STUDIES ON THE INVOLVEMENT OF ENKEPHALIN IN STIMULATION PRODUCED ANALGESIA

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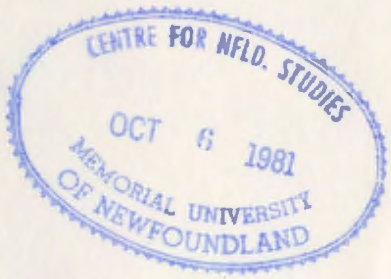
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STUDIES ON THE INVOLVEMENT OF ENKEPHALIN IN STIMULATION PRODUCED ANALGESIA

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ABSTRACT

Similarities between stimulation and opiate analgesia, together with the recent identification of central nervous system opiate receptors and endogenous opiate peptides has led to the proposal that periaqueductal gray (PAG) endorphins modulate pain perception. The specific hypothesis that PAG stimulation induces analgesia by the release of enkephalin was evaluated in rats using a specific competitive enkephalinase inhibitor (Gly-Gly-Phe-Met).

In three studies the enkephalinase inhibitor was administered intracerebrally two millemeters caudal to an analgesia producing stimulation electrode in the PAG. Analgesia was assessed at two minute intervals using the tail flick test. Study I demonstrated that the enkephalinase inhibitor potentiated in intensity and duration the analgesic action of submaximal PAG stimulation in a naloxone reversible manner. In Study II the potentiation effect on stimulation produced analgesia was shown to be directly dependent on enkephalinase inhibitor dose and was completely reversed by naloxone. In Study III using specific norepinephrine (6-OHDA) and serotonin (5, 7-DHT) neurotoxins a serotonergic mechanism was shown to mediate enkephalinase inhibitor action. Additionally in these studies administration of enkephalinase inhibitor in the absence of stimulation was found to produce an attenuated naloxone reversible, dose dependent analgesia. This antinociceptive action was also abolished by a spinal serotonin lesion.

It was concluded that enkephalin within the PAG was released by stimulation and activated opiate receptors thereby producing analgesia. Thus endogenous enkephalin modulates the perception of pain.

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Pain is a subjective experience which is commonly correlated with noxious stimulation. Due to the subjective nature of pain phenomena both scientific experimentation and clinical therapy have remained at a primitive stage. The stimuli inducing many intractable pain states are still unknown as is the mode by which noxious stimuli express themselves in the central nervous system (CNS). Pain is most commonly combated with opium derivatives, a group of drugs which has been used for several millenia. Only within the past decade has the molecular action of opiates been elucidated. Recent knowledge gained from opiate and related research has opened a new era in the scientific study of the perception of pain.

Ascending Spinal Pathways Involved in the Perception of Pain

The receptor (nociceptor), the activation of which induces the sensation of pain, has not been morphologically defined (Iggo, 1976). Physiologically nociceptors are preferentially sensitive to noxious stimuli, demonstrating a high threshold to natural stimuli or an increased response to stimuli increasing in intensity, and often undergoing sensitization (Perl, 1976). Activation of nociceptors by noxious stimuli excites fine A delta or C type (unmyelinated) peripheral sensory fibers (Zotterman, 1939). Neural activity in these fibers has been correlated with the perception of pain (Van Hees, 1976); however the specific sensations represented by the activity of the two types of fiber have not been differentiated (Dubner and Beitel, 1976) (Price, 1972). Noxious information carried by A delta and C fibers is transmitted to cells located in several laminae of the dorsal horn of the

spinal cord. Christensen and Perl (1970) demonstrated that a significant proportion of lamina (L) I cells are driven specifically by mechanical and thermal stimuli in the noxious range. Kaumazawa and Perl (1977) proposed and Light, Perl and Trevino (1979) demonstrated that A delta fibers are responsible for the stimulation of the marginal zone (L I) while the substantia gelatinosa (L II) is stimulated by nociceptive and thermoceptive C fibers. Lamina V (spreading into L IV and VI in most physiological studies) contains polymodal neurons many of which are stimulated maximally by noxious stimuli (Price and Mayer, 1974). Mayer et al (1975) have proposed that activation of lamina V neurons is sufficient to induce pain in man and primate. Finally, neurons responding maximally to noxious stimulation have been observed as far ventrally as lamina VII and VIII (Trevino and Carsten, 1975).

Classically, noxious information was thought to ascend from cell bodies of the spinal gray to the brain via two crossed ventrolateral spinal pathways. However, in recent studies nociceptive stimulation has been demonstrated to be transmitted rostrally via six spinal tracts. Table 1 presents a summary of spinal tracts conveying noxious stimulation, including the location of cell bodies and, where possible, the loci of terminal fields. The spinothalamic tract is a contralateral fiber system located in the ventrolateral spinal cord which is commonly related to nociception. This tract may be subdivided into the neospinothalamic tract (a) and the paleospinothalamic tract (b). It has been demonstrated

TABLE 1

Ascending Spinal Tracts Transmitting Noxious Stimulation

Spinal Tract	Lamina of Origin	Terminal Field	
Ventral			
Contralateral			
a) Neo-Spinothalamic	I, II, V	Lateral Thalamus (Ventrobasal, Posterior Subthalamic Nuclei)	
b) Paleospinothalamic	VII, VIII	Medial and Intralaminar Thalamic Nuclei	
Ipsilateral			
c) Spinoreticular	VII, VIII	Brainstem Reticular Formation, Central Gray	
Dorsal			
Ipsilateral			
d) Spinocervical	V	Lateral Cervical Nucleus	Lateral Thalamus
e) Dorsal Column	V	Rostral Dorsal Column Nucleus	Lateral Thalamus
Dorsal and Ventral			
f) Propriospinal	VII, VIII	Rostral Spinal Cord; Possibly Cells of Tracts b and c	

that the neospinothalamic system originates in cells of lamina I, II and V while the paleospinothalamic tract originates in lamina VII and VIII (Trevino and Carstens, 1975). The former system terminates in the lateral thalamus (including ventrobasal, posterior and subthalamic nuclei) while the phylogenetically older system projects to midline and intralaminar thalamic nuclei (Dennis and Melzack, 1977).

The reticulospinal tract (c) is an uncrossed ventral system arising from cells of lamina VII and VIII and projecting to the brainstem reticular formation and central gray (Kerr and Lippman, 1973). This system is larger than the paleospinothalamic tract but is multisynaptic and lacks somatotopic representation (Bell, 1964).

The spinocervical tract (d) courses rostrally in the lateral cord synapsing in the lateral cervical nuclei. This uncrossed tract is especially prominent in the feline but is diminished in the primate possibly due to the dominance of the neospinothalamic tract (Albe-Fessard et al, 1974). The ventral aspect of the dorsal column is also capable of transmitting noxious information. The dorsal column post synaptic tract (e) relays in the rostral dorsal column nuclei and projects with the spinocervical fibers via the medial lemniscus to nuclei of the neospinothalamic tract. The cells of origin of nociceptive spinal cervical and dorsal column post synaptic tract are found in lamina V. Finally the diffuse multisynaptic propriospinal system (f) may transmit noxious information to more rostral sites within the cord (Fields et al,

1970; Hancock, 1973). The fibers of the propriospinal system arise from cells located in the ventral cord (L VII and VIII) and may influence cells of the spinoreticular tract and the paleospinothalamic tract thereby exerting a cerebral representation explaining the presence of noxious sensation following lesion of spinal axons projecting directly to supraspinal sites (Basbaum, 1973).

In conclusion noxious stimuli activate nociceptors which transmit input via A delta and C fibers of the peripheral nervous system to spinal cells of lamina I, II, V, VII and VIII. The spinal cells of lamina I, II send noxious information via axons in the neospinothalamic tract and cells of lamina V via the neospinothalamic, the spinocervical and the dorsal column post synaptic tract to the lateral thalamic nuclei. The paleospinothalamic and spinoreticular tracts arise from ventral lamina (VII and VIII) and terminate in medial thalamic and reticular sites respectively. Finally the propriospinal system relays noxious information from ventral lamina to rostral cells of the ventral tracts.

Opiate Analgesia: Locus and Mechanism of Action

Opiate derivative drugs have classically been considered to eliminate the perception of pain primarily by inhibiting the transmission of noxious input to cerebral and thalamic sensory fields (Jaffe and Martin, 1975). Lim et al (1964) first demonstrated that the site of the antinociceptive action of opiate compounds is the CNS. The most effective

sites of analgesic action have been found in the medial brainstem including the periventricular gray (Tsou and Tang, 1964) and the periaqueductal gray (PAG) (Herz and Teschemacher, 1971). Although other sites in the brainstem including the floor of the IV ventricle (Teschemacher et al, 1973), the ventral surface of the brainstem (Dey and Feldberg, 1976), the nucleus reticularis paragigantocellularis (NRPG) (Akaike et al, 1978), and the nucleus raphe magnus (NRM) (Dickenson et al, 1979) have been shown to be sensitive to the analgesic effects of microinjection of morphine, the PAG is the locus from which analgesia is most consistently reported (Yaksh et al, 1976). Discrete microinjection of morphine (5 microgram/0.5 microliter) into the PAG repeatedly produces naloxone reversible analgesia equivalent to high doses of systemically administered opiate agonists (morphine 10 mg/kg) (Yaksh et al, 1976). Yaksh and Rudy (1977) have also demonstrated a direct spinal analgesic action of morphine. However the reversal of the antinociceptive action of systemically administered morphine by naloxone microinjection to the PAG (Yaksh and Rudy, 1974) or by PAG lesion (Dostrovsky and Deakin, 1977) demonstrates the importance of a supraspinal locus of opiate analgesia.

