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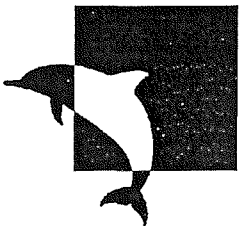

The *in vitro* Rat Spinal Cord -
An Investigation Into The Role Of Excitatory Glutamate In Nociception Using Electrophysiological and Immunohistochemical Techniques.

by

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Doctor of Philosophy Thesis

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For my Mum and Dad

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE
DIVISION OF CELL SCIENCES

Doctor of Philosophy

An Investigation Into The Role Of Glutamate In Nociception Using Electrophysiological and Immunohistological Techniques

by Elise Morgan

In this study, *in vitro* spinal cord preparations have been used to investigate the possible role of excitatory amino acids in nociceptive mechanisms. The *in vitro* spinal cord dorsal horns showed good viability which was demonstrated by the development of spontaneous dorsal root activity and an evoked dorsal root reflex (DRR) 2-3 hours following dissection. Both these activities were blocked by the addition of manganese to the bathing medium, demonstrating them to be of synaptic origin. The DRR evoked by stimulation of an adjacent dorsal root showed evidence of both excitatory and inhibitory phases, similar to that described for hamster spinal cords (Bagust et al. 1982; Bagust et al. 1985a; Bagust et al. 1989).

Stimulation of a lumbar dorsal root gave rise to dorsal horn field potentials. Investigations were made into the effects of the N-methyl-D-aspartate (NMDA) antagonists, D-AP5 and 7-Cl KYNA, arcaine sulphate, MK-801 and the non-NMDA antagonist CNQX on fast and slow dorsal horn field potentials elictied by low and high intensity dorsal root stimulation respectively. None of the NMDA antagonists had any significant effect on fast wave dorsal root field potentials at concentrations up to 100μM, but D-AP5 7-Cl KYNA and MK-801 significantly inhibited the slow wave field potential. The non-NMDA antagonist CNQX significantly inhibited the synaptic components of both the fast and slow wave dorsal horn field potentials. It was concluded that low-threshold, non-noxious sensory transmission is mediated by non-NMDA glutamate receptors and that NMDA glutamate receptors have a role in the transmission of high-threshold, noxious sensory information in the dorsal horn of the spinal cord.

The metabotropic glutamate receptor (mGluR) group I agonist *trans*-ACPD inhibited the synaptic components of the fast wave field potential. However, neither the group I antagonist L-AP3 nor the group II antagonist EGLU had any significant effect. The group III mGluR agonist L-AP4 significantly reduced the fast wave synaptic components. These results suggested a possible involvement of group I, II and III mGluRs in the processing of non-noxious information in the dorsal horn. The slow wave field potential elicited by high threshold dorsal root stimulation was inhibited by trans-ACPD, L-AP3 and EGLU. The group I mGluR agonist DHPG facilitated the slow wave at low concentrations. L-AP4 had no significant effect. It was therefore concluded that group I and II mGluRs but not group III mGluRs have a role in the processing of noxious sensory information in the spinal cord.

Expression of c-*fos* has previously been demonstrated in the dorsal horn of *in vitro* spinal cord preparations taken from 19-23 day old rats following high intensity dorsal root stimulation (Zhang et al. 1998). The expression of c-*fos* occurred only when dorsal root stimulation was sufficient to excite C fibres. Addition of 0.5μM capsaicin to the bathing medium induced intense c-*fos* staining in the absence of dorsal root stimulation. This provided a positive control suggesting that C fibre activation induces c-*fos* expression. Cords obtained from animals treated at 1 day old with capsaicin (50mg/kg) to destroy afferent C fibres showed a significant decrease in the number of Fos-positive cells induced by high intensity dorsal root stimulation when compared to vehicle treated animals. The effect of D-AP5, 7-Cl KYNA and CNQX on pain-related expression of the immediate early gene c-*fos* was also investigated. Both D-AP5 and 7-Cl KYNA significantly reduced the number of Fos-positive cells suggesting that NMDA receptors have a role in mediating expression. CNQX had no significant effect.

This study has confirmed that the *in vitro* spinal cord is a valuable tool for the investigation of nociceptive processing and modulation in the dorsal horn of the spinal cord. The results support the suggestion that NMDA and group I and II metabotropic glutamate receptors acting at glutamatergic synapses in the dorsal horn of the spinal cord have a role in processing or modulating nociceptive input from C-fibre afferents.

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I declare that the data presented in this thesis is my own original work.

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Abbreviations:

aCSF	artificial cerebrospinal fluid
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazole
D-AP5	D(-)-2-Amino-5-phosphonopentanoic acid
c-*fos*-ir	c-*fos*-immunoreactivity
cAMP	cyclic adenosine monophsphate
cGMP	cyclic guanosine monophosphate
7-Cl KYNA	7-Chlorokynurenic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
DAB	diaminobenzidine
DAG	diacylglycerol
DHPG	(RS)-3,5-dihydroxyphenylglycine
DRG	dorsal root ganglia
DRR	dorsal root reflex
EGLU	(2S)-α-ethylglutamic acid
IEG	immediate early gene
IgG	immunoglobulin
IP_3	1,4,5-triphosphate
KA	kainate
L-AP3	L-(+)-2-amino-3-phosphonopropionic acid
L-AP4	L-(+)-2-amino-3-phosphonobutyric acid
mGluR	metabotropic glutamate receptor
MK-801	5R, 10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine / dizocilpine
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
PAD	primary afferent depolarisation
PKA	protein kinase A
PKCγ	protein kinase C gamma
PKG	protein kinase G

PLC	phospholipase C
PBS	phosphate buffered salts
PSTH	peri-stimulated time histogram
SG	substantia gelatinosa
T	threshold
trans-ACPD	(±)-1-Aminocyclopentane-*trans*-1,3,-dicarboxlic acid
WDR	wide dynamic range

Aims of the Project.

Until recently it was thought that the spinal cord was merely a relay system between the peripheral nervous system and higher centres in the brain. It is now known that the spinal cord has a role in the modulation and processing of sensory information. Modulation and processing of nociceptive information occurs in the dorsal horn area and involves many neurotransmitters. Spinal mechanisms of pain are therefore complex but allow diverse therapeutic possibilites for the pharmacological control of the transmission of nociceptive information to the brain.

Spinal cord research in recent years has made it apparent that *generating* mechanisms of pain rather than inhibiting mechanisms may be more important to study in the search for novel strategies to alleviate pain. Instead of activating antinociceptive processes or inhibiting pro-nociceptive processes it would be better to eliminate altogether diseases that cause chronic pain, such as multiple sclerosis, cancer and arthritis. However, so far this has not been possible and remains a goal for the next century. In the meantime, research into the mechanisms of induction and maintenance of chronic pain in the spinal cord will make a major contribution to the search for novel, improved analgesics.

The excitatory amino acids, especially glutamate, are not the only major class of excitatory transmitter in the central nervous system, but are released by primary afferent fibres and have an important role in the spinal mechanisms of pain transmission. The aim of this project is therefore to identify the specific roles of the different classes of glutamate receptor (ionotropic and metabotropic) in pain processing and modulation within the dorsal horn of the spinal cord.

Chapter 1

Introduction

1.1 Cytoarchitectural Organisation of the Spinal Cord.

In the 1950s, Rexed divided the spinal grey matter of the cat into ten layers, most of which extend the entire length of the spinal cord (Rexed 1952). The laminae are of varying thickness and shape, but it is only in the dorsal horn (laminae I - VI) in which the laminae are arranged in a layered sequence (Fig. 1.1). The divisions were based on a purely cytoarchitectural scheme without reference to dendritic branching or axonal terminations but it has still turned out to be of great value in studies of connectivity, both with anatomical and physiological techniques, and in immunocytochemical and enzyme histochemical studies. Most, if not all, of the major features that Rexed described in cats can be seen in the rat spinal cord. However in the rat spinal cord, rather than the almost perfectly horizontal placement of most of the laminae found in cat lumbar segments, there is a more dorsolateral slant to the laminae in the rat lumbar enlargement (Wall 1968).

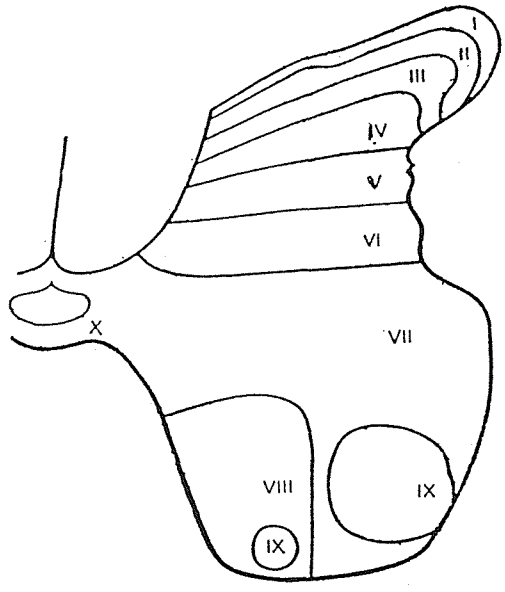


Fig 1.1: Cytoarchitecture according to Rexed. This diagram shows the laminae of Rexed in the spinal grey matter of a lower lumbar segment of a cat. From Wilkinson (1992) The spinal cord. In *Neuroanatomy for medical students*. Butterworth-Hienemann.

1.2 Primary Afferent Projections to the Spinal Cord.

1.2.1 Major functional groups of primary afferent fibres.

On the basis of size and structure, peripheral afferent fibres are divisible into two main groups, myelinated and unmyelinated, and these two categories may be further subdivided into three principal conduction velocity subsets of cutaneous and visceral afferent fibres: Aβ (large diameter myelinated afferents), Aδ (small diameter myelinated afferents), and C (unmyelinated afferents). Primary afferent neurones with fibres in peripheral nerves encode sensory information from many tissues and organs, including the skin, muscle and viscera. Cutaneous Aβ afferents are typically activated by low intensity mechanical stimuli (brush, touch, pressure, vibration), while the most abundant type of cutaneous Aδ afferent is the high threshold mechanoreceptive afferent, which is responsive to noxious mechanical stimuli (pinch, prick). A second type of Aδ afferent, a low threshold mechanoreceptive afferent responsive to brushing hairs, has been termed the delta hair (D-hair) follicle afferent. In various mammalian species, at least six functionally different kinds of cutaneous primary afferent unmyelinated (C) fibres have been identified. These include three types of nociceptive afferents (polymodal, high threshold mechanical and mechanical-cold), in addition to two types of thermoreceptors (cooling and warming) and a single type of low threshold mechanoreceptor. Large numbers of unmyelinated afferents innervate viscera, joints and skin that, in healthy tissue, are unresponsive to any form of acute stimulus. But, in certain chronic pathological states, such as inflammation, these nociceptive afferents may become responsive to a variety of mechanical or chemical stimuli (McMahon and Koltzenburg 1990).

1.2.2 Nociceptive afferents.

The primary afferent nociceptor is generally the initial structure involved in nociceptive processes. Nociceptors respond to chemical, mechanical and thermal stimuli. Depending on the response characteristics of the nociceptor, stimulation results in propagation of impulses along the afferent fibre toward the spinal cord. Two main fibre types, the faster-

conducting myelinated Aδ fibres and the slower-conducting unmyelinated C fibres, are involved in the transmission of nociception and if conduction in these afferent fibres is blocked, so is nociception; however, these fibres do not act simply as inert conductors of sensory information but undergo a number of physiological, morphological and biochemical changes in response to damage of a peripheral nerve. These changes themselves act as a focus of pain sensation.

1.2.3 Laminar organisation of primary afferent projections in the rat spinal cord.

Most nociceptive primary afferent fibres enter the spinal cord via the dorsal roots, each of which is thought to represent the sensory channel of one body wall segment. The cell bodies of primary afferent fibres are located in the dorsal root ganglion, which contains both large (60 to 100 μm) and small (14 to 30 μm) cells. The small unipolar cells give rise to small myelinated and unmyelinated axons, whereas the large cells are connected to larger myelinated fibres. After leaving the dorsal root ganglion, axons fork into a peripheral fibre, which adjoins with a sensory receptor in skin, viscera or muscle, and a centrally directed fibre, which enters the spinal cord (Fig. 1.2).

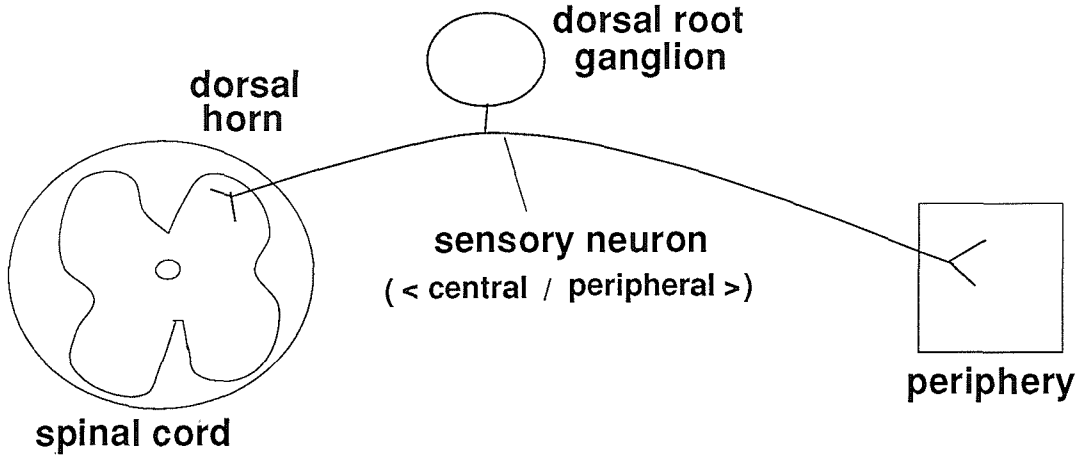


Fig. 1.2: Schematic diagram to show that from the dorsal root ganglia, the axon forks into a peripheral fibre and a central fibre. Adapted from Dray et al. (1994) *Trends Pharmacol. Sci.* **15,** 190-197.

Dorsal roots in the rat are grouped into pairs comprising of 31 segments which consist of 8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3 caudal (Fig. 1.3). The number of dorsal

root ganglion cells in single pairs of dorsal roots may vary considerably between the two sides (Ygge et al. 1981). A similar variation may therefore be expected to exist in the number of dorsal root axons, but their actual number can be assumed to be larger than the number of ganglion cells (Langford and Coggeshall 1979).

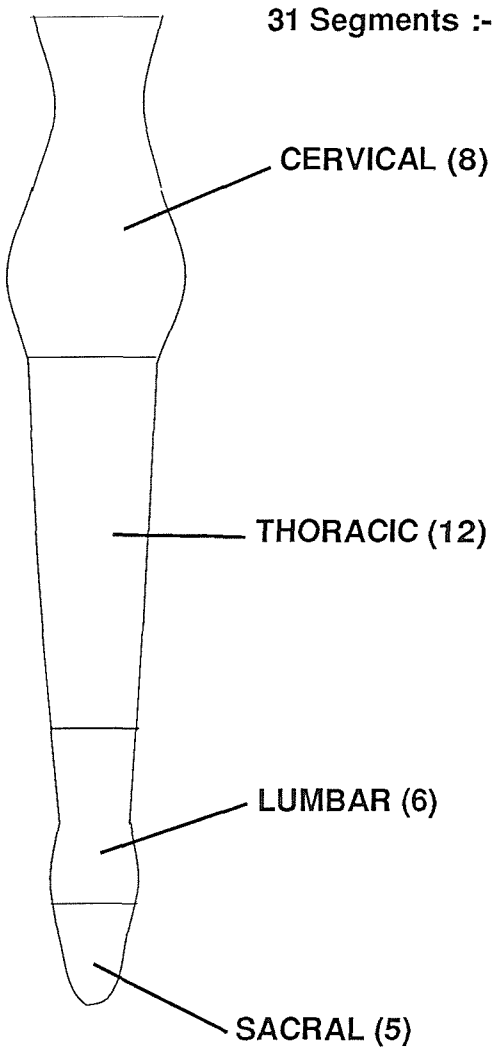


Fig. 1.3: The rat spinal cord comprises 31 segments of which 8 are contained in the cervical region, 13 in the thoracic, 6 in the lumbar, 4 in the sacral and 3 in the caudal region.

Termination of the primary afferents in the spinal grey matter largely follow two principles of organisation in the rat. First, fine calibre fibres distribute in superficial laminae of the dorsal horn, whereas coarse calibre fibres distribute more ventrally (Light and Perl 1979a). Second, the primary afferents terminate somatotopically along a mediolateral axis in the dorsal horn (Ygge and Grant 1983).

Laminae I-IV, near the apex of the dorsal horn, are receptive zones for cutaneous afferents. Both laminae I and II (substantia gelatinosa) receive unmyelinated as well as fine myelinated dorsal root afferents in the rat (Nagy and Hunt 1983). Lamina I is a major target for small diameter myelinated and unmyelinated nociceptive and visceral afferents whereas lamina II receives projections from small diameter afferents signalling both noxious and innocuous stimuli. Within lamina II, the finely myelinated afferents, supposedly Aδ fibres with high threshold mechanoreceptors, appear to terminate preferentially in the superficial parts (Nagy and Hunt 1983). Some fine myelinated fibres, presumably D-hair, may in addition, reach the deepest part of lamina II, from the more ventrally located lamina III. The unmyelinated, C fibre, afferents have been claimed to have their main termination slightly deeper than the first group of fine myelinated fibres, within lamina II (Nagy and Hunt 1983).

The dorsal root afferents to the deep parts of the dorsal horn, which include laminae II-V and the enlargements in lamina VI, are, generally, of coarser calibre than those to the superficial laminae. Exceptions to this seem to be certain types of afferents, including visceral afferents, terminating in lamina V (Cervero and Connell 1984; Light and Perl 1979b). Laminae III-VI receive projections from myelinated mechanoreceptors and muscle afferents: laminae V also contains terminations of high threshold mechanoreceptors and visceral afferents. Figure 1.4 below illustrates the neuronal organisation of the superficial dorsal horn.

The larger diameter primary afferents enter the dorsal horn from the dorsal funiculus and have branches with a recurrent course distributing arborizations in lamina III and the most ventral part of lamina II. Lamina IV has been found to have a dense plexus of primary afferent fibres at thoracic levels in the rat (Smith 1983), but it is clear that lamina IV receives primary afferents also at other levels of the spinal cord (Taylor et al. 1982). Lamina V and VI also receive primary afferent projections in the rat and at least part of the projection to lamina VI has been shown to be derived from muscle afferents (Smith 1983).

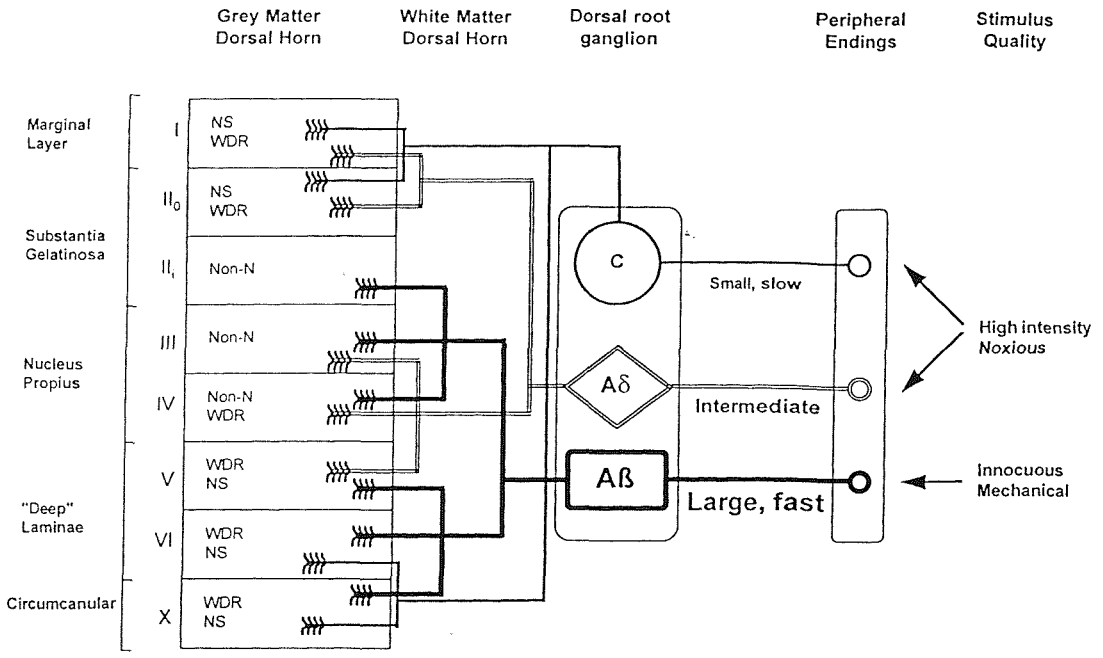


Fig 1.4: Schematic diagram of the neuronal organisation of the superficial dorsal horn. The organisation of cutaneous, primary afferent input to the dorsal horn of the spinal cord is shown. NS=nociceptive-specific; WDR=wide-dynamic range; Non-N=non-nociceptive. From Millan (1999) *Prog. Neurobiol.* **57,** 1-164.

Laminae VII and IX, have been shown to be major targets of muscle spindle primary afferents in the rat and, therefore, also receive primary afferent projections from muscle nerves (Smith 1983). The sacral parasympathetic nucleus, at the dorsolateral border of lamina VII and located in L6 and S1 in the rat, has been shown to have visceral primary afferent connections (Nadelhaft and Booth 1984). Lamina X, including the dorsal commissural nucleus, has also been shown to receive visceral primary afferents in the rat (Nadelhaft and Booth 1984) as well as input from high threshold afferents.

Within the developing spinal cord the somatotropic arrangement of Aβ and C fibre afferent terminals is established early in development. However, the laminar projections are not fully developed until the end of the third postnatal week (Fitzgerald et al. 1994). Aβ fibres in the developing spinal cord project throughout laminae I to V, including the substantia gelatinosa (lamina II). This widespread termination of Ab fibres is gardually withdrawn over the first three postnatal weeks. At the end of the third postnatal week, the terminal field becomes restricted to the normal laminae III to V (Fitzgerald et al. 1994). The widespread large Aβ fibre terminations in the developing spinal cord make synaptic

contacts with postsynaptic targets that process nociceptive information. Therefore, non-nociceptive information will cause a nociceptive sensation in neonates (Fitzgerald et al. 1994). The small unmyelinated C fibres in the developing spinal cord grow specifically to laminae I and II, so that for a considerable postnatal period, the substantia gelatinosa is occupied by both Aβ and C fibre terminals (Fitzgerald and Jennings 1999).

It is apparent from the above that the principle of organisation of the spinal cord primary afferent fibres, which states that fine calibre fibres terminate in superficial laminae of the dorsal horn and coarse calibre fibres terminate more ventrally in the spinal gray matter, is not an absolute one. Fine calibre primary afferents are found in lamina V and visceral fibres, terminate both in laminae VII and X. This led to the proposal in 1979 by Light and Perl that a highly specialised central projection of primary afferent endings related to sensory function and not to fibre diameter occurs in the spinal cord.

1.2.4 Projection neurones.

Second order projection neurones in the dorsal horn, as well as some in the ventral horn and central canal region, project to supraspinal structures through several tracts. These include the spinothalamic, spinoreticular, and spinomesencephalic tracts that ascend the spinal cord in the contralateral anterolateral quadrant.

Spinothalamic tract: The importance of the spinothalamic tract is that it appears to be the main pathway for information from receptors signalling pain and temperature. It consists of the ascending axons of neurones located in the gray matter of the opposite half of the cord. The cells of origin are mostly in lamina V, although smaller numbers are present in laminae I, VII, and VIII. The axons cross the midline in the ventral white commisure close to the central canal and then transverse the ventral horn to enter the ventrolateral and ventral funiculi. The fibres of the spinothalamic tract end in the thalamic nuclei. As they pass through the brain stem, these axons give off collateral branches to the reticular formation in the medulla and pons and to the periaqueductal gray matter in the midbrain. In the rat, spinothalamic cells have laminar locations in the spinal cord which depend on

their terminations in the thalamus. This difference in laminar location seems to be correlated with a difference in afferent input, so that those neurones projecting to the medial thalamus, which are said to belong to the "medial" spinothalamic tract, are activated by deep pressure and joint movement, while those projecting in the "lateral" spinothalamic tract are activated by cutaneous stimuli, both noxious and non-noxious.

Spinoreticular tract: The spinoreticular tract originates in laminae V - VIII. It includes crossed fibres that terminate in the pontine reticular formation and uncrossed fibres that end in the medullary reticular formation. These fibres are involved in the perception of pain and of various sensations originating in the internal organs. In fact, the spinoreticular neurones located in the dorsal horn have similar physiological properties to spinothalamic neurones in the rat: most respond to cutaneous input and have large receptive fields (respond to a wide range of stimuli), while a minority are activated only by noxious mechanical stimuli.

Spinomesencephalic tract: The spinomesencephalic tract originates in laminae I, V and X at all levels of the spinal cord and the fibres terminate in the midbrain reticular formation.

Sectioning of these tracts using an anterolateral cordotomy has been suggested as a useful way of abolishing or relieving pain. However, results are variable and often transient as there is an ipsilateral pathway that progressively takes over from the contralateral spinothalamic pathway after cordotomy.

1.3 Spinal Pain Mechanisms in the Dorsal Horn.

1.3.1 Nociceptive primary afferent terminations.

The dorsal horn of the spinal cord provides the first relay for structures that transmit nociceptive information from the periphery. As described above, primary afferent nociceptors in the rat terminate primarily in laminae I, II and V of the dorsal horn. Here they terminate on several classes of neurones that either transmit or modulate nociceptive signals. The first two classes are projection neurones that transmit information to

supraspinal structures. The first class of projection neurones are termed "nociceptive-specific" and appear to respond preferentially, although not exclusively, to noxious stimuli. The second class of projection neurones are termed "wide dynamic range" and, as their name suggests, they respond to a range of stimuli in the non-noxious and noxious range. The third class of neurones comprise excitatory and inhibitory interneurones within the spinal cord itself. Activation of these interneurones results in either an enhanced or diminished responsiveness to sensory input from the periphery.

1.3.2 Neurotransmitters.

Pharmacological studies have been important in identifying the many neurotransmitters and neuromodulators that act on receptors in the spinal cord dorsal horn. These receptors include opioid (μ, κ, and δ), α-adrenergic, γ-aminobutyric acid (GABA), serotonin (5-HT), adenosine, neurokinin, N-methyl-D-aspartate (NMDA) and non-NMDA receptors such as AMPA (α-amino-3-hydoxy-5-methyl-4-isoxazolepropionic acid) receptors and kainate receptors.

These receptors are located both presynaptically and postsynaptically at the termination of primary nociceptive afferents. Glutamate and Substance P coexist in some primary afferent terminals (Battaglia and Rustioni 1988), and primary afferent stimulation results in release of substance P, glutamate, and calcitonin gene-related peptide, either singly or in combination (co-release). Following release, glutamate acts at AMPA receptors, and substance P acts at neurokinin receptors on the postsynaptic membrane. It appears that non-NMDA receptors such as the AMPA receptor may mediate responses in the physiological processing of sensory information. With prolonged release of glutamate or activation of neurokinin receptors, however, a secondary process occurs that appears to be crucial in the development of abnormal responses to further sensory stimuli. This sustained activation of non-NMDA or neurokinin receptors "primes" the NMDA receptor so that it is in a state ready for activation (Urban et al. 1994).

Fibre Class	Threshold for activation	Principal transmitters	Principal receptors engaged	Neuron types targeted	Sensation mediated	
					Physiological	Pathological
C	High	SP/NKA/NKB CGRP Glutamate	$NK_{1/3}$ $CGRP_{1/2}$ NMDA AMPA mGluR	NS WDR	Noxious	Hyperalgesia Cold Allodynia
Aβ	Low	EAAs	AMPA	NON-N WDR	Non-Noxious	Mechanical Allodynia

Fig 1.5: Comparative properties of primary afferent C and Aβ fibres under normal condidtions showing the principal neurotransmitters involved. SP=substance P; NKA=neurokinin A; CGRP=calcitonin-gene related peptide; EAA=excitatory amino acid; NS=nociceptive specific; WDR=wide dynamic range; NON-N=non-nociceptive. Adapted from Millan 1999.

There is strong evidence that NMDA receptors are involved in a number of phenomena that may contribute to the medium or long-term changes that are observed in chronic pain states. These phenomena include the development of "windup" (Davies and Lodge 1987; Dickenson and Sullivan 1987), facilitation, central sensitisation (Woolf and Thompson 1991; Coderre and Melzack 1992), changes in peripheral receptive fields which may include the recruitment of previously ineffective inputs (Woolf and King 1990), induction of oncogenes (Chapman et al. 1995), and long-term potentiation (Pockett 1995). Long-term potentiation, in particular, refers to the changes in synaptic efficacy that occur as part of the process of memory and may play a role in the development of a cellular "memory" for pain or enhanced responsiveness to noxious inputs. It appears that NMDA antagonists can attenuate these responses, indicating a role for NMDA antagonists in the prevention of chronic pain states.

1.3.3 Central Sensitisation and Windup.

Nociception is a specialised form of sensory signalling which conveys information to the CNS about impending (or actual) tissue damage. Pain perception is related to heightened sensitivity to sensory information from the region of the body which experienced the *nociceptive stimuli.* This phenomenon is termed sensitisation and it consists of an increased neuronal responsiveness set up by sustained noxious stimulation. Once sensitisation has developed it may last for long periods and is characterised by enhanced responses to weaker stimuli and continues even when the initial nociceptive stimuli ceases. A major part of the sensitisation to pain occurs in the spinal cord and is termed the "central component" (Woolf 1983). Although studies on spinothalamic tract neurones indicate that there are other areas of the CNS that contribute to central sensitisation (Dougherty and Willis 1992; Lin et al. 1996), most research on the mechanisms of pain-induced sensitisation focus on the spinal cord.

Changes occur in the periphery after trauma that lead to the phenomena of peripheral sensitisation and primary hyperalgesia. However, the sensitisation that occurs can only be partly explained by the changes in the periphery. After injury, there is also an increased responsiveness to normally innocuous mechanical stimuli (allodynia) and a

zone of "secondary hyperalgesia" in uninjured tissue surrounding the site of injury. These changes are believed to be a result of central sensitisation (Woolf and Thompson 1991). These changes indicate that, in the presence of pain, the central nervous system is not hard-wired, but plastic. A barrage of nociceptive input, such as that which occurs with surgery, results in changes to the response properties of dorsal horn neurones and increases their excitability (Woolf 1983).

Central sensitisation in the spinal cord manifests as *"a decrease in the threshold and an increase in the responsiveness and size of the cutaneous receptive fields of dorsal horn neurones"* (Sivilotti and Woolf 1994). Changes which have been noted to occur in the dorsal horn with central sensitisation include an expansion in the receptive field size (Woolf and King 1990). There is also an increase in the magnitude and duration of the response to stimuli that are above the C fibre threshold level and a reduction in threshold so that stimuli that are not normally noxious activate neurones that would normally transmit nociceptive information. The experimental model used for the study of central sensitisation was developed by Woolf and his colleagues (Woolf 1983; Woolf and Wall 1986; Thompson et al. 1990). However, it was first observed by Mendell and Wall (1965) who found that when a peripheral nerve was stimulated at sufficient intensity to activate C fibres, repetition of the same stimulus at low frequencies resulted in a progressive build-up in the amplitude of the response. They gave the phenomenon the vivid name "windup". Windup is the increase in the number of spikes generated by a neuron after each successive stimulus (of appropriate strength) during a pulse train (Fig 1.6). It has been demonstrated that a noxious stimulus at a level sufficient to activate C fibres not only activates dorsal horn neurones, but also results in a progressive increase in the neuronal activity throughout the duration of the stimulus (Mendell 1966). Therefore, with nociceptive input, there is not a simple stimulus-response relationship, but a "windup" of spinal cord neuronal activity (Davies and Lodge 1987; Dickenson and Sullivan 1987).

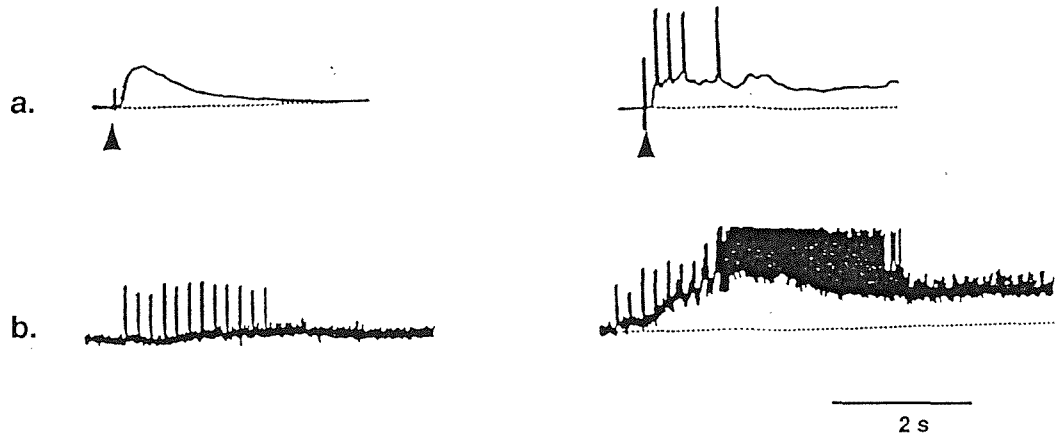


Fig 1.6: Low frequency stimulation response (a) and the response to high frequency stimulation (b) which causes an activity dependent hyperexcitability known as windup. From Urban et al. (1994) *Trends Neurosci.* **17,** 432-438.

The definition of windup is similar to sensitisation and both appear to be related to nociception although the two are not equivalent. Windup only occurs in a particular and artificial situation, in response to a slowly repeated stimulus with a frequency of greater than 0.3Hz, which synchronously activates many C-fibres (Fig. 1.7).

Central sensitisation is more general than windup and can be produced asynchronously by the activation of skin, joint, muscle or visceral afferents in response to chemical irritants, heat, inflammation, nerve ligation etc. Central sensitisation produces no detectable pattern of progressively increasing action potential discharge (Woolf 1996). The synaptic processes that produce windup are sufficient to produce central sensitisation and therefore, windup is likely to lead to the development of central sensitisation (Wall and Woolf 1986; Woolf and Wall 1986). However, the assumption that central sensitisation does not occur in the absence of windup is incorrect. Windup can initiate central sensitisation because it produces an elevation of intracellular calcium not because of the progressive increase in action potential discharge (Woolf 1996).

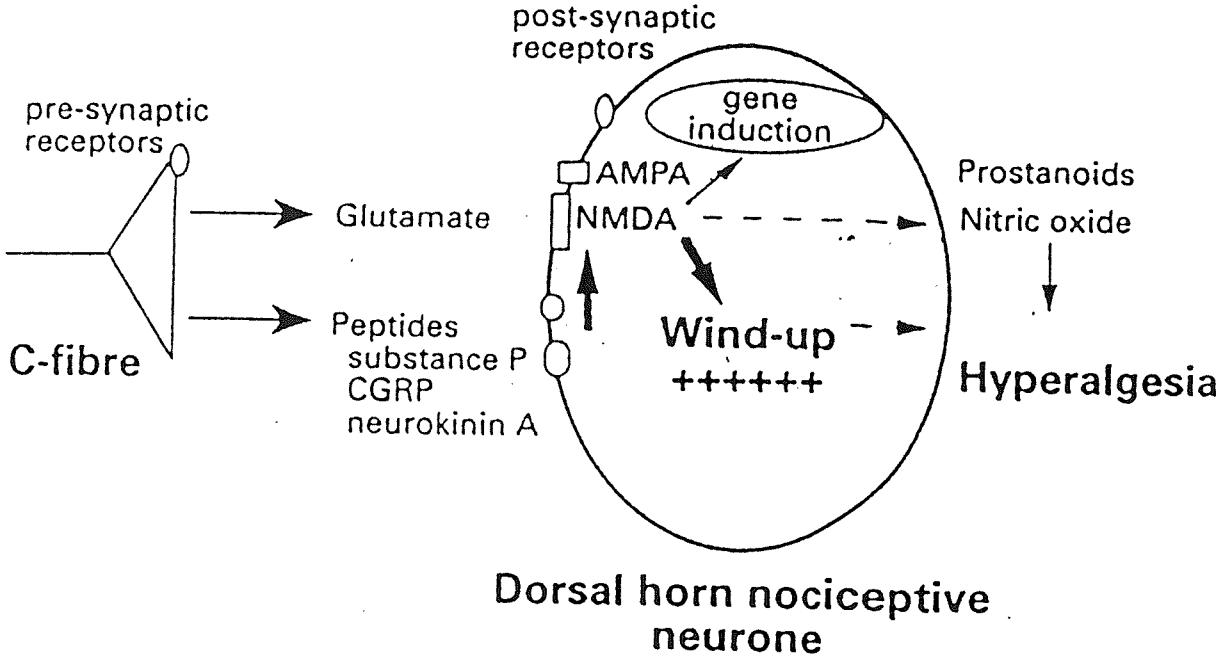


Fig. 1.7: Diagram illustrating the release of excitatory transmitters from C fibres and the subsequent effects on a dorsal horn nociceptive neuron. From Dickenson (1995) *Brit. J. Anaesth.* **75**, 193-200.

The major distinction between central sensitisation and windup is that windup does not persist following a conditioning stimulus whereas central sensitisation is very long lasting. Windup is more analogous to being one particular component of a highly complex pathway which will ultimately lead to hyperalgesia. Evidence suggests there is a relationship between central sensitisation and windup (Cook et al. 1997) but the ability of windup to trigger central sensitisation has only recently been investigated by Li et al. (1999) using receptive fields of wide dynamic range neurones. This study concluded that although central sensitisation and windup are separate phenomenons, they share several mechanisms and stimuli which cause windup also produce central sensitisation. Stimulation at 0.5Hz, sufficient to induce windup, caused an expansion of the receptive field size which also occurs after central sensitisation. However, central sensitisation is associated with an increase in response to Aβ fibre activity. This is thought to contribute to allodynia, and windup did not alter responses to stimulation of Aβ fibres. It was therefore concluded that windup is not sufficient to produce all the characteristics of central sensitisation. However, it remains a useful tool for investigating mechanisms which lead to central sensitisation and hence to chronic pain states. Figure 1.8 shows the mechanism by which windup is thought to trigger central sensitisation. It explains why NMDA receptor antagonists continue to be active once central sensitisation has been established.

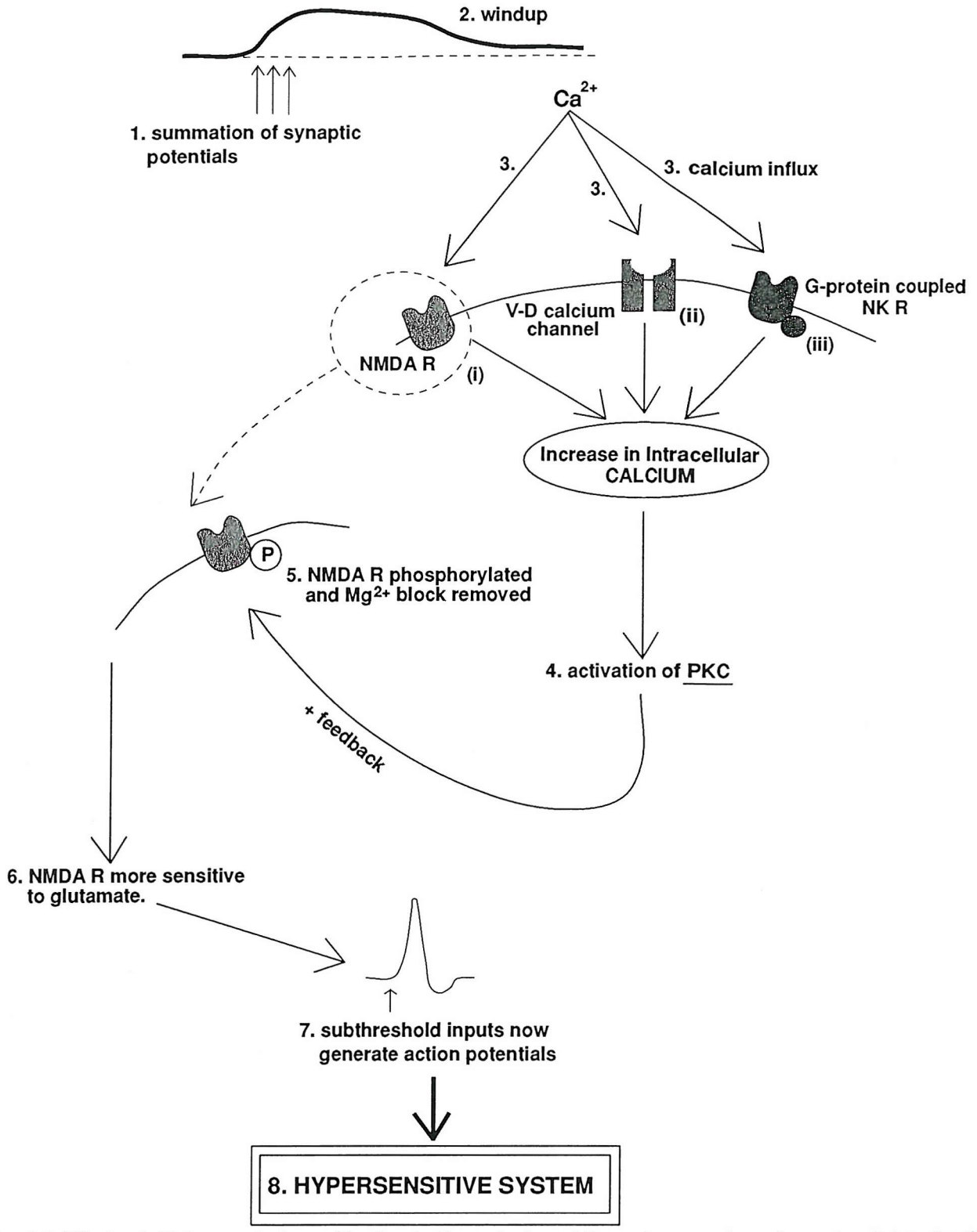


Fig. 1.8: Windup initiates central sensitisation in the spinal cord through a number of events. Primarily, the summation of synaptic potentials causes windup. The resultant cumulative depolarisation produces a substantial increase in the intracellular calcium via (i) the activation of NMDA receptor channels, allowing a direct calcium influx, (ii) the activation of voltage-dependent calcium channels, and (iii) the activation of G-protein coupled neurokinnin (NK) receptors which cause the release of calcium through intracellular stores. The increase in calcium causes the activation of protein kinases (in particular protein kinase C). PKC then phosphorylates the NMDA receptor which partially removes the Mg**2+** channel block causing an increased glutamate sensitivity and a generation of an inward current. Subthreshold inputs now generate action potentials and the whole system becomes hypersensitive, although NMDA antagonists will continue to inhibit the sensitised NMDA receptor and hence inhibit the hypersensitive system.

Central sensitisation can also manifest itself as a change in synaptic efficacy following inflammation which is generated by Aβ sensory neurones innervating the inflammed area. Aβ fibre input in inflammed animals generates an action potential after-discharge in dorsal horn neurones which only Aδ and C fibres normally evoke (Neumann et al. 1996). Aβ fibre-mediated ventral root potentials have also been shown to produce windup, normally only seen in C fibres, in an *in vitro* spinal cord preparation from inflammed neonatal rats (Thompson et al. 1994). These changes in synaptic efficacy are a result of an increased sensitivity of Aδ and C fibres following inflammation which causes a phenotypic switch of Aβ fibres to a phenotype resembling pain fibres as they begin to express substance P, thereby enhancing synaptic transmission in the spinal cord and exaggerating the central response to innocuous stimuli (Neumann et al. 1996). Nerve injury and the resulting phenotypic switch of Aβ fibres also results in the terminals of myelinated afferents sprouting into neighbouring regions of the dorsal horn. Thus, the central changes involved in inflammation may result in the facilitation of Aβ-mediated synaptic input to neurones in the superficial dorsal horn, especially lamina II. This means that nerves which do not normally transmit pain sprout into a more superficial region of the dorsal horn, which normally acts as a relay in pain transmission (Woolf et al. 1992). The novel recruitment of low threshold Aβ-evoked synaptic potentials into areas which predominantly consist of Aδ and C fibres alters sensory processing and contributes to abnormal hypersensitivity. If functional contact is made between these terminals that normally transmit non-noxious information and neurones that normally receive nociceptive input, this may provide a framework for the pain and hypersensitivity to light touch that is seen after nerve injury. These pathophysiological changes may also be important in acute pain states as well as chronic pain states.

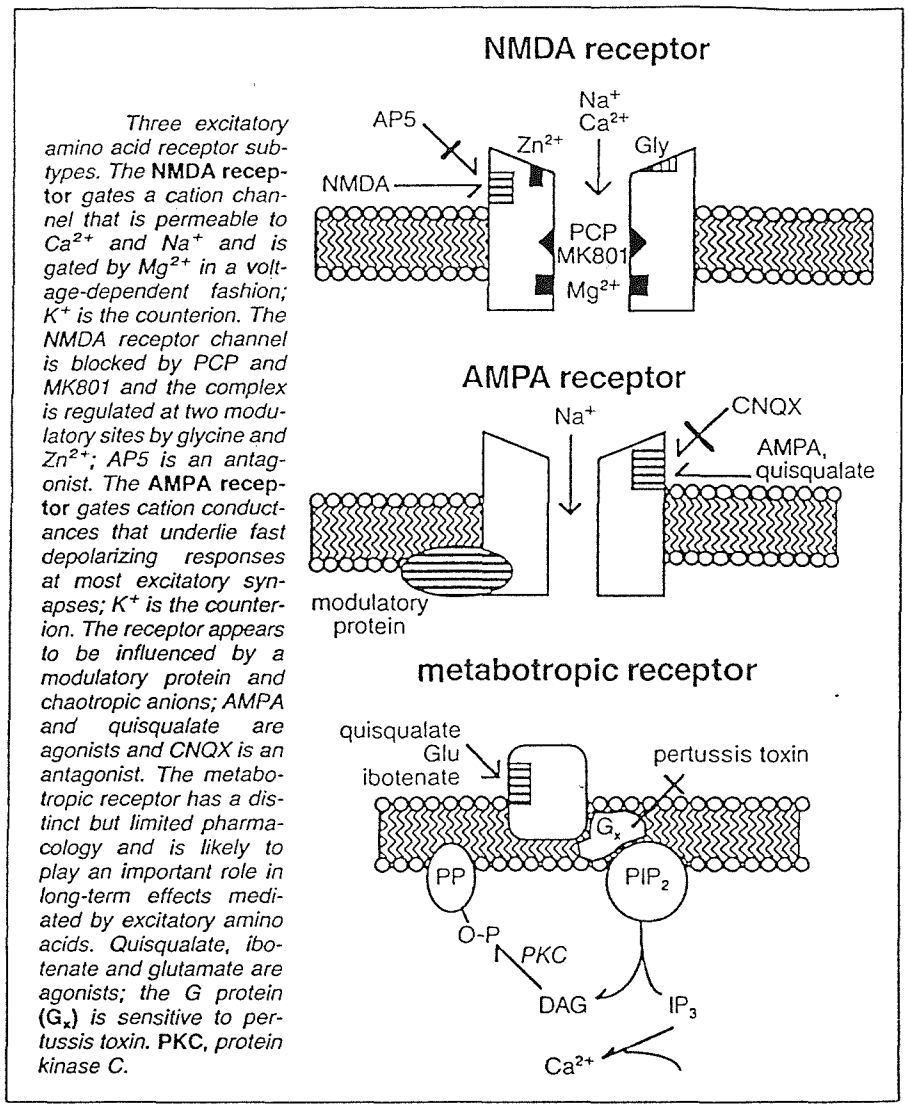
There are two possible mechanisms for the recruitment of fast Aβ fibre-evoked synaptic responses in the substantia gelatinosa. The first mechanism could be that pre-existing inefficient or silent synapses are strengthened and the second is a possible establishment of novel synaptic connections by a structural alteration in synaptic connectivity.

Ineffective synapses result from a small postsynaptic current that fails to depolarise the cell to threshold for an action potential or from a cell with normal postsynaptic current but an increased threshold for action potentials. Silent glutamatergic synapses have been

reported in various parts of the central nervous system (Malenka and Nicoll 1997). However, the existence of silent glutamatergic synapses in the spinal cord has only recently been reported (Li and Zhuo 1998). The neurotransmitter 5HT, important in descending modulation of nociceptive processing, transforms silent glutamatergic synapses into functional ones. Transformation of silent glutamatergic synapses may therefore be part of a cellular mechanism for central plasticity in the spinal cord. Recruitment of silent synapses on a wide dynamic range cell enhances its response to sensory stimuli, especially non-noxious stimuli. Recruitment on a nociceptive-specific neuron may change the phenotype and cause a response to non-noxious stimuli. Understanding the mechanism by which silent glutamatergic receptors become functional will contribute to the design of new drugs to alleviate chronic pain.

1.4 Excitatory Amino Acids in the Spinal Cord.

Since the discovery that glutamate exerts a powerful excitatory action on neurones (Curtis and Watkins 1950s) L-glutamate has been considered to be the major excitatory neurotransmitter in the CNS (Fagg and Foster 1983; Fonnum 1984). Most neurones use the excitatory amino acid glutamate for communication with other neurones. As has been previously mentioned, glutamate plays a key role in the spinal cord along with peptides, in determining the levels of pain transmission. In the dorsal horn, the metabotropic, AMPA and NMDA receptors for glutamate have distinct actions (Fig. 1.9).



Three excitatory amino acid receptor subtypes. The **NMDA receptor** *gates a cation channel that is permeable to Ca^{2+} and Na^{+} and is gated by Mg^{2+} in a voltage-dependent fashion; K^{+} is the counterion. The NMDA receptor channel is blocked by PCP and MK801 and the complex is regulated at two modulatory sites by glycine and Zn^{2+}; AP5 is an antagonist. The* **AMPA receptor** *gates cation conductances that underlie fast depolarizing responses at most excitatory synapses; K^{+} is the counterion. The receptor appears to be influenced by a modulatory protein and chaotropic anions; AMPA and quisqualate are agonists and CNQX is an antagonist. The metabotropic receptor has a distinct but limited pharmacology and is likely to play an important role in long-term effects mediated by excitatory amino acids. Quisqualate, ibotenate and glutamate are agonists; the G protein (G_x) is sensitive to pertussis toxin.* **PKC**, *protein kinase C.*

Fig. 1.9: Diagram to show the three different subtypes of excitatory amino acid receptors. From Young and Fagg (1990) *Trends Pharmacol. Sci.* **11,** 126-133.

1.5 Ionotropic Glutamate Receptors

Ionotropic glutamate receptors can be divided into two groups: N-Methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. Both are coupled to ion channels, though their conductances and ion selectivites are very different. Of the four different glutamate receptor subtypes, the NMDA receptor channel is the most clearly defined. This is because of the development of highly selective ligands such as N-methyl-D-aspartate (NMDA) (Watkins 1962) and D-2-amino-5-phosphono-valerate (D-AP5) (Davies et al. 1981). The NMDA receptor differs from the non-NMDA receptors in some very important

ways. First, the NMDA glutamate receptor is highly permeable to calcium (Ascher and Nowak 1986; MacDermott et al 1986). It also has a voltage-dependence which is brought about by a magnesium ion in the receptor channel that is only removed with sufficient depolarisation (Mayer et al 1984; Nowak et al. 1984). The NMDA receptor also requires glycine as a co-agonist (Johnson and Ascher 1987; Thomson 1989) (Fig 1.10). The non-NMDA receptors, in contrast, do not gate calcium, have a greater selectivity for sodium and potassium and are not voltage-dependent (Monaghan et al. 1989).

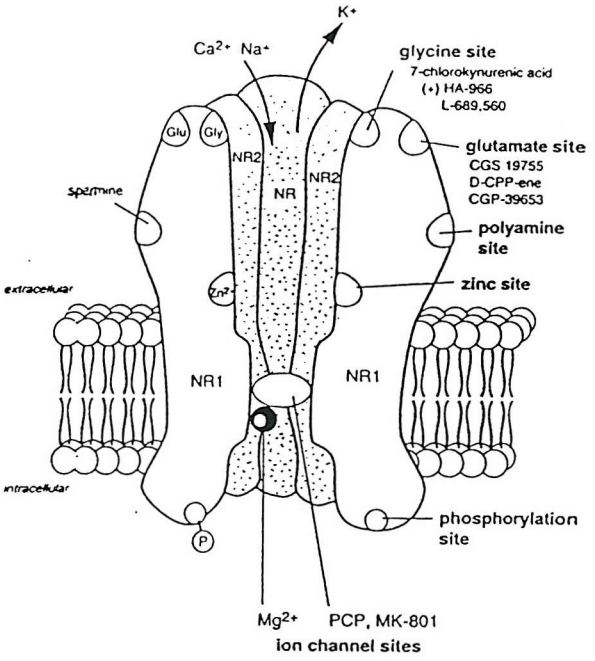


Fig. 1.10: Diagram of the NMDA receptor showing sites for antagonist action. From Leeson and Iversen (1994) *J. Med Chem.* **37**, 4053-4064.

Non-NMDA receptors are further divided into AMPA and kainate receptors by their selective binding of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) (Cotman and Iversen 1987). Like NMDA receptors they gate monovalent ions (Na^+ and K^+) but unlike NMDA receptors they have a low permeability to calcium (Seeburg 1993). AMPA receptors were initially characterised by rapid activation kinetics and fast desensitisation, and kainate receptors were differentiated from AMPA receptors in that they were non-desensitising (Mayer and Westbrook 1987). Studies using molecular biology have indicated that all non-NMDA glutamate receptor channels can be assembled from structurally homologous subunits which can be grouped into subfamilies according to sequence characteristics (Sommer and Seeburg 1992). On the basis of ligand affinity, the GluR A-D (GluR 1-4) subunits are constituents of AMPA receptor

channels (Kienanen et al. 1990; Boulter et al. 1990; Nakanishi et al. 1990; Sakimura et al. 1992), while the GluR 5, GluR 6, GluR 7 and KA subunits form recombinant receptors with a high affinity for kainate (Bettler et al. 1990; Herb et al. 1992; Sommer et al. 1992). However, kainate can gate AMPA receptors and AMPA can gate kainate receptors formed by particular combinations of the high-affinity kainate receptor subunits (Sommer and Seeburg 1992).

A subpopulation of AMPA receptors have been found which are permeable to calcium (Kyrozis et al. 1995). These calcium permeable AMPA receptors were found to induce reversible inhibition of NMDA receptors which was mediated via an increase in the intracellular calcium concentration (Kyrozis et al. 1995). Indeed, calcium entry through the calcium permeable AMPA receptors alone was found to be sufficient for NMDA receptor desensitisation (Kyrozis et al. 1995). The GluR2 appears to be the dominant determinant of calcium permeability for AMPA receptors (Ozawa and Rossier 1996) and it is the GluR2 subunit which confers low calcium permeablility (Petralia et al. 1997). Calcium permeable AMPA receptors are often co-localised with NMDA receptor channels on dorsal horn neurons indicating that the calcium mediated interaction between AMPA and NMDA receptors may occur within small dendritic domains (Petralia et al. 1997).

The NMDA glutamate receptor appears to be involved in complex physiological phenomena in the CNS including long-term potentiation (Nicoll et al. 1988). The high calcium permeability of the NMDA receptor underlies its role in all these phenomena, the increase in intracellular calcium being thought to trigger the biochemical processes responsible. The cloning of cDNA encoding rat NMDA receptor subunits has revealed five subfamilies of subunits designated as NMDA NR1 and NR2A-D (Ishii et al. 1993). The NMDA NR1 subunit yields eight possible splice variants by three alternative splicing events (Mori and Mishina 1995; Sugihara et al. 1992), and is the essential component for the formation of functional NMDA receptors (Mori and Mishina 1995).

The NMDA receptor channel complex has a number of regulatory sites that are targets for modulation by endogenous as well as exogenous compounds. The regulatory sites (Figure 1.11) include a binding site for the endogenous agonist glutamate (and the synthetic agonist NMDA), a high-affinity binding site at which glycine acts to allow agonist-

induced channel opening, which is different from the strychnine-sensitive, chloride-permeable, glycine receptor; and sites within the channel lumen where magnesium and phenylcyclidines (PCP, MK-801, TCP, Ketamine) bind to produce a voltage-dependent open channel block. There is an additional site or sites where Zn^{2+} acts to inhibit allosterically the agonist-induced response in a voltage-independent manner. The endogenous polyamines, spermine and spermidine, allosterically potentiate the response to NMDA acting on an additional site. (For refs. see Lerma et al. 1998). Studies investigating the localisation of NMDA receptor subunit mRNAs in the spinal cord indicate that NMDA receptors are abundant in the substantia gelatinosa of the dorsal horn (Furuyama et al. 1993) and that the functional receptors consist of mainly NMDAR1 and NMDAR2B subunits (Yung 1998).

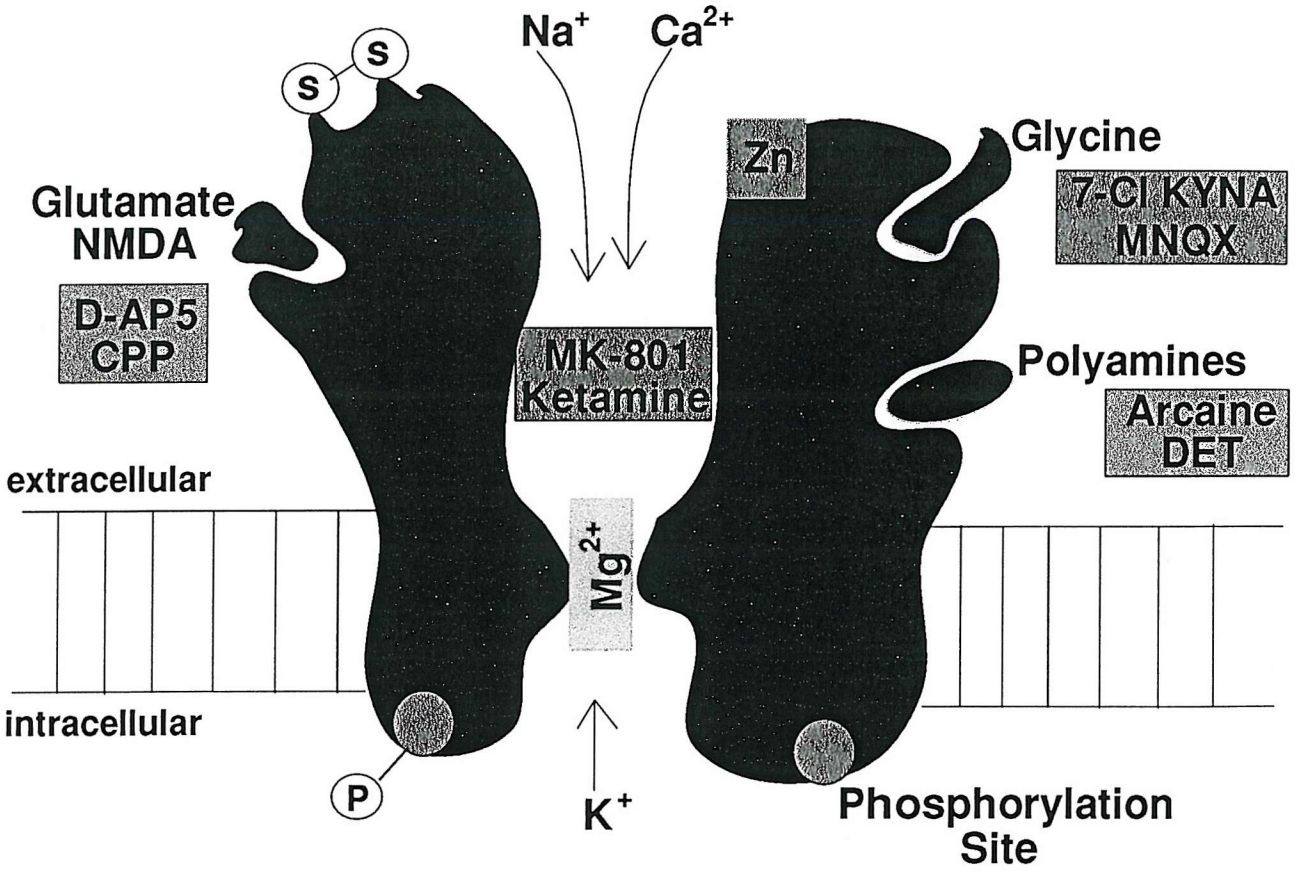


Fig. 1.11: The NMDA receptor complex and its sites of modulation. At least six sites of modulation can be distinguished on the NMDA receptor: the agonist site, where glutamate and NMDA bind to operate the channel; the glycine site, where glycine acts to allow channel opening and to decrease desensitisation; the polyamine site, where spermine and spermidine act to regulate desensitisation and glycine affinity; the channel, where Mg2+ and phenylcyclidines (PCP, MK-801, ketamine) block permeation in a voltage-dependent manner. In addition, there is a site (S-S) which confers receptor sensitivity to redox agents. Antagonists at each site are indicated within shaded boxes. Modified from Lerma et al. (1998) Excitatory amino acid-activated channels. In *Ion Channel Pharmacology* (Ed. Soria, B. and Cena, V.) Oxford University Press.

The neurophysiological functions of the ionotropic glutamate receptors can be summarised as follows: AMPA receptors, found in the majority of excitatory synapses, mediate almost all fast excitatory neurotransmission. Their fast kinetics make them ideally suited for this purpose. The excitatory post-synaptic potential (EPSP) mediated by NMDA receptor channels displays a prolonged time course relative to that mediated by non-NMDA channels due to the slower kinetics of the channel (Dale and Roberts 1985). In NMDA channels, calcium flux is an important consequence of glutamate activation. This receptor-mediated calcium signal can trigger different processes ranging from movement of neuronal growth cones to an activity dependent resetting of the synaptic strength underlying some forms of learning and memory. In the spinal cord, NMDA receptors are involved in phenomena such as windup (Dickenson and Sullivan 1987; Davies and Lodge 1987) and central sensitisation (Woolf and Thompson 1991; Coderre and Melzack 1992) and are responsible for the maintenance of persistent pain states.

1.5.1 Ionotropic Glutamate Receptors and Nociception:

Numerous findings suggest that spinal cord NMDA receptors play a role in the development and expression of acute and chronic pain states (Wong et al. 1995; Yaksh et al. 1995; Dickenson et al. 1997). The first experimental evidence for the involvement of excitatory amino acids in nociception came from behavioural studies in which NMDA agonists induced nociceptive effects and the competitive NMDA antagonist D-AP5 had analgesic effects (Cahusac et al. 1984). Other evidence for the physiological contribution of the NMDA receptor to spinal nociception was shown with the use of subcutaneous formalin as an inflammatory stimulus (Haley et al. 1990). The spinal nociceptive neuronal and behavioural responses to formalin were found to be reduced by blocking NMDA function. It is only the prolonged, second phase of the formalin response, where tissue damage occurs, that is affected by NMDA receptor inhibition (Coderre and Melzack 1992; Haley et al. 1990; Dickenson and Aydar 1991; Hunter and Singh 1994; Millan and Seguin 1994). This indicates that acute pain can be distinguished from inflammatory pain by sensitivity to NMDA antagonism.

NMDA receptors are found in the spinal cord of all species including man. If a C-fibre

stimulus is maintained with enough frequency and intensity to activate NMDA receptors, the resultant amplification and prolongation of the response will underlie many forms of central hyperalgesia (Coderre et al 1993; Dickenson and Sullivan 1990; Dray et al 1994; Dubner and Ruda 1992; McMahon et al. 1993; Woolf 1994). The channel of the NMDA receptor is normally blocked by magnesium so that acute noxious inputs into the spinal cord pain circuits are transmitted via the AMPA and not the NMDA receptor, so a baseline level of transmission is established. The conditions required for NMDA receptor activation are much more complex and only occur by repeated C-fibre activation (Dickenson and Sullivan 1987; Dickenson and Sullivan 1990). Activation of the NMDA receptor requires not only the release of glutamate and its binding to the receptor but also the presence of glycine and the means to remove the magnesium block of the channel, which can only be overcome by sufficent depolarisation of the membrane. If the painful stimulus is continued, peptides start to accumulate and it is likely that the depolarisation produced by the co-release of tachykinins with glutamate from C-fibres cause peptide receptor-mediated depolarisations which remove the magnesium block of the NMDA channel (Dickenson and Sullivan 1987; Urban et al. 1994). Thus, the cooperation between these spinally released peptides and glutamate activates the NMDA receptor. Once the magnesium block is removed, and the NMDA receptor is activated, massive neuronal depolarisations occur resulting from the increased calcium influx into the cell. This causes a delayed increase in activity that is superimposed on the baseline activity and so marked increases occur in the level of excitability of the neuron.

The first evidence that NMDA receptors were also involved in the generation of central hypersensitivity was when Woolf and Thompson (1991) showed that high frequency stimulation of C fibres causes a marked and prolonged increase in the flexion withdrawal reflex in rats. These experiments provided the first evidence for the existence of central sensitisation. NMDA receptor antagonists have since been shown to prevent the sensitisation of spinal cord neurones evoked by high-frequency stimulation of sensory fibres (Liu and Sandkuhler 1995), by experimental nerve injury (Seltzer et al. 1991; Leem et al. 1996) and by the treatment of sensory nerves with noxious agents (Woolf and Thompson 1991; Neugebauer et al. 1993a; Ma and Woolf 1995; Chaplan et al 1997). Also, spinal application of NMDA receptor antagonists reduce pain-related behaviours that are evoked by noxious agents or peripheral nerve injury (Coderre and Melzack 1992;

Kristensen et al. 1992, 1993; Mao et al. 1993; Rice and McMahon 1994; Eisenberg et al. 1994,1995; Chaplan et al. 1997).

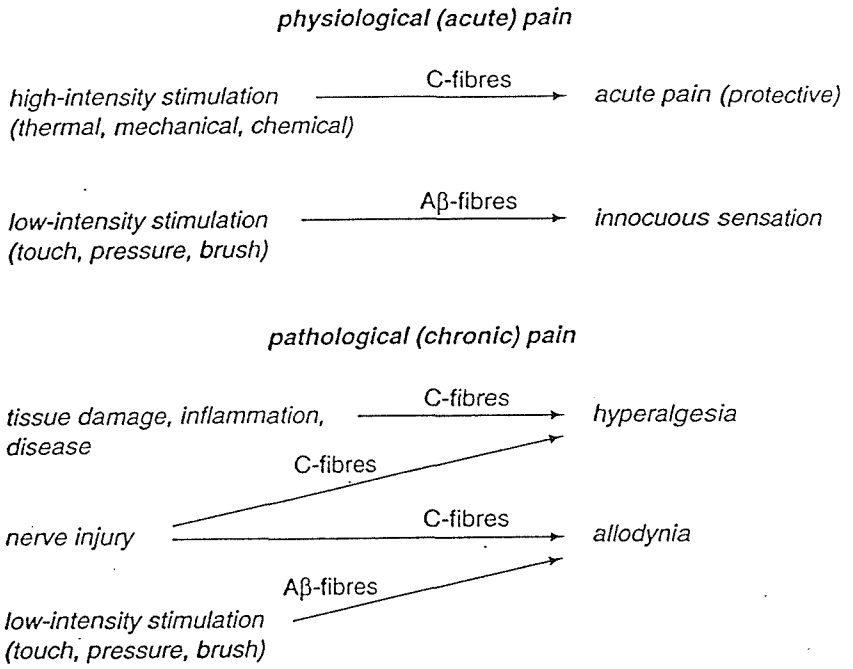
In addition, other studies provided evidence for a contribution of the NMDA receptor to the central hyperalgesia associated with the carrageenan model of inflammatory pain (Eisenberg et al. 1994; Ren et al. 1992). A common role of the NMDA receptor has now been found regardless of the inflammatory model (Schaible et al. 1991). Hypersensitivity in models of peripheral and central ischemia is also reduced by NMDA antagonism (Sher and Mitchell 1990). In all these cases, NMDA antagonists remove the hyperalgesia without influencing the baseline responses, so that activity is markedly reduced without being completley abolished. This concurs with the hypothesis that the NMDA receptor enhances rather than transmits noxious information.

Nerve injury pain (neuropathic pain) is another common clinical problem. Studies in animals with peripheral nerve damage provide evidence for a role of NMDA receptors in these neuropathic pain states. The initial discharges resulting from the injury involve windup and consequently NMDA activation which contribute to pain states associated with nerve injury (Seltzer et al. 1991). Again, the use of antagonists of the NMDA receptor has shown that the hyperalgesia in animal models of nerve injury as well as allodynia (touch-evoked pain) are mediated by the receptor (Bennett 1994; Mao et al 1993; Yaksh 1989).

There is therefore evidence for an involvement of the NMDA receptor in all types of persistent clinically important pains, namely inflammatory pain, neuropathic pain, allodynia and ischemic pain (Fig. 1.12). Since NMDA systems seem to be implicated in pathological rather than acute physiological pain, they may be useful in the treatment of chronic clinical pain. NMDA antagonists have the potential not to abolish pain, but to prevent central hypersensitive states associated with chronic clinical pain states.

NMDA activation may be a site through which further profound changes in nociceptive processing occur. The influx of calcium into neurones through the NMDA channel can induce transcription of certain immediate early genes such as c-*fos* (Wisden and Hunt 1989). It is possible that NMDA is involved in triggering the expression of c-*fos* since the NMDA receptor gates calcium, and it is this cation which is thought to be the intracellular

mediator of transcriptional activation (Morgan 1991). The role of NMDA receptors in the induction of c-*fos* is particularly interesting in view of the evidence for the involvement of NMDA receptors in the production of C-fibre evoked slow depolarisations and in the sensitisation to the repeated C-fibre stimulation in the spinal cord, both of which are likely to contribute to post-injury pain states. This evidence suggests that NMDA receptors are exaggeratedly activated during chronic pain states, and contribute to the development of hyperalgesia at the spinal level.



Under normal **physiological** *conditions, nociceptive signals generated by* **C-fibres** *initiate* **acute pain** *and a* **protective** *reflex. Activation of low-threshold* **Aβ-fibres** *by innocuous stimuli does not induce pain. In* **pathological pain** *following various types of injury or nerve damage, C-fibres become hypersensitive and induce hypersensitivity in sensory pathways, resulting in an enhanced and persistent response following sensory nerve stimulation. Under these conditions, low-intensity Aβ-fibre stimulation induces pain* **(allodynia)**.

Fig. 1.12: Physiological and pathological pain states. From Dray et al. (1994) *Trends Pharmacol. Sci.* **15**, 190-197.

1.5.2 Interactions of glutamate and neuropeptides:

The central terminals of small calibre primary afferents contain both neurokinins (NK) and excitatory amino acids (EAA) (Battaglia and Rustioni 1988) which can be released stimulateously to activate EAA and NK receptors in the spinal dorsal horn (Gerber and Randic 1991). Some dorsal horn cells express both receptor types and interactions

between the two have been indicated (for refs. see Haley and Wilcox 1992). The combined administration of NK and NMDA antagonists produce a greater inhibitory effect than either antagonist alone suggesting a synergism between the two (Coderre and Melzack 1991; Rusin et al. 1992). Upon release, neurokinins act predominantly at the NK-2 receptor in the rat dorsal horn (Nagy et al. 1993; Dalsgaard et al. 1985). Studies using the protein kinase C (PKC) inhibitor staurosporine, which blocks the NK enhancement of NMDA responses, have suggested that NKs enhance actions of excitatory amino acid-evoked currents in the spinal cord dorsal horn via the activation of PKC (Urban et al. 1994). It is hypothesised that the activation of NK receptors induce PKC activity and the subsequent changes initiated, such as protein phosphorylation, produce a modulation of the NMDA receptor activity (Chen and Huang 1992). Enhancement of NMDA receptor activity will then cause the maintenance of spinal hyperexcitability via a heightened sensitivity of the receptor to glutamate.

Presynaptic NMDA receptors have recently been found in afferent terminals in the dorsal horn (Liu et al. 1994) and it is these autoreceptors which are thought to control the release of neuropeptides (Marvizon et al. 1997; Liu et al. 1994; Liu et al. 1997). The duration and intensity of pain behaviour produced by intrathecal injection of substance P in rats is less than that produced by NMDA, emphasising that the substance P released in response to NMDA injection is not the exclusive mediator of pain behaviour. Rather, the release of substance P exacerbates and prolongs the pain behaviour generated when NMDA interacts with postsynaptic receptors (Liu et al. 1997). Presynaptic NMDA receptors located on the terminals of small-diameter pain fibres are therefore thought to facilitate and prolong the transmission of nociceptive information via enhanced release of substance P and glutamate. The glutamate released from C fibres following tissue or nerve injury not only depolarises postsynaptic neurones, but also acts on the presynaptic NMDA autoreceptors to maintain depolarisation of the presynaptic terminal, increasing intracellular calcium and prolonging the release of and the postsynaptic effects of substance P, and possibly further enhancing the effect of glutamate via the activation of PKC. Therapies directed at the presynaptic NMDA receptor could therefore provide a novel target for the treatment of persistent pain states. A model for the synergistic action of neurokinins and glutamate in the dorsal horn is shown in Figure 1.13.

