ELECTROPHYSIOLOGICAL STUDIES ON THE PHARMACOLOGY OF ADENOSINE RELATED TO NOCICEPTIVE PROCESSING IN THE RAT SPINAL CORD.

by
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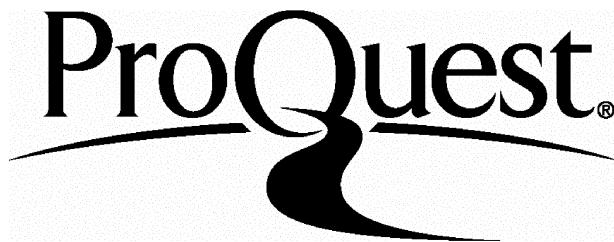
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Abstract.

These *in vivo* electrophysiological experiments establish the role of the purine, adenosine, in spinal cord processing of sensory information.

Activation of the A_1-receptor, by various agonists, selectively attenuated high-threshold inputs, namely the C-fibre evoked responses, wind-up and post-discharge, whilst sparing the low-threshold ($A\beta$-fibre evoked) responses. The $A\delta$-fibre evoked responses showed concurrent facilitations over the same dose range and time-course. The A_{2a}-receptor appeared not to be important in spinal cord sensory processing.

In models of acute inflammation, activation of the A_1-receptor was effective in controlling both the first and second phase of the formalin induced response and agonist actions showed no change in potency following carrageenan induced inflammation compared to normal animals.

Protecting endogenously released adenosine, with two novel adenosine kinase inhibitors, demonstrated that adenosine is released at the spinal cord level during noxious stimulation and that this results in antinociception. The predominant inhibitory effects of protected adenosine were on the post-discharge and wind-up.

Spinally protected adenosine inhibited both the first and the second phase of the formalin evoked response, predominantly attenuating the tonic second phase.

The results with protected adenosine suggest that adenosine is released in response to, and predominantly controls NMDA-receptor mediated events.

After carrageenan induced inflammation, there was no change in the antinociception produced by the intrathecal kinase inhibitor compared to normal animals. However, when adenosine was protected by a kinase inhibitor given by the systemic route there was a marked increase in antinociception, suggesting an additional non-spinal site of antinociception after carrageenan.

A_1-receptor activation was effective in controlling bicuculline induced changes in neuronal responses, a model for certain neuropathic symptoms.

These results extend our knowledge of the role of adenosine as a neuromodulator of spinal somatosensory processing, particularly nociception.

Acknowledgements.

Contents.

Chapter 1.

Introduction.

1.1.1: Adenosine as a Novel Target for Analgesia?

The dorsal horn of the spinal cord has long been established as a key site for the transmission and modulation of sensory afferent inputs (see Besson and Chaouch 1987; Cervero and Iggo 1980; Iggo 1974; Mendell 1966; Menetrey et al. 1977; Pearson 1968; Wall 1967b). Systems, both intrinsic to the spinal cord and projecting into the spinal cord can be manipulated to achieve analgesia. This may be achieved by dampening down excitatory systems or by enhancing inhibitory systems. There has been much interest in the controlling of the excitatory amino acid systems due to their roles in certain pain states. Targeting these systems may be beneficial in altered pain states, however since the spinal processing of noxious information can involve many excitatory systems, inhibiting only one of these systems may not be enough to give rise to adequate analgesia. Increasing the function of inhibitory systems may be a better approach, since it may provide a means of prevention of the function of many excitatory systems. This has been the approach with classical analgesics such as the opioids. However, it has become increasingly apparent that the use of opioids has many associated problems. Side effects such as respiratory depression, constipation and tolerance with long term use can limit the use of opioids. Another problem is that opioids in certain pain states do not always give rise to adequate pain relief. For these reasons much research has been focused on finding other possible targets for analgesia. The purine, adenosine may well be one such target. This thesis is concerned with establishing the role of adenosine and the receptors for this purine in the spinal control of nociceptive processing.

A role for the purines in somatosensory processing has become established since the original observations by Holton and Holton (1954) that both extracts of dorsal and ventral spinal roots contained adenosine triphosphate (ATP) and that this and its breakdown products had vasodilator activity when injected into the rabbit ear. Antidromic stimulation of the auricular nerve in rabbits, causes vasodilatation, similar to that produced by chemicals containing purine and pyrimidine rings (Holton 1959). ATP was subsequently established as the mediator being released from these nerves (Holton 1959). A role for the purines in nociception was only established at a much later date by Paalz ow and Paalz ow (1973) who showed that the methylxanthines, caffeine and theophylline (adenosine-receptor antagonists as well as phosphodiesterase inhibitors) reduced the thresholds for nociception in behavioural studies involving electrical shock to the tail of the rat.

Adenosine is a ribonucleoside, formed from the purine base, adenine by an addition of a ribose sugar. An addition of a phosphate group onto the hydroxyl

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group, C-5 of the sugar, forms the nucleoside, adenosine monophosphate (AMP). Combination with further phosphate groups forms adenosine diphosphate (ADP) and ATP. The deoxyribose nucleotides, lacking an oxygen on the sugar ring, form the nucleotides of DNA. The ribose nucleotides form RNA as well as being an integral part of cellular respiration (oxidative phosphorylation). For these reasons the role of establishing purines as neurotransmitters or neuromodulators has been hampered since they are a ubiquitous component of all cells.

Although there is growing evidence that the purines, especially ATP, do in fact act as independent neurotransmitters in many areas of the body (Braas et al. 1987; Holton 1959) the role of ATP and adenosine in the central nervous system is still open to much investigation and discussion (Dragunow and Faull 1988; Feldberg and Sherwood 1954; Fredholm and Hedqvist 1980; Fyffe and Perl 1984; Jahr and Jessell 1983; Kostopoulos and Phillis 1977; Meghji 1991; Phillis and Kostopoulos 1975; Phillis and Wu 1981; Salter et al. 1993; Snyder 1985; Stone 1981). Increasing knowledge of the effects of both ATP and adenosine per se in the dorsal horn of the spinal cord (Fyffe and Perl 1984; Golembiowska et al. 1995, 1996; Keil and DeLander 1992, 1994, 1995; Phillis and Wu 1981; Salter et al. 1993; Sawynok and Sweeney 1989; Snyder 1985; Stone 1981) provides strong evidence for a role of purines in the modification of sensory perception. Whilst it is clear that ATP can act as a neurotransmitter (Fyffe and Perl 1984; Hongo et al. 1968; Hunt 1961; Salter and Henry 1985, 1987, 1989) it is likely that adenosine is playing a more subtle modulatory role; the reasons for this will be discussed later in this chapter.

1.1.2: Synthesis of Adenosine.

The levels of intracellular adenosine increase when cells undergo high levels of metabolic activity. This is especially the case in conditions of ischemia, hypoxia and hypoperfusion, where the demand ratio for oxygen outweighs the supply available. Here the available adenosine may come from the conversion of cytoplasmic ATP and ADP by the cleaving of phosphate via the enzymes ATPase, ADPase and 5'-nucleotidases.

The enzyme 5'-nucleotidase hydrolyses nucleotides but not adenosine 3'-monophosphate or ATP. There are several 5'-nucleotidases (Novikoff 1958; Scott 1965), two cytosolic forms of 5'-nucleotidases, one specific for AMP the other specific for inosine monophosphate (IMP) (Truong et al. 1988) as well as a plasma membrane bound enzyme. The AMP-preferring enzyme is believed to be responsible for the production of adenosine during states of metabolic stress (Truong et al. 1988). These enzymes have been located in the rat heart (Truong et al. 1988) and in

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neuronal cells, in both the cytoplasm, nerve fibres and Schwann cells (Grondal et al. 1988; see Kreutzberg et al. 1986; Nagata et al. 1984; Nagy and Daddona 1985; Pull et al. 1972; Scott 1965, 1967; Suran 1974a). Activity in glial cells has also been shown to be high (Schoen et al. 1987). Although the spinal cord overall exhibits moderate activity of 5'-nucleotidase the substantia gelatinosa has very high levels of activity in mice (Scott 1967). However, no reactivity was seen in this area in cats (Wolman et al. 1970) and studies by Suran (1974b) showed very low activity of this enzyme in cats compared to mice. In the rat 5'-nucleotidases have been shown to be in the DRG of small primary afferent neurones (Nagy and Daddona 1985) as well as in other areas of the brain (Schoen et al. 1987).

Another intracellular source of adenosine is the conversion of s-adenosylmethionine (SAM) to s-adenosylhomocysteine (SAH) by the enzyme SAH-hydrolase. Hydrolysis of SAH can also give rise to adenosine.

Production of adenosine can also occur extracellularly from the release of ATP into the intracellular space (via release in a calcium dependent manner, exocytosis, or cell leakage due to damage). Once in the interstitial space ATP can be converted to adenosine via ecto-ATPase, ecto-ADPase and ecto-5'-nucleotidase. Ecto-5'-nucleotidase is a homodimer which is linked to the plasma membrane

(see Meghji 1991). The active site of this enzyme therefore faces the cytosol. Ecto-5'-nucleotidase has been shown in the brain of rats (Fastbom et al. 1987a; Schoen et al. 1987) and humans (Fastbom et al. 1987b). The spinal cord of humans, especially the substantia gelatinosa, has been shown to have high levels of activity of this enzyme (Fastbom et al. 1987b). Ecto-5'-nucleotidase has also been shown to be associated with the plasma membrane of glial cells and astrocyte processes (see Kreutzberg et al. 1986; Schoen et al. 1987; Schubert et al. 1983) as well as neuronal cells (Kreutzberg and Hussain 1984; Kreutzberg et al 1986; Marani 1977).

However, although ATP has been demonstrated to be stored and released from synaptic bound vesicles in a calcium dependent manner (Blaschko et al. 1956; Poisner and Douglas 1968; Unsworth and Johnson 1990; White and MacDonald 1990; White et al. 1985; Winkler and Westhead 1980; Zimmermann 1978, 1988) the evidence for the storage and release of adenosine in this fashion is far less clear (MacDonald and White 1985; Sweeney et al. 1989; White and MacDonald 1990).

1.1.3: Storage and Release of Purines.

ATP is stored in synaptic vesicles (Poisner and Douglas 1968; Unsworth and Johnson 1990; Winkler and Westhead 1980; Zimmermann 1978, 1988). Traces

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of adenosine have been shown to be stored in vesicles in the Torpedo electric organ (Zimmermann 1978) but apart from this one study evidence is lacking.

ATP was originally shown to be released in the periphery from antidromic stimulation of sensory nerves in the rabbit ear (Holton 1959). This has subsequently been demonstrated for a variety of other peripheral tissues (Burnstock et al. 1970; see Hoyle and Burnstock 1991; White and MacDonald 1990). Release of ATP in the dorsal horn of the spinal cord has been shown in response to peripheral vibration and activation of the Pacinian corpusc les as well as to hair deflection (Fyffe and Perl 1984; Hongo et al. 1968; Hunt 1961; Salter and Henry 1985, 1987, 1989) suggesting release from myelinated, low-threshold fibres. Rat spinal cord synaptosomes have also been shown to release ATP evoked by capsaicin, K^+ or veratridine in a calcium dependent manner (Hoehn and White 1989; Sweeney et al. 1989; White et al. 1985).

Adenosine has been shown to be released from small non-myelinated fibres of the vagus nerve (Straub 1979). However, the evidence for the production and release of adenosine *per se* from either primary afferent terminals or intrinsic cells within the dorsal horn is far from clear. Whilst *in vitro* studies by Sweeney et al. (1989) have shown a capsaicin induced release of adenosine from dorsal horn synaptosome preparations, this release was inhibited by up to 80% by the addition of inhibitors of ecto-5'-nucleotidases, suggesting that the majority of capsaicin evoked release of adenosine results from the breakdown of nucleotides (Sweeney et al 1989). However, in rat brain synaptosomes the addition of inhibitors of ecto-5'-nucleotidase did not fully abolish the basal extra-synaptosomal accumulation of adenosine (reduced by 74%) the veratridine-evoked accumulation (reduced by 46%) or the K^+ evoked accumulation (reduced by 33%) (MacDonald and White 1985). This strongly suggests that, like the spinal cord, the major source of adenosine is from the conversion of ATP, although a greater proportion of adenosine accumulation in the brain is as a consequence of release of adenosine *per se* (MacDonald and White 1985).

The release of adenosine from dorsal horn synaptic vesicles has also been shown in response to morphine (Sweeney et al. 1987a; Sweeney et al. 1989). However, inhibitors of ecto-5'-nucleotidase were not used in the former study (Sweeney et al. 1987a) so it is not certain that a proportion of adenosine did not arise from the release of ATP.

Evidence for the release of purines from small diameter neurones comes from capsaicin studies. 1). The release of adenosine *in vitro* was reduced by the subcutaneous pretreatment of capsaicin in neonatal animals, suggesting that the source of adenosine is from capsaicin sensitive, unmyelinated afferents. 2). Direct application of capsaicin onto synaptosomes caused a Ca^{++} dependent release of

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adenosine from dorsal but not ventral horn synaptosomes (Sweeney et al. 1989). However, again this was blocked by the addition of ecto-5'-nucleotidase inhibitors, suggesting that the initial release is of ATP, which is subsequently converted to adenosine. Interestingly, the neonatal and adult pretreatment with capsaicin only decreased adenosine release by 50% suggesting an additional source of adenosine (or ATP) must therefore come from cells intrinsic to the spinal cord or from myelinated neurones, which are not affected by the capsaicin treatment. Note it has been shown that glial cells can release purines (Caciagli et al. 1988). This would also fit with the immunocytochemical studies of Braas et al. (1986) in that the majority of the staining for adenosine was in terminals and fibres within the substantia gelatinosa. This could either be in the terminals of primary afferents or in terminals of intrinsic cells at this location.

There is conflicting evidence for the role of the nucleoside transporter in the release of adenosine, since these transporters are bi-directional (Fredholm et al. 1983; see Geiger and Fyda 1991; Phillis and Wu 1981; Sweeney et al. 1993; Young and Jarvis 1983). It has been suggested that cytoplasmically formed adenosine must have a nucleoside transporter in the cell membrane in order to facilitate its passage into the extracellular space (Fredholm et al. 1980; Meghji and Newby 1990; Phillis and Wu 1981; Sweeney et al. 1993; White and MacDonald 1990) further suggesting that adenosine is not being released from vesicles in a Ca²⁺-dependent manner. It has also been reported that the morphine induced release of adenosine also occurs via the dipyridamole-sensitive, but (NBI) nitrobenzylthioinosine -insensitive nucleoside carrier (Sweeney et al. 1993). Other groups have reported indirect evidence that transporters are not required for the release of adenosine (see refs in Geiger and Fyda 1991). Since these transporters are known to be bi-directional and since they also aid in the reuptake of adenosine, as discussed later in 1.1.6.2, they may have a dual function.

1.1.4: Purine Containing Neurones.

Although the evidence for the release of adenosine per se is still a subject of a great deal of speculation, work by Braas et al (1986) has demonstrated adenosine-like immunoreactivity within select areas of the central nervous system. This immunoreactivity does not appear to be solely due to accumulation due to hypoxia or stressful events, since in these states, although the levels of adenosine did increase, the distribution stayed the same (Braas et al. 1986). Adenosine was shown to be located within neuronal fibres and axon terminals of neurones within the substantia gelatinosa (Braas et al. 1986). This suggests two possible locations for adenosine, a pre-synaptic location of adenosine within the primary afferent terminal

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and within the terminals of interneurones within the substantia gelatinosa. However, a location within the primary afferent terminals is favoured by many, since in addition to the immunocytochemistry for adenosine, the uptake site shown by nitrobenzylthioinosine binding and adenosine deaminase, (processes by which adenosine is removed from the synapse and deaminated to inosine), have been shown to be located in greater levels in the dorsal compared to the ventral roots, and decrease with capsaicin pretreatment (Geiger and Nagy 1985, 1986; Nagy and Daddona 1985).

Additional evidence also suggests a post-synaptic location of adenosine within cells intrinsic to the dorsal horn. It has been demonstrated in other areas of the brain that adenosine per se and ATP are released in response to activation of AMPA and N-methyl-D-aspartate-receptor (NMDA) activation (Craig and White 1992, 1993; Hoehn and White 1990a, b, c; Hoehn et al. 1990; Manzoni et al. 1994). Although the NR1 subunit for the NMDA-receptor is located in primary afferent neurones (Tohyama et al. 1994) this configuration is thought not to be functional as the receptor must be in the heteromeric, dimeric form of NR1/NR2 (see Hollmann and Heinemann 1994). Since predominantly the functional NMDA-receptor is known only to be located on cells intrinsic to the spinal cord, if adenosine release was controled by the NMDA-receptor then this would place adenosine in interneurones within the spinal cord.

It is probable that adenosine may have a heterogeneous location in both the primary afferent terminals as well as intrinsic neurones of the substantia gelatinosa. Whatever the precise location, it is most definitely found in the dorsal horn of the spinal cord, in the most superficial laminae, so it is located at a prime site for modulation of sensory inputs (Dunwiddie 1990; Fredholm and Hedqvist 1980; Pearson 1968) from probably at least two different locations.

1.1.5: Adenosine Receptors.

The purino-receptors are classified on their ability to be activated by adenosine or ATP (Burnstock 1978). The receptors for ATP are known as the P_2 and for adenosine, P_1.

There are currently known to be at least 15 cloned receptors for ATP, linked to ion channels and G-proteins. These will not be discussed in the context of this thesis. To date there are four cell surface receptors for adenosine (Collis and Hourani 1993; Fredholm et al. 1994; Jacobson et al. 1992; Linden 1994; Palmer and Stiles 1995; Ribeiro and Sebastiao 1986; Sebastiao and Ribeiro 1996). The P_1 receptors are further subdivided on the basis of inhibition or stimulation of cyclic-

AMP formation (Londos and Wolff 1977; Londos et al. 1980; Van Calker et al. 1979).

The A_1-receptor for adenosine is linked to the G-proteins G_i and G_o (Jockers et al. 1994; Murayama and Ui 1984; Ramkumar and Stiles 1988) and inhibits the formation of cAMP. Using purified G-proteins, the G-proteins $G_i\alpha$ -1-3 and $G_o\alpha$ were shown in the presence of $\beta\gamma$-subunits to~~~facilitate the high affinity binding of~~~agonists~~~ to bovine A_1-receptors (Freissmuth et al 1991; Munshi et al. 1991). The $G_i\alpha$-3 appeared to react with the highest affinity. The interactions between the G_i-subunits and the A_1-receptors can differ between species (Jockers et al. 1994). This implies that since the A_1-receptor can couple to a variety of G-proteins there is no simple signal transduction mechanism for this receptor (see Palmer and Stiles 1995). Whilst the evidence for an A_1-receptor inhibition of adenylate cyclase is well known (Londos and Wolff 1977; Londos et al. 1980; Van Calker et al. 1979) there is also a reported action on phosphatidylinositol-specific phospholipase C. This is somewhat confusing since the A_1-receptor has been shown to inhibit PLC (Long and Stone 1987) to stimulate (Gerwins and Fredholm 1992) or to have no effect on this second messenger (Nanoff et al. 1990) depending on which cell type is being investigated.

The A_2-receptors are linked to G_s and cause the formation of cAMP (Daly et al. 1983; Sattin and Rall 1970; Van Calker et al. 1979). There are two subclasses of the A_2 receptors the A_{2a} and the A_{2b} (Brackett and Daly 1994; Daly et al. 1983) and possibly further subtypes of the A_{2a}-receptor (Palmer et al. 1994). Both have been cloned in a variety of species (see Sebastiao and Ribeiro 1996). The A_{2a}-receptor causes activation of adenylate cyclase as does the A_{2b}-receptor (Fredholm et al. 1994; see Sebastiao and Ribeiro 1996). The A_{2a}-receptor is linked to both G_s and G_{olf} and has more discrete distribution than the A_{2b}-receptor.

The A_{2b}-receptor is present in low abundance in most cells (Daly et al. 1983). High concentrations of adenosine are required to activate this receptor, activation of which causes the accumulation of cAMP via the G-protein Gs as well as activation of calcium channels via the activation of phospholipase C (Yakel et al. 1993). In astrocytes the A_{2b}-receptor is responsible for the adenosine-induced accumulation of cAMP (Peakman and Hill 1994; Van Calker et al. 1979).

There is also an A_3-receptor. This receptor shows a large species difference in terms of structure / pharmacology / distribution. Although this receptor has been cloned in the rat (see Linden 1994; Zhou et al. 1992) to date there is no evidence for this receptor in the rat's central nervous system (Linden 1994). This receptor is also G-protein linked to G_i or G_o, but does not couple to adenylate cyclase and may be linked to inositol 1, 4, 5-triphosphate production and the increase in

intracellular calcium (Linden 1994; Ramkumar et al. 1993; Ribeiro and Sebastiao 1986). There is also a "P" site for adenosine, which requires the presence of the purine ring for activation and is believed to have an intracellular location (see Reddington and Lee 1991).

The A_1 and A_2 receptors have been shown to be located in the central nervous system (Goodman and Snyder 1982) and within the spinal cord in both the ventral and dorsal horns (Geiger et al. 1984; Goodman and Snyder 1982). The A_1-receptors have been shown to be located primarily on intrinsic cells within the spinal cord, since pretreatment with neonatal capsaicin, dorsal rhizotomy and hemitransections did not alter the binding of [^{3}H]cyclohexyladenosine (CHA) (Geiger et al. 1984). Kainic acid injections into the dorsal horn of the spinal cord decreased binding by 33% and so the A_1-receptors are believed to be located, in the main, on intrinsic cells within the dorsal horn (Geiger at al. 1984). It is surprising that since the techniques employed to reduce the number of primary afferent terminals did not decrease binding at all, that the loss of second order neurones by kainic acid destruction, did not give rise to a more dramatic decrease in binding. It may be that due to the uniform low level binding seen throughout the spinal cord (Choca et al. 1988) that only a small decrease in binding is able to be detected after kainic acid destruction.

Other binding studies have demonstrated low levels of binding of tritiated Phenylisopropyladenosine (PIA) and NECA. PIA is selective for the A_1-receptor whereas 3'-N-ethyl-carboxamidoadenosine (NECA) is equipotent for the A_1 and A_2 receptors (Fredholm et al. 1994). It was found that the highest binding densities were in the substantia gelatinosa, followed by laminae X, with the rest of the spinal cord having a uniform low levels of binding sites (Choca et al. 1988). With the use of N6-cyclopentyladenosine (CPA) to block the binding of NECA to the A_1-receptors it was shown that binding with NECA was now more predominant in the ventral horn, demonstrating a more ventral location for the A_2-receptor (Choca et al. 1988). As with the findings of Geiger et al. (1984) unilateral dorsal rhizotomy, hemitransection and complete transection of the spinal cord failed to decrease binding with the above agonists (Choca et al. 1988), again demonstrating a post-synaptic location of these P_1-receptors. Kainic acid injection into the dorsal horn of the spinal cord decreased binding by about 40% at the site of injection and in some cases kainic acid injection decreased the binding of tritiated PIA and NECA within the substantia gelatinosa (Choca et al. 1988).

In other areas of the rat central nervous system adenosine receptors labeled with [^{3}H]CHA are found on excitatory cells, such as granule cells within the cerebellum (Goodman et al. 1983). In the spinal cord it is known that the majority of labelling for the A_1-receptors is located with a dense area of binding within the

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substantia gelatinosa (Choca et al. 1988; Geiger et al. 1984) suggesting that these receptors are probably not on the terminals of primary afferent neurones but on cells intrinsic to the spinal cord. However, it can not be confirmed from these studies if the A_1-receptors are on inhibitory or excitatory neurones.

There are also known to be adenosine and ATP receptors on astroglial cells (Murphy and Pearce 1987). Adenosine has been shown to stimulate and inhibit the formation of cAMP in astrocytes, suggesting that both the A_1 and A_2-receptors are present. As well as the P_1-receptors, the P_2-receptors are also located on these cells, since it has been shown that stimulation of ATP receptors can give rise to an increase in intracellular calcium, from stores located in the astocyte, to release adenosine and other purines (Salter and Hicks 1994, 1995). It has been postulated that this subsequent release of adenosine could then have a neuroprotective role in ischemia (Salter and Hicks 1994, 1995) as in other areas of the CNS (Dragunow and Faull 1988; Phillis et al. 1979a;White 1994; Zhang and Murray 1991; Zhang et al. 1994).

Electrophysiological studies using patch clamp techniques have demonstrated both a pre and post-synaptic effect of adenosine in inhibiting excitations evoked by dorsal root stimulation (Li and Perl 1994). The pre-synaptic effects are to depress the excitatory post-synaptic currents and the post-synaptic effects are to hyperpolarize the post-synaptic membrane via the activation of a potassium current. This functional study places a proportion of the A_1-receptors on the primary afferent terminals, especially those concerned with the activation of poly-synaptic pathways (Li and Perl 1994). The pre-synaptic role comes from analysis of synaptic currents, in that the excitatory post-synaptic current does not decrease in amplitude but does decrease in frequency (Li and Perl 1994) suggesting there is less quantal release from the primary afferent terminals.

Human studies on post-mortem brain samples from subjects without known neurological disease showed low levels of binding of A_1-receptors in the substantia gelatinosa with [^{3}H]CHA (Fastbom et al. 1987b). Note that there were shown to be distribution differences in areas of the brain in humans compared to rats. Along with this receptor high levels of staining for the enzyme 5'-nucleotidase was also observed in human substantia gelatinosa (Fastbom et al. 1987b) as in other areas of the brain (Lee et al. 1986).

From all these observations the location of the A_1-receptors appear to be located within the dorsal horn of the spinal cord, primarily on cells intrinsic to the substantia gelatinosa (Choca et al. 1988; Geiger et al. 1984) with the majority of the binding for the A_2-receptor being placed in the ventral horn on intrinsic neurones (Choca et al. 1988). Functional studies have demonstrated a location of the A_1-

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receptor on a proportion of the primary afferent terminals (Li and Perl 1994).

1.1.6: Inactivation Processes.

The effects of adenosine can be halted by several inactivation processes. It can be taken up into neighbouring cells, by the high and low affinity uptake sites. It can be converted extracellularly by the ecto-enzyme adenosine kinase to 5'-AMP. Once taken up into cells it can be converted to inosine via adenosine deaminase (this may also be present extracellularly although this is not fully clear) converted to 5'-AMP by cytoplasmic-adenosine kinase or incorporated into s-adenosylhomocysteine by SAH-hydrolase. These will be discussed below.

1.1.6.2: Adenosine Transporters.

Adenosine can be taken up into a variety of cells via membrane bound transporters which are bi-directional (Fredholm et al. 1980; Young and Jarvis 1983). Transport of adenosine across plasma membranes in the CNS was first described by Santos et al. (1968) and has subsequently been demonstrated in synaptosomes from the Torpedo electric organ (Zimmermann et al. 1978) rat cerebral cortical synaptosomes (Bender et al. 1980; Marangos et al. 1982; Shank and Baldy 1990) cultured neurones (Thampy and Barnes 1983a) as well as glial cells (Trampy and Barnes 1983b).

Nucleoside transport can be by simple diffusion due to concentration gradients (Bender et al. 1980; Young and Jarvis 1983) or via a carrier mediated diffusion process (Bender et al. 1980; Geiger and Nagy 1984; Gu et al. 1993; Jones et al. 1992; Shimizu et al. 1972). Adenosine may be transported in a sodium dependent manner which has been described in a number of peripheral tissues (Johnston and Geiger 1989; Williams et al. 1989). This has also been demonstrated in nervous tissue along with a requirement for Ca^{++} (Bender et al. 1980; Johnston and Geiger 1989).

There are two main nucleoside transporters labelled by nitrobenzylthioinosine (NBI) and dipyridamole (DPR) and are classified by the sensitivity of these two compounds in binding to the uptake site. In mammalian brains there are DPR- and NBI-sensitive uptake sites as well as a DPR-sensitive but NBI-insensitive site (see Deckert et al. 1988b). However, there are marked species differences. NBI has been shown to be a potent inhibitor of nucleoside uptake in the rat (Marangos et al. 1982). Whilst DPR labelled sites have been demonstrated in

man, dogs and are prevalent in the guinea pig, where it is twice as abundant as the NBI labelled site, (Deckert et al. 1988a; Marangos and Deckert 1987; Marangos et al. 1985) DPR has not been shown to bind to any extent in the rat brain (Davies and Hambley 1983; Marangos and Deckert 1987).

In the guinea pig at least, DPR binding demonstrates an abundant low affinity binding site, since the DPR sensitive site transports adenosine in the millimolar range, compared to the NBI site which transports the major proportion of adenosine in the micromolar range (although a proportion of NBI inhibited uptake is in the nanomolar range) (Deckert et al. 1988a; Marangos et al 1982, 1985). However, since DPR is highly lipophilic it may bind to other plasma bound transporters as well as intracellular transporters and this may account for its increased binding sites compared to NBI (Torres et al. 1987).

The NBI labeled uptake site has a high affinity for adenosine over the other purines and pyrimidines (Geiger and Nagy 1984; Geiger et al. 1985). This site has been demonstrated in rat brains (Geiger and Nagy 1984; Geiger 1986). Both the dorsal and ventral spinal cord along with their associated roots exhibit a single class of high affinity binding site labeled by $[^3H]NBI$ (Geiger and Nagy 1984, 1985). The dorsal grey matter of the cervical and lumber enlargements have high levels of binding, especially concentrated in the substantia gelatinosa. This binding in the dorsal roots is decreased by 35% after neonatal pretreatment with capsaicin which did not affect binding in the dorsal spinal cord. This suggests that a proportion of these transporter sites are located on small diameter afferents fibres but that the majority of the binding (65%) must be located on myelinated fibres or other cell types that are intrinsic to the dorsal root and not affected by the capsaicin (Geiger and Nagy 1985). Recent human post-mortem studies have also shown NBI labelled uptake sites in the spinal cord, in the substantia gelatinosa (Glass et al. 1996).

Further evidence to support a location on intrinsic cells is that uptake sites have been demonstrated on astrocytes and glial cells in primary cultures (Hertz 1978; Meghji et al. 1989; Thampy and Barnes 1983a, b). In astrocytes Hertz (1978) demonstrated saturable and non-saturable uptake mechanisms. The saturable uptake showed high affinity.

Geiger and Nagy (1984) have also shown that $[^3H]NBI$ binding sites in the rat brain correlate closely to the immunoreactivity for the enzyme adenosine deaminase. However, unfortunately there appears to be little correlation in the brain between NBI labelled sites and adenosine receptors labeled by $[^3H]CHA$ (Geiger and Nagy 1984). An explanation for this is that CHA binds mainly to the A_1 receptor and a correlation between NBI sites and the A_2 receptors was not undertaken due to the lack of radio-ligands for this receptor.

Since a proportion of [^{3}H]NBI binding sites are on the unmyelinated primary afferents and within the substantia gelatinosa, a function of adenosine in sensory processing, especially of high-threshold inputs, has been suggested (Bisserbe et al. 1985; Geiger and Nagy 1985).

1.1.6.3: Adenosine Deaminase.

The enzyme adenosine deaminase converts adenosine to the inactive inosine. This enzyme is thought to be mainly cytosolic (Meghji and Newby 1990) and there is some evidence for an ecto-enzyme in the brain (Franco et al. 1986). Correlation between uptake sites and adenosine deaminase suggests that the presence of an uptake site is paramount for intracellular enzymes to inactivate adenosine (Geiger and Nagy 1984). It is thought that cells that have an elevated concentration of this enzyme over basal levels may use adenosine as a neurotransmitter (Nagy et al. 1990). However, problems may arise in using this enzyme as a neuronal marker, as there are marked species differences (see Phillis 1990).

Adenosine deaminase activity has been shown in the dorsal roots as well as Lissauers tract and in the spinal cord, especially small type B cells in dorsal root ganglia (Geiger and Nagy 1986; Nagy and Daddona 1985; Nagy et al. 1984). Immunoreactivity in the spinal cord was confined to fibres and neurones in lamina I and IIo (Nagy and Daddona 1985). The adenosine deaminase containing neurones were shown to coexist with somatostatin but not substance P or fluoride resistant acid phosphatase (FRAP), an important purine handling enzyme associated with the formation of ATP. FRAP has also been shown in a separate population of small type B cells in the dorsal root ganglia (see Knyihar-Csillik and Csillik 1981; Nagy and Daddona 1985; Nagy et al. 1984) as well as in nerve terminals in deeper layers of the substantia gelatinosa (Nagy and Hunt 1982).

Since capsaicin pretreatment abolished all adenosine deaminase immunoreactivity in lamina IIo but only partially depleted staining in lamina I this suggests that a proportion of adenosine deaminase containing neurones may be in thinly-myelinated afferents or in cells intrinsic to the spinal cord (Nagy and Daddona 1985).

1.1.6.4: Adenosine Kinase.

As well as being removed from the synaptic cleft by uptake, adenosine can also be inactivated by adenosine kinase, an enzyme found extracellularly as well as in the cytoplasm. Adenosine kinase converts adenosine to 5'-AMP predominantly

via an ecto enzyme. This enzyme has high affinity for adenosine and works in the micromolar range (Santos et al. 1968).

Adenosine kinase has been purified from rat heart (Fisher and Newsholme 1984). Adenosine kinase is regulated by adenosine itself,
Sinceas the adenosine concentration increases the enzyme becomes inactive and then the main pathway for metabolism is then via adenosine deaminase. Although there is a large species and tissue variation in the activity of adenosine kinase and adenosine deaminase the Kms for these enzymes fits with the above, in that the Km for adenosine kinase is in the order of 1-2 magnitudes lower than the Km for adenosine deaminase (Arch and Newsholme 1978). So under normal physiological conditions the steady state reaction for the formation and removal of adenosine results from the breakdown of 5'-AMP to adenosine via the activity of 5'-nucleotidases and the conversion of adenosine to 5'-AMP by adenosine kinase. Only when the concentration of adenosine increases and adenosine kinase is inhibited by this rise in adenosine does the conversion of adenosine to inosine via adenosine deaminase take place, see Figure 1.

1. 5'-nucleotidase
2. ADO-Kinase
3. ADO-Deaminase
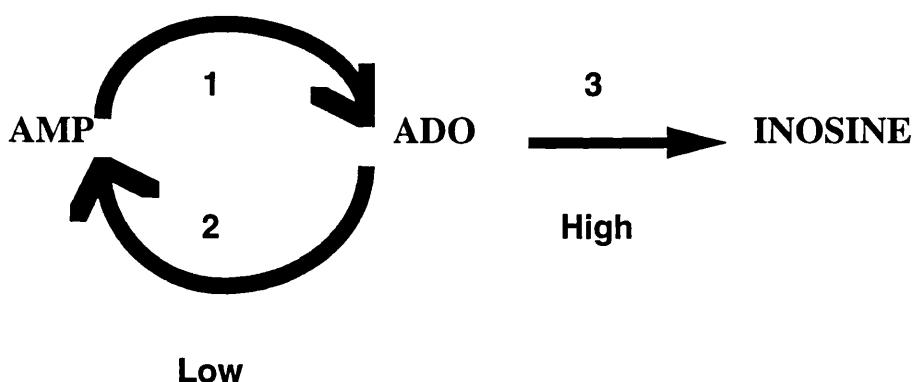
Figure 1. Under normal physiological conditions adenosine, converted from adenosine monophosphate (AMP), is converted back to AMP via phosphorylation by Adenosine kinase. If the concentration of adenosine increases, this enzyme is inhibited and adenosine deaminase takes over to deaminate adenosine to inosine.

It is known that adenosine kinase is present in the brain tissue of rats but the precise anatomical location of this enzyme has not been undertaken (Arch and Newsholme 1978). It has however been shown in other areas of the brain that this

enzyme is important in regulating adenosine levels (Pak et al. 1994; Pedata et al. 1994; White 1994, 1996) and is located as an ecto-enzyme (Ehrlich et al. 1986, 1990). However, since adenosine kinase inhibitors have been shown to produce antinociceptive effects in rats and mice this would suggest that this enzyme is important in regulating the activity of endogenous adenosine at the spinal cord level (Golembiowska et al. 1966; Keil and DeLander 1992; Poon and Sawynok 1995).

1.1.6.5: S-adenosylhomocysteine hydrolase.

Once adenosine has been taken up into cells it can also be converted to s-adenosylhomocysteine by SAH-hydrolase. Although this is believed to only be a minor pathway for the conversion of adenosine it has been demonstrated in nervous tissue (Reddington and Pusch 1983).

1.1.7: Conclusions.

There are many important indicators to be considered in the role of a chemical as a putative neurotransmitter or neuromodulator. The enzymes responsible for its synthesis must be demonstrated to be present, it may be stored in synaptically bound vesicles (although this is not always the case, as with nitric oxide, which is synthesized de novo), it can be released in a quantal fashion, the enzymes or inactivation processes are available close to its release site and the receptors are also present.

At the spinal cord level adenosine fulfils many of these criteria. The enzymes responsible for adenosine synthesis are known to be in the dorsal root ganglia. There is also ample evidence for the presence of the ecto-enzymes responsible for the cleaving of adenosine from the release of ATP in many neuronal tissues. The enzyme 5'-nucleotidase, which converts AMP to adenosine is found in neuronal tissue (Kreutzberg et al 1986; Scott 1967; Suran 1974a) on glial cells (Schubert et al 1983) and also in primary afferents (Nagy and Daddona 1985) and has been shown in the spinal cord (Goodman and Snyder 1982; Scott 1967). The substantia gelatinosa shows high immunoreactivity for ecto-5'-nucleotidase (Fastbom et al. 1987b). The evidence for the release of adenosine per se is not at all together watertight, and it is possible that the main source of spinally released adenosine may come from the conversion of ATP, It is important that ecto-5'-nucleotidase is present, to convert ATP to adenosine.

Adenosine immunoreactivity has been shown to be located within the substantia gelatinosa of the dorsal horn of the spinal cord (Braas et al. 1986). There

is a distinct banding of adenosine immunoreactivity within the Substantia gelatinosa compared to other lamina of the spinal cord. This suggests that adenosine may be present above general background levels in either the terminals of the primary afferents or in cells intrinsic to the substantia gelatinosa.

The release of adenosine per se in the spinal cord is anything but clear. The basal release and a capsaicin and morphine evoked release of adenosine has been shown in spinal cord synaptosomes by Sweeney et al. (1989). However, in the two former experiments the accumulation of adenosine from synaptosomes was significantly inhibited by the addition of an inhibitor of ecto-5'-nucleotidase (Sweeney et al. 1989) suggesting that in fact the major source of adenosine was from the conversion of ATP. Indeed, it would seem surprising that cells would convert AMP back to adenosine before being released. Whole brain synaptosome studies by MacDonald and White (1985) have shown a more convincing release of adenosine *per se*, in that addition of ecto-5'-nucleotidase caused only a 50% inhibition in adenosine accumulation suggesting that a greater proportion of adenosine was being released from neuronal tissue. Figure 2 shows the probable release sites of adenosine in the dorsal horn.
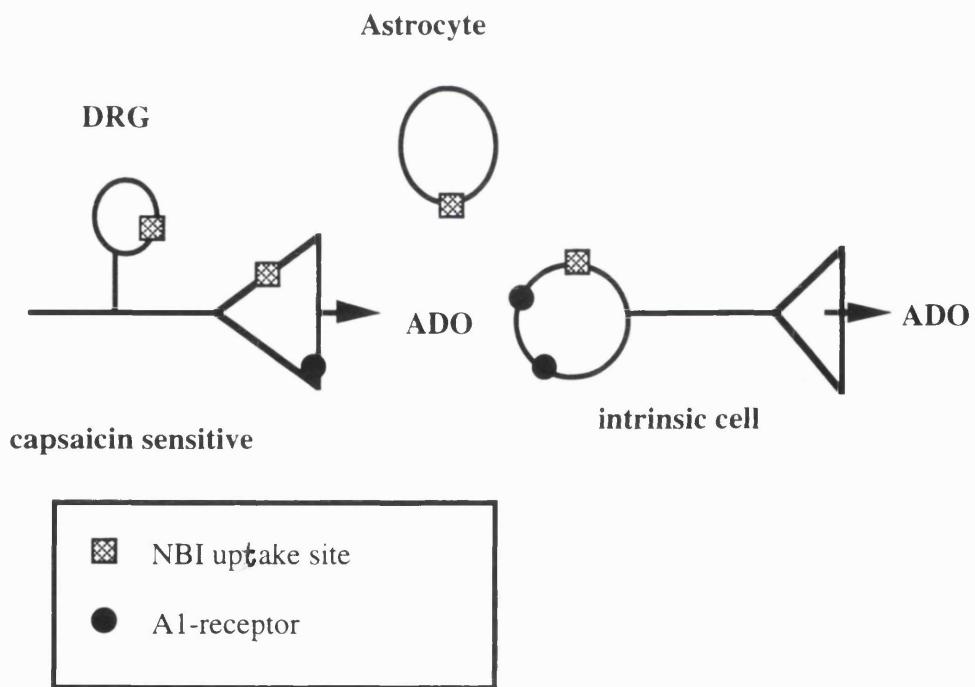
Figure 2. *The A_1-receptor has a predominantly post-synaptic location with a functionally demonstrated pre-synaptic location. The uptake-sites labelled by NBI are known to be present on astrocytes, DRG, afferent terminals as well as neurones intrinsic to the spinal cord. Adenosine could be released from primary afferent terminals and cells intrinsic to the spinal cord, especially in the substantia gelatinosa.*

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However adenosine may be released, the receptors for adenosine (A_1 and A_2) are known to be located within the spinal cord. The A_1-receptor is located predominantly in the dorsal horn of the spinal cord of rats, on cells that are intrinsic to the substantia gelatinosa (Choca et al. 1988; Geiger et al. 1984; Goodman and Snyder 1982). In human studies on post-mortem brains the A_1-receptors have also been placed within the substantia gelatinosa (Fastbom et al. 1987b). Functional studies have also demonstrated that the A_1-receptors may also have a presynaptic location (Li and Perl 1994). However, the majority of the the receptors and its effects come from a post-synaptic located receptor (Choca et al. 1988; Goodman and Snyder 1982; Geiger et al. 1984; Li and Perl 1994) see figure 2.

The uptake sites for adenosine are also known to be located within the dorsal horn of the spinal cord both on myelinated and unmyelinated primary afferent terminals and their associated roots (Geiger and Nagy 1984, 1985) as well on intrinsic cells, such as glia (Hertz 1978; Meghji et al. 1989; Thampy and Barnes 1983a, b). The main cytosolic enzyme for adenosine conversion to inosine, adenosine deaminase, has also been located in the dorsal root ganglia of small diameter afferents (Geiger and Nagy 1986; Nagy and Daddona 1985; Nagy et al. 1984) as well as in myelinated afferents (Nagy and Daddona 1985).

The evidence presented here shows that adenosine is present along with its associated receptors, within the outer lamina of the dorsal horn of the spinal cord, a site well documented to influence somatosensory processing. Thus a role in the processing of sensory inputs can be inferred. I believe that adenosine has a neuromodulator role. In that, due to the predominant processes to convert adenosine back to AMP it is highly doubtful that adenosine is released as such to any degree. However, this does not mean that adenosine does not have an important role in the spinal cord. In fact it is quite the opposite, adenosine has a very important role in the the maintenance of a balanced state between excitations and inhibitions.

1.2.1: Mechanisms of Pain Transmission.

The perception of "pain" is a signal that damage has been done, or about to be done to a part of the body. Although we try to dampen down or inhibit the sensations of pain, it must be remembered that initially these sensations are important, in that they protect the body from further damage. "Pain" is a combination of both a physical sensory perception and an emotional response. It is therefore not a simple process, in that it the stimulus received is not always the same as the stimulus perceived.

There are receptors throughout the body that can convey these external stimuli into   electrical signals. These receptors can be evenly distributed throughout the tissue or can be located in discrete clusters known as sense organs, such as the eye and ear. The external stimuli that make up the sensations perceived by the body (sound, sight, smell, taste, touch and proprioperception) enable the body to be aware of its surroundings. These stimuli, can be light, chemicals, sound waves and are known as the adequate stimulus. This means that a sensory receptor will respond only to a specific quality. The appropriate, adequate stimulus can produce changes in the membrane potential of a sensory receptor by converting the energy of the stimulus to a change in the receptor permeability to ions. The signal is "transduced" from one form into another, thus causing an action potential. The information this action potential conveys is determined by the type of receptor which has been activated along with the frequency and strength of the signal.

The specialized sense receptors that are located in tissue are known as somatovisceral sensory receptors. In the skin and viscera mechano-receptors which respond to changes in pressure, thermo-receptors, responding to changes in temperature and nociceptors, which respond to stimuli in the noxious range (noxious temperature, chemical or mechanical) can be found. These were first described by Sherrington (1906) who defined a sensory receptor as "one which signalled damage or threat of damage" to the skin. These C-nociceptors innervate the epidermis and their ending are free, in that they are not encapsulated. This is in contrast to the endings that make up specialized sense organs, which respond to low threshold stimuli as well as the $A\delta$-nociceptors, which may be encapsulated or partially covered by Schwann cells or keratinocytes respectively (Kruger et al. 1981; see Meyer et al. 1994).

1.2.2: Cutaneous Receptors.

These receptors as the name suggests are located in the skin and surrounding tissue. They include mechano-, chemo, and thermoreceptors. The classification of these receptors is not always easy to define. The terminology of "receptor" is usually thought of as a protein structure embedded in a cell membrane. However, the terminology describing "receptors" for transmission of somatosensory information can incorporate the fibre type they are innervating, the proposed transduction mechanism, as well as the adequate stimulus which activates them, often the term "unit" is used to encompass this. Generally there are "units" which respond to non-painful, low threshold stimulation and these are conveyed by the $A\beta$-fibres and associated endings. The $A\delta$-fibres can be nociceptive or non-nociceptive. The

nociceptors associated with C-fibres are often thought of as encoding only noxious stimulation, and are often termed polymodal since they can respond to a variety of adequate stimuli. The transduction mechanism associated with the free endings of these fibres has still to be ascertained. Some C-fibres however, also convey low-threshold information. The Aδ-fibres have also been shown to behave as polymodal receptors in their own right as well as Aδ-mechanoreceptors behaving like C-polymodal afferents after sensitization. Often the situation (i e after inflammation) has an effect on the transmission of information.

1.2.2.2: Mechanoreceptors.

Mechanoreceptors respond to mechanical changes in the skin such as indentation and displacement and may be activated by stimuli in the non-noxious range or respond to noxious mechanical stimuli (Burgess and Perl 1967). The cutaneously located receptors can be classified by their adequate stimulus and by their adaptation to the duration of the stimulus. Thus the non-noxious activated mechanoreceptors may be divided into two groups, the slowly adapting and rapidly adapting mechanical receptors. They consist of a specially adapted end-organ associated with an afferent nerve fibre terminal.

The slowly adapting receptors can be further categorized into slowly adapting I (SAI) and slowly adapting II (SAII). The SAI receptors respond to skin deflection and displacement and are associated with Merkel's disk and tactile domes in the epidermis of the skin (for review see Gebhart 1995). The SAII receptors respond to indentation and stretch of the skin, associated with the activation of the Ruffini endings in the dermis. SAI tend to have a small receptive fields in comparison to the SAII. Both these classes of receptors continue to discharge as long as the stimulus is maintained. The fast adapting receptors respond to skin indentation but they signal only the velocity of the stimulus, responding to tapping and vibration and not to sustained pressure on the skin. They respond only briefly when the stimulus is applied and in some cases when it is removed (see Willis and Coggeshall 1991b). The sensory end-organs associated with these stimuli are the Meissner's corpusc les in glabrous (non-hairy skin) and the Pacinian corpuscules in both the subdermal glabrous and hairy skin. As discussed in 1.1.3, activation of the Pacinian corpusc les gives rise to the release of ATP in the dorsal horn of the spinal cord from fast conducting, low threshold fibres and results in an/EPSP followed by [excitatory post-synaptic potential] an IPSP [inhibitory post-synaptic potential] by the subsequent conversion of ATP to adenosine (Fyffe and Perl 1984; Hongo et al. 1968; Hunt 1961; Salter and Henry 1985, 1987, 1989).

The sense organs described above all respond to stimuli in the non-

noxious range and are associated with Aβ-myelinated afferents. However, it must be noted that under certain circumstances the Aβ-fibre evoked responses have been shown to cause enhanced responses, usually associated with C-fibre driven activity, and so may be be responsible for allodynia (Woolf 1991). A recent study has shown that the Aβ-fibres have the capacity to change their phenotype under certain conditions (Neumann et al. 1996).

There are also mechanoreceptors which respond to noxious stimuli. These are the C-mechanoreceptors associated with unmyelinated afferent fibres (Bessou and Perl 1969; Bessou et al. 1971; Georgopoulos 1976; Iggo 1960). They have been shown to make up only 10% of mechanically activated receptors (Georgopoulos 1976). A further complication in classification of mechanoreceptors associated with C-fibres is that they can also be activated by low-threshold stimulation (Bessou et al. 1971). The majority of noxious mechanoreceptors are associated with myelinated afferents (Burgess and Perl 1967; Kruger et al. 1981). These have been shown to be Aδ-high threshold mechanoreceptors (HTM) (Burgess and Perl 1967; Georgopoulos 1976; Lynn and Shakhanbeh 1988). These do not respond to non-noxious mechanical stimulation or to noxious stimulus other than mechanical (Burgess and Perl 1967; Campbell et al. 1979; Lynn and Shakhanbeh 1988) although there are receptors which respond to both noxious mechanical and temperature, these are a separate class and show different properties to the mechano-only receptors (Beck et al. 1974; Georgopoulos 1976; Iggo and Ogawa 1971; Lynn and Shakhanbeh 1988). Both the C-fibre associated, and the Aδ-fibre mechanoreceptors respond to similar thresholds (Georgopoulos 1976) and after sensitization the Aδ-mechanoreceptor can also respond like a C-polymodal receptor (Fitzgerald and Lynn 1977).

1.2.2.3: Thermoreceptors.

There are receptors that convey information on cold and warm-temperatures in the non-noxious range as well as noci-thermoreceptors which respond to temperatures in the noxious range (Georgopoulos 1976; see Willis and Coggeshall 1991b).

The cold receptors are usually associated with non-noxious temperatures and have associated myelinated afferents (Long 1977). There are cold-receptors associated with unmyelinated afferent terminals, but these are few (see Gebhart 1995; Georgopoulos 1976; Willis and Coggeshall 1991b). The warm receptors are believed to be active at normal body temperature; their firing increases as the body

temperature increases and slows as it cools. Their activity is inhibited by temperatures in the noxious range, which activate free nerve endings associated with unmyelinated fibres (see Willis and Coggeshall 1991b). In the rat it has been shown that only the C-fibres are responsible for conveying information on noxious temperature (Lynn and Shakhanbeh 1988) whereas in other species high threshold mechanical Aδ-fibres can convey this information under certain conditions (Fitzgerald and Lynn 1977; Georgopoulos 1976). There are also Aδ-fibres which are sensitive to lower temperatures compared to the Aδ-mechano-thermal fibres (Beck et al. 1974; Georgopoulos 1976; Iggo and Ogawa 1971).

1.2.2.4: Cutaneous Nociceptors.

There are two main categories of cutaneous nociceptor, the Aδ-mechanical and C-polymodal (Bessou and Perl 1969; Perl 1968). These are classified by both the fibre type they are associated with as well as the adequate stimulus required to activate them. Along with these receptors present in the skin which are available to be activated at all times, there is also a group of nociceptors which, for the majority of the time are not available for recruitment. These are known as "silent" nociceptors. Under normal conditions these receptors do not respond to thermal or mechanical stimulus (Lynn and Carpenter 1982). It is believed that this population of receptors must first become sensitized before they can be activated. This may occur after inflammation and their activity appears to be associated with hyperalgesia (Reeh et al. 1987).

The Aδ-mechanoreceptors are believed not to have free endings in that their fine endings are covered by Schwann cells (Kruger et al. 1981). There is also evidence that they may lose the association with the Scwann cells and instead be surrounded by keratinocytes (Kruger et al. 1981; see Meyer et al. 1994). Their endings are located in the epidermis of the skin and are activated by mechanical stimuli in most instances (Burgess and Perl 1967; Georgopoulos 1976; Handwerker et al. 1987; Lynn and Shakhanbeh 1988). Application of noxious heat to these receptors sensitizes them to heat (Campbell et al. 1979; see Dubner 1991; Fitzgerald and Lynn 1977). This characteristic is also thought to play a role in hyperalgesia. Repeated application of intense mechanical stimuli to the same location can decrease the responsiveness of this receptor to mechanical stimuli and abolish it in some instances (Lynn and Carpenter 1982; Perl 1968). Some Aδ nociceptors also respond to mechanical, thermal and chemical noxious stimuli without having to be sensitized first and are known as Aδ polymodal nociceptors (Adriaensen et al. 1980).

The C polymodal nociceptor is associated with the unmyelinated C-fibre.

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As the name suggests these receptors respond to a variety of adequate noxious stimuli (see Bessou and Perl 1969; Croze et al. 1976). Their receptive fields are smaller than the Aδ-nociceptors, ranging from 1mm^2 to 1cm^2, although often the receptive field of these fibres will overlap. Polymodal nociceptors show both adaption and fatigue where the response can diminish or after a repetitive stimulus to the same area (Torebjork and Hallin 1974; Van Hees and Gybels 1972). There are also nociceptors which are activated whilst the stimulus is still in the non-noxious range, and progressively respond as the stimulus intensity is increased (Torebjork and Hallin 1974). As discussed in 1.2.2.3. there is also a population of nociceptors which respond to intense cold temperatures although in comparison to the polymodal nociceptors there are few of these receptors in the skin. Usually, at extreme cold temperatures, pain will arise from changes in the vasculature and release of endogenous mediators.

Along with these nociceptors found in the skin there are also receptors responding to noxious stimuli found in skeletal muscle, joints and visceral tissue, along with receptors which respond to non-noxious stimuli and convey position, movement, as well as mechanical forces (Clark and Burgess 1975; Grigg et al. 1986).

In skeletal muscle there are pressure-pain endings which are associated with unmyelinated afferents, thinly myelinated afferents as well as a few associated with the larger myelinated afferents (see Willis and Coggeshall 1991b). In joints there are receptors associated with both Aδ- and C-fibres which respond to movement of a joint beyond its usual range and mechanical stimulation (Grigg et al. 1986; Schaible and Schmidt 1983a, b). A proportion of these are silent, especially those associated with C-fibres, and are activated only when a joint has become inflamed, especially becoming sensitive to mechanical stimulation (Coggeshall et al. 1983; Grigg et al. 1986). The visceral organs, such as the heart, reproductive organs, respiratory system and gastrointestinal tract have also been shown to be innervated by C-fibres (see Coleridge and Coleridge 1984; Meller and Gebhart 1992; Meyer et al. 1994). It is interesting to note that adenosine has been implicated in cardiac pain arising from ischemia (Meller and Gebhart 1992; Olsson and Pearson 1990) although it has also recently been demonstrated that adenosine can also be antinociceptive in myocardial ischemia (Sylven et al. 1996). Unlike the receptors in the skin, which give a very precise location of the stimulus, pain arising from the activation of deep visceral nociceptors is often very diffuse and poorly localized (Cervero 1991).

1.2.3: Chemicals Contributing to the Activation of Nociceptors.

The peripheral terminal of small diameter neurones, especially in conditions of inflammation may be excited by a number of endogenous chemical mediators (see Rang et al. 1991). These can be released from non-neuronal cells, the afferent fibres themselves and from products triggered by activation of the body's defense mechanisms.

The immune response is split into two components, one which is immunologically non-specific, the innate response and an immunologically specific response, the adaptive response. It is the innate response which results in a wide range of chemical mediators being released from non-neuronal cells and from the conversion of arachidonic acid. These mediators can either activate or sensitize the nociceptors. They include bradykinin, protons, serotonin, histamine, arachidonic acid metabolites, substance P, ATP and adenosine.

Bradykinin and Kalladin are kinins, one found in tissue and one located in plasma. It is cleaved from kininogens in response to tissue injury and can cause vasodilatation, oedema, and mobilizes the arachidonic acid metabolites. There are two receptors for bradykinin, which are both linked to G-proteins. Bradykinin receptors have been shown in the dorsal root ganglia as well as peripheral to the DRG cells (Steranka et al. 1988). The B_1-receptor is expressed less than the B_2-receptor and appears to be induced in chronic inflammation, when its expression has been shown to be upregulated (Hall 1992; Perkins et al. 1993). Pain may arise via the activation of the B_2-receptor, which is abundant in most tissues and causes the activation of the C-polymodal units and to a less extent heat-insensitive units (Lang et al. 1990; Rang et al. 1991; Steranka et a. 1988). The response to bradykinin can be enhanced by prostaglandin E_2, heat and serotonin (Lang et al. 1990).

Protons are found to increase in inflammation and ischaemia and may activate the nociceptors directly as well as sensitizing them to mechanical stimulation (Steen et al. 1992). Protons cause a brief depolarization in many neuronal cells by increasing the membrane permeability to Na^+ and K^+ ions (Krishtal and Pidoplichko 1980) as well as causing a sustained depolarization in sensory neurones which are sensitive to capsaicin (Bevan and Yeats 1991). The proton site is believed to be similar to the one activated by capsaicin (Rang et al. 1991).

