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Eicosanoid Mediation/Modulation in Spinal Nociceptive Mechanisms in the Normal Rat and in a Rat Model of Chronic Pain

by

Graham M. Pitcher

Department of Physiology
Faculty of Medicine
McGill University
Montreal, Quebec, Canada

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Abstract

The studies presented in this thesis are concerned with physiological and neurochemical characterization of on-going and stimulation-elicited synaptic transmission of sensory information in the dorsal horn of the rat spinal cord.

Historically, since the use of willow tree bark extract for pain relief, the recognition that it and related nonsteroidal anti-inflammatory drugs (NSAIDs) express their analgesic and anti-inflammatory effects by inhibiting cyclooxygenase (COX) and more recently the identification of two forms of COX, inducible (COX-2) and constitutive (COX-1), efforts have focussed on exploring the therapeutic potential of this class of drugs.

In normal rats, the response to noxious peripheral stimulation involves at least two distinct discharge responses of dorsal horn neurones, an initial discharge which lasts only for the duration of the stimulus and a slowly-decaying afterdischarge which persists beyond the end of the stimulus. Innocuous mechanical stimulation produces excitation of dorsal horn neurones lasting only for the duration of the stimulus. Characterization of these excitatory responses reveals that the initial discharge in response to noxious or innocuous mechanical stimulation involves only limited activation of the eicosanoid pathway via COX, while the afterdischarge is sustained to a major extent via COX activation, at least in part by COX-2. On-going discharge seems not to involve activation of COX.

An animal model of neuropathic pain was induced by implantation of a 2 mm polyethylene cuff around one sciatic nerve in rats. This lowers the nociceptive threshold in the von Frey hair test and induces spontaneous pain behaviour. In cuff-implanted rats, on-going activity is greater compared to that in normal rats. Furthermore, noxious

mechanical stimulation evokes an afterdischarge response of dorsal horn neurones which is markedly greater than that in normal rats but the initial discharge is not different. Importantly, a switch in the response to innocuous mechanical stimulation is observed also such that an afterdischarge occurs after the end of the initial discharge, which is not seen in normal rats.

Characterization of the synaptically-elicited responses in cuff-implanted rats to noxious and innocuous mechanical stimulation reveals that the initial discharge, and to a greater extent the afterdischarge, are subject to a COX-2-mediated or modulatory mechanism. The elevated on-going discharge in cuff-implanted rats may also be sustained via tonic COX-2 activity.

These results demonstrate participation of unique and identifiable physiological and neurochemical mechanisms in the mediation and modulation of on-going and synaptically-elicited responses in the dorsal horn of the spinal cord in normal sensory processing and in sensory processing associated with peripheral neuropathy. Ultimately, the data in this thesis may advance understanding of the neurophysiological basis of chronic pain syndromes.

Résumé

Les travaux présentés dans cette thèse se rattachent à la caractérisation physiologique et neurochimique d'une transmission synaptique continue et obtenue par stimulation sensorielle de la corne dorsale de la moelle épinière du rat.

Il est reconnu que l'extrait de l'écorce du saule, autrefois utilisé pour soulager la douleur, ainsi que les médicaments non-stéroïdes et anti-inflammatoires (NSAID) qui y sont liés, produisent des effets analgésiques et anti-inflammatoires en inhibant le cylooxygénase (COX) et deux formes récemment identifiées de COX: une inducible (COX-2) et l'autre constitutive (COX-1). Des efforts ont porté sur l'exploitation du potentiel thérapeutique de cette catégorie de médicaments.

Chez les rats normaux, la réponse à une stimulation nocive et périphérique donne lieu à au moins deux types de réponse des neurones de la corne dorsale: une réponse qui ne dure que la durée de la stimulation et l'autre plus lente et prolongée. Une stimulation inoffensive et externe produit une excitation des neurones de la corne dorsale ne durant que la durée de la stimulation. La caractérisation de ces réponses excitatoires nous révèle qu'une décharge initiale due à une stimulation externe, nocive ou inoffensive, implique une activation limitée de la voie eicosanoïde par COX, tandis qu'une décharge secondaire est maintenue en grande partie par l'activation du COX, du moins en partie par COX-2. Une réponse prolongée ne semble pas impliquer l'activation du COX.

Un modèle de douleur neuropathique consiste à implanter une sangle de 2 millimètres en polyéthylène autour d'un nerf sciatique chez des rats; ceci fait baisser le seuil nociceptif au test capillaire de von Frey et provoque un comportement de douleur

spontanée. Avec des rats implantés avec une sangle, la période d'activité semble plus importante que celle des rats normaux. De plus, une stimulation externe et nocive produit une décharge secondaire des neurones de la corne dorsale qui semble plus importante que celle des rats normaux mais la réponse initiale ne semble pas différente. Plus important encore, le changement observé dans la réponse à une stimulation inoffensive et externe ne suivent qu'à la fin de la décharge initiale, ce qui ne survient pas chez les rats normaux.

La caractérisation des réponses obtenues par voie synaptique chez des rats implantés d'une sangle suite à une stimulation inoffensive et externe, et en grande partie la réponse secondaire, sont sujettes au mécanisme modulatoire ou au mécanisme lié au COX-2. La décharge élevée et maintenue chez des rats implantés d'une sangle peut être prolongée par l'activité tonique du COX-2.

Ces travaux démontrent la participation de mécanismes physiologiques et neurochimiques spécifiques et identifiables dans la médiation et la modulation de réponses suivies et obtenues par voie synaptique à l'intérieur de la corne dorsale de la moelle épinière, dans le traitement normal de données sensorielles et dans celui lié à la neuropathie périphérique. Finalement, les données présentées dans cette thèse pourraient bien contribuer à une meilleure compréhension du fondement neurophysiologique des syndromes de douleur chronique.

Preface

In accordance with the *Guidelines for Submitting a Doctoral or a Master's Thesis*, McGill University, the candidate has chosen the option of including as part of his thesis the text of original papers submitted or suitable for submission to learned journals for publication. The text relating to this option is as follows:

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> 1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)
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> 2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
>
> 3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.
>
> The thesis must include the following:
>
> (a) a table of contents;
> (b) an abstract in English and French;
> (c) an introduction which clearly states the rational and objectives of the research;
> (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
> (e) a final conclusion and summary;
>
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where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

Contribution of Authors

Chapter 1:

Pitcher, G.M. and Henry, J.L. Mediation and modulation by eicosanoids of responses of spinal dorsal horn neurones to glutamate and substance P receptor agonists - results with the NSAID indomethacin in the rat *in vivo. Neuroscience 93:1109-21, 1999.*

Chapter 2:

Pitcher, G.M. and Henry, J.L. NSAID-induced cyclooxygenase inhibition differentially depresses long-lasting vs. brief synaptically-elicited responses of rat spinal dorsal horn neurones *in vivo. Pain 82:173-86, 1999.*

Chapter 3:

Pitcher, G.M. and Henry, J.L. COX-2 inhibitor meloxicam preferentially depresses the afterdischarge vs.the initial discharge in the response of rat dorsal horn neurons to noxious cutaneous stimulation. *(Submitted for publication)*

Chapter 4:

Pitcher, G.M., Ritchie, J. and Henry, J.L. Paw withdrawal threshold in the von Frey hair test is influenced by the surface on which the rat stands. *Journal of Neuroscience Methods. 87:185-193, 1999.*

Chapter 5:

Pitcher, G.M., Ritchie, J. and Henry, J.L. Nerve constriction in the rat: model of neuropathic, surgical and central pain. *Pain. 83:37-46, 1999.*

Chapter 6:

Pitcher, G.M. and Henry, J.L. Cellular mechanisms of hyperalgesia and spontaneous pain in a spinalized rat model of peripheral neuropathy: changes in myelinated afferent inputs implicated. *European Journal of Neuroscience 12:2006-2020, 2000.*

Chapter 7:

Pitcher, G.M. and Henry, J.L. Cellular mechanisms of tactile allodynia in a spinalized rat model of peripheral neuropathy: changes in myelinated afferent inputs implicated. *(Submitted for publication)*

Chapter 8:

Pitcher, G.M. and Henry, J.L. Spinal neural correlate of spontaneous pain, mechanical hyperalgesia and tactile allodynia in an *in vivo* rat model of neuropathic pain: implication of the eicosanoid pathway via COX. (*Submitted for publication*)

For the study described in Chapter 4, the conception, design and construction of the testing platform was by myself. Miss Ritchie contributed to the collection of data in this study. Respectfully, her name was included as a co-author of this paper.

For the study described in Chapter 5, Miss Ritchie contributed to the collection of data in this study. Respectfully, her name was included as a co-author of this paper.

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
FID	fast initial discharge
i.p.	intraperitoneal
IP_3	inositol-1,4,5-triphosphoate
i.v.	intravenous
L	lumbar
NK-1	neurokinin-1
NMDA	N-methyl-D-aspartic acid
NSAIDs	nonsteroidal anti-inflammatory drugs
RCS	rabbit-contracting substance
SDAD	slowly-decaying afterdischarge
T	thoracic

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Introduction

I. Aims and Scope of Thesis

Exposure of the skin and other organs to injurious or potentially injurious stimuli evokes the unpleasant sensation of pain. Importantly, spanning between the initial presentation of a noxious cutaneous stimulus and the cognitive realization of pain is a series of physiological and neurochemical mechanisms by which the effects of the stimulus are integrated as a nociceptive (pain) message and progressively relayed to and processed in supraspinal structures. These events occur in the periphery, in the dorsal horn of the spinal cord and in supraspinal structures.

This thesis is based on studies of processing of sensory information, in particular nociceptive information, in the region of the spinal dorsal horn where the central terminals of primary afferent sensory neurones project onto second order spinal sensory neurones. This region is of particular importance in the transfer of sensory information because it is the first site of synaptic integration of sensory input. The overall objective of the studies in this thesis is identification and characterization of the physiological and neurochemical mechanisms involved in synaptic transmission in sensory processing at this level. It is hypothesized that specificity of synaptic function mediating the inputs of different sensory modalities relies on unique and identifiable physiological and neurochemical mechanisms.

Rationale for the Study of the Eicosanoid Signal Transduction Pathway via Cyclooxygenase

Historically, alleviation of pain related to inflammation, sickness or injury was achieved by administering an extract from willow tree bark, salicin, which is considered today to be the first nonsteroidal anti-inflammatory drug or 'NSAID' (*see Early History of NSAIDs* in part II.). Since the use of salicin and related derivatives, numerous NSAIDs have been developed which have been shown to be indispensable in the treatment of an extensive variety of pain syndromes. For instance, NSAIDs are an effective therapeutic regimen to alleviate protracted pain states associated with rheumatoid (Walker et al. 1997) and osteo- (Ravaud et al. 1998; Spencer-Green and Spencer-Green 1998) arthritis. Cancer pain treatment is also shown to benefit from NSAIDs (Joishy and Walsh 1998; Minotti et al. 1998; Portenoy and Lesage 1999). In fact, the World Health Organization emphasizes NSAIDs as an effective therapeutic approach in cancer pain management (Grond et al. 1999). There is also an increasing use of NSAIDs in the routine management of postoperative pain (O'Brien et al. 1996; Mikawa et al. 1997; Apt et al. 1998; Dionne et al. 1999). Importantly, adjuvant NSAID treatment is also effective in reducing opioid requirements (Vetter and Heiner 1994; Kokki et al. 1999; Perttunen et al. 1999; Tarkkila and Saarnivaara 1999) thus minimizing opioid-associated side effects (Vetter and Heiner 1994). Thus, NSAID treatment is important and in some cases an essential therapeutic strategy in pain management. However, given that NSAIDs express their effects by inhibiting cyclooxygenase which catalyzes the formation of prostanoids from arachidonic acid (*see* The Eicosanoid Pathway in part II.), these studies are also interpreted to indicate

that the eicosanoid signal transduction pathway via cyclooxygenase may be implicated in sensory mechanisms, in particular altered sensory processing, specifically nociception, associated with a chronic disorder.

What is not known is the involvement of the eicosanoid signal transduction pathway, via cyclooxygenase, in sensory processing at the cellular level in the spinal dorsal horn *in vivo*, in particular on-going activity and early and late components of peripheral stimulation-induced discharge of dorsal horn neurones.

Rationale for the Study of Acute and Chronic Pain

In addition to studies on sensory processing in normal animals, this thesis includes studies on an animal model of chronic pain, in particular 'neuropathic' pain induced by damage of peripheral nerve tissue. A prominent feature of peripheral nerve injury in humans is an enhancement in pain sensation. The persistent pain which accompanies peripheral neuropathy may be characterized by its spontaneous nature (not elicited by extrinsic stimuli) and by the presence of hyperalgesia (an increase in the pain elicited by a noxious stimulus) and allodynia (pain elicited by normally innocuous stimuli). Of these changes, spontaneous pain and mechanical allodynia comprise the most striking alterations of sensation in syndromes associated with peripheral nerve injury (Ochoa and Torebjörk 1980; Gracely et al. 1992; Mailis et al. 1997).

An important question concerning the maintenance of chronic pain is how peripheral neuropathy sustains changes in sensory processing. This is of particular

importance given that chronic pain may be considered to serve no beneficial physiological function. Acute pain, for example, serves a protective function by evoking a motor withdrawal reflex thus ending the noxious stimulus and as a result stopping the pain. Additionally, subchronic pain maintains guarding behaviour and recuperation which allows recovery and alleviation of a subchronic pain condition. The question arises then, what is the teleological advantage of specific mechanisms not evolved to counteract chronic pain? Importantly, the lack or inadequate drive for any such evolution in conditions in which chronic pain is endured puts forth the compelling argument to investigate the neurophysiological and neurochemical basis of chronic pain. Thus, another important objective in this thesis is to investigate in addition to the physiological and neurochemical basis of acute or normal sensory processing due to peripheral stimulation, that of chronic pain, in particular chronic neuropathic pain due to afferent sensory nerve injury.

Rationale for Electrophysiological and Reflex Experimental Approaches

The present thesis is comprised of electrophysiological and nociceptive reflex studies designed to investigate sensory information processing in normal and in chronic pain states. The electrophysiological approach enables extracellular single cell recording of spinal dorsal horn neurones. This is directed towards characterizing specific synaptic responses in the spinal dorsal horn by activation of defined sensory inputs with the purpose of identifying physiological responses including on-going discharge of dorsal horn neurones and the effects of peripheral stimulation-evoked synaptic input. Reflex experiments serve

as a functional assay which reflect a measure of sensory processing at a systems level and, importantly, provide a reflex correlate of the cellular phenomena. Accordingly, these complementary approaches provide unique and identifiable characterization of the neurophysiological basis of sensory processing in acute and in chronic pain states.

Identification and characterization of the neurochemical basis of synaptic transmission can be elucidated in part by examination of the presence, release, action and metabolism of the neurochemical or enzyme in question. However, to elucidate the neurochemical specificity of a particular synaptically-elicited event(s) in which a specific chemical mechanism may be involved, the effect(s) of an agent which selectively interferes with the activation of the respective chemical mechanism can be determined on the synaptically-elicited event(s). In this thesis, the objective is to determine the role of the eicosanoid signal transduction pathway via cyclooxygenase in on-going and peripheral stimulation-elicited activity of spinal dorsal horn neurones. Thus, certain electrophysiological experiments are also run in the presence of the cyclooxygenase inhibitor indomethacin. Importantly, the recent discovery of cyclooxygenase-2 (Fu et al. 1990; Sirois and Richards 1992) has prompted the development of selective cyclooxygenase-2 inhibitors which are anticipated to improve treatment of chronic pain syndromes. During the course of my PhD program we were given access to the cyclooxygenase-2 selective inhibitor, meloxicam (Boehringer Ingelheim, Canada, Ltd.). Two chapters in this thesis are devoted to investigation of the effects of meloxicam on on-going activity and peripheral stimulation-elicited responses of dorsal horn neurones.

Collectively, these studies serve to identify neurochemical specificity in the mediation and/or modulation of on-going discharge and responses of dorsal horn neurones which underlie the transfer of sensory information.

The work in this thesis is focused predominantly on cutaneous sensory input. It is considered beneficial in a practical sense to investigate the effects of cutaneous sensory input given that the protective function of pain is most relevant to the skin which is most exposed to the effects of damaging or potentially damaging exogenous stimuli. Furthermore, chronic pain syndromes including in particular neuropathic pain are often associated with hyperalgesia and allodynia evoked by stimulation of the cutaneous receptive field.

Scope of Thesis

Each study in this thesis is intended to be self-sufficient in nature, yet each is also interdependent in that collectively they yield a progressive understanding of the physiological and neurochemical mechanisms extending from normal sensory information processing to that in a chronic pain state. Using an electrophysiological approach, Chapters 1-3 investigate the eicosanoid signal transduction pathway via cyclooxygenase in sensory processing in the normal rat. Specifically, Chapter 1 considers the concept that eicosanoids may play a role in both mediating and modulating excitation of dorsal horn neurones. Importantly, there remains to be determined the functional specificity of the eicosanoid pathway via cyclooxygenase in the effects of synaptic input on neuronal activity

in the spinal dorsal horn, the possible role of cyclooxygenase-2 in functional specificity and physiological and neurochemical specificity in sensory processing in chronic pain. Thus, Chapter 2 examines the functional role of the eicosanoid pathway via cyclooxygenase in the brief and long-lasting excitatory effects of peripheral stimulation-induced synaptic input in the spinal dorsal horn. Chapter 3 examines the possible role of cyclooxygenase-2 in on-going activity and the brief and long-lasting neuronal responses to peripheral stimulation-induced synaptic input. Chapters 4-7 establish and characterize a chronic pain model using electrophysiological and nociceptive reflex experimental approaches. In particular, Chapters 4 and 5 report setting up and validating, respectively, a rat model of chronic neuropathic pain. Chapter 5 provides the foundation for the electrophysiological studies in Chapters 6-8 in that it determines the magnitude and time course of alterations in the threshold of the paw withdrawal reflex associated with the peripheral neuropathy. Chapters 6 and 7 determine whether there is a neural correlate of the behavioural changes to nociceptive and non-nociceptive inputs, respectively, by examining the effects of peripheral neuropathy on spinal dorsal horn neuronal activity at the selected time points (established in Chapter 5) after onset of the nerve injury. Finally, based on the results obtained in Chapters 6 and 7, Chapter 8 determines the role of the eicosanoid pathway via cyclooxygenase in mediating and/or modulating the cellular events in the spinal dorsal horn sensory processing in a chronic neuropathic pain state.

Thus, collectively, these studies examine the **hypothesis** that *specificity of synaptic function mediating and/or modulating the inputs of different sensory modalities relies on*

unique and identifiable physiological and neurochemical mechanisms.

II. NSAIDs, Cyclooxygenase, Eicosanoids and Mechanisms of Nociceptive Processing: an Historical Integrative Perspective

Early History of NSAIDs

The medicinal effect of certain plants, herbs and trees has been known to several cultures for many centuries. This is certainly the case of the origin of modern NSAIDs. In ancient Greece, Hippocrates (460-377 B.C.) administered a wide variety of broths derived from plant extracts (*see* Rollin 1828) which, by accident or experiment, he had discovered were capable of producing upon the body 'medicinal effects'. In particular, juice made from the bark of the willow tree (*Salix alba*) reduced fever and eased the pain of child birth (the active ingredient now known to be salacin). During the first century A.D., Celsus used willow tree leaves to alleviate the four classic signs of inflammation: 'rubor', 'calor', 'dolor' and 'tumor' (or redness, heat, pain and swelling). Also around this time, the therapeutic effect of willow bark extract was recognized by the Greek physician Dioscorides to reduce inflammation and mild to moderate pain.

During the Middle Ages, further uses of willow tree leaves and bark extract included administration to wounds, relief of menstrual pain and also discomfort associated with dysentery. Herbalists of the time also grew meadowsweet (*Spiraea ulmaria*) which contains salacin and served as a herbal remedy. The therapeutic effects of *Salix* and *Spiraea* species were also known in Asia, South Africa and also to the early native

inhabitants of North America (*see* Cobbs 1996).

Documentation of the Effects of Willow Tree Bark Extract in a 'Clinical Trial'

It was not until late in the eighteenth century that a more systematic approach was carried out to investigate the therapeutic potential of willow tree bark. In England, Reverend Edward Stone (or Edmund Stone; both names appear in the original manuscript) conducted what may be the first 'clinical study' to investigate and document the therapeutic effect of willow tree bark extract on fever and various other maladies (Stone 1763). In his report he states that "if there was any considerable virtue in this bark, it must have been discovered from its plenty. My curiosity prompted me to look into the dispensatories and books of botany, and examine what they said concerning it; but there it existed only by name. I could not find, that it has, or ever had, any place in pharmacy, or any such qualities, as I suspected ascribed to it by the botanists". Stone held the "general maxim that many natural maladies carry their cures along with them, or that their remedies lie not far from their causes". In other words he reasoned that as the "[willow] tree delights in a moist or wet soil, where agues [fever] chiefly abound", it may possess therapeutic properties appropriate to that condition. According to his report, Stone collected a pound of this bark, dried it in a baker's oven for three months, ground it into a powder and treated successfully approximately 50 patients. On June 2, 1763, Stone read his "account of the successes of the bark of willow in the cure of agues" to the Royal Society of London. Interestingly, according to legend, when news of Stone's discovery reached

North America, the colonists gained new respect for the use of willow tree bark in Native American medicine.

Synthetic Salicylic Acid

It is an attractive notion that Stone's exciting results may have provoked greater recognition of the therapeutic capacity of willow tree bark. In fact, near the end of the eighteenth century and during the nineteenth century, European scientists began meticulous analysis of various 'medicinal' plants in attempts to discover the specific chemicals in them that were responsible for their therapeutic properties. In fact, it was demonstrated that the active ingredient of the powder derived from dried willow tree bark and leaves was salacin (Leroux 1830a,b; von Esenbeck 1832; Brandes et al. 1833) which, in its isolated and pure form, was shown to be antipyretic (Leroux 1830a,b; Buss 1875) and effective in treating rheumatoid arthritis (Maclagan 1876). Oil extracted from the meadowsweet (*Spiraea ulmaria*) and wintergreen (*Gaultheria procumbens*) plants (Pagenstecher 1835; Löwig 1836) was also found to contain salacin (Dumas 1839a,b). This was refined into salicylic acid (Ettling 1840) which was shown to be similar (Dumas 1839a,b) to that obtained from the *Salix* species (Piria 1839a,b,c). Although salacin and salicylic acid were effective in treating rheumatoid arthritis, associated pain and fever (Geiger and Liebig 1834; Pleischl 1835; Kolbe 1874; Maclagan 1876), alternative synthesis processes were sought because the purification process from natural sources was apparently tedious and costly, and provided only limited product, due in part to limited plant supply (Dumas 1839b).

Furthermore, there were also undesirable side effects including a very bitter taste, nausea and irritation of the gastrointestinal tract (Buchner 1830).

In the mid 1800's, Charles Frédéric Gerhardt, a French chemist, modified salicylic acid by combining it with acetyl chloride which resulted in a crude form of acetylsalicylic acid (ASA) (Gerhardt 1853a,b). ASA was synthesized again a few years later (von Gilm 1859). Not long after, a German chemist named Karl Johann Kraut replicated and refined the methods of Gerhardt and von Gilm, synthesizing a more pure form of ASA (Schröder et al. 1869). Again, the synthesizing process was found to be tedious and lengthy and was consequently abandoned.

During the latter part of the nineteenth century, the director of the German chemical dye company *Farbenfabriken vormals Friedrich Bayer & Co.*, Dr. Carl Duisberg, noted that one of the waste products of their dye manufacturing was chemically similar to a recently introduced antipyretic by a competing firm. Thus, realizing the tremendous potential in drug development and given their multitude of by-products, Duisberg decided to promote a separate pharmaceutical division in the company. Dr. Arthur Eichengrün was appointed Pharmaceutical Research Director at Bayer on October 1, 1896 and began actively searching for a derivative of comparable or superior therapeutic efficacy to salicylic acid. Eichengrün assigned this task to a young chemist by the name of Felix Hoffmann. Thus, Hoffman undertook the task to prepare acetylsalicylic acid from synthetic sources in an attempt to develop a version of salicylic acid with fewer side effects. Hoffmann apparently came across the work done by Gerhardt and decided that the

acetyl chloride process was a method worth trying. Hoffmann not only developed an improved synthesis process, but on October 10, 1897 produced an even more pure and stable ASA preparation than his predecessors. Importantly, ASA turned out to be very effective in treating diabetes mellitus (Williamson 1902), pain, inflammation and fever associated with gout and rheumatism (Floeckinger 1899; Witthauer 1899; Wohlgemuth 1899; Mackey 1903), carcinoma and neuralgia (Breuss 1903) and exhibited less gastrointestinal irritation than previous drug treatments. Bayer's Pharmacology Research Director Dr. Heinrich Dresser recognized, after initial indifference, that ASA was an important new drug and introduced it in 1899 as *Aspirin* (Wakley and Wakley 1899; *see* Vane and Botting 1992), at the same time writing a scientific paper suggesting that aspirin was a superior formulation in that it was less toxic and convenient way of supplying the body with the active substance salicylic acid (Dreser 1899). (The name Aspirin is said to have been derived from *Spiraea ulmaria*, the plant species from which salicylic acid was once prepared. *A* comes from acetyl chloride, *spir* from *Spiraea* and *in* was the common ending for medicines at that time. Intriguingly, according to one legend, the preparation was named after Saint Aspirinus, a Neapolitan bishop who was said to be the patron saint of headache sufferers.)

Mechanism of Action of Aspirin, Salicylic Acid and Indomethacin - Link to the Eicosanoid Pathway

Despite its known anti-inflammatory, antipyretic and analgesic qualities, for several

decades the mechanism of action of aspirin was not established. Interestingly, in the late 1960's, Priscilla Piper and her supervisor John Vane found that in addition to the release of histamine during anaphylaxis, they also found, using a bioassay system developed earlier by Vane (Vane 1964), some previously unreported substances namely prostaglandins E_2 and $F_{2\alpha}$ from the guinea pig lung tissue assay and a substance called 'rabbit aorta-contracting substance' (RCS) which was later identified as thromboxane A_2 (Hamberg et al. 1975). The first clue to the association between aspirin and prostaglandins came about in a subsequent study in which RCS release in isolated guinea pig lungs during anaphylaxis was blocked by aspirin (Palmer et al. 1970). In addition to RCS, given that chemical or mechanical stimuli in their experiments also lead to the induction of prostaglandin release (Ferreira and Vane 1967; Piper and Vane 1969a,b), Vane postulated that the various stimuli that induce prostaglandin release were perhaps inducing the biosynthesis of these compounds and that aspirin may also block this synthesis process. There was also at this time speculation that prostaglandins participated in the pathogenesis of inflammation and fever, which reinforced the hypothesis that inhibition of the biosynthesis of these eicosanoids could explain some of the clinical actions of this drug. Using the supernatant of a broken cell homogenate from guinea pig lung as a source of 'prostaglandin synthase', Vane demonstrated a dose-dependent decrease in prostaglandin E_2 and $F_{2\alpha}$ levels following administration of aspirin, salicylic acid and indomethacin but not morphine (Vane 1971). In the same issue of *Nature*, two other reports confirmed and extended his finding that aspirin decreases prostaglandin release from aggregating human platelets (Smith and Willis 1971) and that aspirin-like drugs decrease prostaglandin release

from the perfused, isolated spleen of the dog (Ferreira et al. 1971).

In 1982, Vane was awarded the Nobel Prize for Physiology or Medicine (Oates 1982) for discovering aspirin's basic mechanism of action, specifically that its effect is due to inhibition of prostaglandin synthesis. This discovery is extremely important because not only did it provide insight into the basis of the therapeutic mechanism of action of aspirin and 'aspirin-like' drugs, NSAIDs, it has also lead to the development of several novel NSAIDs which serve as important tools for examination of the specific role of eicosanoids and the eicosanoid pathway in physiological and pathophysiological mechanisms.

The Eicosanoid Pathway

Eicosanoids consist of prostaglandins, prostacyclin, thromboxane A_2 and leukotrienes. These chemicals are called eicosanoids because they are derived from 20-carbon polyunsaturated essential fatty acids including: *8,11,14-eicosatrienoic acid* (dihomo-γ-linoleic acid), *5,8,11,14-eicosatetraenoic acid* (arachidonic acid) and *5,8,11,14,17-eicosapentaenoic acid*, which contain three, four and five double bonds, respectively (*see* Wolfe and Horrocks 1994). In humans, the most abundant precursor is arachidonic acid which is derived primarily from dietary linoleic acid.

Arachidonic acid is normally esterified to cell membrane phospholipids including in particular phosphatidylcholine and phosphatidylethanolamine and therefore its concentration as free arachidonic acid in the cell is generally quite low. Thus, the biosynthesis of eicosanoids (ie. prostaglandins and leukotrienes) depends primarily upon

the availability of arachidonic acid to the eicosanoid-synthesizing enzymes. Availability of arachidonic acid occurs from its liberation from the cell membrane predominantly via the action of acyl hydrolases, to some extent via diacylglycerol lipase (Williams et al. 1994; Rapuano and Bockman 1997), but predominantly via phospholipase A_2 (Ball et al. 1999; Balsinde et al. 1999) which has been demonstrated in a variety of cells including human ciliary cells (Yousufzai and Abdel-Latif 1997), human basophils (Miura et al. 1998), mast cells (Reddy and Herschman 1997), neutrophils (Marshall et al. 1995; Tithof et al. 1998) as well as in nervous tissue (Farooqui et al. 1997; Saluja et al. 1997). A wide variety of physical and chemical stimuli can stimulate biosynthesis of the different families of eicosanoids (*see* Chapters 1, 2, 3 and 8).

Once liberated from these membrane lipids via the action of phospholipase A_2, arachidonic acid may either be re-esterified to membrane lipids (Li et al. 1994; Nelson et al. 1997; Crabtree et al. 1998; Grange et al. 1998; Denizot et al. 1999) or metabolized via a variety of oxidative enzymes (Farina et al. 1994; Tramposch et al. 1994; Reddy and Herschman 1997; Yousufzai and Abdel-Latif 1997; Miura et al. 1998; Saunders et al. 1999). The two main important oxidative pathways include the cyclooxygenase pathway which generates prostaglandins (Tramposch et al. 1994; Reddy and Herschman 1997; Yousufzai and Abdel-Latif 1997), thromboxanes (Hamberg et al. 1975) and prostacyclin (Moncada et al. 1976), and the lipoxygenase pathway which synthesizes leukotrienes (Samuelsson 1983, 1997), lipoxins, hydroxyeicosatetraenoic acids and the mono- and di-hydroxyeicosatetraenoic acids (mono- and di-HETEs) (Rao et al. 1994; Macchia et al.

1995; Ghosh and Myers 1998). The focus in this thesis is the eicosanoid signal transduction pathway via cyclooxygenase.

Prostaglandins

In the early 1930's Maurice W. Goldblatt in England and Ulf S. von Euler in Sweden independently found that human seminal fluid induces vasodilatation and contraction of smooth muscle (Goldblatt 1933, 1935; von Euler 1934, 1935a,b, 1937). Using extracts from the vesicular glands of male sheep (von Euler 1937), von Euler observed similar effects and upon examination of the extracts he determined that this activity was associated with the fraction of the extract containing lipid-soluble acids (von Euler 1935b). Von Euler called this active substance 'prostaglandin' (von Euler 1934, 1935b, 1937; von Euler and Hammarström 1937).

For several years little investigation was directed towards prostaglandin until the late 1950's and early 1960's when two lipid acids (named prostaglandin E_1 and prostaglandin $F_{1\alpha}$) were elucidated (Bergström and Sjövall 1957, 1960a,b; Bergström et al. 1960) and their structures determined to be 20-carbon cyclized fatty acids (Abrahamsson et al. 1962; Bergström et al. 1962c,d, 1963a,b; Nugteren et al. 1966). This important finding was almost immediately followed by characterization of several other prostaglandins, including specifically prostaglandins E and F, isolated from human seminal plasma and sheep vesicular glands (Bergström et al. 1962a,b; Samuelsson 1963a,b,c, 1964; Hamberg and Samuelsson 1965, 1966). What became evident over the next few years was

that all of the prostaglandin structures were 20-carbon unsaturated carboxylic acids with a cyclopentane ring which suggested they were a family of unique compounds derived, it was suspected, from eicosanoic essential fatty acids (Bergström et al. 1964a,b; Van Dorp et al. 1964a). In fact, in 1964 it was demonstrated using sheep seminal vesicles that prostaglandin E_2 is synthesized from the eicosanoic fatty acid, arachidonic acid (Bergström et al. 1964c; Van Dorp et al. 1964b) which is now known to be the major endogenous source of prostaglandins in mammalian tissues. In 1982, Bergström received the Nobel Prize for Physiology or Medicine (Oates 1982) for elucidation and characterization of the prostaglandin structures using gas chromatography and mass spectrometry, and for determining that the biosynthetic origin of the 20-carbon fatty acid skeleton of all prostaglandins derives from eicosanoic fatty acids, specifically arachidonic acid.

The first suggestion that eicosanoids may be involved in the transmission of peripheral stimulation-induced synaptic input at the level of the spinal cord was by Ramwell in 1966. It was demonstrated that the perfused spinal cord of the frog liberates prostaglandin E_1 (also released spontaneously) and $F_{1\alpha}$ evoked by electrical stimulation of the hind limbs (Ramwell et al. 1966).

Cyclooxygenase

The demonstration that prostaglandins are synthesized from arachidonic acid (Bergström et al. 1964a,c; Van Dorp et al. 1964b) and that NSAIDs interrupt this conversion (Ferreira et al. 1971; Smith and Willis 1971; Vane 1971) roused keen interest

to elucidate the enzymatic mechanism(s) underlying prostaglandin synthesis. In the mid 1970's, cyclooxygenase (or prostaglandin endoperoxide synthase) was isolated (Hemler and Lands 1976) and in the 1980's was cloned (Dewitt and Smith 1988; Merlie et al. 1988; Yokoyama et al. 1988). This membrane-bound haemo- and glycoprotein is found predominantly in the endoplasmic reticulum and to a more limited extent in the nuclear envelope of prostanoid-forming cells (Smith 1986; Goetzl et al. 1995; Morita et al. 1995; Spencer et al. 1998) and has two distinct activities: an *endoperoxide synthase (cyclooxygenase)* activity that oxygenates and cyclizes the unesterified precursor fatty acid, arachidonic acid, to form the cyclic endoperoxide prostaglandin G_2 (Hamberg et al. 1974), and a *peroxidase* activity that reduces prostaglandin G_2 to H_2 (Samuelsson 1972; Hamberg et al. 1974). Both cyclooxygenase and peroxidase activities occur within the same dimeric protein molecule but are spatially distinct (Picot et al. 1994). Although, prostaglandins G_2 to H_2 are chemically unstable they can be transformed enzymatically into a variety of products, including prostaglandin E_2, F_2, D_2, prostacyclin (prostaglandin I_2) and thromboxane A_2 (Hamberg et al. 1975; Samuelsson et al. 1975; Needleman et al. 1986; Sigal 1991; Smith 1992) depending on the presence and abundance of the particular downstream prostaglandin synthase. In 1982, Dr. Bengt Samuelsson received the Nobel Prize in Physiology or Medicine (Oates 1982) for his work isolating and identifying the two labile endoperoxides, prostaglandin G_2 and H_2, and demonstrating that biosynthesis of prostaglandins D, E and F occurs via a common endoperoxide, prostaglandin H_2.

Function of Cyclooxygenase-1 and -2

Only recently has it been appreciated that there are at least two forms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The existence of a different, specifically 'inducible' COX was first suspected by Needleman when his group discovered that bacterial lipopolysaccharide induced prostaglandin synthesis in human monocytes *in vitro* (Fu et al. 1990) and in mouse peritoneal macrophages *in vivo* (Masferrer et al. 1990). Not long after, this 'inducible' COX (called COX-2) was identified as a distinct isoform encoded by a different gene from the 'constitutively-expressed' COX-1 (Masferrer et al. 1990; Kujubu et al. 1991; O'Banion et al. 1991; Xie et al. 1991; Hla and Neilson 1992; Masferrer et al. 1992; Sirois and Richards 1992). Although the amino acid sequence of its cDNA shows 60% homology with the sequence of COX-1 enzyme and both enzymes have a molecular weight of approximately 71 kDa, there appear to be important distinguishing features of the two isoforms. In particular, some of these include in addition to differences in structure (Picot et al. 1994; Garavito 1996; Kurumbail et al. 1996) and location in the endoplasmic reticulum and nuclear envelope (Goetzl et al. 1995; Morita et al. 1995; Willingale et al. 1997), dissimilarities in kinetic (Spencer et al. 1998; Gierse et al. 1999) and catalytic (Kulmacz and Wang 1995) activity, distinct prostaglandin synthesis (Smith et al. 1997) and selectivity of NSAIDs (Mitchell et al. 1994). Importantly, the active sites for the natural substrate and for blockade by NSAIDs are slightly different (Gierse et al. 1996; Greig et al. 1997; Rieke et al. 1999) which may be due to dissimilarity in the expression of the specific amino acid

residues in the active sites (Gierse et al. 1996; Landino et al. 1997; Wong et al. 1997; Rieke et al. 1999). Interestingly, it is reported that the active site in COX-1 is a long, hydrophobic channel (Picot et al. 1994; Garavito 1996) and it is suggested that NSAIDs inhibit COX-1 by excluding arachidonic acid from the upper portion of this channel. The active site of COX-2 is slightly larger and can accommodate bigger structures than those which are able to reach the active site of COX-1. Specifically, the larger volume of the active site of COX-2 is reported to allow access for the more bulky selective COX-2 inhibitors (Wong et al. 1997).

Thus, the discovery of the inducible COX-2 isoform has provided insight into the distinct pharmacological properties of the COX-1 and -2 isoforms. Importantly, the implications of the different features are emphasized at a clinical level by means of the effects of NSAID-induced COX inhibition. In particular, it is reported that the extent of inhibition of standard NSAIDs against the two enzymes ranges from a high selectivity towards COX-1 (Meade et al. 1993; Vane and Botting 1995a) to equal inhibition of both (Akarasereenont et al. 1994). Importantly, comparison of the epidemiologic data on the side effects of standard NSAIDs shows that they can be accompanied by gastrointestinal toxicity (Lanza 1989; Garcia Rodriguez and Jick 1994). What has emerged is the concept that COX-1 is homeostatic and serves the beneficial physiological functions such as prostacyclin biosynthesis, which has a cytoprotective effect on the gastric mucosa (Vane and Botting 1995b). On the other hand, COX-2 is proposed to be proinflammatory and may be induced by inflammatory stimuli including in particular cytokines (Mino et al.

1998; Mollace et al. 1998; Noguchi et al. 1999). Thus, it is suggested that the anti-inflammatory actions of NSAIDs are due to the inhibition of COX-2, whereas the unwanted side effects such as irritation of the gastrointestinal tract (Sigthorsson et al. 1998; Singh and Ramey 1998; Bjarnason and Thjodleifsson 1999; Singh and Triadafilopoulos 1999) are due to inhibition of the constitutively-expressed isoform, COX-1 (Kargman et al. 1996; Robinson 1997). Presently, there is tremendous interest and effort to further understand the therapeutic potential of COX-2 inhibitors and possibly expand their therapeutic application.

III. Rationale for the Study of COX in Mechanisms of Nociceptive Processing

This historical background is of interest in that it provides an evolutionary perspective on the treatment of pain and various maladies by the means of a specific class of drug, NSAIDs, beginning with the therapeutic effects of crude extracts from willow tree bark and leaves to the cusp of modern day technology with selective COX-2 inhibitors. Importantly, this historical background also emphasizes the involvement of the eicosanoid signal transduction pathway via COX in pain associated with various chronic disorders. Over the last few years, increasing awareness has emerged concerning the role of the eicosanoid pathway via COX in sensory processing. Many of these important contributions are cited in Chapters 1 to 8 in this thesis.

However, when the present study was initiated significant information concerning the eicosanoid pathway was not known. For example, indirect evidence had suggested that

NSAIDs might induce their antinociceptive effects via effects on mechanisms of sensory processing in the spinal cord (Malmberg and Yaksh 1992a,b, 1993; Masue et al. 1999). However, this had not been demonstrated on the basis of direct evidence on sensory processing. Second, it was not known if such antinociceptive effects could be demonstrated at the cellular level in the spinal cord. Third, there was no evidence as to whether any spinal effects were modality-related. This is particularly important because if these effects are not modality-specific, then they cannot be considered to be related to the analgesic effects in humans, as NSAIDs are not anaesthetics but are analgesics. Fourth, there was no direct evidence that any modality-specific effects of NSAIDs were different or similar in models of chronic pain vs. in normal animals. Fifth, there was no information on whether modality-specific effects are altered in a chronic pain model. In light of the results to come, this turns out to be an important point because we note a change in phenotype of sensory neurones in the model studied in this thesis, and this would not have been picked out without this specific experimental approach. Finally, another exciting development which occurred during the progress of this study was the development of drugs which selectively inhibit COX-2, and therefore this study was able to address whether the modality-specific antinociceptive effects observed were attributable to inhibition of COX-2.

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Chapter 1

Mediation and Modulation by Eicosanoids of Responses of Spinal Dorsal Horn Neurons to Glutamate and Substance P Receptor Agonists - Results with the NSAID Indomethacin in the Rat *in vivo*

Abstract

In view of the widespread use of NSAIDs for treatment of inflammatory pain, we determined the effects of the NSAID, indomethacin, on dorsal horn neurons in the rat spinal cord *in vivo*. At 2.0-12.0 mg/kg, i.v., indomethacin depressed the responses of spinal dorsal horn neurons to the effects of iontophoretic application of substance P, N-methyl-D-aspartic acid, quisqualate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. As indomethacin inhibits cyclooxygenase, these are the first data linking prostanoids and possibly arachidonic acid and other eicosanoids to the effects of substance P and glutamate in the spinal dorsal horn. As responses to iontophoretic application can be assumed to have been postsynaptic and as indomethacin had an effect generalized to all excitatory responses, we suggest a postsynaptic site for cyclooxygenase. We also suggest that elements in the cyclooxygenase signal transduction pathway may thus mediate at least some of the effects of substance P and glutamate receptor activation. Activation of the cyclooxygenase pathway in CNS neurons is Ca^{2+}-dependent, and activation of both N-methyl-D-aspartic acid and substance P receptors increases intracellular Ca^{2+}. This led to the expectation that indomethacin would have a greater effect on responses to N-methyl-D-aspartic acid than to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, but the reverse was observed. Thus, in addition to a mediator role, we hypothesize that an element(s) of the cyclooxygenase pathway may regulate the efficacy of excitation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and perhaps other membrane-bound receptors.

The cyclooxygenase signal transduction pathway thus appears to play at least two major roles in regulation of sensory processing in the spinal cord. Therefore, nonsteroidal anti-inflammatory drugs, via cyclooxygenase inhibition, may have multiple actions in control of spinal sensory mechanisms.

Introduction

Effector mechanisms activated by excitatory synaptic inputs to dorsal horn sensory neurons are diverse in nature and several mechanisms have been implicated in synaptic transmission at the first sensory synapse, particularly in nociceptive pathways. One such intracellular signal transduction mechanism which has been implicated is the pathway where cyclooxygenase (COX) leads to the conversion of arachidonic acid to prostanoids. Interest in COX has arisen principally because of the widespread use of inhibitors of COX, also called nonsteroidal anti-inflammatory drugs, or NSAIDs, in the treatment of peripheral inflammation and associated pain; some of the better known NSAIDs include aspirin, diclofenac, ibuprofen and indomethacin.

COX inhibitors were at one time thought to act primarily in the periphery. However, recently, the landmark discovery was made that NSAIDs may act in the spinal cord to bring about at least some of their analgesic effects (Jurna and Brune 1990; Malmberg and Yaksh 1992a) and it was suggested that NSAIDs have a powerful effect on spinal nociceptive processing. Since that time, additional evidence has accumulated supporting this concept. A C-fiber reflex recorded from the biceps femoris muscle is inhibited by intrathecal administration of indomethacin (Bustamante et al. 1997). Expression of mRNA of both the constitutive and inducible isoforms of COX, COX-1 and -2, respectively, are reported in the dorsal horn (Beiche et al. 1996, 1998; Goppelt-Struebe and Beiche 1997; Willingale et al. 1997) and prostaglandin synthase expression has been shown in the superficial laminae (Vesin et al. 1995). Inhibition of COX at the spinal level

suppresses the second phase of the formalin test (Malmberg and Yaksh 1992b). Intrathecal administration of prostaglandin E_2 produces hyperalgesic effects in the paw pressure test and allodynia to innocuous stimuli in mice (Minami et al. 1995; Ferreira and Lorenzetti 1996), possibly due to enhanced release of primary afferent transmitters, such as substance P (Hingtgen et al. 1995; White 1996). Although it appears that COX is involved in processing of nociceptive information in the spinal cord, little is known about the effects of inhibition of COX at the cellular level in the spinal dorsal horn, especially on the responses of dorsal horn neurons to activation of glutamate and NK-1 (substance P) receptors.

We have been studying intracellular transduction mechanisms mediating excitatory synaptic inputs to dorsal horn neurons in the spinal cord *in vivo* (Radhakrishnan et al. 1995; Yashpal et al. 1995b) and it was a logical extension of these studies to include COX inhibitors as a means of beginning to examine the role of eicosanoids in spinal sensory mechanisms. This study therefore examines the effects of indomethacin, a COX inhibitor, on dorsal horn neurons *in vivo*. Indomethacin was used because it is one of the most potent COX inhibitors (Frölich 1997) and prevents prostanoid synthesis by both the inducible and the constitutive forms of COX. As both glutamate and NK-1 receptor activation have been implicated in sensory mechanisms at the spinal level (De Koninck and Henry 1991; Yashpal et al. 1995a; Onaka et al. 1996), it was imperative in this study to determine whether administration of indomethacin altered the responses to specific glutamate receptor agonists, including N-methyl-D-aspartic acid (NMDA), quisqualate, α-

amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and to the NK-1 receptor agonist, substance P, on dorsal horn neurons.

Some results have been reported in abstract form (Pitcher and Henry 1996).

Materials and Methods

Animal preparation

Experiments were performed on adult, male Sprague-Dawley rats from Charles River (St. Constant, Quebec). Guidelines in *The Care and Use of Experimental Animals* as outlined by the Canadian Council on Animal Care (Vols. I and II) were strictly followed. Rats (350-375g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Abbott Laboratories Ltd, Montreal, Quebec) followed by supplements of 10 mg/kg/hr, i.v, or as needed (criteria for additional anesthetic included increased heart rate and/or arterial pressure, pupil dilation, piloerection, excessive salivation or spontaneous movements). The right common carotid artery and jugular vein were catheterized for continuous monitoring of arterial pressure and for injection of drugs, respectively. Spinal cord segments L_1 to L_3 were exposed for recording as these levels receive inputs from the hind limb. The spinal cord was transected at the T_9 vertebral level to eliminate supraspinal influences on the activity of lumbar dorsal horn neurons. Xylocaine (0.05 ml of 1%; Astra, Mississauga, Ontario) was first injected into the spinal cord at the level of transection to minimize spinal shock. The rats normally breathed spontaneously and if the breathing pattern became irregular or if respiratory arrest occurred, the animal was paralyzed with pancuronium bromide (Pavulon, Organon, Scarborough, Ontario; 1 mg/kg i.v. supplemented as necessary) and mechanically ventilated. The spinal cord was covered with mineral oil (Marcol 72, Imperial Oil Limited; Montreal, Quebec) at 37.5°C to prevent drying. Temperature of the rat was maintained at 37.5°C using a heating lamp.

Electrical recording and data acquisition

Single unit spikes were recorded extracellularly using seven-barrelled micropipettes (overall tip diameter 4-5 μm). A solution of 2.7 M NaCl was placed in the central recording barrel (impedance 2-4 MΩ measured at 1 kHz with the tip submerged in saline). Single unit recordings were made at depths ranging from 250 to 1300 μm in the dorsal horn. The raw data were amplified using a unity-gain preamplifier built in-house, displayed on an oscilloscope (Tektronix 5111) and stored on video cassette tapes using a digital data recorder that incorporated a digital pulse code modulation technique (VR-100A, Instrutech Corporation) and a conventional video cassette recorder. The signals were also relayed to a frequency counter/gating unit which discriminated single units, based on spike height, and which counted the number of spikes per unit time (bin widths were 1 s). The rate of discharge (the output of the gating unit) was displayed continuously on a Grass 79D polygraph. Sampling of extracellular recordings was done using the electrophysiological data acquisition program, *Spike 2* (Version 2.02; *Cambridge Electronic Design* 1996) on an IBM Pentium computer.

Functional classification of units

Functional classification of neurons was based on the response to stimulation of their receptive field in the ipsilateral hind limb by both noxious and innocuous stimuli. The following natural peripheral stimuli were used in this study as search stimuli to elicit synaptic input while penetrating the dorsal horn and to characterize functionally the dorsal

horn neurons: (i) air stream passed over the receptive field sufficient to move only the hairs, (ii) light touch, (iii) moderate pressure (0.2 N), (iv) noxious mechanical stimulation using a calibrated clip (21 N) and (v) noxious thermal cutaneous stimulation using radiant heat (measured to be 50°C at the skin surface). The thermal stimulation was applied for a duration of 8 s and was cycled automatically at a fixed interval of 1 or 2 min. The mechanical stimulation was applied for 3 s every 3 or 4 min. Classification of the identified neurons was in three categories (Henry 1976): (i) non-nociceptive neurons that responded only to non-noxious stimuli such as hair, touch and/or pressure stimulation (some receptive fields on the rat hind limb did not have hair), (ii) wide dynamic range neurons that responded to both noxious and non-noxious stimuli and (iii) nociceptive-specific neurons that responded only to noxious stimuli such as noxious mechanical and/or thermal stimulation. In addition, all the units that responded to the "noxious" range of mechanical and/or thermal stimulation showed a characteristic afterdischarge, as described previously (Henry 1976; De Koninck and Henry 1991). Each neuron was classified functionally before any tests of chemical sensitivity were run. For each neuron the excitatory receptive fields for hair movement, light touch, moderate pressure, noxious mechanical and noxious thermal stimulation were represented on a schematic diagram of the hind limb. Although the cutaneous receptive field was determined for each neuron tested, receptive field sizes generally remained unchanged throughout the experiments and were not investigated further in this study.

Iontophoresis

Indomethacin was tested on the responses of single neurons to iontophoretic application of excitatory neuroactive agents because these responses are generally considered to constitute postsynaptic responses. Each of the outer barrels of the micropipette was filled with one of the following solutions: N-methyl-D-aspartic acid (NMDA; 50 mM in 100 mM NaCl), quisqualate (2.5 mM in 100 mM NaCl), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 1 mM in 100 mM NaCl), substance P (0.8 mM in 165 mM NaCl, pH 5.5) and a control solution (165 mM NaCl). The pH of each solution except substance P was adjusted to 7.4. With the exception of substance P (+95 nA for 90 s), iontophoretic application was with inward current (negative polarity). Current strengths ranged from -40 to -80 nA for iontophoretic administration of NMDA and quisqualate and from -70 to -110 nA for AMPA, and currents were applied for 8 to 10 s. To determine whether the current used influenced neuronal activity, control ejections of NaCl of the same magnitude and duration as the test applications were run.

Drug administration

Indomethacin was administered intravenously because it is an uncharged molecule and therefore cannot be applied by iontophoresis. Indomethacin was dissolved in 2% sodium bicarbonate and this solution was titrated to pH 7.4 using sodium monophosphate. Control animals were administered vehicle, 2% sodium bicarbonate, in a similar fashion.

Indomethacin is generally considered to be a non-selective COX-1/-2 inhibitor and

was used in this study to inhibit activation of both COX isoforms (Harada et al. 1998). In other studies examining nociceptive mechanisms in the spinal cord, systemic administration of indomethacin has been used effectively at doses ranging from 1 to 50 mg/kg (Honoré et al. 1995; Bustamante et al. 1996). In the present study, indomethacin was found to be effective in the range 2 to 12 mg/kg, i.v.

Data analysis

The total number of spikes in the responses of dorsal horn neurons to iontophoretic drug administration was determined in each case for a duration of 8-10 s from the onset of NMDA, quisqualate or AMPA responses. In the case of iontophoretic application of substance P, the sample time was increased due to the longer duration of the response to the peptide.

On-going baseline activity was determined as the number of spikes over an 8 or 10 s period for NMDA, quisqualate or AMPA or 2 min immediately preceding the response to substance P. This number was subtracted from the number of spikes occurring during the drug response.

For statistical comparisons, three responses before and three responses after administration of indomethacin were averaged. An effect of indomethacin was calculated by taking the magnitude of the response to stimulation before indomethacin administration minus the magnitude of the response after administration, over the magnitude of the response before indomethacin. This was multiplied by 100 to yield a percent of the

response prior to indomethacin administration. For example, no effect of indomethacin was considered 0% inhibition while complete inhibition of a response by indomethacin was considered 100% inhibition. As the possibility must be considered that not all dorsal horn neurons express COX or are perhaps in some cases insensitive to the effects of COX inhibition, neurons unresponsive to indomethacin were not included in calculation of the percent inhibition; rather, this was done using the responses of dorsal horn neurons that were inhibited by indomethacin.

Only neurons that were sensitive to the effect of indomethacin and showed full recovery following indomethacin were included in this study. Neurons that were insensitive to indomethacin were reported in the ratios of the dose-response histograms. Some neurons were tested more than once with indomethacin. This was done only using a higher subsequent dose and only following full recovery from the lower dose, to ensure that there was no remaining effect of the drug. As a result, each dose group was derived from different neurons.

To calculate significance, the mean ($\pm$SEM) percent inhibition following indomethacin was compared to the mean effect of vehicle administration which was tested on the same testing parameter, for example, substance P, NMDA, quisqualate or AMPA in the same group of neurons. Statistical analysis of the data was done using one-way ANOVA and Student-Newman-Keuls test. A difference between test and control responses was considered significant with a P value <0.05.

Materials

Indomethacin was obtained from Research Biochemicals International, Natick, Massachusetts, USA. NMDA and quisqualate were obtained from Sigma-Aldrich Canada Ltd., Oakville, Ontario, AMPA from Tocris Cookson, Ballwin, Missouri, USA, and substance P from Peninsula Laboratories Inc., Belmont, California, USA.

Results

Effects of indomethacin on on-going activity

As can be seen in the figures, on-going baseline activity was generally unaltered by administration of indomethacin.

Effect of substance P in the presence of indomethacin

Iontophoretic application of substance P produced the typical delayed, slow and prolonged excitatory effect (Henry 1976) on 6 wide dynamic range neurons and 1 nociceptive specific neuron. Figure 1Aa shows one example of an excitatory effect of substance P on a wide dynamic range neuron. This response persisted for approximately 5 min after the end of iontophoretic application.

An effect of indomethacin on a response to substance P is shown in Figure 1A. The response to substance P was attenuated as soon as 4 min following administration of indomethacin (4 mg/kg, i.v.; Figure 1Aa). Indomethacin attenuated both the magnitude and the duration of the excitatory effect of substance P. Figure 1Ab shows that the response to substance P had recovered partially 50 min after indomethacin was given.

Indomethacin was tested on substance P-induced excitation in a total of 4 wide dynamic range neurons and 1 nociceptive specific neuron; in all 5 neurons indomethacin depressed the response to substance P. For the remaining two neurons testing was not considered to be complete. In all cases, full or partial recovery of the response to

substance P was observed. The earliest recovery occurred approximately 80 to 100 min after administration of indomethacin. The effects of indomethacin were dose-related. Maximum inhibition of the responses to substance P generally occurred 10 to 30 min after administration of indomethacin. The data are summarized in Figure 1B. In the cases where more than one dose was tested on one neuron, a subsequent dose was given only after full recovery from the previous dose had occurred. The ratios in the histograms in the figures represent the number of different neurons inhibited by indomethacin over the number tested at that particular dose. In five neurons, 2% Na-bicarbonate was tested on the response to substance P and it was without effect in all cases.

Effect of NMDA in the presence of indomethacin

NMDA was tested on 14 non-nociceptive and 6 wide dynamic range neurons. It produced a typical excitatory effect which was rapid in onset and which ended abruptly following termination of iontophoresis.

Figure 2Aa illustrates an effect of 4 mg/kg of indomethacin on one non-nociceptive neuron. The amplitude of the response to NMDA was depressed within 2 min of administration. Figure 2 Ab shows that recovery had occurred by 50 min after administration. Figure 2B illustrates an effect of 8 mg/kg of indomethacin on one non-nociceptive neuron. Maximum inhibition of the NMDA response occurred approximately 15 to 20 min after indomethacin was given.

Indomethacin was tested on the response to NMDA of both non-nociceptive (n = 11)

and wide dynamic range (n=4) neurons; in the remaining 5 neurons testing was not considered to have been complete. The data are summarized in Figure 2C; some neurons were tested with more than one injection of indomethacin, but full recovery from the first dose was always obtained before administration of the second dose. Indomethacin depressed the excitatory effect of NMDA on both wide dynamic range and non-nociceptive neurons. As the inhibitory effect of indomethacin was similar in both kinds of neuron the mean percent inhibition for each dose was calculated including data from the both nociceptive and non-nociceptive neurons. Figure 2C shows that responses of only 4 out of 9 neurons were depressed by 4 mg/kg; this dose depressed the responses to substance P in all neurons tested. Depression of NMDA-induced excitation typically lasted approximately 60 min after indomethacin administration. In all cases where depression occurred, full or partial recovery of the response to NMDA was seen.

Neuronal responses to NMDA were unaffected by 2% Na-bicarbonate (n=5).

Effect of quisqualate in the presence of indomethacin

Iontophoretic administration of quisqualate evoked a transient excitatory effect with a rapid onset and a rapid offset following the end of iontophoresis. This was observed with both non-nociceptive (n=14) and wide dynamic range (n=5) neurons.

Figure 3Aa shows that 4 mg/kg indomethacin decreased the response of a wide dynamic range neuron to iontophoretic application of quisqualate beginning approximately 2 min following the i.v. injection. Figure 3Ab shows recovery of the quisqualate-induced

excitatory effect 80 min after indomethacin administration.

Both non-nociceptive (n=11) and wide dynamic range (n=3) neurons were tested with indomethacin. Some neurons were tested for dose-dependency. In the remaining 5 neurons testing was not considered to have been complete. Indomethacin-induced depression of the response to quisqualate was dose-dependent (Figure 3B). This was observed in both wide dynamic range and non-nociceptive neurons. As responses of both types of neuron were affected similarly by indomethacin, the data analysis grouped them together. Maximum inhibition of the response to quisqualate occurred approximately 10 to 30 min after indomethacin administration. Generally the quisqualate responses recovered approximately 70 to 90 min after indomethacin administration.

Neuronal responses to quisqualate were unaffected by 2% Na-bicarbonate (n=6).

Effect of AMPA in the presence of indomethacin

AMPA was tested on 21 non-nociceptive and 13 wide dynamic range neurons. Iontophoretic application of AMPA produced a transient excitatory effect similar to that of NMDA and quisqualate.

Figure 4Aa shows the depressive effect of 6 mg/kg of indomethacin on the response of a non-nociceptive neuron to AMPA. The response to AMPA was depressed as soon as 5 to 10 min after indomethacin administration. Figure 4Ab shows that the inhibitory effect of indomethacin was reversible (recovery was 90 min later).

Indomethacin was tested on both non-nociceptive (n=18) and wide dynamic range (n=10) neurons; in the remaining 6 neurons testing was not considered to have been complete. The depressant effects of indomethacin were dose-related in both wide dynamic range and non-nociceptive neurons. As responses of both types of neuron were affected similarly by indomethacin, the data analysis grouped them together. Maximum inhibition generally occurred 10 to 30 min after administration of indomethacin. Generally the AMPA response recovered approximately 80 to 100 min after administration of indomethacin.

Figure 4Ba shows an extracellular recording of a non-nociceptive neuron. After 6 mg/kg indomethacin, the onset of the AMPA response was delayed and the duration was attenuated.

Figure 4C illustrates that the response of most neurons to AMPA were significantly depressed by indomethacin in a dose-related manner. Some neurons were tested with more than one dose of indomethacin.

Comparison of the effects of indomethacin on responses to glutamate receptor agonists

Due to the apparent variability in the effects of indomethacin on the responses to activation of the different glutamate receptors, some neurons were tested with both NMDA and AMPA (n=11) with different doses of indomethacin. A selective inhibitory effect of indomethacin is apparent in Figure 5A, where indomethacin (6 mg/kg) depressed the excitatory effect of AMPA more than the NMDA response. This preferential inhibitory

effect of indomethacin on the response to AMPA vs. the response to NMDA was observed in each of the 5 neurons tested.

Eleven neurons were tested with both quisqualate and AMPA. Figure 5Ba demonstrates that indomethacin (8 mg/kg) was roughly equipotent on both kinds of response (n=8).

Discussion

The objective in this study was to examine the involvement of eicosanoids on responses to activation of NK-1 and glutamate receptors in the spinal dorsal horn. Evidence is revealed suggesting multifunctional roles of this pathway on these responses. Iontophoretic administration of substance P, NMDA, quisqualate and AMPA each produced excitation of neurons, probably by activation of NK-1 and glutamate receptors, respectively. Inhibition of COX dose-dependently depressed the excitatory effects of each chemical. Furthermore, as indomethacin depressed the excitatory effects on non-nociceptive, wide dynamic range and nociceptive specific neurons, it is suggested that COX is present in the spinal dorsal horn and that eicosanoids have effects on the activity of the three classes of neuron. The data also provide evidence that the eicosanoid signal transduction pathway is involved in responses to both non-nociceptive and nociceptive inputs.

Although eicosanoids may also be involved in mechanisms of vasodilatation or vasoconstriction, it is unlikely that the inhibitory effects of indomethacin on NK-1 and glutamate receptor activation reported here were due to local vasodilatation or vasoconstriction in the dorsal horn of the spinal cord as this would affect blood pressure and subsequently cause cell movement in the recording environment. Extracellular traces revealed constant spike amplitude throughout the experiments, indicating stable recording. Furthermore, there were no changes in arterial pressure, heart rate or respiratory rate following administration of indomethacin. Finally, if changes in circulation had been a

factor, one would have seen changes in on-going activity, but this did not occur.

These effects of indomethacin may have been due to decreased levels of prostanoids because of inhibition of COX. The notion that cyclooxygenase inhibition, via indomethacin, decreases prostanoid levels specifically in the spinal cord is not without support. For example, it is known that indomethacin decreases the capsaicin-induced increase in prostaglandin E_2 in the spinal cord slice (Malmberg and Yaksh 1994) as well as the formalin injection-induced increase in spinal prostaglandin E_2 *in vivo* (Yang et al. 1996). However, inhibition of COX could also have increased the substrate, arachidonic acid, or even shunted arachidonic acid metabolism through another eicosanoid pathway deriving from arachidonic acid, such as the lipoxygenase pathway (Vaughan et al. 1997; Kirchner et al. 1997; Gilroy et al. 1998) or the isoprostanes. Although not yet investigated in spinal dorsal horn neurons, there is evidence that indomethacin increases the level of free arachidonic acid in human colorectal cancer cells (Chan et al. 1998) and in *Tetrahymena pyriformis* (Kovács and Csaba 1997). Therefore, it is reasonable to suggest that the effect(s) of indomethacin in the present study may have involved not only a decrease in prostanoid levels but also an increase in the arachidonic acid level. In fact, it is speculated that COX inhibition increases eicosanoid synthesis via the lipoxygenase pathway in the midbrain periaqueductal grey (Vaughan et al. 1997). Indomethacin is also reported to produce elevated levels of leukotriene B_4 in the rat gastric mucosa (Kirchner et al. 1997) and the COX-2 inhibitor, NS-398, is reported to increase the level of leukotriene B_4 in the rat (Gilroy et al. 1998). Although there is a suggestion that

isoprostanes, such as 8-epi-prostaglandin $F_{2\alpha}$ (F_{-2} isoprostane), are generated *in vivo* from the free radical-catalyzed peroxidation of arachidonic acid independent of the COX enzyme (Morrow and Roberts 1996), there is evidence that isoprostane synthesis in rat platelets, monocytes and mesangial cells may in fact occur via a COX-dependent mechanism (Bachi et al. 1997; Jourdan et al. 1997; Klein et al. 1997). Thus, although arachidonic acid is generally thought to be present only transiently in central nervous tissue (Wolfe and Horrocks 1994), there is no reason to exclude any involvement of arachidonic acid, the lipoxygenase pathway and other components of the lipid cycle in the excitatory effects of glutamate and NK-1 receptor activation.

Spinal site of action of indomethacin

It was initially determined that indomethacin is not well suited for iontophoretic application, and all subsequent administrations of indomethacin were via a systemic route. This was considered reasonable in view of the penetration of indomethacin across the blood-brain barrier (Bannwarth et al. 1990) and the short time for effects to be seen centrally after systemic administration (Bustamante et al. 1996). While indomethacin was thus undoubtedly having a peripheral effect, this study was concerned with the effects of indomethacin on the responses of dorsal horn neurons to iontophoretic application of substance P, NMDA, quisqualate and AMPA. As these chemicals were applied directly into the vicinity of dorsal horn neurons, it can be concluded that the effects of indomethacin reported here were due to a spinal action. In fact, by virtue of the nature of

the technique of iontophoresis, the effects of indomethacin reported here would likely have been postsynaptic on each neuron studied. Neurons in the spinal dorsal horn are reported to express both COX-1 and -2 (Beiche et al. 1996, 1998; Goppelt-Struebe and Beiche 1997; Willingale et al. 1997).

Eicosanoids as mediators of the response to substance P

Our evidence appears to be the first to link eicosanoids to the effects of substance P in the spinal dorsal horn. This link may be via an increase in intracellular Ca^{2+}. Ca^{2+} is essential for activation of phospholipase A_2 (Lichtenbergova et al. 1998) and COX in CNS neurons (Stephenson et al. 1994; Kim et al. 1995; Kramer and Sharp 1997). NK-1 receptor activation induces Ca^{2+} influx in dorsal horn neurons *in vitro* (Murase et al. 1986; Womack et al. 1988; Marvizón et al. 1998) and increases intracellular levels of inositol-1,4,5-trisphosphate (IP_3) in the dorsal horn (Igwe 1994). IP_3 inhibits a Ca^{2+}-ATPase in the endoplasmic reticulum and thus releases Ca^{2+} into the cytosol. A substance P-induced release of Ca^{2+} from intracellular stores has been reported in a number of tissues including spinal dorsal horn neurons (Womack et al. 1988).

Thus, it is reasonable for us to suggest that effects of NK-1 receptor activation in our experiments were mediated at least partially via eicosanoids. In rat intrapulmonary bronchi, substance P is reported to evoke prostaglandin E_2 release (Szarek et al. 1998); this release was decreased following administration of the NK-1 receptor antagonist, RP-67580, or the COX inhibitor, meclofenamate.

Eicosanoids as mediators of responses to NMDA

Ours appears also to be the first evidence linking eicosanoids to the effects of NMDA receptor activation in the spinal dorsal horn. In view of the central role of increased intracellular levels of Ca^{2+} argued above and in view of the well-known increased intracellular levels of Ca^{2+} upon NMDA receptor activation, it was interesting to note that there was less of an effect of indomethacin on the response to iontophoretic application of NMDA than was observed on the responses to the other drugs. Differential regulation of membrane-bound receptors is discussed below, but this lesser effect of COX inhibition on the response to NMDA is consistent with the earlier report that NMDA-induced thermal hyperalgesia is unaffected by COX inhibition (Meller et al. 1996).

Activation of NMDA receptors on rat spinal dorsal horn neurons has been shown to increase intracellular Ca^{2+} concentration (Kawamata and Omote 1996) and we have reported that hyperalgesia induced by intrathecal administration of NMDA is attenuated by inhibition of intracellular Ca^{2+} release. The NMDA receptor has a higher Ca^{2+} permeability than the non-NMDA ionotropic receptors (Burnashev et al. 1995; Wollmuth and Sakmann 1998). NMDA receptor activation has been shown to induce the release of arachidonic acid via a Ca^{2+}-dependent phospholipase A_2 in central neurons (Dumuis et al. 1988; Lazarewicz et al. 1990). It has also been shown that preincubation of human cultured astroglial cells with NMDA produces a dose-dependent increase in the level of prostaglandin E_2 in the supernatant (Jefferys and Funder 1994) and this release is decreased by administration of indomethacin. Furthermore, *in vivo* microdialysis of the rabbit

hippocampus revealed that NMDA receptor activation resulted in an eightfold increase in 6-keto-prostaglandin $F_{1\alpha}$ concentration (Lazarewicz and Salinska 1995). Extrusion of Ca^{2+} from the dialysis medium inhibited the release by about 50%. In addition, administration of quinacrine, a phospholipase A_2 inhibitor, decreased the NMDA-evoked eicosanoid release by 30%, whereas indomethacin completely suppressed the release. Finally, NMDA receptor activation has been shown to induce prostaglandin $F_{2\alpha}$-mediated c-Fos expression in neurons in the dentate gyrus (Lerea et al. 1997).