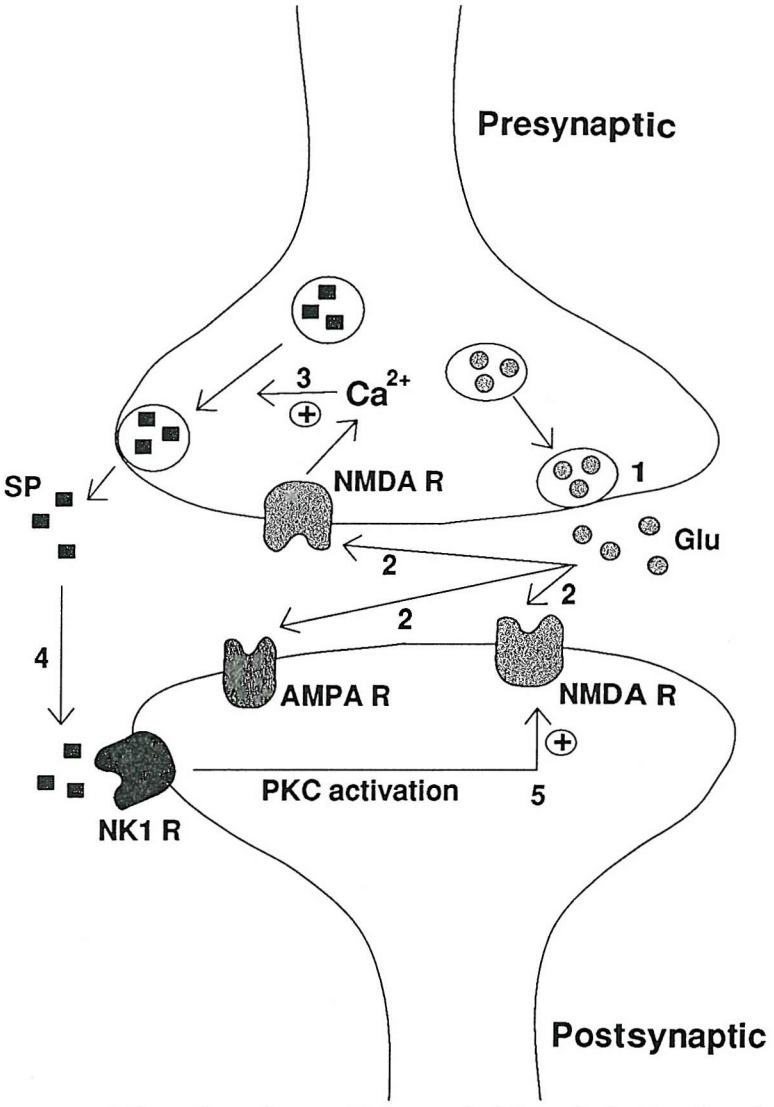


Fig. 1.13: Model for the synergistic action of neurokinins and glutamate in the dorsal horn. Noxious stimuli induce high frequency action potentials in Aδ and C fibres, producing presynaptic depolarisation and glutamate (Glu) release (1). Extracellular glutamate activates presynaptic NMDA autoreceptor channels allowing the influx of calcium (2). The increased intracellular calcium triggers the release of substance P (SP) from core vesicles (3), and substance P then binds to neurokinin 1 (NK1) receptors in surrounding neurones triggering internalisation (4). Activated NK1 receptors increase postsynaptic responses to glutamate through the activation of protein kinase C, leading to an increase in neuronal excitability via the phosphorylation of NMDA receptors. Adapted from Marvizón et al. (1997) *J. Neurosci.* **17,** 8129-8136.

1.5.3 The role of non-NMDA receptors in sensory transmission:

Synaptic activation of dorsal horn neuons by C fiber primary afferents is qualitatively different from that produced by A fibers. Large fiber afferents usually evoke a brief EPSP in dorsal horn neurones (Jessell et al. 1986) which can be blocked by non-NMDA receptor antagonists (Yoshimura and Jessell 1990). It is well known that glutamate acting on NMDA receptors in the spinal cord is involved in triggering the development of chronic

pain states. But how are the non-NMDA receptors involved? The formalin response (a well characterised model of inflammatory pain) is biphasic and lasts for about 60 minutes. Studies on the effect of intrathecal administration of the selective AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX) (Honore et al. 1988) reveals that AMPA receptor activation during the first phase initiates events which prime or sensitise the dorsal horn neurones to the second phase of nociceptive inputs. Furthermore, again using the formailn model of inflammatory pain Chapman and Dickenson (1995a) showed that pre-administration of the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) dose-relatedly inhibited the first and second phases of the response. In contrast, post-administration of the same doses of CNQX were less effective at inhibiting the second phase. These results agree with another behavioural study (Hunter and Singh 1994) which revealed that post-administration of CNQX failed to inhibit the associated hyperalgesia whereas pre-administered CNQX effectively inhibited the hyperalgesic response. It could be concluded from these results that AMPA receptor activation primes or sensitises dorsal horn neurones to the nociceptive inputs so contributing to the induction of central NMDA receptor mediated hypersensitivity.

However, Harris et al. (1996) reported that non-NMDA, AMPA receptors are upregulated in the dorsal horn following sciatic nerve ligation in the rat which suggests that they are directly involved in the nociceptive response. This upregulation was not blocked by the NMDA receptor antagonist MK-801, and since it has been reported that NMDA receptors do not contribute to mechanical hyperalgesia (Tal and Bennett 1994), it is suggested that AMPA receptors are selectively involved in the mechanical hyperalgesia which was induced by sciatic nerve ligation. Consistent with this hypothesis, AMPA receptors have been shown to underlie transmission of both nociceptive and non-nociceptive mechanical stimulation in the spinal cord (Cumberbatch et al. 1994). Non-NMDA receptor upregulation causing alterations in the density and distribution of these receptors could therefore lead to the lowering of nociceptive thresholds so that consequently innocuous mechanical stimuli come to evoke nociceptive responses.

If AMPA receptors are involved in mechanical hyperalgesia, then AMPA receptor antagonists could be used as therapy to supress pain states in which mechanical hyperalgesia is involved. AMPA receptor antagonists have indeed been shown to inhibit

pain behaviours in a rat model of post-operative pain (Zhan et al. 1998). A common cause of persistent pain and hyperalgesia in humans is post-operative pain. Mechanical stimuli and hence mechanical hyperalgesia are the most relevent to this type of pain state. Consistent with previous results indicating that NMDA receptor antagonists do not affect mechanical hyperalgesia, Zahn et al. (1998) found their model of post-operative pain was not affected by intrathecal NMDA receptor antagonist injections. However, a marked supression of mechanical hyperalgesia and pain behaviours in response to i.t. administration of the non-NMDA antagonists NBQX and DNQX was observed. In the future, the development of non-NMDA receptor antagonists could improve postoperative pain management. However, because the AMPA receptor plays an important role throughout the CNS (Barnard and Henley 1990), the use of AMPA antagonists for pain control is likely to produce widespread side-effects unless the spinal receptor differs from those elsewhere.

1.5.4 NMDA receptor antagonists as potential analgesics:

The administration of opioids have traditionally been used for the treatment of chronic pain and are effective as analgesics. However, these drugs are not free from side-effects. Opioids have a risk of severe, potentially lethal respiratory depression, and there is also the tendency for tolerence to develop. It is therefore important to find alternative therapies for the treatment of chronic pain.

The use of NMDA receptor antagonists for the relief of pain in humans has so far been limited to only a small number of clinical trials, mainly because of the high toxicity and the numerous side effects of the drugs (Kristenson et al.1992; Backonja et al.1994; Gordh et al. 1995; Price et al. 1994; Eisenburg and Pud 1998). The predicted side effects of NMDA receptor antagonists, such as amnesia and psychosis could also prove to be a problem. The regulatory sites on the NMDA receptor-channel complex, such as the glycine or polyamine binding sites may prove to be better targets. However, some 'old' drugs used for other purposes have been found to be NMDA receptor antagonists.

Dextromethorphan is a widely used non-opioid cough supressant and has a wide margin

of safety. It does not have psychotic side effects that many other NMDA receptor antagonists possess and is therefore a promising drug for use in the treatment of pain control in humans. Studies in rats show that dextrorphan, the primary metabolite of dextromethorphan, alleviates neuropathic hyperalgesia in rats (Mao et al. 1993; Tal and Bennett 1993) and in humans, dextromethorphan is effective in reducing temporal summation ('windup') pain (Price et al. 1994). Another drug possessing NMDA receptor antagonist activity is memantine which has previously been used to treat Alzheimers and Parkinsons disease and is clinically avaliable. Memantine has been shown to reduce the responses of spinal neurones to noxious stimuli (Neugebauer et al. 1993b) and suppresses neuropathic pain in rats (Eisenberg et al. 1994; Eisenburg et al. 1995).

A more recent study has used amantadine, an agent avaliable for long-term use in humans that has recently been shown to have NMDA antagonist activity (Eisenburg and Pud 1998). Amantadine (and memantine) exerts its effects by binding at a site on the NMDA receptor where it gets trapped after channel closure and agonist unbinding. This is known as a "trapping channel block" (Blanpied et al. 1997). Amantadine has fewer side effects than other "channel blocking" drugs such as ketamine, PCP and MK-801. The resons for these very diverse side-effect profiles are not known but it is plausible that this variation arises in part from a diversity of mechanisms by which the channel is blocked. Three patients suffering from chronic neuropathic pain were given an acute administration of amatadine which completely resolved not only the pain but also the associated hyperalgesia and allodynia (Eisenburg and Pud 1998). Results using amantadine in this clinical trial were highly significant and suggest that further investigation should be carried out.

1.6 Metabotropic Glutamate Receptors.

In 1985 it became apparent that glutamate had more complex roles than was first thought since it was reported to stimulate phospholipase C (PLC) in cultured striatal neurones via a receptor that did not belong to the NMDA, AMPA or KA receptor families (Sladeczek et al. 1985). Soon after, a similar effect of glutamate was described in hippocampal slices (Nicoletti et al. 1986a, b), cultured cerebellar granule cells (Nicoletti et al. 1986c) and

cultured astrocytes (Pearce et al. 1986). These results strongly suggested that glutamate, like GABA, 5-HT and acetylcholine, not only activated ligand-gated channel receptors but also receptors coupled to GTP-binding proteins (G proteins). The existence of such novel glutamate receptors, now known as metabotropic glutamate receptors (mGluRs), was then confirmed using the *Xenopus* oocyte model and new pharmacological tools (Sugiyama et al. 1987).

It has been shown that metabotropic glutamate receptor (mGluR) activation in the spinal cord can result in hyperalgesia (Meller et al. 1993) and sustained activation of dorsal horn neurones (Young et al. 1994). The mechanisms underlying such mGluR-mediated effects are not known and indeed, there is relatively little research into the role of mGluRs in spinal sensory processing. The potential importance of these receptors is highlighted by evidence for their role in gating NMDA-receptor-dependent and -independent forms of long-term potentiation (LTP) in the hippocampus (Bortolotto and Collingridge 1993; Bortolotto et al. 1994) and by the facilitation of AMPA and NMDA responses in dorsal horn neurones by the mGluR agonist (1*S*,3*R*)-1-Aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD] (Bleakman et al. 1992; Cerne and Randic 1992).

Molecular cloning of the G-protein coupled mGluRs has identified eight subtypes (mGluR1-8) whose heterogeneity is increased by the existence of several splice variants (Fig 1.14). On the basis of sequence homology, agonist potency and signal transduction mechanisms the mGluRs have been subdivided into three different groups (Pin and Duvoisin 1995) (Fig. 1.15). Group I (mGluR1a-d and mGluR5a,b) receptors are characterised by the stimulation of phospholipase C (PLC) which is revealed by an increase in phosphoinositide turnover, and calcium release from internal stores (Pin and Duvoisin 1995; Nakanishi 1992; Simoncini 1993). The G proteins involved in the activation of PLC by group I mGluRs have not been clearly identified. However, stimulation of PLC by mGluR1a is found to be partly sensitive to pertussis toxin (PTX) in chinese hamster ovary (CHO) cells, indicating that G proteins of the G_i-G_o family are involved (Aramori and Nakanishi 1992). Activation of PLC catalyses the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) to yield diacylglycerol (DAG) and free inositol-1,4,5-triphospahe (IP_3), both of which act as second messengers. IP_3 formation stimulates the release of calcium from intracellular stores, while DAG activates protein kinase C (Fig.

1.16). The group I receptors have been found to be a class of postsynaptic mGluRs which may mediate neuronal depolarisation and increased excitability in transfected cells, and rat thalamic and hippocampal neurones (see refs. in Watkins and Collingridge 1994).

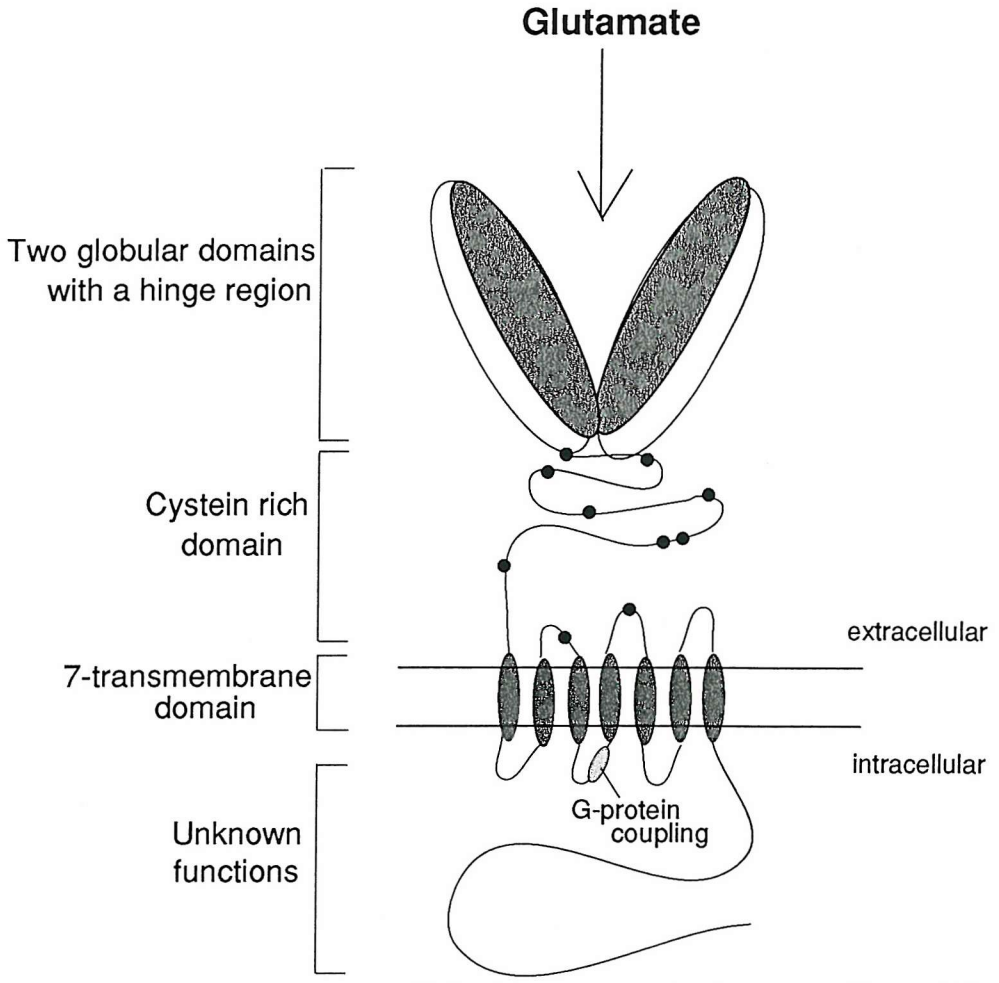


Fig. 1.14: Schematic representation of an mGluR. The segment in the second intracellular loop that is important for G-protein coupling specificity is indicated in grey. Modified from Conn and Pin (1997) *Ann. Rev. Pharmacol. Toxicol.* **37,** 205-237.

Expression in mammalian cells reveals that group II mGluRs (mGluR2 and mGluR3) inhibit adenylyl cyclase which catalyses the conversion of ATP to cAMP. Group II mGluRs repress cAMP formation stimulated by either forskolin or a Gs-coupled receptor (Tanabe et al. 1992; Tanabe et al. 1993). This effect is blocked by PTX treatment of the cells, which indicates the involvement of a G_i-type of G-protein. Group II mGluRs have also been shown to potentiate cAMP independently of kinase activity in the hippocampus (Winder and Conn 1995).

Like group II mGluRs, group III mGluRs (mGluR4a,b, mGluR6, mGluR7a,b and mGluR8) also inhibit adenylyl cyclase via a PTX-sensitive G-protein when expressed in CHO cells or baby hamster kidney (BHK) cells (Okamoto et al. 1994; Saugstad et al. 1994; Tanabe et al. 1993). The inhibition observed, however, is often smaller than that obtained with group II mGluRs (50%), which suggests that either group III mGluRs do not have a very high density of expression in the membrane of these cells or that there is an inappropriate coupling of these receptors to this transduction pathway. However, the tranduction is totally inhibited by PTX suggesting that the G-protein in this coupling is of the G_i family (Pin and Duvoisin 1995). The mGluR6 subtype which is found exclusively in retinol cells, however, is thought to have a different transduction pathway since it is thought to couple to a cGMP phosphodiesterase in its native environment (Shiells and Falk 1992).

subtype	*transduction*	*group*
mGluR1 **mGluR5**	**+PLC**	**I**
mGluR2 **mGluR3**	**-AC**	**II**
mGluR7 **mGluR4** **mGluR8** **mGluR6**	**-AC**	**III**

Figure1.15: The classification and transduction pathways of the metabotropic glutamate receptors.

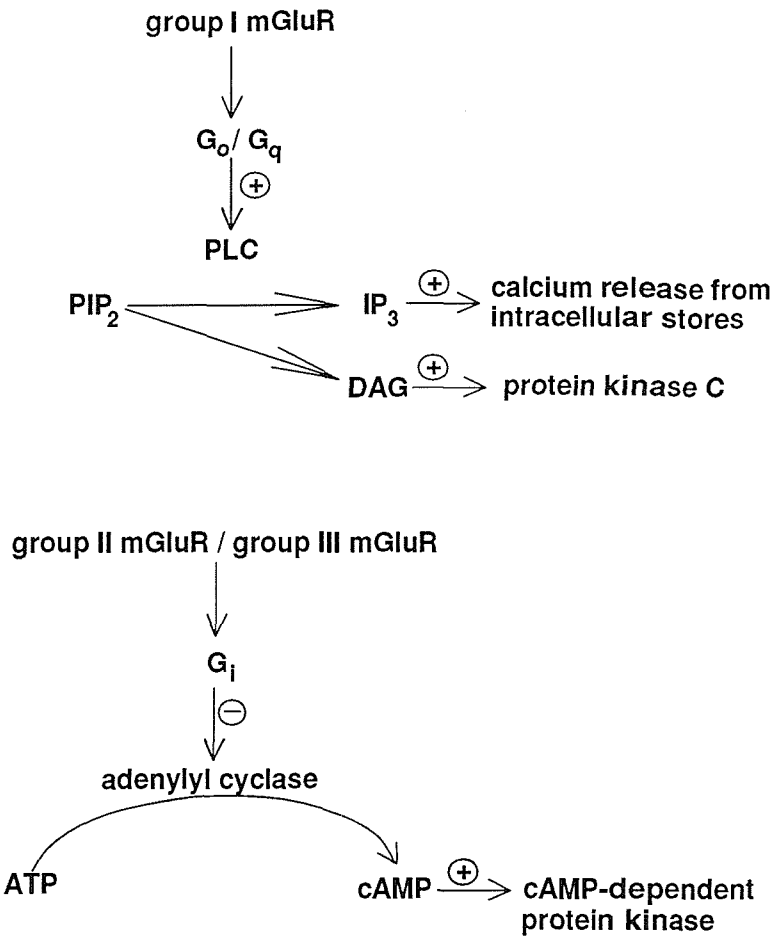


Fig. 1.16: Diagram showing the effector systems of mGluRs.

1.6.1 Localisation of mGluRs in the Spinal Cord.

To aid the characterisation of the various subtypes of mGluRs in the spinal cord, a range of highly selective agonists and antagonists are required. Since there is only a limited availability of these selective ligands, molecular studies aimed at detecting the expression and distribution of the different mGluR isoforms are of great importance. Using a reverse transcriptase-polymerase chain reaction (RT-PCR) methodology, Valerio et al. (1997) demonstrated the presence of mGluR1a, mGluR2, mGluR3, mGluR4, mGluR5 a and b, and mGluR7 mRNAs in the rat spinal cord. The selective expression of the mGluR1a subtype in the spinal cord is the only known example of selective mGluR1 splicing in the CNS. In all other mGluR1-expressing tissues examined, both isoforms of the subtype are expressed albeit with differing intensities (Hampson et al. 1994). This is of particular importance since it has recently been shown that the long carboxyl-terminal domain which

characterises the mGluR1a isoform is able to confer specific properties to the receptor, in comparison with the shorter mGluR1b-d variants, including a better coupling efficiency to the G proteins, and an agonist-independent activity (Prezeau et al. 1996).

Expression of the different group I mRNAs have been shown to have a different distribution within the spinal cord. While the level of mGluR1 mRNA is relatively low and broad in spinal grey matter, the expression of mGluR5 mRNA is very intense and localised to the dorsal horn (Anneser et al. 1995; Boxall et al. 1998). Other studies using antibodies specific for mGluR5 have found the receptor to be located in the superficial laminae of the rat and human spinal cord, mainly in laminae I and II of the dorsal horn (Vidnyanszky et al. 1994). Of all the mGluR subtypes, the mRNA for the mGluR5 subtype shows the greatest expression in the dorsal horn. This is closely followed by the group III subtypes mGluR7 and mGluR4 (Boxall et al. 1998). mGluR7 has been found to be located on primary afferent fibres in the dorsal horn (Ohishi et al. 1995) and has been described using immunocytochemical methods in a broad population of dorsal root ganglia (DRG) cells containing glutamate/aspartate (Li et al. 1996). Based on the avaliable data, mGluR7 receptors have been suggested to act as presynaptic autoreceptors. It has not yet been possible to determine if mGluR4 receptors are located pre- or postsynaptically although it is thought they have a postsynaptic location in the DRG (Boxall et al. 1998).

Although Valerio et al. (1997) found mRNA encoding for mGluR2 and mGluR3 (group II) receptors in the rat spinal cord, expression levels were low. Conversely, Boxall et al. (1998) and Ohishi et al. (1993) found strong expression of mGluR3 mRNA in the superficial medial edge of the dorsal horn. The mRNA for mGluR3 has been shown to be upregulated after UV induced peripheral inflammation in all areas of the spinal cord except motorneurones (Boxall et al. 1998). This may indicate a specific role for the mGluR3 receptor in the modulation of primary sensory transmission. No expression of mGluR3 has been found in the motoneurones of the spinal cord.

Expression of mGluR4 and mGluR7 receptor mRNAs in the rat spinal cord show differing distributions. mGluR4 mRNA is expressed in a disperse manner over the grey matter. In contrast, mGluR7 is localised with a high concentration in the superficial dorsal horn, and

to a lesser extent in the deep dorsal horn and the ventral horn (Boxall et al. 1998). mRNA for mGluR6 and mGluR8 was not detected at all in any area of the spinal cord (Valerio et al. 1997). Thus, group II and group III mGluRs are relatively poorly represented on intrinsic neurones in the spinal cord of normal animals. However, electrophysiological studies have suggested that group II and/ or III mGluRs may be distributed presynaptically on primary afferent terminals and could inhibit transmitter release (Jane et al. 1994; Kemp et al. 1994). Specifically, presynaptic location of mGluR7 receptors has been reported in the spinal cord (Ohishi et al. 1995; Valerio et al. 1997). The predominant presynaptic location of the group II and III receptor subtypes means that mRNA coding would not be expected to be found in the dorsal horn of the spinal cord. Antibody studies have yet to demonstrate whether primary afferent neurones entering the dorsal horn of the spinal cord do show staining for these other group II and III mGluRs.

Figure 1.17 summarises the location of the different metabotropic glutamate receptors in the spinal cord, while figure 1.18 shows their pre- and postsynaptic locations.

Receptor	Group	Location in the Spinal Cord
mGluR1	I	• low expression • broadly expressed throughout the grey matter
mGluR5	I	• very highly expressed • localised in the superficial laminae of the dorsal horn
mGluR2 / mGluR3	II	• low expression • mGluR3 localised to the medial edge of the dorsal horn and upregulated after peripheral UV inflammation • none found on motorneurones
mGluR7	III	• localised in high concentrations in the superficial dorsal horn • located on primary afferent fibres in the dorsal horn and in DRG cells containing glutamate • presynaptic
mGluR4	III	• disperse location in the grey matter • postsynaptic (?) in DRG
mGluR6 / mGluR8	III	• none found in any area of the spinal cord

Fig. 1.17: a summary showing the locations of the different metabotropic glutamate receptors in the spinal cord.

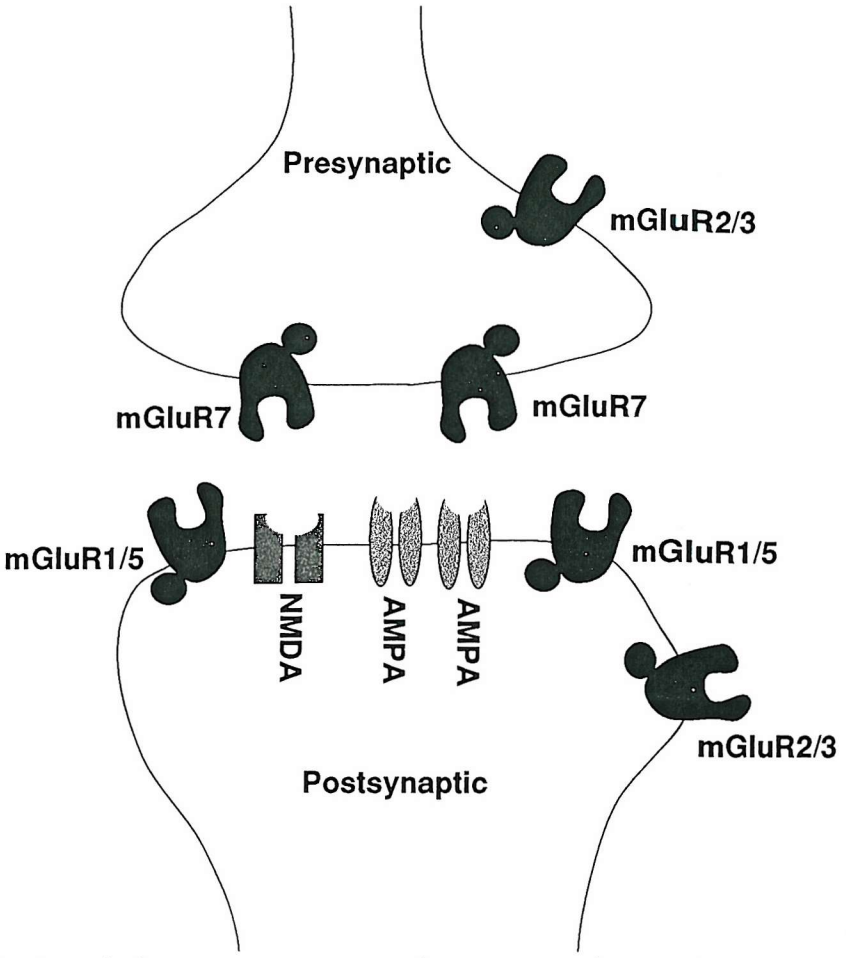


Fig. 1.18: Organisation of glutamate receptors at the synapse. Ionotropic receptors are located in the central postsynaptic specialisation while group I mGluRs are found at the perisynaptic annulus. mGluR7 (group III) is found presynaptically at the active zone while group II mGluRs (mGluR2 and mGluR3) occur at extrasynaptic membrane domains both pre- and postsynaptically. Group I mGluRs and several types of ionotropic receptor have also been reported to be located presynaptically; these are not included as the precise distribution is unknown. Modified from Ottersen and Landsend (1997) *Eur. J. Neurosci.* **9,** 2219-2224.

1.6.2 The involvement of mGluRs in sensory transmission in the spinal cord.

It is well known that modulation and processing of nociceptive information occurs in the dorsal horn area. The prominence of mGluR mRNA in the superficial dorsal horn of the spinal cord suggests that these receptors may play an important role in nociceptive processing (Young and Fagg 1990). Evidence of glutamate uptake into small ganglion cells in 1983 was a strong indicator of a sensory transmitter role for glutamate in fine afferent fibres (Duce and Keen 1983). The different classes of mGluRs have a variety of potential roles depending on their differing locations and effector mechanisms. Activation of mGluRs may induce excitation (McBain et al. 1994) or inhibition (Gereau and Conn

1994), depending on the presence of the subtype and location of the mGluR. A major role for the mGluR has been suggested in plastic changes within the CNS. Both long-term potentiation (LTP) (Bashir et al. 1993) and long-term depression (LTD) (Aiba et al. 1994; Pin and Duvoisin 1995) may depend upon the activation of mGluRs.

In vivo observations have revealed that mGluR activation contributes to the development of spinal hyperexcitability (Cerne and Randic 1992; Meller et al. 1993; Neugebauer et al. 1994; Young et al. 1994). Also, the potentiating effect of the mGluR agonist (1*S*,3*R*)-ACPD occurs at concentrations lower than that by which it causes an increased calcium influx (Bleakman et al. 1992). The effect is, therefore, dissociated from its ability to increase intracellular calcium and is thus a direct effect. These observations presented the first evidence that mGluR activation during hyperalgesia is associated with peripheral inflammation. Such potentiation could represent a key link in the activity dependent synaptic plasticity seen in the spinal cord. However, until recently the pharmacological tools avaliable could not adequately distinguish between the different receptor types involved in these changes. With the advent of newer, more specific drugs it has been possible to investigate the separate roles of the three mGluR subgroups.

The effect of mGluR compounds on activity at the NMDA receptor channel is of particular interest, as this receptor has an important role in spinal nociceptive modulation. Several studies have demonstrated an interaction between the NMDA receptor and mGluRs. In electrophysiological experiments *in vitro* (Bleakman et al. 1992; Cerne and Randic 1992) and *in vivo* (Bond and Lodge 1995; Palacek et al. 1994), NMDA-evoked responses are enhanced when mGluR agonists are applied in combination. Also, in the behavioural studies using the formalin model, the co-administration of NMDA with mGluR agonist ((±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD) results in a much greater increase in nociceptive behaviours than seen with either agonist given alone (Coderre and Melzack 1992).

Investigations using selective ligands which differentiate between the different mGluR subgroups have found that it is the group I receptors which have the most important role in inducing and sustaining nociceptive behaviour. A study by Fisher and Coderre (1996a) using the formalin model of pain revealed that an increase in nociceptive behaviour is

induced by the group I mGluR agonist (*R,S*)-dihydroxyphenylglycine (DHPG) and the non-selective agonist (1*S*, 3*S*)-ACPD, but not by the group II agonist (2*S*,1'*R*,2'*R*,3'*R*)-2-(2',3'-dicarboxy-cyclopropyl)-glycine (DCG-IV). The study also showed that this effect could be blocked by the administration of the NMDA antagonist D-AP5 prior to testing. Other studies using electrophysiological as well as behavioural techniques demonstrated how antagonists acting preferentially at group I receptors have a much more pronounced effect in an inflammed state (Young et al. 1997; Neugebauer et al. 1994). A previous electrophysiological study also demonstrated that the mGluR antagonists L(+)-2-amino-3-phophophonopropionic acid (L-AP3) and (*R,S*)-4-carboxy-3-hydroxyphenyl-glycine (CHPG) attenuated the responses of single dorsal horn neurones to sustained noxious input (Young et al. 1994).

In order to distinguish between the roles of the different mGluR group I receptors, Young et al. (1997) tested both a selective mGluR5 agonist, trans-azetidine dicarboxylic acid (t-ADA) and an drug described to selectively inhibit mGluR1 receptors compared to mGluR5 receptors, cyclothiazide (CTZ). In elecrophysiological experiments, it was found that t-ADA activated a smaller proportion of dorsal horn cells than was activated by the mGluR group I agonist DHPG implying that mGluR1 receptors and to a lesser extent mGluR5 receptors have a role in the activation of dorsal horn neurones. These results agree with another study which reported that intrathecally administered 3,5-DHPG, but not t-ADA, elicits spontaneous nociceptive behaviour (Fisher and Coderre 1996b). CTZ was shown to have no effect on innocuous stimulation but did attenuate the response of single dorsal horn neurones to repeated topical application of mustard oil, which suggests that mGluR1 receptors are selectively involved in mediating a sustained C fibre input to dorsal horn neurones, but not an innocous Aβ fibre input (Young et al. 1997).

The mechanism by which mGluRs may contribute to sustained nociceptive input still remains unsolved. Numerous downstream cellular changes mediated by mGluRs have been described (Chavis et al. 1994; Pin and Duvoisin 1995; Swartz and Bean 1992; Swartz et al. 1993). Enhancement of NMDA receptor activity has been described both in electrophysiolgical experiments *in vitro* (Bleakman et al. 1992; Cerne and Randic 1992) and *in vivo* (Baker et al. 1993; Bond and Lodge 1995; Jones and Headley 1995) and also in behavioural studies (Fisher and Coderre 1996a). Examinations of the NMDA

(Moriyoshi et al 1991) and the AMPA/KA (Hollmann et al 1989) receptors reveal the presence of a large number of concensus phosphorylation sites, suggesting the possibility that these receptors may be modulated through second messenger-mediated mechanisms. In fact, potentiation of NMDA and KA/AMPA responses after activation of protein kinase C (Kelso et al. 1992; Cerne et al. 1993) and cAMP dependent protein kinases (Wang et al. 1991; Keller et al 1992), has been demonstrated previously. However, the effects of (1*S*, 3*R*)-ACPD appear to be dissociated from its ability to increase intracellular calcium and potentiation is rather short lived (Bleakman et al. 1992). Previous reports on the potentiation of NMDA responses showed it to be long lasting, as might be expected for a phosphorylation-mediated effect (Chen and Huang 1991; Rusin and Randic 1991; Rusin et al. 1992).

Activity at group I mGluRs may result in initiation of a number of downstream signal transduction steps, including PKC activation (Manzoni et al. 1990) and inhibitors of PKC (as well as Ca/Calmodulin-dependent kinase II and phospholipase A2) have been shown to block mGluR agonist evoked activation of dorsal horn neurones (Young et al. 1995). PKC inhibitors can block long-term potentiation in the hippocampus (Lovinger et al. 1987; Malenka et al. 1989; Manlinow et al. 1988), as well as the sustained activity in dorsal horn neurones elicited by C fibre activation (Munro et al. 1994). PKC inhibitors, however, do not block activity due to innocous brushing (Munro et al. 1994). Binding assays using [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) have shown how sustained noxius inputs result in the translocation of PKC to the membrane compartment of spinal cord dorsal horn cells in a mustard-oil model (Munro et al. 1994) and also in formalin-evoked hyperalgesia (Yashpal et al. 1995). These results have been shown to correlate with behavioural studies in which hyperalgesia is observed with PKC activation (Mao et al. 1992; Mao et al. 1993) and hyperalgesia induced by subcutaneous formalin is reduced if PKC activation is prevented (Coderre and Yashpal 1994; Yashpal et al. 1995).

PKC-mediated enhancement of the activation of NMDA receptors has been reported by several groups (Ben-Ari et al. 1992; Cheng and Huang 1992; Tingley et al. 1993). Behavioural studies using PKCγ knockout mice showed a concomitant reduction of the behavioural and anatomical response to nerve injury and a decrease in the second phase of the formalin response (Malmberg et al. 1997), indicating an involvement in chronic pain

states. Furthermore, Fisher and Coderre (1996a; 1996b) found that the spontaneous nociceptive behaviour and facilitation of formalin responses elicited by mGluR1 agonists are inhibited by the NMDA receptor antagonist D-AP5. Thus it seems possible that mGlu group I receptors play a role in sustained noxious input into the dorsal horn of the spinal cord by inducing the phosphorylation and hence enhancement of the NMDA receptor response to glutamate.

Although these findings support a modulatory role for mGluRs in the sensory processing in the spinal cord, other studies have failed to confirm this hypothesis (Jones and Headley 1995; Birse et al. 1993). This discrepancy was explained by Neugebauer et al. who showed the functional presence of mGluRs in the spinal cord dorsal horn by iontophorizing (1*S*,2*R*)-ACPD, and revealed that their contribution to reflex transmission was dependent on peripheral inflammation. Similarly, the contribution of mGluRs to spinal nociceptive processing was also observed following mustard oil-skin inflammation (Young et al. 1994). It is apparent for these studies that mGluR activation may play an important role in the maintenance of spinal hyperexcitability, resulting in hyperalgesia and pain.

If synaptic activation of the metabotropic receptors during high frequency stimulation does indeed potentiate nociceptive processing, it could form part of a positive feedback loop whereby increased production of retrograde messengers causes increased glutamate release and additional postsynaptic effects, thus enhancing synaptic responses.

1.7 Transgenic Mice and Pain.

Early gene knockout studies with a neurobiological focus were directed at very obvious target genes and added very little to the knowledge of behavioural neuroscience. However the fact that the predicted behaviour was indeed seen in these knockout mice confirmed that the technology was working. Many different types of knockout mice have now been produced such as mice with a targeted disruption of the gene for NO synthase, mice that lack protein kinase C gamma (PKCγ), mice with targeted mutations deleting the preprotachykinin-A gene that produces substance P and neurokinin A, mice lacking the

substance P receptor NK1 (De Felipe et al. 1998) and mice lacking the group I metabotropic glutamate receptor mGluR1. These particular strains of knockout mice could be used to study the function of particular neurotransmitters and their receptors in the processing and modulation of nociceptive information in the spinal cord. However, it is only mice that lack protein kinase C gamma (PKCγ), mice with targeted mutations deleting the preprotachykinin gene that produces substance P and neurokinin A (which act on NK1 and NK2 receptors respectively), and mice lacking the substance P receptor NK1 (De Felipe et al. 1998) which have been used for this purpose.

1.7.1 The preprotachykinin-A and the NK1 knockout mice.

Both the NK1 knockout mice and the preprotachykinin-A (PPT-A) gene knockout mice have been used to answer the question: is substance P the pain neurotransmitter? Despite the fact that ligand knockouts can not be directly compared to receptor knockouts, some common effects can be found when comparing the two sets of animals. The two different knockout groups of animals appear healthy, are fertile and have altered pain sensitivity. A number of changes in the knockout animals were consistent with effects seen with NK1 and NK2 receptor antagonists - neurogenic inflammation was significantly abolished, and mechanical sensitivity was comaprable to the wild-type animals. Suprisingly, no changes in the mechanical hypersensitivity induced by inflammation were seen in the NK1 and PPT-A knockout animals.

Knocking out the production or the action of substance P via the preprotachykinin A or the NK1 receptor genes disturbs the reaction to somatic and visceral noxious stimuli but not in a universal or consistent manner. Pharmacological studies have shown no role for the NK1 receptor antagonists on normal cutaneous mechanical or thermal noxious stimulation. Substance P is therefore not the pain transmitter. Instead the role of substance P in the role of pain processing within the CNS remains elusive. It is certain that substance P does have a a role in the transmission of nociceptive information, and it is possible it may be involved in severe pain. However, the role of substance P may be indirect such that eliminating its action will not eliminate pain. A polypharmacological approach may be more effective than blocking the action of substance P alone - for

example using a combination of antagonists to block NMDA receptors, mGluRs and NK1 receptors which could improve analgesic efficacy.

1.7.2 The PKCγ knockout mouse.

Transgenic mice lacking PKCγ have been used to study the contribution of this specific isoform of PKC on neuropathic and acute pain (Malmberg et al. 1997). Some studies suggest that nerve injury leads to neuropathic pain because it induces NMDA receptor-mediated hyperexcitability of dorsal horn neurones. Events downstream of the NMDA receptor, including the activation of PKCs, are presumed to underlie the persistence of pain (Manzoni et al. 1990; Young et al. 1995; Munro et al. 1994; Mao et al. 1992; Mao et al. 1993; Coderre and Yashpal 1994; Yashpal et al. 1995). However, the conclusions of these studies are very limited since PKC inhibitors are not specific and they can not differentiate between the different isoforms of PKC. By using mice with a deletion of the gene specifically encoding the neuronal-specific (gamma) isoform of PKC, the influence of PKCγ on acute and neuropathic pain can be investigated.

PKCγ deficient mice have a normal appearance, slight ataxia, modest learning and memory deficiency and some motor incoordination. Syanapic transmission appears normal but LTP is impaired (see refs in Malmberg et al. 1997). Both the wild-type and the mutant mice showed the same response to thermal or mechanical stimulation in the absence of nerve injury, therefore transmission of acute nociceptive information is not affected by PKCγ. However, pain behaviour after sciatic nerve ligation (neuropathic pain) is altered in the PKCγ deficient mice. Mechanical hypersensitivity is the predominant symptom of nerve injury, yet although wild type mice showed mechanical allodynia 3 days following partial sciatic nerve ligation, the PKCγ deficient mice showed no significant change in the mechanical threshold. Therefore, it can be concluded that PKCγ is essential for the production of mechanical allodynia after nerve injury.

Peripheral nerve injury not only produces a neuropathic nerve syndrome but also significantly alters the neurochemistry of the ipsilateral dorsal root ganglion (DRG) and the

spinal cord dorsal horn (Hokfelt et al. 1994; Nahin et al. 1994). The neurochemical consequences of nerve injury on substance P, the NK1 receptor and neuropeptide Y immunoreactivity in the spinal cord dorsal horn and also the DRG were also investigated by Malmberg et al. (1997). Neuropeptide Y in normal animals is only found in the dorsal horn and not in the DRG. However, following nerve injury, the mRNA and the peptide itself are expressed in DRG neurones. In wild type mice, partial nerve injury produced a marked decrease in substance P and an increase in neuropeptide Y and NK1 receptor immunoreactivity in laminae I and II of the dorsal horn ipsilateral to the nerve injury. A smaller nerve injury-induced alteration of substance P, NK1 and neuropeptide Y occured in the mutant mice compared with the wild-type. The failure of PKCγ deficient mice to develop any neuropathic pain syndrome after nerve injury was paralleled by a very limited neurochemical reorganisation in the dorsal horn of the spinal cord. The PKCγ deletion is therefore manifest as a significant reduction of the neurochemical response of postsynaptic neurones to nerve injury.

Although the other PKC isoforms (α, βI and βII) are fairly evenly distibuted in the superficial layers of the spinal cord dorsal horn and the DRG, the PKCγ isoform is highly restricted to a subset of interneurones in the medial part of lamina II in the substantia gelatinosa of the dorsal horn (Mori et al. 1990; Malmberg et al. 1997). This suggests that the mechanism of action of PKCγ in neuropathic pain involves these interneurones in lamina II. Furthermore, since primary afferent fibres do not express the NK1 receptor for substance P (Liu et al. 1994; Brown et al. 1995) the up-regulation of NK1 in the dorsal horn after nerve injury (which was absent in the mutant mice) must involve the PKCγ-containing interneurones that are downstream of the primary afferents. The functional target of these interneurones must include neigbouring dorsal horn neurones that express the NK1 receptor. While the involvement of other PKC isoforms in this process can not be ruled out, the PCKγ-mediated phosphorylation of substrate proteins in interneurones of the medial lamina II area is critical for the full development of a neuropathic pain state produced by nerve damage. The central lamina II neurones respond preferentially to non-noxious stimulation. PKCγ-mediated processing of non-noxious inputs by dorsal horn neurones may therefore be critical to the development of neuropathic pain.

1.7.3 The mGluR1 knockout mouse.

Mice lacking the group I metabotropic receptor mGluR1 have been used to study synaptic plasticity in the hippocampus (Conquet et al. 1994; Bordi et al. 1997). The mGluR1 mice have a motor coordination deficit with impaired balance. Action tremor and errors in metric movement were constantly observed in the mGluR1-/- but not the mGluR+/- mice. The absence of mGluR1 therefore causes a severe motor deficit which becomes apparent between the second and third week following birth (Conquet et al. 1994). The mutant mice also have major learning impairments (Conquet et al. 1994).

The mGluR1 knockout mouse has not yet been used to study the involvement of the specific metabotropic glutamate receptor. However, using this transgenic model it has been found that cerebellar LTD and mossy fibre LTP involve mGluR1 but do not involve NMDA receptor activation. In contrast, the four hippocampal pathways that do not require mGluR1 for the induction of LTP all exhibit the NMDA receptor-dependent form of LTP. These pathways may require an alternative metabotropic receptor such as mGluR5. These results could represent a general association between NMDA receptors and specific mGluR subtypes for the induction of synaptic plasticity. This could also occur in the spinal cord as investigations using selective ligands which differentiate between the different mGluR subgroups have found that it is the group I receptors (mGluR1 and mGluR5) which are thought to act on NMDA receptors to induce and sustain nociceptive behaviour (Fisher and Coderre 1996a; Young et al. 1997; Neugebauer et al. 1994).

1.8 Inhibitory Mechanisms of Pain.

Melzack and Wall first described the existence of a specific pain modulatory system in 1965 (Fig. 1.19). This theory was proposed as the 'Gate Control Theory of Pain'. In simple terms, the gate control theory states that the transmission from spinal nerves to higher brain centres is dependent upon the relative activities in large and small diameter afferents. It is thought that the activity in the large diameter, low threshold fibres (e.g. from touch receptors) inhibits nociceptive transmission via activation of inhibitory

interneurones of the substantia gelatinosa: these "close the gate". Small (nociceptive) fibres de-activate these inhibitory interneurones and "open the gate" to the nucleus proprius. Therefore, artificially increased activity in low threshold cutaneous receptors (e.g. those stimulated by rubbing the skin) can "close the gate" and then less information from nociceptors is transmitted supraspinally. A major nociceptive barrage "opens the gate". This dorsal horn complex also receives corticospinal fibres (originating in the sensory cortex), and reticulospinal fibres, providing paths to influence sensory input if required. The detailed mechanisms of the "gate" concept have always been poorly understood (particularly regarding presynaptic and postsynaptic inhibition, and the precise role of the substantia gelatinosa), and the gate control theory has shown limited evidence for the existence of any descending control of nociception.

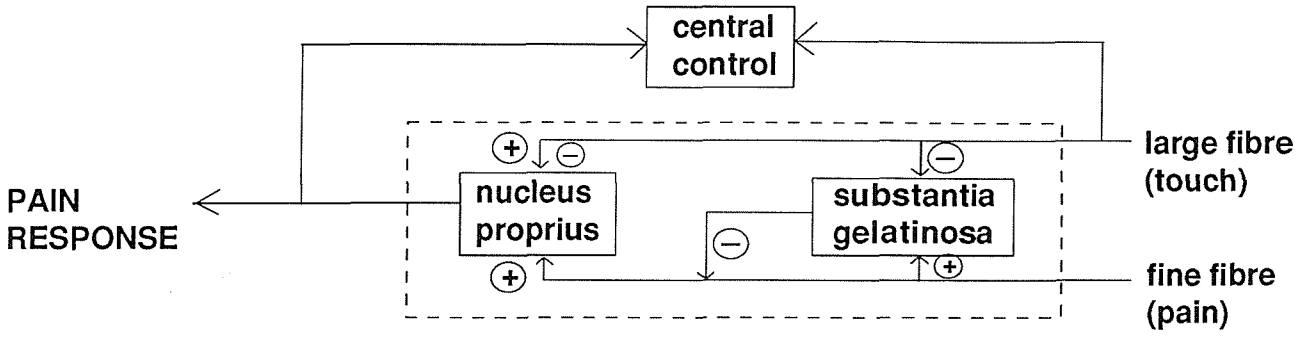


Fig. 1.19: Schematic diagram of the theoretical gate control mechanism in the perception and response to painful stimuli. Adapted from Melzack and Wall (1965).

Transmission of nociceptive information in the spinal cord is subject to inhibitory influences from cells that are located within it (local interneurones) and those that originate in higher regions of the nervous system (descending inhibitory pathways). Descending projections arise from several structures, including the hypothalamus, periaqueductal gray matter (PAG) of the midbrain, locus coeruleus, nucleus raphe magnus (NRM), and ventrolateral medulla to form descending inhibitory pathways. Projections from these regions directly or indirectly terminate at the spinal level to modulate incoming nociceptive signals.

Evidence to support the hypothesis of descending modulation of nociception occurred with the discovery of the stimulation-produced analgesia (SPA) phenomenon (Reynolds 1969; Mayer and Liebeskind 1974). Electrical stimulation of discrete brain sites was shown to suppress responses to noxious stimulation and inhibit nociceptive reflex responses, such as the tail flick, which are associated with intraspinal connections. Thus the phenomenon of SPA indicated that descending pathways to the spinal cord were causing antinociception via the electrical stimulation of the periaqueductal grey (PAG) area of the brain. Stimulation of the PAG causes analgesia in a variety of different animal species and therefore confirms the importance of this brain area as a specific pain-modulating system.

A variety of neurotransmitters have been implicated in descending inhibition. These include the endogenous opioid peptides (β-endorphin, enkephalin, and dynorphin) as well as other neurotransmitters such as 5-HT and noradrenaline. More recently, the neuropeptide nociceptin/orphanin FQ has been found to have a role in pain modulation. A dense plexus of orphanin FQ/nociceptin positive fibres was observed to be present in the superficial layers of the dorsal horn and other areas relating to pain perception, including the PAG (Rield et al. 1996). Direct examination of the spinal cord in the rat has shown that nociceptin inhbits sensory input. In the hemisected spinal cord preparation, nociceptin depressed both C fibre-mediated and A fibre-mediated synaptic responses in a naloxone insensitive manner. In the dorsal horn, locally applied nociceptin inhibited the windup of C fibre-evoked responses but had no effect on single C fibre-evoked responses (Riedl et al 1996).

In the spinal cord, opiate receptors and enkephalin occur in the substantia gelatinosa and act as pain suppressors. Cells in the periaqueductal mesencephalic gray matter and nucleus raphe magnus can be activated by collaterals from ascending or descending pathways and the axons from the nucleus raphe magnus project via a small dorsolateral spinal tract (of which the neurotransmitter is 5-HT) to interneurones of the substantia gelatinosa, some of which release enkephalin. This, in turn, causes inhibition in the pain pathway. This raphe-spinal pathway forms part of a complex descending system that can profoundly affect the transmission of nociceptive information. Many of the traditional strategies involved in pain management, such as the use of opioids (both spinal and

systemic), act via these inhibitory mechanisms.

1.9 Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-noneamide) is the main irritant and pungent ingredient in hot capsicum chilli peppers and elicits a sensation of burning pain by selectively activating a subset of mammalian sensory neurones that convey noxious information to the CNS (Fig. 1.20). In mammals exposure of nociceptor terminals to capsaicin leads to an initial excitation of the neuron and the subsequent perception of pain and release of local inflammatory mediators. With prolonged exposure, nociceptor terminals become insensitive to capsaicin, as well as other noxious stimulators. This phenomenon following prolonged exposure to capsaicin has led to capsaicin being used as an analgesic treatment for many pain related disorders such as rheumatoid arthritis. Following activation, capsaicin rapidly makes sensory neurones inexcitable. Consequently, noxious stimuli are no longer effective. Capsaicin exerts its antinociceptive effect by activating capsaicin receptors on the afferent nerve terminals in the spinal cord. Spinal neurotransmission is subsequently blocked by a prolonged inactivation of sensory neurotransmitters. Spinal administration of capsaicin has been shown to inhibit the release of sensory neuropeptides (Dray 1992).

The specific capsaicin receptor has recently been cloned (Caterina et al. 1997) and has been termed the vanilloid receptor 1 (VR1). It is a proteinacious ion channel and is structurally related to the transient receptor potential (TRP) family of ion channels that have been proposed to mediate the influx of extracellular calcium in response to the depletion of intracellular calcium stores. An antagonist of the capsaicin receptor, capsazepine, has been shown to reduce inflammation-induced hyperalgesic responses in the rat without affecting the inflammation itself (Kwak et al. 1998). This study provided evidence for the presence of an endogenous capsaicin-like substance that mediates pain associated with inflammation. Identification and characterisation of this substance and the associated receptor/channels may aid in the development of novel analgesics.

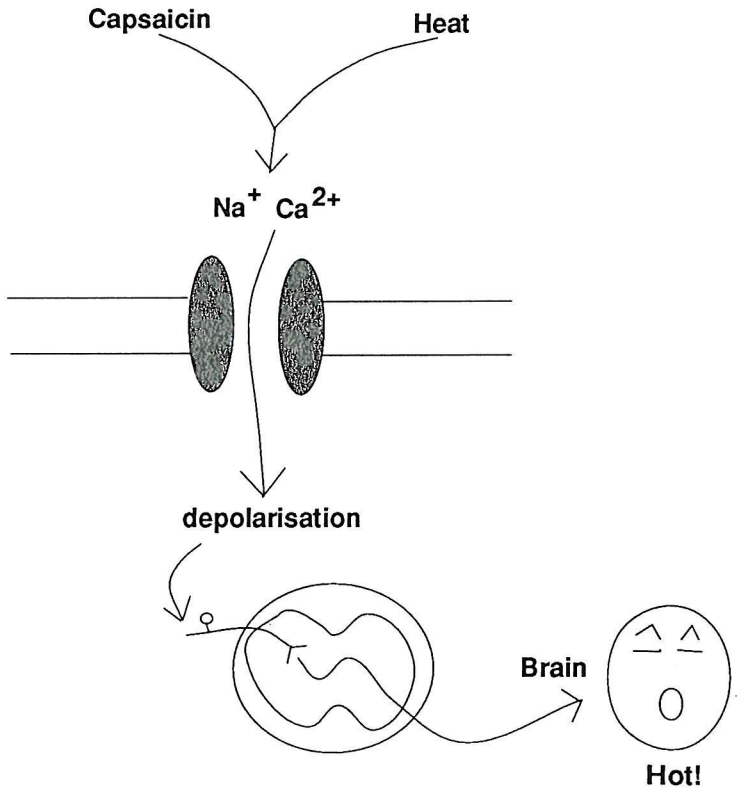


Fig. 1.20: Hot peppers and painful heat both activate sensory fibres through an ion channel. The channel is known as the VR1 receptor and is activated by binding capsaicin. When activated, the channel opens, allowing an influx of calcium and sodium ions. The influx depolarises neuronal pain fibres initiating a nerve impulse through the dorsal root ganglion (DRG) to the brain. Noxious temperature uses the same pathway.

1.10 c-*fos*

The Fos protein is the product of the proto-oncogene c-*fos* which belongs to a large family of "immediate early onset" genes. Following trans-synaptic stimulation mRNAs of c-*fos* and other immediate early genes (IEGs) such as c-*jun* accumulate in the cytoplasm, and the corresponding proteins (Fos and Jun) are translated and then translocated to the nucleus where they form a heterodimeric protein complex that binds to the AP-1 binding site, a DNA regulatory element. Because Fos and Jun proteins are transiently expressed they have the characteristics of a "third messenger" system which may regulate the expression of target genes. When the c-*fos*/c-*jun* heterodimer binds to the AP1 DNA site it regulates downstream expression of target genes (see refs in Herrera and Robertson 1996). Fos is therefore related to long-term intracellular changes affected by extracellular events.

Spinal expression of c-*fos* and its co-regulated genes, such as c-jun, are followed by a prolonged increased expression in the ipsilateral dorsal horn of several genes whose products could affect sensory processing. For example, prodynorphin, preprotachykinin A, and preproenkephalin (Draisci and Iadarola 1989; Noguchi et al. 1992). Procedures that cause hyperalgesia and allodynia induce the expression of dynorphin, an opioid peptide with presumed analgesic effects in the dorsal horn of the spinal cord (Iadarola et al. 1988a; Iadarola et al. 1988b; Ruda et al. 1988). The expression of c-*fos* is co-localised with dynorphin: following carageenin-induced inflammation of a hind-paw, over 80% of spinal neurones immunoreactively labelled for dynorphin were also labelled for Fos (Noguchi et al. 1991). Also, the temporal sequence of Fos and dynorphin expression suggests that Fos may regulate the expression of the preprodynorphin gene (Draisci and Iadorola 1989).

The critical evidence that Fos acts to promote the expression of the preprodynorphin gene comes from a recent study by Hunter et al. (1995). In this study, rats were treated with an antisense oligodeoxynucleotide to c-*fos* mRNA 4 hours before receiving an injection of dilute formalin into a hind-paw. Intrathecal administration of the antisense oliginucleotide blocked the synthesis of the Fos protein in spinal neurones that would otherwise have been induced by the formalin injection. Treatment with the antisense oligonucleotide also prevented the expression of the preprodynorphin mRNA and increased the level of pain response during the tonic phase of the formalin test. Therefore by inducing synthesis of opioid peptides in the spinal cord, Fos appears to be involved in the inhibition of heightened or prolonged nociception. It remains to be shown that these events depend on c-*fos* induction, but at present it seems very likely that they occur in the same population of neurones and may be causally related. The function of increased peptide synthesis within the dorsal horn remains unclear but, it could reflect the activity of an endogenous pain control system. However, some neurones expressing c-*fos* do not contain any of these opioid peptides, suggesting that the increase in the activity of c-*fos* following noxious stimulation has effects other than the regulation of opioid levels.

It is known that morphine markedly depresses the electrophysiological responses of unmyelinated C-fibres and myelinated high-threshold Aδ sensory fibres without affecting responses due to the activation of low-threshold Aα and Aβ fibres. Since morphine also

dose-dependently blocks c-*fos* expression (Tolle at al. 1990), it can be concluded that C-fibres and Aδ fibres are primarily involved the expression of c-*fos*.

Following the suggestion that certain genes from the immediate early onset family might play a part in the establishment of long-term functional change in the nervous system (Goelet et al. 1986), it was found that c-*fos* could be transiently induced in the spinal cord of the anaesthetised rat near the central terminals of C-fibres when stimulated by noxious radiant heat or mustard oil applied to the skin (Hunt et al. 1987, Bullitt 1990). The data suggested that c-*fos* can be used as a transynaptic marker *in vivo* for neuronal activity following peripheral noxious stimulation. The expression of c-*fos* provides an excellent marker for neuronal activity *in vivo* because its expression is rapid and transient in neurones following peripheral stimulation (Bullitt 1989; Bullitt 1990). Fos activation provides a good marker for noxious activity and its expression in the brain and spinal cord after noxious stimulation matches known routes which carry pain sensation (Bullitt 1991). A study in 1990 (Tolle et al.) confirmed previous findings that c-*fos* expression can be induced in spinal cord dorsal horn neurones by noxious peripheral stimulation and also that morphine can effectively suppress this induction in a dose-dependent manner.

The amount of c-*fos* protein produced by dorsal horn neurones is probably proportional to the degree of synaptic activation and the effect of morphine can thus be attributed to its well documented depressant action on neuronal discharge activity. It has been observed that the appearance of Fos protein within the spinal cord was dependent upon the activation of small diameter high-threshold primary afferents, and that the pattern of labelling within the first few hours following stimulation closely followed the pattern of termination of particular sensory afferents within the dorsal horn (Hunt et al. 1987). It was assumed that the molecular events involved in the induction of c-*fos* may be causally related to the changes in spinal cord physiology that follow peripheral injury or tetanic stimulation of C-fibres, which can be both complex and persist for many hours following the initial stimulation. However, c-fos expression has been found to be related to the level of C fibre activity only and not to the level of nociceptive intensity in the spinal cord. A study by Doyle and Hunt (1999), using immunostaining for both Fos protein and neurokinin-I in the rat spinal cord, found that lamina I neurokinin-I receptor containing neurons are involved in intensity discriminative aspects of nociception but deeper neurons

also containing neurokinin-I receptors are involved in spatial localisation or the detection of particular nociceptive types.

Electron microscopy has shown that Fos immunoreactivity is restricted to neuronal cells (Hunt et al. 1987). Following high intensity dorsal root stimulation, both dorsal horn and dorsal root ganglion cells have shown distinct Fos-positive staining (Zhang 1997).

The physiological significance of rapid c-*fos* expression in the dorsal horn cells of the spinal cord by trans-synaptic stimulation of fine primary afferents has remained obscure. It has been suggested that modifications of IEG expression might trigger changes in the phenotype of neurones which may lead to long term changes of nerve cell function (Neumann et al. 1996). Inflammation has been shown to result in Aβ fibres aquiring the capacity to increase the excitability of spinal cord neurones due to a phenotypic switch in a subpopulation of these fibres so that they now, like C fibres, express substance P. Inflammation not only results in an increase in substance P, but also an increase in the level of preprotachykinin A mRNA. It is the expression of IEG genes which are thought to trigger the expression of preprotachykinin A as well as other opioid precursors (Draisci and Iadarola 1989; Noguchi et al. 1992). It is therefore likely that the enhanced expression of c-*fos* and other IEGs lead to plastic changes in spinal nociception and can be used as an indicator of neurones involved in mediating allodynia and hyperalgesia. Conversely, a study in 1996 suggested that the massive expression of c-*fos* is not sufficient to induce plasticity in spinal nociception as electrical intrathecal stimulation failed to produce a change in thermal and mechanical nociceptive thresholds (Sandkuhler et al. 1996).

The link between nociception and c-*fos* expression is further confirmed by the consistency across studies in the reported distribution of spinal neurones that express the gene after noxious stimulation (Harris 1998) and the fact that most manipulations that affect nociceptive processes and other pain-related behaviours also modify the expression of c-*fos*. For example, c-*fos* expression in the dorsal horn is supressed by the administration of analgesic drugs including morphine (Gogas et al. 1991; Jasmin et al. 1994; Presley et al. 1990), indomethacin (Honore et al. 1995) and ketoprophen (Buritova et al. 1996) as well as by other drugs that interfere with nociceptive processing at the spinal level such as

noradrenaline (Jones 1992).

Apart from its common use as a marker of spinal nociceptive processing, c-*fos* has provided a valuble insight into the spinal nociceptive processes themselves. For example, spinal c-*fos* expression induced by different types of noxious stimulation is differentially sensitive to different analgesic drugs. Expression of c-*fos* in spinal neurones induced by noxious thermal stimulation (immersion of a paw in 52°C water) is profoundly suppressed by morphine but not by an NMDA antagonist (Tolle et al. 1991). Also, NMDA antagonists have been found to reduce c-*fos* expression in superficial layers of the spinal cord but not the deeper layers after an injection of formalin (Kehl et al. 1991). These differences may not only reveal how analgesic compounds modulate nociception at the spinal level, but may also relate to differences in the contribution made by deep and superficial neurones in the NMDA-mediated sensitisation of nociceptive processes that underlie chronic pain and hyperalgesia.

1.11 The *in vitro* Spinal Cord.

1.11.1 Advantages of the in vitro spinal cord.

Research on the spinal cord *in vivo* allows only limited pharmacological studies. Peripheral nociceptive systems, descending modulation of spinal cord activities, and the complicating effects of anaesthetics may influence the effect of pharmacological compounds. However, in the isolated preparation these external influences are removed and known concentrations of drugs can be applied directly to the spinal cord tissue. Removal of external influences also means that movement of the animal during respiration or blood pressure pulses is avoided. Visualisation of the electrodes in electrophysiology experiments is also improved and electrodes can be moved several times until a suitable place in the tissue is found. It is for these reasons that this preparation has the potential for the detailed investigation of the pharmacology of pain pathways at the spinal level.

1.11.2 Disadvantages of the in vitro spinal cord.

The dissection of the spinal cord from the animal may cause extensive mecahnical damage to the tissue and the tissue will have cut axons and dendrites. Also, there is no input from descending input from higher brain centres, hence the activity in the spinal cord may be diminshed or be functioning in an abnormal way.

1.11.3 c-fos in the in vitro rat spinal cord.

Expression of c-*fos* has been demonstrated in the dorsal horn of lumbar segments of isolated spinal cord preparations taken from 19 to 21 day old rats (Zhang et al. 1993). Stimulation of a dorsal root for 10 minutes led to c-*fos* immunoreactivity present in both the ipsilateral and contralateral dorsal horns. The greatest density was found on the ipsilateral side. The experiments *in vitro* show that c-*fos* can be detected in the spinal cord at low levels 30 minutes following high intensity (20 times the threshold) stimulation, with the amount of Fos-like immunoreactivity increasing up to 2 hours following stimulation. The expression of c-*fos* does not significantly increase at longer intervals. The optimum incubation temperature for the *in vitro* expression of the c-*fos* gene is 27°C, with few Fos positive cells being seen when the incubation temperature is decreased to 20°C (Zhang et al. 1998).

As Fos activation can only be seen in the isolated spinal cord after high intensity noxious stimulation, it follows that c-*fos* expression can be used to map the effectiveness of therapies which supress the noxious activity of cells in the dorsal horn and to examine the efficacy of potential novel analgesics. One advantage of Fos expression over standard neurophysiological techniques is that the responses of large numbers of cells can be assessed and a whole section of the spinal cord can be examined.

1.11.4 The dorsal root reflex (DRR)

The major pathway for sensory activity entering the vertebrate central nervous system is along the dorsal spinal roots into the spinal cord, with motor activity emerging via the ventral roots. However, as early as 1891, Gotch and Horsley reported that under some circumstances activity could be detected emerging from the cord in the dorsal roots, i.e. travelling antidromically from the spinal cord towards the periphery in the sensory fibres. This "backwards directed discharge" of Gotch and Horsley (1891) is known as the dorsal root reflex (DRR).

The DRR was rediscovered by Barron and Matthews in 1935 in their study of intermittent conduction in the spinal cord. They initially attributed the discharges to collaterals of afferent fibres entering the spinal cord which passed out again via an adjacent root, but their later results showed that the reflex occurred on the rising phase of the electrotonically conducted dorsal root potential. Toennies (1938), investigated the latency of the DRR in the cat and found that it was in excess of 3-4ms - a delay that was incompatible with uninterrupted conduction through the cord.

The DRR is now a subject of interest especially because of its possible contribution to pain pathology and sensory physiology. It has been reported that natural stimulation including light touch, pressure, vibration and muscle stretch all induced the DRR in cats (Millar 1979). During locomotion, DRRs were recorded in normal and thalamic cats (Dubuc et al. 1985; Dubuc et al. 1988; Duenas et al. 1990). Also, paw, foot, joint movement and pressure at joints which are inflammed has been found to induce the DRR in cats and monkeys (Rees et al. 1995; Sluka et al. 1995).

Intra-axonal recordings have confirmed that the DRR is triggered by a rapid change of potential at the terminal regions of afferent fibres (primary afferent depolarisation). The DRR is thought to arise on the rising phase of primary afferent depolarisation (PAD) generated within terminals of afferent fibres in the cord by a synchronous afferent volley arriving in the cord after electrical stimulation of sensory nerves and roots (Eccles et al. 1962). This causes depolarisation of the terminals of the afferent fibres within the cord beyond their threshold, producing action potentials which travel antidromically out of the

cord along the dorsal roots. This is illustrated in Figure 1.21.

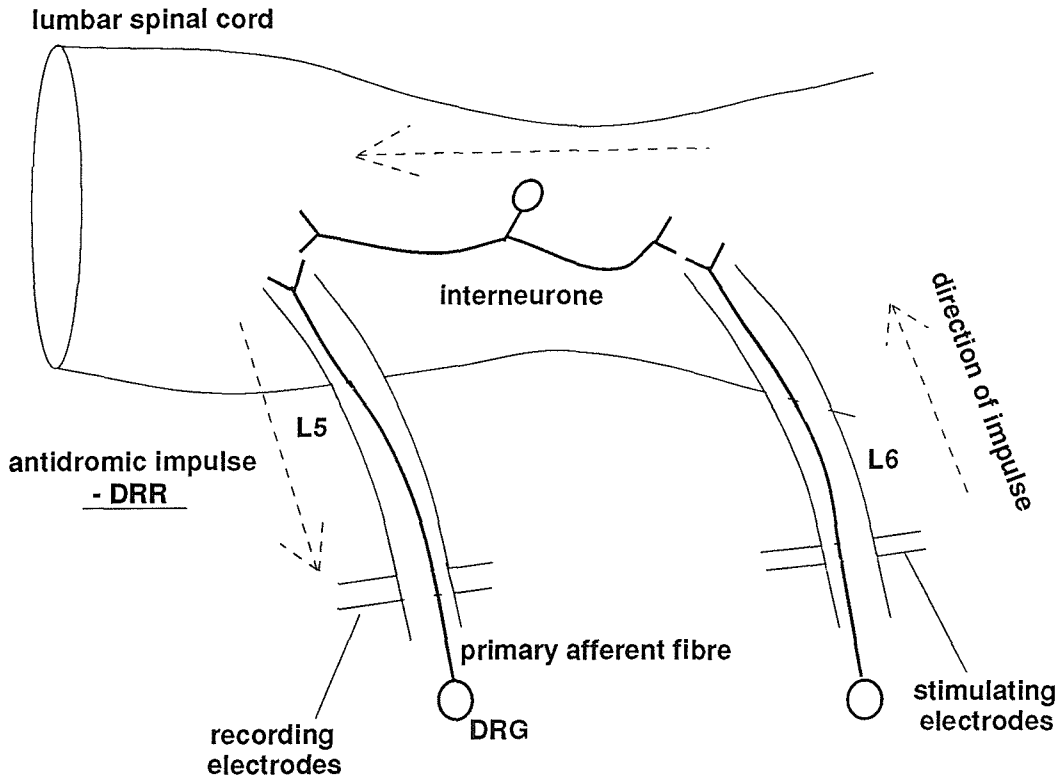


Fig. 1.21: The dorsal root reflex. An action potential will travel from the site of stimulation before synapsing with an interneuron. The action potential then travels antidromically down the adjacent dorsal root where it reaches the recording electrode.

Group II afferents are the main fibre type responsible for eliciting DRRs, while group III are involved in DRR transmission in the rat spinal cord (Lin and Fu 1998a). It is suggested that the neuronal pathway for group II afferents to induce a DRR at the spinal level of rats involves at least two possible pathways. The first is trisynaptic, where group II afferents activate two excitatory interneurones (E1 and E2) to induce the DRR (Fig. 1.22) (Lin and Fu 1998a). The other possible pathway is disynaptic, in which only one neuron is involved (E3) (Fig 1.22). However, this E3 interneuron is of a higher threshold. At a stimulus intensity of 5 times the DRR threshold, not all the group II afferents will be activated. E1 and E2 are therefore activated to allow the DRR to be induced by the trisynaptic pathway, but the intensity is insufficient to activate E3. At an intensity of 20 times the threshold, all group II afferents are activated and the E3 interneuron will recieve more excitatory inputs and will elicit the DRR via the disynaptic pathway (Lin and Fu 1988a).

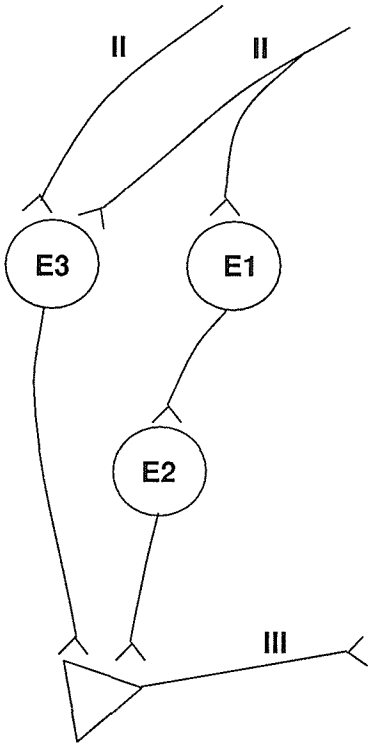


Fig. 1.22: Schematic diagram to show how group II afferents may induce the DRR. Adapted form Lin and Fu (1998a) *Neurosci. Lett.* **247,** 75-78.

The DRR may not be just of a reflex nature but an effective way for the higher brain centres to modulate afferent inputs by: (1) blocking out unnecessary sensory impulses, and (b) adjusting the sensitivity of sensory receptors and therefore controlling the effectiveness of sensory inputs (Lin and Fu 1998b).

The isolated spinal cord demonstrates the DRR very well. The spinal cord is set up with a series of dorsal roots on both sides of the cord, on separate recording/stimulating electrodes. If one dorsal root is stimulated, antidromic activity can be recorded in adjacent ipsilateral and contralateral dorsal roots (Bagust et al. 1985a,b; Bagust 1989). The evoked DRR spreads along the cord in both directions from the point of stimulation (Bagust et al. 1993), showing that there is a strong linkage between the systems responsible for generating the DRR at all levels throughout the thoracic and lumbar segments of the cord.

Chapter 2

Materials and Methods

2.1 Preparation of the isolated spinal cord:

The experiments described in these investigations were performed on *in vitro* spinal cords taken from 19–22 day old wistar rats, male and female, weighing between 35 and 55 grams. Animals were killed by halothane overdose and rapidly perfused transcardially with 100ml of chilled Tyrodes solution according to the formula (in mM): NaCl 136.9, KCl 2.7, $MgCl_2.6H_2O$ 0.5, $NaHCO_3$ 11.9, $NaH_2PO_4.2H_2O$ 0.4, Glucose 5.6, $CaCl.2H_2O$ 0.2. The Tyrodes solution used had a pH of 7.2-7.4 and a temperature of 4°C. The transcardiac perfusion was performed by cutting the left ventricle of the rat heart and inserting a 19 gauge hypodermic needle. The Tyrodes solution flowed through the systemic circulation and was removed by an incision in the right aorta of the heart. Perfusion of the animal both rapidly cooled the tissue and removed all blood from the circulation and spinal cord. This kept the tissue fresh, improved the reflex responses and also eliminated non-specific staining during histology work.

