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. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

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.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

THE PHARMACOLOGICAL EFFECTS OF INTRATHECALLY ADMINISTERED EXCITATORY AMINO ACID RECEPTOR LIGANDS IN THE FORMALIN TEST

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Barry James Connell

Dalvay, P.E.I.

December, 1996

CONDITION OF USE

SIGNATURE PAGES

iii-iv

REMOVED

ABSTRACT

The endogenous excitatory amino acids (EAAs) glutamate (GLU) and aspartate (ASP) are widely recognized as being involved in the spinal processing of peripheral nociceptive inputs. EAA receptors have been divided into two major families: ionotropic receptors, which are ligand gated ion cation channels, and metabotropic receptors, which are G-protein coupled receptors. Ionotropic receptors have been further divided into three subfamilies: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA) receptors. KA receptors have been further divided into both high- and low-affinity receptor subtypes. Each type of EAA receptor has been localized within the spinal dorsal horn and each has been shown to be involved with spinal mechanisms of nociceptive transmission. To date, however, the hypothesis that EAAs are involved in nociceptive transmission within the spinal cord has not been thoroughly tested in established whole animal models of experimental pain. The aim of the current studies was to clarify the role of EAAs in the transmission of nociceptive signals produced by injection of formalin (1.0% or 2.5%) in conscious rats. Studies were conducted by identifying the responses to EAAs administered via chronically implanted intrathecal (i.t.) cannulae. I.t. administration of both GLU and ASP produced significantly increased nociceptive responses when injected prior to 1.0% formalin. Phase 1 (0-10 min) nociception was more sensitive to the spinal administration of both GLU (5, 50, 100 and 200 nmol) and ASP (100 and 200 nmol), while Phase 2 (10-60 min) nociception was only increased following the administration of higher doses of GLU (100 and 200 nmol). Thus, GLU and ASP appear to have a role in the spinal processing of nociceptive inputs. I.t. administration of NMDA produced significant hyperalgesia during Phase 1 (0.75 and 1.0 nmol) but was without effect on Phase 2 nociception, suggesting that ASP-induced Phase 1 hyperalgesia was mediated via an NMDA receptor. I.t. administration of AMPA (100 to 500 pmol) or the EAA metabotropic receptor agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (tACPD) (0.3 to 6.0 nmol) were without effect on formalin-induced nociception in either Phase. I.t. administration of both KA (10 to 400 pmol and domoic acid (DOM) (0.05 to 10 pmol) prior to 2.5% formalin produced significant antinociceptive effects. The selective low-affinity KA receptor antagonist NS-102 (7.5 μg) significantly reversed KA- and DOM-induced antinociception indicating that the antinociception produced by both KA and DOM was mediated via low-affinity KA receptors. It is proposed that KA and DOM mediate their antinociceptive effects via low-affinity KA receptors located on the spinal dorsal roots whose activation blocked C-fibre input into the spinal dorsal horn. The blockage of C-fibre conduction by the activation of low-affinity KA receptors peripheral to the spinal dorsal horns represents a potential mechanism for analgesic drug development.

ACKNOWLEDGMENTS

I would like to thank my supervisory committee members Dr. Andy Tasker, Dr. John Burka, Dr. Bill Ireland, Dr. Alan Donald and Dr. Jamie Amend for their guidance during my time as a Master student.

I would like to especially acknowledge and thank my supervisor Dr. Andy Tasker for his invaluable insights and his extreme patience. Thanks Andy for allowing me to do things my way!

Also, thanks go to Dr. Tarek Saleh for reviewing the thesis and helping me get the story right.

Finally, I would like to thank my wife, Frances, without her love, support and encouragement this thesis would never have been completed.

DEDICATION

For My Children,

STEPHANIE ELIZABETH and CHELSEA ANN,

who will learn that

Success does not come by wishing
But by hard work bravely done.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviation	Term
ACPD	(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid
AMPA	D,L-α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid
ANOVA	analysis of variance
AP3	L(+)-2-amino-3-phosphonopropionic acid
AP4	L-amino-4-phosphonobutanoic acid
AP5	D-amino-5-phosphonopentanoic acid
AP7	(±)-2-amino-7-phosphonoheptanoic acid
ASP	aspartic acid
Asp-T	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
Ca^{2+}	calcium cation
CGS-19755	4-phosphonomethyl-2-peperidinecarboxylic acid
4C3HPG	(S)-4-carboxy-3-hydroxyphenylglycine
cm	centimetre
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione

Abbreviation	Term
CNS	central nervous system
CPP	((3)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid
CRC	concentration-vs-response curve
DAMGO	Tyr-D-Ala,Gly,Me,Phe,Gly-ol
d.f.	degrees of freedom
DGG	gamma-D-glutamylglycine
DNQX	6,7-dinitroquinoxaline-2,3-dione
DOM	domoic acid
DRC	dose response curve
EAA	excitatory amino acid
ED_{30}	30% of the maximally effective dose
ED_{50}	50% of the maximally effective dose
et al.	and others
F	variance ratio
g	gram
GABA	gamma-aminobutyric acid
GLU	glutamic acid
gluR1 to gluR4	AMPA selective binding proteins
gluR5 to gluR7	low-affinity kainate binding proteins

Abbreviation	Term
HA-966	(RS)-3-amino-1-hydroxypyrrolid-2-one
HCL	hydrochloric acid
i.p.	intraperitoneal
i.t.	intrathecal
I-V	current-voltage relationship
K	potassium atom
K^+	potassium cation
KA	kainic acid
KA1 to KA2	high-affinity kainate binding proteins
kg	kilogram
LTP	long term potentiation
M	molar
mg	milligram
µg	microgram
Mg^{2+}	magnesium cation
mGluR	metabotropic glutamate receptor
mGlu1 to mGlu6	metabotropic binding proteins
mRNA	messenger ribonucleic acid
min	minute

Abbreviation	Term
MK-801	(5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-10-imine
µl	microliter
µm	micrometer
ml	milliliter
mm	millimeter
NSAID	non-steroidal anti-inflammatory drug
Na^+	sodium cation
NaCl	sodium chloride
NaOH	sodium hydroxide
NBQX	6-nitro-7-sulphamobenzo(f)quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartate
nmdar1, 2A-2D	NMDA selective binding proteins
nmol	nano-mole
NS-102	5-nitro-6,7,8,9-tetrahydrobenzo[G] indole-2,3-dione-3-oxime
%	percent
p	probability
PBN	parabrachial nucleus

Abbreviation	Term
PBS	phosphate-buffered saline
PCP	phencyclidine
pH	hydrogen ion activity (neg. log)
pK_A	dissociation constant (neg. log)
±	plus-minus
pmol	picomole
PS	physiological saline
* and t	probability less than 0.05
** and tt	probability less than 0.01
q	Studentized variance ratio
QUIS	quisqualic acid
s.c.	subcutaneous
s.e.m.	standard error of the mean
SG	substantia gelatinosa
Sh	sham
SNK	Student-Newman-Keuls test
SSR	sum of squares due to regression
SST	treatment sum of squares
t-test	Student's t-test
T-cell	transmission cell

Abbreviation	Term
v/v	volume/volume
w/v	weight/volume
X	magnification indicator
Zn^{2+}	zinc cation

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORICAL OVERVIEW OF PAIN THEORIES

The manner and mechanisms in which the nervous system differentiates among the various forms of sensory stimulation are central to the study of sensory physiology. In particular, how the body responds to noxious stimuli has been, and continues to be, a controversial issue among the scientific community. The word 'pain' is commonly used to describe a large range of noxious sensory experiences. At one end of the spectrum are pain sensations such as those produced by a pin prick or by a small burn. Except for very rare pathological conditions (congenital insensitivity to pain), these types of stimuli are perceived by all people and are thought to be a protective and necessary component of our sensory repertoire (Melzack and Wall 1965). At the other end of the spectrum are abnormal or pathological pain syndromes such as phantom limb pain (which may occur following the amputation of a limb), the neuralgias (which may occur following peripheral nerve infections or degenerative nerve diseases) and the causalgias (which may occur following traumatic peripheral nerve damage). These pains are experienced by only a minority of people and are generally the consequence of damage or disease in the peripheral or central nervous systems (Melzack and Wall 1965). Chronic and abnormal pain states have no functional or protective significance and result in severe suffering to those afflicted.

In the past, most studies concerned with the mechanisms of pain perception have argued in favour of one of two opposing theories: Specificity Theory or Pattern Theory. Specificity Theory, first proposed by von Frey in 1894, and as described by Melzack and Wall (1982), proposes that pain is a specific and unique sensory modality (such as vision or hearing) and is produced by the activation of pain receptors responsive only to noxious stimuli. Impulses are then transmitted along specific peripheral and central pain pathways to a pain centre in the brain and the intensity of pain is proportional to the amount of tissue damage (Melzack and Wall 1982). Implicit in this model is the idea that stimulation of a pain receptor must always and only result in the sensation of pain. The assumption that there is a one-to-one relationship between stimulus intensity and the perceived pain is the weakness of the Specificity Theory (Melzack and Wall 1982).

The Pattern Theory, developed by Goldscheider in 1894, and as described by Melzack and Wall (1982), argues against the idea of pain specific systems and proposes that the perception of pain is the result of intense stimulation of nonspecific receptors and is maintained centrally by spatial and temporal summation of nerve impulse patterns that travel along nonspecific sensory pathways. Implicit in the Pattern Theory is that there is a high degree of specialization within the somaesthetic system. The various types of specialized receptors (free nerve endings, von Frey hairs, etc.) found in both the skin and viscera are maximally responsive to different types of sensory stimuli (touch, heat, cold, etc.; Melzack and Wall 1982). Most receptors, however, are responsive to different types of stimuli if the stimuli are of

sufficient intensity. These are known as polymodal receptors (Melzack and Wall 1982). The individual receptor response-threshold to differing stimuli most likely represent a continuum, or distribution, of receptor-fibre systems that differ by the stimulus threshold necessary to elicit a response.

Some receptor-fibre systems respond to only high intensity or noxious stimuli. In contrast to the Specificity Theory, the Pattern Theory proposes that neural systems (receptor-fibre systems) responsive to only intense or noxious stimuli do not necessarily result in the perception of pain (Melzack and Wall 1982). Rather, it is the central summation of all sensory inputs that define whether any particular stimulus will be perceived as painful.

Many aspects of the pain experience may be understood in relation to either the Specificity or Pattern Theory. There are, however, many facets of pain perception that neither theory can explain (Melzack and Wall 1982). For example, neither theory provides a convincing explanation of the mechanisms underlying abnormal pain states where: a) excruciating pain may occur spontaneously or be triggered by normally innocuous stimuli (causalgia and neuralgia); or b) severe pain may be perceived in a nonexistent limb (phantom limb pain).

In an attempt to explain chronic and pathological pain states, Livingston (1943) proposed that intense pathological stimulation sets up reverberating circuits within pools of spinal interneurons that can be triggered by normally non-noxious inputs. These then generate abnormal volleys that are interpreted centrally as pain. Noordenbos (1959) proposed the presence of a specialized input controlling system

that under normal conditions prevents summation from occurring. The destruction of such a controlling system would then lead to pathological pain states (Noordenbos 1959). This theory is based on the idea that a rapidly conducting fibre system can inhibit synaptic transmission in a more slowly conducting system that transmits the neural signals necessary for pain perception. These two systems are identified as the fast and slow (myelinated and non-myelinated) fibre systems. Under pathological conditions, the non-myelinated system becomes dominant over the myelinated system leading to the sensation of pain and often hyperalgesia (Noordenbos 1959). Under such conditions, the rapidly conducting system is unable to significantly attenuate the input pattern transmitted in the slow conducting fibres. It is important to note that there are no associations of psychological qualities attached to either system. Rather, it is the relative contribution of the two systems to the spinal output system that is important to pain perception (Melzack and Wall 1965).

Melzack and Wall (1965) were responsible for the next major advance in pain perception theory with the introduction of the Gate Control Theory. Melzack and Wall (1965) argued that, in humans, pain is a highly personal experience that is influenced by cultural learning, the meaning of the situation, attention, and other cognitive activities. The Gate Control Theory proposed that neural mechanisms in the dorsal horn of the spinal cord act like a gate that can increase or decrease the flow of nerve impulses from peripheral fibres to the spinal cord cells that project to the brain. Sensory input is subjected to the modulating influence of the 'gate' before it evokes pain perception and response. The introduction of the concept of a

"spinal gate" represents an evolution of the Pattern Theory since the concepts of temperal and spatial summation are retained within the Gate Control Model. A diagrammatic representation of the Gate Control Theory is shown in FIGURE 1.1.

The Gate Control Theory further proposed that noxious peripheral stimuli result in nerve impulses that are transmitted toward the spinal cord, and upon arrival, are processed by three separate systems: a) the Spinal Gate Control System, b) the Central Control System and c) the Central Action System (see FIGURE 1.1). The Central Action System represents the supraspinal areas and their associated neural processes involved with both the perception and response to a perceived pain. The appropriate supraspinal areas that comprise the Central Action System are deemed to be activated by ascending Transmission (T) cells that are spinal in origin. Prior to the activation of the Central Action System, afferent nociceptive impulses are modified by both the Spinal Gate Control System and the Central Control System. The Gate Control Theory proposes that the pain experience is determined by the interaction of these three systems (Melzack and Wall 1965).

The substantia gelatinosa region of the spinal dorsal horn (laminae I and II of Rexed; FIGURE 1.2.B) is believed to be the location of the spinal gate, while the ascending fibres of the dorsal and lateral columns are thought to act as the first component of the Central Control System (Melzack and Wall 1965). The fibres that comprise the ascending dorsal and lateral columns terminate on supraspinal nuclei which are a) a part of the Central Action System; or b) a part of the Central Control

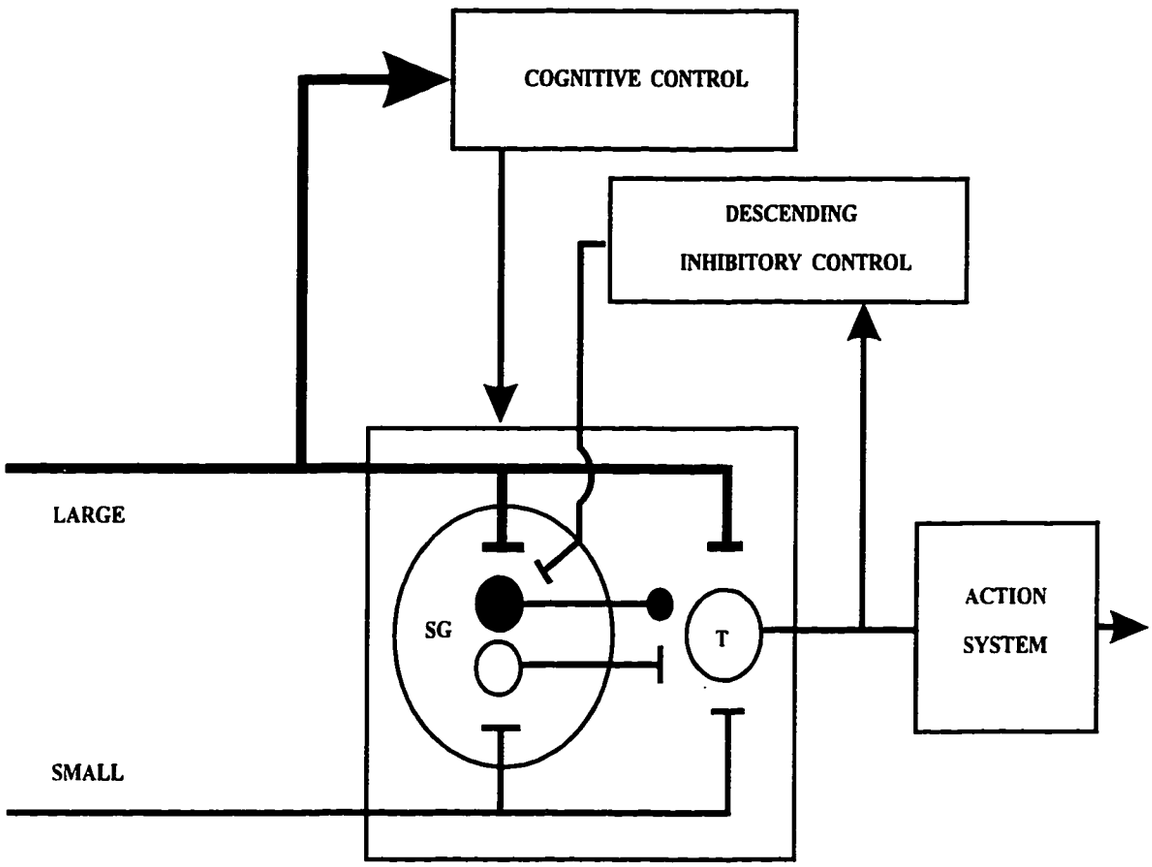


FIGURE 1.1 The Gate Control Theory: This model includes excitatory (white circle) and inhibitory (black circle) links from the substantia gelatinosa (SG) to the Transmission (T) cells as well as descending inhibitory control from brainstem systems. The round knob at the end of the inhibitory link implies that its action may be presynaptic, postsynaptic, or both. All connections are excitatory, except the inhibitory link from SG to T cell. LARGE - large diameter myelinated afferent fibres; SMALL - small diameter myelinated and non-myelinated afferent fibres (adapted from Melzack and Wall 1982).

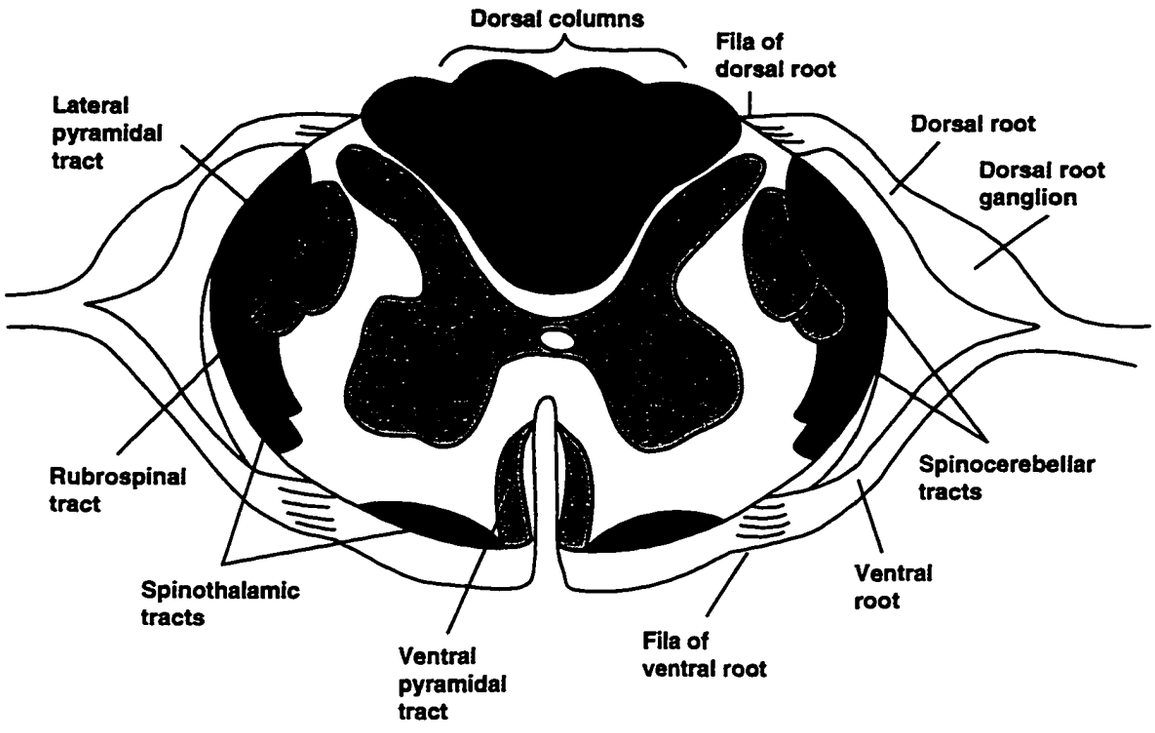
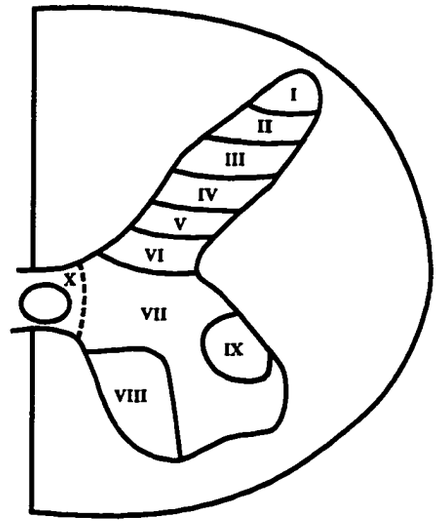


FIGURE 1.2.A A schematic cross section through the spinal cord, showing the principle ascending sensory (dark grey areas) and descending motor (light grey areas) tracts. ***(adapted from Willis and Coggeshall 1978; drawing by F. Connell).***

FIGURE 1.2.B A schematic indicating the location of Rexed's laminae (I through X) from the lumbar spinal cord of a rat. ***(adapted from Willis and Coggeshall 1978; drawing by F. Connell).***



System that may modulate the Spinal Gate Control System via descending spinal pathways (Melzack and Wall 1965). The Central Control System makes it possible for central nervous system (CNS) activities subserving attention, emotion, and memories of prior experiences to exert control over incoming sensory input (Melzack and Wall 1965).

By far the most important contribution of the Gate Control Theory of pain perception is the idea that sensory input from the periphery is modulated in the spinal cord at the level of the substantia gelatinosa (Melzack and Wall 1965). The modulatory role of the substantia gelatinosa is thought to involve three factors: a) the on-going or baseline activity which precedes a nociceptive stimulus; b) the stimulus evoked activity; and c) the relative balance of activity in large versus small afferent fibres (Melzack and Wall 1965). Afferent impulses affect the T cells and Central Action System only following modulation of the incoming sensory signals by the Gate Control System; and modulation of the Gate Control System by the Central Control System (Melzack and Wall 1965). Only when the activity of the T cells reaches an appropriate threshold level will the Central Action System respond and a painful stimulus be perceived (Melzack and Wall 1965).

The stimulation of peripheral sensory receptors does not mark the beginning of the pain process. Rather, stimulation produces neural signals that enter an active nervous system that has developed according to past personal experiences and cultural philosophies. These psychological processes actively participate in the selection and synthesis of information from the total sensory input (Melzack and

Wall 1965). Pain is not simply the end product of a linear sensory transmission system, rather it is a dynamic process that involves continuous interactions among complex ascending and descending systems. The Gate Control Theory of pain provided researchers with a theoretical model whereby afferent nociceptive signals could be modified (attenuated or enhanced) at many different nervous system levels. Specifically, the Gate Control Theory predicted that strategies could be developed to treat both acute and chronic pain states by trying to "close the gate" at the level of the spinal cord. Since the publication of the Gate Control Theory (Melzack and Wall 1965), attempts to both validate and invalidate the theory have resulted in an explosion of scientific data on pain perception mechanisms. For the purpose of the current document. studies pertaining to the spinal mechanisms of sensory transmission will mainly be discussed.

1.2 SPINAL MECHANISMS OF NOCICEPTION

With the exception of a very small minority of afferent fibres that enter the spinal cord via the ventral roots, all sensory afferents terminating in the spinal cord enter through the dorsal roots (Paxinos 1985). The spinal dorsal roots are, therefore, considered to be the route of sensory input while the ventral roots are considered to be involved mainly in motor output (Paxinos 1985). A representation of the spinal cord in cross section is shown in FIGURE 1.2.A.

The cells in the dorsal root ganglia give rise to afferent fibres having a broad spectrum of sizes, including large and medium diameter myelinated axons (Aα, Aß,

Aγ fibres), small myelinated axons (Aδ fibres) and small non-myelinated axons (C fibres) (for review see Willis and Coggeshall 1978). These bipolar neurons project to both the periphery and the spinal dorsal horn. The characteristics of the major afferent fibre types are summarized in TABLE 1.1.

The larger myelinated primary afferents (A fibres) are the most rapidly conducting axons (TABLE 1.1). These fibres may take one of three routes upon entering the spinal cord: a) terminate in lamina V or VI, b) send short collateral branches to the substantia gelatinosa, or c) send longer branches into the ascending dorsal column system (Paxinos 1985; FIGURE 1.2). Axonal projections ascending in the dorsal column system terminate in the brainstem in the dorsal column nuclei (Paxinos 1985). Axonal projections originating in the dorsal column nuclei form the medial lemniscus which provides a direct route to the thalamus and the somatosensory cortex (Paxinos 1985). The fibres of the medial lemniscus are considered to comprise the first link in the Central Control System of the Gate Control Theory (Melzack and Wall 1965).

As previously stated, the spinal dorsal horns are comprised of several distinct layers or laminae (FIGURE 1.2), each of which is thought to have specialized functions (for review see Schonburg 1990). The substantia gelatinosa (laminae I and II) is of particular interest because it represents a unique system on each side of the spinal cord that appears to have a role in modulating the sensory input from peripheral fibres to spinal cells. The substantia gelatinosa is considered within the Gate Control Theory (Melzack and Wall 1965) to be the site of the spinal gate.

FIBRE TYPE	FUNCTION	FIBER DIAMETER (μm)	CONDUCTION VELOCITY (ms)
A α	proprioception, somatic motor	12-20	70-120
ß	touch, pressure	5-12	30-70
γ	motor to muscle spindles	3-6	15-30
δ	pain, temperature, touch	2-5	12-30
B	preganglionic autonomic	<3	3-15
C dorsal root	reflex responses, pain	0.4-1.2	0.5-2
sympathetic	postganglionic sympathetic	0.3-1.3	0.7-2.3

TABLE 1.1 Physiological characteristics of afferent sensory fibres in the mammalian nervous system, (adapted from Ganong 1985).

Many sensory afferents terminate within the substantia gelatinosa, as do the dendrites of many cells found within the deeper laminae (known collectively as the nucleus proprius) whose axons ascend to the brain (Melzack and Wall 1965).

The substantia gelatinosa consists of small densely packed cells that form a functional unit extending the length of the spinal cord (Schonburg 1990). These cells connect to one another by short interneurons (Lissauer's tract) and by longer fibres within the dorsolateral fasciculus (Paxinos 1985). The axons within Lissauer's tract do not project outside of the substantia gelatinosa. The composition of Lissauer's tract (ie., the fiber type, origin, and destination) has been a subject of major controversy. Lissauer's tract is, however, regarded by many researchers to be composed of the axons of dorsal horn interneurons that originate within the substantia gelatinosa (for review see Brown 1982). In addition, many small non-myelinated primary afferent fibres directly enter Lissauer's tract and terminate within the substantia gelatinosa at higher or lower levels of the spinal cord (Chung and Coggeshall 1979). It has been estimated that up to two thirds of the axons in Lissauer's tract are in fact primary afferent fibres (Chung et al. 1979). Lesions of Lissauer's tract produced degeneration within the substantia gelatinosa in lamina I and the outer part of lamina II (LaMotte 1977). This degeneration extended only a few millimetres rostral and caudal to the lesion.

According to the Gate Control Theory, the fibres in Lissauer's tract provided at least one explanation as to the origins of the often seen clinical syndrome of referred pain (Melzack and Wall 1965). Referred pain occurs when pain is felt in

body areas where there is no presence of tissue injury. The presence of Lissauer's tract provided a means whereby activity at one level of the spinal cord might alter neural activity in spinal cord areas above or below the activated region (Melzack and Wall 1965). The control and modulation of neural activity within the substantia gelatinosa is providing clinicians and researchers with a focus for efforts to control clinical pain.

1.3 TESTS OF NOCICEPTION

A certain class of cutaneous receptors respond to relatively high-threshold stimuli such as heat, mechanical or cooling stimuli. Since these receptors respond preferentially to noxious (injurious or potentially injurious) stimuli, they have been termed nociceptors (Sherrington 1906, in Campbell et al. 1989). Hence, a stimulus that affects a response following the activation of a nociceptor is termed a nociceptive stimulus (Hammond 1989). An animal's response to a nociceptive stimulus is termed nociception. Tests of animal nociception have been developed primarily to screen compounds that might provide clinical analgesia, to elucidate the mechanisms of action of a drug within the CNS, or to identify the pathways and physiological mechanisms involved in the peripheral and central transmission of nociceptive information (for review see Hammond 1989; Franklin and Abbott 1989). The following section will review the characteristics of the most frequently used tests of nociception, with particular attention given to the formalin pain test.

1.3.1 THE FORMALIN TEST

The formalin pain test, developed by O'Keefe (1964) and Dubuisson and Dennis (1977), is a model of nociception whereby pain and inflammation are produced by a subcutaneous injection of dilute formalin into an animal's paw. Consequently, the formalin test involves a non-escapable, tissue injury-induced cutaneous pain. Subcutaneous injections of dilute formalin have produced a pattern of pain related behavior in a variety of animal species. Formalin pain has been characterized in rats (Dubuisson and Dennis 1977), cats (O'Keefe 1964), mice (Hunskaar et al. 1985) and primates (Alreja et al. 1984).

The nociception produced by subcutaneous formalin injections is considered moderate to severe and may last up to 2 hours (Dubuisson and Dennis 1977). Formalin-induced nociceptive responses can be further divided into two distinct phases based on an animal's behavioral responses. A transient early phase, characterized by an acute or "phasic" period of nociception, develops during the first 5-10 min following formalin injection. Responses then decrease or disappear for a 5-10 min period. A longer lasting late phase, characterized by a "tonic" period of nociception, follows and continues steadily for approximately 60 to 90 min (Dubuisson and Dennis 1977). The response in rodents to formalin pain involves favouring, lifting, licking and/or biting the injected paw (O'Keefe 1964; Dubuisson and Dennis 1977). In rats, the responses are usually rated according to four objective categories (Dubuisson and Dennis 1977; Cohen et al. 1984), although a number of alternative scoring systems are described in the literature (for review see

Franklin and Abbott 1989). The biphasic profile of formalin pain has been observed in all species studied, although there are inter-species differences in the time frame of observed behaviors (O'Keefe 1964; Dubuisson and Dennis 1977; Alreja et al. 1984; Hunskaar et al. 1985). Further, a number of experimenters have themselves experienced the pain produced by subcutaneous injections of dilute formalin. In humans, formalin-induced sensations were also experienced as two distinct phases. The initial phase was described as having a 'burning' quality while the second phase developed as a 'deep ache' (Chen et al. 1989; Franklin and Abbott 1989). The pain was described as moderate in intensity at its peak and decreased over a 90 min period (Franklin and Abbott 1989). It has been argued that this type of pain is a good simulation of various types of clinical pains observed in patient populations (Franklin and Abbott 1989).

A number of investigations have attempted to characterize the basis of the two phases of the formalin response. It has been suggested that the early phase is due to direct stimulation of nociceptors, while the late phase is due to a subsequent inflammation (Dubuisson and Dennis 1977). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin, and steroidal anti-inflammatory agents, such as hydrocortisone and dexamethasone, produced analgesia in the late phase but had little or no effect on the early phase (Hunskaar et al. 1986; Hunskaar and Hole 1987; Shibata et al. 1989). This pharmacological dissociation of the early and late phases of the formalin response supports the notion that the two phases represent two independent processes which rely on a) separate neural systems mediating

hyperalgesia, b) separate chemical mediators of hyperalgesia, or c) independent and separate responses of the same system to the different stages of inflammation.

Shibata and colleagues (1989) have suggested that the participation of bradykinin, histamine, serotonin and prostaglandins are important for manifestation of the second phase of responding. This was based on evidence obtained following the administration of peripherally acting non-narcotic drugs such as bromelin (a specific depletor of high molecular weight kininogens, such as bradykinin), compound 48/80 (a specific depletor of histamine and serotonin), and indomethacin, aspirin and dexamethasone (prostaglandin synthesis inhibitors). Each of these drugs was potent and efficacious in inhibiting nociceptive behaviours during the second phase (Shibata et al. 1989).

A number of studies have challenged the idea that the two phases of nociceptive responses are completely independent. Dickenson and Sullivan (1987) have demonstrated that spinal applications of the μ-opiate agonist DAMGO (Tyr-D-Ala,Gly,Me,Phe,Gly-ol) significantly inhibited second phase formalin-induced increases in dorsal horn activity. The inhibition of dorsal horn activity, however, occured only when the drug was given prior to the formalin injection, with no effect when the drug was administered within the interval between the first and second phases. This implied that formalin-induced dorsal horn activity in the second phase was dependent upon the presence of the first phase, since dorsal horn activity during the second phase was only affected when DAMGO was administered prior to the first phase.

Coderre and colleagues (1990) have also suggested that the central neural changes which occur coincident to the early phase of responding, following a formalin injection, were essential for the expression of nociceptive behavior during the second phase. Using spinal injections of the local anaesthetic lidocaine, Coderre and colleagues (1990) demonstrated that the late phase of formalin pain was significantly reduced when lidocaine was injected 5 min prior to, but not 5 min following, the formalin injection. Further, using the local anaesthetic bupivicaine, the second phase pain response to formalin was attenuated only when the injected hindpaw was locally anaesthetized prior to the time of formalin injection (Coderre et al. 1990). Rats that experienced the early phase of formalin pain and whose formalin-injected paws were locally anaesthetized only prior to the beginning of the late phase, had late phase pain scores significantly less than their control groups. However, the pain scores were significantly greater than groups whose formalin-injected paws were anaesthetized prior to the early phase (Coderre et al. 1990). These results implied that both local inflammation and the inflammation-induced neural inputs contributed to the late phase pain response. Coderre and colleagues (1990) hypothesize that the inflammation-induced neural inputs were in fact low-level (or low threshold) inputs from the inflamed foot and contributed to late phase responses by activating cells in the CNS that had become sensitized during the early phase (Coderre et al. 1990).

There are further reasons to expect that local inflammation would play only a relatively minor role in the late phase of the formalin response. First, the formalin-

induced edema does not reach its peak until 4-5 hours following injection (Brown et al. 1968; Wheeler-Aceto et al. 1990) while the peak behavioral response is exhibited 20-35 min post-injection (Dubuisson and Dennis 1977; Wheeler-Aceto et al. 1990). Second, inflammatory compounds which produced a much greater degree of inflammation than formalin, such as yeast and carrageenin, produced hyperalgesia but little or no spontaneous pain behavior (Wheeler-Aceto et al. 1990). It is noteworthy that formalin, but not yeast or carrageenin, caused an immediate and intense increase in the activity of C-fibre afferents (Headly et al. 1987). Third, although anti-inflammatory agents were effective in reducing pain during the second phase of the formalin test, NSAIDs were weak inhibitors of formalin edema, as were other agents that produced antinociception in the formalin test (Brown et al. 1968; Tasker et al. 1992). Over the time course of a formalin test, centrally acting narcotic analgesics were more effective at inhibiting formalin-induced edema than NSAIDs (Brown et al. 1968). It was possible, however, that NSAIDs produced at least some of their antinociceptive effects through central mechanisms (Jurna and Brune 1990).

Analgesia can be produced in the late phase of the formalin test by agents that do not affect the early phase such as NSAIDs and steroidal anti-inflammatory drugs (Hunskaar et al. 1986; Hunskaar and Hole 1987; Shibata et al. 1989). However, analgesia restricted to the early phase without affecting the late phase of formalin-induced nociception has not been demonstrated. Centrally acting narcotic analgesics such as morphine, pentazocine and meperidine (Hunskaar et al. 1986; Hunskaar and Hole 1987; Shibata et al. 1989; Vaccarino et al. 1989) or α-adrenergic

agents administered systemically (Tasker and Melzack 1989; Tasker et al. 1992) produced analgesia during the first phase and were also analgesic during the second phase. Furthermore, as previously mentioned, Dickenson and Sullivan (1987) have demonstrated that intrathecal administration of the μ-opiate agonist DAMGO significantly antagonized formalin-induced increases in dorsal horn activity. This inhibition, however, occured only when the drug was administered prior to the formalin injection and not when the drug was given after (Dickenson and Sullivan 1987). These results imply that the dorsal horn activity associated with the late phase of formalin pain depend not only on local inflammatory changes in the formalin-injected foot, but also depend on spinal or central nervous system activation during the early phase (Dickenson and Sullivan 1987). If formalin-induced early and late phase responses are interdependent then antinociception would have been seen during both the early and late phases. This is because the magnitude of the late phase response would have been affected by changes in early phase nociception.

In summary, the biphasic response to formalin appears to involve an interaction between nociceptor stimulation, inflammation, and changes in central nervous system function (CNS plasticity) induced by neural activity generated during the early phase following formalin injection.

1.3.2 THERMAL PAIN THRESHOLD TESTS

Animal models of thermal-pain were developed following the observations of D'Amour and Smith (1941) that humans consistently demonstrated low variability in

heat pain thresholds. The tail-flick response in rats and mice is a simple spinal reflex elicited as a result of a phasic and escapable stimulus: the animal's tail is partially immersed in hot water (44-55°C) or placed under a radiant light heat source (Franklin and Abbott 1989). The time taken for the rat or mouse to twitch the tail is the experimental end-point. An increase in the time taken to register a tail twitch relative to the animal's baseline response would indicate a possible analgesic effect of a drug, or other experimental manipulations.

The most widely used alternative heat-pain threshold test is the hot-plate test (Woolfe and McDonald 1944). In this test, mice or rats are placed onto a heated metal plate (55-60°C). Initially, an animal's response is to lick the hindpaws and the latency to this behavior is the usual experimental end-point (Espejo and Mir 1993). However, the latency to other noxious-evoked behaviors (hindleg withdrawal, jumping, stamping) have also been employed as the experimental end-point (Franklin and Abbott 1989). As in the tail-flick test, the hot-plate test also measures an animal's response to a thermal stimulus, but differs from the tail-flick test in that a coordinated motor movement is required. This would imply that supraspinal nuclei would be involved in coordinating an animal's response, while in the tail-flick test only spinal mechanisms of control are involved. For a drug to be considered a putative analgesic in the hot-plate test, the drug should produce an increase in the animal's latency to response as compared to the baseline latency.

Tests of thermal nociception are the most popular animal models of nociception since the animal does not experience either permanent or temporary

tissue injury. However, when using tests of thermal nociception it is impossible to determine whether the animal feels 'real' pain or is simply reacting once a level of discomfort is reached. It is for this reason that animal models of continuous 'tonic' pain (the formalin test) are pharmacologically distinct from those of acute 'phasic' pain (tail-flick and hot-plate tests) (Dennis and Melzack 1979; Abbott et al. 1982).

1.3.3 CAUDALLY-DIRECTED BITING AND SCRATCHING

In rats and mice, intrathecal administration of a wide variety of neuroexcitatory compounds elicits a behavioral syndrome characterized by hindlimb scratching, caudally-directed biting and licking, and sometimes vocalization and myoclonic twitching (Aanonsen and Wilcox 1986). This array of behaviors may be indicative of a nociceptive or hyperalgesic action of the injected compound; that is, the action of the injected compound may mimick the central effect of a peripheral noxious stimulus (Aanonsen and Wilcox 1986). This test has been used to elucidate the mechanisms of interaction between behavior producing ligands such as EAAs and compounds that have antagonized these behaviors such as EAA antagonists (Aanonsen and Wilcox 1987), opioids (Kellstein et al. 1990) and GABAmimetics (Aanonsen and Wilcox 1989).

1.4 IDENTIFICATION OF GLUTAMATE AND ASPARTATE AS CANDIDATES FOR SPINAL NEUROTRANSMISSION

The concept of synaptic chemical transmission arose from the classic

experiments performed by Otto Loewi in 1921 and were described by Henry H. Dale (1938; in McGeer et al. 1987). Loewi demonstrated chemical neurotransmission by transferring the ventricular fluid of a stimulated frog heart onto a non-stimulated frog heart, thereby showing that the effects of the nerve stimulus on the first heart were reproduced by chemical activity in the solution flowing onto the second heart. Since Loewi's experiments, the study of synaptic biochemistry has grown substantially and many putative CNS neurotransmitters have been identified and characterized.

Chemical neuroanatomical studies using immunocytochemistry, biochemistry, histochemistry, and receptor autoradiography techniques in animals have revealed the presence of a wide variety of putative neurotransmitters in the mammalian spinal cord. Each spinal cord system such as the primary afferents, the intrinsic interneurons, the spinal projection neurons, and the descending fibers each has differing and common neurochemicals that are released into the spinal dorsal horn. Thus, it must be assumed that each neurochemical plays a functional role in the spinal processing of primary afferent information. A partial list of neurochemicals present within the mammalian spinal dorsal horns include the excitatory amino acids (glutamate and aspartate), inhibitory amino acids (GABA and glycine), the monoamines (noradrenalin, adrenalin, serotonin, dopamine, histamine), acetylcholine, peptides (opioids - dynorphins, enkephalins, endorphins; tachykinins; somatostatin, neurotensin, oxytocin, etc.) (for review see Salt and Hill 1983; Faull et al. 1991; Weihe 1992; Todd and Spike 1993).

With respect to the acidic amino acids, L-glutamate (GLU) and L-aspartate (ASP), evidence has accumulated over the years that these compounds are neurotransmitter candidates in the mammalian spinal cord (Watkins and Evans 1981). GLU and ASP are part of a family of agents known collectively as excitatory amino acids (EAAs). The earliest suggestions of a putative neurotransmitter role for these compounds occurred in 1959 when microiontophoretic application of GLU was shown to excite spinal cord neurons (Curtis et al. 1959). Subsequently, the presence of GLU in spinal nerve terminals was demonstrated by immunocytochemistry (Ryall 1964).

In order to consider a chemical to be a neurotransmitter a number of strict criteria must be met: a) the compound must be present in the nervous system in adequate concentrations; b) the substance should be unevenly distributed, this being the anticipated consequence of an association with particular neurons; and c) the candidate neurotransmitter should decrease in concentration following lesions of known or suspected neural pathways in which it is involved (McGeer et al. 1987). There is a considerable literature indicating that GLU and ASP meet at least some of these main criteria and thereby qualify as neurotransmitters in the spinal cord (for review see Shinozaki 1988; and Collingridge and Lester 1989).

Since the original demonstrations by Curtis and colleagues (1959) and Ryall (1964) that GLU was a potential neurotransmitter candidate, experimenters have found that higher concentrations of GLU are present in the dorsal roots as compared to either the ventral roots or their respective peripheral nerves (Duggan and Johnston

1970; Johnson 1977). Supraspinal afferent neurons that originate within the spinal cord and receive inputs from dorsal root sensory neurons have postsynaptic receptors for acidic amino acids (Johnson 1978; Watkins and Evans 1981). Furthermore, a higher concentration of GLU binding sites have been found in the dorsal horn than the ventral horn (Greenamyre et al. 1984). In the rabbit spinal cord, the highest concentration of GLU is found to occur within the spinal dorsal grey matter (Berger et al. 1977). Collectively, the above results suggest that GLU meets some of the criteria for being identified as a spinal neurotransmitter and has a specific physiological and functional role within the spinal dorsal horn.

Neurotransmitters must be stored in nerve terminals for there to be an adequate physiological supply. Failure to find a preferential concentration in this compartment is regarded as important negative evidence with regard to the chemical being a neurotransmitter (McGeer et al. 1987). In nerve endings isolated from mammalian spinal cord and brain, GLU has been found to be concentrated within synaptic vesicles (Storm-Mathisen et al. 1983). Further, GLU is taken up into the vesicles through an energy, or adenosine triphosphate (ATP), dependent mechanism (Naito and Ueda 1983; Naito and Ueda 1985; Maycox et al. 1988; Fykse et al. 1989; Kish et al. 1989; Christensen and Fonnum 1991) using specific high-affinity glutamate transport mechanisms (Naito and Ueda 1985). Debler and Lajtha (1987) provided evidence that both GLU and ASP share at least some of the same vesicular uptake mechanisms.

Any candidate neurotransmitter substance should decrease in concentration

following lesions of known or suspected neural pathways in which it is involved. Such a concentration change is strong evidence of a selective association of a substance with a pathway (McGeer et al. 1987). Several authors have reported that sectioning the dorsal roots (dorsal rhizotomy) resulted in a reduced level of both GLU and ASP in the spinal cord (Jones et al. 1974; Potashner and Dymczyk 1986). Further, when dorsal rhizotomy was followed by sensory nerve stimulation, the release and uptake of D-[2,3-^{3}H]aspartate, a metabolically stable analogue of GLU and ASP (Streit 1980), was significantly depressed (Potashner and Tran 1984). However, another investigation found that dorsal root ligation did not significantly alter GLU concentrations in the spinal cord (Roberts and Keen 1974). This result neither supports nor rules out the possibility that GLU is a primary afferent neurotransmitter. It has been suggested that because the spinal interneuronal concentration of GLU is high, sectioning or ligating the dorsal roots may not significantly decrease spinal cord GLU levels (Johnson 1978).

Immunocytochemical studies have found that GLU is localized in small dorsal root ganglion cells (Battaglia et al. 1987) and that 15-30% of rat dorsal root ganglion neurons were immunoreactive to GLU (Battaglia and Rustioni 1988). Further, GLU immunoreactivity was found in 8.2% of unmyelinated and 2.3% of myelinated neurons in dorsal root axons, and ASP immunoreactivity was present in 15% of unmyelinated and 4.0% of myelinated dorsal root axons (Westlund et al. 1989a; Westlund et al. 1989b). Further, immunocytochemical studies have shown that GLU and ASP are localized in sensory afferent terminals in the spinal dorsal

horn (Maxwell et al. 1990). The most intense distribution of GLU binding sites in the rat spinal cord is reported to occur within the substantia gelatinosa (Greenamyre et al. 1984; Monaghan and Cotman 1985). This distribution is consistent with the suggestion that GLU may be a neurotransmitter released by primary afferent fibres in the dorsal horn of the spinal cord (Graham et al. 1967).

The results of many functional studies also support the premise that GLU and ASP are neurotransmitters released by primary afferents. Electrical or chemical stimulation of peripheral sensory neurons in anaesthetized rats resulted in an increase in the concentrations of GLU and ASP in spinal cord perfusate (Hopkin and Neal 1971; Kangrga et al. 1990). In awake rats, noxious peripheral stimulation produced a significant increase in the release of ASP and GLU in the spinal cord dorsal horn (Skilling et al. 1988; Smullin et al. 1990). Furthermore, organotypic cultures of dorsal root ganglion cells showed an increased release of GLU and ASP following depolarization with potassium (Jeftinija et al. 1991). Other experiments have suggested that GLU, rather than ASP, is the principle excitatory neurotransmitter released by primary afferent neurons in newborn rats and in the frog spinal cord. For example, electrical stimulation of dorsal roots produced a large calcium dependent release of GLU whereas ASP release was only marginally increased (Kawagoe et al. 1985; Kawagoe et al. 1986).

GLU metabolism in the mammalian CNS is thought to be segregated between neural and glial compartments (van den Berg et al. 1969; Minchin and Beart 1975). A glutamine cycle has been proposed as a mechanism for maintaining pre-synaptic

neuronal GLU levels in the face of continual loss due to transmitter release (Benjamin and Quastel 1976; Shank and Aprison 1981). In this model, GLU released from neurons is taken up by glial cells where it is metabolized to glutamine. Glutamine is then released from glial cells and taken up by neurons where it is converted to GLU for re-use (FIGURE 1.3). Thus, it has been hypothesized that glutamine is the major substrate for the neurotransmitter GLU (Bradford et al. 1978; Hamberger et al. 1979). It follows, therefore, that if a population of dorsal root ganglion cells does utilize GLU as a transmitter, then these cells should contain the elements of the glutamine cycle and, in particular, neurons and their satellite glial cells should accumulate glutamine and GLU respectively. This would provide strong evidence that GLU is in fact a neurotransmitter within the spinal dorsal horn.