Thus, NMDA receptor-induced activation of dorsal horn neurons may be occurring by a sequence of intracellular events similar to the mechanism described above for substance P and it is reasonable to suggest that effects of NMDA receptor activation may be mediated in part by eicosanoids.

Eicosanoids as mediators of responses to quisqualate

The present study demonstrates that the quisqualate-induced excitatory response of dorsal horn neurons was dose-dependently inhibited by indomethacin. Similar to NK-1 or NMDA receptor activation, quisqualate-induced receptor activation is also linked to an increase in intracellular Ca^{2+}. Quisqualate induces Ca^{2+} influx in leech glial cells (Hochstrate and Schlue 1994) as well as release of Ca^{2+} from intracellular stores in cells from the cochlear nucleus as well as basolateral amygdala neurons (Zirpel et al. 1995; Keele et al. 1997).

However, the link between the quisqualate receptor and eicosanoids may be

different from that of NK-1 and NMDA receptor activation. For example, stimulation of quisqualate receptors induces arachidonic acid release from striatal neurons via phospholipase C activation (Dumuis et al. 1990). Quisqualate has also been shown to stimulate hydrolysis of inositol phospholipids in rat striatal, cerebellar granule and hippocampal neurons (Nicoletti et al. 1988; Schoepp and Johnson 1988) and one of the products of phospholipase C activation is diacylglycerol, which is metabolized to arachidonic acid by diacylglycerol lipase (Wolfe and Horrocks 1994). In fact, it has even been suggested that phospholipase A_2 and phospholipase C are differentially activated via activation of different glutamate receptors. In astroglial cells activation of phosphoinositide metabolism, specifically the breakdown of phosphoinositide by phospholipase C, is induced by quisqualate but not by NMDA receptor activation (Milani et al. 1989). NMDA-induced release of arachidonic acid is reduced by quinacrine at concentrations that inhibited phospholipase A_2 but this does not affect either the activity of phospholipase C or the hydrolysis of phosphoinositides induced by quisqualate (Lazarewicz et al. 1990), suggesting that the effects of quisqualate receptor activation on eicosanoids may not necessarily involve phospholipase A_2 activation. In fact, there may be a differential generation of arachidonic acid via the phospholipase A_2 and phospholipase C pathways; specifically, it was determined that phospholipase A_2 activation in platelets requires higher levels of intracellular Ca^{2+} than phospholipase C (Simon et al. 1986). Similarly, in another study using cerebellar granule cells, even in the absence of extracellular Ca^{2+}, activation of inositol phospholipid metabolism occurred after quisqualate administration (Nicoletti et al. 1986). Thus, it is suggested that quisqualate

receptor-mediated activation of eicosanoids, via phospholipase C, may be distinct from the phospholipase A_2-mediated pathway and may also require less Ca^{2+} for activation.

Ours thus appears to be the first evidence associating quisqualate receptor activation with eicosanoids in the spinal dorsal horn. This effect is consistent with the effects discussed above regarding activation of NK-1 and NMDA receptors and this intracellular signal transduction pathway.

COX inhibition and the responses to AMPA

Intriguingly, AMPA-induced excitation of dorsal horn neurons was depressed following COX inhibition. This observation was surprising because although AMPA receptor activation has been reported to increase Ca^{2+} concentration in some brain and cerebellar neurons (Iino et al. 1994; Hack and Balázs 1995) in most CNS neurons AMPA receptor activation causes little Ca^{2+}-permeability (Hollmann and Heinemann 1994; Burnashev et al. 1995; Geiger et al. 1995). In the heteromeric AMPA receptor the Ca^{2+}-impermeability properties of the GluR2 (GluR-B) subunit are dominant (Geiger et al. 1995).

At the spinal dorsal horn level, the majority of AMPA receptors are reported to be Ca^{2+}-impermeable (Furuyama et al. 1993; Tölle et al. 1993; Tachibana et al. 1994). Although a Ca^{2+}-permeable AMPA receptor may exist in specific laminae in the dorsal horn (Gu et al. 1996; Engelman et al. 1999), several other reports demonstrate the predominance of AMPA receptors containing the Ca^{2+}-impermeable GluR2 subunit in the

spinal dorsal horn (Goldstein et al. 1995; Bonnot et al. 1996; Tomiyama et al. 1996; Ye and Westlund 1996). Therefore, in our experiments, while AMPA-induced excitation may have been mediated in part via a Ca^{2+}-dependent mechanism similar to that described for NK-1 receptor activation, a Ca^{2+}-independent mechanism cannot be overlooked.

If Ca^{2+}-impermeable receptors were involved in eliciting the effects of AMPA in the present study, it is intriguing, considering the argument that eicosanoids may be linked to NK-1 or NMDA receptor activation via increased intracellular Ca^{2+}, that COX inhibition altered the excitatory effect of AMPA. Four possible explanations for this are suggested for consideration. One is that AMPA may have been activating eicosanoids via a Ca^{2+}-independent mechanism. While there is evidence of Ca^{2+}-independent phospholipase A_2 (Yang et al. 1996; Farooqui et al. 1997), to our knowledge there is no link to activation of this enzyme following AMPA receptor activation at the spinal level. Second, eicosanoid synthesis may result from depolarization *per se* induced in this case by AMPA receptor activation. However, this is unlikely because depolarization by itself does not seem to be sufficient to increase the level of arachidonic acid in the hippocampus (Sanfeliu et al. 1990). Third, AMPA may be acting on non-NMDA ionotropic receptors, as discussed above for quisqualate receptor activation, with subsequent activation of phospholipase C and hydrolysis of inositol phospholipids. However, the effects of ionotropic quisqualate receptor activation on phospholipase C activity as well as phospholipid hydrolysis are not mimicked by AMPA administration in hippocampal and striatal neurons (Schoepp and Johnson 1988; Dumuis et al. 1990). A fourth possibility is

that an element in the eicosanoid pathway regulates the AMPA receptor. That is, increased levels of arachidonic acid or decreased levels of prostanoids, both of which could result from COX inhibition, might decrease the efficacy of activation of the AMPA receptor.

Thus, to account for our observations, we hypothesize that indomethacin administration produces decreased levels of prostanoids and/or increased levels of intracellular arachidonic acid (Kovács and Csaba 1997; Chan et al. 1998) and/or possibly other eicosanoids, and that this change alters the efficacy of AMPA receptor activation. Interestingly, in rat hippocampal and dorsal root ganglion neurons, docosahexaenoic acid or arachidonic acid decreases the peak current elicited by kainate (Wilding et al. 1998). Furthermore, in cerebellar granule cells and pyramidal neurons, arachidonic acid decreases AMPA-induced current (Kovalchuk et al. 1994; Nishikawa et al. 1994), providing support for the idea that indomethacin may depress the response to AMPA by altering intracellular levels of eicosanoids.

Modulator role of eicosanoids

Support for this idea of a modulatory role comes from evidence that arachidonic acid has an effect on membrane-bound proteins including chemically-sensitive receptors. NMDA receptors, for example, seem to be affected by arachidonic acid. In acutely dissociated rat cerebral cortex pyramidal neurons arachidonic acid potentiates a peak NMDA-induced current (Nishikawa et al. 1994; Horimoto et al. 1996). In isolated cerebellar granule cells arachidonic acid increases an NMDA current by increasing channel

open probability without a change in open channel current, and it was suggested that this might have occurred by binding directly to a novel site on the receptor or by altering the lipid environment of the receptor (Miller et al. 1992). In fact, the excitatory effect of arachidonic acid on NMDA responses is reported to occur via mechanical deformation of the plasma membrane (Casado and Ascher 1998). Furthermore, in xenopus oocytes, the ϵ 1/zeta 1 heterodimer of the NMDA receptor is reported to be more sensitive to arachidonic acid than the ϵ 2/zeta 1 heterodimer (Tabuchi et al. 1997). There is no obvious reason to exclude other membrane-bound, ligand-sensitive receptors from such a modulation by arachidonic acid. If modulation of the AMPA receptor by arachidonic acid contributed to the results of the present study, we must consider that there may have been a regulation of other membrane-bound receptors as well. Accordingly, some of the effects of indomethacin on responses to substance P, NMDA and quisqualate may have been due to this additional regulatory, or modulatory, role rather than only to the mediator role discussed above via alterations in the levels of particular eicosanoids which may have been sufficient to alter the effects of receptor activation.

Arachidonic acid and its non-metabolizable analogue, eicosatetraynoic acid, inhibit a K_{ATP} current in dog coronary artery smooth muscle cells *in vitro* (Xu and Lee 1996). Other K^+ channels also appear to be affected by arachidonic acid. Arachidonic acid alters the amplitude and duration of Ca^{2+} transients and myocyte shortening in rat ventricular myocytes by inhibition of voltage-gated K^+ channels (Damron and Summers 1997). A-type K^+ currents in myocytes of guinea pig vas deferens, ureter and proximal colon and

in rabbit vas deferens are reduced by arachidonic acid (Nagano et al. 1997). It has been reported that arachidonic acid modulates the K^+ M-current (I_M) in bullfrog sympathetic neurons (Villarroel 1994).

Ca^{2+} channels are also affected. Voltage-dependent Ca^{2+} channel currents in single smooth muscle cells freshly isolated from vas deferens of the guinea pig are decreased by arachidonic acid and the suggestion was made that arachidonic acid may be expressing its effects directly on Ca^{2+} channels or on membrane phospholipids (Nagano et al. 1995).

A voltage-gated Na^+ conductance in rat skeletal and cardiac muscle cells is depressed by arachidonic acid, the mechanism proposed being a decrease in the total gating charge and alteration of fast-inactivation kinetics (Bendahhou et al. 1997).

With regard to transmitter transporters, uptake of glutamate into astrocytes is inhibited by arachidonic acid (Volterra et al. 1994), and Ca^{2+}-induced inhibition of kainate-evoked glutamate release from cultured chick retina cells is partially reversed by inhibition of phospholipase A_2 (Duarte et al. 1996). Arachidonic acid has also been reported to inhibit uptake of D-aspartate into rat brain synaptosomes (Lundy and McBean 1996). As inhibition of transporters of excitatory neurotransmitters has been suggested to lead to excessive excitation (Tong and Jahr 1994), arachidonic acid may thus play a pivotal role in regulation of excitability in spinal nociceptive mechanisms as well as other central nervous system functions (Rothstein et al. 1992)}.

Some evidence supports the idea that prostanoids may also regulate the efficacy of membrane-bound proteins. The arachidonic acid-induced increase in K^+ currents in rat

neocortical neurons is inhibited by indomethacin; administration of prostaglandin E_2 or carba-prostaglandin I_2 increases these currents (Zona et al. 1993). Prostaglandin E_2 and carba-prostaglandin I_2, but not prostaglandin $F_{2\alpha}$, have also been reported to suppress an outward K^+ current in embryonic rat sensory neurons (Nicol et al. 1997). Prostaglandin E_2 and carba-prostaglandin I_2, but not prostaglandin $F_{2\alpha}$, have also been reported to suppress an outward K^+ current in embryonic rat sensory neurons (Nicol et al. 1997).

Prostaglandin E_2 modulates the tetrodotoxin-resistant Na^+ channel in rat dorsal root ganglion neurons to increase the magnitude of the response to a constant suprathreshold stimulus (England et al. 1996) and to increase the rate of activation and inactivation (Gold et al. 1996a; Cardenas et al. 1997). This modulatory effect may involve phosphorylation as the prostaglandin E_2-induced effect on the tetrodotoxin-resistant Na^+ channel in rat dorsal root ganglion neurons is depressed by inhibitors of both protein kinase C and A (Gold et al. 1998). Prostaglandin E_2 decreases the Ca^{2+}-dependent slow afterhyperpolarization in cultured dorsal root ganglion neurons and increases the number of action potentials generated in response to depolarizing current injection (Gold et al. 1996b).

Evidence is also available indicating an effect of prostanoids on neurotransmitter release. Prostaglandin I_2 stimulates the release of substance P and CGRP from rat sensory neurons (Hingtgen et al. 1995) and prostaglandin E_2 and carba-prostaglandin I_2 facilitate glutamate and peptide release from these neurons (Hingtgen et al. 1995; Ferreira and Lorenzetti 1996), perhaps by binding to a G-protein-coupled binding site (White 1996).

Mediation vs. modulation

Therefore, our suggestion that some of the effects of indomethacin on responses to substance P, NMDA and quisqualate may have been due to an additional regulatory, or modulatory, role is not without other supporting evidence. This regulation may be indirect, on effector mechanisms, but our vision here is a more direct one, whereby intracellular arachidonic acid and other eicosanoids including those in the lipoxygenase pathway, increased by inhibition of COX, or prostanoids, decreased by inhibition of COX, regulates the efficacy of chemically-sensitive membrane-bound receptors. The greater effect of indomethacin on non-NMDA vs. NMDA receptor-mediated responses suggests that the modulator role may predominate for some membrane-bound receptors over others.

Just how important each of these mechanisms is to nociceptive neurotransmission remains to be determined. However, it is important to point out here that there is some evidence that AMPA and kainate receptor activation does not stimulate arachidonic acid release, diluting arguments for a mediator role in these cases. For example, in a study in which NMDA induced arachidonic acid release, it was found that kainate and quisqualate were ineffective (Dumuis et al. 1988). Thus, our results could be explained exclusively by COX inhibition altering eicosanoid levels to where the responses to AMPA and quisqualate were depressed. We are not saying that this is necessarily the case, but considering the data together, the hypothesis most consistent with the literature is that for all mechanisms eliciting the effects seen here (ie. membrane-bound receptors) the effects may be at least equally explainable by this modulation vs. the mediation role of

eicosanoids.

Conclusions

As responses to iontophoretic application of substance P, NMDA, quisqualate and AMPA can be assumed to have been postsynaptic and as indomethacin had an effect generalized to all excitatory responses, we suggest a postsynaptic site for COX. We also suggest that elements in the COX pathway may thus mediate at least some of the effects of NK-1 and glutamate receptor activation. In addition to a mediator role, we hypothesize that an element of the COX pathway may regulate the efficacy of excitation of membrane-bound proteins, including chemically sensitive receptors. The COX pathway thus appears to play at least two major roles in regulation of sensory processing in the normal spinal cord. Therefore, NSAIDs or COX inhibition may have multiple actions in control of sensory processing, specifically in the transmission of non-nociceptive and nociceptive information in the spinal dorsal horn.

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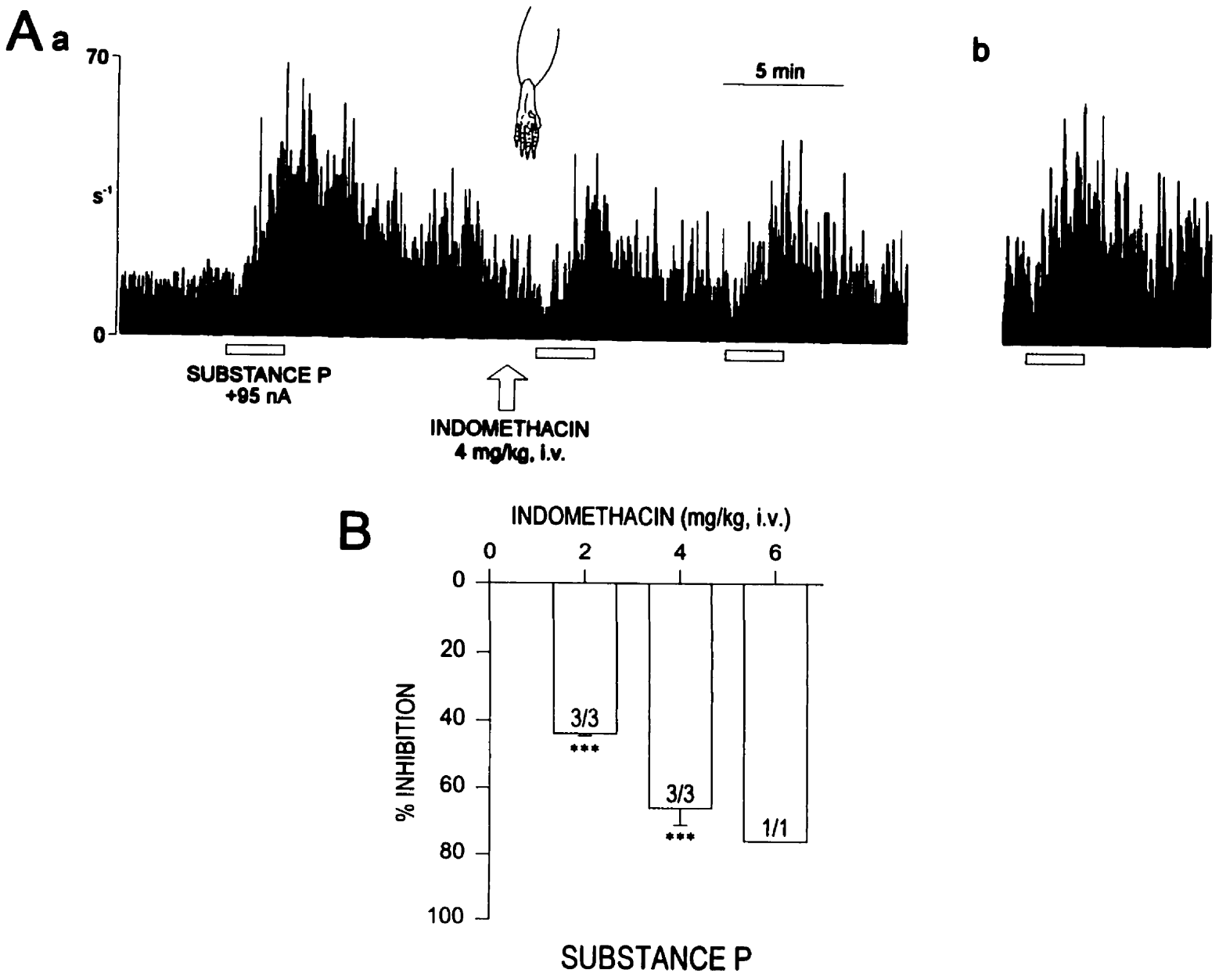
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70
s⁻¹
0
5 min
SUBSTANCE P
+95 nA
INDOMETHACIN
4 mg/kg, i.v.
b
B
INDOMETHACIN (mg/kg, i.v.)
0
2
4
6
0
20
40
60
80
100
% INHIBITION
3/3
***
3/3
***
1/1
SUBSTANCE P

Figure 1. Indomethacin depresses the response to substance P. *A(a)* Inhibitory effect of indomethacin on the response of a wide dynamic range neuron (633 μm deep from the dorsal surface of the spinal cord) to iontophoretic application of substance P. *(b)* The substance P response had recovered partially 50 min later. The vertical axis represents the firing frequency (s^{-1}). The time and duration of the substance P applications are shown by the shaded bars below the histograms. The inset shows the cutaneous receptive field on the left hind paw of the rat. *B* Dose-response histogram summarizing the maximum effects of different doses of indomethacin on neuronal responses to iontophoretic application of substance P. The horizontal axis depicts the different doses of indomethacin tested. The vertical axis represents the mean ($\pm$SEM) maximum percent inhibition expressed as a percent of the rate of discharge prior to the administration of indomethacin, normalized to 100%. The ratio in each histogram is for each dose, the number of dorsal horn neurons inhibited over the number tested. To calculate significance, the mean percent inhibition was compared to the mean effect of vehicle administration. *** $P < 0.001$

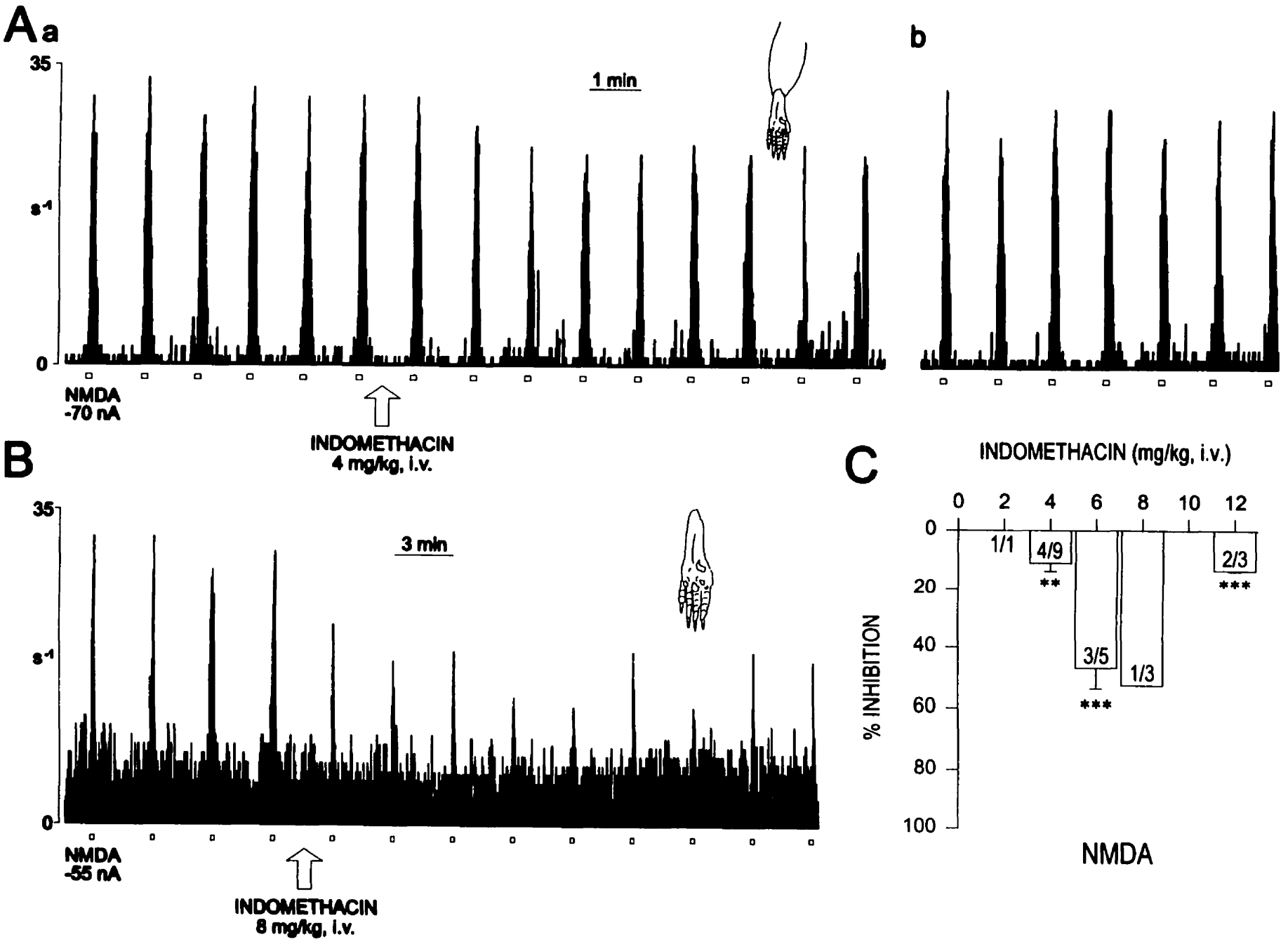
A a
35
s-1
0
1 min
NMDA
-70 nA
INDOMETHACIN
4 mg/kg, i.v.
b
B
35
s-1
0
3 min
NMDA
-55 nA
INDOMETHACIN
8 mg/kg, i.v.
C
INDOMETHACIN (mg/kg, i.v.)
0 2 4 6 8 10 12
0
20
40
60
80
100
% INHIBITION
1/1
4/9
**
3/5
***
1/3
2/3
***
NMDA

Figure 2. *A(a)* Effect of NMDA (-70 nA; indicated by the clear rectangles below the histogram) cycled at 1 min intervals on a non-nociceptive neuron (754 μm) in the presence of indomethacin (4 mg/kg, i.v.). *(b)* The NMDA response recovered 50 min later. ***B*** Effect of NMDA (-55 nA; indicated by the clear rectangles below the histogram) cycled at 3 min intervals on a non-nociceptive neuron (1162 μm) in the presence of indomethacin (8 mg/kg, i.v.). ***C*** Dose-response histogram summarizing the maximum effects of different doses of indomethacin on neuronal responses to iontophoretic application of NMDA. ** $P < 0.05$ or *** $P < 0.001$

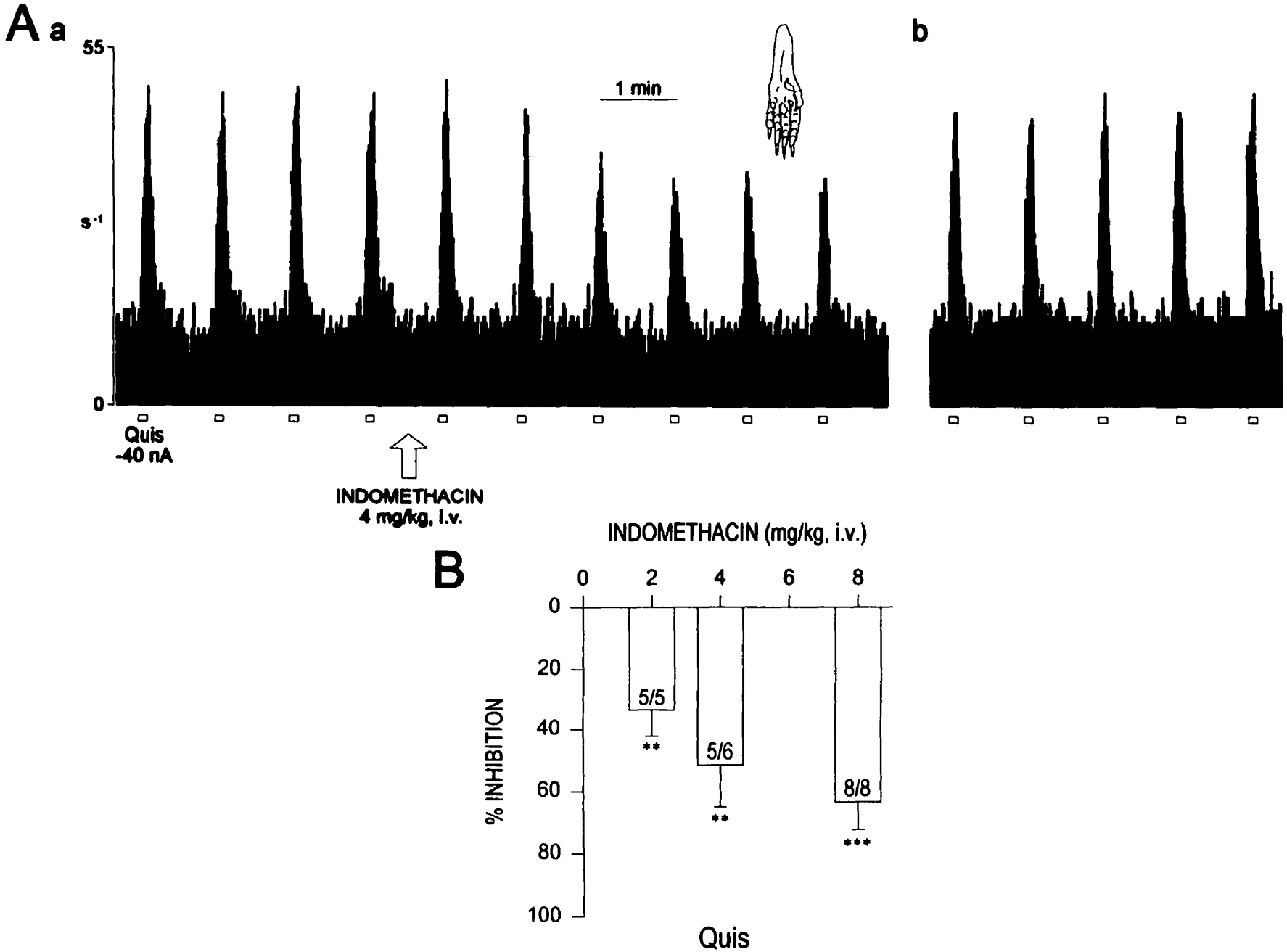
A a
55
s⁻¹
0
1 min
b
Quis
-40 nA
INDOMETHACIN
4 mg/kg, i.v.
B
INDOMETHACIN (mg/kg, i.v.)
0
2
4
6
8
0
20
40
60
80
100
% INHIBITION
5/5
**
5/6
**
8/8
***
Quis

Figure 3. ***A(a)*** Inhibitory effect of indomethacin (4 mg/kg, i.v.) on the response of a wide dynamic range neuron (726 μm) to quisqualate (-40 nA) cycled at 1 min intervals. ***(b)*** The quisqualate response had recovered approximately 80 min later. ***B*** Dose-response histogram summarizing the maximum effects of different doses of indomethacin on neuronal responses to iontophoretic application of quisqualate. ** $P < 0.01$ or *** $P < 0.001$

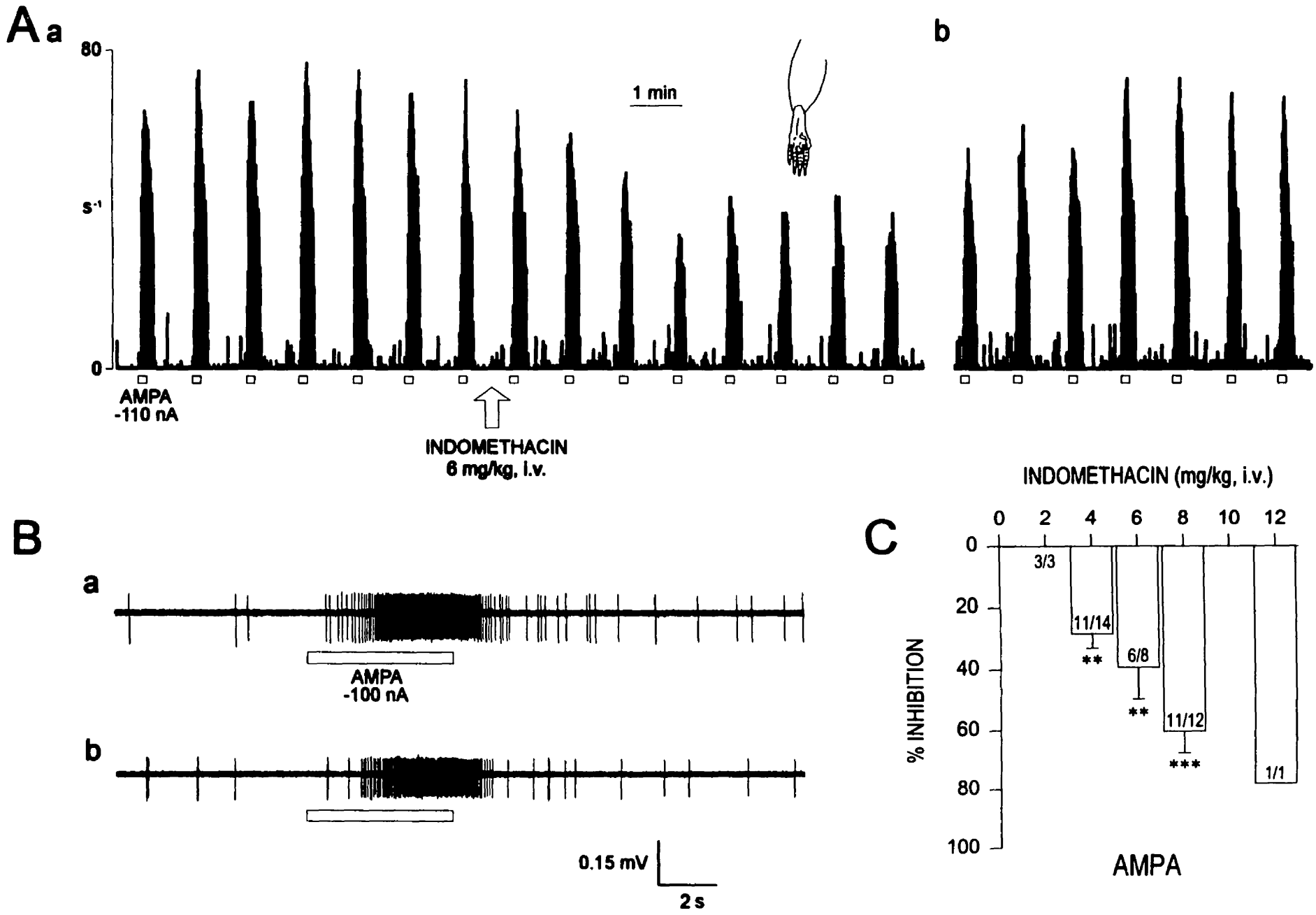
A a
80
s⁻¹
0
1 min
AMPA
-110 nA
INDOMETHACIN
6 mg/kg, i.v.
b
B
a
AMPA
-100 nA
b
0.15 mV
2 s
C
INDOMETHACIN (mg/kg, i.v.)
0 2 4 6 8 10 12
% INHIBITION
0
20
40
60
80
100
3/3
11/14
**
6/8
**
11/12
***
1/1
AMPA

Figure 4. *A(a)* Inhibitory effect of indomethacin (6 mg/kg, i.v.) on the response of a non-nociceptive neuron (514 μm) to AMPA (-110 nA) cycled at 1 min intervals. *(b)* The AMPA response had recovered approximately 90 min later. *B(a)* Extracellular recordings showing representative single unit excitatory responses to iontophoretic application of AMPA (-100 nA; shown by the clear rectangles below each trace) of a non-nociceptive neuron (834 μm). *(b)* Note the increase in the latency of onset of the effect of AMPA and the decrease in the number of spikes in the response to AMPA 5 min after administration of 6 mg/kg indomethacin, i.v. Also notice the earlier offset of the response after the end of AMPA administration. *C* Dose-response histogram summarizing the maximum effects of different doses of indomethacin on neuronal responses to iontophoretic application of AMPA. ** $P < 0.01$ or *** $P < 0.001$

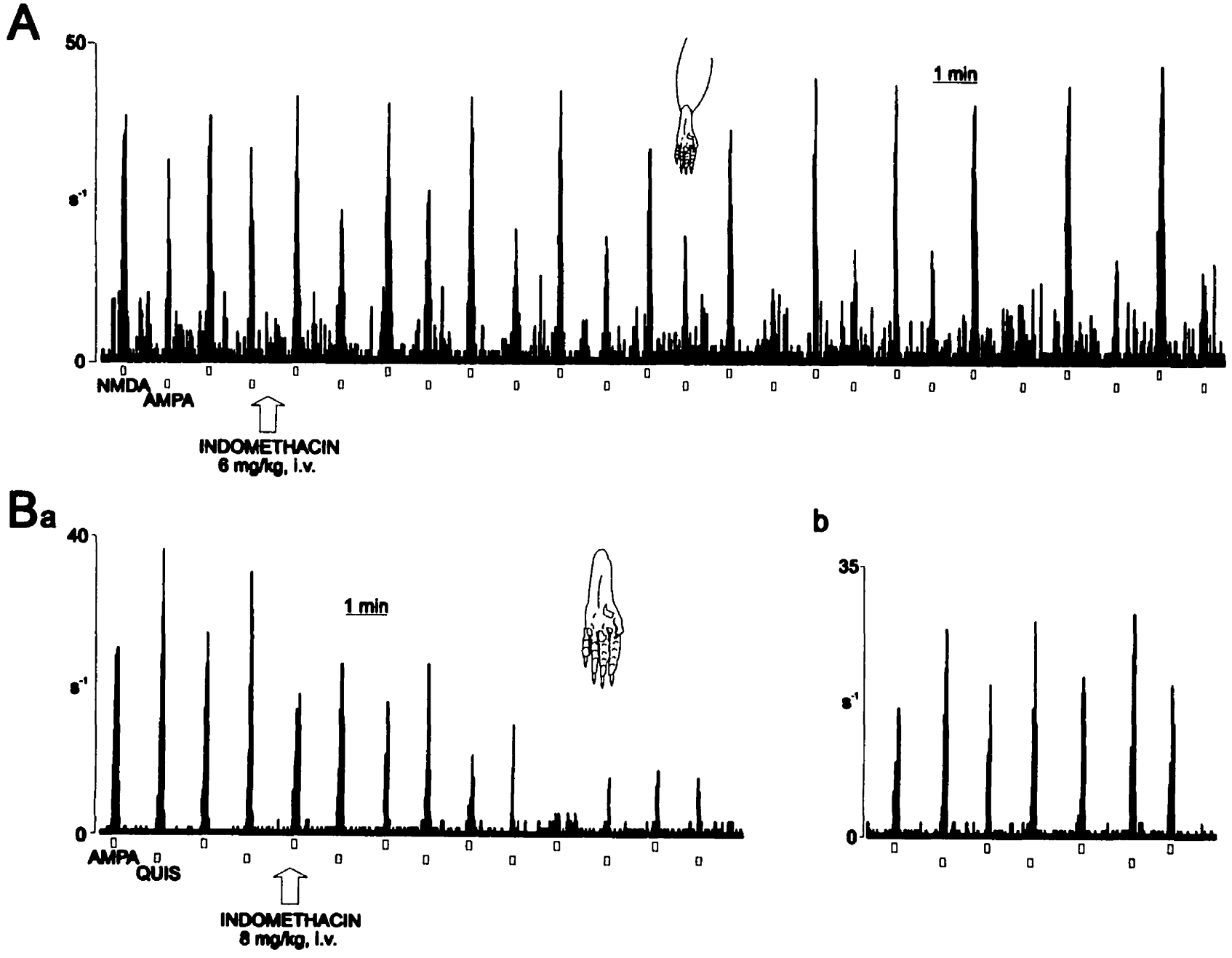
A
50
s⁻¹
0
1 min
NMDA
AMPA
INDOMETHACIN
6 mg/kg, i.v.
Ba
40
s⁻¹
0
1 min
AMPA
QUIS
INDOMETHACIN
8 mg/kg, i.v.
b
35
s⁻¹
0

Figure 5. ***A*** Effects of indomethacin (6 mg/kg, i.v.) on the responses of a non-nociceptive neuron (516 μm) to iontophoretic application of both AMPA (-45 nA) and NMDA (-55 nA). Note the selective inhibitory effect of indomethacin on the response to AMPA. ***B(a)*** Firing rate histogram showing the maximum inhibitory effects of indomethacin (8 mg/kg, i.v.) on the response of a non-nociceptive neuron (1014 μm) to iontophoretic application of both quisqualate (-40 nA) and AMPA (-60 nA). *(b)* Responses to both quisqualate and AMPA on this neuron had partially recovered 50 min later. The times and durations of iontophoretic application are shown by the bars below the histograms. Note the lack of effect of indomethacin on baseline firing frequency. The insets show the cutaneous receptive fields.

Unifying Statement

Thus, the major finding of Chapter 1 was that eicosanoids may play a role in both mediating and modulation of synaptic inputs. Major questions remain however, such as is there a functional specificity to this involvement (addressed in Chapter 2), is the major enzyme COX-1 or COX-2 (addressed in Chapter 3) and is there a similar involvement in sensory mechanisms in an animal model of chronic pain (addressed in Chapters 4-8)?

Chapter 2

NSAID-induced Cyclooxygenase Inhibition Differentially Depresses Long-lasting vs. Brief Synaptically-elicited Responses of Rat Spinal Dorsal Horn Neurons *in vivo*

Abstract

This electrophysiological study examined the effects of NSAID administration on synaptically-elicited responses of rat single spinal dorsal horn neurons to natural stimulation of peripheral receptive fields. Nociceptive responses consisted of a fast initial discharge during the stimulus followed by a slowly-decaying afterdischarge. The cyclooxygenase inhibitor, indomethacin (2.0-8.0 mg/kg, i.v.), was without effect on the on-going rate of discharge but dose-dependently inhibited synaptically-elicited responses to noxious cutaneous mechanical stimulation (fast initial discharge: n = 3/3 with 2 mg/kg, 5/8 with 4 mg/kg, 5/6 with 8 mg/kg; slowly-decaying afterdischarge: n = 3/3 with 2 mg/kg, 6/8 with 4 mg/kg, 6/6 with 8 mg/kg) and thermal (fast initial discharge: n = 7/9 with 8 mg/kg; slowly-decaying afterdischarge: n = 3/4 with 4 mg/kg, n = 7/9 with 8 mg/kg). The inhibitory effect of indomethacin started within 2-4 min and lasted up to 120 min. To eliminate any effect of indomethacin via cutaneous sensory receptors it was tested on the responses of some neurons to electrical stimulation of the sciatic nerve; indomethacin depressed these evoked responses (fast initial discharge: n = 5/6 with 2 mg/kg, n = 7/7 with 4 mg/kg; slowly-decaying afterdischarge: n = 6/6 with 2 mg/kg, n = 7/7 with 4 mg/kg). The brief excitatory responses to innocuous pressure (fast initial discharge: n = 2/3 with 2 mg/kg, n = 6/8 with 4 mg/kg, n = 4/6 with 8 mg/kg) and hair (n = 2/7 with 2 and 4 mg/kg, respectively) stimulation in both non-nociceptive and wide dynamic range neurons were also depressed but to a lesser extent. However, the prolonged excitation of 3 wide dynamic range neurons to continuous hair stimulation was almost

entirely inhibited by indomethacin.

Overall, inhibition of the afterdischarge and the excitatory effect of long-lasting synaptic input were greater than inhibition of the fast synaptic input-evoked initial discharge. The evidence supports the suggestion that systemically-administered indomethacin has an effect in the spinal cord and demonstrates an action specifically in the dorsal horn. The data are interpreted to suggest that sensory inputs are more involved than input-independent excitation of dorsal horn neurons in leading to *de novo* synthesis of eicosanoids and that the time course of this synthesis brings the levels to a point where COX inhibition can have an observable effect during prolonged excitation. Although the data suggest that COX inhibition differentially inhibits nociceptive vs. non-nociceptive mechanisms at the cellular level, irrespective of the modality of the stimulus, this is the first direct demonstration that prolonged activation of synaptic mechanisms is preferentially inhibited. According to this it would be predictable that NSAIDs would be more effective on nociceptive types of pain characterized by time or prolonged inputs of primary afferents.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used clinically for decreasing peripheral inflammation as well as for depressing inflammation-induced pain. These effects occur by inhibition of cyclooxygenase (COX), which catalyzes arachidonic acid to prostaglandins. While an obvious site of action of NSAIDs is in the periphery, at the site of the inflammation, recent work has suggested that a site of the analgesic action of COX inhibitors may also be centrally (Jurna and Brune 1990), specifically at the level of the spinal cord (Jurna et al. 1992).

This suggestion has been supported by a variety of studies. COX mRNA expression is observed in the dorsal horn (Beiche et al. 1996, 1998a; Hay and De Belleroche 1997; Willingale et al. 1997). Prostaglandin synthase is expressed in neurons in laminae II and III (Vesin et al. 1995, 1996). Intrathecal administration of NSAIDs inhibits the tail-flick reflex (Wang et al. 1994), the second phase of nociceptive scores in the formalin test (Malmberg and Yaksh 1992b, 1994b), withdrawal of the hind paw to noxious thermal stimulation (Malmberg and Yaksh 1992a) and responses in the writhing test and the colorectal distension test (Björkman 1995). Thus, it is well established that antinociceptive effects of NSAIDs can be elicited at the level of the spinal cord.

While it is intuitively likely that these effects are expressed by an action in the dorsal horn, this has not yet been established experimentally. Although evidence has indicated that intrathecal administration of a COX inhibitor inhibits the tail flick reflex without any motor effects (Wang et al. 1994), prostaglandin D synthase and COX

immunoreactivity is found not only in the dorsal horn but also in motoneurons in the ventral horn (Vesin et al. 1995; Vesin 1996; Willingale et al. 1997). Thus, it is possible that at least some of the antinociceptive effects of NSAIDs administered intrathecally may occur via an action in the ventral horn.

It has also not been established whether the spinal effects of prostaglandins are expressed selectively on nociceptive vs. non-nociceptive mechanisms. Functional studies on COX inhibitors at the spinal level have generally used only pain tests (Uda et al. 1990; Minami et al. 1992, 1994, 1995; Ferreira and Lorenzetti 1996). One paper reported an inhibitory effect of intravenous administration of R(-)- or S(+)-flurbiprofen on dorsal horn neuronal responses to innocuous stimuli in rats with acute knee joint inflammation, although the specific components of the responses of dorsal horn neurons to nociceptive and non-nociceptive stimuli were not determined (Neugebauer et al. 1995). Otherwise, effects of NSAIDs in functionally specific pathways are not clearly known.

Therefore, the purpose of the present study was to investigate the role of COX in the transmission of sensory information in normal animals, specifically in the responses of dorsal horn neurons to peripheral stimulation-induced synaptic input. At the cellular level, an important question that remains is whether the action of COX mediates responses to only noxious stimulation or whether it also mediates responses to non-noxious stimuli. Furthermore, as the duration of responses of dorsal horn neurons to non-nociceptive or nociceptive input are different, we also investigated the effect of COX inhibition in long-lasting vs. brief synaptically-elicited responses. Thus, the present study determined the

effects of COX inhibition on synaptically-elicited responses of dorsal horn neurons to natural stimulation of the cutaneous receptive field in the rat and determined whether there is a preferential effect on nociceptive vs. non-nociceptive inputs and prolonged vs. brief responses. To eliminate any effect of indomethacin via cutaneous sensory receptors it was also tested on responses to electrical stimulation of the sciatic nerve.

Some of the results in this study have been reported previously in abstract form (Pitcher and Henry 1996).

Materials and methods

2.1. Animal preparation

Experiments were done on adult, male Sprague-Dawley rats from Charles River (St. Constant, Quebec, Canada). Guidelines regarding *The Care and Use of Experimental Animals* as outlined by the Canadian Council on Animal Care (Vols. I and II) were strictly followed. Rats (350-375g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Abbott Laboratories Ltd, Montreal, Quebec, Canada) followed by supplements of 10 mg/kg/hr, i.v. The right common carotid artery and jugular vein were catheterized for continuous monitoring of arterial pressure and for injection of drugs, respectively. Temperature of the rat was maintained at 37.5°C using an infrared heating lamp when required.

Spinal cord segments L_1 to L_3 were exposed for recording as this is the location of synaptic input from the cutaneous receptive fields in the hind limbs. The spinal cord was transected at the T_9 vertebral level to eliminate supraspinal influences on the activity of lumbar dorsal horn neurons; to minimize spinal shock xylocaine (0.05 ml of 1%; Astra Pharma, Mississauga, Ontario, Canada) was injected into the cord at the level of transection just prior to transection. The spinal cord was covered with mineral oil (Marcol 72, Imperial Oil Limited; Montreal, Quebec, Canada) at 37.5°C to prevent drying.

The left sciatic nerve was exposed by blunt dissection through the biceps femoris muscle and was isolated from surrounding connective tissue using glass probes. Bipolar stainless steel hook electrodes were then inserted under the isolated nerve.

Each rat normally breathed spontaneously during the experiment. However, in experiments where electrical stimulation was used, the anesthetized animal was also paralysed with pancuronium bromide (1 mg/kg i.v. supplemented as necessary; Pavulon, Organon, Scarborough, Ontario, Canada) and ventilated mechanically according to standard parameters (Kleinman and Radford 1964).

2.2. Electrical recording and data acquisition

Single unit spikes were recorded extracellularly using seven-barrelled or single barrelled micropipettes (overall tip diameter 4-5 or 1-2 μm, respectively). The multi-barrelled electrodes were used because iontophoretic drug experiments were also run in some cases. A solution of 2.7 M NaCl was placed in the central recording barrel (impedance 2-4 MΩ measured at 1 kHz with the tip submerged in saline). Single unit recordings were made at depths ranging from 250 to 1300 μm in the dorsal horn of the spinal cord. The raw data were amplified using a unity-gain preamplifier built in-house, displayed on an oscilloscope (Tektronix 5111) and stored on video cassette tapes using a digital data recorder that incorporated a digital pulse code modulation technique (VR-100A, Instrutech Corporation, Great Neck, NY, U.S.A.) and a conventional video cassette recorder. The signals were also relayed to a frequency counter/gating unit which counted single unit spikes per unit time (bin widths were 1 s). The output of the gating unit, recorded as the rate of discharge, was displayed continuously on a Grass 79D polygraph. Sampling and analysis were done using the data acquisition program, *Spike 2* (Version

2.02; *Cambridge Electronic Design*, Cambridge, England) and an IBM Pentium computer.

2.3. Functional classification of dorsal horn neurons

Functional classification of neurons was based on the responses to natural stimulation of their respective receptive fields in the ipsilateral hind limb using both noxious and innocuous stimuli. The following natural stimuli were used as search stimuli to elicit synaptic input while penetrating the dorsal horn and to characterize functionally a neuron once stable single unit recording was obtained: (i) an air stream passed over the receptive field sufficient to move only the hairs continuously or for 3 s periods, (ii) light touch, (iii) moderate pressure (0.2 N for 3 s), (iv) noxious mechanical stimulation using a calibrated clip (21 N for 3 s) and (v) noxious radiant heat (measured to reach 50°C at the skin surface). The thermal stimulation was applied for a duration of 8 s and was cycled at a fixed interval of 1 or 2 min. The mechanical stimulation was applied for 3 s every 3 or 4 min.

Classification of the identified neurons was in three categories (Henry 1976): (i) non-nociceptive neurons that responded only to non-noxious stimuli such as hair, touch and/or pressure stimulation (some receptive fields on the rat hind limb did not have hair), (ii) wide dynamic range neurons that responded to both noxious and innocuous stimuli and (iii) nociceptive-specific neurons that responded only to noxious stimuli. In addition, all the units that responded to the noxious range of mechanical and/or thermal stimulation showed a characteristic slowly-decaying afterdischarge, as described previously (Henry

1976; De Koninck and Henry 1991).

For each neuron the receptive fields for hair movement, light touch, moderate pressure, noxious mechanical and noxious thermal stimulation were represented on a schematic diagram of the hind limb. Receptive field sizes generally remained unchanged throughout the experiments and were not investigated further in this study.

Some neurons were also tested for their response to a train of electrical stimuli applied directly to the exposed left sciatic nerve using a bipolar electrode. The stimulus consisted of 8 mA current pulses of 1 ms duration given at 20 Hz for 3 s. These parameters have been used in our laboratory to evoke excitation of afferent fibers. This was considered to include activation of nociceptive primary afferents as it produced a slowly-decaying afterdischarge similar to that appearing in response to noxious mechanical or noxious thermal stimuli. The response in wide dynamic range neurons thus consisted of a fast initial discharge followed by a slowly-decaying afterdischarge.

2.4. Drug administration

Indomethacin (RBI, Natick, Massachusetts) was dissolved in 2% sodium bicarbonate and this solution was titrated to pH 7.4 using sodium monophosphate. Indomethacin was administered intravenously at a dose of 1 ml/kg body weight. As a control, the vehicle, 2% sodium bicarbonate (pH 7.4 using sodium monophosphate), was administered in a similar fashion.

Indomethacin is generally considered a non-selective COX-1/-2 inhibitor (Mitchell

et al. 1994; Gierse et al. 1995; Yamamoto and Nozaki-Taguchi 1996; Riendeau et al. 1997; Harada et al. 1998) and was used in this study to inhibit activation of both COX isoforms. Previous studies used doses ranging from 1 to 50 mg/kg (Jurna and Brune 1990; Chapman and Dickenson 1992; Hu et al. 1994; Buritova et al. 1995; Honoré et al. 1995; Bustamante et al. 1996). In the present study, pilot experiments indicated that 2 mg/kg of indomethacin was effective in depressing the effects of noxious as well as innocuous stimulation-induced synaptic input to dorsal horn neurons. Furthermore, in a previous study, we found this dose to be effective in depressing the responses to iontophoretic application of the glutamate receptor agonists, N-methyl-D-aspartic acid (NMDA) and quisqualate and α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA), and of the NK-1 receptor agonist, substance P (Pitcher and Henry 1996).

2.5. Data analysis

Quantization of the magnitude of a response to stimulus-induced synaptic input was done as follows. The total number of spikes was calculated over a period of 3 s, from the onset of the neuronal responses to hair and pressure. As the responses to pinch, noxious radiant heat and electrical stimulation consisted of not only a fast initial discharge but also a slowly-decaying afterdischarge, sampling for these responses included the two respective periods. Thus, the number of spikes in the fast initial discharge in response to pinch and electrical stimulation was determined over the 3 s period of the stimulus. The number of spikes in response to noxious thermal stimulation was determined over the 3 s period at the

end of the stimulus. For the slowly-decaying afterdischarge the sample period was 60 sec, beginning immediately after the fast initial discharge. The magnitude of the response was then calculated for each sample period by subtracting the number of spikes over the 3 or 60 s period immediately preceding the stimulus.

To quantitate the effects of indomethacin, three responses before administration of indomethacin were averaged and three responses at the maximum inhibition after administration of indomethacin were averaged. The mean value during was subtracted from the mean value before and this was divided by the mean value before indomethacin. This was then multiplied by 100 to yield a percent inhibition. Thus, no effect of indomethacin would be 0% inhibition while complete inhibition of a response by indomethacin would be 100% inhibition. Some neurons were tested more than once with indomethacin to determine the dose-response relationship. This was done only using a higher subsequent dose and only following full recovery from the lower dose tested to ensure that there was no remaining effect of the drug. As a result, each dose group was derived from different neurons. Responses of neurons that were insensitive to indomethacin were included in the ratios of the dose-response histograms.

In the case of continuous hair stimulation the total number of spikes was calculated over a period of 3 min before the stimulation and during the continuous air stream. The magnitude of the response was the difference in the number of spikes. A similar difference was calculated at the maximum inhibition after administration of indomethacin. To quantitate the effect of indomethacin, the value after indomethacin administration was

subtracted from the value before and this was divided by the value before and multiplied by 100 to yield a percent inhibition.

To calculate significance, the mean ($\pm$SEM) percent inhibition following a particular dose of indomethacin was compared to the mean percent inhibition following vehicle administration (0% inhibition). Statistical analysis of the data was done using one-way ANOVA and Student Newman Keuls test. A difference between test and control responses was considered significant with a P value < 0.05.

Results

Indomethacin was tested on responses of dorsal horn neurons to stimulation of the cutaneous receptive field and to electrical stimulation of the sciatic nerve in 39 rats. Indomethacin could not be given iontophoretically due to its high resistance property in the ejection barrel of the electrode. Accordingly, the data to follow were all obtained from systemic administration of indomethacin. Data from a neuron were included only if full testing could be completed. Only neurons that showed full recovery from the effects of indomethacin were included in this study. Indomethacin was found to be consistently without effect on spike amplitude. It was also without effect on arterial pressure and respiration.

3.1. Tests of indomethacin on on-going activity

On-going baseline activity was unaltered by administration of indomethacin. The records in the figures illustrate this lack of effect.

3.2. Effect of indomethacin on response to electrical stimulation of the sciatic nerve

Electrical stimulation of the exposed sciatic nerve produced a transient excitatory response (fast initial discharge) followed by a slowly-decaying afterdischarge which persisted for about 1 min after the end of the stimulus (Figure 1A). This effect was observed in all 17 wide dynamic range neurons tested.

The effect of indomethacin on the response of a neuron to this stimulation is shown in Figure 1A. The firing frequency of the fast initial discharge and the slowly-decaying afterdischarge were both attenuated following indomethacin administration (4 mg/kg, i.v.). The predominant effect of indomethacin was attenuation of the duration and amplitude of the slowly-decaying afterdischarge.

Spike activity of another single wide dynamic range neuron illustrating excitatory responses to electrical stimulation of the sciatic nerve is shown in Figure 1B. The fast initial discharge and the slowly-decaying afterdischarge are clearly seen, the fast initial discharge being the high frequency firing during the electrical stimulus (Figure 1Ba and b). The slowly-decaying afterdischarge persisted until approximately 30 to 40 s after the end of the stimulus of this particular neuron (Figure 1Ba). Thirty minutes after administration of indomethacin (4 mg/kg, i.v.) both the initial discharge (Figure 1Bc and d) and the afterdischarge (Figure 1Bc) were attenuated.

The effect of indomethacin (2 or 4 mg/kg, i.v.) on the response to electrical stimulation was tested in a total of 13 wide dynamic range neurons. Twelve exhibited a depression of the fast initial discharge. All 13 showed inhibition of the slowly-decaying afterdischarge. Figure 1Ca shows that 2 and 4 mg/kg indomethacin depressed the fast initial discharge by 23.72 ± 10.22 % ($n = 5$, $P < 0.05$ vs. control) and 37.83 ± 7.05 % ($n = 7$, $P < 0.01$), respectively. The slowly-decaying afterdischarge was also depressed by 2 and 4 mg/kg indomethacin (61.46 ± 11.71 %; $n = 6$, $P < 0.01$ and 62.51 ± 10.94 %; $n = 7$, $P < 0.001$), respectively (see Figure 1Cb). The maximum percent inhibition of

the fast initial discharge and the slowly-decaying afterdischarge at 2 mg/kg indomethacin are significantly different ($P < 0.05$). In all cases, full recovery of the response to peripheral electrical nerve stimulation was observed. Indomethacin-induced inhibition of the response of dorsal horn neurons to peripheral nerve stimulation typically began at about 2-4 min after administration and generally lasted for 90 to 120 min following injection.

Neuronal responses to electrical nerve stimulation were unaffected by 2% sodium bicarbonate (n=5).

3.3. Effect of indomethacin on response to noxious mechanical stimulation

Noxious mechanical stimulation of the peripheral cutaneous receptive field produced a typical excitatory response in all 18 wide dynamic range neurons tested. This response consisted of a fast initial discharge occurring throughout the duration of the stimulus, and a slowly-decaying afterdischarge lasting beyond the end of the stimulus (Henry 1976).

Figure 2Aa shows the response of a wide dynamic range neuron to noxious pinch applied to the cutaneous receptive field on the left hind paw of the rat. The fast initial discharge lasted for the duration of the 3 s pinch and the slowly-decaying afterdischarge persisted approximately 1 min after the end of the pinch stimulus. Both the fast initial discharge and the slowly-decaying afterdischarge were attenuated by 4 mg/kg of indomethacin i.v. (Figure 2Ab). While the fast initial discharge was decreased by about 30%, the record shows an almost complete block of the slowly-decaying afterdischarge.

Indomethacin depressed the pinch-induced fast initial discharge and the slowly-decaying afterdischarge in a dose-related manner. Doses of 2, 4 and 8 mg/kg were given to a total of 14 wide dynamic range neurons. Indomethacin at a dose of 2 mg/kg depressed the fast initial discharge of all three wide dynamic range neurons tested (21.73 ± 9.09 %, $P < 0.05$ vs. control). Higher doses of 4 and 8 mg/kg indomethacin depressed the fast initial discharge of five out of eight neurons (30.61 ± 10.07 %, $P < 0.05$) and five out of six neurons (29.94 ± 8.58 %, $P < 0.01$), respectively. Indomethacin at doses of 2, 4 and 8 mg/kg depressed the slowly-decaying afterdischarge of three out of three (74.39 ± 11.67 %, $P < 0.001$), six out of eight (64.72 ± 13.97 %, $P < 0.01$) and six out of six (65.44 ± 11.53 %, $P < 0.001$) wide dynamic range neurons, respectively. The maximum percent inhibition of the fast initial discharge and the slowly-decaying afterdischarge at 2 and 8 mg/kg indomethacin, respectively, are significantly different ($P < 0.05$). In cases where inhibition occurred, full recovery of the response to a noxious pinch was always observed.

Generally, the firing frequency of the fast initial discharge was attenuated as soon as 4 min following administration of indomethacin and the slowly-decaying afterdischarge as soon as 2 min. Indomethacin attenuated not only the amplitude but also the duration of the slowly-decaying afterdischarge (see Figure 2Ab). Responses of wide dynamic range neurons to repeated noxious stimulation recovered from indomethacin-induced depression approximately 90 to 120 min after higher doses were given.

As a control, 2% sodium bicarbonate was tested on the pinch response in five

neurons and was always without effect.

3.4. Effect of indomethacin on response to noxious thermal cutaneous stimulation

The effect of noxious thermal stimulation of the cutaneous receptive field on the hind paw of the rat was tested on 11 wide dynamic range neurons. This stimulus produced the usual response consisting of a fast initial discharge followed by a slowly-decaying afterdischarge which persisted after the end of the noxious thermal stimulus (Figure 3Aa). The fast initial discharge typically started 4 to 5 s after the bulb was turned on. The firing rate continued to increase throughout the application of the stimulus and reached a peak approximately 1 s after the stimulus ended. This typical response was seen in all 11 wide dynamic range neurons tested with noxious heat.

Figure 3A shows the effect of 8 mg/kg of indomethacin on the response of one wide dynamic range neuron to noxious thermal stimulation of the cutaneous receptive field. Inhibition of the fast initial discharge and the slowly-decaying afterdischarge occurred within 10 min of administration of indomethacin (Figure 3Ab). Figure 3Ac shows that full recovery of both types of discharge in the response of this neuron to noxious thermal stimulation had occurred by approximately 100 min after administration of indomethacin.

Spike activity of another single wide dynamic range neuron demonstrating excitatory responses to noxious thermal stimulation of the cutaneous receptive field is shown in Figure 3B. Five min after administration of indomethacin (8 mg/kg, i.v.; Figure 3Bb) the initial discharge was noticeably decreased and the afterdischarge was almost

entirely inhibited. Thirty min after administration of indomethacin there remained considerable inhibition of both the initial discharge and the afterdischarge (Figure 3Bc). Approximately 100 min after indomethacin there was complete recovery of both components (Figure 3Bd).

Indomethacin was tested on each of the 11 wide dynamic range neurons. Full recovery was observed with each dose. Figure 3C illustrates the dose-dependent inhibition by indomethacin of the initial discharge (Figure 3Ca) and the afterdischarge (Figure 3Cb) of wide dynamic range neurons tested with noxious thermal stimulation. At 4 mg/kg of indomethacin, the slowly-decaying afterdischarge was depressed in 3 out of 4 neurons (61.71 ± 19.84 %, $P < 0.01$ vs. control). Although the 4 mg/kg dose of indomethacin depressed the fast initial discharge (36.62 ± 23.18 %), comparison of the mean percent inhibition with the mean percent effect of vehicle indicated that the response at this dose was not significant. However, 8 mg/kg of indomethacin significantly depressed the afterdischarge in 7 out of 9 neurons (80.69 ± 11.17 %, $P < 0.01$) as well as the initial discharge in 7 out of 9 neurons (72.13 ± 13.98 %, $P < 0.001$). Neuronal responses to noxious thermal stimulation were unaffected by administration of vehicle (n=5).

3.5. Effect of indomethacin on response to innocuous mechanical stimulation

On neurons responding to innocuous peripheral mechanical stimulation, moderate pressure (n = 14) or touch (n = 14) evoked transient excitatory effects which were rapid in onset and termination (Figure 4Aa and C).

The response to pressure was depressed by indomethacin. This effect is illustrated in Figure 4Aa, where 8 mg/kg of indomethacin decreased the response of a non-nociceptive neuron to pressure stimulation of the cutaneous receptive field. The onset of the inhibitory effect occurred within 2-4 min following administration.

Indomethacin was tested on the responses to pressure of 9 non-nociceptive and 5 wide dynamic range neurons. Responses of 2 out of 3 non-nociceptive neurons were depressed by 2 mg/kg of indomethacin (8.87 ± 3.10 % $P < 0.01$ vs. control), 4 out of 5 non-nociceptive and 2 out of 3 wide dynamic range neurons by 4 mg/kg indomethacin (16.04 ± 3.61 %, n = 6, $P < 0.01$) and 2 out of 2 non-nociceptive and 2 out of 4 wide dynamic range neurons (24.17 ± 8.28 %, n = 4, $P < 0.05$) by 8 mg/kg of indomethacin. Some dorsal horn neurons were insensitive to indomethacin (see Figure 4Ab). It is also important to note that the inhibitory effect of indomethacin on pressure responses of wide dynamic range neurons was not greater than the inhibitory effect of indomethacin on the responses of non-nociceptive neurons to this stimulus. Generally, the response to pressure recovered 60 to 100 min after administration of indomethacin.

Vehicle had no effect on responses of neurons to pressure stimulation-induced synaptic input (n=5).

3.6. Effect of indomethacin on response to hair stimulation

Hair stimulation, using an air stream, also produced a transient excitatory response occurring only for the duration of the stimulus. The response to brief pulses of air was

tested with indomethacin on 3 non-nociceptive and 3 wide dynamic range neurons. Figure 4Ba shows the response of a non-nociceptive neuron to brief periods of hair movement. Only this neuron was depressed by 4 mg/kg of indomethacin and this occurred in a dose-dependent manner using 4 and 8 mg/kg (22.59 % and 45.98 %, respectively; see Figure 4Bb). The response of this neuron to hair stimulation recovered approximately 70 min after indomethacin administration. Vehicle had no effect on the response to hair-induced synaptic input to dorsal horn neurons (n=5).

In 3 wide dynamic range neurons tested, continuous hair stimulation elicited an on-going increase in the firing frequency. Figure 5A shows that this excitation was decreased by 93% at 30 min after administration of indomethacin (8 mg/kg, i.v.). Figure 5B shows the recovery of the excitatory effects of continuous application of hair stimulation approximately 90 to 100 min after the administration of indomethacin.

Indomethacin at a dose of 8 mg/kg decreased the response to constant hair stimulation of 3 wide dynamic range neurons. The excitation was decreased maximally by $91.95 \pm 0.72\%$ at approximately 25 min after administration of indomethacin. Vehicle had no effect on the response to hair-induced synaptic input to dorsal horn neurons (n=3).

3.7. Comparison of effects of indomethacin on noxious and innocuous sensory inputs

Figure 4Ca shows the effects of touch, pressure and noxious pinch stimuli on the activity of a wide dynamic range neuron. A slowly-decaying afterdischarge remained after the end of application of the noxious pinch stimulus. Indomethacin (8 mg/kg) decreased

predominantly the slowly-decaying afterdischarge response of this neuron to the pinch while having only a slight inhibitory effect on the fast initial discharge (Figure 4Cb). Responses of this neuron to touch and pressure stimuli were also less affected by indomethacin. Figure 4Cc illustrates the number of spikes per response to touch (T), pressure (Pr) or pinch (P) before and after administration of indomethacin (8 mg/kg, i.v.). The slowly-decaying afterdischarge of this wide dynamic range neuron was more sensitive to the effect of indomethacin (51.6 % inhibition) than the response to touch (14.6 % inhibition), pressure (13.0 % inhibition) or the fast initial discharge to the pinch stimulus (16.1 % inhibition).

Discussion

This study demonstrates that systemic administration of the cyclooxygenase inhibitor, indomethacin, selectively and dose-dependently depresses nociception-induced responses of spinal dorsal horn neurons, particularly the slowly-decaying afterdischarge of the response to noxious cutaneous stimuli or to electrical stimulation of the sciatic nerve. The fast initial discharge responses of wide dynamic range neurons to brief noxious or innocuous stimulation, and of non-nociceptive neurons to innocuous stimulation, were depressed considerably less by indomethacin. Importantly, in 3 wide dynamic range neurons tested, the prolonged excitatory effect of continuous hair stimulation of the receptive field was inhibited by indomethacin.

The inhibitory effects of indomethacin on synaptically-induced activation of dorsal horn neurons cannot be accounted for by changes in arterial pressure. Such changes would cause movement of the cord vis-à-vis the electrode tip, resulting in a change in extracellular spike amplitude. However, the extracellular traces had constant spike amplitude throughout experiments, indicating stable recording. In addition, in the present study, blood pressure and respiratory rate remained unchanged following administration of indomethacin.

4.1. Spinal vs. peripheral site of action of indomethacin

Indomethacin does not lend itself well to iontophoretic application, and administration was necessarily via a systemic route. This seemed reasonable in view of

the report that indomethacin crosses the blood-brain-barrier (Bannwarth et al. 1990) and the short time for effects to be seen centrally after systemic administration (Bustamante et al. 1996). However, as application was systemically in the present study, this raises the possibility that at least some of the effects reported here may have been due to a peripheral site of action, particularly in the cutaneous receptive field. COX products of arachidonic acid metabolism can alter the threshold of cutaneous nociceptors in the rat (Taiwo and Levine 1990). In fact, it has been suggested that prostaglandins are directly involved in sensitizing cutaneous nociceptors (Gold et al. 1994, 1996). While we shall not argue against this possibility, it is reasonable to expect that at least some of the effects seen here were due to an action in the spinal cord. This is substantiated by the fact that we have completed experiments (Pitcher and Henry 1996) in which indomethacin given systemically depressed the excitatory effects of iontophoretic application of excitatory neurotransmitters onto dorsal horn neurons.

