and 500ms in different experiments).

3.3. RESULTS

3.3.1. Reflexes

After dissection and placing of all electrodes, the majority of *in vitro* preparations displayed tonic activity in units of the promotor and anterior levator muscle roots, units of remotor, posterior levator and depressor being silent. In such quiescent preparations, CSD_2 mechanical stimulation mainly led to excitation of anterior levator and promotor by enhancing the firing frequency of already active units and eliciting action potentials in formerly silent units. This excitation occurred during the 'on' stage of the stimulation, i.e. when the receptor strand is shortened (Fig.3.1B).

In contrast, CSD_1 stimulation mostly led to a powerful excitation in the depressor by eliciting action potentials in formerly silent units and resulted in inhibition of active units in the anterior levator (Fig.3.1A). Both excitation in depressor and inhibition in anterior levator occurred during the 'on' phase of the stimulation. Responses in promotor, remotor and posterior levator to CSD_1 stimulation were mostly of an excitatory nature, also occurring during the 'on' phase of the stimulation.

Not infrequently, the reflex responses in promotor, anterior levator and depressor varied from the above, generalized description and Table 3.1 summarizes all extracellular responses observed during mechanical stimulation of the CSD receptor organs. In the majority, CSD_1 stimulation led to depressor excitation and anterior levator inhibition, whereas CSD_2 stimulation led to anterior levator and promotor excitation.

It often occurred that particular motorneurone groups were silent, so that inhibitory reflex responses could not be noticed in the extracellular trace. Even excitatory reflex responses may have passed unnoticed, in cases of low level motorneurone excitability (i.e. very negative membrane potential) when the excitatory postsynaptic potentials (epsps) resulting from afferent inputs were not able to raise the membrane potential beyond spiking threshold. It was also observed that different units, recorded simultaneously in one and the same motor root, responded differently to CSD stimulation, demonstrating the very complex effects CSD stimulation has on the motor output in the proximal muscle groups (Fig.3.2).

For these reasons, it was important to record directly from the motorneurones (Fig.3.3). These results are summarized in Table 3.2 and in a more simplified way for anterior levator and depressor motorneurones in Fig.3.4. The

results from the intracellular recordings back up the main observations made in the extracellular recordings. All depressor motorneurones are excited and most of the anterior levator motorneurones are inhibited during the 'on' phase of CSD_1 stimulation. When the CSD_2 strand shortens during the 'on' phase of mechanical stimulation, most promotor and anterior levator motorneurones respond with an epsp as seen in extracellular recordings. In contrast to the extracellular data, all three motorneurone groups of promotor, anterior levator and depressor show an increased percentage of their units to be inhibited by CSD_2 stimulation. These reflex responses were probably not obvious in the extracellular recordings due to the reason mentioned above.

In motorneurones, excitatory responses (epsps) were observed both to pressure applied to the soft cuticle of the receptor ('on') and to its release ('off'). In contrast, inhibitory responses (inhibitory postsynaptic potentials, ipsps) were only observed when 'on' units were activated (Fig.3.5).

3.3.2. Conduction velocity

The conduction velocity of most motorneurones was measured. When correlating the response obtained (i.e. epsp or ipsp as seen in Table 3.2) with the conduction velocity, it was found that all fast conducting anterior levator motorneurones responded with ipsps to stimulation of CSD_2, but all slower conducting anterior levator motorneurones were excited by stimulation of CSD_2. No such relationship was revealed for CSD_1 stimulation of anterior levator motorneurones or for the other motorneurone groups with either CSD activity (Fig.3.6). The clear link between reflex response and conduction velocity in anterior levator motorneurones indicates that different units in a pool of synergistic motorneurones control different behavioural acts performed by the same muscle, such as locomotion or posture, and that CSD_2 sensory feedback differs accordingly.

3.3.3. Synaptic connections

A number of experimental tests exist that can be used to decide whether reflex connections are monosynaptic or polysynaptic (Berry and Pentreath, 1976). Some of these were used to test CSD - motorneurone connections for mono- and polysynaptic components.

3.3.3.1. Correlation between intracellular epsp and extracellular sensory spike

Epsps recorded intracellularly in motorneurones could be correlated to

extracellularly recorded units in the CSD sensory nerve root. In 13 cases, latencies of 3-12ms were measured. This variation corresponds with the wide range of conduction velocities in the different sensory fibres (conduction velocities of 0.5 - 5ms^{-1} and conduction distances of 8 - 12mm were measured). Only in cases where the latency between extracellular CSD spike and intracellular epsp was constant and the epsp amplitude consistent, was the connection thought to be monosynaptic (Fig.3.7). Small variations in latency and epsp size were thought to stem from additional synapses interposed in the pathway between CSD sensory terminals and motorneurones, and hence indicated a polysynaptic pathway (Fig.3.8).

3.3.3.2. High frequency electrical stimulation

The ability of the postsynaptic potential to follow each presynaptic spike without loss at high frequency is a good indicator of monosynaptic connections, since the presence of intercalated neurones would substantially increase the likelihood of transmission failures. Most of the short latency epsps follow each electrical shock of a train of electrical stimuli at frequencies of 100Hz and more. Short latency ipsps (5-6ms) were observed which followed trains of 90Hz in a one-to-one fashion, but failed to do so at higher frequencies (Fig.3.9).

Often only a pair of shocks were used in order to identify whether epsps could follow CSD stimulation in a 1:1 fashion. This method made it easier to decide whether longer latency responses had monosynaptic components or not. It is difficult doing so when early and late postsynaptic potentials (psps), resulting from different stimuli in the same train of shocks, summate to a complex response (Fig.3.10). Most of the short latency epsps followed twin shocks 3-5ms apart.

3.3.3.3. Effect of high Ca^{++}/high Mg^{++} saline

Increasing the concentration of calcium and magnesium ions in the saline raises the firing threshold of neurones and thus tends to block polysynaptic, but not monosynaptic reflexes. As the application of high Ca^{++}/high Mg^{++} was restricted to the fifth ganglion (see Methods, section 3.2.4.), the physiological properties of the sensory fibres in the periphery were unchanged and any change in the motorneurone response was due to central effects. Any epsp occurring in the motorneurone in response to electrical stimulation in normal saline and disappearing later in high Ca^{++}/high Mg^{++} saline was considered to result from polysynaptic connections (Fig.3.11). In most cases, it was the epsps with the longer latencies that disappeared 20-40 minutes after being bathed in this saline, thus indicating that they originated from polysynaptic pathways.

3.3.3.4. Effect of Ca++ free saline

Replacing the normal saline with Ca++ free solution steadily removed all Ca++ ions from the surroundings of the fifth ganglion, thus continuously decreasing the amount of transmitter being released at all chemical synapses. As a consequence, epsp size would continually diminish. This process should be gradual for monosynaptic epsps, whereas polysynaptic epsps should disappear suddenly at one point in time when the interneurone fails to fire. Epsps with latencies shorter than 7ms were found to decrease continuously in size when the normal saline was replaced by Ca++ saline. In contrast, all long latency epsps and all ipsps disappeared abruptly with the same treatment. These results support all prior findings that short latency epsps resulted from monosynaptic connections, whereas all ipsps and a lot of the later epsps were of polysynaptic origin (Fig.3.12).

3.3.3.5. Correlation between intracellular epsp and intracellular sensory spike

Simultaneous intracellular recordings of correlated sensory impulses in CSD terminals and epsp in motorneurones allows measurement of reflex transmission time within the ganglion. A direct connection is evident if each sensory impulse in the CSD afferent is followed by a psp in the motorneurone at a short constant latency. Obviously, the latency depends upon the location of the two recording electrodes within the ganglion in relation to the site of the sensorimotor synapse. The latency is measured as the time lapse between the two observations of firstly a CSD spike in the sensory afferent and secondly an epsp in the postsynaptic motorneurone. The further away from the site of synaptic contact the recordings are made, both in pre- and postsynaptic direction, the longer is the latency. Simultaneous recordings of the pre- and postsynaptic cell were achieved in three cases. Latencies of 0.4 to 0.85ms were measured (Fig.3.13).

Application of the above described tests indicated that most of the excitatory responses, but none of the inhibitory responses, had monosynaptic components. All early epsps with latencies between 3-5ms resulted from monosynaptic connections. Later epsps could either result from monosynaptic or polysynaptic pathways. All these tests do not exclude the possible presence of non-spiking interneurones which are known to exist in the thoracic ganglia of crayfish (Chrachri and Clarac, 1989).

3.3.4. Number of sensory units connecting onto each motorneurone

The correlation of epsps in motorneurones with extracellular spikes in the sensory nerve root revealed that there were often several CSD units connected to a single motorneurone, each eliciting a different postsynaptic potential (psp) (Fig.3.14). During mechanical stimulation, the input from all these different CSD units would contribute to the compound response of the motorneurone (see Fig.3.3). In order to estimate the number of CSD units involved in such a compound response, electrical stimulation was used. Starting with a subthreshold stimulus and increasing the intensity of the stimulation continuously, it was possible to stimulate gradually more and more CSD units. Thus each distinctive increase in psp size results from one more CSD unit activated by the increased stimulation (Fig.3.15). Obviously, each stepwise increase in psp size could result from more than one CSD unit being stimulated at the same threshold value (remember the large number of CSD_1 and CSD_2 units seen in the cross sections, Chapter 2, part 2.3.2.). For this reason, the counts and all the numbers quoted in the following represent the minimum number of CSD units making connections with motorneurones.

When the above described tests for distinguishing between mono- and polysynaptic pathways were applied, in addition to determining the numbers of sensory fibres connecting onto a single motorneurone, it was possible to separate the connections into three categories; monosynaptic epsps, polysynaptic epsps and ipsps (all of which are polysynaptic). Figure 3.16 summarizes the results showing that an average of 3.75 (of which 0.6 are monosynaptic connections) CSD_1 fibres connect to anterior levator motorneurones and an average of 4.6 (of which 1.4 are monosynaptic connections) CSD_1 fibres connect to depressor motorneurones. Anterior levator motorneurones receive sensory input from an average of 5.8 (2.2 monosynaptic) CSD_2 fibres and depressor motorneurones from an average of 4.2 (1.9 monosynaptic) CSD_2 units. The nine promotor cells recorded when either stimulating CSD_1 or CSD_2 electrically show that less CSD fibres make a connection with the promotor motorneurones, an average of 3.2 (of which 0.7 are monosynaptic) and 4.1 (of which 1.4 are monosynaptic) were found for CSD_1 and CSD_2, respectively. The average numbers of 3.8 (0.7 monosynaptic) CSD_1 and of 5.2 (2.2 monosynaptic) CSD_2 sensory fibres for remotor motorneurones were surprisingly high, probably due to the small sample size.

It is worth pointing out that in all muscle groups the average number of CSD units per motorneurone producing an epsp is larger than the average number of CSD units producing an ipsp. This is not surprising when considering that there are more motorneurones being excited than inhibited by CSD stimulation in all but the

anterior levator motorneurone pools. The latter respond in 60% of all cases with a compound ipsp to CSD_1 stimulation. Hence, an average number of only 1.46 inhibiting CSD_1 fibres per anterior levator motorneurone - this is equal to 39% of the overall average number - is surprisingly small.

Strong electrical stimulation of a CSD receptor organ often led to the production of action potentials in the motorneurones. In most cases it was epsps originating from polysynaptic pathways rather than from monosynaptic connections which were able to raise the membrane potential above spiking threshold. Therefore, the impression was gained that polysynaptic inputs are rather more powerful in eliciting an effective reflex response than monosynaptic inputs. Psp size could be augmented due to a kind of amplification mechanism in the interneurones. However, it is to be remembered that most preparations were only tonically active and most motorneurones quite hyperpolarized. In a more active state, the membrane potentials could be more depolarized, thus allowing even the smaller inputs from monosynaptic CSD units to succeed in eliciting an action potential, at shorter latencies and more reliably than the polysynaptic inputs could achieve it. In this context, it is interesting to observe that the ratio of monosynaptic to polysynaptic connections is fairly equal for CSD_2 fibres. In contrast, there are more CSD_1 fibres involved in polysynaptic than in monosynaptic transmission.

3.3.5. Entrainment

Rhythmic activity in the isolated thoracic preparation always showed the same pattern of reciprocal activity in antagonistic muscles as seen in intact animals. Nevertheless, bursts and cycle periods were longer than observed in freely moving animals (Barnes, 1977). 4-80s cycle periods were found in the *in vitro* preparation, compared to 0.8-3s cycle periods in walking crayfish (Klärner and Barnes, 1986; Chapter 2).

When either of the two Cuticular Stress Detectors was stimulated in an intermittent, rhythmic fashion, a change in the timing of the rhythmic activity was often obvious in the first few bursts after stimulation began. If the frequency of the periodic stimulation approximated that of the endogenous rhythm, 1:1 entrainment was observed, i.e. for each stimulus period, being measured from the onset of one stimulus to the onset of the next, one burst of each of the entrained motor groups would occur (Fig.3.17). When the stimulus cycle was much shorter than the inherent rhythm in the motor roots, 2:1 entrainment was observed, meaning two stimulus cycles would occur while the motor roots bursted only once (Fig.3.18).

Nevertheless, in the majority of preparations, entrainment of the motor

rhythm did not occur within the first few bursts after stimulation was begun, but rather took a longer time to become obvious. Another difficulty in observing the ability of CSD sensory feedback to entrain the centrally generated motor rhythm was the occurrence of rather unpatterned rhythms with changing burst and period durations (Fig.3.19A). These irregular rhythms could also comprise a number of short bursts in one motor root reciprocally occurring with single rather longer bursts in the antagonistic motor root (Fig.3.19B).

For these reasons, fairly long bouts of rhythmic motor activity (20-80 cycles) were analysed. The onset of each motor burst was determined by the criteria described in the Methods (section 3.2.6.) and correlated to the onset of its nearest preceding stimulus cycle. This correlation was expressed as the phase at which the burst onset occurred within the stimulus cycle. Phase histograms were drawn to show the distribution of all phase relationships found during sensory stimulation (Fig.3.20). Such phase histograms are circular distributions as a phase of 0 is the same as a phase of 1. Thus circular statistics (Watson's modification of a test by Smirnov, Batschelet, 1965) were applied to test whether the distribution of phases was significantly different from an equal distribution (i.e. the same number of points in each bin of the histogram). If a significant difference was found (at a level of at least 5%), the circular mean for all phases was calculated.

As a control, fictive stimulus cycles were interpolated before the real stimulation had started and phase relationships were measured as before. In all cases no significant distributions of phase relationships existed between the onset of bursts in the motor roots and the imaginary beginning of the stimulus cycles, clearly indicating that the CSD stimulation had altered the timing of the spontaneous rhythm. Thus, entrainment of the motor rhythm takes place by changing the timing of the motor bursts with regard to the stimulus cycle by phase locking the motor to the sensory burst (Fig.3.20).

94% of all analysed bouts of rhythmic activity were found to be entrained by either CSD_1 of CSD_2 stimulation. All mean values of significantly different distributions are summarized in Fig.3.20. Entrainment of anterior levator and depressor motorneurones occurred mostly at opposite phases of the stimulus cycle. CSD_1 stimulation induced depressor units to fire at mean phase of 0.07 relative to the stimulus cycle, whereas anterior levator neurones preferred to burst later at around a phase of 0.45. CSD_2 entrainment of depressor bursts led to phase relationships around a mean of 0.63 and to a mean around 0.53 in promotor bursts. CSD_2 entrainment of anterior levator motorneurones often showed two preferential phases (see Fig.3.20B). These could either constitute a 1:2 relationship where two anterior levator bursts

occurred for each stimulus cycle, or indicate different entrainment patterns for different anterior levator units. The latter appears to be the case in Fig.3.20B. Here, calculating the mean value as done normally would not represent the true phase relationships. Hence, the two phase bins containing the maximum numbers of occurrences were used instead and are summarized in Fig.3.21F.

In order to record from the CSD_2 sensory nerve root, the basipodite region of the leg had to be dissected. During this process, the CSD_1 receptor organ is usually damaged, although the CSD_1 nerve root can be kept intact. Hence, simultaneous stimulation of CSD_1 and CSD_2 is possible and achieved by either stimulating both nerves electrically or by mechanically stimulating CSD_2 when electrically stimulating CSD_1. It is known that CSD_1 and CSD_2 bursts occur at different phases of the stepping cycle in normal locomotion, CSD_1 bursts earlier than CSD_2 bursts (Chapter 2). In addition, they entrain anterior levator and depressor motor units at opposite phases (Fig.3.20). When stimulating both receptor organs simultaneously, the attempt was made to take this into consideration and set stimulation parameters accordingly. 8 different bouts of locomotor activity were analysed by measuring phase relationships as described above. All 8 showed entrainment to preferred phases (Data not shown). However, for technical reasons it was not possible to provide CSD_1 and CSD_2 stimulation of equal strength at any other phase difference than zero. Hence, no conclusions can be drawn from the above data. The only consistent result was that anterior levator and depressor units were always entrained to approximately reciprocal phases.

In addition to monitoring phase relationships, the period durations before and during stimulation were measured. Imposed stimulation with cycle durations shorter than the endogenous motor periods often increased the speed of the rhythm. Correspondingly, slower stimulation could slow down the rhythm. Nevertheless, due to the often rather irregular bursting in the motorneurones, variations of period were so large that no quantitative analysis was attempted.

3.4. DISCUSSION

3.4.1. Proximal muscle groups and their innervation

The CSDs supply some of the proprioceptive feedback which is so important in the shaping of the final motor output in crustacean legs. They are known to exert control over all the different leg muscles (Clarac, 1976). Here, only their sensory connections and their influence on rhythmic motor output to the proximal muscle groups were studied. The proximal muscle groups of promotor, remotor, levator and depressor are fundamental to the execution of forward and backward walking in normal locomotion (Evoy and Ayers, 1982). As the CSDs are force-sensitive mechanoreceptors and also respond to tension in the muscles which move the leg dorso-ventrally (Clarac, 1977), the majority of experiments were concerned with the levator and depressor muscle groups, but data for other proximal muscle groups are also shown.

In order to be sure that the experiments present an adequate summary of the synaptic connections between the cuticular stress detectors and the different groups of proximal muscles, it is necessary to compare the number of intracellular recordings made during CSD stimulation with estimates of the total size of the motorneurone pools. This information is presented in Table 3.3. The number of motorneurones in each group is determined from cobalt backfills, with the exception of the posterior levator. Here, an approximate figure was derived from the number of units that can be recognized from extracellular recordings. These numbers are estimates for the following reason. As has already been discussed in Chapter 2, both the cobalt backfill and methylene blue staining techniques may miss some smaller motorneurones. Parson (1982), for example, re-examined the innervation pattern of the flexor muscle in *Carcinus* using histological techniques. He found two additional axons which had not been described before (van Harreveld and Wiersma, 1939; Wiersma and Ripley, 1952). Thus, it is important to note that Cattaert *et al.* (1992b), using histological techniques, give a higher number for anterior levator and depressor motorneurones in crayfish walking legs. They counted 20 and 15 axons respectively. Barnes and Kidd (unpublished data) found that the anterior levator nerve in *Astacus leptodactylus* contained over 30 axons, 14 of which had diameters of more than 12μm and were thus presumed to be motorneurones. It is also known that all crayfish leg muscles are innervated by at least one inhibitory motorneurone, the common inhibitor (Cooke and Macmillan, 1983). Neither of the staining techniques used in previous studies is able to distinguish between excitatory or inhibitory motorneurones. Thus the

number of excitatory motorneurones might be less than the numbers given in Table 3.3, whereas the number of motorneurone recordings made in this study do not include the common inhibitor. The numbers of intracellular recordings from anterior levator and depressor motorneurones in response to either CSD_1 or CSD_2 stimulation (which range from 19-27, Table 3.3) is probably insufficient to be sure that every motorneurone was penetrated at least once. However, they are thought to present a reasonably clear picture of the variety of synaptic connections present. The numbers of recordings from the other motor roots (promotor, remotor and posterior levator) were thought to be insufficient to allow firm conclusions to be drawn, but provide useful information on the nature of at least some of the CSD inputs to these muscles.

3.4.2. *In vitro* preparation

The crayfish *in vitro* preparation of the thoracic nervous system made it possible to monitor motor and sensory activity simultaneously, not only on an extracellular level, but also intracellularly in single units. The latter approach makes it possible to obtain a much clearer picture of all reflex responses, for they are often subthreshold, not being able to raise the membrane potential above firing threshold. They would thus not be recorded in any extracellular trace. The intracellular recording technique also allowed a distinction to be made between excitatory and inhibitory connections, and between mono- and polysynaptic pathways.

3.4.3. Reflex connections

As a first step in assessing the influence of sensory feedback from the CSDs onto the final motor output, the circuitry involved has been investigated in quiescent preparations. Here, no phasic input from the CNS changes the occurrence of reflex responses. The diversity of responses is rather confusing, but all the reflex connections of the CSDs can be divided into two functional groups, which were called "levation" and "depression" reflexes. A "levation reflex" is defined as indentation of the CSD soft cuticle leading to levator excitation or depressor inhibition, or release of pressure on soft cuticle leading to depressor excitation. All are compatible with an increase in cuticular stress producing increased levator firing. In contrast, the "depression reflex" is defined as CSD indentation leading to depressor excitation or levator inhibition, or CSD release leading to levator excitation. This is shown in Table 3.4 which is derived from data presented in Table 3.2.

It is interesting to note that both feedback loops from CSD_1 and CSD_2 onto anterior levator and depressor motorneurones contain monosynaptic components. Only presynaptic inhibition of the sensory signal in the afferent terminal would

prevent a response in the motorneurone.

3.4.4. Compartmentalization

In vertebrates, it is known that many biarticular muscles exhibit some degree of compartmentalization, in that a part of the muscle exerts its action primarily on one joint while another part has the greater effect on the other joint. It is noteworthy that the incoming sensory information is also rather compartment specific, the same sensory organ having different effects in different compartments of one and the same muscle (Hasan and Stuart, 1988).

In crustaceans, leg movement in all directions is performed by the simultaneous movement of several joints, each of which moves in a single plane under the control of an antagonistic pair of muscles. Each of these muscles was thought to be homogeneous in its action. However, in *Brachyura* at least, it is known that the anterior levator muscle can be subdivided into three separate portions which pull the tendon in different directions. It has been suggested that these different heads are independently active in different motor programmes. In *Carcinus*, some of the anterior levator motorneurones innervate only one of the anterior levator heads, whereas others innervate all three parts (Moffett *et al.*, 1987). In the fifth leg of the swimming crab, *Portunus*, this segregation is complete in that the 10 different anterior levator units, in groups of 3 or 4, each supply a different head (Hoyle and Burrows, 1973). In crayfish, it is known that pools of synergistic motorneurones can be classified into subgroups depending on their reflex response to sensory feedback. Skorupski *et al.*, (1992) studied the reflex responses in promotor and remotor motorneurones to the thoracic-coxal muscle receptor organ (TCMRO) and the thoraco-coxal chordotonal organ (TCCO) in the isolated crayfish preparation. They identified two subgroups in each of the motorneurone pools, which they named group 1 and group 2. Motorneurones belonging to group 1 display only positive feedback reflexes. In contrast, motorneurones of group 2 respond with negative feedback reflexes to TCMRO and TCCO stimulation.

Such compartmentalization in motorneurone pools has not been reported for the anterior levator and depressor motorneurones in studies on their reflex responses to stimulation of the coxo-basipodite chordotonal organ (CBCO) and the TCMRO (ElManira *et al.*, 1991a; Head and Bush, 1992). However, the reflex connections for anterior levator and depressor motorneurones in response to CSD stimulation do show such a diversity of responses within a pool of synergistic motorneurones that neither anterior levator nor depressor motorneurones can be treated as a single group of homogeneous cells. Thus, as far as CSD stimulation is

concerned, anterior levator and depressor motorneurones can be separated into two subgroups, those responding in the "levation reflex" and those responding in the "depression reflex".

3.4.5. Are reflexes preferentially involved in tonic or phasic activities?

Compartmentalization in anterior levator motorneurones is not only established by the positive or negative sign of their reflex response, classifying them as "levation or depression reflex" motorneurones, but also extends to their conduction velocity. With CSD_2 stimulation, all fast conducting anterior levator motorneurones responded with an ipsp to stimulation of CSD_2 and so were involved in the "depression reflex", but all slow (i.e. slower than $2ms^{-1}$) conducting units were excited by stimulation of CSD_2 and so are classed as "levation reflex" motorneurones. In studies on the closer muscle in lobster legs (Govind *et al.*, 1981; Costello and Govind, 1983), the lobster swimmeret system (Davis, 1971) and the flexor muscle in the walking legs of *Carcinus* (Parson, 1982), it has been pointed out that there is a tendency for fast motorneurones to innervate fast contracting muscle fibres which are mainly recruited in phasic motor acts, and slow motorneurones to innervate slow contracting muscle fibres which are active in tonic contractions. Correspondingly, it is thought possible that the compartmentalization of anterior levator motorneurones based on reflex response and conduction velocity reveals the trend in the anterior levator muscle to respond with a "levation reflex" during more tonic activity, e.g. when the crayfish stands, whereas a "depression reflex" would result from CSD_2 stimulation during more phasic activity, e.g. when the crayfish walks.

Three further points need, however, to be borne in mind. Firstly, there are a lot of intermediate muscle fibres which do not fall clearly into either of the above-mentioned fast contracting or slow contracting groups. Secondly, many muscle fibres are innervated both by slow and fast conducting motorneurones. Such muscle fibres will be involved in both tonic and phasic contractions. Finally, the way in which fast and slow motorneurones are used in different behaviours may vary. For instance, it is known that both the tonic and phasic posterior levator motorneurone are active during walking in *Carcinus* (Clarac and Wales, 1970), while the two largest and therefore fastest flexor motorneurones are not active in normal locomotion in crayfish (Barnes, 1977). This means that it will only become clear whether different behaviours determine the overall reflex response within a muscle when the involvement of the different motorneurones in specific motor acts (e.g. locomotion, posture) is known.

For CSD_1, the possible distinction between the "depression reflex" being involved in locomotion and the "levation reflex" being involved in more tonic activity

cannot be made in the same fashion as for CSD_2. This is because most coxopodite motorneurones respond with a "depression reflex" to CSD_1 stimulation and no relationship between reflex response and conduction velocity has been found. However, the suggestion that CSD_1 rather plays a more important role in phasic activities such as locomotion is supported by the finding that the reflex response to CSD_1 stimulation habituates within a short time (see Chapter 2). This would make any CSD_1 involvement in long term load control of posture ineffective. Hence, the involvement of most coxopodite motorneurones in a "depression reflex" in response to CSD_1 stimulation can be linked to the predominant role of CSD_1 in phasic rather than tonic activities. Thus, in both CSD_1 and CSD_2 the "depression reflex" would be rather involved in the control of locomotion.

3.4.6. Assistance and resistance reflexes

CSD_2 sensory feedback encodes information about ground contact and load distribution in each crayfish leg (Klärner and Barnes, 1986). With CSD_2, the "levation reflex" is thus compatible with an increase in cuticular stress producing increased levator firing and as such is a negative feedback effect. In a standing crayfish such effects would compensate for inequalities of load distribution between appendages. The "depression reflex" in contrast is an assistance reflex where increase in cuticular stress leads to increased activity in the depressor motorneurones, thus increasing tension in load-bearing muscles. This might enhance propulsion during locomotion.

CSD_1 is stimulated earlier than CSD_2 in each stepping cycle. Its sensory cells start to fire at the time when the anterior levator motorneurones have reached their highest firing frequencies during the return stroke (first sub-burst) and continue to fire during the power stroke (second sub-burst) (Chapter 2). In an overwhelming majority, both anterior levator and depressor motorneurones are involved in the "depression reflex" when CSD_1 is stimulated, anterior levator motorneurones being inhibited and depressor motorneurones excited.

In locomotion, the influence of CSD_1 activity in the first sub-burst could be interpreted as preparing for the switch from return to power stroke by supplying hyperpolarizing inputs in highly excited levator motorneurones and depolarizing inputs in deeply depressed depressor motorneurones. Its influence is thus a rather subtle one, not initiating a sudden phase switch, but rather preparing for it. Clarac *et al.* (1971) have shown that the reflex response in the anterior levator and depressor motorneurones to CSD_1 stimulation is not as strong as the reflex response to CBCO stimulation, and fewer CSD than CBCO units connect onto the coxopodite muscles

(compare data of Fig.3.16 with ElManira *et al.*, 1991a). Hence, the reflex effect could be described as assisting the central pattern generator in supplying smoothing inputs during extreme levels of membrane potentials when changes are imminent. In addition to being already active during the return stroke, CSD_1 sensory fibres are stimulated during the power stroke for a similar time duration as CSD_2 fibres (second sub-burst). Here, the "depression reflex" in response to CSD_1 activity is also an assistance reflex, since the depressor motorneurones which are active during the power stroke receive additional excitatory inputs, while the inactive anterior levator motorneurones are additionally inhibited by CSD_1 feedback. Thus for CSD_1 as for CSD_2, the "depressor reflex" constitutes an assistance reflex.

3.4.7. Entrainment

The influence of CSD sensory feedback was further studied in rhythmic preparations. Entrainment, the ability of afferent signals to modify the timing of the central pattern generator (Hasan and Stuart, 1988), has been demonstrated in the crustacean walking system in intact animals walking on a treadmill and in isolated preparations. Clarac and Chasserat (1983) compared walking patterns in freely moving rock lobsters with patterns observed in animals fixed above a power driven treadmill. They found that walking parameters, such as phase relationships of motor bursts, stepping speed or instantaneous discharge frequencies, were more regular in animals on the treadmill than in freely moving animals. Indeed, animals always matched their walking speed to the speed of the treadmill and were able to make adjustments, when the treadmill speed was changed, within a period as short as one step cycle, even when the new speed was almost three times as fast as the original speed (e.g. from $3cms^{-1}$ to $8cms^{-1}$) (Chasserat and Clarac, 1983). They also reported cases when the animal stopped walking when a speed change occurred, to resume moving after a brief period at the adjusted speed. In isolated preparations, it has been possible to link entrainment with the activity of particular sensory organs. The thoraco-coxal muscle receptor organ (TCMRO), comprising only very few afferent cells, has been shown to entrain the centrally generated rhythmic motor output with the remotor phase of the rhythm entraining to TCMRO stretch, the promotor phase to release (Sillar and Skorupski, 1986; Elson *et al.*, 1992). Since the receptor strand lies in parallel with the promotor muscle, excitation of remotor motorneurones by TCMRO stretch constituting a positive feedback or assistance reflex. However, promotor motorneurones are not affected by the release of the TCMRO strand, but are rather excited by TCMRO stretch or by release of the TCCO which lies in parallel with it (Skorupski *et al.*, 1992). Thus, Elson *et al.* (1992) concluded that the

entrainment of promotor and remotor motorneurones by rhythmic stimulation of the TCMRO results from both positive and negative feedback effects.