The presence of a selective, high-affinity transport system for the rapid removal of GLU and ASP from the synaptic cleft following stimulated release has been demonstrated (Larsson et al. 1986), and a high-affinity GLU uptake site has been localized in rat spinal cord and brain (Henn et al. 1974). Further, satellite cells of the dorsal root ganglion have been shown to accumulate GLU (Schon and Kelly 1974; Duce and Keen 1983). Glutamine synthetase has been localized in glial cells (Norenberg and Martinez-Hernandez 1979) where GLU is metabolized to glutamine (Duce and Keen 1983). Further, glutamine synthetase activity has been shown to reach high levels in rat astrocyte cultures (Patel et al. 1985). There exists only preliminary evidence that asparagine synthetase, the enzyme hypothesized to convert ASP to asparagine, is present in neural glial cells (Reubi et al. 1980). Cultured rat

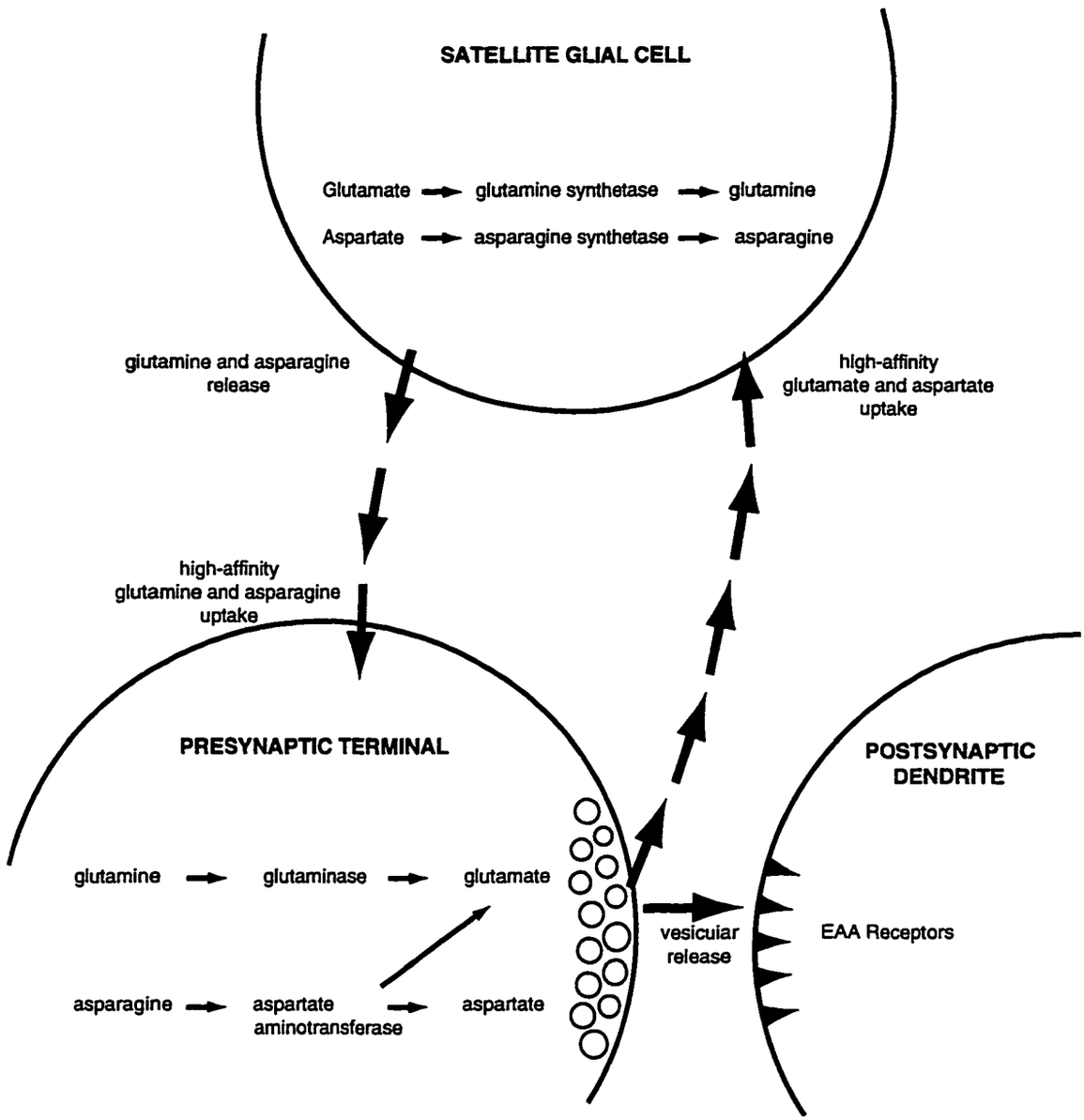


FIGURE 1.3 Glutamine Cycle: Possible mechanisms for glutamate and aspartate metabolism following vesicular release from the presynaptic terminal ***(Drawing by F. Connell).***

cortical astrocytes have also been shown to have the capacity to transport exogenous GLU, metabolize it into glutamine, and return glutamine to the incubation solution via a high-affinity transport mechanism (Waniewski and Martin 1986). Finally, in rat cortical slices, inhibition of glutamine synthetase decreases the amount of GLU released (Okamoto and Quastel 1972).

With respect to spinal processing of sensory information, dorsal root neurons are able to take up glutamine via a high-affinity uptake mechanism (Roberts and Keen 1974; Duce and Keen 1983). In fact, glutamine has been shown to be taken up to a greater extent by small dorsal root ganglion cells than by larger ones (Cangro et al. 1985). Evidence is lacking for the presence of a high-affinity asparagine uptake mechanism in presynaptic terminals. However, such a high-affinity mechanism is postulated due to the presence of asparagine in cortical presynaptic terminals (Cox and Bradford 1978). Following glutamine uptake in presynaptic terminals, small dorsal root neurons have the capacity to metabolize glutamine into GLU through the activity of the enzyme glutaminase (Weiler et al. 1979; Duce and Keen 1983). With respect to asparagine, transamination to either aspartate or glutamate has been shown to take place via the actions of the enzyme aspartate aminotransferase (Asp-T) (Fonnum 1968). This enzyme has been localized in nerve terminals throughout the CNS (Fonnum 1968; Heydorn et al. 1985). Interestingly, Flint and colleagues (1981) hypothesized that if Asp-T was the enzyme responsible for the synthesis of neurotransmitter pools of either GLU or ASP, it might be possible for some neurons to actively secrete both amino acids. This

would provide a mechanism for a single nerve terminal to regulate the relative levels of storage and release of both GLU and ASP. To complete the cycle, GLU is taken up into synaptic vesicles through an energy dependent mechanism as noted above.

Collectively, these observations provide strong evidence that mammalian neuronal populations in general, and dorsal root neurons in particular, have the functional machinery required for synthesis, metabolism, vesicular storage, and neuronal release of GLU. For ASP, the evidence is equivocal. However, the findings described previously suggest that both GLU and ASP function as neurotransmitters released from primary afferent neurons within the dorsal horn of the mammalian spinal cord.

1.5 EAA RECEPTORS

1.5.1 OVERVIEW

Neurotransmitters act on receptors that are membrane bound (McGeer et al. 1987). Such receptors are highly specialized glycoproteins that are present in perisynaptic membranes at concentrations considerably below those of the neurotransmitter (McGeer et al. 1987). Neurons have a multiplicity of membrane receptors and this repertoire of receptors provides for neuronal versatility.

Neuronal receptors can be broadly classified as either metabotropic or ionotropic. Metabotropic receptor activation leads to the initiation of second messenger mediated chemical reactions (McGeer et al. 1987). The activation of

ionotropic receptors produce changes in the membrane potential by a direct action on ion channels allowing the passage of ions of suitable charge and size (McGeer et al. 1987). Pharmacological and molecular biological studies have demonstrated that for EAA's, both metabotropic and ionotropic receptors exist in the mammalian CNS (for review see Monaghan et al. 1989; Nakanishi 1992; Cunningham et al. 1993; Hollmann and Heinemann 1994; Jorgensen et al. 1995). The major EAA receptor subtypes along with a list of prominent agonists and antagonists are presented in TABLE 1.2.

1.5.2 IONOTROPIC EAA RECEPTORS

Traditionally, the ionotropic group of EAA receptors have been divided into two main classes, N-methyl-D-aspartate (NMDA) and non-NMDA receptors, based on their sensitivity to specific EAA agonists (for review see Watkins and Evans 1981). The non-NMDA receptor class has been also divided into separate receptor subclasses, DL-α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) or kainic acid (KA) receptors depending on the relative affinity of the receptor for each prototype agonist. Recent evidence supports a further dissociation of KA receptors into high-affinity KA or low-affinity KA subtypes depending on the affinity of either KA or domoic acid (DOM) for the receptor recognition site (London and Coyle 1979; Johansen et al. 1993; Verdoorn et al. 1994).

Numerous non-NMDA receptor subunit genes have been cloned and isolated. These proteins can be combined into different subfamilies of non-NMDA receptor

RECEPTOR	GENE	AGONIST	ANTAGONIST
IONOTROPIC			
NMDA			
competitive site	nmdar1 nmdar2A-nmdar2D	NMDA	AP5 CPP CGS19755
modulatory site		glycine serine	HA966
channel			Mg^{2+} PCP MK-801
non-NMDA			
AMPA	gluR1 gluR2 gluR3 gluR4	AMPA>KA	NBQX CNQX DNQX
KAINATE			
high affinity	KA-1 KA-2	KA>AMPA	
low affinity	gluR5 gluR6 gluR7	DOM>KA	NS-102
METABOTROPIC			
mGLU	mGlu1 mGlu2 mGlu3 mGlu4 mGlu5 mGlu6	QUIS>ACPD>>AP4 ACPD>QUIS>>AP4 ACPD>QUIS>>AP4 AP4>ACPD>>QUIS QUIS>ACPD>>AP4 unknown	AP3 4C3HPG

TABLE 1.2 Listing of the major Excitatory Amino Acid receptor subtypes, the corresponding genes, and the prominent receptor agonists and antagonists.

Chemical names: **ACPD** (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; **AMPA** D,L-α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid; **AP3** L(+)-2-amino-3-phosphonopropionic acid; **AP4** L-amino-4-phosphonobutanoic acid; **AP5** D-amino-5-phosphonopentanoic acid; **4C3HPG** (S)-4-carboxy-3-hydroxyphenylglycine; **CGS19755** 4-phosphonomethyl-2-piperidinecarboxylic acid; **CNQX** 6-cyano-7-nitroquinoxaline-2,3-dione; **CPP** ((3)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; **DNQX** 6,7-dinitroquinoxaline-2,3-dione; **DOM** domoic acid; **HA966** (RS)-3-amino-1-hydroxypyrrolid-2-one; **KA** kainic acid; **Mg^{2+}** magnesium cation; **MK-801** (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-10-imine; **NBQX** 6-nitro-7-sulphamobenzo(f)quinoxaline-2,3-dione; **NMDA** N-methyl-D-aspartic acid; **NS-102** 5-nitro-6,7,8,9-tetrahydrobenzo[G]indole-2,3-dione-3-oxime; **PCP** phencyclidine; **QUIS** quisqualic acid (adapted from TIPS - 1996 Receptor and Ion Channel Nomenclature Supplement).

subunits that bind either KA or AMPA (TABLE 1.2). Non-NMDA receptor protein genes are classified as GluR1 - GluR4 (AMPA-preferring) (Hollman et al. 1989; Keinänen et al. 1990; Boulter et al. 1990), GluR5 - GluR7 (low-affinity KA subclass), and KA1 and KA2 (high-affinity KA subclass) (Bettler et al. 1990; Egebjerg et al. 1991; Werner et al. 1991; Bernard and Henley 1991; Bettler et al. 1992; Herb et al. 1992; Seeburg 1993). Further, splice variants and post-transcriptional modifications by RNA editing have increased the pharmacological and functional diversity of non-NMDA receptor proteins (Hume et al. 1991; Sommer et al. 1991; Köhler et al. 1993).

The pharmacological and electrophysiological properties of GluR subunits have been investigated by expressing GluR proteins in various cell lines and in Xenopus oocytes. Electrophysiological studies have demonstrated that GluR1-4 receptor subunits could each form functional homomeric channels (Hollmann et al. 1989; Keinänen et al. 1990; Boulter et al. 1990). Functional homomeric receptors of GluR1, GluR3, and GluR4 were all Ca^{2+} permeable, whereas GluR2 formed Ca^{2+} impermeable channels (Hume et al. 1991; Verdoorn et al. 1991). When GluR2 was coexpressed with any of the other AMPA receptor subunits, channels with permeability properties similar to homomeric GluR2 receptor proteins were formed (Hollmann et al. 1991). All homomeric and heteromeric combinations of GluR1-4 have demonstrated a higher affinity for AMPA binding than KA binding (TABLE 1.2; Keinänen et al. 1990; Keinänen et al. 1994). Within the CNS, AMPA receptors are found in the majority of excitatory synapses and are thought to mediate fast

excitatory neurotransmission (for review see Sommer and Seeburg 1992; Seeburg 1993).

[^{3}H]KA binding studies have revealed that both high- and low-affinity KA binding sites exist in the CNS (TABLE 1.2; London and Coyle 1979). Electrophysiological studies on low-affinity KA binding proteins have demonstrated that GluR5 and GluR6 were capable of forming functional homomeric channels with DOM being the most potent agonist (Bettler et al. 1990; Egebjerg et al. 1991). Further, the coexpression of GluR5 or GluR6 with KA2 were capable of forming functional heteromeric channels (Herb et al. 1992). The presence of GluR6 defines whether a functional channel is permeable to Ca^{2+} (Köhler et al. 1993), whereas all functional KA channels, regardless whether GluR6 is present or not, have been shown to be permeable to Na^+ and K^+ (for review see Sommer and Seeburg 1992; Hollmann and Heinemann 1994).

NMDA receptors now appear to be a complex of multiple binding sites (for review see Dingledine and McBain 1994; McBain and Mayer 1994). Genes that code for NMDA receptor proteins are classified as nmdar1 and nmdar2A - nmdar2D subunits (for review see Sommer and Seeburg 1992; Seeburg 1993). Activation of the NMDA receptor results in the opening of a nonspecific cation channel with high Ca^{2+} permeability (Dingledine 1983; FIGURE 1.4). However, the binding of GLU, ASP or NMDA to the NMDA receptor is not in itself sufficient to open the cation channel. The binding of the amino acid glycine at a strychnine insensitive binding site is also required (Johnson and Ascher 1987; for review see Kemp and Leeson

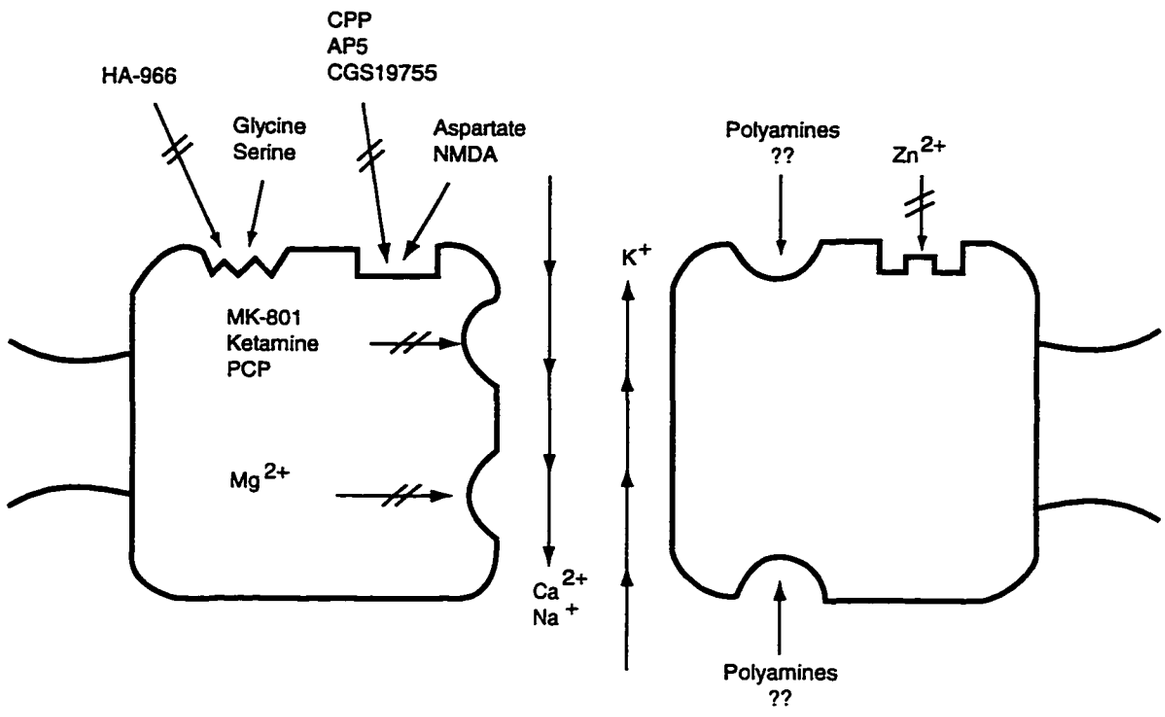


FIGURE 1.4 A schematic showing the NMDA receptor with its associated ion channel and modulatory sites. The NMDA receptor is coupled to a cationic channel that is permeable to the inflow of Na^+ and Ca^{2+}, and the outward exchange of K^+. Arrows represent agonist or positive modulatory actions. Crossed arrows represent antagonist or negative modulatory actions. ***(Adapted from Wong and Kemp 1991; Drawing by F. Connell).***

Chemical names: **AP5** D-amino-5-phosphonopentanoic acid; **CGS-19755** 4-phosphonomethyl-2-piperidinecarboxylic acid; **CPP** ((3)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; **HA966** (RS)-3-amino-1-hydroxypyrrolid-2-one; **MK-801** (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-10-imine; **PCP** phencyclidine.

1993). Under normal physiological conditions there is a sufficient concentration of glycine present in the extracellular synaptic space to provide a high probability of glycine binding (Johnson and Ascher 1987). The NMDA and glycine binding sites are distinct and are considered the competitive recognition and modulatory sites respectively of the NMDA receptor complex (Kemp and Leeson 1993; Dingledine and McBain 1994).

The activity of the NMDA receptor can also be modulated through the actions of divalent cations. Magnesium (Mg^{2+}) reversibly blocks NMDA channels in a voltage dependent manner by binding to a site within the channel opening (Mayer and Westbrook 1984; Nowak et al. 1984). The Mg^{2+} block can be overcome by membrane depolarization produced by the activation of other receptors such as AMPA or KA (Mayer et al. 1992). Low concentrations of the divalent cation zinc (Zn^{2+}) will also reversibly inhibit NMDA receptor responses but in a voltage independent manner, presumably via a site located on the extracellular domain of the NMDA receptor complex (Ascher 1988). The presence of a sufficient quantity of Zn^{2+} can result in a decrease in either the probability of channel opening or the duration of channel opening time (Ascher 1988).

The endogenous polyamines, spermine and spermidine, can also enhance NMDA responses via a voltage independent mechanism (Yoneda and Ogita 1991). Electrophysiological evidence suggests that polyamines are released presynaptically and exert their effects through a unique postsynaptic binding site attached to the NMDA receptor (Yoneda and Ogita 1991). It is not known, however, if the

polyamines exert their effects through an intracellular or extracellular binding site (FIGURE 1.4). The binding of polyamines is thought to increase the affinity of the NMDA receptor for glycine and therefore result in an enhancement of the NMDA effect (Yoneda and Ogita 1991).

There is a further NMDA inhibitory modulatory site that exists within the cation channel. Dissociative anaesthetics such as PCP (phencyclidine), ketamine and MK-801 ((5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-10-imine) bind non-competitively at a site distinct from the Mg^{2+} binding site, with MK-801 being the most potent non-competitive channel blocking agent to date (Kemp et al. 1987; Wong and Kemp 1991). Further, the blockade of the NMDA channel by MK-801 was agonist-dependent, requiring repeated applications of NMDA to fully develop the block (Kemp et al. 1987).

The combination of NMDA receptor recognition and modulatory sites makes the NMDA receptor truely unique among the different classes of EAA receptors. The increasing availability of new pharmacological tools is allowing for continued research into the physiological and functional nature of the interaction among these sites.

1.5.3 METABOTROPIC EAA RECEPTORS

Metabotropic glutamate receptors (mGluRs; TABLE 1.2) are a novel class of recently cloned G protein coupled receptors (for review see Schoepp et al. 1990). Characterization of mGluRs as a unique receptor class has generally involved the

measurement of phosphoinositide hydrolysis or intracellular calcium mobilization induced by mGluR agonists in different cell types in the central nervous system (Schoepp et al 1990). Recent molecular studies have firmly established mGluRs as a unique receptor family comprised of six different structurally unique cloned receptor proteins: mGluR1 - mGluR6 (TABLE 1.2; Bockaert et al. 1993). Each receptor protein can be pharmacologically characterized by the relative binding affinities of the different EAA ligands quisqualic acid (QUIS); (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD); or L-amino-4-phosphonobutanoic acid (AP-4) for each receptor protein (Schoepp et al. 1990; Schoepp and Conn 1993).

1.6 THE ROLE OF EAAs IN FORMALIN-INDUCED NOCICEPTION

Accumulated evidence strongly suggests that the acidic amino acids, GLU and ASP, are neurotransmitter candidates in the mammalian spinal cord (Section 1.4). Increased pain sensitivity (hyperalgesia) often follows peripheral tissue injury. The increase in pain sensitivity depends in part on central nervous system sensitization (Coderre et al. 1990). This sensitization can be described as an increase in the excitability of central neurons to normally innocuous peripheral stimuli (Woolf and Thompson 1991). Experimental evidence exists that central sensitization and the subsequent increased pain sensitivity are dependent on a spinal

action of GLU and ASP at EAA receptors (Schneider and Perl 1985; Coderre and Melzack 1992a).

Curtis and colleagues (1959) were among the first to demonstrate that the iontophoretic application of GLU onto the spinal cord resulted in a depolarization of dorsal horn neurons. It was later demonstrated that a subcutaneous injection of formalin into a rat's hindpaw evoked an increased release of GLU and ASP in the spinal dorsal horn (Smullin et al. 1988; Sorkin et al. 1992). Further, following spinal iontophoretic application of EAAs and EAA agonists, it was found that most of the neurons that were excited by EAA compounds were also excited following the application of tactile or electrical stimuli of sufficient strength to cause C-fibre stimulation (Schneider and Perl 1985). In addition, repetitive C-fibre afferent stimulation produced a 'wind-up' of dorsal horn neurons which was mimicked by spinal iontophoretic application of GLU (Zieglgansberger and Herz 1971). Wind-up of dorsal horn neurons can be described as a frequency-dependent amplification of neuronal responses seen upon recurrent C-fibre stimulation (Zieglgansberger and Herz 1971; Dickenson 1990). These results imply that following formalin-induced tissue injury, the persistent or long-lasting nociception depends on a prolonged alteration in the excitability of spinal nociceptive neurons. Further, such prolonged changes may be mediated by EAA receptors following the release of endogenous GLU and ASP in the spinal cord.

There have been very few behavioral studies that have examined the effect of spinal applications of the endogenous EAAs, GLU and ASP, on nociception.

However, Coderre and Melzack (1992a) examined nociception in the formalin test with rats pretreated with GLU and ASP. GLU and ASP were administered via lumbar puncture while the rats were under brief ether anaesthesia. These authors found that nociceptive responses were not elevated during the first 5 min of the formalin test, but were significantly enhanced from 5 min onward. These results were interpreted by Coderre and Melzack (1992a) to indicate that the drugs did not produce an additive or increased overall hyperalgesia. Finally, nociceptive responses observed during the late phase of the formalin test were also noted during the normally non-nociceptive intermediate phase. It appeared, therefore, that EAA pretreatment shifted the late phase of the formalin test to an earlier time point. These results were interpreted by these authors as evidence that EAA receptors in the spinal cord played a critical role in mediating the tonic response to subcutaneous formalin injection.

To elucidate the role of EAA receptor subtypes in the spinal transmission of formalin-induced nociception, many researchers have applied EAA receptor agonists and/or antagonists intrathecally (i.t.) in rats or mice. The role of the NMDA receptor in mediating the early and late phase response of formalin-induced nociception remains controversial. Haley and colleagues (1990) recorded electrical activity from cells in the superficial and deep dorsal horn of halothane anaesthetized rats. These authors observed that formalin-induced neural activity was nearly completely antagonized by prior i.t. application of the non-selective EAA receptor antagonist gamma-D-glutamylglycine (DGG). Further, from 10 to 60 min (late

phase) following the formalin injection, the neuronal discharges of spinal neurons were attenuated by the i.t. application of the competitive NMDA receptor antagonist AP5 (2-amino-5-phosphonopentanoic acid) (Haley et al. 1990). It is important to note that the i.t. application of AP5 did not effect the neuronal activity of spinal cord neurons from 0 to 10 min (early phase) following formalin injection. In a comparable experimental paradigm, Dickenson and Aydar (1991) described a similar effect on the late phase response following the spinal application of the competitive antagonist of the NMDA associated glycine recognition site, 7-chlorokynurenate (see FIGURE 1.4). The results reported by Haley and colleagues (1990) and by Dickenson and Aydar (1991) indicated that the antagonism of spinal NMDA receptors had a greater effect on formalin-induced late phase responses compared to early phase responses. King and colleagues (1988) reported that the spinal application of NMDA in anaesthetized rats produced wind-up-like activity in spinal dorsal horn cells, resembling the neural activity observed following repetitive C-fibre stimulation. Further, Dickenson and Aydar (1991) and Thompson and colleagues (1990) reported that the spinal application of 7-chlorokynurenate and AP5 inhibited the wind-up of dorsal horn neurons produced by the repetitive C-fibre stimulation. Systemic injections of the non-competitive NMDA receptor channel blockers MK-801 and ketamine were also found to decrease formalin-induced late phase responses recorded in spinal cord dorsal horn neurons (Haley et al. 1990). Unlike the non-selective EAA antagonist DGG, i.t. administration of both competitive and non-competitive NMDA receptor antagonists had little or no effect on the formalin-

induced activity evoked in spinal neurons during the early phase but attenuated formalin-induced neuronal activity only during the late phase (Haley et al. 1990; Thompson et al. 1990; Dickenson and Sullivan 1990; Dickenson and Aydar 1991). The above results suggest that spinal NMDA receptors play an important role in mediating formalin-induced late phase nociception, either through a direct agonist-induced action on spinal NMDA receptors during the late phase or through sensitization of spinal nociceptive neurons during the early phase.

Behavioral studies with formalin-induced tissue injury have also been used to elucidate the role of EAA receptor subtypes in spinal processing of nociceptive information. Administration of NMDA, AMPA, or ACPD prior to formalin injection produced differing results (Coderre and Melzack 1992a). The i.t. administration of both NMDA and ACPD produced a significant increase in nociception between 5 and 15 min following formalin, while AMPA had no effect on formalin-induced responses (Coderre and Melzack 1992a). These results were interpreted to suggest that the observed hyperalgesia between 5 and 15 min was mediated via an action at either the NMDA or metabotropic EAA receptor. Co-administration of half doses of NMDA and ACPD or NMDA and AMPA produced a greater increase in nociception as compared to the full dose of either NMDA or ACPD alone, indicating that there was an interaction between the hyperalgesic effect of NMDA and non-NMDA receptor activation (Coderre and Melzack 1992a). Similar results, implicating the NMDA receptor in the spinal transmission of nociception have been described in several studies. The i.t. administration of the

competitive NMDA receptor antagonists AP5 (Murray et al. 1991; Coderre and Melzack 1992a) and CGS-19755 (4-phosphonomethyl-2-piperidinecarboxylic acid; Hunter and Singh 1994), the non-competitive NMDA channel blocker MK-801 (Coderre and Melzack 1992a; Vaccarino et al. 1993), the NMDA receptor glycine site antagonists HA-966 (Hunter and Singh 1994) and ACEA-1011 (Vaccarino et al. 1993) all resulted in a dose-dependent decrease of formalin-induced nociceptive responses during the intermediate and late phase with no antinociceptive effects observed during the early phase. The results of these behavioral studies were consistent with the results of electrophysiological studies (Haley et al. 1990; Thompson et al. 1990; Dickenson and Sullivan 1990; Dickenson and Aydar 1991).

Non-NMDA receptor antagonists have rarely produced antinociceptive effects in the formalin test. The spinal administration of the competitive metabotropic receptor antagonist AP3 (2-amino-3-phosphopropionic acid; Coderre and Melzack 1992a), and the non-selective KA receptor antagonist urethane (Murray et al. 1991) did not significantly attenuate formalin-induced nociceptive responses at any point during the formalin test. In contrast to the non-analgesic effects of AP3 and urethane, the i.t. administration of the competitive non-NMDA receptor antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and DNQX (6,7-dinitro-quinoxaline-2,3-dione) produced significant antinociception during the early phase of the formalin-induced response (Näsström et al. 1992). Goettl and Larson (1994) also described an antinociceptive effect during the early phase following spinal administrations of DNQX, but did not find an antinociceptive effect of i.t.

administered DNQX on the late phase. Finally, the systemic administration of the competitive AMPA receptor antagonist NBQX (6-nitro-7-sulphamobenzo(f)quinoxaline-2,3-dione) produced significant early phase antinociception, but was without effect in the late phase (Hunter and Singh 1994).

Given the above results, NMDA receptor antagonists appear to suppress the neuronal expression of central sensitization in spinal dorsal horn neurons which leads to persistent nociception (late phase responding). Early phase responding appears to be mediated via non-NMDA receptors following the endogenous release of the EAAs, GLU and ASP. However, both NMDA and non-NMDA receptor activation appears to be involved in late phase responding, which would explain the synergistic nociceptive effects found by Coderre and Melzack (1992a) following the co-administration of either AMPA or ACPD with NMDA. Coderre and Melzack (1992a) have hypothesised that repetitive AMPA receptor activation is necessary to produce sufficient dorsal horn neuronal depolarization to counter the voltage-dependent NMDA receptor cation channel Mg^{2+} block. Further, Coderre and Melzack (1992a.b) suggested that upon removal of the block, Ca^{2+} enters the cell. In combination with subsequent second messenger effects, which are mediated via activation of the metabotropic receptor, changes occur in cellular function. These changes, which include the release of intracellular calcium and hydrolysis of ionositol phospholipids, may mediate central sensitization and persistent nociception (Coderre 1994). Finally, Coderre and Melzack (1992b) hypothesized that the central neuronal changes that occur during the early phase are essential for the expression of

nociception during the late phase. Specifically, NMDA receptors are necessary for the maintenance but not the induction of central sensitization and persistent nociception (Coderre et al. 1990; Coderre 1992, 1994; Coderre and Melzack 1992a,b).

Contrary to the aforementioned studies, several investigators have found that NMDA receptors are indeed necessary for both the induction and the maintenence of central sensitization and persistent nociception. Woolf and Thompson (1991) have examined whether primary afferent-induced hypersensitivity in flexor motorneurons is dependent on a) the activation of NMDA receptors, and whether b) wind-up is a possible trigger for the production of central hypersensitivity. Woolf and Thompson (1991) used an animal model where either C-fibre stimulation of the sural nerve or the application of a cutaneous irritant modified a hindlimb flexion withdrawal reflex from a brief non-tissue damaging mechanical stimulus applied to the toe. These authors noted that the spinal application of either the competitive NMDA receptor antagonist CPP ((3)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid) or the non-competitive NMDA receptor channel blocker MK-801, at doses that did not modify baseline reflexes, reduced the facilitation of the flexor reflex. Further, following the induction of central sensitization (wind-up) produced by the application of a cutaneous irritant, the spinal administration of CPP or MK-801 resulted in the facilitated reflex returning to baseline levels. These results indicated that dorsal horn NMDA receptors are involved in both the induction and maintenence of central sensitization (Woolf and Thompson 1991).

Behavioral evidence from several laboratories also support the hypothesized role of spinal NMDA receptors in both the induction and maintenance of central sensitization. Kristensen and colleagues (1994) and Goettl and Larson (1994) reported significant antinociception during both the early and late phase of the formalin response following i.t. administration of CPP. Further, Goettl and Larson (1994) found that the non-competitive NMDA channel antagonist MK-801 was equally antinociceptive during both the early and late phases of the formalin response. Näsström and colleagues (1992) also reported significant early phase antinociception following the spinal applications of the competitive NMDA receptor antagonists CPP, AP5, and AP7, or the NMDA receptor glycine site antagonist HA-966. Unfortunately, only results from the early phase were reported. However, Millan and Seguin (1993) found antinociceptive effects during both phases of the formalin response following systemic administration of HA-966. Further, Millan and Seguin (1993) described significant antinociception during the late phase when HA-966 was administered following the early phase, but prior to the late phase.

The results from both electrophysiological and behavioral experiments discussed above indicated that spinal NMDA receptor activation contributes to both the early and late phase of the formalin response, and supports the hypothesis that NMDA receptors are necessary for both the induction and maintenance of persistent nociception (Woolf and Thompson 1991; Näsström et al. 1992; Millan and Seguin 1993; Goettl and Larson 1994; Kristensen et al. 1994). The spinal application of NMDA receptor antagonists, whose actions may be competitive or non-competitive.

antagonized formalin-induced nociception during both the early and late phase.

Most experimenters agree that spinal NMDA receptors are involved in the maintenance of central sensitization. However, there are disagreements concerning the role of spinal NMDA receptors in the induction of central sensitization. Additional research is required to further elucidate the role that spinal NMDA and non-NMDA receptors have in the transmission of nociceptive information.

1.7 STATEMENT OF THE PROBLEM

A great deal of research has been directed toward defining the role of neurotransmitters in primary sensory afferents, particularly those involved in nociception. It is widely hypothesized that the putative endogenous EAA neurotransmitters GLU and ASP are released from nociceptive primary afferents. Further, there is evidence that EAAs act via specific receptors within the spinal dorsal horns to transmit and/or modify incoming nociceptive information arising from noxious peripheral stimulation.

To date, however, the hypothesis that EAAs are involved in nociceptive transmission within the spinal cord has not been thoroughly tested in established whole animal models of experimental pain. It is the aim of this thesis and the studies described herein to elucidate the role of EAAs in the transmission of nociceptive signals produced by the injection of formalin in the conscious rat. Specifically, the objectives are as follows:

1. To determine whether the i.t. administration of the endogenous EAA receptor ligands GLU and ASP mediate hyperalgesia in the formalin test;

2. To determine whether the i.t. administration of non-NMDA and NMDA receptor ligands mediate hyperalgesia in the formalin test.

Determining which EAA receptor subtypes mediate nociception and hyperalgesia in the formalin test is clinically relevant. Compounds that block or modulate neurotransmitter release from nociceptive primary afferents are potential analgesics.

REFERENCES

CHAPTER 1

AANONSEN LM, WILCOX GL. Muscimol, gamma-aminobutyric $acid_A$ receptors and excitatory amino acids in the mouse spinal cord. J Pharmacol Exp Ther 1989; 248: 1034-1038.

AANONSEN LM, WILCOX GL. Nociceptive action of excitatory amino acids in the mouse: effects of spinally administered opioids, phencyclidine and sigma agonists. J Pharmacol Exp Ther 1987; 243: 9-19.

AANONSEN LM, WILCOX GL. Phencyclidine selectively blocks a spinal action of N-methyl-D-aspartate in mice. Neurosci Lett 1986; 67: 191-197.

ABBOTT FV, MELZACK R, LEBER BF. Morphine analgesia and tolerance in the tail flick and formalin tests: dose-response relationships. Pharmacol Biochem Behav 1982; 17: 1213-1219.

ALREJA M, MUTALIK P, MAYAR U, MANCHANDA SK. The formalin test: a tonic pain model in the primate. Pain 1984; 20: 97-105.

ASCHER P. Divalent cations and the NMDA channel. Biomed Res 1988; 9: 31-37.

BATTAGLIA G, RUSTIONI A. Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. J Comp Neurol 1988; 277: 302-312.

BATTAGLIA G, RUSTIONI A, ALTSCHULER RA, PETRUSZ P. Glutamic acid coexists with substance P in some primary sensory neurons. In: Schmidt RF, Schaible HG, Vahle-Hinz C, eds. Fine afferent nerve fibres and pain. Weinheim: VCH, 1987: 77-84.

BENJAMIN AM, QUASTEL JH. Cerebral uptakes and exchange diffusion *in vitro* of L- and D-glutamate. J Neurochem 1976; 26: 431-441.

BERGER SJ, CARTER JG, LOWRY OH. The distribution of glycine, GABA, glutamate and aspartate in rabbit spinal cord, cerebellum and hippocampus. J Neurochem 1977; 28: 149-158.

BERNARD EA, HENLEY JM. The non-NMDA receptors: types, protein structure and molecular biology. TIPS 1991; Special Report: 82-89.

BETTLER B, BOULTER J, HERMANS-BORGMEYER I. O'SHEA-GREENFIELD A, DENERIS ES, MOLL C, BORGMEYER U, HOLLMANN M, HEINMANN SF. Cloning of a novel glutamate receptor subnunit, GluR5: expression in the nervous system during development. Neuron 1990; 5: 583-595.

BETTLER B, EGEBJERG J , SHARMA G, PECHT G, HERMANS-BORGMEYER I, MOLL C, STEVENS CF, HEINMANN SF. Cloning of a putative glutamate receptor: a low affinity kainate-binding subunit. Neuron 1992; 8: 257-265.

BOCKAERT J, PIN J, FAGNI L. Metabotropic glutamate receptors: an original family of G protein-coupled receptors. Fundam Clin Pharmacol 1993; 7: 473-485.

BOULTER J, HOLLMANN M O'SHEA-GREENFIELD A, HARTLEY M, DENERIS E, MARON C,HEINEMANNSF. Molecular cloning and functional expression of glutamate receptor subunit genes. Science 1990; 249: 1033-1037.

BRADFORD HF, WARD HK, THOMAS AJ. Glutamine: a major substrate for nerve endings. J Neurochem 1978; 30: 1453-1459.

BROWN AG. The dorsal horn of the spinal cord. J Exp Physiol 1982; 67: 193-212.

BROWN JH, KISSEL JW, LISH PM. Studies on the acute inflammatory response: I. Involvement of the central nervous system in certain models of inflammation. J Pharmacol Exp Ther 1968; 160: 231-242.

CAMPBELL JN, RAJA SN, COHEN RH, MANNING DC, KHAN AA, MEYER RA. Peripheral neural mechanisms of nociception. In: Wall PD, Melzack R, eds. Textbook of Pain. New York: Churchill Livingston, 1989: 22-45.

CANGRO CB, SWEETNAM PM, WRATHALL JR, HASBER WB, CURTHOYS NP, NEALE JH. Localization of elevated glutaminase immunoreactivity in small DRG neurons. Brain Res 1985; 336: 158-161.

CHEN ACN, DWORKIN SF, HAUG J, GEHRIG J. Human pain responsivity in a tonic pain model: physiological determinants. Pain 1989; 37: 143-160.

CHRISTENSEN H, FONNUM F. The ontogeny of the uptake systems for glycine, GABA and glutamate in synaptic vesicles isolated from rat spinal cord-medulla. Dev Brain Res 1991; 64: 155-159.

CHUNG K, COGGESHALL RE. Primary afferent axons in the tract of Lissauer in the cat. J Comp Neurol 1979; 186: 451-464.

CHUNG K. LANGFORD LA, APPLEBAUM AE, COGGESHALL RE. Primary afferent fibres in the tract of Lissauer in the rat. J Comp Neurol 1979; 184: 587-598.

CODERRE TJ. The role of excitatory amino acid receptors and intracellular messengers in persistent nociception after tissue injury in rats. Molec Neurobio 1994; 7: 229-246.

CODERRE TJ. Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. Neurosci Lett 1992; 140: 181-184.

CODERRE TJ, MELZACK R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 1992a; 12: 3665-3670.

CODERRE TJ, MELZACK R. The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. J Neurosci 1992b; 12: 3671-3675.

CODERRE TJ, VACCARINO AL, MELZACK R. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 1990; 535: 155-158.

COHEN SR, ABBOTT FV, MELZACK R. Unilateral analgesia produced by intraventricular morphine. Brain Res 1984; 303: 277-287.

COLLINGRIDGE GL, LESTER RAJ. Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Review 1989; 40: 143-210.

COX DWG, BRADFORD HF. Uptake and release of excitatory amino acid neurotransmitters. In: McGeer EG, ed. Kainic acid as a tool in neurobiology. New York: Raven, 1978: 71-93.

CUNNINGHAM MD, FERKANY JW, ENNA SJ. Excitatory amino acid receptors: a gallery of new targets for pharmacological intervention. Life Sci 1993; 54: 135-148.

CURTIS DR, PHILLIS JW, WATKINS JC. Chemical excitation of spinal neurones. Nature (Lond.) 1959; 183: 611-612.

DALE HH. Acetylcholine as a chemical transmitter substance of the effects of nerve impulses: The William Henry Welch Lectures, 1937. J Mt Sinai Hosp 1938; 4: 401-429. In: McGeer PL, Eccles JC, McGeer EG, eds. Molecular neurobiology of the mammalian brain. New York: Plenum Press, 1987.

D'AMOUR FE, SMITH DL. A method for determining loss of pain sensation. J Pharmacol Exp Ther 1941; 72: 74-79.

DEBLER EA, LAJTHA. High-affinity transport of γ-aminobutyric acid, glycine, taurine, L-aspartic acid, and L-glutamic acid in Synaptosomal (P_2) tissue: a kinetic and substrate specificity analysis. J Neurochem 1987; 48: 1851-1856.

DENNIS SG, MELZACK R. Comparison of phasic and tonic pain in animals. In: Bonica JJ, ed. Advances in pain research and therapy. New York: Raven, 1979: 747-760.

DICKENSON AH. A cure for windup: NMDA receptor antagonists as potential analgesics. Trends Pharamac Sci 1990; 11: 307-309.

DICKENSON AH, AYDAR E. Antagonism at the glycine on the NMDA receptor reduces spinal nociception in the rat. Neurosci Lett 1991; 121: 262-266.

DICKENSON AH, SULLIVAN AF. Differiential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurones in rat. Brain Res 1990; 506: 31-39.

DICKENSON AH, SULLIVAN AF. Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. Pain 1987; 30: 349-360.

DINGLEDINE R. N-methyl aspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cells. J Physiol 1983; 343: 385-405.

DINGLEDINE R, MCBAIN CJ. Excitatory amino aced transmitters. In: Siegel GJ, ed. Basic neurochemistry: molecular, cellular, and medical aspects, 5th ed. New York: Raven Press, 1994.

DUBUISSON D, DENNIS SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977; 4: 161-174.

DUCE IR, KEEN P. Selective uptake of [^{3}H] glutamine and [^{3}H] glutamate into neurons and satellite cells of dorsal root ganglia in vitro. Neurosci 1983; 8: 861-866.

DUGGAN AW, JOHNSTON GAR. Glutamate and related amino acids in cat spinal roots, dorsal root ganglia and peripheral nerves. J Neurochem 1970; 17: 1205-1208.

EGEBJERG J, BETTLER B, HERMANS-BORGMEYER I, HEINEMANN SF.Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. Nature 1991; 351: 745-748.

ESPEJO EF, MIR D. Structure of the rat's behaviour in the hot plate test. Behav Brain Res 1993: 56: 171-176.

FAULL RLM, JANSEN KLR, WALDVOGEL HJ, VILLIGER JW, BULLOCK JY. WILLIAMS MN, DRUGUNOW M. Receptors in the human spinal cord. In: Receptors in the human nervous system. New York: Academic Press Inc., 1991: 207-243.

FLINT RS, REA MA, McBRIDE WJ. *In Vitro* release of endogenous amino acids from granule cell- satellite cell- and climbing fibre- deficient cerebella. J Neurochem 1981; 37: 1425-1430.

FONNUM F. The distribution of glutamate decarboxylase and aspartate transaminase in subcellular fractions of rat and guinea-pig brain. Biochem J 1968; 106: 401-412.

FRANKLIN KBJ, ABBOTT FV. Techniques for assessing the effects of drugs on nociceptive responses. Neurometh 1989; 13: 145-216.

FYKSE EM, CHRISTENSEN H, FONNUM F. Comparison of the properties of γ-aminobutyric acid and L-glutamate uptake into synaptic vesicles isolated from rat brain. J Neurochem 1989; 52: 946-951.

GANONG WF. Review of medical physiology. 12th.ed. East Norwalk, CT: Appleton-Century-Crofts, 1985: 42.

GOETTL VM, LARSON AA. Antinociception induced by 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), and N-methyl-D-aspartate(NMDA) competitive antagonist, plus 6,7-dinitroquinoxaline-2,3-dione (DNQX), a non-NMDA antagonist, differs from that induced by MK-801 plus DNQX. Brain Res 1994; 642: 334-338.

GOLDSCHEIDER A. Ueber den Schmerz. In: Physiologischer und klinischer Hinsicht. Berlin: Hirschwald, 1894. In: Melzack R, Wall P, eds. The challenge of pain. Middlesex, Eng: Penguin, 1982.

GRAHAM LT Jr., SHANK RP, WERMAN R, APRISON MH. Distribution of some synaptic transmitter suspects in cat spinal cord. J Neurosci 1967; 14: 465-472.

GREENAMYRE JT, YOUNG AB, PENNEY JB. Quantitative autoradiographic distribution of L-[^{3}H] glutamate-binding sites in rat central nervous system. J Neurosci 1984; 4: 2133-2144.

HALEY JE, SULLIVAN AF, DICKENSON AH. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. Brain Res 1990; 518: 218-226.

HAMBERGER AC, CHIANG GH, NYLEN ES, SCHEFF SW, COTMAN CW. Glutamate as a CNS transmitter - I. Evaluation of glucose and glutamine as precursors for the synthesis of preferentially-released glutamate. Brain Res 1979; 168: 513-530.

HAMMOND DL. Inference of pain and its modulation from simple behaviors. In: Chapman CR, Loeser JD, eds. Issues in pain management. New York: Raven Press Ltd., 1989: 69-91.

HEADLY PM, PARSONS CG, WEST DC. The role of N-methylaspartate receptors in mediating responses of rat and cat spinal neurones to defined sensory stimuli. J Physiol 1987; 385: 169-188.

HENN FA, GOLDSTEIN MN, HAMBERGER A. Uptake of the neurotransmitter candidate glutamate by glia. Nature 1974; 249: 663-664.

HERB A, BUNASHEV N, WERNER P, SAKMANN B, WISDEN W, SEEBURG PH. The KA-2 subunit of excitatory amino acid receptors show widespread expression in brain and forms ion channels with distantly related subunits. Neuron 1992; 8: 775-785.

HEYDORN WE, CREED GJ, WADA H, JACOBOWITZ DM. Immunological evidence for existence of two subforms of soluble glutamate oxaloacetic transaminase (sGOT) in human and rat brain. Neurochem Int 1985; 7: 833-841.

HOLLMANN M, HEINEMANN S. Cloned glutamate receptors. Annu Rev Neurosci 1994; 17: 31-108.

HOLLMANN M, HARTLEY M, HEINEMANN SF. Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. Science 1991; 252: 851-853.

HOLLMANN M, O'SHEA-GREENFIELD A, ROGERS SW, HEINEMANN SF. Cloning by functional expression of a member of the glutamate family. Nature 1989; 342: 643-648.

HOPKIN J, NEAL MJ. Effect of electrical stimulation and high potassium concentrations on the efflux of [^{14}C] glycine from slices of spinal cord. Br J Pharmacol 1971; 42: 215-223.

HUME RI, DINGLEDINE R, HEINEMANN SF. Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 1991; 253: 1028-1031.

HUNSKAAR S, HOLE K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987; 30: 103-114.

HUNSKAAR S, BERGE OG, HOLE K. Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicylic acid and indomethacin in the formalin test. Pain 1986; 25: 125-132.

HUNSKAAR S, FASMER OB, HOLE K. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Meth 1985; 14: 69-76.

HUNTER JC, SINGH L. Role of excitatory amino acid receptor in the mediation of the nociceptive response to formalin in the rat. Neurosci Lett 1994; 174: 217-221.

JEFTINIJA A, JEFTINIJA K, LIU F, SKILLING SR, SMULLEN DH, LARSON AA. Excitatory amino acids are released from rat primary afferent neurons in vitro. Neurosci Lett 1991; 125: 191-194.

JOHANSEN TH, DREJER J, WATJEN F, NIELSEN EO. A novel non-NMDA receptor antagonist shows selective displacement of low-affinity [^{3}H]kainate binding. Eur J Pharmacol 1993; 246: 195-204.

JOHNSON JL. The excitant amino acids glutamic and aspartic acid as transmitter candidates in the vertebrate central nervous system. Prog Neurobiol 1978; 10: 155-202.

JOHNSON JL. Glutamic acid as a synaptic transmitter candidate in the dorsal sensory neuron: reconsiderations. Life Sci 1977; 20: 1637-1644.

