INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

QUANTIFYING FORMALIN-INDUCED BEHAVIOURS AND MORPHINE ANALGESIA

Rok Ocvirk
Department of Psychiatry, McGill University
Montreal, Canada
July, 1999

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the Master of Science degree.

ABSTRACT

The formalin test in the rat is a frequently used model of acute tissue injury-induced pain, but data collection is time-consuming and labour intensive, and very wide ranges of formalin concentrations and behavioural indices of pain are used in the literature. The present investigation validated a time-sampling method of scoring formalin-induced pain-specific behaviours. The method improves efficiency by at least a factor of five, and also provides measures of other aspects of behaviour, without the loss of statistical power of the results. Using the time-sampling method, formalin-induced behaviours were examined over the entire range of commonly used formalin concentrations (1-10%), for a prolonged period of time (16 hours postformalin). The best predictors of the log of formalin concentration were found to be the sum of lifting and licking, biting or shaking the injected paw, and the weighted means scores. The pain response increased dose-dependently up to 2% formalin in the first phase and up to 10% in the second phase if behaviour was scored for at least 90 minutes postformalin. Significant residual pain occurred only at 10% formalin. The effect of formalin on the behavioural state was most prominent in the first hour after formalin administration. At concentrations up to 2%, formalin induced inactivity (lying and sitting still), at the expense of grooming and exploration. This effect was reversed at 5 and 10%, where activity increased and agitation occurred. After the second phase terminated, all treatment groups showed significant ptosis, hunched posture and/or piloerection. Sleep was reduced dose-dependently and became significant at 10% formalin. Behaviour in all treatment groups appeared to become normalised within 10 hours postformalin. Rats that were habituated to the testing environment were more sensitive to formalin than unhabituated rats in the first phase. The onset of the pain response in unhabituated animals was delayed and there was a trend for lower pain scores in the second phase of the test. When morphine dose-effect relationships were examined at varying formalin concentrations, there was a systematic rightward shift in the morphine dose-effect relationships up to about 2% formalin, at which point, further increases in formalin concentration did not produce any further shift, and morphine appeared to non-competitively antagonize formalin-induced pain. 8 mg/kg morphine blocked all pain.

RÉSUMÉ

Le test du formol est un modèle de douleur aigüe causée par un dommage aux tissus qui est fréquemment utilisé chez le rat, mais la collecte des données est ardue et une large variété de concentrations de formol ainsi que d'indices comportementaux de la douleur sont utilisés dans la littérature scientifique. La présente étude a validé une méthode non-continue de mesurer les comportements douloureux spécifiques causés par le formol. La méthode est au moins cinq fois plus efficace, et permet également la collecte de données sur d'autres aspects du comportement, sans une réduction de pouvoir statistique. En utilisant la méthode 'time-sampling', les comportements induits par toutes les concentrations de formol fréquemment utilisées (1-10%) ont été examinés durant une période temporelle prolongée (16 heures post injection de formol). La somme des soulèvements et lèchements, mordillements ou agitation de la patte injectée, ainsi que la moyenne de ces comportements prédisaient le mieux la concentration de formol sous forme logarithmique. La douleur observée a augmenté d'une manière dépendante de la concentration de formol jusqu'à 2% durant la première phase, et jusqu'à 10% durant la seconde phase si le comportement était mesuré au moins jusqu'à 90 minutes après l'injection de formol. On peut observer de la douleur résiduelle lorsqu'une concentration de 10% de formol est injectée. L'effet du formol sur l'état comportemental était le plus marqué durant la première heure après l'administration du formol. Les concentrations de formol allant jusqu'à 2% ont causé de l'inactivité (les rats étaient couchés ou assis), au détriment du toilettage et de l'exploration. Cet effet a été complètement éliminé aux concentrations de 5 et de 10% car à ces concentrations le niveau d'activité a augmenté et de l'agitation est apparue. À la fin de la seconde phase, les animaux dans tous les groupes expérimentaux avaient le dos courbé, et ont éprouvé de façon significative de la ptosis et/ou de la piloérection. Le sommeil a été réduit d'une façon dépendante de la concentration de formol adminitrée, et était réduit de façon significative à une concentration de formol de 10%. Le comportement des rats appartenant à tous les groupes expérimentaux a semblé se normaliser 10 heures après l'injection du formol. Les rats qui ont été habitués aux lieux et appareil où le test du formol est effectué étaient plus sensibles au formol durant la première phase du test que ne l'étaient

les rats qui n'avaient reçu aucune séance d'habituation. Le déclenchement de l'expression de la douleur chez les animaux non-habitués était retardé, et il semblait que ces animaux exprimaient moins de douleur durant la seconde phase du test. Quand la relation entre la dose de morphine et son effet a été examinée à différentes concentrations de formol, il y avait un déplacement systématique vers la droite de la relation entre la dose et l'effet de la morphine et ce, jusqu'à une concentration de formol allant jusqu'à 2%. D'additionnelles augmentations de la concentration de formol n'ont pas produit d'autre déplacement de la courbe, et la morphine a semblé être un antagoniste non-compétitif de la douleur causée par le formol. 8mg/kg de morphine ont bloqué toute douleur indépendamment de la concentration de formol.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%MPE	Percent maximal possible effect
µL	Microlitre
14C-2DG	14C-2-deoxyglucose
5-HT	5-hydroxytriptamine (serotonin)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Anaiysis of variance
BK	Bradykinin
BLM	Backwards locomotion (retropulsion)
Ca^{++}	Calcium
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
cm	Centimetre
CNS	Central nervous system
DLTP	Dorsolateral pontomesencephalic tegmentum
EAA	Excitatory amino acids
EEG	Electroencephalography
E_{max}	Maximal effect
EMG	Electromyography
E_{min}	Minimal effect
IEG	Immediate-early gene
IL	Interleukin
kg	Kilogram
log	Logarithm
LSD	Least significant difference
LT	Leukotriene
mg	Milligram
mL	Millilitre

mm	Millimetre
MPE_{50}	Half-maximal possible effect
NA	Neurokinin A
NaCl	Sodium chloride
NK-1	Neurokinin-1
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NRM	Nucleus raphe magnus
PAG	Periaqueductal grey
PG	Prostaglandin
RMC	Nucleus reticularis magnocellularis
RVM	Rostral ventromedial medulla
SEM	Standard error of the mean
SFW	Shake, flinch or whole body flinch
SP	Substance P
WBF	whole body flinching
WMS	Weighted means score

ACKNOWLEDGEMENTS

I would like to thank Dr. Frances V. Abbott for having given me the opportunity to pursue a graduate degree under her supervision. She has provided me with a challenging and enjoyable learning environment. I look forward to collaborating with her in the future.

I would also like to thank my parents and family for their support and encouragement.

I have very much enjoyed working with my colleagues, Marie-Claire Albanese and Robert H. Najafee. I would like to thank them for their help and pleasant company.

This research was supported by the Natural Science and Engineering Research Council of Canada grant to Dr. Frances V. Abbott.

Chapter 1

INTRODUCTION

1.1 THE STUDY OF PAIN

Pain is the most common symptom of disease or injury (Adams & Victor, 1996). Although intuitively it may seem to be merely a nuisance, pain is essential to an organism's survival. Its purpose is to signal that injury has occurred, or is about to occur, and it allows the organism to react and minimize the injury. Pain also serves as a negative reinforcer so organisms can avoid similar injury in the future. In addition, it leads to protection of injured tissue and frequently reduces activity and induces rest, which allows for faster recovery from the insult. The fact that experiencing pain is critical for survival can be illustrated by individuals who suffer from congenital analgesia, in which the lack of pain sensation results in frequent and repetitive injury, such as biting into the tongue while eating, burning one's self with boiling water, and not noticing that one's skin has been damaged. Humans, who can communicate threats and dangers with other people, are less affected by the inability to experience pain, but in animals this condition is catastrophic (Melzack & Wall, 1996).

1.1.1 The need for pain research

When pain no longer performs its biological function, it becomes an unnecessary burden. This is true when pain is not a symptom of injury or disease, or in the case of chronic pain where nothing can be gained from the pain, and the pain itself becomes a medical problem (Melzack & Wall, 1996). In the United States alone, at any point in time there are more than two million working people incapacitated by pain (Jessel & Kelly, 1991). In some cases, the origin of pain is understood, and there are effective means of alleviation. However, in many cases, even when the cause of pain has been identified, there is no remedy (such as in rheumatoid arthritis), and in others, the etiology is not fully understood. A prominent example of the latter is phantom limb pain, where the part of body

that seems to hurt has been amputated. It is, therefore, essential that we investigate how pain arises, and how we can intervene in that process to improve the sufferers' quality of life (Melzack & Wall, 1996).

1.1.2 Complexity of pain research

Pain is a multidimensional phenomenon, and all aspects of it need to be studied. Firstly, the noxious stimulus activates afferent neural fibres, which convey a message to the central nervous system (CNS). This process is referred to as nociception, and forms the basis of the sensory-discriminative component of pain (Melzack & Wall, 1996), which is experienced as the sensation of pain. The stimulus also activates CNS systems that extract information about the motivational-affective and cognitive-evaluative significance of the stimulus. Motivational-affective elements of pain are subserved primarily by reticular and limbic structures, while the cognitive-evaluative aspects arise from neocortical processes. The ultimate response consists of behavioural responses, which are presumed to improve the probability of survival (Adams & Victor, 1996; Melzack & Katz, 1994).

1.1.2.A *Nociception*

The noxious stimuli act directly on peripheral cutaneous and deep receptors. These are the free nerve endings of high-threshold primary afferent nerve fibres, dispersed throughout peripheral and deep tissue (Melzack & Wall, 1996). The activation of these receptors initiates trains of action potentials which travel along the sensory afferents and enter the spinal cord. Two kinds of neurons that transmit pain information to the spinal cord have been identified. Small-diameter, lightly myelinated, fast conducting Aδ fibres, which are activated by high-intensity thermal and mechanical stimuli, and transmit to the CNS information on sharp, pricking, transient pain ('phasic pain': Jessel & Kelly, 1991). The small-diameter, slow-conducting, unmyelinated C-fibres are activated by high-intensity thermal, mechanical and chemical stimuli, and mediate persistent or 'tonic' pain (Jessel & Kelly, 1991).

Afferent neurons enter the spinal cord via the dorsal horns, and synapse with other neurons in the marginal zone (lamina I) and substantia gelatinosa (lamina II). Some Aδ fibres project more deeply, and terminate in lamina V. At the point of termination, primary afferents synapse with other neurons. Neurons terminating at the level of the substantia gelatinosa convey the message to the ventral horns within the same or in adjacent spinal segments and mediate somatic and autonomic reflexes (Adams & Victor, 1996). Neural input to other laminae is conveyed to higher CNS structures (Jessel & Kelly, 1991).

1.1.2.B *Pain sensation*

In the dorsal horns sensory afferents synapse, directly or through interneurons, with neurons that project rostrally to higher brain centres, primarily in five neural tracts. Most of the projection neurons cross the midline and ascend along the contralateral side of the spinal cord. Projection neurons originating in laminae I and V-VII of the dorsal horn ascend in the spinothalamic tract to the thalamus. From laminae VII and VIII, neurons of the spinoreticular tract project to the thalamus and the reticular formation. The spinomesencephalic tract originates in laminae I and V and terminates in the periaqueductal grey (PAG), mesencephalic reticular formation and other regions of the midbrain. From laminae III and IV the nociceptive input is conveyed to the ipsilateral cervical nucleus of the spinal cord. From here, axons cross the midline and project to the thalamus and midbrain nuclei. From laminae III and IV axons also project to the cuneate and gracile nuclei in the medulla (Jessel & Kelly, 1991). The ascending pathways deliver the information about the noxious stimulation directly to the brain structures where they terminate, and to other brain areas indirectly. The nociceptive input enters an active brain, so that further processing depends on the nature of ongoing activity. The ultimate result is the pain sensation with its sensory-discriminative, cognitive, evaluative, motivational and affective components (Melzack & Wall, 1996).

1.1.2.C *Pain behaviours*

Noxious stimulation can result in simple reflexive responses. These do not require supraspinal processing or conscious sensation of pain, and include withdrawal of a limb exposed to radiant heat. Responses that require processing in higher CNS structures and are more complex are referred to as organised unlearned responses (Chapman et al., 1985). Organised learned responses occur when a subject has experienced a particular painful stimulus in the past, and has learned to avoid it (Chapman et al., 1985; Dubner, 1994).

1.1.3 Mechanisms of pain modulation

The magnitude of pain sensation is not always a direct function of the intensity of the noxious stimulation. It is subject to modulatory mechanisms along the entire pathway of transmission of the nociceptive input. Peripheral nociceptors can undergo changes in their activity patterns as a consequence of other activity in the nervous system (Meyer et al. 1994). In the CNS, transmission can be affected at almost every synaptic junction by local events, as well as by centrifugal input from other CNS centres (Melzack & Wall, 1996).

1.1.3.A *States of altered pain processing*

Pain stimuli can evoke responses whose magnitude is expected considering the intensity of the noxious stimulus. This is referred to as normoalgesia. Only stimuli sufficient or nearly sufficient to produce tissue injury evoke pain (Yaksh & Malmberg, 1994). The state of increased sensitivity to stimuli (excluding the special senses) is called hyperaeshesia. The latter includes hyperalgesia and allodynia. Hyperalgesia occurs when there is an increased response to a stimulus that normally produces pain. Allodynia describes the state in which stimuli, whether thermal, tactile or other, that normally do not produce, result in a painful sensation (Merskey & Bogduk, 1994). Hypoalgesia is an increase in stimulus intensity required to produce pain. In other words, the magnitude of the pain response is reduced as compared to the magnitude of the response to the same stimulus in the state of normoalgesia, resulting in pain insensitivity to a tissue-damaging stimulus (Yaksh & Malmberg, 1994).

1.1.3.B *Mechanisms underlying pain modulation*

1.1.3.B.i Changes in nociceptor sensitivity

Activity patterns of peripheral nociceptors can change after they are repeatedly stimulated. In the periphery, C-fibre nociceptors have been found to desensitize after repeated stimulation, while Aδ-fibres become sensitized. A prominent example of afferent sensitization is in the case of peripheral inflammation, during which chemical mediators are released from the nociceptors themselves, the injured tissue or blood cells. These mediators, e.g. bradykinin (BK), prostaglandins (PG) and leukotrienes (LT), 5-hydroxytriptamine (5-HT), interleukins (IL), histamine, substance P (SP), adenosine and others, act on the afferent neurons and decrease the threshold of their activation (Meyer et al., 1994).

1.1.3.B.ii Central nervous system plasticity

Neuronal circuitry in the CNS can dramatically alter the transmission of the noxious input, and thus change the relationship between the noxious stimulus and response. The incoming events can be enhanced or attenuated. The underlying mechanism of this plasticity is that the noxious input elicits release of neurotransmitters from the afferent, intrinsic and descending neural fibres, and subsequently affects local events of neurotransmission. These changes have been best characterized at the level of the dorsal horn (Dickenson, 1995). Several neuropeptides have been implicated in altering nociceptive transmission. SP originates from unmyelinated primary afferents, as well as intrinsic neurons and descending fibres. It exerts its enhancing effect on nociceptive transmission by acting on neurokinin-1 (NK-1) receptors. Neurokinin A (NA), may play a similar role. Calcitonin gene-related peptide (CGRP), somatostatin and galanin are other neuropeptides released from nociceptors into the dorsal horn. CGRP appears to enhance, while galanin and somatostatin inhibit transmission of noxious input (Dickenson, 1995). Excitatory amino acids (EAA) glutamate and aspartate, thought to be released by small-diameter myelinated primary afferents, interneurons and projection neurons, produce conditions favouring nociceptive transmission. This occurs due to their action on N-methyl-D-aspartate (NMDA) receptors, as well as non-NMDA receptors, such as alpha-amino-3-hydroxy-5-methyl-

4-isoxazolepropionic acid (AMPA), metabotropic glutamate, and kainate receptors. NMDA receptor activation has been implicated in chronic pain states in addition to the acute physiological phenomena. NMDA receptor activation has been associated with Ca^{++} influx which activates nitric oxide synthase (NOS), the enzyme responsible for generation of nitric oxide. This gas may enhance facilitated reflexes and promote nociceptive transmission. The actions of EAA on NMDA receptors, and SP on NK receptors promote prostanoid synthesis in the spinal cord, which may also facilitate transmission of nociceptive stimulation (Dickenson, 1995).

1.1.3.B.iii Input from other brain centres (descending control)

The somatosensory cortex, the paraventricular hypothalamic nucleus, nucleus raphe magnus and the pontine lateral tegmental field have all displayed the ability to modulate the firing of ascending nociceptive neurons and spinal reflex responses to pain. Their action is, however, indirect. Medullary and midbrain structures have been shown to be the origin of centrifugal control of ascending nociceptive pathways. The midbrain PAG, which receives input from other midbrain and forebrain structures, as well as from the spinal cord, sends its projections down to the rostral ventromedial medulla (RVM). The latter includes the nucleus raphe magnus (NRM) and nucleus reticularis magnocellularis (RMC). The most important origin of descending fibres travelling to the spinal dorsal horn, via the dorsolateral funiculus, originate in the RVM. These projections terminate in the superficial laminae and lamina V of the dorsal horns. The neurotransmitter involved in their action appears to be 5-HT. The dorsolateral pontomesencephalic tegmentum (DLTP) also appears to supress nociception in the spinal ascending pathways, primarily by means of descending noradrenergic projections to the dorsal horn (Fields & Basbaum, 1994).

The site at which the descending inhibitory pathways originate is rich in opioid receptors and endogenous opiates. They appear to be one site of action of opioid narcotics. In addition, these systems allow that the level of incoming pain sensation is altered by certain complex CNS conditions such as motivation, stress, anticipation, attention, emotion etc. (Melzack & Wall, 1996).

1.2 ANIMAL MODELS OF PAIN

Advances in the knowledge of how to alleviate clinical pain rely almost exclusively on animal experimentation. Over fifty different animal pain models have been developed, all of which rely on the assumption that the behavioural and physiological responses that occur in the animal are similar to those that would occur in a human presented with the same noxious stimulus. The employment of animal models of pain serves two main purposes: it allows us to inflict pain and study the way in which noxious stimulation produces pain; similarly, it provides us with the opportunity to study the mechanisms producing analgesia, as well as to identify new analgesic agents (Dubner, 1994; Franklin & Abbott, 1989).

It is important to be aware of the limitations associated with each pain model, and the conclusions that can be inferred from experimental observations. Different paradigms employ different types of noxious stimuli, and these may elicit different physiological and behavioural responses and underlying painful experiences. Each test may reflect a dissimilar neural or pharmacological mechanism. It is essential that the model of pain chosen be relevant to the clinical problem being addressed (Melzack and Wall, 1996). In the same way that different pain stimuli activate different neuronal pathways, different types of pain may be differentially susceptible to different analgesic treatment (Franklin & Abbott, 1989). Some pain models measure simple reflex responses, such as tail-flick or limb-withdrawal, others require supraspinal processing. The latter may quantify unlearned or learned behaviours (Dubner, 1994). Examples of the different types of pain models are summarized below.

1.2.1 Simple reflex models

Simple reflex models include the tail-flick test and the limb-withdrawal tests. In the tail-flick test (D'Amour & Smith, 1941), a heat stimulus is applied to the animal's tail, and the time the animal takes to withdraw its tail from the heat source is taken as a pain measure. Similarly, in the limb-withdrawal test (Bennett & Peterson, 1975) a limb is stimulated thermally or electrically until the animal withdraws it. As in the tail-flick test,

the withdrawal latency is taken as a measure of pain. These tests measure pain thresholds rather than quantifiable pain. The animal has control over the noxious stimulus since it can withdraw its tail or limb and terminate the noxious stimulation. The stimulus produces little or no tissue damage. Although the change in withdrawal latencies due to administration of analgesic agents in these pain models is generally a good predictor of analgesia in humans, reflex behaviours are not a measure of pain *per se*. Limb-withdrawal and tail-flick occur in decerebrate or spinalised animals where the noxious stimulus does not reach the brain and a pain sensation can not occur (Dubner, 1994).

1.2.2 Models requiring supraspinal processing

These models of pain require that the noxious stimulation be processed by higher CNS structures before a response occurs. These responses, unlike simple reflexes, will not occur in decerebrate animals (Dubner, 1994; Chapman et al., 1985).

1.2.2.A *Organised unlearned response paradigms*

Unlearned response paradigms involve noxious stimuli-induced behaviours more complex than a simple reflex. These are voluntary responses and require supraspinal sensory processing, and have not been learned prior to testing. With respect to the duration of the noxious stimulus they can be grouped into phasic pain models, in which the stimulus is of relatively short duration (in the order of seconds), and tonic pain models, in which the noxious stimulation is inflammation-induced and prolonged (in the order of several minutes to several weeks; Dubner, 1994).

1.2.2.A.i Phasic pain paradigms

In the hot-plate test, primarily used with rodents, a rat or a mouse is placed on a heated plate. The measure of pain is the time required for the animal to begin licking its hind paws. This test is similar to the simple reflex pain models in that the pain is of short duration, and that the animal has control over the pain and can terminate the noxious

stimulation. The measure of pain is latency, and the test measures pain thresholds (Dubner, 1994).

1.2.2.A.ii Tonic pain paradigms

Several paradigms involving longer-lasting, inflammatory pain, have been developed, and are preferred models because the nature of the induced pain is similar to most clinical pains (Abbott, 1997; Dubner, 1994). The animals do not have control over the stimulus intensity or duration. The pro-inflammatory agents used to induce pain produce tissue damage. Another advantage of these models over the simple reflex paradigms is that multiple behavioural measures are available for pain quantification (Dubner, 1994). Complete Freund's adjuvant (CFA), injected intradermally in the tail, the plantar surface of a rear paw or the skin on the back, produces a generalized systemic disease. The irritant, comprised of tuberculin bacilli (myobacterium) cell walls in mineral oil and emulsifiers, produces an autoimmune reaction (Franklin & Abbott, 1989; Coderre & Wall, 1987). Injected into the tail, CFA produces arthritis in multiple joints. In addition to inflammation and hyperalgesia in the joints it also produces damage to other tissues such as eyes, ears, genitals, skin, bone and liver. Subjects experience pain over a course of several weeks, as well as weight loss and reduced motor activity (Coderre & Wall, 1987). Pain is measured by observing scratching, locomotor activity, weight loss and vocalization upon mechanical stimulation of the affected limbs (Dubner, 1994). Although this is the only model in which pain occurs over the course of several weeks, there are serious ethical considerations regarding the use of the model, due to severe discomfort produced over a relatively long time period (Franklin & Abbott, 1989).

As an alternative to CFA, Coderre & Wall (1987) introduced the ankle joint urate arthritis model. Sodium urate crystals are injected directly into one ankle joint. Inflammation and hyperalgesia remain localized to the injected limb, peak within 24 hours of injection, and persist over the course of approximately one week. This model does not produce severe systemic disease, and the discomfort is shorter in duration.

The formalin test (Dubuisson & Dennis, 1977) is another model where a pro-inflammatory agent is injected into an animal's paw, which produces pain lasting approximately one hour, and edema lasting several days. Pain responses are complex (favouring, lifting, licking or biting of the injected paw and flinching) and easily quantifiable (Tjølsen et al., 1992).

The writhing test (Vyklický, 1979) involves an intraperitoneal (IP) injection of an irritant, which activates visceral afferent neurons. This is a model of visceral pain, and the pain measures are various behavioural responses, such as internal rotation of a paw, rolling on the side, arching of the back and abdominal contractions (Dubner, 1994).

1.2.2.B *Learned or operant response paradigms*

Operant response paradigms are models of pain where the animal has control over the noxious stimulus intensity or duration. It can terminate the aversive experience by performing a learned behaviour, such as pressing a lever. The measure of pain is the latency of the behavioural response. These paradigms involve complex supraspinal sensory processing and performance of subjects in the tasks involve attention, motivation and learning (Dubner, 1994).

1.2.3 Ethical considerations

The general notion regarding pain research in animals is that this kind of investigations are necessary. Physiological and pharmacological manipulations of pain mechanisms in humans are considered unethical and therefore not possible (Chapman et al., 1985). Animal experimentation is virtually the only means by which we can advance our knowledge of pain control (Dubner, 1994). However, it is necessary to minimise the use of animals, and the degree of pain and injury inflicted upon them. The responsibility investigators have to display begins in the choice of paradigms and careful planning of their experiments, since badly designed or performed experiments cause unnecessary pain and provide little or no useful information (Franklin and Abbott, 1989). The least ethical concern regards pain testing on animals under anaesthesia, or those that have undergone

surgery that minimizes pain sensation, e.g. decerebration. These experiments do not involve conscious sensation of pain. More care has to be taken in experimentation on awake, conscious animals, and particularly in models of persistent and chronic pain and tissue injury. The level of induced pain should be closely monitored and kept to a minimum. The level of pain should never exceed that which would be tolerated in humans (Dubner, 1994; Bowd, 1980). In addition, the experimental design should permit the use of the smallest possible number of subjects to obtain results of sufficient statistical power.

1.3 FORMALIN TEST IN THE RAT

The formalin test is a model of acute tissue injury-induced cutaneous pain (Franklin & Abbott, 1989). It was introduced by Dubuisson & Dennis in 1977. Dilute formalin is injected into one of the animals' paws. The animal is then placed in an observation chamber and the behavioural responses are observed.

Dubuisson & Dennis (1977) performed the test on rats and cats. Since then formalin induced behaviours have been studied in several species, such as guinea pigs (Wheeler-Aceto & Cowan, 1991), rabbits (Carli et al, 1981), monkeys (Alreja et al., 1984), octodon degus (Pelissier et al, 1989), domestic fowl (Hughes & Sufka, 1991), mice (Hunskaar et al, 1985) and crocodiles (Kanui et al., 1990). It is, however, most frequently employed in rats and mice (Tjolsen et al., 1992). In the following review of the formalin test the focus will be on the rat, unless otherwise indicated.

1.3.1 Formalin as a pro-inflammatory agent

The term formalin refers to 37-39% formaldehyde, which usually contains 10-15% methanol as a stabiliser, mixed with normal physiological saline. Formaldehyde belongs to the group of chemical compounds called aldehydes, which have been found to exert a dose-dependent toxic effect on cells (Harvey, 1975). Formalin's cytotoxicity arises from its ability to bind free amino groups of proteins, forming cross-links (Alberts et al., 1994) and causing protein precipitation (Harvey, 1975). When applied to tissue in vivo, formaldehyde denatures cellular proteins and induces an inflammatory response (Brown, 1968;

Miampamba et al., 1992), which manifests itself in pain responses and swelling of the paw (Dubuisson & Dennis, 1977). Pain develops just seconds after formalin administration and precedes the development of histological changes associated with acute inflammation. The initial pain is probably due to the interaction of formaldehyde with ion channel proteins on afferent neurons, leading to an influx of sodium and eventual failure of all ionic currents over a period of hours (Hille, 1992; Margineanu, 1990). The inflammatory phase begins minutes after formalin application (Millitzer, 1976). During this period, pain probably results from activation of neuronal afferents by inflammatory mediators. Edema and extravasation develop at a slower rate than pain, peak within a day after formalin injection, and can last several weeks. Depending on the amount of formalin, a small blister may develop, which is then replaced with scar tissue (Dubuisson & Dennis, 1977).

1.3.2 Formalin-induced pain

As implied by the preceding discussion, the behavioural response to formalin-induced pain is biphasic. In humans, formalin administration induces initially a sharp, short-lasting pain, followed by a prolonged, poorly localized, burning pain (Dubuisson & Dennis, 1997; Franklin & Abbott, 1989). In rodents, two distinct phases of pain are separated by a period where no pain appears to be experienced, the so-called interphase (Tjolsen et al, 1992; Franklin & Abbott, 1989). In other species, such as rabbits, the pain is monophasic. The different profiles of painful responses in different species may be due to activation of different underlying physiological processes (Tjolsen et al., 1992). In rats, favouring, lifting, shaking, flinching, licking or biting the injected paw begins immediately after the injection and lasts some 3 to 5 minutes. This 'first' or 'acute' phase of pain responses is followed by a period where no pain responses are observed and rats tend to be inactive (interphase). The pain responses reappear 15-20 minutes post-formalin, accompanied by an increase in locomotor activity ('second' or 'tonic' phase; Dubuisson & Dennis, 1997; Wheeler-Aceto et al., 1990). The two phases of formalin-induced pain display different sensitivities towards specific analgesic agents (Wheeler-Aceto et al., 1990;

Coderre et al., 1990), supporting the notion that the mechanisms underlying pain in the two phases is different.

1.3.2.A *First phase*

Initially, an intraplantar injection of formalin appears to induce pain by directly stimulating peripheral sensory receptors (Dubuisson & Dennis, 1997; Hunskaar & Hole, 1987), and thus activating peripheral sensory afferents (Brown et al., 1968). Studies in mice have led to the conclusions that direct stimulation of peripheral sensory afferents by formalin results in SP and BK release. SP and BK may synergistically participate in inducing first phase pain (Shibata et al., 1989).

1.3.2.B *Second phase*

Pain emerging some 15 or 20 minutes post-formalin is due to developing inflammation (Dubuisson & Dennis, 1977; Hunskaar & Hole, 1987). Histamine, 5-HT, prostoglandin E2 (PGE2), BK and noradrenaline have been shown to play a role in the induction of the second phase pain (Abbott et al., 1996; Hong & Abbott, 1996; Shibata et al., 1989).