In CSD_1 the "depression reflex" constitutes a positive feedback loop. Indeed, in the entrainment experiments, the bursts of depressor units are phase linked to the beginning of the stimulus cycle, probably resulting from the excitatory inputs from CSD_1. Most anterior levator units are inhibited by CSD_1 stimulation. In addition, the anterior levator bursts reciprocally to the depressor at around mid cycle, when CSD stimulation ceases.

The entrainment experiments involving CSD_2 tell a rather different story. Firstly, different anterior levator motorneurones behave in different ways during rhythmic activity. Elson *et al.* (1992, Fig.2C1) observed rhythmic patterns with two antiphasically modulated sets of units in the anterior levator muscle nerve. This was confirmed in the present study. CSD_2 sensory feedback entrains depressor motorneurones at a phase of 0.6. Anterior levator bursts, however, often show two different preferred phases (0.0 and 0.7), a feature not observed during CSD_1 stimulation. Secondly, the entrainment of some anterior levator units at phase 0.0 and depressor motorneurones at phase 0.6 points to an influence from of the "levation reflex" pathways and thus negative feedback control. Only the anterior levator units bursting at around phase 0.7 might have been influenced by inhibitory inputs from CSD_2 sensory fibres. Thus entrainment in these fibres results from a positive feedback loop.

3.4.8. Central pattern generator

Since both CSDs and the TCMRO can entrain fictive locomotor rhythms, they must have an input to the central pattern generating network. This is because they reset the rhythm rather than simply amplify or gate the output. Whether this is a direct effect or occurs via interneurones is not known. Most authors favour the theory that at least some of the motorneurones are part of the central pattern generator (CPG) in the crustacean central nervous system and thus play a part in generating the locomotor rhythm. It is known that motorneurones are part of the CPG in the swimmeret system (Heitler, 1978) and in the pyloric network of the stomatogastric system in crustaceans (Maynard and Selverston, 1975). Also some of the motorneurones innervating proximal leg muscles have been shown to exhibit plateau properties and could be part of the central pattern generator (Chrachri and Clarac, 1990; Sillar and Elson, 1986). Thus, the influence of sensory feedback onto the CPG might be achieved by direct connections from sensory afferents onto motorneurones. In this context, it is interesting to note that in some preparations in this study antagonistically active

motorneurones often exhibited different rhythms (see Fig.3.19B). This suggests that the CPG in crayfish walking legs comprises an assembly of different motorneurone pools, each of which can generate its own bursting pattern, but all of which are in some way coupled, e.g. by interneurones, and can produce a common rhythm.

It is also rather important to mention that not only does sensory feedback influence the timing of the CPG, but that the CPG itself can modulate the efficacy of incoming sensory information at a presynaptic level. The occurrence of primary afferent depolarizations (PADs) are known to be influenced by phasic locomotor programmes in cats (Gossard *et al.*, 1989). In crayfish, both the T and S fibre of the TCMRO receive central inputs to gate sensory information during phasic motor output (Skorupski and Sillar, 1986), while primary afferent depolarizations can be recorded in the central terminals of CBCO afferents that occur at particular times in the fictive locomotor cycles (Cattaert *et al.*, 1990; 1992b).

Central pattern generators are able to produce the motor outputs generating rhythmic movements independently of any afferent information. However, sensory feedback is necessary in order that the motor output can be adjusted to the situation faced by the animal. For instance, the change in load a crayfish experiences when walking in water or on land is instantaneously detected, processed and the final motor output adequately adjusted without causing a major disruption in the continuous locomotor cycle. Thus, sensory feedback influences the final motor output and is itself under the control of the central pattern generator.

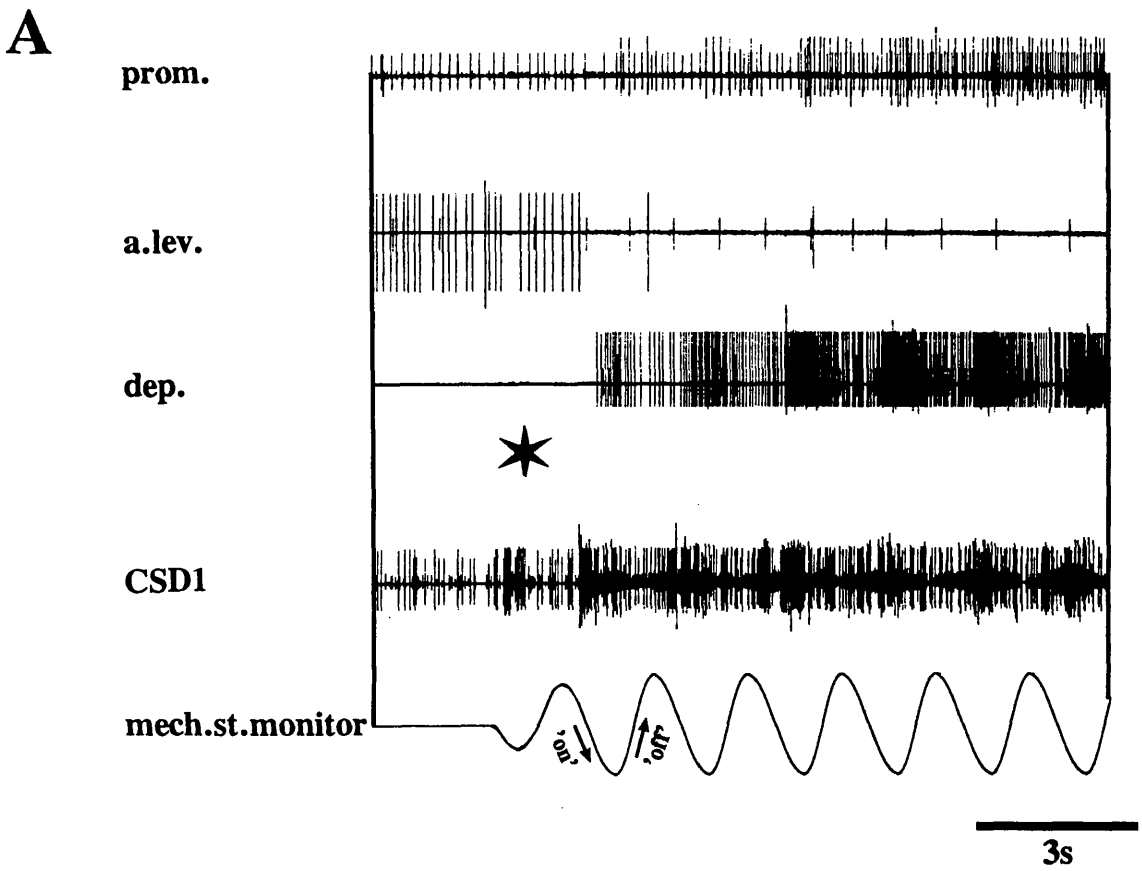
FIGURE 3.1:

Typical recording of CSD reflexes in the proximal muscle groups in response to mechanical stimulation.

A: CSD_1
B: CSD_2

Notice the smaller reflex response in the motor roots at the onset of the mechanical stimulation when fewer sensory fibres are stimulated (asterisk). At the beginning of each mechanical stimulation, the blunt probe had to be brought close to the soft cuticle of the receptor organ. Often, this was not accomplished in one stimulation cycle, so that there was a transient phase when the probe was touching the window intermittently.

The amplitude of the sinusoidal waveform in the figures (mech.st. monitor) depends on the amplification chosen when reproducing the mechanical stimulation monitor trace with the paper recorder. Thus it cannot be used to compare stimulus amplitudes between different figures.


A
prom.
a.lev.
dep.
CSD1
mech.st.monitor
'on'
'off'
3s

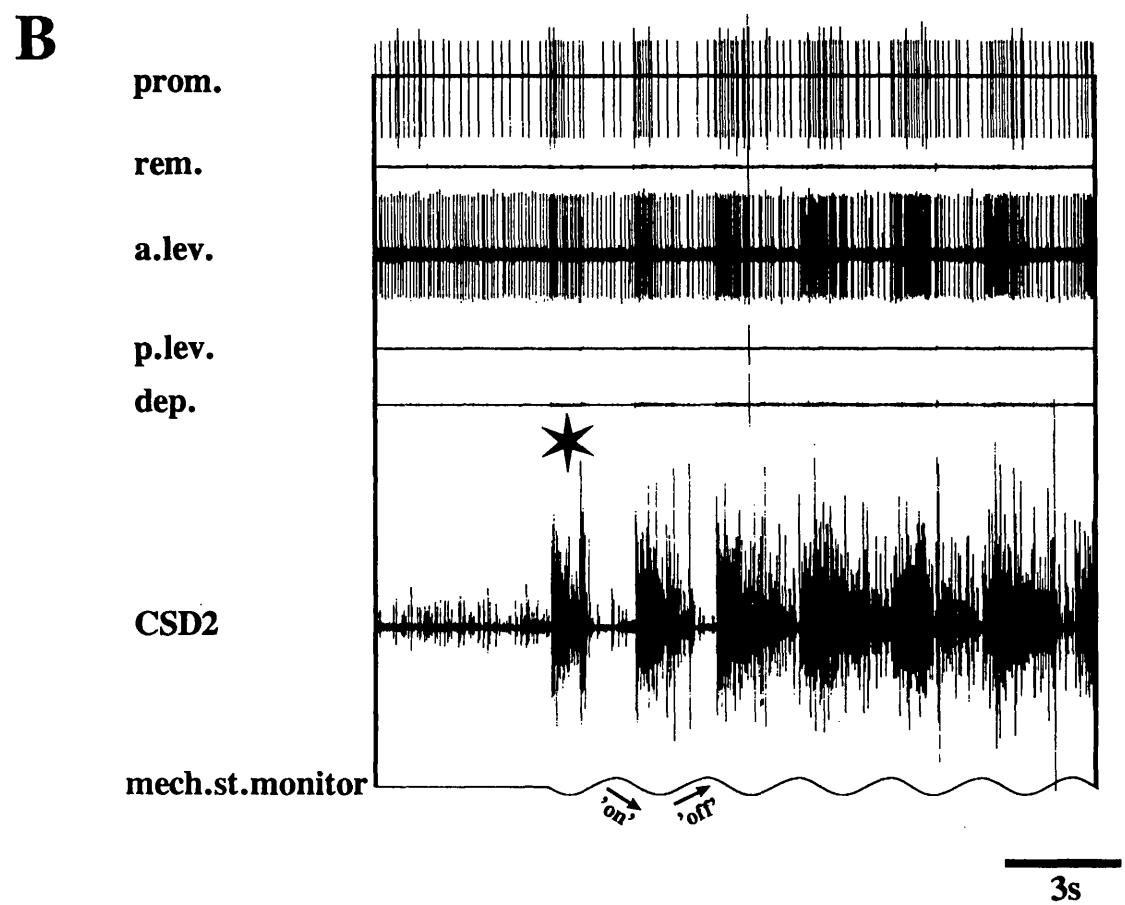

B
prom.
rem.
a.lev.
p.lev.
dep.
CSD2
mech.st.monitor
'on'
'off'
3s

FIGURE 3.2:

Reflex responses to mechanical stimulation of CSD_2 in three different anterior levator units recorded extracellularly in the motor root.

Before stimulation, unit I is silent, while units II and III are tonically active (not shown). Units I and III are excited and unit II is inhibited when the receptor strand is shortened during the 'on' stage of the stimulation. Notice that the inhibition in unit II lasts longer than the excitation in unit I.

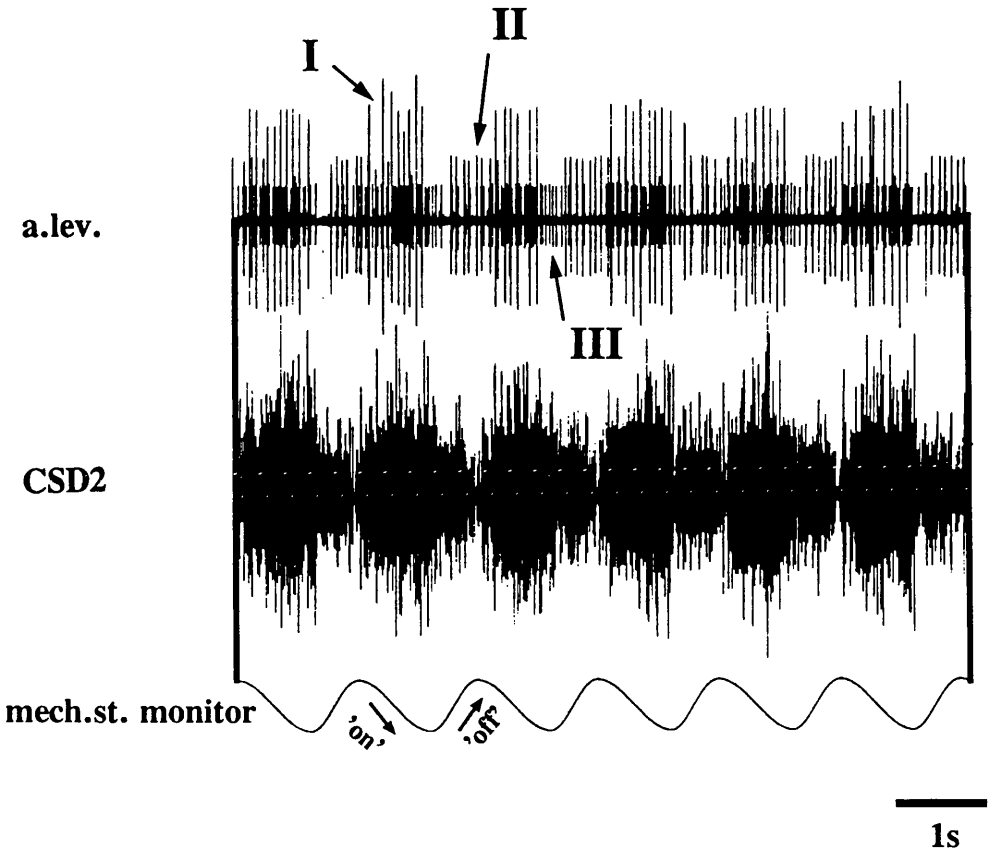

I
II
III
a.lev.
CSD2
mech.st. monitor
'on'
'off'
1s

FIGURE 3.3:

Intracellular recordings showing compound responses in different motor-neurones to mechanical stimulation of the CSDs.

A: Depressor and promotor motorneurones
B: Remotor motorneurone
C: Posterior levator motorneurone
D: Anterior levator motorneurone

The motorneurone membrane potentials (which ranged from -70mV to -40mV) were often too close to the chloride equilibrium potential to be certain that an excitatory postsynaptic potential (epsp)(as seen in A, B, C) was not in fact a reversed inhibitory postsynaptic potential (ipsp). Thus, constant positive current was injected into all motorneurones at one point in the recording in order to depolarize the membrane potential. Any excitatory input from CSD afferents could then raise the membrane potential above firing threshold and the motorneurone would spike in response to CSD stimulation (as seen in D where a +4nA constant current was injected into the anterior levator motorneurone). This procedure eliminated any doubt about the sign of the response, since under such conditions ipsps would be clearly hyperpolarizing.

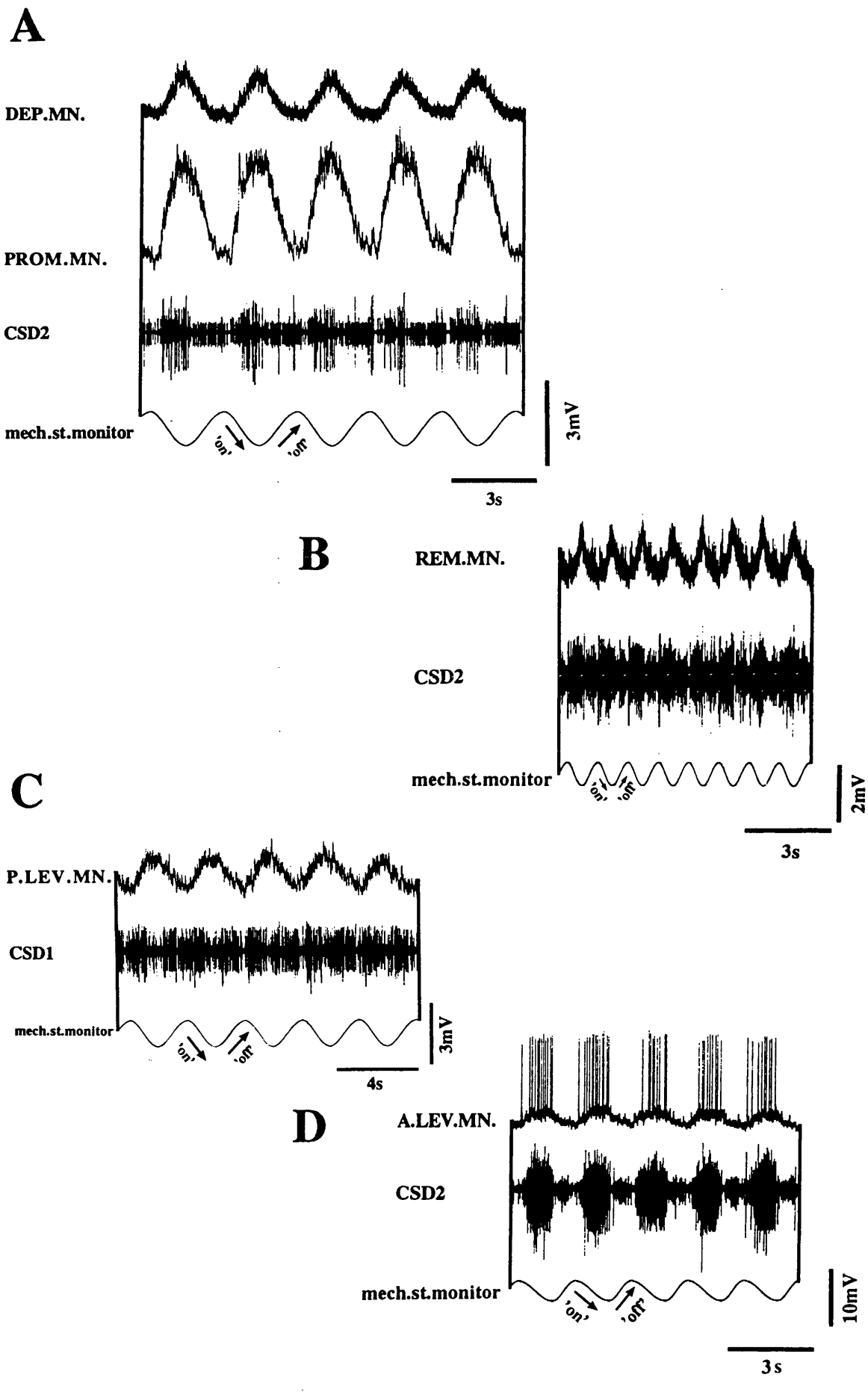

A
DEP.MN.
PROM.MN.
CSD2
mech.st.monitor
'on'
'off'
3mV
3s
B
REM.MN.
CSD2
mech.st.monitor
'on'
'off'
2mV
3s
C
P.LEV.MN.
CSD1
mech.st.monitor
'on'
'off'
3mV
4s
D
A.LEV.MN.
CSD2
mech.st.monitor
'on'
'off'
10mV
3s

FIGURE 3.4:

Wiring diagrams showing the percentages of motorneurones responding either with an epsp (arrow heads indicate excitatory connections) or an ipsp (filled circles represent inhibitory connections) to 'on' or 'off' CSD_1 or CSD_2 units. All percentages pointing to one motorneurone pool should add up to 100%. However, if there is a difference, e.g. for CSD_1 only 95% of all recorded anterior levator motorneurones showed a reflex response), it will indicate that some motorneurones of that pool did not respond to the stimulation ('no response' category in Table 3.2).

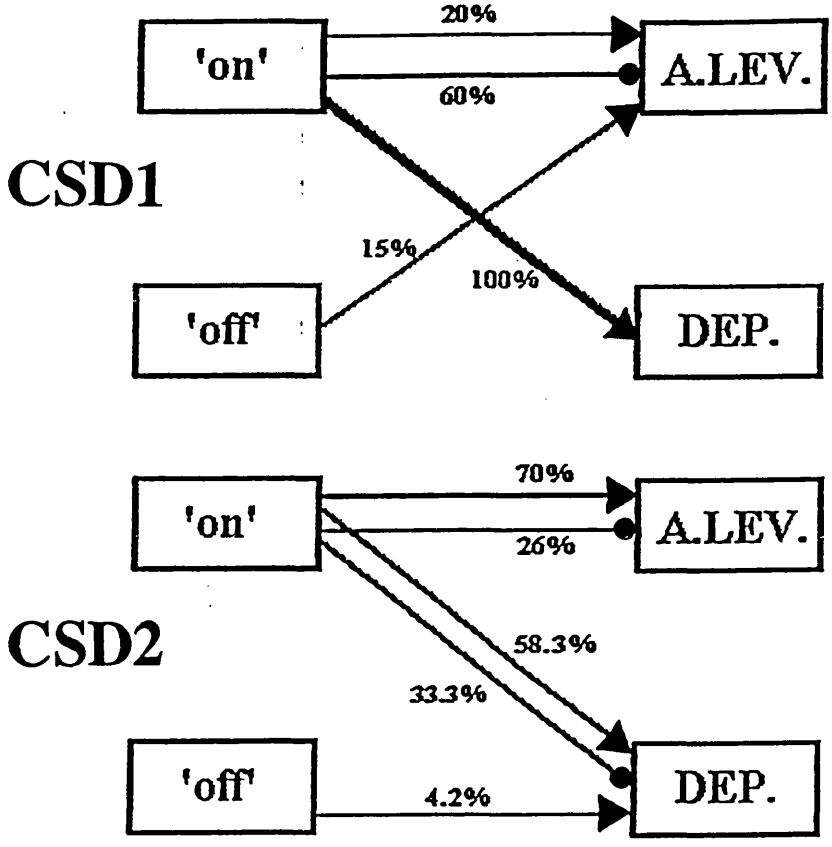

CSD1
'on'
'off'
A.LEV.
DEP.
20%
60%
15%
100%
CSD2
'on'
'off'
A.LEV.
DEP.
70%
26%
58.3%
33.3%
4.2%

FIGURE 3.5:

Intracellular recordings of two motorneurones during mechanical stimulation of CSD_2.

A: The depressor motorneurone responds with a compound epsp (resulting from the summation of several epsps occurring closely together) when the mechanical pusher releases the soft window, thus stimulating the 'off' fibres of CSD_2. As can be seen from the CSD sensory root recording, the CSD_2 'off' fibre spikes are noticeably smaller in size than CSD_2 'on' fibre spikes.

B: The anterior levator motorneurone responds with a compound ipsp during the 'on' phase of CSD_2 mechanical stimulation. Notice the smaller amplitude of the compound ipsp at the beginning of stimulation when fewer CSD_2 units are activated (see legend of Fig.3.1).

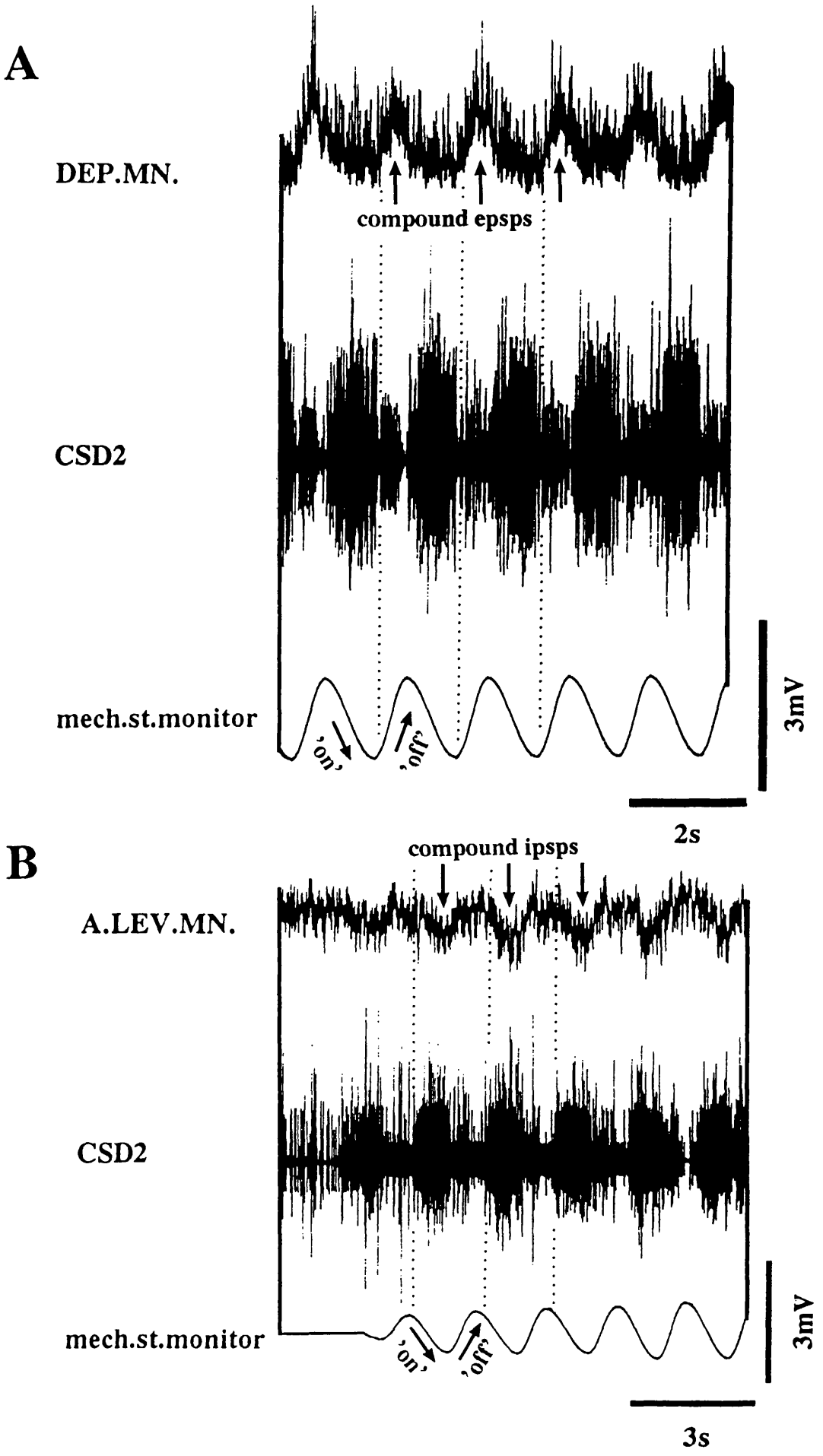

A
DEP.MN.
compound epsps
CSD2
mech.st.monitor
'on'
'off'
3mV
2s
B
compound ipsps
A.LEV.MN.
CSD2
mech.st.monitor
'on'
'off'
3mV
3s

FIGURE 3.6:

Each of the five histograms shows reflex responses in motorneurones of one of the three motor pools - promotor, anterior levator or depressor - to either CSD_1 or CSD_2 mechanical stimulation. Each motorneurone is classified accordingly to its type of response (striped = epsp, cross-hatched = ipsp) and its conduction velocity. Each conduction velocity bin covers a 0.5ms-1 range of velocities.

All depressor motorneurones responded with an epsp to CSD_1 stimulation. In all other cases both epsps and ipsps were observed. Only the anterior levator motorneurones show a distinct correlation between conduction velocity and type of reflex response. This occurs only with CSD_2 stimulation. All slow anterior levator motorneurones ($\leq 2.0ms^{-1}$) respond with an epsp, all fast ones ($> 2.0ms^{-1}$) with an ipsp.

Histograms for similar correlations in respect of promotor motorneurones to CSD_1 stimulation, and for remotor and posterior levator motorneurones in response to stimulation of either CSD, are not shown due to insufficient data.