After perfusion the animal was decapitated. A block of tissue containing the vertebral column and spinal cord was then rapidly excised and chilled by immersion in cold (4°C) artificial cerebrospinal fluid (aCSF) according to the formula (in mM): NaCl 118; KCl 3; $NaHCO_3$ 24; $MgCl_2$ 1; $CaCl_2$ 2.5; glucose 12. By chilling the verebral column as rapidly as possible the death of spinal neurones by anoxia was prevented. The vertebral column and the spinal cord was then transferred to a dissection chamber. Under a continuous flow of cold aCSF, the cord was exposed by removing laminae from the ventral surface, using a pair of fine scissors working from the rostral to the caudal end of the cord. The cord was removed by cutting the spinal nerves distal to the dorsal root ganglia, particular care being taken not to damage the lumbar dorsal roots which were cut peripheral to the dorsal root ganglia. The entire length of the cord complete with all lumbar roots was then floated free

from the vertebrae. Any adhering dura was carefully dissected away and the ventral and dorsal roots in the lumbar region were separated. The dorsal root ganglion remained attached to the lumbar dorsal roots. All the ventral roots were severed to ensure that only dorsal root activity was recorded during subsequent electrophysiological and immunochemical experiments.

Immunohistochemical experiments were carried out using whole, intact spinal cords *in vitro*. However, electrophysiological experiments were carried out using hemisected cords. In order to hemisect the rat spinal cord, the cord was torn apart which gave a clean midline split. The rostral end of the cord was held with two sets of forceps and the right and left halves were pulled apart using a steady lateral pull. The rostral end of the cord, damaged by the forceps, was cut off.

After dissection, which usually took between 30 and 40 minutes to complete, the spinal cord was mounted in the experimental bath. The cord was pinned to the base of the experimental bath using fine tugsten pins with the dorsal surface facing upwards for stimulation and recording.

2.1.1 The experimental bath:

The experimental bath consisted of a groove 1cm wide and 9cm long, milled in a block of Perspex (Fig. 2.1). The base of this channel was filled with a silicone rubber compound (Dow Corning Encapsulant 3112 TVR) providing a soft base into which pins could be driven. Perfusion fluid (aCSF) flowed into the chamber at a low level at one end and was sucked out of a reservoir at the other end, the level of the bath fluid being determined by the level of the outlet suction probe (a 25 gauge hypodermic needle).

The aCSF in the main 500ml reservoir was bubbled with a 95% oxygen-5% carbon dioxide gas mixture and was fed into the experimental chamber at a flow rate of 20–40ml/min. Inflow into the bath was by gravity feed from the 500ml reservoir providing a hydrostatic pressure head of approximately 20cm of water. A peristaltic pump was used to suck the fluid out of the bath via the suction probe after it had passed over the tissue and return it to

the reservoir, thereby maintaing a constant level. The temperature of the circulating aCSF could be raised by passing through a heated water jacket prior to entering the experimental chamber and this bath temperature was continuously monitored by means of a thermocouple. A system of three–way stopcock taps enabled test solutions, which had different fluid compositions, to be run through the bath from a second 100ml reservoir with no change in flow rate or temperature.

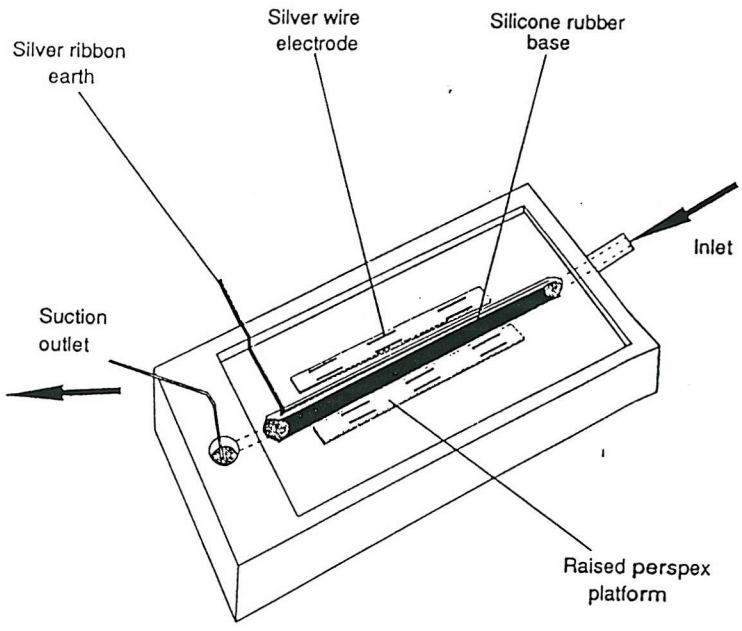


Fig. 2.1: Diagram of the experimental bath. From Bagust (1993) The spinal cord as an in vitro preparation. In D.I. Wallis (Ed). *Electrophysiology: a practical approach.* Oxford University Press.

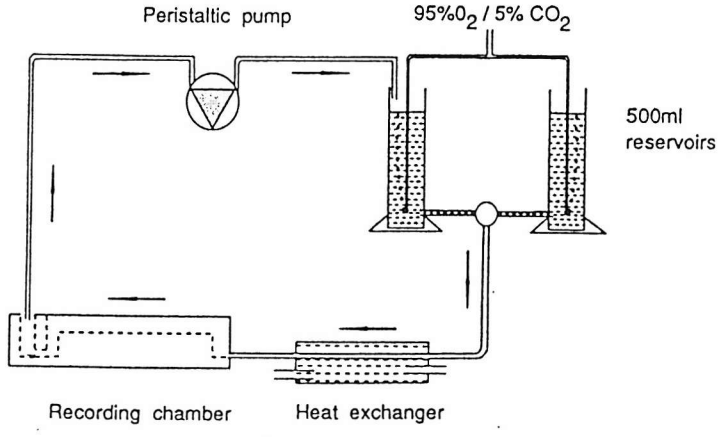


Fig. 2.2: The set up of the recirculating aCSF system. From Bagust (1993) The spinal cord as an in vitro preparation. In D.I. Wallis (Ed). *Electrophysiology: a practical approach.* Oxford University Press.

2.1.2 Recovery:

Dorsal roots set up for recording immediately following dissection of the spinal cord showed only a small amount of spontaneous activity. One or two units fired continuously and this was thought to be due to damage of the roots (Chen et al. 1993). However, the continuous firing subsided within the first hour following dissection and after 2–3 hours, a characteristic pattern of bursts of spontaneous activty developed which gradually increased in both frequency and in the number of spikes within a burst. Approximately three hours after the dissection, a stable pattern of activity was reached which continued for up to 15 hours (Bagust et al. 1989). Therefore, any stimulation and/or recordings made on the spinal cord preparation were carried out 3 hours after the dissection was finished.

2.2 Stimulation and recording:

Stimulation and recordings were made by draping two adjacent lumbar dorsal roots over silver wire electrodes in the side chamber of the experimental bath. One dorsal root was used for stimulation and the other was used to allow dorsal root activity to be monitored during the experiment. Although it was only possible to record from one root and to stimulate another at any one time, the apparatus was designed to provide maximum flexibility and to permit easy changes in the combination of stimulation and recording electrodes used. Dessication of the roots draped over the silver wire electrodes was prevented by covering all exposed tissue with a layer of petroleum jelly mixed with liquid paraffin.

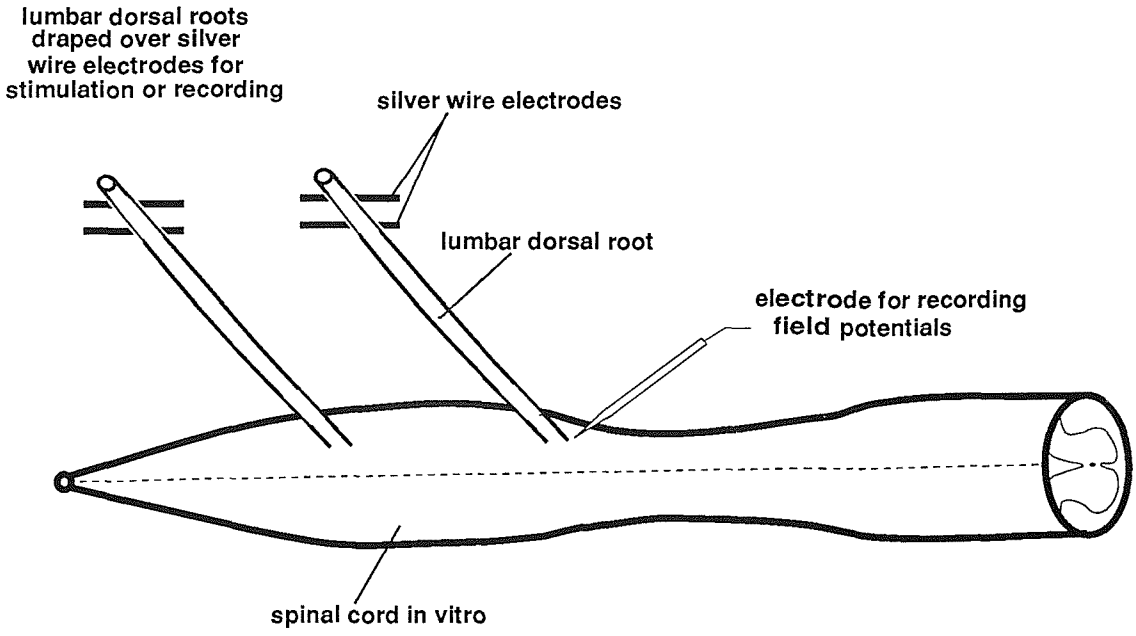


Fig. 2.3: The *in vitro* rat spinal cord arrangement for stimulation and recording.

Recordings were made simultaneously from pairs of electrodes and delivered to a microcomputer–based recording system via a multichannel interface unit (Grafitek, SP9) and an IBM–PC compatible computer (Amstrad). Incoming data was amplified and digitized by the Data Logging Interface (Grafitek, SP9) and delivered to the computer. The programs used in the experiments described were EROS (evoked response on–line recording system), SAP (signal average program) and SPIKE (Bagust 1989).

EROS superimposes software–generated threshold levels upon the signal and redisplays the signal through digital–to–analogue converters (DACs) to a dual–channel non–storage oscilloscope (Hitachi, V–152F). By adjusting the input amplification and the threshold level through the keyboard of the computer, baseline noise was eliminated, ensuring that only events exceeding a certain amplitude were recorded, although care was taken to set the threshold discriminator levels as close to the baseline noise as possible to enable the smallest action potentials to be recorded. A sound generator in the interface unit was used to provide auditory confirmation. Negative going events were recorded by inverting the incoming signal. The Data–Logging Interface also provided a software–driven trigger output which was used to drive external equipment such as stimulators. Stimuli in the experiments described were applied from two Grass S44 stimulators to the dorsal roots using the silver wire electrodes. Computer–driven stimuli produced in this way were

recorded by EROS or SAP and used to generate dorsal horn field potentials or produce peri-stimulus time histogram (PSTH) plots of evoked activity.

Using the EROS program, trains of up to 50,000 spikes could be recorded simultaneously from the two input channels, together with a third channel containing information on computer-generated stimuli and keyboard-activated event markers. Recordings were stored on disc for later analysis. The recorded data was used to produce displays of spike count against time, amplitude histograms and peri-stimulus time histogram (PSTH) plots of evoked activity. Each spike recorded was identified by number, allowing easy location of the start and finish of any section of data analysed. The PTSH analyses allowed pretriggering in order to "look back" and investigate the events occuring before stimulation. Figure 2.4 shows a diagram of the recording/stimulating system.

Alternatively, extracellular field potentials generated by stimulation of a dorsal root were recorded on SAP (signal average program) (Bagust 1989). The SAP program allowed stored data from the Grafitek Transient Signal Recorder (TSR) to be read and displayed on the computer screen for detailed examination and measurement. The Grafitek TSR acted as a "buffer" stage, capturing fast analogue signals and storing them in a digital form. The sample rate was 40KHz when measuring fast wave dorsal horn field potentials and 1KHz when measuring slow wave dorsal horn field potentials. When the data capture was complete an interrupt signal was sent to the computer which copied the data into its own memory and displayed it on the monitor screen. This data was then manipulated by the computer. Incoming data was averaged with another 10 samples to improve the signal/noise ratio, and the data displayed on the screen was stored on disc for later examination. Sections of the data store were expanded and examined in detail, measurements were made using cursors, and a hardcopy of the captured field potential was also obtained. Measurements of shape, latency, duration and amplitude of the evoked potentials could be made using the cursor function in SAP.

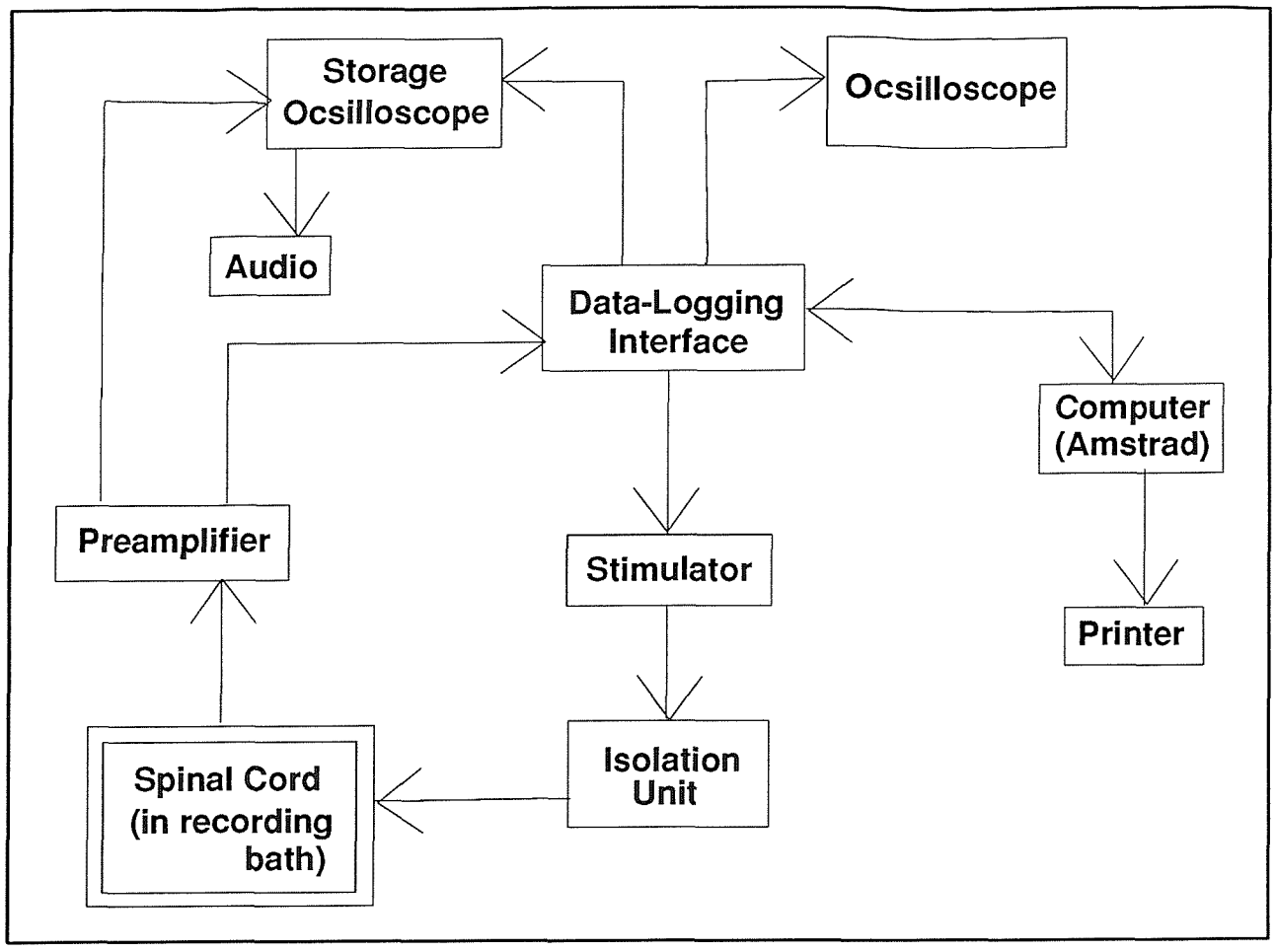


Fig. 2.4 : diagram to show the set up of the stimulating/recording equipment.

2.2.1 Temperature:

To investigate the effect of temperature upon spontaneous dorsal root activity, the circulating aCSF and hence the preparation was either cooled externally using ice or heated via a waterbath and a heated water jacket enclosing the circulating aCSF. The number of spikes were measured every 30 seconds for a 15 minute period at different temperatures ranging from 16°C to 26°C. At each temperature, a 15 minute period was allowed before the spontaneous activity was recorded in order to allow for equilibration.

2.2.2 Evoked Activity:

Lumbar dorsal roots L5 and L6 were set up for stimulation and recording. Normal spontaneous activity was recorded for 30 minutes after which time single stimuli were given at 15 second intervals for a 1 hour period. Stimuli given had a duration of 0.5ms and an intensity of 1.5 times the voltage necessary to evoke a DRR (i.e. 1.5 x threshold voltage, 1.5T). All data was recorded on EROS for later analysis. Temperature was kept constant at 20°C.

2.2.3 Addition of Cations to the Medium:

Lumbar dorsal roots L5 and L6 were set up for stimulation and recording. For 30 minutes prior to the experiment, normal spontaneous activity was recorded at a temperature of 18°C. Single stimuli at an intensity of 1.5 times the DRR threshold voltage and a duration of 0.5ms were given at 15 second intervals. This evoked activity was recorded for a further 15 minutes. The circulating aCSF was then modified so that the calcium in the medium was replaced by 2mM manganese. After 5 minutes the manganese was washed from the preparation by replacing the original aCSF containing calcium and no manganese. The data was recorded on EROS throughout the experiment and the temperature remained at 20°C.

2.3 Dorsal Horn Field Potentials:

Field potentials were used to measure the response in a population of cells rather than in a single cell. Transient potential differences occuring within interneurones as a result of electrical activity were measured by placing a recording electrode into the superficial dorsal horn of the spinal cord.

Field potential recordings were achieved by placing a blunt glass microelectrode filled with a 1M solution of NaCl (resistance <5MΩ) into the superficial laminae of the lumbar dorsal horn. The blunt glass microelectrode was placed into the superficial dorsal horn using a head stage. The microelctrode was placed caudal to the L5 or L6 dorsal root and the head stage manipulator was turned 720 degrees which placed the microelectrode 2mm deep in the superficial laminae.Stimulation every 15 seconds at 5 times the threshold voltage necessary to evoke a DRR in an adjacent dorsal root (for fast waves) or 20 times the DRR threshold voltage every 30 seconds (for slow waves) gave rise to a complex field potential consisting of at least 4 components (Fig. 2.5). A fifth component occurs with stimulation of the dorsal root at 20 times the DRR threshold voltage (Fig. 2.6).

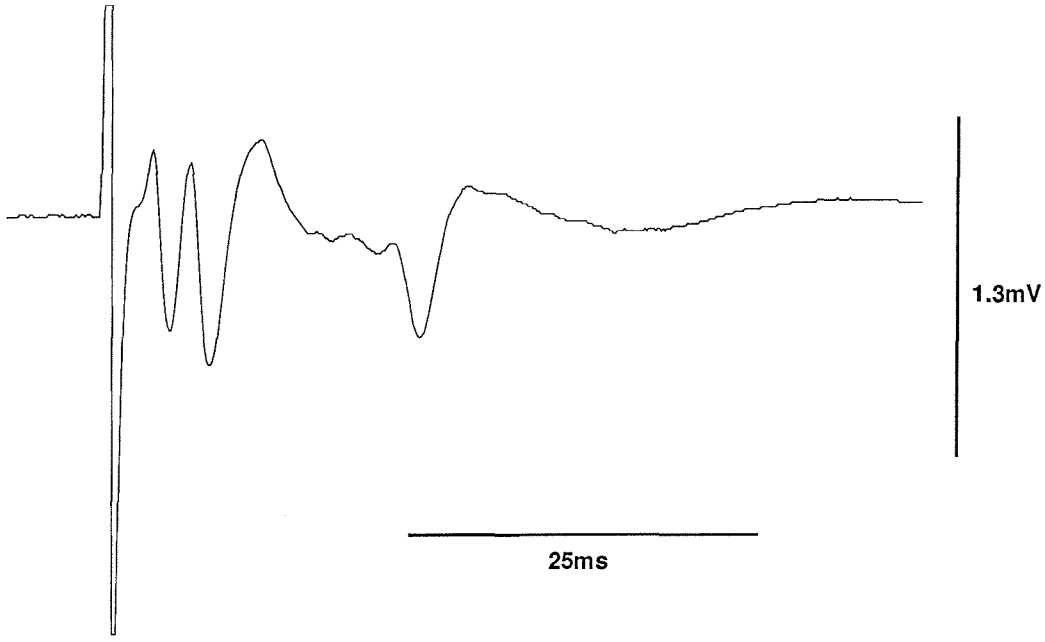


Fig. 2.5: The fast wave dorsal horn field potential evoked by stimulation of a dorsal root at 5 times the DRR threshold voltage.

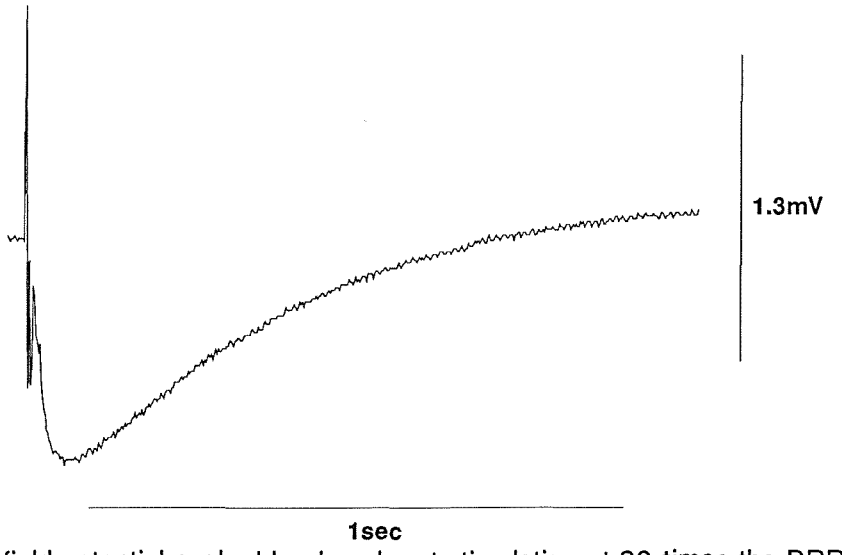


Fig. 2.6. The slow field potential evoked by dorsal root stimulation at 20 times the DRR threshold voltage. The "tail" of the slow field potential lasted for approximately 500ms.

Recordings in the dorsal horn reached a maximum negative potential in the superficial laminae (approximately laminae I and II) before becoming positive in the deeper, more ventral laminae (laminae III to X). This ensured the correct positioning of the electrode for recording from the substantia gelatinosa (laminae I and II).

2.3.1 Dorsal Root Fibres Involved in the Dorsal Horn Field Potentials

To investigate which class of fibres were recruited when a dorsal root was stimulated, the conduction velocitites of the activated fibres were calculated. A dorsal root was draped over the silver wire electrodes of the experimental bath for stimulation, and a suction electrode was attached to the same dorsal root near to the cord itself to record the resulting afferent volley. The stimulation-recording arrangement is shown in Fig. 2.7.

The suction electrode used to measure the afferent volley was manufactured from a glass capillary tube and filled with aCSF. To record activity, the dorsal root was sucked up into the tube by negative pressure so that it made contact with the aCSF inside the electrode. Before any recordings of the afferent volley were made, the calcium in the medium was replaced with 2mM of manganese to ensure that no antidromic activity in the dorsal root was recorded. All recordings were made at a bath temperature of 20-22°C.

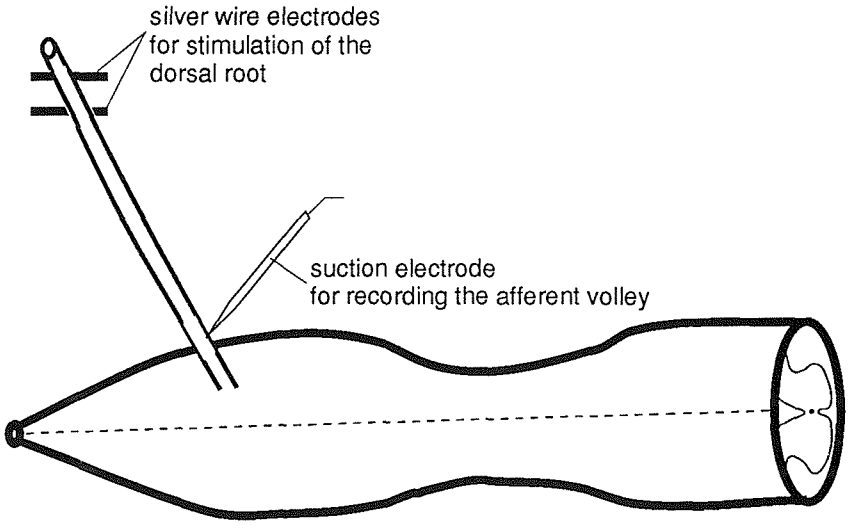


Fig. 2.7: Diagram to show the arrangement of silver wires for stimulation of a dorsal root and the suction electrode attached at the end of the same dorsal root to measure the resulting afferent volley.

2.3.2 The Effect of Chemical Agents on Dorsal Horn Field Potentials

The effect of various chemical agents on the dorsal horn field potential responses were examined. Test substances were added as cumulative doses to create a cumulative concentration-response curve for each experiment. A 1mM solution of each drug was made and varying volumes were added to the 100ml drug bath. Each amount added gave a final concentration in the aCSF bathing the tissue of between 0.1 and 100µM. The protocol below was followed:

Volume of 1mM solution:	Total volume in bath	Final bath concentration:
0.01ml	0.01ml	0.1µM
0.09ml	0.1ml	1.0µM
0.9ml	1ml	10µM
9ml	10ml	100µM

The slow/fast wave dorsal horn field potentials were recorded after each dose of drug. Recordings were made 20 minutes after the addition of the drug and lasted for 5 minutes. 10 samples were recorded and averaged to give a final result for each dose which was stored on disc for later analysis.

2.3.1 Electrophysiology Analysis:

To investigate the effect of adding the drugs to the medium, the dorsal horn field potentials were measured by two different methods:

(i) the amplitude of the S1 and the S2 synaptic components of the fast wave dorsal horn field potentials were measured using the cursor function of the SAP program (Fig. 2.8). Amplitudes were expressed as a value in mV. The percentage increase or decrease of the control amplitude was then calculated before the results were analysed.

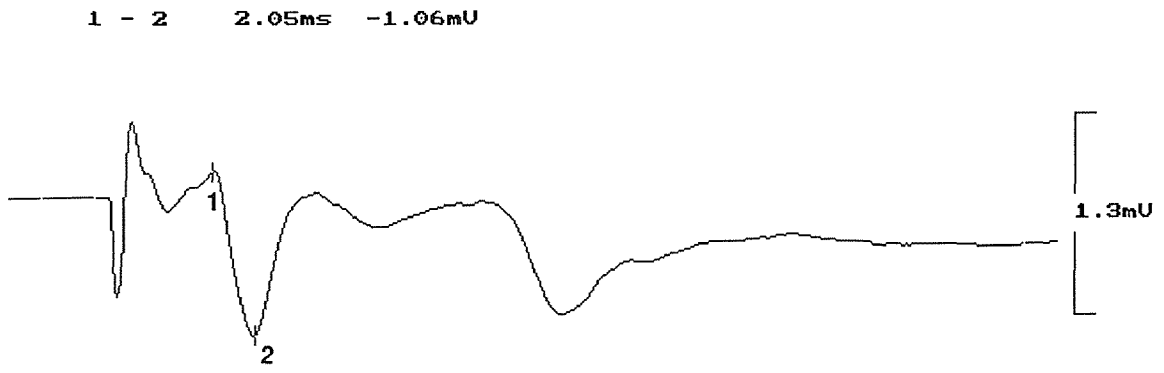


Fig. 2.8: Measurement of the S1 component amplitude using the cursor function of the SAP program. Cursors are marked 1 and 2.

(ii) the slow wave field potential was measured as an area. The area between the baseline (pre-stimulation) and the end of the slow wave was measured and expressed as an arbitary value (Fig 2.9). By measuring the area of the slow wave any effects of added ligands were calculated as a percentage increase or decrease of the control area.

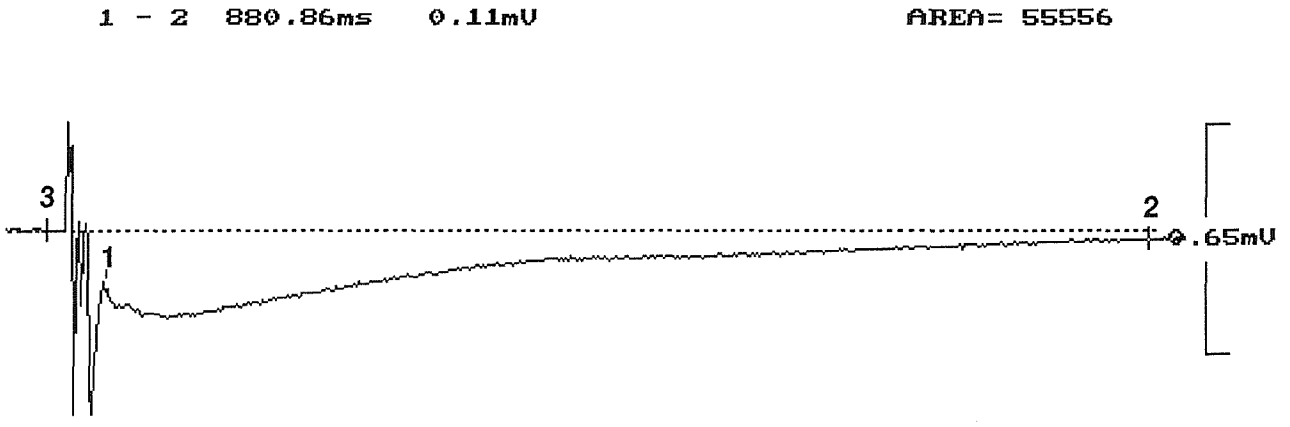


Fig. 2.9: measurement of the area of the slow wave using cursors. The arbitary value for the area is given at the top right side of the screen. Cursors are marked 1, 2 and 3.

The significance of the results were calculated using the Students paired t-test. Statistics were carried out on raw data and the result for each concentration of drug (n=4-8) was considered to be significantly different from the control if $p<0.05$. The one sample t-test was used to confirm certain significant results. The one sample t-test compares the mean of a set of data with a theoretical mean value entered. For example, the significance of the results after the addition of 100μM of a drug was calculated using the experimental

results (as a percentage of the control) and a theoretical mean of 100. Data for each concentration of drug was displayed in a line graph as a percentage of the control.

2.4 Induction of Fos expression

Lumbar dorsal roots (L5 and L6) were mounted on the silver wire electrodes for stimulation or recording. The temperature of the circulating aCSF was then increased to 27°C and test chemicals were added to the bathing medium before commencing experimentation. Various methods were then used to investigate the induction of c–*fos*.

High intensity stimulation: Evoked responses were obtained by stimulating one adjacent dorsal root at an intensity of 20 times the DRR threshold voltage, 10Hz frequency and 0.5ms pulse duration. Stimulation was given for 10 minutes.

Low intensity stimulation: Evoked responses were obtained by stimulating one adjacent dorsal root at an intensity of 1.5 times the DRR threshold voltage, 10Hz frequency and 0.5ms pulse duration. Stimulation was given for 10 minutes.

Capsaicin added to the medium: 0.5μM capsaicin dissolved in alcohol was added to the bathing medium. No stimulation was given.

Addition of chemicals to the medium: Test substances were added to the experimental bath at least 40 minutes before a train of high intensity dorsal root stimulation (10Hz, 0.5mspulse duration, 20 times the DRR threshold voltage for 10 minutes) was delivered to the cord.

An incubation period of 2 hours following stimulation (or addition of capsaicin) was allowed before the spinal cord in each experiment was fixed in 4% paraformaldehyde (pH 7.3–7.4) overnight and subsequently cryoprotected in 20% sucrose in PBS for 24 hours.

2.4.1 Capsaicin Pretreatment of Animals:

In order to destroy C fibres, neonatal animals were injected subcutaneously with capsaicin (50mg/kg) within 24 hours of birth. Capsaicin was injected in 10% ethanol, 10% tween 80 and 80% saline. An equal number of littermates were injected with a saline vehicle control. At 19-22 days of age the spinal cord of capsaicin pretreated and control animals were removed and subjected to a train of stimuli (0.5ms duration, 10Hz frequency for 10 minutes) at 20 times the DRR threshold voltage before being processed for Fos immunoreactivity.

2.4.2 Immunohistochemistry:

Tissue sections were cut transversely from spinal cord segments L1-L6 using a 2800 Frigocut E cryostat at 7μm. Every tenth tissue section was mounted onto a chrome gelatin coated slide for immunohistochemical location of the Fos protein. The primary antibody to the Fos oncoprotein (Genosys Biotechnologies Inc.) was polyclonal and was raised from sheep. It was used at a dilution of 1:800 and was left in incubation with the tissue sections overnight at a temperature of 4°C. To enhance antibody penetration, the antibody was diluted with PBS buffered saline (Inflow Laboratories) containing 0.2% Triton.

Following overnight incubation with the primary antibody, the tissue sections were washed with PBS for 10 minutes. Next, an immunoperoxidase technique known as the ABC technique was employed. The use of the avidin-biotin reaction, known as the ABC method is a simple and sensitive method for localising antigens in formalin-fixed tissues. The technique involves the addition of an unlabelled primary antibody, followed by the application of a biotin labelled secondary antibody and then a preformed avidin-biotin horseradish peroxidase complex. Biotin-binding site avaliability in the complex is created by the incubation of a relative excess of avidin with biotin-labelled peroxidase. During formation of the complex, avidin acts as a bridge crosslinking biotin-labelled peroxidase molecules. Consequently a 'lattice' complex containing several peroxidase molecules is formed. Binding of this complex to the biotin moieties associated with secondary antibody results in a high staining Intensity (Hsu et al.1981).

The biotinylated antibody (dilution 1:200) was incubated with the tissue sections for 30 minutes at room temperature. The sections were then washed in PBS for 10 minutes before incubating with the avidin–biotin complex for a further 30 minutes at room temperature. The ABC kit used was purchased from Vector and used sheep IgG. The sections were again washed in PBS for 10 minutes. The next step involved developing the sections for 5–7 minutes in a substrate hydrogen peroxidase solution which consisted of a 1:1 mixture of 0.02% hydrogen peroxidase and 1mg/ml 3,3–Diaminobenzidine in 0.1M Tris buffer. The reaction was terminated by washing the sections in PBS.

The method used for the immunohistochemical location of the Fos protein was carried out using a Sequenza Immunostaining Centre.

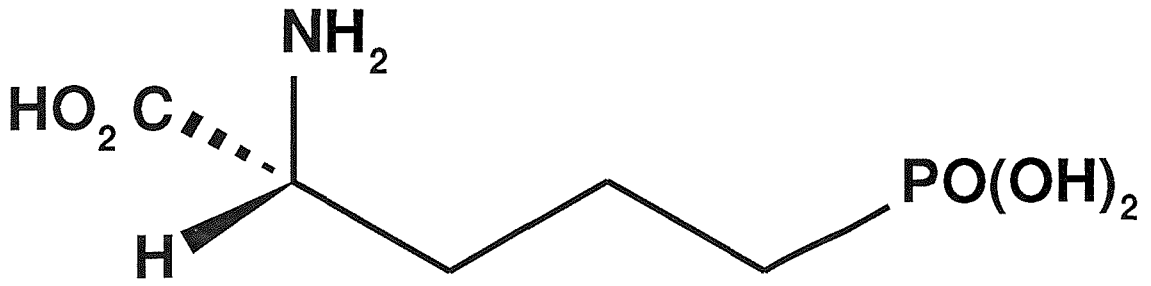
In order to mount sections on the slides they were first dehydrated with alcohol in the series 50%, 70%, 90% and 100% (x2) before being rinsed with inhibisol and covered with DePX and cover slips. All the slides were stored at room temperature for subsequent analysis.

Immunohistochemically labelled tissue sections were examined microscopically at a magnification of x20. The Fos immunoreactive nuclei were seen as dark, round to oval structures. For quantitative analysis all Fos–positive cells were counted separately, regardless of staining intensity. The number of Fos-positive cells were counted in a total of 20 tissue sections (10 L5 sections and 10 L6 sections) per animal. The mean number of Fos-positive cells per section for each experiment was then calculated.

Results were expressed as the mean number of cells per section.

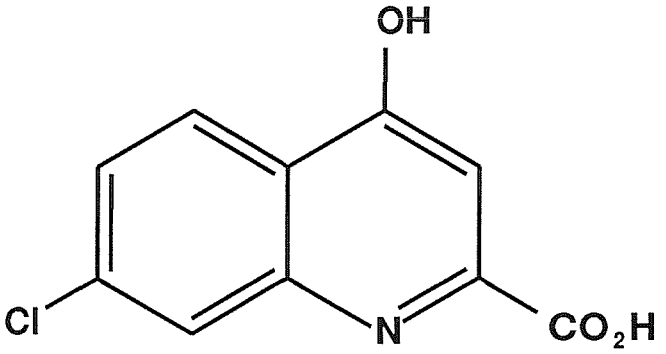
2.5 Chemicals:

D- AP5 (D(-)-2-amino-5-phosphonopentanoic acid)



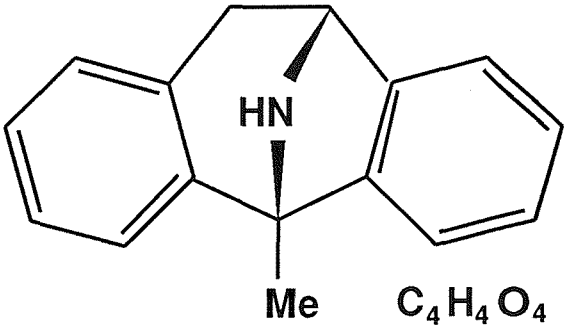
A potent and competitive NMDA receptor antagonist acting at the NMDA/glutamate site of the receptor. D-AP5 was purchased from Tocris.

7-Cl KYNA (7-chlorokynurenic acid)



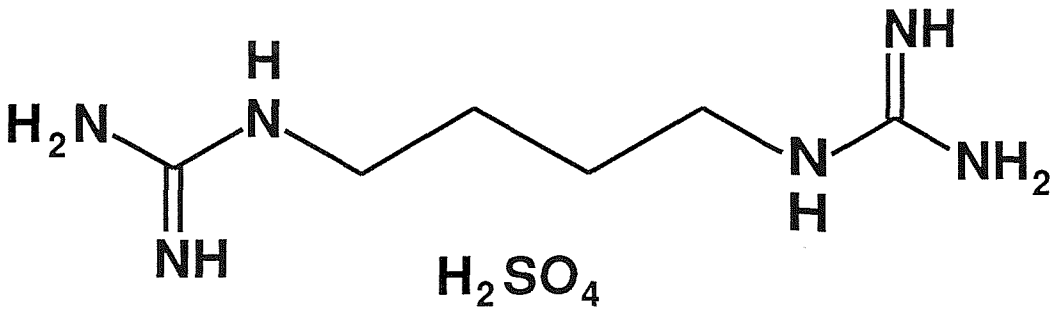
7-Cl KYNA ia an NMDA receptor antagonist acting at the glycine site. Purchased from Tocris.

(+)-MK-801 ((5R, 10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine / dizocilpine)



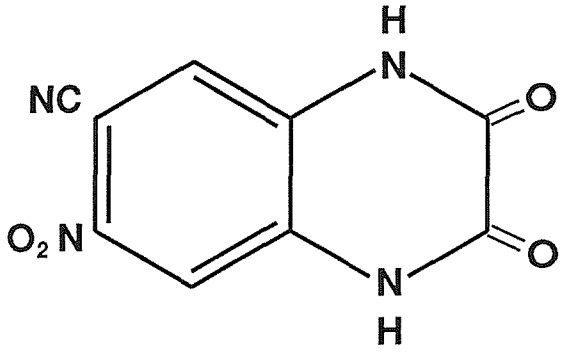
A potent, selective and non-competitive NMDA receptor antagonist. It acts by binding to a site located within the NMDA associated ion channel and therefore prevents calcium influx. MK-801 was purchased from Tocris.

Arcaine Sulphate (N, N'-1,4-butanediylbisguanidine)



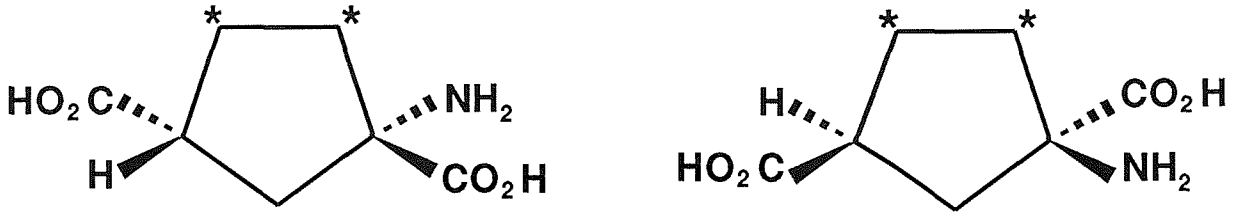
An NMDA receptor antagonist acting as a competitive inhibitor at the polyamine site. It also has an antagonist action at the enzyme nitric oxide synthase (NOS). Arcaine sulphate was purchased from Tocris.

CNQX (6-cyano-7-nitroquinoxaline-2,3-dione)



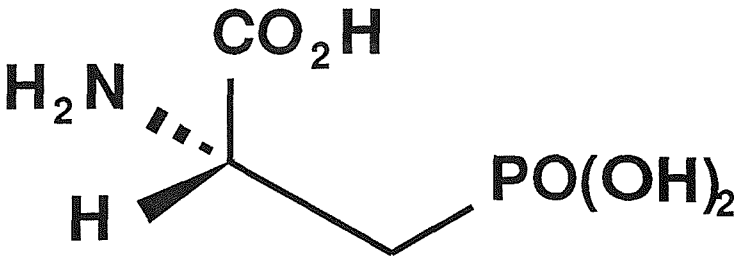
A potent AMPA/kainate receptor antagonist. Also an antagonist at the glycine modulatory site of the NMDA receptor complex at high concentrations. CNQX was purchased from Tocris.

***trans*-ACPD** ((±)-1-Aminocyclopentane-*trans*-1,3,-dicarboxlic acid)



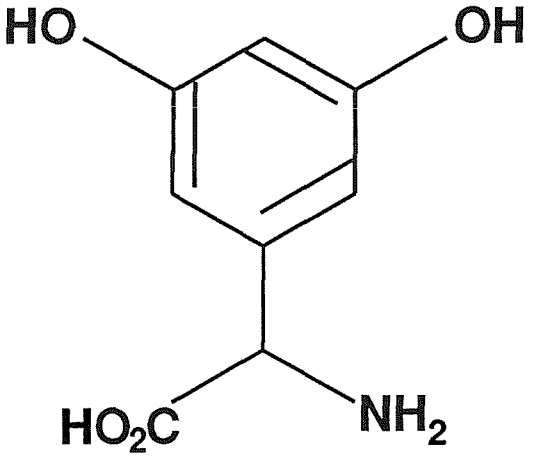
Equimolecular mixture of (1S, 3R)- and (1R,3S)-ACPD. Selective agonist for metabotropic glutamate receptors; active at both group I and group II metabotropic glutamate receptors. *Trans*-ACPD was purcahsed from Tocris.

L-AP3 (L-(+)-2-amino-3-phosphonopropionic acid)



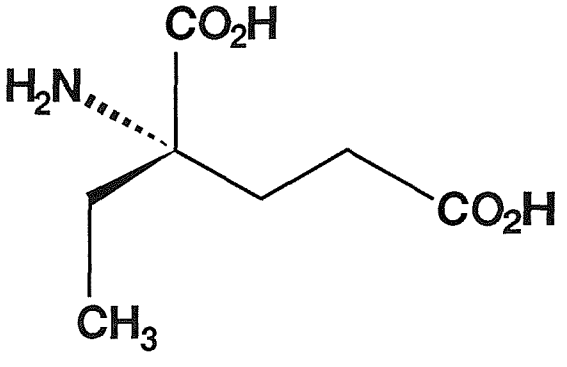
Antagonist of metabotropic glutamate receptor-mediated phosphoinositide responses. Acts at the group I metabotropic glutamate receptors. L-AP3 was purchased from Tocris.

(RS)-3,5-DHPG ((RS)-3,5-Dihydroxyphenylglycine)



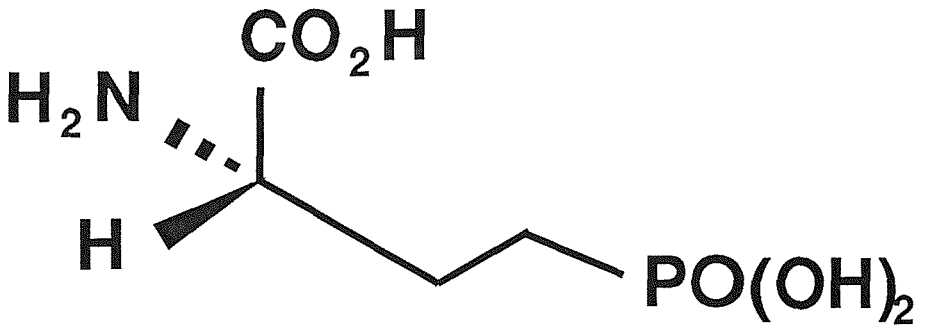
Selective group I metabotropic glutamate receptor agonist which activates both $MGluR_1$ and $MGluR_5$. Also reported to be an antagonist at metabotropic glutamate receptors linked to phospholipase D. DHPG was purchased from Tocris.

EGLU ((2S-α-Ethylglutamic acid)



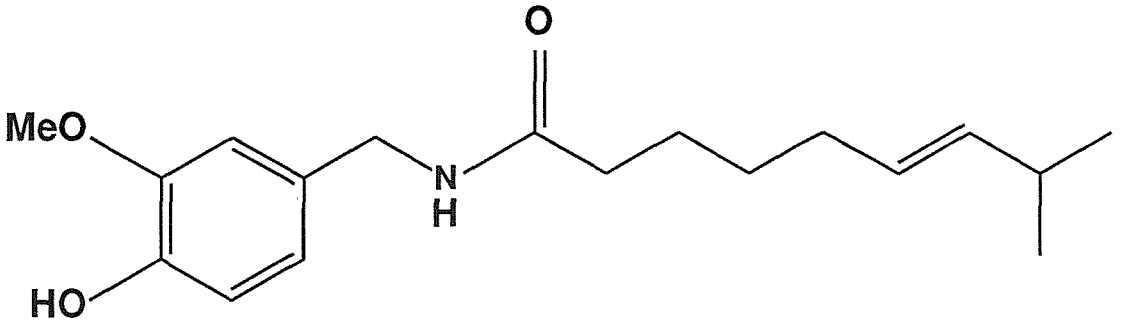
EGLU is a selective group II metabotropic glutamate receptor antagonist. Purchased from Tocris.

L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid)



Selective group III metabotropic glutamate receptor agonist. L-AP4 was purchased from Tocris.

Capsaicin (8-methyl-N-vanillyl-6-nonenamide)



A potent neurotoxin. It is the consituent part of cayenne peppers and activates sensory neurones that give rise to unmyelinated C-fibres. Topical application desensitises the sensory endings giving a paradoxical antinociceptive effect. Systemic activation can be neurotoxic to capsaicin-sensitive cells especially in newborn animals. Capsaicin was purchased from Sigma UK.

Fos antibody:

The Fos antibody used was a sheep polyclonal antibody to Fos. It was raised against the synthetic peptide Met-Phe-Ser-Gly-Phe-Asn-Ala-Asp-Tyr-Glu-Ala-Ser-Ser-Ser-Arg-Cys selected from a conserved region of mouse and human Fos. It was human and rodent reactive and immunoprecipitated a 62KD protein. It did not react with the Jun protein. The Fos antibody was purchased from Genosys.

Chapter 3

Characteristics of the in vitro Spinal Cord

3.1 Introduction

Experiments using *in vivo* spinal cord preparations allow only limited pharmacological studies and results may be misinterpreted due to the complicating effects of anesthetics, blood pressure and descending modulatory systems that affect activity. In the *in vitro* spinal cord preparation these external influences are removed which allows more detailed pharmacological studies of pain and nociceptive processing in the spinal cord.

The aims of the following experiments were:

1) to investigate the characteristics of the *in vitro* spinal cord preparation.

2) to validate the preparation as a viable tool for the investigation of nociceptive mechanisms that occur within the dorsal horn of the spinal cord.

3.2 Results:

3.2.1 Spontaneous activity:

At 18-20°C, spontaneous activity could be detected in the lumbar dorsal roots, which had a characteristic pattern of bursts of action potentials, separated by a silent period of approximately 10 seconds. This spontaneous activity developed over a period of 2-3 hours following dissection and persisted for 8-10 hours. The spontaneous activity recoreded in root L5 of an *in vitro* spinal cord preparation is shown in Fig. 3.1.

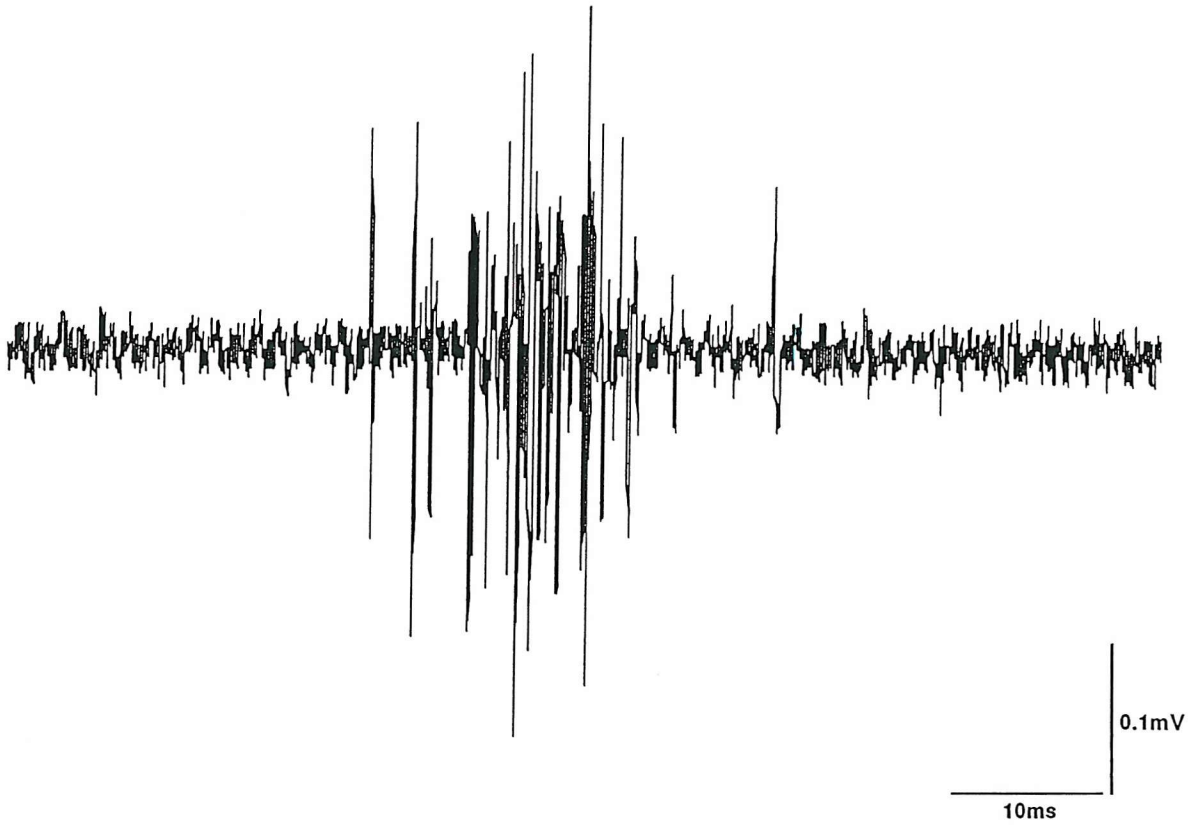


Fig 3.1: Recording of spontaneous activity in dorsal root L5 made from an isolated rat spinal cord preparation showing the characteristic change in the pattern of activity.

Inter-spike interval analysis of spontaneous activity (Fig. 3.2) recorded from the dorsal roots showed a bimodal distribution of intervals, characteristic of a bursting pattern of activity. One peak had a mode at short intervals (1-4 ms), representing the time between action potentials within the burst. The second mode occured at longer intervals (3900-7000 ms), representing the time between successive bursts.

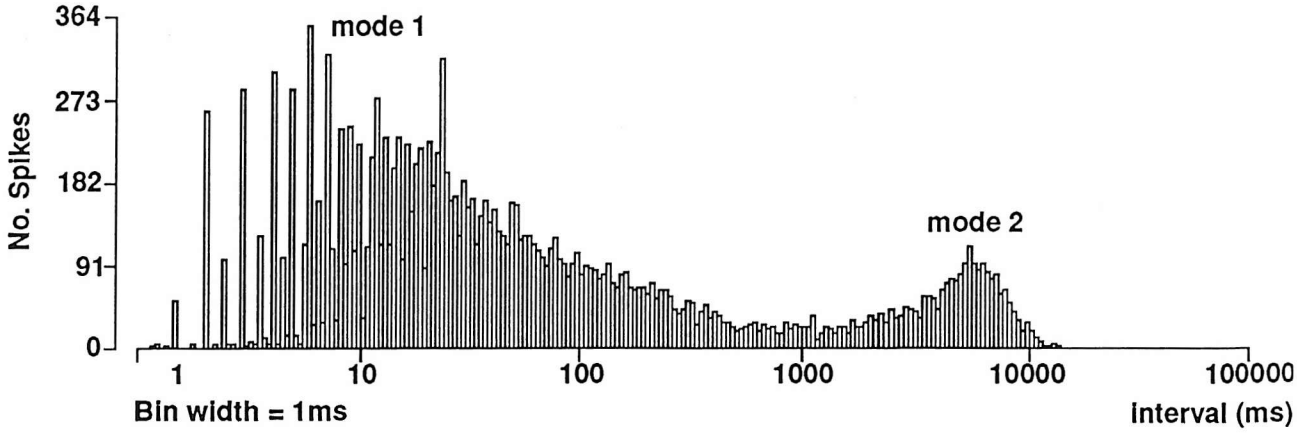


Fig 3.2: Inter-spike interval plot of spontaneous dorsal root (L5) activity in a cord taken form a 19 day old rat demonstrating the bimodal distribution of intervals.

Previous investigations (Bagust et al. 1985a) have shown that the spontaneous bursts of activity are due to synaptically generated action potentials emerging from the spinal cord and travelling antidromically along the dorsal roots. A continuous pattern of firing without silent periods between bursts was considered to be caused by damage to either the roots or the cord, and preparations in which this persisted were discarded.

3.2.2 Evoked dorsal root activity

Stimulation of a dorsal root adjacent to the one being used for recording, at an intensity of 1.5-2 times the DRR threshold voltage, evoked a burst of action potentials in the root under test. This was the classic DRR as described by Toennies in 1938. The DRR activity usually consisted of more action potentials than were observed in the bursts of spontaneous activity. Peri-stimulus time histogram (PSTH) plots (Fig. 3.3) showed that the evoked burst had a duration up to 50ms with a latency in the range of 25ms, at 20°C.

A period of inhibition following the initial excitatory component, shown by a reduction in spontaneous activity was observed approximately 5 seconds following the reflex.

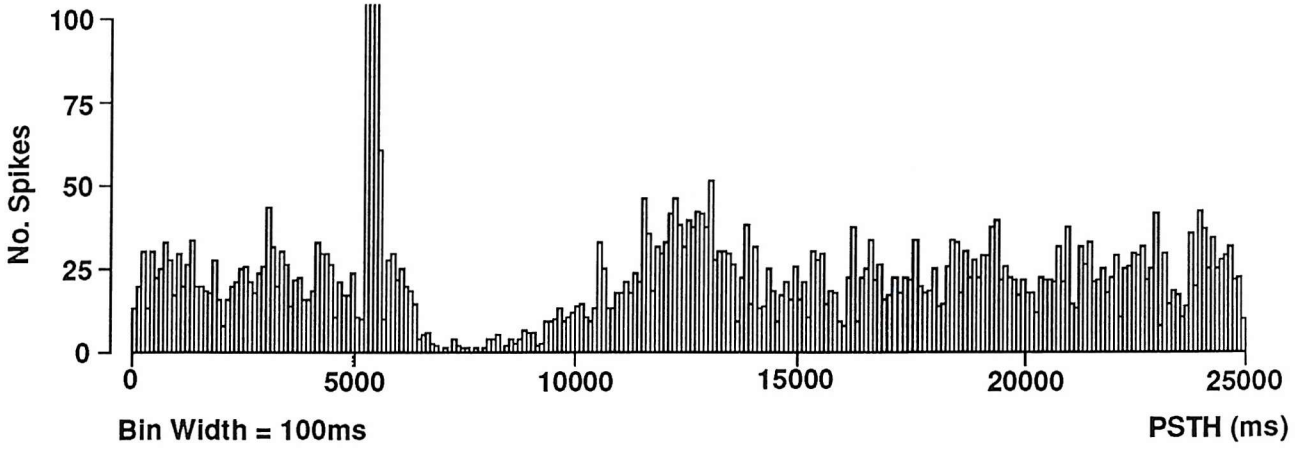


Fig. 3.3: Peri-stimulus time histogram (PSTH) plot of dorsal root activity following stimulation of an adjacent dorsal root. This PSTH shows the depression of firing following the reflex response.The (PSTH) above has bin widths of 100msec.

3.2.3 *The effect of temperature on spontaneous dorsal root activity*

Experiments performed on the DRR *in vivo* have consistently demonstrated an increase in activity at low temperatures (Toennies 1938; Brooks et al. 1955), but it is difficult to reduce the cord temperature to less than 32°C under *in vivo* conditions. The *in vitro* preparation allows greater flexibility in manipulating the environmental conditions than are possible *in vivo*, and therefore the effects of temperatures between 16°C and 28°C, upon the frequency of the spontaneous dorsal root activity, have been examined. An example of such an experiment is shown in Fig. 3.4 and a summary of the results of 4 experiments is shown in Fig. 3.5, where the frequency of dorsal root activity is expressed as a percentage of the control activity at 19°C in each experiment. The spontaneous activity was found to reach a peak at temperatures between 16°C and 18°C, falling away steeply at temperatures above this range.

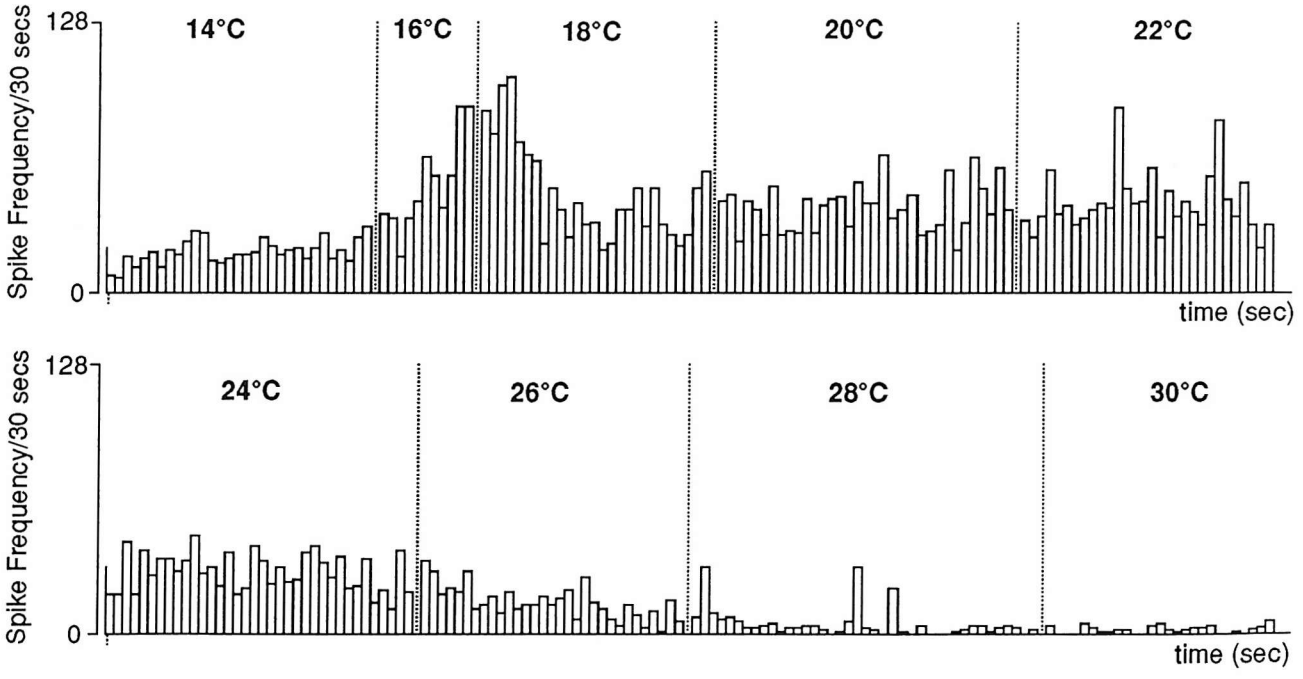


Fig. 3.4: Raw data to show how temperature effects spontaneous activity. Spike frequency was decreased as the temperature was increased. The optimum temperature was found to be 16-18°C.

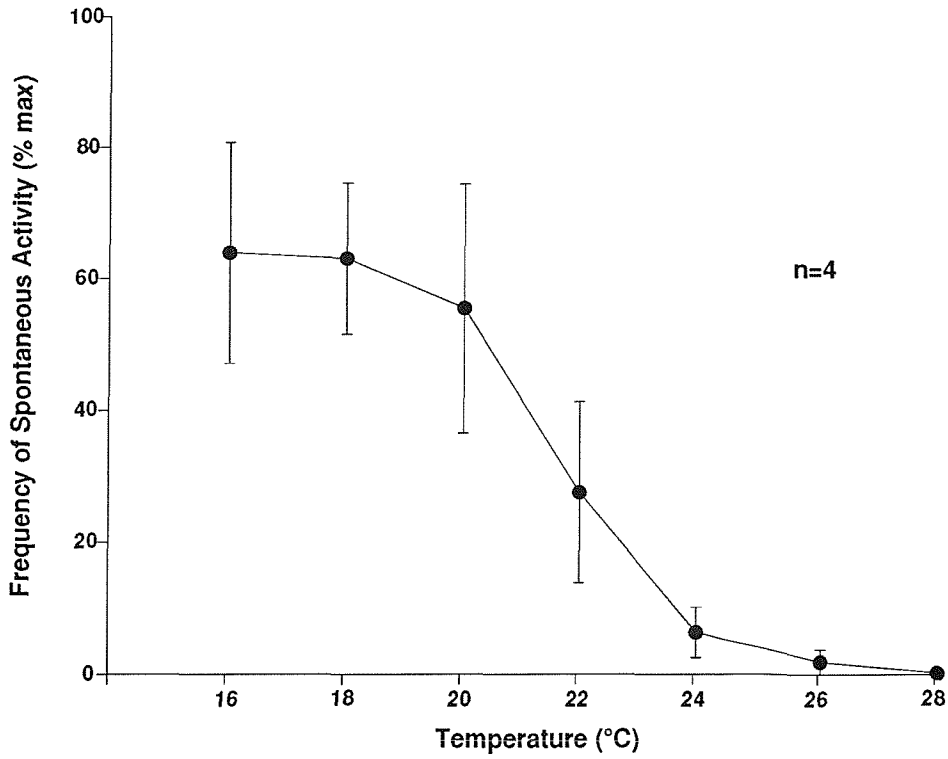


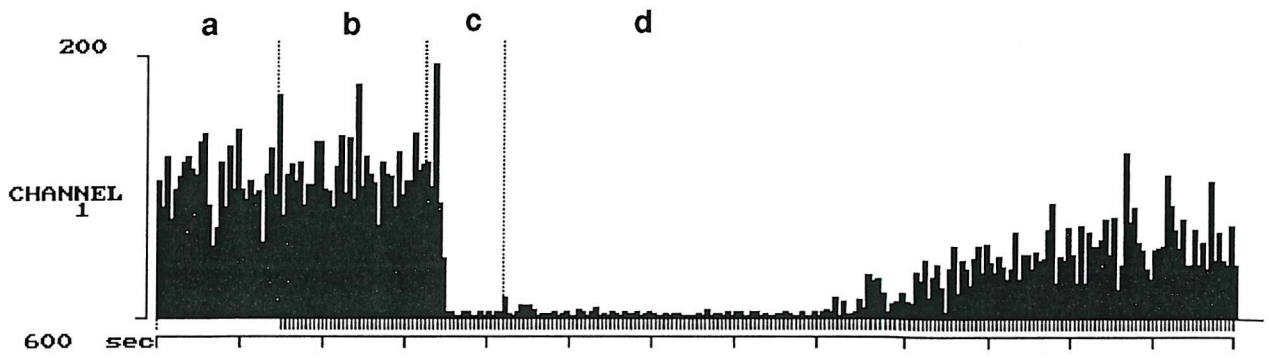
Fig. 3.5: Graph showing the effect of changes in temperature upon the frequency of spontaneous activity in 4 experiments. Optimum temperature was found to be 16-18°C.

3.2.4 The synaptic origin of spontaneous and evoked dorsal root activity:

In order to differentiate between electrical activity due to conduction in directly stimulated afferent nerves and activity following synaptic transmission, the calcium in the bathing medium was omitted and replaced by manganese. It is known that the release of presynaptic transmitter is calcium dependant, and the omission of this ion from the perfusion medium, or replacement by an ion such as manganese, is frequently used as a means of reversibly blocking synaptic activity in isolated preparations (Kerkut and Bagust 1995). Manganese prevents the release of transmitter vesicles from the pre-synaptic terminal by blocking the voltage-dependent calcium channels involved in the release mechanism.

The effect on the spontaneous activity in the dorsal root of replacing the calcium in the aCSF with 2mM manganese is shown in Fig. 3.6. The 2mM manganese effectively blocked all synaptically mediated events in the isolated spinal cord, and this treatment reversibly blocked both the DRR evoked by stimulation of an adjacent dorsal root and the

spontaneous dorsal root firing. Recovery of synaptic activity was obtained within 30 minutes of returning to a perfusion medium containing normal levels of calcium and no manganese.



a = spontaneous activity control
b = evoked activity control
c = addition of 2mM manganese
d = wash

Fig 3.6: an experiment illustrating blockade of spontaneous and evoked activity recorded from a lumbar dorsal root (L6) by replacement of calcium in the medium by 2mM manganese.

3.2.5 Dorsal Horn Field Potentials

Stimulation of the lumbar dorsal roots evoked a complex pattern of field potentials which was recorded using a glass microelectrode. At least 4 components were identified in the lumbar dorsal horn elicited by low intensity dorsal root stimulation (5 x voltage required to evoke a DRR in an adjacent dorsal root) (Fig. 3.7).

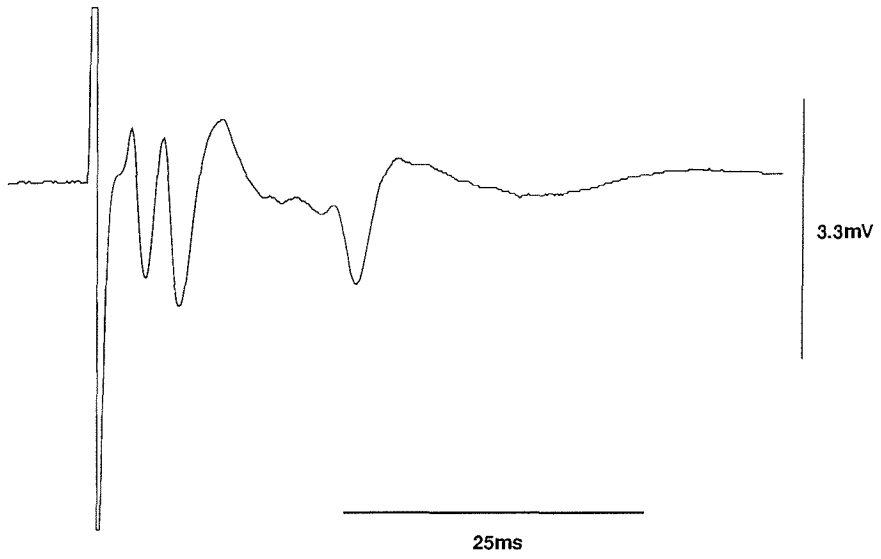


Fig. 3.7: The fast wave dorsal horn field potential evoked by stimulation of a dorsal root at 5 x DRR threshold voltage.

At a higher stimulation (20T), the initial 4 fast wave components occur along with a slow "tail" which took 500-1000ms to return to the baseline (Fig. 3.8).

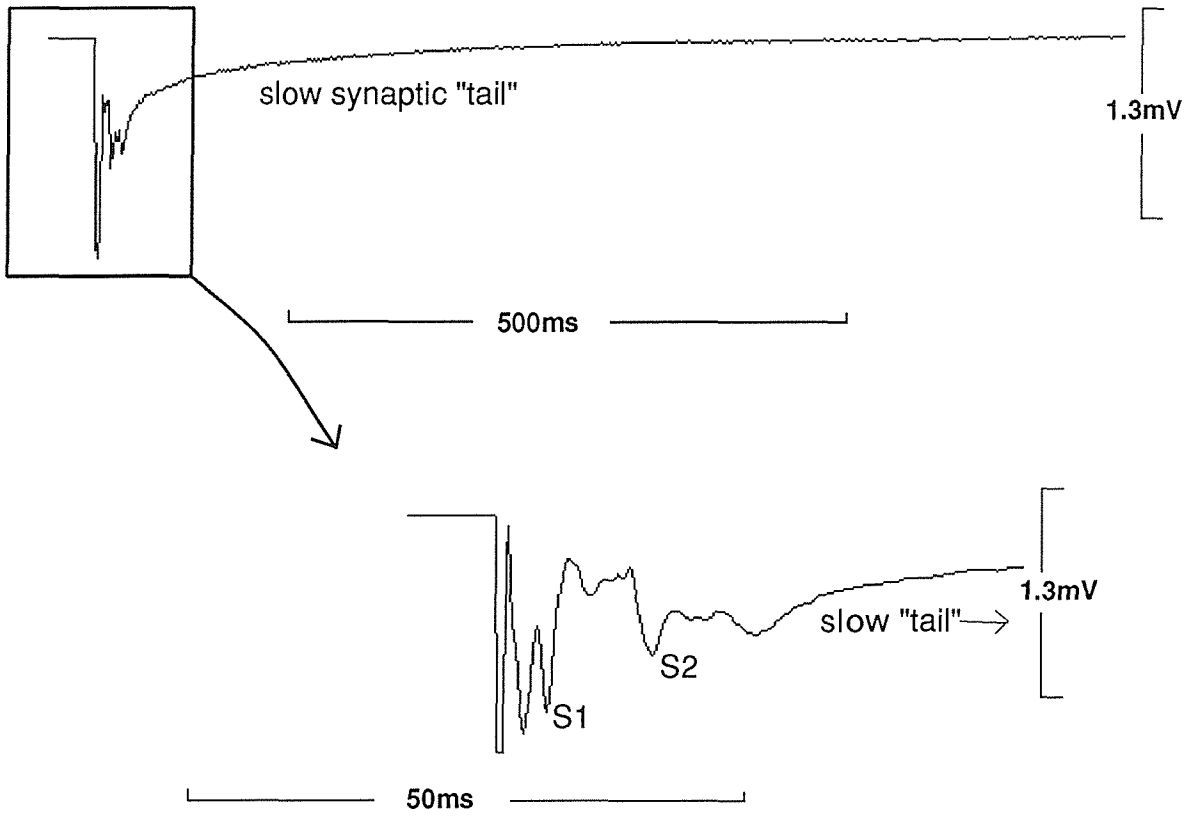


Fig. 3.8: The slow wave field potential was superimposed upon the first four components of the fast wave field potential as well as a synaptic "tail" which takes 500-1000ms to return to the baseline.

To investigate which of these components were of synaptic origin, the synaptic activity in the spinal cord was completley inhibited by replacing the calcium in the bathing medium with a 2mM concentration of manganese (Figs. 3.9 and 3.10).

Blocking the synaptic activity of the cord reversibly inhibited the last two components of the fast dorsal horn field potential which had a latency of 5-10ms and 20-25ms respectively (Fig. 3.9). These components were, therefore, concluded to be of synaptic origin.