1.3.2.C *Beyond peripheral stimulation*

Formalin injected into a rat's paw has been shown to activate C- and Aδ -nerve fibres (Puig & Sorkin, 1994; McCall et al., 1996) of the three branches of the sciatic nerve: saphenous (Heapy et al., 1987), sural (Puig & Sorkin, 1993) and tibial (Carli et al., 1987). The activation of the nerve fibres displayed a similar time-course to the behavioural responses to subcutaneous injection of formalin (Porro & Cavazzuti, 1993). The sciatic nerve enters the spinal cord at the fourth and fifth lumbar segment. Neuronal activation in the spinal cord has been demonstrated mainly in the dorsal horns of the spinal cord, ipsilateral to the injected paw (laminae I, II, V and VI; Abbadie et al., 1997). Prolonged and widespread neuronal activity was also observed in the deep laminae VII and VIII. Activity

in both the superficial and deep lamina display a time course similar to the behavioural responses (Porro & Cavazzuti, 1993).

1.3.3 Methods of assessing pain and analgesia

The formalin test has been used to study many different aspects of pain. Formalin induces inflammation at the site of injection, and permits the study of the inflammatory process and inflammation-induced nociception (Abbott et al., 1996; Hong & Abbott, 1996; Shibata et al, 1989). Electrophysiological studies have been used to study the transmission of noxious information along afferent neuronal pathways, as well as the transmission in the spinal cord and brainstem (Porro & Cavazzuti, 1993). Electromyographic (EMG) techniques can monitor muscle activity after administration of formalin. Electroencephalography (EEG) has been used to investigate activity in various parts of the cerebral cortex following formalin injection in cats and rabbits (Carli et al., 1976). CNS activity has also been measured by means of radioactive tracers, such as 14C-2-deoxyglucose (14C-2DG). Activation of nerve fibres can be traced from the periphery to the central nervous system using electrophysiology. Neuronal projections in the spinal cord and brainstem that are activated by formalin can be identified by immediate-early gene (IEG) mapping. IEGs, such as c-*fos*, are expressed upon neuronal activation, and can be identified by immunohistocemical methods (Porro & Cavazzuti, 1993). Microdialysis and microperfusion have been used to elucidate neurotransmitter and neuropeptide release in the brain and spinal cord (Tjolsen et al., 1992). The biochemical studies during nociceptive stimulation by formalin have examined the release of neuropeptides, neurotransmitters and other neurochemical changes, as well as CNS processing and plasticity (Coderre et al., 1990). Pharmacological studies employed the formalin test to study the mechanism of analgesia mediated by drugs such as opiates (Abbott & Palmour, 1988), non-steroidal anti-inflammatory drugs (NSAIDs: Abbott & Hellemans, submitted for publication), local anaesthetics (Coderre et al., 1990), dopaminergic (Franklin, 1989), serotonergic (Abbott et al., 1996), GABA-ergic (Carmody et al., 1991) and adrenergic (Coderre et al, 1984) agonists and antagonists, tricyclic antidepressants (Fasmer et al., 1989), excitatory amino acids

(Coderre & Melzack, 1992a), calcium channel blockers (Coderre & Melzack, 1992b) and other pharmacological agents (Porro & Cavazzuti, 1993).

Appendix I is a list of scientific reports of studies using the formalin test to study pain and related phenomena, published between January 1997 and December 1998. It contains details of behavioural rating and other pain assessment, formalin volume and concentration, site of injection and other experimental variables.

1.3.4 Behavioural studies

Behavioural studies use behavioural responses to measure the degree of painful sensation induced by a formalin injection. A variety of behavioural responses have been observed after subcutaneous administration of formalin, and they can be grouped in two distinct classes. Pain-specific behaviours, which are presumed to be a direct function of the painful experience, are quantified and used to determine a pain index to estimate the magnitude of the pain. Other behaviours reflect the complex effects of pain on more general behavioural patterns, such as sleeping, grooming and exploring. Monitoring general behaviour can also provide information on the action of drugs studied in the formalin test, such as sedative effects of opioid analgesics (Abbott et al., 1995).

1.3.4.A *Pain-specific behaviours*

Formalin injection in the paw induces several different behaviours that can be quantified and used as a measure of the magnitude of pain sensation. Dubuisson and Dennis (1977), described formalin induced behaviours that are still commonly used today (Tjolsen et al., 1992). The behavioural categories used are 'normal', 'favour', 'lift' and 'lick, bite or shake' the injected paw. When all paws rest on the floor, with the animal's weight equally distributed between them, a score of '0' is recorded (normal). When both paws rest on the floor, but a rat puts more weight on the paw that did not receive the formalin injection as compared to the one that received the formalin, it is said to favour the injected paw (score '1'). When the injected paw is held above the ground, whether the rat is lying, sitting, walking or grooming, a score of '2' is assigned ('lift'). The behavioural score of '3' is

recorded when the injected paw is licked, bitten or shaken (Dubuisson & Dennis, 1977). Another pain response frequently employed to quantify pain is 'flinching'. This includes shaking the injected paw and convulsive flexion of the limb, paw and or the animals' whole hindquarters (Ryan et al., 1985; Wheeler-Aceto & Cowan, 1990).

1.3.4.B *Methods of quantification*

Behavioural responses can be quantified in several different ways. Some investigators record the amount of time formalin-treated animals spend displaying one or more of the behavioural responses (Dubuisson & Dennis, 1977; Abbott et al., 1995). This is referred to as 'continuous rating', and is the most commonly used method of quantification when favouring, lifting, licking, biting and or shaking behaviour are used to determine pain levels. An alternative to pain measures expressed in terms of time is 'time-sampling', where behaviour is recorded at regular time intervals, and pain scores are expressed as frequencies of behaviours with respect with the total number of observations (Altier & Stewart, 1998; Teng & Abbott, 1998; Farneslow, 1984). Another method of quantification is counting the number of times a certain behaviour is displayed in a pre-defined time-period. This method uses mostly the flinching response (Ryan et al., 1985).

1.3.4.C Methods of determining pain indices

Pain assessment methods can record a single behavioural response to obtain a pain score (single measure methods), or a combination of two or more responses (multiple-measure methods).

1.3.4.C.1 Single-measure pain assessment

Observing a single behaviour rather then distinguishing among several behavioural categories is less demanding and may represent an easier-to-perform task. The amount of time spent lifting (Abbott et al., 1981) or licking (Sugimoto et al., 1986) the formalin-treated paw has been used as an index of pain. The number of flinches has also

been proposed to be a good indicator of formalin-induced pain, sensitive to analgesic treatment (Ryan et al., 1985; Wheeler-Aceto et al., 1990; Tjølsen et al., 1991a, b).

Several studies have analysed pain measures by investigating the dose-dependence of pain indices with respect to formalin concentration, and the sensitivity of the pain assessment method to analgesic agents and non-analgesic drugs with behavioural effects. Favouring has not been used as a pain index since it correlates negatively with formalin dose in the second phase of the test. It is also a measure more prone to inter-observer variability. Lifting does not significantly correlate with formalin dose in the second phase of the test (Abbott et al, 1995). Licking, and particularly flinching, have been extensively used as indicators of pain. Abbott et al. (1995) concluded that licking is, among the single

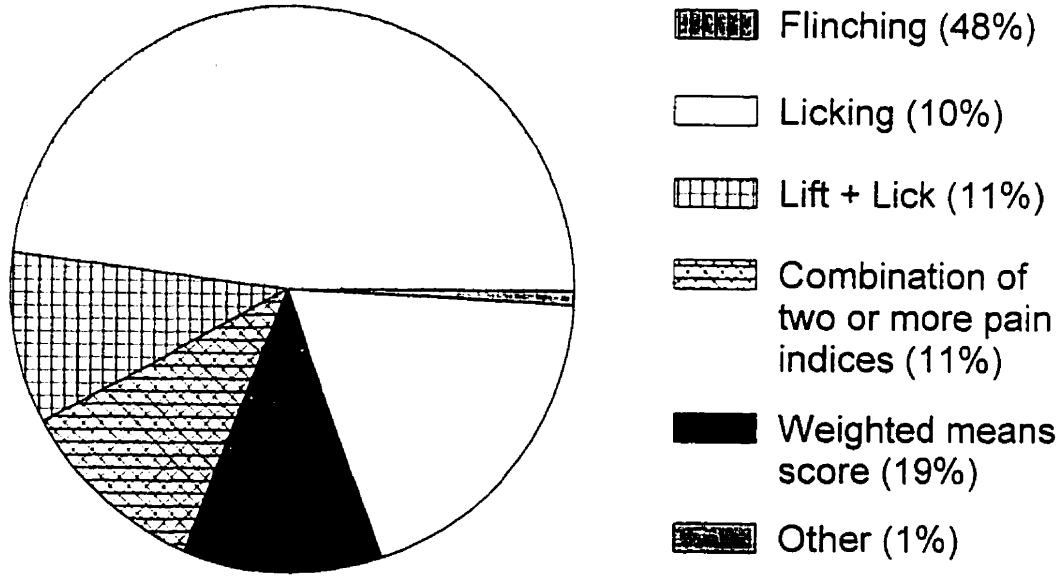

Figure 1

Methods of assessing pain in the literature using behavioural rating in the formalin test, published in 1997 and 1998.

pain measures, the best index of pain. It was found to be a good predictor of formalin dose and morphine analgesia and was unaffected by sedative non-analgesics. It was, however, only marginally sensitive to non-sedative analgesics (Abbott et al., 1995). Wheeler-Aceto & Cowan (1991) analysed lifting and flinching behaviours. They also concluded that licking is affected by non-analgesic agents that either stimulate or suppress locomotor activity. They proposed that flinching is a better measure to be used. However this category of behaviour failed to explain 50% of variance of the effect of formalin in the study by Abbott et al. (1995).

Ninety percent of the scientific reports using formalin to induce pain, that were published in 1997 and 1998 (Appendix I), employed behavioural rating to asses the magnitude of painful sensation. Nearly 60% of the behavioural reports scored a single behavioural category. Approximately half of all behavioural studies used flinching exclusively, and approximately 10% scored licking the injected paw (Figure 1).

1.3.4.C.2 Composite pain assessment

Abbott et al. (1995) systematically analysed all frequently employed behavioural measures, and concluded that pain scoring methods that take into account more than one behavioural response generally correlate better with formalin concentration and analgesic treatment. Approximately 40% of behavioural studies published in the years 1997 and 1998 used two or more behavioural measures. 19% of the studies used the weighted means score (WMS) method and 11% used the sum of time spent lifting and licking or biting the injected paw. Close to 11% of the studies used two or more different pain assessment methods, mostly the number of flinches and time spent licking (7%; Appendix I)

1.3.4.C.2.i Weighted means scores

Several variations of this method exist. As proposed initially by Dubuisson & Dennis (1997), behaviour is rated continuously, and is categorized in four mutually exclusive categories, as they were initially defined: 'normal' (no pain), 'favour', 'lift' and 'lick' (i.e. lick, shake or bite the formalin treated paw). The mean of time each group of

animals spends in each category is assigned a weight. The weights are supposed to account for different pain intensities the respective behaviours indicate. The pain index is calculated by summing the products of the amount of time in each behavioural category with the weight assigned to the category. Dubuisson & Dennis (1977) assigned weights 1, 2 and 3 to favour, lift and lick, respectively, and proposed that the pain index be calculated in the following way:

$$\text{pain} = \frac{1*T_1 + 2*T_2 + 3*T_3}{\text{total time}}. \qquad \text{(Eq. 1)}$$

where T_1, T_2 and T_3 are times spent favouring, lifting and licking, respectively. Favouring was thought to reflect lower, and lifting and licking progressively higher, levels of pain.

Watson et al. (1997) proposed a different assignement of weights: 0, 1 and 2, for the means of time spent favouring, lifting and licking, respectively:

$$\text{pain} = \frac{1*T_2 + 2*T_3}{\text{total time}}. \qquad \text{(Eq. 2)}$$

where T_2 and T_3 are means of times spent lifting and licking, respectively. The sum of weighted means are superior to all single measure methods (Coderre et al., 1993, Watson et al., 1997; Abbott et al., 1995). Watson et al. (1997) compared the weights proposed by Dubuisson and Dennis (1977; 1,2,3) to the weights 0,1,2 and concluded that the latter are optimal weights as determined by Pearson's correlations and multiple regression analyses obtained from behavioural studies of formalin-induced pain and morphine analgesia. This conclusion is obvious, since inclusion of a variable that correlates negatively with formalin concentration will necessarily reduce the value of r^2.

1.3.4.C.2.ii Simple sum of means

A sum of means of time spent lifting and licking (behavioural categories 2 and 3, Dubuisson & Dennis, 1977) may also be used as a pain index. This method appears to be equally valid as the weighted means method (Abbott et al., 1995; Watson et al., 1997). In theory, this method may, however, fall into the category of weighted means, with weights 0.1 and 1 corresponding to behavioural categories 1,2 and 3, respectively (Watson et al., 1997).

1.3.4.C.3 Automated systems

An attempt has been made in recent years to develop an automated system for determining the pain intensity in the formalin test. This is primarily due to the fact that manual scoring is time-consuming. Most scoring methods use continuous rating (see Table 1), which only permits one or two subjects to be tested simultaneously. In addition, continuous careful observation of the animals is a demanding, vigilant task. Recording the number of flinches during the testing period does not require continuous recording of behavioural codes. However, it does require continuous observation and does not represent an improvement over time sampling in terms of saving time.

The first automated system was developed by Jett & Michelson (1996). After the formalin injection, rats are placed in a clear plastic tube 16 cm in length, with an internal diameter of 8 cm. The apparatus measures 'dynamic force changes' (Jett & Michelson, 1996), and records it automatically as a single-measure agitation index. The apparatus yields a bi-phasic, dose-dependent response to formalin and is susceptible to analgesic agents, and the results obtained by this method seem to be consistent with a manual scoring method. However, the pain index is based on a single parameter (agitation), and can not resolve any specific pain responses (Jourdan et al., 1997). In addition, it is likely that sedative non-analgesic agents would appear to produce analgesia in a system determining pain by measuring agitation.

A second automated apparatus was introduced by Jourdan et al. (1997). A camera which records the rats' behaviour, is connected to a computer. The computer analyses the

images continuously created by the camera. This system is somewhat sensitive towards pain-specific and general behaviours. Grooming, licking or biting can be distinguished from locomotor activity such as walking across the testing chamber. Like the automated system by Jett & Michelson (1996), the apparatus produces biphasic pain indices comparable to those determined by manual scoring, both for different formalin concentrations and analgesic treatments. The authors of both automated devices present their inventions as clearly advantageous over the manual scoring designs, in that the scoring is completely objective, does not require investigators' presence or performance of vigilant tasks, and that many animals can be tested simultaneously (Jett & Michleson, 1996; Jourdan et al., 1997).

1.3.4.D *Quantification of general behaviour*

In addition to pain-specific behaviours, several studies have attempted to record behavioural responses other than those used to assess the level of pain, such as exploration, grooming, motor performance etc. (Tjolsen et al., 1992). Scoring changes in general behavioural patterns may provide valuable information on the effect of different levels of pain on general behaviour. Certain changes in general behaviour may even be indicative of the level of pain (Abbott et al., 1995). In addition, such information can disclose side effects of drugs being tested in the formalin paradigm, such as sedative effects of opiates (Abbott et al., in press).

Whether pain is being assessed by means of continuous rating to obtain a single or composite pain score, or by means of counting of flinches, other behavioural measures can only be scored with difficulty. Abbott et al. (1995) videotaped formalin tests while pain behaviours were scored, and rescored other behaviours by observing the videotaped footage. Farneslow (1984) used a time-sampling procedure, where a rat's behaviour was recorded at regular time intervals. If a pain specific behaviour was displayed (lifting or licking), a 'recuperative' score was assigned, and if not, general behaviour was scored (freezing, grooming or rearing). The sum of recuperative scores served to compute the pain index.

To measure the effect of pain and pharmacological agents on general behaviour, other methods have been used, such as measuring cardiovascular parameters (Bhatnagar et

al., 1998), or the rotorod test of motor performance (Simmons et al., 1998). In the case of the latter, pain can not be measured concomitantly. Cardiovascular parameters can be measured simultaneously with pain measurement, however not without complicating the procedure. Watson et al. (1997) measured activity during formalin tests using ultrasonic movement detectors. Although this procedure does provide a measure of activity, the output is univariate and non-specific, and requires costly equipment. The automated system by Jourdan et al. (1997) provides a similar output and may also be financially unfavourable.

1.3.4.E *Parameters affecting formalin-induced pain*

Behavioural responses to formalin treatment have been shown to vary with the site of injection, the volume and concentration of formalin, and ambient temperature. In addition, the familiarity of the subjects with the testing environment has been shown to have an effect on the behavioural responses. Appendix I provides information on these variables in recent studies employing the formalin test.

1.3.4.E.i Site of injection

Formalin has been injected subcutaneously into both forepaws and hind paws. Initially, the forepaws were used (Dubuisson & Dennis, 1977). However, the use of hind paws is preferred, since licking of the hind paws rarely occurs during normal grooming behaviour (Tjolsen et al., 1992). Formalin may be injected either in the dorsal or the plantar surface of the paws. Plantar injections produce more pain than dorsal surface injections (Neoh et al., 1992). In rats, formalin is usually injected into the plantar surface of a rear paw when licking, sum of lifting and licking, or WMS is used as the index of pain. When flinching is scored, investigators inject formalin in either the dorsal or plantar surface of a hind paw (see Table 1). Formalin has also been used to study orofacial pain in the rat. In these studies, formalin was injected into the upper lip (Luccarini et al., 1998; Cadet et al., 1998).

1.3.4.E ii Formalin volume and concentration

Formalin-induced pain increases with formalin concentration. The volume of injection used ranges from 10 to 200 µl (Appendix I).The most commonly used volume of injection is 50 µl. The concentrations have varied greatly, and have been as high as 100 µl of 12% (equivalent to 24% in 50 µl: Machelska et al., 1997), and as low as 0.25% (Takahashi et al., 1984). The mean concentration of formalin used is 5.14% (standard deviation 4.02%), and the most commonly used is 5%. Figure 2 shows the usage of different formalin concentrations in the literature published in 1997 and 1998 (Appendix I).

1.3.4.E iii Ambient temperature

Temperature of the testing environment affects the pain scores inferred from behavioural testing. An increase in temperature increases the pain scores (Abbott et al.,

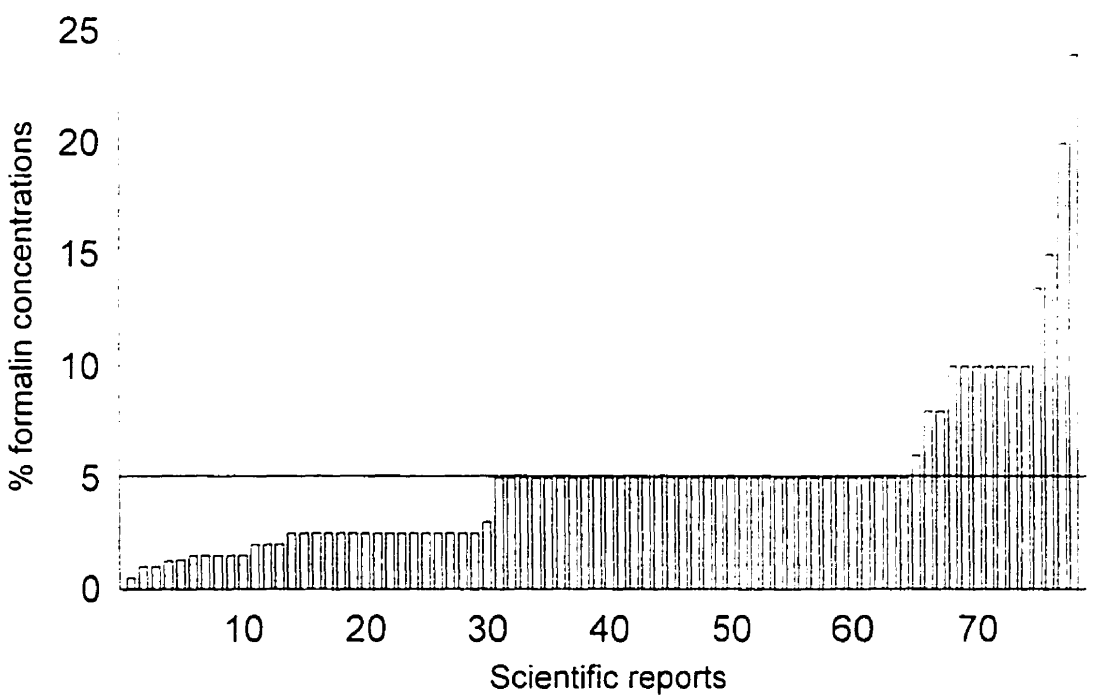

Figure 2

Formalin concentrations used in scientific reports using the formalin test, published in 1997 and 1998.

1995). In particular, the second phase of the test is affected, probably because of the effect of temperature on peripheral blood flow. An increase in temperature increases peripheral blood flow and hence promotes the development of inflammation (Tjolsen et al., 1992).

1.3.4.E.iv Stress

Stress has been shown to decrease pain sensation in humans and laboratory animals (Terman et al., 1984). Rats that are familiarized with the formalin apparatus prior to the testing show higher pain responses, as compared to those that are exposed to the test environment for the first time when tested. Similarly, rats that are restrained during the behavioural scoring may show less pain than those who are not restrained show that the neurochemical bases of the pain reduction produced by testing in an unfamiliar environment are similar to those mediating the effects of restraint. Stress due to novel environment or restraint are believed to enhance endogenous pain-suppressing mechanisms. Serotonergic and endogenous opiate systems appear to play a role in stress-induced analgesia (Abbott et al., 1986a).

1.3.5 Advantages of the formalin test over other animal pain models

The pain paradigms used in investigations of mechanisms of pain and analgesia should be chosen on the basis of similarities between the noxious stimulation and the nature of the painful experience in the experiments and the clinical problem being addressed. Different noxious stimuli may evoke different pains by activating different physiological and psychological processes (Melzack & Wall, 1996). Most commonly used animal pain tests, like the hot-plate and the tail-flick tests, apply brief, transient painful stimuli. The threshold of pain is measured rather than the quality and quantity of the painful sensation. In addition, the animals have to be restrained during the procedure, and they have control over the noxious stimulus (Dubner, 1994).

The formalin test in many ways mimics acute clinical pain that would be induced by tissue injury. The animals are not restrained during testing procedures and do not have control over the noxious stimulus or duration of the painful experience. Investigators who

have volunteered to experience the formalin-induced pain have described it as initially burning, then poorly localized deep pain of moderate intensity. The site of tissue injury is surrounded by a region where mild spontaneous pain and hyperaesthesia occur (Franklin & Abbott, 1989; Dubuisson & Dennis, 1977). In addition, the formalin-induced pain is limited in duration (pain generally does not persist more than two hours after injection even when high doses of the irritant are administered; Dubner, 1994). The tissue injury associated with formalin administration is localized to the paw of injection and does not appear to cause prolonged distress or systemic effects. This paradigm therefore presents fewer ethical concerns than other animal models of tonic pain.

1.4 OBJECTIVES

In the present investigations, methodological issues concerning the formalin test are addressed. The purpose of investigations was threefold. Initially, a time-sampling method of behavioural rating was introduced, which would be easier to perform and more time-efficient, and would record pain-specific as well as general behavioural responses (Experiment 1). The new method was validated and then used to examine all formalin-induced behaviours over the range of formalin concentrations most commonly employed (Experiment 2). Finally, the relationship between morphine analgesia and formalin-induced pain was investigated (Experiment 3).

Chapter 2

MATERIALS AND METHODS

The methods that apply to all experiments are outlined below. Details of materials and methods that apply only to individual experiments are described in the introductory section of each experiment.

2.1 Subjects

Male Long Evans rats were used in all experimental procedures. They were obtained from Charles River, Quebec. The weight of the animals, when received, ranged from 150 to 200 grams. They were housed, two or three per cage, in a colony room with standard living conditions, and a 12-hour light - 12-hour dark cycle with lights on at 7 am. Food and water were available *ad libitum*. All experimentation and animal handling was conducted during the light phase of the animal's cycle.

2.2 Ethical Considerations

All animal procedures were approved by the Ethics Subcommittee of the Animal Facilities Coordinating Committee of McGill university, and for the higher formalin concentration in the absence of analgesia, by the Animal Facilities Coordinating Committee. These committees operate under the guidelines of the Canadian Council of Animal Care.

Care was taken to minimise the amount of induced pain and suffering to the animals by carefully planning the experiments. The smallest possible number of subjects was assigned to each treatment group. It was agreed with the Animal Facilities Coordinating Committee that any rats that continued to display continuous pain behaviours together with agitation for more than 3 hours would be immediately withdrawn from the experimentation and have a therapeutic dose of morphine administered. However, this was never necessary.

2.3 Animal handling and habituation to the testing environment

Upon arrival to the colony room, animals were allowed to rest for two days. After, rats that were to be tested without habituation were left undisturbed until the testing day. Those that were to be tested after habituation, were brought to the laboratory and introduced to the formalin apparatus for a period of ten to fifteen minutes on five consecutive days. On the first three days the rats were placed in the formalin boxes, two or three at a time (same as in housing arrangements), and on the last two days singly. A sweet treat was placed in the formalin box, to serve as a positive reinforcer to the testing environment. In addition, immediate eating of the treat indicated a satisfactory level of familiarity with the formalin apparatus, since rats will not eat in a novel or threatening environment, even when food-deprived (Broadhurst, 1957).

It has been shown that the formalin test can be repeated on the other paw of the same animal, producing virtually the same results (Matthies & Franklin, 1992). Therefore, both hind paws of each rat were used except for rats that received over 2.5% formalin. Animals that were tested on both paws were allowed to recover for two days after the first formalin test, and were given another three habituation sessions, one rat per formalin box, prior to testing on the second paw. Rats were killed using carbon dioxide intoxication after the last formalin treatment they received.

2.4 Drugs

Dilute concentrations of formalin were prepared from a 37% formaldehyde stock solution containing 10% methanol as preservative (Fischer Scientific). The 37% formaldehyde corresponded to 100% formalin, and was diluted with a sterile, preservative-free physiological saline solution (0.9% NaCl) to yield the desired concentrations of formalin, which ranged from 0.5 to 13.75%. Injection volume of formalin solutions was 50 μL. Morphine Sulphate (gift of Sabex, Quebec) was dissolved in sterile water to produce morphine concentrations ranging from 0.25 to 8 mg/ml. Injection volume was 1 ml kg.

2.5 Drug administration

Formalin was injected subcutaneously into the plantar surface in one of the animal's hind paws with a 300 µl disposable insulin syringe with an attached 29 Ga. needle. While the rat was lightly restrained, the paw to be injected was marked for easier observation. The needle was then inserted into the paw and run rostrally under the skin for at least 5 mm. 50 µL of the appropriate formalin dilution was injected. Immediately following the injection, the rat was placed into the formalin box, and the scoring began.

Morphine was administered subcutaneously under the skin of the rat's back, with a 1 cc syringe and a 26 ½ Ga. needle, 30 minutes before the injection of formalin to assure that the peak effect of morphine would occur during the second phase of the formalin test (Abbott et al., 1986b).

2.6 Behavioural Rating

The formalin tests were conducted in 30 cm × 30 cm × 30 cm clear plexiglass boxes to allow visual observation of the subjects' behaviour. A mirror was placed underneath the glass floor at a 45° angle, to allow an unobstructed view of the paws. The rater observed the behavioural responses to administered agents and recorded them by entering behavioural codes into a personal computer. Computer programs for continuous rating and time sampling were written by Hiloma Programming Inc.

2.6.A Continuous rating

This method of behavioural rating records the amount of time the rats spend displaying behaviours coded in mutually exclusive categories. To allow analysis of the time-course of behavioural responses, the test was divided into five-minute bins. The number of animals tested by this method is restricted to two, since it is not possible to follow continuously and closely the behaviours of more than two animals, and at the same time record behavioural codes in the computer.

2.6.B Time-sampling

This scoring method relies on the statistical principle that the time-course and ratio of behaviours can be deduced from observations of behaviours sampled at regular time intervals. The computer was programmed to produce a beep that was only audible to the rater - i.e., outside the hearing range of rats - at pre-defined time intervals. At this time the rater entered the behavioural codes that describe the behaviour of the rats at the time of the beep. Time-sampling allows for behavioural rating on more than one axis. On the first axis, the pain scale identical to the one used in continuous rating was used. On a different axis a scale was defined that described, more generally, the behavioural state of the animal.

2.7 Data analysis

Formalin-induced behaviours for the continuous rating method were expressed as percentage of time each behaviour was displayed with respect of the total time in a bin (5 minutes). Time-sampling scores of each behavioural response were expressed as the proportion of the total number of observations.

The effect of formalin was expressed as percent maximal possible effect (%MPE) according to the following formula:

$$\%MPE = \frac{(E - E_{min})}{(E_{max} - E_{min})} \quad \text{(Eq. 3)}$$

where E_{min} was defined as 0 (no pain), E_{max} as the total possible amount a behaviour can be displayed for continuous rating and the total number of observations for time-sampling, and E as the sum of time each subject spent lifting and licking for continuous rating and total number of observations of lift or lick for time sampling. Morphine analgesia was determined using the same formula. E_{min} was defined as the mean pain score of controls receiving no morphine and the same formalin concentration, and E_{max} as 0 (no pain, i.e. maximal analgesia).