CSD1

CSD2

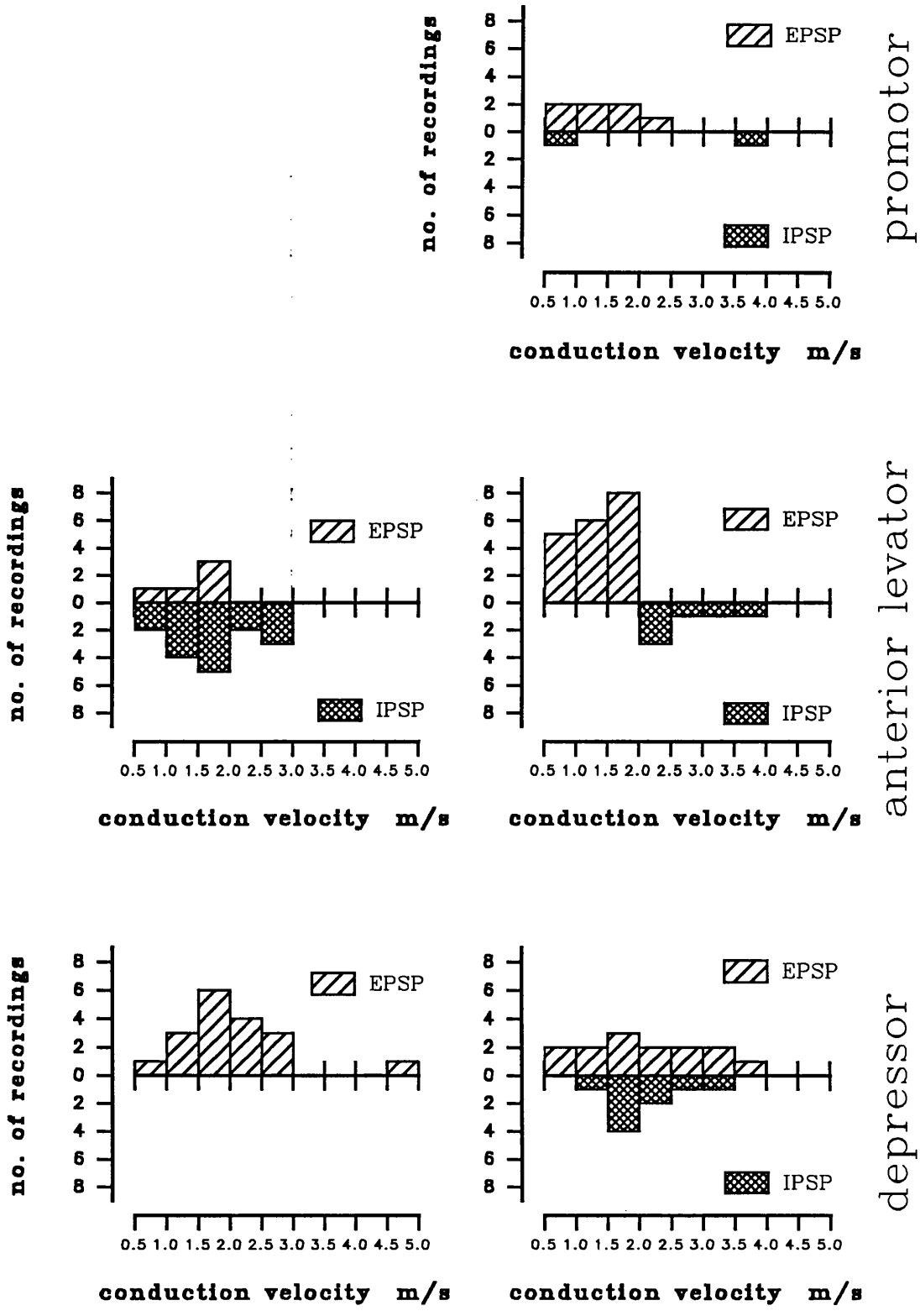

no. of recordings
EPSP
IPSP
0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
conduction velocity m/s
promotor
anterior levator
depressor

FIGURE 3.7:

Several sweeps triggered on the epsps in the depressor motorneurone are superimposed to show the correlation between epsps and one sensory unit in the CSD_1 trace. Distances, conduction times and conduction velocities were measured as following:

	distance		conduction time		conduction velocity
CSD_1 - adr	3.2mm	/	1.0ms	=	$3.2ms^{-1}$
CSD_1 - ganglion	8.0mm	/	2.8ms	=	$2.9ms^{-1}$
adr - ganglion	4.8mm	/	1.8ms	=	$2.7ms^{-1}$

Assuming that the conduction velocity within this particular CSD_1 fibre was the same all the way from the periphery to the ganglion ($3.2ms^{-1}$), then the slower conduction velocities measured between the CSD_1/adr electrodes and the intracellular electrode in the ganglion indicated the synaptic delay and/or a delay caused by interneurones in case of a polysynaptic connection. Here, the delay was 0.3ms. This is so short that the presence of an interpolated neurone can be excluded, thus proving that the connection was a monosynaptic one.

The possibility that the epsps originate from a different sensory unit which fires at exactly the same time as the one seen in the figure is thought to be very small, because all the sensory spikes of the unit under observation produce an epsp in the motorneurone at fixed latency. Nevertheless, the ultimate proof of a connection between sensory and motor unit would be to inject positive current pulses into the sensory unit in order to make it fire and then to observe epsps in the motorneurone as consequence.

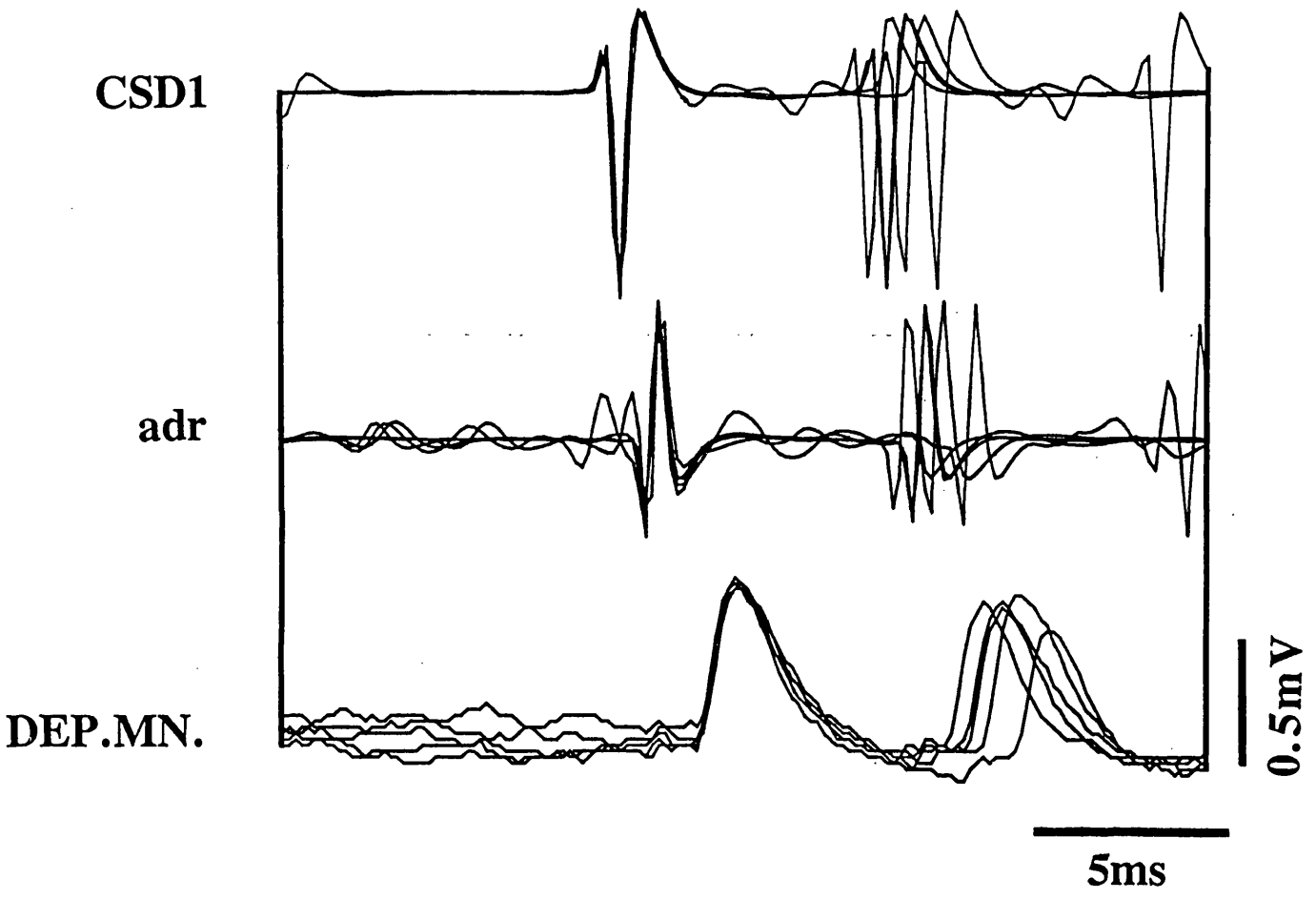

CSD1
adr
DEP.MN.
0.5mV
5ms

FIGURE 3.8:

Superimposing several sweeps which had been triggered on epsps in the depressor motorneurone (solid arrows) were realigned in the computer in order to show that the epsps could be correlated with single units in the sensory nerve (open arrows).

In B, the fixed latency between epsps in the motorneurone and CSD_1 extracellular spikes and the unchanged epsp size indicate a direct connection. By contrast, in A, the variation in latency (range: 1.3ms) and amplitude (range: 0.8mV) indicates the presence of an intercalated neurone or neurones.

Here, the possibility that the correlation is the consequence of a volley of co-incident afferent impulses has to be considered, because there is generally more activity in the CSD_1 sensory units (than for example seen in Fig.3.7). Also impulses of the two marked units (open arrows) might be missed, when they do not produce an epsp in the motorneurone. Thus the argument that no failures were seen in the connection from the sensory to the motorneurone cannot be applied.

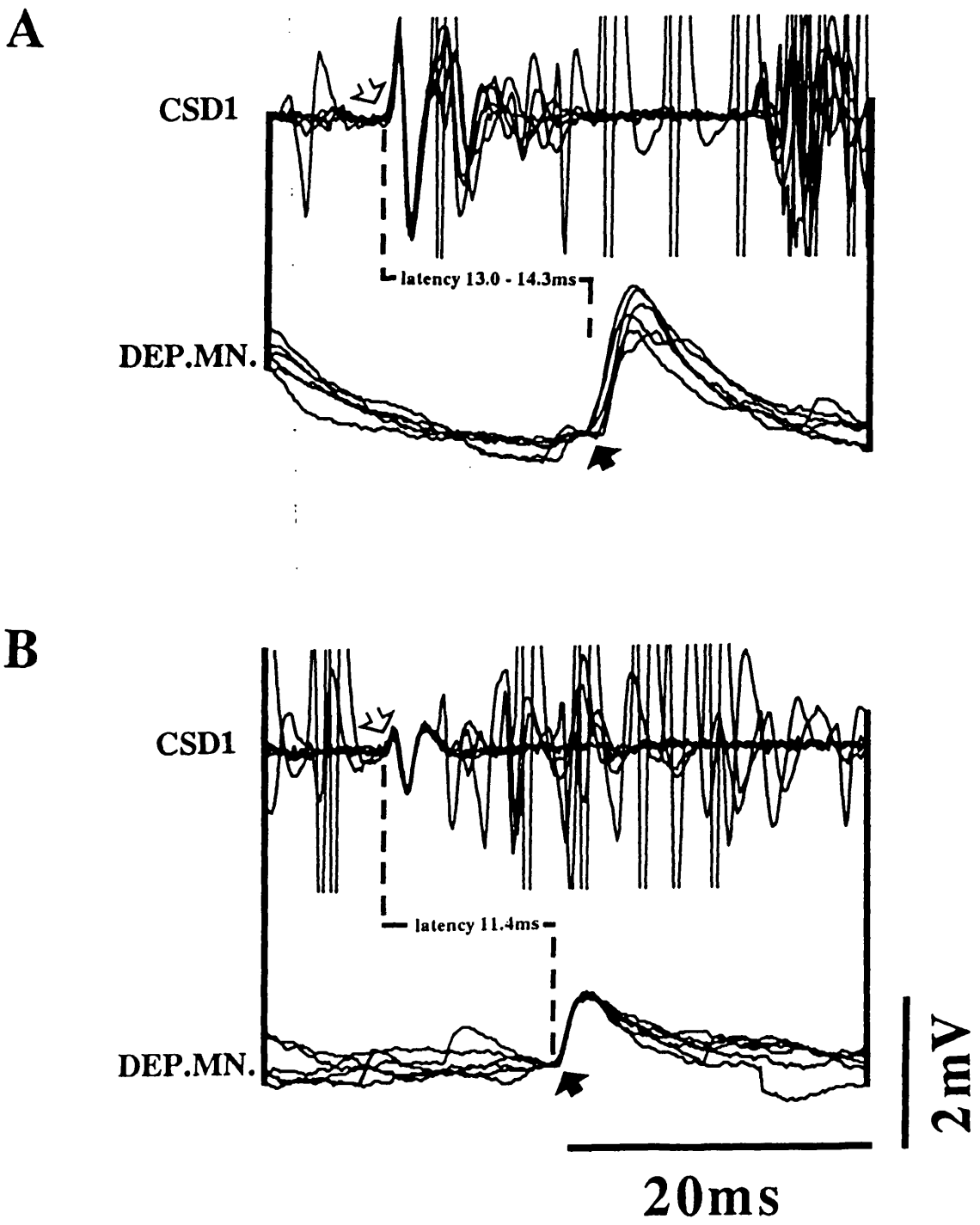

A
CSD1
latency 13.0 - 14.3ms
DEP.MN.
B
CSD1
latency 11.4ms
DEP.MN.
2mV
20ms

FIGURE 3.9:

Responses of an anterior levator and a depressor motorneurone to electrical stimulation of the CSD_1 sensory nerve root.

All sweeps were triggered on the first stimulation artifact of any train of stimuli. Stimulation artifacts are indicated by solid triangles.

A: 5 superimposed sweeps showing a short latency (6ms) ipsp in response to electrical stimulation of CSD_1. Stimulation parameters: 3V, 0.3ms.

B: The same ipsp as observed in A follows in a 1:1 fashion a train of 6 shocks administered at a rate of 90Hz. Notice the reduction of ipsp size to consecutive shocks in each train. Stimulation parameters for each shock as in A.

C: The epsps (arrows) in the depressor motorneurone follow in a 1:1 fashion a train of 8 shocks administered to the CSD_1 sensory nerve at a rate of 150Hz. Stimulation parameters for each shock: 2.4V, 0.3ms.

In A and B, constant, positive current of +2.9nA was injected into the motorneurone.

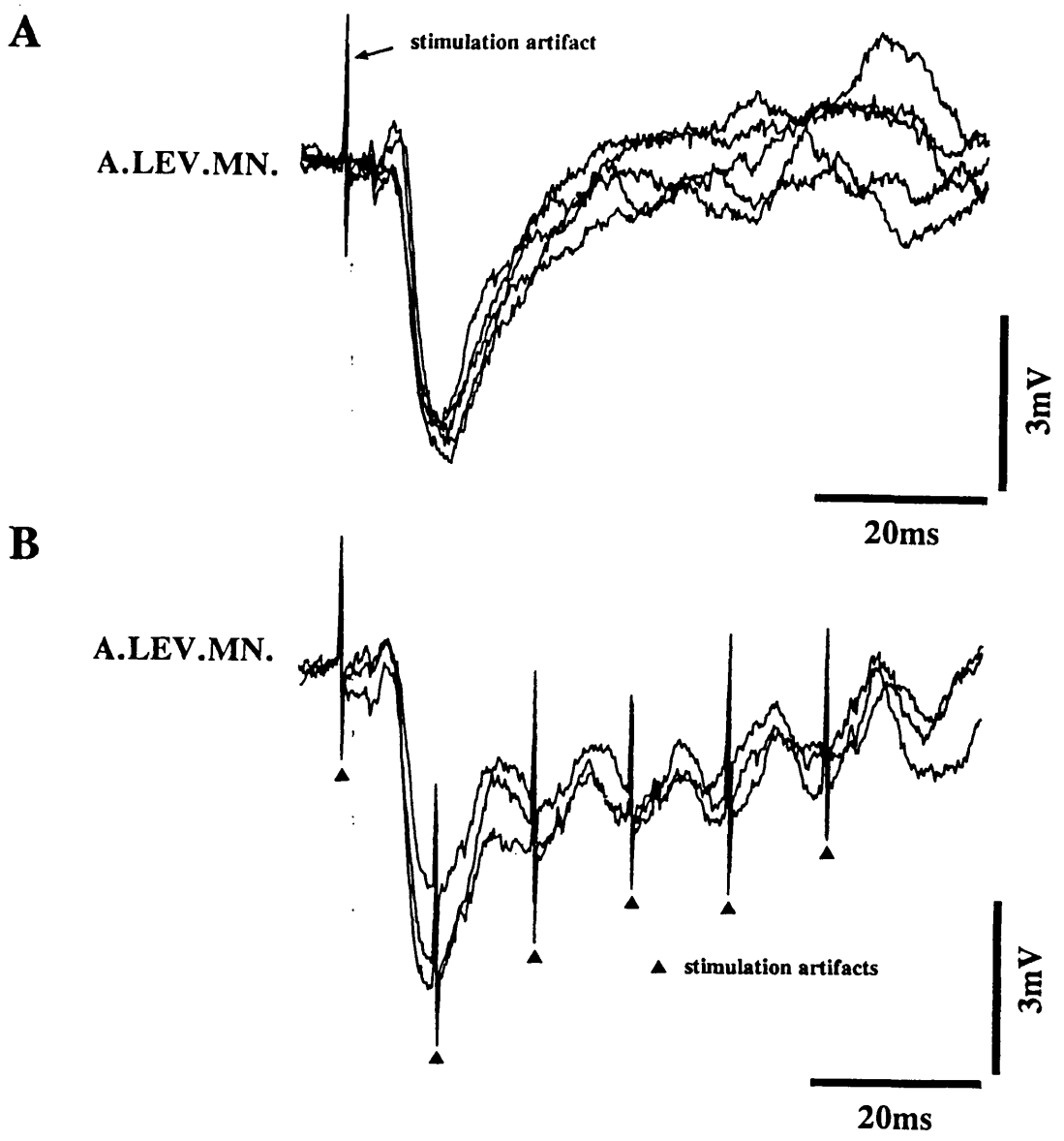

A
stimulation artifact
A.LEV.MN.
3mV
20ms
B
A.LEV.MN.
stimulation artifacts
3mV
20ms
C
▼stimulation artifacts
DEP.MN.
epsps
2mV
20ms

FIGURE 3.10:

Double shock electrical stimulation of CSD_2 at two different intensities showing different psp components in an anterior levator motorneurone and their connectivity to CSD_2 afferents. All sweeps were triggered on the electrical stimulation artifact.

A to E: stimulation parameters per shock: 3V, 0.3ms.

A: 4 superimposed sweeps showing the response to double shock stimulation. Stimuli followed each other with a delay of 5ms. Each stimulus evokes an epsp in the motorneurone.

B: 4 superimposed sweeps showing the response to a single shock.

C and D: Averages of A and B, respectively.

E: Subtraction of the single shock response from the double shock response demonstrates that the second shock produces basically the same response as the first one. Latency for the second shock is exactly the same as for the first. The amplitude is different due to the raised membrane potential of the postsynaptic neurone when the second epsp occurred. This ability to follow a pair of shocks separated by a short interval indicates a direct connection.

A' to E' show equivalent responses in the same anterior levator motor-neurone to stimulation of CSD_2 with the same stimulation parameters as above with the exception of stimulus intensity, which was raised to 5V. Analysis was carried out as in A-E.

The response in the anterior levator motorneurone consists now of three components (B'). Subtraction of single from twin shock responses (E') demonstrates that the second epsp (latency 6ms, arrow in B' and D') does not occur in the response to the second shock (arrow in E' indicating where it should have been) and can therefore be assumed to be of polysynaptic origin.

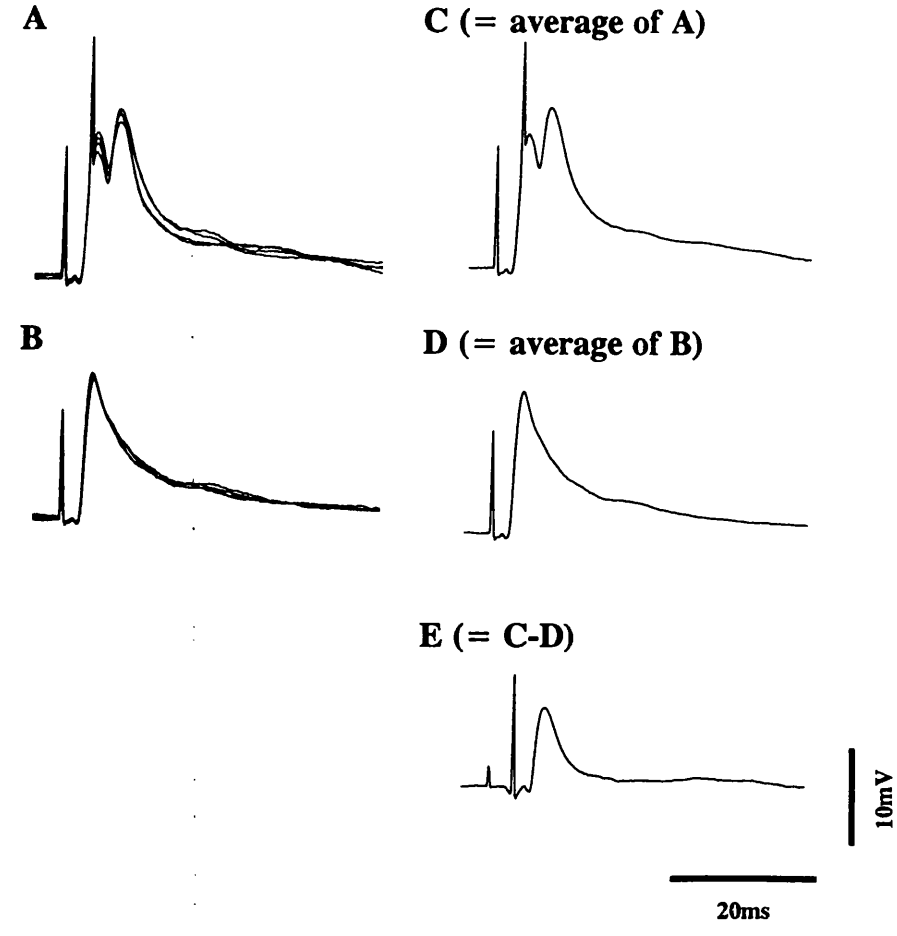

A
C (= average of A)
B
D (= average of B)
E (= C-D)
10mV
20ms
A'
C' (= average of A')
B'
D' (= average of B')
E' (= C'-D')
10mV
20ms

FIGURE 3.11:

Effect of high Ca^{++}/high Mg^{++} saline on reflex responses of a depressor motorneurone to electrical stimulation of CSD_1.

A: In normal saline, the reflex can be seen to have a late (see arrow) as well as an early component.

B: In the high Ca^{++}/high Mg^{++} saline, the late component disappears indicating that it is polysynaptic, while the early component is unaffected and may thus be presumed to be monosynaptic. The size of the epsp in B is increased due to the increased Ca^{++} concentration of the saline.

Stimulation parameters: 2.6V, 0.3ms

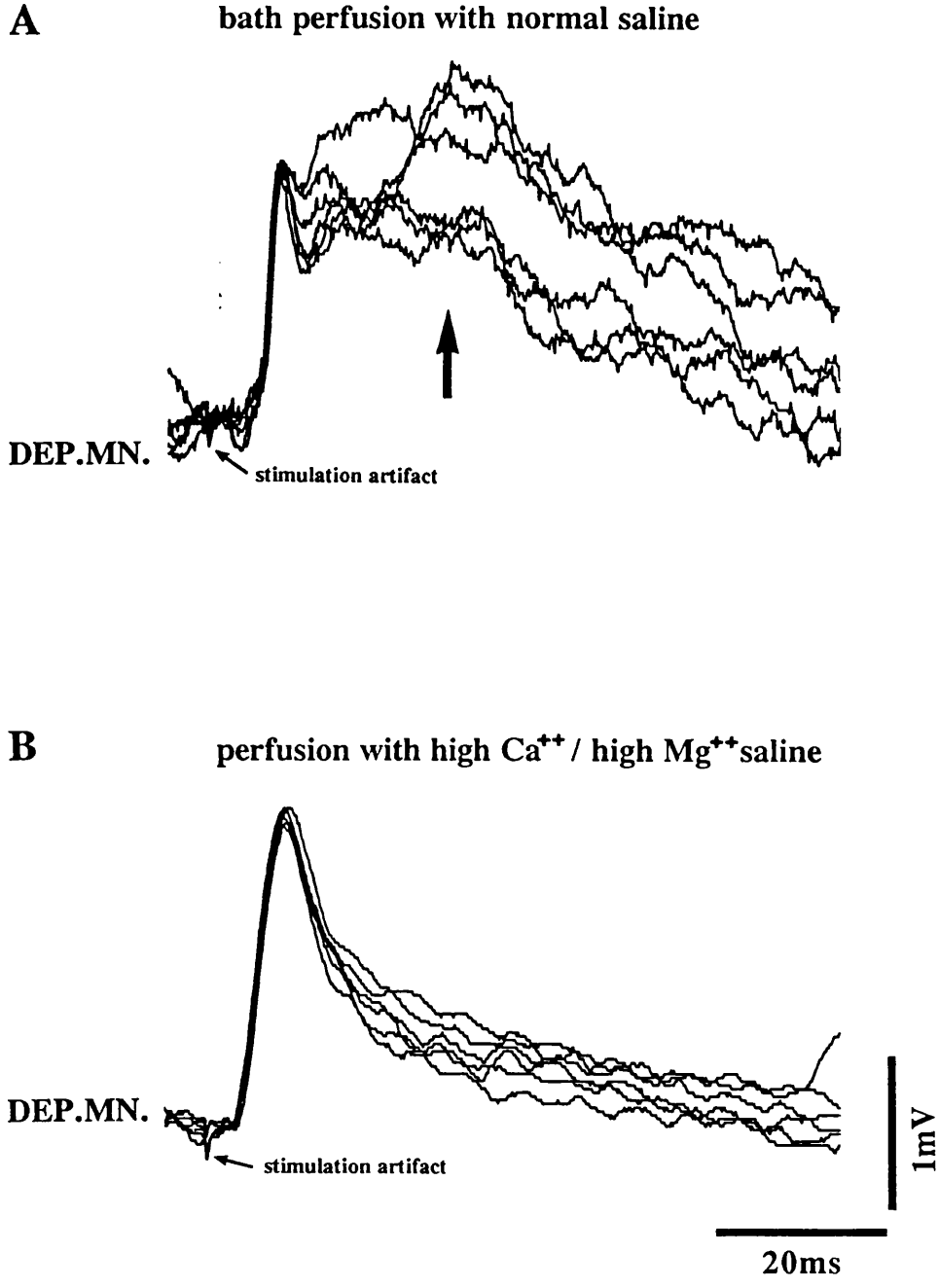

A
bath perfusion with normal saline
DEP.MN.
stimulation artifact
B
perfusion with high Ca++ / high Mg++saline
DEP.MN.
stimulation artifact
1mV
20ms

FIGURE 3.12:

Effect of calcium-free saline on the reflex response of an anterior levator motorneurone to CSD_2 electrical stimulation.

Each stimulus produces a compound epsp consisting of an early and a late component. Superfusing the preparation with Ca^{++} free saline will block any transmitter release, thus gradually decreasing the size of monosynaptic epsps with time. Polysynaptic epsps will abruptly disappear at the point in time when the interneurone fails to fire.

The CSD sensory fibres were stimulated every 50s.

The figure shows all sweeps beginning at a time 35min after superfusion with Ca^{++} free saline. Both the early and the late epsp show a gradual decline in size (indicated by arrow). After 55min of superfusion with Ca^{++} free saline, the late epsp suddenly disappears (diamond) indicating a polysynaptic connection.

Stimulation parameters: 5V, 0.3ms.

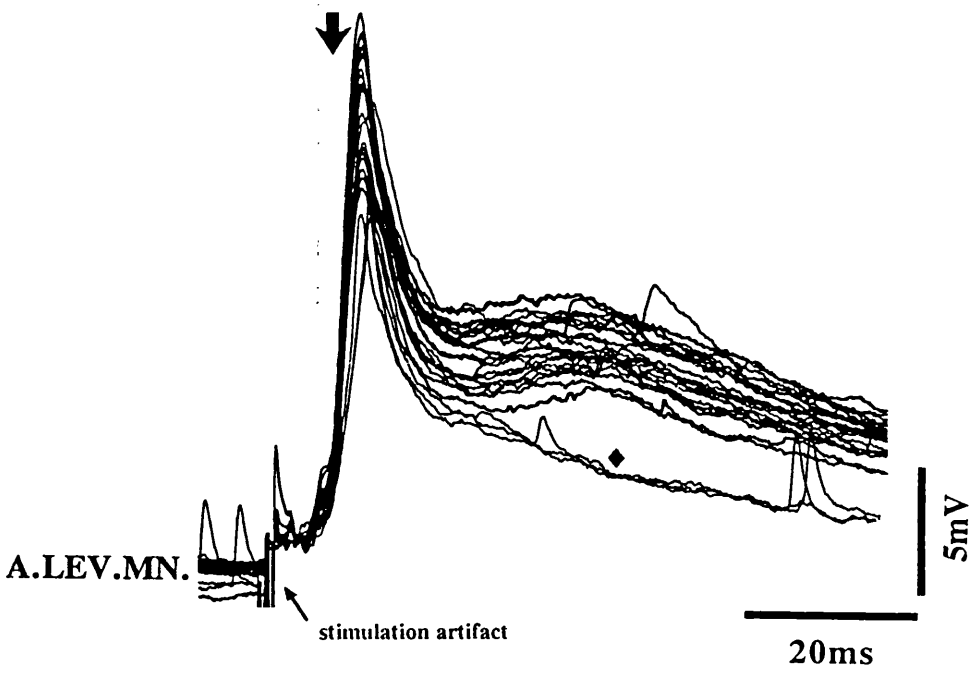

A.LEV.MN.
stimulation artifact
5mV
20ms

FIGURE 3.13:

Correlation between epsps recorded intracellularly in a depressor motorneurone and intracellular sensory spikes in a CSD_1 terminal. Sensory spikes were elicited by mechanical stimulation of CSD_1.

A: 14 superimposed sweeps triggered on the sensory impulses in the CSD terminal show a direct connection between the terminal and the motor-neurone. Each epsp in the motorneurone follows at a constant latency of 0.4ms, clearly indicating a monosynaptic reflex.

Notice the presence of other epsps in the motorneurone, probably the result of sensory input from other fibres.

B: Average of the 14 sweeps in A.

C: The 12 superimposed sweeps are triggered on the first of the three successive sensory spikes. The epsps in the motorneurone follow at a constant latency as seen in A. Absolutely no failures are observed, thus clearly showing a monosynaptic connection.

D: Average of the 12 sweeps in C. The averages of the second and third sensory spikes are smaller, because they do not all occur at exactly the same time.