A second line of evidence supporting a role for COX in sensory mechanisms in the spinal cord is that responses to electrical stimulation of the sciatic nerve were inhibited by indomethacin. As this electrical stimulation bypassed cutaneous receptors, the data indicate that at least some of the inhibitory effects of indomethacin had to be at the level of the spinal dorsal horn.

A third line of support for a central site of action in the present study is the evidence of COX expression in the spinal cord (Beiche et al. 1996, 1998b; Hay and De Belleroche 1997; Goppelt-Struebe and Beiche 1997) and of prostaglandin synthase in

superficial laminae (Vesin et al. 1995, 1996). This evidence provides support for the existence of a mechanism at the spinal level by which the effects of indomethacin can be expressed.

Finally, another reason that the effects may have been due to an action in the spinal cord is physiological evidence that intrathecal administration of NSAIDs blocks nociceptive responses in the formalin test (Malmberg and Yaksh 1992b, 1993) and depresses hyperalgesia in response to intrathecal administration of glutamate and substance P (Malmberg and Yaksh 1992a).

Thus, while we must entertain the possibility of a peripheral contribution to the present results, this study also strongly leans toward a central involvement of eicosanoids, specifically in mediating the effects of sensory inputs in the spinal dorsal horn in the normal rat.

4.2. COX involvement in on-going vs. evoked activity of dorsal horn neurons

The lack of effect of indomethacin, at least with the doses used in this study, on the on-going discharge of dorsal horn neurons may be interpreted to indicate that eicosanoids are normally not involved in mediating or regulating the basal activity or excitability of these neurons. It is not implied that arachidonic acid or prostaglandins are not present during on-going activity, but it is suggested that eicosanoids are not normally synthesized at levels which are functionally significant at least in terms of contribution to or regulating on-going spiking activity.

Responses to synaptic input, on the other hand, were sensitive to indomethacin. The effects of eicosanoids appear to be stimulus-dependent and thus synaptic input may cause eicosanoid synthesis to levels which have physiological effects. Therefore, the effects of COX inhibition would be observed only during the period that eicosanoids are above the basal levels. This possibility is consistent with the data of Malmberg *et al.* who reported increased prostaglandin E_2-like immunoreactivity from the superfused spinal cord slice upon addition of capsaicin to the perfusate (Malmberg and Yaksh 1994a).

Thus, from these combined data we propose that eicosanoids may not participate as a major component in mediating or regulating on-going activity, but that indomethacin is expressing its effects mainly upon altered levels of excitability coincident with synaptic input to dorsal horn neurons. It follows that synaptic activation of neurons in the spinal dorsal horn provokes *de novo* synthesis of eicosanoids presumably via activation of phospholipase A_2 and COX.

4.3. Involvement of COX in nociceptive vs. non-nociceptive processing

We report here that indomethacin depresses the responses to both noxious and innocuous stimulation in wide dynamic range neurons as well as the effects of innocuous stimulation in non-nociceptive neurons. The involvement of eicosanoids in central nociceptive processing is not a novel idea, as indicated above. Different systemically administered COX inhibitors including indomethacin have been shown to dose-dependently decrease responses of thalamic neurons to the effects of high intensity electrical stimulation

of C fibers in the hind limb sural nerve (Jurna and Brune 1990). Various systemically- (Bustamante et al. 1996) and intrathecally- (Bustamante et al. 1997) administered NSAIDs including indomethacin also depress the electrical stimulation-induced C fiber reflex in the rat. Therefore, our findings support the idea that eicosanoids are involved in nociceptive mechanisms in the spinal cord.

To the best of our knowledge a role of eicosanoids in the transmission of nociceptive vs. non-nociceptive information has not been investigated directly. One study has demonstrated that while the COX inhibitor, salicylic acid, depressed the activation of single neurons in the thalamus induced by electrical stimulation of C fibers in the sural nerve (Jurna et al. 1992), no effect of this NSAID was observed on the response to electrically-evoked activation of low threshold sensory afferents. Although low intensity stimulation was used, presumably to mimic the effect of non-nociceptive input, it is not clear whether or not non-nociceptive mechanisms were actually activated in this earlier study because the thalamic neurons that responded to electrical stimulation of the sural nerve did not respond to touch, gentle stroking or air puffs applied to the skin. Ours thus appears to be the first directed specifically to whether COX is associated only with nociceptive mechanisms in the central nervous system.

4.4. Selective involvement of eicosanoids in synaptic input-induced prolonged discharge

While our results show that COX inhibition is not associated exclusively with nociceptive mechanisms at the spinal level it is shown in this study that COX inhibition

depresses the long-lasting discharge more than the fast initial discharge of nociceptive dorsal horn neurons in response to noxious stimuli. The transient responses to innocuous stimuli, of both nociceptive and non-nociceptive neurons, were also depressed by COX inhibition, but this depression was of similar magnitude as the depression of the fast initial discharge in response to noxious stimulation.

An explanation for this preferential effect does not come readily to mind. If eicosanoids are synthesized *de novo* when synaptic input occurs, as we suggest above, then in its simplest form, both types of input should be depressed equally. In the case of the transient responses, however, if the time of synthesis of eicosanoids is at all delayed, it could quite well be that the synaptically-induced excitation is largely over by the time that eicosanoids reach their maximum level. On the other hand, if the synaptically-induced excitation is more prolonged, then eicosanoids are being synthesized continuously and one would therefore expect a greater effect of COX inhibition on the more prolonged types of excitation. However, given the relatively rapid turnover of eicosanoids in neurons (Wolfe and Horrocks 1994), the data may be interpreted to suggest that during the transient responses eicosanoid synthesis is short-lasting thus yielding presumably a less than maximal level of eicosanoids. In other words, brief excitation in the spinal dorsal horn evokes limited eicosanoid synthesis. It follows that prolonged synaptically-induced excitation would result in increased eicosanoid synthesis as there is long-lasting activation of the eicosanoid signal transduction pathway. This could apply to any type of prolonged excitation, whether the action of slowly-acting chemical mediators of synaptic transmission

or sustained input of fast-acting chemical mediators. This in turn would account for the preferential effect on the afterdischarge of the response of nociceptive neurons to noxious stimuli and to electrical stimulation of sensory fibers. It would also account for the rather strong effect of COX inhibition to the prolonged excitation induced by continuous activation of hair receptors.

The concept of an association between the tonic effects of peripheral stimulation-induced synaptic input and activation of the eicosanoid signal transduction pathway is not without support. For example, it is demonstrated that formalin injection increases the prostaglandin E_2 and excitatory amino acid diasylate levels in the lumbar spinal cord (Malmberg and Yaksh 1995a,b). Furthermore, the increases in the levels of both of these chemicals correlate temporally with the behavioral nociceptive responses in the formalin test (Malmberg and Yaksh 1995a). As the long-lasting behavioral effects of noxious formalin injection are attributed to continuous C fiber afferent activity (Dallel et al. 1995; McCall et al. 1996; Puig and Sorkin 1996), the finding that the increased diasylate levels of prostaglandin E_2 and excitatory amino acids correlates with the two phases of the formalin test suggests further that eicosanoids may be synthesized *de novo* and persist only as long as there is on-going primary afferent activity.

In the present study, in the case of the afterdischarge, substance P is a likely candidate mediating this prolonged excitation as administration of the substance P (NK-1) receptor antagonists including CP-96,345 and CP-99,994 have been shown to attenuate this type of afterdischarge (De Koninck and Henry 1991; Radhakrishnan and Henry 1991,

1995). Whether the tonic activation of NK-1 receptors is due to tonic release of substance P from primary afferents, presumably from C fibers, throughout the afterdischarge, to persistence of the ligand in the synaptic cleft, to a slow removal or breakdown of substance P or to any other mechanism is not specifically revealed in this study. Whichever the case, the tonic effects of substance P and maybe other slow-acting neurochemical mediators may be at least one mechanism which participates in increased activation of the eicosanoid pathway during the noxious stimulation-induced afterdischarge.

In the case of hair stimulation, the response to brief stimulation was affected to a minor degree while that to sustained stimulation was depressed to a major degree. Both NMDA and non-NMDA receptor activation have been shown to mediate the effects of non-nociceptive inputs to neurons in the spinal dorsal horn (Radhakrishnan and Henry 1993). Furthermore, we have found that COX participates in the excitatory responses to NMDA, AMPA and quisqualate receptor activation in dorsal horn neurons (Pitcher and Henry 1996). Thus, the preferential effects of COX inhibition do not seem linked so much to whether the type of chemical mediating the excitation is fast or slow acting; rather, the effect of COX inhibition is more closely tied to the time course of this excitation; we propose that the time course of *de novo* synthesis of the eicosanoids is such that they reach physiologically significant levels during the effect of prolonged excitatory input.

Conclusions

This electrophysiological study demonstrates that eicosanoids are involved in processing of sensory input in the spinal dorsal horn in the normal rat. It is revealed that indomethacin depresses the excitatory effect of electrical stimulation of the sciatic nerve on wide dynamic range neuronal activity, demonstrating a central mechanism of eicosanoids, specifically in the spinal dorsal horn. As indomethacin has no effect on on-going discharge of single dorsal horn neurons but inhibits the excitatory effects not only of nociceptive but also non-nociceptive peripheral cutaneous stimulation on both wide dynamic range and non-nociceptive neurons, it is suggested that the role of eicosanoids in synaptically-elicited excitation of these neurons involves *de novo* synthesis of eicosanoids via COX. Although the data suggest that eicosanoids may be involved specifically in the effects of nociceptive rather than non-nociceptive sensory input, the data also suggest that irrespective of the modality of the stimulus, long-lasting synaptically-elicited responses may be selectively mediated by eicosanoids. The finding that expression of COX-2 mRNA and protein in rat spinal dorsal horn neurons is increased in a model of chronic peripheral inflammation (Goppelt-Struebe and Beiche 1997; Beiche et al. 1998a) suggests that the eicosanoid pathway can become upregulated during the effects of prolonged sensory input. Therefore, we propose that the eicosanoid signal transduction pathway is involved in normal sensory processing but is predominantly involved in the effects of long-lasting synaptic transmission with the potential to become upregulated. These specific properties of the eicosanoid pathway in the spinal dorsal horn may be the mechanisms underlying the

chronic pain conditions observed clinically which are sensitive to the effects of NSAIDs.

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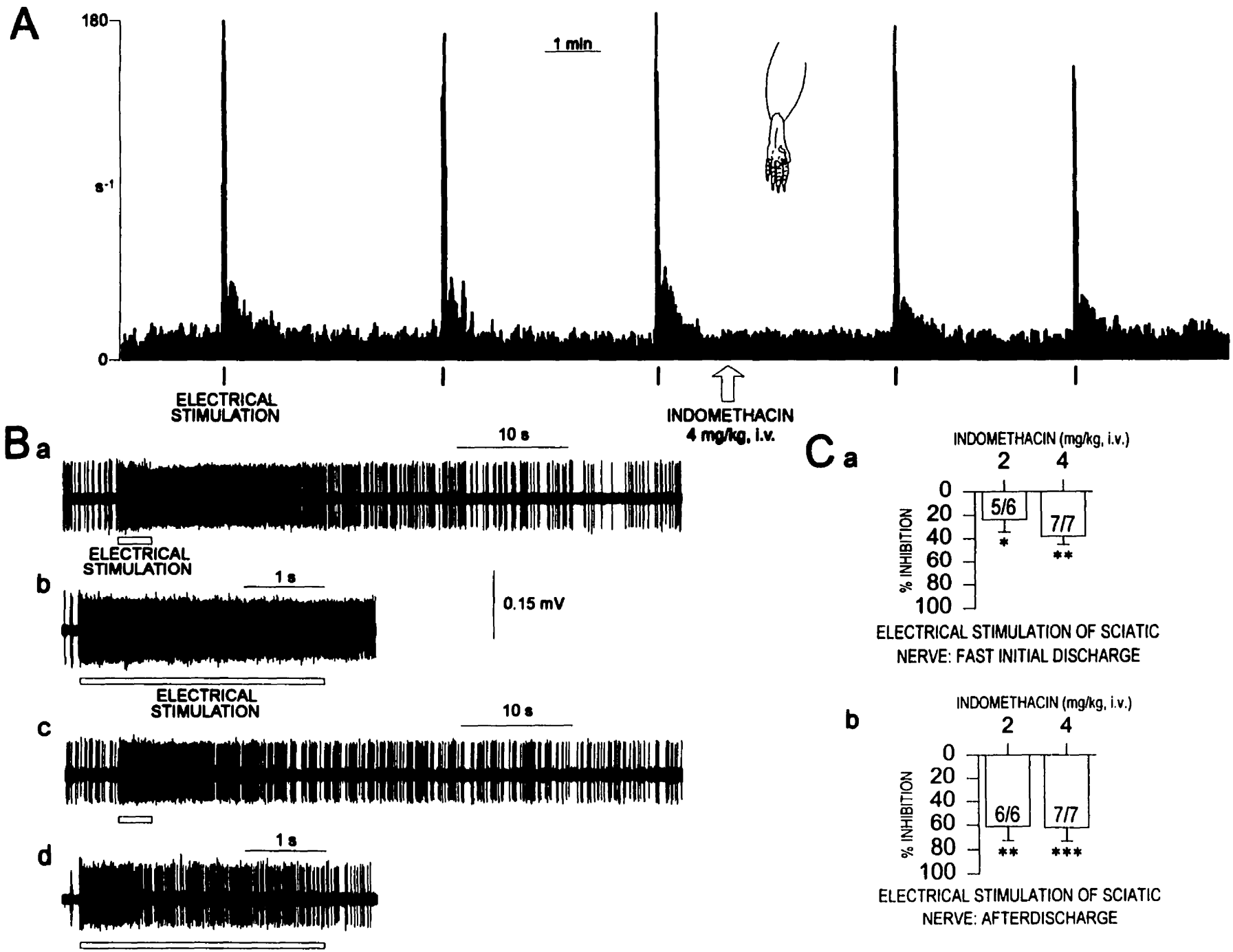
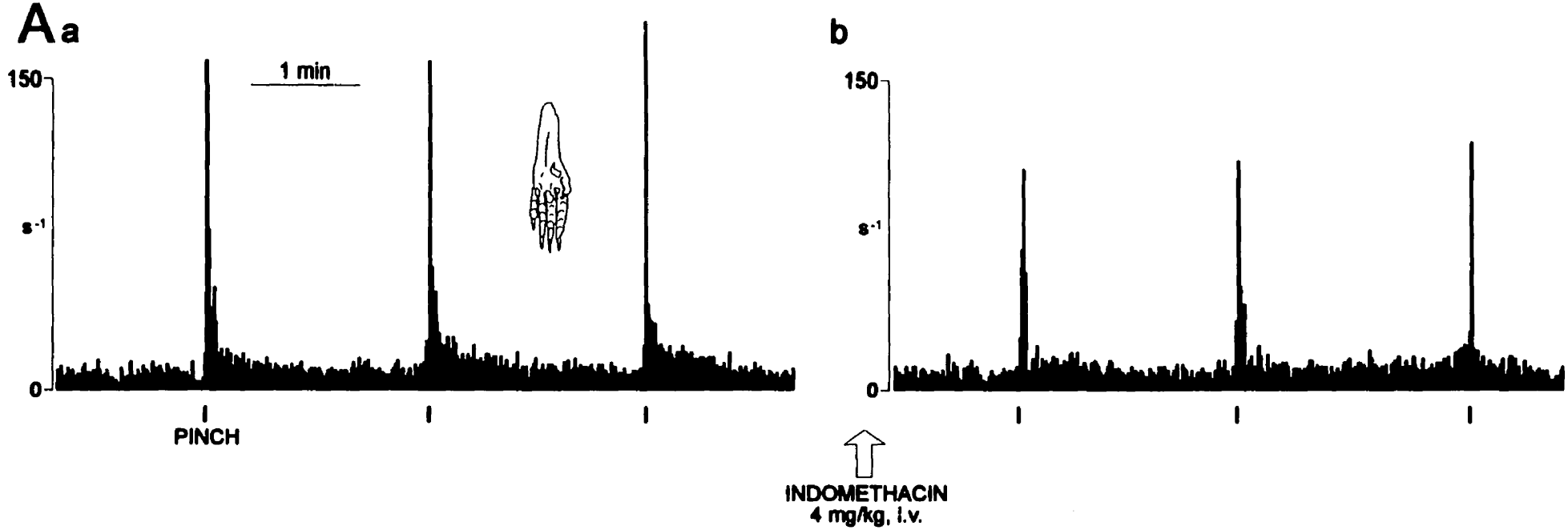

A
180
s-1
0
1 min
ELECTRICAL STIMULATION
INDOMETHACIN 4 mg/kg, i.v.
B a
10 s
ELECTRICAL STIMULATION
b
1 s
0.15 mV
ELECTRICAL STIMULATION
c
10 s
d
1 s
C a
INDOMETHACIN (mg/kg, i.v.)
2
4
% INHIBITION
0
20
40
60
80
100
5/6
7/7
*
**
ELECTRICAL STIMULATION OF SCIATIC NERVE: FAST INITIAL DISCHARGE
b
INDOMETHACIN (mg/kg, i.v.)
2
4
% INHIBITION
6/6
7/7
**
***
ELECTRICAL STIMULATION OF SCIATIC NERVE: AFTERDISCHARGE

Figure 1. Indomethacin depresses the response to electrical stimulation of the sciatic nerve. (A) Electrical stimulation (1 ms rectangular pulses of 8 mA at 20 Hz for 3 s) to the exposed left sciatic nerve produced a fast initial discharge which lasted only for the duration of the stimulus and a slowly-decaying afterdischarge which persisted for up to approximately 1 min. The vertical axis represents the firing frequency in spikes/s. The horizontal axis is time. Indomethacin inhibited the fast initial discharge and the slowly-decaying afterdischarge, with a preferential depression of the latter. The time of administration of indomethacin is depicted by the open arrow. The time and duration of the electrical stimulus are shown by the narrow rectangles below the histogram. The neuron was found 1148 μm deep from the dorsal surface of the spinal cord. The inset shows the cutaneous receptive field on the left hind paw of the rat. (B) Extracellular recordings showing representative single unit excitatory responses to a electrical stimulus (shown by the clear rectangle below each trace) of another wide dynamic range neuron (700 μm). (a) The fast initial discharge is demonstrated by the high frequency firing rate during the stimulus. This is followed by a relatively slowly-decaying afterdischarge lasting approximately 30 to 40 s. (b) Magnified representation of the fast initial discharge shown in (a). Note the increase in firing rate during the electric train compared to the prestimulus baseline firing rate. (c) At 30 min after administration of indomethacin (4 mg/kg, i.v.) firing frequency of the fast initial discharge and the slowly decaying afterdischarge are noticeably decreased. Note that the duration of the afterdischarge is also attenuated compared to the duration of the afterdischarge shown in (a) before indomethacin was given. (d) Magnified representation of the fast initial discharge shown in (c) after

administration of indomethacin. A considerable decrease in firing rate is demonstrated. Note that while the firing frequency was decreased by indomethacin, spike amplitude remained unaltered. (C) Dose-response histogram summarizing the effects of indomethacin on (a) the fast initial discharge and (b) the slowly-decaying afterdischarge. Each vertical axis represents the mean ($\pm$SEM) percent inhibition expressed as a percent of the rate of discharge prior to the administration of indomethacin. Each ratio is the number of dorsal horn neurons inhibited over the number tested. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ vs. vehicle (0% inhibition). The maximum percent inhibition of the fast initial discharge and the slowly-decaying afterdischarge at 2 mg/kg indomethacin are significantly different ($P < 0.05$).


A a
1 min
150
s-1
0
PINCH
b
150
s-1
0
INDOMETHACIN
4 mg/kg, i.v.

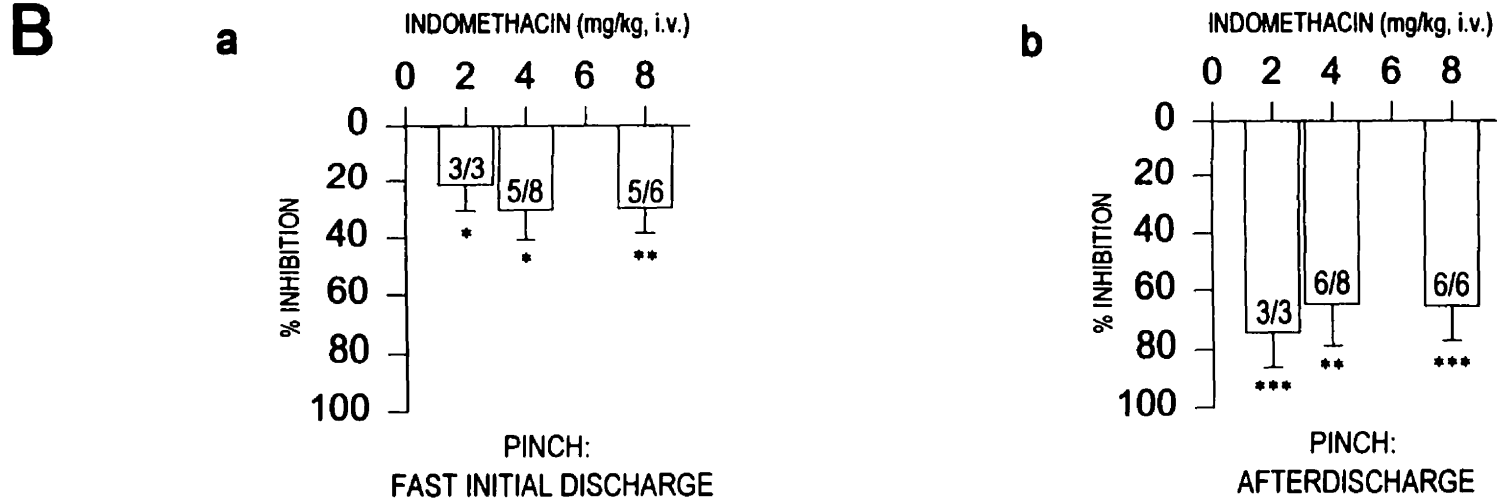

B
a
INDOMETHACIN (mg/kg, i.v.)
0 2 4 6 8
% INHIBITION
0
20
40
60
80
100
3/3
5/8
5/6
*
*
**
PINCH:
FAST INITIAL DISCHARGE
b
INDOMETHACIN (mg/kg, i.v.)
0 2 4 6 8
% INHIBITION
0
20
40
60
80
100
3/3
6/8
6/6
***
**
***
PINCH:
AFTERDISCHARGE

Figure 2. Indomethacin depresses the response to noxious mechanical stimulation of the cutaneous receptive field. A(a) Noxious mechanical stimulation (pinch) produced a fast initial discharge which lasts only for the duration of the stimulus and a slowly-decaying afterdischarge which persisted for up to 1 min in a wide dynamic range neuron (647 μm deep from the dorsal surface of the spinal cord). The time and duration of the pinch applications are shown by the narrow rectangles below the histogram. The inset shows the cutaneous receptive field on the left hind paw of the rat. (b) Inhibitory effect of indomethacin on the fast initial discharge and slowly-decaying afterdischarge responses of this neuron. This record was taken 20 min after the end of (a). (B) Dose-response histogram summarizing the effects of different doses of indomethacin on (a) the fast initial discharge and (b) the slowly-decaying afterdischarge. Each vertical axis represents the mean ($\pm$SEM) percent inhibition expressed as a percent of the rate of discharge prior to the administration of indomethacin. To calculate significance, the mean percent inhibition was compared to the mean effect of vehicle administration. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ vs. vehicle. The maximum percent inhibition of the fast initial discharge and the slowly-decaying afterdischarge at 2 and 4 mg/kg indomethacin, respectively, are significantly different ($P < 0.05$).

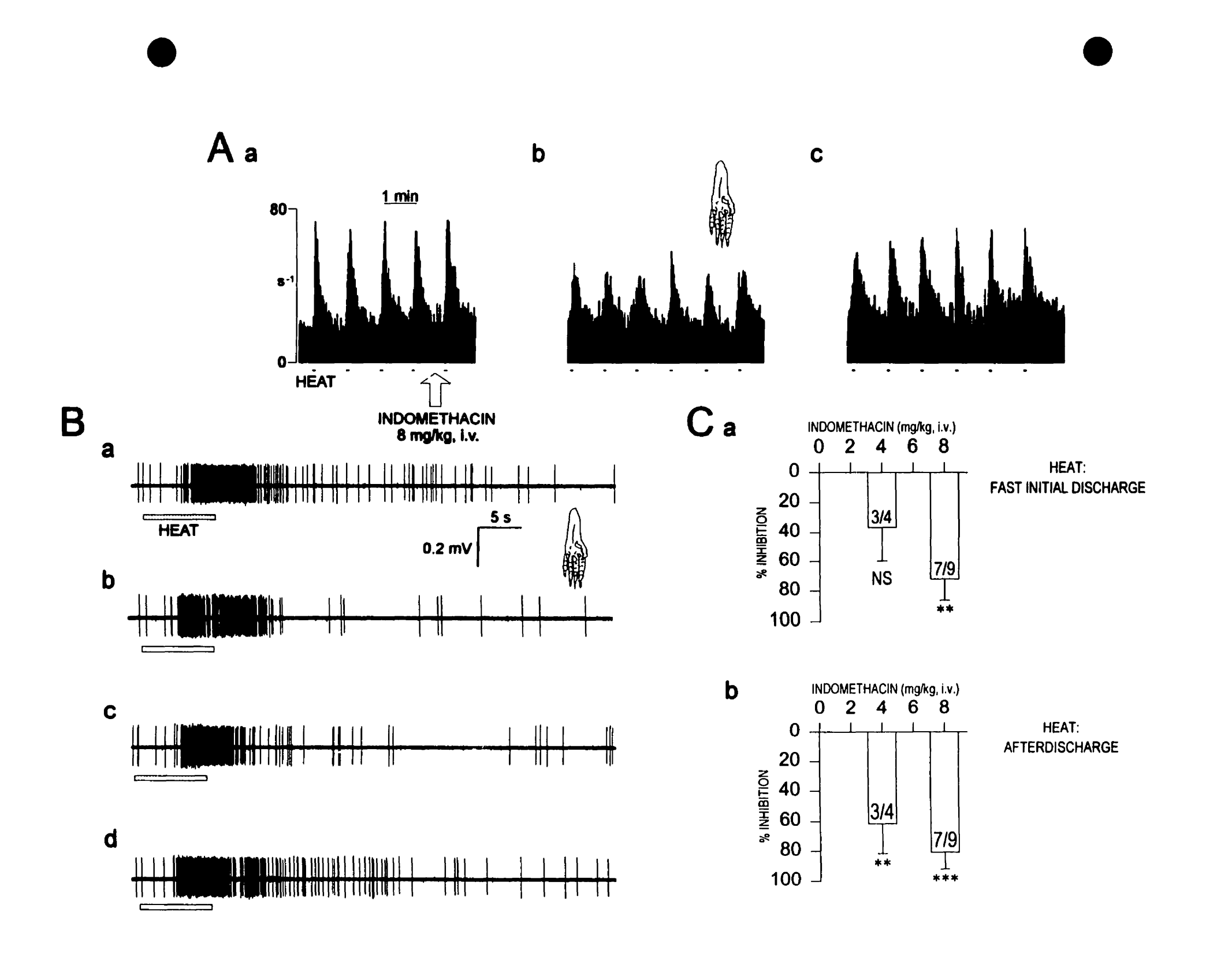
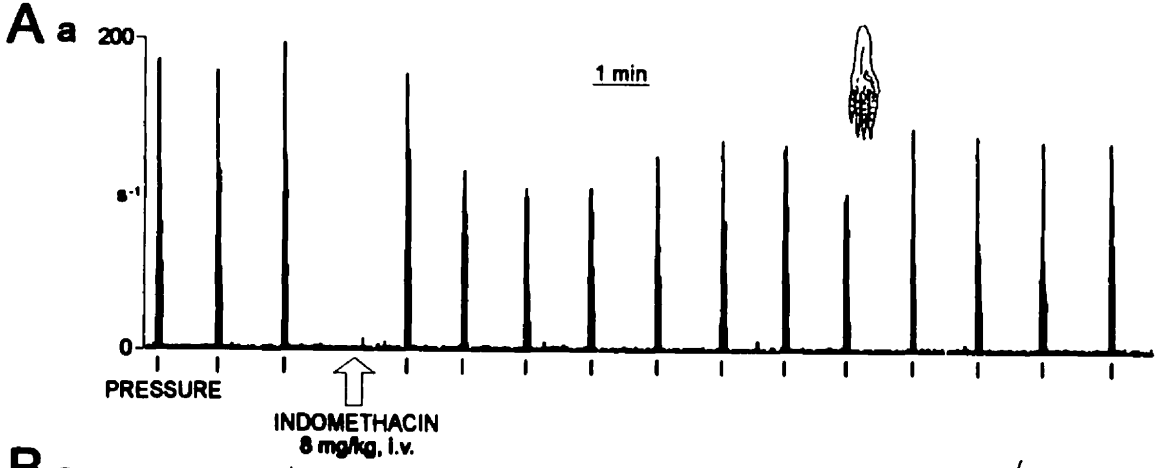
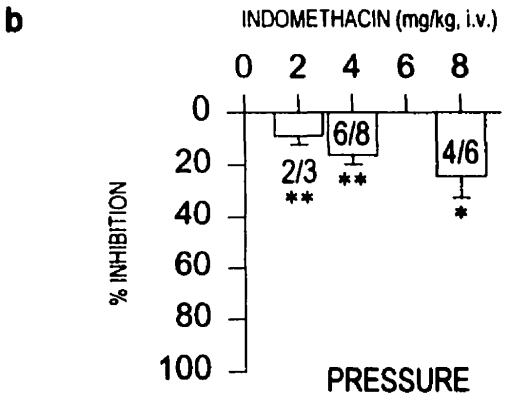
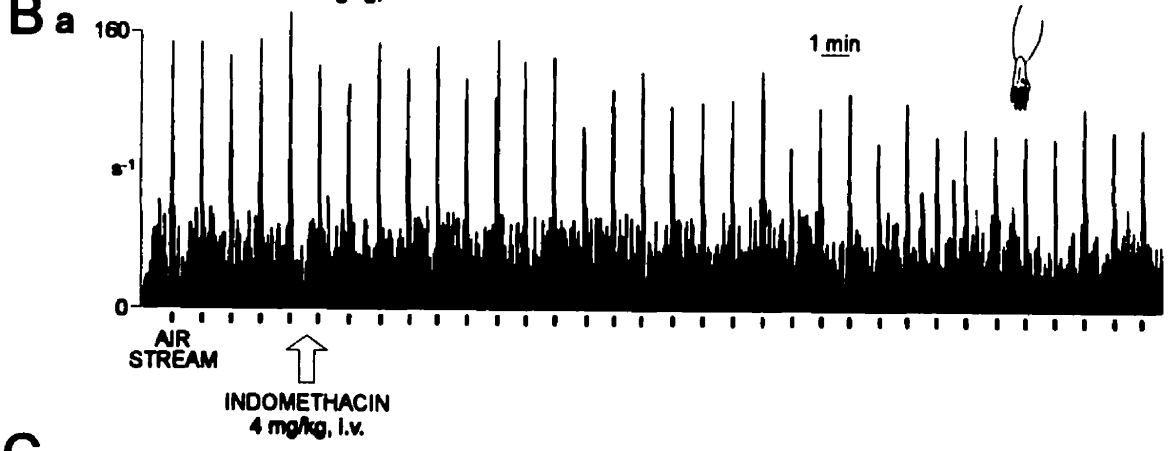

A a
1 min
80
s⁻¹
0
HEAT
INDOMETHACIN
8 mg/kg, i.v.
b
c
B
a
HEAT
5 s
0.2 mV
b
c
d
C a
INDOMETHACIN (mg/kg, i.v.)
0 2 4 6 8
% INHIBITION
0
20
40
60
80
100
3/4
NS
7/9
**
HEAT:
FAST INITIAL DISCHARGE
b
INDOMETHACIN (mg/kg, i.v.)
0 2 4 6 8
% INHIBITION
3/4
**
7/9
***
HEAT:
AFTERDISCHARGE

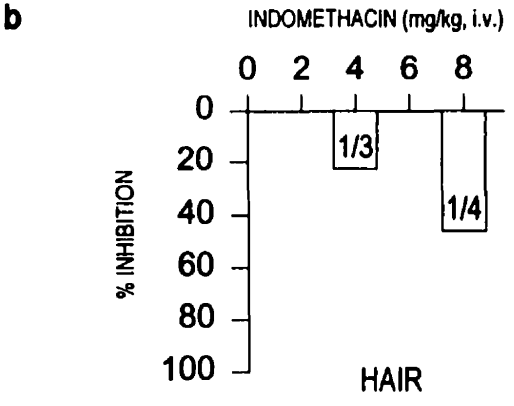
Figure 3. Indomethacin attenuates the response to noxious thermal stimulation (radiant heat) of the cutaneous receptive field. A(a) Noxious thermal stimulation (indicated by the narrow rectangles below the histogram) cycled at 1 min intervals elicited a fast initial discharge followed by the slowly-decaying afterdischarge in a wide dynamic range neuron (410 μm). (b) Inhibitory effect of indomethacin (8 mg/kg, i.v.) on the fast initial discharge and persistent responses. The record was taken 10 min after administration of indomethacin. The inset shows the cutaneous receptive field. (c) The response to noxious thermal stimulation had recovered by 90 min later. (B) Example of extracellular recordings showing representative single unit excitatory responses to noxious thermal stimulation (shown by the shaded rectangles below each trace) of another wide dynamic range neuron (938 μm). (a) The fast initial discharge is revealed by high frequency firing rate toward the end and slightly after application of the noxious thermal stimulus. This is followed by a relatively slowly-decaying afterdischarge. (b) At 5 min after administration of indomethacin (8 mg/kg, i.v.) firing frequency of the fast initial discharge is noticeably decreased and the slowly-decaying afterdischarge is almost entirely inhibited. Also the offset of the fast initial discharge after the end of the thermal stimulus is sooner. (c) At 30 min after indomethacin was given there remained a considerable inhibition of both the fast initial discharge and slowly-decaying afterdischarge. Note the increase in the latency of onset of the effect of noxious thermal stimulation in (c) compared to (a) and the decrease in the number of spikes. (d) At 100 min after indomethacin administration, there was a complete recovery of both the fast initial discharge and the slowly-decaying afterdischarge. Note that while the firing frequency was decreased by indomethacin, spike amplitude

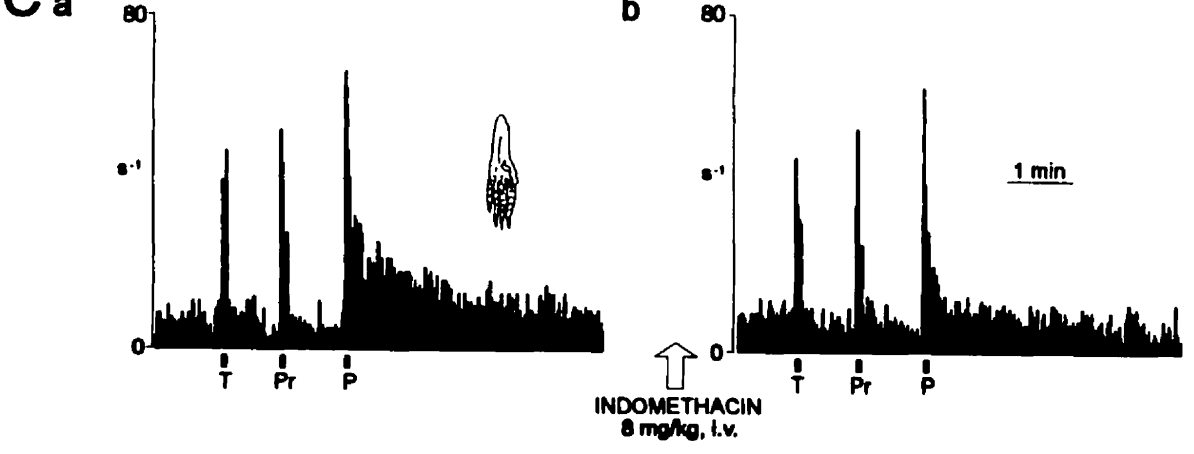
remained unaltered. (C) Dose-response histogram summarizing the effects of different doses of (a) indomethacin on the fast initial discharge and (b) the slowly-decaying afterdischarge neuronal responses to noxious thermal stimulation of the cutaneous receptive field. NS not significant, ** $P < 0.01$ or *** $P < 0.001$ vs. vehicle.









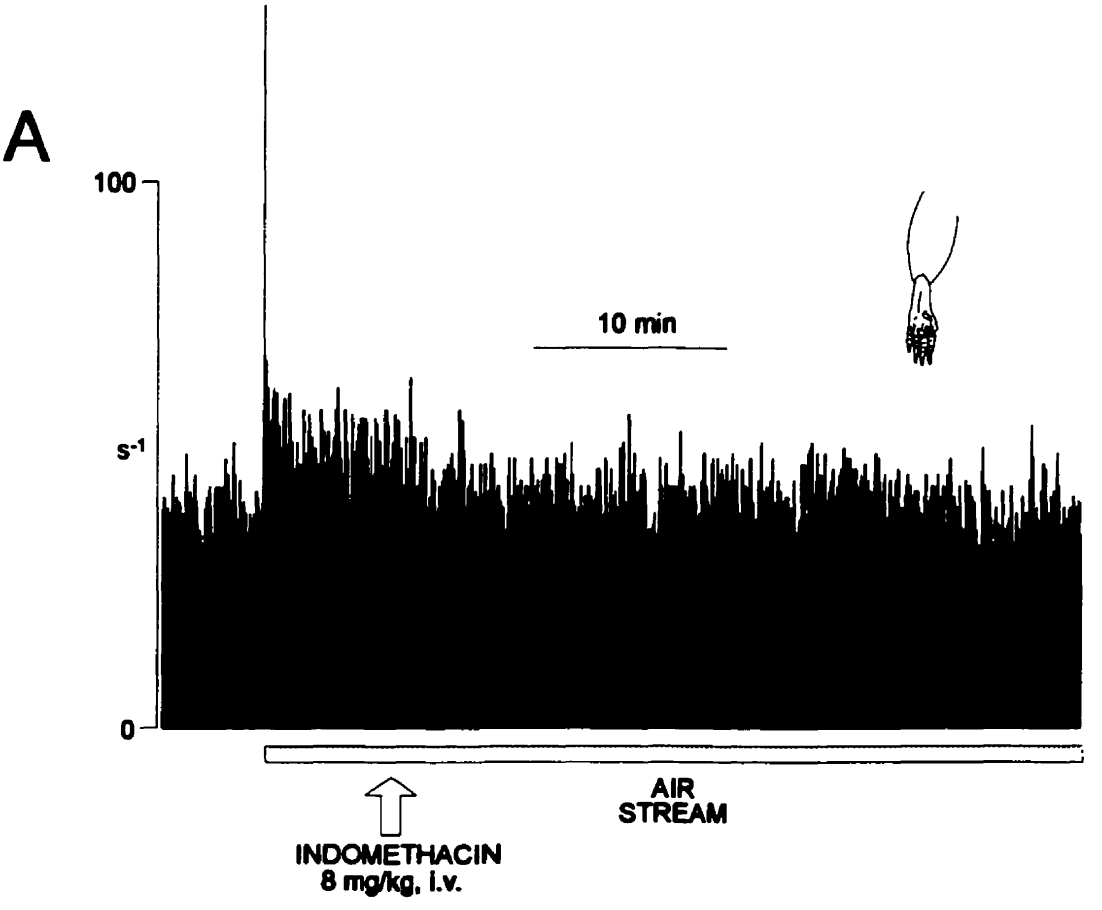


c

NUMBER OF SPIKES IN RESPONSE:	BEFORE	AFTER	% INHIBITION
T	130	111	14.6 %
Pr	115	100	13.0 %
P(FID)	186	156	16.1 %
P(SDAD)	996	482	51.6 %

Figure 4. Indomethacin induces relatively weak inhibition of responses to innocuous stimuli. A(a) Inhibitory effect of indomethacin on the response of a non-nociceptive neuron (935 μm) to moderate innocuous pressure (3 s duration) applied to the cutaneous receptive field (shown on inset of left hind paw of a rat) at 1 min intervals. (b) Dose-response histogram summarizing the effects of different doses of indomethacin on neuronal responses to pressure stimulation, * $P < 0.05$ or ** $P < 0.01$ vs. vehicle. (B) Indomethacin induces relatively weak inhibition of the response to hair stimulation (air-stream for 3 s duration) of the receptive field in one neuron. (a) Effects of indomethacin on the responses of a non-nociceptive neuron (446 μm) to an air stream applied to the hairy skin of the left hind paw of a rat (receptive field depicted on inset of hind limb). (b) Dose-response histogram summarizing the effects of different doses of indomethacin on neuronal responses to hair stimulation, * $P < 0.05$ or ** $P < 0.01$ vs. vehicle. C(a) Touch (T), pressure (Pr; 3 s) or noxious pinch (P; 3 s) stimuli on the cutaneous receptive field produced a fast initial discharge which lasted only for the duration of the stimulus in a wide dynamic range neuron (645 μm). A slowly-decaying afterdischarge following the fast initial discharge response is also is also produced by the pinch stimulus. (b) Indomethacin induced a relatively weak inhibitory effect on the fast initial response of this wide dynamic range neuron to touch, pressure and pinch applied to the cutaneous receptive field (shown on inset of left hind paw of a rat) 25 min after administration. However, indomethacin decreased noticeably the magnitude and duration of the slowly-decaying afterdischarge. (c) Number of spikes per response of this particular wide dynamic range neuron to touch (T), pressure (Pr) and pinch (P) before and after administration of indomethacin. Notice

that while there is only a slight decrease in the number of spikes per response to touch, pressure or the fast initial discharge (FID) to pinch stimulation after indomethacin administration, the number of spikes in the slowly-decaying afterdischarge (SDAD) is decreased to less than half of the number of spikes before indomethacin was given (51.6 % inhibition).


A
100
s-1
0
10 min
AIR
STREAM
INDOMETHACIN
8 mg/kg, i.v.

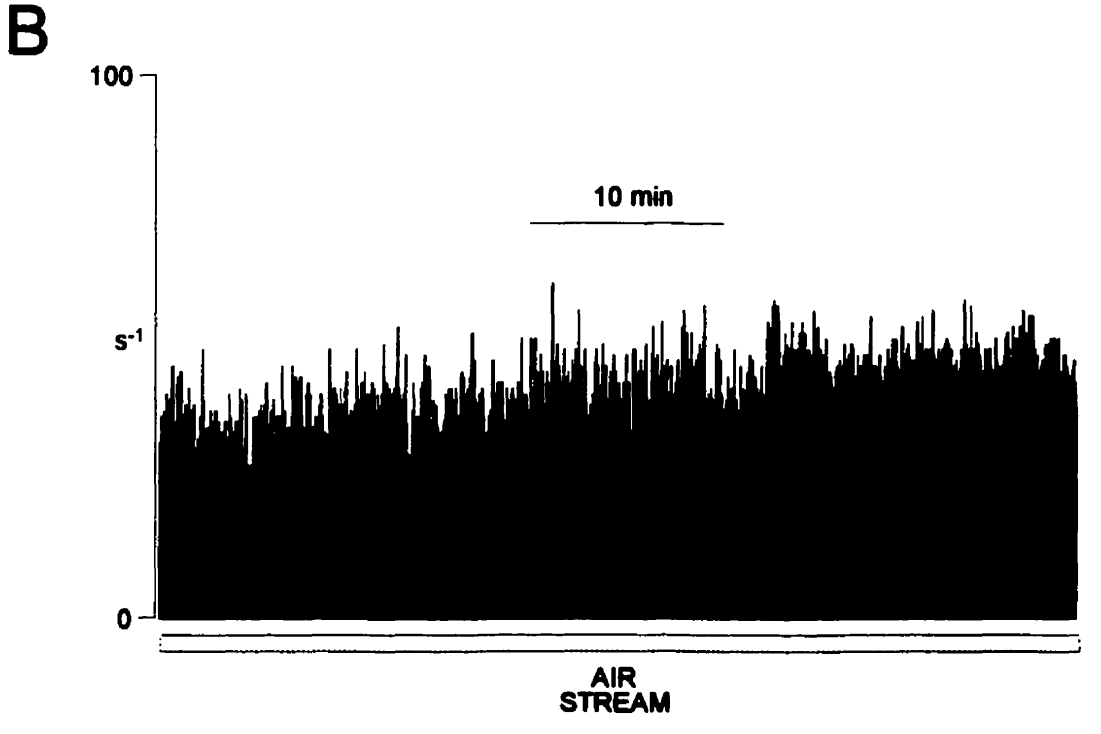

B
100
s-1
0
10 min
AIR
STREAM

Figure 5. Indomethacin depresses the on-going excitation induced by prolonged hair stimulation. (A) Continuous air stream (indicated by the hatched rectangle below the histogram) applied to the hairy receptive field increases firing frequency of a wide dynamic range neuron (844 μm). The pre-hair stimulation baseline firing frequency of 34 Hz is represented by the white dotted line spanning the record. Administration of indomethacin (8 mg/kg, i.v.) inhibits the hair stimulation-evoked increase in firing rate by 93% at 30 min after administration of indomethacin. (B) Gradual recovery of continuous hair stimulation-induced activity. The end of the trace is approximately 90 to 100 min after administration of indomethacin.

Unifying Statement

It is demonstrated in Chapter 2 that COX inhibition preferentially depresses nociceptive vs. non-nociceptive mechanisms, in particular, long-lasting, synaptically-elicited responses. These data suggest an association between tonic effects of peripheral stimulation-induced synaptic input and activation of the eicosanoid signal transduction pathway via COX.

However, is this effect mediated via COX-1 or via COX-2? Given the recent availability of COX-2 selective inhibitors, it was of fundamental importance and interest to determine the possible role of COX-2 in on-going and peripheral stimulation-induced synaptic input in the spinal dorsal horn. We were given access to the selective COX-2 inhibitor meloxicam and examined its effect on on-going activity and the initial and afterdischarge responses of spinal dorsal horn neurons to pinch stimulation of the cutaneous receptive field. This is shown in the next chapter, Chapter 3.

Chapter 3

COX-2 Inhibitor Meloxicam Preferentially Depresses the Afterdischarge vs. the Initial Discharge of the Response of Rat Spinal Dorsal Horn Neurons to Noxious Cutaneous Stimulation

Abstract

This study examined the effects of the COX-2 inhibitor, meloxicam, on responses of spinal dorsal horn neurons to synaptic inputs *in vivo*. Experiments were run using male Sprague Dawley rats (350-375 g) anesthetized with Na-pentobarbital (50 mg/kg, i.p.) and acutely spinalized at T9. Spinal segments L1-4 were exposed for extracellular single unit recording of on-going and stimulation-evoked activity of wide dynamic range neurons. On-going activity of 8 wide dynamic range dorsal horn neurons was unaffected by meloxicam (0.1 mg/kg, i.v.). However, responses to noxious mechanical stimulation (21 N for 3 s) of the cutaneous receptive field of the hind paw were depressed by meloxicam in particular the slowly-decaying afterdischarge ($34.50 \pm 6.06\%$ inhibition) in all 8 neurons studied. The brief initial discharge in response to stimulation was depressed less ($15.31 \pm 4.89\%$ inhibition, $n=7/8$). Specifically, the data indicate that the percent inhibition of the afterdischarge was significantly greater than that of the initial discharge ($P < 0.05$). Given the selectivity of meloxicam for COX-2, the data suggest that COX-2 may be involved in mediating and/or modulating excitatory effects of nociceptive inputs to dorsal horn neurons, in particular the prolonged stimulation-evoked afterdischarge.

Introduction

The discovery of the inducible isoform of cyclooxygenase COX-2 (Hla and Neilson 1992) prompted the development of selective inhibitors which are anticipated to be useful in the treatment of pain. The COX-2 inhibitors rofecoxib (Morrison et al. 1999) and meloxicam (Lund et al. 1998) are reported to be analgesic in humans suffering from rheumatoid arthritis and osteoarthritis. In animal pain models, the COX-2 inhibitor, SC58125, is reported to reduce thermal hyperalgesia induced by peripheral administration of carrageenan (Dirig et al. 1998). Meloxicam depresses capsaicin- and formalin-induced nociceptive behavior in mice (Santos et al. 1998) and reduces thymulin injection-induced c-*fos*-like-immunoreactivity in the rat spinal dorsal horn (Saadé et al. 1999). In an *in vitro* rat spinal cord preparation, meloxicam also inhibits 'wind-up' (Lopez-Garcia and Laird 1998).

We have recently demonstrated that systemic administration of the non-selective COX-1/-2 inhibitor, indomethacin, preferentially depresses the afterdischarge of the response of dorsal horn neurons to noxious peripheral stimulation (Pitcher and Henry 1999). We were subsequently given access to meloxicam and determined its effects on on-going activity of rat spinal dorsal horn neurons and on the initial and afterdischarge excitatory responses to noxious stimulation-induced synaptic input. The purpose was to determine the specific role of COX-2 in brief and long-lasting synaptically-elicited neuronal responses.

Materials and Methods

Experiments were done using male Sprague Dawley rats (350-375 g; Harlan Sprague Dawley, Inc., Indianapolis, USA) anesthetized with Na-pentobarbital (initial dose 50 mg/kg, i.p., supplemented with 10 mg/kg/hr, i.v.; Abbott Laboratories Ltd., Montreal, Canada). Preparation was similar to that described elsewhere (Pitcher and Henry 1999) following guidelines in *The Care and Use of Experimental Animals* by the Canadian Council on Animal Care (Vols. I and II). Each rat normally breathed spontaneously during the experiment. Spinal cords were transected at T9 to eliminate descending controls and segments L1-4 were exposed for recording extracellular single units using single-barrelled micropipettes (1-2 μm tip diameter; 3 M NaCl solution, impedance 2-3 MΩ at depths of 150 to 1000 μm).

Neurons were classified functionally based on their excitatory responses to natural stimulation of the cutaneous receptive field (Pitcher and Henry 1999, 2000). Responses to the noxious range of mechanical stimulation of the hind paw (pinch, 21 N for 3 s) included the fast initial discharge and slowly-decaying afterdischarge which characterize responses of nociceptive neurons to noxious stimulation (De Koninck and Henry 1991; Pitcher and Henry 1999, 2000). The initial discharge was measured for the duration of the 3 s pinch stimulus. The afterdischarge began immediately after the initial discharge and ended once the firing rate returned to the prestimulus discharge level. These responses were quantified as the total number of spikes after subtracting the background discharge. Pinch stimuli were given at 10 min intervals.

Meloxicam (generously supplied by Boehringer Ingelheim, Canada, Ltd.) was dissolved in 100 μl of 2M NaOH and distilled water. This stock solution (10 mg/ml) was diluted to 0.1 mg/ml using distilled water (pH 7.4 using 2% $NaHCO_3$ and $NaH_2PO_4 \bullet H_2O$) and was administered i.v. at a dose of 0.1 mg/kg. As a control, the vehicle was administered 90 min prior to meloxicam administration. Only one neuron was studied per rat.

The maximum inhibitory effects of meloxicam on on-going and pinch-evoked activity occurred between 40 and 70 min and were expressed as percent inhibition as described elsewhere (Pitcher and Henry 1999). Statistical analysis was done using the Mann-Whitney Rank Sum Test. The mean ($\pm$SEM) percent inhibition following meloxicam was compared to that following vehicle administration and a difference was considered significant with a P value < 0.05.

Results

Meloxicam could not be given iontophoretically due to its high resistance in an ejection barrel. Accordingly, the following data were obtained from the effects of systemic administration. Meloxicam and its vehicle were consistently without effect on spike amplitude (Figure 1). Similarly, arterial pressure and respiratory rate were not altered. Vehicle was also consistently without effect on on-going discharge or on peripheral stimulation-evoked responses.

On-going activity of a wide dynamic range neuron is shown prior to the stimulation-evoked discharge (Figure 1A). Meloxicam had no effect on on-going activity. This is illustrated by the similar levels of on-going activity before (Figure 1A) and after (Figure 1B) administration. Figure 2 shows the cumulative data from the 8 wide dynamic range neurons tested.

Noxious pinch to the cutaneous receptive field produced a typical excitatory response in all 8 wide dynamic range neurons tested. This response consisted of an initial discharge lasting only the duration of the stimulus and an afterdischarge persisting beyond the end of the pinch stimulus.

Figure 1A shows the response of one neuron to pinch to the ipsilateral hind paw. The initial discharge lasted for the duration of the 3 s pinch and the afterdischarge persisted 2-3 min after the end of the stimulus. Figure 1B shows the response to the same stimulus after administration of meloxicam. There was a depression of both the afterdischarge and the initial discharge; spike activity of this neuron during the afterdischarge is illustrated

before (Figure 1Aa) and after (Figure 1Bb) administration.

The effect of meloxicam on the response to noxious mechanical stimulation was determined in a total of 8 neurons. Figure 2 shows the cumulative data. While 7 out of 8 neurons tested exhibited a statistically significant depression of the initial discharge ($P < 0.05$ vs. control) the afterdischarge of all of the 8 neurons ($P < 0.01$ vs. control) was depressed. Figure 2 also demonstrates that the percent inhibition of the afterdischarge ($34.50 \pm 6.06\%$) was significantly greater ($P < 0.05$) than that of the initial discharge ($15.31 \pm 4.89\%$).

Discussion

The data demonstrate that meloxicam, at the dose used, depresses the responses of wide dynamic range neurons to noxious stimulation of the cutaneous receptive field but does not influence on-going activity. These data are interpreted to suggest that COX-2 may be involved in mediating and/or modulating excitatory effects of noxious stimulation-induced synaptic input on neuronal activity, in particular, longer-lasting excitatory effects. As experiments were run in acutely spinalized rats the inhibitory effects of meloxicam are interpreted to have occurred independently of a supraspinal site and were therefore at a peripheral and/or spinal level.

The absence of an effect of meloxicam on on-going activity is interpreted to suggest that eicosanoid synthesis via COX-2 may not be at a level which is functionally significant in terms of sustaining or regulating on-going activity in the normal rat. This lack of effect is not unexpected as the non-selective COX-1/-2 inhibitor, indomethacin, is also without effect on on-going discharge of rat dorsal horn neurons (Pitcher and Henry 1999).

In contrast, responses to pinch of the receptive field were inhibited by meloxicam, which is consistent with the preferential depressant effect of indomethacin on the excitatory effects of peripheral stimulation-evoked synaptic input (Pitcher and Henry 1999). Given that meloxicam depresses prostaglandin E_2 synthesis (Engelhardt et al. 1996) and is selective for COX-2 (Engelhardt et al. 1996; Ogino et al. 1997; Blanco et al. 1999), it is proposed that COX-2 is involved in the excitatory effects of noxious mechanical stimulation on spinal nociceptive neurons. Thus, eicosanoid synthesis via COX-2 may be

interpreted to be stimulus-dependent. Specifically, COX-2 may be involved in elevating eicosanoid levels, particularly prostaglandins, during stimulation-induced synaptic input such that they exhibit physiological effects on dorsal horn neuronal activity.

The preferential inhibitory effect of meloxicam on the afterdischarge supports the concept of an association between the tonic effects of peripheral stimulation-evoked synaptic input and COX-2 activity. In a recent study, based on the selective inhibitory effect of indomethacin on the noxious stimulation-evoked afterdischarge, we put forth the concept that prolonged vs. short-lasting stimulation-induced excitation may result in increased eicosanoid synthesis as there may be longer-lasting activation of the eicosanoid signal transduction pathway (Pitcher and Henry 1999). Additionally, in a rat model of tonic pain, the formalin test, prostaglandin E_2 diasylate levels are increased in the lumbar spinal cord following formalin injection (Malmberg and Yaksh 1995) and these levels change temporally with the biphasic nociceptive responses in the formalin test. Peripherally, COX is also involved in each nociceptive phase (Chapman and Dickenson 1992; Damas and Liégeois 1999). Importantly, as the long-lasting behavioral effects of noxious formalin injection are attributed to continuous afferent input (Puig and Sorkin 1996; Henry et al. 1999) and COX-2 inhibition depresses both excitatory phases in the formalin test (Santos et al. 1998), it is suggested that eicosanoids may be derived from COX-2 and persist only as long as there is sustained primary afferent activity. Thus, it is not unreasonable to hypothesize that the long-lasting excitatory effects of pinch stimulation on dorsal horn neuronal activity are mediated and/or modulated, at least in part, via

elevated levels of eicosanoids derived from prolonged activation of COX-2.

The proposed role of COX-2 in prolonged neuronal responses may be relevant to sustained elevated synaptic transmission demonstrated in rat models of chronic pain and, ultimately, may advance understanding of the neurophysiological basis of chronic pain syndromes.

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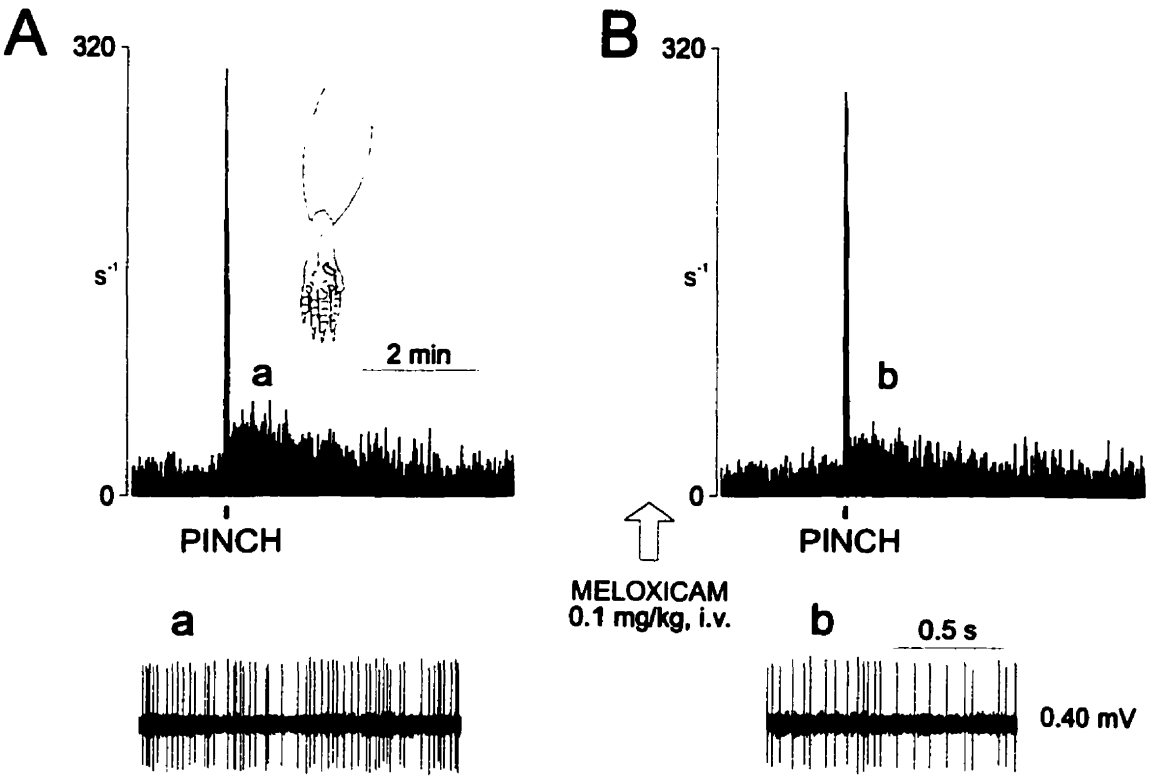
A
320
s⁻¹
0
a
2 min
PINCH
a
B
320
s⁻¹
0
b
PINCH
MELOXICAM
0.1 mg/kg, i.v.
b
0.5 s
0.40 mV

Figure 1. Meloxicam depresses the response to noxious mechanical stimulation of the cutaneous receptive field of one hind paw. *A*: Noxious mechanical (pinch) stimulation produces an initial discharge lasting only duration of the stimulus and a slowly-decaying afterdischarge (peak firing rate represented by the dotted line) which persists 2-3 min in a wide dynamic range neuron (413 μm deep from the dorsal surface of the spinal cord). The time and duration of the pinch stimulus are shown by the narrow rectangle below the histogram. The inset shows the cutaneous receptive field. *a*: Spike activity of this neuron during the afterdischarge before meloxicam administration. *B*: Meloxicam (0.1 mg/kg, i.v.) preferentially depresses the afterdischarge. Note that the peak firing rate is less than that prior to meloxicam administration (represented by the dotted line). The fast initial discharge is considerably less sensitive to the effect of meloxicam and on-going activity is not altered. This record was taken approximately 60 min after the end of *A*. *b*: Spike activity during the afterdischarge after meloxicam administration. Note that spike amplitude remains unaltered. In all cases, the vehicle alone was administered prior to meloxicam administration and in none did this have any observable effect.

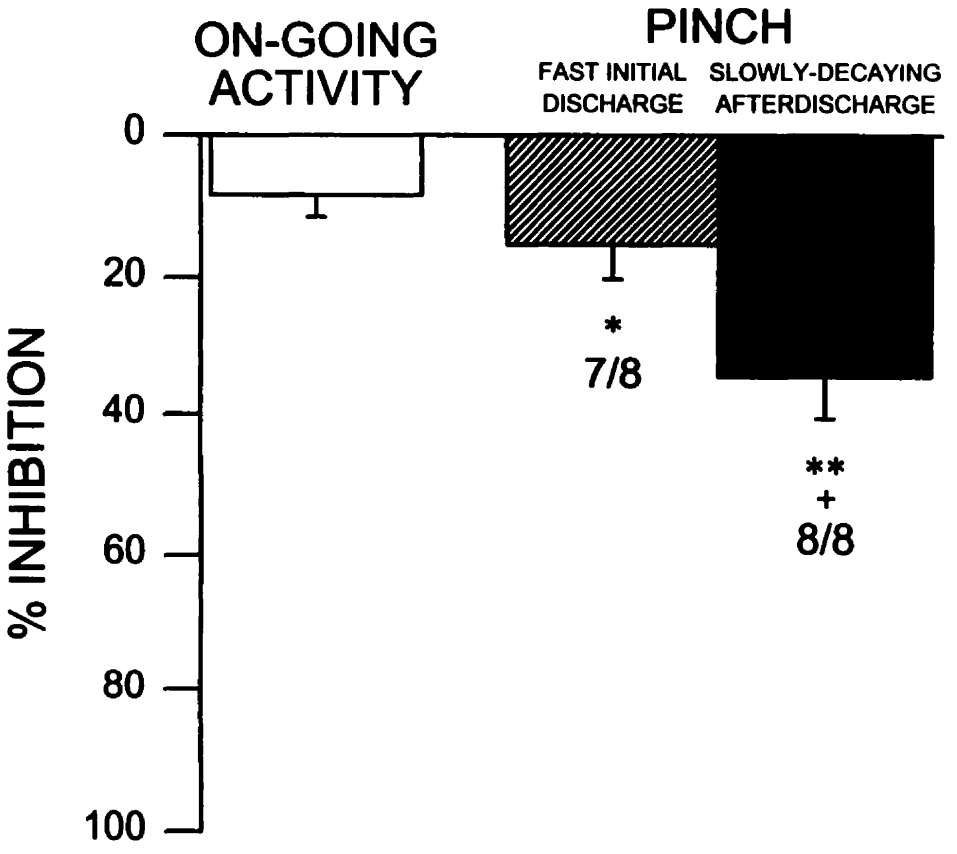
ON-GOING ACTIVITY
PINCH
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
% INHIBITION
0
20
40
60
80
100
*
7/8
**
+
8/8

Figure 2. Effects of meloxicam on on-going activity, the initial discharge and the afterdischarge of the 8 neurons. The vertical axis represents the mean (±SEM) percent inhibition expressed as a percentage of the magnitude (number of spikes) of the response prior to administration of meloxicam. Each ratio is the number of dorsal horn neurons inhibited over the number tested. * $P < 0.05$ and ** $P < 0.01$ vs. vehicle. The maximum percent inhibition of the afterdischarge is significantly greater than that of the initial discharge (+ $P < 0.05$).

Unifying Statement

The selective depressive effects of indomethacin (Chapter 2) and meloxicam (Chapter 3) on long-lasting peripheral stimulation-elicited discharge of dorsal horn neurons suggest an association between the tonic effects of peripheral stimulation-induced synaptic input and activation of the eicosanoid signal transduction pathway.

Thus, given the chronic nature of some kinds of pain, investigation of the eicosanoid pathway was extended in a rat model of chronic pain. However, prior to examination of the effects of indomethacin and meloxicam on dorsal horn neuronal activity in a chronic pain model, it is necessary to have an appropriate testing paradigm such that the magnitude and time course of behavioral effects of chronic pain could be determined reliably. This would provide a basis for the time course of electrophysiological examination of dorsal horn neuronal activity. Therefore, the next chapters report setting up (Chapter 4) and validating (Chapter 5) an animal model of peripheral neuropathy.

Chapter 4

Paw Withdrawal Threshold in the von Frey Hair Test is Influenced by the Surface on which the Rat Stands

Abstract

The effect of testing surface on the rat hind paw withdrawal threshold in the von Frey hair test is investigated in this study. The data indicate that wire mesh, which is typically used when von Frey hairs are applied, may have an effect on the paw withdrawal threshold. In control rats tested on the wire mesh, variability in the withdrawal threshold was observed between the left and the right hind paws (51.04 ± 12.29 g and 64.31 ± 9.37 g, respectively) and on different days of testing (35.24 ± 9.54 g and 45.83 ± 12.97 g for the left and right hind paws, respectively, 7 days later). In an attempt to reduce this variability a customized platform was used to measure the von Frey hair-induced paw withdrawal in the rat. It consists of an opaque, flat-surfaced plastic platform with holes through which von Frey hairs are inserted and applied to the plantar surface of the paw. In control rats tested with von Frey hairs using this customized platform, variability in the paw withdrawal thresholds between the left and right hind paws in single rats over time as well as between different rats was reduced (49.86 ± 6.97 g and 49.29 ± 6.56 g for the left and right hind paws, respectively, on day 0; 48.29 ± 5.82 g and 53.00 ± 4.59 g for the left and right hind paws, respectively, 7 days later). Furthermore, in rats in which a 2 mm polyethylene cuff was used to constrict the left common sciatic nerve, the ipsilateral as well as the contralateral hind paw withdrawal thresholds were decreased (2.45 ± 0.65 g and 26.09 ± 5.86 g, respectively, 7 days later). In similar rats tested on the wire mesh, the ipsilateral but not the contralateral paw withdrawal threshold decreased (12.80 ± 2.21 g and 65.00 ± 10.28 g, respectively, at 7 days). The data suggest that the flat surface and opaque

properties of the customized platform enable accurate, reliable and repeatable measurements of ipsilateral and contralateral paw withdrawal threshold using von Frey hairs in normal and nerve-injured rats.

Introduction

von Frey hairs are important tools for the study of mechanisms of cutaneous stimulation-induced sensory input. Mechanical force is exerted via application of a particular hair to the cutaneous receptive field until buckling of the hair occurs. To date, wire mesh is the commonly used apparatus to allow their application. Specifically, the elevated wire mesh floor supports a rat while the hairs are applied from below, through the wire mesh, to the plantar surface of the paw. In addition, applying hairs of different force is done to establish the paw withdrawal threshold. Surprisingly, in many of the studies that report using wire mesh, the dimensions and characteristics of the surface on which the rats are tested with von Frey hairs are seldom reported, presumably because any effect the surface may have on paw withdrawal threshold is of little or no concern. To the best of our knowledge, there seems to be no set standard for the dimensions of wire mesh used except in a few studies that report using a 6×6 mm grid to examine von Frey hair-induced paw withdrawal in neuropathic rats (DeLeo et al. 1996; Wagner and DeLeo 1996; Wagner and Myers 1996). We have tested rats on wire mesh and find it convenient for the experimenter to use. However, we also find that there is a relatively large variability in the paw withdrawal threshold values between control rats tested on the wire mesh as well as from single rats over different days of testing.

In an effort to determine whether the characterisitics of the surface might influence the amount of variability, we ran a pilot study which indicated that variability could be reduced by allowing the rats to stand on a flat surface. This paper is a report on a full

study using a customized testing platform which consists of an opaque flat-surfaced plastic platform which contains holes through which von Frey hairs are easily inserted and applied to the plantar surface of the paw. To determine the effect of the testing surface and to verify the platform's applicability to testing the hairs, we compared withdrawal thresholds of control and nerve-injured rats measured on the customized platform and on the wire mesh.