The mechanism by which opiates induce analgesia from PAG loci has been studied under two hypotheses: (1) a direct inhibition of neural components of the nociceptive system as they course through the PAG or (2) an excitation of a PAG originating inhibitory pathway. The former hypothesis

of a direct inhibition of ascending noxious input has been studied by attempts to mimic the proposed depressant action of morphine with discrete brainstem lesion and local anaesthetic application. Kelly and Glusman (1968) and Liebman et al (1970) found slight decreases in pain threshold and increases in pain reactivity following electrolytic lesion of the PAG, as did Yaksh et al (1975) following microinjection of local anaesthetics. Therefore, since physiological or pharmacological manipulations which directly attenuate neuronal transmission fail to increase pain threshold, a mechanism by which opiates directly inhibit ascending fibers in the PAG appears untenable.

On the other hand, the hypothesis of an opiate excited PAG originating inhibitory pathway has withstood experimental study. Morphine administered iontophoretically has been demonstrated to excite cells of the PAG (Frederickson and Norris, 1976; Gent and Wolstencroft, 1976; Bradley and Bramwell, 1975). Systemically and intraventricularly administered morphine produces a naloxone reversible excitation of multiunit activity within the PAG (Cannon et al, 1978). Recent evidence that iontophoretically applied morphine excites cells of the NRPG (Satoh et al, 1979) and the NRM (Anderson, et al, 1978) supports proposals that these nuclei also participate in morphine analgesia. Irwin et al (1951) first postulated a bulbospinal mechanism of morphine analgesia following the demonstration of reduced antinociceptive potency of opiate agonists in spinal animals. Satoh and Takagi (1971) found that high spinal transection blocked the inhibitory effects

of morphine on pain evoked potentials in the cord. Recently specific lesions of the dorsolateral funiculus (DLF) have been shown to attenuate the analgesic action of PAG administered morphine (Murfin, 1976) and to reduce the potency of systemically administered morphine (Basbaum et al, 1977, Hayes et al, 1978). On the basis of these findings the presently accepted mechanism of opiate induced analgesia is an excitation of PAG neurons which activate inhibitory fibers descending in the DLF thereby attenuating noxious input within the spinal cord. (For reviews see Basbaum 1976; Fields and Basbaum, 1978; Yaksh and Rudy, 1978; Mayer and Price, 1976).

Alternatives to morphine analgesia have been sought in hopes of reducing the pharmacological shortcomings of tolerance, dependency and sedative side effects present in all opiate derivatives. Although a more efficient pharmacological agent has not been formulated, a physiological manipulation has been demonstrated to be effective in elimination of pain with a minimum of side effects. Stimulation of specific brainstem loci is a potent therapeutic means of dealing with a majority of pain patients (Akil, personal communication).

Stimulation Produced Analgesia

Focal electrical stimulation induced analgesia was first demonstrated by Reynolds (1969). Laparotomy was performed on the undistressed rat in the absence of chemical anaesthesia. Efficacious sites centered around the dorsolateral PAG, the stimulation of which produced analgesia in the absence of

sedation while retaining normal reactivity to non-noxious stimuli. Subsequently stimulation produced analgesia (SPA) has been observed in the rat (Mayer and Liebeskind, 1974; Mayer et al, 1971; Akil and Liebeskind, 1975), cat (Melzack and Melinkoff, 1974; Oliveras et al, 1974), monkey (Ruda et al, 1976; Goodman and Holcombe, 1976) and man (Adam, 1976; Boethius et al, 1978; Gybels and Cosyns, 1978; Hosobuchi et al, 1977; Richardson and Akil, 1977; Akil et al, 1978 a and b; Hosobuchi et al, 1979). Analgesia in some cases has been equivalent to 50 mg/kg of morphine, a dose which produces significant side effect and sedation not seen in SPA (Mayer and Liebeskind, 1974). Mayer et al (1971) have described cases in which PAG stimulation removed all behavioural signs of pain to tail pinch regardless of the force applied. The temporal relations of stimulation to analgesia are still obscure. Analgesia is usually evoked after a few seconds of stimulation and continues for several minutes to hours following current termination (Liebeskind, et al, 1978). In this regard SPA appears to be extremely effective in man, as five minutes of stimulation can produce 24 hours of relief from previously intractable pain (Akil et al, 1978). The locus of stimulation is an important parameter in SPA. Original attempts at SPA used electrodes sited in the septum (Gol, 1976). This site is associated with many undesirable side effects and has been shown to be related to

analgesia only after epileptic seizure activity (Abbott and Melzack, 1978). Brain areas which support self-stimulation behaviour such as the lateral hypothalamus have been shown to induce analgesia in some cases (Mayer et al, 1971); however this finding has not been consistently replicated (Mayer and Liebeskind, 1974) and there is not a complete correlation between self-stimulation supporting sites and SPA inducing brain loci (Liebeskind et al, 1978). Analgesia is consistently observed upon stimulation of the PAG region, extending from the dorsal raphe to the paraventricular region of the caudal thalamus and posterior hypothalamus (Mayer and Liebeskind, 1974; Rhodes and Liebeskind, 1978). In man, rostral sites produce excellent analgesia and reduce the intensity of side effects (Akil, 1978a).

In the rat, stimulation of specific PAG loci may induce analgesia restricted to portions of the body while leaving other areas unaffected (Balagura and Ralph, 1973, Mayer et al, 1971). It has been postulated that a somatotopic representation may exist within the PAG which would account for analgesia observed in a specific locus (Yaksh et al, 1975). Recently it has been shown that stimulation of two other brainstem loci induces analgesia similar to that observed following PAG stimulation. These two sites are the NRPG (Akaike et al, 1978) and the NRM (Oliveras et al, 1978).

Attempts to determine the mechanism by which stimulation of the PAG induces analgesia have tested hypotheses identical to those formulated to account for the mechanism of opiate analgesia. Thus stimulation may produce a functional lesion

thereby directly inhibiting the transmission of noxious information through the PAG or stimulation may excite cells in the PAG which activate distal neurons which are inhibitory on nociceptive pathways. As mentioned above, electrolytic lesions (Bevan and Pert, 1975) and microinjection of local anaesthetics (Yaksh,1975; Rhodes, 1975) to the PAG induces a state of hyper-reactivity to noxious stimuli thereby discounting the hypothesis of direct inhibition. Supportive evidence for the second hypothesis is the wide yet specific extent of SPA action.

Stimulation produced analgesia has been demonstrated to inhibit the nociceptive responses of neurons from the spinal cord to the thalamus. Stimulation of the ventrocaudal PAG of the cat has been found to suppress responding of wide range dynamic neurons in lamina V to noxious stimulation but not that of cells in lamina IV to light touch (Liebeskind et al, 1973; Oliveras et al, 1974; Carstens et al, 1979). Morrow and Casey (1976) demonstrated a specific block of noxious stimulation induced activity in cells of the nucleus gigantocellularis reticularis following PAG stimulation. Similarly multiple unit responses in the ventrobasal thalamus are attenuated by SPA while non-noxious responses are augmented (Oleson and Liebeskind, 1976). Finally Basbaum et al (1977) demonstrated that lesions of the DLF abolish analgesia induced by PAG stimulation. Therefore it has been hypothesized that SPA acts by stimulating cells of the PAG which activate spinal tracts which are selectively inhibitory to nociceptive cells of the spinal gray receiving noxious stimuli (Fields

and Basbaum, 1978).

The Relationship Between Opiate and Stimulation Analgesia

As a result of the similarities in locus and hypothesized mechanism of action, comparisons between opiate and stimulation produced analgesia have become an active topic in scientific research (Cannon et al, 1978). The first direct study of a common mechanism of opiate and stimulation produced analgesia was performed by Akil and colleagues (1972) who demonstrated that the opiate antagonist, naloxone reversed SPA in the rat. Subsequently laboratory studies of the inhibitory action of naloxone on SPA in the rat have produced mixed results probably due to methodological differences. Yaksh et al (1976) found naloxone ineffective in reversing SPA during stimulation, while Pert and Walter (1976) observed a partial reversal of analgesia by naloxone following stimulation at current levels considerably higher than those normally used to produce SPA. Akil et al (1976) replicated the demonstration of significant reduction of post stimulation analgesia by naloxone. Clinical studies using human patients have found naloxone completely reverses stimulation produced analgesia (Akil et al, 1978a, Adams, 1976; Hosobuchi et al, 1977, 1979). Additionally, Mayer and Hayes (1975) have demonstrated the development of tolerance to repeated SPA trials and a cross tolerance to morphine. Two studies, (Lewis and Gehart, 1977; Yeung et al, 1977) have extensively examined the loci which are effective in producing SPA and opiate analgesia. Both these studies found analgesia can be induced from identical sites within the PAG although some loci tested were sensitive to only one manipulation. It has been concluded that until the histology of PAG is

further investigated and stimulation techniques refined, the coincidence of morphine and SPA sensitive sites will remain only partially comprehended.

A further similarity between stimulation and opiate analgesia derived from the PAG is a spinal mechanism involving a descending bulbospinal serotonergic pathway inhibitory on nociceptive neurons of the spinal gray.