Serotonin is released from non-neuronal cells such as platelets, Mast-cells and specialized cells within the digestive tract. Serotonin can cause excitation of

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nociceptive afferents via the activation of its own membrane bound receptors as well as sensitize the nociceptors, especially to bradykinin (Lang et al. 1990; see Rang et al. 1991). _this may be by increasing $[Ca^{2+}]i$ as well as other responses downstream to the receptor._ Serotonin has many subclasses of receptors, most are linked to G-proteins apart from one, the 5-HT$_3$-receptor which is linked to an ion channel. It is this receptor which is believed to be responsible for the activation of sensory afferents and may play a large role in the pain associated with migraine.

Mast cells, as well as releasing 5-HT can also release histamine which causes vasodilatation, oedema and produces an itch sensation (Simone et al. 1991). Adenosine, via the A$_3$-receptor can also cause mast cells to degranulate, thus being pro-inflammatory under these conditions (see Linden 1994). Substance P released from the peripheral terminals of primary afferents (axon reflex) can also cause the mast cells to degranulate giving rise to a red appearance, swelling and itch sensation around the site of damage. Histamine can act at its own receptor, mainly the H$_1$-receptor to activate the afferent fibres by increasing the calcium permeability of the membranes (see Rang et al. 1994).

An important and more complex component in inflammation is the release of arachidonic acid metabolites. Arachidonic acid, _from the conversion of phospholipd_ by phospholipase A$_2$, is subsequently metabolized by two main pathways controlled by two enzymes, cyclo-oxygenase and lipoxygenase. This metabolism gives rise to the leukotrienes, thromboxanes, prostacyclins, and prostaglandins. These are collectively known as the eicosanoids. They do not activate nociceptors directly but sensitize them to other mediators and stimuli. Many agents are employed to prevent their effects and production, such as steroids and the non-steroidal anti-inflammatory (NSAIDs) group of drugs. Whilst the NSAIDs may be effective in acute inflammatory situations they are not in more chronic situations. As the above drugs prevent the conversion of arachidonic acid to these mediators they can only prevent further conversion and can not halt the effects of mediators that have already been released from sensitizing nociceptors.

The afferent fibres themselves can also release mediators. These can be peptides, such as substance P, CGRP and other mediators such as ATP.

The consequences of ATP release from the peripheral terminals are complex. ATP release can excite P$_2$-receptors which can activate the sensory neurones themselves via activation of a mono- and divalent sensitive ion channel (Krishtal et al. 1983). ATP receptors are also present on macrophages and may result in the release of cytokines. ATP can also be converted to adenosine via ecto-enzymes which can activate the P$_1$ receptors. The P$_1$-receptors may be pronociceptive (A$_2$), pro-inflammatory (A$_3$) or antinociceptive (A$_1$). The role of peripheral adenosine is not at all clear and will depend on the relative distribution of

the receptors and levels of adenosine. It has been demonstrated that peripherally administered adenosine can give rise to pain sensations (Taiwo and Levine 1990). However, adenosine can have a dual effect on sensory transmission, since low levels of adenosine activate the A_1-receptor, which will inhibit transmission and higher levels will activate the A_2-receptor which will result in excitation (Fredholm et al. 1994). The A_3-receptor has also been implicated in inflammation via the degranulation of Mast cells (see Linden 1994). The resulting histamine release can contribute to inflammation as discussed above.

1.2.4: From the Periphery to the Spinal Cord.

Whatever the mechanism of activation and transduction, the activation of the peripheral terminals of the afferent fibres results in the generation of an action potential by altering the membrane permeability to ions. The propagation of action potentials relays the electrical signal to the central terminal of the afferent fibre, where neurotransmitters can be released into the dorsal horn of the spinal cord. The use of different types of peripheral stimulation can be employed to study the consequences of activation of these fibre types on the dorsal horn of the spinal cord. Acute peripheral electrical stimulation can be used to observe the different fibre evoked responses before and after pharmacological manipulation. Since electrical stimulation activates the nerve axons directly there is no sensitization of transduction mechanisms as can occur with repetitive natural stimulation. Models of inflammation (carrageenan and formalin) can also be used to observe the changes that occur in the periphery and at the spinal cord level.

1.2.5: Afferent Nerve Types.

As discussed in 1.2.2, the classification of nerve cells that convey sensory information often includes the transduction process that these cells innervate. The nerve fibres that convey the somatosensory information have also been shown to have different properties.

The nerve fibre type associated with the relay of non-noxious information are known as the $A\beta$-fibres. These fibres innervate various receptors in the dermis and are myelinated by Schwann cells so that these fibres have a rapid conduction velocity, between 30-100m/s. The $A\delta$-fibres are thinly myelinated and therefore their conduction velocities are slower than the $A\beta$-fibre responses, 4-30m/s. As discussed previously their peripheral endings may not be free but covered by Schwann cells or keratinocytes (Kruger et al. 1981; see Meyer et al. 1994). These fibres have the

capacity to convey both non-noxious and noxious information. The C-fibres are not myelinated so their conduction velocity is the slowest of these three fibre types, <2.5m/s and in most cases are activated by high-threshold stimuli, although this is not always the case. There are also afferent fibres innervating muscle and joints. There are three classes of myelinated axons: Group I, conduction velocity of 72-120m/s; Group II, 24-71m/s; Group III, 6-23m/s. There are also unmyelinated axons associated with these tissues with conduction velocities of less than 2.5m/s, known as group IV axons. Group I includes the muscle spindle afferents, Group II corresponds to the Aβ-fibres and group III and IV to the Aδ- and C-fibres respectively. There are few large myelinated axons associated with the viscera and the Aδ- and C-fibres largely convey information from these tissues (Gebhart 1995).

Since these nerve cells have differing patterns of myelination they can be distintinguished visually due to the fact that they have different diameters. They are often described by their diameter, large fibres being those which conduct low-threshold information and small diameter conveying high-threshold inputs. The threshold for activation of these fibre types is generally inversely related to their diameter size. Weak stimuli activate the large fibres and more intense stimuli activate the smaller fibres. All these neuronal cells are bi-polar in that they have both a peripheral terminal and a centrally located terminal. Their cell bodies are found in the dorsal root ganglion. It is known in the case of the C-fibres that they are capable of releasing substances at both their peripheral and central terminals.

1.2.6: Dorsal Root Ganglia.

The cell bodies in the dorsal root ganglia are responsible for synthesis of the cells requirements, such as enzymes, precursors for neurotransmitters, receptors etc.. Once synthesized these substances can be transported both to the peripheral as well as to the central terminals of the cell. It has been shown that under normal conditions opioid receptors are expressed but non-functional at the peripheral terminal. However, after inflammation the perineurium surrounding the free endings is broken down making the opioid receptors accessible (see Stein 1993).

The cells in the DRG can be separated on size. The large diameter axons tend to have large cell bodies and make up the Aβ-fibres, group I and II from muscles and joints. The smaller fibres have smaller cell bodies and make up Aδ-, C-fibres as well as group III and IV afferents. These cell bodies can be further subdivided depending on which peptides and enzymes they are positive for. All the cell bodies stain for the excitatory amino acid, glutamate. Many cells have CGRP,

Vasopressin and Oxytocin (30%) with between 10-30% staining for Substance P (SP) (see Willis and Coggeshall 1991c). A smaller percentage (10%) stain for somatostatin (SOM), cholecystokinin (CCK), bombesin (BOM), vasoactive intestinal polypeptide (VIP), galanin (GAL), dynorphin (DYN), endorphin (END), enkephalin (ENK) and corticotrophin-releasing factor (CRF). As well as establishing that cells can contain these substances it has also been shown that co-localization can occur (Lundberg and Hokfelt 1986). Immunocytochemical studies have demonstrated that substance P can co-localize with CGRP (Wiesenfeld-Hallin et al. 1984). Substance P appears to   be associated with CGRP in small type cells, whereas CGRP can be located without substance P (Wiesenfeld-Hallin et al. 1984). Substance P also co-localizes with CCK, SOM, BOMB, VIP and DYN or ENK (see Willis and Coggeshall 1991c).

The precise function of these transmitters is open to debate. Many of the small fibres stain predominantly for these peptides compared to the larger fibres. This suggests that the transmission of noxious information is open to a greater degree of fine tun ing, if all of these substances can act as neurotransmitters or neuromodulators.

1.2.7: Entering the Spinal Cord.

The central process of the axon passes to the spinal cord via the dorsal root. A single dorsal root is responsible for information from a particular area of the body. This area is known as the dermatome. The spinal cord is split up into 31 spinal segments. A segment contains the dorsal root (containing afferents) as well as the ventral root (containing efferents). The segments are the cervical, thoracic, lumbar, sacral and coccygeal.

It is generally thought that there is functional separation between the dorsal and ventral roots, in that the dorsal roots contain afferent sensory fibres and the ventral roots contain efferent motor fibres. However, a variety of studies have shown that this separation is not absolute. In a study in cats a small proportion of the afferent fibres, predominantly small in diameter, enter not by the dorsal roots but via the ventral root and have been shown to terminate in the marginal zone and substantia gelatinosa (Light and Metz 1978). In this same study some larger diameter fibres were also shown to enter the spinal cord via this route but terminate in the nucleus proprius (Light and Metz 1978). It has been suggested by Kim et al. (1987) that these afferents entering the ventral root may be a possible third branch of these neurones or could be afferents arising from the pia matter. Stimulation of these ventral root afferents have been shown to give rise to changes attributed to the

response to painful sensations in awake animals (Longhurst et al. 1980).

The large fibres enter via the dorsal columns and the smaller fibres via a bundle known as Lissauer's tract (Molander et al. 1984). Lissauer's tract also contains axons of cells originating in the dorsal horn, from marginal as well as SG neurones. Both large and small fibre types split into two and may project both rostral and caudal to the dorsal root before entering the spinal cord.

1.2.8: The Spinal Cord.

The spinal cord is divided into white and grey matter. The white matter contains axons which tend to be myelinated, whereas the grey matter contains cell bodies and their processes. The grey matter is further divided into ten laminae, first described in the cat by Rexed (1952) and subsequently by others in the rat (Molander et al. 1984; Wall 1967b). When viewed in section these laminae can be seen as layers of functionally distinct cells. They also form columns of functionally related cells that extend the length of the cord. The different laminae contain different intrinsic cells, receive different inputs and may also have outputs to the ventral horn and to the brain.

These laminae are not separated by hard distinct borders. Often the cell types making up each proposed lamina may be integrated to some extent. Lamina I forms a rim around the most dorsal part of the grey matter and white matter. This lamina contains large horizontal neurones known as the marginal cells of Waldeyer and historically has been called the Waldeyer's layer or marginal zone. These cells are large but few. They have long dendrites which mainly pass over the surface of the dorsal horn, occasionally entering the SG and form a vague boundary between the white and grey matter. The dendrites, as well as covering the outer surface of the dorsal horn also join Lissauer's tract for up to 5-6 segments where they join the grey matter again (Cervero and Iggo 1980). There are also more smaller neurones located here which may make up the more dorsally located SG cells. These cells are more prolific in number than the marginal cells. As well as these intrinsic, propriospinal cells the various laminae also receive afferent inputs. The marginal plexus contains the processes of afferent neurones, superficial neurones as well as the processes of deeper cells. The Aδ-fibres terminate in lamina I entering via this marginal plexus (Light and Perl 1979). Some of the visceral inputs terminate here as well as laminae IIo, V and X. Visceral inputs usually synapse onto the same cells that concurrently receive inputs from the skin and muscle. This is known as convergence, since a variety of inputs project to the same second order neurone. This is one of the reasons that visceral pain is often diffuse and hard to localize as well as the fact that visceral

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afferents only represent 10% of the afferent input into the spinal cord and they also tend to have a greater rostro-caudal distribution than afferents from the skin.

Lamina II of the spinal cord is also known as the substantia gelatinosa and historically has been called Rolandi substance (see Cervero and Iggo 1980; Pearson 1968; Rexed 1952). The substantia gelatinosa comprises of lamina IIouter (which is more dorsal and contains densely packed cells) and lamina IIinner (less compact and more ventrally located). The literature is somewhat confusing as lamina IIi has also been called lamina III; here it will be referred to as lamina IIi. The intrinsic cells here are predominantly stalk and islet cells but there are also arboreal cells, border cells and spiny cells.

The stalk cells have classically been called limiting cells, since their spines are short. Their cell bodies are in IIo near the border with lamina I and they send their dendrites towards the ventral horn. The stalk cells act to relay transmission from the superficial lamina to deeper laminae, and are presumed to be mainly excitatory, although the presence of enkephalin has been shown in some of these cells (Bennett et al. 1980).

The islet cells have their cell bodies in IIo and inner and their dendrites extend in a rostro-caudal plane. The islet cells are inhibitory cells and play an important role in the control of the presynaptic terminals of afferent inputs, via axo-axonic connections as well as a post-synaptic inhibitory control via axo-dendritic connections. It is presumably activation of these cells by the collaterals of the large diameter fibres that control the inputs of predominantly the Aδ-fibre terminals and to a lesser extent the C-fibre terminals and underlies the "Gate Theory" of control first put forward by Melzack and Wall (1965) (Alvarez et al. 1992; Bernardi et al. 1995; Melzack and Wall 1965).

The other intrinsic cells are the arboreal cells found in lamina IIo and have extensive dendritic branches in IIo as well as IIi and I. The border cells are not very common and their dendrites arborize in laminae IIo and IIi. The spiny cells send dendrites to laminae I-III.

As well as these cells intrinsic to the spinal cord, afferents also terminate in these laminae. Hair follicle afferents are the only large diameter afferents to terminate in lamina IIi although the medial part of lamina II is crossed by large nerve fibres entering from the dorsal white column (Molander et al. 1984). The innocuous C-fibres terminate in lamina IIi and the noxious C-fibre terminals are in lamina IIo. However, it should be noted that although most C-fibres do not terminate in deeper lamina, as well as deeper laminae cells located in laminae V and VI can receive C-fibre inputs since they extend their dendrites into more dorsal laminae (Fitzgerald and Wall 1980). Afferent terminals from Aδ-fibres are few in this lamina, though some have been shown to terminate in

lamina IIo (Molander et al. 1984).

The border between lamina IIi and III is irregular and often hard to visualize since it contains densely packed cells. Lamina III-VI make up the deep dorsal horn. Cells located here send their dendrites to deeper laminae or into superficial lamina. Inputs arising from cutaneous mechano and proprioception terminate in lamina III and IV (SAI mechano, FAI, FAII and hair follicles). Lamina IV forms the base of the head of the dorsal horn. The cells found here are diffusely arranged, are large and extend their dendrites into more superficial lamina. This means that they can receive inputs from afferents terminating more dorsally as well as receiving inputs directly into this lamina. There are a few fine afferents terminating in this lamina. Muscle stretch receptors, joint receptors and SAII terminate in lamina IV to VII and IX. Lamina V forms the neck of the dorsal horn and Lamina VI forms the base of the dorsal horn, both receives inputs from thick myelinated fibres. More ventral laminae (VII-IX) have mainly efferents of visceral and somatic motor neurones. Lamina X is located close to the central canal.

Cells within the spinal cord can send projections to other areas of the spinal cord (propriospinal) or out of the spinal cord to areas of the brain, projection neurones. The large fibres, as well as sending collaterals into the dorsal horn at their point of entry, ascend ipsilaterally in the dorsal columns to terminate on second order neurones in the medullary dorsal column nuclei. They then cross over to the contralateral side to the thalamus and cortex. The small fibres may project both rostral and caudal along the dorsal root before entering the spinal cord where they terminate in the dorsal horn of the spinal cord in superficial lamina. These afferents synapse with interneurones which relay their information to deeper projection neurones, to dendrites of more ventrally located projection neurones or back to spinothalamic neurones in laminae I. Projection neurones from laminae I, III and IV transmit noxious and thermal sensations to the brain in the anterolateral white matter. This incudes the spinoreticular, spino-mesencephalic and the spinothalamic tracts. These project to the reticular formation of the brain stem (spinoreticular) or the parabrachial area in the midbrain (spinomescenphalic) then on to the thalamus and limbic systems.

As well as relaying information from the periphery to supraspinal sites the spinal cord is also under the control of supraspinal structures. Descending pathways from the brain stem provides tonic inhibition of cells responding to noxious stimuli within the spinal cord (Wall 1967b). Ascending tracts can activate these descending controls which arise from activation of the periaqueductal gray, rostral ventromedial medulla and dorsolateral pontomesencephalic tegmentum. These descending controls release serotonin, noradrenaline and opioids to inhibit

nociresponsive neurones.

1.2.9: Pharmacology of the Spinal Cord.

The pharmacology of the spinal cord is very complex. The neurotransmitters and modulators found here can be split into two categories, those that cause excitations and those that result in inhibitions. By this it is meant the effect of the transmitter on the adjacent synapse, since in the spinal cord it is possible for an excitatory neurotransmitter to have an inhibitory effect. This may be by the excitation of inhibitory interneurones, or as in the case of primary afferent depolarization described by Eccles et al. (1963) an inhibitory effect results from a previous excitation of a terminal.

Neurotransmitters released from primary afferent terminals are synthesized in the cell bodies in the DRG or in the terminal by enzymes produced in the DRG. They are then also carried to the central primary afferent terminals. Note that transmitters are translocated to the peripheral terminal and under certain conditions can be released here as well.

The stimulation of afferents have been shown to cause the excitation of intrinsic dorsal horn cells (Hongo et al. 1968). Neurotransmitters released from afferents have been characterized by their ability to produce EPSPs in second order neurones. These EPSPs may be fast and of a short duration or slow and longer lasting. The fast EPSPs have been shown to result from the effects of the amino acid glutamate (Jessell et al. 1986) and the slow EPSPs from peptides (Murase and Randic 1984; Murase et al. 1989).

Glutamate is the main neurotransmitter in the CNS, (Watkins and Evans 1981) including the primary afferent terminals in the dorsal horn of the spinal cord, regardless of whether they are are small or large diameter. The excitatory effects of amino acids was first demonstrated by ionophoretically applied glutamate and aspartate which caused repetitive discharges of a variety of interneurones of the dorsal horn of the cat (Curtis et al. 1959, 1960; Curtis and Watkins 1960). Endogenous glutamate has been shown to be stored in small open core vesicles of the central terminals of afferents (De Biasi and Rustioni 1988; Hokfelt 1991) and to be released from spinal cord slices by stimulation of the dorsal root (Jessell et al. 1986). The dorsal roots have been shown to contain more glutamate than the ventral roots (Duggan and Johnston 1970; Salt and Hill 1983). Once released glutamate has an excitatory effect on the post-synaptic cell, causing a depolarization via three distinct receptor subclasses. The ionotrophic AMPA- and NMDA-receptors and the G-

protein linked metabotropic group of receptors (see Hollmann and Heinemann 1994; Monaghan et al. 1989).

Amino acids are released in response to acute and more sustained noxious inputs (Kangrga and Randic 1991; Skilling et al. 1988). During low-frequency stimulation of primary afferents, glutamate released into the synaptic space is available to activate AMPA-receptors, which are responsible for the vast majority of excitatory neurotransmission in the CNS (see Dickenson 1994a). In the case of spinal somatosensory processing, AMPA-receptor activation is responsible for components of acute and tonic noxious inputs, as well as tactile transmission (Davies and Watkins 1983; Dickenson and Aydar 1991; Dickenson and Sullivan 1990, 1991; Hunter and Singh 1994; Jessell et al. 1986; Skilling et al. 1988). However, during repetitive high frequency stimulation of noxious inputs there is an enhanced response of convergent neurones (Mendell 1966). This enhanced activity has been attributed to the recruitment and activation of another class of excitatory amino acid receptors, the NMDA-receptors (Davies and Lodge 1987; Dickenson and Sullivan 1987a, 1990) and is known to underlie hyperalgesia and more persistent and altered pain states (see Dickenson 1994a; Dubner and Ruda 1992; Hunter and Singh 1994; Raigorodsky and Urca 1987; Ren and Dubner 1993; Ren et al. 1992; Sluka et al. 1994; Yaksh 1989).

During circumstances of acute noxious inputs (low-frequency) the activation of the NMDA-receptor is not possible, since under normal physiological conditions the ion channel of this receptor is blocked by Mg^{++} (Mayer et al. 1984). The NMDA-receptor channel is ligand gated but it is unique in that the Mg^{++} plug also means that it requires concurrent membrane depolarization to become activated. The removal of the Mg^{++} is achieved by membrane depolarization due to the activation of other non-NMDA receptors including those of the peptides. It is known that there is an upregulation of peptide production and an increase in spinal peptide release, predominantly substance P and CGRP during inflammation and more tonic pain states (Lembeck et al. 1981; Noguchi et al. 1988; Oku et al. 1987a, b; Randic and Miletic 1977; Smith et al. 1992). This peptide release is responsible for the slow EPSPs in post-synaptic neurones (Hosli et al. 1981; Murase et al. 1989; Urban and Randic 1984) and has been shown to enhance the responses of excitatory amino acids (Kangrga and Randic 1990; Murase et al. 1989; Randic et al. 1990). This increase in dorsal horn excitability is blocked by NMDA-receptor antagonists (Coderre and Melzack 1992; Dickenson and Sullivan 1987a; Haley et al. 1990; Murray et al. 1991; Sher and Mitchell 1990; Vaccarino et al. 1993; Yamamoto and Yaksh 1992) but these antagonists have no effect on the input or underlying basal transmission (Dickenson and Sullivan 1987a, 1990, 1991; Sher and Mitchell 1990). The slow EPSPs caused by peptides are thought to result in these enhanced responses, since as the membrane potential

moves away from a hyperpolarized state to a more positive transmembrane potential the affinity of Mg^{++} for the ion channel of the NMDA-receptor is reduced, thus the Mg^{++} block is removed (MacDonald and Nowak 1990).

There are many peptides located in the superficial layers of the dorsal horn (De Lanerolle and LaMotte 1983; Holfelt 1991; Hope et al. 1990). These have been found in dense core vesicles in the primary afferent terminals (Holfelt 1991) which include the tackykinins (substance P, neurokinin A), CGRP, bombesin, somatostatin and VIP. The two most prevalent peptides are substance P and CGRP.

Substance P has been shown to be released in response to noxious stimulation (Duggan et al. 1987, 1988; Go and Yaksh 1987; Kuraishi et al. 1985a; Lembeck and Donnerer 1981; Nicoll 1980). The majority of binding of the receptors for substance P, the NK1-receptors are located post-synaptically in laminae I and II (Yashpal et al. 1990) although effects of substance P have been shown on the presynaptic terminals themselves (Randic et al. 1982). The effect of substance P is to provide a longer lasting depolarization by inactivating K^{+} currents (Hosli et al. 1981; Nowak and MacDonald 1982) or by increasing Na^{+} or Ca^{++} currents (Murase et al. 1989; Murase and Randic 1984). Neurokinin 1-receptor antagonists have been shown to block the initiation of wind-up and enhanced spinal responses (DeKonick and Henry 1989; Yamamoto and Yaksh 1991), since as discussed above the effects of NK1-receptor activation is vital for the recruitment of NMDA-receptor driven events. The enhanced responses observed result in the increase in intracellular calcium by mobilization of $[Ca^{++}]i$ (by NK1-receptor activation) or an increase in calcium influx, via activation of the NMDA-receptor.

Another tachykinin is neurokinin A which acts at the NK2-receptor. Neurokinin A has been shown to be released in the dorsal horn and there are high basal levels of NKA in the absence of stimulation (Duggan et al. 1990; Hope et al. 1990). Unlike substance P, NKA is found throughout the dorsal horn (Duggan et al 1990; Hope et al. 1990). However, NKA is also released in response to noxious stimulation (Duggan et al. 1990) antagonists of which have been shown to attenuate the effects of noxious inputs (Fleetwood-Walker et al. 1990). Since NKA can be detected for up to thirty minutes after noxious stimulation has ceased it has been suggested that this peptide is responsible for enhanced responses of other neurones (Duggan et al. 1990). NKA has also been shown to spread more diffusely after release (volume transmission) even being present in the white matter (Duggan et al. 1990). The other neurokinin, neurokinin B acts at the NK3-receptor but is practically absent from dorsal roots and appears to be derived from intrinsic cells (Ogawa et al. 1985).

Another peptide is calcitonin gene related peptide (CGRP).

Dorsal rhizotomies significantly reduce CGRP immunoreactivity in Lissauer's tract, and in laminae I, II and V (Gibson et al. 1984; Traub et al. 1989) whilst in monoarthritic animals, CGRP increases in the DRG (Smith et al. 1992). CGRP can be co-localized with substance P (Gibson et al. 1984) and CGRP itself has been shown to attenuate the breakdown of substance P and enhance its effects (Le Greves et al. 1985; Wiesenfeld-Hallin et al. 1984) as well as potentiating the release of substance P from the afferent terminals, possibly by the mobilization of calcium (Oku et al. 1987a). CGRP has also been shown to cause a slow depolarization of post-synaptic cells which may also contribute to the enhanced responses of these cells to further stimulations (Ryu et al. 1988).

Other peptides in the dorsal horn are also excitatory. Cholecystokinin enhances the release of glutamate from primary afferent terminals by mobilizing intracellular calcium stores (see Stanfa et al. 1994) and has been shown to decrease in inflammatory states thus enhancing the potency of morphine (Stanfa and Dickenson 1993; Stanfa et al. 1992).

As well as these excitatory peptides there are also inhibitory ones, such as the endogenous ligands for the opioid-receptors. There are three main subgroups of opioid receptors, the mu, delta and kappa-receptors. Each receptor has an endogenous ligand, namely endorphins, enkephalins and dynorphins for the above receptors respectively. Note however that these opioid peptides are not specific for any one receptor. Since peptides, like adenosine are broken down quickly, the role of the endogenous ligands have been observed by preventing their degradation with peptidase inhibitors and thus prolonging their half life (Dickenson et al. 1987a).

It has clearly been demonstrated that the opioids, especially those acting at the μ-receptor are capable of producing antinociception (Dickenson 1991; Dickenson and Sullivan 1986, 1987c; Dickenson et al. 1987b; Fleetwood-Walker et al. 1988; Yaksh 1993; Yaksh and Rudy 1977) and have little if any effect on non-noxious inputs (Dickenson and Sullivan 1986; Duggan and North 1984). The μ-receptors are predominantly located in the substantia gelatinosa (Morris and Herz 1987), especially on primary afferent terminals with a smaller portion of receptors on interneurones (see Dickenson 1994b; LaMotte et al. 1976; see Yaksh 1993). This is the opposite to adenosine, where the majority of the receptors are located on interneurones with only a smaller proportion on primary afferent terminals (Choca et al. 1988; Geiger et al. 1984). Evidence for the location of opioid-receptors comes from electrophysiological studies that have demonstrated that the initial input onto cells by small diameter fibre stimulation are decreased with the spinal application of opioids, indicative of a pre-synaptic location of the receptors (Dickenson and Sullivan 1986). Evidence from a variety of other studies that have a shown a

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decrease in substance P and glutamate release from primary afferent terminals with spinal application of opioid-receptor agonists (see Dickenson 1994b) as well as a decrease in opioid binding after peripheral nerve damage (Stevens et al. 1991), further demonstrates a presynaptic location.

At the primary afferent terminal level, activation of opioid-receptors, whether by increasing potassium conductance or by switching off of calcium channels will result in less influx of calcium and therefore less neurotransmitter being released from afferents (see Duggan and North 1984). A pre-synaptic location is beneficial since it can prevent the effects of excitatory amino acids and peptides at one go by preventing the release of any neurotransmitters from the afferent terminals. However, opioids appear to be less effective, although not altogether ineffective in controlling phenomenon associated with wind-up and activation of the NMDA-receptor (Dickenson and Sullivan 1986; Jadad et al. 1992). This is probably due to the fact that once wind-up has been set up the opioid-receptors are not so well located to control the activity of intrinsic cells, since only a small proportion of receptors are found post-synaptically and activation of these requires higher doses (see Dickenson 1994b). There are also other peptides located in the spinal cord such as somatostatin and bombesin (Randic and Miletic 1978; De Koninck and Henry 1989).

Along with the excitatory amino-acids there are also the inhibitory amino-acids glycine and γ-amino-butyric acid (GABA) found in intrinsic cells in the spinal cord. These neurotransmitters have been shown to have an important role in the control of sensory inputs (Basbaum 1988; Bohlhalter et al. 1994; Game and Lodge 1975; Van den Pol and Gorcs 1988; see Willis and Coggeshall 1991a). GABA has been shown to be located in the superficial laminae of the spinal cord (Magoul et al. 1987; Powell and Todd 1992; Todd and McKenzie 1989) especially in the islet cells that run in a rostro-caudal direction in lamina IIo together with glycine (Aprison and Werman 1965; Magoul et al. 1987; Powell and Todd 1992; Spike and Todd 1992; Todd 1990; Todd and McKenzie 1989; Todd and Sullivan 1990; Todd et al. 1996). The GABAergic neurones have been shown to make axo-axonic connections (Barber et al. 1978; Magoul et al. 1987) as well as axo-dendritic connection (Alvarez et al. 1992; Baba et al. 1994; Magoul et al. 1987; Powell and Todd 1992), implying that GABA can have controlling effects on both the primary afferent terminals themselves as well as on intrinsic neurones. The receptors for these transmitters (GABA$_A$, GABA$_B$ and glycine) are known to be on both primary afferent terminals and interneurones (Bowery et al. 1987; Desarmenien et al. 1984; Price et al. 1987; Van den Pol and Gorcs 1988). The actions of GABA on the primary afferent terminals may be responsible for primary afferent depolarization (PAD). Although this also results in inhibitory effects it differs in mechanism to the IPSPs produced by GABA (see Willis and Coggeshall 1991d).

Spinal applications of antagonists of both GABA and glycine have shown to cause both cardiovascular changes and agitation indicative of noxious stimulation but in response to low threshold stimulation (Roberts et al. 1986; Sherman and Loomis 1994; Yaksh 1989). Electrophysiological experiments have also demonstrated that there are altered patterns of neuronal firing in the presence of antagonists of these receptors (Curtis 1969; Curtis et al. 1968; Duggan et al. 1981; Game and Lodge 1975; Sivilotti and Woolf 1994).

Along with the peptides there is another "neuromodulator" that can increase the responses to glutamate, the gas nitric oxide (NO) (Garthwaite et al. 1989). The enzyme responsible for the synthesis of NO, NO synthase (NOS) has been located in the dorsal horn of the spinal cord as well as DRG cells (Anderson 1992; Morris et al. 1992). It has been demonstrated that NO is synthesized on demand in response to increased activity in intrinsic cells. An increase in NMDA-receptor activation can result in the increase in calcium influx (Garthwaite et al. 1989). This rise in intracellular calcium can result in many processes being switched on, such as the regulation of genes (namely cFos and cJun) and the activation of calcium dependent processes. One such calcium dependent process is the production of NO. The enzyme NOS is activated by a calmodulin-sensitive site on the enzyme, which has previously been activated by the increase in intracellular calcium. NOS catalyses the conversion of L-arginine and molecular oxygen to NO and L-citrulline. Once produced NO can diffuse into neighbouring cells or can have effects in the cell it is produced from. One target is the primary afferent terminals where NO activates soluble guanylate cyclase to increase cGMP which in turn can cause the further release of glutamate. For this reason NO is a possible candidate for retrograde transmission in the dorsal horn (see Meller and Gebhart 1993). Inhibitors of nitric oxide synthase have been shown to decrease both the enhanced responses of spinal neurones and behavioural measures of nociception after inflammation (Haley et al. 1992; Malmberg and Yaksh 1993b; see Meller and Gebhart 1993; Moore et al. 1993).

1.2.10: Aims of this Project.

There has been considerable interest in the involvement of purines in the transmission and modulation of somatosensory processes. To date the majority of the studies undertaken have been behavioural (DeLander and Keil 1994; Karlsten et al. 1990; Keil and DeLander 1992, 1994, 1995; Malmberg and Yaksh 1993a; Sawynok et al. 1989; Sosnowski and Yaksh 1989; Sosnowski et al. 1989) apart from the studies of Salter and Henry (1985, 1987, 1989) who only considered low-

threshold stimuli. Since purines are known to effect motor behaviour this may hamper the interpretation of results in awake animals (Karlsten et al. 1990; Sosnowski et al 1989). These studies presented in this thesis were undertaken to assess the role of the P_1 purino-receptors for adenosine, the A_1 and the A_2-receptors in spinal dorsal horn processing in acute and more persistent pain states. Here I used selective and non-selective agonists and antagonists in studies on various types of nociception - acute, short and longer term inflammation. In addition I attempted to elucidate the function of adenosine itself in the control of spinal cord processing by protecting endogenously released adenosine (from whatever source) by use of agents that inhibit adenosine kinase which converts adenosine back to 5'-AMP. It is hoped that work undertaken in this thesis will aid in the understanding of the involvement of adenosine in spinal cord processing of somatosensory inputs and hopefully this will be translated into a rational basis for novel therapy based on manipulation of the effects of this interesting purine.

Chapter 2.

Experimental Methods.

2.1: Introduction.

The methods here describe the surgical preparation and recording techniques for the evaluation of the activity of single dorsal horn neurones.

The animals used for all experiments were male rats (200-250g), Sprague-Dawley and obtained from University College London Biological Services.

2.2: Anaesthetics.

The anaesthetic used for induction and maintenance of anaesthesia during the experimental procedure was the volatile anaesthetic, halothane (1, 1, 1-trifluorobromochloro ethane). This was used in conjunction with a gaseous mixture of N_2O and O_2 in a 66/33% mixture.

There are several techniques that can be employed to record the activity of neurones in systems related to pain and analgesia. Recordings can be made in anesthetised animals (Dickenson and Sullivan 1986; Herrero and Headley 1995) in animals that have had their nervous system modified (Fitzgerald and Wall 1980; LeBars et al. 1976; Sivilloti and Woolf 1994) or in animals that are awake, physiologically intact and drug free (Collins 1985; Herrero and Headley 1995). It has been demonstrated that many substances used to maintain a pain and stress free environment can effect the recordings of spinal neurones and so it must be of consideration that the recordings observed are under a degree of modification by the anesthetic used. However, a major concern is also a ethical one, in that the animal at no point undergoes unnecessary suffering. It is for this reason that anesthetized animals were used for all experimental recordings. In fact in a study of awake versus halothane-anesthetized sheep the differences observed in neuronal activity between the two techniques were surprisingly subtle (Herrero and Headley 1995).

The choice of anaesthetic requires consideration. It would appear that anesthetics exert their effects not by a common mechanism of action but by a variety of effects that all serve to stabilize a variety of synapses (Wall 1967a). This implies that the different agents have distinct mechanisms of action. The anaesthetic ketamine (N-methyl-D-aspartate receptor antagonist) has been shown to inhibit cells in lamina 1 and 5 but not 4 and 6 of the dorsal horn of the spinal cord in decerebrated cats (Conseiller et al. 1972; Kitahata et al. 1973). As these lamina are of particular relevance to inputs from noxious stimuli (Cervero and Iggo 1980; Fitzgerald and Wall 1980) and the NMDA-receptor is known to be involved in many of the spinal cord processing of noxious information (Dickenson 1994a) as well as the enhanced

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response of wind-up (Davies and Lodge 1987; Dickenson and Sullivan 1987a) ketamine was excluded from use in this study.

Halothane has been shown to suppress spontaneous and evoked activity of dorsal horn cells (deJong et al. 1969, 1970). This may be due to a reduction in the amplitude of low-voltage-activated (LVA) calcium currents (T current) which has been shown in dorsal root ganglion cells (Takenoshita and Steinbach 1991). This current is believed to be important for the after-potentials that produce bursting and influences neuronal excitability, whereas the other types of calcium currents (high-voltage-activated, N and L) are important for the release of neurotransmitters (Bean 1989). Although the use of this anaesthetic could cause a decrease in neuronal excitability, the induction of wind-up caused reliable enhanced responses in most cells. The animals could have been spinalized, but this can cause two problems. The cells could then display a degree of enhanced evoked and spontaneous activity, as well as occluding the study of descending controls on the spinal cord, which are also of interest.

Nitrous oxide is included in the gaseous mixture since it prevents the animal from becoming hyper-oxygenated by maintaining the correct PCO_2 levels within the blood. _If the animal was maintained on high O_2 levels there would be no drive to_ As the levels of PCO_2 drop _and_ the respiratory centre has no _respiration,_ stimulus to maintain respiration. Nitrous oxide also prevents hypoxia, due to halothane poisoning. If the levels of halothane rise significantly in the blood then the flow of halothane across the alveoli will be from the blood to the lungs. This then prevents oxygen from being adequately taken up into the blood from the lungs. _It should be noted that nitrous oxide can suppress the activity of spinal neurones._

2.3: Surgery.

The rat was placed in a perspex sealed box and the anaesthetic mixture passed into the container by means of plastic tubing inserted into a hole the diameter of the tubing. The halothane level was about 3%. Once the animal had no righting reflex it was taken out of the container and placed, back down onto a heating blanket with the nose placed into a cone, through which the anaesthetic was now administered. This was now reduced to 2.5% in order not to cause an overdose.

Once the animal had no withdrawal reflex, in that it did not respond if either hind-paw was pinched, an incision was made with a pair of scissors to remove the hairy skin covering the trachea. The exposed muscle was teased apart with mouse-tooth forceps to expose the trachea. A fine pair of forceps was placed under the trachea and the end rested on a raised surface in order not to bend and occlude the airway. A piece of silk thread, folded double, about 10cm in length (Pearsalls sutures braided untreated silk) was then placed under the trachea. The folded end

was cut and the two separate pieces of thread now tied loosely above and below the forceps. The trachea was cut in between two rings of cartilage and a cannula (non-sterile polythene tubing, inside diameter 1.57mm, outside diameter 2.08mm) of about 4cm in length was inserted into the opened trachea (about 2cm was left above the incision). This was held in place by tightening the silk thread taking care not to completely collapse the trachea. The animal was reconnected to the anaesthetic, by placing the tubing over the cannula and the halothane level was now reduced slightly. At no point did the animal become "light" during this procedure.

The animal was now held in a stereotaxic frame by means of ear bars. The skin on the back of the animal was cut to expose the covered spinal cord. Using a scalpel an incision was made either side of the vertebral column to allow the spinal cord to be clamped. This was first done rostral to the lumbar spinal cord. The skin covering the spinal cord was removed to expose the dorsal surface of the vertebral column. The spinal process was removed about segments L_1-L_2, to allow exposure of the preferred recording area L_4-L_5. The transverse process was left in order to maintain stability. The cord was now clamped caudal to the lumbar segments. After surgery the anaesthetic level was turned down to 1.5% halothane or at a level where no withdrawal reflex was observed to noxious stimuli.

2.4: Temperature Regulation.

Through out the surgery and the duration of the experiment the animals temperature was maintained at 37°C via means of a rectal probe which fed back to a heating blanket.

2.5: Isolating Cells.

Glass-coated tungsten electrodes were inserted into the most superficial dorsal horn and then moved into more deeper laminae via means of a microdrive. This moved the electrode in 10μm steps at a time. The glabrous skin of the hind paw was tapped with a finger whilst moving the electrode to stimulate the peripheral receptive field. Cells that responded to touch (non-noxious) and to pinch (noxious) stimulation were then stimulated electrically in order to determine the cells characteristics. Cells that had Aβ-fibre evoked inputs as well as C-fibre evoked inputs were included for evaluation. The Aβ-fibre evoked inputs were activated by low-threshold stimulation and appeared at latencies of 0-20ms after stimulation (see Chapter 3). The Aδ-fibres had higher threshold than the Aβ-fibres and appeared at latencies of 20-90ms after stimulation. These fibre evoked responses were followed

by the high-threshold C-fibre evoked inputs at 90-300ms after the initial stimulation. Post-discharge of cells appeared at latencies of 300-800ms and are associated with the increased response of a cell to a constant train of inputs (Davies and Lodge 1987: Dickenson and Sullivan 1987a). All cells were characterized electrically.

Once the cell characteristics had been measured and it fulfilled the criteria of having a C-fibre response, tests were done at ten minute intervals until the cells response was stable. A test consisted of a train of 16 stimuli given at three times the Aβ- or C-fibre threshold at 0.5Hz with a 2ms interval. Subsequent drug evaluations were expressed as percent of initial control responses of the cells for the acute studies. Typically tests were made every ten minutes for 40-60 minute blocks for each doses. Doses were evaluated in a cumulative manner.

2.6: Recording Procedures.

After a cell had been isolated by natural and electrical stimulation the signal it generates is sent to the head stage. The A lead is the signal from the electrode, including the neuronal response as well as the background activity of the spinal cord. The B lead carries the activity from electrical interference, mains hum and cardiovascular and respiratory activity.

The output of the headstage is amplified by the preamplifier, differentiated (A minus B), amplified further by a AC/DC Amp and filtered. The resulting signal is sent to the auditory amp so there is an auditory representation of the signal, the oscilloscope, so the signal can be visualized and to the window discriminator. The window discriminator can be set so that action potentials over a certain degree of amplitude can be counted. If the spike height of the action potential fits the set criteria then it triggers a Brit brightening pulse (displayed as a dot on the oscilloscope). This pulse is sent to the interface (1401) and to the computer, where it is displayed as a post-stimulus histogram or rate recording using CED MRATE software.

The stimulus given into the peripheral receptive field of the neurones which make up a test is delivered by means of stimulus isolator. This isolator is driven by three parts of the Neurolog system. On commencement of a test, the period generator gives rise to a stimulus every 2 seconds, the width and amplitude of which are controlled by the digital width and pulse buffer respectively. The period generator also activates the delay width which controls the latch counters, which in turn control the counting periods. In most experiments this was set to between 90-800ms after the stimulation is applied to the receptive field, to record action potentials

generated by the C-fibres and the after discharge (post-discharge) generated by C-fibre stimulation.

A schematic representation of the processes involved in the generation of a test can be seen in Figure 1.
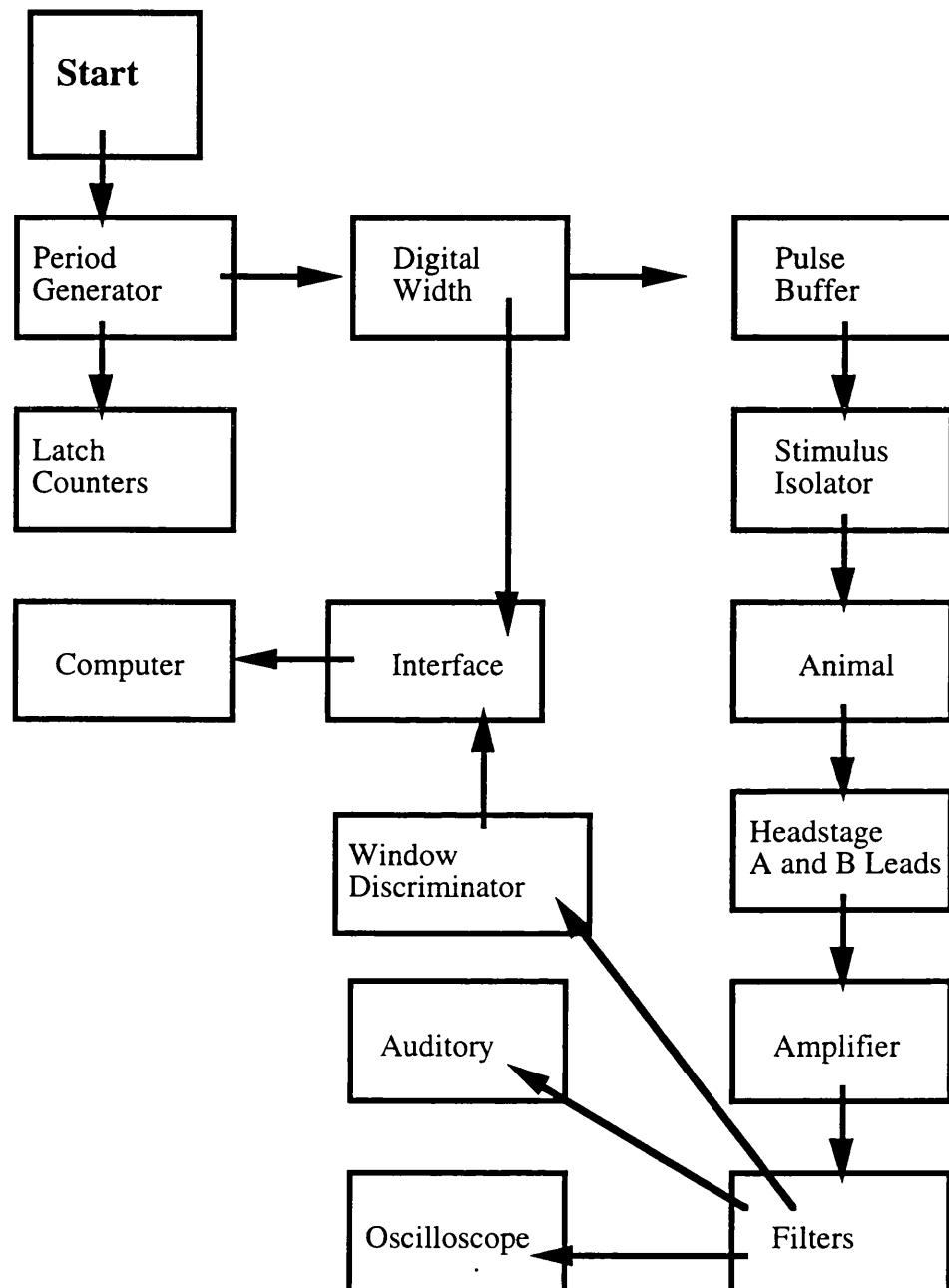

Figure 1. *Schematic representation of the stimulation and recording processes involved in the generation of neuronal responses and their analysis.*

2.7: Formalin Induced Inflammation.

Cells included in formalin studies were first characterized electrically to establish if they received a C-fibre input. Once assessed, 50μl of formalin was injected into the peripheral receptive field, always a toe. Formalin was made up in saline to a 5% concentration. The introduction of formalin into the paw resulted in immediate firing of dorsal horn cells which had previously displayed little if any spontaneous activity. This firing was observed and recorded for one hour. The activity was quantified on the basis of the two phases of the response. The first (phasic) phase occurred after injection and lasted for about ten minutes. This was followed by a silent phase which was then followed by another period of firing, the second or tonic phase, which generally lasted for up and beyond sixty minutes post-injection. Action potentials occurring in the first phase (10 minutes) and second phase (50 minutes) were evaluated over these time periods. The overall time-course was also split into ten minute blocks.

For every series of experiments, control responses for formalin were established due to the large degree of variability in this response. Every day that a drug was to be evaluated against formalin a control response would first be established in that animal. Subsequent drug effects were only done if adequate controls could be obtained. Drugs in these studies were given as a pretreatment to formalin injection. The time of the pretreatment depended on the time course of the drugs which were first established from separate experiments using electrically evoked responses. Drug effects were expressed as percentages of control formalin responses. Only one dose per formalin response was evaluated.

2.8: Carrageenan Induced Inflammation.

The response of cells included in the carrageenan studies was first characterized with electrical stimulation. λ-carrageenan (2%) made up in saline, in a volume of 200μl, was injected into the glabrous skin of the pad around the centre of the receptive field of the cell. The response of the cells was observed for three hours at ten minute intervals with electrical stimulation before drug administration. Unlike the formalin induced inflammation, the cells had little or no response to the introduction of carrageenan. The neuronal responses to electrical stimulation during the three hours pretreatment with carrageenan were expressed as percentages of initial controls. Drug effects were expressed as both percentages of these initial controls and as percentages of the final two responses after three hours carrageenan.

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2.9: Bicuculline Methobromide Studies.

The response of cells was characterized to electrical as well as to non-noxious stimulation, prod and brush, until stable responses were observed. The brush response was produced by lightly stroking the skin of the receptive field with a fibre brush for ten seconds. The response to prod was observed by placing a blunt probe on the main part of the receptive field so that there was gentle deflection of the skin. The pressure applied was 4 Newtons per cm^2 _calculated from weight and radius of probe._ The $GABA_A$-receptor antagonist, bicuculline was applied spinally as a pretreatment of forty minutes in order to observe the effects of drugs on the changes in neuronal responses evoked by antagonism of this receptor. The response of the cell to the bicuculline pretreatment was expressed as a percentage of initial controls and the subsequent drug effects on these changes was expressed as both percentages of initial controls and as percentages of the bicuculline effects.

2.10: Drugs.

Drugs were added by systemic (s.c.) or spinal routes. In the latter case the drugs were applied in a volume of 50μl to the exposed surface of the spinal cord. This would be equivalent to an intrathecal route since it was subdural.

The following drugs were obtained from Research Biochemical International; 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-(p-sulfophenyl) theophylline (8pSPT) and 2-p-(2-Carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680). 7-chlorokynurenate (7-CK) and (-)-bicuculline methobromide were obtained from Tocris Cookson. 1,3-dimethyxanthine-anhydrous (theophylline), Caffeine, Lambda carrageenan, 6-amino-2-chloropurine riboside (2-Chloroadenosine, 2ClAdo), N^6-cyclopentyladenosine (CPA) and Naloxone were obtained from SIGMA. Morphine was obtained from Thornton and Ross. Formalin was from BDH chemicals, UK. GPA and GPB were kindly donated by Gensia Pharmaceuticals, San Diego, USA. Bicuculline was kept in the dark at all times. DPCPX was made up in 50% absolute ethanol/50% distilled water and GPA was made up in 100% Dimethyl sulphoxide (DMSO). All other drugs were made up in saline. 7-CK was buffered with 22.5μl of 3M HCl to pH 7.3. All drugs once made up were kept at a temperature of 4^oC apart from CGS-21680 which was made up and then kept in the freezer. 8pSPT was made up every week.

2.11: Analysis.

All results are expressed as a mean ± standard error of the mean for each

population of neurones, apart from wind-up graphs which are examples of single neurones. Student's t-test was used to test for significance; a paired and two tailed test was used in all cases. The nonparametric, Mann-Whitney, unpaired, 2 tailed test was also used to analyse some of the data where comparisons of treatment groups was required. Drug effects for the time course of bicuculline were statistically analysed using a 1-way analysis of variance with repeated measures, followed by Fisher protected least squares difference (PLSD) post hoc test. Significant level was set at a P value of 0.05.

Chapter 3.

Dorsal horn convergent neurones: Their
responses to peripheral transcutaneous electrical
stimulation, carrageenan and formalin induced
inflammation and the changes produced by
spinal bicuculline.

3.1: Introduction.

Cells within the dorsal horn of the spinal cord were recorded in response to a variety of peripherally evoked stimulation.

The cells we observed are termed convergent or wide-dynamic range neurones, since they responded to a variety of peripheral stimulation in both non-noxious and noxious ranges. It has been previously demonstrated in a variety of species that cells within the dorsal horn can be divided into categories depending on their adequate stimulus. Cells that respond to nonnoxious stimuli only have been categorized as class 1 cells (Menetrey et al. 1977). If stimulated with electrical stimulation they have a short latency and duration of response. Increasing the stimulation intensity does not give rise to an increase in response. Cells can also respond to noxious and nonnoxious stimuli (Fitzgerald and Wall 1980; Mendell 1966; Menetrey et al. 1977; Schouenborg and Sjolund 1983). Since our search techniques involve natural stimuli of both nonnoxious as well as noxious intensity, touch and pinch respectively, the cells selected for recording appear to correspond to class 2A described previously in the rat (Menetrey et al. 1977). These cells have been shown to display a prolonged discharge to noxious pinch, and with electrical stimulation a response to $A\beta$-, $A\delta$- as well as C-fibre components (Menetrey et al. 1977; Schouenborg and Sjolund 1983). When locating cells it was found that they would often respond vigorously to touch applied to the receptive field but when pinch was applied gave only a short burst of activity which could not be sustained. When electrically stimulated these cells usually only had an A-fibre component. For a cell to be included in this study they had to respond with an A-fibre component as well as a reproducible, stable C-fibre component.

The neurones selected fit the criteria of giving reproducible responding to both high and low threshold stimuli, similar to Class 2 neurones. These neurones have been shown to have projections to supraspinal sites via the spinocervical and spinothalamic tracts (Hongo et al. 1968; Iggo 1974; Mendell 1966). Secondly the depth of cells would also fit with previous studies (Menetrey et al. 1977). Convergent neurones have been shown to be located in the marginal zone (Lamina I) as well as in a wide area throughout the middle of the dorsal horn. The cells selected for recording were between depths of 1-1000μm from the surface of the dorsal horn.

However, no cells were selected for between 250-500μm, since this area represents the substantia gelatinosa (see Cervero and Iggo 1980; Rexed 1952). Since this area contains intrinsic cells, many of which are inhibitory (Magnuson and Dickenson 1991; Woolf and Fitzgerald 1981) they were not included in our studies.

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I did not observe any cells that responded to noxious only inputs. Cells which responded to joint movement (Class 4) or nonnoxious only (Class 1) were often seen.

3.2: The Response of Cells to Electrically Evoked Activity.

The responses to transcutaneous electrical stimulation, given as a train of sixteen stimuli applied to the centre of the peripheral receptive field were recorded as action potentials evoked by various fibre types. It has previously been shown that trains of between 16-20 stimuli are optimal to evoke maximum C-fibre responses (Schouenborg and Sjolund 1983). The fibre evoked responses can be seen in Figure 1A, B, C, D.
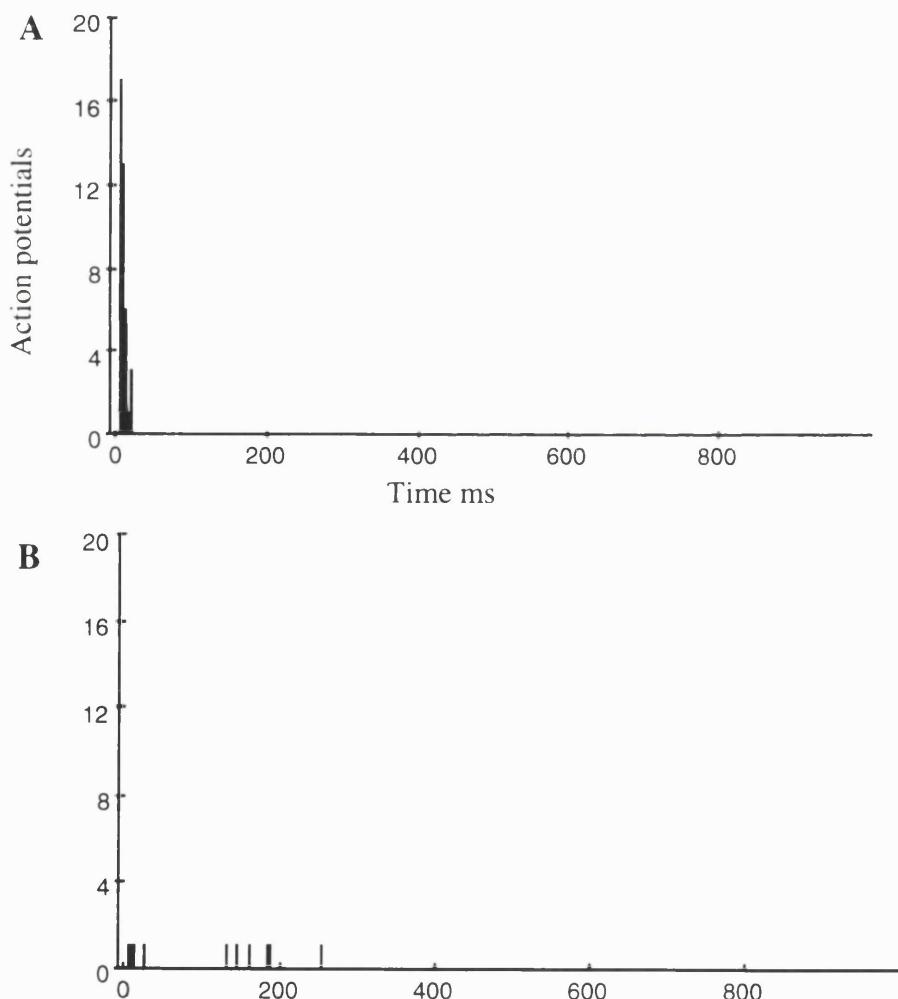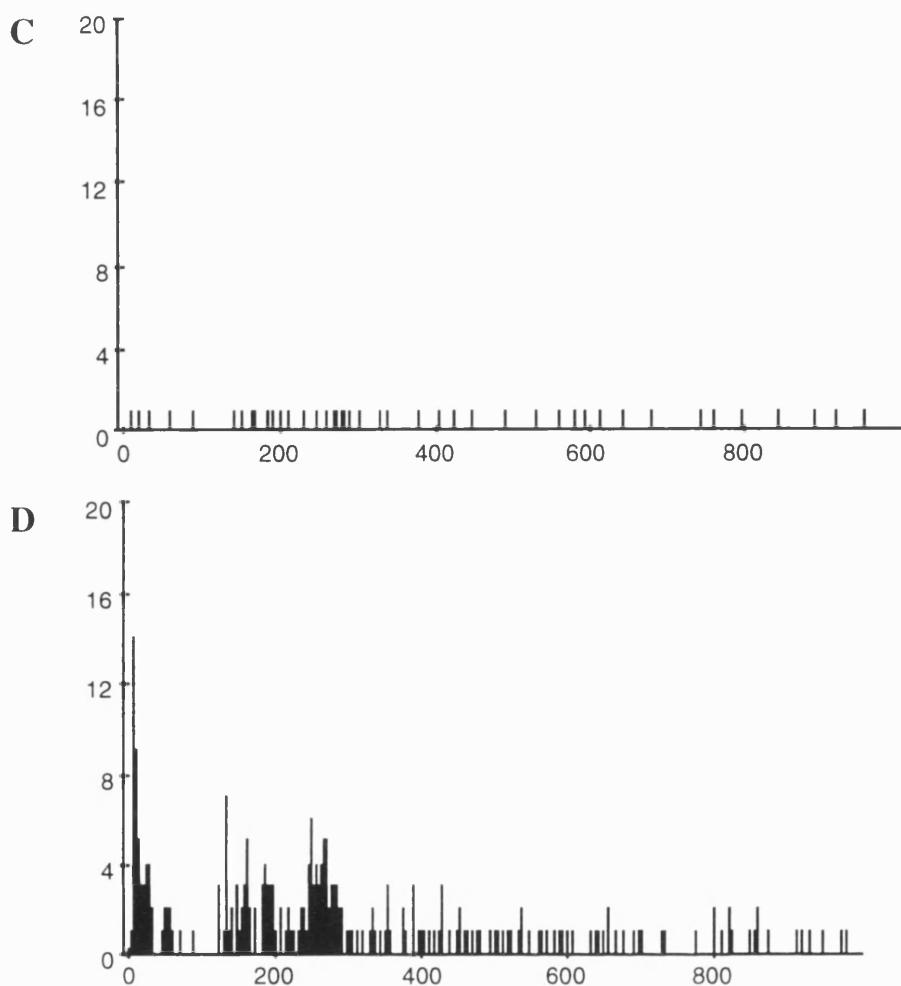
Figure 1. The response of dorsal horn cells to peripheral transcutaneous electrical stimulation. (A) The evoked neuronal response to a train of sixteen stimuli at three times Aβ-fibre threshold. (B) The initial response at three times C-fibre threshold in a train of sixteen stimuli. (C) The evoked responses to the sixteenth stimulus in a train at three times C-fibre threshold. (D) The cumulative response to all sixteen stimuli at three times C-fibre threshold.