The data in this study show that properties of the surface on which rats are tested may influence sensory processing and subsequently affect the withdrawal threshold to von Frey hair application. It is suggested further that characterization of mechanisms of sensory input using von Frey hairs may be obtained more accurately and reliably in rats tested on a smooth opaque surface rather than on a see-through and irregular surface such as wire mesh.

Materials and Methods

2.1. Animals

Experiments were performed on adult, male Sprague-Dawley rats (375-425g) from Harlan Sprague Dawley (Indianapolis, Indiana). They were housed in plastic cages containing beta-chip bedding (Hardwood Laboratory Bedding, Northeastern Products Corp., Warrensburg, N.Y., USA) and maintained on a 12:12 h light:dark cycle (lights on at 06:00 h) with access to food and water *ad libitum*. Only 2 rats from the same testing group (ie. control or nerve-injured) were together in the cages. Experiments were conducted during the light component of the cycle. Guidelines outlined in *The Care and Use of Experimental Animals* by the Canadian Council on Animal Care (Vols. I and II) were strictly followed.

2.2. Surgical procedure

Sciatic nerve constriction was done using a variation of the technique reported by Mosconi and Kruger which consists of an enclosure of the sciatic nerve using a polyethylene cuff (Mosconi and Kruger 1996). Under Na-pentobarbital anaesthesia (50 mg/kg, i.p., Abbott Laboratories, Limited, Montreal, Canada), the left common sciatic nerve was exposed by blunt dissection through the biceps femoris muscle and was isolated from surrounding connective tissue using glass probes. The sciatic nerve was slightly elevated and held in place using a sterilized glass probe. Using fine forceps a 2 mm section of split PE-90 polyethylene tubing (Intramedic PE-90, Clay Adams, Division of

Becton Dickinson and Company, Parsippany, New Jersey, USA) was placed around the nerve thus constricting the nerve.

After cuff implantation, the sciatic nerve was kept moist using sterile saline at 37.5°C. The muscle layer was sutured using 3-O silk thread (Ethicon Inc., Montreal, Quebec, Canada) and the shaved skin layer was closed using 3 stainless steel suture clips (Fine Science Tools, Inc., North Vancouver, British Columbia, Canada). Nitrofurazone ointment 0.2% (Univet Pharmaceuticals Ltd., Milton, Ontario, Canada) was placed on the skin suture to minimize any risk of infection. Rats were allowed to recover for 24 h before testing was begun. Autotomy was not observed at any time during the study in any rat. The rats in each of the groups appeared equally well groomed throughout the study.

2.3. Experimental setting and design of testing surface

During testing, a rat was placed on the customized platform (see Figures 1 and 2) which was fixed in a transparent plexiglass chamber with dimensions of 30×30×30 cm. (see Figure 2). The customized platform is 30×30 cm and is made of 3 mm thick plexiglass. It contains 1.5 mm diameter holes in a 5 mm grid of perpendicular rows throughout the entire area of the platform (see Figure 1). This platform is also opaque in appearance so that von Frey hairs are applied from underneath without distracting the rat (see Figure 2). Although objects distal to the surface of the customized platform were not visible, the plantar surface of a hind paw was visible as it was in direct contact with the platform surface. Therefore, no difficulty was experienced by the experimenter in

applying the von Frey hairs accurately and reliably to the plantar surface of the hind paws in rats tested on the customized platform.

Measurement of mechanical threshold measurements using von Frey hairs was also determined in rats tested on an irregular surface. The same protocal was used as described above but a wire mesh platform was used instead of the customized platform. It consists of 1 mm diameter wire with an 8×8 mm square grid pattern which we considered large enough to be physically extremely different compared to the customized platform but small enough so that there was no risk of a rat placing its paw through the mesh.

2.4. *Measurement of mechanical hind paw withdrawal threshold*

The hind paw withdrawal threshold was determined using von Frey hairs and was expressed in grams. Eleven hairs ranging from 0.23 to 91.0 g were used. The value of each hair was confirmed weekly by measuring the magnitude in grams exerted by the hair when applied to a Mettler AE 100 electronic balance. This was done because it was determined that slight fluctuation in the value of a hair may occur after use. If this was the case for a particular hair, the new value in grams, determined using the electronic balance, was used in determining the paw withdrawal threshold.

The protocol used in this study was a variation of that described previously (Takaishi et al. 1996). A testing session for a particular rat began after 5 min of habituation or as soon as the rat stopped exploring and appeared acclimatized to the testing environment. The series of von Frey hairs was applied from below the customized

platform or the wire mesh to the plantar surface of the left hind paw in ascending order beginning with the lowest hair (0.23 g). Application was to the central region of the plantar surface avoiding the foot pads. A particular hair was applied until buckling of the hair occurred. This was maintained for approximately 2 s. The hair was applied only when the rat was stationary and standing on all four paws. A withdrawal response was considered valid only if the hind paw was completely removed from the platform. Although infrequent, if a rat walked immediately after application of a hair instead of simply lifting the paw, the hair was reapplied. On rare occasions, the hind paw only flinched after a single application; as the hind paw was not lifted from the platform, this was not considered a withdrawal response.

A trial consisted of application of a von Frey hair to the hind paw 5 times at 5 s intervals or as soon as the hind paw was placed appropriately on the platform. If withdrawal did not occur during 5 applications of a particular hair, the next larger hair in the series was applied in a similar manner. When the hind paw was withdrawn from a particular hair either 4 or 5 times out of the 5 applications, the value of that hair in grams was considered to be the withdrawal threshold.

Once the threshold was determined for the left hind paw, the same testing procedure was repeated on the right hind paw after 5 min. Second and third testing sessions were run for each of the left and right hind paws. If the withdrawal threshold in the second or third session did not match the withdrawal threshold of the previous testing session(s) in a given hind paw, the next larger hair in the series was tested. This was done until the withdrawal

thresholds in 3 successive trials matched. Only hind paw withdrawal thresholds that remained consistent in the second and third successive trials in the control or cuff-implanted rats were used in the data analysis. The total testing time for each rat usually lasted 35 to 40 min.

The baseline withdrawal thresholds of each of the hind paws using von Frey hairs were determined for each rat prior to surgical manipulation (day 0). Measurement of the paw withdrawal threshold was measured next on day 4 and then on day 7. Testing with the wire mesh and the customized platform was done on the same days.

2.5. Statistical analysis

Hind paw withdrawal thresholds were analyzed using Kruskal-Wallis one way ANOVA on ranks. Student-Newman-Keuls test was used for post-hoc comparisons between or within groups of rats following ANOVA. The mean ($\pm$standard error of the mean) withdrawal threshold values between different groups of rats or within the same group at different time points were considered significantly different with a P value <0.05.

Results

3.1. Paw withdrawal threshold in control rats

The control groups were comprised of rats which received no surgical manipulation. One group was tested with von Frey hairs on the wire mesh and the other was tested on the customized platform.

In rats tested on the wire mesh (n=7), the minimal von Frey hair stimulus used to evoke withdrawal at day 0 (baseline) was 51.04±12.29 and 64.31±9.39 g for the left and right hind paws, respectively (see Figure 3A). On day 4 the mean withdrawal thresholds were 39.09±9.67 and 32.60±10.43 g for the left and right hind paws, respectively, and 35.24±9.54 and 45.83±12.97 g for the left and right hind paws, respectively, on day 7. Figure 3A illustrates that although the mean hind paw withdrawal thresholds were not significantly different compared to the baseline mean paw withdrawal threshold values, there was considerable variability between rats as well as within single rats on different days of testing.

In control rats tested on the customized platform, the mean withdrawal thresholds on different days of testing were similar. For example, on day 0, the withdrawal thresholds were 49.86±6.97 and 49.29±6.56 g for the left and right hind limbs, respectively (see Figure 3B). On day 4 the mean withdrawal thresholds were 53.43±4.88 and 48.71±6.11 g for the left and right hind paws, respectively, and 48.29±5.82 and 53.00±4.59 g for the left and right hind paws, respectively, on day 7.

3.2. Paw withdrawal threshold in cuff-implanted rats

One group of neuropathic rats was tested on the wire mesh and the other was tested on the customized platform in a manner similar to that done in the control groups.

In the group tested on the wire mesh on day 0 (n=7), the minimal von Frey hair stimulus used to evoke paw withdrawal was 75.86±7.80 and 65.71±6.86 g for the left and right hind paws, respectively (see Figure 3A). These values were not significantly different compared to paw withdrawal values obtained from the control group tested on wire mesh. Four days after cuff implantation, the mean withdrawal threshold of the cuff-implanted hind paw was decreased to 10.56±2.16 g ($P < 0.05$) and to 12.80±2.21 g ($P < 0.05$) on day 7. In the same group of rats the mean withdrawal threshold of the contralateral hind paw was 63.17±11.39 g on day 4 and 65.00±10.28 g on day 7. The withdrawal thresholds obtained from the right hind paw on days 4 and 7 were not significantly different compared to the right paw withdrawal thresholds on day 0 (see Figure 3A).

In the group of cuff-implanted rats tested on the customized platform, the mean baseline withdrawal thresholds of the left and right hind paws were 53.86±5.14 and 53.86±5.14 g, respectively (see Figure 3B). These values were not significantly different compared to the mean paw withdrawal thresholds of the control group of rats. After cuff implantation, the mean withdrawal threshold of the left hind paw was decreased to 3.83±0.73 g ($P < 0.05$) on day 4 and decreased further on day 7 to 2.45±0.65 g ($P < 0.05$). However, unlike the group of neuropathic rats tested on the wire mesh, the mean

right hind paw withdrawal threshold was decreased on day 4 (36.00±7.55 g, $P < 0.05$) and on day 7 (26.09±5.86 g, $P < 0.05$; see Figure 3B).

3.3. Effects of testing surface on rat behaviour

3.3.1. Customized platform

Before surgery, upon being placed in the testing chamber, rats from each of the groups engaged in general exploration. However, habituation occurred always before the end of the prescribed 5 min acclimatization time period. Less frequent exploratory behaviour in the control and cuff-implanted rats occurred after the initial exposure to testing on day 0. Vocalization did not occur in response to application of any of the von Frey hairs.

Behavioural differences between control and cuff-implanted rats became apparent as soon as 1 day after surgery. On days 1 to 7 after surgery the cuff-implanted rats spontaneously lifted the ipsilateral hind paw. Teeth-chattering also occurred during this time. Furthermore, after cuff implantation, the rats held up the ipsilateral hind limb longer and higher while walking than rats in the control group. In fact, while walking, the cuff-implanted rats often hopped on the contralateral limb. Furthermore, the low threshold mechanical stimuli applied to the hind paws in this group of rats sometimes induced abrupt paw withdrawal with the paw remaining elevated for 10 to even 30 s which was followed by licking and shaking of the stimulated paw. This was not observed in control rats. Another interesting abnormal behaviour common to all cuff-implanted rats was their

tendency to curve or "cup" the ipsilateral hind paw. In addition, many of these rats showed ventral eversion of the cuff-implanted hind paw as well as calloused skin on the heel and side of the cuff-implanted paw.

3.3.2. Wire mesh

The physical characteristics of the rats described above, specifically the effects of nerve injury on the posture of the paw as well as the prolonged elevation of the nerve-injured paw following stimulation were also observed in cuff-implanted rats tested on the wire mesh.

Rats tested on the wire mesh engaged in general exploratory behaviour on day 0. However, unlike the rats on the customized platform, rats tested on the wire mesh often required up to 10 to 15 min to become habituated. Consistent paw withdrawal thresholds during the second or third trials were less easily obtained in cuff-implanted and control rats tested on the wire mesh. In addition, although it was not specifically quantified, it was noted that while the control and cuff-implanted rats were being tested on the wire mesh little if any time was spent grooming compared to rats tested on the customized platform.

Interestingly, a few of the control rats but many of the cuff-implanted rats shifted their weight continuously from one hind paw to the other. Cuff-implanted rats supported their weight predominantly on the heels of the paw rather than on the plantar surface.

Furthermore, it was also observed that the experimenter's hand approaching the wire mesh from below in order to apply the von Frey hairs often provoked the rat to look

towards the hand.

Discussion

The purpose of this study is to report a customized testing platform, as an alternative to wire mesh, to measure reliably and accurately the threshold of von Frey hair-induced paw withdrawal reflex in the rat. It consists of an opaque flat-surfaced plastic platform which contains small holes through which von Frey hairs are easily inserted and applied to the plantar surface of the rat paw. It is well suited to support rats while they are being tested and, in conjunction with von Frey hairs, it yields accurate characterization of changes in paw withdrawal threshold in both control and nerve-injured rats over time. Furthermore, the platform does not interfere with buckling of the von Frey hair while it is applied to the hind paw.

It is shown in this study that in control rats tested on the customized platform the mean paw withdrawal thresholds of the left or right hind paws remain predominantly unchanged on different days of testing. However, in similar rats tested on wire mesh, the mean withdrawal thresholds are less consistent. Furthermore, in the cuff-implanted rats tested on the customized platform, the mean withdrawal threshold ipsilateral to nerve constriction decreases to values that are less than the values obtained from similar rats tested on the wire mesh. In addition, a contralateral decrease in withdrawal threshold is shown which is not observed in cuff-implanted rats tested on the wire mesh. The data suggest that properties of the surface on which the paw withdrawal reflex is measured using von Frey hairs may be a source of variability in the withdrawal threshold.

4.1. von Frey hair-induced hind paw withdrawal in control rats: wire mesh vs. customized platform

Conventionally in studies where von Frey hairs are applied to the plantar surface of the hind paw in rats, a wire mesh platform is used. The main purpose that it serves is to maintain the rat at an elevated level such that the plantar surface of a paw is exposed and easily accessible to mechanical stimulation using the hairs.

In the present study, the hind paw withdrawal threshold remained constant in rats tested on the customized platform while the threshold was less consistent in rats tested on the wire mesh. With all other experimental parameters equal between the two groups with the exception of the testing surface, it is suggested that the wire mesh has an effect on the paw withdrawal threshold. This agrees with the recent finding that exposure to wire mesh may induce hind paw tactile hypersensitivity (Mizisin et al. 1998).

An important consideration of the wire mesh surface is that by virtue of its structure, constant focused mechanical stimulation is exerted at the points of contact between the individual wires and cutaneous receptive field of the plantar surface of the paws. This may be particularly critical when one hind paw is elevated in response to von Frey hair stimulation because the animal shifts its weight to the contralateral paw which therefore endures an increase in sustained mechanical stimulation due to its contact with the individual wires of the mesh surface. Recently, it has been reported that weight bearing of hind limbs is an important confounding factor in the assessment of the effects of tactile stimulation of the hind paws in rats (Schött et al. 1994; Kauppila et al. 1998). Thus, in

the present study, the paw withdrawal threshold may not be entirely dependent on the hair stimulus, but also on tactile hyperesthesia and perhaps on the level of stress of the rat while supporting its weight on individual wires. It is not unreasonable to speculate that any discomfort or stress due to the weight bearing of the hind paws on the wire mesh may incite changes in sensory processing.

Furthermore, as a result of the wire mesh testing surface being irregular, different aspects of the plantar surface of the hind paws support the rat on the wire mesh at any given time while the rat is being tested. This is critical because the testing surface to which the paw is returned after withdrawal is inevitably different. Therefore, the wire mesh may incorporate inconsistent testing conditions and consequently have an effect on paw withdrawal threshold throughout the time course of the experiment.

4.2. von Frey hair-induced hind paw withdrawal in cuff-implanted rats: wire mesh vs. customized platform

It is evident in this study that the hind paw ipsilateral to the nerve injury is very sensitive to mechanical stimulation as application of the lower force von Frey hairs is shown to be effective in evoking paw withdrawal. This is observed not only in nerve-injured rats tested on the customized platform or the wire mesh in the present study but is supported by numerous other studies using wire mesh, including those using the Bennett and Xie (1988) (Meyerson et al. 1995; Cui et al. 1996, 1997; Ren et al. 1996; Eaton et al. 1997; Kim et al. 1997; Ramer and Bisby 1997; Ramer et al. 1997; Sawin et al. 1997),

Kim and Chung (1992) (Kim and Chung 1991; Chung et al. 1993, 1996, 1997; Sheen and Chung 1993; Qian et al. 1996; Lee and Chung 1996; Na et al. 1996; Yoon et al. 1996; Kim et al. 1997) and the Seltzer *et al.* (1990) (Meyerson et al. 1995; Ren et al. 1996; Stiller et al. 1996; Kim et al. 1997), models of neuropathic pain. Interestingly, it is reported that while there is a hypersensitivity to the effect of von Frey hair stimulation ipsilateral to the nerve injury, there appears to be a lack of effect of nerve injury on the contralateral paw withdrawal threshold. One obvious interpretation is that there are no physiological effects of the nerve injury on contralateral sensory mechanisms. However, due to the physical characteristics of the wire mesh, as detailed above, it may be difficult to assess any change in sensory mechanisms of the hind paw contralateral to the nerve injury. For example, any physiological change in sensory processing may remain unnoticed as the rat may be reluctant to elevate the contralateral limb in response to even a large von Frey hair stimulus because any weight supported by the ipsilateral nerve-injured limb may be unbearable. This is not unreasonable as discomfort would be exacerbated in rats already hypersensitive to tactile stimulation. Therefore, increased mechanical force via application of greater force hairs may be required to induce withdrawal of the contralateral paw and it may therefore appear that there is no effect of nerve-injury in mechanisms of contralateral sensory processing.

This lack of effect on the contralateral hind paw may be expected to persist as long as the nerve-injured paw shows decreased paw withdrawal threshold, which is consistent with rats tested on wire mesh. However, in rats tested on the customized platform, this

is not the case. The mean withdrawal thresholds of the contralateral hind paw decreased significantly over time as revealed on days 4 and 7 after cuff implantation. Sensitization of sensory mechanisms is not the case as control rats tested on the customized platform exhibit a stable level of withdrawal threshold at the same testing times. Therefore, the data suggest that rats may be more inclined to support their weight on the ipsilateral nerve-injured paw while on the customized platform rather than on the wire mesh. Furthermore, changes in sensory mechanisms governing the contralateral hind paw may in fact develop which result in a significant decrease in the contralateral withdrawal threshold.

Further evidence suggesting an effect of the testing surface on sensory processing is that rats tested on the wire mesh required consistently more habituation time than rats tested on the customized platform. Rats examined on the customized platform were less restless and appeared to be well habituated. It is difficult to account unequivocally for the reasons for the differences in habituation times as well as the general behaviour of the rats during the testing period. Nonetheless, the increased habituation time concurs with the suggestion that wire mesh may exacerbate discomfort especially in animals with particularly sensitive sensory mechanisms such as in nerve-injured rats (Bennett and Xie 1988).

4.3. Other possible stress-associated features of the wire mesh testing surface

In rats tested on wire mesh, stress or anxiety as a consequence of the mesh being elevated and see-through may also influence the paw withdrawal threshold. Using the

elevated plus-maze which is an animal test of anxiety, it has been shown that rats experience anxiety derived from elevation (File 1993). Further evidence that elevation promotes anxiety in rats comes from studies in which anxiolytic drugs such as chlordiazepoxide, diazepam, stracozolate or clonidine reduced elevation-induced stress (Handley and Mithani 1984; Pellow et al. 1985; Pellow and File 1986). Not only does there seem to be no habituation of the anxiogenic response with repeated exposure to the *elevated plus-maze* (Pellow et al. 1985), an increased anxiogenic response may occur with repeated elevation (Rodgers et al. 1992). In studies using wire mesh to test von Frey hairs, any impact that elevation may have on sensory mechanisms and consequently on the paw withdrawal threshold is not known. However, based on the data obtained in both the control and cuff-implanted rats tested on the customized platform, it is suggested here that the opaque property of this testing surface may be important in reducing any influence that elevation-induced stress may have on sensory processing.

It is worthwhile noting that the effect of the experimenter carefully raising the von Frey hair from below the wire mesh to the plantar surface may evoke behavioural responses in rats including movement and alertness which are responses unrelated to application of the von Frey hair. For example, when the experimenter's hand, holding the von Frey hair, approached the mesh floor from below, in many cases the rats moved their heads towards the hand. This was not observed in rats tested using the customized platform. If anything, the rats sometimes moved their heads towards the origin of the von Frey hair stimulus on the surface of the platform. Therefore, it is suggested that the

opaque property of the customized platform may also deter any visually introduced cues to the rats thus avoiding inadvertent conditioning of the withdrawal response to von Frey hair application.

Recently, alternative methods to wire mesh to test von Frey hairs have been used. For example, a coated wire mesh to test mice is reported (Chaplan et al. 1997), as well as perforated metal sheets (Tal and Bennett 1994; Xiao and Bennett 1995; Mao et al. 1997), or perforated floor (Xiao and Bennett 1994), to apply von Frey hairs. Yet, to date, no extensive examination of control or nerve-injured rats has been done using these devices to uncover the effects of nerve injury on the ipsilateral and contralateral sensory mechanisms over time.

Conclusion

It is revealed in this study that physical attributes of the customized platform provide accurate and reliable measurement of paw withdrawal threshold using von Frey hairs. Its smooth flat surface eliminates focused points that exert force to the plantar surface of the rats' paws which is unavoidable with the wire mesh. In this respect, regardless of the position or placement of the hind paw the customized platform provides a surface which is equivalent to each paw. We suggest that while von Frey hairs can be applied using wire mesh, a flat surface such as the customized platform is a suitable alternative as it provides improved assessment of the changes that may occur bilaterally in sensory processing in animal models of chronic pain.

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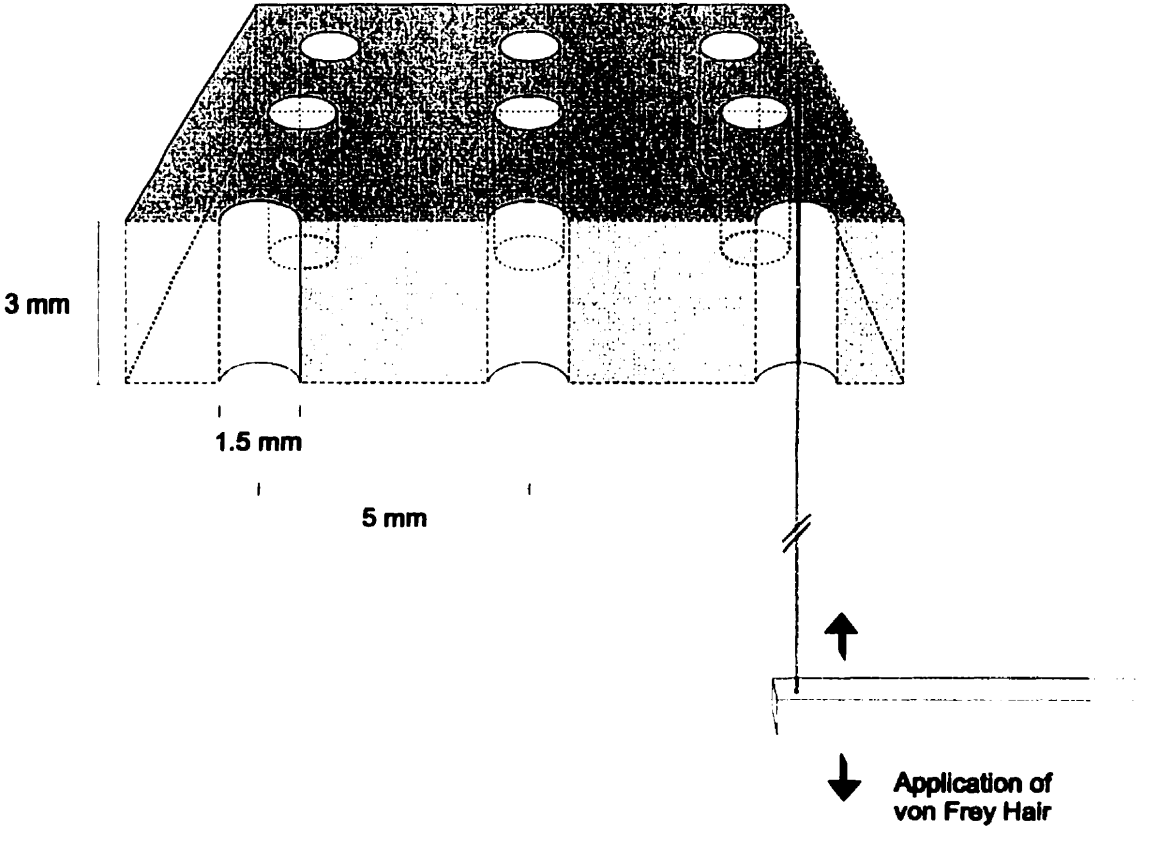

3 mm
1.5 mm
5 mm
Application of
von Frey Hair

Figure 1. Three-dimensional schematic diagram of the customized von Frey hair testing platform showing magnified aspect of one corner. The platform is a 3 mm thick slightly opaque plastic platform (30×30 cm), containing 1.5 mm diameter holes. The holes are 5 mm apart and cover symmetrically the entire surface of the platform. Application of a von Frey hair through a cross-section of one of the holes is demonstrated. The von Frey hair is truncated in this view in order to be shown in the schematic representation of the platform.

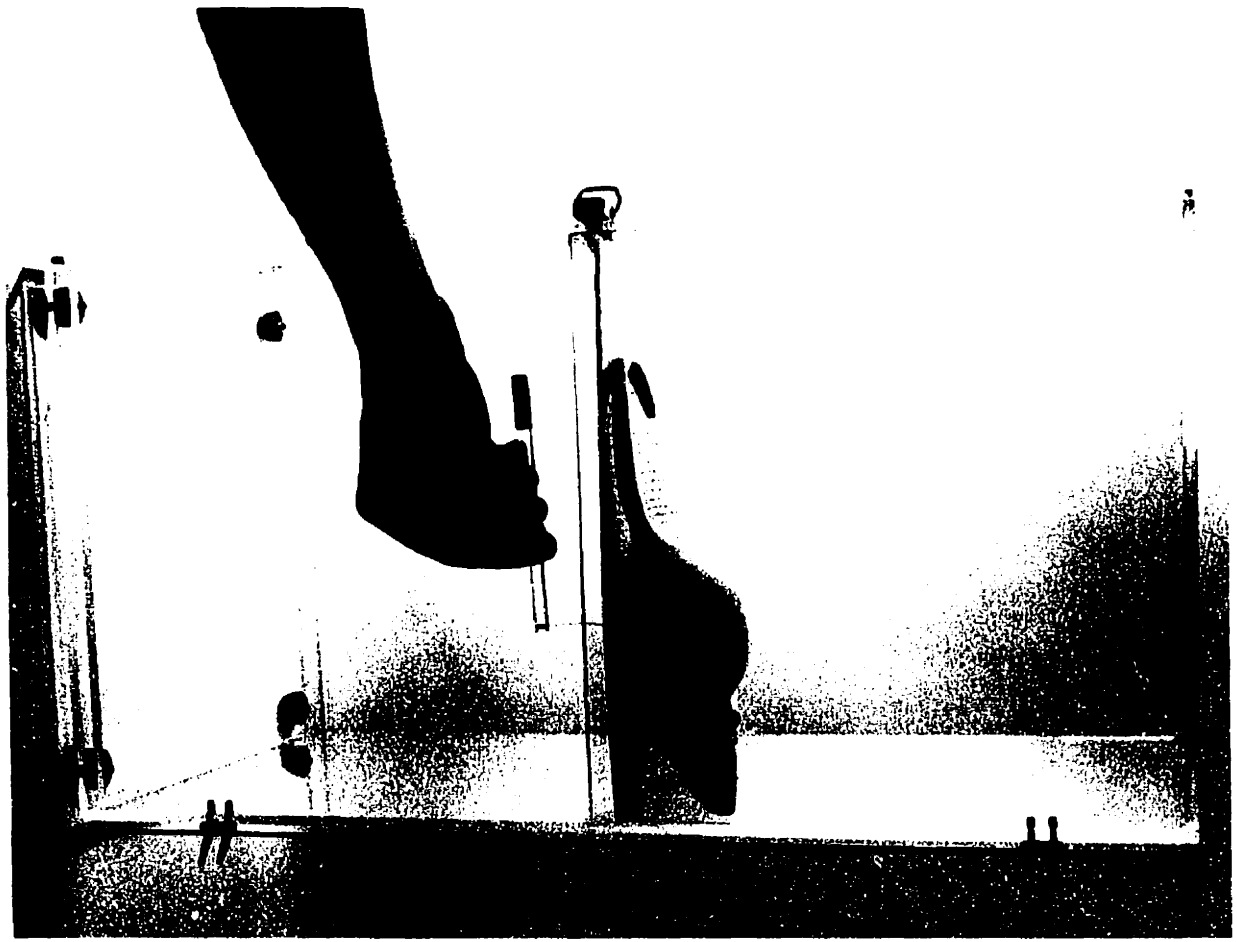
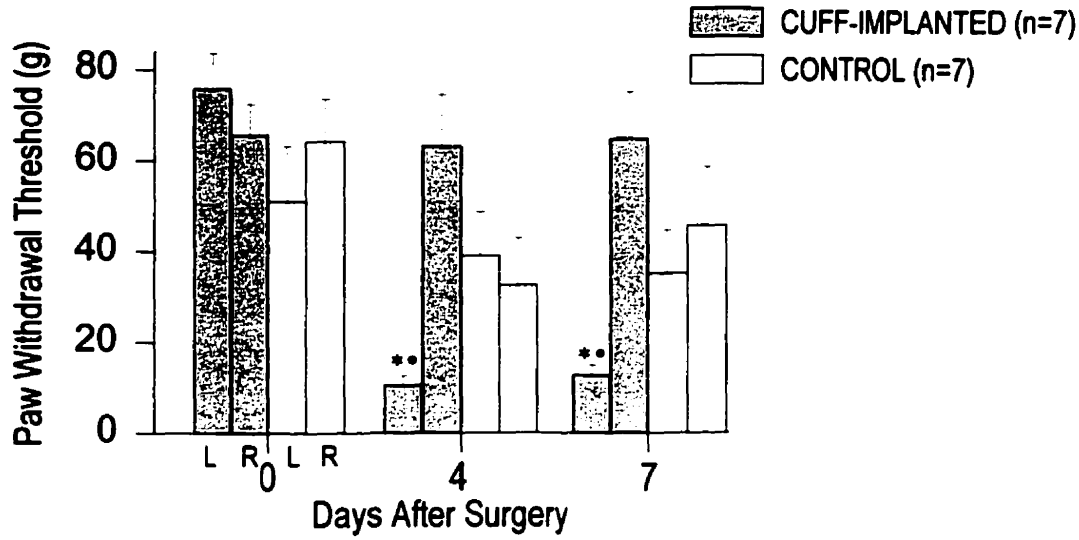


Figure 2. Photograph showing a control rat on the customized platform (30×30 cm) in the testing chamber (30×30×30 cm). Application of a von Frey hair from below the platform to one hind paw of the rat is shown. Notice that the rat is standing quietly on all four paws and is unaware of the experimenter's hand.

A WIRE MESH



B CUSTOMIZED PLATFORM

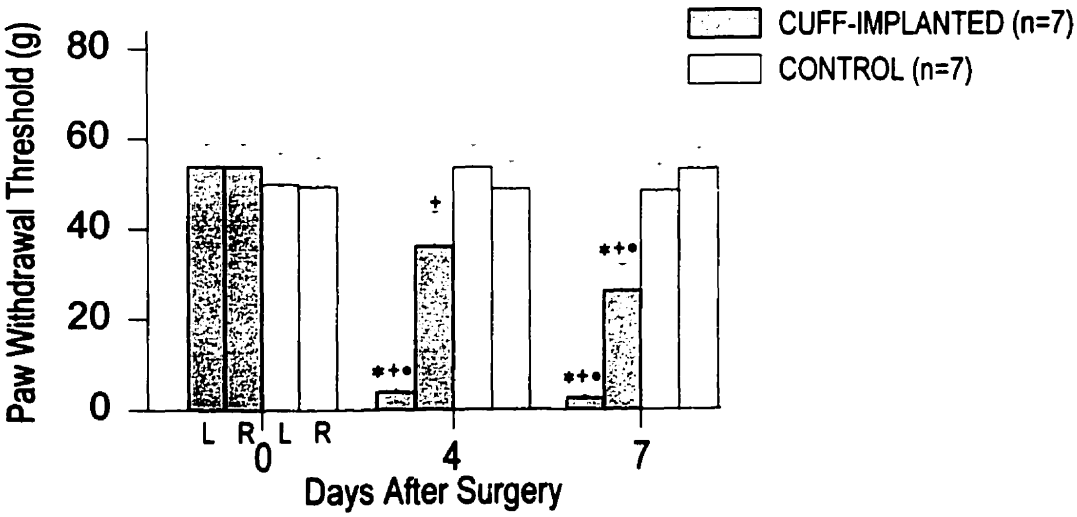


Figure 3. Histograms illustrating the effects of the wire mesh (A), or the customized platform testing surface (B) on the mean paw withdrawal thresholds of the left (ipsilateral; cuff implanted on the left sciatic nerve) and right (contralateral) hind paws of control rats (clear bars) and cuff-implanted rats (hatched bars). The vertical axis represents the paw withdrawal threshold using von Frey hairs measured in grams (g). The horizontal axis represents the day of testing at baseline (day 0) and on days 4 and 7. Notice that the mean baseline withdrawal threshold values of the ipsilateral and contralateral hind paws of rats tested on the customized platform are lower than the values obtained from rats tested on wire mesh. In addition, control rats tested on the customized platform show stable mean withdrawal thresholds at days 0, 4 and 7 which is not the case in control rats examined on the wire mesh. Furthermore, on days 4 and 7, not only is the mean withdrawal threshold of the nerve-injured hind paw lower in rats tested on the customized platform compared to that of rats tested on wire mesh, the mean withdrawal threshold of the contralateral hind paw on day 7 is also lower in rats tested on the customized platform compared to baseline and the wire mesh, respectively. (* $P < 0.05$ vs. control withdrawal threshold, + $P < 0.05$ vs. withdrawal threshold measured on wire mesh and • $P < 0.05$ vs. baseline withdrawal threshold)

Unifying Statement

The paw withdrawal reflex experiments in Chapter 4 demonstrate that physical attributes of the customized platform provide accurate and reliable measurement of paw withdrawal threshold in the von Frey hair test. It is suggested that while von Frey hairs can be applied using conventional wire mesh, a flat surface such as the customized platform may provide improved assessment of changes in sensory processing in animal models of chronic pain. Importantly, alterations in the hind paw withdrawal threshold associated with peripheral neuropathy, by placing a 2 mm polyethylene cuff around the sciatic nerve, can be characterized using this novel platform.

In Chapter 5 we undertook to expand our studies on the transmission of sensory information in normal animals to a rat model of chronic pain. Ultimately, the objective was to investigate the effects of peripheral neuropathy on spinal dorsal horn neuronal activity. However, one of the main concerns was the optimal time to do testing after peripheral neuropathy was begun. Therefore, Chapter 5 is concerned with determining the time course and quantifying the tactile hypersensitivity in the von Frey hair test after induction of peripheral neuropathy in the rat. The effect of surgery alone without cuff implantation on the hind paw withdrawal threshold was also examined.

Chapter 5

Nerve Constriction in the Rat: Model of Neuropathic, Surgical and Central Pain

Abstract

In preparation for a series of electrophysiological experiments in a model of neuropathic pain, the present spinal reflex study was done to determine the optimal time after sciatic nerve constriction in the rat for tactile allodynia and to determine also the appropriate 'control' for the nerve constriction model. Therefore, this study focused on the magnitude and time course of change in paw withdrawal threshold following unilateral sciatic nerve constriction in the rat. Male Sprague Dawley rats (375-425g) were used. Nerve constriction was done by placing a 2 mm polyethylene cuff (PE-90) around the left sciatic nerve (n = 8). A second group of rats (n = 8) received unilateral sham surgery and a third group (n = 8) was unoperated. The ipsi- and contralateral hind paw withdrawal thresholds in each of the 3 groups were measured using von Frey hairs. In unoperated rats, the withdrawal threshold of each of the hind paws remained unchanged at approximately 50 g throughout the entire time course of the study, which lasted 145 days. However, in cuff-implanted rats, the withdrawal threshold of the nerve-injured hind paw decreased as soon as 1 day after surgery, reached as low as 1 to 2 g by 5 days and remained low throughout the test period. Threshold in sham-operated rats showed a bilateral decrease starting on days 1-3, which stabilized at about 30 g until about day 40, after which values returned gradually toward the unoperated withdrawal thresholds. In nerve-constricted rats the withdrawal threshold of the hind paw contralateral to the cuff followed the same change seen in sham-operated rats until about day 37, after which the withdrawal threshold matched that of the cuff-implanted hind paw. The data show that the

cuff-induced sciatic nerve constriction produces a sustained hypersensitivity to normally innocuous tactile sensory input and that a relatively constant ipsilateral mechanical hyperalgesia can be found from days 5 to 27. It is also demonstrated that the contralateral hind paw and either hind paw in sham-operated rats are inappropriate as 'controls' in which there may be expected to be no change from the baseline withdrawal threshold. The data in this study suggest that three distinct types of allodynia are expressed. Ipsilateral allodynia may be representative of a model of neuropathic pain. The contralateral allodynia may be a model of central pain, arising from changes in central and/or peripheral sensory processing. Allodynia in sham-operated rats was also expressed bilaterally and may be a model of long-term postoperative pain.

Introduction

Clinically, many different nerve injury or neuropathic conditions exist. Some are diagnosed as causalgia, postherpetic neuralgia or reflex sympathetic dystrophy to mention only a few. In some cases, patients afflicted with one of these neuropathic syndromes express continuous intense pain which is exacerbated by stimuli that are normally innocuous such as light touch (Price et al. 1989; Gracely et al. 1992). Recently, animal models have been developed in an attempt to mimic the effects of nerve injury clinically and thus provide a better understanding of the physiological mechanisms involved in producing and maintaining neuropathic pain. We undertook to expand our studies on the chemical basis of synaptic transmission in spinal sensory mechanisms in normal animals to a nerve constriction model of neuropathic pain. However, during the selection process of a suitable model a number of questions were encountered which were not satisfactorily answered in the literature. These centred mainly on the optimal time to do testing after the nerve constriction was applied and what was appropriate to use as a control.

The first widely accepted animal model of neuropathy was developed by Bennett and Xie (1988), in which loose ligatures were placed around a sciatic nerve in the rat. This rendered an ipsilateral increase in sensitivity to noxious and innocuous mechanical stimuli and a favoring of the respective paw. Since that report, three additional principal models have been reported, by Seltzer et al. (1990), in which only part of the sciatic nerve is tightly ligated, by Kim and Chung (1992) in which tight ligatures are placed around the L5 and 6 spinal nerves, and by Mosconi and Kruger in which short cuffs of polyethylene

tubing are inserted around the sciatic nerve (Mosconi and Kruger 1996). This most recent model provides presumably little if any variation in the magnitude of constriction of the sensory nerve between rats of the same size and weight. As our long-term studies are to involve both reflex testing in one project and electrophysiological recording in another, it was important to minimize variation in the degree of nerve constriction and the Mosconi and Kruger model was considered appropriate to accomplish this.

In this study, a 2 mm polyethylene cuff was used to constrict the left common sciatic nerve and the effect of this on the withdrawal threshold of the ipsilateral hind paw was examined using von Frey hairs. Specifically, we determined changes in the withdrawal threshold from the presurgery baseline level and the time course of any such changes. To establish the appropriate control, withdrawal thresholds were also measured in the contralateral hind limb, in both hind limbs of sham-operated rats as well as in both hind limbs of unoperated rats.

Materials and Methods

2.1. Animals

Experiments were done using adult, male Sprague-Dawley rats (375-425g) from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). They were housed in plastic cages containing wood chip bedding (Hardwood Laboratory Bedding, Northeastern Products Corp., Warrensburg, N.Y.) and maintained on a 12:12 h light:dark cycle (lights on at 07:00 h) with access to food and water *ad libitum*. Experiments were conducted during the light component of the cycle. Only two rats from the same testing group (i.e. unoperated, sham-operated or cuff-implanted) were together in any cage. Guidelines in *The Care and Use of Experimental Animals* by the Canadian Council on Animal Care (Vols. I and II) were strictly followed. All experiments were approved by the *McGill University Animal Care Committee*.

2.2. Surgical procedure

All surgery was done under aseptic conditions. Under Na-pentobarbital anesthesia (50 mg/kg, i.p., Abbott Laboratories, Limited, Dorval, Montreal, Quebec), the left common sciatic nerve (n=8) was exposed by blunt dissection through the biceps femoris muscle. The nerve was isolated from surrounding connective tissue using glass probes. Approximately 4 to 6 mm of the nerve was elevated minimally and held in place using a sterilized glass probe in order to place on the nerve a 2 mm section of split PE-90

polyethylene tubing (Intramedic PE-90, Fisher Scientific Ltd., Montreal, Quebec). The nerve was kept moist using sterile saline at 37.5°C throughout exposure, which lasted approximately for 10 s. The muscle layer was closed using 3-O silk suture thread (Ethicon Inc., Montreal, Quebec) and the shaved skin layer was closed using 3 stainless steel suture clips (Fine Science Tools, Inc., North Vancouver, British Columbia). Nitrofurazone ointment 0.2% (Univet Pharmaceuticals Ltd., Milton, Ontario) was placed on the skin suture to control any infection and rats were then allowed to recover for 24 h before testing. Sham-operated rats (n=8) underwent the same surgical procedure as described above but without implantation of the cuff. During the post-operative period the animals were monitored several times a day. Particular attention was paid to general behavior and appearance. Body weight was measured once per day.

To examine any effect of Na-pentobarbital anesthesia on responses of rats on the paw withdrawal threshold, 4 of the 8 unoperated control rats were anesthetized as described above, at the time of surgery of the cuff-implanted and the sham-operated rats. The remaining 4 untreated rats were not taken from their cages until testing was begun.

2.3. Measurement of mechanical hind paw withdrawal threshold

The hind paw withdrawal threshold was determined using von Frey hairs and was expressed in grams. Ten hairs ranging from 0.23 to 59.0 g were used. The value of each hair was confirmed weekly by measuring the magnitude in grams exerted by the hair when applied to a Mettler AE 100 electronic balance. This was done because it was determined

that slight fluctuation in the value of a hair may occur after use. If this was the case for a particular hair, the new value in grams, determined using the electronic balance, was used in determining the paw withdrawal threshold.

Application of the von Frey hairs was done using a platform designed and constructed specifically for von Frey hair testing (Pitcher et al. 1999). Briefly described, the platform was made of plexiglass 3 mm thick. It was slightly opaque in appearance and contained 1.5 mm diameter holes in perpendicular rows, 5 mm apart throughout the entire area of the platform. For testing, a rat was placed on this platform which was fixed in a transparent plexiglass observation chamber (30×30×30 cm).

Testing was blind such that the experimenter was not aware of the kind of rat being tested, i.e. unoperated, sham-operated or cuff-implanted. The protocol used in this study was a variation of that described by Takaishi et al. (1996). A testing session for a particular rat began after 5 min of habituation or as soon as the rat stopped exploring and appeared acclimatized to the testing environment. The series of von Frey hairs was applied from below the platform to the plantar surface of the left hind paw in ascending order beginning with the lowest rated hair (0.23 g). Application was to the central region of the plantar surface avoiding the foot pads. A particular hair was applied until buckling of the hair occurred. This was maintained for approximately 2 s. The hair was applied only when the rat was stationary and standing on all four paws. A withdrawal response was considered valid only if the hind paw was completely removed from the platform. Although infrequent, if a rat walked immediately after application of a hair instead of

simply lifting the paw, the hair was reapplied. On rare occasions, the hind paw only flinched after a single application; as the hind paw was not lifted from the platform, this was not considered a withdrawal response.

A trial consisted of application of a von Frey hair to the hind paw 5 times at 5 s intervals or as soon as the hind paw was placed appropriately on the platform after 5 s. If withdrawal did not occur during 5 applications of a particular hair, the next larger hair in the series was applied in a similar manner. When the hind paw was withdrawn from a particular hair either 4 or 5 times out of the 5 applications, the value of that hair in grams was considered to be the withdrawal threshold.

Once the threshold was determined for the left hind paw, the same testing procedure was repeated on the right hind paw after 5 min. Second and third testing trials were run for the left and right hind paws, respectively. If the withdrawal threshold in the second or third trial did not match the withdrawal threshold of the previous testing trial(s) in a given hind paw, the next larger hair in the series was tested. This was done until the withdrawal thresholds in 3 successive trials matched. Only hind paw withdrawal thresholds that remained consistent in the second and third successive trials in unoperated, sham-operated or cuff-implanted rats were used in the data analysis. The total testing time for each rat usually lasted 35 to 40 min.

The baseline withdrawal thresholds of each of the hind paws to von Frey hair application were determined for each rat prior to surgical manipulation (day 0). Testing commenced the following day (day 1). Subsequent testing occurred each day for 2 weeks,

then every 2 to 3 days until day 32, every 5 days until day 47, every 7 days until day 61 and then every 14 days until day 145, the last day of testing.

2.4. Statistical analysis

Hind paw withdrawal thresholds were analyzed using Kruskal-Wallis one way ANOVA on ranks. Student-Newman-Keuls test was used for post-hoc comparisons between or within groups of animals following ANOVA. Hind paw withdrawal threshold values between different groups of rats or within the same group at different time points were considered significantly different with a P value <0.05.

Results

3.1. Effects of cuff-implantation or sham surgery on rat behavior

Before surgery, upon being placed in the testing chamber, rats from the 3 groups engaged in general exploration. However, habituation occurred always before or at most shortly after the end of the prescribed 5 min acclimatization time period. During testing vocalization did not occur in response to application of any of the von Frey hairs nor was autotomy observed at any time in rats in any of the 3 groups. Furthermore, throughout the study, rats in each of the groups were well groomed. General appearance, weight as well as the stools were normal throughout the study. However, during approximately the second and third weeks of testing, the claws of the cuff-implanted hind paw were curved and noticeably longer than normal, which is consistent with previous reports (Bennett and Xie 1988). In some of the cuff-implanted rats, elongation of the claws was observed bilaterally.

Behavioral differences between the unoperated, sham-operated and cuff-implanted rats were also apparent. For example, between days 1 and 30 the cuff-implanted rats spontaneously lifted the ipsilateral hind paw in their housing cages as well as in the testing chamber. 'Teeth-chattering' also occurred during this time in these rats. Spontaneous lifting of the injured hind paw was less frequently displayed in sham-operated rats and not at all in unoperated rats. Furthermore, after surgery the cuff-implanted rats held up the ipsilateral hind limb longer and higher while walking; this was not observed in rats in the unoperated or the sham-operated groups. In fact, while walking, the cuff-implanted

animals often hopped on the contralateral limb. This was particularly noticeable during the first 2 weeks of the study. Application of even the low threshold von Frey hairs to the hind paws in this group also evoked abrupt paw withdrawal, with the paw remaining elevated for 10 to 30 s in some cases. This was often followed by licking and shaking of the stimulated paw which was not observed in the unoperated nor in the sham-operated rats. Interestingly, common to all of the cuff-implanted rats was their tendency to curve or 'cup' the ipsilateral hind paw. Some of these rats even demonstrated 'ventralflexion' of the toes of the ipsilateral hind paw and developed calloused skin on the heel and side of the paw which have also been reported in other models of neuropathy in the rat (Kim and Chung 1992; Na et al. 1996).

Postmortem examination of each of the rats in the cuff-implanted group showed that each implanted cuff continued to constrict the common sciatic nerve. It is important to note that there was fibrous tissue development on the sciatic nerve extending 2 to 3 mm on each side of the cuff. In a few of the sham-operated rats, examination revealed some fibrous tissue. However, this was substantially less than that observed in the cuff-implanted rats.

3.2. Baseline hind paw withdrawal threshold in unoperated, sham-operated and cuff-implanted rats

Figure 1A shows that on day 0, before cuff implantation or sham surgery, the hind paw withdrawal threshold in rats in the unoperated (44.63 ± 6.39 g and 43.63 ± 5.56 g for

the left and right hind paws, respectively), sham-operated (51.88±4.30 g for each of the hind paws) and cuff-implanted (53.63±4.46 g for each of the hind paws) groups were not statistically different.

3.3. Unoperated rats

The withdrawal thresholds of each of the hind paws obtained from the 4 rats that were given Na-pentobarbital on day 0 were not significantly different compared to the paw withdrawal thresholds obtained from the 4 unoperated rats that did not receive the anesthetic. Figure 1A, B and C show that left and right hind paw withdrawal threshold values of the 8 rats as one group were not statistically different at any of the testing days compared to the baseline paw withdrawal threshold at day 0.

3.4. Sham-operated rats

On day 1, the mean withdrawal threshold of the left hind paw, i.e. ipsilateral to the surgery, was at baseline. However, by day 3 it had decreased to 28.51±6.31 g ($P < 0.05$ vs. left hind paw withdrawal threshold of the unoperated group) and remained approximately at this level up to day 42 (26.96±5.03 g, $P < 0.05$ vs. unoperated). Between days 9 and 23, the mean threshold showed some fluctuation possibly due to day to day handling of the rats. However, between days 47 and 145 the withdrawal thresholds were not significantly from those of the unoperated group (see Figure 1B and C).

The mean withdrawal threshold of the right hind paw, i.e. contralateral to the surgery, decreased to 30.45±4.85 g on day 1 ($P < 0.05$ vs. right hind paw withdrawal threshold of the unoperated group) and to 14.48±2.21 g on day 3 ($P < 0.05$ vs. unoperated). Figure 1A and B show that by day 5 the mean withdrawal threshold was 31.06±7.16 g and remained approximately at this level up to day 37 (28.68±5.74 g, $P < 0.05$ vs. unoperated). From day 42 to the end of the study, the right hind paw withdrawal thresholds of the sham-operated and the unoperated groups were not significantly different (see Figure 1B and C).

3.5. Cuff-implanted rats

The mean withdrawal threshold of the left hind paw, i.e. ipsilateral to the cuff, decreased shortly after surgery (see Figure 1A). For example, it was 30.38±8.58 g on day 1 ($P < 0.05$ vs. hind paw withdrawal thresholds of the unoperated and the sham-operated groups), 5.51±1.57 g on day 3 ($P < 0.05$ vs. unoperated, sham-operated and the contralateral), 2.95±0.77 g on day 5 ($P < 0.05$ vs. unoperated, sham-operated and contralateral), and remained approximately at this level up to day 23 (3.78±0.45 g, $P < 0.05$ vs. unoperated, sham-operated and contralateral). Figure 1B and C show that from day 26 (6.15±1.36 g, $P < 0.05$ vs. unoperated, sham-operated and contralateral) to day 145 (26.71±5.67 g, $P < 0.05$ vs. unoperated and sham-operated) the mean threshold returned gradually towards pre-cuff values.

Figure 1A shows that the right hind paw withdrawal threshold became different

from that of the unoperated group only on day 6 (23.35±5.62 g, $P < 0.05$). On day 10 the mean withdrawal threshold was 15.21±2.38 g ($P < 0.05$ vs. unoperated) and remained approximately at this level until day 61 (15.58±2.43 g, $P < 0.05$ vs. unoperated and sham-operated). Figure 1C shows that between days 60 and 75, the mean threshold began recovering gradually and that on day 145 the mean right hind paw withdrawal threshold had reached 32.16±5.49 g ($P < 0.05$ vs. unoperated). Between days 37 and 145, Figs. 1B and 1C show that the withdrawal thresholds of each of the hind paws were not significantly different.

Discussion

It is demonstrated in this study that unilateral constriction of the left common sciatic nerve, using a variation of the Mosconi and Kruger (1996) technique, gives rise to a marked increase in sensitivity to normally innocuous tactile stimuli in both the nerve-injured as well as the intact contralateral hind paws. Although less in magnitude and duration, surgery alone without nerve constriction also produces a decrease in withdrawal threshold of each of the hind paws. As unoperated rats showed no change from the normal hind paw withdrawal threshold throughout the study, we interpret our findings to suggest that nerve constriction and even the effects of surgery alone establish sustained modifications in sensory processing which maintain long-lasting tactile allodynia ipsi- and contralateral to the nerve constriction or surgical injury.

4.1. No effect of testing protocol on hind paw withdrawal

In unoperated rats the withdrawal threshold of each of the hind paws to the effects of application of von Frey hairs remained unchanged throughout the entire time course of the study. This is interpreted to suggest that a number of important aspects of our testing protocoi do not have adverse effects on the paw withdrawal reflex. For example, three tests per hind paw per session yielded the same threshold. Secondly, there was no variation in the thresholds measured once a day every 1, 2, 3, 5, 7 or 14 days which demonstrates that the period between testing sessions also has no effect on the paw withdrawal threshold. Thirdly, there was no change in the threshold over 145 days which

reveals no effect of duration of testing on threshold. Thus, the period between testing rats, the duration of testing and the experimental set-up including the surface on which the rats stand while being tested with von Frey hairs provide measurement of stable paw withdrawal thresholds.

4.2. Hind paw withdrawal threshold ipsilateral to cuff implantation

The present study shows that constriction of the common sciatic nerve using a 2 mm polyethylene cuff produces a partial and long-lasting hypersensitivity to normally innocuous tactile stimuli. The onset of this effect occurs as soon as 1 day after cuff implantation and is sustained for at least 145 days with maximal hypersensitivity, measured at 1 to 2 g using von Frey hairs, occurring between 4 and 27 days. Although the cuff-implanted rats appeared well groomed and showed no autotomy or vocalization during testing, there was a persistent favoring and abrupt lifting and licking of the ipsilateral paw in response to application of von Frey hairs during the first 3 to 4 weeks of testing. Therefore, constriction of the sciatic nerve produces changes in sensory processing which are expressed as decreased withdrawal thresholds to normally innocuous stimuli and as nociceptive behavior such as paw favoring.

Increased sensitivity of the nerve-injured hind paw in the rat to von Frey hair application is observed in numerous other studies including those using the Bennett and Xie (1988) (Cui et al. 1996, 1997; Ramer and Bisby 1997; Ramer et al. 1997; Sawin et al. 1997), the Seltzer et al. (1990) (Meyerson et al. 1995; Ren et al. 1996; Stiller et al. 1996;

Kim et al. 1997) and the Kim and Chung (1992) (Kim and Chung 1991; Chung et al. 1993, 1996; Sheen and Chung 1993; Qian et al. 1996; Lee and Chung 1996; Na et al. 1996; Yoon et al. 1996; Chung et al. 1997) models of neuropathic pain. Some differences exist though. For example, using the Bennett and Xie technique (1988) to induce nerve injury, a maximum decrease in the withdrawal threshold to 5-10 g was reported and this decrease persisted up to the end of the study at 28 days (Ramer and Bisby 1997). Using both the Bennett and Xie (1988) and the Seltzer et al. (1990) techniques, it is reported that an increase in sensitivity of the nerve-injured hind paw to repetitive application of an 0.8 g von Frey hair occurs beginning at least 1 day after surgery and this effect persists for approximately 28 days with full recovery by 84 days (Kim et al. 1997). Using their own model, Seltzer et al. (1990) report a decrease in withdrawal threshold of the nerve injured paw to approximately 2 g as soon as 1 h after surgery and this effect persists up to the end of the study at 54 days. Also in the Seltzer model, a decrease in the withdrawal threshold to approximately 5 g is reported at 7 and 112 days after surgery (Takaishi et al. 1996). In studies in which the Kim and Chung model of neuropathic pain is used, the onset of the decrease in paw withdrawal threshold to repetitive application of an 0.8 g von Frey hair is reported to occur as soon as 1 (Chung et al. 1997) to 3 (Sheen and Chung 1993) days after surgery and persist for up to 56 (Chung et al. 1996; Na et al. 1996), 84 (Kim and Chung 1992) or 140 (Kim et al. 1997) days after surgery.

Therefore, peripheral nerve injury induces a decrease in paw withdrawal threshold which is early in onset and persists for at least several weeks. The differences in

magnitude and time course of the effects of nerve injury in the different studies as well as in our own are postulated to be due, at least in part, to the effects of the different kinds of nerve injury techniques on sensory input processing. In fact, in the chronic constriction injury model, there is suggestion that resorption of the chromic gut, which is used for ligatures in some studies, allows the process of recovery to occur (Coggeshall et al. 1993). In addition, there is accumulating evidence that the testing surface, specifically wire mesh, on which rats are tested with von Frey hairs, may produce discomfort for the rat (Kauppila et al. 1998; Mizisin et al. 1998). While many studies report using wire mesh to apply the von Frey hairs, very few indicate the dimensions of the mesh. Therefore, differences in the kinds of testing surfaces used may be another source of inconsistency between studies.

In the present study, the long-lasting tactile hypersensitivity in the cuff-implanted hind paw is considered an appropriate model of neuropathic pain and may be representative of the clinical effects of neuropathy (Price et al. 1989; Gracely et al. 1992).

4.3. Hind paw withdrawal threshold contralateral to cuff implantation

The decrease in the withdrawal threshold of the intact contralateral hind paw was gradual with the maximum mechanical sensitivity occurring approximately 37 days after surgery. It was not considered prior to this time because the sustained decrease in the withdrawal threshold before day 37 was statistically similar to the sham surgery-induced decrease in the contralateral hind paw withdrawal threshold. Therefore, prior to day 37, although we cannot entirely exclude any influence of nerve constriction on the withdrawal

threshold of the hind paw contralateral to nerve constriction, such an effect appears to be minimal during this time and is likely predominantly expressed between days 32 and 77 after cuff-implantation.

The bilateral decrease in the hind paw withdrawal threshold shown here is distinct from the almost invariable unilateral decrease reported in most studies to date. Seltzer et al. reported a decrease in the withdrawal threshold of the contralateral hind paw beginning 1 hour after surgery at the same von Frey hair threshold as that of the ipsilateral nerve-injured hind paw and this decrease persisted for at least 54 days (Seltzer et al. 1990). Takaishi et al. reported also a contralateral decrease in the threshold of hind paw withdrawal at 1 and 16 weeks after nerve injury (Takaishi et al. 1996). In one study, peripheral nerve constriction is also reported to decrease the hind paw withdrawal latency in the Randall Selitto test (Yu et al. 1996). Bilateral tactile hyperesthesia is also reported following peripheral nerve cryoneurolysis (DeLeo et al. 1994; Willenbring et al. 1994), carrageenan injection into one hind paw (Kissin et al. 1998), transection of the ventral ramus of the spinal nerve L5 (Blenk et al. 1997), and bilateral autotomy has been observed following dorsal rhizotomy (Lombard et al. 1979). Moreover, clinically, the pain associated with causalgia in humans is sometimes found to be manifested opposite to that of the nerve injury (Kozin et al. 1976; Procacci and Maresca 1987). Thus, in addition to changes in sensory processing ipsilateral to nerve constriction, our data endorse the notion that the physiological mechanisms governing sensory input via the intact contralateral hind paw are also subject to modulation.

Although, at present, we have no comprehensive answer to account for the contralateral effect observed in the present study, several potential explanations may provide some insight to how contralateral sensory input may be modified such that normally innocuous stimuli become painful. At present, both central and peripheral mechanisms must be considered. Perhaps one of the first indications that ipsilateral sensory input may influence sensory processing contralaterally was from Culberson et al. and Light and Perl who reported that the central terminals of primary afferent nerve fibers may project to the contralateral dorsal horn (Culberson et al. 1979; Light and Perl 1979). However, intriguingly, in the cat and in the opossum these projections were rarely seen in the lumbosacral level of the spinal cord compared to the cervical/brachial levels. More recently, bilateral expression of 'dark neurons', presumably the result of transynaptic degeneration subsequent to unilateral nerve constriction, is shown in the superficial laminae of the lumbar spinal dorsal horn (Sugimoto et al. 1990; Hama et al. 1994, 1996). Interestingly, GABAergic neurons which are normally found in the superficial laminae in the lumbar dorsal horn under normal conditions become decreased significantly ipsi- and contralaterally following peripheral nerve constriction (Ibuki et al. 1997). However, whether decreased inhibitory mechanisms centrally contribute to nociceptive behavior and a decreased withdrawal threshold bilaterally remains to be clarified. Persistent noxious thermal stimulation as well as the tonic effects of formalin injection are also reported to produce bilaterally in the spinal dorsal horn significant increases in membrane-associated protein kinase C, as assayed by quantitative autoradiography of the specific binding of [^{3}H]-phorbol-12,13-dibutyrate (Yashpal et al. 1995). Furthermore, unilateral formalin

injection is also reported to increase bilaterally in the spinal dorsal horn increased metabolic activity measured by [^{14}C]-2-deoxyglucose uptake (Porro et al. 1991; Aloisi et al. 1993). Constriction injury of the sciatic nerve is also shown to increase bilaterally both [^{3}H]-phorbol-12,13-dibutyrate binding and [^{14}C]-2-deoxyglucose uptake in the spinal dorsal horn (Mao et al. 1993). Surprisingly, constriction of the rat sciatic nerve is also reported to induce a vasodilator response in the contralateral hind paw (Kurvers et al. 1996) which is also referred to as 'reflex neurogenic inflammation' and is presumably mediated via connections across the spinal cord (Levine et al. 1985). Furthermore, unilateral nerve injury depresses mRNA levels of a specific Na^+ channel subunit, SCN10A, in the ipsilateral as well as the contralateral rat dorsal root ganglia (Oaklander and Belzberg 1997) but increases neuropeptide Y-like immunoreactivity (Rydh-Rinder et al. 1996). Unilateral Freund's complete adjuvant induces an ipsilateral and a delayed contralateral ankle arthritis and bilateral increases in preprotachykinin and calcitonin gene-related peptide in dorsal root ganglia (Donaldson et al. 1995). How these contralateral changes, peripheral and central, come about and influence sensory input is not yet evident. Nevertheless, the concept is put forward here that sensory processing contralateral to nerve injury is manifested via the effects of altered central and perhaps peripheral sensory mechanisms.

The data in this study show that unilateral nerve injury evokes an onset of decreased paw withdrawal threshold followed by a plateau phase and then recovery of the withdrawal thresholds in each of the hind paws. Given that the onset of the contralateral effect follows that of the ipsilateral effect and given also that the commencement of the recovery of the

contralateral effect is subsequent to that of the ipsilateral recovery (50 to 55 days later), it is conceivable that altered contralateral modulation of sensory input develops over time and may be mediated primarily by altered sensory processing occurring ipsilaterally. In other words, ipsilateral changes in sensory processing may render contralateral sensory mechanisms sensitive to modulation, the consequence of which results in a decrease in the threshold to the effects of innocuous tactile stimuli. This effect is considerable as the withdrawal threshold of the nerve-constricted and the contralateral hind paws are similarly decreased in the mid and end portions of the study. Thus, a model of central pain is proposed here in which persistent nociceptive input elicits changes in sensory processing at a central level, perhaps bilaterally such that the effects of ipsi- and even contralateral sensory input are altered.

Although bilateral effects of nerve injury as yet appear to be relatively uncommon, it must be considered that a contralateral effect of unilateral nerve injury may potentially dispute any rationale for sham surgery or 'control' testing done on the intact limb contralateral to the nerve-injured paw in the experimental animal. Furthermore, careful interpretation is particularly critical of data obtained from nociceptive tests which incorporate bilateral hind paw stimulation such as the hot plate or cold water tests in which both hind paws are simultaneously stimulated. For example, nociceptive behavior in these cases may be a manifestation of abnormal bilateral nociceptive input rather than of the nerve-injured hind paw only.

4.4. Hind paw withdrawal threshold ipsi- and contralateral to sham operation

The main reason for running a group of rats which received only sham surgery without cuff implantation was to determine whether surgical manipulation without nerve constriction is an appropriate control group for comparison to the cuff-implanted rats. Specifically, we are inquiring whether any effect(s) of surgery alone has the capacity to alter the processing of sensory information such that normally unperceived innocuous tactile stimuli evoke a paw withdrawal reflex. It is demonstrated in this study that sham surgery is sufficient to produce a relatively long-lasting increase in sensitivity of the hind paws to the effects of application of von Frey hairs. However, the magnitude is less and the duration is not as long-lasting as that observed in the cuff-implanted rats. Interestingly, the intact contralateral hind paw was also sensitive to the effects of application of von Frey hairs and this was identical to that of the ipsilateral hind paw. Therefore, the effects of surgery are adequate in altering mechanisms of sensory processing.

Surprisingly, cutaneous and muscular incision of the hind limb is generally reported to be devoid of effect on the paw withdrawal threshold. However, both Seltzer et al. (1990) and Takaishi et al. (1996), using the Seltzer et al. model of neuropathic pain demonstrate a bilateral decrease persisting for several days in a substantial number of rats revealed by the decreased mean and the large standard error of the mean in the paw withdrawal threshold in the sham-operated group. Kim and Chung and Blenk et al. report also a decrease in the withdrawal threshold of the sham-operated hind limb (Kim and Chung 1992; Blenk et al. 1997). However, unequivocal evidence that surgical

manipulation without direct nerve injury can influence sensory mechanisms comes from Brennan et al. who reported that surgical incision of the rat foot induces a reliable and quantifiable mechanical allodynia lasting for several days after surgery (Brennan et al. 1996, 1997; Zahn and Brennan 1998; Zahn et al. 1998). As even sites remote from the wound showed persistent mechanical allodynia, this observation demonstrates that sensitisation of sensory mechanisms may be induced by surgery without manipulation of the sensory nerve. Similar observations have also been made clinically. For example, it is reported that surgery may induce 'spinal sensitisation' (Lascelles et al. 1995; Wilder-Smith et al. 1996). Thus, the effects of surgery alone appear to have a substantial effect on sensory input perhaps by upregulating or sensitising sensory information processing. Involvement of central mechanisms at least in part in mediating surgery-induced pain is not unreasonable as this concept concurs with the report that a surgical procedure that does not include major nerve damage induces transynaptic degeneration in laminae I-III of the spinal dorsal horn (Nachemson and Bennett 1993). It is presumed that nociceptor-driven excitotoxic insult in the dorsal horn impairs neurons resulting in postoperative pain. For example, intrathecal non-NMDA receptor antagonists are reported to inhibit pain behaviors in a rat model of postoperative pain (Zahn et al. 1998).

It is important to note that in the present study, the decreased hind paw withdrawal thresholds in the sham-operated group were significantly lower than the baseline threshold as well as the threshold from the unoperated group. Therefore, it is suggested that a sham-operated group of rats or sham surgery done to the hind paw contralateral to nerve injury

may be inappropriate to use as 'control' in which there is expected to be no change from the baseline withdrawal threshold.

If one considers here that sham surgery involved not only exposure of the sciatic nerve but also slight elevation of the nerve for a very short period of time equivalent to that done necessarily in rats in the neuropathic group in order to place the cuff on the sciatic nerve, then the data must also interpreted to suggest that even minimal physical contact even without constriction of the sciatic nerve may also be sufficient to induce hind limb tactile allodynia.

Presently, we have no definitive explanation for the decreased withdrawal threshold of the contralateral hind paw. Nonetheless, a contralateral effect is at least consistent with the increased sensitivity of the hind paw contralateral to cuff implantation. Therefore, possible involvement of surgery-evoked mechanical hyperesthesia in the decreased withdrawal threshold in the cuff-implanted group of rats is not without consideration. However, given that the effect of sham surgery on withdrawal threshold is significantly less in magnitude than the effect of nerve constriction in the cuff-implanted group and that the effects of sham surgery on the paw withdrawal threshold abate several weeks prior to the effects of cuff-induced nerve constriction, the effects of sham surgery are probably less representative of 'neuropathic' pain derived from nerve constriction. Rather, the effects of sham surgery observed here are more indicative of surgical sensitisation as contralateral in addition to the ipsilateral allodynia were detected. It is suggested that the effect of sham surgery may be considered representative of postoperative pain.

Conclusions

In summary, striking evidence is revealed in this study of three types of allodynia in the sciatic nerve-constricted or surgically-injured rat. Each type has a distinct onset, time course, magnitude and recovery. The marked decrease in the withdrawal threshold of the cuff-implanted hind paw may be indicative of neuropathic allodynia characterized by the remarkably long-lasting tactile hypersensitivity accompanied by a combination of nociceptive behaviors. The second type of allodynia was seen in operated but not in unoperated controls and is therefore suggested to be a model of surgical pain. It is also induced unilaterally but expressed bilaterally. The third type of allodynia may be a model of central pain and it is demonstrated by the decreased withdrawal threshold of the intact hind paw contralateral to the cuff-implanted paw. Although it is initially less in magnitude compared to that of the neuropathic allodynia, it is slower in onset and later in recovery. Therefore, it is speculated that central pain may be established and maintained via the peripheral and/or central effects of nerve constriction.