To estimate formalin and morphine dose-effect parametres (MPE_x and slope) a jackknifing procedure was used. The latter is an iterative procedure that computes an unbiased estimate of the MPE_{x} and its variability, by interpolating on a dose-effect line fitted to the raw data points by linear regression, as well as the slope. Details of the procedure are described in Abbott et al. (in press).

Chapter 3

VALIDATION OF THE TIME-SAMPLING METHOD

3.1.A OBJECTIVES

Conducting the formalin test is a demanding, vigilant task. Continuous monitoring of behaviour is not only difficult, but also requires that a small number of animals are tested at one time, which makes obtaining results time-consuming. Here we introduce a time-sampling method which records behaviours instantaneously, at regular time intervals. This allows that more than one behavioural code is recorded per animal, which permits us to score other behaviours, such as locomotor activity, exploring, grooming, sleeping etc. The scoring of additional quantifiable behaviours would provide a more complete description of the behavioural response to formalin and other treatments.

The time-sampling method was validated by comparing behavioural responses of favour, lift and lick, shake or bite, as they were recorded with the continuous scoring and the time-sampling method. Dose-effect relationships for formalin and morphine were established from data sets obtained by both methods. The time-sampling results were then compared to those obtained by the continuous rating. In addition, the effect of formalin and morphine on general behaviour of animals was examined from the time-sampling data.

3.1.B EXPERIMENTAL DESIGN

Rats were assigned to two groups. One was used to establish the formalin dose-effect relationship and time-course, and the other to determine the morphine dose-effect relationship at a single formalin concentration.

3.1.B.i Formalin concentration-effect relationship

15 rats were randomly assigned to three groups of 5 subjects. Each group received one of three concentrations of dilute formalin (0.5%, 1% or 2%). Scoring commenced at the

time of the formalin injection and continued for 60 minutes. Continuous rating collected data in 12 bins, five minutes each, and time-sampling at 60 one-minute intervals.

3.1.B.ii Morphine dose-effect relationship

40 subjects were randomly assigned to six groups. The control group received only 1% formalin (n=5). Other groups (seven subjects in each) were pretreated with 1, 2, 4, 6 or 8 mg/kg morphine, 30 minutes before the injection of 1% formalin. Scoring began 15 minutes after the formalin injection and lasted 30 minutes to obtain data on the effects of morphine on pain in the second phase of the formalin test. Behavioural responses were sampled at one-minute intervals, and rated continuously in six five-minute bins. After one formalin test, rats were randomly reassigned to another condition, and testing was scheduled such that all groups had rats that were receiving the first and the second formalin test.

3.1.B.iii Behavioural rating

All subjects were habituated to the testing environment. Two rats at a time were rated simultaneously by two individuals, one using the continuous scoring and the other the time-sampling method. The behavioural scale used is described in Table I.

The behavioural categories used to record pain-specific behaviours were as described by Dubuisson & Dennis (1997), and were identical for both scoring methods. The general behaviour was only scored with the time-sampling.

3.1.B.iv Data analysis

Data analysis was performed as described in section 2.7. One-minute time-sampling scores were, in addition, transformed into two-minute time-sampling by omitting every other reading from the one-minute time-sampling.

The sum of time spent lifting and licking in continuous rating, and the number of observations of lift and lick in time-sampling were used to determine the pain index.

Table I

Behavioural scale used in Experiments I and III. The pain-specific behavioural categories were identical for continuous rating and time-sampling. General behaviours were only scored with time-sampling.

Behavioural score	**Description of behavioural category**
Continuous rating and time-sampling Axis I: Pain-specific behaviours	
0	both hind paws rest on the floor, bearing approximately equal amounts of the rat's weight
1	the injected paw rests on the floor, but bears less weight than the uninjected paw; limping is observed during walking
2	the injected paw is held above ground, whether the animal is lying, sitting or moving around
3	the injected paw is licked, bitten or shaken
Time-sampling Axis II: General behaviours	
0	sleeping
1	lying with eyes open
2	sitting or standing still
3	walking
4	grooming
5	rearing
6	sniffing
7	licking the walls or floor

3.2 RESULTS

3.2.1 Qualitative comparisons - time courses

Time courses of pain-specific responses to different formalin concentrations were constructed from data collected by both scoring methods. Figure 3 shows the time courses plotted from continuous rating scores. Pain behaviours in each treatment group are averaged over 5-minute bins and expressed as percentage of time the behaviours were displayed. Time courses from time-sampling are plotted in Figure 4. Pain behaviours are averaged over each formalin treatment group for every 1-minute time-sampling period and are expressed as percent of the total number of observations.

Time courses for both rating methods displayed similar biphasic pain response to formalin. There is an initial period of favouring, lifting and licking in the first 5 minutes of the test (first phase). Between 5 and 10 minutes of the test (interphase), little or no pain is observed. After the interphase, pain scores increase again (second phase). The amount of lifting and licking of the injected paw in the second phase increases with formalin concentration in an apparently dose-dependent manner. The amount of favouring decreases with formalin concentration. Lifting and licking in the second phase persists longer at higher formalin concentrations. At 2 % formalin, pain scores are near-maximal from the onset of the second phase until the end of the testing period.

3.2.2 Quantitative comparisons

Behavioural responses recorded by the two rating methods were compared quantitatively by calculating correlation coefficients between them. Dose-effect relationships were constructed for first and second phase of the formalin-induced pain responses and morphine analgesia at 1% formalin. The log of formalin concentration and morphine dose were used. In the case of the latter, this is a standard procedure for analysis of receptor-mediated interactions (Ross, 1996). Dose-effect relationships obtained from both methods were superimposed on the same graphs, and curve parameters (MPE_{50} and slope) were compared.

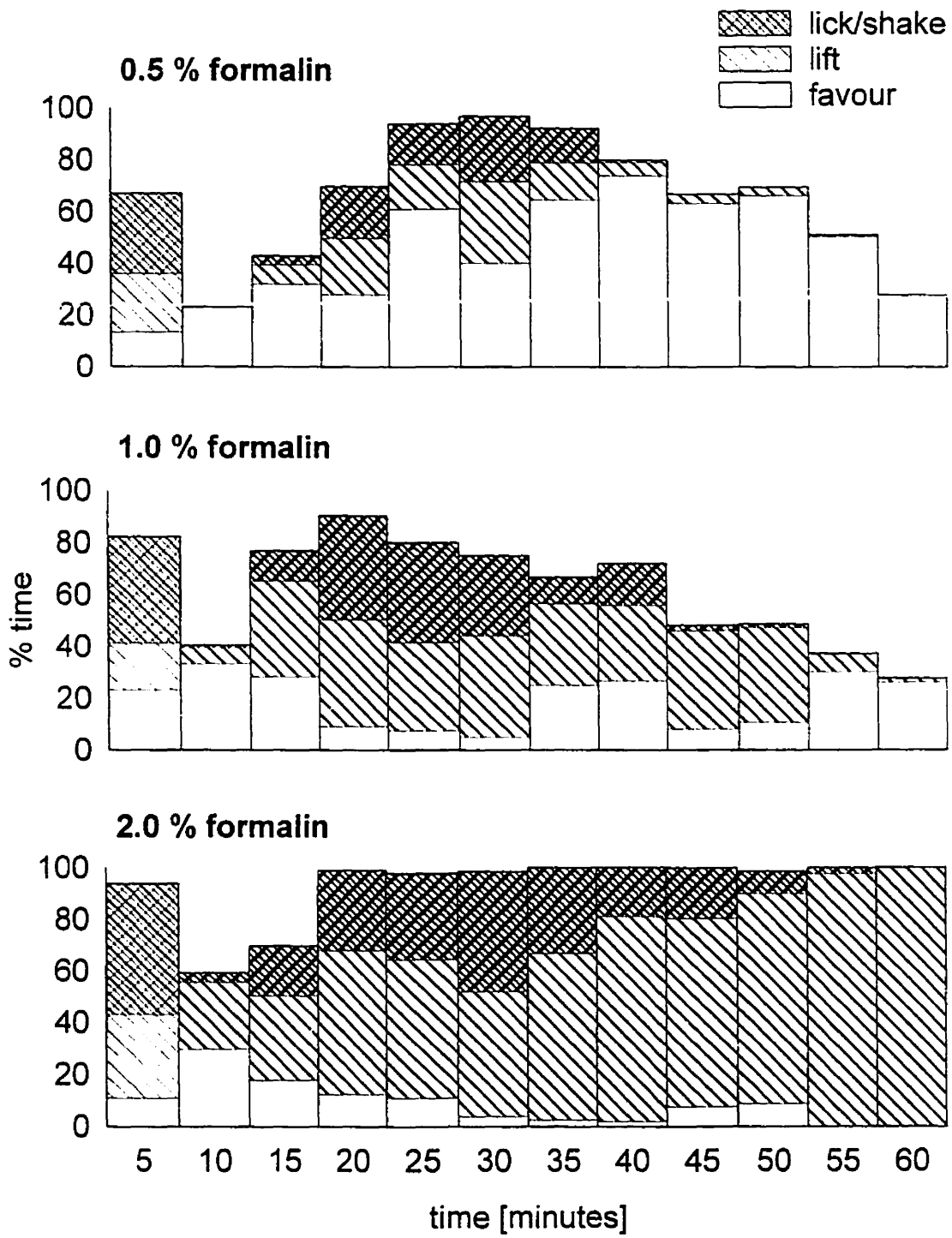

Figure 3

Time courses of formalin-induced pain-specific behaviours constructed from continuous rating data. Responses are averaged over five-minute bins.

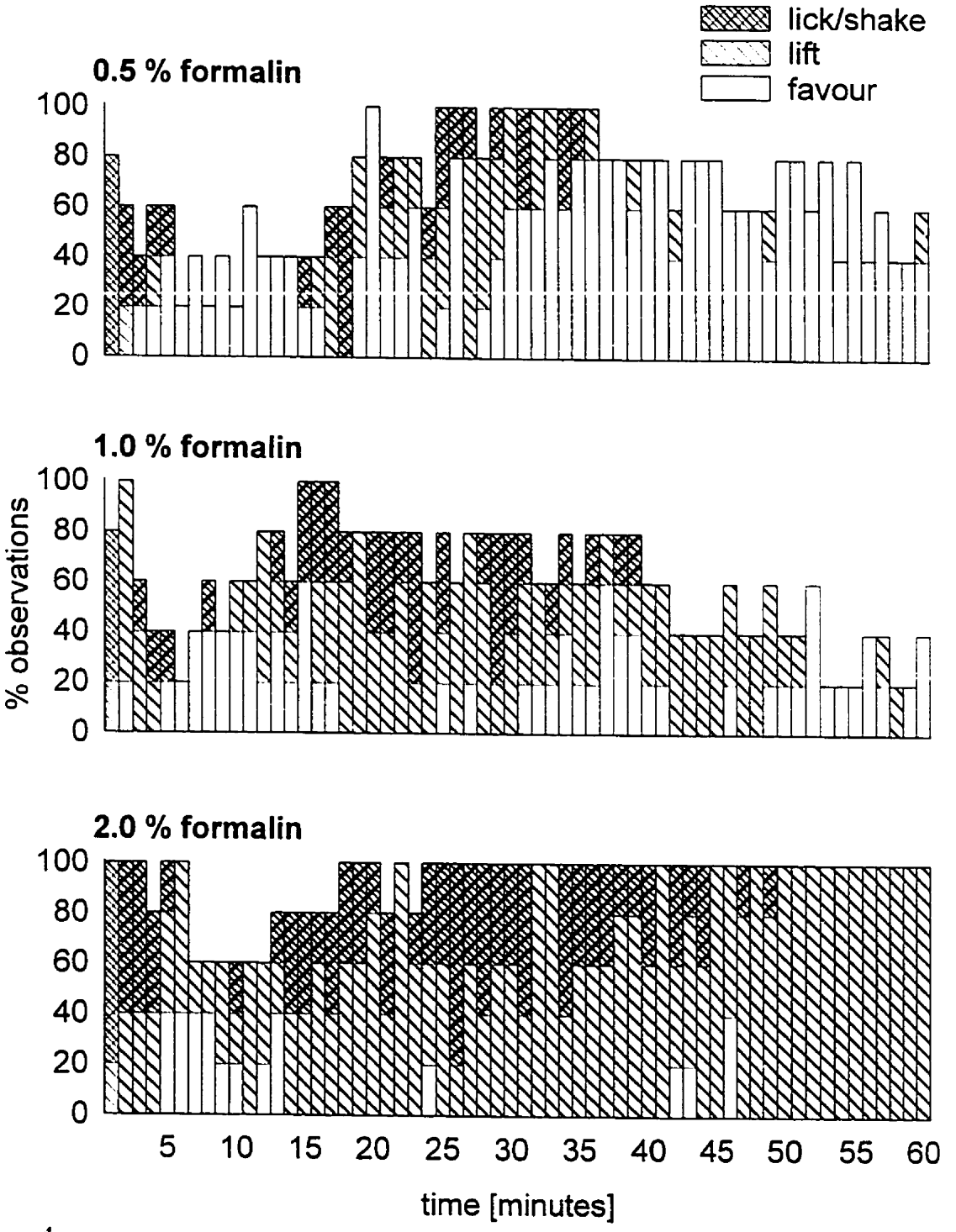

Figure 4

Time courses formalin-induced pain-specific behaviours constructed from one-minute time-sampling data. Responses are averaged over one-minute bins.

3.2.2.i Bivariate correlations of behavioural scores

Pain responses, summed over the first phase and the interphase (1 to 10 minutes) and the second phase (11 to 60 minutes) of the formalin test, were compared by computing bivariate correlation coefficients between the continuous rating scores and the time-sampling every minute. Pearson's correlation coefficients are listed in Table II. Favouring, lifting and licking responses in both phases of the formalin test were significantly positively correlated for the time-sampling and continuous rating ($p<0.01$). Two-minute time-sampling scores of favour, lift and lick, shake or bite, are also shown in Table II. They were also found to correlate significantly with the continuous rating scores ($p\leq0.01$).

The correlations between pain indices (the sum of lifting and licking, shaking or biting) determined by the continuous rating and both one- and two-minute time-sampling were also highly significant ($p<0.01$; Table II). The pain indices determined by all scoring methods also correlated highly with the logarithm of formalin dose ($p<0.05$ for first phase and $p<0.001$ for second phase). They are shown in Table III. The pain scores determined from all scoring methods explained more than 50 % of variance in the second phase ($R = 0.84$). The correlation between pain scores and log of formalin dose did not decrease when behaviour was rated at regular time intervals as compared to continuous rating.

Table II

Pearson's correlation coefficients between pain-specific behaviours of the formalin concentration-effect relationship as scored by the continuous rating method and time-sampling at 1 and 2 minute intervals.

behavioural category	phase of formalin test	**Pearson's correlation coefficients (two-tailed test of significance)**	
		Continuous Rating vs. Time Sampling at 1 minute intervals	Continuous Rating vs. Time Sampling at 2 minute intervals
Favour	phase I	0.76**	0.82**
	phase II	0.99**	0.99**
Lift	phase I	0.98**	0.97**
	phase II	0.88**	0.68**
Lick	phase I	0.98**	0.88**
	phase II	0.96**	0.89**
Pain [1]	phase I	1.00**	0.99**
	phase II	1.00**	0.93**

** Correlation is significant at the 0.01 level (two tailed).

[1] Pain is calculated as the sum of time spent lifting and licking for continuous rating, and as the sum of observations of lift and lick for time sampling

Table III.

Pearson's correlation coefficients between pain indices determined from continuous rating and time-sampling every minute and every two minutes, and log of formalin dose. Correlations of pain indices explaining more that 50% of variance ($R > 0.71$) are bolded.

	Continuous rating	Time-sampling	
		every minute	every two minutes
Phase I	0.52*	0.63*	0.58*
Phase II	**0.84****	**0.84****	**0.84****

* indicates significance at the 0.05 level

** indicates significance at the 0.01 level

3.2.2.ii Dose-effect relationship parameters

Figure 5 shows concentration-effect relationships for %MPE of formalin-induced pain (first and second phase) and dose-effect relationship for morphine analgesia at 1% formalin (second phase only). Each dose-effect relationship was plotted as determined from continuous rating and one-minute time-sampling. Formalin and morphine doses are plotted on a logarithmic scale. In this analysis the sum of lift and lick, bite or shake was taken as the measure of pain.

Slope and MPE_{50} values of the formalin concentration-effect relationship are listed in Table IV. MPE_{50} values for the first phase and second phase of the formalin test are virtually identical for continuous rating and time sampling. The concentration of formalin that produced a half-maximal response in the first phase was approximately 1.3%, and in the second phase approximately 1.0%. Slope values obtained by both methods are also not significantly different, particularly for the second phase of the test. The second phase slope is, however, significantly steeper than that of the first phase (approximately 29 for first phase and approximately 53 for second phase). The 95% confidence intervals of MPE_{50}'s from both rating methods are almost identical. Similarly, the standard errors of the means of slopes for both phases are very similar in value. This suggests that there is no loss of statistical power when behaviour is sampled at 1-minute intervals instead of continuous

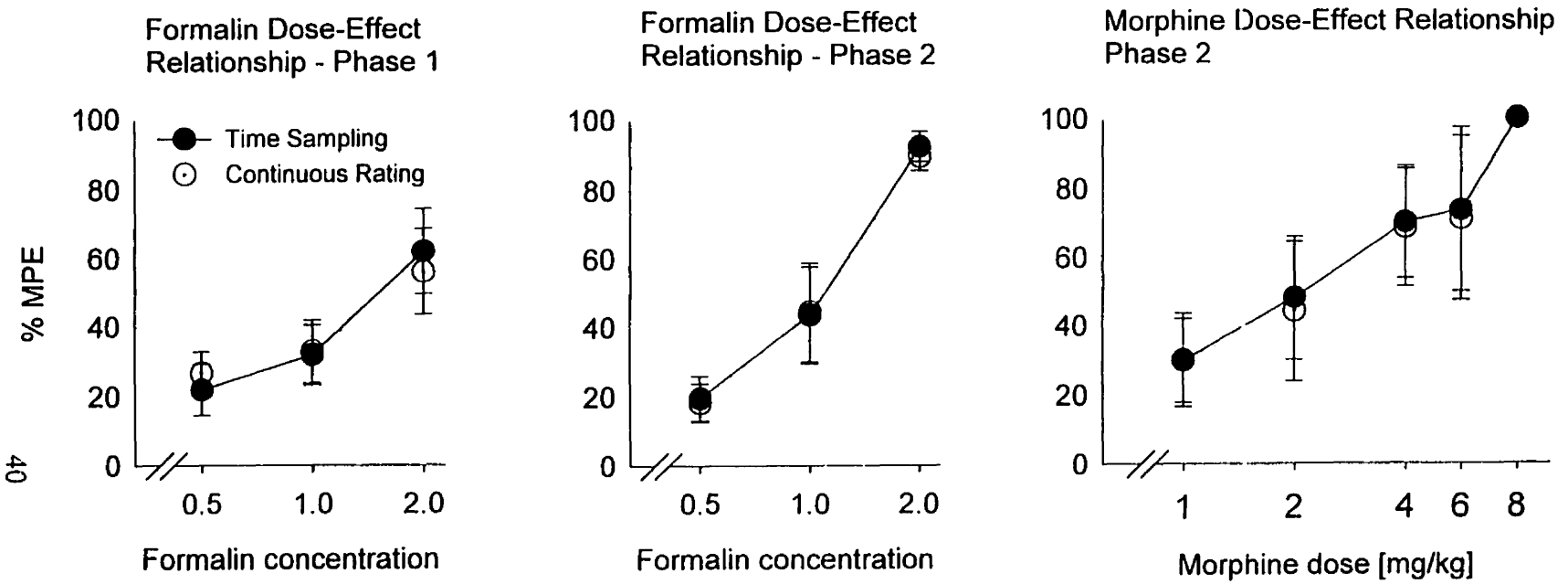

Figure 5

Formalin concentration-effect relationships for the first and second phase of the formalin test, and morphine dose-effect relationship for the second phase of the test, constructed from continuous rating and one-minute time-sampling data. The sum of lifting and licking, biting or shaking the injected paw was taken as the index of pain.

Table IV

MPE_{50} and slope values of formalin concentration-effect relationships obtained by continuous rating and one- and two-minute time-sampling. The sum of lift and lick, bite or shake was used as the index of pain.

		Continuous Rating	Time Sampling	
			every minute	every two minutes
Phase I	**MPE_{50}**[1]	**1.30**	**1.33**	**1.34**
	95% confidence interval	(0.95 - 4.83)	(0.65 - 2.71)	(0.58 - 3.09)
	SLOPE	**21.20**	**28.99**	**28.99**
	standard error of mean	10.54	10.98	12.65
Phase II	**MPE_{50}**[1]	**0.98**	**0.96**	**0.97**
	95% confidence interval	(0.79 - 1.22)	(0.77 - 1.20)	(0.78 - 1.19)
	SLOPE	**51.68**	**52.75**	**54.49**
	standard error of mean	5.42	6.24	6.43

[1] MPE_{50} is expressed in % formalin.

rating. Table IV also shows MPE_{50} and slope values for the formalin concentration-effect relationships determined from 2-minute time-sampling. The MPE_{50}'s and slopes are virtually identical to those obtained from continuous rating and one-minute time-sampling.

The morphine concentration-effect relationships obtained by continuous rating and time-sampling every minute and every two minutes are also almost identical. MPE_{50} and slope values are listed in Table V. 95% confidence intervals are smaller in time-sampling, and the standard errors of means indicate that the slopes constructed from time-sampling data have a tighter fit. No loss of statistical power was observed using the time-sampling

method. The morphine dose that produced 50% analgesia (using 1% formalin) was approximately 2.1 mg kg. The slope of the dose-effect relationship was approximately 30.

Table V.

MPE_{50} and slope values of morphine dose-effect relationships at 1% formalin concentration, obtained by continuous rating and one- and two-minute time-sampling. The sum of lift and lick, bite or shake was taken as the index of pain.

	Continuous Rating	Time Sampling	
		one-minute intervals	two-minute intervals
MPE_{50}[1]	**2.22**	**2.10**	**2.08**
95% confidence interval	(1.29 - 3.84)	(1.27 - 3.49)	(1.21 - 3.58)
SLOPE	**30.79**	**30.89**	**29.73**
standard error of mean	8.16	7.51	7.71

[1] MPE_{50} is expressed in mg kg.

3.2.3 Effect of formalin on general behaviour

The time-courses of general behaviour of untreated rats (they received no formalin, but were habituated to the testing environment) and those that received 1 and 2% formalin, are plotted in Figure 6. Behaviours considered 'inactive' (sleeping, lying down with eyes open and sitting or standing still) appear at the bottom of the stacked bars, while 'active' behaviours (walking, grooming, sniffing and rearing) are plotted above the inactive behaviours. Untreated rats (upper panel) displayed a cyclical pattern of activity. They were more active in the beginning, middle and towards the end of the 1-hour session. Rats treated with formalin did not display this regular pattern of activity. In addition, formalin appeared to depress activity. Particularly in the second half of the session, the 1% formalin rats tend

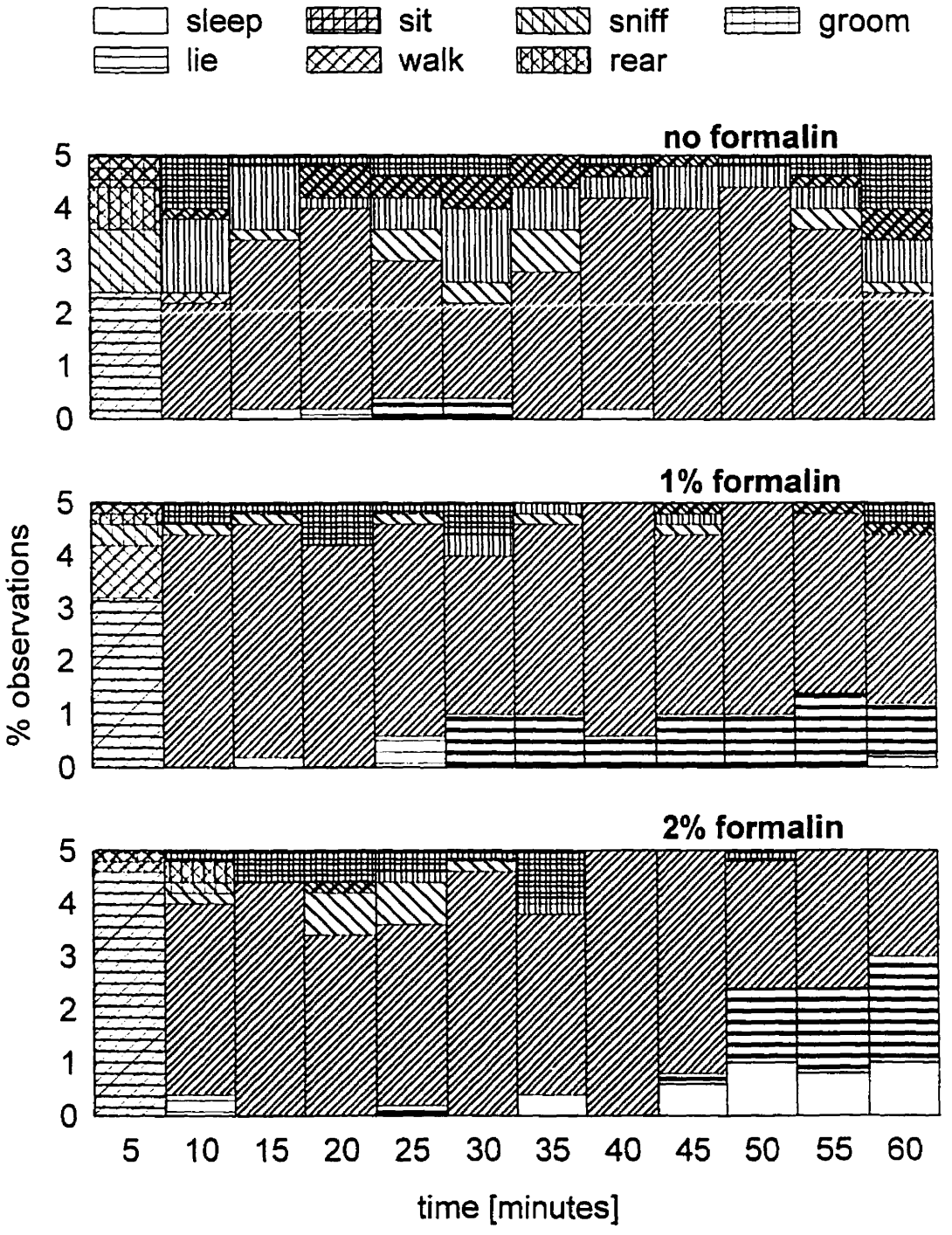

Figure 6

Time-courses of general behaviours observed in no-formalin controls and 1 and 2% formalin-treated rats. The responses are averaged over five-minute bins.

to lie down, and the 2% rats to lie down or sleep. The latter behaviours did not occur in rats not treated with formalin.

The active and inactive behaviours are complementary since they add up to the total number of observations. This allows that only one variable is analysed. For the purpose of the analysis of general activity, the 60 minute session was divided into first phase (1-10minutes), and second phase (11-60 minutes). The second phase was further divided into phase 2A (11-35 minutes) and phase 2B (36-60 minutes).

Pain-specific behaviours and general behaviours for each phase are plotted in Figure 7, pain responses in the upper and general behaviours in the lower panel. In the latter, active behaviours are plotted above the abscissa and inactive behaviours below the abscissa. A repeated measures ANOVA was used for the analysis, with formalin dose as the between-subject factor, and phase as the within-subject measure. The interaction of phase and formalin dose was not significant. There was a significant difference in activity across the three phases ($p<0.001$). The between-subject factor (formalin dose) was also significant ($p<0.01$). Least significant difference (LSD) post-hoc analyses revealed that in the first phase formalin dose-dependently reduced activity. While the 0.5% formalin group was not significantly less active than controls ($p=0.095$), the 1 and 2% groups were ($p<0.05$ and $p<0.01$, respectively). In phase 2A there were no significant differences between the groups. In phase 2B, the 0.5, 1 and 2% formalin groups were significantly less active than controls ($p<0.001$ for all groups vs. controls).

In all three phases the explorative behaviours were depressed dose-dependently (p's ranging from 0.09 to 0.02 for 0.5% formalin vs. controls, and p's ranging from 0.01 to 0.001 for 2% vs. controls). Grooming was only significantly depressed in phase 2B ($p<0.001$ for all formalin-treated groups vs. controls). Of the inactive behaviours, only sitting was dose-dependently depressed in first phase ($p=0.11$ for 0.5% vs. controls, $p=0.012$ for 1% formalin vs. controls, and $p=0.004$ for 2% formalin vs. controls).