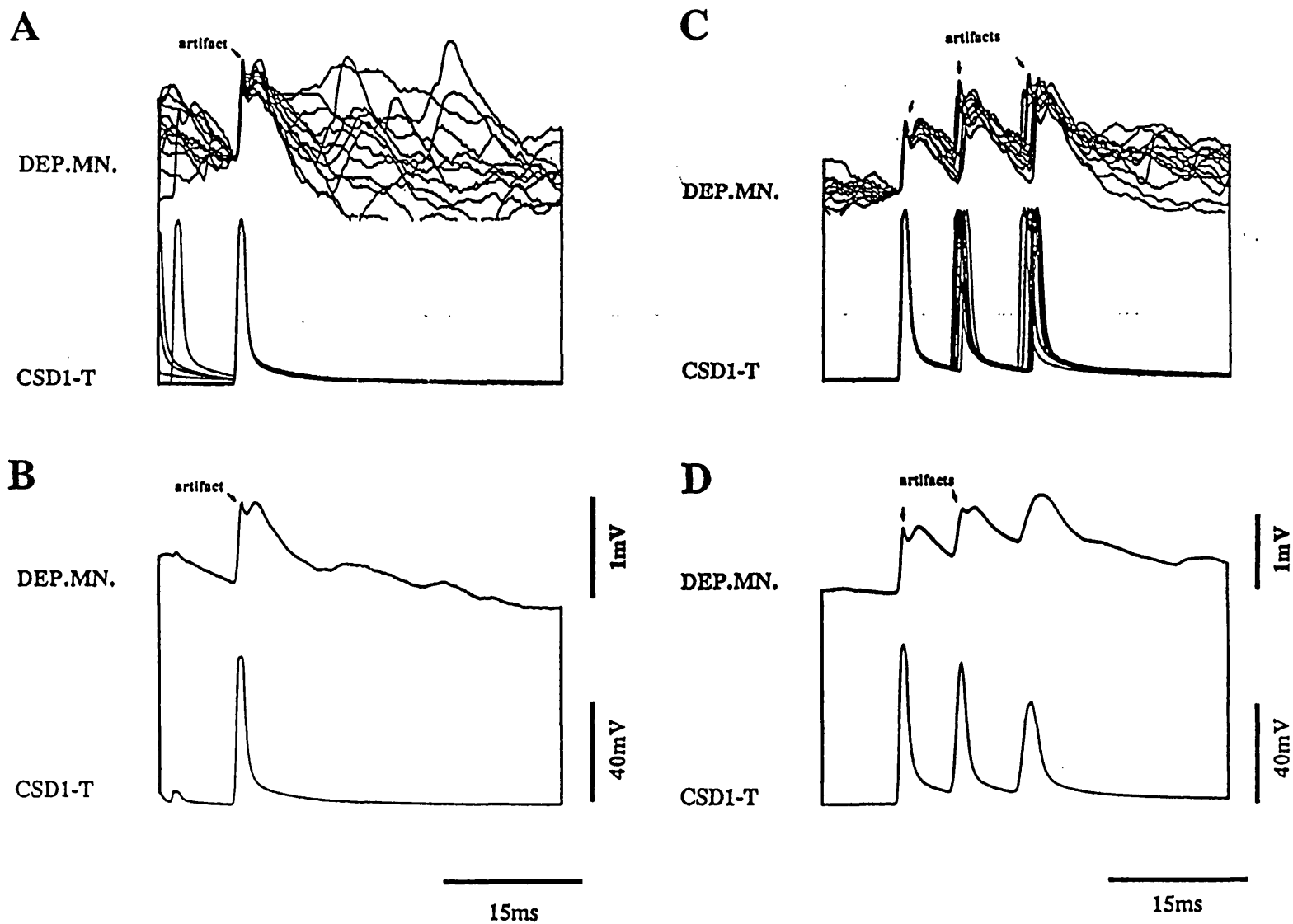

A
artifact
DEP.MN.
CSD1-T
B
artifact
DEP.MN.
CSD1-T
1mV
40mV
15ms
C
artifacts
DEP.MN.
CSD1-T
D
artifacts
DEP.MN.
CSD1-T
1mV
40mV
15ms

FIGURE 3.14:

Correlation of extracellular CSD_2 spikes (upper traces) with intracellularly recorded epsps in an anterior levator motorneurone (lower traces) indicating that each motorneurone receives inputs from several CSD afferents. All six figures are averages of several sweeps and thus do not allow a distinction to be made between monosynaptic and polysynaptic connections. The sweeps were triggered on the epsps in the motorneurone, superimposed, and sorted into different categories according to single recognizable sensory units in the extracellular recording. Each category was then averaged. Sensory spikes were elicited by mechanical stimulation of CSD_2.

Latencies range from 5.6 to 7.3ms.

A: Average of 9 sweeps.
B: Average of 8 sweeps.
C: Average of 11 sweeps.
D: Average of 8 sweeps.
E: Average of 6 sweeps.
F: Average of 9 sweeps.

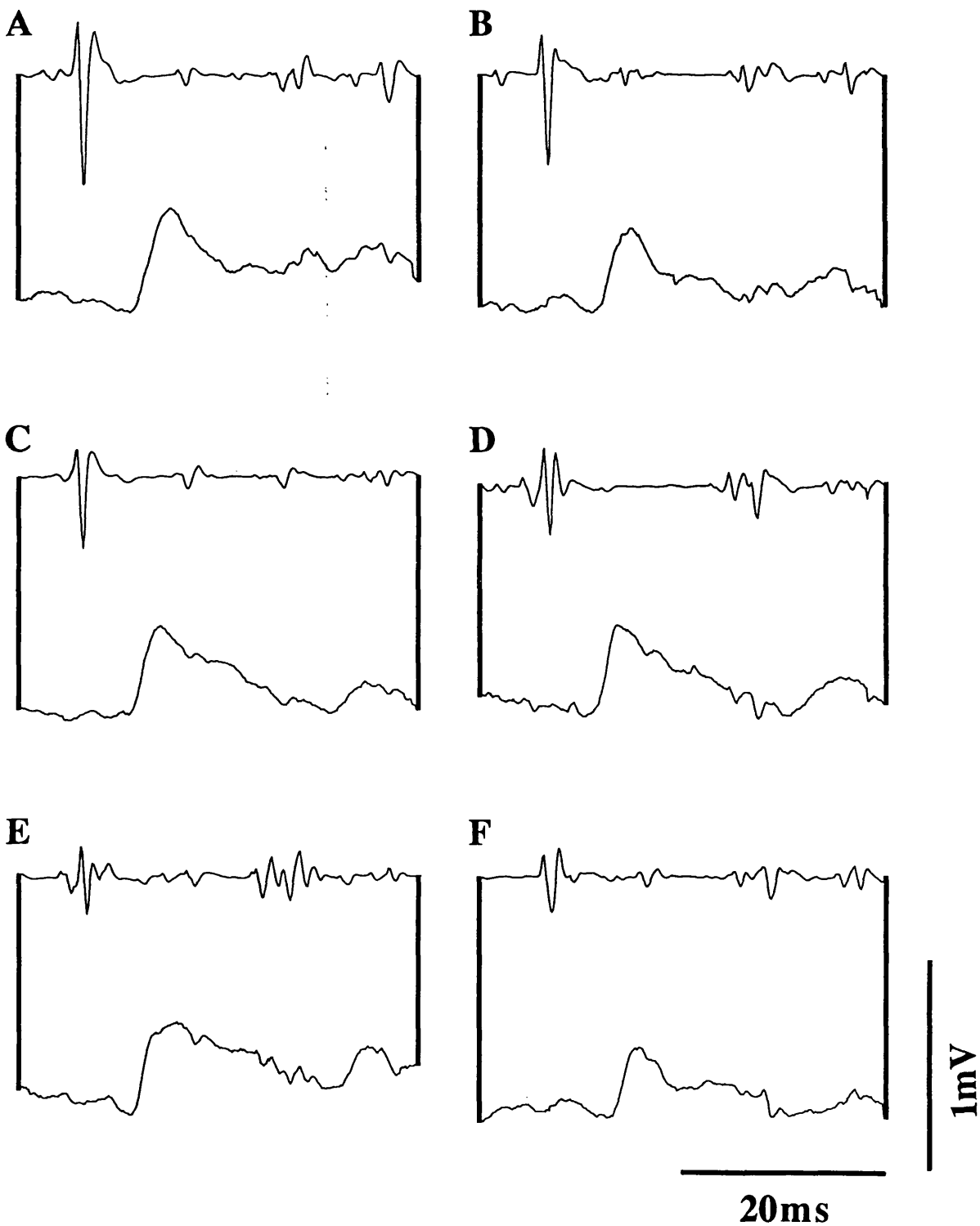

A
B
C
D
E
F
1mV
20ms

FIGURE 3.15:

Compound response in an anterior levator motorneurone to electrical stimulation of CSD_2 at different intensities.

A: Superimposed sweeps triggered on the stimulation artifact show a single epsp in response to electrical stimulation of 2.4V and 3V intensity, but a more complex response at 4V, 5V and 6V. At 6V, the summation of all the epsps resulting from CSD_2 sensory input raises the membrane potential above spiking threshold and the motorneurone fires.

Each electrical shock had a duration of 0.3ms.

B: Averages of the sweeps in A.

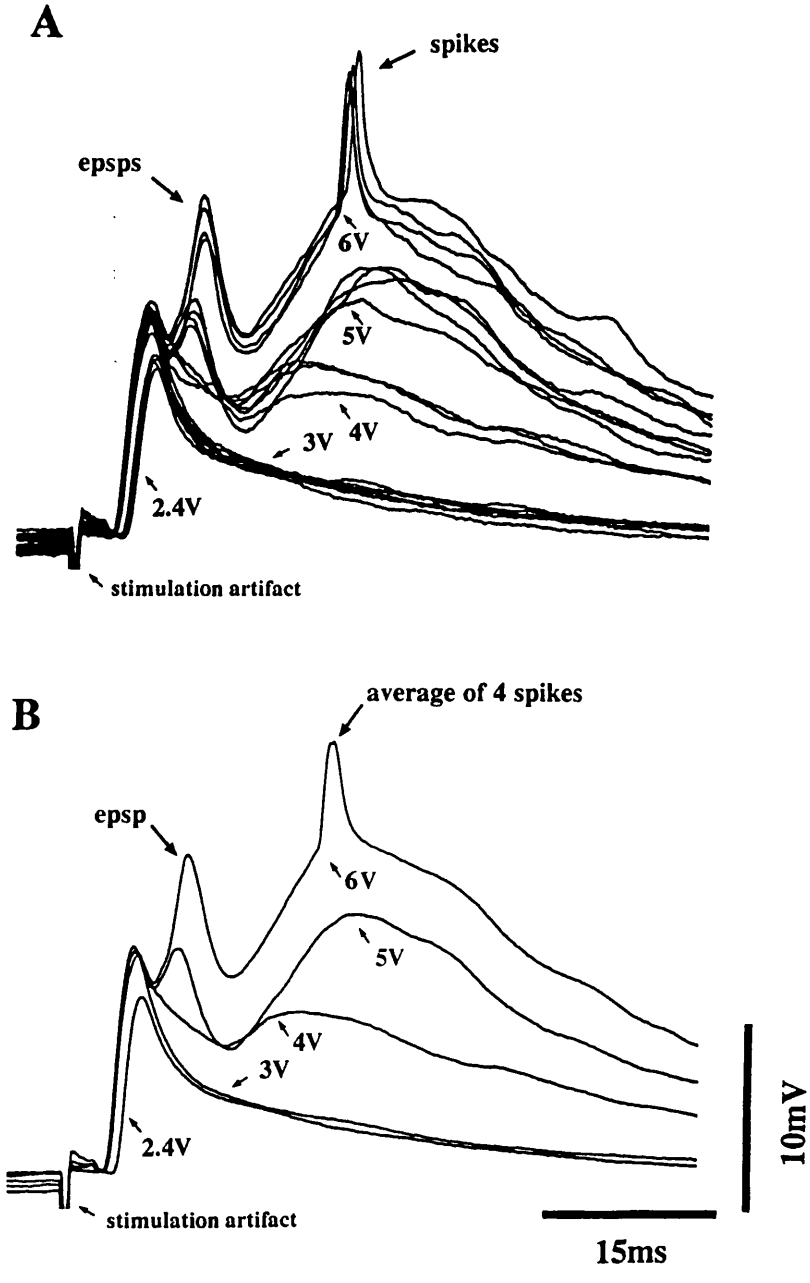
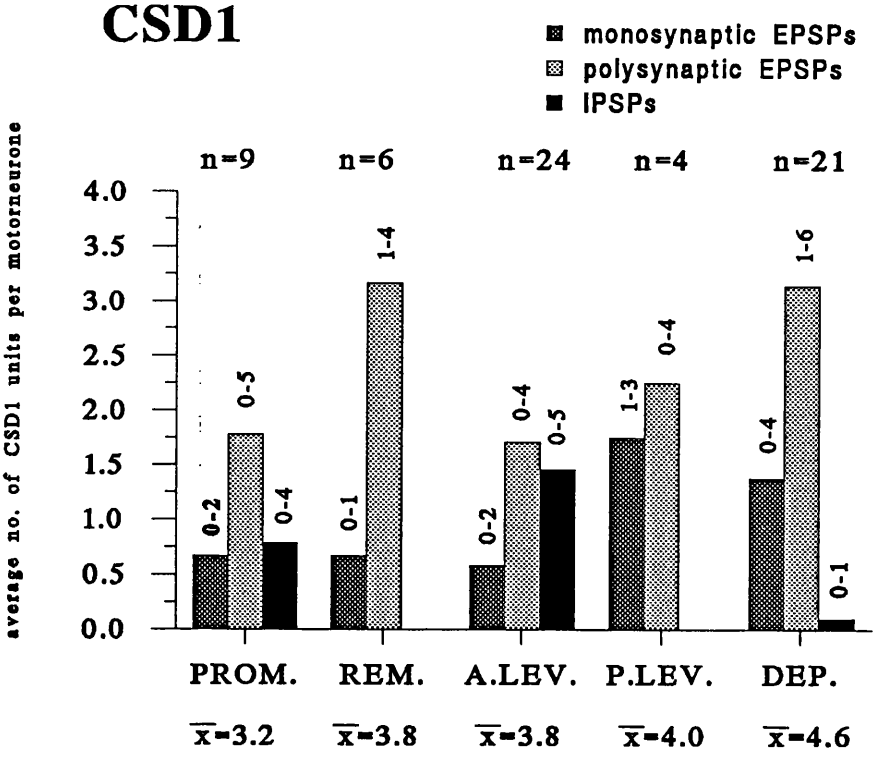

A
spikes
epsps
6V
5V
4V
3V
2.4V
stimulation artifact
B
average of 4 spikes
epsp
6V
5V
4V
3V
2.4V
stimulation artifact
10mV
15ms

FIGURE 3.16:

Histograms summarizing the average numbers of psps produced by CSD inputs in each motorneurone during electrical stimulation of the CSD nerve roots. A distinction is made between monosynaptic epsps, polysynaptic epsps and ipsps. The mean number ($\bar{x}$) of sensory fibres innervating each motorneurone was obtained by dividing the total number of psps in each motorneurone pool by the total number of motorneurones recorded for each motorneurone pool (n). The numbers written vertically above each bar represent the minimum and maximum number of psps recorded in each class for a single motorneurone.


CSD1
monosynaptic EPSPs
polysynaptic EPSPs
IPSPs
average no. of CSD1 units per motorneurone
n=9
n=6
n=24
n=4
n=21
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0-2
0-5
0-4
0-1
1-4
0-2
0-4
0-5
1-3
0-4
0-4
1-6
0-1
PROM.
REM.
A.LEV.
P.LEV.
DEP.
x̄=3.2
x̄=3.8
x̄=3.8
x̄=4.0
x̄=4.6

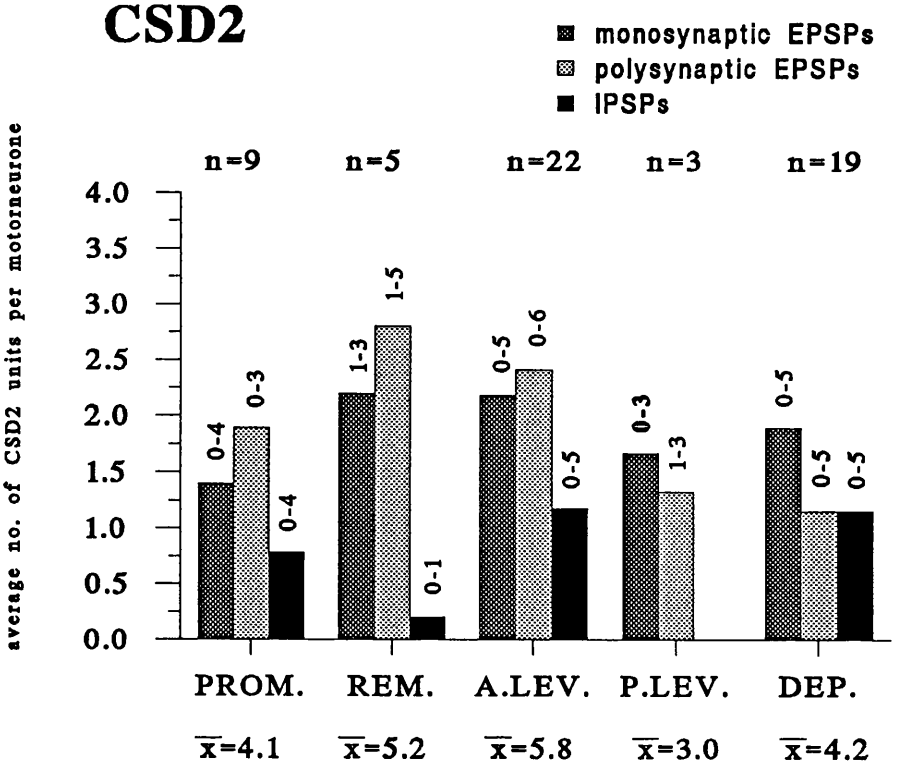

CSD2
monosynaptic EPSPs
polysynaptic EPSPs
IPSPs
average no. of CSD2 units per motorneurone
n=9
n=5
n=22
n=3
n=19
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0-4
0-3
0-4
1-3
1-5
0-1
0-5
0-6
0-5
0-3
1-3
0-5
0-5
0-5
PROM.
REM.
A.LEV.
P.LEV.
DEP.
x̄=4.1
x̄=5.2
x̄=5.8
x̄=3.0
x̄=4.2

FIGURE 3.17:

Extracellular recordings showing a fictive locomotor rhythm where anterior levator and depressor motorneurones fire reciprocally. Since the promotor units fire in phase with the anterior levator units, the pattern is that of forward walking. The cycle periods vary around a mean of about 8s. When CSD_2 is repetitively stimulated every 6 seconds (sinusoidal stimulus of period 6s during which the probe touched the soft cuticle of CSD_2 for a duration of 2.5s), the rhythm speeds up with cycle periods now reduced to about 6s. Thus the entrained relationship is 1:1.

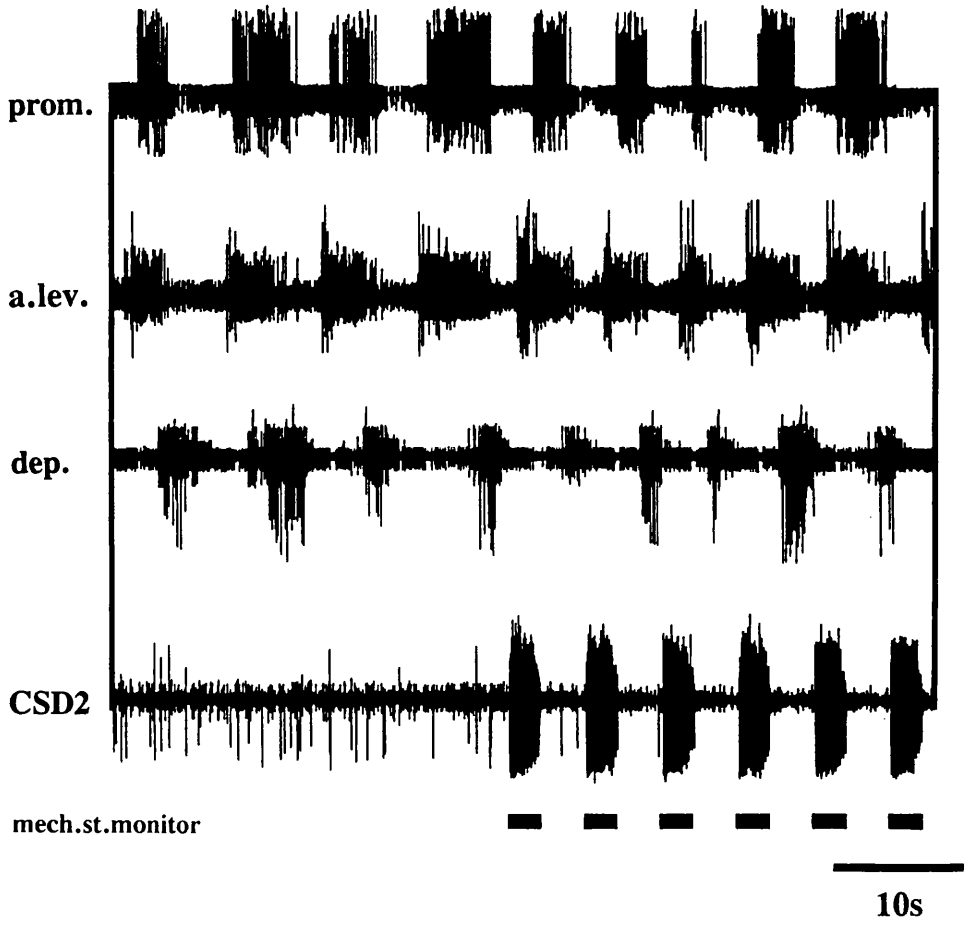

prom.
a.lev.
dep.
CSD2
mech.st.monitor
10s

FIGURE 3.18:

Extracellular recordings showing a fictive locomotor rhythm where levator and depressor motorneurones fire reciprocally. Since the remotor units fire in phase with the anterior levator units, the pattern is that of backward walking. The cycle periods vary around a mean of about 60s. When CSD_2 is repetitively stimulated every 11 seconds (sinusoidal stimulus of period 11s during which the probe touched the soft cuticle of CSD_2 for a duration of 4s), the rhythm speeds up with cycle periods now reduced to about 22s. Thus the entrained relationship is 2:1; i.e. two stimulus cycles occur for each cycle of the fictive locomotor rhythm.

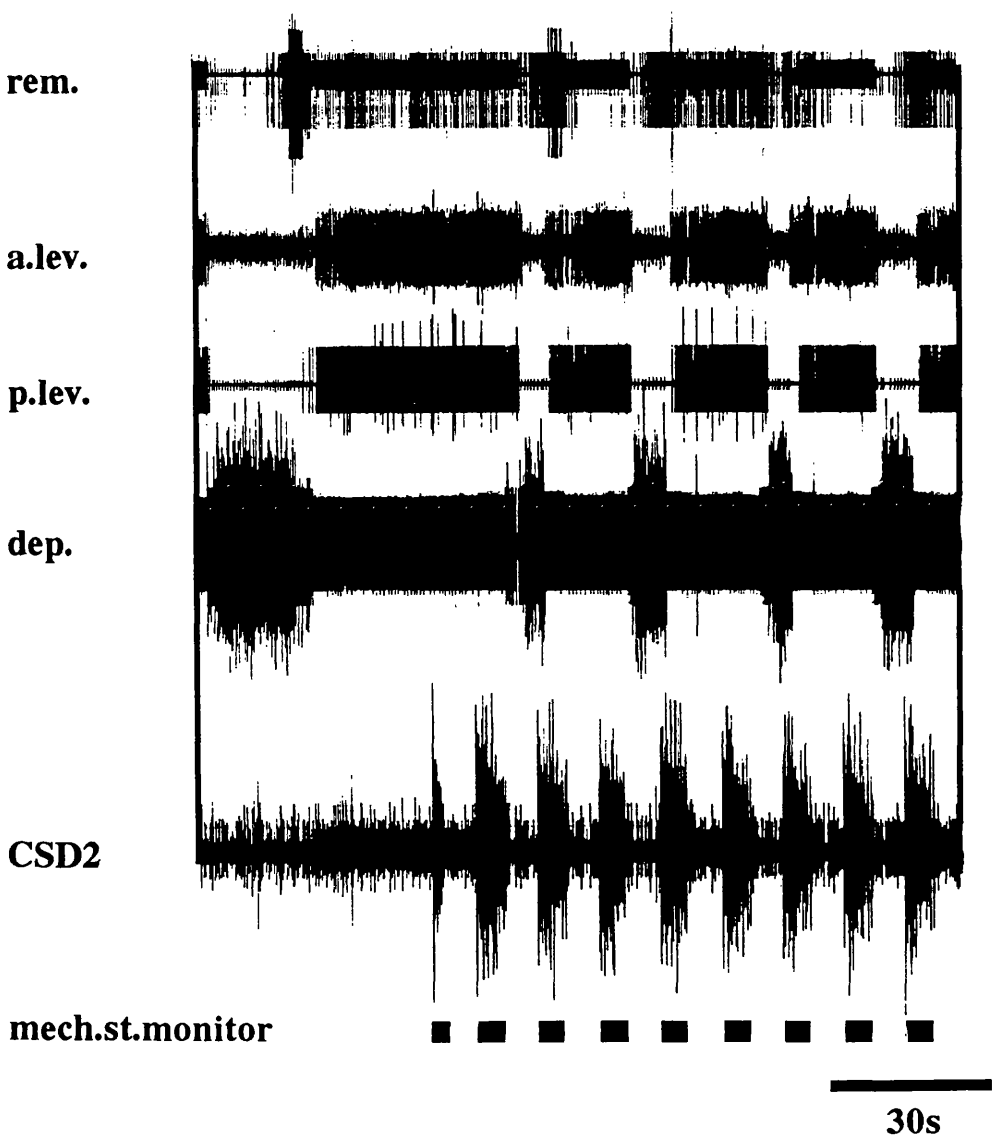

rem.
a.lev.
p.lev.
dep.
CSD2
mech.st.monitor
30s

FIGURE 3.19:

Extracellular recordings showing irregular fictive locomotor rhythms in anterior levator and depressor motorneurones.

A: In this record, anterior levator and depressor motor units burst reciprocally. A sudden change in cycle period occurs in both traces at about the same time. Note that the burst duration in the depressor bursts is not affected by this change, whereas anterior levator bursts become shorter when the cycle period is decreased.

B: Spontaneously occurring rhythm in which anterior levator and depressor motorneurones have different cycle periods. Thus these two pools of motorneurones can, at times, be part of separate central pattern generators. Nevertheless, they are still co-ordinated as levator and depressor bursts never occur at the same time. Thus the principle of antagonistically active motor-neurones bursting reciprocally is still preserved.

A

B

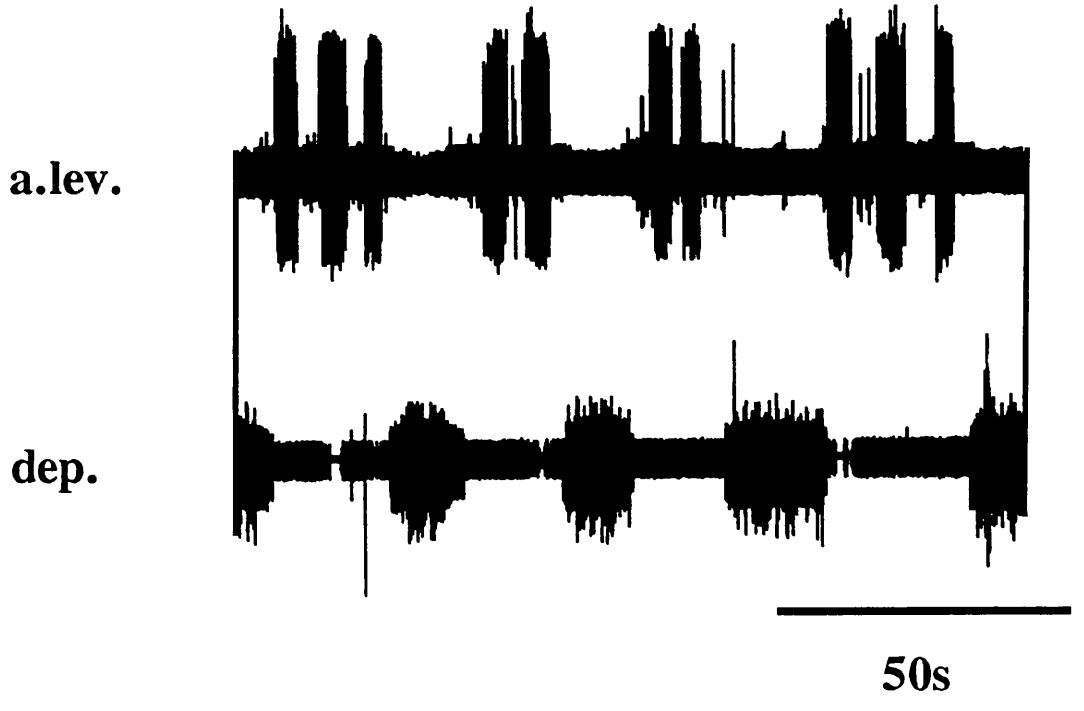


FIGURE 3.20:

In A and B, the electrophysiological recordings show fictive locomotion in a motor root (promotor in A, anterior levator in B) just before and during sinusoidal mechanical stimulation of CSD_2 where the probe touched the soft cuticle of CSD_2 for the period indicated by the black bars. White bars represent imaginary stimuli interpolated backwards from the first real stimulus. The distances between the beginning of each motor burst (marked by finely dotted lines) and the beginning of its nearest preceding stimulus cycle were measured (d) and divided by the stimulus period (p), thus yielding the phase of each motor burst within a stimulus cycle. Separate histograms were drawn for phase values measured for the 'before stimulation' and 'during stimulation' situations. Histograms were then analysed for any significant difference from a random distribution (Smirnov test).

