# Studies of inflammatory hyperalgesia with especial reference to the viscera.

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# Abstract:

This project examines the possibility that peripheral modulatory influences acting upon the transmission of nociceptive stimuli, arising in visceral structures, may be exploited in the development of novel analgesic compounds. An established model of persistent visceral inflammation (the turpentine-inflamed rat urinary bladder) was utilised and the phenomenon of a referred viscero-somatic hyperalgesia in response to thermal and mechanical stimulation was demonstrated. This further supports the model's relevance in the investigation of visceral pain states. The referral of sensory features was demonstrated to be modulated by agents exerting activity primarily in peripheral tissues. Assessment of potentially active molecules was made by observing the effects upon reflex responses of the urinary bladder and expression of an immediate early gene in the dorsal horn of the spinal cord.

Reflex responses of the bladder were attenuated by prophylactic administration of a capsaicin-like molecule, a bradykinin B2 receptor antagonist and a cannabinoid CB1 receptor agonist. Additionally, bradykinin receptor antagonists (acting at both B1 and B2 receptor subtypes) and cannabinoid receptor agonists (exerting actions through CB1 and CB2 receptor sites) were associated with reversal of turpentine-associated hyper-reflexic changes observed in the urinary bladder.

Agents with positive inhibitory actions in the hyper-reflexia model were further assessed, observing reductions in the c-Fos-like immunoreactivity which occurs in the dorsal horn of the spinal cord following noxious chemical stimulation of the urinary bladder. Bradykinin B2 receptor antagonists and cannabinoid CB2 receptor agonists were demonstrated to reduce this staining, further suggesting an attenuation of nociceptive traffic.

The evidence presented here provides support for the postulate that novel therapeutic strategies in the management of pain arising in the viscera may be developed. This might potentially procure a major clinical impact, in view of the common nature of pain arising in visceral structures.

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# Abbreviations:

| | |
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| ABC | Avidin biotin complex. |
| ACPD | 1-Amino-1,3-cyclopentanedicarboxylic acid. |
| ALIA | Autacoid local inflammation antagonism. |
| AMPA | 2-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid |
| ANA | Anandamide. |
| ANOVA | Analysis of variance. |
| AP-5 | 2-amino-5-phosphonovaleric acid. |
| AUC | Area under the curve. |
| B1 | Bradykinin 1 receptor. |
| B2 | Bradykinin 2 receptor. |
| BDNF | Brain derived neurotrophic factor. |
| $BF_{Thresh}$ | Baseline withdrawal threshold of fore paw. |
| $BH_{Thresh}$ | Baseline withdrawal threshold of hind paw. |
| BK | Bradykinin. |
| CB1 | Cannabinoid 1 receptor. |
| CB2 | Cannabinoid 2 receptor. |
| *c-fos* | Immediate early gene *c-fos*. |
| c-Fos | Protein product of *c-fos*. |
| CMG | Cystometrogram. |
| CPS-$WST_{0,1,2.}$ | Composite pain score. |
| DAB | 3,3'-Diaminobenzidine. |
| D-CPP | 3(R-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid. |
| DMSO | Dimethyl sulphoxide. |
| $Dur_{mic}$ | Cumulative duration of micturition. |
| $\Delta^9$-THC | Delta 9 tetrahydrocannabinol. |

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| $\Delta_{Thresh}$ | Difference between fore and hind paw withdrawal thresholds as a % of baseline values. |
| $F_{thresh}$ | Withdrawal threshold of fore paw. |
| $H_{thresh}$ | Withdrawal threshold of hind paw. |
| IASP | International Association for the Study of Pain. |
| i.a. | Intra-arterial. |
| i.p. | Intra-peritoneal. |
| L-NAME | $N^{\omega}$-nitro-L-arginine methyl ester. |
| L-NAPNA | L-$N^{g}$-nitro arginine *p*-nitroanilide. |
| MK-801 | Dizocilpine. |
| NGF | Nerve growth factor. |
| NMDA | N-methyl-D-aspartate. |
| PBS | Phosphate buffered saline. |
| PEA | Palmitoylethanolamide. |
| s.c. | Sub-cutaneous. |
| trk-A | Tyrosine kinase receptor for nerve growth factor. |
| Trk-A-IgG | Nerve growth factor sequestering molecule. |
| $V_{mic}$ | Micturition threshold of the urinary bladder. |
| VR1 | Vanilloid receptor (subtype 1). |
| VSH | Viscero-somatic hyperalgesia. |
| VVH | Viscero-visceral hyper-reflexia. |

# Chapter 1:

# Introduction

When compared to pain consequent upon damage to somatic structures, pain arising from the viscera demonstrates profound differences in both clinical and neurophysiological features [216]. This might be considered to be unimportant if visceral pain were a rare occurrence. However, since pain arising from internal structures is an extremely common clinical complaint and in addition is often poorly responsive to conventional analgesic therapies, further investigation into the transmission and modulation of such pain might be expected to have a major clinical impact. Whilst much discussion has centred around the importance of central processing in the subjective perception of visceral nociceptive events [221], drug treatments, such as opioids, directed at modulating these processes are likely to be associated with a multitude of adverse side effects [85,226]. These may range from the merely unpleasant (such as nausea and itching) to the frankly dangerous (including respiratory and cardiovascular depression). Therefore, the possibility that peripheral events may be of at least as great an importance as central processing and might also be amenable to therapeutic manipulation is intriguing. The work presented in this thesis was undertaken to assess the effects of modulation of peripheral events in an accepted animal model of persistent visceral pain - the turpentine inflamed rat urinary bladder.

## 1.i. Physiology of pain

The nociceptive response consists of a series of related neurophysiological events, subsequent upon the stimulation of receptors demonstrating preferential sensitivity to tissue damaging stimuli [1]. In contrast, pain is a subjective sensation, defined by The International Association for the Study of Pain (IASP) as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' [1].

### 1.i.a <u>Acute, persistent and chronic pain:</u>

Acute pain (usually of limited duration) commonly signals actual tissue damage, allowing the individual to elude further harm by means of avoidance behaviour [62]. Persistent pain occurs when tissue damage continues over a prolonged period of time, whilst chronic pain may result from ongoing tissue damage, but is also recognised as a disease in its own right. Under such circumstances, chronic pain is often associated with no clear tissue diagnosis, or it persists for a prolonged period beyond the normal time required for healing [282]. Abnormal sensory perceptions, such as allodynia and hyperalgesia, are common features of persistent and chronic pain states.

**Allodynia:**

Allodynia refers to the perception of pain following a stimulus that is not normally pain provoking [1]. A clinical example of this is the pain perceived by individuals suffering from post herpetic neuralgia in response to gentle stroking of the skin of the affected dermatome [288]. Under these conditions the normal specificity of the sensory mechanism is modified, such that cells in the dorsal horn of the spinal cord respond to activity in low threshold afferent neurones in a manner usually reserved for responding to

activity transmitted via high threshold nociceptors [340]. Further evidence supporting this modification in the specificity of neurophysiological processing is provided by the description of a patient known to have a severe large fibre neuropathy. Following the intra-dermal injection of capsaicin, an area where punctate hyperalgesia was demonstrated failed to exhibit allodynia [238]. Thus allodynia, unlike hyperalgesia, is dependent upon activity in neurones, which under normal circumstances, respond only to innocuous stimuli.

**Hyperalgesia:**

Hyperalgesia is defined as an increased response to a stimulus which is normally painful (see figure 1) [1,326]. This increase in response may be measured by a reduction in latency, or an increase in magnitude, of the evoked response. Moreover, this enhanced response may be observed not only at the site of the original injury (primary hyperalgesia) but also in undamaged areas of tissue surrounding the original injury (secondary hyperalgesia). The neural mechanisms associated with primary and secondary hyperalgesia differ and are discussed below. When considering these neuronal mechanisms it is important to consider not only the location and type of the tissue (such as whether it is somatic or visceral in nature), but also the type of stimulus to which a hyperalgesic response is observed (for example whether it is thermal or mechanical in nature).

Hyperalgesia in response to thermal stimuli occurs only at the site of tissue injury (i.e. primary hyperalgesia). It is at least in part, mediated by a sensitisation of nociceptors [238,273] - that is 'a leftward shift of the stimulus-response function that relates magnitude of neural response to stimulus intensity'. From a teleological standpoint, this increased sensitivity

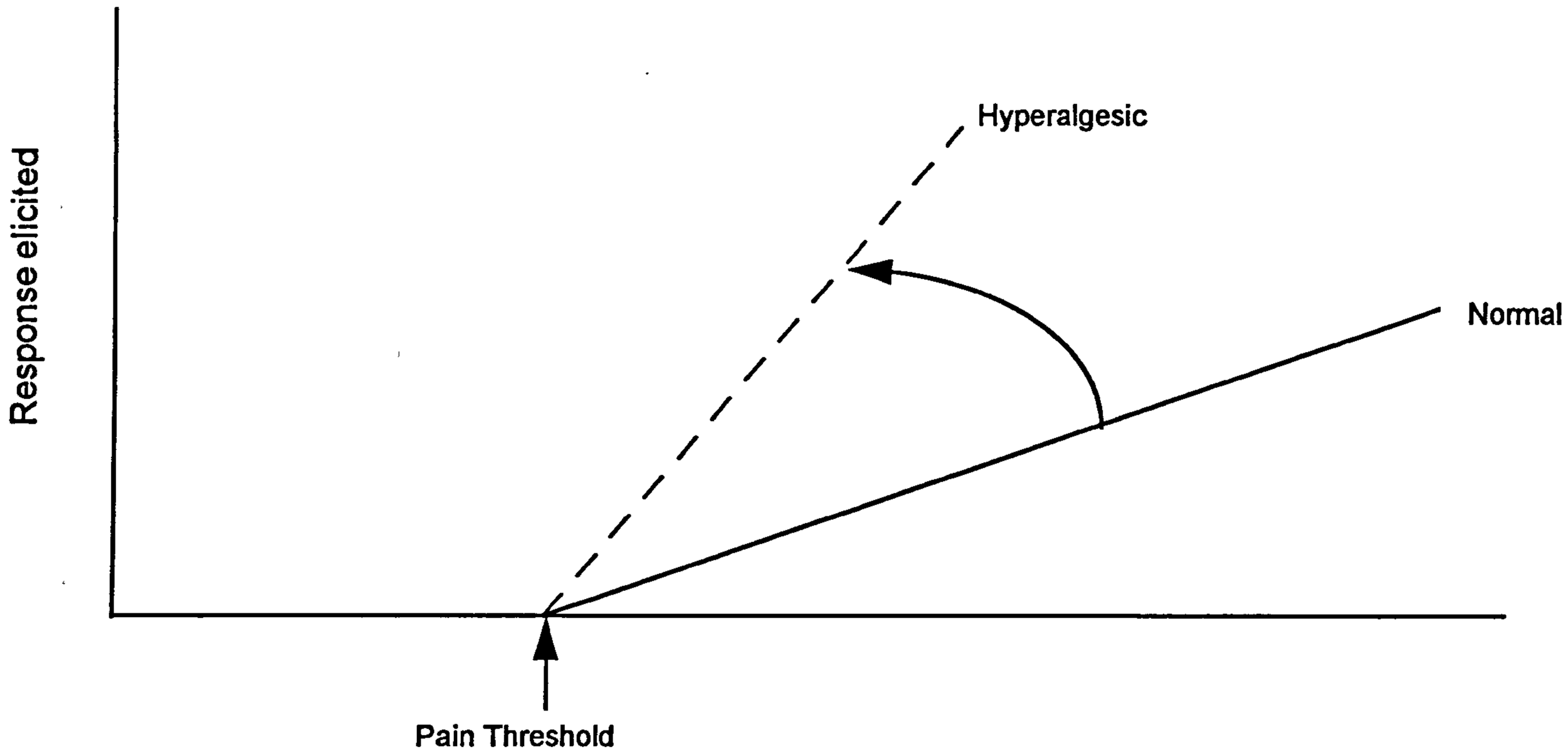



Figure 1.
Diagram demonstrating the alteration in responses (either neurophysiological or behavioural) that are observed when hyperalgesia occurs. Note the leftward shift of the stimulus - response curve in the hyperalgesic state.

may lead the individual to protect the injured area, thus promoting healing [192]. A common example of primary hyperalgesia in human volunteers is observed after exposure of the skin to a thermal injury. Such thermally induced damage is commonly followed by the subjective reporting of a localised increase in the magnitude of pain perceived in response to subsequent thermal stimulation [238]. Similar findings, of an increased response to thermal stimuli are observed locally following intra-dermal administration of chemicals such as capsaicin [97].

This subjective reporting of a rapid development of increased pain sensations by individuals [335] is matched by an enhanced responsiveness to thermal stimulation of mechano-heat receptors in animal models. Under these circumstances, electrophysiological experiments have determined that Aδ nociceptive afferent neurones supplying the skin in monkeys exhibit [238,326]:

- a decreased threshold stimulus required to elicit a given response.
- an increased response to application of supra-threshold stimuli.
- a novel spontaneous activity.

The increased responsiveness of these primary afferent neurones appears to be resultant upon the localised release of a multitude of pro-inflammatory mediators, including bradykinin [39,159,165,310], histamine [94], serotonin [165,317], prostaglandins [165], leukotrienes [238], protons [30,165], substance P [325], neurotrophins [14,182,284] and cytokines [342]. Such mediators are released from inflammatory components, such as mast cells and immunocytes [94,238]. Moreover, application of these pro-inflammatory molecules to isolated tissues, *in vitro*, is associated with increases in physiological responses such as muscle contraction [261]. In addition, when exogenously administered to the skin of human volunteers, the chemicals elicit hyperalgesic-type responses [266].

Whilst peripheral events are important in the production of hyperalgesic responses to thermal stimuli, central events also play their part. When NMDA (N-methyl-D-aspartate) is administered intra-thecally, a reduced latency of the tail flick response to radiant heat, indicative of hyperalgesia is observed [161]. This augmentation of response appears to be mediated by nitric oxide, since prophylactic treatment with the nitric oxide synthase inhibitor L-NAME ($N^{\omega}$-nitro-L-arginine methyl ester), prevents the onset of such hyperalgesia. Furthermore, intra-thecal administration of nitric oxide donor compounds (such as sodium nitroprusside) results in the production of similar hyperalgesic responses to thermal stimuli [161].

Hyperalgesia occurring in response to mechanical stimulation is observed both at the site of injury, but also in normal tissue, distant from the area of damage (i.e. secondary hyperalgesia) [326]. An example of secondary hyperalgesia in response to mechanical stimulation is observable in human volunteers when a thermal or chemical (capsaicin) injury is applied to the skin. Such injury is followed by an increased responsiveness to mechanical stimulation in undamaged tissue of the surrounding the area [8,291]. This area of secondary hyperalgesia is not only distinct from the site of primary injury, but also exceeds that in which inflammatory changes, such as oedema and hyperaemia, occur. Moreover the observed hyperalgesia outlasts any such inflammatory tissue changes [8,142,326].

Alterations in peripheral events may influence this spreading of the hyperalgesic response [192]. When an area of skin is injured (by either thermal or mechanical means) the amplitude of the response of individual Aδ high threshold nociceptive units, to a subsequent mechanical stimulus (applied by von Frey hairs) is unchanged. However, the size of the receptive field of individual nociceptive fibres expands. Thus the potential for spatial summation (as distinct from temporal summation) in response to subsequent

punctate mechanical stimulation might provide adequate explanation for the increase in pain experienced in the injured area [238]. Moreover, low threshold mechanoreceptors provide an inhibitory input to the dorsal horn of the spinal cord, that is by activity in (described by Melzack and Wall in the 'gate control theory' [236]). This peripheral inhibition appears to decrease following injury to tissue. Attenuation of such peripheral inhibitory activity would tend to lead to a central disinhibition of input from primary afferent nociceptors, increasing perceived pain [238].

However, alterations in central events appear to be of particular importance in the phenomenon of secondary hyperalgesia [221,238,340]. If the nociceptive input to the central nervous system from Aδ high threshold nociceptive units is prevented, by prior neuronal blockade (using local anaesthetic agents) the primary tissue response continues unchecked. In contrast, the secondary hyperalgesic response is totally ablated, suggesting that alterations in neural mechanisms at a purely peripheral level are unlikely to entirely account for secondary hyperalgesia [238]. However, the same data might also suggest that peripheral events are required to initiate the phenomenon of central sensitisation. Further evidence for this is provided by the observation that if capsaicin is administered peripherally, a marked secondary hyperalgesia results, that is somewhat reduced if the injection area is subsequently cooled or anaesthetised [238,326].

The hyperalgesic response, to mechanical stimulation, following tissue injury appears to demonstrate two distinct time courses - possibly with different underlying mechanisms. The acute onset of a transient hyperalgesia seems to depend upon synergistic responses to AMPA (2-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid) and metabotropic glutamate receptor activation [234,235]. When AMPA and trans-ACPD (trans-1-Amino-1,3-cyclopentanedicarboxylic acid) are administered intrathecally to rats a reduction in the mechanical force that needs to be

applied before the limb is removed, is observed. Moreover, intra-thecal administration of quisquilate, which activates both AMPA sensitive ionotropic and trans-ACPD sensitive metabotropic receptors, is associated with an early hyperalgesic response to mechanical stimuli. Conversely, the hyperalgesic response to quisquilate is inhibited by the co-administration of AMPA and metabotropic receptor antagonist molecules.

In contrast to these early events, hyperalgesia which demonstrates a delayed onset, appears to rely, at least in part, upon NMDA-dependent mechanisms [168]. When intra-plantar carrageenan is administered, tissue inflammation and hyperalgesia to mechanical stimulation develop over a period of several hours. The hyperalgesia is dose dependently attenuated by the systemic administration of receptor antagonists acting at the glycine regulatory site of the NMDA receptor. The transduction mechanism by which NMDA activation exerts effects may well be the nitric oxide system [241]. Following intra-plantar carrageenan, tissue inflammation is reduced by the systemic administration of nitric oxide synthase inhibitors [285]. Furthermore, following formalin injection into the rat paw (the responses to which are partially mediated by NMDA [132]) an increase in the number of neurones in the dorsal horn of the spinal cord expressing nitric oxide synthase is observed [169]. Additionally, in a rat model of visceral pain, administration of both NMDA [275] and nitric oxide synthase inhibitors [274] is associated with a reduction in the hyper-reflexic changes observed.

However, non-NMDA mediated effects may also be important. Intra-plantar zymosan injection is associated with a similar delayed mechanical hyperalgesia, that appears to involve activation of not only NMDA receptors, but also of metabotropic glutamate receptors. Activation of these receptors may effect changes in the production of an alternate gaseous neurotransmitter, carbon monoxide. This is produced in a reaction catalysed by the neuronally associated enzyme, haem oxygenase. When this enzyme is

blocked by the administration of metalloporphyrins, mechanical hyperalgesia is attenuated [235]. It is possible that these non-NMDA mediated effects may be instituted by a nerve growth factor (NGF)-associated mechanism, since systemic NGF administration is followed by a similar delayed onset of mechanical hyperalgesia, that is not attenuated by the administration of NMDA receptor antagonists [182].

## 1.i.b. Differences in visceral & somatic pain:

The classical theory of pain transmission based upon Descartes description, relies on a hard wired system of anatomically distinct neurones, which transmit nociceptive information from damaged tissue to the sensory cortex, by way of relay stations in the dorsal horn of the spinal cord and the thalamic nuclei [152] (see figure 2). In this system, no distinction is made between acute, persistent or chronic pains, or pain arising from somatic and visceral structures. However, it is now clear that, the nervous system shows plasticity of response (as exemplified by hyperalgesia), with current nociceptive responses being influenced by prior events [330]. In addition to this variability across time observed in both domains, pain arising from somatic structures exhibits different clinical and pathophysiological features to that arising from visceral structures [221].

### Effective stimuli:

The roles of the nociceptive processes in the two domains are rather different. Somatic structures are exposed to external threats, to which avoidance behaviour may be mounted, whilst visceral organs are much more influenced by internal disease processes, against which it is not possible to mount such a defence. It is therefore unsurprising that the stimuli associated with the perception of pain are different in the two domains. As regards

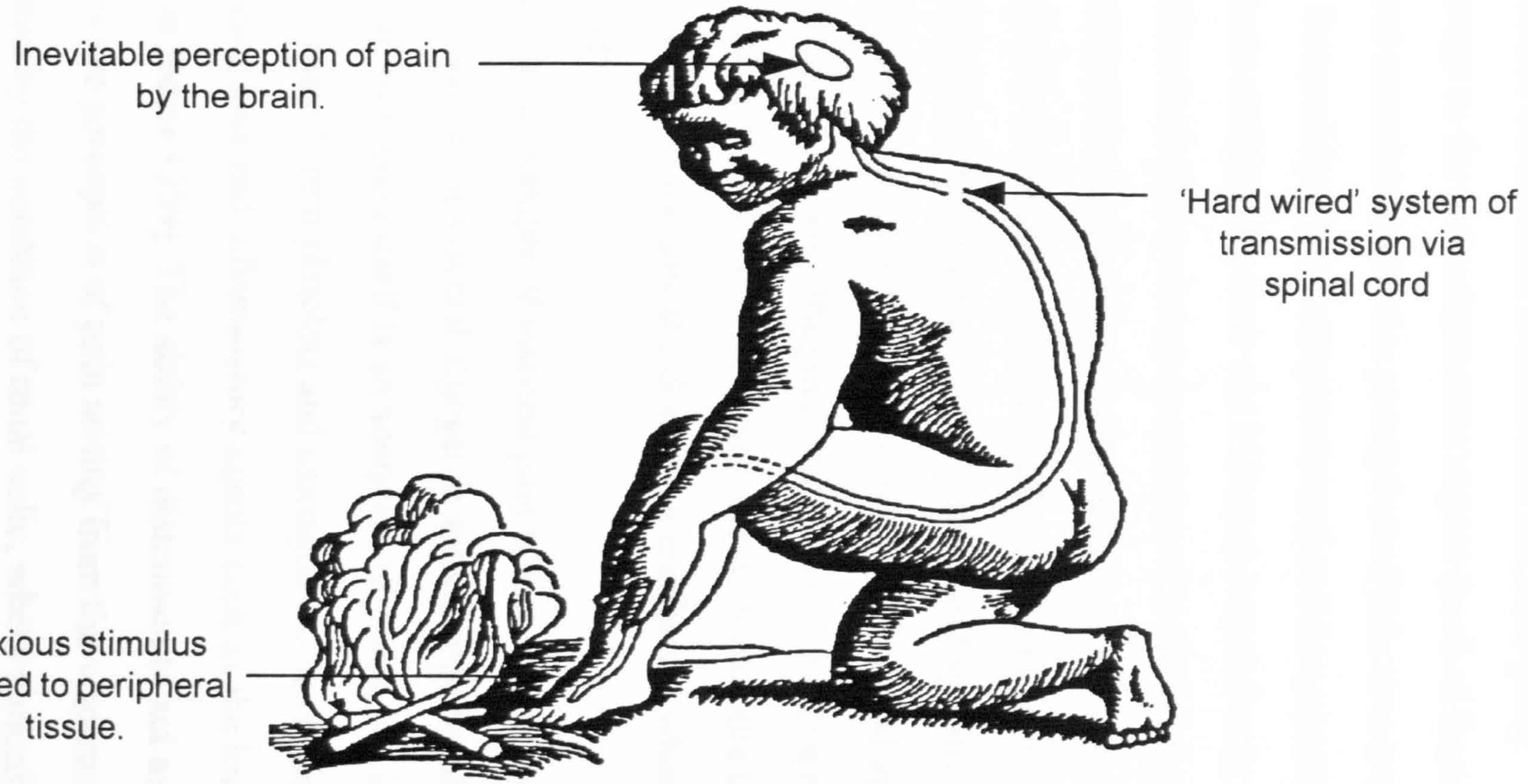



Figure 2.
Diagram to demonstrate Descarte's concept of the direct neural thread between skin and brain, leading to the inevitable perception of pain following application of a noxious stimulus to peripheral tissue (1664).

somatic structures, direct thermal, mechanical, electrical and chemical stimuli may all be effective in producing pain once a threshold level is reached (and these commonly lead to defence reactions). However, in the visceral domain the situation is somewhat different [216].

Damage to the parenchymatous organs (such as hepatic and renal tissue) is not associated with the perception of pain or any other sensory response. Deep viscera (including the heart) and the parenchymal capsules (such as those surrounding liver and kidneys) respond only to painful stimuli, whilst the hollow viscera in contact with the external environment (such as urinary bladder, colon and stomach) respond to both innocuous and noxious stimuli. The stimuli which are effective in inducing pain arising from these organs appear to be somewhat restricted and specific to the viscera. As such, distension, contraction, ischaemia and chemical application are all adequate stimuli in evoking the perception of pain when applied to visceral tissues [17,135,209,250,268]. However, the direct trauma that is an adequate stimulus in the somatic domain is ineffective when applied to the viscera [221].

A common example of visceral pain is that arising from myocardial infarction. A variety of visceral algogenic stimuli may be of importance in this situation: ischaemia itself is an adequate stimulus, but in addition chemicals released from platelets and damaged tissue (including hydrogen and potassium ions and inflammatory agents such as the kinins) may also be algogenic in nature [269]. The ability of distension to act as an adequate stimulus for the perception of pain arising from the viscera is clearly demonstrated by the condition of renal colic, where abnormal distension of the ureter (commonly following obstruction by a stone) is associated with severe colicky-type pain [99].

Although autonomic reactions are the most frequently recognised of the clinical signs associated with pain arising in the viscera, there have also been descriptions of hyperalgesia 'referred' to superficial structures, distant from the site of tissue damage [17,121,123,135]. In the case of myocardial infarction, it is recognised that tenderness may occur in the muscles of the chest, inter-scapular region and forearm - an obvious description of referred hyperalgesia to mechanical stimulation [269].

**Neurophysiology:**

Whilst clinicians have described differing patterns of symptomatology, scientists have observed that there are also differences in the neural processing of noxious stimuli arising from somatic and visceral domains.

a) Primary afferent nociceptors:

In the somatic domain non-noxious afferent information is transmitted by large Aβ myelinated fibres (demonstrating rapid transmission rates), which are selectively sensitive to warmth, cold or pressure. In contrast, noxious information travels in specific nociceptive pathways composed of small, myelinated Aδ and unmyelinated C fibres (with much slower conduction velocities), which are distinguished by the requirement for a high threshold for stimulation prior to any active response [206,238]. Thus, for sensations arising in somatic structures specificity of neuronal pathways is the norm.

In contrast, the primary afferent neurones supplying visceral organs are entirely composed of small fibres. These small fibres must therefore encode both noxious and innocuous information. The classical teaching regarding this has been that the central nervous system interprets alterations in the frequency or pattern of electrical activity to distinguish between qualitatively different sensations. Summation of activity in separate neuronal

**Characteristics:**

Unlike pain arising from somatic structures (which tends to be perceived as focal in origin, with well defined boundaries) early pain arising from the viscera is usually extensive and poorly localised. A common example of such pain is that associated with myocardial infarction. This is most frequently 'deep' in nature and described in terms that exclude the use of the word 'pain' but include phrases such as 'squeezing', 'tightening' or 'constricting' [269].

Whilst such early perceptions are described as arising from deep tissues later sensations are often localised to more superficial structures, frequently in areas distant from the site of origin of the initiating stimulus [135]. This referral of pain commonly results from repeated or prolonged visceral algogenic processes and is usually segmental in nature [268]. It is most frequently localised to structures innervated by the same spinal nerves as the damaged viscus [216]. For example, in patients suffering a myocardial infarction, pain is commonly perceived as arising from the left arm (an area supplied by spinal nerves C8 and T1) or less frequently the neck (supplied by C2 and C3) [2,269]. A fuller discussion of referred pain is contained in section 2.iv..

In addition to the characteristic early deep pain and later referral patterns, visceral pain is frequently associated with 'autonomic' symptoms. A common example of clinical symptoms associated with such autonomic activity is the nausea, vomiting and sweating described by patients suffering with myocardial ischaemia or infarction. This may be exceptionally intense and associated with alarm reactions which are extremely distressing to the individual [269]. Furthermore, autonomic reactions may include vasomotor responses such as profound alterations in heart rate and blood pressure, which precede the onset of pain itself and are themselves pathological in nature [268].

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fibres (rather than anatomical separation of pathways) is required to achieve such a wide range of information transmission [51,222]. Such 'intensity coding' and spatial summation has been termed the 'pattern theory', in contrast with the specificity theory described above for encoding sensory information arising in the somatic domain [51,222]. More recently however, it has been demonstrated that this 'pattern theory' is not the sole explanation for the ability to distinguish between innocuous and noxious stimulation applied to visceral tissue - a degree of specificity of pathway is also present in visceral tissues. When activity in the pelvic nerve (supplying the urinary bladder) of both the cat and rat is recorded, it is possible to demonstrate the presence of two distinct populations of primary afferent neurones, sharing similar conduction velocities and threshold responses. One group (comprising approximately 80% of the neurones) has a low stimulus threshold and increasing intra-vesical pressures (from 0 to 20mmHg) are reflected by alteration in the frequency of action potentials transmitted (intensity coding). However, the second, smaller population of neurones, exhibit a high stimulation threshold prior to the observation of any response (mean intra-vesical pressure +/- sem, 34mmHg +/- 2.5). This suggests that in addition to the graded intensity coding neurones previously described, a group of specific nociceptors is also present [129,292].

Furthermore, it is known that up to ninety percent of primary afferent neurones supplying the visceral domain demonstrate no response to any physiological stimuli under normal circumstances [223]. However, under conditions of inflammation, these so called 'silent afferents' respond to physiological stimulation. This recruitment involves the development of a novel mechanosensitivity to stimuli which usually lie outside the noxious range [51,129,223,238]. Thus, whilst only approximately three percent of unmyelinated primary afferent neurones supplying the cat urinary bladder demonstrate electrical responses to distension (and none are spontaneously

active), following induction of inflammation, a high proportion of fibres develop a spontaneous, resting activity and also demonstrate electrical activity in response to distension of the bladder [128]. In contrast, in somatic tissues the prevalence of these mechanically insensitive afferents is of the order of only approximately thirty percent [238]. Thus the novel activity in 'silent' afferent neurones is likely to be of particular importance in the interpretation of nociceptive information arising from the viscera [128-130,223].

b) Dorsal horn processing:

Primary afferent neurones supplying the viscera of the pelvis enter the spinal cord in the thoraco-lumbar region, in association with efferent autonomic neurones [216].Retrograde tracing techniques demonstrate that synaptic terminations occur mainly in Rexed laminae I, V and X [79] (a similar distribution to capsaicin sensitive neurones [151]). Although the absolute number of primary afferent neurones arising from the viscera is low, because of widespread ramifications, a relatively high proportion of spinal neurones are activated following peripheral stimulation [216]. Upon stimulation of visceral organs by distension, it is possible to identify subsets of dorsal horn neurones which demonstrate different minimum thresholds prior to response [216]. As is the case when electrophysiological responses of primary afferent neurones are examined, both intensity coding and spatial summation are observed [163,249]. Moreover, alterations in the electrical responses of dorsal horn neurones are observed following visceral inflammation. Again, both a leftward shift of the stimulus-response curve [215] and a novel mechanosensitivity of previously unresponsive neurones [216] can be observed - changes reflective of neuronal sensitisation. This sensitisation is likely consequent upon a barrage of neuropeptide release from the central terminals of primary afferent neurones [79,216]. Since a

high proportion of the dorsal horn neurones which are activated following visceral stimulation, also exhibit excitatory responses to convergent inputs from receptive fields arising in somatic tissues [249], the possibility of referral of responses to structures in the somatic domain, can be imagined.

## 1.ii. Neuroimmune interactions

It is well known that the immune system is important in the recognition and removal of pathogens and tumours. However, it has more recently become clear that interactions between the immune and neurological systems occur and that these may be important in the modulation of nociception [331]. This holds possibly important implications for the management of persistent pain states. Immune processes consequent upon tissue damage, occur primarily in peripheral tissues. Agents acting to inhibit these effects might be less likely to be associated with problematic central side effects, such as those associated with the opioid drugs [226].

### 1.ii.a. <u>The inflammatory response:</u>

Inflammation is the common defence reaction of the body in response to a wide range of tissue insults. The triple response (first described by Lewis in 1927) consists of erythema, swelling and an increase in temperature in the area of damage. Both non-specific and specific immune activity occurs. Non-specific activity includes:

a) the phagocytic action of cells, including the monocyte derived macrophages.
b) the release reaction of mast cells.
c) the activation of kinin, complement, coagulation and fibrinolytic cascades.

Specific immunity involves the recognition of foreign antigens with the subsequent attraction of B and T lymphocytes, leading to opsonisation of 'non-self' tissue by antibodies and subsequent cytotoxic removal.

## 1.ii.b. Mast cells:

Although mast cells were first described by Friedrich von Recklinghausen and Paul Erlich in the late 1880s, there is still no consensus of opinion regarding any specific biological functions attributable to them [237]. These cells comprise a heterogeneous population, extensively distributed in the body and most commonly observed in perivascular and perineural locations in peripheral tissues [320]. However, mast cells have also been identified in sites such as the central nervous system, for example in the chiasmal plaques (examined at autopsy) of patients suffering from multiple sclerosis [255].

Upon exposure to a wide variety of stimuli, mast cells responses include: antigen processing, cytokine production and the release of vasoactive and nociceptive molecules. Algogenic and pro-hyperalgesic molecules which are released from mast cells include histamine, serotonin and nerve growth factor (NGF) and application of these molecules to peripheral neurones is known to result in excitation of the primary afferent nerve terminals [238].

Mast cells express a number of different receptors including the trkA receptor for NGF [179,251] and the cannabinoid CB2 receptor [101,115]. Whilst stimulation of the trkA receptor for NGF has been observed to promote mast cell survival *in vitro* [141], it has also been demonstrated to induce a release reaction resulting in the localised secretion of pro-inflammatory molecules, including NGF itself [238]. Thus, molecules such as NGF, released during inflammation, provide a mechanism by which positive feedback of inflammatory processes may result in ongoing tissue

damage. Similarly, it is possible that stimulation of alternate inhibitory receptors, such as the CB2 cannabinoid receptor may provide a negative, downregulatory mechanism on the inflammatory cascade (this is discussed further in section 6.iv.).

In the clinical setting, mast cells appear to be of importance in chronic painful conditions (such as interstitial cystitis and irritable bowel syndrome) afflicting visceral organs including the urinary bladder and gastro-intestinal system. In patients suffering with these conditions, a profound localised mastocytosis is observed. Such areas exhibit a proliferation of substance P containing neurones [258] further suggesting the potential for interactions between the nervous and immune systems. Once an inflammatory reaction has been initiated, for example by an episode of bacterial gastro-enteritis [247], a self-sustaining response may result. Thus, discharge of pro-inflammatory mediators from damaged cells might result in the release of pro-inflammatory neuropeptides (such as substance P and calcitonin gene related peptide) from the distal terminals of associated primary afferent neurones, producing ongoing stimulation of inflammatory cells. These neuropeptides are also released from the central terminals of such primary afferent neurones, possibly resulting in central (in addition to peripheral) sensitisation [56,79,328].

## 1.iii. The urinary bladder

### 1.iii.a. <u>Innervation of the urinary bladder:</u>

The urinary bladder is a hollow pelvic organ, the normal function of which is the storage of urine produced by the kidneys. This is achieved by the gradual distension of the viscus, prior to urine excretion during active intermittent micturition contractions. The normal resting intra-vesical

pressure has been shown to vary from 0 - 15mmHg in rats, but during micturition contractions this may rise to approximately 25mmHg [221]. Such contractions are not normally associated with the perception of pain [250].

The afferent neuronal supply of the bladder is transmitted with both sympathetic and parasympathetic nerve trunks. Retrograde tracing experiments using horseradish peroxidase have demonstrated the spinal target sites of these neurones [183]. An intact sympathetic supply is not essential for micturition to occur. However, it appears to be of importance in the maintenance of bladder neck closure and the inhibition of parasympathetic activity during the storage of urine. The afferent neurones which travel with the sympathetic supply from the hypogastric and lumbar splanchnic nerves, project to the grey matter of the intermediolateral area of the spinal cord at the T10 to L2 levels

In contrast, the presence of functional afferent neurones travelling with the parasympathetic supply is an absolute requirement for the normal micturition process to occur. These neurones project to Lissauer's tract and the intermediolateral grey of the parasympathetic nucleus located in the S2 to S4 spinal cord levels (in humans) and the L5, L6 and S1 levels (in rats) [79]. In addition to these capsaicin sensitive neurones [151], information also reaches the spinal parasympathetic nucleus from the micturition centre in the rostral pontine portion of the brainstem [183]. If the central portion of the reflex pathway is severed (such as after cord transection), an early phase of urinary retention is followed by the appearance of automatic, spinal, micturition contractions, which are often hyper-reflexic in nature [109] and are propagated by electrical activity occurring in unmyelinated C-fibres [78]. A similar hyper-reflexic response is observed during inflammation of the urinary bladder, when abnormal input is received from the periphery [106,138].

## 1.iii.b. Pain arising from the urinary bladder:

A number of pathophysiological conditions affecting the urinary bladder may result in the perception of pain. This is commonly perceived as an unpleasant sensation of distension of the bladder and supra-pubic region [99]. Additionally, as with pain arising from other visceral structures, a component of the pain is referred to distant, superficial sites. Referred pain arising from the bladder is commonly perceived as discomfort occurring in the inner thighs [214]. However, it may also be reported as arising from structures as far distant as the knees [250] or feet [135].

The most common causes of pain of vesical origin are inflammatory conditions (of an acute or chronic nature), where pain is often associated with symptoms including frequency of micturition. Acute cystitis usually presents as a result of bacterial infection and rapid resolution of symptoms, following appropriate antibiotic therapy is the norm. However, chronic inflammation of the bladder is less commonly associated with a particular causative factor and is correspondingly less responsive to specific therapies [43].

Whilst normal micturition contractions are rarely described as painful, pathological changes resulting in abnormal micturition activity may be associated with the perception of pain. When micturition contractions occur against an outflow obstruction, such as an enlarged prostate, the intra-vesical pressures may exceed the noxious threshold (of approximately 30 - 40mmHg) [221,250]. Similarly, during inflammatory processes involving the urinary bladder, intra-vesical pressures exceeding 40mmHg may be attained. Under these abnormal conditions associated with raised intra-vesical pressure, pain may be perceived. Furthermore, the presence of inflammation of the bladder mucosa is known to be associated with a reduced pain threshold to mechanical and electrical stimuli and an increased response to such stimuli (in addition to altered reflex responses) [214,250]. This

alteration in sensory perception, observed following inflammation of the urinary bladder demonstrates analogies with responses observed following inflammation of cutaneous tissue, suggesting the concept of a visceral hyperalgesic response. Thus, an increased responsiveness to both thermal and mechanical stimulation is associated with increased activity in both primary afferent and dorsal horn neurones. These features are discussed more fully in section 1.iv.a.

Experimental evidence derived from somatic tissues, demonstrates that electrical activity in capsaicin-sensitive neurones is crucial in the transmission of noxious information from the skin [271,316]. There is evidence to suggest that this is also the case for pain arising from the urinary bladder. Capsaicin-sensitive neurones form a dense plexus in the sub-mucosal layer of the bladder (in close association with blood vessels). These neurones appear to be both chemosensitive and mechanosensitive [190]. Pain-related behaviour (licking of the lower abdominal wall and perineum) is observed following intra-vesical instillation of xylene and this is abolished by the prophylactic administration of capsaicin [190]. Similarly, when vesical distension exceeds a critical threshold, reflex active contractions of the urinary bladder result, which are impaired by the prior administration of systemic capsaicin [190]. This mechanosensitivity may be prostanoid mediated since levels of prostanoids are increased in pelvic venous blood during bladder distension and prophylactic treatment with cyclo-oxygenase inhibitors increases bladder capacity [190]. Thus, distension of the bladder might lead to local prostanoid production and activity in primary afferent neurones of the pelvic nerve. This is an effect that, given the increased level of prostanoids observed following tissue injury, would be likely to be enhanced during inflammation [114,298].

*In vivo* work demonstrates that intra-vesical administration of chemicals (including bradykinin, prostanoids, 1% acetic acid and turpentine)

both facilitates the micturition reflex [79,173,190,218] and also increases activity in pelvic nerve afferent neurones, supplying vesical tissue [215]. This facilitation of vesical reflexes is also attenuated by the prophylactic administration of systemic capsaicin [173]. Moreover, noxious stimulation of the bladder by 1% acetic acid is associated with an increased expression of the immediate early gene *c-fos* (a nociceptive marker) in the areas to which parasympathetic-associated neurones project [36] and this upregulation is greatly attenuated by the prior administration of systemic capsaicin [67].Thus, inflammation of the rat urinary bladder appears to result in responses commonly associated with nociceptive events.

## 1.iv. Models of visceral pain:

### 1.iv.a. <u>The turpentine inflamed rat urinary bladder model:</u>

Because of the relative difficulty of gaining access to and providing effective noxious stimuli to internal organs, useful animal models of persistent visceral pain are less common than those of pain arising from somatic structures [118]. However, in 1987 McMahon and Abel described a model which mimics many of the features observed in painful conditions arising from the urinary bladder in human patients [218]. This model (the chemically inflamed rat urinary bladder) was originally described in the chronically decerebrate rat, but I have used similar techniques in the urethane anaesthetised rat. Since the early experiments utilising this model, turpentine oil has been used as the sole inflammatory agent, since the instillation of the alternative agents - 2.5% croton oil or 2.5% mustard oil - is associated with extensive tissue damage [218]. Following intra-vesical instillation of turpentine oil a number of reproducible features have been documented [89-91,215,218,221,275]:

i) An inflammatory response is observed as evidenced by:

a) Protein extravasation into bladder tissue [218].

This has been documented by observing the increase in the amount of albumin bound Evan's blue dye extravasated into vesical tissue, in animals treated with intra-vesical turpentine oil. The time course of this extravasation appears to be biphasic, with an early leakage (evident by one hour post-turpentine instillation) followed by a diminution and a subsequent sustained increase, until forty eight hours post-induction of inflammation.

b) Invasion of bladder tissue by leukocytes [218].

This has been documented by examining haematoxylin-eosin stained sections of vesical tissue under light microscopy. Whilst emigration of leukocytes is extremely variable, in general, a raised level of leukocytes is observed in bladders from animals previously treated with intra-vesical turpentine oil (in comparison to control animals).

c) Vesical tissue oedema [215,218].

This is partially a neurogenic phenomenon to which primary afferent neurones contribute. The importance of the presence of an intact neuronal supply has been demonstrated by the reduction in such oedema formation when tissue is chronically denervated [225].

ii) Viscero-visceral hyper-reflexia (VVH) (see figure 3) [91,218,275]:

When the normally compliant urinary bladder is gradually filled with fluid within the physiological range, initial distension of the viscus prevents any striking rise in measured intra-vesical pressures. However, once a critical threshold volume is reached a series of large, regular waves of increased pressure are observed, which continue for

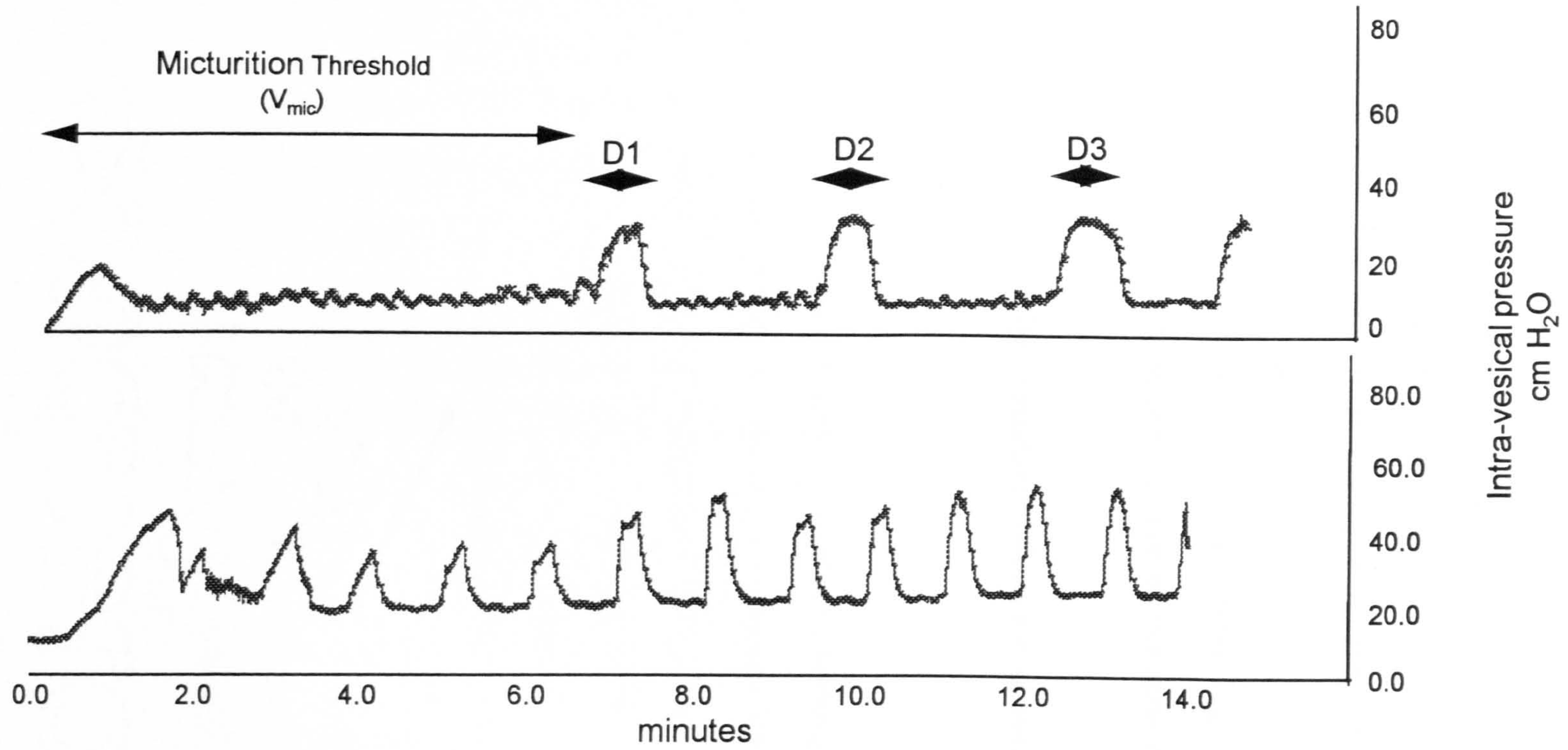



Figure 3.

Example of serial cystometrograms (CMGs) taken from the same animal. The upper trace is a baseline CMG demonstrating the relationship between intra-vesical volume (read on the time scale, since rate of distension of the bladder is constant at 0.05ml/min) and pressure. Note that the micturition threshold ($V_{mic}$) is reduced and the cumulative duration of micturition ($Dur_{mic}$ - calculated from D1+D2+D3) is increased following induction of inflammation with agents such as turpentine oil (see lower trace).

the duration of filling. These can be recorded by cystometrography (CMG) and are thought to reflect the onset of active micturition contractions. Following inflammation of the urinary bladder, these reflexes are observed to be hyper-excitable, the features of which are:

a) An elevation in intra-vesical pressures for a given intra- vesical volume, reflecting an increase in bladder wall tension (i.e. an upward and leftward sift of the stimulus-response curve - see figure 1 on page 20).

b) A reduction in the micturition threshold, $V_{mic}$ (the intra-vesical volume at which the onset of active micturition contractions occurs).

c) An elevation in the cumulative duration of micturition contraction times, $Dur_{mic}$.

These alterations in bladder reflex responses following inflammation are thought to a great extent to be secondary to unmasking of spinally mediated micturition reflexes. Such hyper-reflexic activity is not observed if the bladder is acutely denervated, either surgically [218] or by the administration of capsaicin [79]. The VVH associated with bladder inflammation is prevented by the administration of intra-thecal NMDA antagonists such as AP-5 (2-amino-5-phosphonovaleric acid) [275] and nitric oxide synthase inhibitors such as L-NAPNA (L-$N^g$-nitro arginine *p*-nitroanilide) [274], at dose ranges which have no effect on normal bladder reflexes. Moreover, alterations in the level of expression of nociceptive markers in the dorsal horn of the spinal cord is observed following inflammation (see below) [89].

iii) Viscero-somatic hyperalgesia (VSH) [218]:

This is defined as hypersensitivity to noxious stimuli applied to somatic structures distant in location, but segmentally appropriate for the inflamed bladder.

Noxious thermal and mechanical stimuli applied to the tail, foot and caudal abdomen are associated with reflex withdrawal responses in the decerebrate rat. Following intra-vesical turpentine treatment, thermal stimulation of tail, foot and caudal abdomen (with water at $49^0$C) is associated with a reduction in latency of withdrawal times, reflective of hyperalgesia. Similar findings have been documented when a punctate mechanical stimulus is applied, by means of pincers attached to a strain gauge.

iv) Alterations in neuronal excitability [215,221]:

a) An increase in excitability of primary afferent neurones in the pelvic nerve, supplying the urinary bladder is observed following intra-vesical administration of inflammatory agents [215,221]. Using extra-cellular recording techniques, myelinated Aδ, mechanosensitive fibres can be shown to be rapidly excited by the intra-vesical application of inflammatory agents, an effect which persists even after removal of the inflammatory stimulus. Moreover, subsequent direct electrical stimulation of the afferent neurones, is associated with a reduction in the stimulus threshold or an increase in the response to supra-threshold stimulation.

Thus an enhanced peripheral afferent barrage occurs in response to mechanical distension of the bladder (primary hyperalgesia).

b) An increased excitability of dorsal horn neurones in the relevant segments of the spinal cord has also been described [215].

The response properties of dorsal horn cells, which respond to supra-maximal stimuli applied to vesical afferents in the pelvic nerve, have been studied. Following turpentine-induced inflammation of the bladder:

- The ongoing activity of these dorsal horn cells, in response to bladder distension, is increased to 184% of baseline.
- In more than half of these dorsal horn cells the response to a single supra-maximal electrical stimulus applied to peripheral vesical afferent neurones is increased.
- A novel mechanosensitivity is induced (or enhanced) in 50% of the dorsal horn cells.

Thus, the afferent barrage produced following vesical inflammation is associated with alterations in the excitability of the central component of the nervous system response, reflecting hypersensitivity.

c) A novel recruitment of previously 'silent' afferents occurs [221]. Following intra-vesical instillation of irritant chemicals, unmyelinated primary afferent neurones, which have previously demonstrated no response to intra-luminal pressures in either the physiological or supra-maximal range, develop a unique mechanosensitivity within the physiological pressure range. This mobilisation of previously unresponsive neurones may as much as double the afferent inflow of information to the spinal cord, under conditions of inflammation.

v) Alterations in levels of immediate early gene expression occur (see also section 1.vi.) [89]:

Under normal conditions, little c-Fos-like immunoreactivity is observed in the spinal cord [52]. However, following chemical

inflammation of the urinary bladder, an increase in staining for such c-Fos-like immunoreactivity in the spinal cord segments in which vesical afferents terminate (L5, L6 and S1 in the rat) is observed [89].

The most consistent of the changes associated with turpentine-induced inflammation of the bladder is the viscero-visceral hyper-reflexia (VVH). Furthermore, whilst the VVH might be expected to be more indicative of micturition frequency and urgency, it is the most predictable monitor of pharmacological interventions in the model. The consistent relationship of the VVH with the other features associated with visceral pain described above, suggest that it might be useful as a method of assessing modulation of visceral pain mechanisms [215,218].

## 1.iv.b. Other models of visceral pain:

For animal models to provide a reliable source of information regarding the transmission and modulation of nociceptive stimuli, key criteria need to be achieved [118]:

- The stimulus applied should be:
  - representative of pathophysiological stimuli.
  - minimally invasive.
  - controllable.
  - reproducible.
  - quantifiable.
- The response observed should be:
  - observable in the unanaesthetised animal.
  - reliable.
  - reproducible.
  - quantifiable.

Unfortunately, whilst numerous models of pain arising from somatic tissues have been developed, the fulfilment of these criteria is an ongoing problem in the modelling of visceral pain. Animal models which utilise chemical inflammation of a viscus are reasonably representative of inflammatory conditions, such as cystitis or inflammatory bowel disease. However, the degree of inflammation elicited (and therefore the expected response) is likely to be rather variable. In addition to the variability in physical application, consequent upon the difficulty in achieving visualisation of the endothelial surfaces of the viscus, the concentration of the inflammatory agent is likely to be reduced by dilution with physiological substances (such as urine). In the case of the urinary bladder, this can be overcome by separation of the inflow tract (the ureters) from the viscus [194], but this requires additional invasive surgery, with a concomitant increase in alternate nociceptive stimuli.

Balloon distension of hollow organs has been used as a nociceptive stimulus and it is certainly more quantifiable and controllable than chemically stimuli. Whilst it is a natural stimulus of hollow organs such as the bladder [221], in the clinical setting it is much less common as a cause of ongoing pain, since by the nature of the model the stimulus is of short duration. An example of such a model, is that of the balloon distension of the rat colon [63,249]. In this model rats quickly learn behaviour designed to avoid the onset of colorectal distension in a place preference paradigm. These behavioural changes are associated with cardiovascular pressor responses and abdominal contractions (both of which are observed in human patients with abdominal pain) [118]. However, even using this model, much of the information derived has followed observation of changes subsequent to inflammation of the colon by chemicals such as zymosan [63]. Under these conditions similar problems (of the reproducibility of the stimulus) as those alluded to in the turpentine inflamed rat urinary bladder model, pertain.

Whilst it is possible to apply direct electrical stimuli to afferent neurones arising from visceral structures [107,167], this is clearly not an 'ideal model' for the study of pain. The stimulus, although entirely reproducible and quantifiable, is hardly 'natural'. Furthermore, to deliver such stimuli in an awake animal, such that behavioural responses can be observed is a challenging problem. Thus although much information regarding anatomical pathways transmitting potentially nociceptive information can be elicited using such models, the true meaning of such findings under pathophysiological conditions is uncertain.

## 1.v. Models of somatic pain:

In addition to the studies pertaining to visceral inflammatory pain, I wished to compare the analgesic effects of some of the molecules being examined in the visceral and somatic domains. Therefore I utilised an established model of persistent cutaneous inflammation - the behavioural response to formalin inflammation of the rat paw - as a comparator.

### 1.v.a. <u>Formalin induced inflammation of the rat paw:</u>

The formalin test was first described by Dubuisson and Dennis in 1977 [98] and has since undergone some modification [323,332]. It is considered to be a reliable model of pain under conditions of persistent somatic inflammation. Both the behavioural and neurophysiological responses (described below) are susceptible to several classes of analgesic drugs [57,87,88,98,196,205]. In investigating mechanisms of pain arising in the somatic domain, the choice of model is important since persistence of pain related behaviour observed over a period of time (not detected in the hyper-acute, reflex response models of nociception such as the tail flick [70] and hot plate tests) is a feature of clinical pain states.

The formalin test relies upon the manifestation of predictable, biphasic, nocifencive behaviour in rodents following the sub-cutaneous injection of formalin into the dorsal aspect of the paw. In addition to the behavioural changes observed, electrophysiological responses in primary afferent neurones and spinal dorsal horn cells in the relevant spinal level (L1, L2 and L3 for the hind paw) demonstrate similar reproducible changes in activity [87].

Although in the original description the formalin was injected into the fore paw, it is now more frequently administered to the hind paw, since it is less common to observe licking (one of the behaviours graded in the nociceptive response) of this limb during normal grooming behaviour, in the rat [323]. The nocifencive behaviour induced by sub-cutaneous formalin has been characterised by four behavioural states [98]:

0 the injected paw is not favoured.

1 little or no weight is borne on the injected paw.

2 the injected paw is held elevated and not in contact with any surface.

3 the injected paw is licked, bitten or shaken.

Recording of the time spent in such altered behavioural states has been used as a marker of pain assessment. Animals are observed for up to one hour following sub-cutaneous injection of formalin into the paw and the duration of time spent displaying each behavioural state is recorded during five minute time periods. A number of scoring systems have been used to assess the pain related behaviour observed:

a) Single parameter recordings, such as paw lick duration, paw flinch frequency or generalised stereotypical behaviour patterns.

b) Weighted scoring techniques (WST), in which specified behaviour patterns are designated a 'weight' based on the impression of pain intensity likely to be present.

Whilst simplicity of scoring is likely to reduce observer errors, Abbott *et al* [3] showed that a weighted scoring system (such as that utilised in the original description [98]) is a superior method of measuring pain behaviour than any single measurement. They administered varying concentrations of formalin to demonstrate a dose-response relationship, which was most strongly correlated when several behaviours were assessed. To produce a 'weighted score' for each five minute time period, the time spent in each of several behaviour patterns is multiplied by a 'weighting factor' assigned to that behaviour and divided by the total time of the scoring period (300 seconds). The appropriate 'weighting' of specific categories has been investigated by several groups. This work suggests that an ordinal ranking system is appropriate [55] and that the optimal weights for the categories are [332]:

0 for behavioural states 0 and 1.

1 for behavioural state 2.

2 for behavioural state 3.

The validation was based on multiple regression analysis of data from the second phase of the response, following administration of either a variable inflammatory stimulus (by increasing the concentration of formalin injected) or variable analgesic treatments following a fixed inflammatory stimulus [312,332]. This weighting system has the great advantage that an observer does not need to make any deductions regarding the level of pressure that the paw can withstand (the 'little' weight, of behavioural state 1), but merely observe whether the paw is elevated or bitten. Thus inter-

observer (and possibly intra-observer) reliability is enhanced and the reliability of the overall measure improves.

When such composite pain scores which have been weighted (CPS-$WST_{0,1,2}$) are plotted against time, a biphasic, nocifencive response to sub-cutaneous formalin injection is observed. Figure 4 shows a typical example of the biphasic response observed in a group of control animals. The x axis denotes time from injection and each point on the y axis is calculated using the equation:

$$\frac{\left(\left[Time \times Weight1\right]+\left[Time \times Weight2\right]+\left[Time \times Weight3\right]\right)}{300}$$

In addition to such response curves, a single measure for each of the two phases (0 - 15 minutes and 20 - 50 minutes) can be calculated to provide an overall CPS-$WST_{0,1,2}$ for each phase. This may provide for greater ease of comparison of the effects of pharmacological agents on the nociceptive behaviour.

The two classical phases of the formalin response are thought to arise from differing mechanisms. The first phase, beginning immediately after the sub-cutaneous injection and lasting for approximately five minutes, is probably due to the direct chemical stimulation of nociceptors. This stimulation appears to be associated with C fibre activity evoked by substance P and bradykinin [98,212,323]. A period of ten to fifteen minutes, during which little nociceptive behaviour is displayed, is followed by the second phase response which lasts up to forty minutes. This second phase (which appears to depend upon both peripheral and central components) seems to be qualitatively different from the first phase, in that a variety of

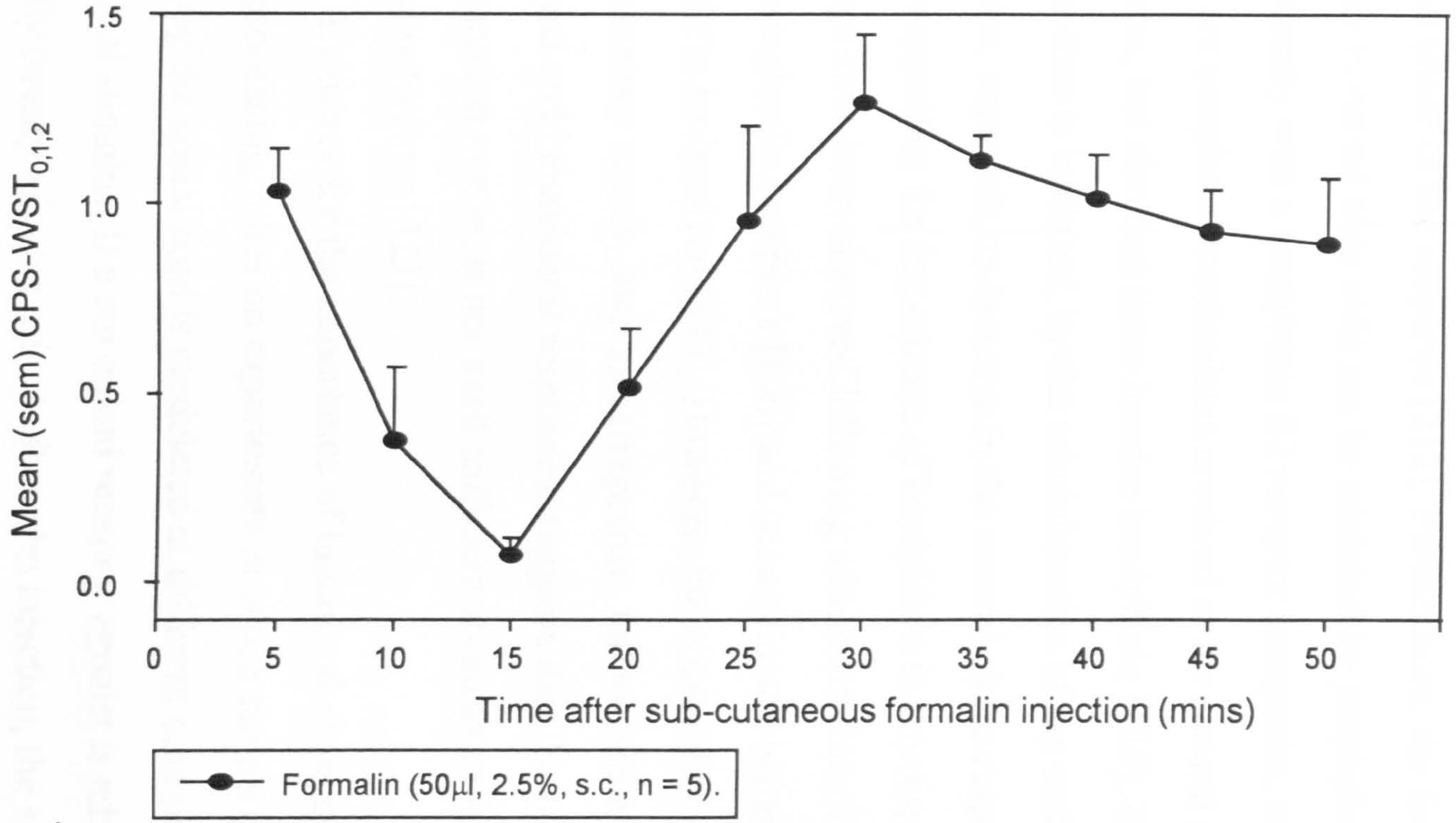



Figure 4.

Plot demonstrating the typical biphasic behavioural response, as measured by the composite pain score described by Watson *et al* ($CPS\text{-}WST_{0,1,2}$), to the injection of sub-cutaneous formalin, in the dorsum of the paw of the rat. Note that there is an initia phase of high pain ratings lasting for approximately 5 minutes. There is a subsequent lull in the observed pain-related behaviour, followed by a second phase response which lasts until the completion of the test period (50 minutes).

drugs which demonstrate inhibitory effects on the second phase response, lack any effect on the first phase [323].

The observation of ongoing activity in primary afferent neurones, which demonstrates a similar biphasic activity pattern to that of the behavioural response, suggests that peripheral input is important in the second phase of the response [212]. Furthermore, the fact that second phase activity in dorsal horn cells can be inhibited by prophylactic treatment peripherally with a bradykinin B2 receptor antagonist, suggests not only that there are peripheral mechanisms involved in the second phase of the response, but also that these involve bradykinin [131]. If bradykinin metabolism is inhibited, by the administration of the carboxypeptidase inhibitor, captopril, an increase in the second phase response is observed [74], supporting the importance of bradykinin in the response. Similar findings have been observed following administration of antagonists acting at prostaglandin receptors [196] and serotonin and histamine are also thought to be involved [323]. However, the observation that alternative inflammatory agents, such as carrageenan, fail to evoke similar biphasic neuronal and behavioural responses, suggests that the presence of peripheral inflammation *per se*, is not itself sufficient stimulus to account for pain related behaviour [323].

Evidence for the importance of functional changes occurring in dorsal horn processing, relies on experiments in which the peripheral information reaching the spinal cord is modulated at different time points in relation to the initial stimulus. If a mu opioid receptor agonist is administered intra-thecally twenty minutes prior to formalin injection, the second phase of the electrical response of dorsal horn neurones is prevented. If the mu receptor antagonist, naloxone, is co-administered then the second phase of the response is present. However, if the naloxone is administered separately, subsequent to formalin injection, the second phase response is only partially

attenuated [87]. This suggests that the peripheral afferent neuronal input is an absolute requirement for the onset of the second phase of the response and that once this has occurred, an inevitable alteration in dorsal horn processing is set in motion, which cannot subsequently be entirely ablated. Similar observations of behavioural responses have been made following the administration of intra-thecal local anaesthetic agents [58]. Moreover, nociceptive behaviour patterns associated with sub-cutaneous formalin are increased by the spinal administration of excitatory amino acids (such as aspartate and glutamate) and NMDA receptor agonists. Prior intra-thecal administration of NMDA receptor antagonists decreases such behaviour [57]. Taken together, this evidence suggests that the central change induced by formalin involves effects at the NMDA receptor.

## 1.vi. The immediate early gene *c-fos*

### 1.vi.a. Immediate Early Genes:

Immediate early genes were first described as proto-oncogenes; that is, the normal cellular counterpart of viral genes capable of inducing tumours (oncogenes). A large number of oncogenes have been identified and their cellular components cloned. Although in general the induction of these proto-oncogenes is rapid and transient [52] (indeed the expression of the immediate early genes *c-fos* and *c-myc* are amongst the earliest events observed when fibroblasts are stimulated with a variety of growth factors and intra-cellular second messengers), this is not the case in all circumstances. In some tissues, such as skin and bone, *c-fos* is continually expressed, whilst in others, upregulation of expression only occurs at more distant time points (up to 12 to 24 hours later) and may persist for weeks or

months. A good example of this is the upregulation of c-*jun* expression following transection of the sciatic nerve [243].

## 1.vi.b. c-Fos:

The protein product, c-Fos, of the immediate early genes *c-fos* is generated in the membrane, cytosol and nucleus of cells. Whilst in certain areas of the brain, such as the hippocampus, there is a basal level of Fos-like staining in adult neuronal nuclei [144], in the spinal cord, basal expression is extremely low [52]. However, any activation of peripheral neurones has been shown to be associated with the rapid increase in expression of c-Fos in neurones of the dorsal horn. Genetic changes such as this might potentially be associated with the establishment of prolonged functional changes, outlasting the initial precipitating stimulus.

### Somatic domain:

Non-noxious stimulation of skin, such as that of gentle brushing, is associated with only a low level of c-Fos-like immunoreactivity in Rexed layers II and IV of the spinal cord (with no significant staining observed in the dorsal root ganglion or layer I of the spinal cord).

In contrast, noxious stimulation is associated with a much greater number of neuronal nuclei stained, predominantly localising in layers I, II, V and X [145]. When noxious chemical stimuli, such as mustard oil, are applied topically to skin, the resultant C and Aδ fibre activation is associated with neurogenic plasma extravasation and an increase in neuronal c-Fos-like immunoreactivity in the ipsilateral spinal cord in dorsal horn layers I, II, IV and V. Noxious thermal stimulation of the skin utilising a radiant heat source with a range from of $40^0$ to $52^0$C, which induces C fibre activation, is associated with similar somatotopically appropriate localisation of immunostaining.

Whilst peripheral noxious stimulation inevitably results in the appearance of c-Fos-like immunoreactivity in the spinal cord, the reverse scenario does not hold true. If c-Fos-like immunoreactivity is induced by intra-thecal stimulation using electrical (0.5msec pulses for 15min, at 15V and 3Hz) or chemical stimuli (25nmol NMDA), alterations in nociceptive responses to peripheral stimuli (either thermal or mechanical) are not inescapable [286]. Thus, whilst the upregulation of *c-fos* may be used as a marker for nociceptive activity, it should not be seen as sufficient in itself to induce long term changes in spinal nociceptive processing.

The findings described above, suggest that although c-Fos is indeed induced at a low level by innocuous stimuli, the technique is probably an appropriate one with which to study spinal nociceptive processes. There is a clear preferential expression following noxious stimuli [52] and this upregulation in expression can be inhibited by treatment with appropriate analgesic agents.

**Visceral domain:**

Similar findings to those described above in somatic models have been observed in the visceral domain. Inflammation of the bladder by chemicals such as acetic acid [36], NGF and turpentine oil [89] has been shown to be associated with increases in *c-fos* expression at appropriate sites in the lumbar enlargement of the spinal cord. Thus, at spinal cord levels L5, L6 and S1, an increase in c-Fos-like immunoreactivity is observed in the dorsal horn. This staining is particularly heavy in layers I, II and X and in the intermediolateral posterior spinal nucleus.

In addition to this upregulation in the expression of *c-fos* following noxious stimulation, a reduction in expression has been observed following: i) neonatal capsaicin treatment (selectively destroying C-fibres) [52].

ii) intra-vesical capsaicin treatment (several hours prior to the induction of inflammation, inducing desensitisation) [67].

iii) activation of the bulbo-spinal descending inhibitory systems of the nociceptive pathway[52].

iv) the pre-administration of analgesic agents (such as opioids and NMDA antagonists) [35,52,327].

In the light of this evidence, the degree of staining for c-Fos-like immunoreactivity appears to be an additional technique by which to assess the effects of molecules on the modulation of visceral pain arising from the urinary bladder.