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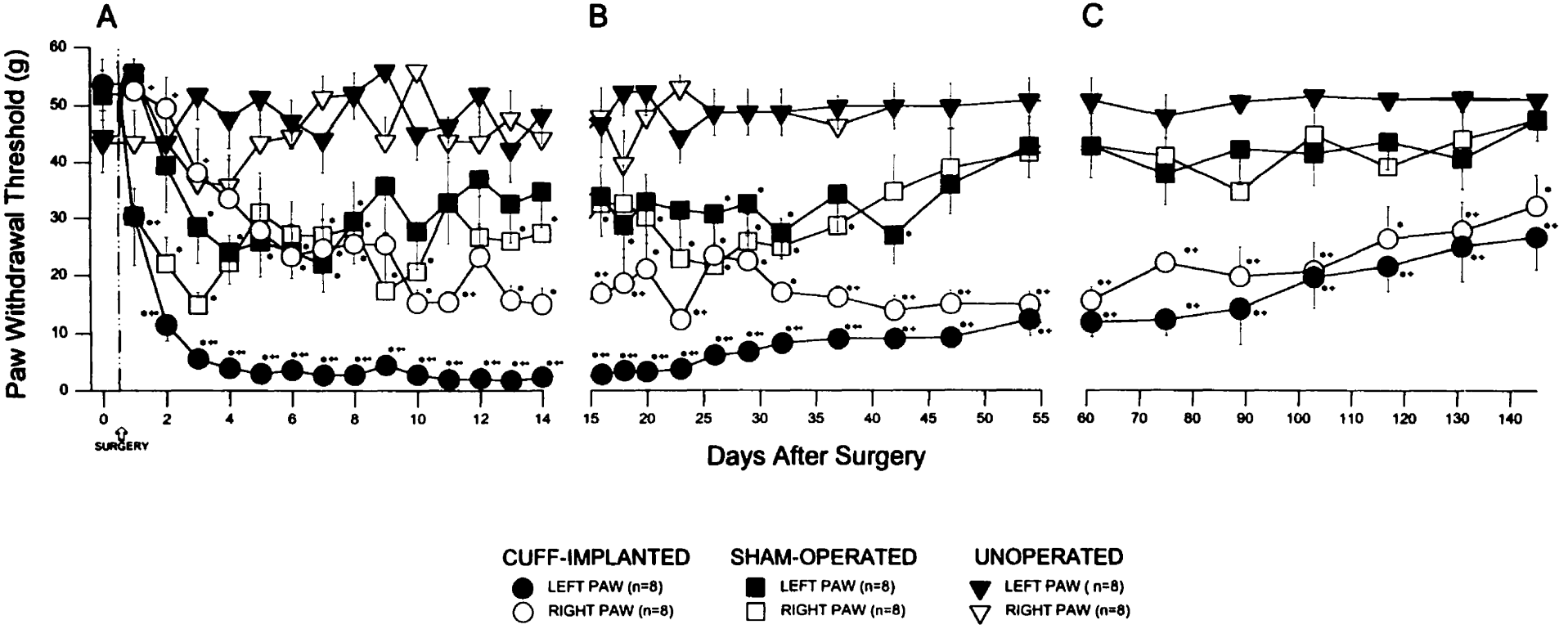
A
B
C
Paw Withdrawal Threshold (g)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
SURGERY
15
20
25
30
35
40
45
50
55
60
70
80
90
100
110
120
130
140
Days After Surgery
CUFF-IMPLANTED
LEFT PAW (n=8)
RIGHT PAW (n=8)
SHAM-OPERATED
LEFT PAW (n=8)
RIGHT PAW (n=8)
UNOPERATED
LEFT PAW (n=8)
RIGHT PAW (n=8)

Figure 1. Effect of cuff implantation, sham surgery or no surgery on the withdrawal threshold of the left and intact contralateral hind paws in rats. The horizontal axis represents time in days and the vertical axis represents the mean (±SEM) paw withdrawal threshold expressed in grams and determined via application of von Frey hairs. (A) Initially, the mean hind paw withdrawal thresholds in each of the 3 groups of rats were measured over 15 consecutive days. On day 0, the baseline left and right hind paw withdrawal thresholds in the unoperated, sham-operated and cuff-implanted groups of rats were statistically similar. Cuff implantation and sham surgery were done after baseline testing. In unoperated rats, the mean withdrawal thresholds of both hind paws remained at baseline. However, in the sham-operated group of rats, the mean withdrawal thresholds of the operated paw and the intact contralateral paw decreased. In cuff-implanted rats, the mean paw withdrawal threshold of the nerve-injured hind paw decreased and was lower than withdrawal thresholds in the sham-operated group. Contralateral hind paw withdrawal thresholds were similar to that in the sham-operated group. (B) Between days 16 and 54, rats were tested every 2, 3, 5 and then 7 days. Mean hind paw withdrawal thresholds in the unoperated group of rats remained at baseline and the sham-operated rats remained approximately at 30 g until the onset of recovery between days 42 and 47. In cuff-implanted rats, onset of recovery of the withdrawal threshold occurred between days 23 and 26 while the withdrawal threshold of the contralateral hind paw remained predominantly unchanged. (C) Rats were tested every 14 days between days 61 and 145, the last day of testing. Mean left and right hind paw withdrawal thresholds remained at baseline in the unoperated and in the sham-operated groups of rats. Onset of recovery of

the intact hind paw contralateral to the nerve constriction occurred between days 60 and 77. During recovery, hind paw withdrawal thresholds were statistically similar. (* $P < 0.05$ vs. unoperated group, + $P < 0.05$ vs. sham-operated group and • $P < 0.05$ vs. contralateral hind paw; SEM bars directed upward on hollow symbols and downward on solid symbols.

Unifying Statement

The paw withdrawal reflex experiments in Chapter 5 were directed towards investigation of the effects of cuff implantation and sham surgery on the hind paw withdrawal threshold using the von Frey hair test. While surgery alone induced a decrease in the hind paw withdrawal threshold lasting approximately 35 days, a 2 mm cuff implanted around the sciatic nerve produced a decrease in the withdrawal threshold of both ipsi- and contralateral hind paws to normally innocuous tactile stimulation in the von Frey hair test lasting at least 145 days, the maximum length of the observation period. This is of particular interest not only with respect to its representation of at least some forms of clinical chronic neuropathic pain, it also demonstrates that altered sensory information processing may be established and maintained bilaterally via the effects of peripheral neuropathy. Specifically, it is demonstrated that the maximum tactile allodynia occurs in rats on days 11-14 and 15-22 after cuff-implantation. Furthermore, maximal contralateral tactile allodynia occurs in rats on days 42-52 after cuff implantation.

However, it remains to be determined whether there is a cellular correlate of these changes at the physiological level. These striking characteristics of cuff-implantation on the rat behavior are interpreted to be indicative of substantial alterations in cellular mechanisms mediating sensory processing. Therefore, this behavioral paradigm provides a basis for the cellular approach used in Chapter 6 to investigate the effects of peripheral neuropathy on spinal dorsal horn neuronal activity at the optimal time points determined in Chapter 5.

Chapter 6

Cellular Mechanisms of Hyperalgesia and Spontaneous Pain in a Spinalized Rat Model of Peripheral Neuropathy: Changes in Myelinated Afferent Inputs Implicated

Abstract

Various hypotheses have been proposed to account for the mechanical hyperalgesia and spontaneous pain seen in animal models of peripheral neuropathy. The purpose of the present study was to determine whether there exists a spinal neuronal correlate to these properties. An experimental neuropathy was induced in male Sprague Dawley rats by placing a 2 mm PE-90 polyethylene cuff around the sciatic nerve. All rats were subsequently confirmed to exhibit mechanical allodynia in the von Frey test. After induction of anaesthesia with pentobarbital and acute spinalization at T_9, electrophysiological experiments were done, recording extracellular single unit activity from ipsi- and contralateral wide dynamic range dorsal horn neurones in spinal segments L_{1-4}. On-going activity was greater in short-term (11-22 days after cuff implantation) and in long-term (42-52 days) cuff-implanted rats; 38 spikes/s in short-term vs. 19 spikes/s in controls; 29 spikes/s in long-term ipsi- and contralateral neurones. Receptive fields in controls were always restricted but in almost all cuff-implanted rats extended over the whole hind paw. Responses to noxious mechanical (pinch) and to noxious heat stimulation of the cutaneous receptive field in controls consisted of a typical fast initial discharge followed by an afterdischarge. In all neurones from cuff-implanted rats the initial discharge resembled that in controls. However, the afterdischarge, particularly that in response to noxious pinch, was markedly greater in both magnitude and duration. It is suggested that the greater on-going discharge is the cellular correlate of spontaneous pain and the potentiation of the afterdischarge in response to noxious stimulation is the correlate

of hyperalgesia. Given that acutely spinalized rats were tested, only peripheral and/or spinal mechanisms can be considered to explain these data. Considering all the data, it can be concluded that there is a greater change in responses to activity in fibers mediating noxious mechanical than noxious thermal inputs. Among different hypotheses, the one with which the present data are most compatible is that which proposes that chronic nerve injury or inflammation induces phenotypic changes predominantly in myelinated afferents. There may be a redistribution of membrane-bound ion channels, predominantly sodium channels, which leads to ectopic activity and thus spontaneous discharge of dorsal horn neurones. With regard to mechanical stimulation-evoked synaptic input, the central terminals of myelinated afferents expand into regions of the spinal cord which normally receive their predominant input from unmyelinated nociceptive afferents. This may be coupled with a change in these myelinated afferents so that they now synthesize and release peptides, primarily substance P, from their central terminals with the result that the effects of their chemical mediators of synaptic transmission add to the effects of nociceptive inputs leading to exaggerated responses to painful stimuli, thus the basis of clinical hyperalgesia.

Introduction

Experimental neuropathy in the rat typically features greater sensitivity of the nerve-injured hind limb to normally innocuous tactile stimulation. Dorsal horn neurones in rats exhibiting tactile allodynia show greater on-going activity ipsilateral to chronic loose sciatic nerve constriction (Palecek et al. 1992b; Laird and Bennett 1993; Sotgiu 1993), partial sciatic nerve ligation (Yakhnitsa et al. 1999), loose ligation of the L_{4-6} spinal nerves (Tabo et al. 1999), tight ligation of the L_{5-6} spinal nerves (Pertovaara et al. 1997; Chapman et al. 1998), or tight ligation of the L_7 spinal nerve in primates exhibiting tactile allodynia (Palecek et al. 1992a). Dorsal horn neurones in neuropathic rats also show greater responses to noxious mechanical stimuli (Palecek et al. 1992a,b; Leem et al. 1995, 1996; Pertovaara et al. 1997). Given that electrophysiological studies to date have focussed predominantly on greater excitatory characteristics of spinal dorsal horn neurones in the spinally intact preparation, processing of sensory information in the spinal cord can be considered to involve a supraspinal component. Therefore, taken together, the data in these studies may be interpreted to suggest hyperexcitability in the spinal-supraspinal neural axis.

Based on studies which show that tactile allodynia in experimental neuropathy is substantially decreased or abolished following spinal cord transection (Kauppila 1997; Bian et al. 1998; Kauppila et al. 1998; Sung et al. 1998) or injection of lidocaine into the brain stem (Pertovaara et al. 1996), it has been argued that greater tactile sensitivity of the nerve-injured hind paw is sustained predominantly via a supraspinal loop with tonic descending

facilitatory properties. To our knowledge, no substantial attempt has been made to examine the effects of experimental neuropathy, including specifically on-going activity and peripheral stimulation-elicited synaptic input, independently of supraspinal modulation. If tonic descending facilitation does in fact govern hyperexcitability in the spinal dorsal horn, specifically that mediating mechanical sensory processing, it is reasonable to speculate that spinal cord transection may result in marked diminution of neuronal hyperexcitability. However, based on studies showing greater peripheral sensory neuronal activity (Kajander and Bennett 1992; Zhang et al. 1997; Ali et al. 1999) and phenotypic changes in afferent sensory neurones (Marchand et al. 1994; Koerber et al. 1999) in different models of neuropathic pain, there appears to be considerable evidence supporting the notion that central hyperexcitability may be sustained via peripheral and/or spinal mechanisms. Therefore, determination of neuronal activity in the spinal dorsal horn independent of supraspinal influence in the nerve-injured rat is essential to identify the contribution of peripheral and spinal sensory processing in experimental neuropathy.

A less common phenomenon reported in the literature is tactile hypersensitivity of the contralateral hind paw in experimental neuropathy. Some studies show contralateral tactile allodynia in rats lasting at least 10 days (Sinnott et al. 1999), 30 days (Shir and Seltzer 1990), 54 days (Seltzer et al. 1990) or approximately 120 days (Takaishi et al. 1996; Tabo et al. 1999) after cuff implantation was induced. Recently, we have shown that implanting a 2 mm polyethylene cuff around the sciatic nerve (Mosconi and Kruger 1996) produces a decrease in the withdrawal threshold of both ipsi- and the contralateral

hind paws to normally innocuous tactile stimulation in the von Frey hair test (Pitcher et al. 1999b) lasting at least 145 days, the maximum length of the observation period (Pitcher et al. 1999a). This evidence is of interest not only with respect to its representation of at least some forms of clinical chronic neuropathic pain (Kozin et al. 1976; Oaklander et al. 1998), it also demonstrates that altered sensory information processing may be established and maintained bilaterally via the effects of peripheral nerve injury or inflammation (Bennett 1999). To date, no study has investigated the effects of chronic sensory nerve injury or inflammation on neuronal activity in the contralateral spinal dorsal horn *in vivo* independent of a supraspinal loop.

Thus, the main objective of this study was to determine the capacity of ipsi- and contralateral spinal dorsal horn neurones *in vivo*, independent of supraspinal processing, to sustain the excitatory effects of chronic peripheral nerve injury or inflammation in a rat model of experimental neuropathy. Chronic implantation of a 2 mm polyethylene cuff around the left sciatic nerve (Mosconi and Kruger 1996) and electrophysiological testing was done in rats exhibiting marked tactile allodynia determined using the von Frey hair test. The specific parameters examined included on-going activity and the effects of noxious mechanical and noxious radiant heat on dorsal horn neuronal activity in acutely spinalized, rats. The purpose was to determine whether a spinal neuronal correlate of the effect of sciatic nerve injury or inflammation is present, and if so, whether it is modality selective.

Preliminary data have been presented in abstract form (Pitcher and Henry 1999a;

Pitcher and Henry 1999b).

Materials and methods

Animals

Experiments were performed using adult, male Sprague-Dawley rats (375-425g) from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana, USA). They were housed in plastic cages containing wood chip bedding (Hardwood Laboratory Bedding, Northeastern Products Corp., Warrensburg, New York, USA) and maintained on a 12:12 h light:dark cycle (lights on at 07:00 h) with access to food and water *ad libitum*. Experiments were conducted during the light component of the cycle. Guidelines in *The Care and Use of Experimental Animals* by the Canadian Council on Animal Care (Vols. I and II) were strictly followed and all experiments were approved by the *McGill University Animal Care Committee*.

Cuff implantation

A variation of the technique of Mosconi and Kruger (1996) was used. Under Na-pentobarbital anaesthesia (50 mg/kg, i.p., Abbott Laboratories, Limited, Montreal, Quebec, Canada) and aseptic conditions, the left hind paw was shaved and an incision in the skin was made above the biceps femoris muscle. The common sciatic nerve was exposed by blunt dissection through the muscle and was isolated from surrounding connective tissue using glass probes. The nerve was elevated minimally using a sterilized glass probe in order for a 2 mm section of split polyethylene tubing (Intramedic PE-90, Fisher Scientific Ltd., Whitby, Ontario, Canada) to be placed around the nerve. The

muscle layer was closed using 3-O silk suture thread (Ethicon Inc., Montreal, Quebec, Canada) and the skin layer was closed using 3 stainless steel suture clips (Fine Science Tools, Inc., North Vancouver, British Columbia, Canada). The topical antibacterial ointment, nitrofurazone (0.2%, Univet Pharmaceuticals Ltd., Milton, Ontario, Canada) was placed on the skin suture to counter risk of infection. The rat was then allowed to recover. Only two rats from the same testing group (ie. unoperated or cuff-implanted) were together in a cage.

von Frey hair test

Before an animal was tested in an electrophysiological experiment it was examined for mechanical sensitivity in the von Frey hair test. Tactile hind paw withdrawal threshold in rats was determined by applying von Frey hairs (Xymotech Biosystems, Inc., Montreal, Quebec, Canada) to the plantar surface of the hind paw. Application of the von Frey hairs to the hind paws was done by placing the rat on a platform designed and constructed specifically for von Frey hair testing (Pitcher et al. 1999b). Described briefly, the platform is made of plexiglass 3 mm thick. It is slightly opaque in appearance and contains 1.5 mm diameter holes in perpendicular rows, 5 mm apart throughout the entire area of the platform. A von Frey hair was applied through a particular hole to a hind paw. For testing, this platform was fixed in a transparent plexiglass chamber (30×30×30 cm).

The mechanical hind paw withdrawal threshold, determined using von Frey hairs, was expressed in grams. Ten hairs ranging from 0.23 to 59.0 g were used. The bending

force of each hair in grams was confirmed periodically by measuring the force exerted by the hair when applied to a Mettler AE 100 electronic balance. The hair was applied in a manner such that the degree of bending was the same as that when applied to the rat hind paw. Confirmation was done because it was determined that slight fluctuation in the bending force of a hair may occur with extended use. If this was the case, the new bending force in grams, determined using the electronic balance, was used as the value.

Testing was blind such that the experimenter was not aware of the kind of rat being tested, ie. unoperated or cuff-implanted. The protocol used in this study is similar to that used previously (Pitcher et al. 1999a; Pitcher et al. 1999b). Briefly described, a testing session for a particular rat began after 5 min of habituation to the testing chamber. The series of von Frey hairs was applied from below the platform to the left hind paw in ascending order beginning with the lowest hair (0.23 g). Hairs were applied only when the rat was stationary and standing on all four paws. Application was to the central region of the plantar surface, avoiding the foot pads. The hair was applied to the paw until bending of the hair occurred. Application of the hair was maintained for approximately 2 s. A withdrawal was considered a valid response only if the hind paw was completely removed from the platform. Although infrequent, if a rat walked immediately after application of a particular hair, the hair was reapplied. On rare occasions, the hind paw only flinched after a single application of the hair. As the hind paw was not lifted from the platform, this was not considered a withdrawal response.

A trial consisted of application of a von Frey hair to the hind paw 5 times at 5 s

intervals. If the hind paw withdrawal persisted beyond the 5 s interval, testing resumed after the hind paw was placed appropriately on the platform. Hind paw withdrawal either 4 or 5 times out of the 5 applications was considered to be the withdrawal threshold. If hind limb withdrawal was not evoked 4 or 5 times using a particular hair, the next larger hair in the series was applied in a similar manner.

Once the threshold was determined for the left hind paw, the same testing procedure was repeated on the right hind limb after an inter-trial interval of 5 min. Second and third trials were determined for each of the left and right hind paws with inter-trial intervals of 5 min. If the withdrawal threshold in the second or third trial did not match that of the previous trial(s) on a particular hind paw, the next larger hair in the series was tested. This was done until paw withdrawal thresholds in the 3 trials were consistent. The total testing time for each rat usually lasted 35 to 40 min. In almost all cases, the first 3 trials were consistent.

The baseline withdrawal threshold of both hind paws in the von Frey hair test was determined in unoperated and in cuff-implanted rats prior to surgery (normalized to day 0). 'Short-term' cuff-implanted rats were tested on days 11-14 and 15-22. Two short-term periods were examined to enable determination of any change in excitability over weeks 2 and 3 after cuff implantation. 'Long-term' cuff-implanted rats were tested on days 42-52. A statistically significant decrease in the hind paw withdrawal threshold was considered indicative of tactile allodynia. The von Frey hair test was done immediately prior to electrophysiological testing or the day before to confirm the absence or presence

of tactile allodynia.

Only hind paw withdrawal thresholds that remained consistent in each of the 3 trials in unoperated or in cuff-implanted rats were used in the data analysis. Comparisons were done using the Mann-Whitney Rank Sum Test and were considered significantly different with a P value < 0.05.

Animal preparation for electrophysiological experiments

Acute electrophysiological experiments were run using unoperated and cuff-implanted rats tested previously in paw withdrawal reflex experiments using von Frey hairs. Rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.; Abbott Laboratories Ltd, Montreal, Quebec, Canada) followed by supplements of 10 mg/kg/h, i.v. The right common carotid artery and the jugular vein were catheterized for continuous monitoring of arterial pressure and for injection of drugs, respectively. Temperature of the rat was maintained at approximately 37.5°C using an infrared heating lamp when required.

Spinal cord segments L_1 to L_4 were exposed for recording from single dorsal horn neurones. The spinal cord was transected at the T_9 vertebral level to eliminate supraspinal influences on the activity of lumbar dorsal horn neurones; to minimize spinal shock xylocaine (0.05 ml of 1%; Astra Pharma, Mississauga, Ontario, Canada) was injected into the cord at the level of transection just prior to transection. Once the rat was stabilized on the stereotaxic frame, the exposed spinal cord was covered with mineral oil (Marcol 72,

Imperial Oil Limited; Montreal, Quebec, Canada) at 37.5°C to prevent drying. Experiments were begun 1.5 to 2 h after spinalization.

Each rat breathed spontaneously during the experiment. However, if breathing became irregular the anaesthetised rat was also paralysed with pancuronium bromide (1 mg/kg i.v. supplemented as necessary; Pavulon, Organon, Scarborough, Ontario, Canada) and ventilated mechanically according to standard parameters (Kleinman and Radford 1964). The animal was sacrificed at the end of the experiment.

Electrical recording

Single unit extracellular spikes were recorded using seven-barrelled or single-barrelled micropipettes (overall tip diameter 4-5 or 1-2 μm, respectively). The multi-barrelled electrodes were used because iontophoretic drug experiments were also run in some cases after testing the effects of synaptic input. A solution of 3 M NaCl was placed in the central recording barrel (impedance 2-4 MΩ measured at 1 kHz with the tip submerged in 0.9% saline). Single unit recordings were made at depths ranging from 150 to 1200 μm in the spinal dorsal horn. The raw data were amplified 10,000 × using a DP-301 Differential Amplifier (*Warner Instrument Corp.*), displayed on an oscilloscope (Tektronix 5111) and stored on video cassette tapes using a digital data recorder that incorporated digital pulse code modulation (VR-100A, Instrutech Corporation, Great Neck, NY, U.S.A.) and a conventional video cassette recorder. The signals were also relayed to a frequency counter/gating unit which counted single unit spikes per unit time (bin

widths were 1 s) and which thus displayed a continuous time histogram of the rate of discharge on a Grass 79D polygraph. Sampling and analysis were done using the data acquisition program, *Spike 2* (Version 2.02; *Cambridge Electronic Design*, Cambridge, England), *SigmaStat* (Version 2.03; *SPSS, Inc.*, USA) and an IBM Pentium computer.

Functional classification of dorsal horn neurones

Functional classification of a lumbar dorsal horn neurone was based on its response to innocuous and noxious stimulation of the cutaneous receptive field of the respective hind paw. The following natural stimuli were used as search stimuli to elicit synaptic input while penetrating the spinal dorsal horn and to characterize a neurone functionally once stable single unit recording was obtained: (i) hair stimulation, (ii) light touch/moderate pressure using a calibrated clip (0.2 N for 3 s), (iii) noxious mechanical stimulation using a calibrated clip (pinch; 21 N for 3 s) and (iv) noxious radiant heat (measured to reach 50°C at the skin surface) applied by a focussed projector bulb through a 10 mm diameter circular hole for a duration of 10 s. In unoperated and in cuff-implanted rats, innocuous stimuli were never tested after noxious stimuli had been tested; results from innocuous testing are the subject of another manuscript.

Classification of the identified dorsal horn neurones was in three categories (Pitcher and Henry 1999c): (i) non-nociceptive neurones that responded only to non-noxious stimuli such as touch and/or pressure stimulation, (ii) wide dynamic range neurones that responded to both noxious and innocuous stimuli or (iii) nociceptive-specific neurones that responded

only to noxious stimuli. In addition, all the units that responded to the noxious range of mechanical and/or heat stimulation showed a characteristic slowly-decaying afterdischarge, as described previously (De Koninck and Henry 1991; Pitcher and Henry 1999c). Only wide dynamic range neurones were tested in this study. Responses of neurones in the left or right dorsal horn were evoked by stimulation of the hind paw ipsi- or contralateral to cuff implantation.

Extreme care was taken to investigate the region in the receptive field corresponding to the same area of the hind paw where von Frey hair testing had been done prior to electrophysiological experiments. In addition, in order to minimize possible differences between neurones located in different mediolateral parts of the spinal dorsal horn, only a specific recording region adjacent to the entry of dorsal roots in spinal segments L_{3-4} was searched for single units. Some neurones were tested with pinch only, some with noxious heat only and some with both.

Controls used were unoperated rats. In our previous study (Pitcher et al. 1999a) we found a surgery-induced tactile allodynia persisting approximately 35 days in sham-operated rats. This could have had an influence on the changes in short-term cuff-implanted rats, but not in long-term rats. In pilot experiments, sham surgery-induced hyperexcitability of spinal dorsal horn neurons was markedly less than that in cuff-implanted rats. Sham surgery was without significant effect in long-term rats. In this regard, we interpret the changes reported here to be mainly due to the cuff implantation. Other electrophysiological studies have used unoperated rats to serve as controls

(Pertovaara et al. 1997; Yakhnitsa et al. 1999).

Analysis of electrophysiological data

Sampling of on-going activity occurred only after at least 5 min of stable on-going discharge and prior to any peripheral stimulation-induced synaptic input. On-going activity was quantified as the total number of spikes during a 60 s period.

Sampling of evoked responses of wide dynamic range dorsal horn neurones included a fast initial discharge followed by a slowly-decaying afterdischarge. The fast initial discharge persisted only for the duration of the 3 s mechanical stimulus and was evaluated during the application of the stimulus. The noxious heat stimulation-evoked fast initial discharge was determined over the 3 s period at the end of the 10 s stimulus. The slowly-decaying afterdischarge was evaluated when a cell continued firing above background on-going activity after the end of the stimulus. The sample period of the slowly-decaying afterdischarge began immediately after cessation of the fast initial discharge and ended once the firing rate returned to the prestimulus discharge level. Evoked responses were quantified as the total number of spikes in the fast initial discharge or slowly-decaying afterdischarge minus the background discharge of equivalent duration as the evoked response.

On-going and synaptic input-elicited activity are expressed as means ($\pm$ S.E.M.) of the number of spikes during the respective sample period. The duration of the slowly-decaying afterdischarge is expressed as the mean $\pm$ S.E.M. number of seconds. Statistical

analysis of the data was done using the Mann-Whitney Rank Sum Test. Data from neurones in cuff-implanted rats were compared to those in unoperated rats and a difference was considered significant with a P value < 0.05.

Results

von Frey Hair Test

Hind paw withdrawal threshold in unoperated rats

In 28 unoperated rats, ipsi- and contralateral hind paw withdrawal thresholds were determined on days 0, 11-14, 15-22 and 42-52. Figure 1A shows that on day 0 ipsi- and contralateral hind paw withdrawal thresholds were 49.57 ± 2.75 and 47.18 ± 2.92 g, respectively and that there was no significant difference between the hind paw withdrawal thresholds on day 0 and on subsequent testing days. As subsequent paw withdrawal thresholds in these unoperated rats were not different they were pooled, to yield ipsi- and contralateral hind paw withdrawal thresholds of 50.14 ± 2.44 and 51.39 ± 2.24 g, respectively.

Hind paw withdrawal threshold in cuff-implanted rats

Every cuff-implanted rat exhibited a marked decrease in hind paw withdrawal threshold after cuff-implantation compared to its own threshold on day 0. In 32 short-term cuff-implanted rats, the ipsi- and the contralateral withdrawal thresholds were determined on days 11-14 and in 26 rats on days 15-22 days. In 67 long-term cuff-implanted rats, the ipsi- and contralateral hind paws were tested on days 42-52.

Figs. 1B and C show that in short-term rats on day 0 withdrawal thresholds were 47.75 ± 2.60 and 49.00 ± 2.45 g on the ipsi- and contralateral sides, respectively, for the

11-14 day group and 52.04 ± 2.51 and 49.19 ± 2.91 g, respectively, for the 15-22 day group. Thresholds were decreased on days 11-14 (5.02 ± 0.64 and 33.38 ± 3.47 g; $P < 0.001$ and $P < 0.01$ vs. day 0, respectively) and on days 15-22 (5.96 ± 0.93 and 26.50 ± 3.39 g; $P < 0.001$ vs. day 0).

Figure 1D shows that in long-term rats ipsi- and contralateral thresholds were 15.03 ± 0.80 and 19.37 ± 1.25 g, respectively, which were decreased ($P < 0.001$) compared to their withdrawal thresholds at day 0 (48.37 ± 1.74 and 47.81 ± 1.74 g, respectively). At the time points studied, the maximum decrease of the ipsilateral hind paw withdrawal threshold occurred in short-term rats ($P < 0.001$ vs. day 42-52) and the maximum decrease of the contralateral hind paw withdrawal threshold occurred in long-term rats ($P < 0.001$ vs. days 11-14).

Several of the cuff-implanted rats, particularly the short-term rats, exhibited an after-effect to innocuous stimulation persisting for several seconds which included lifting and licking of the hind paw in response to von Frey hair application. These data are consistent with previous findings (Pitcher et al. 1999a).

Electrophysiological Experiments

(i) On-going activity

On-going activity of spinal wide dynamic range neurones in short- and long-term cuff-implanted rats was compared to that in unoperated rats. In 28 unoperated rats, on-going activity was recorded from 66 wide dynamic range neurones. In short-term cuff-

implanted rats, on-going activity was recorded from 51 ipsilateral neurones in 32 rats on days 11-14 and from 46 ipsilateral neurones in 26 rats on days 15-22. In long-term cuff-implanted rats, on-going activity was recorded from 78 ipsilateral neurones in 38 rats and from 60 contralateral neurones in 29 rats on days 42-52.

Figure 2 shows the combined data. On-going activity in short-term cuff-implanted rats (38.17 $\pm$ 3.78 spikes/s and 38.06 $\pm$ 4.75 spikes/s) was greater than that in unoperated rats ($P < 0.001$ vs. 19.82 $\pm$ 2.39 spikes/s). In long-term cuff-implanted rats, on-going activity of ipsi- (29.56 $\pm$ 3.12 spikes/s) and of contralateral neurones (29.28 $\pm$ 3.25 spikes/s) was also greater than that in unoperated rats ($P < 0.05$). In addition, on-going activity in day 11-14 short-term rats was greater ($P < 0.05$) than that in long-term rats tested ipsilaterally.

(ii) Cutaneous receptive field size

The cutaneous receptive field to tactile stimulation was examined for each wide dynamic range neurone investigated in unoperated and in cuff-implanted rats. Although specific measurements of the cutaneous receptive field size were not systematically determined in this study, it was noted that the receptive field to tactile stimulation was unequivocally and universally larger in cuff-implanted rats than in unoperated rats. It is important to note that while characterizing dorsal horn neurones, the receptive field to touch stimulation in cuff-implanted rats consisted almost invariably of the entire ipsi- or contralateral hind paw. In normal rats, the receptive field to touch stimulation was

typically on the plantar surface and rarely extended above the ankle joint. This is illustrated in the figures.

For each ratemeter histogram two schematic diagrams are shown illustrating the cutaneous receptive fields. The receptive field to innocuous tactile stimulation is depicted in the left schematic diagram. The right schematic diagram shows the area of the receptive field on which natural stimulation including pinch or heat was applied to evoke dorsal horn neuronal responses for the purpose of analysis.

(iii) Responses to noxious mechanical stimulation

In 18 unoperated rats, the effect of pinch stimulation was tested on 34 neurones. A representative response of a neurone to pinch is illustrated in Figure 3A. Typically, pinch elicited a fast initial discharge, high frequency activity during the pinch stimulus, and a slowly-decaying afterdischarge, increased activity persisting 2 to 3 min after the end of the stimulus.

In short-term cuff-implanted rats, pinch was tested on 31 neurones in 22 rats on days 11-14 and on 31 neurones in 19 rats on days 15-22. Figure 3B shows a representative response of a neurone to pinch in the receptive field in a rat tested on day 12. The striking feature of this response is the markedly greater magnitude and duration of the slowly-decaying afterdischarge compared to those recorded from a wide dynamic range neurone in the unoperated rat (Figure 3A).

In long-term cuff-implanted rats, pinch was tested on 35 ipsilateral neurones in 26

rats and on 26 contralateral neurones in 21 rats. Figs. 4A and B illustrate representative responses of ipsi- and contralateral neurones to pinch stimulation in 2 rats tested on day 42. In each neurone, the magnitude and duration of the slowly-decaying afterdischarge were markedly greater than in controls.

Figs. 5A and B show the cumulative data of the responses to pinch stimulation. In short-term rats, the pinch-induced fast initial discharge (779.53 ± 95.59 and 785.24 ± 96.38 spikes per response) was similar to that in unoperated rats (686.04 ± 88.47 spikes). However, the magnitude of the slowly-decaying afterdischarge (15298.35 ± 3421.01 and 13241.32 ± 3880.66 spikes) was greater ($P < 0.01$ vs. unoperated, 2487.83 ± 267.55 spikes). In addition, the duration of the slowly-decaying afterdischarge (729.31 ± 114.35 s and 729.02 ± 116.25 s) was greater ($P < 0.001$ vs. unoperated, 232.78 ± 24.03 s).

In long-term rats, the pinch-induced fast initial discharge in ipsi- and contralateral neurones (674.21 ± 70.95 and 804.99 ± 121.76 spikes) was also similar to that in unoperated rats. The magnitude (6177.50 ± 896.69 and 5135.43 ± 788.99 spikes; $P < 0.01$ vs. unoperated) and the duration (459.40 ± 48.37 s and 358.68 ± 53.98 s; $P < 0.001$ and $P < 0.05$ vs. unoperated) of the slowly-decaying afterdischarge were greater compared to slowly-decaying afterdischarge responses in unoperated rats.

In day 11-14 short-term rats, the magnitude and duration of the slowly-decaying afterdischarge were greater ($P < 0.01$ and $P < 0.05$, respectively) than the magnitude and duration, respectively, in long-term rats tested ipsilaterally. In addition, the duration of the slowly-decaying afterdischarge in day 15-22 short-term rats was greater ($P < 0.05$)

than that in long-term rats tested ipsilaterally.

(iv) Responses to noxious heat stimulation

In 17 unoperated rats, the effect of noxious heat stimulation was tested on 33 neurones. A representative response of a neurone to noxious heat is illustrated in Figure 6A. The fast initial discharge is shown by the high frequency activity at the end of the heat stimulus and the slowly-decaying afterdischarge is depicted by greater activity persisting after the end of the stimulus.

In 24 short-term cuff-implanted rats, heat was tested on 35 neurones on days 11-14. Figs. 6B and C show representative responses of 2 neurones to heat stimulation of the receptive fields in a rat tested on day 12. In each case, regardless of the notable high level of on-going activity, augmentation of the magnitude of the heat-evoked slowly-decaying afterdischarge was limited.

In long-term cuff-implanted rats, heat was tested on 33 ipsilateral neurones in 25 rats and on 31 contralateral neurones in 20 rats. Figs. 6D and E illustrate representative responses of ipsi- and a contralateral neurones, respectively, to heat stimulation in 2 rats tested on day 42. In each neurone, cuff implantation appears to have had a negligible effect on the magnitude of the slowly-decaying afterdischarge and the duration appears to have been similar to that in unoperated rats.

Figs. 7A and B illustrate cumulative responses to heat stimulation. In short term rats, the heat-induced fast initial discharge (374.65 $\pm$ 46.50 spikes) was similar to that in

unoperated rats (282.18 ± 39.42 spikes). The magnitude of the slowly-decaying afterdischarge (1548.34 ± 334.54 spikes) was greater by a small degree ($P < 0.05$ vs. unoperated, 707.78 ± 118.68 spikes) but the duration of the afterdischarge (87.76 ± 15.98 s) was not significantly different compared to that in unoperated rats (59.10 ± 12.27 s).

In long-term rats, the heat-evoked fast initial discharge in ipsi- and contralateral neurones (354.61 ± 66.39 and 305.77 ± 34.04 spikes) was also similar to that in unoperated rats. Furthermore, the magnitude of the slowly-decaying afterdischarge (1050.85 ± 166.87 spikes and 1109.59 ± 189.87 spikes, respectively) was slightly greater ($P < 0.05$ vs. unoperated). However, the slowly-decaying afterdischarge duration (99.97 ± 19.98 and 56.37 ± 10.65 s, respectively) was not greater than that in unoperated rats.

Discussion

The data from the present electrophysiological experiments indicate that chronic implantation of a 2 mm PE-90 polyethylene cuff around the sciatic nerve (Mosconi and Kruger 1996) induces important changes in some of the physiological properties of spinal nociceptive neuronal activity *in vivo* in the acutely spinalized rat. In particular, these changes include greater on-going, or spontaneous, activity, an expanded receptive field size in response to natural cutaneous stimuli and a greater magnitude and duration of the afterdischarge in response to noxious mechanical stimulation of the cutaneous receptive field. Interestingly, the response to noxious heat stimulation was also greater, but to a much lesser extent. It is speculated that these changes can best be accounted for by changes in the properties of myelinated sensory afferent fibers in the sciatic nerve. The specific arguments supporting this position are presented below.

The present cellular approach was used to examine the effects of an experimental neuropathy because it has several advantages over behavioral testing, which has received the bulk of attention experimentally. One is that in electrophysiological experiments changes induced in the excitability of spinal dorsal horn sensory neurones can be identified. For example, early and late components of neuronal responses to different stimulus modalities can be evaluated, which cannot be identified or measured in behavioral studies. Interestingly, these have also not been addressed in previous electrophysiological studies. Another advantage of this cellular approach is that changes in receptive field size can be determined for single spinal dorsal horn neurones. Behavioral studies on the other hand

are unable to provide information of this type. Third, changes in sensory vs. motor mechanisms are difficult to differentiate in behavioral experiments, whereas in electrophysiological experiments recording can be done in the sensory dorsal horn only, thus yielding information exclusively on sensory processing. Finally, although some behavioral studies have compared responses to noxious mechanical and noxious heat stimuli in the same spinally-intact (Luukko et al. 1994; Ossipov et al. 1999) or spinalized animal (Kauppila 1997; Bian et al. 1998; Kauppila et al. 1998), there is no information on whether this is sustained by the same population of spinal neurones.

As all rats tested in the electrophysiological experiments had exhibited tactile allodynia in response to von Frey hair stimulation prior to the acute experiment, our data suggest a correlation between the changes observed in excitability of dorsal horn mechanisms and the behavioral tactile allodynia. Comparison of neuronal hyperexcitability and tactile allodynia in 11-14 and 15-22 day rats indicates that there was no significant difference between these two groups in the changes induced by cuff implantation. This is interpreted to suggest that the so-called neuropathic condition imposed by the cuff implantation was stable over the time covered by the two periods. The greatest increase in excitability of spinal nociceptive neurones and the maximum tactile allodynia in the von Frey experiments occurred in rats tested at these time points. Furthermore, a greater excitability of contralateral spinal neurones and a contralateral tactile allodynia were both observed in the long-term rats. Thus, the data are interpreted to suggest that hyperexcitability in spinal dorsal horn neurones may be the neurophysiological basis of the

mechanical hyperalgesia observed in neuropathic pain patients.

Changes induced in sensory processing-significance

(i) Altered spontaneous activity

Greater spontaneous activity of dorsal horn neurones is a common observation in electrophysiological experiments in spinally-intact rats exhibiting tactile allodynia, including the chronic loose sciatic nerve constriction model (Palecek et al. 1992b; Laird and Bennett 1993; Sotgiu 1993), the partial sciatic nerve ligation model (Yakhnitsa et al. 1999), the loose ligation of the L_{4-6} spinal nerves model (Tabo et al. 1999), the tight ligation of the L_{5-6} spinal nerves model (Pertovaara et al. 1997; Chapman et al. 1998), and the tight ligation of the L_7 spinal nerve model in primates (Palecek et al. 1992a). Thus, the elevated spontaneous activity observed here in acutely spinalized rats is consistent with previous observations in intact animals. In fact, this greater spontaneous activity may be the cellular correlate of the spontaneous pain which characterizes neuropathic pain in humans (Gracely et al. 1992).

(ii) Altered receptive field size

Although most electrophysiological studies on animal models of neuropathic pain have not focused on cutaneous receptive field size, enlarged receptive fields have been shown in spinally-intact (Behbehani and Dollberg-Stolik 1994; Takaishi et al. 1996) and

in spinally-transected rats (Cumberbatch et al. 1998). The significance of this observation to clinical neuropathic pain is less obvious but the greater receptive field size may be due to a number of different mechanisms, which are described below.

(iii) Altered response to noxious pinch stimulation

We were surprised that the initial discharge of dorsal horn neurones in response to noxious pinch stimulation of the receptive field was similar in cuff-implanted rats and in unoperated control rats. The greater response can be accounted for exclusively by the greater afterdischarge in response to the noxious mechanical stimulation. In this case, both the magnitude and the duration of the afterdischarge were greater. Previous electrophysiological studies report only increases in the response to noxious cutaneous mechanical stimulation (Palecek et al. 1992a,b; Leem et al. 1995, 1996; Pertovaara et al. 1997) but have not examined specifically different components of the nociceptive response. This greater afterdischarge which we have observed may account for the mechanical hyperalgesia which characterize neuropathic pain in human patients. The possible mechanisms underlying this greater afterdischarge are discussed below.

(iv) Altered response to noxious heat stimulation

It was notable that the initial discharge in response to noxious heat stimulation of the cutaneous receptive field was the same in the cuff-implanted rats as in the unoperated rats. The afterdischarge was greater, although to a considerably lesser extent than the

afterdischarge in response to noxious mechanical stimulation. In previous studies it has also been observed in spinally intact rats that neuronal responses to noxious heat are largely unaffected by peripheral nerve constriction (Palecek et al. 1992b; Laird and Bennett 1993; Pertovaara et al. 1997). This resembles the clinical condition, in which heightened sensitivity is usually more pronounced and more common with tactile stimuli than with heat stimuli (Bouhassira et al. 1999; Sieweke et al. 1999). The lack of a pronounced increase in the response to noxious heat stimulation shown here may also be interpreted to indicate that small diameter afferent inputs are relatively less potentiated than are myelinated fiber inputs in this model of neuropathic pain.

(v) Altered response of contralateral neurones

Neurones contralateral to the cuff were studied in long-term rats because our previous behavioral study demonstrated the late but marked development of a mechanical allodynia in the contralateral hind paw (Pitcher et al. 1999a). The results from the present electrophysiological study support the earlier results in that contralateral neurones showed greater spontaneous activity, greater receptive field size and greater afterdischarge in response to noxious mechanical and to noxious heat stimulation. Studies using spinally-intact rats report enlarged hind paw receptive fields bilaterally (Behbehani and Dollberg-Stolik 1994; Takaishi et al. 1996) and greater mechanical stimulation-evoked activity of ipsi- and contralateral spinal dorsal horn neurones (Pertovaara et al. 1997) in models of peripheral neuropathy. This contralateral effect may be the basis for clinical

disorders that exhibit "mirror" pains (Kozin et al. 1976; Procacci and Maresca 1987).

Mechanisms underlying these changes

A number of different mechanisms could underlie the changes observed in the physiological properties of ipsilateral dorsal horn neurones in the present study. The mechanisms discussed below could be acting in conjunction with each other, or one or more may predominate. The data in this study tend to support the concept that the changes we are reporting here in ipsilateral dorsal horn neurones were due to a change in the properties of sensory afferents, particularly myelinated afferents.

(i) Central sensitization

It has been suggested that nerve injury-induced changes in the properties of sensory afferent fibers could yield a difference in the properties of spinal neurones, such that a central sensitization occurs (Koltzenburg et al. 1994; Mailis et al. 1997; Sandkühler and Liu 1998). However, spontaneous nociceptive afferent input from a peripheral focus has been reported to maintain altered central processing in clinical neuropathic pain (Gracely et al. 1992; Campero et al. 1998). Even long-term potentiation in the isolated peripheral nerve-spinal cord preparation is dependent on C fiber afferent input for induction and expression (Lozier and Kendig 1995).

In any case, while central sensitization of spinal neurones to synaptic inputs may

be perceived to be able to account for the greater spontaneous activity of dorsal horn neurones, this cannot explain the preferential effect on the afterdischarge. One might expect that a central sensitization would have been manifested as including an increase in both the initial discharge and afterdischarge components of the response to noxious cutaneous stimulation. Instead, what was observed was a selectively greater afterdischarge only. Furthermore, a change in central sensitization also cannot account for the preferential effect on mechanical vs. heat nociceptive inputs. In the spinalized rat, if we consider central mechanisms alone, sensitization of wide dynamic range neurones, without supraspinal modulation, would presumably result in modality-independent alteration in sensory processing. On the other hand, if we consider different inputs projecting onto a single wide dynamic range neurone as in the present study, it follows that in cuff-implanted rats, as noxious mechanical and heat responses were each unique in terms of magnitude and duration, differential changes in afferent sensory processing may be more accountable for the different responses and may arise from alteration specifically in A and C fiber expression and function.

(ii) Decreased spinal inhibitory mechanisms

Greater excitability could be attributable to decreased inhibition, or disinhibition. A decrease in GABAergic interneurones (Ibuki et al. 1997; Eaton et al. 1998) and a decrease in extracellular GABA (Stiller et al. 1996) have been reported in the spinal cord in animal models of neuropathic pain. Reduced glycine receptors (Simpson and Huang

1998) and glutamate decarboxylase-immunoreactive cells (Eaton et al. 1998) and increased expression of dark neurones (Sugimoto et al. 1990; Hama et al. 1994, 1996; Mao et al. 1997) have also been reported in the spinal dorsal horn in neuropathic rats. However, again there is no indication of how such a loss of inhibition in the dorsal horn can account for more than an elevated spontaneous discharge of dorsal horn nociceptive neurones. Furthermore, there is no obvious explanation of how such a loss could account for the selectively greater afterdischarge rather than an overall nonselective increase in the total response to nociceptive inputs.

(iii) Spinal vs. supraspinal involvement

Previous behavioral studies have argued that increased tactile sensitivity of the cuff-implanted hind paw is sustained predominantly via a supraspinal loop producing a descending facilitation. This is based on observations that tactile allodynia in nerve-injured rats is substantially decreased or abolished following spinal transection (Pertovaara et al. 1996; Kauppila 1997; Bian et al. 1998; Kauppila et al. 1998) or lidocaine injection into the rostral ventral medulla or the periaqueductal grey (Pertovaara et al. 1996). The data in the present study can be interpreted to suggest that processing of the excitatory effects of mechanical stimulation does not rely exclusively on tonic descending facilitation. Rather it appears to be sustained via peripheral and/or spinal mechanisms. This is consistent with earlier data showing that pinch stimulation produces a withdrawal reflex in spinalized, spinal nerve-ligated rats (Bian et al. 1998).

(iv) Changes in myelinated fibers

Myelinated fibers have been implicated in mechanical hyperalgesia associated with nerve injury (Campbell et al. 1988; Gautron et al. 1990; Shir and Seltzer 1990), postherpatic neuralgia (Nurmikko et al. 1991; Baron and Saguer 1993; Baron et al. 1997) and peripheral neuropathic pain (Ochoa and Yarnitsky 1993; Mailis et al. 1997); C fibers do not seem to be involved in mechanical allodynia in animal models of neuropathic pain (Mosconi and Kruger 1996; Ossipov et al. 1999). In the present study, whether cuff implantation-induced changes derived from *de novo* mechanisms or modification of existing sensory neurones was not determined. However, there are three changes which have been proposed by other investigators to occur in myelinated afferents which might account for the observations in the present study and for neuropathic pain in humans, altered expression of ion channels and a change in phenotype. Each will be discussed as each appears able to contribute to the observations in the present study.

Increased Na^+ channel expression would be expected to lead to greater excitability. Ectopic activity in primary afferents has been documented extensively (Kajander and Bennett 1992; Kajander et al. 1992; Yoon et al. 1996; Zhang et al. 1997; Ali et al. 1999; Lee et al. 1999; Pan et al. 1999). This may derive specifically from dorsal root ganglion cells (Xie et al. 1995; Study and Kral 1996), from the site of the nerve injury (Matzner and Devor 1994; Tal and Eliav 1996) or even from the peripheral receptive field (Gracely et al. 1992). A reasonable mechanism by which this might occur is an altered expression of Na^+ channels in dorsal root ganglion cells (Novakovic et al. 1998; Porreca et al. 1999;

Waxman et al. 1999) as well as at the site of injury in these fibers (Matzner and Devor 1994; Novakovic et al. 1998). Therefore, it is not unrealistic to suggest that altered ion channel expression may be the source of altered activity which, in turn, provides a barrage of tonic excitatory input to spinal dorsal horn neurones. This may be interpreted to account for the greater spontaneous activity shown here in short- and long-term rats as well as the for the occurrence of ectopic activity from the periphery in neuropathic pain patients (Gracely et al. 1992), specifically in myelinated afferents (Campero et al. 1998) and the spontaneous pain seen with peripheral neuropathy in human patients. An increased density of these channels along an axon may also lead to repetitive action potentials or high frequency firing (Campero et al. 1998; Waxman 1999) perhaps arising from a single action potential (Campero et al. 1998) arriving at the region of abundant channels.

An additional possible mechanism which may account for the results in the present study is that a phenotypic change took place in large diameter sensory neurones (Koerber et al. 1999; Zhou et al. 1999). A predominance of degenerative/regenerative changes in large, myelinated fibers vs. small, unmyelinated fibers following sciatic nerve constriction (Basbaum et al. 1991; Munger et al. 1992; Nuytten et al. 1992; Coggeshall et al. 1993; Guilbaud et al. 1993) may account for the relatively greater effect of cuff implantation on the response to pinch vs. that to noxious heat stimulation in the present study, and to the greater incidence of mechanical vs. heat hyperalgesia in neuropathic patients (Bouhassira et al. 1999; Sieweke et al. 1999). Chronic sciatic nerve constriction, partial nerve ligation (Marchand et al. 1994; Ma and Bisby 1998), spinal nerve ligation (Fukuoka et al. 1998)

and axotomy (Noguchi et al. 1994; Noguchi et al. 1995; Miki et al. 1998) induce the expression of preprotachykinin gene, substance P and calcitonin gene-related peptide mRNA in large diameter sensory neurones. Destruction of dorsal horn cells expressing the NK-1 receptor decreases mechanical hyperalgesia in the chronic sciatic nerve model (Benoliel et al. 1999) and NK-1 receptor antagonists block hyperalgesia in animal models of neuropathic pain (Campbell et al. 1998; Coudoré-Civiale et al. 1998; Cumberbatch et al. 1998; Walpole et al. 1998). Given that mechanical stimulation, whether innocuous or noxious in intensity, may activate large diameter sensory neurones, the possibility is considered here that activation of Aβ fibers may have contributed to the pinch stimulation-evoked afterdischarge via the slow, prolonged excitatory effects of substance P (De Koninck and Henry 1991).

It has also been proposed that retraction of central terminals of unmyelinated fibers after nerve injury allows the projection of myelinated fibers into regions in the spinal dorsal horn containing an abundance of nociceptive second order neurones (McMahon and Kett-White 1991; Kitchener et al. 1994; Coggeshall et al. 1997; Doubell and Woolf 1997), with the result that normally non-nociceptive afferents now project to nociceptive neurones (Lekan et al. 1996; Lekan et al. 1997; Nakamura and Myers 1999). A reorganization of the central terminals of Aβ fibers has been reported following nerve constriction (Lekan et al. 1997; Nakamura and Myers 1999) so that they project into the superficial laminae of the dorsal horn (Lekan et al. 1996; Wilson and Kitchener 1996). Although reorganization of the central terminals of sensory neurones was not specifically examined

in this study, the occurrence of a retraction of unmyelinated fibers and sprouting of large diameter myelinated fibers onto nociceptive spinal dorsal horn neurones is certainly consistent with the preferential effect on mechanical vs. heat nociceptive inputs.

(v) Contralateral mechanisms

The mechanisms by which modification of sensory processing is induced and sustained contralaterally remain elusive and are speculative to date. Subcutaneous injection of 0.15 ml of normal saline into the footpad on the plantar surface of one hind paw has been shown to induce swelling and hyperalgesia of the contralateral hind paw (Levine et al. 1985). Interruption of venous circulation to the injured limb by vein ligation did not alter the response in the contralateral hind paw demonstrating that the contralateral neurogenic inflammation and hyperalgesia were likely neurally mediated. However, as experiments in these earlier studies were run in spinally-intact rats, supraspinal modulation cannot be excluded. The concept of bilateral changes may be substantiated by bilateral expression of dark neurones (Sugimoto et al. 1990; Hama et al. 1994; Hama et al. 1996; Mao et al. 1997) and bilateral decreases in numbers of GABA- (Ibuki et al. 1997; Eaton et al. 1998) and glutamate decarboxylase-immunoreactive cells (Eaton et al. 1998) and a bilateral decrease in glycine receptors (Simpson and Huang 1998) in the spinal cord in nerve injury models.

It is difficult to reconcile how alterations in sensory processing in the contralateral dorsal horn can account for the preferential effect of cuff implantation on mechanical vs.

heat nociceptive inputs, and fast initial discharge vs. slowly-decaying afterdischarge responses. It should be pointed out that in addition to contralateral changes centrally, contralateral changes may also occur peripherally. For example, unilateral postherpetic neuralgia may be associated with bilateral sensory neurone damage (Oaklander et al. 1998), and unilateral nerve injury in the rat down-regulates Na^+ channel SCN10A mRNA bilaterally in rat dorsal root ganglia (Oaklander and Belzberg 1997). Moreover, transneuronal effects evoked by saphenous nerve injury in the rat are reported to be restricted specifically to neurones of the contralateral homologous nerve (Kolston et al. 1991). Therefore, to account for the effects of cuff implantation on hyperexcitability in the contralateral dorsal horn, peripheral changes contralaterally must also be considered.

Conclusions

The present study has been directed towards understanding the effects of experimental peripheral neuropathy on sensory processing in the spinal dorsal horn. Specifically, the data in this study demonstrate that chronic implantation of a 2 mm PE-10 polyethylene cuff around the sciatic nerve results in markedly elevated on-going activity and a greater slowly-decaying afterdischarge in response to noxious mechanical stimulation. The effect of cuff implantation on the noxious heat-induced slowly-decaying afterdischarge was considerably less. As acutely spinalized rats were used, cuff implantation-evoked hyperexcitability in the spinal dorsal horn does not rely exclusively on sensory processing via a supraspinal loop and tonic descending excitation, as has been proposed elsewhere. Considering all the data, it can be concluded that there is a greater change in fibers mediating noxious mechanical than noxious thermal inputs.

What is visualized to have occurred in the present study, and what is most consistent with both our data as well as data from basic and clinical studies, is that the cuff implantation or compromize of peripheral sensory nerves alters protein synthesis in the cell body in the dorsal root ganglion. Three particular changes appear significant to neuropathic pain, at least on the basis of our data. One change may be an altered regulation of different ion channels, perhaps Na^+ channels, rendering the primary afferents spontaneously active and exhibiting greater discharge in response to afferent signals (Waxman et al. 1999). The second change may be that large diameter afferent sensory neurones, which normally do not produce slow-acting chemical mediators such as

substance P, now begin to do so. Thus, when activated by their adequate stimulus, in this case noxious pinch stimulation, they release from their central terminals not only glutamate, as they normally do, but now also substance P. In addition, the retraction of central terminals of unmyelinated fibers allows the projection of myelinated fibers into regions containing an abundance of nociceptive second order neurones, so that normally non-nociceptive afferents now project to nociceptive neurones. Given this scenario, activation of myelinated afferents by noxious stimuli would not be expected to elicit an initial discharge any different in cuff-implanted rats than in normal rats. This is what we observed in the present study. Rather, one would expect to see a greater afterdischarge as a result of the subsequent release of more substance P, in this case from the additional population of afferents, that is myelinated afferents. Thus, we interpret our data to be most consistent with the hypothesis that chronic cuff implantation in the rat induces phenotypic changes in myelinated sensory fibers. In light of these findings, it is speculated that these alterations in sensory processing may conceivably constitute the neurophysiological bases for the mechanical hyperalgesia and spontaneous pain of clinical neuropathic pain. Appreciation of the preferential influence of experimental neuropathy on on-going discharge and on mechanical stimulation-elicited activity of dorsal horn neurones in the present study may provide important insight into the development of novel approaches in the clinical management of chronic neuropathic pain, which remains infamously refractory to medical treatment.

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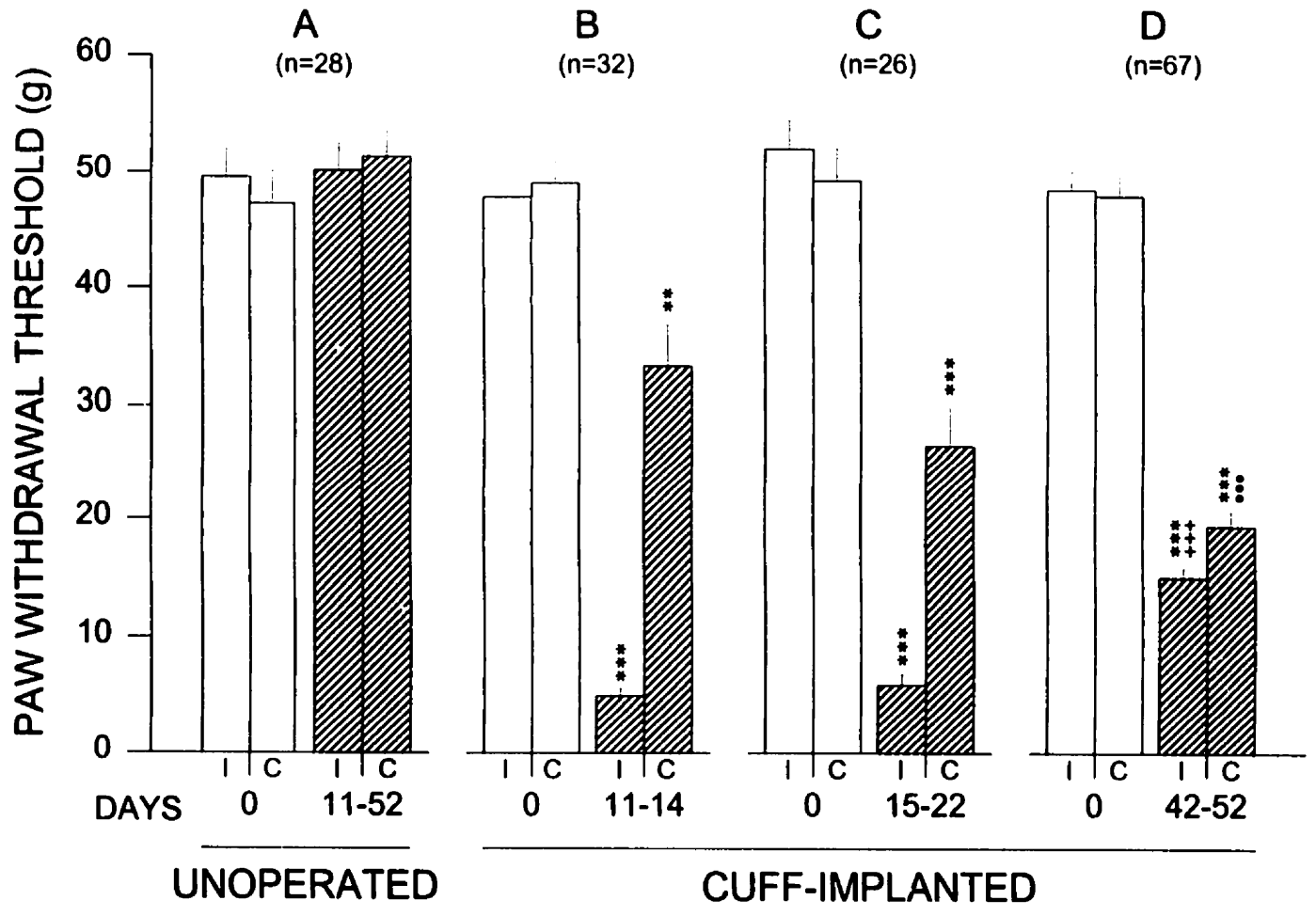
A
(n=28)
B
(n=32)
C
(n=26)
D
(n=67)
PAW WITHDRAWAL THRESHOLD (g)
60
50
40
30
20
10
0
I C I C
DAYS
0
11-52
0
11-14
0
15-22
0
42-52
UNOPERATED
CUFF-IMPLANTED

Figure 1. Hind paw withdrawal thresholds in the von Frey hair test. The vertical axis represents the withdrawal threshold measured in grams. The horizontal axis shows hind paw withdrawal thresholds ipsi- (I) and contralateral (C) to the cuff-implanted sciatic nerve in short- (days 11-14 and 15-22 shown by hatched bars) and in long-term (days 42-52 shown by hatched bars) rats. Thresholds from unoperated rats are also included. (A) In 28 unoperated rats, there was no significant difference between the hind paw withdrawal thresholds on day 0 and the pooled paw withdrawal thresholds measured at time points on days 11-14, 15-22 or 42-52. (B) In 32 short-term rats tested on days 11-14, the ipsilateral hind paw withdrawal threshold was markedly decreased. The contralateral hind paw withdrawal threshold was also decreased but to a lesser degree. (C) In 26 short-term rats tested on days 15-22, reduction of the ipsi- and contralateral hind paw withdrawal threshold was similar to that in (B). (D) In 67 long-term rats, the ipsilateral hind paw withdrawal threshold was greater (+++ $P < 0.001$ vs. respective hind paw withdrawal threshold on days 11-14 and 15-22) while the contralateral hind paw withdrawal threshold was reduced further (••• $P < 0.001$ vs. respective hind paw withdrawal threshold on days 11-14; ** $P < 0.01$ and *** $P < 0.001$ vs. respective hind paw withdrawal threshold at day 0)

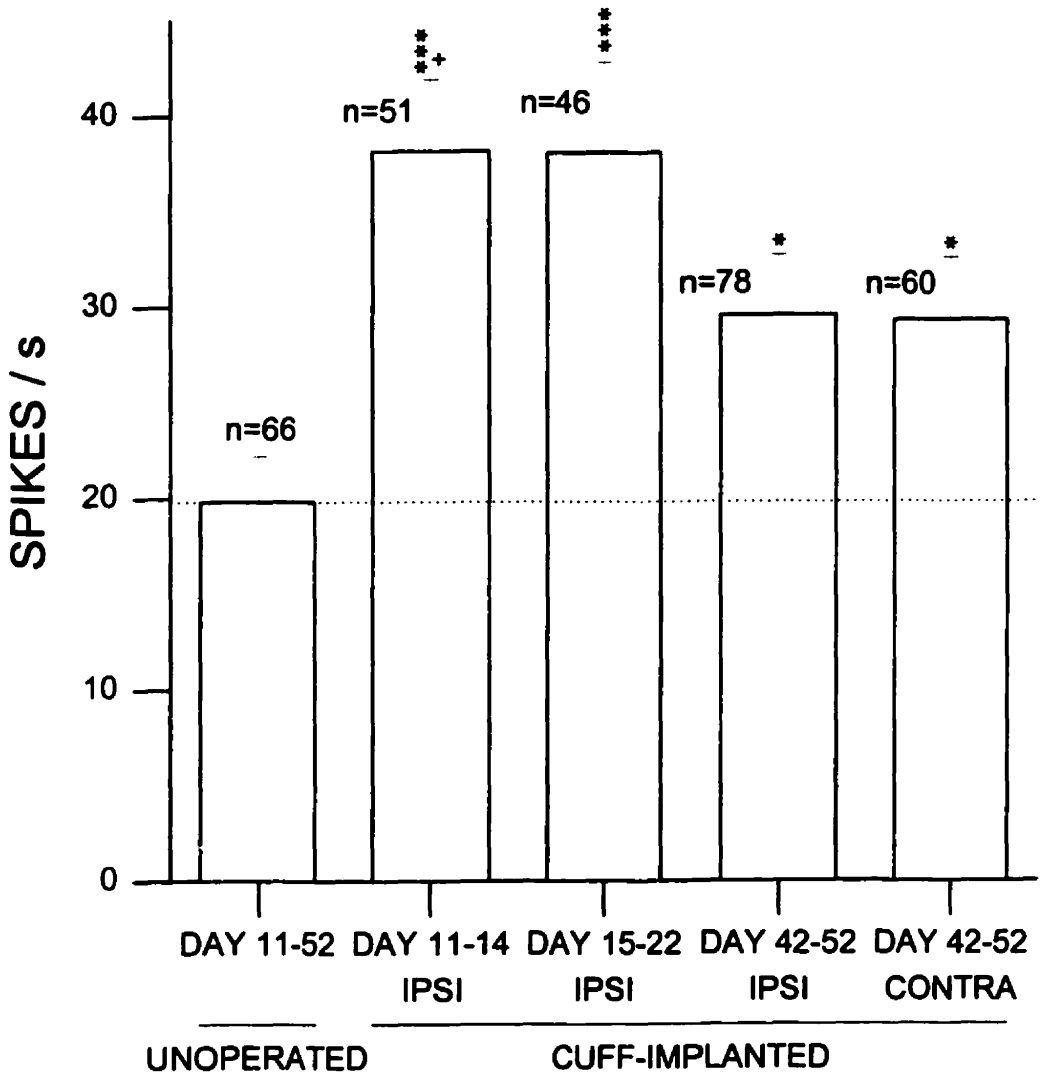
SPIKES / s
40
30
20
10
0
n=66
n=51
n=46
n=78
n=60
DAY 11-52
DAY 11-14 IPSI
DAY 15-22 IPSI
DAY 42-52 IPSI
DAY 42-52 CONTRA
UNOPERATED
CUFF-IMPLANTED

Figure 2. On-going activity of wide dynamic range spinal dorsal horn neurones in unoperated and in cuff-implanted rats. The vertical axis shows the number of spikes per second of stable on-going neuronal discharge. The horizontal axis shows on-going activity in unoperated rats (days 11-52) and in short- (days 11-14 or 15-22) and in long-term (days 42-52) cuff-implanted rats. In unoperated rats, mean on-going activity is illustrated by the dotted line. In cuff-implanted rats, mean on-going activity in ipsilateral (IPSI) neurones in short- and long-term rats and in contralateral (CONTRA) neurones in long-term rats was greater than that in unoperated rats. Furthermore, mean on-going activity in day 11-14 short-term rats was greater than that in long-term rats tested ipsilaterally. The number of neurones tested in each group is indicated above each histogram. (* $P < 0.05$ and *** $P < 0.001$ vs. unoperated; + $P < 0.05$ vs. IPSI on days 42-52)

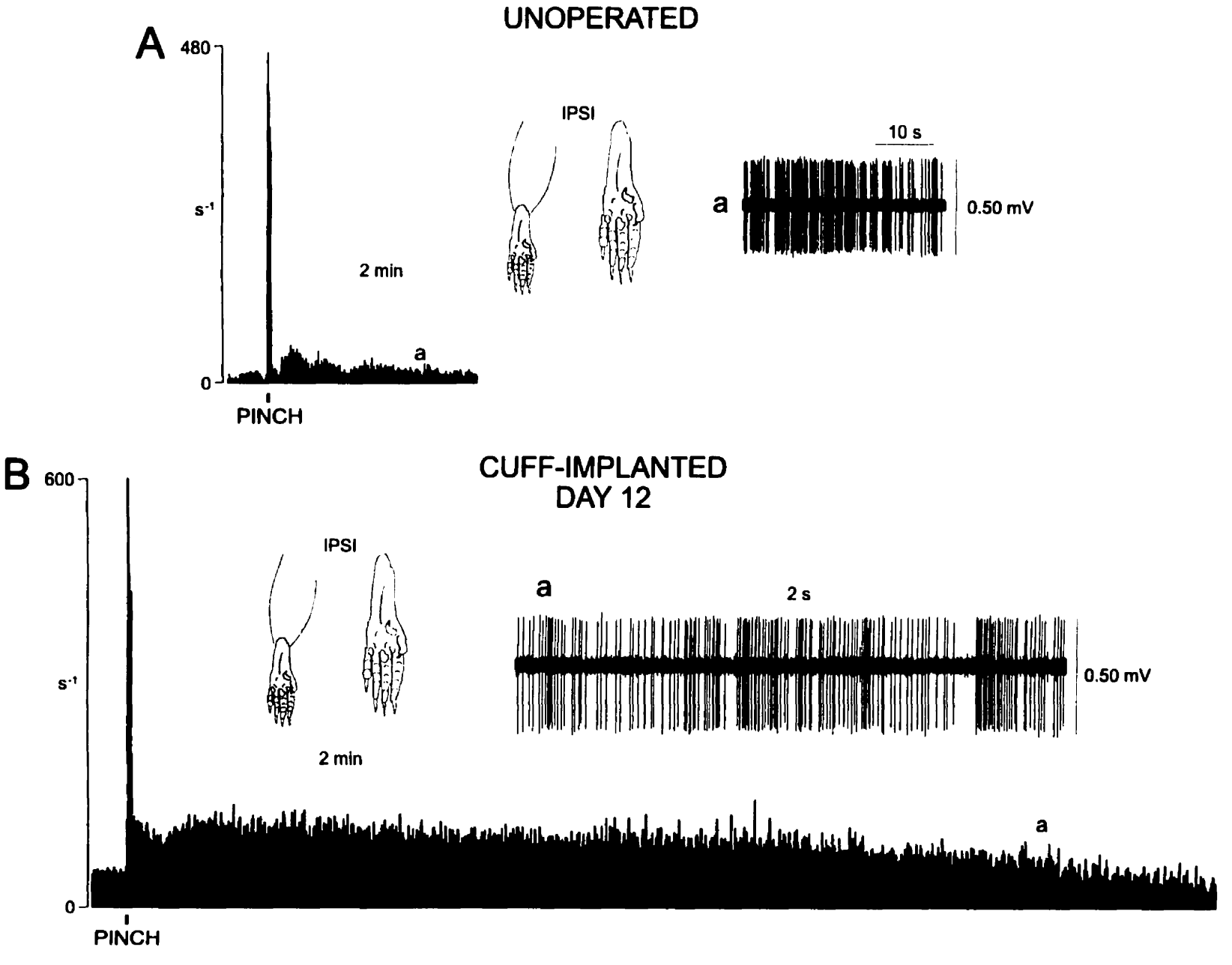
UNOPERATED
A
480
s⁻¹
0
2 min
a
PINCH
IPSI
10 s
a
0.50 mV
B
CUFF-IMPLANTED
DAY 12
600
s⁻¹
0
IPSI
a
2 s
0.50 mV
2 min
a
PINCH

Figure 3. Effect of pinch stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. (A) The ratemeter record on top shows that in an unoperated rat pinch stimulation (21 N for 3 s) produced a fast initial discharge which lasted only for the duration of the stimulus followed by a slowly-decaying afterdischarge which persisted for 1.5 to 2 min. The neurone was 1106 μm deep from the dorsal surface of the spinal cord. The horizontal axis represents time and the vertical axis represents frequency of spikes (1 s bin width). The time and duration of the pinch application are shown by the narrow rectangle below the ratemeter histogram. The inset on the left shows the cutaneous receptive field to touch stimulation, depicted by the shaded area. The inset on the right shows the area subjected to pinch stimulation. The extracellular record at right shows single unit activity taken at 'a' in the ratemeter record. (B) In a short-term cuff-implanted rat tested on day 12, pinch stimulation of the ipsilateral hind paw (IPSI) produced a fast initial discharge which lasted only for the duration of the stimulus and a slowly-decaying afterdischarge which persisted for approximately 18 min (1162 μm). Descriptions of the insets are otherwise similar to those in (A). The extracellular record shows the spike activity taken at 'a'.