The evoked responses were separated into fibre bands based on threshold and latency. It is known that the Aβ-fibre evoked responses have a short latency, since their axons are myelinated which increases conduction velocity as electrical propagation can jump between the Nodes of Ranvier. These fibres therefore have a rapid conduction velocity, between 30-100m/s and appear between 0-20ms after peripheral stimulation (Figure 1A). The Aβ-fibre evoked responses were the first to be evoked with electrical stimulation of low-intensity; as the intensity was increased

the response to Aδ-fibre evoked responses now became apparent, in the latency band 20-90ms (see Figure 1B, C, D). This has previously been shown by Schouenborg and Sjolund (1983) who showed that increased intensity recruited additional early spikes that were still below the threshold for C-fibre evoked responses. These Aδ-responses appeared after the responses to Aβ-fibres. The Aδ-fibres are thinly myelinated and therefore their conduction velocities are slower than the Aβ-fibre responses, 4-30m/s.

The C-fibre evoked responses appear in response to high-threshold stimulation and have the slowest conduction velocity since their axons are not myelinated and the electrical signal must propagate along the whole axon. These fibre evoked responses appeared after the Aδ-fibre responses in the latency band 90-300ms. The responses I recorded have latencies in the same range previously shown by direct sural nerve activation to be attributed to C-fibres and give comparable latencies of the "late component" of transcutaneous electrical stimulation (Mendell 1966; Menetrey et al. 1977; Schouenborg and Sjolund 1983).

Figure 1B represents the first response to the train of sixteen stimuli at C-fibre intensity and Figure 1C shows the sixteen response to the same train. The cumulative response to all sixteen stimulations can be seen in Figure 1D. This repetitive stimulation resulted in the appearance of post-discharge in the latency band 300-800ms. This firing is indicative of "wind-up" which was first observed and termed by Mendell (1966). Single unit recordings of spinocervical tract neurones in the anesthetized cat showed enhanced activity to a volley of un-myelinated fibre stimulation occurring after the late-discharge (C-fibre evoked activity) (Mendell 1966). This was shown to be a phenomenon driven by the activity of centrally located cells, since there was no change in the size of the C-fibre volley with a repetitive stimulus, even though the central effects increased (Mendell 1966). This increase in activity has subsequently been shown to be due to the activation of the NMDA-receptor, which results in increased excitation (Davies and Lodge 1987; Dickenson and Sullivan 1987a). The enhanced response to a constant stimulus can be seen in Figure 2A, B. Here the response to initial stimuli (observed from 90-800ms after stimulation) gave rise to relatively constant numbers of action potentials, but as the stimulation proceeds the action potentials evoked increase. The example in Figure 2A shows a cell winding-up to only a small degree. The slight increase in excitation can not be maintained and the cell, towards the end of the train of stimulations begins to wind-down. This example is from the cell shown in the post-stimulus histograms above. The example in Figure 2B is of a more "classic" wind-up response. The increased activity reaches a plateau towards the end of the train of stimulations.
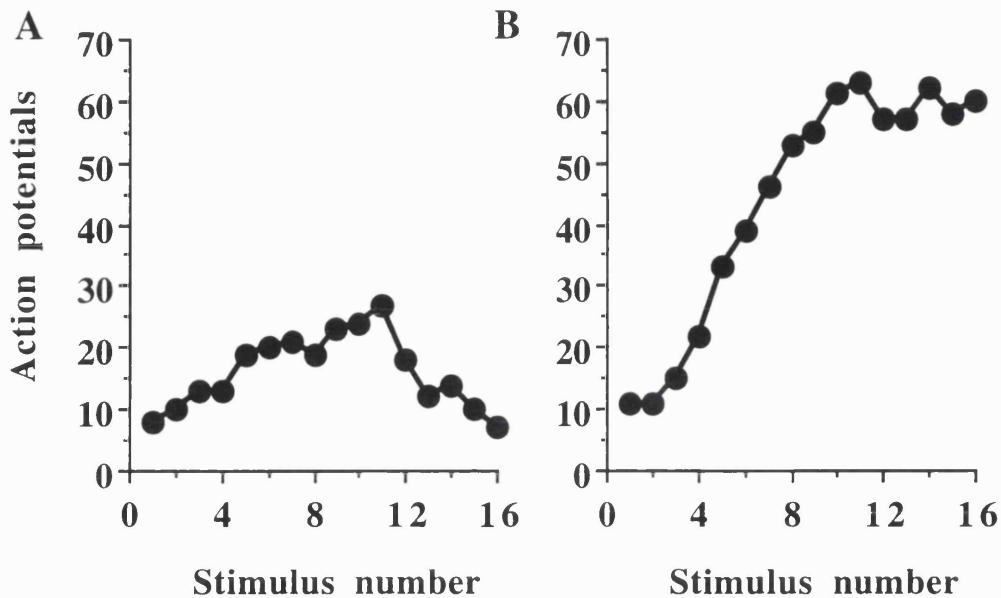
Figure 2. *Action potentials evoked in response to a train of stimulation, three times C-fibre threshold, 0.5Hz with a 2ms wide pulse, given into the peripheral receptive field of two individual neurones. (A) This cell shows only a marginal increase in excitability which cannot be maintained. (B) A more "classic" wind-up response. The action potentials evoked increase with each stimuli until they reach a plateau.*

Overall, the responses of 198 cells were characterized to peripheral electrically evoked stimulation. The mean depth was 761±16μm, with an average C-fibre threshold of 1.89±0.05mA. The average action potentials evoked by the train of sixteen stimuli attributed to the three classes of fibre evoked responses were 359±12, 75±3, 82±2 action potentials for the C-fibres, Aδ- and Aβ-fibres respectively (Figure 3A). The post-discharge response was 229±13 action potentials with the excess action potentials over the predicted range from the input in a train of 16 stimuli being 303±20 (Figure 3B). The post-discharge is a measurement of the action potentials occurring in the latency band 300-800ms and is generated by activity of the C-fibres (Schouenborg and Sjolund 1983). The excess action potentials is calculated by the C-fibre evoked input onto the cell which is multiplied by sixteen. This figure is then subtracted from the action potentials evoked by the final sixteenth

stimuli, to give the number of action potentials over the predicted range.
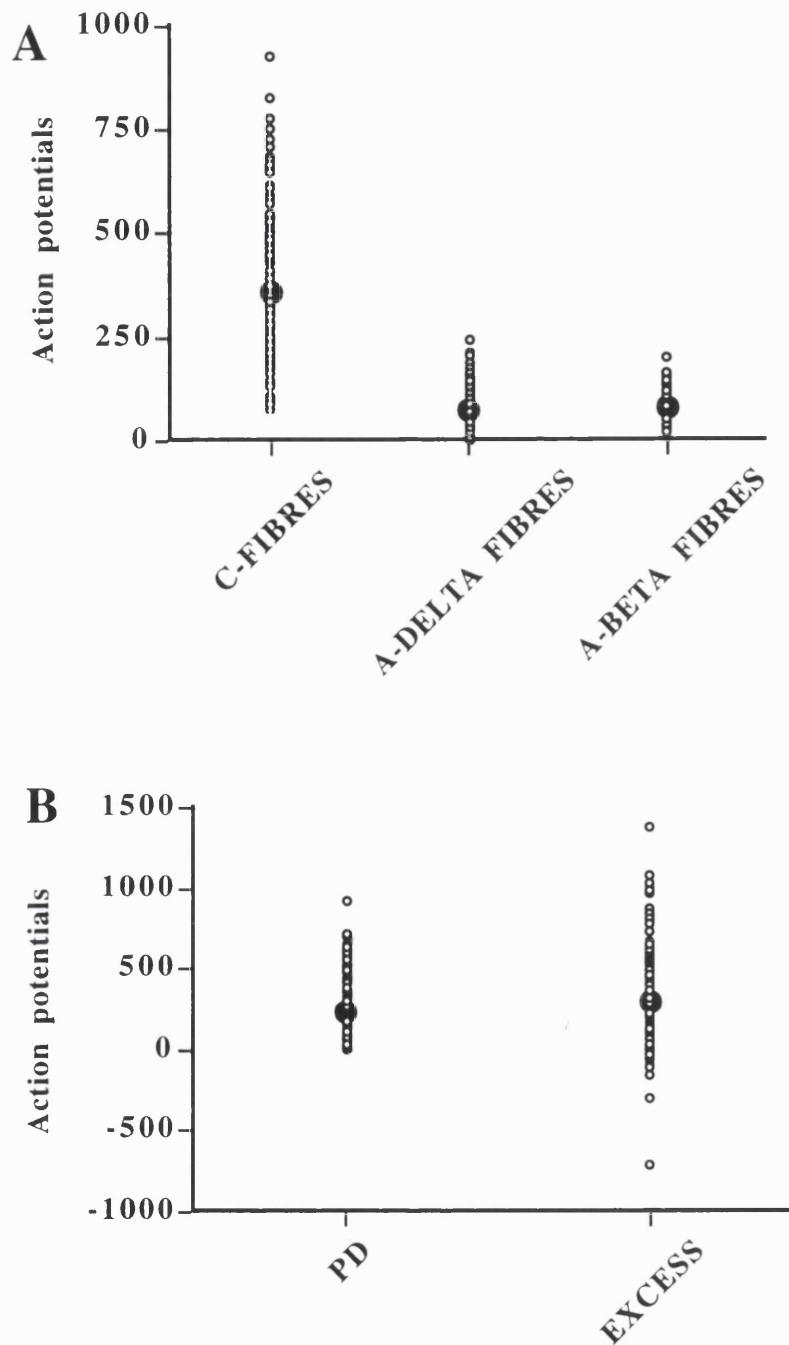
Figure 3. *(A) Action potentials produced by C-, Aδ- and Aβ-fibre evoked stimulation for 198 neurones. (B) The post-discharge (300-800ms) and the excess action potentials. The mean responses are indicated as filled circles.* The fibre evoked responses are separated by conduction velocity and threshold, Aβ (0-20ms); Aδ (20-90ms); c-fibre evoked responses (90-300ms) after stimulation.

The input response gave a mean of 21±1 action potentials (see Figure 4)

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with the final response to a train of 16 stimulations being 619±26 action potentials.
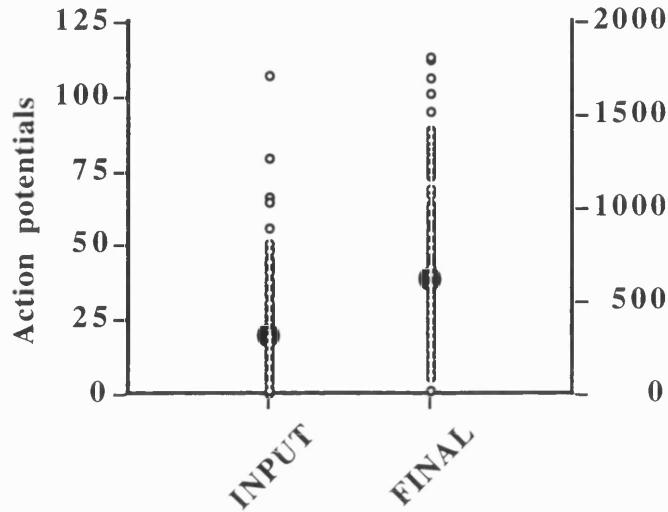

Figure 4. *Action potentials evoked by the input response in a train of sixteen stimuli and the final response in the same train (90-800ms). Data is for a population of cells (n=198) and the mean responses are indicated by the filled circles.*

There was found to be no correlation between the excitability of the cells measured by their ability to wind-up and their response to C-, Aδ- and Aβ-fibre responses, the post-discharge as well as the excess action potentials. There was also no correlation between the depth of the cells with any of the fibre evoked responses or the measures of the increased excitability of the cells (post-discharge and excess action potentials). Note it has been shown in other studies that class 2 cells found in deeper laminae have a greater response to C-fibre evoked after discharge (post-discharge) than more superficially located cells (Schouenborg and Sjolund 1983). This was not found to be the case in this study.

There was however a correlation between the C-fibre evoked responses and the action potentials occurring in the latency band 300-800ms, indicative of the post-discharge of the cells (Spearman's correlation r=0.69, with a two tailed P value of <0.0001) (Figure 5). This would indicate that the greater the C-fibre evoked responses the greater the post-discharge. This is not surprising since the increased excitability of the cells, measured as an after discharge is known to be generated by C-fibre evoked activity (Mendell 1966; Schouenborg and Sjolund 1983).
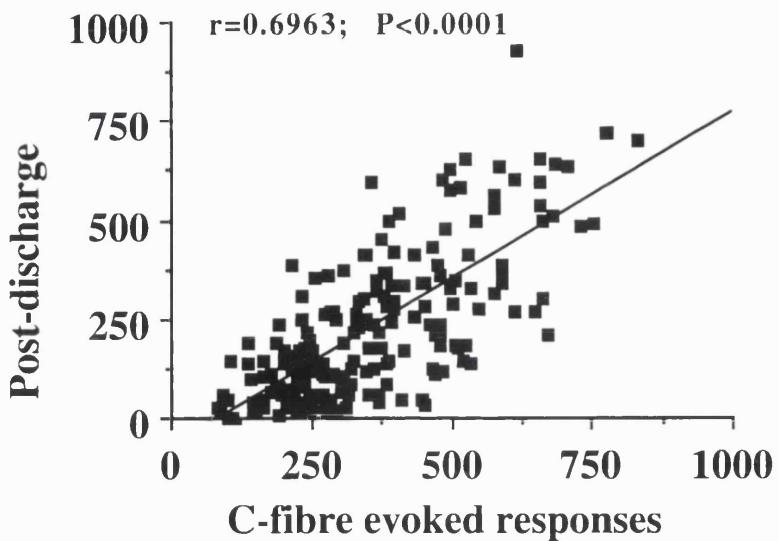
Figure 5. *The response to electrically stimulated C-fibre evoked activity of a population of 198 cells within the dorsal horn of the spinal cord compared to the corresponding post-discharge of the same cells. It was found that the C-fibre evoked responses related to the magnitude of the post-discharge response.*

3.3: The Responses After Carrageenan Induced Inflammation.

The response to electrically evoked activity of cells within the dorsal horn of the spinal cord was observed before and after three hours carrageenan induced inflammation. Forty eight cells were studied after three hours carrageenan. At this stage drug effects were then observed.

There was found to be no correlations between the response of the cell before and after carrageenan with any of the cells responses. This is surprising since it has been clearly demonstrated that the response of neurones can alter (increase or decrease) depending on how excitable cells are before the administration of carrageenan (Stanfa et al. 1992). It has previously been demonstrated that if a cell is winding down (i.e. it cannot maintain its initial firing rate over the predicted response in a train of 16 stimuli) or has a low level of wind-up, the response of the cell becomes increased after carrageenan induced inflammation. If the cell is very excitable (i.e. the responses are increased over the predicted range) then the responses after carrageenan induced inflammation have been shown to decrease (Stanfa et al. 1992). Although I found no correlations between the excitability of the

cells before carrageenan and their responses three hours after carrageenan, there were clear increases and decreases in the responses compared to controls indicating that inflammation can both increase and decrease neuronal responses (see Figures 6A, B and 7). The lack of significance may be due to the low numbers in this study.
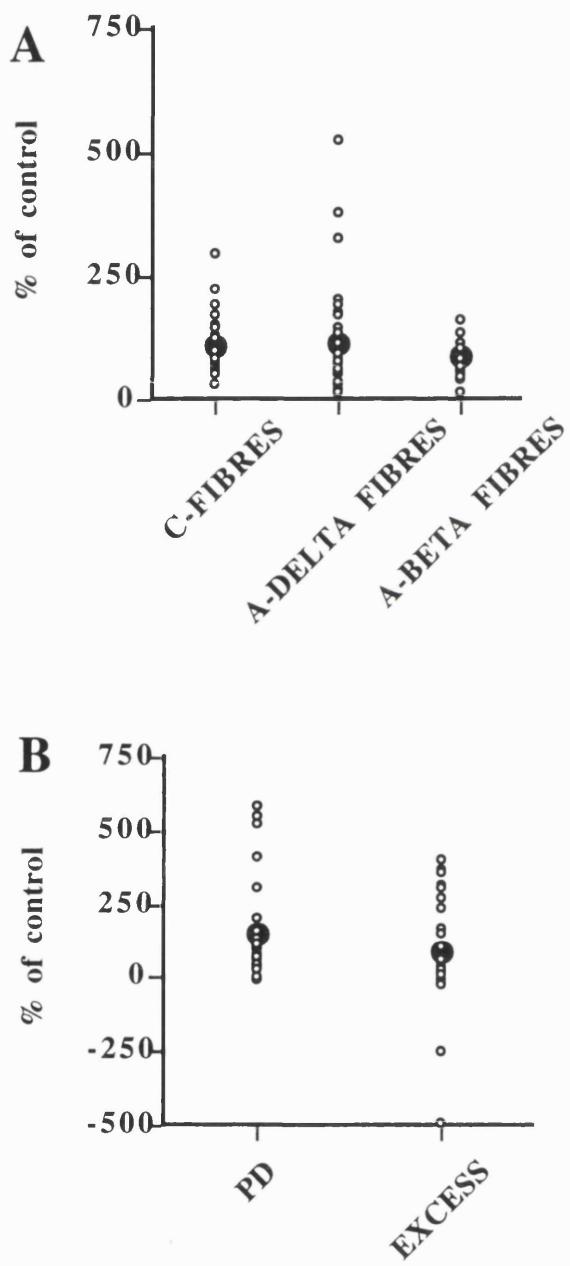

Figure 6. *The response of cells after three hours carrageenan induced inflammation (n=33). The responses are expressed as percentage of initial controls. (A) The C-, Aδ- and Aβ-fibre evoked responses. (B) Post-discharge (PD) and the excess response. The mean responses are indicated by filled circles..*

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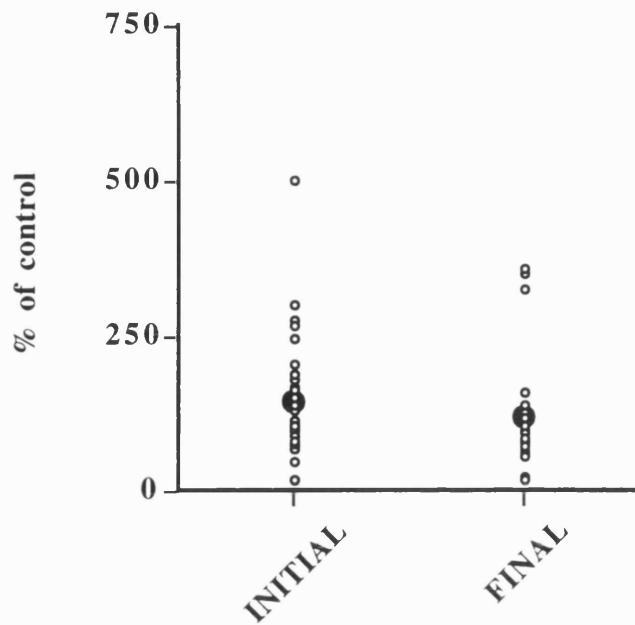
Figure 7. Action potentials evoked by the input response in a train of sixteen stimuli and the final response in the same train (90-800ms) after three hours carrageenan induced inflammation. Data is for a population of cells (n=33) and is expressed as percentage of initial controls. The means are shown as filled circles.

3.4: The Responses to Formalin Induced Activity.

The responses of fifty two cells with an average depth of $751\pm34\mu m$ were recorded after formalin. These cells acted as controls. An example can be seen in Figure 8. The cells included in the formalin studies were first tested with electrical stimulation to observe their characteristics. The average C-fibre threshold was $2.7\pm0.6mA$. The average $A\beta$-, $A\delta$- and C-fibre evoked responses were 91 ± 4, 79 ± 7, 313 ± 20 action potentials respectively.

The first phase of the control formalin response consisted of 5311 ± 641 action potentials and the second phase of the response, 18348 ± 2547 action potentials. There were found to be no correlations between the C-fibre threshold and the magnitude of the first and second phases, the depth of the cells and any of the electrically evoked responses.
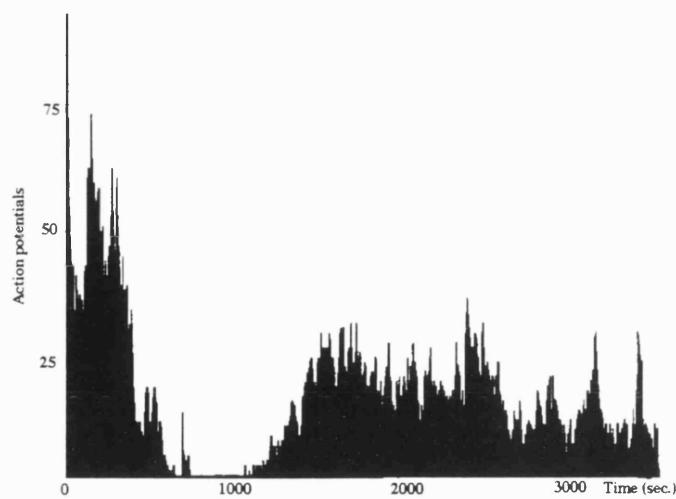
Figure 8. Rate recording showing the action potentials evoked by formalin injected into the peripheral receptive field of a single neurone.
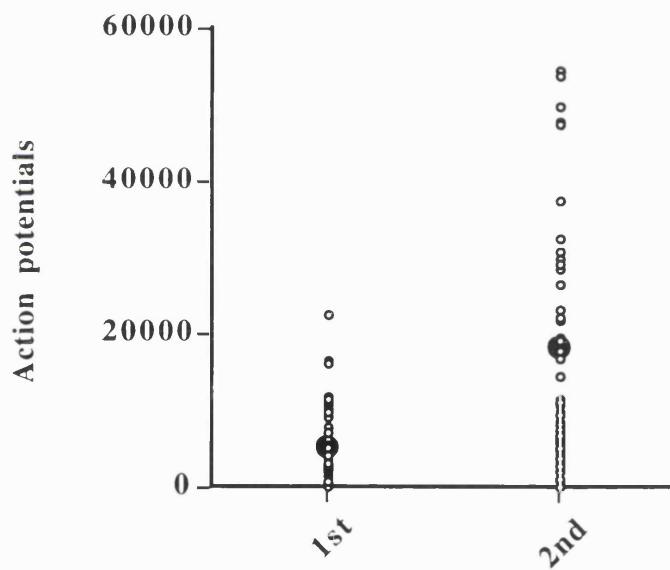
Figure 9. *The response of 52 cells to formalin injected into the peripheral receptive field. Action potentials evoked in the first phase (0-10 minutes) and the second phase (10-60 minutes) are shown. The mean response for this population of cells is indicated by filled circles.*

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3.5: The Response of Cells After Intrathecal Application of Bicuculline.

The responses of fifty cells were observed after intrathecal bicuculline (50μg) with an average depth of 818±27μm from the surface of the spinal cord.

The application of spinal bicuculline was found to cause a significant increase in the response to the natural stimulation of prod. The responses to electrically evoked Aβ-fibre stimulation were altered in a complex manner. After bicuculline it was found that there was additional firing later than the normal Aβ-fibre latency band, especially in latencies associated with Aδ-fibres (see Figure 10A, B).

A
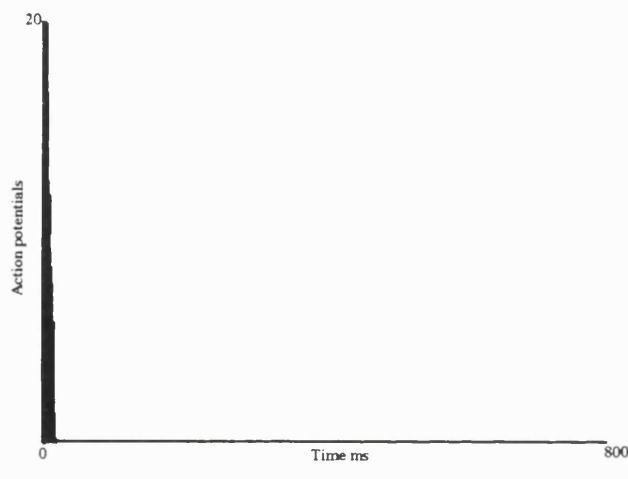
B
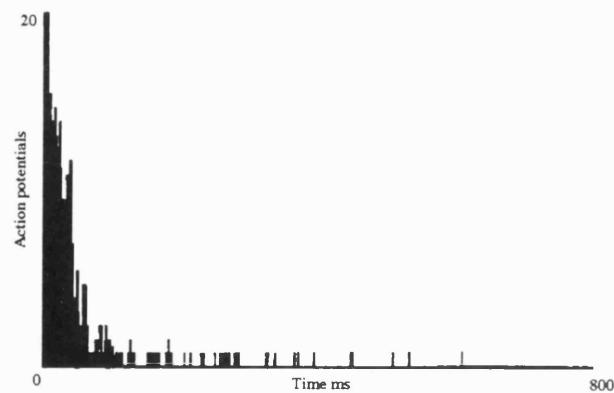
Figure 10. *The response of a single cell to a train of transcutaneous electrical stimulation at three times Aβ-fibre threshold. (A) Control response. (B) The response of the same cell to the above stimulation after 50μg bicuculline.*

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There was also enhanced responses to electrical stimulation at C-fibre strength, and an increase in the responses associated with the Aδ-fibre latency band (see Figure 11A, B).

A
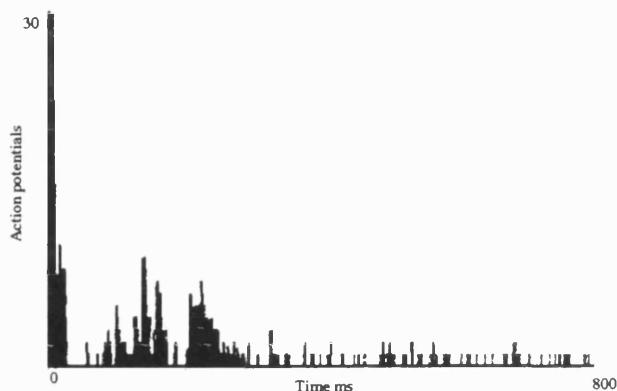
B
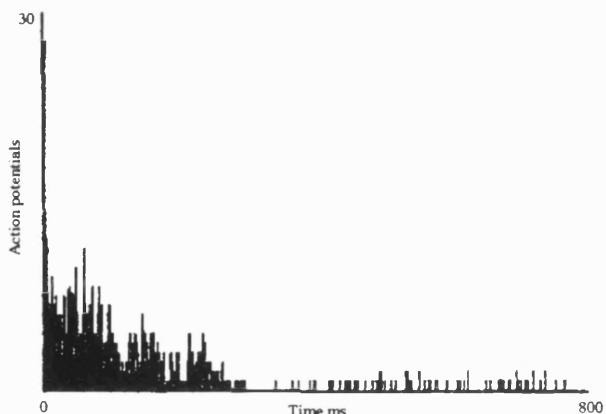
Figure 11. *The response of a single cell to a train of transcutaneous electrical stimulation at three times C-fibre threshold. (A) Control response. (B) The response of the same cell to the above stimulation after 50μg bicuculline.*

3.6: Conclusions.

The responses of single dorsal horn neurones to electrical stimulation in the rat gives rise to stable, reproducible responses that can be easily quantified. For this reason this preparation is ideal to study the effects of spinal cord processing.

Since the animals are intact (not spinalized) the neuronal responses observed very rarely show any degree of spontaneous activity. This means that changes observed by pharmacological manipulation are not affected by bursts of activity, that may influence interpretation of results. The fact that the animal is intact also means that it is possible to observe somatosensory processing as a whole, since this is not a closed system, consisting of just a spinal cord, but also has peripheral and supraspinal afferent pathways, intact and functional.

Acute electrical stimulation delivered to the peripheral receptive field of a neurone is a good model for the study of the responses to discrete fibre evoked stimulations. As the responses to non-noxious and noxious stimulation can be separated due to the differences in threshold and latency, it is easy to observe the effects of different pharmacological manipulations on low and high-threshold inputs. This is important since it is beneficial for analgesic drugs to be as specific for noxious inputs as possible, since the inhibition of non-noxious inputs can give rise to numbness, which can be potentially harmful for patients.

The use of models of altered and more prolonged pain states is also of importance to study, since few, if any clinically untreatable pain state, are acute. Peripheral inflammation gives rise to changes occurring at the site of injury as well as to central changes. Understanding these changes will hopefully aid in more beneficial treatment.

Chapter 4.

The effects of A_1-agonists, an A_2-agonist, adenosine receptor antagonists and kinase inhibitors on the acute responses of dorsal horn neurones.

4.1: Introduction.

Adenosine has long been used for the treatment of cardiovascular disorders (Mangano 1990; Mullane and Young 1993; Olsson and Pearson 1990). However, it has only been in the last few decades that an increase in research in the therapeutic benefits of the use of adenosine in many other disorders has occured. The possibilities of adenosine being used as an anti-epileptic, in sleep disorders, as a neuroprotective agent in cerebral ischemia and as an analgesic, as well as antagonists of adenosine being used in the treatment of asthma, have been explored (de Mendonca et al. 1995; see Marangos 1991; Salter et al. 1993; Snyder 1985). It is probable in many of these conditions that adenosine has a presynaptic effect in inhibiting neurotransmitter release and thus decreasing excitations (Fastbom and Fredholm 1985; Fredholm and Dunwiddie 1988; Fredholm and Hedqvist 1980; Goodman et al. 1983; Manzoni et al. 1994; Peris and Dunwiddie 1986; Snyder 1985). However, in the spinal cord the effects of adenosine are in fact mainly post-synaptic (Choca et al. 1988; Hongo et al. 1968; Li and Perl 1994) and this site of action has been observed in other areas of the brain (Craig and White 1992; Hoehn et al. 1990a; de Mendonca et al. 1995).

The function of adenosine as an analgesic has been a subject of much consideration (see Salter et al. 1993; Sawynok and Sweeney 1989; Snyder 1985; Stone 1981 for reviews). However, there have been many confusions and conflicts in the historical literature, on adenosine.

The first clues that the purinergic system may be involved in antinociception was gained from studies by Contreras et al. (1972) and by Paalz ow and Paalzlow (1973). The non-specific adenosine antagonist, theophylline when given i.p. was shown to decrease the reaction time in the hot plate test in mice (Contreras et al. 1972). In separate studies theophylline and caffeine were again administered i.p. to rats, and found to lower the threshold for vocalization and tail withdrawal from transcutaneous electrical stimulation to the tail (Paalz ow and Paalzlow 1973). However, the algesic effects of these methylxanthines in these experiments was not attributed to the antagonism of endogenous adenosine at a receptor level, but their effects on phosphodiesterase activity and possible alteration in serotonin turnover and accumulation of cyclic adenylate (Paalzlow and Paalzlow 1973; Contreras et al. 1972) respectively.

Since these studies there have been many behavioural studies observing the effects of analogues of adenosine in nociceptive tests (Ahlijanian and Tackemori 1985; DeLander and Hopkins 1986; Holmgren et al. 1983, 1986; Karlsten et al.

1990; Malmberg and Yaksh 1993a; Post 1984; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Vapaatalo et al. 1975; Yarbrough and McGuffin-Clineschmidt 1981). However, unlike the behavioural studies there have been few electrophysiological studies (Salter and Henry 1985, 1987, 1989; Salter et al. 1993). Behavioural studies are hampered by the possibility of motor effects of purines, which may not only influence interpretation of the results and prevent a full dose response relationship from being explored (Karlsten et al. 1990; Malmberg and Yaksh 1993a; Sosnowski et al. 1989). This is especially so in the case of the agonist N-ethylcarboxamide-adenosine (NECA), equipotent at A_2 and A_1-receptors (Fredholm et al. 1994). *Since the A_2-component may also give rise to* Early *motor effects.* studies attributed antinociceptive effects to the A_2-receptor using this agonist (Sawynok et al. 1986) but a study by Karlsten et al. (1990) has shown that motor effects, which are predominantly mediated by the A_2-receptor, occur at low doses of NECA; doses which fall within the dose-range used in the early behavioural studies.

In view of these limitations and lack of electrophysiological evidence I examine here the role of the A_1-receptor agonists, N^6-cyclopentyladenosine (CPA) and 2-chloroadenosine (2-Cl-Ado) on the electrically evoked A- and C-fibre evoked activity of dorsal horn neurones. The reversal of the effects of CPA and 2-Cl-Ado was observed using the non-specific antagonist theophylline and the effects of CPA was also measured in the presence of 8(p-Sulphophenyl) theophylline (8pSPT).

The effect of the A_{2a}-receptor agonist, 2-p-(2-Carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680), was also observed on the responses of dorsal horn neurones to electrical stimulation. Due to the lack of water soluble specific A_{2a}-receptor antagonists, the effects with CGS-21680 could not be reversed.

The effects of antagonists alone were also evaluated on the electrically evoked responses of dorsal horn neurones to gauge any effects of endogenous adenosine. Here the non-specific antagonists, theophylline and 8pSPT as well as the more specific, but less soluble antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) were used.

Finally, the use of two novel adenosine kinase inhibitors, known here as *(Hill et al. 1995; Foster et al. 1995)* GPA and GPB, were also evaluated in order to protect endogenously released adenosine by preventing its breakdown. This was to ascertain the role of endogenous adenosine in the control of nociceptive inputs.

4.2.1: N^6-cyclopentyladenosine.

The effects of CPA were observed on a total population of 18 cells, with a mean depth of 740±42µm from the surface of the dorsal horn of the spinal cord. CPA was given directly onto the exposed spinal cord via the intrathecal route. The doses of CPA evaluated were 0.05, 0.5, 5, 50 and 250µg in 50µl, with each dose being evaluated for forty minutes. The results for each dose were expressed as a mean of the population ± the standard error of the mean. In the calculations cells were omitted if they showed spontaneous activity throughout the experiment; here two cells were omitted.

The Aβ-fibre evoked activity was not significantly affected with any dose of CPA (Figure 1A). Even with the top dose (250µg) this response was still 77±24% of control. The effects of CPA were dose dependent on all other fibre evoked responses as well as the enhanced responses of the cells, post-discharge and wind-up.
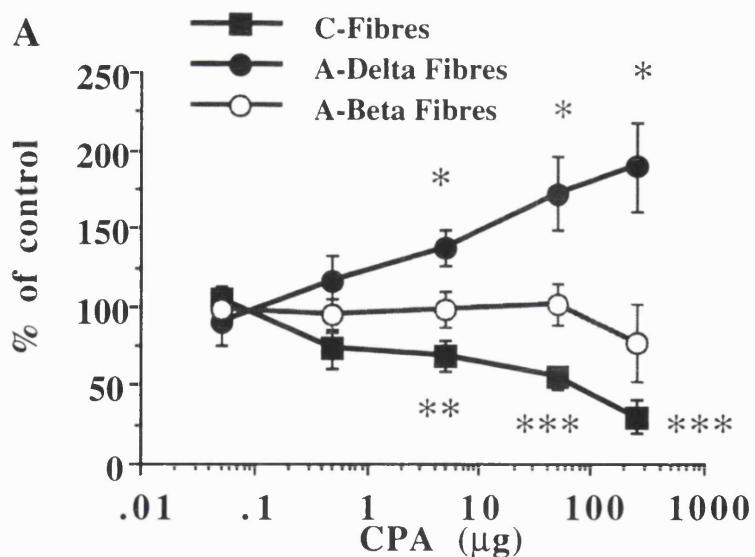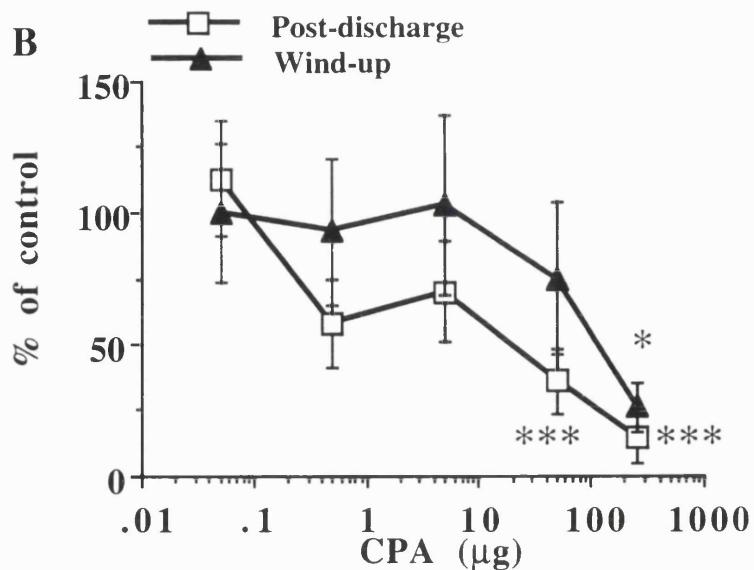
Figure 1. Dose-response relations for CPA. (A) Effects of 0.05, 0.5, 5, 50 and 250μg CPA applied intrathecally on the mean responses of a population of dorsal horn neurones to electrical C-fibre, Aδ-fibre and Aβ-fibre stimulation. (B) Effects on post-discharge and wind-up of the same population of neurones. *P<0.05, **P<0.01, ***P<0.005.

The C-fibre evoked responses were significantly inhibited by 5μg CPA and subsequent doses, so that the C-fibre evoked activity was 69±10, 55±7 and 30±11% of control for 5, 50 and 250μg CPA respectively (see Figure 1A for P values). However, the Aδ-fibre evoked response was concurrently facilitated over the same time-course and dose range (see Figure 1A and 2A). Thus, the response of the same population of cells were facilitated to 137±11, 172±24 and 189±29% of control with 5, 50 and 250μg CPA. All these doses produced significant facilitations (P<0.05) whereas lower doses were not significant.

The enhanced responses of the cells to the constant stimulation, the post-discharge and wind-up, were also inhibited. Post-discharge was significantly inhibited by 50μg and above with wind-up only being significantly inhibited with 250μg CPA (see Figure 1B and 2B). The post-discharge response was 36±12 and 15±10% of control after 50 and 250μg CPA respectively. Wind-up was reduced to 26±9% of control with 250μg CPA (also see Figures 3 and 4).

All these reduced responses were reversed by the antagonist theophylline, at a high dose of 1000μg (Figures 2A, B). This was given at forty minutes after 250μg CPA and the effects followed for a further forty minutes.
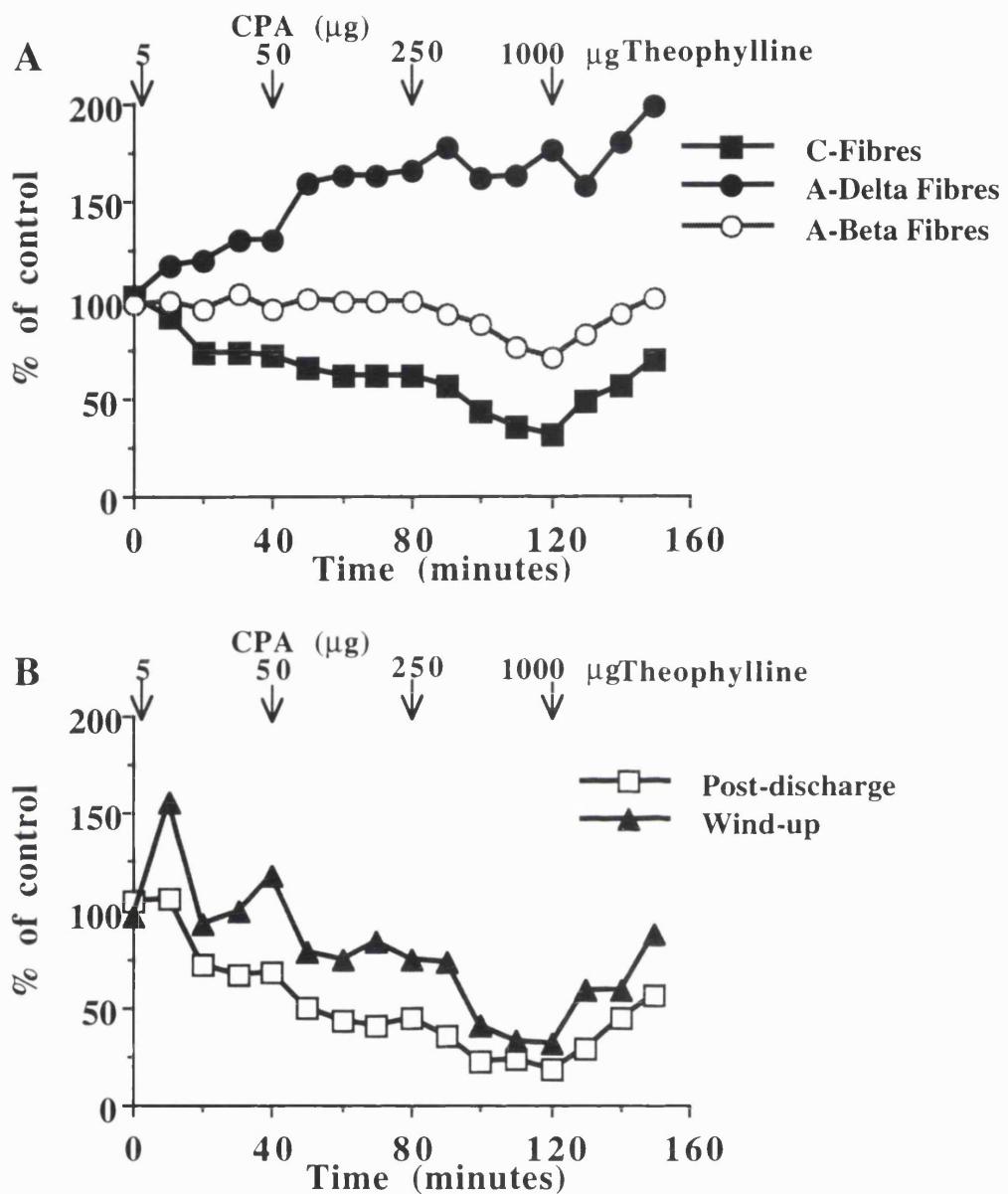
Figure 2. Time course of the effects of CPA and reversal by theophylline
(1000μg). Effects of 5, 50 and 250μg CPA applied intrathecally (indicated by
arrows) on the responses of dorsal horn neurones to (A) C-fibre, Aδ-fibre and Aβ-
fibre evoked activity. (B) Post-discharge and wind-up.

The non significant inhibition of the Aβ-fibre evoked responses was
77±24% of control with 250μg CPA and this was reversed to 102±15% of control
and 1000μg theophylline respectively. The C-fibre evoked responses were also
reversed from 30±11% of control with 250μg CPA to 72±14% of control with

1000μg theophylline. The facilitated Aδ-fibre evoked response which was 189±29% of control with 250μg CPA was not altered by theophylline and was 199±33% of control after exposure to 1000μg theophylline.

Individual examples of the effects of CPA on the wind-up of the cells can be seen in figures 3A, B.
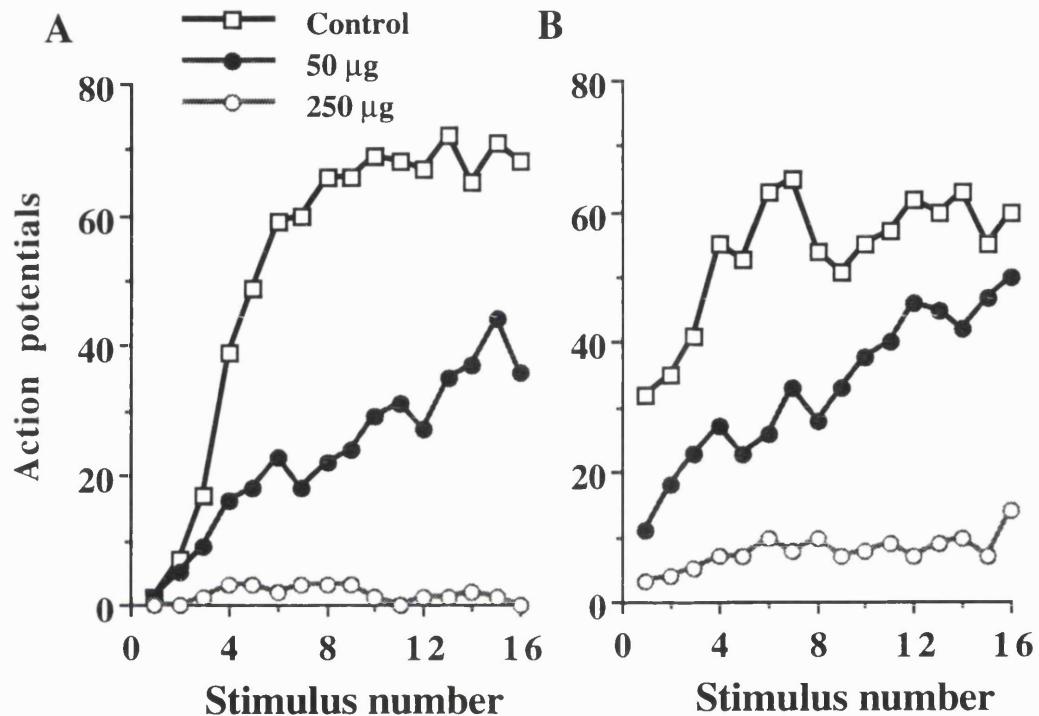
Figure 3. *Examples of wind-up responses of two individual cells. Wind-up is plotted as action potentials against stimulus number. The control response before drug application is shown, compared to 50 and 250μg CPA. Note in (B) that the input is also decreased along with the wind-up response.*

Generally 50μg CPA reduced the increased excitability of the cells as measured by the wind-up response, although there was a degree of variability between individual cells. Increasing the dose of CPA to 250μg abolished the wind-up of some cells and dramatically reduced others. As well as decreasing wind-up, CPA also reduced the initial input response of the cells as can be seen in the example in Figure 3B. The effects of CPA on the initial and final response to a train of stimuli can also be seen in Figure 4. As you can see CPA reduced both the initial response (as can be seen in Figure 3B) and the final response to a similar extent. Although 5μg CPA and above gave significant effects on the initial input, only 50μg and above

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significantly inhibited the final response to a train of stimuli *(P<0.05)*.

Theophylline reversed the effects of CPA on both the wind-up and post-discharge. The inhibited post-discharge (15 ± 10) was $60\pm16\%$ of control, and wind-up (26 ± 9) was $95\pm29\%$ of control after $1000\mu g$ theophylline (Figure 2B).
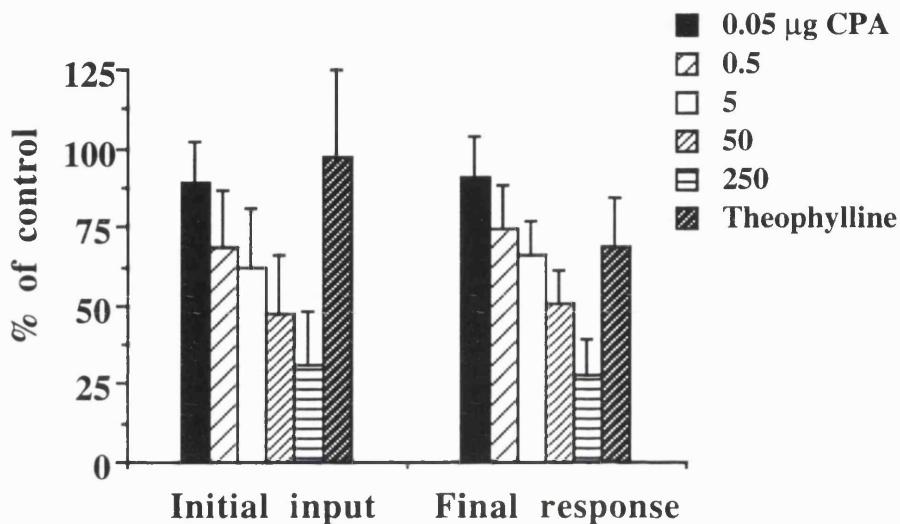
Figure 4. *The response of a population of cells to the initial (input) in a train of 16 stimuli at C-fibre stimulation and the final response in the same train. The subsequent inhibitory effects of CPA (0.05, 0.5, 5, 50 and 250μg) on these responses are shown. Reversal with theophylline (1000μg) is also shown.*

4.2.2: 8(p-Sulphophenyl) Theophylline Pretreatment to CPA.

The effect of the maximal dose studied in the dose response relations for CPA, ($250\mu g$), was also observed after a pretreatment with $400\mu g$ 8pSPT. Ten dorsal horn neurones with a depth of $745\pm60\mu m$ were observed in this study; previously the same population of cells had been used to establish the dose response relations for 8pSPT alone.

The effect of CPA alone ($250\mu g$) on the C-fibre evoked response was an inhibition of $70\pm11\%$. However, the same dose of CPA in the presence of 8pSPT only inhibited the C-fibre evoked responses by $33\pm7\%$. This attenuation of the inhibition was significant *(P<0.05)*. The inhibitory effects of CPA on the wind-up responses of the same cells were also significantly attenuated by 8pSPT *(P<0.05)*

(see Figure 5). However, the post-discharge and the Aβ- and Aδ-fibre evoked responses were not significantly changed.
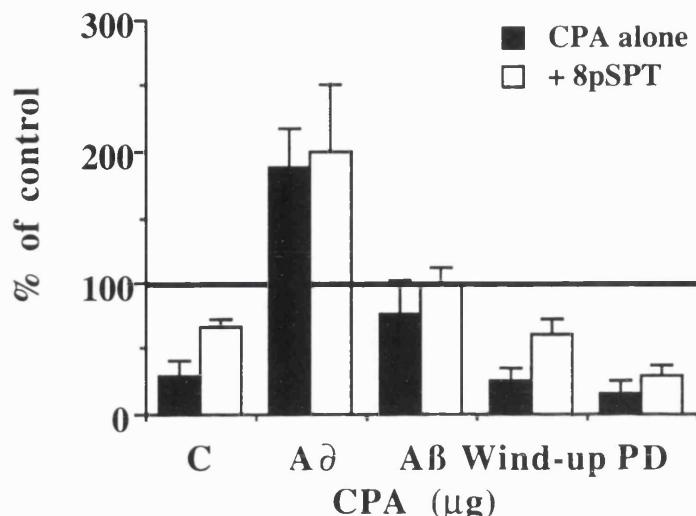
Figure 5. *The effects of CPA alone (250μg) are compared to the effects in the presence of 8pSPT (400μg). Pretreatment with 8pSPT was 40 minutes prior to CPA. The C-fibre evoked responses and wind-up were significantly attenuated by pretreatment with the antagonist.* n = 10, P<0.05

Since wind-up is calculated as the difference between the initial input onto the cells and the overall response to a train of stimulation (action potentials evoked between 90-800ms after stimulation) the differences between the effects of the antagonists on wind-up and post-discharge can be explained on the basis of the calculation of these responses. As can be seen in Figure 6, the addition of 8pSPT decreases the ability of CPA to reduce the initial input onto the cells, but not the last response in the train. Thus, the inhibition of the wind-up response appears to be significantly attenuated by the presence of 8pSPT whereas in reality it reflects the reduced effect of CPA on the initial input.
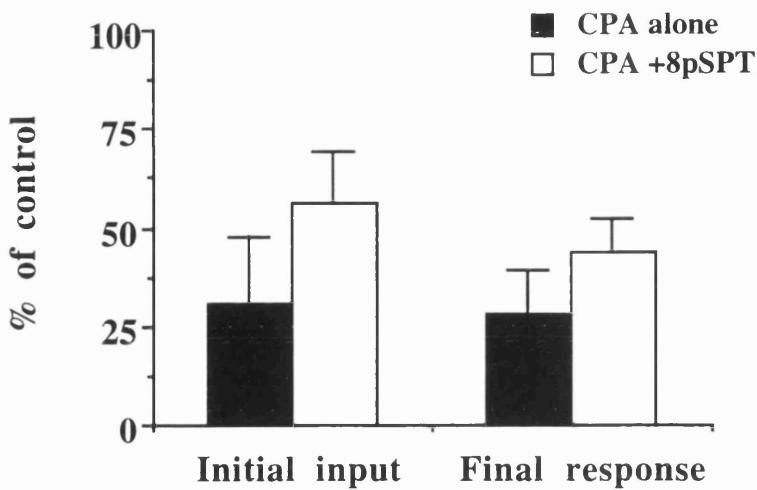
Figure 6. The initial response (input) to a train of 16 stimuli at C-fibre stimulation and the final response to the same train is shown for the population of cells. The inhibitory effects of CPA alone (250μg) and the same dose of CPA in the presence of 8pSPT (400μg) on these responses are shown.

4.2.3: 2-chloroadenosine.

The effects of 2-Cl-Ado (50, 250 and 500μg) were evaluated on six neurones with an average depth of $702\pm82\mu m$ from the surface of the dorsal horn. Unlike the effects of CPA there were no clear dose-dependent effects of 2-Cl-Ado. For example, the C-fibre evoked responses were 66 ± 10, 65 ± 9 and $56\pm13\%$ of control after 50, 250 and 500μg of 2-Cl-Ado respectively. The inhibitory effects of 50 and 250μg 2-Cl-Ado on the C-fibre responses were significant when compared to controls ($p<0.05$). However, the inhibition after 500μg was not significant reflecting a degree of variability with this dose. In addition, there was no significant dose-dependency (see Figure 7). There were comparable effects with 2-Cl-Ado on the post-discharge and wind-up responses, in that the first dose tested (50μg) produced a near maximal change whereas increasing the dose (250 and 500μg) produced no greater change in response (Figure 7). With both these measured responses only 50μg gave significant change in response compared to controls ($P<0.05$). Again, as with CPA the Aδ-fibre evoked responses were concurrently facilitated by the same doses and time course as the inhibited responses. As with the inhibited responses with 2-Cl-Ado, there was a ceiling effect on this Aδ-fibre evoked

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facilitation with the lowest dose tested giving near maximal effects. Both 50 and 250µg gave significant effects when compared to controls ($P<0.05$). The Aβ-fibre evoked responses were not significantly effected by any dose of 2-Cl-Ado.
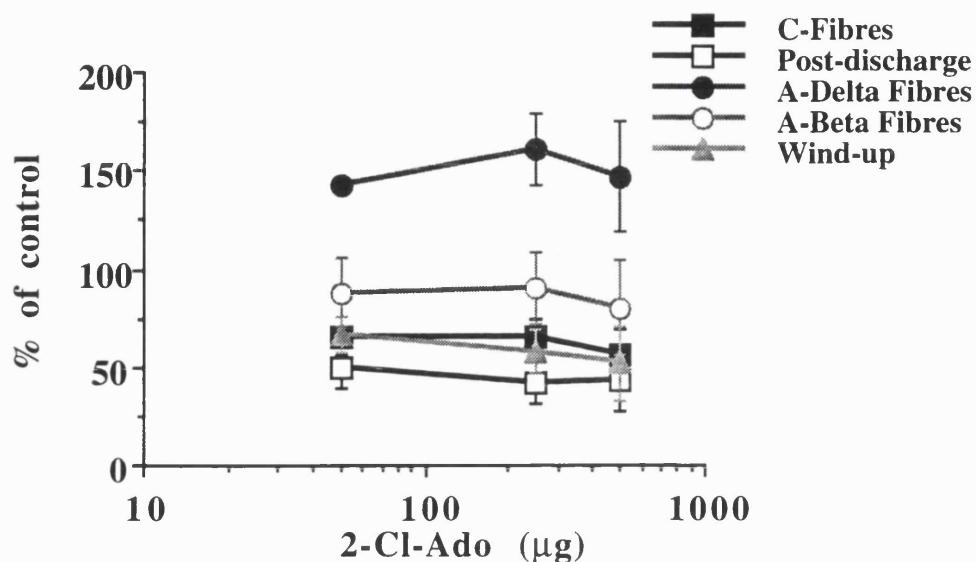
Figure 7. *Dose response relations for 2-Cl-Ado (50, 250 and 500µg) on the response of a population of deep dorsal horn neurones. The wind-up and post-discharge were significantly inhibited by 50µg 2-Cl-Ado as were the C-fibre evoked responses (all doses). The Aδ-fibre evoked responses were significantly facilitated by all dose of 2-Cl-Ado.*

Thus, although some individual doses showed significant effects when compared to control values, there were no significant dose-dependent effects on any of the fibre evoked responses with 2-Cl-Ado or on post-discharge and wind-up. It may be that the doses chosen were at the top end of the dose response curve, but in view of the weaker effects compared to CPA, further studies with 2-Cl-Ado were not undertaken.