## 1.vii. Hypothesis

The work presented here was designed to assess the effects of chemical mediators, with the potential to act at peripheral sites in the neuro-immune systems, upon the modulation of inflammatory pain. However, the turpentine-inflamed rat urinary bladder model was itself first examined. Further assessment of whether it accurately models visceral pain, as opposed to merely vesical hyper-reflexia was undertaken. Symptoms and signs referred to distant sites are characteristic features of visceral pain in humans. However, this has yet to be fully documented in many animal models. Thus, the data presented in chapters 2 and 3 were designed to document the presence, or absence, of referred signs following vesical inflammation, in distant, but somatotopically appropriate, cutaneous sites.

Hyperalgesic responses are at least partially mediated by sensitisation of nociceptors [238,273]. This sensitisation occurs following the production and release of chemical mediators from both injured tissue and immune cells [93] (see figure 5). The neurotrophin, nerve growth factor (NGF) appears to play a pivotal role in the sensitisation of nociceptive pathways (discussed

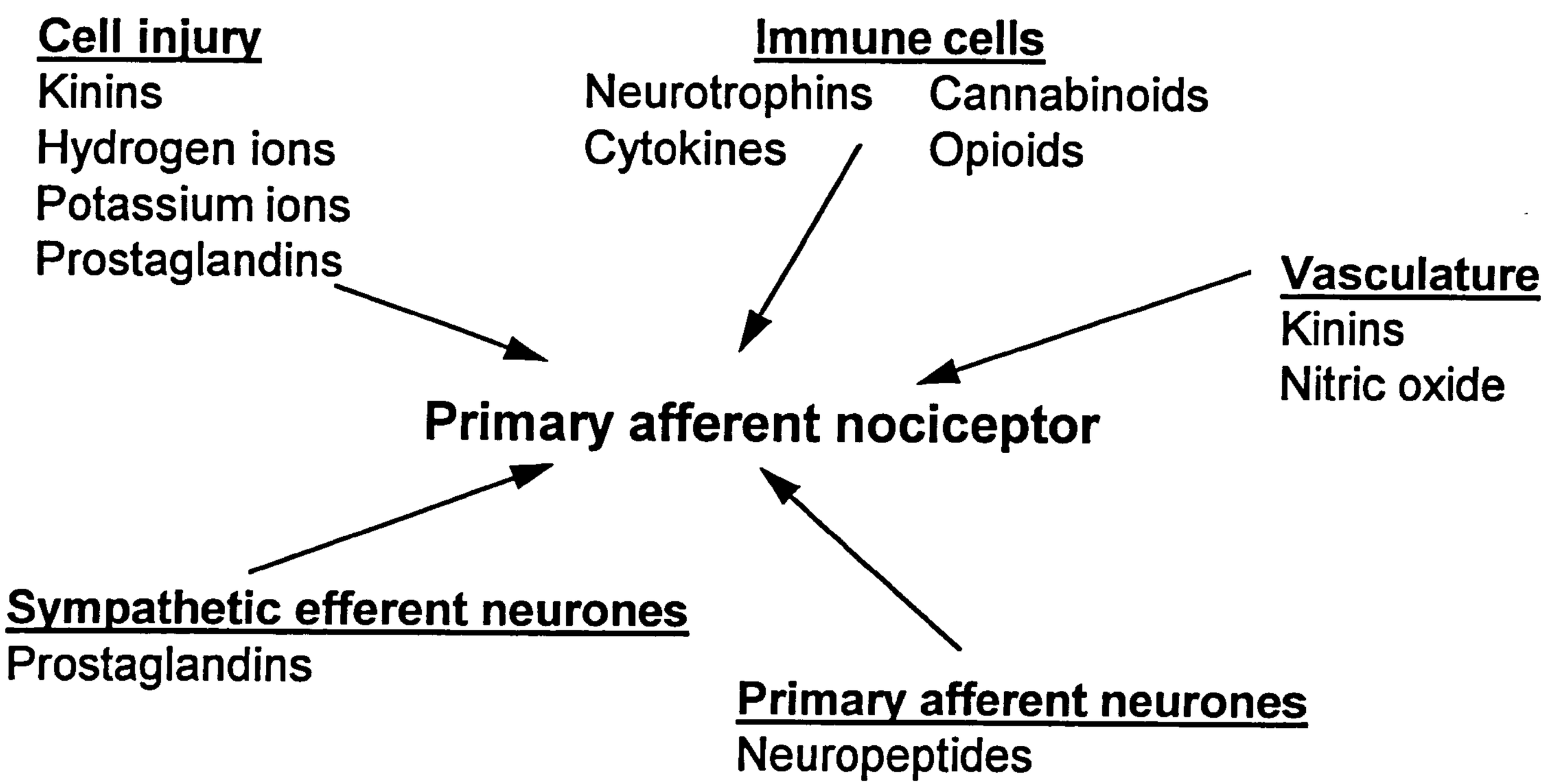



Figure 5.
Diagram demonstrating the multiplicity of molecules influencing the response of the primary afferent nociceptor during the inflammatory process. Note that immune cells release both excitatory (neurotrophins and cytokines) and inhibitory (opioids and cannabinoids) molecules.

fully in chapter 3). NGF is capable of binding to trkA and p75 receptors [162], located on both primary afferent neurones and mast cells [19,251]. This results in increased activity in nociceptive pathways both by direct action on neuronally located receptors, but also by the release of alternative algogens from mast cells. It is therefore in a prime position to orchestrate neuro-immune interactions [180]. The data presented in chapter 3 address the NGF-dependence of the referred signs documented in chapter 2.

Receptors activated by the pro-algesic molecule bradykinin [338,339] have been localised to both primary afferent nociceptors and immune system components, such as the mast cell. Moreover, NGF regulates the sensitivity of neurones to bradykinin [27,265]. Thus, the potential for neuro-immune responses to be modulated in parallel by either bradykinin, or NGF, produced during inflammation, is clear. The possibility that inhibition of such bradykinin-mediated effects may attenuate the integrated reflex responses consequent upon such sensitisation is addressed in chapter 4.

Capsaicin-sensitive neurones have long been associated specifically with the transmission of nociceptive information. Recently many of the effects of capsaicin have been demonstrated to be mediated by the VR1 vanilloid receptor [49]. This receptor is localised exclusively to primary afferent neurones. Therefore, changes associated with administration of compounds acting at the VR1 receptor will be entirely mediated at peripheral sites. The possibility that chemicals, such as NGF, released during inflammation may effect changes in the sensitivity of the VR1 receptor, leading to alterations in nociceptive activity, is discussed. The potential effects of compounds which lead to desensitisation of such VR1-expressing neurones upon the modulation of visceral pain are explored in chapter 5.

Inhibition of the sensitising effects of pro-inflammatory mediators would be anticipated to attenuate responses in the nociceptive pathway. Like

trkA receptors, cannabinoid receptors have been localised both to NGF-dependent primary afferent neurones [113] and also to immune cells [10]. Activation of such cannabinoid receptors appear to be associated with inhibitory effects [103]. Since endogenous cannabinoids have been demonstrated to be released under conditions of inflammation [246], the potential for physiological down-regulation of inflammation-associated, NGF-dependent responses exists. Therefore, the role of endogenous cannabinoids is examined in models of both somatic and visceral pain (presented in chapter 6).

In summary, I have sought to investigate the possibility that both up- and down-regulation of hyperalgesic responses following visceral inflammation, may be achieved by means of agents targeted at peripheral sites, particularly those with a relationship to NGF-associated activity. The possible development of therapeutic compounds acting predominantly at such sites has the potential to impact in a major fashion on clinical practice, since central side effects of drugs such as opioids currently limit clinical use.

# Chapter 2:

# Referred viscero-somatic hyperalgesia (VSH)

## 2.i. Introduction

A cardinal feature of visceral pain in humans is the perception by the sufferer of pain arising away from the area of injury to an often distant somatic site, so called 'referred pain' (see chapter 1.i.b.) [216,221]. Because of the subjective nature of pain [65,333] and by necessity the objective nature of work with animal models, it is impossible to ascertain the presence or absence of referred pain *per se* in the rat. Assumptions have therefore been made about the perception of pain, on the basis of observed behavioural patterns. However, whilst it is not possible to document the presence or absence of pain, it is possible to observe altered responses to a standard stimulus applied to the skin. Thus if in addition to pain, visceral inflammation is also associated with referral of hyperalgesia (as has been described by Head [135]) this should be demonstrable in animal models.

### 2.i.a. Experimental rationale:

The experiments presented in this chapter were undertaken in an effort to elucidate the presence or absence of an hyperalgesia referred to the skin in the turpentine inflamed rat urinary bladder model of visceral inflammation. I have compared the difference in withdrawal thresholds (from both thermal and mechanical stimuli) of the fore and hind paws of rats treated with intra-vesical turpentine, to that of control animals.

The hind paw was chosen as the somatotopically appropriate site of referral for bladder inflammation in the rat since both areas are supplied by the L5 neuronal root [35,89,318]. Additionally (and of significance in the context of the experimental protocol) the distance of the test site from the stimulus source could easily be maintained at a constant level, with minimal

interference to the animal. Although the L6 nerve root lies in the middle of the range of the neuronal supply of the bladder (L5, L6 and S1) it would be more difficult to supply a constant stimulus to the skin area that is innervated by it. The L6 dermatomal area, as determined by the antidromic stimulation of C fibres in spinal nerves, is covered with course hair on the back and tail base areas [318]. The distance of this area from the floor of the test chamber would be more difficult to standardise (during the thermal stimulation experiments) than is the observation of contact between the paw and the base of the cage. Furthermore, the outcome measure utilised (limb withdrawal) was easily discernible, providing less potential for variations in observation of outcome.

## 2.ii. Methods

All experiments conformed with British Home Office Regulations. Animals were housed according to national guidelines in colony cages of six animals per cage with a dark : light cycle of 12 hours : 12 hours. They were provided with free access to food and water.

Surgical preparation:

Female Wistar rats (Bantin and Kingman) were anaesthetised by the administration of a single dose of 40mg/kg intra-peritoneal (i.p.) pentobarbitone. The urinary bladder was catheterised trans-urethrally with a sterile, lubricated, nylon catheter (external diameter 0.75mm - Portex, U.K., 200/300/020). Vesical inflammation was achieved by the instillation of 0.5ml, 50% turpentine oil left in place for one hour. Once anaesthesia had been achieved, tail vein cannulation was performed with a plastic cannula (external diameter 0.6mm, Abbocath-T, Abbott Ireland Ltd., Sligo, Republic of Ireland).

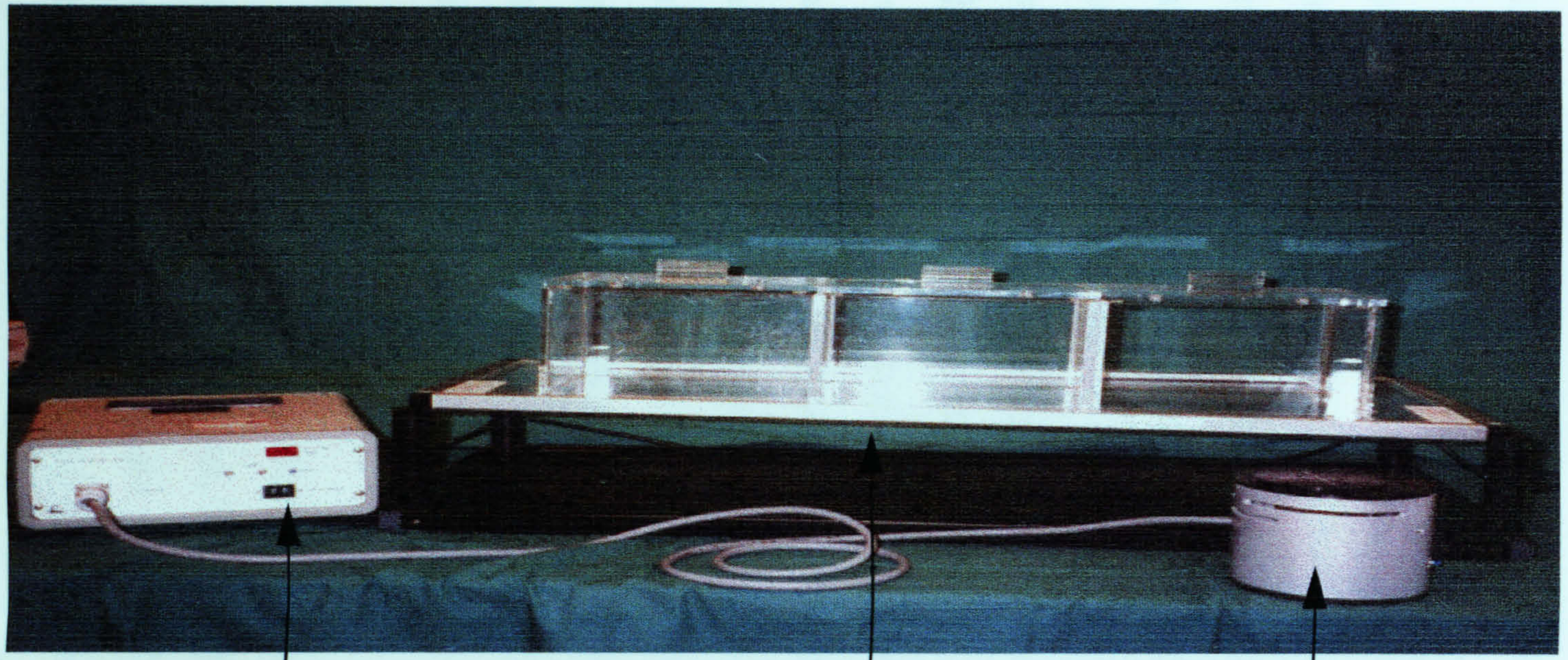
Emptying the bladder of turpentine and urine prior to removal of the catheter was achieved by the application of gentle pressure on the anterior

abdominal wall. Compete emptying was not confirmed by the direct visual inspection of the bladder described previously [218] since I aimed to minimise direct stimulation of somatic tissues. However, since the urethra itself does have a somatic neuronal supply (arising from the same spinal segments as the bladder) the potential for neuronal input to the spinal cord from this source could not be avoided [79].

Testing of responses to the application of thermal stimuli [133]:

Animals were acclimatised to the testing environment (a clear plexiglas box measuring 23 x 18 x 14cm, with a glass floor of low infra-red absorption characteristics - see photograph in figure 6) until explorative behaviour ceased. Once this occurred, baseline limb withdrawal thresholds (in seconds) from a constant intensity, infra-red thermal stimulus (maximum temperature $46^0$C) applied to the palmar or plantar skin of the right fore and hind paw, were measured, using a Hargreave's device (Ugo Basile, Italy). This device both supplied a thermal stimulus and then automatically terminated it if the overlying tissue was moved [133]. An integral timer recorded the duration of the applied stimulus to the nearest one tenth of a second. Additionally, so as to limit tissue damage, the stimulus was automatically terminated at 21.4 seconds if the animal had not already withdrawn the limb. The device was calibrated regularly using a calibration sensor at infra-red intensity 50nm, which ensured reproducibility over the range 10 - 90nm. Furthermore the temperature attained immediately adjacent to the source was consistently measured as $46^0$C. Thus, the temperature supplied by the device was consistently in the range capable of exciting C (but not A) mechano-heat nociceptors [238].

Three consecutive baseline readings (approximately one minute apart) were measured and the mean of these was taken as the baseline limb withdrawal threshold. Animals were subsequently anaesthetised and



Timer device automatically stops when movement occurs above the detector.

Glass floor of cage with low infra-red absorption characteristics.

Mobile infra-red output system and movement detector.

Figure 6.
Photograph to show the Hargreaves device, used to measure withdrawal latency from a thermal stimulus.

experimental interventions applied. Further readings were taken at four, six, eight and twenty four hours post-intervention (each recorded value being the mean of three consecutive tests). Animals were then sacrificed by overdose of anaesthetic and cervical dislocation.

Testing of the responses to application of mechanical stimuli:

After animals had been acclimatised to the testing environment (a clear plexiglas box measuring 22 x 17 x 14cm with a plastic floor containing perforations of 0.8cm diameter - see photograph in figure 7), baseline limb withdrawal thresholds from a punctate mechanical stimulus applied using standard von Frey hairs (see photograph in figure 8) were measured. The hairs were applied to the plantar surface of the right fore and hind paws in unrestrained animals. The hair from which the paw of the animal was actively removed on at least two out of three occasions was documented. Hairs were applied in ascending fashion from minimum (0.0174gm) to maximum (263gm) force applications. To ensure that the hairs delivered a constant stimulus the environmental temperature and humidity was controlled at a constant level [16]. After recovery from anaesthesia subsequent readings were taken at four, six, eight and twenty four hours post-intervention. At the completion of each experiment, animals were sacrificed by overdose of anaesthetic and cervical dislocation.

Test protocols (see figure 9):

The effect of turpentine instillation into the bladder on limb withdrawal thresholds was observed, to assess if this was associated with a referred viscero-somatic hyperalgesia (VSH). Two separate control groups were used. Animals in the first group underwent no intervention other than general anaesthesia (controlling for the effect of urethral cannulation), whilst those in the second control group underwent intra-vesical instillation of saline via a trans-urethral catheter (0.5ml,1 hour) to control for the effect of

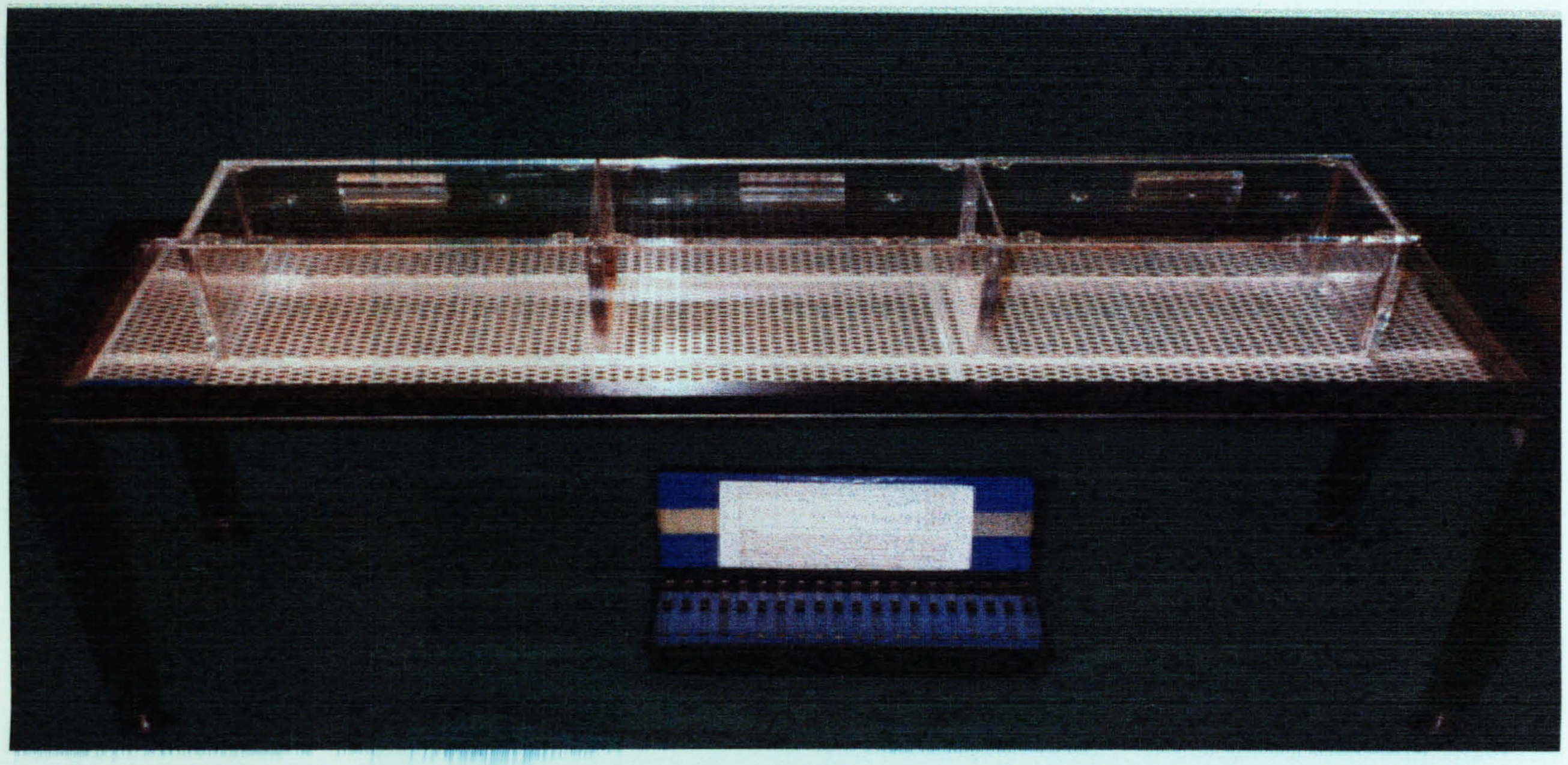


Figure 7.
Photograph showing the animal environment for the mechanical hyperalgesia testing with von Frey hairs. Note the plastic floor, which has perforations (diameter 0.6mm) to allow testing to proceed with minimal handling of the animals.

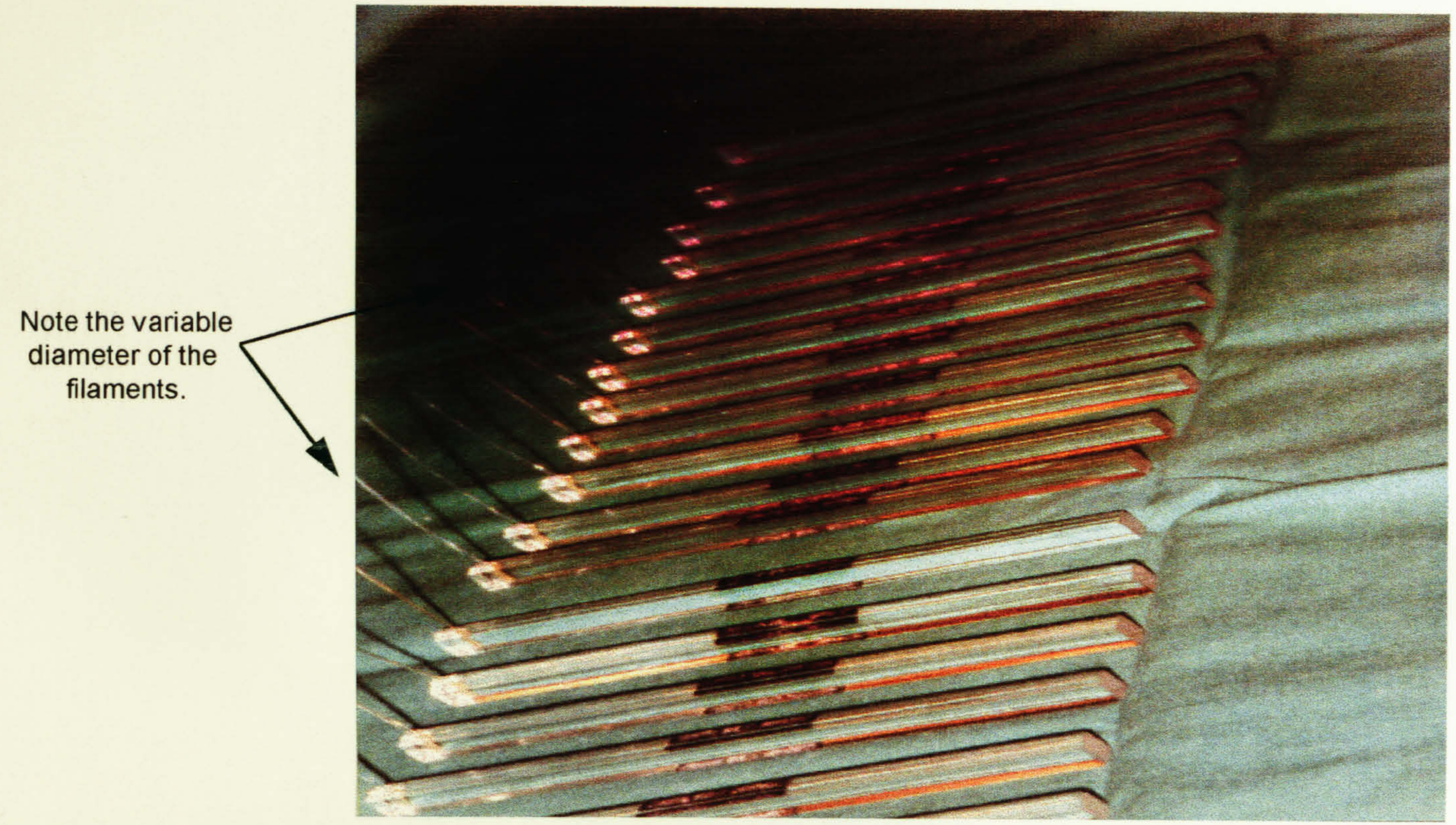



Figure 8.
Photograph of a set of von Frey hairs, used to assess withdrawal latency from a punctate mechanical stimulus.

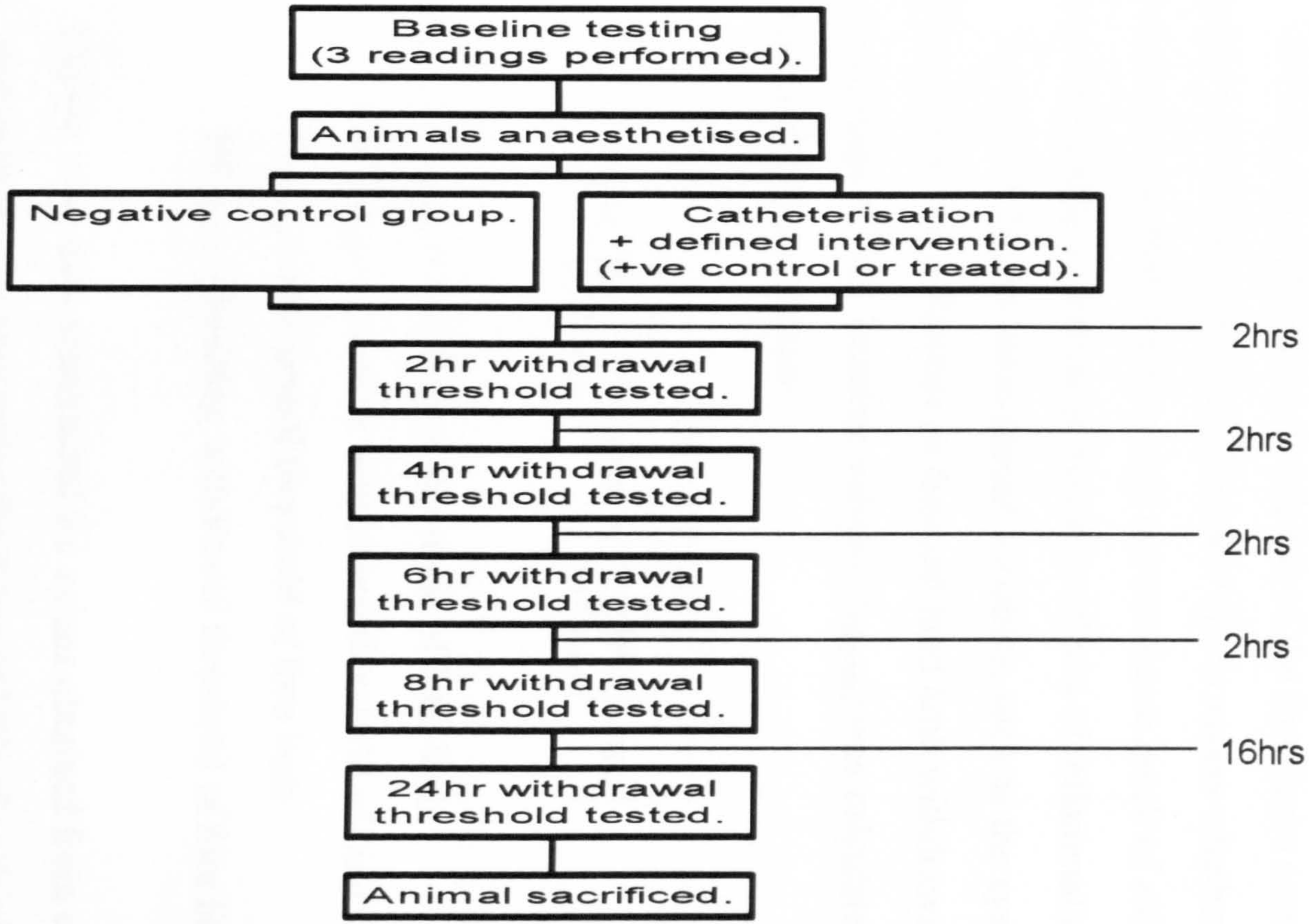



Figure 9.
Flow diagram outlining the testing protocol for somatic withdrawal threshold testing (in the original experiments no tests were performed at 2 hours since the animals remained unresponsive as a result of the anaesthetic).

turpentine. Animals in the test group were subjected to both trans-urethral cannulation and intra-vesical turpentine instillation, as an inflammatory stimulus (0.5ml, 1 hour).

Outcome measures:

Initially, the withdrawal thresholds of the fore limbs across time were compared to baseline (ANOVA [Dunnett's]), to assess whether any systematic change from baseline had occurred as a result of either passage of time, habituating to testing or systemic effects of inflammation.

To control for any intra-animal variables, such as the systemic effect of inflammation, the difference in fore and hind limb withdrawal thresholds (as a percentage of their baseline values) ($\Delta_{Thresh}$) was calculated for each time point using the equation:

$$\Delta_{Thresh} = \left[\left(\frac{H_{Thresh}}{BH_{Thresh}}\right) \times 100\right] - \left[\left(\frac{F_{Thresh}}{BF_{Thresh}}\right) \times 100\right]$$

Where: $H_{Thresh}$ = withdrawal threshold of hind limb.

$BH_{Thresh}$ = baseline withdrawal threshold of hind limb.

$F_{Thresh}$ = withdrawal threshold of fore limb.

$BF_{Thresh}$ = baseline withdrawal threshold of fore limb.

Curves were then constructed for values obtained from each animal of $\Delta_{Thresh}$ against time. The area under the curve (AUC) of each of these curves was calculated using the trapezium rule (Sigmaplot version 3.0, Jandel Scientific Software). Comparison of the AUCs of the two intervention groups, against the no catheter control group was undertaken by means of the unpaired t-test (Sigmastat version 2.0, Jandel Scientific Software).

## 2.iii. Results

### 2.iii.a. Responses to the application of thermal stimuli:

The experiments were performed on thirty three animals, mean weight 214.8gm (range 200 - 250gm). The withdrawal latency of fore and hind limbs from a standard thermal stimulus was recorded. There was no significant variation ($p > 0.05$) across time, from baseline latencies for the fore limb withdrawal thresholds (ANOVA [Dunnett's]) in any of the three treatment groups.

The curves plotting mean (sem) $\Delta_{Thresh}$ against time are shown in figure 10. In the no catheter control group ($n = 11$) there was minimal variation around the zero line, indicative of the lack of significant change in withdrawal thresholds from baseline values. In both the intra-vesical saline instillation group ($n = 11$) and the intra-vesical turpentine induced inflammation group ($n = 11$) a negative deflection of the $\Delta_{Thresh}$ against time curve was observed, indicating a relative hyperalgesia of the hind limb in relation to the fore limb. This negative deflection was greater in the turpentine-induced inflammation group.

The area under the curve (AUC) was calculated for each $\Delta_{Thresh}$ against time plot using the trapezium rule (Sigmaplot 3.0, Jandel Scientific Software). No significant difference between the AUCs was observed (t-test, $p > 0.05$) when the no catheter (+53.6 +/- 190.0) control group was compared to the saline instillation (-100.5 +/- 363.1) control group (see figure 11). Thus, catheterisation *per se* was not associated with a significant referred somatic hyperalgesia to thermal stimulation. However, there did appear to be a trend in the direction of hyperalgesia, probably reflective of the presence of the catheter itself irritating the bladder mucosa - an effect previously observed when c-Fos-like immunoreactivity in the dorsal horn of

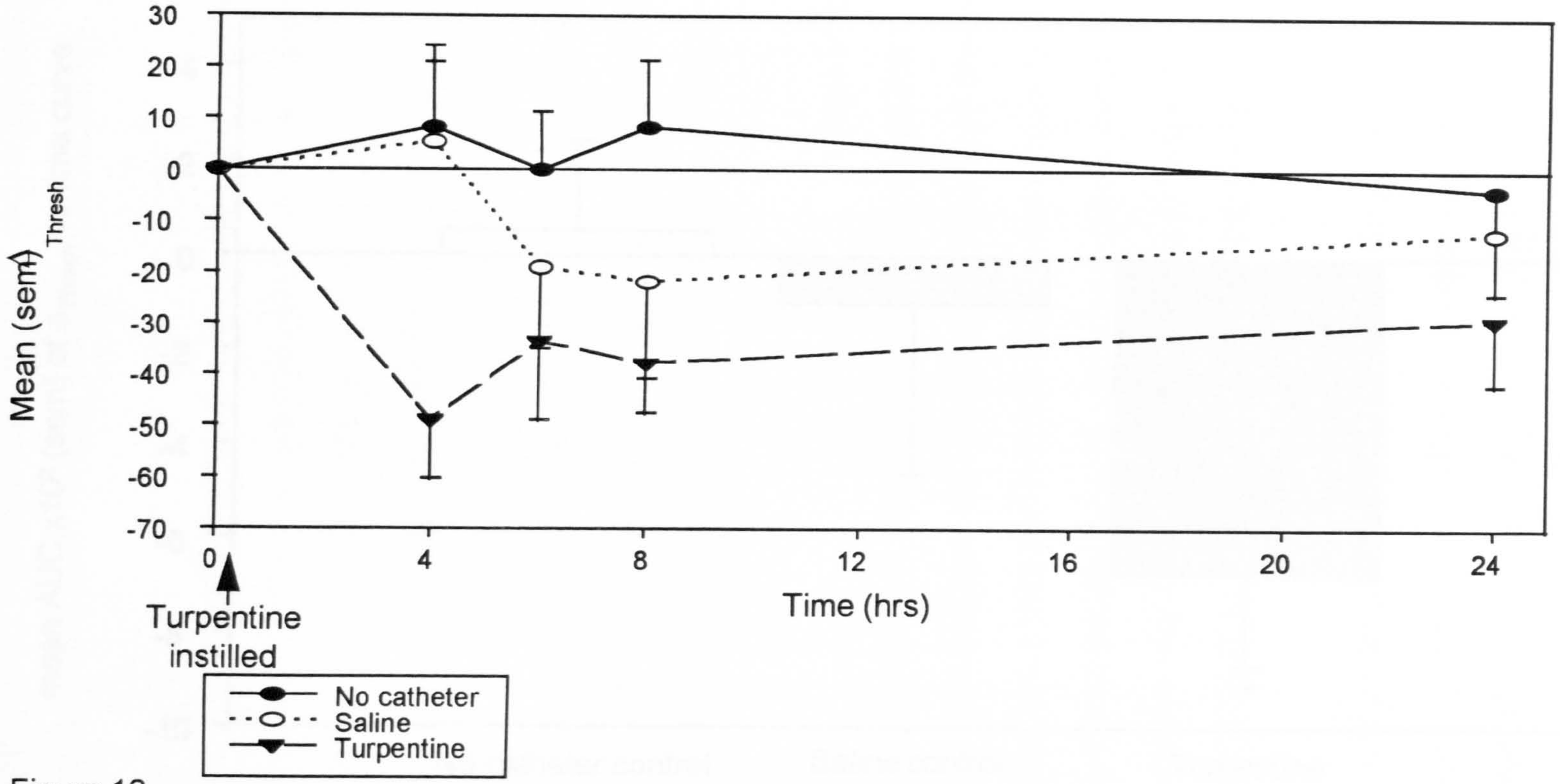



Figure 10.

Graph demonstrating the difference between fore and hind paw withdrawal thresholds from a thermal stimulus, over a 24hr period following intervention. Note that for the no catheter control group the curve approximates to zero, indicating no difference in withdrawal thresholds. However the curve for the turpentine inflamed group shows a negative deflection, indicating a relative hyperalgesia of the hind paw. The curve for the catheterised (but non-inflamed) group lies between the 2 extremes.

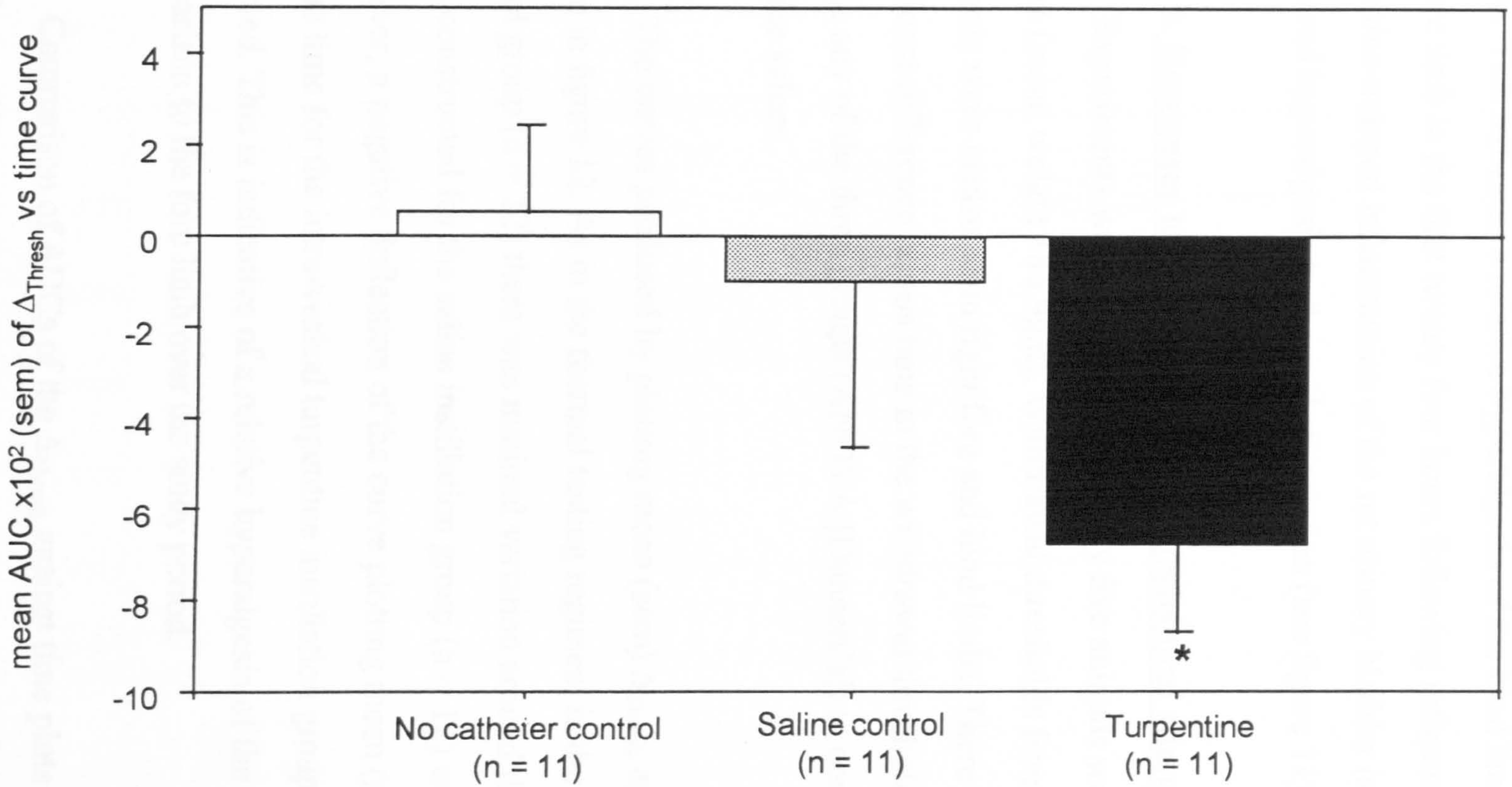



Figure 11.
Bar chart of the mean (sem) area under the curve (AUC) of the $\Delta_{Thresh}$ against time plot for thermal stimulation.
* $p < 0.05$ in comparison to the no catheter control group (t-test).

the spinal cord is used a s the outcome measure [36]. However, a significant difference (t-test, $p < 0.05$) was observed between the AUCs for the no catheter (+53.6 +/- 190.0) and turpentine inflammation (-676.8 +/- 193.2) groups. This indicates a relative hyperalgesia of the hind limb in relation to the fore limb in the first twenty four hours following inflammation. Thus, turpentine-induced inflammation of the rat urinary bladder is associated with a referred hyperalgesia to thermal stimulation (see figure 11).

### 2.iii.b. Responses to the application of mechanical stimuli:

Experiments were performed on thirty five animals weighing 145 - 245gm (mean weight 186.9gm). Withdrawal thresholds from von Frey filaments were measured in right fore and hind limbs. There was no significant difference across time in the withdrawal threshold of the fore limb in any of the three groups (ANOVA [Dunnett's]) in comparison to baseline values.

The curves produced by plotting mean (sem) $\Delta_{Thresh}$ against time are shown in figure 12. As in the thermal testing regimen, in the no catheter control group (n = 12) there was minimal variation around the zero line. The curve constructed for the saline instillation group (n = 12) was similar. However, a negative deflection of the curve plotting mean (sem) $\Delta_{Thresh}$ against time for the intra-vesical turpentine instillation group (n = 11) was observed. This is indicative of a relative hyperalgesia of the hind limb in comparison to the fore limb over the study period.

Comparison of AUCs of the $\Delta_{Thresh}$ against time plots demonstrated no significant difference between the no catheter ($+2.4 \times 10^3$ +/- $2.4 \times 10^3$) and saline ($-0.77 \times 10^3$ +/- $0.86 \times 10^3$) control groups (see figure 13). However, the AUC for the turpentine inflamed group ($-13.3 \times 10^3$ +/- $7.0 \times 10^3$) was

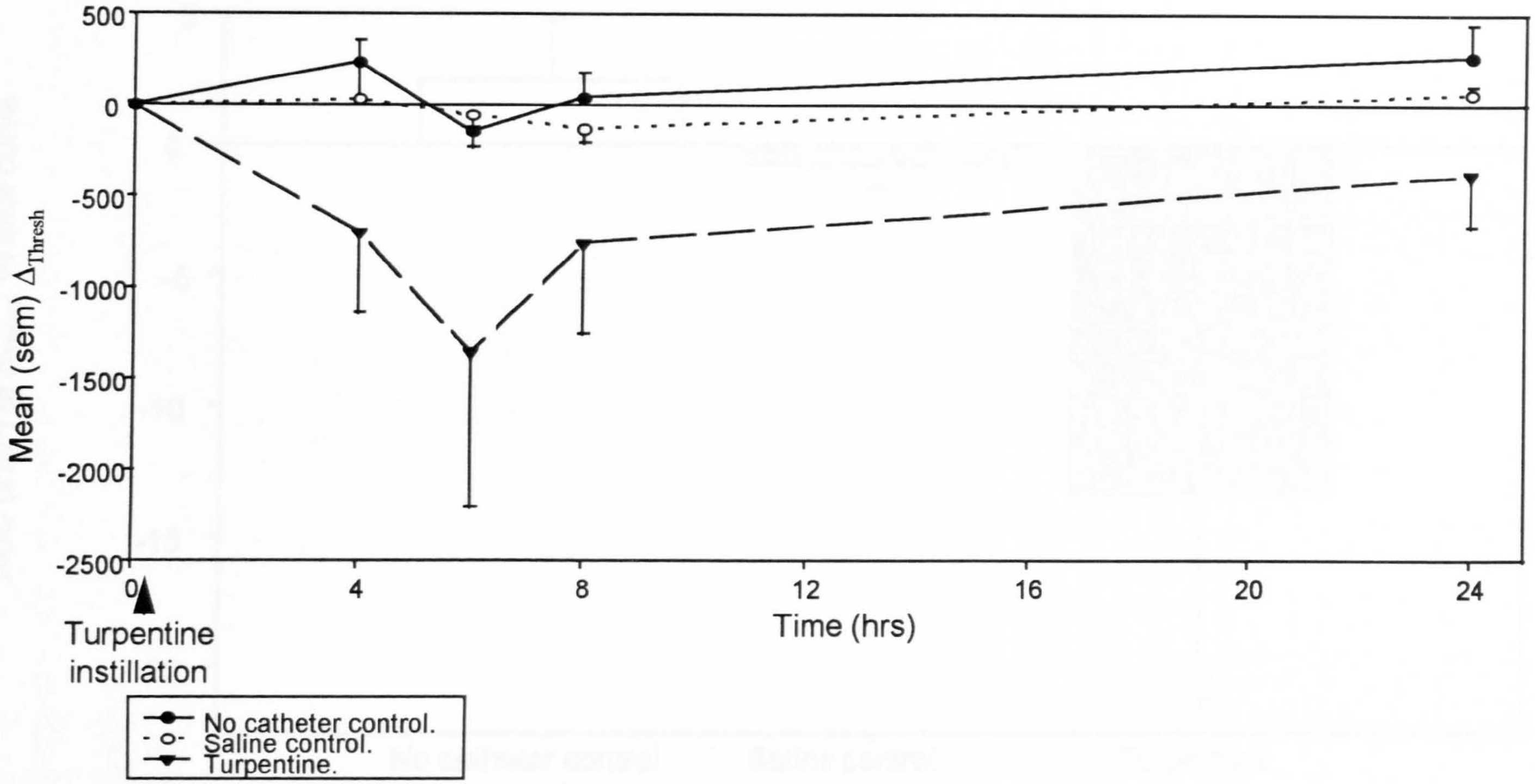



Figure 12.

Graph demonstrating $\Delta_{Thresh}$ for mechanical stimuli (applied by von Frey hairs) against time. Note the curve produced for the control group approximates to zero, but that for the turpentine inflamed group show a negative deflection. This is indicative of a hyperalgesia of the hind paw relative to the fore paw.

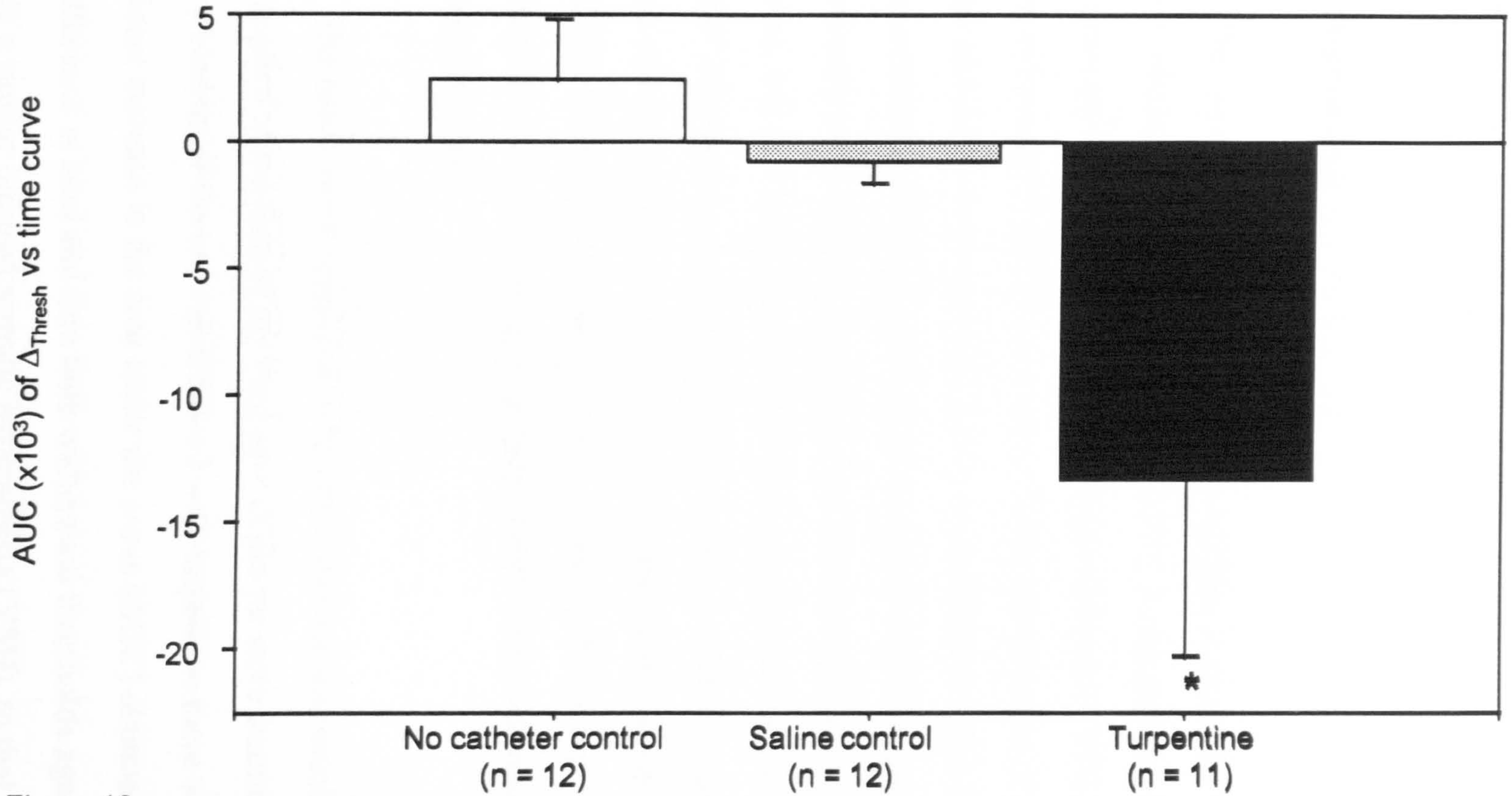



Figure 13.

Bar chart of the mean (sem) area under the curve (AUC) of the $\Delta_{Thresh}$ against time plot for mechanical stimulation with von Frey hairs.

* $p < 0.05$ in comparison to the no catheter control group (t-test).

significantly different to that for the no catheter control (t-test, $p < 0.05$). Thus a significant somatic hyperalgesia to mechanical stimulation referred to the hind limb is present following turpentine-induced inflammation of the urinary bladder.

## 2.iv. Discussion

The turpentine inflamed rat urinary bladder is an established model of visceral inflammation [12,89,90,215,218,219]. It has previously been shown to be associated with a viscero-visceral hyper-reflexia (VVH) [218], plasma protein extravasation into vesical tissue [218], sensitisation of both primary afferent and dorsal horn neurones [215], an increase in c-Fos-like immunoreactivity in the dorsal horn of the spinal cord [36] and recruitment of previously mechanically insensitive (or silent) nociceptors [222,224]. However, one cardinal feature of visceral injury in humans is that of the 'referral' of symptoms to distant sites located in somatic tissues [216]. Since no such referral has been convincingly described in this model, it might be argued that the model is more reflective of urinary frequency, than of visceral pain. In the experiments described here I have addressed this fundamental point.

The behavioural responses when both thermal and mechanical stimuli were applied to the skin of the hind paw of the rat were examined. When the urinary bladder of the rat was inflamed with turpentine there was a significant increase in the area under the curve (AUC) obtained by plotting the difference in hind and fore limb withdrawal thresholds against time. This reflects a similar referred somatic hyperalgesia (VSH), to that observed in human patients [135]. When a thermal stimulus was applied, the AUC was increased more than five fold in the animals in which the urinary bladder

had previously been inflamed with turpentine oil (when compared to control animals). When a punctate mechanical stimulus was applied the increase was more than thirteen fold. In neither case was this alteration entirely explicable by the presence of the urinary catheter *per se*. Although catheterisation was associated with an increase in the calculated AUC, this was only a trend and did not reach significance ($p > 0.05$). In the clinical scenario, this may reflect the discomfort, rather than pain, described by many patients in whom a trans-urethral catheter has been passed.

In the rat, both the urinary bladder and plantar aspect of the hind paw are supplied by afferent neurones which enter the cord at the L5 spinal level [35,89,318]. When the urinary bladder is chemically inflamed with agents such as acetic acid, not only is an increase in the neuronal activity of peripheral afferent neurones entering the spinal cord at L5, L6 and S1 root levels observed [221], but an increase in c-Fos-like immunoreactivity is also noted in the dorsal horn cells of the spinal cord at these levels [35]. Moreover, when antidromic stimulation of the L5 nerve root is applied, the result is extravasation of Evan's Blue-labelled albumin into the tissues of the hind paw [318]. This suggests that in the rat the hind paw is a peripheral somatic site sharing sensory spinal root innervation with the urinary bladder. Therefore the data, relating to hind paw sensory changes, presented here document the referral of hyperalgesia (in response to both thermal and mechanical stimuli) to a distant, but somatotopically appropriate site.

The referral of pain (arising from damage to visceral organs) to distant somatic structures often follows a dermatomal pattern. Several theories have been advanced to explain this [221]. In 1948 Sinclair suggested that some axons could innervate both visceral and somatic targets [299] making it impossible for the central nervous system to determine the source of the input to the spinal cord. There is now some evidence, from double labelling and electrophysiological studies, available to support this. However, the

relative frequency of dorsal root ganglia cells demonstrating double labelling following intra-neural injections of dye into somatic and visceral nerve trunks is low - levels of up to 2% are most commonly described [9,77,267]. Moreover, electrophysiological recordings from the dorsal root of S2, following stimulation of pelvic and pudendal nerves (looking for evidence of collision of action potentials in the dorsal root) was only able to demonstrate dichotomizing of less than 0.5% of afferent neurones [127]. Even lower levels of dichotomization were observed in a range of somatic afferent neurones [84]. Perhaps more importantly, such pre-spinal convergence is unable to explain either the delay in the onset of referred pain or the presence of a referred hyperalgesia (since antidromic activation of C-fibres is not associated with sensitisation [239]). An alternative theory was postulated by Ruch in 1946 when he suggested that primary afferent neurones from visceral and somatic targets might converge onto common dorsal horn neurones in the spinal cord. These neurones might then misconstrue the incoming visceral inputs as arising from a somatic source [283]. Whilst this theory might explain the referral of pain, it does not directly address that of referred hyperalgesia.

However, the earliest and most intriguing theory was advanced by Mackenzie in 1909 [188]. He postulated that the viscera were intrinsically insensitive to pain, but that noxious activity arising from them were capable of setting up an 'irritable focus' in the dorsal horn of the spinal cord. He suggested that this could facilitate somatosensory inputs to such an extent that the threshold for the perception of pain was lowered such that normally innocuous stimuli could be perceived as painful in nature, thus providing some explanation for referral of hyperalgesia. This theory has obvious correlates with the current ideas regarding central sensitisation (or mode 3 of the physiological states of the dorsal horn described by Woolf [340]). Furthermore, it might explain the delay in onset of referred pain, since the

'irritable focus' would take time to develop. Indeed, electrophysiological studies such as those described below have provided evidence that activity in visceral and somatic domains can impinge upon identical spinal neurones and that plastic changes in spinal processing occur.

Visceral afferent neurones are known to display wide arborisations in the dorsal horn of the spinal cord [242,245]. Whilst only approximately 15% of dorsal root ganglion cells are able to respond to visceral stimulation, this rises to up to 75% of spinal neurones, implying the presence of multiple sites of interaction between visceral neuronal input and the spinal cord. Indeed spinal neurones at all levels in the cervical, thoracic and lumbar cord have been demonstrated to be excited by stimulation of the greater splanchnic nerves [248]. In addition to this widespread effect of visceral inputs upon the spinal cord a high degree of viscero-somatic convergence has been observed in spinal neurones responding to afferent stimulation of urinary bladder [248], oesophagus [117], rectum [249] and myocardium [108,117]. This has been documented in: the superficial dorsal horn [249], spinothalamic tract cells [107,108], the deep dorsal horn [117] and areas surrounding the parasympathetic nuclei [248] and central canal [163], all areas known to be of importance in the processing of visceral nociceptive input [248]. Furthermore viscero-somatic convergence is most marked in regions of the spinal cord, such as the thoraco-lumbar expansions, where a large visceral input is observed [50,248]. Of particular interest, in the context of the referral of pain, is the observation, as indicated by conduction velocity studies, that the afferent neuronal input arising from somatic sites which demonstrates convergence with afferent neuronal input from visceral organs is predominantly of C and Aδ fibre origin [50].

Not only has it been demonstrated that visceral and somatic neuronal inputs effect responses in identical spinal cord neurones, but plasticity of response of spinal cord neurones has been shown to occur. When dorsal

horn neurones in the L4 and L5 spinal levels are pre-conditioned by excitation of C-fibres in the gastrocnemius-soleus nerve an expansion in size of the cutaneous receptive field able to elicit a reflex response in motor neurones is observed [60]. Furthermore, when the physiological properties of lamina I nociceptive specific, projection neurones at the L4/L5 level were investigated following adjuvant-induced inflammation, alterations that could not be accounted for by alterations in peripheral processing were observed. Specifically, the size of the receptive field of such spinal neurones is increased (in comparison to control) in the absence of any increase in activity in dorsal root ganglion cells. In addition, an increase in the spontaneous activity of such cells is observed, even when input from the peripheral receptive field is ablated by the injection of local anaesthetic agents[146].

A less well established effect than the referral of pain is the referral of hyperalgesia, to locations distant from the site of origin of the pain. Since the original clinical descriptions by Head (as long ago as 1893 [135]) this has been documented in both animal models of ureteric colic [122] and patients with proven calculosis [17,121,123]:

- When noxious electrical stimuli are administered to the ureter of the awake rat, hyperalgesia in response to similar noxious electrical stimulation of the ipsilateral latissimus dorsi muscle is observed [122].
- In a more clinically relevant model (the prior implantation of a ureteric stone composed of dental cement) a similar hyperalgesia following noxious electrical stimulation of the external oblique muscle of the anterior abdominal wall is observed [124]. This hyperalgesia lasts for several days, the duration being increased by the continuing presence of the stone in the ureteric lumen.

- In human patients suffering with ureteric colic, non-invasive elimination of the intra-luminal stone (by extracorporeal shock-wave lithotripsy) has been shown to be associated with a reduction in the hyperalgesic response. This has been observed following both mechanical and electrical stimulation of either skin or muscle in the ipsilateral lumbar region - areas innervated by neurones arising in the same spinal cord levels as those supplying the ureter [121]. As in the rat model described above, this attenuation of the referred hyperalgesic responses following stone removal is time dependent and most profound in the skin. Prior examination of a small series of similar (untreated) patients demonstrated that sensory thresholds in the area of referral are most profoundly reduced in ipsilateral muscles (in comparison to the contralateral unaffected area) with least effect being observed in the skin [329]. Thus, it would appear that, following inflammation of visceral organs, not only is an associated hyperalgesia observed which is referred to distant sites, but that this hyperalgesia demonstrates plasticity of response, in terms of both site and duration.

As discussed in section 1.i.a., secondary hyperalgesia appears to be consequent upon alterations in central processing, with peripheral effects alone being unable to explain the phenomenon. However, in cutaneous tissue, activity generated in peripheral nociceptors appears to play a critical role in both generating and maintaining such referred effects. One such example of this is observed when the generation of a zone of secondary hyperalgesia in skin following capsaicin administration is examined. The zone of cutaneous hypersensitivity away from the injection site can be ablated by the prior administration of a proximal nerve block, with no effect being observed on the peripheral flare response, which continues unabated. Furthermore, if the capsaicin injection site is cooled or anaesthetised

following the injection, the extended zone of secondary hyperalgesia observed is markedly attenuated [238]. Thus, altered activity in the periphery appears to be associated with changes in central processing that result in the perception of secondary hyperalgesia.

In the visceral domain, previous work with the inflamed rat urinary bladder model has demonstrated that inflammation is associated with an increase in the electrical activity observed in peripheral afferent neurones [221]. This might be expected to occur consequent to the production of an inflammatory soup of chemical mediators released from immune components and damaged cells. However, sensitisation of the dorsal horn component of the neuronal response has also been documented [215]. Such sensitisation has been characterised by Woolf as a shifting of neuronal response from mode 1 (the control or normal state) to mode 3 (the sensitised state) [340]. If such a shift were to occur it would tend to allow facilitation of somatosensory inputs at the appropriate dorsal horn level (in this case L5, L6 and S1). Thus visceral inflammation, with an associated peripheral sensitisation would lead to central sensitisation and subsequent somatic hyperalgesic responses might then be observed in appropriate dermatomes. Thus, alterations in peripheral processing would be limited to the visceral afferent neurones. Central sensitisation, rather than antidromic sensitisation of somatic afferent neurones would explain apparent alterations in perceptions of peripheral somatic tissue effects.

As described above, such plastic changes in the activity of dorsal horn neurones have been observed following electrical stimulation of primary afferent neurones. Repetitive cutaneous C-fibre stimulation is associated with a highly augmented response to later C-fibre stimulation has been documented [345]. This 'wind up' appears to be, at least in part, mediated by glutamate acting at the NMDA receptor. Not only has the 'wind up' of wide dynamic range dorsal horn cells been prevented by the administration

of NMDA receptor blocking drugs, such as ketamine [73], but facilitation of the α motor neurone reflex response is also blocked by NMDA receptor antagonists including MK-801 (Dizocilpine) and D-CPP (3(R-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid) [344]. 'Wind up', albeit of a much lesser degree (only approximately 20% enhancement, in comparison to 190% in the somatic domain) has also been observed following stimulation of visceral afferent neurones in the splanchnic nerve [167]. Again this 'wind-up' of response is dose-dependently inhibited by ketamine. Moreover, the reflex responses to distension of visceral organs are upregulated following inflammation of both the bladder [275] and the colon [63]. Pre-emptive administration of the competitive NMDA antagonist AP-5 prevents such changes in the bladder [275], whilst MK-801 reverses the visceral hyperalgesia observed following intra-colonic instillation of zymosan [63]. In addition, upregulation of *c-fos* expression in the spinal cord following bladder inflammation is also attenuated by MK-801 [35]. Thus, multiple strands of evidence point to the importance of the excitatory amino acid, glutamate, in the production of sensitisation of central neuronal elements, in both somatic and visceral domains.

The presence of symptoms and signs referred to distant somatic sites is a feature characteristic of pain arising in the viscera. As such, if a model is to be accepted as appropriate for the study of visceral pain it is important to document referred signs. I believe that the data presented here provides such documentation. Not only are the alterations in visceral reflex responses in accord with those observed in human patients, but the somatic sensory effects identified here are also consistent. Thus it supplies a new strand of evidence supporting the chemical inflammation of the rat urinary bladder as an appropriate animal model in which to study persistent visceral pain.

# Chapter 3:

# The relevance of nerve growth factor to referred hyperalgesia

## 3.i. Introduction

### 3.i.a. Nerve growth factor (NGF):

The neurotrophin nerve growth factor (NGF), was initially described as a target derived growth factor, important for the survival and phenotypic differentiation of sympathetic and small diameter sensory neurones in foetal animals [66,220,280]. As the animal matures the proportion of neurones that depend upon NGF for survival decreases, such that in the adult deprivation is not associated with any neuronal loss [181]. However, whilst neurone survival becomes independent of NGF, the phenotypic expression of neurones may continue to depend upon it into later life [341]. In addition, there is accumulating evidence that NGF is pivotal in the modulation of pain-signalling pathways in adults:

1) Low levels of NGF (or its mRNA) are observed in many peripheral tissues under basal conditions. However, expression is increased both in a variety of animal models of inflammation [217] and also in inflamed tissues taken from human patients [11,186].
2) When exogenous NGF is administered to animals, it has been shown to:
    a) produce hyperalgesia in response to thermal [319] and mechanical stimulation [182].
    b) be associated with the onset of high pressure waves observed in the cystometrogram, following intra-vesical instillation [91].
    c) sensitise primary afferent nociceptive neurones [90,217].

d) upregulate the production of sensory neurotransmitters in the dorsal root ganglia [184].

e) sensitise dorsal horn neurones in the spinal cord [15].

f) induce the expression of the immediate early gene *c-fos* in the dorsal horn of the spinal cord [89].

3) In contrast, if endogenous NGF is sequestered by the exogenous administration of the trkA-IgG fusion molecule (see below) or an anti-NGF antibody, the result is:

a) hypoalgesia in response to application of both thermal and chemical stimuli [220].

b) the prevention of hyperalgesia associated with inflammatory states affecting both somatic [220,343] and visceral tissues (including the reversal of turpentine-induced VVH) [91].

c) avoidance of the inflammation-associated upregulation of sensory neurotransmitters (or downregulation under basal conditions) [220,343].

d) the reduction of inflammation associated *c-fos* expression in the dorsal horn of the spinal cord [343].

4) NGF 'knockout' mice (in which the gene encoding NGF has been deleted) fail to develop nociceptive neurones. Furthermore, such mice also exhibit an increased threshold to nociceptive stimuli [66].

5) Mice which overexpress the NGF gene product exhibit hyperalgesia to noxious mechanical stimulation [75].

## 3.i.b. NGF Receptors:

The effects of NGF are mediated via two different classes of receptor: the high affinity trkA receptor and the low affinity p75 receptor [19]. The high affinity trkA receptor is a member of the tyrosine specific protein kinase family and is principally expressed in neural tissue. It was first

identified as a result of an oncogenic transformation at the catalytic domain, which led to the expression of the protein product gp140$^{trk}$. NGF was demonstrated to preferentially bind to this product in a variety of cell lines. This binding led to the rapid phosphorylation of tyrosine residues and an increase in kinase activity of the gp140$^{trk}$ product [162]. Alternate neurotrophins, such as brain derived neurotrophic factor and neurotrophin 3 also interact with the p75 receptor but they also preferentially bind to their own specific high affinity receptors, trkB and trkC [66]. Interactions between the various neurotrophins are complex [19].

Transgenic mice which fail to express the trkA receptor demonstrate a wide variety of sensory defects, including the failure to respond to a wide variety of noxious stimuli. These features correspond to a neuronal loss in sensory and sympathetic ganglia, for which NGF is a specific survival requirement in the neonate. In neonatal animals trkA is expressed on seventy to eighty percent of dorsal root ganglia cells. However, in the adult peripheral nervous system the trkA receptor is selectively expressed on only a subset of the small diameter primary afferent neurones, comprising approximately forty percent of the total [25]. These account for ninety percent of visceral afferent neurones (such as those in the pelvic nerve) but only approximately forty five percent of somatic afferents measured in peripheral nerves [219] and demonstrate a high degree of co-localisation with calcitonin gene related peptide (known to be contained in many nociceptors) [18]. Thus, NGF induced responses are likely to be particularly important in the modulation of responses to sensory stimuli applied to the viscera.

In addition to its neuronal localisation, trkA is also expressed on cells of immune origin, such as the mast cell [251], where it is associated with changes in the release reaction following inflammatory stimuli. When NGF is applied to mast cells *in vitro*, it increases both mast cell survival [141] and

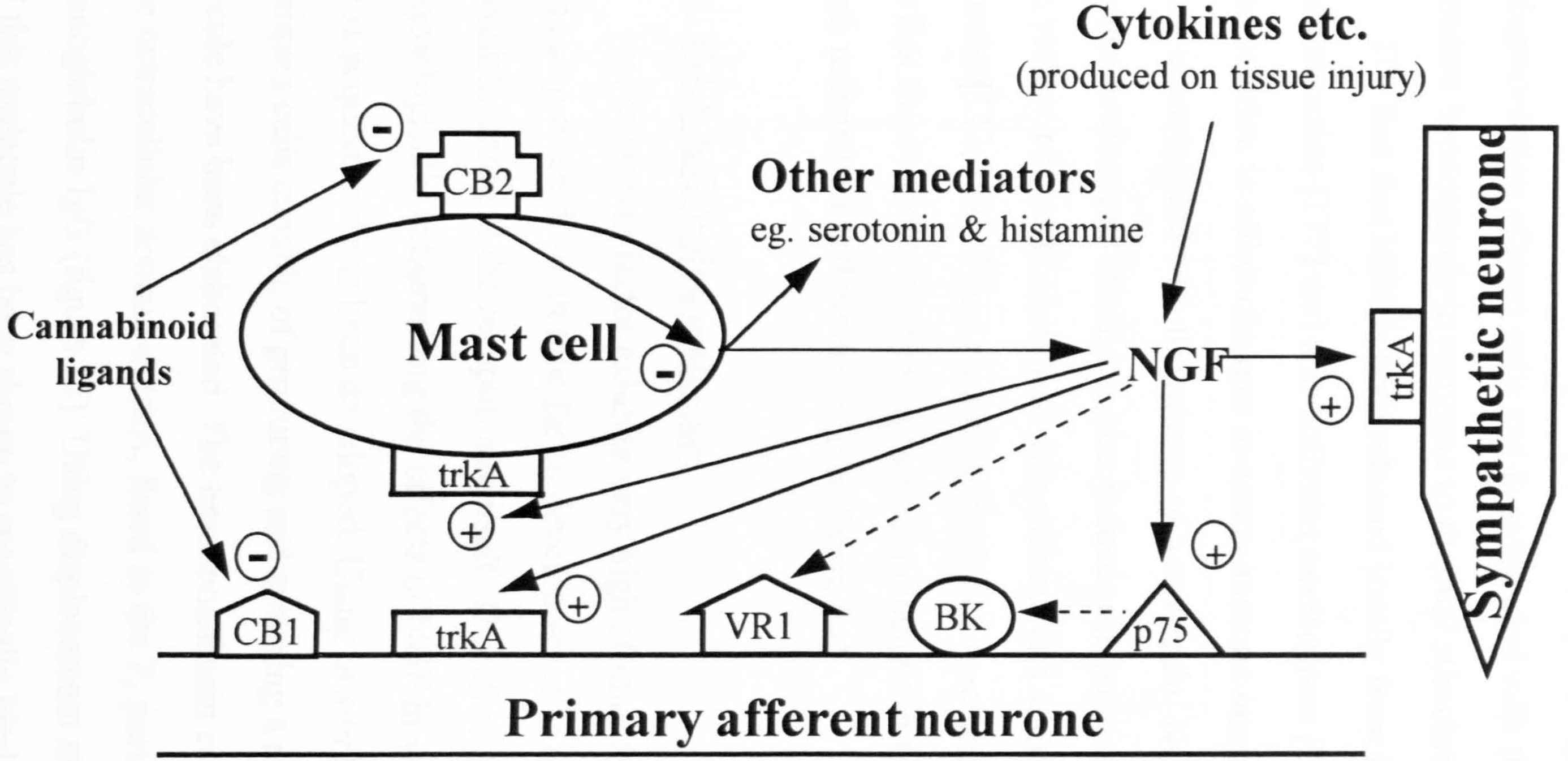



Figure 14.
Diagram demonstrating the putative interaction between nerve growth factor (NGF) and other molecules produced during the inflammatory response. Via its interaction with the trkA receptor NGF is capable of increasing primary afferent neurone activity, both directly and indirectly. Interaction with the p75 receptor may be associated with alteration in receptor responses to both bradykinin (BK) and agents acting at the vanilloid receptor (VR1). Cannabinoids released during inflammation may downregulate inflammatory responses.

the release of inflammatory mediators, such as serotonin [142]. *In vivo* administration of NGF has been demonstrated to increase the number and size of mast cells in the local area in addition to increasing vascular permeability in the local area [179]. Moreover, NGF administration triggers the degranulation of mast cells and is associated with the onset of a dose-dependent hyperalgesia in response to thermal stimulation [319].