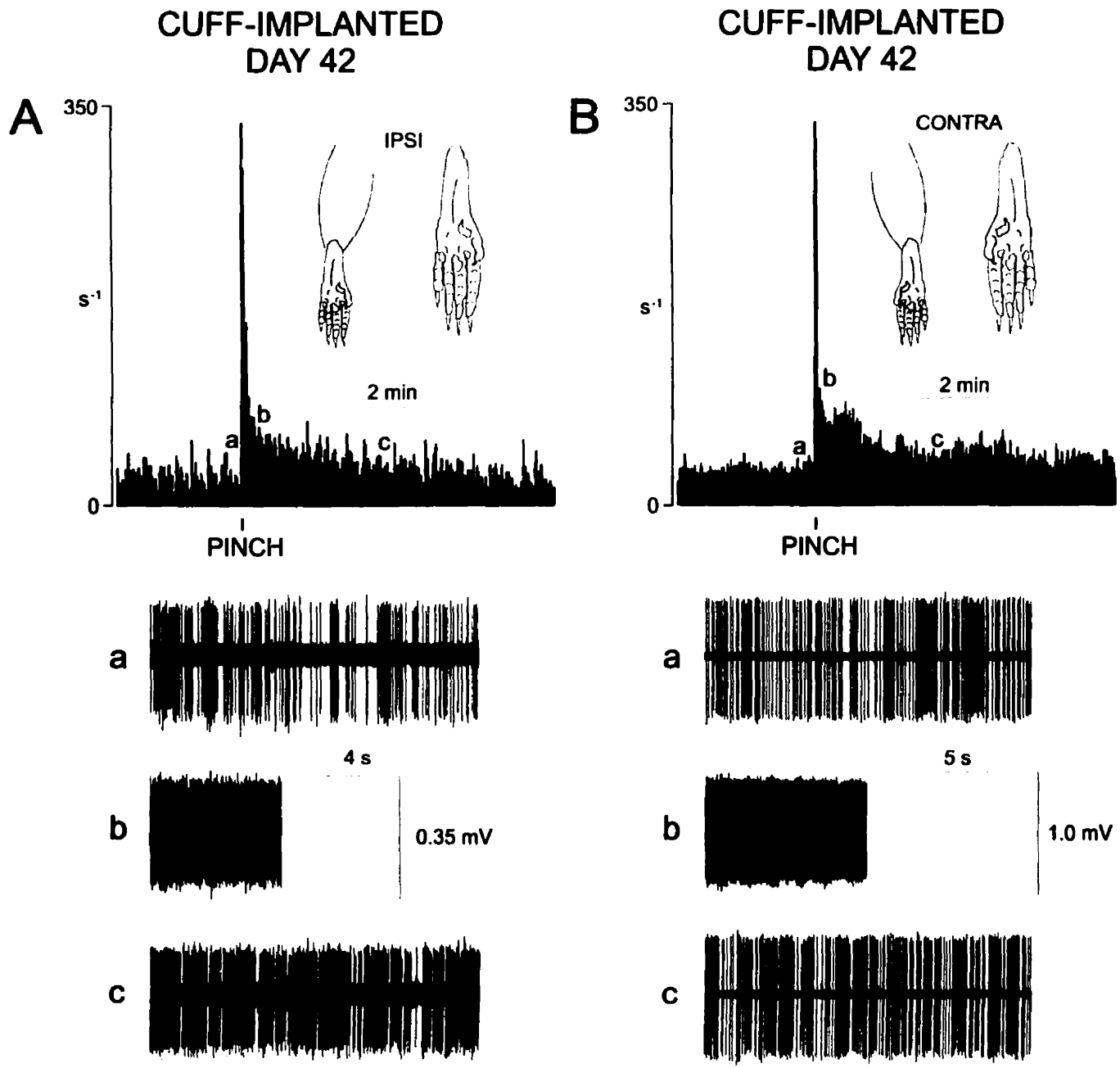
CUFF-IMPLANTED
DAY 42
A
350
s-1
0
IPSI
2 min
a
b
c
PINCH
a
4 s
b
0.35 mV
c
CUFF-IMPLANTED
DAY 42
B
350
s-1
0
CONTRA
2 min
b
a
c
PINCH
a
5 s
b
1.0 mV
c

Figure 4. Effect of pinch stimulation on wide dynamic range dorsal horn neuronal activity in long-term cuff-implanted rats. (A) In an ipsilateral (IPSI) neurone, pinch stimulation (21 N for 3 s) of the receptive field of the ipsilateral hind paw produced a fast initial discharge which lasted for the duration of the stimulus and a slowly-decaying afterdischarge which persisted for 4 to 5 min (722 μm). Extracellular records show single unit activity at times selected at 'a', 'b' and 'c' in the ratemeter histogram. Details of the insets are otherwise the same as in Figure 3. (B) In a contralateral (CONTRA) neurone, pinch stimulation of the receptive field of the contralateral hind paw evoked a fast initial discharge and a slowly-decaying afterdischarge which also persisted for approximately 4 to 5 min (604 μm).

PINCH

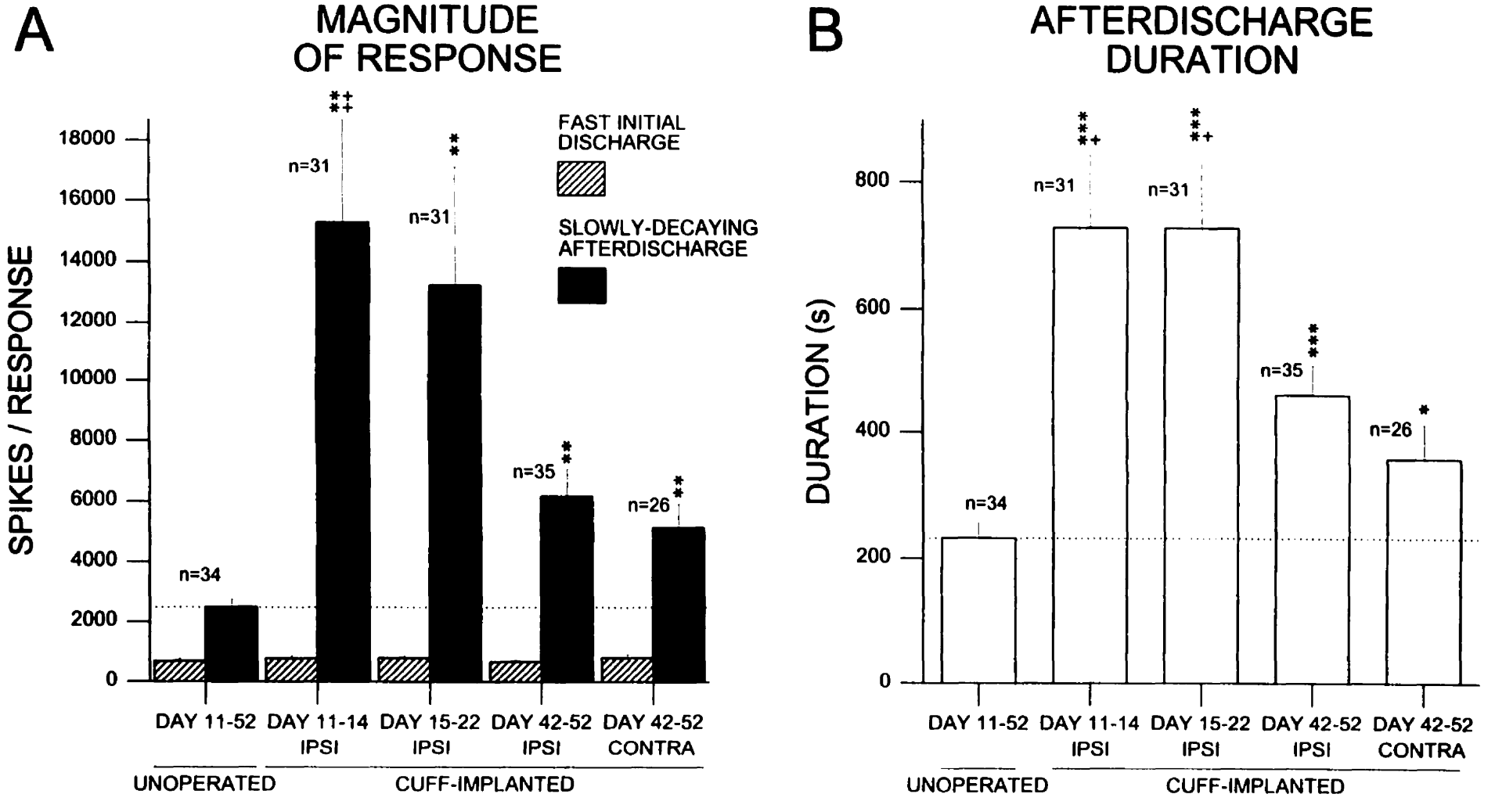
A
MAGNITUDE OF RESPONSE
SPIKES / RESPONSE
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
n=34
n=31
n=31
n=35
n=26
DAY 11-52
DAY 11-14 IPSI
DAY 15-22 IPSI
DAY 42-52 IPSI
DAY 42-52 CONTRA
UNOPERATED
CUFF-IMPLANTED
B
AFTERDISCHARGE DURATION
DURATION (s)
800
600
400
200
0
n=34
n=31
n=31
n=35
n=26
DAY 11-52
DAY 11-14 IPSI
DAY 15-22 IPSI
DAY 42-52 IPSI
DAY 42-52 CONTRA
UNOPERATED
CUFF-IMPLANTED

Figure 5. Summary of the effects of pinch stimulation on wide dynamic range neuronal activity in unoperated and in cuff-implanted rats. The vertical axis shows the number of spikes per pinch-evoked fast initial discharge and slowly-decaying afterdischarge response. The horizontal axis shows fast initial discharge and slowly-decaying afterdischarge responses in unoperated rats (days 11-52) and in short- (days 11-14 and 15-22) and long-term (42-52) cuff-implanted rats. (A) In unoperated rats, the mean number of spikes per slowly-decaying afterdischarge response is depicted by the dotted line. In cuff-implanted rats, the mean magnitude of the slowly-decaying afterdischarge in ipsilateral (IPSI) neurones in short- and long-term rats and in contralateral (CONTRA) neurones in long-term rats was greater than that in unoperated rats. Furthermore, the mean magnitude of the slowly-decaying afterdischarge in day 11-14 short-term rats was greater than that in long-term rats tested ipsilaterally. Cuff implantation had no effect on the fast initial discharge response. (** $P < 0.01$ vs. unoperated; ++ $P < 0.01$ vs. IPSI on days 42-52) (B) Effect of cuff implantation on the duration of the pinch-evoked slowly-decaying afterdischarge. The vertical axis represents duration expressed in seconds. The horizontal axis shows the duration of the slowly-decaying afterdischarge in unoperated rats and in short- and long-term cuff-implanted rats at the time points shown in (A). In unoperated rats, the mean duration of the slowly-decaying afterdischarge is illustrated by the dotted line. In cuff-implanted rats, the mean duration of the slowly-decaying afterdischarge in ipsilateral neurones in short- and long-term rats and in contralateral neurones in long-term rats was greater than that in unoperated rats. In addition, in short-term rats, the mean duration of the slowly-decaying afterdischarge was greater than that in long-term rats tested

ipsilaterally. (* $P < 0.05$ and *** $P < 0.001$ vs. unoperated; + $P < 0.05$ vs. IPSI on days 42-52)

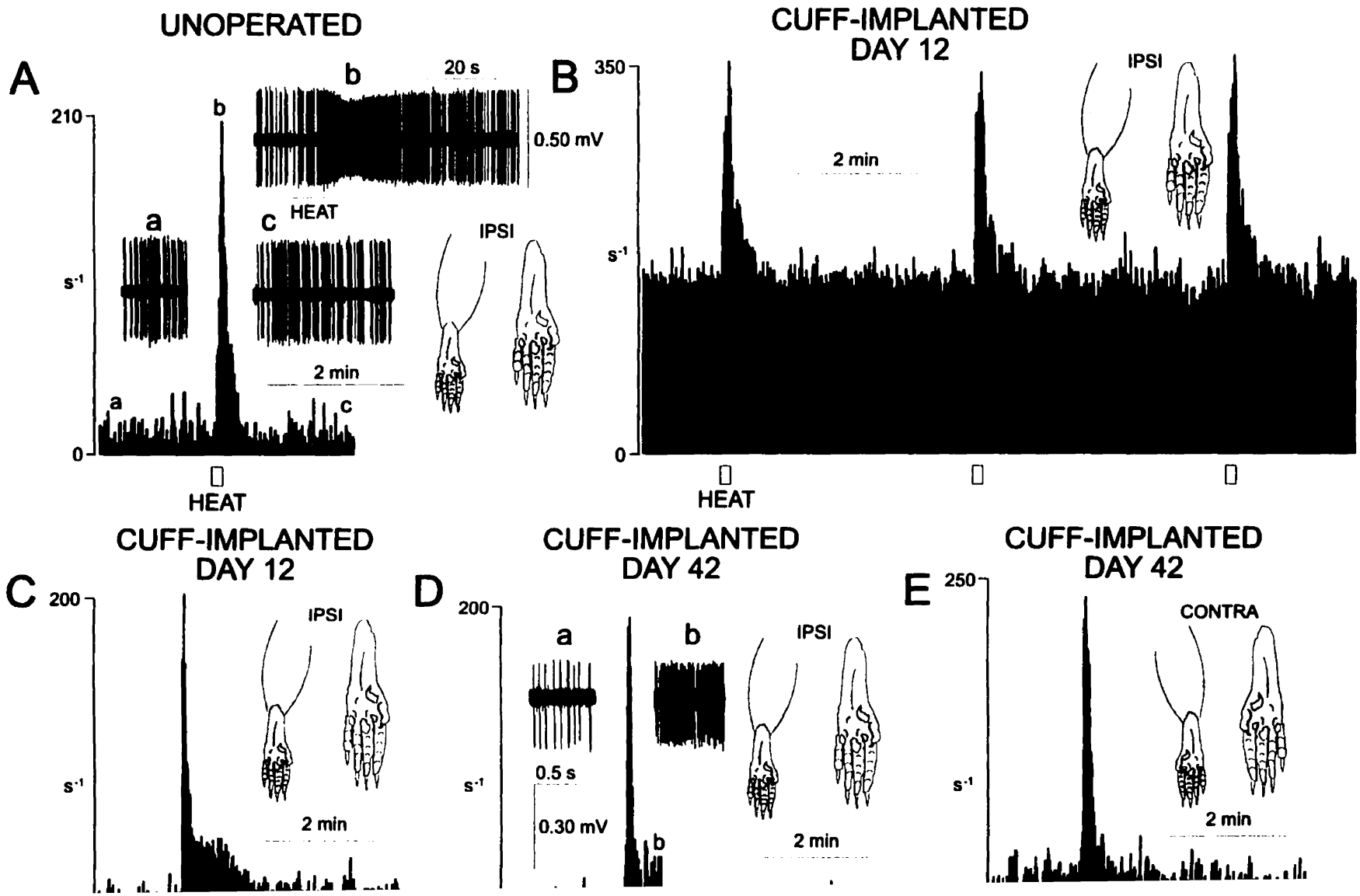
UNOPERATED
A
210
0
s-1
a
b
c
20 s
0.50 mV
HEAT
IPSI
2 min
HEAT
CUFF-IMPLANTED
DAY 12
B
350
0
s-1
IPSI
2 min
HEAT
CUFF-IMPLANTED
DAY 12
C
200
s-1
IPSI
2 min
CUFF-IMPLANTED
DAY 42
D
200
s-1
a
b
IPSI
0.5 s
0.30 mV
2 min
CUFF-IMPLANTED
DAY 42
E
250
s-1
CONTRA
2 min

Figure 6. Effect of noxious heat stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. (A) The ratemeter record at the top left shows that in an unoperated rat heat stimulation (50°C for 10 s) produced a fast initial discharge which lasted until the end of the stimulus and a slowly-decaying afterdischarge which persisted for 0.5 to 1 min (782 μm). The time and duration of the heat stimulus are shown by the narrow rectangle below the ratemeter histogram. The inset on the left shows the cutaneous receptive field to touch stimulation, depicted by the shaded area. The inset on the right shows the area subjected to heat stimulation. Extracellular records show single unit activity at times selected at 'a', 'b' and 'c', in the ratemeter histogram. (B) In a short-term cuff-implanted rat tested on day 12 heat stimulation of the ipsilateral hind paw (IPSI) produced a fast initial discharge and a slowly-decaying afterdischarge which persisted for 0.5 to 1 min (1182 μm). Note the greater on-going activity and stable responses of this neurone to repetitive heat application. Descriptions of the insets are otherwise similar to those in (A). (C) Neuronal response in another short-term rat tested on day 12 (692 μm). (D) In a long-term cuff-implanted rat tested on day 42 heat stimulation of the ipsilateral hind paw (IPSI) produced a fast initial discharge followed by a slowly-decaying afterdischarge which persisted for approximately 1 min (722 μm). Extracellular records show single unit activity at times selected at 'a' and 'b' in the ratemeter histogram. (E) In another long-term rat tested on day 42 heat stimulation of the contralateral hind paw (CONTRA) produced a fast initial discharge followed immediately by a slowly-decaying afterdischarge which persisted for 0.5 to 1 min (552 μm).

NOXIOUS HEAT

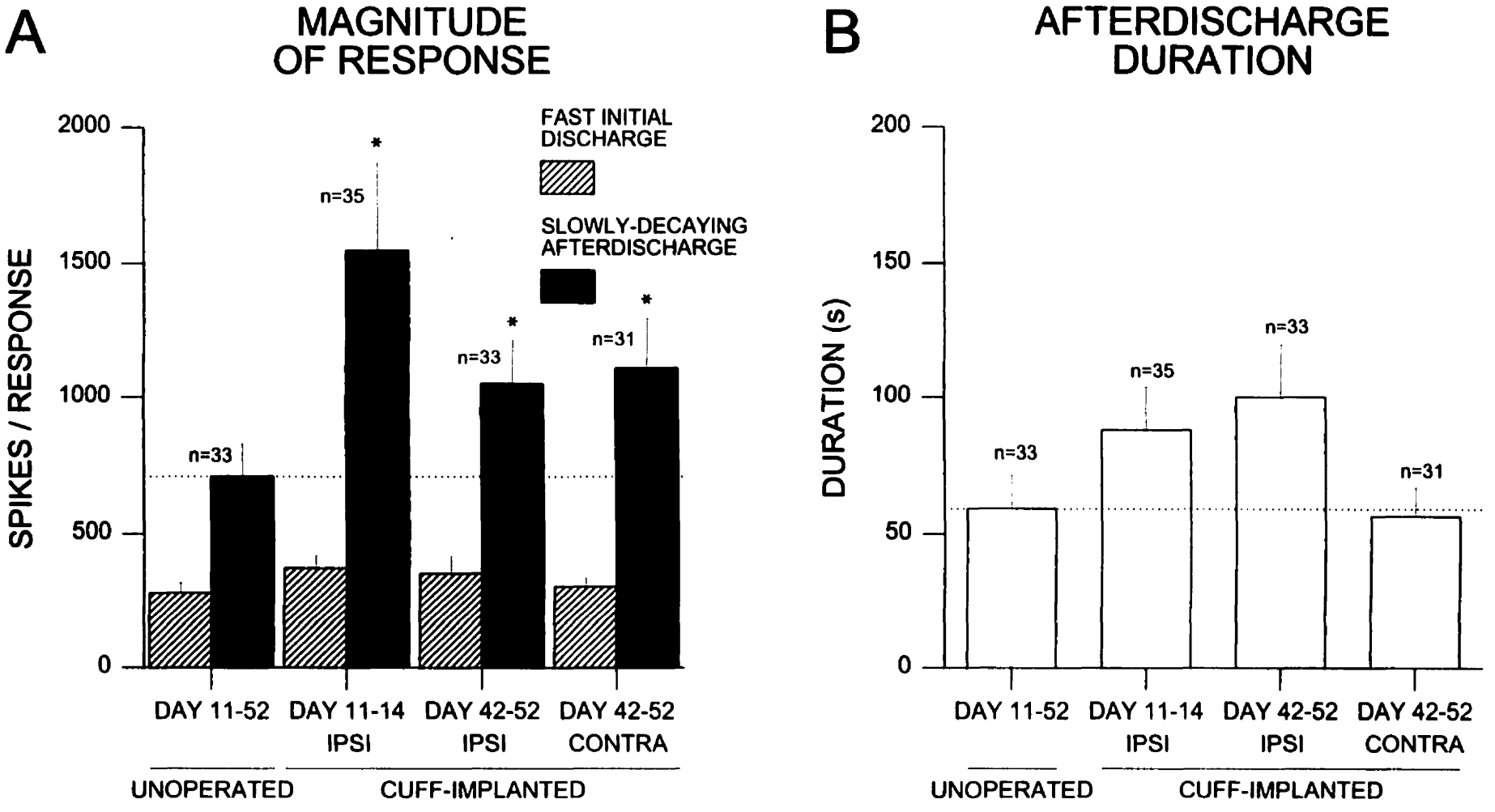

Figure 7. Summary of the effects of noxious heat stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. The vertical and horizontal axis are otherwise similar to those shown previously. (A) In unoperated rats, the mean number of spikes per slowly-decaying afterdischarge response is depicted by the dotted line. In cuff-implanted rats, the magnitude of the slowly-decaying afterdischarge in ipsilateral (IPSI) neurones in short- and long-term rats and in contralateral (CONTRA) neurones in long-term rats was greater than that in unoperated rats. Cuff implantation had no effect on the fast initial discharge response. (* $P < 0.05$ vs. unoperated) (B) Effect of cuff implantation on the duration of the heat-evoked slowly-decaying afterdischarge. In unoperated rats, the mean duration of the slowly-decaying afterdischarge is illustrated by the dotted line. In cuff-implanted rats, the mean duration of the slowly-decaying afterdischarge in ipsilateral neurones in short- and long-term rats and in contralateral neurones in long-term rats was not significantly different compared to that in unoperated rats.

Unifying Statement

Chapter 6 has confirmed that in this animal model of peripheral neuropathy there is an increased on-going discharge of dorsal horn neurons and an increased response of nociceptive neurones to noxious stimuli. This may be the neural correlate to the spontaneous pain and hyperalgesia seen in human patients suffering from neuropathic pain. However, allodynia, a painful response to a normally innocuous stimulus also characterizes these patients. The next chapter, Chapter 7, examines the effect of innocuous pressure stimulation again at the cellular level in acutely spinalized, cuff-implanted rats at the optimal time points established in Chapter 5. Electrical stimulation of afferent sensory neurons was also carried out in an attempt to identify the different fiber types involved in mediating the hyperexcitability of dorsal horn neurons.

Chapter 7

Cellular Mechanisms of Tactile Allodynia in a Spinalized Rat Model of Peripheral Neuropathy: Changes in Myelinated Afferent Inputs Implicated

Abstract

The purpose of the present study was to document the spinal cellular correlate of the mechanical allodynia and spontaneous pain seen in an animal model of peripheral neuropathy. Experimental neuropathy was induced in male Sprague Dawley rats by placing a 2 mm polyethylene cuff around the sciatic nerve. von Frey hair testing confirmed tactile allodynia in cuff-implanted rats before electrophysiological testing. Anesthetised rats were spinalized for extracellular recording. On-going activity of single dorsal horn neurons was greater in cuff-implanted than in control rats (n=70 neurons). Cuff-implanted rats were tested short-term (43 neurons tested on days 11-14, 53 on days 15-22) and long-term (days 42-52, 82 neurons ipsilateral and 49 contralateral). In control rats innocuous pressure stimulation (0.2 N for 3 s) evoked a typical brief excitation (n=20 neurons). However, in cuff-implanted rats, every neuron exhibited also a nociceptive-like afterdischarge persisting 2-4 min (23 neurons short-term, 25 long-term ipsilateral and 17 long-term contralateral). This afterdischarge was never observed in control rats. Electrical stimulation of the sciatic nerve at 4 and at 20 Hz produced an initial discharge that was the same in control and cuff-implanted rats. However, the afterdischarge was markedly greater in the latter group. It is suggested that the greater on-going discharge is the cellular correlate of spontaneous pain and the afterdischarge in response to innocuous mechanical stimulation is the correlate of tactile allodynia. Given that acutely spinalized rats were studied, only peripheral and/or spinal mechanisms can be considered to explain these data. The particularly exaggerated afterdischarge response to 20 Hz stimulation suggests a

predominant role of afferent neurons that respond preferentially to higher frequency nerve stimulation. Among different hypotheses, the one with which the present data are most compatible is that which proposes that chronic nerve injury or inflammation induces phenotypic changes predominantly in myelinated afferents. There may be a redistribution of membrane-bound ion channels in myelinated afferents, predominantly sodium channels, which leads to ectopic activity and thus greater discharge of dorsal horn neurons. With regard to innocuous mechanical stimulation-elicited synaptic input, a change in myelinated afferents occurs such that they now synthesize and release peptides, for example substance P, from their central terminals with the result that these myelinated afferents excite nociceptive neurons. This is the first electrophysiological study to identify the spinal correlates of mechanical allodynia and spontaneous pain in an animal model of peripheral neuropathy.

Introduction

Allodynia, defined as a pain or a nociceptive response provoked by an innocuous stimulus, is a common feature of chronic neuropathic pain. Yet it remains an enigma in terms of its underlying mechanism. Several hypotheses have been proposed to account for this allodynia. For example, changes within the central nervous system have been proposed (Jett et al. 1997; Goff et al. 1998; Lin et al. 1999; Röyttä et al. 1999), including increased excitability of dorsal horn neurons (Sandkühler and Liu 1998), altered signal transduction mechanisms (Inoue et al. 1998; Lin et al. 1999) and decreased inhibitory mechanisms (Porreca et al. 1998; Toda et al. 1998; Idänpään-Heikkilä and Guilbaud 1999). Based on behavioral studies, which show that tactile allodynia is decreased or abolished by spinal transection (Pertovaara et al. 1996; Kauppila 1997; Bian et al. 1998; Kauppila et al. 1998; Sung et al. 1998), it has been argued that this allodynia is sustained predominantly via a supraspinal loop with tonic descending facilitatory properties. To our knowledge, no attempt has been made at the cellular level to examine effects of experimental neuropathy independently of supraspinal modulation.

Based on studies showing increased peripheral sensory neuronal activity (Kajander and Bennett 1992; Zhang et al. 1997) in different models of neuropathic pain, there appears to be considerable evidence supporting the notion that central hyperexcitability may be sustained via peripheral mechanisms. A novel suggestion has also been made that a phenotypic change takes place in large diameter, non-nociceptive sensory neurons (Koerber et al. 1999) in models of peripheral neuropathy, so that their activation leads to the central

release of additional chemical mediators of synaptic transmission, such as peptides, which normally activate nociceptive rather than non-nociceptive second order neurons. Chronic sciatic nerve constriction, partial nerve ligation (Marchand et al. 1994; Ma and Bisby 1998) and axotomy (Noguchi et al. 1994, 1995; Fukuoka et al. 1998; Miki et al. 1998) markedly increase the expression of preprotachykinin gene, substance P and calcitonin gene-related peptide mRNA expression in large diameter sensory neurons. According to this, it would be expected that activation of Aβ fibers by low threshold, innocuous mechanical stimulation of the peripheral receptive field would elicit a nociceptive type of response, specifically a response with an afterdischarge, which normally is observed only in response to noxious stimulation. There is presently no direct evidence for this.

Therefore, the present electrophysiological study was done with two purposes in mind: to determine any cellular correlate at the spinal level of the behavioral changes seen in an animal model of neuropathic pain and to determine whether the response to myelinated fiber inputs is altered in this model.

Preliminary data have been presented in abstract form (Pitcher and Henry 1999a).

Materials and Methods

Animals

Experiments were performed using adult, male Sprague Dawley rats (375-425g) from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana, USA). They were housed in plastic cages containing wood chip bedding (Hardwood Laboratory Bedding, Northeastern Products Corp., Warrensburg, New York, USA) and maintained on a 12:12 h light:dark cycle (lights on at 07:00 h) with access to food and water *ad libitum*. Experiments were conducted during the light component of the cycle. Guidelines in *The Care and Use of Experimental Animals* by the Canadian Council on Animal Care (Vols. I and II) were strictly followed and all experiments were approved by the *McGill University Animal Care Committee*.

Cuff implantation

A variation of the technique of Mosconi and Kruger (1996) was used. Under Na-pentobarbital anesthesia (50 mg/kg, i.p., Abbott Laboratories, Limited, Montreal, Quebec, Canada) and aseptic conditions, the left hind paw was shaved and an incision in the skin was made above the biceps femoris muscle. The common sciatic nerve was exposed by blunt dissection through the muscle and was isolated from surrounding connective tissue using glass probes. The nerve was elevated minimally using a sterilized glass probe in order for a 2 mm section of split polyethylene tubing (Intramedic PE-90, Fisher Scientific Ltd., Whitby, Ontario, Canada) to be placed around the nerve. The

muscle layer was closed using 3-O silk suture thread (Ethicon Inc., Montreal, Quebec, Canada) and the skin layer was closed using 3 stainless steel suture clips (Fine Science Tools, Inc., North Vancouver, British Columbia, Canada). The topical antibacterial ointment, nitrofurazone (0.2%, Univet Pharmaceuticals Ltd., Milton, Ontario, Canada), was placed on the skin suture line to counter the risk of infection. The rat was then allowed to recover. Only two rats from the same testing group (ie. unoperated or cuff-implanted) were together in a cage.

von Frey hair test

The von Frey hair test was done immediately prior to electrophysiological testing or the day before to confirm the absence or presence of tactile allodynia. Tactile hind paw withdrawal threshold in rats was determined by applying von Frey hairs (Xymotech Biosystems, Inc., Montreal, Quebec, Canada) to the plantar surface of the hind paw. Application of the von Frey hairs to the hind paws was done by placing the rat on a platform designed and constructed specifically for von Frey hair testing (Pitcher et al. 1999a). Described briefly, the platform is made of plexiglass 3 mm thick. It is slightly opaque in appearance and contains 1.5 mm diameter holes in perpendicular rows, 5 mm apart throughout the entire area of the platform. A von Frey hair was applied through a particular hole to a hind paw. For testing, this platform was fixed in a transparent plexiglass chamber (30×30×30 cm).

The mechanical hind paw withdrawal threshold, determined using von Frey hairs,

was expressed in grams. Ten hairs ranging from 0.23 to 59.0 g were used. The bending force of each hair in grams was confirmed periodically by measuring the force exerted by the hair when applied to a Mettler AE 100 electronic balance. The hair was applied in a manner such that the degree of bending was the same as that when applied to the rat hind paw. Confirmation was done because it was determined that slight fluctuation in the bending force of a hair may occur with extended use. If this was the case, the new bending force in grams, determined using the electronic balance, was used as the value.

Testing was blind such that the experimenter was not aware of the kind of rat being tested, ie. unoperated or cuff-implanted. The protocol used in this study is similar to that used previously (Pitcher et al. 1999a,b). Briefly described, a testing session for a particular rat began after 5 min of habituation to the testing chamber. The series of von Frey hairs was applied from below the platform to the left hind paw in ascending order beginning with the lowest hair (0.23 g). Hairs were applied only when the rat was stationary and standing on all four paws. Application was to the central region of the plantar surface, avoiding the foot pads. A hair was applied to the paw until bending of the hair occurred. Application of the hair was maintained for approximately 2 s. A withdrawal was considered a valid response only if the hind paw was completely removed from the platform. Although infrequent, if a rat walked immediately after application of a particular hair, the hair was reapplied. On rare occasions, the hind paw only flinched after a single application of the hair. As the hind paw was not lifted from the platform, this was not considered a withdrawal response.

A trial consisted of application of a von Frey hair to the hind paw 5 times at 5 s intervals. If the hind paw withdrawal persisted beyond the 5 s interval, testing resumed after the hind paw was placed appropriately on the platform. Hind paw withdrawal either 4 or 5 times out of the 5 applications was considered to be the withdrawal threshold. If hind limb withdrawal was not evoked 4 or 5 times using a particular hair, the next larger hair in the series was applied in a similar manner.

Once the threshold was determined for the left hind paw, the same testing procedure was repeated on the right hind limb after an inter-trial interval of 5 min. Second and third confirmation trials were determined for each of the left and right hind paws with inter-trial intervals of 5 min. If the withdrawal threshold in the second or third confirmation trial did not match that of the previous trial(s) on a particular hind paw, the next larger hair in the series was tested. This was done until paw withdrawal thresholds in the 3 trials were consistent. The total testing time for each rat usually lasted 35 to 40 min. In almost all cases, the first 3 trials were consistent.

The baseline withdrawal threshold of both hind paws in the von Frey hair test was determined in unoperated and in cuff-implanted rats prior to surgery (normalized to day 0). 'Short-term' cuff-implanted rats were tested on days 11-14 and 15-22. Two short-term periods were examined to enable determination of any change in excitability over weeks 2 and 3 after cuff implantation. 'Long-term' cuff-implanted rats were tested on days 42-52. A statistically significant decrease in the hind paw withdrawal threshold was considered indicative of tactile allodynia.

Only hind paw withdrawal thresholds that remained consistent in each of the 3 trials in unoperated or in cuff-implanted rats were used in the data analysis. Comparisons were done using the Mann-Whitney Rank Sum Test. Differences were considered significant with a P value < 0.05.

Animal preparation for electrophysiological experiments

Acute electrophysiological experiments were run using unoperated and cuff-implanted rats tested previously in paw withdrawal reflex experiments using von Frey hairs. Rats were anesthetised with sodium pentobarbital (50 mg/kg, i.p.; Abbott Laboratories Ltd, Montreal, Quebec, Canada) followed by supplements of 10 mg/kg/h, i.v. The right common carotid artery and the jugular vein were catheterized for continuous monitoring of arterial pressure and for injection of drugs, respectively. Temperature of the rat was maintained at 37.5°C using an infrared heating lamp when required.

Spinal cord segments L_1 to L_4 were exposed for recording from single dorsal horn neurons. The spinal cord was transected at the T_9 vertebral level to eliminate supraspinal influences on the activity of lumbar dorsal horn neurons; to minimize spinal shock xylocaine (0.05 ml of 1%; Astra Pharma, Mississauga, Ontario, Canada) was injected into the cord at the level of transection just prior to transection. Once the rat was stabilized on the stereotaxic frame, the exposed spinal cord was covered with mineral oil (Marcol 72, Imperial Oil Limited; Montreal, Quebec, Canada) at 37.5°C to prevent drying. Experiments were begun 1.5 to 2 h after spinalization.

In experiments in which the effects of electrical stimulation were tested on dorsal horn neuronal activity, the left or right sciatic nerve was exposed by blunt dissection through the biceps femoris muscle and was isolated from surrounding connective tissue using glass probes. Bipolar stainless steel hook electrodes were then inserted under the isolated nerve. When stimulating the cuff-implanted nerve, the electrodes were placed between the cuff and the spinal cord.

Each rat breathed spontaneously during the experiment. However, in experiments where electrical stimulation was used, the anesthetised rat was also paralysed with pancuronium bromide (1 mg/kg i.v. supplemented as necessary; Pavulon, Organon, Scarborough, Ontario, Canada) and ventilated mechanically according to standard parameters (Kleinman and Radford 1964). The anesthetised animal was sacrificed at the end of the experiment.

Electrical recording and data acquisition

Single unit extracellular spikes were recorded using seven-barrelled or single-barrelled micropipettes (overall tip diameter 4-5 or 1-2 μm, respectively). The multi-barrelled electrodes were used because iontophoretic drug experiments were also run in some cases after testing the effects of synaptic input. A solution of 3 M NaCl was placed in the central recording barrel (impedance 2-4 MΩ measured at 1 kHz with the tip submerged in 0.9% saline). Measurements of the depths of microelectrode penetrations allowed us to conclude that the majority of neurons studied were located in laminae II-V.

Single unit recordings were made at depths ranging from 150 to 1200 μm in the spinal dorsal horn. The raw data were amplified 10,000 $\times$ using a DP-301 Differential Amplifier (*Warner Instrument Corp.*), displayed on an oscilloscope (Tektronix 5111) and stored on video cassette tapes using a digital data recorder that incorporated digital pulse code modulation (VR-100A, Instrutech Corporation, Great Neck, NY, U.S.A.) and a conventional video cassette recorder. The signals were also relayed to a frequency counter/gating unit which counted single unit spikes per unit time (bin widths were 1 s) and which thereby displayed a continuous time histogram of the rate of discharge on a Grass 79D polygraph. Sampling and analysis were done using the data acquisition program, *Spike 2* (Version 2.02; *Cambridge Electronic Design*, Cambridge, England), *SigmaStat* (Version 2.03; *SPSS, Inc.*, USA) and an IBM Pentium computer.

Functional classification of dorsal horn neurons

Functional classification of a lumbar dorsal horn neuron was based on its response to innocuous and noxious stimulation of the cutaneous receptive field of the respective hind paw. The following natural stimuli were used as search stimuli to elicit synaptic input while penetrating the spinal dorsal horn and to characterize a neuron functionally once stable single unit recording was obtained: (i) hair stimulation, (ii) light touch/moderate pressure using a calibrated clip (0.2 N for 3 s), (iii) noxious mechanical stimulation using a calibrated clip (pinch; 21 N for 3 s) and (iv) noxious radiant heat (measured to reach 50°C at the skin surface) applied by a focussed projector bulb through a 10 mm diameter

circular hole for a duration of 10 s. In unoperated and in cuff-implanted rats, innocuous stimuli were never tested after noxious stimuli had been tested; results from natural noxious testing are the subject of another manuscript.

Classification of the identified dorsal horn neurons was in three categories (Pitcher and Henry 1999b): (i) non-nociceptive neurons that responded only to non-noxious stimuli such as touch and/or pressure stimulation, (ii) wide dynamic range neurons that responded to both noxious and innocuous stimuli or (iii) nociceptive-specific neurons that responded only to noxious stimuli. In addition, all the units that responded to the noxious range of mechanical and/or thermal stimulation showed a characteristic slowly-decaying afterdischarge, as described previously (De Koninck and Henry 1991; Pitcher and Henry 1999b). Only wide dynamic range neurons were tested in this study. Responses of neurons in the left or right dorsal horn were evoked by stimulation of the hind paw ipsi- or contralateral to the cuff implantation. The response of a neuron to natural stimulation (ie. pressure) of the most responsive part of the receptive field was recorded. Some neurons were also tested for their response to a train of electrical stimuli. Using a bipolar electrode applied directly to the exposed left or right sciatic nerve, a stimulus consisting of 5 mA current pulses of 1 ms duration was given at 4 or 20 Hz for 3 s.

Care was taken to investigate neurons whose receptive fields corresponded to the same area of the hind paw where von Frey hair testing had been done prior to electrophysiological experiments. In addition, in order to minimize possible differences between neurons located in different mediolateral parts of the spinal dorsal horn in

unoperated and in cuff-implanted rats, only a specific recording region adjacent to the entry of dorsal roots in spinal segments L_{3-4} was searched for single unit recording.

Controls used were unoperated rats. In our previous study (Pitcher et al. 1999b) we found a surgery-induced tactile allodynia persisting approximately 35 days. This could have had an influence on the changes in short-term cuff-implanted rats, but not in long-term rats. In pilot experiments, sham surgery-induced hyperexcitability of spinal dorsal horn neurons was markedly less than that in cuff-implanted rats. Sham surgery was without significant effect in long-term rats. In this regard, we interpret the changes reported here to be mainly due to the cuff implantation. Other electrophysiological studies have used unoperated rats to serve as controls (Pertovaara et al. 1997; Yakhnitsa et al. 1999).

Analysis of electrophysiological data

Sampling of on-going activity occurred only after at least 5 min of stable on-going discharge and prior to any peripheral stimulation-induced synaptic input. On-going activity was quantified as the total number of spikes during a 60 s period.

Sampling of evoked responses of wide dynamic range dorsal horn neurons included a fast initial discharge or a fast initial discharge followed by a slowly-decaying afterdischarge. The fast initial discharge persisted only for the duration of the 3 s mechanical or electrical stimulus and was evaluated during the application of the stimulus. The slowly-decaying afterdischarge was evaluated when a cell continued firing above

background on-going activity after the end of the stimulus. The sample period of the slowly-decaying afterdischarge began immediately after cessation of the fast initial discharge and ended once the firing rate returned to the prestimulus discharge level. Evoked responses were quantified as the total number of spikes in the fast initial discharge or slowly-decaying afterdischarge minus the background discharge of equivalent duration as the evoked response.

On-going and synaptic input-elicited activity are expressed as means ($\pm$ S.E.M.) of the number of spikes during the respective sample period. The duration of the slowly-decaying afterdischarge is expressed as the mean $\pm$ S.E.M. number of seconds. Statistical analysis of the data was done using the Mann-Whitney Rank Sum Test. Data from neurons in cuff-implanted rats were compared to those in unoperated rats and a difference was considered significant with a P value < 0.05.

Results

Hind paw withdrawal experiments:

Unoperated rats

In 24 unoperated rats, ipsi- and contralateral hind paw withdrawal thresholds were determined on days 0, 11-14, 15-22 and 42-52. Figure 1*A* shows ipsi- (50.00 ± 3.22 g) and contralateral (47.54 ± 3.46 g) hind paw withdrawal thresholds on day 0. Subsequent paw withdrawal thresholds were not different and were pooled together to yield ipsi- and contralateral hind paw withdrawal thresholds of 49.46 ± 3.06 and 50.92 ± 2.85 g, respectively. Figure 1*A* shows that there was no significant difference between the hind paw withdrawal thresholds on day 0 and on subsequent testing days.

Cuff-implanted rats

Every cuff-implanted rat exhibited a marked decrease in hind paw withdrawal threshold after cuff-implantation compared to its own threshold on day 0. In 27 short-term cuff-implanted rats, the ipsi- and the contralateral withdrawal thresholds were determined on days 11-14 and in 20 rats on days 15-22 days. In 56 long-term cuff-implanted rats, the ipsi- and contralateral hind paws were tested on days 42-52.

Figs. 1*B* and *C* show that in short term rats on day 0 the ipsi- and the contralateral withdrawal thresholds were 49.76 ± 3.18 and 48.43 ± 3.32 g, respectively for the 11-14 day group and 52.90 ± 2.92 and 51.00 ± 3.27 g, respectively for the 15-22 day group.

Thresholds were decreased on days 11-14 (4.71 ± 0.39 and 31.26 ± 4.26 g; $P < 0.001$ and $P < 0.05$ vs. day 0, respectively) and on days 15-22 (5.11 ± 0.75 and 26.20 ± 4.24 g; $P < 0.001$ vs. day 0).

Figure 1*D* shows that ipsi- (15.10 ± 1.05 g) and contralateral (18.54 ± 1.49 g) thresholds in long-term rats were decreased ($P < 0.001$) compared to their withdrawal thresholds at day 0 (47.68 ± 2.06 and 47.89 ± 1.06 g, respectively). At the time points studied, the maximum decrease of the ipsilateral hind paw withdrawal threshold occurred in short-term rats ($P < 0.001$ vs. days 42-52) while the maximum decrease of the contralateral hind paw withdrawal threshold occurred in long-term rats ($P < 0.05$ vs. days 11-14).

Several of the cuff-implanted rats, in particular the short-term rats, exhibited an after-effect to innocuous stimulation persisting for several seconds which included lifting and licking of the hind paw in response to von Frey hair application. These observations are consistent with previous findings (Pitcher et al. 1999b).

Electrophysiological experiments:

Cutaneous receptive field size

The cutaneous receptive field to tactile stimulation was examined for each wide dynamic range neuron investigated in unoperated and in cuff-implanted rats. Although specific measurements of the cutaneous receptive field size were not systematically determined in this study, it was noted that the receptive field to tactile stimulation was

unequivocally and universally larger in cuff-implanted rats than in unoperated rats. It is important to note that while characterizing dorsal horn neurons, the receptive field to touch stimulation in cuff-implanted rats consisted almost invariably of the entire ipsi- or contralateral hind paw. In normal rats, the receptive field to touch stimulation was typically on the plantar surface and rarely extended above the ankle joint. This is illustrated in the figures.

For each ratemeter histogram two schematic diagrams are shown illustrating the cutaneous receptive fields. The receptive field to innocuous tactile stimulation while characterizing a dorsal horn neuron is depicted in the left schematic diagram. The right schematic diagram shows the area of the receptive field on which natural stimulation was applied to evoke dorsal horn neuronal responses for the purpose of analysis.

On-going activity

On-going activity of spinal wide dynamic range neurons in short- and long-term cuff-implanted rats was compared to that in unoperated rats. In 24 unoperated rats, on-going activity was recorded from 70 wide dynamic range neurons. In short-term cuff-implanted rats, on-going activity was recorded from 43 ipsilateral neurons in 27 rats on days 11-14 and from 53 ipsilateral neurons in 20 rats on days 15-22. In long-term cuff-implanted rats, on-going activity was recorded from 82 ipsilateral neurons in 35 rats and from 49 contralateral neurons in 21 rats on days 42-52.

On-going activity in short-term cuff-implanted rats (37.81 $\pm$ 4.52 spikes/s and

37.37 ± 4.13 spikes/s) was greater than that in unoperated rats ($P < 0.001$ vs. 19.13 ± 2.01 spikes/s). In long-term cuff-implanted rats, on-going activity of ipsi- (28.08 ± 2.77 spikes/s) and of contralateral (27.92 ± 3.21 spikes/s) neurons was also greater than that in unoperated rats ($P < 0.05$). In addition, on-going activity in day 11-14 short-term rats was greater ($P < 0.05$) than that in long-term rats tested ipsilaterally. Figure 2 shows the combined data.

Responses to peripheral stimulation

Responses of spinal wide dynamic range neurons to peripheral stimulation were investigated in unoperated and in short- and long-term cuff-implanted rats. In short-term cuff-implanted rats, the effects of stimulation were investigated on ipsilateral wide dynamic range neuronal activity. In long-term cuff-implanted rats, the effects of stimulation were investigated on neurons ipsi- and contralateral to the cuff implantation.

(i) Pressure

In 6 unoperated rats, the effect of pressure stimulation was tested on 20 neurons. A representative response of a neuron to pressure is illustrated in Figure 3*A*. The fast initial discharge is shown by high frequency activity which persisted only as long as the duration of the pressure stimulus. In 14 short-term cuff-implanted rats, pressure was tested on 23 neurons on days 11-14. Figure 3*B* shows a representative response of a wide dynamic range neuron to pressure stimulation in the receptive field in a rat tested on day

12. The striking feature of this response is the pressure-evoked slowly-decaying afterdischarge which persisted for several minutes. In long-term cuff-implanted rats, pressure was tested on 25 ipsilateral neurons in 16 rats and on 17 contralateral neurons in 8 rats. Figs. 3*C* and *D* illustrate representative responses of an ipsi- and a contralateral neuron to pressure stimulation of the cuff-implanted and the contralateral hind paws, respectively, in 2 rats tested on day 42. A pressure-evoked slowly-decaying afterdischarge was seen in each of these neurons. Figs. 4*A* and *B* show the cumulative data of the responses to pressure stimulation. In short-term rats, the pressure-induced fast initial discharge (541.17 ± 93.33 spikes) was similar to that in unoperated rats (356.01 ± 50.81 spikes). However, slowly-decaying afterdischarge responses which were not seen after pressure stimulation in unoperated rats (91.65 ± 24.17 spikes over 4.58 ± 2.02 s) occurred in short-term rats (1263.48 ± 370.77 spikes, $P < 0.001$ vs. unoperated) and persisted approximately 2 to 3 min after the end of the pressure stimulus (147.29 ± 31.53 s, $P < 0.001$ vs. unoperated). In long-term rats, although the pressure-induced fast initial discharge also remained unchanged in ipsi- and in contralateral neurons (433.03 ± 62.89 and 587.12 ± 150.88 spikes, respectively) compared to that in unoperated rats, slowly-decaying afterdischarge responses were also elicited in ipsi- (1128.02 ± 376.83 spikes, $P < 0.001$ vs. unoperated) and in contralateral neurons (846.30 ± 284.36 spikes, $P < 0.001$ vs. unoperated) and persisted for several seconds (98.11 ± 29.20 s and 81.76 ± 17.49 s, respectively; $P < 0.001$ vs. unoperated).

(ii) 4 Hz electrical nerve stimulation

In 13 unoperated rats, the effect of 4 Hz electrical stimulation was tested on 26 neurons. A representative response of a neuron to 4 Hz electrical stimulation is illustrated in Figure 5*A*. The fast initial discharge is shown by high frequency activity during electrical stimulation and the slowly-decaying afterdischarge is depicted by the increased activity persisting approximately 1 min after the end of the stimulus. In short-term cuff-implanted rats, 4 Hz electrical stimulation was tested on 30 neurons in 19 rats on days 11-14. Figure 5*B* shows a representative response of a neuron to 4 Hz electrical stimulation in a rat tested on day 12. There was a greater magnitude and duration (approximately 10 min) of the slowly-decaying afterdischarge than in unoperated rats. In long-term cuff-implanted rats, 4 Hz electrical stimulation was tested on 26 ipsilateral neurons in 14 rats and on 23 contralateral neurons in 11 rats. Figs. 5*C* and *D* illustrate representative responses of an ipsi- and a contralateral neuron, respectively, to 4 Hz electrical stimulation in 2 rats tested on day 42. The magnitude and duration of the slowly-decaying afterdischarge were greater. Figs. 6*A* and *B* show cumulative data of the response to 4 Hz electrical stimulation. In short-term rats, the 4 Hz electrical stimulation-induced fast initial discharge (546.89 $\pm$ 82.86 spikes) was similar to that in unoperated rats (412.51 $\pm$ 63.16 spikes). However, the magnitude of the slowly-decaying afterdischarge (2181.89 $\pm$ 347.75 spikes) was much greater ($P < 0.001$ vs. unoperated, 923.82 $\pm$ 246.76 spikes). In addition, the duration of the slowly-decaying afterdischarge (299.95 $\pm$ 77.48 s) was greater ($P < 0.01$ vs. unoperated, 140.38 $\pm$ 29.18 s). In long-term rats, the 4 Hz

electrical stimulation-induced fast initial discharge in ipsi- and contralateral neurons (617.16 ± 106.14 and 428.06 ± 84.78 spikes, respectively) was also similar to that in unoperated rats. However, while the magnitude (1960.07 ± 384.36 spikes; $P < 0.01$ vs. unoperated) and the duration (241.30 ± 35.45 s; $P < 0.05$ vs. unoperated) of the slowly-decaying afterdischarge were greater ipsilaterally, contralaterally the magnitude (1578.10 ± 329.62 spikes; $P < 0.05$ vs. unoperated) but not the duration of the slowly-decaying afterdischarge (201.00 ± 29.98 s) was greater compared to that in unoperated rats.

(iii) 20 Hz electrical nerve stimulation

In 15 unoperated rats, the effect of 20 Hz electrical stimulation was tested on 27 neurons. A representative response of a neuron to 4 Hz electrical stimulation is illustrated in Figure 7*A*. The fast initial discharge is shown by high frequency activity during electrical stimulation and the slowly-decaying afterdischarge is depicted by the increased activity persisting approximately 1 min after the end of the stimulus. In short-term cuff-implanted rats, 20 Hz electrical stimulation was tested on 38 neurons in 27 rats on days 11-14. Figure 7*B* shows a representative response of a neuron to 20 Hz electrical stimulation in a rat tested on day 12. A markedly greater magnitude and duration (approximately 18 min) of the slowly-decaying afterdischarge is shown. In long-term cuff-implanted rats, 20 Hz electrical stimulation was tested on 34 ipsilateral neurons in 19 rats and on 20 contralateral neurons in 9 rats. Figs. 7*C* and *D* illustrate representative responses of an ipsi- and a contralateral neuron, respectively, to 20 Hz electrical stimulation in 2 rats tested

on day 42. A greater magnitude and duration of the slowly-decaying afterdischarge is seen. Figs. 8*A* and *B* show cumulative data of the response to 20 Hz electrical stimulation. In short-term rats, the 20 Hz electrical stimulation-induced fast initial discharge (959.32 ± 81.69 spikes) was similar to that in unoperated rats (732.18 ± 89.31 spikes). However, the magnitude of the slowly-decaying afterdischarge (4725.81 ± 886.66 spikes) was markedly greater ($P < 0.001$ vs. unoperated, 1122.72 ± 244.29 spikes). In addition, the duration of the slowly-decaying afterdischarge (395.43 ± 41.12 s) was greater ($P < 0.001$ vs. unoperated, 165.24 ± 32.30 s). In long-term rats, the 20 Hz electrical stimulation-induced fast initial discharge in ipsi- and contralateral neurons (860.44 ± 90.17 and 755.17 ± 114.67 spikes, respectively) was similar to that in unoperated rats. However, the magnitude (2547.82 ± 496.66 and 2463.88 ± 643.99 spikes, respectively; $P < 0.01$ and $P < 0.05$ vs. unoperated, respectively) and the duration (300.07 ± 36.34 s and 340.21 ± 54.90 s, respectively; $P < 0.01$ vs. unoperated) of the slowly-decaying afterdischarge were greater. In short-term rats, the magnitude of the slowly-decaying afterdischarge was greater ($P < 0.05$) than that in long-term rats tested ipsilaterally.

(iv) Comparison of effects of 4 and 20 Hz stimulation

Figs. 7*A* and 8*A* show that in unoperated rats, the fast initial discharge induced by 20 Hz stimulation was greater ($P < 0.01$) than that induced by 4 Hz stimulation. Slowly-decaying afterdischarge responses were not significantly different. However, in short-term rats, both the 20 Hz-evoked fast initial discharge and the afterdischarge responses were

greater ($P < 0.001$ and 0.01, respectively) than the respective 4 Hz-induced responses. In ipsi- and contralateral neurons in long-term rats, only the fast initial discharge evoked by 20 Hz stimulation was greater ($P < 0.05$ and 0.01, respectively).

Discussion

The data from the present electrophysiological experiments indicate that chronic implantation of a 2 mm polyethylene cuff around the sciatic nerve (Mosconi and Kruger 1996), induces changes in physiological properties of spinal nociceptive mechanisms, including greater on-going, or spontaneous, activity, an expanded receptive field size in response to natural cutaneous stimuli, a nociceptive-type response to an innocuous mechanical stimulus and a greater magnitude and duration of the afterdischarge in response to 4 Hz and even more so 20 Hz electrical stimulation of sensory afferents. As acutely spinalized rats were used, the data are interpreted to suggest that cuff implantation-evoked hyperexcitability in the spinal dorsal horn does not rely exclusively on sensory processing via a supraspinal loop. An argument will be developed below which suggests that these changes can best be accounted for by changes in the properties of sensory afferents, particularly myelinated fibers, in the sciatic nerve.

All rats tested in the electrophysiological experiments had exhibited tactile allodynia in response to von Frey hair stimulation prior to the acute electrophysiological experiment. Neuronal hyperexcitability and tactile allodynia were greatest in short-term cuff-implanted rats. Two short-term periods were studied to determine how stable the model is in electrophysiological terms, and the data indicate that there was no difference in the changes induced between these two periods. This indicates that the so-called neuropathic condition imposed by the cuff implantation was stable over the time covered by the two periods. In addition, a greater excitability contralaterally of spinal neurons and a contralateral tactile

allodynia both occurred predominantly in long-term rats. These data are consistent with previous findings (Pitcher et al. 1999b) and suggest a correlation between the changes observed in excitability of dorsal horn mechanisms and the behavioral tactile allodynia. Thus, the greater excitability of neurons bilaterally may constitute the neurophysiological basis for the allodynia in animal models of peripheral neuropathy and perhaps some forms of clinical neuropathic pain.

The electrophysiological approach used in this study to examine sensory processing has several advantages over behavioral testing. For example, alterations in the excitability of spinal dorsal horn neurons can be determined, including early and late components of neuronal responses to different stimulus modalities. In addition, sensory mechanisms can be differentiated from motor mechanisms, thus yielding information exclusively on sensory processing.

Significance of larger receptive field size

Although most electrophysiological studies on animal models of neuropathic pain have not focussed on cutaneous receptive field size, enlarged receptive fields have been shown in spinally-intact (Behbehani and Dollberg-Stolik 1994; Takaishi et al. 1996) and in spinally-transected rats (Cumberbatch et al. 1998). The significance of this observation to clinical neuropathic pain is less obvious but the larger receptive field size may be due to a number of different mechanisms, which are presented below.

Significance of greater on-going activity

Greater on-going activity of dorsal horn neurons is a common observation in electrophysiological experiments in spinally-intact rats exhibiting tactile allodynia, including the chronic loose sciatic nerve constriction model (Palecek et al. 1992b; Laird and Bennett 1993; Sotgiu 1993), the partial sciatic nerve ligation model (Yakhnitsa et al. 1999), the loose ligation of the L_{4-6} spinal nerves model (Tabo et al. 1999), the tight ligation of the L_{5-6} spinal nerves model (Pertovaara et al. 1997; Chapman et al. 1998), and the tight ligation of the L_7 spinal nerve model in primates (Palecek et al. 1992a). Thus the elevated spontaneous activity observed here in acutely spinalized rats is consistent with previous observations in intact animals. In fact, this greater on-going activity may be the cellular correlate of the spontaneous pain which characterizes neuropathic pain in humans (Gracely et al. 1992).

Significance of afterdischarge in response to innocuous pressure

The most surprising observation in this study was the afterdischarge which occurred after innocuous pressure stimulation of the cutaneous receptive field in the cuff-implanted rats. This type of effect was not seen in control rats run in conjunction with the cuff-implanted rats and this has not been seen by one of the authors (JLH) in over 25 years of recording from dorsal horn neurons in normal animals. Importantly, the fast initial discharge in the cuff-implanted rats was not different from that in the unoperated rats. This suggests that the large diameter fiber-induced initial discharge was unaltered in the

cuff-implanted rats. It is a reasonable assumption that this initial discharge is due to glutamate acting on ionotropic receptors (Radhakrishnan and Henry 1993). Thus, if fast synaptic transmission from myelinated fibers is unaltered in this model, the change must have been in either small diameter afferents being recruited by the innocuous stimulus or the myelinated afferents must have been releasing a chemical mediator of synaptic transmission in the cuff-implanted rats that was not being released in unoperated controls. Resolution to these two possibilities is argued below, where a position is taken supporting the second possibility.

The change in the response to innocuous stimulation indicates that the neuron is responding as it would to only a noxious stimulus to the receptive field in a normal rat (Pitcher and Henry 1999b). This pressure-induced afterdischarge may be the neural correlate of the after-effect persisting after von Frey hair application and tactile allodynia which characterizes neuropathic pain in humans.

Significance of response to 4 Hz vs. 20 Hz stimulation

The fast initial discharge in response to 4 Hz stimulation of the sciatic nerve in the cuff-implanted rats was the same as that in the unoperated rats. However, the afterdischarge was different in the two groups of rats. Both the magnitude and the duration of the afterdischarge were greater in cuff-implanted rats. This difference may be the neural correlate of the hyperalgesia which characterizes neuropathic pain in humans.

Another striking observation in this study was that while the response to 20 Hz

stimulation in unoperated control rats was not markedly greater than that to 4 Hz stimulation, the same cannot be said of the difference in the cuff-implanted rats, where the afterdischarge induced by 20 Hz stimulation was significantly greater than that induced by 4 Hz. Given that 1 to 5 Hz electrical stimulation is typically used to evoke C fiber responses (Molander et al. 1992; Thompson et al. 1994; Gozariu et al. 1997) and that the synaptic responses to C fibers fail at 5 Hz (Seltzer et al. 1991), in the present study the 20 Hz-elicited afterdischarge is interpreted to suggest that cuff implantation on the sciatic nerve produced changes predominantly in myelinated afferents. Compared to unmyelinated fibers, large myelinated fibers are reported to undergo substantially greater degenerative/regenerative changes following peripheral nerve constriction (Basbaum et al. 1991; Munger et al. 1992; Nuytten et al. 1992; Coggeshall et al. 1993; Guilbaud et al. 1993).

Spinal vs. supraspinal mechanisms

Previous reports have argued that increased tactile sensitivity of the nerve-injured hind paw is sustained predominantly via a supraspinal loop with tonic descending facilitatory control. This is based on observations that tactile allodynia in nerve-injured rats is substantially decreased or abolished following spinal transection (Pertovaara et al. 1996; Kauppila 1997; Bian et al. 1998; Kauppila et al. 1998; Sung et al. 1998) or lidocaine injection into the rostral ventral medulla or the periaqueductal grey (Pertovaara et al. 1996). Conceivably, this mechanism may explain brush or pressure stimulation-elicited

spinal dorsal horn neuronal activity in spinally-intact nerve-constricted rats (Leem et al. 1995, 1996; Yakhnitsa et al. 1999). In this regard, if tonic descending facilitation maintains central effects of tactile stimulation, spinal cord transection may be expected to attenuate, if not abolish, pressure stimulation-induced dorsal horn neuronal hyperexcitability. However, this was not the case here. In both short- and long-term cuff-implanted rats, an afterdischarge was evoked even by innocuous pressure stimulation, which cannot be accounted for by tonic descending facilitation. Therefore, processing of excitatory effects of innocuous pressure appears to be sustained, at least in part, via peripheral and/or spinal mechanisms.

Central sensitization

It has been suggested that nerve injury-induced changes in the properties of sensory afferent fibers could yield a difference in the properties of spinal neurons, such that a central sensitization occurs (Koltzenburg et al. 1994; Mailis et al. 1997; Sandkühler and Liu 1998). However, spontaneous nociceptive afferent input from a peripheral focus has been reported to maintain altered central processing, including allodynia, spontaneous pain and other sensory abnormalities in clinical neuropathic pain (Gracely et al. 1992; Campero et al. 1998). Even long-term potentiation in the isolated peripheral nerve-spinal cord preparation is dependent on C fiber afferent input for induction and expression (Lozier and Kendig 1995).

Furthermore, while greater sensitivity of spinal neurons to synaptic inputs may be

able to account for the greater on-going activity of dorsal horn neurons, this cannot explain the appearance of an afterdischarge in response to innocuous cutaneous stimulation, particularly when the initial discharge is unaltered in this model. One possibility is that the stimulation could be supramaximal, in which case an effect on the fast initial discharge is not observed due to a "ceiling effect". However, in pilot experiments (Pitcher and Henry 1999a) we noted that mechanical stimulation approximately 100 times the mechanical stimulus intensity used here evoked a fast initial discharge greater in magnitude. Given that this was the case in both unoperated and in cuff-implanted rats, the stimulus intensity and neuronal responses reported here were not supramaximal. Therefore, we conclude that while there may be a change in excitability of dorsal horn neurons in this model, central sensitization is likely not the main component underlying the observed differences in the responses of these neurons particularly to peripheral stimulation-elicited synaptic input.

Decreased spinal inhibitory mechanisms

Hyperexcitability could be attributable to decreased inhibition, or disinhibition. A bilateral decrease in GABAergic interneurons (Ibuki et al. 1997; Eaton et al. 1998) and a decrease in extracellular GABA (Stiller et al. 1996) have been reported in the spinal cord in animal models of neuropathic pain. Reduced glycine receptors (Simpson and Huang 1998) and glutamate decarboxylase-immunoreactive cells (Eaton et al. 1998) and increased expression of dark neurons (Sugimoto et al. 1990; Hama et al. 1994, 1996; Mao et al.

1997) have also been reported in the spinal dorsal horn in nerve-constricted rats. In addition, presynaptic inhibitory controls are also reported to be impaired following constriction injury of the sciatic nerve (Laird and Bennett 1992). However, there is no indication of how such a loss of inhibition in the dorsal horn can account for more than an elevated spontaneous discharge of spinal nociceptive neurons and there is no obvious means by which such a loss could account for the occurrence of an afterdischarge in the response to pressure stimulation without a concomitant greater initial discharge. For reasons argued above, a supramaximal response of the fast initial discharge is likely not the case here.

Changes in myelinated afferents

Novel proposals with a peripheral basis have been put forth to account for the spontaneous pain and tactile allodynia in neuropathic pain. Given that myelinated rather than unmyelinated fibers are implicated (Shir and Seltzer 1990; Gracely et al. 1992; Baron and Saguer 1993; Na et al. 1993; Ossipov et al. 1999; Tal et al. 1999), it has been suggested that the principal changes occur predominantly in these fibers to sustain the painful symptoms of peripheral neuropathy.

Ectopic activity in primary afferents, including myelinated fibers, has been reported in neuropathic pain patients (Campero et al. 1998) and in animal models of neuropathic pain (Kajander and Bennett 1992; Tal et al. 1999). This activity has been proposed to arise from dorsal root ganglia (Xie et al. 1995; Study and Kral 1996; Zhang et al. 1997) or from the site of nerve injury (Tal and Eliav 1996). Ectopic generation of bursts of spontaneous

action potentials may be triggered by a preceding action potential (Campero et al. 1998). A reasonable mechanism by which this ectopic activity might arise is by an excessive density of Na^+ channels expressed in afferent sensory neurons (Matzner and Devor 1994; Kral et al. 1999; Waxman et al. 1999a,b). Thus it seems reasonable that this source of abnormal activity may provide a barrage of tonic excitatory input to spinal dorsal horn neurons to account for the greater spontaneous activity in the present study and perhaps for spontaneous pain in human patients with neuropathic pain.