Common Involvement of Serotonin

It has been proposed that morphine analgesia and SPA share a common bulbospinal pathway (Basbaum et al, 1976). The PAG receives fibers from the anterolateral quadrant of the spinal cord (Mehler et al, 1969) and has been shown to receive fibers involved in nociception (Becker et al, 1969, Liebeskind and Mayer, 1971). However the PAG does not project directly into the spinal cord. Ruda (1975) using tritiated amino acid techniques and Gallager et al (1978) using microiontophoresis of horseradish peroxidase have demonstrated a prominent anatomical connection from the PAG to the nucleus raphe magnus. The NRM is a serotonin (5-HT) containing nucleus that projects via the dorsolateral funiculus (DLF) to lamina I and V of the spinal cord (Basbaum et al, 1977b). The evidence for a physiological link between the PAG and the NRM is provided by studies in which cellular excitants such as glutamate and tetanus toxin applied to the PAG stimulate cells of the NRM while inducing analgesia (Cannon et al, 1978; Urca 1978; Urca et al, 1977). Similarly systemic (Urca et al, 1977; Oleson et al, 1978) and PAG (Fields and Anderson, 1978) injection of morphine excites in a naloxone reversible manner multi-unit activity and single cell activity

within the NRM.

Lesions of the NRM reduce morphine analgesia from both systemic (Proudfit and Anderson, 1975; Yaksh et al, 1977; Basbaum et al, 1977a) and PAG administration (Murfin et al, 1976). Stimulation of the NRM alone produces potent analgesia (Oliveras et al, 1975; Oleson and Liebeskind, 1975; Proudfit and Anderson, 1975). Stimulation produced analgesia from the NRM has been shown to specifically inhibit the response of spinal cells (McCreery et al, 1979; Beall et al, 1976; Fields et al, 1977) and the response of neurons of the spinothalamic tract (Willis et al, 1977) to noxious stimuli.

Manipulation of serotonin levels in the brainstem and spinal cord have been demonstrated to influence reactivity to noxious stimuli and influence the analgesic action of morphine and electrical stimulation of the PAG (see Messing and Lytle, 1977 for review). Specifically, serotonin depletion with 5, 6-Dihydroxytryptamine or p-Chlorophenylalanine (PCPA), or receptor blockade with methysergide, attenuates morphine analgesia, while administration of the re-uptake blocking drug, fluoxatine, enhances morphine analgesia (Vogt, 1974; Cheney and Goldstein, 1971; Yaksh et al, 1976; Duncan and Spencer, 1973). Yaksh and Tyce (1979) have demonstrated that antinociception induced by PAG administered morphine is correlated with a significant increase in spinal 5-HT release, and is inhibited by the intrathecal administration of methysergide (Yaksh, 1979). Similarly, 5-HT receptor blockade with LSD, and 5-HT depletion with PCPA, reduces the analgesia induced by PAG stimulation (Akil and Mayer, 1977; Guilbaud et al, 1973; Hayes et al, 1976). Interestingly,

tolerance to SPA following repeated stimulation of the NRM can be reversed by administration of the 5-HT precursor, 5-hydroxytryptophan (Oliveras et al, 1977). Further, intrathecally administered 5-HT produces significant analgesia which is potentiated by fluoxetine and inhibited by methysergide (Yaksh and Wilson, 1979). Finally iontophoretically applied 5-HT inhibits nociceptive spinal neurons of lamina I and IV (Randic and Yu, 1976; Duggan and Headley, 1978; Belcher, 1978). On this basis it has been hypothesized that stimulation of, and morphine administration to, the PAG acts to inhibit nociception, by a serotonergic pathway. Activation of the PAG, by these manipulations, stimulates the serotonergic cells of the NRM. The axons of these cells descend in the DLF, terminating in laminae which contain nociceptive neurons. The release of 5-HT, from NRM originating axons, specifically inhibits the response of spinal gray cells to noxious stimulation. Therefore PAG induced activation of NRM spinal serotonergic cells reduces nociception (Fields and Basbaum, 1978).

The physiological significance of a pain attenuating system which is activated by electrical stimulation or exogenous opiate derivatives must be considered. Takagi et al (1955) suggested that descending inhibition of noxious input may be part of a feedback loop that is activated in the brainstem by ascending noxious information. Postulating that the PAG is the initiation center of the descending component of this loop, the effect of PAG excitation by electrical stimulation is reasonable. However, the role of opiates in this antinociceptive mechanism is less obvious, but has begun to be

understood concomitantly with the isolation of CNS opiate receptors and endorphins.

Endorphins

Stereospecific opiate receptors in brain and gut nerve plexus tissue were first identified in 1973 (Pert and Snyder, 1973; Simon et al, 1973; Terenius, 1973). The CNS location of stereospecific receptors for opiates is similar in primate and rodent. The amygdala, hypothalamus, caudate and PAG demonstrate the highest concentrations of receptors in the brain while the substantia gelatinosa possesses the highest concentration of opiate receptors in the spinal cord. (Kuhar et al, 1973; Hiller et al, 1973; Pert et al, 1973). Endogenous peptide ligands to this receptor were identified in the brain (Hughes, 1975; Pasternak et al, 1975) and pituitary gland (Cox et al, 1976) (for reviews, see Golstein, A., 1976; Hughes and Kosterlitz, 1977; and Snyder and Simantov, 1977). Several opiate peptides with an N terminal of the amino acids tyrosine, glycine, glycine, phenylalanine, identical to α-lipotropine (α-LPH) (61-64) are collectively termed endorphins. Met and Leu enkephalin are pentapeptides with methionine and leucine C terminal amino acids respectively Hughes et al, 1975). β-endorphin, (α-LPH 61-91), found concomitant with ACTH, is confined to cells of the basal tuberal hypothalamus (Watson et al, 1978; Bloom et al,1978). Met and Leu enkephalin is found more extensively throughout the brain and spinal cord with a distribution similar to that of opiate receptors including the amygdala, corpus striatum, PAG and reticular formation (Sar et al, 1978).

Consistent with the role of endogenous opiates, endorphins mimic morphine pharmacologically in an ability to inhibit electrically induced contrations of mouse vas deferens and guinea pig ileum, and inhibit ^{3}H naloxone receptor binding (Hughes, 1976). Enkephalins are released by potassium and electrical stimulation in a calcium ion dependent manner from brain slices and synaptosomal fractions (Hughes, 1978). Enkephalin demonstrates an ability to depress the activity of specific neurons in a naloxone reversible manner following iontophoretic application. Cells sensitive to the depressant action of enkephalin are also inhibited by morphine (Frederickson and Norris, 1976; Gent and Wolstencroft, 1976; Henry, 1976). Cells which demonstrate excitation following morphine application also demonstrate enkephalin induced excitation. These include hippocampal pyramidal cells, Renshaw cells, cells of the PAG (Gent and Wolstencroft, 1976, Frederickson and Norris, 1976; Davies and Dray, 1976; Nicoll et al, 1977) and neurons in the NRPC (Satoh et al, 1979). Interestingly, microinjection of enkephalin into the PAG has been demonstated to stimulate multi-unit activity in the NRM (Urca et al, 1977). Enkephalin is rapidly broken down by peptidases while β-endorphin is more slowly catabolized. Despite the lack of information concerning synthesis, enkephalin has been proposed to act as a CNS neuromodulator (Hughes, 1978).

The role of the endogenous opiate peptide enkephalin in analgesia has been proposed due to the pharmacological similarities of enkephalin to morphine as described above and due to the high concentration of enkephalin containing cell bodies and terminals in the PAG, the site at which morphine induces

an analgesic action. It has therefore been hypothesized that enkephalin acts as a neuromodulator in the PAG to activate descending pathways inhibitory on nociception, ultimately modulating the perception of pain. The excitation of PAG neurons by iontophoretic application of enkephalin is an important step in the demonstration of enkephalin involvement in analgesia but a study on the cellular level fails to demonstrate the behavioural aspects which are critically required in a study of nociceptive modulation.

A study of the involvement of an endogenous compound in a behaviour attempts to demonstrate, using a minimum of extraneous and nonspecific manipulations, that the compound acts at a specific receptor site to modify the behaviour in the whole animal. In pain research it is common to study the effect of a pharmacological or physiological manipulation on the response to stimuli that have consistently been demonstrated noxious in the species studied. Pharmacological manipulations used in studies of the modulation of nociception are aimed at altering synaptic transmission in circuits responsible for the modifying of pain perception. Physiological manipulations attempt to selectively activate axons of pathways involved in the transmission or inhibition of noxious stimulation. The conclusions which a study can formulate about a mechanism underlying a behaviour are limited by the specificity of the manipulation utilized. Despite the attractiveness of the hypothesis of an endogenous opiate peptide modulating nociception by a mechanism similar to that described for opiate analgesia there is a dearth of direct experimental

evidence in support of a role of enkephalin in antinociception.

Opiate peptides exogenously administered intracerebrally (to PAG) or intraventricularly have been demonstrated to induce naloxone reversible analgesia in rodents (Bucher et al, 1976; Belluzzi et al, 1976, Malick and Goldstein, 1977). The analgesic potency of β-endorphin similarly administered is significantly greater than that of enkephalin despite an equality of 'in vitro' receptor binding affinity (Feldberg and Smith, 1976; Loh et al, 1976). This discrepancy of 'in vivo' potency may be accounted for by the rapid catabolism of enkephalin in the brain (Hambrook et al, 1976; Meek et al, 1977). These studies, using exogenously administered peptides, do not demonstrate release of enkephalin to sites responsible for antinociception. The similarity of pharmacological action between enkephalin and morphine at the cellular level may account for the observed analgesia which is the action of enkephalin on opiate receptors normally not receiving enkephalin from endogenous sources.