The fact that NGF is both released locally from immune tissue at sites of inflammation [177] and also activates nociceptors [217], places it in a prime position to effect changes in neuro-immune interactions. Thus, local trauma is associated with the release of a molecule, NGF, that not only activates nociceptors itself, but also induces the release from mast cells, of a wide variety of chemicals which also activate and sensitise primary afferent neurones [142,319]. Thus, a positive feedback mechanism is set in motion, such that the concentration of algogenic molecules in the area of nociceptors is both maintained and increased (see figure 14).

### 3.i.c. TrkA-IgG fusion molecule:

Nerve growth factor exhibits very high affinity for the trkA receptor and it has proved difficult thus far to develop specific competitive antagonists acting at the receptor site itself. Therefore an alternate approach to physiologically antagonising the effects of NGF in which endogenous NGF is sequestered has been developed. Using *in situ* hybridisation techniques cells capable of producing and secreting a novel immunoadhesin molecule have been elaborated. The immunoadhesin consists of two copies of the extracellular domain of trkA, fused to the $F_c$ portion of the immunoglobulin IgG (figure 15). Using displacement studies with iodinated NGF this molecule has been shown to specifically bind to NGF [293]. *In vitro* studies indicate that not only does this immunoadhesin bind to NGF, but it also alters the biological activity of NGF. The survival of chick

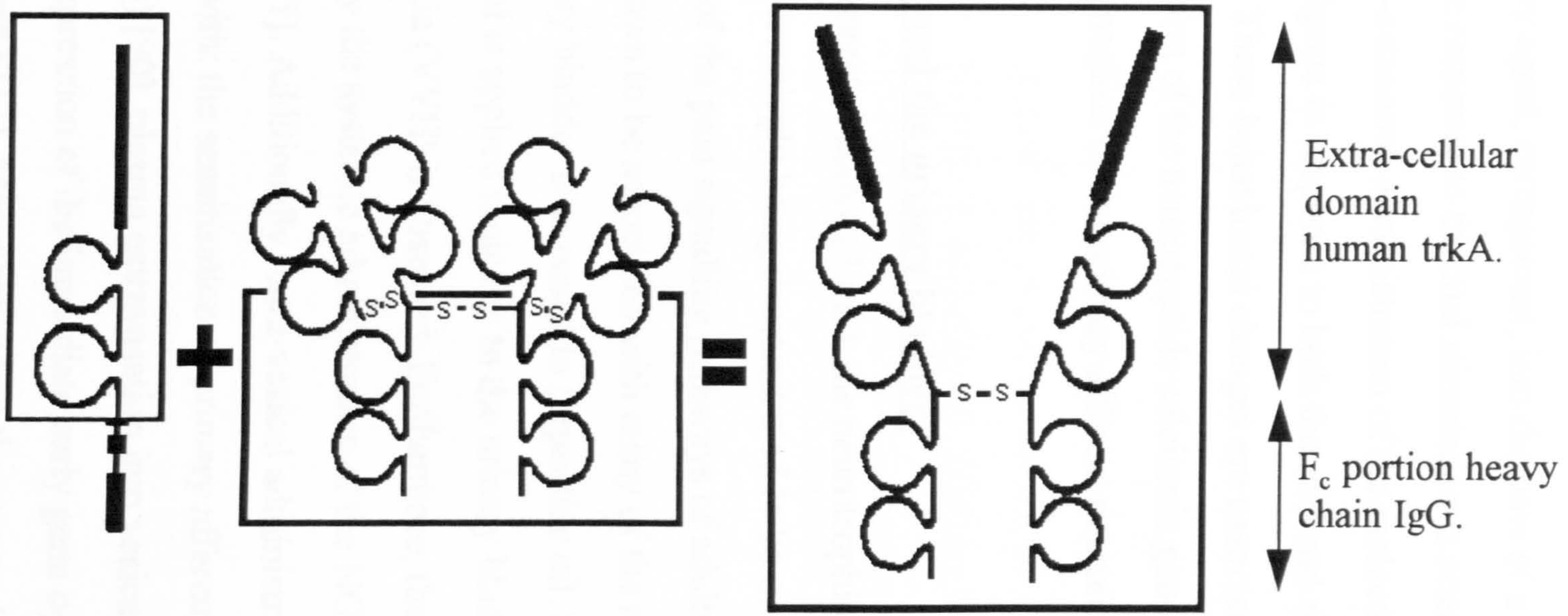



Figure 15.
Schematic diagram representing the fomulation of the soluble immunoadhesin molecule trkA-IgG.
Human nerve growth factor (NGF) binds avidly to the trkA portion of the molecule and is thus rapidly sequestered.

neurones derived from both dorsal root and sympathetic ganglia are inhibited by the presence of trkA-IgG [293]. Furthermore, *in vivo* studies indicate the potential of this molecule to provide new information in the study of pain mechanisms [220]. When trkA-IgG is co-administered with the inflammatory agent, carrageenan, into the paw of a rat, the expected hyperalgesic response to thermal stimulation is attenuated. Moreover, chronic sub-cutaneous administration of the molecule is associated with a local hypoalgesia in response to both thermal and chemical (capsaicin) stimulation. These behavioural changes are associated with a downregulation of the neuropeptide calcitonin gene related peptide in the dorsal root ganglion of the primary afferent neurones supplying the treated area.

### 3.i.d. NGF and the urinary bladder:

As suggested above (3.i.a.), the neurotrophin nerve growth factor (NGF), is increasingly being demonstrated to be of major importance in the modulation of the pain signalling pathways of adults [220]. Furthermore, it has been shown to be associated with many of the responses observed when the rat urinary bladder is exposed to turpentine oil. Specifically, when turpentine oil is applied topically to the urinary bladder a viscero-visceral hyper-reflexia (VVH) is observed. Furthermore, the presence of this VVH is prevented by the systemic administration of the NGF sequestering agent trkA-IgG [91]. Additionally, intra-vesical administration of NGF is associated with: the sensitisation of primary afferent neurones (located in the pelvic nerve) [90], plasma extravasation into vesical tissue [90] and an increased expression of the immediate early gene c-*fos* in the dorsal horn of the spinal cord [217]. Moreover, when the urinary bladder is inflamed by the instillation of 50% turpentine oil, an increased level of NGF is observed in vesical tissue [13].

Not only has NGF been implicated in the pathophysiological responses to urinary bladder inflammation in animal models (as described above), but there is some evidence that correlates may be found with human patients. In individuals suffering with painful conditions of the urinary bladder, increased levels of NGF in bladder tissue biopsies have been demonstrated [186]. Increased numbers of mast cells have also been demonstrated in bladder biopsies from patients suffering with the chronic, painful, inflammatory condition interstitial cystitis [178]. These mast cells are more responsive to a range of secretogogues (of which NGF is one such [141,142,319]), than are those isolated from normal vesical tissue [111]. Furthermore, cultured human mast cell lines have been demonstrated to express both functional trkA receptors (which demonstrate high affinity for NGF) and also the mRNA encoding for NGF itself [251]. Thus NGF appears to be of critical importance in inflammatory conditions of the urinary bladder, in both animal models and human patients.

### 3.i.e. Experimental rationale:

Since many of the features associated with turpentine inflammation of the rat urinary bladder are known to be NGF dependent, the relevance of NGF to the referred viscero-somatic hyperalgesia (VSH) was also investigated. Therefore, the fore and hind limb withdrawal thresholds (from both thermal and mechanical stimuli) were compared between control animals and animals in which the bladder was inflamed with NGF or turpentine (in the presence or absence of an NGF sequestering agent).

## 3.ii. Methods

All experiments conformed with British Home Office Regulations. Animals were housed according to national guidelines in colony cages of six

animals per cage with a dark : light cycle of 12 hours : 12 hours. They were provided with free access to food and water.

Surgical preparation:

In view of the prolonged recovery time noted in experiments described in chapter 2, when animals were anaesthetised with pentobarbitone, the anaesthetic technique was modified to allow observations to be made at earlier time points. Animals were anaesthetised in a clear perspex induction box with 5% isoflurane in a carrier supply of nitrous oxide and oxygen (in a 2 : 1 ratio). Maintenance was achieved with 0 to 2% Isoflurane (in the same carrier) administered via head masks (see figure 16). The urinary bladder was catheterised with a sterile, lubricated, nylon catheter (external diameter 0.75mm - Portex, U.K., 200/300/020). Vesical inflammation was achieved by the instillation of 0.5ml, 50% turpentine oil or 10μgm of NGF, both for a duration of one hour. Venous access was achieved via a tail vein (0.6mm external diameter, Abbocath-T, Abbott Ireland Ltd., Sligo, Republic of Ireland).

Treatment protocols:

Four different treatment groups were utilised:

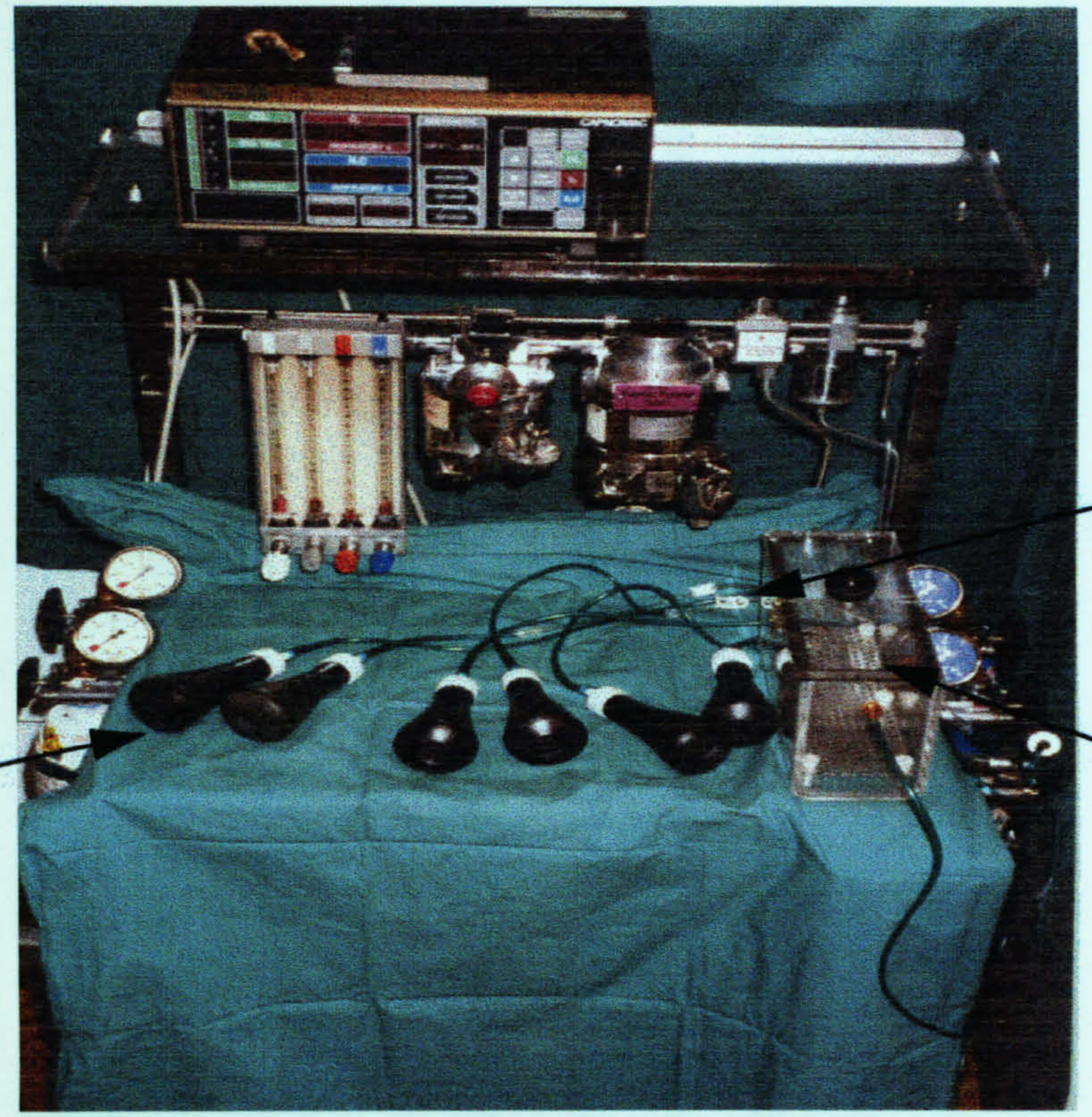
I. Administration of general anaesthesia alone.

II. Instillation of intra-vesical turpentine, pre-treatment with 1mg/kg of the test compound trkA-IgG (i.v.).

III. Instillation of intra-vesical turpentine, pre-treatment with 1mg/kg of an IgG-control molecule, as a control for the trkA-IgG test compound (i.v.).

IV. Instillation of intra-vesical NGF (10μgm, 0.5ml DMSO : saline carrier 1:19dilution). No other treatment.

Systemic test compounds were administered (i.v.) immediately prior to the instillation of the inflammatory agent (0.5ml 50% turpentine oil, or 10μgm NGF) into the bladder. Observation of the right fore and hind limb



Clip system on gas tubing allows control of flow to head masks.

Head masks to allow the administration of anaesthetic to 6 rats concurrently.

Induction box connected to supply of isoflurane in a carrier gas of nitrous oxide and oxygen (2 : 1 mixture).

Figure 16.
Photograph of equipment used to anaesthetise rats in experiments assessing the presence of a somatic hyperalgesia following inflammation of the urinary bladder.

withdrawal latencies from either thermal or mechanical stimuli were made prior to any intervention and at two, four, six, eight and twenty four hours post-intervention. The additional early observation (at two hours post-intervention) was possible since the refined anaesthetic technique appeared to be associated with a more rapid recovery than the pentobarbitone anaesthetic previously utilised (in the experiments described in chapter 2). The testing regimens were identical to those described in chapter 2, section ii and figure 9 on page 66. Animals were culled by overdose of anaesthetic (i.p. phenobarbitone) and cervical dislocation at completion of experiments.

Outcome measures:

As in the experiments described in chapter 2, the withdrawal thresholds of the fore limbs across time were compared to baseline (ANOVA [Dunnett's]), to assess whether any change in this parameter had occurred secondary to the systemic effects of bladder inflammation.

The difference in fore and hind limb withdrawal thresholds, as a percentage of their baseline values ($\Delta_{Thresh}$) was calculated for each time point using the same equation as in chapter 2:

$$\Delta_{Thresh} = \left[\left(\frac{H_{Thresh}}{BH_{Thresh}}\right) \times 100\right] - \left[\left(\frac{F_{Thresh}}{BF_{Thresh}}\right) \times 100\right]$$

Curves were constructed for values obtained from each animal of $\Delta_{Thresh}$ against time and the area under the curve (AUC) for each of these curves was calculated using the trapezium rule (Sigmaplot version 3.0, Jandel Scientific Software). Comparison of the AUCs of each group was undertaken using ANOVA with *post-hoc* Dunnett's testing (Sigmastat version 2.0, Jandel Scientific Software).

## 3.iii. Results

### 3.iii.a. <u>Modulation of the observed thermal hyperalgesia:</u>

Experiments were performed on twenty four animals weighing 225 - 275gm (mean weight 229.7gm). No significant difference across time ($p > 0.05$, ANOVA [Dunnett's]) was observed in the fore limb withdrawal thresholds (for any of the four groups) when compared to baseline withdrawal latencies from the thermal stimulus.

Curves of the mean (sem) $\Delta_{Thresh}$ against time for the no catheter, negative control group animals ($n = 6$) and the intra-vesical NGF group ($n = 6$) are shown in figure 17. Whilst the curve for the no catheter control group shows a small variation around the zero point, the administration of intra-vesical NGF is associated with a negative deflection of the curve, indicating a relative hyperalgesia of the hind limb.

Comparison of the AUCs for these curves demonstrated a significant difference (ANOVA [Dunnett's], $p < 0.05$) between the mean AUC of the no catheter control group ($+0.4 \times 10^3$, sem $0.3 \times 10^3$) and that of the intra-vesical NGF instillation treated group ($-1.4 \times 10^3$, sem $0.6 \times 10^3$) (see figure 18). The degree of response observed is similar to that observed in the previous experimental protocol presented in chapter 2 (inflammation with turpentine alone). Thus, it would appear that intra-vesical NGF treatment is associated with a similar VSH to that observed after intra-vesical turpentine instillation. However the response observed following intra-vesical NGF is larger by a single order of magnitude than that observed following turpentine instillation. This probably reflects the rapid negative deflection of the $\Delta_{Thresh}$ against time curve in the first two hours. Thus, when the first observation made is at four hours the AUC would tend to be underestimated. However, a significant relative hyperalgesia to thermal stimulation of the hind limb (in

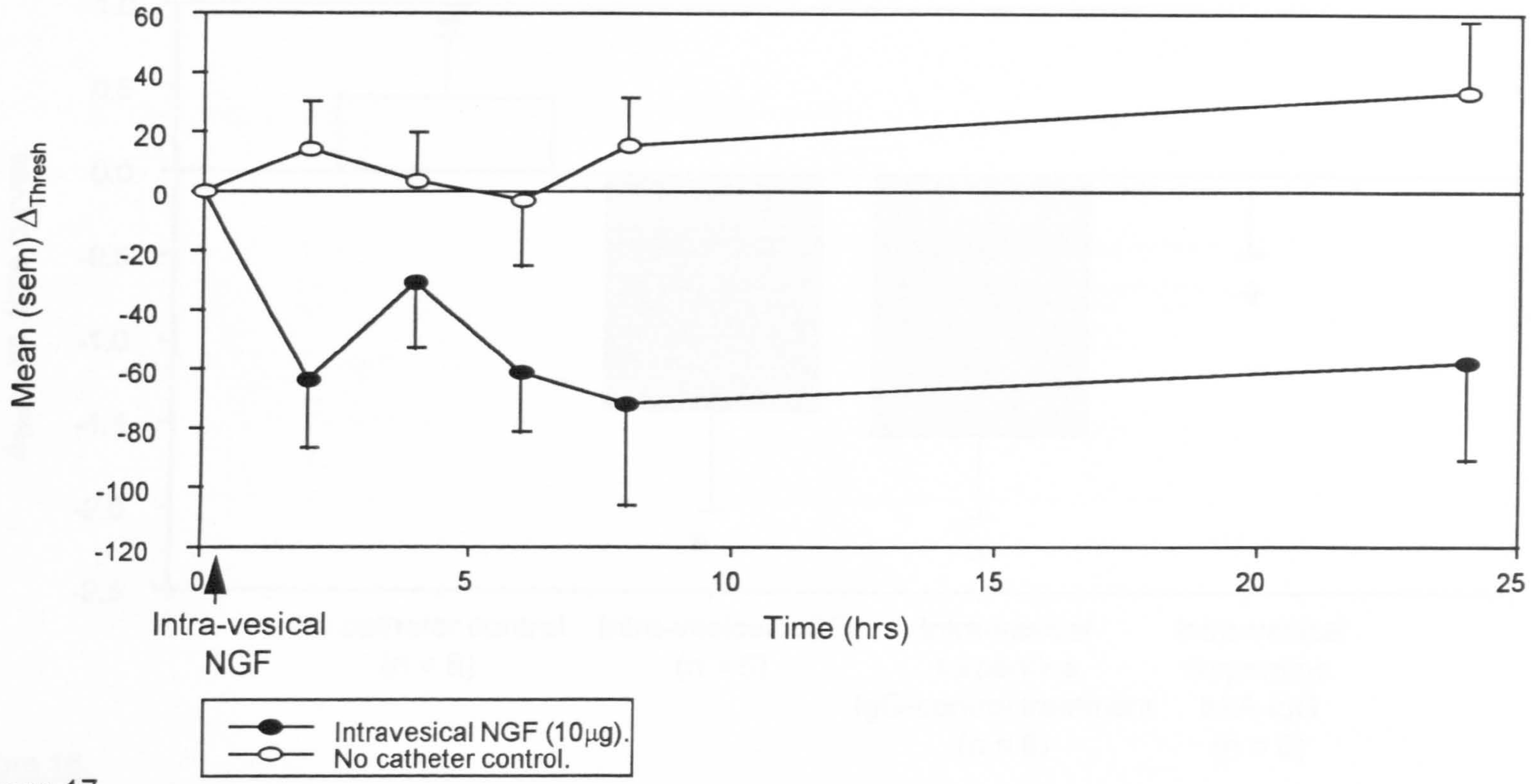



Figure 17.

Graph showing the difference between fore and hind paw withdrawal thresholds ( $\Delta_{Thresh}$) from a thermal stimulus (46$^0$C) following inflammation of the urinary bladder with intra-vesical nerve growth factor (NGF). Note the negative deflection of the curve in the NGF-treated animals, indicative of a relative hyperalgesia of the hind limb. This is maintained for the full 24 hours of the observation period.

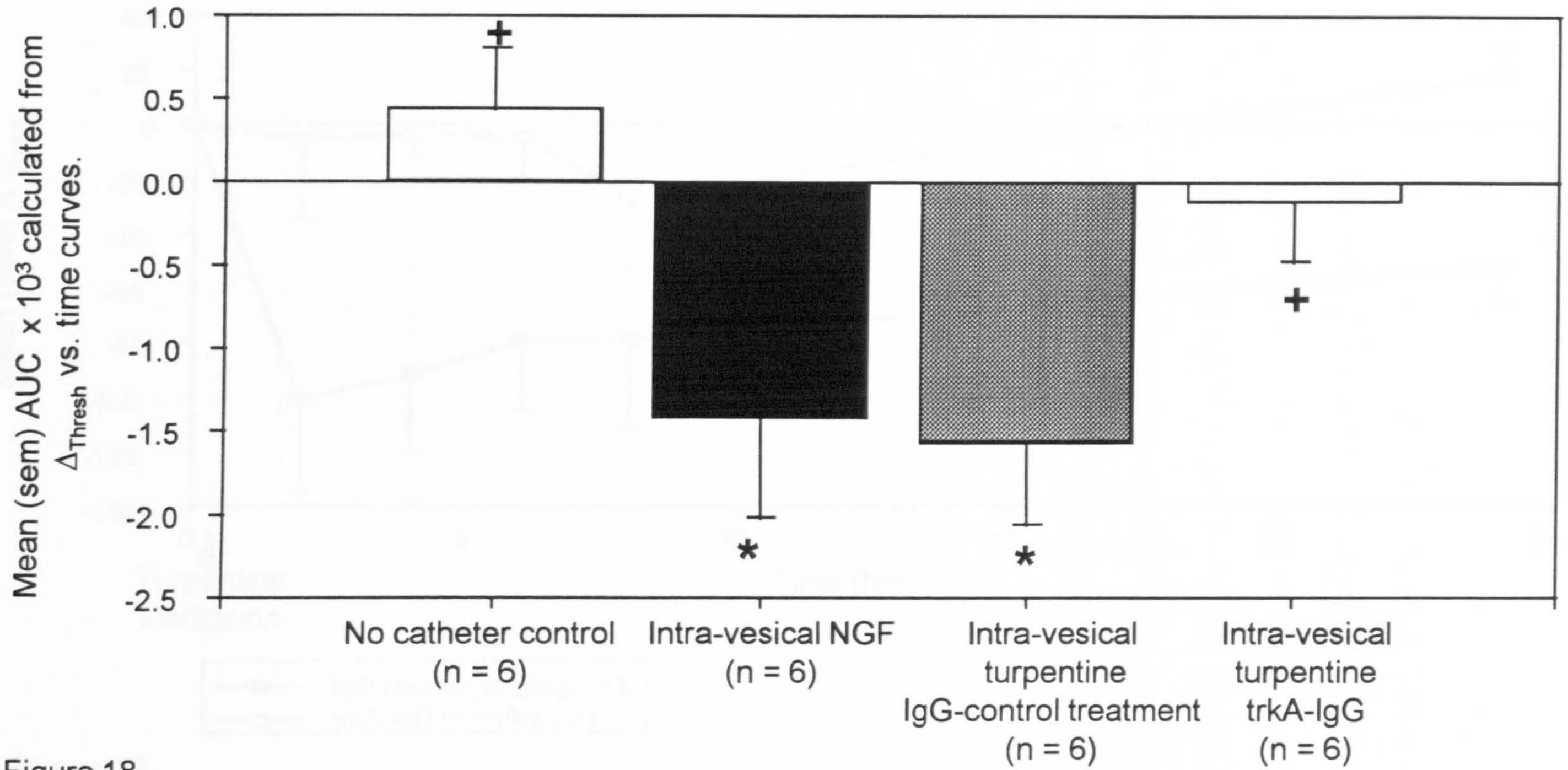



Figure 18.

Bar chart showing the mean area under the the $\Delta_{Thresh}$ against time curve (AUC), when a thermal stimulus (46$^0$C) is applied to the paw. Note that when the urinary bladder was treated with NGF or turpentine oil a large negative AUC is observed. When prophylactic treatment with an NGFsequesterent (trkA-IgG) is administered this effect is attenuated.

* $p < 0.05$ compared to the no catheter control group (t-test).

\+ $p < 0.05$ compared to the IgG control treated group (t-test).

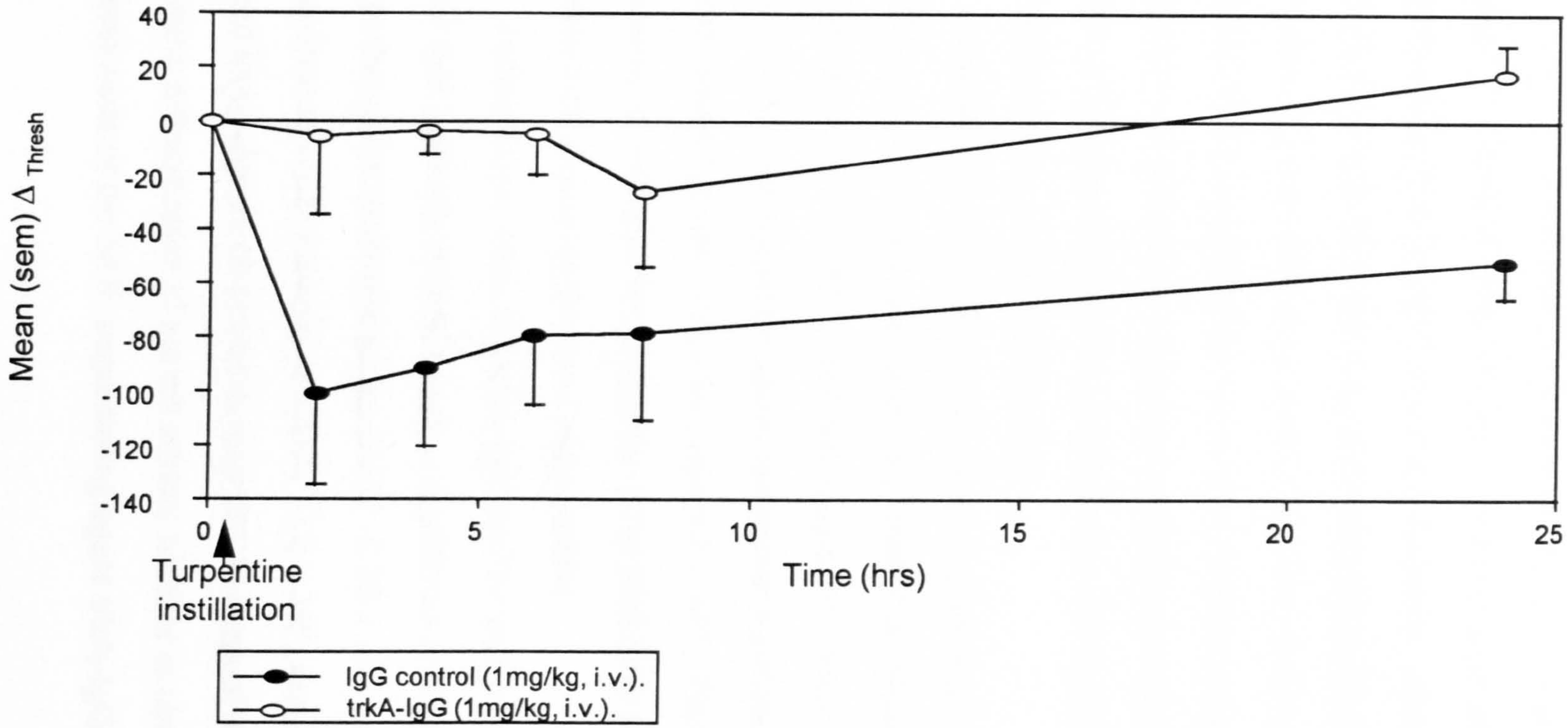



Figure 19.

Graph of $\Delta_{Thresh}$ against time (for withdrawal from a thermal stimulus) following systemic prophylactic treatment with either trkA-IgG or a control antibody (IgG), with subsequent instillation of intra-vesical turpentine oil. Note that the curve for the active (NGF sequestering) trkA-IgG antibody treated group approximates to zero, whilst that for the control antibody treated group demonstrates a negative deflection indicative of a relative hyperalgesia of the hind paw in comparison to the fore paw.

relation to the fore limb) is evident in the first twenty four hours following treatment of the urinary bladder with the neurotrophin NGF.

When the difference between fore and hind limb withdrawal latencies is plotted for the turpentine inflamed, control immunoglobulin treated group, a negative deflection is again observed. However, when the NGF sequestering agent trkA-IgG was administered prophylactically no such associated negative deflection of the curve was observed (see figure 19). Thus the relative hyperalgesia of the hind limb (in comparison to the fore limb) observed following turpentine instillation into the urinary bladder appears to be prevented by the prophylactic, systemic administration of the NGF sequestering molecule trkA-IgG.

Comparison of the AUCs for these curves was undertaken (see figure 18). There was a significant difference (ANOVA [Dunnett's], $p < 0.05$) between the AUCs of the no catheter, negative control group ($+0.4 \times 10^3$, sem $0.3 \times 10^3$) and the intra-vesical turpentine instillation and IgG control antibody treated group ($-1.6 \times 10^3$, sem $0.5 \times 10^3$). This confirms the association of a relative hyperalgesia of the hind limb when the bladder is inflamed with topical application of turpentine.

Furthermore, when the trkA-IgG treated group is compared to the control IgG antibody treated group, a significant reduction in the AUC is seen in the active treatment group (AUC $-0.08 \times 10^3$, sem $0.3 \times 10^3$) in comparison to control treatment (AUC $-1.6 \times 10^3$, sem $0.5 \times 10^3$). Thus the referred hyperalgesia observed in response to thermal stimulation, following turpentine inflammation of the rat urinary bladder is obtunded by the administration of the NGF sequestering agent trkA-IgG.

### 3.iii.b. Modulation of mechanical hyperalgesia:

Experiments were performed on twenty four animals weighing 200 - 235gm (mean weight 212.5gm). The measurements of the withdrawal thresholds of the fore limb across time show no significant difference ($p > 0.05$, ANOVA [Dunnett's]) from baseline withdrawal thresholds in three of the four experimental groups. Somewhat surprisingly, when IgG-control antibody treatment (i.v.) preceded intra-vesical turpentine instillation a significant difference across time in the fore paw withdrawal thresholds, (indicative of a hypoalgesia in response to mechanical stimulation) was observed. The reason for this is unclear.

Curves of the mean (sem) $\Delta_{Thresh}$ against time for the no catheter control group and the intra-vesical NGF instillation group are shown in figure 20. Intra-vesical NGF instillation following systemic administration of a control antibody is associated with a negative deflection of the curve, suggestive of a hyperalgesia of the hind limb relative to the fore limb.

Comparison of the AUCs calculated from the curves plotted for each animal are shown in the bar graph in figure 21. The administration of intra-vesical NGF is associated with a large negative AUC, with a clear trend toward hyperalgesia of the hind limb. The results are of a similar order of magnitude as in the previous experiments, utilising intra-vesical turpentine oil (see chapter 2) with a mean AUC $-8.2 \times 10^3$ (sem $3.5 \times 10^3$). However, the difference between the AUC for the group treated with intra-vesical NGF and the no catheter control group (mean AUC $-0.5 \times 10^3$, sem $1.2 \times 10^3$) does not quite attain significant levels ($p = 0.065$) with a sample size of $n = 6$.

As shown in figure 22, i.v. treatment with an IgG control molecule prior to turpentine inflammation of the bladder is associated with a negative

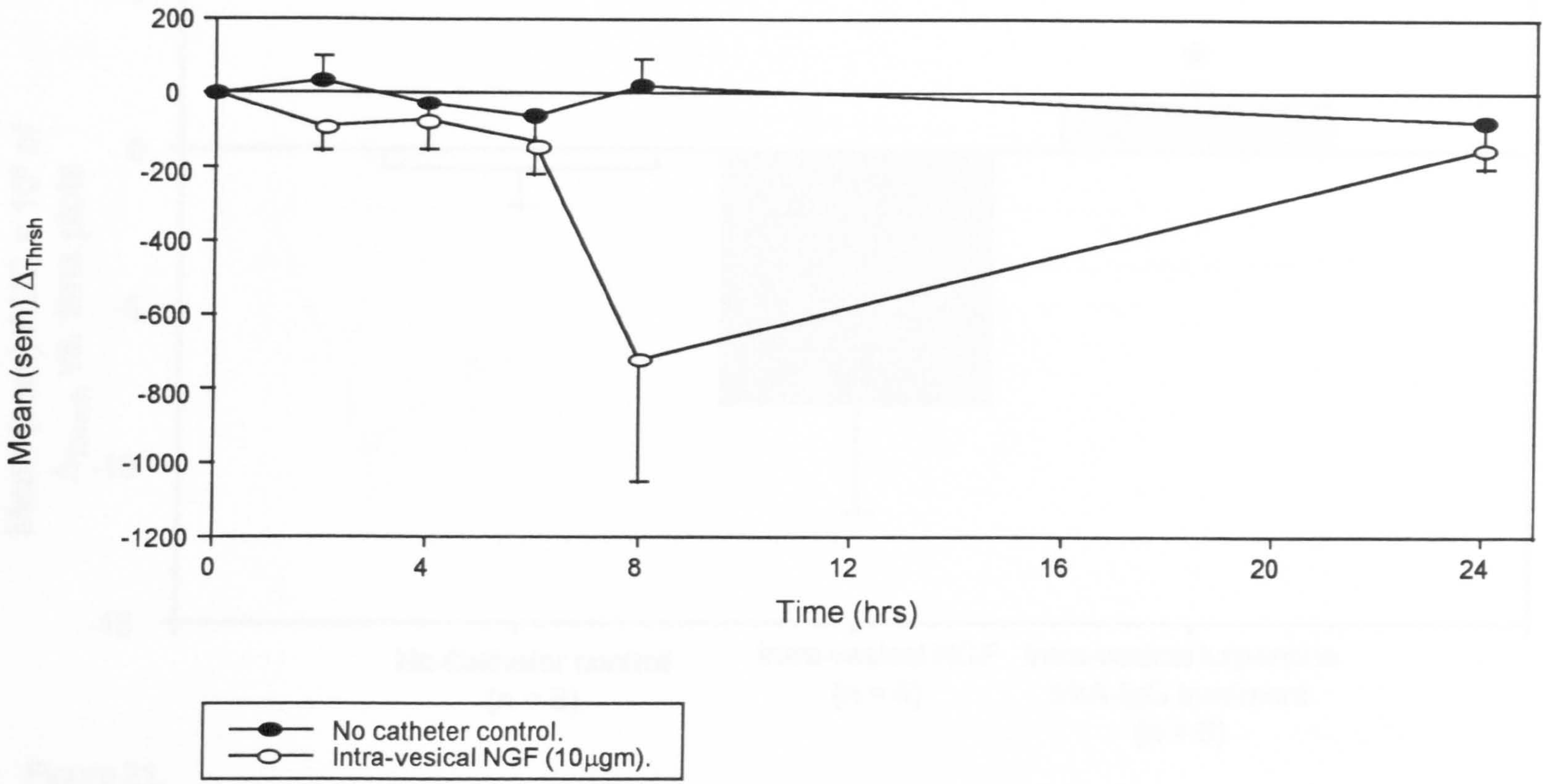



Figure 20.

Graph of the difference between fore and hind limb withdrawal thresholds ( $\Delta_{Thresh}$) from a mechanical stimulus (von Frey hairs) following intra-vesical instillation of nerve growth factor (NGF). Note there is minimal deflection from the zero line in the control group. However, a large negative deflection of the curve is observed following NGF instillation. This suggests the presence of hyperalgesia of the hind paw relative to the fore paw.

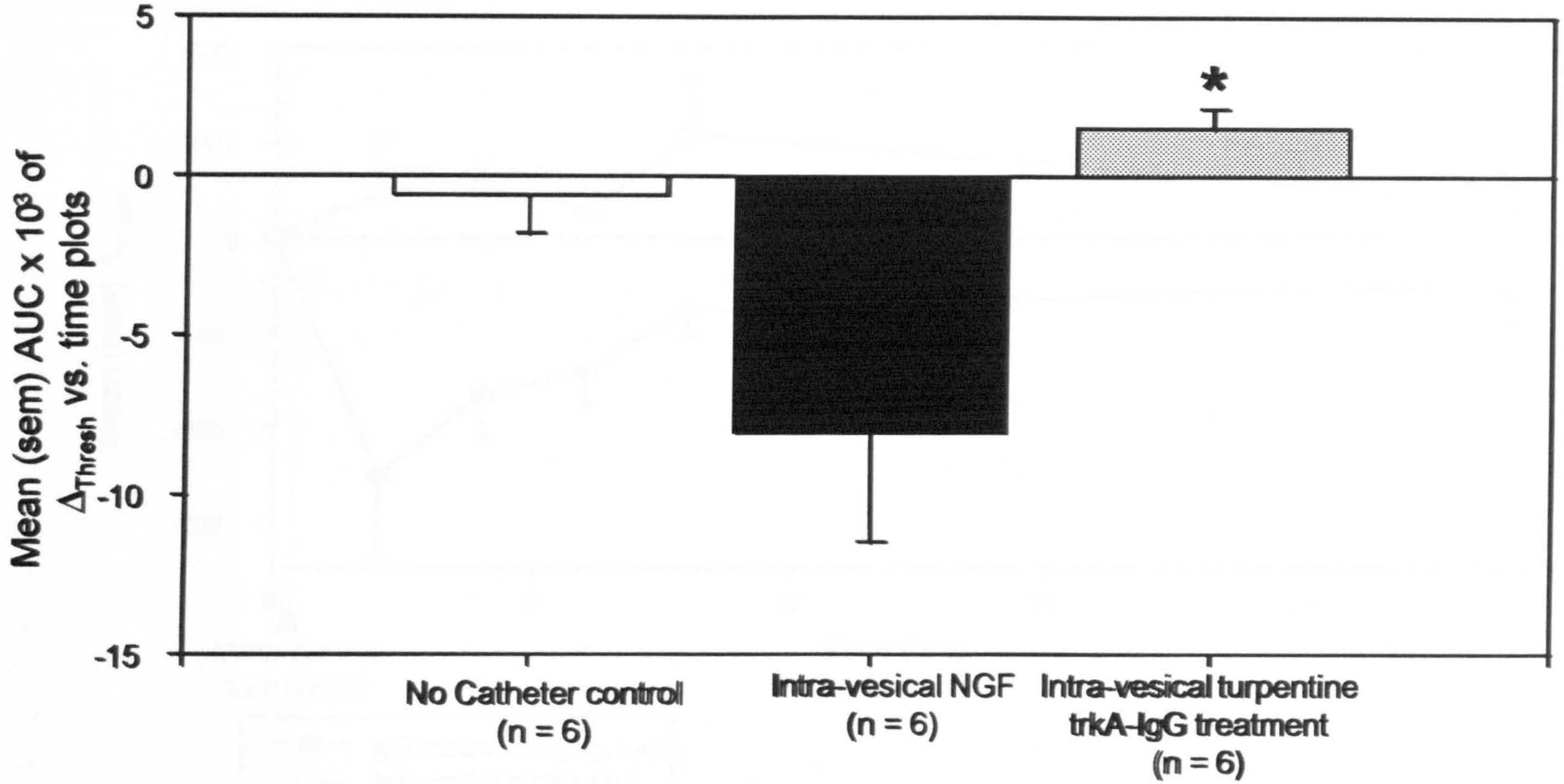



**Figure 21.**

**Bar chart demonstrating the mean (sem) area under the curve (AUC) of $\Delta_{Thresh}$ against time plots following stimulation with von Frey hairs. Not only is treatment with the active nerve growth factor (NGF) sequestering agent (trkA-IgG) not associated with a negative deflection, but it also demonstrates a significant difference from the group treated with intra-vesical NGF.**

* $p < 0.05$ in comparison to the NGF treated group.

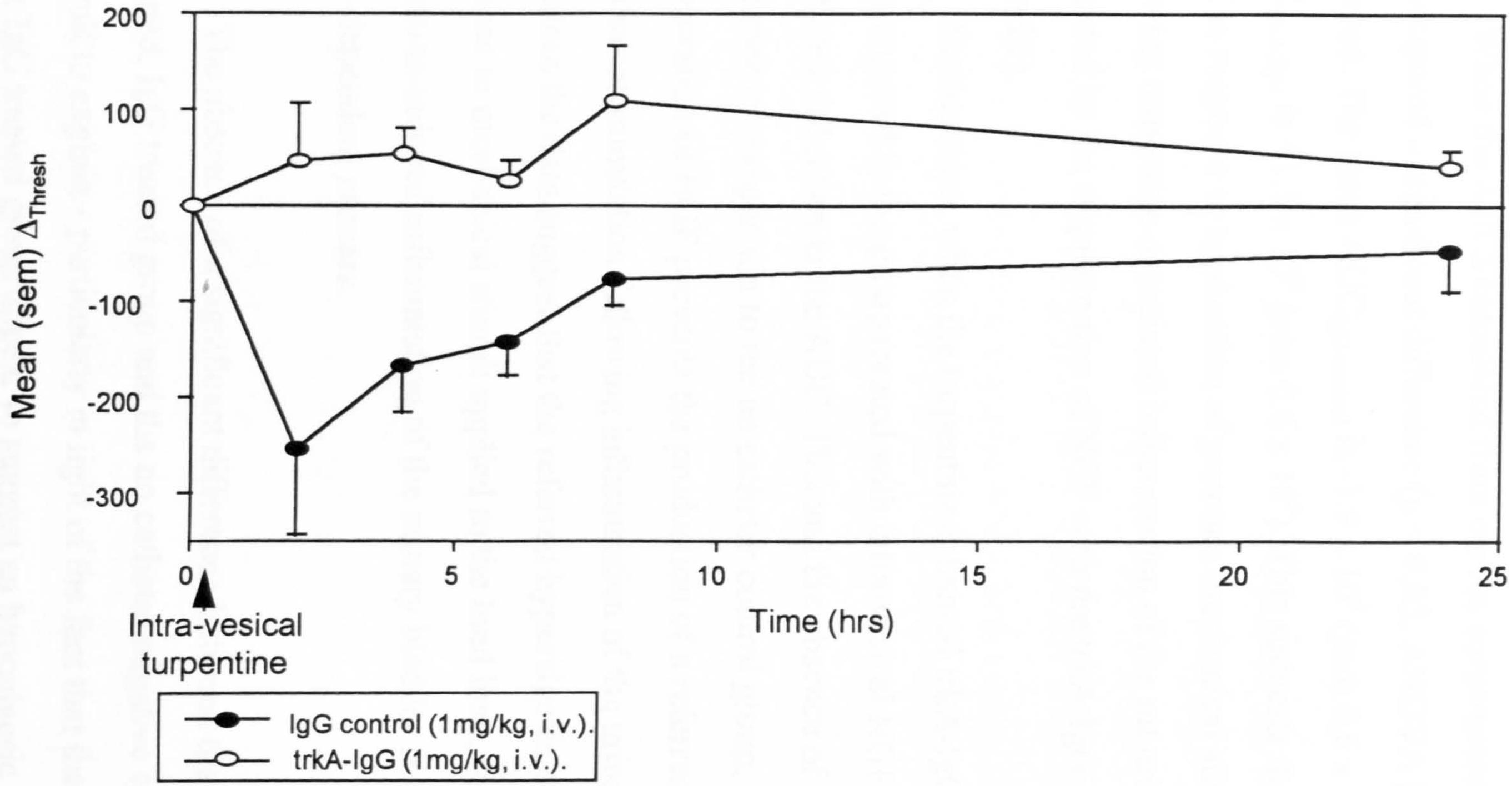



Figure 22.

Graph of the difference between fore and hind limb withdrawal thresholds ( $\Delta_{Thresh}$) against time, from von Frey hairs, following treatment with intra-vesical turpentine oil. Note the negative deflection of the curve following treatment with the control antibody (IgG), indicative of a relative hyperalgesia of the hind paw. This is prevented by prophylactic treatment with the active NGF sequestering molecule ( trkA-IgG).

deflection of the mean (sem) $\Delta_{Thresh}$ against time curve. In contrast, prophylactic treatment with systemically administered trkA-IgG active molecule, is not associated with any such a deflection of the curve.

When the AUCs calculated from curves constructed for each animal are compared a significant difference ($p < 0.05$, ANOVA [Dunnett's]) is observed. The mean $AUC_{IgG\ control}$ is $-1.9 \times 10^3$ (sem $0.6 \times 10^3$) and the mean $AUC_{trkA\text{-}IgG}$ is $+1.5 \times 10^3$ (sem $0.6 \times 10^3$). This suggests that the referred VSH in response to application of punctate mechanical stimuli observed following turpentine-associated inflammation of the rat urinary bladder, is prevented by the sequestration of NGF with the trkA-IgG molecule (see figure 21).

Furthermore, when the turpentine-inflamed trkA-IgG treated group was compared to the group treated with intra-vesical NGF there was a significant reduction in the AUC. This and the absence of a significant difference in comparison to the no catheter control group, suggests that the sequestration of NGF prevents the production of a referred VSH to mechanical stimulation following inflammation of the urinary bladder. Therefore the data suggest that the referred hyperalgesia observed in response to mechanical stimuli applied to the hind limb, following turpentine-induced inflammation of the urinary bladder is mediated via an NGF-dependent process.

The absence of a significant difference between the turpentine inflamed, IgG treated group and the no catheter negative control group is difficult to explain - particularly in light of the fact that the fore limb values in the IgG treated group appear to suggest an hypoalgesic response to the treatment over the time course of the experiment. This could be due to a failure to produce an adequate inflammatory stimulus with turpentine in this group (although this has proved to be a robust technique) or a true effect of

the control antibody preventing the mechanical VSH. It is unclear why a control antibody should have such an effect, particularly when a similar effect was not observed in the thermal stimulation group (see section 3.iii.a.).

## 3.iv. Discussion

The neurotrophin nerve growth factor (NGF), has been shown to be of importance in the turpentine inflamed rat urinary bladder model of visceral pain. Previous work has documented the onset of a vesical hyper-reflexia (VVH) which is NGF-dependent [91]. Whilst this VVH is a feature associated with clinical conditions in which pain arising from the urinary bladder is prominent, it is not necessarily inextricably linked with it. The presence of a referred somatic hyperalgesia (VSH), documented in the previous chapter, suggests that sensory changes also occur following instillation of intra-vesical turpentine oil, further validating the model as an appropriate one in which to study visceral pain mechanisms. The work presented here investigated the role of NGF in the observed VSH.

I have demonstrated that the VSH observed in response to thermal stimulation, following inflammation of the urinary bladder of the rat, is NGF-dependent. This is evidenced by a five fold increase in the area under the curve (obtained by plotting the difference in fore and hind limb withdrawal thresholds ($\Delta_{Thresh}$) against time) for animals in which the bladder was inflamed with NGF, in comparison to control animals. Furthermore, the referred VSH observed in response to thermal stimulation following intra-vesical turpentine instillation, was attenuated by the prophylactic, systemic administration of the NGF sequestering molecule, trkA-IgG (but not a control molecule).

In addition to these changes in response to thermal stimuli, the VSH observed in response to the application of mechanical stimuli to the hind

limb also appears to be critically linked to the presence of NGF. Although, following the instillation of intra-vesical NGF the calculated mean AUC did not differ significantly from that derived from the no catheter control group ($p = 0.065$), there was a definite trend towards a referred hyperalgesia to punctate mechanical stimulation (as observed by a negative deflection of the $\Delta_{Thresh}$ against time curve). However, sequestration of NGF, by the trkA-IgG fusion molecule, did significantly attenuate the increase in the AUC observed following the instillation of intra-vesical turpentine oil. Thus, the findings suggest that NGF is an important factor in the induction of sensory changes following turpentine-induced inflammation of the rat urinary bladder.

The evidence currently available suggests that interactions of algogens with both the nervous and immune systems are of importance in the modulation of activity in pain pathways [331]. During tissue inflammation, accumulation of mast cells in the affected tissue is observed [204,237] and these mast cells are particularly prevalent in peri-neural locations [320]. Thus, molecules released from mast cells are in a prime position to interact with receptors located on neuronal tissue. One such algogenic molecule, known to be released by mast cell degranulation, is the neurotrophin, nerve growth factor (NGF) [238]. There is accumulating evidence that this molecule is pivotal in mechanisms underlying the perception of visceral pain in particular.

In the animal model of bladder inflammation utilised in these experiments, the endogenous production of both the mRNA encoding for NGF and also the NGF protein itself is upregulated in vesical tissue [217,253]. Moreover, in human subjects suffering with chronic inflammatory conditions of the bladder, tissue levels of NGF have been demonstrated to be elevated [186]. Although NGF is released from mast cells [177], the

presence of NGF is also associated with induction of the degranulation reaction of mast cells [319]. Therefore, both autocrine and paracrine effects mediated by NGF may occur. This mast cell degranulation reaction, instigated by NGF, is transduced via the interaction of NGF with [19] and subsequent phosphorylation of [251] the high affinity trkA receptor, which is expressed on the mast cell membrane of both rats [142] and humans [251]. Moreover, in addition to NGF, a cocktail of other pro-inflammatory mediators, including: cytokines, histamine, serotonin, leukotrienes and prostaglandins, are also released from mast cells [204,320]. Thus a rapidly acting, positive feedback mechanism, inducing upregulation of activity in the immune system may be set in motion by the action of NGF upon mast cells (see figure 14 on page 85).

In addition to its location on mast cells, the trkA receptor has also been demonstrated (by both *in situ* hybridisation and retrograde labelling techniques) to be expressed on primary afferent neurones arising in both somatic and visceral tissues. However, whilst between 20 and 50% of somatic afferent neurones express the trkA receptor, in visceral afferent neurones of the pelvic nerve this level rises to between 75 and 90% [26,219]. Thus, NGF is likely to exhibit particularly profound effects on visceral tissues such as the urinary bladder. Furthermore, the intra-vesical instillation of NGF is associated with both activation and sensitisation of primary afferent neurones travelling in the pelvic nerve [90]. This effect has been observed to be rapid in onset (within ten minutes), suggesting that it is, at least in part, directly mediated, probably via trk-A receptors expressed on primary afferent neurones. However, rapid release of inflammatory mediators from local mast cells, leading to indirect sensitisation of primary afferent neurones and subsequent VVH cannot be excluded. Indeed the increased sensitivity of the bladder to distension with time following NGF instillation might support such an indirect effect. Further support for the

neuronally mediated effect of NGF arises from the fact that approximately 90% of the primary afferent neurones in the pelvic nerve demonstrate an increase in activity following the instillation of intra-vesical NGF [90]. This is approximately the same proportion of pelvic nerve neurones known to express the trk-A receptor [219]. Moreover, when NGF is instilled intra-vesically, plasma extravasation into bladder tissue is observed unless animals have been pre-treated with capsaicin. Since capsaicin exerts effects via receptors located exclusively on primary afferent neurones [31,337], this suggests a direct neuronal effect of NGF [91]. Thus, if the instillation of turpentine oil into the urinary bladder is associated with a local release of NGF from vesical tissue, this might be expected to be associated with an increase in activity in afferent neurones (by both direct and indirect means) and thus enhancement of neural inputs to the dorsal horn of the spinal cord.

In addition to effects on the immune system (including the degranulation of mast cells [319]) and the activation of primary afferent neurones [90], the administration of exogenous NGF *in vivo* is also associated with: the elevation of neuropeptides in primary afferent neurones [343], the sensitisation of dorsal horn neurones [15] and the induction of both thermal [182,266,319] and mechanical [182,266,321] hyperalgesia. Furthermore, the upregulation of endogenous NGF production in transgenic mice has been associated with the presence of hyperalgesia [75]. Thus, pathophysiological increases in the endogenous release of NGF, observed during inflammation, might be expected to be associated with similar hyperalgesic responses. Whilst increases in the contractile activity of the bladder itself is reflective of visceral mechanical hyperalgesia and might be explicable entirely by local NGF actions, the explanation of effects observed at somatotopically appropriate, but distant sites requires alterations in more central processes. Such central sensitisation may well be, not only instituted, but also maintained by activity occurring in the periphery. Evidence from a

model of somatic pain, the sub-cutaneous injection of formalin into the paw of a rat, would tend to support this. The activity in C-fibres mimic the biphasic response observed in behavioural observations [212]. Furthermore, maximal inhibition of phase 2 nocifensive behaviour following both intra-thecal administration of mu receptor agonists [87] and peripheral injection of local anaesthetic agents [58] is only observed when treatments both, precede phase 1 of the response and also extend into phase 2. Thus ongoing activity in peripheral neurones is associated with and required for both induction and maintenance of central sensitisation. There is evidence that NGF is involved in both hyper-reflexia following chemical inflammation of the bladder [90] and hyperalgesia to thermal and mechanical stimuli following cutaneous inflammation [182]. In both these circumstances central sensitisation has been implicated. However, the inter-relationship between NGF and NMDA is unclear. Whilst both VVH [275] and thermal hyperalgesia [182] appear to be NMDA-dependent, mechanical hyperalgesia seems to be an NMDA-independent process [182]. This disparity in the inhibition, by NMDA receptor antagonists, of hyperalgesic responses is supported by observations following the administration of NMDA receptor agonists. Under these circumstances, thermal, but not mechanical, hyperalgesia results [45]. More studies will be necessary to elucidate the reasons for such discrepancies.

There is now some evidence that another neurotrophin, brain derived neurotrophic factor (BDNF) may have some role to play in the modulation of pain arising from the viscera. The high affinity receptor for BDNF is another of the tyrosine kinase family of receptors, trkB [19]. This is co-expressed with the NGF receptor trkA, on both small diameter cutaneous fibres and approximately 80% of visceral afferent neurones projecting in the pelvic nerve [219]. In the turpentine-inflamed rat urinary bladder model, a rapid, short duration rise in the levels of mRNA encoding BDNF is observed in bladder tissue [253]. Following exogenous administration of NGF, levels

of both the mRNA encoding BDNF and also the protein itself are increased specifically in the dorsal root ganglia of trkA-expressing neurones [158,240]. Furthermore, following the administration of NGF the anterograde transport of BDNF in both peripheral and central processes of these trkA-expressing neurones is increased, BDNF being predominantly observed in central arbors which also express the excitatory neuropeptide, calcitonin gene related peptide [240]. This elevation in BDNF is associated with potentiation of both spinal reflexes (whether elicited by direct electrical stimulation or NMDA administration) and c-Fos-like immunoreactivity in the spinal cord [158]. Moreover, exogenous administration of BDNF (and an alternate endogenous trkB agonist, NT-4/5) has been demonstrated to sensitise C fibres to thermal stimuli *in vitro* and in behavioural tests a thermal hyperalgesia is elicited [296].

Sequestration of BDNF by intra-thecal delivery of the trkB-IgG fusion molecule inhibits both the *in vitro* upregulation of spinal reflexes and also the thermal hyperalgesia observed following intra-plantar carrageenan administration [158]. In contrast however, systemic administration of the trkB-IgG fusion molecule has not been found to reverse the hyper-reflexia observed in the turpentine-inflamed rat urinary bladder model in the same manner as the NGF sequesterant trkA-IgG [91]. Thus, it would appear that although BDNF is probably involved in the modulation of NGF-associated nociceptive events, its effects are more likely to be occurring at a central level.

Taken together, the work presented here and previous reports documenting hyper-reflexic changes in bladder motility [91], demonstrate that the sequestration of NGF by the trkA-IgG fusion molecule prevents both the local (VVH) and the referred (VSH) hyperalgesic phenomena associated with intra-vesical turpentine instillation. The importance of NGF

in the central changes is further supported by the documentation of inhibition of the upregulation of *c-fos* by prior administration of trkA-IgG [89]. In the somatic domain, hypoalgesia in response to both thermal and mechanical stimuli have been reported following treatment with the NGF sequestering molecule [220]. Furthermore, prevention of the upregulation of both sensory neurotransmitters and the expression of the immediate early gene *c-fos* in the spinal cord have been described [343]. Thus, NGF appears to be of importance in both peripheral and central components of hyperalgesia, observed in skin following a variety of inflammatory insults applied to both cutaneous and vesical tissues.

However, since trkA-IgG is a large molecule it is unable to cross the blood-brain barrier and must exert its effects via a peripheral site of action. Thus a possible explanation of the effects of administration of trkA-IgG in this model is as follows. Sequestration of endogenous NGF, produced in bladder tissue following intra-vesical turpentine instillation, might attenuate both the degranulation of mast cells and the sensitisation of primary afferent neurones. Electrical activity in primary afferent neurones of the pelvic nerve would be decreased, preventing the inflammation-associated increase in excitability of dorsal horn neurones which results in central sensitisation. Thus, spinally mediated VVH, referred sensory events (VSH) and *c-fos* induction in the spinal cord, consequent upon such central sensitisation, might be attenuated or avoided.

### 3.iv.a. Clinical implications:

The experiments described here (and in chapter 2) suggest that the turpentine inflamed rat urinary bladder model may be truly reflective of pain arising from the urinary bladder, rather than merely the hyper-reflexic response to inflammation. Furthermore, NGF appears to play a role in both

the primary and secondary hyperalgesic responses. In the clinical scenario, patients suffering with hyper-reflexic syndromes such as interstitial cystitis commonly complain of pain [257] which is resistant to current therapies. Thus animal models which can be used to investigate new treatments would be helpful. Bladder biopsies taken from hyper-reflexic patients demonstrate an increase in the endogenous production of the mRNA encoding NGF [186] (as is the case in animal models [254]). In a small phase one study, when NGF was administered sub-cutaneously to healthy human volunteers, the result was local hyperalgesia to later application of both thermal and mechanical stimuli. Interestingly female subjects in this study described a longer lasting, but mild myalgia, that persisted for several days despite an absence of measurable plasma levels of NGF [266]. This sex-related difference is particularly enticing in light of the fact that interstitial cystitis occurs almost exclusively in the female population. Furthermore, bladder mast cells, which degranulate following exposure to NGF, express high affinity receptors for oestrogen [257]. Thus, a modulatory effect of female sex hormones on the NGF mediated response might potentially explain the observed clinical distribution of interstitial cystitis.

Nerve growth factor appears to occupy a central role in the events associated with inflammation and manipulation of such responses might prove to be a novel therapeutic tool. Conventional competitive antagonists for the trkA receptor have as yet not been developed. However, immunoadhesin molecules, comparable to the trkA-IgG molecule used here, could provide an attractive avenue for research, if they could be proven to be safe for use in the human population. Not only might such molecules be expected to attenuate the distressing symptom of urinary frequency, but the work presented here suggests that they might reduce unpleasant sensory perceptions, such as referred pain.

# Chapter 4:

# Bradykinin and visceral inflammatory pain

## 4.i. Introduction

### 4.i.a. Biochemistry:

Bradykinin (BK) is a nonapeptide, produced *de novo* in response to tissue injury and inflammation. It is cleaved from a high molecular weight kininogen precursor by the action of plasma kallikrein. Plasma kallikrein is an enzyme produced in the liver via a factor XII dependent pathway and activated on plasma contact with an injured surface [94,260,270]. An equipotent peptide - Lys-bradykinin (or kallidin) is produced by the action of tissue kallikrein (an acidic glycoprotein produced in a multitude of tissues) on low molecular weight kininogen. The precursors of both high and low molecular weight kininogen are $\alpha_2$-globulins, produced predominantly in the liver, but also detectable in endothelial and smooth muscle cells in the absence of serum [203]. The transportation of kininogens in the plasma (bound to high affinity sites on the membranes of platelets and neutrophils) provides a reservoir for rapid kinin release following activation by kallikrein [203].

Bradykinin exhibits a short duration of action (plasma $t_{1/2}$ = 10 to 30 seconds). Effects produced following its production are terminated by the activity of abundant proteolytic enzymes including kininases and endopeptidases. There do not appear to be any enzymes which act specifically on the kinins, but rather a wide variety of carboxypeptidases (including angiotensin converting enzyme and enkephalinase) cleave the carboxy-terminal $Phe^8$-$Arg^9$ from the active kinin (see figure 23), resulting in inactivation [203]. Whilst many inert peptide (and amino acid) metabolites

Bradykinin

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

HOE140

H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH

des-Arg$^{9}$,[Leu$^{8}$]-BK

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-OH

Figure 23.
Diagram of the amino acid sequences of bradykinin (upper) and the B1 (lower) and B2 (middle) receptor antagonists utilised in the viscero-visceral hyper-reflexia and immunohistochemical experimental protocols.

are produced, one of its major metabolites, [des-Arg$^9$]-bradykinin does exhibit activity consequent upon binding to specific receptor sites [270,309].

### 4.i.b. Pathophysiological effects [22]:

Bradykinin is known to exert effects on a wide variety of tissues, including many types of smooth muscle which respond either by contraction or relaxation. Hypotension may be observed and this is likely to occur secondarily to the potent arterial vasodilatation that has been documented in tissue preparations. Tissue oedema (of both skin and mucous membranes including those of the nasal passages and bronchial tree) results from the profound increase in vascular permeability observed after exposure of the vasculature to bradykinin. Epithelial ion transport systems in the respiratory and gastro-intestinal tracts and the renal tubule are stimulated and an increase in nasal, bronchial and gastro-intestinal secretions and both diuresis and naturesis may result [203].

In addition to these physiological responses, bradykinin has a major pathological role in inflammation [96]. In both an animal model of chronic inflammation (the carrageenan inflamed rat paw) and human subjects exhibiting an acute (oral surgery) or chronic (rheumatoid arthritis) inflammatory response, plasma levels of circulating bradykinin have been shown to be increased [134]. When applied to blister bases or administered intra-dermally bradykinin is associated with the observation of pain related behaviour in animals [310] and the perception of acute pain in human subjects [93]. Thus, not only are levels of endogenous bradykinin increased under inflammatory conditions known to be associated with pain, but the molecule has also been shown to be a potent algogen [96].

Bradykinin is an efficient activator of nociceptors supplying both somatic [93,273] and visceral tissues [322]. In the visceral domain, cardiac sympathetic afferents, known to be sensitive to ischaemia have been

observed to respond with an increased rate of discharge to intra-cardiac administration of bradykinin [273].

Additionally however, bradykinin has been shown to be capable of sensitising nociceptors, thus enhancing their response to a variety of alternate stimuli [260]. Skin-nerve preparations, in which both heat and bradykinin stimuli are applied to the skin and recordings of activity are determined in the primary afferent neurone supplying the region, demonstrate that the response to thermal stimulation is increased by the direct application of bradykinin at concentrations incapable of producing neuronal excitation *per se* [164]. Similar findings have been observed in visceral preparations. If the primary afferent neurones travelling in the spermatic nerve of the dog are exposed to a direct thermal stimulus (by heating of the organ bath containing the neuronal preparation) the excitatory response is enhanced following the addition of bradykinin [166]. Moreover, single neurone preparations of dissociated dorsal root ganglia cells reflect these findings. When inward currents are measured using patch clamp techniques, the response to hydrogen ions is increased when bradykinin is also present in the perfusate [165]. Thus bradykinin is associated with the production of phenomena important in the generation of hyperalgesia.

### 4.i.c. <u>Bradykinin receptors:</u>

It has recently become clear that the effects of bradykinin are mediated via at least two specific G-protein coupled receptors (B1 and B2), each with seven trans-membrane domains [96,202,203]. Bradykinin itself does not discriminate between the B1 and B2 receptors (although its affinity for the B2 receptor is greater than for the B1). However, the active metabolite, [des-Arg$^9$]-bradykinin demonstrates relative selectivity for the B1 receptor [120,260]. Activation of these receptors is associated with the

generation of intra-cellular second messengers, including: inositol triphosphate, calcium ions and cGMP [110,273].

The B2 receptor is constitutive and expressed in a wide range of tissues, subserving a multitude of physiological functions [22]. Receptors are expressed both on sensory neurones in the periphery [339] and also in the dorsal root ganglion and superficial regions of the dorsal horn [310]. Bradykinin - B2 receptor binding appears to be important in the acute activation of nociceptors in both somatic and visceral domains [338]. One example of such effects in the somatic domain is observed when bradykinin is administered sub-cutaneously into the paw of a rat. This results in a mechanical hyperalgesia, which is reversed by the B2 receptor antagonist NPC17731 [160]. In healthy human subjects the administration of another antagonist (NPC349) is associated with a decrease in the pain experienced when bradykinin is applied to a blister base [309]. In the visceral domain, the activation of sympathetic afferent neurones by intra-cardiac injection of bradykinin is blocked by the systemic administration of an alternative B2 receptor antagonist HOE140 [322]. Furthermore, the electrical responses of polymodal nociceptors in another *in vitro* preparation, that of testis-superior spermatic nerve, to application of thermal stimuli, are augmented by the administration of bradykinin and attenuated following application of the B2 receptor antagonist NPC349 [166].

Not only are the pain related responses induced by bradykinin itself inhibited B2 receptor antagonists, but the inhibition of pain-related behaviour following administration of alternative chemical algogens is also observed. When formalin is administered sub-cutaneously to the paw of a rodent, a biphasic response (see 1.v.a.) of both the pain related behaviour and also the electrophysiological responses of primary afferent and dorsal horn neurones is observed [87,212,323]. The B2 receptor antagonist

HOE140, when administered either locally or systemically at appropriate concentrations, inhibits both phases of the behavioural response [29,61]. An alternative B2 receptor antagonist (B4162) has been demonstrated to inhibit the second phase of the electrophysiological response observed in primary afferent neurones supplying the paw to which intra-plantar formalin has been administered [131].

Transgenic B2 receptor 'knockout mice' (in which the gene encoding the B2 receptor is disrupted) have been developed and appear to support the role of the B2 receptor in acute nociception. Although these animals exhibit normal chronic hyperalgesic responses, they fail to exhibit acute nociceptive responses to bradykinin. However, responses to alternative algogenic stimuli, such as heat or intra-plantar carrageenan, remain unaffected [39].

In contrast to the B2 receptor, the B1 receptor has a much more limited distribution and appears to be expressed almost exclusively in inflamed tissues [22,38]. Isolated tissues, such as the rabbit aorta and rat bladder, develop an increasing responsiveness to the B1 agonist [des-Arg$^9$]-bradykinin, as a function of time from isolation [102,202]. Similar findings have been observed in human tissues, such as the isolated ileum and umbilical vein [273]. However, the upregulation of such receptor-mediated effects is observed at earlier time points if cytokines (the increased production of which is a prominent feature of inflammation) or agents known to induce inflammation (such as lipopolysaccharide or Triton-X-100) are directly applied to the isolated preparation [38], or administered to the animal twenty four hours prior to sacrifice [102,202]. This enhanced sensitivity of tissue to the effects of [des-Arg$^9$]-bradykinin is inhibited by the administration of glucocorticoids, such as dexamethasone, which act as anti-inflammatory agents [201].

A variety of *in vivo* responses support the upregulation of B1 receptor expression under inflammatory conditions. Whilst administration of the B1 receptor antagonist des-Arg$^9$,[Leu$^8$]-BK is not associated with the inhibition of the hyperalgesia induced in the naïve rat by intra-dermal bradykinin, attenuation of the response is observed if the animal has been pre-treated with Freunds complete adjuvant [74,160]. In addition the mechanical hyperalgesia observed following either the intra-articular injection of Freunds complete adjuvant, or the previous exposure of skin to an ultra-violet burn, is reversed by the administration of des-Arg$^9$,[Leu$^8$]-BK [261,262]. Similar effects have been demonstrated on the thermal hyperalgesia of the rat paw observed following intra-peritoneal NGF administration [284]. Pain related behaviour observed following intra-dermal injection of formalin into the dorsum of the paw of mice is also reduced following administration of a B1 receptor antagonist [61].