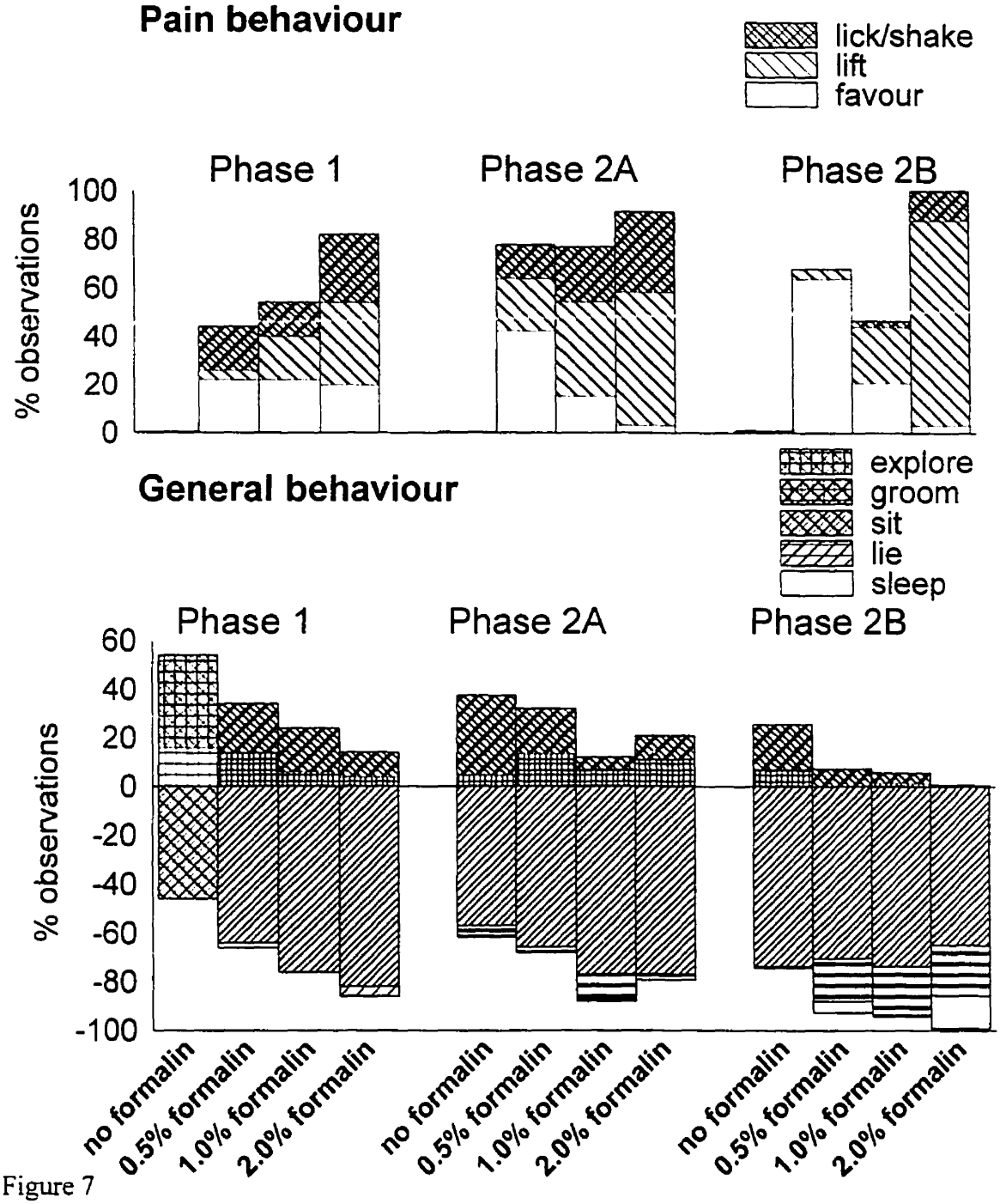

Figure 7

Formalin-induced pain-specific (upper panel) and general behaviours (lower panel) in the first (0 to 10 minutes) and second phase (11 to 35 and 36 to 60 minutes postformalin; phase 2A and 2B, respectively) of the test. Inactive general behaviours are plotted below the abscissa.

3.2.4 Effect of morphine on general behaviour

The activity in rats receiving different morphine doses (1, 2, 4, 6 and 8 mg kg) and a single formalin concentration (1%) was compared to uninjected habituated controls and rats receiving only 1% formalin. Figure 8, lower panel, shows the behavioural state of all groups, with active behaviours (walking, grooming, sniffing, rearing and licking floor) stacked above the abscissa, and inactive behaviours (lying down, sleeping and sitting or standing still) below the abscissa. The upper panel shows the pain-specific behaviours of the corresponding treatment groups.

ANOVA was used to analyse general behaviours. The effect of morphine was significant ($p<0.001$). Post-hoc (LSD) comparisons indicated that injection of 1% formalin significantly depressed activity compared to uninjected controls ($p<0.05$). This depression of activity was reversed by lower doses of morphine (1 and 2 mg kg). These groups were not significantly different from uninjected controls. Groups that received high doses of morphine (4, 6 and 8 mg kg) were significantly less active than uninjected controls. The 2 mg kg morphine group was significantly more active than the 1% formalin group that received no morphine.

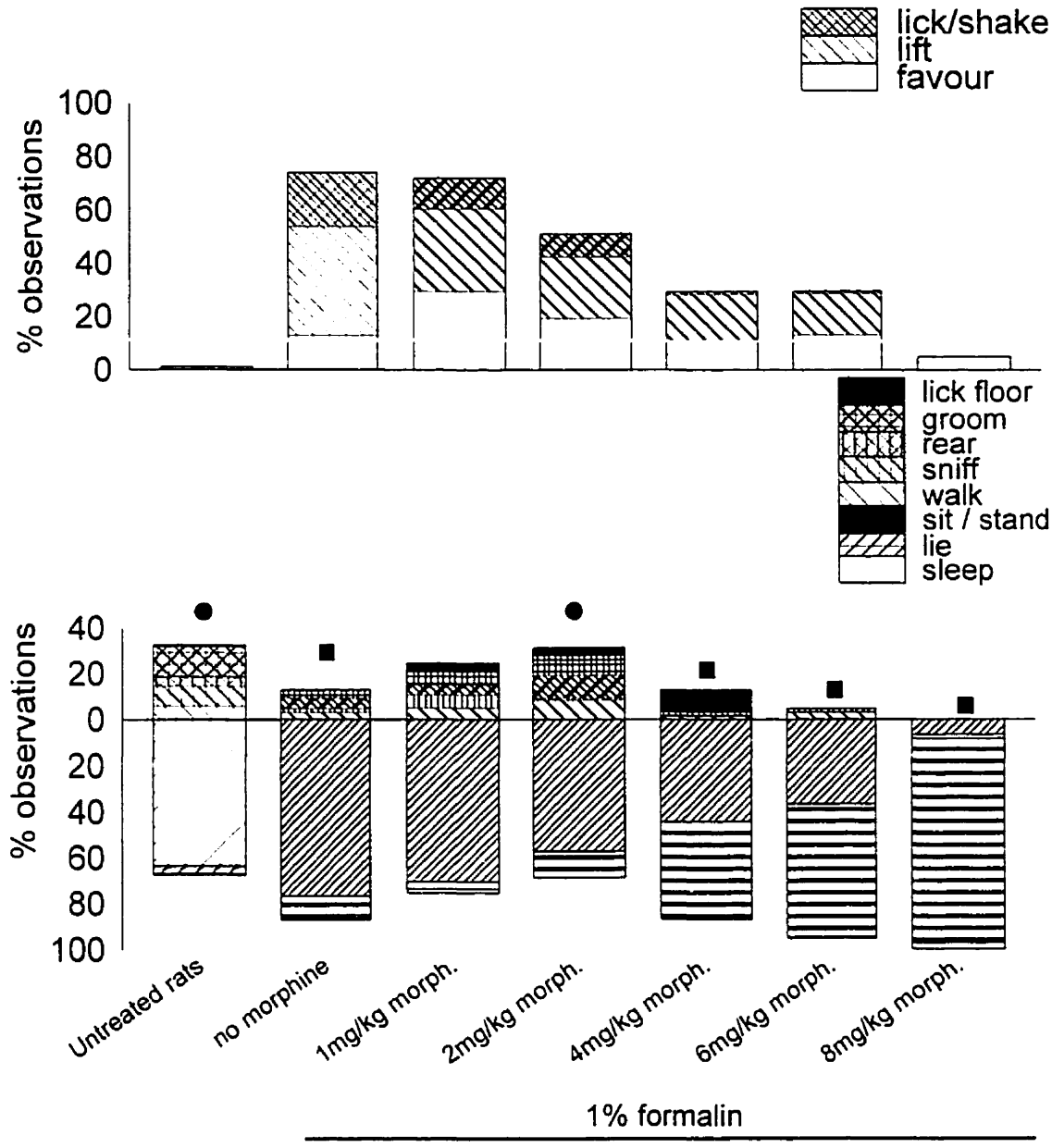

Figure 8

Effect of 1% formalin and different doses of morphine on pain-specific (upper panel) and general behaviours (lower panel) in the second phase of the formalin test. Inactive general responses are plotted below the abscissa. Square indicates that the mean activity of a group was significantly depressed as compared to untreated rats (first column). Circle indicates that the mean activity was significantly higher than that of the 1% formalin group.

Chapter 4

ANALYSIS OF FORMALIN-INDUCED BEHAVIOURS

4.1 Objectives

The concentration of formalin used in the literature varies greatly, as do the behavioural indices used to determine the level of pain. No study has, however, examined all pain-specific behavioural measures over the wide range of formalin concentrations that are routinely administered. This study examines all pain-specific, as well as general behaviours, over the range of formalin concentrations most commonly employed (1 to 10%). In addition, in the literature there is no information on behaviours after the acute pain response has ended. Therefore, the time course of formalin-induced behaviours was recorded for a prolonged period of time (16 hours), to determine if any residual pain or disruption of normal behaviour occurs beyond the time most studies terminate the observation period (60 minutes).

Habituation of animals to the observers and the testing apparatus is not frequently performed. This study also addressed the effects of habituation to the testing environment on formalin induced behaviours, to determine whether stress of novel environment in non-habituated subjects produces significant stress-induced analgesia.

4.2 Experimental design

48 subjects were randomly assigned to two groups, 24 rats in each. One group was subjected to habituation prior to testing, and the other was not handled until the testing day. From each of the two groups, rats were assigned to five treatment groups: control (no formalin), and 1%, 2%, 5% and 10% formalin (five subjects per group for no-formalin controls, 1, 2 and 5% formalin, and 4 in the 10% formalin group). Behavioural scoring commenced at the time of formalin injection and lasted 16 hours. Responses were sampled every two minutes. The behavioural categories used are described in Table VI.

Table VI

Behavioural scale used in Experiment II. Pain specific behaviours include all behaviours used for determining pain indices in scientific reports employing the formalin test. The scale of general behaviour was adapted for testing with higher formalin concentrations and for longer periods of testing.

Behavioural score	**Description of behavioural category**
Axis I: Pain-specific behaviours	
0	both hind paws rest on the floor, bearing approximately equal amount of the rat's weight
1	the injected paw rests on the floor, but bears less weight than the uninjected paw; limping is observed during walking
2	the injected paw is held above ground, whether the animal is lying, sitting or moving around
3	the injected paw is licked or bitten
4	the injected paw is shaken
5	flinching of the injected paw (the paw is vibrated rapidly)
6	whole-body or hindquarter flinching (WBF)
Axis II: General behaviour	
0	sleeping
1	lying with eyes open
2	sitting or standing still
3	walking
4	grooming
5	rearing
6	sniffing
7	licking the walls or floor

Behavioural score	Description of behavioural category
8	backwards locomotion: this behaviour is associated with high levels of pain
9	eating or drinking
Axis III: morbidity or agitation	
0	no morbidity or agitation
1	morbidity (ptosis, hunch and or piloerection)
2	agitation

The behavioural scale was similar to the one used in experiment one. It was, however, altered such that all pain-specific behaviours used in the literature were scored as mutually exclusive behavioural categories (favour, lift, lick or bite, shake, flinch and whole-body flinch (WBF)). Two additional categories were added to the general behavioural scale: backwards locomotion (BLM), which has been observed when higher formalin concentrations were administered (F.V. Abbott, unpublished observations), and eating or drinking. A third axis was introduced to describe the observer's subjective impression of the rats' behaviour. A morbidity score was assigned if the rat displayed ptosis, hunched posture and/or piloerection, in other words, appeared 'sick'. If the animal rapidly changed posture, appeared restless or agitated by the pain, frantically licked or bit the injected paw, an agitation score was recorded.

Up to ten subjects were tested simultaneously. Food and water were provided to the rats during the testing. Water bottles were mounted on the outside of the formalin boxes with the spout protruding through the wall of the formalin box. Pre-weighed food pellets were provided one hour before the time lights turned off daily in the colony room (7pm).

To obtain statistical validation of results, this experiment was treated as a two-way one-repeated-measure ANOVA with formalin dose and habituation as the between-subject factors and phase of formalin test as the repeated measure. Correlational analysis was used to determine the relationship between the natural logarithm of formalin dose and the individual behavioural measures, as well as their combinations and weighted means scores.

4.3 Results

4.3.1 Pain-specific behaviours

Behavioural responses of favour, lift, lick or bite, shake or flinch, and WBF were plotted on a time-course for each treatment group. Individual pain-specific behaviours, as well as combinations of them and weighted-means scores, were examined to find the best predictor of the log of formalin concentration. The pain measure that performed best as a predictor of the log of formalin concentration was then applied to determine the dose-effect relationships of formalin in habituated and unhabituated animals. Finally, the effect of habituation on formalin-induced pain was investigated.

4.3.1.A Time course of pain-specific behaviours

Time courses of pain responses that occurred during the first two hours after formalin injection are shown in Figure 9 for unhabituated rats and in Figure 10 for habituated rats. The behavioural responses in each treatment group were averaged for each time-sampling bin (2 minutes). Time plots of habituated and unhabituated no-formalin controls are not shown since virtually no pain-specific behaviours occurred.

Inspection of Figures 9 and 10 indicated that all treatment groups displayed a bi-phasic response to formalin. In all treatment groups the first phase occurred within the first 6 minutes after formalin. The second phase began as early as 8 minutes into the test, after a brief interphase. In both habituated and unhabituated animals, the amount of pain behaviours shown in the first hour increased up to 5%, where it was near-maximal and did not increase further at 10% formalin. In the same time-period, the frequency of favouring decreased, while the frequency of other pain behaviours increased with formalin concentration. The duration of the pain responses in the second phase became longer with increasing formalin concentration. In habituated and unhabituated animals, an injection of 1% formalin resulted in pain responses that terminated almost entirely within 60 minutes after injection. Rats receiving 10% formalin showed almost continuous pain behaviours until approximately 90 minutes after the injection.

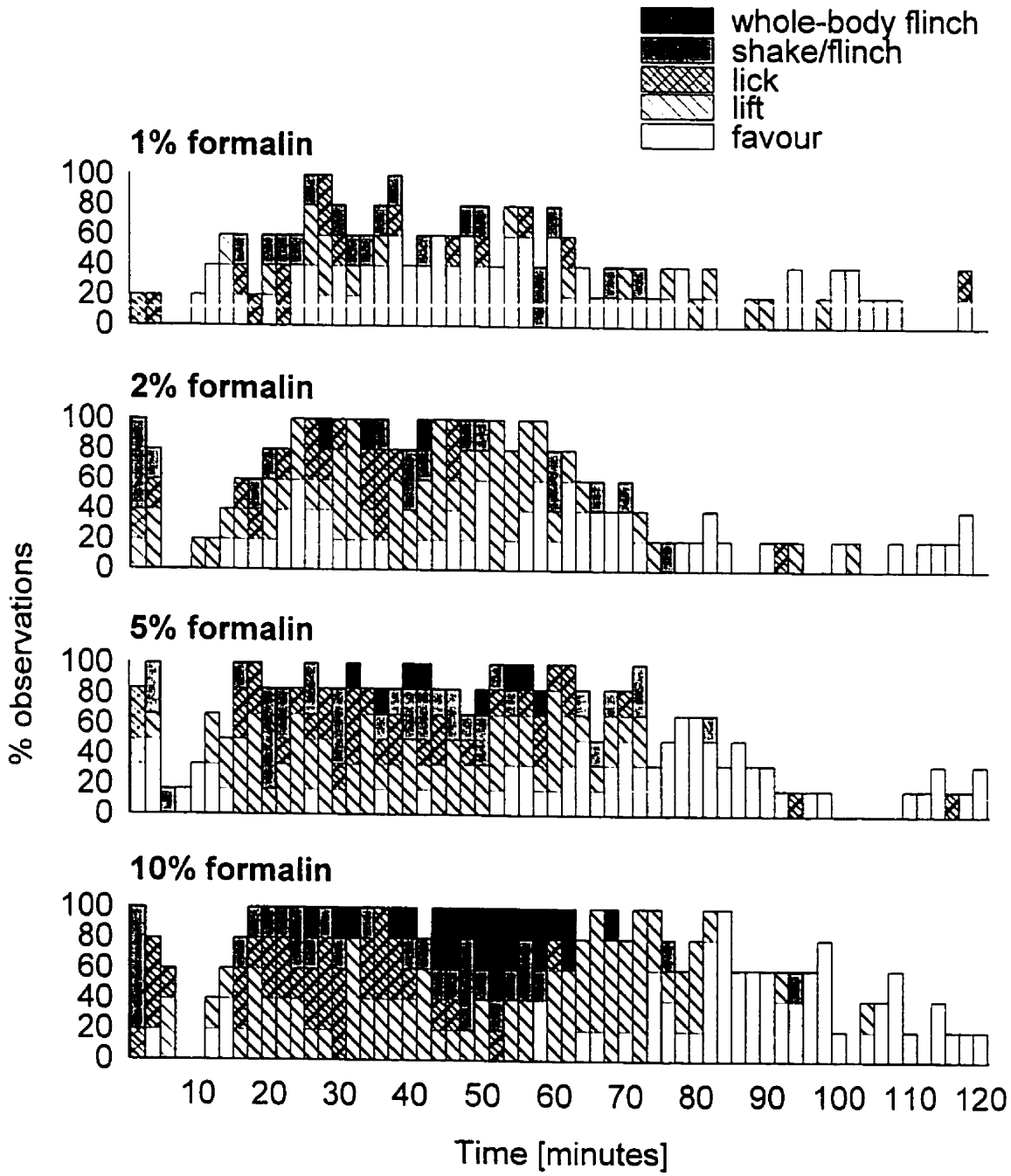

Figure 9

Time-courses of formalin-induced pain-specific responses of unhabituated animals during the first two hours after formalin injection. Behaviours in each treatment group are averaged over two-minute observation bins. Responses of the no-formalin group are not shown since virtually no pain behaviours occurred.

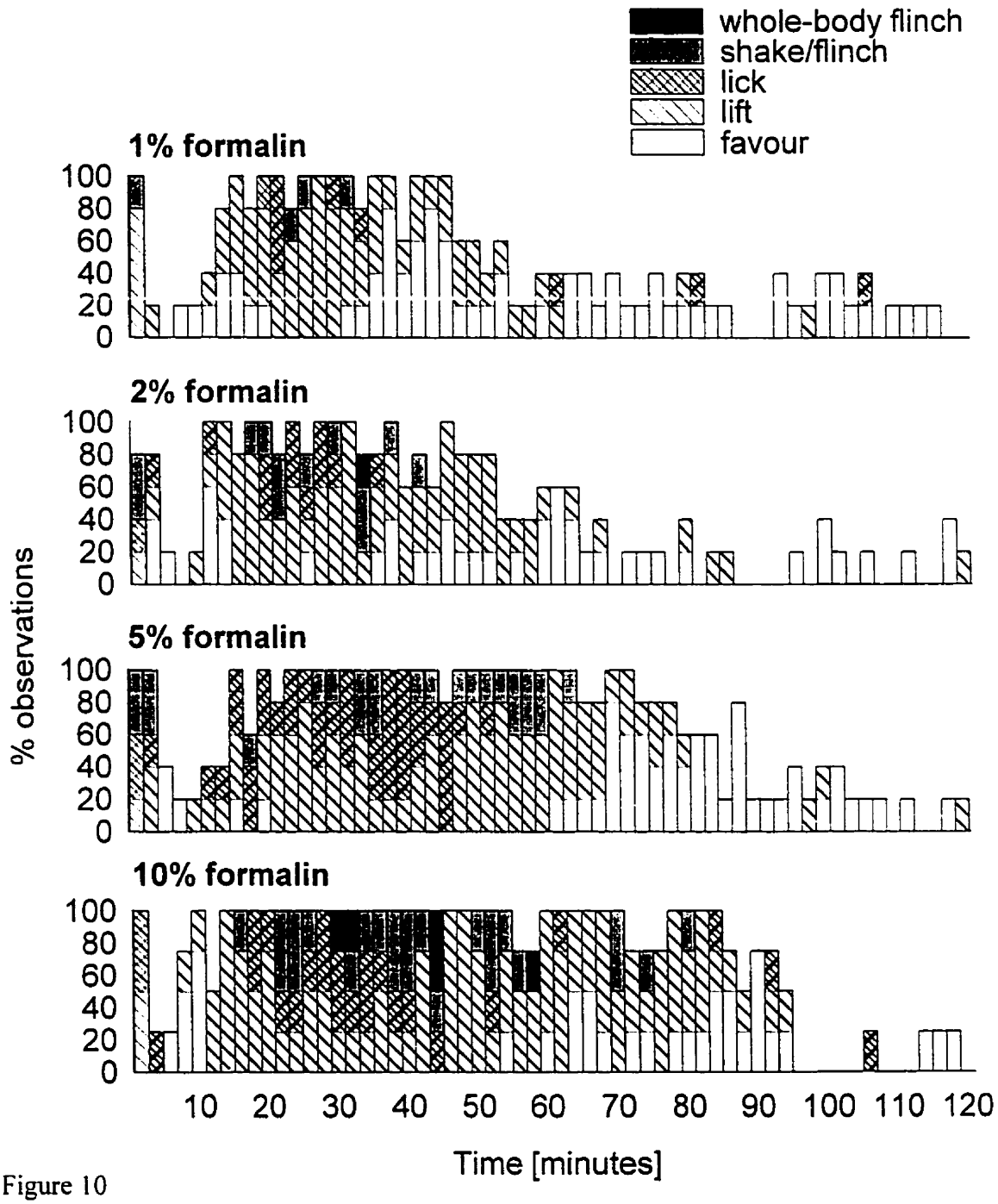

Figure 10

Time-courses of formalin-induced pain-specific responses of habituated animals during the first two hours after formalin injection. Behaviours in each treatment group are averaged over two-minute observation bins. Responses of the no-formalin group are not shown since virtually no pain behaviours occurred.

Figures 11 and 12 show time courses of pain responses for unhabituated and habituated rats, respectively, for the entire 16-hour session. Here pain responses were averaged over 30-minute bins for each treatment group. High levels of pain occurred in the first hour of the test in all treatment groups. Rats receiving higher formalin concentrations (5 and 10%) also showed pain behaviours in the third half-hour. Little pain occurred after 90 minutes postformalin, even at 10% formalin. Occasional favouring and lifting was observed in all treatment groups during the third phase of the test (2-16 hours after formalin injection). This residual pain appeared to increase with formalin concentration.

For the purpose of further analyses, the first phase of the formalin session was defined as 0 to 6 minutes postformalin, the second phase as 8 to 120 minutes, and the third phase as the remaining time of the 16-hour session (2-16 hours).

4.3.1.B Analysis of pain measures

Formalin-induced pain responses of habituated and unhabituated animals in the three phases are shown in Figure 13. Shaking and flinching are plotted as a single response category (*vide infra*).

Pearson's bivariate coefficients of correlation between pain-specific responses and log of formalin concentration were computed for each phase of the formalin test (first, second and third), for unhabituated and habituated rats separately. Correlations were also computed for the period between 10 and 60 minutes after formalin injection, which is the time period most commonly defined as the second phase in the literature (Phase 2[1]). Table VII shows correlation coefficients between the log of formalin dose and single pain-specific behaviours, their combinations and weighted means scores. To consider a pain response or composite pain measure a good predictor of the logarithm of formalin dose, the correlation coefficient was required to be equal to or greater than 0.71, such that at least 50% of the variance was explained by the pain measure.

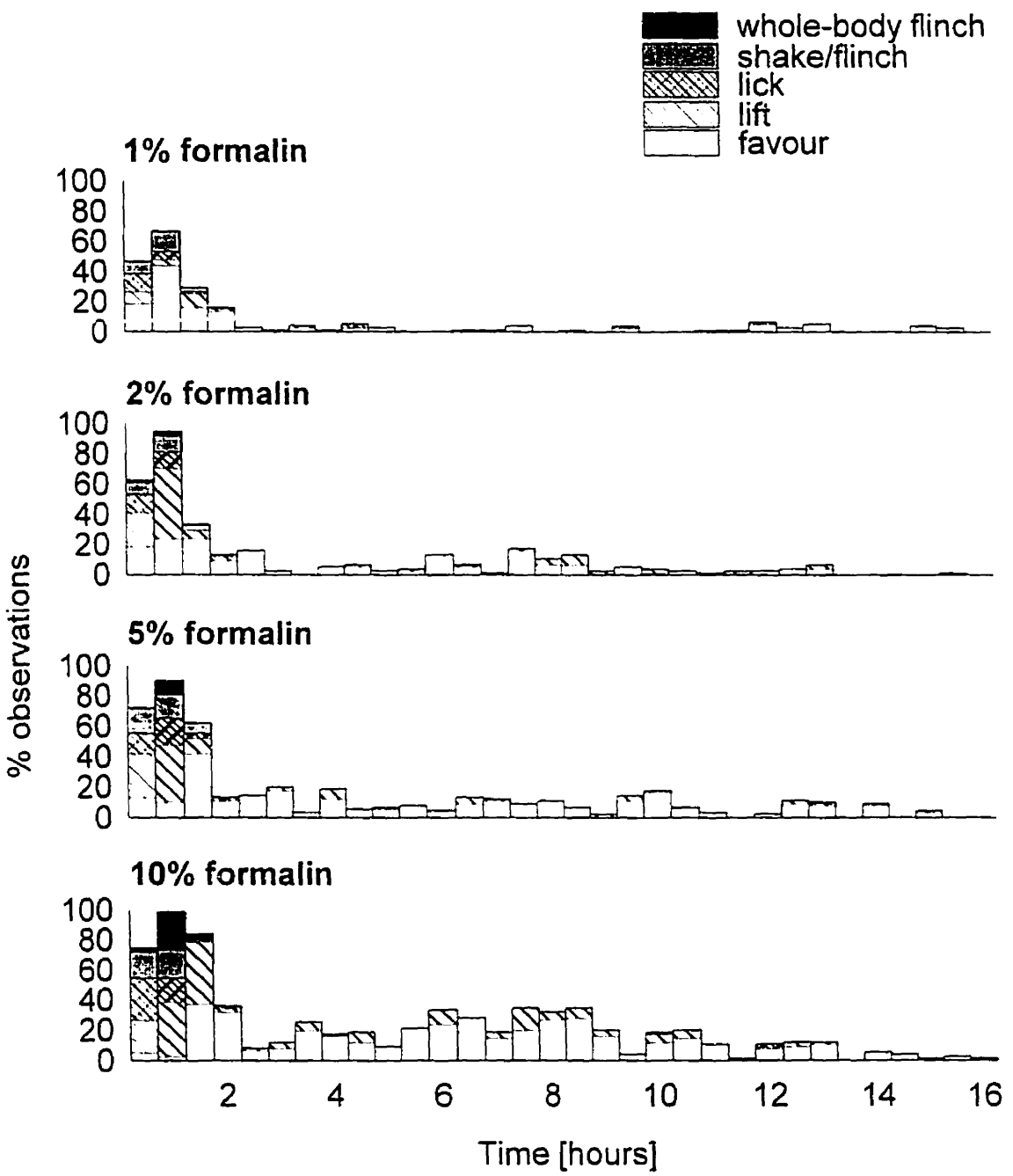

Figure 11

Time-course of pain responses for unhabituated animals during 16 hours after formalin administration. Pain scores are averaged over 30-minute time periods. Responses of the no-formalin group are not shown.