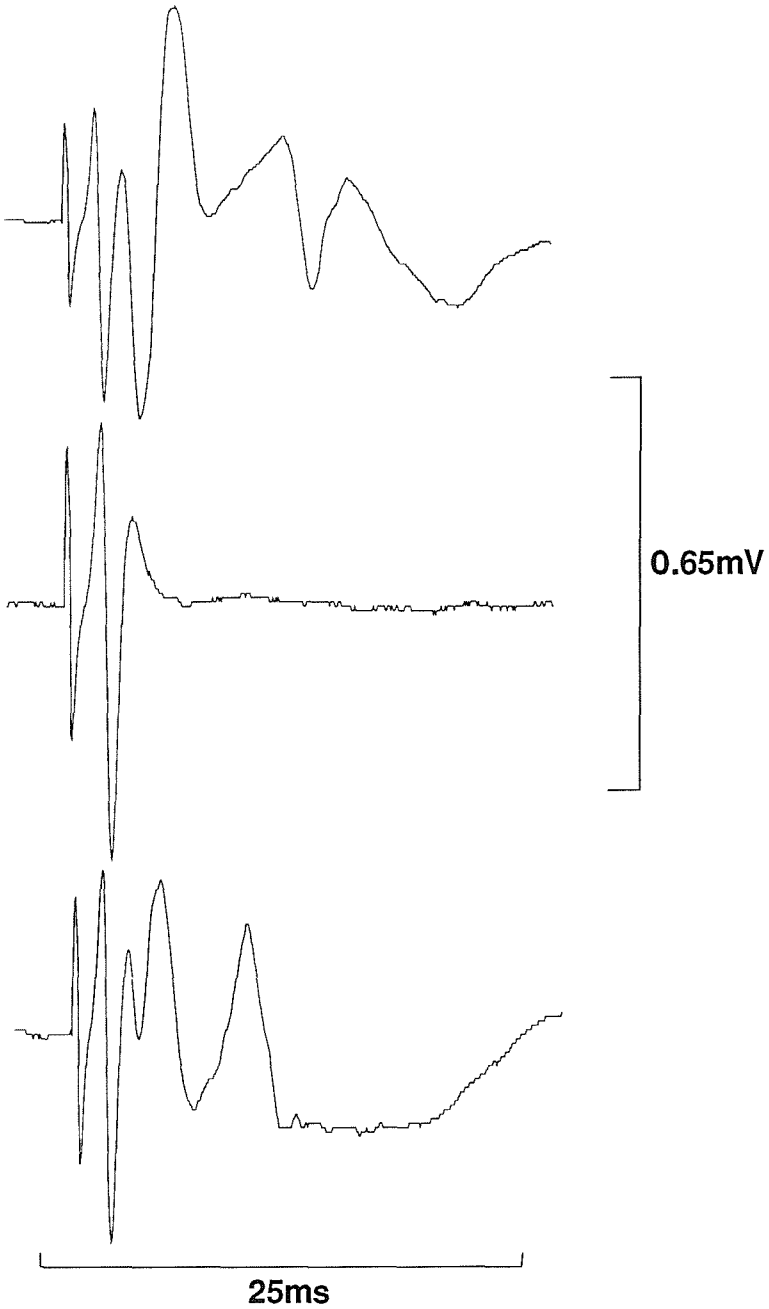


Fig. 3.9: An example of one experiment (averaged recording of 10 cycles) showing the replacement of calcium in the bathing medium with a 2mM concentration of manganese. This inhibited the two later components of field potentials elicited by low intensity (5T) dorsal root stimulation. These are the synaptic components and are termed S1 and S2.

The 4 components of the fast wave field potential can therefore be concluded to consist of:

(a) the stimulus artifact;

(b) the conducted component representing the incoming volley of the stimulated afferent fibres;

(c) the first synaptic component occuring as synaptic activation excites post-synaptic cells and having a latency of 5-10msec;

(d) a second "fast" synaptic component can also occur at 20-25ms.

The slow "tail" elicited by stimulation at 20 times the DRR threshold voltage was also inhibited by the addition of manganese to the bathing medium. It was therefore concluded that this tail was of a synaptic origin (Fig. 3.10).

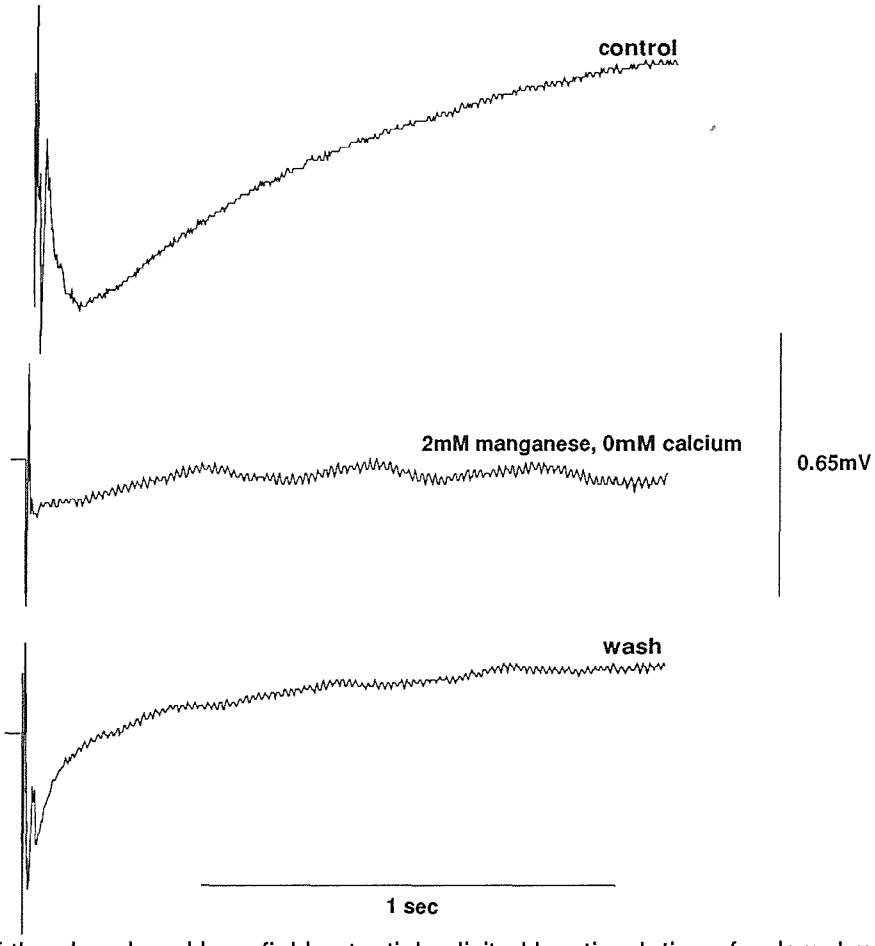


Fig 3.10: The "tail" of the slow dorsal horn field potentials elicited by stimulation of a dorsal root at 20T was found to be of a synaptic origin since it was blocked by the addition of 2mM manganese to the bathing medium. This diagram shows an example of one such experiment (average of 10 cycles).

3.2.6 The Dorsal Root Afferent Volley

A suction electrode, manufactured from a glass capillary tube, was used to record the afferent volley of the fibres activated by stimulation of a lumbar dorsal root at differing intensities. The dorsal root to be stimulated was mounted over a pair of silver wire electrodes and the end nearest the cord was sucked into the electrode by negative pressure. This part of the dorsal root then made contact with aCSF inside the glass and allowed recordings to be made of the afferent volley entering the spinal cord. The calcium in the medium was replaced with a 2mM concentration of manganese before recordings were made to ensure recordings were not due to antidromic activity in the root. All recordings were made at a bath temperature of 20-22°C.

Stimulation of a dorsal root at 5 times the DRR threshold voltage yielded two groups of peaks. The first and highest peak represented both the stimulus artifact and stimulation of the fibres with very fast conduction velocities. Because the dorsal roots of the rat spinal cord were so short, it was very difficult to separate the stimulus artifact from the fast conducting fibres. The second group of peaks (peaks 1-4) had a conduction velocity of between 2.20±0.13 m/s and 0.78±0.03 m/s (n=5).

Stimulation of a dorsal root at 20 times the DRR threshold voltage yielded both the first group of peaks (which represented the stimulus artifact and the very fast conducting fibres) and the second group of peaks (peaks 1-4). However, a third group of peaks was also recorded. This third group of peaks (peaks 5-8) had much slower conduction velocities of between 0.56±0.03 m/s and 0.25±0.01 m/s (n=5).

The results of these experiments are shown in Figs. 3.11 and 3.12.

Peak	Conduction Velocity ± SEM
1	2.20± 0.13 m/s
2	1.47± 0.06 m/s
3	1.01± 0.05 m/s
4	0.78± 0.03 m/s
5	0.56± 0.03 m/s
6	0.48± 0.01 m/s
7	0.36± 0.02 m/s
8	0.25± 0.01 m/s

Fig. 3.11: The conduction velocities of the peaks after stimulation of a dorsal root. Peaks 1-4 occurred after stimulation of a dorsal root at 5 times the DRR threshold voltage. Stimulation of a dorsal root at 20 times the DRR threshold voltage yielded 8 peaks. Peaks 5-8 had slower conduction velocities. (n=5).

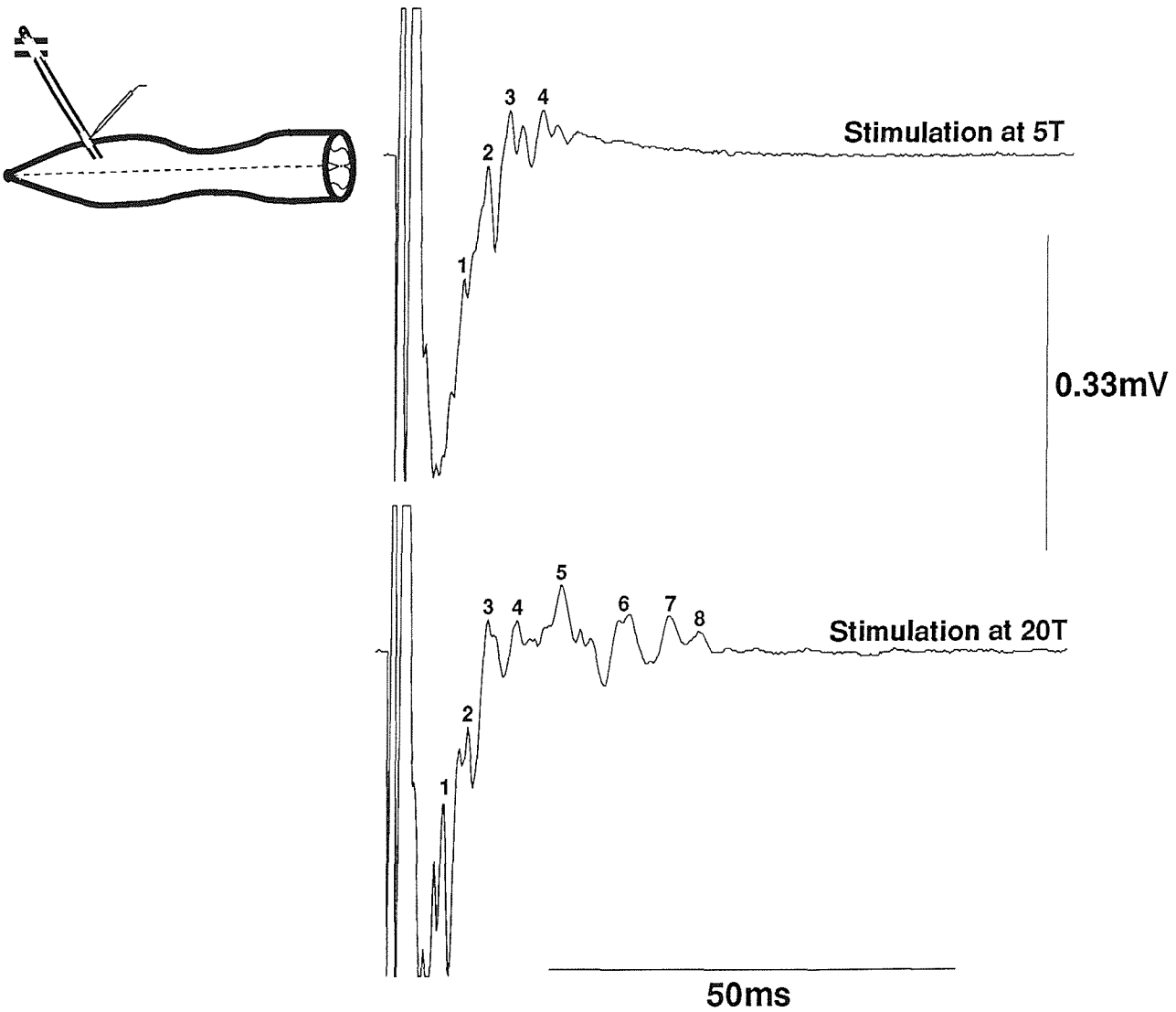


Fig 3.12: An example of the dorsal root volley evoked by low intensity (5 times the DRR threshold - 5T) and high intensity (20 times the DRR threshold - 20T) dorsal root stimulation. The dorsal root volley was measured using a suction electrode. The peaks are numbered and represent activation different groups of afferent fibres.

3.2.7 Stimulus-Response Experiments

The diagram below (Fig. 3.13) illustrates the results from a set of experiments in which the intensity of dorsal root stimulation was increased. The resulting response amplitude of the S1 and S2 components of the fast wave field potential were measured and plotted in a graph of stimulation against response (Fig. 3.14). The results of the stimulus-response experiments clearly show that the fast wave field potential conducted component (representing the incoming volley of the stimulated afferent fibres) increases proportionally to the amount of dorsal root stimulation, while the first and second synaptic components do not increase in such a manner. The amplitude of the first and second components remain relatively constant regardless of the intensity of the dorsal root stimulation at intensities up to 12 times the threshold voltage required to induce a DRR in an adjacent dorsal root.

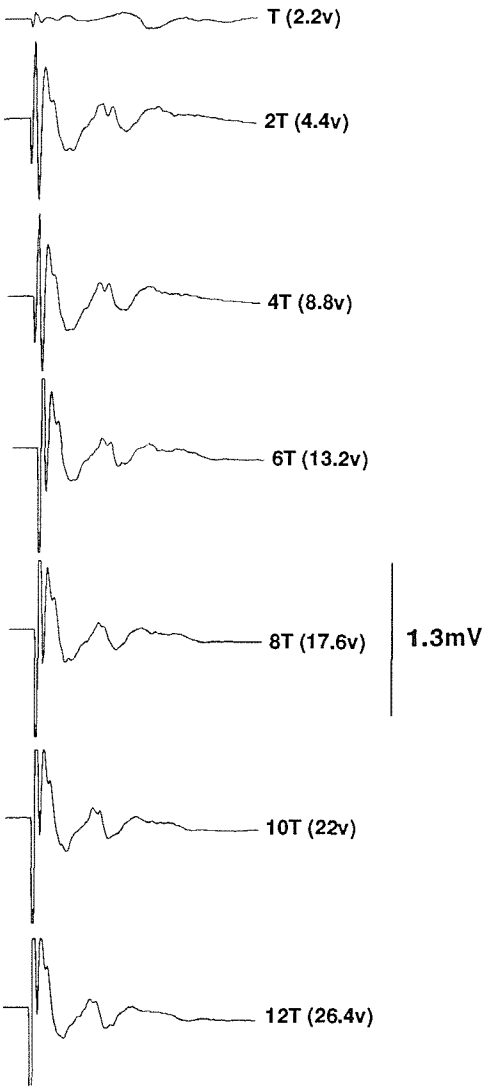


Fig. 3.13: Raw data illustrating that the S1 and S2 components do not increase as the conducted component increases.

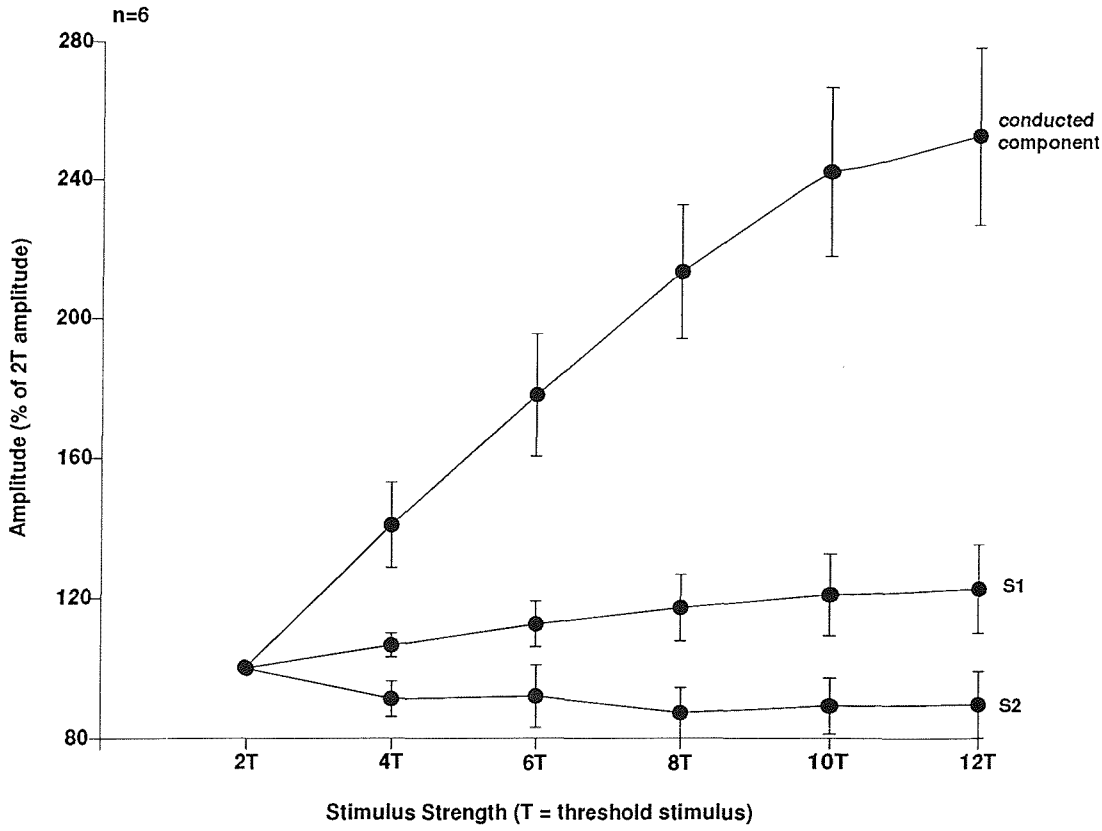


Fig. 3.14: Graph to show that as the dorsal root stimulation increased, so the conducted component increased. However, the S1 and S2 synaptic components did not increase proportionally to the intensity of dorsal root stimulation. n=6.

3.3 Discussion:

Two to three hours following dissection, spontaneous bursts of action potentials were observed emerging from the cord along the dorsal roots. Such activity has only rarely been reported *in vivo*, e.g. in the dorsal root discharge recorded from the dorsal roots of anesthetised spinal cats (Barron and Matthews 1935). However, when the effects of temperature are considered, this is not surprising. In the isolated spinal cord, spontaneous dorsal root activity was found to be abolished at temperatures approaching normal body temperature. In addition, anaesthetic and descending nervous activity effects, which might also interfere with dorsal root activity, were excluded from the preparation. Evidence based upon measurements of variations of excitability *in vivo* has illustrated fluctuations in the degree of depolarisation of primary afferent fibres (Rudomin and Dutton 1969). It is probable that the spontaneous dorsal root activity seen *in vitro* is caused by an increase in amplitude and duration of these membrane potential fluctuations under the conditions of reduced temperature, and this enables the firing threshold of the fibres to be reached and results in an antidromic discharge along the afferent fibres of the dorsal roots.

3.3.1 The Dorsal Root Reflex (DRR)

The *in vitro* spinal cord preparation demonstrates the dorsal root reflex (DRR) very well and has enabled detailed studies to be made of the effects of the environment of the cord upon both the spontaneous and evoked dorsal root activity. It is known that the evoked activity in *in vitro* hamster spinal cord preparations has an optimum temperature of between 25 and 27°C, above and below which the activity declines (Bagust et al. 1985a; Bagust et al. 1989). The frequency of the spontaneous activity in the *in vitro* rat spinal cord is much lower than that of the *in vitro* hamster spinal cord and has been found to reach a maximum at 18°C. At temperatures above 18°C, the frequency of activity declines. It is not known why there is a difference in optimum temperature for spontaneous activity and DRR in different species, but it may be an evolutionary difference due to differing natural environmental conditions. The decline of activity over

the higher temperature ranges means that within the normal physiological range of 36°C to 38°C, spontaneous activity would not be seen. This effect might be attributed to an increasing level of anoxia with increasing temperature in this preparation. However, this is proved to be untrue, as experiments in hamster isolated spinal cord preparations, in which the temperature was raised to 36°C, thus abolishing dorsal root activity showed a return of the spontaneous activity when the temperature was brought back to its original level (Bagust et al. 1985a). These experiments indicate that no observable permanent changes occur at higher temperatures.

Simultaneous recordings made from two ipsilateral dorsal roots taken from *in vitro* hamster spinal cords showed positive correlations between the activity recorded from both (Bagust et al. 1993). This evidence suggests that the spread of the evoked DRR along the cord was not an artifact caused by the electrical stimulation, but is indicative of a functional pathway within the cord which might be responsible for the spread of primary afferent depolarisation (PAD). The physiological significance of the DRR is obscure. The observation that it is optimally seen at temperatures below that usually found in the intact animal and is best evoked by synchronous volleys of action potentials entering the cord (Bagust et al. 1985a; Bagust et al. 1989), suggest that the reflex itself occurs very seldomly *in vivo*.

It is possible that the DRR can only be observed under favourable conditions and may have little significance; it does, however, reflect an underlying mechanism within the cord which probably has a role in the normal physiology of the intact animal. Indeed, the DRR which was once thought to be an electrical artifact, only seen under experimental conditions is now thought to have a physiological role in sensory physiology and locomotion. It has been reported that natural stimulation including light touch, pressure, vibration and muscle stretch all induced the DRR in cats (Millar 1979). DRRs have also been recorded during locomotion, in cats (Dubuc et al. 1985; Dubuc et al. 1988; Duenas et al. 1990). In addition, paw, joint movement and pressure at joints which are inflammed has been found to induce the DRR in cats and monkeys (Rees et al. 1995; Sluka et al. 1995).

The DRR is important as a convenient method of investigating the mechanisms

underlying PAD, since the DRR is thought to be caused by intense PAD following the simultaneous activation of afferent inputs by an electrical stimulus. Intrafibre recordings in the dorsal horn of *in vitro* hamster spinal cord preparations (Bagust et al. 1985b) have demonstrated the presence of spontaneous PAD, which is capable of generating antidromic action potentials in the afferent fibres. PAD is of particular interest because it is thought to be responsible for presynaptic inhibition and provides a means for controlling the relative effectiveness of particular sensory inputs.

Both the evoked and the spontaneous DRR was found to be followed by a period of inhibition lasting more than 5 seconds in the *in vitro* rat spinal cord. In addition, the spontaneous activity was inhibited by an evoked DRR. This suggests that there are more complex events underlying the generation of the DRR than a simple depolarisation of the terminals of the afferent fibres. The excitability of the afferent fibres within the cord appear to be determined by a balance between excitatory and inhibitory inputs with the duration of the inhibition being a major factor in determining the frequency of the spontaneous bursts of activity. This could explain the observation of inhibition following a DRR after both spontaneous and evoked dorsal root activity. Whether the trigger for firing is a spontaneous increase in excitability of a single segment above its threshold, or is provided by activation of afferent inputs by stimulation of the roots, once activated, the mechanism of spread would be the same. Waves of excitation travel outward from the point of activation to the ends of the cord, generating dorsal root reflexes as they passed, to be followed by a period of reduced activity due to inhibition.

The *in vitro* preparation has also allowed investigations to be made into the origin of the events underlying the generation of spontaneous and evoked activity, since the composition of the solution bathing the tissue can be easily modified. Manganese, which is known to block synaptic activity, blocked both the spontaneous and the evoked DRR, demonstrating them to be of synaptic origin and not the result of conduction in collaterals nor due to a build up of potassium released from the stimulated afferent fibres.

The similarity in the responses of the dorsal root reflex and the spontaneous dorsal root firing to temperature and manipulation of the composition of the bathing medium suggests that a common mechanism may be responsible for the generation of both. This was

confirmed by intracelluar recordings in which both spontaneous and evoked PAD was observed to give rise to action potentials in primary afferent fibres (Bagust et al. 1989). However, it has been observed that the pattern of spontaneous activity is largely unchanged when the cord is transected between the two roots, although the correlation between them disappears (Bagust et al. 1989). This suggests that the spontaneous dorsal root activity can be generated at a segmental level, and is not produced by a single central generator. However, the mechanism responsible for generating the spontaneous activity is not known. The isolated spinal cords are not connected to any sense organs and there is no evidence of any activity in the dorsal roots other than the spontaneous activity which is generated within the cord. The mechanism responsible for generating the spontaneous dorsal root activity therefore, must lie within the cord itself.

3.3.2 Dorsal Horn Field Potentials

Stimulating a lumbar dorsal root at a low intensity (5 times the voltage necessary to evoke a DRR in an adjacent dorsal root) gives rise to a complex dorsal horn field potential consisting of at least 4 components. Replacement of calcium in the bathing medium with a 2mM concentration of manganese reversibly blocks the last two components of the field potential, which occur at 5-10ms and 20-25ms. Since manganese is known to inhibit all synaptic activity by blocking voltage-dependent calcium channels, it can be concluded that these last two components are of a synaptic origin. The synaptic component occurring 5-10ms following stimulation has been termed S1 and the second synaptic component occurring at 20-25ms has been termed S2 (Zhang 1997). The first component not affected by the addition of manganese is not of a synaptic origin and has been termed the conducted component. The conducted component represents the volley of incoming afferent activity following dorsal root stimulation. The fast wave field potential is shown in Figure 3.15.

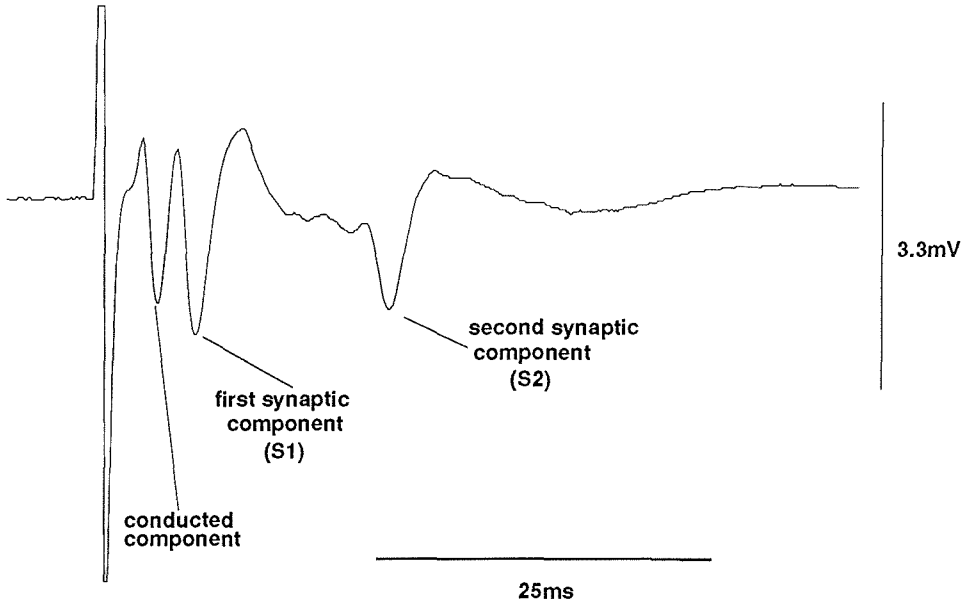


Fig 3.15: The fast wave field potential showing the conducted component and the first (S1) and second (S2) synaptic components.

Stimulation of a dorsal root at 20 times the voltage necessary to evoke a DRR in an adjacent dorsal root produces the four characteristic components of the fast wave field potential (stimulus artifact, conducted component, S1 and S2) as well as a slow 'tail' which takes 0.5 to 1 second to return to baseline. The slow wave field potential was reversibly blocked by replacement of calcium in the medium by 2mM manganese. This indicates that the slow wave is also synaptic in origin.

Stimulation of a dorsal root at 5 times the voltage necessary to evoke a DRR in an adjacent root activated fibres with conduction velocities of between 2.20±0.13 m/s and 0.78±0.03 m/s (n=5) which were recorded using a suction electrode. These conduction velocities corresponded to the conduction velocity that would be expected of the low-threshold, myelinated, non-noxious Aβ fibres. Stimulation of a dorsal root at 20 times the threshold necessary to evoke a DRR in an adjacent dorsal root activated fibres that had conduction velocities approximately equal to that of Aβ fibres as well as fibres with slower conduction velocities of between 0.56±0.03 m/s and 0.25±0.01 m/s (n=5). These fibres, activated only when the dorsal root was stimulated at 20 times the DRR threshold voltage, had conduction velocities which corresponded to that expected of small diameter, unmyelinated C fibres. The threshold of C fibres is approximately 20 times that of the fast conducting Aβ fibres (Gasser 1955), and high threshold responses are indicitative of the presence of C fibres.

The conduction velocities obtained for Aβ and C fibres in the *in vitro* spinal cord were relatively low. This was due to several factors: (1) the temperature of the circulating aCSF (20°C) was much lower than the normal body temperature of the rat, (2) the laminar projections of Aβ and C fibres in the developing rat spianl cord are not fully developed until the end of the third postnatal week (Fitzgerald et al. 1994). Aβ fibres in the developing spinal cord project throughout laminae I to V, including the substantia gelatinosa (lamina II). This widespread termination of Ab fibres is gardually withdrawn over the first three postnatal weeks. At the end of the third postnatal week, the terminal field becomes restricted to the normal laminae III to V (Fitzgerald et al. 1994). The small unmyelinated C fibres in the developing spinal cord grow specifically to laminae I and II, so that for a considerable postnatal period, the substantia gelatinosa is occupied by both Aβ and C fibre terminals (Fitzgerald and Jennings 1999). This means that the recordings conduction velocities after dorsal root stimulation contain a mixture of fibre types, (3) the length of the rat dorsal roots are very short making recordings difficult.

The response of the first and second synaptic components (S1 and S2) has been shown to be unaffected by the amplitude of the conducted component at dorsal root stimulations up to 12 times the DRR threshold voltage. As the dorsal root stimultation was increased, the amplitude of the of the conducted component also increased. However, the amplitude of the S1 and S2 components was not increased. This is because the class of fibres recruited when the dorsal root was stimulated at 12 times the DRR threshold voltage were the same class of fibres being recruited at 2 times the DRR threshold voltage. From the previous experiments using a suction electrode, it seems likely that it was Aβ fibres which were being activated at 2 to 12 times the DRR threshold voltage.

The investigations which have been described illustrate the advantages of the complete *in vitro* mammalian spinal cord preparation over *in vivo* and other more conventional tissue slice preparations. It has been demonstrated that this preparation is a valuable tool for spinal cord pharmacology research.

Chapter 4

Ionotropic Glutamate Receptors.

4.1 Introduction:

Ionotropic glutamate receptors can be divided into NMDA receptors and non-NMDA receptors. The first experimental evidence for the involvement of ionotropic glutamate receptors in nociception came from behavioural studies in which NMDA agonists induced nociceptive effects and the competitive NMDA antagonist D-AP5 had analgesic effects (Cahusac et al. 1984). It is now known that the NMDA, AMPA and metabotropic glutamate receptors each have distinct actions in determining the level of pain transmission in the spinal cord. The AMPA receptor appears to set the baseline level of nociception and faithfully transmits the intensity and duration of the peripheral stimulus (see refs in Dickenson et al. 1997). NMDA receptors are found in the spinal cord of all species including man. The channel of the NMDA receptor is normally blocked by magnesium so that acute noxious inputs into the spinal cord pain circuits are transmitted via the AMPA and not the NMDA receptor, and hence the baseline level of transmission is established. The conditions required for NMDA receptor activation are much more complex and only occur by repeated C-fibre activation (Dickenson and Sullivan 1987; Dickenson and Sullivan 1990).

While NMDA receptor antagonists have the potential for being useful analgesics, the lack of selectivity of current antagonists for spinal cord NMDA receptors diminishes their clinical use. The finding that D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) and ±-3-amino-1-hydroxypyrrolide-2-one (HA-966) had moderate selectivity for spinal cord receptors presents a potential objective for further studies (Wei et al. 1997). Therefore the aims of the following investigations were:

(i) To confirm that NMDA receptors are not involved in the synaptic transmission of low intensity sensory stimulation in the dorsal horn of the spinal cord and to confirm that non-

NMDA receptors do have a role in low intensity sensory stimulation in the dorsal horn.

(ii) To confirm that NMDA receptors have a role in the synaptic transmission of high intensity sensory stimulation in the dorsal horn of the spinal cord.

(iii) To determine which NMDA receptor antagonists best inhibit the transmission of high intensity sensory stimulation and would therefore be of most clinical benefit.

4.2 Results:

Various antagonists were used for the following investigation whose sites of action are shown in Figure 4.1. Significance values (p) were calculated using the raw data (n=4-8) and the Students paired t-test. Certain results were confirmed using the one sample t-test. The significance of results were calculated using the data (percentage of control values) and a theoretical mean of 100. All results are illustrated in the following graphs as percentages of the control value.

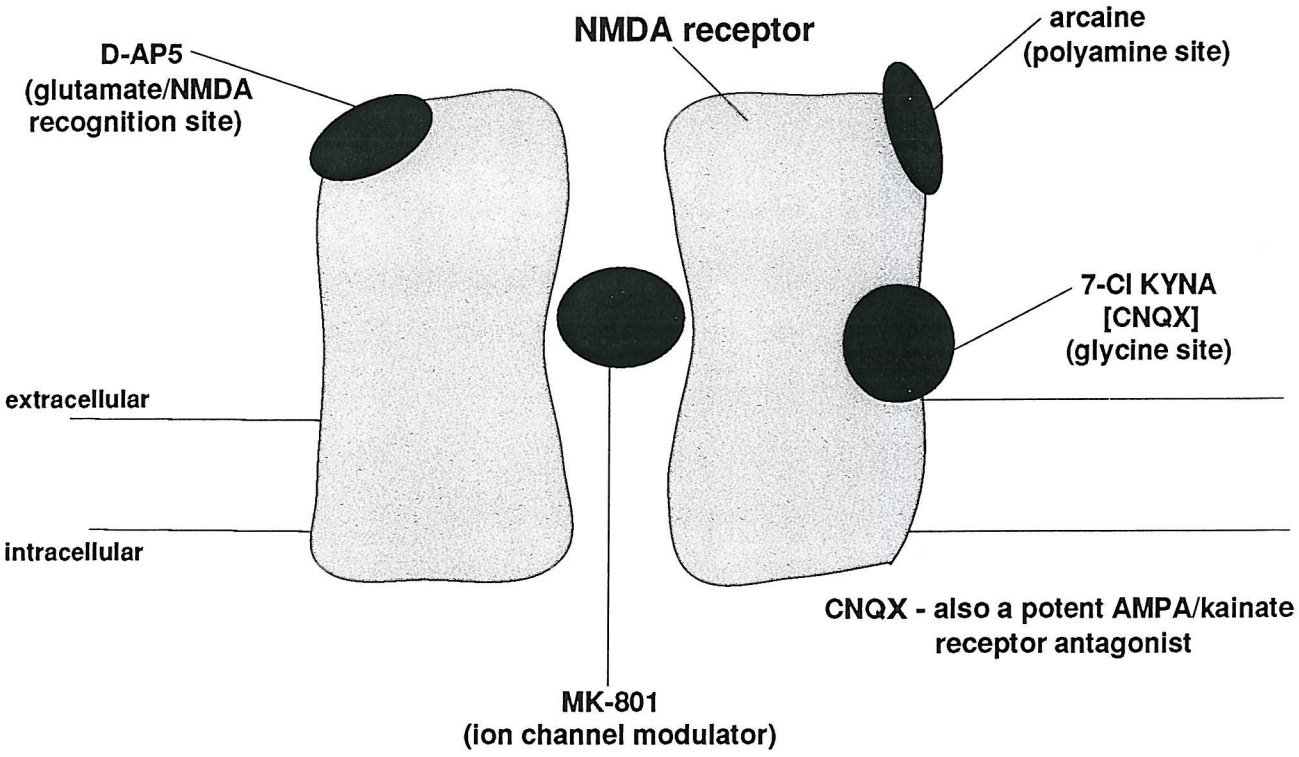


Fig. 4.1: diagram to show the sites of action of the ionotropic glutamate receptor antagonists used.

4.2.1 Antagonism at the NMDA site of the NMDA receptor.

D-AP5 is a potent and competitive antagonist of the NMDA glutamate receptor which binds at the L-glutamate/NMDA recognition site (Davies and Watkins 1982). Application of D-AP5 to the in *vitro* rat spinal cord had no significant effect on the S1 component of the fast wave field potential at concentrations up to 10μM (Fig. 4.2/4.3). However, at a concentration of 100μM the amplitude of the S1 component was significantly increased to 119.8±9.31% (mean ± S.E.M) of the control amplitude ($p<0.05$, paired t-test using raw data, n=5). The result following the addition of 100μM D-AP5 was then tested using the one sample t-test and a hypothesis that the mean = 100%. The result was confirmed to be not significant. The S2 component of the fast wave field potential was not significantly affected by the addition of D-AP5 to the bathing medium at concentrations up to 100μM (n=5) (Fig. 4.2/4.4). The area of the slow "tail" evoked by stimulation at 20 times the DRR threshold voltage was significantly reduced at concentrations of 1μM to 100μM ($p<0.005$, paired t-test, n=4) (Fig. 4.5/4.6). At 100μM, the slow wave was reduced to 71.0±8.19% of the control.

The IC_{75} (drug concentration which produces a 75% response) of D-AP5 acting on the slow wave dorsal horn field potential was calculated to be 8.5μM.

4.2.2 Antagonism of the glycine site on the NMDA receptor.

7-Chlorokynurenic acid (7-Cl KYNA) is a potent and highly selective NMDA antagonist acting at the strychnine-insensitive glycine site (Kemp et al. 1988). Apart from its co-agonist action, glycine acts to reduce the amount of desensitisation of the receptor (Kemp and Leeson 1993) and has a physiological role in the modulation of synaptic responses mediated by NMDA receptors.

7-Cl KYNA had no significant effect on the fast wave S1 component at concentrations of up to 10μM. However, at a concentration of 100μM the S1 component was significantly reduced to 64.96±14.87% of the control ($p<0.05$, paired t-test, n=8) (Fig. 4.7/4.8). Using the one-sample t-test and a hypothesis that the mean = 100%, the result was calculated

to be very significant. The S2 component was not significantly affected by the addition of 7-Cl KYNA at concentrations up to 100μM (n=8) (Fig. 4.7/4.9). Interestingly, the fast wave response to 7-Cl KYNA showed an "uncoupling" of the S1 and S2 components. While the S1 component remained at an approximately constant amplitude at concentrations up to 100μM, the S2 component was reduced. In one atypical experiment, the S2 component was completley abolished at 100μM while the S1 component continued to have a high response amplitude. The results of this particular experiment can be seen in Fig. 4.10.

The slow wave area of the field potential was significantly reduced at 7-Cl KYNA concentrations of 1μM, 10μM and 100μM ($p<0.05$ paired t-test, n=4) (Fig. 4.11/4.12). The IC_{75} was found to be 0.83μM.

4.2.3 Antagonism of the ion-channel site of the NMDA receptor.

(+)-MK-801 malate (MK-801) is a potent, selective and non-competitive NMDA receptor antagonist. It acts by binding to a site located within the NMDA associated ion channel to prevent Ca^{2+} influx (Wong et al. 1986).

The S1 component of the fast wave field potential elicited by stimulation of a dorsal root at 5 times the DRR threshold voltage was not significantly affected by the addition of MK-801 to the bathing medium at concentrations up to 100μM (n=5) (Figs. 4.13/4.14). The S2 component, however, was significantly reduced at concentrations of 1μM and 10μM to 69.10±5.99% and 69.43±14.85% of control results respectively ($p<0.05$ paired t-test, n=4) (Figs. 4.13/4.15). The one sample t-test confirmed that these differences were statistically significant.

MK-801 inhibited the slow wave dorsal horn field potential after the addition of 10μM and 100μM of the drug ($p<0.05$ paired t-test, n=6) (Fig. 4.16/4.17). The IC_{75} was calculated to be 68μM.

4.2.4 Antagonism of the polyamine site of the NMDA receptor.

Arcaine is an antagonist of the NMDA receptor acting as a competitive inhibitor at the polyamine site (Reynolds 1990). Arcaine was found no have no significant effect on either the fast wave dorsal horn field potential S1 component (n=5) (Fig 4.18/4.19) or the S2 component (n=5) (Fig. 4.18/4.20) at concentrations up to 100μM. In contrast with previous results using other NMDA receptor antagonists, arcaine had no significant effect on the slow wave dorsal horn field potential (n=4) (Fig. 4.21/4.22) up to concentrations of 100μM.

Since unexpected results were obtained after the addition of arcaine to the medium, a concentration of 100μM was added to the bathing medium for a total period of 30 minutes and the response was recorded every 10 minutes during this time. Arcaine was then washed from the medium for another 30 minutes and recorded every 10 minutes. The addition of 100μM arcaine to the spinal cord for 30 minutes and the wash for 30 minutes did not have any significant effect on the S1 or S2 components of the fast wave field potential (n=4) (Figs. 4.23/4.24/4.25) In contrast, the slow wave field potential was significantly increased by 100μM arcaine after 20 minutes to 110±1.12% of the control ($p<0.05$ paired t-test, n=4) and was significantly increased to 114.2±4.49% of control 30 minutes after the drug was washed from the system ($p<0.05$ paired t-test, n=4) (Figs. 4.26/4.27).

4.2.5 Antagonism of non-NMDA receptors.

CNQX is a potent antagonist of AMPA/Kainate receptors and has a less potent effect as an antagonist at the glycine site of NMDA receptors (Honore et al. 1988; Long et al. 1990). CNQX significantly inhibited both the S1 component (Fig. 4.28/4.29) and the S2 component (Fig. 4.28/4.30) of fast wave dorsal horn field potentials. The S1 component was significantly reduced to 46.78±13.19% of the control response at 10μM ($p<0.05$ paired t-test, n=5) and 31.40±10.61% of the control response at 100μM ($p<0.05$ paired t-test, n=5). The S2 component was significantly reduced to 27.0±12.46% of the control response at 10μM ($p<0.05$ paired t-test, n=5) and 2.8±2.8% of the control response at

100μM ($p<0.05$ paired t-test, n=5). The amplitude of the S1 and S2 components remained significantly low even after the drug was washed out from the medium ($p<0.05$ paired t-test, n=5 for both S1 and S2). The IC_{75} for the S1 component of the fast wave dorsal horn field potentials was calculated to be 4.5μM and for the S2 component it was calculated to be 1.2μM.

In order to validate the cumulative dose-response curves, the effect of 10μM of CNQX on the fast wave dorsal horn field potentials was measured every 10 minutes over a total period of 30 minutes. The CNQX was then washed off and the effect of washing off the drug on the S1 and S2 components of the fast waves was measured every 10 minutes for another 30 minutes. The results are illustrated in Fig. 4.31/4.32 for the S1 component and in Fig. 4.31/4.33 for the S2 component. The S1 component was significantly decreased to 42.27±8.05% and 35.25±5.44% of the control following the addition of 10μM CNQX for 20 minutes and 30 minutes respectively ($p<0.05$ paired t-test, n=4) and to 69.9±9.23% of control 10 minutes following the wash ($p<0.01$ paired t-test, n=4). The S2 component was not significantly inhibited (n=4).

The experimental protocol used for the cumulative dose-response curve involves adding each dose of the drug/wash for a period of 20 minutes before recording the response over a period of 5 minutes. Fig. 4.34 compares the results of 10μM CNQX after the cumulative addition of the drug and the results after the direct addition of a single dose of 10μM CNQX for 20 minutes.

CNQX significantly inhibited the slow wave dorsal horn field potential to 89.83±2.39% of control at a concentration of 0.1μM ($p<0.05$ paired t-test, n=4), 68.55±6.27% of control at a 10μM concentration ($p<0.05$ paired t-test, n=4) and to 48.98±9.69% of the control response at a concentration of 100μM ($p<0.05$ paired t-test, n=4). The slow wave continued to be significantly inhibited after CNQX was washed out from the system ($P<0.01$ paired t-test, n=4). The IC_{75} was calulated to be 6.9μM.

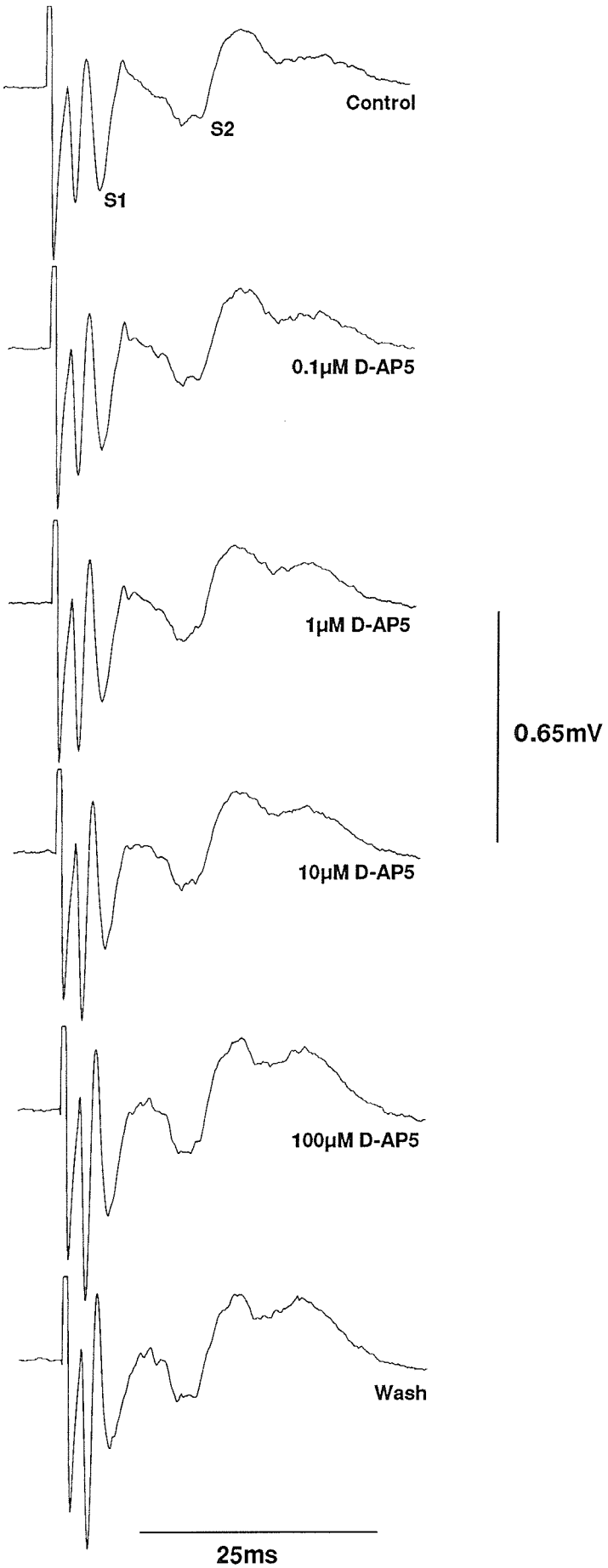


Fig. 4.2: Traces to show the effect of the NMDA antagonist D-AP5 on the fast wave field potentials evoked by stimulation of a dorsal root at 5 times voltage necessary to evoke a DRR in an adjacent root.

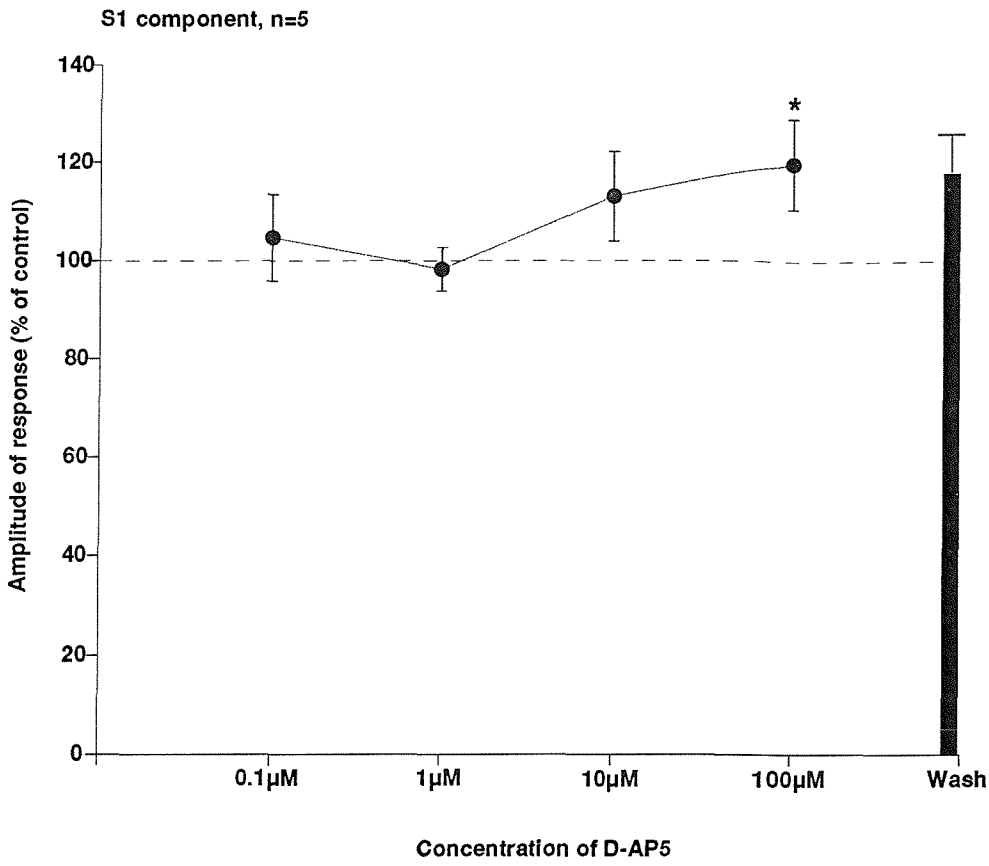


Fig 4.3: Dose-response graph to illustrate the effect of D-AP5 on the mean amplitude of the S1 component of the fast wave dorsal horn field potential (±SEM). Significance values (*) calculated using the paired t-test. n numbers (n) show the number of experiments.

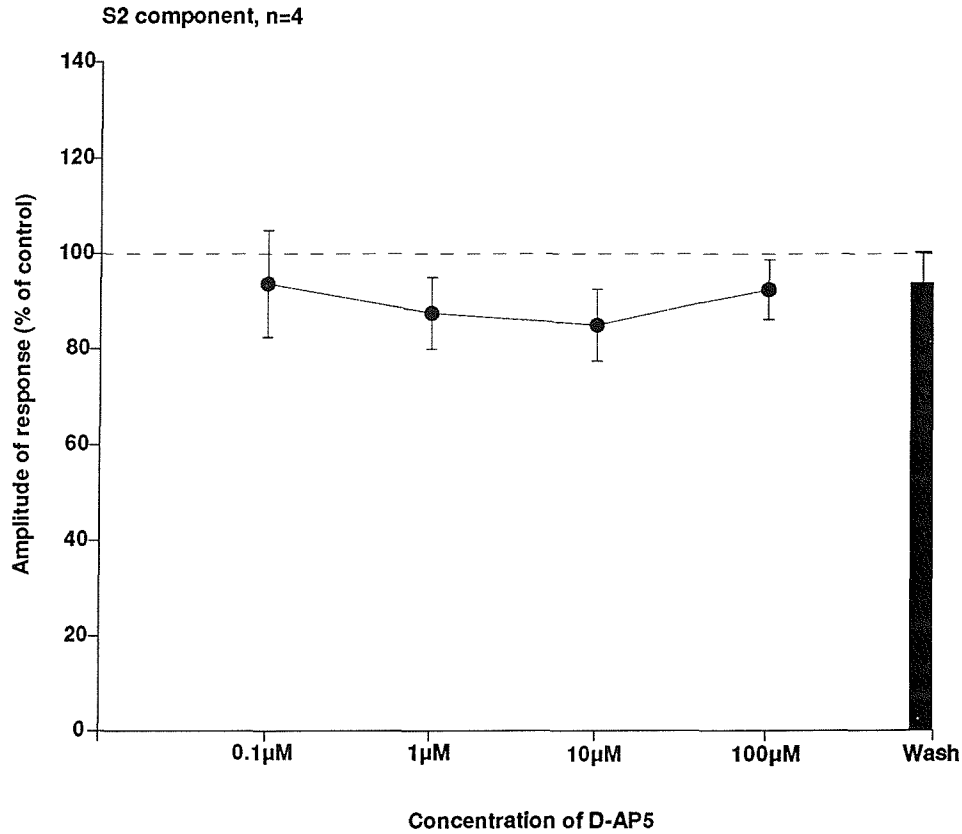


Fig. 4.4: Dose-response graph to show how D-AP5 did not significantly affect the amplitude of the S2 fast wave dorsal horn field potential component. One experiment did not show an S2 component therefore n=4.

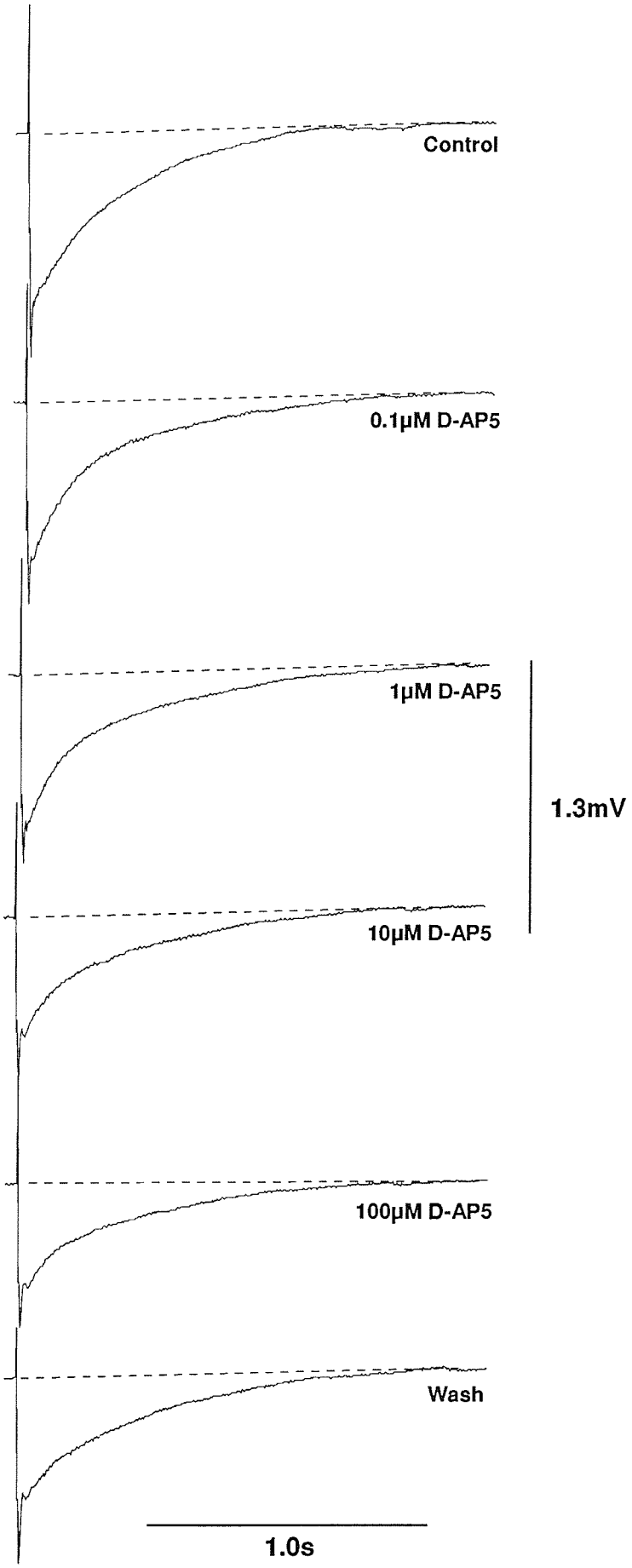


Fig. 4.5: Data to show the slow wave dorsal horn field potential was decreasd by increasing concentrations of D-AP5.

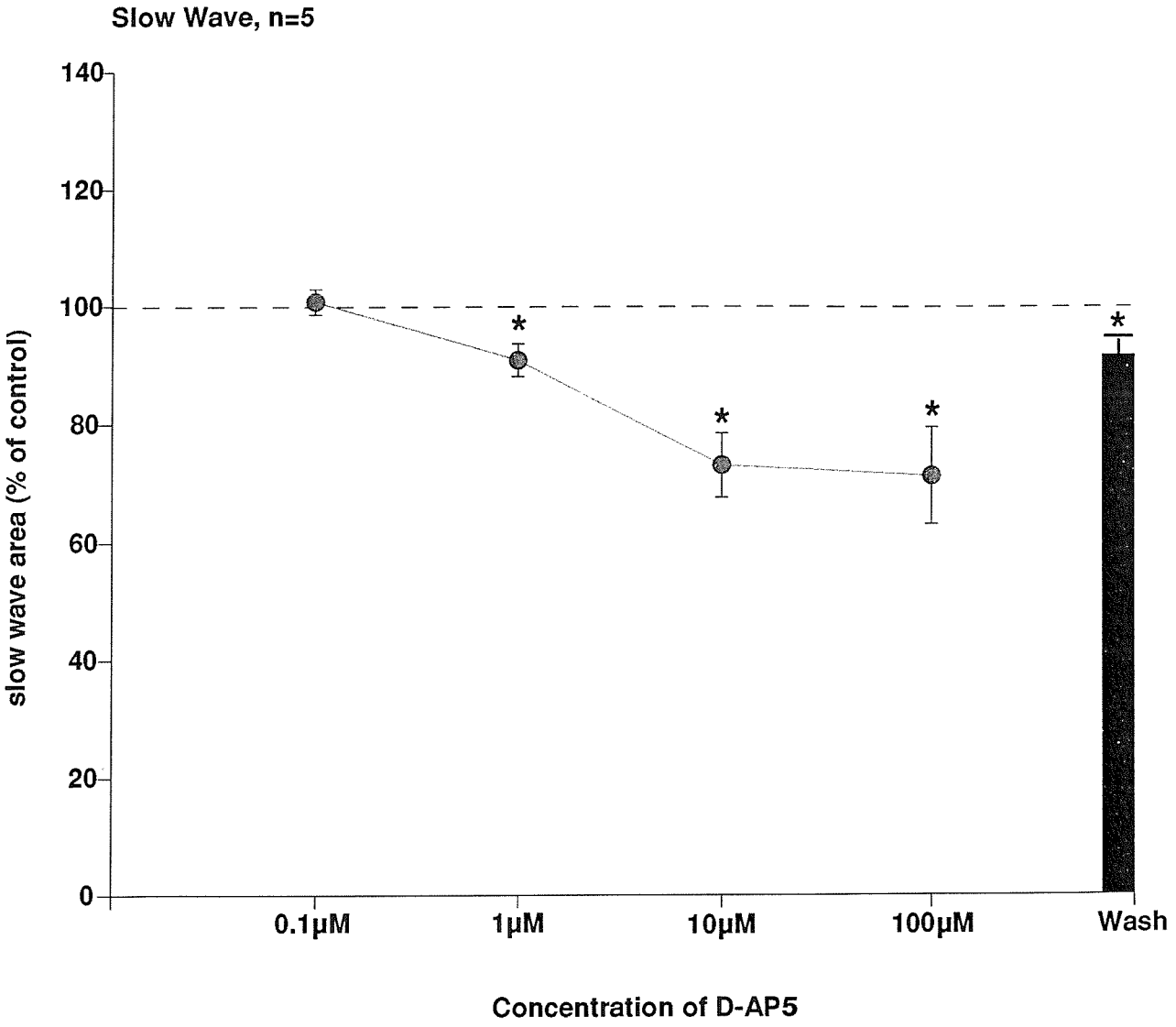


Fig. 4.6: Dose-response graph to show how D-AP5 significantly inhibited the slow wave at concentrations of 1µM, 10µM and 100µM. significance values (*) calculated using the paired t-test. n=4.

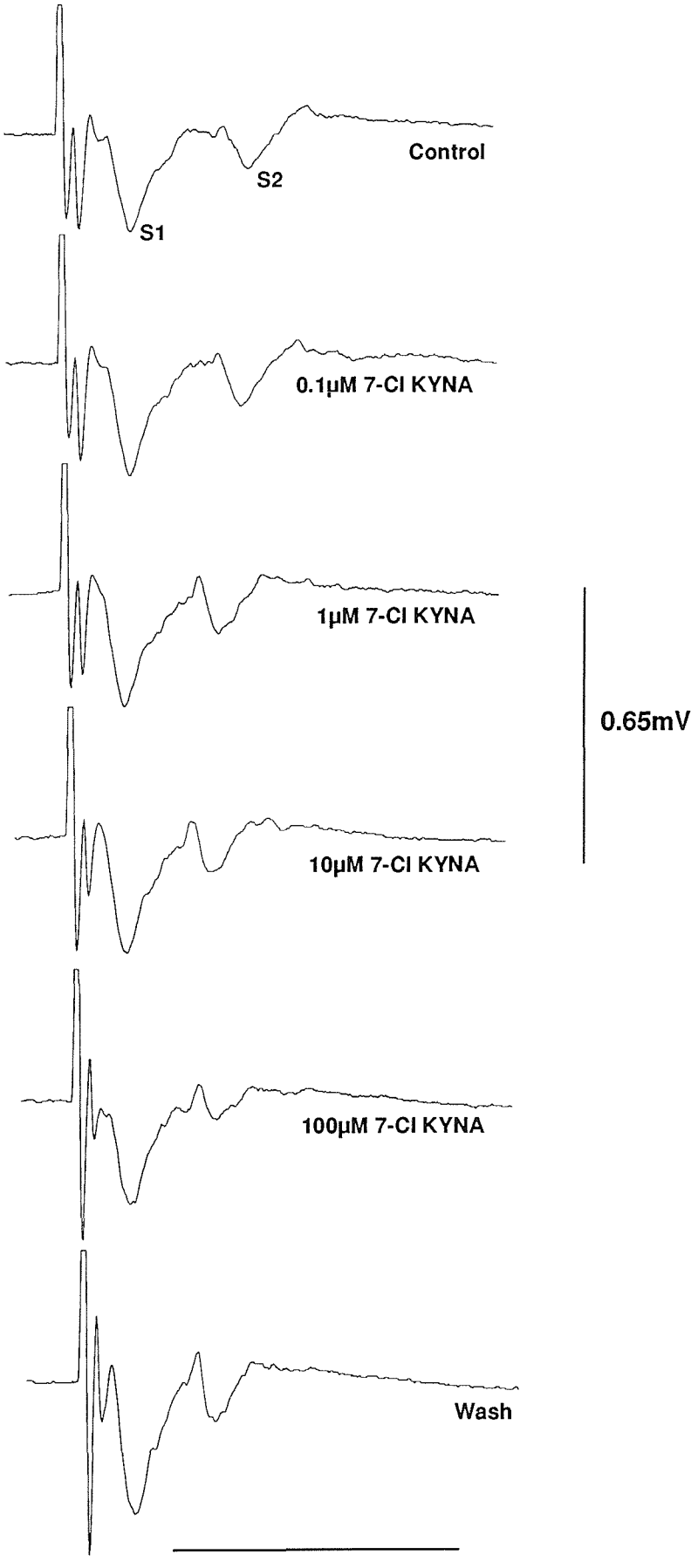


Fig. 4.7: Data showing the effect of the NMDA antagonist 7-Cl KYNA on the fast wave dorsal horn field potentials.

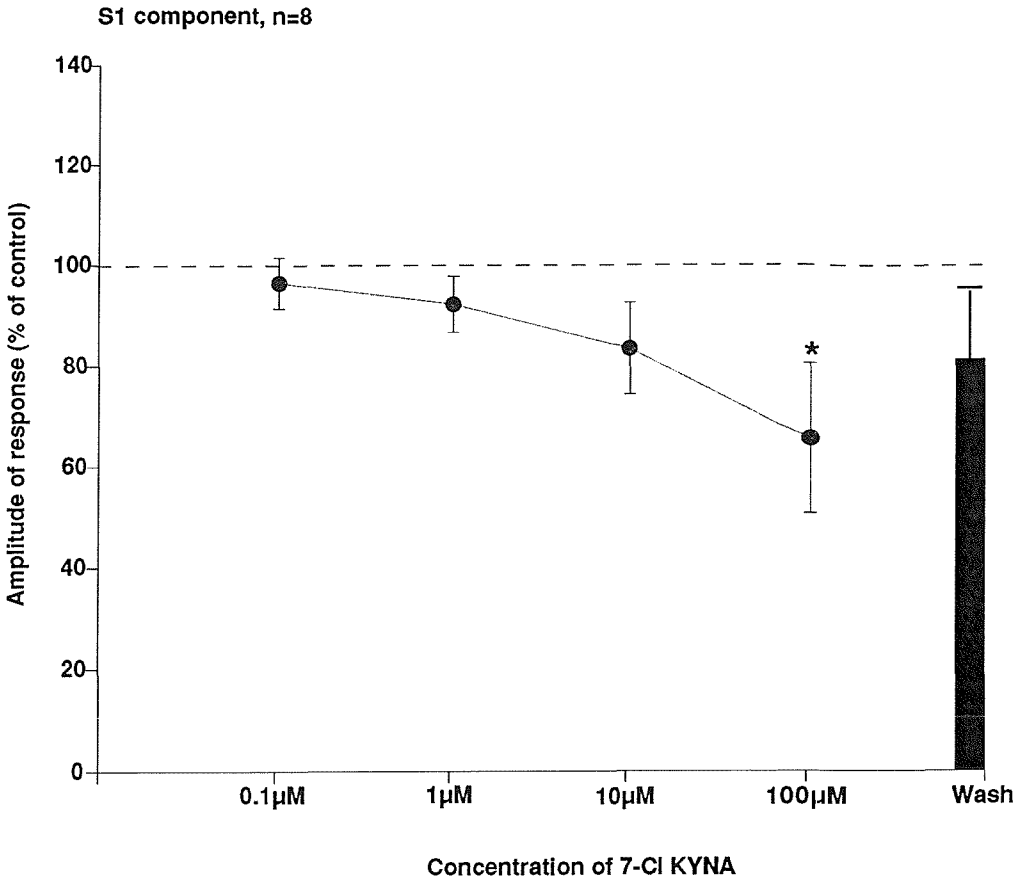


Fig. 4.8: Dose response graph showing that 7-Cl KYNA an inhibitory effect on the fast wave dorsal horn field potential S1 component at 100µM. n=8.

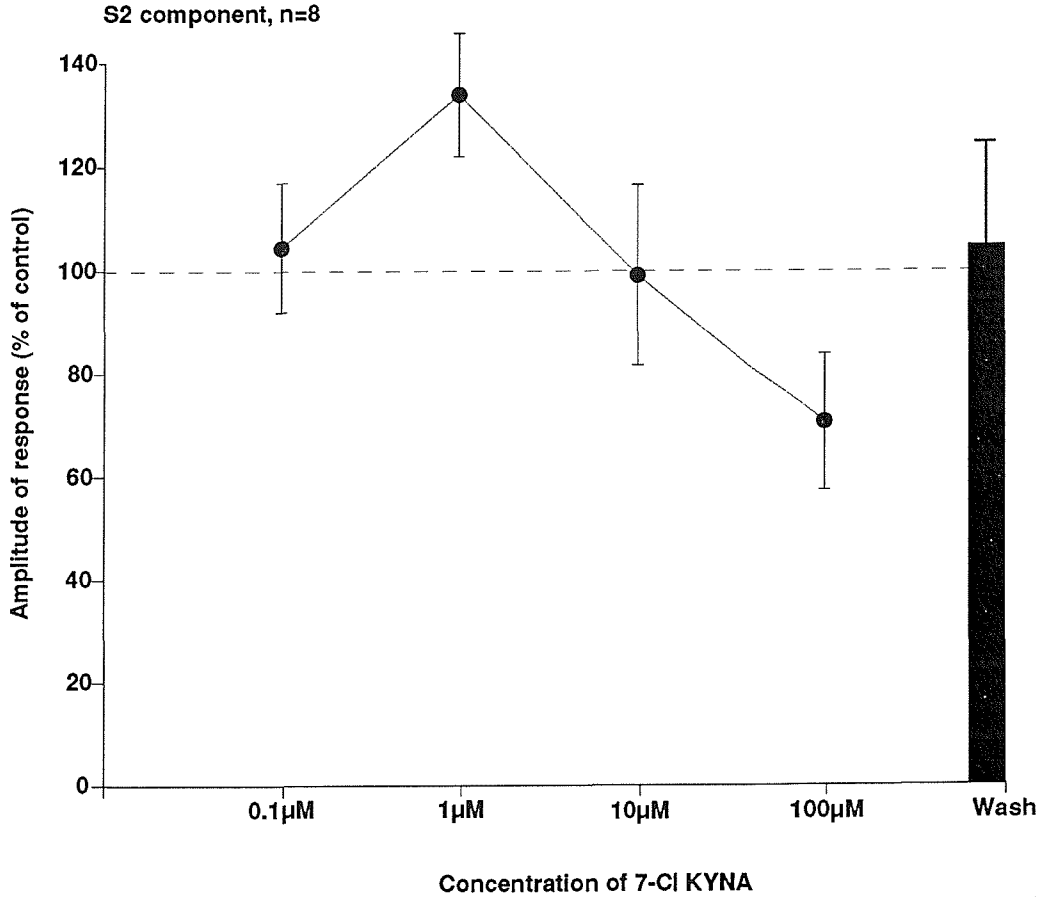


Fig. 4.9: 7-Cl KYNA had no significant effect on the S2 component of the fast wave field potentials. n=8.

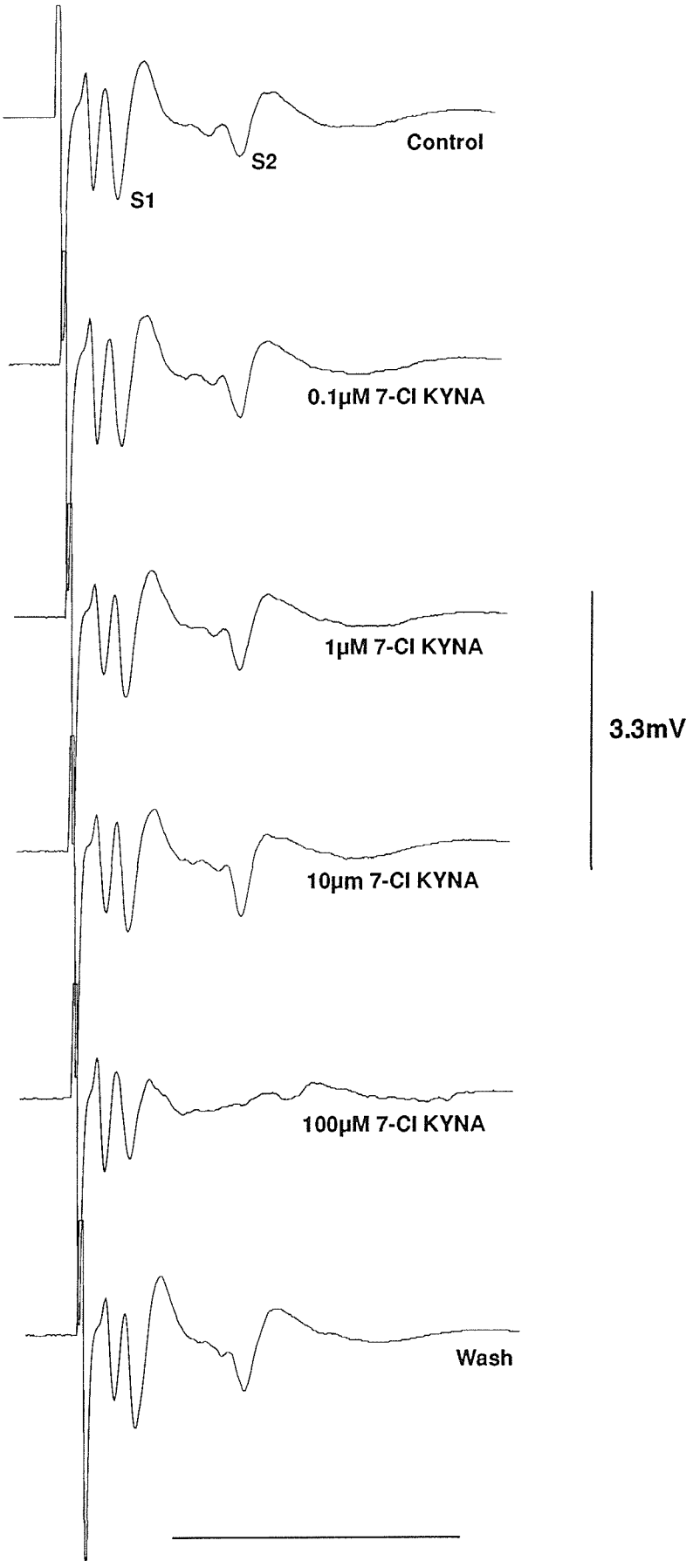


Fig. 4.10: In this atypical experiment, at a concentration of 100µM 7-Cl KYNA, the S2 component was no longer present. The S1 and S2 components became uncoupled showing that they are independent events.