The B1 receptor itself has, as yet, not been located on primary sensory afferent neurones [76]. It is possible that induction of expression of the receptor is limited to inflammatory [201] and smooth muscle cells [102] and neurones of the sympathetic nervous system, through which indirect sensitisation of the pain pathway might occur [76]. Since tissue and circulating levels of bradykinin are increased during inflammation [96,134], the release of further mediators (such as cytokines, prostanoids and neurotrophins) from inflammatory cells in response to bradykinin could act as a positive feedback mechanism. Thus the enhanced release of algogenic agents such as hydrogen ions, ATP and serotonin, in addition to sensitising agents, including histamine, prostanoids and leukotrienes, might be expected to further exacerbate the nociceptive response of neurones in the damaged area [273].

## 4.i.d. Bradykinin receptor antagonists:

Once bradykinin receptors had been identified it was possible to develop specific antagonists acting at the bradykinin binding sites. To date these have been mainly peptide analogues of bradykinin, which are subject to metabolism by tissue peptidases, often resulting in a very short half life [22]. An additional problem has been the difficulty in producing compounds that show selectivity, since many such compounds act as inhibitors of common signal transduction pathways. However, two of the more selective and potent agents were utilised during these experiments.

HOE140 is a structural analogue of bradykinin (see figure 23), containing the iminoacids D-Tic and Oic [338], which confer high stability ($t_{1/2}$ = 5 hours) and affinity ($IC_{50}$ in the nanomolar range). There appears to be some selectivity exhibited for the B2 receptor [96], in that no inhibition of B1 agonist induced contraction of the rat aorta is observed following HOE140 administration [176].

des-Arg$^9$,[Leu$^8$]-BK, is a more recent development and is thought to act as a specific B1 receptor antagonist. Following its administration there is no observable antagonism of the actions of bradykinin on tissues such as rabbit jugular vein, that only express B2 receptors [22,96]. Moreover its administration is associated with attenuation of the hyperalgesia induced by the administration of Freunds adjuvant or ultra-violet light, in conditions where B2 receptor antagonists have no attenuating effects [261,262]. However, there is some evidence to suggest that, at least in the visceral domain, des-Arg$^9$,[Leu$^8$]-BK may exhibit some partial agonist activity [231]. The isolated longitudinal smooth muscle of the rat ileum responds with a brisk contractile response when either bradykinin or the B1 receptor agonist [des-Arg$^9$]-bradykinin are applied topically (responses to a selective B2 receptor agonist are less profound). Whilst HOE140 partially inhibits the contractile response of the ileum to bradykinin (but not [des-Arg$^9$]-

bradykinin), des-Arg$^{9}$,[Leu$^{8}$]-BK, far from inhibiting the response, appears to act as a partial agonist at early time points and a full agonist at five hours after experimental set-up. This is the time delay that would be expected prior to be maximal induction of B1 receptors, suggesting that the effect is receptor mediated [202]. The pharmacokinetics of des-Arg$^{9}$,[Leu$^{8}$]-BK are less clear, than those for HOE140. The aminoacid sequence for this compound is also shown in figure 23.

## 4.i.e. Experimental rationale:

Bradykinin is upregulated during inflammation [134] and receptors activated by it and its metabolites have been localised to primary afferent sensory neurones and the dorsal horn of the spinal cord [310,339]. However, the majority of the *in vivo* data relates to rat models of persistent somatic pain, such as the hyperalgesia induced by the intra-dermal [160] or intra-articular [262] administration of Freunds complete adjuvant, the intra-venous administration of NGF [284] or the ultra-violet irradiation of the paw [261]. Since (as documented in section 1.i.b.) the neurophysiological events associated with visceral inflammation are associated with significant differences when compared to those arising from inflammation in the somatic domain, I was interested to assess the possibility of alternative bradykinin associated effects in the turpentine inflamed rat urinary bladder model of visceral pain. Furthermore, pain arising in the viscera is both common and poorly responsive to current therapies. Therefore the possibility of demonstrating an alternative target for therapeutic interventions is exciting.

## 4.ii. Methods

All experiments conformed with British Home Office Regulations. Animals were housed according to national guidelines in colony cages of six animals per cage with a dark : light cycle of 12 hours : 12 hours. They were provided with free access to food and water.

### 4.ii.a. Urinary bladder reflex experiments:

Surgical preparation [147,148,215,218]:

Animals were anaesthetised with a single dose of 1.25gm/kg intra-peritoneal (i.p.) urethane and the trachea was cannulated, with polythene tubing of internal diameter 1.67mm (Portex, UK., 800/100/460/100). The bladder was cannulated trans-urethrally with a sterile, lubricated, nylon catheter, with external diameter 1.02mm (Portex, U.K., 200/300/030). This was secured in position with 3-0 silk ties around the external urethral orifice and a free flow of clear urine was ensured. The carotid artery was cannulated with a sterile nylon catheter, external diameter 0.75mm (Portex, U.K., 200/300/020) and again secured with 3-0 silk ties. A mid-line, ventral laparotomy allowed visual confirmation of completion of bladder emptying between cystometrograms (CMGs). To ensure maintenance of normothermia, the animals were placed on a heated operating table, the wounds were covered by saline soaked swabs and temperature was continually monitored during the experiments.

Measurement of bladder motility [147,148,215,218]:

The outcome measure used in these experiments was the cystometrogram, which is the pressure-volume relationship for the urinary bladder. Cystometrograms were performed by the infusion of warm, sterile, 0.9% saline at a constant rate (0.05ml/min) to a maximum of 0.7ml, via an

infusion pump (sp230iw, Worcester Polytechnic Institute, USA). Under these circumstances the bladder gradually fills, with a concomitant gradual increase in intra-vesical pressure. At a critical threshold volume (usually approximately 0.25 - 0.5ml) the cystometrogram trace begins to exhibit large pressure waves, which are associated with active, reflex micturition contractions of the bladder [218]. These reoccur on a regular basis during the remainder of the filling of the bladder. The intra-vesical pressure during distension was monitored continually via a pressure transducer (Neurolog 108T, Digitimer, U.K.) attached to the side arm of the infusion system (see figure 24). The signal was subsequently amplified using the Neurolog NL108 system (Digitimer, U.K.) and displayed on a Viglen genie 4SX PC. Data were analysed and stored using the MP100 system (Biopac, Santa Barbara, CA, USA) and AcqKnowledge software (version 3.02).

<u>Outcome measures:</u>

Two separate measures were extracted from the cystometrogram (see figure 25):

i) The micturition threshold, $V_{mic}$.

This was defined as the intra-vesical volume at which active micturition contractions first appeared. These waves are associated with intra-vesical pressures greater than 20 - 30mmHg. This is an intra-vesical pressure which has previously been shown to be associated with discomfort in human subjects [223,250]. A lowering of the micturition threshold may provide an explanation for the low bladder volumes (prior to a sense of urgency) that patients with cystitis experience.

ii) The cumulative duration of micturition, $Dur_{mic}$.

This was calculated as the total sum of the duration of each micturition contraction observed. Since this increase is related to a reduction in compliance of vesical tissue, the increase in $Dur_{mic}$ is probably analogous to

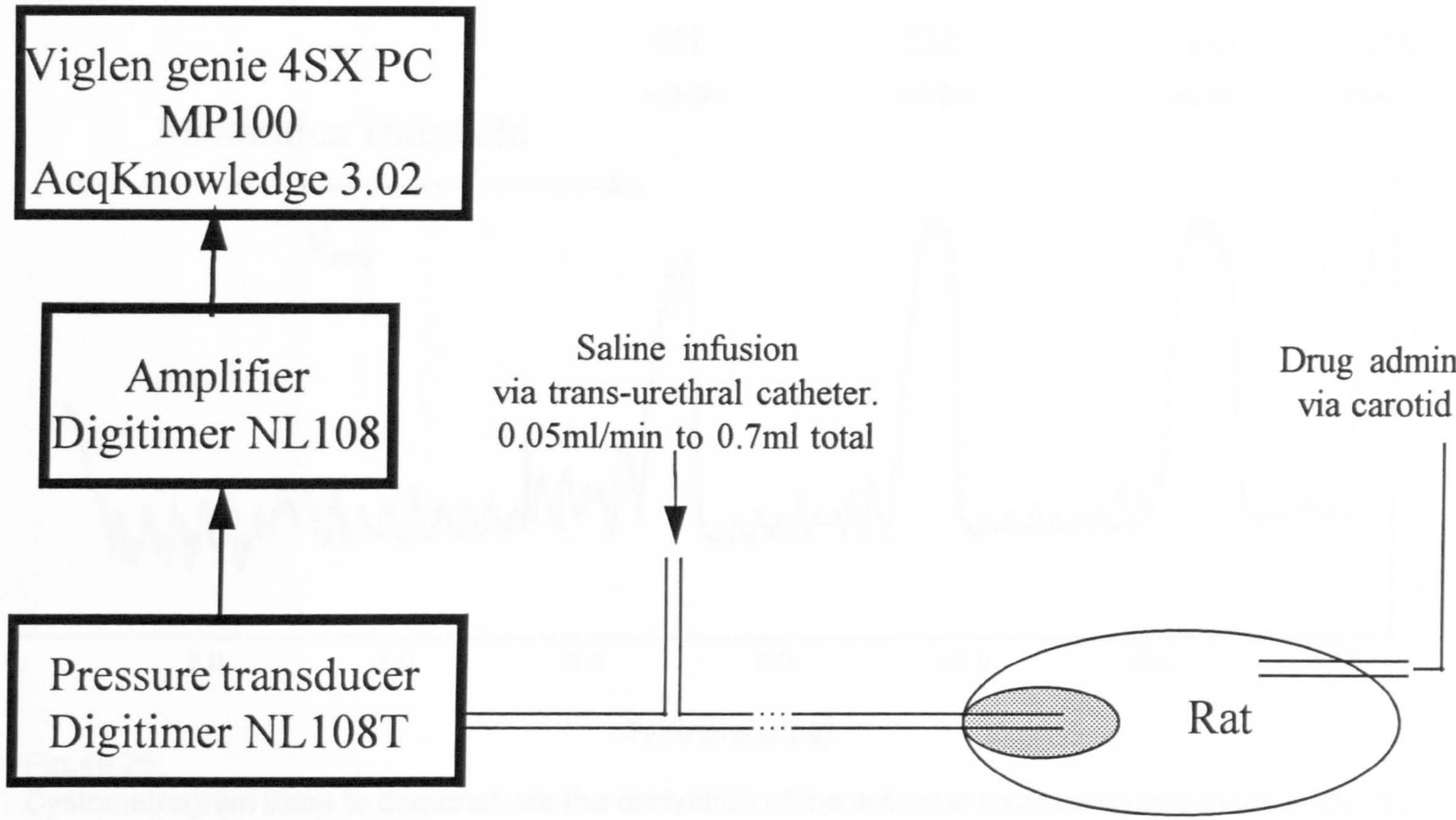



Figure 24.
Diagram to demonstrate the setup of the cystometrography equipment for the bladder reflex experiments.

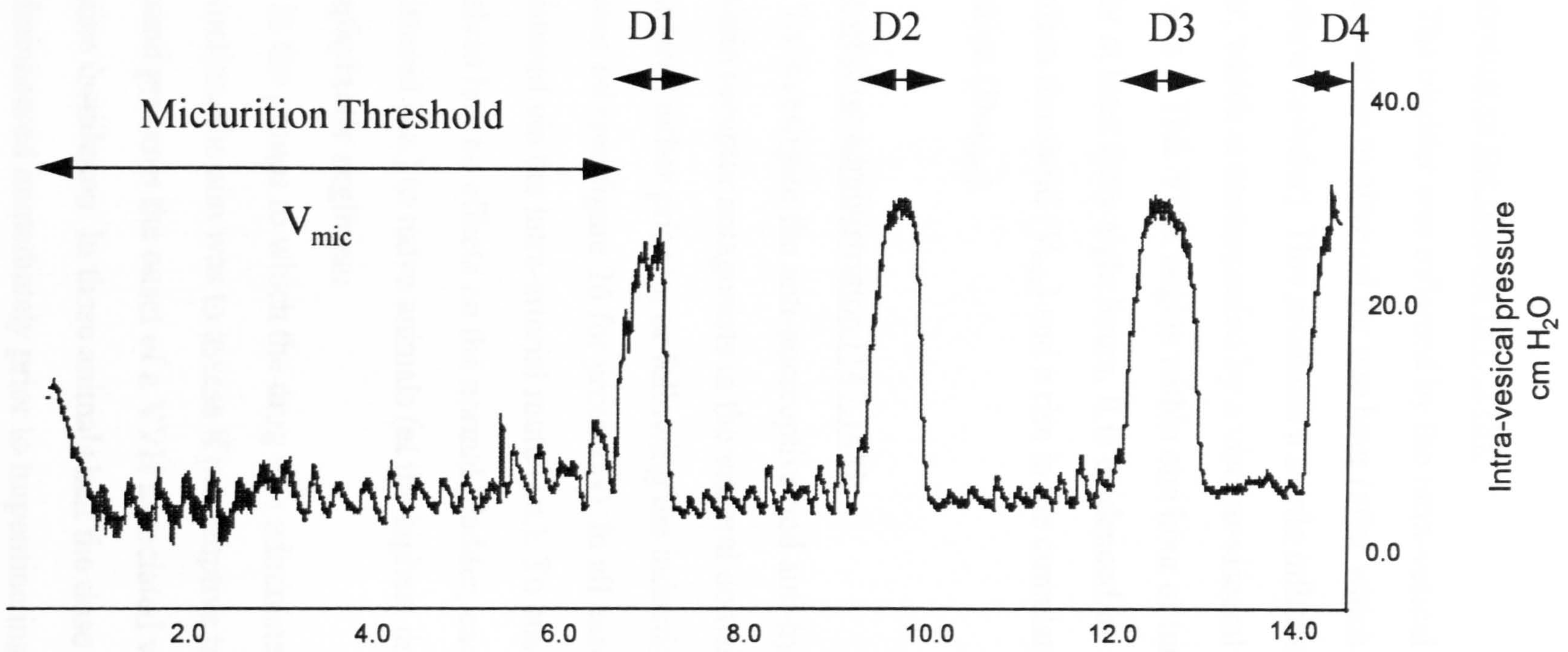



Figure 25.

Cystometrogram trace to demonstrate the derivation of the outcome measures used in the micturition reflex experiments.

The micturition threshold ($V_{mic}$) is calculated as: 0.05 x time in minutes to first high pressure wave (since the bladder is distended at a constant rate of 0.05ml/minute.

The cumulative duration of micturition ($Dur_{mic}$) is calculated from D1+D2+D3+D4 and is measured in seconds.

the increased frequency of micturition observed in human subjects suffering with inflammation of the bladder [223].

Inflammation of the bladder [215,218]:

The bladder was inflamed by the intra-vesical instillation of 0.5ml of 50% turpentine in olive oil for one hour (after which time it was removed via the urethral catheter). This produces a sterile inflammatory response in the bladder, which is accompanied by a viscero-visceral hyper-reflexia (VVH) (see 1.iv.a.). This VVH begins within one hour of turpentine instillation and lasts for at least forty eight hours. It is evidenced by a lowering in the micturition threshold ($V_{mic}$) and a rise in the cumulative duration of micturition ($Dur_{mic}$).

Effects of drug administration [147,148]:

To investigate the anti-nociceptive and anti-hyper-reflexic potential of bradykinin receptor antagonists in the visceral domain, compounds were administered either prior to, or following the induction of inflammation with turpentine oil (see figure 26 for protocols). In all cases compounds were administered via the intra-arterial route (i.a.). To ensure that the drugs themselves had no effects on the normal bladder, each drug was administered (i.a.) to naïve animals (at the highest dose level investigated).

**a) Prophylactic regimen:**

In the groups to which the drug was administered prior to induction of inflammation, the aim was to assess if pre-emptive treatment with a compound prevents the onset of a VVH associated with intra-vesical turpentine instillation. In these animals half the dose of the test compound was administered immediately prior to turpentine instillation, with the remainder being delivered thirty minutes later. This protocol was chosen as full pharmacokinetic data (particularly the elimination half time) was not

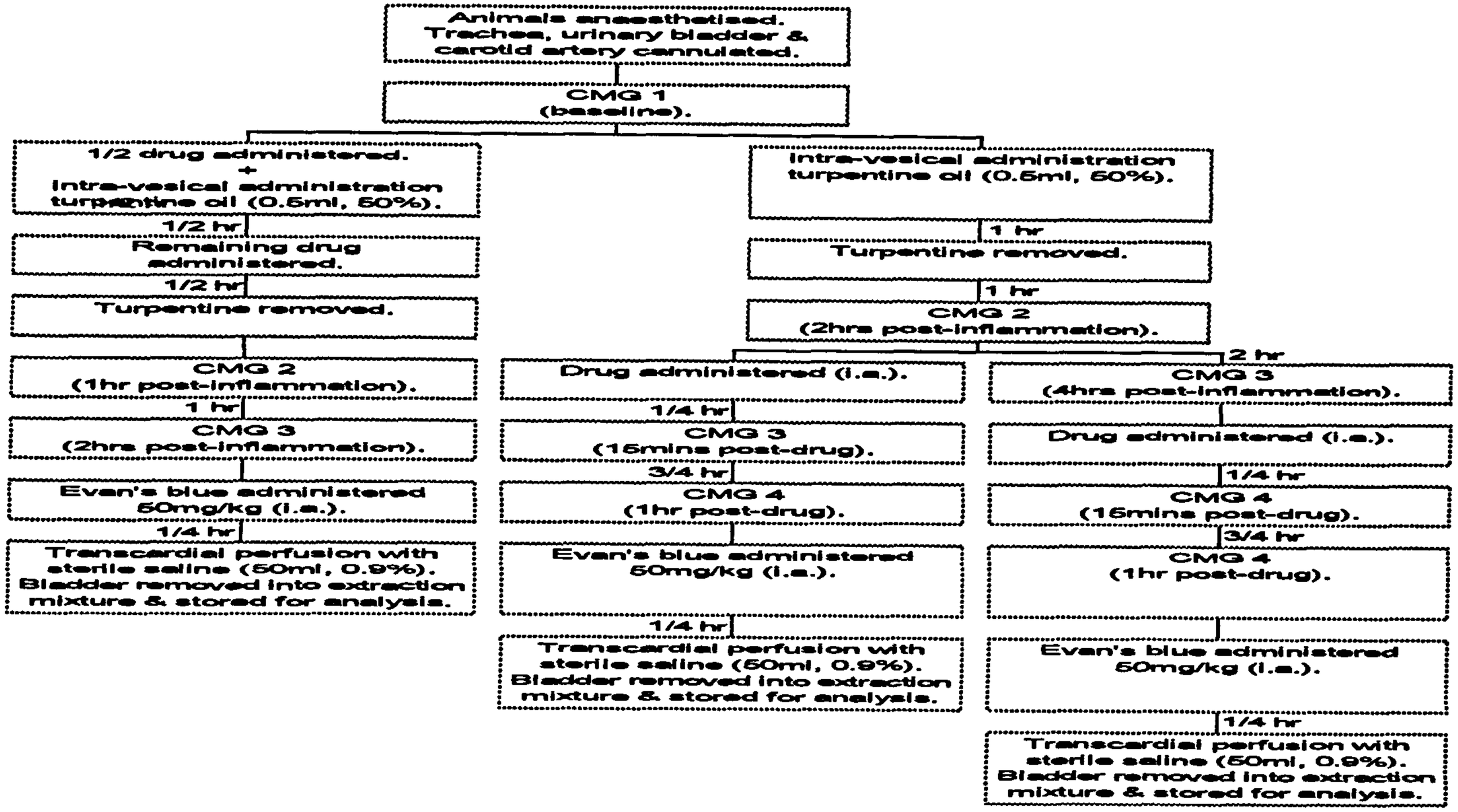



Figure 26.

Flow diagram outlining the protocols for pre- and post-inflammation drug administration in the micturition reflex experiments.

available for both the compounds under test and I wished to avoid observation of an apparent loss of effectiveness in the event that the elimination half time of a compound was short.

**b) Treatment regimen:**

The post-inflammation administration group evaluated the ability of the compounds to reverse an established VVH. In the post-administration protocol, two time points for administration were used:

i) Early administration - drug was delivered two hours after intra-vesical turpentine instillation.

ii) Late administration - drug was administered five hours following intra-vesical instillation of turpentine oil. At this time maximal induction of B1 receptors would be expected to have occurred [202].

<u>Bradykinin receptor antagonists assessed:</u>

Storage and preparation details for all compounds are given in appendix 1.

a) HOE140 (a B2 receptor antagonist) [a gift of Hoechst A.G.].

Dose range: 1mg/kg - 7.5mg/kg.

This dose range corresponds to a range of 0.8μmol/kg - 5.7μmol/kg. Investigations previously undertaken in animal models of somatic inflammation [29,47,61,71,80,154] indicate that doses ranging from 0.5nmol/kg to 3.8μmol/kg are efficacious in attenuating hyperalgesia, oedema and pain-related behaviour. Furthermore in human volunteer trials doses of 76nmol/kg (i.v.) have been observed to inhibit bradykinin induced vasodilatation [54], whilst 2.3μmol/kg administered via a nebuliser improve pulmonary function tests [7]. Thus, any significant inhibitory effects of HOE140 on inflammation-associated changes observed in this model would be expected to be evident in the dose range chosen.

b) des-Arg$^9$[Leu$^8$]-bradykinin (a B1 receptor antagonist) [Sigma].

Dose range: 1mg/kg - 7.5mg/kg.

This dose range corresponds to a range of 1.1μmol/kg - 8.6μmol/kg. Previous investigations in the somatic domain [47,160,261,262,284,312] have demonstrated significant inhibitory effects on hyperalgesia, oedema and pain-related behaviour at doses ranging from 0.5nmol/kg to 0.5μmol/kg. Thus any potential inhibitory effects occurring in this experimental protocol would be likely to be demonstrated at the dose range administered.

Data presentation:

Statistical comparisons all utilised the Sigmastat package, version 2.0 (Jandel Scientific Software) and a significance level of $p < 0.05$ was employed. Graphs were produced using the Sigmaplot program, version 3.0 (Jandel Scientific Software).

Protocols (see figure 26) and statistical comparisons:

**a) Prophylactic regimen:**

When compounds were administered prior to inflammation the post-inflammation measures were compared to the baseline measurements (paired t-test) and the results expressed as a percentage of baseline values. Since inflammation of the bladder with turpentine oil has previously been shown to induce hyper-reflexic changes, evident from one hour post intra-vesical instillation [218], initial measurements were made one hour after induction of inflammation. A final measurement was undertaken after a further hour to assess the continued efficacy of the compound under test. Compounds were taken to be effective when the post-inflammation measures did not differ significantly from baseline.

**b) Treatment regimen:**

When compounds were administered after induction of inflammation the post-treatment values (at both fifteen minutes and one hour) were compared to both the post-inflammation (two hours for early administration, or four hours for late administration) and the baseline values. Comparisons utilised analysis of variance testing (ANOVA [*post hoc* Tukey or Dunnett's as appropriate]). Since by one hour after instillation of turpentine oil inflammatory hyper-reflexia is well established, the early measurement allowed assessment of the onset time prior to any therapeutic effects of the test compound becoming evident. The later measurement at one hour post treatment assessed the ability of the compound to maintain its effect.

Compounds were assessed as at least partially effective if there was a significant difference between post-inflammation and post-treatment measures. If in addition there was no significant difference between baseline and post-treatment measures, compounds were taken to be effective at fully reversing the inflammatory VVH.

## 4.ii.b. Plasma Extravasation experiments:

Outcome measure [218]:

In animals utilised in the bladder reflex experiments, a further measure of the efficacy of the compounds (decrease in the inflammation-associated plasma extravasation into bladder tissue) was assessed. In each case, after the final cystometrogram had been performed, the animal was slowly perfused (i.a.) with 50mg/kg of Evan's Blue Dye (EB). Fifteen minutes later the animal was terminally perfused with 50ml of 0.9% saline trans-cardially to ensure clearance of the Evan's Blue Dye from the vascular compartment. The bladders were then excised, weighed and stored in 4ml of filtered extraction mixture (70/30 acetone + 0.5gm/L sodium sulphide) for a

minimum of five days. Since Evan's Blue Dye binds to albumin it acts as a marker for plasma extravasation into bladder tissue. The quantity of Evan's Blue Dye extravasated was measured by spectrophotometry at $\lambda = 620nm$ using the Unicam 5625 UV/VIS spectrophotometer (ATI Unicam, Cambridge, UK). The tissue concentration of dye in mg / mg of vesical tissue was calculated, using the equation:

$$\frac{(Measured \quad [EB]) \times 4000}{Bladder \quad weight}$$

Tissue concentrations of Evan's Blue Dye were compared between control and treated groups by means of either an unpaired t-test (prophylactic administration of compounds) or ANOVA testing [*post hoc* Dunnett's] (treatment protocol). All comparisons were performed using the Sigmastat package, version 2.0 (Jandel Scientific Software). Compounds were taken to be effective if the treated group demonstrated a lower level of plasma extravasation than the control group.

## 4.ii.c. *C-fos* immunohistochemistry:

The protocol for these experiments is shown in figure 27. Sources for all chemicals are given in appendix 1.

Surgical preparation:

All animals were anaesthetised with urethane (1.25gm/kg i.p.) and the trachea, urethra and carotid artery cannulated. Test compounds were administered thirty minutes prior to inflammation of the urinary bladder. This was induced by intra-vesical instillation of turpentine oil (0.5ml, 50%, 1hour). Animals were culled two hours after induction of inflammation (by

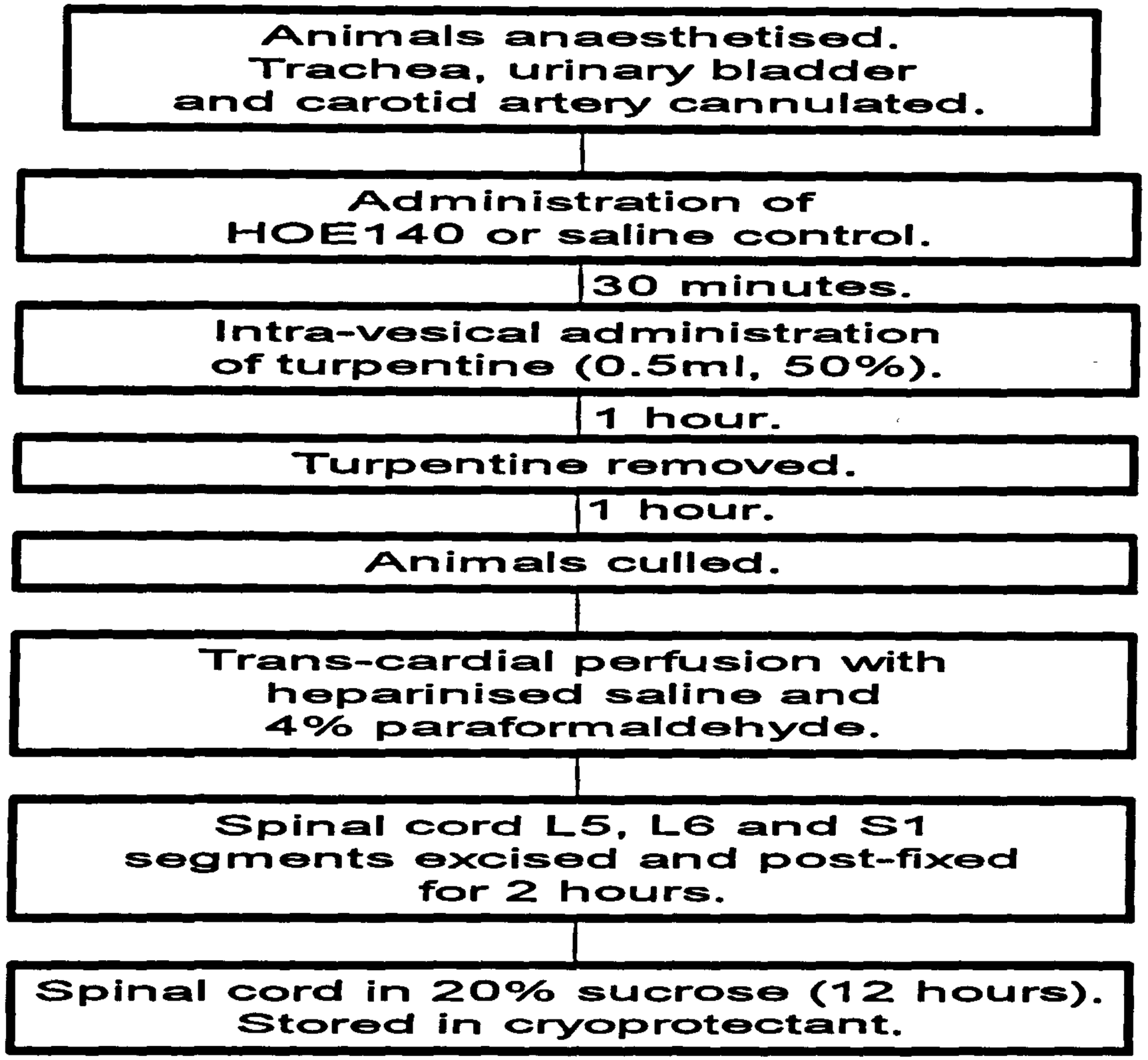



Figure 27.
Flow diagram outlining the protocol for collection of tissue from animals used in the *c-fos* immunohistochemical experiments.

terminal anaesthesia) since previous immunohistochemical studies of bladder inflammation have shown that induction of *c-fos* expression is maximal two hours after urothelial exposure to a noxious chemical [36].

Drug administration:

The HOE140 or (0.9% saline control) was administered (i.a.) thirty minutes prior to turpentine instillation intra-vesically. A dose of 5mg/kg was chosen since it was well within the dose range shown to be effective both in the VVH protocol presented here and in previous work involving inflammation of somatic structures [29,71,312].

Tissue fixation [232]:

In order to achieve immunolocalisation of the intra-cellular c-Fos protein, cells were permeabilized to allow entry of the antibodies. To prevent the c-Fos antigen leaving the cell once this process had occurred, the tissue was first fixed. Therefore, animals were initially, trans-cardially perfused with 0.9% heparinised saline at $4^0$C to clear the vascular compartment of blood and then immediately with 4% paraformaldehyde to fix the tissues prior to dissection. The spinal cord was further subjected to two hours in 4% paraformaldehyde as a post-dissection fixative.

Tissue dissection:

Spinal cords were exposed through a dorsal laminectomy and the dura was incised and separated along its length. Dorsal roots were identified for the L6 level (the largest dorsal root ganglion) and tracked rostrally. This allowed accurate identification of the lumbar enlargement containing cord levels L5, L6 and S1 - the relevant cord section for receiving afferent input from the urinary bladder [36,90,188]. These sections were excised and post-fixed for two hours (as described above). They were then placed in 20%

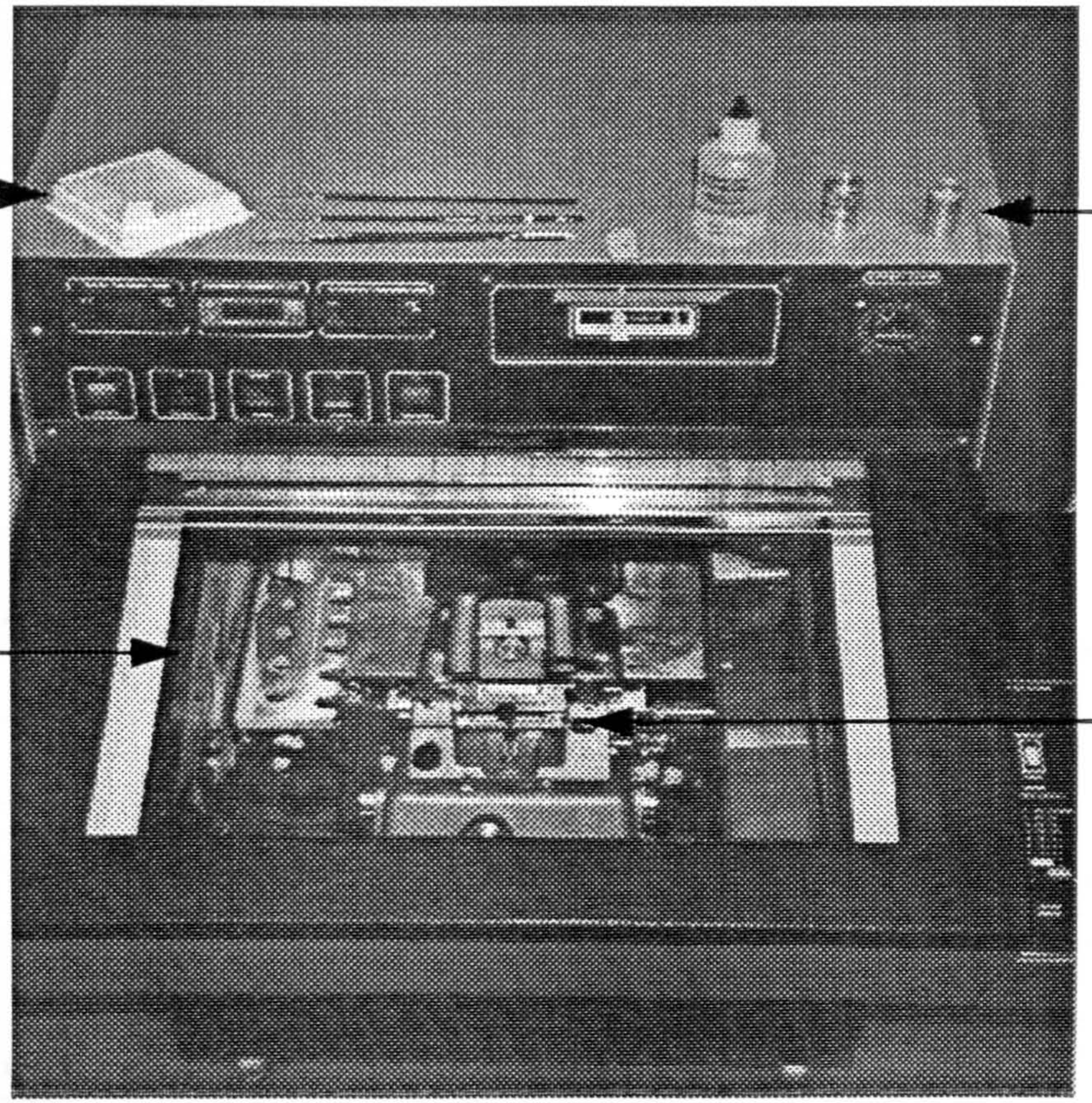



Figure 28.
Photograph of the cryostat used to prepare 20 μm spinal cord slices for immunohistochemical experiments.

sucrose for twelve hours as a cryoprotectant (preventing ice crystal formation during the freezing process). Subsequently the sections were blocked in OCT mounting medium at -70$^0$C. Cords were cut into 20μm thick coronal sections at intervals of 100μm apart using a cryostat (Bright Instrument Company Ltd., Huntingdon, Cambs. - see figure 28). Sections were mounted on Superfrost Plus slides (BDH laboratory supplies, England). Prior to staining these sections were stored at -20$^0$C in a cryoprotectant solution (see appendix 1 for composition). Slides stored in cryoprotectant were given six, five minute washes in phosphate buffered saline (PBS) prior to staining, to remove all the cryoprotectant solution from the samples.

Tissue staining (see figure 29):

Tissue was initially incubated with a blocking antibody (in this case 10% normal goat serum), to reduce cross reactivity reactions to non-specific protein epitopes. The primary antibody (rabbit anti-c-Fos) was applied (200μl/slide) and incubated for forty eight hours, followed by a ninety minute incubation with the secondary antibody (biotinylated goat anti-rabbit). Avidin biotin complex (ABC) reagent was applied and incubated for a further two hours prior to developing the reaction with 3,3'-Diaminobenzidine (DAB).

After staining, slides were cleared by dehydration through increasing alcohol concentrations (from 30% to 100%) and xylene. DePeX was used as a mounting medium, prior to protection of stained sections with a coverslip.

Outcome measures:

Neurones positive for c-Fos-like immunoreactivity were identified by light microscopy, as a black stain against a clear background. Each tissue slice was counted under 'blinded' conditions. Slides were labelled only with

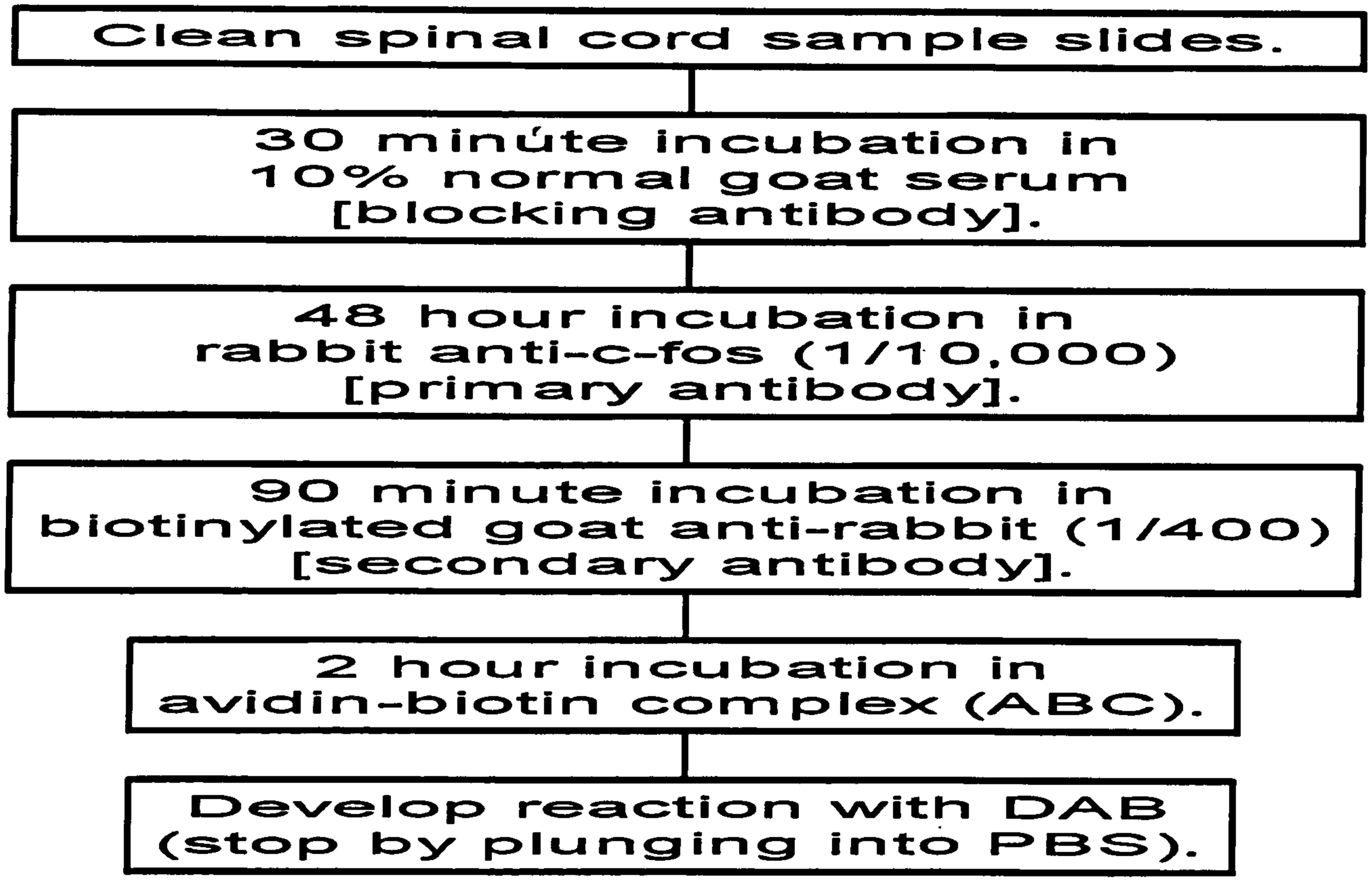



Figure 29.
Flow diagram outlining the protocol used for c-Fos immunolocalisation in spinal cord samples.

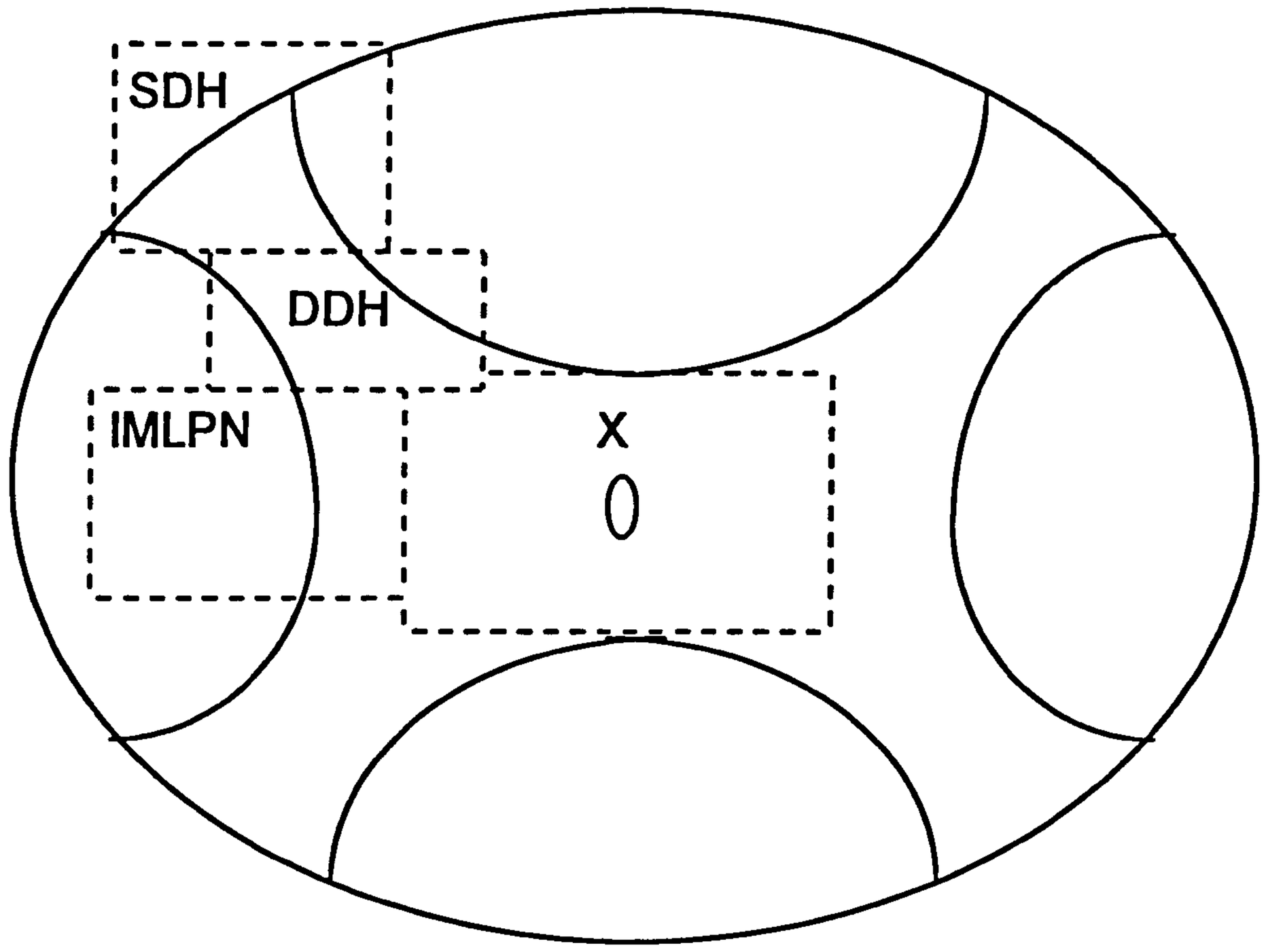



Figure 30.
Diagram to demonstrate the sub-areas of the spinal cord in which the c-Fos-like immunoreactivity was counted. Since counting in lamina X (X) was bilateral, counts for the superficial dorsal horn (SDH), deep dorsal horn (DDH) and the intermediolateral posterior nucleus (IMLPN) were multiplied by two for comparison.

code numbers to avoid unblinding of the observer to the treatment that had been administered. Cells positive for c-Fos-like immunoreactivity were counted in four separate areas (see figure 30) which have previously been shown to be of importance in nociceptive processing (see 1.vi.b.):

The superficial dorsal horn (SDH).

The deep dorsal horn (DDH).

Lamina X (X).

The intermediolateral posterior nucleus (IMLPN).

Spinal cord levels and areas were identified by comparison with a standard anatomical text [259].

Statistical comparison:

All statistical tests were performed using the Sigmastat package, version 2.0 (Jandel Scientific Software) and a significance level of $p < 0.05$ was employed. Data were presented in graphical form using the Sigmaplot package, version 3.0 (Jandel Scientific Software).

Data were separated by:

a) cord level.

b) area of the cord.

Within the control group, comparison of the number of neurones positive for c-Fos-like immunoreactivity across the cord levels L5, L6 and S1 was made (unpaired t-test). This provided an assessment of the relative importance of each of the neuronal roots in transmission of nociceptive inputs arising from the urinary bladder.

Subsequently, comparisons were made (unpaired t-test) between control and treatment groups at each of the three spinal cord levels (L5, L6 and S1):

i) for total staining.

ii) for staining in each sub-area of the dorsal horn of the spinal cord.

This provided an assessment of the efficacy of HOE140 in attenuating activity in the nociceptive pathways, following chemical inflammation of the urinary bladder.

## 4.iii. Results

### 4.iii.a. Urinary bladder reflex experiments:

Experiments were performed on 118 female Wistar rats weighing 195 - 300gm (mean weight 225gm). Using data pooled from all animals having a cystometrogram performed at two hours post-turpentine instillation, but prior to drug treatment (n = 74), turpentine instillation into the bladder was observed to be associated with a viscero-visceral hyper-reflexia (VVH). This was demonstrated by a significant reduction in the micturition threshold $V_{mic}$ to 39.83% (+/- 2.14) of baseline and a significant elevation in the cumulative duration of micturition $Dur_{mic}$ to 294.88% (+/- 26.07) of baseline (ANOVA [Tukey], ($p < 0.05$)).

Compound administration in the absence of inflammation (see table 1 in appendix 2):

When the B2 receptor antagonist HOE140 (7.5mg/kg) was administered in the absence of inflammation (n = 5) no significant effect (paired t-test, $p > 0.05$) on the cystometrogram was observed. At one hour post administration the $V_{mic}$ was 88.4% (+/-13.6) of baseline and the $Dur_{mic}$ was 119.0% (+/- 10.2).

Similarly, administration of the B1 receptor antagonist, des-$Arg^9[Leu^8]$-BK, (7.5mg/kg, n = 6) was not associated with any significant effects (paired t-test, $p > 0.05$). The $V_{mic}$ was 119.1% (+/-13.8) of baseline and the $Dur_{mic}$ was 126.3% (+/- 13.3) at one hour post-administration.

Saline (control) administration:

Pre-inflammation administration of 0.7ml of 0.9% saline (n = 6) had no significant preventative effect on the inflammation induced changes in the cystometrogram. At one hour post-turpentine instillation the $V_{mic}$ was significantly reduced to 48.8% (+/- 10.3) and $Dur_{mic}$ was significantly elevated to 262.0% (+/- 43.6) of baseline (paired t-test, $p < 0.05$).

Similarly, neither early (n = 6) nor late (n = 5) post-inflammation administration of saline reversed the observed changes. At one hour after early administration of saline (two hours following induction of inflammation) the $V_{mic}$ was 52.2% (+/- 12.2) and the $Dur_{mic}$ 310.3% (+/- 20.1) of baseline values. In the late administration group (five hours following induction of inflammation), values for $V_{mic}$ and $Dur_{mic}$ of 44.9% (+/- 8.9) and 501.3% (+/- 240.6) of baseline were observed (ANOVA [Tukey], $p < 0.05$). The more profound changes observed in the late administration protocol reflect the ongoing inflammatory process, rather than the effect of saline itself.

The effect of pre-inflammation administration of receptor antagonists:

The prophylactic administration of the B2 receptor antagonist HOE140 (n = 6 all groups), prevented the inflammation associated decline in $V_{mic}$ (paired t-test) at all dose levels (see figure 31). The inflammation-associated elevation in $Dur_{mic}$ was also attenuated (see table 2 in appendix 2).

In contrast, pre-inflammation administration of the B1 receptor antagonist, des-$Arg^9$[$Leu^8$]-BK (n = 6 each group) failed to prevent the reduction in $V_{mic}$ (paired t-test, $p < 0.05$) (see figure 31) or the elevation of $Dur_{mic}$ (paired t-test, $p < 0.05$) observed in the control groups, at any of the doses tested (ranging from 1mg/kg to 7.5mg/kg) (see table 3 in appendix 2).

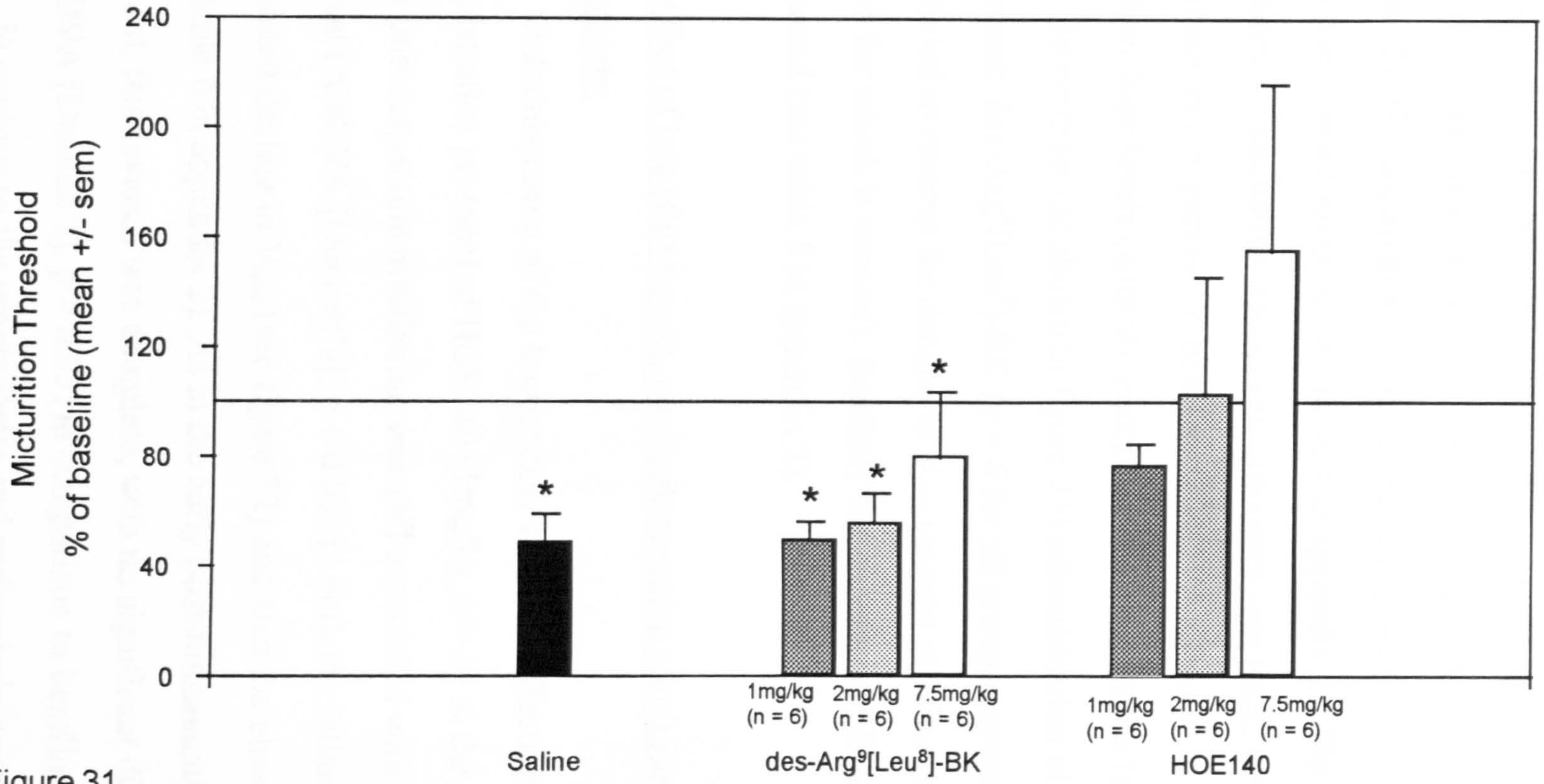



Figure 31.

Box plot demonstrating the differing effects of bradykinin B1 (des-Arg$^9$[Leu$^8$]-BK) and B2 (HOE140) receptor antagonists upon the micturition threshold ($V_{mic}$) when administered (i.a.) prior to the induction of inflammation. Note the reduction in $V_{mic}$, indicative of hyper-reflexia, when the control substance (saline) or the B1 receptor antagonist are administered. When the B2 receptor antagonist is administered this reduction is prevented.

* $p < 0.05$ in comparison to baseline (paired t-test).

The effect of early (two hours) post-inflammation administration of receptor antagonists:

The B2 receptor antagonist, HOE140 (n = 6 for all groups, except 1mg/kg, where n = 8) reversed the inflammation associated decline in $V_{mic}$ at all doses administered (1 to 7.5mg/kg) (ANOVA [Tukey], $p < 0.05$) (see figure 32). The attenuation of the rise in $Dur_{mic}$ was less consistent, but the trend was toward reversal (see table 4 in appendix 2). The reversal of all the measured inflammation-associated outcomes was total, with no significant difference in comparison to baseline (ANOVA [Tukey], $p > 0.05$) at any of the three dose levels (with the exception of $Dur_{mic}$ at the 1mg/kg dosage).

In contrast (as shown in figure 33) administration of the B1 receptor antagonist, des-$Arg^9$[$Leu^8$]-BK (n = 6 for all groups except 1mg/kg, where n = 9) failed to reverse the decline in $V_{mic}$ (except at a dose of 1mg/kg, the reason for which is unclear). Similarly the elevation in $Dur_{mic}$ was not attenuated (see table 5 in appendix 2).

The effect of late (five hours), post-inflammation administration of receptor antagonists:

Administration of the lowest dose that was effective in the early administration protocol of HOE140 (1mg/kg, n = 6) at the later time of five hours post-turpentine instillation, was still associated with a significant reversal (ANOVA [Dunnett's], $p < 0.05$) in both the inflammation-associated decline in $V_{mic}$ (see figure 32) and also the elevation in $Dur_{mic}$ (see table 6 in appendix 2). As in the early post-inflammation administration protocol, this reversal was complete, with no significant difference (ANOVA [Dunnett's], $p > 0.05$) in comparison to baseline.

In contrast to the prophylactic and early administration regimens, when the B1 receptor antagonist, des-$Arg^9$[$Leu^8$]-BK, was ineffective at attenuating inflammation-associated alterations in the cystometrogram trace,

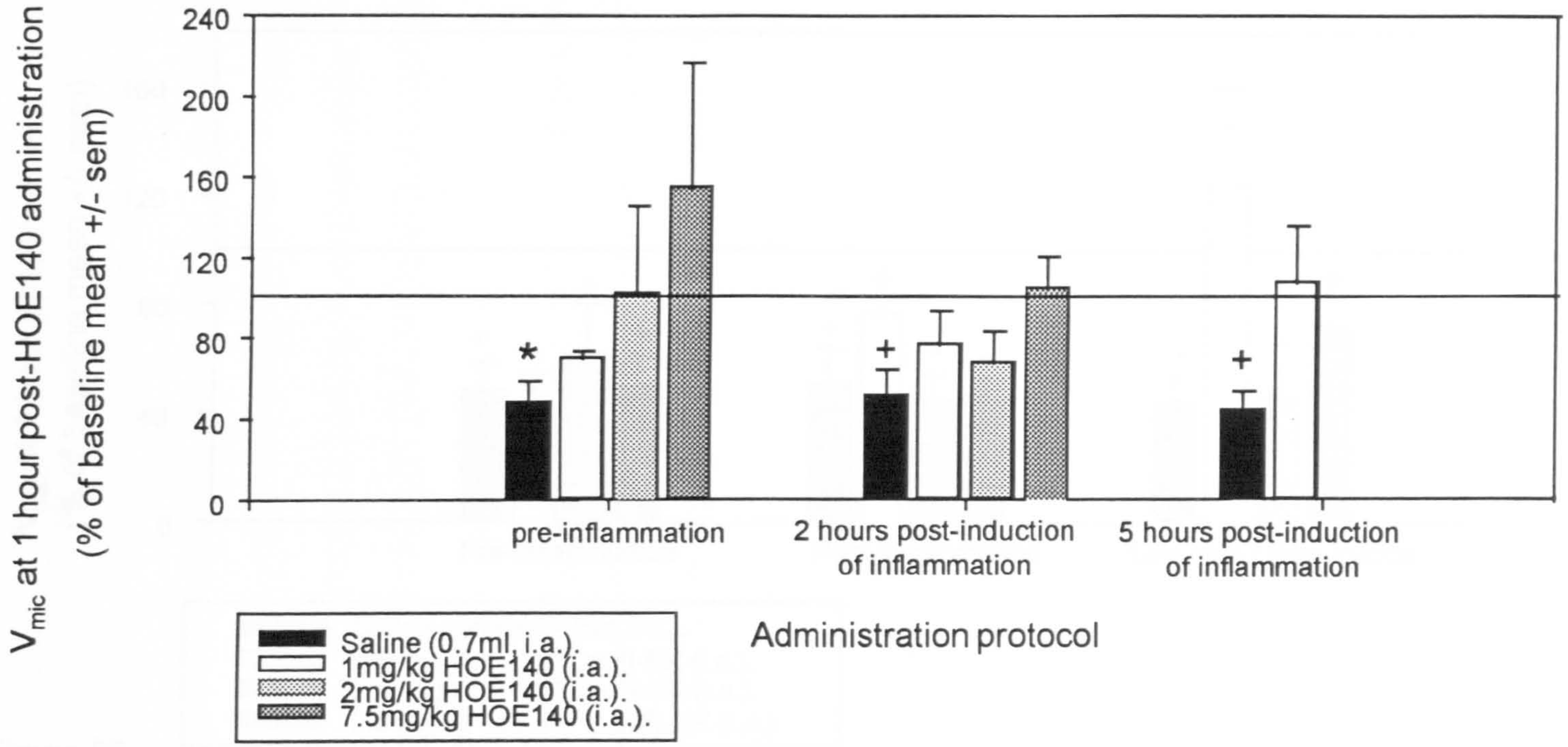



Figure 32.

Bar chart showing the effect of the timing of administration of HOE140 (in relation to induction of inflammation) on the micturition threshold, $V_{mic}$ at 1 hour after administration. Note that all control groups are associated with a significant reduction in $V_{mic}$ in comparison to baseline, whilst none of the treatment groups are associated with any significant difference from baseline (irrespective of time of administration).

* $p < 0.05$ in comparison to baseline (paired t-test).

\+ $p < 0.05$ in comparison to baseline (ANOVA [Tukey]).

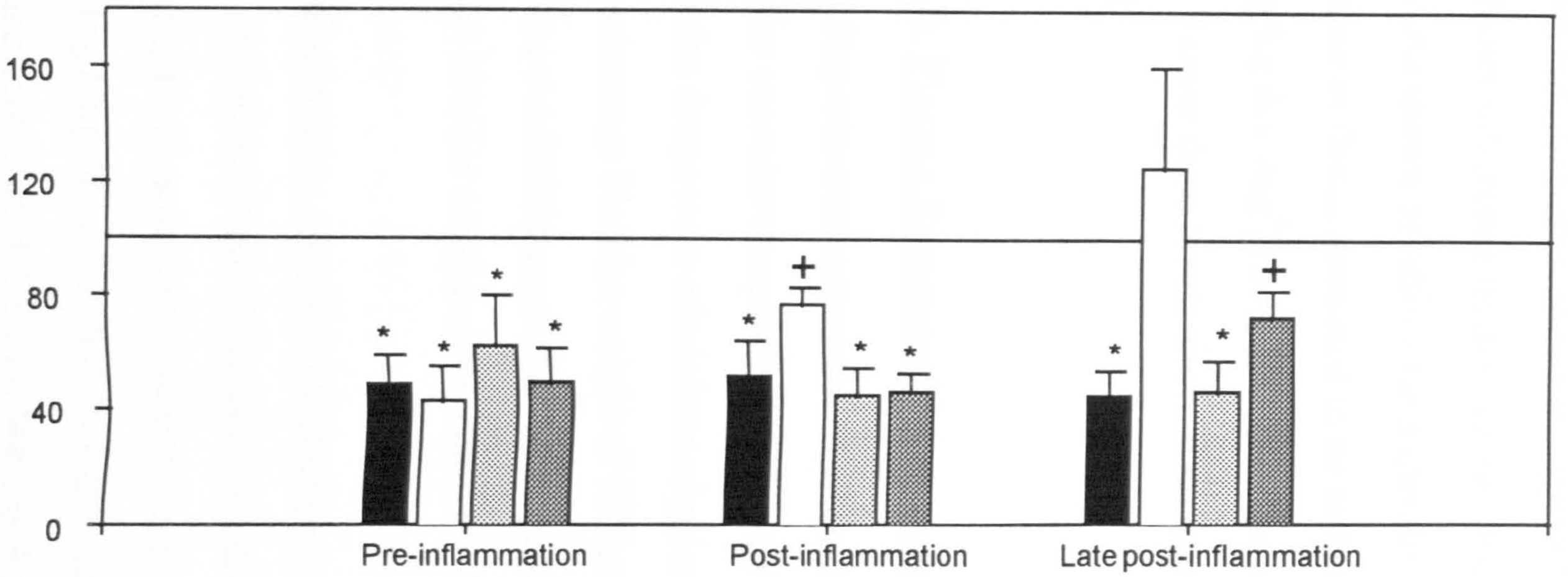



Figure 33.

Bar chart showing the effect of time of administration (in relation to induction of inflammation), of the B1receptor antagonist des,Arg$^9$[Leu$^8$]-BK, on the micturition threshold, $V_{mic}$. Note the reduction in $V_{mic}$ in all the prophylactic treatment and early post-inflammation treatment groups. This indicates a failure to prevent or reverse the effect of chemical inflammation. In the late post-inflammation treated groups there is a significant difference in comparison to the post-inflammation $V_{mic}$ in the 7.5mg/kg dose group.

* $p < 0.05$ in comparison to baseline (paired t-test in prophylactic regimen and ANOVA [Tukey] in treatment protocol).

\+ $p < 0.05$ in comparison to post-inflammation $V_{mic}$.

administration five hours after the commencement of inflammation, at a dose of 7.5mg/kg (n = 6), was associated with a significant attenuation of the micturition threshold $V_{mic}$ (ANOVA [Dunnett's], $p < 0.05$) (see figure 33). Furthermore this attenuation amounted to a complete reversal, with no significant difference from baseline observed (ANOVA [Dunnett's], $p > 0.05$). As shown in table 7 (in appendix 2) the inflammation associated elevation in $Dur_{mic}$ appeared to be reduced by the administration of 7.5mg/kg des-$Arg^9$[$Leu^8$]-BK, but this was not significant at the $p < 0.05$ level. Lower dose levels showed no attenuating effect on the observed VVH.

## 4.iii.b. Plasma Extravasation experiments:

Experiments were performed on animals in which it was possible to clear the vascular compartment of Evan's Blue Dye (n = 55). Animals in which the drugs were administered prior to turpentine inflammation (n = 24) had an average bladder weight of 93.1gm (+/- 4.5). Those in which the drugs were administered after turpentine inflammation (n = 31) had an average bladder weight of 98.7gm (+/- 2.6).

Pre-inflammation administration of receptor antagonists:

Administration of the saline control (0.7ml, 0.9%, i.a.) solution was associated with a mean tissue concentration of Evan's Blue Dye of 0.75 (+/- 0.12) mg / mg of vesical tissue. Intra-arterial administration of neither the B2 receptor antagonist HOE140, nor the B1 receptor antagonist des-$Arg^9$[$Leu^8$]-BK, was associated with a significant change (unpaired t-test, $p < 0.05$) in the mean concentration (see table 8 in appendix 2).

Post-inflammation administration of receptor antagonists:

Saline control administration (0.7ml, 0.9%, i.a.) following turpentine induced inflammation was associated with a mean tissue concentration of Evan's Blue Dye of 0.74 (+/- 0.13) mg / mg of vesical tissue. Neither administration of HOE140, nor des-Arg$^9$[Leu$^8$]-bradykinin, was associated with a significant difference (ANOVA [Dunnett's], $p < 0.05$) in mean tissue concentration of Evan's Blue Dye from this control treated group (see table 8 in appendix 2).

## 4.iii.c. *C-fos* immunohistochemistry:

Experiments were performed on thirteen animals, mean weight 221.9gm (range 200 - 240gm). Analysis of counts from control animals (n = 6) demonstrated a significant increase in the total number of neurones staining positive for c-Fos-like immunoreactivity at the L6 and S1 levels in comparison to the L5 level (ANOVA [Dunns], $p < 0.05$) (see figure 34). As shown in table 9 (in appendix 2) this pattern was reflected in all sub-areas counted. The heavy staining for c-Fos-like immunoreactivity is demonstrated in a typical spinal cord section shown in figure 35, by the high density of black granules.

Comparison of control and treated animals demonstrated that pre-inflammation administration of 5mg/kg HOE140 was associated with a significant reduction in the total number of cells staining positive for c-Fos-like immunoreactivity at both the L6 and S1 levels (see figures 34 and 35). Furthermore, there was a significant reduction ($p < 0.05$, unpaired t-test) at all spinal cord levels in lamina X and the intermediolateral posterior nucleus and also in the superficial dorsal horn at level L6 (see table 9 in appendix 2). An example of the reduced level of staining for c-Fos-like immunoreactivity

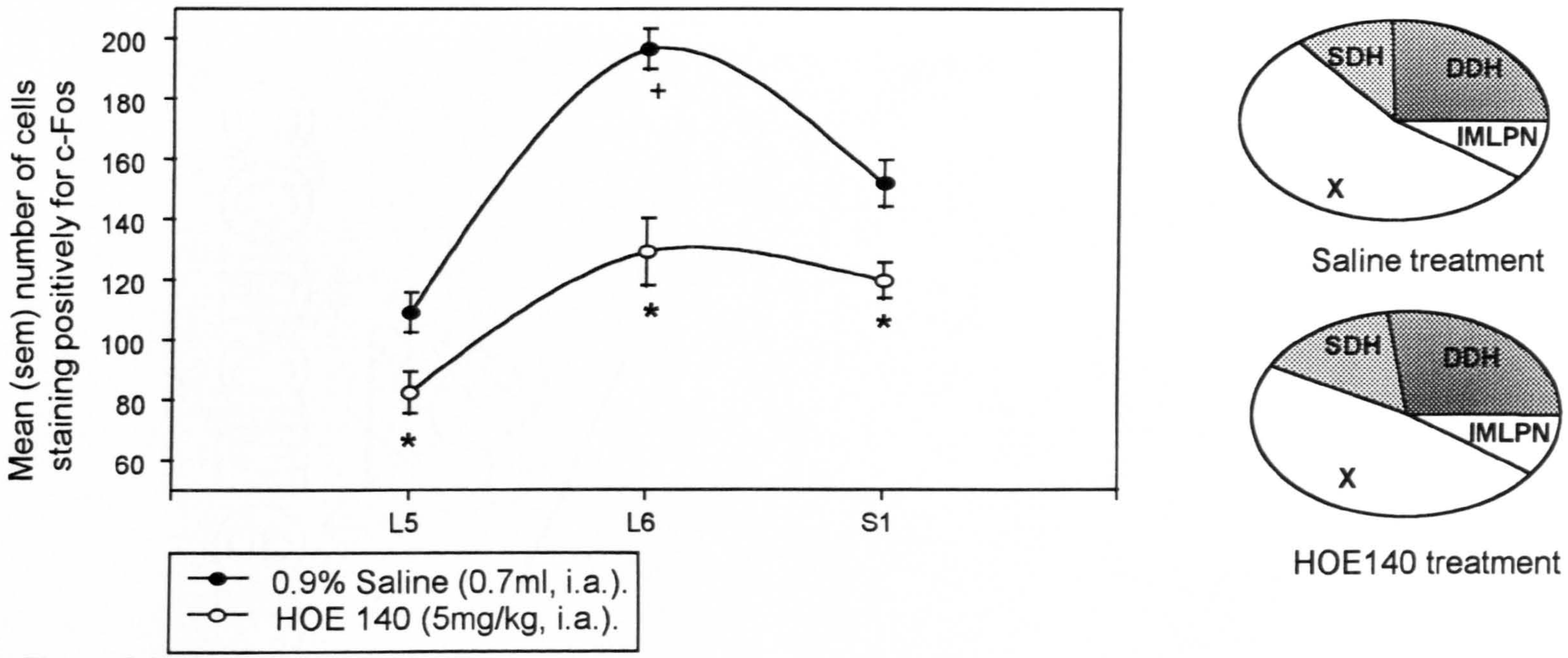



Figure 34.

Graph to demonstrate the total level of c-Fos-like immunoreactivity in sections of the spinal cord following turpentine-induced inflammation of the urinary bladder. The increased level of staining at the L6 and S1 levels is indicative of activity in nociceptive pathways. Note the reduction in staining observed when prophylactic treatment with a B2 receptor antagonist is administered.

\+ $p < 0.05$ in comparison to staining at the L5 spinal level.

* $p < 0.05$ in comparison to staining observed in control animals, at the same level (unpaired t-test).

The pie charts to the right demonstrate the proportion of staining in each of the subareas counted at the L6 spinal level. Note that the pattern of staining is little changed by the administration of HOE140 (5mg/kg).