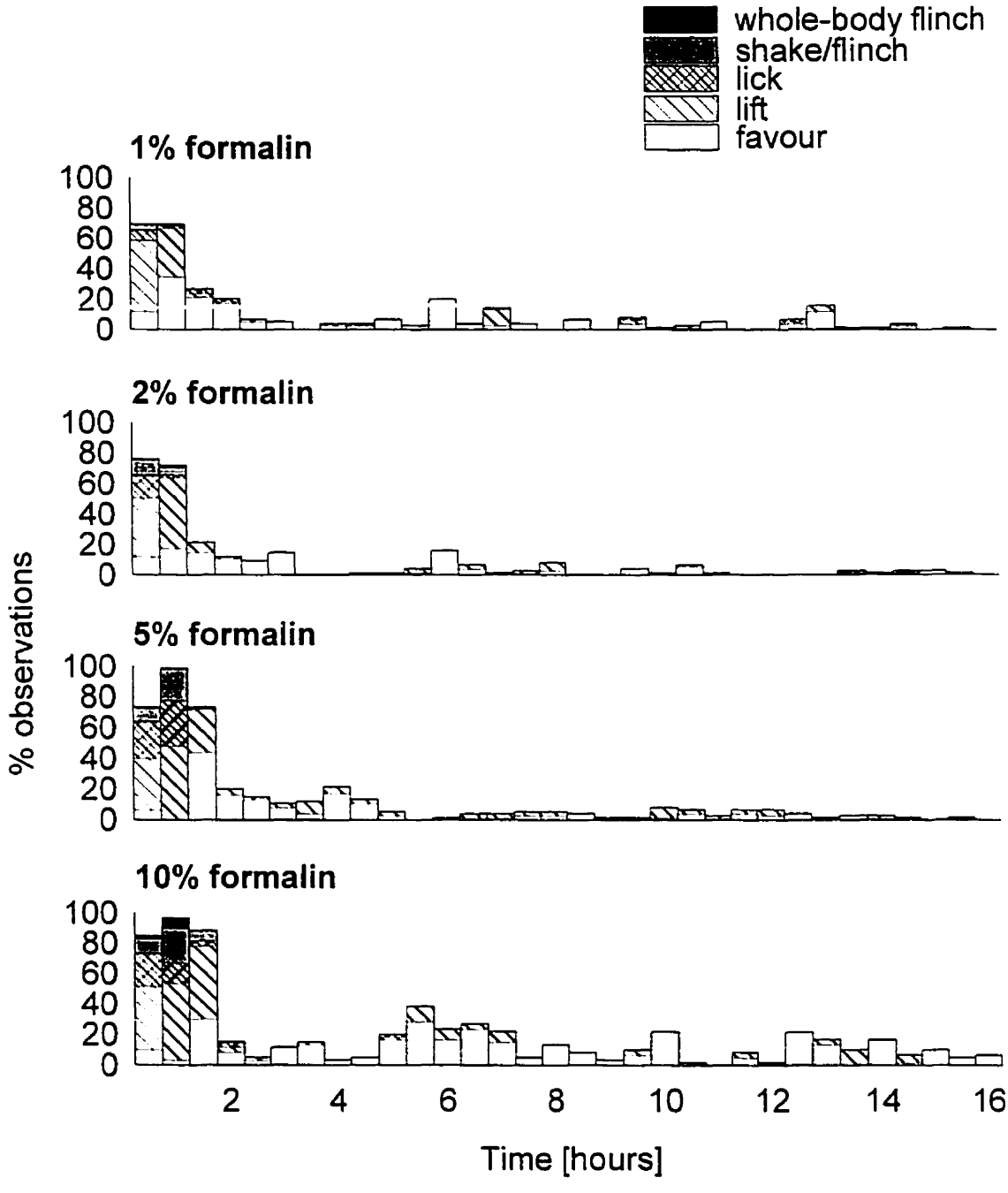

Figure 12

Time-course of pain responses for habituated animals during 16 hours after formalin administration. Pain scores are averaged over 30-minute time periods. Responses of the no-formalin group are not shown.

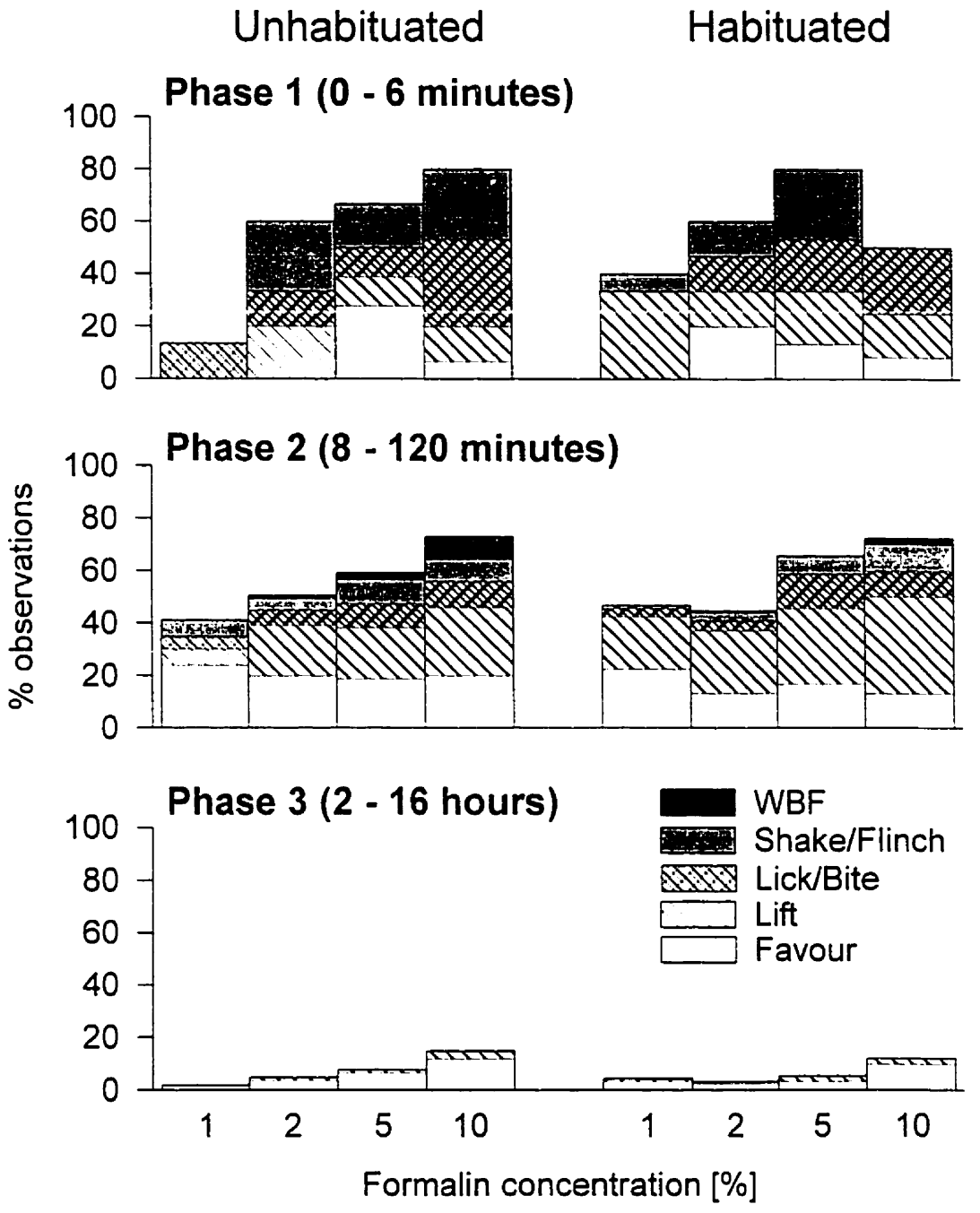

Figure 13

Pain-specific responses summed over the three phases of the formalin test, for unhabituated and habituated rats.

Table VII.

Pearson's correlation coefficients of pain measures vs. natural logarithm of formalin concentration in the three phases of the formalin test. Correlations are shown also for phase 2[1]. Correlations explaining at least 50% of the variance are bolded ($R > 0.71$).

Pain measure	**Unhabituated**				**Habituated**			
	Phase 1	Phase 2		Phase3	Phase 1	Phase 2		Phase 3
Time period[min]:	0-6	10-60	8-120	122-960	0-6	10-60	8-120	122-960
Single measures								
Favour	0.34	-0.70**	-0.19	**0.71****	0.14	-0.68**	-0.30	0.54*
Lift	0.20	0.41	0.58**	0.62**	-0.29	0.11	0.52*	0.46*
Lick	0.24	0.43	0.42	-0.03	0.40	0.60**	0.64**	-0.35
Shake	0.43*	0.04	0.05	0.29	0.02	0.52*	0.56*	-0.30
Flinch	-	0.35	0.37	-	-	0.63**	0.70**	-0.11
WBF[b]	-	0.64**	0.64**	0.29	-	0.53*	0.53*	-0.11
Composite measures								
Shake + Flinch	0.43*	0.26	0.26	0.29	0.02	0.63**	0.66**	-0.32
Shake + WBF[b]	0.43*	0.59**	0.58**	0.43	0.02	0.60**	0.61**	-0.24

* indicates significance of correlation coefficient at the $p < 0.05$ level

** indicates significance of correlation at the $p < 0.01$ level

[b] whole-body flinching

Table VII. (continued)

Pain measure	Unhabituated				Habituated			
	Phase 1	Phase 2		Phase3	Phase 1	Phase 2		Phase 3
Time period [min]:	0-6	10-60	8-120	122-960	0-6	10-60	8-120	122-960
Flinch + WBF[b]	-	0.59**	0.63**	0.29	-	0.68**	**0.73****	-0.16
Shake +Flinch+WBF[b]	0.43*	0.55**	0.56**	0.43	0.02	0.67**	0.69**	-0.28
Favour + Lick	0.35	-0.25	0.43	**0.76****	-0.11	-0.52	0.35	0.60**
Lift + Lick	0.35	0.59**	0.69**	0.61**	0.26	0.43	**0.71****	0.43
Lick + SFW[@]	0.50*	0.65**	0.65**	0.29	0.37	**0.80****	**0.81****	-0.42
Lift+Lick+SFW[@]	0.54*	**0.75****	**0.77****	0.62**	0.24	0.67**	**0.81****	0.40
Favour+Lift+Lick	0.51*	0.04	0.59**	**0.75****	0.24	-0.03	0.61**	0.59**
Weighted means								
Lift+2*Lick/SFW[@]	0.54*	**0.76****	**0.77****	0.62**	0.33	**0.78****	**0.86****	0.34
Favour + 2*Lift + 3*Lick/SFW[@]	0.65**	**0.74****	**0.80****	**0.76****	0.33	**0.74****	**0.85****	0.57*

* indicates significance of correlation coefficient at the p < 0.05 level

** indicates significance of correlation at the p < 0.01 level

[b] whole-body flinching (WBF)

[@] refers to general shaking behaviour (shake, flinch or whole-body flinching; SFW).

Of the single behavioural responses, none performed satisfactorily as a pain measure (Table VII). Shaking, flinching and WBF during the second phase correlated highly among each other, particularly in habituated animals (r's ranging from 0.51 to 0.79). These behaviours are also less distinguishable and they were collapsed together into a single category of shaking, flinching or whole-body flinching (SFW). The latter measure performed better, however did not meet the required criteria. Adding licking to SFW improved the correlation with log of formalin concentration. In habituated animals, this measure explained more than 50% of the variance in second phase and phase 2^{+}. It correlated highly significantly with the log of formalin concentration in the second phase and phase 2^{+} in unhabituated animals ($p<0.01$), however did not explain 50% of the variance. Lifting and licking performed well only in the second phase of habituated animals. Lifting, licking and SFW performed best of all the simple-sum measures, explaining more than 50% of the variance in second phase and phase 2^{+} in unhabituated, and second phase in habituated animals. It correlated highly significantly with phase 2^{+} in habituated animals ($p<0.01$). The weighted means scores as introduced by Dubuisson & Dennis (1977; Eq. 1) and Watson et al. (1996; Eq. 2) performed equally well and explained 50% of the variance in second phase and phase 2^{+} in unhabituated and habituated animals.

4.3.1.C Formalin concentration-effect relationships and effect of habituation on pain behaviours

The sum of lifting, licking or biting, and SFW was used as the pain index in the analysis of the formalin concentration-effect relationships, and the effect of habituation on pain responses. Figures 14-16 show the concentration-effect relationships for formalin in the first phase, second phase and phase 2^{+}, and the third phase, respectively, for both habituated and unhabituated animals. Two-way repeated measures ANOVA revealed that the three-way interaction between phase, habituation and the logarithm of formalin concentration was significant ($p<0.05$). This implies that the relation between the effect of habituation at different formalin concentrations differs across the phases of the formalin response. This interaction was further investigated by analysing the interaction of formalin

dose and habituation at each level of the repeated measure (phase of formalin test). For each phase of the test, concentration-effect parameters (MPE_{50} and slope) were computed for habituated and unhabituated rats. They are shown in Table VIII.

Table VIII

Slope and MPE_{50} of formalin concentration-effect relationships for the first and second phase of the formalin test, in habituated and unhabituated animals. Concentration-effect parameters are also computed for phase 2^{L}. Values could not be obtained for the third phase due to low pain scores, and are therefore not shown.

		MPE_{50} (upper and lower limits)	**Slope (SEM of slope)**
Phase 1 (excluding 10% formalin groups)	unhabituated	3.55 (0.56 - 21.95)	12.94 (9.83)
	habituated	2.33 (1.34 - 4.04)	17.24 (4.42)
Phase 2^{L} (10-60 minutes)	unhabituated	1.83 (1.19 - 2.81)	25.00 (3.83)
	habituated	1.05 (0.34 - 3.24)	20.30 (5.77)
Phase 2 (8 - 120 minutes)	unhabituated	8.38 (6.00 - 11.71)	15.02 (2.26)
	habituated	5.56 (3.90 - 6.84)	15.69 (3.12)

4.3.1.C.i First phase (0-6 minutes)

The formalin concentration-effect relationships for the first phase of the test can be observed in Figure 14. The main effect of formalin was highly significant ($p<0.001$). Post Hoc comparisons revealed that all formalin treatments produced significant levels of pain as compared to no-formalin controls ($p<0.01$). Significant increases in pain responses were observed between the no-formalin vs. 1% formalin group ($p<0.01$) and 1% vs 2% group ($p<0.01$). No significant differences were found between the 2, 5 and 10% groups.

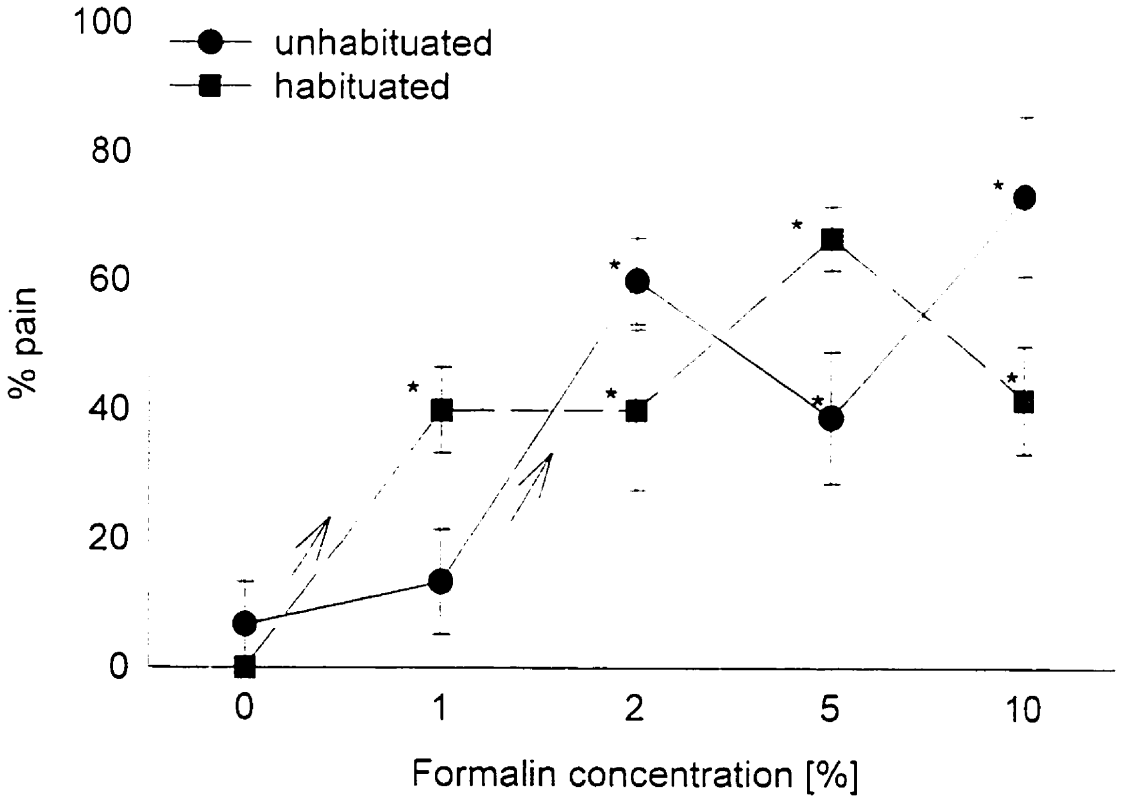

Figure 14

Formalin concentration-effect relationship of habituated and unhabituated rats in the first phase of the formalin test. Arrow indicates a significant increase in pain between two contiguous formalin concentrations. Star indicates that the pain level is significantly higher than that of no-formalin controls.

The effect of habituation was not significant, but the interaction between formalin concentration and habituation was highly significant ($p=0.001$). To examine the formalin dose x habituation interaction, the means of unhabituated and habituated rats were compared at each formalin concentration using 2-tailed t-tests with Bonferroni corrections. At 1 and 5% formalin, habituation significantly increased first phase pain ($p<0.05$).

In unhabituated animals, the 1% formalin group did not show significantly more pain than uninjected controls, while other groups did. Pain increased significantly only between 1 and 2% formalin ($p<0.05$). In habituated rats, all formalin treatment groups showed significantly more pain than controls. The pain score only increased significantly between no formalin and 1% formalin ($p<0.05$).

MPE_{50} and slope were determined for habituated and unhabituated animals separately. They are shown in Table VIII. The concentration-effect parameters were calculated using only the pain scores of animals that received formalin concentration up to 5%, due to the lack of increase in pain between the 5 and 10% groups, where the maximal pain response may already have been reached. The formalin concentration producing 50% of the maximal pain responses was higher in unhabituated animals (3.55%) as compared to habituated ones (2.33%). The slope in unhabituated animals was lower (12.94 ± 9.83) than in habituated rats (17.25 ± 4.42). Although these differences were not statistically significant, the concentration-effect parameters imply that in the first phase, a lower formalin concentration is required to obtain half the maximal possible response in habituated animals, and that the pain responses in habituated animals increase faster with increasing formalin concentration.

4.3.1.C.ii Second phase (8-120 minutes)

Figure 15 shows the formalin concentration-effect relationships for the phase 2b (10 - 60 minutes) in the upper panel, and the entire second phase (8 - 120 minutes) in the lower panel. For the second phase (8 - 120 minutes), the main effect of formalin concentration was highly significant ($p<0.001$). A significant increase was observed between all contiguous formalin concentrations ($p<0.05$). When only the observations in the period

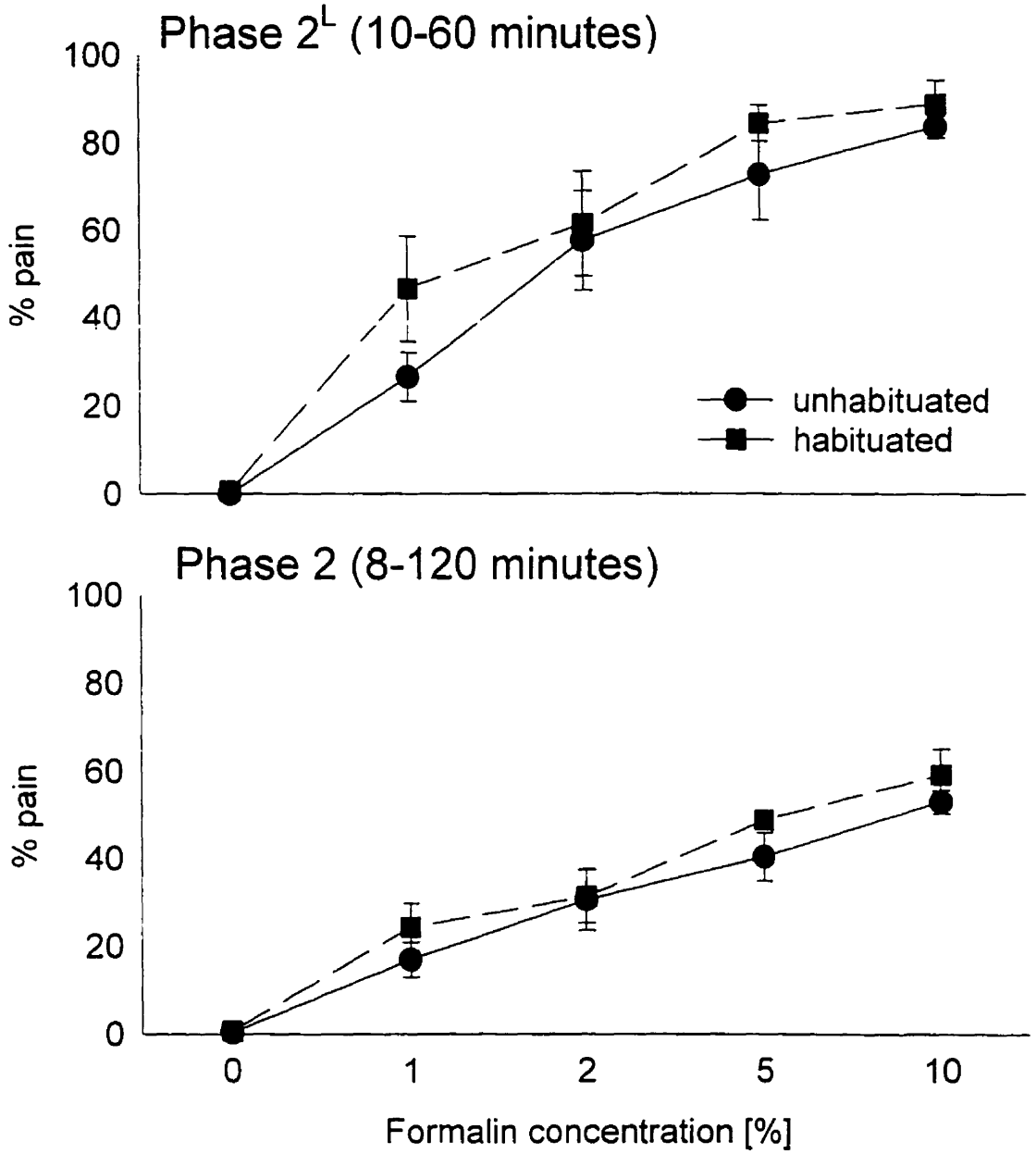

Figure 15

Formalin concentration-effect relationships for phase 2^L (10-60 minutes; upper panel) and second phase (8-120 minutes postformalin; lower panel). In phase 2L pain increases dose-dependently ($p<0.05$) up to 5% formalin. In the period from 8 to 120 minutes, pain increases significantly ($p<0.05$) between all contiguous formalin concentrations.

between 10 and 60 minutes after formalin administration were used to determine the pain scores, the effect of formalin increased dose-dependently up to 5% formalin ($p<0.05$ between contiguous concentrations). The difference between 5 and 10% formalin was not significant ($p>0.35$).

The slope and MPE_{50} of the concentration-effect relationships for formalin in the second phase (8 to 120 minutes) are shown in Table VIII; the values calculated for habituated and unhabituated groups did not differ significantly. The MPE_{50} was approximately 8.4% in unhabituated animals, and the slope was approximately 15. The concentration-effect relationship for habituated rats had an MPE_{50} of 5.6% with a slope of 15.7. Values calculated for phase 2[b] (10 to 60 minutes) are also found in Table VIII. The MPE_{50} values are significantly lower than those for the entire second phase ($p<0.05$), which means that a much lower formalin concentration (approximately 1.8% in unhabituated and approximately 1.0 % in habituated rats) produced the half-maximal behavioural pain response. The slope estimate for unhabituated animals was significantly steeper in phase 2[b] as compared to second phase ($p<0.05$), while the slopes for the habituated rats did not differ significantly.

The main effect of habituation in the second phase was not significant, nor was the interaction between formalin dose and habituation. However, as was observed in the first phase, the MPE_{50} of the fomalin concentration-effect relationship for habituated animals had a slightly lower value than that of unhabituated.

To investigate the rate of onset of pain in the second phase, the area under the pain response vs. time curve was computed for the first ten minutes of the second phase (8 to 18 minutes) for each rat. The mean area under the curve for each treatment group is shown in Figure 16. Two-way ANOVA (with formalin and habituation treatments as between-subjects factors) was used to analyse the onset of the second phase response. While the two-way interaction and the effect of fomalin were not significant, habituation had a significant effect on pain in this period of time ($p<0.05$). Second phase pain arose more rapidly in habituated animals as compared to unhabituated ones. This can also be observed by inspecting the time courses in Figures 9 and 10.

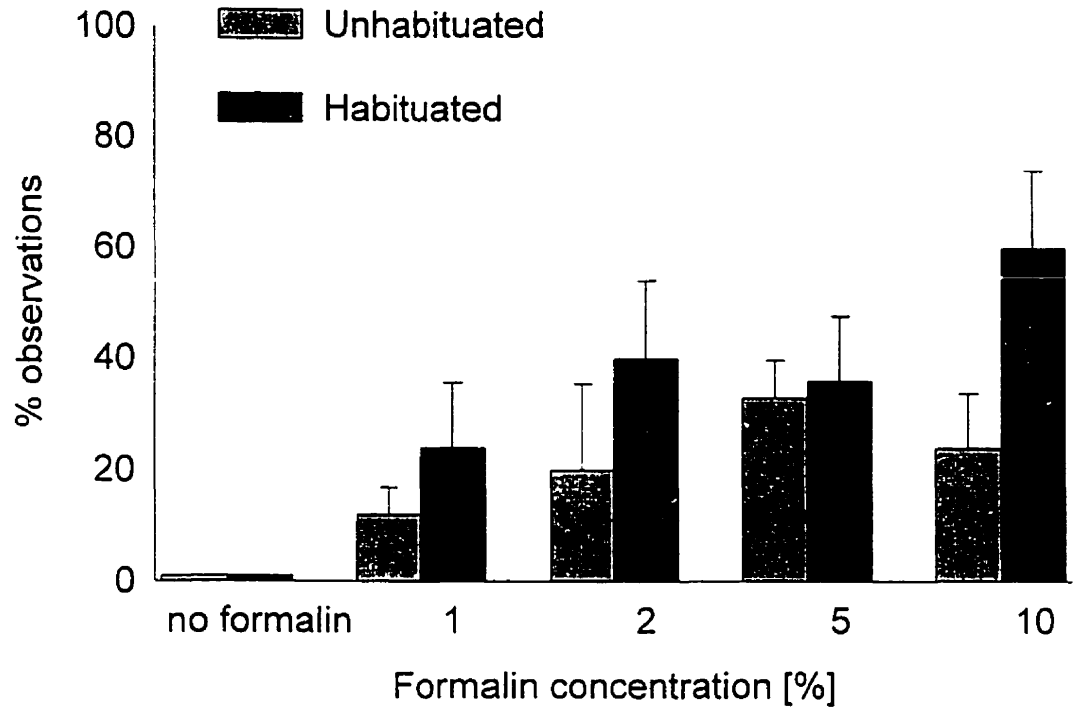

Figure 16

Area under the pain vs. time curve for the onset of the second phase pain response in the formalin test (8 to 18 minutes).

4.3.1.C.iii Third phase (2 - 16 hours)

Pain scores observed in unhabituated and habituated animals are shown in figure 17. The interaction between formalin concentration and habituation was not significant for the third phase of the test. Habituation did not produce significant differences in pain responses. The effect of formalin concentration was significant ($p<0.001$). Post hoc comparisons showed that only 5 and 10% formalin produced significantly more pain than no-formalin controls in the period between 2 and 16 hours post-formalin.

MPE_{50} and slope values could not be computed for this part of the formalin test. Pain levels were too low to obtain a slope and MPE_{50} estimate.

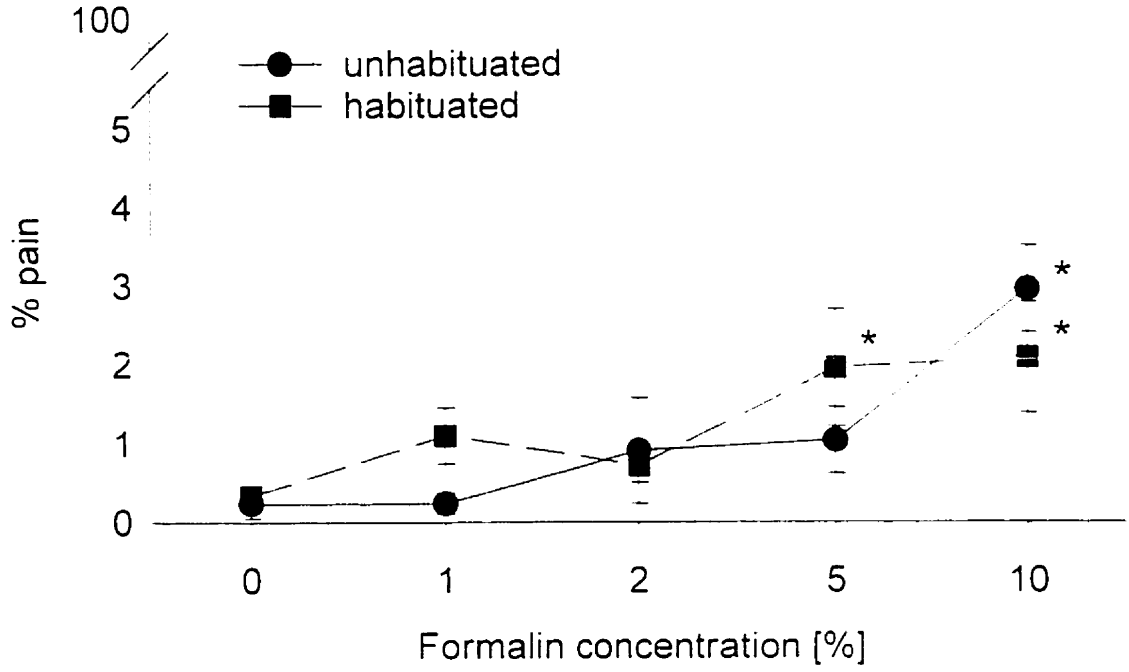

Figure 17

Formalin concentration-effect relationship for habituated and unhabituated rats in the third phase (2 to 16 hours after formalin injection) of the formalin test. Star indicates that the pain level is significantly higher than that of no-formalin controls.