The criticism of failure to demonstrate enkephalin release in studies using receptor agonists is eliminated in studies of pain perception following opiate antagonist administration. In studies of this nature it is hypothesized that if enkephalin inhibits nociception, receptor blockade with naloxone, the most specific opiate antagonist, will decrease the pain threshold. Naloxone has been demonstrated to lower the pain threshold in several studies using the hot plate as test of analgesia. (Jacob et al, 1974; Jacob and Ramabadran, 1978; Grevert and Goldstein, 1977). However, other studies using the tail flick test (Yaksh, Yeung and Rudy, 1976; Goldstein et al, 1976) and

the formalin test (North, 1978) have failed to demonstrate a hyperalgesic action of naloxone. The lack of opiate antagonistic effect in some laboratories may be a result of diurnal fluctuation in CNS enkephalin concentration, which alters the effect of naloxone, when compared to saline, administration in the hot plate test (Frederickson et al, 1976) and is correlated with nociceptive sensitivity (Wesche and Frederickson, 1979). Recently naloxone has been demonstrated to lower pain threshold in post-operative patients in a dose dependent manner implicating an action of endorphins in post surgical trauma (Levine et al, 1979). The systemic administration of naloxone used as a pharmacological manipulation in above cited studies does not provide the specificity of location required to demonstrate an action of PAG enkephalin in antinociception. Naloxone exhibits affinities for all opiate receptors in the body and inhibits the action of β-endorphin in addition to enkephalin. Therefore despite the demonstration of naloxone induced hyperalgesia these studies of opiate antagonist action fail to fortify the hypothesis of PAG enkephalin modulation of nociception.

Another means by which the importance of endorphins in pain modulation can be studied is by an inhibition of peptide catabolism. Patthy and collaborators (1977) demonstrated a significant increase in pain threshold in the tail flick test following intraventricular administration of bacitracin. The duration of action of bacitracin in this test was related to the duration of inhibition of enkephalin catabolism but not of β-endorphin catabolism (Miller et al, 1977;

Smyth et al, 1978). This study suggests that the inhibition of enkephalin catabolism decreases the perception of pain. Again the pharmacological manipulation did not localize the site of antinociceptive action as the agent was administered intraventricularly. The other major criticism of this experiment is the non-specific action of bacitracin on many brain peptidases which may act on other peptides involved in the response required by the analgesic test.

The use of opiate antagonists and catabolic inhibitors in a demonstration of enkephalin involvement in modulation of nociception is dependent upon the assumption that enkephalins are released phasically in response to noxious stimuli or stress. A phasic release of enkephalin by stress has been demonstrated by Wesche and Frederickson (1979) and release by noxious stimulation is implied in the study by Levine et al (1979). The lack of consistent results in studies using opiate antagonists as a pharmacological manipulation may be due to an inconsistent control of enkephalin release in the PAG due to variances in procedure of analgesic testing.

Stimulation of enkephalin release in the loci in which enkephalins are hypothesized to modulate pain perception is a physiological means by which enkephalin mediation of antinociception can be studied. Electrical stimulation has been demonstrated to release enkephalin 'in vitro' from brain slices (Hughes, 1978) and can therefore be used to activate release of enkephalin from 'in vivo' sites containing high concentrations of opiate receptors and enkephalin cell bodies

and terminals such as the PAG (Uhl et al, 1979). As discussed above, electrical stimulation of the PAG produces profound analgesia. In studies designed to determine if this analgesia produced by stimulation is mediated by enkephalins Mayer and Hayes (1975) demonstrated a cross-tolerance between SPA and morphine, similar to the cross-tolerance between morphine and enkephalin demonstrated by Waterfield (1976). Similarly, the opiate receptor antagonist, naloxone, has been shown to inhibit the analgesic action of PAG stimulation in experimental studies using the rat (Akil et al, 1976) and in human clinical studies (Adams, 1976; Hosobuchi et al, 1977 and 1979; Akil et al, 1978a). These studies imply an opioid mediation of SPA, but do not directly test the involvement of PAG located enkephalin in antinociception due to the lack of specificity in the locus of the naloxone effect, and a failure to demonstrate a release of enkephalin from the PAG.

Studies which have directly attempted to demonstrate the release of endorphin by analgesia producing stimulation have identified two different opiate peptides dependent upon the species studied and the biochemical assay performed. Akil et al (1976) demonstrated a significant elevation of whole brain opiate-like factors as measured by an inhibition of ^{3}H naloxone binding assay following analgesia producing stimulation of the rat PAG. Similarly Stein (cited in Liebeskind et al, 1978) observed an increase in enkephalin levels in cerebral perfusate collected from a push pull cannula located two mm caudal to a PAG analgesia producing stimulating electrode. Similar increases of endorphins in cerebrospinal fluid (CSF) of human patients have been observed

during SPA (Meyerson et al, 1977; Hughes, 1977; Akil et al, 1978a and b; Hosobushi et al, 1979). Akil et al (1978a) have isolated an enkephalin-like peptide from human ventricular CSF during SPA. Akil et al (1978a) and Hosobuchi et al (1979) have also described increases in β-endorphin in human ventricular CSF as determined by radioimmunoassay upon analgesic electrical stimulation. The collection of peptide from the CSF would favour the presence of the larger endorphin due to the resistance of β-endorphin to catabolism in both the CSF and cerebral tissue as compared with enkephalin.

It has been demonstrated in the above mentioned studies that electrical stimulation of the PAG in the rat and human, releases an opiate peptide during periods in which stimulation induces analgesia. It cannot be concluded however, that the observed analgesia is due to the demonstrated release of endorphin. In combination with the results of studies of naloxone effects on SPA, it may be concluded that stimulation of the central gray releases opiate peptides into the CSF, and that the analgesia produced by the stimulation is blocked by naloxone and therefore is mediated by opiate receptors. The above described studies have not demonstrated that the released opiate peptides induce analgesia. Nor can it be concluded that SPA is mediated by enkephalin release as the observed enkephalin released by PAG stimulation of the rat (Stein cited in Liebeskind et al, 1978) was not demonstrated responsible for the analgesic action of the stimulation.

A simple method of determining the importance of enkephalin

in modulation of nociception and in the analgesia produced by PAG stimulation is the demonstration of the potentiation of the analgesic effect of stimulation by the administration of an inhibitor of enkephalin catabolism. Conceptually this experiment would involve the release of enkephalin by PAG stimulation and an assessment of the potentiation of analgesia induced by the increased enkephalin levels resulting from catabolic inhibition. The potentiation of analgesia as a result of inhibition of catabolism is proposed, based on the demonstration of increased analgesia potency of centrally administered enkephalin structural analogs correlated with reduced susceptibility to degradation.

Synthetic analogs of Met enkephalin have been produced which are catabolized slowly 'in vitro' and demonstrate equal or greater analgesic potency than β-endorphin when administered intracerebrally (PAG), intraventricularly or intravenously (Pert et al, 1976; Bajusz et al, 1976, Coy et al, 1976). Effective chemical alterations involve the incorporation of a D amino acid (usually alanine at the β-LPH_{62} position substituting for glycine) and the addition of an amino group (NH_3) to the C terminal amino acid. Opiate receptor affinities require five amino acids and a tyrosine residue which need not be N terminal as β-LPH_{60-65} but not β-LPH_{62-65}, β-LPH_{62-66}, nor β-LPH_{61-64} demonstrates receptor affinity and agonist efficacy. Marks (1978) states the importance of both N and C terminal protection in synthetic analogs as enkephalins are subject to a vast number of proteolytic enzymes in cerebral tissue.

The specific enzyme which is responsible for enkephalin catabolism following synaptosomal release has not yet been conclusively identified. The discrepancy in the literature appears to arise from the enzyme preparation which is used for 'in vitro' enkephalin degradation. Using crude brain homogenate (Vogel and Alstein, 1978; Marks et al, 1977), membrane fractions (Meeks et al, 1977; Miller et al, 1977), plasma (Vogel and Altstein, 1979; Hambrook, 1976) or intraventricular administration (Meeks et al, 1977) enkephalins are rapidly cleaved at the tyrosine-glycine bond probably by an arylamidase (Hayashi, 1978). This proteolysis is inhibited by puromycin and to a lesser degree bacitracin (Vogel and Alstein, 1979; Marks, 1978). Sullivan et al (1978) and Malfroy et al (1978) have argued that the N terminal degradation of enkephalin is non-specific as it is performed by most tissues of the body with low affinity kinetics and is not specific to particulate membrane fractions. Sullivan et al (1978) first described the specific catabolism of enkephalin, by a washed membrane preparation containing opiate receptors, as a removal of the C terminal amino acid. Enzymes isolated from particulate fractions of striatal tissue which catabolize Met and Leu enkephalin with high affinity (50-150 times that found in soluble fraction enzymes) have recently been described by Malfroy et al, (1978) and Swertz et al (1979). This enzyme cleaves the C terminal amino acid from both Met and Leu enkephalin. The concentration of this enzyme is heterogenous within various brain regions and appears correlated to opiate receptor concentration. Chronic morphine

administration increases the V max of enkephalin catabolism implicating an increased enzyme number in tolerant subjects. Stereospecificity of the C terminal structure appears important for peptide affinity to this enzyme which is now termed enkephalinase. The tetrapeptide Gly-Gly-Phe-Met has been demonstrated to be a selective competitive enkephalinase inhibitor. Bacitracin is the only classical peptidase inhibitor with significant potency in inhibiting this particulate fraction enzyme explaining the effects observed by Patthy et al (1977).