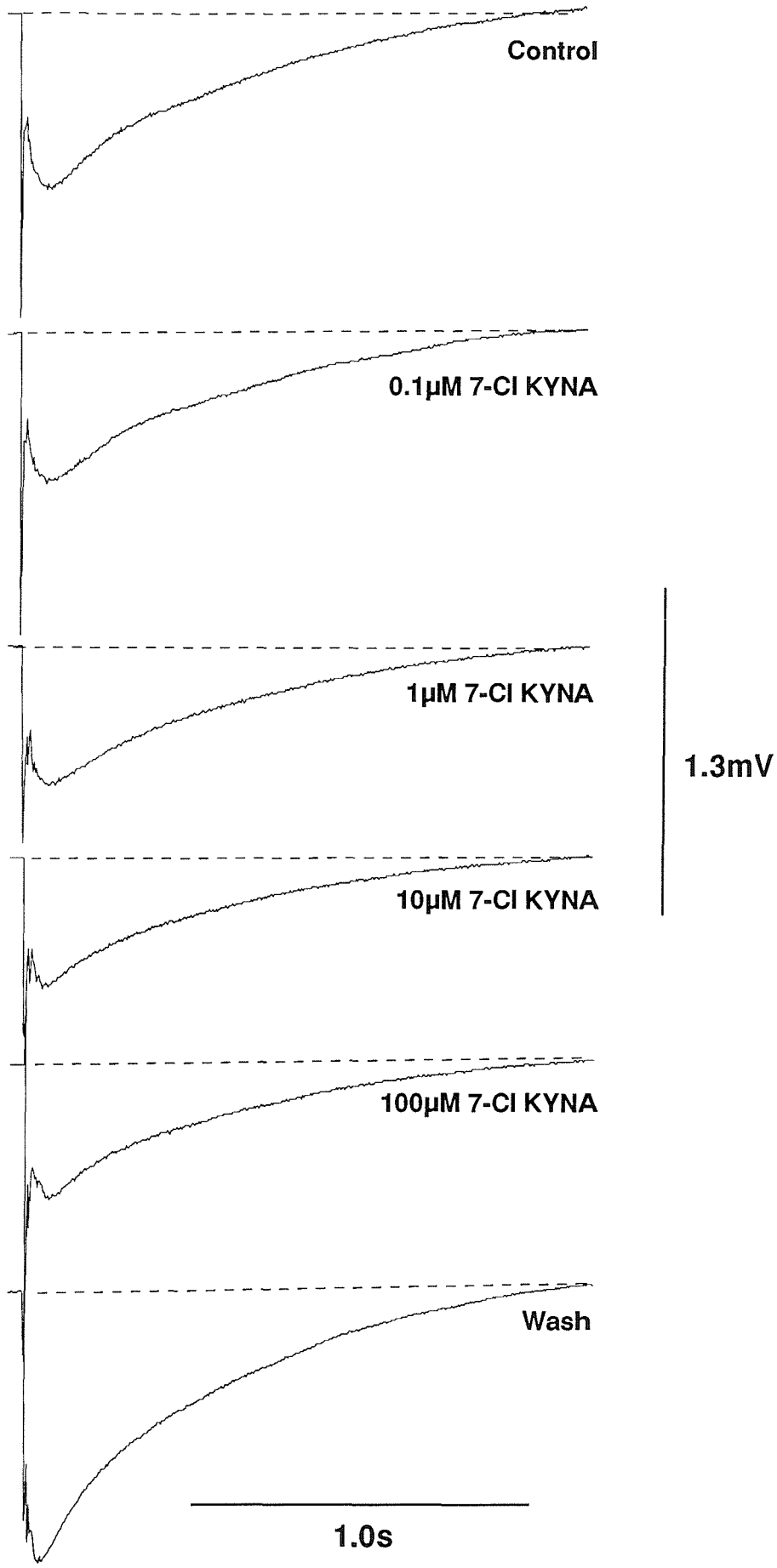


Fig. 4.11: Slow wave data from one experiment to show that 7-Cl KYNA was decreased in a concentration-dependent manner.

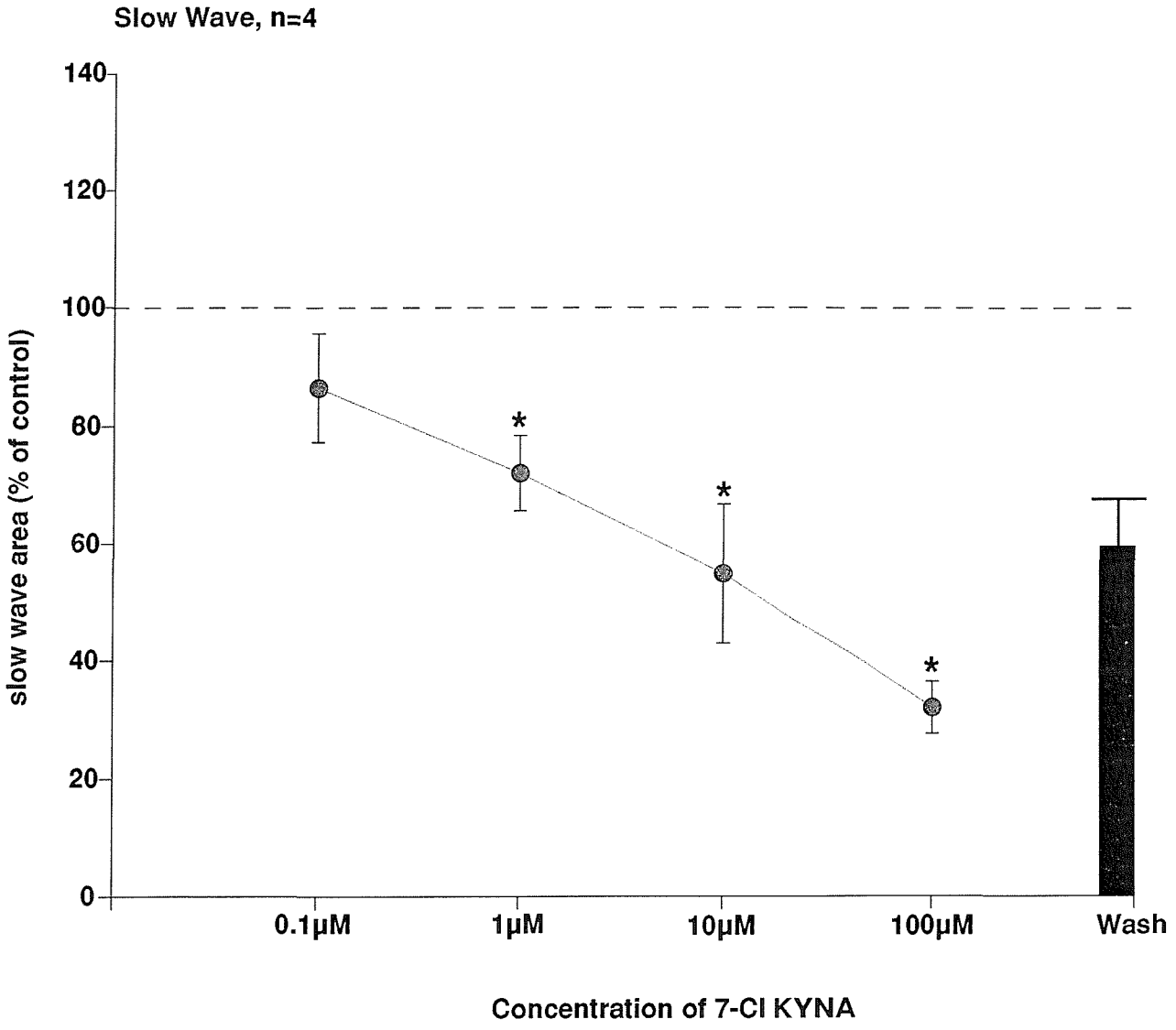


Fig. 4.12: Dose-response curve showing the effect of the NMDA antagonist 7-Cl KYNA on the slow wave dorsal horn field potential. At concentrations of 1µM and above 7-Cl KYNA significantly inhibited the slow wave ($p<0.05$ Paired T-test). n=4.

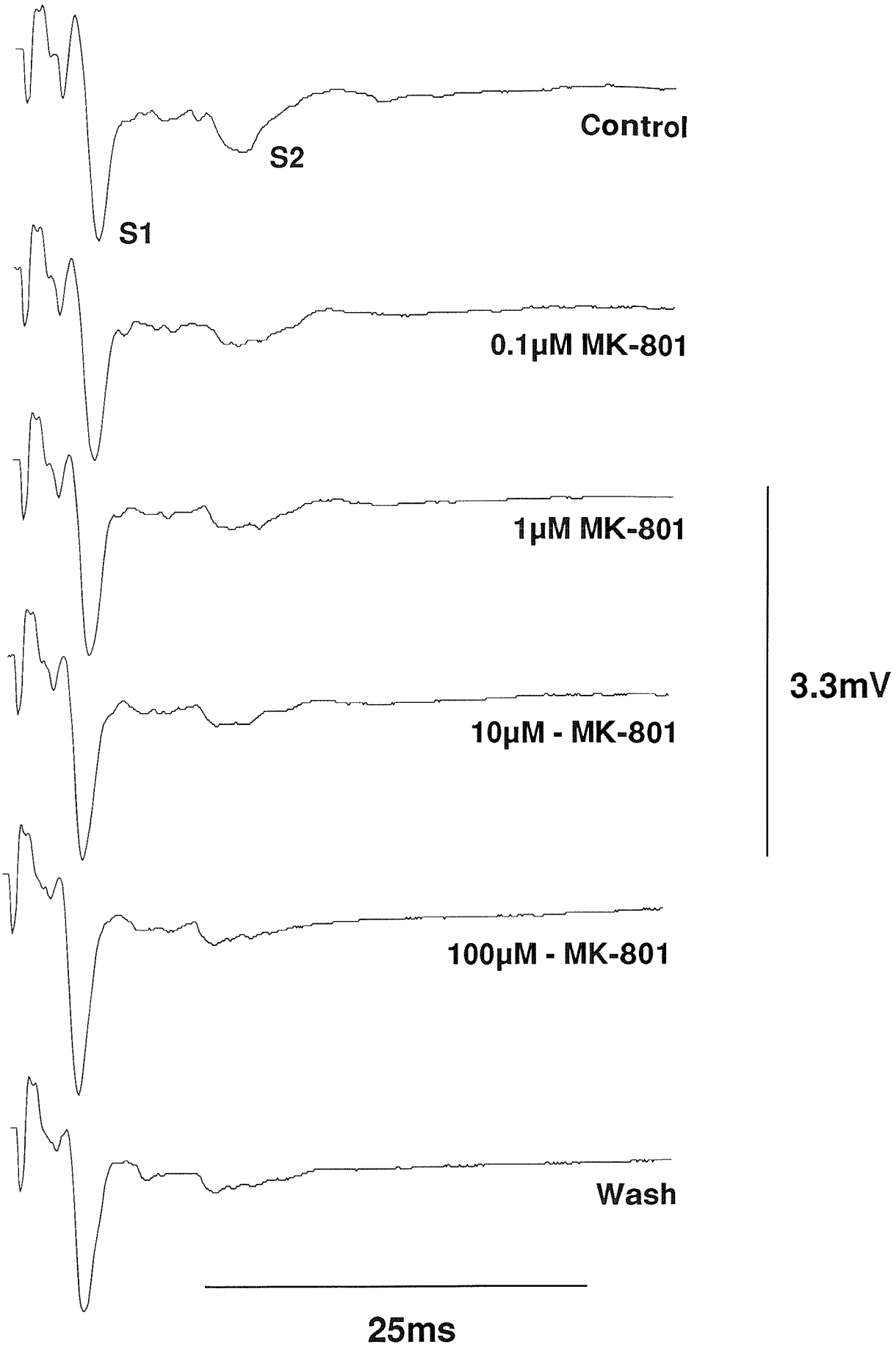


Fig. 4.13: Fast wave field potentials showng the effect of the NMDA antagonist MK-801 which acts as a channel blocker, on the amplitude of the S1 and S2 components.

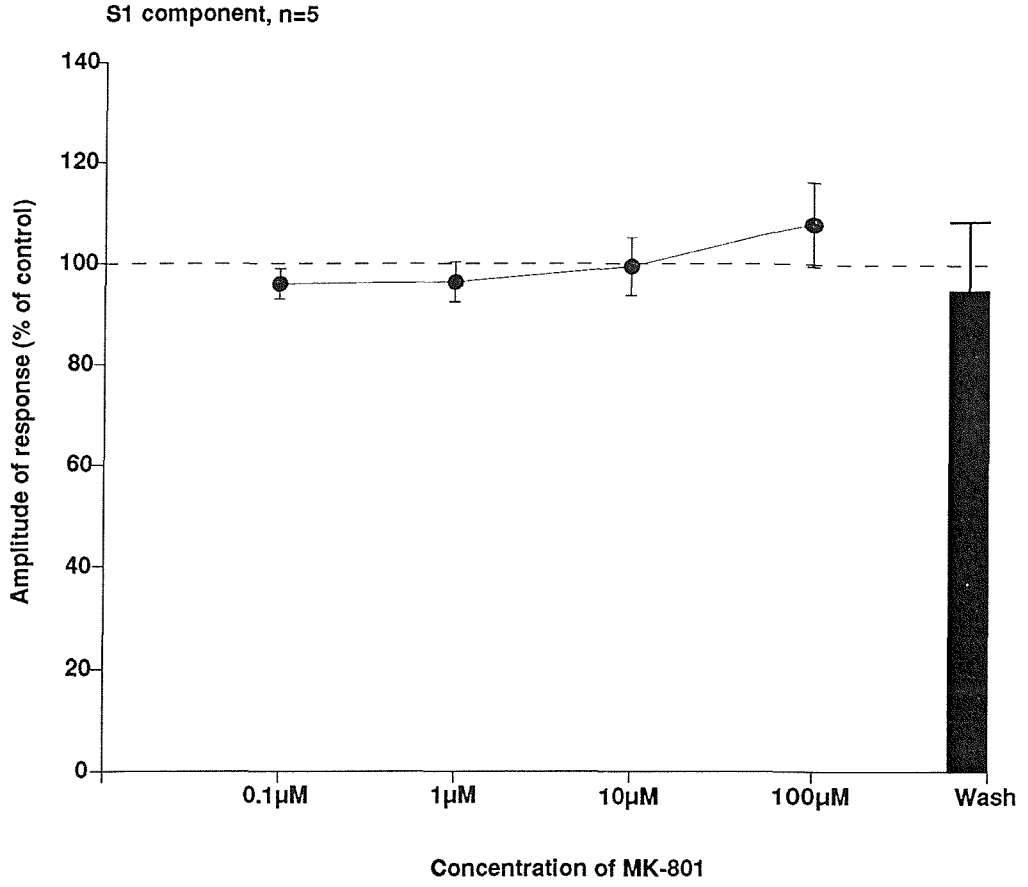


Fig. 4.14: Dose-response curve showing that MK-801 had no effect on the S1 component fast wave field potential.

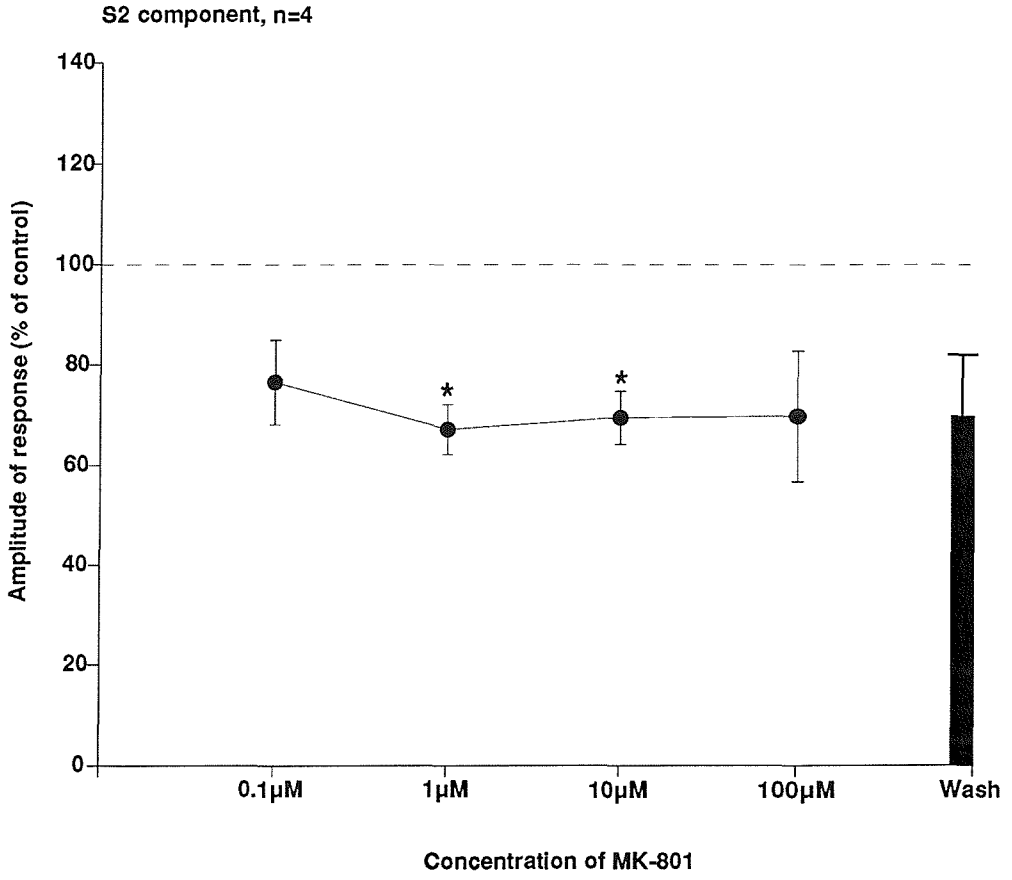


Fig. 4.15: Dose-response curve showing that MK-801 inhibited the S2 component fast wave field potential at concentrations of 1μM and 10μM ($p<0.05$, paired T-test).

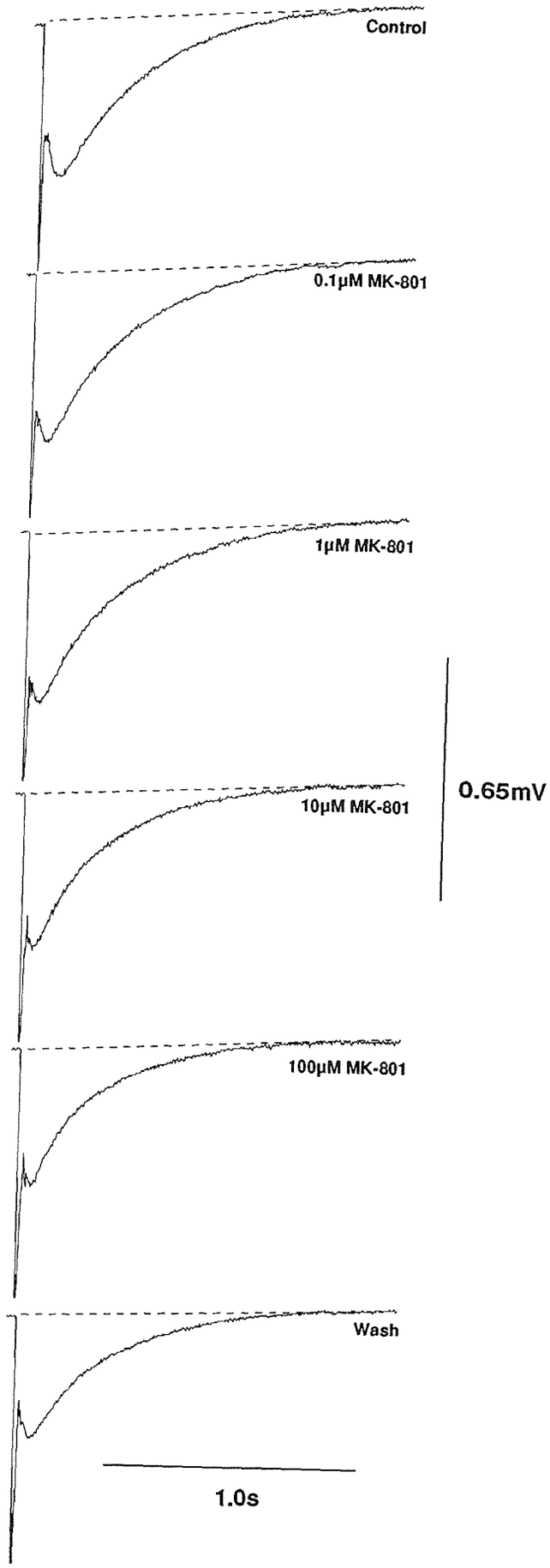


Fig. 4.16: Raw data showing the effect of MK-801 on the slow wave dorsal horn field potential.

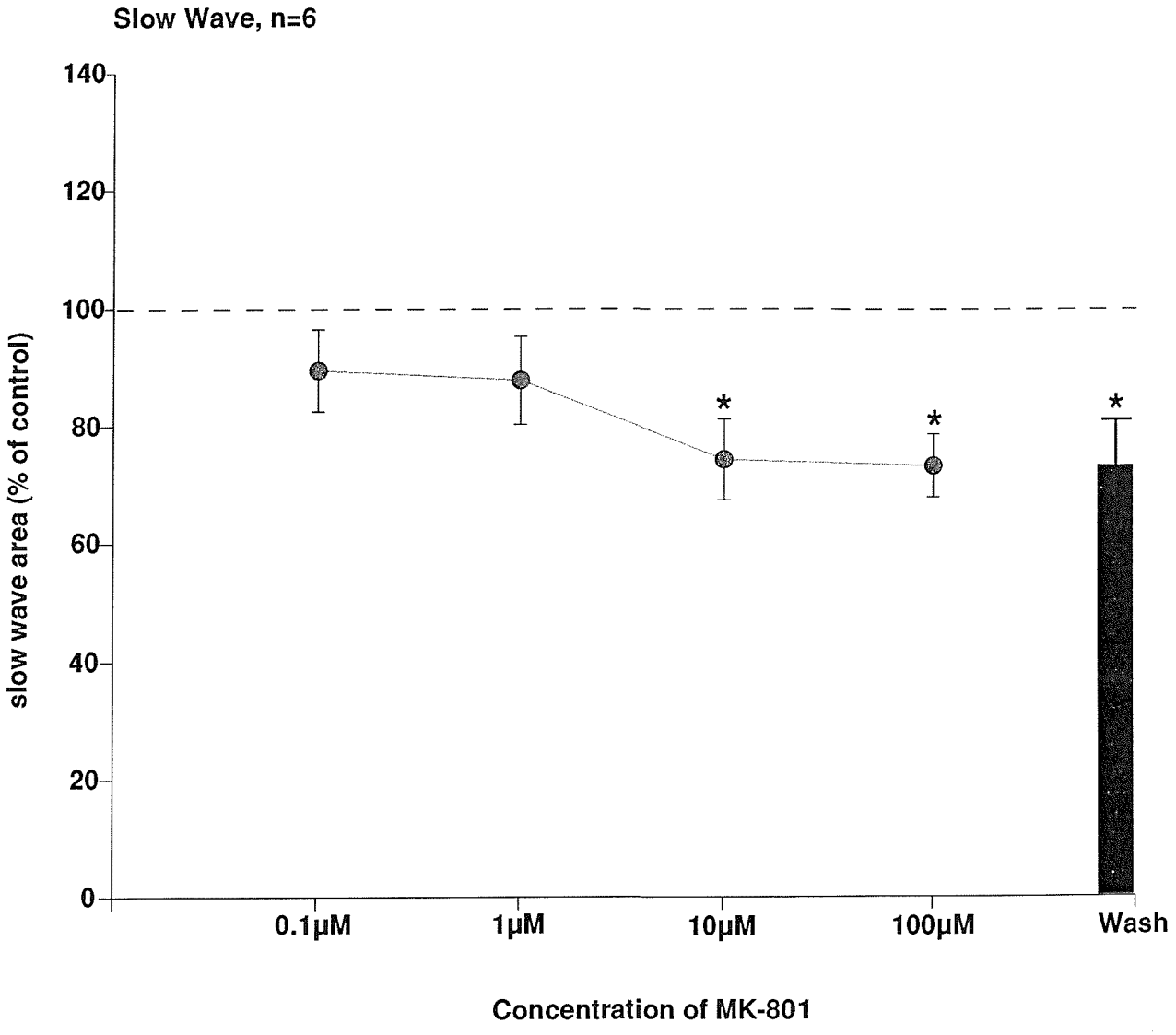


Fig. 4.17: Dose response curve to show the effect of MK-801 on the slow wave dorsal horn field potential. The paired t-test calculated that concentrations of 10µM, 100µM and the wash were significantly decreased when compared to the slow wave. n=6.

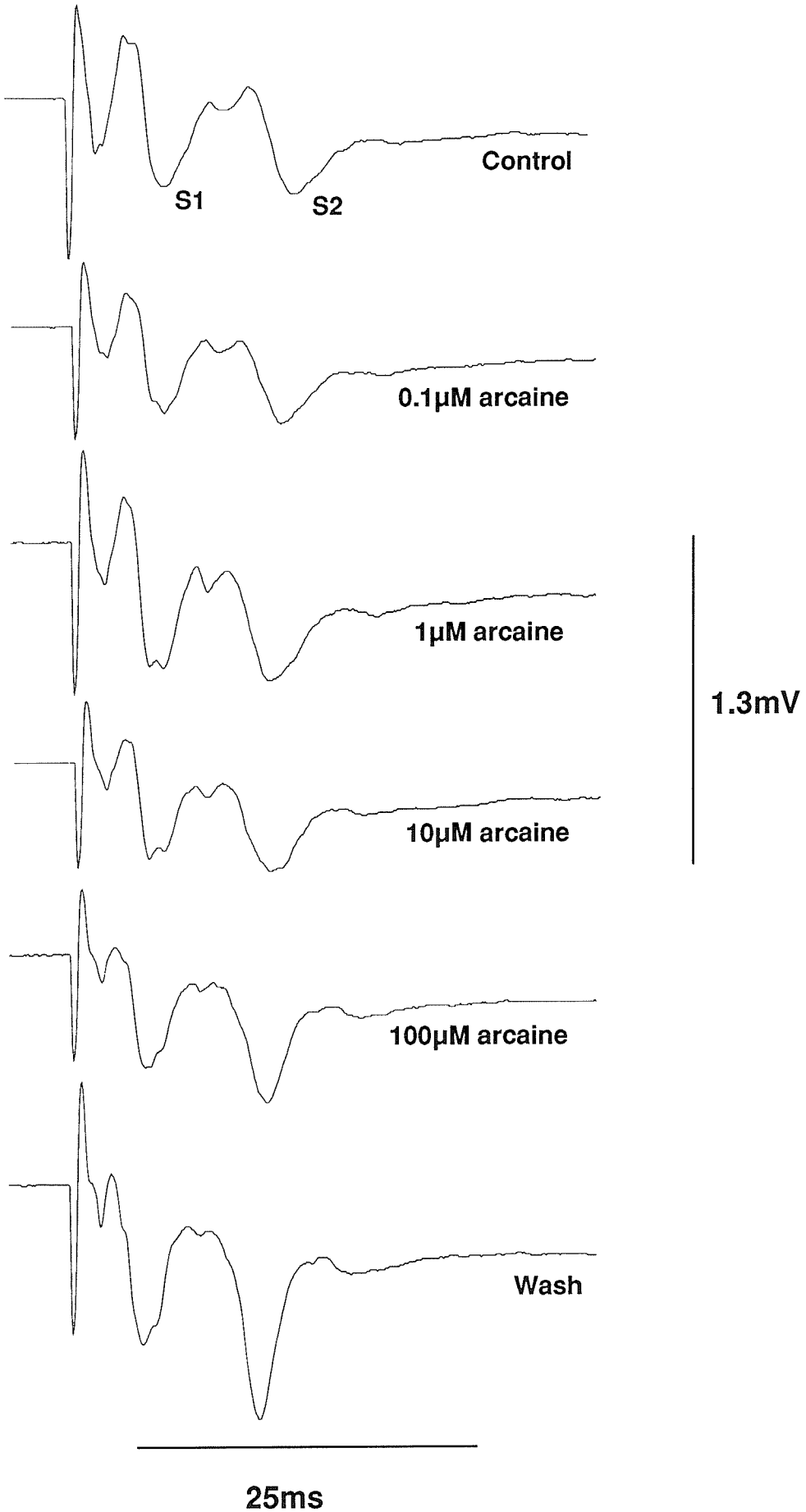


Fig. 4.18: The addition of arcaine to the spinal cord and the effect on the fast wave dorsal horn field potential in one experimental example.

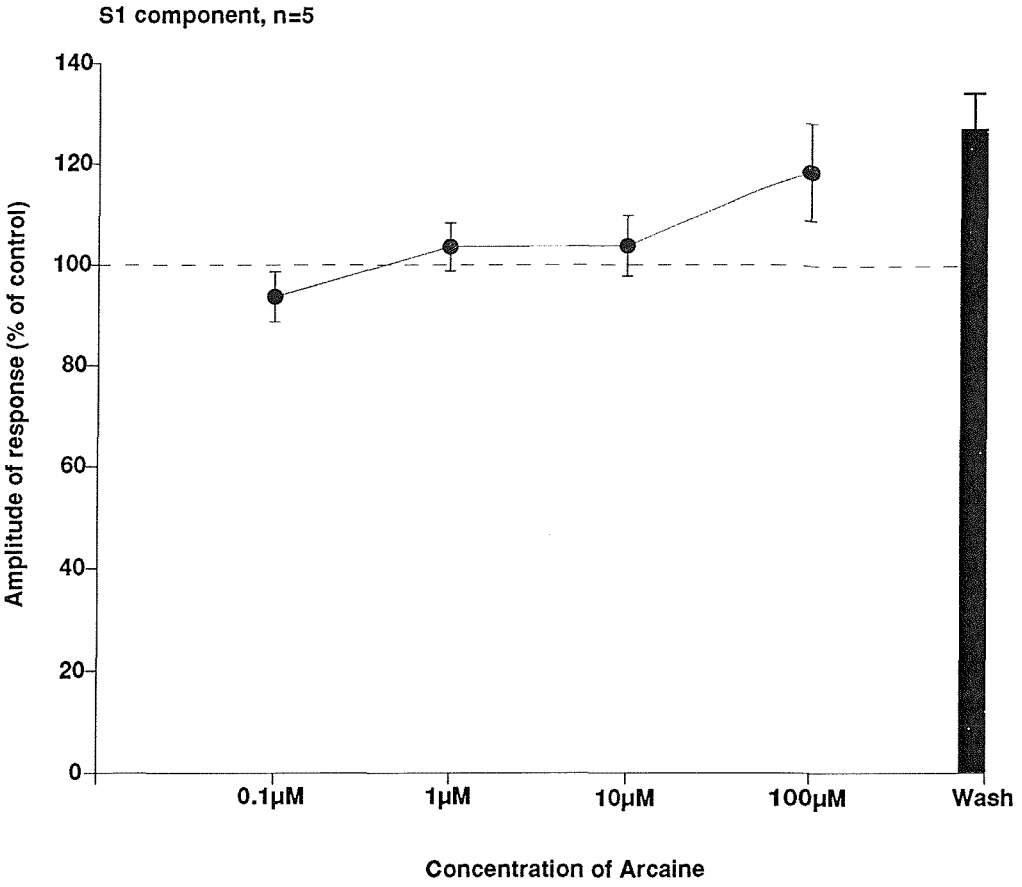


Fig. 4.19: The change in the S1 component of the fast wave dorsal horn field potential was not significant at concentrations up to 100μM. n=5

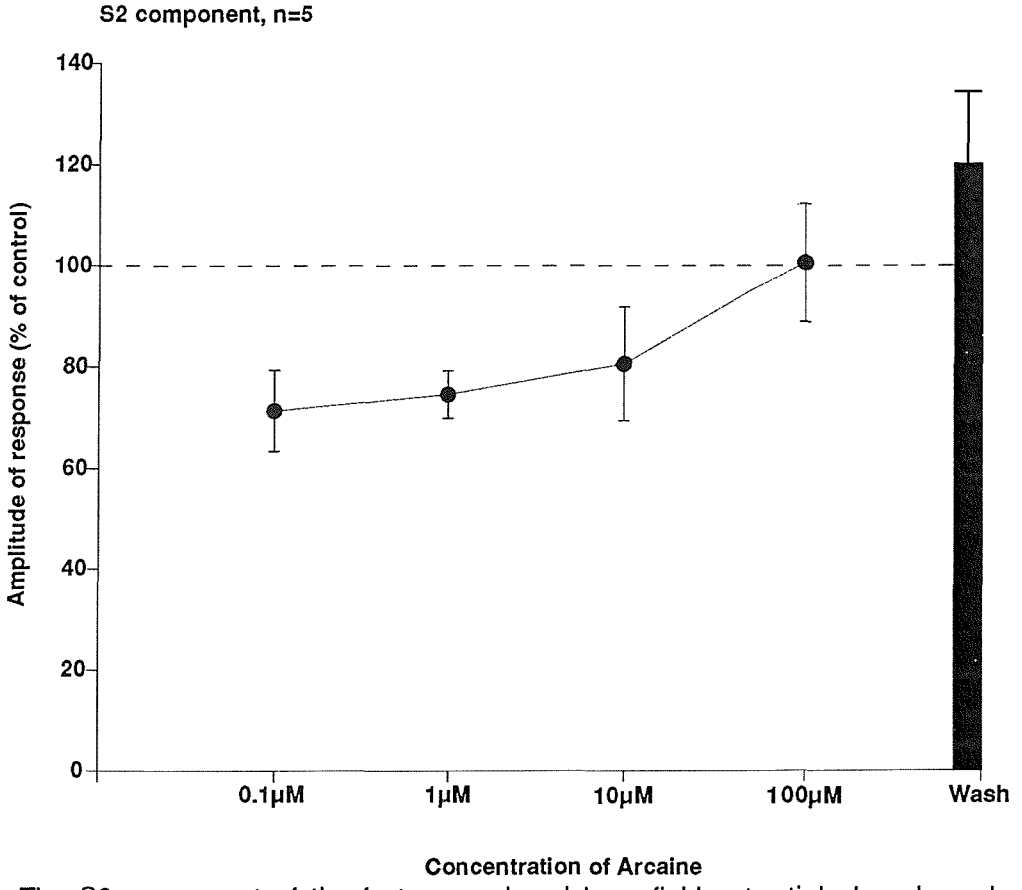


Fig. 4.20: The S2 component of the fast wave dorsal horn field potential also showed no significant changes at concentrations up to 100μM. n=5 .

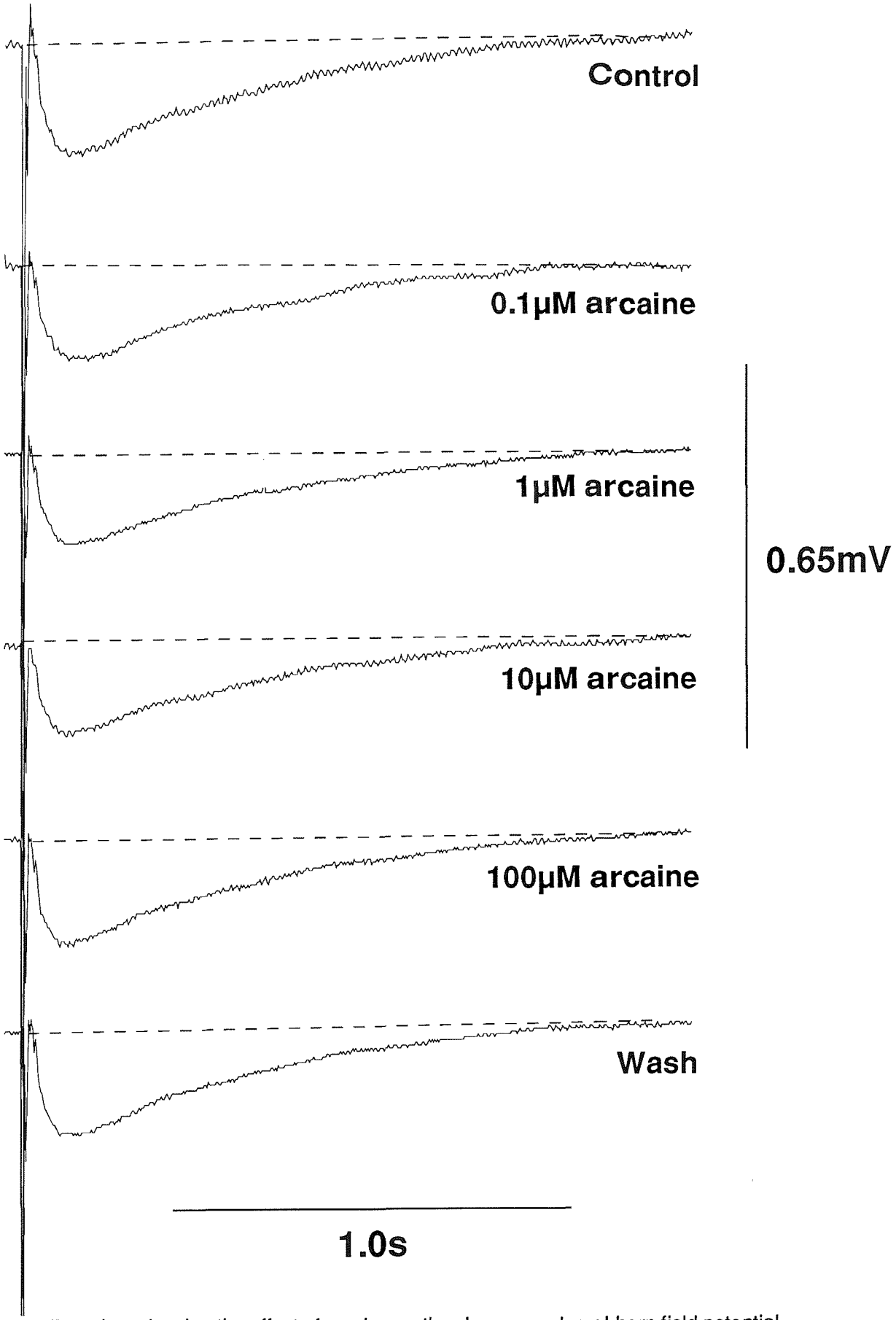


Fig. 4.21: Raw data showing the effect of arcaine on the slow wave dorsal horn field potential.

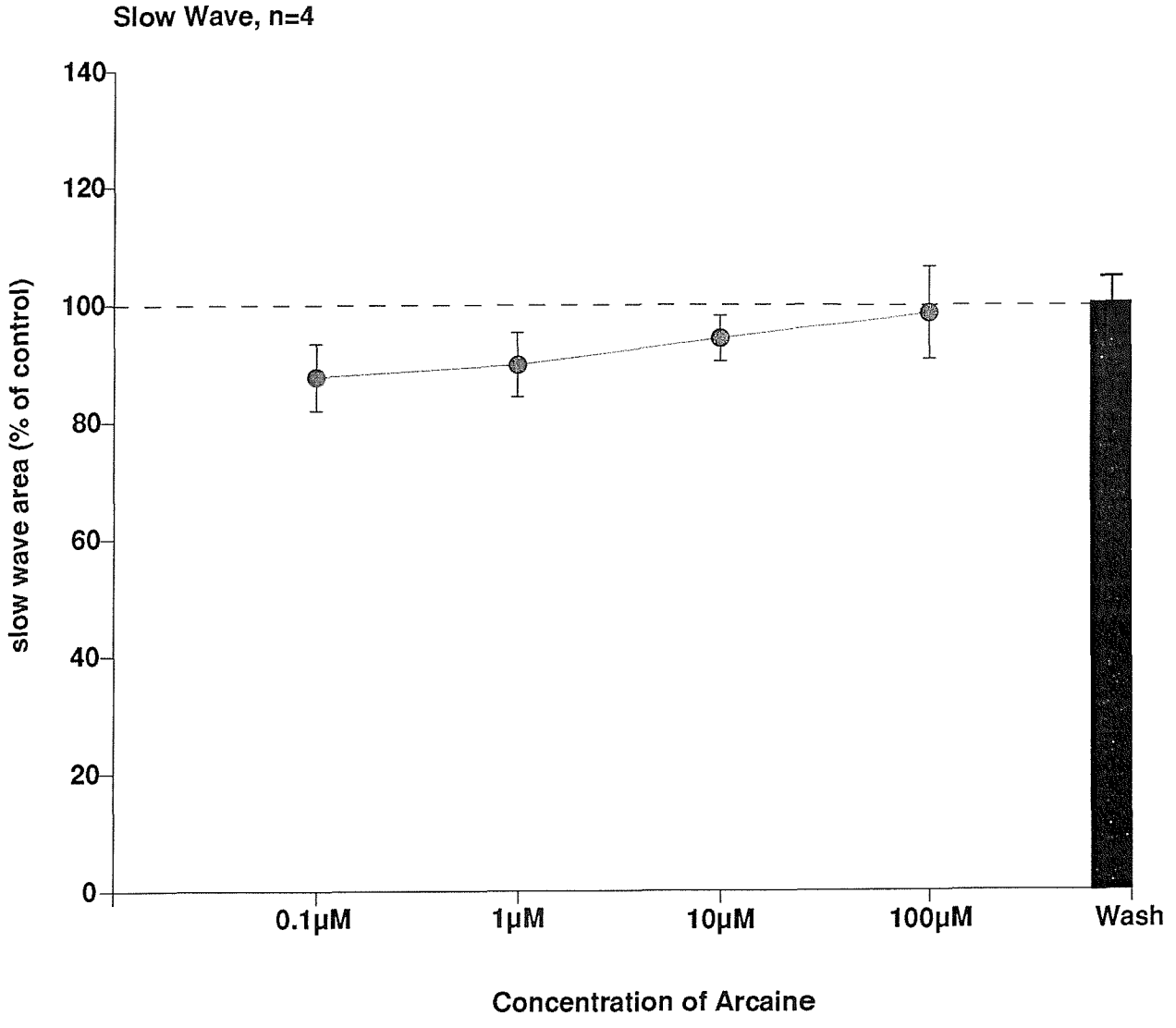


Fig. 4.22: Dose-response curve to illustrate the effect of the NMDA antagonist arcaine which acts at the polyamine site of the receptor. Arcaine had no significant effect on the slow wave dorsal horn field potential at concentrations up to 100µM. n=4.

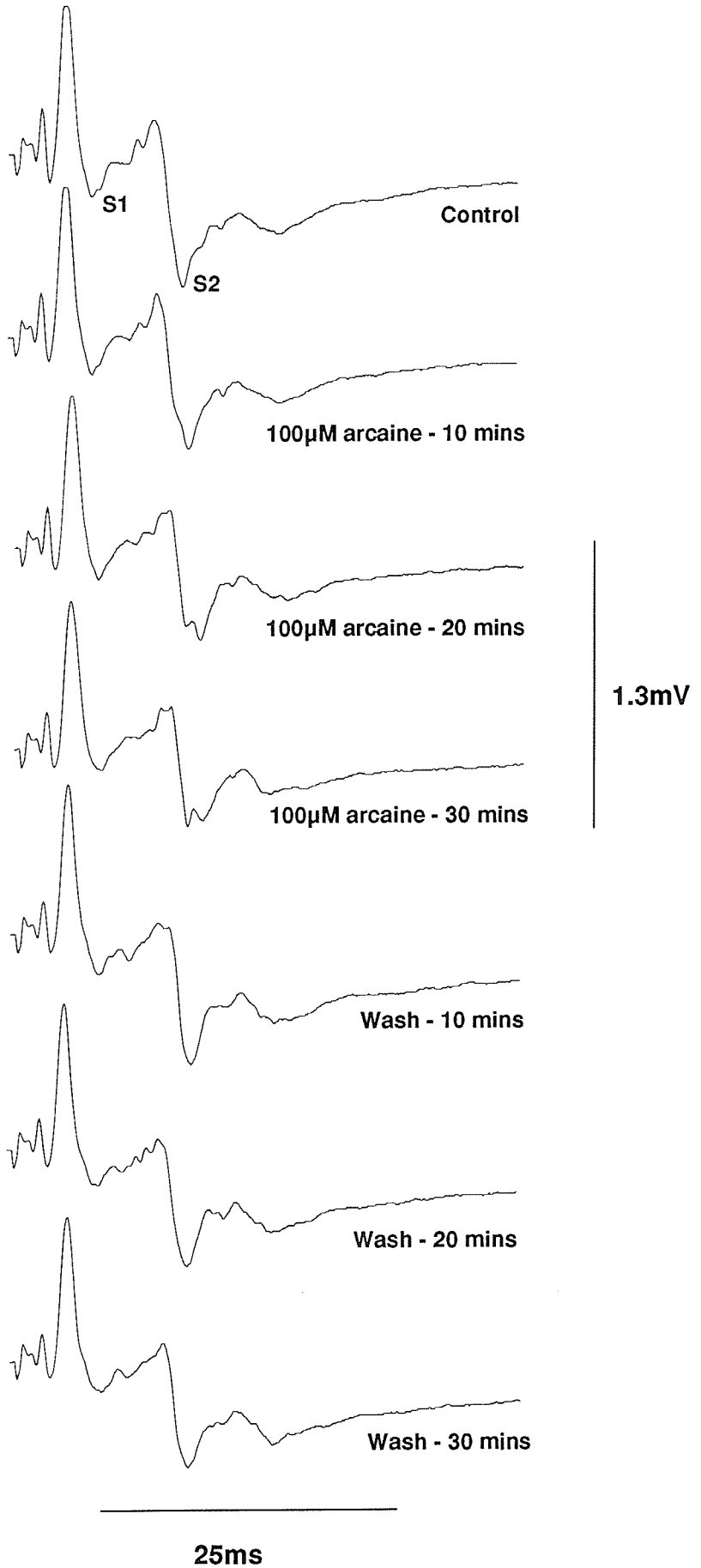


Fig. 4.23: Data showing the effect of adding 100µM arcaine to the medium on the fast wave dorsal horn field potentials.

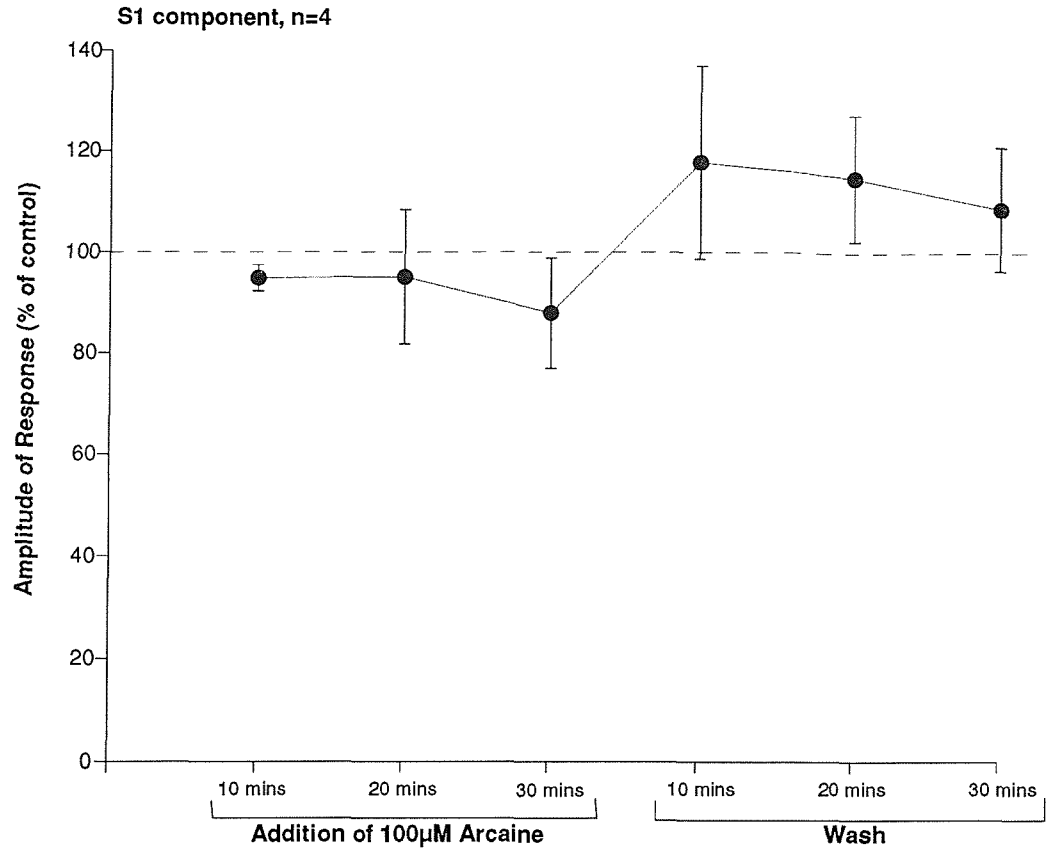


Fig. 4.24: The graph shows that100µM arcaine had no significant effect on the fast wave dorsal horn field potential S1 component. n=8.

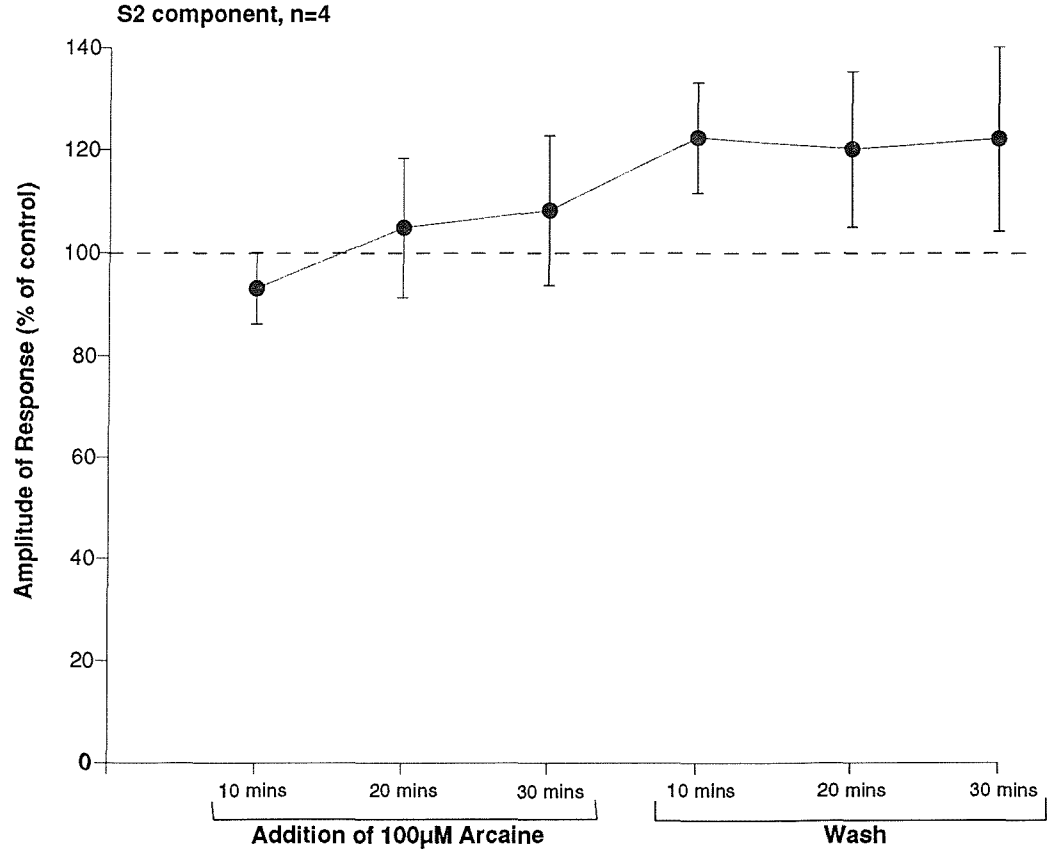


Fig. 4.25: 100µM arcaine had no significant effect on the S2 component of the fast wave field potentials after being added to the medium for 30 mins. n=8.

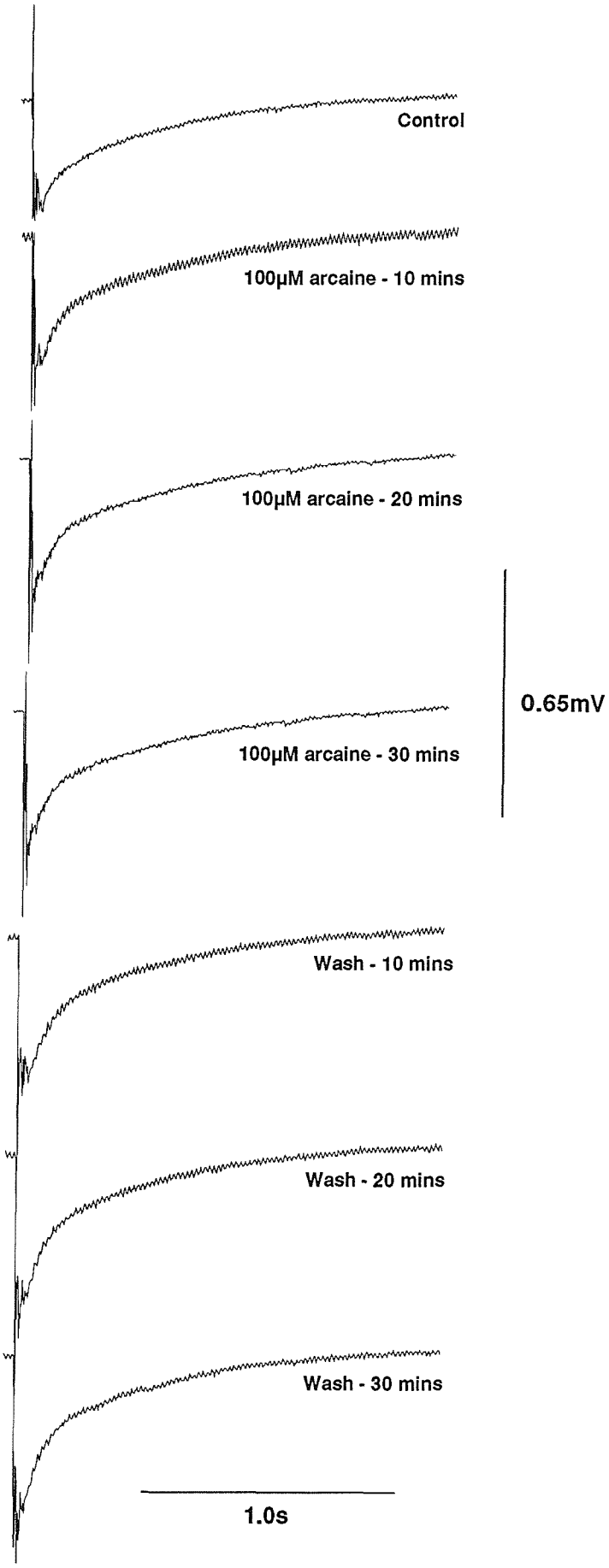


Fig. 4.26: In this experiment example 100µM arcaine was added to the medium for 30 mins and then washed out for 30 minutes. The slow wave field potential was measured every 10 minutes.

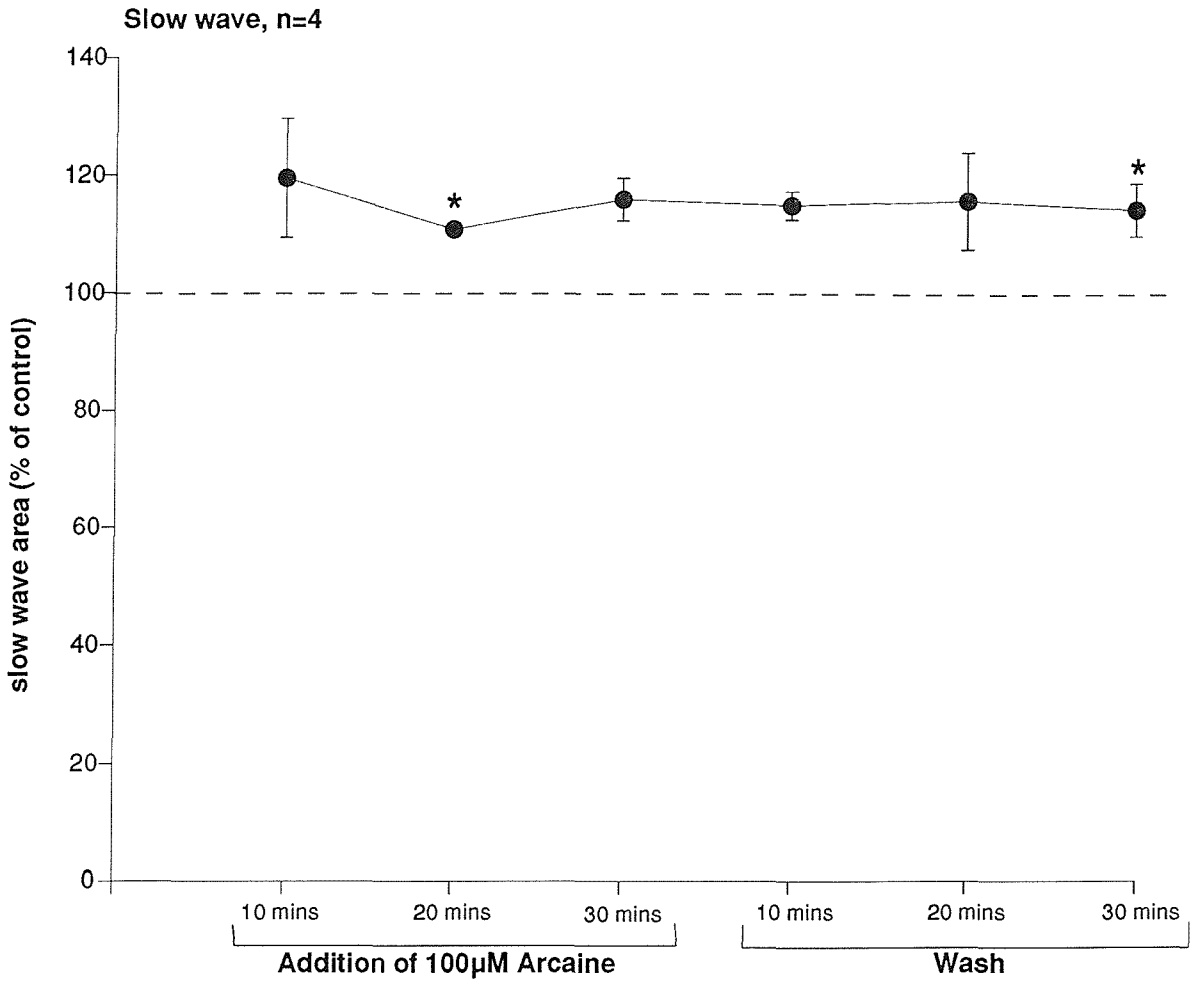


Fig. 4.27: Arcaine (100µM) significantly increased the slow wave field potential after 20 mins ($p<0.05$ paired T-test) and the slow wave was significantly increased after 30 mins wash out ($p<0.05$ paired T-test). n=4

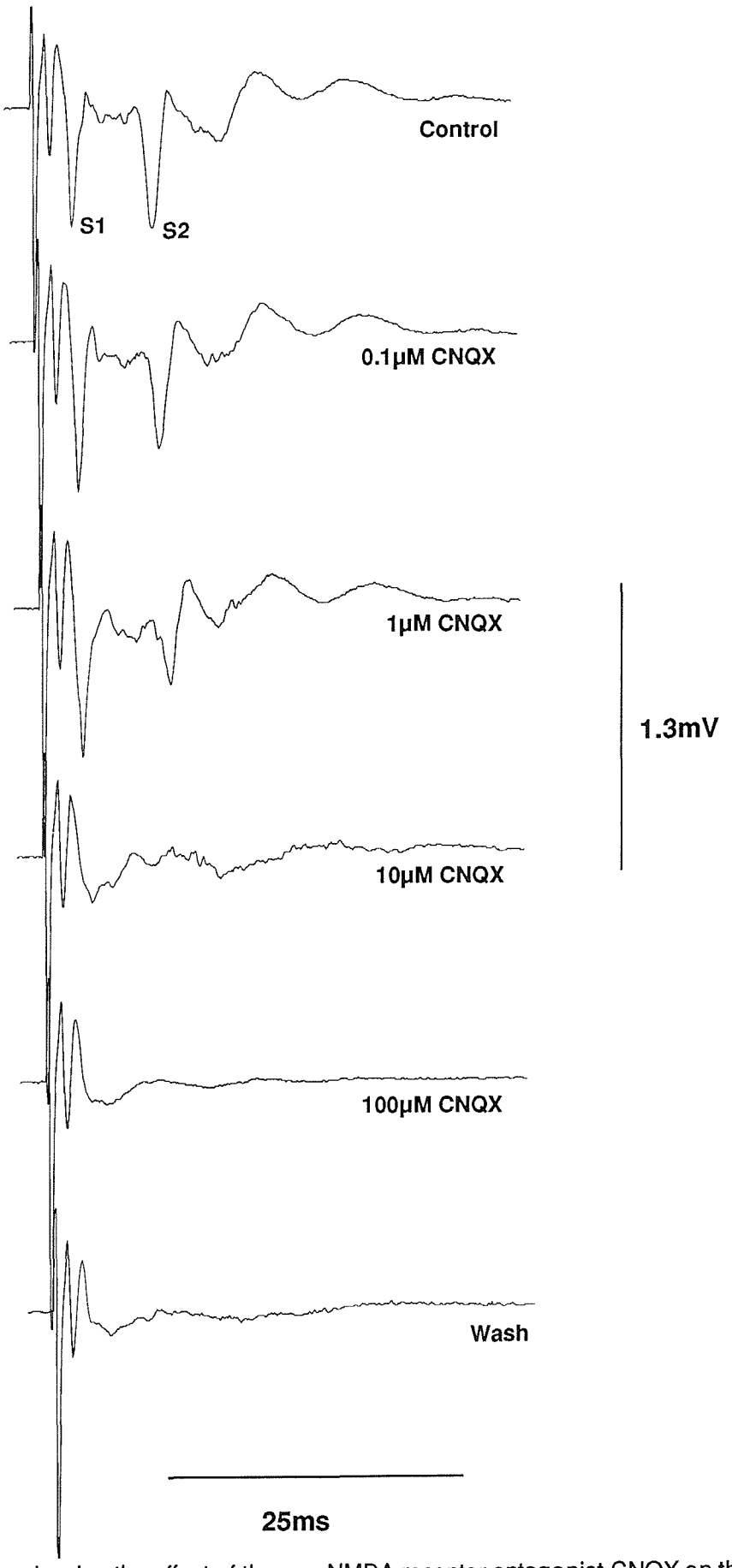


Fig. 4.28: Raw data showing the effect of the non-NMDA receptor antagonist CNQX on the fast wave dorsal horn field potentials. At a concentration of 100µM the S1 and S2 components have been completley inhibited.

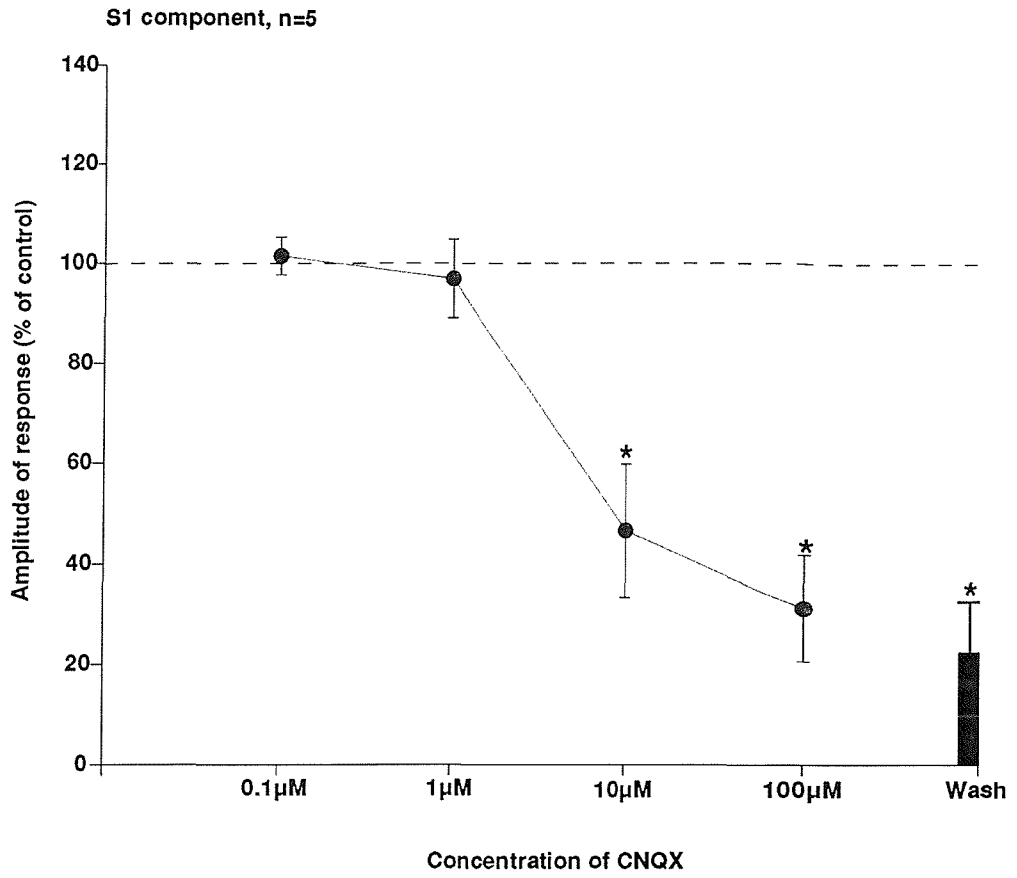


Fig. 4.29: The dose-response curve indicates that the inhibition of S1 by CNQX at 10µM, 100µM and after a wash was significant (paired T-test). n=5.

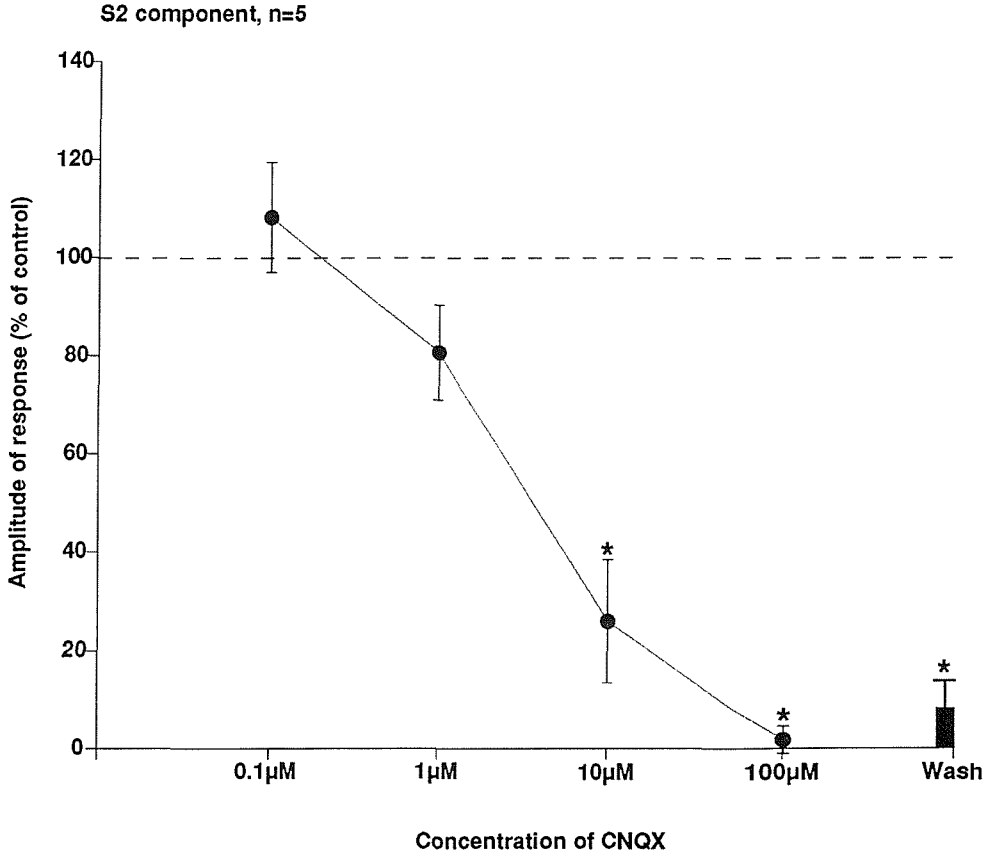


Fig. 4.30: The S2 component of the fast wave field potentials was significantly inhibited by CNQX at concentrations of 10µM, 100µM and after the wash (paired T-test). n=5.

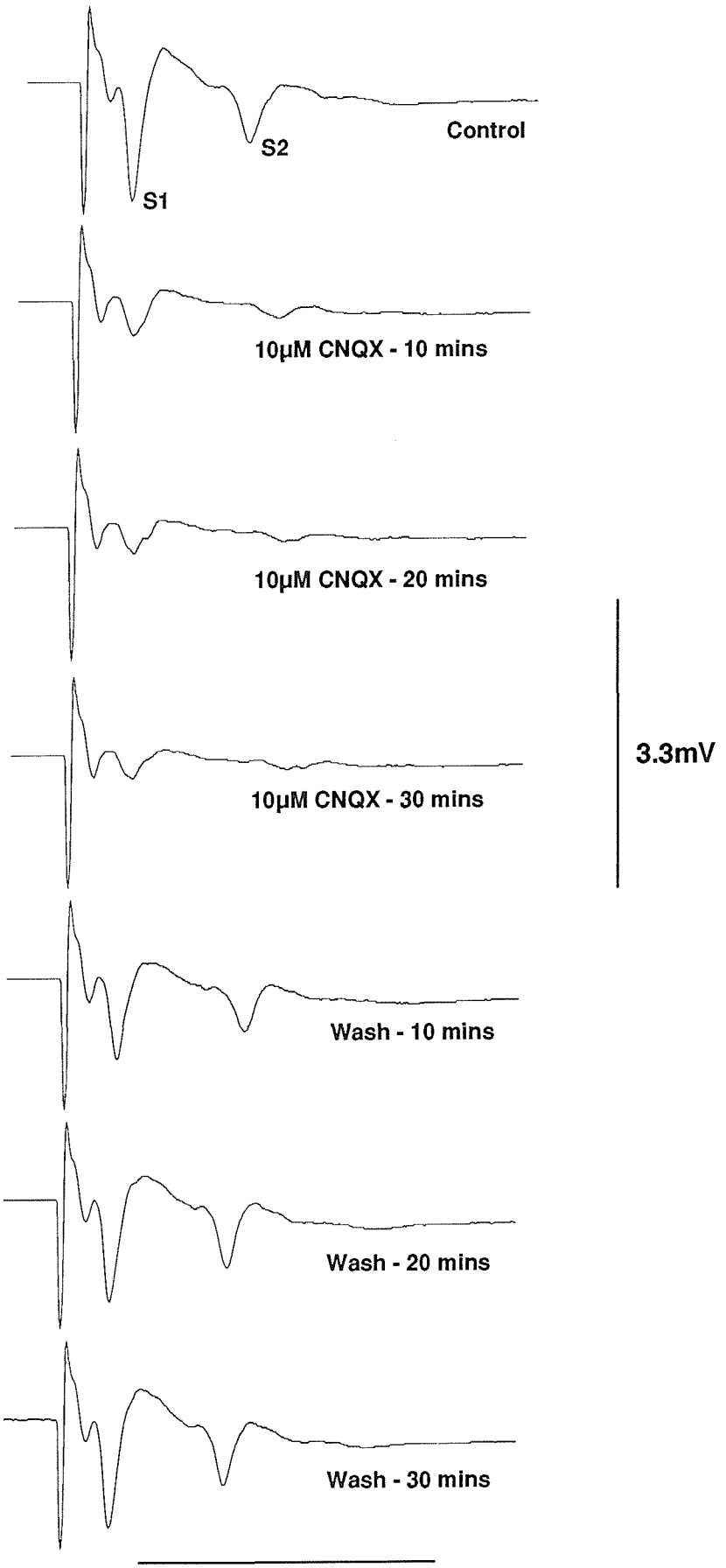


Fig. 4.31: Raw data to show the effect of adding 10µM CNQX for 30 mins before washing it from the tissue for another 30 mins.