4.3.2 General behaviours

General behavioural categories observed were sleeping, lying, sitting or standing still, walking, grooming, sniffing, rearing, licking floor, backwards locomotion and eating or drinking. Sniffing, rearing and walking were analysed as one variable, 'exploring'. Figures 18 and 19 display time courses of general behaviour for the entire 16-hour formalin session. The behaviours that occurred during the first phase are shown separately. The second phase is divided in four bins, the first spanning 8 to 30 minutes postformalin. The responses that occurred during the remaining time of the session are averaged for each treatment group, over 30-minute periods . Figure 18 shows time courses of unhabituated rats receiving no formalin, 1, 2, 5 and 10% formalin, and figure 19 of habituated rats receiving the same formalin concentrations.

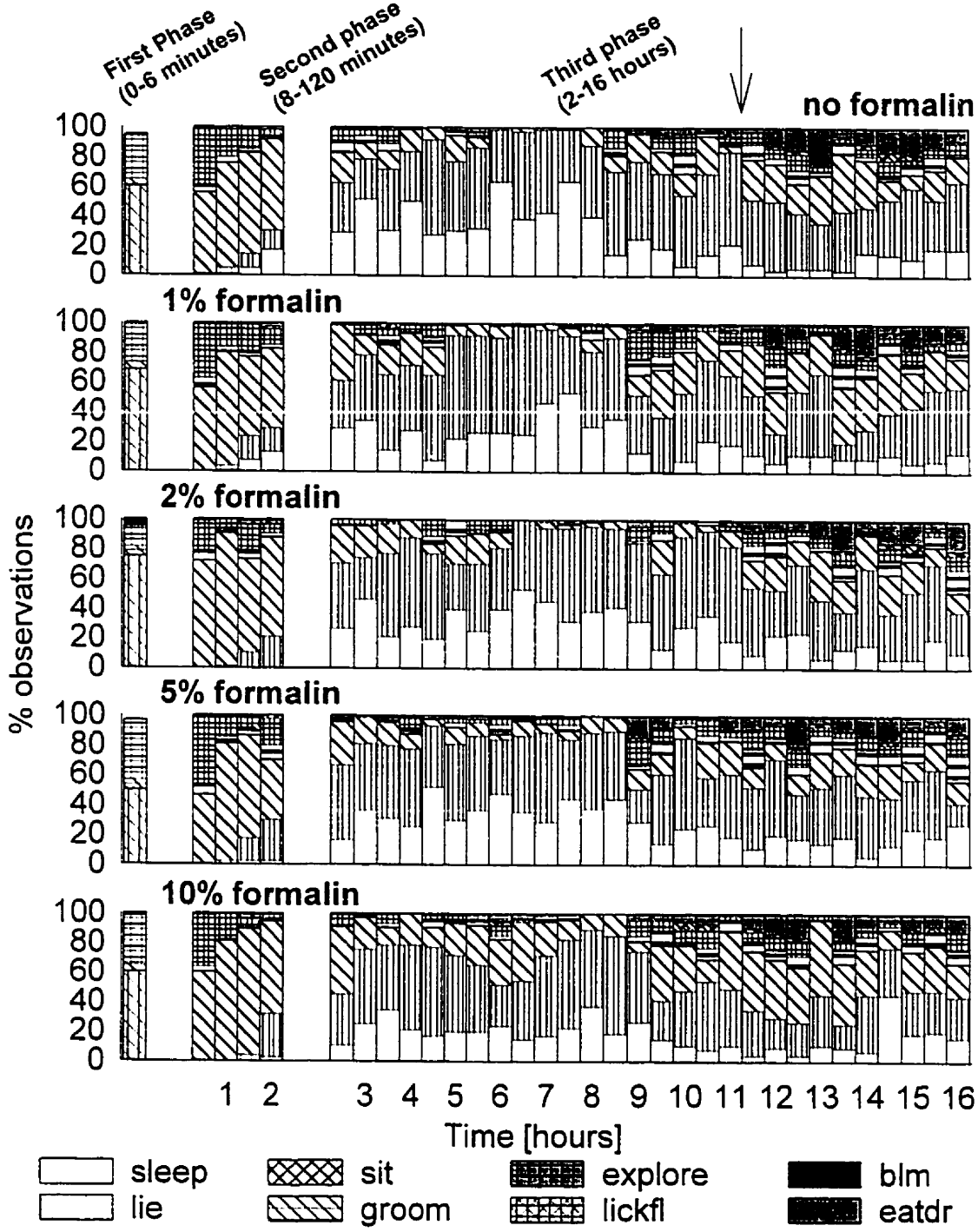

Figure 18

Time-courses of general behaviours in the first, second and third phase of the formalin test in unhabituated animals. First phase responses are averaged over the first six minutes of the test. The second phase is divided into four bins (8 to 30; 32 to 60; 62 to 90 and 92 to 120 minutes). The third phase is divided in 30-minute bins. Arrow indicates the time at which lights were turned off in the colony room (7pm).

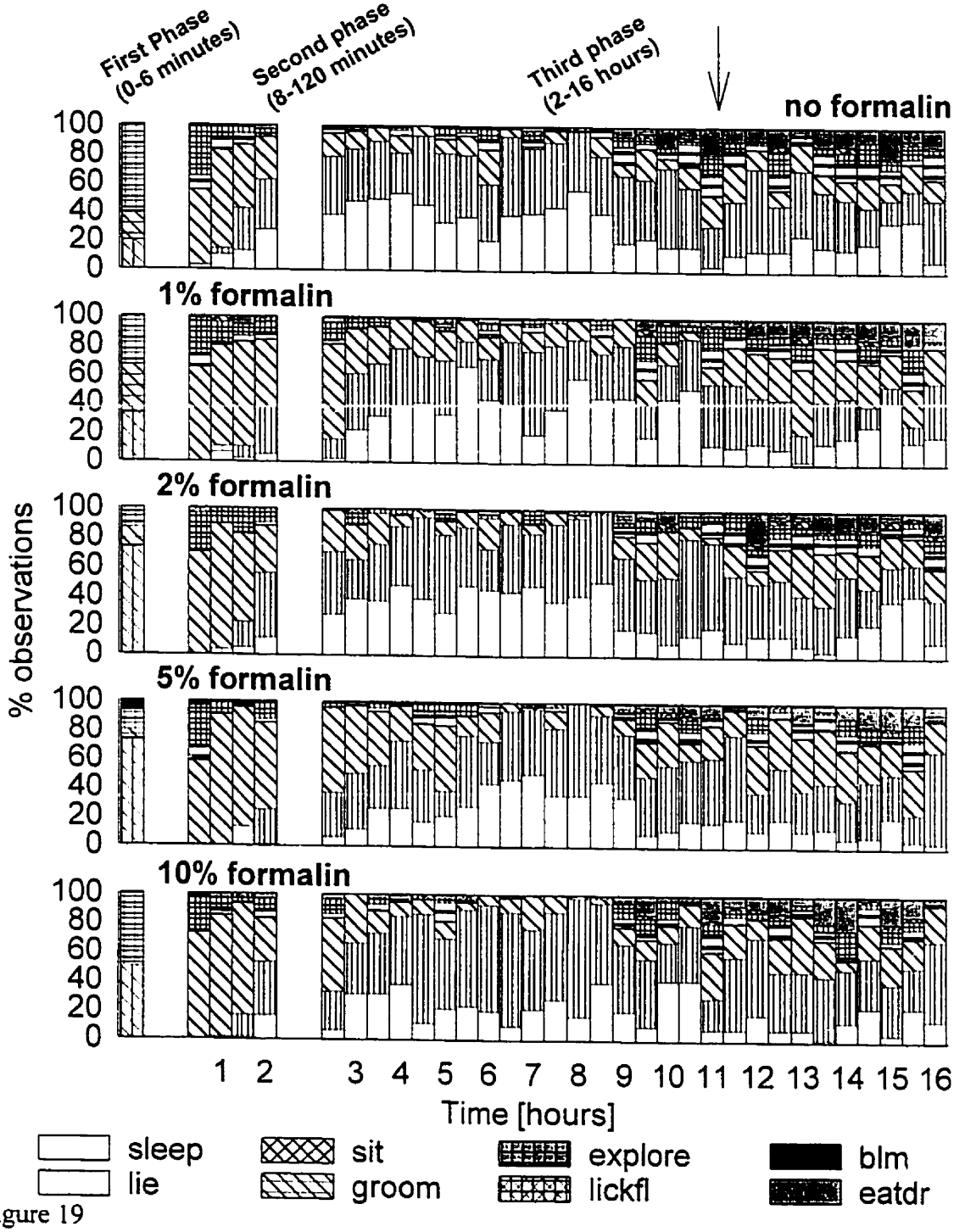

Figure 19

Time-courses of general behaviours in the first, second and third phase of the formalin test in habituated animals. First phase responses are averaged over the first six minutes of the test. The second phase is divided into four bins (8 to 30; 32 to 60; 62 to 90 and 92 to 120 minutes). The third phase is divided in 30-minute bins. Arrow indicates the time at which lights were turned off in the colony room (7pm).

The effect of formalin on general behaviour was analysed within three distinct phases of the experiment. The first and second phase corresponded to the two pain phases (0 to 6 and 8 to 120 minutes, respectively). The third phase (from 2 to 16 hours postformalin), was, in addition, divided in phase 3A and 3B. Phase 3A was defined as the time period immediately following the second phase and lasting until the time of food administration, which occurred approximately at the time of the animals' waking time. Phase 3B spanned the time between the waking hour until the end of the testing period.

Behaviours were examined quantitatively using ANOVA with behavioural categories as repeated measures, separately for each of the four phases (first, second, 3A and 3B), and habituation and formalin concentration as between-subject factors. To further reduce the number of behavioural categories, lying and sitting or standing still were grouped together as 'still'.

4.3.2.1 First phase (0-6 minutes)

The interaction effects between behavioural category and habituation, and between behavioural category and formalin concentration, were significant at the $p<0.01$ level and were further examined by t-tests with Bonferroni correction, and LSD post hoc analyses, respectively.

Sleeping and feeding behaviours did not occur during the first phase, while backwards locomotion was infrequent. Lying and sitting or standing still was increased in the 2, 5 and 10% formalin groups as compared to rats that did not receive any formalin ($p<0.05$). Exploring was depressed in the 2% and 5% formalin groups compared to uninjected controls ($p<0.05$). Exploration was depressed in habituated animals as compared to unhabituated ones (the difference was significant in the 1% and 5% groups ($p<0.05$), and approaching significance in the 2% group ($p<0.10$). Grooming increased from the no formalin to 1% formalin group, the difference approaching significance ($p<0.07$) and was then dose-dependently depressed through the 2, 5 and 10 % groups (p's decreasing from 0.025 for 1% vs. 2% formalin groups and 0.002 for 1% vs. 10% formalin groups). Particularly in habituated animals, grooming decreased progressively up to 5% formalin,

and was not observed in the 10% group.

4.3.2.ii Second phase (8-120 minutes)

Behaviours during the second phase of the formalin test did not differ significantly between the habituated and non-habituated animals, or between the different formalin treatments. The ratios of different responses changed markedly during the second phase, hence the latter was divided into four time periods (8 to 30, 32 to 60, 60 to 90 and 92 to 120 minutes; Figures 18 and 19) for the purpose of further analyses. ANOVA of the second phase now revealed that behaviour was significantly different between the four time slots ($p<0.001$), and the interaction between formalin concentration and phase was approaching significance ($p=0.067$).

In the first part of the second phase (8 to 30 minutes) the activity in all treatment groups was very high. The main difference between the higher and lower formalin concentration was the occurrence of BLM (10% unhabituated and 5% and 10% of habituated rats). This behavioural response may augment the increase in activity of the higher formalin groups. Activity during the next time period (32 to 60 minutes) was depressed as compared to the first part of the second phase. In the period of 62 to 90 minutes, the activity was lower in the 5 and 10% groups. This may have been due to the fact that in these animals pain persisted through this time period, while the pain responses in others had ceased. Sleeping occurred only in rats that received lower formalin concentrations . During the last part of the second phase (92 to 120 minutes), an increase in sleeping can be observed in all treatment groups. The decrease in activity throughout the second phase can be observed in Figures 18 and 19.

4.3.2.iii Third phase (2-16 hours)

The interaction between formalin concentration and behavioural category was significant in phase 3A. Sleeping was significantly depressed in the 10% formalin group as compared to the no formalin controls ($p<0.01$). In the same treatment group inactive behaviours (lying, sitting or standing still) were significantly increased ($p<0.05$). During

the waking period of the test (10 to 16 hours postformalin, phase 3B) the effects of formalin and habituation on general behaviour was not significant. No effect of formalin and habituation on general behaviour was observed. The rater did not observe any additional differences between the treatment groups.

4.3.3 Morbidity and agitation

Scores of morbidity (ptosis, hunched posture and or piloerection) and agitation were treated as mutually exclusive categories. When behaviour was normal a score of '0' was recorded. Figure 20 shows agitation in the first and second phase of the test and morbidity in the second and third phase. Agitation did not occur during the third phase while morbidity was not seen in the first phase.

The effect of agitation was not analysed by ANOVA since the behaviour did not occur in no-formalin or 1% formalin groups, and in the 2% group it only occurred infrequently in one subject per group. Therefore only the agitation scores in the 5% and 10% groups were tested against the no-formalin group using t-test. In these groups agitation was significant in the second phase ($p<0.05$ for 5% formalin groups and $p<0.01$ for 10%). During scoring, agitation correlated highly with backwards locomotion.

Repeated measures ANOVA revealed that there was a significant interaction between formalin dose and phase of the formalin test and a significant effect of phase, while the effect of habituation and formalin dose was not significant. The significant interaction was investigated by investigating the effect of formalin on morbidity at each phase of the formalin test. In the first phase no morbidity was scored. In the second phase only the 2% formalin group had a morbidity score significantly higher than no-formalin controls. In the third phase, rats that received any formalin treatments were significantly morbid ($p<0.05$ for the 2% formalin group, and $p<0.01$ for the 1, 5 and 10% formalin groups. These effects are depicted in Figure 20.

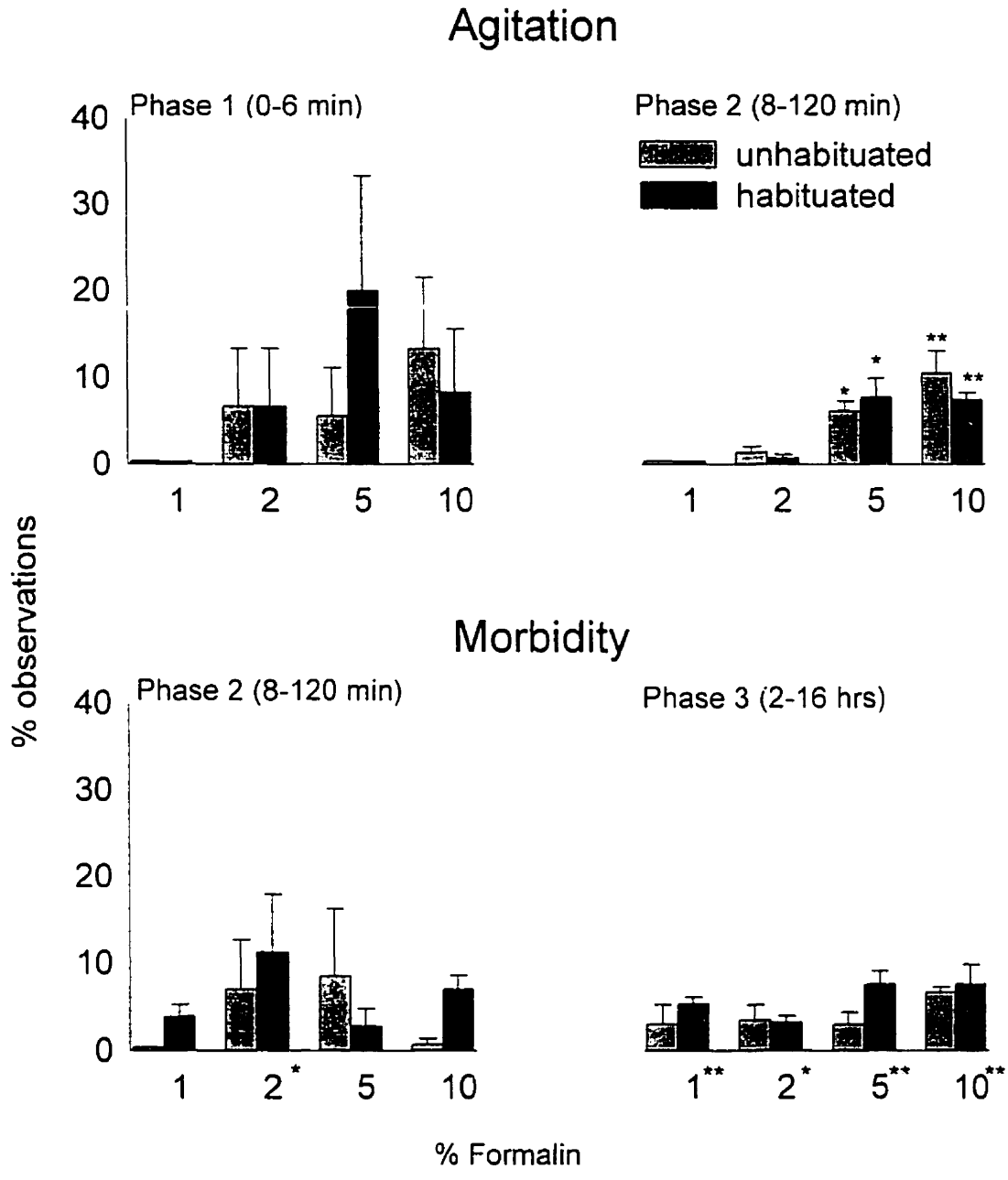

Figure 20

Morbidity and agitation scores in the formalin test. Morbidity was only observed in the second and third phase, while agitation only occurred in the first and the second phase.

* indicates the score is significantly greater than that of no-formalin controls.

** indicates that the score is significantly higher than that of controls at the $p<0.01$ level.

Chapter 5

Analysis of effects of morphine

5.1 Objectives

In this experiment, the relationship between the amount of pain and the analgesic potency of morphine was investigated. Morphine dose-effect relationships were established over the full range of frequently used formalin concentrations. They are shown in Figure 20. Formalin concentration-effect relationships at fixed morphine doses were established from the same data sets and are shown in Figure 21.

5.2 Experimental design

Rats were randomly assigned into 6 groups, each representing one of six formalin concentrations (0.75, 1.5, 2.25, 6.75 and 13.25%). From each of these groups, the animals were further assigned to three or four subgroups of five subject, corresponding to different doses of morphine. Morphine doses were determined such that the pain scores were submaximal (i.e. animals did not display continuous pain behaviours throughout the test), and were administered 30 minutes before formalin. The doses ranged from 0.25 to 8 mg kg.

Behaviour was sampled every two minutes, for 60 minutes after the administration of formalin. The pain behaviours displayed in the time-period between 12 and 60 minutes post-formalin was used to determine the pain index. The behavioural scale used was identical to that used in Experiment I, and is described in Table I.

The delay in obtaining permission from the Animal Ethics Committee to carry out experiments using formalin concentrations over 5% without administration of analgesic agents did not permit all experiments to be conducted in the desired order. Therefore, the investigation of morphine dose-effect relationships at different formalin concentrations was conducted prior to the analysis of formalin-induced behaviours over the full range of formalin concentrations. Because of this, the behavioural rating scheme used was that

described by Abbott et al. (1995) for formalin doses ranging from 0.5 to 2.5%. The assumption was made that formalin-induced pain increased dose-dependently up to 10% formalin, and only a strong analgesic (morphine) was used in the analysis. It was also not possible to run no-morphine control subjects. The baseline pain level occurring at each formalin concentration in the absence of morphine was therefore estimated by interpolating the morphine dose-effect relationships.

5.3 Results

Morphine dose-effect relationships at different formalin concentrations are shown in Figure 20. Each line connects the mean pain scores obtained of rats that received different morphine doses at one formalin concentration. The range of morphine doses at each formalin concentration was chosen in a way that the lowest morphine dose permitted a pain response that was submaximal, i.e. the animals did not show continuous pain behaviours, and the highest morphine dose alleviated virtually all pain. At any formalin concentration, an increase in morphine dose resulted in a decrease in the pain response. The exception to this was 0.5 mg/kg morphine at 1% formalin. Within the range of lower morphine doses (up to 1.5 mg/kg), an increase in formalin concentration increased the requirement for morphine to maintain the same level of pain control. This can be observed by a progressive rightward shift in the morphine dose-effect relationships at fixed formalin concentrations. At formalin concentrations higher than 1.5%, the requirement for morphine no longer increased with increasing formalin concentrations, which can be concluded from the lack of a rightward shift in morphine dose-effect relationships. The 8 mg/kg morphine dose appeared to block pain induced by any formalin concentration (up to 13.5%).

Mean pain indices produced by different formalin concentration at 2, 4 and 8 mg/kg morphine were replotted in Figure 21, to show formalin dose-effect relationships at fixed morphine doses. At 8 mg/kg morphine, no formalin concentration produced a significant pain response. At morphine doses lower than 8 mg/kg, in the lower range of formalin concentrations (less or equal to 2.25%), an increase in formalin concentration increased the pain response. However, as the formalin concentration increased further, the pain response

became asymptotic. The level of maximal pain that can be produced at each morphine dose appeared to decrease with an increase in morphine dose.

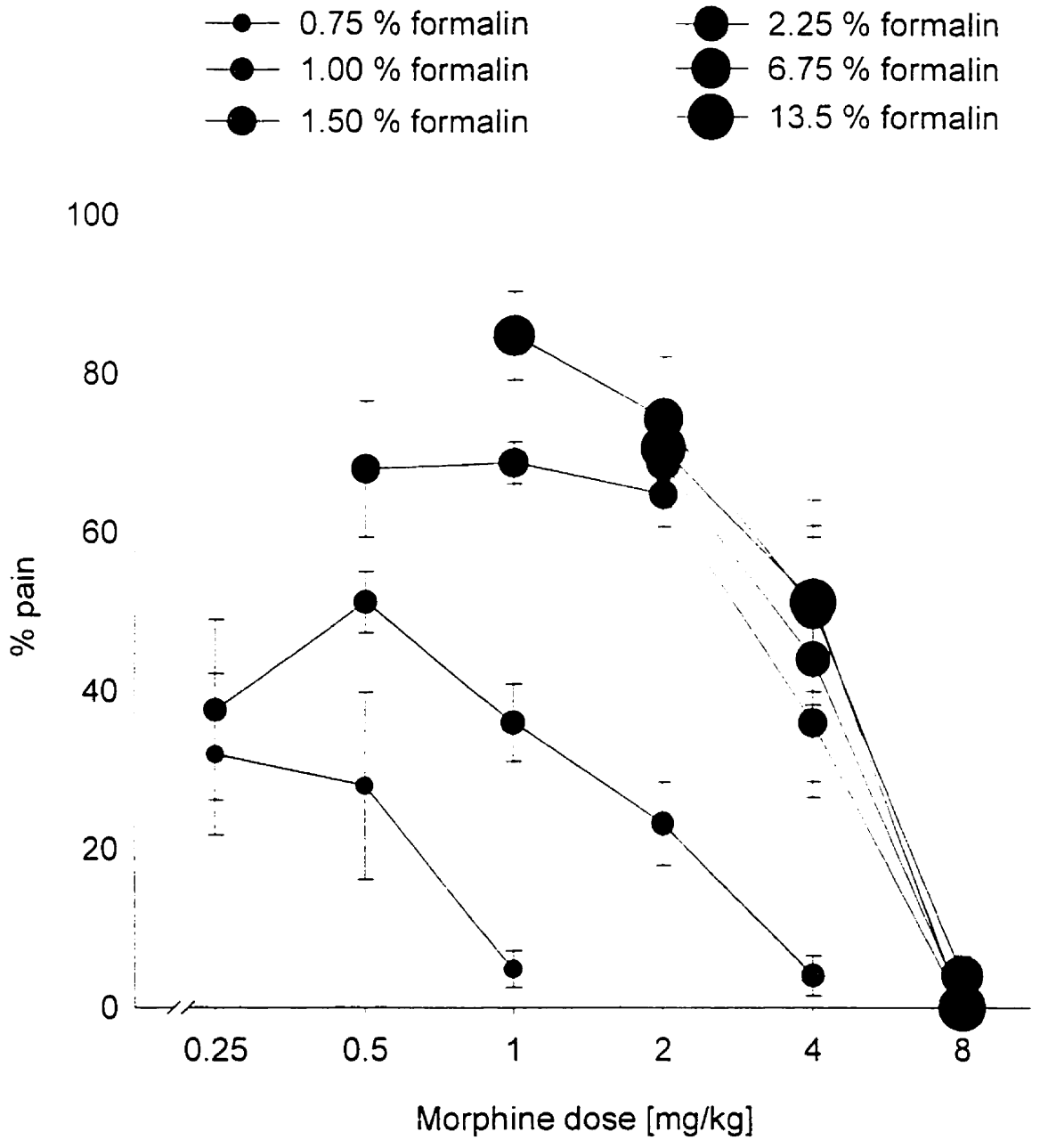

Figure 21

Morphine dose-effect relationships at different formalin concentrations.

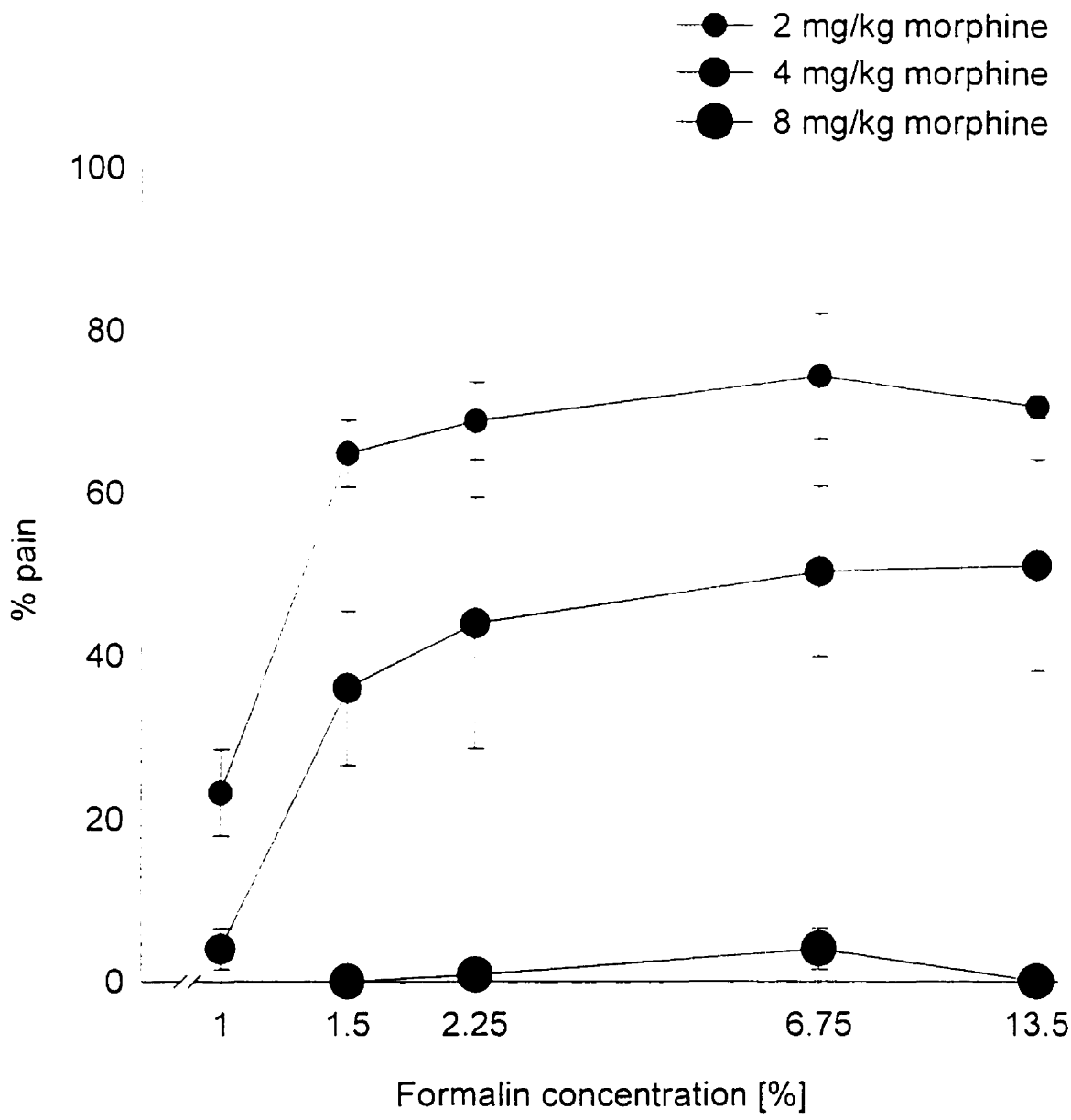

Figure 22

Formalin concentration-effect relationships at different morphine doses.

Chapter 6

DISCUSSION

The formalin test is a useful animal pain model. Formalin-induced pain occurs due to tissue injury, and resembles many types of clinical pain, as opposed to other pain models in which transient painful stimuli are presented and pain thresholds are measured. There are, however, considerable inconsistencies regarding the methodology of the test. The time-sampling method validated in the present study is an attempt to simplify the scoring procedure of the test. It makes it less labour-intensive and time consuming. It also provides the opportunity to score other behaviours and provide additional quantifiable information on pain and pharmacological treatments. The second objective was to examine all commonly observed behaviours induced by the range of concentrations most commonly used, and examine the effect of stress due to novel environment on responses to subcutaneous formalin. The last experiment investigated the requirement of morphine by formalin concentration.