To account for tactile allodynia, a phenotypic change is proposed to take place in large diameter sensory neurons (Zhou et al. 1999) so that they begin to express preprotachykinin mRNA (Marchand et al. 1994; Ma and Bisby 1998). Their activation then leads to the central release of chemical mediators of synaptic transmission, in particular substance P. While under normal circumstances innocuous stimulation causes selective activation of non-nociceptive neurons, the altered central terminal arborization coupled with the release of substance P, under these circumstances causes activation of nociceptive neurons. Therefore, activation of myelinated afferents by their adequate stimulus, innocuous mechanical stimulation of the skin, would lead to activation of nociceptive neurons, which would in turn exhibit the typical nociceptive response to synaptic activation by nociceptive afferents in normal rats.

Altered response of contralateral neurons

Neurons contralateral to the cuff were studied in long-term rats because our

previous behavioral study demonstrated the late but marked development of a mechanical allodynia in the contralateral hind paw (Pitcher et al. 1999b). The results from the present electrophysiological study support the earlier results in that contralateral neurons showed greater spontaneous activity, greater receptive field size and greater afterdischarge in response to noxious mechanical and to noxious heat stimulation. Studies using spinally-intact rats report enlarged hind paw receptive fields bilaterally (Behbehani and Dollberg-Stolik 1994; Takaishi et al. 1996) and greater mechanical stimulation-evoked activity of ipsi- and contralateral spinal dorsal horn neurons (Pertovaara et al. 1997) in models of peripheral neuropathy. This contralateral effect may be the basis for clinical disorders that exhibit "mirror" pains (Kozin et al. 1976; Procacci and Maresca 1987).

The mechanisms by which modification of sensory processing is induced and sustained contralaterally remain elusive and are speculative to date. Subcutaneous injection of 0.15 ml of normal saline into the footpad on the plantar surface of one hind paw has been shown to induce swelling and hyperalgesia of the contralateral hind paw (Levine et al. 1985). Interruption of venous circulation to the injured limb by vein ligation did not alter the response in the contralateral hind paw demonstrating that the contralateral neurogenic inflammation and hyperalgesia were likely neurally mediated. However, as experiments in these earlier studies were run in spinally-intact rats, supraspinal modulation cannot be excluded. The concept of bilateral changes may be substantiated by bilateral expression of dark neurons (Sugimoto et al. 1990; Hama et al. 1994; Hama et al. 1996; Mao et al. 1997) and bilateral decreases in numbers of GABA- (Ibuki et al. 1997; Eaton

et al. 1998) and glutamate decarboxylase-immunoreactive cells (Eaton et al. 1998) and a bilateral decrease in glycine receptors (Simpson and Huang 1998) in the spinal cord in nerve injury models.

It is difficult to reconcile how alterations in sensory processing in the contralateral dorsal horn can account for the preferential effect of cuff implantation on fast initial discharge vs. slowly-decaying afterdischarge responses. It should be pointed out that in addition to contralateral changes centrally, contralateral changes may also occur peripherally. For example, unilateral postherpetic neuralgia may be associated with bilateral sensory neuron damage (Oaklander et al. 1998), and unilateral nerve injury in the rat down-regulates Na^+ channel SCN10A mRNA bilaterally in rat dorsal root ganglia (Oaklander and Belzberg 1997). Moreover, transneuronal effects evoked by saphenous nerve injury in the rat are reported to be restricted specifically to neurons of the contralateral homologous nerve (Kolston et al. 1991). Therefore, to account for the effects of cuff implantation on hyperexcitability in the contralateral dorsal horn, peripheral changes contralaterally must also be considered.

Conclusions

The data in the present study demonstrate that chronic cuff implantation around the sciatic nerve induces greater on-going activity, a nociceptive type of response to an innocuous mechanical stimulus and a greater magnitude and duration of the afterdischarge in response to 4 and even more so 20 Hz electrical stimulation of sensory afferents. As acutely spinalized rats were used, cuff implantation-evoked hyperexcitability in the spinal dorsal horn does not rely exclusively on sensory processing via a supraspinal loop and tonic descending excitation, as has been proposed elsewhere. Only peripheral and/or spinal mechanisms may be considered to explain these findings. The data are interpreted to suggest that alterations in sensory afferent neurons in particular myelinated fibers may underlie the changes observed in spinal dorsal horn neuronal activity. These changes at the spinal level may be the cellular neural correlate of spontaneous pain and mechanical allodynia in neuropathic pain.

What is visualized to have occurred in the present study, and what is most consistent with both our data as well as data from basic and clinical studies cited in this report, is that compromise of peripheral sensory nerves alters the signal returning to the cell body in the dorsal root ganglion, with the effect that the synthesis of proteins is altered. Two particular changes in protein synthesis appear significant to neuropathic pain. One may be that there is an increase in production and subsequent transport of Na^+ channels; this may result in ectopic discharge and amplification of the afferent signal. The other is that these neurons, which normally do not produce substance P, may now begin

to do so. Thus, when activated by their adequate stimulus, they release from their central terminals not only glutamate, as they normally do, but now also substance P. Given this scenario, activation of myelinated afferents by innocuous stimulation would not be expected to elicit an initial discharge any different in cuff-implanted rats than in normal rats. This is what we observed in the present study. Rather, one would expect to see an afterdischarge as a result of the subsequent release of substance P, in this case from the additional population of afferents which release it. Thus, we interpret our data to be most consistent with the hypothesis that chronic cuff implantation in the rat, and neuropathic pain in humans, induces significant physiological changes in sensory afferents, in particular myelinated fibers. This model may provide important insights into the development of new approaches to the treatment of neuropathic pain.

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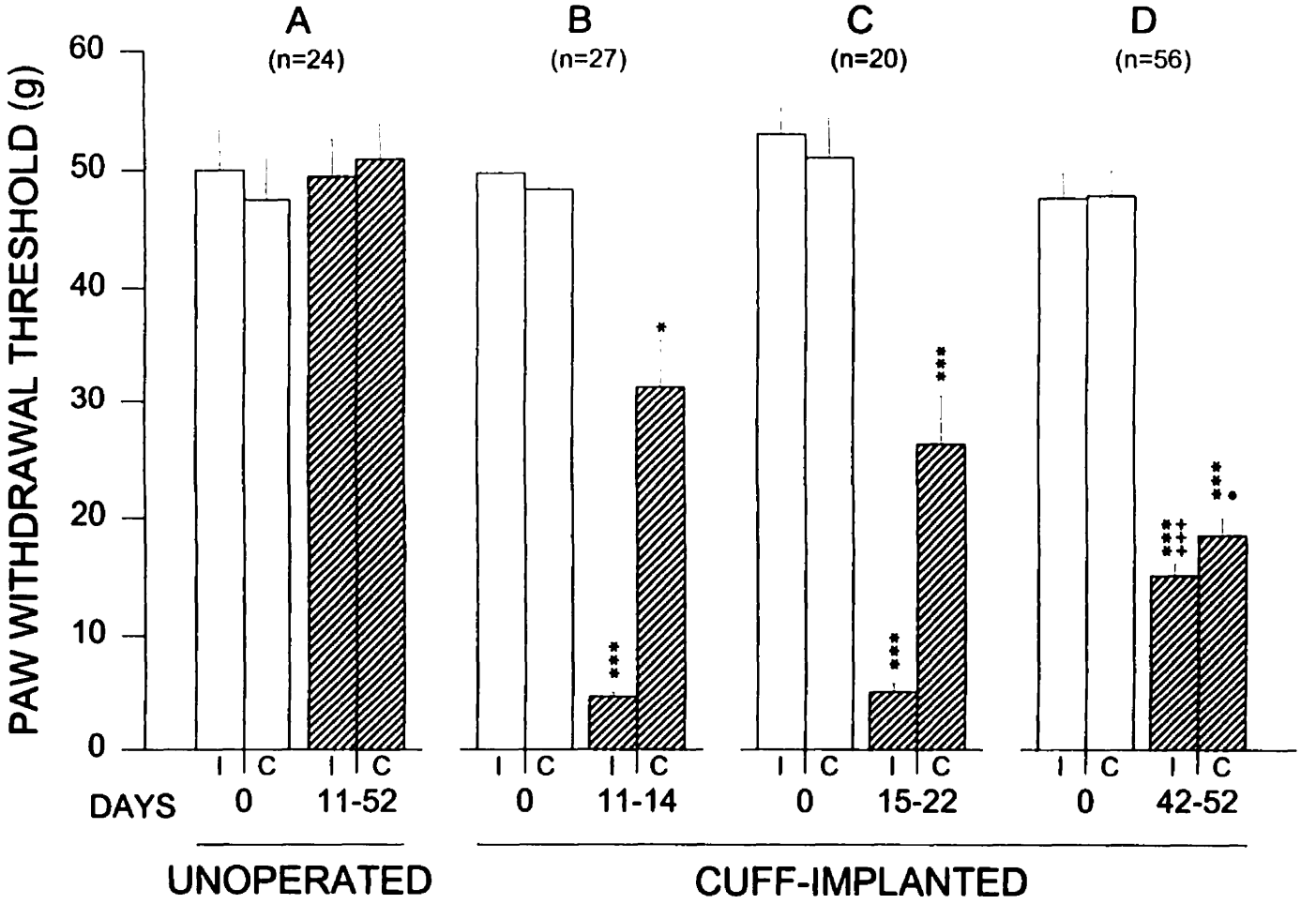
A
(n=24)
B
(n=27)
C
(n=20)
D
(n=56)
PAW WITHDRAWAL THRESHOLD (g)
60
50
40
30
20
10
0
I C I C
DAYS
0
11-52
0
11-14
0
15-22
0
42-52
UNOPERATED
CUFF-IMPLANTED

Figure 1. Hind paw withdrawal thresholds in the von Frey hair test. The vertical axis represents the withdrawal threshold measured in grams. The horizontal axis shows hind paw withdrawal thresholds ipsi- (I) and contralateral (C) to sciatic cuff implantation in short- (days 11-14 and 15-22 shown by hatched bars) and in long-term (days 42-52 shown by hatched bars) rats. Thresholds from unoperated rats are also included. *A*: In 24 unoperated rats, there was no significant difference between the hind paw withdrawal thresholds on day 0 and the pooled paw withdrawal thresholds measured at time points on days 11-14, 15-22 or 42-52. *B*: In 27 short-term rats tested on days 11-14, the ipsilateral hind paw withdrawal threshold was markedly decreased. The contralateral hind paw withdrawal threshold was also decreased but to a lesser degree. *C*: In 20 short-term rats tested on days 15-22, reduction of the ipsi- and contralateral hind paw withdrawal threshold was similar to that in *B*. *D*: In 56 long-term rats, the ipsilateral hind paw withdrawal threshold was greater (+++ $P < 0.001$ vs. respective hind paw withdrawal threshold on days 11-14 and 15-22) while the contralateral hind paw withdrawal threshold was reduced further (• $P < 0.05$ vs. respective hind paw withdrawal threshold on days 11-14; * $P < 0.05$ and *** $P < 0.001$ vs. respective hind paw withdrawal threshold at day 0)

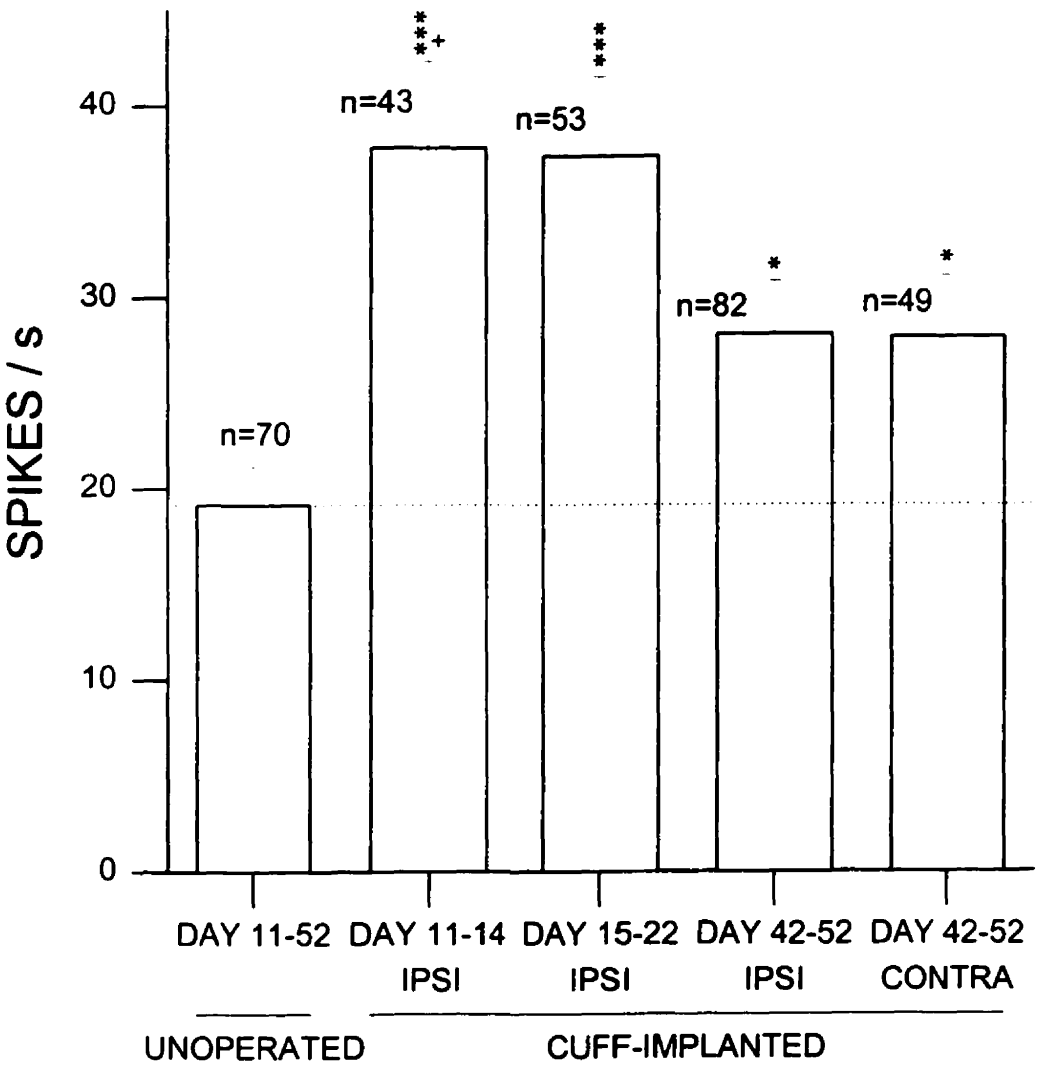
SPIKES / s
40
30
20
10
0
n=70
n=43
n=53
n=82
n=49
DAY 11-52
DAY 11-14 IPSI
DAY 15-22 IPSI
DAY 42-52 IPSI
DAY 42-52 CONTRA
UNOPERATED
CUFF-IMPLANTED

Figure 2. On-going activity of wide dynamic range spinal dorsal horn neurons in unoperated and in cuff-implanted rats. The vertical axis shows the number of spikes per second of stable on-going neuronal discharge. The horizontal axis shows on-going activity in unoperated rats (days 11-52) and in short- (days 11-14 or 15-22) and in long-term (days 42-52) cuff-implanted rats. In unoperated rats, mean on-going activity is illustrated by the dotted line. In cuff-implanted rats, mean on-going activity in ipsilateral (IPSI) neurons in short- and long-term rats and in contralateral (CONTRA) neurons in long-term rats was greater than that in unoperated rats (* $P < 0.05$ and *** $P < 0.001$ vs. unoperated). Furthermore, mean on-going activity in day 11-14 short-term rats was greater than that in long-term rats tested ipsilaterally (+ $P < 0.05$). The number of neurons tested in each group is indicated above each histogram.

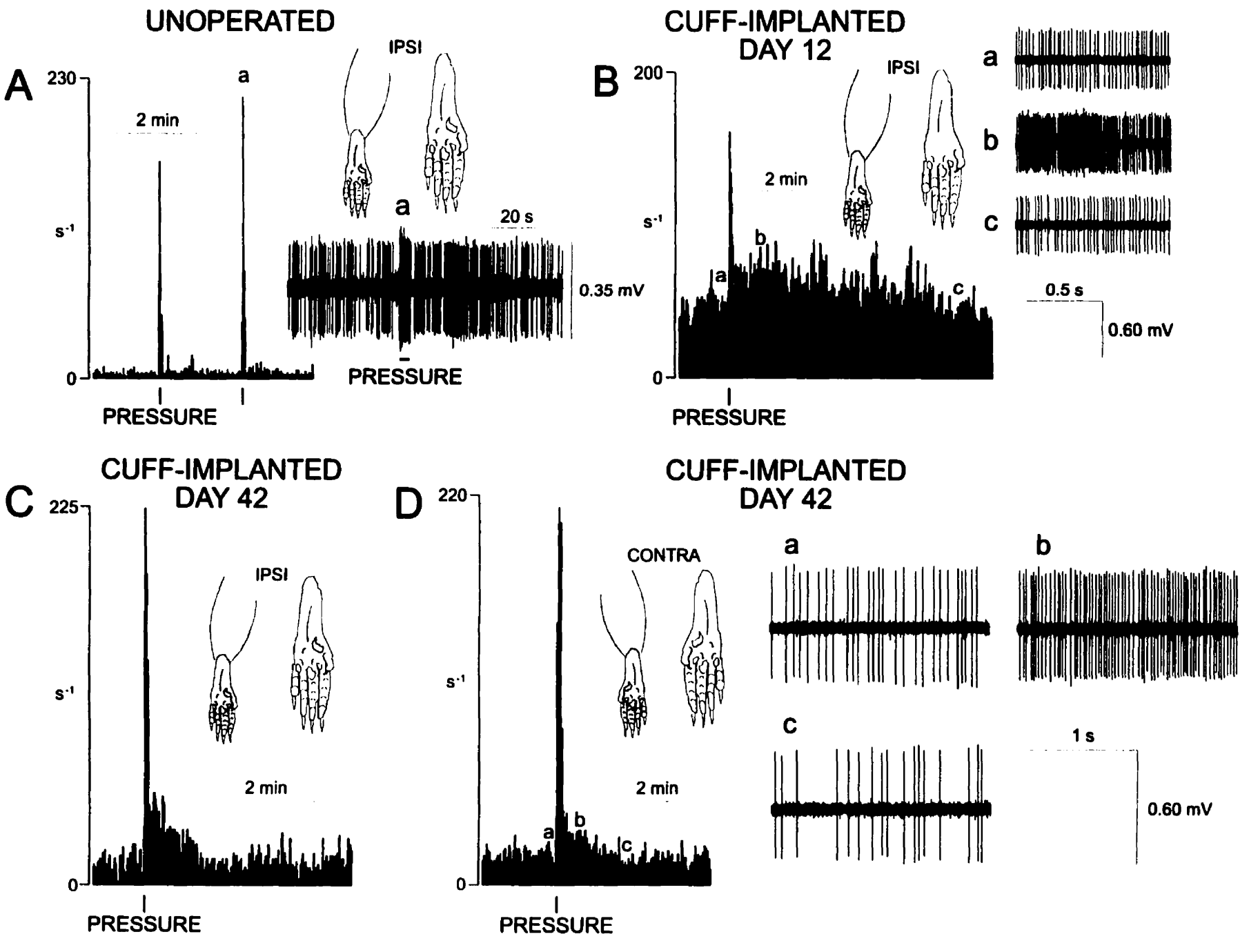
UNOPERATED
A
230
2 min
a
IPSI
a
20 s
s⁻¹
0.35 mV
0
PRESSURE
PRESSURE
CUFF-IMPLANTED
DAY 12
B
200
IPSI
a
b
2 min
s⁻¹
a
c
0
PRESSURE
0.5 s
0.60 mV
CUFF-IMPLANTED
DAY 42
C
225
IPSI
s⁻¹
2 min
0
PRESSURE
CUFF-IMPLANTED
DAY 42
D
220
CONTRA
s⁻¹
2 min
a
b
c
0
PRESSURE
a
b
c
1 s
0.60 mV

Figure 3. Effect of pressure stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. *A*: The ratemeter record, top left, shows that in an unoperated rat innocuous pressure stimulation (0.2 N for 3 s) produced a fast discharge which lasted only for the duration of the stimulus. The neuron was 990 μm deep from the dorsal surface of the spinal cord. The horizontal axis represents time and the vertical axis represents frequency of spikes (1 s bin width). The time and duration of the pressure stimulus are shown by the narrow rectangle below the ratemeter histogram. The inset to the left shows the cutaneous receptive field to touch stimulation, depicted by the shaded area. The inset to the right shows the area subjected to pressure stimulation. The extracellular record shows single unit activity taken at 'a' in the ratemeter histogram. *B*: In a short-term cuff-implanted rat tested on day 12 pressure stimulation of the ipsilateral hind paw (IPSI) produced a fast initial discharge which lasted only for the duration of the stimulus followed immediately by a slowly-decaying afterdischarge which persisted for 4 to 5 min (1124 μm). Details of the insets are otherwise similar to those in *A*. Extracellular records shown at the top right depict single unit activity at times selected at 'a', 'b' and 'c' in the ratemeter histogram. *C*: In a long-term cuff-implanted rat tested on day 42 pressure stimulation of the ipsilateral hind paw (IPSI) produced a fast initial discharge which lasted for the duration of the stimulus followed by a slowly-decaying afterdischarge which persisted for 1 to 1.5 min (408 μm). *D*: In another long-term rat tested on day 42 innocuous pressure stimulation of the contralateral hind paw (CONTRA) produced a fast initial discharge which lasted only for the duration of the stimulus followed immediately by a slowly-decaying afterdischarge which lasted 1 to 1.5 min (604 μm). Extracellular

records show single unit activity at times selected at 'a', 'b' and 'c' in the ratemeter histogram.

PRESSURE

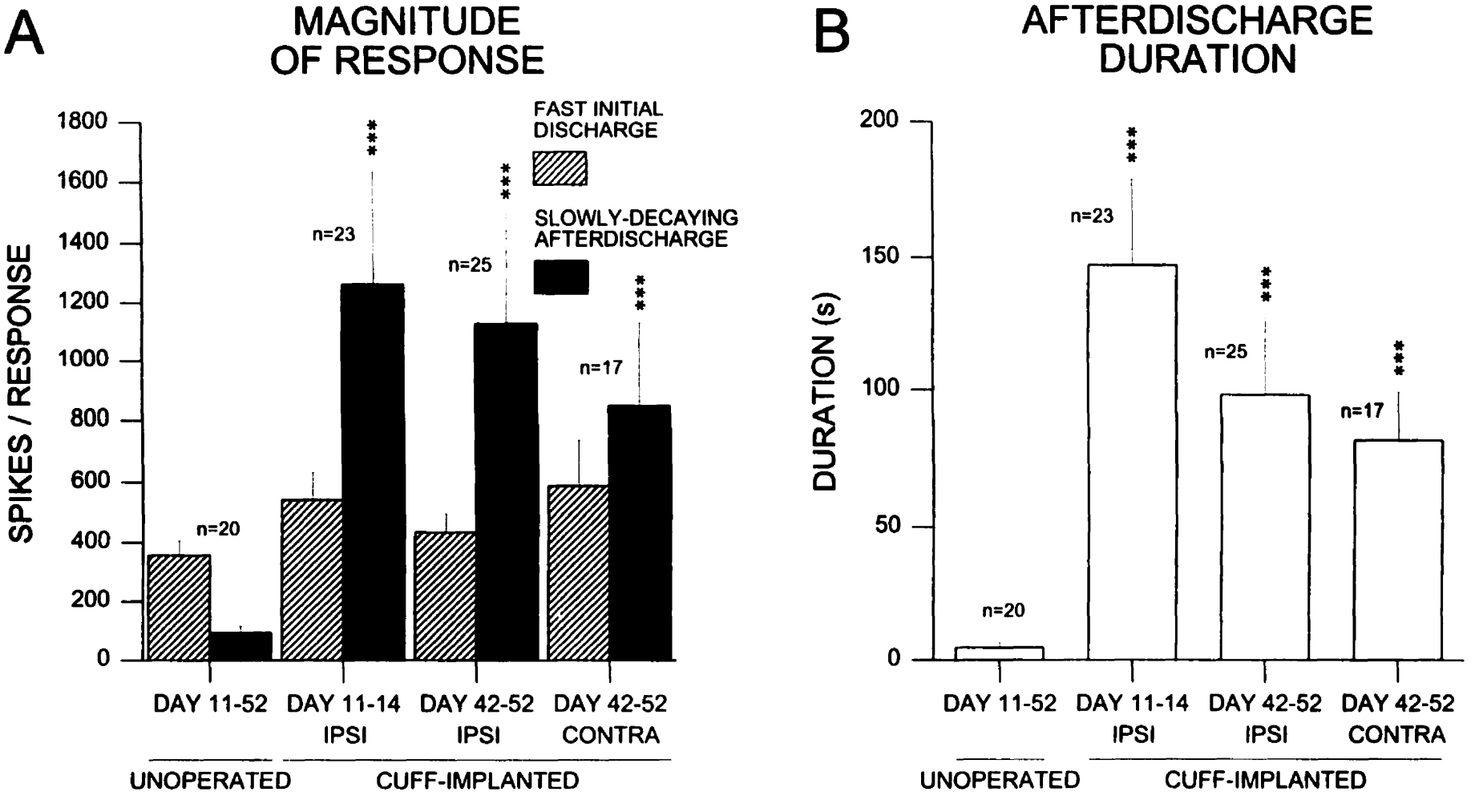
A
MAGNITUDE OF RESPONSE
SPIKES / RESPONSE
1800
1600
1400
1200
1000
800
600
400
200
0
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
n=20
n=23
n=25
n=17
***
DAY 11-52
DAY 11-14 IPSI
DAY 42-52 IPSI
DAY 42-52 CONTRA
UNOPERATED
CUFF-IMPLANTED
B
AFTERDISCHARGE DURATION
DURATION (s)
200
150
100
50
0
n=20
n=23
n=25
n=17
***
DAY 11-52
DAY 11-14 IPSI
DAY 42-52 IPSI
DAY 42-52 CONTRA
UNOPERATED
CUFF-IMPLANTED

Figure 4. Summary of the effects of innocuous pressure stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. The vertical axis shows the number of spikes per pressure-evoked fast initial discharge and slowly-decaying afterdischarge response. The horizontal axis shows fast initial discharge and slowly-decaying afterdischarge responses in unoperated rats (days 11-52) and in short- (days 11-14) and long-term (days 42-52) cuff-implanted rats. *A*: In unoperated rats, afterdischarge activity was normally not present or at most of negligible magnitude. In cuff-implanted rats, slowly-decaying afterdischarge responses were evoked in ipsilateral (IPSI) neurons in short- and long-term rats and in contralateral (CONTRA) neurons in long-term rats (*** $P < 0.001$ vs. unoperated). Cuff implantation had no effect on the fast initial discharge response. *B*: Effect of cuff implantation on the duration of the pressure-evoked slowly-decaying afterdischarge. The vertical axis represents duration expressed in seconds. The horizontal axis shows the duration of the slowly-decaying afterdischarge in unoperated rats and in short- and long-term cuff-implanted rats at the time points shown in *A*. In short- and long-term cuff-implanted rats, the slowly-decaying afterdischarge responses persisted for several seconds. (*** $P < 0.001$ vs. unoperated)

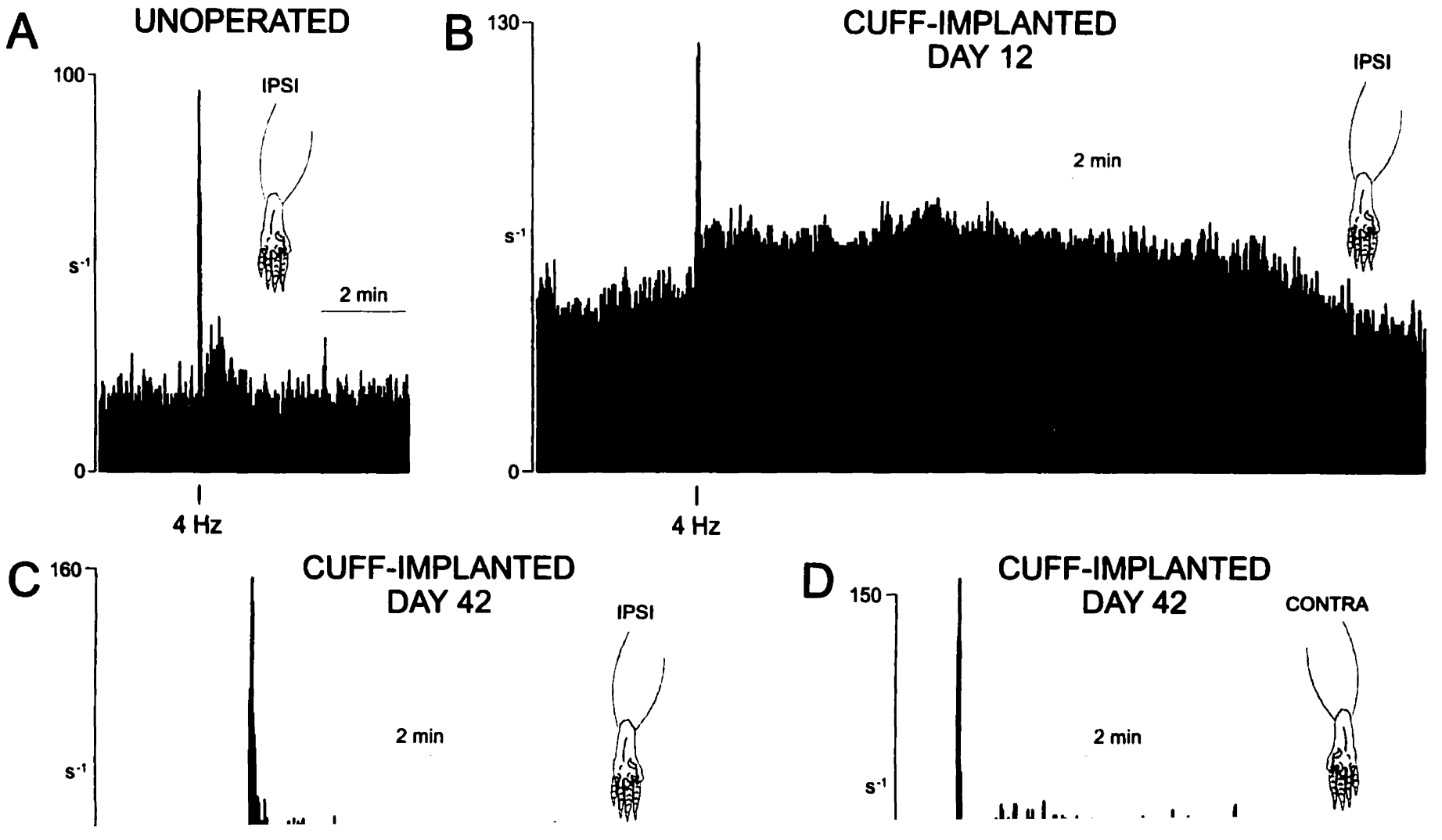
A
UNOPERATED
100
s-1
0
IPSI
2 min
4 Hz
B
130
s-1
0
CUFF-IMPLANTED
DAY 12
IPSI
2 min
4 Hz
C
160
s-1
CUFF-IMPLANTED
DAY 42
IPSI
2 min
D
150
s-1
CUFF-IMPLANTED
DAY 42
CONTRA
2 min

Figure 5. Effect of 4 Hz electrical stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. *A*: The ratemeter record, top left, shows that in an unoperated rat 4 Hz stimulation of the sciatic nerve (5 mA pulse of 1 ms duration given for 3 s) evoked a fast initial discharge which lasted only for the duration of the stimulus and a slowly-decaying afterdischarge which persisted 1.5 to 2 min (968 μm). The time and duration of electrical stimulation are shown by the narrow rectangle below the ratemeter histogram. The inset shows the cutaneous receptive field to touch stimulation, depicted by the shaded area. *B*: In a short-term cuff-implanted rat tested on day 12, 4 Hz stimulation of the ipsilateral sciatic nerve (IPSI) produced a fast initial discharge which lasted only for the duration of the stimulus and a slowly-decaying afterdischarge which persisted approximately 10 min (1294 μm). Description of the inset is otherwise similar to that in *A*. *C*: In a long-term cuff-implanted rat tested on day 42, 4 Hz stimulation of the ipsilateral nerve (IPSI) produced a fast initial discharge and a slowly-decaying afterdischarge which persisted for approximately 4 min (1062 μm). *D*: In a long-term cuff-implanted rat tested on day 42, 4 Hz stimulation of the contralateral sciatic nerve (CONTRA) produced a fast initial discharge and a slowly-decaying afterdischarge which persisted for approximately 4 min (1166 μm).

4 Hz ELECTRICAL STIMULATION

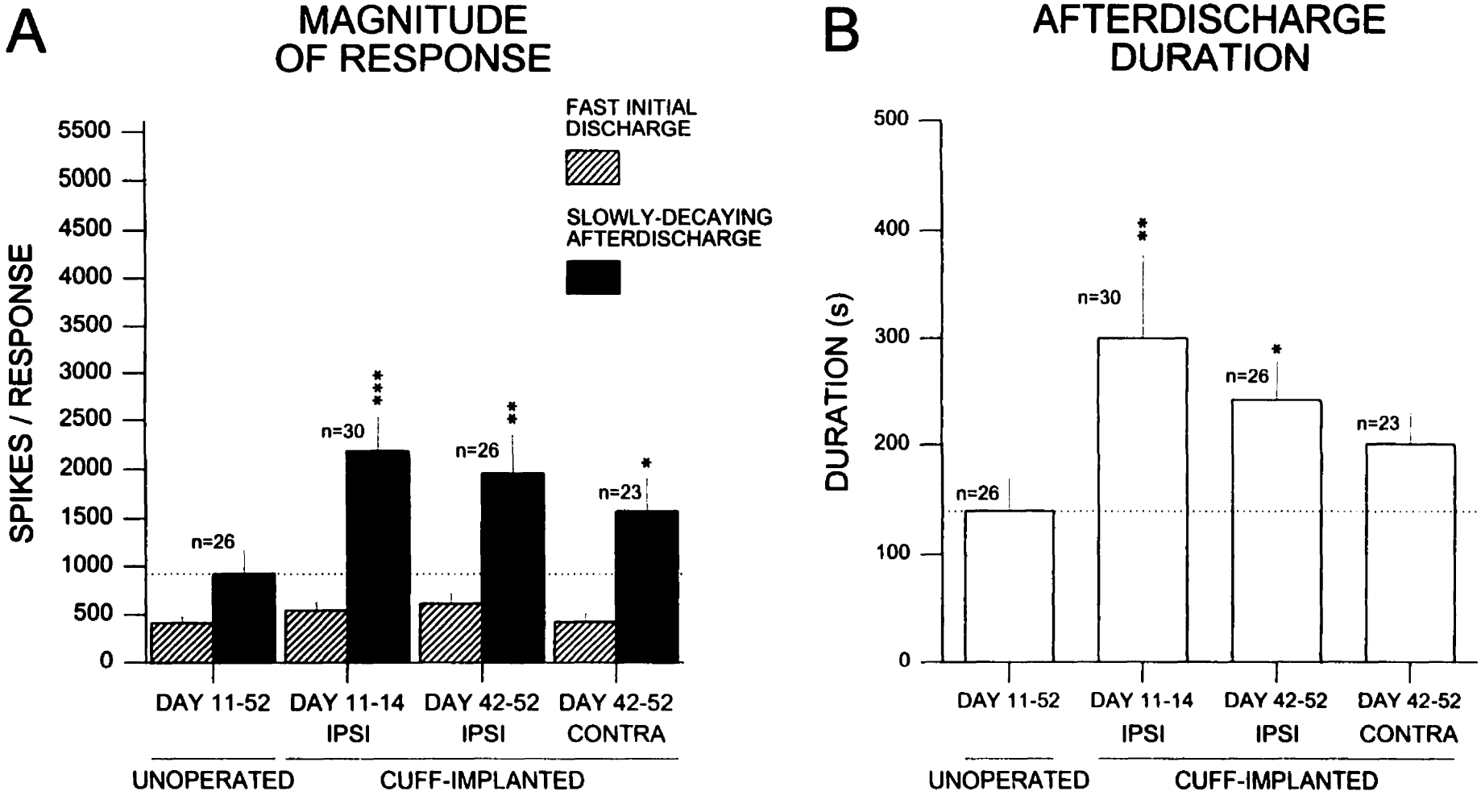

Figure 6. Summary of the effects of 4 Hz nerve stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. The vertical and horizontal axis are otherwise similar to those shown previously. *A*: In unoperated rats, the mean number of spikes per slowly-decaying afterdischarge is depicted by the dotted line. In cuff-implanted rats, the magnitude of the slowly-decaying afterdischarge in ipsilateral (IPSI) neurons in short- and long-term rats and in contralateral (CONTRA) neurons in long-term rats was greater than that in unoperated rats (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. unoperated). Cuff implantation had no effect on the fast initial discharge response. *B*: Effect of cuff implantation on the duration of the 4 Hz stimulation-evoked slowly-decaying afterdischarge. In unoperated rats, the mean duration of the slowly-decaying afterdischarge is illustrated by the dotted line. In cuff-implanted rats, the mean duration of the slowly-decaying afterdischarge in ipsilateral neurons in short- and long-term rats was greater than that in unoperated rats. (* $P < 0.05$ and ** $P < 0.01$ vs. unoperated)

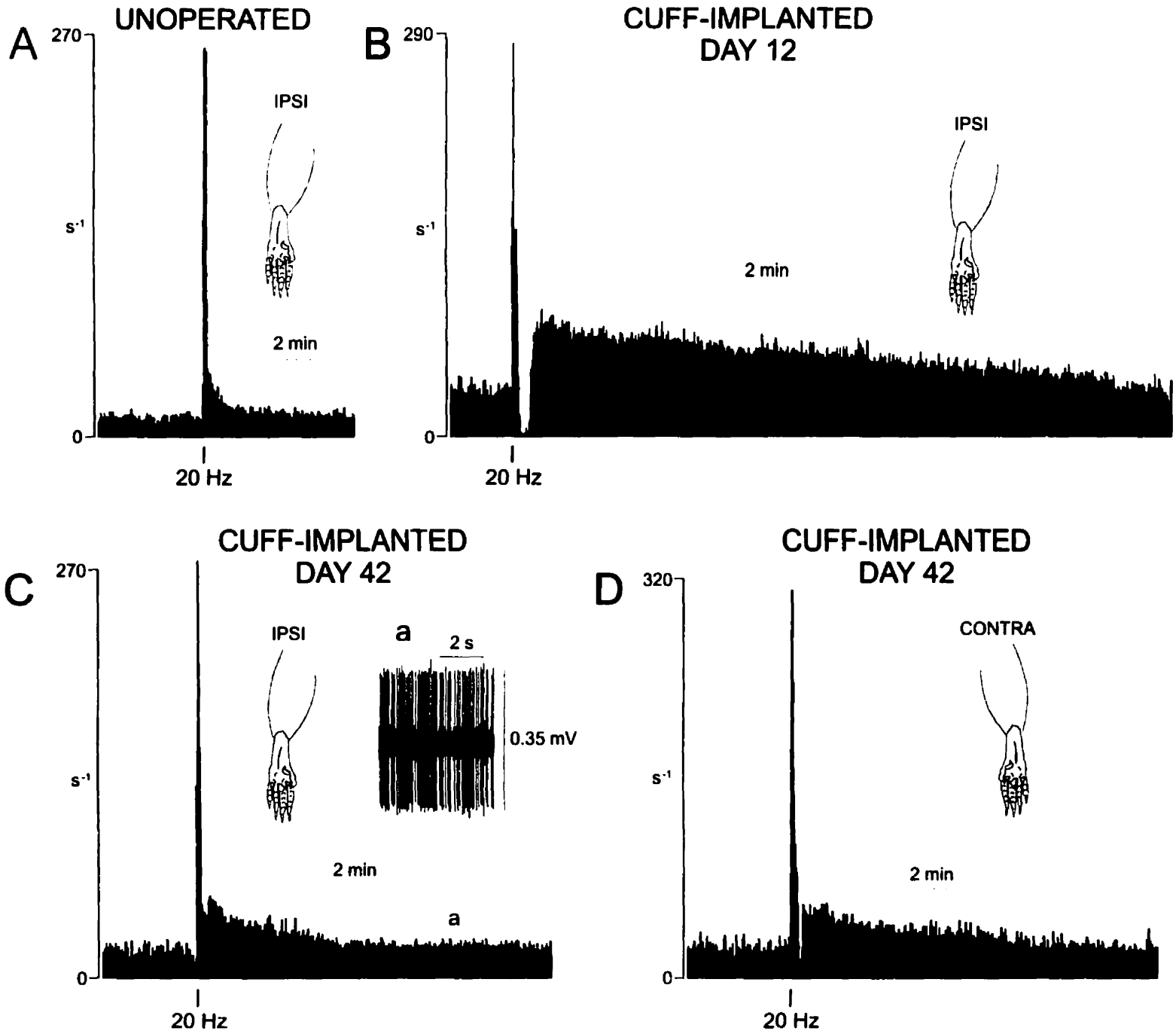
A
UNOPERATED
270
s-1
0
IPSI
2 min
20 Hz
B
CUFF-IMPLANTED
DAY 12
290
s-1
0
IPSI
2 min
20 Hz
C
CUFF-IMPLANTED
DAY 42
270
s-1
0
IPSI
a
2 s
0.35 mV
2 min
a
20 Hz
D
CUFF-IMPLANTED
DAY 42
320
s-1
0
CONTRA
2 min
20 Hz

Figure 7. Effect of 20 Hz electrical stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. *A*: The ratemeter record, top left, shows that in an unoperated rat 20 Hz stimulation of the sciatic nerve (5 mA pulse of 1 ms duration given for 3 s) evoked a fast initial discharge which lasted only for the duration of the stimulus and a slowly-decaying afterdischarge which persisted 0.5 to 1 min (876 μm). The time and duration of electrical stimulation are shown by the narrow rectangle below the ratemeter histogram. The inset shows the cutaneous receptive field of the ipsilateral hind paw to touch stimulation, depicted by the shaded area. *B*: In a short-term cuff-implanted rat tested on day 12, 20 Hz stimulation of the ipsilateral sciatic nerve (IPSI) produced a fast initial discharge and a slowly-decaying afterdischarge which persisted approximately 18 min (692 μm). Description of the inset is otherwise similar to that in *A*. *C*: In a long-term cuff-implanted rat tested on day 42, 20 Hz stimulation of the ipsilateral sciatic nerve (IPSI) produced a fast initial discharge and a slowly-decaying afterdischarge which persisted 3 to 4 min (382 μm). The extracellular record shows single unit activity taken at 'a' in the ratemeter record. *D*: In a long-term cuff-implanted rat tested on day 42, 20 Hz stimulation of the contralateral sciatic nerve (CONTRA) produced a fast initial discharge and a slowly-decaying afterdischarge which persisted for approximately 4 min (618 μm).

20 Hz ELECTRICAL STIMULATION

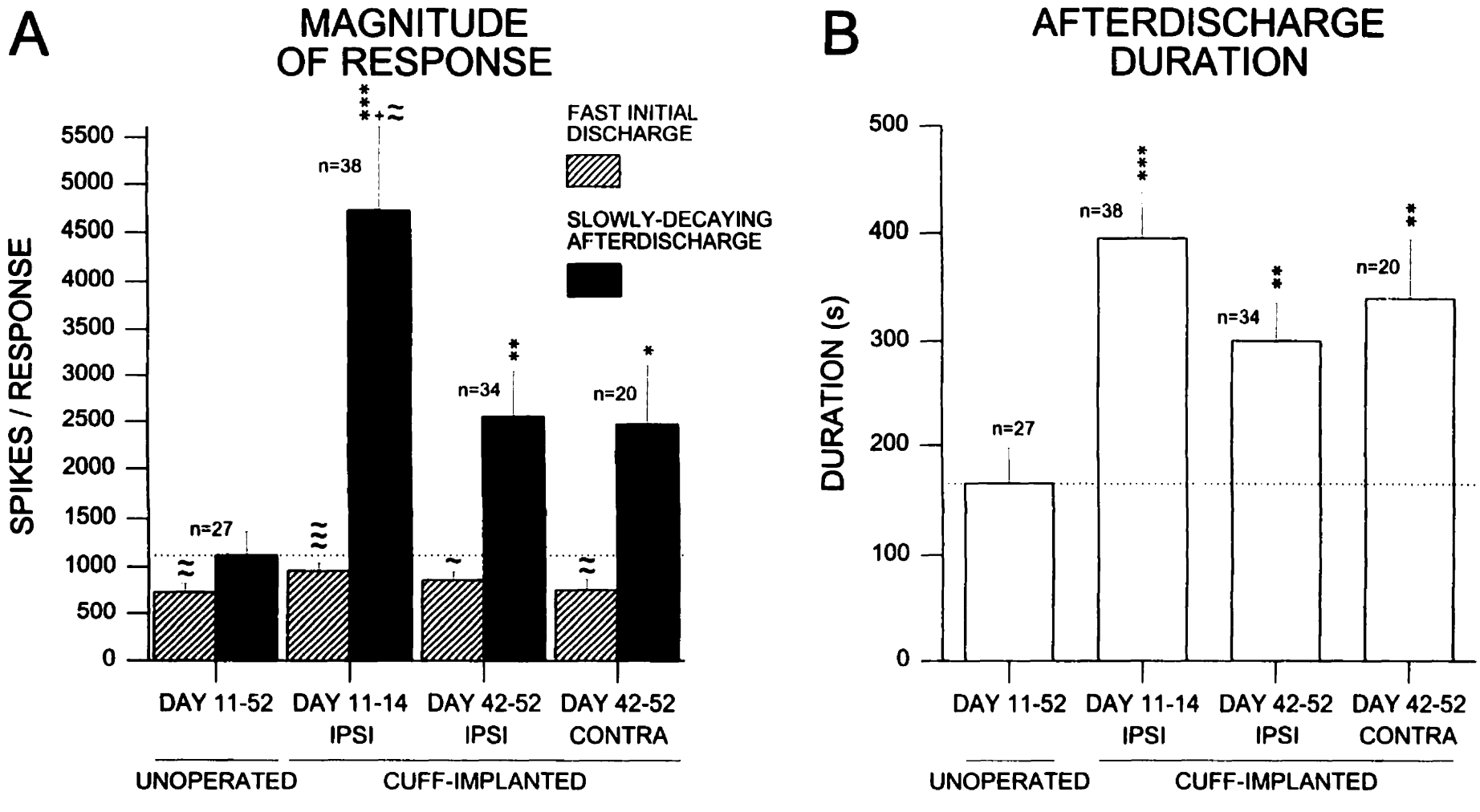

Figure 8. Summary of the effects of 20 Hz nerve stimulation on wide dynamic range dorsal horn neuronal activity in unoperated and in cuff-implanted rats. The vertical and horizontal axis are otherwise similar to those shown previously. *A*: In unoperated rats, the mean number of spikes per slowly-decaying afterdischarge is depicted by the dotted line. In cuff-implanted rats, the magnitude of the slowly-decaying afterdischarge in ipsilateral (IPSI) neurons in short- and long-term rats and in contralateral (CONTRA) neurons in long-term rats was greater than that in unoperated rats (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. unoperated). The mean magnitude of the slowly-decaying afterdischarge in short-term rats was also greater than that in long-term rats tested ipsilaterally (+ $P < 0.05$). Cuff implantation had no effect on the fast initial discharge response. In addition, the mean magnitude of the slowly-decaying afterdischarge in short-term rats was greater than that in short-term rats tested with 4 Hz stimulation (˜ ˜ $P < 0.01$). Furthermore, the mean magnitude of the fast initial discharge in cuff-implanted rats was greater than that in cuff-implanted rats tested with 4 Hz stimulation (˜ $P < 0.05$, ˜ ˜ $P < 0.01$ and ˜ ˜ ˜ $P < 0.001$). *B*: Effect of cuff implantation on the duration of the 20 Hz electrical stimulation-evoked slowly-decaying afterdischarge. In unoperated rats, the mean duration of the slowly-decaying afterdischarge is illustrated by the dotted line. In cuff-implanted rats, the mean duration of the slowly-decaying afterdischarge in ipsilateral neurons in short- and long-term rats and in contralateral neurons in long-term rats was greater than that in unoperated rats. (** $P < 0.01$ and *** $P < 0.001$ vs. unoperated)

Unifying Statement

These last two chapters have confirmed at the cellular level that the model chosen is characterized by spontaneous pain, mechanical hyperalgesia and tactile allodynia, and these results are consistent with the data from Chapter 5, that this animal model exhibits similar behavioural changes. Thus, we can now return to the last half of the global question, whether there is a role of the eicosanoid pathway in cellular events mediating chronic pain? This is answered in the last chapter, Chapter 8.

Chapter 8

Spinal Neural Correlate of Spontaneous Pain, Mechanical Hyperalgesia and Tactile Allodynia in an *in vivo* Rat Model of Neuropathic Pain: Implication of the Eicosanoid Pathway via COX

Abstract

The purpose of the present electrophysiological study was to characterize involvement of the eicosanoid pathway via COX in the spinal neural correlate of pain associated with peripheral neuropathy. In male Sprague Dawley rats, peripheral neuropathy was induced by placing a 2 mm PE-90 polyethylene cuff around the left sciatic nerve. Rats were subsequently confirmed to exhibit mechanical allodynia in the von Frey hair test. After anaesthesia with pentobarbital and acute spinalization at T9 extracellular single unit activity was recorded from wide dynamic range dorsal horn neurons in spinal segments L1-4. Nociceptive responses to noxious (pinch) and innocuous (pressure) mechanical cutaneous stimulation of the cuff-implanted hind paw consisted of a fast initial discharge during the stimulus followed by a slowly-decaying afterdischarge. The effects of the non-selective COX-1/-2 inhibitor, indomethacin, and the selective COX-2 inhibitor, meloxicam, were determined on synaptically-elicited activity including on-going discharge and pinch and pressure stimulation-evoked responses.

Indomethacin (2.0 mg/kg, i.v.) depressed on-going discharge ($33.45 \pm 4.33\%$ inhibition; $P < 0.01$ vs. vehicle) of 10 neurons examined. In 6 out of 7 neurons tested with pinch, indomethacin decreased the initial discharge ($29.68 \pm 6.72\%$; $P < 0.01$ vs. vehicle) and the magnitude ($67.61 \pm 6.27\%$; $P < 0.01$ vs. vehicle) and duration ($56.27 \pm 6.84\%$; $P < 0.01$ vs. vehicle) of the afterdischarge. Inhibition of the magnitude of the afterdischarge was greater than that of the initial discharge ($P < 0.01$). Of 5 neurons tested with pressure, indomethacin decreased the initial discharge of only 3

neurons (27.37±7.20%; $P < 0.05$ vs. vehicle) while the magnitude (47.02±13.62%; $P < 0.01$ vs. vehicle) and duration (48.71±9.12%; $P < 0.01$ vs. vehicle) of the afterdischarge were depressed in all 5 neurons. Meloxicam (0.1 mg/kg, i.v.) decreased on-going discharge (52.96±4.48%; $P < 0.01$ vs. vehicle) of 16 neurons examined. Of 12 out of 14 neurons tested with pinch, meloxicam depressed the initial discharge (33.56±5.36%; $P < 0.01$ vs. vehicle) while the magnitude (63.87±4.96%; $P < 0.01$ vs. vehicle) and duration (49.50±7.23%; $P < 0.05$ vs. vehicle) of the afterdischarge were decreased in 13 neurons. Inhibition of the afterdischarge was greater than that of the initial discharge ($P < 0.01$). Of the 6 neurons tested with pressure the initial discharge was decreased by meloxicam in all 6 neurons (28.10±5.85%; $P < 0.01$ vs. vehicle) while the magnitude (75.74±4.74%; $P < 0.01$ vs. vehicle) and duration (55.39±14.39%; $P < 0.01$ vs. vehicle) of the afterdischarge were decreased in 5 neurons. Notably, inhibition of the afterdischarge was significantly greater than that of the initial discharge ($P < 0.001$).

These data are interpreted to suggest that the eicosanoid pathway via COX, in particular COX-2, is involved in mediating and/or modulating spinal nociceptive processing associated with neuropathic pain. Specifically, the predominant depressive effect of indomethacin and meloxicam on on-going discharge and on the afterdischarge of the response to pinch and pressure stimulation suggest participation of the eicosanoid pathway via COX-2 in the spinal neural correlate of spontaneous pain, mechanical hyperalgesia and tactile allodynia. Based on these data, it is suggested that COX

inhibitors, in particular those selective for COX-2, may be a tenable strategy in the pharmacological management of at least some kinds of neuropathic pain.

Introduction

The impact of peripheral nerve injury on mechanisms of sensory processing is extensive and diverse. Specifically, examination of both clinical and basic science literature over the last few years indicates several potential peripheral and central physiological and pharmacological mechanisms which can be influenced by nerve injury to provoke a dysfunctional or abnormal pain state. In this regard, pain management in neuropathy-related disorders in many cases requires a battery of different drug treatment strategies to achieve satisfactory pain relief. The prevailing trend for neuropathic pain therapy tends to involve in some cases utilization of opioids (Portenoy et al. 1990; Rowbotham et al. 1991; Dellemijn and Vanneste 1997; Watson and Babul 1998; Dellemijn 1999; Suzuki et al. 1999), but more often N-methyl-D-aspartate glutamate receptor antagonists such as ketamine (Backonja et al. 1994; Eide et al. 1995; Mathisen et al. 1995; Klepstad and Borchgrevink 1997; Eisenberg and Pud 1998; Rabben et al. 1999), local anaesthetics including lidocaine (Rowbotham et al. 1991, 1996; Galer et al. 1999) and even compounds not conventionally recognized as analgesics including anticonvulsant, antidepressant, and/or antiepileptic drugs (McQuay et al. 1996; MacFarlane et al. 1997; Hansen 1999). Unfortunately, in a number of cases the neuropathic pain is intractable due to unresponsiveness or unfavourable side effects (Arner and Meyerson 1988; Eide et al. 1995; McQuay et al. 1996; MacFarlane et al. 1997; Rabben et al. 1999). In these instances, novel therapeutic approaches may be beneficial and in some cases vital in order to achieve adequate pain control.

Historically, non-steroidal anti-inflammatory drugs (NSAIDs) have remained relatively inconspicuous in the management of neuropathic pain. This is possibly due to the presumption that pain associated with nerve injury is unresponsive to NSAIDs or fear of side effects associated with NSAID use. Nonetheless, some recent clinical studies indicate that NSAIDs are in fact useful in some neuropathic pain control regimens. For example, in some cases cancer-induced neuropathic pain which is unresponsive to opioid and anticonvulsant or antidepressant adjuvant drugs is reported to be depressed by continuous subcutaneous infusion of the NSAID, ketorolac (Ripamonti et al. 1996). Another NSAID, ibuprofen, has been shown to be effective in managing neuropathic pain associated with human immune-deficiency virus (Klaus 1996). Furthermore, topically-administered NSAIDs including aspirin or acetylsalicylic acid have been reported to provide pain relief in patients suffering from acute herpes zoster and postherpetic neuralgia (De Benedittis and Lorenzetti 1996; Bareggi et al. 1998). Thus, taken together these clinical findings suggest that NSAIDs may be effective in the management of some kinds of neuropathic pain syndromes. Importantly, given that NSAIDs exert their effects via inhibition of cyclooxygenase (COX) which catalyses the first step in metabolism of arachidonic acid to prostanoids, it is not unreasonable to speculate from these studies that the eicosanoid signal transduction pathway may be implicated in altered sensory processing associated with peripheral nerve injury.

Recent work in our laboratory has shown that in rats in which a 2 mm polyethylene cuff (Mosconi and Kruger 1996) is placed around the sciatic nerve, on-going activity of

spinal dorsal horn wide dynamic range neurons is greater than that in normal rats (Pitcher and Henry 2000). Additionally, the magnitude and duration of the pinch-elicited slowly-decaying afterdischarge is markedly greater than that in normal rats and innocuous pressure stimulation induces a slowly-decaying afterdischarge in cuff-implanted rats which does not occur in normal rats (Pitcher and Henry 1999b). As these different aspects of neural hyperexcitability were obtained in cuff-implanted rats exhibiting spontaneous pain and tactile allodynia in the von Frey hair test, the exaggerated on-going and peripheral stimulation-evoked activity is interpreted to represent, at least in part, the neurophysiological basis of spontaneous pain, mechanical hyperalgesia and tactile allodynia, respectively. We have recently demonstrated selective effects of NSAID-induced COX inhibition on neuronal activity in the spinal dorsal horn, in particular a preferential effect of the COX inhibitor indomethacin on long-lasting versus brief cutaneous stimulation-elicited excitation of dorsal horn neurons, and no effect on on-going neuronal discharge (Pitcher and Henry 1999c). Thus, the main objective of this study was to determine the effect(s) of COX inhibition on on-going, pinch- and pressure-evoked activity of dorsal horn neurons *in vivo* in rats previously implanted with a 2 mm polyethylene cuff. The results of these experiments would provide insight into the involvement of the eicosanoid signal transduction pathway in the etiology of neuropathic pain.

Despite the widespread use of NSAIDs in the treatment of various rheumatic disorders, their non-selective COX-1/-2 inhibitory effect (Riendeau et al. 1997; Kawai et

al. 1998; Lora et al. 1998) is in some cases associated with adverse side effects, most frequently gastrointestinal, renal and/or haematological (Schachter et al. 1998; Sigthorsson et al. 1998; Singh and Ramey 1998; Singh and Triadafilopoulos 1999). The discovery of COX-2 (Fu et al. 1990; Sirois and Richards 1992) has prompted the development of selective COX-2 inhibitors which are anticipated to revolutionize the pharmacological treatment of chronic inflammation and pain by selectively inhibiting COX-2 activity while sparing the beneficial physiological functions of COX-1 activity (Kargman et al. 1996; Robinson 1997). We were given access to the COX-2 selective inhibitor, meloxicam, which is reported to decrease both early and late phases of formalin-induced nociception (Santos et al. 1998), carrageenan-evoked spontaneous pain behavior (Laird et al. 1997) and 'wind-up' of dorsal horn neurons (Lopez-Garcia and Laird 1998). Thus, another aim in this study was to investigate the effects of meloxicam on spinal dorsal horn neuronal activity in rats with peripheral neuropathy.

Preliminary data have been presented in abstract form (Henry and Pitcher 1999d).

Materials and Methods

2.1. Hind paw withdrawal reflex experiments

2.1.1. Animals

Experiments were done using adult, male Sprague-Dawley rats (375-425g) from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana, USA). They were housed in plastic cages containing wood chip bedding (Hardwood Laboratory Bedding, Northeastern Products Corp., Warrensburg, N.Y., USA) and maintained on a 12:12 h light:dark cycle (lights on at 07:00 h) with access to food and water *ad libitum*. Experiments were conducted during the light component of the cycle. Guidelines in *The Care and Use of Experimental Animals* by the Canadian Council on Animal Care (Vols. I and II) were strictly followed and all experiments were approved by the *McGill University Animal Care Committee*.

2.1.2. Cuff implantation

A variation of the technique of Mosconi and Kruger (1996) was used. Under Na-pentobarbital anaesthesia (50 mg/kg, i.p., Abbott Laboratories, Limited, Montreal, Canada) and aseptic conditions, the hind paw was shaved and an incision in the skin was made above the biceps femoris muscle. The common sciatic nerve was exposed by blunt dissection through the muscle and was isolated from surrounding connective tissue using glass probes. The nerve was elevated minimally using a sterilized glass probe in order for a 2 mm section of split polyethylene tubing (Intramedic PE-90, Fisher Scientific Ltd.,

Whitby, Ontario, Canada) to be placed around the nerve. The muscle layer was closed using 3-O silk suture thread (Ethicon Inc., Montreal, Quebec, Canada) and the skin layer was closed using 3 stainless steel suture clips (Fine Science Tools, Inc., North Vancouver, British Columbia, Canada). The topical antibacterial ointment, nitrofurazone (0.2%, Univet Pharmaceuticals Ltd., Milton, Ontario, Canada) was placed on the skin suture line to counter the risk of infection.

2.1.3. von Frey hair test

The von Frey hair test was done immediately prior to electrophysiological testing or the day before to confirm the absence or presence of tactile allodynia. Tactile hind paw withdrawal threshold in rats was determined by applying von Frey hairs (Xymotech Biosystems, Inc., Montreal, Quebec, Canada) to the plantar surface of the hind paw. Application of the von Frey hairs to the hind paws was done by placing the rat on a platform designed and constructed specifically for von Frey hair testing (Pitcher et al. 1999a). Described briefly, the platform is made of plexiglass 3 mm thick. It is slightly opaque in appearance and contains 1.5 mm diameter holes in perpendicular rows, 5 mm apart throughout the entire area of the platform. A von Frey hair was applied through a particular hole to a hind paw. For testing, this platform was fixed in a transparent plexiglass chamber (30×30×30 cm).

The mechanical hind paw withdrawal threshold, determined using von Frey hairs, was expressed in grams. Ten hairs ranging from 0.23 to 59.0 g were used. The bending

force of each hair in grams was confirmed periodically by measuring the force exerted by the hair when applied to a Mettler AE 100 electronic balance. The hair was applied in a manner such that the degree of bending was the same as that when applied to the rat hind paw. Confirmation was done because it was determined that slight fluctuation in the bending force of a hair may occur with extended use. If this was the case, the new bending force in grams, determined using the electronic balance, was used as the value.

The protocol used in this study is similar to that used previously (Pitcher et al. 1999a,b). Briefly described, a testing session for a particular rat began after 5 min of habituation to the testing chamber. The series of von Frey hairs was applied from below the platform to the left hind paw in ascending order beginning with the lowest hair (0.23 g). Hairs were applied only when the rat was stationary and standing on all four paws. Application was to the central region of the plantar surface, avoiding the foot pads. A hair was applied to the paw until bending of the hair occurred. Application of the hair was maintained for approximately 2 s. A withdrawal was considered a valid response only if the hind paw was completely removed from the platform. Although infrequent, if a rat walked immediately after application of a particular hair, it was reapplied. On rare occasions, the hind paw only flinched after a single application of the hair. As the hind paw was not lifted from the platform, this was not considered a withdrawal response.

A trial consisted of application of a von Frey hair to the hind paw 5 times at 5 s intervals. If the hind paw withdrawal persisted beyond the 5 s interval, testing resumed after the hind paw was placed appropriately on the platform. Hind paw withdrawal either

4 or 5 times out of the 5 applications was considered to be the withdrawal threshold. If hind limb withdrawal was not evoked 4 or 5 times using a particular hair, the next larger hair in the series was applied in a similar manner.

Once the threshold was determined for the left hind paw, the same testing procedure was repeated on the right hind limb after an inter-trial interval of 5 min. Second and third trials were determined for each of the left and right hind paws with inter-trial intervals of 5 min. If the withdrawal threshold in the second or third trial did not match that of the previous trial(s) on a particular hind paw, the next larger hair in the series was tested. This was done until paw withdrawal thresholds in the 3 trials were consistent. The total testing time for each rat usually lasted 35 to 40 min. In almost all cases, the first 3 trials were consistent.

The baseline withdrawal threshold of both hind paws in the von Frey hair test was determined in cuff-implanted rats prior to surgery (normalized to day 0). Rats were tested next on days 42-52. A statistically significant decrease in the hind paw withdrawal threshold was considered indicative of tactile allodynia.

Only hind paw withdrawal thresholds that remained consistent in each of the 3 trials in cuff-implanted rats were used in the data analysis. Comparisons were done using the Mann-Whitney Rank Sum Test. Differences were considered significant with a P value < 0.05.

2.2. *Electrophysiological experiments*

2.2.1. Animal preparation

Acute electrophysiological experiments were run using rats 42-52 days after cuff implantation and tested previously in paw withdrawal reflex experiments using von Frey hairs. In pilot experiments, sham surgery was without significant effect on dorsal horn neuronal activity in rats tested 42-52 days after surgery which is consistent with the absence of sham surgery-induced tactile allodynia after approximately day 35 after sham surgery (Pitcher et al. 1999b). In this regard, the effects reported here are interpreted to be mainly due to the effects of cuff implantation.

Rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.; Abbott Laboratories Ltd, Montreal, Quebec, Canada) followed by supplements of 10 mg/kg/h, i.v. The right common carotid artery and the jugular vein were catheterized for continuous monitoring of arterial pressure and for injection of drugs, respectively. Temperature of the rat was maintained at approximately 37.5°C using an infrared heating lamp when required.

Spinal cord segments L1 to L4 were exposed for recording from single dorsal horn neurons. The spinal cord was transected at the T9 vertebral level to eliminate supraspinal influences on the activity of lumbar dorsal horn neurons; to minimize spinal shock xylocaine (0.05 ml of 1%; Astra Pharma, Mississauga, Ontario, Canada) was injected into the cord at the level of transection just prior to transection. Once the rat was stabilized on the stereotaxic frame, the exposed spinal cord was covered with mineral oil (Marcol 72, Imperial Oil Limited; Montreal, Quebec, Canada) at 37.5°C to prevent drying.

Experiments were begun 1.5 to 2 h after spinalization.

Each rat normally breathed spontaneously during the experiment. However, if the respiratory rate became irregular, the anaesthetised rat was also paralysed with pancuronium bromide (1 mg/kg i.v. supplemented as necessary; Pavulon, Organon, Scarborough, Ontario, Canada) and ventilated mechanically according to standard parameters (Kleinman and Radford 1964). The anaesthetised animal was sacrificed at the end of the experiment.

2.2.2. Electrical recording

Single unit extracellular spikes were recorded using seven-barrelled or single-barrelled micropipettes (overall tip diameter 4-5 or 1-2 μm, respectively). The multi-barrelled electrodes were used because iontophoretic drug experiments were also run in some cases after testing the effects of synaptic input. A solution of 3 M NaCl was placed in the central recording barrel (impedance 2-4 MΩ measured at 1 kHz with the tip submerged in 0.9% saline). Single unit recordings were made at depths ranging from 150 to 1200 μm in the spinal dorsal horn.

The raw data were amplified 10,000 $\times$ using a DP-301 Differential Amplifier (*Warner Instrument Corp.*), displayed on an oscilloscope (Tektronix 5111) and stored on video cassette tapes using a digital data recorder that incorporated digital pulse code modulation (VR-100A, Instrutech Corporation, Great Neck, NY, U.S.A.) and a conventional video cassette recorder. The signals were also relayed to a frequency

counter/gating unit which counted single unit spikes per unit time (bin widths were 1 s) and which thereby displayed a continuous time histogram of the rate of discharge on a Grass 79D polygraph. Sampling and analysis were done using the data acquisition program, *Spike 2* (Version 2.02; *Cambridge Electronic Design*, Cambridge, England), *SigmaStat* (Version 2.03; *SPSS, Inc.*, USA) and an IBM Pentium computer.

2.2.3. Functional classification of dorsal horn neurons

Functional classification of a lumbar dorsal horn neuron was based on its response to innocuous and noxious stimulation of the cutaneous receptive field of the ipsilateral hind paw. The following natural stimuli were used as search stimuli to elicit synaptic input while penetrating the spinal dorsal horn and to characterize a neuron functionally once stable single unit recording was obtained: (i) hair stimulation, (ii) light touch/moderate pressure using a calibrated clip (0.2 N for 3 s), (iii) noxious mechanical stimulation using a calibrated clip (pinch; 21 N for 3 s) and (iv) noxious radiant heat (measured to reach 50°C at the skin surface) applied by a focused projector bulb through a 10 mm diameter circular hole for a duration of 10 s.

Classification of the identified dorsal horn neurons was in three categories (Pitcher and Henry 1999c, 2000): (i) non-nociceptive neurons that responded only to non-noxious stimuli such as touch and/or pressure stimulation, (ii) wide dynamic range neurons that responded to both noxious and innocuous stimuli or (iii) nociceptive-specific neurons that responded only to noxious stimuli. All the units that responded to the noxious range of

mechanical and/or thermal stimulation showed a characteristic slowly-decaying afterdischarge, as described previously (De Koninck and Henry 1991; Pitcher and Henry 1999c, 2000). Only wide dynamic range neurons were tested in this study. Stimuli were given at 20 to 25 min intervals.

Care was taken to investigate the region in the receptive field corresponding to the same area of the hind paw where von Frey hair testing had been done prior to electrophysiological experiments. In addition, in order to minimize possible differences between neurons located in different mediolateral parts of the spinal dorsal horn in cuff-implanted rats, only a specific recording region adjacent to the entry of dorsal roots in spinal segments L3-4 was searched for single units.

2.2.4. Drug administration

Indomethacin and meloxicam could not be given iontophoretically due to high resistance properties when in the ejection barrel of the electrode. Accordingly, the following data were obtained from the effects of systemic administration. Only one neuron was studied per rat.