Theophylline (1000µg) was also used to attempt to reverse the effects of 500µg 2-Cl-Ado. The C-fibre evoked responses were reversed from 56±13 to 84±8% of control with 2-Cl-Ado and theophylline respectively. The Aδ-fibre evoked responses were 147±28% of control with 2-Cl-Ado and after theophylline the response was 133±42% of control. The Aβ-fibre evoked responses were unchanged after either agonist or antagonist. The post-discharge was not reversed by theophylline. After theophylline the response was 45±21% of control whereas

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previously it had been 43±16% of control with the agonist. Wind-up also was not fully reversed. With 2-Cl-Ado the response was 52±19 and after theophylline it was 76±15% of control.

4.2.4: 2-*p*-(2-Carboxyethyl) Phenethylamino-5'-*N*-Ethylcarboxamidoadenosine Hydrochloride.

The effects of CGS-21680 were studied on eight dorsal horn neurones with a mean depth of 842±60μm from the surface of the dorsal horn. CGS-21680 was given intrathecally at doses of 0.75, 7.5, 25 and 75μg. Each dose was followed for forty minutes. Higher doses could not be evaluated due to solubility problems.

The effects of CGS-21680 did not distinguish between high and low threshold inputs so that all evoked responses were inhibited to some extent (see Figure 8).
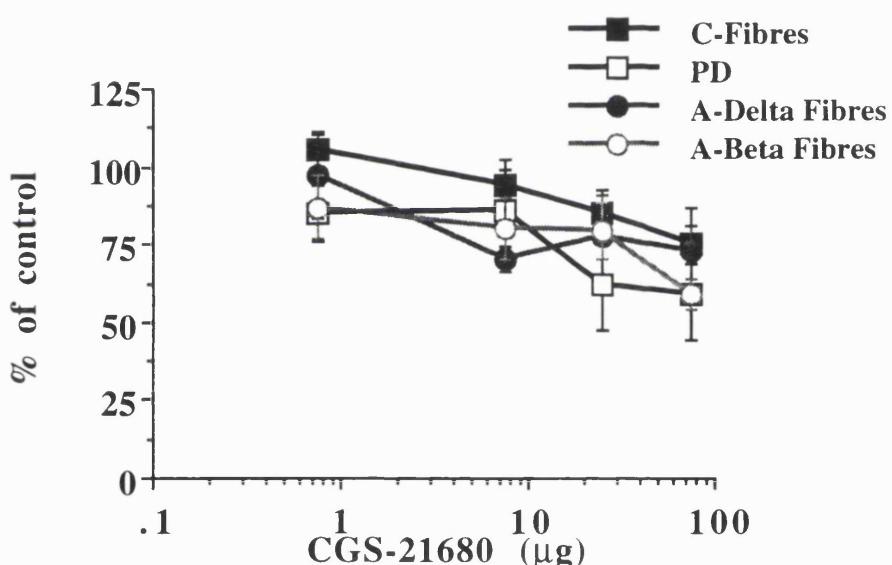
Figure 8. Dose response relationship for CGS-21680. Inhibition by CGS-21680 of the high and low threshold inputs was not distinguishable. Post-discharge is shown as PD.

With 75μg CGS-21680 the C-fibre evoked response was 75±6% of control, which was significant inhibition (*P*<0.05). Although the post-discharge responses tended to be inhibited no effect was significant. The Aδ-fibre evoked responses were also significantly inhibited by 7.5 and 25μg (*P*<0.05). The Aβ-fibre evoked responses were only inhibited by 75μg CGS-21680 (*P*<0.05).

Wind-up could not be calculated as often in the control observations the

cells were winding down and thus giving negative recordings. Here this measure was calculated by observing the initial input onto the cell and the final response in a train of stimulation. With the top dose tested, 75μg CGS-21680, there was a reduction in both the input onto the cell and final response. Figure 9 shows the effects of CGS-21680 on these responses for the population of cells (n=6).
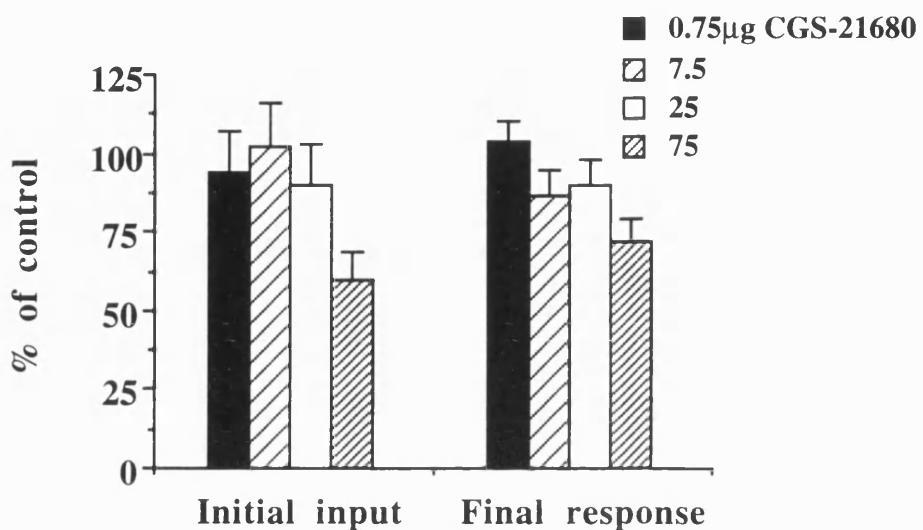
Figure 9. The response of a population of cells to the initial (input) in a train of 16 stimuli at C-fibre stimulation and the final response in the same train. The effects of CGS-21680 (0.75, 7.5, 25 and 75μg) are shown. n= b

Although CGS-21680 had some inhibitory effects they were weak and non-selective for both nociceptive and non-nociceptive responses in contrast to the selective effects of CPA for noxious evoked activity.

4.2.5: 8(p-Sulphophenyl) Theophylline.

The effects of 8pSPT were observed on ten dorsal horn cells, with a mean depth of 745±60μm from the surface of the dorsal horn. The antagonist was given intrathecally at doses of 1, 10, 100 and 400μg. Although some responses tended to be inhibited there were no significant effects with 8pSPT on any of the fibre evoked responses or the measures of increased excitability, post-discharge and wind-up (see Figure 10).
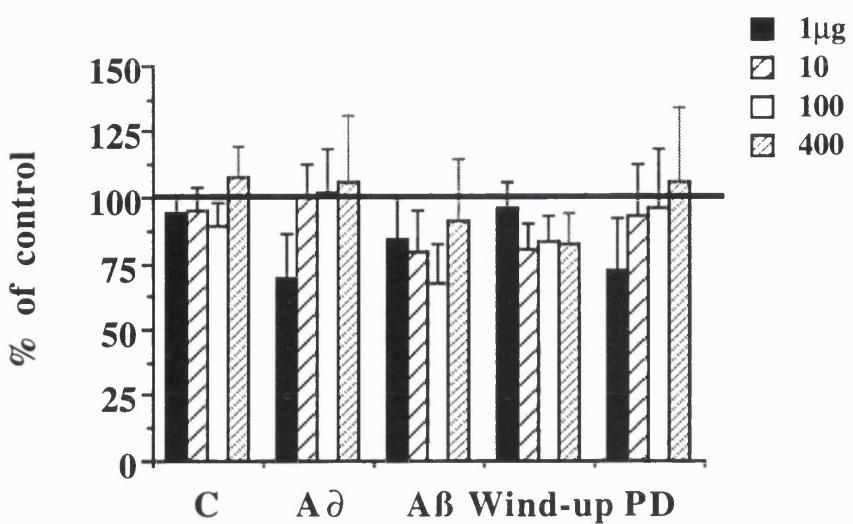
Figure 10. Dose-response relations for the antagonist, 8pSPT (1, 10, 100 and 400μg) given intrathecally. Although some doses appeared to inhibit the Aδ-fibre and Aβ-fibre evoked responses as well as post-discharge (PD) there were no significant effects of this antagonist. n = 10

4.2.6:Theophylline.

A population of 6 cells with a mean depth of 788±110μm from the surface of the dorsal horn was studied. Theophylline was given intrathecally at doses of 500, 1000, and 2000μg.

The C-fibre evoked responses tended to be facilitated with all doses of theophylline, but due to variability in the responses the facilitations were not significant (see Figure 11). This was also the case for the post-discharge and wind-up of the cells. The Aδ- and Aβ-fibre evoked responses were unchanged after any of the doses of theophylline.
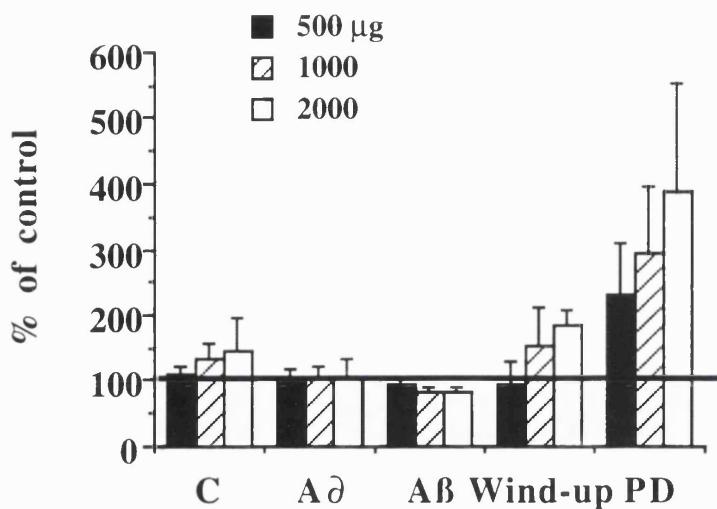
Figure 11. *Dose-response relations for the antagonist, theophylline (500, 1000 and 2000µg) given intrathecally. Although the C-fibre evoked response, post-discharge and wind-up tended to be facilitated these were not significant, reflecting the variability of responses.* n = 6

4.2.7: 8-cyclopentyl-1, 3-dipropylxanthine.

The effects of the antagonist DPCPX (0.5, 5 and 50µg) were tested on 9 dorsal horn cells with an average depth of 929±78µm. This antagonist had to be made up in a vehicle of 50% absolute ethanol / 50% distilled water. Five control cells were observed for the vehicle alone with an average depth of 852 ±127µm.

The vehicle did have inhibitory effects itself and was by no means ideal. Other vehicles were tried but were found not to give adequate solubility. The vehicle reduced the C-fibre response to 72±7% of control ($P<0.05$), Aδ-fibre evoked responses to 49±12% of control ($P<0.05$) and the Aβ-fibre responses to 68±15% of control. Post-discharge was also reduced to 47±10% of control values ($P<0.05$). Wind-up could not be calculated due to the cells not being able to maintain their excitability (see Figure 12) so the response was instead evaluated as initial input onto the cells and the final response to a train of constant stimuli. The vehicle had no effect on the initial input but did reduce the final overall response in the train of stimuli. The initial response was 113±14% of control whereas the final response was 75±10% of control.
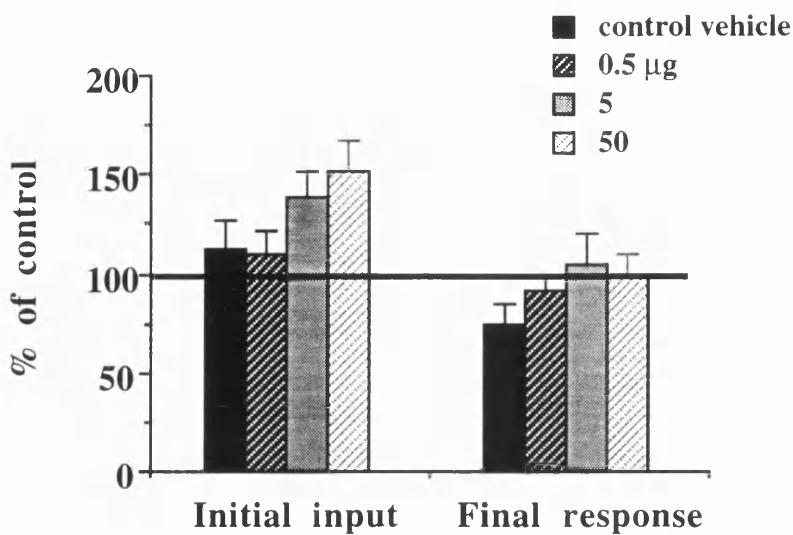
Figure 12. *The response of a population of cells to the initial (input) in a train of 16 stimuli at C-fibre stimulation and the final response in the same train. The effects of the vehicle for DPCPX can be seen to decrease the overall excitability of the cells (final response) but did not effect the initial input. The effects of DPCPX in the same vehicle (0.5, 5 and 50µg) can also be seen.* n= 9

These marked inhibitory effects of the vehicle will have masked some of the effects of the antagonist. However, there were clear significant drug effects of DPCPX compared to the vehicle alone. The C-fibre and Aδ-fibre evoked responses as well as post-discharge were all significantly increased by all doses of DPCPX ($P<0.05$). The Aβ-fibre evoked responses were not significantly inhibited by the vehicle alone or increased by DPCPX (see Figure 13). Nevertheless, in view of the undoubted neurotoxic effects of the vehicle, it would be unwise to extrapolate these results to an interpretation of physiological function .

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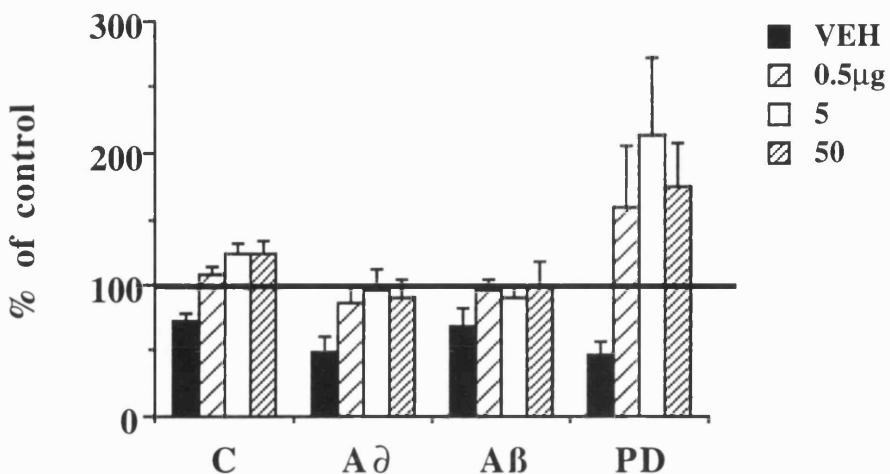
Figure 13. *Dose response relations for DPCPX. Vehicle controls are shown for intrathecally applied 50% absolute ethanol/ 50% distilled water. These are expressed as percentage of initial controls. DPCPX (0.5, 5 and 50μg) were given in the same vehicle and expressed as a percentage of the vehicle controls.* n= 9

4.2.8: GPA-An Adenosine Kinase Inhibitor.

The effects of GPA were observed on 8 cells with a mean depth of 854±71μm from the surface of the dorsal horn. GPA was given subcutaneously, the doses being 1, 5 and 10mg/kg in a volume of 250μl. GPA was dissolved in 100% DMSO and controls for the vehicle were evaluated on five cells with a mean depth of 862±102μm.

The vehicle controls were found to have no significant effect on any of the responses evaluated after DMSO was given subcutaneously (see Figure 14). So that the C-fibre evoked responses were 94±11% of control, the Aδ-fibres were 87±12 and the Aβ-fibres were 97±7 with the vehicle alone. The post-discharge and wind-up responses were reduced to 74±5 and 72±11% of control respectively for DMSO alone.

GPA dose dependently inhibited the C-fibre evoked responses, with 10mg/kg only being significant when compared to the vehicle controls (*P*<0.05). So that the C-fibre evoked responses were reduced to 68±8, 67±6, 55±5% of control for 1, 5 and 10mg/kg GPA respectively. The Aδ-fibres were significantly inhibited by all doses of GPA when compared to controls, the Aβ-fibre responses were not significantly inhibited with any dose of GPA. Post-discharge was significantly

inhibited by 10mg/kg GPA only, whereas wind-up responses were not significantly inhibited with any dose of GPA when compared to the vehicle controls (see Figure 14).
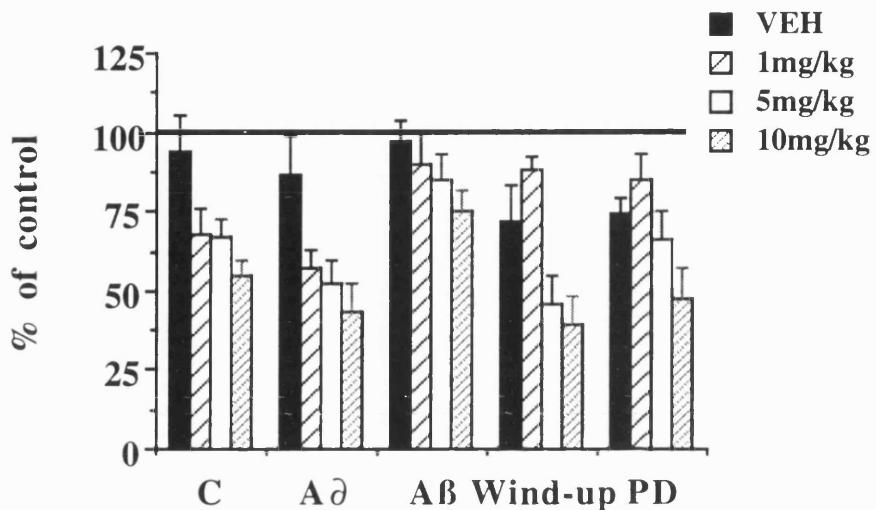
Figure 14. *Dose response relations for the systemically given kinase inhibitor,* (n= 8) *GPA. The effect of the vehicle, 100% DMSO can also be seen(n= 5).*

The excitability of the cells was also measured in the initial input onto the cells as well as the final overall response. The vehicle given alone did not cause any significant change in the input or the overall final response, see Figure 15.

All doses of GPA (1, 5, 10mg/kg) reduced both the input and the overall, final response to a similar extent. However, when comparing these measures to the vehicle controls none of these responses were significant.
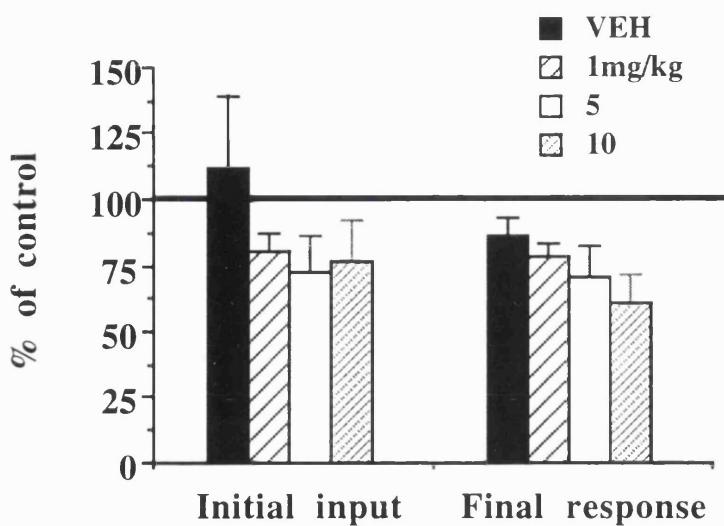
Figure 15. *The response of a population of cells to the initial (input) in a train of 16 stimuli at C-fibre stimulation and the final response in the same train. The effects of vehicle and GPA (1, 5, 10mg/kg) are shown.*

4.2.9: GPB-An Adenosine Kinase Inhibitor.

Seven dorsal horn neurones with a mean depth of $577\pm102\mu$m from the surface of the dorsal horn were studied after 5, 50 and 500μg of GPB given intrathecally, and made up in saline.

The C-fibre evoked responses were inhibited by GPB to 93 ± 14, 90 ± 12 and $70\pm8\%$ of control with 5, 50 and 500μg of GPB respectively. Only the effects of 500μg GPB were significant ($P<0.05$) see figure 16A. The Aδ-fibre evoked responses were only significantly inhibited by 500μg GPB to $73\pm10\%$ of control. The Aβ-fibre evoked responses were not significantly changed with any dose of GPB. Post-discharge of the cells was only significantly inhibited by 500μg GPB ($48\pm9\%$ of control) ($P<0.05$). Although the wind-up responses tended to be facilitated with the lower doses of GPB (5 and 50μg) these were not significant. Increasing the dose of GPB (500μg) significantly inhibited wind-up to $46\pm10\%$ of control ($P<0.05$) see Figure 16B.
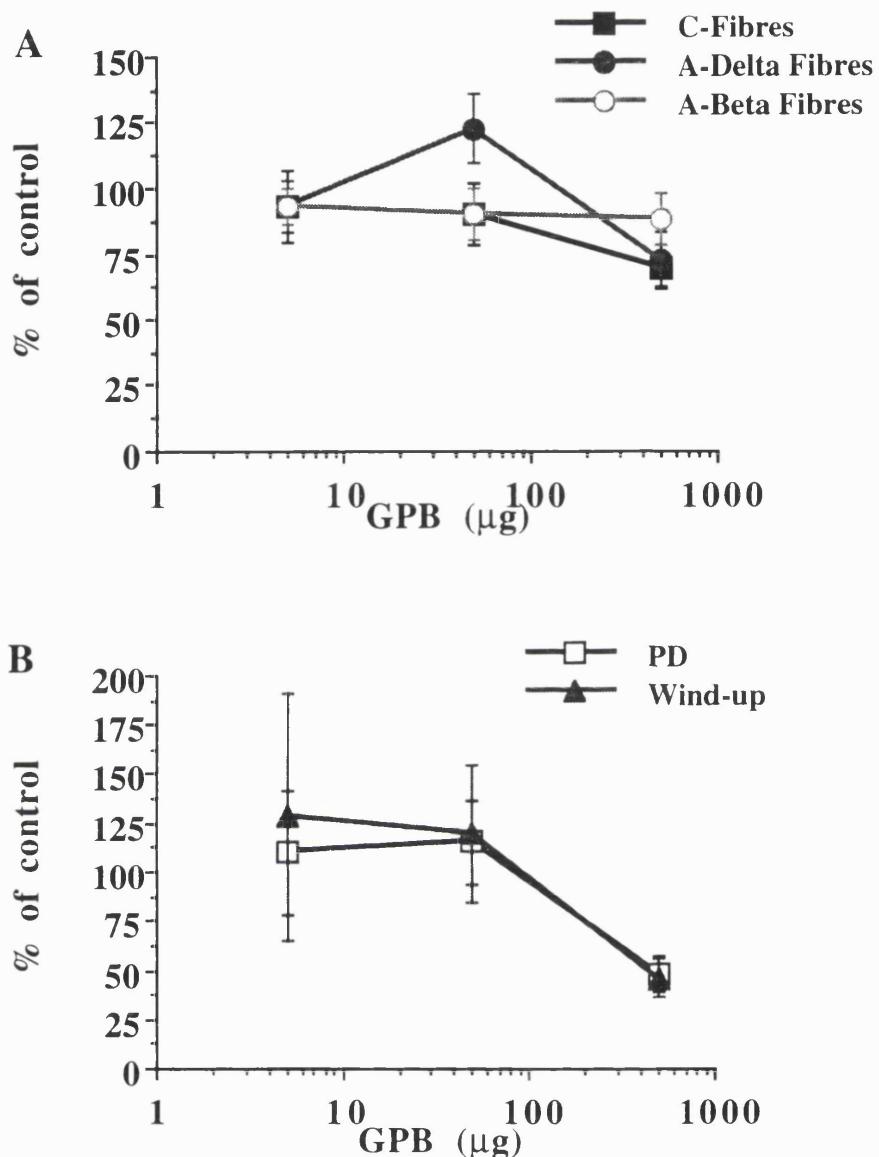
Figure 16. (A) Effects of 5, 50 and 500μg GPB applied intrathecally on the response of a mean population of dorsal horn neurones to electrically evoked C-fibre, Aδ-fibre and Aβ-fibre evoked activity. (B) Effects on post-discharge and wind-up of the same population of neurones. ⒩=7

The effects of 500μg GPB were challenged with 1000μg intrathecal caffeine. There was no significant reversal of GPB with caffeine with any of the responses. The C-fibre evoked responses were still 73±8% of control, Aδ-fibre responses were 58±7% and the Aβ-fibre responses were 102±5% of control after GPB in the presence of 1000μg caffeine. The post-discharge was 58±7% of control,

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the input onto the cells was 57±11% and the final overall response was 62±7% of control.

The excess action potentials over the predicted range were also calculated as the input and final overall response of the cells as there was a degree of variability in the wind-up responses. The input responses tended to be facilitated with 5 and 50μg GPB but increasing the dose to 500μg GPB significantly inhibited the input onto the cells (P<0.05) see Figure 17. The overall, final response in a train of stimuli showed a slight facilitation with 5μg GPB and then tended towards an inhibition with 500μg giving significant inhibition of this response.
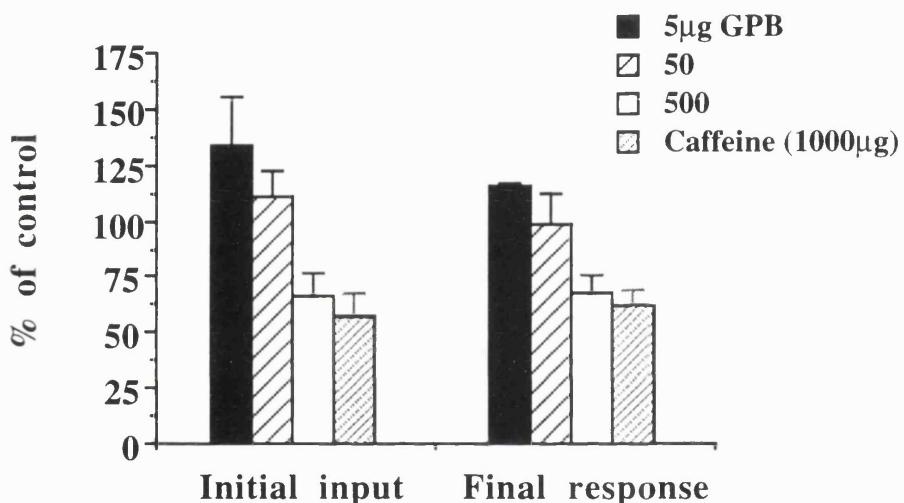
Figure 17. *The response of a population of cells to the initial (input) in a train of 16 stimuli at C-fibre stimulation and the final response in the same train. The effect of 500μg GPB only was significant on the input onto the cells and the overall final response. There was no significant reversal with caffeine (1000μg).* n= 7

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4.3: Discussion.

These results support the behavioural findings that A_1-receptor agonists are antinociceptive at the spinal cord level. Various studies with different A_1-receptor agonists have shown analgesic actions when given systemically (Ahlijanian and Takemori 1985; Holmgren et al. 1983, 1986; Vapaatalo et al. 1975: Yarbrough and McGuffin-Clineschmidt 1981), orally (Herrick-Davis et al. 1989) or into the brain or spinal cord (DeLander and Hopkins 1986; Doi et al. 1987; Herrick-Davis et al. 1989; Karlsten et al. 1990; Malmberg and Yaksh 1993a; Post 1984; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Yarbrough and McGuffin-Clineschmidt 1981). It has been shown that adenosine agonists have a far greater potency when given into the CNS~~especially intrathecally~~(than by peripheral routes (Herrick-Davis et al. 1989; Holmgren et al. 1986; Yarbrough and McGuffin-Clineschmidt 1981) suggesting that the majority of analgesic action of these drugs is at the spinal cord level. There is still much experimental evidence lacking in this area: for example, the relative expression of A_1/A_2 receptors is not known in the periphery and studies observing agonist effects on the afferent drive have still to be undertaken. In some humans studies adenosine is algesic (Bleehen and Keele 1977; Conradson et al. 1987) whilst other human studies have shown adenosine to be analgesic (Belfrage et al. 1995; Ekblom et al. 1995; Karlsten and Gordh 1995; Sergerdahl et al. 1994, 1995, 1996; Sollevi 1992; Sollevi et al. 1995, 1996; Sylven et al. 1996). These differences may well relate to a peripheral algesic action yet a central analgesic effect. Whatever the case, I have conclusively shown that A_1-receptor agonists are analgesic in this model, independently of motor or other non-specific effects, at the spinal cord level.

The A_1-receptor agonists tested (CPA and 2-Cl-Ado) were selective for the noxious inputs, as they inhibited C-fibre evoked responses but had no significant effects on the $A\beta$-fibre evoked responses. 2-Cl-Ado was not as potent and efficacious as CPA. It may well be that the doses used were at the top of the dose response curve and lowering the doses would have given better dose response relations. However, 2-Cl-Ado had a low ceiling in effectiveness at inhibiting the noxious inputs into the spinal cord so further studies were not undertaken with this drug.

The effects of CPA in inhibiting the C-fibre evoked responses as well as significantly inhibiting the wind-up and post-discharge of the cells demonstrates that the use of A_1-receptor agonists at the spinal cord level could be a useful alternative target for analgesia. The wind-up of dorsal horn cells, enhancing the responses to a constant stimulation, involving the recruitment of the NMDA-receptor (Davies and

Lodge 1987; Dickenson and Sullivan 1987a; Mendell 1966) has been implicated in the spinal maintenance of more persistent and chronic pain states (see Dickenson 1994a). CPA dose dependently inhibited (and often abolished) the wind-up of many of the cells. Not only did it inhibit this enhanced response but CPA also reduced the initial inputs onto the cells.

Other agents for spinal analgesia are the classical opioids or NMDA-receptor antagonists. However, both of these classes of drugs have limitations. Opioids have relatively low effectiveness in inhibiting wind-up in animals unless high doses are used and their predominant action is on the initial inputs onto the neurones (Dickenson and Sullivan 1986). In humans with more persistent pain states, opioids are effective but in neuropathic pains they need to be used at doses that are not acceptable to the patient (Jadad et al. 1992). There is however an opioid, ketobemidone, that can control wind-up by merit of additional NMDA-receptor antagonistic properties (Andersen et al. 1996).

NMDA-receptor antagonists have been shown in animal studies to be very good at controlling wind-up of dorsal horn cells but the baseline activity is not inhibited by these class of drugs due to AMPA-receptors still being available for activation (Dickenson and Sullivan 1987a). Thus, NMDA-receptor antagonists do not decrease the input onto the cells, only the enhanced responses. It is very interesting to note that A_1-receptor agonists controlled both the enhanced responses as well as decreasing the input onto the cells. This is probably due to the location of the A_1-receptors. It is known that the majority of A_1-receptors are located on second order spinal neurones (Choca et al. 1988; Geiger et al. 1984) and at this location, activation of A_1-receptors hyperpolarize the neurones, making it harder for the membrane potentials to reach threshold (thus hyperexcitability is reduced). There is also a smaller proportion of A_1-receptors located on primary afferent terminals (Li and Perl 1994). Here, the activation of the A_1-receptor will result in a decrease in neurotransmitter release, possibly due to an inhibition of calcium influx (Li and Perl 1994) as has been demonstrated in other areas of the central nervous system (Arch and Newsholme 1987; Fastbom and Fredholm 1985; Fredholm and Dunwiddie 1988; Goodman et al. 1983; Snyder 1985). This will result in an inhibition of quantal neurotransmitter release from afferents (Li and Perl 1994). I observed a decrease in input onto the cells, supporting a pre-synaptic inhibition of neurotransmitter release. These results (the attenuation in wind-up and decreasing input) bears out the idea of a heterogeneous location of A_1-receptors.

The effects of both CPA and 2-Cl-Ado were reversed to varying extents by theophylline. The dose of antagonist used was high compared to behavioural studies and reflects the higher agonist doses used in our experiments. This

difference in doses may be due to the fact that behavioural studies involve only threshold stimulation (i.e. withdrawal, as soon as the animal shows signs of discomfort). In anaesthetized animals suprathreshold stimulation (i. e. three times C-fibre threshold) can be used. Thus, in our studies we use suprathreshold stimuli sufficient to induce wind-up and so a more realistic model of clinical pain. Threshold stimulation may not be very clinically relevant and is an obvious limitation to behavioural studies. As we are driving the neurones harder, larger doses of agonists and therefore higher doses of antagonists to reverse the effects may be required.

The concurrent facilitatory effects of both A_1-receptor agonists on $A\delta$-activity was somewhat of a surprise in that there were clear dose dependent effects that mirrored the inhibitions in time-course, magnitude and duration. It is only possible to propose tentative explanations for this phenomenon. It could be considered that the effects are due to the activation of an excitatory A_3-receptor on $A\delta$-fibre terminals themselves or on interneurones interposed in $A\delta$ pathways. This idea is supported by the fact that the facilitations appeared to be insensitive to reversal by theophylline and also were not blocked by a pretreatment with 8pSPT. The A_3-receptor is known to be insensitive to methylxanthines (Linden et al. 1994). However, this receptor does not appear to be present in the rat spinal cord (see Linden et al. 1994). Another possibility is that these agonists are also acting on the A_{2a}-receptor, again in the $A\delta$-fibre pathway. However, although 2-Cl-Ado can have effects on the A_{2a}-receptor in about the same dose range as A_1-receptor effects, CPA has a 1000 fold greater affinity for the A_1-receptor compared to the A_{2a}-receptor (Fredholm et al. 1994). Differential effects of the two agonists would therefore be expected if the effects were mediated by A_{2a}-receptor effects. Furthermore, the A_{2a}-receptor agonist CGS-21680 inhibited rather than facilitated the $A\delta$-fibre evoked response, albeit not to any great extent.

What is more probable is disinhibition. The $A\delta$-fibre terminals (Alvarez et al. 1992; Bernardi et al. 1995) and indeed interneuronal pathways (Baba et al. 1994) are known to be under inhibitory control, especially by GABAergic interneurones (Alvarez et al. 1992; Bernardi et al. 1995). The A_1-receptor is primarily located on second order neurones (Choca et al. 1988; Geiger et al. 1984). Thus, A_1-receptor activation could hyperpolarize these inhibitory neurones with a net result of disinhibition of $A\delta$-fibre responses. Since this was seen with both CPA and 2-Cl-Ado a common mechanism such as this is likely

. Generally, the A_{2a}-receptor agonist CGS-21680 was not as selective or efficacious at controlling the responses of dorsal horn neurones as the A_1-receptor agonists. This agonist showed only weak and non-selective inhibitions of both low and high-threshold inputs. It has been reported that the A_2-receptor at the spinal cord

level is antinociceptive (Sawynok et al. 1986). However, these studies employed the use of the agonist 5'-N-ethyl-carboxamidoadenosine (NECA) which is eqipotent for the A_1-receptor and the A_{2a}-receptor (Fredholm et al. 1994). Therefore, analgesic actions of this agonist can not be attributed to the A_{2a}-receptor alone. Another complication with the use of NECA in behavioural studies is that it has been shown that there are motor impairments caused by activation of the A_2-receptor at doses in the low nanomolar range (Karlsten et al. 1990). Unfortunately this was not controlled for in study by Sawynok et al. (1986) and as the top doses employed overlapped those which have been shown to cause inhibition of motor function, antinociception in this dose range must be viewed with caution. In the periphery adenosine may be pro nociceptive via the activation of the A_2-receptor. In a study by Bleehen and Keele (1977) where adenosine given to a human blister base caused pain, the concentrations of adenosine given were in the dose range that would activate A_2 receptors.

Although we found that CGS-21680 did produce inhibitions of the C-fibre evoked responses as well as wind-up and post-discharge, they were weak compared to the inhibitory effects of CPA. Therefore, at the spinal cord level it is quite clear that the major antinociceptive effects are through activation of the A_1-receptor.

Early work with methylxanthine antagonists suggested that a role of purines in analgesia, since methylxanthines were shown to decrease the threshold for nociceptive responses (Contreras et al. 1972; Paalz ow and Paalz ow 1973). However, these algesic effects were not attributed to the antagonism of endogenous adenosine at a receptor level (Contreras et al. 1972; Paalz ow and Paalz ow 1973). However, many studies have since demonstrated that methylxanthines do have affinity at the A_1 and A_2-receptors (see Fredholm et al. 1994; Snyder et al. 1981; see Williams 1991). Pretreatment or reversal with a variety of methylxanthines receptor antagonists have shown to attenuate the analgesic effects of adenosine agonists (DeLander and Hopkins 1986; Herrick-Davis et al. 1989; Holmgren et al. 1983, 1986; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Sosnowski et al. 1989; Yarbrough and McGuffin-Clineschmidt 1981) in a variety of behavioural tests.

We have demonstrated that antagonists acting at A_1-receptors can prevent the effects of CPA as well as reverse the effects of this A_1-receptor agonist and to some extent, the effects of 2-Cl-Ado. Reversal of the intrathecally applied kinase inhibitor GPB with caffeine was not significant. Unfortunately caffeine was a poor choice of antagonist and other studies with these kinase inhibitors has demonstrated clear receptor effects with the use of other antagonists (see chapter 7). However,

even though we saw reversal and attenuation with 8pSPT and theophylline, these antagonists are by no means ideal. Both are fairly weak antagonists (Fredholm et al. 1994) and theophylline also has inhibitory phosphodiesterase activity. 8pSPT has less phosphodiesterase activity, since it does not pass across the cell membrane as readily as theophylline (Smellie et al. 1979). It is interesting to note that theophylline tended to cause greater facilitation of responses than 8pSPT alone. This may be due to the additional phosphodiesterase inhibition produced by theophylline. Whatever the case, neither of these antagonists produced significant facilitations of the responses of dorsal horn neurones. This is not surprising given that adenosine has a half life of only a few seconds. The utility of the more specific A_1-receptor antagonist, DPCPX, was also hampered by its poor solubility. Although we did see significant facilitatory effects with this antagonist, the vehicle problems made Interpretation difficult.

The problems associated with trying to assess endogenous systems with antagonists is not unique to adenosine. Naloxone, the mu-receptor antagonist has little effect when given alone in our model (see LeBars et al. 1981). However, protecting endogenously released opioids by preventing their breakdown has shown that indeed endogenous opioids are released at the spinal cord level and can be antinociceptive (Dickenson et al. 1986; Dickenson et al. 1987a).

To establish the role of endogenous adenosine we employed a similar tactic, by protecting adenosine with the use of spinally and peripherally given adenosine kinase inhibitors which prolong the half life of adenosine.

Adenosine deaminase has been shown to be present both in the dorsal horn of the spinal cord (Geiger and Nagy 1986) and in small diameter primary afferents (Nagy and Daddona 1985). In a spinal superperfusion model adenosine deaminase inhibitors were found to be more effective at protecting endogenous adenosine than adenosine kinase inhibitors (Golembiowska et al. 1995). However, this study contradicts a large body of evidence, which has shown that the enzyme adenosine kinase is more important in protecting endogenous adenosine *in vivo*, which can then act to give rise to antinociception (Golembiowska et al. 1996; Keil and DeLander 1992, 1994, 1996; Poon and Sawynok 1995). It may be that in the study of Golembiowska et al. (1995) the adenosine kinase was saturated so that adenosine deaminase assumed greater importance. Thus, this release study may not be as physiological as *in vivo* studies. In other areas of the central nervous system adenosine kinase inhibitors have been shown to be more effective at protecting adenosine released from NMDA-receptor activation than adenosine deaminase inhibitors (White 1996).

The kinase inhibitors tested, whether given spinally or systemically were

antinociceptive. The spinally given kinase inhibitor (GPB) reduced the C-fibre evoked responses, as well as wind-up and post-discharge. However, when compared to the effects of an A_1-receptor agonist, such as CPA, GPB was not as as effective. The C-fibre evoked responses were markedly inhibited with CPA and although GPB produced significant inhibitions, these were only with the highest dose and were still about forty percent less than the effects of CPA. This difference may result from the site of release and the location of receptor target for the released adenosine. Whilst many people favour a release of adenosine from primary afferent terminals, as Braas et al. (1986) demonstrated that adenosine levels were high in terminals in the substantia gelatinosa, this study did not rule out that the terminals stained for were in fact those of second order neurones. There is also strong evidence in the rest of the central nervous system that adenosine is released in response to the activation of AMPA- and NMDA-receptors (Craig and White 1992, 1993; White 1996). Thus if this is the case in the spinal cord then adenosine may be released from intrinsic cells (since these are known to contain functional NMDA-receptors) and so adenosine would be released in a location more likely to activate the post-synaptic receptors. The effects on the initial input onto the cells also back this up, since again there is a dramatic difference in the ability of protected adenosine and an exogenous agonist at controlling this response. The kinase inhibitor although reducing the input was some thirty percent less effective than CPA. Thus this demonstrates that protected adenosine is not as effective at pre-synaptic sites ~on primary afferent terminals~ since input is primarily a measure of afferent activity.

The systemically given kinase inhibitor was also shown to have antinociceptive effects similar to that of the inhibitor given by the intrathecal route; although no statistical analysis was undertaken to compare GPB directly to GPA. However, the exact site of release of adenosine and antinociception cannot be ascertained from these experiments. It cannot be ruled out that in addition to a spinal site, analgesia could also be mediated by supraspinal actions as well as in the periphery. Supraspinally protected adenosine could activate the A_2-receptors and so activate descending inhibitory controls. In the periphery protected adenosine could be antinociceptive by activating the A_1-receptor and inhibiting transmission at the level of the afferents.

Chapter 5.

The spinal interactions between an adenosine A_1-receptor agonist, CPA and the μ-receptor agonist, morphine.

5.1: Introduction.

The effects of spinal opioids in the production of analgesia have been known for many years (see Dickenson 1994b; see Yaksh 1993). The opioids exert their effects via three classes of opioid receptors, the μ-, δ- and κ-receptors (Martin et al. 1976). The μ-receptor is known to have effects predominantly on the primary afferent terminals of nociceptive neurones and has an analgesic action at this location by inhibiting transmitter release from primary afferents (Duggan and North 1984). There is also evidence for post-synaptic action of opioids, which results in hyperpolarization of nociceptive neurones in the dorsal horn (Duggan and North 1984; Hylden and Wilcox 1983).

There is well documented evidence that the consequences of μ-receptor activation may alter as a result of interactions with other pharmacological systems such as the noradrenergic, via the α_2-receptor, with CCK, via the CCK_B-receptor, F8a (a peptide, FLFQPQamide) and with the NSAIDs (Dickenson and Sullivan 1993; Kalso et al. 1993; Stanfa and Dickenson 1993; Stanfa et al. 1994; see Yaksh 1993). These interactions may enhance or attenuate opioid analgesic effects.

It has been extensively reported that adenosine may be released in response to activation of the μ-receptor and the subsequent effects of the purine have been proposed to be partly responsible for the analgesic actions of opioids (Ahlijanian and Takemori 1985; Crain and Shen 1990; DeLander and Keil 1994; DeLander and Wahl 1989; DeLander et al. 1992; Keil and DeLander 1995; Sawynok and Sweeney 1989; Sawynok et al. 1989; Sweeney et al. 1987a, b, 1993; Yang et al. 1994, 1995). This opioid induced release is not exclusive to adenosine, since it has also been shown that substance P and somatostatin may be released from small diameter afferents in response to opioids (Kuraishi et al. 1985a, b; Solomon et al. 1989; Tiseo et al. 1990; Wiesenfeld-Hallin et al. 1991). If this opioid evoked transmitter release has physiological relevance, then opioids would have to produce excitatory effects on transmitter release.

In fact, low doses of μ-receptor agonists result in facilitatory effects when given intrathecally, with inhibitory effects then predominating as the dose is increased (Crain and Shen 1990; Dickenson and Sullivan 1986; Dickenson et al. 1987b; Wiesenfeld-Hallin et al. 1991). A study by Crain and Shen (1990) demonstrated that the μ-receptor may differentially couple to the G-proteins Gs and Gi: with low doses of morphine (nanomolar concentrations) the μ-receptor may be coupled to Gs which results in facilitatory effects whilst increasing the dose now allows the μ-receptor to be coupled to the inhibitory Gi-protein.

Another possibility for the dual action of opioids is that at low doses, disinhibition of intrinsic inhibitory cells occurs, with the net result being an increase in excitation. Increasing the dose of μ-receptor agonists then causes direct inhibitory effects on transmission (Dickenson and Sullivan 1986; Magnuson and Dickenson 1991).

Whatever the case, it is possible that these low dose facilitations could result in release of substances which could then serve to modulate opioid analgesia. In the case of adenosine the net result would be to enhance analgesia, since it is clearly established that spinal adenosine and activation of the A_1-receptor will result in antinociception (see Chapter 4; Ahlijanian and Takemori 1985; DeLander and Hopkins 1986; Herrick-Davis et al. 1989; Holmgren et al. 1983, 1986; Karlsten et al. 1990; Malmberg and Yaksh 1993a; Post 1984; Reeve and Dickenson 1995; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Vapaatalo et al. 1975; Yarbrough and McGuffin-Clineschmidt 1981).

The effects of two doses of morphine were observed alone and in the presence of CPA on the acute electrically evoked activity of dorsal horn neurones to observe if there were any interactions between the inhibitory effects of morphine and an A_1-receptor agonist.

5.2.1: Morphine.

As a control, the effects of the μ-receptor agonist, morphine (1 and 5μg), followed by 1μg naloxone, were observed on a population of nine dorsal horn neurones with an average depth of 663±102μm from the surface of the dorsal horn of the spinal cord.

The C-fibre evoked responses were reduced to 83±4 and 46±11% of control by 1 and 5μg morphine respectively. The effects of 5μg only were significant on the C-fibre evoked responses ($P<0.05$). The Aδ- and the Aβ-fibre evoked responses were 70±7 and 39±8 and 89±4 and 79±8% of control for the above doses respectively. The effects of 1 and 5μg morphine significantly reduced the Aδ-fibre evoked responses ($P<0.05$) with no significant effect on the Aβ-fibre responses being observed. The post-discharge was reduced to 50±14 and 30±12% of control for 1 and 5μg morphine respectively, with 5μg being significant ($P<0.05$). The wind-up of the cells was also reduced with the same doses to 58±14 and 45±9% of control again with 5μg only giving a significant inhibition ($P<0.05$). The input and final overall responses were also evaluated. The input was significantly inhibited after both 1 and 5μg morphine ($P<0.05$) to 79±7 and 37±11% of control, respectively. The final response was reduced to 79±6 and 46±11% of control for 1 and 5μg morphine respectively with only the effects of 5μg morphine being significant. The inhibitory effects of 5μg morphine were reversed by naloxone (1μg). The C-fibres were reversed to 87±9% of control, the Aδ-fibres were reversed to 101±24% of control and the Aβ-responses were 106±6% of control after naloxone. Post-discharge and wind-up were reversed to 111±18 and 148±32% of control respectively.

5.2.2: Morphine and N^6-cyclopentyladenosine.

The effects of CPA (5μg) have previously been described in 4.2.1. Here CPA was given as a ten minute pretreatment prior to the same doses of morphine (1 and 5μg) on a separate population of nine dorsal horn cells with an average depth of 687±89μm from the surface of the spinal cord. Morphine (5μg) was followed by theophylline (1000μg).

The pretreatment with CPA reduced the C-fibre evoked responses to 67±7% of control ($P<0.05$). The post-discharge was also significantly reduced to 67±13% of control with CPA. The Aδ-fibre evoked responses were facilitated with

this dose of CPA to 136±13% of control. The Aβ-fibre evoked responses along with wind-up were not significantly changed by this dose of CPA, so that the responses were 95±5 and 86±13% of control respectively. The input and final overall responses were both significantly inhibited by CPA so that the initial response was 48±11 and overall response was 62±9% of control (*P<0.05*).

Morphine (1 and 5μg) in the presence of CPA now inhibited the C-fibre evoked responses to 42±7 and 39±8% of control (*P<0.05*) (see Figure 1A).
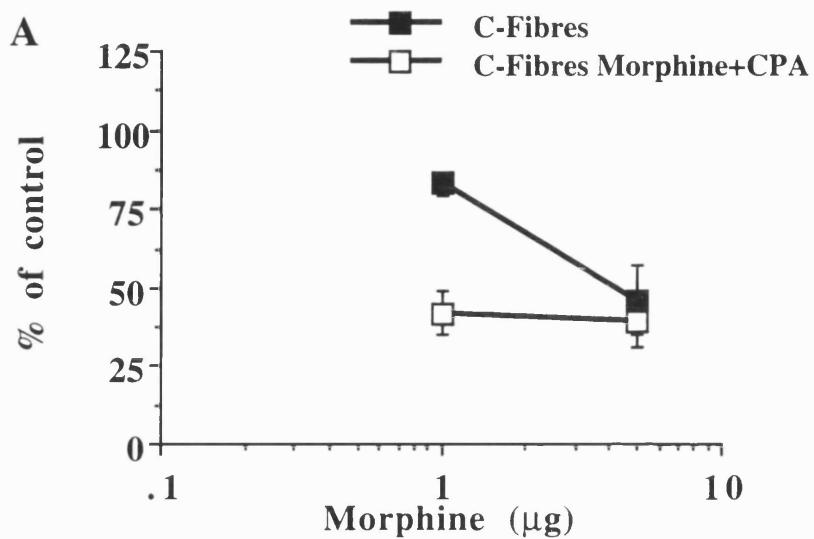
Figure 1. *(A) The C-fibre evoked responses in the presence of morphine alone (1 and 5μg) and the same doses of morphine in the presence of CPA (5μg).*
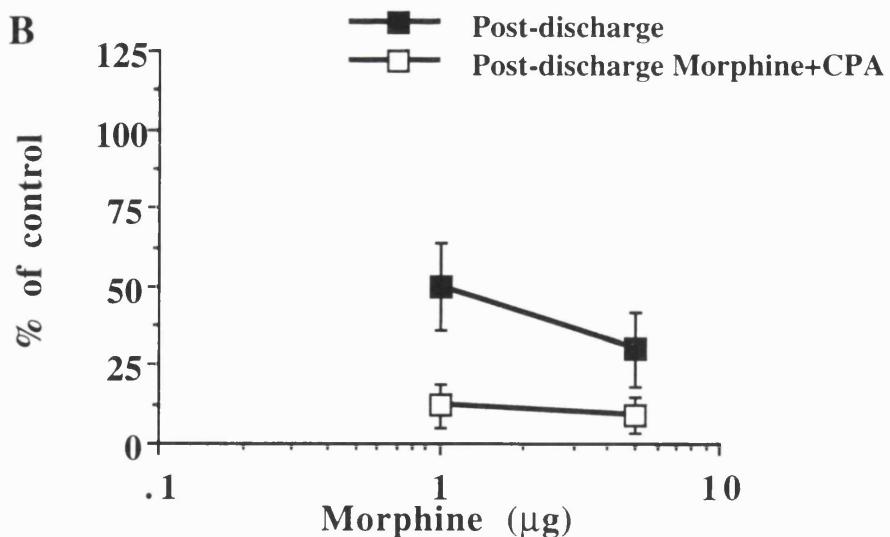
Figure 1. *(B) The effects on post-discharge in the presence of morphine alone (1 and 5μg) and the same doses of morphine in the presence of CPA (5μg).*

The post-discharge and wind-up responses were inhibited to 12±7 and 9±6; 36±10 and 43±10% of control for 1 and 5µg respectively (*P<0.05*) (Figures 1B and C).
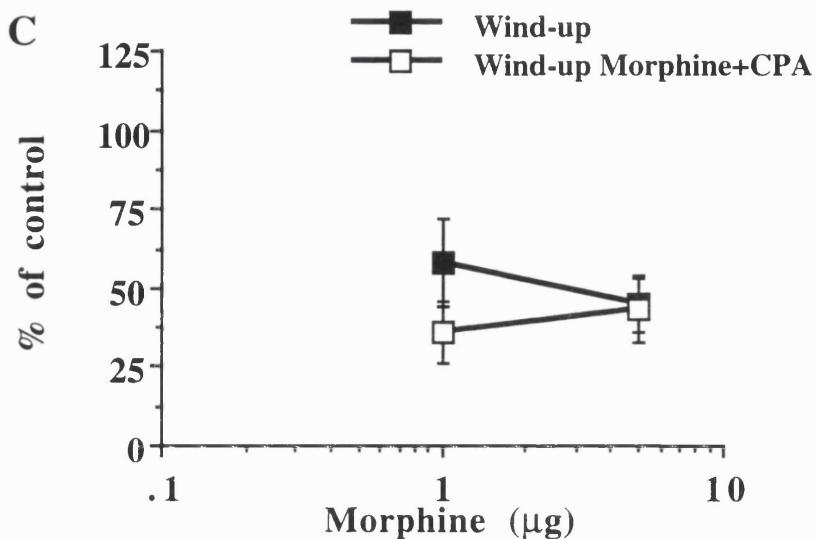
Figure 1. *(C) The effects on wind-up in the presence of morphine alone (1 and 5µg) and the same doses of morphine in the presence of CPA (5µg).*

The Aβ-fibre evoked responses were not significantly different when morphine and CPA were combined, compared to morphine alone. The combination did give significant effects when compared to controls with 1µg morphine. However, the response was only reduced to 72±8 % of control (Figure 1D).
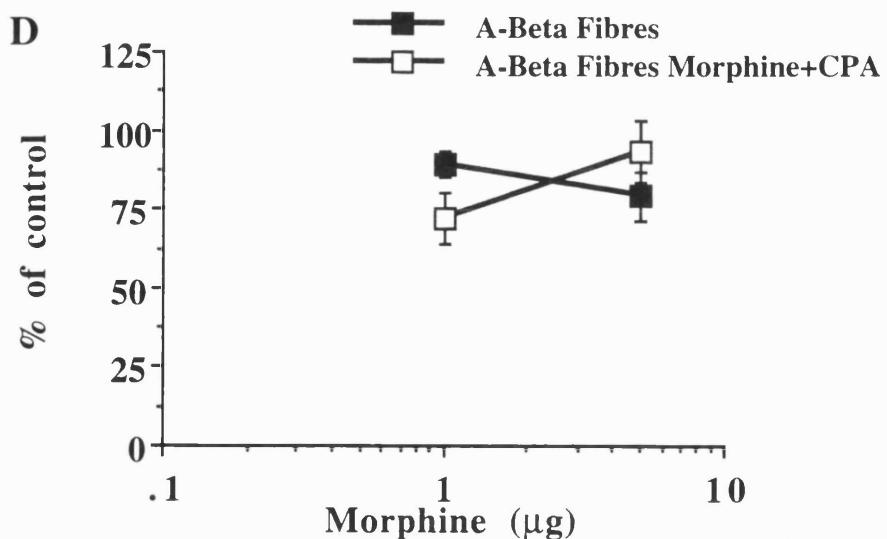
Figure 1. *(D) The Aβ-fibre evoked responses in the presence of morphine alone (1 and 5µg) and the same doses of morphine in the presence of CPA (5µg).*

The Aδ-fibre evoked responses were now facilitated by morphine plus CPA, so that the responses were 172±14 and 175±16% of control (*P<0.05*) (Figure 1E).
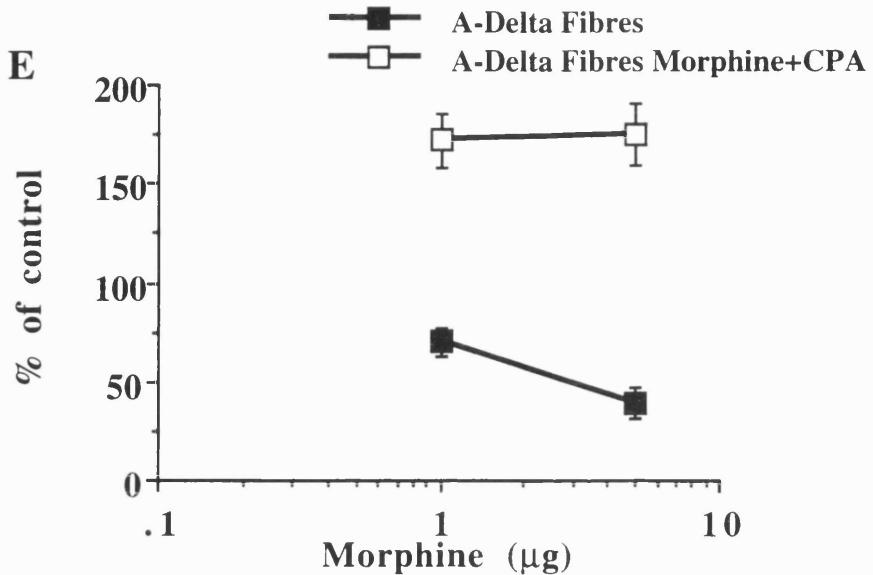
Figure 1. *(E) The Aδ-fibre evoked responses in the presence of morphine alone (1 and 5μg) and the same doses of morphine in the presence of CPA (5μg).*

The input and final response to a train of stimuli were reduced by morphine in the presence of CPA to 26±6, 17±4 and 28±7, 26±5% of control for 1 and 5μg respectively (*P<0.05*) (Figure 2).
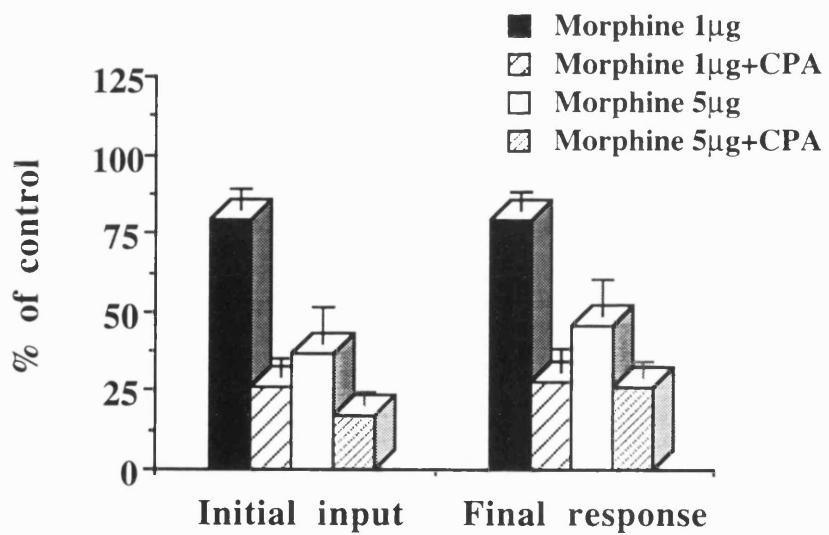
Figure 2. *The initial input and final overall response to a train of stimuli. The response after morphine alone (1 and 5μg) and the same doses of morphine in the presence of CPA (5μg) are shown.*

These effects of morphine and CPA were reversed with theophylline (1000μg) to varying extents. The inhibited C-fibre evoked responses were reversed to 79±11% of control, the facilitated Aδ-fibre responses to 101±12% of control. The Aβ-fibres were 105±6% of control after theophylline. Post-discharge was still inhibited, 60±11% of control after theophylline, and wind-up was 83±11% of control. The initial input and final responses to a train of stimuli were still inhibited in the presence of theophylline, 53±14 and 62±9% of control respectively.