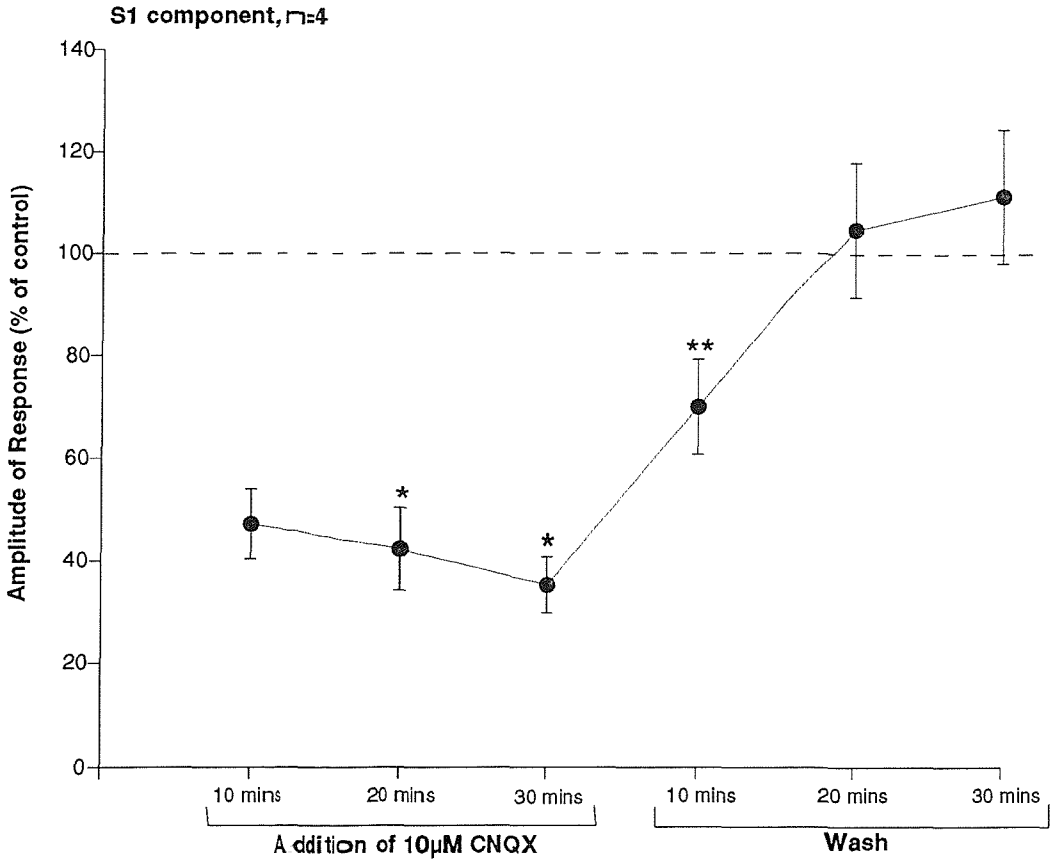


Fig. 4.32: Graph to show the effect of adding 10µM CNQX to the medium for 30 mins and washing it off for a further 30 mins on the S1 component of the fast wave dorsal horn field potential. n=4.

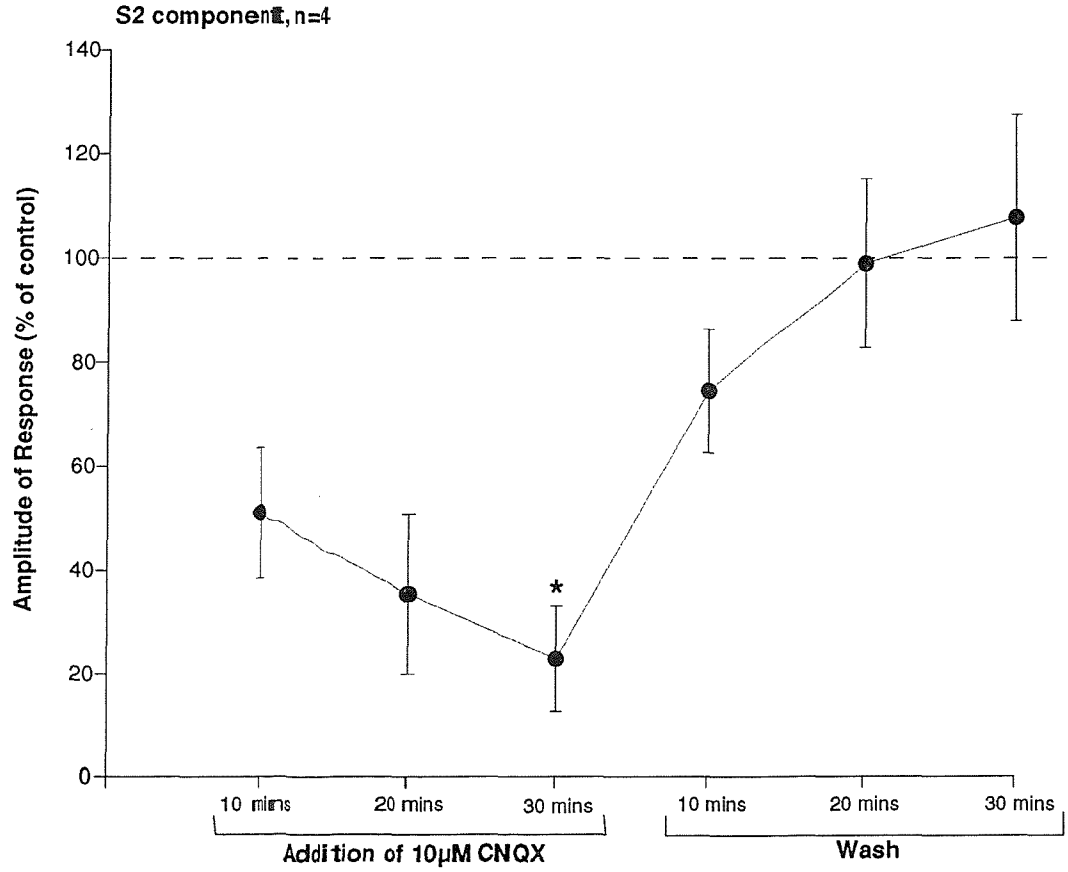
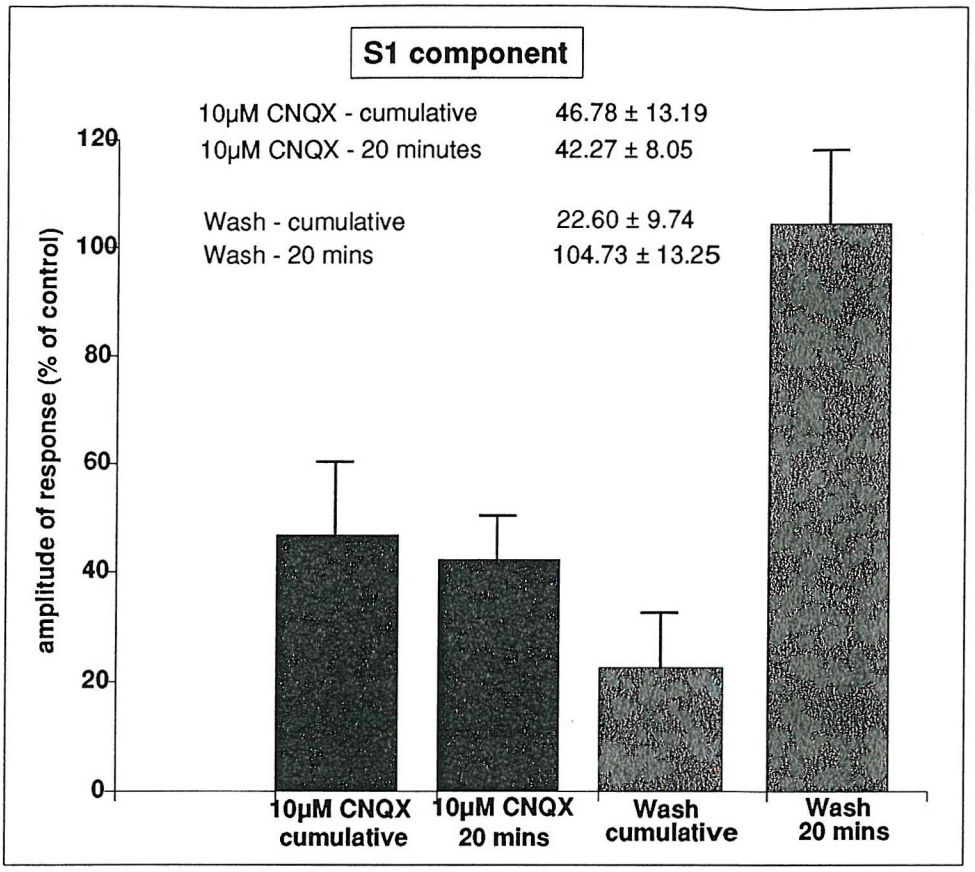


Fig. 4.33: Graph showing the effect of adding 10µM CNQX to the medium for 30 mins and washing it off for a further 30 mins on the S2 component of the fast wave dorsal horn field potential. n=4.



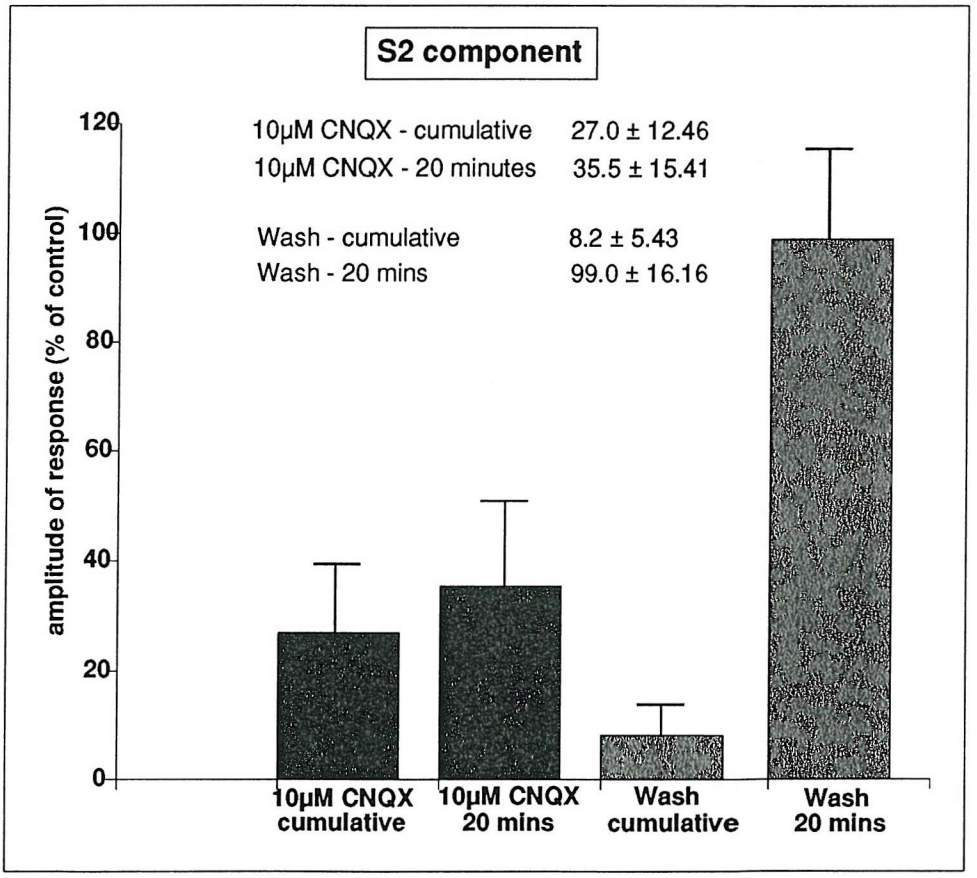


Fig. 4.34: These graphs compare the amplitude of the S1 and S2 components after the cumulative and the direct addition of 10µM CNQX. The amplitudes after washing the drug from the medium is also compared. (cumulative, n=5; 20 mins direct, n=4).

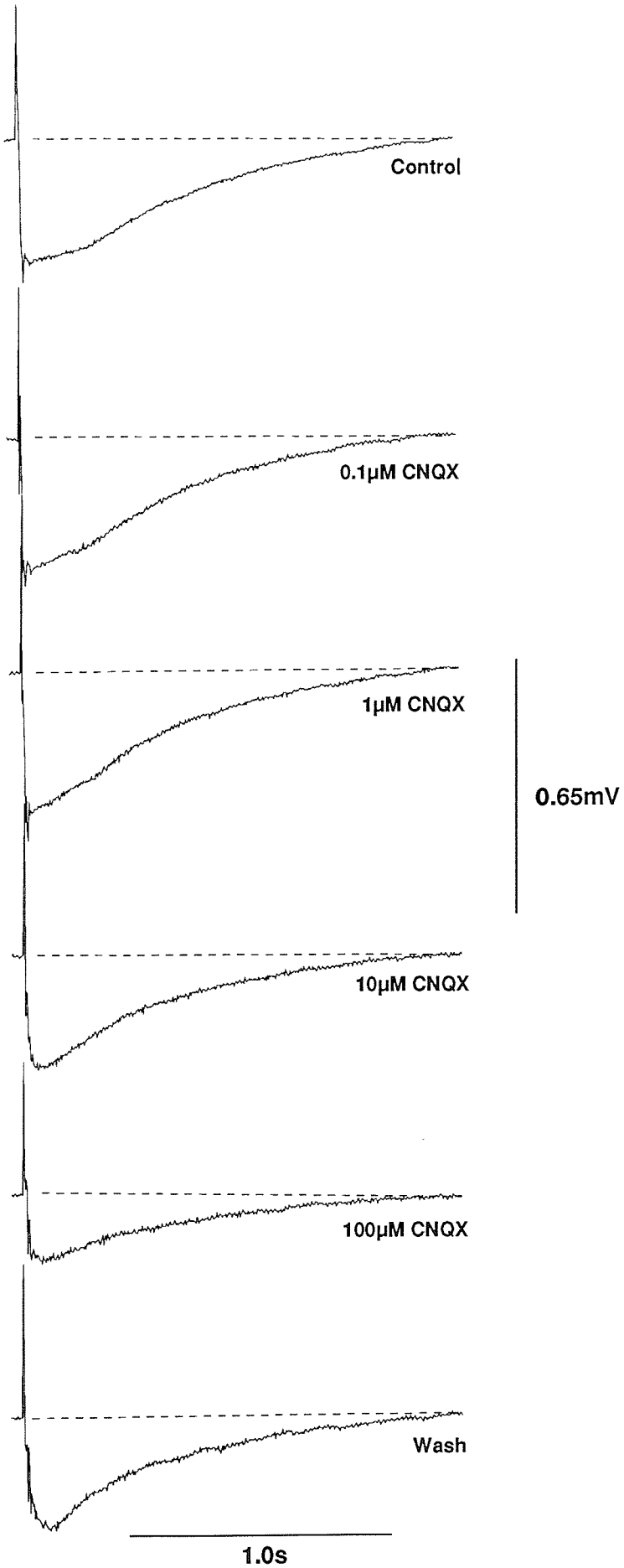


Fig. 4.35: Raw data to show that CNQX potently inhibited the slow wave field potential.

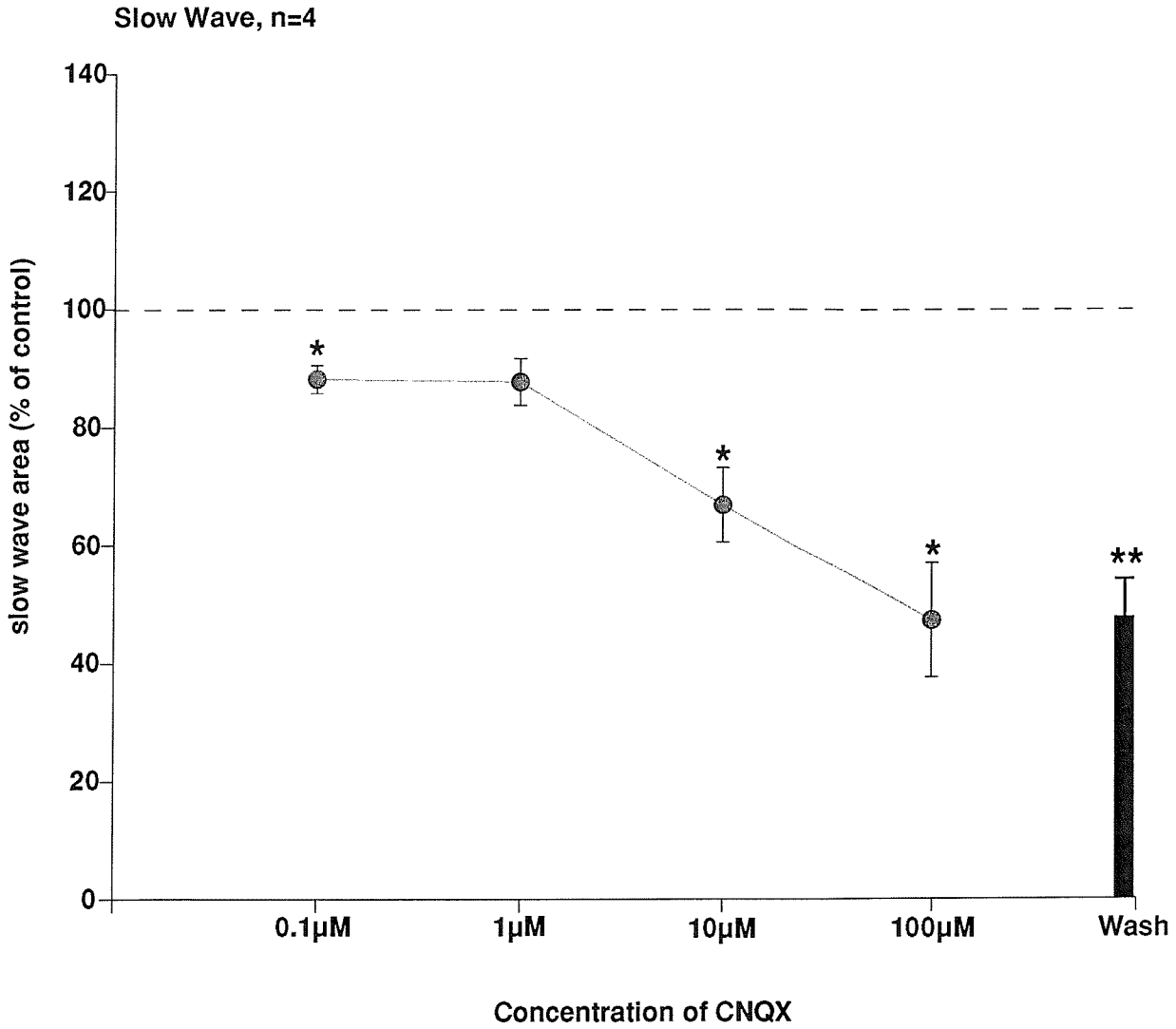


Fig. 4.36: The graph illustrates that the non-NMDA receptor antagonist CNQX significantly inhibited the slow wave field potential at concentrations of 0.1µM, 10µM and 100µM and also after the drug was washed from the system.

4.2.6 Summary of Results

	D-AP5		7-Cl KYNA		MK-801		Arcaine		CNQX	
	<100µM	100µM	<100µM	100µM	<100µM	100µM	<100µM	100µM	<100µM	100µM
S1	ns	↑	ns	↓	ns	ns	ns	ns	↓	↓
S2	ns	ns	ns	ns	↓	↓	ns	ns	↓	↓
Slow	↓	↓	↓	↓	↓	↓	ns	ns	↓	↓

Fig. 4.37: Table to summarise the effects of ionotropic glutamate receptor antagonists on the S1 and S2 components of the fast wave field potential and on the slow wave field potential.

4.3 Discussion:

The results of this study using the *in vitro* spinal cord agree with other studies conducted in intact animals (Coderre et al 1993; Dickenson and Sullivan 1990; Dray et al 1994; Dubner and Ruda 1992; McMahon et al. 1993; Woolf 1994; Yoshimura and Nishi 1992). These experiments in the *in vitro* spinal cord have therefore validated the use of the preparation as a useful tool in the study of spinal pain mechanisms. The results of these experiments indicated that non-NMDA and NMDA receptors mediate both high and low threshold transmission to dorsal horn neurones. While NMDA receptors have a major role in the mediation of high threshold transmission to dorsal horn neurones and a minor role in low threshold transmission, the reverse is true for the mediation of low threshold transmission to the dorsal horn. Low threshold transmission is mediated principally by non-NMDA receptors with a small NMDA receptor component.

It has been shown in chapter 3 that the stimulation of a dorsal root at 5 times the voltage necessary to induce a DRR in an adjacent root activates nerve fibres with a conduction velocity approximately equal to that of low threshold, myelinated Aβ fibres. This low intensity stimulation of a dorsal root produces a fast wave field potential with two synaptic components (S1 and S2). Stimulation of a dorsal root at 20 times the DRR threshold voltage activates fibres with differing conduction velocities. Fibres with conduction velocities expected of Aβ fibres are stimulated and in addition fibres with conduction velocities expected of high threshold, unmyelinated C fibres are also excited. High intensity stimulation of a dorsal root has been shown to produce both a fast wave field potential and also a slow wave field potential that returns to baseline after 500-1000ms.

4.3.1 Mediation of Fast Wave Field Potentials

4.3.1.1 NMDA Receptors

D-AP5 is an NMDA receptor antagonist that acts at the glutamate-binding site. This antagonist had no significant effect on the S1 component of the fast wave field potential at

low concentrations (≤10μM), although this was significantly increased at 100μM (paired t-test). This result suggests that NMDA receptors may have a minor role in the mediation of fast wave field potentials. D-AP5 had no significant effect on the S2 component at concentrations up to 100μM.

The results using D-AP5 suggest that NMDA receptors have a minor role in the transmission of non-noxious information via low threshold Aβ fibres to dorsal horn neurones in the spinal cord. Other experiments in this study using different NMDA receptor antagonists confirmed the hypothesis that NMDA receptors mediate Aβ fibre transmission. The NMDA receptor antagonist 7-Cl KYNA, which exerts its action via the glycine site, did not have any significant effects on the S1 component of the fast wave field potential at concentrations up to 10μM, although at 100μM, the S1 component was significantly decreased. This was most likely due to the non-selective actions of 7-Cl KYNA at high concentrations. At high concentrations, 7-Cl KYNA is known to inhibit kainate receptors (Kemp et al. 1988; Kleckner and Dingledine 1989). This could account for the decrease in the S1 component amplitude as the experiments with CNQX suggests that non-NMDA receptors have a major role in mediating the fast wave field potential.

MK-801, which is also an NMDA receptor antagonist and acts as an ion channel modulator (Wong et al. 1986) had no significant effect on the S1 component of the fast wave field potential but at concentrations of 1μM and 10μM, it significantly decreased the S2 component. MK-801 is also known to be a sodium channel blocker. The inhibition of the fast wave field potential by MK 801 may therefore be due to a sodium channel block.

The NMDA receptor antagonist arcaine, which acts at the polyamine site of the receptor (Reynolds 1990), had no significant effects on either the S1 or the S2 components of the fast wave field potential.

These results using NMDA receptor antagonists suggest that NMDA receptors are involved in mediating the S1 or S2 components of the fast wave field potential and therefore probably have a minor involvement in the transmission and/or processing of non-noxious information in the dorsal horn of the spinal cord.

4.3.1.2 Non-NMDA Receptors

The application of the non-NMDA receptor antagonist CNQX significantly inhibited the S1 and S2 components of the fast wave field potential at concentrations of 10μM and 100μM. CNQX is known to have effects on NMDA receptors as well as non-NMDA receptors (Honore et al. 1988; Long et al. 1990). However, since antagonists acting exclusively on NMDA receptors were shown to have significant effects in mediating the S1 or S2 components of the fast wave dorsal horn field potentials at high concentrations it can be concluded that the inhibition seen after the addition of low concentrations of CNQX was mediated solely by non-NMDA receptors. It is not known which type of non-NMDA receptor is primarily involved in the mediation of the fast wave field potentials because using CNQX does not allow the effects of either AMPA receptors or kainate receptors to be distinguished. The anatomical evidence, however, suggests it is the AMPA receptors which are primarily involved since they are found in greater abundance in the dorsal horn than kainate receptors (Furuyama et al. 1993).

4.3.3 Further Observations - Arcaine

To further test the effects of arcaine, a concentration of 100μM was added to the medium for a total period of 30 minutes and recordings were made every 10 minutes. The addition of 100μM arcaine to the medium for 30 minutes did not significantly effect either the S1 or the S2 component of the fast wave field potential. The one-sample t-test calculated the increase of the S1 component observed after arcaine was washed out of the tissue to be significantly different to a mean of 100% ($p<0.05$).

As well as having an NMDA receptor antagonist activity, arcaine also has an inhibitory effect on the enzyme nitric oxide synthase (NOS) (Yokoi et al. 1994). NOS activation and hence nitric oxide (NO) are produced in response to NMDA receptor activation. Activation of the NMDA receptor results in an influx of intracellular Ca^{2+}. This increase in intracellular Ca^{2+} triggers a cascade of events that include the stimulation of phospholipases to produce diacylglycerol (DAG) and ecoisanoids, stimulation of the

production of 1,4,5-triphosphate (IP_3), the activation of PKC and, importantly, the activation of the NOS enzyme (Li et al. 1994; Meller et al. 1996). NMDA activation therefore results in an increase in NO (Fig. 4.37). These changes in Ca^{2+} and NO may cause central sensitisation and/or windup in the spinal cord (Meller et al. 1992). NO is generated by the catabolism of L-arginine to L-citrulline by the enzyme NOS. There are several different isoforms of the NOS enzyme including neuronal NOS (nNOS) and epithelial NOS (eNOS) both of which are found in neurones (Bredt et al. 1990; Forstermann et al. 1995). nNOS is the major source of neuronal pools of NO. It shows constitutive (ongoing) activity, but its action can be enhanced by an increase in intracellular Ca^{2+} triggered, for example, by NMDA receptor activation (Li et al. 1994). In situations of tissue damage, the inducible form of NOS (iNOS) becomes important and is also stimulated by an increase in intracellular Ca^{2+}. NO is coupled to several intracellular conduction mechanisms, including the generation of cyclic guanidine monophosphate (cGMP) which subsequently activates a specific protein kinase G (PKG) (for refs. see Millan 1999).

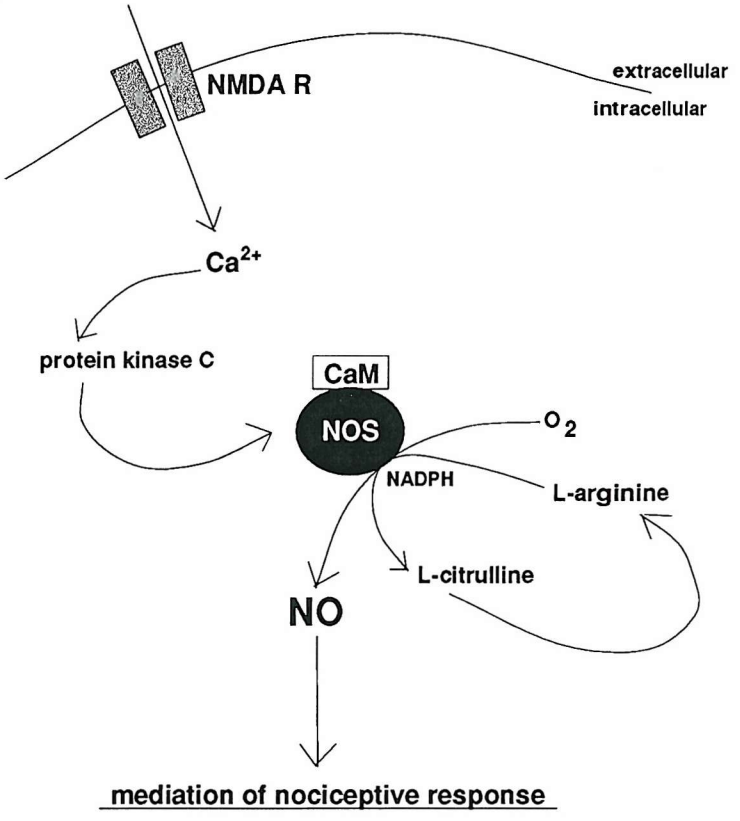


Figure 4.37: Diagram to illustrate the interaction between NMDA receptors, the activation of the enzyme nitric oxide synthase (NOS) and the production of nitric oxide (NO). Abbreviations: NMDA R = NMDA glutamate receptor, NOS=nitric oxide synthase, NO = nitric oxide, CaM = calmodulin. Adapted from Meller and Gebhart (1993) *Pain* **52,** 127-136.

The increase in amplitude of the fast wave response after arcaine has been washed out of the system could be due to a "rebound phenomenon". If the constitutive form of nNOS is present in the dorsal horn of the spinal cord, then adding arcaine would have inhibited the enzyme and blocked its actions. When arcaine was washed out from the system the enzyme appeared to become sensitised and its actions therefore enhanced. The actions of NOS subsequently caused the increase in the amplitude of the S1 component of the dorsal horn field potential. Indeed, sensitisation of nociceptive primary afferent fibres and the enhanced release of neuropeptides in response to NO has previously been observed (Lawland et al. 1997; Miyasaka and Hirata 1997). The mechanism of sensitisation most probably involves the activation of a cGMP-dependent PKG (Qian et al.1996).

4.3.4 The First and Second Synaptic Components as Independent Events

The NMDA receptor antagonist 7-Cl KYNA which acts at the glycine site was shown to be capable of uncoupling the S1 and S2 components of the fast wave dorsal horn field potential (Fig. 4.10). This suggests that the first (S1) and the second (S2) synaptic components are independent events. Other results showing a significant increase or reduction in one component but not the other confirms that S1 and S2 are independent events. It is therefore possible that S1 and S2 represent two different synapses in a pathway.

4.3.5 Cumulative and Single Dose Addition of Drugs to the Bathing Medium

The protocol underlying the cumulative dose-response curve for CNQX was that each dose of the drug was added to the bathing medium for a period of 20 minutes before the dorsal horn field potentials were recorded for a further 5 minutes. A comparison was made of the effect of 10μM CNQX on the fast wave field potentials after the cumulative addition of the drug and the effect of 10μM being added directly to the bathing medium for a period of 20 minutes. The decrease in amplitude of the S1 and the S2 components by

CNQX being added as both a cumulative and a direct dose showed no significant difference (Fig. 4.34). Therefore, results obtained after drug doses are added in a cumulative manner are shown to be valid. However, the increase in amplitude of the S1 and S2 components of the fast wave field potentials when a direct application of 10μM CNQX was washed from the bathing medium was significantly different to that after a cumulative dose protocol. After the direct dose of CNQX was washed out of the bathing medium the amplitude of the S1 and S2 components returned to 104.73±13.25% and 99.0±16.16% of the control response respectively. Following the cumulative addition of CNQX, the amplitudes of the S1 and S2 components only returned to 22.60±9.74% and 8.2±5.43% respectively. The cumulative addition of CNQX involves adding doses up to a concentration of 100μM and this higher dose may not be as easily removed from the tissue as a dose of 10μM, explaining why the amplitudes of the S1 and S2 components will not return to control values after the removal of the drug from the bathing medium. The drug was also applied to the tissue for a longer period of time following cumulative addition. Therefore CNQX has probably had a greater penetration into the spinal cord and been added at a higher concentration was harder to remove. The results after the drug was added directly to the medium, however, indicate that CNQX does inhibit the fast synaptic waves of the dorsal horn field potential and that this effect is reversible.

4.3.6 Mediation of Slow Wave Field Potentials

The NMDA receptor antagonists D-AP5, 7-Cl KYNA and MK-801 significantly inhibited the slow wave field potential elicited by stimulation of a dorsal root at 20 times the DRR threshold voltage. These results indicate that the slow wave field potential is mediated by NMDA receptors. However, even at high concentrations of these antagonists, the slow wave field potential was not completley abolished, suggesting that other transmitters/ receptors are involved, for example, substance P. The non-NMDA receptor antagonist CNQX, which also has a low affinity for the glycine site of the NMDA receptor (Long et al. 1990) significantly reduced the slow wave field potential at a concentration of 100μM. However, because of the nature of the drug it is not known if this effect is due to (i) the direct involvement of non-NMDA receptors, (ii) the inhibition of AMPA receptors which

thereby inhibit the "priming/sensitisation" of the NMDA receptors to noxious input, or (iii) the antagonist action of CNQX at the glycine site of the NMDA receptor. Previous studies have shown that mediation of high threshold inputs are mediated by a non-NMDA receptor element (Yoshimura and Nishi 1992) suggesting that the effect of CNQX may be due to its non-NMDA receptor antagonist properties. Indeed, other studies have confirmed a role for the non-NMDA receptors in attenuating nociceptive behviours in rat models of pain (Zahn et al. 1998; Procter et al. 1998; Cumberbatch et al. 1994).

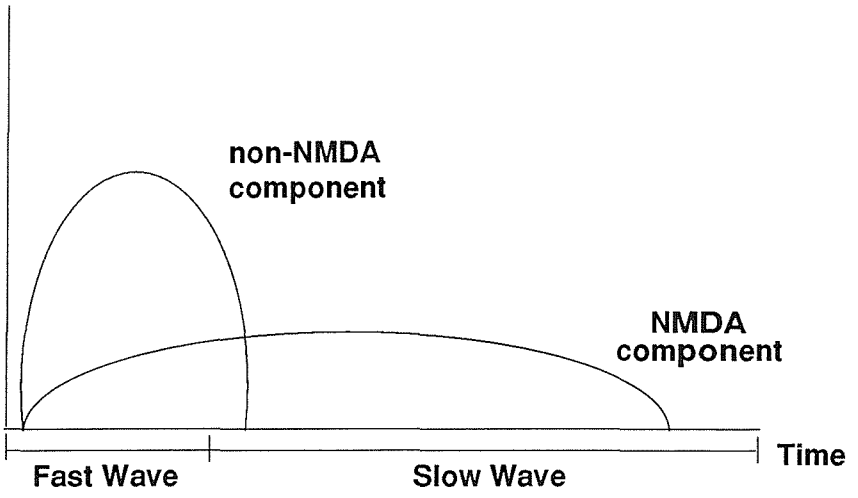


Fig. 4.83: This diagram illustrates the involvement of both non-NMDA and NMDA receptors in the fast and slow wave field potentials.

Alternatively, a study by Procter et al. (1998) has suggested that kainate receptors are located presynaptically on rat C fibres and are negatively coupled to glutamate at neonatal ages. Glutamate release is inhibited in response to nociceptive inputs. However, in adult rats kainate autoreceptors appear to become positively linked to glutamate release, hence kainate antagonists rather than agonists become antinociceptive. Since CNQX is also a kainate receptor antagonist, it may be acting at these presynaptic sites to further decrease the slow wave response amplitude. The slow potential may also be further down stream of the synaptic chain to non-NMDA receptors.

The experiments investigating the effect of NMDA receptor antagonists on the slow wave field potential in this study gave a potency rating of 7-Cl KYNA > D-AP5 > MK-801. A study by Chaplan et al. (1997) tested the effectiveness of several NMDA antagonists at inhibiting the painful response to (i) the formalin test, and (ii) nerve ligation. For the nerve

injury model the order of efficacy was:- memantine = D-AP5 > dextrorphan = dextromethorphan > MK-801 > ketamine. For the formalin model the efficacy order was:- D-AP5 > memantine >> MK-801 > dextrorphan > dextromethorphan > ketamine. The effectiveness of MK-801 at inhibiting painful behaviour in both of these models was relatively low. MK-801 showed limitations in each of the models tested. The results of the present investigation seem to agree with those of Chaplan et al (1997).

Cloning of the NMDA receptor has revealed that there are a number of subtypes, and *in situ* hybidisation has mapped a different distribution for several subtypes in the brain. The pharmacology and functional diversity of NMDA receptors has been described in the brains of both mouse and rat (for refs see Chaplan et al. 1997), and it is possible that the described subtypes which exist in the spinal cord have a distinctive pharmacology compared with other CNS regions. Also, the selectivity of ligands studied for sensory and motor receptors may vary. This could explain why MK-801 appears to be less effective at inhibiting the slow wave dorsal horn field potential when compared with other NMDA receptor antagonists. A selectivity in the spinal cord for the NMDA antagonists D-AP5 and HA-966 has previously been found (Wei et al. 1997). If NMDA receptors do have differing pharmacological profiles, then definition of discrete NMDA receptor populations is required in order to better define the molecular target for spinal cord selective NMDA receptor antagonists.

The NMDA receptor antagonist arcaine was expected to significantly inhibit the slow wave dorsal horn field potential. Arcaine not only antagonises the NMDA receptor but also inhibits the enzyme NOS (Yokoi et al. 1994). As has already been mentioned, activation of NMDA receptors leads to an increase in intracellular calcium. The increased calcium level then activates the inducible form of the neuronal NOS enzyme and/or upregulates the consitutive form (Li et al 1994). The resulting increase in NO and the potentiation of synaptic transmission that results effects spinal nociceptive processing. For example; hyperalgesia, facilitation, an expansion of receptive fields, central sensitisation and/or windup (see refs in Millan 1999). Therefore because both NMDA receptors and NO are involved in the spinal cord nociceptive response (Meller and Gebhart 1993) the slow wave was expected to be significantly inhibited. However, arcaine was shown to have no significant effect on the slow wave field potential at concentrations up to 100μM. This may

be due to the poor penetration of the drug into the tissue. The poor penetration hypothesis would also partly explain why the effects of arcaine on the fast wave dorsal horn field potentials were seen so late on in the experiment, i.e. after the wash.

A concentration of 100μM arcaine was added to the medium for 30 mins and a recording of the slow wave field potential was made every 10 mins. The slow wave was significantly increased 20 minutes following arcaine addition and also 30 minutes after the drug had been washed from the medium. This was unexpected as evidence suggests that activation of spinal NMDA receptors by a nociceptive stimulus can induce persistent enhancement of nociception via the production of NO (Li et al. 1994; Meller et al. 1996). Arcaine would, therefore, be expected to inhibit the slow wave field potential because it is known to inhibit both NMDA receptors and the enzyme NOS.

The unexpected results observed in this study may be explained by recent data suggesting that NO causes a tonic depression of resting impulse activity in spinal neurones (Hoheisel et al. 1995). A pharamacological block of nNOS led to an increase in activity in spinal dorsal horn neurones of the rat (Hoheisel et al. 1995), and conversely application of an NO donor reduced the activity of dorsal horn neurones (Schmid and Pehl 1996; Pehl and Schmid 1997). However, the effects of NO in the spinal cord appear to be region specific. In contrast to neurones in the dorsal horn, the majority of spinal neurones around lamina X increased their activity following application of an NO donor (Schmid and Pehl 1996; Pehl and Schmidt 1997). The NO effects on the resting activity of spinal neurones are probably mediated by an NO-induced increase in the synthesis of cGMP (Schmid and Pehl 1996; Pehl and Schmidt 1997).

Coderre and Empel (1994) showed that effective analgesia following formalin injury in rats can be obtained by combined treatment of D-AP5 with glycine acting as an agonist at the allosteric strychnine-insensitive glycine site, and the ion channel modulator MK-801 acting with the polyamine site inhibitor spermine. These antagonist interactions dramatically enhance the analgesic effects of D-AP5 and MK-801 without increasing their side-effects demonstrating that NMDA receptor antagonists can be synergistic and could be useful as therapeutic agents for the treatment of clinical pain. In this investigation the NMDA antagonist acting at the glycine site of the receptor, 7-Cl KYNA, was shown to have a

significant effect on the slow wave field potential as well as significantly inhibiting the expression of c-fos in the dorsal horn (Chapter 6: c-*fos* expression in the in vitro spinal cord). Thus it is possible that this glycine site may be a useful target for agents in reducing the consequences of NMDA-mediated events in sensory processing in the spinal cord, including modulation/transmission of nociceptive information and c-fos expression. The glycine site of the NMDA receptor could therefore be a potential drug target for analgesic therapy.

Chapter 5

Metabotropic Glutamate Receptors

5.1 Introduction:

The metabotropic glutamate receptors (mGluRs) were discovered in the mid-1980s when it was shown that glutamate could stimulate phosphoinositide turnover (Sladeczek et al. 1985; Nicoletti et al. 1986a, b; Pearce et al. 1986). Molecular cloning of the G-protein coupled mGluRs has identified eight subtypes (mGluR1-8) whose heterogeneity is increased by the existence of several splice variants. On the basis of sequence homology, agonist potency and signal transduction mechanisms the mGluRs have been subdivided into three different groups (Pin and Duvoisin 1995). Group I includes mGluR1 (with its splice variants a, b, c and d) and mGluR5 (a and b), which are positively coupled to phospholipase C (PLC) and lead to an increase in diacylglycerol (DAG) and inositol triphosphate (IP_3), revealed by an increase in phosphoinositide turnover, and calcium release from internal stores (Pin and Duvoisin 1995; Nakanishi 1992; Simoncini 1993). Expression in mammalian cells reveals that group II mGluRs (mGluR2 and mGluR3) inhibit adenylyl cyclase which catalyses the conversion of ATP to cAMP. Like group II mGluRs, group III mGluRs (mGluR4a,b, mGluR6, mGluR7a,b and mGluR8) also inhibit adenylyl cyclase.

The presence of mRNAs for all mGluR subunits except mGluR6 and mGluR8 have been demonstrated in the rat spinal cord by *in situ* hybridisation, reverse transcriptase-polymerase chain rection (RT-PCR) and immunocytochemistry techniques (Berthele et al. 1999; Valerio et al. 1997; Vidnyanszky et al. 1994; Anneser et al. 1995; Boxall et al. 1998). However, little is known about the functional role of the mGluRs and especially the roles of the seperate mGluR subtypes in the spinal cord. Evidence suggests that mGluRs are involved in the spinal processing of somatosensory information, and that they contribute to development of spinal hyperexcitability following noxious stimulation (Bleakman et al. 1992; Lodge et al. 1996; Bond and Lodge 1995; Boxall et al. 1996;

Boxall et al. 1998; Budai and Larson 1998).

Drugs that act through the mGluRs may be able to modulate glutamatergic synapses and hence inhibit the transmission of nociceptive signals in the spinal cord. Using these drugs to treat pain in a more selective manner would reduce the potential side-effects and may therefore be more useful than the ionotropic glutamate receptor antagonists. This class of glutamate receptors could provide a unique opportunity for designing more selective pain therapies.

The aims of the investigations in this chapter were:

1) To identify which mGluR receptor groups are involved in the processing of noxious information in the spinal cord.

2) To determine if the mGluRs are involved in the transmission of non-noxious information in the spinal cord.

5.2 Results:

The role of mGluRs in sensory processing in the spinal cord was investigated using various mGluR agonists and antagonists selective for a particular mGluR receptor group. The table below (Fig. 5.1) gives a summary of the various agonist and antagonist actions of ligands used in this study. Fast wave field potentials were elicited by stimulating a dorsal root at 5 times the stimulus necessary to evoke a dorsal root reflex (DRR) in an adjacent dorsal root. Slow wave field potentials were evoked by stimulation at 20 times the DRR threshold voltage.

Ligand	Action
trans-ACPD	mGluR group I and II agonist
L-AP3	mGluR group I antagonist
DHPG	mGluR group I agonist
EGLU	mGluR group II antagonist
L-AP4	mGluR group III agonist

Fig. 5.1: Table summarising the actions of ligands used in this study.

5.2.1 Effect of trans-ACPD on dorsal horn field potentials.

Trans-ACPD is an equimolecular mix of (1S, 3R)-ACPD and (1R, 3S)-ACPD and is a selective agonist for metabotropic glutamate group I and II receptors (Pin and Duvoisin 1995). The effect of trans-ACPD on the S1 component of the fast wave field potential is shown in Figs. 5.2/5.3. The concentration-response curve shown in Fig. 5.3 illustrates that *trans*-ACPD significantly reduced the S1 component of the fast wave field potential to 85.6±4.3% and 82.9±6.3% of the control response at concentrations of 0.1μM and 1μM respectively ($p<0.05$, paired t-test) (n=5). Higher concentrations of *trans*-ACPD continued to inhibit the amplitude of the S1 component, but larger SEMs rendered these results non significant.

Trans-ACPD significantly reduced the fast wave S2 component to 62.9±7.2% and 32.8±8.6% of the control response at concentrations of 10μM and 100μM respectively ($p<0.01$ paired t-test) (n=5) (Figs. 5.2/5.4). The IC_{75} of trans-ACPD (the concentration of the drug that produces a 75% response) was found to be 18μM for the S1 component and 0.6μM for the S2 component. These results show that trans-ACPD had a much more potent effect on the S2 component.

The slow wave field potential was significantly inhibited by trans-ACPD at concentrations

of 0.1μM and 100μM. At 0.1μM, the response was reduced to 86.6±2.4% of the control ($p<0.01$ paired t-test). At 100μM it was reduced to 70.8±9.4% of the control ($p<0.05$ paired t-test) (n=4). Figures 5.5 and 5.6 illustrate the inhibition of the slow wave field potential by the addition of *trans*-ACPD to the spinal cord bathing medium.

5.2.2 Effect of L-AP3 on dorsal horn field potentials.

L-AP3 is an antagonist of mGluR-mediated phosphoinositide responses (Schoepp et al. 1990). The group I antagonist had no significant effect on either the S1 or the S2 components of the fast wave dorsal horn field potential at concentrations of up to 100μM. This is shown in Figs. 5.7, 5.8 and 5.9.

The slow wave field potential was significantly inhibited by L-AP3 at concentrations of 10μM and 100μM. At 10μM the slow wave amplitude was decreased to 89.0±2.7% ($p<0.001$ paired t-test) of the control response and at 100μM it was further reduced to 76.1±4.5% ($p<0.05$ paired t-test) of the control (n=4). This result is illustrated in Figs. 5.10 and 5.11.

5.2.3 Effect of (RS)-3,5-DHPG on the slow wave dorsal horn field potential.

(RS)-3,5-DHPG is a selective group I mGluR agonist which activates both the mGluR1 and mGluR5 subtypes (Ito et al. 1992). At a concentration of 1μM, DHPG significantly increased the amplitude of the slow wave field potential to 119.4±4.7% of the control response ($p<0.05$) (n=4 paired t-test). Concentrations of 0.1μM, 10μM and 100μM had no significant effect. This is shown in Figs. 5.12 and 5.13.

5.2.4 Effect of EGLU on dorsal horn field potentials.

EGLU is a highly selective group II mGluR antagonist (Thomas et al. 1996). EGLU did not have any significant effects on the fast wave field potentials. The S1 and S2

components were not significantly affected by the group II antagonist (Figs 5.14 , 5.15 and 5.16).

The slow wave field potential was significantly inhibited by EGLU. At 1μM the slow wave field potential was reduced to 83.5±6.0% of the control response ($p<0.05$ paired t-test), at 10μM it was reduced to 81.5±7.5% of the control response ($p<0.05$ paired t-test) and at 100μM the response was reduced to 80.5±9.6% of control (n=4). These results are shown in Figs. 5.17 and 5.18.

5.2.5 Effect of L-AP4 on dorsal horn field potentials.

L-AP4 is a selective group III metabotropic glutamate receptor agonist (Nakanishi 1992). L-AP4 had a significant effect on the fast wave but not the slow wave dorsal horn field potential.

At 10μM and 100μM the S2 component of the fast wave field potential was significantly inhibited (n=4). At 10μM the response was decreased to 57.2±1.2% of the control ($p<0.05$ paired t-test) and at 100μM it was decreased to 37.9±11.1% of the control (n=4). After L-AP4 was washed out of the bath the response remained significantly low and was recorded as 33.3±3.0% of the control (Figs. 5.19 and 5.21). The S1 component of the fast wave field potential was unaffected by L-AP4 (n=4) (Figs. 5.19 and 5.20).

L-AP4 had no significant effect on the slow wave dorsal horn field potential (n=4) (Figs. 5.22 and 5.23).

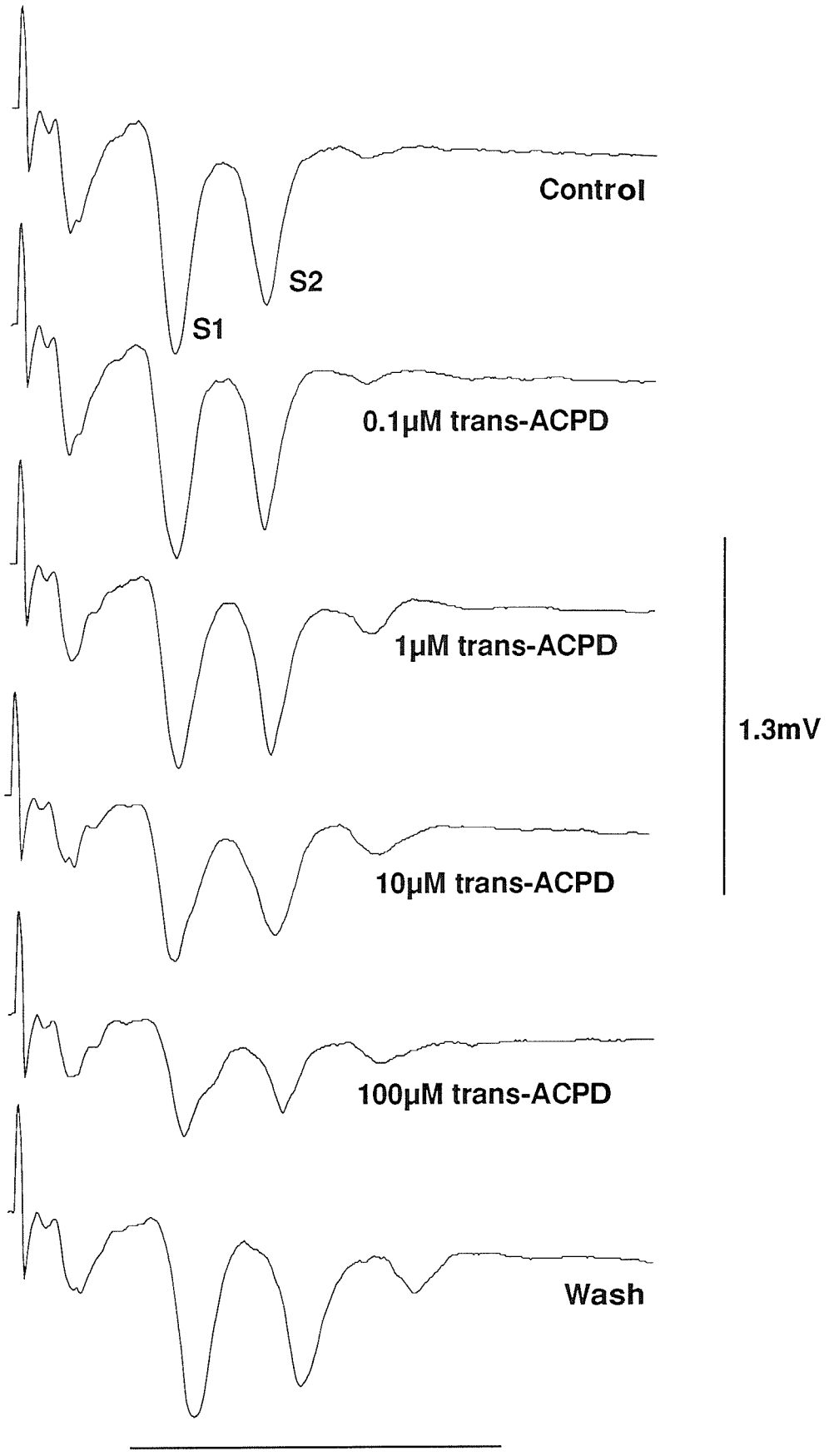


Fig. 5.2: The S2 component of the fast wave dorsal horn field potential was significantly inhibited by 10µM and 100µM *trans*-ACPD.

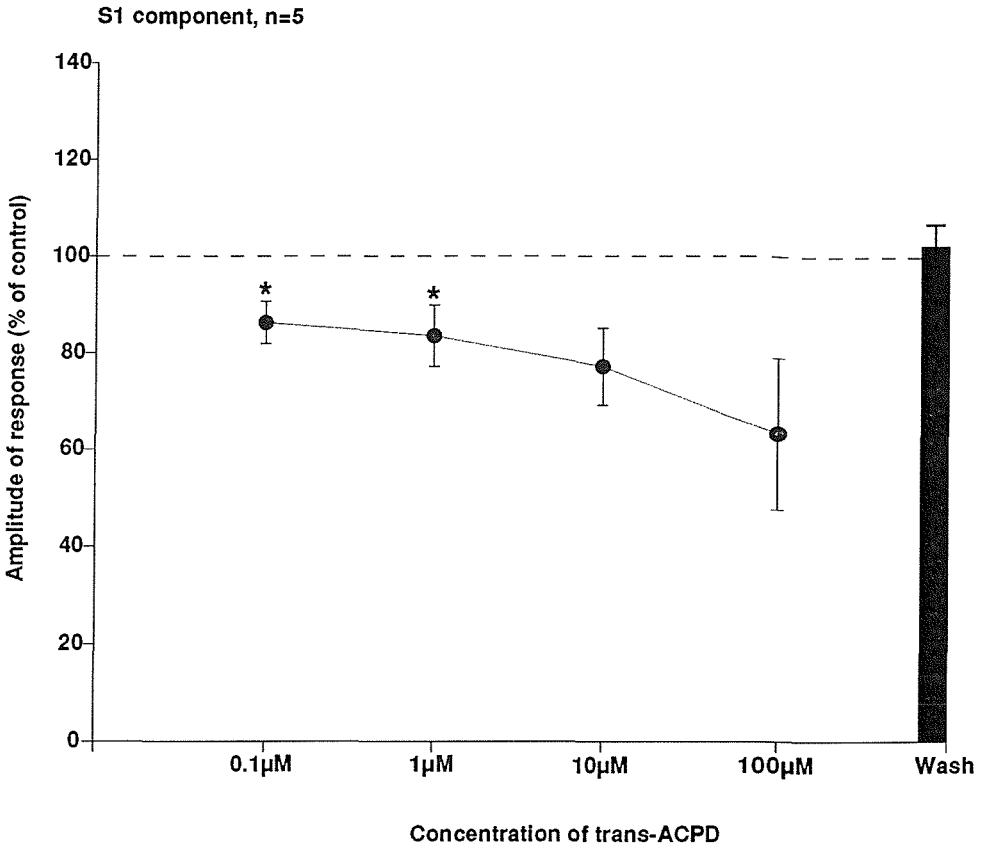


Fig. 5.3: The graph shows that *trans*-ACPD significantly inhibits the S1 component of fast wave field potentials at low concentrations (n=5). Significance values ($p<0.05$ = *) were calculated using the paired t-test.

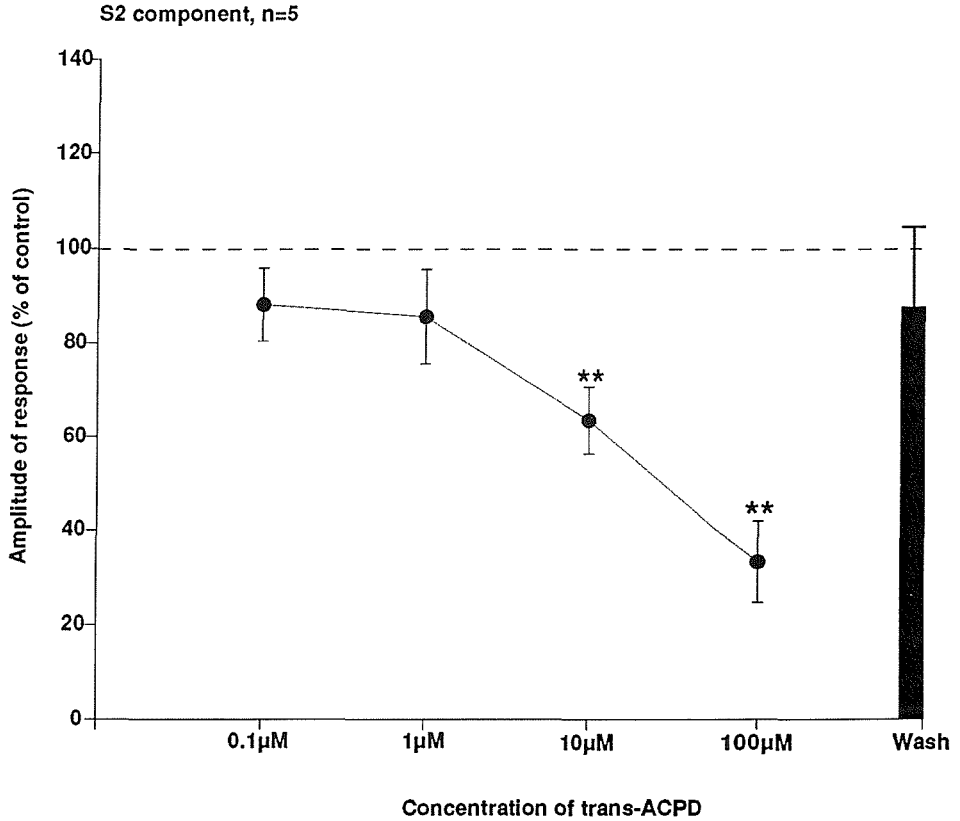


Fig. 5.4: This graph illustrates that *trans*-ACPD significantly reduces the S2 component of fast wave dorsal horn field potentials at high concentrations (n=5). Significance values ($p<0.01$ = **) were calculated using the paired t-test.

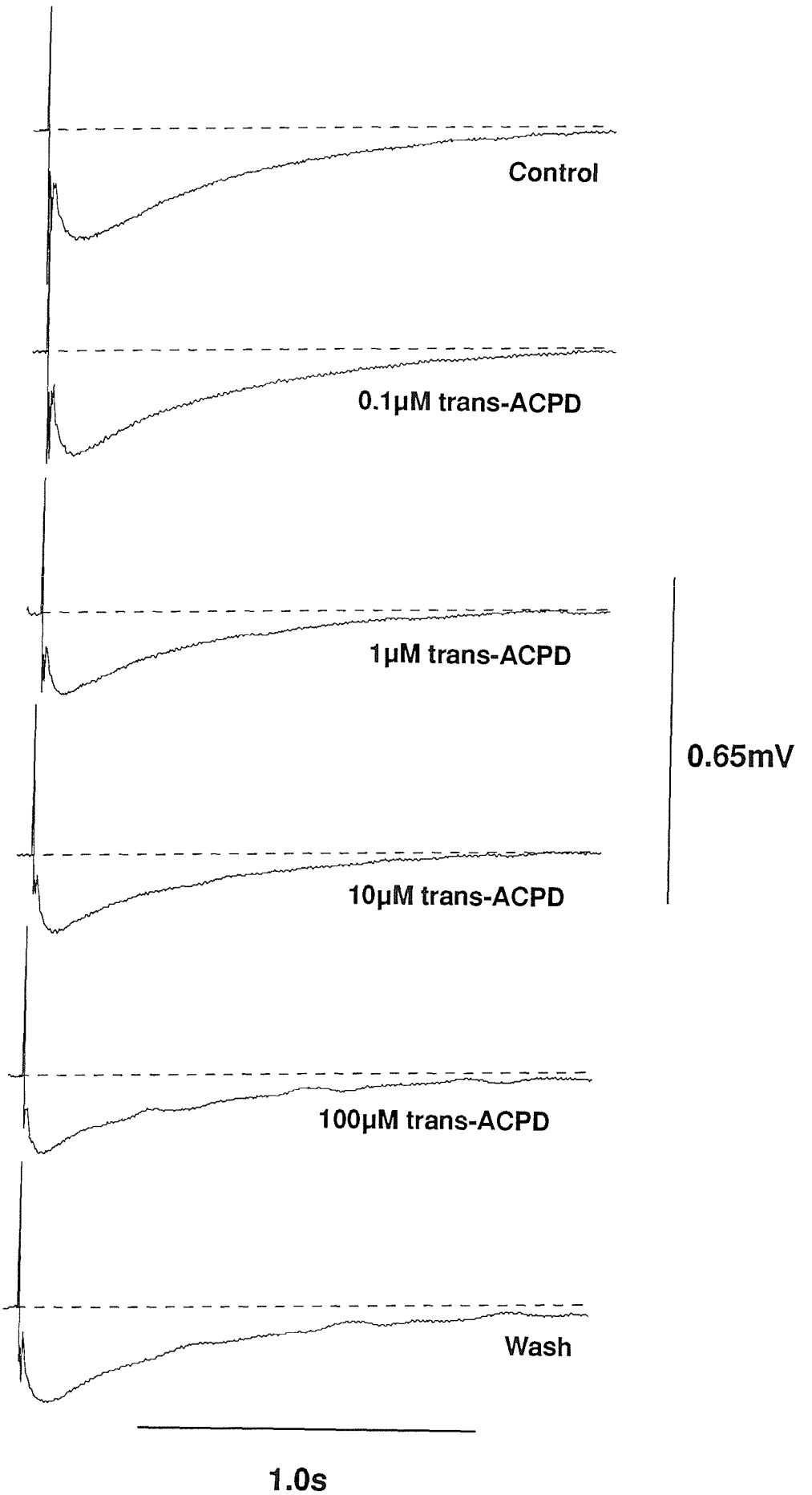


Fig. 5.5: An example of raw data showing trans-ACPD reduced the area of the slow wave field potential.

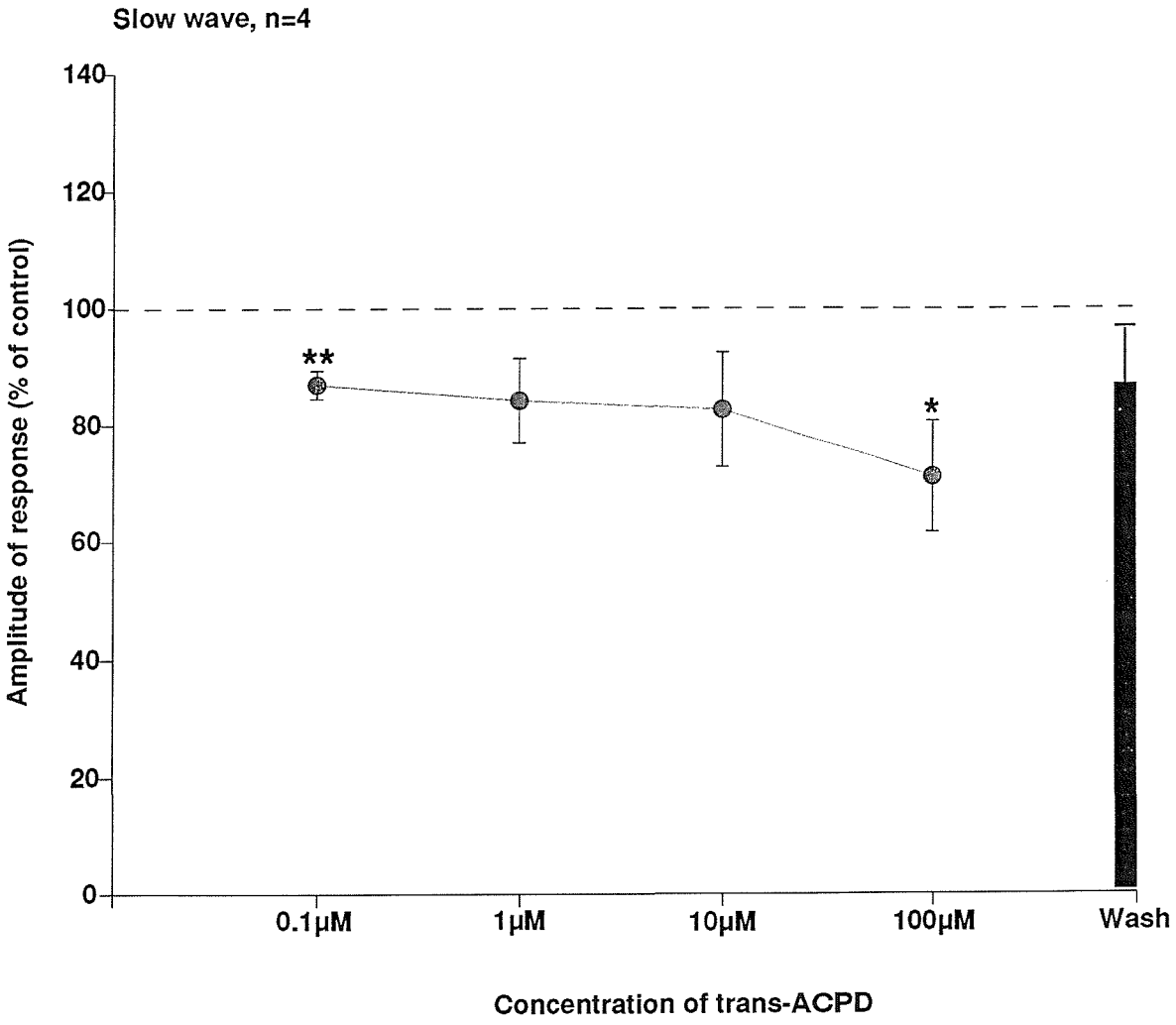


Fig. 5.6: This graph shows that the group I and group II mGluR agonist *trans*-ACPD significantly reduces the area of the slow wave at concentrations of 0.1µM and 100µM (n=4). Significance values ($p<0.05$ = *, $p<0.01$ = **) were calculated using the paired t-test.

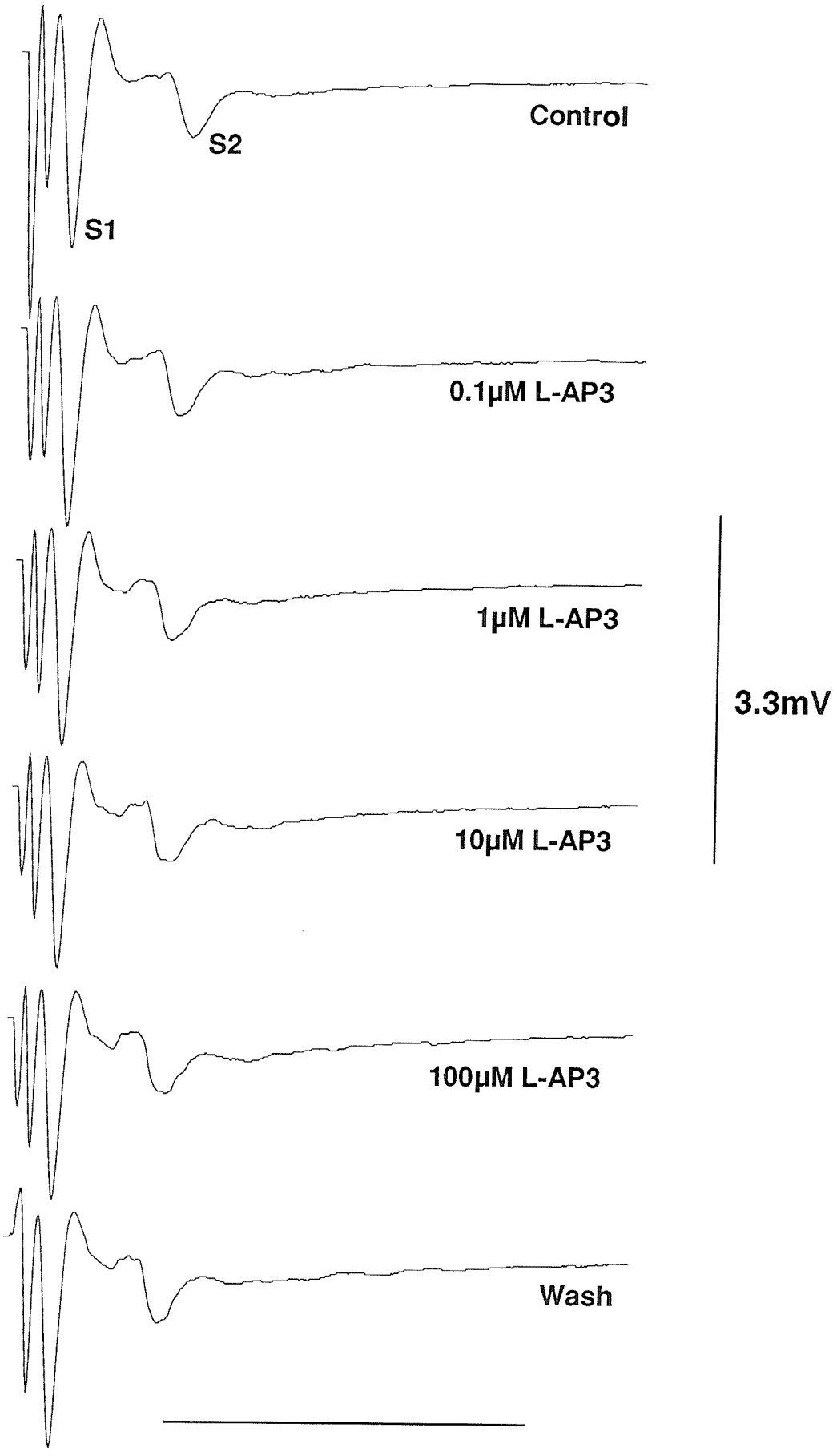


Fig. 5.7: Raw data showing that L-AP3 had no significant effect on the fast wave dorsal horn field potential.

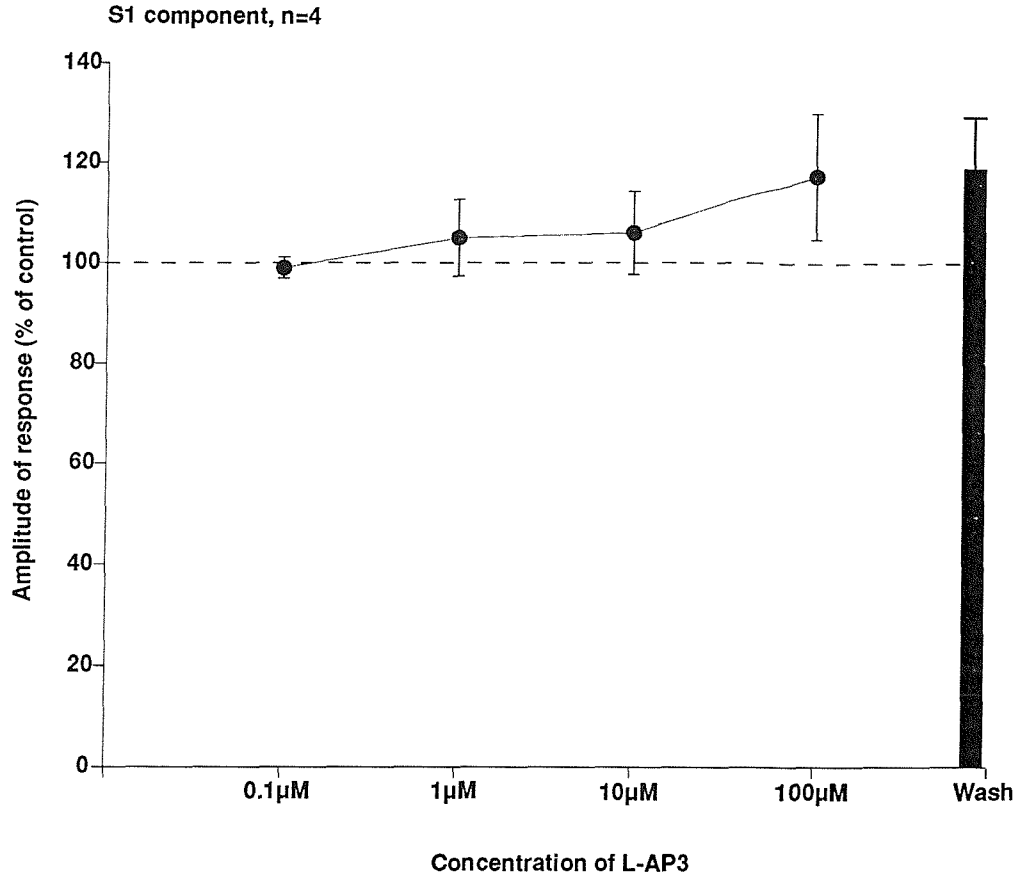


Fig. 5.8: The graph shows that L-AP3 had no significant effect on the amplitude of the fast wave field potential (n=4).

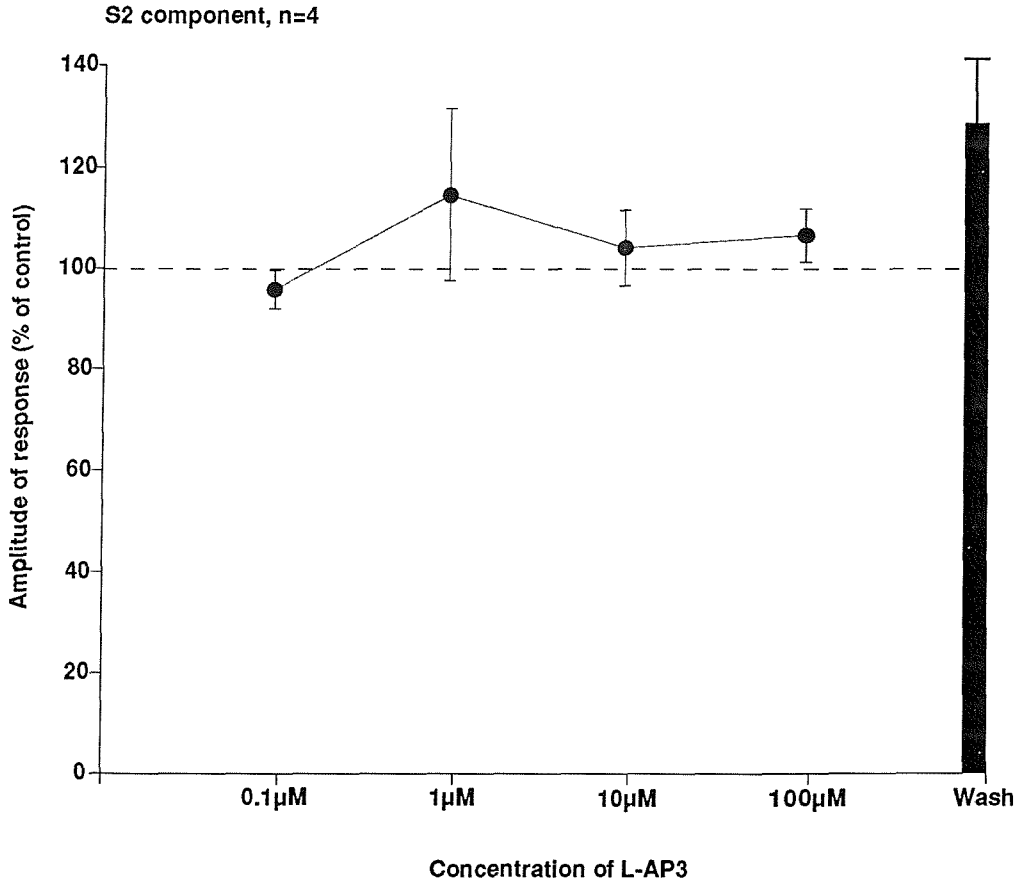


Fig. 5.9: This graph ilustrates that L-AP3 had no significant effect on the S2 component of dorsal horn field potentials (n=4).