A: Entrainment is demonstrated for promotor bursts during CSD_2 stimulation, since phases measured before stimulation were randomly distributed, whereas phases measured during stimulation show a clear phase preference. During stimulation bursts tended to begin halfway through each stimulus cycle, at a mean phase value (m) of 0.63; n represents the number of bursts analysed.

The means (m) of the histograms were obtained following the procedure described in Batschelet (1965) for the calculation of empirical mean vectors in circular distributions.

B: Equivalent demonstration of entrainment for anterior levator bursts. In addition to the preferred phase at 0, there is clearly a secondary smaller peak occurring at around a phase of 0.6. That this might arise by different anterior levator fibres being entrained to different phases is supported by the observation that smaller levator units sometimes (as in this recording, see asterisks) burst at different phases to larger ones.

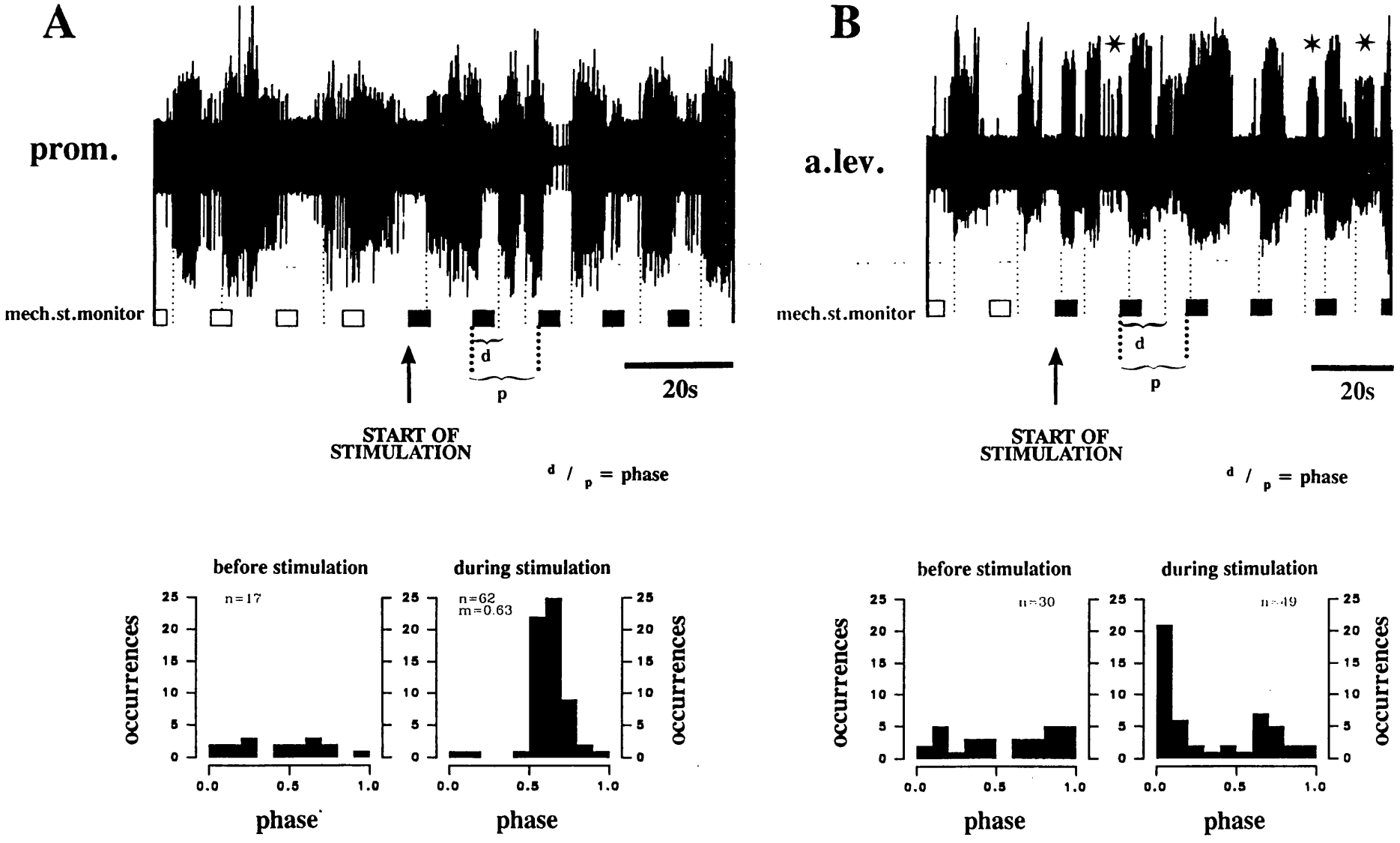

A
prom.
mech.st.monitor
d
p
20s
START OF STIMULATION
d / p = phase
before stimulation
during stimulation
n=17
n=62
m=0.63
occurrences
phase
B
a.lev.
mech.st.monitor
d
p
20s
START OF STIMULATION
d / p = phase
before stimulation
during stimulation
n=30
n=49
occurrences
phase

FIGURE 3.21:

A to E: Histograms showing all mean phase values calculated in experiments where entrainment was observed. A and B: anterior levator and depressor motorneurones in response to CSD_1 entrainment. C, D and E: promotor, anterior levator and depressor motorneurones in response to CSD_2 stimulation.

F: When the anterior levator motor bursts showed two preferred phases, the peak values were used for constructing the histogram, since the arithmetic mean value would have been meaningless.

n = number of 'fictive locomotor' bouts which showed entrainment to CSD stimulation. Obviously in F, n represents twice this number, for each analysis yielded two phase values.

m = circular mean of histogram.

r = concentration parameter. (N.B. the closer r is to 1, the tighter is the distribution).

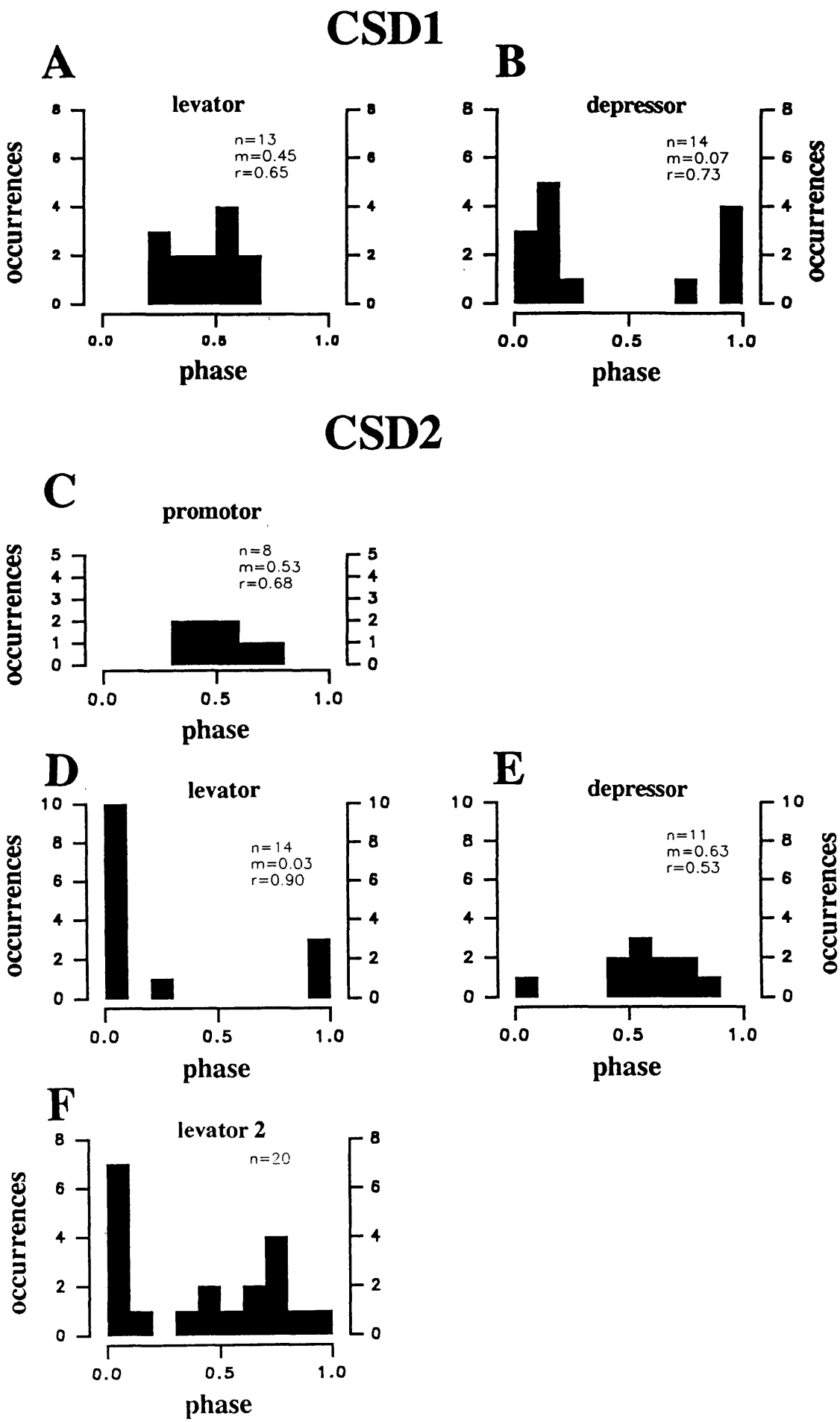

CSD1
A
levator
n=13
m=0.45
r=0.65
occurrences
phase
B
depressor
n=14
m=0.07
r=0.73
occurrences
phase
CSD2
C
promotor
n=8
m=0.53
r=0.68
occurrences
phase
D
levator
n=14
m=0.03
r=0.90
occurrences
phase
E
depressor
n=11
m=0.63
r=0.53
occurrences
phase
F
levator 2
n=20
occurrences
phase

TABLE 3.1:

Summary of all reflexes observed in the motor roots leading to the promotor, remotor, anterior levator, posterior levator and depressor muscles in response to CSD mechanical stimulation.

For each motor root, data is shown as a percentage of the total number of experiments (n) in which activity was observed in the motor root either prior to or in response to stimulation. Thus a silent root that was not excited by CSD stimulation was not included in the counts. Hence the total number of experiments is smaller for motor roots which were often silent (e.g. remotor and posterior levator) than for more active motor roots (e.g. anterior levator and promotor).

Notice that each row adds up to 100%.

Definitions: excitation 'on', increase in activity during the 'on' phase of stimulation; excitation 'off', increase in activity during the 'off' phase of stimulation; inhibition 'on', decrease in activity during the 'on' phase of stimulation; no response, no change in firing frequency of active motorneurones during either 'on' or 'off' phases of stimulation.

TABLE 3.2:

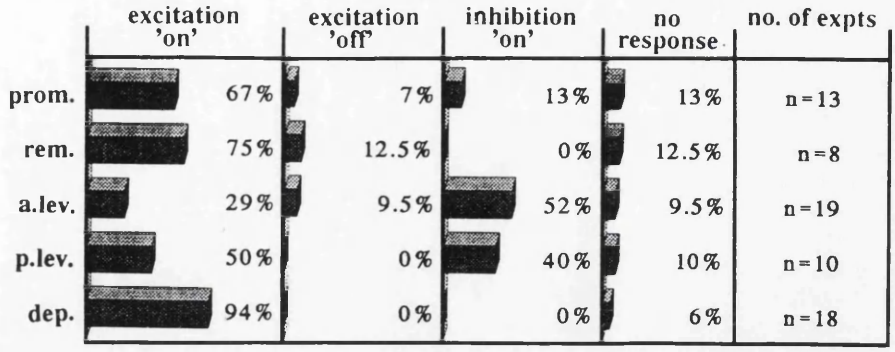
Summary of all responses recorded in proximal leg muscle motorneurones in response to CSD mechanical stimulation.

The total number of cells recorded for each motorneurone pool (no. of MN.s) comprises all cells where the type of response was clearly established. Again, as in Table 3.1, the rows add up to 100%.

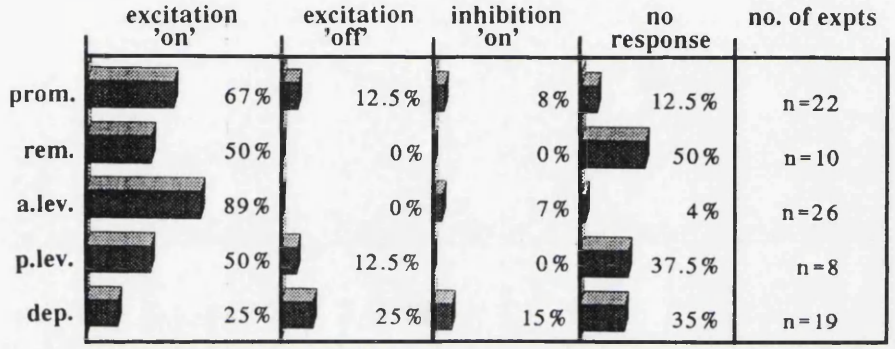
Note that the 25% of 'no response' promotor motorneurones in CSD_1 experiments resulted from 1 recording. In contrast, 1 anterior levator motorneurone not responding to CSD_1 stimulation constituted only 5% within its pool.

Definitions: EPSP 'on', compound epsp recorded during 'on' phase of stimulation; EPSP 'off', compound epsp recorded during 'off' phase of stimulation; IPSP 'on', compound ipsp recorded during 'on' phase of stimulation; no response, no psps recorded.

TABLE 3.1

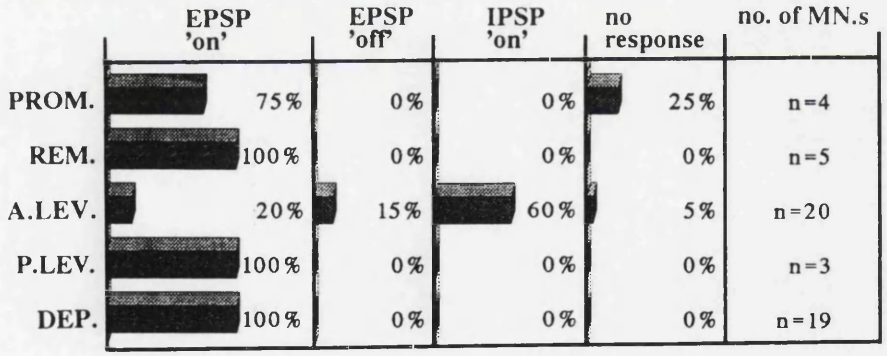


CSD1

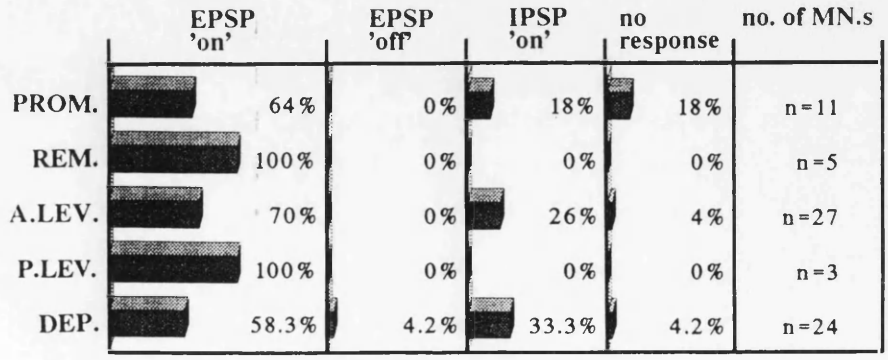


CSD2

TABLE 3.2



CSD1



CSD2

TABLE 3.3:

Numbers of motorneurone innervating each of the proximal muscle groups in crayfish legs (no. of motorneurones), counted from cobalt backfills by previous workers, are listed to allow a comparison to be made with the number of motorneurones recorded intracellularly in this study (no. of cells penetrated whilst stimulating).

TABLE 3.4:

Data on responses of anterior levator and depressor motorneurones to CSD mechanical stimulation taken from Table 3.2 are reclassified in terms of their functional response to CSD stimulation into 'levation reflex', 'depression reflex' and no response categories. For explanation see text.

Table 3.3

MUSCLE GROUPS	NO. OF MOTORNEURONES	no.of cells penetrated whilst stimulating	
		CSD1	CSD2
promotor	10 - 13 [1,3,4]	4	11
remotor	7 [2,4]	5	5
anterior levator	11 - 13 [1,2]	20	27
posterior levator	about 5	3	3
depressor	10 [2]	19	24

[1] Chrachri and Clarac (1989)
[2] ElManira *et al.* (1991a)
[3] ElManira *et al.* (1991b)
[4] Skorupski and Sillar (1988)

Table 3.4

CSD1

	A.LEV.MN.s	DEP.MN.s
"levation reflex"	20%	
"depression reflex"	75%	100%
no response	5%	

CSD2

	A.LEV.MN.s	DEP.MN.s
"levation reflex"	70%	38%
"depression reflex"	26%	58%
no response	4%	4%

Chapter 4

FORCE DEPENDENT REFLEX RESPONSE REVERSAL IN A CRAYFISH LIMB MUSCLE - DIFFERING ROLES FOR CUTICULAR STRESS DETECTOR 1 IN LOCOMOTION AND LIMB AUTOTOMY.

4.1. INTRODUCTION

It has long been accepted that reflexes play an important part in the control and co-ordination of motor output. Diverse reflexes are mediated by various sensory structures. In invertebrate systems, many sense organs have been investigated in order to study the sensory characteristics, the motor properties and the connectivity within the reflex arcs at extracellular and intracellular levels. Such detailed studies have shown that single components of one and the same sense organ often encode different information (e.g. Kalmring *et al.*, 1978; Matheson, 1990; Mill, 1976; Mill and Lowe, 1972; Wiersma and Boettiger, 1959). In crustaceans, for example, the T fibre of the TCMRO signals velocity, whereas the S fibre encodes amplitude of the receptor displacement (Sillar and Skorupski, 1986). It has also been shown that some sense organs, which had always been treated as a single unit, are indeed compositions of two functionally different sub-parts. The femoral chordotonal organ (fCO) functions as a transducer for the femur-tibia angle in orthopteran legs. In all legs of the stick insect and in the middle leg of the locust, it has been shown that two sub-units are morphologically distinguishable and that these two sub-units serve different functions (Field and Pflüger, 1989; Kittmann and Schmitz, 1992). The smaller scoloparium elicits the known resistance reflexes in extensor and flexor tibiae motorneurones. The larger scoloparium has no detectable function in the femur-tibia angle control loop, but has been suggested to be involved in reception of vibrations (Field and Pflüger, 1989). In this particular case, a morphological difference between the two scoloparia exists and could be an indication of the functional specialization of the two sub-sets.

In this chapter, a possible distinction between two sub-groups of CSD_1 sensory cells is made based on their different activation properties and the different reflex responses they elicit in a leg muscle. Increased stimulus strength causes a reversal in the reflex response in particular motorneurones. This is proposed to play a role in the control of autotomy.

In Decapod Crustacea, autotomy, the self-amputation of limbs, results from powerful contraction of the anterior levator muscle, a proximal muscle in the second segment of the walking legs (Frédéricq, 1892). By exerting force onto a plug within a preformed breakage plane, the rupture at the latter is initiated and the limb falls off. Since it was first described in the 18th Century (de Réaumur, 1731), this phenomenon has intrigued many a researcher (for reviews see: Wood and Wood, 1932; Bliss, 1960; McVean, 1982). Nevertheless, the exact mechanism underlying

autotomy is far from being completely understood. Questions concerning the exact stimulus and pathways eliciting the process (Needham, 1947; Easton, 1972), its purpose and function in behaviour (Paul, 1915; Robinson *et al.*, 1970) and the precise role of the muscles proximal to the breakage plane (McVean, 1974; Moffett, 1975) are not yet fully answered.

Since its discovery in 1970 (Wales *et al.*, 1970), the proximity of the cuticular stress detector one (CSD_1) to the breakage plane suggested an involvement in the control of autotomy. CSD_1 is a mechanoreceptor stimulated by pressure applied to the cuticle in the basi-ischiopodite region of the leg (see Chapter 2). Work on the reflex connections from CSD_1 to the proximal muscles demonstrated, *inter alia*, inhibitory connections to the anterior levator. As the anterior levator not only effects autotomy, but is also involved in normal, routine limb elevation during locomotion, those are thought to play a role in preventing autotomy from occurring when it is not required (Findlay, 1978). However, the reflex has to be overcome if autotomy is to take place. In the following, a force-dependent response reversal in the CSD_1-anterior levator reflex pathway will be described. Its involvement in the control of anterior levator function in both normal locomotion and autotomy will be discussed.

4.2. MATERIALS AND METHODS

A reduced preparation consisting of the isolated thoracic central nervous system and the 5th walking leg of the crayfish, *Procambarus clarkii*, was used. The preparation and the stimulation of the cuticular stress detector one are described in detail in Chapter 2.

In all experiments described here, care was taken to ensure that the only sensory information reaching the thoracic central nervous system came from CSD_1 receptor cells. This was achieved by cutting all other sensory roots to the ganglia.

4.3. RESULTS

4.3.1. High threshold fibres

When mechanically stimulating CSD_1 by sinusoidally indenting and releasing the soft window, thus shortening and lengthening the receptor strand which is attached to it, sensory cells will discharge if the stimulating parameters meet their specific characteristics (Clarac *et al.*, 1971). There are phasic, phaso-tonic and tonic fibres and a fourth class which was first described in Chapter 2. The characteristic feature of fibres of the fourth class is their high stimulation threshold. Indeed, activation of these fibres requires a combination of higher frequencies and larger amplitudes of the sinusoidal movement of the mechanical stimulator than are needed for activation of fibres in the other three classes (Fig.4.1). Since spike size in extracellular traces is positively correlated to the size of the axon diameter and high threshold fibres have large amplitude extracellular spikes, it is assumed that the axons of the high threshold fibres are of large diameter (Chapter 2). Thus, it is likely that penetration of these fibres is more easily achieved than for the other fibre types with smaller axon diameters. This could mean that the value of 50% (14 out of 28, see Chapter 2) for the number of high threshold fibres (as a percentage of all CSD_1 fibres) does not represent the true ratio of low and high threshold fibres in CSD_1, but is biased towards large diameter, high threshold fibres.

4.3.2. Response reversal

4.3.2.1. Extracellular recordings

When monitoring the nerve roots leading to the proximal, antagonistically operating muscle groups of anterior levator and depressor, mechanical stimulation of the CSD_1 receptor organ with frequencies of about 0.6Hz and amplitudes which represented forces of about 2mN lead in most preparations to inhibition of active anterior levator motorneurones and excitation of formerly silent depressor motorneurones (Fig.4.2A). In cases where a more powerful stimulation of CSD_1 was applied, by increasing frequency and/or amplitude of the sinusoidal stimulation, the response in the anterior levator reversed its sign and anterior levator motorneurones were now excited (7 out of 10 experiments). Such strong CSD_1 stimulation also led to activation of the very large, high threshold CSD_1 units (described above). A reversal in the anterior levator response never occurred before activation of these particular CSD_1 fibres was observed in the extracellular trace. The depressor generally showed no change in the sign of its reflex response. Sometimes, a weak decrease in firing frequency of depressor motorneurones was observed, but was attributed to inhibition

from the active anterior levator motorneurones, since antagonistic motorneurone pools are known to inhibit each other reciprocally (Chrachri and Clarac, 1989).

4.3.2.2. Intracellular recordings

Response reversal, the change from an inhibitory to an excitatory reflex response in the anterior levator motorneurones caused by a more forceful stimulation of CSD_1, was also observed in intracellular recordings. In 60% of cases, intracellular recordings of anterior levator motorneurones in the 5th thoracic ganglion showed that normal mechanical stimulation of CSD_1 results in hyperpolarization of the membrane potential by the summed effect of inhibitory inputs stemming from CSD_1 receptor cells (Table 3.2; Fig.4.3C). In seven recordings of anterior levator motorneurones, the reflex response was not only examined during the rather weak CSD_1 mechanical stimulation which was generally used, but also with a more forceful stimulation. The latter stimulation led to depolarization of the membrane potential in five of the anterior levator motorneurones and as such reversed the sign of the reflex response. This depolarization could lead to firing in the motorneurone (Fig.4.3B). In the two other recordings, the anterior levator motorneurones responded with a compound epsp to weak stimulation. As such these motorneurones fell into the group of 20% of anterior levators responding with excitation to such stimulation. Strong stimulation resulted in a compound epsp in one of the motorneurones, whereas the membrane potential of the other motorneurone was hyperpolarized by sensory inputs from CSD_1. This is a response reversal but in the opposite direction to the one described above.

4.3.3. Low threshold fibres

Intracellular recordings of CSD_1 afferent terminals which belonged to one of the three classes of low threshold fibres showed a remarkable decrement in spike size when the receptor organ was strongly stimulated and high threshold fibres were active (Fig.4.4). As will be discussed later, possible explanations include presynaptic inhibition and non-linear properties of the terminal membrane. This phenomenon was observed in all five cases where the CSD_1 receptor organ was subjected to strong stimulation while recordings were made from the terminal of a low threshold afferent.

4.4. DISCUSSION

4.4.1. Response reversal

Described here is the reversal of a reflex response in the locomotor system of crayfish dependent upon the force with which the receptor organ transmitting the reflex responses is stimulated. It is shown that the response reversal in the anterior levator motorneurones only occurs when a special set of CSD_1 sensory units is activated. Activation of this special set of sensory fibres depends upon the strength of the stimulation. Thus, the high threshold fibres constitute a functionally separate group in the CSD_1 receptor organ in that their threshold is about an order of magnitude higher than that of the low threshold fibres. Such functional specialization of different cells in one sense organ is known for chordotonal organs in crustaceans and insects (see Introduction). Nevertheless, for example, the position and movement fibres of chordotonal organs are mostly complementary in their effect on the central motor output and not opposing as in the case of the low and high threshold fibres of CSD_1. In contrast, the T and S fibres of the TCMRO are similar to CSD_1 in that they have opposing reflex effects on promotor and remotor motorneurones (Bush and Cannone, 1985). It has been shown that in inactive crayfish preparations the S fibre mediated reflex is subthreshold and has as such no influence on the resulting reflex response. In active preparations, however, both T and S fibre elicit reflex responses in the promotor and remotor motorneurones. Stimulation of the TCMRO leads to excitation of the promotor and remotor motorneurones during their active phases, but to inhibition during their reciprocal phases. It has been proposed that this reflex modulation is achieved by gating of the sensory information at the appropriate times within the rhythm cycle (Skorupski and Sillar, 1986). Such phase-dependent reflex effects have also been reported in the locomotor systems of tadpoles (Sillar and Roberts, 1992), cats (Forssberg *et al.*, 1975) and humans (Duysens *et al.*, 1992) and are termed reflex reversals. The term, reflex reversal, is thought not to be appropriate to describe the present phenomenon, because two different kinds of stimuli (distinguishable by the different intensities of force needed in order to activate the sensory units) are used. Therefore it is not the state of the preparation or the phase in the rhythm cycle which is the deciding factor for the sign of the reflex response (Bässler, 1976; 1986; Sillar and Roberts, 1992; Skorupski and Sillar, 1986), but the force acting on the soft cuticle of CSD_1. In another crustacean preparation, the lobster stomatogastric system, such an force-dependent response modulation is also known. The reflex pathway from a single mechanoreceptor neurone, the anterior gastric

receptor (AGR), onto motorneurones (GM) which innervate the power stroke muscle of the medial gastric tooth involves only one intercalated neurone, the interneurone CG. The receptor cell encodes information about passive stretch of the muscle and about its active contractions. At low stimulation strengths, activation of AGR leads to excitation of GM motorneurones, while at high stimulation strengths, it leads to inhibition of the motorneurones (Combes et al., 1993). This reflex reversal is achieved by the non-linear membrane properties of the intercalated neurone CG. It has a maximum as well as a minimum excitation threshold, so that only a particular bandwidth of firing frequencies of the receptor cell elicit excitation in the motorneurones. Very weak and very strong stimuli will activate the receptor cell, but will not lead to excitation of the motorneurone, rather to their inhibition (Simmers and Moulins, 1991).

4.4.2. Mechanisms

Since inputs from low and high threshold fibres have opposing effects on anterior levator motorneurones, it is of interest to know what happens to the low threshold CSD_1 fibre-anterior levator motorneurone reflex loops when the reflex response reverses during high intensity stimulation. Three mechanisms which could influence the reflex loops either pre- or postsynaptically were observed in some experiments and are listed below.

In one instance [Fig.4.1 terminal CSD1-T (a)], a CSD_1 unit was recorded that was activated during weak stimulation, but fell silent when CSD_1 was strongly stimulated. In such cases, low threshold CSD_1 units mediating inhibitory reflexes would not interfere with the excitatory inputs from high threshold CSD_1 units.

Secondly, low threshold spikes arriving in the thoracic ganglion are shunted or at least diminished in size due to either presynaptic inhibition or to non-linear membrane properties of the sensory terminals (see Fig.4.4). Its effect would be the complete absence of the reflex response or a decrease in the reflex response in postsynaptic neurones. Primary afferent depolarizations (PADs), known to mediate presynaptic inhibition in crayfish afferent terminals (Cattaert *et al.*, 1992b), are indeed observable in recordings from low threshold CSD_1 terminals subject to strong stimulation (small potentials in CSD1-T trace in Fig.4.4). Such PADs could arise from high threshold fibres via an intercalated inhibitory neurone.

Thirdly, the inhibitory response in anterior levator motorneurones is overridden, because the ipsps resulting from weak stimulation are swamped by the epsps resulting from strong stimulation (see Fig.4.3).

A further possible mechanism is that modulation of the reflex response

occurs in interneurones intercalated between sensory afferents and motorneurones. This is reasonable since all inhibitory reflex responses are transmitted via a polysynaptic pathway.

In summary, stimulation of CSD_1 evokes two opposing reflex responses in anterior levator motorneurones depending on intensity of the stimulation. Inhibition occurs with low intensity stimuli, excitation with high intensity stimuli.