In comparing the effects of morphine alone and in the presence of CPA there was a significant difference in the inhibitions of the C-fibre evoked responses, post-discharge and the input and final response to a train of stimuli with 1μg morphine alone compared to 1μg morphine in the presence of CPA ($P<0.05$). As seen in Figures 1A, B and Figure 2 there was an increase in inhibitory effects of morphine at this dose. Increasing the dose of morphine to 5μg in the presence of CPA did not give significant enhanced inhibitory effects when compared to the same dose of morphine alone for the above responses. The Aδ-fibre evoked responses were facilitated in the presence of morphine and CPA, whereas morphine alone inhibited these fibre evoked responses (Figure 1E). The change in effect of morphine was significant for both 1 and 5μg ($P<0.05$). The Aβ-fibre evoked responses were not significantly different when comparing morphine alone to the combination of the A_1-receptor agonist and morphine.

5.3: Discussion.

Whilst these results presented here do not ascertain whether adenosine is released in response to mu-receptor activation or not, they do show that there are possible complex interactions between the two receptors, probably at the level of common spinal circuitry.

The effects of morphine were greater in the presence of CPA. However, the interaction appeared to be very simple in that additive inhibitory effects were seen. This is in keeping with the study of DeLander and Keil (1994). However, increasing the dose of morphine did not give further inhibitory effects, when compared to morphine alone, so that there was no longer a dose dependent effect. These results make it unlikely that the opioid and adenosine act entirely by common mechanisms since potentiation would then be expected.

The change in direction of the Aδ-fibre evoked responses from inhibitions with morphine alone, to facilitatory effects with morphine in the presence of CPA demonstrates an independent action of adenosine A$_1$-receptors and μ-opioid receptors. I have shown that transmission through Aδ-fibre pathways is enhanced in the presence of A$_1$-receptor agonists (Reeve and Dickenson 1995). The intrinsic cells in the Aδ-pathway responsible for the facilitatory effects are not under the control of the μ-receptor since in the presence of morphine the facilitations still occurred.

There has been much debate on the relationship between adenosine and morphine analgesia. There can be no doubt that opioids can exert powerful inhibitory responses at a number of sites in their own right (see Dickenson 1991, 1994b; Stein 1993; Yaksh 1993) and at certain doses opioids have been shown to release substances from primary afferent neurones (Kuraishi et al. 1985b; Tiseo et al. 1990; Wiesenfeld-Hallin et al. 1991) along with a reported release of adenosine (Sawynok et al. 1989; Sweeney et al. 1987a, b, 1989, 1993; Yang et al. 1994). It has also been well established that adenosine at the spinal cord level is analgesic in its own right (Ahlijanian and Takemori 1985; DeLander and Hopkins 1986; Herrick-Davis et al. 1989; Holmgren et al. 1983, 1986; Karlsten et al. 1990; Malmberg and Yaksh 1993a; Post 1984; Reeve and Dickenson 1995; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Vapaatalo et al. 1975; Yarbrough and McGuffin-Clineschmidt 1981).

However, although these systems can act independently, it may also be possible that they can interact together to produce analgesia. It has been shown that CCK, F8a, and noradrenaline can also influence opioid analgesia at the spinal cord

level via interactions with their own receptors (Devillers et al. 1994; Dickenson and Sullivan 1993; Kalso et al. 1993; Stanfa and Dickenson 1993; Wiertelak et al. 1994). These interactions are not always positive and some of them can attenuate opioid analgesia.

The relationship between adenosine and morphine analgesia was first suggested by the reversal of morphine analgesia by aminophylline and various other methylxanthines (Ahlijanian and Takemori 1985; DeLander and Hopkins 1984; Ho et al. 1973a, b; Jurna 1981, 1984; Sweeney et al. 1987b; Yang et al. 1994, 1995). Morphine has been shown to release ^{3}H-purines from the cortex in vivo (Phillis et al. 1979b, 1980) as well as from the dorsal horn of the spinal cord *in vivo* and *in vitro* (Sweeney et al. 1987a, b, 1993). Morphine given i c v produced analgesia at the spinal level which was reversed by theophylline (DeLander and Hopkins 1986; DeLander and Wahl 1989; DeLander et al. 1992). Note that it has also been shown that morphine can attenuate descending inhibitory controls (Duggan et al. 1980).

The use of methylxanthines as adenosine receptor antagonists is hampered by the fact that this class of drugs can also inhibit the enzyme phosphodiesterase (which usually inactivates cyclic-AMP to 5'-AMP) as well as being adenosine receptor antagonists (see Williams 1991). As both adenosine A_1-receptors and the μ-receptor can have the same second messenger systems which involve the inhibition of cAMP (formation) via inhibition of adenylate cyclase (Childers 1993; Collis and Hourani 1993; Fredholm et al. 1994; Jacobsen et al. 1992; Linden 1994; Olah and Stiles 1992; Palmer and Stiles 1995; Ribeiro and Sebastiao 1986; Sebastiao and Ribeiro 1996) interpretation of the use of methylxanthines in reversing morphine analgesia as an involvement of adenosine must be viewed with caution.

In the study undertaken by Sweeney et al. (1987b) they demonstrated that K^+ and veratridine caused the release of adenosine from synaptosomes from the dorsal horn of the spinal cord and to a less extent from the ventral horn of the spinal cord. However, much of this release was blocked by inhibitors of 5'-nucleotidase, suggesting that much of adenosine came from the conversion of ATP. Morphine also caused a release of adenosine in a dose dependent manner, some of which was blocked by 5'-nucleotide inhibitors (see Sawynok et al. 1989; Sweeney et al. 1987b). This release was blocked by naltrexone. What is surprising is that morphine caused a dose-dependent release of adenosine for the following reasons discussed below.

Firstly there appears to be more than one site of adenosine location in the dorsal horn. The study by Braas et al. (1986) demonstrated a clear band of adenosine-like immunoreactivity in the substantia gelatinosa, and in terminals within this area. Many have interpreted this to mean that adenosine is located in the terminals of primary afferents, ignoring the possibilities that adenosine could also be

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in terminals of intrinsic cells. Neonatal and adult pretreatment with capsaicin only decreased adenosine release by 50% suggesting an additional source of adenosine (or ATP) must therefore come from cells intrinsic to the spinal cord, which are not affected by the capsaicin treatment. Work I have done with the adenosine kinase inhibitors (Chapter 4 and 6) would indicate that adenosine may be released in response to increased activity of the NMDA-receptor and act as a negative feedback mechanism to control further NMDA-receptor driven events, as has been demonstrated in other areas of the CNS (Craig and White 1992, 1993; Hoehn and White 1990a, b; Manzoni et al. 1994). This would place adenosine in intrinsic cells, since to date there are no functionally known NMDA-receptors on afferent terminals (see Hollmann and Heinemann 1994). So adenosine may have a heterogeneous location.

What is somewhat troubling is the proposed mechanism for release of adenosine by morphine by Sweeney and colleagues. Crain and Shen (1990) have demonstrated that at various doses of agonist the μ-receptor may differentially couple between G_S and G_i-proteins; therefore at low doses of agonist the μ-receptor is excitatory *but* as the dose of agonist is increased the μ-receptor now couples to the G_i-protein and results in inhibitory effects. Certainly in animal and human studies this dual opioid effect is seen. In animal studies with low doses of opiates the response of neurones are enhanced (Dickenson and Sullivan 1986; Magnuson et al. 1991; Sastry and Goh 1983; Woolf and Fitzgerald 1981) and in humans, patients report sensations of itching around the periphery of an epidural. However, these responses could be attributed to disinhibition of intrinsic inhibitory neurones, which are overcome as the doses are increased. If the release studies by Sweeney et al. (1987a, b) was representing a release of adenosine from primary afferent terminals, it would be more plausible if only low doses of morphine evoked adenosine release and increasing the dose inhibited the release. The enhancement of the electrically evoked release of enkephalin has been observed with low doses of opiates (nanomolar concentrations) yet higher concentrations attenuate this release (Xu et al. 1989); this has also been seen with substance P release (Wiesenfeld-Hallin et al. 1991).

Disinhibition of intrinsic inhibitory cells may be a more representative physiological explanation for the release of adenosine. As discussed above adenosine may also be located in intrinsic cells as well as primary afferents. Low dose disinhibition by morphine could indeed cause the release of adenosine from the primary afferent location. However, if this was the only source of adenosine release I would expect that this would be a bimodal response, with higher doses of morphine inhibiting adenosine release. However, there is a clear dose dependent release of adenosine by morphine (see Sawynok et al. 1989; Sweeney et al. 1987b).

As the dose of morphine is increased it is known to also activate receptors which are post-synaptic (see Dickenson 1994b). If these receptors were on inhibitory neurones which usually control purinergic neurones, then this disinhibition could result in the release of adenosine, from an intrinsic neuronal source. Since higher doses of morphine release more adenosine (see Sawynok et al. 1989) then a second-order neuronal source of adenosine fits with what is known about opioid receptor activity, see Figure 1.

Inhibitory   Purinergic
Interneurone   Interneurone

● Mu-receptors

● A1-receptors
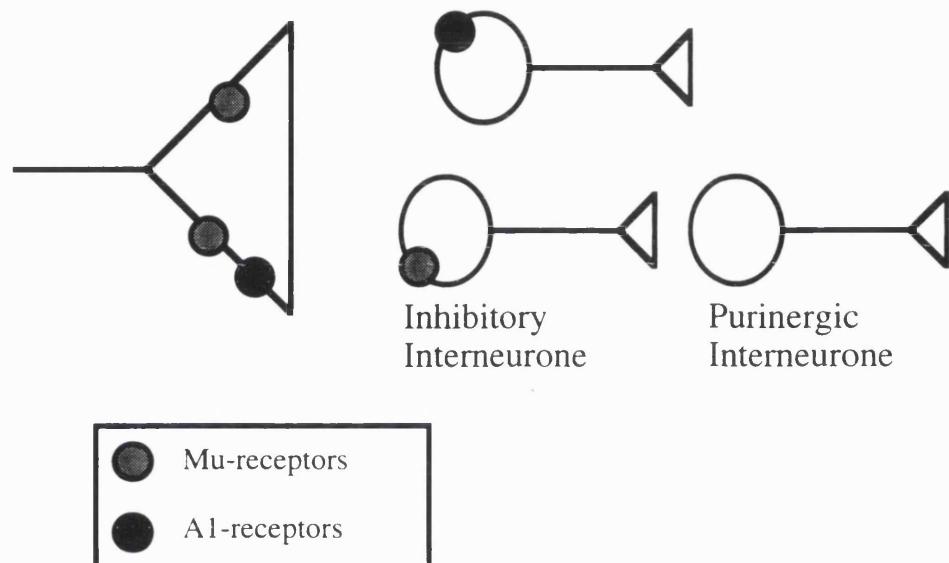
Figure 1. *Schematic representation of the possible mechanism of the release of purines by morphine. Adenosine may be released per se from primary afferents or from intrinsic cells or come from the conversion of ATP. Disinhibition of inhibitory intrinsic neurones, which control purinergic neurones could facilitate the release of adenosine by morphine if mu-receptors control these inhibitory neurones.*

The results presented here show that a low dose of an A_1-receptor agonist and a low dose of a mu-receptor agonist can have additive inhibitory effects, as has been demonstrated in the elegant study by DeLander and Keil (1994). It is possible that combined therapy of adenosine and morphine may therefore be beneficial, since it may give adequate analgesia reducing the associated side effects. The $A\delta$-fibre evoked responses continue to be facilitated in the presence of morphine and an A_1-receptor agonist. Whether these responses would result in a nociceptive event in an awake animal, would depend on whether they represent high or low-threshold inputs. Since behavioural studies with adenosine analogues alone (Ahlijanian and Takemori 1985; DeLander and Hopkins 1986; Herrick-Davis et al.

1989; Holmgren et al. 1983, 1986; Karlsten et al. 1990; Malmberg and Yaksh 1993a; Post 1984; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Vapaatalo et al. 1975; Yarbrough and McGuffin-Clineschmidt 1981) and adenosine A_1-receptor agonist combined with opioids are antinociceptive (DeLander and Keil 1994) this would suggest that enhanced $A\delta$-fibre evoked activity is not nociceptive in awake animals. Specific testing of $A\delta$ vs C mediated responses in awake animals would further clarify this point.

Chapter 6.

The effects of an A_1-agonist and kinase
inhibitors on the formalin evoked responses of
dorsal horn neurones.

6.1: Introduction.

Since few if any clinically relevant pain states involve acute pain, models by where tissue damage can be induced may be more clinically relevant. The formalin inflammatory model can be used as a model of tonic tissue-injury induced changes (Abbott et al. 1982, 1995; Dubuisson and Dennis 1977; Tjolsen et al. 1992; Wheeler-Aceto and Cowan 1991). Since many pain states involve continuous pain rather than transient, the use of various models of tonic pain may shed light on changes which occur in both pain signalling and analgesic systems.

The changes that occur in inflammation may result from an enhanced peripheral afferent drive (Dickenson and Sullivan 1987b), changes occurring in dorsal root ganglion cells (Lembeck et al. 1981; Noguchi et al. 1988; Oku et al. 1987b; Smith et al. 1992) and from central changes (Hunter and Singh 1994; Presley et al. 1990; Ren et al. 1992; Sugimoto et al. 1990; Woolf 1983) evoked by an increase in afferent drive.

Formalin evoked activity has been studied in a number of species including the rat (Abbott et al. 1982; Chapman and Dickenson 1992; Dickenson and Sullivan 1987b; Dubuisson and Dennis 1977; Haley et al. 1990; Malmberg and Yaksh 1993a), mice (Murray et al. 1991), cats (Dubuisson and Dennis 1977), primates (Alreja et al. 1984) and humans (Dubuisson and Dennis 1977).

The introduction of formalin into the peripheral receptive field is believed to activate the afferents directly, probably by activation of chemoreceptors (Dubuisson and Dennis 1977; See Tjolsen et al. 1992) that gives rise to an acute/phasic response. Generally it is only the C-fibre afferents which will respond to chemical activation, although some Aδ-polymodal afferents have been shown to respond to chemicals (Adriaensen et al. 1980). However, it has been shown that formalin evokes activity predominantly in C-fibres and not in Aδ-fibres (Heapy et al. 1987). This direct effect of formalin results in an immediate barrage of activity. In electrophysiological experiments in the rat the phasic response comprises the firing of dorsal horn neurones from the point of formalin injection until about ten minutes after administration (Chapman and Dickenson 1992; Dickenson and Sullivan 1987b; Haley et al. 1990) and behaviorally results in paw-flinching over the same period (Abbott et al. 1982, 1995; Dubuisson and Dennis 1977; Malmberg and Yaksh 1993a). This is followed by a short period where the firing activity of dorsal horn neurones becomes less or is zero and corresponds exactly to a decrease in behavioural responses suggestive of nociception (Dickenson and Sullivan 1987b; Dubuisson and Dennis 1977). After this quiet phase the dorsal horn cells begin to fire again or the animal

resumes its characteristic behavioural responses (Dickenson and Sullivan 1987b; Dubuisson and Dennis 1977; Malmberg and Yaksh 1993a). This phase of activity is known as the second or tonic phase and again originates from an afferent drive, this time produced by a complex series of biochemical and cellular events in the periphery (peripheral sensitization) and from enhanced and altered central responses (central sensitization) (Coderre and Melzack 1992; Dickenson and Sullivan 1987c; Dubuisson and Dennis 1977; see Tjolsen et al. 1992).

The two phases differ in their central consequences. It has been shown that the first/acute phase of the formalin induced activation is mediated by activation of the AMPA-receptor (Hunter and Singh 1994) whilst this receptor appears to have a reduced role in the tonic/second phase. This latter phase is driven centrally by activation of the NMDA-receptor (Coderre and Melzack 1992; Haley et al. 1990; Hunter and Singh 1994; Vaccarino et al. 1993). This would at first suggest that the phasic response primarily activates mono-synaptic pathways and the tonic phase involves poly-synaptic pathways, since NMDA-receptor antagonists have less effect on inhibiting mono-synaptic pathways pathways compared to polysynaptic pathways (Davies and Watkins 1983; Dickenson and Aydar 1991). There is some evidence that the NMDA-receptor may set up changes during the later stages of the first phase which maintain the tonic responses of the second phase (Haley et al. 1990; Malmberg and Yaksh 1995; Vaccarino et al. 1993). The tonic phase also is accompanied by an increased release of SP and CGRP (Murray et al. 1991; Noguchi et al. 1988). The release of peptide neurotransmitter is imperative for the recruitment of the NMDA-receptor, since the magnesium block of the channel in its resting state (Mayer et al. 1984; MacDonald and Nowak 1990) is thought to be removed by peptide actions.

It has been shown in behavioural studies that the A_1-receptor agonists N^6-[L-2-phenylisopropyl]-adenosine (L-PIA) and N^6-cyclohexyladenosine (CHA) reduce the second phase of the formalin evoked response (Malmberg and Yaksh 1993a; Poon and Sawynok 1995) but have little effect on the acute phase.

Here we examine the effects of an exogenous A_1-receptor agonist, CPA on the formalin evoked responses of dorsal horn neurones as well as using kinase inhibitors (GPA and GPB) to ascertain if adenosine is released in response to formalin evoked activity and if protecting endogenous adenosine can inhibit the acute and tonic phases of this inflammatory response.

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6.2.1: N^6-cyclopentyladenosine.

41 neurones were studied after peripheral injection of formalin (5%) . Twenty one cells with an average depth of 758±44μm were used as controls. The first phase of the formalin response (0-10mins) gave a response of 5248±1170 action potentials. The second phase of the formalin evoked response, measured from 10-60 minutes, produced a count of 14530±3014 action potentials (Figure 1 and 2).

Four doses of CPA were given intrathecally as pretreatments to formalin. One dose of CPA was tested per formalin response and the pretreatment was for a twenty minute period. Five cells were observed for each dose of CPA (0.01, 0.1, 1 and 5μg). The lowest dose tested (0.01μg) had no significant effect on either the first or the second phase of the formalin response. Increasing the dose of CPA to 0.1μg significantly inhibited the first phase of the formalin response to 35±13% of control (*P<0.05*). However, at this dose although the second phase tended to be reduced to 34±14% of control, this was not significant (*P=0.059*). At 1μg of CPA and above both the first and second phase of this response was significantly inhibited, so that the first phase was 17±6 and 5±2% of control for 1 and 5μg respectively (see Figure 1 and 2). The second phase was reduced to 5±2% and 0.4±0.19% of control for the above doses, respectively.
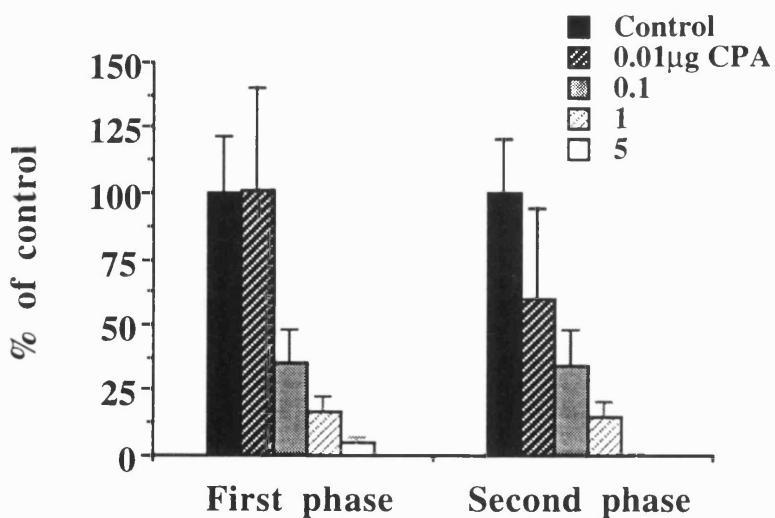
Figure 1. *Dose response relations for CPA (0.01, 0.1, 1 and 5μg) given as a pretreatment to peripheral formalin injections. Both the first and the second phase of formalin evoked activity was significantly inhibited by CPA. (n=21 for control, n=5 for all doses of CPA).*

The duration of the effects of the agonist increased with the dose, so that the partial recovery of the second phase of the formalin response seen with the lower doses of CPA was lost as the doses increased. With 5µg CPA there was a complete inhibition of this response (see Figure 2).
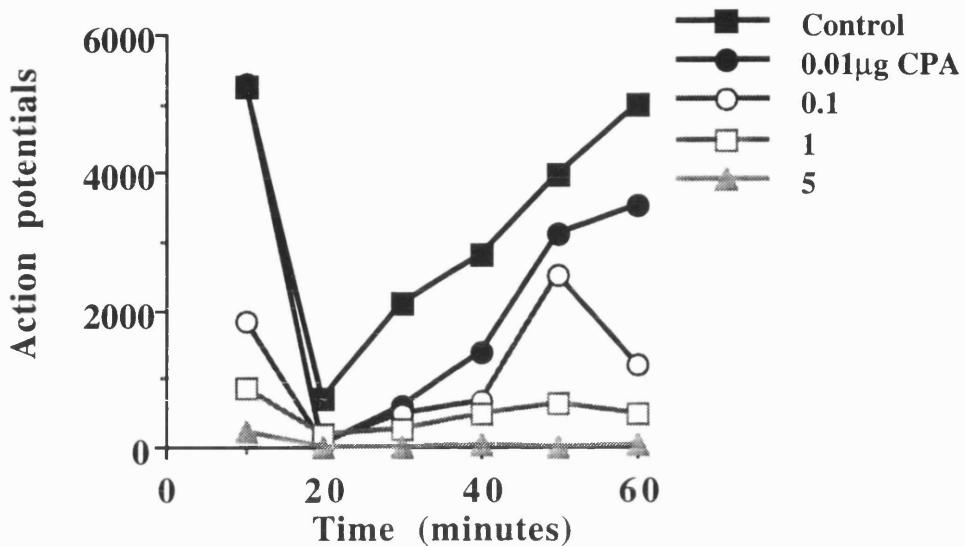
Figure 2. Time-course of the formalin evoked response shown as action potentials counted in ten minute blocks, against time. Increasing the dose of CPA increased the duration of inhibitory action, so that there was no recovery of the second phase with 5µg CPA.

6.2.2: GPA-An Adenosine Kinase Inhibitor.

The effects of GPA on the formalin response could not be evaluated as the vehicle controls, systemic DMSO pretreatment (0.25ml) were found to inhibit the formalin evoked responses. The control formalin responses for these experiments without any vehicle pretreatment (n=7) gave a mean of 6539 ± 1537 and 23137 ± 8722 action potentials for the first and second phase respectively. After DMSO, four cells were inhibited and two were similar in second phase responses to formalin controls alone, but still showed a reduced first phase. The responses of the four inhibited cells were 2074 ± 817 and 905 ± 587 action potentials for the first and second phase respectively and the other two cells gave a response of 3475 ± 705 and 35973 ± 7596 action potentials respectively. In view of this vehicle problem the effects of GPA on the formalin response could not be evaluated.

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6.2.3: GPB-An Adenosine Kinase Inhibitor.

Control formalin responses for GPB were first established (n=11). The *subsequent evaluation with* control values were 5739±1157 and 28275±6090 action potentials for the first phase and the second phase respectively. GPB was given intrathecally as a twenty minute pretreatment prior to formalin at doses of 0.5, 5, 50 and 500µg (n=5 for each dose). Only one dose was tested per cell. The first phase was only significantly inhibited with 500µg GPB ($P<0.05$) with a mean response of 1540±493 action potentials for the first phase which was 27±9% of controls. The second phase of the formalin response was significantly inhibited by 5, 50 and 500µg of GPB ($P<0.05$) to give responses of 7411±3114, 4000±2200, 1609±878 action potentials respectively. Figure 3 shows the effects of GPB on the first and second phase of the formalin evoked response as percentages of the control formalin responses for these experiments.
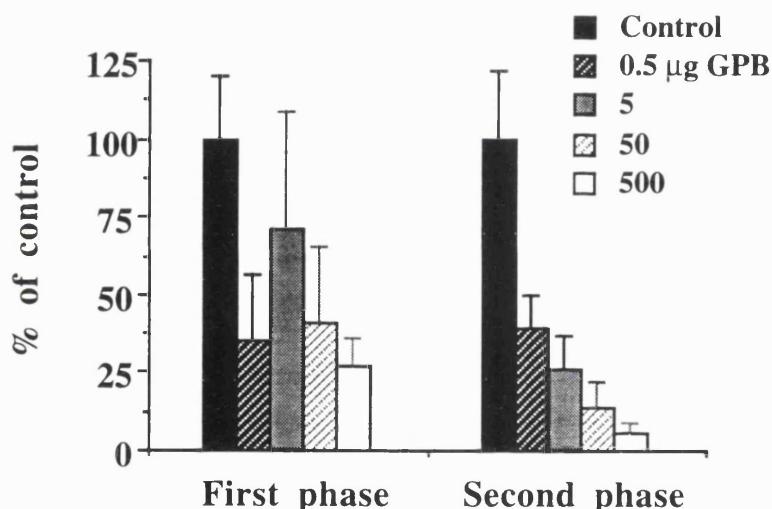
Figure 3. Dose response relations for the kinase inhibitor, GPB on the first and second phase of the formalin response. There is a dose dependent inhibition of the second phase but not the first, suggesting that predominantly adenosine is released during tonic activity.

From Figure 4 it can be seen that increasing the dose of GPB reduced the second phase although the time-course of the response remained the same. With 500µg GPB towards the end of this response there was partial recovery of the formalin evoked activity, this is in contrast to CPA (see Figures 2 and 4).
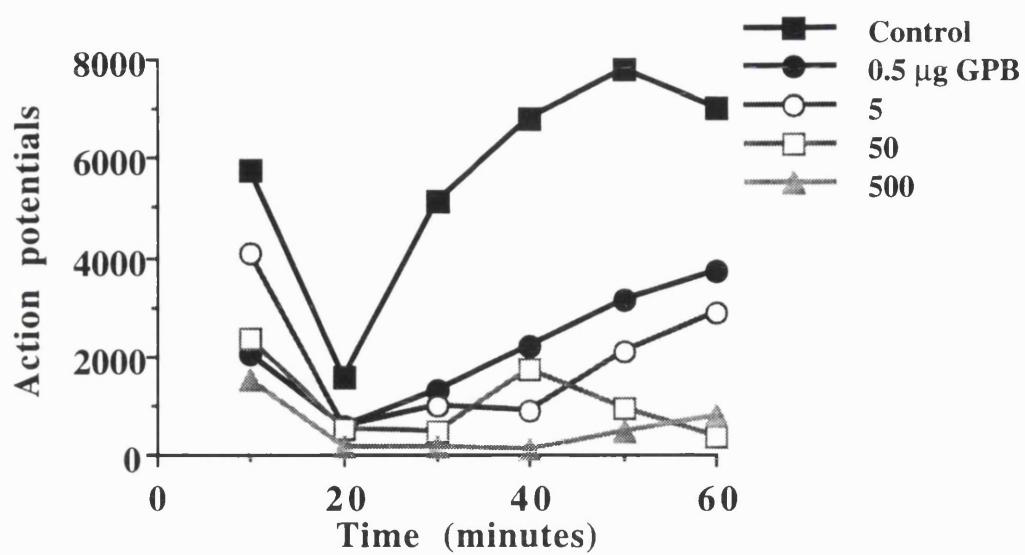
**Figure 4.** *Time-course of the formalin evoked response shown as action potentials counted in ten minute blocks, against time. Increasing the dose of GPB increased the duration of inhibitory action, however, the second phase did show some recovery.*

6.3: Discussion.

Since many clinical pain states have an associated tonic inflammatory component induced by tissue damage, the study of agents which can cause inflammation may be employed to observe the peripheral and central changes which occur. Behavioural tests in formalin treated animals have studied the central and peripheral roles of adenosine in controlling formalin evoked activity (Doak and Sawynok 1995; Malmberg and Yaksh 1993a; Poon and Sawynok 1995). However, behavioural testing can be hampered by motor effects of agonists (Malmberg and Yaksh 1993a; Poon and Sawynok 1995).

We found that the intrathecally applied A_1-receptor agonist, CPA, dose-dependently inhibited both the first and second phase of the formalin evoked response to a similar extent.

This is in contrast to the study of Malmberg and Yaksh (1993a) and Poon and Sawynok (1995) who found that the first phase was not altered by the application of L-PIA and CHA respectively, but there was a dose dependent decrease in the second phase. This discrepancy between behavioural and electrophysiological experiments may be due to the different agonist used; L-PIA is slightly less potent than CPA (Jacobson et al. 1992) whereas L-PIA and CHA have similar affinities. In addition, motor effects could have prevented doses being increased so that the effects on the first phase may have been missed. However, when comparing doses between our experiments and the studies of Malmberg and Yaksh (1993a) and Poon and Sawynok (1995) the doses are all in the low nanomolar range, so this can not explain the differences observed. In addition, we see similar effects on the first and second phase of the formalin response with low doses of CPA, in the exact range tested with L-PIA and CHA, so the differences must arise from more subtle differences between behavioural and electrophysiological testing.

It must be noted that there are also differences between the behavioural testing between the two groups. In the study by Malmberg and Yaksh (1993a) they record the flinching/shaking of the paw and found that this was inhibited some 80% by L-PIA. Poon and Sawynok (1995) measured flinching/shaking and biting/licking as separate observations. They found with CHA that there was only a 50% of flinching/shaking but a complete inhibition of the licking/biting during the tonic phase. This demonstrates the limitations of behavioural testing when different which may result in different interpretations of the same experiment criteria of the same test are used (Abbott et al. 1995; Tjolsen et al. 1992). The biting/licking is a sporadic behaviour and has been suggested by Poon and Sawynok (1995) to represent phasic (acute) pain. However, if this is the case then since the first phase of the formalin response is a phasic/acute phase then this phase should also be inhibited, yet both behavioural studies report no effects on the first phase of the formalin evoked response (Malmberg and Yaksh 1993a; Poon and Sawynok 1995).

The inhibition of the first and second phase I observed with CPA

indicates possible pre- and post-synaptic effects of this drug. This was also the case when considering the effects of CPA on the electrically evoked activity (see chapter 4). Thus the inhibition of the electrically evoked wind-up and post-discharge, which are predominantly driven by recruitment of neurones in poly-synaptic pathways and thus a post-synaptic phenomenon, indicate post-synaptic controls by the A_1-receptor. The second phase of the formalin response has been shown to be centrally maintained by the NMDA-receptor (Coderre and Melzack 1992; Haley et al. 1990; Hunter and Singh 1994; Vaccarino et al. 1993). The inhibition of the acute/first phase of the formalin response seen with CPA could also indicate a pre-synaptic action. It is known that the majority of the A_1-receptors are predominantly located on second order neurones (Choca et al. 1988; Li and Perl 1994) this would suggest that the post-synaptically located A_1-receptors also are influential in controlling mono-synaptic inputs into the dorsal horn, since there are few receptors on the primary afferent terminals themselves.

It is known that there is an up-regulation of peptide production and an increase in spinal peptide release, predominantly SP and CGRP during inflammation (Lembeck et al. 1981; Noguchi et al. 1988; Oku et al. 1987b; Smith et al. 1992). This peptide release may result in an enhancement of the responses of excitatory amino acids (Kangrga and Randic 1990; Murase et al. 1989; Randic et al. 1990). This increase in dorsal horn excitability is blocked by NMDA-receptor antagonists. The release of peptides in more tonic responses may well be responsible for the subsequent recruitment of the NMDA-receptor, since they cause slow long-lasting depolarizations which results in the removal of the magnesium block of the ion channel associated with this receptor (Mayer et al. 1984). It is therefore not surprising that NMDA-receptor antagonists can reduce the enhanced responses associated with tonic activity and so inhibit the second phase of the formalin response (Coderre and Melzack 1992; Haley et al. 1990; Hunter and Singh 1994; Vaccarino et al. 1993).

The inhibitory effects of the A_1-receptor agonists in controlling the second/tonic phase of the formalin response may also be due to the inhibition of peptide release. It has been shown that CHA inhibits the electrical field stimulated release of CGRP and SP from the dorsal portion of the rat spinal cord (Santicioli et al. 1992, 1993). This would indicate a pre-synaptic location of the A_1-receptor which is known to only represent a small proportion of the receptor population (Choca et al. 1988; Li and Perl 1994). This inhibition of peptide release has not been consistently observed, since Vasko and Ono (1990) found that CHA and L-PIA did not decrease substance P release. This is also born out by human studies measuring the concentrations of peptides in the CSF. It was found that intravenous adenosine infusion did not decrease substance P, Neurokinin A, Neuropeptide Y or CGRP in the CSF (Sollevi et al. 1996).

The kinase inhibitors were used to evaluate the conditions for release and subsequent actions of adenosine in formalin evoked responses in this model of tonic

pain. It has been shown that protecting endogenous adenosine can be effective in a model of septic shock (Firestein et al. 1994) and may also have anti-inflammatory properties in the periphery. However, it has also been shown that adenosine can lower the threshold for mechanical nociception when given into the periphery (Taiwo and Levine 1990). The possible role of adenosine in influencing afferent drive will depend on the concentration of adenosine and will be discussed in more detail in chapter 7.

Unfortunately the vehicle controls for GPA (DMSO) inhibited the formalin evoked responses so this kinase inhibitor could not be evaluated. It is known that DMSO can inhibit peripheral nerve C-fibres (Evans et al. 1993) and this may well be responsible for the reduced formalin evoked response. This vehicle was not found to be a problem when electrically evoked activity was used (see chapter 4) and is probably due to the fact that transcutaneous electrical stimulation at three times threshold for the C-fibre afferents can activate the nerve axon directly and so overcomes any vehicle effects.

The spinally applied kinase inhibitor, GPB, inhibited both the first and second phase of the formalin evoked response although the second phase was inhibited in a clear dose dependent manner and to a much greater extent. The fact that there was inhibition of the formalin evoked activity with a kinase inhibitor is consistent with the hypothesis that adenosine is being released at the spinal cord level in response to this form of stimulation and that protecting adenosine from metabolism results in inhibition of the response.

Behavioural studies have reported that protecting endogenously released adenosine at the spinal cord level has no effect on the first or the second phase of the formalin evoked response (Poon and Sawynok 1995). However if the formalin concentration was reduced to 2% then an inhibition of the second phase was observed. The authors attributed this to an enhanced sensitivity of the formalin test when a lower concentration was used but there are major problems with this assumption. What is causing the release of adenosine? It seems unlikely that lower intensity stimulation would result in a greater release of adenosine. It is likely that adenosine is released in the dorsal horn of the spinal cord in response to NMDA-receptor activity and then acts as a negative feedback mechanism to control further NMDA-receptor activity. Evidence to support this in our model comes from the inhibitory effects of the same kinase inhibitor on the electrically evoked wind-up and post-discharge, phenomena mediated by activation of the NMDA-receptor (Davies and Lodge 1987: Dickenson and Sullivan 1987a). Since the kinase inhibitor appeared to be more effective in inhibiting these responses compared to other responses of the neurones, this is consistent with the idea that adenosine is being released in response to NMDA-receptor activation. This has been shown in other areas of the central nervous system (Craig and White 1992, 1993; Hoehn et al. 1990a, b; White 1994, 1996) and is believed to be the mechanism behind the neuroprotective role of adenosine. The use

of kinase inhibitors has been shown especially effective at protecting NMDA-receptor dependent release of adenosine (White 1996). It would therefore seem unlikely that lowering the concentration of formalin would result in an increased release of adenosine at the spinal cord level as seen by Poon and Sawynok (1995).

It is possible that NMDA-receptor evoked adenosine release may influence differentially the first and second phase of the formalin response. It is known that glutamate release sets up the second phase of the formalin response (Malmberg and Yaksh 1995) and antagonists of the NMDA-receptor predominantly attenuate the second phase of the response (Haley et al. 1990; Hunter and Singh 1994) so that it can be presumed that this receptor plays an important role in the maintenance of the tonic phase. Thus in this second phase the clear dose-dependent inhibitory effects of the kinase inhibitor GPB lends strong support to the idea that NMDA-receptor activity is a primary stimulus for the release of adenosine. However, effects of GPB on phase I as well suggests that functionally relevant adenosine is not only released by NMDA-receptor-mediated mechanisms. We have clearly demonstrated that adenosine A_1-receptor activation can control both the phasic and tonic components of the formalin evoked response. In addition, under conditions of protection of endogenously released adenosine, the present data suggests that adenosine may be released at the spinal cord level in response to tonic activity which involve the activation of the NMDA-receptor. Thus adenosine may have the capacity to act as a negative feedback mechanism in the spinal cord in response to enhanced activity, as has been seen in other areas of the brain.

Chapter 7.

The effects of an A_1-agonist, adenosine receptor
antagonists and kinase inhibitors after
carrageenan inflammation.

7.1: Introduction.

The use of carrageenan (a mucopolysaccharide from the sea moss, *Chondrus*) to induce inflammatory changes, was first described by Robertson and Schwartz 1953 and later by Winter et al. (1962). This latter study found that swelling of the injected site reached a maximum 3-5 hours after administration and this degree of oedema was then maintained for many hours (Winter et al. 1962). Studies by Kayser and Guilbaud (1987) showed that there was a decrease in vocalization threshold for mechanical stimulation which developed over the first hour after the administration of carrageenan and persisted for some 96 hours after administration. Further studies by Hargreaves et al. (1988) demonstrated that a decrease in paw withdrawal latencies to mechanical pressure corresponded to decreased in nociceptive thresholds to thermal stimulation observed over a time-course of one to seven hours after carrageenan induced inflammation. This resulting hyperalgesia was confined to stimuli applied to the side of injection and did not extend to the contralateral paw (Hargreaves et al. 1988).

This method of inducing inflammation results in an acute inflammatory response, more prolonged than formalin induced inflammation, but still within a time-course compatible with recording from anaesthetized animals.

The inflammatory changes that occur in the periphery can have consequences for events at the spinal level. The levels of opioid peptides and transcription markers have been shown to be elevated after carrageenan inflammation (Iadarola et al. 1988). The effects of exogenous opioids have been shown to be enhanced when given spinally (Hylden at al. 1991: Stanfa et al. 1992) but also have a novel peripheral effect (Stein 1993; Stein et al. 1988) after inflammation.

Alterations, such as increased activity of the NMDA-receptor after inflammation have also been well documented (Dubner 1991: Ren et al. 1992). It has been shown that intrathecal NMDA can be both pronociceptive as well as antinociceptive and that this is probably a spinal event since spinalization does not alter the ability of NMDA to cause analgesia, which was reversed by naloxone (Raigorodsky and Urca 1987). In other areas of the CNS increased activity of the NMDA-receptor can result in the release of adenosine per se as well as from the breakdown of ATP (Craig and White 1992: Hoehn et al. 1990b) Thus it is possible that adenosine is released at the spinal cord level and acts as an endogenous negative feedback mechanism to prevent cell death or damage from excitotoxity.

In the periphery it has been shown that the activation of systems that stimulate adenylate cyclase and increase cyclic AMP produce hyperalgesia possibly via the A_2-receptor (Taiwo

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and Levine 1989) and adenosine is capable of this effect (Taiwo and Levine 1990). Systems that inhibit adenylate cyclase and thus cAMP have been shown to inhibit hyperalgesia associated with inflammation (Levine and Taiwo 1989: Taiwo and Levine 1990). Interestingly these inhibitory systems includes the opioids as well as adenosine.

It is possible that adenosine levels may alter in the periphery as well as centrally in response to peripheral inflammation. The A_1-receptor agonist, CPA and the antagonists 8pSPT and DPCPX were observed three hours after carrageenan induced inflammation. The use of systemic and intrathecal adenosine kinase inhibitors were also employed to protect endogenously released adenosine to try to ascertain the role and sites of action of endogenous adenosine in a longer lasting acute inflammatory model.

7.2.1: N^6-cyclopentyladenosine.

The effects of CPA (0.5, 5, 50 and 250µg) were evaluated after three hours carrageenan induced inflammation on seven dorsal horn neurones with an average depth of 840±68µm from the surface of the spinal cord.

The effects of CPA after carrageenan induced inflammation were to dose dependently inhibit C-fibre evoked responses, post-discharge and wind-up with a tendency to facilitate the Aδ-fibre evoked responses and the Aβ-fibre evoked responses.

The C-fibre evoked responses were significantly inhibited by 50µg and 250µg of CPA ($P<0.05$), responses being 54±11 and 46±11% of control respectively (Figure 1A). The same doses (50 and 250µg) also significantly inhibited post-discharge and wind-up responses of the same population of cells (see Figure 1B) to 35±17, 6±2; 27±10 and 20±7% of control respectively. Although both the Aβ- and Aδ-fibre evoked responses tended to be facilitated none of these responses were significant.
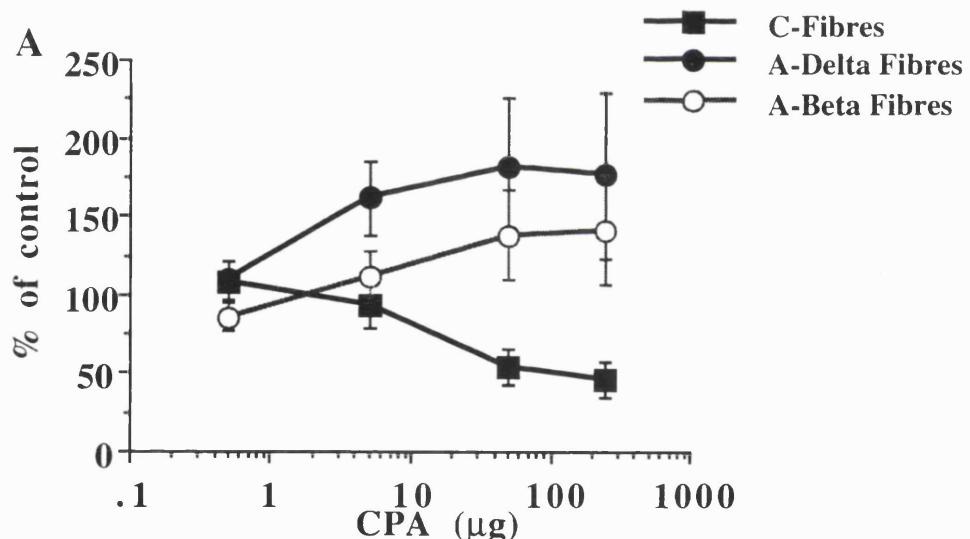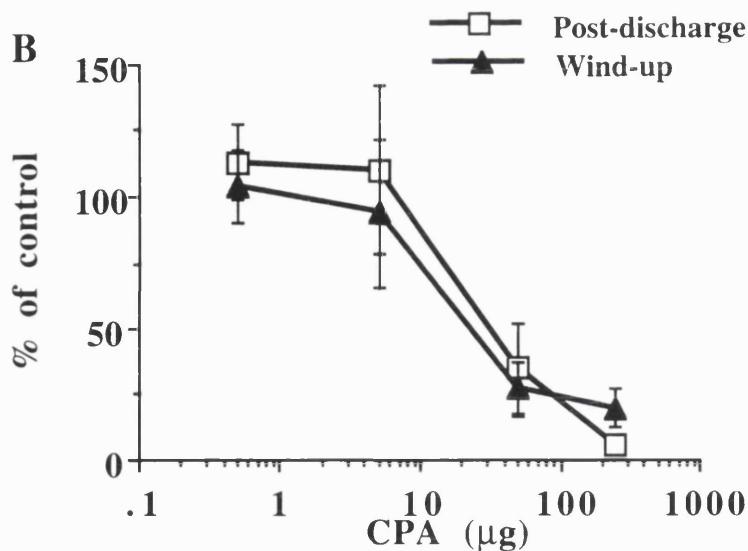
Figure 1. Dose-response relations for 0.5, 5, 50 and 250µg CPA after three hours carrageenan induced inflammation. (A) The response of C-fibre evoked activity, Aδ- and Aβ-fibres. (B) The responses of wind-up and post-discharge.

The effects of the antagonist 8pSPT (10 and 1000µg) were evaluated for thirty minutes each after the highest dose of CPA (250µg). The lowest dose of 8pSPT (10µg) did not reverse any of the effects of 250µg CPA, so that the C-fibre evoked response which was 46±11 with CPA was 29±15% of control with 10µg 8pSPT (see Figure 2A). The post-discharge response was 6±2% of control with 250µg CPA and after 10µg 8pSPT was 12±10% of control. The wind-up of the cells was 20±7% of control with 250µg CPA, after 10µg 8pSPT the wind-up responses were still inhibited, 27±14% of control (Figure 2B). The enhanced responses of Aβ- and Aδ-fibre evoked responses did not return to control values with 10µg 8pSPT.

However, increasing the dose of 8pSPT to 1000µg
now
ᴸreversed the inhibitory effects of 250µg CPA . The C-fibre evoked responses were reversed to 77±21% of control and the post-discharge returned to 70±22% of control. The enhanced Aδ- and Aβ-fibre responses returned to 107±43 and 109±26% of control (Figure 2A). Increasing the dose of 8pSPT restored the wind-up responses of the cells to 97±43% of control.
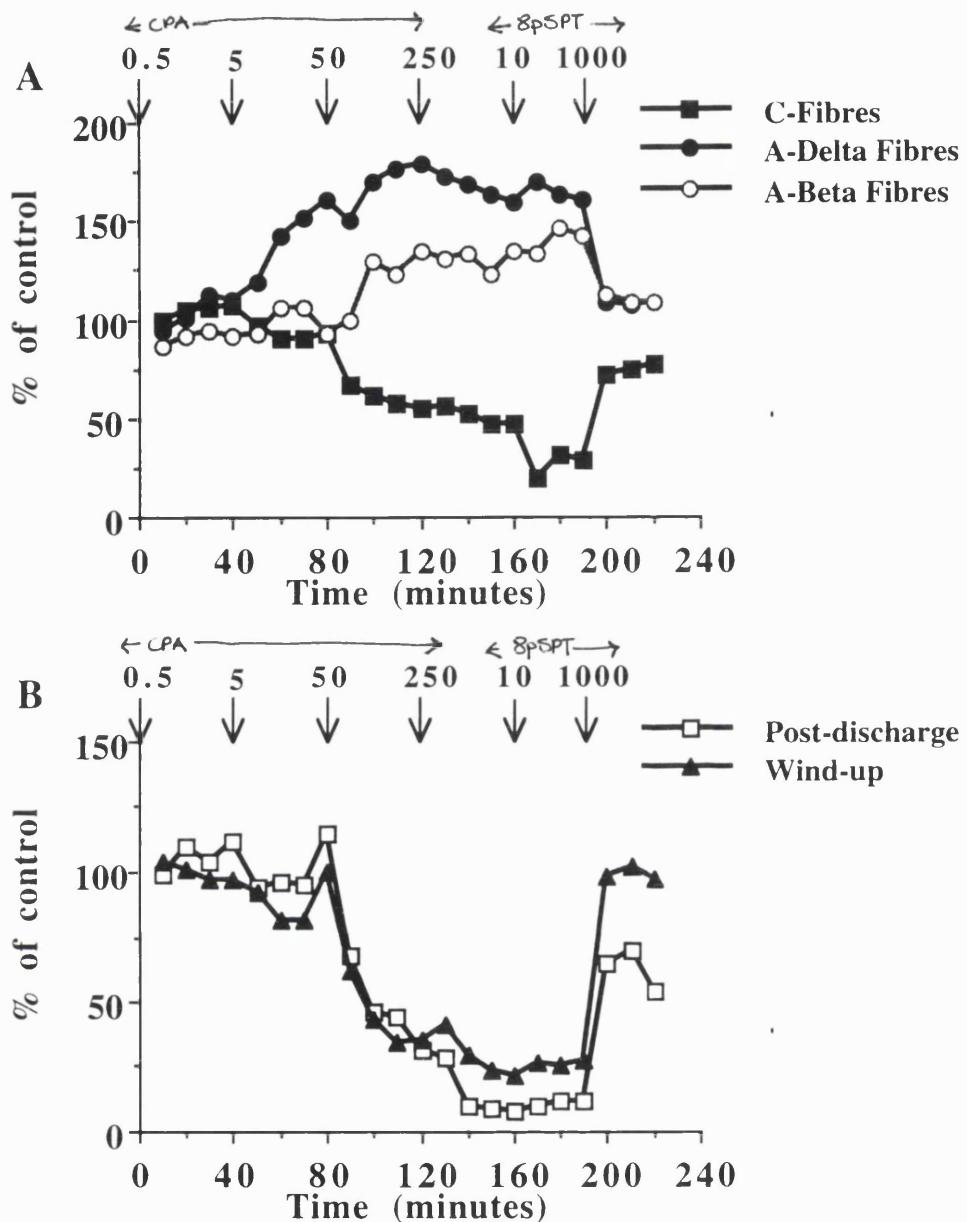
Figure 2. Time-course of CPA after three hours carrageenan induced inflammation. The arrows indicate the application of CPA (0.5, 5, 50 and 250μg) followed by 10 and 1000μg 8pSPT. (A) The effects of the CPA and 8pSPT on the C-, Aδ- and Aβ-fibre evoked responses. (B) The post-discharge and wind-up responses.

The measures of input and final overall response to a train of stimuli were shown to increase over the three hour carrageenan pretreatment and decrease with CPA (Figure 3). The dose of 5μg CPA tended to inhibit the input response and subsequent doses (50 and 250μg) significantly reduced the input onto the cells. The

final overall response was also significantly inhibited by 50 and 250µg CPA ($P<0.05$). 8pSPT (10 and 1000µg) was then used to attempt to reverse the agonist effects. The lowest dose of 8pSPT did not reverse the effects of CPA on the input and final response. However, increasing the dose to 1000µg 8pSPT reversed the input response from 19±10 to 88±29% of control. The final response to a train of stimuli was reversed from 21±10 to 74±17% of control with this dose of 8pSPT.
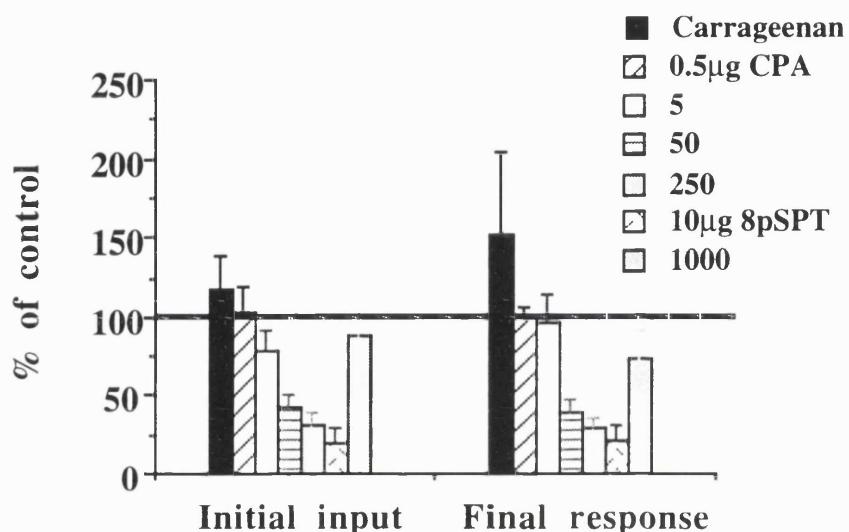
Figure 3. *The effects of three hours carrageenan induced inflammation on the initial input and final overall response to a train of stimuli. The carrageenan effects are as a percentage of initial, pre-carrageenan controls. The subsequent effects of doses of CPA (0.5, 5, 50 and 250µg) are expressed as percentage of the carrageenan effects. CPA reduced both the input and final response. The effects of the antagonist 8pSPT (10 and 1000µg) are also shown.*

7.2.2: Comparison of the Effects of CPA in Untreated Animals Compared to Animals Three Hours Post Carrageenan.

Comparing the dose response curves (0.5, 5, 50 and 250µg CPA) in untreated animals compared to those animals which had received carrageenan induced inflammation showed no significant difference in the effect of any dose of CPA on the Aδ-fibre evoked responses, the Aβ-fibre responses or wind-up. The effects of 0.5µg CPA only was significantly different on the C-fibre evoked responses and post-discharge between the two groups of animals tested ($P<0.05$). In untreated animals the C-fibre evoked responses were inhibited to 73±12% of control whereas

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after carrageenan induced inflammation the C-fibre responses with the same dose of CPA were still 108±5% of control (Figure 4A).
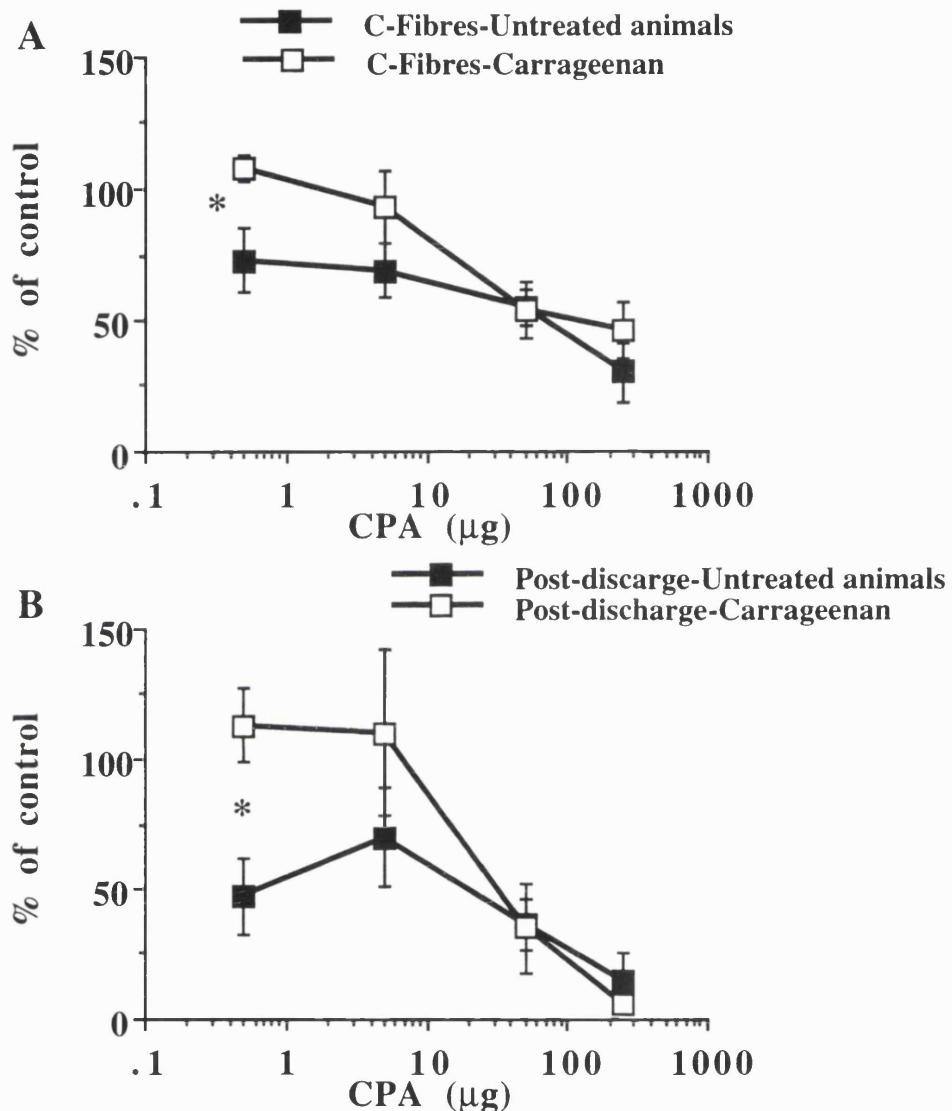
Figure 4. *Dose-response relations for CPA in animals three hours post carrageenan induced inflammation and animals without any pretreatment. (A) C-fibre evoked activity. (B) Post-discharge responses. * (P<0.05).*

The post-discharge in untreated animals was reduced to 47±15% of control after 0.5µg CPA whereas the same dose after carrageenan on the post-discharge produced a response of 113±14% of control (Figure 4B).