JOHNSON JW, ASCHER P. Glycine potentiates the NMDA response of mouse central neurones. Nature 1987; 325: 529-531.

JONES IM, JORDAN CC, MORTON IKM, STAGG CJ, WEBSTER RA. The effect of chronic dorsal root section on the concentration of free amino acids in the rabbit spinal cord. J Neurochem 1974; 23: 1239-1244.

JORGENSEN M, TYGESEN CK, ANDERSEN PH. Ionotropic glutamate receptors - focus on non-NMDA receptors. Pharmacol Tox 1995; 76: 312-319.

JURNA I, BRUNE K. Central effects of the non-steroid anti-inflammatory agents, indomethacin, ibuprofen and diclofenac, determined in C fibre-evoked activity in single neurones of the rat thalamus. Pain 1990; 41: 71-80.

KANGRGA I, LAREW JSA, RANDIC M. The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neurosci Lett 1990; 108: 155-160.

KAWAGOE R, ONODERA K, TAKEUCHI A. The release of endogenous glutamate from the newborn rat spinal cord induced by dorsal root stimulation and substance P. Biomed Res 1986; 7: 253-259.

KAWAGOE R, ONODERA K, TAKEUCHI A. Release of endogenous glutamate from the frog spinal cord following dorsal root stimulation. Biomed Res 1985; 6: 239-245.

KEINÄNEN K, KÖHR G, SEEBURG PH, LAUKKANEN ML, OKER-BLOOM C. High level expression of functional glutamate channels in insect cells. Bio-Tech 1994; 12: 802-806.

KEINÄNEN K, WISDEN W, SOMMER B, WERNER P, HERB A, VERDOORN TA, SAKMANN B, SEEBURG PH. A family of AMPA-selective glutamate receptors. Science 1990; 249: 556-560.

KELLSTEIN DE, COGHILL RC, FRENK H, BOSSUT DF, MAYER FJ. Opioid inhibition of kainic acid-induced scratching: mediation by mu and sigma but not delta and kappa receptors. Pharmacol Biochem Behav 1990; 35: 1-5.

KEMP JA, LEESON PD. The glycine site of the NMDA receptor - five years on. TIPS 1993; 14: 20-25.

KEMP JA, FOSTER AC, WONG EHF. Non-competitive antagonists of excitatory amino acid receptors. TINS 1987; 10: 294-298.

KING AE, THOMPSON SWN, URBAN L, WOOLF CJ. An intracellular analysis of amino acid-induced excitations of deep dorsal horn neurons in the rat spinal cord slice. Neurosci Lett 1988; 89: 286-292.

KISH PE, KIM SY, UEDA T. Ontogeny of glutamate accumulating activity in rat brain synaptic vesicles. Neurosci Lett 1989; 97: 185-190.

KÖHLER M, BURNASHEV N, SAKMANN B, SEEBURG PH. determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. Neuron 1993; 10: 491-500.

KRISTENSEN JD, KARLSTEN R, GORDH T, BERGE OG. The NMDA antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) has antinociceptive effect after intrathecal injection in the rat. Pain 1994; 56: 59-67.

LAMOTTE C. Distribution of the tract of Lissauer and the dorsal root fibers in the primate spinal cord. J Comp Neurol 1977; 172: 529-562.

LARSSON OM, GRIFFITHS R, ALLEN IC, SCHOUSBOE A. Mutual inhibition kinetic analysis of γ-aminobutyric acid, taurine, and β-alanine high-affinity transport into neurons and astrocytes: evidence for similarity between the taurine and β-alanine carriers in both cell types. J Neurochem 1986; 47: 426-432.

LIVINGSTON WK. Pain Mechanisms. New York: Macmillan, 1943.

LONDON ED, COYLE JT. Specific binding of [^{3}H]kainic acid receptor sites in rat brain. Molec Pharmacol 1979; 15: 492-505.

MAXWELL DJ, CHRISTIE WM, SHORT AD, STORM-MATHISEN J, OTTERSEN OP. Central boutons of glomeruli in the spinal cord of the cat are enriched with L-glutamate-like immunoreactivity. Neurosci 1990; 36: 83-104.

MAYCOX PR, DECKWERTH T, HELL JW, JAHN R. Glutamate uptake by brain synaptic vesicles. J Biol Chem 1988; 263: 15423-15428.

MAYER ML, WESTBROOK GL. Mixed-agonist of excitatory amino acids on mouse spinal cord neurones under voltage clamp. J Physiol 1984; 354: 29-53.

MAYER ML, BENVENISTE M, PATNEAU DK, VYKLICKY L Jr. Pharmacologic properties of NMDA receptors. Ann NY Acad Sci: Neurotoxins and Neurodegenerative Disease 1992; 648: 194-204.

MCBAIN CJ, MAYER ML. N-methyl-D-aspartate receptor structure and function. Physiol Rev 1994; 74: 723-760.

MCGEER PL, ECCLES JC, MCGEER EG. Molecular neurobiology of the mammalian brain. New York: Plenum Press, 1987.

MELZACK R, WALL PD. The challenge of pain. Middlesex, Eng: Penguin, 1982: 223-239.

MELZACK R, WALL PD. Pain mechanisms: a new theory. Science 1965; 150: 971-979.

MILLAN MJ, SEGUIN L. (+)-HA 966, a partial angonist at the glycine site coupled to NMDA receptors, blocks formalin-induced pain in mice. Eur J Pharmacol 1993; 238: 445-447.

MINCHIN MCW, BEART PM. Compartmentation of amino acid metabolism in the rat dorsal root ganglion; a metabolic and autoradiographic study. Brain Res 1975; 83: 437-449.

MONAGHAN DT, COTMAN CW. Distribution of N-methyl-D-aspartate-sensitive L-[^{3}H] glutamate-binding sites in rat brain. J Neurosci 1985; 5: 2909-2919.

MONAGHAN DT, BRIDGES RJ, COTMAN CW. The excitatory amino acid receptors: Their classes, pharmacology, and distinct properties in the function of the central nervous system. Annu Rev Pharmacol Toxicol 1989; 29: 365-402.

MURRAY CW, COWAN A, LARSON AA. Neurokinin and NMDA antagonists (but not kinic acid antagonist) are antinociceptive in the mouse formalin model. Pain 1991; 44: 179-185.

NAITO S, UEDA T. Characterization of glutamate uptake into synaptic vesicles. J Neurochem 1985; 44: 99-109.

NAITO S, UEDA T. Adenosine triphosphate-dependent uptake of glutamate into protein 1-associated vesicles. J Biol Chem 1983; 258: 696-699.

NAKANISHI S. Molecular diversity of glutamate receptors and implications for brain function. Science 1992; 258: 597-603.

NÄSSTRÖM J, KARLSSON U, POST C. Antinociceptive actions of different classes of excitatory amino acid receptor antagonists in mice. Eur J Pharmacol 1992: 212: 21-29.

NOORDENBOS W. Pain. Amsterdam: Elsevier, 1959.

NORENBERG MD, MARTINEZ-HERNANDEZ A. Fine structural localization of glutamine synthetase in astrocytes of rat brain. Brain Res 1979; 161: 303-310.

NOWAK L, BREGESTOVSKI P, ASCHER P, HERBET A, PROCHIANTZ A. Magnesium gates glutamate-activated channels in mouse central neurones. Nature 1984; 307: 462-465.

OKAMOTO K, QUASTEL JH. Uptake and release of glutamate in cerebral cortex slices from the rat brain. Biochem J 1972; 128: 1117-1124.

O'KEEFE J. Spinal cord mechanisms subserving pain perception. M.Sc. Thesis, McGill University, 1964.

PATEL AJ, WEIR MD, HUNT A, TAHOURDIN CSM, THOMAS DGT. Distribution of glutamine synthetase and glial fibrillary acidic protein and correlation of glutamine synthetase with glutamate decarboxylase in different regions of the rat central nervous system. Brain Res 1985; 331: 1-9.

PAXINOS G. The rat nervous system. Volume 2: Hindbrain and spinal cord. Australia: Academic Press, 1985.

POTASHNER SJ, DYMCZYK L. Amino acid levels in the guinea pig spinal gray matter after axotomy of primary sensory and descending tracts. J Neurochem 1986; 47: 412-422.

POTASHNER SJ, TRAN PL. Decreased uptake and release of D-aspartate in the guinea pig spinal cord after dorsal root section. J Neurochem 1984; 42: 1135-1144.

REUBI JC, TOGGENBURGER G, CUENOD M. Asparagine as precursor for transmitter aspartate in corticostriatal fibers. J Neurochem 1980; 35: 1015-1017.

ROBERTS PJ, KEEN P. Effect of dorsal root section on amino acids of rat spinal cord. Brain Res 1974; 74: 333-337.

RYALL RW. The subcellular distribution of acetylcholine, substance P, 5-hydroxytryptamine, γ-aminobutyric acid and glutamic acid in brain homogenates. J Neurochem 1964; 11: 131-145.

SALT TE, HILL RG. Neurotransmitter candidates of somatosensory primary afferent fibres. Neurosci. 1983; 10: 1083-1103.

SCHNEIDER SP, PERL ER. Selective excitation of neurons in the mammalian spinal dorsal horn by aspartate and glutamate in vitro: correlation with localization and excitatory input. Brain Res 1985; 360: 339-343.

SCHOEPP DD, CONN PJ. Metabotropic glutamate receptors in brain function and pathology. TIPS 1993; 14: 13-20.

SCHOEPP D, BOCKAERT J, SLADECZEK F. Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. TIPS 1990; 11: 508-515.

SCHONBURG ED. Spinal sensorimotor systems and their supraspinal control. Neurosci Res 1990; 7: 265-340.

SCHON F, KELLY JS. Autoradiographic localization of [^{3}H]GABA and [^{3}H]glutamate over satellite glial cells. Brain Res 1974; 66: 275-288.

SEEBURG PH. The TIPS/TINS Lecture: The molecular biology of mammalian glutamate receptor channels. TIPS 1993; 14: 297-303.

SHANK RP, APRISON MH. Minireview: present status and significance of the glutamine cycle in neural tissues. Life Sci 1981; 28: 837-842.

SHERRINGTON CS. The integrative action of the nervous system. New York: Scribner, 1906. In: Wall PD, Melzack R, eds. Textbook of Pain. New York: Churchill Livingston, 1989: 22-45.

SHIBATA M, OHKUBO T, TAKAHASHI H, INOKI R. Modified formalin test: characteristic biphasic pain response. Pain 1989; 38: 347-352.

SHINOZAKI H. Pharmacology of the glutamate receptor. Prog in Neurobiol 1988; 30: 399-435.

SKILLING SR, SMULLEN DH, BEITZ AJ, LARSON AA. Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following vertridine and nociceptive stimulation. J Neurochem 1988; 51: 127-132.

SMULLIN DH, SKILLING SR, LARSON AA. Interactions between substance P, calcitonin gene-related peptide, taurine and excitatory amino acids in the spinal cord. Pain 1990; 42:93-101.

SOMMER B, SEEBURG PH. Glutamate receptor channels: novel properties and new clones. TIPS 1992; 13: 291-296.

SOMMER B, KÖHLER M, SPRENGEL R,SEEBURG PH. RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. Cell 1991; 67: 11-19.

SORKIN LS, WESTLUND KN, SLUKA KA, DOUGHERTY PM, WILLIS WD. Neural changes in acute arthritis in monkeys. IV. Time course of amino acid release into the lumbar dorsal horn. Brain Res Rev 1992; 17: 39-50.

STORM-MATHISEN J, LEKNES AK, BORE AT, VAALAND JL, EDMINSON P, HAUG FMS, OTTERSEN OP. First visualization of glutamate and GABA in neurones by immunocytochemistry. Nature 1983; 301: 517-520.

STREIT P. Selective retrograde labeling indicating the transmitter of neuronal pathways. J Comp Neurol 1980; 191: 429-463.

TASKER RAR, MELZACK R. Different alpha receptor subtypes are involved in clonidine produced analgesia in different pain tests. Life Sci 1989; 44: 9-17.

TASKER RAR, CONNELL BJ, YOLE MJ. Systemic injections of alpha-1 adrenergic agonists produce antinociception in the formalin test. Pain 1992; 49: 383-391.

THOMPSON SWN, KING AE, WOOLF CJ. Activity-dependent changes in rat ventral horn neurones in-vitro; summation of prolonged afferent evoked postsynaptic depolarizations produce d-APV sensitive windup. Eur J Neurosci 1990; 2: 638-649.

TODD AJ, SPIKE RC. The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog. Neurobiol. 1993; 41: 609-645.

VACCARINO AL, TASKER RAR, MELZACK R. Analgesia produced by normal doses of opioid antagonists alone and in combination with morphine. Pain 1989; 36: 103-109.

VACCARINO AL, MAREK P, KEST B, WEBBER E, KEANA JFW. LIEBESKIND JC. NMDA receptor antagonists, MK-801 and ACEA-1011, prevent the development of tonic pain following subcutaneous formalin. Brain Res 1993; 615: 331-334.

VAN DEN BERG CJ, KRZALIC LJ, META P, WAETSCH H. Compartmentation of glutamate metabolism in brain: evidence for the existence of two different tricarboxylic acid cycles in brain. Biochem J 1969; 113: 281-290.

VERDOORN TA, JOHANSEN TH, DREJER J, NIELSEN EO. Selective block of recombinant glur6 receptors by NS-102, a novel non-NMDA receptor antagonist. Eur J Pharmacol 1994; 269: 43-49.

VERDOORN TA, BURNASHEV N, MONYER H, SEEBURG PH, SAKMANN B. Structural determinants of ion flow through recombinant glutamate receptor channels. Science 1991; 252: 1715-1718.

VON FREY M. Ber. Kgl. Sächs. Ges. Wiss. 1894, 46: 185. In: Melzack R, Wall P, eds. The challenge of pain. Middlesex, Eng: Penguin, 1982.

WANIEWSKI RA, MARTIN DL. Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. J Neurochem 1986; 304-313.

WATKINS JC, EVANS RH. Excitatory amino acid transmitters. Annu Rev Pharmacol Toxicol 1981; 21: 165-204.

WEIHE E. Neurochemical anatomy of the mammalian spinal cord: functional implications. Ann Anat 1992; 174: 89-118.

WEILER CT, NYSTROM B, HAMBERGER A. Characteristics of glutamine vs. glutamate transport in isolated glia and synaptosomes. J Neurochem 1979; 559-565.

WERNER P, VOIGHT M, KAINÄNEN K, WISDEN W, SEEBURG PH. Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. Nature 1991; 351: 742-744.

WESTLUND KN, McNEILL DL, COGGESHALL RE. Glutamate immunoreactivity in rat dorsal root axons. Neurosci Let 1989a; 96: 13-17.

WESTLUND KN, McNEILL DL, PATTERSON JT, COGGESHALL RE. Aspartate immunoreactive axons in normal rat L_4 dorsal roots. Brain Res 1989b; 489: 347-351.

WHEELER-ACETO H, PORRECA F, COWAN A. The rat paw formalin test: comparison noxious agents. Pain 1990: 40: 229-238.

WILLIS WD, COGGESHALL RE. Sensory mechanisms of the spinal cord. New York: Plenum, 1978.

WONG EHF, KEMP JA. Sites for antagonism on the N-methyl-D-aspartate receptor channel complex. Annu Rev Pharmacol Toxicol 1991; 31: 401-425.

WOOLF CJ, THOMPSON CWN. The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation: implications for the treatment of post-injury pain hypersensitivity states. Pain 1991: 44: 293-299.

WOOLFE G, McDONALD AD. The evaluation of the analgesic action of pethidine hydrochloride (Demerol). J Pharmacol Exp Ther 1944; 80: 300-307.

YONEDA Y, OGITA K. Neurochemical aspects of the N-methyl-D-aspartate receptor complex. Neurosci Res 1991; 10: 1-33.

ZIEGLGÄNSBERGER W, HERZ A. Changes of cutaneous receptive field of spinocervical tract neurons and other dorsal horn neurons by micro-electrophorectically administered amino acids. Exp Brain Res 1971; 13: 111-126.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF EXPERIMENTAL TECHNIQUES

2.1 FORMALIN CONCENTRATION-RESPONSE CURVE

2.1.1 INTRODUCTION

The formalin test was originally developed to measure the analgesic properties of morphine in the presence of a 'tonic', non-escapable, inflammatory nociceptive stimulus (O'Keefe 1964). Pain and inflammation produced by a subcutaneous injection of dilute formalin into an animal's paw is moderate to severe and lasts up to 2 hours. In rats (Dubuisson and Dennis 1977), cats (O'Keefe 1964), mice (Hunskaar et al. 1985) and primates (Alreja et al. 1984), nociceptive responding to formalin follows a biphasic time course. There is an initial early phase of moderate to intense responding (0-10 min) followed by a period of about 10 min where nociceptive responses are greatly reduced or completely absent. A late phase of moderate responding continues for approximately 1.0 to 1.5 hours (Dubuisson and Dennis 1977). The early phase (Phase 1) is considered to be due to a direct action of formalin on cutaneous nociceptors while the late phase (Phase 2) is thought to be at least partly due to formalin-induced edema and inflammation (Dubuisson and Dennis 1977; see Chapter 1, Section 1.3.1). The histological changes surrounding the formalin injection site have been characterized (Rosland et

al. 1990; Wheeler-Aceto and Cowan 1991) and found to be consistent with current theories of inflammation (for review see Rote 1990; Fantone and Ward 1990). Recent evidence (Coderre et al. 1990) has suggested that, in addition to inflammation-induced nociception, the late phase of responding is, in part, due to spinal neural plasticity; there are changes in the CNS processing of Phase 2 nociceptive inputs as a result of previous processing during Phase 1. Evidence in support of these theories has been described in greater detail in CHAPTER 1 (see Section 1.3.1).

Because the formalin test is used to measure the analgesic properties of various therapeutic and experimental agents, a standard nociceptive stimulus of sufficient intensity and duration has been adopted by many investigators. In rats, the injection of 2.5% formalin provides a consistent level of responding adequate for measuring the relative analgesic properties of various agents (Dubuisson and Dennis 1977; Tjølsen et al. 1992; Tasker et al. 1992; Coderre et al. 1993). While the response to 2.5% formalin allows easy detection of decreased nociception (analgesia), the stimulus intensity is close to maximal thereby precluding the testing of drugs that increase nociception (hyperalgesia). To characterize the mechanisms involved in producing nociception in rats, a less intense stimulus is needed.

In the studies described in Chapter 3, different EAA ligands were used to characterize the role of EAA receptor subtypes in the spinal mechanisms of formalin-induced nociception. To test the theory that some of the EAA agonists and/or antagonists either increased or decreased pain as measured by the formalin

test, two different concentrations of formalin were required: one which caused a consistent low level of nociception and one which caused a consistent high level of nociception. This would allow the detection of both increases and decreases in pain behavior.

The objectives of the following experiment, therefore, were a) to establish a formalin concentration-response curve in rats, b) to determine two doses of formalin which reliably provide statistically different levels of formalin-induced nociception, and c) to characterize the acute inflammatory response to formalin in the rat hindpaw.

2.1.2 METHODS

2.1.2.1 ANIMALS

Male Long-Evans rats (300-450 g) obtained from Charles River Laboratories (Montreal, PQ) were housed in groups of 6 with food and water available ad libitum except during habituation and testing. Only male rats were used to avoid any potential complications of drug interactions with the stage of esterous. Lighting in the colony room was maintained on a 12 hour light / 12 hour dark cycle with all testing taking place between 8 AM and 2 PM. All rats were allowed a minimum of 5 days for acclimatization before any experimental manipulations were performed. No animal was tested more than twice (different hindpaws) and a minimum of 7 days was allowed between tests. In all cases, each data point represents a mean

obtained from a group of 6 rats. Further, each data point represents a combination of test results from 3 right feet and 3 left feet. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.1.2.2 FORMALIN TEST

The formalin test (O'Keefe 1964; Dubuisson and Dennis 1977) was used according to previously described modifications (Cohen et al. 1984). Testing took place in clear Plexiglas^{R} boxes (32 x 32 x 30 cm) with a mirror placed at a 45° angle beneath the floor to allow an unobstructed view of the formalin-injected paw. Prior to each formalin test, rats were habituated to the testing box for a minimum of 30 min on the day prior to the test and for 30 min immediately prior to formalin administration. To inject the formalin, rats were loosely wrapped in a cloth towel and one of the hindpaws was removed from the end of the cloth wrapping. Subcutaneous (s.c.) injections of formalin (0.05 ml) at concentrations of 0.5%, 1.0%, 1.5%, 2.5%, 5.0% and 10.0% (v/v with physiological saline (PS), 0.85% NaCl) or of PS were made into the plantar surface of one hindpaw.

Behavioral rating began immediately following formalin or PS administration and lasted for 60 min. A weighted-pain score was determined for each 5 min block by measuring the amount of time spent in each of 4 behavioral categories as follows (Cohen et al. 1984):

0 - The injected hindpaw was not favoured and the rat's weight was equally distributed between the two hindpaws;

1 - The injected hindpaw had little or no weight on it but was still in contact with the testing box floor;

2 - The injected hindpaw was elevated and not in contact with the testing box floor;

3 - The rat was licking or biting the injected hindpaw.

The weighted-pain score for each 5-min block was calculated as follows:

$$\text{Pain Score} = \frac{(a)(0) + (b)(1) + (c)(2) + (d)(3)}{300}$$

a = # of seconds with pain score of 0

b = # of seconds with pain score of 1

c = # of seconds with pain score of 2

d = # of seconds with pain score of 3

2.1.2.3 STATISTICAL ANALYSIS

Pain behavior was continuously rated using the previously described 4-point scale (Cohen et al. 1984). For all concentrations of formalin, an average, weighted-pain score for each 5 min block was used to calculate an area under the pain-score vs time curve (AUC). AUC was calculated according to the Trapezoid Rule for each time period of interest: a) the complete test period (0-60 min); b) Phase 1 (0-10

min); and c) Phase 2 (10-60 min). The AUC values were used to generate a concentration-vs-response curve (CRC), where higher AUC values were indicative of higher levels of nociception. A one-way analysis of variance (ANOVA) was used to determine if formalin concentration produced a significant nociceptive effect as measured by the AUC of the different formalin concentrations. Post-hoc analysis was carried out with the Student-Newman-Keuls (SNK) test to determine which formalin concentrations produced significantly different AUC values over the full test, and for each of Phase 1 and Phase 2.

2.1.2.4 HISTOLOGY

The formalin test measures the response to an acute, non-escapable noxious stimulus produced by localized tissue injury. It has been well documented that subcutaneous injections of formalin produce a characteristic inflammatory response and many experimenters have hypothesized that the inflammation has a role in the ensuing nociceptive response (Dubuisson and Dennis 1977; Hunskaar et al. 1986; Hunskaar and Hole 1987; Shibata et al. 1989). In order to demonstrate the type of histological changes that occur following formalin administration in the hindpaw of rats, both a formalin (2.5%) injected and non-injected hindpaw from one rat were removed and processed for light microscopy as described below.

Sixty min following the administration of formalin into the left hindpaw (an interval that coincides with the behavioral test period), the rat was euthanized with 1.0 ml sodium pentobarbitol (65 mg/ml i.p.; Somnotol, M.T.C. Pharmaceuticals,

Cambridge, ON). Both injected and non-injected hindpaws were removed and placed in 10% phosphate-buffered (0.1 M) formalin for 48 hours and then decalcified in Bouin's fixative for one month. The hindpaws were then washed with several changes of 50% ethyl-alcohol and were returned to 10% phosphate-buffered formalin (0.1 M). The tissues underwent routine processing with an automatic tissue processor (model 66-MP, Fisher Scientific Ltd., Ottawa, ON) followed by paraffin embedding. The paraffin blocks were trimmed and sectioned at 6-10 μm (Spencer Microtome, model 820, American Optical Co., Buffalo, NY). Mounted sections were then treated with Cal-Ex II (Fisher Scientific Ltd., Ottawa, ON) to allow further decalcification and stained with haematoxylin and eosin (Luna 1968). Tissue sections were examined and photographed with light microscopy (Zeiss D-7082 Transmitted-Light Photo-microscope, Carl Zeiss, Canada Ltd., ON).

2.1.3 RESULTS

2.1.3.1 FORMALIN CONCENTRATION-RESPONSE CURVE

Formyl-saline (formalin) is derived from 37% formaldehyde mixed with PS. Subcutaneous injections of PS into the plantar surface of one hindpaw produced minimal nociceptive responding over a 60 min testing period (mean score = 0.61 ± 0.30; data not shown).

The injection of formalin into the hindpaw of a rat produced nociceptive behavior that was rated according to the previously described 4-point scale (see

Section 2.1.2.2 above). Formalin-induced nociception always progresses in order from behaviors of a lower ranking to behaviors of a higher ranking. Thus, an animal will always favor the injected paw (score of 1) prior to lifting (score of 2) or licking (score of 3) the injected paw. The injection of various concentrations of formalin into the plantar surface of a rat's hindpaw produced a sigmoidal concentration-response curve over the 60 min test period (FIGURE 2.1). There was a significant formalin concentration effect over the 60 min test period ($F\{5,30\}=33.42$, $p<0.01$). The AUC value produced by 2.5% formalin was not significantly different from that of the highest formalin concentration (10.0%) tested ($q\{3,30\}=3.49$, $p>0.05$; FIGURE 2.1) while the AUC produced by 1.5% formalin was significantly less than the AUC produced by 10.0% formalin ($q\{4,30\}=3.85$, $p<0.05$). Thus, 2.5% formalin was the lowest concentration that produced nociception not significantly different from the observed maximum level of nociception seen following 10.0% formalin injections. 1.0% formalin was the closest concentration to 2.5% formalin that resulted in a significantly lower AUC as compared to the AUC of 2.5% formalin ($q\{3,30\}=4.45$, $p<0.01$; FIGURE 2.1).

When the 60 min AUC scores were analyzed as Phase 1 and Phase 2 components, both the first and second phases of response increased as the concentration of formalin increased (FIGURE 2.2). There was a significant formalin concentration effect for both Phase 1 and Phase 2 ([$F\{5,30\}=24.84$, $p<0.01$] and [$F\{5,30\}=28.47$, $p<0.01$] respectively). As with the 60 min comparisons, Phase 1 and Phase 2 responses following 2.5% formalin injections did not result in

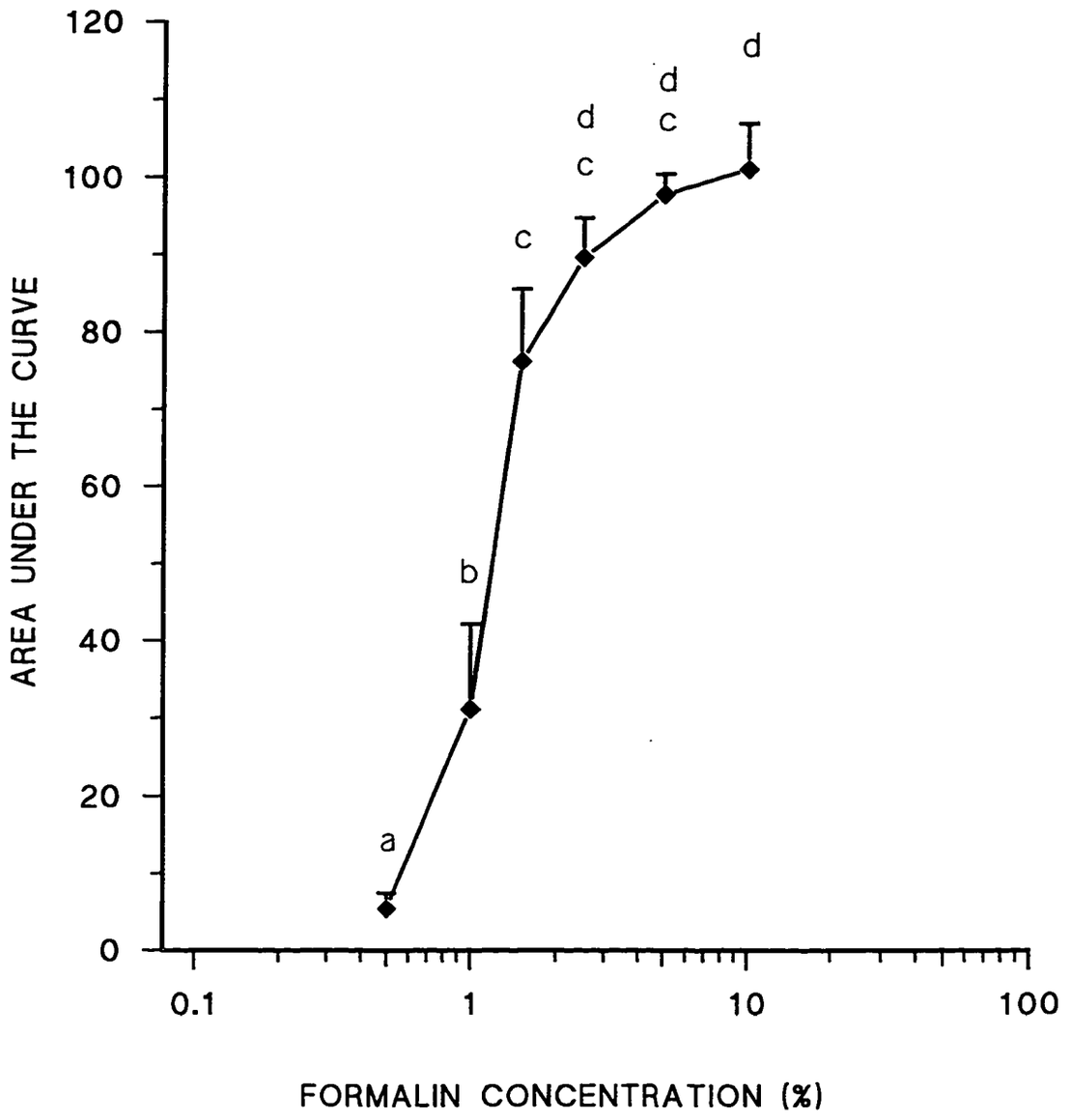


FIGURE 2.1 Concentration-vs-Response Curve for differing concentrations of formalin in the formalin test. Area-Under-the-Curve (AUC) values (mean + s.e.m.) are representative of the level of nociceptive behavior measured over a 60 minute test period. All data points represent the mean AUC from a group of 6 rats. Identical letters (a,b,c or d) above data points represent mean AUC values not significantly different from each other.

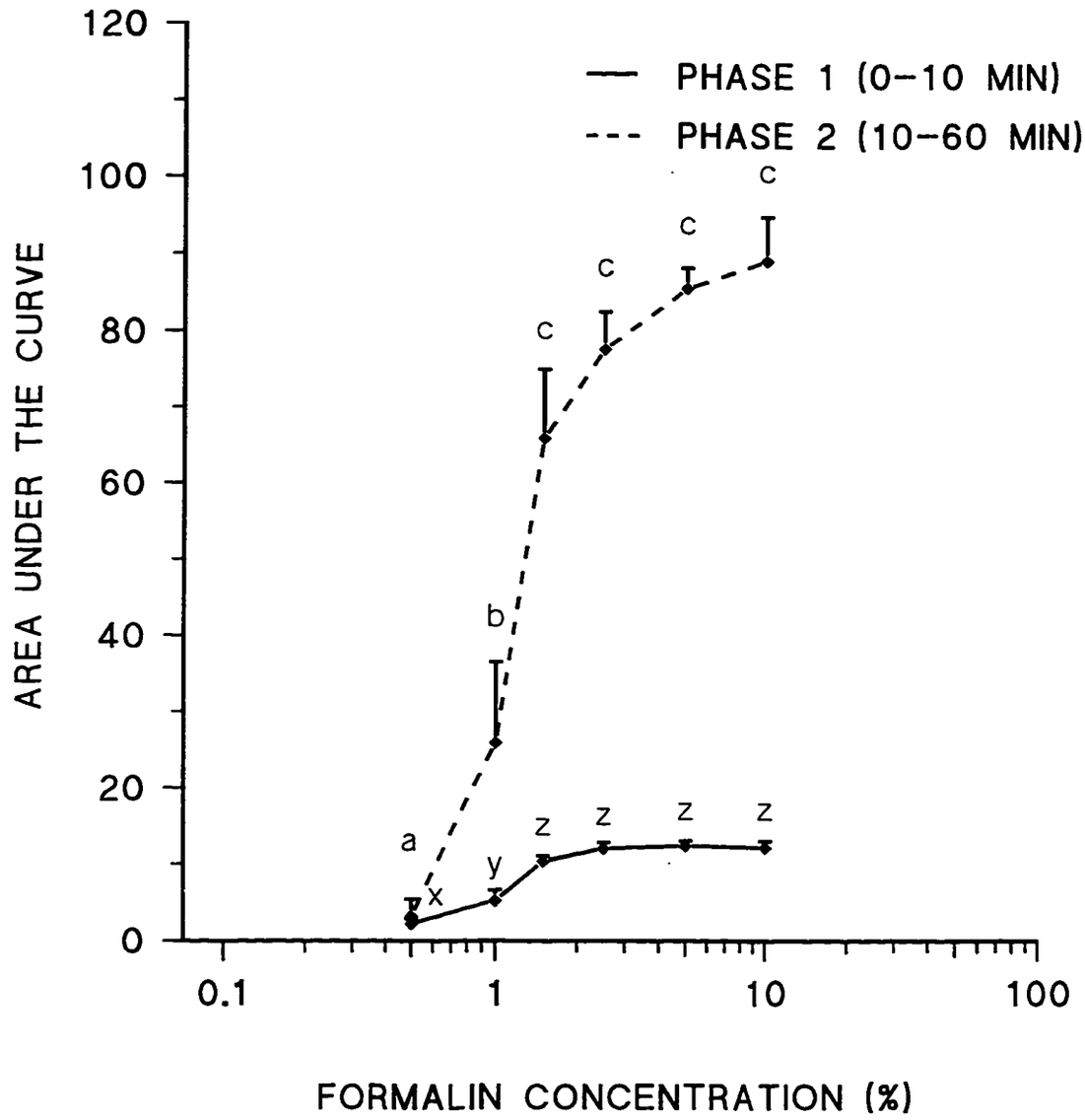


FIGURE 2.2 Concentration-vs-Response Curve for differing concentrations of formalin in the formalin test. Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociceptive behavior measured over the first phase (0-10 minutes) and second phase (10-60 minutes). All data points represent the mean AUC from a group of 6 rats. Identical letters (a,b,c,x,y or z) above data points of each curve represent mean AUC values not significantly different from each other.

significantly different AUC values as compared to 10.0% formalin injections ([q{3,30}=3.49, p>0.05] and [q{3,30}=3.49, p>0.05] respectively). However, in contrast to the 60 min results, 1.5% formalin rather than 2.5% formalin was the lowest injected concentration of formalin that was not significantly different from 10.0% formalin injections for both Phase 1 and Phase 2 ([q{4,30}=3.85, p<0.05] and [q{4,30}=3.85, p>0.05] respectively; FIGURE 2.2). As with the 60 min comparisons, AUC values following 1.0% formalin injections for Phase 1 and Phase 2 were statistically less than AUC values following 2.5% formalin injections ([q{3,30}=4.45, p<0.01] and [q{3,30}=4.45, p<0.01] respectively; FIGURE 2.2).

Since the AUC values following 2.5% formalin injections were not significantly different from maximal AUC values during all the time periods of interest, and because the AUC values following 2.5% and 1.0% formalin injections were significantly different from each other during the complete test period and during both Phase 1 and Phase 2, concentrations of 1.0% and 2.5% formalin were selected for future use as low and high intensity stimuli. A direct comparison of the mean AUC values following 1.0% and 2.5% formalin injections for the full test period, and for Phase 1 and Phase 2 components are presented in FIGURE 2.3. The time course of responding over a 60 min testing period for 1.0% and 2.5% formalin is shown in FIGURE 2.4. Both concentrations demonstrated a characteristic biphasic response profile.

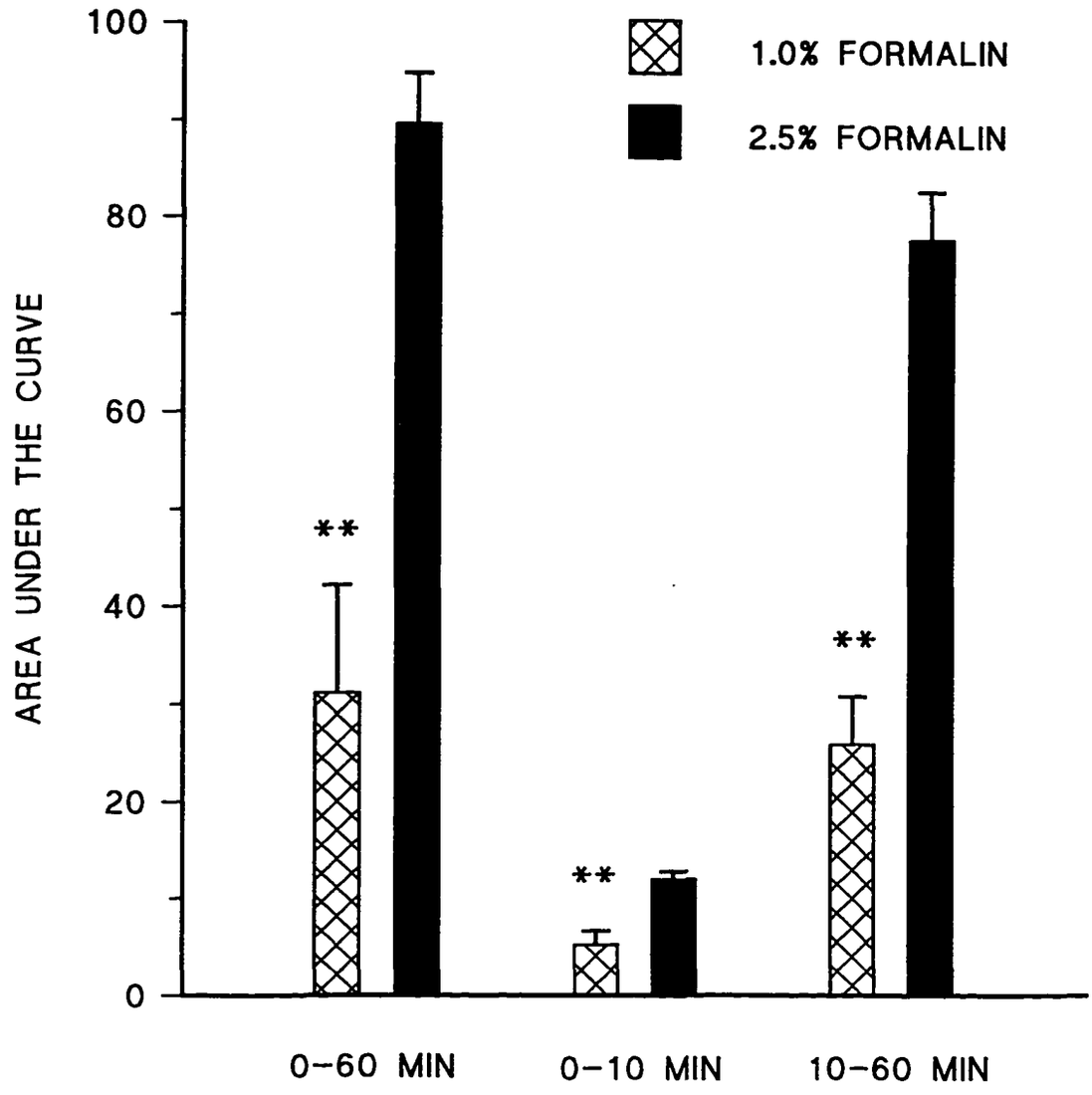


FIGURE 2.3 Effect of 1.0% and 2.5% formalin concentrations on nociception in rats. Data are expressed as the Area-Under-the-Curve (AUC; mean + s.e.m.) of the pain-score vs time curve for 0-60 minutes, Phase 1 (0-10 minutes) and Phase 2 (10-60 minutes). All data points represent the mean from a group on 6 rats ($p<0.01$ relative to the AUC for 2.5% formalin).**

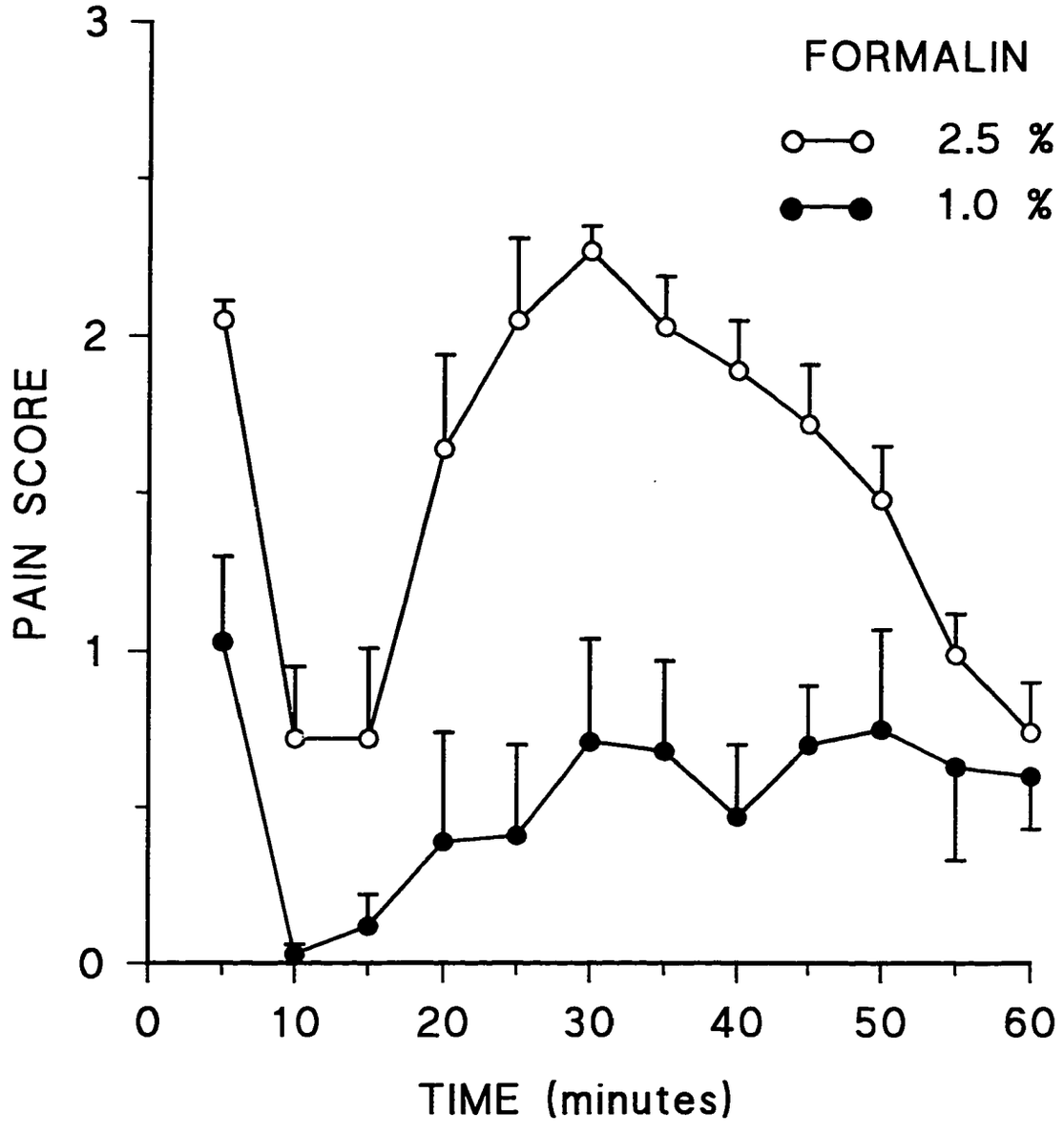


FIGURE 2.4 Pain scores (mean ± s.e.m.) in the formalin test over a 60 minute test period for two concentrations of formalin (1.0% and 2.5%). The characteristic biphasic response curve is seen following the administration of both concentrations of formalin. All data points represent the mean from a group of 6 rats.

2.1.3.2 HISTOLOGY

As described previously (Section 2.1.2), a formalin-injected (2.5%) and non-injected hindpaw of a rat were removed and processed for light microscopy 60 min following injection. Under low power magnification (25X), obvious differences could be seen between the control foot and the formalin-injected foot (FIGURE 2.5). The formalin-injected foot was characterized by edema and disruption of the integrity of the tissue, while these characteristics of inflammation were completely absent in the control foot. Further, within the section of the formalin-injected foot a vast number of leucocytes and red blood cells could be seen. Under higher magnification (800X), differences could again be observed between the control and the formalin-injected foot (FIGURE 2.6). The inflammatory response within the formalin-injected tissue could be observed as edema and a loss of tissue integrity. Mast cell degranulation was evident in the edematous tissue in contrast to the control tissue where non-degranulated mast cells were evident. Further, the formalin-injected tissue was characterized by an invasion of leucocytes and red blood cells.

FIGURE 2.5 Photomicrographs of a control and a formalin-injected rat hindpaw. (A) Section of a non-injected control rat hindpaw 60 minutes following an injection of 2.5% formalin to the contralateral hindpaw. (B) Section of a formalin-injected rat hindpaw 60 minutes following an intraplantar injection of 2.5% formalin (0.05 ml). Note the edema and infiltration of neutrophils along with the presence of red blood cells in the formalin-injected section. Both tissues were fixed in 10.0% phosphate-buffered formalin followed by post immersion in Bouin's fixative. Sections are 6-10 μm thick (25X magnification; scale bar = 400μm).

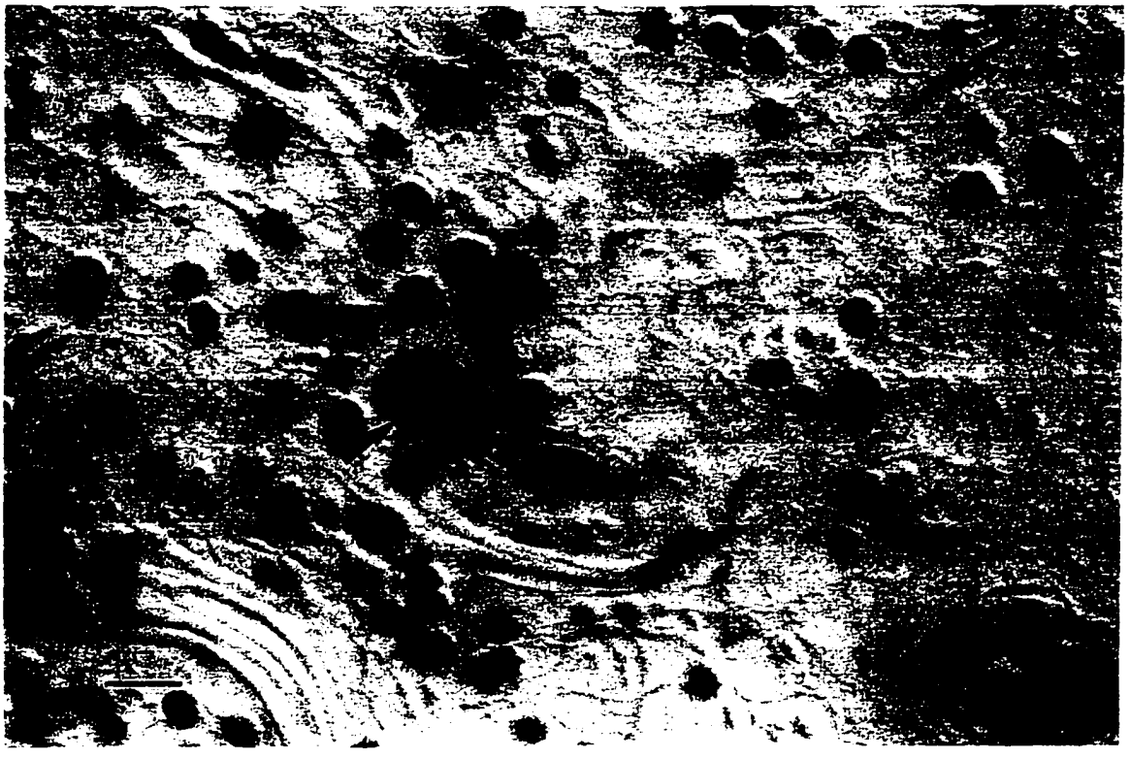
FIGURE 2.5.A

FIGURE 2.5.B

FIGURE 2.6 Photomicrographs of a control and a formalin-injected rat hindpaw. (A) Section of a non-injected rat hindpaw 60 minutes following an injection of 2.5% formalin to the contralateral hindpaw. (B) Section of a formalin-injected rat foot one hour following an intraplantar injection of 2.5% formalin (0.05 ml). Note the edema, degranulated mast cells and infiltration of neutrophils along with the presence of red blood cells in the formalin-injected tissue. Note the prominent granulated mast cell in (A) in contrast to the degranulated mast cell in (B) (arrows). Both tissues were fixed in 10.0% phosphate-buffered formalin followed by post immersion in Bouin's fixative. Sections are 6-10 μm thick (800X magnification; scale bar = 1.25μm).