The present experiment uses the rationale of Patthy et al (1977). Inhibition of enkephalin catabolism will potentiate the pharmacological action of the opiate peptide if enkephalin is present at the receptor site. If enkephalin is released during the test situation, application of specific enkephalinase inhibitor to the PAG will potentiate the action of enkephalin which is proposed to be analgesic at this locus. If enkephalin is not released in the test situation but requires neuronal stimulation, application of enkephalinase inhibitor to the PAG will only induce analgesia if the enkephalin system is activated. Therefore it is possible to test the hypothesis that SPA is mediated through the activation of PAG located enkephalin neurons by evaluating the potentiation of SPA following application of a specific enkephalinase inhibitor.

The enkephalinase inhibitor chosen for this experiment is Gly-Gly-Phe-Met. This tetrapeptide has been demonstrated to specifically and competitively inhibit enkephalin

C terminal catabolism, and enkephalin C terminal catabolism is the most likely mechanism of specific degradation following neuronal release (Malfroy et al, 1978). This experiment differentiates between the antinociceptive effect of β-endorphin and enkephalin, since β-endorphin is cleaved by an endopeptidase at the Leu-Phe (β-LPH 77-78) bond, followed by N terminal exopeptidase action (Marks, 1978). The tetrapeptide only inhibits the catabolism of enkephalin, and therefore any potentiation of analgesia will be due to the increased concentration of enkephalin in the PAG.

The present experiment is designed to study (I) the effect of enkephalinase inhibitor administration on analgesia produced by PAG stimulation, and the naloxone reversibility of this effect. It is hypothesized that tetrapeptide administration to the PAG 2 mm caudal to the site of stimulation (to coincide with the study of Stein cited in Liebeskind, 1978), will inhibit the catabolism of any enkephalin present. The increased concentrations of enkephalin will potentiate the intensity of SPA, and the retardation of catabolism will prolong the duration of analgesia. Similarly, the duration and intensity of analgesia induced by PAG administration of the tetrapeptide without electrical stimulation will determine if enkephalin released in the test situation by natural stimuli is responsible for modulating antinociception. The naloxone reversibility of these parameters will determine the validity of the hypothesis that potentiation of analgesia is mediated by opiate receptors. This experiment also studies (II) the relationship between enkephalinase inhibitor

dose and the magnitude of the potentiation of stimulation produced analgesia. It is hypothesized that since the tetrapeptide is a competitive enkephalinase inhibitor increasing the concentration of Gly-Gly-Phe-Met will increase amount of enkephalin present at the receptor site by further reducing enkephalin catabolism. It is proposed that the potentiation will be in duration and intensity of SPA, that the analgesic effect will be naloxone reversible, and dependent on enkephalinase inhibitor dose. Finally the experiment studies (III) the mechanism of SPA potentiation. Stimulation and opiate analgesia have been demonstrated to involve a common bulbospinal serotonergic pathway. Specific lesion of this pathway will eliminate the potentiation of SPA by the tetrapeptide if the observed analgesic effect of enkephalinase inhibition is mediated by an identical mechanism.

This experiment in three studies directly tests the hypothesis that enkephalin within the PAG is responsible for modulating the perception of pain.

STUDY I

THE ACTION OF ENKEPHALINASE INHIBITOR

The aim of this experiment is to study the effect of intracerebral (IC) administration of the enkephalinase inhibitor (Gly-Gly-Phe-Met) on analgesia produced by PAG stimulation. It is hypothesized that IC administration of enkephalinase inhibitor will potentiate the intensity and the duration of stimulation induced analgesia in a naloxone reversible manner. The analgesic effect of IC injection of enkephalinase inhibitor in the absence of stimulation is also studied. The experiment design is similar to that used by Akil and Liebeskind (1975) in which the action of a drug on the analgesia produced by a submaximal current is tested in a crossover design with saline or naloxone pretreatment separated by one week.

METHOD

Subjects

Twenty-two male albino rats of the Sprague Dawley strain were obtained from the Canadian Breeding Laboratories. Subjects (Ss) were housed individually on a 12 hour light, 12 hour dark schedule and maintained on ad lib food and water throughout the experiment.

Surgery

Subjects weighing 180-200 g at the time of surgery were implanted with chronic bipolar stimulating electrodes and 26 gauge cannulae under Nembutal anaesthesia (45 mg/kg).

Electrodes obtained from Plastic Parts (#MS-202) were constructed of two teflon coated stainless steel wires, 0.2 mm in diameter, twisted together, cut to equal length and bared of insulation only at the cross section of the tip. Guide cannulae were made from 26 gauge Yale disposable stainless steel needles cut on a bevel to within 4 mm from the plastic barrel. A 32 gauge teflon coated stylet was placed in the guide and cut to protrude 0.25 mm from the tip of the cannulae, to avoid contamination. Electrodes were aimed at the rostral ventral PAG, a region demonstrated to be efficacious for SPA by Rhodes and Liebeskind (1977); Yeung, Yaksh and Rudy (1977) and as reviewed by Mayer and Price (1976). Co-ordinates AP 1.0 (4.8 from bregma), L $\pm$ 0.25, DV -2.0 (-6.0 from surface of the brain), as derived from Pelligrino and Cushman (1967) were used. Guide cannulae were aimed at the ventrolateral PAG ipsilateral and 2 mm caudal to the stimulating electrode to correspond with Stein (cited in Liebeskind, 1978); Yaksh and Rudy (1977); Yeung, Yaksh and Rudy (1977) and as reviewed by Mayer and Price (1976). The co-ordinates used were AP -1.0 (6.8), L $\pm$ 0.5, DV -1.0 (-4.5), again derived from Pelligrino and Cushman (1967). Both electrodes and guide cannulae were fixed in place with dental cement. All Ss were allowed 10 days for recovery from surgery.

Analgesic Testing: Procedure and Apparatus

The D'Amour and Smith (1947) tail flick test was chosen as the test of analgesia because of its relative reliability compared with other forms of measuring analgesia. The equipment used in this test is described below. In the tail flick test, radiant heat is focused on the tip of the S's tail. The latency between the activation of the heat source and the response of tail movement is measured. It is generally accepted that alteration in latency of the tail flick response is indicative of a modification in pain perception and that modulation of the response is limited to opiate agonist and PAG stimulation manipulations (Grumback, 1977; Mayer and Price, 1976).

During testing animals were restrained in a cardboard box (16 x 7 x 10 cm^3) cut away to allow free access to the head and with the tail protruding from the other end. A 300 watt incandescent projection bulb backed by a concave metallic reflector was focused above the distal one cm of tail which was resting in a groove in an asbestos board. Below the distal one cm of tail, encased in the asbestos was a photocell which, when activated closed a circuit running a timer and the heat source. A trial was initiated by opening the circuit which activated the timer and the lamp. The tail flick response closed the circuit by allowing the lamp to shine on the photocell, thereby shutting off the timer and the heat source. If no response occurred the lamp was automatically turned off after 7.0 seconds. The lamp intensity was initially regulated by a rheostat and was held constant

throughout the experiment. The lamp intensity used in all studies, was adjusted prior to the experiment with twenty same age, same strain control Ss, to produce a 3.5 s tail flick latency. The experimental room temperature was maintained at 75°F and was illuminated by a dim 100 watt house light. All experiments were performed during the latter part of the 12 hour light cycle.

Determination of Median Analgesic Current of Stimulation

During recovery from surgery all subjects were habituated to the testing restraint box over three periods of ten minutes in which the S was placed in and removed from the box. Following recovery from surgery Ss were tested for baseline response and analgesia produced by electrical stimulation. Tail flick responses were measured at two minute intervals. Baseline response latency was first calculated from the average of the last 3 responses of 4 trials. For the remaining trials 20 seconds of brain stimulation immediately preceded the tail flick test. Brain stimulation consisted of 100 ms trains of 60 Hz sine waves delivered at 3 pulses per second. Brain stimulation current was initiated at 10 microamps and was increased in each subsequent trial in 10 microamp steps until total analgesia, aversive effects or 100 microamps had been reached. Total analgesia was defined as a response latency equal to 7.0 seconds. From these latencies, recorded at each current level, a percentage analgesia (A%) could be determined for each current level using the following formula:

$$A\% = 100 \times \frac{T - BL}{7.0 - BL}$$

where BL = baseline latency before stimulation and

T = test latency following stimulation.

A current intensity midway between the current required to produce 10% and 100% analgesia was termed the median analgesic current (MAC). A median analgesic current was assigned to each animal and was used in all subsequent tests. Subjects demonstrating no analgesic effects of maximal electrical stimulation currents or intense aversive reactions to lower current levels were eliminated from the experiment at this stage. Eight of the twenty-two subjects were removed from the experiment at this stage.