The measures of input and overall, final, response to a train of stimuli were not significantly different between the two groups of animals with any dose of

7.2.3: 8(p-Sulphophenyl) Theophylline.

The effects of 8pSPT alone were observed after three hours post carrageenan on a population of ten cells with an average depth of $708\pm88\mu$m from the surface of the spinal cord. The doses of 8pSPT evaluated were 1, 10, 100 and 400μg.

The effects of 8pSPT on the population of ten cells were split into two groups since seven cells tended to have inhibited responses, whilst three cells showed facilitated responses. The average depth of the inhibited cells was $856\pm83\mu$m and the facilitated population, $680\pm36\mu$m. The statistical analysis could only be undertaken for the inhibited cells as the facilitated population was too small (n=3).

The results discussed here are for the larger, inhibited population of cells. The C-fibre evoked responses were only significantly inhibited by 1μg 8pSPT ($P<0.05$), so that the responses were 83 ± 4, 90 ± 10, 94 ± 10 and $91\pm8\%$ of control for 1, 10, 100 and 400μg 8pSPT respectively. Although the Aδ- and Aβ-fibre evoked responses also tended towards inhibition no effect of any dose of 8pSPT was significant (see Figure 5A). The post-discharge and wind-up responses were significantly inhibited by 10μg 8pSPT and subsequent doses (Figure 5B). The post-discharges were 80 ± 19, 58 ± 15, 48 ± 10 and $42\pm10\%$ of control for 1, 10, 100 and 400μg 8pSPT respectively. Wind-up was 65 ± 14, 72 ± 16, 60 ± 9 and $47\pm12\%$ of control for the same above doses.

A

C-Fibres
A-Delta Fibres
A-Beta Fibres

% of control

150
100
50
0

.1   1   10   100   1000

8pSPT (μg)

B

Post-discharge
Wind-up

% of control

150
100
50
0

.1   1   10   100   1000

8pSPT (μg)
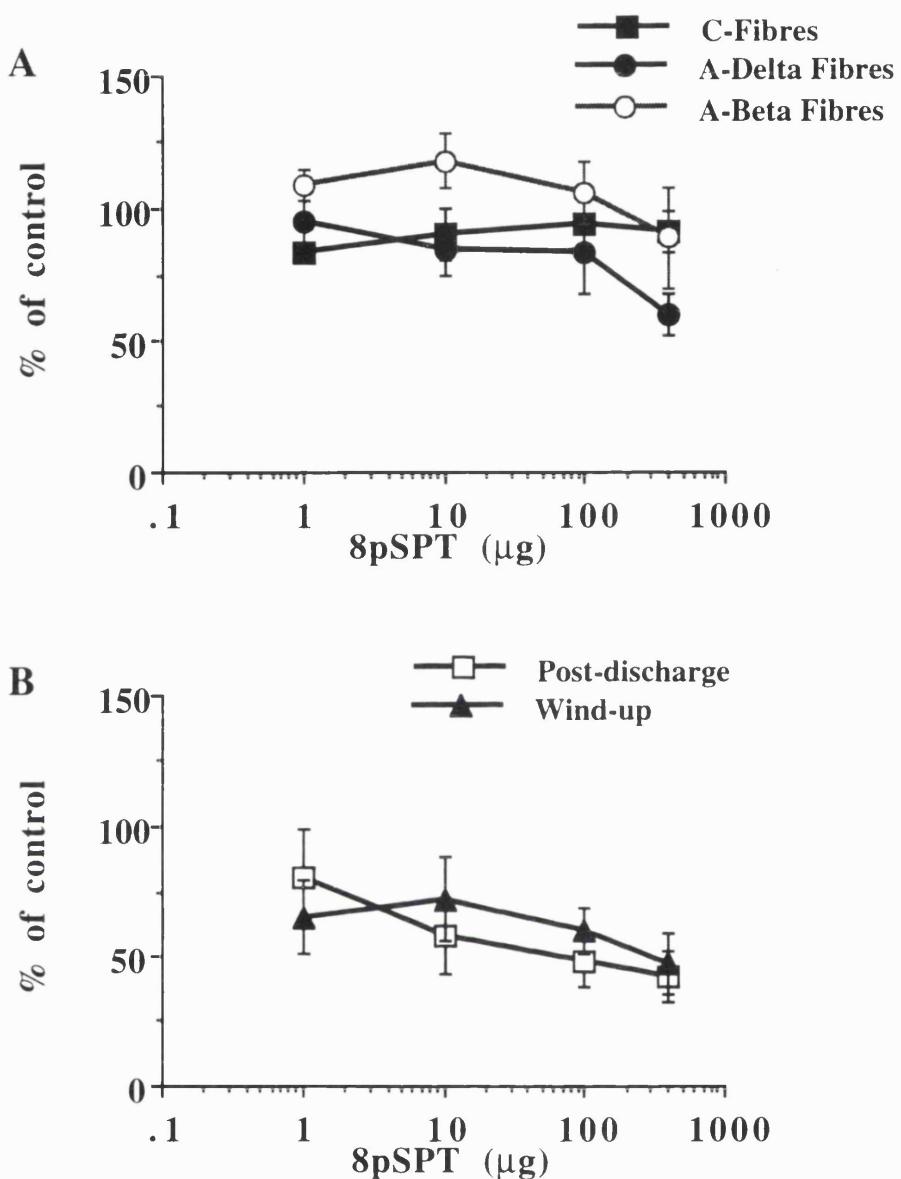
Figure 5. *Dose-response relations for 8pSPT (1, 10, 100 and 400μg) after carrageenan induced inflammation. (A) The effects on the C-, Aδ- and Aβ-fibre evoked responses. (B) The responses of wind-up and post-discharge.*

The input and final overall response were evaluated for the two groups of neurones. The cells inhibited by 8pSPT had enhanced inputs and overall responses three hours after carrageenan. The input was 204±63 and the final response was 139±39% of control responses. After the antagonist 8pSPT, the input responses were 103±11, 95±8, 108±21 and 93±30% of carrageenan controls for 1, 10, 100 and 400μg 8pSPT respectively. The final responses were 65±10, 76±7, 62±12 and 95±31% of control for the above same doses (see Figure 6A).
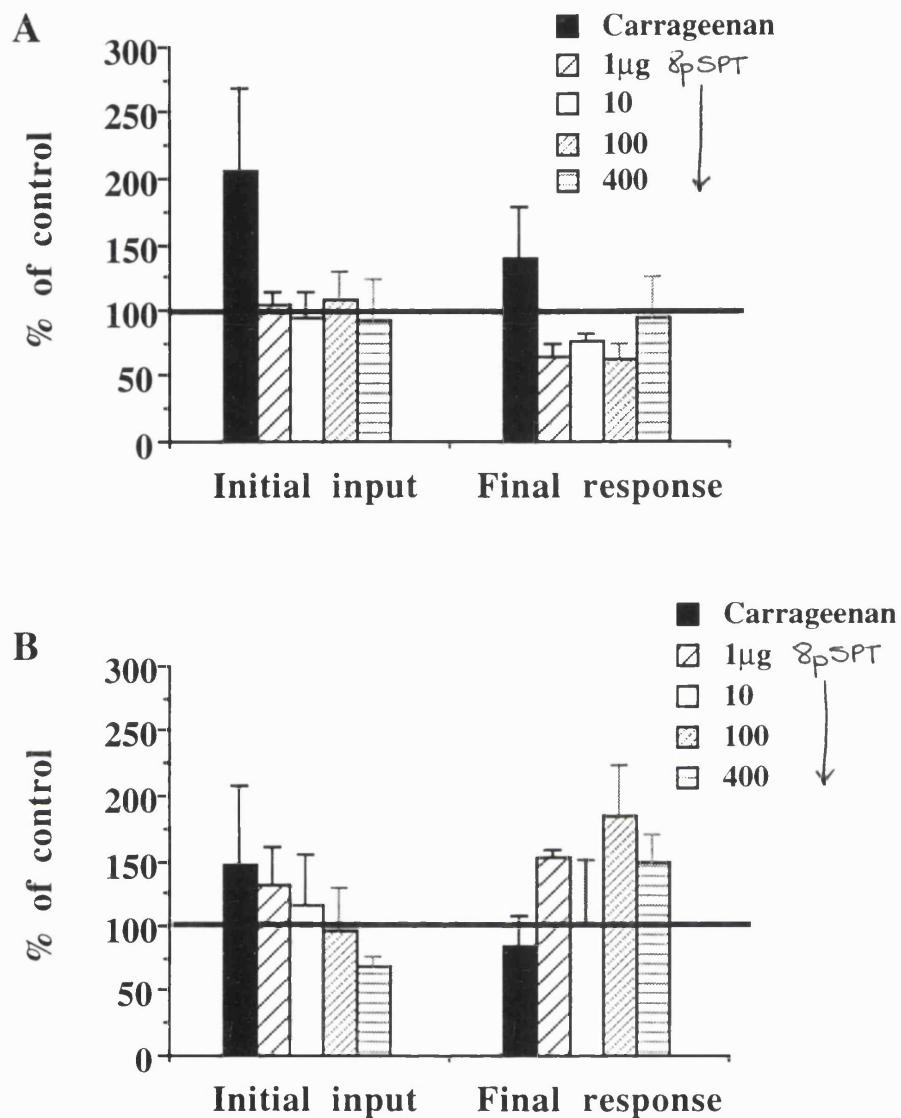
Figure 6. *The effects of three hours carrageenan induced inflammation on the initial input and final overall response to a train of stimuli. The carrageenan effects are expressed as a percentage of initial controls. The effects of subsequent doses of 8pSPT (1, 10, 100 and 400μg) are expressed as percentage of carrageenan effects. (A) The effects of 8pSPT on the inhibited population of cells (n=7). The enhanced responses after carrageenan were returned back to control levels and in the case of the final response, also tended to be inhibited. (B) The cells facilitated by 8pSPT (n=3). The responses tended to be enhanced, apart from 400μg on the input.*

The facilitated cells with 8pSPT (n=3) after three hours carrageenan

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induced inflammation had an input response of 148±60 and a final response to a train of stimuli of 85±23% of control. After 8pSPT the input responses were 131±30, 115±40, 96±33 and 69±75% of carrageenan controls for 1, 10, 100 and 400μg respectively. The final responses were 153±6, 100±51, 184±39 and 149±21% of control for the above same doses (Figure 6B). When these two populations were taken together there was no change in input or final response with any dose of 8pSPT, so that the carrageenan controls and 1, 10, 100 and 400μg 8pSPT had input responses of 190±49, 109±10, 100±16, 95±18, 85±20% of controls respectively. The final response for the total population of cells was 126±30, 106±18, 101±15, 99±23 and 94±16% of control for three hours carrageenan and doses of 8pSPT respectively. There was found to be no obvious correlations between inhibited or facilitated responses with cell depth or the excitability of the cells before carrageenan.

7.2.4: Comparison of the Effects of 8pSPT in Untreated Animals Compared to Animals Three Hours Post Carrageenan.

In comparing the dose response curves (1, 10, 100 and 400μg 8pSPT) in untreated animals compared to those animals which had received carrageenan induced inflammation there was found to be no significant difference in the Aδ-fibre evoked responses, the C-fibre responses or wind-up with any dose of 8pSPT. The wind-up responses of cells were inhibited after carrageenan and in untreated animals the wind-up responses were unchanged with 8pSPT, however, this was found not to be significant. The effects of 10μg 8pSPT only was significantly different on the Aβ-fibre evoked responses and with 10 and 100μg on the post-discharge ($P<0.05$). In untreated animals the Aβ-fibre evoked responses were inhibited to 80±10% of control and after carrageenan induced inflammation the Aβ-fibre responses with the same dose of 8pSPT were 118±10% of control. The post-discharge in untreated animals was 99±6 and 102±17% of control with 10 and 100μg 8pSPT, the same doses after carrageenan on the post-discharge gave responses of 58±15 and 48±10% of control. The measures of initial input and final response to a train of stimuli were not significantly different between the two groups of animals.

7.2.5: 8-cyclopentyl-1, 3-dipropylxanthine.

The effects of DPCPX was evaluated three hours post-carrageenan

induced inflammation with 0.5, 5 and 50µg on eight dorsal horn neurones with an average depth of 726±64µm from the surface of the spinal cord.

The vehicle for DPCPX (50% absolute ethanol/50% distilled water) was followed for sixty minutes directly after three hours carrageenan and had no significant effect on any of the measured responses. This was then followed by DPCPX in the same vehicle. None of the doses of DPCPX significantly changed any of the responses. The time-course showing the vehicle and effects of DPCPX can be seen in Figure 7A and B.
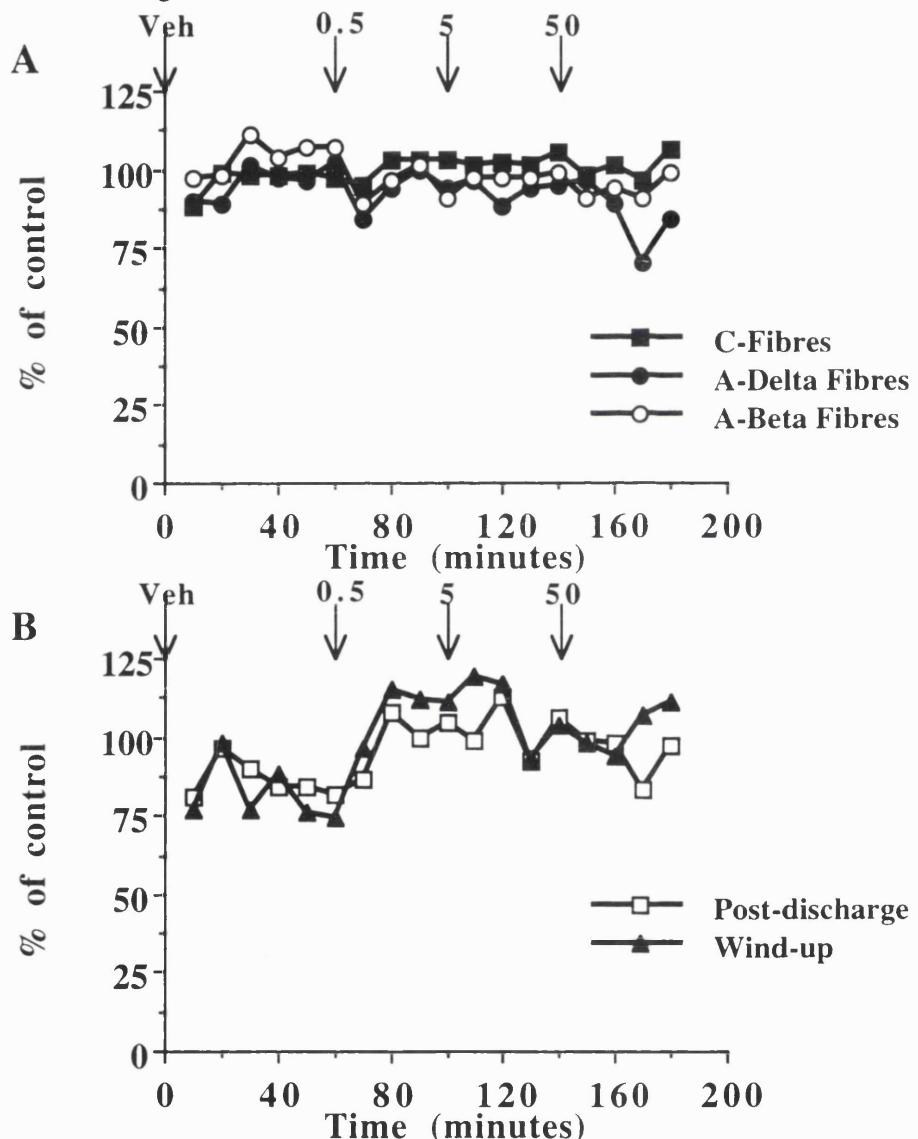
Figure 7. *The effects of the vehicle (50% absolute ethanol/50% distilled water) after three hours carrageenan induced inflammation. The vehicle (Veh) was followed for one hour, this was followed by the antagonist, DPCPX (0.5, 5 and 50µg). (A) The effects on the C-, Aδ- and Aβ-fibre evoked responses. (B) The response of wind-up and post-discharge.*

7.2.6: Comparison of the Effects of DPCPX in Untreated Animals Compared to Animals Three Hours Post Carrageenan.

In comparing the dose response curves (0.5, 5 and 50µg DPCPX) in untreated animals compared to those animals which had received carrageenan induced inflammation there was found to be no significant difference between the two groups of animals with any of the responses evaluated.

7.2.7: GPA-An Adenosine Kinase Inhibitor.

The effects of GPA, an adenosine kinase inhibitor were evaluated three hours after carrageenan induced inflammation on a population of eight dorsal horn neurones with an average depth of $647\pm38\mu m$ from the surface of the spinal cord. GPA was given systemically at doses of 0.01, 0.1, 1 and 10mg/kg.

Figure 8 shows the dose-response relations for GPA after carrageenan induced inflammation on the C-fibre evoked responses, as well as a comparison to untreated animals and the vehicle controls(DMSO, for forty minutes).
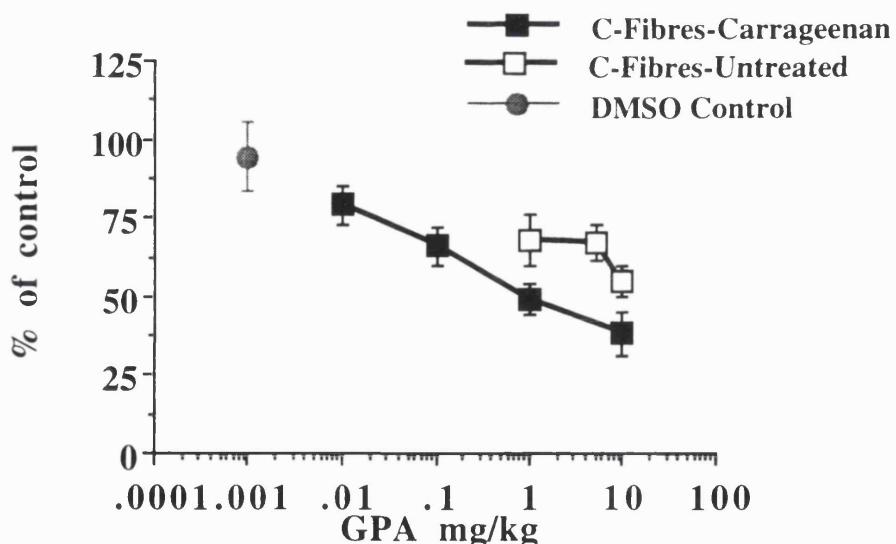
Figure 8. The effects of the kinase inhibitor GPA on the C-fibre evoked responses. The responses in untreated animals, those with no carrageenan induced inflammation are shown, as well as the dose-response relations for GPA after carrageenan. Also shown is the vehicle effect on the C-fibre evoked response.

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The C-fibre evoked responses were inhibited to 79±6, 66±6, 49±5 and 38±7% of control after 0.01, 0.1, 1 and 10mg/kg respectively. The effects of 0.01mg/kg and above gave significant inhibition of the C-fibre evoked responses when compared to carrageenan controls (*P<0.05*). Comparing the effects of GPA after carrageenan to the vehicle controls it was found that doses of 0.1mg/kg and above gave significant inhibition of the C-fibre evoked responses.

The Aδ-fibre evoked responses were also inhibited to 55±11, 43±11, 27±8 and 29±11% of control with 0.01, 0.1, 1 and 10mg/kg respectively. All doses gave significant inhibition when compared to carrageenan controls (*P<0.05*) see Figure 9. When comparing the effects on the Aδ-fibres to the vehicle controls the effects of 0.1mg/kg and above gave significant inhibition (*P<0.05*).
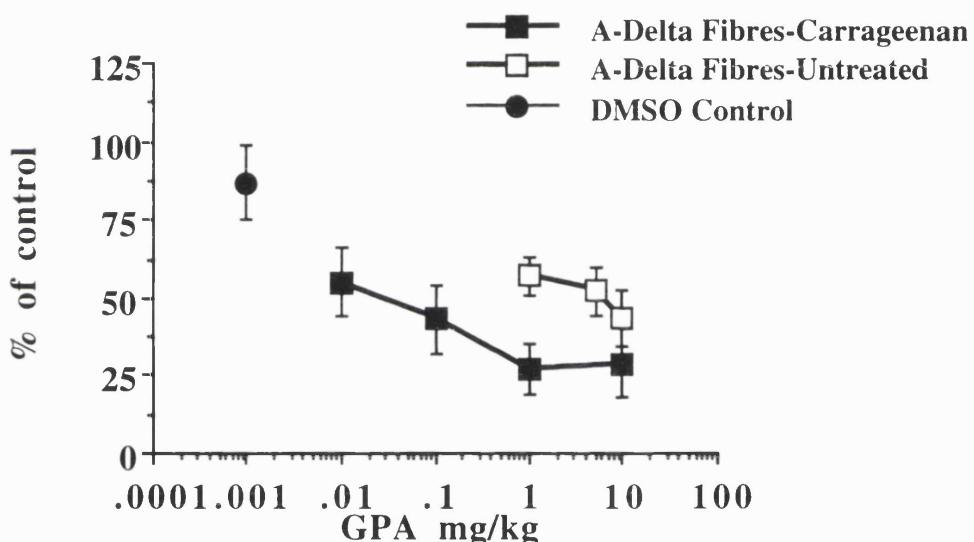
Figure 9. The effects of the kinase inhibitor GPA on the Aδ-fibre evoked responses. The responses in untreated animals, those with no carrageenan induced inflammation are shown, as well as the dose-response relations for GPA after carrageenan. Also shown is the vehicle effect on the Aδ-fibre evoked response.

The Aβ-fibre evoked responses were not significantly changed from control values with 0.01, 0.1, 1 mg/kg but were significantly inhibited to 81±7% of control with 10mg/kg (*P<0.05*) see Figure 10. If compared to the vehicle controls, again the Aβ-fibre evoked responses were not significantly effected with any dose of GPA.

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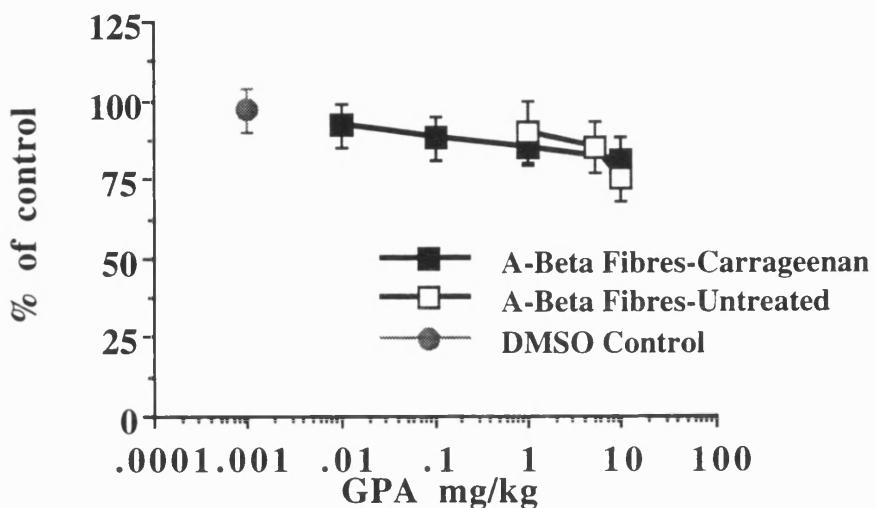
Figure 10. The effects of GPA on the Aβ-fibre evoked responses. There was no significant difference after carrageenan compared to untreated animals.

Both the post-discharge and wind-up were significantly inhibited by all doses of GPA when compared to carrageenan controls (*P<0.05*) see Figures 11 and 12.
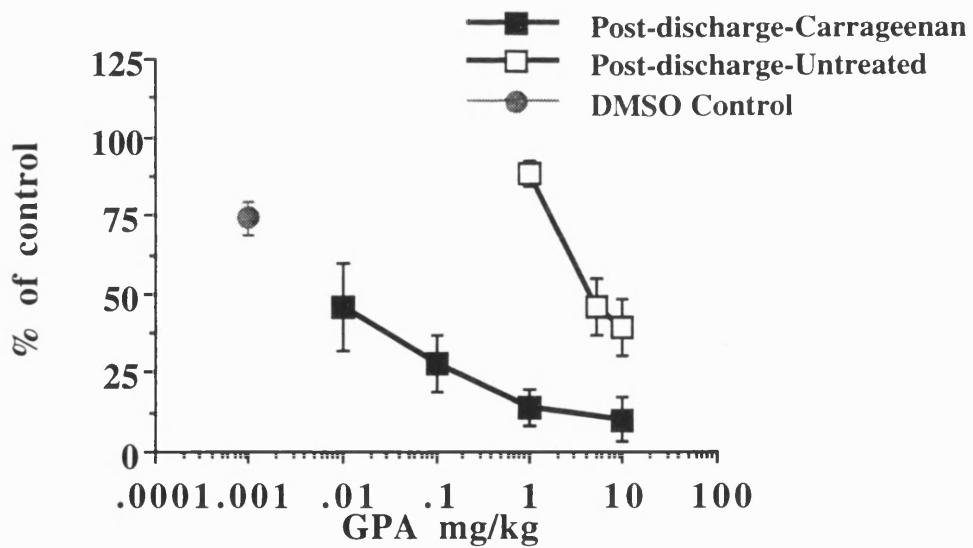
Figure 11. The effects of GPA on the post-discharge. The responses in untreated animals as well as the dose-response relations for GPA after carrageenan. Also shown is the vehicle effect.

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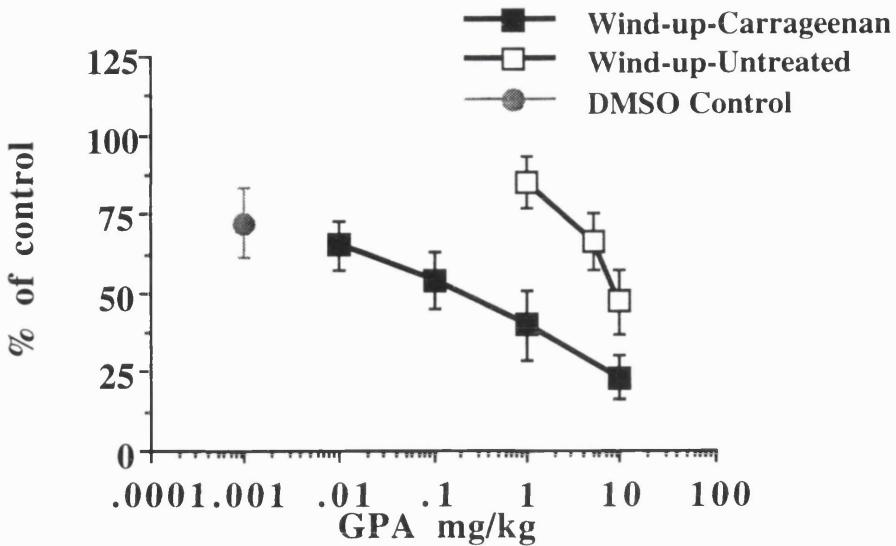
Figure 12. *The effects of GPA on wind-up. The responses in untreated animals as well as the dose-response relations for GPA after carrageenan. Also shown is the vehicle effect.*

The post-discharge was reduced to 46 ± 14, 28 ± 9, 14 ± 6, $10\pm7\%$ of control for 0.01, 0.1, 1 and 10mg/kg GPA respectively. When compared to vehicle controls the effects of 0.1mg/kg and above gave significant inhibition of the post-discharge response.

Wind-up was reduced in a similar manner to the post-discharge responses. The responses of wind-up were 65 ± 8, 54 ± 9, 40 ± 11 and $23\pm7\%$ of control for the 0.01, 0.1, 1 and 10mg/kg respectively. Although this response was significantly inhibited by all doses compared to carrageenan controls, only 10mg/kg was significant when compared to the vehicle effects.

The responses of input and final, overall response to a train of stimuli were also measured. After three hours carrageenan pretreatment the initial input was $168\pm33\%$ of control and the final response to $136\pm34\%$ of control. GPA dose dependently inhibited both the input and final responses (Figure 13). However, only 10mg/kg produced significant inhibition of the input response ($50\pm15\%$ of control) ($P<0.05$). All doses of GPA significantly inhibited the overall, final response to a train of stimuli when compared to carrageenan controls ($P<0.05$). When compared to vehicle controls none of the doses of GPA on the input response had significant effects. The final overall response was significantly inhibited by 1 and 10mg/kg when

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compared to the vehicle controls (*P<0.05*).
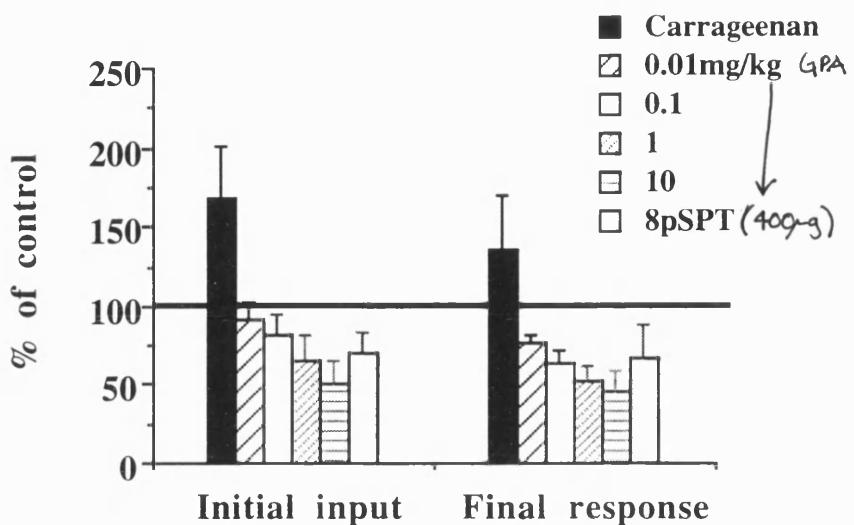
Figure 13. *The responses of initial input and overall final response to a train of stimuli after three hours carrageenan induced inflammation. The carrageenan effects are as a percentage of initial controls. The subsequent doses of GPA (0.01, 0.1, 1 and 10mg/kg) are expressed as percentage of carrageenan effects. The inhibitory effects of GPA are only partially reversed with intrathecally given 8pSPT (400μg).*

The effects of systemic GPA were reversed to a varying extent, with the intrathecal 8pSPT (400μg). The C-fibre evoked responses were 38±7% of control with 10mg/kg GPA, after 8pSPT this was reversed to 65±17% of control. The inhibited Aδ-fibre evoked responses were not reversed by 8pSPT, so that with GPA they were 29±11% and after 8pSPT they were 37±20% of control (Figure 14A). Wind-up was 23±7 and 68±28% of control with GPA and 8pSPT respectively. Post-discharge showed a great deal of variation after 8pSPT, so that some cells were facilitated to well above control levels and some cells were not reversed at all. With 10mg/kg GPA wind-up was 23±7 and after 8pSPT the response was initially 70±62% of control reflecting the vast degree of variability (Figure 14B). Over the forty minutes the effects of 8pSPT began to wear off (see Figure 14A and B).

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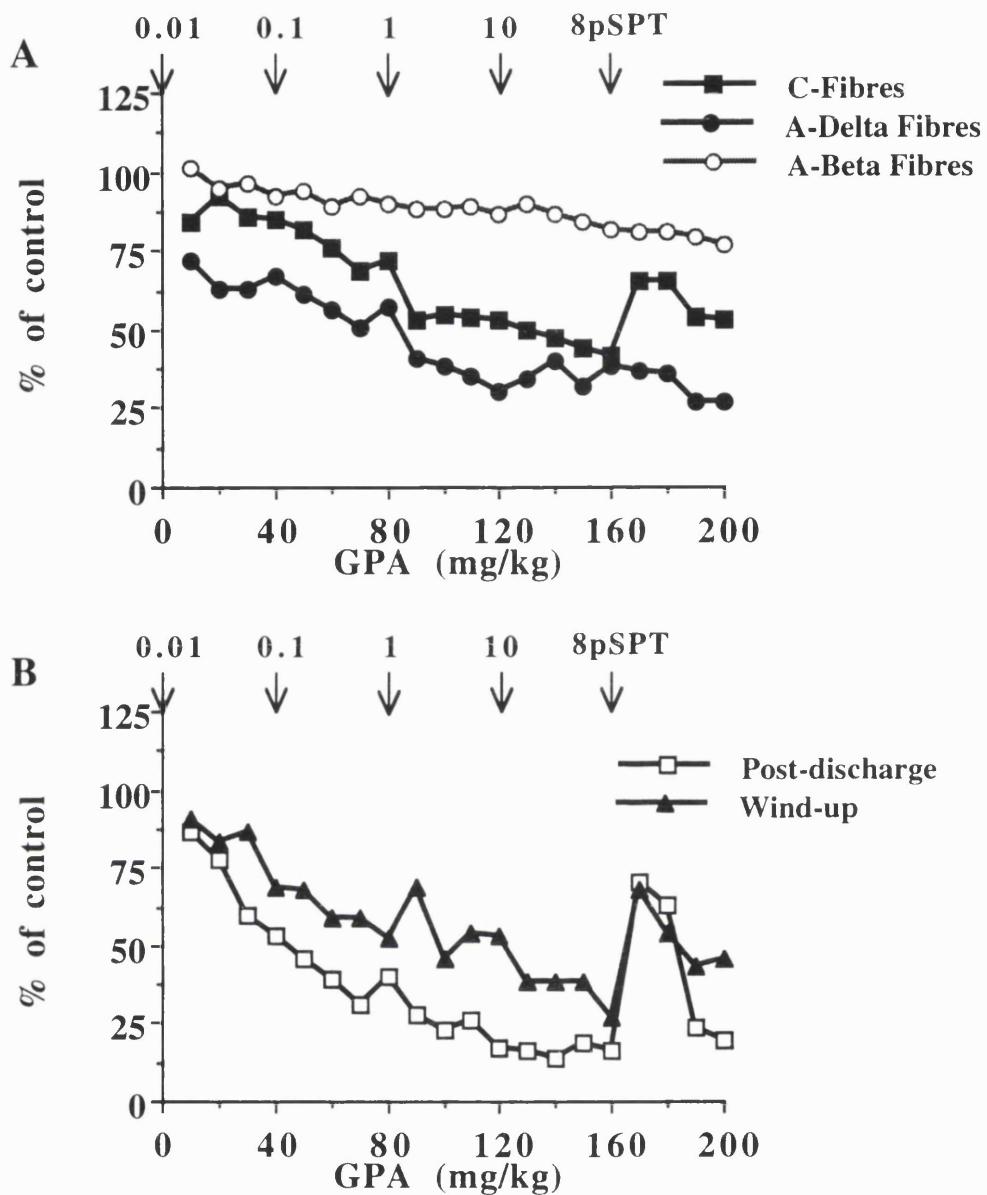
Figure 14. Time-course showing the effects of GPA given systemically at (0.01, 0.1, 1 and 10mg/kg) followed by the intrathecally applied 8pSPT (400μg). (A) The responses evoked by C-, Aδ- and Aβ-fibres. (B) The post-discharge and wind-up. Note that the reversal of these responses was maximal within the first twenty minutes which then wore off.

As the reversal of GPA with 8pSPT was incomplete, the effects of 10mg/kg of GPA was also evaluated after pretreatment with intrathecally applied 8pSPT (400μg), also in the presence of carrageenan induced inflammation, to test whether the effect of the antagonist was greater when given prior to the kinase inhibitor.

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After pretreatment with 8pSPT, GPA inhibited the C-fibre evoked responses to 68±26% of control, whereas GPA alone inhibited these responses to 38±7% of control. The Aδ-fibre evoked responses were 33±6 and 29±11% of control with GPA in the presence of the antagonist and GPA alone respectively. The Aβ-fibre evoked responses were 83±11 and 81±7% of control with the above same combinations. The post-discharge and wind-up responses were 14±7 and 67±36% of control respectively for GPA in the presence of 8pSPT. Alone GPA inhibited these response to 10±7 and 23±7% of control for post-discharge and wind-up respectively.

7.2.8: Comparison of the Effects of GPA in Untreated Animals Compared to Animals Three Hours Post Carrageenan.

In comparing the dose response curves for GPA in untreated animals compared to those animals which had received carrageenan induced inflammation there was found to be a leftward shift in the dose response curve for the C-, Aδ-fibre evoked responses, post-discharge and wind-up (see Figures 8, 9, 11 and 12 above).

The C-fibre evoked responses and post-discharge were only significantly inhibited with 10mg/kg when compared to vehicle controls in untreated animals. However, after carrageenan induced inflammation doses of 0.1mg/kg GPA gave significant inhibition of the C-fibre evoked responses and post-discharge when compared to vehicle effects. The Aδ-fibre evoked responses were significantly inhibited by 1mg/kg in untreated animals whereas after carrageenan, 0.1mg/kg gave significant inhibition of the Aδ-fibre responses. The Aβ-fibre evoked responses did not show any significant effect in untreated animals or in animals post-carrageenan induced inflammation when compared to vehicle controls (Figure 10).

The input responses to a train of stimuli were not significantly effected with any doses of GPA, when compared to vehicles, in either untreated animals or carrageenan treated animals. In untreated animals none of the doses of GPA had a significant effect on the final overall responses when compared to vehicles, whereas after carrageenan 1mg/kg and above significantly inhibited this responses.

7.2.9: GPB-An Adenosine Kinase Inhibitor.

The effects of GPB, an adenosine kinase inhibitor, given intrathecally on a population of eleven dorsal horn neurones with an average depth of 729±66μm.

The doses of GPB were 0.5, 5, 50 and 500μg given after three hours carrageenan induced inflammation.

The C-fibre evoked responses were only significantly inhibited with the top dose of GPB (500μg); the responses were 102±8, 97±9, 102±10 and 77±10% of control after 0.5, 5, 50 and 500μg respectively (see Figure 15A).
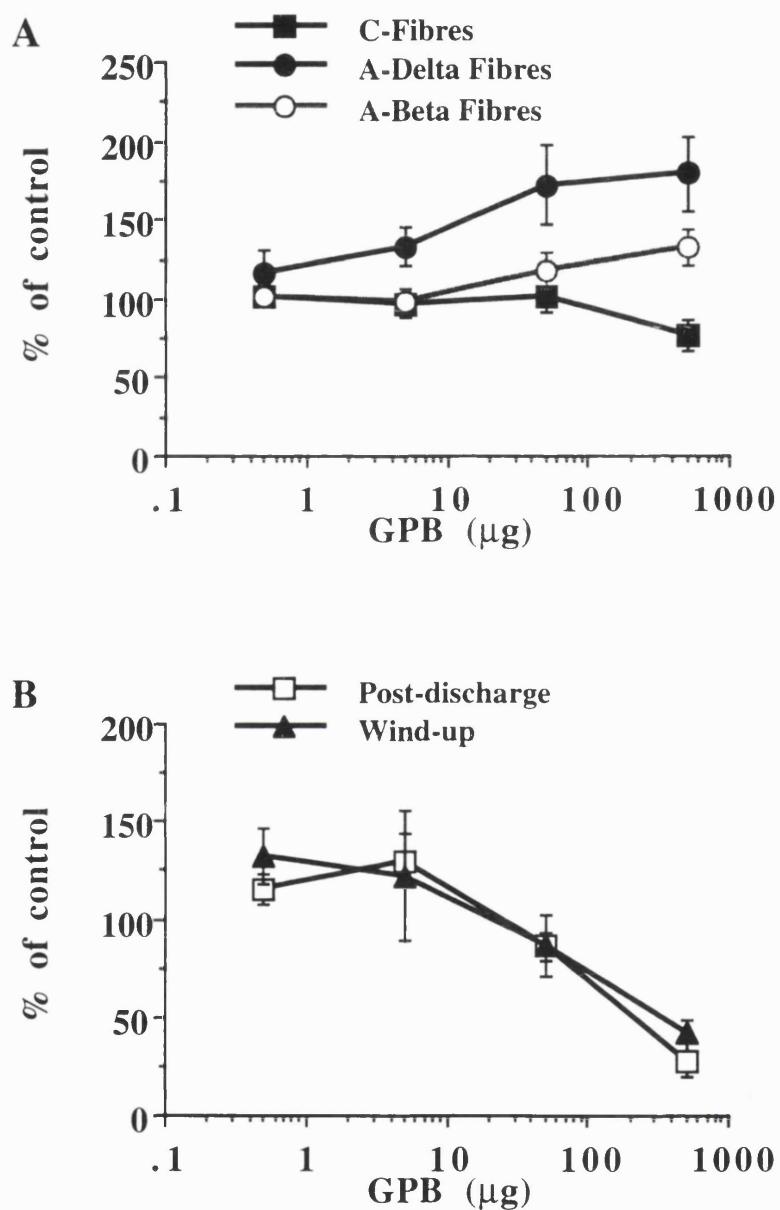
Figure 15. Dose-response relations for GPB after three hours carrageenan induced inflammation. (A) The effects on C-, Aδ- and Aβ-fibre evoked responses. (B) The post-discharge and wind-up responses.

Post-discharge and wind-up were only significantly inhibited by 500µg GPB (*P<0.05*) see Figure 15B. The Aβ-fibre evoked responses were not significantly inhibited by any dose of GPB. The Aδ-fibre responses were facilitated with GPB, with 50 and 500µg giving significant responses. The Aδ-fibre responses were 116±14, 133±12, 172±25 and 179±24% of control with 0.5, 5, 50 and 500µg respectively (see Figure 15A).

The effects of 500µg GPB were attenuated to varying extents with 400µg 8pSPT. The C-fibre evoked responses were ~slightly attenuated~ from 77±10 to 87±18% of control with 400µg 8pSPT (see Figure 16 for time course and reversal). The Aδ-fibre evoked responses which were facilitated (179±24%) were brought back to and below control values with 8pSPT (Figure 16). The Aβ-fibre evoked responses were not significantly effected by GPB or by 8pSPT. Post-discharge was not reversed with 8pSPT. The effects of 8pSPT could not be evaluated on the wind-up responses of these cells since after 8pSPT the cells would wind down. Instead the initial response and final response to the train of stimuli were evaluated (Figure 17).
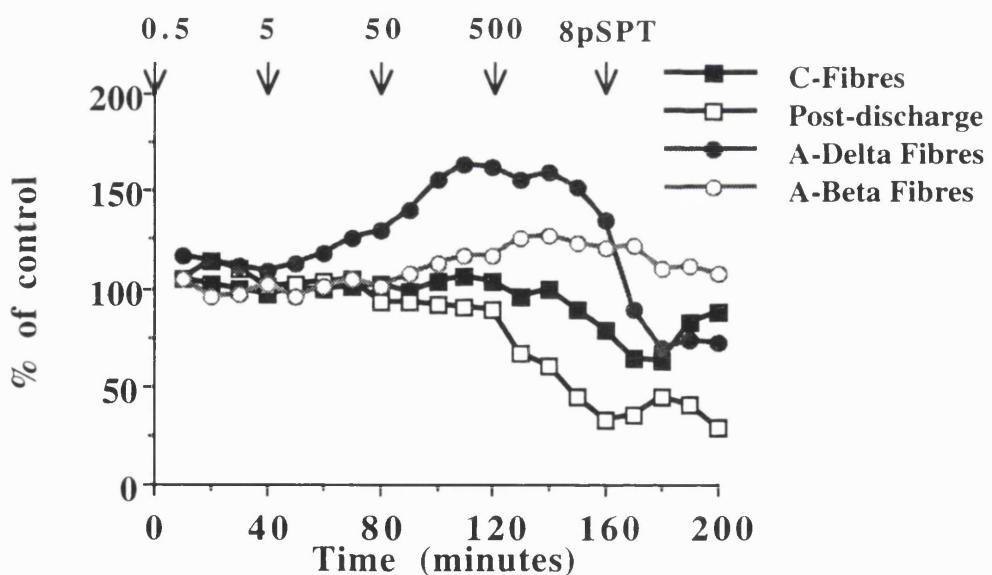
Figure 16. *Time-course of the effects of GPB (0.5, 5, 50 and 500µg) after carrageenan induced inflammation, as well as the reversal with 8pSPT (400µg). Application is indicated by the arrows.*

The input onto the cell of the latency corresponding to C-fibres, was significantly inhibited by all doses of GPB, (*P<0.05*) but inhibition only occurred to any extent with 500µg GPB. The input responses were 85±3, 86±6, 86±7 and 42±6% of control with 0.5, 5, 50 and 500µg

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respectively (see Figure 17). The final overall response to the same train of stimuli was significantly inhibited by 500μg GPB only. The antagonist, 8pSPT after GPB (500μg) gave a response of 63±11 and 69±14 for the initial input and overall response to a train of stimuli.
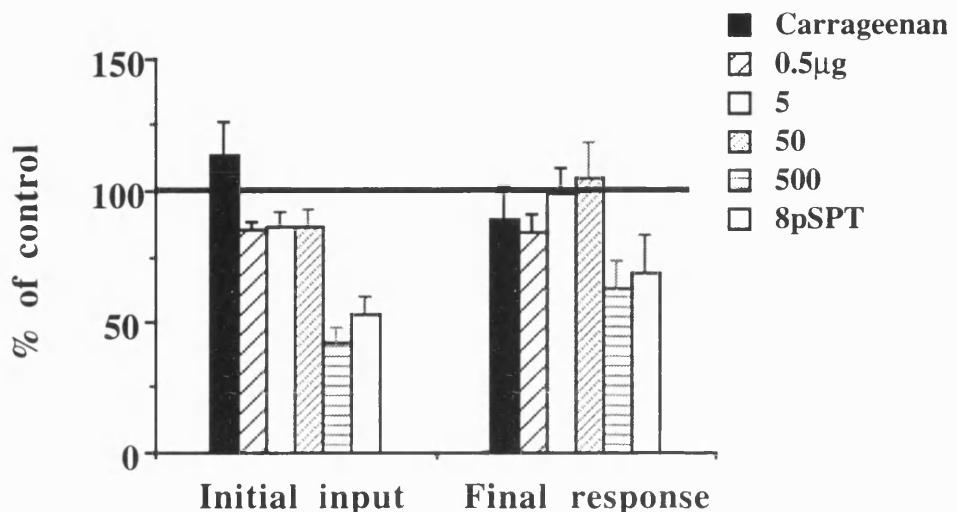
Figure 17. The effects of three hours carrageenan induced inflammation on the initial input and final overall response to a train of stimuli. The carrageenan effects are as a percentage of initial controls. The subsequent doses of GPB (0.5, 5, 50 and 500μg) are expressed as percentage of carrageenan effects. The effects of 8pSPT are also shown on these responses.

7.2.10: Comparison of the Effects of GPB in Untreated Animals Compared to Animals Three Hours Post Carrageenan.

The effects of GPB were compared before and after carrageenan induced inflammation. The only significant difference between the two groups of animals was with the effect on the Aδ-fibre evoked responses and input responses. In untreated animals although the lower doses of GPB tended to facilitate the Aδ-fibre evoked responses the only significant effect was with 500μg, which was to inhibit these responses (see Chapter 4.2.10). After carrageenan induced inflammation all doses of GPB tended to facilitate the Aδ-fibre evoked responses (see Figure 15A this Chapter) see Figure 18.

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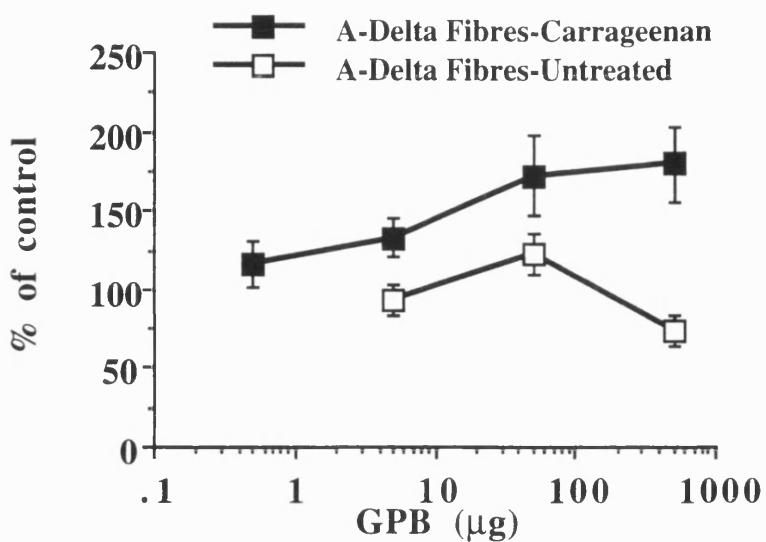
Figure 18. *The effect of GPB on the Aδ-fibre evoked responses in normal animals and animals after carrageenan induced inflammation.*

The input responses to a train of stimuli were significantly different with 5 and 50μg GPB only. In untreated animals these doses tended to facilitate the input response, whereas after carrageenan induced inflammation the inputs were inhibited, albeit to only a small extent, which was significantly different to the untreated animals (*P<0.05*).

7.3: Discussion.

These results suggest that after inflammation, following the peripheral administration of carrageenan into the plantar region of the paw, the activation of the A_1-receptor is still as effective in controlling noxious inputs into the dorsal horn of the spinal cord at the spinal cord level as compared to normal animals. Thus there is no change in the receptor such as down-regulation after carrageenan. The antagonist studies could not be interpreted in terms of a simple role of endogenous adenosine and were also hampered by solubility problems. Thus protection of endogenous adenosine at the spinal cord level was employed to asses the role of adenosine. The results with the spinally applied adenosine kinase inhibitor were consistent with release of adenosine three hours after carrageenan induced inflammation and showed that when adenosine is protected the end result is nociception. However, there is no increase in the levels of adenosine in the spinal cord since the effects of the spinally administered inhibitor were identical to those seen in normal animals. The effects of the systemically administered kinase inhibitor would be consistent with the idea that after inflammation there is another site of adenosine release which results in enhanced antinociception as this compound was much more effective than in normal animals.

The administration of CPA significantly inhibited the C-fibre evoked responses, post-discharge and wind-up. Only with the lowest dose of CPA (0.5µg) was there any difference in effect after carrageenan. Thus, the C-fibre evoked responses and post-discharge were facilitated in animals with inflammation, whereas in normal animals this dose tended to inhibit these responses. One possible explanation is that endogenous adenosine which may be released in response to inflammation is competing with CPA. As the doses of CPA are increased this is overcome, since CPA has a greater affinity for the A_1-receptor than adenosine itself.

The action of the antagonists 8pSPT and DPCPX were not easy to explain. It was postulated before the experiments that if adenosine was being released in response to inflammation then the cells responses may go down during the three hours pretreatment and become enhanced after antagonist application. However, there was no obvious inhibitory trend in the cell responses after inflammation. It has been shown that cells that are not very excitable before inflammation have enhanced responses as the inflammation develops and cells which are excitable before inflammation have reduced responses after carrageenan induced inflammation (Stanfa et al. 1992). Obviously the response of cells to an inflammatory challenge is not simple and clear since the

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response of a cell to peripheral inflammation appears dependent on the level of excitability of the cell before the inflammation is induced.

If adenosine was being released in response to inflammation at the spinal cord level then the antagonists may facilitate the responses of dorsal horn neurones, however, the effects of the antagonists were not this clear cut. Firstly, most of the evoked responses were not significantly changed with 8pSPT after carrageenan induced inflammation, as observed with the acute electrically evoked responses.

There also appeared to be two population of cell responses with 8pSPT. One major group showed inhibition of wind-up and post-discharge responses whilst in a much smaller population these responses were facilitated. The differences in these responses could not be explained by the cells excitability before carrageenan induced inflammation or any difference in their depth.

The inhibition of wind-up and post-discharge is hard to explain. As an A_1-receptor agonist under normal conditions and indeed after inflammation inhibits these responses, to an equal extent between the two groups of animals, you would not expect an antagonist of the same receptor to also have inhibitory effects. It has been reported that caffeine has analgesic properties (Sawynok and Yaksh 1993). However, given the increasing knowledge of purinopharmacology, especially at the spinal cord level, the analgesic effect of caffeine can not be a matter of simple receptor antagonism but may result from more complex circuitry effects. It has been shown that caffeine, when given with an adenosine A_1-agonist, in the presence of an non-steroidal anti-inflammatory (NSAID) after formalin induced inflammation, resulted in enhanced inhibition of nociceptive responses (Malmberg and Yaksh 1993a). However, when given with the NSAID alone it did not enhance the antinociception. What appeared to be critical was the presence of an A_1-agonist as well (Malmberg and Yaksh 1993a). When given orally, caffeine can enhance the action of other analgesic drugs by increasing their uptake from the stomach and also decreasing first pass metabolism (Sawynok and Yaksh 1993). Adenosine in the periphery can also be pronociceptive, since it causes degranulation from mast cells and can directly activate the peripheral terminals of afferents via the A_2-receptor (Taiwo and Levine 1990). Thus, an adenosine antagonist may be antinociceptive in the periphery by reducing the above events. However, this does not explain the spinal inhibitory effect of 8pSPT, or the selective inhibition of the post-discharge and wind-up responses.

It could be that 8pSPT is antagonizing the effects of an excitatory A_2-receptor, since it can also have affinity for this receptor (Fredholm et al. 1994). However, as I have shown in Section 4.2.5, an A_2-receptor agonist actually reduced the enhanced

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excitability of dorsal horn neurones, albeit to a lesser extent than an A_1-receptor agonist.

Studies with the more specific A_1-receptor antagonist did not show any significant effect on any of the evoked responses after carrageenan induced inflammation. However, studies with DPCPX were problematic due to its poor solubility and hence associated vehicle problems. Although the vehicle was not shown to have any significant effect after carrageenan induced inflammation the vehicle effects were different when compared to acute studies. Due to these problems with the antagonist studies, the use of adenosine kinase inhibitors were employed to see if protecting endogenously released adenosine may shed more light on the release and role of adenosine during carrageenan evoked changes.

The effects of the intrathecally applied kinase inhibitor, GPB showed no significant change in degree of inhibition of dorsal horn cells after carrageenan induced inflammation compared to animals with no inflammation. Since from the formalin studies (Chapter 6) and in acute studies (Chapter 4) were consistent with a release of adenosine by NMDA-receptor activation, it was expected that GPB may have enhanced effects after carrageenan. However, the results with the intrathecally applied kinase inhibitor suggests that either adenosine levels are no higher in the cord after peripheral inflammation or that it can not be protected further by this kinase inhibitor. Interestingly, despite claims that peripheral inflammation results in enhanced central NMDA-receptor activity (Ren et al. 1992) in this model we have no evidence for any overall increase in NMDA dependent responses (Stanfa et al 1994). It is also possible that other mechanisms of metabolism take over from the kinase pathway, since this enzyme is active in the low micromolar range (Santos et al. 1968); as the concentration of adenosine increases this enzyme is saturated and adenosine deaminase takes over. It is possible that adenosine deaminase activity may prevent further actions of adenosine at the spinal cord level, where this enzyme is known to be present (Geiger and Nagy 1986; Nagy et al. 1984; Nagy and Daddona 1985).

However, when GPA, another adenosine kinase inhibitor was given systemically there was a clear leftward shift in the dose-response curve for GPA after carrageenan induced inflammation. The C-fibre evoked responses, Aδ-fibre evoked responses, wind-up and post-discharge responses showed inhibition with GPA at lower doses compared to animals with no inflammation. The C-fibre evoked responses and post-discharge responses were significantly inhibited by a hundred fold lower dose than untreated animals. The Aδ-fibre evoked responses had significant inhibitory effects with GPA at a ten fold lower dose than untreated animals. The effects of GPA on the wind-up responses of cells were only significant

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after carrageenan inflammation. Protecting adenosine had no effect on the $A\beta$-fibre evoked responses in either groups of animals whether the kinase inhibitors were given systemically or intrathecally. This would indicating that protecting endogenous adenosine acts only to inhibit noxious responses, making it an potential analgesic target.