FIGURE 2.6.A

FIGURE 2.6.B



2.1.4 DISCUSSION

2.1.4.1 FORMALIN CONCENTRATION-RESPONSE CURVE

The results of the analysis of the formalin CRC described above demonstrated that over the 60 min test period, injections of formalin in the rat hindpaw produced concentration-dependent nociceptive responding (FIGURE 2.1). Further, when the 60 min test period was separated into Phase 1 and Phase 2, concentration-dependent increases in nociceptive responses are preserved (FIGURE 2.2). These results represent the first systematic study of the effect of formalin concentration on nociceptive responses in rats occurring over a full 60 min test period. However, it has been previously noted in rats that nociceptive responses in the formalin test from 30 to 50 min were also directly dependent on formalin concentration (Coderre et al. 1993).

The AUC values following the injection of formalin at concentrations of 2.5% and 1.0% were statistically different from each other over the 60 min test period (FIGURES 2.1 to 2.3). Since 1.0% and 2.5% formalin concentrations, respectively, allow statistically measurable increases and decreases in nociceptive responding during all time periods of interest, they were used as the nociceptive stimuli in all further experiments (see CHAPTER 3).

Administration of either 1.0% or 2.5% formalin resulted in a characteristic biphasic response profile over the 60 min test period (FIGURE 2.4). The observed biphasic response allowed the total 60 min test period to be divided into two

separate response components. The Phase 1 and Phase 2 time intervals were chosen based on the observation that for any given formalin concentration, the second phase of the behavioral response did not begin until at least 15 min (average pain score from 10-15 min) following formalin injection.

In mice, formalin concentrations (0.02% to 5.0%) produced a graded increase in nociceptive responding when the time spent licking or biting the injected foot was used as a nociceptive index (Rosland et al. 1990). The results reported by Rosland and colleagues (1990) parallelled those reported above with rats (FIGURE 2.1) except that Rosland and colleagues (1990) described statistically greater nociceptive responses during the first phase (0-5 min) as compared to the second phase (20-30 min). The results of Rosland and colleagues (1990) were in contrast to the current observations (FIGURES 2.2 and 2.3) where greater nociception occurred during Phase 2. There were two major methodological differences in their experimental protocol and the protocol used here. Firstly, using the total time spent licking and/or biting the formalin-injected foot (mice; Rosland et al. 1990) neglected the contribution of low-level nociceptive behaviors (pain score = 1 or 2) to the overall nociceptive experience. It must be emphasized that it is difficult to consider only one type of behavior as being representative of the perceived pain in the formalin test. It is a better strategy to record and evaluate several behaviors in order to obtain a more accurate picture of an animal's behavior. On the other hand, it is difficult to measure low-level nociceptive behaviors in mice as the speed of responding in this species makes the accurate recording of behavioral changes impractical. Secondly,

Rosland and colleagues (1990) reported results from isolated time periods (0-5 and 20-30 min) even though formalin-induced nociception in mice, as in rats, may last between 60 and 90 min.

The formalin concentration response profile differed (was not parallel) during the two phases of nociceptive responding (FIGURE 2.2) indicating that the mechanisms underlying the processing of nociceptive information in each phase differ. Examination of the data presented in FIGURE 2.2 revealed that, following the administration of formalin concentrations greater than 1.5%, Phase 2 AUC values continued to increase even though the Phase 1 response had plateaued. Therefore, it was reasonable to assume that first phase activation of spinal and supraspinal systems did not increase with formalin concentrations greater than 1.5%. The AUC values at the first phase plateau were approximately 50% of the maximum score that can theoretically be obtained during this phase of responding. Two possible explanations for the further increase in second phase AUC in the absence of increasing first phase AUC are a) increased inflammatory responses due to increased formalin concentrations and b) progressive recruitment of non-nociceptive spinal and supraspinal systems by increased formalin concentrations. The contribution of each of these possibilities requires further study.

2.1.4.2 HISTOLOGY

The observed histological changes were consistent with previously reported observations (Rosland et al. 1990) and represent tissue changes that were typical of

an acute inflammatory response (for review see Rote 1990; Fantone and Ward 1990). Unfortunately, the contribution of physiological saline to the inflammatory response, if any, could not be determined because a non-injected foot was used as the control. Histological changes that occurred following a saline injection into a hindpaw of a mouse were previously studied (Rosland et al. 1990) and it was found that there were no histological indicators of an inflammatory response up to one hour following the saline injection. Therefore, based on the observations of Rosland and colleagues (1990), it was reasonable to suggest that the observed histological changes following a formalin injection were due to formalin-induced tissue responses. Other biochemical responses might include the release of one of several chemical mediators such as histamine, kinins, serotonin, and prostaglandins (for review see Fantone and Ward 1990). Further, these mediators have been demonstrated to be an integral part of the inflammatory response and may contribute to the stimulation and sensitization of peripheral nociceptors (Fantone and Ward 1990).

2.2 THE EFFECT OF DRUG VEHICLE ON THE FORMALIN RESPONSE

2.2.1 INTRODUCTION

Possibly the best way to examine drug action at the spinal level in the conscious, unrestrained animal is to introduce the drug directly into the spinal

subarachnoid fluid. The method of direct spinal injection via an acute needle puncture into the lumbar subarachnoid space was developed for both mice (Hylden and Wilcox 1980) and rats (Coderre and Melzack 1992; Coderre and Van Empel 1994). However, the technique of spinal puncture suffers from one main disadvantage: the animal must be tightly restrained (mice) or anaesthetized (rats) prior to the spinal puncture. The effect of restraint stress or anaesthetics on the ability of EAAs or other ligands to bind or activate receptors is, for the most part, unknown. Certain anaesthetics, however, are known to interact with EAA receptors (Carlà and Moroni 1992; Lees 1995; Porter and Greenamyre 1995). Further, stress-induced analgesia in the formalin test has been reported (Abbott et al. 1986; Vaccarino et al. 1992).

To minimize potential complications due to restraint stress and anaesthetic administration, a modified version of the surgical procedure introduced by Yaksh and Rudy (1976) was developed. This method consists of inserting a chronic indwelling intrathecal polyethylene cannula via a puncture in the atlanto-occipital membrane and is described below. Radiographs were used to verify that the modified surgical procedure actually placed the cannula at the rostral edge of the lumbar enlargement.

GLU and ASP, two endogenous EAA ligands, and many EAA receptor agonists and antagonists are acidic in nature. A pH of 7.0 represents the approximate pH of the cerebral-spinal fluid while a pH of 2.5 represents the approximate pH of PS containing GLU or ASP (1000 nmol/μl) solutions. The

concentration of GLU or ASP used to determine this pH, as determined in a pilot experiment, was a much greater concentration than that expected to be used in subsequent behavioral studies.

The pH of an injected drug solution is an important consideration when interpreting behavioral results. The final pH of the solution is reflected by the pK_A of the drug. The pK_A of a compound indicates the pH level at which a compound exists equally in the ionized and non-ionized state. Thus, drugs with a low pK_A are 50% ionized at a low pH.

Both GLU and ASP are amino acids with low pK_As and carry negatively charged carboxyl groups at pH 6.0 to 7.0. At approximately neutral pH, the two amino acids have an overall negative charge which increases the hydrophilic nature of the molecule. The more hydrophilic the molecule, the less likely it is to diffuse across the lipid membranes into an area where it can be pharmacologically active. In a more acidic environment, pH 2.0 to 4.0, the molecules will be more lipophilic and better able to diffuse across a lipid membrane, such as the spinal meningeal membrane, and act at the level of the synapse. It is, therefore, an advantage to administer a compound under conditions where it has the greatest chance to reach its presumed site of action. Unfortunately, administering a drug at a pH that is different from the surrounding systemic pH may cause behavioral side effects that would interfere with the study.

The objectives of this section were three-fold. Firstly, to determine the reliability of the chronic cannulation procedure. Secondly, to determine whether the

intrathecal cannulation procedure changed the baseline nociceptive response to formalin. Thirdly, to determine whether intrathecal injections of potential vehicles (PS or phosphate-buffered saline (PBS)) at either physiological or acidic pH produced changes in the nociceptive response compared to sham intrathecal injections.

2.2.2 METHODS

2.2.2.1 ANIMALS

Male Long-Evans rats (300-450 g) obtained from Charles River Laboratories (Montreal, PQ) were housed in groups of 6 with food and water available ad libitum. All rats were acclimatized to the housing area for a minimum of 5 days prior to surgery. Following catheter implantation (see below) the rats were individually housed with food and water available ad libitum except during habituation and testing. All animals were allowed 7 - 10 days to recover from surgery prior to experimental manipulation. Lighting in the colony room was maintained on a 12 hour light / 12 hour dark cycle with all testing taking place between 8 AM and 2 PM. No animal was tested more than twice (each hindpaw) and a minimum of 7 days was allowed between tests. In all cases, each data point represented a mean obtained from 3 right feet and 3 left feet from a group of 3 rats. All experiments were conducted in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2.2.2 SURGICAL PROCEDURE

Rats were anaesthetized with sodium pentobarbital (65 mg/kg i.p.; Somnotol, M.T.C. Pharmaceuticals, Cambridge, ON) and the skin on the caudal part of the head and the rostral part of the neck was shaved. Each rat was fitted into a conventional stereotaxic frame (David Kopf Instruments, Tajunga, CA) and the shaved portion of the head and neck was cleaned with chlorohexidine gluconate (4.0% w/v; Cyanamid Canada Inc, Montreal, PQ). A drop of mineral oil was applied to each eye to prevent drying as the eye-blink reflex is inhibited under the influence of the anaesthetic. The incisor bar of the stereotaxic instrument was pushed snugly against the dorsal surface of the head so that the snout was held downward at approximately a 90° angle in relation to the spinal cord. A midline incision approximately 1 cm in length was made perpendicular to the occipital crest of the skull resulting in a split of the underlying neck muscles. Retraction of the exposed muscles with forceps exposed the atlanto-occipital membrane. A small puncture in the membrane directly at the base of the occipital crest of the skull was made with fine forceps. Considerable care was required to avoid damage to the dorsal surface of the medulla oblongata which lies immediately ventral to the membrane. The atlanto-occipital membrane is actually made up of two layers: a very tough outer fascial layer and the underlying dura. It was possible to cut the outer fascia and not penetrate the dura. When the incision had been carried out correctly, there was leakage of a small amount of clear cerebrospinal fluid through the punctures in the two membranes. The rat was then ready for insertion of the

catheter into the subarachnoid space.

The catheter consisted of a 13.5 cm length of polyethylene tubing (PE-10) (Intramedic, Clay Adams, Parsippany, NJ) stretched to approximately 130% of its length so that the internal volume of the catheter was consistently between 6.0 μl and 7.0 μl and the outer diameter of the tubing (0.55 mm) was reduced by 20% - 30%. The catheter was stored overnight in sterile 70% ethyl-alcohol, flushed with PS and the external end was plugged with a 1 cm piece of small gauge stainless steel wire. The catheter was slowly inserted through the incision in the atlanto-occipital membrane while being slightly rotated with the thumb and forefinger. This rotation facilitated the entry of the catheter into the subarachnoid space. Once the catheter had been advanced 1-2 cm, care was required to insure that the spine remained straight: a small book, approximately 2.5 cm thick, was placed under the animal and a slight tension was applied to the tail. The catheter was then inserted 8.5 cm from the membrane opening. If resistance was felt during the insertion procedure, the catheter was slightly withdrawn and reinserted. Insertion of the catheter 8.5 cm placed the catheter tip at the rostral margin of the lumbar enlargement (T-12 to L-1; see Section 2.2.3 - Results).

Once the insertion of the cannula was completed, the head of the rat was freed of the downward pressure and the jaw was secured with the incisor bar. With the head placed in a more upward position, the caudal part of the skull became visible through the previous incision. The periosteum covering the skull was cut with fine scissors and retracted with small bulldog clamps. Two holes.

approximately 1 mm apart, were drilled through the skull with a sharp 23 gauge needle, and a stainless steel jeweller's screw (length=3.4 mm, thread diameter=0.9 mm; Lomat, Montreal, PQ) was inserted into each hole. The catheter was then placed between the screws. The catheter and screws were further secured with a covering of acrylic dental cement (Canada Dental Supply, Halifax, NS). Once the cement was dry, a slow injection of PS (7.0 µl) was made to clear the catheter of any debris that may have accumulated during insertion.

Following surgery, each rat was individually housed and allowed a minimum of 7 days to recover from the surgery before any experimental manipulations were performed. Any rat showing spontaneous or induced neurological or motor deficits following recovery from the anaesthetic was immediately euthanized. Such deficits were observed in approximately 20% of the cannulated animals.

To demonstrate the reliability of the surgical procedure, and to verify that the catheter tip was located at the T-12 to L-1 region of the spinal cord, radiographs were made of two rats that had undergone catheterization. Two rats (approx 350 g) that had recovered for at least one week following catheterization were randomly chosen and anaesthetized with 0.5 ml of sodium pentabarbitol (65 mg/kg). Each catheter was filled with 7 µl of radio-opaque contrast agent (CONRAY-60; 60% othalamate-meglumine; Mallinckrodt Canada Inc, Montreal, PQ) and then dorsal and sagittal radiographs were taken of each rat.

2.2.2.3 DRUG INJECTION

Five min prior to drug administration each rat was placed into a cloth restrainer (modified version of Owen et al. 1984) to restrict movement of the rat during the intrathecal injection. This type of restraint is less stressful to the rat compared to other traditional types of rat restraints (Owen et al. 1984) making analgesic measurements less variable by decreasing the interaction between the drug effect and stress. The external portion of the catheter was visualized by threading it through a small hole cut into the cloth directly above the head of the rat. The stainless steel wire used to plug the external end of the catheter was removed and the tip of a Hamilton syringe (10 µl) was inserted into the end of the catheter. A 10 µl volume of PS or PBS (0.1M; pH 2.5 or 7.2), followed by a 7 µl volume of PS to flush the catheter, was injected over a two min period. The two vehicle solutions were adjusted to the appropriate pH with either hydrochloric acid (HCl: 0.1 N) or sodium hydroxide (NaOH: 0.1 N). The Hamilton syringe was removed and the stainless steel wire plug was returned to the end of the catheter. The animal was then returned to the testing box.

Two additional groups of 6 rats were implanted with intrathecal catheters as described above (Section 2.2.2.2). These two groups underwent all injection handling procedures as previously described except that no fluid was injected (sham). One group was tested with 1.0% formalin and the other with 2.5% formalin. Finally, the data from two previously described groups of non-operated rats that received either 1.0% or 2.5% formalin injections (Section 2.1) were

compared to the data from the sham and different vehicle groups in order to characterize any effects due to surgical manipulation (sham) and vehicle injection.

2.2.2.4 FORMALIN TEST

The formalin test (O'Keefe 1964; Dubuisson and Dennis 1977) was used according to modifications described by Cohen and colleagues (1984) as previously described (Section 2.1.2). Briefly, rats were continuously rated according to a 4-point rating scale (Cohen et al. 1984) as described in section 2.1.2.2. Prior to each formalin test, rats were habituated to the testing box for 30 min on the day prior to testing and for 30 min immediately prior to testing. Further, each rat was habituated to the restraint apparatus for 10 min on the day prior to testing. Ten min following the end of the intrathecal injection, formalin (1.0% or 2.5%, 0.05ml) was injected into the plantar surface of a rat's hindpaw. To inject the formalin, rats were loosely wrapped in a cloth towel and one of the hindpaws was freed from the end of the cloth wrapping. Behavioral rating began immediately following formalin administration and lasted for 60 min.

2.2.2.5 STATISTICAL ANALYSIS

Pain behavior was continuously rated using a previously described 4-point scale (Cohen et al. 1984; Section 2.1.2.2) and average weighted-pain scores were used to calculate AUCs.

A two sample (independent) t-test was used to determine if a statistical

difference was present between sham-injected and non-operated groups following both 1.0% and 2.5% formalin injections. A two sample (independent) t-test was again used to determine if the previously seen statistical difference between 1.0% and 2.5% formalin in non-operated rats was preserved in the sham-injected control groups.

A one-way ANOVA was used to determine if a significant difference existed between any of the groups at either formalin concentration.

Within a regression analysis model, the sum of squares can be partitioned so that the sum of squares due to regression (SSR) is broken down into the various components that reflect the relative contributions of the various terms in the fitted model (Kleinbaum et al. 1988). In an ANOVA framework, it is possible, via the use of orthogonal contrasts, to partition the treatment sum of squares (SST) into meaningful components associated with certain specific comparisons of interest (Kleinbaum et al. 1988). Therefore, post hoc analysis was carried out with orthogonal contrasts to determine if a statistical difference existed between the sham-injected control group and either of the PS or the PBS groups (pH 2.5 or 7.0) following injections of either 1.0% or 2.5% formalin. The main-effect mean-square-error term needed for each orthogonal contrast was determined from the appropriate ANOVA calculations.

2.2.3 RESULTS

2.2.3.1 CANNULATION PROCEDURE

To demonstrate the reliability of the surgical procedure, and to verify that the catheter tip was located at the T-12 to L-1 region of the spinal cord, dorsal and sagittal radiographs were made of two rats that had undergone catheterization (FIGURE 2.7). The radiographs verified that the catheters terminated at the level of the T-12 to L-1 spinal vertebrae. This location corresponds to the rostral edge of the lumbar enlargement of the spinal cord in rats.

2.2.3.2 EFFECT OF INTRATHECAL CANNULATION ON NOCICEPTION

To determine if the cannulation procedure caused undetected changes in sensory perception, as measured in the formalin test, a group of rats underwent a sham-injection prior to formalin administration and the results were compared against the results of non-operated groups from Section 2.1.3.

Following the administration of 2.5% formalin, there were no significant differences in AUC values between the non-operated and sham-injected groups for the 60 min period, Phase 1 and Phase 2 ([$t=0.93$, $p>0.05$, d.f.$=10$], [$t=0.96$, $p>0.05$, d.f.$=10$] and [$t=0.56$, $p>0.05$, d.f.$=10$] respectively; FIGURE 2.8).

Similarly, following the administration of 1.0% formalin, AUC values were not significantly different between the non-operated and sham-injected groups for the

FIGURE 2.7 Sagittal (A) and dorsal (B) radiographs of a rat having a chronically implanted spinal catheter. The polyethylene catheter has been made visible by the injection of 7μl of Conray-60. The catheter tip terminates in the area between T-12 and L-1 at the rostral face of the lumbar enlargement (arrow) (scale bar = 10 mm).

FIGURE 2.7.A

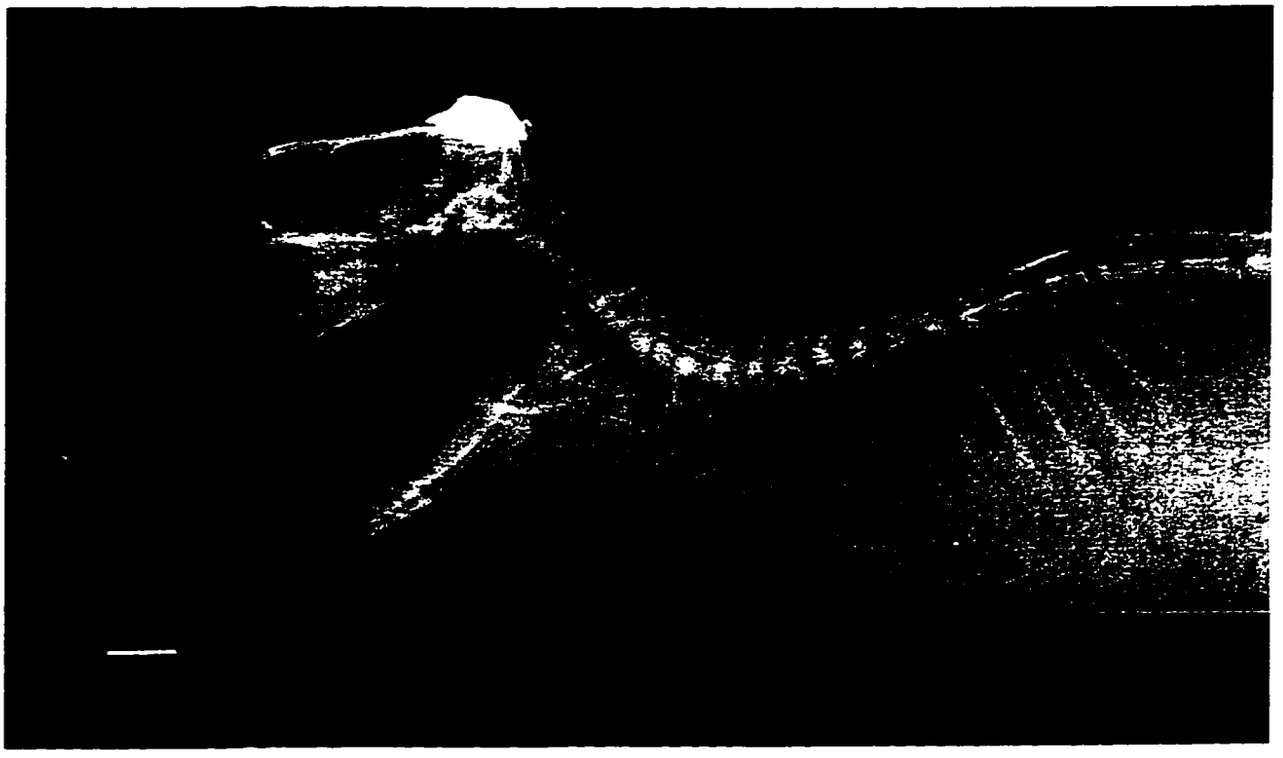
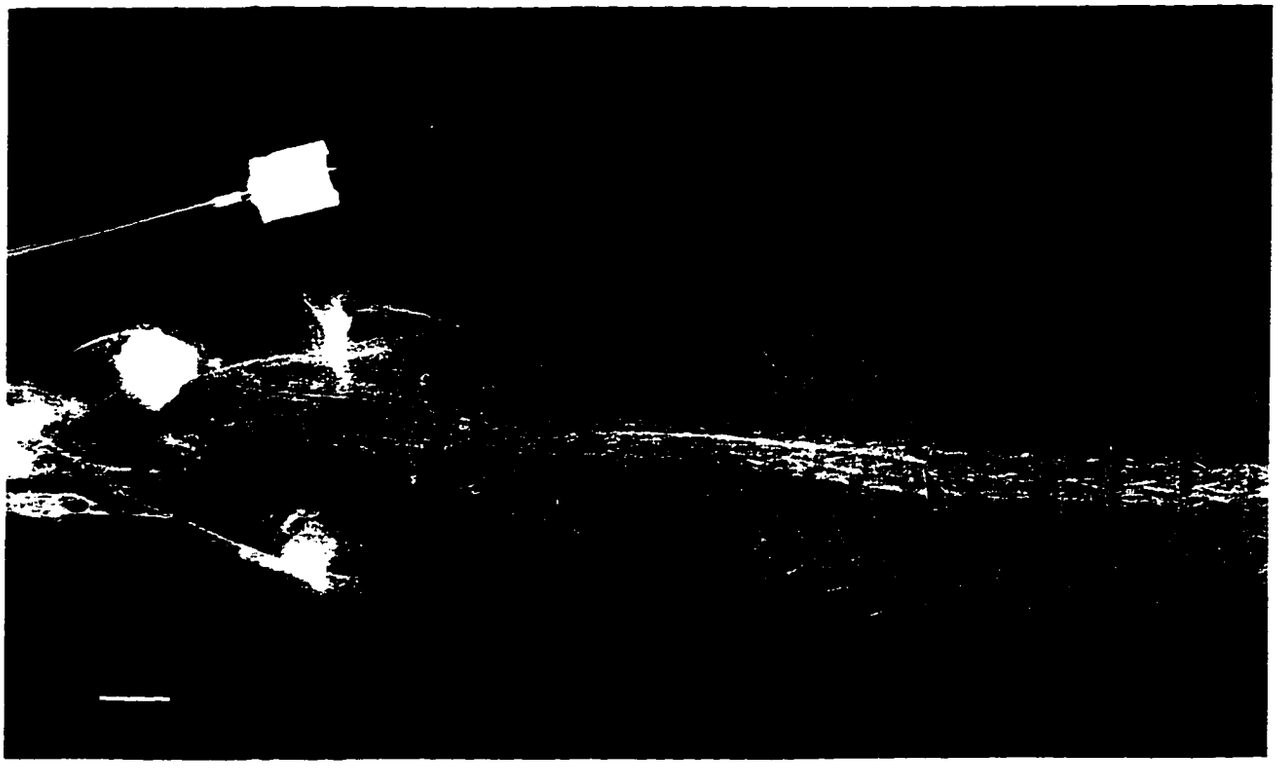


FIGURE 2.7.B



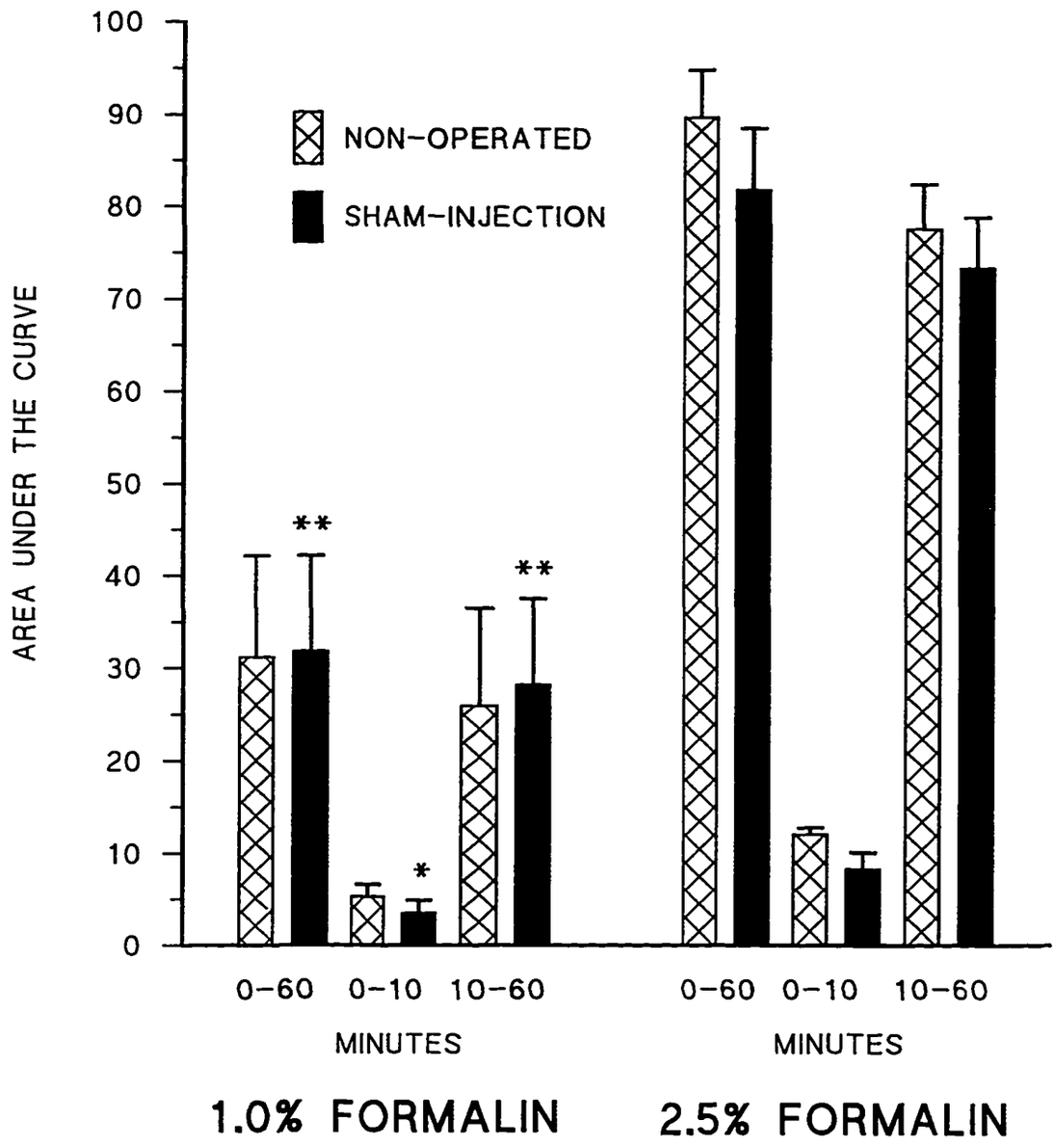


FIGURE 2.8 Effect of 1.0% and 2.5% formalin on nociception in both non-operated and sham-injected rats. Data are expressed as the Area-Under-the-Curve (AUC; mean + s.e.m.) of the pain-score vs time curve for 0-60 minutes, Phase 1 (0-10 minutes) and Phase 2 (10-60 minutes). All data points represent the mean AUC from a group of 6 rats ($p<0.01$, *$p<0.05$ relative to the AUC for 2.5% formalin in sham-injected group).**

60 min test period, Phase 1 and Phase 2 ([t=0.05, p>0.05, d.f.=10], [t=0.87, p>0.05, d.f.=10] and [t=0.17, p>0.05, d.f.=10] respectively; FIGURE 2.8).

The two different concentrations of formalin (1.0% and 2.5%) continued to produce significantly different levels of nociception based on the AUCs of the two sham-injected groups. AUC values for the complete test period, Phase 1 and Phase 2 were all significantly different from each other ([t=4.13, p<0.01, d.f.=10], [t=2.27, p<0.05, d.f.=10] and [t=4.26, p<0.01, d.f.=10] respectively; FIGURE 2.8). These results parallelled those reported above for non-operated rats (Section 2.1: FIGURE 2.3).

The time-profile of the mean pain score for each 5 minute block over the 60 min test period for sham-injected rats could be described by a characteristic biphasic response curve (FIGURE 2.9). The curves for both 1.0% and 2.5% formalin administration for sham-injected rats were similar to the curves presented previously (Section 2.1; FIGURE 2.4) for non-operated rats.

2.2.3.3 EFFECT OF VEHICLE AND VEHICLE pH ON NOCICEPTION

PS or PBS at pH 2.5 or pH 7.0 were administered through chronic indwelling intrathecal catheters and the level of nociception (AUC) was compared against that of sham-injected rats following injection of either 2.5% or 1.0% formalin. The AUC values for the sham, PS and PBS injected groups for the 60 min, Phase 1 and Phase 2 periods following either 2.5% or 1.0% formalin administration are presented in FIGURES 2.10 and 2.11.

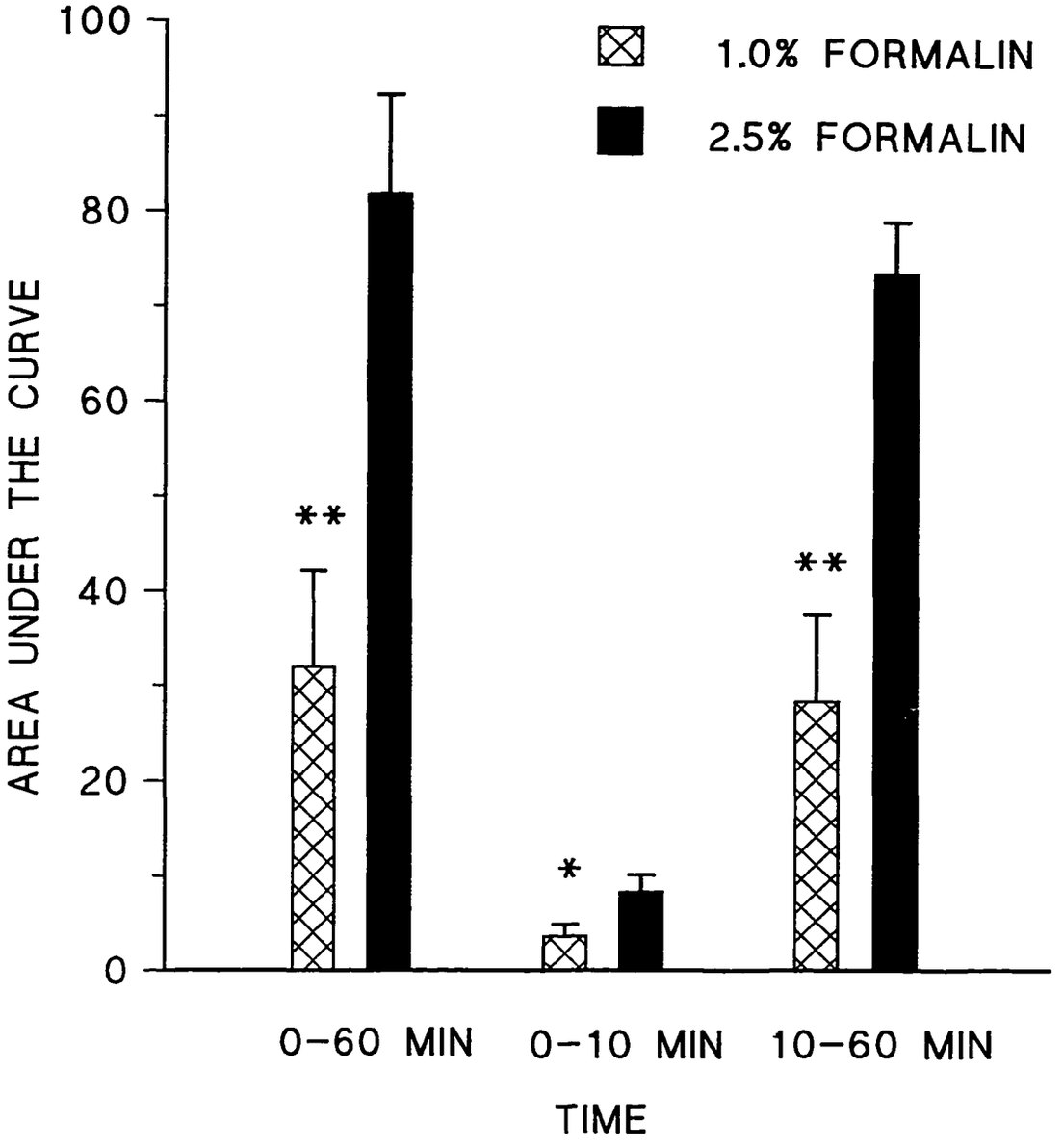


FIGURE 2.9. Effect of 1.0% and 2.5% formalin concentrations on nociception in sham-injected rats. Data are expressed as the Area-Under-the-Curve (AUC) (mean + s.e.m.) of the pain-score vs time curve for 60 minutes, Phase 1 (0-10 minutes) and Phase 2 (10-60 minutes). All data points represent the mean from a group of 6 rats ($p<0.01$, * $p<0.05$ relative to the AUC for 2.5% formalin).**

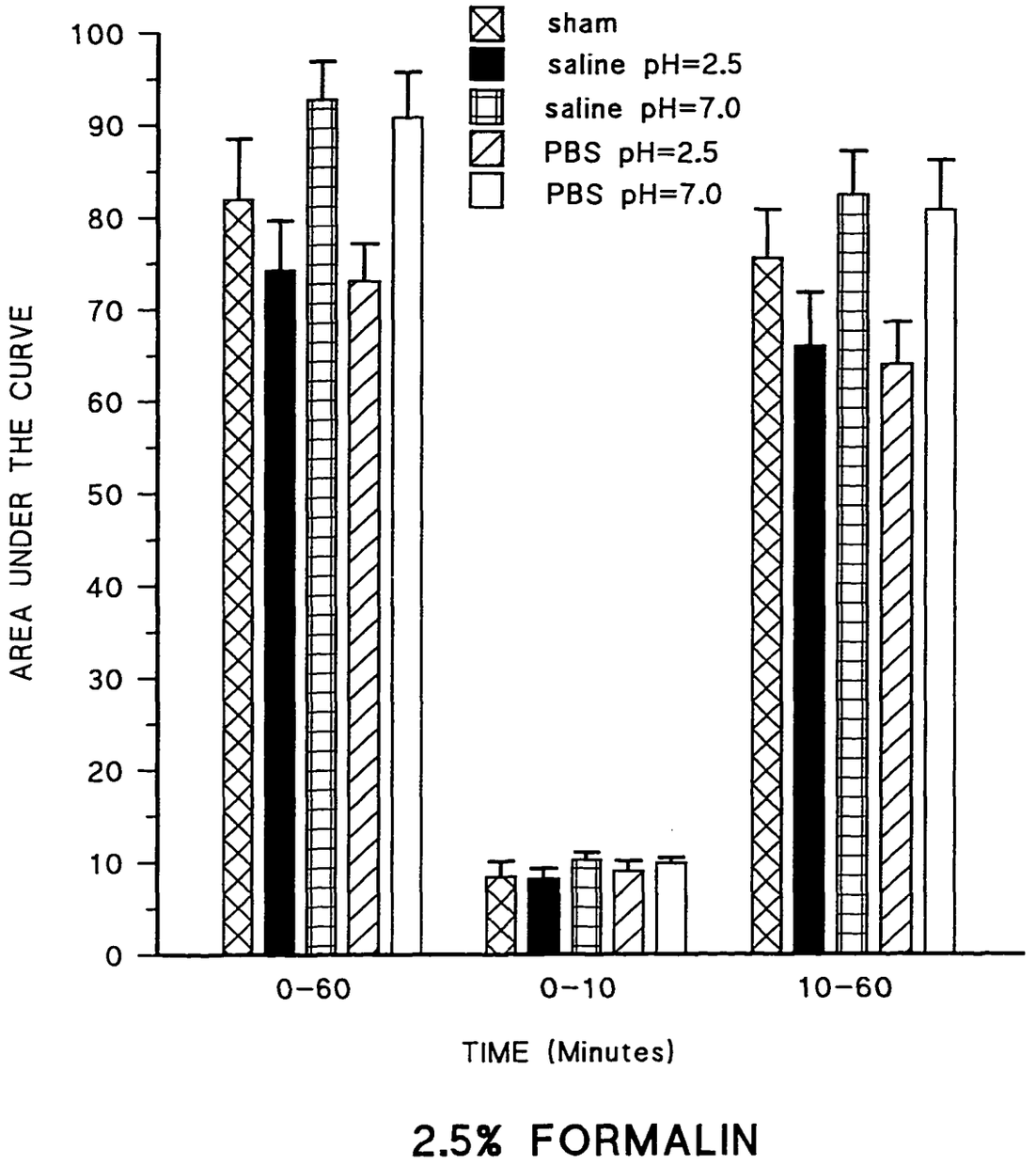


FIGURE 2.10 Effect of physiological saline (PS), phosphate-buffered saline (PBS) and sham-injection on nociception in rats following an injection of 2.5% formalin. Data are expressed as the Area-Under-the-Curve (AUC; mean + s.e.m.) of the pain-score vs time curve for 60 minutes, Phase 1 (0-10 minutes) and Phase 2 (10-60 minutes). All data points represent the mean from a group of 6 rats.

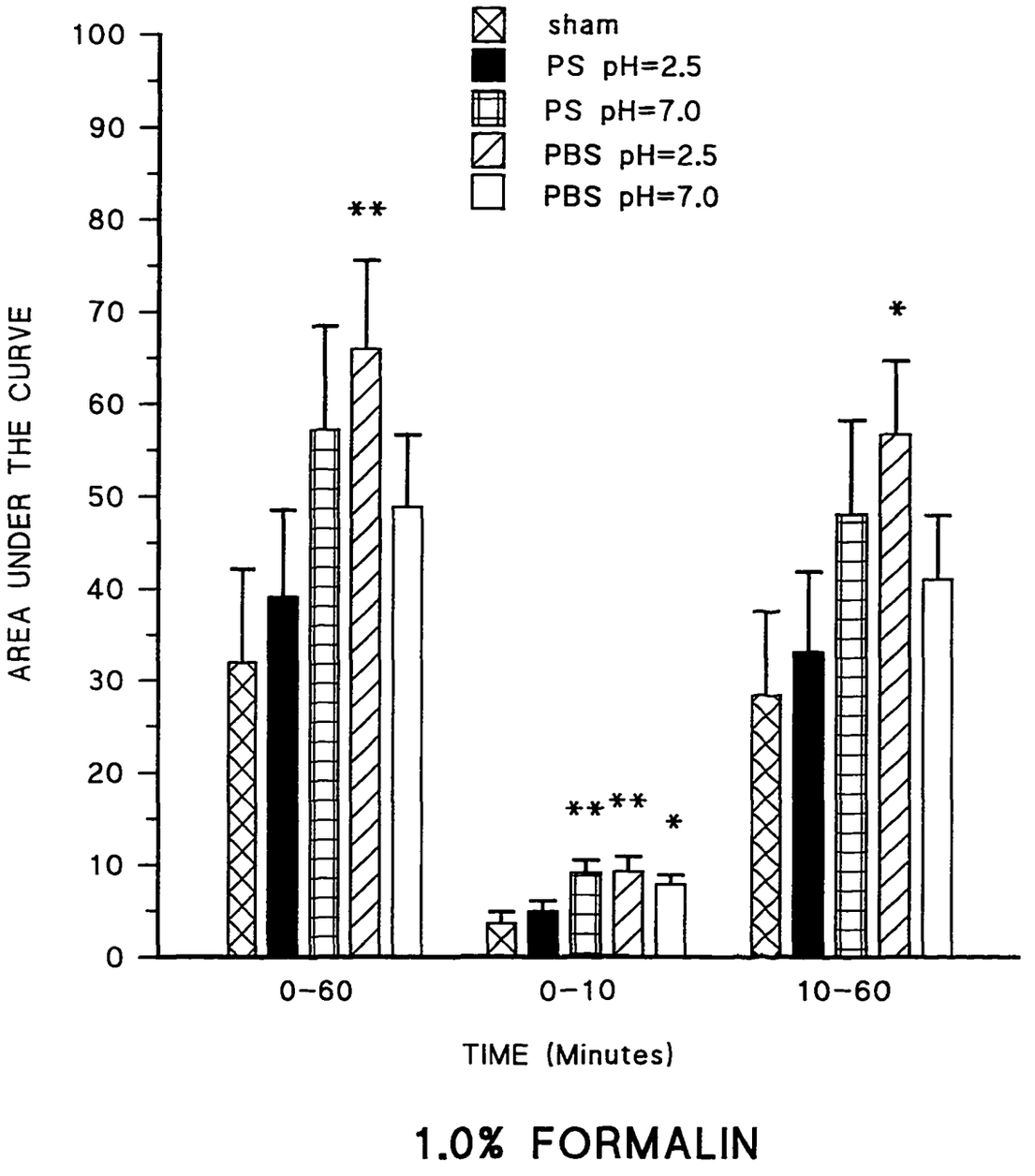


FIGURE 2.11 Effect of physiological saline (PS), phosphate-buffered saline (PBS) and sham-injection on nociception in rats following an injection of 1.0% formalin. Data are expressed as the Area-Under-the-Curve (AUC; mean + s.e.m.) of the pain-score vs time curve for 60 minutes, Phase 1 (0-10 minutes) and Phase 2 (10-60 minutes). All data points represent the mean from a group of 6 rats ($p<0.01$, * $p<0.05$ relative to the AUC for sham-injected rats).**

Results obtained following injection of 2.5% formalin are shown in FIGURE 2.10. The AUC values of the sham group were not statistically different from the AUC values of the PS (pH 2.5) group over the 60 min, Phase 1 or Phase 2 test periods ([F{1,15}=0.97, p>0.05], [F{1,15}=0.002, p>0.05] and [F{1,15}=1.00, p>0.05] respectively). Similar results were obtained with PS at pH 7.0 ([F{1,15}=0.22, p>0.05], [F{1,15}=1.11, p>0.05] and [F{1,15}=1.42, p>0.05] respectively; FIGURE 2.10). With respect to injections of PBS, the AUC values of the sham group were not statistically different from the AUC values of the PBS groups at either pH 2.5 or 7.0 during the 60 min, Phase 1 and Phase 2 periods ([F{1,15}=1.42, p>0.05], [F{1,15}=1.30, p>0.05] and [F{1,15}=1.78, p>0.05] respectively for pH 2.5) and ([F{1,15}=1.42, p>0.05], [F{1,15}=0.72, p>0.05] and [F{1,15}=1.07, p>0.05] respectively for pH 7.0; FIGURE 2.10).

Results obtained following 1.0% formalin injections are shown in FIGURE 2.11. The AUC values of the sham group were not statistically different from either the AUC values of the PS group (pH 2.5) over the 60 min, Phase 1 or Phase 2 test periods ([F{1,15}=0.19, p>0.05], [F{1,15}=0.65, p>0.05] and [F{1,15}=0.14, p>0.05] respectively), or the AUC values of the PS group (pH 7.0) for the 60 min and Phase 2 periods ([F{1,15}=3.00, p>0.05] and [F{1,15}=2.22, p>0.05] respectively; FIGURE 2.11). However, the AUC value of the PS group for Phase 1 was statistically different from the sham group ([F{1,15}=9.84, p<0.01]; FIGURE 2.11).

PBS (pH 2.5) treated rats responded with significantly more nociception than

sham-injected rats during all 3 time periods: 60 min, Phase 1 and Phase 2, ([F{1,15}=6.75, p<0.01], [F{1,15}=8.86, p<0.01] and [F{1,15}=6.15, p<0.05] respectively; FIGURE 2.11). Further, PBS (pH 7.0) treated rats also had demonstrated significantly more nociception as compared to sham-injected rats during Phase 1 (F{1,15}=4.99, p<0.05; FIGURE 2.11). However, the AUC values of the PBS (pH 7.0) and sham groups were not statistically different from each other during the 60 min and Phase 2 periods (F(1,15)=1.68, p>0.05 and F(1,15)=1.24, p>0.05 respectively; FIGURE 2.11).

Intrathecal injections of PS at pH 2.5 did not produce significant changes in baseline nociception as compared to sham-injected rats when PS was administered prior to either 2.5% or 1.0% formalin (FIGURES 2.10 and 2.11). Further, intrathecal injections of PS (pH 2.5) prior to 2.5% formalin, resulted in AUC values significantly greater than AUC values of the corresponding 1.0% formalin-injected group for 60 min, Phase 1 and Phase 2 ([t=3.39, p<0.01, d.f.=10], [t=2.28, p<0.05, d.f.=10] and [t=3.16, p<0.01, d.f.=10] respectively). The significant differences between 2.5% and 1.0% formalin-injected groups following intrathecal injections of PS at pH 2.5 parallel the differences found in sham-injected rats (FIGURE 2.8). The AUC values of PS (pH 2.5) treated rats for the full test period, Phase 1 and Phase 2 following 2.5% and 1.0% formalin (FIGURES 2.10 and 2.11) have been redrawn in FIGURE 2.12 to clearly demonstrate the differences between these two groups. The time course of responding following 2.5% and 1.0% formalin for PS (pH 2.5) treated groups over the 60 min test period is presented in FIGURE 2.13.