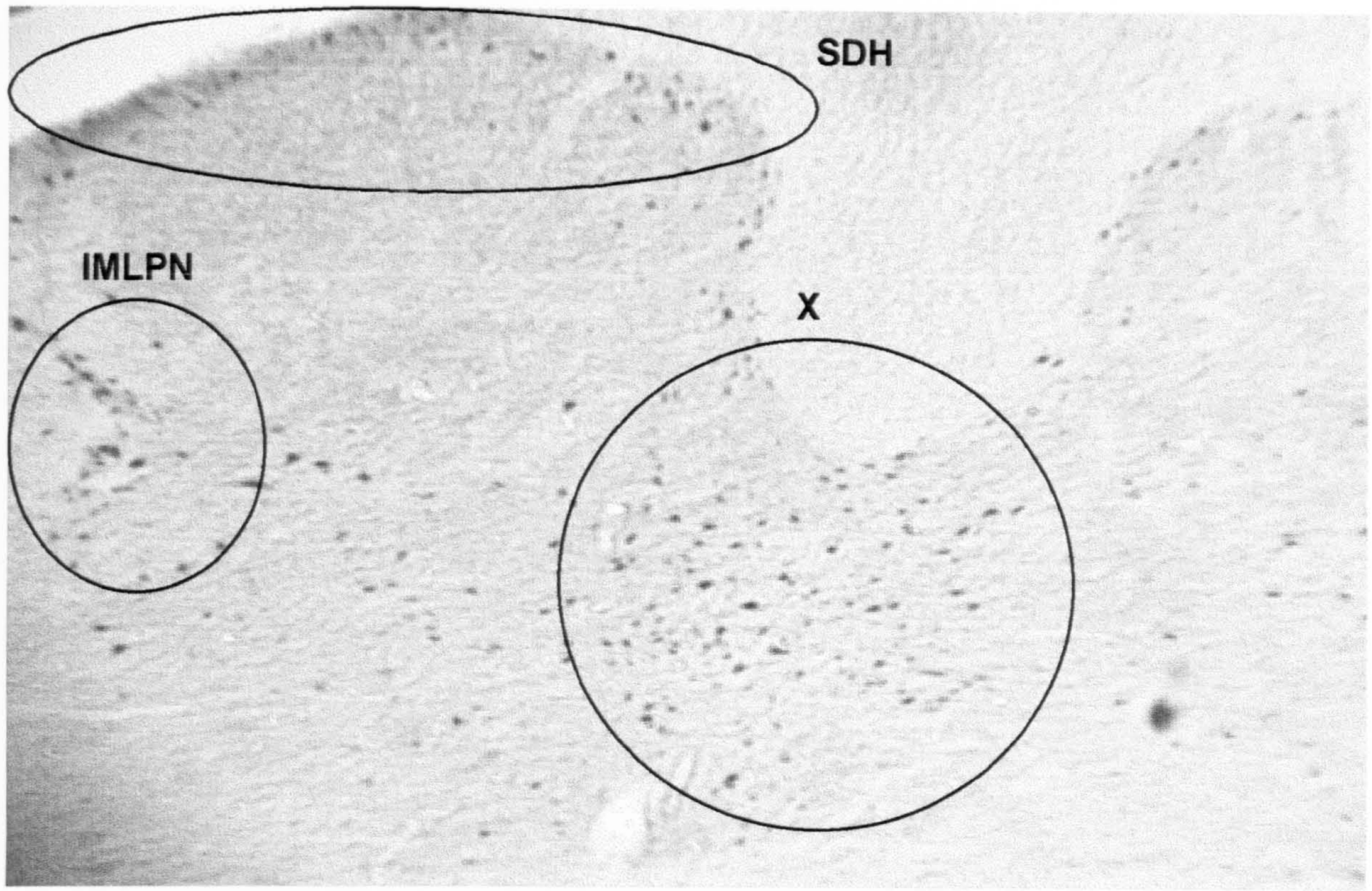



Figure 35.
Photograph of a slice of sacral spinal cord taken from an animal treated with saline (0.7ml, i.a.) prior to the intra-vesical instillation of turpentine oil (0.5ml, 50%, 1hr). Note the heavy staining for c-Fos-like immunoreactive protein observed in the superficial dorsal horn (SDH), intermediolateral posterior nucleus (IMLPN) and lamina X (X). This reflects activity in nociceptive pathways arising from the urinary bladder.

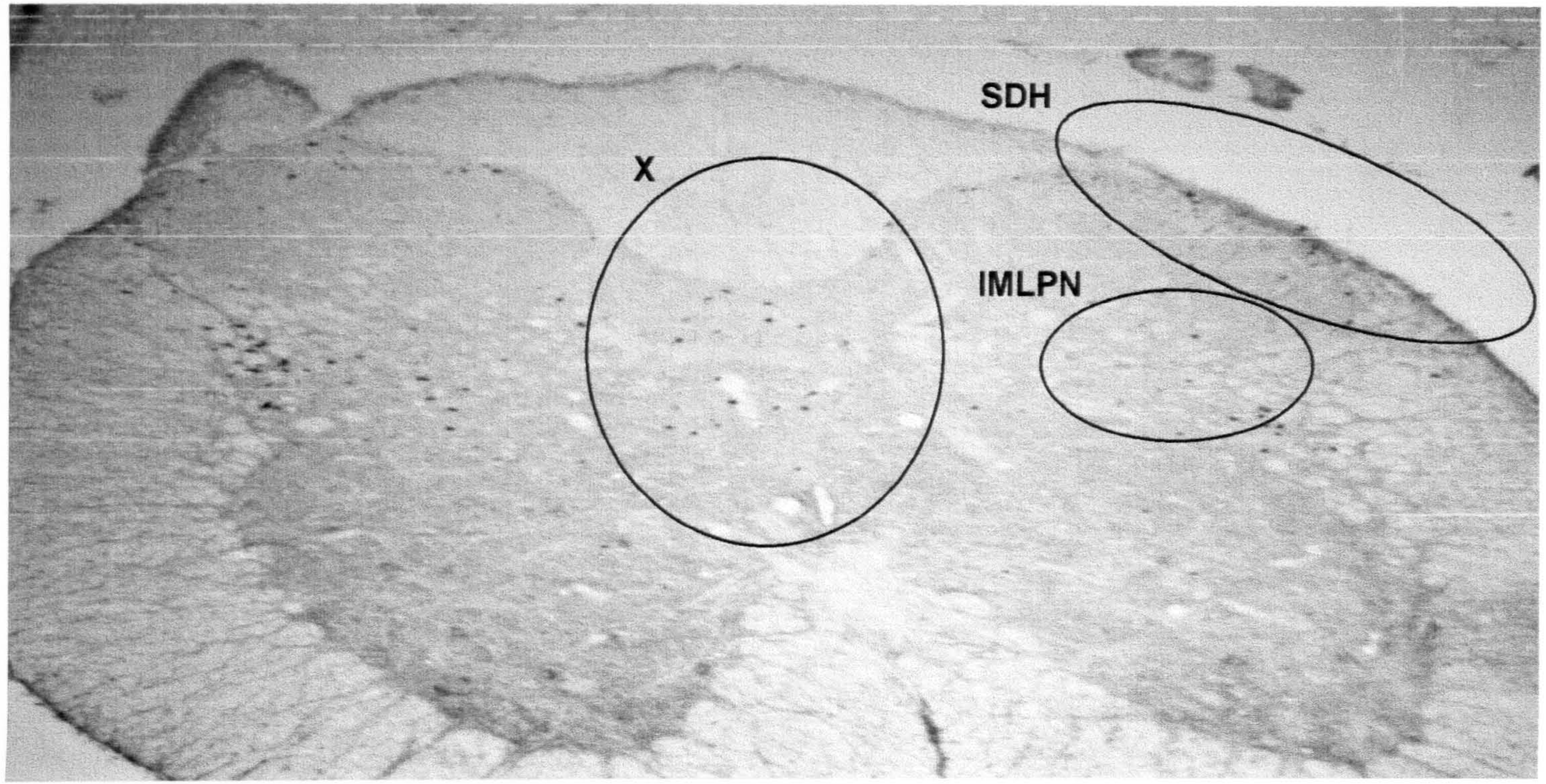



Figure 36.
Photograph of a slice of sacral spinal cord, taken from an animal treated with 5mg/kg HOE140 30 minutes prior to intra-vesical instillation of turpentine oil (0.5ml, 50%, 1hr). Note the reduction in c-Fos-like immunoreactivity observed in the superficial dorsal horn (SDH), lamina X (X) and the intermediolateral posterior nucleus (IMLPN). This reflects reduced activity in nociceptive pathways arising from the urinary bladder.

observed in spinal cord slices obtained from a typical animal treated with HOE140, prior to chemical inflammation of the bladder, is shown in figure 36.

## 4.iv. Discussion

The work presented here, confirms the occurrence of an hyper-reflexic response of the rat urinary bladder (VVH) following the instillation of intra-vesical turpentine oil [215,218]. This is demonstrated by a reduction in the micturition threshold ($V_{mic}$) and an elevation in the cumulative duration of micturition ($Dur_{mic}$). Furthermore, the results suggest that bradykinin may exert an important role in mediating these events.

Despite the lack of any significant effects on the micturition reflexes of the uninflamed bladder, the prophylactic administration of the bradykinin B2 receptor antagonist HOE140 was associated with the inhibition of the VVH observed following subsequent intra-vesical turpentine instillation. Moreover, such prophylactic administration of the compound inhibited the increase in c-Fos-like immunoreactivity, previously demonstrated to occur in the spinal cord [89]. In addition, HOE140 reversed an established VVH whether it was administered at early or late time points after the induction of inflammation. However, in none of the experimental protocols was the attenuation of the VVH associated with any reduction in plasma extravasation into vesical tissue.

In contrast to the activity observed in all treatment regimens where HOE140 was utilised, the B1 receptor antagonist des-Arg$^9$[Leu$^8$]-bradykinin was only associated with reversal of an established VVH. Moreover, the attenuation was only evident when the drug was administered five hours after the induction of inflammation. As with HOE140, no effect on the plasma extravasation into vesical tissue was observed.

Although bradykinin B2 receptors are expressed in the dorsal horn of the spinal cord [310] and the VVH observed following chemical inflammation of the bladder is mediated via a spinal reflex (see 1.iv.a. for in depth discussion) [218,274,275], the effects of HOE140 could be explained by activity at peripheral sites. Bradykinin receptors are expressed on dorsal root ganglion cells [310] and in vesical tissue [200]. Quantitative autoradiography has demonstrated the localisation of bradykinin receptors in the sub-epithelial lamina propria - an area rich in small diameter unmyelinated neurones. Whilst unmyelinated neurones innervate both the sub-mucosal and muscular layers of the bladder [72,138,190], in inflammatory conditions an increased presence of substance P positive fibres is only observed in the sub-mucosal layer [258]. These fibres are commonly associated with the presence of mast cells [187], which are also known to express bradykinin receptors [96]. Thus, under conditions of inflammation, bradykinin might be expected to exert effects on both nociceptive neurones and immune elements in vesical tissue.

Topical administration of exogenous bradykinin induces rapid onset of both tonic and phasic contractions of the urinary bladder in animal models, whether the bladder is naive [173] or has been inflamed by either the intra-vesical instillation of xylene or systemic cyclophosphamide [194]. Bradykinin-associated tonic contractions are unaffected by capsaicin pre-treatment, suggestive of non-neuronal, smooth muscle effects [173]. However, the phasic hyper-reflexic changes are prevented by the pre-treatment of animals with capsaicin [173,194], an agent specifically evoking desensitisation of small primary afferent neurones [31,313,337]. Since such neurones are known to express the bradykinin receptor [310] the bradykinin-associated VVH could be explained by a direct, neuronally mediated mechanism. Support for these bradykinin-induced phasic contractions of the bladder being neuronally mediated arises from a number of *in vitro* models.

Application of bradykinin to the isolated urinary bladder is associated with a rapid increase in the calcitonin gene related peptide-like immunoreactivity detected in the fluid perfusing the bladder [194]. Since calcitonin gene related peptide is released by the peptidergic subset of capsaicin sensitive neurones [92,273,345], this would indicate that the bradykinin-associated VVH is specifically mediated by NGF-dependent, capsaicin sensitive, afferent neurones. Furthermore, since this bradykinin-associated neuropeptide release is inhibited by the presence of HOE140 in the perfusate, the effect would appear to be receptor mediated [194]. Electrophysiological evidence is provided by an alternative visceral *in vitro* model - the testis-superior spermatic nerve preparation. Bradykinin addition to the perfusate is associated with sensitisation of polymodal nociceptors and a leftward shift of the stimulus response curve [166], further supporting the direct neuronal effects of bradykinin.

However, non-neuronal effects may also have a role to play in the response to bradykinin. Bradykinin receptors are expressed not only on neuronal elements, but also on immune tissues, including mast cells [96]. Binding to such mast cell-associated receptors results in degranulation [204,320]. The consequent release of additional algogens, including NGF [177], histamine [175], cytokines and serotonin [237], each capable of binding to and initiating activation, or sensitisation of afferent neurones, would tend to exacerbate the nociceptive response. Thus, a positive feedback cycle may be set in motion, with amplification of the primary neuronal response being initiated by elements of the immune system involved in inflammation.

I have demonstrated that the systemic administration of HOE140 is effective in exerting inhibitory effects on the integrated neuronal response associated with intra-vesical turpentine instillation, namely the VVH. This is

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evident whether the compound is administered by either a prophylactic or a treatment paradigm. Since the bradykinin B2 receptor is constitutive [22] such antagonist effects, possibly mediated at peripheral level by interaction at both neuronal and immune-associated receptor sites, would explain these findings.

The dose of HOE140 observed to inhibit the reflex responses to intra-vesical turpentine (0.77μmol/kg to 5.75μmol/kg) is somewhat higher than that found to be effective in inhibiting similar vesical contractions, evoked by the systemic administration of bradykinin (35nmol/kg) [173]. However, a dose-relationship between bradykinin and the incidence of vesical contractions has been documented. Furthermore, administration of a higher dose of HOE140 (35nmol/kg) was noted to be associated with attenuation of the contractile response in a higher proportion of cases than that following a lower dose (10nmol/kg) [173]. Thus if intra-vesical turpentine instillation were to be associated with a massive local release of bradykinin, greater than the highest dose of bradykinin exogenously administered by Lecci *et al*, the requirement for larger doses of the B2 antagonist to attenuate the hyper-reflexic response would not be unexpected. In support of this, when vesical hyper-reflexia is induced by the systemic administration of cyclophosphamide, a dose of 100nmol/kg of HOE140 is required to attenuate the VVH response [194]. Cyclophosphamide-induced inflammation occurs in response to a renally excreted metabolite, acrolein [64]. It is possible that the level of bradykinin released in response to acrolein is greater than the doses administered by Lecci *et al* [173], but less than that in response to topically administered turpentine oil. This would explain the intermediate dose of HOE140 effective in the cyclophosphamide-induced VVH [194].

Somewhat surprisingly, I have been unable to demonstrate any reduction in inflammation-associated plasma protein extravasation into

vesical tissue following treatment with HOE140. As in the visceral domain [218], local administration of bradykinin, or a variety of other inflammatory agents, into cutaneous tissue is associated with oedema formation [29,71]. In contrast to the results presented here, somatic inflammation is associated with a reduction in the volume of oedema following prophylactic administration of nmol/kg doses of HOE140. However, in view of the differences in neurophysiology between the visceral and somatic domains (see 1.i.b.) it is possible that tissue responses to HOE140 might differ. In addition, although the anti-hyperalgesic and anti-inflammatory effects of compounds often parallel each other, differential effects have been described. One such example is observed when hyperalgesia (in response to mechanical stimuli) and localised tissue oedema are induced by the sub-cutaneous injection of brewers yeast. Whilst the presence of hyperalgesia is inhibited by low doses of paracetamol, no such inhibitory effect is observed upon the tissue oedema [34].

Inflammation of the urinary bladder of the rat is known to be associated with an increase in c-Fos-like immunoreactivity in the spinal cord [35,36,171,223]. These experiments confirm that following such inflammation, staining is most markedly increased in cells located at the L6 level of the rat spinal cord. Furthermore, such spinal cord staining is heaviest in the areas of the superficial dorsal horn and peri-aqueductal grey areas of laminae I, II and X. These are areas that contain the central projection terminals for capsaicin-sensitive afferent neurones particularly involved in the transmission of nociceptive, as a pose to innocuous information [36,151].

The experiments presented here further demonstrate that administration of HOE140 is associated with a reduction in the c-Fos-like immunoreactivity observed in the spinal cord, in response to vesical inflammation. Since the upregulation of expression of the immediate early

gene *c-fos* is used as a marker for nociceptive activity [52] this reduction in staining suggests that HOE140 is capable of interrupting the flow of nociceptive traffic following inflammation of the rat urinary bladder. Whilst such effects have been documented to occur at a central level [35], pharmacological manipulation of peripheral events might also effect such a change [52]. Thus both the reflex response experiments and the immunohistochemical findings support the postulate that peripherally located B2 receptors play an important role in the modulation of inflammation-associated hyper-reflexic responses of the urinary bladder.

Radioligand studies have failed to demonstrate the expression of the inducible bradykinin B1 receptor [201] on dorsal root ganglia cells of sensory neurones [76]. The distribution is limited almost exclusively to vascular and inflamed tissues [22]. Expression of the B1 receptor is known to be time dependent in both somatic models of inflammatory pain [102] and isolated tissues, such as the rabbit aorta [231]. Furthermore, such B1 receptor expression is greatly enhanced in the presence of inflammatory stimuli [38,102]. Therefore, the observation that administration of des-Arg$^{9}$[Leu$^{8}$]-bradykinin was only associated with a positive effect when treatment was instituted at five hours following induction of inflammation is not entirely surprising.

*In vitro* evidence from observations of the contractile response of rabbit aorta and bladder tissue, in response to the B1 receptor agonist des-Arg$^{9}$-bradykinin, suggests that B1 receptor induction takes at least three hours [38,46]. Such time dependency is also observed in a wide variety of human vasculature [201]. In addition, the accumulation of intra-cellular second messengers (such as the inositol phosphates) in response to des-Arg$^{9}$-bradykinin mirrors this time dependency [46]. *In vivo* evidence arising from work in the somatic domain, also supports the proposition that

induction of B1 receptors is associated with a time delay, reflected by a lag time before inhibition of pathophysiological responses is observed. When hyperalgesia to thermal stimulation is induced by administration of NGF, the inhibitory effect of B1 receptor antagonists takes several hours to develop [284]. Furthermore, when pain related behaviour is observed following the administration of sub-cutaneous formalin, much higher doses of des-Arg$^{9}$[Leu$^{8}$]-bradykinin are required to inhibit the first phase of the response when compared to the second phase. Data such as this suggests modification in the B1 receptor population with time following an inflammatory stimulus [312].

The possibility that upregulation of the B1 receptor is controlled via immune mediators suggests again the importance of neuro-immune interactions in the modulation of pain. Expression of the B1 receptor has been demonstrated to be upregulated in human vascular tissue following administration of lipopolysaccharide or interleukin-1β. This upregulation is inhibited, not only by anti-inflammatory steroids, but also by the administration of protein synthesis or trafficking inhibitors [287]. Thus it would appear that the protein expression of different genetic populations of receptors may depend upon the presence of inflammation. Experiments investigating both tissue responses and behavioural activity support this notion. *In vitro* contraction of smooth muscle in response to B1 receptor agonists is increased, not only by the local application of inflammatory agents including lipopolysaccharide [38], but also by the induction of an inflammatory response in the animal prior to removal of the tissue [102]. Furthermore, behavioural indices of pain, such as the thermal hyperalgesia associated with ultra-violet irradiation of the paw [261,262] and the mechanical hyperalgesia associated with intra-dermal injection of Complete Freunds Adjuvant [160] are enhanced by administration of the B1 receptor agonist des-Arg$^{9}$-bradykinin. These effects are inhibited by the concurrent

administration of B1 receptor antagonists such as des-Arg$^9$[Leu$^8$]-bradykinin. My observation that the efficacy of des-Arg$^9$[Leu$^8$]-bradykinin was limited to late time points when inflammation was well developed mirrors these findings.

As in the experiments in which the B2 receptor antagonist HOE140 was administered, I failed to demonstrate any effect of the B1 receptor antagonist, des-Arg$^9$[Leu$^8$]-bradykinin, on the inflammation-associated plasma extravasation into vesical tissue. Similar findings have been reported in the somatic domain by Corrêa and Calixto, who noted that paw oedema associated with sub-cutaneous formalin injection is not attenuated by the co-administration of a B1 receptor antagonist [61]. This apparent anomaly of a positive inhibitory effect of des-Arg$^9$[Leu$^8$]-bradykinin upon the turpentine-associated VVH and the lack of effect on plasma extravasation might be explained by factors involving the actions of the neurotrophin NGF. In a somatic model of inflammation, in which Complete Freunds Adjuvant is injected sub-cutaneously, hyperalgesia is associated with upregulation of both neuropeptides in dorsal root ganglion cells and genetic markers such as *c-fos* in the dorsal horn neurones. Both the behavioural responses and the upregulation of nociceptive markers are attenuated by the administration of anti-NGF antibodies. However, tissue changes, including the associated sub-cutaneous oedema and erythema are unchanged [343]. Thus, although both the reflex and neuronal changes observed following turpentine inflammation of the rat urinary bladder [12,13,89,91,254] and paw [343] depend to some degree upon NGF, the tissue changes might reasonably be considered to be secondary to effects acting via an NGF-independent pathway. This is of particular interest in regard to the turpentine-inflamed rat urinary bladder model used here, since there is evidence suggesting that responses to NGF and bradykinin are related. This derives from experiments, described below,

in which both administration of exogenous NGF and sequestration of endogenous NGF were examined.

When dissociated rat dorsal root ganglion cells are incubated in the presence of NGF there is an increase in both the proportion of cells expressing bradykinin binding sites (from 50% to 80%) and the density of such binding sites [265]. If this were also true *in vivo*, the increase in tissue NGF observed during inflammation [13,92,186,253] would tend to increase the effects of local bradykinin release. Rather surprisingly the upregulation of bradykinin binding sites appears to be dependent not upon the high affinity NGF receptor trkA, but upon the low affinity, p75 receptor. Not only does the upregulation of bradykinin sites on small diameter neurones not occur when cells are grown in the presence of both NGF and an anti-p75 antibody, but furthermore, binding sites are also observed on larger neurones [265] which, whilst expressing the p75 receptor do not express the trkA receptor to any great extent [219].

In the *in vitro* rat skin-nerve preparation, the effect of sequestration of endogenous NGF has been examined [27]. When trkA-IgG is infused into skin, the proportion of small diameter nociceptors responding to bradykinin administration is reduced from 28% to 8%. This reduction cannot be explained as a result of cell death, since in the adult rat nociceptors are no longer dependent upon NGF for survival [181,297]. An alternative explanation is the downregulation of bradykinin binding sites on cutaneous nociceptors consequent upon the sequestration of endogenous NGF. Thus, the endogenous levels of a pivotal inflammatory mediator may regulate tissue responses to an alternate algogen, in this case bradykinin.

*In vivo* experiments also support an interaction between NGF and bradykinin. Systemic administration of NGF to adult rats results in hyperalgesia in response to both thermal and mechanical stimuli. The late component of the thermal hyperalgesia is inhibited by the administration of a

B1 receptor antagonist des-Arg$^{9}$[Leu$^{8}$]-bradykinin [284]. The lack of any effect of bradykinin antagonists on the NGF-induced mechanical hyperalgesia might be explicable since intra-dermal bradykinin is associated with hyperalgesia to thermal but not mechanical stimuli [199].

Whilst the experiments described above were all performed utilising stimuli applied to the somatic domain, the differences in the neurophysiology pertaining to visceral and somatic tissues (discussed in section 1.i.b.) indicate that the potential interactions between NGF and bradykinin might be of even greater significance in pain arising from visceral tissues. The proportion of primary afferent neurones which express the trkA receptor for NGF is much higher in the viscera than for other tissues (of the order of 90%, in comparison to 50% of cutaneous and 20% of muscle afferent neurones) [26,219]. Furthermore, NGF appears to play a pivotal role in the production of features associated with the inflamed bladder. Intra-vesical instillation of NGF is associated with both local (VVH) [91] and referred (VSH) [described in chapter 3] hyperalgesic responses, in addition to sensitisation of primary afferent [90] and dorsal horn neurones [15] and upregulation of the immediate early gene *c-fos* in the spinal cord [89]. Thus, the possibility arises, that the increase in NGF associated with inflammation [11,217], may alter bradykinin binding site expression to an even greater extent in visceral than somatic tissues [265], increasing neuronal excitability in response to bradykinin and inducing pain and hyperalgesia. This effect, like B1 receptor induction, would be expected to increase with time, as NGF release from mast cells increases. Thus the lack of efficacy of des-Arg$^{9}$[Leu$^{8}$]-bradykinin until late time points may be a result of NGF-dependent upregulation of bradykinin receptor expression.

## 4.iv.a. Clinical Implications:

Bradykinin has long been known to be a potent algogen in humans [96] and circulating levels are increased under conditions of chronic inflammation, such as rheumatoid arthritis [134]. Therefore, it would not be unreasonable to propose that compounds antagonising the effects of bradykinin might prove efficacious in the treatment of pain or inflammation.

The bradykinin B2 receptor antagonist HOE140 (approved name Icatibant) is currently undergoing clinical trials in human volunteers. In a small phase 1 study, the effect of Icatibant on bradykinin-induced vasodilatation of the human forearm has been examined. It demonstrated that bradykinin increases regional blood flow and that this increase is dose-dependently inhibited by the prophylactic, systemic administration of Icatibant at doses ranging between 20 and 100μg/kg [54]. Moreover, no adverse reactions were reported. Further work has suggested that administration of Icatibant is (as might be expected from experimental studies [22]) associated with a rise in systemic blood pressure, consequent upon an increase in systemic vascular resistance [338].

Therapeutic trials reported thus far have mainly assessed the potential impact of such treatment in the management of allergic conditions such as asthma [7]. In a multi-centre, double blind, randomised, placebo controlled, phase 2, study nebulised Icatibant has been shown to be associated with a dose dependent improvement in pulmonary function tests, whilst being well tolerated by patients with moderate to severe asthma [7]. Thus, Icatibant is being developed as an alternate second line therapy in allergic conditions.

The studies presented here suggest that HOE140 may show efficacy, not only in allergic conditions, but also in those inflammatory conditions arising from the viscera in which pain is a prominent feature. Administration of HOE140 is associated with both prevention and reversal of hyper-reflexic changes in addition to the attenuation of altered gene expression observed in

an animal model of persistent visceral pain. This raises the possibility that it (or agents with a similar spectrum of activity) might provide a new tool in the medical armamentarium, for the treatment of such conditions in the clinic.

As in animal models of visceral pain [12,254], the endogenous production of NGF has been shown to be upregulated in patients suffering with inflammatory conditions affecting the urinary bladder (discussed more fully in chapter 3) [251]. Since bradykinin B1 receptor interactions with NGF are implicated in the response of sensory neurones to noxious stimuli [284], this might provide a further mechanism by which antagonists acting at this receptor site could be accomplishing analgesic effects. Thus, inhibition of bradykinin mediated effects might be expected to attenuate the positive feedback response associated with NGF. This is of particular relevance in conditions affecting the viscera, since approximately 90% of visceral afferent neurones express the trkA receptor [26,219]. Moreover, in view of the fact that the B1 receptor is induced only under conditions of inflammation [22,38] drugs acting at this site would be expected to be associated with fewer unwanted effects.

# Chapter 5:

# Capsaicin and visceral inflammatory pain

## 5.i. Introduction

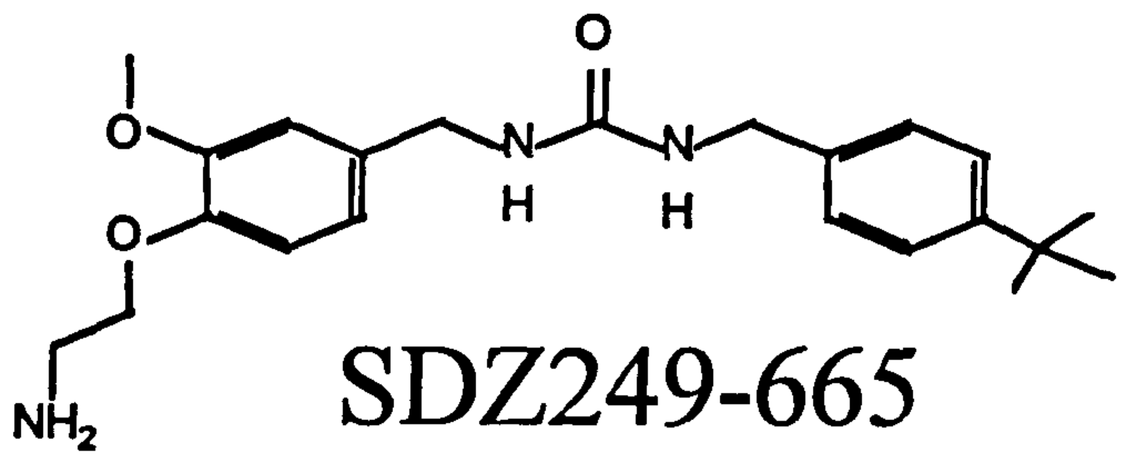
### 5.i.a. Capsaicin:

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the main pungent ingredient in 'hot' chilli peppers of the *Capiscum* family (see figure 37). Its topical analgesic action has been recognised and used for centuries in countries where the plant is indigenous [271]. When applied topically to skin capsaicin induces an initial burning sensation to which tolerance rapidly develops, with an associated reduction in pain sensitivity. This selective local analgesia is consequent upon selective activation and subsequent functional desensitisation of the terminals of nociceptive neurones [316].

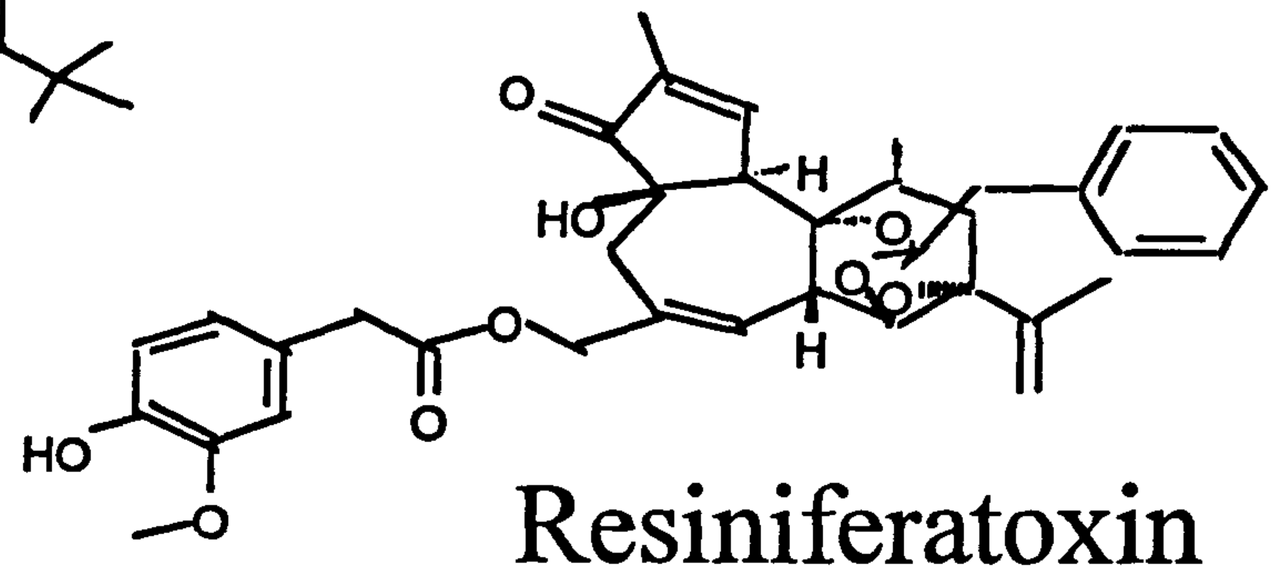
The initial intensity and duration of the burning pain correlates well with the nociceptor discharge which evokes release of neuropeptides, including substance P, neurokinin A and calcitonin gene related peptide, in the dorsal horn of the spinal cord [193,337]. Efferent functions of these axons encompass:

- The peripheral release of neurogenic mediators, such as substance P.
- A localised neurogenic inflammatory response associated with erythema and oedema.
- The ability to elicit smooth muscle contractions in a variety of visceral organ systems including the bladder [31,316].

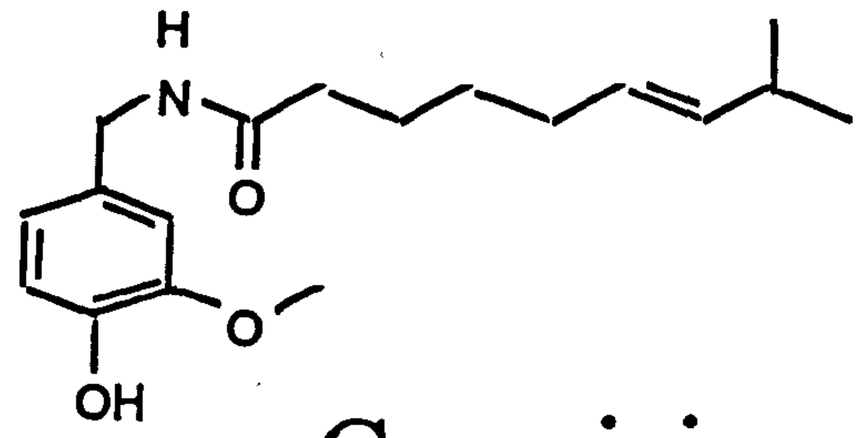
The later, non-specific, functional desensitisation to a variety of stimuli appears to be secondary to a calcium-dependent stimulation of the cytosolic protein phosphatase calcineurium. The desensitisation is abolished



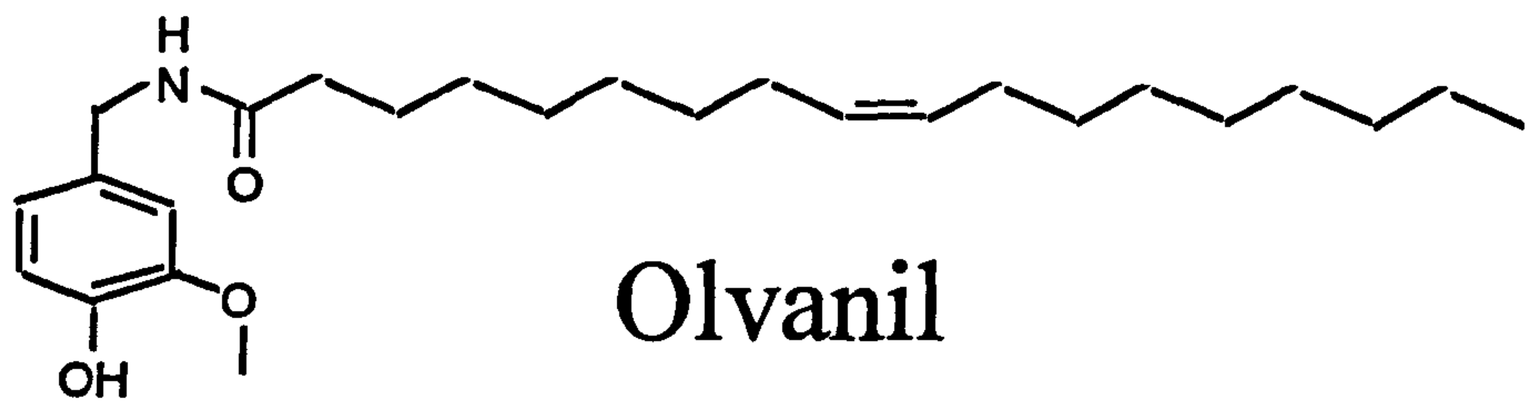

SDZ249-665




Resiniferatoxin




Capsaicin




Olvanil

Figure 37.
Diagram demonstrating the chemical structures of capsaicin an some vanilloid analogues.

by the removal of either calcium ions or calcineurium (by complexing with cyclosporin A) [337]. In the context of pain arising from the viscera, it is of particular interest that *in vitro* studies of sensory neurones suggest that the expression of the capsaicin binding site and the intra-cellular accumulation of calcium are entirely dependent upon the presence of the neurotrophin NGF [149].

The pharmacokinetics of capsaicin are unclear and there is no evidence to support per-cutaneous absorption in humans. However, animal studies suggest that after systemic administration capsaicin is distributed throughout the body, prior to hepatic metabolism by mixed function oxidases and excretion in the urine [271].

## 5.i.b. Effect of developmental state:

The effects elicited by capsaicin depend not only upon the dose, route and timing of drug administration, but also upon the developmental state of the animal to which it is administered. High doses of capsaicin are neurotoxic, an effect that is particularly profound after systemic administration to the neonate [31,337]. This neurotoxicity involves the same sub-population of nociceptive sensory neurones which are stimulated by single topical doses and appears to be related to osmotic swelling and subsequent lysis of cells, which occurs after entry of sodium and calcium ions [33]. Whilst in the neonate a profound cellular loss is observed, in the adult animal most cell bodies survive and respond vigorously with attempts at regeneration and subsequent somatosensory reorganisation [31,337].

## 5.i.c. The vanilloid (VR1) receptor:

The vanilloid VR1 receptor, with which capsaicin interacts, has recently been identified and cloned [49]. It is a six trans-membrane domain, ion channel protein, showing homology with a family of calcium channels. It

appears to be exclusively expressed on polymodal primary sensory afferent neurones involved in the nociceptive pathway [313] and has been demonstrated to be present in the urinary bladder of the rat [315]. In addition to being activated by capsaicin and related chemical agonists (such as resiniferatoxin), it is strongly activated by temperatures in the nociceptive range [49]. Although no endogenous agonists have as yet been described, hydrogen ions appear to act as modulators [30].

Receptor activation by agonist ligands is associated with a robust inward current of sodium and calcium ions, producing membrane depolarisation of both neuronal cells [31] and non-neuronal (human embryonic kidney-derived HEK293) cells transfected with the VR1 receptor [49]. The unique nature of the ion channel is indicated by the observation that the influx of cations is insensitive to conventional calcium and sodium channel blocking agents (such as ω-conotoxin and tetrodotoxin) [337], whilst being sensitive to the polyvalent cationic dye Ruthenium Red. As with the physiological response (of a burning sensation), membrane depolarisation desensitises with continuous vanilloid exposure.

## 5.i.d. <u>VR1 receptor agonists:</u>

Resiniferatoxin (see figure 37) is an ultra-potent, naturally occurring capsaicin analogue, derived from the cactus-like plant *Euphorbia resinifera*. As with other compounds demonstrating agonist activity at the VR1 receptor, the homovanillyl subgroup in the ligand appears to be crucial, although more forgiving of alteration than is the case with capsaicin [314]. Activation of nociceptors occurs in an identical fashion to that associated with capsaicin: namely by instigating the entry of sodium and calcium ions into cells [337]. However, the kinetics of the response are somewhat different, in that whilst opening of the cation channel after binding of resiniferatoxin appears to proceed at a slow rate, once open the channel

continues to conduct for a prolonged period of time. In contrast, capsaicin itself evokes rapid, but transient currents [193,314].

More recently, non-pungent capsaicin-like compounds have been developed, the best characterised of which is long acyl chain analogue olvanil (see figure 37). Whilst this compound is equipotent with capsaicin in terms of calcium influx into dorsal root ganglion cells [139], it appears to exhibit less potency for nociceptor activation and release of neuropeptides from spinal cord. It has therefore been suggested that olvanil binds to a subtype of vanilloid receptor, demonstrating preferential activity for slowly activatable channels [185].

## 5.i.e. VR1 receptor antagonists:

In addition to the description of agonists, a specific competitive antagonist at the VR1 receptor has been developed [337]. Capsezapine inhibits the inward flow of cations induced by capsaicin, in both voltage-clamped neurones and dorsal root ganglia cultures. Furthermore, *in vitro* tail-spinal cord preparations demonstrate inhibition of capsaicin-induced depolarisations of ventral root, whilst the antinociceptive effects of capsaicin in whole animal models are reversed by the administration of capsazepine [149].

## 5.i.f. SDZ249-665 [personal communication]:

SDZ249-665 is a systemically active capsaicin analogue recently developed by Novartis (see figure 37). It can be dissolved in DMSO and then further diluted in 0.9% saline. Under *in vitro* conditions SDZ249-665 displaces resiniferatoxin from binding sites on dorsal root ganglia membranes with an $IC_{50}$ 0.9 +/- 0.22μM. Like capsaicin, it acts as a full agonist at vanilloid receptors, blocking calcium uptake into cultured dorsal root ganglia cells ($EC_{50}$ 0.92 +/- 0.04μM) - an effect blocked by the addition

of the VR1 receptor antagonist capsazepine. *In vivo*, attenuation of pain related behaviour in a variety of animal models (including the tail flick mouse model of hyper-acute pain and the intra-dermal administration of turpentine to rat paw) is observed.

Evidence from both *in vitro* and *in vivo* models suggests that SDZ249-665 is non-stimulatory in nature. In the isolated vagus nerve, SDZ249-665 demonstrates a decreased potency (in comparison to capsaicin) in causing depolarisation. Similarly, it shows less potency in activating ventral root reflexes in the rat spinal cord-tail preparation. Moreover, a decreased potency is observed in a variety of *in vivo* models, including: induction of bronchoconstriction and stereotypic nose wiping in guinea pigs and elevation of pulse rate and blood pressure in rats. Thus it would appear that, unlike capsaicin, there is a therapeutic window between doses required for analgesia and unwanted side effects of 10 to 30 fold.

### 5.i.g Experimental rationale:

The sensory innervation of the urinary bladder is predominantly provided by small unmyelinated polymodal C fibres arising from the lumbo-sacral spinal cord [183]. When capsaicin is applied topically to the bladder, the immediate effect is stimulatory, provoking the onset of urinary bladder contractions and thence micturition [193,195]. This presumably occurs via stimulation of the VR1 receptor, located on primary afferent neurones and demonstrated to be present in the urinary bladder of the rat [315].

Additionally, intra-vesical administration of capsaicin has been shown to be associated with induction of the expression of the immediate early gene *c-fos* in the superficial dorsal horn of the rat at appropriate segmental levels [67]. The prophylactic (or neonatal) administration of capsaicin prevents the onset of similar hyper-reflexic responses associated with the administration of alternate irritant agents such as acetic acid and cyclophosphamide

[79,192]. Furthermore, case reports in humans of the administration of intra-vesical capsaicin suggest that a similar inhibition of hyper-reflexia occurs [109,252].

Chronic diseases of the urinary bladder in humans are associated with a variety of clinical symptoms including pain, the relief of which is problematic [221]. The traditional therapy for the relief of associated symptoms such as urgency and frequency of micturition consists of parasympathetic blockade with anti-cholinergic agents. Unfortunately, such treatment is associated with a variety of distressing side effects, including: dryness of the mouth, flushing, visual disturbances and constipation [252]. Therefore, there is a pressing need for the development of efficacious new drugs devoid of these problems.

Topical administration of capsaicin appears to be associated with a degree of symptomatic relief, but may require the administration of sedation for the procedure, necessitating hospital admission and careful monitoring [20,68,119,172,191]. Therefore, the development of a systemically active capsaicin analogue provides an exciting opportunity for a more clinically acceptable alternative. Thus the experiments presented here assess the influence of one such novel analogue, SDZ249-665, on the hyper-reflexic and hyperalgesic responses observed in the turpentine inflamed rat urinary bladder.

## 5.ii. Methods

All experiments conformed with British Home Office Regulations. Animals were housed according to national guidelines in colony cages of six animals per cage with a dark : light cycle of 12 hours : 12 hours. They were provided with free access to food and water.

## 5.ii.a. Urinary bladder reflex experiments:

Surgical preparation [147,148,215,218]:

Animals were prepared as for the bladder reflex experiments in which the bradykinin receptor antagonists des-Arg$^9$,[Leu$^8$]-BK and HOE140 were assessed (see 4.ii.a.). After anaesthesia the trachea, urethra and carotid artery were cannulated.

Measurement of bladder motility [147,148,215,218]:

As in the previous experiments (see chapter 4), the outcome measure used was the cystometrogram (the pressure-volume relationship for the urinary bladder). Again, cystometrograms were performed, by the infusion of warm, sterile, 0.9% saline at a constant rate (0.05ml/min, to a maximum of 0.7ml) into the bladder. The intra-vesical pressure was monitored continually via a pressure transducer and the signal was amplified and displayed on a PC. Data were analysed and stored using AcqKnowledge software (version 3.02).

Outcome measures:

The same two measures as were used in the bradykinin receptor antagonist experiments were extracted from the cystometrogram (see figure 25 on page 123):

i) The micturition threshold, $V_{mic}$.

ii) The cumulative duration of micturition, $Dur_{mic}$.

Inflammation of the bladder [215,218]:

As with the previous experiments the bladder was inflamed by the intra-vesical instillation of 0.5ml of 50% turpentine in olive oil for one hour.

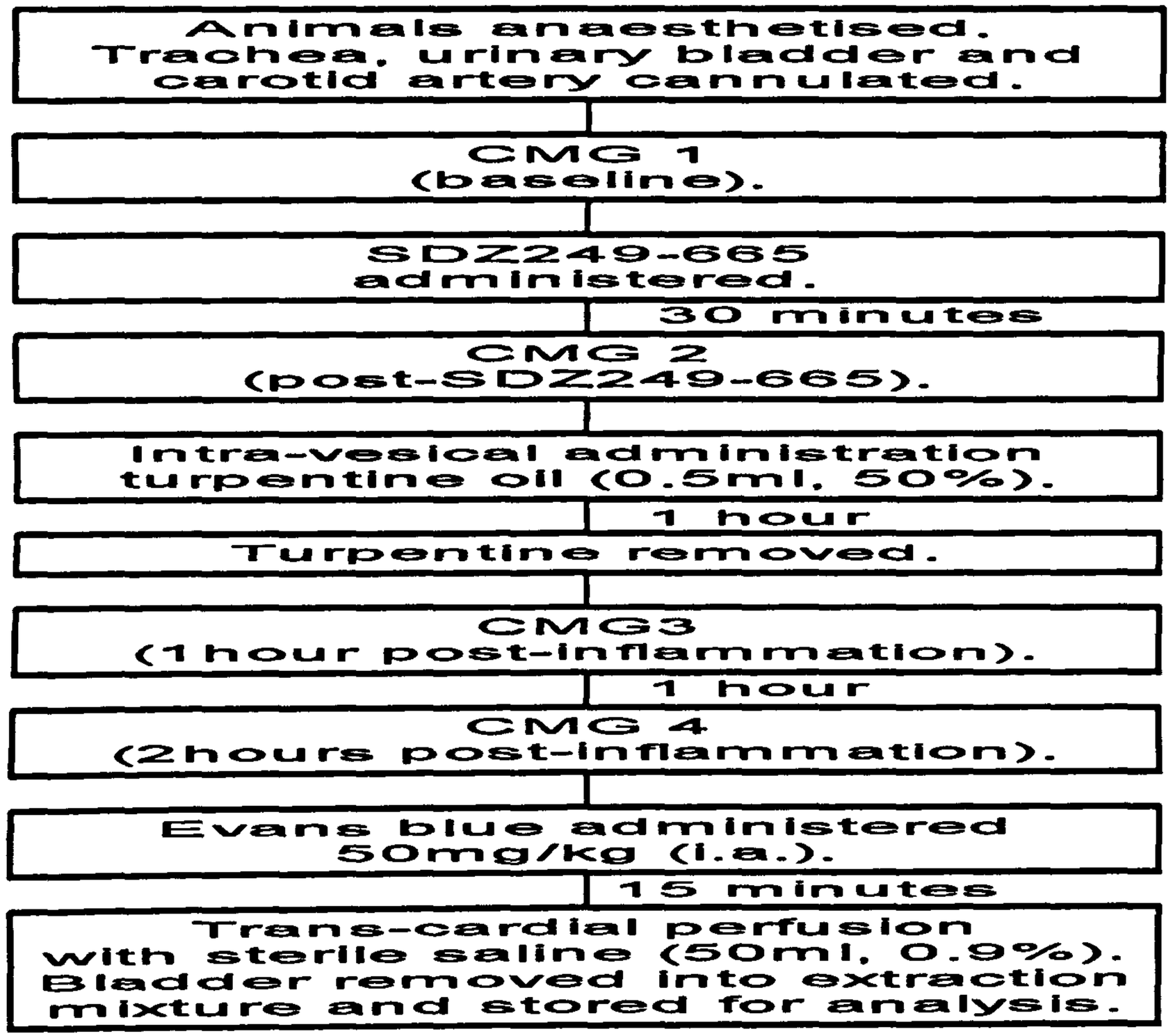



Figure 38.
Protocol for SDZ249-665 administration in the viscero-visceral hyper-reflexia (VVH) experiments.

Data presentation:

Statistical comparisons were performed using analysis of variance with *post hoc* Dunnett's testing. All utilised the Sigmastat package, version 2.0 from Jandel Scientific Software and significance levels of $p < 0.05$ were accepted. Graphs were produced using the Sigmaplot program, version 3.0 (Jandel Scientific Software).

Effects of drug administration (see figure 38 on page 168):

Baseline cystometrograms were performed, after which SDZ249-665 (or a control solution) was administered intra-arterially. A second cystometrogram was performed half an hour later to assess the effects of SDZ249-665 on bladder reflexes in the absence of inflammation. Immediately after this the bladder was inflamed as described above. Two further cystometrograms were performed, the first immediately after removal of the turpentine from the bladder and a final recording one hour thereafter.

Five test groups (each n = 6) were assessed:

I Solvent control (DMSO : saline, 1 : 19, 0.7ml, i.a.).

II SDZ249-665 0.05mg/kg (i.a.).

III SDZ249-665 0.1mg/kg (i.a.).

IV SDZ249-665 0.5mg/kg (i.a.).

V SDZ249-665 1mg/kg (i.a.).

Statistical comparisons were made using the raw data to demonstrate:

a) The effect of SDZ249-665 on the uninflamed bladder

- the data recorded at thirty minutes post-drug administration and at baseline were compared (ANOVA [Dunnett's]).

b) Any effect of SDZ249-665 in preventing the inflammation-associated viscero-visceral hyper-reflexia.

- the data recorded at one and two hours post-induction of inflammation were compared with baseline data (ANOVA [Dunnett's]).

Values were expressed as a percentage of baseline values for each group.

## 5.ii.b. Plasma Extravasation experiments:

Outcome measure [218]:

As in the bradykinin receptor antagonist experiments, the possibility that SDZ249-665 administration might decrease the inflammation associated plasma extravasation into bladder tissue, was assessed. Again, after the final cystometrogram had been performed, the animal was slowly perfused with Evan's Blue Dye (50mg/kg) and fifteen minutes later terminally perfused with 0.9% saline, to ensure clearance of the dye from the vascular compartment. The bladders were excised, weighed and stored in an extraction mixture of 70/30 acetone + 0.5gm/L sodium sulphide for a minimum of five days. The quantity of Evan's Blue Dye extravasated was measured by spectrophotometry at $\lambda = 620nm$ (using the Unicam 5625 UV/VIS spectrophotometer, ATI Unicam, Cambridge, UK) and the tissue concentration in mg / mg of vesical tissue was calculated using the equation:

$$\frac{(Measured \quad [EB]) \times 4000}{Bladder \quad weight}$$

Tissue concentrations of Evan's Blue Dye were compared between control and treated groups by means of the unpaired t-test (Sigmastat package, version 2.0, Jandel Scientific Software). Compounds were deemed to be effective if the treated group was associated with a lower level of plasma extravasation ($p < 0.05$) than the control group.

## 5.ii.c. Referred viscero-somatic hyperalgesia experiments:

Surgical preparation:

As in the previous experiments (see section 3.ii.) animals were anaesthetised with Isoflurane in a carrier gas of nitrous oxide and oxygen in a ratio of 2 : 1. The urinary bladder was catheterised and vesical inflammation induced by the instillation of 0.5ml, 50% turpentine oil for one hour. Venous access was achieved via a tail vein.

Test protocols

The groups of animals utilised for thermal and mechanical test protocols are described below:

I. General anaesthesia alone, maintained for 105 minutes (the time required to allow for both drug administration and bladder inflammation in the intervention groups).
II. Intra-vesical turpentine instillation after pre-treatment with solvent control (DMSO : saline, 1 : 19, 0.7ml, i.v.).
III. Intra-vesical turpentine instillation after pre-treatment with 0.1mg/kg SDZ249-665 (i.v.) (in the mechanical hyperalgesia protocol only).
IV. Intra-vesical turpentine instillation after pre-treatment with 0.5mg/kg SDZ249-665 (i.v.).
V. Intra-vesical turpentine instillation after pre-treatment with 1mg/kg SDZ249-665 (i.v.).

All test compounds were administered forty five minutes prior to the induction of bladder inflammation. Observation of the right fore and hind limb withdrawal latencies from thermal and mechanical stimuli was made prior to intervention and at then again at two, four, six, eight and twenty four hours post-intervention. The testing regimens were exactly as those

described in chapter 2 (see figure 9 on page 66). Just as previously, animals were sacrificed by overdose of anaesthetic (i.p. phenobarbitone) and cervical dislocation at completion of experiments.

Data presentation:

As in previous experiments, the withdrawal thresholds of the fore limbs across time were compared to baseline (ANOVA), to assess whether any systematic change from baseline had occurred.

$\Delta_{Thresh}$ - the difference in fore and hind limb withdrawal thresholds (as a percentage of their baseline values) was calculated for each time point using the same equation as previously:

$$\Delta_{Thresh} = \left[\left(\frac{H_{Thresh}}{BH_{Thresh}}\right) \times 100\right] - \left[\left(\frac{F_{Thresh}}{BF_{Thresh}}\right) \times 100\right]$$

Curves were constructed, using the values obtained from each animal, of $\Delta_{Thresh}$ against time. The area under the curve (AUC) for each of these curves was calculated using the trapezium rule (Sigmaplot, version 3.0, Jandel Scientific Software). Comparison of the AUCs of each group was undertaken using ANOVA [Dunnett's] testing with the Sigmastat programme, version 2.0 (Jandel Scientific Software). A significance level of $p < 0.05$ was instituted.

## 5.iii. Results

### 5.iii.a. Urinary bladder reflex experiments:

Experiments were performed on thirty animals weighing 175 - 270gm (mean weight 217.2gm). The mean baseline value for the micturition threshold $V_{mic}$ was 0.37mls (+/- 0.03) and that for the cumulative duration of

micturition $Dur_{mic}$ was 186.6secs (+/- 15.06). These values are consistent with previous work in this model [218].

The effect of SDZ249-665 administration on the uninflamed bladder:

Administration of the solvent control (DMSO + saline) alone, prior to bladder inflammation, was associated with a trend toward a reduction in the micturition threshold, $V_{mic}$ and elevation in the cumulative duration of micturition, $Dur_{mic}$. However, this was not significantly different ($p > 0.05$) from baseline values (ANOVA [Dunnett's]) (see table 10 in appendix 2). This statistically insignificant reduction in $V_{mic}$ and elevation in $Dur_{mic}$ is not unusual, although it is more common to observe values in the range of 80% (for $V_{mic}$) and 150% (for $Dur_{mic}$) of baseline values. It is probably related to the process of trans-urethral cannulation and the irritant presence of the catheter itself (see section 2.iv.).

There also appears to be a dose related effect (although the changes fail to reach statistical significance) of SDZ249-665 on the cystometrogram when administered in the absence of bladder inflammation, with a trend toward increase of the micturition threshold and decrease in the cumulative duration of micturition (see table 10 in appendix 2). This might be taken to imply a primary effect of SDZ249-665 on the micturition reflex. However, whilst administration of SDZ249-665 did demonstrate this inhibitory trend, there was certainly no trend towards a stimulatory effect such as that observed after administration of capsaicin [195]. If such a stimulatory effect were to be present one would expect to observe a reduction in the micturition threshold ($V_{mic}$) and an increase in cumulative duration of micturition ($Dur_{mic}$).

The effect of solvent control administration:

Pre-inflammation administration of the solvent control (DMSO : saline, 1 : 19) did not prevent a VVH following subsequent intra-vesical turpentine instillation. A reduction in the micturition threshold $V_{mic}$ to 43.7% (+/- 6.3) of baseline at one hour post-inflammation and 51.6% (+/- 8.8) at two hours post-inflammation was observed. The cumulative duration of micturition, $Dur_{mic}$ at these time points was 413.0% (+/-138.1) and 458.3% (+/- 153.0) respectively.

The effect of SDZ249-665 administration:

In contrast to the administration of the solvent control, the prior administration of SDZ249-665 (at all doses ranging between 0.05 and 1mg/kg) prevented any significant reduction in $V_{mic}$ or elevation in $Dur_{mic}$ at both one and two hours after the induction of inflammation (ANOVA [Dunnett's], $p > 0.05$). An example of the cystometrogram traces is shown in figure 39. Figure 40 shows the grouped data for the effect on $V_{mic}$, whilst the analogous data for $Dur_{mic}$ is given in figure 41.

## 5.iii.b. Plasma Extravasation experiments:

Experiments were performed on twenty six animals with an average bladder weight of 93.1gm (+/- 3.5). Administration of the DMSO + saline (1 : 19) solvent control (0.7ml, i.a.) was associated with an Evan's Blue Dye concentration in bladder tissue of 1.1 (+/- 0.21) mg / mg of vesical tissue. Administration of SDZ249-665 was not associated with any significant difference ($p > 0.05$) from control values, although as shown in table 11 (in appendix 2) there was a trend towards a reduction in plasma extravasation.

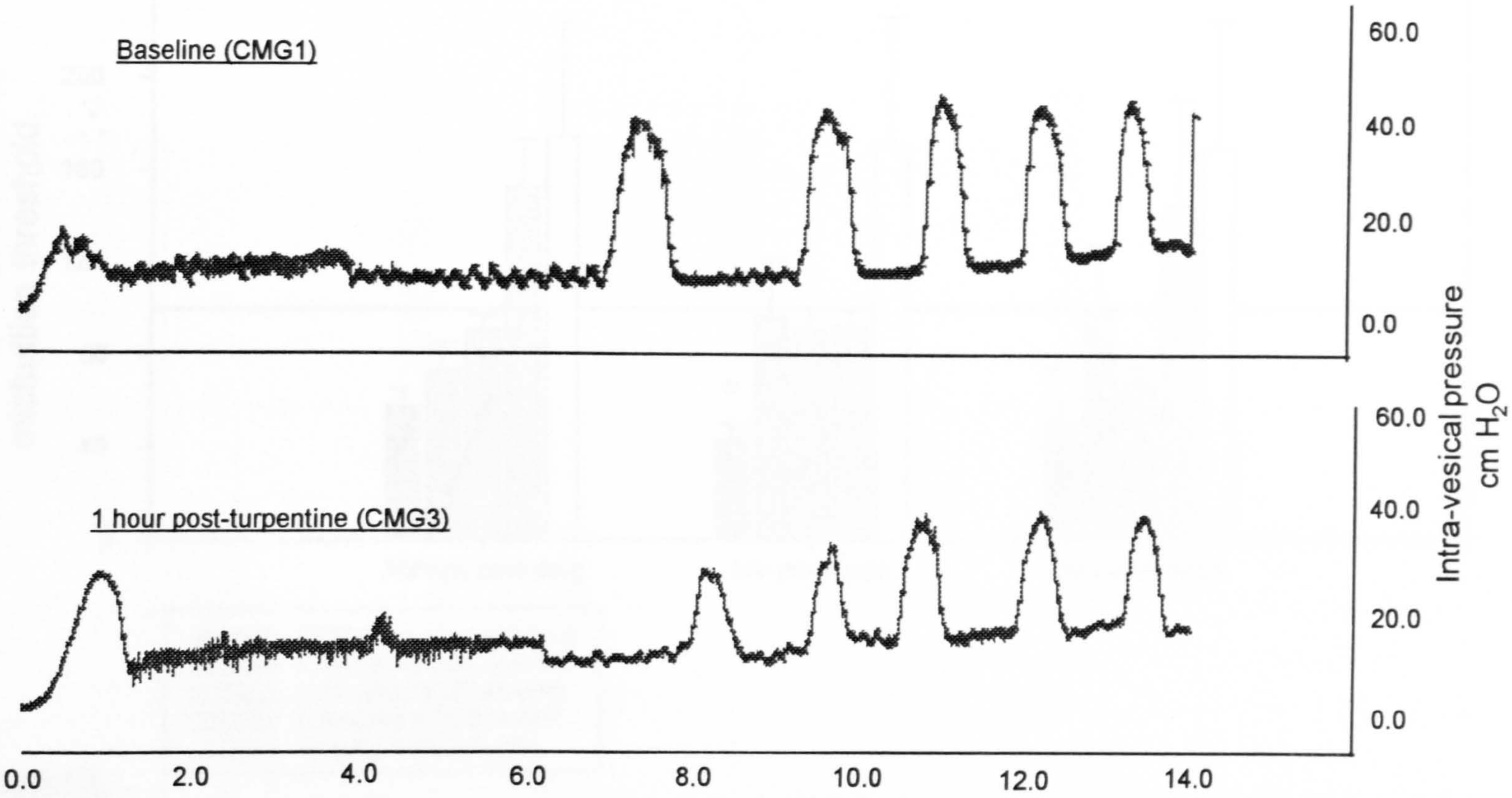



Figure 39.

Serial cystometrograms (CMGs) recorded from the same animal, treated with 0.5mg/kg SDZ249-665. Note that there is essentially no change in the micturition threshold ($V_{mic}$) or cumulative duration of micturition ($Dur_{mic}$) following instillation of intra-vesical turpentine.

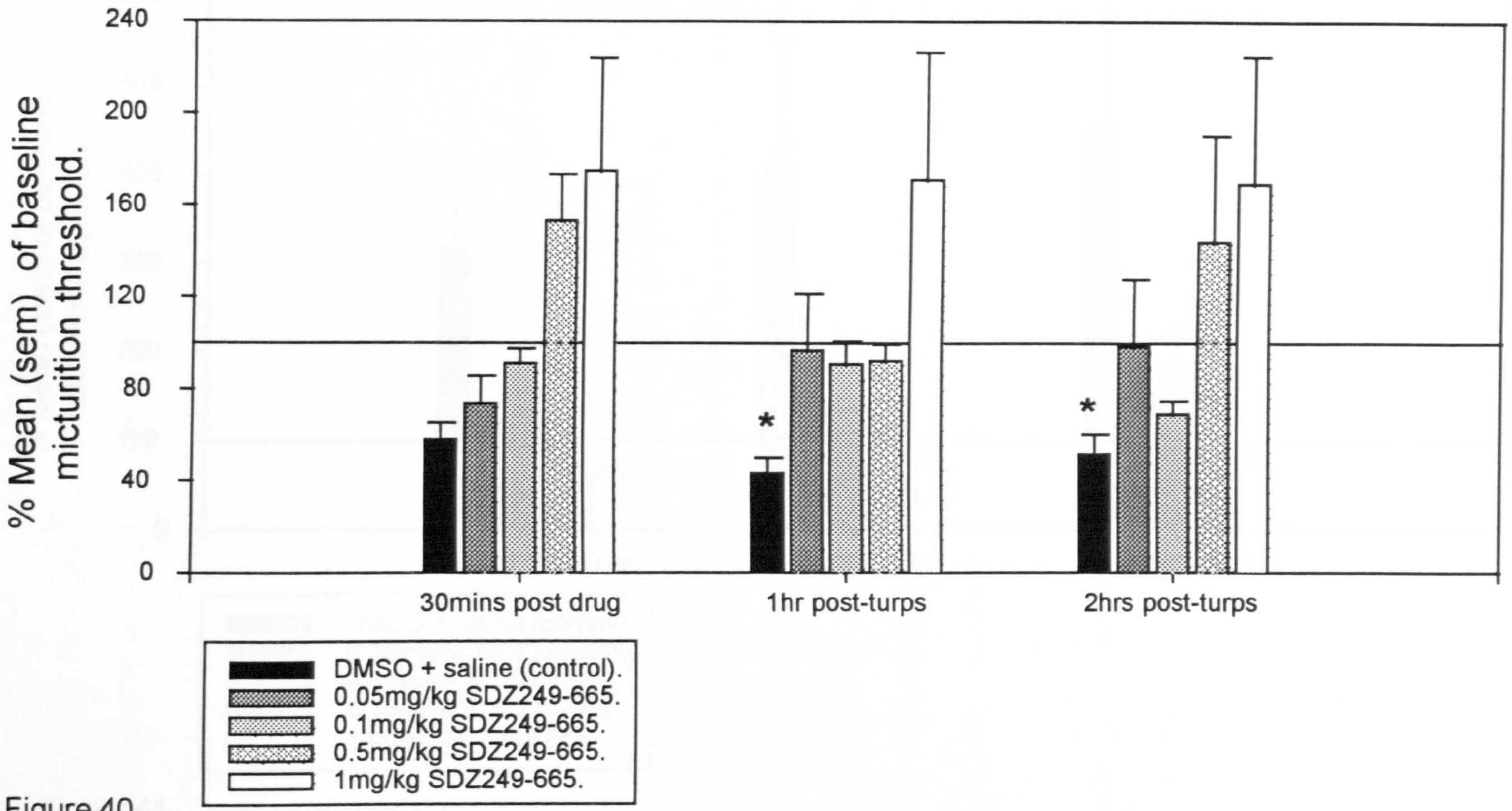



Figure 40.

Diagram demonstrating the effect of the capsaicin analog SDZ249-665 on the micturition threshold $V_{mic}$. Note that there is a significant reduction in $V_{mic}$ at both 1 and 2hrs after turpentine administration, unless prophylactic treatment with SDZ249-665 has been instituted. In addition SDZ249-665 administration is associated with a trend towards an increase in $V_{mic}$ in the absence of inflammation.

* $p < 0.05$ in comparison to baseline (ANOVA [Tukey]).

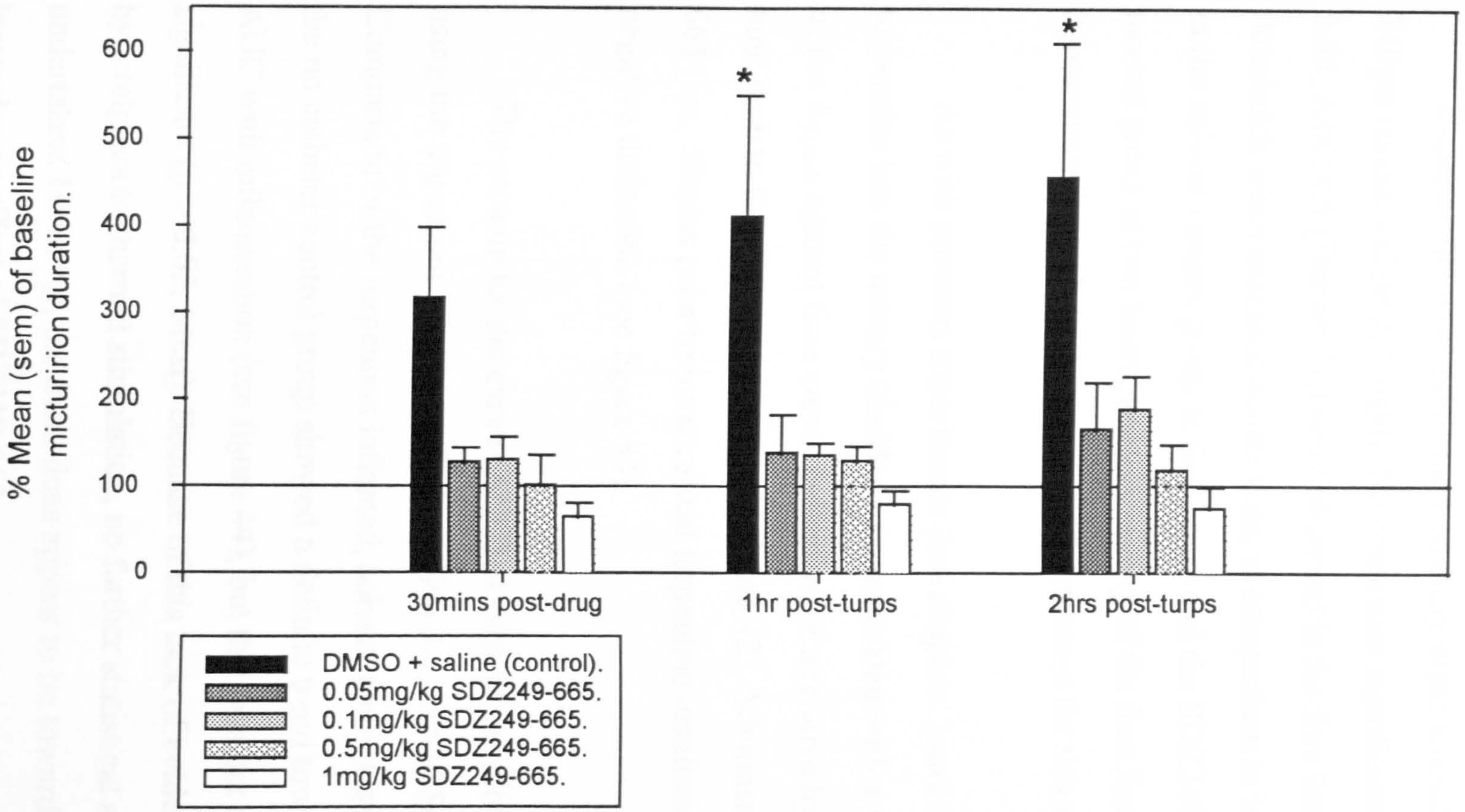



Figure 41.

Bar graph demonstrating the effects of SDZ249-665 on the cumulative duration of micturition following turpentine inflammation of the rat urinary bladder. Note that in treated animals the micturition duration is not significantly different to baseline, whereas in control animals the micturition duration is significantly increased.