Since the results with intrathecal GPB suggest that there is no enhanced release of adenosine at the spinal cord level, the resulting increase in antinociception, seen when kinase inhibitor was given systemically, may result from increased release of adenosine either in the periphery or at a supraspinal site. The exact site can not be deduced from these experiments. In the periphery it is known that adenosine is a component of inflammation and has a direct effect on the peripheral terminals of the primary afferents, lowering nociceptive thresholds via activation of the A_2-receptor (Taiwo and Levine 1990). This receptor and the A_3-receptor can also cause mast cells to degranulate, thus adding to the inflammatory "soup". It is known in situations of hypoxia and ischemia that adenosine is produced in large amounts and is a component of cardiac pain experienced during such ischaemic episodes. However, there is also evidence to suggest that adenosine in humans is also analgesic in these events (Sylven et al. 1996). Biphasic responses may result from variations in the absolute levels of adenosine produced. High levels of adenosine must be produced to activate the A_2 and A_3-receptors receptors (Fredholm et al. 1994). If lower levels of adenosine are produced then the A_1-receptor will be activated and the excitatory receptors spared. Thus, when the injury/inflammation/hypoxia is acute, the low levels of adenosine released will inhibit the pain transmitting fibres and dampen down the noxious signal. However, if the same injury states are of a greater intensity then the increased levels of adenosine that are released will activate the excitatory receptors. After inflammation the levels of adenosine could be in the range that activates the A_1-receptors, thus inhibiting afferent drive by hyperpolarizing the afferent fibres.

Another site may a be supraspinal one. Here it would seem more probable that the activation of excitatory receptors (the A_2, since to date the A_3 has not found in the brain of the rat) (Linden 1994) may activate descending inhibitory controls. Thus the resulting enhanced antinociception seen with the peripheral kinase inhibitor may still be a spinal event, but activated from an additional site. However, from these studies we can not ascertain the exact site.

In summary these results clearly demonstrate that the A_1-receptor, post carrageenan induced peripheral inflammation, can still produce antinociception to the same extent as in an animal without any inflammation. Since the dose-response curves to CPA are identical. The role of endogenous adenosine at the spinal cord level from the kinase inhibitor studies show that there may be a

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release of adenosine in the spinal cord after peripheral inflammation but this is no greater than in normal animals. These results are consistent with the hypothesis that a novel site(s) of action of adenosine, either peripheral or supraspinal may be revealed after inflammation.

Chapter 8.

Neuronal responses after bicuculline-
methobromide: modulation by N^6-
cyclopentyladenosine, 7-chlorokynurenate and
morphine.

8.1.1: Introduction.

It has been established that the inhibitory amino acids, γ-aminobutyric acid (GABA) and glycine play an important role within the dorsal horn of the spinal cord in a variety of species (Basbaum 1988; Bohlhalter et al. 1994; Game and Lodge 1975; Van den Pol and Gorcs 1988).

Somatosensory inputs into the dorsal horn, the first site of relay of afferent information, have been shown to modulate other subsequent inputs. This was first demonstrated by Melzack and Wall (1965) who found that noxious inputs could be attenuated by low-threshold non-noxious inputs. In this way it was postulated that the relay of somatosensory inputs was not a fixed event and that it could undergo a degree of modulation at the spinal cord level. This was known as "the Gate Theory".

Stimulation of afferent fibres (Curtis et al. 1968; Duggan et al. 1981; Game and Lodge 1975) and descending pathways (McGowan and Hammond 1993) have since been shown to inhibit activity of neurones within the spinal cord (Cullheim and Kellerth 1981; see Curtis 1969; Duggan et al. 1981; Game and Lodge, 1975; Rudomin et al. 1990; Schneider and Fyffe 1992; Yoshimura and Nishi 1995). Inhibitions produced by some of these means have been postulated to arise from activation of intrinsic islet cells in lamina II (Todd and McKenzie 1989). The inhibitions produced by afferent stimulation have been demonstrated to be both strychnine and bicuculline sensitive (Duggan et al. 1981; Game and Lodge 1975). Strychnine is an antagonist of the glycine-receptor and bicuculline an antagonist of the picrotoxin -sensitive $GABA_A$-receptor.

Both GABA and glycine are located within various intrinsic cells in the dorsal horn (Aprison and Werman 1965; Magoul et al. 1987; Powell and Todd 1992; Spike and Todd 1992; Todd 1990, 1991; Todd and McKenzie 1989; Todd and Sullivan 1990; Todd and Spike 1993; Todd et al. 1991, 1996). Glutamic acid decarboxylase (GAD), the enzyme responsible for the synthesis of GABA has also been shown to be located in intrinsic cells within the dorsal horn (Barber et al. 1978; Hunt et al. 1981; McLaughlin et al. 1975).

GABA can have inhibitory effects at both pre-and post-synaptic levels. Immunocytochemical studies have demonstrated axo-axonal synapses with primary afferent terminals (Alvarez et al. 1992; Barber et al. 1978 ; Bernardi et al. 1995; Hunt et al. 1981; Magoul et al. 1987; Spike and Todd 1992) implicating an inhibitory role onto the pre-synaptic terminals. Axo-dendritic synapses, implying a post-synaptic inhibition have also been shown (Alvarez et al. 1992; Baba et al. 1994; Magoul et al.

1987; Powell and Todd 1992). As well as immunocytochemical studies for the transmitter itself the receptors for GABA; $GABA_A$ and $GABA_B$ have also been shown to be located at both a pre- and post-synaptic level (Bowery et al. 1987; Desarmenien et al. 1984; Price et al. 1987).

The location of these inhibitory neurotransmitters and their receptors make them ideal candidates for the attenuation of afferent inputs and neuronal responses at the spinal cord level.

8.1.2: Allodynia.

Allodynia, defined as states where non-noxious stimuli give rise to feelings of pain may result from the loss of tonic inhibitions within the dorsal horn of the spinal cord resulting in miscoding of somatosensory inputs.

Intrinsic neurones, containing both GABA and glycine may be responsible for this, since, experimental studies in animals have shown that loss of these controls can result in changes resulting in the phenomenon that may represent allodynia in humans (Hao 1992a, b; Roberts et al. 1986; Woolf 1981; Yaksh 1989). The most important observation is that spinally applied bicuculline in awake animals results in agitation, vocalization and cardiovascular changes, indicative of allodynia (Roberts et al. 1986; Yaksh 1989).

Since spinal controls exerted by inhibitory neurotransmitter systems usually result from the activation of low threshold primary afferent input (Melzack and Wall 1965; Roberts et al. 1986; Todd et al. 1991; Yaksh 1989) it is possible to speculate that the loss of these controls now causes a hyperesthesia due to the central miscoding of afferent inputs. Since both GABA and glycine stabilize the membrane potential to near resting potential (Desarmenien et al. 1984) loss of these controls will result in the ability of primary afferent terminals and second order neurones to reach their threshold potentials more readily. Thus, the release of excitatory neurotransmitter from primary afferents is more probable. This increase in available excitatory neurotransmitters may result in increased recruitment of inputs onto convergent neurones, thus producing an enhanced response to stimulation (Game and Lodge 1975; Hao 1992a, b).

In experimental studies spinal levels of GABA have been shown to decrease after spinal cord injury (Demediuk et al. 1989; Martiniak et al. 1991; Zhang et al. 1994) and after peripheral nerve injury (Castro-Lopes et al. 1993). Since patients with these types of pathology have reported changes in their sensory perception, especially pain to a variety of non-noxious stimuli the role of GABA as a tonic and ongoing inhibitory system is of great interest in the phenomenon of

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allodynia.

8.1.3: The Role of Adenosine in the Control of Allodynia.

Adenosine, has been established as an important modulator of noxious inputs at the spinal cord level (Ahlijanian and Takemori 1985; DeLander and Hopkins 1986; Doi et al. 1987; Herrick-Davis et al. 1989; Holmgren et al. 1983, 1986; Karlsten et al. 1990; Malmberg and Yaksh 1993a; Post 1984; Reeve and Dickenson 1994; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Vapaatalo et al. 1975; Yarbrough and McGuffin-Clineschmidt 1981) in both behavioural and electrophysiological studies. Indeed activation of primary afferent terminals, evoked by vibration and activation of Pancinian corpuscules in the periphery, results in these low threshold inputs directly inhibiting their own transmission at a second-order neuronal level. This is mediated by the release of ATP causing a post-synaptic EPSP followed by the conversion of ATP to adenosine and a subsequent IPSP of the same neurone due to the effects of adenosine (Fyffe and Perl 1984; Hongo et al. 1968; Salter and Henry 1985, 1987,1989: Salter et al. 1993).

In human studies with patients with neuropathic pain and associated allodynia, adenosine has been shown to be a useful analgesic in controlling the allodynia (Belfrage et al. 1995; Karlsten and Gordh 1995; Sergerdahl et al. 1995; Sollevi et al. 1995, 1996). However, the mechanism of action of adenosine in reducing these pain symptoms is not altogether clear, since surprisingly a single infusion of adenosine gives relief of the allodynia lasting from between 4 hours to 48 hours (Sollevi et al. 1995). The interpretation of this is hard to reconcile with the half life of adenosine being a few seconds. Clinical studies so far have been few and more data is required to establish how effective adenosine is as a treatment of allodynia.

It is possible to propose a mechanism of action of adenosine which has parallels with other studies in the CNS, demonstrating that adenosine is released per se and also broken down from the release of ATP. Release appears to be due to activation of the NMDA- and AMPA-receptors and adenosine then acts as a negative feedback mechanism to dampen down further NMDA evoked activity (Craig and White 1992, 1993; Manzoni et al. 1994; Reeve and Dickenson 1995). NMDA-receptor antagonists have been shown to alleviate allodynia in both humans and animal models (Persson et al. 1995; Sherman and Loomis 1994; Sosnowski and Yaksh 1989; Tal and Bennett 1994; Yamamoto and Yaksh 1993) and can decrease "wind-up like" phenomenon (Kristensen et al. 1992) it is possible therefore that activation of this receptor may be responsible for the miscoding of low-threshold

somatosensory information. It is plausible that adenosine acts to control the increased excitation by regulating excitatory transmission at both pre and post-synaptic levels (Li and Perl 1994; Reeve and Dickenson 1995). Since adenosine may provide an inhibitory threshold which has to be overcome before transmission can maximally proceed again, the differences in duration of action of adenosine, seen in the clinic may be due to the differing levels of altered central excitability of each patient.

For these reasons the effects of the adenosine A_1-receptor agonist, N^6-cyclopentyladenosine was gauged on the hyper-responsiveness of dorsal horn neurones after pretreatment with bicuculline.

8.1.4: The Role of the NMDA-Receptor in Allodynia.

It has long been established that the NMDA-receptor is important in wind-up, the amplification of a response evoked by a constant peripheral stimulus (Davies and Lodge 1987; Dickenson and Sullivan 1987a). A role of the receptor in more persistent and chronic pain states (see Dickenson 1994a; Dubner and Ruda 1992; McMahon et al. 1993; Price et al. 1994) has also been proposed on the basis of the ability of the NMDA-receptor to enhance nociception. Allodynia is often associated with neuropathic pain (see Bennett 1994) and neuropathic pain can be controlled by NMDA-receptor antagonists (Eide et al 1994; Kristensen et al. 1992).

An enhanced release of excitatory amino acid neurotransmitters from pre-synaptic terminals due to the loss of control of the pre-synaptic terminals by GABA is plausible given the anatomical location of the receptors and the axo-axonal connections made by islet cells containing GABA (Alvarez et al. 1992; Barber et al. 1978 ; Bernardi et al. 1995; Bowery et al. 1987; Desarmenien et al. 1984; Hunt et al. 1981; Magoul et al. 1987; Spike and Todd 1992). The loss of controls acting on presynaptic terminals could increase the release of excitatory neurotransmitters and increase recruitment of the NMDA-receptor (Ma and Woolf 1995; Yaksh 1989) as well as inducing neuronal damage (Sugimoto et al. 1990). Glutamate receptor antagonists decrease hyperesthesia and hyperalgesia (Backonja et al. 1994; Tal and Bennett 1994; Yamamoto and Yaksh 1993), allodynia in humans and in animal models (Backonja et al. 1994; Eide et al 1994; Persson et al. 1995; Sherman and Loomis 1994; Yaksh 1989) and decrease 'wind-up like" phenomenon in humans with neuropathic pain (Eide et al 1994; Kristensen et al. 1992).

Given the implication for this receptor in both central hyper-excitability and maintenance of more prolonged pain states (Backonja et al. 1994; see Dickenson 1994a; Dubner and Ruda 1992; Eide et al 1994; Kristensen et al. 1992; McMahon et

al. 1993; Persson et al. 1995; Price et al. 1994; Sherman and Loomis 1994; Tal and Bennett 1994; Yaksh 1989; Yamamoto and Yaksh 1993) and since previous studies have shown that the NMDA-receptor antagonist at the glycine site, 7-chlorokynurenate is a useful tool for the study of the NMDA-receptor (Chapman and Dickenson 1992; Dickenson and Aydar 1991) the effects of 7-CK were observed after pretreatment with spinally applied bicuculline.

8.1.5: Morphine in the Control of Allodynia.

It has also long been established that activation of opioid-receptors within the spinal cord and at supraspinal sites is a widely used analgesic strategy (see Dickenson 1991, 1994b; Yaksh 1993). However, there is still much confusion on the use of opioids in more persistent pain states, such as neuropathically driven pain with accompanying allodynia (Arner and Meyerson 1988; Jadad et al. 1992; Lee et al. 1994; Lee et al. 1995; Portenoy et al. 1990; Yaksh 1989). Neuropathic pains are less responsive than other pains to opioids but patients are not "opioid unresponsive" (Jadad et al. 1992). Conversely it is interesting to note that high doses of morphine have been shown to cause symptoms usually attributed to allodynia (Woolf 1981; Yaksh and Harty 1988). Since opioids are not as effective in treating wind-up and events associated with the activation of the NMDA-receptor (see Dickenson 1994a) and it is established that the NMDA-receptor has a role in allodynia (Backonja et al. 1994; Eide et al 1994; Ma and Woolf 1995; Persson et al. 1995; Sherman and Loomis 1994; Yaksh 1989) the effects of morphine, the mu-opioid-receptor agonist, are also observed after bicuculline pretreatment. Although opioids have been shown to exert a degree of control in neuropathic and allodynic pain states (Desmeules et al. 1993; Jadad et al. 1992) it may be at doses that also give unsatisfactory side-effects.

8.2.1: Dose-Dependent Effects of Bicuculline-Methobromide on Neuronal Activity.

The effects of intrathecally applied 0.5, 5, 50 and 250µg bicuculline was studied on the electrically evoked responses of deep dorsal horn neurones to C-, Aδ-, Aβ-fibre stimulation, including the post discharge and wind-up of the neurones. The effects of bicuculline on the responses to prod and brush were also measured.

The effects of the doses of 0.5 and 250µg were observed on nine cells with an average depth of 812±55µm from the surface of the spinal cord. The effects of 5µg was tested on twenty cells with an average depth of 826±46µm and 50µg (used in the subsequent studies on the effects of CPA, 7-CK and morphine) was observed on fifty two cells with an average depth of 811±28µm.

The C-fibre evoked responses were facilitated in a dose dependent manner, so that the responses were 90±7, 104±7, 114±7 and 166±44% of control for 0.5, 5, 50 and 250µg respectively, with the effects of 50µg and above being significant ($P<0.05$) (Figure 1A).
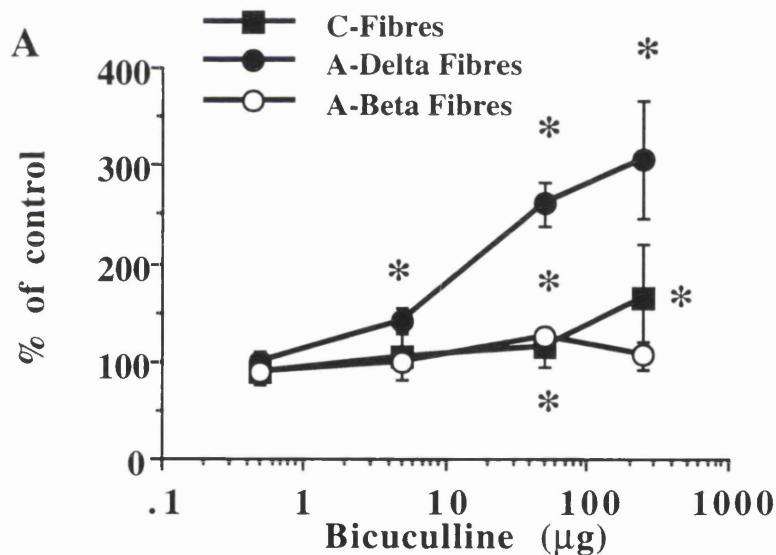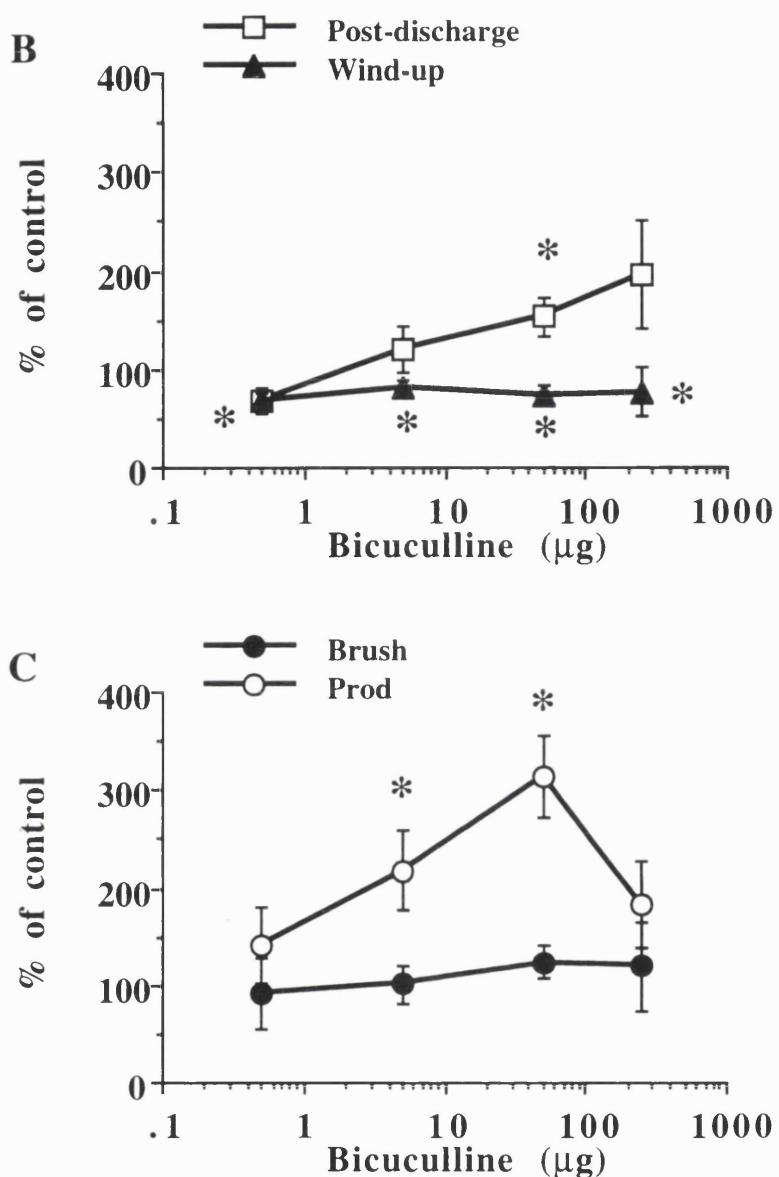
Figure 1. *Dose response relationships for bicuculline (0.5, 5, 50 and 250µg) on the mean maximal response of a population of dorsal horn neurones. (A) The responses to electrical stimulation of the Aβ-, Aδ- and C-fibres. (B) Post-discharge and wind-up. (C) The responses to natural stimulation, brush and prod on the same population of neurones. *P≤0.05. The n values range from 9-52.*

The Aδ-fibre evoked responses were also facilitated in a dose dependent manner with the effects of 5µg and above being significant. The degree of facilitation was far greater for these evoked responses than the C-fibre evoked responses. The doses of 0.5, 5, 50 and 250µg bicuculline produced Aδ-fibre evoked responses of

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99.7±10, 142±12, 261±22 and 305±60 % of controls respectively ($P<0.05$).

The responses to brush showed no significant change from controls with any of the doses of bicuculline (Figure 1C). Although all neurones responded to pinch and prod, often the same cells did not respond to brush and this is reflected by the lower numbers for this part of the study. Here 0.5μg bicuculline was tested on only 3 cells. The response to brush was 91±36% of control after this dose. Increasing the doses to 5μg and above gave responses to brush of 101±20 (n=8), 123±17 (n=17) and 120±46% (n=5) respectively.

Vigorous responses to innocuous prod on the other hand were always observed for all the neurones in the study. The responses to prod were facilitated by all doses of bicuculline, although with the highest dose of 250μg, the responses showed less facilitation than with 5 and 50μg bicuculline. The responses after bicuculline (0.5, 5, 50 and 250μg) were 142±39, 218±40, 314±42 and 151±47 % of control respectively but only the effects of 5 and 50μg bicuculline were significant ($P<0.05$). The Aβ-fibre responses showed little change from control measures after bicuculline so that 0.5, 5, 50 and 250μg produced responses of 90±8, 100±6, 126±6, 106±14 5% of control respectively, with only the effects of 50μg bicuculline being significant (Figure 1A). However, stimulation at three times Aβ-fibre evoked threshold after doses of 5μg bicuculline and above, caused firing at latencies after the main Aβ-fibre response, falling primarily in the Aδ range. These additional responses were variable from cell to cell but no cell ever exhibited any consistent firing in this range before bicuculline with Aβ-strength stimulation. This can be seen in the examples of post stimulus histograms in Chapter 3 (Figure 10A, B). Figure 10A is the response of a single neurone to Aβ-fibre suprathreshold stimulation. There is no firing after the Aβ-fibre range of 0-20ms. However, after application of 50μg bicuculline firing occurred in later latencies, especially in the latency of 20-90ms which is usually associated with the Aδ-fibre evoked activity. After bicuculline, responses of between 20 and 200 action potentials per trial were observed in the latency band 20-90ms and these increases were dose-dependent. As well as the changes of firing patterns associated with Aβ-fibre stimulation changes also occurred at C-fibre suprathreshold stimulation. Usually, in control responses the banding between the fibre types can be clearly observe (see Chapter 3, Figure 11A). However, after bicuculline the distinctions between the fibres was lost (Figure 11B). This example is with 50μg bicuculline but there was an even more pronounced loss of banding with 250μg bicuculline.

The measures of increased excitability, namely post-discharge and wind-up, showed opposing effects, in that post-discharge tended to be facilitated and

wind-up was significantly inhibited with all doses of bicuculline (Figure 1B-this Chapter). The post-discharge of the cells was inhibited by the lowest dose of 0.5μg bicuculline (68±14% of control) although increasing the doses to 5μg and above caused facilitations of 121±24, 153±20, 195±55% of control respectively. Due to this variability only the effects of 50μg were significant ($P<0.05$). The wind-up responses were inhibited in a non dose-dependent manner, so that wind-up was inhibited to 67±8, 80±10, 73±11 and 77±25 % of control respectively, all dose effects being significant. However, we do not feel that this reduction in wind-up represents an inhibition, but is due to the effects of bicuculline on the initial responses of the neurones to the first stimulus in the train (Figure 4 and 5B). Thus, bicuculline increased the initial response to C-fibre stimulation to 109±16, 168±12, 253±24 and 603±231% of control with 0.5, 5, 50 and 250μg of bicuculline respectively. However, the same doses did not cause a parallel increase in the overall final responses which were 96±6, 113±9, 126±7 and 264±110% of control respectively (Figure 2).
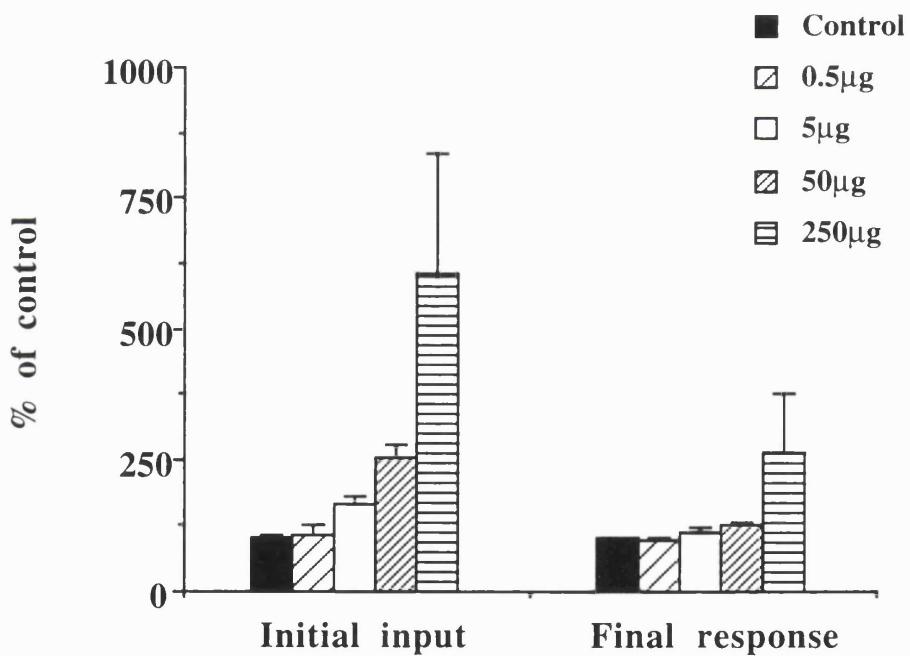
Figure 2. *The response of a mean population of cells to the initial and final response in a train of 16 stimuli at C-fibre suprathreshold stimulation. The effects of 0.5, 5, 50 and 250μg bicuculline, expressed as % of control responses ± SEM.*

Since wind-up was calculated as the overall difference (recorded from

90-800ms) between the initial and the overall response, the much greater increases in the initial responses produced an apparent decrease in wind-up. As can be seen with the example in figure 3B, after bicuculline wind-up was simply imposed upon a higher baseline. The response to Aβ-fibre stimulation was measured over the entire duration of recording, from 0-800ms. Even after bicuculline the responses to Aβ-fibre stimulations did not show a "wind-up like" effect with any dose of bicuculline. The control responses to pved were robust and of a sufficiently high magnitude for the drug effects not to be overemphasised. The same holds true for the Aδ-responses.
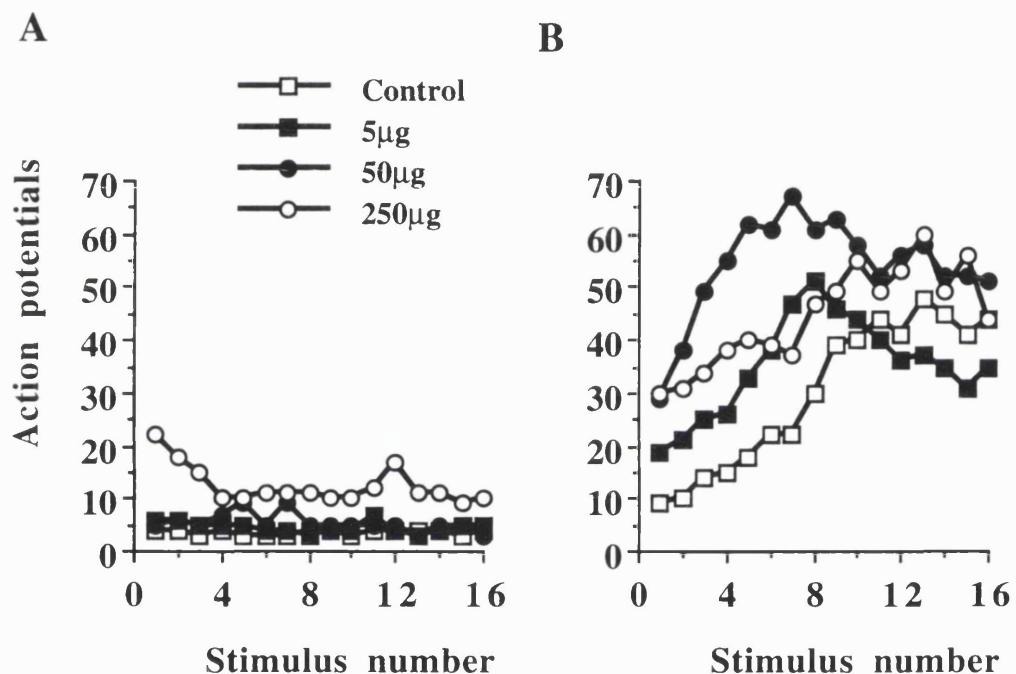
Figure 3. *Examples of wind-up. Graphs of the response of single neurones to a train of 16 stimuli, given at 0.5Hz with a 2ms wide pulse, plotted as action potentials against stimulus number. (A) The response to Aβ-fibre evoked stimulation given at suprathreshold stimulation, here responses from 0-800ms are plotted. The responses before (control) and after intrathecal administration of bicuculline (5, 50 and 250µg) can be seen. (B) The response to C-fibre suprathreshold stimulation at C-fibre latencies and beyond (90-800ms).*

8.2.2: Time-Course of the Effects of Bicuculline-Methobromide.

On the basis of the above results the dose of 50µg was chosen for the subsequent pharmacological studies, since it produced significant effects on all measures except for the Aβ responses. The time-course of the enhanced effects of bicuculline were also evaluated for this dose. A separate population of ten deep dorsal horn neurones with an average depth of 804±71µm from the surface of the cord was therefore studied after this single dose of intrathecal bicuculline (50µg) for forty minutes. This was then followed by 50µl of intrathecal saline, as a control for the other drugs to be tested. The neuronal responses were then followed for a further ninety minutes (Figure 4A, B).

The overall time course was then statistically evaluated both as a whole and as separate blocks of forty minutes, from 0-40 minutes, 41-80 minutes and 81-120 minutes. These latter periods corresponded to the times of application of the subsequent drugs.

Only two cells responded to brush and therefore their responses were not statistically evaluated. (a blunt probe 4N/cm²)

The responses to prod were significantly facilitated in the first ($F_{1,86}$=11.089, PSLD Fisher test, P=0.001) and second block ($F_{1,84}$=11.822, P=0.0009) with only the effects over the last forty minutes not being significant. However, the overall time course was significant ($F_{1,262}$=24.544 $P<0.0001$) (Figure 4B). The C-fibre evoked responses were also significant in the first and second block ($F_{1,78}$=5.239 P=0.02; $F_{1,78}$=4.58 P=0.03 respectively) but not the last. However, again when the entire time-course was taken into account the C-fibre evoked responses were significantly facilitated compared to controls ($F_{1,250}$=11.59; P=0.0008) (Figure 4A).
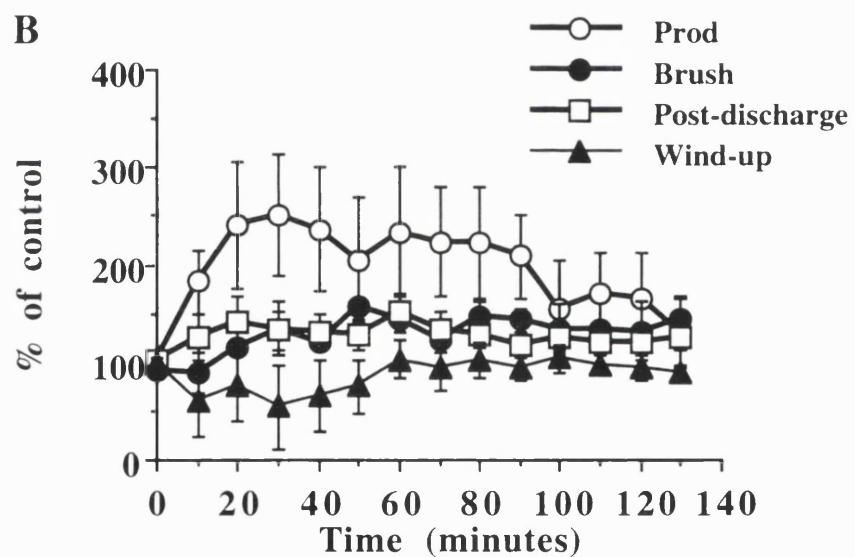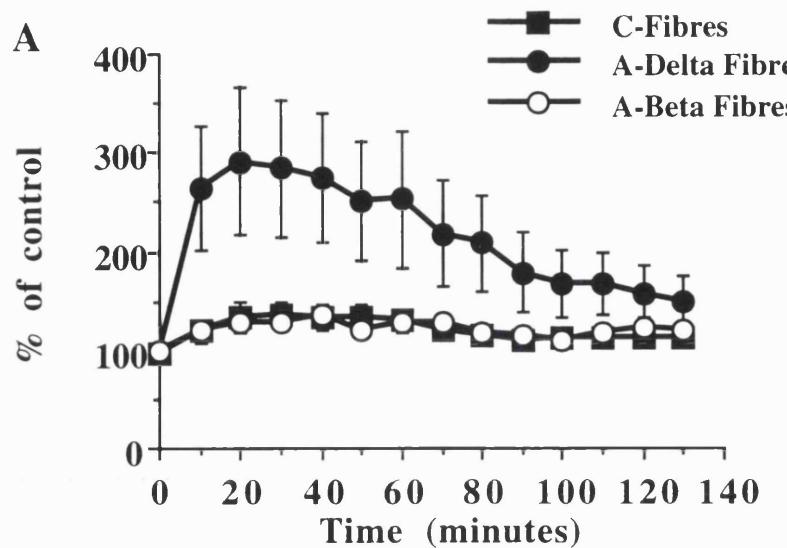
Figure 4. Time course of the responses of a population of dorsal horn neurones to 50μg bicuculline applied at time zero, followed by 50μl of saline after 40 minutes. (A) The responses to Aβ-, Aδ- and C-fibre evoked activity. (B) The responses of post-discharge, wind-up, brush and prod responses of the same cells. The overall time course was significantly changed from controls for all the fibre-evoked responses as well as prod. Note that the effects of bicuculline persist for up to two hours (n=10).

Post-discharge and wind-up were not significantly changed in any block of the time-course.

The $A\delta$-fibre evoked responses were significantly facilitated in every time block ($F_{1,78}=45.118$ $P<0.0001$; $F_{1,78}=25.986$ $P<0.0001$; $F_{1,72}=9.204$ $P=0.003$ respectively) and again the entire time-course was significantly facilitated ($F_{1,248}=72.570$ $P=0.0001$) (Figure 6A).

The $A\beta$-fibre evoked responses were significantly facilitated as an overall time-course ($F_{1,248}=19.966$ $P<0.0001$) with the first two blocks also being significantly facilitated ($F_{1,78}=10.733$ $P=0.001$; $F_{1,78}=6.17$ $P=0.01$) respectively.

8.2.3: Receptive Fields of the Neurones.

The receptive fields of all neurones were evaluated on the ipsilateral hindpaw. The receptive fields for brush, prod and pinch were observed by giving the appropriate stimuli to the paw and observing the evoked firing of the neurone. However, since there was no obvious change in the size of the receptive field to any of the stimuli observed, after any dose of bicuculline, this was not quantified.

8.2.4: N^6-cyclopentyladenosine.

In these and all subsequent studies the effects of CPA, 7-CK and morphine were observed after forty minutes pretreatment with 50μg of intrathecal bicuculline. The results were expressed as percentages of the last two tests with bicuculline, for the studies with CPA, 7-CK and morphine. A summary of the cellular responses with each drug combination can be seen in table 1.

The effects of CPA, the A_1-adenosine agonist, was observed on a population of eight deep dorsal horn neurones with an average depth of 836±85μm.

The dose related effects of CPA after bicuculline can be seen in Figure 5A. Doses of 5 and 50μg CPA were observed for 40 minutes each which was then followed by 250μg 8pSPT, an A_1-adenosine receptor antagonist (Figure 5B and C). There was a significant increase from controls with 50μg bicuculline alone on the responses to prod, C- and $A\delta$-fibre evoked responses (Figure 5B, C).
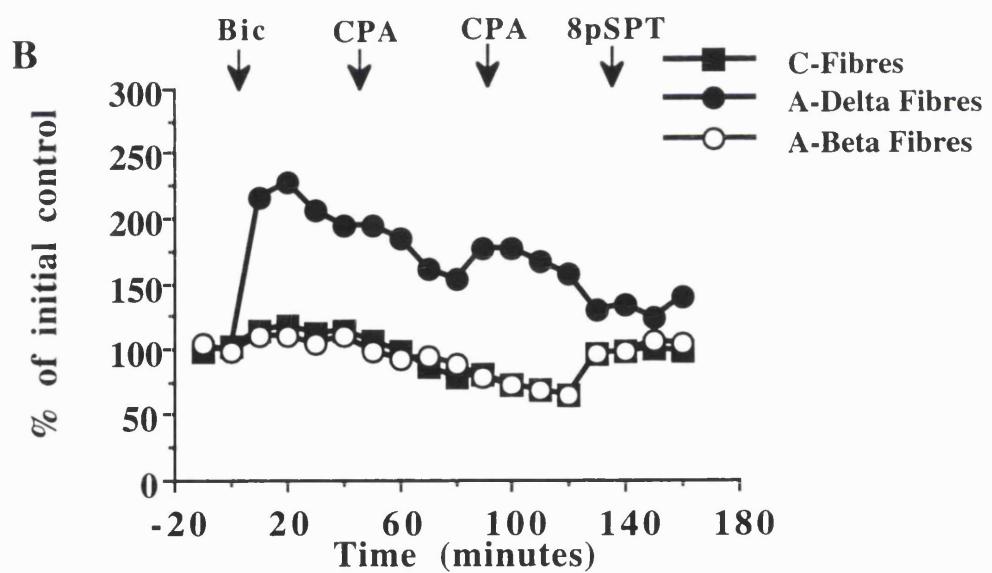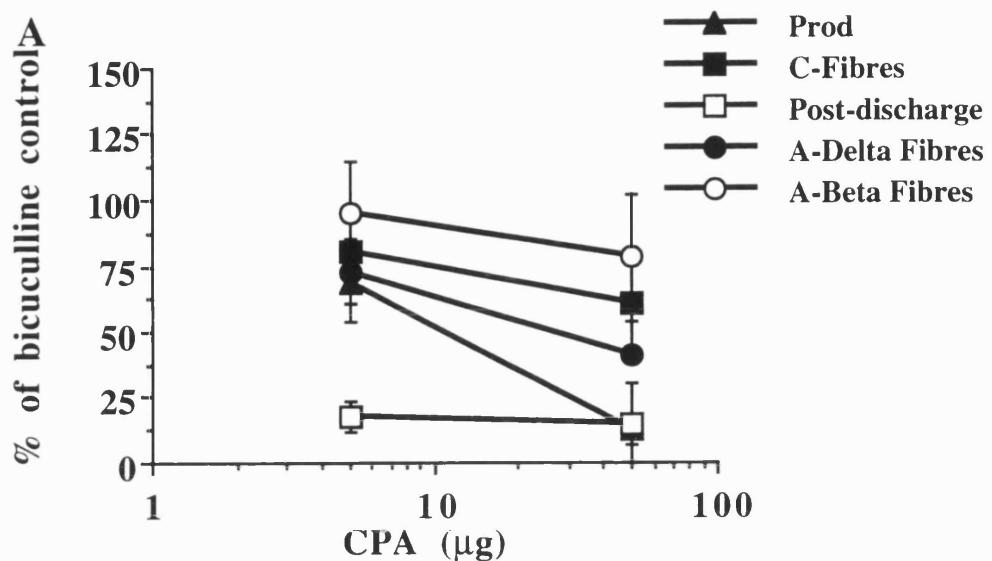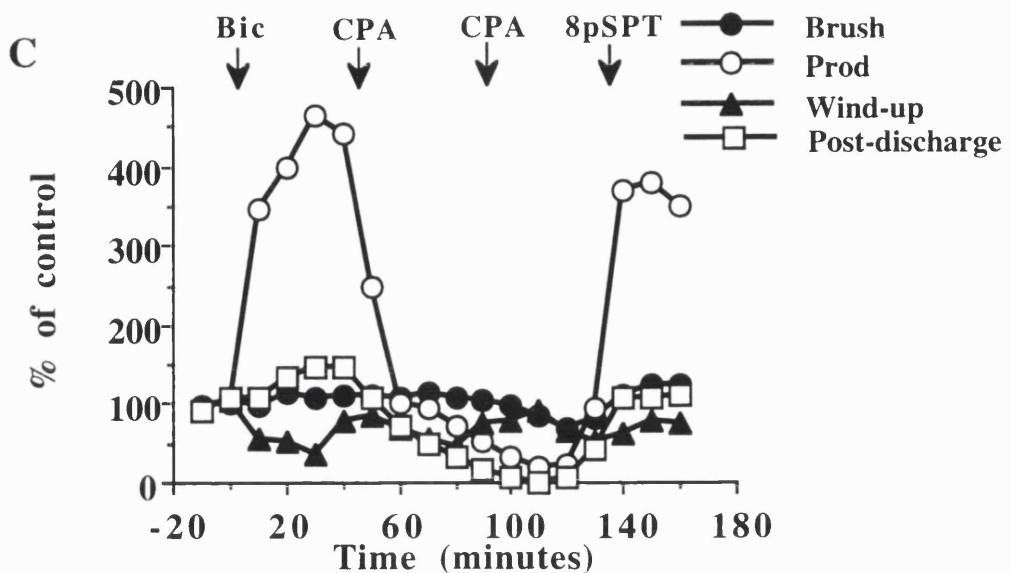
Figure 5. *The effects of 5 and 50µg CPA on the effects of intrathecal bicuculline, 50µg (Bic) on neuronal responses. (A) The mean maximal effects of CPA, expressed as percentages of bicuculline pretreatment. (B and C) Time courses showing the effects of the same doses of CPA, after bicuculline and reversal of CPA with 8pSPT (250µg). Results are expressed as percentage of initial control responses.* Error bars have been omitted for clarity.

Both prod (a blunt probe 4N/cm²) and the C-fibre evoked responses were significantly inhibited by CPA after bicuculline and reversed with 8pSPT (*P<0.05*). Cyclopentyladenosine inhibited the Aδ-fibre evoked responses of six cells but in two cells the responses remained facilitated. The former cells were significantly inhibited (*P<0.05*) by CPA and the effects reversed by the antagonist (Figure 5A, B). However, when compared to the initial controls the response of the cell to Aδ-fibre inputs were still marginally facilitated (see Figure 5B).

The Aβ-evoked responses at C-fibre stimulation were significantly inhibited by both 5 and 50µg CPA and the reversal with 8pSPT was also significant (*P<0.05*). The response to brush was only observed with four cells and was not significantly changed with any of the drugs. The post-discharge of three cells was facilitated by CPA and for five cells, this response was inhibited, although on the total population these effects were not significant. However, if the two populations were taken separately the inhibitions were significant with 5µg CPA only. The reversal with 250µg 8pSPT was also significant.

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Figure 6 also shows the firing of individual cells at three times Aβ-fibre threshold, which after bicuculline, also occurred in the latency usually associated with the Aδ-fibres (20-90ms). Cyclopentyladenosine decreased or abolished this excess firing.
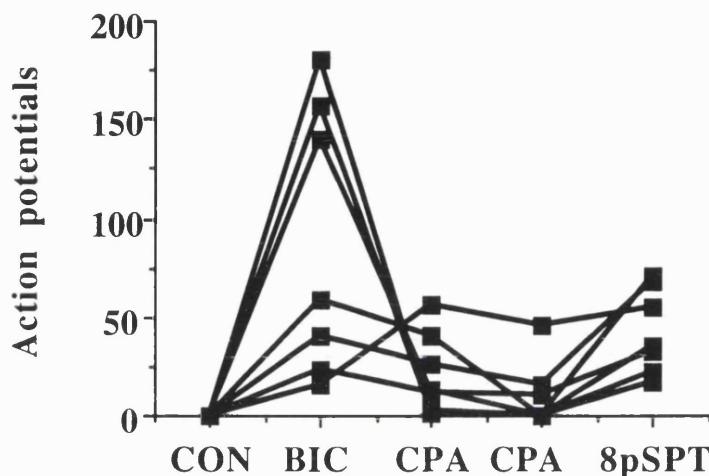
Figure 6. *Firing in the Aδ-fibre latency band of seven single neurones to Aβ-fibre suprathreshold stimulation. After 50μg bicuculline (BIC) activity was produced in this band (20-90ms) whereas in the controls (CON) no firing in this range was observed. This altered activity was markedly reduced and/or abolished by 5 and 50μg CPA (CPA) in a dose dependent manner and the effects were partly reversed by the antagonist 8pSPT (250μg).*
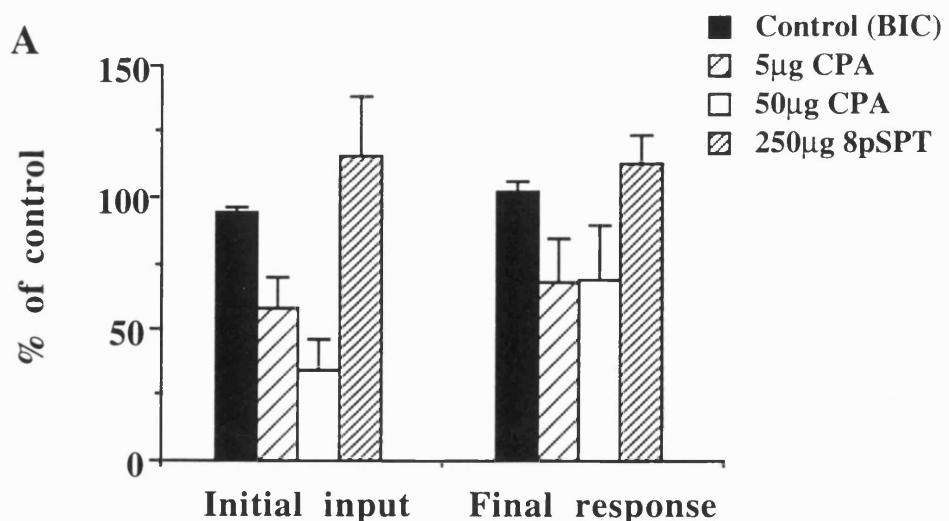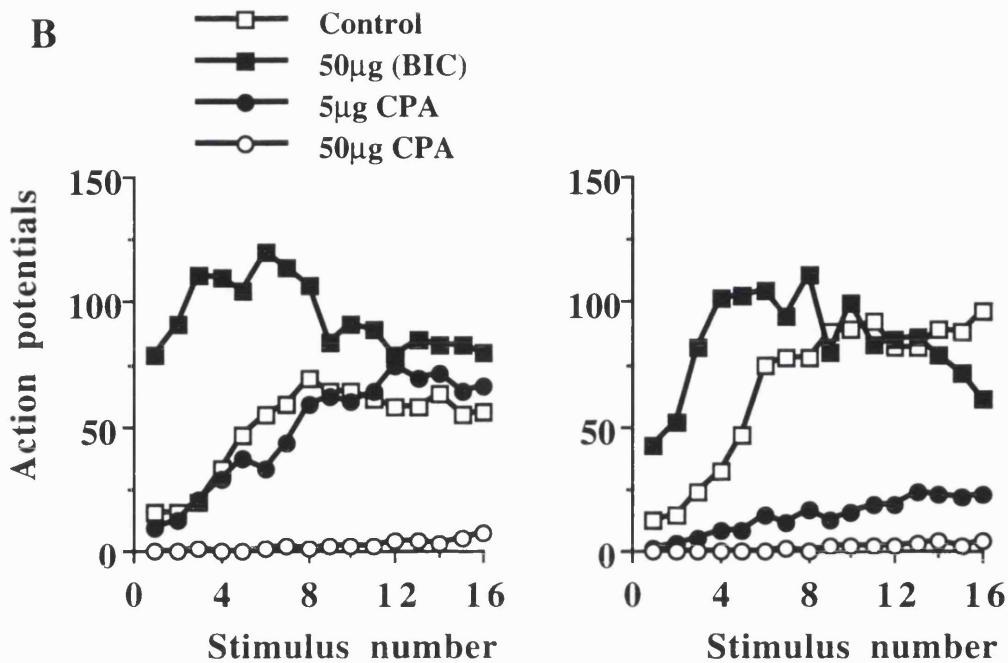
Figure 7. (A) The responses of a population of cells showing the initial (input) to a train of 16 stimuli at C-fibre stimulation and the final response to the same train. The mean final effects after pretreatment with bicuculline (50μg) forms the control (BIC). The subsequent inhibitory effects of CPA (5 and 50μg) and reversal with 8pSPT (250μg) are shown. (B) Wind-up. Two examples of the response of neurones to a train of stimuli at C-fibre stimulation, plotted as action potentials against stimulus number. The response before pretreatment with bicuculline is shown as the control. The increased response with 50μg bicuculline (BIC) was inhibited by CPA. Note that both the input and overall excitability were reduced by CPA.

After bicuculline there was an increased initial response of the cell compared to controls. Cyclopentyladenosine (5 and 50μg) decreased this to 58±12 and 34±12% of control respectively (Figure 7A). However, although the overall final response onto the cell was decreased by CPA the maximal effect was seen after 5μg only, so the responses were 68±16 and 69±20% of control respectively for 5 and 50μg. Examples of wind-up of single cells can be observed in Figure 7B. Although 50μg abolished wind-up of one of the cells shown, the overall responses of wind-up were not significantly inhibited (see Figure 7C). The inhibitions of the

initial, overall C-fibre evoked responses and post-discharge were reversed by 8pSPT.

8.2.5: Morphine.

The effects of the mu opioid agonist, morphine (0.25 and 1µg) were tested on seven deep dorsal horn neurones with an average depth of 902±57µm. The effects of the antagonist naloxone (0.5µg) given after morphine were also studied.
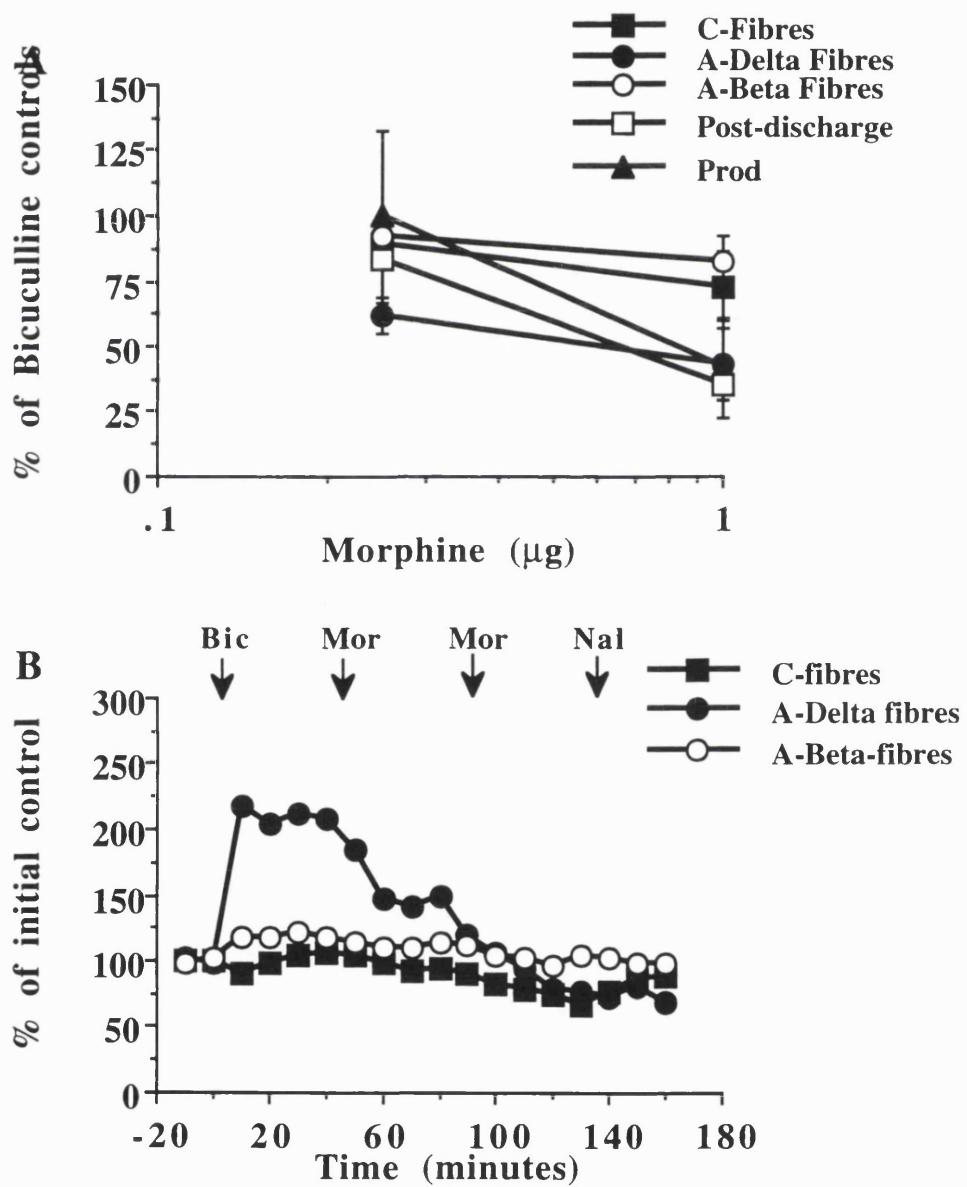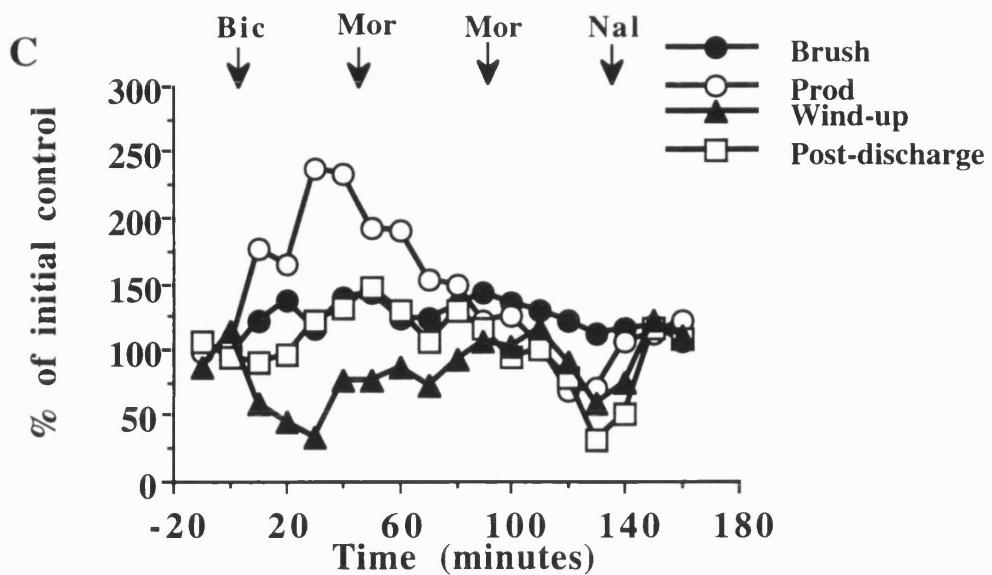
Figure 8. *The effects of morphine (0.25 and 1μg) on the effects of 50μg bicuculline (Bic) on neuronal responses. (A) The mean maximal effects, expressed as percentages of bicuculline pretreatment. (B and C) Time courses showing the effects of the same doses of morphine and reversal with naloxone (0.5μg). Results are expressed as percentage of initial control responses.*

The C- and Aβ-fiber evoked responses were only significantly inhibited by 1μg morphine (*P<0.05*) and naloxone reversal was not significant. However, although inhibition was significant for the above responses, they were not inhibited to the extent as the responses to prod (see below) so that the effect of 1μg morphine was 73±13 and 82±10 % of control for the C- and Aβ-fibre evoked responses respectively (Figure 8A, B). The responses to brush were not significantly changed with any of the doses of morphine (n=4). The responses to prod tended to be facilitated by bicuculline (see figure 8C). Morphine tended to inhibit the responses to prod, but this was only significant with 1μg morphine (42±19 % of control) (*P<0.05*). Reversal of the response to prod, with naloxone, was apparent but not significant. The Aδ-fibre evoked responses at C-fibre stimulation were significantly facilitated with bicuculline and significantly inhibited by both doses of morphine (*P<0.05*). Reversal was not significant with naloxone.

The additional firing seen in the latency range 20-90ms, after bicuculline at suprathreshold Aβ-fibre evoked activity was inhibited by 0.25 and 1μg morphine, (Figure 9). The control responses to prod were robust and of a sufficiently high magnitude for the drug effects not to be overemphasised.

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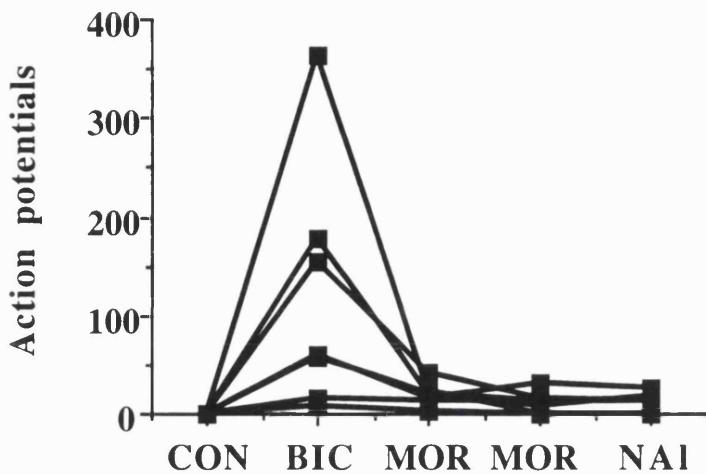
Figure 9. Examples of firing in the Aδ-fibre latency band of six single neurones to Aβ-fibre stimulation. After 50μg bicuculline (BIC), activity was produced in the band 20-90ms with none having occurred in the controls (CON). This altered activity was markedly reduced and/or abolished by morphine (0.25 and 1μg) and was only marginally reversed by the antagonist naloxone (0.5μg).