6.1 Validation of the time-sampling method

Performing the formalin test is a demanding task, mainly because behaviour is rated for a longer period of time than in other pain tests, and some of the behavioural responses must be scored continuously. In a typical pharmacological study, where three doses of a drug are tested along with a no-drug control group, with only two subjects rated at one time, ten hours of testing are required to obtain mean pain scores for five animals in each treatment group.

Investigators have attempted to simplify the scoring of the formalin test, usually by rating only a subset of the behavioural responses. Observing fewer behavioural responses may be an easier task for the observer. However, the present study, which is in agreement with that of Abbott et al. (1995), shows that the pain indices that best correlate with the natural logarithm of formalin concentration require the scoring of lift and lick, bite, shake

and flinch. Moreover, methods employing simplified behavioural scoring are not more efficient than continuous rating of all pain-specific behaviours, because constant observation of animals is still required. In some publications researchers report scoring behaviours only during short time periods. As was shown in the second experiment, the pain response to different formalin concentrations varies in duration and magnitude. Scoring only a part of the response may therefore be less reliable for estimating the level of pain, and does not provide information on the temporal aspects of behaviour.

Two automated systems have been developed to decrease the amount of labour required, and to increase time-efficiency. They do not, however, distinguish between different pain-specific behaviours. The pain intensity is inferred from less specific activity measures. As a result, certain pain-specific behaviours may not be detected by the automated methods. Neither of the automated systems (Jett & Michelson, 1996; Jourdan et al., 1997) can detect, for example, pain behaviours such as lying still with the injected paw lifted above the floor. In addition, in the automated system by Jourdan et al (1997), diazepam appeared to produce analgesia, while Jett & Michelson (1996) did not test their system for the effect of non-analgesic sedative agents on the pain scores. It is probable that sedative agents would produce pseudo-analgesia in the dynamic force change apparatus.

The time-sampling method of scoring formalin-induced behaviours introduced here has many advantages over the traditional continuous rating. When behaviour is sampled every minute or every two minutes, the scoring is considerably less demanding for the observer. The amount of time required to describe an animal's instantaneous behaviour by entering two or three behavioural codes is in the order of seconds. Up to ten animals have been tested using this method when behaviour was sampled every two minutes, provided the rater is experienced and can maintain a constant look-and-enter rhythm. An experienced observer will finish rating ten animals in less than one minute and will be able to relax until the next rating bin. The number of animals that can be tested simultaneously is limited primarily by the number of animals that can be injected during the time between two contiguous ratings. The program is started at the time of the first injection, hence all rats to be tested have to be injected before the first rating one or two minutes later. It is important

that rats are injected in the same order they are observed. A study using two-minute time-sampling to rate ten rats simultaneously will require five times less scoring time than if continuous rating were used.

Results obtained by the time-sampling compared well to those from continuous rating, and the time course of pain-specific behaviours obtained from sampling every minute or every two minutes was almost identical to that of continuous rating. The correlation between individual behavioural pain-specific responses scored by both methods was highly significant, as were the correlations between pain indices computed from results of both methods. The concentration-effect relationships established from time-sampling data were virtually identical to continuous rating ones, particularly for the second phase of the test. Most importantly, the statistical power was not reduced, so that the same number of subjects per group yielded equally robust results. Similarly, both methods were equally sensitive to morphine analgesia and revealed almost identical morphine dose-effect relationships.

Another important advantage of the time-sampling method is that other behaviours can be rated in addition to the pain-specific ones. This is simply done by entering one or more additional behavioural codes each time a rat's behaviour is being described. In fact, this method is very flexible and can be used to describe many different aspects of behaviour, such as the degree to which a pharmacological agent is sedative or excitatory. It also allows the extent to which the behaviour is normalised to be used as an additional index of analgesia. In the present study it was observed that rats that were habituated to the testing environment, and did not receive any formalin, displayed a cyclical pattern of behaviour that was not observed in formalin-treated rats (Figure 6). Formalin-treated animals, in addition, displayed less exploratory behaviour and more inactive behaviours. Administration of morphine reversed the depression of activity at lower doses. At 2 mg kg, behaviour appeared to be normalised, although this dose of morphine did not produce complete analgesia if only pain-specific behaviours were considered. With a progressive increase in morphine dose an increasing degree of sedation was observed. Complete block of pain responses occurred at 8 mg/kg morphine. It is probable that effective clinical

analgesia is obtained at the dose that normalizes behaviour. This would be consistent with the very low doses of buprenorphine, which was shown, in a study by Liles & Flecknell (1994), to reduce post-surgical adipsia and aphagia in rats.

6.2 Analysis of formalin-induced behaviours and the effect of habituation

A wide range of formalin concentrations is used in the formalin test, and there is fragmented use of behavioural indices. The best behavioural measure used to infer the magnitude of pain may depend on such testing conditions as the concentration of formalin used or whether or not the animals have been familiarized with the observer and the testing environment, and during which time period the behaviour is scored. The present study was designed to investigate the effect of these factors on pain measures commonly used and the formalin concentration-effect relationships. In addition, a profile of general behaviours induced by formalin was established.

All formalin concentrations (1 to 10%) produced the typical biphasic response. In agreement with most reports, the first phase was terminated within the first 5-6 minutes. The interphase appeared very brief, and most rats began to show continuous pain behaviours as early as 8-10 minutes after formalin injection, although in the literature the interphase has been reported to last approximately 10-15 minutes (Tjolsen et al., 1992). The duration of the second phase varied with formalin concentration. The response terminated within 60 minutes in rats receiving 1% formalin, and lasted progressively longer in rats that received higher concentrations (approximately 75 minutes in the 5% formalin group and 90 minutes in the 10% group). This observation is not in agreement with Tjolsen et al. (1992), who report that the second phase pain behaviours last until approximately 40 minutes after formalin injection. They do not, however, refer to what formalin concentrations this conclusion was based on. Most researchers observe behaviour only during one hour after formalin injection (Appendix I), and at the same time the most commonly used concentration is 5%, the pain responses displayed past 60 minutes post-formalin are not accounted for. During the remaining time of the 16-hour test session (third phase), most rats displayed occasional favouring and lifting. This residual pain increased

gradually with increasing formalin concentration and became significant as compared to uninjected animals in the 10% formalin group.

The best predictor of the level of pain was determined by the analysis of correlations between pain-specific responses and the log of formalin concentration. The findings are in general agreement with those of Abbott et al. (1995) and Coderre et al. (1993): no single behaviour managed to explain 50% of the variance; favouring correlated negatively with formalin concentration in the second phase of the test. The best simple pain measures were general shaking (shake, flinch or whole-body flinch; SFW), which is most commonly included in 'flinching'. The combination of two measures combined (flinch-lick) performed well in the second phase, where they explained 50% of the variance in habituated rats (*vide infra*). The best pain indices were the sum of lifting and licking, biting or paw shaking (SFW), as well as the weighted means score by Dubuisson & Dennis (1977) and Watson et al. (1997). These measures have been previously found to be more robust than any other measure (Abbott et al., 1995; Coderre et al, 1993; Watson et. al, 1997).

A good pain measure should be resistant to sedative agents, and be able to discriminate between sedative and analgesic effects of a sedative analgesic. The weighted means scores have been tested using non-analgesic sedatives for formalin concentrations up to 2%. They were found to be relatively resistant to the sedative effects of pentobarbital, and were not affected by amphetamine (Abbott et al., 1995). It was not possible to conduct the same tests in this study, due to ethical concern of not using a strong analgesic with 5 and 10% formalin.

Acute stress has been shown to reduce clinical pain (Melzack et al., 1982). Similarly, stress can affect the behavioural responses in animal pain models. However, chronic stress has been found to aggravate prolonged pain (discussed below). Abbott & Franklin (1986) have shown that acute stress due to novel environment reduces formalin pain. Here we show that rats that were not habituated to the testing environment show somewhat different responses to formalin. The effect of habituation on the behavioural response to formalin was investigated by comparing formalin concentration-effect relationships for different periods of the test, as well as its effect on general behavioural

state.

The effect of habituation was most prominent during the first half-hour of the formalin test. In the first phase, habituated animals that received 1% formalin displayed significant pain, while the unhabituated animals did not. In unhabituated animals pain responses only rose to significant levels at 2% formalin. At higher concentrations habituation did not have an effect, however this may be due to our observations that behavioural indices in the first phase become asymptotic at around 2% formalin (*vide infra*). The onset of the second phase was significantly delayed in unhabituated animals, although the area under the pain vs. time curve was not affected. The proportion of pain behaviours in the second and third phase did not differ between the groups, however a slight trend was observed in habituation producing a slight rightward shift in formalin concentration-effect relationships. The magnitude of the effect of habituation may hence decrease with familiarization to the environment during the test. The effect of habituation on general behaviour also occurred at the beginning of the test. In the first phase unhabituated rats explored more and groomed less than habituated ones. During the second phase of the test the prominent difference between habituated and unhabituated rats was a more frequent occurrence of whole-body flinching in unhabituated animals. This appeared occasionally at concentrations as low as 2%, and progressively increased to 10% formalin, while in habituated animals only a small amount of WBF was observed in the 10% formalin group. This implies that WBF is a specific pain behaviour. The subjective appearance of this response suggests the presence of a higher level of pain.

Since habituation conditions produce a significant effect on pain-specific behaviours it is important to be aware that ignoring this variable may produce false interpretation of specific results. For example, in experiments with pharmacological treatments that alter the degree of stress due to novel environment, such as opioids, rats that have positive drug experiences associated with handling (e.g. repeated administration prior to testing) will experience less stress on the day of the test, and may show a decrease in the pain response due to familiarity with the experimenter and/or the testing environment.

Another important observation was made. As mentioned above, WBF occurred

more in the second phase in unhabituated animals. As was mentioned earlier, WBF may indicate higher levels of pain. In the second phase an index of higher pain sensation could be related to the fact that in prolonged clinical pain stress aggravates the pain. In some animal studies it has also been shown that, while acute stress tends to decrease pain (Abbott et al., 1986a), prolonged stress may enhance it (King et al. 1996).

To investigate formalin concentration-effect relationships the sum of lifting and licking, biting or shaking was used as the index of pain. In the first phase, formalin-induced behaviours increased significantly from no formalin up to 2% formalin, where pain-specific behaviour appeared to become asymptotic since concentration-dependent increase was no longer observed. A similar asymptote in the behavioural responses in the first phase has been observed in mice, where pain-specific responses in the first phase plateaued at 0.2% formalin, while second phase behavioural responses to pain continued to increase with formalin concentration (Rosland et al., 1990). This may explain the poor correlation of all behavioural measures with log of formalin dose in the first phase. In fact, the correlations somewhat improved when the high formalin dose (10%) was not included in the calculation of the coefficients of correlation. It is clear that, to obtain statistical power in studies concerning the first phase, larger samples than five rats per group would be necessary.

In the second phase of the formalin test (8 to 120 minutes), formalin produced a dose-dependent increase in pain responses with formalin concentration. This increase in pain response appeared to be log-linearly related formalin concentration. The pain response increased with formalin concentration in two dimensions: it became more intense and lasted longer. Within the first part of the second phase (up to 60 minutes - 'phase 2b'), with increasing formalin concentration, rats spent more time displaying lifting, licking or biting, flinching, shaking and WBF. However, these behaviours became asymptotic at 5% formalin and did not increase at 10%. The difference between 5 and 10% formalin could be quantified by the increase in the duration of the response. This can be best observed by the shape of the concentration-effect relationship depicted in Figure 15. Most researchers using the formalin test use 5% formalin and score behaviour for only 60 minutes. Whether the formalin test is performed under these conditions, it is not sensitive to treatments that

produce hyperalgesia. This problem can be solved by either extending the observation period or decreasing the formalin concentration.

During the second phase the difference between 5 and 10% formalin lies mainly in the duration of the response. As mentioned above, at 5% formalin the behaviours are already saturated. However, several observations point towards the fact that by looking at the complete behavioural response there is indication that pain does increase from 5 to 10% formalin but the increase can not be detected by the behavioural pain indices. WDT is almost non-existent at formalin concentrations up to 2%. The amount showed at 5% was not significantly bigger than zero, while at 10% it was. Of all the pain behaviours scored in the formalin test, this behaviour appears to the observer the most severe and may indicate higher levels of pain. Further evidence for this increase comes from the observation that there was concentration-dependent increase in subjective agitation scores between 5 and 10% formalin. In addition, BLM (retropulsion) occurred almost exclusively in the 10% formalin group. The latter behaviour has been associated with higher levels of clinical pain in man (Bergouignon et al., 1968).

The analysis of general behaviour revealed that formalin affected the behavioural state of the animals. Similar effects of formalin were observed during the first phase of the test (0 to 6 minutes) and the first quarter of the second phase (8 to 30 minutes postformalin). At 1 and 2% formalin, there was a concentration-dependent decrease in exploration. This is in agreement with the observations of Abbott et al. (1995), for concentrations up to 2.5%. In addition, formalin appeared to reduce activity by increasing lying and sitting still, but not sleeping, which did not occur in the first half-hour of the test. In the 5 and 10% formalin groups the effect on activity was reversed. This is due partly to the occurrence of backwards locomotion in these groups of rats, and also because of an increase in exploratory behaviours that occurred because of agitation produced by the high level of pain. In the first phase formalin appeared to dose-dependently depress grooming. This is also in agreement with Abbott et al. (1995), however no effect of formalin on grooming was observed during the second phase. In the second quarter of the second phase (32 to 60 minutes) the effect of formalin on general behaviour was less pronounced. Sleeping began to occur in the no-

formalin controls and lower formalin concentrations. During the third quarter activity was lower in the 5 and 10% groups vs. the other treatment groups. During this period of time the rats that received 5 and 10% formalin were still experiencing pain which may be related to the depression in activity. Moreover, the observer noted on several occasions that these animals displayed ptosis, piloerection and sometimes hunched posture, more so than other animals which appeared to have already recovered from the pain. Until the end of the second phase, no major differences were observed in general behaviour among the different formalin groups except a dose-dependent depression in sleeping during the first part of the third phase (2 to 10 hours postformalin): the amount of time spent sleeping was significantly lower in the 10% formalin group versus the uninjected controls. This implies that the residual pain at 10% formalin is behaviourally significant.

Pain-specific and general behaviour point towards questioning the use of formalin concentrations commonly used in the scientific literature. Five percent formalin produces maximal pain responses in the period of time most commonly observed (0 to 60 minutes), and as mentioned above, formalin treatments in excess of 5% don't produce greater pain levels when behaviour is rated one hour postformalin. This does not mean that pain does not increase when the formalin concentration is raised to 10% formalin. As a matter of fact, the change in general behaviour, e.g. an increase in backwards locomotion, whole-body flinching and subjective agitation in the 10% formalin group support the idea that pain does increase but is not detectable by the pain-specific indices due to ceiling effects. Since formalin produces an almost linear increase in pain behaviour up to 5% in the first hour, a lower formalin concentration will be more susceptible to treatments that alter pain behaviours, i.e. an increase in sensitivity of the test. Additional support for the use of lower formalin concentrations is provided by the analysis of general behaviour which indicates that formalin concentrations of 5% and higher produce subjective distress which is not observed at lower formalin concentrations. Moreover, 5 and 10% formalin produce pain responses lasting over an hour. Pain behaviours that are displayed past 60 minutes after formalin injection and are not scored, produce unnecessary pain and suffering and raise ethical concerns. Similarly, 10% formalin produces significant behavioural disturbance and

pain up to ten hours postformalin. Use of such high formalin concentrations should be avoided unless there is specific justification.

6.3 Analysis of effects of morphine

Morphine dose-effect relationships at a wide range of formalin concentrations (0.25 to 13.5%) were constructed and the morphine requirement by formalin concentration was examined. At concentrations up to 1.5%, an increase in formalin concentration increased the requirement for morphine. This can be observed by rightward shifts of morphine dose-effect curves in Figure 21. At higher formalin concentrations, an increase in formalin concentration no longer resulted in a rightward shift of the dose-effect curves. This ceiling effect is not to due to a lack of increase in pain when formalin concentration are raised. As discussed above, pain increases dose-dependently at least until 10% formalin. The phenomenon is therefore more likely to be related to the mechanism of action of morphine. As can be observed in Figure 22, the interaction between formalin pain and morphine analgesia mimics that of a typical non-competitive antagonism. It appears that morphine acts by a mechanism resembling a valve that controls the flow of a liquid. At each morphine concentration there appears to be a maximum possible amount of pain that can be experienced, and this amount decreases with increasing morphine dose. 8 mg/kg morphine appeared to block all pain.

At the lower formalin concentrations it may be that a different neuronal mechanism underlies the morphine analgesia. It has been shown that an injection of the GABA agonist muscimol into the ventro-caudal brainstem blocks the effects of high doses of morphine, but not of low doses (Gilbert & Franklin, 1998). The present data would be consistent with the morphine effects observed at higher formalin concentrations being mediated by bulbospinal descending systems. The effects at low formalin doses could either depend on forebrain mechanisms (Franklin, 1998) or peripheral effects (Hong & Abbott, 1995).

Chapter 7

CONCLUSIONS

Time-sampling is a less demanding and more efficient method of behavioural rating. It also enables the observer to score non-specific behaviours. These provide useful additional information about the pain-related phenomena under study.

When 1 to 10% formalin concentration are used, the best pain indices are the sum of lifting and licking, biting or shaking, and the weighted means scores. In the first phase pain responses to formalin concentrations over 2% are not distinguishable using any rating scheme studied. In the second phase, pain increases concentration-dependently between 1 and 10% formalin. Behaviour should be scored for at leat 90 minutes when concentrations of 5% and over are used. 10% formalin produces significant residual pain and sleep disturbance. Habituation to the testing environment increases the sensitivity to formalin in the first phase and increases the rate of rise of the second phase.

Properties of morphine analgesia change with the dose used and the formalin concentration. There may be two different mechanisms by which morphine alleviates formalin-induced pain.

REFERENCES

Abbadie, C., Taylor, B.K., Peterson, M.A. and Basbaum, A.I., Differential contribution of c-fos expression in the rat spinal cord: studies with remifentanil and lidocaine. Pain, 69 (1997) 101-110.

Abbott, F.V. and Hellemans K.G.C., Phenacetin, Acetaminophen and Dipyrone: Analgesic and Rewarding Effects, submitted for publication, subject to revision.

Abbott, F.V. and Bonder, M., Options for managment of acute pain in the rat. Veterinary Record, 140 (1997) 553-557.

Abbott, F.V. and Palmour, R.M., Morphine-6-glucoronide: analgesic effects and receptor binding profile in rats. Life Sciences, 43 (1988) 1685-1695.

Abbott, F.V., Franklin, K.B.J. and Connell, B., The stress of a novel environment reduces formalin pain: possible role of serotonin. European Journal of Pharmacology, (1986a) 141-144.

Abbott, F.V., Franklin, K.B.J. and Libman R.B., A dose-ratio comparison of mu and kappa agonists in formalin and thermal pain. Life Sciences, 39 (1986b) 2017-2024.

Abbott, F.V., Franklin, K.B.J., Ludwick, R.J. and Melzack, R., Apparent lack of tolerance in the formalin test suggests different mechanisms for morphine analgesia in different types of pain. Pharmacology, Biochemistry and Behaviour, 15 (1981) 637-640.

Abbott, F.V., Franklin, K.B.J. and Westbrook, R.F., The formalin test: scoring properties of first and second phases of the pain response in rats. Pain, 60 (1995) 91-102.

Abbott, F.V., Hong, Y. and Blier, P., Activation of 5-HT2A Receptors Potentiates Pain Produced by Inflammatory Mediators. Neuropharmacology, 35:1 (1996) 99-110.

Abbott, F.V., Oevirk, R., Najafee, R.H. and Franklin, K.B.J., Improving the efficiency of the formalin test. In press.

Adams, R.D. and Victor, M., Principles of neurology, McGraw-Hill (1993) 111-129.

Ahmadiani, A., Fereidoni, M., Semnanian, S., Kamalinejad, M. and Saremi, S., Antinociceptive and anti-inflammatory effects of sambucus ebulus rhizome extract in rats. Journal of Ethnopharmacology, 61 (1998) 229-235.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. And Watson, J.D., Molecular biology of the cell. Garland Publishing Inc., 1994.

Aloisi, A.M., Casamenti, F., Scali, C., Pepeu, G. and Carli, G., Effects of novelty, pain and stress on hippocampal extracellular acetylcholine levels in male rats. Brain Research, 748 (1997) 219-226.

Alreja, M., Mutalik, P., Nayar, U. and Manchanda, S.K., The formalin test: a tonic pain model in the primate. Pain, 20 (1984) 97-105.

Altier, N. and Stewart, J., Dopamine receptor antagonists in the nucleus accumbens attenuate analgesia induced by ventral tegmental area substance P or morphine and by nucleus accumbens amphetamine. Journal of Pharmacology and Experimental Therapeutics, 285 (1998) 208-215.

Altier, N. and Stewart, J., Neuropeptide FF in the VTA blocks the analgesic effects of both intra-VTA morphine and exposure to stress. Brain Research, 758 (1997a) 250-4.

Altier, N. and Stewart, J., Tachykinin NK-1 and NK-3 selective agonists induce analgesia in the formalin test for tonic pain following intra-VTA or intra-accumbens microinfusions. Behavioural Brain Research, 89 (1997b) 151-165.

Bannon, A.W., Decker, M.W., Curzon, P., Buckley, M.J., Kim, D.J., Radek, R.J., Lynch, J.K., Wasicak, J.T., Lin, N.H., Arnold, W.H., Holladay, M.W., Williams, M. and Arneric, S.P., ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective antinociceptive agent acting via neuronal nicotinic acetylcholine receptors: II. In vivo characterization. Journal of Pharmacology & Experimental Therapeutics, 285 (1998) 787-794.

Bardin, L., Bardin, M., Lavarenne, J. and Eschalier, A., Effect of intrathecal serotonin on nociception in rats: influence of the pain test used. Experimental Brain Research, 118 (1997) 81-87.

Barr, G.A., Maturation of the biphasic behavioral and heart rate response in the formalin test. Pharmacology, Biochemistry & Behavior, 60 (1998) 329-335.

Bennett, K.A. and Peterson, K.E., A modification of the jump-flinch technique for measuring pain sensitivity in rats. Pharmacology, Biochemistry and Behaviour, 3 (1975) 1-47.

Bhatnagar, S., Dallman, M.F., Roderick, R.E., Basbaum, A.I. and Taylor, B.K., The effects of prior chronic stress on cardiovascular responses to acute restraint and formalin injection. Brain Research, 797 (1998) 313-320.

Bowd, A.D., Ethics and animal experimentation. American Psychologist, 35 (1980) 224-225.

Broadhurst, P.L., Determinants of emotionality in the rat: I. Situational factors. British Journal of Psychology, 48 (1957) 1-12.

Brown, J.H., Kiessel, J.W. and Lish, P.M., Studies on the acute inflammatory response. I. Involvement of the central nervous system in certain models of inflammation. Journal of Pharmacology and Experimental Therapeutics, 160 (1968) 231-242.

Buerkle, H., Marsala, M. and Yaksh, T.L., Effect of continuous spinal remifentanil infusion on behaviour and spinal glutamate release evoked by subcutaneous formalin in the rat. British Journal of Anaesthesia, 80 (1998) 348-353.

Cadet, R., Pajot, J., Papon, A. and Woda, A., Is there a correlation between scores of nociceptive behavioral responses to formalin injections given at different body sites in the rat? Neuroscience Letters, 242 (1998) 123-126.

Carli, G., Farabollini, F. And Fontani, g., Effects of pain, morphine and naloxone on the duration of animal hypnosis. Behavioural Brain Research, 2 (1981) 373-385.

Carli, G., Lefebvre, L., Silvano, G. and Vierucci, S., Suppression of accompanying reactions to prolonged noxious stimulation during animal hypnosis in the rabbit. Experimenta Neurology, 53 (1976) 1-11.

Carli, G., Montesano, A., Rapezzi, S. And Paluffi, G., Differential effects of persistent nociceptive stimulation on sleep stages. Behavioural Brain Research, 26 (1987) 89-98.

Carlton, S.M. and Zhou, S.T., Attenuation of formalin-induced nociceptive behaviors following local peripheral injection of gabapentin. Pain, 76 (1998) 201-207.

Carmody, J., Knodler, L and Murray, S., Paradoxical modulation of nociception in mice by barbiturate agonism and antagonism: is a GABA site involved in nociception? European Journal of Neuroscience 3 (1991) 833-838.

Chaplan, S.R., Malmberg, A.B. and Yaksh, T.L., Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. Journal of Pharmacology & Experimental Therapeutics, 280 (1997) 829-838.

Chapman, C.R., Casey, K.L., Dubner, R., Foley, K.M., Gracely, R.H. and Reading, A.E., Pain measurement: an overview. Pain, (1985) 1-31.

Coderre, T.J. and Melzack, R., The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. Journal of Neuroscience 12 (1992a) 3665-3670.

Coderre, T.J. and Melzack, R., The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. Journal of Neuroscience, 12 (1992b) 3671-3675.

Coderre, T.J. and Wall, P.D., Ankle join urate arthritis (AJUA) in rats: an alternative animal model of arthritis to that produce by Freund's adjuvant. Pain, 28 (1987) 379-393.

Coderre, T.J., Abbott, F.V. and Melzack, R., Behavioural evidence in rats for a peptidergic-noradrenergic interaction in cutaneous sensory and vascular function. Neuroscience Letters 47 (1984) 113-118.

Coderre, T.J., Fundytus, M.E., McKenna, J.E., Dalal, S. and Melzack, R., The formalin test: a validation of the weighted-scores method of behavioural pain rating. Pain, 54 (1993) 43-50.

Coderre, T.J., Vaccarino, A.L. and Melzack, R., Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Research 535 (1990) 155-158.

Courteix, C., Bourget, P., Caussade, F., Bardin, M., Coudore, F., Fialip, J. and Eschalier, A., Is the reduced efficacy of morphine in diabetic rats caused by alterations of opiate receptors or of morphine pharmacokinetics? Journal of Pharmacology & Experimental Therapeutics, 285 (1998) 63-70.

Culman, J., Ritter, S., Ohlendorf, C., Haass, M., Maser-Gluth, C., Spitznagel, H. and Unger, T., A new formalin test allowing simultaneous evaluation of cardiovascular and nociceptive responses. Canadian Journal of Physiology & Pharmacology, 75 (1997) 1203-1211.

D'Amour, F.E. and Smith D., A method for determining loss of pain sensation. Journal of Pharmacology and Experimental Therapy, 72 (1941) 74-79.

Dalal, S. and Melzack, R., Psychostimulant drugs potentiate morphine analgesia in the formalin test. Journal of Pain & Symptom Management, 16 (1998) 230-239.

Davidson, E.M., Coggeshall, R.E. and Carlton, S.M., Peripheral NMDA and non-NMDA glutamate receptors contribute to nociceptive behaviors in the rat formalin test. Neuroreport, 8 (1997) 941-946.

Dickenson, A.H., Central acute pain mechanisms. Annals of Medicine, 27 (1995) 223-227.

Dirig, D.M., Konin, G.P., Isakson, P.C. and Yaksh, T.L., Effect of spinal cyclooxygenase inhibitors in rat using the formalin test and in vitro prostaglandin E2 release. European Journal of Pharmacology, 331 (1997) 155-160.

Doak, G.J. and Sawynok, J., Formalin-induced nociceptive behavior and edema: involvement of multiple peripheral 5-hydroxytryptamine receptor subtypes. Neuroscience, 80 (1997) 939-949.

Dubner R., Methods of assessing pain in animals. In: Textbook of Pain, Wall, P.D. and Melzack, R. (Eds), Churchill Livingstone, 1994.

Dubuisson, D. and Dennis, S., The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain, 4 (1977) 161-174.

Erb, K., Liebel, J.T., Tegeder, I., Zeilhofer, H.U., Brune, K. and Geisslinger, G., Spinally delivered nociceptin/orphanin FQ reduces flinching behaviour in the rat formalin test. Neuroreport, 8 (1997) 1967-1970.

Euchenhofer, C., Maihofner, C., Brune, K., Tegeder, I. and Geisslinger, G., Differential effect of selective cyclooxygenase-2 (COX-2) inhibitor ns 398 and diclofenac on formalin-induced nociception in the rat. Neuroscience Letters, 248 (1998) 25-28.

Farneslow, M.S., Shock-induced analgesia on the formalin test: effects of shock severity, naloxone, hypophysectomy, and associative variables. Behavioural neuroscience, 98 (1984) 79-95.

Fasmer, O.B., Hunskaar, S., and Hole, K., Antinociceptive effects of serotonergic reuptake inhibitors in mice. Neuropharmacology, 28 (1989) 1363-1366.

Field, M.J., Oles, R.J., Lewis, A.S., McCleary, S., Hughes, J. and Singh, L., Gabapentin (neurontin) and S-(+)-3-isobutylgaba represent a novel class of selective antihyperalgesic agents. British Journal of Pharmacology, 121 (1997) 1513-1522.

Fields, H., and Basbaum, A. Endogenous pain control mechanisms. In: Textbook of Pain, 3rd edn., Wall, P.D., and Melzack, R. (Eds), Churchill Livingston, 1994.

Franklin, K.B.J., Analgesia and the neural substrate of reward. Neuroscience and Biobehavioral Reviews 13 (1989) 149-154.