* $p < 0.05$ in comparison to baseline (ANOVA [Tukey]).

## 5.iii.c. Referred viscero-somatic hyperalgesia experiments:

Effect of SDZ249-665 administration on thermal hyperalgesia:

Experiments were performed on thirty eight animals weighing 175 - 240gm (mean weight 221.6gm). No consistent significant differences ($p > 0.05$, ANOVA [Dunnett's]) were observed in the fore limb withdrawal thresholds when assessed across time, in comparison to baseline. However, in the solvent control group at four hours and the SDZ249-665, 0.5mg/kg treated group at two hours, sensory testing of the fore limb appeared to demonstrate hypoalgesic responses. The reasons for this are unclear.

As with previous experiments (see chapters 2 and 3) the instillation of turpentine into the urinary bladder was associated with a negative deflection of the $\Delta_{Thresh}$ against time curve, indicative of a relative hyperalgesia of the hind limb to thermal stimulation (see figure 42). Administration of SDZ249-665 (i.v., 30mins prior to intra-vesical turpentine instillation) reduced this negative deflection (see figure 43).

The area under the curve (AUC) of each of the plots was calculated using the trapezium rule (SigmaPlot version 3.0, Jandel Scientific Software). Comparison of the turpentine inflamed, solvent control treated group with the no catheter control group showed a definite trend towards an increase in AUC with inflammation (see figure 44), but this was not statistically significant ($p > 0.05$, t-test). Because of this lack of evidence of a significant hyperalgesia to thermal stimulation, no further statistical analysis was undertaken. However, the trend does appear to be towards an anti-hyperalgesic effect of SDZ249-665.

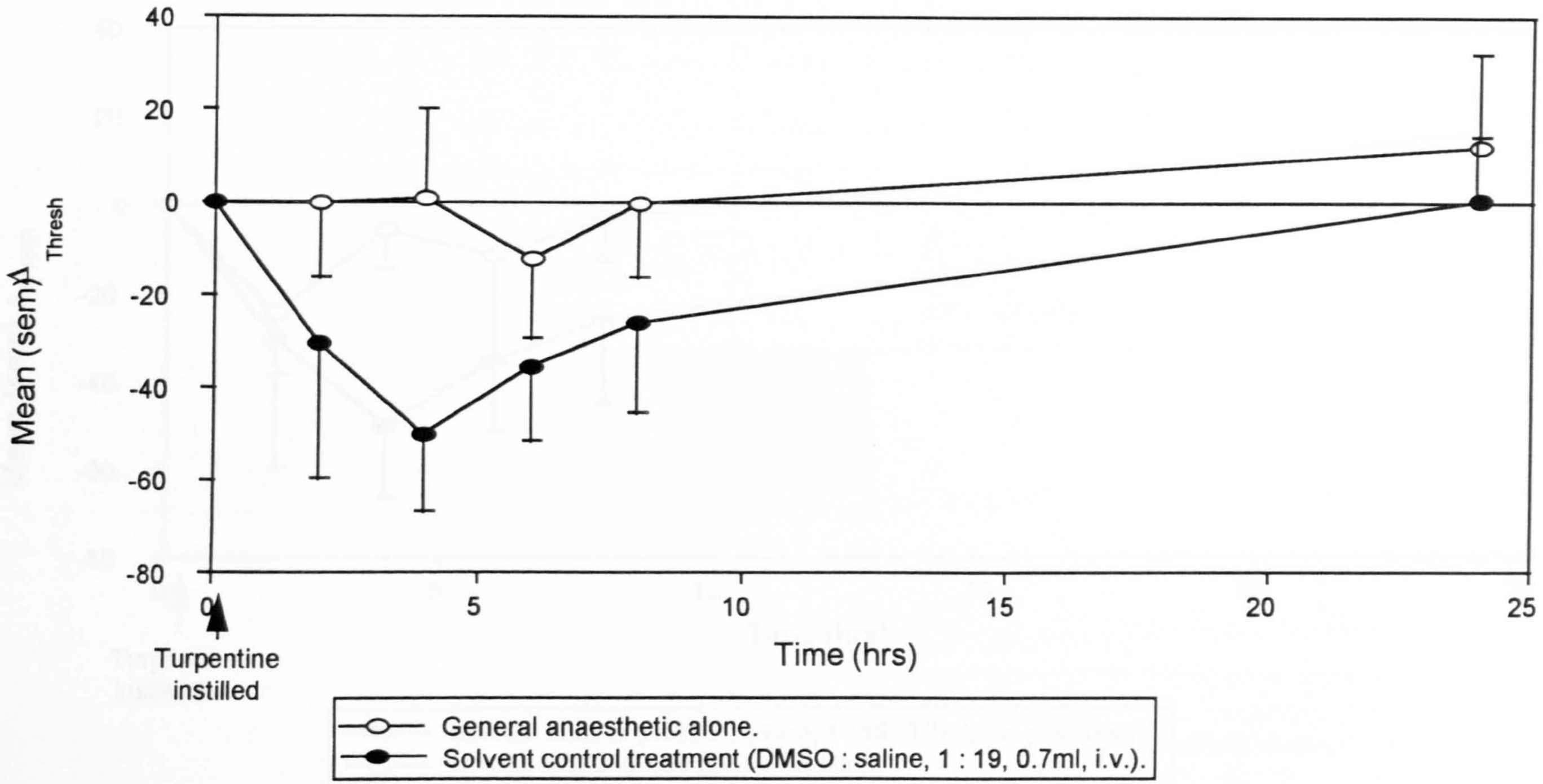



Figure 42.

Graph showing the difference in hind and fore limb withdrawal thresholds ( $\Delta_{Thresh}$) from a thermal stimulus, in animals treated with a solvent control solution after turpentine inflammation of the bladder. Note the negative deflection of the curve (in comparison to the general anaesthesia alone group) indicative of a relative hyperalgesia of the hind limb.

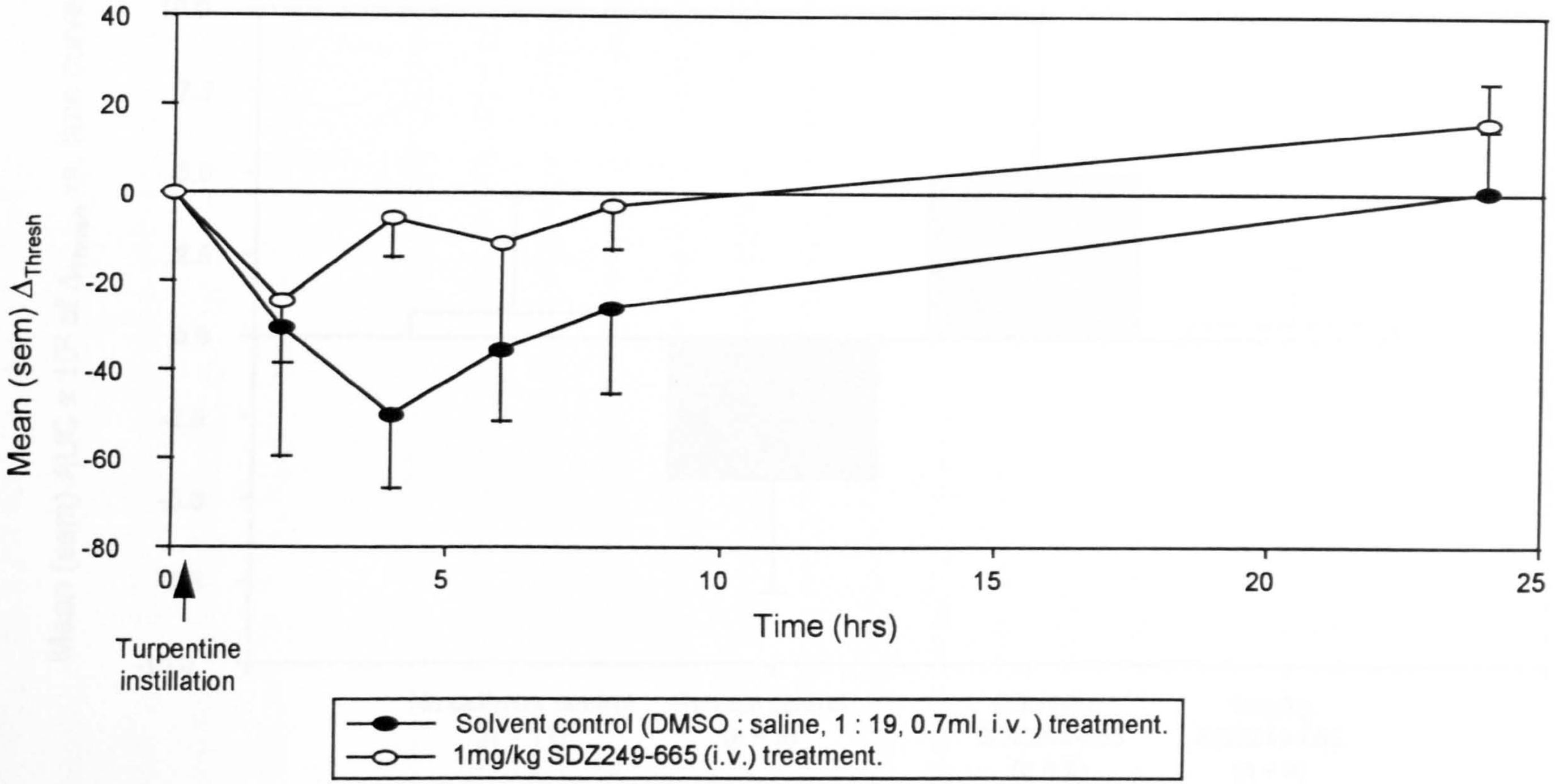



Figure 43.

Graph of difference in fore and hind limb withdrawal thresholds ($\Delta_{Thresh}$) against time (for thermal stimulation) following turpentine inflammation of the rat urinary bladder. Note the negative deflection of the graph for the group treated with the solvent control substance, indicating a relative hyperalgesia of the hind limb. Animals pre-treated with 1mg/kg SDZ249-665 (i.v.) did not demonstrate such a response.

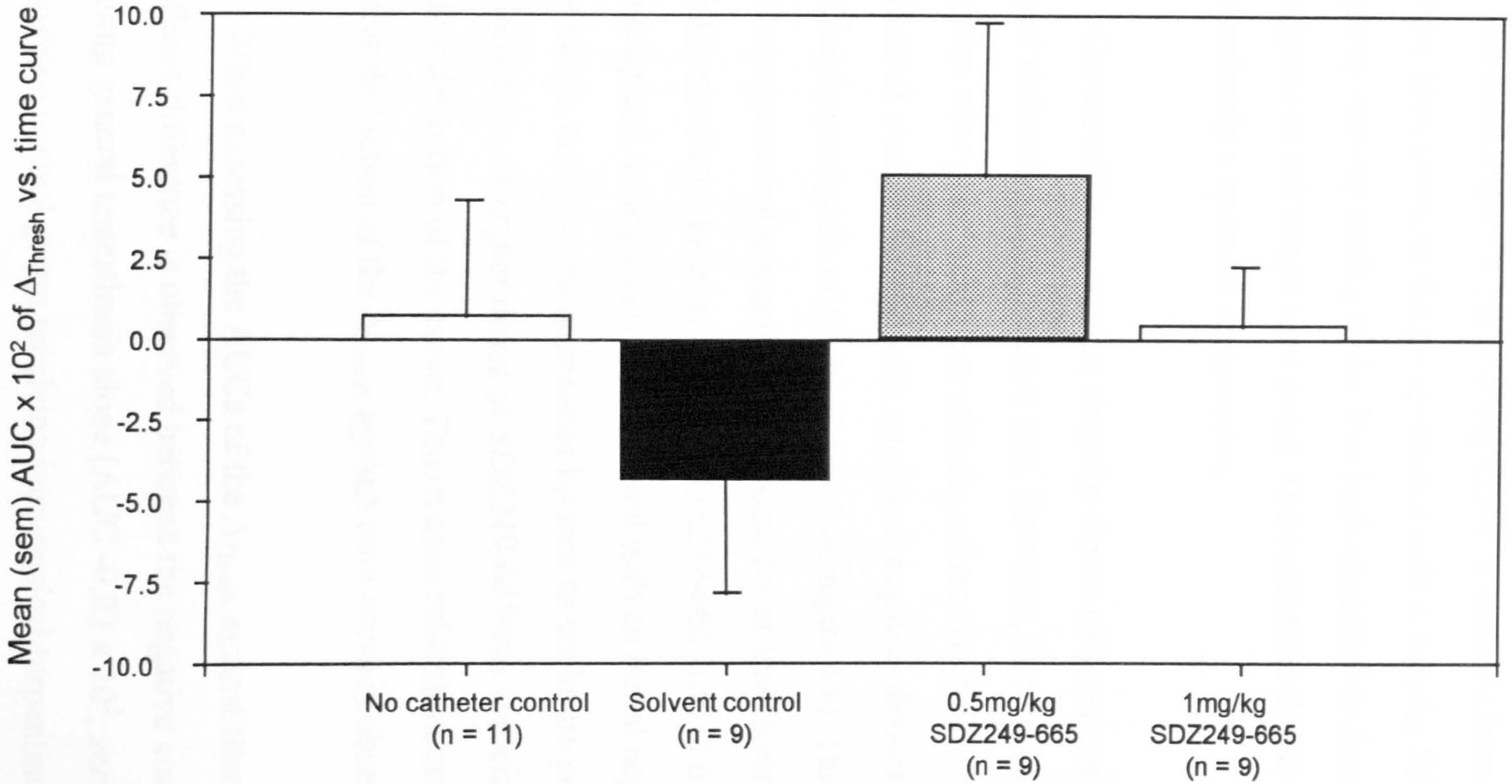



Figure 44.
Bar chart showing the area under the curve (AUC) for the $\Delta_{Thresh}$ against time plots for thermal stimulation, when animals were treated with the capsaicin analogue SDZ249-665 prior to turpentine inflammation of the urinary bladder.

Effect of SDZ249-665 administration on mechanical hyperalgesia:

Fifty five animals, mean weight 221gm (range 175 - 275gm) were used in these experiments. No significant difference was observed in the fore limb withdrawal thresholds in comparison to baseline latency (except at the two hour time point, in the group treated with 0.1mg/kg SDZ249-665). In this group sensory testing of the fore limb appeared to demonstrate hypoalgesia at this single time point. The reason for this is unclear, but it is most probably a spurious observation.

Curves of $\Delta_{Thresh}$ against time for the no catheter control group show minimal deflection from the zero line. However, instillation of intra-vesical turpentine after prior treatment with the solvent control (DMSO + saline, 1 : 19 dilution) was associated with a marked negative deflection, indicative of a relative hyperalgesia of the hind paw (see figure 45). This is maximal at eight hours post-inflammation and persists for at least twenty four hours. Pre-inflammation administration of. SDZ249-665 (i.v) at the lower doses (0.1mg/kg and 0.5mg/kg) was associated with an initial negative deflection of the curve, but this was attenuated by four to six hours post-inflammation. Administration of higher doses of SDZ249-665was associated with no such negative deflection of the curve. Thus a dose related reduction in the negative deflection of the $\Delta_{Thresh}$ against time curve is observed.

When assessing the AUCs of the $\Delta_{Thresh}$ against time curve, a significant difference is observed between the negative control group receiving general anaesthesia alone (AUC $-0.02 \times 10^3$, sem $0.6 \times 10^3$) and the positive control group receiving intra-vesical turpentine and solvent control substance (AUC $-5.2 \times 10^3$, sem $1.7 \times 10^3$). This indicates the presence of a referred hyperalgesia to mechanical stimulation following chemical inflammation of the urinary bladder.

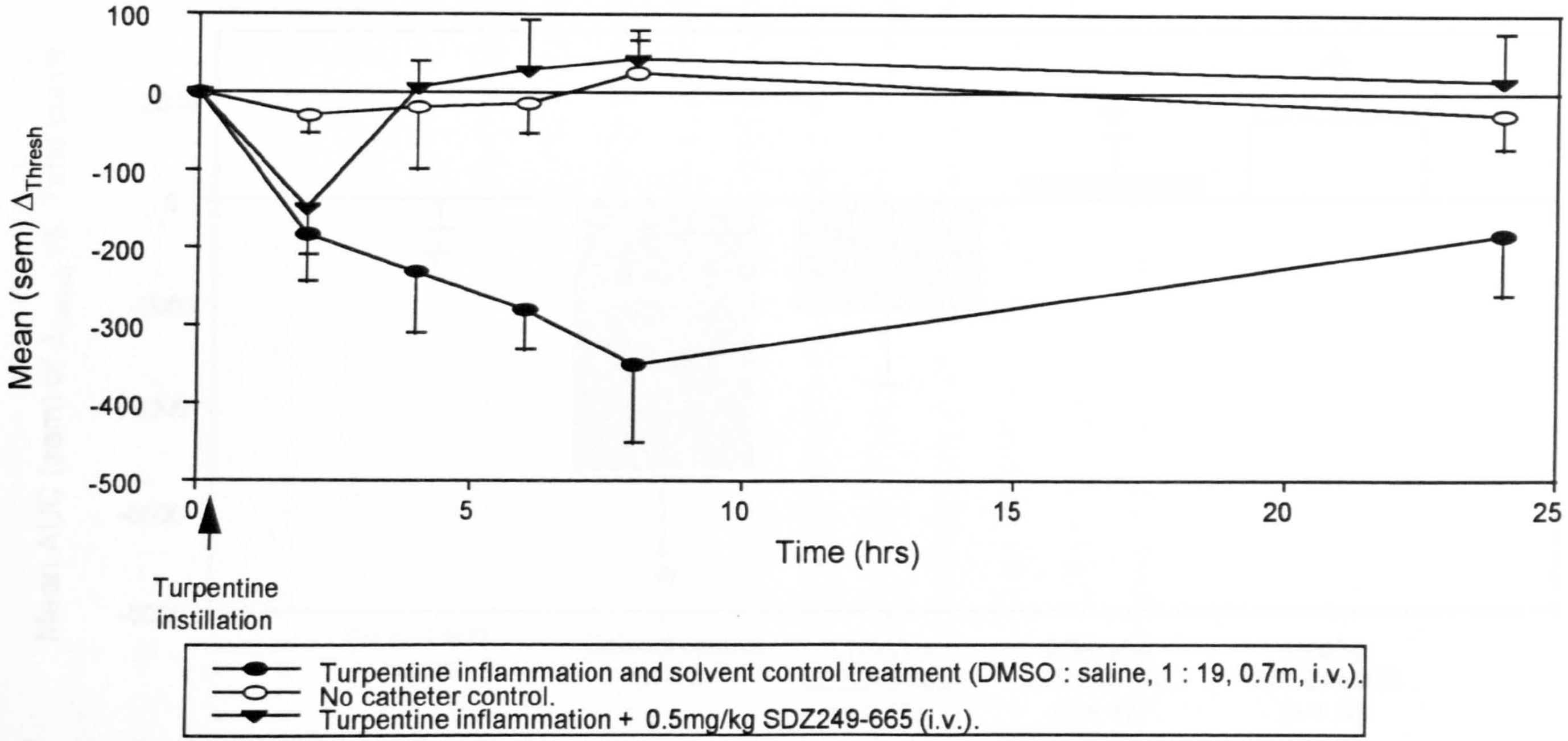



Figure 45.

Graph to demonstrate the difference in hind and fore limb withdrawal thresholds ( $\Delta_{Thresh}$) from a mechanical stimulus (delivered by von Frey hairs) after turpentine instillation into the urinary bladder. Note the negative deflection of the curve when the bladder is inflamed with intra-vesical turpentine. This is reversed, towards the curve for the no catheter control group, when pre-treatment with the capsaicin analogue SDZ249-665 is instituted. For clarity, only one dose level of SDZ249-665 is plotted.

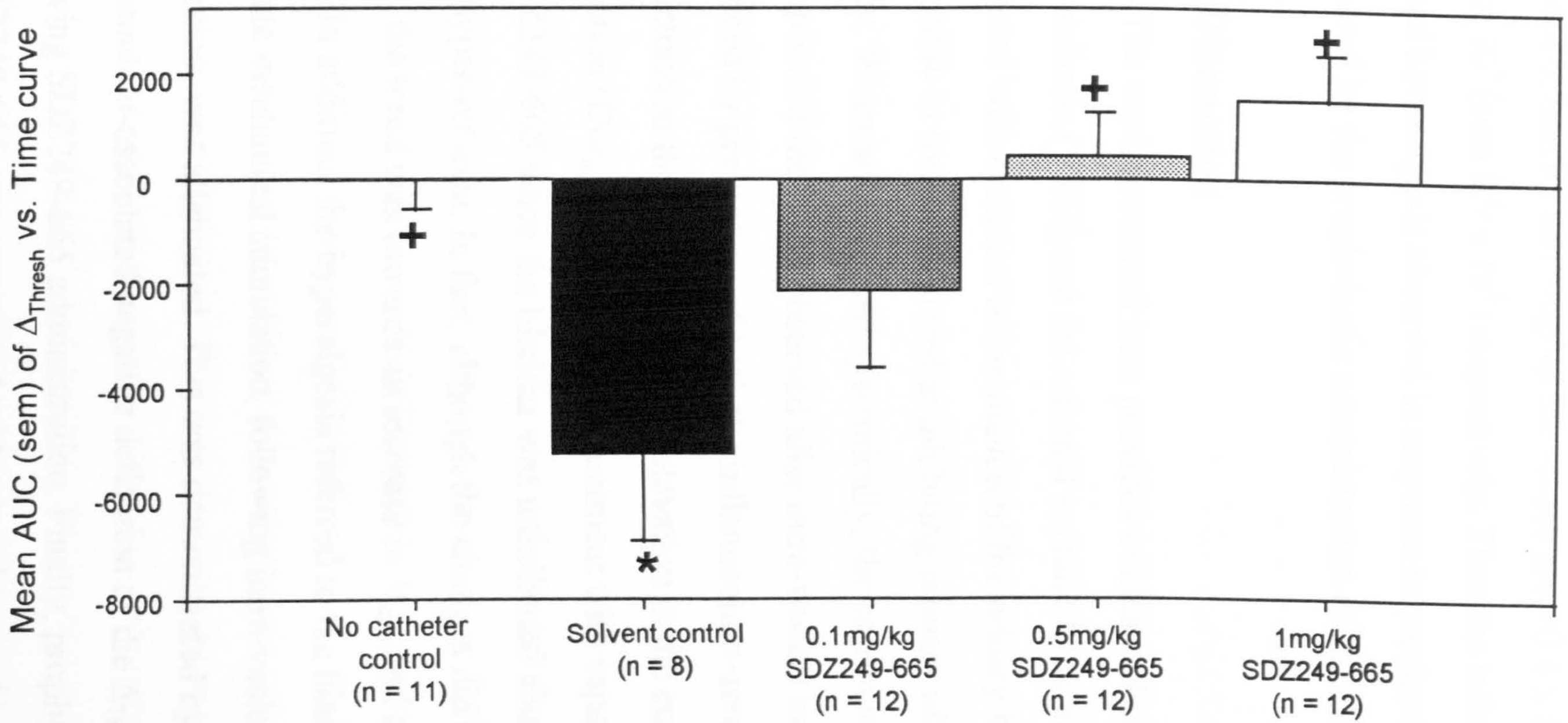



Figure 46.

Bar chart showing the area under the curve (AUC) of the $\Delta_{Thresh}$ against time curve (for stimulation by von Frey hairs) following turpentine-induced inflammation of the urinary bladder. Note the large negative AUC in the +ve control group, administered 0.7ml DMSO+saline (1:19 dilution) as a solvent control. This is attenuated, in a dose related manner, by the administration of the capsaicin analogue SDZ249-665.

* $p < 0.05$ in comparison to the no catheter control (ANOVA [Dunnett's]).

\+ $p < 0.05$ in comparison to the solvent control group (ANOVA [Dunnett's]).

There is a dose related attenuation of the inflammation-associated decline in AUC, for all SDZ249-665 treatment groups (see figure 46). At a dose of 0.1mg/kg the AUC is -2.1 x $10^3$ (sem 1.5 x $10^3$) whilst with higher doses of 0.5mg/kg and 1mg/kg the values are +0.4 x $10^3$ (sem 0.8 x $10^3$) and +1.5 x $10^3$ (sem 0.9 x $10^3$) respectively. Thus the inflammation associated referred hyperalgesia observed in response to mechanical stimulation is prevented by the prophylactic administration of SDZ249-665.

## 5.iv. Discussion

The work presented here provides evidence that both the visceral hyper-reflexia (VVH) and the referred somatic hyperalgesia (VSH), associated with chemical inflammation of the urinary bladder may be susceptible to treatment aimed at inhibiting primary afferent neurone activity. When administered systemically, the capsaicin analogue SDZ249-665 repressed the VVH observed after intra-vesical turpentine instillation, as evidenced by prevention of both the inflammation-associated reduction in the micturition threshold ($V_{mic}$) and elevation in the cumulative duration of micturition ($Dur_{mic}$). In contrast to treatment with capsaicin, administration of SDZ249-665 when the bladder was uninflamed showed no evidence of early hyper-reflexia. In fact, although the changes did not reach significance levels, the trend was towards an increase in $V_{mic}$ and decrease in $Dur_{mic}$.

In addition, the hyperalgesia referred to the hind paw, in response to punctate mechanical stimulation, following intra-vesical turpentine instillation, was attenuated. This was demonstrated by the attenuation of the inflammation-associated negative deflection of the $\Delta_{Thresh}$ against time curve following SDZ249-665 administration. Finally, prophylactic administration of SDZ249-665 was associated with a trend towards a decrease in the plasma extravasation into vesical tissue associated with inflammation.

Capsaicin exerts its effects via an interaction with the vanilloid receptor, VR1 [49]. This receptor is expressed almost exclusively on unmyelinated, polymodal, primary afferent sensory neurones, involved in the nociceptive pathway [313]. Electrophysiological and *in situ* hybridisation studies have demonstrated that 88% of small to medium sized, isolated, dorsal root ganglia cells are sensitive to capsaicin and that these neurones express high levels of the mRNA encoding VR1 [136]. Furthermore, VR1 has been immunolocalised to axons projecting to the rat urinary bladder [324]. Immunocytochemical analysis of the spinal cord has demonstrated the VR1 protein to be most heavily expressed in areas in which nociceptive afferent neurones make central synaptic connections [324]. Specifically, intense staining is observed in laminae I, II, V and X. In the lumbo-sacral spinal cord, these areas have been demonstrated (by both transganglionic transport of horseradish peroxidase [151,242] and staining for c-Fos-like immunoreactivity [89]) to be of particular relevance to the modulation of afferent information arising from the bladder.

Activation of VR1 occurs following administration of ligands including capsaicin [136]. Capsaicin administration is associated with a rapid influx of sodium ions via tetrodotoxin sensitive channels [316] and the resultant action potential is perceived as pain [314]. However, impairment of neuronal activity and functional desensitisation appears to be related to accumulation of cytoplasmic calcium [33]. When capsaicin is applied to dorsal root ganglia neurones a rise in cytoplasmic calcium is observed only in the presence of extra-cellular calcium. This despite the presence of intra-cellular stores capable of releasing sufficient calcium to provide adequate intra-cellular levels [53]. Furthermore, this finding is replicated in VR1 transfected cells of both mammalian and non-mammalian origin. When capsaicin is applied to *Xenopus* oocyte or human embryonic kidney-derived, HEK293 cells, transfected with VR1, desensitisation does not occur in the

absence of extra-cellular calcium ions [49,324]. Thus, both receptor occupancy and elevation in cytoplasmic calcium are required for desensitisation to occur.

In addition to responding to chemical stimuli, activation of the VR1 receptor appears to be associated with the application of physical stimuli such as heat [49,324]. When VR1-transfected HEK293 cells are subjected to sudden increases in temperature, several measures of activation, including the inward evoked current [324] and calcium uptake [49] are observed. Furthermore these effects are blocked by antagonists, including capsazepine and ruthenium red.

Much interest has been displayed in the search for potential endogenous ligands of the VR1 receptor. Speculation as to the role of hydrogen ions followed observations that protons stimulate a similar proportion of visceral afferents as does capsaicin. Furthermore, proton-evoked leakage of plasma proteins into skin is decreased if animals are pre-treated with capsaicin [30]. *In vitro* application of protons to slices of urinary bladder is associated with release of neuropeptides (including calcitonin gene related peptide and substance P) from capsaicin-sensitive neurones. As with capsaicin application, these are only detected in the perfusate if calcium is present in the culture medium [30]. Surprisingly, whilst potentiation of both capsaicin [49] and heat [324] evoked responses have been documented, at room temperature, protons alone are unable to activate the VR1 receptor and generate an inward current [49]. However, activation of the VR1 receptor demonstrates variability, with inward current differing as a function of temperature, across both physiological and noxious ranges. Consequently, at normal body temperature ($37^0$C) protons alone are able to elicit activity [324]. Thus, under physiological conditions it would appear that the VR1 receptor is capable of integrating responses to physical and chemical stimuli. These effects are likely to be of particular relevance

under inflammatory conditions, when tissue pH is reduced and temperature increased [273].

The VR1 protein has been demonstrated to co-localise with the plant lectin IB4, on glial derived neurotrophic factor-dependent neurones. However, an even higher degree (85% compared to approximately 60%) of co-localisation is observed with substance P, expressed by NGF-dependent neurones [324]. Furthermore, the presence of NGF is required to maintain the long term sensitivity of dorsal root ganglia neurones *in vitro* to both capsaicin and protons, as measured by either electrophysiological [32] or radioactive labelled calcium uptake [336] methods. Indeed, blockade of signal transduction by the trkA receptor (with the alkaloid K252a) inhibits the NGF-dependent upregulation of such sensitivity [41]. Furthermore, sequestration of endogenous NGF ablates the thermal hyperalgesia associated with intra-dermal capsaicin [220]. Since NGF itself is associated with thermal hyperalgesia [182], any documentation of an NGF- dependency of heat sensitivity of VR1 expressing neurones, would be intriguing, suggesting the possibility of a leftward shift of the normal dose-response curve for heat application.

Sensitivity to heat stimuli is a feature of approximately half the population of both substance P and IB4 positive neurones. Furthermore, VR1 is expressed on both sub-populations of small diameter neurones [324]. Addition of NGF to the culture medium for 24 hours increases the proportion of both IB4 and substance P-positive dorsal root ganglia neurones responding to heat [311]. Since a proportion of IB4 positive neurones also express the trkA receptor for NGF [18] it would be appealing to suggest that these cells correspond to the group which co-localise VR1 and IB4. Furthermore, since substance P-expressing neurones exhibit a lower threshold for heat responsiveness, in addition to larger heat-evoked

currents, the possibility that there is a higher level of VR1 expression per neurone in this group is attractive.

Reflex and behavioural models of both visceral and somatic pain have demonstrated the early excitatory effects associated with administration of capsaicin [8,97,193,195]. Intra-vesical application of capsaicin to a previously uninflamed bladder is associated with an initial stimulatory effect. This is evidenced by a reduction in micturition threshold and the onset of active micturition contractions [195], in addition to an increase in substance P-like immunoreactivity detectable in both vesical tissue and the spinal cord [193]. In patients with hyper-reflexia, the initial aggravation of symptoms may be associated with the perception of a burning pain or warmth [20,68,191].This early reduction in bladder capacity is followed by an increase in micturition threshold, the reason for which is unclear. Both desensitisation of the primary afferent neurones [68,95,195,337] and reduction in neurone density in the lamina propria of the urinary bladder [72] have been suggested as possible mechanisms.

Following intra-dermal injection of capsaicin, a similar perception of pain, of immediate onset and lasting approximately fifteen minutes is observed. This is associated with a zone of primary hyperalgesia to both thermal and mechanical stimuli [238]. A visible flare extends from this area and is surrounded by an even greater region of secondary hyperalgesia, exclusively to mechanical stimuli [8]. Thus, in both visceral and somatic domains, excitatory effects, explicable on the basis of peripheral sensitisation (primary hyperalgesia) have been described. Furthermore, in the somatic domain, secondary hyperalgesia, probably mediated by central sensitisation, occurs. Since the zone of secondary hyperalgesia exists only in response to mechanical stimulation [8] it would be interesting to investigate if a referred hyperalgesia, demonstrating similar separation of responses to

thermal and mechanical stimuli is observed following intra-vesical capsaicin. In the somatic domain this separation has been postulated to result from either the sensitisation of mechano-specific channels, or the loss of inhibitory influences provided by low threshold peripheral mechanoreceptors following noxious stimulation [8].

The early stimulatory effect of capsaicin, described above, has also proven to be problematic in clinical practice, since patient compliance with treatment is reduced [227]. Agents including resiniferatoxin and olvanil appear to stimulate different end points (such as excitation and desensitisation) with different potencies [313,314], suggesting that separation of these effects may be achievable. It has been suggested that this disconnection between excitatory and desensitisation responses may be entirely explained by pharmacokinetic effects, since the response to resiniferatoxin is delayed and if the compound is in contact with the tissue for insufficient time the response may be missed [193]. However, others claim that such separation is consequent upon mediation of responses by different subtypes of receptor [6].

Although it appears likely that resiniferatoxin and olvanil act via the VR1 receptor, definitive evidence of binding of these agents (or indeed SDZ249-665) to the cloned VR1 receptor is currently lacking. Furthermore, activation of the VR1 receptor following chemical inflammation of the bladder has yet to be described. However, agents acting at the VR1 receptor would appear to provide an exciting new avenue for research into visceral pain mechanisms.

**5.iv.a. Clinical Relevance:**

Hyper-reflexic disorders of the bladder present a problem for the clinical management of patients suffering with certain neurological disorders. Patients with upper motor neurone lesions following cerebro-vascular accidents, spinal cord injury and diseases such as multiple sclerosis, constitute a major clinical workload and may suffer considerable morbidity associated with detrusor hyper-reflexia and incontinence [252]. Agents capable of inducing the afferent desensitisation observed following capsaicin administration, whilst avoiding the early stimulatory effects, have the potential to provide an effective treatment for such disorders, whilst remaining devoid of uncomfortable side effects. Furthermore a systemic and bioavailable agent might be more tolerable to patients than agents requiring recurrent urethral catheterisation for their topical administration.

Although the results presented here were observed in an inflammatory model of hyper-reflexia, clinical experience with intra-vesical capsaicin instillation to the hyper-reflexic bladder has proved helpful irrespective of whether the primary cause is inflammatory or neurogenic [20,68,72,109,119,191]. Thus, having demonstrated that SDZ249-665 is effective under inflammatory conditions, it is possible that it would also be efficacious in treating hyper-reflexic responses secondary to neurogenic conditions. If this were the case, SDZ249-665-like agents, with a more acceptable side effect profile and more tolerable administration regimen than capsaicin might be developed. This is of particular significance in the context of an ageing population (data available from the Office for National Statistics on: http://www.statistics.gov.uk), since this group are particularly susceptible to vesical hyper-reflexia related to cerebro-vascular events and prostatism.

Pain arising from the urinary tract is a common phenomenon associated with chronic conditions including interstitial cystitis and the loin pain / haematuria syndrome. It is often perceived by the sufferer as arising from somatic sites, distant from the primary locus of injury [135], but sharing the same spinal dermatomes as the injured viscera [221]. Although historically it has proved difficult to treat effectively [44,138] there have been several case reports (although no blinded trials) of the instillation of intra-vesical or intra-ureteric capsaicin being associated with a reduction in painful symptoms [20,44,68,119,172,191]. Unfortunately, such treatments appear to provide only a relatively short lived response, requiring repeated applications several times *per annum*. The possibility that a systemically administered alternative might be achievable is alluring, since hospital admission might be avoided.

The data presented here provide evidence that (at least in an animal model of chemical inflammation of the urinary bladder) a referred punctate mechanical hyperalgesia may be attenuated by the means of a capsaicin analogue. This presumably exerts effects via interaction with the VR1 receptor, located on primary afferent nociceptive neurones [49]. The possibility that attenuation of referred hyperalgesia (and by analogy, perhaps pain itself) can be achieved by a systemically administered drug, acting at peripheral sites, is clinically intriguing. The possible development of new therapeutic strategies for painful conditions affecting the viscera, that have previously proved somewhat resistant to treatment is raised.

Similarly, painful neuropathic conditions affecting somatic tissues have proven somewhat resistant to conventional therapies [28]. In view of the intractable nature of such pains and the possible advantages of reducing neurotransmitter release from aberrantly active afferent neurones, there would appear to be some logic in clinical trials of topically applied capsaicin. As expected, pain is rapidly perceived when capsaicin is applied

topically to the skin [150,271]. Indeed, this has been the cause of failure of compliance with treatment. However, evidence from systematic reviews of the literature would suggest that there is a rationale for the clinical use of capsaicin in conditions including diabetic neuropathy and osteoarthritis [227]. Unfortunately, early pain is also a problem in the 'blinding to treatment' of both patients and staff. This may account for the paucity of quality trials investigating the use of capsaicin in post-herpetic neuralgia and post-mastectomy pain [227]. Therefore the development of analogues such as SDZ249-665, that demonstrate the desensitising effects of capsaicin, in the absence of early stimulatory effects, have the potential to both improve patient compliance (in conditions where capsaicin is of proven efficacy) and also ease clinical trial design for investigation of further indications for agents acting via the vanilloid receptor.

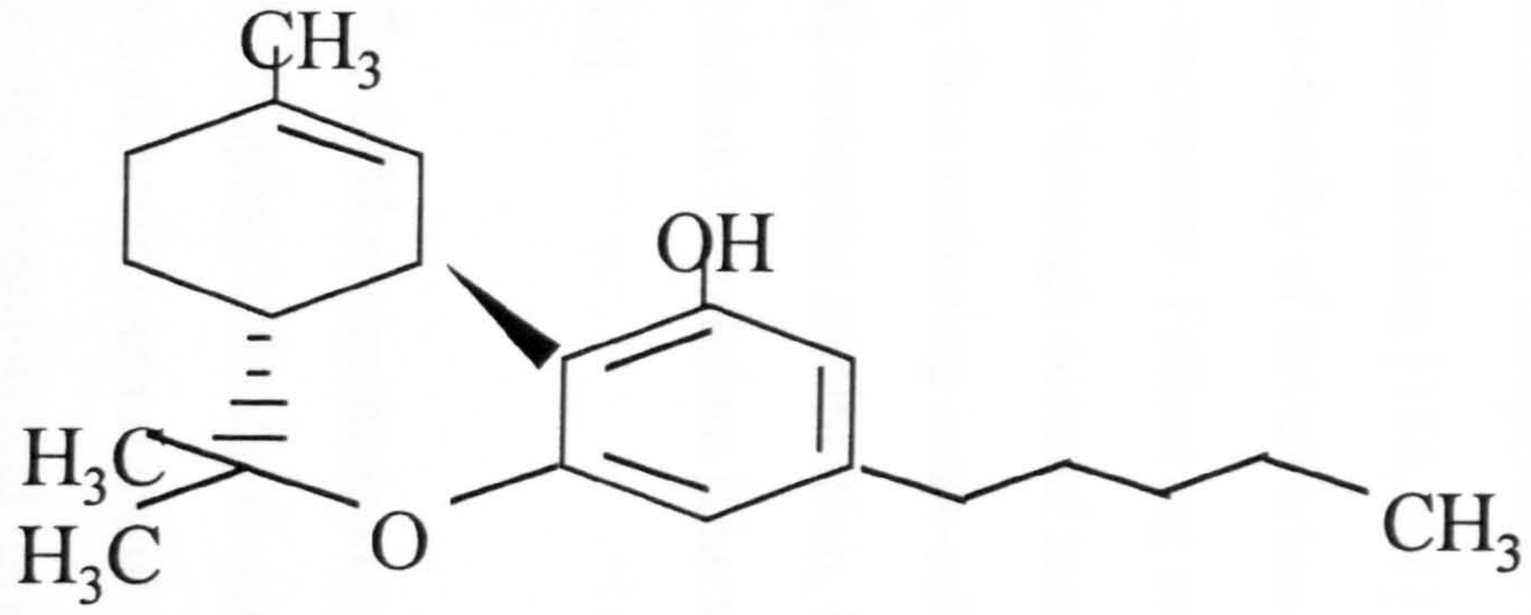
# Chapter 6:

# Cannabinoids and visceral pain.

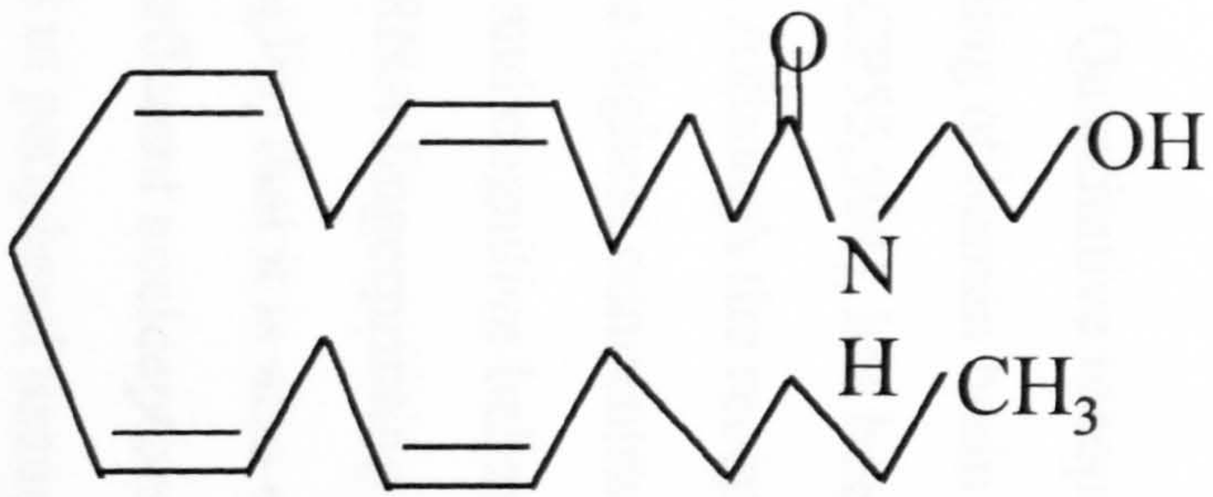
## 6.i. Introduction

Extracts derived from the *Cannabis Sativa* plant have been used both socially and medicinally for thousands of years [4,228]. The major pharmacologically active compound of the plant has been identified as $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), although at least sixty active compounds, termed cannabinoids, have been isolated from 'hemp' [40]. Until the 1920s, when the use of such compounds became illegal, the medicinal use of crude extracts of the plant (administered both orally and by inhalation) included the treatment of such wide ranging conditions as malaria, dysentery, insomnia, pain, convulsions and glaucoma [103]. As the abuse potential of the narcotic drugs, such as cocaine and morphine, became clear, the legal use of cannabis waned, although it was still possible to legally prescribe in it Great Britain until as late as 1971. However, with the discovery of specific cannabinoid receptors and endogenous ligands, interest in the drug has been reawakened. The potential for separating the therapeutic and non-therapeutic effects of agents acting via these receptors has been recognised. Once more the use of agents with $\Delta^9$-THC - like effects to provide symptomatic relief, including anti-nociception and anti-emesis, has been suggested [100].

**Δ⁹-THC**




**ANA**




**PEA**

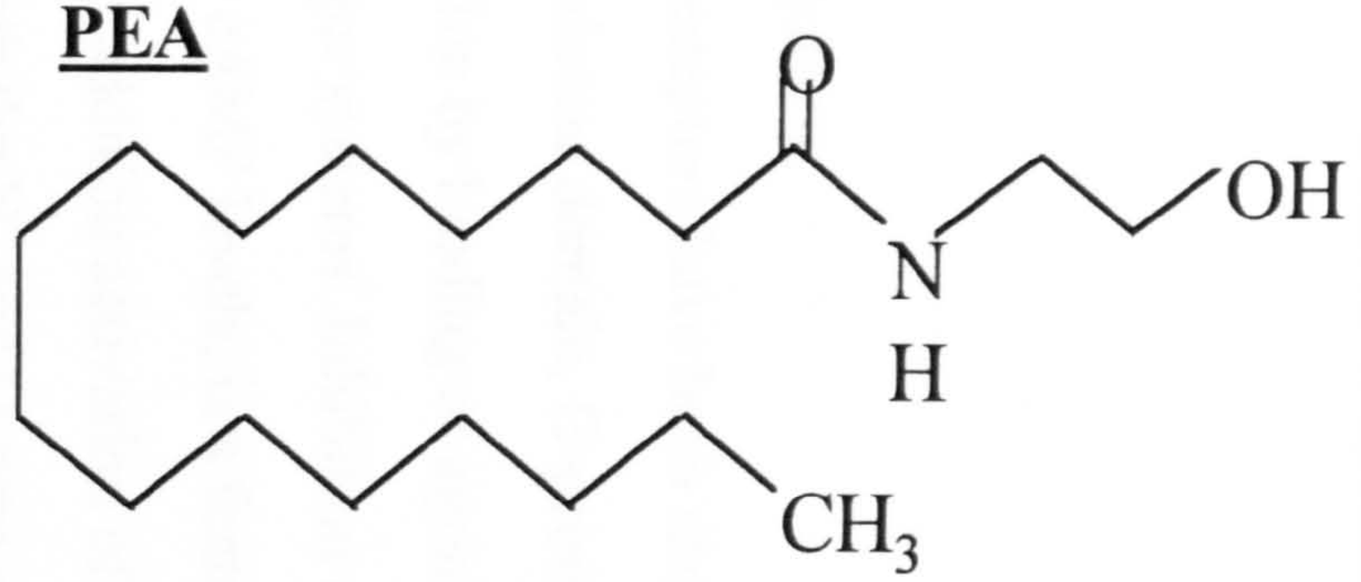



Figure 47.
Diagram to demonstrate the profound difference in structure between classical cannabimoids, such as $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and the endogenous cannabinoid ligands, anandamide (ANA) and palmitoylethanolamide (PEA).

## 6.i.a. Cannabinoid receptors:

Two cannabinoid receptors have been identified and cloned [115,156,208,244]. They fulfil criteria indicative of specific receptors [230,263], such as:

1. A structure-activity relationship.
2. Stereoselectivity.
3. Potency in an appropriate range.
4. The efficacy of competitive antagonists.

Since cloning [156,208] the receptors have been shown to belong to the super-family of seven trans-membrane domain, G-protein linked, cell surface receptors. Following activation by binding of agonist ligands, effects are exerted through second messenger systems. Inhibition of adenylate cyclase, thus reducing intra-cellular cAMP levels, is a feature of both CB1 and CB2 receptor stimulation, but in addition activation of the CB1 receptor inhibits influx of calcium ions via both the N- and Q-type channels [103,143].

The CB1 receptor is constitutive. Quantitative receptor autoradiography, utilising *in vitro* labelling of human brain sections with a tritiated synthetic cannabinoid agonist (CP55,594), has been used to demonstrate its anatomical localisation. Although the receptor is distributed throughout the brain and spinal cord, the highest concentrations are found in regions of the brain important to motor and cognitive behaviour [125]. More recently, evidence has emerged (using RNA fingerprinting techniques in dissociated lumbosacral dorsal root ganglia) that it is also expressed on the cell bodies of NGF-dependent primary afferent nociceptors [113]. Lower concentrations have also been observed in peripheral tissues, including autonomic neurones and the bladder [103,264,277].

The CB2 receptor exhibits only 44% overall homology with the CB1 receptor [103], although this rises to 68% in helical regions, where structure is highly conserved amongst the whole family of G-protein-linked receptors [294]. Most presently available evidence suggests that it is expressed solely in the periphery, with localisation to cells of immune origin (including lymphocytes, mast cells and macrophages) [101,115,264], the tonsils and the marginal zone of spleen [244]. However, there has been one report of the presence of CB2 receptors located on primary cultures of rat microglial cells, which form part of the reticuloendothelial system in the brain [157]. This predominance of expression of CB2 receptors on immunocytes makes it unsurprising that expression is induced under conditions of immune cell activation [5]. Consequently, it would appear that activation of this receptor is more involved in the downregulation of the inflammatory response, than in analgesia *per se* [10,101,211]. However, there is some evidence that compounds acting at this site may also inhibit the mechanical hyperalgesia associated with chronic constriction injury of peripheral nerves [210].

### 6.i.b. Endogenous cannabinoid ligands:

The identification of specific cannabinoid receptors suggests the likely presence of endogenous ligands for these receptors. The unsaturated N-acylethanolamide, anandamide (ANA) has been extracted from porcine, rat and human brain tissue [83,105] and shown to be produced by neurones of the central nervous system [86]. It fulfils criteria suggesting that it is an endogenous ligand [230] at cannabinoid receptors, in that:

- It is present at brain sites known to be associated with cannabamimetic activity.
- It exhibits a structure-activity relationship for such cannabamimetic activity.

- It has been observed to displace the CB1 receptor probe HU-210 from binding sites in brain tissue, in a competitive fashion, with $K_i$ of 39 +/- 5nM (as compared to 46 +/- 3nM for $\Delta^9$-THC) [83].

In animal models it has been shown to produce a wide range of central effects known to be associated with classical cannabinoid compounds [82,302,308]. These include: a reduction in spontaneous motor activity, hyper-responsiveness to sensory stimuli and hypothermia. Furthermore, in models of acute somatic pain, such as the tail flick [302,334] and hot plate tests [308], anti-nociceptive effects have been observed.

The structure of anandamide is quite unlike that of classical cannabinoids such as $\Delta^9$-THC (see figure 47 on page 195). Two possible mechanisms for its synthesis have been proposed [5]:

a) The condensation of arachadonic acid and ethanolamide (derived from N-arachidonyl-phosphatidylethanolamine) [272].

b) The phosphodiesterase mediated cleavage of N-arachidonyl-phosphatidylethanolamine derived from lipids of the neuronal cell membrane [86].

As with the endogenous opioids the duration of action of anandamide is limited [5,272]. In this case termination of activity appears to occur via rapid cellular re-uptake [24] accompanied by hydrolytic degradation, by an amidohydrolase [81], to release ethanolamine and arachidonate. These molecules can subsequently be reincorporated into phospholipids of the neuronal cell membrane [86,272]. In addition to binding to the CB1 receptor, anandamide has also been shown to bind to the peripheral CB2 receptor, albeit with a lesser degree of affinity (receptor $K_i$ ratio CB2/CB1 of 3.6) [23,101,104,294]. However, there is controversy over whether this binding to the CB2 receptor is associated with functional agonist activity [23,101,104,294,300]. When the human CB2 receptor has been expressed in chinese hamster ovary cells both inhibition [104,294] and failure of

inhibition [23] of forskolin-induced adenylate cyclase activity has been described. Additionally, whilst anandamide has been demonstrated to bind to CB2 receptors expressed on rat RBL-2H3 mast cells (by inhibition of the binding of tritiated WIN55,212-2) there appears to be no associated functional activity. This is demonstrated by the fact that the serotonin release induced from such cells in response to dinitrophenylated human serum albumin is inhibited by WIN55,212-2 (a compound known to exhibit activity at the CB2 receptor site), but not by anandamide. Furthermore, co-administration of anandamide reduces the inhibition of activity associated with WIN55,212-2 administration [101]. Similar functional results have been observed in primary cultures of mouse cerebellar granule cells expressing the mRNA for both the CB1 and CB2 receptor. Following acute exposure of granule cells to glutamate, WIN55,212-2 administration result in a reduction of the observed neuronal loss, which is thought to be NMDA mediated. However, the protective effect of this compound is inhibited by the co-administration of anandamide. Moreover, when anandamide is administered as a sole agent there is no reduction in the neuronal loss induced by glutamate [300]. Thus, whilst anandamide is accepted as demonstrating agonist activity at the CB1 receptor, its status regarding activity at the CB2 receptor is currently unclear.

Palmitoylethanolamide (PEA), produced by the N-acylation of cell membrane phospholipids (see figure 47 on page 195), is known to accumulate in inflamed tissue [246] and has been proposed as an endogenous ligand active at the CB2 receptor. The molecule, which does not bind to the CB1 receptor [143,180], binds weakly to cloned CB2 receptors in chinese hamster ovary cells [295]. However, in a rat mast cell line RBL-2H3 (rat basophilic leukaemia) which is used to assess inflammatory mediator release during inflammation, an $IC_{50}$ of 1.0 +/-0.6nM was found.

This compares with an $IC_{50}$ of 33 +/- 29nM for anandamide in the same preparation. Furthermore, agonist activity has been demonstrated following such PEA binding [101,300,301]. *In vitro* protection of neuronal cell death, induced by glutaminergic activity [300] or mast cell activation [301], has been demonstrated to be associated with PEA administration. Functionally, PEA administration is associated with a reduction in mast cell degranulation and plasma extravasation *in vivo* in a variety of mouse models of inflammation [211]. In whole animal models a dose dependent inhibition of the inflammatory hyperalgesia associated with sub-cutaneous administration of carrageenan has been demonstrated [211], as has a reduction in the mechanical hyperalgesia associated with a model of neuropathic pain (that of sciatic nerve constriction in the rodent) [210]. Therefore, it is postulated that PEA acts as a weak agonist at the CB2 receptor, whilst lacking major CB1 receptor-mediated effects. Since CB2 receptor expression appears to be limited to immune tissues, the actions of PEA would be expected to relate particularly to inflammatory conditions.

Autocrine activity of NGF, following release from mast cells would tend to produce a positive feedback response with increased release of other sensitising molecules including serotonin and pro-inflammatory cytokines [177,237,251]. The paracrine function of NGF, along with a soup of inflammatory mediators leads to excitation and sensitisation of primary afferent neurones [238], producing pain and hyperalgesia which may be associated with the onset of protective behaviour allowing healing to occur. However, the presence of a high concentration of inflammatory mediators is also associated with tissue damage, including demyelination of both peripheral and central nervous system neurones demonstrated *in vitro* [153,180]. This poses a clear risk of long term damage to the organism, particularly since myelin basic protein is capable of instituting mast cell degranulation [153], with concomitant upregulation of inflammation. It is

therefore reasonable to expect, that in addition to the positive feedback cycles described, negative feedback control mechanisms might also be present. Autacoid local inflammation antagonism (ALIA), perhaps acting via a CB2 mediated response, might provide just such a control [10,180]. Whilst PEA accumulates in inflamed tissue [246], the relatively low affinity that it demonstrates for the CB2 receptor [295] would require that high concentrations were reached before inhibition of mast cell activity occurred. This would therefore avoid inappropriate downregulation of the inflammatory response, whilst still providing a natural brake against the tissue damaging effects of uncontrolled inflammation.

In addition to the ethanolamide compounds described above, acyl-glycerol esters, including 2-arachidonyl glycerol (2-AG), have been postulated to demonstrate endogenous cannabinoid activity. 2-arachidonyl glycerol, which has been isolated from the brain, the gastro-intestinal tract, the pancreas and the spleen, co-localises with anandamide, but is present in higher concentrations than anandamide in all tissues. Like anandamide, its effects (such as vasorelaxation and hypotension) are of short duration and appear to be mediated through the CB1 receptor [229].

### 6.i.c. <u>Cannabinoid receptor agonists:</u>

Drugs with cannabinoid receptor agonist activity have been under development since before the description of the cannabinoid receptors. Agents of a variety of different chemical structures have been described [21,103]:

i) The classical cannabinoids (see figure 47 on page 195).

The structure of these agents is based on the dibenzopyran ring structure of $\Delta^9$-THC and include such agents as HU-210.

ii) The bicyclic and tricyclic derivatives of $\Delta^9$-THC.

These agents include compounds such as CP 55,940, which binds to both CB1 and CB2 receptors with similar affinity.

iii) The aminoalkylindoles and their derivatives.

Amongst these agents is WIN 55,212-2, which shows moderate selectivity for the CB2 receptor ($K_i$ 0.3nM as compared to 1.9nM for the CB1 receptor). This group of compounds was developed when it was observed that some non-steroidal anti-inflammatory drugs acted as cannabinoid receptor agonists as well as cyclo-oxygenase inhibitors.

iv) The aromatic amide derivatives.

Many of these recently described molecules (the pharmacology of which has yet to be reported in detail) show selectivity for the CB2 receptor.

v) The endogenous ligands (see figure 47 on page 195).

Including anandamide, palmitoylethanolamide and 2-arachidonyl glycerol (described above). Analogues of these, such as methanandamide, have also been developed.

## 6.i.d. Cannabinoid receptor antagonists:

A specific competitive CB1 receptor antagonist (SR141716A) has recently been described [279]. *In vitro* blocking of the binding of tritiated CP55,940 to both brain synaptosomes and chinese hamster ovary cells transfected with the CB1 receptor occurs. No such activity is demonstrated when spleen cells or cells transfected with the CB2 receptor are used. Additionally, a competitive antagonism of the inhibition of forskolin induced adenylate cyclase activity and mouse vas deferens contraction associated with a variety of cannabinoids, has been observed [279]. *In vivo*,

SR141716A has been shown to reverse both the antinociceptive effect of $\Delta^9$-THC and WIN55,212-2 in the tail flick test [59] and also the hypothermia and immobility induced by administration of WIN55,212-2 in rodents [279]. The possible importance of endogenous cannabinoids under physiological conditions was suggested following the observation that administration of SR141716A is followed by hyperalgesic responses to thermal stimuli [276] and an increase in basal locomotor activity observed in rodents [59]. However, there is some evidence (described below) that SR141716A is not truly a pure antagonist, but shows a degree of inverse agonism [37,170,189]. If this is true, then deductions regarding endogenous cannabinoid tone, made from data accumulated using this compound, are invalid since observations may be secondary to a direct effect of the compound itself. When isolated chinese hamster ovary cells expressing the CB1 receptor are exposed to WIN55,212-2 an increase in binding of labelled GTPγS is observed, indicating CB1 receptor activation. This is inhibited by administration of SR141716A. However, in addition to this antagonism of the effects of exogenously applied cannabinoids, SR141716A administered alone to the isolated system is associated with a reduction in the basal rate of GTPγS incorporation, indicating inverse agonist activity [170]. Furthermore, in isolated rat sympathetic ganglion neurones expressing the CB1 receptor (following mRNA injection) SR141716A not only antagonises the inhibition of inward calcium current induced by WIN55,212-2, but also increases the basal current when applied alone [256]. Data such as this makes it difficult to assess the possible importance of any basal effects of endogenous cannabinoid tone until a new CB1 receptor antagonist, devoid of inverse agonist effects, has been developed.

More recently a specific CB2 receptor antagonist (SR144528) has been described [278]. This molecule displaces the cannabinoid receptor agonist CP55,940 from both mouse spleen cells and chinese hamster ovary

cells transfected with human CB2 receptors. It has a seven hundred fold higher affinity for CB2 than CB1 receptors, but no detectable affinity for a wide range of other receptors. Functionally it demonstrates blockade of agonist induced cAMP accumulation within chinese hamster ovary cells expressing CB2 receptors and also attenuation of immune proliferation evoked in human tonsillar B lymphocytes. So far no whole animal experiments have been reported.

### 6.i.e. <u>CB1 and CB2 receptor 'knockout' mice:</u>

A CB1 receptor knockout mouse, in which the gene encoding the CB1 receptor has been invalidated, has been described [174]. In models of acute pain, such as the 'hot plate test', the effect of cannabinoid (but not opioid) administration is obliterated, suggesting a crucial role for CB1 receptors in anti-nociceptive responses. In the 'tail immersion test', where a degree of tissue injury may result in cannabinoid activation of CB2 (in addition to CB1) receptors, the antinociceptive effects of cannabinoids are reduced, but not obliterated. This supports a role for both cannabinoid receptor types in anti-nociceptive responses. Further investigations using this mouse strain may shed light on the role of CB1 receptors in other pain states.

Recently a CB2 receptor knockout mouse has been developed [42]. Since it has been shown to be both healthy and fertile, it is hoped that further experimentation may elucidate more clearly the role of CB2 receptor activation in immune responses. Such experiments may include the investigation of the importance of CB2 receptor activation in the instigation and maintenance of hyperalgesia.

### 6.i.f. Experimental rationale:

Cannabinoid receptors have been localised to both primary sensory afferent neurones [113] and also to cells of immune origin [115]. However, the *in vivo* data available to date supporting the possible peripheral actions of cannabinoids is limited [277]. In view of the widespread belief amongst the lay public that *cannabis sativa* has beneficial analgesic actions [40,207] and that these actions are separate from the well described psychotropic effects, I was interested to examine the possibility that cannabinoid agonists which exhibit a degree of receptor specificity might provide a novel clinical approach. This is of especial interest in the visceral domain, where the effects of peripherally active molecules, such as NGF, are of particular importance [91,219] and current therapeutic options are limited [221].

## 6.ii. Methods

All experiments conformed with British Home Office Regulations. Animals were housed according to national guidelines in colony cages of six animals per cage with a dark : light cycle of 12 hours : 12 hours. They were provided with free access to food and water.

### 6.ii.a. Urinary bladder reflex experiments:

Surgical preparation [147,148,215,218]:

Animals were prepared as for the reflex experiments in which the bradykinin receptor antagonists and capsaicin analogue were assessed (see sections 4.ii.a. and 5.ii.a.). After anaesthesia the trachea, urethra and carotid artery were cannulated.

Measurement of bladder motility [147,148,215,218]:

As in the previous experiments, the outcome measure used was the cystometrogram (the pressure-volume relationship for the urinary bladder). Again, cystometrograms were performed, by the infusion of warm, sterile, 0.9% saline at a constant rate (0.05ml/min, to a maximum of 0.7ml) via an infusion pump. The intra-vesical pressure was monitored continually via a pressure transducer and the signal was amplified and displayed on a PC. Data were analysed and stored using AcqKnowledge software (version 3.02).

Outcome measures:

The same two measures used in the bradykinin receptor antagonist and capsaicin analogue experiments were extracted from the cystometrogram (see figure 25 on page 123):

i) The micturition threshold, $V_{mic}$.

ii) The cumulative duration of micturition, $Dur_{mic}$.

Inflammation of the bladder [215,218]:

The bladder was inflamed by the intra-vesical instillation of 0.5ml of 50% turpentine in olive oil for one hour. The sterile inflammatory response induced in the bladder is accompanied by a lowering of the micturition threshold ($V_{mic}$) and a rise in the cumulative duration of micturition ($Dur_{mic}$).

Data presentation:

Statistical comparisons all utilised the Sigmastat package, version 2.0 (Jandel Scientific Software) and a significance level of $p < 0.05$ was employed. Graphs were produced using Sigmaplot version 3.0 (Jandel Scientific Software).

Protocols (see figure 26 on page 125) and statistical analysis:

**a) Prophylactic regimen:**

As with the bradykinin receptor antagonist experiments, when compounds were administered prior to inflammation the post-inflammation measures (at one and two hour post-turpentine instillation) were compared to the baseline measurements (paired t-test). The results were then expressed as a percentage of baseline values. Compounds were assessed as effective when the post-inflammation measures did not differ significantly from baseline.

**b) Treatment regimen:**

When compounds were administered after induction of inflammation the post-treatment values (at both fifteen minutes and one hour) were compared to both the post-inflammation and the baseline values by analysis of variance testing (ANOVA [*post hoc* Tukey or Dunnett's as appropriate]).

Compounds were assessed as at least partially effective if there was a significant difference between post-inflammation and post-treatment measures. If in addition there was no significant difference between baseline and post-treatment measures, compounds were taken to be effective at fully reversing the inflammatory vesical hyper-reflexia.

Cannabinoid receptor agonists assessed:

Storage and preparation details for all compounds are given in appendix 1.

a) Anandamide (ANA) (an endogenous CB1 receptor agonist) [Sigma].

Dose range: 10mg/kg - 25mg/kg.

This dose range was chosen because previous experiments undertaken in acute pain models (tail flick and hot plate) demonstrated analgesic activity at doses ranging between 5 and 25mg/kg [112,302,308]. Therefore any significant inhibitory effects in this visceral model were likely to be detected by this dosing regimen.

b) Methanandamide (a longer acting CB1 receptor agonist).

[Sigma]. Dose: 5mg/kg.

This dose was chosen because it is well within the range previously demonstrated to have cannabamimetic activity (inhibition of motor activity in rats) [281].

c) Palmitoylethanolamide (PEA) (a putative CB2 receptor agonist)

[Tocris Cookson]. Dose range: 10mg/kg - 30mg/kg.

This dose range was chosen since 10mg/kg has been shown to be efficacious in modulating inflammatory responses in the somatic domain [211].

## 6.ii.b. Plasma Extravasation experiments:

Outcome measure [218]:

As with the previously assessed molecules, animals utilised in the bladder reflex experiments, were used to investigate the ability of the compounds to decrease the inflammation-associated plasma extravasation into vesical tissue. In each case, after the final cystometrogram had been performed, the animal was slowly perfused with Evan's Blue Dye (50mg/kg) prior to terminal perfusion with 0.9% saline (to ensure clearance of the dye from the vascular compartment). The bladders were excised, weighed and stored in an extraction mixture of 70/30 acetone + 0.5gm/L sodium sulphide, for a minimum of five days. The quantity of Evan's Blue Dye extravasated was measured by spectrophotometry at $\lambda = 620nm$ and the tissue concentration of dye in mg / mg of vesical tissue was calculated, using the equation:

$$\frac{(Measured \quad [EB]) \times 4000}{Bladder \quad weight}$$

Tissue concentrations of Evan's Blue Dye were compared between control and cannabinoid treated groups by means of the unpaired t-test (prophylactic administration of compounds) or ANOVA [*post hoc* Dunnett's] (treatment protocol). All comparisons were performed using the Sigmastat package, version 2.0, Jandel Scientific Software. Compounds were taken to be effective if the treated group showed a significantly lower level of plasma extravasation than the control group ($p < 0.05$).

## 6.ii.c. Formalin test:

Pain behaviour observations [57,98,323]:

Animals were acclimatised to the testing environment (a clear plexiglas box measuring 23 x 18 x 14cm, with a clear plastic floor), until explorative behaviour ceased (see figure 48). With a minimum of restraint, 50μL of 2.5% formalin was administered sub-cutaneously (s.c.) into the dorsum of the right hind paw. A mirror angled at $45^0$ below the floor of the cage allowed an unobstructed view of the paws after return of the animal to the testing environment. Observations of the animals' behaviour were made in consecutive five minute periods for the fifty minutes following formalin injection.

Effect of cannabinoid administration:

Anandamide and palmitoylethanolamide were administered i.p. immediately prior to the sub-cutaneous formalin injection. The doses for anandamide ranged from 5mg/kg to 15mg/kg, a range which has previously been shown to be effective in rodents models of hyper-acute pain, such as the tail flick and hot plate tests [112,302]. Similarly, palmitoylethanolamide doses were chosen after review of the literature suggested that doses in the range of 1 to 10mg/kg are effective when administered systemically in a

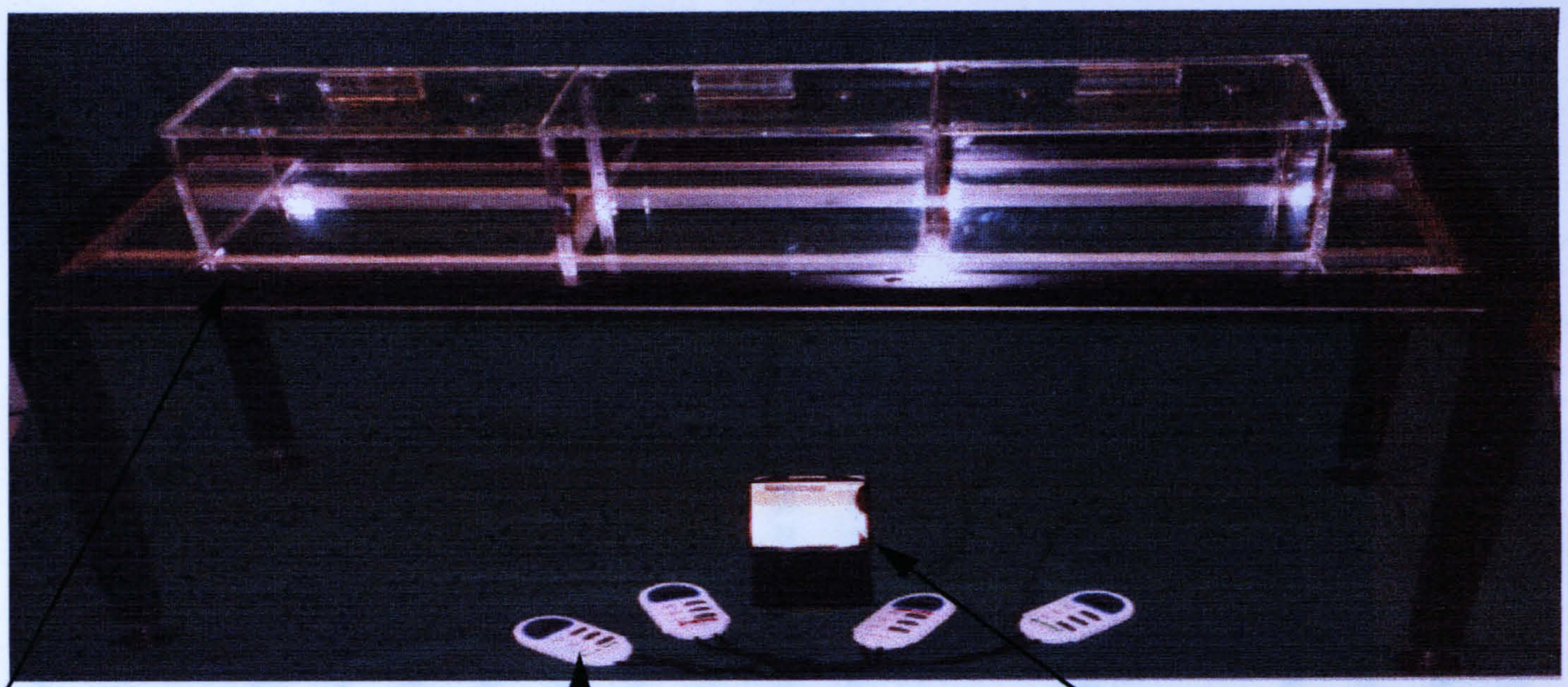



Figure 48.
A photograph of the testing environment for the sub-cutaneous formalin-induced pain behaviour experiments.

variety of rodent models, including carrageenan induced inflammation of the rat paw [211].