4.4.3. Autotomy

The CSD_1 receptor organ situated in the proximal part of the walking legs is stimulated by both internal and external forces, indeed anything that stresses the exoskeleton of the leg and hence deforms the pliable cuticle of the CSD_1 window where the receptor strand is attached (Clarac *et al.*, 1971). As discussed in the previous chapter, activation of CSD_1 receptor cells leads mainly to assistance reflexes in anterior levator and depressor motorneurones and aids in the co-ordination of their motor output in locomotion. It is thus astonishing to find a response reversal in anterior levator motorneurones which does not seem to make much sense in terms of the locomotory requirements of the walking legs. The response reversal, elicited by an extremely strong CSD_1 stimulation which probably would not occur during normal walking, brings to mind the unsolved problem of how inhibition of anterior levator motorneurones in decapod crustaceans is overcome, so that autotomy can occur. Moffett (1975) suggested that the reflex loops from CSD_1 to anterior and posterior levator were centrally inhibited, while Findlay (1978) advanced a second possibility where CSD_1-anterior levator motorneurone reflex loops remained intact, but the anterior levator motorneurones were also excited by an alternative pathway. This study suggests that there is indeed an alternative pathway in that a separate group of CSD_1 fibres produces excitation in the anterior levator muscle, whereas inputs from all other fibres lead to inhibition in the muscle. Such effects would prevent autotomy from occurring when CSD_1 stimulation was weak, but aid in its production when CSD_1 stimulation was very strong.

However, it is generally agreed that Reptantian walking legs (i.e. legs of crayfish and lobsters) do not show true autotomy, but rather autospasy. Autospasy (Piéron, 1907; Wood and Wood, 1932) is defined by Bliss (1960) as "the separation of an appendage at a preformed breakage plane when the appendage is pulled by an outside agent against resistance provided by the animal's weight or its efforts to escape". This definition negates any involvement of muscular activity to aid in the process, but Paul (1915) showed levator muscle involvement in the walking legs of Reptantia by monitoring the levator muscle contraction during limb severance. Wood

and Wood (1932) were able to "autotomize" the legs of lobsters mechanically by pulling at the anterior levator tendon. They stated that more force was needed than in Brachyuran walking legs which show true autotomy, but the fact that it was possible remained. They also reported an incident involving a young crayfish of the genus *Cambarus* which instantly threw its claws and legs off when injury was inflicted on them. The only difference between this animal and all other crayfish being experimented on is the fact that it was freshly caught from a stream and the experiment was performed on the spot. Thus the large discrepancy of reports on occurrences of true autotomy in this group might be heavily influenced by the condition of the experimental animals (Wood and Wood, 1932).

Wood and Wood (1932) were the last workers who studied autotomy, not only in crabs, but also extensively in other decapod crustaceans. Their conclusion concerning lobsters and crayfish is summarized in the following statement: "The levator muscle appears to be in the line of development of a true autotomizer. Autotomy seldom occurs in the walking legs of lobsters, but this muscle apparently assists in the act. ..... The crayfish is more advanced than its close relative, the lobster. Its degree of specialization as to autotomy more nearly resembles a distantly related, more specialized group, the Palinuridae", which show true autotomy in all their walking legs.

Thus, it is believed that the anterior levator is involved in the process of autospasy in crayfish walking legs. This then requires a mechanism by which the forces acting on the leg can be monitored and the final motor output adjusted in order to prevent autospasy during normal locomotion, but allow its occurrence when it is required. Such a mechanism is found in the different effects which different kinds of CSD_1 stimulation have on the anterior levator motorneurones depending on the strength of the stimulus. Inhibition of the anterior levator motorneurones by weak to moderate stimulation of the CSD_1, as might occur when the leg involuntarily comes up against a resistance during the swing phase, will insure that any accidental loss of a limb is prevented. Also, these reflex loops will assist in the production of a smooth locomotor output as described in Chapter 3. Extreme forces acting on the CSD_1, for example when an outside agent pulls on the leg, lead to activation of the large CSD_1 fibres and excitation of the anterior levator motorneurones, thus aiding in the process of limb separation.

Crayfish show true autotomy in their first pair of pereiopods, the chelae, and it is not clear whether the absence of true autotomy in the rest of the walking legs constitutes an evolutionary precursor stage or whether they originally possessed the ability and subsequently lost it.

It would be interesting to know whether the above described mechanism of a response reversal being possibly used both to overcome CSD_1's role in the prevention of autospasy and to aid autospasy itself also holds true for autotomy in Brachyuran Decapods.

FIGURE 4.1:

Intracellular recordings of two CSD_1 afferent terminals in the 5th thoracic ganglion [CSD1-T(a) and CSD1-T(b)] and extracellular recording of the pooled activity in the CSD_1 receptor nerve (CSD1) during mechanical stimulation of CSD_1. Mechanical stimulation parameters were a frequency of 30Hz and amplitudes which constituted forces of 0.6mN to 2.5mN peak-to-peak. Dots represent omitted parts of the originally continuous record.

Modification of the amplitude reveals the activating characteristics of the two CSD_1 units. CSD1-T (a) responds to low frequency (not shown here) and high frequency, small amplitude stimulation of CSD_1 (A). When the amplitude of the stimulation is increased, thus exerting more force on the pliable cuticle of CSD_1, CSD1-T (b) starts firing, whereas CSD1-T(a) falls silent (B). Decreasing the amplitude again leads to activation of CSD1-T(a), while CSD1-T(b) falls silent (C). Thus, CSD1-T(b) is a high threshold fibre, whereas CSD1-T(a) is a low threshold fibre.

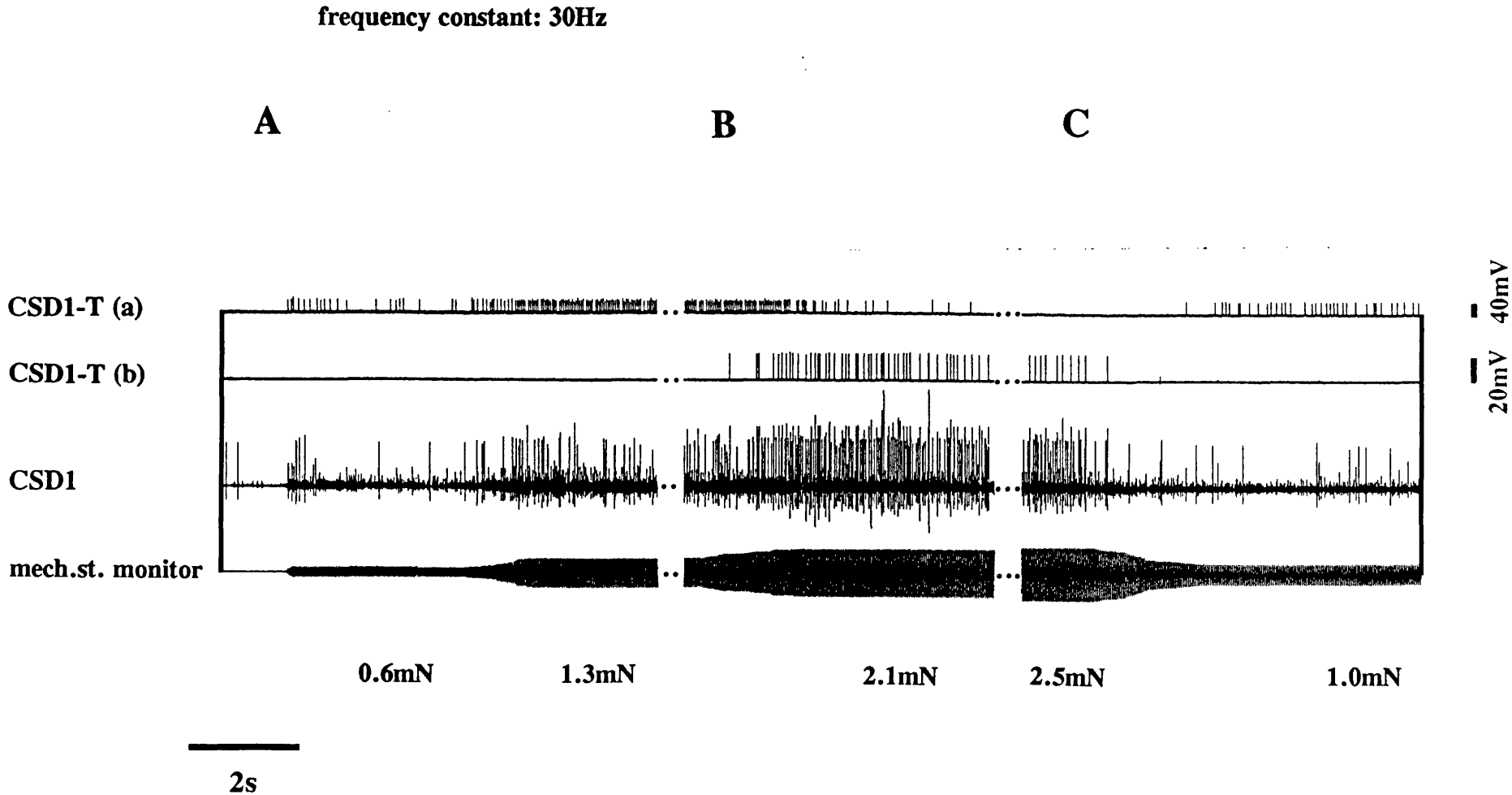

frequency constant: 30Hz
A
B
C
CSD1-T (a)
CSD1-T (b)
CSD1
mech.st. monitor
40mV
20mV
0.6mN
1.3mN
2.1mN
2.5mN
1.0mN
2s

FIGURE 4.2:

Response reversal in an extracellular recording of anterior levator motorneurones during mechanical stimulation of CSD_1.

A: Anterior levator motorneurones respond with inhibition to CSD_1 mechanical stimulation, depressor motorneurones with excitation. Stimulation parameters: frequency, 0.6Hz; amplitude, equivalent to a force of 1.9mN peak-to-peak.

B: When the frequency of the sinusoidal stimulation is increased to 20Hz and the amplitude kept relatively small at values equivalent to forces between 3.1 and 3.9mN peak-to-peak, the reflex responses are the same as in A in that anterior levator motorneurones are inhibited and depressor motorneurones excited. Increasing the amplitude to about 7.4mN leads to a response reversal in that anterior levator motorneurones are now excited. The response in the depressor motorneurones does not change in sign.

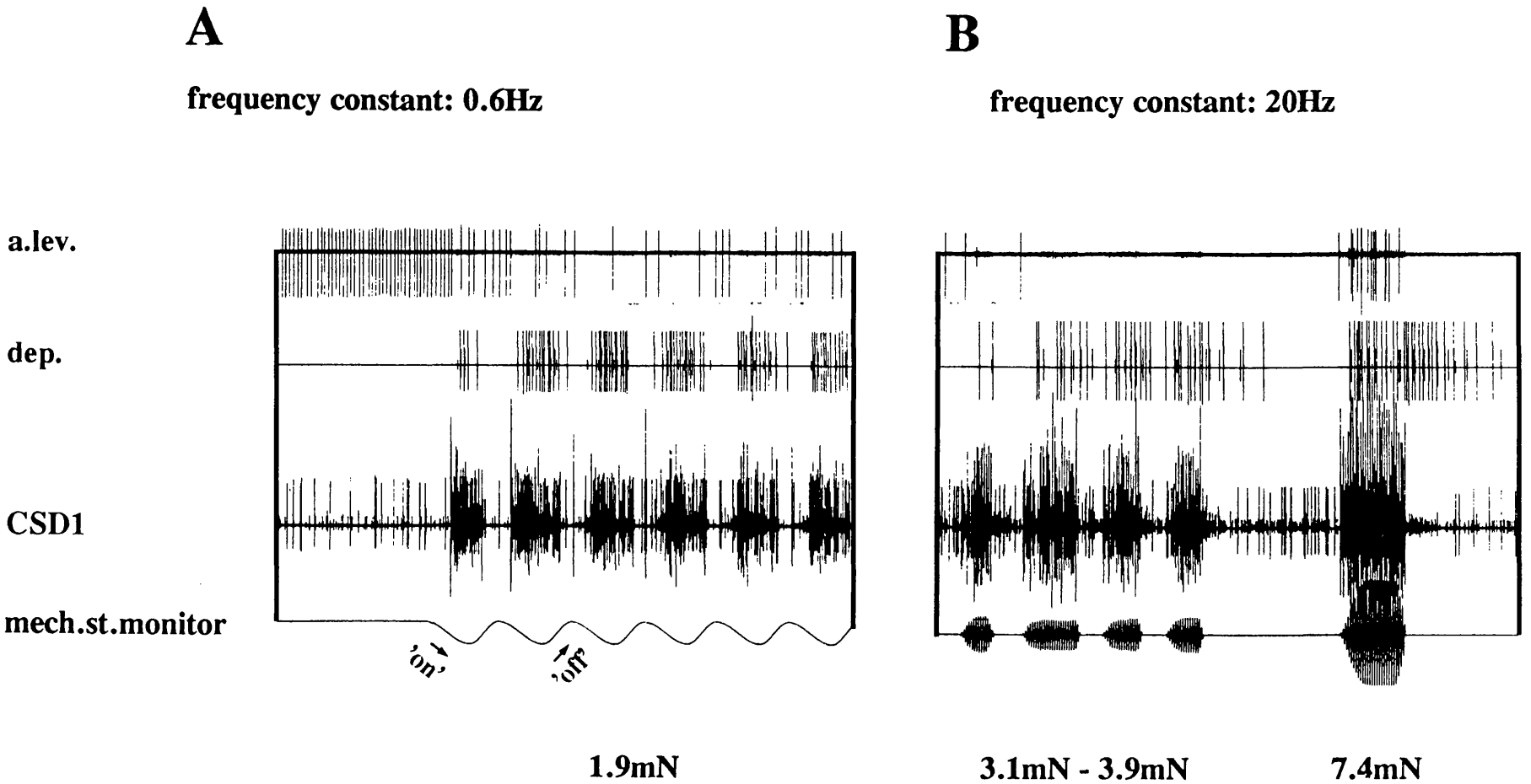

A
frequency constant: 0.6Hz
B
frequency constant: 20Hz
a.lev.
dep.
CSD1
mech.st.monitor
'on'
'off'
1.9mN
3.1mN - 3.9mN
7.4mN
2s

FIGURE 4.3:

Response reversal in the intracellular recording of an anterior levator motorneurone during mechanical stimulation of CSD_1.

Large amplitude, high frequency stimulation (B) results in excitation of the anterior levator motorneurone (see spikes in B which are illustrated in the left hand inset at a magnified timebase). Simultaneously, it activates large CSD_1 units. Reducing the amplitude (C) leads to hyperpolarization of the membrane potential, resulting from inhibition of the motorneurone. The large CSD_1 units are now no longer activated. Following a further reduction of the stimulus amplitude, inhibitory inputs are still recognizable in the anterior levator motorneurone, but to a lesser extent (D). When CSD_1 stimulation is completely abolished (E), the membrane potential returns to its original level (as in A). Notice that excitation occurs only in B (marked by double-headed arrow above the anterior levator trace), while ipsps (illustrated in the right hand inset at the same magnified timebase as the other inset) are visible during strong (B) and weak (C and D) CSD_1 stimulation (see double-headed arrow beneath the anterior levator trace).

The depressor motorneurone responds with excitation to CSD_1 stimulation. The extent of excitation is positively correlated to the degree of force applied to the CSD_1 receptor organ. A response reversal is not observed.

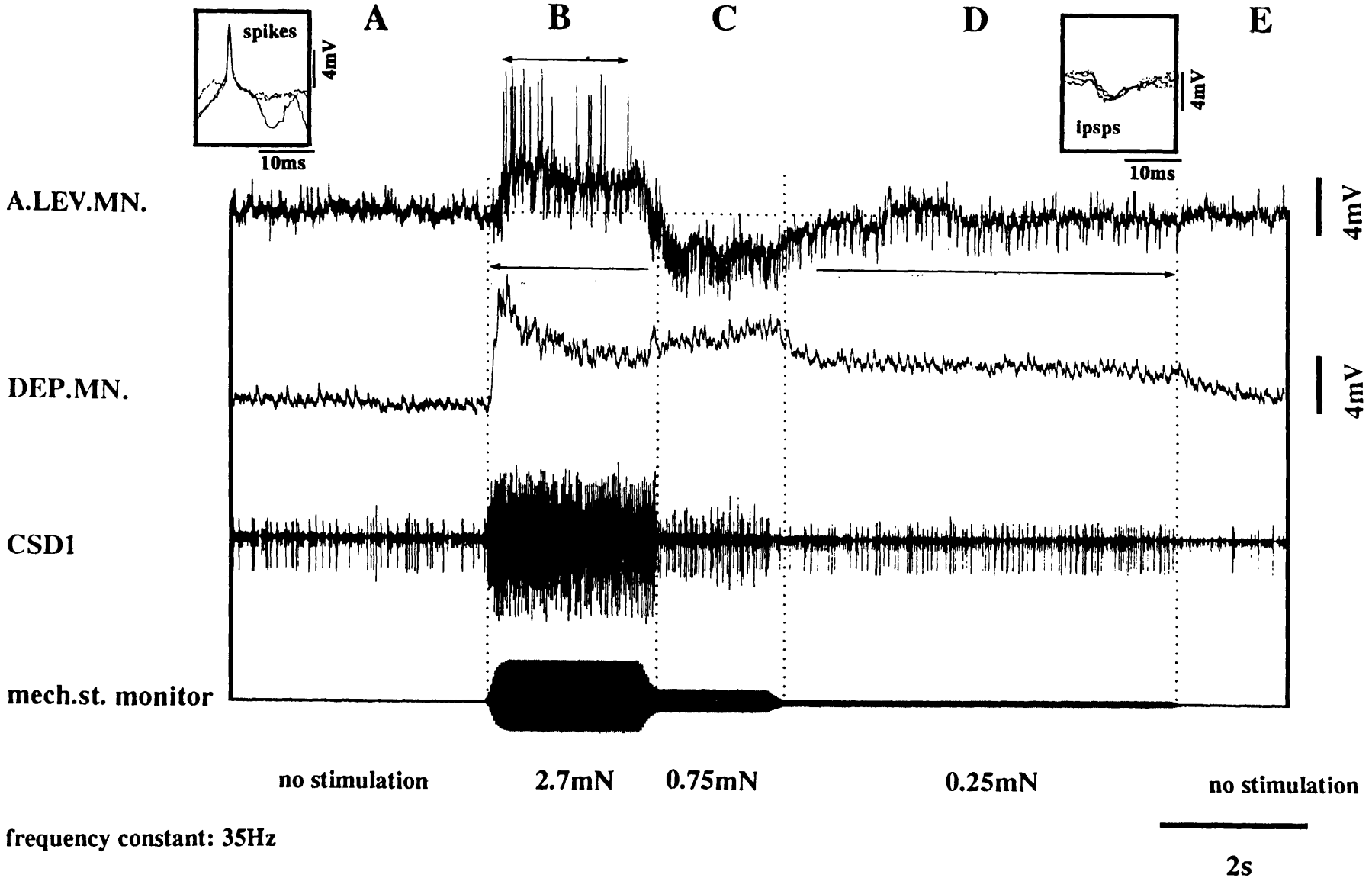
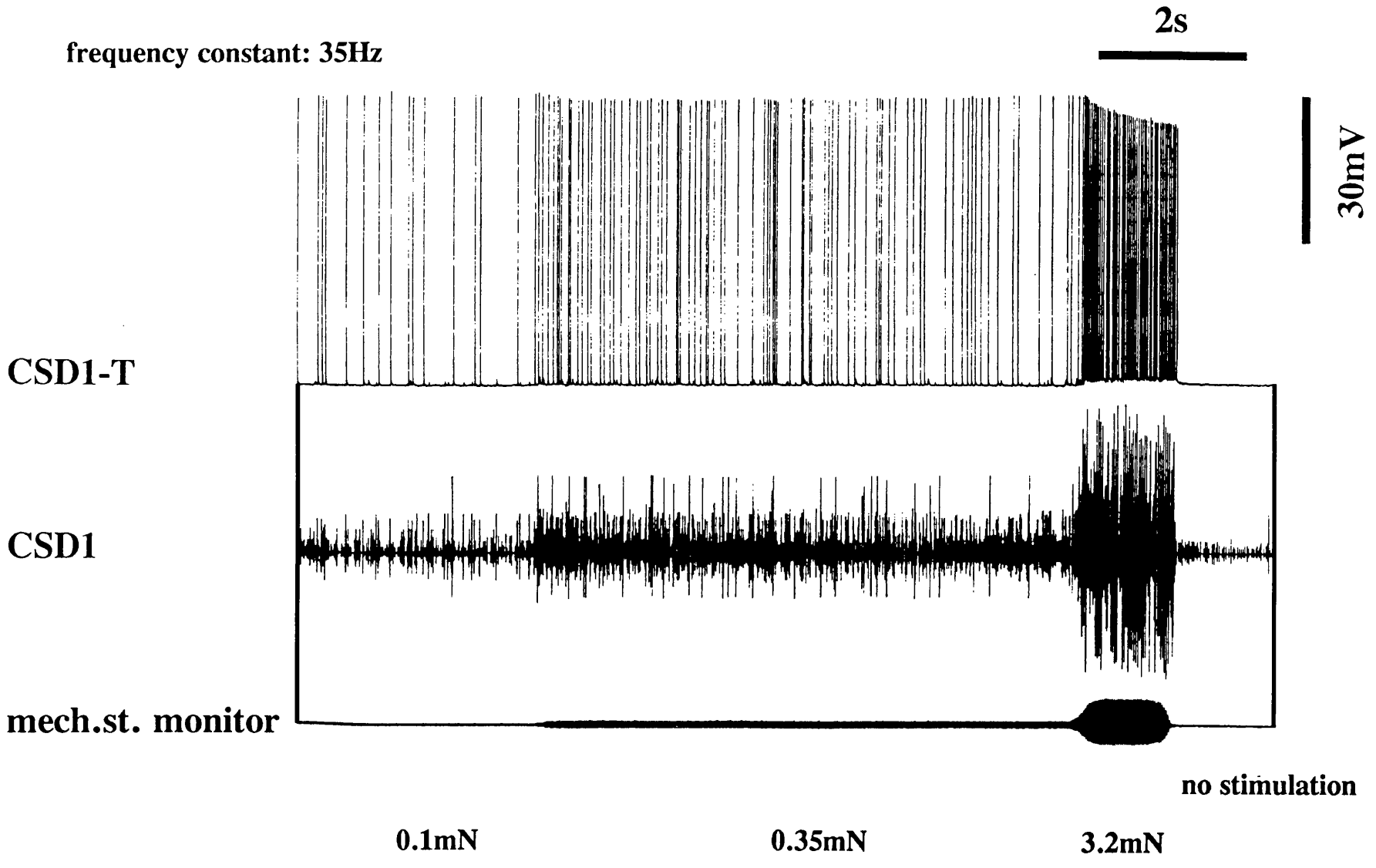

A
B
C
D
E
spikes
4mV
10ms
ipsps
4mV
10ms
A.LEV.MN.
4mV
DEP.MN.
4mV
CSD1
mech.st. monitor
no stimulation
2.7mN
0.75mN
0.25mN
no stimulation
frequency constant: 35Hz
2s

FIGURE 4.4:

Intracellular recording from the terminal of a low threshold CSD_1 fibre during mechanical stimulation of CSD_1. When high threshold CSD_1 fibres (seen as large extracellular spikes in the CSD_1 trace) were active (at an applied force of about 3.2mN), the amplitude of the impulses in the terminal was reduced. The same is true (but to a much smaller extent) during an applied force of about 0.35mN.

Stimulation parameters: frequency, 35Hz; amplitudes constituting forces of 0.1mN to 3.2mN.


2s
frequency constant: 35Hz
30mV
CSD1-T
CSD1
mech.st. monitor
no stimulation
0.1mN
0.35mN
3.2mN

Chapter 5

CENTRAL COUPLING OF MECHANORECEPTOR SENSORY TERMINALS IN CRAYFISH.

5.1. INTRODUCTION

Since 1959, when Furshpan and Potter described the first electrical synapse, electrical transmission between neurones has been reported in many invertebrates (Kaneko *et al.*, 1978; Koester, 1989; McCrohan and Winlow, 1985), lower vertebrates (Kaneko, 1971; Korn and Bennett, 1972; Sonnhof *et al.*, 1977), and in mammals (Baker and Llinás, 1971; Gogan *et al.*, 1974; Gutnik and Prince, 1980; Kettenmann *et al.*, 1983; Korn *et al.*, 1973; Walker and Hild, 1969). In mammals this phenomenon has been studied mainly in cell cultures.

Electrical transmission occurs at the "electrotonic synapse", so called because it generally behaves like a simple resistance connecting the cell interiors, and is essentially the same as electrotonic spread along a core conductor (Bennett, 1972). Morphologically, electrical synapses are gap junctions (Bennett, 1978; Payton *et al.*, 1969), specialized cell-to-cell contacts consisting of an aggregate of aqueous channels ('connexons'). These channels are delineated by a cylindrical assembly of molecules spanning the intermembrane cleft to connect the cell cytoplasm of coupled cells (Caspar *et al.*, 1977; Makowski *et al.*, 1977).

Electrotonic transmission has generally been found to be involved in rather rigid stereotyped behaviour, but many studies have demonstrated that they do have variable functional capabilities. These derive from the ability of electrical synapses to perform integrative operations similar to chemical synapses. For example, some electrical synapses can show facilitation, the postsynaptic event increasing in size during repetitive presynaptic firing (Getting and Willows, 1974; Kaneko *et al.*, 1978). Another example is rectification at some electrical synapses which transmit signals in only one direction (Heitler *et al.*, 1991) or, as in the case of the touch cells in leech, let only depolarizing but not hyperpolarizing current pass in both directions (Baylor and Nicholls, 1969). All integrational mechanisms depend upon the transjunctional conductance and the extrajunctional membrane resistance at the electrical synapses (Furshpan and Potter, 1959a; Giaume and Korn, 1983; Getting, 1974; Getting and Willows, 1974). These can, for example, be influenced by hyperpolarizing or depolarizing inputs from chemical synapses (Furshpan and Potter, 1959b; Leitch *et al.*, 1990). Thus, electrical synapses allow for a greater plasticity than was originally thought to be possible (Berry and Pentreath, 1977).

In Crustacea, electrical coupling is widespread and well documented in several neural systems. In the giant fibre system, which co-ordinates escape behaviour, each of the pair of lateral giant axons consists of a chain of electrically

coupled cells. Both lateral and medial giant fibres are electrically coupled to abdominal interneurones which themselves make electrical synapses with motorneurones. Lateral and medial fibres are also electrically coupled to motorneurones in the thorax and abdomen (Fraser and Heitler, 1989; Furshpan and Potter, 1959a; Heitler and Fraser, 1989; Macmillan *et al.*, 1985). Electrical synapses are also known to exist in the lobster stomatogastric system (Hartline *et al.*, 1988), the crayfish abdominal swimmeret system (Heitler, 1978) and between synergistic thoracic motorneurones innervating the proximal leg muscles in crayfish (Chrachri and Clarac, 1989; Skorupski and Sillar, 1988). There are also strong indications that the T fibre, one of the non-spiking afferent neurones of the TCMRO, is electrically coupled to some, but not all, remotor and promotor motorneurones in the thoracic ganglia of crayfish (Skorupski, 1992).

In Crustacea, coupling has not yet been described in any spiking sensory neurone as it has, for example, in the cell bodies of sensory neurones in the mesencephalic fifth nucleus of the rat (Baker and Llinás, 1971) and in cutaneous mechanoreceptor neurones in the leech (Baylor and Nicholls, 1969; DeRiemer and Macagno, 1981; Nicholls and Purves, 1970). Here, all three ipsilateral touch cells of one ganglion are electrically coupled to each other and to the three contralateral touch cells via an electrical 'coupling interneurone' (Muller and Scott, 1981). In addition, touch cells are also electrically coupled to postsynaptic motorneurones (Nicholls and Purves, 1970; 1972).

This chapter demonstrates the presence of electrotonic coupling between the central terminals of CSD mechanosensory afferents in the fifth thoracic ganglion of the crayfish. Possible functional implications will be discussed.

5.2. MATERIAL AND METHODS

Crayfish of the species *Procambarus clarkii* were used.

Recording and stimulation procedures, staining techniques and data analysis were as described in Chapter 2.

Experiments utilized an *in vitro* preparation as previously described, with the exception that extracellular recordings were made only from the sensory nerve roots and the two main leg nerves (adr and pdr), which lead to the distal part of the leg. Often two electrodes were placed against each of them.

5.3. RESULTS

5.3.1. Spike transmission in sensory afferents

The sensory fibres of either CSD_1 or CSD_2 were stimulated by indenting and releasing the soft cuticle in the basi-ischiopodite region of the leg where the receptor strands are attached. Thus, action potentials were initiated in the bipolar cells of the receptor organs. Sensory spike transmission from the periphery to the fifth thoracic ganglion was monitored by a combination of extracellular and intracellular recordings. Firstly, extracellular electrodes were placed against the CSD sensory nerve and, secondly, against the appropriate distal root (adr for CSD_1, pdr for CSD_2). Thirdly, single sensory fibres were penetrated with microelectrodes within the ganglion. Such recordings revealed the presence of large amplitude, short duration potentials which followed the afferent spikes observed in the CSD extracellular traces at a fixed latency. These action potentials travelling within the ganglion are not overshooting (Fig.5.1A).

Spike transmission within the ganglion is not an active process as it is within the nerve, but occurs via electrotonic spread. Penetration of the same afferent terminal at two different places within the ganglion revealed that the impulses change shape during their passage through the ganglion. One microelectrode was placed close to the periphery of the ganglion where the axons enter. Intracellular recordings here show fairly large spikes of short duration as described above. A second electrode was placed more centrally. The impulses recorded from this second site had a smaller amplitude and were of longer duration. This can only be explained if conduction within the ganglion occurs decrementally by electrotonic spread (Fig.5.1B). As previously mentioned, spike conduction within the CSD afferent axons from their somata up to the ganglion is active. Thus the last place where active propagation in the CSD afferent fibres occurs is very probably close to the point of their entry into the ganglion.