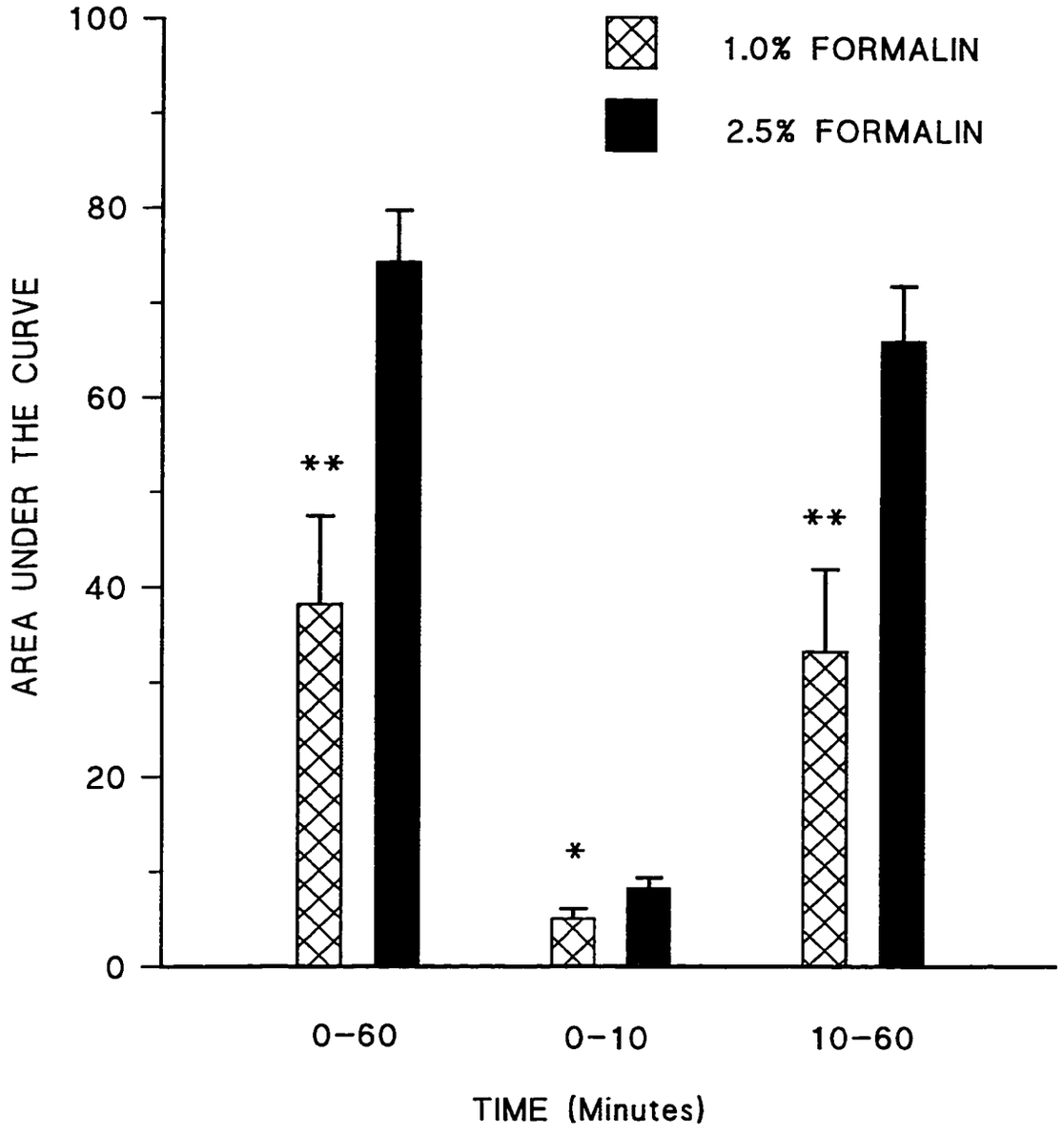


FIGURE 2.12 Effect of 1.0% and 2.5% formalin concentrations on nociception in rats following intrathecal injections of physiological saline (PS; pH 2.5). Data are expressed as the Area-Under-the-Curve (AUC; mean + s.e.m.) of the pain-score vs time curve for 60 minutes, Phase 1 (0-10 minutes) and Phase 2 (10-60 minutes). All data points represent the mean from a group of 6 rats ($p<0.01$, * $p<0.05$ relative to the AUC for 2.5% formalin).**

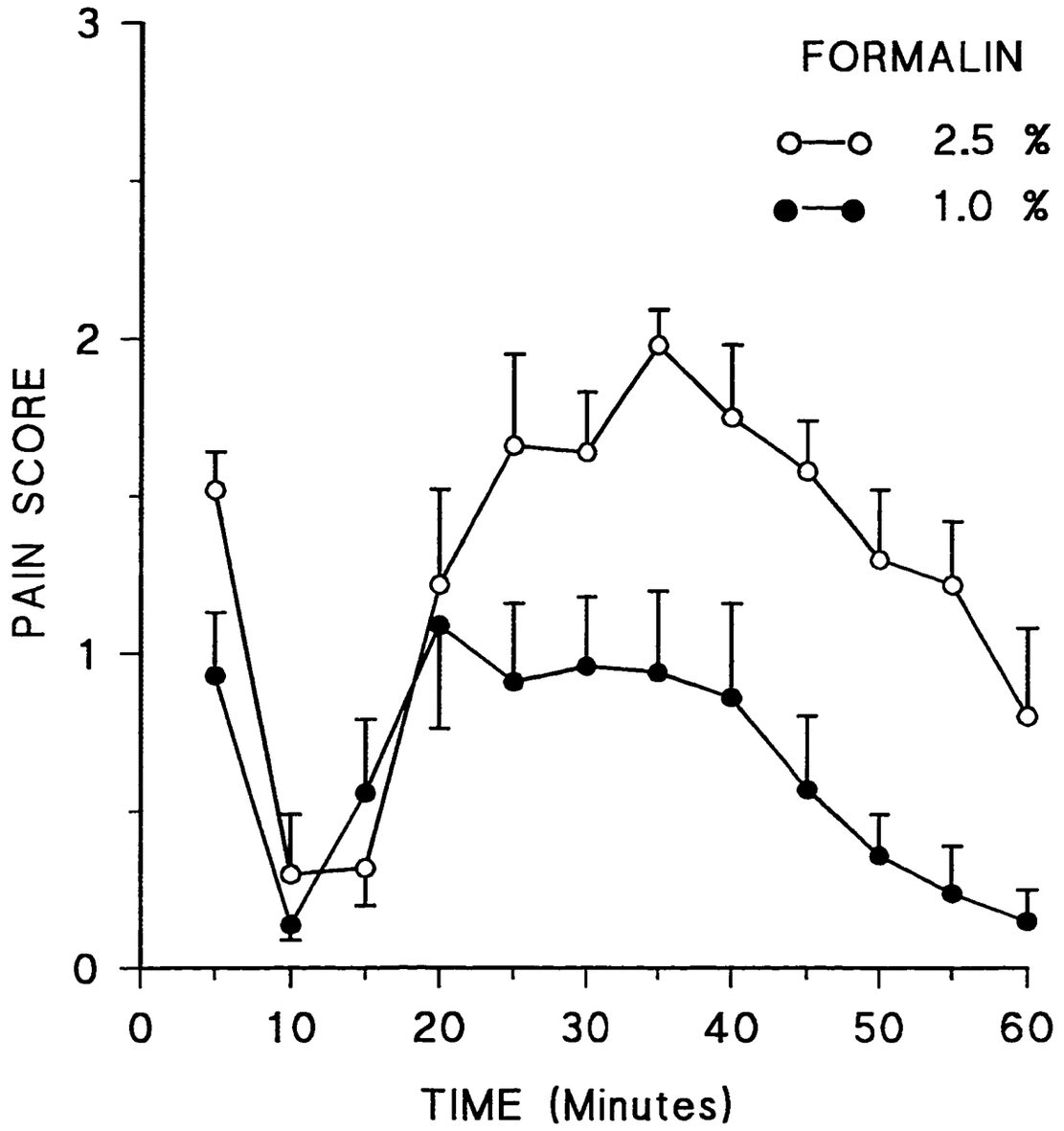


FIGURE 2.13 Pain scores (mean ± s.e.m.) in the formalin test over a 60 minute test period for two concentrations of formalin (1.0% and 2.5%) following intrathecal administration of physiological saline (PS; pH 2.5). The characteristic biphasic response curve is seen following the administration of both concentrations of formalin. All data points represent the mean from a group of 6 rats.

The characteristic biphasic response profile is maintained for both formalin concentrations following intrathecal injections of PS at pH 2.5.

2.2.4 DISCUSSION

One concern with the spinal catheterization procedure is whether the injected compound remains in the vicinity of the catheter tip or whether it spreads freely away from the injection site. Stated more specifically, does the injected compound reach supraspinal structures in any significant quantity following injection? This question is of particular importance in the use of EAA analogues. Antinociceptive actions of EAAs have been documented following administration in the medulla (Aimone and Gebhart 1988; Jensen and Yaksh 1989), in the periaqueductal grey (Jacquet 1988; Jacquet and Squires 1988; Jensen and Yaksh 1989), in the nucleus tractus solitarius and in the nucleus reticularis ventralis (Randich et al. 1988). The antinociceptive actions of EAAs in supraspinal nuclei could confound the results of any intrathecal administration study if EAAs were able to reach supraspinal sites.

Yaksh and Rudy (1976) have shown that following intrathecal injections (10μl) of either histological dyes or radio-labelled naloxone or morphine, the extent of rostra-caudal diffusion along the spinal axis into supraspinal structures was minimal for measurement periods up to an hour following injection. Further, Milne and colleagues (1985) injected methylene-blue into a chronically implanted intrathecal catheter that terminated in the region of the rostral lumbar enlargement. These authors found no trace of the marker farther than 1 cm distant from the

injection site for up to 2 days post injection. Assuming that the distribution of EAA ligands is similar to the dyes and radioligands, it is probable that any effect of intrathecally administered EAAs is due to actions at the lumbar spinal level and not due to actions on supraspinal structures.

Intact sensory and motor systems are required to properly measure the effect of a drug in the formalin test. Following the intrathecal cannulation procedure, rats that demonstrated observable neurological or motor abnormalities were immediately euthanized. It may have been possible that some of the remaining rats had undetected sensory abnormalities which may have altered a rat's baseline response to a nociceptive stimulus.

One way to determine if the cannulation procedure caused any such sensory abnormalities is to measure the nociceptive response to formalin in a group of intrathecally cannulated rats that underwent a sham-injection prior to formalin administration. These results would then be compared to the baseline results obtained from a group of non-operated rats. If the baseline results were not statistically different, it could be concluded that the cannulation surgery did not alter the rat's capacity to perceive a nociceptive stimulus.

The AUC values for sham-injected groups, following hindpaw injections of either 1.0% or 2.5% formalin, were not statistically different from the corresponding non-operated control groups (FIGURE 2.8). It is, therefore, reasonable to suggest that the procedure of chronic intrathecal cannulation did not cause a change in the baseline response to a nociceptive stimulus regardless of whether the stimulus was

of high intensity (2.5%) or low intensity (1.0%). It would be expected that baseline responses would change if the cannula was in some way interfering with the transmission of afferent or efferent information to or from supraspinal structures. Such interference might derive from mechanical pressure by the cannula on the spinal cord, interference in the spinal cord's ability to absorb and utilize nutrients, or the presence of infection that might be toxic to the spinal nerves (Yaksh and Rudy 1976). Apparently none of these considerations were a significant factor in the current studies since the baseline responses of the sham-injected rats were not different from those of non-operated controls (FIGURE 2.8). These results parallel those of Yaksh and Stevens (1986) who described a systematic study where the spinal cords of rats were examined histologically for any evidence of lesions following chronic intrathecal catheterization. Yaksh and Stevens (1986) found no mechanical, anoxic or bacterial damage to the spinal cord following periods of up to 6 weeks.

In sham-injected groups of rats (FIGURE 2.8), as in non-operated controls (Section 2.1; FIGURE 2.3), 1.0% or 2.5% formalin injections produced significant differences between AUC values during each time period of interest. Again, this confirms the choice of these two formalin concentrations as appropriate nociceptive stimuli (Section 2.2) for studying EAAs that may either increase or decrease nociception in the formalin test (see CHAPTER 3).

The AUC values of the different vehicle groups were not as consistent following 1.0% formalin injections (FIGURE 2.11). The group that had been given

PS at pH 2.5 was the only group that did not show a significant difference compared to sham-injected groups during any of the three time periods of interest. No explanation was immediately apparent that might have accounted for the differences observed from the sham-injected rats following PS (pH 7.0) or PBS (pH 2.5 or 7.0) (FIGURES 2.10 and 2.11). However, the osmolarity of each solution was not determined. It is possible that the differences in ionic strength due to varying osmolarities could have affected both primary afferent activity and post-synaptic responses within the dorsal horns. Further, a considerable volume of NaOH would have been needed to alter the pH of the PBS solutions. The increase in the concentration of Na^+ ions could have conceivably altered dorsal horn neural function, resulting in increased nociception.

Based on the observations that a) PS at pH 2.5 mediated significantly different AUC values following both 1.0% and 2.5% formalin during each of the time periods of interest (FIGURE 2.12), and b) baseline AUC values of both 2.5% and 1.0% formalin in PS (pH 2.5) treated rats were not significantly different from AUC values in corresponding groups of sham-injected rats (FIGURES 2.10 and 2.11), PS at pH 2.5 was chosen as the vehicle for the experiments presented in Chapter 3.

REFERENCES

CHAPTER 2

ABBOTT FV, FRANKLIN KBJ, CONNELL B. The stress of a novel environment reduces formalin pain: possible role of serotonin. Eur J Pharmacol 1986; 126: 141-144.

AIMONE LD, GEBHART GF. Serotonin and/or an excitatory amino acid in the medial medulla mediates stimulation-produced antinociception from the lateral hypothalamus in the rat. Brain Res 1988; 450: 170-180.

ALREJA M, MUTALIK P, MAYAR U, MANCHANDA SK. The formalin test: a tonic pain model in the primate. Pain 1984; 20: 97-105.

CARLÀ V, MORONI F. General anaesthetics inhibit the responses induced by glutamate receptor antagonists in the mouse cortex. Neurosci Lett 1992; 146: 21-24.

CODERRE TJ, MELZACK R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 1992; 12: 3665-3670.

CODERRE TJ, VAN EMPEL I. The utility of excitatory amino acid (EAA) antagonists as analgesic agents. I. Comparison of the antinociceptive activity of various classes of EAA antagonists in mechanical, thermal and chemical nociceptive tests. Pain 1994; 59: 345-352.

CODERRE TJ, VACCARINO AL, MELZACK R. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 1990; 535: 155-158.

CODERRE TJ, FUNDYTUS ME, McKENNA JE, DALAL S, MELZACK R. The formalin test: a validation of the weighted-scores method of behavioural pain rating. Pain 1993; 54: 43-50.

COHEN SR, ABBOTT FV, MELZACK R. Unilateral analgesia produced by intraventricular morphine. Brain Res 1984; 303: 277-287.

DICKENSON AH, SULLIVAN AF. Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. Pain 1987; 30: 349-360.

DUBUISSON D, DENNIS SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977; 4: 161-174.

FANTONE JC, WARD PA. Inflammation. In: Rubin E, Farber JL, eds. Essential pathology. Philadelphia: JB Lippincott, 1990: 20-35.

HUNSKAAR S, HOLE K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987; 30: 103-114.

HUNSKAAR S, BERGE OG, HOLE K. Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicylic acid and indomethacin in the formalin test. Pain 1986; 25: 125-132.

HUNSKAAR S, FASMER OB, HOLE K. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Meth 1985; 14: 69-76.

HYLDEN JLK, WILCOX GL. Intrathecal morphine in mice: a new technique. Eur J Pharmacol 1980; 67: 313-316.

JACQUET YF. The NMDA receptor: central role in pain inhibition in rat periaqueductal gray. Eur J Pharmacol 1988; 154: 271-276.

JACQUET YF, SQUIRES RF. Excitatory amino acids: role in morphine excitation in rat periaqueductal gray. Behav Brain Res 1988; 31: 85-88.

JENSEN TS, YAKSH TL. Comparison of the antinociceptive effect of morphine and glutamate at coincidental sites in the periaqueductal grey and medial medulla in rats. Brain Res. 1989; 476: 1-9.

KLEINBAUM DG, KUPPER LL, MULLER KE. Applied regression analysis and other multivariable methods. Boston: PWS-Kent, 1988.

LEES GJ. Influence of ketamine on the neuronal death caused by NMDA in the rat hippocampus. Neuropharmacology 1995; 34: 411-417.

LUNA LG. Manual of histological staining methods of the Armed Forces Institute of Pathology - 3rd Edition. New York: The Blakiston division, McGraw-Hill, 1968.

MILNE B, CERVENKO FW, JHAMANDAS K, SUTAK M. Intrathecal clonidine: analgesia and effect on opiate withdrawal in the rat. Anaesthesiology 1985; 62: 34-38.

O'KEEFE J. Spinal cord mechanisms subserving pain perception. M.Sc. Thesis. McGill University, 1964.

OWEN JA, TASKER RAR, NAKATSU K. A simple, less stressful rat restrainer. Experientia 1984; 40: 306-308.

PORTER RH, GREENAMYRE JT. Regional variations in the pharmacology of NMDA receptor channel blockers: implications for therapeutic potential. J Neurochem 1995; 64: 614-623.

RANDICH A, ROOSE MG, GEBHART GF. Characterization of antinociception produced by glutamate microinjection in the nucleus tractus solitarius and the nucleus reticularis ventralis. J Neurosci 1988; 8: 4675-4684.

ROSLAND JH, TJØLSEN A, MÆHLE B, HOLE K. The formalin test in mice: effect of formalin concentration. Pain 1990; 42: 235-242.

ROTE NS. Inflammation. In: McCance KL, Huether SE, eds. Pathophysiology: the biologic basis for disease in adults and children. St Louis: CV Mosby, 1990: 217-237

SHIBATA M, OHKUBO T, TAKAHASHI H, INOKI R. Modified formalin test: characteristic biphasic pain response. Pain 1989; 38: 347-352.

TASKER RAR, CONNELL BJ, YOLE MJ. Systemic injections of alpha-1 adrenergic agonists produce antinociception in the formalin test. Pain 1992; 49: 383-391.

TJØLSEN A, BERGE OG, HUNSKAAR S, ROSLAND JH, HOLE K. The formalin test: an evaluation of the method. Pain 1992; 51: 5-17.

VACCARINO AL, PREZEMYSLAW M, LIEBESKIND JC. Stress-induced analgesia prevents the development of the tonic, late phase of pain produced by subcutaneous formalin. Brain Res 1992; 572: 250-252.

WHEELER-ACETO H, COWAN A. Neurogenic and tissue-mediated components of formalin-induced edema: evidence for supraspinal regulation. Agents and Actions 1991; 34: 264-269.

YAKSH TL, RUDY TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976; 17: 1031-1036.

YAKSH TL, STEVENS CW. Simple catheter preparation for permitting bolus intrathecal administration during chronic intrathecal infusion. Pharmacol Biochem Behav 1986; 25: 483-485.

CHAPTER 3

EFFECT OF INTRATHECAL ADMINISTRATION OF EXCITATORY AMINO ACID LIGANDS IN THE FORMALIN TEST

3.1 INTRODUCTION

Glutamic acid (GLU) and aspartic acid (ASP) are widely recognized as excitatory amino acid neurotransmitters involved in the spinal transmission of nociceptive information. Consistent with this, neuroanatomically, both GLU and ASP have been immunocytochemically localized within primary sensory afferents (Johnson 1978; Potashner and Tran 1985; Westlund et al. 1989a,b) and ASP has been immunocytochemically localized within spinal dorsal horn interneurons (Madl et al. 1987).

Electrophysiological studies have described a depolarizing action of GLU on spinal cord neurons (Curtis et al. 1959). Further, both GLU and ASP selectively excited dorsal horn neurons that were responsive to C-fibre stimulation (Schneider and Perl 1985). Other studies using anaesthetized rats (Aanonsen et al. 1990) and monkeys (Dougherty et al. 1992) have shown that EAA receptor agonists selectively enhanced the response of spinal neurons to noxious stimuli.

In studies using conscious animals, intrathecal injections of EAAs are often associated with the onset of aversive behaviors (Aanonsen and Wilcox 1987; DeLander and Wahl 1988). Furthermore, direct lumbar injections of GLU, ASP and

various EAA ligands have been reported to produce hyperalgesia in a variety of conventional tests of nociception, such as the formalin, tail-flick and hot-plate tests (Aanonsen and Wilcox 1987; Coderre and Melzack 1992).

Although administration of EAA agonists has produced hyperalgesia in many animal models of nociception, another approach to investigating the role of EAA receptor subtypes in nociception has been to examine the antinociceptive actions of various EAA antagonists. The actions of both competitive and non-competitive EAA receptor antagonists have been described in a variety of chemical and thermal animal models of nociception (Aanonsen et al. 1990; Mao et al. 1992; Näsström et al 1992; Coderre and Van Empel 1994; Coderre 1994). However, results from different laboratories have not consistently elucidated the specific roles of different EAA receptor subtypes in mediating the various modalities of nociception.

The formalin test is an increasingly popular model for both behavioral and electrophysiological studies. Originally described by O'Keefe (1964) and Dubuisson and Dennis (1977), this test involves the subcutaneous injection of a small quantity of dilute formalin into an extremity (usually a hindpaw) resulting in a characteristic biphasic nociceptive response that can be quantified either according to a behavioral scale (Cohen et al. 1984) or in the form of direct recordings from spinal neurons (Haley et al. 1990).

EAAs are thought to play a role in the spinal transmission of formalin-induced nociceptive information, partly based on the results obtained by Skilling and colleagues (1988), who observed that injections of formalin into the hindpaw of a rat

caused an immediate increase in the levels of GLU and ASP in the extracellular fluid obtained from the spinal dorsal horn. While the two phases of the formalin response have historically been considered to be distinct (Dubuisson and Dennis 1977), recent evidence supports the idea that the late phase of responding is at least partially dependent on EAA receptor-mediated central sensitization (Coderre and Melzack 1991; Coderre and Melzack 1992; Vaccarino et al. 1993). Further, central changes induced by spinal neural activity, or sensitization generated during the early phase, led to persistent nociception as measured by the presence of nociception in the second phase (Dickenson and Sullivan 1987; Coderre et al. 1990). Other evidence has indicated that the two phases of formalin-induced nociception are independent of each other but still involve EAA receptor activation (Woolf and Thompson 1991).

The experiments described in this chapter will attempt to systematically characterize the spinal mechanisms of EAA receptor modulation on formalin-induced nociception. To accomplish this, different EAA receptor agonists and antagonists will be administered via an intrathecal catheter prior to an injection of formalin. The format of these studies will be to establish dose-response relationships for different combinations of EAA agonists and antagonists.

3.2 METHODS COMMON TO ALL EXPERIMENTS

3.2.1 EAA LIGANDS

GLU (L-glutamic acid), ASP (L-aspartic acid), KA (kainic acid) and NMDA (N-methyl-D-aspartic acid) were purchased from Sigma Chemical Company (St. Louis, MO). DOM (Domoic acid) was purchased from Diagnostic Chemicals Ltd. (Charlottetown, PE). AMPA ((±)-α-amino-[3-hydroxy-5-methyl-4-isoxazole] propionic acid) was purchased from Research Biochemicals Inc. (Natick, MA). ACPD (trans-(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid) and CPP (3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid) were purchased from Tocris Neuramin (Bristol, UK). NBQX (6-nitro-7-sulphamoyl-benzo(f)quinoxaline-2,3-dione), NS-102 (5-nitro-6,7,8,9-tetrahydrobenzo(g)indole-2,3-dione-3-oxime) and Cremophore were gifts from NeuroSearch Inc. (Copenhagen, DK).

All drugs except NS-102 were dissolved in physiological saline. NS-102 was dissolved in 10% (w/v) Cremophore in saline. The pH of all drug solutions and vehicle solutions was adjusted to pH 2.5 with hydrochloric acid (HCl; 0.1 M) or sodium hydroxide (NaOH; 0.1 M).

3.2.2 ANIMALS

Male Long-Evans rats (300-450 g) obtained from Charles River Laboratories (Montreal, PQ) were housed in groups of 6 with food and water available ad libitum. All rats were acclimatized to the housing area for a minimum of 5 days

prior to surgery. Lighting in the colony room was maintained on a 12 hour light / 12 hour dark cycle with all testing taking place between 8 AM and 2 PM. Following intrathecal catheter implantation, the rats were individually housed with food and water available ad libitum except during habituation and testing. No animal was tested more than once. In all cases, each data point represents a mean obtained from a group of 6 or 10 rats. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.3 INTRATHECAL CANNULATION

Rats were implanted with chronic indwelling catheters in the spinal subarachnoid space as described previously (Section 2.2.2.2). Briefly, under sodium pentobarbital anaesthesia, a 13.5 cm. length of stretched PE-10 catheter was advanced through an incision in the atlanto-occipital membrane to a position 8.5 cm caudal to the membrane. This placed the tip of the catheter at the level of the lumbar enlargement (T12-L1; see Section 2.2.3.1). The catheter was fastened to the skull with acrylic dental cement and plugged with stainless steel wire. Following surgery, each rat was individually housed and allowed a minimum of 7 days to recover from the surgery prior to experimental manipulations. Any rat showing spontaneous or induced neurological or motor deficits, following recovery from the anaesthetic, was immediately euthanized.

3.2.4 INJECTION PROTOCOL

Intrathecal (i.t.) injections were carried out according to the protocol described previously (Section 2.2.2.3). Briefly, each rat was placed into a cloth restraining system (modified version of Owen et al. 1984) 5 min prior to i.t. drug administration. The external portion of the catheter was visualized by threading it through a small hole cut into the cloth directly above the head of the rat. The stainless steel plug was removed and a Hamilton micro-syringe (10 μl) containing a drug solution was inserted.

In all experiments, including co-administration studies, the total injection volume (10 μl) of drug solution remained constant and was delivered over two min. An additional 7 μl of saline was injected to flush the cannula. The Hamilton syringe was removed following the injection and the stainless steel wire plug was returned to the end of the catheter. The animal was then returned to the testing box. The rat was removed from the testing box 10 min following the end of the i.t. injection and wrapped loosely in a cloth towel. Formalin (1.0% or 2.5%; 0.05 ml) was injected into the plantar surface of one hindpaw and testing began immediately and lasted for 60 min.

3.2.5 FORMALIN TEST

The formalin test (O'Keefe 1964; Dubuisson and Dennis 1977) was used according to modifications described by Cohen and colleagues (1984). Testing took place in a clear Plexiglas^{R} box (32 x 32 x 30 cm) with a mirror placed at a 45°

angle beneath the floor to allow an unobstructed view of the formalin-injected paw. Each rat was habituated to the testing box for 60 min on the day prior to being tested. Further, each rat was habituated to the testing box for between 30 and 60 min immediately prior to drug and formalin administration. Behavioral rating began immediately following formalin administration and lasted for 60 min. An average, weighted-pain score was determined for each 5 min block by measuring the amount of time spent in each of 4 behavioral categories, as described in Section 2.1.2.2.

3.2.6 STATISTICAL ANALYSIS

Pain behavior was continuously rated using the previously described 4-point scale (Section 2.1.2.2). An average, weighted-pain score for each 5 min block was used to calculate an area under the pain-score vs time curve (AUC). AUC was calculated according to the Trapezoid Rule for each time period of interest: a) the 60 min test period, b) Phase 1, and c) Phase 2.

The AUC values for differing drug concentrations were used to generate dose-response curves (DRCs), where higher AUC values were indicative of higher levels of nociception. A one-way analysis of variance (ANOVA) was used to determine whether there were any significant drug effects. Post-hoc analysis was conducted with orthogonal comparisons to determine which doses produced the significant effects as compared to vehicle injection. When a single dose of a drug was administered, paired t-tests were performed.

A two-way ANOVA was used in all co-administration studies to determine if

there were agonist, antagonist or interaction main-effects. Main effects were analyzed with one-way ANOVA and post-hoc analysis was conducted with orthogonal contrasts to determine which doses produced significant effects as compared to vehicle injections.

When appropriate, DRCs were compared for potency and parallelism by multiple partial regression analysis with dummy variables (Kleinbaum et al. 1988).

3.3 EXPERIMENT 1 - STUDIES WITH THE ENDOGENOUS EAA RECEPTOR AGONISTS GLU AND ASP

3.3.1 RATIONALE

Hyperalgesia has been defined as a state of enhanced responsiveness to noxious stimuli (Haley and Wilcox 1992). Coderre and Melzack (1992) reported that both GLU and ASP, when injected intrathecally via lumbar puncture into briefly anaesthetized rats, enhanced formalin-induced nociception for both the intermediate and late phases but did not affect nociception in the early phase. The hyperalgesia noted by Coderre and Melzack (1992) was described based on a single dose of either GLU or ASP.

Experiment 1 was performed to determine whether GLU- and ASP-induced hyperalgesia in the formalin test could be replicated in rats where GLU and ASP injections were made via a chronic indwelling intrathecal catheter, and to determine whether GLU- and ASP-induced hyperalgesia was dose-dependent. A low dose of

formalin (1%) was used to allow for the detection of EAA-induced hyperalgesia.

3.3.2 RESULTS

Intrathecal administration of either GLU or ASP produced a typical biphasic response at all doses tested (data not shown). DRCs based on AUC values over the 60 min test period are shown in FIGURE 3.1. Both 100 nmol and 200 nmol GLU produced significant hyperalgesia compared to control, ([$F\{1,30\}=12.88$, $p<0.01$] and [$F\{1,30\}=10.61$, $p<0.01$] respectively). However, only the 200 nmol dose of i.t. administered ASP produced significant hyperalgesia ($F\{1,30\}=5.16$, $p<0.05$; FIGURE 3.1). Lower doses of either amino acid were without significant nociceptive or antinociceptive effect as compared to vehicle.

When the 60 min response was divided into Phase 1 and Phase 2 components, the nociceptive response following GLU administration differed between the two phases (FIGURE 3.2). The analysis of Phase 2 AUC values provided comparable results to those of the 60 min test period, with 100 nmol and 200 nmol producing significant nociception compared to control, ([$F\{1,30\}=11.75$, $p<0.01$] and [$F\{1,30\}=5.96$, $p<0.05$] respectively). In contrast, Phase 1 AUC values following the i.t. administration of all doses of GLU, with the exception of the 10 nmol dose, produced a significantly greater AUC compared to control ([$F\{1,30\}=5.85$, $p<0.05$], [$F\{1,30\}=5.51$, $p<0.05$], [$F\{1,30\}=16.25$, $p<0.01$] and [$F\{1,30\}=8.32$, $p<0.01$] for 5.0 nmol, 50 nmol, 100 nmol and 200 nmol respectively).

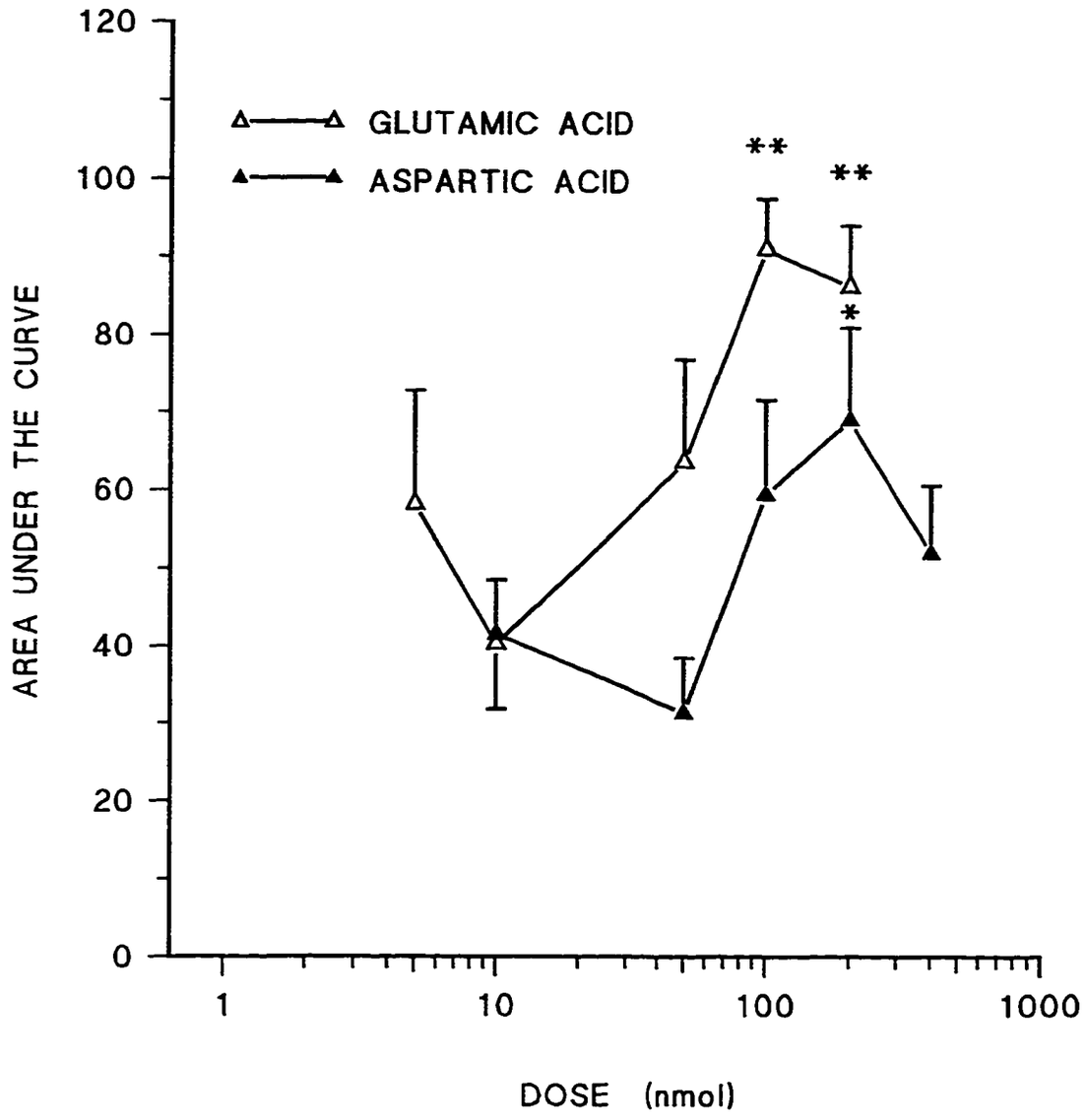


FIGURE 3.1 Log dose-response curves for intrathecal Glutamic Acid and Aspartic Acid in the formalin test (1% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded over the 60 min test period. All data points represent the mean AUC from a group of 6 rats (** $p<0.01$ and * $p<0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: 60 min = 38.22 ± 9.3).

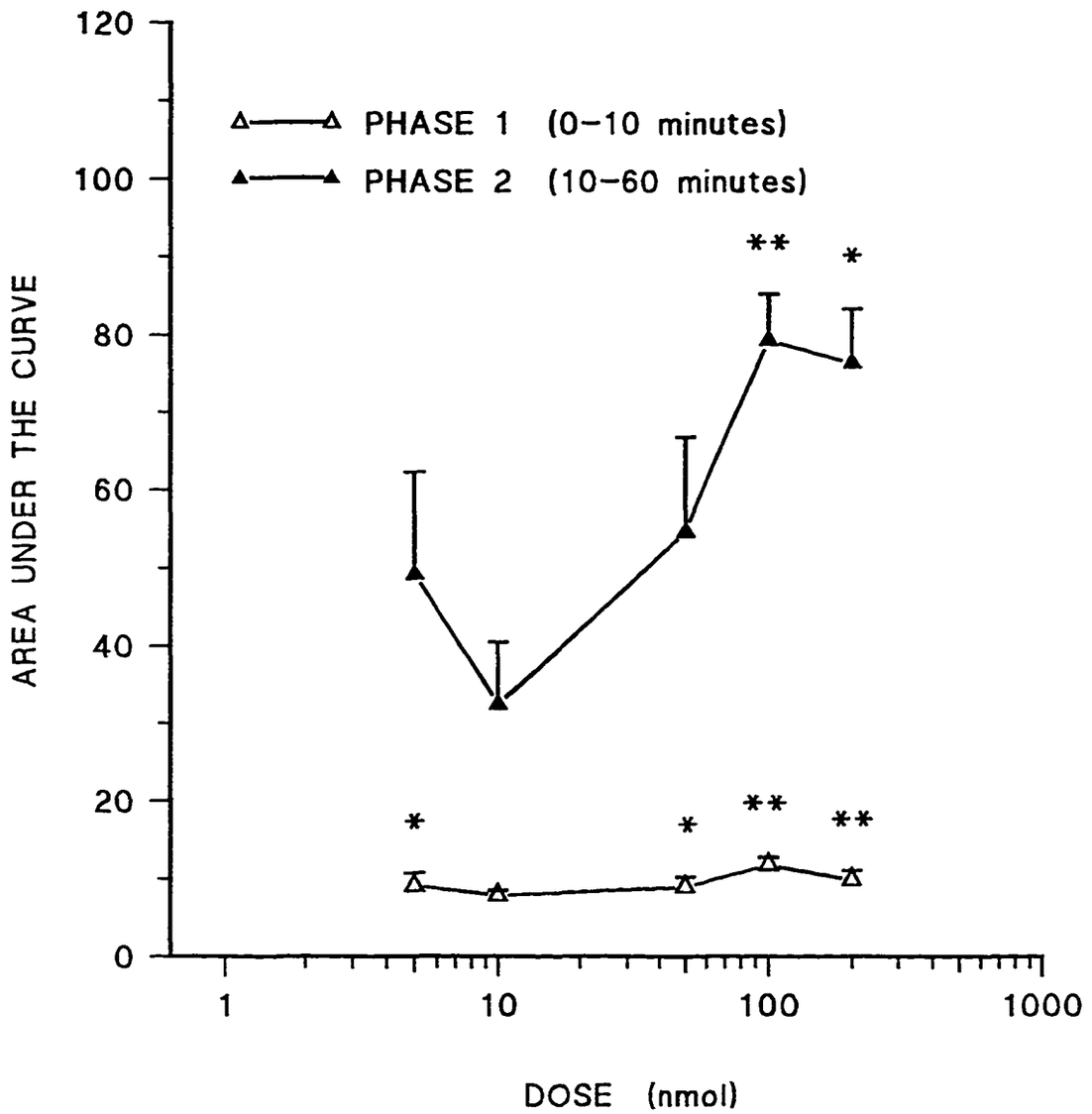


FIGURE 3.2 Log dose-response curves for intrathecal Glutamic Acid for both Phase 1 and Phase 2 in the formalin test (1% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (** $p<0.01$ and * $p<0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: Phase 1 = 5.0 ± 1.0, Phase 2 = 33.2 ± 8.6).

ASP administration resulted in no significant differences compared to vehicle control for Phase 2 AUC values. However, Phase 1 AUC values were significantly greater than control at two of the ASP doses tested (100 nmol and 200 nmol; [$F\{1,30\}=7.52$, $p<0.05$] and [$F\{1,30\}=9.97$, $p<0.01$] respectively; FIGURE 3.3).

It appeared that the Phase 1 response was more sensitive to the hyperalgesic actions of both GLU and ASP than was the Phase 2 response since lower concentrations of each agonist were needed to produce a hyperalgesic effect (FIGURES 3.2 and 3.3). Further, GLU appeared to be the more potent of the two agonists.

Neither motor nor sensory abnormalities were observed following i.t. administration of either 100 nmol GLU or 200 nmol ASP.

3.3.3 DISCUSSION

Formalin-induced nociceptive behaviors were enhanced by prior i.t. administration of the endogenous amino acids, GLU and ASP. These results support the theory that dorsal horn EAA receptors play a role in formalin-induced central sensitization which occurs during Phase 1, and in the persistent Phase 2 nociception that follows. I.t. administration of GLU produced a significant increase in nociception during both Phase 1 and Phase 2 of the formalin test. Statistical analysis of the data revealed that 5, 50, 100 and 200 nmol GLU produced significant hyperalgesia in Phase 1 whereas only the two highest doses were hyperalgesic in Phase 2. Phase 1 nociception appeared more sensitive to the hyperalgesic effects of

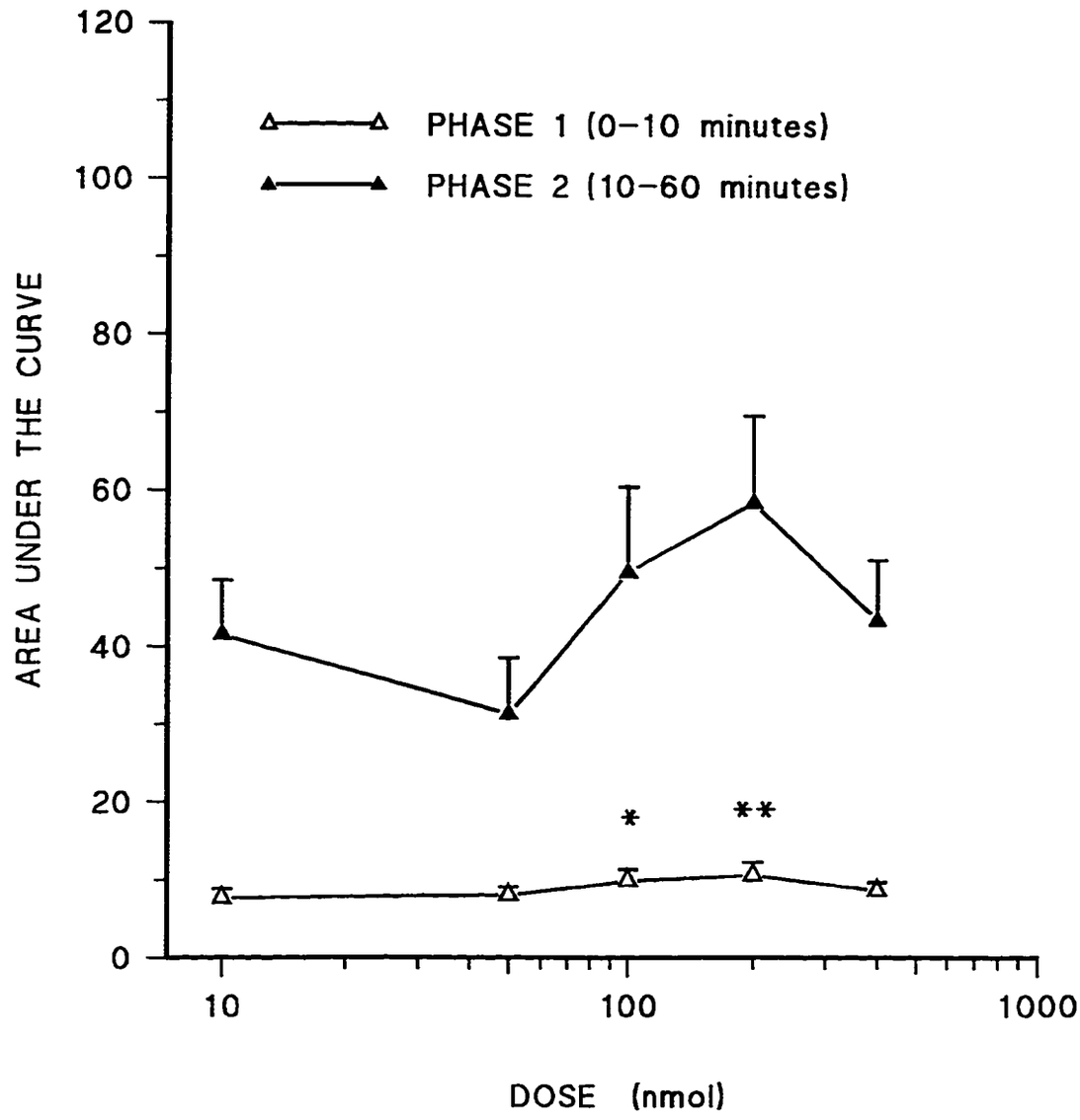


FIGURE 3.3 Log dose-response curves for intrathecal Aspartic Acid for both Phase 1 and Phase 2 in the formalin test (1% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (** $p<0.01$ and * $p<0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: Phase 1 = 5.0 ± 1.0, Phase 2 = 33.2 ± 8.6).

i.t. GLU than Phase 2 since significantly increased Phase 1 nociception was recorded with lower doses (5 nmol vs 100 nmol; FIGURE 3.2). However, since 10 nmol GLU did not produce a significant Phase 1 hyperalgesia while both higher and lower doses were hyperalgesic, care must be taken when interpretting these results. In the case of i.t. ASP administration, Phase 1 was clearly more sensitive than Phase 2 to enhanced hyperalgesia (FIGURE 3.3). Both 100 nmol and 200 nmol ASP resulted in significant hyperalgesia in Phase 1 while none of the doses tested resulted in increased Phase 2 nociception. However, it was clear that GLU was more potent than ASP in mediating Phase 1 hyperalgesia since lower doses of GLU than ASP produced hyperalgesia.

Coderre and Melzack (1992) reported an enhanced formalin response in rats pretreated with either GLU or ASP. The results of Experiment 1 also provided evidence for an enhanced 60 min formalin response when rats were pretreated with either GLU or ASP (FIGURE 3.1). However, the results of Experiment 1 also differed from those of Coderre and Melzack (1992). These authors reported that GLU- and ASP-induced hyperalgesia occurred only during what was defined as the intermediate (5-15 min) and late (15-50 min) phases. In Experiment 1, however, both GLU- and ASP-induced hyperalgesia occurred during Phase 1 (0-10 min). The difference in the results may be explained by the difference in the time frames used to measure early and late phase nociception.

Other methodological differences might have accounted for the inconsistencies in the results obtained in Experiment 1 and those obtained by

Coderre and Melzack (1992). It has been reported that stressful stimuli introduced prior to a formalin injection resulted in a measurable stress-induced analgesia (Abbott et al. 1986; Vaccarino et al. 1992). Coderre and Melzack (1992) did not report if precautions were undertaken to minimize stress-induced analgesia. In addition, these authors administered GLU and ASP in rats under ether anaesthesia via direct lumbar puncture. Several anaesthetic agents, particularly ether, have been reported to interact with the NMDA receptor cation channel, perhaps at the phencyclidine (PCP) recognition site (Carlà and Moroni 1992; Porter and Greenamyre 1995; Lees 1995; FIGURE 1.4). Hence, stress-induced analgesia or the use of an anaesthetic agent at the time of drug administration might have accounted for the difference in the effect of GLU and ASP on Phase 1 formalin-induced nociception.

GLU- and ASP-induced hyperalgesia was not strictly dose-dependent. However, there was a trend toward increased hyperalgesia over the 60 min test period as the dose of either ligand was increased (FIGURE 3.1). Coderre and Melzack (1992) used only one dose of GLU and ASP to test for hyperalgesia in the formalin test (20μg [136 nmol] and 25μg [188 nmol] respectively). These doses fall within the range of doses that produced significant hyperalgesia in Experiment 1. The lack of a true dose-dependent relationship resulting from i.t. administration of GLU and ASP in the formalin test, might indicate that both GLU and ASP mediate enhanced nociception via actions at more than one EAA receptor-subtype. NMDA vs non-NMDA EAA receptor subtypes. Further studies to elucidate the EAA receptor

subtypes involved in GLU- and ASP-induced hyperalgesia in both Phase 1 and Phase 2 are necessary.

3.4 EXPERIMENT 2 - STUDIES WITH THE NON-NMDA RECEPTOR AGONISTS KA AND AMPA

3.4.1 RATIONALE

Very few studies have determined the extent of KA and AMPA receptor involvement in the spinal transmission of nociceptive information. The effects of i.t. administration of a KA ligand in the formalin test have been reported by only one laboratory (Murray et al. 1991). These authors found that urethane, a KA receptor antagonist, did not mediate an antinociceptive response in the late phase of formalin-induced nociception in mice. The effect of urethane on early phase nociception was not discussed.

Evidence implicating KA receptors as having a role in the spinal processing of nociceptive information in other behavioral paradigms comes mainly from experiments where KA was administered spinally and the frequency of aversive behaviors were noted in the first one or two minutes following KA injection (Aanonsen and Wilcox 1987, 1989; Hornfeldt and Larson 1989; Kellstein et al. 1990; Urca and Urca 1990; DeLander and Wahl 1991) (See Section 1.6).

The role of AMPA receptors in the spinal transmission of formalin-induced nociceptive information has not yielded consistent results between laboratories.

Näsström and colleagues (1992) observed significant antinociception during both early and late phases following the i.t. application of the non-NMDA, AMPA-selective receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in mice. Further, Hunter and Singh (1994) and Goettl and Larson (1994) described a significant antinociceptive effect of the non-NMDA, AMPA-selective receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) only during the formalin-induced early phase response. These results indicated that in the formalin test, antagonists of spinal AMPA receptors mediated antinociception. Electrophysiological experiments support this hypothesis. Chapman and Dickenson (1995) reported that in anaesthetized rats, i.t. injections of CNQX prior to hindpaw formalin injections decreased early and late phase dorsal horn neuronal responses. A decrease in dorsal horn neuronal responses is presumed to correspond to an antinociceptive effect (Dickenson and Sullivan 1987; Chapman and Dickenson 1995). However, others have reported that the i.t. pretreatment of rats with either AMPA or CNQX did not significantly change formalin-induced nociception (Coderre and Melzack 1992).