The initial response of the cell, after bicuculline was reduced by both 0.25 and 1μg morphine (Figure 10A, B; table 1) The overall final response of the cell was inhibited but there was no clear dose-dependency. Neither influences were reversed fully with naloxone (Figure 10A).
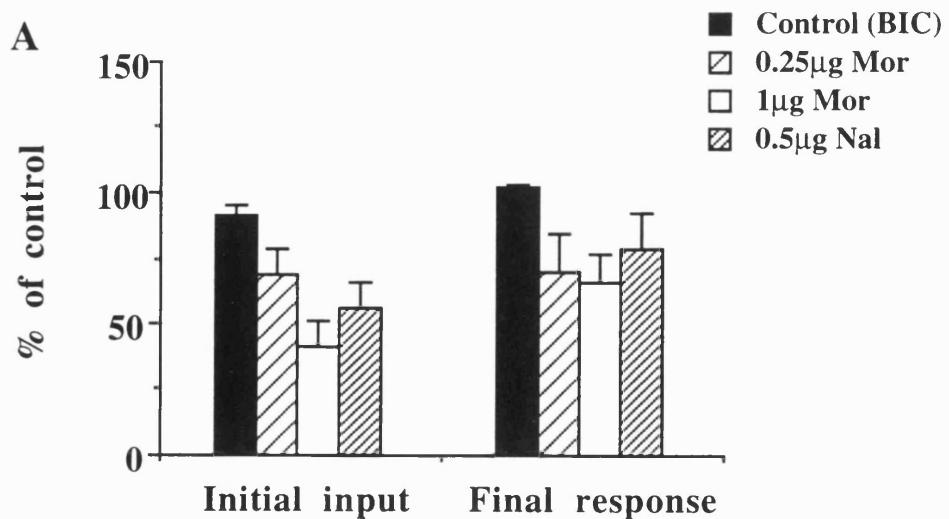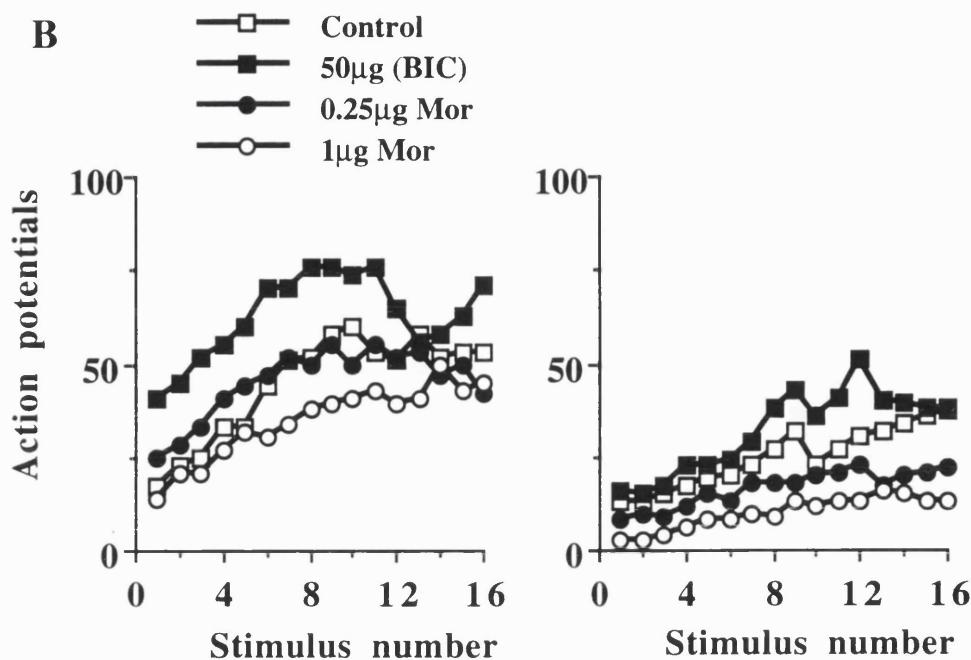
Figure 10. (A) The responses of a population of cells showing the initial response (input) in a train of 16 stimuli at C-fibre stimulation and the final response to the same train. The mean final effects after pretreatment with bicuculline (50μg) forms the control (BIC). The subsequent inhibitory effects of morphine (0.25, 1μg) and reversal with naloxone (0.5μg) are shown. (B) Wind-up. Two examples of the response of single neurones to a train of stimuli at C-fibre stimulation, plotted as action potentials against stimulus number. The control response is that prior to bicuculline pretreatment. The increased response with 50μg bicuculline (BIC) was partly inhibited by morphine.

8.2.6: 7-chlorokynurenate.

The effects of 7-CK was observed on seven cells with an average depth of 826±64μm from the surface of the spinal cord.

There was a significant increase in response compared to controls after 50μg bicuculline for the post-discharge and Aδ-fibre evoked responses. Apart from brush responses (n=3) all responses were significantly inhibited by both 10 and 50μg 7-CK (Figure 11A, B, C; table 1).

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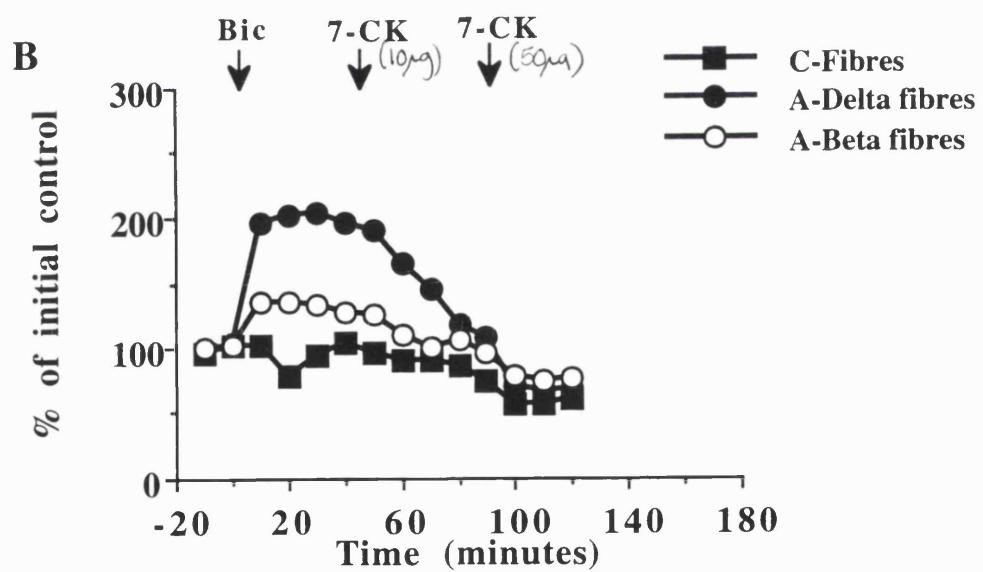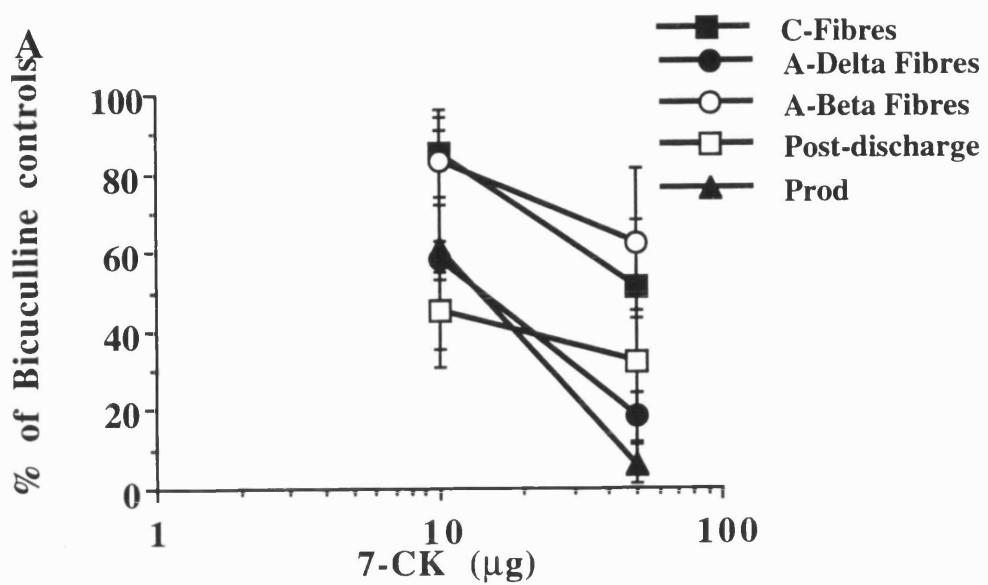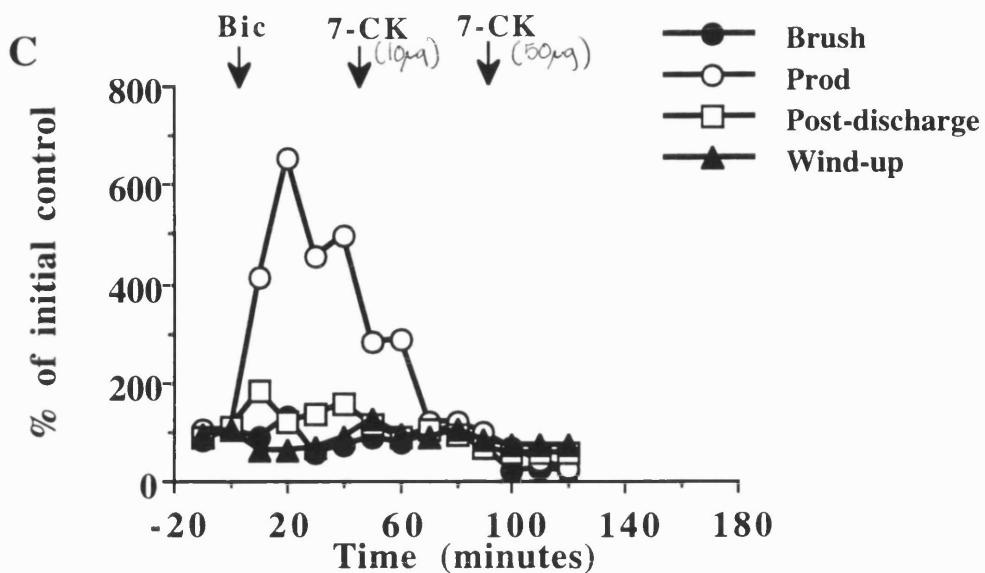
Figure 11. The effects of 7-CK (10 and 50μg) on the effects of 50μg bicuculline (Bic) on neuronal responses. (A) The mean maximal effects, expressed as percentages of bicuculline pretreatment. (B and C) Time courses showing the effects of the same doses of 7-CK. Results are expressed as percentage of initial control responses.

However, there was a greater inhibition of some of the responses compared to others. The rank order of inhibition with 50μg 7-CK was prod, Aδ-, post-discharge, C-, and Aβ-fibres (Figure 11A; table 1).

The Aβ-fibre evoked activity occurring in the latency 20-90ms after bicuculline was decreased or abolished after 10 and more often 50μg 7-CK (Figure 12).
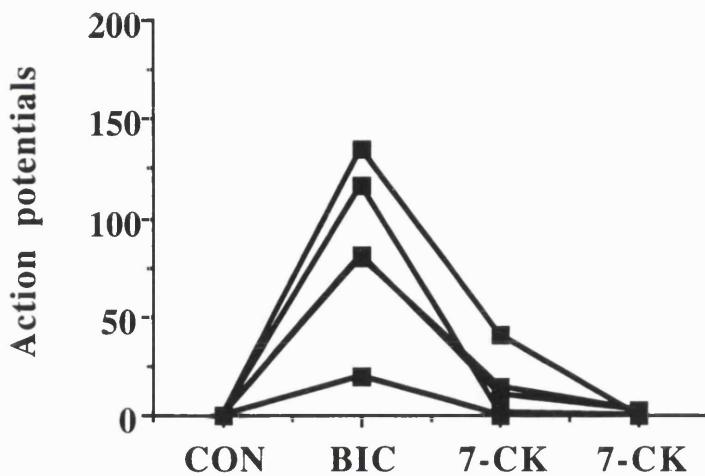
Figure 12. *Examples of firing of five single neurones to Aβ-fibre threshold evoked activity now occurring in the Aδ-fibre latency band after 50μg bicuculline (BIC). This was decreased by 7-CK in a dose-dependent manner.*

Overall, both doses of 7-CK inhibited both the initial response and the final response of the cell (see Figure 13A). However, it appears that the initial response, as compared to the final response, was more sensitive to the inhibitory effects of 7-CK. It was apparent from the individual wind up curves that although 7-CK reduced the initial response, there was a more limited effect on wind up, which in turn contributes to the final response of the cell (Figure 13B). This was the case for five of the cells with the response of only one cell being abolished.

B

Legend:
- □ Control
- ■ 50μg (BIC)
- ● 10μg 7-CK
- ○ 50μg 7-CK
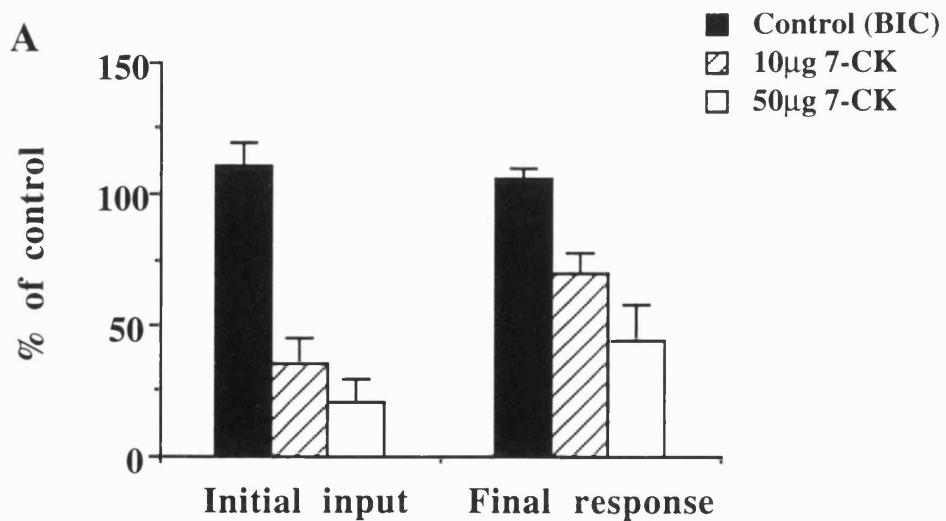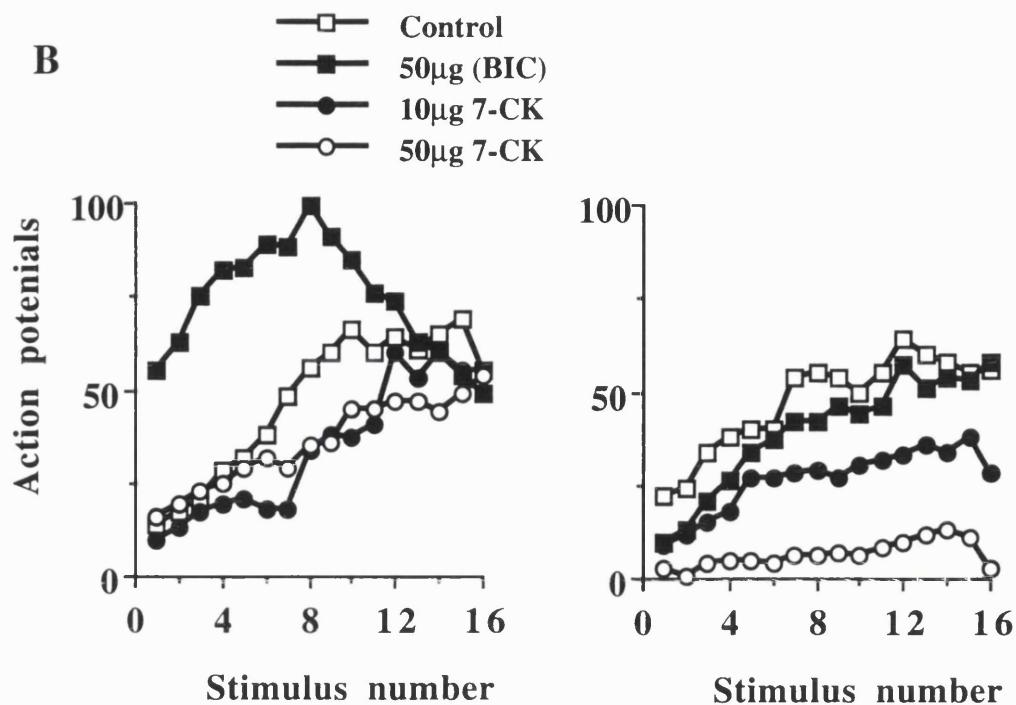
Figure 13. (A) The responses of a population of cells showing the initial (input) to a train of 16 stimuli at C-fibre stimulation and the final response in the same train. The mean final effects after pretreatment with bicuculline (50μg) forms the control response (BIC). The subsequent inhibitory effects of 7-CK (10 and 50μg) on these responses are shown. (B) Wind-up. The response of single neurones to a train of stimuli at C-fibre strength, plotted as action potentials against stimulus number. The response before treatment with bicuculline is shown as the control The increased response with 50μg bicuculline (BIC) was inhibited by 7-CK.

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Cell response	7-CK (50μg)	CPA (50μg)	Morphine (1μg)
	% Response of bicuculline pretreatment.		
Prod	6±5	13±6	42±19
C-Fibres	52±17	61±20	73±13
Aδ-Fibres	18±6	41±2	43±14
Aβ-Fibres	62±19	78±24	82±10
Post-discharge	32±13	15±5 (109±44)	35±5 (81±27)
Initial response	21±8	34±12	41±10
Overall response	44±14	69±20	66±10
n number	7	8	7

Table 1. The mean maximal inhibitory effects of the NMDA-antagonist, 7-CK, the A₁-adenosine agonist, CPA and the mu-opioid agonist, morphine expressed as percentages of the final effects of 50μg bicuculline pretreatment. The results for the post-discharge shown are for the inhibited cells only. The bracketed responses show the overall response for post-discharge for the population of inhibited and facilitated cells.

8.3: Discussion.

These results extend the evidence that GABA acts as a modulating neurotransmitter, within the dorsal horn of the spinal cord, via the activation of the $GABA_A$-receptor. This study also shows that the removal of $GABA_A$-receptor control can lead to the loss of distinction between fibre evoked responses which under normal circumstances is very precise. This "miscoding" was seen with both low threshold inputs as well as high-threshold evoked inputs; the low-threshold inputs could in an awake animal be perceived as painful (allodynia). There was also shown to be an amplification of noxious inputs (hyperalgesia).

Bicuculline (50μg) was shown to antagonize the inhibitory effects of muscimol (5μg) (a $GABA_A$-receptor agonist) in separate experiments done at the same time in the laboratory (M. Green). In my experiments intrathecal application of bicuculline onto the exposed spinal cord produced a moderate increase in the Aβ- and C-fibre evoked responses of deep dorsal horn neurones. There was also a more dramatic increase in firing of these cells occurring in the Aδ-fibre latency bands and in response to innocuous pressure.

The increased activity in the Aδ-fibre latency band/reflect a prolongation of firing produced by Aβ-fibre inputs or disinhibition of true Aδ-fibre evoked activity. In the control measures, the banding of the evoked activity produced by the various fibre types could easily be distinguished, but after bicuculline these distinctions were harder to observe and a merging of activity between the A and C bands became apparent. This has also been observed by others (Duggan et al. 1981; Game and Lodge 1975; Hao et al. 1992a).

There is ample human evidence that allodynia, at least to mechanical stimuli, is evoked by activation of low threshold A-fibre afferents as gauged by a number of experimental approaches (Campbell et al. 1988; Dubner et al. 1987; Price et al. 1989, 1992; Torebjork et al. 1992). There is also evidence that high-threshold mechanical allodynia can also be experienced by patients with sympathetically maintained pain (Price et al. 1992) thus implicating the high-threshold mechano-Aδ-fibre. Note that these patients reported burning pain and other characteristic responses of C-polymodal evoked responses to high-threshold mechanical stimulation. However, there is no clear evidence to determine whether the activation of low-threshold Aδ-fibres can also be responsible for this phenomenon. Although we observed an increase in firing in the established Aδ-fibre latency band at C-fibre threshold, as well as the appearance of firing in this band at Aβ-fibre threshold, we cannot determine if this is due to an increase in firing of the Aδ-fibres or an

unmasking of Aβ-fibre inputs now running into the Aδ-fibre latency band. A complication is the lack of comparative data on response properties of myelinated afferents in the rat in different skin types. Our study used the glabrous skin of the hindpaw. In humans allodynia and hyperalgesia are not restricted to any particular body surface. It has been shown that both Aδ- and C-fibre nociceptors in hairy skin in cats respond to a range of pressure (Garell et al. 1996). Not only was our stimulus innocuous to the experimenters but our stimulus would appear to be below the pressure threshold for Aδ-nociceptors (Garell et al. 1996).

For all peripherally evoked responses.

We found the proportion of cells exhibiting brush responses were few. Although the cells recorded from are convergent dorsal horn neurones which can respond to a variety of stimuli (low-and high-threshold) the absence of a response to brush does not mean that these cells are not convergent neurones, since they still respond to innocuous touch and prod (see Besson and Chaouch 1987; Fitzgerald and Wall 1980; Le Bars and Chitour 1983; Menetrey et al. 1977). Another reason that the response to brush was not often observed may have been due to a low number of brush receptors in glabrous skin of the rat paw.

The cells that did respond to brush showed unchanged responses after bicuculline. As stated above, cells always responded to innocuous prod, as well as to noxious pinch and to electrically evoked C-fibre activity. These prod responses were found to be markedly and significantly facilitated after bicuculline. It is tempting to speculate that the enhanced responses to innocuous pressure result from the elevated Aβ or Aδ latency responses of the neurones seen after bicuculline. Some Aδ-fibres respond to low-threshold mechanical stimuli (see Besson and Chaouch 1987) and have been suggested to underlie allodynia in humans (Persson et al. 1995).

The spinal application of antagonists of the amino acids GABA and glycine has been reported to cause agitation and cardiovascular activation to non noxious stimulation such as hair deflection in behavioural testing (Roberts et al. 1986; Sherman and Loomis 1994; Yaksh 1989). Electrophysiological experiments have demonstrated enhancements in the evoked firing patterns of cells produced by both low and high threshold stimulation in the presence of antagonists of these two receptor systems (Curtis et al. 1968; Duggan et al. 1981; Game and Lodge 1975; Sivilotti and Woolf 1994). In the latter case (Sivilotti and Woolf 1994) not only were low threshold stimuli enhanced to a greater extent than pinch, but were able to activate the nociceptive reflex in motorneurones after antagonist treatment (Sivilotti and Woolf 1994). These observed changes have been likened to the phenomenon of allodynia and have been attributed to the loss of these inhibitions within the dorsal horn of the spinal cord

(Hao et al. 1992a, b; Sivilotti and Woolf 1994; Yaksh 1989; Yaksh and Harty 1988). No previous study has concentrated on neuronal sensory responses.

There is ample evidence for a GABA control of low-threshold afferent activity in the spinal cord, but importantly, recent immunohistochemical studies have demonstrated that the Aδ-fibre primary afferents are under a greater degree of innervation by GABAergic neurones at a presynaptic level compared to C-fibres (Alvarez et al. 1992; Bernardi et al. 1995). These GABA controls of the presumed Aδ-fibre pathways may be at both the pre and post-synaptic level. It has been shown that cells post-synaptic to the Aδ-fibre primary afferent terminals can be controlled by inhibitions evoked by stimulation of these afferents (Baba et al. 1994; Shimizu et al. 1995; Yoshimura and Nishi 1993, 1995). These inhibitions are mainly strychnine and bicuculline sensitive (Baba et al. 1994; Yoshimura and Nishi 1995). Hyperesthesia resulting from a loss of these controls could be due to an increase in the ability of these primary afferent terminals to reach threshold potentials (since both GABA and glycine stabilize the membrane potential to near resting potentials) (Desarmenien et al. 1984).

Although the post-discharge of the cells generated by suprathreshold C-fibre stimulation was observed to increase after GABA$_A$-receptor antagonism, indicating an increase in excitability to noxious stimuli, paradoxically, the wind-up of the same cells was shown to decrease. Wind-up is measured as the excess firing over the predicted response based on the initial C-fibre evoked activity. The reduction in wind-up was found to be a reflection of an increased input onto the cells, so that the response to the first C-fibre stimulus was markedly increased. Further increases in the neuronal responses during the train did not occur so that a "wind-up like" response could not be produced. There was also an increase in the initial response onto the cell after Aβ-fibre stimulation, but this was not as great as that seen with suprathreshold C-fibre stimuli. The response of cells to Aβ-fibre stimulation did not show a "wind-up like" response with any dose of bicuculline. Note, it has been reported elsewhere that the Aβ-fibre evoked activity can produce a "wind-up" like effect (Price et al. 1989, 1992; see Woolf 1991). Aβ-fibres can change their phenotype after inflammation (Neumann et al. 1996).

Essentially, our results show that bicuculline markedly enhances low-threshold responses of these dorsal horn neurones, with clear but less pronounced increases in C-fibre evoked activity. It could be that these changes underlie allodynia and hyperalgesia. In behavioural studies, similar or higher concentrations of bicuculline produce reactions indicative of allodynia but the presence of this hypersensitivity precluded nociceptive testing (Roberts et al. 1986; Sherman and Loomis 1994; Yaksh 1989).

The enhancement of responses of deep dorsal horn neurones caused by

spinal antagonism of GABAergic controls with bicuculline were subsequently pharmacologically modulated by administration of the spinally applied adenosine A_1-receptor agonist, N^6-cyclopentyladenosine, the mu opioid-receptor agonist, morphine and the NMDA-receptor antagonist 7-chlorokynurenate. A previous study measuring behavioural agitation observed a relatively short duration of action of antagonists of the inhibitory amino acids (Yaksh 1989). We found that effects plateaued between 20-40 minutes after initial bicuculline administration. However, these effects were still significantly enhanced over a longer time course (up to 2 hours) which justified our subsequent pharmacological studies on the modulation of the responses. The differences in duration between studies may reflect differences between electrophysiological and behavioural approaches.

After bicuculline treatment, the A_1-agonist, CPA, inhibited the enhanced $A\delta$- and C-fibre responses and the post-discharge of the cells. The response to prod was also inhibited by CPA. These inhibitions were reversed with the antagonist 8pSPT. With the exception of the $A\delta$-fibre evoked responses observed at C-fibre strength stimuli, all the inhibitions produced by CPA fully countered the enhanced responses caused by bicuculline, reducing them to below the pre-bicuculline controls. Only the $A\delta$ responses, although reduced by CPA, still remained above control values. However, the increased firing of cells with suprathreshold $A\beta$-stimulation, which now occurred at latencies usually associated with the $A\delta$-fibre evoked responses, was dose dependently and almost fully inhibited by CPA and restored to a lesser extent by 8pSPT.

The increased excitability of the cells, manifest in the post-discharge response, fell into two populations with regard to the direction of effects of CPA. A small proportion had facilitated responses but in the main this response was inhibited after bicuculline. The calculation of wind-up was hampered after bicuculline as discussed in the results section. Consequently, to ascertain the effects of bicuculline on the enhanced excitability of a cell, the effect of CPA was observed on the basis of the initial input and the last response of the cell to a train of stimuli. Cyclopentyladenosine decreased the initial input onto the cell that had previously been increased by bicuculline as well as reducing, to a lesser extent, the overall final response.

Adenosine has well documented analgesic properties in both animal and human studies (Belfrage et al. 1995; Karlsten and Gordh 1995; Malmberg and Yaksh 1993a; Reeve and Dickenson 1995; see Salter et al. 1993; Sawynok and Sweeney 1989; Sergerdahl et al. 1994, 1995; Sollevi et al. 1992, 1995, 1996). Animal studies with adenosine analogues (Sosnowski and Yaksh 1989) and recent human studies

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with adenosine have indicated that adenosine infusion can attenuate allodynia induced either by peripheral stimuli or as a result of peripheral nerve damage (Belfrage et al. 1995; Karlsten and Gordh 1995; Sergerdahl et al. 1995; Sollevi et al. 1995). As previously discussed, the main receptor for adenosine ~influencing spinal transmission of somatosensory inputs,~ in the dorsal horn of the spinal cord is the inhibitory A_1-receptor, located primarily on interneurones with a smaller population on primary afferent terminals (Choca et al. 1988; Fastbom et al. 1987a; Geiger et al. 1984; Goodman and Snyder 1982; Li and Perl 1994). Acute studies in normal animals have shown inhibitory effects of A_1-receptor agonists on wind-up and C-fibre evoked responses, yet concurrent facilitatory effects on $A\delta$-fibre evoked responses, indicative of pre- and post-synaptic effects (Chapter 4). With the exception of this latter response, CPA was extremely effective at reducing all enhanced responses of the neurones after bicuculline, thus providing a possible basis for the use of adenosine in neuropathic states. The enhanced $A\delta$-fibre activity produced by adenosine A_1-receptor agonists in normal animals is probably not ~perceived as~ noxious in an awake animal, since behavioural studies with A_1-receptor agonists give rise to antinociception (Ahlijanian and Takemori 1985; DeLander and Hopkins 1986; Herrick-Davis et al. 1989; Holmgren et al. 1983, 1986; Karlsten et al. 1990; Malmberg and Yaksh 1993a; Post 1984; Sawynok et al. 1986; Sosnowski and Yaksh 1989; Vapaatalo et al. 1975; Yarbrough and McGuffin-Clineschmidt 1981).

Activation of inhibitory interneurones by $A\delta$-fibres has been shown to require an initial non-NMDA receptor depolarization by glutamate (Shimizu et al. 1995; Yoshimura and Nishi 1993, 1995). GABA inhibitory neurones can then control the release of glutamate from afferents (Alvarez et al. 1992; Bernardi et al. 1995). Bicuculline would disinhibit this $GABA_A$ control of glutamate release from afferents, so that peripheral stimuli would evoke a greater response. In fact, this is exactly what we observed, in that the response to the first stimulus at C-fibre strength was massively elevated after bicuculline, with a lesser increase in the initial response to $A\beta$ stimuli. This loss of control of the release of excitatory neurotransmitters could then increase via the recruitment of the NMDA-receptor (Ma and Woolf 1995; Yaksh 1989).

The NMDA-receptor has been implicated as a possible target in neuropathic pain (see Dickenson 1994a) since its activation underlies hyperalgesia in a number of persistent pain states. The reasons for this are that the receptor is required for wind-up, whereby C-fibre stimulation produces an amplified and prolonged neuronal response (Davies and Lodge 1987; see Dickenson 1994a; Dickenson and Sullivan 1987a; Dubner and Ruda 1992; McMahon et al. 1993; Price et al. 1994). Evidence to support that the loss of control of excitatory neurotransmitters and subsequent recruitment of the NMDA-receptor can result in

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miscoding of inputs, comes from reports that glutamate receptor antagonists decrease hyperesthesia and hyperalgesia (Backonja et al. 1994; Tal and Bennett 1994; Yamamoto and Yaksh 1993): allodynia in humans and in animal models (Backonja et al. 1994; Bennett 1994; Eide et al 1994; Persson et al. 1995; Sherman and Loomis 1994; Yaksh 1989): decreases 'wind-up like" phenomenon in humans with neuropathic pain (Eide et al 1994; Kristensen et al. 1992) as well as controlling neuropathically driven pain in animals (Mao et al. 1993; Tal and Bennett 1994; Yamamoto and Yaksh 1993).

The effects of the NMDA-receptor glycine site antagonist, 7-CK, were used to gauge both the involvement of the receptor complex in the post-bicuculline responses and also to investigate the ability of agents acting at this site to modulate altered nociception. 7-CK has been found to be a useful and consistent tool for the study of the NMDA-receptor in this model (Chapman and Dickenson 1992, 1994; Dickenson and Aydar 1991). 7-CK profoundly inhibited all responses of the deep dorsal horn cells to electrically and naturally applied stimuli. The responses to prod were inhibited to the greatest extent, followed by the Aδ-, post-discharge, C- and Aβ-fibre evoked responses. The Aδ-fibre evoked responses at C-fibre strength were brought back down to levels below the initial baseline with 7-CK, unlike the responses with CPA. The increased activity in the Aδ-fibre latency band at suprathreshold Aβ-stimulation after bicuculline was abolished by 7-CK. The input onto the cells evoked by C-fibre suprathreshold activation was decreased by 7-CK to a greater extent than the cumulative responses, so that wind-up in the majority of cells was paradoxically restored. Interestingly, in normal animals 7-CK has no effect on the initial responses but blocks wind-up (Chapman and Dickenson 1992; Dickenson and Aydar 1991) suggesting that bicuculline reveals an NMDA component to the baseline responses of the neurones. It is apparent from these results that the enhanced responses to non-noxious prod after bicuculline, are now influenced by an NMDA-receptor antagonist, implying that the increased responses to this stimulus are due to the activation and recruitment of this receptor. Note that after ultraviolet A light radiation, resulting in inflammation, 7-CK also was shown to have an effect on non-noxious, electrically evoked responses (Chapman and Dickenson 1994).

The opioids have been long established as analgesics. However, their effectiveness can alter in different pain states (see Dickenson 1991, 1994b; Yaksh 1993). A consensus would be that opioids have reduced effectiveness in neuropathic conditions (Arner and Meyerson 1988; Jadad et al. 1992). In particular, it has been reported that opioids do not decrease hypersensitivity attributed to allodynia (Arner and Meyerson 1988; Lee et al. 1994; Lee et al. 1995; Sherman and Loomis 1994; Yaksh 1989) although positive effects have also been reported (Desmeules et al.

1993; Eide et al. 1994).

We found that although morphine did weakly inhibit the response to prod, C-, Aδ-fibre evoked responses and the initial responses of the cells it was the least effective of the three drugs observed. As with CPA, the inhibition by morphine of the post-discharge of the cells was not clear cut, in that there were both inhibited and facilitated responses. By far the most noticeable difference between morphine, CPA and 7-CK was the inhibition of the responses evoked by prod. Cyclopentyladenosine and 7-CK inhibited this response to a similar extent but morphine was markedly less effective. There is some evidence that GABA$_A$ mechanisms are required for opioid disinhibitions of inhibitory neurones in the substantia gelatinosa (Magnuson and Dickenson 1991). As bicuculline interferes with this mechanism, the reduced effects of morphine that we have observed could also result from reductions in this disinhibition.

Overall these results lend   support to a role for low threshold, possibly Aβ- or Aδ-fibre evoked responses, in the hypersensitivity of the spinal cord resulting in allodynia. In fact, behavioural studies by Yaksh (1989) show that pretreatment with capsaicin did not alter the strychnine evoked allodynia. We observed an increase in the C-fibre evoked responses but this was less marked than the facilitation of the Aβ/Aδ-fibre evoked responses. These low-threshold fibre evoked responses were clearly under the highest degree of control by intrinsic GABAergic inhibitory systems within the dorsal horn of the spinal cord and we believe that they are responsible for the enhanced responses to innocuous pressure, evoked by prod. This would agree well with the finding that Aδ-fibre primary afferents are under the greatest degree of control by GABAergic neurones from immunohistochemical studies (Alvarez et al. 1992; Bernardi et al. 1995).

Our findings also might indicate that there is an increase in excitatory neurotransmitter release from the primary afferent terminals, in that the initial response of the cell to a train of C-fibre strength stimuli was increased after bicuculline (reflecting this increase in release). In addition, we saw enhanced C-fibre evoked responses including post-discharges which may result from the increased initial activation of the neurones. Whether these changes, seen at C-fibre strength stimuli, are due to increased glutamate release from C- or Aδ-fibres cannot be determined.

Although the literature is somewhat contradictory it would appear that NMDA-receptor antagonists and A$_1$-receptor agonists are effective at treating allodynia (Belfrage et al. 1995; Bennett 1994; Karlsten and Gordh 1995; Ma and Woolf 1995; Persson et al. 1995; Sergerdahl et al. 1995; Sollevi et al. 1995; Tal and Bennett 1994; Yaksh 1989; Yamamoto and Yaksh 1993) whereas opioids are not as

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effective *at influencing allodynia* compared to nociceptive pain in humans and animals (Arner and Meyerson 1988; Lee et al. 1994; Lee et al. 1995; Sherman and Loomis 1994, Yaksh 1989). This is in keeping with our findings in that although morphine was effective it was not as potent as 7-CK or CPA at decreasing changes evoked by GABAergic disinhibition, *on all measures of neuronal excitability.*

Electrophysiological studies cannot be used to demonstrate allodynia in the sense of experiencing distress and presumably pain to non-noxious stimulation. However, hyperalgesia is seen in these experiments, in that the responses to C-fibre evoked stimulation increases. We also saw an increased response to innocuous prod as well as a change in the evoked firing patterns of deep dorsal horn neurones at both C- and Aβ-fibre stimulation. It has been suggested, based on many different models of evoked hyper-responsiveness produced by antagonism of inhibitory mechanisms (Game and Lodge 1975; Roberts et al. 1986; Sosnowski and Yaksh 1989; Yaksh 1989; Yamamoto and Yaksh 1993) high doses of opioids (Woolf 1981; Yaksh and Harty 1988) peripherally induced changes (Ma and Woolf 1995; Sugimoto et al. 1987, 1990) and spinal ischemia (Hao et al 1992a) that a loss of ongoing inhibitions results in the miscoding of somatosensory information by deep dorsal horn neurones. In this sense I feel that the observed changes of deep dorsal horn neurones in these experiments do reflect changes that represent hyperalgesia and could result in allodynia in awake animals.

Thus, the evidence here further supports the idea of an important inhibitory role of GABA within the dorsal horn and indicates a basis for the use of NMDA-receptor complex antagonists and adenosine in the control of both allodynia and hyperalgesia as observed in behavioural studies with this and other models (see Bennett 1994; Sosnowski and Yaksh 1989; Yaksh 1989).

Chapter 9.

Discussion:
Adenosine as a novel target for analgesia-mechanisms and implications.

The spinal cord is a fascinating system. It is hard for many to comprehend the plasticity that this system is capable of. The ingenious ways that the body has evolved to cope with processing as well as acting upon the wealth of sensory information that surrounds us.

It is increasingly apparent that although our knowledge of spinal processing over the last few decades has resulted in a wealth of new information we still have a lot to uncover and understand. Pain is a component of everyone's lives. Hopefully it most circumstances it can be adequately controlled and maintained and many of us will not have to suffer the devastating effects i n our lives of "intractable pain". For a proportion of people the pain they suffer will completely control their lives, since for a variety of reasons the pain is poorly managed. Unfortunately scientific knowledge and advances often takes years to reach the clinical level and it is only through education and a greater understanding between clinicians and scientists that advancements will be made. However much knowledge we uncover it is meaningless if we never act upon it.

The spinal cord processes many sensory inputs and is therefore, open to a great deal of modulation. There are systems that can amplify responses (one of the most widely studied is the NMDA-receptor and NMDA - receptor system) and systems that can reduce these inputs, such as the opioids, adrenergic, serotonergic, GABAergic and also the purinergic systems. Controlling noxious inputs can be achieved in two ways, either by reducing excitatory systems or by activating inhibitory systems. One of the main problems with the removal of one excitatory systems is due to the fact that the spinal cord has a rich pharmacology there are plenty of alternative excitatory systems that can still be activated. Inhibiting neurones, either at the primary afferent terminal or intrinsic neuronal level may be a better strategy, since this has the possibility of controlling many excitatory systems at one go. The opioid system to date has well documented spinal analgesic effect (see Dickenson 1991, 1994b; Yaksh 1993; Yaksh and Rudy 1977). However, the use of opioids in certain pain states does not rise to adequate pain control. This may be due to loss of central primary afferent terminals and so a reduction in opioid receptors; physiological antagonism by other mediators, such as CCK and F8a; as well as alterations in spinal processing, which lead to Aβ-fibre mediated wind-up, which usually are not under the control of opioids (Dickenson and Sullivan 1986). For this reason it is of interest to identify other systems which may be a suitable target for analgesia especially in pain states that are poorly responsive to opioids

There are several reasons why the purinergic system may be one such suitable target. Firstly the main receptor for adenosine found in the dorsal horn, the A_1-receptor, is linked to the G-protein G_i and therefore is likely to result in

hyperpolarization. The location of this receptor is also important, since the bulk of binding shows that it has a post-synaptic location. This would mean that unlike opioids, A_1-receptor function will not be compromised by damage to and loss of primary afferent terminals. Thirdly behavioural studies have indicated that spinal administration can result in antinociception. For these reasons the effects of A_1- and A_2-receptor agonists, as well as protecting endogenously released adenosine, were evaluated on the response of dorsal horn neurones believed to be responsible for processing of noxious and non-noxious information.

The first step was to evaluate the responses of these cells by using transcutaneous electrical stimulation. The response to electrical stimulation is a good basis to evaluate the role of a system since this method directly activates the axons of neurones therefore bypassing the transduction mechanisms. The responses to heat or mechanical stimuli can result in peripheral changes due to tissue damage which can alter the sensitivity of subsequent stimulations. Another reason for using transcutaneous stimulation is that activation of the $A\beta$- and C-fibre evoked responses can be easily distinguished, due to threshold and latency, thus it is a fairly straight forward process to ascertain the effects on noxious and innocuous inputs.

From the acute studies it was apparent that the spinal activation of the A_1-receptor was more important in controlling and differentiating between noxious and innocuous inputs into the dorsal horn the spinal cord compared to the A_{2a}-receptor. This would seem to contradict previous behavioural study that have implied that the A_2-receptor also has analgesic effects when given spinally (Sawynok et al. 1986). However, this study used the non-selective agonist, NECA, which is equipotent for both the A_1- and A_2-receptor (Fredholm et al. 1994) and at doses shown to cause motor impairment (Karlsten et al. 1990).

The enhanced responses of post-discharge and wind-up, evoked by transcutaneous stimulation were reduced to the greatest extent by A_1-receptor agonists. These enhanced responses to a constant stimulation are known to be driven by activation and recruitment of the NMDA-receptor (Davies and Lodge 1987; Dickenson and Sullivan 1987a). It is also known that activation of this receptor underlies more persistent pain states (Dickenson 1994a) and it has been established that the NMDA receptor has an enhanced role in altered pain states (Backonja et al. 1994; Chapman and Dickenson 1994; Eide et al 1994; Ma and Woolf 1995; Persson et al. 1995; Sherman and Loomis 1994; Yaksh 1989). Although the use of conventional analgesics, such as the opioids can control these pain states, it is at doses that give unacceptable side-effects (Desmeules et al. 1993; Jadad et al. 1992).

I have shown that A_1-agonists are very effective at controlling the enhanced responses of wind-up and post-discharge. Unlike NMDA-receptor

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antagonists and μ-receptor agonists, A_1-agonists have a dual effect on wind-up, reducing both the input and increased excitability. Note that NMDA-receptor antagonists only decrease the enhanced excitability and opioids decrease input but wind-up can still break through. Indirect evidence for this control of NMDA-receptor mediated events with purines also comes from clinical studies, in that patients report a slow build-up of noxious sensations over a period of many hours after adenosine infusion has ceased (Sollevi et al. 1995). Thus suggesting that activation of the NMDA-receptor must first overcome the purinergic inhibitory threshold before transmission can proceed maximally again, as has been suggested in other areas of the brain (White 1994, 1996).

The inhibition of these experimentally produced enhanced responses, a reduction in C-fibre evoked responses, along with a lack of effect on the Aβ-fibre evoked responses, provides a sound scientific basis for the use of adenosine or analogues acting at the A_1-receptor in the treatment of more persistent, and altered pain states. Of course, as with any drug there will always be associated side-effects. However, often these unwanted effects are dose-related. Adenosine also has cardiovascular effects and is clinically the drug of choice in the treatment of supra-ventricular tachycardia (SVT) (Conradson 1987). However, it would appear that doses of adenosine required to inhibit noxious inputs are much lower than those required to treat SVT and cardiovascular side effects can be contained. This has been demonstrated to be the case in human studies (Belfrage et al. 1995; Ekblom et al. 1995; Karlsten and Gordh 1995; Sergerdahl et al. 1994, 1995, 1996; Sollevi 1992; Sollevi et al. 1995, 1996; Sylven et al. 1996).

The concurrent Aδ-fibre evoked facilitation, seen with A_1-receptor activation, was dose dependent and followed the same time-course as the inhibitions. Since these facilitations were not reversed with theophylline or blocked by

8pSPT it was considered that the facilitations may be driven by activation of the excitatory A_3-receptor on Aδ-fibre terminals themselves or on interneurones found in Aδ pathways. The A_3-receptor is known to be insensitive to methylxanthines (Linden 1994; Zhou et al. 1992). However, it has been reported that this receptor is not found in the spinal cord of the rat (see Linden et al. 1994).

Another explanation is disinhibition. The Aδ-fibre terminals (Alvarez et al. 1992; Bernardi et al. 1995) and interneuronal pathways (Baba et al. 1994) are known to be under major inhibitory control, especially by GABAergic interneurones (Alvarez et al. 1992; Bernardi et al. 1995). The A_1-receptor is primarily located on second order neurones (Choca et al. 1988; Geiger et al. 1984; Li and Perl 1994). These facilitated responses could therefore be due to A_1-receptor hyperpolarization of these inhibitory interneurones, controlling the Aδ-fibre terminals and pathways.

Since these facilitations were seen with both A_1-receptor agonists used a common mechanism such as this is likely.

What is important is what these enhanced responses would mean in an awake animal. Since $A\delta$-fibres can convey both high and low-threshold inputs these facilitations could result in nociception in an awake animal. However, since behavioural studies with A_1-receptor agonists, as well as human studies with adenosine, give rise to antinociception to a variety of stimuli, this would suggest that the facilitated responses seen in these experiments reflect low-threshold inputs.

As well as the response of neurones to acute stimulation it is also important to study their responses in models of altered pain states. The perception of sensations is not a static event. Alterations in the activity and sensitivity of primary afferents can give rise to an altered perception of sensations, where the stimulus perceived bears little relation to the actual quality of the adequate stimulus.

Inflammation can give rise to such altered perception, since chemicals released in response to tissue damage can change the way afferents are activated, either by sensitizing them to mediators that they would not usually respond to or by lowering their thresholds to mediators that they already respond to. Since tissue injury by a noxious stimulus will always result in inflammation, the role of A_1-receptors was observed in two models of acute inflammation. The role of endogenous adenosine in inflammation is a very complex one, since in the periphery at least it can have a variety of effects. ATP has been shown to be released from sensory neurones (Holton and Holton 1954; Holton 1959). The adenosine converted from ATP can add to adenosine that may be released from surrounding tissues during damage and ischemia. The consequences of release, regardless of source will depend on the concentration of adenosine at any one time, since low concentrations will activate the A_1-receptor and higher concentrations activate the A_2-receptor (Fredholm et al. 1994). Adenosine can also add to the inflammation by causing mast cells to degranulate via activation of the A_3-receptor (Linden 1994). The effects of adenosine at the site of damage can therefore be antinociceptive or pronociceptive depending on which receptors are activated.

Formalin and carrageenan are well documented models of chemically induced inflammation, resulting in changes in the periphery along with central changes (Abbott et al. 1982; Dubuisson and Dennis 1977; Hargreaves et al. 1988; Kayser and Guilbaud 1987; Robertson and Schwartz 1953; Tjolsen et al. 1992; Wheeler-Aceto and Cowan 1991; Winter et al. 1962).

It was found that spinal A_1-receptor activation was effective at controlling both the first and second phase of the formalin evoked response. This is

in contrast to behavioural studies which found that other A_1-receptor agonists only had a significant effect on the second phase of this response (Malmberg and Yaksh 1993a; Poon and Sawynok 1995). These results would imply that A_1-receptor activation can control phasic as well as tonic activity.

After carrageenan induced inflammation there was shown to be no difference in the activation of A_1-receptor between normal and carrageenan animals, demonstrating that activation of the A_1-receptor is still as effective three hours after inflammation. Other systems have been shown to have enhanced activity after carrageenan induced inflammation (Stanfa et al. 1992; Stanfa and Dickenson 1993) however, this was not found to be the case with activity at the A_1-receptor.

Another clinically relevant pain state is allodynia. Although most people associate this with sun-burn, allodynia can also be a component of neuropathic pain. Allodynia may result from changes in afferent drive, which in turn lead to central changes. Generally an altered afferent drive can result in the loss of inhibitory systems since the resultant excitotoxicity can kill off or render inactive inhibitory intrinsic neurones (Sugimoto et al. 1990). This can result in further enhanced activity which can lead to the miscoding of afferent inputs. This "miscoding" is where the usual discrimination between low- and high-threshold inputs is lost, hence non-noxious inputs are now perceived as noxious, allodynia. Spinal application of bicuculline was employed to try to evoke a pharmacologically induced state akin to allodynia in an awake animal. I can not categorically say that the changes I found, enhanced responses to noxious stimuli (hyperalgesia), loss of distinction between the banding of fibre evoked responses as well as an unmasking of responses not usually present, would give rise to a response indicative of allodynia in an awake animal. Although behavioural studies indicate that a change in perception is occurring they can not tell us exactly how the convergent neurones are responding. For this reason I feel that this study was justified as it complements the behavioural work.

It was found that A_1-receptor activation was effective at reducing and controlling the changes evoked by $GABA_A$-receptor antagonism. This backs up recent clinical studies with adenosine and analogues in patients with allodynia resulting from nerve damage (Karlsten and Gordh 1995; Sollevi et al. 1995) and from allodynia induced by mustard oil (Sergerdahl et al. 1995). What is interesting is that the effects of adenosine infusion in humans persisted long after the concentration of adenosine in the CSF was shown to decrease (Sollevi et al. 1996).

Since many pain states are multipharmacological events the use of combined therapy may be appropriate. This regime may also eleviate associated side-effects since lower doses can give enhanced analgesia (Dickenson and Sullivan 1993). I have also shown that a combined low dose of the A_1-receptor agonist, CPA

and morphine gave additive inhibitory effects. This is in keeping with the behavioural study of DeLander and Keil (1994). However, in humans when morphine and adenosine were combined no additional antinociceptive effects were seen against noxious heat (Ekblom et al. 1995). It may be that the doses in this study were still too high as in my study the top combination tested did not result in any enhanced inhibitory responses.

So far the evidence presented here has been on the role of the A_1-receptor. This does not tell us anything about the role of the endogenous ligand, adenosine. Antagonist studies were hampered by

the poor solubility of selective antagonists. Antagonists that are water soluble[and presently available] tend to be less selective, have activity at the A_2-receptor, as well as phosphodiesterase inhibitory effect. For these reasons the use of two novel adenosine kinase inhibitors were employed to try to protect endogenously released adenosine, prolonging its half life so its effects may be more easily detectable.

When given systemically (s.c.) or intrathecally, adenosine kinase inhibitors were shown to produce antinociception in response to acute electrical stimulation. The main inhibitory effects seen were on the wind-up and post-discharge responses with less of an inhibitory effects on the C-fibre evoked responses. The non-noxious inputs evoked by $A\beta$-fibre evoked responses were not affected by either kinase inhibitor. This profile is the same as the exogenously applied A_1-receptor agonists albeit to a lesser extent.

To gauge the role of adenosine in inflammation the[effects of the] kinase inhibitors were observed three hours after carrageenan induced inflammation as well as the intrathecally applied kinase inhibitor as a pretreatment to formalin evoked activity. In the formalin response both the first and the second phase were inhibited, with a greater inhibitory effect on the second phase. This would suggest that there is a greater release of adenosine during the tonic phase of this response or during the very latter stages of the first phase, thus preventing the second phase from being set-up. Note that the exogenous A_1-receptor agonist reduced both the first and second phase of the formalin response to an equal extent.

The predominant inhibitory effects on wind-up and post-discharge with protected adenosine, along with a greater inhibition of the second phase of the formalin evoked response, would suggest that adenosine (or ATP) is released in response to phenomena known to be driven by activation of the NMDA-receptor (Coderre and Melzack 1992; Davies and Lodge 1987; Dickenson and Sullivan 1987a; Haley et al. 1990; Hunter and Singh 1994; Vaccarino et al. 1993). This backs-up the

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findings that adenosine is released per se and converted from released ATP in a calcium-dependent manner in response to NMDA-receptor activation (Craig and White 1992, 1993; Hoehn and White 1990a, b; Hoehn et al. 1990; White 1996) as seen in other areas of the CNS. The effects of protected adenosine on the first phase of the formalin response as well as C-fibre evoked responses suggests that adenosine may also be released in response to AMPA-receptor activation, this has also been shown in the CNS (Craig and White 1993; Hoehn and White 1990a,b)

. It has been well documented that endogenous adenosine is released in response to ischemia and under conditions of increased excitotoxicity, such as in cases of stroke (see Meghji 1991). Adenosine then acts as a negative feedback mechanism to control further NMDA-receptor activity. For activation the NMDA-receptor requires membrane depolarization to remove the Mg^{++} block from the ion channel. The A_1-receptor hyperpolarizes membranes via increasing K^+ channel activity. Thus the NMDA-receptor may be physiologically antagonized by the hyperpolarization caused by A_1-receptor activity. If this mechanism also applies to the spinal cord it could account for the enhanced duration of action of adenosine seen in the clinic (Sollevi et al 1996), since adenosine may provide an inhibitory threshold which has to be overcome before transmission can maximally proceed again.

Spinally released adenosine showed no changes in inhibitory action [protected from degradation] three hours after carrageenan induced inflammation. This would imply that either adenosine release was not changed or that the kinase inhibition could not protect adenosine any further. Since this enzyme is active in the low-micromolar range if the levels of adenosine increase then this enzyme becomes saturated and other mechanisms take over such as the enzyme adenosine deaminase. Even if the levels of endogenous adenosine were to increase it may not necessarily result in enhanced effects of adenosine.

The kinase inhibitor given systemically after three hours carrageenan induced inflammation showed a leftward shift in inhibition of all responses apart from the $A\beta$-fibre evoked responses.

This would suggest that there is an additional site of antinociception other than at the spinal level. It can not be ascertained from these experiments where this additional site is. It may in the periphery (in which case the levels of adenosine would be low to activate the A_1-receptor, sparing the A_2- and A_3-receptors since these would be pronociceptive and pro-inflammatory). In addition it is possible that supraspinal activation of descending inhibitory controls could also result in enhanced antinociception.

The effects of both kinase inhibitors were reversed with the antagonists, 8pSPT. Whilst not being the most selective antagonist, it is water soluble and has

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reduced phosphodiesterase activity (Smellie et al. 1979). The effects of the kinase inhibitors were reversed to a varying extent with this antagonist and often reversal was not complete. This could imply several things. The antinociception seen was not mediated by adenosine alone thus this component could not be reversed. It is known that a variety of agonists can block the uptake site for adenosine (Geiger and Fyda 1991). The kinase inhibitors could also have an effect on the uptake sites for adenosine which also would not be reversed by an A$_1$-receptor antagonist.

The evidence presented here extends our understanding of the role of adenosine in the spinal cord. I believe adenosine is acting as a neuromodulator at the spinal cord level for a variety of reasons. To date there have been no distinct "adenosinergic" pathways identified. It is not at all clear that adenosine is stored in a classical way, although release of adenosine has pointed to a certain degree of calcium dependency. The only strong evidence for storage of adenosine comes from the one study by Braas et al. (1986) who showed that there was a distinct banding of adenosine contained within the substantia gelatinosa. However, this was shown to increase after ischemia, so may not represent a true 'adenosinergic' neuronal source, simply a metabolic origin. Although storage of a neurotransmitter is not an absolute requirement, since nitric oxide has been shown to be synthesized on demand (see Garthwaite et al. 1989). What makes the identification of distinct purinergic pathways hard is that adenosine and related nucleotides are an integral part of every cell. Adenosine, unlike ATP has not been shown to be stored in vesicles. Another factor is that a host of ecto-enzymes exist for the conversion of ATP to adenosine. If adenosine is released per se in any great quantity these enzymes would be superfluous.

In the CNS adenosine per se and that derived from the conversion of ATP has shown to be released in response to increases in excitation which could be potentially damaging to surrounding cells. Adenosine then appears to act to control and dampen down the increased activity thus preventing excitotoxicity. Such a negative feedback can provide a valuble mechanism for physiological homeostasis.

In the spinal cord we know that the increase in the release of excitatory neurotransmitters can lead to enhanced responses which if not kept in check can also result in cell damage and death. The consequences of these events may be altered pain states. Since A$_1$-receptor agonists and protected adenosine primarily inhibit wind-up, the phenomena of enhanced responses this would point to endogenous adenosine having a role in the modulation of enhanced sensory events.

The use of adenosine or analogues at the spinal level may therefore be of clinical use both in acute and more persistent states. If given to a patient with acute pain adenosine may prevent the pain state developing in to a more damaging state. In a patient who has already reached a state of altered pain

processing, adenosine may be able to give relief where other analgesics, which are not as effective at controlling wind-up, fail.

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