Enkephalinase Inhibitor Test Procedure

Seven days after the determination of the appropriate median analgesic current intensity for each subject, enkephalinase inhibitor tests were performed. Half of the subjects were injected with saline (IP) and half were injected with naloxone (IP) (5.0 mg/kg) 15 minutes prior to baseline tail flick testing. The baseline response latency was calculated from the last two of three tail flick tests. Analgesic tests of the effect of each condition were performed under the following schedule: Intracerebral injection (60 s) (if appropriate), stimulation (20 s) (if appropriate), tail flick tests. Tail flick tests were repeated at 2 minute intervals until the response latency was within 0.5 seconds of the individual baseline latency. Conditions tested in each session were stimulation, enkephalin inhibitor + stimulation, saline + stimulation, enkephalinase inhibitor. Table 2 provides the procedural scheme of the overall study and describes the

procedure of the enkephalinase test session. (Both schema are left to right with respect to time).

Immediately after baseline testing, stimulation was administered at the appropriate median current using the previously mentioned parameters for 20 s followed by a tail flick test, and the latency recorded. Tail flick tests were repeated at 2 minute intervals until the response latency was within 0.5 s of the pre-stimulation baseline latency. Enkephalinase inhibitor (Gly-Gly-Phe-Met), (7 micrograms in 0.5 microliters saline neutralized with phosphate buffer to a pH 7.1) was then administered via a 32 gauge cannula, connected to a Hamilton 5 microliter syringe, by polyethylene tubing, at a rate of 0.5 microliter/minute. A dose of 7 micrograms of enkephalinase inhibitor was used, to coincide with the minimal analgesic dose of PAG administered enkephalin demonstrated by Malick and Goldstein (1976). The injection was performed by removing the stylet from the guide cannula and inserting the injection cannula, which extended 0.5 mm from the guide tip. Immediately following the IC injection PAG stimulation at the median analgesic current level was again initiated for 20 s. Tail flick tests separated by 2 minutes were again repeated until baseline latency ($\pm$ 0.5 s) was observed. One half of a microliter of saline (0.9%, pH 7.3) was identically administered via a 32 gauge cannula followed by 20 s of stimulation at the median analgesic current. Again immediately upon termination of PAG stimulation tail flick tests were repeated at two minute intervals until the baseline latency criterion was met. Enkephalinase inhibitor (7 micrograms) was again administered as described

TABLE 2

Scheme of Experimental Procedure

Surgery	Median Analgesic Stimulation Current (MAC) Determination	Session A Enkephalinase Inhibitor (Enk.ase I) Test	Session B Enkephalinase Inhibitor Test

7 day period between each segment

Enkephalinase Inhibitor Test

Pretreatment	Baseline	Stimulation	Enkephalinase Inhibitor + Stimulation	Saline + Stimulation	Enkephalinase Inhibitor
Saline or Naloxone IP	3 Tail Flick Tests	20 s stimulation at MAC	IC injection of Enk.ase I (60 s)	IC injection of saline (60 s)	IC injection of Enk.ase I (60 s)
Wait 15 minutes		Tail Flick Tests to Baseline	20 s stimulation at MAC	20 s stimulation at MAC	Wait 20 s
			Tail Flick Tests to Baseline	Tail Flick Tests to Baseline	Tail Flick Tests to Baseline

above. Twenty seconds following the termination of the injection, tail flick tests were performed at 2 minute intervals until baseline latency was reached. Each group of tail flick tests constituted a test of the effect of a condition.

Seven days later the identical design was repeated, with the exception of the reversal of the naloxone and saline IP pretreatment administration, so that subjects receiving saline in Session A received naloxone in Session B. Naloxone was obtained from Endo Laboratories, the peptide Gly-Gly-Phe-Met was obtained from Beckman Laboratory.

Histology

After completion of Test Session B subjects were sacrificed with an overdose of Nembutal and perfused with saline (50 ml) and 10% formalin. Brains were extracted and placed in 30% sucrose formalin overnight. Forty micron coronal sections were taken from each brain on a freezing microtome. These slices were stained in thionin and used to identify electrode and cannula placements for each subject.

Analysis of Data

Two dependent measures were taken for each experimental condition following baseline response latency determination. Baseline latency was calculated as the mean of the last two of three tail flick response latencies in the baseline segment of the test session. Intensity of analgesia was determined from the latency in the tail flick test immediately preceded by stimulation (or 20 s after IC administration in the enkephalinase inhibitor condition) in each condition.

(Pilot studies had shown this to be the period of maximal effect). The duration of analgesic effect was calculated as the product of the number of tail flick tests in which response latency exceeded the baseline latency by 0.5 s and the interval between trials (2 minutes). Finally in order to allow comparisons with studies in the SPA literature (Akil and Liebeskind, 1974) latency data was normalized for individual baseline differences by conversion to maximum percent effect using the formula:

$$MPE = \frac{\text{Post Drug latency} - \text{Pre Drug latency}}{\text{Cutoff time} - \text{Pre Drug latency}} \times 100\%$$

where cutoff time was equal to 7 seconds and pre drug latency was equal to the baseline latency. (Since these data are simply a transformation of intensity scores they are presented in Appendix B).

Since the experiment was performed as a crossover design with the pretreatment (naloxone, saline) variable reversed within subjects over Sessions A and B, the significance of replication effects was determined by individual t-tests on latency, duration and MPE data. If differences were not significant ($p > .05$), data were pooled across test sessions for each pretreatment in each condition.

A two (pretreatment) by five (condition) within subject analysis of variance was performed on the intensity data. From this analysis within cell variance (MSW) was calculated. The MSW was then used to calculate the significance of differences between means using tests of simple effects (Wiener, 1962). Comparisons between means were determined *a priori* to test the experimental hypothesis.

All conditions were compared to baseline latency and against the pretreatment counterpart. Stimulation and saline stimulation conditions were compared to enkephalinase inhibitor + stimulation and to each other, within each pretreatment factor.

Comparisons between baseline and each condition were used to demonstrate analgesia produced by the parameters of the conditions. Comparison between naloxone and saline pretreatment within conditions demonstrated the naloxone reversibility of the observed analgesia. Stimulation and saline + stimulation conditions were used as controls for stimulation and saline IC injection on the enkephalinase inhibitor + stimulation condition. Therefore comparisons between enkephalinase inhibitor + stimulation and stimulation, and between enkephalinase inhibitor + stimulation and saline + stimulation were used to demonstrate the potentiation effect of enkephalinase inhibitor on SPA.

Using the same method of comparison with removal of baseline data the significance of differences between the means of duration data and MPE results were calculated. In these latter cases the MSW was calculated from a two by four within subject analysis of variance.

RESULTS

Threshold: The mean baseline tail flick latency in the threshold session was 3.7 s. Electrical stimulation produced analgesia in fourteen Ss without adverse effects and at currents below 100 microamps. The mean median analgesic current was 30.7 microamps with a standard error (Se) of 5.88 microamps. No differences were apparent in Ss requiring high and low MACs in any subsequent tests. The tail flick latency of 3Ss was not altered by currents up to 100 microamps while 5 Ss demonstrated aversive and escape behaviour to lower currents of brain stimulation.

Enkephalinase Inhibitor Test

Replication Factor: t-tests were performed to determine differences in results between Session A and Session B. There were no significant differences between results of Ss run under saline during Session A and Session B or of Ss run under naloxone pretreatment in Session A and Session B as determined by individual t-tests performed on each condition, with latency, duration, or MPE data. Therefore results from tests performed in Session A were pooled with results (under the appropriate condition) obtained in Session B.

Intensity: Table 3 presents the mean latency of tail flick response in seconds under each test condition. Table 4 demonstrates the F ratios of the specific a priori

comparisons of the simple effects of differences between test conditions. Pretreatment with naloxone was not found to significantly alter baseline tail flick latency. The combined naloxone and saline pretreatment baseline was 3.60 s. In the saline pretreated Ss stimulation significantly increased tail flick latency to 4.71 s ($F(1,52)$ 82.65, $p < .01$) as did saline + stimulation to 4.38 s ($F(1,52)$ 43.5, $p < .01$). The differences in latency between these two conditions was significant ($F(1,52)$ 6.22, $p < .05$). Administration of enkephalinase inhibitor + stimulation increased latency to 6.91 s which is significantly greater than baseline ($F(1,52)$ 656.4, $p < .01$), stimulation ($F(1,52)$ 273.2, $p < .01$), and than saline + stimulation ($F(1,52)$ 361.9, $p < .01$). Intracerebral injection of enkephalinase inhibitor augments response latency to 4.09 s also significantly different from the baseline ($F(1,52)$ 19.78, $p < .01$).

Following naloxone pretreatment stimulation, saline + stimulation, and enkephalinase inhibitor + stimulation increased tail flick latency to 4.24, 4.28, and 4.36 respectively. These latencies are all significantly greater than naloxone pretreatment baseline ($F(1,52)$ 15.74, 18.21 and 23.56 respectively, $p < .01$ all), but do not differ significantly from each other. Enkephalinase inhibitor administration following naloxone injection did not significantly alter tail flick latency from baseline (Mean ($\bar{X}$) = 3.69).