Two control groups were utilised:

a) A formalin alone group.

b) A solvent control group, in which 0.7ml of DMSO + 0.9% saline in a 1 : 19 dilution were administered (i.p.) immediately prior to the injection of sub-cutaneous formalin.

Outcome measures:

The duration of time spent involved in each of the nociceptive behaviour responses was recorded and weighted using the method of Watson *et al.* (see section 1.v.) [332]. The 'weighting factors' were:

0 the injected paw is not favoured.

1 the injected paw is elevated and not in contact with any surface.

2 the injected paw is licked, bitten or shaken.

Thus a single measure, the weighted composite pain score (CPS-$WST_{0,1,2}$) for each of the five minute observation periods was calculated using the equation:

$$\frac{\left(\left[Time \times Weight1\right]+\left[Time \times Weight2\right]+\left[Time \times Weight3\right]\right)}{300}$$

In addition, an overall CPS-$WST_{0,1,2}$ was calculated for the first (zero to fifteen minutes) and second (twenty to fifty minutes) phases of the behavioural response. The overall CPS-$WST_{0,1,2}$ for the first and second phases of the behavioural response were compared, using analysis of variance with *post-hoc* Dunnett's testing, against the formalin alone control group (Sigmastat version 2.0, Jandel Scientific Software). Plots of CPS-

$WST_{0,1,2}$ were produced using Sigmaplot, version 3.0, Jandel Scientific Software.

## 6.ii.d. *C-fos* immunohistochemistry:

The protocol for these experiments is shown in figure 27 on page 130.

Surgical preparation:

As in the bradykinin B2 receptor antagonist experiments (see section 4.ii.c.) animals were anaesthetised with urethane and the trachea, urethra and carotid artery cannulated. Inflammation of the bladder was induced by intra-vesical instillation of turpentine oil and animals were culled two hours after induction of inflammation (by terminal anaesthesia).

Drug administration:

All compounds were administered (i.a.) thirty minutes prior to turpentine instillation intra-vesically. Compounds administered were chosen to assess the effects of both CB1 and CB2 receptor agonists on the expression of *c-fos* in the dorsal horn of the spinal cord. Compounds tested were:

a) Methanandamide (or 35% alcohol, solvent control) - this was chosen to replace anandamide as it is more stable and of longer duration of activity [281]. A dose of 5mg/kg was administered, as this has previously been demonstrated to be active in behavioural tests [281].

b) Palmitoylethanolamide (or DMSO + saline, 1 : 19 dilution, solvent control). A dose of 20mg/kg was administered, as this was within the dose range found to be active in the bladder reflex experiments.

c) WIN55,212-2 (or DMSO + saline, 1 : 19 dilution, solvent control). A dose of 5mg/kg was administered, since this has

previously been demonstrated to be effective in an attenuating the expression of c-Fos-like immunoreactivity in the spinal cord following an inflammatory stimulus (sub-cutaneous formalin injection) applied to tissues of the somatic domain [327].

Tissue fixation [232]:

As with the previous experiments (described in section 4.ii.), animals were trans-cardially perfused with 0.9% heparinised saline at $4^0$C and then 4% paraformaldehyde to fix the tissues prior to dissection. The spinal cord was further subjected to two hours in 4% paraformaldehyde as a post-dissection fixative.

Tissue dissection:

The L5, L6 and S1 spinal roots and segments were identified and excised prior to post-fixing. They were stored in 20% sucrose for twelve hours and subsequently the sections were blocked in OCT mounting medium at -$70^0$C. 20μm thick sections of spinal cord were cut at intervals of 100μm. Prior to staining these sections were stored at -$20^0$C in a cryoprotectant solution.

Tissue staining (see figure 29 on page 134):

As in the previous experiments, tissue staining was performed using10% normal goat serum as a blocking antibody, rabbit anti-c-Fos as the primary antibody and biotinylated goat anti-rabbit as the secondary antibody. ABC reagent was used to enhance the staining prior to development of the reaction with DAB. Slides were cleared by dehydration through alcohol and xylene and mounted using DePeX.

Outcome measures:

Neurones positive for c-Fos-like immunoreactivity were identified by light microscopy and both total staining and sub-area staining (described in section 4.ii.c.) were counted by a 'blinded' observer.

Statistical comparison:

Data were separated by:

a) cord level.

b) area of the cord (described in section 4.ii.c.).

Within the control groups, comparison of the number of neurones positive for c-Fos-like immunoreactivity across the cord levels L5, L6 and S1 was made (unpaired t-test). This provided an assessment of the relative importance of each of the spinal roots in transmission of nociceptive inputs arising from the urinary bladder.

Subsequently, comparisons were made (unpaired t-test) between both control and treatment groups at each of the three spinal cord levels (L5, L6 and S1):

i) for total staining.

ii) for staining in each sub-area of the dorsal cord.

All statistical tests were performed using the Sigmastat package (version 2.0, Jandel Scientific Software) and a significance level of $p < 0.05$ was employed. Data was presented in graphical form using the Sigmaplot package (version 3.0, Jandel Scientific Software).

## 6.iii. Results

### 6.iii.a. Urinary bladder reflex experiments:

Experiments were performed on ninety eight animals weighing 165 - 250gm (mean weight 216gm). The mean baseline micturition threshold ($V_{mic}$) and cumulative duration of micturition ($Dur_{mic}$) were 0.42mls (+/- 0.01) and 179.9 seconds (+/- 8.1) respectively. The effect of turpentine-induced inflammation of the urinary bladder was assessed using data from animals in which a cystometrogram was performed after induction of inflammation, but prior to drug administration (n = 46). A viscero-visceral hyper-reflexia (VVH) was demonstrated by a reduction in $V_{mic}$ and an elevation in $Dur_{mic}$, to 43.9% (+/-2.5) and 297.8% (+/- 30.2) of baseline respectively (ANOVA [Tukey], $p < 0.05$) at two hours following instillation.

The effect of administration of receptor agonists in the absence of inflammation:

Administration of the CB1 receptor agonist, anandamide (ANA), at the highest dose investigated (25mg/kg, n = 5) was not associated with any significant alteration in the reflex responses to increasing intra-vesical volume in the uninflamed bladder (paired t-test, $p > 0.05$). The mean (sem) $V_{mic}$ and $Dur_{mic}$ were respectively observed to be 131.3% (+/- 46.4) and 88.3% (+/- 17.4) of baseline values.

Similarly, the CB2 receptor agonist, palmitoylethanolamide (PEA), was not associated with any significant alterations in the responses observed in the cystometrogram in the uninflamed bladder (n = 5): $V_{mic}$ remained at 91.1% (+/- 21.3) and $Dur_{mic}$ was 97.8% (+/- 18.0) of baseline values.

The effect of solvent control administration on micturition reflexes:

Pre-inflammation administration of the solvent control (n = 5, 0.7ml, DMSO + saline, 1 : 19 dilution, i.a.) did not prevent the onset of

inflammation-associated changes in either the micturition threshold $V_{mic}$ or the cumulative duration of micturition $Dur_{mic}$. The $V_{mic}$ at one hour post-administration was significantly reduced to 58.37% (+/- 12.32) and $Dur_{mic}$ was elevated to 215.4% (+/- 58.0) of baseline values (paired t-test, $p < 0.05$).

Similarly, post-inflammation administration of solvent control was not associated with any significant reversal of the inflammation-associated changes (ANOVA [Dunnett's]). The micturition threshold, $V_{mic}$ remained depressed at 43.9% (+/-9.3) of baseline values and the respective value for $Dur_{mic}$ was 211.9% (+/- 40.6). Thus the solvent control had no significant effect on the inflammation-associated viscero-visceral hyper-reflexia (VVH) ($p < 0.05$) when administered in either the prophylactic or the treatment regimen.

<u>The effect of pre-inflammation administration of receptor agonists:</u>

When anandamide was administered prophylactically (n = 6 in 10mg/kg and 15mg/kg groups and n = 5 in 25mg/kg group) the changes in the cystometrogram associated with VVH were prevented in a dose related manner, with a dose threshold of 25mg/kg (see figures 49 and 50). A clearly visible trend in prevention of the inflammation associated decline in $V_{mic}$ and elevation in $Dur_{mic}$ is observed in the bar charts. However, this trend only reaches significant levels (paired t-test, $p < 0.05$) in both outcome measures, at the highest tested dose of 25mg/kg (see table 12 in appendix 2).

Table 13 (in appendix 2) and figures 49 and 50, demonstrate that the administration of the CB2 receptor agonist palmitoylethanolamide, was not associated with any significant alteration in the onset of VVH if administered prior to the instillation of intra-vesical turpentine (n = 6 for all treatment groups). Both a significant reduction in $V_{mic}$ and a significant

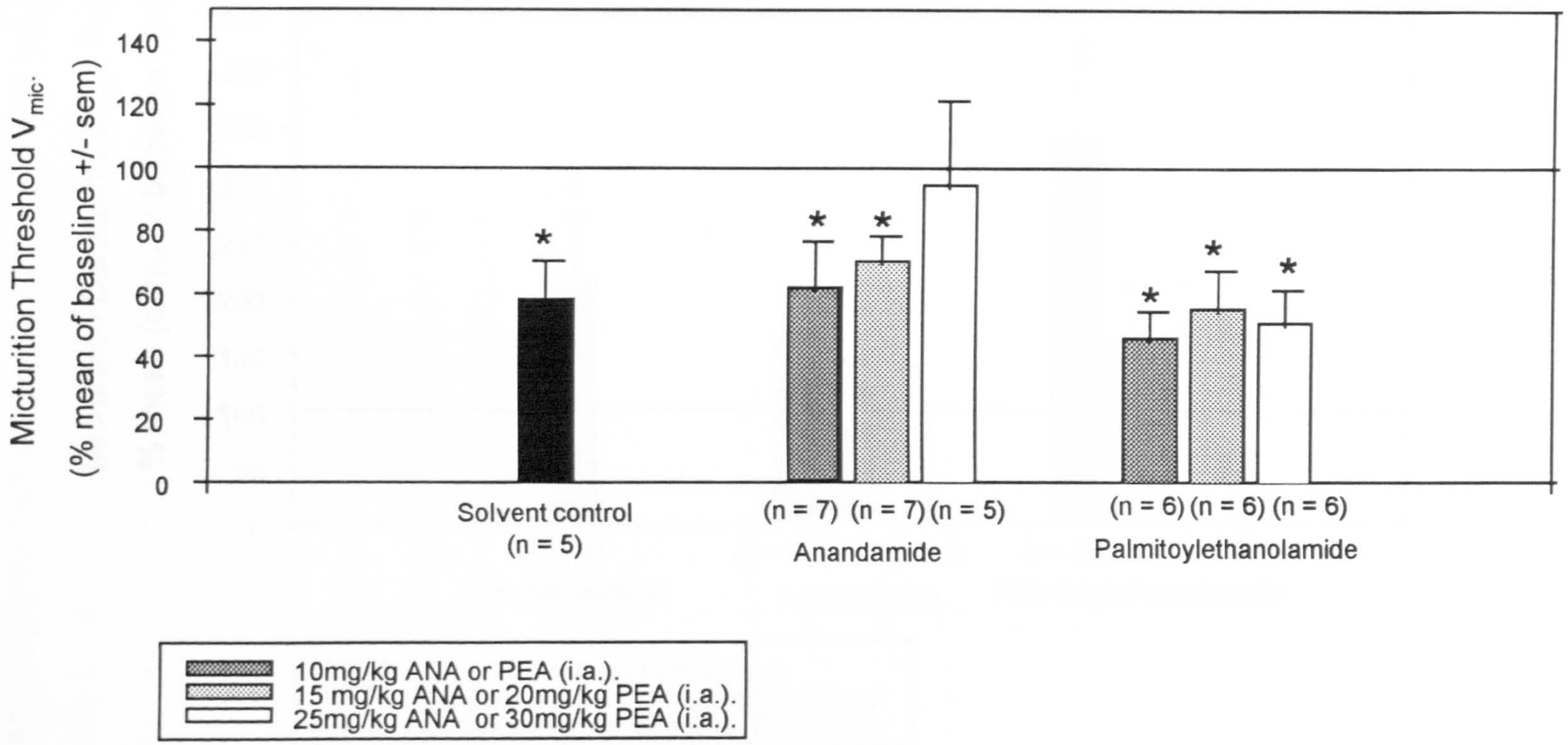



Figure 49.

Bar chart demonstrating the different effects of prophylactic treatment with anandamide (ANA) and palmitoylethanolamide (PEA) on the micturition threshold ($V_{mic}$), 1hour after administration. Note that treatment with the solvent control, PEA and low doses of ANA are associated with a significant reduction in $V_{mic.}$ Administration of 25mg/kg of ANA prevents this inflammation-associated reduction.

* t-test, $p < 0.05$ in comparison to baseline.

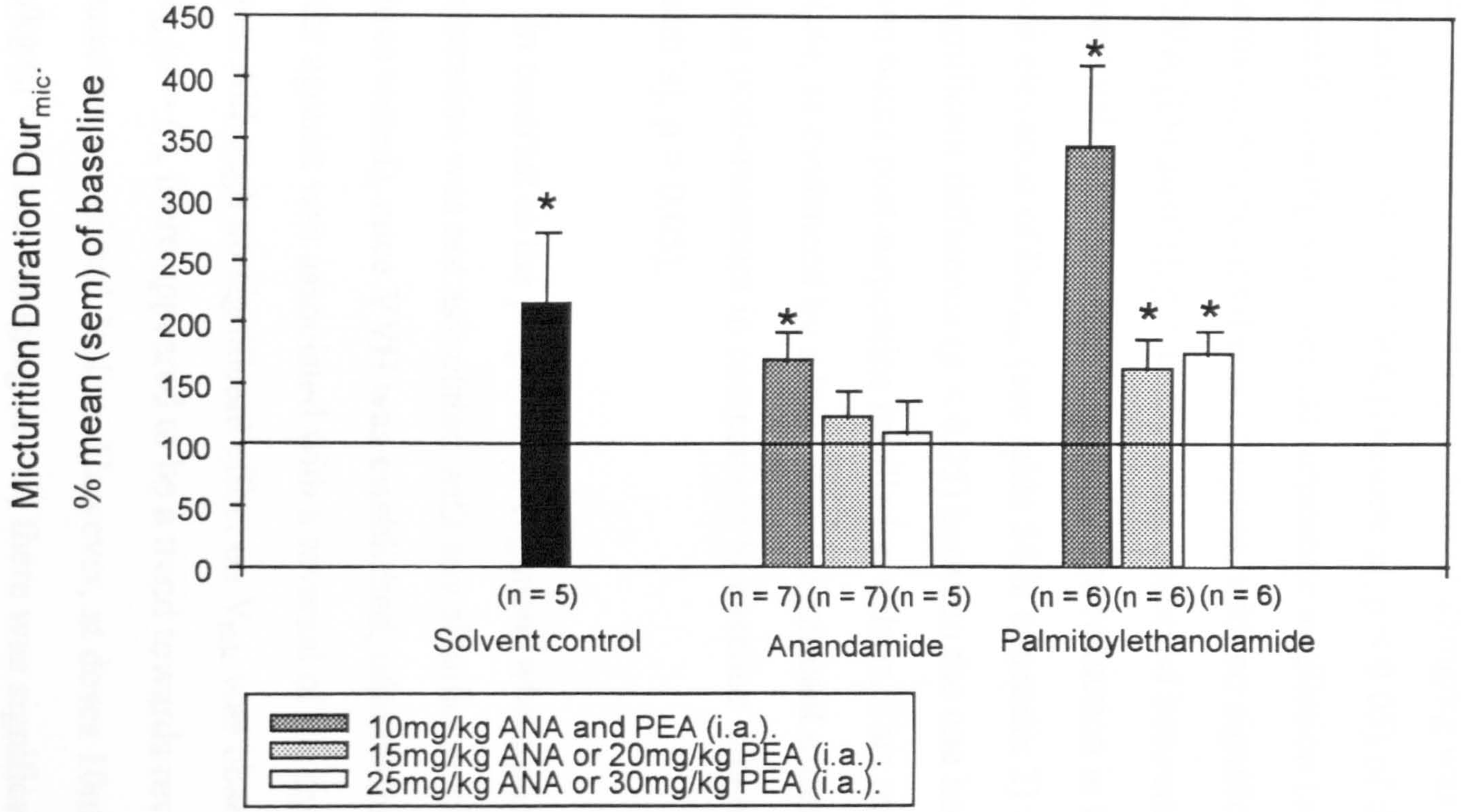



Figure 50.

Graph to show the effect of prophylactic administration of anandamide (ANA) and palmitoylethanolamide (PEA) on the cumulative duration of micturition ($Dur_{mic}$), 1hour after administration. Note the significant elevation in $Dur_{mic}$ after prophylactic treatment with solvent control and PEA. However, when ANA is administered this elevation is attenuated in a dose dependent manner.

* t-test, $p < 0.05$ in comparison to baseline.

elevation in $Dur_{mic}$ were observed in comparison to baseline (paired t-test, $p < 0.05$).

<u>The effect of early post-inflammation administration of receptor agonists:</u>

When anandamide was administered after the induction of inflammation (n = 6 each group) a dose of 25mg/kg was associated with significant reversal (ANOVA [Dunnett's], $p < 0.05$) of the reduction in $V_{mic}$ observed following intra-vesical turpentine instillation (see figure 51). Furthermore, this reversal was complete, with no significant difference (ANOVA [Dunnett's], $p > 0.05$) being observed between baseline and post-treatment values. Moreover, the associated reduction in the turpentine-induced elevation of $Dur_{mic}$ (see table 14 in appendix 2) was demonstrated by a significant difference ($p < 0.05$) between the one hour post-treatment and two hours post-turpentine instillation values. This reversal was also complete, as evidenced by a lack of any significant difference in $Dur_{mic}$ at one hour post-treatment in comparison to baseline values (ANOVA [Dunnett's], $p > 0.05$).

In contrast to the prophylactic regimen (where palmitoylethanolamide administration was not associated with any significant effects in the outcome measures tested), once VVH was established, administration of the CB2 receptor agonist was associated with a reversal of the hyper-reflexic changes. Although no significant effect on $V_{mic}$ was observed at a dose of 1mg/kg (n = 6), there appeared to be a trend towards reversal of the inflammation associated decline. However, at doses 10mg/kg (n = 5), 20mg/kg (n = 6) and 30mg/kg (n = 5) there was significant reversal (ANOVA [Dunnett's], $p < 0.05$) toward baseline values of $V_{mic}$ (see figure 52). Additionally, except at the 20mg/kg dose this reversal of the reduction in $V_{mic}$ was complete, with no significant difference from baseline (ANOVA

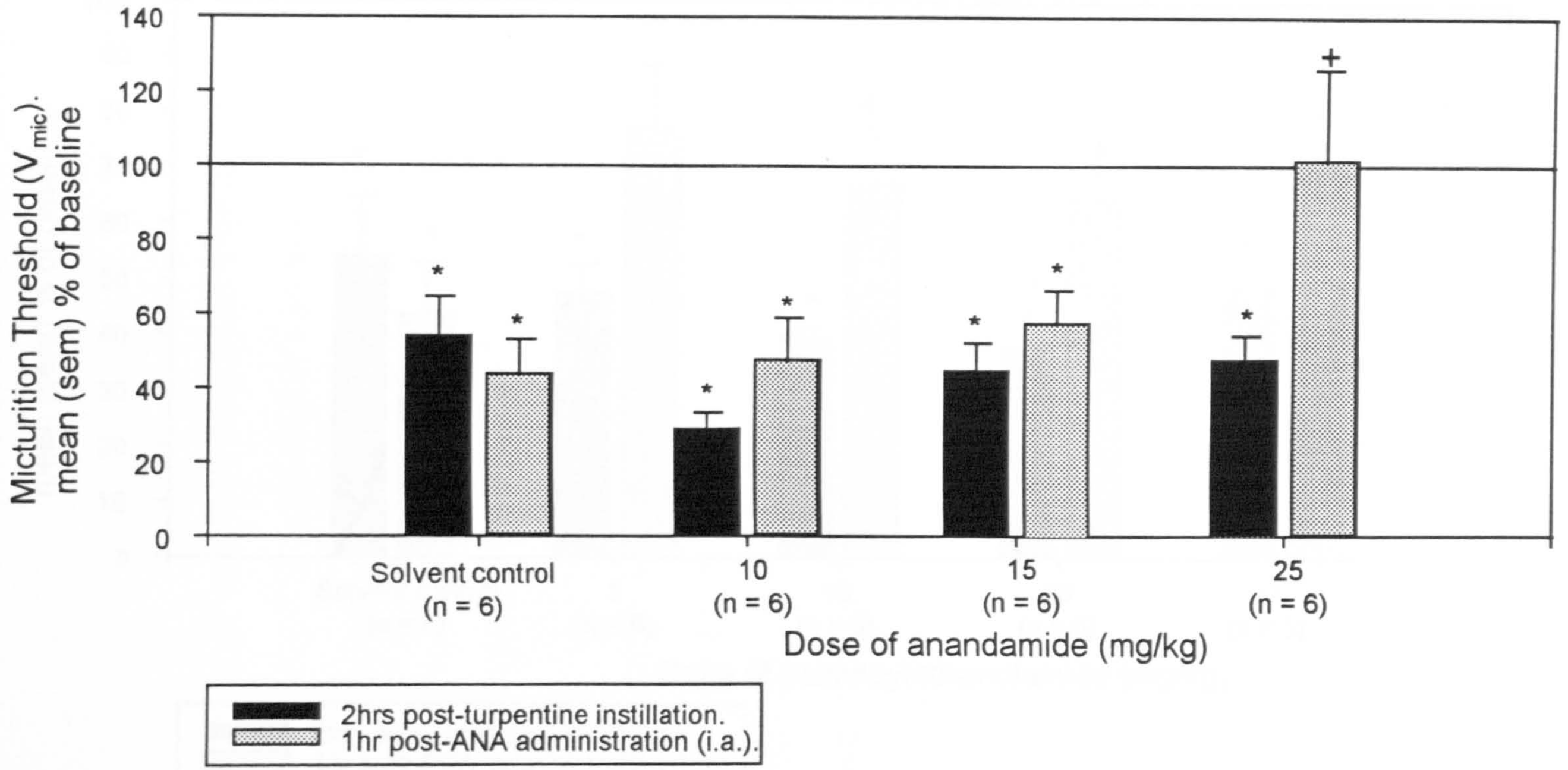



Figure 51.

Bar chart demonstrating the reduction of the micturition threshold ($V_{mic}$) which follows intra-vesical turpentine instillation. Note the further reduction in $V_{mic}$ after treatment with solvent control. This is most likely consequent upon ongoing inflammation. The reduction in $V_{mic}$ is reversed by 25mg/kg anandamide (ANA).

* $p < 0.05$ in comparison to baseline $V_{mic}$ (ANOVA [Dunnett's]).

\+ $p < 0.05$ in comparison to $V_{mic}$ 2 hours after turpentine instillation (ANOVA [Dunnett's]).

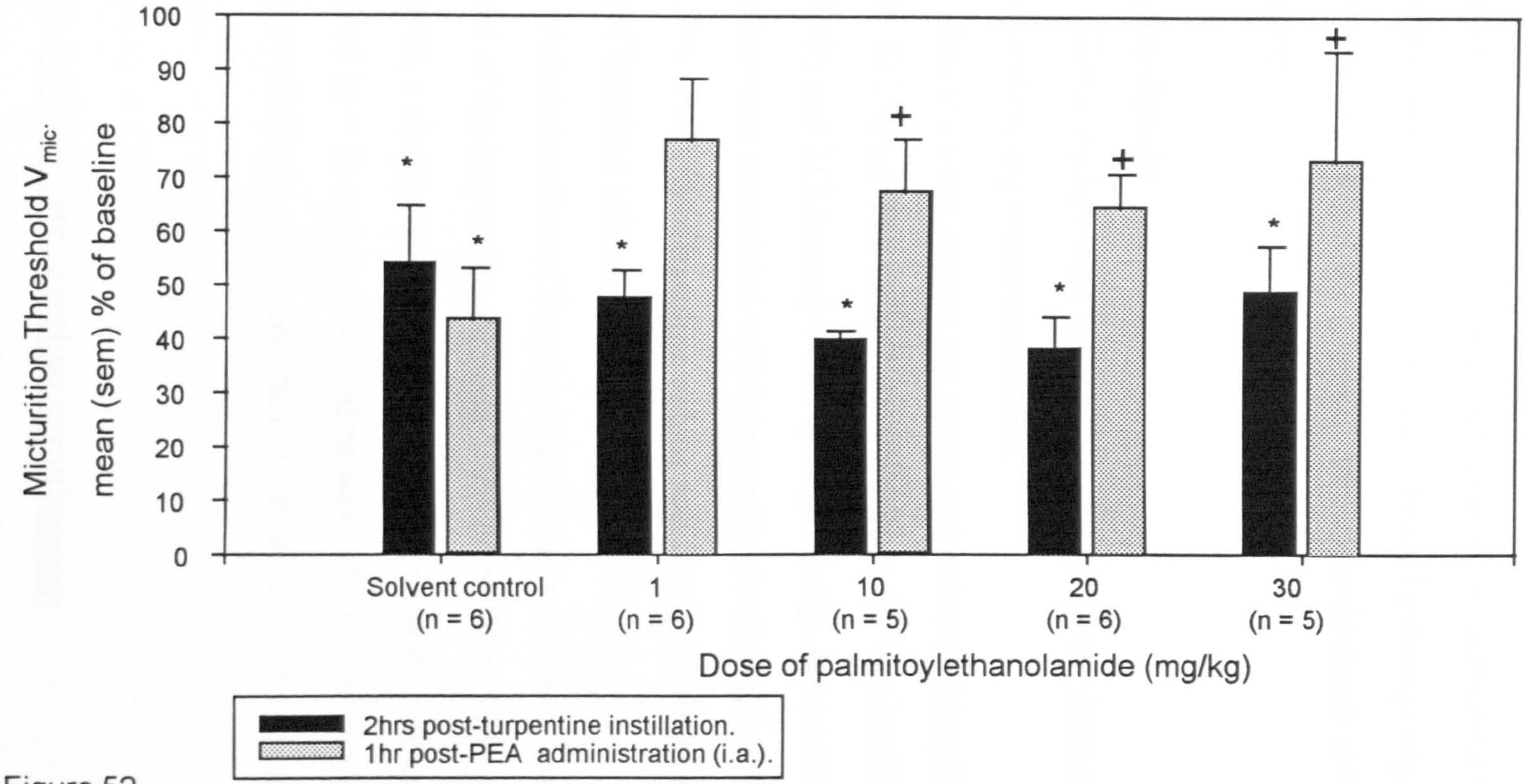



Figure 52.

Bar chart demonstrating the effect of post-inflammation treatment with palmitoylethanolamide (PEA). Note the reduction in micturition threshold ($V_{mic}$) following turpentine instillation. This is significantly reversed towards baseline levels following treatment with PEA.

* $p < 0.05$ in comparison to baseline (ANOVA [Dunnett's]).

\+ $p < 0.05$ in comparison to 2 hours post-turpentine instillation (ANOVA [Dunnett's]).

[Dunnett's], $p > 0.05$). As with results from all other drug experiments performed, the results of $Dur_{mic}$ were less consistent than those for $V_{mic}$ (see table 15 in appendix 2). However, at doses of 1, 20 and 30mg/kg a significant reduction ($p < 0.05$) in the cumulative duration of micturition $Dur_{mic}$ was observed in comparison to post-inflammation levels. The absence of such an attenuation in the 10mg/kg dosage regimen is probably related to the fact that the post-inflammation elevation in $Dur_{mic}$ was less in this group.

Methanandamide:

These studies were undertaken following the immunohistochemical experiments using methanandamide.

Studies were carried out on ten animals weighing 200 - 260gm (mean weight 223.5gm). The mean baseline values for $V_{mic}$ and $Dur_{mic}$ were 0.3mls (+/- 0.03) and 212.6secs (+/- 25.4) respectively. Intra-vesical instillation of turpentine was associated with hyper-reflexic changes - $V_{mic}$ being reduced to 34.9% (+/- 3.6) of baseline values and $Dur_{mic}$ elevated to 245.4% (+/- 41.3) of baseline values.

Administration of 5mg/kg MetANA ($n = 4$) was associated with reversal of the turpentine-induced VVH (ANOVA [Tukey], $p < 0.05$). However, a similar reversal was observed when the solvent (35% alcohol) was used ($n = 6$) and further studies were abandoned since the compound was unavailable in alternative form (see table 16 in appendix 2).

## 6.iii.b. Plasma extravasation experiments:

Experiments were performed on the fifty nine animals in which it was possible to clear the vascular compartment of Evan's Blue Dye. Animals to which the drugs were administered before onset of turpentine associated

inflammation (n = 30) had an average bladder weight of 94.9gm (+/- 5.6), whilst those to which compounds were administered after the induction of inflammation (n = 29) had an average bladder weight of 88.8gm (+/- 2.9).

<u>The effect of pre-inflammation administration of receptor agonists:</u>

Administration of the DMSO + saline (1 : 19) solvent control (0.7ml, i.a.) was associated with a bladder tissue concentration of Evan's Blue Dye of 0.63 (+/- 0.12) mg / mg of vesical tissue. Administration of neither anandamide nor palmitoylethanolamide had any significant effect ($p > 0.05$) on the level of concentration of the dye in vesical tissue (see table 17 in appendix 2).

<u>The effects of post-inflammation administration of receptor agonists:</u>

Solvent control (0.7ml, DMSO + saline, 1 : 19, i.a.) administration after induction of inflammation was associated with a tissue concentration of Evan's Blue Dye of 0.62 (+/- 0.08) mg / mg of vesical tissue. This was not significantly altered by administration of either anandamide or palmitoylethanolamide ($p > 0.05$) (see table 17 in appendix 2).

### 6.iii.c. <u>Formalin test experiments:</u>

Experiments were carried out on forty four animals, weight range 175 - 245gm (mean 211.0gm). The formalin control group confirmed the previously described, characteristic biphasic behavioural response to sub-cutaneous formalin injection (see figure 53). There was no significant difference in CPS-$WST_{0,1,2}$ for either phase, between the formalin control group (n = 5) and the solvent control (DMSO + Saline, 1 : 19) group (n = 6).

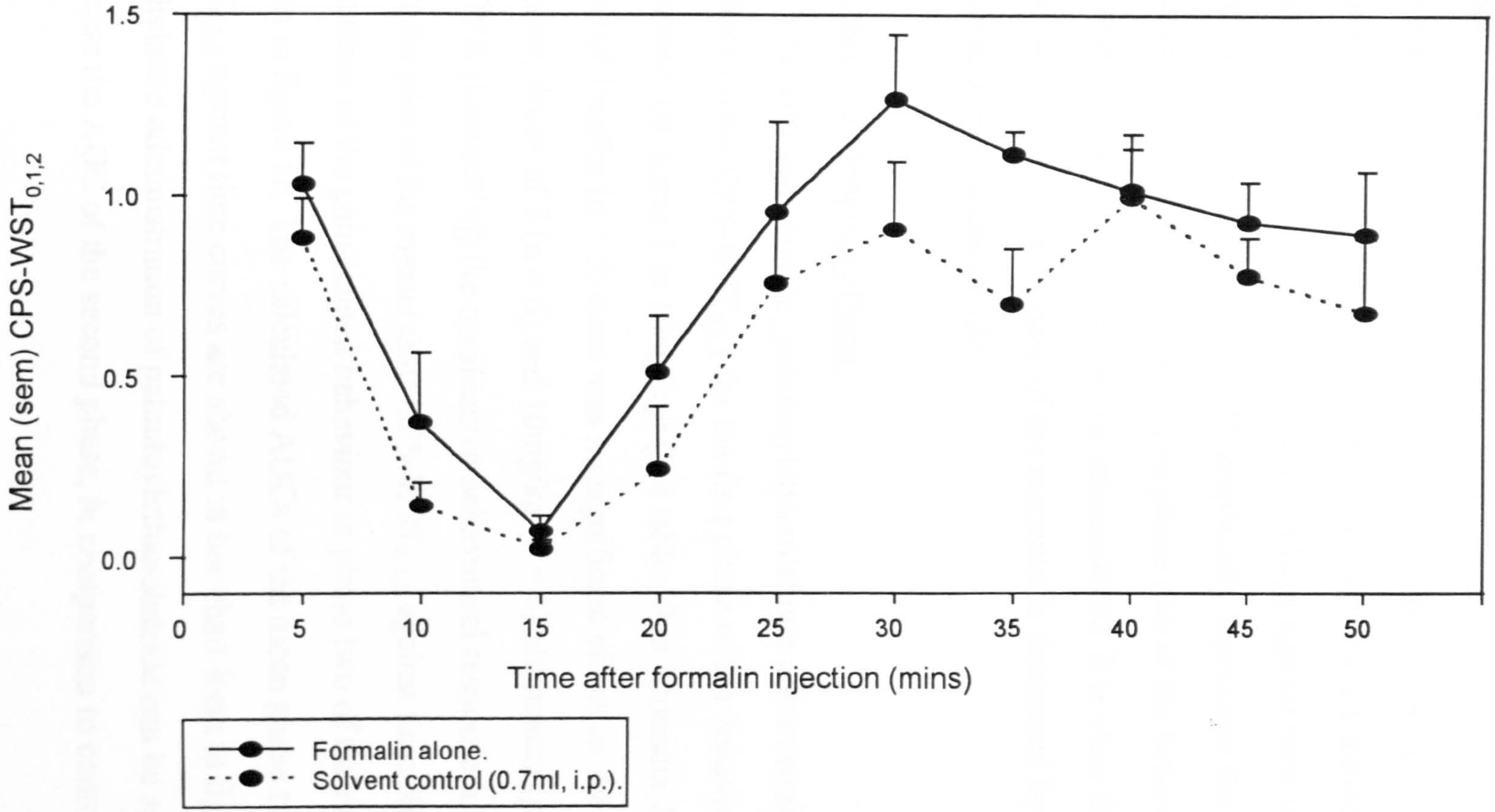



Figure 53.

Graph demonstrating the typical biphasic behavioural response to sub-cutaneous formalin injection into the paw. Note that prior administration of the solvent control substance (DMSO + saline, 1 : 19) did not significantly alter the behavioural response to formalin, as measured by the weighted composite pain score ($CPS\text{-}WST_{0,1,2}$).

CB1 receptor agonist mediated effects:

Administration of anandamide (i.p.) at doses of 5 (n = 5), 10 (n = 5) or 15mg/kg (n = 6) had no effect on the CPS-$WST_{0,1,2}$ for the first phase of the behavioural response (see table 18 in appendix 2). However, all doses of anandamide were associated with a significant reduction ($p < 0.05$, ANOVA [Dunnett's]) in the CPS-$WST_{0,1,2}$ for the second phase behavioural response. A representative plot of the mean CPS-$WST_{0,1,2}$ against time is shown in figure 54. The bar chart in figure 55 graphically represents the calculated AUCs (mean +/- sem) for the effect on phase two of the behavioural response of all doses of anandamide administered. It is clear that the AUC of the plot of the second phase of the response is decreased by the prior administration of anandamide.

CB2 receptor mediated effects:

As with anandamide, palmitoylethanolamide administration (i.p.) had no effect on the CPS-$WST_{0,1,2}$ for the first phase of the behavioural response to formalin (at doses 1 to 10mg/kg) (see table 19 in appendix 2). Although at a dose of 1mg/kg (n = 5) there was no significant effect on the second phase response, doses of 5 (n = 6) and 10mg/kg (n = 6) attenuated ($p < 0.05$, ANOVA [Dunnett's]) the nocifencive behavioural response to formalin. An exemplar plot of the mean (sem) CPS-$WST_{0,1,2}$ against time showing attenuation of the pain-related behaviour in phase two of the response, is shown in figure 56. The calculated AUCs of the mean phase two CPS-$WST_{0,1,2}$ against time curves are shown in bar chart form in figure 57. The prophylactic administration of palmitoylethanolamide can be seen to decrease the AUC of the second phase, in comparison to control values.

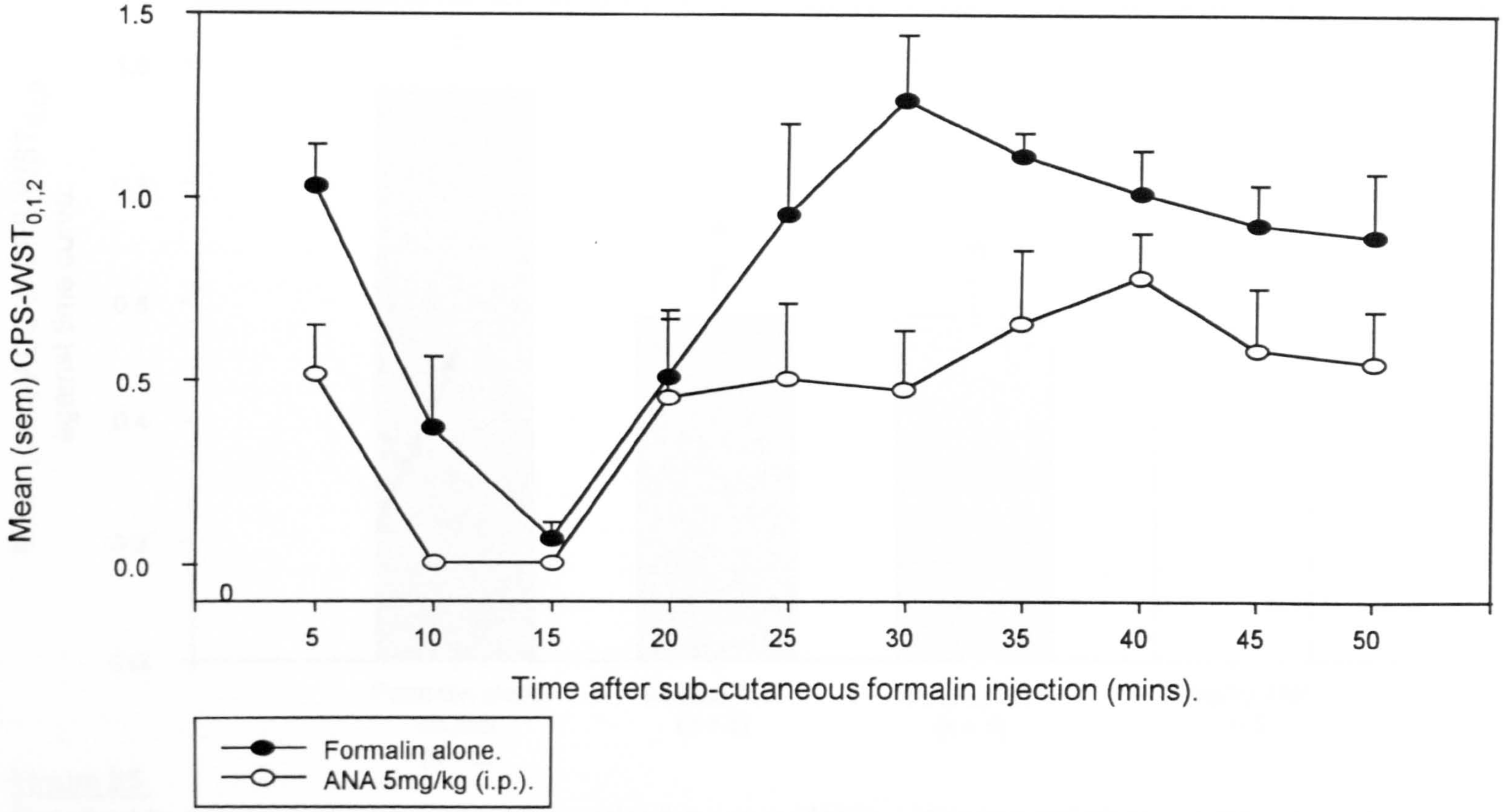



Figure 54.

Graph demonstrating the biphasic behavioural response to sub-cutaneous formalin. Note the reduction in observed pain related behaviour - measured using the composite pain score ($CPS\text{-}WST_{0,1,2}$) - after the administration of anandamide (ANA).

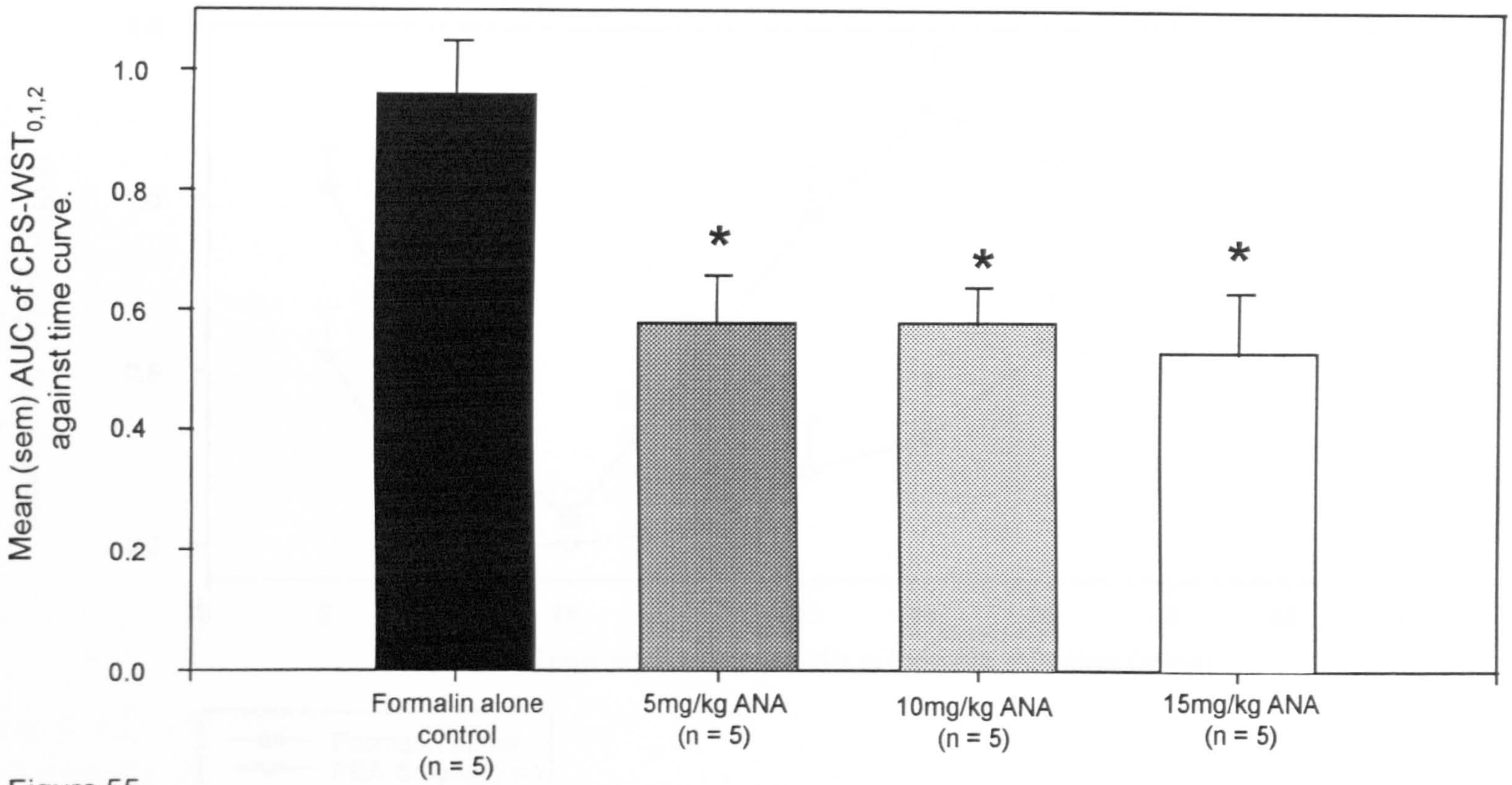



Figure 55.

Bar chart to demonstrate the mean area under the curve (AUC), of the second phase of the plot of the composite pain score ($CPS\text{-}WST_{0,1,2}$) against time curve. In animals treated with i.p. anandamide (ANA) prior to administration of sub-cutaneous formalin into the hind paw, there is a reduction in the AUC.

* $p < 0.05$ in comparison to control (ANOVA [Dunnett's]).

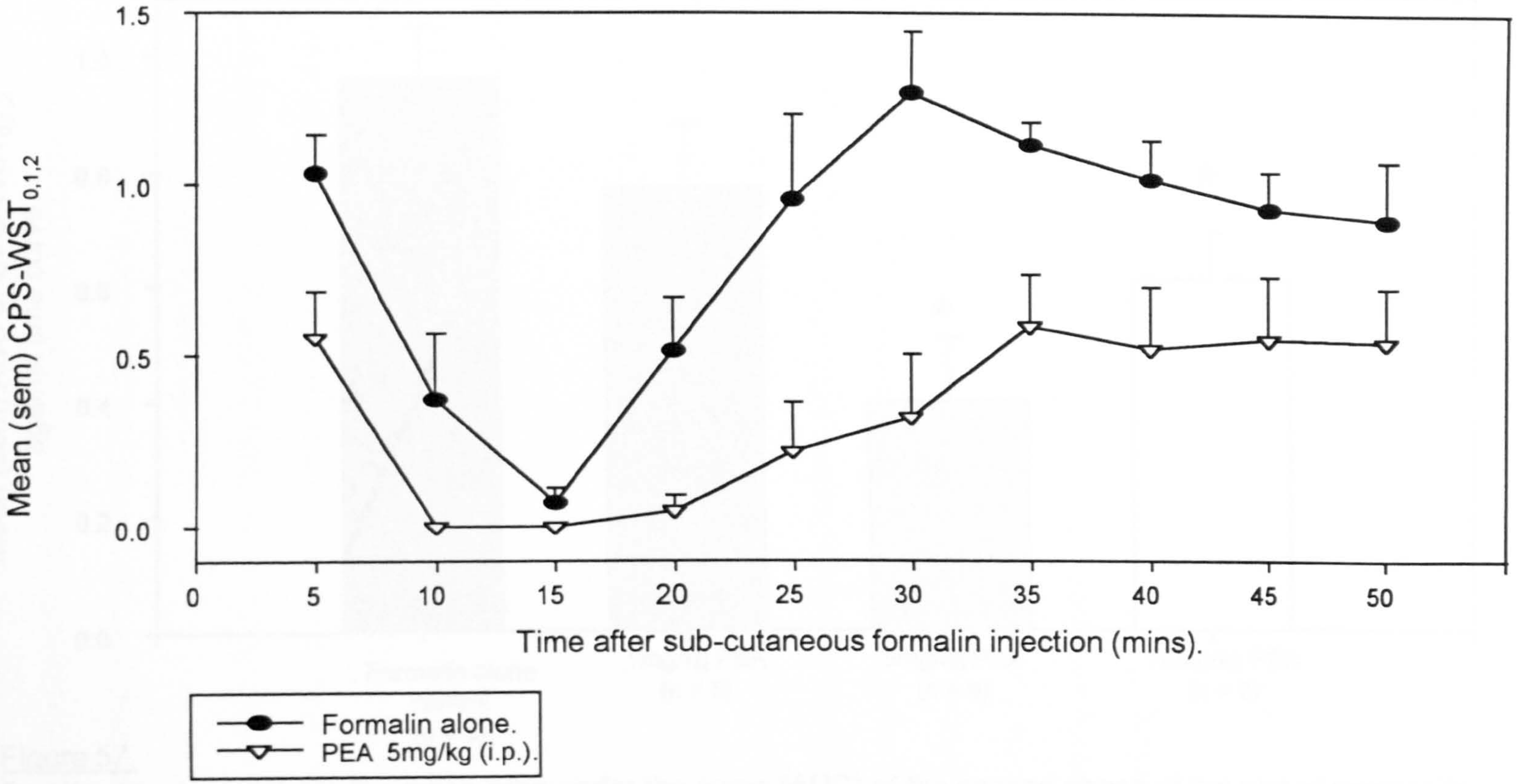



Figure 56.
Graph to show the effect of palmitoylethanolamide (PEA) on the biphasic behavioural response to sub-cutaneous formalin injection. Note the flattening of the curve in phase 2 of the response.

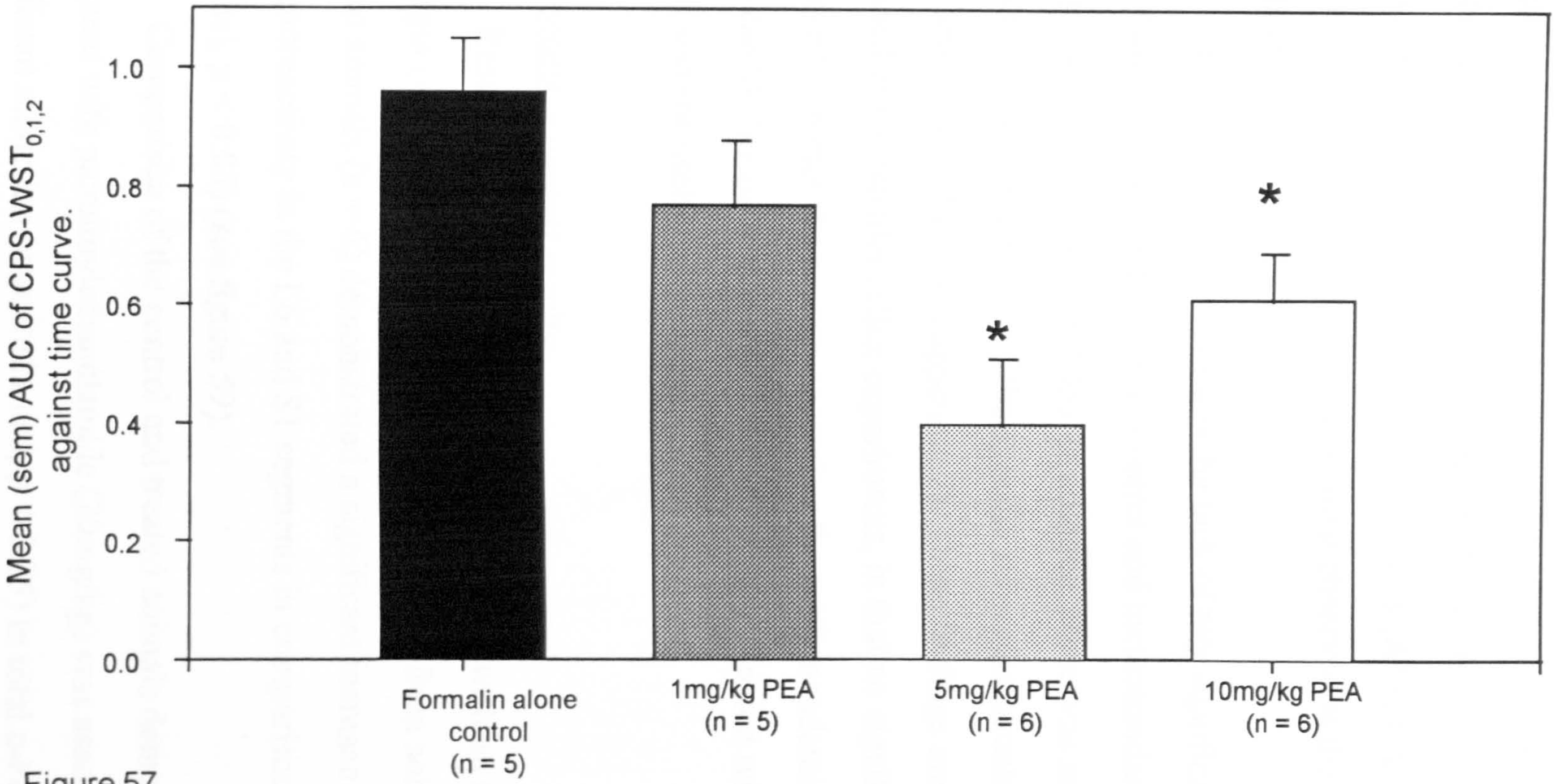



Figure 57.

Bar chart to demonstrate the mean area under the curve (AUC) of the second phase of the plot of composite pain score ($CPS\text{-}WST_{0,1,2,}$) against time, following sub-cutaneous injection of formalin. Note the reduction in AUC following administration of palmitoylethanolamide (PEA).

* $p < 0.05$ in comparison to control (ANOVA [Dunnett's]).

## 6.iii.d. *c-fos* immunohistochemistry experiments:

The effect of CB1 receptor agonist administration:

Results were obtained from ten animals with average weight 215.5gm (range 190 - 280gm). Analysis of counts from control animals (n = 5) demonstrated that there was a significant difference in total c-Fos-like immunoreactivity between all three cord levels (ANOVA [Dunns], $p < 0.05$), with the maximal degree of staining observed at the L6 and S1 spinal levels (see figure 58).

Figure 58 also demonstrates the lack of any significant difference in total staining between the solvent control and methanandamide treated groups (unpaired t-test, $p > 0.05$). In addition, there was no significant reduction in staining in any of the specific sub-areas counted, at any of the cord levels (see table 20 in appendix 2). These findings are similar to those obtained in the bladder reflex experiments, in that no significant VVH was observed in either the solvent control or the methanandamide treated groups. Thus the likelihood is that a positive effect is associated with administration of the solvent itself.

CB2 receptor agonist results:

Results were obtained from twelve animals with an average weight of 218.8gm (range 200 - 260gm). Analysis of counts from solvent control treated animals (n = 6) demonstrated a significant increase in total c-Fos-like immunoreactivity in the L6 and S1 segments in comparison to L5 (ANOVA [Dunns], $p < 0.05$) (see figure 59).

Comparison of the control and treated animals demonstrated that pre-treatment with palmitoylethanolamide (20mg/kg) was associated with a significant reduction (unpaired t-test, $p < 0.05$) in total c-Fos-like immunoreactivity at both L6 and S1 levels (see figure 59). An example of the level of c-Fos-like immunoreactivity observed is shown in figure 60.

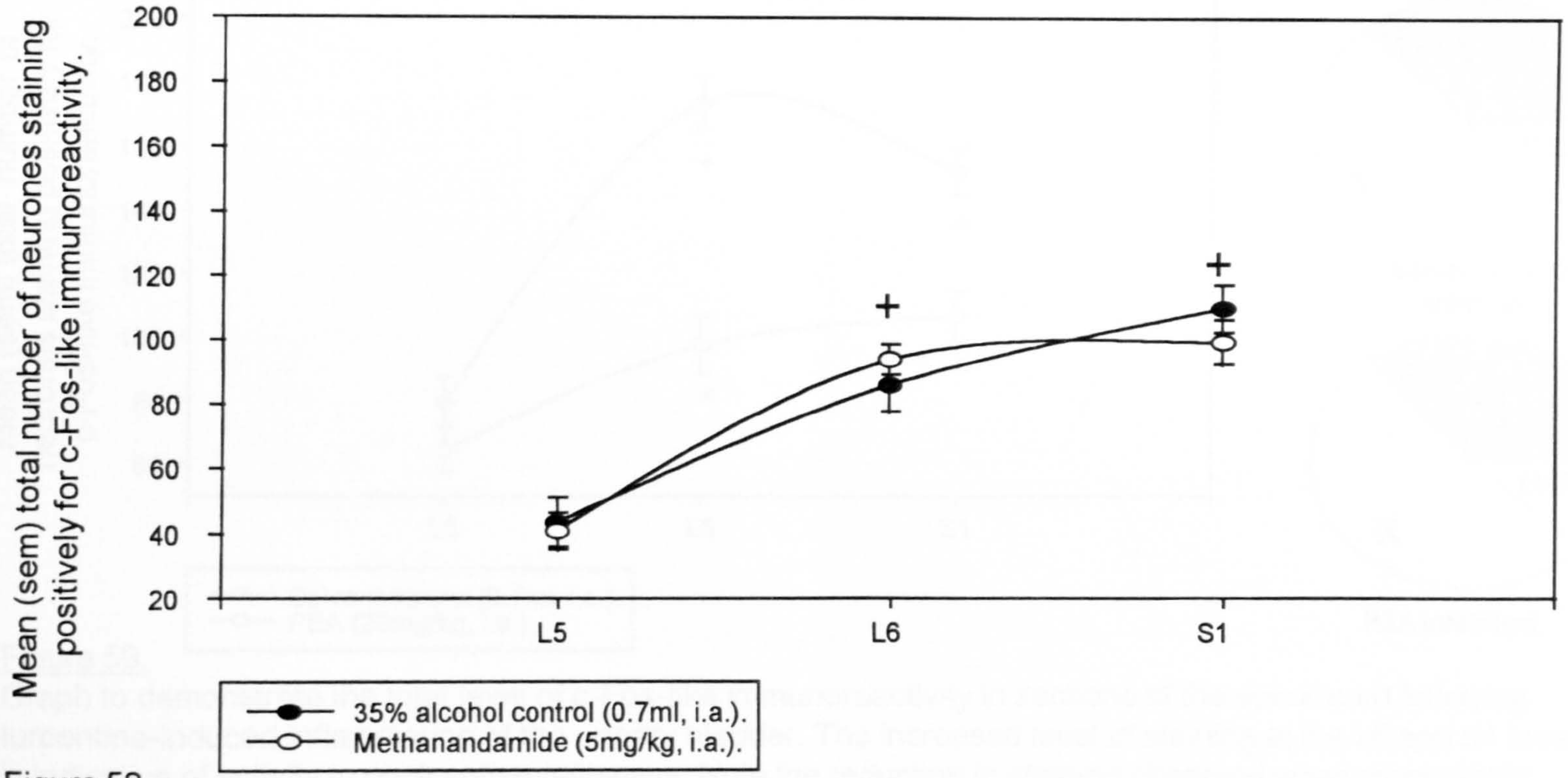



Figure 58.

Graph to show the total number of neurones in sections of the spinal cord demonstrating c-Fos-like immunoreactivity following turpentine-induced inflammation of the urinary bladder. An increased level of staining in the L6 and S1 spinal cord levels is observed. Also, note that the prophylactic administration of methanandamide is not associated with any significant reduction in staining, in comparison to control.

+ $p < 0.05$ in comparison to staining in the L5 spinal cord level (ANOVA [Dunns]).

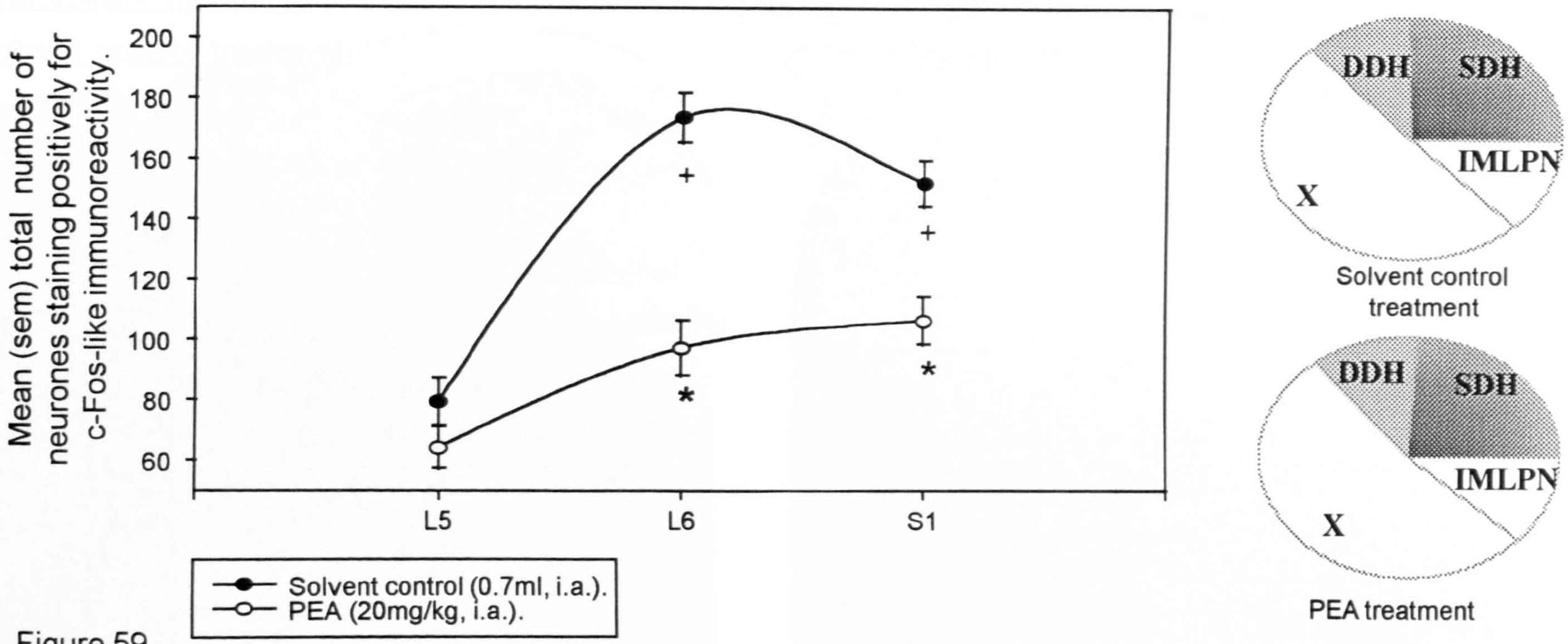



Figure 59.

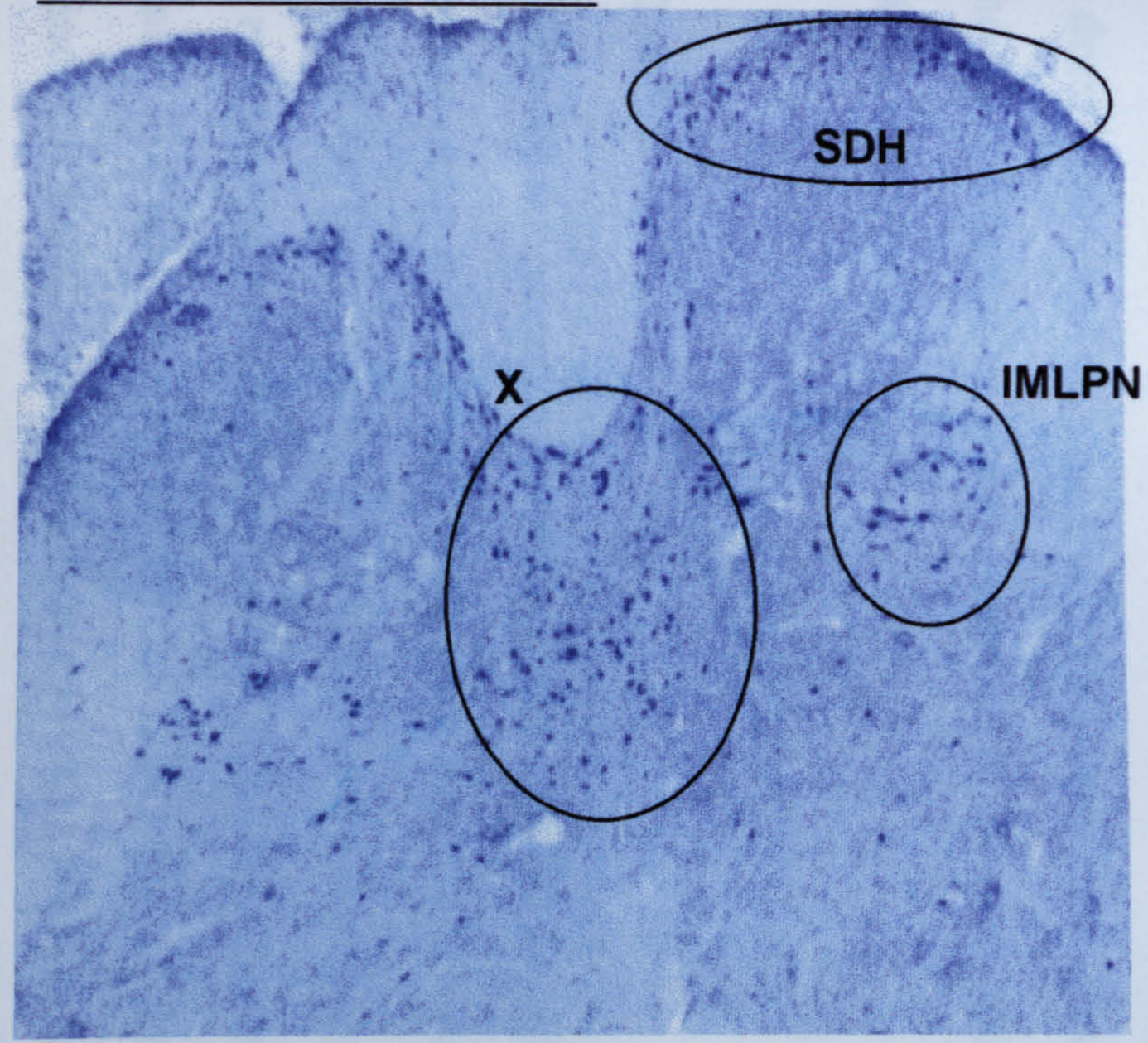
Graph to demonstrate the total level of c-Fos-like immunoreactivity in sections of the spinal cord following turpentine-induced inflammation of the urinary bladder. The increased level of staining at the L6 and S1 levels is indicative of activity in nociceptive pathways. Note the reduction in staining observed when prophylactic treatment with a CB2 receptor agonist has been administered.

\+ $p < 0.05$ in comparison to count at the L5 spinal level.

* $p < 0.05$ in comparison to staining observed in control animals, at the same level (unpaired t-test).

The pie charts to the right demonstrate the proportion of staining in each of the subareas counted at the L6 spinal level. Note that the pattern of staining is little changed by the administration of PEA (20mg/kg).

Solvent control treatment:




Palmitoylethanolamide treatment:

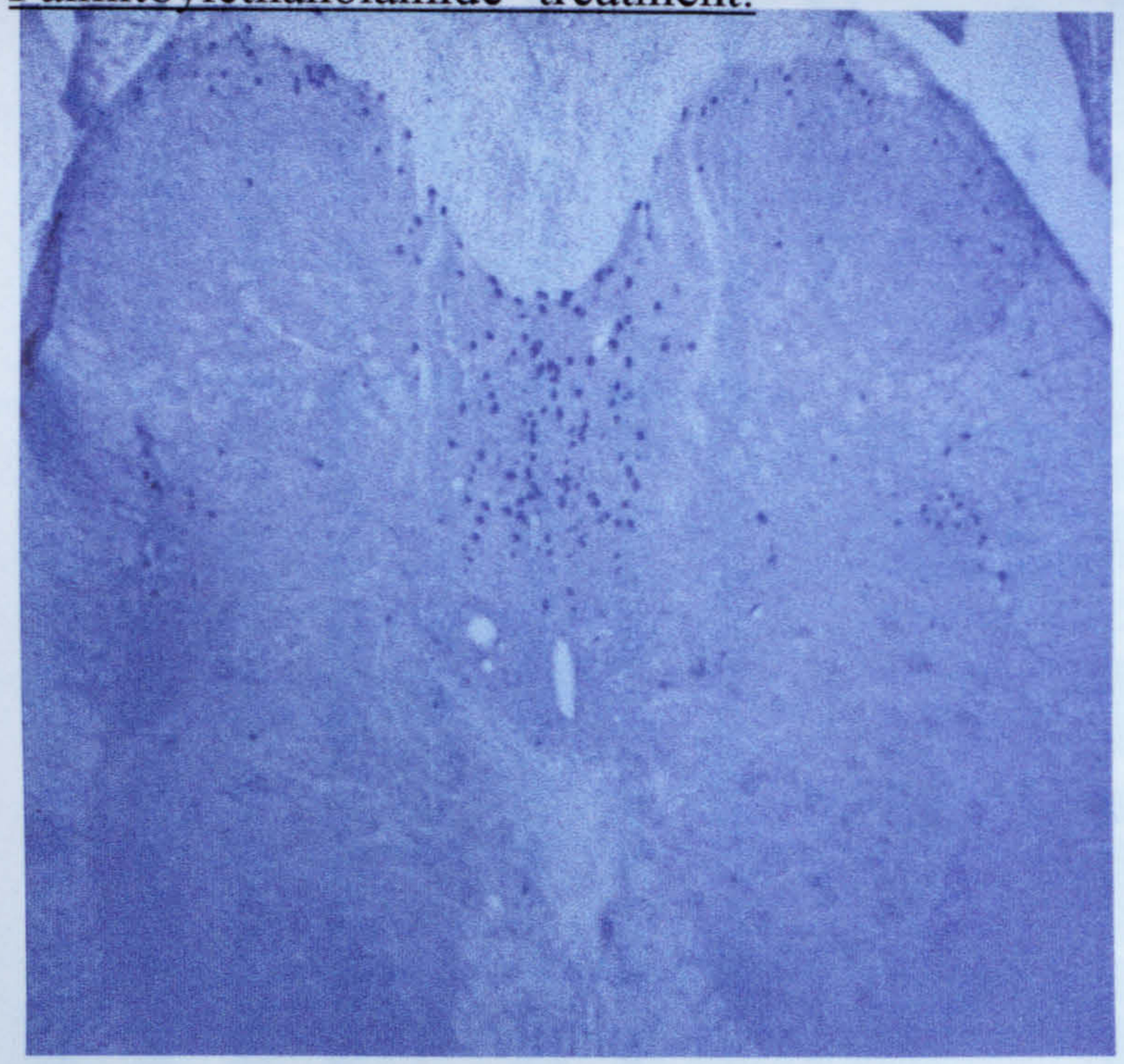


Figure 60:
Photographs of representative slices of the lumbar enlargement of the spinal cord stained to demonstrate c-Fos-like immunoreactivity. Note the heavy staining (black) in the superficial dorsal horn (SDH), intermediolateral posterior nucleus (IMLPN) and lamina X (X) when treatment with a solvent control substance was administered. This is reduced when active treatment with palmitoylethanolamide (20mg/kg, i.a.) is administered prophylactically.