5.3.2. Coupling

5.3.2.1. Electrophysiology

Out of 60 penetrated terminals, 21 (35%) showed additional impulses of smaller size. These are thought to be spikes transmitted via electrotonic junctions from other afferents, since they can be time linked to spikes in the extracellular traces (Fig.5.2A). Other small potentials observed in the recordings of sensory terminals are

primary afferent depolarizations (PADs). The possibility of confusing them with coupled spikes is remote for they are of longer duration and cannot be linked to spikes in the extracellular traces (Fig.5.2B).

Coupled neurones did not necessarily have the same physiological properties; they often had different conduction velocities and 'on' units were sometimes seen to be coupled to 'off' units (Fig.5.3).

5.3.2.2. Dye coupling

Dye-coupling has been used to demonstrate the presence of electrical synapses. Lucifer Yellow, an intensely fluorescent 4-aminonaphthalimide, is known to cross gap junctions in vertebrates and invertebrates. It has been widely used to identify cell-to-cell contacts by observing the spread of the dye *in vivo* from one injected cell to others nearby (Stewart, 1981). Iontophoretic injection of the dye into single CSD afferents resulted in the staining of the elaborate dendritic tree-like ramifications of the afferent terminals within the ganglion (see Chapter 2). It is not possible to determine from the appearance of these ramifications whether branches of one or several CSD units were stained simultaneously when injecting the dye into a single cell. However, due to retrograde axonal transport mechanisms, Lucifer Yellow also spreads into the axons. Thus the presence of more than one filled axon within the nerve roots is evidence for the filling of more than one cell and indicates that dye-coupling via gap junctions took place (Fig.5.4).

5.3.3. Antidromic spikes

Although afferent conduction is passive within the ganglion, small impulses from coupled afferents were sometimes seen to elicit action potentials in otherwise silent fibres. Stronger stimulation of the receptor organ will activate more units and increase the firing frequency of single units. Thus the probability of several impulses from different, but coupled fibres arriving at the same time (or at least close together) in a sensory terminal within the ganglion is increased (Fig.5.5). Such impulses will summate because the axon membrane within the ganglion is electrically unexcitable (Fig.5.5B). They will be conducted passively to the point where propagated impulses can occur, so long as they are not cancelled out by incoming sensory impulses. Thus, in silent fibres, they will give rise to action potentials if they raise the membrane potential above firing threshold. Action potentials thus created are seen to sit on top of the coupled impulses in the intracellular recording. They will travel in both directions from their site of initiation (towards the end of the terminal and towards the periphery), so that antidromic spikes can be seen in the extracellular

traces (Fig.5.6).

5.3.4. Antidromic spikes in extracellular recordings

In some experiments, the conduction of sensory spikes was observed extracellularly in the nerve roots in preparations where the ganglion had not been penetrated by microelectrodes. Although the normal direction of spike conduction in sensory fibres is from the periphery to the ganglion, impulses were sometimes seen which travelled from the central ganglion to the periphery (i.e. antidromically). The occurrence of these antidromic impulses was increased with increased stimulus intensity. About 150 sweeps (each 80ms long) were captured at random at different stimulation intensities. Antidromic spikes were identified and counted in all of these. With weaker stimulation, only 2-5% of the sweeps contained an antidromic impulse. In contrast, with stronger stimulation, up to 24% of the sweeps contained an antidromic impulse (Fig.5.7).

5.4. DISCUSSION

5.4.1. Electrotonic coupling

Electrophysiological recordings from single CSD sensory afferents show the existence of electrical coupling between afferent terminals in the crayfish thoracic nervous system. The existence of electrotonic synapses between neurones is known for many different nervous systems. The criteria by which they can be distinguished from chemical synapses comprise morphological, electrophysiological and chemical tests (Bennett, 1972). In most cases, electrical coupling between two or more cells has been demonstrated by microelectrode penetration of two coupled cells simultaneously. Thus an action potential in either of the cells can be observed as a decrementally conducted, reduced impulse in the other cell. Current injected into one of the cells will spread to the other as if they were connected by a resistance. The coupled impulses occur at very short latencies and are unchanged by application of chemical substances, such as Mn^{++} or increased concentrations of Mg^{++}, both of which block chemical synapses.

The crayfish isolated thoracic nervous system preparation used here makes it possible to correlate intracellularly recorded sensory and motor impulses with spikes recorded extracellularly in the nerve roots. Thus all electrical events recorded by the intracellular microelectrode in a sensory terminal could be checked for correlation with peripheral spikes. Both large and small impulses, but not PADs, could be correlated with impulses in a distal root and a CSD sensory nerve. Since all small impulses recorded in these experiments were correlated to extracellular CSD spikes, they must be derived from coupled CSD afferent terminals. The existence of electrical coupling can thus be demonstrated without the use of a second microelectrode. In these experiments, coupled spikes were present in 35% of penetrated afferent terminals.

Dye-coupling is another criterion which suggests the presence of electrical coupling among neuronal cells. Particular dyes, such as Lucifer Yellow and Procion Yellow, are known to cross gap junctions, which are the morphological structures constituting electrical synapses. Thus the occurrence of dye-coupling proves the existence of gap junctions, which are a necessary prerequisite for transmission at electrical synapses. When using Lucifer Yellow to fill single CSD afferents within the ganglion, the dye was observed to have spread into one or more afferents. The existence of dye-coupling indicates that gap junctions between primary afferents exist and supports the electrophysiological finding that electrical synapses indeed occur

between CSD afferent terminals.

It might be argued that coupling is artificially induced by microelectrodes penetrating the ganglion, electrotonic junctions being the result of mechanical damage. Three reasons for concluding this is not so are as follows.

First, out of 132 motorneurones recorded intracellularly in the fifth ganglion in this study, only 15 (11.4%) were found to be coupled to another motorneurone. If coupling were the result of mechanical damage and one assumed that no electrical synapses existed between cells in the thoracic ganglia of crayfish, then this 11.4% would constitute the average probability of mechanically inducing links between neurones. Since 35% of CSD afferents were found to be coupled, this would still leave 23.6% (35% minus 11.4%) of additional CSD recordings, all showing coupled impulses, to be accounted for. Since the existence of electrical synapses between motorneurones innervating the proximal muscles is not in question (Chrachri and Clarac, 1989; Skorupski and Sillar, 1988), the probability of causing coupling by damaging neuronal membranes by microelectrode penetration must be even less than 11.4%, and is very probably close to 0%. Therefore, the 35% of CSD units showing coupled impulses do so because of their physiological properties and not because the experimenter induced an artifact by using microelectrodes.

Secondly, when dye was released into the extracellular space rather than into a neurone, it did not fill any neurones, even after repeated penetration of the ganglion had taken place. Thus, microelectrodes stabbing through cells within the ganglion do not cause any permanent damage to neuronal membranes separating intra- and extracellular space.

Thirdly, as will be discussed fully below, the existence of antidromic spikes in CSD sensory afferents in preparations that had not been penetrated by microelectrodes is good evidence for the true nature of coupling, since such antidromic spikes arise from coupling between afferents.

5.4.2. Functional significance of electrical coupling

Bennett (1972) has stated that there are few situations where one mode of transmission - electrical or chemical - is of a distinct advantage. However, one factor greatly in favour of electrical transmission is its speed and reliability. Indeed, electrical synapses are often found where very fast reactions are essential for the survival of animals, as for example, in escape responses (Furshpan and Potter, 1959a). Each electrotonic synapse will save a few tenths of a millisecond in conduction time. Another function which is often linked with the occurrence of electrical coupling is synchronization of groups of neurones in order to produce simultaneous volleys (Korn

and Bennett, 1972). For example, the production of synchronous activity in the electromotor neurones in electric catfish increases efficiency by maximizing the voltage and power output (Bennett, 1972).

Electrical coupling is not usually associated with sensory structures. However, some studies have shown that electrical coupling is a phenomenon that also occurs in sensory neurones. For example, Baylor and Nicholls (1969) reported the existence of electrotonic synapses in the touch cells of the leech nervous system. As a functional explanation, they tentatively suggested that in intact animals a number of excitatory inputs from coupled cells might be sufficient to reach firing threshold and initiate action potentials. Thus the afferent signal would be amplified. However, they did not observe spikes being centrally initiated in the touch cells of the isolated leech preparation which was used in their experiments. In another sensory system, such spike production was actually demonstrated. Baker and Llinás (1971) reported electrical coupling in stretch receptor afferents in the mesencephalic fifth nucleus of the rat and showed that in some cases the small coupled impulses elicited action potentials. They themselves did not try to interpret this phenomenon functionally, but Bennett (1972) proposed that it could provide some positive feedback between stretch receptor afferents and increase the efficiency of the stretch reflex.

In the crayfish thoracic nervous system preparation, it is possible to observe action potentials which are initiated, in otherwise silent cells, by impulses from active cells which are electrically coupled to them. As the cells in question are primary afferent terminals which convey sensory information from the periphery to the motorneurones in the central nervous system, this mechanism constitutes an amplification of the incoming sensory information.

As such a system constitutes a positive feedback loop, the question arises as to how the system limits itself in order to avoid over-amplification and subsequent instability. Two reasons are presented in the following to show that the system is self-limiting and instability is no imminent threat. First, not all coupled impulses which were seen in CSD afferent terminals elicited additional spikes, only in silent fibres were antidromic spikes and hence extra, centrally generated impulses seen. Spikes being centrally generated within active fibres would collide with incoming impulses, thus cancelling out the incoming information. Hence, amplification of sensory information occurs only in silent fibres. Secondly, when the active fibres fall silent, i.e. when they are not stimulated any longer, their depolarizing effect on coupled, silent units ceases and no more additional spikes are generated within the ganglion. Therefore the amplification effect is thought to be a subtle one, mainly occurring, when several active units are coupled to one silent one, and being terminated when

stimulation ceases. In another crustacean preparation, such positive feedback between sensory neurones has also been described. In crabs, the third, the small diameter P fibre of the TCMRO receives centrally excitatory inputs from one of the two large diameter fibres, the T fibre. This synaptic T-to-P fibre excitation is thought to extend the range of the dynamic sensitivity of the P fibre, because at low velocity stretches the response of the P fibre to the onset of stretch may be insufficient to evoke a spike, but a T fibre induced spike may still be present in the receptor potential of the P fibre (Wildman and Cannone, 1991). It has not been reported whether the excitatory connection is based on a chemical or electrical synapse. Both systems, the CSD afferent terminals and the TCMRO fibres, seem to have evolved mechanisms to increase the range of their sensory efficiency.

In the experiments, 'on' units were seen to be coupled to 'off' units, thus conveying apparently conflicting information. This would seems to exclude the theory of a possible amplification effect of coupled units. However, inhibitory input from sensory fibres during the 'on' phase can have the same effect as excitatory input from fibres during the 'off' phase (see Chapter 3) and is thus not contradictory in its effect on postsynaptic neurones.

Biocytin anterograde fills of the afferent neurones (see Chapter 2) not only revealed the location of the central terminals within the ganglion, but also showed the absence of any cell bodies. Thus all fibres in the CSD nerves are sensory, and all action potentials within the sensory roots travelling towards the periphery are truly antidromic. The occurrence of antidromic spikes is thought to be the direct result of current, spreading from other active neurones via electrotonic synapses, eliciting action potentials in otherwise quiescent fibres. These centrally produced spikes travel in both directions from their place of origin. The orthodromically travelling spike will amplify the sensory message of the coupled cell, while the other spike will be seen as an antidromic spike in a CSD nerve root. Thus the occurrence of antidromic spikes is linked with the existence of electrotonic synapses in CSD afferent terminals.

Both orthodromic and antidromic CSD impulses were observed in preparations that had not been subject to penetration by microelectrodes. Thus, no mechanical damage mimicking electrical synapses and resulting in current spread from one cell to another could have occurred. Hence, antidromic impulses seen in preparations where no microelectrodes had been used are an indication of the true nature of electrotonic synapses in CSD afferent terminals. This is the third reason mentioned above in support of coupling being a true physiological property of CSD sensory afferents.

Cattaert *et al.* (1992b) showed that antidromic spikes occur in the sensory

fibres of the crustacean coxo-basipodite chordotonal organ (CBCO). The function of the antidromic spikes in CBCO afferents is not linked with an amplification mechanism, as has just been done for CSD terminals, but rather with an involvement in presynaptic inhibition. Primary afferent depolarizations (PADs) play an important role in the integration of sensory information in afferent terminals by supplying inhibitory inputs to the terminals. They elicit action potentials in the central ganglion which travel orthodromically to the ends of the terminals, but were shown not to produce any psp in the postsynaptic neurones. These PAD-induced action potentials also travel antidromically to the periphery where they are believed to aid in inhibition of sensory information by colliding with orthodromic impulses, thus preventing them from reaching the ganglion. The effect which centrally produced spikes have on postsynaptic cells has not been tested for the CSD receptor cells, but since the spikes are here not initiated from PADs (which shunt the membrane), there is no reason to believe that they would have any less effect than spikes originating in the periphery. Simultaneous recordings of pre- and postsynaptic cells would be necessary to confirm this point.

Other possible functions of electrical coupling can equally be dismissed. For instance, the function most often attributed to electrotonic synapses is synchronization of activity in a group of cells in order to maximize their effect. In the CSD receptor cells, synchronization could only be effectively produced if the somata or dendrites in the periphery close to the spike initiating zone were electrically coupled. Thus, synchronization is rejected as a possible function for coupling in CSD receptor cell.

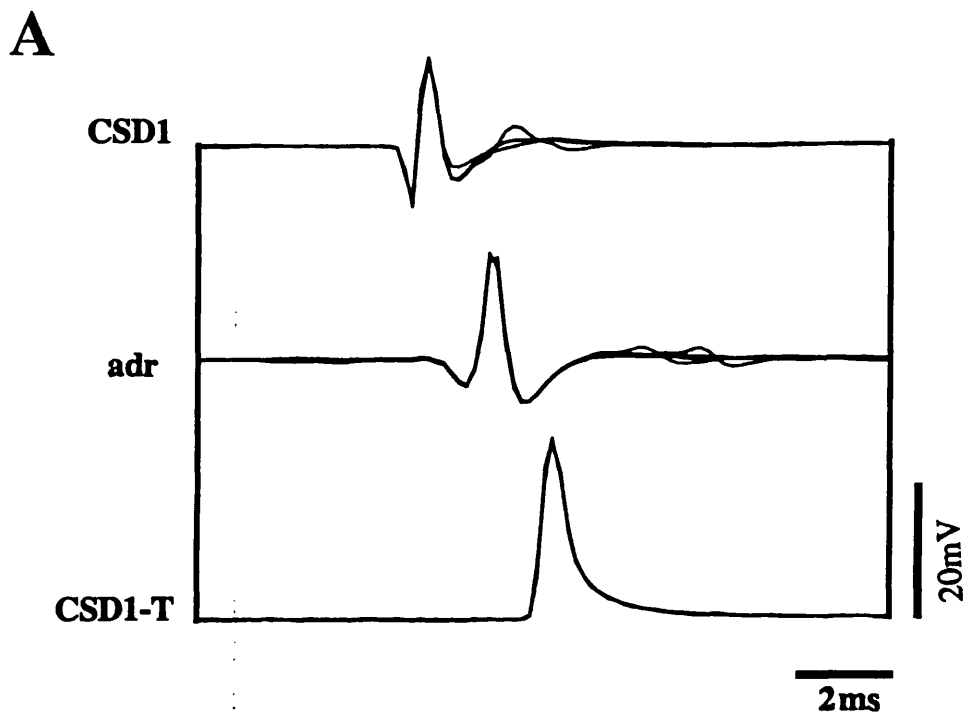
Another possible function which should be considered is that the gap junctions are present to allow an exchange of low molecular weight molecules between afferent fibres. This could be a simple mechanism for exchange of material, but it also could constitute a kind of chemical communication system between coupled neuronal cells as has been proposed by Bennett *et al.* (1981) as a possible function for gap junctions in embryonic neurones. This would imply that the transmission of electrical impulses across gap junctions is a mere by-product of metabolic co-operation between sensory afferents.

Such a metabolic function cannot be rejected on the basis of these experiments, but there is positive evidence that coupling of sensory terminals in crayfish is a mechanism for amplification of the sensory message, since coupling of units can lead to central spike initiation in otherwise silent fibres. This theory is supported by the positive correlation between the occurrence of antidromic spikes and the intensity of stimulation.

FIGURE 5.1:

A: Four superimposed sweeps triggered on an intracellularly recorded spike (CSD1-T) showing it to be time locked with an extracellular unit recorded in the CSD_1 sensory nerve and anterior distal nerve root (adr). This identifies it as an CSD terminal. Since the resting membrane potential was -78mV, the 23mV spikes are not overshooting.

B: Intracellular recording from a single CSD_2 terminal (note phase-locking with extracellular spikes in CSD_2 and pdr) with two microelectrodes simultaneously. The second electrode [CSD2-T(b)] penetrated the terminal further towards the midline of the fifth thoracic ganglion than the first electrode [CSD2-T(a)]. The spike in the second electrode is flatter and longer, thus demonstrating that conduction within the ganglion occurs via electrotonic spread.


A
CSD1
adr
CSD1-T
20mV
2ms

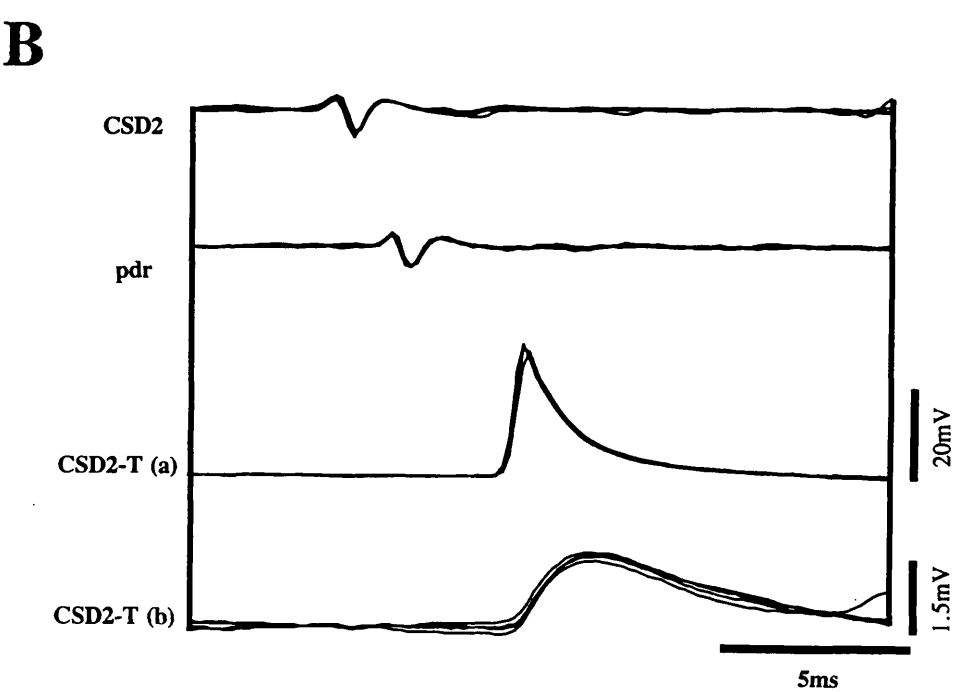

B
CSD2
pdr
CSD2-T (a)
CSD2-T (b)
20mV
1.5mV
5ms

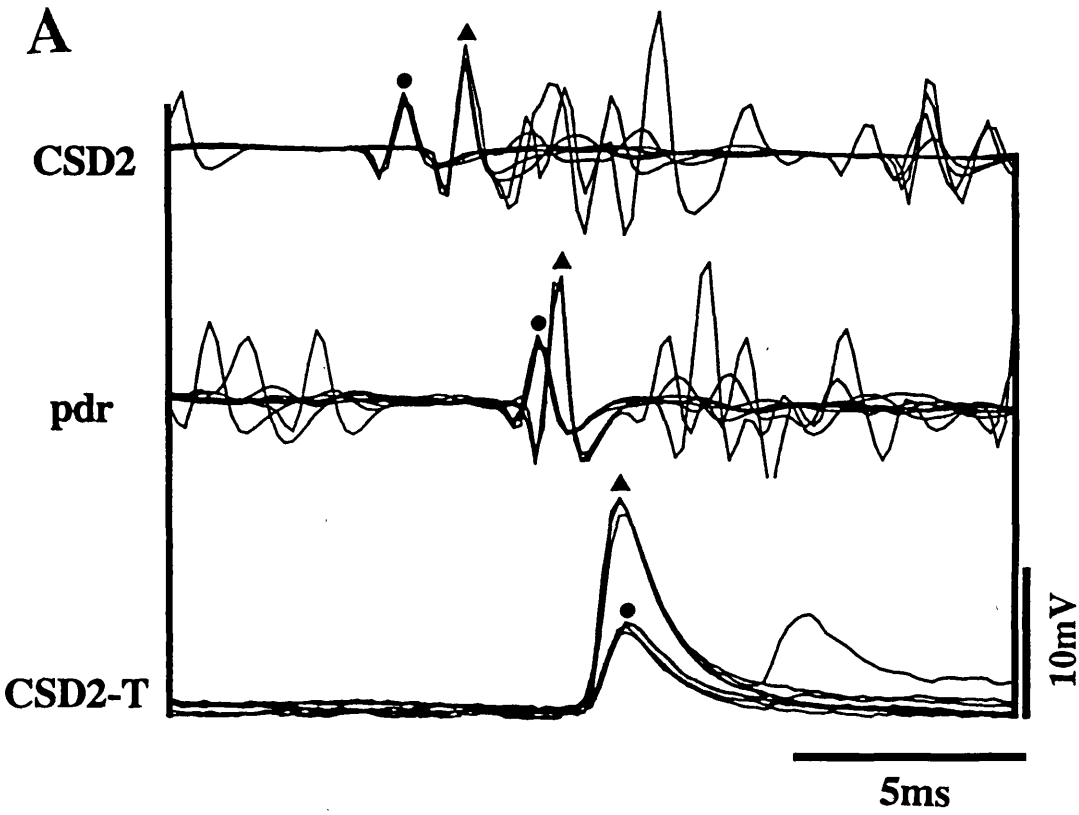
FIGURE 5.2:

A: Intracellular recordings of a CSD_2 sensory terminal showing small and large impulses. In this plot, 3 sweeps triggered on the large impulses (triangles) in the CSD_2 terminal (CSD2-T) and 3 sweeps triggered on the small impulses (circles) are superimposed and identified as two different CSD_2 units which can both be seen in the extracellular CSD_2 and pdr traces as indicated by the triangles and circles, respectively.

Notice the faster conduction velocity of the larger of the two CSD units.

B: 9 superimposed sweeps triggered on small depolarizing events in a CSD_1 afferent terminal. The impulses show no time link to any extracellular CSD_1 and adr spikes and are therefore identified as primary afferent depolarizations (PADs).

Note that the PAD has a duration of about 20ms, much longer than that of coupled impulses.


A
CSD2
pdr
CSD2-T
10mV
5ms

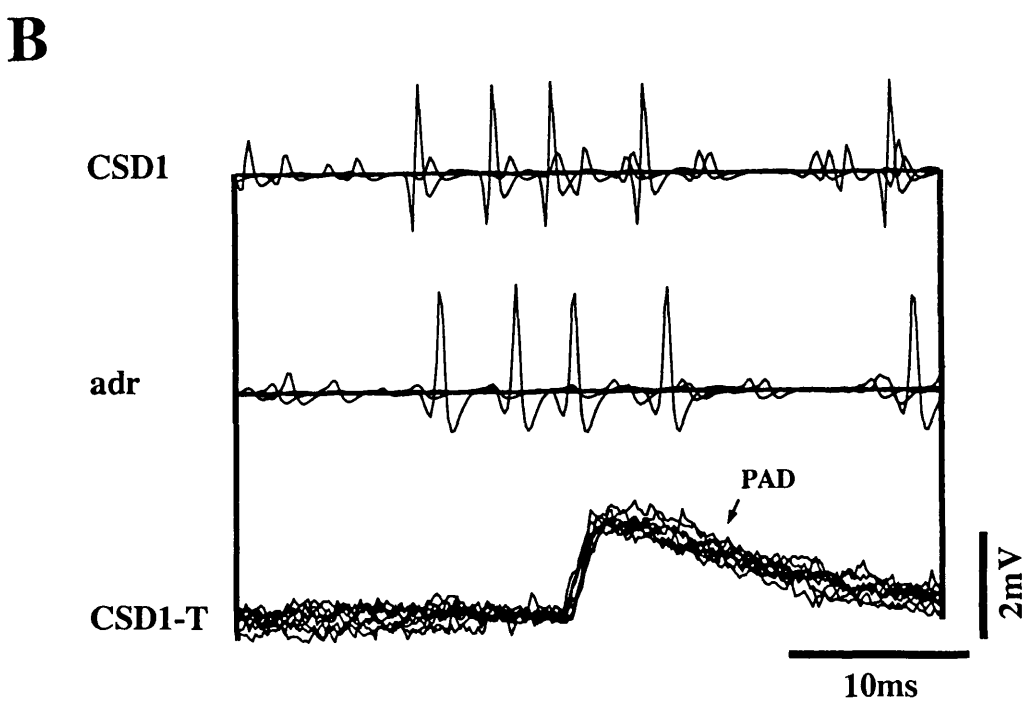

B
CSD1
adr
PAD
CSD1-T
2mV
10ms

FIGURE 5.3:

Coupled units conveying similar as well as conflicting information.

A: Both the large and small impulses in the intracellular record (CSD1-T) respond during 'on' and 'off' phases of the mechanical stimulus when the strand is stretched and released, respectively. Thus they are both bidirectional movement sensitive units.

B: In contrast, the large impulses in this CSD_2 primary afferent (CSD2-T) are from a movement sensitive 'on' fibre, whereas the small impulses respond tonically to CSD stimulation.

C: The CSD_2 coupled units (CSD2-T) are active during opposite phases of the stimulus cycle, the large during the 'off' phase, the small during the 'on' phase of the mechanical stimulus.

CSD_1 and CSD_2 extracellular recordings are shown for reference.

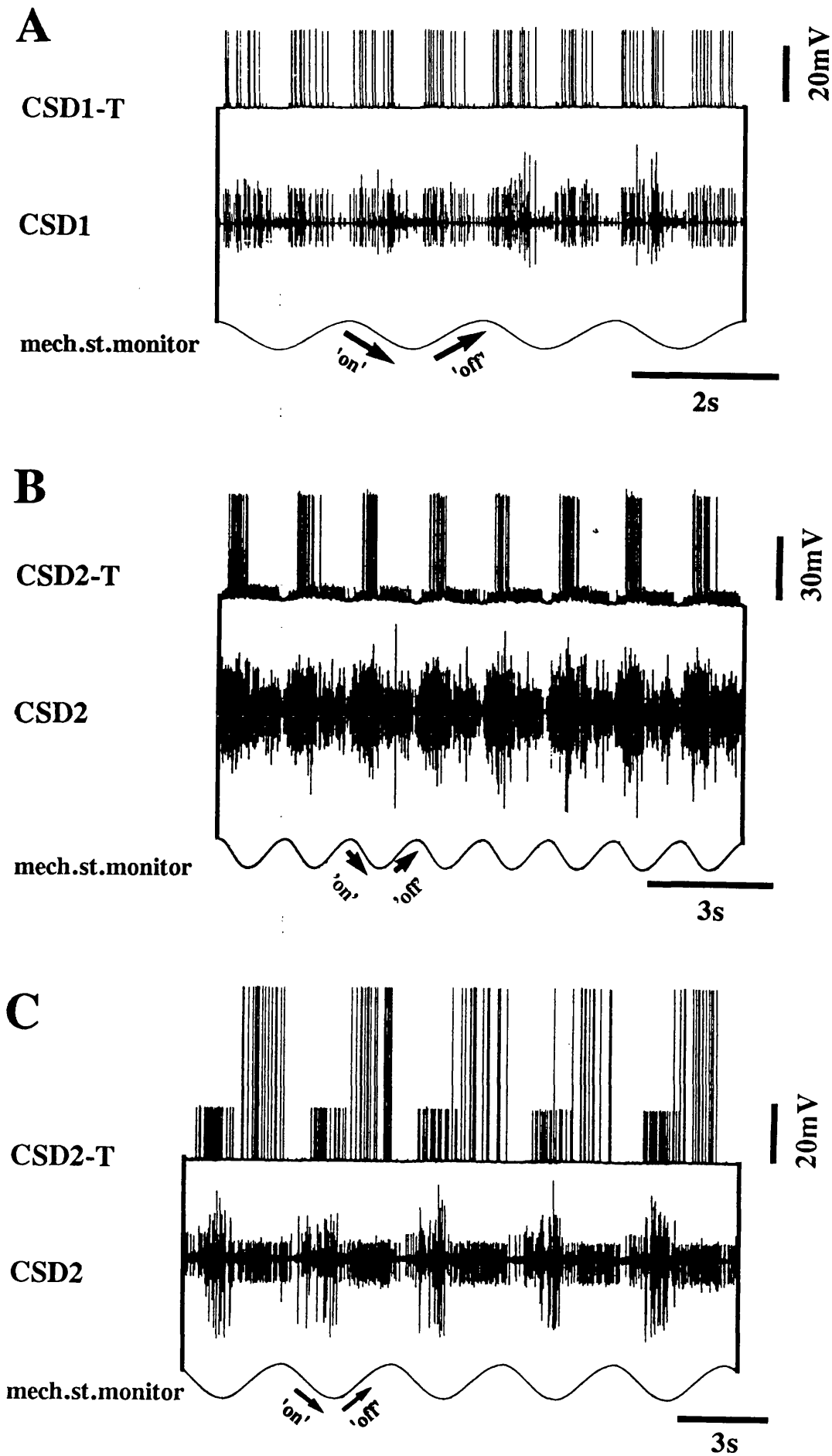

A
CSD1-T
CSD1
mech.st.monitor
'on'
'off'
20mV
2s
B
CSD2-T
CSD2
mech.st.monitor
'on'
'off'
30mV
3s
C
CSD2-T
CSD2
mech.st.monitor
'on'
'off'
20mV
3s

FIGURE 5.4:

Drawing of CSD_2 primary afferent branching within the fifth left thoracic hemiganglion from a Lucifer Yellow fill. The dye was injected intracellularly into a single fibre. Three axons are filled within the leg nerve (arrows), thus indicating three coupled units.