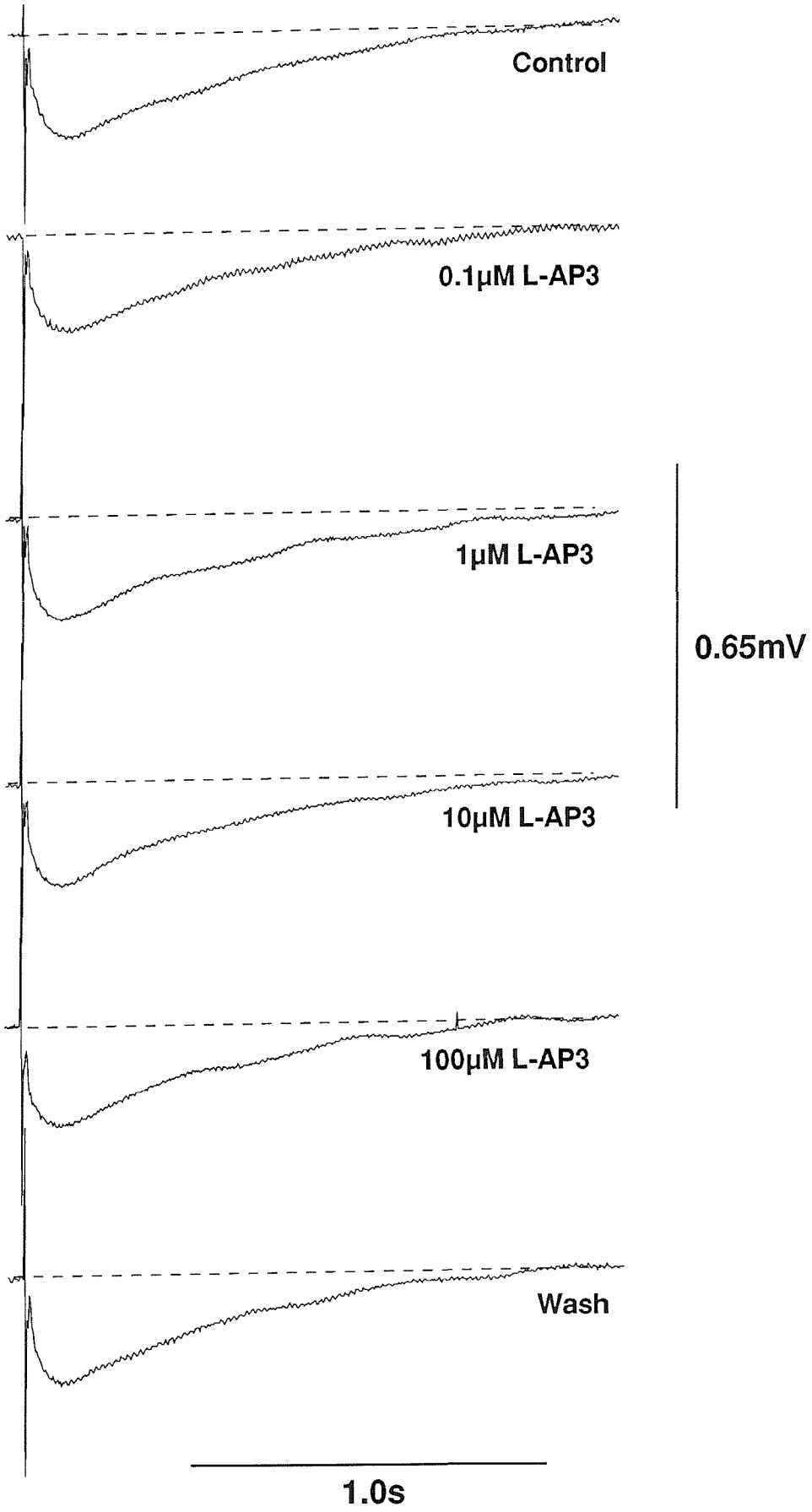


Fig. 5.10: Raw data illustating that L-AP3 significantly reduced the area of the slow wave field potential at high concentrations.

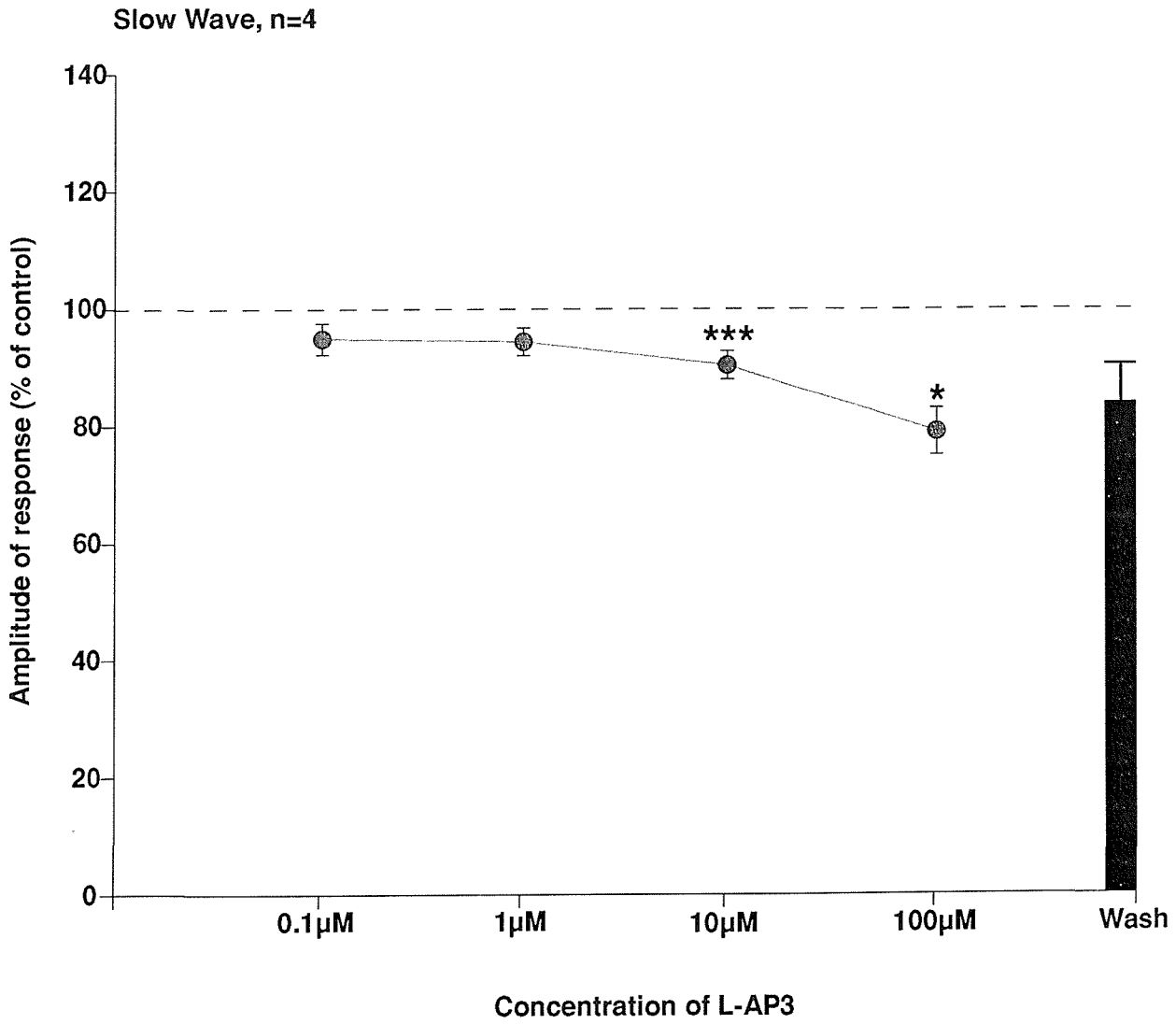


Fig. 5.11: The graph illustrates that L-AP3 significantly reduced the area of the slow wave at high concentrations (n=4). Significance values ($p<0.05$ = *, $p<0.001$ = ***) were calculated using the paired t-test.

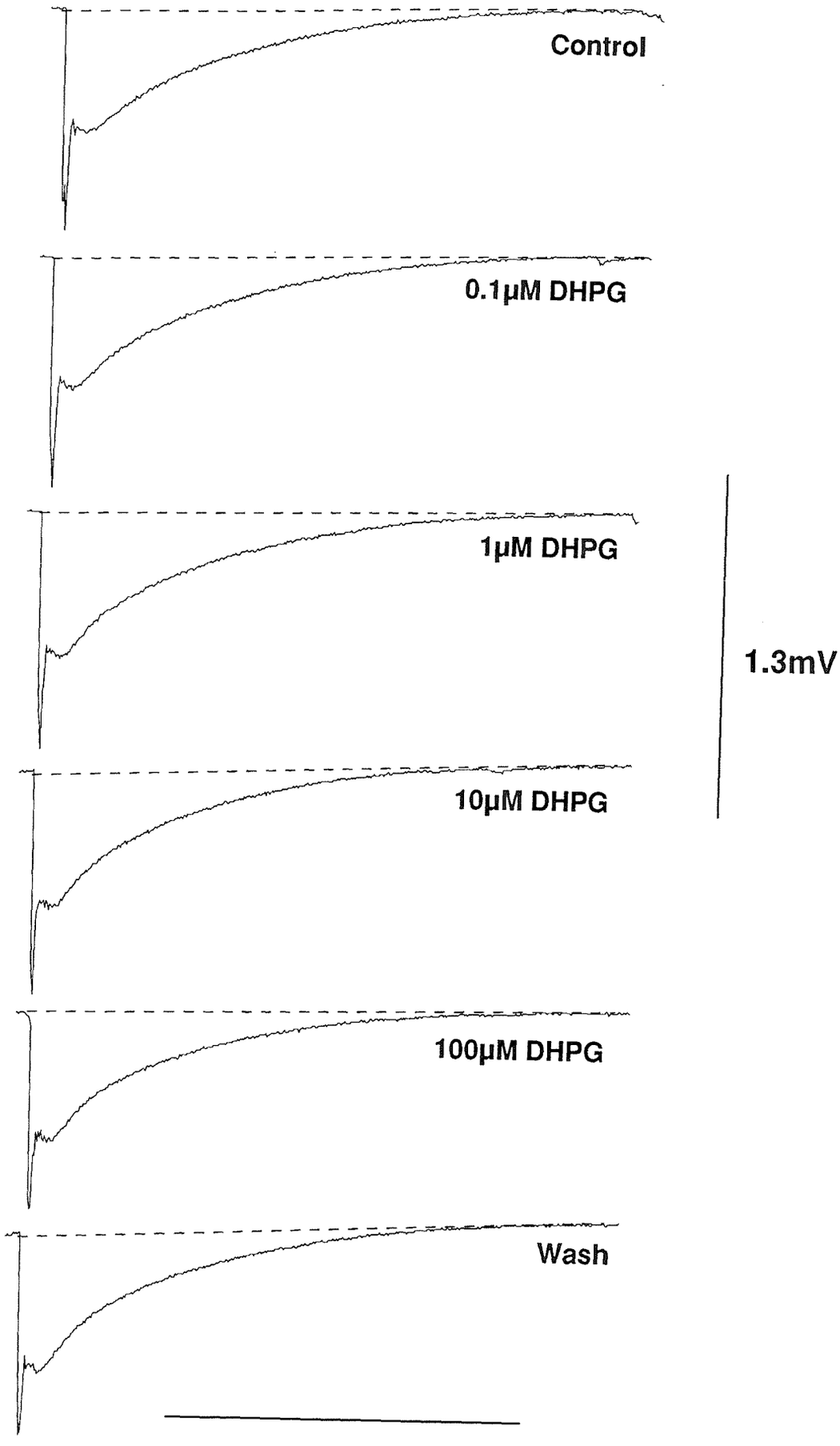


Fig. 5.12: Raw data to illustrate that DHPG facilitated the slow wave field potential at low concentrations.

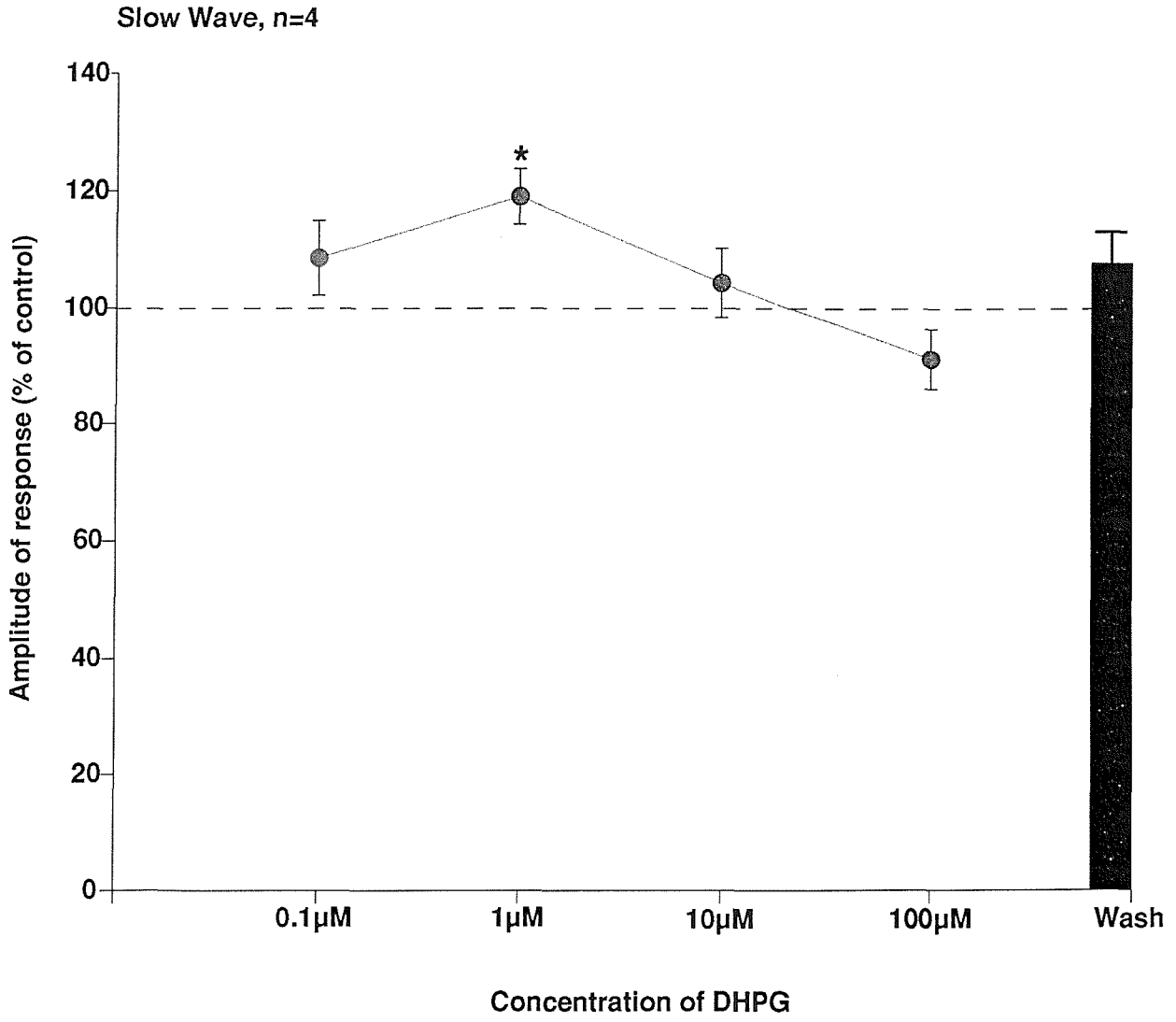


Fig. 5.13: The graph illustrates that the slow wave field potential was significantly increased at a concentration of 1µM DHPG (n=4). Significance values ($p<0.05$ = *) were calculated using the paired t-test.

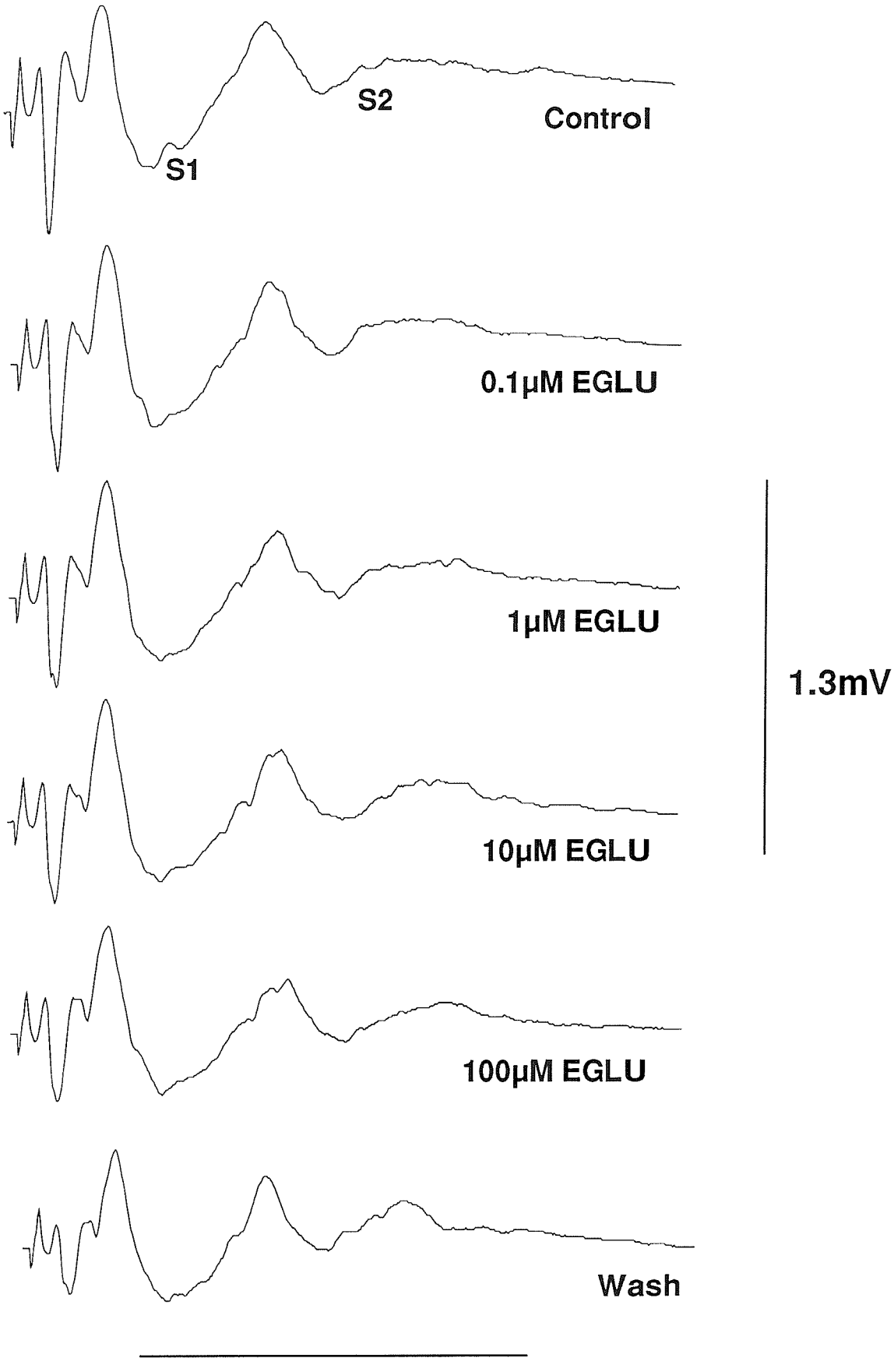


Fig. 5.14: Raw data to show the effect of the group II mGluR antagonist EGLU on the fast wave field potential. EGLU had no significant effect on the fast wave field potentials.

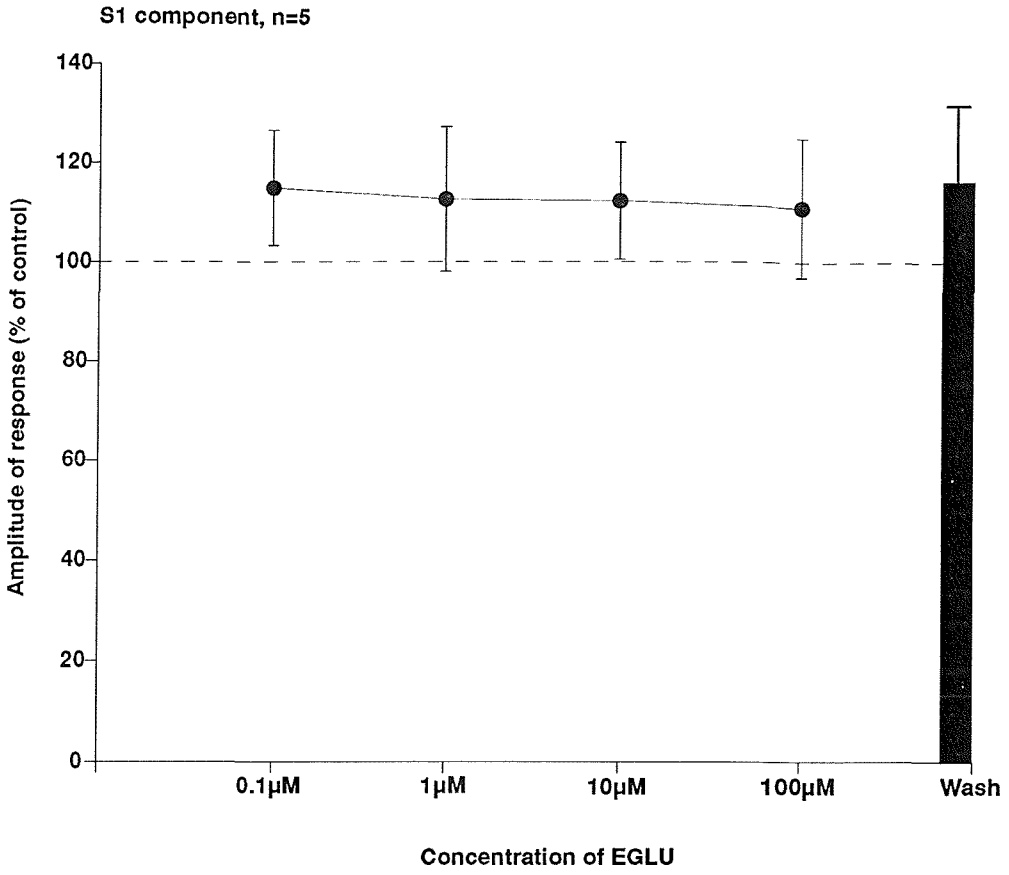


Fig. 5.15: The group II mGluR antagonist did not significantly increase the S1 fast wave field potential component (n=5).

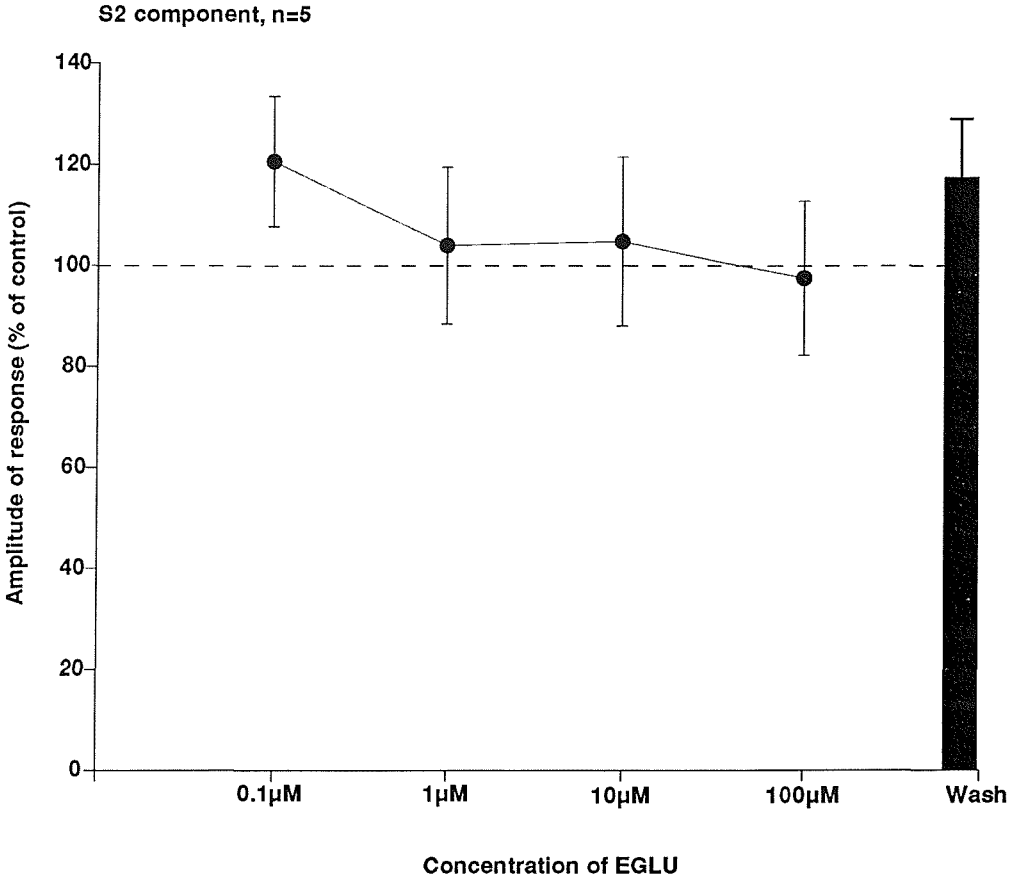


Fig. 5.16: EGLU had no significant effect on the S2 component of the fast wave field potential (n=5).

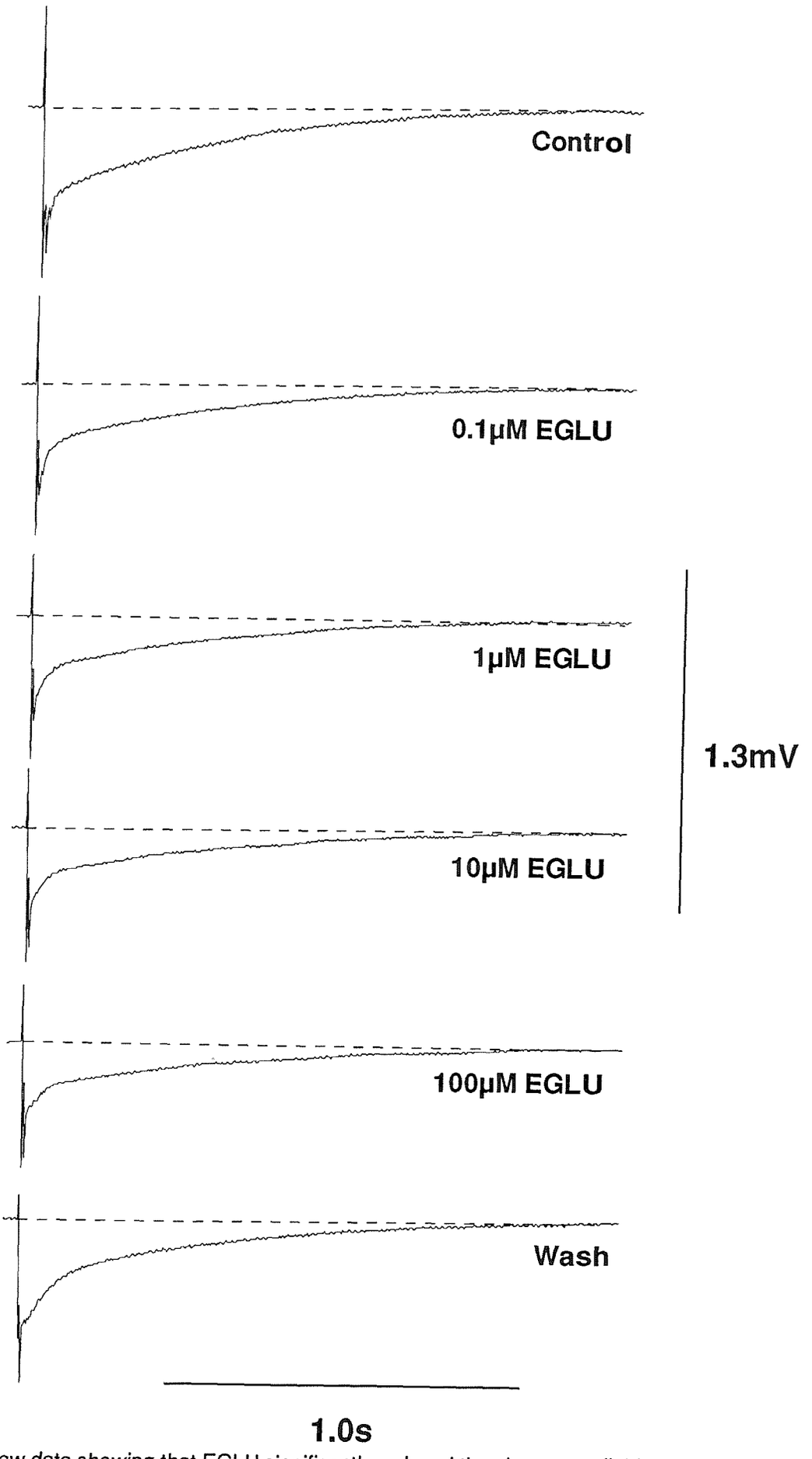


Fig. 5.17: Raw data showing that EGLU significantly reduced the slow wave field potential.

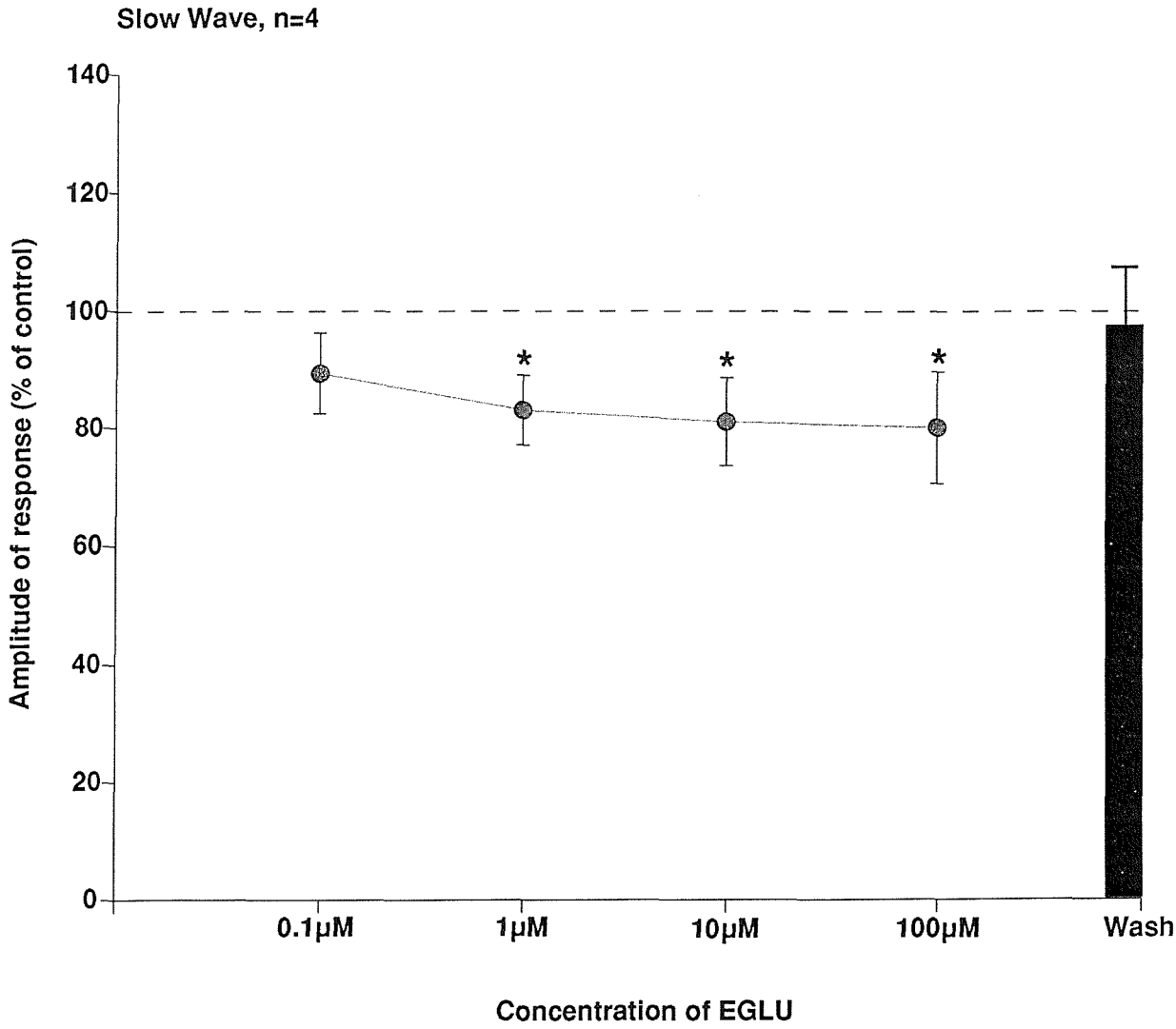


Fig. 5.18: EGLU significantly inhibited the slow wave field potential at concentrations of 1µM, 10µM and 100µM (n=4). Significance values ($p<0.05$ = *) were calculated using the paired t-test.

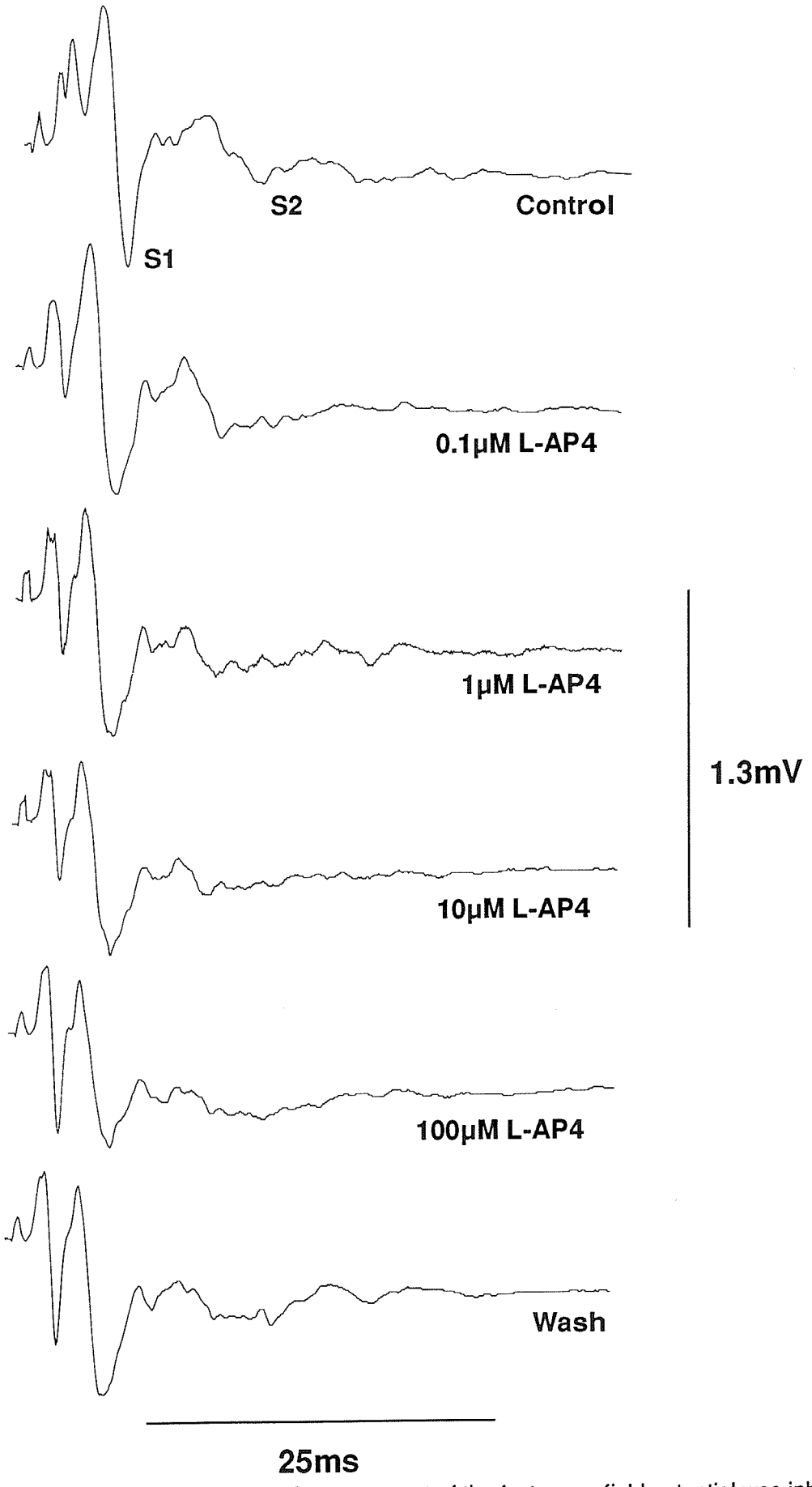


Fig. 5.19: Raw data to show that only the S2 component of the fast wave field potential was inhibited by the mGluR group III agonist L-AP4.

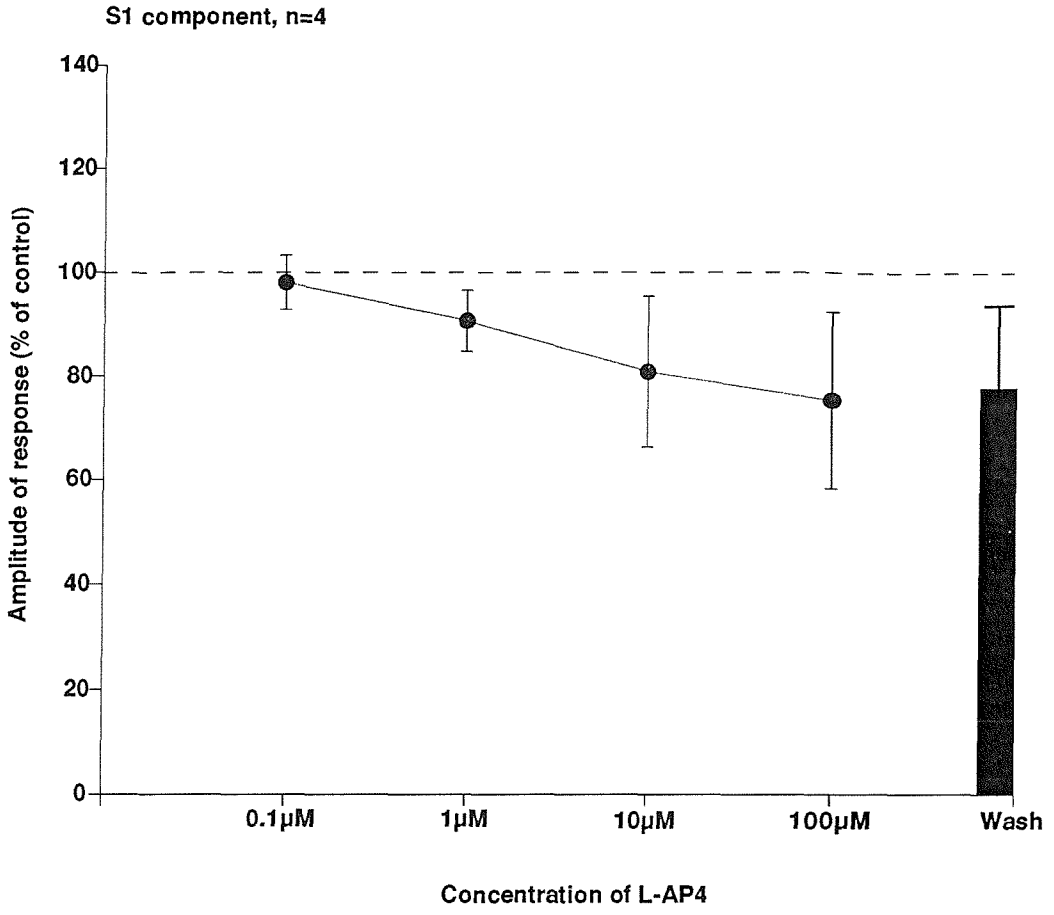


Fig. 5.20: L-AP4 had no significant effect on the fast wave S1 component (n=4).

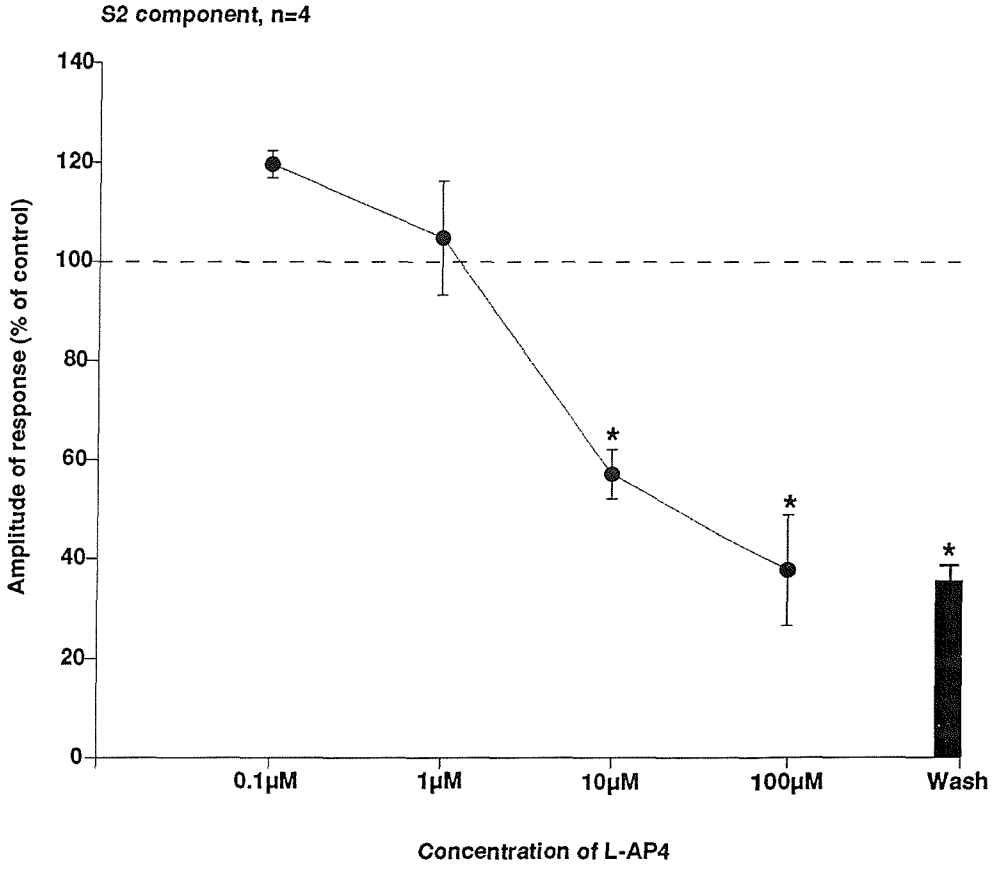


Fig. 5.21: the S2 component of the fast wave field potential was significantly inhibited by the group III mGluR agonist L-AP4 (n=4).

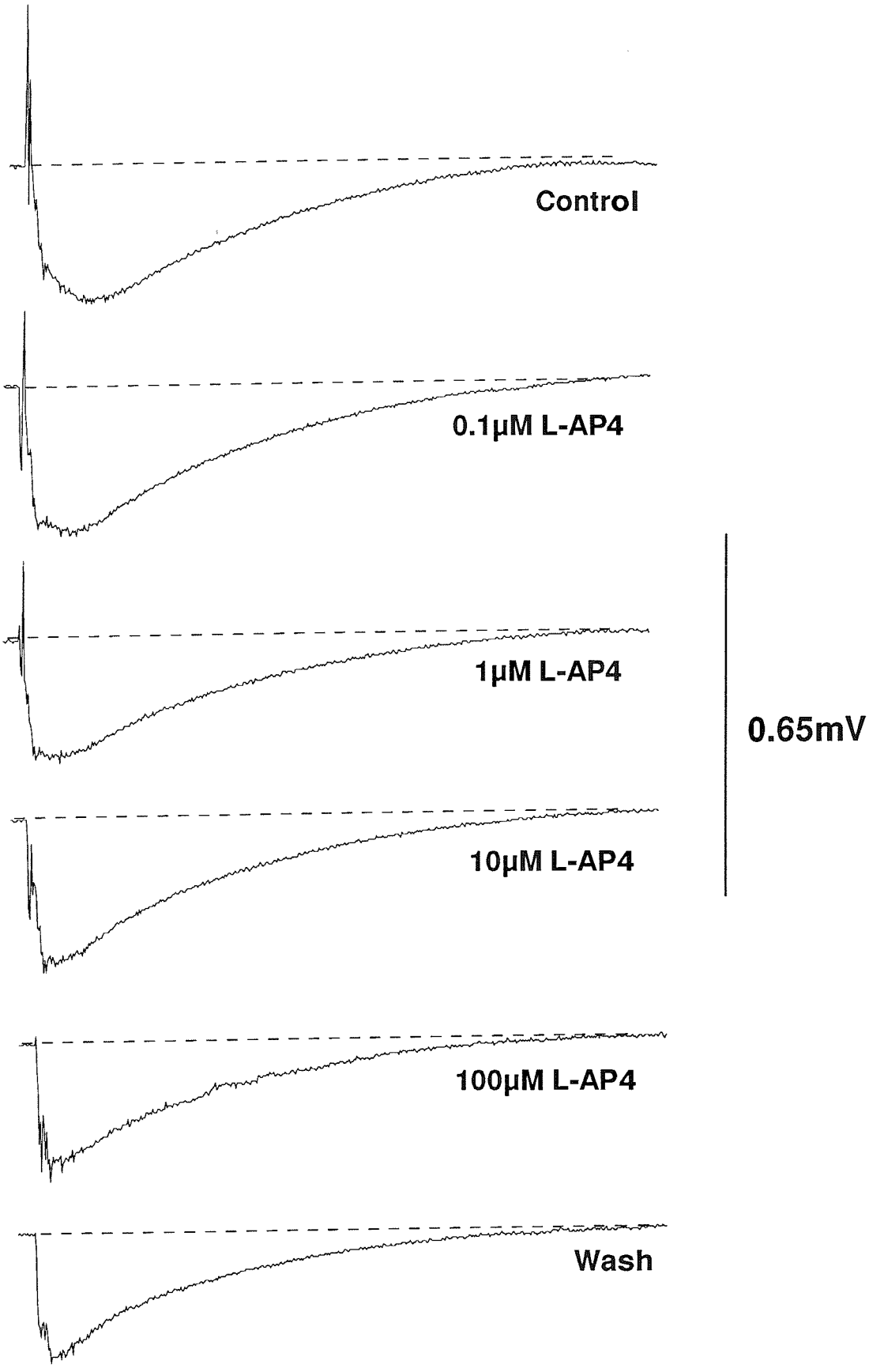


Fig. 5.22: raw data to show that L-AP4 had no significant effect on the slow wave field potential.

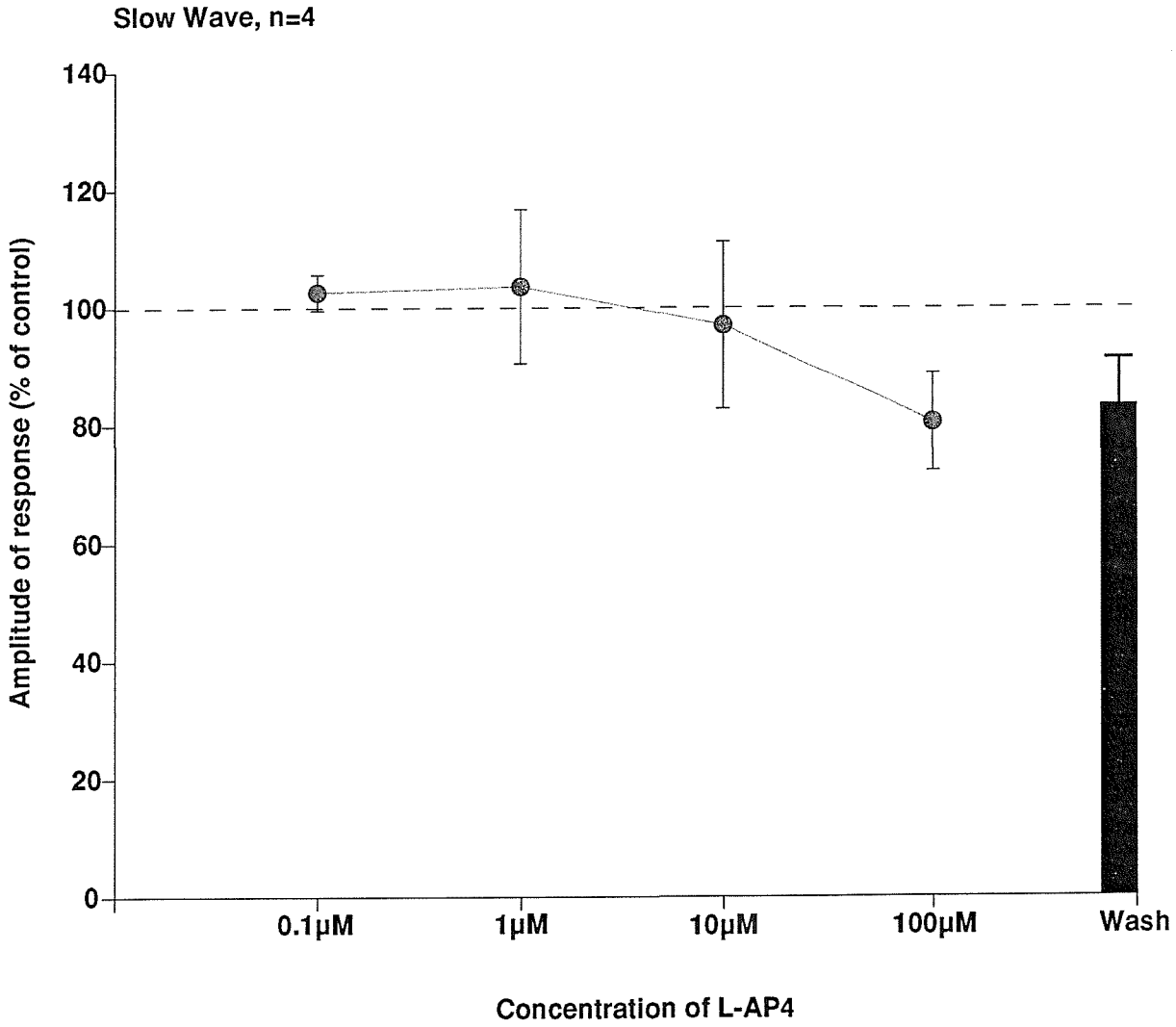


Fig. 5.23: This graph illustrates that the slow wave was not significantly changed by the addition of L-AP4 to the medium (n=4).

5.2.6 Summary of Results

	trans-ACPD (grp I/II antag.)		L-AP3 (grp I antag.)		DHPG (grp I agonist)		EGLU (grp II antag.)		L-AP4 (grp III agonist)	
	<100µM	100µM	<100µM	100µM	<100µM	100µM	<100µM	100µM	<100µM	100µM
S1	↓	ns	ns	ns	–	–	ns	ns	ns	ns
S2	↓	↓	ns	ns	–	–	ns	ns	↓	↓
Slow	↓	↓	↓	↓	↑*	ns	↓	↓	ns	ns

*** at a concentration of 1µM only**

Fig. 5.24: Table to summarise results of the effect of metabotropic glutamate receptor agonists and antagonists on dorsal horn field potentials.

5.3 Discussion:

The results of this investigation indicate that the different groups of mGluRs might have different roles in the transmission and processing of sensory information in the spinal cord.

Stimulation of a dorsal root at 5 times the voltage necessary to evoke a dorsal root reflex in an adjacent dorsal root has been shown in chapter 3 to activate fibres with conduction velocities expected of Aβ fibres. The results of the following experiments using mGluR ligands indicated that the mGluRs are involved in mediating the response elicited by the activation of Aβ fibres in the dorsal horn.

When a dorsal root was stimulated at 5 times the DRR threshold voltage, the mGluR group I and II agonist *trans*-ACPD significantly inhibited the S1 and S2 components of the fast wave dorsal horn field potential. As *trans*-ACPD is an agonist of both group I and group II mGluRs, the results suggest the possibility of group I and/or group II mGluR involvement. Either of these receptor groups may act as presynaptic autoreceptors to inhibit further glutamate release. This hypothesis is illustrated in Figure 5.25. However, this hypothesis is uncertain since neither the group I antagonist L-AP3 nor the group II antagonist EGLU had any significant effect on the fast wave field potential response following stimulation of a dorsal root at 5 times the voltage required to elicit a DRR in an adjacent root. Selective agonists of group II receptors have previously been shown to inhibit spinal cord responses and are therefore thought to be located presynaptically in the spinal cord (Jane et al. 1994; Kemp et al. 1994). Hence, the use of a group II antagonist may not reveal the actions of this receptor type as the concentration of glutamate at the synapse would have to be high before an antagonist would reveal the effects of presynaptic receptor inhibition. A group II agonist such as (2S, 3S, 4S)-CCG/(2S, 1'S, 2'S)-2-(Carboxycyclopropyl) glycine (L-CCG-I) may, therefore, be more useful in determining any involvement of group II mGluRs in the inhibition of the S1 and S2 components of the fast wave field potential. However, it was not possible to use a group II mGluR agonist in this study because of financial constraints.

Recent studies have also confirmed the presence of presynaptic group I mGluRs in the CNS (Thomas et al. 1998). However, in the spinal cord dorsal horn the group I mGluR receptor mGluR5 has been shown to be located post-synaptically (Vidnyanszky et al. 1994). Using ligands selective for mGluR1 and also mGluR5, it has been shown that the group I receptor subtype mGluR1 is not involved in mediating Aβ fibre input to dorsal horn neurones (Young et al. 1997). It is therefore likely that any effect being mediated by presynaptic autoreceptors is due to the action of mGluR5 or group II mGluRs.

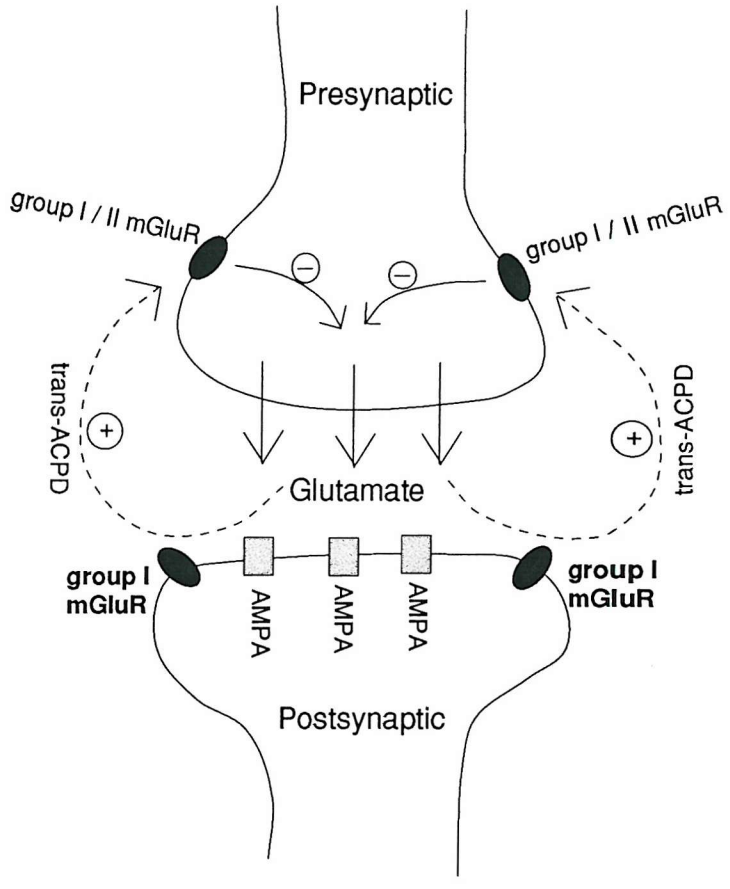


Fig. 5.25: The existence of presynaptic group I or group II mGlu receptors on Aβ fibres may explain why the group I and II mGluR agonist *trans*-ACPD decreased the amplitude of fast wave field potentials.

An alternative hypothesis is that there may have been a receptor interaction between the group I and II mGluRs and the net effect of agonising both group I and group II mGluRs by *trans*-ACPD was more than the additive effect of using a selective agonist for either receptor group alone would be. The inhibition of the S1 and S2 components would therefore not have been observed by the addition of a group I or a group II antagonist alone. Synergistic effects of group I and group II receptor agonists on the activation of

second messenger systems have previously been described in the rat hippocampus (Schoepp et al. 1996), and in the neonatal rat spinal cord, Lodge et al. (1996) have shown a synergistic action between group I and group II mGluRs. Lodge et al. (1996) showed that potentiation of ionotropic glutamate responses were enhanced by the addition of both a group I and a group II mGluR agonist. To prove this hypothesis, the amplitude of the fast wave S1 and S2 components after the separate addition of a group I mGluR agonist, a group II mGluR agonist, and both the mGluR group I agonist and the group II agonist together, would need to be compared. If the response to the addition of both agonists was greater than either agonist alone, it could be concluded that a receptor interaction was occurring.

Another possibility is that the fast wave field potentials are inhibited by group I/II mGluRs located on inhibitory spinal cord interneurones. Activation of these interneurones would lead to an inhibition of the subsequent dorsal horn neurones and hence the fast wave field potential would be inhibited. This is shown in Figure 5.26. Evidence suggesting that the activation of mGluRs facilitates the inhibitory effects of GABA and glycine in the spinal cord *in vivo* (Baker et al. 1993; Bond and Lodge 1995) supports this hypothesis. Inhibition could therefore dominate excitation in some neurones leading to an inhibition of fast wave dorsal horn field potentials. An inhibition of electrically evoked Aβ-fibre neuronal responses in the dorsal horn of normal animals by the application of the mGluR group I and group II agonist (1*S*, 3*R*)-ACPD (50μg) has been previously seen in vivo (Stanfa and Dickenson 1998). The inhibition of electrically evoked non-noxious responses has not before been seen in *in vitro* spinal cord preparations. This may be due to the age of the animals used for most *in vitro* spinal cord experiments which range from 5-16 days. These animals are anatomically and physiologically immature and do not have the sophisticated pain circuits which are thought to have matured by 21 days of age in the rat (Fitzgerald and Jennings 1999). This study used rats that were 21-23 days old and allowed the investigation of more advanced pain circuitry within the spinal cord.

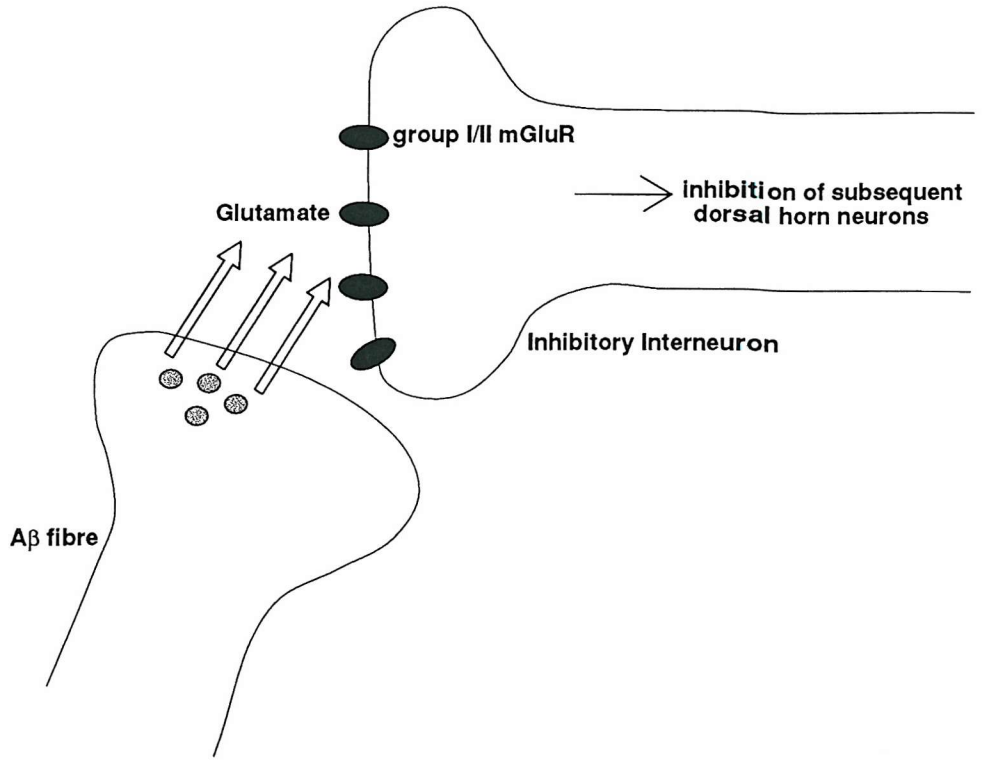


Fig. 5.26: Group I/II mGluR receptors may be located on inhibitory interneurones such that activation by glutamate will cause an inhibition of the subsequent dorsal horn neurones.

The group III mGluR agonist L-AP4 did not significantly affect the S1 component of the fast wave field potential (n=4) but did significantly inhibit the S2 component at concentrations of 10μM and 100μM ($p<0.05$ paired t-test, n=4). Group III mGluRs consist of the mGluR4, mGluR6, mGluR7 and mGluR8 receptor subtypes. The presence of mGluR8 in the spinal cord has not yet been investigated and it is known that mGluR6 is exclusively located in retina cells (Nakajima et al. 1993). In the spinal cord, mGluR7 receptors have been found to be located on primary afferent fibres in the dorsal horn (Ohishi et al. 1995) and have been suggested to act as presynaptic autoreceptors. It has not yet been possible to determine if mGluR4 receptors are located pre- or postsynaptically although it is thought they have a postsynaptic location in the DRG (Boxall et al. 1998). Group III mGluRs have been found to be located presynaptically in the spinal cord (Jane et al. 1994; Kemp et al. 1994). It is therefore possible that the reduction of the fast wave S2 component is mediated by the mGluR7 subtype acting as presynaptic autoreceptors (Fig. 5.27). However, more selective pharmacological tools which differentiate between the different receptor subtypes would be required to investigate this hypothesis. Although L-AP4 modestly reduced the amplitude of the S1 component response it significantly inhibited the S2 component of the fast wave field

potential. The S2 component may therefore represent a second synapse, possibly that of an interneuron. If a greater density of group III receptors is located on the second synapse in this pathway, it follows that the addition of L-AP4 to the medium will inhibit this second synapse to a greater degree. Alternatively, If L-AP4, acting at the S1 synapse, blocked further presynaptic release of glutamate by inhibiting the group III autoreceptors, this would cause a greater subsequent reduction of the S2 component due to (i) the decreased postsynaptic depolarisation via the S1 synapse, and (ii) the action of L-AP4 on the S2 presynaptic group III autoreceptors.

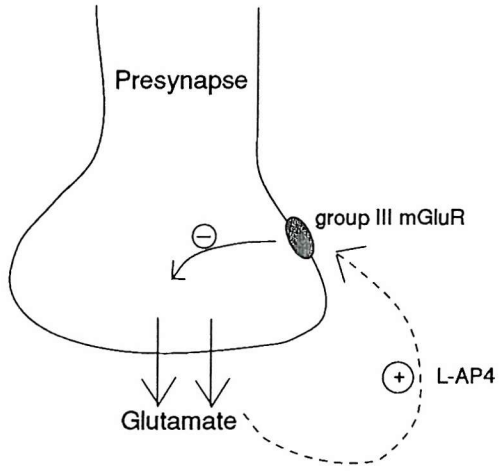


Fig. 5.27: Diagram to show the presynaptic action of group III mGluRs in the dorsal horn of the spinal cord. The group III mGluR agonist increases the inhibition of glutamate release and hence reduces the fast wave field potential.

Stimulation of a dorsal root at 20 times the voltage required to evoke a DRR in an adjacent root is known to activate fibres with a conduction velocity corresponding to that of high threshold, unmyelinated C fibres (see chapter 3). Addition of *trans*-ACPD to the bathing medium significantly inhibited the slow wave dorsal horn field potential elicited by stimulating a dorsal root at 20 times the DRR threshold voltage at concentrations of 0.1µM and 100µM. Concentrations of 1µM and 10µM were reported to be 'not quite' significant using the Student's paired t-test. Using the one sample t-test, the effect of adding 1µM of *trans*-ACPD to the medium was not quite significant. However, the response following the addition of 10µM *trans*-ACPD was calculated to be significantly different from 100%. These results indicate the involvement of group I and/or group II mGluRs in mediating C fibre input to dorsal horn neurones. However, the results conflicted with other experiments in this study. The selective group I antagonist L-AP3 significantly reduced the slow wave at concentrations of 10µM and 100µM, while the selective group II

antagonist EGLU significantly inhibited the slow wave response at concentrations of 1μM, 10μM and 100μM. An effect similar to the inhibition of the slow wave field potential by trans-ACPD has previously been seen *in vivo.* A reduction in monosynaptic events by the mGluR receptor agonists (1S, 3R)-ACPD and L-CCG-1 has previously been observed in the spinal cord (Pook et al. 1992; Ishida et al. 1993; Shinozaki and Ishida 1993; Jane et al. 1994). A smaller reduction in long-latency presumed polysynaptic components of the extracellular ventral root reflex by (1S, 3R)-ACPD (active isomer of trans-ACPD) has also been reported (Ishida et al. 1993; Shinozaki and Ishida 1993; Jane et al. 1994). This attenuation of polysynaptic responses by group I mGluR agonists occurred despite offsetting the mGluR agonist-induced depolarisations. Therefore, it is not likely to be a postsynaptic mechanism that is depressing synaptic transmission. The depression of the slow wave field potential by *trans*-ACPD may not be a postsynaptic mechanism and might be elicited via another mechanism.

The selective and potent mGluR group I agonist DHPG significantly increased the slow wave field potential at a low concentration (0.1μM). At higher concentrations, no significant increase of the slow wave occured and this was possibly due to the depression of synaptic transmission that has been reported to occur after the addition of a group I mGluR agonist (King and Liu 1996). The inhibition of the slow wave by trans-ACPD may also be due to a general depolarising block reported by King and Liu (1996) after the addition of group I agonists. The slow wave facilitation by the group I agonist DHPG at low concentrations corresponded to the slow wave inhibition observed after the addition of the mGluR group I antagonist L-AP3. This data, along with that showing that the group II antagonist EGLU reduced the slow wave field potential, indicated the involvement of both group I and group II receptor types in the transmission of noxious C fibre impulses in the spinal cord. EGLU is a more potent drug than L-AP3 and had a significant effect on the slow wave field potential at a concentration of 1μM. L-AP3 had a significant effect on the slow wave at a concentration of 10μM. However, the addition of L-AP3 to the medium reduced the slow wave to 76.10±4.55% of the control whereas the addition of EGLU reduced the slow wave to only 80.55±9.60% of the control. These results suggest that group I mGluRs have a predominant involvement in the transmission and/or processing of noxious information in the spinal cord. This predominant involvement of group I mGluRs has previously been noted in *in vivo* experiments (Young et al. 1997; Neugebauer et al.

1994; Fisher and Coderre 1996a). In contrast to L-AP3 and EGLU, the group III mGluR agonist L-AP4 had no effect on the slow wave field potential and hence was concluded to have no role in the synaptic transmission of high threshold C fibres.

Previous studies have suggested that it is the group I mGluRs which are more important in the transmission of nociceptive information (Baker et al. 1993; Bond and Lodge 1995; Cerne and Randic 1992; Fisher and Coderre 1996b; Meller et al. 1993; Stanfa and Dickenson 1998; Young et al. 1995; Young et al. 1997). The importance of group I mGluRs in processing somatosensory information is supported by the location of these receptors in the spinal cord. Several studies have shown that group I mGluR protein and mRNA is localised in the superficial dorsal horn (Berthele et al. 1999; Anneser et al. 1995; Boxall et al. 1998; Vidnyanszky et al. 1994). Group II mGluRs, although important, play a less significant role in nociception (Young et al. 1997; Neugebauer et al. 1994; Fisher and Coderre 1996a). Correspondingly, group II mGluRs have less prominence in the spinal cord. The mRNA of mGluR2 has been reported to be absent in the spinal cord of rats aged 21 days which may be due to low levels of expression (Berthele et al. 1999). The other group II receptor, mGluR3, shows expression throughout the dorsal horn (Berthele et al. 1999; Ohishi et al. 1993). However, mGluR3 is upregluated after UV induced peripheral inflammation (Boxall et al. 1998) indicating a role for these receptors in the modulation of sensory transmission in the spinal cord after the onset of a chronic pain state.

The activation of group I and II mGluRs in spinal neurones has previously been shown to be involved in activity dependent spinal hyperexcitability during wind-up in vitro (Boxall et al. 1996). However, the exact mechanism and the involvement of specific mGluRs is not known at present. Application of ionotropic glutamate receptor agonists directly onto spinal neurones elicited pronounced and and rapid postsynaptic excitation as well as high frequency cell firing (King et al. 1992). In contrast to the effects of ionotropic agonists, mGluR agonists did not cause a significant increase in the firing rate of spinal neurones (King and Liu 1996). This suggests that the postsynaptic activation of mGluRs does not result in the transmission of impulses to subsequent spinal neurones within a polysynaptic pathway. Instead, the results suggest that mGluRs have a modulatory role and influence postsynaptic excitability and hence subsequent afferent inputs. Other evidence suggests

that mGluR activation enhances the response of ionotropic glutamate receptor activation (Bleakman et al. 1992).

PKC-mediated enhancement of the activation of NMDA receptors has been reported by several groups (Ben-Ari et al. 1992; Cheng and Huang 1992; Tingley et al. 1993). Behavioural studies using PKCγ knockout mice showed a concomitant reduction of the behavioural and anatomical response to nerve injury and a decrease in the second phase of the formalin response (Malmberg et al. 1997), indicating an involvement in chronic pain states. Furthermore, Fisher and Coderre (1996a; 1996b) found that the spontaneous nociceptive behaviour and facilitation of formalin responses elicited by mGluR1 agonists are inhibited by the NMDA receptor antagonist D-AP5. Thus it seems possible that mGlu group I receptors play a role in sustained noxious input into the dorsal horn of the spinal cord by inducing the phosphorylation and hence enhancement of the NMDA receptor response to glutamate.

The results of this study demonstrate that the actions of glutamate in the spinal cord acting at metabotropic glutamate receptors are complex. Experiments using this *in vitro* spinal cord preparation have shown results not previously seen in other similar preparations. Most in vitro preparations use spinal cords taken from neonatal rat pups. However, the spinal cord is not thought to be fully mature until 21 days of age (Fitzgerald and Jennings 1999). Group I, II and III metabotropic receptors are involved in processing non-noxious information as they mediate the transmission from low threshold Aβ fibres. The group III mGluRs are not involved in the transmission of noxious information via high threshold C fibres. Transmission of noxious information is mediated primarily by group I receptors although group II receptors also play a role. The mechanism of how mGluRs contribute to nociceptive transmission is unknown. However, one possible mechanism is that group I mGluRs activate PKCγ which in turn modulates NMDA receptors so that they are more susceptible to activation by glutamate. This is shown in Fig 5.28.

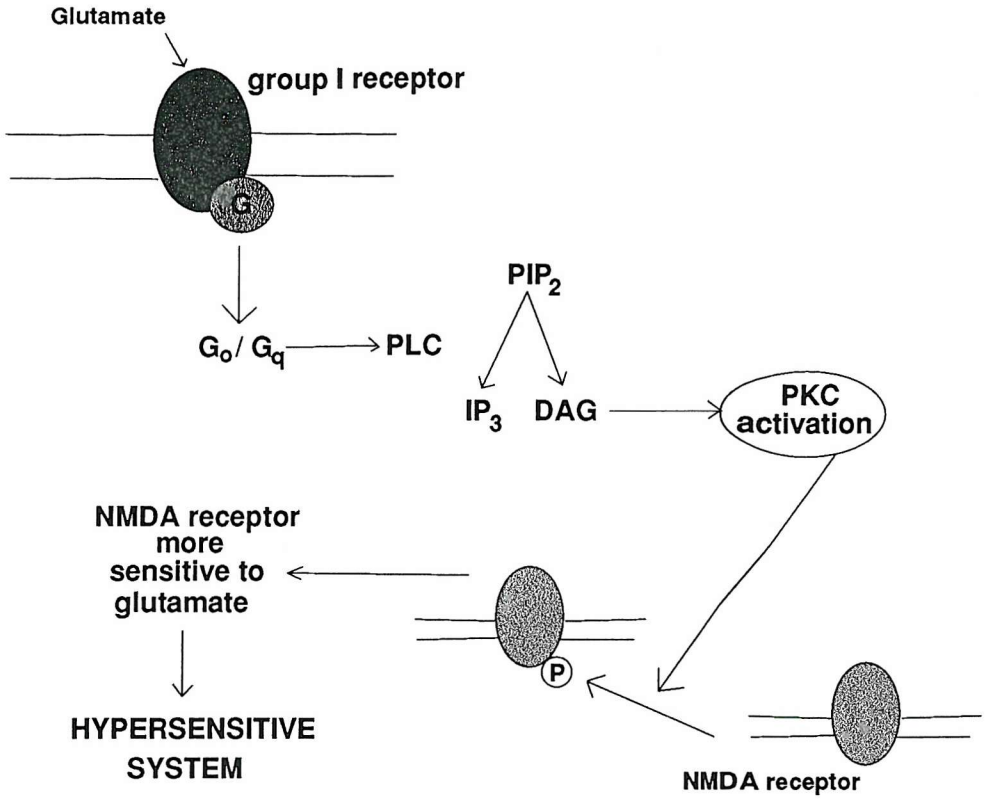


Fig. 5.28: When the group I mGluRs activate G proteins of the G_o/G_q family, the activated α subunit stimulates phospholipase C (PLC) which leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and the production of the calcium mobiliser molecule inositol-1,4,5-trisphosphate (IP_3) and the production of the protein kinase C (PKC) activator diacylglycerol (DAG). PKC phosphorylates the NMDA receptor and makes it more sensitive to glutamate. The whole system becomes hypersensitive and nociception is therefore increased.