Franklin, K.B.J., Analgesia and abuse potential: an accidental association or a common substrate? Pharmacology, Biochemistry and Behaviour, 59 (1998) 993-1002.

Franklin, K.J.B. and Abbott, F.V., Techniques for assessing the effects of drugs on nociceptive responses. In: Neuromethods: Vol. 13, Psychopharmacology I, pp., A.A. Boulton, C.B. Baker and A.J. Greenshaw (Eds), Humana Press (1989) 145-216.

Fuchs, P.N. and Melzack, R., Repeated cold water swim produces delayed nociceptive responses, but not analgesia, for tonic pain in the rat. Experimental Neurology, 145 (1997) 303-307.

Giardina, W.J., Decker, M.W., Porsolt, R.D., Roux, S., Collins, S.D., Kim, D.J.B. and Bannon, A.W., An evaluation of the gaba uptake blocker tiagabine in animal models of neuropathic and nociceptive pain. Drug Development Research, 44 (1998) 106-113.

Gilbert, A.K. and Franklin, K.B.J., Modulatory effects of muscimol administered into the rostroventral medulla on formalin pain and morphine analgesia: a dose-response analysis. Neuroscience abstracts (1998) 251.11.

Granados-Soto, V., Rufino, M.O., Gomes, L.L.D. and Ferreira, S.H., Evidence for the involvement of the nitric oxide-cGMP pathway in the antinociception of morphine in the formalin test. European Journal of Pharmacology, 340 (1997) 177-180.

Hao, S.L. and Ogawa, H., Naltrexone, but not, atropine or yohimbine, antagonizes suppression of formalin-induced spinal sensitization by intrathecal nociceptin. Life Sciences, 63 (1998a) PL167-PL173.

Hao, S.L. and Ogawa, H., Sevoflurane suppresses behavioral response in the rat formalin test - combination with intrathecal lidocaine produced profound suppression of the response. Neuroscience Letters, 248 (1998b) 121-126.

Harvey, S.C., Antiseptics and disinfectants: fungicides, ectoparasiticides. In: The pharmacological basis of therapeutics, 5th edition. Goodman, L.S. and Gilman, A. (Eds). Macmillan (1975) 987-1017.

Hermanson, O. and Blomqvist, A., Differential expression of the AP-1 CRE-binding proteins FOS and CREB in preproenkephalin mRNA-expressing neurons of the rat parabrachial nucleus after nociceptive stimulation. Molecular Brain Research, 51 (1997) 188-196.

Hermanson, O., Telkov, M., Geijer, T., Hallbeck, M. and Blomqvist, A., Preprodynorphin mRNA-expressing neurones in the rat parabrachial nucleus: subnuclear localization, hypothalamic projections and colocalization with noxious-evoked fos-like immunoreactivity. European Journal of Neuroscience, 10 (1998) 358-367.

Hille, B. Ionic channels of excitable membranes, 2nd ed., Sounderland: Sinaur associates Inc., 1992.

Hong, Y. and Abbott F.V., Contribution of peripheral 1α-adrenoreceptors to pain induced by formalin or by -methyl-5-hydroxytryptamine plus noradrenaline. European Journal of Pharmacology, 301 (1996) 41-48.

Hou, W.Y., Shyu, B.C., Chen, T.M., Lee, J.W., Shieh, J.Y. and Sun, W.Z., Intrathecally administered c-fos antisense oligodeoxynucleotide decreases formalin-induced nociceptive behavior in adult rats. European Journal of Pharmacology, 329 (1997) 17-26.

Hua, X.Y., Chen, P., Hwang, J. and Yaksh, T.L., Antinociception induced by civamide, an orally active capsaicin analogue. Pain, 71 (1997) 313-322.

Hughes, R.A. and Sufka, K.J., Morphine hyperalgesic effects on the formalin test in domestic fowl (*Gallus gallus*). Pharmacology, Biochemistry and Behaviour, 38 (1991) 247-251.

Hunskaar, S. and Hole, K., The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain, 30 (1987) 103-114.

Hunskaar, S., Fasmer, O.B. and Hole, K., Formalin test in mice, a useful technique for evaluating mild analgesics. J. Neurosci. Meth. 14 (1985) 67-76.

Iyengar, S., Hipskind, P.A., Gehlert, D.R., Schober, D., Lobb, K.L., Nixon, J.A., Helton, D.R., Kallman, M.J., Boucher, S., Couture, R., Li, D.L. and Simmons, R.M., LY303870, a centrally active neurokinin-1 antagonist with a long duration of action. Journal of Pharmacology & Experimental Therapeutics, 280 (1997) 774-785.

Jaggar, S.I., Hasnie, F.S., Sellaturay, S. and Rice, A.S., The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. Pain, 76 (1998) 189-199.

Jessel, T.M. and Kelly D.D., Pain and analgesia. In: Principles of neural science, Kandel E.R., Schwartz, J.H. and Jessel, T.M. (Eds), Appleton & Lange, 1991.

Jett, M.F and Michelson, S., The formalin test in the rat: validation of an automated system. Pain, 64 (1996) 19-25

John, J.M., Kaneko, M. and Hammond, D.L., Intrathecal bicuculline does not increase formalin-induced inflammation. Brain Research, 794 (1998) 320-324.

Jourdan, D., Ardid, D., Bardin, L., Bardin, M., Neuzeret, D., Lanphouthacoui, L. And Eschalier, A., A new automated method of pain scoring in the formalin test in rats. Pain, 71 (1997) 265-270.

Kang, W., Wilson, M.A., Bender, M.A., Glorioso, J.C. and Wilson, S.P., Herpes virus-mediated preproenkephalin gene transfer to the amygdala is antinociceptive. Brain Research, 792 (1998) 133-135.

Kanui, T.I., Hole, K. and Miaron, J.O., Nociception in crocodiles - capsaicin instillation, formalin and hot plate tests. Zoological Science, 7 (1990) 537-540.

Kauppila, T., Jyvasjarvi, E., Hamalainen, M.M. and Pertovaara, A., The effect of a selective alpha2-adrenoceptor antagonist on pain behavior of the rat varies, depending on experimental parameters. Pharmacology, Biochemistry & Behavior, 59 (1998) 477-485.

King, T.E., Joynes, R.L., Meagher, M.W. and Grau, J.W., Impact of shock on pain reactivity: II. Evidence for enhanced pain. Journal of Experimental Psychology: Animal Behaviour Processes, 22 (1996) 265-278.

Kwak, J.Y., Jung, J.Y., Hwang, S.W., Lee, W.T. and Oh, U., A capsaicin-receptor antagonist, capsazepine, reduces inflammation-induced hyperalgesic responses in the rat - evidence for an endogenous capsaicin-like substance. Neuroscience, 86 (1998) 619-626.

Li, T., Gao, W. and Rao, Z.R., Noxious somatic stimulation-induced expression of Fos-like immunoreactivity in catecholaminergic neurons with habenular nucleus projection in the medullary visceral zone of rat. Brain Research, 783 (1998) 51-56.

Liles, J.H. and Flecknell, P.A., A comparison of the effects of buprenorphine, carprofen and flunixin following laparotomy in rats. Journal of Veterinary Pharmacology and Therapeutics, 17 (1994) 284-290.

Liu, R.J., Qiang, M. and Qiao, J.T., Nociceptive c-fos expression in supraspinal areas in avoidance of descending suppression at the spinal relay station. Neuroscience, 85 (1998) 1073-1087.

Luccarini, P., Cadet, R., Duale, C. and Woda, A., Effects of lesions in the trigeminal oralis and caudalis subnuclei on different orofacial nociceptive responses in the rat. Brain Research, 803 (1998) 79-85.

Machelska, H., Labuz, D., Przewlocki, R. and Przewlocka, B., Inhibition of nitric oxide synthase enhances antinociception mediated by mu, delta and kappa opioid receptors in acute and prolonged pain in the rat spinal cord. Journal of Pharmacology & Experimental Therapeutics, 282 (1997) 977-984.

Manning, B.H. and Franklin, K.B., Morphine analgesia in the formalin test: reversal by microinjection of quaternary naloxone into the posterior hypothalamic area or periaqueductal gray. Behavioural Brain Research, 92 (1998) 97-102.

Margneanu, D.G., and Van Driessche, W., Effects of millimolar concentrations of glutaraldehyde on the electrical properties of frog skin. Journal of Physiology, 427 (1990) 567-581.

Matthies, B.K., and Franklin, K.B., Formalin pain is expressed in decerebrate rats but not attenuated by morphine. Pain, 51 (1992) 199-206.

McCall, W.D., Tanner, K.D. and Levine, J.D., Formalin induces biphasic activity in C-fibers in the rat. Neuroscience Letters, 208 (1996) 45-48.

Mcnally, G.P. and Westbrook, R.F., Test type influences the expression of lithium chloride-induced hyperalgesia. Pharmacology, Biochemistry & Behavior, 61 (1998) 385-394.

Melzack, R. and Patrick D. Wall, The Challenge of pain, Penguin Books Ltd., 1996.

Melzack, R. and Katz, J., Pain measurement in persons in pain. In: Textbook of pain, Wall, P.D. and Melzack, R. (Eds), Churchill Livingstone, 1994.

Melzack, R., Wall, P.D. and Ty, C. T., Acute pain in an emergency clinic: latency of onset and descriptor patterns related to different injuries. Pain, 14 (1982) 33-43.

Merskey, H. and Bogduk, N. (Eds), Classification of chronic pain (2nd Ed.), IASP Task Force on Taxonomy, IASP Press, 1994.

Meyer, R.A., Campbell, J.N. and Raja, S.N., Peripheral neural mechanisms of nociception. In: Textbook of pain, Wall, P.D. and Melzack, R. (Eds), Churchill Livingstone, 1994.

Miampamba, M., Chery-Croze, S. and Chayvialle, J.A., Spinal and Intestinal Levels of Substance P, Calcitonin Gene-related Peptide and Vasoactive Intestinal Polypeptide Following Perendoscopic Injection of Formalin in Rat Colonic Wall, Neuropeptides, 22 (1992) 73-80.

Militzer, K., Histometrische Untersuchungen an Epidermis und Korium der Rattenpfote nach experimentellem Entzunungsreiz. Arch. Derm. Res., 256 (1976) 151-166.

Morrow, T.J., Paulson, P.E., Danneman, P.J. and Casey, K.L., Regional changes in forebrain activation during the early and late phase of formalin nociception: analysis using cerebral blood flow in the rat. Pain, 75 (1998) 355-365.

Nencini, P., Fraioli, S., Pascucci, T. and Nucerito, C.V., (-)-Norpseudoephedrine, a metabolite of cathinone with amphetamine-like stimulus properties, enhances the analgesic and rate decreasing effects of morphine, but inhibits its discriminative properties. Behavioural Brain Research, 92 (1998) 11-20.

Neoh, C.-A., Jawan, B. And Lee, J.H., Does site of injection play any important role in the formalin pain test. Chin. J. Pain, 2 (1992) 51-60.

Nozaki-Taguchi, N. and Yamamoto, T., The interaction of FK409, a novel nitric oxide releaser, and peripherally administered morphine during experimental inflammation. Anesthesia & Analgesia, 86 (1998a) 367-373.

Nozaki-Taguchi, N. and Yamamoto, T., Involvement of nitric oxide in peripheral antinociception mediated by kappa- and delta-opioid receptors. Anesthesia & Analgesia, 87 (1998b) 388-393.

Omote, K., Kawamata, T., Kawamata, M. and Namiki, A., Formalin-induced release of excitatory amino acids in the skin of the rat hindpaw. Brain Research, 787 (1998) 161-164.

Ortega-Alvaro, A., Gibert-Rahola, J., Mellado-Fernandez, M.L., Chover, A.J. and Mico, J.A., The effects of different monoaminergic antidepressants on the analgesia induced by spinal cord adrenal medullary transplants in the formalin test in rats. Anesthesia and Analgesia, 84 (1997) 816-820.

Pelissier, T., Bustamante, D., Saavedra, H., Tampier, L., Vergara, V. and Paeile, C., New differences between the Wistar rat and Octogun degus, a putative laboratory animal resistant to morphine. Comparative Biochemistry and Physiology, 93 (1989) 359-366.

Peterson, M.A., Basbaum, A.I., Abbadie, C., Rohde, D.S., McKay, W.R. and Taylor, B.K., The differential contribution of capsaicin-sensitive afferents to behavioral and cardiovascular measures of brief and persistent nociception to Fos expression in the formalin test. Brain Research, 755 (1997) 9-16.

Pini, L.A., Vitale, G. and Sandrini, M., Serotonin and opiate involvement in the antinociceptive effect of acetylsalicylic acid. Pharmacology, 54 (1997) 84-91

Pini, L.A., Vitale, G., Ottani, A. and Sandrini, M., Naloxone-reversible antinociception by paracetamol in the rat. Journal of Pharmacology & Experimental Therapeutics, 280 (1997) 934-940.

Porro, C.A. and Cavazzuti, M., Spatial and temporal aspects of spinal cord and brainstem activation in the formalin pain model. Progress in Neurobiology, 41 (1993) 565-607.

Przesmycki, K., Dzieciuch, J.A., Czuczwar, S.J. and Kleinrok, Z., An isobolographic analysis of drug interaction between intrathecal clonidine and baclofen in the formalin test in rats. Neuropharmacology, 37 (1998) 207-214.

Przesmycki, K., Dzieciuch, J.A., Czuczwar, S.J. and Kleinrok, Z., Isobolographic analysis of interaction between intrathecal morphine and clonidine in the formalin test in rats. European Journal of Pharmacology, 337 (1997) 11-17.

Puig, S. and Sorkin, L.S., Subcutaneous formalin evoked activity in single fibers of rat sural nerve. Society of Neuroscience abstracts (1994) 319.12.

Randolph, B.C. and Peters, M.A., Analgesic effectiveness of ketorolac compared to meperidine in the rat formalin test. Anesthesia Progress, 44 (1997) 11-16.

Ross, E.M., Pharmacodynamics. Mechanisms of Drug Action and the Relationship Between Drug Concentration and Effect. In: Goodman & Gilman's The Pharmacological Basis of Therepeutics. Eds: Hardman, J.G., Limbird, L.E., Molinoff P.B., Ruddon, R.W. and Gilman, A.G., McGraw-Hill, 1996.

Ryan, S.M., Watkins, L.R., Mayer, D.J. and Maier, S.F., Spinal pain suppression mechanisms may differ for phasic and tonic pain. Brain Research, 334 (1985) 172-175.

Sandrini, M., Ottani, A., Vitale, G. and Pini, L.A., Acetylsalicylic acid potentiates the antinociceptive effect of morphine in the rat - involvement of the central serotonergic system. European Journal of Pharmacology, 355 (1998) 133-140.

Sawynok, J. and Reid, A., Peripheral adenosine 5'-triphosphate enhances nociception in the formalin test via activation of a purinergic p2X receptor. European Journal of Pharmacology, 330 (1997) 115-121.

Sawynok, J., Reid, A. and Poon, A., Peripheral antinociceptive effect of an adenosine kinase inhibitor, with augmentation by an adenosine deaminase inhibitor, in the rat formalin test. Pain, 74 (1998) 75-81.

Shibata, M., Ohkubo, T., Takahashi, H., and Inoki, R., Modified formalin test: characteristic biphasic pain response. Pain, 38 (1989) 347-352.

Shimoyama, N., Shimoyama, M., Davis, A.M., Inturrisi, C.E. and Elliott, K.J. Spinal gabapentin is antinociceptive in the rat formalin test. Neuroscience Letters, 222 (1997) 65-67.

Shimoyama, N., Shimoyama, M., Elliott, K.J. and Inturrisi, C.E., d-Methadone is antinociceptive in the rat formalin test. Journal of Pharmacology & Experimental Therapeutics, 283 (1997) 648-652.

Simmons, R.M.A., Li, D.L., Hoo, K.H., Deverill, M., Ornstein, P.L. and Iyengar, S., Kainate glur5 receptor subtype mediates the nociceptive response to formalin in the rat. Neuropharmacology, 37 (1998) 25-36.

Sugimoto, M., Kuraishi, Y., Satoh, M. and Takagi, H., Involvement of medullary opioid-peptidergic and spinal noradrenergic systems in the regulation of formalin-induced persistent pain. Neuropharmacology, 25 (1986) 481-485.

Takahashi, H., Ohkubo, T., Shibata, M. and Naruse, S., A modified formalin test for measuring analgesia in mice. Jpn. J. Oral. Biol., 26 (1984) 543-548.

Taylor, B.K., Akana, S.F., Peterson, M.A., Dallman, M.F. and Basbaum, A.I., Pituitary-adrenocortical responses to persistent noxious stimuli in the awake rat: endogenous corticosterone does not reduce nociception in the formalin test. Endocrinology, 139 (1998) 2407-2413.

Taylor, B.K., Peterson, M.A. and Basbaum, A.I., Continuous intravenous infusion of naloxone does not change behavioral, cardiovascular, or inflammatory resonses to subcutaneous formalin in the rat. Pain, 69 (1997) 171-177.

Teng, C.J. and Abbott, F.V., The formalin test: a dose-respone analysis at three developmental stages. Pain, 76 (1998) 337-347.

Terman, G.W., Shavit, Y., Lewis, J.W., Cannon, J.T. and Liebeskind, J.C., Intrinsic mechanisms of pain inhibition: activation by stress. Science, 226 (1984) 1270-1277.

Tjølsen, A., Berge, O.-G. and Hole, K., Lesions of bulbo-spinal serotonergic or noradrenergic pathways reduce nociception as measured by the formalin test. Acta Physiol. Scandinav., 142 (1991a) 229-236.

Tjølsen, A., Berge O.-G., Hunskaar, S., Rosland, J.H. and Hole, K., The formalin test: an evaluation of the method. Pain, 51 (1992) 5-17.

Tjølsen, A., Lund, A., Berge, O.-G. and Hole, K., Antinociceptive effect of paracetamol in rats is partly dependent on the spinal serotonergic systems. European Journal of Pharmacology, 193 (1991b) 193-201.

Vaccarino, A.L., Clemmons, H.R., Mader, G.J.J. and Magnusson, J.E., A role of periaqueductal grey NMDA receptors in mediating formalin-induced pain in the rat. Neuroscience Letters, 236 (1997) 117-119.

Vitale, G., Pini, L.A., Ottani, A. and Sandrini, M., Effect of acetylsalicylic acid on formalin test and on serotonin system in the rat brain. General Pharmacology, 31 (1998) 753-758.

Vyklicky, L., Techniques for the study of pain in animals. In: Advances in pain research and therapy, Vol. 3., Bonica, J.J., Liebeskind, J.C. and Albe-Fessard, D.G. (Eds). Raven Press, New York, (1979) 727-745.

Watson, G.S., Sufka, K.J. and Coderre, T.J., Optimal scoring strategies and weights for the formalin test in rats. Pain, 70 (1997) 53-58.

Wheeler-Aceto, H. and Cowan, A., Standardization of the rat paw formalin test for the evaluation of analgesics. Psychopharmacology, 104 (1991) 35-44.

Wheeler-Aceto, H., Porreca, F. and Cowan, A., The rat paw formalin test: comparison of noxious agents. Pain, 40 (1990) 229-238.

Yaksh, T.L., and Malmberg, A.B., Central pharmacology of nociceptive transmission. In: Textbook of Pain, 3rd edn., Wall, P.D., and Melzack, R. (Eds), Churchill Livingston, Edinburgh, 1994, pp. 165-200.

Yamamoto, T., Nozaki-Taguchi, N. and Kimura, S., Analgesic effect of intrathecally administered nociceptin, an opioid receptor-like1 receptor agonist, in the rat formalin test. Neuroscience, 81 (1997) 249-254.

Yashpal, K., Mason, P., McKenna, J.E., Sharma, S.K., Henry, J.L. and Coderre, T.J., Comparison of the effects of treatment with intrathecal lidocaine given before and after formalin on both nociception and Fos expression in the spinal cord dorsal horn. Anesthesiology, 88 (1998) 157-164.

Zangen, A., Herzberg, U., Vogel, Z. and Yadid, G., Nociceptive stimulus induces release of endogenous beta-endorphin in the rat brain. Neuroscience, 85 (1998) 659-662.

Zhu, C.B., Cao, X.D., Xu, S.F. and Wu, G.C., Orphanin FQ potentiates formalin-induced pain behavior and antagonizes morphine analgesia in rats. Neuroscience Letters, 235 (1997) 37-40.

APPENDIX I

Scientific reports published in the world literature in 1997-1998 using the rat formalin model of acute injury-induced pain. The table shows the authors, the formalin concentration and volume, the pain measure used if any, the time for which pain was recorded and whether the rats were habituated prior to testing.

Appendix I

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Abbadie et al. 1997	2	50	2	s.c., plantar surface of hind paw	flinching "	-	-	-
Abbott & Bonder 1997	2.5	50	2.5	s.c., plantar surface of hind paw	lift-lick '	-	50	15 min x 5 days
Abbott et al. 1997	1	50	1	s.c., plantar surface of hind paw	lift-lick '	-	40	15 min x 5 days
Ahmadiani et al. 1998	5	50	5	-	flinching "	-	45	-
Aloisi et al. 1997	10	50	10	s.c., dorsal surface of hind paw	licking ' ; flexing ' ; jerking	-	90	-
Altier & Stewart 1998	2.5	50	2.5	s.c., plantar surface of hind paw	WMS 1S	-	60	2 x 60 min
Altier & Stewart 1997b	2.5	50	2.5	s.c., plantar surface of hind paw	WMS 1S	-	60	-
Altier & Stewart 1997a	2.5	50	2.5	s.c., plantar surface of hind paw	WMS 1S	-	60	2 x 60 min

First Author	forma-lin [%]	µl	equiva-lents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	dura-tion [min]	habitua-tion
Bannon et al. 1998	5	50	5	s.c., dorsal surface of hind paw	flinching "; lick '	-	60	-
Bardin et al. 1997	5	50	5	s.c., plantar surface of hind paw	licking '	-	30	-
Barr G.A. 1998	10	10	2	s.c., plantar surface of hind paw	WMS (TS)	-	60	-
Bhatnagar et al. 1998	1.25	50	1.25	s.c., hind paw	flinching "; lick '	blood pressure, heart rate	70	14 hours
Bhatnagar et al. 1998	10	100	20	s.c., distal part of tail	-	-	-	-
Buerkle et al. 1998	5	50	5	s.c., hind paw	flinching "	-	60	-
Cadet et al. 1998	1.5	50	1.5	paw or upper lip	flinching / rubbing	-	75	15 min x 2 days
Carlton & Zhou 1998	2	20	1.3	-	lift-lick 1; flinching "	-	60	15 min x 2 days

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Chaplan et al. 1997	5	50	5	s.c., dorsal surface of hind paw	flinching [#]	-	60	-
Courteix et al. 1998	5	50	5	s.c., dorsal surface of forepaw	WMS [§]	-	45	15 min
Culman et al. 1997	2.5	50	2.5	s.c., hind paw	licking [§]	-	15	-
Dalal & Melzack 1998	2.5	50	2.5	s.c., plantar surface of hind paw	WMS [§]	-	50	-
Davidson et al. 1997	5	15	1.5	s.c., plantar surface of hind paw	lift+lick [§]	-	45	3 x 30 min
Dirig et al. 1997	5	50	5	s.c., dorsal surface of hind paw	flinching [#]	PGE_2 radioimmuno assay	60	-
Doak & Sawynok 1997	2.5	50	2.5	s.c., dorsal surface of hind paw	flinching	-	60	-
Erb et al. 1997	5	50	5	s.c., dorsal surface of hind paw	flinching [#]	-	60	15 min

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Euchenhofer et al. 1998	5	50	5	s.c., dorsal surface of hind paw	flinching #	-	60	15 min
Field et al. 1997	5	50	5	s.c., plantar surface of hind paw	licking #	-	45	15 min
Fuchs & Melzack 1997	2.5	50	2.5	s.c., plantar surface of hind paw	WMS ‘	-	120	20 min
Giardina et al. 1998	5	50	5	s.c., hind paw	flinching #	-	35	-
Granados-Soto et al. 1997	5	50	5	s.c., hind paw	flinching #	-	60	30 min
Hao & Ogawa 1998b	5	50	5	s.c., dorsal surface of hind paw	flinching #	-	60	-
Hao & Ogawa 1998a	5	50	5	s.c., dorsal surface of hind paw	flinching #	-	60	-
Hermanson et al. 1998	5	100	10	-	-	C-fos	-	3 hours
Hermanson & Blomqvist, 1997	5	100	10	-	-	C-fos	-	3 hours

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Hou et al. 1997	5	50	5	s.c., plantar surface of hind paw	WMS [1]; flinching [2]; licking/biting [3]	-	60	none
Hua et al. 1997	5	50	5	s.c., dorsal surface of hind paw	flinching [4]	-	60	-
Iyvengar et al. 1997	5	50	5	s.c., dorsal surface of hind paw	licking T [5]	-	50	60 min
Jaggar et al. 1998	2.5	50	2.5	s.c., dorsal surface of hind paw	WMS [1]	-	60	15 min x 4 days
John et al. 1998	5	100	10	-	-	extravasation	-	-
Jourdan et al. 1997	5	50	5	s.c., plantar surface of hind paw	WMS (automated)	-	60	-
Kang et al. 1998	1	50	1	s.c., dorsal surface of hind paw	flinching [2]	-	60	-
Kauppila et al. 1998	5	50	5	s.c., plantar surface of hind paw	lift-lick [1]	-	30	-

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Kwak et al. 1998	5	150	15	-	-	C-fos	-	-
Li et al. 1998	8	50	8	-	-	C-fos	-	-
Liu et al. 1998	2	200	8	-	-	C-fos	-	-
Luccarini et al. 1998	1.5	50	1.5	upper lip	rubbing	-	45	-
Machelska et al. 1997	12	100	24	-	flinching #	-	75	-
Manning & Franklin 1998	2.5	50	2.5	s.c., plantar surface of hind paw	lift-lick °	-	50	-
Mcnally & Westbrook, 1998	1.5	50	1.5	s.c., plantar surface of hind paw	lift-lick °	-	40	15 min x 4 days
Morrow et al. 1998	2.5	50	2.5	s.c., hind paw	-	cerebral blood flow	25	1-2 weeks
Nencini et al. 1998	5	50	5	s.c., dorsal surface of hind paw	flinching	-	60	-

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Nozaki-Taguchi & Yamamoto 1998a	2.5	50	2.5	s.c., plantar surface of hind paw	flinching [#]	-	60	-
Nozaki-Taguchi & Yamamoto 1998b	2.5	50	2.5	s.c., plantar surface of hind paw	flinching [#]	-	60	-
Omote et al. 1998	5	50	5	-	-	-	-	-
Ortega-Alvaro et al. 1997	13.5	100	13.5	-	licking [‡]	-	50	20-30 min
Peterson et al. 1997	5	100	10	s.c., dorsal surface of hind paw	flinching [#]	-	50	18 hours
Pini et al. 1997	5	50	5	s.c., dorsal surface of hind paw	flinching [#]	-	60	-
Pini et al. 1997	5	50	5	s.c., dorsal surface of hind paw	flinching	-	60	2 hours
Przesmycki et al. 1998	5	50	5	s.c., plantar surface of hind paw	flinching [#]	rotorod test of motor performance	-	-

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Przesmycki et al. 1997	5	50	5	s.c., plantar surface of hind paw	flinching "	-	60	-
Randolph & Peters 1997	5	50	5	s.c., hind paw	lick '	-	50	24 hours
Sandrini et al. 1998	5	50	5	s.c., dorsal surface of hind paw	flinching "	-	40	-
Sawynok & Reid 1997	0.5	50	0.5	s.c., dorsal surface of hind paw	flinching "	-	60	30 min
Sawynok et al. 1998	1.5	50	1.5	s.c., dorsal surface of hind paw	flinching '	-	60	20 min
Shimoyama et al. 1997	5	50	5	s.c., hind paw	flinching "	-	60	-
Shimoyama et al. 1997	5	50	5	s.c., hind paw	flinching "; licking '	-	60	-
Simmons et al. 1998	5	50	5	s.c., dorsal surface of hind paw	licking '	rotorod test of motor performance	50	1 hours

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Taylor et al. 1998	5	50	5	-	flinching [#]; licking [‡]	-	90	-
Taylor et al. 1997	2	50		s.c., plantar surface of hind paw	flinching [#]	-	70	-
Teng & Abbott 1998	10	50		s.c., plantar surface of hind paw	lift+lick FS	-	60	-
Vaccarino et al. 1997	2.5	50	2.5	s.c., plantar surface of hind paw	WMS [‡]	-	70	10 min
Vitale et al. 1998	5	50	5	-	flinching [#]	-	60	2 hours
Watson et al. 1997	5	50	5	s.c., plantar surface of hind paw	WMS [‡]	ultrasound to measure activity	50	-
Yamamoto et al. 1997	5	50	5	-	flinching [#]	-	60	-
Yashpal et al. 1998	2.5	50	2.5	s.c., plantar surface of hind paw	WMS [‡]	-	60	-

First Author	formalin [%]	µl	equivalents*	site of injection	behavioural pain measure	other behavioural/ physiological measures	duration [min]	habituation
Zangen et al. 1998	10	50	10	s.c., hind paw	-	microdyalisis - arcuate nucleus	-	-
Zhu et al. 1997	5	60	6	-	WMS [C]	-	60	-

* Formalin concentration computed for a 50µl volume
WMS = weighted means score
[#] indicates simple counting quantification
[TS] indicates time-sampling method of quantification
[C] indicates continuous rating