The results of Experiment 1 demonstrated that i.t. administration of both GLU and ASP mediated enhanced formalin-induced nociception. Further, Phase 1 responding was more sensitive to the hyperalgesic effects of both ligands. Experiment 2 was conducted to determine whether spinal KA or AMPA receptors played a role in mediating the enhanced nociception observed following i.t. administration of GLU or ASP.

3.4.2 RESULTS

To determine whether KA or AMPA receptor activation in the spinal cord would produce hyperalgesia in the formalin test, various doses of KA and AMPA were administered i.t. prior to formalin (1.0%). Unexpectedly, i.t. administration of KA produced a dose-dependent decrease in nociception (FIGURE 3.4). Administration of 400 nmol KA produced significant antinociception over both the full 60 min test period and during Phase 2 ([$F\{1,20\}=4.38$, $p<0.05$)] and [$F\{1,20\}=5.87$, $p<0.05$] respectively; FIGURE 3.4). However, administration of KA prior to formalin did not produce significant changes in Phase 1 nociception (FIGURE 3.4).

In contrast to the results obtained with KA, i.t. administration of 100 pmol, 250 pmol or 500 pmol of AMPA had neither antinociceptive nor nociceptive effects during any of the three time periods examined when 1% formalin was used as the stimulus (FIGURE 3.5).

Aversive-type behaviors, such as caudally-directed biting and scratching, were not observed in any of the rats following the i.t. administration of any dose of KA or AMPA.

3.4.3 DISCUSSION

The i.t. administration of KA prior to a formalin (1.0%) injection in the rat produced a significant antinociception (decreased pain) that was due entirely to actions during Phase 2 (FIGURE 3.4). These results are in contrast to other findings

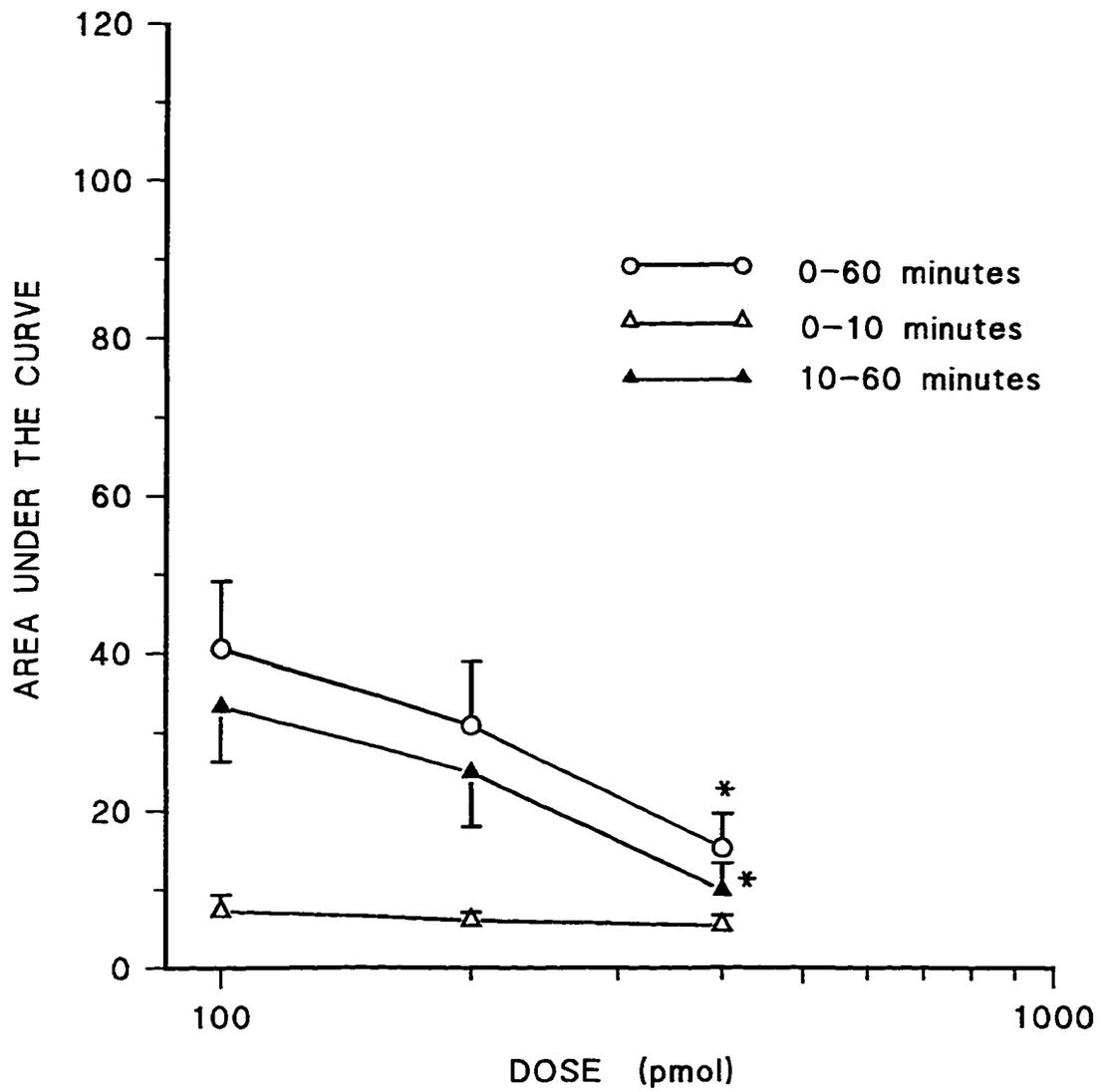


FIGURE 3.4 Log dose-response curves for intrathecal Kainic Acid for 60 min, Phase 1 and Phase 2 in the formalin test (1% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (* $p < 0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: 60 min = 38.2 ± 9.3, Phase 1 = 5.0 ± 1.0, Phase 2 = 33.2 ± 8.6).

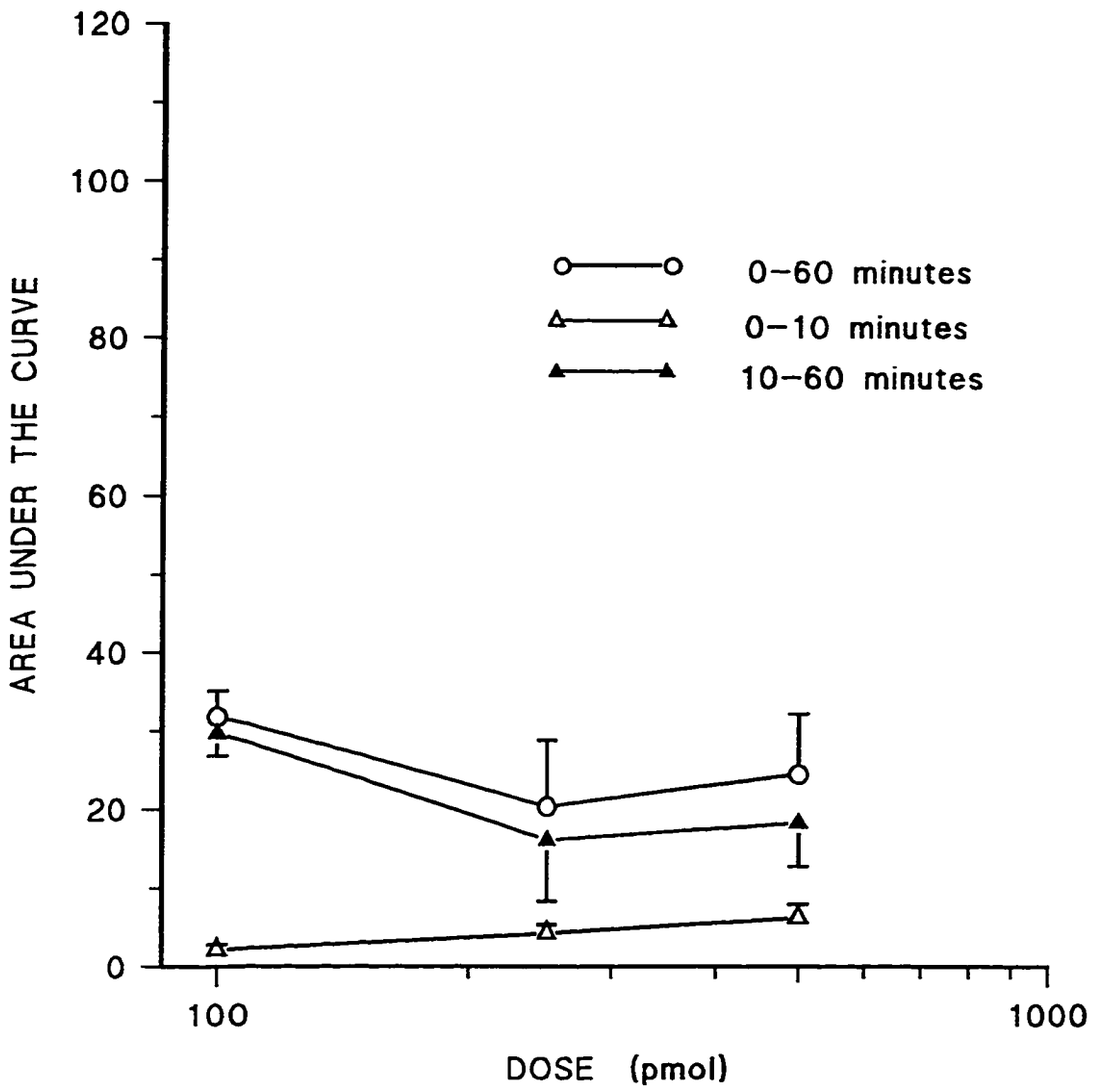


FIGURE 3.5 Log dose-response curves for intrathecal AMPA for 60 min, Phase 1 and Phase 2 in the formalin test (1% formalin). Area-Under-the-Curve (AUC) values (± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (Saline AUC: 60 min = 38.2 ± 9.3, Phase 1 = 5.0 ± 1.0, Phase 2 = 33.2 ± 8.6).

where non-NMDA receptor agonists have produced increased aversive behaviors following i.t. administration (Aanonsen and Wilcox 1987, 1989; Hornfeldt and Larson 1989; Kellstein et al. 1990; Urca and Urca 1990; DeLander and Wahl 1991). These results suggest that it is unlikely that the GLU- and ASP-induced hyperalgesia observed in Experiment 1 resulted from KA receptor activation (see FIGURES 3.1, 3.2, 3.3).

The i.t. administration of AMPA produced neither enhanced nor decreased nociception during the formalin test. These results are consistent with those reported by Coderre and Melzack (1992), who found that there was no change in nociceptive responses in the formalin test when rats were pretreated with i.t. AMPA (270 pmol). The dose administered by Coderre and Melzack (1992) was within the range of doses of AMPA administered in the current study (100 - 500 pmol; FIGURE 3.5).

The antinociceptive effects of KA could not be explained by either overt sensory or motor deficits since none of the rats tested in this experiment with KA demonstrated these behaviors. Kellstein and colleagues (1990) observed caudally-directed biting and scratching behaviors following the spinal administration of 0.5 nmol KA, and only following a dose of 0.9 nmol KA did they report vocalizations and myoclonic twitching. These doses of KA were slightly higher than those that produced antinociception in the current study (0.4 pmol).

3.5 EXPERIMENT 3 - STUDIES WITH THE NON-NMDA RECEPTOR AGONISTS DOM, KA AND AMPA

3.5.1 RATIONALE

The results of Experiment 2 demonstrated that the non-NMDA receptor agonist KA mediated a significant antinociceptive response following a low dose formalin stimulus (1%). Further, the antinociception observed during the 60 min test period could be explained solely by an effect during Phase 2 since i.t. KA administration did not significantly affect Phase 1 nociception (FIGURE 3.4). However, a potential antinociceptive effect in Phase 1 could have been masked by a "floor effect" because a low-intensity stimulus (1.0% formalin) was used. Therefore, Experiment 3 was carried out with a higher concentration of formalin (2.5%) as the nociceptive stimulus.

In addition to KA and AMPA, domoic acid (DOM), a non-NMDA low-affinity KA receptor agonist (Johansen et al. 1993; Tasker et al. 1996), was also administered i.t. There is increasing evidence that while DOM and KA share similar actions, the two drugs are not identical in their pharmacological properties. Differences in electrophysiological (Verdoorn et al. 1994), behavioral (Tasker et al. 1991; Tasker and Strain 1992) and histopathological (Strain and Tasker 1991) properties of the two non-NMDA receptor ligands provide indirect evidence that their neural actions are dissociable. Therefore, the results of experiment 3 would also determine if the i.t. administration KA and DOM would have similar actions in

the formalin test.

3.5.2 RESULTS

The effects of i.t. administration of a range of KA, DOM and AMPA doses on AUC values for the 60 min test period, Phase 1 and Phase 2 following 2.5% formalin injections are shown in FIGURES 3.6, 3.7 and 3.8, respectively. In this paradigm, 50 pmol and 400 pmol KA produced significant antinociception over the 60 min test period ([F{1,35}=7.25, p<0.05] and [F{1,35}=8.73, p<0.01] respectively; FIGURE 3.6). Further, both doses of KA produced significant antinociception during Phase 2 only ([F{1,35}=7.99, p<0.01] and F{1,35}=8.95, p<0.01] respectively; FIGURE 3.6). Antinociception following 400 pmol KA is consistent with the result obtained using 1% formalin (see FIGURE 3.4; 50 pmol KA was not tested with 1% formalin). It is unclear, however, why doses between 50 and 400 pmol failed to produce a significant effect following either 1% or 2.5% formalin.

A similar dose-response profile was obtained using DOM, as shown in FIGURE 3.7. Doses of 0.25 pmol and 0.5 pmol DOM produced significant antinociception over the 60 min test period when compared to saline AUC values ([F{1,45}=4.08, p<0.05] and [F{1,45}=15.22, p<0.01] respectively). With the 0.25 pmol dose, the 60 min effect was due exclusively to an action on the Phase 2 response (F{1,45}=4.05, p<0.05; FIGURE 3.7). However, 0.50 pmol DOM produced significant antinociception in both Phase 1 and Phase 2 ([F{1,45}=14.08, p<0.01] and [F{1,45}=6.92, p<0.05)] respectively).

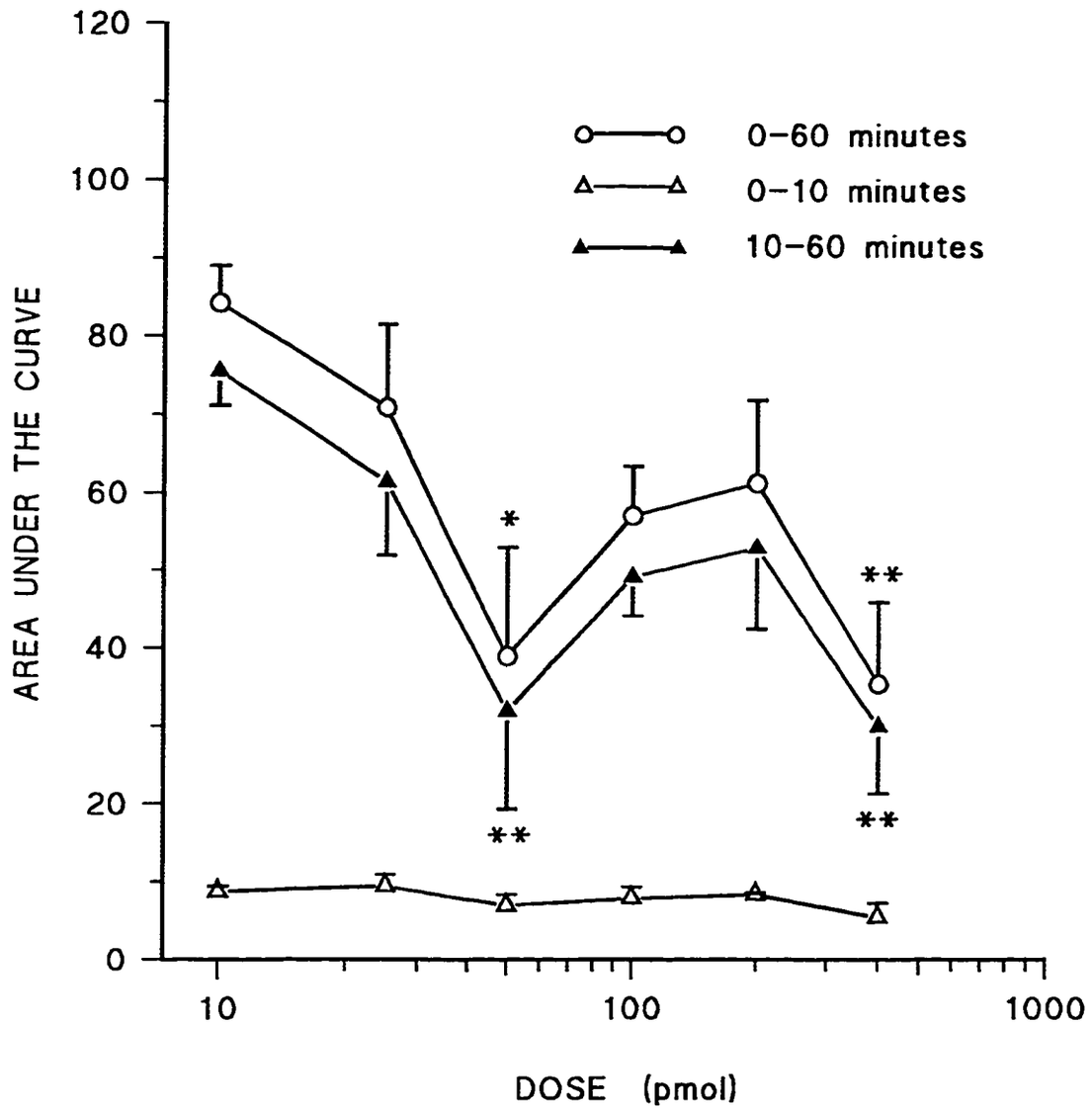


FIGURE 3.6 Log dose-response curves for intrathecal Kainic Acid for 60 min, Phase 1 and Phase 2 in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (** $p < 0.01$ and * $p < 0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: 60 min = 74.4 ± 5.3, Phase 1 = 8.3 ± 1.0, Phase 2 = 66.1 ± 5.8).

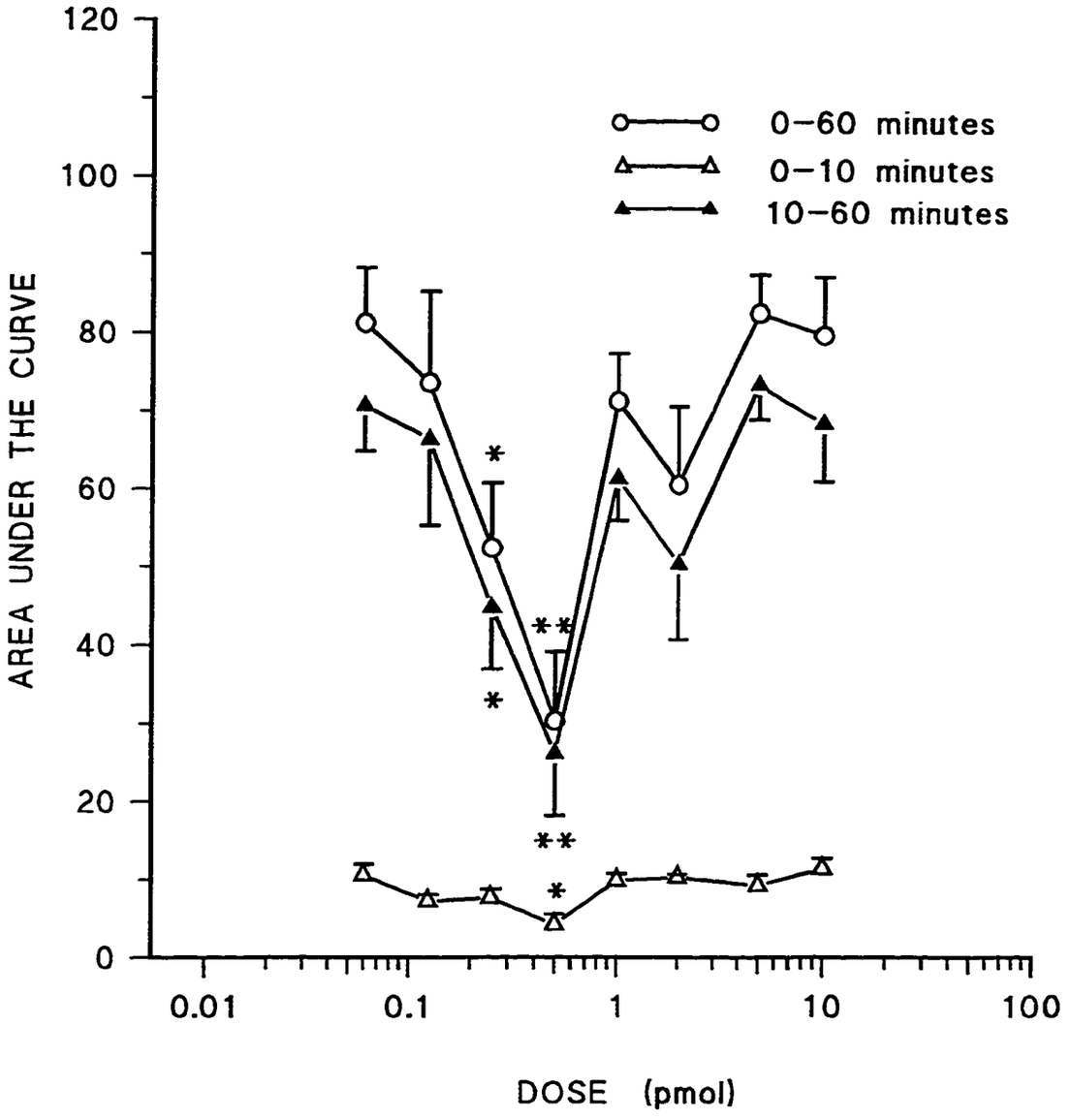


FIGURE 3.7 Log dose-response curves for intrathecal Domoic Acid for 60 min, Phase 1 and Phase 2 in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats ($p<0.01$ and * $p<0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: 60 min = 74.4 ± 5.3, Phase 1 = 8.3 ± 1.0, Phase 2 = 66.1 ± 5.8).**

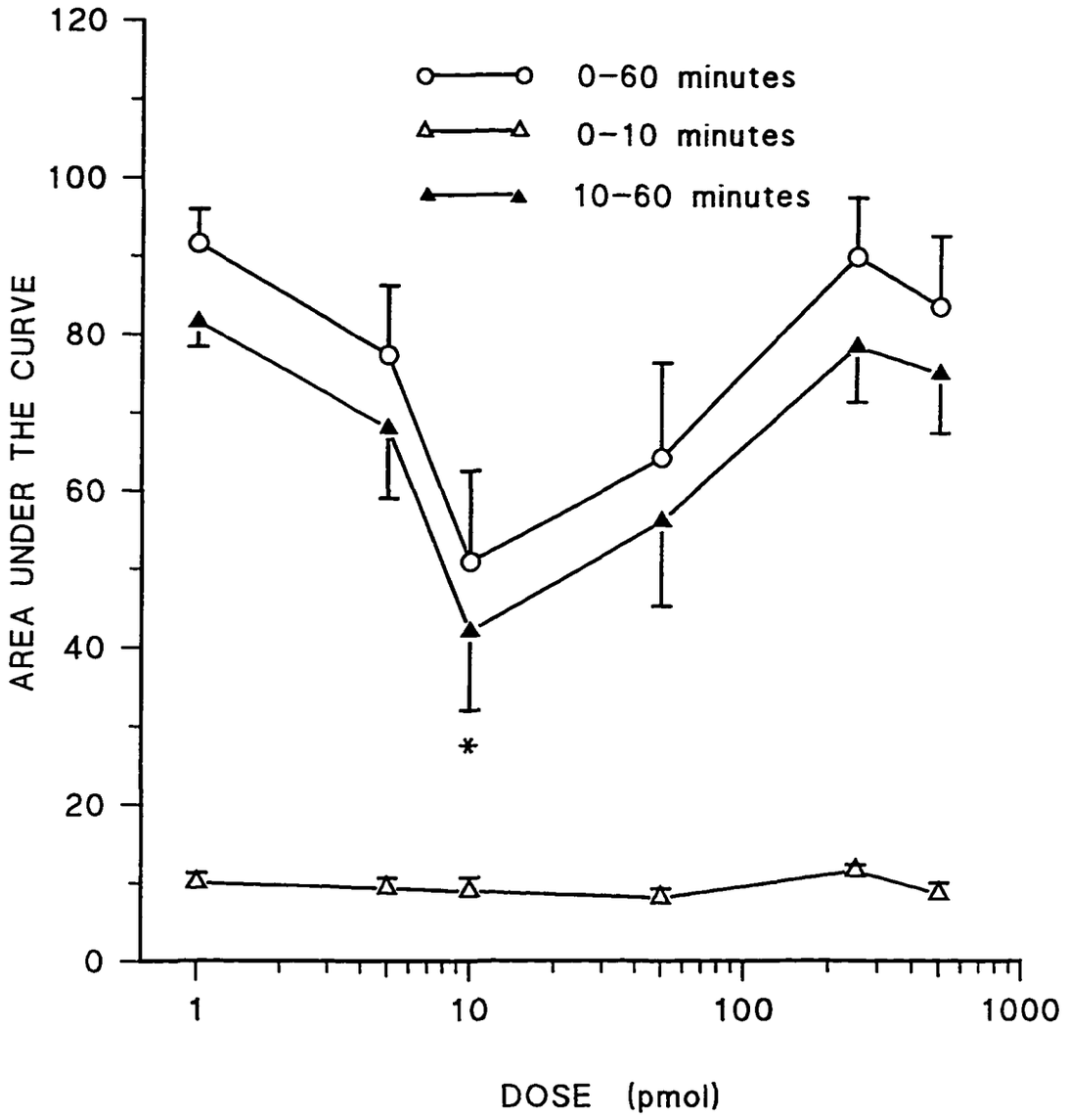


FIGURE 3.8 Log dose-response curves for intrathecal AMPA for 60 min, Phase 1 and Phase 2 in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (* $p<0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: 60 min = 74.4 ± 5.3, Phase 1 = 8.3 ± 1.0, Phase 2 = 66.1 ± 5.8).

The antinociceptive effects observed following AMPA administration were minimal in contrast to the effects seen following the i.t. administration of KA and DOM. Only one of the i.t. administered doses of AMPA (10.0 pmol) produced significant antinociception and that effect was seen only during Phase 2 ($F\{1,35\}=4.5$, $p<0.05$; FIGURE 3.8). Neither the 60 min nor Phase 1 AUC values were significantly affected by any of the concentrations of AMPA used (FIGURE 3.8).

Interestingly, 1.0 pmol and 10.0 pmol administrations of AMPA, following 2.5% formalin injections, produced significantly different AUC values when compared to each other for both the 60 min and Phase 2 responses ([$F\{1,35\}=10.63$, $p<0.01$] and [$F\{1,35\}=12.11$, $p<0.01$] respectively). However, as noted above, only during the Phase 2 response did 10 pmol AMPA produce significant antinociception compared to control (FIGURE 3.8). It was also notable that the mean AUC value for 1.0 pmol AMPA was greater than the mean AUC value for saline for both the 60 min test period (91.5 vs 74.4) and for Phase 2 (81.5 vs 66.1). However, neither of these differences reached significance.

The analgesic portions of the dose-response curves for the three agonists (DOM, KA and AMPA) were compared by multiple regression for the Phase 2 response (TABLE 3.1). Phase 2 responses were used because AMPA did not produce significant antinociception during the 60 min test period (FIGURE 3.5). DOM appeared to be the most efficacious of the three agonists tested since the maximally effective dose (0.5 pmol) produced approximately 63% analgesia. KA

EFFECT	DRUG	DOSE (pmol)	95% CI (pmol)	RELATIVE POTENCY
OBSERVED MAXIMUM	DOM	0.5	------	100
	AMPA	10.0	------	5
	KA	50.0	------	1
INTERPOLATED MAXIMUM	DOM	0.4	0.18 - 0.93	116
	AMPA	9.7	5.98 - 15.85	4.8
	KA	46.3	10.34 - 207.49	1
INTERPOLATED ED_{30}	DOM	0.2	0.13 - 0.47	139
	AMPA	7.4	4.83 - 11.25	4.7
	KA	34.9	10.36 - 117.33	1

TABLE 3.1 Dose-response data for intrathecal DOM, KA and AMPA for Phase 2 response in the formalin test (2.5% formalin). Dose (pmol), 95% Confidence Interval (95% CI) and Relative Potency values for the Observed Maximum, Interpolated Maximum and Interpolated 30% Effective Dose (ED_{30}) are based on the data presented in FIGURES 3.6, 3.7 and 3.8.

appeared to be more efficacious than AMPA; the maximally effective dose of KA (50.0 pmol) produced approximately 54% analgesia while the maximally effective dose of AMPA (10.0 pmol) produced approximately 44% analgesia. Since AMPA was unable to produce more than 50% analgesia, the 30% effective dose (ED_{30}) was used as a basis of comparison of potency between the three agonists rather than the conventional 50% effective dose (ED_{50}) (TABLE 3.1). DOM was the most potent of the agonists, approximately 140 times more potent than KA based on ED_{30} and significantly more potent than either KA or AMPA ($F\{1,35\} = 23.14$, $p<0.01$). AMPA was approximately 5 times more potent than KA in response to an injection of 2.5% formalin. However, AMPA did not produce a significantly different measure of analgesia than KA based on ED_{30}s ($F\{1,35\}=0.63$, $p>0.01$). The slopes of the analgesic portions of the Phase 2 dose-response curves for KA, DOM and AMPA were compared and were found to be not significantly different ($F\{2,4\}=2.00$, $p>0.05$; see FIGURES 3.6, 3.7 and 3.8).

Concentrations higher than 10 pmol DOM, 400 pmol KA, or 500 pmol AMPA resulted in both motor and sensory abnormalities such as caudally-directed biting and scratching, tail twitches, foot stomping and/or some degree of hindlimb paralysis.

3.5.3 DISCUSSION

The results of the current study parallel the results from Experiment 2, where it was reported that i.t. administration of KA produced significant analgesia in the formalin test following a low dose formalin stimulus (1%). Further, i.t. administration of DOM, a putative low-affinity KA receptor agonist, also produced significant analgesia. However, confirmation of the receptor subtypes involved in KA- and DOM-induced antinociception requires co-administration studies with selective antagonists (see EXPERIMENT 4).

In general, the non-NMDA receptor agonists mediated analgesic effects by decreasing nociception during Phase 2 while having little or no effect on Phase 1 responses (with the exception of 0.5 pmol DOM). The effect of AMPA on Phase 2 responses was not consistent with results reported by Coderre and Melzack (1992), who found that AMPA (270 pmol) was without effect on nociceptive responses at any time during a formalin test. Further, the results of the current study were inconsistent with most other results that have indicated that AMPA receptor antagonists, rather than agonists, have mediated antinociception in either the early or late phases of the formalin test (Näsström et al. 1992; Hunter and Singh 1994; Goettl and Larson 1994; Chapman and Dickenson 1995). It is difficult to speculate as to the mechanisms involved in AMPA-induced antinociception as observed in Experiment 3 especially since AMPA receptor antagonists mediated an analgesic effect in other studies.

Selective doses within the lower range of KA, DOM and AMPA doses tested

produced significant analgesia during Phase 2 responding (FIGURES 3.6, 3.7, 3.8). Higher doses of DOM and AMPA caused nociceptive responses to return to baseline control levels (FIGURES 3.7 and 3.8), whereas higher doses of KA initially produced a non-significant increase in nociception (increased AUC values) followed by a second significant decrease in nociception (decreased AUC values) (FIGURE 3.6).

The maximum analgesic effect for each drug was consistently within the picomole (pmol) range (TABLE 3.1) suggesting a receptor-selective action. Further, parallel DRCs and similar efficacies are consistent with a common mechanism of action (Kenakin 1993). The slopes of the analgesic portions of the dose-response curves for DOM, KA and AMPA during Phase 2 were not significantly different. Although the relative analgesic efficacies of each agonist appeared different, the general "U" shape of the dose-response curves were similar (FIGURES 3.6 and 3.7). The similarities of the shapes of the dose-response curves, along with the not-significantly different slopes over the analgesic portion of the DRCs, was another indication that KA, DOM and AMPA were interacting at the same receptor. If all three non-NMDA agonists mediated antinociception via the same receptor subtype, the apparent differences in antinociceptive efficacies might indicate that KA and AMPA are partial agonists relative to DOM's receptor actions. This hypothesis could be studied in an experiment where KA or AMPA are co-administered i.t. with DOM. If a decreased antinociceptive effect is found compared to DOM administration alone, partial agonist actions of KA or AMPA would be suggested.

Results of this nature have not been previously reported. Considering the evidence from some laboratories that spinal non-NMDA receptor activation led to nociceptive behavior or enhanced responsiveness from dorsal horn neurons (Jahr and Jessell 1985; Aanonsen and Wilcox 1987; DeLander and Wahl 1988), the results from the current study are difficult to explain. Also, the results of the current study certainly do not support the hypothesis that non-NMDA receptor activation in the spinal cord mediated the GLU- or ASP-induced hyperalgesia as described in Experiment 1 (see FIGURE 3.1).

3.6 EXPERIMENT 4 - STUDIES WITH THE NON-NMDA RECEPTOR ANTAGONISTS NS-102 AND NBQX

3.6.1 RATIONALE

It has been known for the last decade that [^{3}H]KA binding can be sub-divided into both high- and low-affinity sites (London and Coyle 1979; Nishikawa et al. 1983). However, the functional significance of this distinction has not been well characterized.

The results of several studies on the molecular biology of non-NMDA receptors have thus far identified nine receptor binding units (see CHAPTER 1, Section 1.5, TABLE 1.2). Four of the non-NMDA receptor binding proteins are selectively activated by AMPA and KA (GluR1-GluR4; Hollmann et al. 1989), and

the remaining five are selectively activated by KA (GluR5-GluR7, KA-1 and KA-2; Sakimura et al. 1990). Receptor binding studies have also determined that DOM is relatively more potent than KA in binding with the GluR5-GluR7 binding proteins (Johansen et al. 1993). The GluR5-GluR7 binding proteins are thought to correspond to a low-affinity [^{3}H]KA binding site (Verdoorn 1994).

The results of Experiment 3 demonstrated that the i.t. administration of KA, DOM and AMPA mediated significant analgesia during Phase 2 of the formalin test (see FIGURES 3.6, 3.7 and 3.8). To test whether the antinociceptive effect of each non-NMDA receptor agonist was mediated via a common EAA receptor subtype, co-administration studies were conducted using the selective non-NMDA receptor antagonists 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide (NBQX) and 5-nitro-6,7,8,9-tetrahydrobenzo(g)indole-2,3-dione-3-oxime (NS-102). NBQX is a competitive antagonist of non-NMDA receptors and has about a 30-fold selectivity for AMPA receptor binding relative to KA receptor binding (Sheardown et al. 1990). NS-102 is a competitive antagonist of non-NMDA receptors and is selective for the low-affinity KA binding site and the GluR6 binding protein (Johansen et al 1993; Verdoorn et al. 1994).

3.6.2 METHODS

See Section 3.2 for general methods. To determine whether the analgesic effects of DOM, KA or AMPA were mediated via a common EAA receptor subtype, the maximally effective antinociceptive dose of each agonist (0.5 pmol DOM; 50

pmol KA: 10 pmol AMPA: see EXPERIMENT 3) was co-administered with either NS-102 (2.5 μg [9.6 nmol] and 7.5 μg [28.7 nmol]) (Tasker et al. 1996) or NBQX (10 nmol) (Hunter andSingh 1994) prior to injection of formalin (2.5%).

3.6.3 RESULTS

NS-102

Analysis by two-way ANOVA determined that there were significant agonist and antagonist main effects, but no significant agonist/antagonist interactions for the three time periods of interest.

Co-administration of NS-102 vehicle, 2.5 μg NS-102 or 7.5 μg NS-102 with saline, did not significantly change baseline nociceptive responses during any of the tested time periods (FIGURES 3.9, 3.10 and 3.11).

Co-administration of 0.5 pmol DOM and NS-102 vehicle produced significant analgesia when AUC values were compared to the saline/NS-102 vehicle group, for both the 60 min time period and for Phase 2 ([$F\{1,19\}=10.87$, $p<0.01$] and [$F\{1,19\}=10.51$, $p<0.01$] respectively; FIGURES 3.9 and 3.11). A significant antinociceptive effect of 0.5 pmol DOM during Phase 1 was not seen when DOM was co-administered with NS-102 vehicle (FIGURE 3.10). Further, co-administration of DOM with 2.5 μg NS-102 produced significant analgesia when AUC values were compared against control for 60 min, Phase 1 and Phase 2 ([$F\{1,15\}=18.99$, $p<0.01$], [$F\{1,15\}=5.11$, $p<0.05$] and [$F\{1,15\}=20.11$, $p<0.01$]

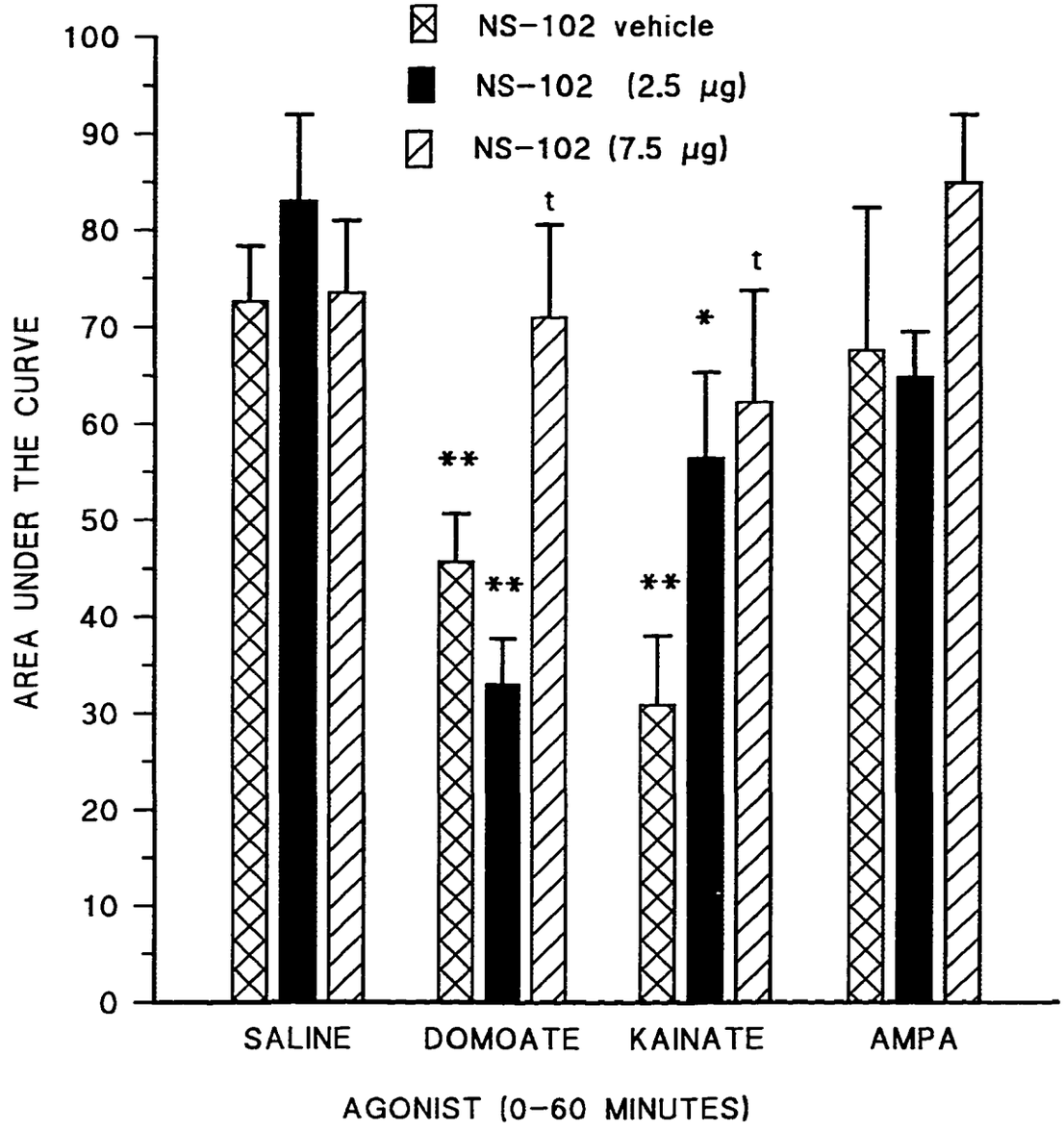


FIGURE 3.9 Effect of intrathecal co-administration of Domoic Acid (0.5 pmol), Kainic Acid (50 pmol) or AMPA (10 pmol) with NS-102 on nociception in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during the 60 min test period. All data points represent the mean AUC of a group of either 6 or 10 rats ($p < 0.01$ and * $p < 0.05$ compared to the corresponding saline/NS-102 group; t $p < 0.05$ when compared to the corresponding agonist/NS-102 vehicle group).**

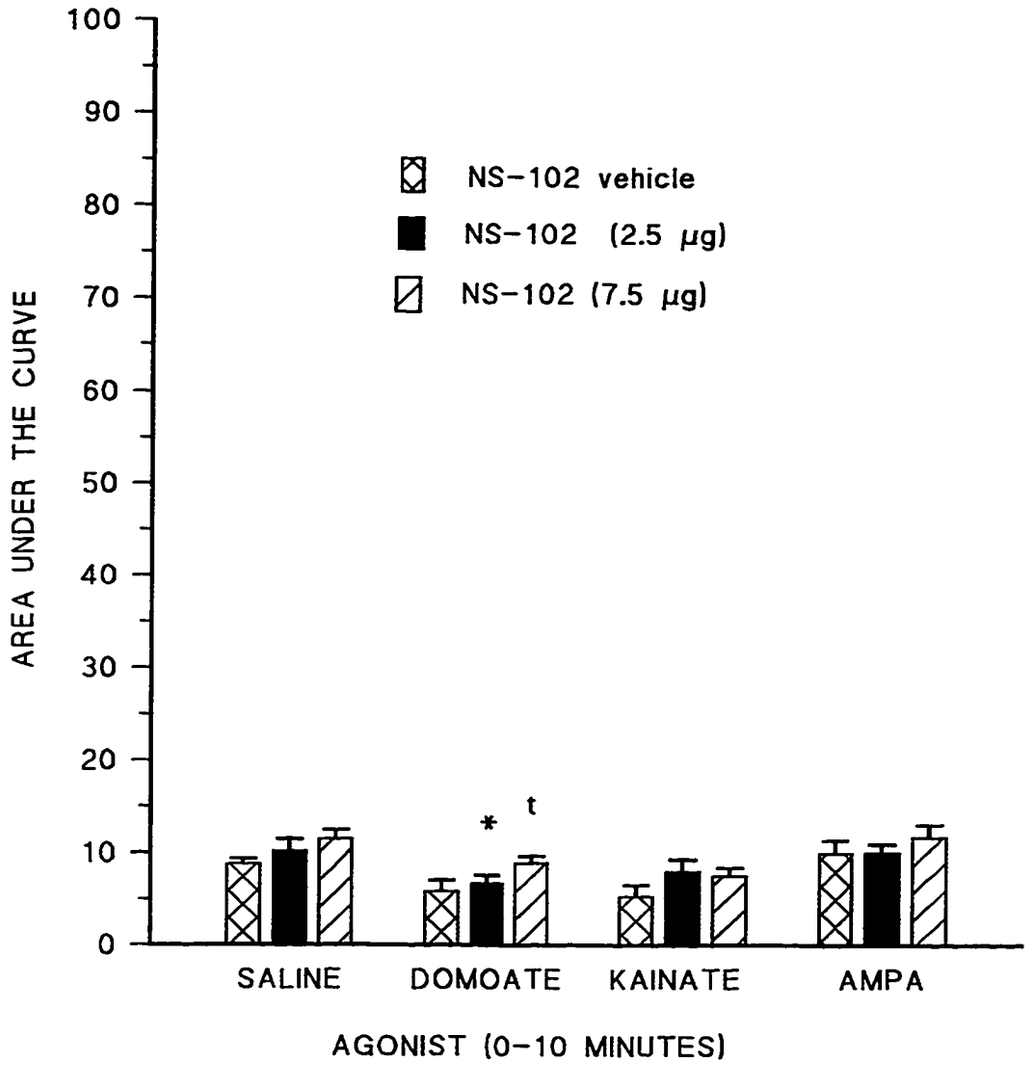


FIGURE 3.10 Effect of intrathecal co-administration of Domoic Acid (0.5 pmol), Kainic Acid (50 pmol) or AMPA (10 pmol) with NS-102 on nociceptive responses in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during Phase 1. All data points represent the mean AUC of a group of either 6 or 10 rats (* $p < 0.05$ when compared to the corresponding saline/NS-102 group; t $p < 0.05$ when compared against the corresponding agonist/NS-102 vehicle group).

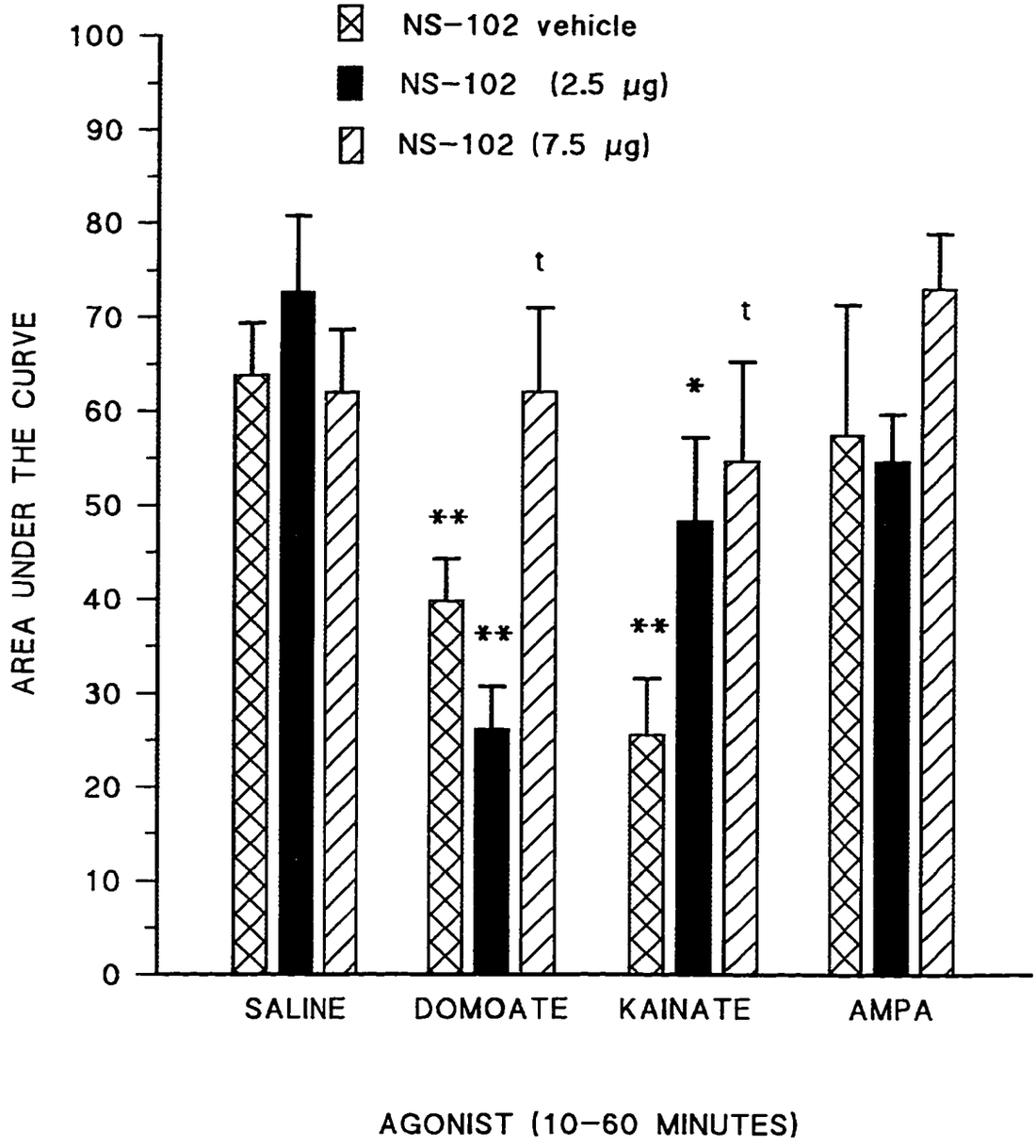


FIGURE 3.11 Effect of intrathecal co-administration of Domoic Acid (0.5 pmol), Kainic Acid (50 pmol) or AMPA (10 pmol) with NS-102 on nociceptive responses in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during Phase 2. All data points represent the mean AUC of a group of either 6 or 10 rats ($p < 0.01$ and * $p < 0.05$ when compared to the corresponding saline/NS-102 group; t $p < 0.05$ when compared to the corresponding agonist/NS-102 vehicle group).**

respectively: FIGURES 3.9, 3.10 and 3.11). Significant DOM-induced antinociception was not found when DOM was co-administered with 7.5 μg NS-102 during any of the three time periods (FIGURES 3.9, 3.10 and 3.11). Co-administration of 7.5 μg NS-102 significantly antagonized DOM-induced antinociception during the 60 min and Phase 2 periods ([$F\{1,23\}=6.70$, $p<0.05$] and [$F\{1,23\}=5.91$, $p<0.05$] respectively: FIGURES 3.9 and 3.11). NS-102 (7.5 μg) significantly enhanced DOM-induced Phase 1 AUC values. However, it must be noted DOM did not produce a significant Phase 1 antinociceptive effect compared to the saline/NS-102 vehicle group (FIGURE 3.10). Finally, 2.5 μg NS-102 did not significantly antagonize the antinociceptive effects of DOM during any of the three time periods (FIGURES 3.9, 3.10 and 3.11).