Chapter 6

c-fos expression in the in vitro spinal cord

6.1 Introduction

Since the report by Hunt et al. in 1987, there have been many studies showing that various types of noxious stimulation, including thermal, mechanical and chemical stimuli induce the expression of the immediate early gene c-*fos* in the dorsal horn of the spinal cord. Each of these studies has confirmed that there is a relationship between nociception and c-*fos* expression, and c-*fos* expression has become a valuable experimental tool for investigating mechanisms of nociceptive processing in the spinal cord. However, research involving the expression of c-*fos* to investigate pain processing and to map pain pathways in the spinal cord has been mainly carried out in *in vivo* preparations. The aims of the following study are, therefore, to investigate the possibility of this technique being used in the *in vitro* spinal cord. Since the *in vitro* preparation has many advantages over *in vivo* preparations, the technique of using c-*fos* expression to investigate the pharmacology of nociceptive processing in the dorsal horn of the spinal cord could prove to be a powerful experimental tool.

Expression of c-fos in the *in vitro* spinal cord preparation has previously been demonstrated by Bagust's research group and some of the data from this and other investigations has been published (Zhang et al. 1998). The number of Fos-positive cells in the lumbar region of the dorsal horn was found to increase when the cords were incubated at 27°C for a period of 0.5 and 1 hour following high intensity stimulation. Expression of the gene reached a maximum at 2 hours. Staining intensity increased at longer incubation periods, however, the number of Fos-positive cells found in the dorsal horn did not significantly increase. When the incubation temperature was reduced from 27°C to 20°C the number of Fos-positive cells found in the dorsal horn did not significantly increase above unstimulated control experiments. An increase in temperature to 33°C did

not change the level of c-*fos* expression from that obtained at an incubation temperature of 27°C. Therefore, in the present study, cords were incubated for 2 hours at a temperature of 27°C following stimulation before being cut into 7μM transverse sections and processed for Fos immunoreactivity. The c-*fos* antibody used was a sheep polyclonal antibody to c-*fos*. It was raised against the synthetic peptide Met-Phe-Ser-Gly-Phe-Asn-Ala-Asp-Tyr-Glu-Ala-Ser-Ser-Ser-Arg-Cys selected from a conserved region of mouse and human c-fos. The c-fos antibody was purchased from Genosys. (see Chapter 2: Materials & Methods).

6.2 Results:

For quantitative analysis all fos–positive cells were counted separately, regardless of staining intensity. The number of Fos-positive cells were counted in a total of 20 tissue sections (10 L5 sections and 10 L6 sections) per animal. The mean number of Fos-positive cells per section for each experiment was then calculated. Results are expressed as the mean number of Fos-positive cells/section per number of experiments (n).

6.2.1 c-fos expression in the dorsal horn

Stimulation of a lumbar dorsal root for 10 minutes at a frequency of 10Hz, pulse duration of 0.5ms and at 20 times the voltage necessary to evoke a DRR in adjacent dorsal root produced dense staining for the protein product of the c-*fos* gene, Fos (Fig. 6.1). Immunoreactive, Fos-positive cellular nuclei appeared as dark round structures in the dorsal horn of the spinal cord. Graduations in labelling density were common, which meant that some immunoreactive cells stood out harshly from the surrounding background, whereas other Fos positive cells in the same region showed less immunostaining. After incubation for 2 hours following stimulation at a bath temperature of 27°C, 76.1±4.7 Fos-positive (mean ± S.E.M) (n=6) were found to be present in each 7μm section of the L5 to L6 region of the dorsal horn. All Fos-positive cells were restricted to what appeared to be the substantia gelatinosa region of the dorsal horn.

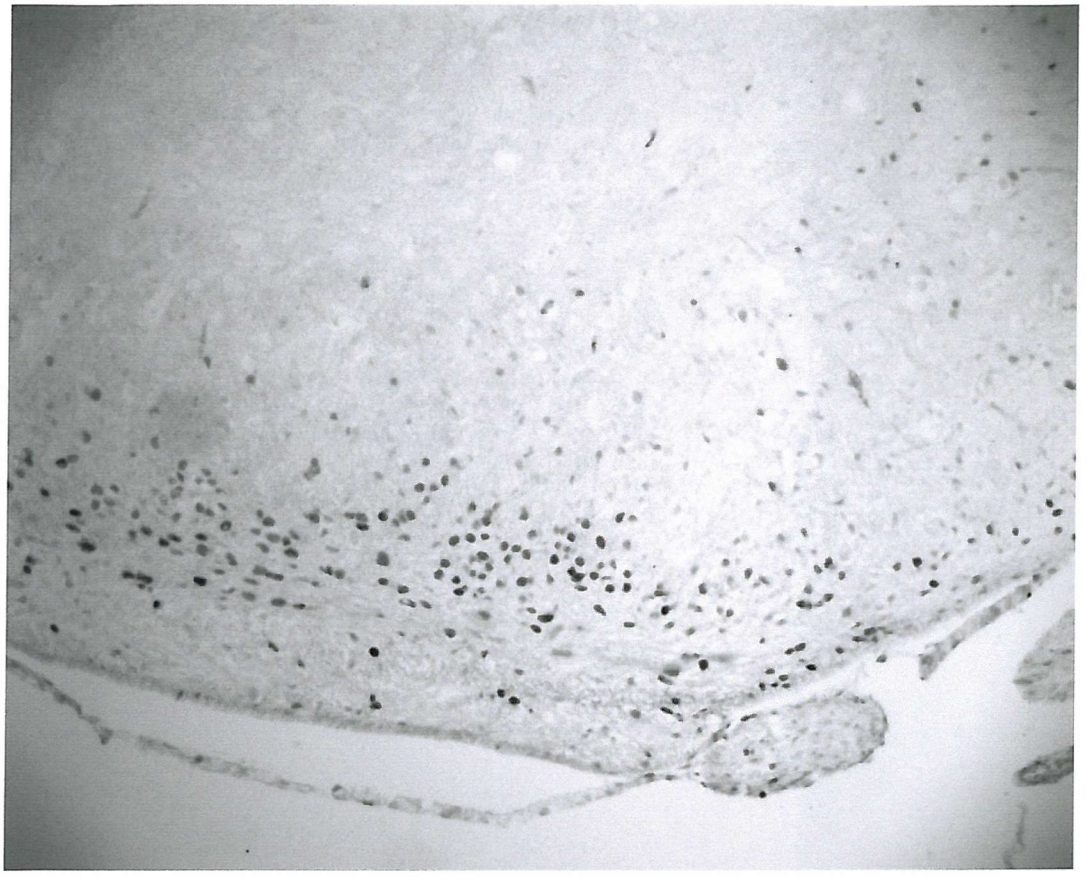


Fig. 6.1 Photomicrograph to show Fos-positive cells in the dorsal horn of a section taken from the L6 region of a rat spinal cord. Fos-positive cells are restricted to the superficial layer of the dorsal horn in the substantia gelatinosa. Magnification = x10.

6.2.2 The effect of stimulus intensity on c-fos expression

To investigate the effect of stimulus intensity upon the expression of c-*fos* in the dorsal horn of the *in vitro* spinal cord, a series of experiments were conducted whereby the stimulus applied to the dorsal root was varied. Experiments in which no dorsal root stimulation was given showed almost no c-*fos* expression in the lumbar spinal cord at any region. The number of Fos-positive cells found in the dorsal horn after no stimulation was given to the dorsal root was 2.99±0.05/section (n=3). The Fos-positive cells observed showed faint staining and were sparse and highly variable (Fig. 6.2). This experiment was used as a negative control to investigate whether handling of the animals prior to surgery or the surgery itself caused the c-*fos* gene to be expressed.

Following low intensity stimulation (1.5 times the DRR threshold voltage (1.5T)), relatively few Fos-positive stained cells were observed in the dorsal horn (Fig. 6.3). Only 12.2±2.4 Fos-positive cells per section were found following low stimulation (n=3) and this was not found to be significantly different to unstimulated controls ($p>0.05$, Tukey-Kramer multiple comparisons test). The number of Fos-positive cells in the dorsal horn was significantly increased after high intensity stimulation (20 times the DRR threshold voltage (20T)). The number of cells in the dosal horn was found to be 76.1±4.7 per section (n=6) and were densely located in laminae II of the dorsal horn (Fig. 6.4). This number was found to be significantly different compared to unstimulated controls ($p<0.001$ Tukey-Kramer multiple comparison test).

6.2.3 The effect of synaptic blockade on c-fos expression

Dorsal roots were stimulated at an intensity of 20 times the DRR threshold voltage and incubated for 2 hours at 27°C in aCSF in which the calcium had been replaced with a 2mM concentration of manganese, before being fixed and processed for Fos immunoreactivity. The inability to induce a DRR in the adjacent root to the stimulated for dorsal root was used to confirm that the addition of manganese had blocked all synaptic activity before the dorsal root was stimulated at a high intensity (20T). Addition of the manganese was shown to suppress c-*fos* expression and only 2.16±1.3 Fos-positive cells

per section were detected in the dorsal horn (Fig. 6.5). This number of Fos-positive cells was not significantly different to that seen in unstimulated controls ($p>0.05$, Tukey-Kramer multiple comparisons test), but was very significantly different from cords subjected to high intensity stimulation in normal aCSF ($p<0.001$ Tukey-Kramer multiple comparison test).

6.2.4 The effect of capsaicin on c-fos expression

Capsaicin is a pain producing neurotoxin which is known to stimulate C-fibres. The effect of capsaicin on c-*fos* expression in the dorsal horn of the *in vitro* spinal cord was used as a positive control. Capsaicin was dissolved in alcohol and added directly to the bathing medium so that the final concentration was 0.5µM. Following the addition of capsaicin, the *in vitro* spinal cord was incubated for a period of 2 hours. No dorsal root stimulation was given. Recordings made from the dorsal roots showed intense afferent activity following the addition of 0.5µM capsaicin to the bathing medium. The number of Fos-positive cells found in the dorsal horn following the addition of 0.5µM capsaicin was 64.0±21.9/section (n=3) and cells exhibited intense staining within lamina II (Fig. 6.6). This number of Fos-positive cells was similar to that seen after high intensity stimulation and was calculated to be significantly different to unstimulated controls ($p<0.01$, Tukey-Kramer multiple comparisons test).

Results of all the above experiments are shown in the photomicrographs and in a table (Fig. 6.7) and a histogram (Fig. 6.8) below.

Fig. 6.2

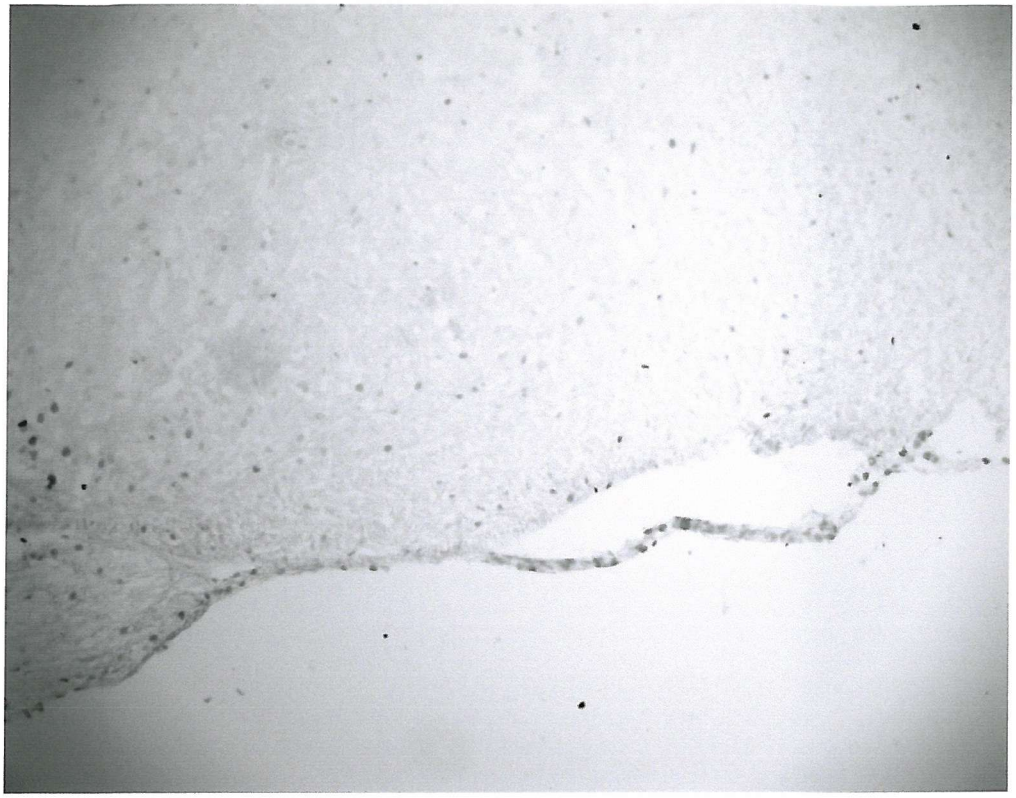
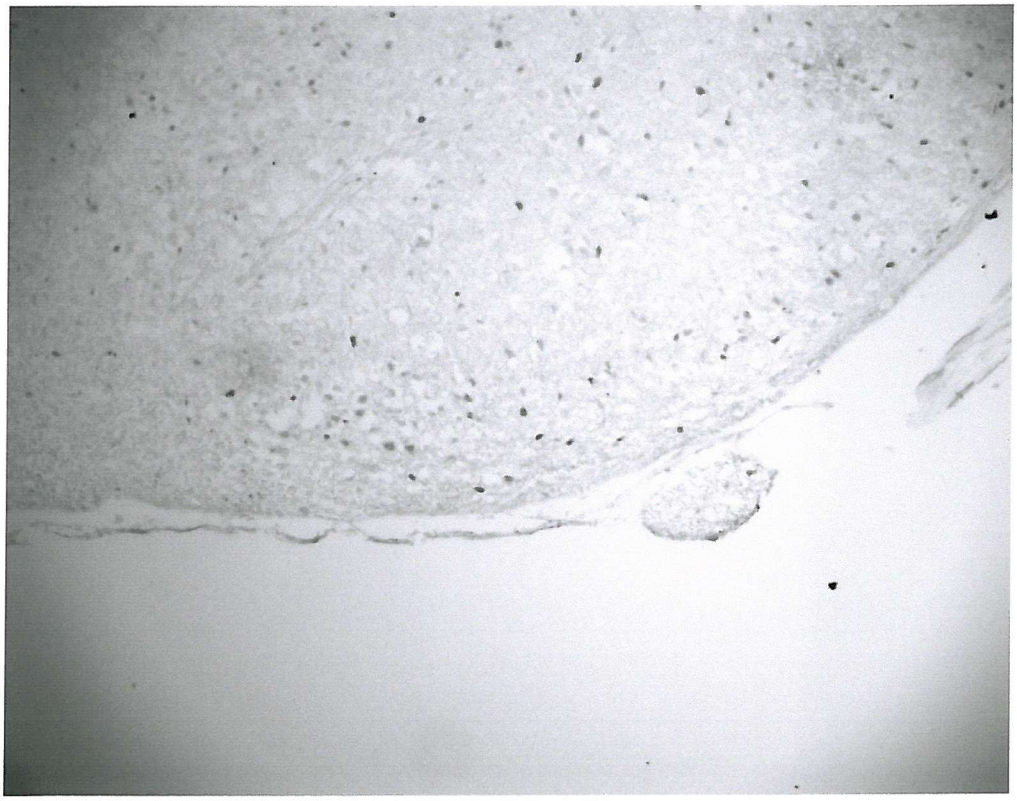


Fig. 6.3



Figs. 6.2 and 6.3: Photomicrographs showing Fos-positive cells in the dorsal horn of sections taken from rat spinal cord in which (Fig. 6.2) no dorsal root stimulation was given and (Fig. 6.3) subjected to low intensity (1.5T) dorsal root stimulation. Magnification = x10.

Fig. 6.4

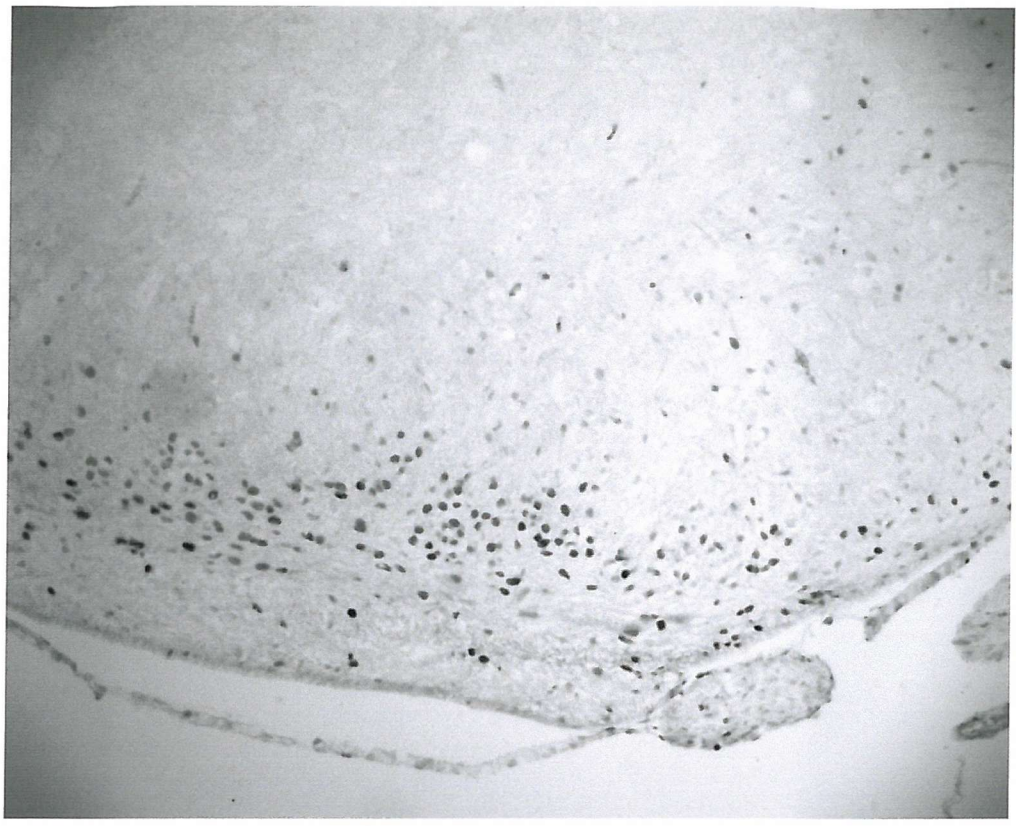
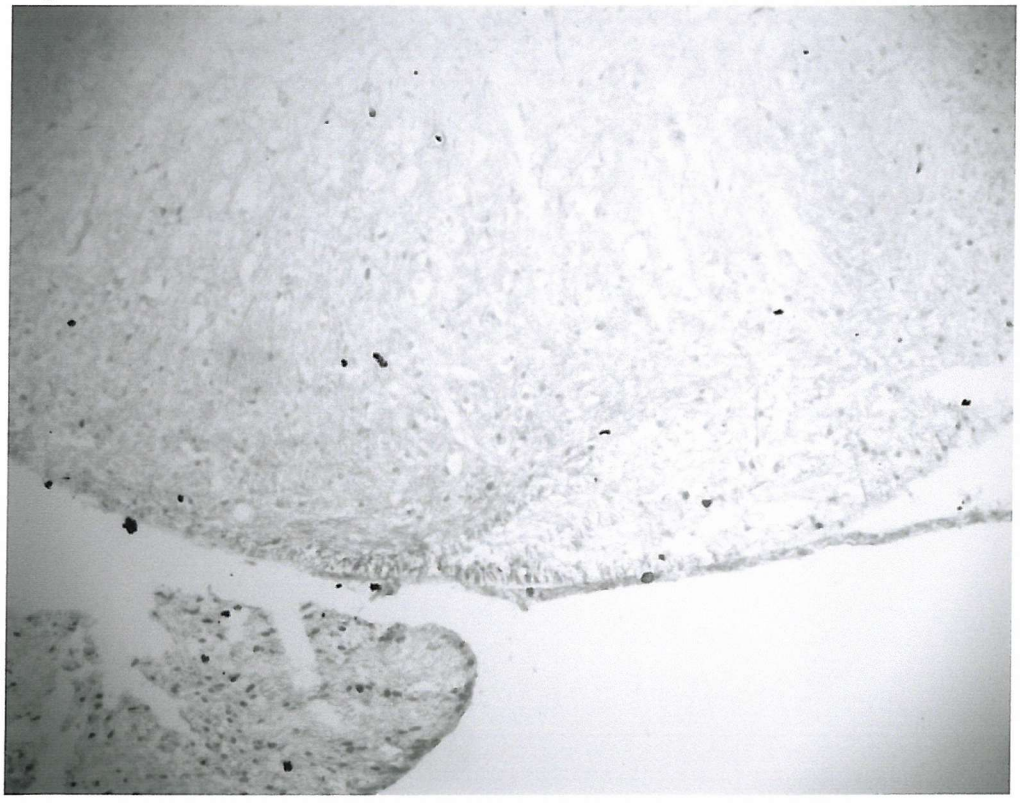
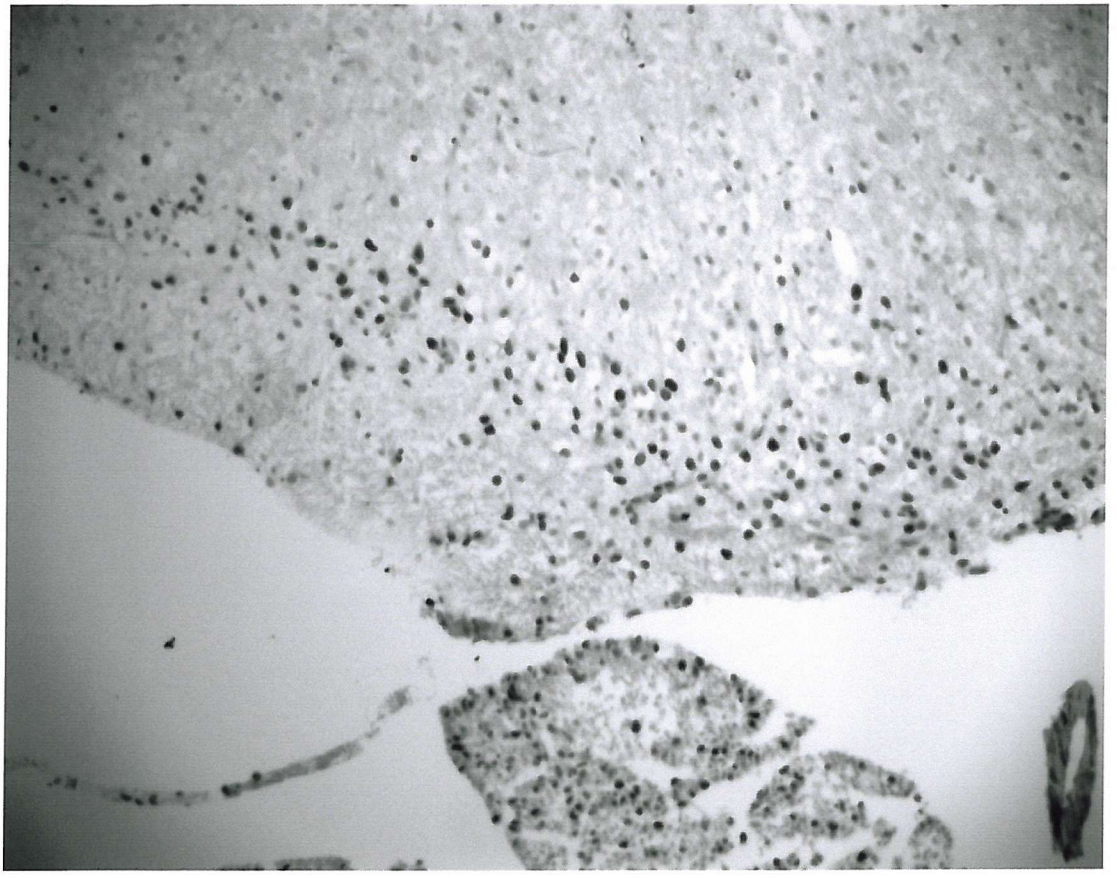


Fig. 6.5



Figs. 6.4 and 6.5: Photomicrographs showing Fos-positive cells in the dorsal horn of sections taken from rat spinal cord (Fig. 6.4) subjected to high intensity (20T) dorsal root stimulation and (Fig. 6.5) subjected to high intensity (20T) dorsal root stimulation but incubated in a medium containing 2mM manganese. Magnification = x10.

Fig. 6.6



Figs. 6.6: Photomicrograph showing Fos-positive cells in the dorsal horn of sections taken from rat spinal cord subjected to the addition of 0.5μM capsaicin (no dorsal root stimulation). The staining can be seen to be very intense in the dorsal horn. Magnification = x10.

Experiment	Mean	SEM	n	p
High Stim.	76.1	4.7	6	p<0.001
Low Stim.	12.2	2.4	3	ns
Unstim.	2.99	0.05	3	-
Capsaicin	64.0	21.9	3	p<0.01
Manganese	2.16	1.3	3	ns

Fig. 6.7: mean number of dorsal horn cells expressing *c-fos* in 7µm transverse sections through the ipsilateral dorsal horn under different experimental conditions. Significance values (p) were obtained using the ANOVA test and the Tukey-Kramer multiple comparison test (post-hoc test) to compare different conditions with the unstimulated control group. (ns=no significant difference)

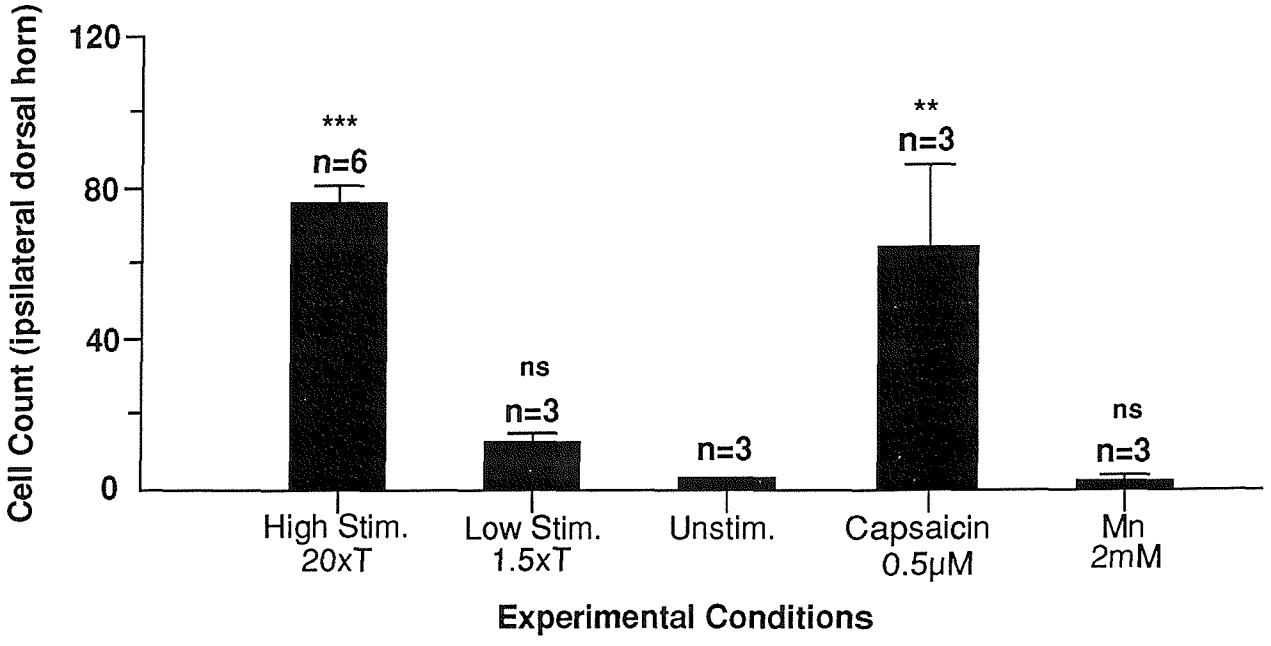


Fig. 6.8: Histogram showing the difference in the number of Fos-positive cells/section in the dorsal horn under different experimental conditions. The bars show the mean cell count (± SEM) and the number of animals (n) is shown over each bar. *c-fos* expression was significant if dorsal roots were stimulated at a high intensity (20T) or if capsaicin was added to the medium. Significance is shown above each bar (** = p<0.01, *** = p<0.001).

6.2.5 Capsaicin pretreatment of animals

To investigate the role of C fibres in the expression of c-*fos*, half of a litter of rat pups (4 animals) were pretreated at birth with 50mg/kg of capsaicin to destroy their C-fibres (Jancsó et al. 1977). The other half of the litter were treated with a vehicle control (4 animals). Spinal cords taken from these animals when they reached 22 days of age were subjected to a high intensity dorsal root stimulation (0.5ms duration, 10Hz frequency, 20 x DRR threshold voltage for 10 minutes) before being processed for c-*fos* immunoreactivity. In the dorsal horn of both capsaicin pretreated animals and vehicle control treated animals, Fos-positive cells were localised to lamina II and had a dark staining intensity (Figs. 6.9 and 6.10). However, less Fos-positive cells were present in the animals pretreated with capsaicin. The cell count of Fos-positive cells in the dorsal horn of spinal cords from the animals pretreated with capsaicin was found to be 42.4 ± 5.7/section (n=4). In animals treated with a vehicle control the cell count was 79.8 ± 3.6/section (n=4). Therefore, the c-*fos* expression in the dorsal horn of animals lacking a percentage of their C-fibres was reduced by 53.1% compared with vehicle treated controls (Figs. 6.10/ 6.11). This reduction was found to be significant ($p<0.05$) when tested using the Tukey-Kramer multiple comparison test.

Fig. 6.9

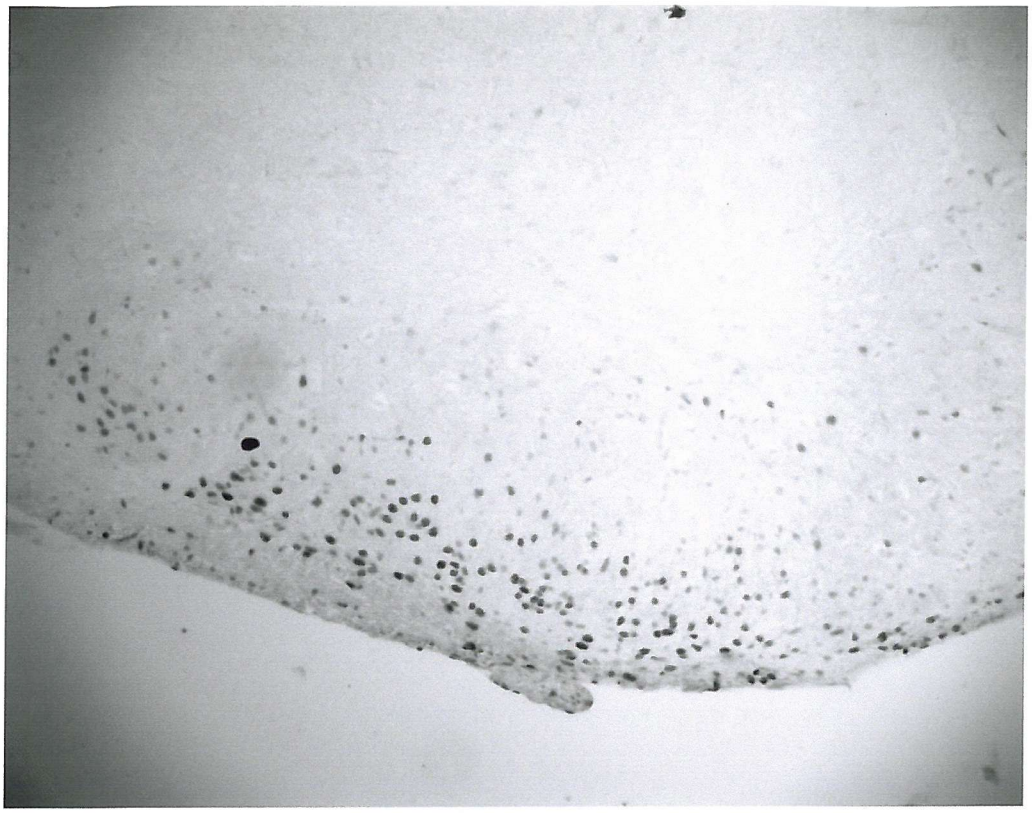
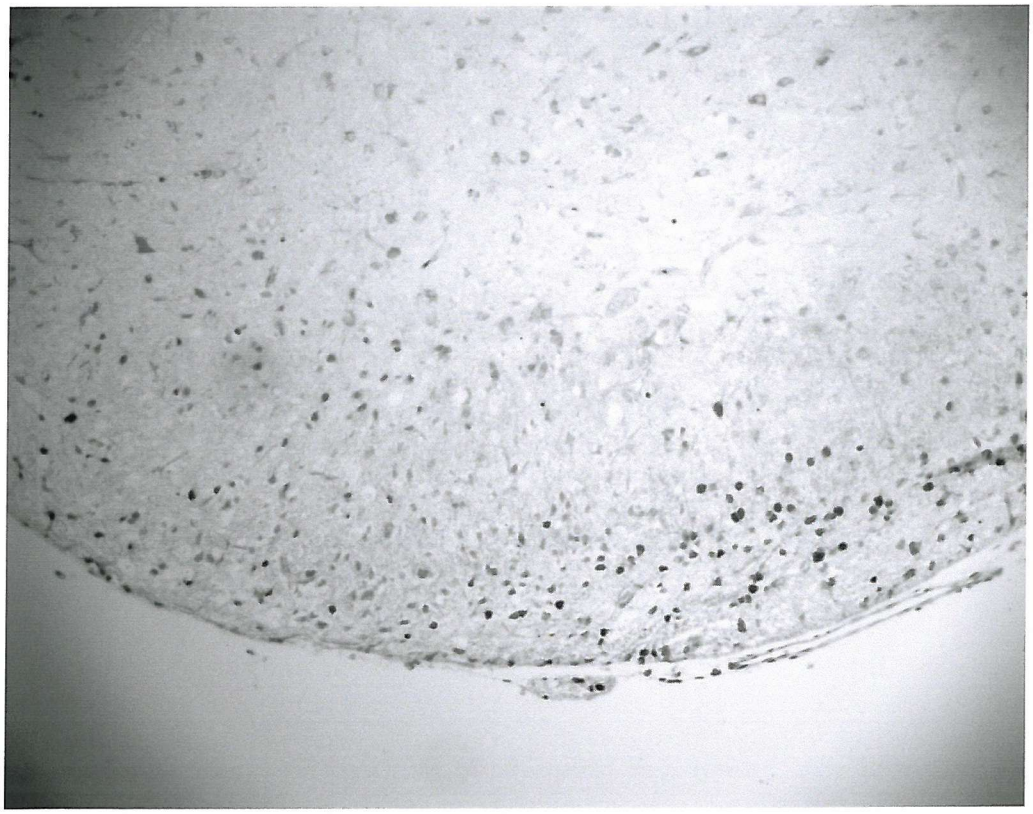


Fig. 6.10



Figs. 6.9 and 6.10: Photomicrographs showing Fos-positive cells in the dorsal horn of sections taken from rat spinal cord of animals pre treated with (Fig. 6.9) a vehicle control, and (Fig 6.10) 50mg/Kg of capsaicin. Magnification = x10.

Experiment	Mean	SEM	n	p
Caps. Pretreat.	42.4	5.7	4	p<0.05
Vehicle Control	79.8	3.6	4	-

Fig. 6.11: this table shows mean number of dorsal horn cells expressing c-*fos* in 7μm transverse sections through the ipsilateral dorsal horn of capsaicin and vehicle treated animals. The significance value (p) was obtained using the Tukey-Kramer multiple comparison test to compare the two groups.

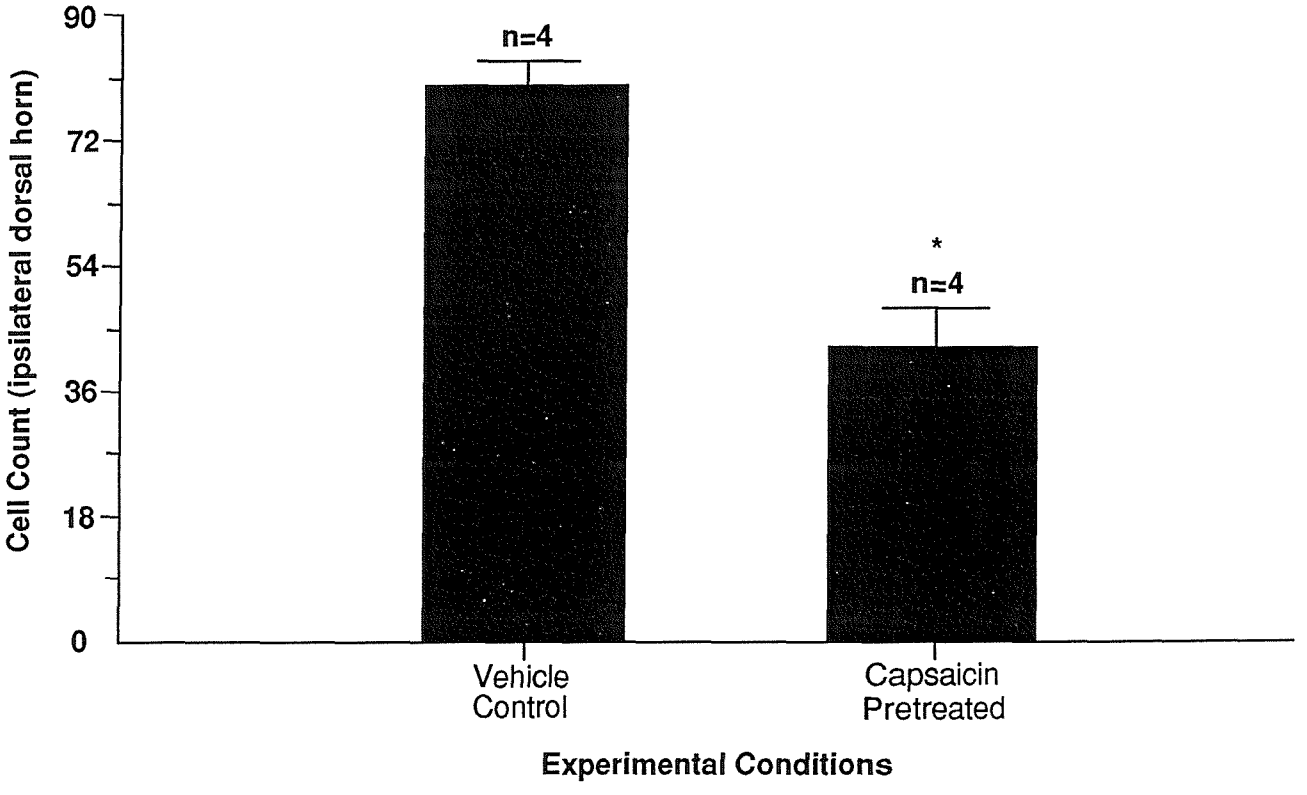


Fig 6.12: Histogram showing the difference in the number of Fos-positive cells between capsaicin and vehicle treated groups. The bars represent the mean cell count per section obtained (± SEM). c-*fos* expression is significantly reduced in animals pretreated with capsaicin. The number of animals (n) is shown over each bar.

6.2.6 The effect of glutamate receptor antagonists on c-fos expression

In order to confirm the *in vitro* spinal cord as a valid pharmacological tool for the study of nociceptive mechanisms and agents that affect them, the following series of experiments were carried out. Ionotropic glutamate receptors are known to be involved in the processing of noxious information in the dorsal horn, therefore the addition of ionotropic glutamate receptor antagonists to the bathing medium should reduce the expression of c-*fos* and hence reduce the number of Fos-positive cells observed. Both NMDA receptor and non-NMDA receptor antagonists were used in this study at a concentration of 50μM. Addition of 50μM of each antagonist was used because this concentration significantly affected dorsal horn field potentials (see Chapter 4: ionotropic glutamate receptors).

Addition of 50μM of the NMDA receptor antagonist D-AP5, which acts at the glutamate binding site, to the bathing medium of the spinal cord inhibited the expression c-*fos* following high intensity dorsal root stimulation (0.5ms duration, 10Hz frequency, 20 x DRR threshold voltage) for 10 minutes. Fos-positive cells showed a more superficial localisation, being located mainly in laminae I of the dorsal horn (Fig. 6.14). The addition of 50μM D-AP5 significantly reduced the number of Fos-positive cells in the dorsal horn from a control cell count of 76.1±4.7 to a cell count of 33.5±8.5 % of the control cell count (Fig. 6.17). A significance value of $p<0.001$ was calculated using the Tukey-Kramer multiple comparison test.

Addition of 50μM of the NMDA receptor antagonist 7-Cl KYNA, which acts at the glycine site, to the bathing medium, significantly inhibited the expression of c-*fos* in the dorsal horn. Fos-positive cells were seen to be located more superficially in the medial dorsal (Fig. 6.15). The number of Fos-positive cells in the dorsal horn were reduced to 48.7± 3.75% (Fig. 6.17) which was calculated to be significant using the Tukey-Kramer multiple comparison test ($p<0.05$, $n=3$).

Fos-like immunoreactivity in the dorsal horn was little changed when cords were incubated with 50μM of the non-NMDA receptor antagonist CNQX, when compared with control results (Fig. 6.16). The reduction to 69.6±6.1 proved to be not significant when tested with the Tukey-Kramer multiple comparison test ($p>0.05$ $n=4$)(Fig. 6.17).

Fig. 6.13

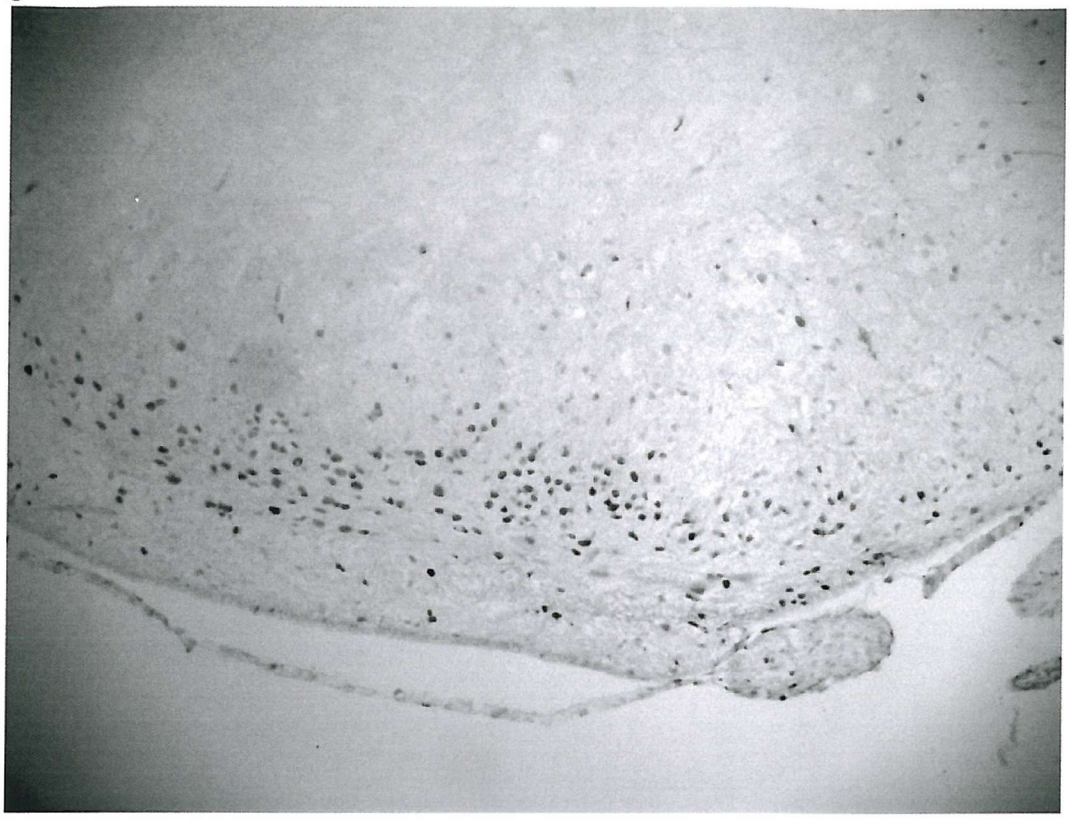
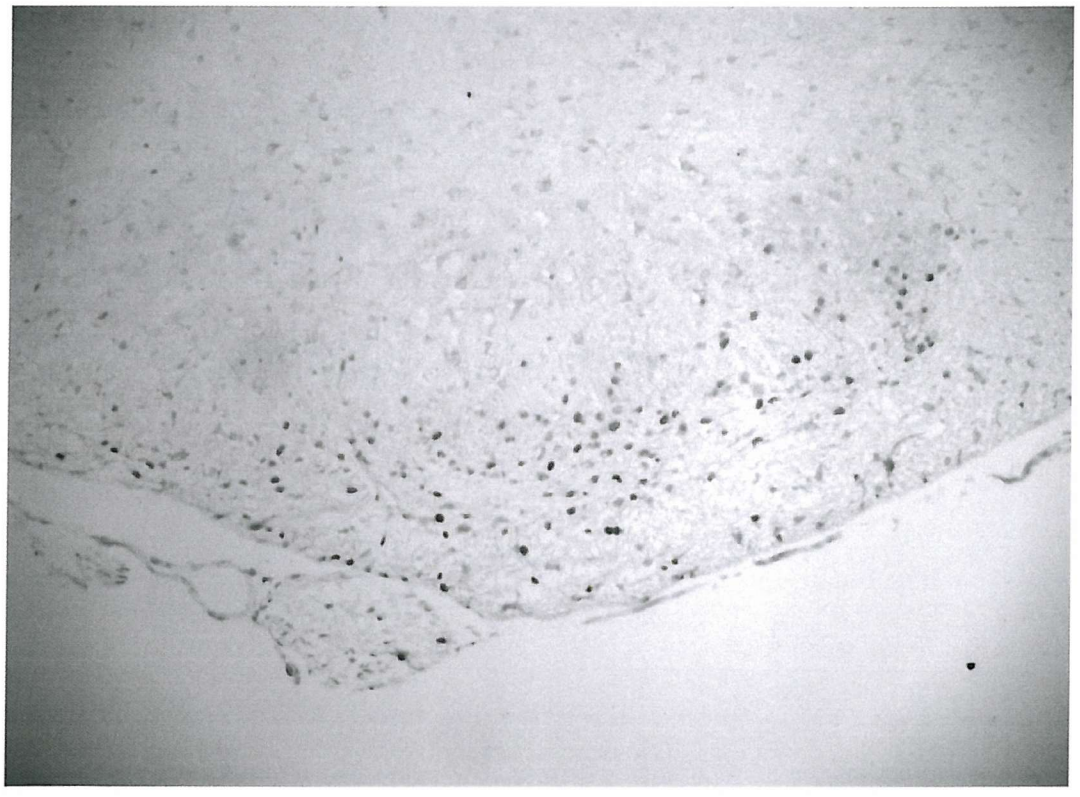


Fig. 6.14



Figs. 6.13 and 6.14: Photomicrographs showing Fos positive cells in the dorsal horn of sections taken from rat spinal cord subjected to high intensity (20T) stimulation (Fig. 6.13) and subjected to high intensity (20T) stimulation and the addition of 50μM D-AP5 to the medium (Fig. 6.14). Magnification = x10.

Fig. 6.15

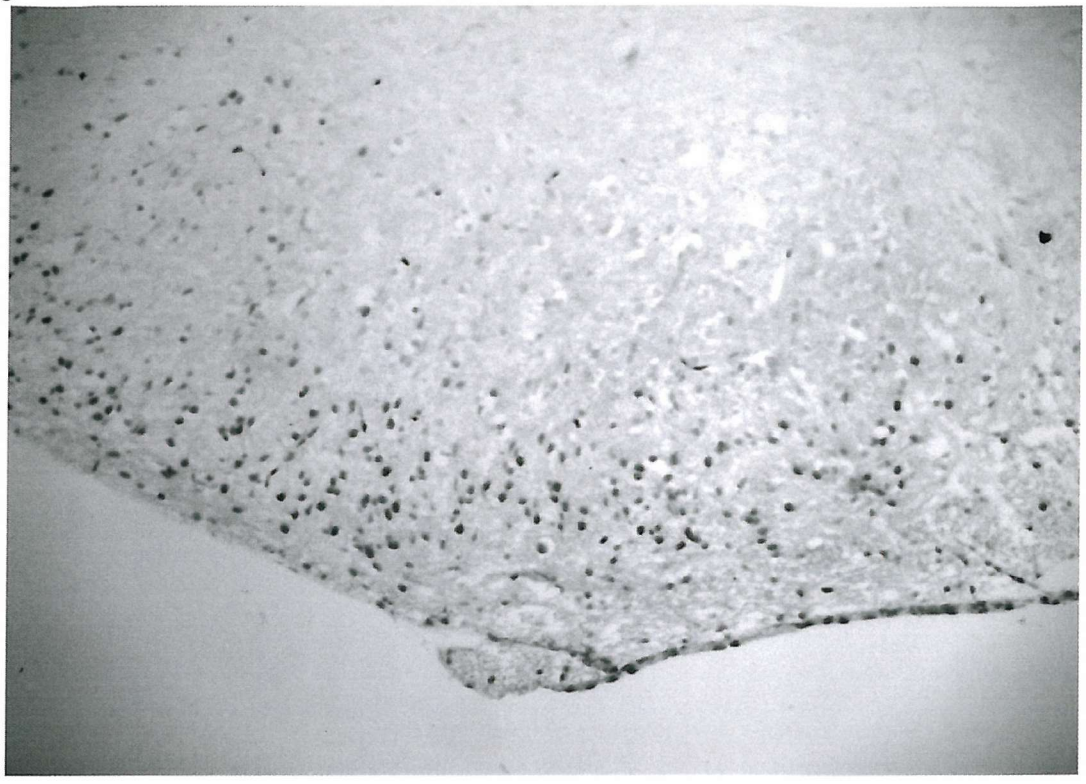
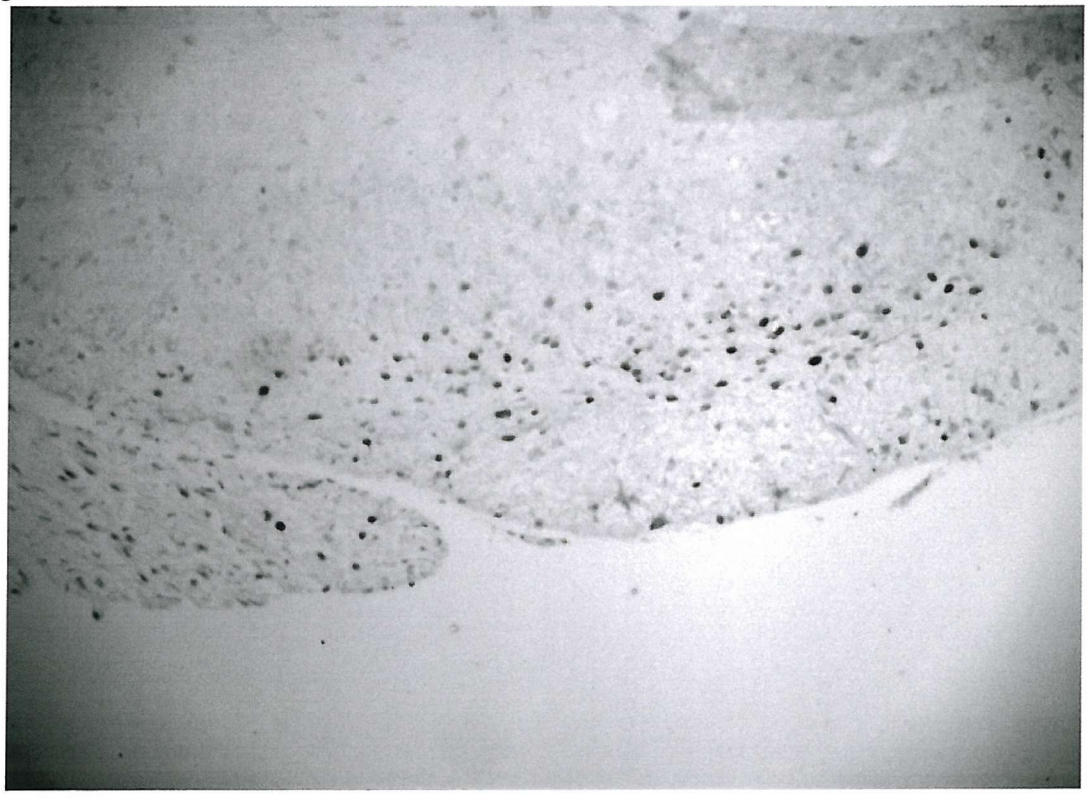


Fig. 6.16



Figs. 6.15 and 6.16: Photomicrographs showing Fos positive cells in the dorsal horn of sections taken from rat spinal cord subjected to high intensity (20T) stimulation and 50μM 7-Cl KYNA (Fig. 6.15) and spinal cords subjected to high intensity (20T) stimulation and the addition of 50μM CNQX to the medium (Fig. 6.16). Magnification = x10.

	Cell Count	SEM	n	p
Control	76.1	4.7	6	-
CNQX	69.6	6.1	4	p>0.05
AP5	33.5	8.5	4	p<0.001
7 Cl-KYNA	48.7	3.75	3	p<0.05

Significance values (p) calculated using the Tukey-Kramer Multiple Comparison Test.

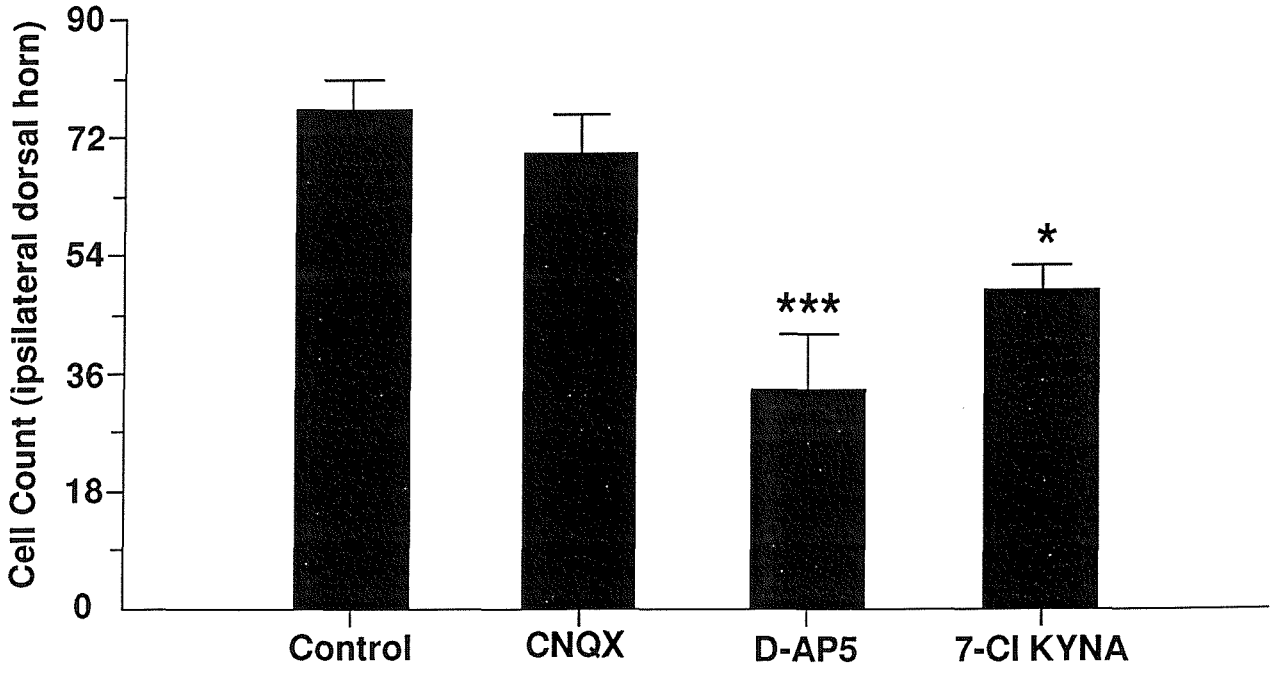


Fig. 6.17: Cell counts of the number of Fos-positive cells in the spinal cord dorsal horn showed that the NMDA antagonists D-AP5 and 7-Cl KYNA significantly inhibited expression when compared to the high intensity dorsal root stimulation control group (**, p<0.01). There was no significant difference between the CNQX treated group and the control group (p>0.05). Significance values (p) were calculated using the ANOVA and post-hoc Tukey-Kramer multiple comparisons test.

6.3 Discussion:

It was initially thought that the surgery involved in removing the spinal cord from the animal would result in expression of the immediate early gene c-*fos* in the *in vitro* spinal cord. However, control experiments in this study involving no dorsal root stimulation showed an almost complete absence of any Fos immunoreactivity in the dorsal horn. This result indicated that neither handling of the animals prior to surgery nor the surgery itself contributed to the expression of the c-*fos* gene. The rapid cooling of the tissue by transcardiac perfusion with Tyrodes solution appeared to effective at inhibiting c-*fos* expression. Fos positive cells detected in the dorsal horn during subsequent experiments were therefore, due to the experimental conditions alone.

Experiments involving electrical stimulation of the dorsal roots showed that c-*fos* induction was only brought about when the intensity of stimulation was sufficient to stimulate high threshold C-fibres i.e. at 20 times the DRR threshold voltage. Stimulation of a dorsal root at 20 times the DRR threshold voltage was previously shown in this investigation to activate slow conducting fibres with a conduction velocity approximately equal to that of C fibres. The number of Fos-positive cells observed in the dorsal horn following high intensity (20T) stimulation (76.1±4.7, n=6) was similar to the number reported in *in vivo* experiments (Bullitt et al. 1992). Spinal cords in which no dorsal root stimulation was given showed a very low level of c-*fos* expression, (2.99±0.05/section, n=3) which was similar to values obtained in control experiments *in vivo* (Abbadie and Besson 1993; Abbadie et al. 1994). However, the results disagree with a study by Jennings and Fitzgerald (1996) which reported that low threshold stimulation resulted in the expression of the Fos protein in the dorsal horn. The Jennings and Fitzgerald (1996) study was performed using neonatal rat spinal cord which may account for the discrepancy between the results of this study. The neonatal rat has shown to have less sensitive reflex sensitivity which is likely to be because of differing central processing mechanisms to that of the adult rat. In the present study using rats which are more developmentally mature, low intensity stimulation, insufficient for C-fibre stimulation, did not significantly increase the number of Fos positive cells above the level of that seen in spinal cords in which the dorsal roots were not stimulated. These experiments indicated that c-*fos* expression in

the dorsal horn is dependent upon high threshold fibre stimulation conducting at C-fibre velocities.

By blocking all synaptic activity in the isolated cord, using manganese to replace the calcium in the perfusion medium (Bagust and Kerkut 1980), it was shown that cells expressing c-*fos* were activated transynaptically by stimulation of the dorsal root afferents. Manganese is thought to prevent the increase in levels of intracellular calcium in the presynaptic axon terminals which trigger the release of transmitter vesicles. Replacement of calcium in the aCSF by manganese was, therefore, expected to block the expression of c-*fos*. The abolition of Fos induction by the addition of manganese to the medium occurred even when high intensity dorsal root stimulation was given. The absence of any Fos immunoreactivity in these experiments confirmed that staining was not a non-specific effect caused by erythrocytes in the preparation, as reported by Metz et al. (1989).

Capsaicin, which is known to stimulate nociceptive C-fibres and cause release of substance P from afferent terminals, increased the number of Fos positive cells seen in the dorsal horn when it was added to the bathing medium (0.5μM). This provided a positive control and proved that c-*fos* was induced in dorsal horn neurones by activity in C-fibre afferents. Capsaicin administered to rats within 24 hours of birth causes the destruction of a large percentage of the C-fibres of the animal (Jancsó et al. 1977). In cords taken from animals that had been preteated with 50mg/kg of capsaicin, the expression of c-*fos* induced by high intensity dorsal root stimulation was significantly reduced compared with animals that had been treated with a vehicle control. These experiments agree with those of *in vivo* experiments in which animals treated with capsaicin at 1 day old showed significantly less Fos positive cells after hind paw inflammation than animals treated with a vehicle control (Hylden et al. 1992). These studies further confirm the hypothesis that c-*fos* is induced in the dorsal horn of the spinal cord by high threshold C-fibre afferent activation.

These experiments investigating the expression of c-*fos* have demonstrated that the isolated spinal cord preparation is a useful tool for researching the processing of high threshold afferent signals in the dorsal horn. It has been shown that induction of the c-*fos* gene is not the result of animal handling nor surgical procedures and that the

immunoreactive staining is not non-specific. In addition, the abolition of c-*fos* expression by manganese and the strong positive response to capsaicin demonstrates that the *in vitro* preparation is a powerful method for the investigation of the pharmacology of the pathways activated by high threshold inputs to the spinal cord.

The immunocytochemical localisation of the c-*fos* gene product Fos is known to be an indicator of activation of the nociceptive neurones at the spinal level (Hunt et al. 1987). The NMDA antagonists D-AP5 and 7-Cl KYNA was shown to significantly inhibit the expression of c-*fos* in the dorsal horn of the spinal cord form a control Fos-positive cell count of 76.1±4.7 to cell counts of 33.5±8.5% and 48.7±3.75% of control values respectively. Inhibition of c-*fos* expression by NMDA receptor antagonists not only decreased the number of Fos-positive cells in the dorsal horn but the pattern of expression became much more superficial. Fos-positive cells in the dorsal horn appeared to be localised in laminae I and II after high intensity dorsal root stimulation experiments. With the addition of 50μM of either D-AP5 or 7-Cl KYNA the expression pattern became much more superficial. This effect of NMDA antagonism on the expression of c-*fos* is interesting since lamina II and the superficial section of lamina III is the termination site for Aβ fibres. Under normal conditions Aβ fibre activation does not evoke nociceptive responses (see refs in Haley and Wilcox 1992) or c-*fos* expression (Zhang et al. 1998). Also, Aβ fibre responses are not normally influenced by NMDA receptor antagonism (Dickenson and Sullivan 1990). However there is mounting evidence that Aβ fibre inputs can evoke nociceptive responses under certain conditions such as inflammation (Woolf and Doubell 1994).

NMDA receptor antagonism inhibited the expression of c-fos in lamina II 2 hours following dorsal root stimulation. It is therefore unlikely that the inhibition reflects a functional change in the Aβ fibres. It is also unlikely that Aβ fibres terminating in laminae III directly activate NMDA receptors. Aβ fibre inputs could, however, be terminating on neurones that are already hyperexcitable and this hyperexcitability could be mediated in part by NMDA receptors. Thus the reduction in the number of Fos-positive cells in lamina III by NMDA receptor antagonism may be due to a general decrease in spinal excitability. This effect has previously been seen *in vivo* (Chapman et al. 1995).

These results demonstrate that NMDA receptor activation contributes to the expression of the c-*fos* gene. The expression of c-*fos* in postsynaptic cells has been shown to be calcium dependant (Greenberg et al. 1992), therefore, it is possible that NMDA antagonism reduces Fos-like immunoreactivity because of the reduced influx of calcium into dorsal horn cells through the NMDA channels. However, the c-*fos* results also agree with previous experiments in this study using electrophysiological techniques. It was found that NMDA receptor antagonists inhibited the slow wave field potential induced by high intensity dorsal root stimulation. Therefore, it can be concluded from both the immunohistochemical and the electrophysiological experiments that NMDA receptors are involved in the processing of noxious information in the dorsal horn of the spinal cord of the rat. Previous experiments *in vivo* have also reported that NMDA receptor antagonism reduces the expression of c-fos (Huang and Simpson 1999; Chapman et al. 1995; Tao and Zhao 1998). NMDA antagonism has also been shown to Inhibit temporal summation of second pain, the equivalent of windup, in humans (Price et al. 1994).

The non-NMDA receptor antagonist CNQX had no effect on c-*fos* expression in the dorsal horn of the spinal cord. This suggests that non-NMDA receptors do not have a role in the processing of noxious information in the spinal cord dorsal horn. Similar results have also been observed *in vivo* using the non-NMDA receptor antagonist DNQX (Tao and Zhao 1998). Results of previous electrophysiology experiments in this study have shown that the non-NMDA receptors have a role in the processing responses to low intensity, non-noxious dorsal root stimulation.

Unfortunately, problems with the quality and supply of the primary antibody meant that it was not possible to carry out further experiments using metabotropic glutamate receptor antagonists.

The investigations described illustrate the advantages of the *in vitro* mammalian spinal cord preparation over *in vivo* and other more conventional tissue slice preparations. It has been demonstrated that the expression of c-*fos* under differing experimental conditions in this preparation agrees with results reported from *in vivo* preparations and also with results from electrophysiological experiments in this study. This preparation is a valuable tool for spinal cord pharmacology research.

Chapter 7

Summary

Experiments carried out in this study have proved the value of the *in vitro* spinal cord as a valuble tool for spinal cord pharmacological research. In particular, the age of the animals used for this preparation has proved to be a major advantage for the investigation of the pharmacology of nociceptive processing in the dorsal horn.

7.1 Ionotropic glutamate receptors and nociception

The addition of ionotropic glutamate receptor antagonists acting at NMDA and non-NMDA receptors to the bathing medium of the *in vitro* spinal cord has demonstrated that NMDA receptors have a minor role and non-NMDA receptors have a major role in the transmission and processing of low-threshold non-noxious sensory information in the dorsal horn. In contrast, the processing of noxious sensory information elictied by the activation of high threshold fibres is mediated, in part, by NMDA receptors but also has a non-NMDA receptor component. However, the response to high threshold fibre activation was not completely inhibited by NMDA receptors suggesting the involvement of other transmitters and/or receptor systems. For example, substance P, which is co-released with glutamate from primary afferent fibres (Battaglia and Rustioni 1988) which is known to have a role in nociception in the spinal cord (for refs. see Haley and Wilcox 1992).

The results of this study using ionotropic glutamate receptor antagonists in the *in vitro* spinal cord agree with other studies conducted *in vivo* (Coderre et al 1993; Dickenson and Sullivan 1990; Dray et al 1994; Dubner and Ruda 1992; McMahon et al. 1993; Woolf 1994). These experiments in the *in vitro* spinal cord have therefore validated the use of the preparation as a useful tool in the study of spinal pain mechanisms.

In this investigation the NMDA antagonist acting at the glycine site of the receptor, 7-Cl

KYNA, was shown to have a significant effect on the slow wave field potential as well as significantly inhibiting the expression of c-fos in the dorsal horn (Chapter 6: c-*fos*). These results suggest that the glycine site of the NMDA receptor may be a possible target for novel analgesic therapies and may be a useful for reducing the consequences of NMDA-mediated sensory events in the spinal cord, including modulation/transmission of nociceptive information and c-fos expression.

7.2 Metabotropic glutamate receptors and nociception

The results of the study using metabotropic glutamate receptor (mGluR) agonists and antagonists suggest that different subgroups of mGluRs have different roles in the transmission and processing of sensory information in the spinal cord.

The addition of the group I and group II mGluR agonist *trans*-ACPD to the *in vitro* spinal cord suggested the possibility of group I and/or group II mGluRs acting as autoreceptors to inhibit further glutamate release after the activation of low-threshold non-noxious fibres. However, neither the group I antagonist L-AP3 nor the group II antagonist EGLU showed any significant effect on responses to low-threshold fibre activation. As the group II mGluRs are thought to be presynaptic (Jane et al. 1994; Kemp et al. 1994), the use of a group II agonist may have been more useful in determining if group II receptors have a role in the transmission and processing of non-noxious sensory information. However, the cost of mGluR group II receptor agonists meant that this was not financially possible.

The group III mGluR agonist L-AP4 significantly affected the S2 component of the fast wave field potential after activation of low-threshold fibres. Group III mGluRs consist of the mGluR4, mGluR6, mGluR7 and mGluR8 receptor subtypes. It is known that mGluR6 and mGluR8 receptors are not found in the spinal cord (Nakajima et al. 1993). In the spinal cord, evidence of presynaptic mGluR7 and postsynaptic mGluR4 receptors has been found (Ohishi et al. 1995; Boxall et al. 1998). It is therefore possible that the reduction of the fast wave S2 component is mediated by the mGluR7 subtype acting as pre-synaptic autoreceptors. However, more selective pharmacological tools which differentiate between the different receptor subtypes would be required to investigate this hypothesis.

Addition of the group I/II agonist *trans*-ACPD to the bathing medium significantly inhibited the slow wave field potential after the activation of high-threshold, noxious fibre activation. This suggested the involvement of group I and/or group II mGluRs in mediating noxious sensory information in the dorsal horn. However, the results conflicted with other experiments in this study. The selective group I antagonist L-AP3 also significantly reduced the slow wave field potential as did the selective group II antagonist EGLU.

To investigate these conflicting results further, the selective and potent mGluR group I agonist DHPG was used. DHPG significantly increased the slow wave at low concentrations. At higher concentrations, no significant increase of the slow wave occured and this may have due to the depression of synaptic transmission that has been reported to occur after the addition of group I mGluR agonists (King and Liu 1996). The facilitation by the group I agonist DHPG corresponded to the inhibition observed after the addition of the mGluR group I antagonist L-AP3. This data, along with that showing that the group II antagonist EGLU reduced the slow wave after high-threshold fibre activation indicated the involvement of both group I and group II receptor types in the transmission and processing of noxious high-threshold fibre impulses in the spinal cord. The addition of L-AP3 to the medium reduced the slow wave to a greater extent than the addition of EGLU. These results suggest that group I mGluRs have a predominant involvement in the transmission and/or processing of noxious information in the spinal cord. This predominant involvement of group I mGluRs has previously been noted in *in vivo* experiments (Young et al. 1997; Neugebauer et al. 1994; Fisher and Coderre 1996a).

In contrast to L-AP3 and EGLU, the group III mGluR agonist L-AP4 had no effect on the slow wave field potential and hence it was concluded that group III mGluRs have no role in the synaptic transmission of noxious sensory information following the activation of high-threshold fibres.

The mechanism of how mGluRs contribute to nociceptive transmission is unknown. However, one possible mechanism is that group I mGluRs activate PKCγ which in turn modulates NMDA receptors so that they are more susceptible to activation by glutamate.

7.3 c-fos expression in the in vitro spinal cord

It was initially thought that expression of c-*fos* in the *in vitro* spinal cord would not be possible as the surgery involved in removing the spinal cord from the animal would result in the expression of the immediate early gene. However, control experiments proved that Fos immunoreactivity did not occur in the preparation unless high-threshold fibre excitation occurred and that the number of Fos-positive cells observed in the dorsal horn was similar to numbers reported in *in vivo* experiments (Bullitt et al. 1992). The rapid cooling of the tissue by transcardiac perfusion with Tyrodes solution appeared to effective at inhibiting c-*fos* expression during the dissection.

Addition to the bathing medium of the neurotoxin capsaicin, which is known to stimulate nociceptive C-fibres and cause release of substance P from afferent terminals, increased the number of Fos-positive cells observed in the dorsal horn. This result suggested that c-*fos* was induced by activity in C-fibre afferents. The expression of c-*fos* induced by high intensity dorsal root stimulation was significantly reduced in cords taken from animals that had a large percentage of their C fibres destroyed by teatment with capsaicin 24h after birth (Jancsó et al. 1977) compared with animals that had been treated with a vehicle control. These studies further confirmed that c-*fos* is induced in the dorsal horn of the spinal cord by high threshold C-fibre afferent activation.

Previous evidence has shown that there is a relationship between nociception and the expression of the immediate early gene *c-fos* (Hunt et al. 1987). The NMDA receptor antagonists D-AP5 and 7-Cl KYNA significantly inhibited the expression of *c-fos* in the dorsal horn of the spinal cord and changed the localisation of the Fos-postive cells so that it became more superficial in the dorsal horn. This strongly suggests that NMDA receptors are involved in the induction of the gene. CNQX had no significant effect on the expression of c-*fos* indicating no role for the non-NMDA receptors in the induction of this gene.

All the investigations described in this study illustrate the advantages of the *in vitro* mammalian spinal cord from juvenille rats over *in vivo* and other more conventional tissue slice preparations. The results agree with those reported *in vivo* and observations have been made which have not been previously reported in other *in vitro* spinal cord preparations.

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