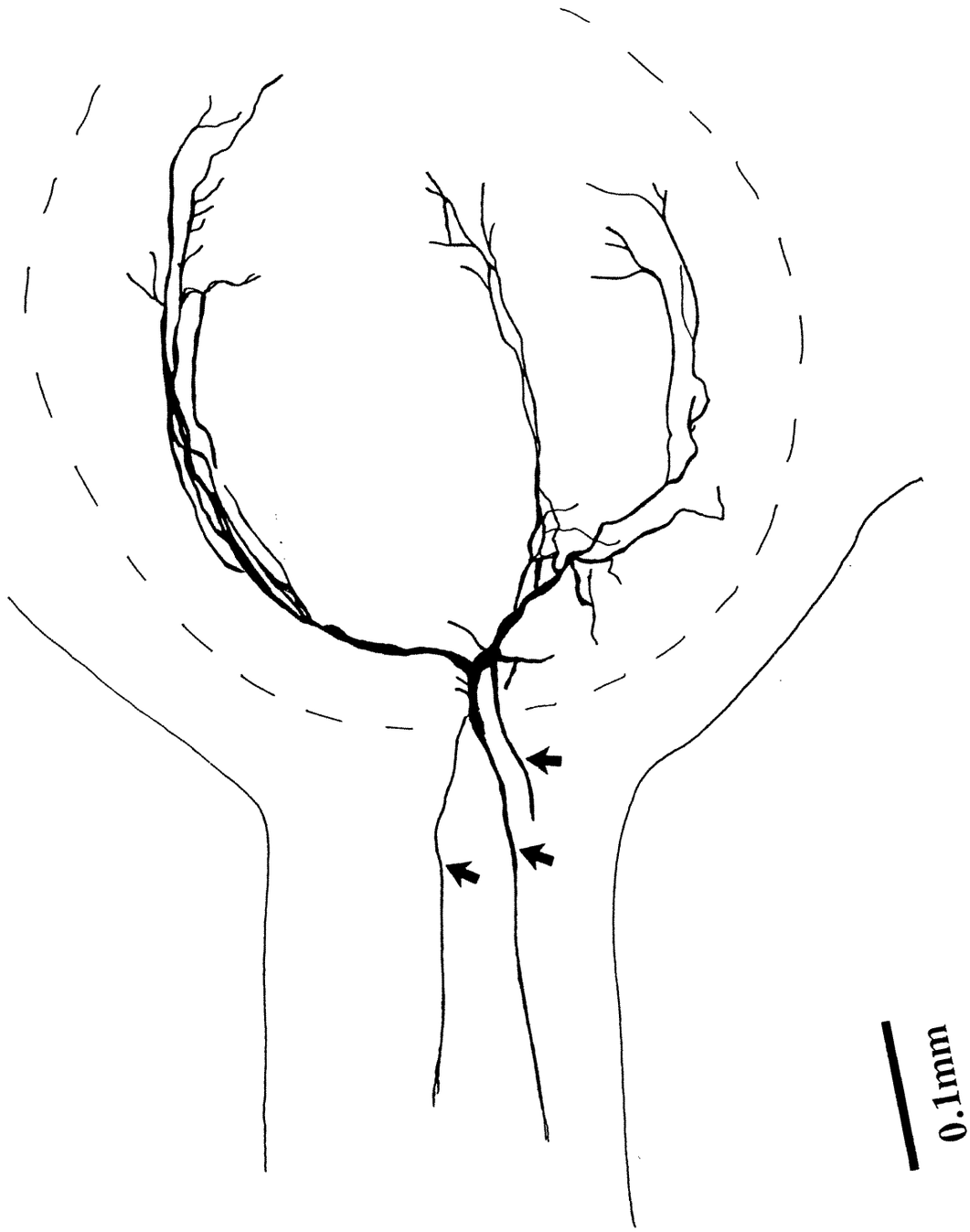

0.1mm

FIGURE 5.5:

A: Four (1-4) single frames showing the increased likelihood of coupled CSD_2 spikes (CSD2-T) arriving simultaneously in the ganglion when the stimulation intensity is increased.

Frequency of stimulus movement:

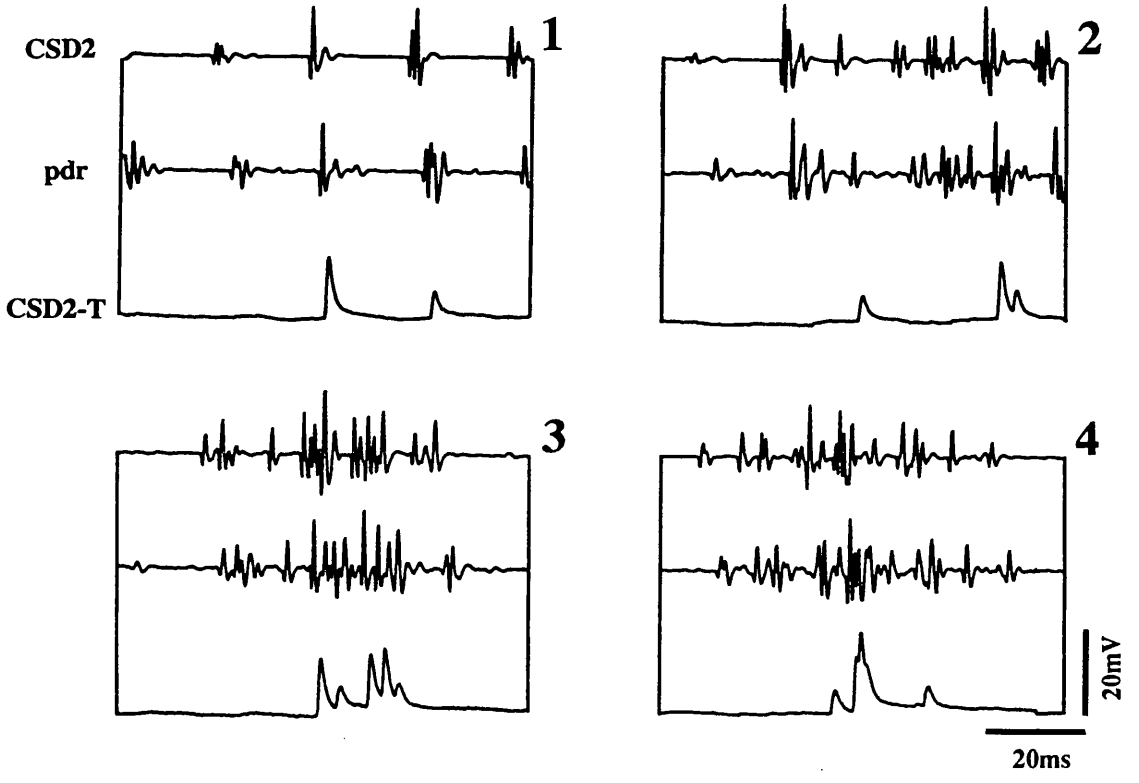
1 - 0.6Hz

2 - 10Hz

3 and 4 - 30Hz

B: The intracellular CSD_2 traces from frames 1 and 4 are superimposed to show the increased depolarization that occurs when several coupled impulses arrive at the same time and summate.

A



B

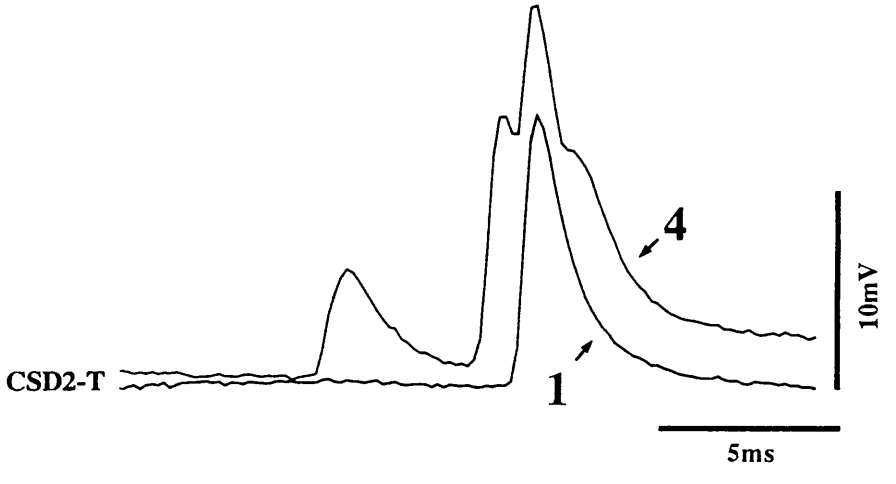
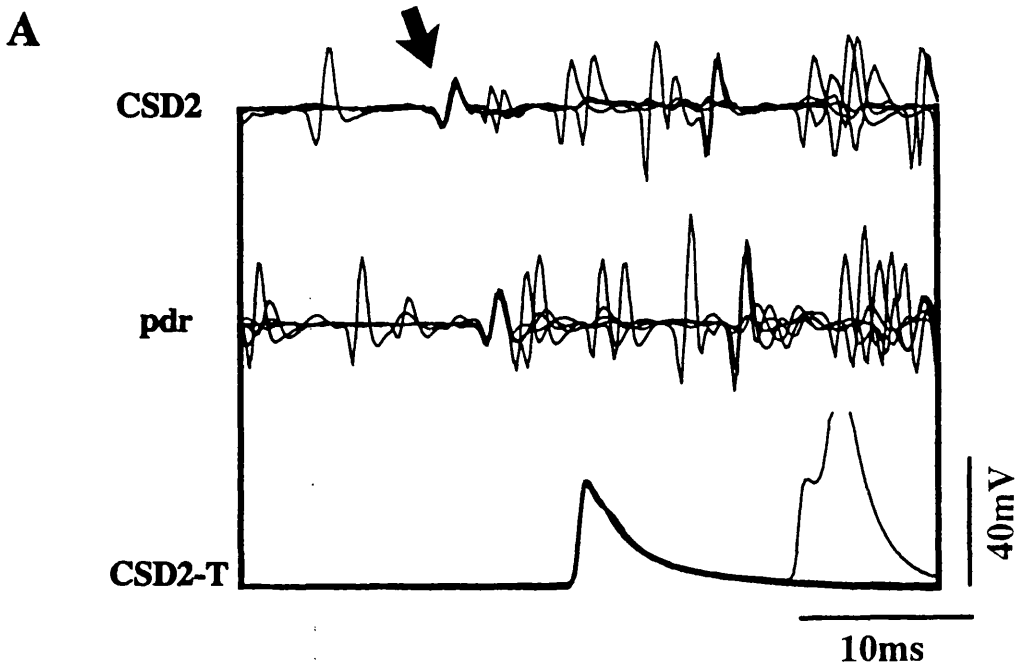


FIGURE 5.6:

Antidromic spikes in a CSD_2 sensory axon.

By superimposing several sweeps of data triggered on impulses in the intracellular trace (CSD2-T), it is possible to identify all units, for the extracellular spike is always time locked to the intracellular spike. In the case of orthodromic impulses (A), the extracellular spikes (arrow) **precede** the intracellular recorded spike at a fixed latency, while with antidromic impulses (B), the extracellular spikes (arrow) **follow** the intracellular spikes, also at a fixed latency.

The cell penetrated in these records is a quiescent CSD_2 unit in which small impulses originating from coupled CSD_2 afferents can be seen in (A). When several of them occur closely together, the membrane potential of the silent fibre is raised beyond spiking threshold and impulses sitting on top of the coupled spikes are seen in the intracellular trace (B).


A
CSD2
pdr
CSD2-T
40mV
10ms

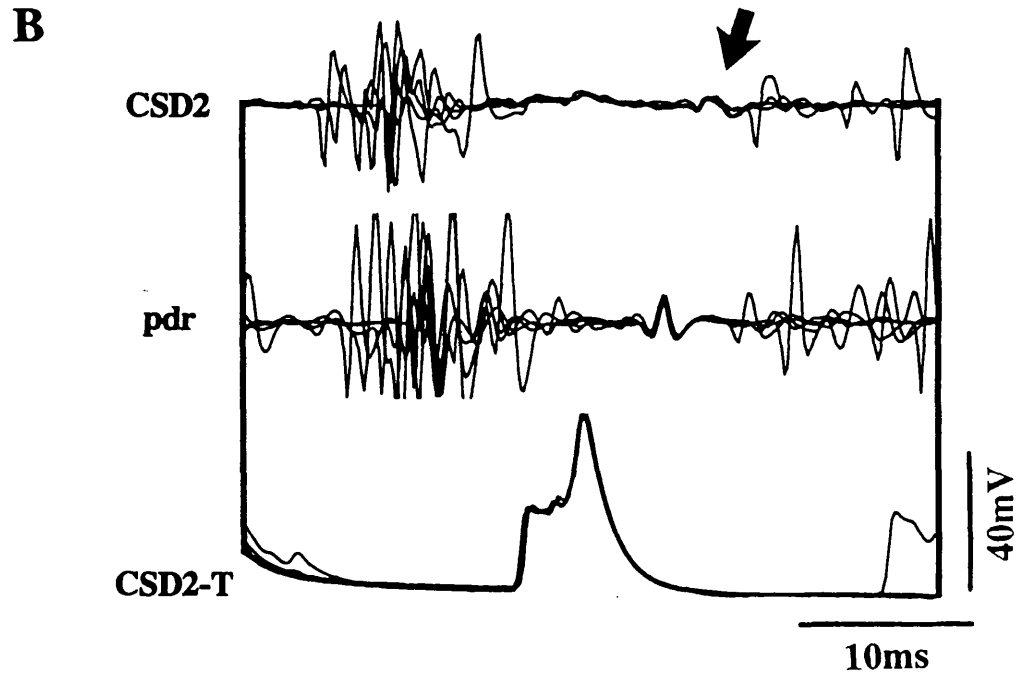

B
CSD2
pdr
CSD2-T
40mV
10ms

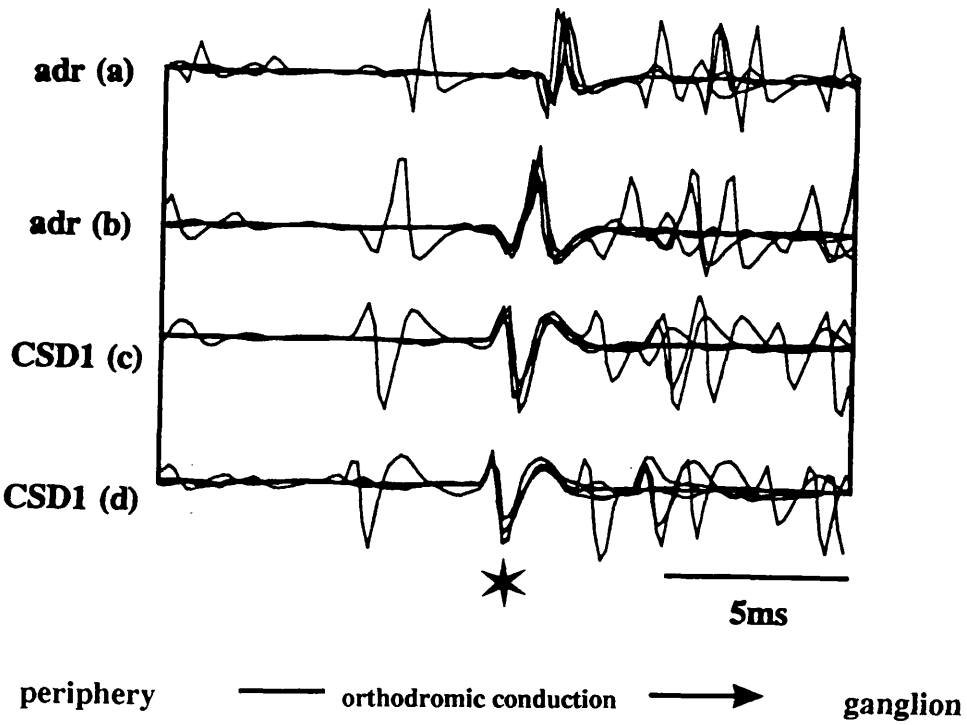
FIGURE 5.7:

Electrophysiological recording with four extracellular electrodes, two placed beside the CSD_1 sensory nerve and two beside the adr, monitoring spike transmission between the CSD_1 receptor organ in the periphery and the 5th thoracic ganglion. The records were produced by triggering on impulses in trace (d) and then superimposing selected traces.

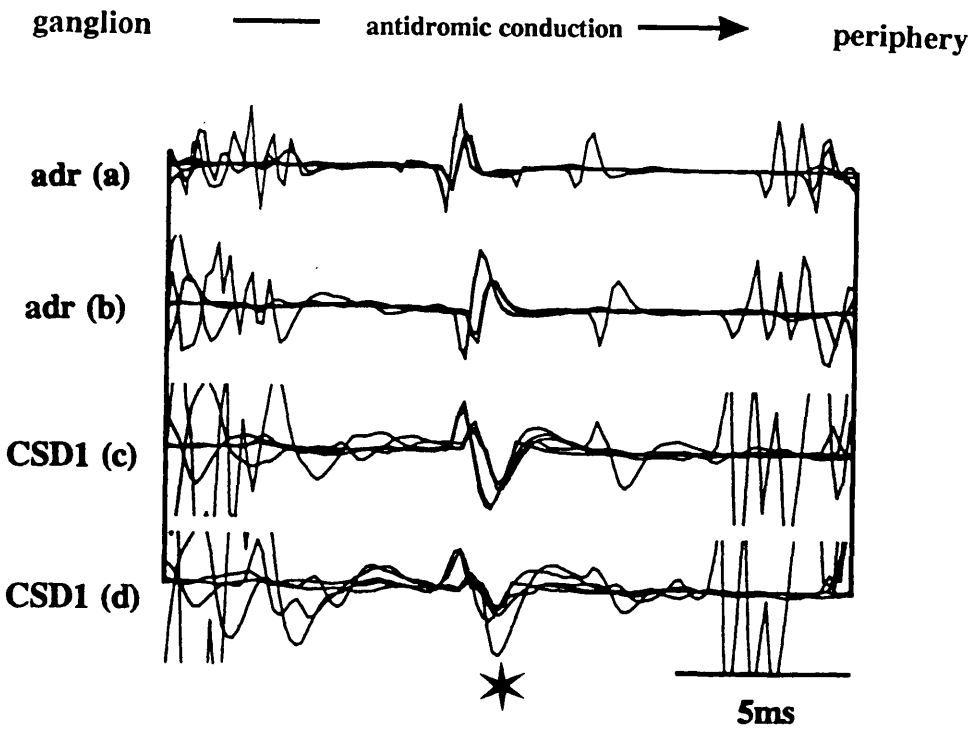
A: Spikes marked in this record by an asterisk occur first in the CSD_1 nerve root, then in the distal root. The spikes travel from the sense organ to the ganglion past each of the four electrodes in turn in their orthodromic direction of conduction.

B: Spikes marked in this record by an asterisk travel from the 5th thoracic ganglion to the CSD_1 sense organ in the periphery. They are therefore antidromic in their direction of conduction.

A


adr (a)
adr (b)
CSD1 (c)
CSD1 (d)
5ms
periphery
orthodromic conduction
ganglion

B


ganglion
antidromic conduction
periphery
adr (a)
adr (b)
CSD1 (c)
CSD1 (d)
5ms

Chapter 6

CONCLUDING REMARKS

When confronted with the question: What is the focal point of this thesis? The answer would be: the cuticular stress detectors in crayfish.

The co-ordination of muscle activity - an essential requirement in the production of meaningful behavioural acts, particular in locomotion - has intrigued many researchers. Animal and human bodies are able to produce very precise, minute and large movements, they are able to employ the same muscles in performing multiple tasks and have mastered the ability of various ways of locomotion. How is all this achieved? It is nowadays accepted that in most cases locomotion is based on the interaction of a centrally produced rhythmic pattern and sensory feedback from the periphery, enabling an animal to walk in different environments, to use different gaits and to avoid obstacles and obstructions in its way. Therefore it is important to have knowledge of all parts involved: central pattern generators, motorneurones, muscle fibres, sense organs and interneurones. The cuticular stress detectors are only two of the many sense organs in the legs of decapod crustaceans which supply the pattern generators in the central ganglia with essential information about the inside and outside world. As such, they themselves, their connections to motorneurones and their influence on the central pattern generators are the subject of this study and hence the focal point. However, such a study not only allows us to learn more about influence and co-ordinating role of two sense organs in crayfish locomotion, but also might allow us to generalize about principles in sensory processing when comparing several known systems with each other.

The two cuticular stress detectors are mechanoreceptors in the legs of decapod crustaceans which respond to pressure applied to or acting upon the cuticle of a leg. They are located in the basi-ischiopodite region of the leg. In contrast to chordotonal or muscle receptor organs whose main target muscles are situated in the same area as the sense organs, it is more likely that the cuticular stress detectors' influence in not focused at a particular location within the leg, because stress can occur everywhere in the leg and be transmitted along the hard cuticle to the CSD receptor organs. Nevertheless, this study has been concentrated on the muscles in the basi- and coxopodite joints for three reasons. First, there is a purely practical consideration. In order to gain access to the CSD nerve roots, the coxo-, basi- and partly ischiopodite are dissected. The additional dissection of the proximal muscle roots to promotor, remotor, depressor and levators situated in these joints is not as much of an extra effort as the dissection of muscle roots in the distal part of the leg would be. In addition, refraining from recording activity in muscle roots distal to the CSD receptor organs made it possible to cut the main leg roots immediately distal to

the joining point of CSD nerve roots to the main leg nerves. This was important to insure that no other sensory than CSD afferent information reached the ganglion. Secondly, it has been shown that an important stimulus for CSD activity - at least for CSD_2 activity - is the load increase the leg experiences when making contact with the ground at the onset of the power stroke (Klärner and Barnes, 1986). Therefore, muscles which are involved in dorso-ventral movements are of interest; of particular interest are the levator and depressor muscles at the base of the leg, because they play a dominant role in generating dorso-ventral leg movements. Thirdly, CSD_1 has been implicated in the control of autotomy from its first discovery by Wales and co-workers (1970) onwards. The anterior levator muscle has been shown to be the executing force in autotomy (Frédériq, 1892), therefore here again it is at the centre of attention.

A connection between CSD_1 and the phenomenon of autotomy or autospasy has been made in the present study (Chapter 4). It has been shown that the fibres of the CSD_1 receptor organ can be divided into two separate groups based on their activation characteristics. The low threshold fibres seem to be involved in the co-ordination of locomotion and as such the prevention of autospasy when it is not needed. 60% of all recorded anterior levator motorneurones were hyperpolarized by such CSD_1 activity. The high threshold fibres on the other hand are activated when strong forces act on the leg and seem to add to the process of autospasy by exciting anterior levator motorneurones. As this connection has only been made when recording either extra- or intracellularly from anterior levator motorneurones and extracellularly from the CSD_1 fibres, it would be desirable to directly show the connection by recording intracellularly from both CSD_1 fibres and motorneurones simultaneously. A second question arising as consequence of this work is: can this phenomenon, that is, different sensory units eliciting different reflex responses in the same motorneurones, be found in crustacean legs which show true autotomy? In order to answer this question, it would be necessary to develop a similar reduced *in vitro* preparation as has been used here, but instead of using crayfish as experimental animals, crustaceans that show true autotomy in their walking legs like, for example, crabs should be used. An *in vitro* preparation using crabs is known in studies on the TCMRO (e.g. Head and Bush, 1992), therefore it is thought to be a feasible project, even considering that the CSD_1 receptor organ is difficult to dissect due to its location and the shortness of its sensory nerve before joining one of the main leg nerves. Another possibility would be using a crayfish preparation where the first pair of walking legs, the chelae, are dissected. This could be a more suitable preparation when using intracellular recording techniques than the reduced crab preparation,

because all thoracic ganglia are locally isolated from each other in the crayfish ladder-like central nervous system, whereas they are stacked one above another in the crab nervous system, thus making intracellular recording from a particular central ganglion more difficult. On the other hand, it is not known, if the dissection of the CSD_1 nerve root in the chelae would not be more difficult due to the more compressed and compact anatomy and thicker cuticle of the chelae.

The last data chapter deals with a phenomenon which will in due time - so the author of this thesis is convinced - be accepted as a general principle in the processing of sensory information in invertebrate systems, but which at present is thought of as a mere artifact by some scientists (Burrows, personal communication). Electrical coupling between sensory neurones is best known in vertebrate photoreceptors. However, it is also known to play a role in the processing of sensory information in some touch sensitive cells in the leech (Baylor and Nicholls, 1969) and has been shown to occur in the CBCO (ElManira *et al.*, 1993) and presently also in the CSDs. New concepts always take time until they are generally accepted, therefore it is believed that after the phenomenon of central coupling in mechanosensory terminals will have been shown to occur in more and more sensory systems in invertebrates it will be acknowledged generally.

Comparisons of experimental procedures have shown that dissecting the CSD receptor organs is more complicated than dissecting other chordotonal sense organs and hence makes it more difficult to use the *in vitro* crayfish preparation. Therefore it is thought advisable to study general phenomena such as electrical coupling between afferent terminals or occurrence of PADs in preparations which are more easily accomplished like, for example, the *in vitro* preparation where the CBCO or TCMRO are included. Nevertheless, it is thought to be of outmost importance that these phenomena have been demonstrated in other sensory structures as well, in order to support their claim that they are general principles in the processing of sensory information.

For the reasons just stated, the crayfish isolated preparation including the CSDs does not seem to be the best model system for studying the two above stated phenomena, electrical coupling and autotomy. Nevertheless, the bulk of the work for this thesis is concerned with the elucidation of the connections between the CSD afferent terminals and the proximal motor groups. The *in vitro* preparation is well suited to pursue this aim - as already discussed in Chapter 3 - and allowed a detailed study of the connections, particularly to anterior levator and depressor motorneurones. The results show that the reflex responses to CSD stimulation are variable, within one and the same motorneurone pool, some motorneurones respond with excitatory

potentials, some with inhibitory. However, it is observable that CSD_1 seems to have a strong excitatory influence on depressor, whereas CSD_2 rather excites anterior levator motorneurones. This trend can also be seen when looking at the entrainment results. Here, in the majority CSD_1 entrained depressors and CSD_2 anterior levators at phase 0.0. These are only generalized trends, because the more detailed picture is more complicated - as has been discussed in Chapter 3. The motorneurone pools seem to consist of subgroups where different motorneurones are employed in executing different tasks and hence respond differently to CSD stimulation. However satisfactory it is to know so much about the connections, it is important to emphasise that connections between sensory and motor units can only be put into context, when the stimulating characteristics for the sensory structures and the modes of employment for the muscular structures are known. Hence *in vivo* experiments are an important part in any studies on the function of sensory feedback systems. For only the behaviour shown by the animal will indicate which pathways are important and will be employed at different times in the course of walking. Comparing the results from both preparations has yielded the best possible interpretation of the phenomena concerning the CSD sense organs and the role they play in the control of locomotion in crustaceans.

It should also be mentioned that the *in vitro* preparation not only allows us to study one sense organ and its postsynaptic effects at a time, but actually as many as one wants to dissect. Thus one can demonstrate their interactions to one another and the effects, similar or opposing, they have on motorneurones. This was tried with the two CSD receptors which could be simultaneously stimulated by electrical stimuli in some preparations. Nevertheless, experiments were not pursued in detail and no data were presented here. The main reason was that time was limited for this project. It is an interesting and exciting thought to imagine the stimulation of, for example, TCMRO, CBCO and the CSDs simultaneously or at appropriate phases and observe their reflex effects on motorneurones or their influence in rhythmic preparations. Such experiments would make it particularly easy to assess the degree of influence each single sense organ has in comparison to the others. Comparisons between different preparation are difficult, but have been mentioned in Chapter 3 where it was pointed out that more CBCO afferents make connections to anterior levator motorneurones than CSD afferents do.

The present study only covers the influence the two CSD receptor organs in the fifth thoracic walking leg have on motorneurones in the fifth thoracic walking leg of crayfish. Previous studies have shown that mechanoreceptors, including CSD_2 (Klärner and Barnes, 1986), also influence the muscle activity in neighbouring,

contralateral or ipsilateral, legs. Therefore a logical continuation of the present study would be the investigation of anterior levator and depressor motorneurone reflex responses to stimulation of the CSDs where input and output components of the reflex arcs are situated in different legs.

LIST OF ABBREVIATIONS

AEP	anterior extreme position
a.lev.	anterior levator nerve root
A.LEV.MN.	anterior levator motorneurone
CBCO	coxo-basipodite chordotonal organ
CNS	central nervous system
CPG	central pattern generator
CSD_1	cuticular stress detector one
CSD_2	cuticular stress detector two
CSD1-T	CSD_1 afferent terminal
CSD2-T	CSD_2 afferent terminal
DC	direct current
dep.	depressor nerve root
DEP.MN.	depressor motorneurone
DSA	dactyl sensory afferents
el.stimulation	electrical stimulation
epsp(s)	excitatory postsynaptic potential(s)
IN.	interneurone
ipsp(s)	inhibitory postsynaptic potential(s)
mech.st.monitor	mechanical stimulus monitor
PDCO	propo-dactylopodite chordotonal organ
PEP	posterior extreme position
p.lev.	posterior levator motor root
P.LEV.MN.	posterior levator motorneurone
prom.	promotor motor root
PROM.MN.	promotor motorneurone
psp(s)	postsynaptic potential(s)
rem.	remotor motor root
REM.MN.	remotor motorneurone
TCCO	thoraco-coxal chordotonal organ
TCMRO	thoraco-coxal muscle receptor organ
TRIS	Tris(hydroxymethyl)aminomethane
VNC	ventral nerve cord

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The roots of education are bitter, but the fruit is sweet.

Aristoteles, Diogenes Laertius