Furthermore, at the L6 level there was a significant reduction (unpaired t-test, $p < 0.05$) in staining observed in all the sub-areas counted, in the palmitoylethanolamide treated group (in comparison to the control group). A similar finding was observed at the S1 level, in three of the four sub-areas counted (see table 21 in appendix 2).

WIN55,212-2 results:

Results were obtained from eleven animals with an average weight of 213.6gm (range 200 - 230gm). Analysis of counts from solvent control treated animals ($n = 6$) are described in the section on CB2 receptor agonist results (since the control animals were the same).

Comparison of the control and treated animals demonstrated that pre-treatment with WIN55,212-2 (5mg/kg) was associated with a significant reduction (unpaired t-test, $p < 0.05$) in total c-Fos-like immunoreactivity at both L6 and S1 levels (see figure 61). Furthermore, at all three spinal levels assessed there was a significant reduction in c-Fos-like immunoreactivity in the deep dorsal horn and intermediolateral posterior nucleus regions and at the L6 and S1 levels this was also observed in the peri-aqueductal region (see table 22 in appendix 2).

Control results:

In view of the unexpected lack of any significant difference from control values observed in the CB1 receptor agonist treated animals, a comparison of the three control groups (including the saline control utilised in the B2 receptor antagonist experiments) was undertaken. This demonstrated that there was no significant difference in the total c-Fos-like immunoreactivity between animals treated with saline ($n = 6$) or DMSO + saline (1 : 19) ($n = 6$) at any of the three spinal levels (figure 62). However,

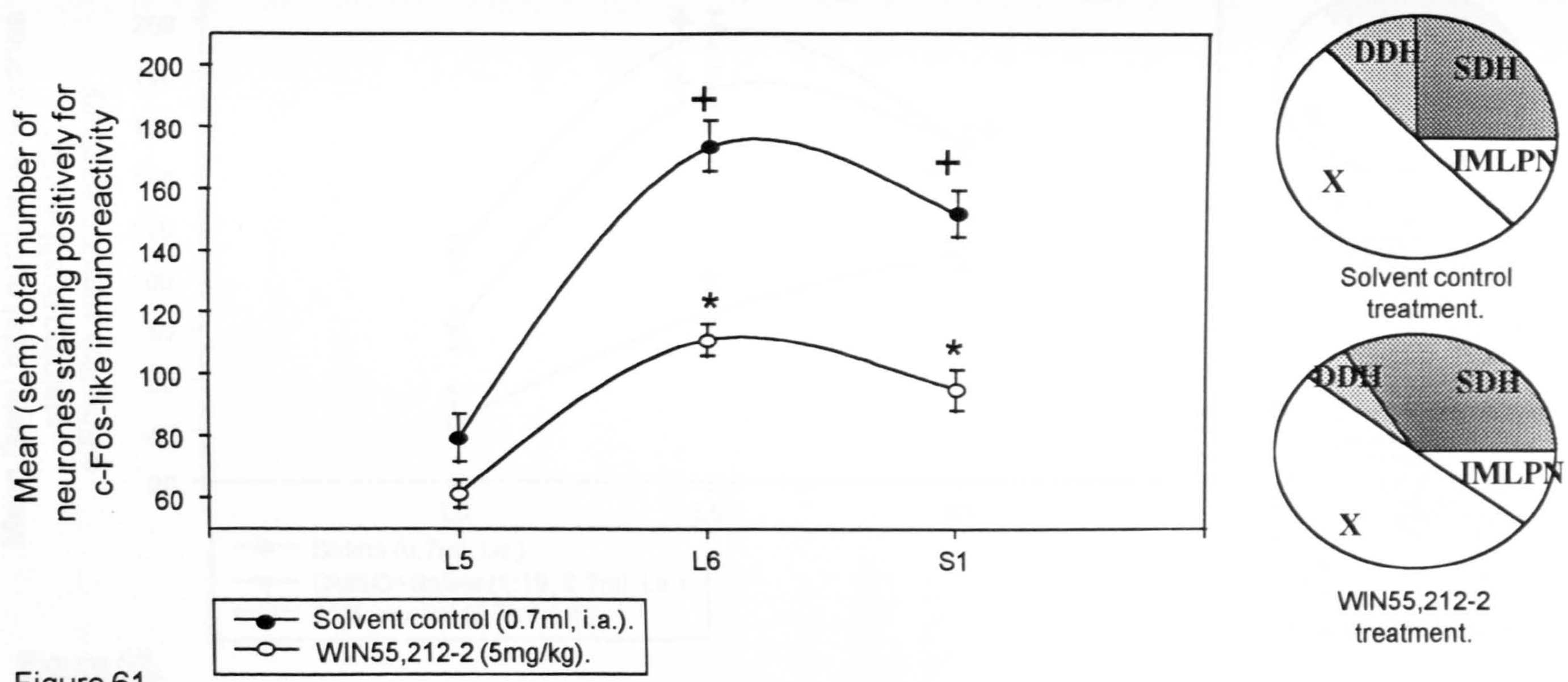



Figure 61.

Graph to demonstrate the total level of c-Fos-like immunoreactivity in sections of the spinal cord following turpentine-induced inflammation of the urinary bladder. The increased level of staining at the L6 and S1 levels is indicative of activity in nociceptive pathways. Note the reduction in staining observed when prophylactic treatment with WIN55,212-2 has been administered.

\+ $p < 0.05$ in comparison to count at the L5 spinal level.

* $p < 0.05$ in comparison to staining observed in control animals, at the same level (unpaired t-test).

The pie charts to the right demonstrate the proportion of staining in each of the subareas counted at the L6 spinal level.

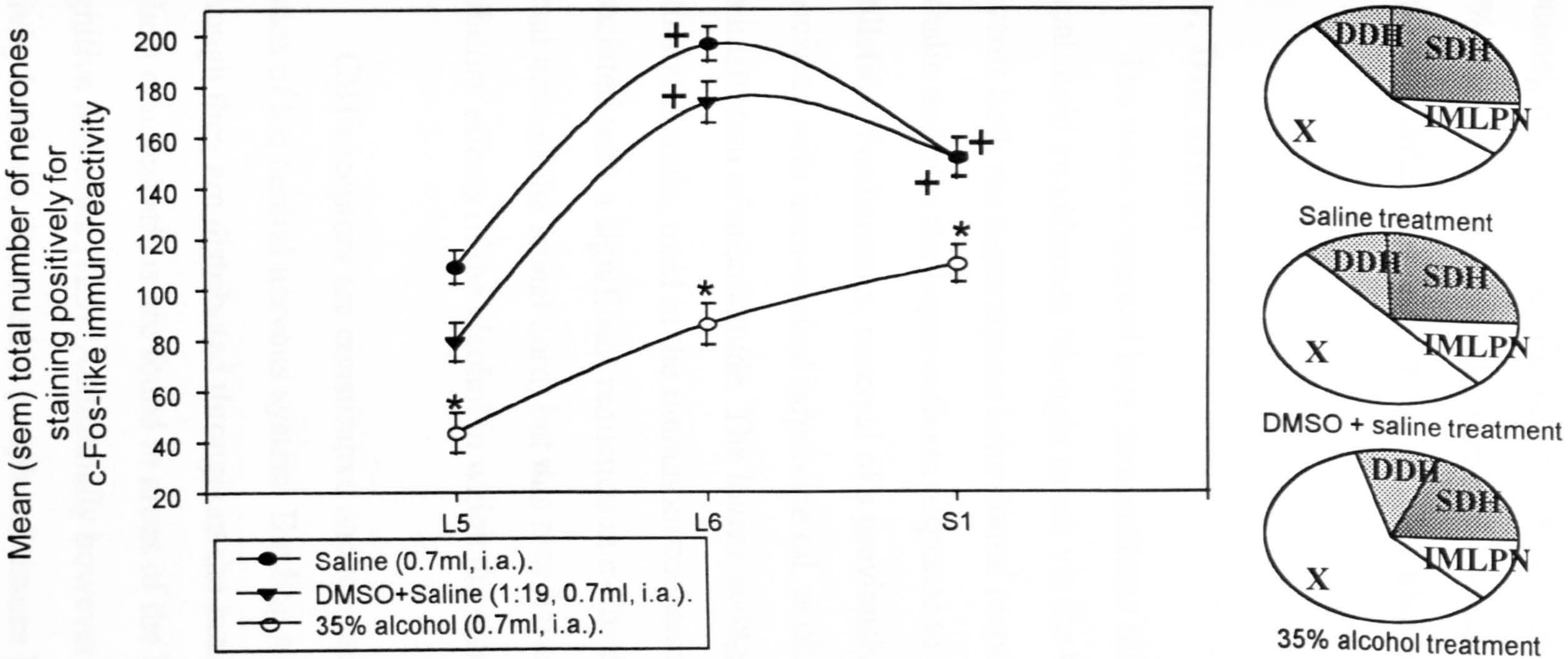



Figure 62.

Graph to demonstrate the total level of c-Fos-like immunoreactivity in sections of the spinal cord following turpentine-induced inflammation of the urinary bladder. The increased level of staining at the L6 and S1 levels is indicative of activity in nociceptive pathways. Note the reduction in staining observed when prophylactic treatment with the control substance 35% alcohol has been administered.

\+ $p < 0.05$ in comparison to count at the L5 spinal level.

* $p < 0.05$ in comparison to staining observed in control animals, at the same level (unpaired t-test).

The pie charts to the right demonstrate the proportion of staining in each of the subareas counted at the L6 spinal level. Note that the pattern of staining is little changed by the administration of 0.7ml, 35% alcohol.

the animals treated with 35% alcohol (n = 5) showed a significant reduction in total c-Fos-like immunoreactivity staining at both L5 and L6 levels in comparison to both saline and DMSO + saline treated groups (ANOVA [Dunns], $p < 0.001$) (see figure 62). Thus, it appears that pre-treatment with 35% alcohol reduces the induction of *c-fos* expression associated with turpentine inflammation of the rat urinary bladder.

## 6.iv. Discussion

The work presented here demonstrates that the endogenous cannabinoid anandamide (thought to act via the CB1 receptor [82,103]) prevents both the hyperalgesic behavioural response to sub-cutaneous formalin and also the hyper-reflexic response to intra-vesical turpentine instillation. Furthermore, reversal of a previously established VVH, associated with intra-vesical turpentine oil, is observed following systemic administration of anandamide. The longer acting synthetic analogue, methanandamide, used in the immunohistochemical studies, was not associated with a significant reduction in c-Fos-like immunoreactivity in the dorsal horn of the spinal cord, but this may have been related to significant inhibitory effects of the alcohol in which the compound was administered.

CB1 receptors are constitutive and are predominantly localised to tissues of the central nervous system. Binding studies have shown that although they are distributed throughout the brain and spinal cord, the highest concentrations are found in areas of the brain associated with higher cognitive functions [125]. Additionally however, CB1 receptor related activity has been observed in peripheral tissues [264] and the mRNA encoding the CB1 receptor has been identified in cell bodies of NGF-dependent primary afferent neurones [113].

The vesical hyper-reflexia observed following turpentine inflammation is mediated via a spinal reflex [218], which is influenced by both spinal glutaminergic (NMDA) [275] and nitric oxide systems [274]. In addition, the second phase of the behavioural response to formalin is, at least in part, a spinal phenomenon - again driven by ongoing afferent activity involving spinal NMDA and nitric oxide systems [132,198]. Therefore, it is possible that anandamide is exerting effects via activity at spinally located CB1 receptors. However, the potential for anandamide to influence events at a more peripheral site under inflammatory conditions, when the expression of CB1 receptors is upregulated [5], has been documented in the carrageenan model of paw inflammation [277]. This has obvious correlates with the peripheral action of opioids under inflammatory conditions [48,69,289,290,304,306,307]. Thus, although the most likely site of action of anandamide is perhaps the brain (where maximal CB1 receptor expression has been demonstrated to occur [125]), there is some evidence that the analgesic effects could, at least in part, be mediated by activity at spinally or peripherally located receptors.

The fact that administration of anandamide was not associated with attenuation of oedema formation in vesical tissue, despite a reduction in VVH, demonstrates a differential effect of CB1 receptor agonists activity on hyperalgesia and tissue inflammation responses. Whilst no such separation of effects is observed when cutaneous inflammation is induced by carrageenan [211], it is not without precedent [213]. Both non-steroidal anti-inflammatory drugs and paracetamol have been widely documented to be associated with separation between analgesic and anti-inflammatory effects [34,213]. Furthermore, as described in section 4.iv., a similar detachment in effects was observed when administering bradykinin receptor antagonists in the turpentine-inflamed rat urinary bladder model. As regards the cannabinoid ligands, the division in effects observed after anandamide

administration could be explained if the anti-hyperalgesic effect was mediated at a spinal. or higher, level, thus leaving unaltered the peripheral inflammatory processes. However, since non-steroidal anti-inflammatory drugs are unable to cross the blood-brain barrier, it seems likely that their action is predominantly peripheral (although there is some data to suggest central sites of action [197]) and they show a similar separation of effects [34,213]. Thus the separation of anti-hyperalgesic and anti-inflammatory responses should not be seen as conclusive proof of a central effect.

The observation that the level of c-Fos-like immunoreactivity (consequent upon upregulation of *c-fos* expression) was not reduced following administration of methanandamide is explained by the positive inhibitory effect of the solvent control solution (35% alcohol) to which the methanandamide treated group was compared. The attenuating effect of the alcohol was intense enough to mask any CB1 receptor mediated effect. Supporting evidence for this comes from my description here of both a profound inhibition of VVH following administration of the 35% alcohol control and also the observation that the total c-Fos-like immunoreactivity detected in the alcohol control group was significantly reduced at all three spinal levels in comparison to both saline and DMSO + saline control treated groups. Previous work sustaining this postulate includes the demonstration that intra-thecal ethanol is associated with attenuation of thermal hyperalgesia in rats, whether this is induced by peripheral (sciatic) nerve damage or central (intra-thecal) administration of chemicals (such as NMDA) [233]. Furthermore, *in vitro* evidence suggests that the function of the NMDA receptor is inhibited by pharmacologically relevant concentrations of ethanol [126]. Thus, in cultured cells, ethanol decreases NMDA channel opening (as demonstrated by patch clamping techniques) and NMDA-evoked calcium ion uptake and catecholamine release [126]. Additionally, *in vivo* evidence suggests an interaction between NMDA

receptors and alcohol. When rats are exposed to high levels of alcohol *in utero*, hippocampal neurones demonstrate a decreased sensitivity to NMDA stimulation when mature. Furthermore, pigeons trained to discriminate ethanol from saline, also respond to NMDA receptor antagonists [126]. Therefore, alcohol may exert an inhibitory effect on the spinal components of VVH, thus masking any cannabinoid effect.

The cannabinoid receptor ligand WIN 55,212-2 has been demonstrated to exhibit high affinity for the CB1 receptor ($K_i$ 1.9nM). When administered prior to inflammation of the paw with sub-cutaneous formalin, a reduction in both pain related behaviour and also c-Fos-like immunoreactivity in the spinal cord is observed [327]. Furthermore, when WIN 55,212-2 is administered prophylactically, the activity in wide dynamic range neurones of the dorsal horn of the spinal cord of anaesthetised rats is decreased in response to noxious thermal stimulation [140]. However, although this agonist has high affinity for the CB1 receptor, it shows relative selectivity for the CB2 receptor ($K_i$ 0.3nM) [21]. Therefore the inhibition of upregulation of c-Fos-like immunoreactivity that was observed here following the intra-vesical instillation of turpentine can be explained by activity at either the CB1 or the CB2 receptor site. This is also the case for the inhibition of upregulation of c-Fos-like immunoreactivity reported by others after the sub-cutaneous injection of formalin [327]. To conclusively exclude CB2 receptor mediated effects, co-administration of CB2 receptor antagonists with WIN 55,212-2 would be required.

The data presented here also demonstrates that whilst palmitoylethanolamide (PEA), a putative CB2 receptor agonist [101], is associated with the reversal of an established VVH, no such effect is observed when the compound is administered in a pre-emptive paradigm. In the light of this it is perhaps surprising that the prophylactic administration

of PEA was associated with a reduction in both pain-related behaviour in the second phase of the response to sub-cutaneous formalin injection and also to the level of c-Fos-like immunoreactivity in the spinal cord following turpentine-induced inflammation of the bladder.

The results in the turpentine-inflamed rat urinary bladder model provide some physiological support for the concept that PEA exerts its effect via actions involving CB2 receptors, perhaps located on mast cells. If the anti-hyperalgesic actions of PEA are exerted via neuronally located receptors, the expectation would be that, like anandamide, both prevention and reversal of the turpentine-associated hyper-reflexia of the urinary bladder would be observed. However, if effects are mediated via a CB2 receptor mechanism, no effect would be expected until time dependent mastocytosis (associated with immune cell entry into inflamed bladder tissue) had occurred. This data is analogous to the findings presented in chapter 4 with regard to the bradykinin B1 receptor antagonists.

The observation that pre-emptive administration of PEA attenuates the second phase of the behavioural response to sub-cutaneous formalin is therefore somewhat at odds with the results from the inflamed bladder model. As with the inflammatory processes occurring in the visceral domain, the accumulation of mediators following formalin injection is a time dependent process, with maximal response being observed at four hours post-injection [323]. Thus, it is difficult to explain how PEA could be exerting its effects via CB2 receptors present on immune cells, unless chemotaxis of critical elements occurs more rapidly in the somatic domain than in the viscera. Conversely, whilst the second phase of the behavioural response to formalin is partially a centrally mediated phenomenon, there remains evidence that ongoing peripheral activity is also important [131,212] and it may be that it is this component that is affected by PEA treatment. Unfortunately, this does not explain the discrepancy between the results of

prophylactic administration of palmitoylethanolamide in the bladder reflex and immunohistochemical experiments.

However, at least in the visceral domain, the results of the reflex experiments utilising palmitoylethanolamide provide support for the importance of the autacoid local inflammation antagonism (ALIA) concept. Autacoid local inflammation antagonism would allow for the negative modulation of the inflammatory process at the level of the mast cell (see figure 63). Agonist activity at the CB2 receptor located on mast cells, would downregulate the release reaction during inflammation, by inhibiting responses to and production of NGF, thus interrupting the positive feedback cycle. Hyperalgesic responses would then be indirectly reduced by a concomitant reduction in the barrage of activity reaching the dorsal horn of the spinal cord via primary afferent neurones.

If PEA only exerts effects via an inhibition of mast cell activity, the reduced expression of the immediate early gene *c-fos* following bladder inflammation seems somewhat surprising. However, the previous description of a cannabinoid receptor ligand with relative selectivity for the CB2 receptor (WIN 55,212-2) [21] producing inhibition of *c-fos* expression in the spinal cord following paw inflammation [327] would tend to support the findings presented here of CB2-mediated inhibition of c-Fos-like immunoreactivity following vesical inflammation.

Whilst CB2 receptors themselves have as yet been identified almost entirely in peripheral tissues [101,115,157,264], a negative regulatory effect of palmitoylethanolamide has been observed in the cerebellar granule cell, where it has been shown to afford protection against glutamate induced neurotoxicity [300]. If CB2 receptors were to be identified on centrally located mast cells (such as those observed in the plaques of multiple sclerosis lesions [255]) the development of novel analgesic agents devoid of

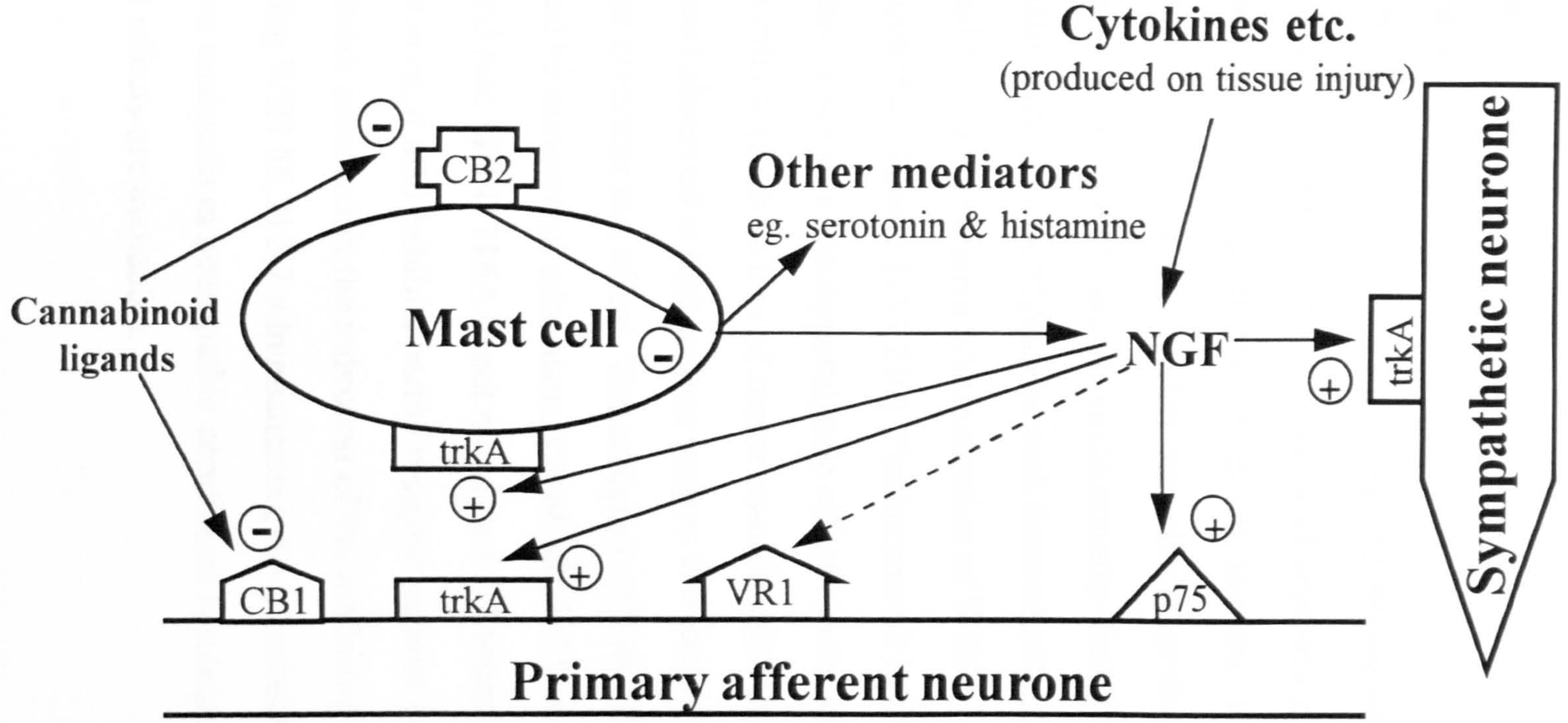



Figure 63.
Diagram demonstrating the putative interaction between nerve growth factor (NGF) and cannabinoids. During inflammation a positive feedback loop results in the rapid production of algogenic mediators such as NGF. Cannabinoid ligands (produced in inflamed tissues) potentially interupt the extreme release reaction of the mast cell by autacoid local inflammation antagonism (ALIA). Furthermore attenuation of the response of the primary afferent neurone, to compounds capable of direct neuronal activation, may further inhibit nociceptive activity in the periphery.

central psychotomimetic side effects might be achieved for use in these difficult clinical scenarios (assuming that receptors are not also expressed on neurones of the central nervous system). There is some early evidence suggesting that CB2 receptors are located on microglial cells of the central nervous system [157], but as yet no record of such receptors on neuronal tissue is available.

It has further been suggested that the development of specific CB2 receptor agonists affords the possibility of separating useful analgesic effects and undesirable central effects. Moreover, this may be an achievable goal in the management of another challenging clinical problem - that of neuropathic pain. In rats with a unilateral mononeuropathy (induced by loose ligation of the sciatic nerve), hyperalgesia to both thermal and mechanical stimulation is reversed by the subsequent administration of WIN 55,212-2 or palmitoylethanolamide [137,210]. Furthermore it has been shown that at moderate doses this anti-hyperalgesic effect can be attained in the absence of side effects (such as loss of motor co-ordination and extreme startle reactions) observed at higher dose ranges. It is currently unclear which receptor mediates this effect. The analgesic effects of WIN 55,212-2 are reversed by concurrent administration of SR141716A. However, it is now accepted that SR141716A is not merely a CB1 receptor antagonist and at least *in vivo*, it also exhibits partial receptor agonist activity. Whatever the mechanism involved in the induction of the inhibitory effects observed following WIN 55,212-2 administration, the possibility of providing effective analgesia in neuropathic conditions is intriguing, particularly if central effects are avoidable.

### 6.iv.a. Clinical Relevance:

The potential for harnessing the positive analgesic effects of derivatives of *Cannabis Sativa*, which lack the pro-emetic features of opioids and the renal toxicity of non-steroidal anti-inflammatory drugs has been speculated upon for centuries. In recent times their potential use in the treatment of resistant pain has been ignored, because of the central psychotomimetic side effects [40]. However, if at least a proportion of the analgesic effects of the plant are consequent upon activity at peripheral receptor sites, the possibility of divorcing the analgesic properties from the less politically acceptable central nervous system effects arises. The data presented here, suggest that, at least in persistent inflammatory conditions (arising from both somatic and visceral structures) specific agonists acting at the immune cell associated CB2 receptor, may be efficacious in the prevention and treatment of associated hyperalgesic responses. Furthermore, since CB2 receptors are known to be located on mast cells in peripheral tissue [101,115] and mast cells have been located in plaques of multiple sclerosis lesions [255], this may provide an explanation for the anecdotal reports of improvement of multiple sclerosis-associated symptoms after inhalation or ingestion of extracts of the hemp plant [40,116,207]. If indeed CB2 receptors are expressed on immune cells of the central nervous system [157], the potential for both the analgesic and primary therapeutic effects to be harnessed is enticing. Furthermore, since these compounds are well absorbed from the bronchial tree, the development of inhalers to deliver such compounds with rapid onset should be possible.

An alternative potential mechanism for achieving analgesia in the absence of central nervous system effects is the development and utilisation of CB1 receptor agonist agents that are incapable of crossing the blood brain barrier. Such agents would, *a priori*, exert any therapeutic effects via peripherally located receptors. The data I have presented here confirm that

CB1 receptor ligands have analgesic effects in persisting somatic inflammation and document a similar effect in persistent visceral inflammation. I provide no concrete evidence as regards the potential site of action of the agents, however, evidence has recently been presented in the literature to suggest that peripheral effects may be of some importance [277]. As with peripheral opioid administration [155,303-305], peripheral application of CB1 receptor ligands as been shown to be associated with analgesic effects at doses which are sub-therapeutic when administered systemically [277]. Thus in the future peripheral application of CB1 receptor agonists, or the systemic administration of CB2 receptor agonists, may provide an alternative therapeutic intervention for patients who are unable to tolerate more conventional analgesic regimens.

# Chapter 7:

# General conclusions and further work.

Pain persisting over prolonged periods of time is a significant problem for individual sufferers. Furthermore, is also potentially of major socio-economic importance. Pain arising from the viscera is, in terms of patient numbers a far greater clinical problem than that arising from cutaneous tissues [221]. Thus, the investigation of the pathophysiological mechanisms underlying the phenomenon of persistent visceral pain and the development of agents that might attenuate such pain, is beguiling. Such agents would ideally not only demonstrate analgesic efficacy, but would also be devoid of the unwanted side effects observed following administration of currently available compounds.

Significant problems consequent upon the administration of analgesic agents include the well documented side effects of nephrotoxicity and bronchoconstriction following non steroidal anti-inflammatory drug administration; whilst those of opioids are mainly related to the inhibitory effects observed over widespread areas of the central nervous system, including the cardiac and respiratory centres (notes included in the British National Formulary). Since these side effects are by no means minor in nature, the development of novel compounds might be expected to encourage alterations in clinical prescribing practices. This would be of particular relevance in the management of pain arising from visceral structures, since historically such pain has been poorly treated. Therefore the work presented here addressed the possibilities provided by agents acting upon bradykinin, vanilloid and cannabinoid receptors sites.

The turpentine-inflamed rat urinary bladder model:

Chemical inflammation of the rat urinary bladder has been exploited, for over a decade, to investigate mechanisms of pain arising from the viscera [35,36,89-91,215,218,253,275]. However little information regarding the referral of symptoms to distant sites is available [218]. Since this is a characteristic feature of pain arising in the viscera, documentation of such effects would further validate the model. Here I have documented the presence of a referred hyperalgesia to both thermal and mechanical stimuli, following vesical inflammation. The sensitisation of dorsal horn neurones already described [215] endorses the convergence-facilitation theory of referred pain. Only a very small proportion of primary afferent neurones have been demonstrated to innervate both somatic and visceral targets [9,77,267]. However, if sensitisation of the L5, L6 and S1 afferent neurones supplying somatic tissues could be demonstrated following vesical inflammation, this would lend support to the axon reflex theory described by Sinclair [221,299]. Absence of any such sensitisation would further bolster the currently popular convergence-facilitation theory.

Many of the features of the turpentine inflamed bladder have been replicated by the intra-vesical instillation of NGF, namely: viscero-visceral hyper-reflexia (VVH) [91], sensitisation of primary afferent neurones [90], upregulation of *c-fos* in the spinal cord [89] and the referred somatic hyperalgesia (VSH) described here in chapter 3. It would therefore be interesting to document the presence of any sensitisation of dorsal horn neurones or absence of sensitisation of afferent neurones innervating somatic tissues, at the L5, L6 and S1 levels, following the intra-vesical instillation of NGF. Such findings would again tend to sustain the convergence-facilitation theory as the physiological mechanism underlying the referral of symptoms and signs to sites distant from the initiating tissue damage.

Bradykinin receptor antagonists:

The work presented in chapter 4 suggests that agents demonstrating antagonist activity at either the bradykinin B2 or B1 receptor sites might usefully be further investigated as potential analgesics in conditions where visceral inflammation is associated with pain. I have demonstrated an inhibition of both the VVH and the immediate early gene expression associated with turpentine-induced inflammation of the urinary bladder. Others have reported that intra-vesical NGF is associated with a similar inflammation to that evoked following intra-vesical turpentine instillation [89-91]. The expression of bradykinin binding sites on dorsal root ganglion cells is regulated by NGF [265] and NGF receptors are most highly expressed on afferent neurones supplying visceral tissues [219]. Further experiments assessing the effects of bradykinin receptor antagonists upon reflex, neuronal and genetic changes associated with intra-vesical NGF instillation would clarify the inter-relationship between the two mediators.

Since NGF demonstrates intimate links with the algogenic responses to bradykinin [265] NGF-sequestering agents might be expected to effect changes in sensitivity to administration of bradykinin receptor antagonists. The possibility that responses to alternate algogens are also NGF-dependent is exciting. If this were the case, treatments aimed at decreasing NGF-mediated effects might demonstrate greater efficacy than those aimed at alternate single targets. Thus, the NGF-dependence of alternate receptor systems (including the vanilloid receptor system) merits investigation in this model. Alternatively, combinations of antagonists of NGF and other mediators (including bradykinin) may demonstrate additive or synergistic effects.

Capsaicin-like agents:

In the work presented in chapter 5, I have demonstrated that a systemically administered capsaicin-like agent is capable of attenuating both the VVH and VSH associated with intra-vesical turpentine instillation. Since at body temperature, the VR1 receptor, located on primary afferent neurones, can be activated by hydrogen ions [324], this provides an alternative mechanism by which features of visceral pain, including VVH, are induced. Thus, under inflammatory conditions, agents acting via the VR1 receptor may prevent the VR1-mediated effects of protons, released locally from damaged tissues. The fact that VR1 is expressed in an NGF-dependent fashion suggests that the synergism (or otherwise) of agents inhibiting the action of NGF and desensitising the primary afferent neurone should be investigated.

Capsaicin itself, is currently administered in the clinical scenario for the treatment of both hyper-reflexia [68,119,191] and pain [20,44] arising from the urogenital system. However, the early pro-algesic effects and the requirement for recurrent catheterisation, limit the acceptability of the treatment to both doctors and patients alike. The production of compounds similar to SDZ249-665, effective following systemic (and preferably oral) administration, might have a major clinical impact. Initial development of such compounds would demonstrate similar efficacy to SDZ249-665 in a variety of animal models of visceral and somatic pain. Following toxicity studies in animals, clinical trials in controlled settings would be appropriate. These trials should be randomised, controlled, double blind studies in large selected patient groups, to ensure efficacy, over and above the placebo response.

Cannabinoid receptor agonists:

The evidence presented in chapter 6 demonstrates that prophylactic administration of anandamide prevents hyper-reflexic and hyperalgesic changes in both visceral and somatic domains. Furthermore, when administered after the induction of inflammation, the compound reverses the hyper-reflexic changes observed in the urinary bladder. Since CB1 receptors have been demonstrated to be expressed outside the central nervous system [103,113,264,277], it would be interesting to question the site of action of anandamide by which the effects I have documented here are produced. Thus the ability of anandamide to inhibit the sensitisation of primary afferent neurones in the pelvic nerve, observed during inflammation of the viscera [90], should be addressed. Furthermore, the specific exclusion of central effects should be attempted. This could be achieved by the administration of intra-thecal cannabinoid receptor antagonists, in conjunction with systemic anandamide administration, whilst monitoring changes in reflex activity of the bladder by means of cystometrography. Topical application of anandamide to the bladder might prove more problematic due to the pharmacokinetic problems associated with variable absorption across bladder mucosa [90]. Additionally, the effects of anandamide upon the referred VSH should be addressed. If anandamide were to prevent VSH (in a similar manner to trkA-IgG and SDZ249-665), the question as to the peripheral or central site of action of such attenuation of central sensitisation should be addressed. Systemic administration of anandamide, with concurrent intra-thecal administration of cannabinoid receptor antagonists would again be an appropriate technique.

In addition to the data relating to anandamide, I have demonstrated that the administration of palmitoylethanolamide is associated with reversal of an established VVH, but not prevention of onset when administered prophylactically. This is somewhat surprising in view of the inhibition,

following such prophylactic administration, of both: the second phase of the behavioural response to formalin and also the upregulation of c-Fos-like immunoreactivity in the dorsal horn of the spinal cord (following intra-vesical turpentine instillation). Therefore, the site and mechanism of action of palmitoylethanolamide requires some elucidation. Initially any peripheral expression of CB2 receptors, particularly under inflammatory conditions (such as those induced by intra-vesical turpentine oil) should be assessed. Demonstration of CB2 receptors in bladder tissue, by the pharmacological means used to document CB1 receptors [264], would be a first step. Immunohistochemical techniques might be attempted, but low levels of receptor expression and the difficulty of producing effective primary antibodies against CB2 would risk false negative results being obtained. *In situ* hybridisation studies, to document the presence of mRNA encoding the CB2 receptor might overcome these problems. As regards central expression of CB2 receptors, further confirmation of early results is required [157]. An intriguing possibility is that of the presence of CB2 receptors on mast cells, present in inflammatory plaques of the spinal cord. Again, *in situ* hybridisation studies might address this problem.

For novel analgesic drugs to be influential in the clinical scenario, the avoidance of common depressant effects involving the cardio-respiratory centre is mandatory. The avoidance of such central effects can be approached in several ways. Drugs unable to cross the blood-brain barrier might be developed, so that even were receptors to be present both peripherally and centrally, inadequate drug levels at the active site in the central nervous system would be attained. This is of particular relevance to CB1 receptor agonist drugs, since maximal levels of CB1 expression has been observed to occur in brain tissue.

Unfortunately, even poorly lipid soluble drugs may reach the central nervous system under conditions of failure of the blood-brain barrier. Therefore the development of compounds acting at receptors that are present at only extremely low levels in the brain and spinal cord is another option. Currently there is only one report of the presence of CB2 receptors in the central nervous system [157] and these are located on non-neuronal elements. If the central expression of CB2 receptors is indeed minimal, agents effecting modulatory changes via action at this receptor may inhibit nociceptive activity by purely peripheral mechanisms and thus avoid central side effects. This is of particular interest, since CB2 receptor expression is upregulated under inflammatory conditions, when the blood brain barrier is most likely to be compromised. Therefore, not only would peripherally mediated side effects be expected to be minimal when inflammation was absent, but central side effects would be avoided, even during inflammatory episodes.

Since the neurotrophin, NGF, appears to be pivotal in the visceral inflammatory response, further investigation of the effects of cannabinoid receptor agonists upon NGF-induced vesical inflammation would be enlightening. Such investigation should involve the assessment of the effects of cannabinoid receptor agonists upon physiological reflex, electrophysiological, somatosensory and immunohistochemical experimental systems.

In summary, the work presented here has:

- Further validated the chemically-inflamed rat urinary bladder, as an appropriate model in which to study visceral pain mechanisms, by the documentation of signs referred to somatic sites distant from the initiating nociceptive stimulus.
- Demonstrated an NGF-dependence of the referred somatic signs, similar to that previously documented for other features of the model, including: viscero-visceral hyper-reflexia, sensitisation of primary afferent neurones and upregulation of the immediate early gene, *c-fos*.
- Demonstrated that manipulation of the responses to chemical mediators, tending to interrupt the positive feedback mechanism in which NGF maintains a pivotal role, may attenuate pain-related responses during visceral inflammation.

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# Appendix 1

# Drug information:

Acetone / Sodium sulphide extraction fluid:

70mls acetone mixed with 3mls of 5% sodium sulphate (white powder supplied by BDH and diluted in distilled water) and 27mls of distilled water. Any precipitate present was filtered prior to use. Solution stored at room temperature in a dark cupboard.

Anandamide (ANA):

An acylethanolamide endogenous cannabinoid ligand of MW 347.5gm, supplied by Sigma as a light yellow oil. Diluted in dimethyl sulphoxide (DMSO) and saline (1:19) to a concentration of 5mg/ml. Stored in 2ml aliquots at -20$^0$C, for no longer than one month.

Biotinylated goat α-rabbit (Secondary antibody):

Supplied by Vector

200μL aliquots stored at -70$^0$C

Diluted in 7.8ml of (PBS + 0.2% Triton-X-100 + 0.1% Azide) producing a 1/400 solution

Cryoprotectant:

240gm sucrose (from BDH Laboratory supplies, as a white crystalline solid) was added to 400ml 0.01M phosphate buffered saline (PBS) and 240ml ethylene glycol (supplied by Sigma as a clear, colourless solution).

3,3'-Diaminobenzidine (DAB):

DAB kit (SK-4100) purchased from Vector Laboratories Ltd (Peterborough, UK). To 5ml of distilled water, 2 drops buffer solution, 4 drops DAB and 4 drops nickel sulphate were added. Just prior to use 2 drops hydrogen peroxide added. The brown product developed in 2 to 3 minutes.

des-Arg$^9$[Leu$^8$]-bradykinin:

A peptide B1 receptor antagonist, MW 972.1gm, supplied by Sigma as a white solid and stored at -20$^0$C. Reconstituted in sterile 0.9% saline to a concentration of 2mg/ml stock solution in 2ml aliquots. Each aliquot stored at -20$^0$C, for no longer than one month.

Formalin:

10% formalin (supplied by Sigma as a clear fluid) was diluted with 0.9% saline to a 2.5% solution.

HOE 140:

A peptide B2 receptor antagonist (D-Arg[Hyp$^3$,Thi$^5$,D-Tic$^7$,Oic$^8$]BK) with MW 1304.6gm, (a gift of Hoechst A.G.) supplied as a white solid and stored at -4$^0$C. Reconstituted in sterile 0.9% saline to a concentration of 2mg/ml and stored at -4$^0$C in 2ml aliquots for no longer than 1 month.

Methanandamide:

An analogue of anandamide of MW 361.57gm, supplied by Sigma as a solution in 70% alcohol. Diluted with saline (1:1) to a concentration of 5mg/ml. Stored in 2ml aliquots at -20$^0$C, for no longer than 1 month.

Nerve growth factor (NGF):

An endogenous neurotrophin presented as a clear, colourless fluid in a sodium acetate / sodium chloride buffer at a concentration of 1mg/ml (a gift from Genentech). This was stored at 4$^0$C and diluted in 10% DMSO (in saline) to a final concentration of 20µg/ml.

10% normal goat serum:

2.5ml aliquots (supplied by Quadratech) were kept at -70$^0$C. This stock solution was diluted in 22.5ml of (PBS + 0.2% Triton-X-100 + 0.1% Azide) and stored at 4$^0$C for no longer than one month

Palmitoylethanolamide (PEA):

An acylethanolamide cannabinoid ligand of MW 299.5gm, supplied by Tocris Cookson as a clear, colourless oil, stable at room temperature.

Diluted in DMSO and saline (1:19) to a concentration of 5mg/ml. Stored in 2ml aliquots at -4$^0$C, for no longer than 1 month.

4% paraformaldehyde fixative:

A cross-linking fixative agent which can therefore induce conformational changes in proteins, hence the possible false negatives (due to modification of reactive groups) and false positives (due to linkage of low MW antigens to structural proteins).

40gm paraformaldehyde (white powder, supplied by TAAB Laboratories Equipment Limited, Berks.) was added to 500mls distilled water and heated *to 60$^0$C. 4-8 drops of 40% sodium hydroxide (NaOH) were added to clear* the solution and finally 500mls of 0.2M phosphate buffer (pH 7.4) was added. The solution was then cooled to 4$^0$C.

0.2M phosphate buffer:

Solution A was added to solution B until pH reached 7.4 (usually approximately 350ml).

Solution A: 15.6gm sodium dihydrogen orthophosphate dihydrate (Sigma) dissolved in 500ml distilled water.

Solution B: 107.55gm disodium hydrogen orthophosphate dodecahydrate (Sigma) dissolved in 1.5L distilled water.

Phosphate buffered saline (PBS):

87.6gm sodium chloride (Sigma) and 2gm potassium chloride (Sigma) were dissolved in 500ml 0.2M phosphate buffer. This was diluted with 9.5L distilled water.

Rabbit α-c-*fos* (Primary antibody):

200μL aliquots (supplied by Oncogene Science) were stored at -70$^0$C. These were diluted in 7.8ml of (PBS + 0.2% Triton-X-100 + 0.1% Azide) producing a 1/10,000 solution, which was stored at 4$^0$C for no longer than one month

SDZ249-665:

A capsaicin analogue donated by Novartis, London, UK). Diluted in DMSO to a stock concentration of 2mg/ml. Stored at $4^0$C for no longer than one month.

20% sucrose:

10gm sucrose (from BDH Laboratory supplies) presented as a white crystalline solid) was added 25ml 0.2M phosphate buffer and 25ml distilled water.

Triton X-100:

A polyoxyethylene, non-ionic detergent which disrupts cell membranes, allowing the entry of Ab into the intra-cellular compartment.

Presented as a clear, colourless solution, kept at room temperature.

trk-A-IgG:

A fusion molecule (see figure 15), sequestering NGF. Presented as clear colourless solution (kindly supplied by Genentech) and stored at -$4^0$C. Diluted in sterile 0.9% saline and stored in 2ml aliquots at -$70^0$C for no longer than one month.

Turpentine oil:

Turpentine diluted 1:1 with extra virgin olive oil (Marks & Spencer) and kept at room temperature for no longer than one week.

Urethane:

An ethyl carbamate anaesthetic agent, molecular weight (MW) 89.09gm. Supplied by Sigma as a white crystalline powder and reconstituted with 0.9% saline to a 25% solution. Once reconstituted it was stored at room temperature for no longer than 1 week.

Vectastain ABC system:

ABC kit PK-6100 supplied by Vector Laboratories Ltd (Peterborough, UK). 20μL of solution A was added to 20μL of solution B and 0.96ml PBS 30min prior to use.

# Appendix 2

# Results tables:


Table 1 The effects of all compounds tested on the mean (sem) micturition reflexes of the uninflamed bladder.

Table 2 The effect of pre-inflammation administration of HOE140 on mean (sem) micturition reflexes.

Table 3 The effect of pre-inflammation administration of des-Arg$^9$[Leu$^8$]-BK on mean (sem) micturition reflexes.

Table 4 The effect of early post-inflammation administration of HOE140 on mean (sem) micturition reflexes.

Table 5 The effect of early post-inflammation administration of des-Arg$^9$[Leu$^8$]-BK on mean (sem) micturition reflexes.

Table 6 The effect of late post-inflammation administration of HOE140 on mean (sem) micturition reflexes.

Table 7 The effect of late post-inflammation administration of des-Arg$^9$[Leu$^8$]-BK on mean (sem) micturition reflexes.

Table 8 Mean (sem) plasma extravasation into vesical tissue following administration of bradykinin receptor antagonists.

Table 9 Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in HOE140 (or control) treated animals.

Table 10 The effect of pre-inflammation administration of SDZ249-665 on mean (sem) micturition reflexes.

Table 11 Mean (sem) plasma extravasation into vesical tissue following administration of the capsaicin analogue SDZ249-665.

Table 12 The effect of pre-inflammation administration of ANA on mean (sem) micturition reflexes.

Table 13 The effect of pre-inflammation administration of PEA on mean (sem) micturition reflexes.

Table 14 The effect of post-inflammation administration of ANA on mean (sem) micturition reflexes.

Table 15 The effect of post-inflammation administration of PEA on mean (sem) micturition reflexes.

Table 16 The effect of post-inflammation administration of methanandamide on mean (sem) micturition reflexes.

Table 17 Mean (sem) plasma extravasation into vesical tissue following administration of cannabinoid receptor agonists.

Table 18 The effect of ANA administration on the weighted composite pain score (CPS-$WST_{0,1,2}$) for the first and second phases of the behavioural response to sub-cutaneous formalin.

Table 19 The effect of PEA administration on the weighted composite pain score (CPS-$WST_{0,1,2}$) for the first and second phases of the behavioural response to sub-cutaneous formalin.

Table 20 Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in methanandamide or 35% alcohol (control) treated animals.

Table 21 Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in PEA or solvent control (DMSO : saline, 1 : 19 dilution) treated animals.

**Table 1:**

**The effects of all compounds tested on the mean (sem) micturition reflexes of the uninflamed bladder:**

| Test compound. | $V_{mic}$ % of baseline at 1hr post-administration. | $Dur_{mic}$ % of baseline at 1hr post-administration. |
|---|---|---|
| HOE140 (n = 5) | 88.4 +/- 13.6 | 119.0 +/- 10.2 |
| des-Arg$^9$[Leu$^8$]-BK (n = 6) | 119.1 +/- 13.8 | 126.3 +/- 13.3 |
| ANA (n = 5) | 131.3 +/- 46.4 | 88.3 +/- 17.4 |
| PEA (n = 5) | 91.9 +/- 21.3 | 97.8 +/- 18.0 |

**Table 2:**

**The effect of pre-inflammation administration of HOE140 on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 1hr post- treatment. | $Dur_{mic}$ % of baseline at 1hr post- treatment. |
|---|---|---|
| Saline (n = 6) | 48.8 +/- 10.3 * | 262.0 +/- 43.6 * |
| 1 (n= 6) | 70.7 +/- 3.0 | 157.1 +/- 14.8 |
| 2 (n= 6) | 102.9 +/- 42.7 | 105.4 +/- 18.5 |
| 7.5 (n= 6) | 155.7 +/- 60.4 | 104.9 +/- 12.5 |

**Table 3:**

**The effect of pre-inflammation administration of des-Arg$^9$[Leu$^8$]-BK on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 1hr post- treatment. | $Dur_{mic}$ % of baseline at 1hr post- treatment. |
|---|---|---|
| Saline (n= 6) | 48.8 +/- 10.3 * | 262.0 +/- 43.6 * |
| 1 (n=6) | 42.83 +/- 12.35 * | 127.15 +/- 15.37 * |
| 2 (n=6) | 62.31 +/- 17.67 * | 156.56 +/- 37.05 * |
| 7.5 (n=6) | 49.5 +/- 12.08 * | 192.19 +/- 21.67 * |

* $p < 0.05$ in comparison to baseline (paired t-test).

**Table 4:**

**The effect of early post-inflammation administration of HOE140 on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15min post HOE 140 treatment. | $V_{mic}$ % of baseline at 1hr post HOE 140 treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15min post HOE 140 treatment. | $Dur_{mic}$ % of baseline at 1hr post HOE 140 treatment. |
|---|---|---|---|---|---|---|
| Saline (n= 6) | 53.8 (3.4) | | 52.2 (12.2) | 289.9 (49.4) | | 310.3 (20.1) |
| 1 (n= 8) | 33.9 (15.0) | 77.93 (11.03) + | 77.9 (16.1) + | 328.3 (100.7) | 223.63 (64.81) + | 220.2 (48.4) + |
| 2 (n= 6) | 20.3 (6.5) | 64.98 (9.13) + | 68.6 (14.8) + | 339.7 (146.8) | 281.89 (120.0) | 294.2 (141.6) |
| 7.5 (n= 6) | 45.5 (7.8) | 110.02 (19.32) + | 105.4 (15.5)+ | 192.9 (27.6) | 128.53 (36.23) | 126.9 (20.2) + |

+ $p < 0.05$ in comparison to 2 hours post-turpentine instillation (ANOVA [Tukey]).

## Table 5:

**The effect of early post-inflammation administration of des-Arg$^9$[Leu$^8$]-BK on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15mins post treatment. | $V_{mic}$ % of baseline at 1hr post treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15mins post treatment. | $Dur_{mic}$ % of baseline at 1hr post treatment. |
|---|---|---|---|---|---|---|
| Saline (n= 6) | 53.8 (3.4) | | 52.2 (12.2) | 289.9 (49.4) | | 310.3 (20.1) |
| 1 (n= 9) | 38.2 (5.1) | 53.19 (6.64) | 77.1 (5.8) + | 214.2 (31.7) | 187.02 (31.63) | 172.2 (35.6) |
| 2 (n= 6) | 48.0 (8.6) | 45.57 (7.80) | 44.9 (9.9) | 235.8 (77.4) | 232.70 (67.61) | 207.5 (74.4) |
| 7.5 (n= 6) | 51.3 (7.3) | 57.50 (11.18) | 46.4 (6.5) | 230.0 (37.9) | 192.89 (30.34) | 229.6 (55.0) |

+ $p < 0.05$ in comparison to 2 hours post-turpentine instillation (ANOVA [Tukey]).

**Table 6:**

**The effect of late post-inflammation administration of HOE140 on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15mins post treatment. | $V_{mic}$ % of baseline at 1hr post treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15mins post treatment. | $Dur_{mic}$ % of baseline at 1hr post treatment. |
|---|---|---|---|---|---|---|
| Saline (n = 5) | 43.1 (9.4) | 53.44 (7.95) | 44.9 (8.9) | 611.6 (312.3) | 464.60 (203.4) | 501.3 (240.6) |
| 1 (n = 6) | 34.8 (5.1) | 48.12 (8.46) | 108.3 (27.7) + | 406.3 (129.0) | 331.72 (105.2) | 267.2 (103.31) + |

+ $p < 0.05$ in comparison to 2 hours post-inflammation (ANOVA [Dunnett's]).

## Table 7:

**The effect of late post-inflammation administration of des-Arg$^9$[Leu$^8$]-BK on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15mins post-treatment. | $V_{mic}$ % of baseline at 1hr post-treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15mins post-treatment. | $Dur_{mic}$ % of baseline at 1hr post-treatment. |
|---|---|---|---|---|---|---|
| Saline (n= 5) | 43.1 (9.4) | 53.44 (7.95) | 44.9 (8.9) | 611.6 (312.3) | 464.60 (203.36) | 501.3 (240.6) |
| 1 (n = 6) | 45.16 (7.0) | 39.90 (7.2) | 124.94 (35.5) | 215.6 (34.9) | 258.60 (38.99) | 104.6 (32.9) |
| 2 (n = 6) | 40.31 (5.8) | 39.75 (8.07) | 49.64 (10.8) | 212.3 (27.0) | 215.67 (32.54) | 205.9 (30.1) |
| 7.5 (n= 6) | 41.8 (6.7) | 47.35 (11.92) | 72.7 (9.3) + | 280.7 (39.6) | 274.63 (68.90) | 189.3 (18.0) |

+ $p < 0.05$ in comparison to 2 hours post-inflammation (ANOVA [Dunnett's]).

**Table 8:**

**Mean (sem) plasma extravasation into vesical tissue following administration of bradykinin receptor antagonists:**

| Drug | (n =) | Dose (mg/kg) | Administration protocol. | Concentration of Evan's Blue Dye in vesical tissue (mg / mg of vesical tissue). |
|---|---|---|---|---|
| Saline | 7 | | Pre-inflammation | 0.75 (0.12) |
| des-Arg$^9$[Leu$^8$]-BK | 3 | 1 | Pre-inflammation | 0.59 (0.04) |
| | 4 | 2 | Pre-inflammation | 0.63 (0.06) |
| | 3 | 7.5 | Pre-inflammation | 0.68 (0.04) |
| HOE140 | 5 | 1 | Pre-inflammation | 0.47 (0.12) |
| | 6 | 2 | Pre-inflammation | 0.50 (0.08) |
| | 3 | 7.5 | Pre-inflammation | 0.58 (0.06) |
| Saline | 7 | | Post-inflammation | 0.74 (0.13) |
| des-Arg$^9$[Leu$^8$]-BK | 4 | 1 | Post-inflammation | 0.82 (0.22) |
| | 6 | 2 | Post-inflammation | 1.03 (0.16) |
| | 6 | 7.5 | Post-inflammation | 0.76 (0.19) |
| HOE140 | 3 | 1 | Post-inflammation | 0.55 (0.09) |
| | 6 | 2 | Post-inflammation | 1.03 (0.21) |
| | 6 | 7.5 | Post-inflammation | 0.62 (0.16) |

**Table 9:**

**Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in HOE140 (or control) treated animals:**

| Cord level. | Drug administered. | Superficial dorsal horn. | Deep dorsal horn. | Lamina X. | Intermediolateral posterior nucleus. |
|---|---|---|---|---|---|
| L5 | Saline (n = 27) | 22.96 (1.99) | 14.44 (1.20) | 60.63 (3.72) | 11.07 (0.95) |
| | HOE140 (n = 27) | 19.93 (1.66) | 12.56 (1.24) | 41.93 * (4.10) | 7.85 * (0.89) |
| L6 | Saline (n = 34) | 49.68 (3.11) | 20.36 (1.01) | 107.09 (3.74) | 19.29 (1.07) |
| | HOE140 (n = 23) | 35.00 * (2.91) | 19.30 (1.88) | 62.26 * (5.56) | 12.74 * (1.78) |
| S1 | Saline (n = 19) | 35.37 (2.64) | 17.58 (1.21) | 80.95 (4.57) | 18.26 (1.45) |
| | HOE140 (n = 34) | 30.38 (1.79) | 15.50 (0.97) | 58.88 * (3.91) | 15.12 * (1.70) |

* $p < 0.05$ in comparison to control group (matched for spinal cord level and sub-area) (unpaired t-test).

## Table 10:

**The effect of pre-inflammation administration of SDZ249-665 on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 30mins post-treatment. | $V_{mic}$ % of baseline at 1hr post-turpentine. | $V_{mic}$ % of baseline at 2hrs post-turpentine. | $Dur_{mic}$ % of baseline at 30mins post-treatment. | $Dur_{mic}$ % of baseline at 1hr post-turpentine. | $Dur_{mic}$ % of baseline at 2hrs post-turpentine. |
|---|---|---|---|---|---|---|
| DMSO (n = 6) | 58.2 (7.0) | 43.7 * (6.3) | 51.6 * (8.8) | 317.9 (80.2) | 413.0 * (138.1) | 458.3 * (153.0) |
| 0.05 (n = 6) | 73.8 (11.8) | 97.1 (24.5) | 98.6 (29.4) | 127.8 (16.0) | 139.4 (43.3) | 167.1 (53.5) |
| 0.1 (n = 6) | 91.1 (6.6) | 90.7 (10.2) | 69.0 (5.8) | 130.4 (26.0) | 136.3 (13.3) | 189.4 * (38.2) |
| 0.5 (n = 6) | 153.1 (20.3) | 92.2 (7.4) | 144.2 (46.3) | 101.5 (33.8) | 130.0 (15.9) | 119.2 (28.9) |
| 1 (n = 6) | 175.0 (48.7) | 171.1 (55.5) | 169.5 (55.4) | 65.2 (15.5) | 80.5 (14.2) | 75.0 (24.3) |

* $p < 0.05$ in comparison to baseline (ANOVA [Tukey]).

**Table 11:**

**Mean (sem) plasma extravasation into vesical tissue following administration of the capsaicin analogue SDZ249-665:**

| Drug | (n = ) | Dose (mg/kg). | Concentration of Evan's Blue dye in vesical tissue (mg / mg of vesical tissue). |
|---|---|---|---|
| Solvent control (DMSO + saline, 1 : 19, 0.7ml) | 5 | - | 1.11 (0.21) |
| SDZ249-665 | 4 | 0.05 | 1.23 (0.30) |
| SDZ249-665 | 6 | 0.1 | 0.96 (0.12) |
| SDZ249-665 | 4 | 0.5 | 0.79 (0.20) |
| SDZ249-665 | 7 | 1 | 0.66 (0.12) |

## Table 12:

**The effect of pre-inflammation administration of ANA on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 1hr post-treatment. | $V_{mic}$ % of baseline at 2hrs post-treatment. | $Dur_{mic}$ % of baseline at 1hr post-treatment. | $Dur_{mic}$ % of baseline at 2hrs post-treatment. |
|---|---|---|---|---|
| Solvent control (n = 5) | 58.37 (12.32) * | 54.36 (12.44) * | 215.43 (57.98) | 205.77 (39.98) * |
| 10 (n = 7) | 61.96 (14.92) * | 50.62 (10.18) * | 169.58 (22.22) * | 195.37 (35.33) * |
| 15 (n = 7) | 70.35 (7.77) * | 60.51 (12.54) * | 122.96 (20.40) | 174.66 (33.28) |
| 25 (n = 5) | 94.49 (26.98) | 50.62 (19.62) | 110.55 (25.10) | 146.00 (23.24) |

## Table 13:

**The effect of pre-inflammation administration of PEA on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 1hr post-treatment. | $V_{mic}$ % of baseline at 2hrs post-treatment. | $Dur_{mic}$ % of baseline at 1hr post-treatment. | $Dur_{mic}$ % of baseline at 2hrs post-treatment. |
|---|---|---|---|---|
| Solvent control (n = 5) | 58.37 (12.32) * | 54.36 (12.44) * | 215.43 (57.98) | 205.77 (39.98) * |
| 10 (n = 6) | 45.82 (8.48) * | 40.41 (10.29) * | 344.82 (65.74) * | 398.67 (77.37) * |
| 20 (n = 6) | 70.35 (7.77) * | 41.95 (7.49) * | 162.32 (24.30) * | 181.33 (16.78) * |
| 30 (n = 6) | 50.62 (10.57) * | 67.17 (13.79) | 175.24 (17.01) * | 184.74 (29.97) * |

* $p < 0.05$ in comparison to baseline (paired t-test).

**Table 14:**

**The effect of post-inflammation administration of ANA on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15mins post treatment. | $V_{mic}$ % of baseline at 1hr post treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15mins post treatment. | $Dur_{mic}$ % of baseline at 1hr post treatment. |
|---|---|---|---|---|---|---|
| Solvent control (n = 6) | 54.22 (10.53) | 60.00 (11.31) | 43.89 (9.26) | 201.58 (39.66) | 198.49 (37.54) | 211.89 (40.62) |
| 10 (n = 6) | 29.25 (4.13) | 42.05 (8.12) | 47.69 (11.47) | 272.93 (24.22) | 191.01 (32.13) | 189.93 (35.55) |
| 15 (n = 6) | 44.79 (7.49) | 52.72 (15.88) | 57.58 (9.01) | 300.00 (43.48) | 268.50 (59.16) | 220.02 (36.18) |
| 25 (n = 6) | 47.69 (6.51) | 79.18 (16.68) + | 101.7 (24.52) + | 213.52 (33.95) | 173.56 (28.97) | 122.73 (20.13) |

+ $p < 0.05$ in comparison to 2hours post-inflammation (ANOVA [Dunnett's]).

**Table 15:**

**The effect of post-inflammation administration of PEA on mean (sem) micturition reflexes:**

| Dose (mg/kg) | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15mins post treatment. | $V_{mic}$ % of baseline at 1hr post treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15mins post treatment. | $Dur_{mic}$ % of baseline at 1hr post treatment. |
|---|---|---|---|---|---|---|
| Solvent control (n = 6) | 54.22 (10.53) | 60.00 (11.31) | 43.89 (9.26) | 201.58 (39.66) | 198.49 (37.54) | 211.89 (40.62) |
| 1 (n = 6) | 47.78 (5.04) | 60.99 (8.27) | 77.16 (11.22) | 330.4 (69.67) | 308.6 (81.46) | 269.86 (77.95) + |
| 10 (n = 5) | 40.13 (1.50) | 51.84 (7.07) + | 67.64 (9.56) + | 185.32 (29.24) | 180.73 (30.56) | 154.82 (23.98) |
| 20 (n = 6) | 38.38 (5.87) | 46.78 (6.86) | 64.68 (6.16) + | 483.25 (134.02) | 475.99 (185.64) | 313.98 (84.69) + |
| 30 (n = 5 ) | 48.87 (8.62) | 44.36 (8.89) | 73.33 (20.33) + | 392.01 (173.95) | 387.34 (177.55) | 329.28 (153.45) + |

+ $p < 0.05$ in comparison to 2hours post-inflammation (ANOVA [Dunnett's]).

**Table 16:**

**The effect of post-inflammation administration of methanandamide on mean (sem) micturition reflexes:**

| Drug | $V_{mic}$ % of baseline at 2hrs post-inflammation. | $V_{mic}$ % of baseline at 15mins post treatment. | $V_{mic}$ % of baseline at 1hr post treatment. | $Dur_{mic}$ % of baseline at 2hrs post-inflammation. | $Dur_{mic}$ % of baseline at 15mins post treatment. | $Dur_{mic}$ % of baseline at 1hr post treatment. |
|---|---|---|---|---|---|---|
| 35% Alcohol (n = 6) | 54.1 * (3.0) | 167.2 (17.6) | 141.3 (18.0) | 163.1 * (17.5) | 83.6 (17.5) | 97.7 (25.2) |
| Methanandamide (5mg/kg) (n = 4) | 34.9 * (3.6) | 104.6 (12.7) | 81.2 (7.8) | 245.4 * (41.3) | 94.7 (11.6) | 120.9 (12.1) |

* $p < 0.05$ in comparison to baseline (ANOVA [Dunnett's]).

**Table 17:**

**Mean (sem) plasma extravasation into vesical tissue following administration of cannabinoid receptor agonists:**

| Drug | (n =) | Dose (mg/kg). | Administration protocol. | Concentration of Evan's Blue dye in vesical tissue (mg / mg of vesical tissue). |
|---|---|---|---|---|
| Solvent control | 5 | | Pre-inflammation | 0.62 (0.12) |
| ANA | 4 | 10 | Pre-inflammation | 0.42 (0.13) |
| | 5 | 15 | Pre-inflammation | 0.54 (0.11) |
| | 4 | 25 | Pre-inflammation | 1.07 (0.14) |
| PEA | 5 | 10 | Pre-inflammation | 0.69 (0.11) |
| | 4 | 20 | Pre-inflammation | 0.75 (0.01) |
| | 3 | 30 | Pre-inflammation | 1.04 (0.17) |
| Solvent control | 5 | | Post-inflammation | 0.62 (0.08) |
| ANA | 3 | 10 | Post-inflammation | 0.51 (0.10) |
| | 6 | 15 | Post-inflammation | 0.75 (0.34) |
| | 4 | 25 | Post-inflammation | 0.58 (0.15) |
| PEA | 4 | 10 | Post-inflammation | 0.64 (0.08) |
| | 3 | 20 | Post-inflammation | 0.69 (0.10) |
| | 4 | 30 | Post-inflammation | 0.82 (0.13) |

**Table 18:**

**The effect of ANA administration on the weighted composite pain score (CPS-$WST_{0,1,2}$) for the first and second phases of the behavioural response to sub-cutaneous formalin.**

| Drug | Dose (mg/kg) | CPS-$WST_{0,1,2}$ (0 - 15min) mean (sem). | CPS-$WST_{0,1,2}$ (20 - 50min) mean (sem). |
|---|---|---|---|
| Formalin alone (n = 5) | - | 0.49 (0.11) | 0.96 (0.09) |
| Solvent control (n = 6) | - | 0.35 (0.05) | 0.73 (0.09) |
| ANA (n = 5) | 5 | 0.18 (0.04) | 0.58 (0.08) * |
| ANA (n = 5) | 10 | 0.30 (0.05) | 0.58 (0.06) * |
| ANA (n = 6) | 15 | 0.36 (0.99) | 0.53 (0.10) * |



* $p < 0.05$ in comparison to formalin (alone) control group (ANOVA [Dunnett's]).

## Table 19:

**The effect of PEA administration on the weighted composite pain score (CPS-$WST_{0,1,2}$) for the first and second phases of the behavioural response to sub-cutaneous formalin.**

| Drug | Dose (mg/kg) | CPS-$WST_{0,1,2}$ (0 - 15min) mean (sem). | CPS-$WST_{0,1,2}$ (20 - 50min) mean (sem). |
|---|---|---|---|
| Formalin alone (n = 5) | | 0.49 (0.11) | 0.96 (0.09) |
| Solvent control (n = 6) | | 0.35 (0.05) | 0.73 (0.09) |
| PEA (n = 5) | 1 | 0.34 (0.05) | 0.77 (0.11) |
| PEA (n = 6) | 5 | 0.19 (0.04) | 0.40 (0.11) * |
| PEA (n = 6) | 10 | 0.32 (0.08) | 0.61 (0.08) * |



* $p < 0.05$ in comparison to formalin (alone) control group (ANOVA [Dunnett's]).

## Table 20:

**Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in methanandamide or 35% alcohol (control) treated animals:**

| Cord level. | Drug administered. | Superficial dorsal horn. | Deep dorsal horn. | Lamina X. | Intermediolateral posterior nucleus. |
|---|---|---|---|---|---|
| L5 | 35% Alcohol (n = 16) | 7.19 (1.97) | 5.81 (1.24) | 27.13 (4.32) | 3.50 (1.03) |
| | ANA (n = 20) | 11.25 (1.58) | 5.10 (0.59) | 21.40 (3.51) | 3.15 (0.84) |
| L6 | 35% Alcohol (n = 24) | 16.25 (2.29) | 9.17 (1.02) | 50.63 (4.52) | 10.33 (1.50) |
| | ANA (n = 24) | 21.29 (1.51) | 12.96 * (1.34) | 50.17 (2.39) | 9.88 (1.22) |
| S1 | 35% Alcohol (n = 13) | 24.62 (2.03) | 15.23 (1.96) | 55.39 (5.56) | 14.85 (1.32) |
| | ANA (n = 21) | 21.43 (2.72) | 9.86 * (1.02) | 55.33 (3.54) | 13.10 (1.76) |

* $p < 0.05$ in comparison to control group (matched for spinal cord level and sub-area) (unpaired t-test).

**Table 21:**

**Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in PEA or solvent control (DMSO + saline, 1 : 19 dilution) treated animals:**

| Cord level. | Drug administered. | Superficial dorsal horn. | Deep dorsal horn. | Lamina X. | Intermediolateral posterior nucleus. |
|---|---|---|---|---|---|
| L5 | Solvent control (n = 27) | 17.22 (2.87) | 9.19 (1.43) | 44.41 (3.91) | 8.76 (0.85) |
| | PEA (n = 33) | 12.70 (1.38) | 7.85 (1.08) | 38.42 (4.40) | 5.39 * (0.74) |
| L6 | Solvent control (n = 24) | 44.25 (2.97) | 19.63 (1.72) | 87.96 (5.04) | 22.21 (3.29) |
| | PEA (n = 28) | 23.46 * (1.99) | 11.36 * (1.26) | 51.07 * (5.34) | 11.54 * (1.54) |
| S1 | Solvent control (n = 29) | 34.28 (2.60) | 14.14 (1.28) | 84.38 (3.94) | 19.48 (1.24) |
| | PEA (n = 15) | 25.07 * (2.27) | 12.00 (1.28) | 56.33 * (5.11) | 13.33 * (1.25) |

* $p < 0.05$ in comparison to control group (matched for spinal cord level and sub-area) (unpaired t-test).

**Table 22:**

**Mean (sem) count of neurones demonstrating c-Fos-like immunoreactivity in sub-areas of the spinal cord in WIN 55,212-2 or solvent control (DMSO + saline, 1 : 19 dilution) treated animals:**

| Cord level | Drug administered | Superficial dorsal horn | Deep dorsal horn | Lamina X | Intermediolateral posterior nucleus |
|---|---|---|---|---|---|
| L5 | Solvent control (n = 27) | 17.22 (2.87) | s9.19 (1.43) | 44.41 (3.91) | 8.76 (0.85) |
| | WIN 55,212-2 (n = 13) | 22.8 (1.6) | 2.8 * (0.6) | 31.8 (3.8) | 3.9 * (0.5) |
| L6 | Solvent control (n = 24) | 44.25 (2.97) | 19.63 (1.72) | 87.96 (5.04) | 22.21 (3.29) |
| | WIN 55,212-2 (n = 20) | 31.8 (3.8) | 6.0 * (0.7) | 56.2 * (2.5) | 11.9 * (1.1) |
| S1 | Solvent control (n = 29) | 34.28 (2.60) | 14.14 (1.28) | 84.38 (3.94) | 19.48 (1.24) |
| | WIN 55,212-2 (n = 18) | 31.9 (2.1) | 5.8 * (1.2) | 45.4 * (3.0) | 11.7 * (1.7) |

* $p < 0.05$ in comparison to control group (matched for spinal cord level and sub-area) (unpaired t-test).