Response latency in the stimulation but not saline + stimulation condition was significantly greater ($F(1,52)$ 12.36, $p < .01$) following saline as compared to naloxone pretreatment. Tail flick latency in the enkephalinase

TABLE 3

Study I: Mean Latency of Tail Flick Response in Enkephalinase Inhibitor Test (Seconds ± Standard Error)

CONDITION	PRETREATMENT	
	Saline	Naloxone
Baseline	3.497 ± 0.087	3.711 ± 0.094
Stimulation	4.707 ± 0.121	4.239 ± 0.117
Enkephalinase Inhibitor + Stimulation	6.907 ± 0.068	4.279 ± 0.121
Saline + Stimulation	4.375 ± 0.138	4.357 ± 0.141
Enkephalinase Inhibitor	4.089 ± 0.168	3.693 ± 0.126

TABLE 4

Study I: Enkephalinase Inhibitor Test: Comparisons in Latency of Response (F Ratios)

		SALINE					NALOXONE				
		Base-line	Stim	Enkase I + Stim	Saline + Stim	Enkase I	Base-line	Stim	Enkase I + Stim	Saline + Stim	Enkase I
SALINE	Baseline	-	13.36*								
	Stim	82.65*	-								
	Enkase I Inhib + Stim	656.4	273.23*	-							
	Saline + Stim	43.5*	6.22+	361.9*	-						
	Enkase Inhibitor	19.78*				-					
NALOXONE	Baseline	2.59					-				
	Stim		12.36*				15.74*	-			
	Enkase I Inhib + Stim			389.8*			18.21*		0.09	-	
	Saline + Stim				0.018		23.56*		0.78	0.34	-
	Enkase Inhibitor					8.85*	0.018				-

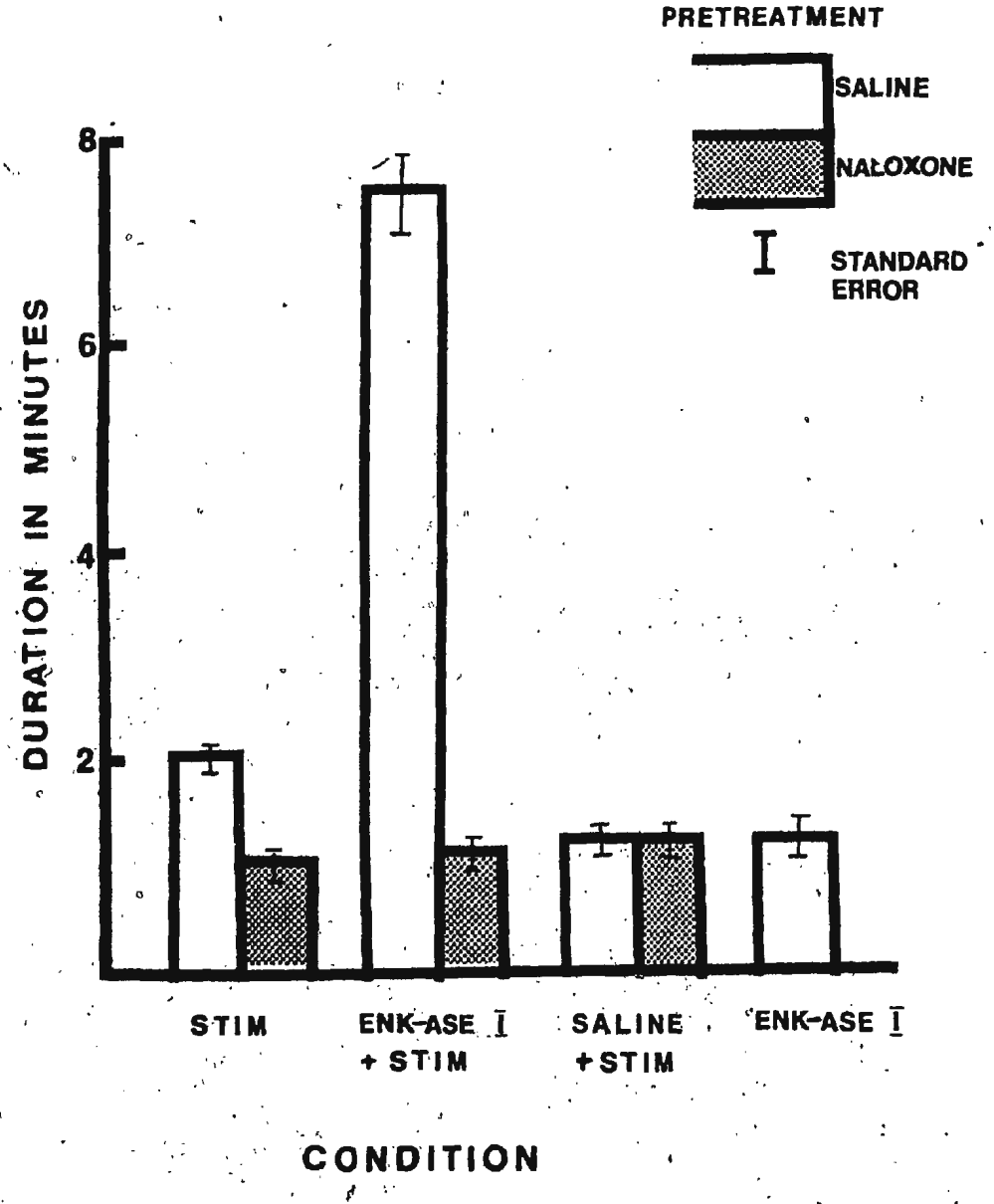
* $p < .01$

+ $p < .05$

inhibitor alone and combined with stimulation conditions was significantly greater in the saline pretreatment as compared to naloxone pretreatment situation ($F(1,52)$ 8.85, $F(1,52)$ 389.8 respectively, $p < .01$ both).

Duration: Figure 1 presents the mean duration of analgesia for subjects in each of the four experimental conditions. Comparisons between appropriate means, using simple effects tests, demonstrates a similar pattern of results as observed in the intensity data. With saline pretreatment, analgesia (as previously defined) occurs for 2.0 minutes (min) following stimulation, and 1.28 min after saline + stimulation. Both of these durations are significantly shorter than the mean duration of analgesia, following enkephalinase inhibitor + stimulation ($\bar{X}$ = 7.43 min) ($F(1,39)$ 102.63, $F(1,39)$ 131.65 compared to stimulation and saline + stimulation respectively $p < .01$ both), but do not differ from each other. Under naloxone pretreatment, durations of analgesia, in stimulation (1.00 min), enkephalinase inhibitor + stimulation (1.14 min), and saline + stimulation (1.28 min) conditions do not significantly differ. In comparison of pretreatment effects under enkephalinase inhibitor + stimulation conditions, the duration of analgesia was significantly longer following saline injection ($F(1,39) = 137.7$, $p < .01$). Neither stimulation nor saline + stimulation duration significantly differed under naloxone as compared with saline injection. Administration of enkephalinase inhibitor alone resulted in a mean duration of analgesia of 1.28 minutes under saline and 0 minutes under naloxone

Figure 1. Duration of analgesic action in minutes for Study I .


PRETREATMENT
SALINE
NALOXONE
STANDARD ERROR
DURATION IN MINUTES
8
6
4
2
STIM
ENK-ASE I + STIM
SALINE + STIM
ENK-ASE I
CONDITION

pretreatment. The difference between these durations is significant ($F(1,39)$ 5.7, $p < .05$). Table 5 presents the F values of appropriate comparisons in duration of analgesia.

Histology: Histological examination of the 14 brains of Ss demonstrating stimulation produced analgesia demonstrated that the tip of the stimulating electrode of each S was near or within the PAG. Figure 2 presents a schematic diagram illustrating the location of effective SPA electrode tips. Effective placements were located within 0.2 mm of AP 1 as described in Pelligrino and Cushman (1967). Figure 3 presents a schematic diagram depicting the placement of guide cannulae. All cannulae tips were found to lie within or adjacent to the caudal PAG. The injection cannulae extended 0.5 mm from the tip of the guide cannulae.

DISCUSSION

Baseline latencies between test session within subjects did not significantly differ, indicating that this dependent measure is reliable in confirmation of Dewey and Harris (1975). Naloxone pretreatment did not significantly alter baseline latency in the tail flick test in agreement with Goldstein et al (1976). Naloxone partially blocked the analgesia produced by stimulation in each condition. The lack of complete naloxone reversibility of SPA is similar to that observed by Akil et al (1976) in the rat suggesting

TABLE 5

Study I: Enkephalinase Inhibitor Test: Comparison in Duration of Analgesia (F ratios)

		SALINE				NALOXONE			
		Stimulation	Enkase I + Stim	Saline + Stim	Enkase I	Stimulation	Enkase I + Stim	Saline + Stim	Enkase I
SALINE	Stimulation	-							
	Enkase I + Stim	102.63*	-						
	Saline + Stim	8.80	131.65	-					
	Enkase Inhibitor				-				
NALOXONE	Stimulation	3.48				-			
	Enkase I + Stim		137.7*			0.068	-		
	Saline + Stim			0		0.068	0.27	-	
	Enkase Inhibitor				5.7+				-

*p < .01
+p < .05

Figure 2. Schematic diagram of electrode tip sites of rats demonstrating stimulation - produced analgesia in Study I [Numbers refer to co-ordinates from the intra-aural line in millimeters. Taken from Pellegrine & Cushman, (1967)].