Indomethacin (RBI, Natick, Massachusetts, USA) was dissolved in 2% sodium bicarbonate and this solution was titrated to pH 7.4 using sodium monophosphate. It was administered intravenously (2 mg/kg body weight). The vehicle, 2% sodium bicarbonate (pH 7.4 using sodium monophosphate) was administered in a similar manner and served as a control. Vehicle was administered approximately 90 min prior to indomethacin

administration.

Meloxicam (generously supplied by Boehringer Ingelheim, Canada, Ltd.) was dissolved in 100 μl 2M NaOH and distilled water. This stock solution (10 mg/kg) was diluted to 0.1 mg/ml using distilled water (pH 7.4 using 2% $NaHCO_3$ and $NaH_2PO_4 \bullet H_2O$) and was administered i.v. at a dose of 0.1 mg/kg. As a control, the vehicle was administered approximately 90 min prior to meloxicam administration.

2.2.5. Analysis of electrophysiological data

Sampling of on-going activity occurred only after on-going discharge had been stable for at least 5 min and prior to any peripheral stimulation-induced synaptic input. On-going activity was quantified as the total number of spikes during a 60 s period.

Sampling of evoked responses of wide dynamic range dorsal horn neurons included a fast initial discharge followed by a slowly-decaying afterdischarge. The initial discharge persisted only for the duration of the 3 s mechanical stimulus and was quantitated during this period. The sample period of the afterdischarge began immediately after the end of the initial discharge and ended once the firing rate returned to the prestimulus discharge level. Evoked responses were quantified as the total number of spikes in the initial discharge or afterdischarge minus the background discharge of equivalent duration as the evoked response.

The maximum inhibitory effects of indomethacin (determined 30-60 min following drug administration) and meloxicam (determined 40-70 min following drug administration)

on on-going and pinch-and pressure-evoked activity were expressed as percent inhibition as described elsewhere (Pitcher and Henry 1999c). Briefly, the percent inhibition was expressed as a percentage of the magnitude (number of spikes) and duration (seconds) of the response prior to administration of indomethacin or meloxicam. Statistical analysis was done using the Mann-Whitney Rank Sum Test; the mean ($\pm$SEM) percent inhibition following indomethacin or meloxicam was compared to that following vehicle administration and a difference was considered significant with a P value < 0.05. No significant effect of vehicle was observed on on-going discharge or on peripheral stimulation-evoked responses of dorsal horn neurons (expressed as 0% inhibition).

Results

3.1. Hind paw withdrawal threshold

Figs. 1A and B show that in the von Frey test, every rat exhibited a marked decrease ($P < 0.001$ vs. day 0) in hind paw withdrawal threshold after cuff implantation (days 42-52) compared to its own threshold before the cuff was implanted (normalized to day 0). These data are consistent with data reported previously (Pitcher et al. 1999b, 2000).

The rats represented in Figure 1A were used to study the effect(s) of indomethacin and those in Figure 1B to study the effect(s) of meloxicam in electrophysiological experiments.

3.2. Electrophysiological experiments

3.2.1. Effects of indomethacin on spinal dorsal horn neuronal activity

3.2.1.1. On-going discharge

The effect of cuff-implantation on on-going discharge of spinal wide dynamic range neurons (35.33 ± 7.26 spikes/s, $n = 10$) is consistent with that reported previously (Pitcher and Henry 2000). Examples of on-going activity of 2 wide dynamic range dorsal horn neurons are shown prior to the stimulation-evoked discharge in the ratemeter records in Figs. 2A and C.

Figs. 2B and D illustrate the depressive effect of indomethacin (2 mg/kg, i.v.) on

the on-going rate of discharge of the 2 neurons shown in Figs. 2A and C, respectively. Extracellular records showing on-going spiking activity before ('a') and after ('d') indomethacin also reflect the decrease in firing frequency. Figure 3 shows that in the 10 neurons tested, indomethacin depressed on-going activity by $33.45 \pm 4.33\%$ ($P < 0.01$ vs. vehicle). Vehicle was without significant effect on on-going discharge.

3.2.1.2. Response to noxious mechanical stimulation

Noxious mechanical stimulation of the cutaneous receptive field of the hind paw was tested on 7 wide dynamic range neurons and produced an excitatory response in each neuron studied. This response consisted of an initial discharge occurring throughout the duration of the pinch stimulus (726.06 ± 160.07 spikes per response) and an afterdischarge (5459.55 ± 900.06 spikes per response) lasting several minutes beyond the end of the noxious stimulus (501.57 ± 135.80 s). The magnitude and duration of these responses to pinch stimulation are consistent with those reported previously (Pitcher and Henry 2000).

The response of a wide dynamic range neuron to pinch stimulation is depicted in Figure 2A. The initial discharge can be seen during the 3 s pinch stimulus and the afterdischarge can be seen persisting approximately 6 min after the end of the pinch stimulus. The effect of indomethacin on the pinch-evoked response is shown in Figure 2B. While the pinch-elicited initial discharge was inhibited by indomethacin, the ratemeter histogram shows an almost complete block of the afterdischarge. Extracellular records of the neuron depicted in Figs. 2A and B show reduced activity during the afterdischarge after

('e') indomethacin compared to that before ('b').

Figure 3 summarizes the effect of indomethacin on the pinch response. Of the 7 neurons tested, 6 were sensitive to indomethacin. The depressive effect of indomethacin was observed on the initial discharge (29.68±6.72%; $P < 0.01$ vs. vehicle) and on the magnitude (67.61±6.27%; $P < 0.01$ vs. vehicle) and duration (56.27±6.84% ($P < 0.01$ vs. vehicle) of the afterdischarge. The depressive effect of indomethacin on the afterdischarge was markedly greater than its effect on the initial discharge ($P < 0.01$). Vehicle was without significant effect on the pinch-elicited response.

3.2.1.3. Response to innocuous mechanical stimulation

Pressure stimulation of the peripheral cutaneous receptive field was tested on 5 wide dynamic range neurons and evoked an initial discharge persisting only as long as the duration of the pressure stimulus (318.76±82.04 spikes) and an afterdischarge (1074.17±484.11 spikes) which lasted for several seconds after the end of the stimulus (129.67±28.29 s). The magnitude and duration of these pressure responses are comparable to previous measurements (Pitcher and Henry 1999b).

A representative response to pressure stimulation applied to the cutaneous receptive field of the cuff-implanted hind paw is illustrated in Figure 2C. The initial discharge can be seen during the 3 s stimulus and the afterdischarge is shown persisting approximately 2 min after the end of the stimulus. The depressive effect of indomethacin on the pressure-induced response is illustrated by the ratemeter histogram in Figure 2D.

Figure 3 summarizes the effect of indomethacin on the pressure-elicited response. Of the 5 neurons tested with indomethacin, 3 exhibited a depression of the initial discharge ($27.37 \pm 7.20\%$; $P < 0.05$ vs. vehicle) while the magnitude ($47.02 \pm 13.62\%$; $P < 0.01$ vs. vehicle) and duration ($48.71 \pm 9.12\%$; $P < 0.01$ vs. vehicle) of the afterdischarge in all 5 neurons were sensitive to the effect of indomethacin. Vehicle was without significant effect on the pressure-elicited response.

3.2.2. Effects of meloxicam on spinal dorsal horn neuronal activity

3.2.2.1. On-going activity

In another group of rats ($n = 16$), the effect of cuff implantation on on-going discharge (34.82 ± 12.55 spikes/s) was examined in 16 wide dynamic range neurons. Examples of on-going activity of 2 of these neurons are shown before the peripheral stimulation-induced discharge in Figs. 4A and C.

The depressive effect of meloxicam (0.1 mg/kg, i.v.) on the on-going activity of each of these neurons is shown in Figs. 4B and D. Extracellular records of the neuron depicted in Figs. 4C and D demonstrate the marked decrease in the rate of on-going discharge after meloxicam ('e') administration compared to that before ('a'). Figure 5 summarizes the inhibitory effect of meloxicam on the on-going discharge of the 16 neurons tested ($52.96 \pm 4.48\%$; $P < 0.01$ vs. vehicle). Vehicle was without significant effect on on-going discharge.

3.2.2.2. Response to noxious mechanical stimulation

Noxious mechanical stimulation of the cutaneous receptive field was tested on 14 wide dynamic range neurons. The initial discharge (736.03±109.09 spikes), the afterdischarge (6363.95±666.94 spikes) and its duration (519.39±44.18 s) are characteristic of pinch-elicited responses (Pitcher and Henry 1999b, 2000).

The ratemeter histogram in Figure 4A depicts a representative response of a wide dynamic range neuron to noxious pinch applied to the cutaneous receptive field on the cuff-implanted hind paw. Figure 4B illustrates the preferential depressive effect of meloxicam on the pinch-evoked afterdischarge.

The data, summarized in Figure 5, indicate that of the 14 neurons tested with meloxicam, 12 exhibited a depressed initial discharge (33.56±5.36%; $P < 0.01$ vs. vehicle) while 13 showed a substantially decreased magnitude (63.87±4.96%; $P < 0.01$ vs. vehicle) and duration (49.50±7.23%; $P < 0.05$ vs. vehicle) of the afterdischarge. The percent inhibition of the afterdischarge was significantly greater than that of the initial discharge ($P < 0.01$). Vehicle was without significant effect on the pinch-elicited response.

3.2.2.3. Response to innocuous mechanical stimulation

Innocuous pressure stimulation was tested on 6 wide dynamic range neurons. Characteristic initial discharge (439.08±186.86 spikes) and afterdischarge (1654.40±650.53 spikes; duration of 81.71±29.80 s) responses were observed. The

ratemeter histograms in Figs. 4C and D illustrate representative responses of a wide dynamic range neuron to pressure stimulation of the cutaneous receptive field before (C) and after (D) meloxicam. It is evident from Figure 4D the preferential inhibitory effect of meloxicam on the afterdischarge. This is also demonstrated by the extracellular records which depict spiking activity during the afterdischarge before ('c') and after ('f') meloxicam administration. The firing frequency in 'f' is noticeably decreased compared to that in 'c'.

The effect of meloxicam on the pressure-evoked discharge of the 6 wide dynamic range neurons tested is summarized in Figure 5. All 6 neurons showed a depressed initial discharge ($28.10 \pm 5.85\%$; $P < 0.01$ vs. vehicle) and 5 neurons exhibited a substantial decrease of the magnitude ($75.74 \pm 4.74\%$; $P < 0.01$ vs. vehicle) and duration ($55.39 \pm 14.39\%$; $P < 0.01$ vs. vehicle) of the afterdischarge. Notably, the percent inhibition of the afterdischarge was significantly greater than its effect on the initial discharge ($P < 0.001$). Vehicle was without significant effect on the pressure-elicited response.

Discussion

The main finding in this study is that NSAID-induced COX inhibition decreases on-going and stimulation-evoked discharge of spinal dorsal horn neurons in rats with peripheral neuropathy. Specifically, the non-selective COX-1/-2 inhibitor, indomethacin, and the selective COX-2 inhibitor, meloxicam, depress the exaggerated afterdischarge response to noxious pinch stimulation. The initial discharge response to this brief pinch stimulus is depressed considerably less. The afterdischarge evoked by innocuous pressure stimulation, which does not occur in normal rats (Pitcher and Henry 1999c, 2000), is also selectively attenuated by the effect(s) of each NSAID. Notably, the exaggerated on-going discharge, which is greater than on-going discharge in normal rats (Pitcher and Henry 2000), also exhibits sensitivity to the effects of indomethacin and meloxicam.

The importance of the electrophysiological approach used in this study is that the effects of these NSAIDs were determined on neuronal activity in the spinal dorsal horn. Given that at the doses used, intravenous administration of indomethacin, meloxicam and their respective vehicles were consistently without effect on spike amplitude and that arterial pressure and respiration were not altered, the data are interpreted to reflect the effects of indomethacin and meloxicam exclusively on mechanisms mediating and/or modulating sensory processing.

The effects of indomethacin on sensory processing are presumed to have occurred via inhibition of COX-1 and -2 activity (Mitchell et al. 1994; Gierse et al. 1995; Yamamoto and Nozaki-Taguchi 1996; Harada et al. 1998) and by decreasing the level of

prostaglandin E_2 (Malmberg and Yaksh 1994). Given the depressive effect of meloxicam on prostaglandin E_2 synthesis (Engelhardt et al. 1996a) and its COX-2-selective properties (Engelhardt et al. 1996a,b; Lazer et al. 1997; Ogino et al. 1997; Blanco et al. 1999), it is presumed that meloxicam exerted its effect on neuronal activity by altering eicosanoid synthesis via inhibition of COX-2.

4.1. On-going discharge

A novel finding in this study was the depressive effect of indomethacin and meloxicam on on-going discharge. As the same doses of these drugs exerted no effect on on-going activity in normal rats it is evident that peripheral neuropathy augmented eicosanoid synthesis to the extent that eicosanoids exceeded normal basal levels giving rise to tonically-elevated on-going activity of dorsal horn neurons. As meloxicam does not influence on-going discharge in normal rats (Henry and Pitcher 1999), its depressive effect on on-going neuronal activity in cuff-implanted rats leads us to speculate that upregulation of this pathway may involve elevated expression and tonic activity of COX-2.

Recently, we put forth the concept that elevated on-going discharge such as that shown here may underlie spontaneous pain behavior in cuff-implanted rats (Pitcher et al. 1999b). In this regard, the effects of indomethacin and meloxicam on the exaggerated on-going activity may be indicative of the mechanisms underlying the therapeutic effects of NSAIDs in alleviating spontaneous pain associated with peripheral neuropathy. Although in some cases NSAID treatment is reported to be ineffective in providing pain relief in

patients with neuropathic pain (Max et al. 1988; Kingery 1997), other reports indicate that they can be very effective in treating pain in patients with diabetic neuropathy (Cohen and Harris 1987), cancer-induced neuropathic pain (Ripamonti et al. 1996) and pain associated with herpes zoster and post herpetic neuralgia (King 1988, 1993; De Benedittis and Lorenzetti 1996).

4.2. Initial discharge

Both indomethacin and meloxicam depressed the initial discharge of the response to noxious mechanical stimulation as well as to innocuous mechanical stimulation of the peripheral receptive field. However, comparison of the data in this study with those of the earlier study of the effects of indomethacin on the initial discharge in normal rats, indicates that the inhibition was not different (Pitcher and Henry 1999c). This suggests that cuff implantation did not alter the involvement of the eicosanoid pathway in bringing about the initial discharge.

4.3. Afterdischarge

The preferential effect of indomethacin and meloxicam on the pinch-elicited afterdischarge is in accord with the concept of an association between the tonic effects of peripheral stimulation-induced synaptic input and activation of the eicosanoid signal transduction pathway. Interestingly, the percent inhibition of the pinch-elicited afterdischarge shown here is comparable to that in normal rats given the same dose of

indomethacin (Pitcher and Henry 1999c). This is interpreted to suggest that peripheral neuropathy leads to upregulation and considerably longer-lasting COX activity which may account for the greater magnitude and duration of the afterdischarge. Based on the inhibitory effect of meloxicam on the pinch afterdischarge, it is reasonable to suggest that COX-2 may have contributed to the exaggerated and prolonged pinch afterdischarge.

Recently, we have shown that the exaggerated afterdischarge is the distinguishing feature of the response of spinal dorsal horn neurons to pinch stimulation (Pitcher and Henry 2000) and suggest that it may be the neural correlate of mechanical hyperalgesia. In this regard, its sensitivity to indomethacin and meloxicam may provide insight into the mechanisms underlying mechanical hyperalgesia in the neuropathic pain state. In particular, the present data may explain the depressive effects of indomethacin and meloxicam on mechanical hyperalgesia reported in partial nerve-ligated rats (Striatowicz et al. 1999).

The pressure-evoked afterdischarge was also selectively depressed by each of these NSAIDs which is consistent with the concept of a preferential involvement of COX in long-lasting elevated synaptic input. Importantly, given that this afterdischarge is the distinguishing feature of the response to innocuous pressure stimulation in cuff-implanted rats (Pitcher and Henry 1999b), its sensitivity to NSAIDs is a novel and important aspect of the neurophysiological mechanisms underlying tactile allodynia. In fact, to the best of our knowledge, the depressive effect of indomethacin on the pressure-elicited afterdischarge shown in the present study is the first identification of the involvement of

the eicosanoid pathway in the neurophysiological basis of tactile hypersensitivity associated with peripheral neuropathy. Importantly, the involvement of the eicosanoid pathway in the pressure afterdischarge may be the cellular correlate of tactile hypersensitivity which characterizes neuropathic pain in humans (Gracely et al. 1992). In fact, the present data may provide insight into the therapeutic effect of topical application of the non-selective NSAID aspirin in treating tactile hypersensitivity in patients with peripheral neuropathy (King 1993). An important clinical implication of the effect of meloxicam on the pressure afterdischarge is the possible involvement of COX-2 in tactile hypersensitivity in patients with peripheral neuropathy.

4.4. Spinal COX

In the present study, the possibility of any drug effect on COX on lumbar dorsal horn neurons via supraspinal structures was eliminated as experiments were run in rats acutely spinalized at the thoracic level. Thus, the effects of indomethacin and meloxicam on COX inhibition are interpreted to have occurred independently of a supraspinal site and were likely at a spinal and/or peripheral level.

Support for a spinal site of action of indomethacin is that it crosses the blood-brain barrier (Bannwarth et al. 1990). In addition, a recent report from our laboratory showed that systemically administered indomethacin depresses the excitatory effects of iontophoretic application of excitatory agents onto rat spinal dorsal horn neurons (Pitcher and Henry 1999a). Importantly, constitutively-expressed COX-1 and -2 protein

(Goppelt-Struebe and Beiche 1997; Willingale et al. 1997; Beiche et al. 1998) in the dorsal horn provides mechanisms at the spinal level by which the effects of indomethacin may have been expressed. Furthermore, L5-6 spinal nerve ligation increases COX-2 protein in the rat spinal dorsal horn (Zhao et al. 2000). Importantly, given the non-selective COX-1/-2 inhibitory properties of indomethacin and the depressive effects of indomethacin on the on-going activity and on pinch- and pressure-evoked neuronal responses of neurons in the spinal dorsal horn, involvement of COX-1 and/or -2 in the spinal dorsal horn may be considered in mediating and/or modulating, at least in part, sensory processing including the neural correlates of mechanical hyperalgesia, tactile allodynia and spontaneous pain associated with peripheral nerve injury.

The data in this study may represent the neurophysiological basis for effects of NSAIDs observed in behavioral studies. For instance, intrathecal administration of the NSAID ketorolac is reported to decrease behavior associated with peripheral nerve injury in rats, including L5-6 spinal nerve ligation (Lashbrook et al. 1999) and sciatic nerve-constriction (Parris et al. 1996) models. In addition, intrathecal administration of the COX-1 selective inhibitor, piroxicam, and the COX-2 selective inhibitor, NS-398, are also reported to depress behavior associated with L5-6 spinal nerve ligation in rats (Lashbrook et al. 1999).

Although meloxicam is reported to inhibit 'wind-up' in an *in vitro* spinal cord preparation (Lopez-Garcia and Laird 1998), unlike indomethacin (Pitcher and Henry 1999a), the possibility of a spinal site of action of systemically-administered meloxicam

appears to be less well supported. This is based in part on the selective effect of systemic administration of meloxicam on peripherally-mediated reflex responses without affecting centrally-mediated reflexes (Laird et al. 1997). Moreover, little if any meloxicam is reported in the rat central nervous system following systemic administration (Busch et al. 1998). Although a spinal site of action is not ruled out in the present study, sensitivity of dorsal horn neuronal activity to meloxicam may be accounted for by a peripheral site of action.

4.5. Peripheral COX

Accumulating evidence reveals increased expression of the eicosanoid pathway at the site of nerve injury in different models of neuropathic pain. This may derive from pathophysiologic mechanisms including inflammation (Clatworthy et al. 1995) and neuroimmune factors (Van Dijk et al. 1997; Clatworthy 1998; Bennett 1999; Eliav et al. 1999) at the nerve injury site. In particular, it is suggested that non-neuronal cells including macrophages, mast cells, neutrophils and Schwann cells release eicosanoids at the site of peripheral nerve injury (Tracey and Walker 1995). Specifically, phospholipase A_2 expression and activity is increased in rat glial cells within the precise region of neuron loss following facial nerve axotomy (Stephenson et al. 1999). In the chronically-constricted sciatic nerve the increase in phospholipase A_2 parallels the time course of mechanical hyperalgesia (Sawin et al. 1997). Mast cell activation induces prostaglandin D_2 and E_2 synthesis (Reddy et al. 1997) which is sensitive to indomethacin (Marshall et al.

1999). In addition, COX-2 expression is elevated in phagocytosing macrophages in the endoneurium in patients with vasculitic neuropathies (Satoi et al. 1998). Furthermore, in neutrophils, which are recruited to injured nerves (Clatworthy et al. 1995) and can lead to hyperalgesia (Levine et al. 1985), tumor necrosis factor increases the expression of COX-2 which is paralleled with prostaglandin E_2 release (Maloney et al. 1998). Thus, taken together, these studies establish a basis for the proposal that eicosanoids can be synthesized, released and likely exert effects on sensory neurons, perhaps altering neuronal activity, as a result of an inflammatory/neuroimmune response at the site of nerve injury.

In the present study, indomethacin- and meloxicam-induced COX-2 inhibition at the site of cuff implantation may account for reduced excitability of sensory neurons and the depressed synaptically-elicited dorsal horn neuronal activity. A peripheral site of action has been reported for systemically-administered indomethacin (Wallace et al. 1999) and meloxicam (Laird et al. 1997; Campos et al. 1998; Santos et al. 1998). Mechanical hyperalgesia associated with partial nerve cut is reported to be depressed following local application of indomethacin onto the site of nerve injury (Striatowicz et al. 1999).

COX expression at the level of the cutaneous receptive field may also be considered to account for the effects of indomethacin and meloxicam in the present study. Injection of meloxicam into the cutaneous receptive field produces relief of mechanical hyperalgesia in partial nerve-ligated rats (Striatowicz et al. 1999), an effect also observed with indomethacin. Furthermore, topically-administered aspirin (Kassirer 1988; King 1988, 1993; De Benedittis et al. 1992; De Benedittis and Lorenzetti 1996; Bareggi et al. 1998)

or piroxicam (Nicholls 1993) is effective in treating pain associated with acute herpes zoster and post herpetic neuralgia.

Collectively, these studies suggest that the eicosanoid pathway, perhaps via COX-2, may be involved in generating and sustaining afferent activity from peripheral loci which may occur in the neuropathic pain state (Gracely et al. 1992). Thus, peripheral COX, specifically COX-2, may be considered in mediating and/or modulating on-going discharge and pinch- and pressure-elicited responses of dorsal horn neurons in the present study.

Conclusions

This study provides the first evidence implicating eicosanoids in the neurophysiological basis of altered sensory processing associated with peripheral neuropathy in the rat. The exaggerated pinch-evoked afterdischarge, which is characteristic of the pinch response in cuff-implanted rats, was selectively depressed by systemic administration of indomethacin and meloxicam while the initial discharge was influenced less. The effects of these drugs on the pinch response may be indicative of the neurophysiological mechanisms underlying mechanical hyperalgesia. The exaggerated on-going activity and the innocuous pressure-evoked afterdischarge, which are not seen in normal rats, were also markedly sensitive to both indomethacin and meloxicam. An important clinical implication of these data is that the eicosanoid pathway via COX-2 may also be involved in different aspects of peripheral neuropathy including specifically spontaneous pain and tactile allodynia.

NSAIDs including selective COX-2 inhibitors still remain a relatively under examined option in the treatment of pain coupled with peripheral neuropathy. The data in this electrophysiological study indicate a specific role of the eicosanoid pathway, perhaps via COX-2, in sustained elevated synaptic transmission associated with peripheral neuropathy. These novel findings encourage further investigation of the involvement of the eicosanoid pathway in pain syndromes associated with peripheral nerve pathology.

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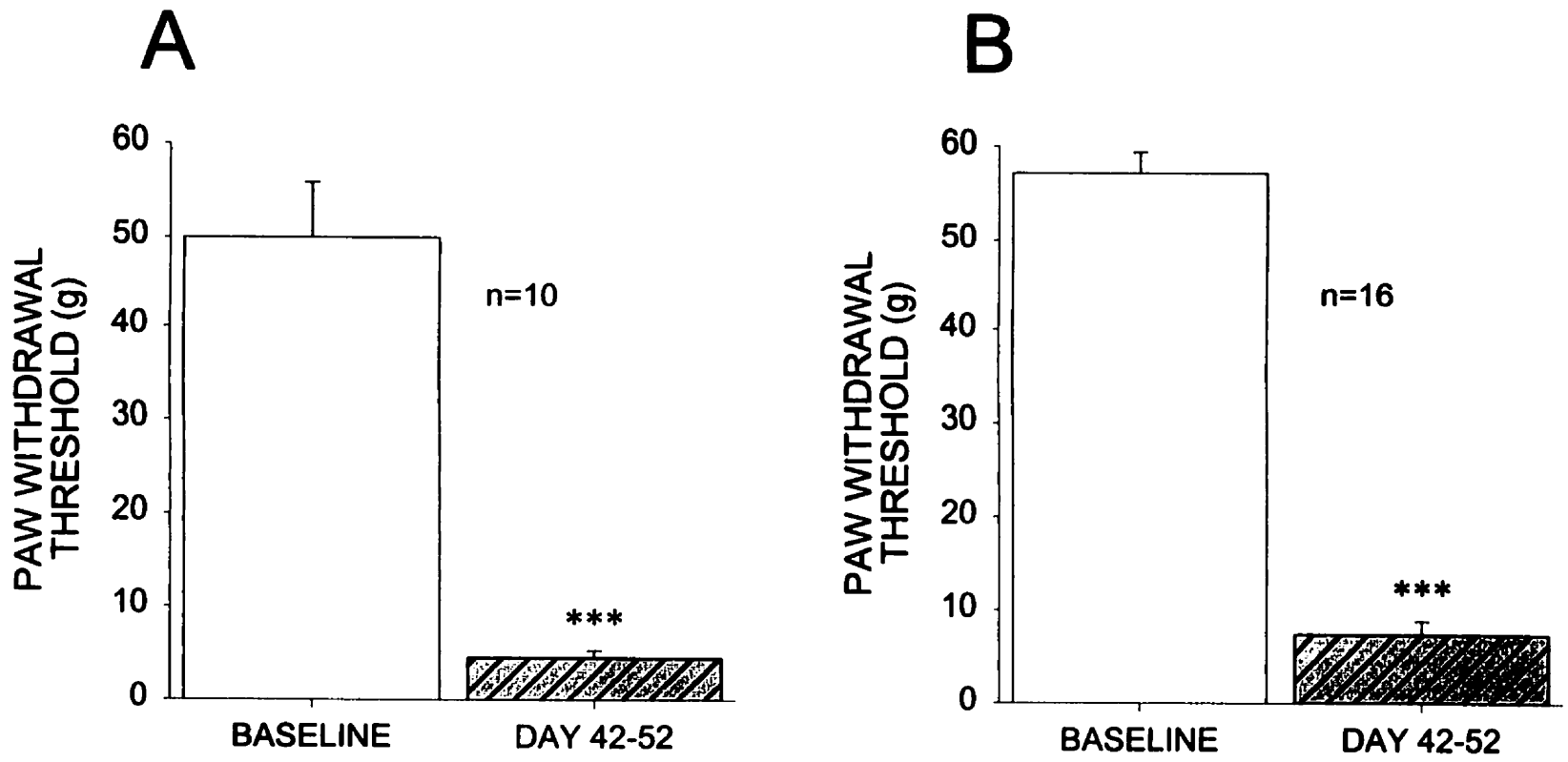
A
B
PAW WITHDRAWAL THRESHOLD (g)
60
50
40
30
20
10
0
n=10
n=16
***
***
BASELINE
DAY 42-52
BASELINE
DAY 42-52

Figure 1. Hind paw withdrawal thresholds in the von Frey hair test. The vertical axis represents the withdrawal threshold measured in grams and the horizontal axis shows left hind paw withdrawal thresholds before (normalized to day 0) and after (days 42-52) implantation of a 2 mm polyethylene cuff on the left sciatic nerve. In 10 (A) and 16 (B) rats tested after cuff implantation, the hind paw withdrawal thresholds were markedly decreased ($P < 0.001$) compared to withdrawal thresholds on day 0. Cuff-implanted rats in groups A and B were tested subsequently in electrophysiological experiments to determine the effects of indomethacin and meloxicam, respectively, on spinal dorsal horn neuronal activity.

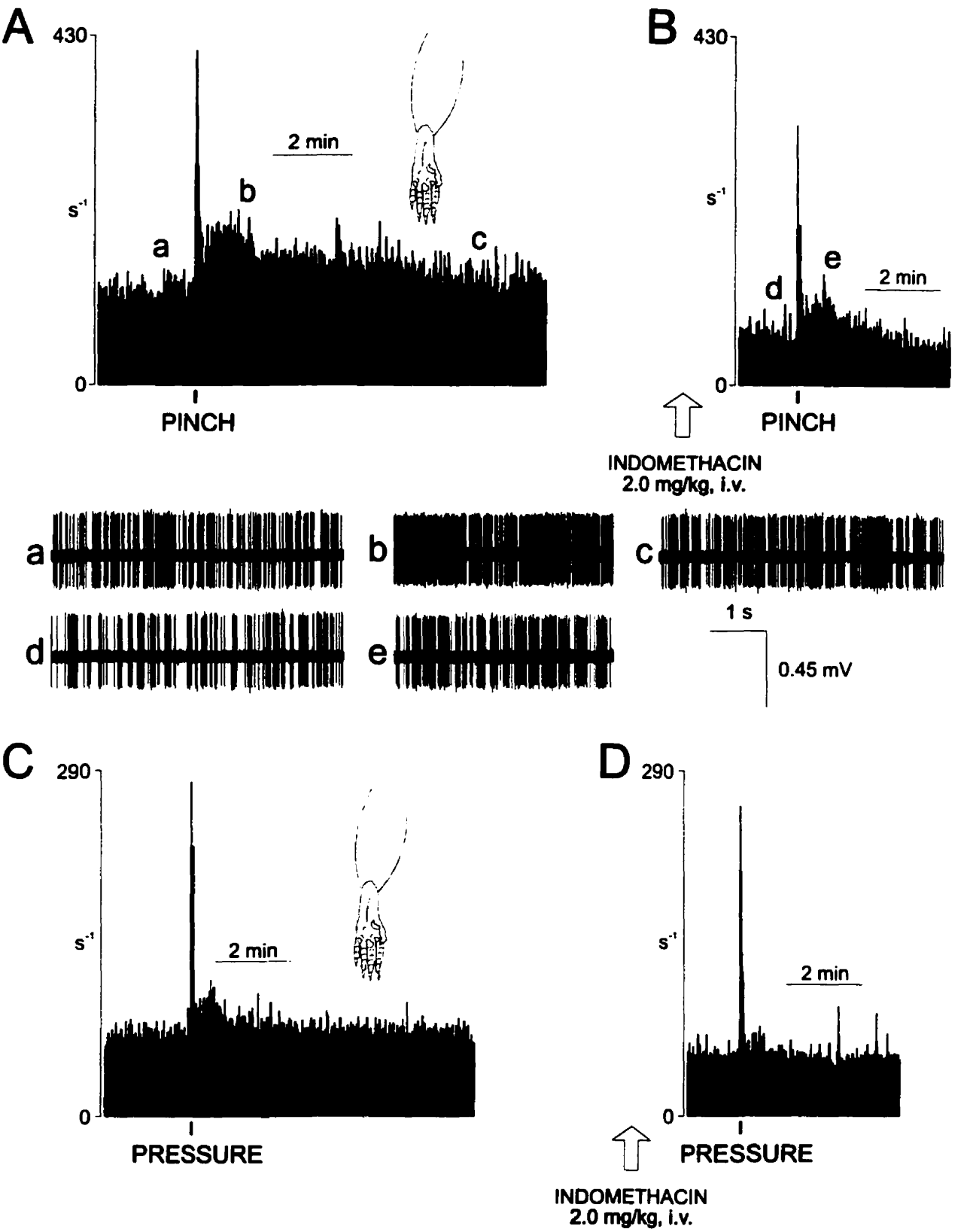
A
430
s⁻¹
0
2 min
a
b
c
PINCH
B
430
s⁻¹
0
d
e
2 min
PINCH
INDOMETHACIN
2.0 mg/kg, i.v.
a
b
c
d
e
1 s
0.45 mV
C
290
s⁻¹
0
2 min
PRESSURE
D
290
s⁻¹
0
2 min
PRESSURE
INDOMETHACIN
2.0 mg/kg, i.v.

Figure 2. Effect of indomethacin (2 mg/kg, i.v.) on on-going discharge and responses to noxious and innocuous mechanical stimulation of the cutaneous receptive field. (A) The ratemeter record shows that in a cuff-implanted rat noxious mechanical stimulation (pinch; 21 N for 3 s) produced a fast initial discharge which lasted only for the duration of the stimulus followed immediately by a slowly-decaying afterdischarge which persisted for approximately 6 min in a wide dynamic range neuron 960 μm deep from the dorsal surface of the spinal cord. The horizontal axis represents time and the vertical axis represents frequency of spikes (1 s bin width). The time and duration of the pinch stimulus are represented by the narrow rectangle below the ratemeter histogram. The inset shows the cutaneous receptive field subjected to pinch stimulation depicted by the shaded area. Extracellular records show single unit activity taken at 'a', 'b' and 'c' in the ratemeter histogram. (B) Depressive effects of indomethacin on on-going discharge and pinch-evoked responses of the neuron shown in (A). While both the magnitude and duration of the pinch-elicited afterdischarge were substantially decreased by indomethacin, the initial discharge was considerably less sensitive to the effect of indomethacin. This record was taken approximately 40 min after the end of (A). Spike activity is illustrated at 'd' and 'e' in the ratemeter histogram. Note the decrease in firing frequency during on-going and stimulation-induced activity compared that prior to administration of indomethacin. Also notice that while the firing frequency of the neuron was decreased by indomethacin the spike amplitude remained unaltered. (C) In another wide dynamic range neuron (1104 μm), pressure stimulation produced an initial discharge which lasted for the duration of the stimulus followed immediately by an afterdischarge which persisted approximately 2 min.

The inset shows the cutaneous receptive field subjected to pressure stimulation depicted by the shaded area. (D) Indomethacin markedly decreased the magnitude and duration of the pressure-evoked afterdischarge while a negligible effect was observed on the initial discharge. On-going discharge showed a decrease in firing frequency after indomethacin administration similar to that shown in (B). This record was taken approximately 40 min after the end of (C). In all cases, the vehicle alone was administered prior to indomethacin and in none did this control injection have any observable effect.

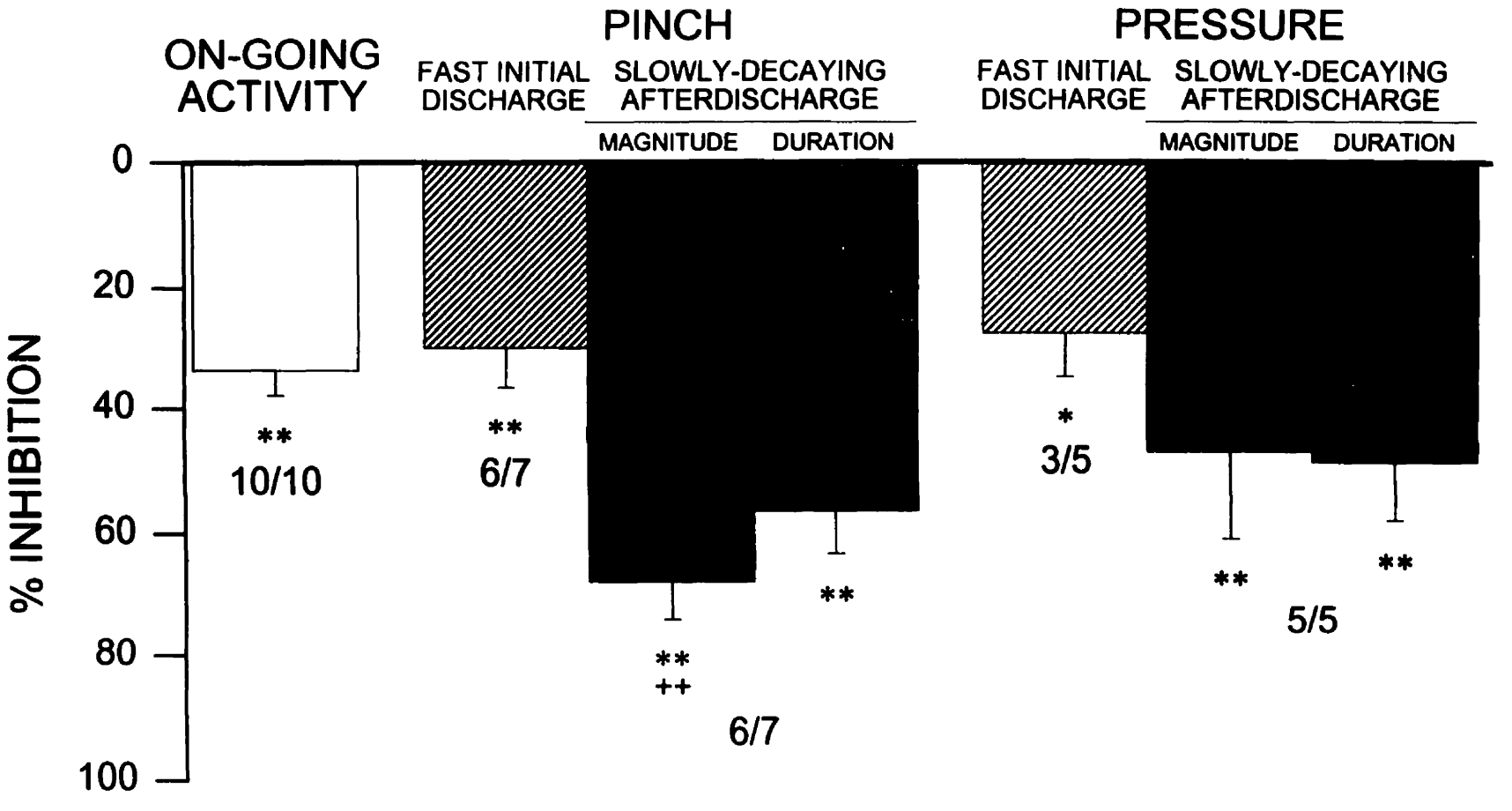
PINCH
PRESSURE
ON-GOING ACTIVITY
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
MAGNITUDE
DURATION
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
MAGNITUDE
DURATION
% INHIBITION
0
20
40
60
80
100
**
10/10
**
6/7
**
++
**
6/7
*
3/5
**
**
5/5

Figure 3. Summary of the inhibitory effects of indomethacin on on-going discharge and on pinch and pressure stimulation-elicited initial discharge and afterdischarge responses of 10 wide dynamic range neurons. The vertical axis represents the mean ($\pm$SEM) percent inhibition expressed as a percentage of the rate of discharge prior to administration of indomethacin. Each ratio is the number of wide dynamic range dorsal horn neurons inhibited over the number tested.* $P < 0.05$ and ** $P < 0.01$ vs. vehicle (0% inhibition). Note that the percent inhibition of the pinch-evoked afterdischarge is greater (++ $P <$ 0.01) than that of the initial discharge.

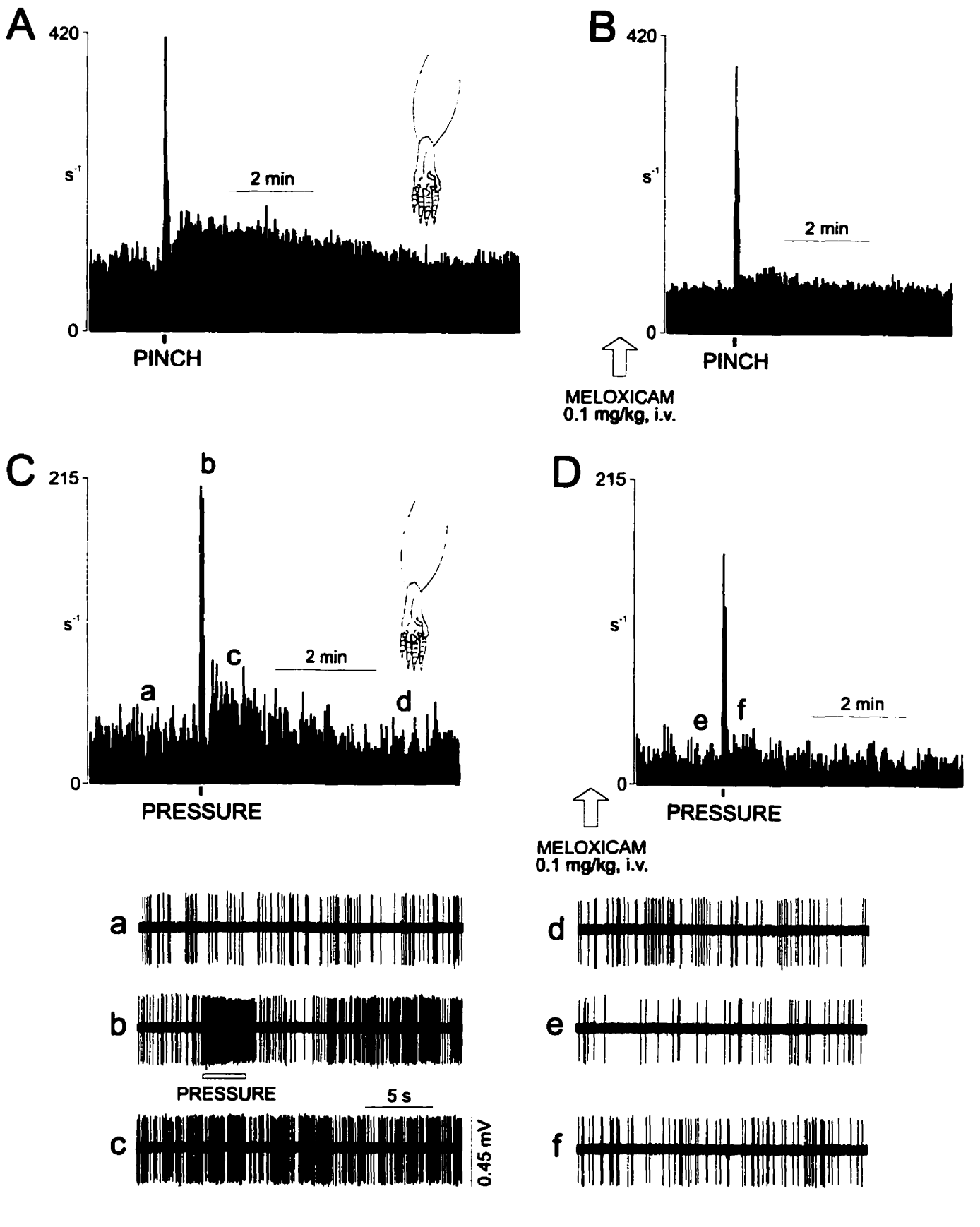
A
420
s⁻¹
0
2 min
PINCH
B
420
s⁻¹
0
2 min
PINCH
MELOXICAM
0.1 mg/kg, i.v.
C
215
s⁻¹
0
a
b
c
d
2 min
PRESSURE
D
215
s⁻¹
0
e
f
2 min
PRESSURE
MELOXICAM
0.1 mg/kg, i.v.
a
b
PRESSURE
5 s
c
0.45 mV
d
e
f

Figure 4. Effect of meloxicam (0.1 mg/kg, i.v.) on on-going discharge and on pinch and pressure stimulation-evoked responses. (A) In a wide dynamic range neuron (879 μm), pinch stimulation produced an initial discharge followed by an afterdischarge which persisted for approximately 5 min. The vertical and horizontal axis are otherwise similar to those shown previously. The time and duration of the pinch stimulus are shown by the narrow rectangle below the ratemeter histogram. The shaded region on the cutaneous receptive field depicts the region of pinch stimulation on the left hind paw. (B) Depressive effects of meloxicam on on-going discharge and pinch-evoked responses of the neuron shown in (A). While both the magnitude and duration of the afterdischarge response were decreased, the initial discharge was considerably less sensitive to the effect of meloxicam. This record was taken approximately 60 min after the end of (A). (C) In another wide dynamic range neuron (934 μm), pressure stimulation evoked an initial discharge followed immediately by an afterdischarge which persisted approximately 2 min. The inset shows the cutaneous receptive field subjected to pressure stimulation depicted by the shaded area. Extracellular records illustrate single unit activity taken at 'a', 'b', 'c' and 'd' in the ratemeter histogram. (D) As shown in (B), on-going activity was decreased by meloxicam. The pressure-evoked afterdischarge was almost entirely inhibited by meloxicam while the initial discharge was decreased considerably less. Spike activity at 'e' and 'f' in the ratemeter histogram demonstrate the depressive effect of meloxicam on on-going and evoked neuronal activity. Note that while the firing frequency of the neuron was decreased by meloxicam the spike amplitude remained unaltered. This record was taken approximately 60 min after the end of (C). In all cases, the vehicle alone was administered

prior to meloxicam and in none did this control injection have any observable effect.

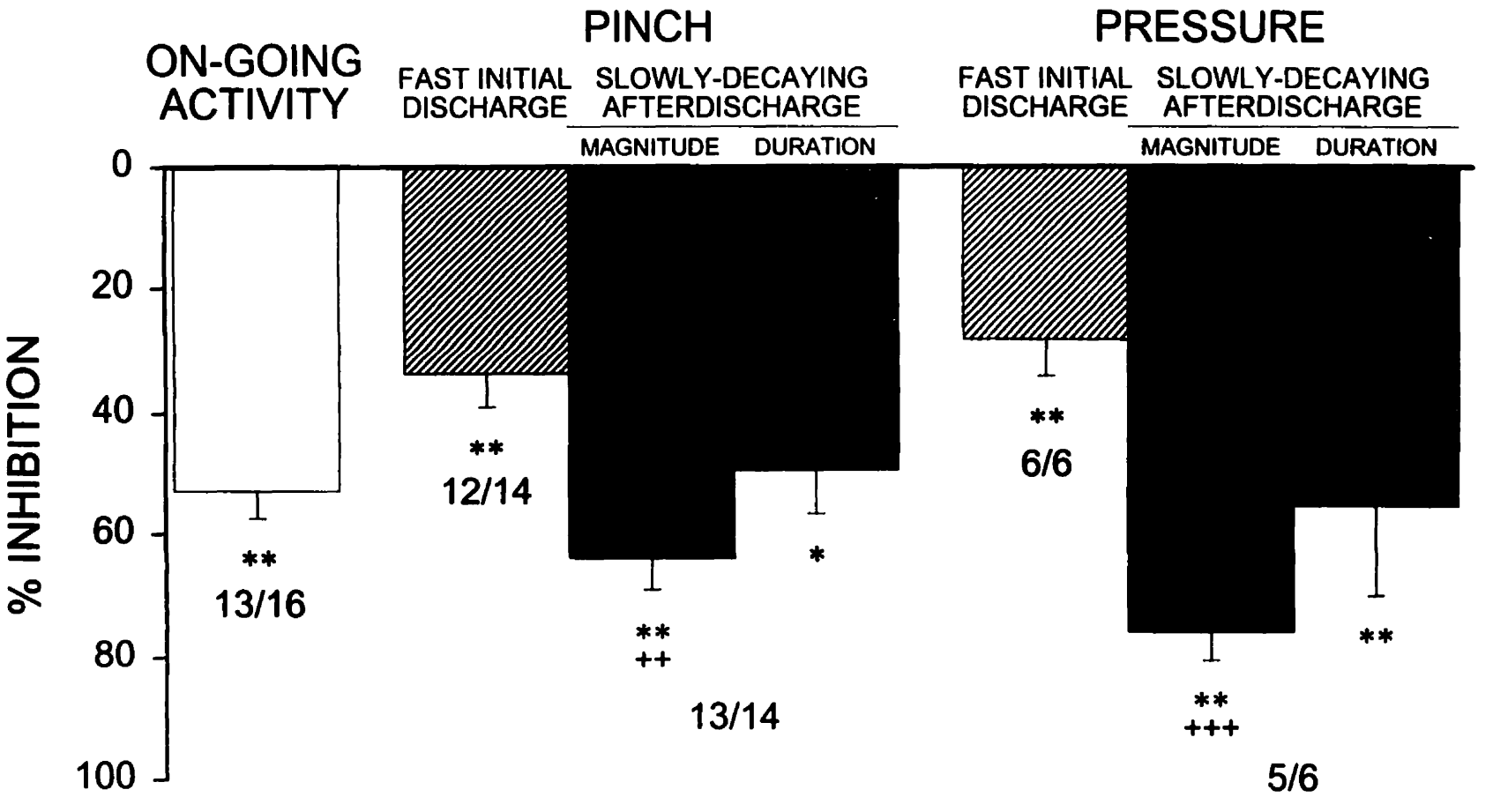
PINCH
PRESSURE
ON-GOING ACTIVITY
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
MAGNITUDE
DURATION
FAST INITIAL DISCHARGE
SLOWLY-DECAYING AFTERDISCHARGE
MAGNITUDE
DURATION
% INHIBITION
0
20
40
60
80
100
**
13/16
**
12/14
**
++
*
13/14
**
6/6
**
+++
**
5/6

Figure 5. Summary of the inhibitory effects of meloxicam on on-going activity and on pinch and pressure stimulation-elicited initial discharge and afterdischarge responses of 16 wide dynamic range neurons. The vertical and horizontal axis are otherwise similar to those shown in Figure 3. Each ratio is the number of wide dynamic range dorsal horn neurons inhibited over the number tested.* $P < 0.05$ and ** $P < 0.01$ vs. vehicle (0% inhibition). Note that the percent inhibition of the pinch- and pressure-induced afterdischarges is greater (++ $P < 0.01$ and +++ $P < 0.001$) than the percent inhibition of the initial discharges.

Summary and Conclusions

The present thesis is comprised of electrophysiological and nociceptive reflex studies designed to investigate and further understand sensory information processing in the region of the first sensory synapse in the mammalian spinal dorsal horn. Specifically, the main interest and focus is the involvement of the eicosanoid signal transduction pathway via COX in sensory processing at the cellular level in the spinal dorsal horn *in vivo*, in particular on-going activity and early and late components of peripheral stimulation-induced discharge.

Each study herein is intended to be self-sufficient in nature, yet each interdependent in that collectively they yield a progressive understanding of the physiological and neurochemical mechanisms extending from normal sensory information processing to that in chronic neuropathic pain. Using an electrophysiological approach, Chapters 1, 2 and 3 investigate the eicosanoid signal transduction pathway via COX in normal sensory processing. Chapters 4, 5, 6 and 7 establish and characterize a model of neuropathic pain using electrophysiological and nociceptive reflex approaches. Chapter 8 investigates electrophysiologically the eicosanoid chemical pathway in rats exhibiting neuropathic pain behaviour. Collectively, these studies thus examine the **hypothesis** that *specificity of synaptic function mediating and/or modulating the inputs of different sensory modalities relies on unique and identifiable physiological and neurochemical mechanisms*.

Briefly summarized below are the main conclusions derived from examination of this hypothesis.

Chapter 1

i. The purpose of this study was to investigate the effect of COX inhibition using indomethacin on glutamate and NK-1 receptor-induced excitation of spinal dorsal horn neurones.

ii. Substance P, NMDA, quisqualate and AMPA each produced excitation of spinal dorsal horn neurones when applied by iontophoresis. It can be assumed that these effects were mediated by activation of NK-1 and glutamate receptors.

iii. Inhibition of COX using the non-selective NSAID, indomethacin, depressed the excitatory effects of each of these chemicals. Given that indomethacin depressed the excitatory effects on non-nociceptive, wide dynamic range and nociceptive specific neurones, it is suggested that COX is present in the spinal dorsal horn and that eicosanoids have effects on the activity of the three classes of neurone. As responses to iontophoretic application can be assumed to have been postsynaptic and as indomethacin had an effect generalized to all excitatory responses, we suggest a postsynaptic site for COX. It is suggested that elements of the eicosanoid signal transduction pathway via COX may thus mediate at least some of the effects of NK-1 and glutamate receptor activation.

iv. Activation of the eicosanoid pathway via COX in CNS neurones is reported to be Ca^{2+}

dependent, and activation of both NMDA and NK-1 receptors increases intracellular Ca^{2+}. This led to the expectation that indomethacin-induced COX inhibition would exhibit a greater effect on NMDA-evoked excitation than on AMPA-evoked excitation of dorsal horn neurones, but this was not observed; both were affected, AMPA more than NMDA. It is thus hypothesized that in addition to a mediator role, elements of the eicosanoid pathway via COX, i.e. arachidonic acid and prostaglandins, may modulate efficacy of activation of NMDA and AMPA receptors and possibly other receptors.

Chapter 2

i. The functional role of the eicosanoid pathway on on-going and peripheral stimulation-evoked activity in normal sensory processing was determined in this study.

ii. Noxious peripheral mechanical, heat or electrical stimulation produced a fast initial discharge of dorsal horn neurones lasting for the duration of the stimulus. This was followed by a slowly-decaying afterdischarge which persisted after the end of the stimulus. Innocuous stimulation including pressure or hair stimulation produced excitation of dorsal horn neurones lasting only for the duration of the stimulus.

iii. At the doses tested, indomethacin was without effect on the on-going rate of discharge

of spinal dorsal horn neurones but inhibited synaptically-elicited responses to noxious cutaneous mechanical and thermal stimulation.

iv. Inhibition of the afterdischarge and of the excitatory effect of long-lasting synaptic input was greater than inhibition of the fast synaptic input-evoked initial discharge.

v. The brief excitatory responses to innocuous pressure stimulation in both non-nociceptive and wide dynamic range neurones were also depressed, but to a lesser extent.

vi. These data are interpreted to suggest that peripheral stimulation-evoked inputs are more involved than input-independent excitation of dorsal horn neurones in leading to *de novo* synthesis of prostaglandins and that the time course of this synthesis brings the levels to a point where COX inhibition can have an observable effect during prolonged excitation.

vii. Although the data suggest that COX inhibition differentially inhibits nociceptive vs. non-nociceptive mechanisms at the cellular level, the data also indicate that irrespective of the modality of the stimulus, COX inhibition decreases prolonged activation of synaptic mechanisms.

viii. In this regard, it is reasonable to suggest that NSAIDs would be more effective on

nociceptive types of pain characterized by prolonged or tonic input of primary afferent neurones.

Chapter 3

i. The functional role of the eicosanoid pathway via COX-2 on on-going and noxious mechanical stimulation-elicited activity in normal sensory processing was determined in this study.

ii. At the dose tested, meloxicam was without effect on the on-going rate of discharge of spinal dorsal horn neurones but inhibited synaptically-elicited responses to noxious cutaneous mechanical stimulation.

iii. Inhibition of the pinch-induced afterdischarge was greater than inhibition of the synaptic input-evoked initial discharge.

iv. The preferential inhibitory effect of meloxicam on the afterdischarge supports the concept of an association between the tonic effects of peripheral stimulation-evoked synpatic input and COX-2 activity.

Chapter 4

i. The objective in this particular study was to determine the effect of the testing surface and to verify the applicability of the novel platform to testing von Frey hairs in normal rats and in rats with peripheral neuropathy.

ii. In normal rats tested on the customized platform which consists of a smooth opaque plastic surface, the mean paw withdrawal thresholds of the left or right hind paw remained unchanged on different days of testing. However, in similar rats tested on wire mesh, the mean withdrawal thresholds were less consistent.

iii. Thus, a flat surface on which rats stand while being tested with von Frey hairs provides stable and repeatable hind paw withdrawal thresholds.

iv. In cuff-implanted rats tested on the customized platform, the mean withdrawal threshold ipsilateral to cuff implantation decreased to values that were less than values obtained from similar rats tested on wire mesh. In addition, a contralateral decrease in the withdrawal threshold was not observed in neuropathic rats tested on wire mesh.

v. These data suggest that the customized platform is an important improvement to using wire mesh to measure hind paw withdrawal thresholds using von Frey hairs. In particular, the physical properties of the platform provide improved assessment and yield accurate

characterization of the expression of the effects of peripheral neuropathy on tactile sensitivity.

Chapter 5

i. Changes in the withdrawal threshold and the time-course of any such changes in rats with peripheral neuropathy were determined in this study. To establish the appropriate control, withdrawal thresholds were also measured in the contralateral hind limb, in both hind limbs in sham-operated rats as well as in both hind limbs of unoperated rats.

ii. Implantation of a 2 mm polyethylene cuff on the common sciatic nerve produced a marked increase in sensitivity to normally innocuous tactile stimuli. The onset of this effect occurred as soon as 1 day after cuff implantation and was sustained for at least 145 days with maximal hypersensitivity, measured at 1 to 2 g using von Frey hairs, occurring between 4 and 27 days.

iii. Unilateral cuff implantation also gave rise to an increase in tactile sensitivity of the contralateral hind paw. The tactile hypersensitivity of the contralateral hind paw was initially less in magnitude compared to that of the cuff-implanted hind paw and was slower in onset. After several weeks of cuff implantation, the ipsi- and the contralateral hind paws were equally sensitive to normally innocuous tactile stimulation.

iv. Surgery alone without cuff implantation also produced a decrease in withdrawal threshold of each of the hind paws. However, this decrease was less in magnitude and duration compared to that in cuff-implanted rats.

v. As unoperated rats showed no change in hind paw withdrawal threshold throughout the study, the data are interpreted to suggest that the testing paradigm itself had no effect on withdrawal threshold and that cuff implantation as well as surgery establish sustained modifications in sensory processing which maintain long-lasting tactile allodynia ipsi- and contralateral to the cuff implantation or surgical injury.

vi. Three types of allodynia are manifested via changes in sensory processing in sciatic cuff-implanted or surgically-injured rats. Each type has a distinct onset, time course, magnitude and recovery. The marked decrease in the withdrawal threshold of the cuff-implanted hind paw may be indicative of neuropathic allodynia characterized by the remarkably long-lasting tactile hypersensitivity accompanied by a combination of nociceptive behaviours. The second type of allodynia was seen in operated but not in unoperated controls and may be of a model of surgical pain. It is also induced unilaterally and expressed bilaterally. The third type of allodynia may be a model of central pain and it is demonstrated by the decreased withdrawal threshold of the intact hind paw contralateral to the cuff-implanted paw. Although it is initially less in magnitude compared to that of the neuropathic allodynia, it is slower in onset and later in recovery. It is

speculated that central pain may be established and maintained via the peripheral and/or central effects of experimental neuropathy.

Chapters 6 and 7

i. The objective in this electrophysiological study was to characterize spinal dorsal horn neuronal activity in rats with experimental neuropathy exhibiting tactile allodynia and spontaneous pain.

ii. Cuff implantation on the sciatic nerve increased on-going and peripheral stimulation-evoked activity of ipsi- and contralateral spinal dorsal horn neurones.

iii. Effects of cuff implantation on the sciatic nerve on ipsilateral on-going and peripheral stimulation-evoked activity of dorsal horn neurones are maximally increased relatively soon after cuff implantation.

iv. Several weeks after cuff implantation, on-going and peripheral stimulation-evoked activity of ipsi- and contralateral spinal dorsal horn neurones were equally increased.

v. Cuff implantation had no effect on initial discharge responses of dorsal horn neurones to natural innocuous and noxious stimulation-induced synaptic input but increased slowly-

decaying afterdischarge responses of dorsal horn neurones to noxious mechanical stimulation.

vi. In addition, cuff implantation induced a switch in the response to innocuous stimuli such that an afterdischarge occurred at the end of the stimulus. In unoperated controls an afterdischarge occurred only following a noxious stimulus. Experimental neuropathy appeared to have less of an effect on responses of dorsal horn neurones to noxious thermal stimulation.

vii. Electrical stimulation of the sciatic nerve at 4 and at 20 Hz produced an initial discharge which was the same in control and in cuff-implanted rats. However, the afterdischarge was potentiated in cuff-implanted rats tested with 20 Hz stimulation. The data are interpreted to support the hypothesis that nerve injury in experimental neuropathy induces a change in myelinated afferents.

viii. Taken together, the data indicate that chronic cuff implantation induces increased excitability in ipsi- and in contralateral spinal dorsal horn neurones independently of supraspinal sensory processing. This bilateral excitability may underlie the tactile hypersensitivity of the cuff-implanted and contralateral hind paws.

. The exaggerated on-going and mechanical stimulation-evoked activity is interpreted

to represent, at least in part, the neurophysiological basis of spontaneous pain, mechanical hyperalgesia and tactile allodynia seen in neuropathic pain in human patients.

ix. The findings in this study may also be interpreted to suggest a peripheral and/or spinal basis for pain and allodynia in at least some forms of clinical neuropathic pain including syndromes characterized with ipsi- and/or bilateral hyperalgesia and allodynia.

Chapter 8

i. Involvement of the eicosanoid signal transduction pathway in the etiology of neuropathic pain was investigated.

ii. In cuff-implanted rats, NSAIDs including the non-selective COX-1/-2 inhibitor, indomethacin, and the selective COX-2 inhibitor, meloxicam, preferentially depressed the pinch-evoked afterdischarge vs. the initial discharge. Both NSAIDs depressed the pressure stimulation-evoked afterdischarge.

iii. Taken together, the data indicate that in cuff-implanted rats, eicosanoids via the COX pathway are involved in sensory processing, specifically in the long-lasting effects of peripheral stimulation-induced synaptic input.

iv. At the doses tested in normal rats (***Chapters 1, 2 and 3***), on-going dorsal horn neuronal activity was predominantly insensitive to the effect of NSAID administration. However, in cuff-implanted rats, the same dose of indomethacin or meloxicam depressed the elevated on-going activity.

v. These data indicate that the eicosanoid signal transduction pathway via COX-2 may be involved in maintaining elevated on-going neuronal activity in rats with experimental neuropathy.

vi. Given that experiments were run in spinally transected rats, the inhibitory effects of the NSAIDs used in this study likely occurred at a spinal and/or peripheral level.

vii. Collectively, the depressive effects of indomethacin and meloxicam on peripheral neuropathy-induced neuronal hyperexcitability may be interpreted to suggest that the eicosanoid signal transduction pathway, perhaps via COX-2, may underlie the etiology of specific aspects of neuropathic pain including spontaneous pain, mechanical hyperalgesia and tactile allodynia.

viii. In this regard, the use of NSAIDs, perhaps those selective for COX-2, may provide effective therapeutic approaches for the treatment of some forms of pain associated with

peripheral neuropathy.

These chapters have addressed the hypothesis in the Introduction, and confirm a pivotal role of the eicosanoid pathway in regulating nociceptive mechanisms involved in both acute and chronic pain. Each of the chapters answers an important question. Yet each question is inexplicably linked to the others, and all are important in addressing the hypothesis developed in the Introduction.

However, a number of important questions remain which can drive this project onward. For example, it is important to know the role of the constitutive COX, COX-1, in these mechanisms. What is the relative significance of pre- vs. postsynaptic COX in regulation of nociceptive mechanisms? Is the eicosanoid pathway similarly implicated in other models of neuropathic pain, such as diabetic neuropathy or post-herpetic neuralgia, and such as the central neuropathic pain following, for example, spinal trauma? This is important because it is not demonstrated clinically that these forms of "neuropathic pain" in humans share a similar etiology despite sharing a common term. In these cases, is the eicosanoid pathway involved in mediating nociceptive inputs at different levels of the neuraxis? Is there a synergism between COX and other intracellular signal transduction pathways, because if this is found to be the case novel strategies may be developed to provide improved management of pain through coupling drug treatments.

I hope that one of the results of my thesis is that it will, through introducing some basic issues, prompt other investigators to take up the challenges I have raised.

Original Contributions

Over the last few years the eicosanoid signal transduction pathway has become recognized as an important chemical pathway in pain processing. This has come about largely via investigation of the effects of different NSAIDs in animal models of acute and chronic pain, with much attention directed to peripheral inflammation-induced chronic pain. However, few studies have examined the effects of NSAIDs on spinal dorsal horn neuronal activity specifically on-going discharge and the early and late components of synaptic input-elicited responses. Moreover, the effects of NSAIDs in other models of chronic pain have not been systematically investigated.

This thesis focuses on the functional role of the eicosanoid pathway, specifically via COX, in sensory processing at the level of the mammalian spinal dorsal horn. In particular, on-going and peripheral stimulation-elicited activity of dorsal horn neurones *in vivo*, in normal rats and in rats with peripheral neuropathy is examined with particular emphasis afforded to the early and late components of the effects of peripheral stimulation-evoked synaptic input.

The experimental results and conclusions reported in this thesis are original and have not appeared elsewhere except as stated specifically in the text and the Preface. The work presented in this thesis is the first detailed electrophysiological account of the functional role of the eicosanoid signal transduction pathway via COX in mediating and/or modulating synaptic transmission of sensory information in the spinal dorsal horn *in vivo* from a physiological and pharmacological perspective in normal rats and in a rat model of

neuropathic pain. With this purpose/objective, the results of the studies in this thesis show that:

1. Components of the eicosanoid signal transduction pathway may play a role in modulating in addition to mediating the effects of glutamate and NK-1 receptor activation at the level of the spinal dorsal horn. This study puts forth the concept of possible implications of the eicosanoid pathway in receptor modulation in the peripheral and central nervous systems.

2. In the normal rat, the eicosanoid pathway via COX is involved in mediating and/or modulating the effects of non-nociceptive sensory input on dorsal horn neuronal activity. However, it is predominantly involved in processing nociceptive sensory information, specifically the effects of long-lasting vs. brief synaptic input.

3. COX-2 may be involved in preferentially mediating and/or modulating the longer-lasting excitatory effects of noxious peripheral stimulation-induced synaptic input on spinal nociceptive neurons in the normal rat. It is suggested that COX-2 may be involved in elevating eicosanoid levels during stimulation-induced synaptic input such that they exhibit physiological effects on dorsal horn neuronal activity.

4. Properties of the surface on which rats are tested may influence sensory processing and subsequently affect the withdrawal threshold to von Frey hair application. In this regard, it is suggested that characterization of mechanisms of sensory input using von Frey hairs may be obtained more accurately and reliably in rats tested on a smooth opaque surface rather than on a see-through and irregular surface such as wire mesh.

5. Three types of tactile allodynia occur in the cuff-implanted rat. Each type has a distinct time course of onset, recovery and magnitude. The model of peripheral neuropathy induced by placing a 2 mm polyethylene cuff around the sciatic nerve is characterized by remarkably long-lasting tactile hypersensitivity accompanied by nociceptive behaviours. Tactile allodynia in operated but not in unoperated rats is suggestive of post-surgical pain. It is induced unilaterally but expressed bilaterally. The third type of allodynia is tactile hypersensitivity of the hind paw contralateral to the cuff-implanted hind limb. It is less in magnitude compared to that of neuropathic allodynia, slower in onset and later in recovery. It is speculated that central pain may be established and maintained via peripheral and/or central effects of peripheral nerve injury.

6. Cuff implantation on the sciatic nerve induces important changes in some of the physiological properties of ipsi- and contralateral spinal nociceptive neuronal

activity *in vivo* in the acutely spinalized rat. In particular, on-going discharge of spinal wide dynamic range dorsal horn neurones is greater in cuff-implanted rats than in unoperated rats. Interestingly, the initial discharge in response to noxious pinch and innocuous pressure stimulation of the cutaneous receptive field is similar in cuff-implanted rats and in unoperated rats. However, the afterdischarge response to noxious mechanical stimulation in cuff-implanted rats is of greater magnitude and duration than that in normal rats. Furthermore, a nociceptive-like afterdischarge in response to innocuous pressure stimulation occurs in cuff-implanted rats which is not observed in unoperated rats. The afterdischarge response to noxious heat stimulation is also greater, but to a much lesser extent. As acutely spinalized rats were used, cuff implantation-induced excitation of spinal dorsal horn neurones does not rely exclusively on sensory processing via a supraspinal loop and tonic descending excitation. It is concluded that there is a greater change in fibers mediating noxious and innocuous mechanical than noxious thermal inputs. The greater on-going and noxious and innocuous mechanical stimulation-evoked afterdischarge may be interpreted to represent the neurophysiological basis of spontaneous pain, mechanical hyperalgesia and tactile allodynia, respectively.

7. The eicosanoid signal transduction pathway via COX-2 may be involved in the etiology of the effects of peripheral neuropathy on spinal nociceptive neurons

including in particular elevated on-going activity and the noxious- and innocuous mechanical stimulation-elicited initial discharge and afterdischarge responses. These data are the first implicating the eicosanoid signal transduction pathway in the neurophysiological basis of altered sensory processing associated with peripheral neuropathy in the rat. An important clinical implication of these data is that the eicosanoid pathway via COX-2 may be involved in different aspects of peripheral neuropathy including specifically spontaneous pain, mechanical hyperalgesia and tactile allodynia.