To determine whether NS-102 would also antagonize the antinociceptive actions of KA, NS-102 vehicle and 2.5 μg or 7.5 μg NS-102 were co-administered with 50 pmol KA. KA produced significant antinociception when AUC values were compared to the saline/NS-102 vehicle group during the 60 min and Phase 2 periods ([$F\{1,19\}=20.87$, $p<0.01$] and [$F\{1,19\}=21.34$, $p<0.01$] respectively: FIGURES 3.9 and 3.11). However, co-administration of KA and NS-102 vehicle did not produce significant antinociception during Phase 1 (FIGURE 3.10). Similarly, KA produced significant antinociception when co-administered with 2.5 μg NS-102 during only the 60 min and Phase 2 periods ([$F\{1,15\}=5.34$, $p<0.05$] and [$F\{1,15\}=5.50$, $p<0.05$] respectively: FIGURES 3.9 and 3.11). Co-administration of KA with 7.5 μg NS-102 did not result in significant antinociception during any of the three time periods

(FIGURES 3.9, 3.10 and 3.11). Co-administration of 7.5 μg NS-102 significantly antagonized KA-induced antinociception during the 60 min and Phase 2 periods ([F{1,15}=5.31, p<0.05] and [F{1,15}=5.64, p<0.05] respectively; FIGURES 3.9 and 3.11). NS-102 (7.5 μg) was without effect on KA-induced Phase 1 nociception (FIGURE 3.10). Finally, 2.5 μg NS-102 did not significantly antagonize KA-induced nociceptive responses during any of the three time periods (FIGURES 3.9, 3.10 and 3.11).

Co-administration of AMPA (10 pmol) and NS-102 (vehicle, 2.5 μg or 7.5 μg) did not produce changes in formalin-induced nociceptive responses during any of the three time periods (FIGURES 3.9, 3.10 and 3.11). It should be noted that in the current study, AMPA (10 pmol) did not produce a significant Phase 2 antinociception as was found in Experiment 3 (FIGURE 3.8).

Collectively, the results of NS-102 co-administration studies suggest that DOM- and KA-induced antinociception in the formalin test were mediated via an action on a NS-102-sensitive receptor since co-administration of 7.5 μg NS-102 significantly antagonized the antinociceptive effects of both DOM and KA.

NBQX

Analysis by two-way ANOVA determined that there were significant agonist main effects for all three time periods. Further, an antagonist main effect was observed over the 60 min and Phase 2 periods only. Finally, there were no significant agonist/antagonist interaction effects found for the three time periods.

Co-administration of NBQX vehicle or 10 nmol NBQX with saline, did not significantly change baseline nociceptive responses during any of the tested time periods (FIGURES 3.12, 3.13 and 3.14).

Co-administration of 0.5 pmol DOM and NBQX vehicle produced significant analgesia when AUC values were compared to those of the saline/vehicle control group for 60 min, Phase 1 and Phase 2 ([$F\{1,20\}=8.92$, $p<0.01$], [$F\{1,20\}=4.63$, $p<0.05$] and [$F\{1,20\}=5.791$, $p<0.05$] respectively; FIGURES 3.12, 3.13 and 3.14). DOM produced significant antinociception when co-administered with 10 nmol NBQX during only the Phase 1 period ([$F\{1,20\}=5.25$, $p<0.05$]; FIGURE 3.13). Co-administration of 10 nmol NBQX did not significantly antagonize DOM-induced antinociception during any of the three time periods (FIGURES 3.12, 3.13 and 3.14).

Co-administration of 50 pmol KA and NBQX vehicle produced significant analgesia when AUC values were compared to those of the saline/vehicle control group for 60 min and Phase 2 ([$F\{1,20\}=5.79$, $p<0.05$] and [$F\{1,20\}=6.53$, $p<0.05$] respectively; FIGURES 3.12 and 3.14). However, KA did not produce significant antinociception when co-administered with 10 nmol NBQX during any of the time periods (FIGURES 3.12, 3.13 and 3.14). Co-administration of 10 nmol NBQX significantly antagonized KA-induced antinociception during all three time periods ([$F\{1,10\}=9.68$, $p<0.01$], [$F\{1,10\}=5.53$, $p<0.05$] and [$F\{1,10\}=10.00$, $p<0.01$] respectively; FIGURES 3.12, 3.13 and 3.14).

Co-administration of AMPA (10 pmol) and NBQX (vehicle or 10 nmol) did not produce changes in formalin-induced nociceptive responses during any of the

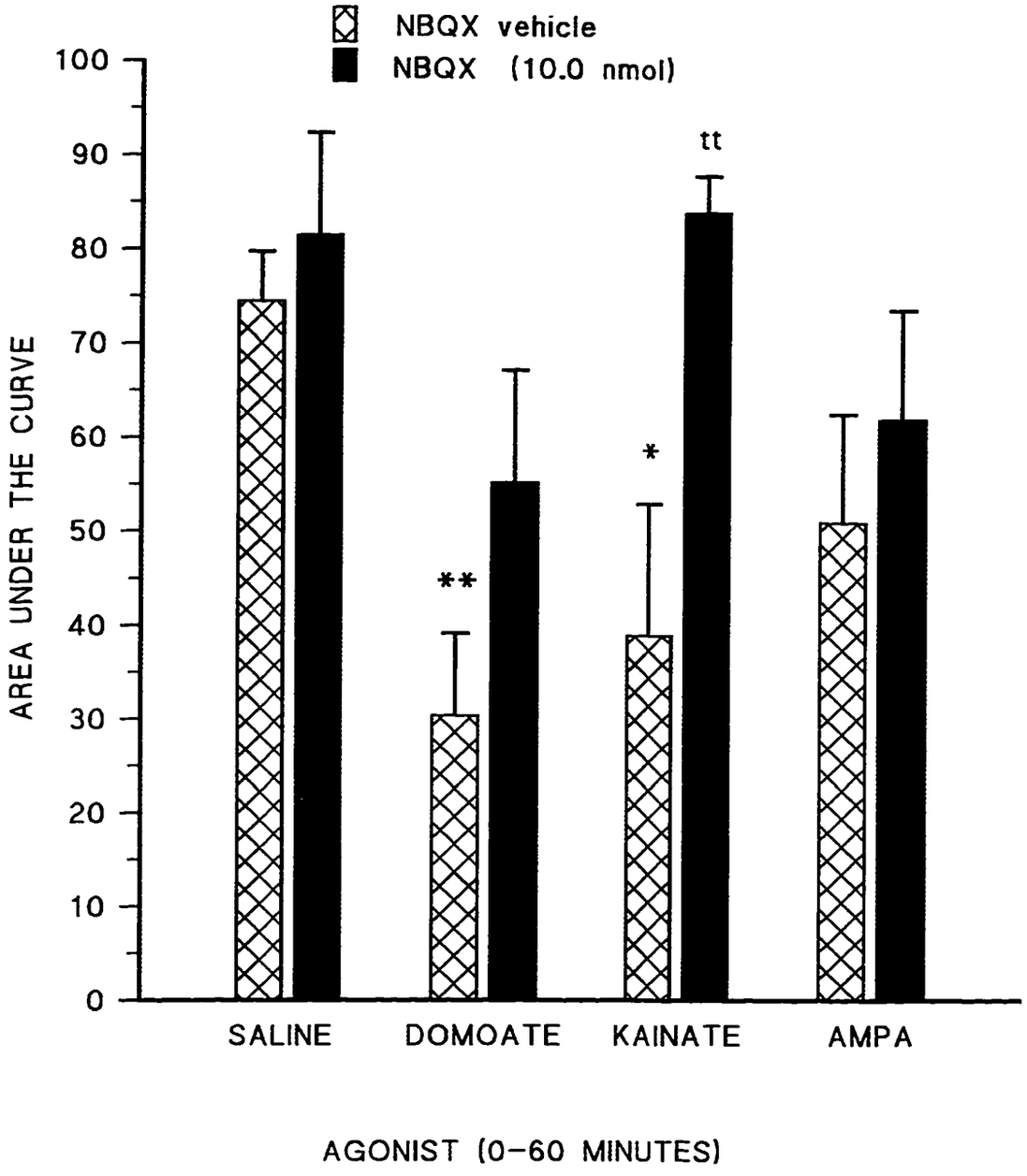


FIGURE 3.12 Effect of intrathecal co-administration of Domoic Acid (0.5 pmol), Kainic Acid (50 pmol) or AMPA (10 pmol) with NBQX on nociception in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during the 60 min test period. All data points represent the mean AUC of a group of 6 rats ($p < 0.01$ and * $p < 0.05$ when compared to the corresponding saline/NBQX group; tt $p < 0.01$ when compared to the corresponding agonist/NBQX vehicle group).**

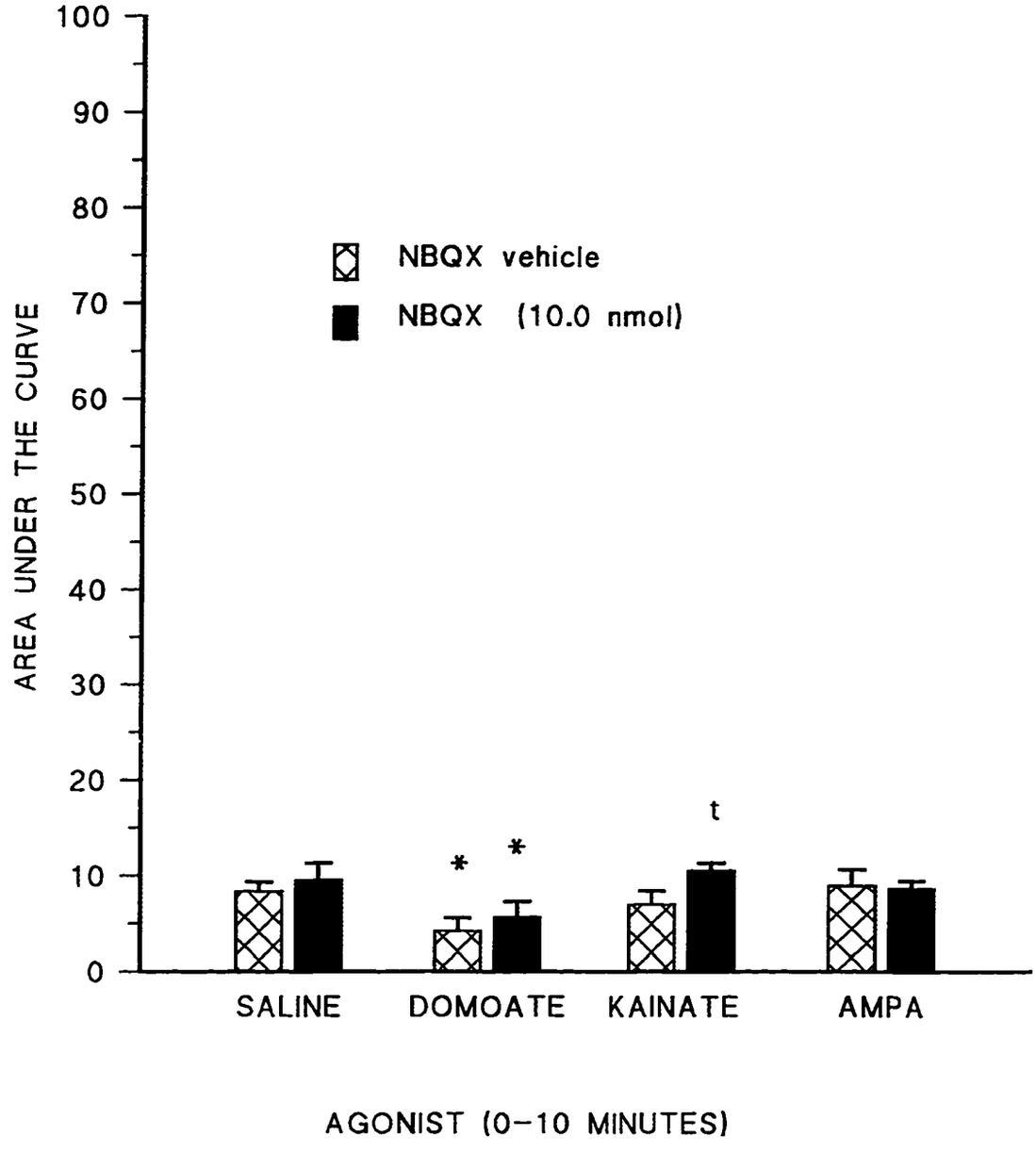


FIGURE 3.13 Effect of intrathecal co-administration of Domoic Acid (0.5 pmol), Kainic Acid (50 pmol) or AMPA (10 pmol) with NBQX (vehicle or 10 nmol) on nociception in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during Phase 1. All data points represent the mean AUC of a group of 6 rats (* $p < 0.05$ when compared to the corresponding saline/NBQX group; t $p < 0.05$ when compared to the corresponding agonist/NBQX vehicle group).

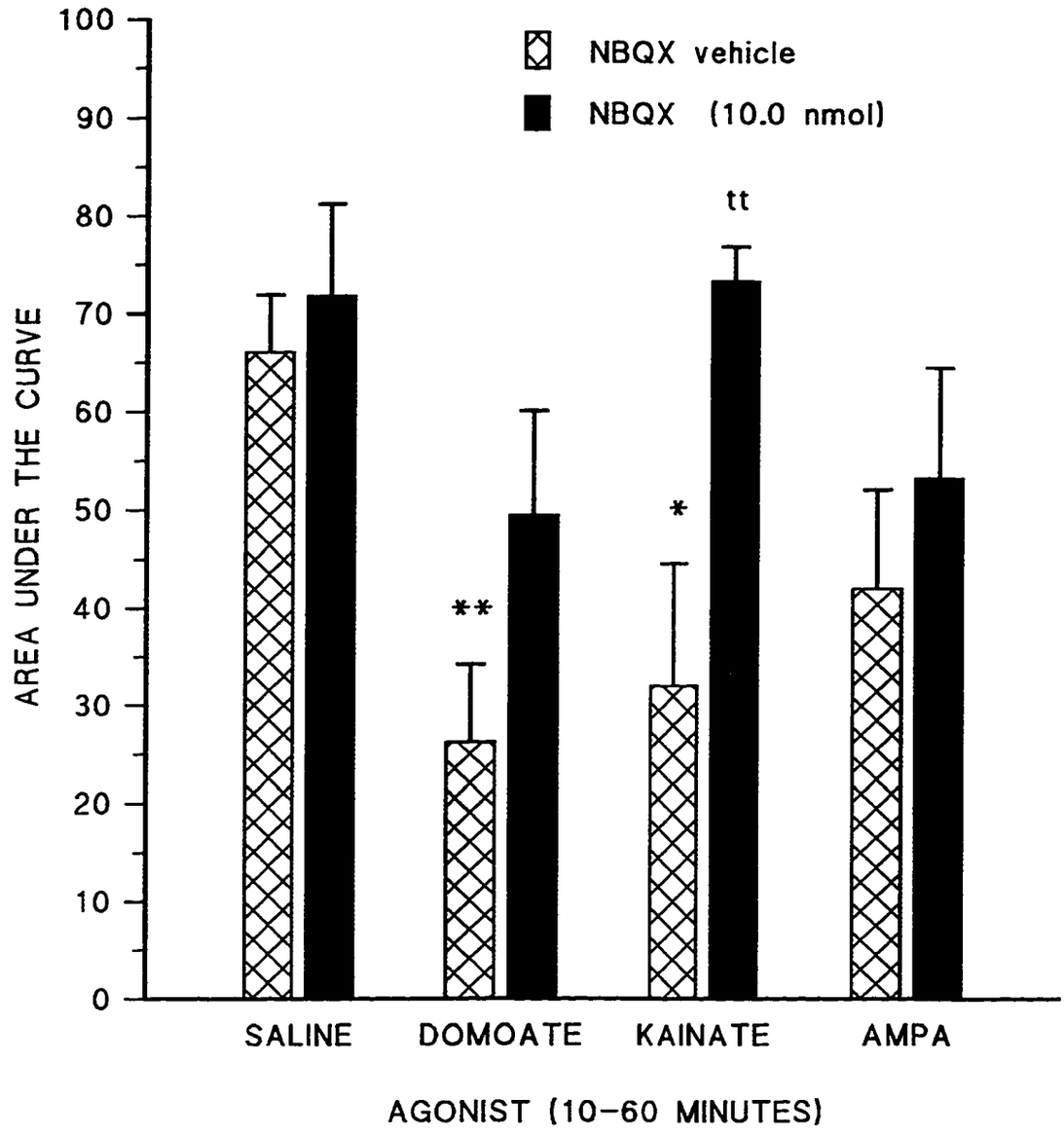


FIGURE 3.14 Effect of intrathecal co-administration of Domoic Acid (0.5 pmol), Kainic Acid (50 pmol) or AMPA (10 pmol) with NBQX on nociceptive responses in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during Phase 2. All data points represent the mean AUC of a group of 6 rats (* $p < 0.05$ and ** $p < 0.01$ when compared to the corresponding saline/NBQX group; tt $p < 0.01$ when compared to the corresponding agonist/NBQX vehicle group).

three time periods (FIGURES 3.12, 3.13 and 3.14). However, it must be noted that in the current study, AMPA (10 pmol) did not produce a significant Phase 2 antinociception as was found in Experiment 3 (FIGURE 3.8).

Collectively, the results of NBQX co-administration studies suggest that KA-induced antinociception in the formalin test was mediated via an action on NBQX-sensitive receptors since co-administration of NBQX significantly reversed the antinociceptive effects of KA. The effect of NBQX on DOM-induced antinociception was less clear.

3.6.4 DISCUSSION

The results of Experiment 4 indicated that i.t. DOM- and KA-induced antinociception were mediated in part via a low-affinity KA receptor. This conclusion was based on the finding that NS-102 antagonized DOM- and KA-mediated antinociception (FIGURES 3.9 and 3.11). Further, the NS-102-mediated antagonism of both DOM- and KA-induced antinociception was due to an action solely on Phase 2 responses (FIGURE 3.14). NS-102 (7.5 μg) significantly antagonized DOM-induced Phase 1 AUC levels. However, the i.t. combination of DOM and NS-102 vehicle failed to produce significant Phase 1 antinociception (FIGURE 3.10). The possibility remains that other spinal receptor mechanisms might have been involved in mediating Phase 1 nociception. In order to determine if high-affinity KA receptors have a function in mediating the observed KA- and DOM-induced analgesia, co-administration of high-affinity KA receptor antagonists

with KA and DOM should be performed. Unfortunately, selective high-affinity KA receptor antagonists have not as yet been developed. Also, experiments with a larger number of animals in each group might provide more conclusive results.

Sheardown and colleagues (1990) have reported that NBQX has a 30-fold selectivity for AMPA receptors as compared to KA receptors. However, using binding assays, Johansen and colleagues (1993) found that besides being a selective AMPA receptor antagonist, NBQX also displaced [^{3}H]KA binding from both a high- and low-affinity KA binding site. Further, Johansen and colleagues (1993) reported that NBQX had a slightly higher affinity for the low-affinity KA binding site as compared to the high-affinity KA binding site. These results suggested that NBQX could have an effect on both KA- and DOM-induced antinociception in the formalin test. However, in the current study NBQX significantly reversed KA-induced antinociception but did not significantly affect DOM-induced antinociception (FIGURES 3.12 and 3.14).

Neither NBQX nor NS-102 significantly enhanced or attenuated AMPA responses suggesting that AMPA receptors were probably not involved in mediating formalin-induced nociceptive responses (FIGURES 3.9, 3.10 and 3.11). Further, AMPA in combination with either NS-102 or NBQX vehicle did not effect nociceptive responses during any time period (FIGURES 3.9 to 3.14). Although AMPA produced significant Phase 2 antinociception when administered alone (FIGURE 3.8), this result did not appear to be robust. This result is supported by Coderre and Melzack (1992), who also reported that the spinal administration of

AMPA did not affect formalin-induced nociception.

3.7 EXPERIMENT 5 - STUDIES WITH NMDA

3.7.1 RATIONALE

The role of the NMDA receptor in mediating the early and the late phase of formalin-induced nociception remains controversial. The results of electrophysiological studies in the rat have indicated that NMDA receptor activation is required to mediate formalin-induced nociceptive responses in the late phase, but not the early phase (King et al. 1988; Haley et al. 1990; Thompson et al. 1990; Dickenson and Aydar 1991). The results of behavioral studies, where selective NMDA receptor agonists or antagonists have been administered i.t., have found that NMDA receptor activation led to a dose-dependent enhancement of nociception only during the late phase of the response (Coderre and Melzack 1992). Also, NMDA receptor antagonists have dose-dependently attenuated formalin-induced nociception specifically during the late phase (Murray et al. 1991; Coderre and Melzack 1992; Vaccarino et al. 1993; Hunter and Singh 1994). The results of the above studies have indicated that spinal NMDA receptor activation might be necessary for late phase nociception but not for early phase nociception.

The results of other electrophysiological studies (Woolf and Thompson 1991) supported the hypothesis that NMDA receptor activation is required for both the induction and maintenance of formalin-induced, early and late phase nociceptive

responses. Further, results of behavioral studies have described significant antinociception during both the early and the late phase of the formalin response following i.t. administration of competitive NMDA receptor antagonist (Näsström et al. 1992; Millan and Seguin 1993; Kristensen and colleagues 1994; Goettl and Larson 1994). These results support the hypothesis that NMDA receptors are necessary for both early and late phase nociception.

The current study was conducted to determine whether the i.t. administration of NMDA would lead to enhanced nociception during either Phase 1 or Phase 2 of the formalin-response. Further, if the spinal application of NMDA resulted in an enhanced nociception, this might provide a possible explanation as to which EAA receptor mediated the GLU- and ASP-induced enhanced nociception found in Experiment 1.

3.7.2 RESULTS

When 1% formalin was administered as the nociceptive stimulus, only 1.0 nmol NMDA produced a significantly enhanced level of nociception for 60 min when compared to control ($F\{1,30\}=4.18$, $p<0.05$; FIGURE 3.15). During Phase 1, both 0.75 nmol and 1.0 nmol NMDA mediated significantly enhanced nociception ([$F\{1,30\}=4.37$, $p<0.05$] and [$F\{1,30\}=4.86$, $p<0.05$] respectively; FIGURE 3.15). Phase 2 nociception, however, was not significantly affected by any of the doses of NMDA administered in the current study (FIGURE 3.15).

0.5 nmol NMDA produced a different response compared to the other

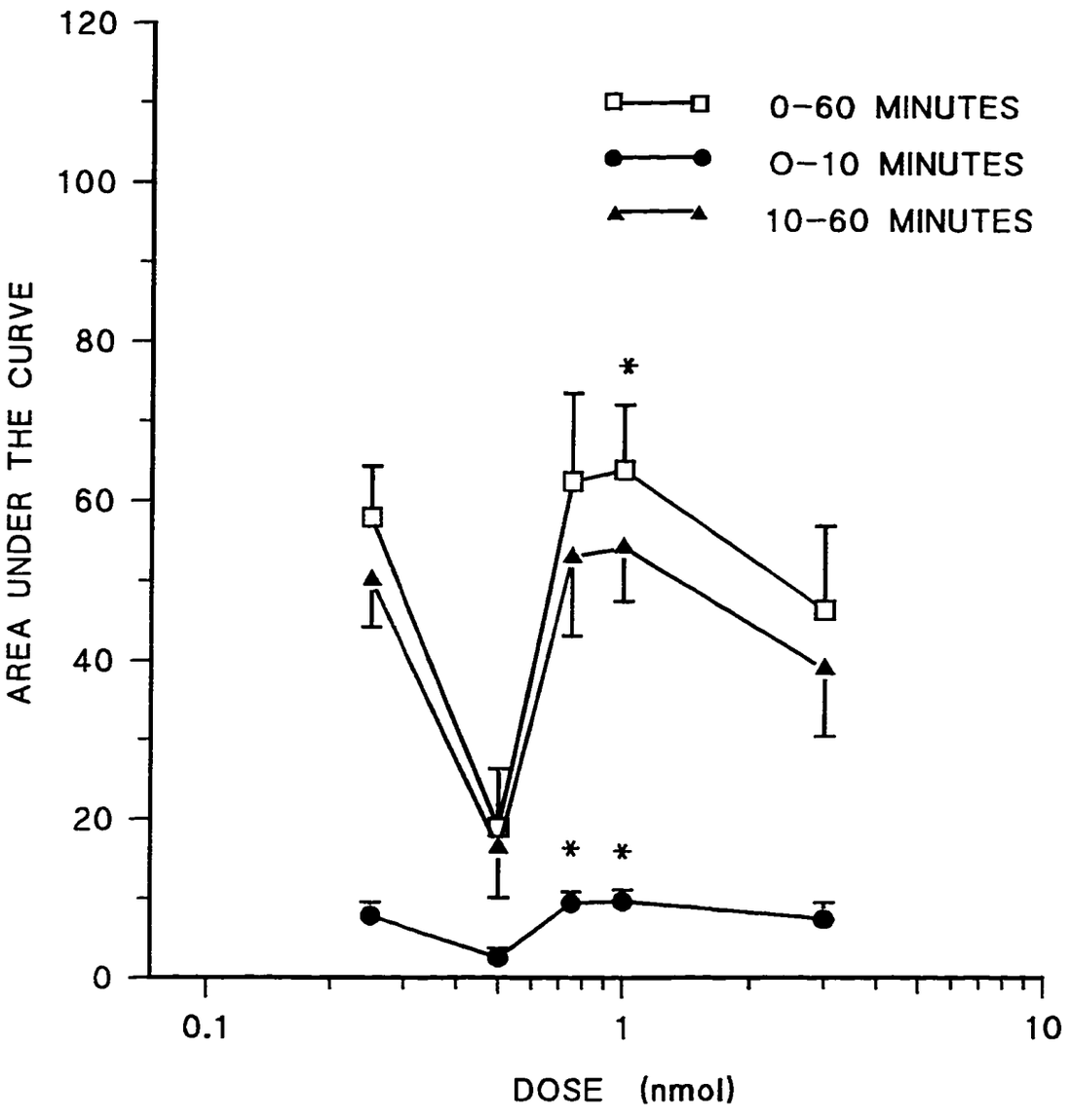


FIGURE 3.15 Log dose-response curves for intrathecal NMDA for 60 min, Phase 1 and Phase 2 in the formalin test (1.0% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (* $p < 0.05$ when compared to groups administered saline at pH 2.5; Saline AUC: 60 min = 38.2 ± 9.3, Phase 1 = 5.0 ± 1.0, Phase 2 = 33.2 ± 8.6).

administered doses of NMDA (FIGURE 3.15). A large but non-significant reduction in the AUC value for the 60 min period was observed (AUC=18.9 ± 7.4 for 0.5 nmol NMDA vs 38.2 ± 9.3 for saline; FIGURE 3.15). To determine whether the decreased AUC value represented a real decrease in nociception, which may have been limited by a 'floor' effect due to the use of a low concentration of formalin (1%), 0.5 nmol NMDA was administered i.t. prior to a higher concentration of formalin (2.5%).

Administration of NMDA (0.5 nmol) prior to a 2.5% formalin injection did not produce a significant change in nociception during the 60 min, Phase 1 or Phase 2 time periods (FIGURE 3.16).

3.7.3 DISCUSSION

The results of the current study demonstrated that the i.t. administration of NMDA prior to a low-level formalin stimulus (1%) mediated a significant increase in nociception during both the 60 min and Phase 1 periods (FIGURE 3.15). Nociception during Phase 2 was not significantly affected by any of the doses of NMDA administered, although mean AUC values were greater than control. Since i.t. administration of NMDA resulted in hyperalgesia only during Phase 1, it could be suggested that spinal NMDA receptors have a role in the induction of persistent nociception. The results of the current study did not provide evidence that spinal NMDA receptors were involved in the maintenance of persistent nociception (Phase 2 nociception). However, the hypothesis that NMDA receptors are involved in

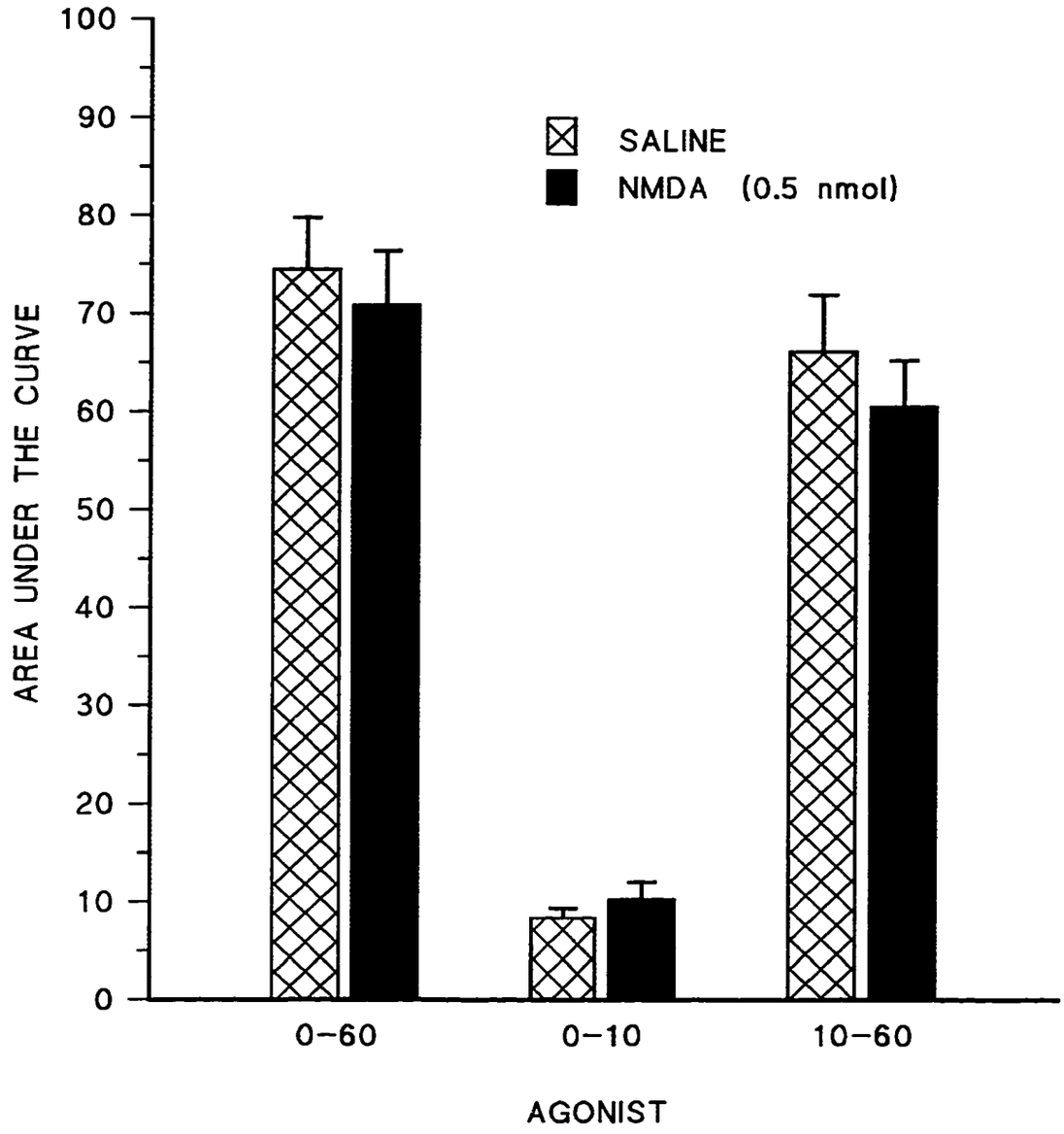


FIGURE 3.16 Effect of intrathecal administration of NMDA on nociceptive responses in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean + s.e.m.) represent the level of nociception recorded during 60 min, Phase 1 and Phase 2. All data points represent the mean AUC from a group of 6 rats.

formalin-induced Phase 2 nociception (maintenance) could not be ruled out based on the above results. Since only one dose of NMDA mediated significant overall hyperalgesia, care must be taken when interpreting these results. It is possible that the observed hyperalgesia was an artifact of the data variability.

When NMDA (0.5 nmol) was administered i.t. prior to a high level formalin stimulus (2.5%) there were no changes in nociception during the three time peiods measured (FIGURE 3.16). This result indicated that the large but non-significant decrease in nociception observed following 1.0% formalin injections was not masked by the use of a low level formalin stimulus. It is also plausable that the decrease in AUC observed following 0.5 nmol NMDA was an artifact of the data variability.

The results of Experiment 1 demonstrated that hyperalgesia could be produced following the i.t. administration of both GLU and ASP (FIGURES 3.1, 3.2 and 3.3). Further, Phase 1 nociception was more sensitive to the hyperalgesic effects of both GLU and ASP. It has been postulated that ASP is a selective agonist of NMDA receptors (Watkins and Evans 1981) while the activity of GLU is mediated via both NMDA and non-NMDA receptors (MacDonald and Porietis 1982; Mayer and Westbrook 1987). The results of the current study suggested that Phase 1 hyperalgesia following i.t. administration of ASP and GLU was mediated via an action at spinal NMDA receptors, since ASP, GLU and NMDA selectively enhanced Phase 1 nociception (FIGURES 3.3 and 3.15). However, co-administration studies with NMDA receptor antagonists would need to be done to confirm this hypothesis.

3.8 EXPERIMENT 6 - STUDIES WITH THE METABOTROPIC RECEPTOR AGONIST tACPD

3.8.1 RATIONALE

Metabotropic glutamate receptors (mGluRs; see TABLE 1.2) are a novel class of recently cloned G-protein-coupled receptors. These receptors are formed by heterogeneous groupings of metabotropic binding proteins and are coupled to a multitude of second messenger systems that include phosphoinositide hydrolysis through phospholipase C, increased cAMP formation, decreased cAMP formation, changes in intracellular Ca^{2+} and changes in ion channel function (Schoepp and Conn 1993). Further, EAA metabotropic receptors are thought to be present both pre- and post-synaptically within the mammalian central nervous system (Schoepp et al. 1990).

The action of EAA metabotropic receptors in the spinal transmission of nociceptive information has not been extensively studied. However, the development of the selective metabotropic receptor agonist trans-(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (tACPD: Schoepp et al. 1990) has provided a pharmacological tool to allow the elucidation of the role of spinal metabotropic receptors.

tACPD has been demonstrated to increase the depolarizing response and to increase inward Ca^{2+} currents in isolated dorsal horn neurons when iontophoretically co-applied with the EAA ionotropic receptor agonists NMDA, AMPA or KA (Cerne

and Randic 1990; Bleakman et al. 1992). Paleček and colleagues (1994) applied by microdialysis low doses of tACPD and increased the responsiveness of spinal thalamic tract (STT) neurons to innocuous mechanical stimuli. Behavioral studies (Coderre and Melzack 1992) found that spinally administered tACPD significantly enhanced nociception during the first 15 min following formalin injection. This result (Coderre and Melzack 1992) was obtained using only one dose of tACPD (0.3 nmol). The aim of the current study, therefore, was to further elucidate the role of spinal metabotropic receptors by the i.t. administration of a range of tACPD doses. Further, both 1.0% and 2.5% formalin stimuli will be used to determine if tACPD has either nociceptive or antinociceptive effects.

3.8.2 RESULTS

The EAA metabotropic receptor agonist tACPD was administered i.t. prior to either 1.0% or 2.5% formalin. None of the doses of tACPD administered, prior to either 1% or 2.5% formalin, resulted in a significant difference in AUC values when compared to saline control during any of the time frames of interest (FIGURES 3.17 and 3.18).

3.8.3 DISCUSSION

Prior administration of tACPD did not significantly increase or decrease nociceptive responses in any of the three time periods (FIGURES 3.17 and 3.18). The results of the current study were in contrast to the findings of Coderre and

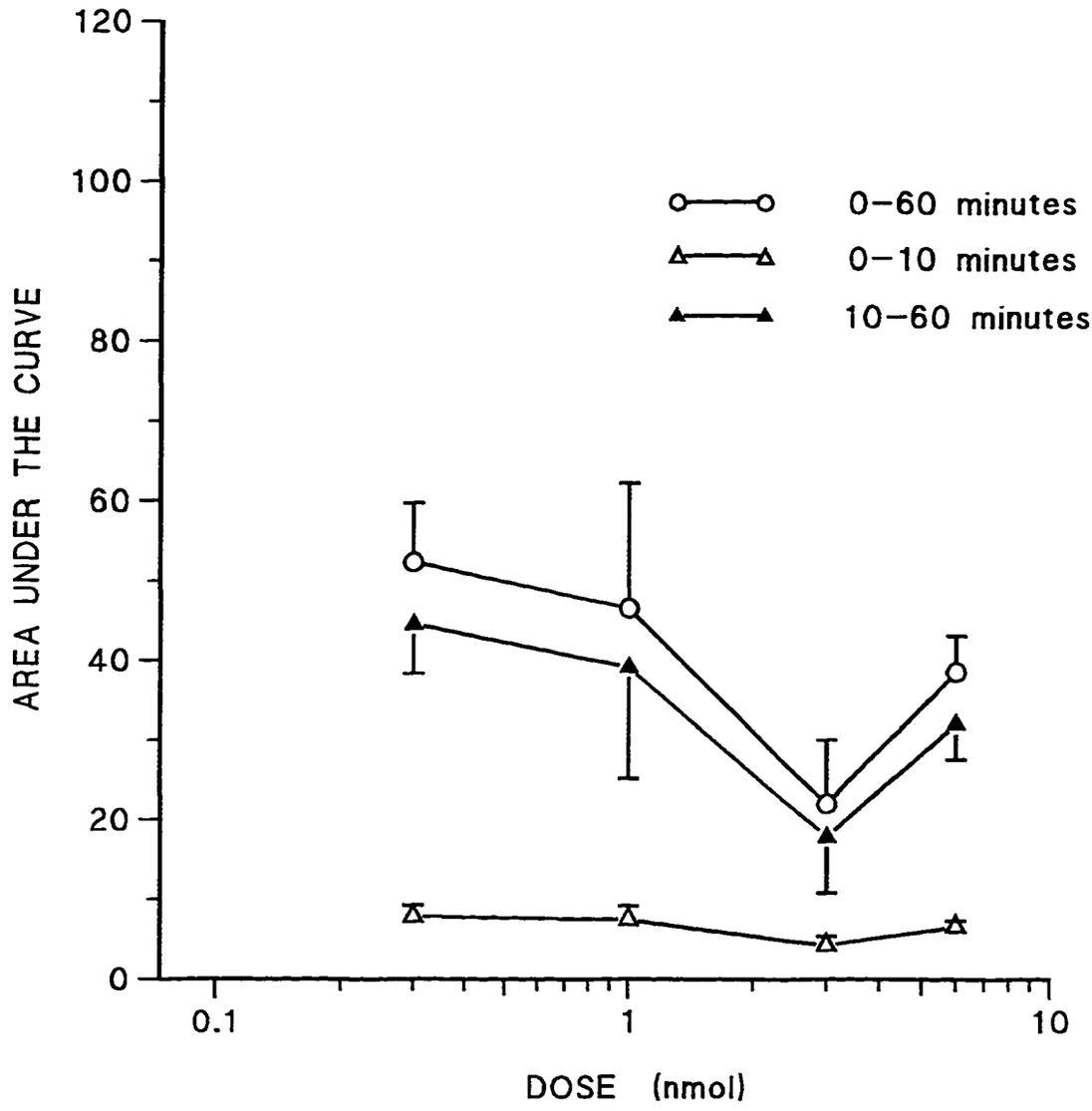


FIGURE 3.17 Log dose-response curves for intrathecal ACPD for 60 min, Phase 1 and Phase 2 in the formalin test (1.0% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (Saline AUC: 60 min = 38.2 ± 9.3, Phase 1 = 5.0 ± 1.0, Phase 2 = 33.2 ± 8.6).

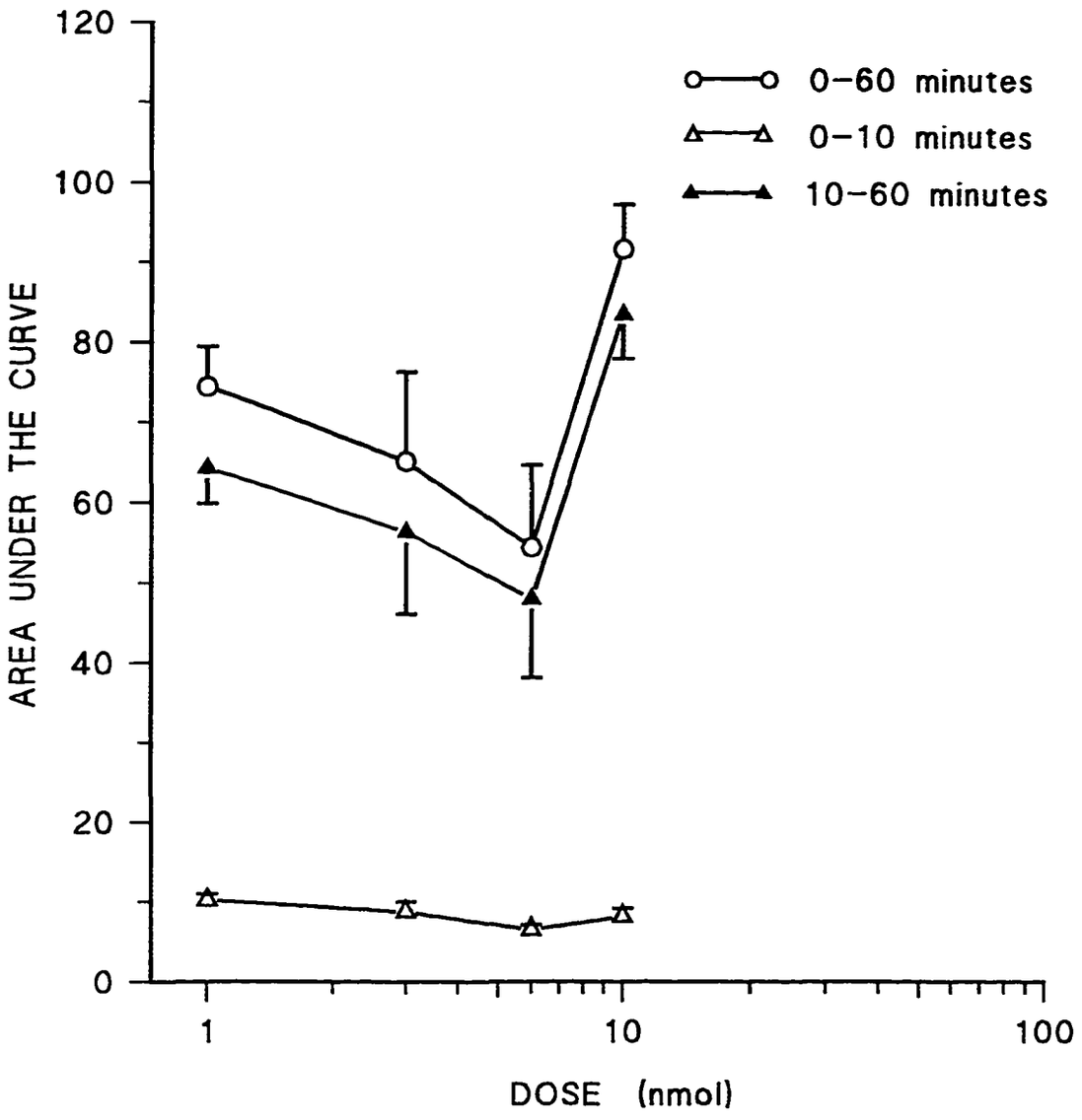


FIGURE 3.18 Log dose-response curves for intrathecal ACPD for 60 min, Phase 1 and Phase 2 in the formalin test (2.5% formalin). Area-Under-the-Curve (AUC) values (mean ± s.e.m.) represent the level of nociception recorded during each phase of response. All data points represent the mean AUC from a group of 6 rats (Saline AUC: 60 min = 74.4 ± 5.3, Phase 1 = 8.3 ± 1.0, Phase 2 = 66.1 ± 5.8).

Melzack (1992), who demonstrated that spinal injections of tACPD significantly enhanced formalin-induced nociception during the early phase. An explanation as to why contrasting results were found is not evident. However, a couple of possible explanations are apparent. Coderre and Melzack (1992) used 0.1 nmol tACPD while the lowest dose of tACPD administered in Experiment 6 was 0.3 nmol. It is possible that the administration of lower doses might have resulted in an enhanced or a decreased analgesia. Further, perhaps methodological differences in the type and use of anaesthetics and in drug injection protocol might have accounted for the discrepancies.

Electrophysiological studies found either no effect or a minimal effect on spinal dorsal horn neuronal responses when tACPD was administered alone (Cerne and Randic 1992; Bleakman et al. 1992). However, tACPD increased dorsal horn neuronal responses when administered in combination with different EAA ionotropic receptor agonists (Cerne and Randic 1992; Bleakman et al. 1992). Consistent with this, in a behavioral study, Coderre and Melzack (1992) found that the co-injection of tACPD and NMDA resulted in a synergistic increase in nociception compared to nociception observed following the i.t. administration of either drug independently. More elaborate co-administration studies of various EAA agonists/antagonists with EAA metabotropic receptor agonists might yield interesting results concerning the spinal processing of nociceptive information.

3.9 SUMMARY AND GENERAL DISCUSSION

The experiments in Chapter 3 have attempted to characterize pharmacologically the EAA receptor subtypes involved in spinal mechanisms of nociception and antinociception in the formalin test in rats. Studies identified the responses to EAAs administered via chronically implanted intrathecal cannulae. The role of EAAs in nociception has been suspected as early as 1960 when Curtis and Watkins found that microinjections of EAAs onto dorsal horn neurons resulted in an increased excitation of these cells. Subsequently, Skilling and colleagues (1988) reported that noxious stimulation produced an increased release of GLU and ASP in the spinal dorsal horn. The results of Experiment 1 provided more evidence that GLU and ASP are involved in the spinal processing of nociceptive inputs. I.t. administration of both GLU and ASP produced significantly increased nociceptive responses when injected prior to a low concentration of formalin (FIGURES 3.1, 3.2 and 3.3). Interestingly, Phase 1 nociception was more sensitive to the spinal administration of both agonists, while Phase 2 nociception was only enhanced following the administration of a higher dose of GLU.

The results of Experiment 5 provided preliminary evidence that NMDA receptor activation mediated the GLU- and ASP-induced Phase 1 hyperalgesia. I.t. administration of NMDA produced significant hyperalgesia during Phase 1 but was without effect on Phase 2 nociception (FIGURE 3.15). ASP has been described as the selective endogenous NMDA receptor agonist while GLU is considered to activate both NMDA and non-NMDA receptors (Watkins and Evans 1981;

MacDonald and Porietis 1982; Mayer and Westbrook 1987). It can be suggested, therefore, that ASP- and GLU-induced Phase 1 hyperalgesia was mediated in part via an NMDA receptor.

The results of several behavioral experiments have demonstrated a role for spinal NMDA receptors in the modification of nociceptive behavior. The spinal application of NMDA has been found to produce hyperalgesia in the tail-flick test (Aanonsen and Wilcox 1987; 1989), the hot-plate test (Aanonsen and Wilcox 1987) and the caudally-directed biting and scratching test (Aanonsen and Wilcox 1987; 1989; DeLander and Wahl 1988; Hornfeldt and Larson 1989). The hyperalgesic effects of i.t. NMDA in each of these nociceptive tests have been found to occur within a time frame consistent with Phase 1 nociception (0-10 min). Further, formalin-induced early phase nociception has been described as the acute or 'phasic' component of the formalin response profile (Dubuisson and Dennis 1977). Therefore, it is consistent that NMDA would enhance the phasic component of formalin-induced nociception. In Experiment 5, the finding of NMDA-mediated hyperalgesia in Phase 1 supports the hypothesis that NMDA receptor activation is important in modifying the spinal transmission of phasic nociceptive stimuli (Aanonsen and Wilcox 1987). Further indications of the role of NMDA receptors in Phase 1 nociception would be given by the co-administration of a NMDA receptor antagonist.

The EAA receptors involved in GLU-induced enhancement of Phase 2 nociception could not be determined by the experiments presented in Chapter 3.

Coderre and Melzack (1992), Cerne and Randic (1992), and Bleakman and colleagues (1992) have provided evidence that Phase 2 nociception might be the result of a coordinated activation of more than one EAA receptor subtype. Future experimentation might, therefore, involve the co-administration of different combinations of EAA agonists and EAA antagonists in order to elucidate the EAA receptor mechanisms involved in Phase 2 nociception.

Collectively, the results of Experiments 2, 3 and 4 demonstrated that the i.t. administration of DOM and KA mediated significant antinociception in the formalin test. Also, DOM- and KA-induced antinociception was mediated specifically via the low-affinity KA receptor since both DOM- and KA-induced antinociception was significantly antagonized following the co-administration of the competitive and selective low-affinity KA receptor antagonist NS-102 (FIGURES 3.9 to 3.14). The mechanism by which the low-affinity KA receptor mediated DOM- and KA-induced antinociception is unknown. The results of Experiments 2, 3 and 4 are the first known reports of DOM- and KA-induced antinociception in any animal model of nociception. There are, however, indications in the literature that DOM and KA might have actions on low-affinity KA receptors located on the spinal dorsal roots (Evans 1985; Agrawal and Evans 1986). The relevance of dorsal root EAA receptors to KA- and DOM-induced antinociception in the formalin test will be discussed in greater depth in Chapter 4.

REFERENCES

CHAPTER 3

AANONSEN LM, WILCOX GL. Muscimol, gamma-aminobutyric acid$_A$ receptors and excitatory amino acids in the mouse spinal cord. J Pharmacol Exp Ther 1989; 248: 1034-1038.

AANONSEN LM, WILCOX GL. Nociceptive action of excitatory amino acids in the mouse: effects of spinally administered opioids, phencyclidine and sigma agonists. J Pharmacol Exp Ther 1987; 243: 9-19.

AANONSEN LM, LEI S, WILCOX GL. Excitatory amino acid receptors and nociceptive neurotransmission in rat spinal cord. Pain 1990; 41: 309-321.

ABBOTT FV, FRANKLIN KBJ, CONNELL B. The stress of a novel environment reduces formalin pain: possible role of serotonin. Eur J Pharmacol 1986; 126: 141-144.

AGRAWAL SG, EVANS RH. The primary afferent depolarizing of kainate in the rat. Br J Pharmacol 1986; 87: 345-355.

BLEAKMAN D, RUSIN KI, CHARD PS, GLAUM SR, MILLER RJ. Metabotropic glutamate receptors potentiate ionotropic glutamate responses in the rat dorsal horn. J Pharm Exp Ther 1992; 42: 192-196.

CARLÀ V, MORONI F. General anaesthetics inhibit the responses induced by glutamate receptor antagonists in the mouse cortex. Neurosci Lett 1992; 146: 21-24.

CERNE R, RANDIC M. Modulation of AMPA and NMDA responses in rat spinal dorsal horn neurons by trans-1-aminocyclopentane-1,3-dicarboxylic acid. Neurosci Lett 1992; 144: 180-184.

CHAPMAN V, DICKENSON AH. Time-related roles of excitatory amino acid receptors during persistent noxiously evoked responses of rat dorsal horn neurones. Brain Res 1995; 703: 45-50.

CODERRE TJ. The role of excitatory amino acid receptors and intracellular messengers in persistent nociception after tissue injury in rats. Molec Neurobiol 1994; 7: 229-245.

CODERRE TJ, MELZACK R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 1992; 12: 3665-3670.

CODERRE TJ, MELZACK R. Central neural mediators of secondary hyperalgesia following heat injury in rats: neuropeptides and excitatory amino acids. Neurosci Lett 1991; 131: 71-74.

CODERRE TJ, VAN EMPEL I. The utility of excitatory amino acid (EAA) antagonists as analgesic agents. I. Comparison of the antinociceptive activity of various classes of EAA antagonists in mechanical, thermal and chemical nociceptive tests. Pain 1994; 59: 345-352.

CODERRE TJ, VACCARINO AL, MELZACK R. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res 1990; 535: 155-158.

COHEN SR, ABBOTT FV, MELZACK R. Unilateral analgesia produced by intraventricular morphine. Brain Res 1984; 303: 277-287.

CURTIS DR, WATKINS JC. The excitation and depression of spinal neurones by structurally related amino acids. J Neurochem 1960; 6: 117-141.

CURTIS DR, PHILLIS JW, WATKINS JC. Chemical excitation of spinal neurones. Nature (Lond.) 1959; 183: 611-612.

DELANDER GE, WAHL JJ. Descending systems activated by morphine (ICV) inhibit kainic acid (IT)-induced behavior. Pharmacol Biochem Behav 1991; 39: 155-159.

DELANDER GE, WAHL JJ. Behavior induced by putative nociceptive neurotransmitters is inhibited by adenosine or adenosine analogs coadministered intrathecally. J Pharmacol Exp Ther 1988; 246: 565-570.

DICKENSON AH, AYDAR E. Antagonism at the glycine site on the NMDA receptor reduces spinal nociception in the rat. Neurosci Lett 1991; 121: 263-266.

DICKENSON AH, SULLIVAN AF. Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. Pain 1987; 30: 349-360.

DOUGHERTY PM, PALECEK J, PALECKOVA V, SORKIN LS, WILLIS WD. The role of NMDA and non-NMDA excitatory amino acid receptors in the excitation of primate spinothalamic tract neurons by mechanical, chemical, thermal, and electrical stimuli. J Neurosci 1992; 12: 3025-3041.

DUBUISSON D, DENNIS SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977; 4: 161-174.

EVANS RH. Kainate sensitivity of C-fibres in rat dorsal roots. J Physiol 1985; 358: 42P.

GOETTL VM, LARSON AA. Antinociception induced by 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), and N-methyl-D-aspartate(NMDA) competitive antagonist, plus 6,7-dinitroquinoxaline-2,3-dione (DNQX), a non-NMDA antagonist, differs from that induced by MK-801 plus DNQX. Brain Res 1994; 642: 334-338.

HALEY JE, WILCOX GL. Involvement of excitatory amino acids and peptides in the spinal mechanisms underlying hyperalgesia. In: Willis WD Jr, ed. Hyperalgesia and allodynia. New York: Raven, 1992: 281-293.

HALEY JE, SULLIVAN AF, DICKENSON AH. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. Brain Res 1990; 518: 218-226.

HOLLMANN M, O'SHEA-GREENFIELD A, ROGERS SW, HEINEMANN S. Cloning by functional expression of a member of the glutamate receptor family. Nature 1989; 342: 643-648.

HORNFELDT CS, LARSON AA. Selective inhibition of excitatory amino acids by divalent cations. A novel means for distinguishing N-methyl-D-aspartic acid-, kainate-, and quisqualate-mediated actions in the mouse spinal cord. J Pharmacol Exp Ther 1989; 251: 1064-1068.

HUNTER JC, SINGH L. Role of excitatory amino acid receptor in the mediation of the nociceptive response to formalin in the rat. Neurosci Lett 1994; 174: 217-221.

JAHR CE, JESSELL. Synaptic transmission between dorsal root ganglion and dorsal horn neurons in culture: antagonism of monosynaptic excitatory postsynaptic potentials and glutamate excitation by kynurenate. J Neurosci 1985; 5: 2281-2289.

JOHANSEN TH, DREJER J, WATJEN F, NIELSEN EO. A novel non-NMDA receptor antagonist shows selective displacement of low-affinity [^{3}H]kainate binding. Eur J Pharmacol 1993; 246: 195-204.

JOHNSON JL. The excitant amino acids glutamic and aspartic acid as transmitter candidates in the vertebrate central nervous system. Prog Neurobiol 1978; 10: 155-202.

KELLSTEIN DE, COGHILL RC, FRENK H, BOSSUT DF, MAYER FJ. Opioid inhibition of kainic acid-induced scratching: mediation by mu and sigma but not delta and kappa receptors. Pharmacol Biochem Behav 1990; 35: 1-5.

KENAKIN T. Pharmacological analysis of drug-receptor interaction. New York: Raven Press, 1993.

KING AE, THOMPSON SWN, URBAN L, WOOLF CJ. An intracellular analysis of amino acid induced excitations of deep dorsal horn neurones in the rat spinal cord slice. Neurosci Lett 1988; 89: 286-292.

KLEINBAUM DG, KUPPER LL, MULLER KE. Applied regression analysis and other multivariable methods. Boston: PWS-Kent, 1988.

KRISTENSEN JD, KARLSTEN R, GORDH T, BERGE O-G. The NMDA antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) has antinociceptive effect after intrathecal injection in the rat. Pain 1994; 56: 59-67.

LEES GJ. Influence of ketamine on the neuronal death caused by NMDA in the rat hippocampus. Neuropharmacology 1995; 34: 411-417.

LONDON ED, COYLE JT. Specific binding of [^{3}H]kainic acid to receptor sites in rat brain. Mol Pharmacol 1979; 15: 492-505.

MACDONALD JF, PORIETIS AV. DL-Quisqualic and L-Aspartic acids activate separate excitatory conductances in cultured spinal cord neurons. Brain Res 1982; 245: 175-178.

MADL JE, BEITZ AJ, JOHNSON RL, LARSON AA. Monoclonal antibodies specific for fixative-modified aspartate: immunocytochemical localization in the rat CNS. J Neurosci 1987; 7: 2639-2650.

MAO J, PRICE DD, HAYES RL, MAYER DJ. Differential roles of NMDA and non-NMDA receptor activation in induction and maintenance of thermal hyperalgesia in rats with painful peripheral mononeuropathy. Brain Res 1992; 598: 271-178.

MAYER ML, WESTBROOK GL. The physiology of excitatory amino acids in the vertebrate central nervous system. Prog Neurobiol 1987; 28: 197-276.

MILLAN MJ, SEGUIN L. (+)-HA 966, a partial agonist at the glycine site coupled to NMDA receptors, blocks formalin-induced pain in mice. Eur J Pharmacol 1993; 238: 445-447.

MURRAY CW, COWAN A, LARSON AA. Neurokinin and NMDA antagonists (but not kainic acid antagonist) are antinociceptive in the mouse formalin model. Pain 1991; 44: 179-185.

NÄSSTRÖM J, KARLSSON U, POST C. Antinociceptive actions of different classes of excitatory amino acid receptor antagonists in mice. Eur J Pharmacol 1992; 212: 21-29.

NISHIKAWA T, TAKASHIMA M, TORU M. Increased [^{3}H]kainic acid binding in the prefrontal cortex in schizophrenia. Neurosci Lett 1983; 40: 245-.

O'KEEFE J. Spinal cord mechanisms subserving pain perception. M.Sc. Thesis, McGill University, 1964.

OWEN JA, TASKER RAR, NAKATSU K. A simple, less stressful rat restrainer. Experientia 1984; 40: 306-308.

PALEČEK J, PALEČKOVÁ V, DOUGHERTY PM, WILLIS WD. The effect of trans-ACPD, a metabotropic excitatory amino acid receptor agonist, on the responses of primate spinothalamic tract neurons. Pain 1994; 56: 261-269.

PORTER RH, GREENAMYRE JT. Regional variations in the pharmacology of NMDA receptor channel blockers: implications for therapeutic potential. J Neurochem 1995; 64: 614-623.

POTASHNER SJ, TRAN PL. Decreased uptake and release of D-aspartate in the guinea pig spinal cord after partial cordotomy. J Neurochem 1985; 44: 1511-1519.

SAKIMURA K, BUJO H, KUSHIYA E, ARAKI K, YAMAZAKI M, MEGURO H, WARASHINA A, NUMA S, MISHINA M. Functional expression from cloned cDNAs of glutamate receptor species responsive to kainate and quisqualate. FEBS Lett 1990; 272: 73-80.

SCHNEIDER SP, PERL ER. Selective excitation of neurons in the mammalian spinal dorsal horn by aspartate and glutamate in vitro: correlation with localization and excitatory input. Brain Res 1985; 360: 339-343.

SCHOEPP D, CONN PJ. Metabotropic glutamate receptors in brain function and pathology. TIPS 1993; 14: 13-20.

SCHOEPP D, BOCKAERT J, SLADECZEK F. Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. TIPS 1990; 11: 508-515.

SHEARDOWN MJ, NIELSON EO, HANSEN AJ, JACOBSEN P, HONORE T. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. Science 1990; 247: 571-574.

SKILLING SR, SMULLEN DH, BEITZ AJ, LARSON AA. Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following vertridine and nociceptive stimulation. J Neurochem 1988; 51: 127-132.

STRAIN SM, TASKER RAR. Hippocampal damage produced by systemic injections of domoic acid in mice. Neurosci 1991; 44: 343-352.

TASKER RAR, STRAIN SM. Morphine differentially affects domoic acid and kainic acid toxicity *in vivo*. NeuroReport 1992; 3: 789-792.

TASKER RAR, CONNELL BJ, STRAIN SM. Pharmacology of systemically administered domoic acid in mice. Can J Physiol Pharmacol 1991; 69: 378-382.

TASKER RAR, STRAIN SM, DREJER J. Selective reduction in domoic acid toxicity in vivo by a novel-non-N-methyl-D-aspartate receptor antagonist. Can J Physiol Pharmacol 1996; 74: 1047-1054.

THOMPSON SWN, KING AE, WOOLF CJ. Activity-dependent changes in the rat ventral horn neurones in vitro; summation of prolonged afferent evoked postsynaptic depolarizations produce a d-APV sensitive windup. Eur J Neurosci 1990; 2: 638-649.

URCA G, URCA R. Neurotoxic effects of excitatory amino acids in the mouse spinal cord: quisqualate and kainate but not N-methyl-D-aspartate induce permanent neural damage. Brain Res 1990; 529: 7-15.

VACCARINO AL, PREZEMYSLAW M, LIEBESKIND JC. Stress-induced analgesia prevents the development of the tonic, late phase of pain produced by subcutaneous formalin. Brain Res 1992; 572: 250-252.

VACCARINO AL, MAREK P, KEST B, WEBBER E, KEANA JFW, LIEBESKIND JC. NMDA receptor antagonists, MK-801 and ACEA-1011, prevent the development of tonic pain following subcutaneous formalin. Brain Res 1993; 615: 331-334.

VERDOORN TA, JOHANSEN TH, DREJER J, NIELSEN EO. Selective block of recombinant glur6 receptors by NS-102, a novel non-NMDA receptor antagonist. Eur J Pharmacol 1994; 269: 43-49.

WATKINS JC, EVANS RH. Excitatory amino acid transmitters. Annu Rev Pharmacol Toxicol 1981; 21: 165-204.

WESTLUND KN, McNEILL DL, COGGESHALL RE. Glutamate immunoreactivity in rat dorsal root axons. Neurosci Let 1989a; 96: 13-17.

WESTLUND KN, McNEILL DL, PATTERSON JT, COGGESHALL RE. Aspartate immunoreactive axons in normal rat L_4 dorsal roots. Brain Res 1989b; 489: 347-351.

WOOLF CJ, THOMPSON CWN. The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation: implications for the treatment of post-injury pain hypersensitivity states. Pain 1991; 44: 293-299.

CHAPTER 4

HYPOTHESIS OF DOMOIC ACID- AND KAINIC ACID-INDUCED ANTINOCICEPTION

4.1 PRELIMINARY REMARKS

Intrathecal administration of DOM and KA produced significant antinociception in the formalin test. Spinally-mediated antinociception due to any EAA receptor agonist in any test of nociception has not been previously described. Therefore, Chapter 4 will present a hypothesis describing a proposed site of action where the antinociceptive actions of DOM and KA are mediated. Further, speculation as to the mechanisms underlying DOM and KA's antinociceptive actions will be presented.

4.2 PERIPHERAL LOW-AFFINITY KA RECEPTORS

The introduction of the Gate Control Theory by Melzack and Wall (1965) represented a major theoretical breakthrough in our understanding of how the spinal cord processes peripheral noxious stimuli. This theory suggested that the relative afferent activity of large rapidly conducting A-fibres and small slowly conducting C-fibres, at the level of the substantia gelatinosa, could determine the strength of spinal output via the activation of Transmission (T) cells, which terminate in supraspinal areas (FIGURE 1.1). Further, these authors suggested that activity in A-fibres could

exert a modulating presynaptic control over C-fibre input to the spinal cord via the action of inhibitory interneurons located within the substantia gelatinosa. Further, A-fibre volleys have been shown to produce increased excitability of C-fibre afferent synaptic terminals in the spinal cord, consistent with mechanisms of presynaptic inhibition (Calvillo 1978; Hentall and Fields 1979; Fitzgerald and Woolf 1981; McGeer et al. 1987). Either direct or indirect A-fibre-induced depolarization of C-fibre synaptic terminals represents an important mechanism controlling noxious peripheral input from sensory receptors supplied by unmyelinated C-fibres.

The results of Experiments 2, 3 and 4 (CHAPTER 3) demonstrated that i.t. administration of both DOM and KA produced significant antinociception in the formalin test. Further, both DOM- and KA-induced antinociception were antagonized by the co-administration of the higher of the two doses of the selective low-affinity KA receptor antagonist NS-102 (FIGURES 3.9-3.11). Finally, KA-induced antinociception was reversed by the co-administration of the non-selective, non-NMDA receptor antagonist NBQX (FIGURES 3.12-3.14). These results suggested that DOM and KA mediated their antinocieptive actions via a low-affinity KA receptor located within or near the spinal dorsal horn.

Within the framework of the Gate Control Theory (Melzack and Wall 1965; FIGURE 1.1), it can be hypothesized that the antinociception produced following low-affinity KA receptor activation was due to A-fibre-induced presynaptic inhibition of afferent C-fibre inputs resulting in a decreased synaptic release of endogenous EAA ligands. Indeed, DOM- and KA-induced presynaptic inhibition of

GLU release has been demonstrated in hippocampal synaptosomal preparations (Chittajallu et al. 1996). The hypothesis that DOM- and KA-induced presynaptic inhibition of GLU release in the spinal dorsal horn in relation to the results of Experiments 2, 3 and 4 (CHAPTER 3) would fit nicely within the theoretical model of the Gate Control Theory (Melzack and Wall 1965; FIGURE 1.1).

However, upon further considerations, the hypothesis that analgesic actions of DOM and KA were mediated via actions within the spinal dorsal horn cannot be supported. Indeed, such a hypothesis would not be consistent with findings reported in the literature. The results of Experiment 1 (CHAPTER 3) and other behavioral studies have described hyperalgesic actions of spinally-administered GLU in the formalin test (Coderre and Melzack 1992). GLU is considered to be the endogenous ligand that activates non-NMDA receptors, including KA receptors (MacDonald and Porietis 1982; Mayer and Westbrook 1987; see TABLE 1.2). Spinally-administered GLU and KA have produced caudally-directed biting and scratching (Hornfeldt and Larson 1989; DeLander and Wahl 1988). Electrophysiological studies have found a depolarizing action of GLU on dorsal horn neurons (Curtis et al. 1959). Also, GLU has been reported to selectively excite dorsal horn neurons that were responsive to C-fibre stimulation (Schneider and Perl 1985). Finally, Skilling and colleagues (1988) observed that injections of formalin into the hindpaw of a rat caused an immediate increase in the levels of GLU in the spinal dorsal horn and in spinal extracellular fluid. Collectively, the results of the above studies, including those of Experiment 1, support the theory that EAA receptors within the dorsal horn mediate

nociception rather than antinociception. Since several electrophysiological and behavioral studies have demonstrated a role of spinal EAA receptors in increasing nociceptive behaviors, the results of Experiments 2, 3 and 4 (CHAPTER 3) are not consistent with the hypothesis that the analgesic actions of DOM and KA were mediated via actions within the spinal dorsal horn.

An alternate synaptic location for EAA receptors outside of the CNS might, therefore, be the site of action for DOM- and KA-induced antinociception. Davies and colleagues (1979) and Agrawal and Evans (1986) found that GLU, DOM and KA directly depolarized isolated dorsal roots from spinal cords of both immature and mature rats. It was also demonstrated that the primary afferent depolarizing effects of GLU, KA and DOM were restricted to the slowest conducting afferent C-fibres (Evans 1985; Agrawal and Evans 1986). Further, DOM was the most potent agent tested for depolarizing the dorsal roots, 20 to 40 times more potent than KA (Agrawal and Evans 1986). Finally, KA was approximately 10 times more potent than GLU in this paradigm (Agrawal and Evans 1986; Shinozaki et al. 1991; Ishida and Shinozaki 1991). Applications of AMPA, NMDA or quisqualic acid at high doses were either without effect or had only minimal depolarizing effects indicating that the actions of GLU, KA and DOM were mediated via a kainate-preferring receptor. Collectively, these results suggest that a certain population of primary afferent C-fibres possess axonal KA receptors that are activated via the endogenous EAA agonist GLU.

The most striking effect of DOM- and KA-induced C-fibre depolarization

was the depression or complete blockage of peripherally stimulated C-fibre-evoked volleys (Evans 1985; Agrawal and Evans 1986; Evans et al. 1987). Dorsal root C-fibre volleys reappeared on washout of KA, indicating that KA-induced depression of C-fibre transmission was not due to KA-induced neurotoxic effects. Evans and colleagues (1987) also demonstrated that KA-induced depression of C-fibre volleys could be antagonized by co-administration of kynurenic acid, a non-NMDA selective receptor antagonist (Birch et al. 1988), indicating that non-NMDA receptors mediated the observed KA-induced depression of C-fibre activity.

Blocking C-fibre volleys can potentially be analgesic as C-fibre activation has been proposed to result from peripheral high-intensity noxious stimulation (Melzack and Wall 1965). DOM- and KA-induced analgesia as described in Experiments 2, 3 and 4 (CHAPTER 3) is proposed to have been mediated via activation of low-affinity KA receptors located on C-fibres external to the spinal dorsal horn, thereby blocking C-fibre conduction prior to dorsal horn activation. DOM and KA have been shown not to induce receptor desensitization, in contrast to GLU (Agrawal and Evans 1986). Therefore, DOM- and KA-induced KA receptor activation could significantly decrease the level of afferent nociceptive input into the spinal dorsal horns. The decrease in afferent C-fibre input received by the spinal dorsal horns would then translate into behavioral analgesia.

If i.t. administration of DOM and KA produced analgesia in the formalin test by actions at low-affinity KA receptors located peripheral to the spinal dorsal horn, the low-affinity KA receptor binding proteins GluR5, GluR6 and GluR7 (TABLE

1.2) must be manufactured within dorsal root ganglion cells and transported to an axonic postsynaptic site where functional KA receptors would be assembled. Using *in situ* hybridization techniques, GluR5 and GluR6 mRNA have been located in the dorsal root ganglion and in the dorsal roots of both embryonic and mature rats (Sommer and Seeburg 1992; Bahn et al. 1994; Petralia et al. 1994). Further, in mature rats, dense immunocytochemical staining of GluR6 and GluR7 has been localized within smaller cell bodies within the dorsal root ganglia (Petralia et al. 1994). The size of the dorsal root ganglion soma have been shown to be positively correlated with signal conduction velocity (Harper and Lawson 1985). Thus, it can be concluded that the smaller cell bodies, where GluR6 and GluR7 staining mainly occurs, are the soma of afferent C-fibres. Therefore, peripheral afferent C-fibres have the potential to manufacture functional low-affinity KA-preferring receptors.

Evidence exists from several laboratories that the low-affinity KA binding subunits GluR5 and GluR6 can form functional homomeric channels when expressed in host cells (Egebjerg et al. 1991; Bettler et al. 1992; Sommer et al. 1992; Burnashev 1993; Seeburg 1993). Several of the electrophysiological properties of homomeric combinations of either GluR5 or GluR6 subunits resembled those of the native KA receptor found on dorsal root ganglion cells (Heuttner 1990; Sommer et al. 1992) and on the peripheral dorsal roots (Agrawal and Evans 1986; Evans et al. 1987). For example, homomeric GluR5 receptor channels have shown strong desensitization following the application of GLU (Trussel et al. 1988; Egebjerg et al. 1991; Bettler et al. 1992; Sommer et al. 1992), and either extremely weak or no

desensitization following DOM or KA application (Mayer and Vlyklicki 1989; Heuttner 1990). Similar results have been described for dorsal root ganglion (Heuttner 1990) and peripheral dorsal root KA receptors (Agrawal and Evans 1986). Also, expressed homomeric receptors comprised of GluR6 subunits have been shown to be insensitive to the application of AMPA (Egebjerg et al 1991; Sommer et al. 1992), as was also demonstrated for dorsal root ganglion (Heuttner 1990) and dorsal root KA receptors (Agrawal and Evans 1986). It can, therefore, be hypothesized that dorsal root KA-preferring receptors are composed of either homomeric GluR5 or GluR6 binding proteins or heteromeric combinations of GluR5 and GluR6 binding proteins. However, the presence of other GluR binding proteins involved in mediating the analgesic effects of DOM and KA cannot be ruled out. These peripheral low-affinity KA receptors are the proposed sites of action mediating DOM- and KA-induced antinociception.

4.3 MECHANISMS OF ACTION OF LOW-AFFINITY KA RECEPTOR ACTIVATION

An electrical signal being transmitted along non-myelinated C-fibres is subject to electrotonic decay whereby the signal weakens as it is transmitted centrally towards the spinal cord (McGeer et al. 1987). Since DOM- and KA-induced activation of dorsal root low-affinity KA receptors results in a depolarization of C-fibres (Evans 1985; Agrawal and Evans 1986), the expected consequence would be strengthened or enhanced centrally propagated C-fibre

signals. However, C-fibre depolarization resulted in a blockage of stimulus-evoked C-fibre volleys (Evans 1985; Agrawal and Evans 1986). Therefore, a mechanism to hyperpolarize, or at least resist, depolarizing alterations of the resting membrane potential must be associated with the C-fibre depolarizing actions of DOM and KA.

Homomeric combinations of the low-affinity KA receptor binding subunits GluR5 and GluR6 have been shown to be permeable to Ca^{2+} upon activation of the receptors with KA (Pruss et al. 1991; Burnashev 1993; Köhler et al. 1993). Further. Type I and Type II KA receptor current responses have been distinguished in cultured hippocampal cells (Iino et al. 1990; Ozawa et al. 1991). Type II KA responses were due to a Ca^{2+} permeant form of KA receptor channels, while Type I KA responses were due to a Na^{+} permeant form of KA receptor channels. Ozawa and colleagues (1991) demonstrated that Type II KA responses were characterized by an inward rectification of the current-voltage (I-V) relationship, indicating an efflux of positive charges. These authors suggested that the associated efflux of positive charge was due to the efflux of K^{+} ions. It is likely that DOM- and KA-induced activation of low-affinity KA receptor subunits GluR5 and GluR6 located peripheral to the dorsal horn mediates a depolarizing influx of Ca^{2+}, consistent with Type II KA responses. Hence, the influx of Ca^{2+} would lead to the efflux of K^{+} (Ozawa et al. 1991).

The activation of inwardly rectifying K^{+} channels normally results in a stabilization of the membrane potential at a relatively polarized level. This action would oppose impulse propagation-induced membrane depolarization which would

ultimately lead to neurotransmitter release at a synapse (McGeer et al. 1987). The influx and cytoplasmic accumulation of Ca^{2+} has been shown to result in the opening of Ca^{2+}-dependent K^+ channels in some neural systems (Hille 1992). The efflux of K^+ mediates a slow hyperpolarization across the axonal membrane. The resulting hyperpolarization could attenuate or block axonal impulse propagation (McGeer et al. 1987).

The proposed mechanism of DOM- and KA-induced antinociception following i.t. administration is via the activation of low-affinity KA receptors located on dorsal root C-fibres allowing the influx of Ca^{2+} resulting in a Ca^{2+}-induced depolarization as measured by Agrawal and Evans (1986). Ca^{2+} would then activate Ca^{2+}-sensitive K^+ channels resulting in an efflux of K^+ ions and an associated axonal hyperpolarization. The associated decrease in axonal membrane excitability inhibits C-fibre signal propagation into the spinal dorsal horns. Such a modulation of C-fibre input to the spinal cord would then translate into antinociception, as was found following i.t. DOM and KA administration (Experiments 2 and 3; CHAPTER 3).

The proposed hypothesis of DOM- and KA-induced antinociception also predicts that enhanced nociception should occur if the activation of Ca^{2+}-sensitive K^+ channels is inhibited. The neuropeptide bradykinin, administered prior to peripheral C-fibre stimulation, has been shown to inhibit the increase of internal Ca^{2+} concentration in the dorsal roots (Ewald et al. 1989; Bleakman et al. 1990). Further, Weinreich (1986) demonstrated that in peripheral C-fibres, bradykinin mediated an inhibition of a Ca^{2+}-activated K^+ current. Given the results of the above studies,

bradykinin may enhance C-fibre membrane excitability via a mechanism which inhibits Ca^{2+}-activated K^+ channels. Inhibition of Ca^{2+}-activated K^+ channels would increase the likelihood of electrical impulses (nociceptive information) reaching the spinal dorsal horns. Consistent with this, bradykinin has been localized within dorsal root ganglion cells (Miller 1987) and has been shown to enhance nociceptive responses in thermal models of nociception (Yaksh and Hammond 1982). The above experiments demonstrated that Ca^{2+}-activated K^+ channels exist within primary afferent C-fibres and that the modulation of C^{2+}-activated K^+ channels could change a behavioral response to a noxious stimulus.

Two types of Ca^{2+}-dependent K^+ conductances have been described: a) mini-K^+ conductance, characterized by a small, voltage insensitive K^+ conductance and selectively blocked by apamin; and b) maxi-K^+ conductance, characterized by a large, voltage-sensitive K^+ conductance and selectively blocked by charybdotoxin (for review see Hille 1992). An apamin-sensitive, Ca^{2+}-activated mini-K^+ conductance has been demonstrated in the parabrachial nucleus (PBN) (Kombian et al. 1996). The iontophoretic administration of the neuropeptide somatostatin in the PBN resulted in a neuronal hyperpolarization and a decrease in the firing rate of PBN cells (Kombian et al. 1996). The observed hyperpolarization was mediated via the activation of a mini-K^+ conductance through apamin-sensitive Ca^{2+}-dependent K^+ channels (Kombian et al. 1996). It can be hypothesized that DOM and KA mediated their antinociceptive effects on spinal dorsal roots via a mechanism similar to that of somatostatin in the PBN. I.t. co-administration of DOM or KA with apamin,

charybdotoxin, or with neuropeptides present in primary afferent fibres, such as bradykinin (Steranka et al. 1988) and somatostatin (Hökfelt et al. 1976), would further elucidate the mechanisms underlying DOM- and KA-induced antinociception.

4.4 MODIFICATION OF THE GATE CONTROL THEORY

The results of Experiments 2, 3 and 4 (CHAPTER 3) suggest that the Gate Control Theory as hypothesized by Melzack and Wall (1965) should be modified to account for DOM- and KA-induced antinociception in the formalin test. A schematic for a modified Gate Control Theory is presented in FIGURE 4.1. The added connection between large diameter A-fibres and small diameter C-fibres represents a functional inhibitory connection peripheral to the spinal dorsal horns.

A-fibre volleys have been demonstrated to produce increased excitability of C-fibre afferent terminals in the spinal dorsal horn, consistent with theories of presynaptic inhibition (Calvillo 1978; Hentall and Fields 1979; Fitzgerald and Woolf 1981; Todd and Spike 1993; Yaksh 1993). Further, A-fibres have been shown to inhibit C-fibre inputs to supraspinal projection cells in the spinal cord (Fitzgerald 1981). It is, therefore, plausible that inhibitory inputs to afferent C-fibres originate from afferent A-fibres. Although C-fibre modulation by A-fibres has been frequently reported, it is possible, however, that functional inhibitory connections do exist between other small diameter fibres and KA-sensitive C-fibres.

It is interesting to speculate about a possible functional role for C-fibre KA receptors which can be activated by L-glutamate. Under conditions where noxious

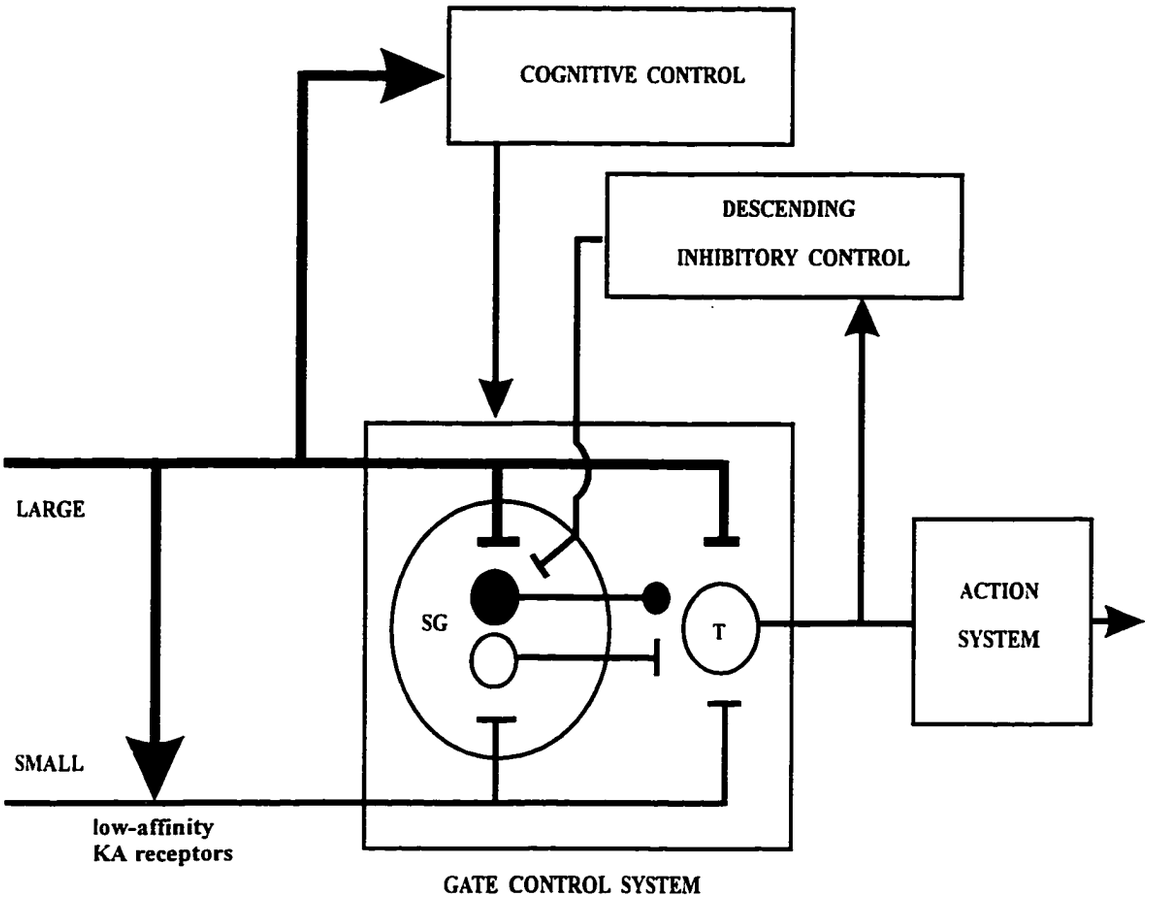


FIGURE 4.1 The Modified Gate Control Theory: The Gate Control Theory schematic of FIGURE 1.1 has been modified to include a functional inhibitory axo-axonic connection external to the spinal dorsal horns, from the large diameter myelinated afferent fibres (LARGE) to the non-myelinated C-fibre component of the small diameter myelinated and non-myelinated afferent fibres (SMALL). This model includes excitatory (white circle) and inhibitory (black circle) links from the substantia gelatinosa (SG) to the Transmission (T) cells as well as descending inhibitory control from brainstem systems. The round knob at the end of the inhibitory link implies that its action may be presynaptic, postsynaptic, or both. All connections are excitatory, except the inhibitory link from SG to T cell, and the axo-axonic connection from LARGE to SMALL (adapted from Melzack and Wall 1982).

peripheral inputs are not present, low-threshold A-fibre activity would result in the release of GLU onto KA receptors located on afferent C-fibres. The physiological effect would be blocked or limited amount of baseline C-fibre activity reaching the spinal cord. When noxious peripheral stimulation is present, the inhibitory effect of GLU on C-fibre KA receptors would be greatly diminished due to the rapidly desensitizing nature of GLU-mediated inhibition. Receptor desensitization would result in a greater number of C-fibre nociceptive inputs reaching the spinal cord and, thus, an enhanced synaptic release of neurotransmitter. However, some attenuation of C-fibre transmission would remain as a result of desensitized receptors returning to an active state. The degree of attenuation of C-fibre input into the dorsal horns might provide central processing systems with information concerning the intensity of a noxious peripheral stimulus.

4.5 SUMMARY

In conclusion, DOM- and KA-induced antinociception is postulated to be mediated via the activation of low-affinity KA receptors located on the spinal dorsal roots. DOM and KA activation of these receptors is proposed to mediate a depolarizing inward Ca^{2+} current through KA receptor activated Ca^{2+} channels. The influx of Ca^{2+} subsequently activates Ca^{2+}-dependent K^+ channels resulting in an efflux of K^+ ions, thereby decreasing C-fibre excitability and limiting the afferent transmission of nociceptive information. The blockage of C-fibre conduction by the activation of low-affinity KA receptors peripheral to the spinal dorsal horns

represents a potential target for analgesic drug development and pain research.

REFERENCES

CHAPTER 4

AGRAWAL SG, EVANS RH. The primary afferent depolarizing action of kainate in the rat. Br J Pharmacol 1986; 87: 345-355.

BAHN S, VOLK B, WISDEN W. Kainate receptor gene expression in the developing rat brain. J Neurosci 1994; 14: 5525-5547.

BETTLER B, EGEBJERG J, SHARMA G, PECHT G, HERMANS-BORGMEYER I, MOLL C, STEVENS CF, HEINEMANN S. Cloning of a putative glutamate receptor: a low affinity kainate-binding subunit. Neuron 1992; 8: 257-265.

BIRCH PJ, GROSSMAN CJ, HAYES AG. 6,7-dinitro-quinoxaline-2,3-dion and 6-nitro-7-cyano-quinoxaline-2,3-dione antagonise responses to NMDA in the rat spinal cord via an action at the strychnine-insensitive glycine receptor. Eur J Pharmacol 1988; 156: 177-180.

BLEAKMAN D, THAYER SA, GLAUM SR, MILLER RJ. Bradykinin-induced modulation of calcium signals in rat dorsal root ganglion neurons *in vitro*. Molec Pharmacol 1990; 38: 785-796.

BURNASHEV N. Recombinant ionotropic glutamate receptors: functional distinctions imparted by different subunits. Cell Physiol Biochem 1993; 3: 318-331.

CALVILLO O. Primary afferent depolarization of C fibres in the spinal cord of the rat. Can J Physiol Pharmacol 1978; 56: 154-157.

CHITTAJALLU R, VIGNES M, DEV KK, BARNES JM, COLLINGRIDGE GL, HENLEY JM. Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. Nature 1996; 379: 78-81.

CODERRE TJ, MELZACK R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J Neurosci 1992; 12: 3665-3670.

CURTIS DR, PHILLIS JW, WATKINS JC. Chemical excitation of spinal neurones. Nature (Lond) 1959; 183: 611-612.

DAVIES J, EVANS RH, FRANCIS AA, WATKINS JC. Excitatory amino acid receptors and synaptic excitation in the mammalian central nervous system. J Physiol (Paris) 1979; 75: 641-654.

DELANDER GE, WAHL JJ. Behavior induced by putative nociceptive neurotransmitters is inhibited by adenosine or adenosine analogs coadministered intrathecally. J Pharmacol Exp Ther 1988; 246: 565-570.

EGEBJERG J, BETTLER B, HERMANS-BORGMEYER I, HEINEMANN S. Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. Nature 1991; 351: 745-748.

EVANS RH. Kainate sensitivity of C-fibres in rat dorsal roots. J Physiol 1985; 358: 42P.

EVANS RH, EVANS SJ, POOK PC, SUNTER DC. A comparison of excitatory amino acid antagonists acting at primary afferent C fibres and motoneurones of the isolated spinal cord of the rat. Br J Pharmacol 1987; 91: 531-537.

EWALD DA, STERNWEIS PC, PANG IH, MILLER RJ. Differential G-protein mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurons *in vitro*. Neuron 1989; 3: 1185-1193.

FITZGERALD M. A study of the cutaneous afferent input into the substantia gelatinosa. Neurosci 1981; 6: 2229-2237.

FITZGERALD M, WOOLF CJ. Effects of cutaneous nerve and intraspinal conditioning on C-fibre afferent terminal excitability in decerebrate spinal rats. J Physiol 1981; 318: 25-39.

HARPER AA, LAWSON SN. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. J Physiol 1985; 359: 31-46.

HENTALL ID, FIELDS HL. Segmental and descending influences on single intraspinal C-fibres. J Neurophysiol 1979; 42: 1527-1537.

HEUTTNER JE. Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockage of desensitization by Con A. Neuron 1990; 5: 255-266.

HILLE B. Ionic channels of excitable membranes (2nd Edition). Sunderland MA: Sinauer, 1992.

HÖKFELT T, ELDE R, JOHANSSON O, LUFT R, NILSSON G, ARIMURA A. Immunocytochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. Neurosci 1976; 1: 131-136.

HORNFELDT CS, LARSON AA. Selective inhibition of excitatory amino acids by divalent cations. A novel means for distinguishing N-methyl-D-aspartic acid-, kainate-, and quisqualate-mediated actions in the mouse spinal cord. J Pharmacol Exp Ther 1989; 251: 1064-1068.

IINO M, OZAWA S, TSUZUKI K. Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. J Physiol 1990; 424: 151-165.

ISHIDA M, SHINOZAKI H. Novel kainate derivatives: potent depolarizing actions on spinal motoneurons and dorsal root fibres in newborn rats. Br J Pharmacol 1991; 104: 873-878.

KÖHLER M, BURNASHEV N, SACKMANN B, SEEBURG PH. Determinants of Ca^{2+} permeability in both TM1 and TM2 of high-affinity kainate receptor channels: diversity by RNA editing. Neuron 1993; 10: 491-500.

KOMBIAN SB, SALEH TM, ZIDICHOUSKI JA, PITTMAN QJ. Somatostatin activates an apamin-sensitive potassium conductance in parabrachial neurons *in vitro*. Soc Neurosci Abst 1996; 22: 89.

MACDONALD JF, PORIETIS AV. DL-Quisqualic and L-Aspartic acids activate separate excitatory conductances in cultured spinal cord neurons. Brain Res 1982; 245: 175-178.

MAYER ML, VLYKLICKI L. Concanavalin A selectively reduces desensitization of mammalian neuronal quisqualate receptors. Proc Nat Acad Sci (USA) 1989; 86: 1411-1415.

MAYER ML, WESTBROOK GL. The physiology of excitatory amino acids in the vertebrate nervous system. Prog Neurobiol 1987; 28: 2639-2650

MCGEER PL, ECCLES JC, MCGEER EG. Molecular neurobiology of the mammalian brain (2nd Edition). New York: Plenum Press, 1987.

MELZACK R, WALL PD. The challenge of pain. Middlesex, Eng: Penguin, 1982: 223-239.

MELZACK R, WALL PD. Pain mechanisms: A new theory. Science 1965; 150: 971-979.

MILLER RJ. Bradykinin highlights the role of phospholipid metabolism in the control of nerve excitability. Trends Neurosci 1987; 10: 226-228.

OZAWA S, IINO M, TSUZUKI K. Two types of kainate response in cultured rat hippocampal neurons. J Neurophysiol 1991; 66: 2-11.

PETRALIA RS, WANG YX, WENTHOLD RJ. Histological and ultrastructural localization of the kainate receptor subunits, KA2 and GluR6/7 in the rat nervous system using selective antipeptide antibodies. J Comp Neurol 1994; 349: 85-110.

PRUSS RM, AKESON RL, RACKE MM, WILBURN JL. Agonist-activated cobalt uptake identifies divalent cation-permeable kainate receptors on neurons and glial cells. Neuron 1991; 7: 509-518.

SCHNEIDER SP, PERL ER. Selective excitation of neurons in the mammalian spinal dorsal horn by aspartate and glutamate in vitro: correlation with localization and excitatory input. Brain Res 1985; 360: 339-343.

SEEBURG PH. The molecular biology of mammalian glutamate receptor channels. Trends Neurosci 1993; 16: 359-365.

SHINOZAKI H, ISHIDA M, KWAK S, NAKAJIMA T. Use of Acromelic Acid for production of rat spinal lesions. Meth Neurosci 1991; 7: 38-57.

SKILLING SR, SMULLIN DH, BEITZ AJ, LARSON AA. Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following vertridine and nociceptive stimulation. J Neurochem 1988; 51: 127-132.

SOMMER B, SEEBURG PH. Glutamate receptor channels: novel properties and new clones. TIPS 1992; 13: 291-296.

SOMMER B, BURNASHEV N, VERDOORN TA, KAINÄNAN K, SAKMANN B, SEEBURG PH. A glutamate receptor channel with high affinity for domoate and kainate. EMBO 1992; 11: 1651-1656.

STERANKA LR, MANNING DC, DEHAAS CJ, FERKANY JW, BOROSKY SA, CONNOR JR, VAVREK RJ, STEWART JM, SNYDER SH. Brakykinin as a pain mediator: receptors are localized to sensory neurons, and antagonists have analgesic actions. Proc Nat Acad Sci 1988; 85: 3245.

TODD AJ, SPIKE RC. The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog Neurobiol 1993; 41: 609-645.

TRUSSELL LO, THIO LL, ZORUMSKI CF, FISCHBACH GD. Rapid desensitization of glutamate receptors in vertebrate central neurons. Proc Nat Acad Sci (USA) 1988; 85: 2834-2838.

WEINREICH D. Bradykinin inhibits a slow spike after-hyperpolarization in visceral sensory neurons. Eur J Pharmacol 1986; 132: 61-63.

YAKSH TL. New horizons in our understanding of the spinal pyhsiology and pharmacology of pain processing. Seminars Oncology 1993; 20: 6-18.

YAKSH TL, HAMMOND DL. Peripheral and central substrates involved in the rostrad transmission of nociceptive information. Pain 1982; 26: 434-481.