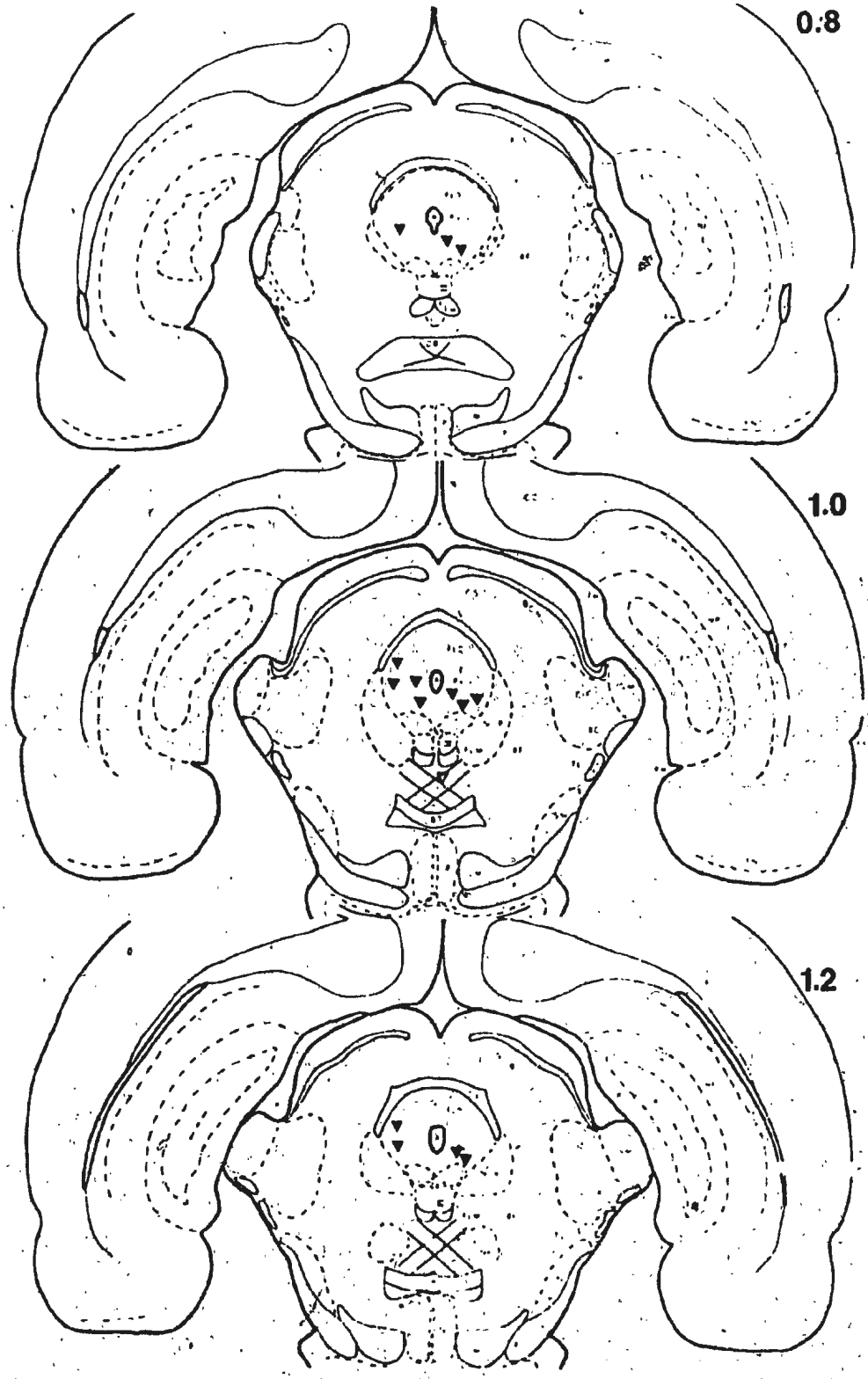
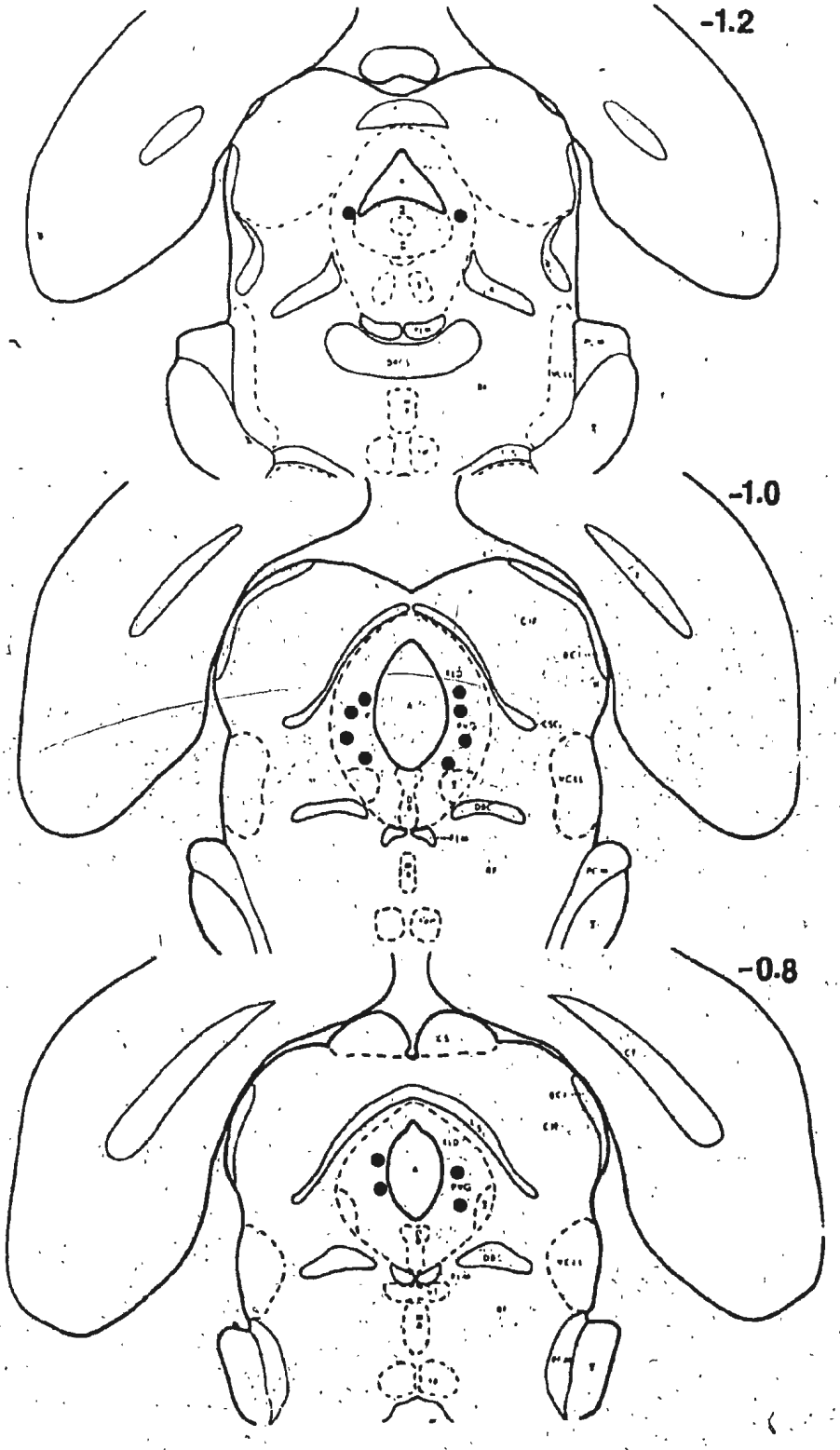

0.8
1.0
1.2

Figure 3. Schematic diagram of guide cannulae tip sites of rats demonstrating stimulation - produced analgesia in Study I [Numbers refer to co-ordinates from the intra-aural line in millimeters. Taken from Pellegrine & Cushman, (1967)].


-1.2
-1.0
-0.8

that non-opiate mechanisms are also important in SPA. It was demonstrated however that all effects of enkephalinase inhibitor were completely naloxone reversible. Therefore it is concluded that the analgesic effects of enkephalinase inhibitor alone and the potentiation of SPA are mediated by opiate receptors. Unexpectedly the antinociception observed in the stimulation condition was greater than that observed in the saline + stimulation condition. Possible explanations for this difference are a rapid development of tolerance to SPA or a nonspecific effect of the IC injection volume. Importantly, this discrepancy in control conditions is small, and acts against the significant effect of enkephalinase inhibitor on stimulation produced analgesia.

The intracerebral administration of enkephalinase inhibitor significantly potentiated stimulation produced analgesia in a naloxone reversible manner. This potentiation was in terms of intensity (in MPE, see Appendix B) and duration of analgesia, augmenting measures of both variables approximately sixfold. Enkephalinase inhibitor administration alone also produced a significant naloxone reversible analgesia as measured in intensity and duration. The effect of enkephalinase inhibitor + stimulation was significantly greater than the sum of analgesic effects of enkephalinase inhibitor and stimulation. It is concluded from the results of this study that enkephalinase inhibitor potentiates the action of

stimulation in a naloxone reversible manner by modulating the mechanism which is responsible for producing analgesia.

STUDY II

THE RELATIONSHIP OF ENKEPHALINASE INHIBITOR DOSE TO ANTINOCICEPTION RESPONSE

In pharmacological studies a relationship between dose and response implies a specificity of drug action. The magnitude of an observed biological effect, under the occupancy assumption of drug action, is directly proportional to the occupation of specific receptors by drug molecules. In the present experiment it is assumed that enkephalinase inhibitor increases enkephalin concentration by competitively inhibiting catabolism (Swertz et al, 1979).

A competitive enzyme inhibitor interacts with the active site of the enzyme, therefore reducing the effective affinity of the substrate for the enzyme. The removal of substrate is thus reduced by the competitive inhibitor, without effecting the potential maximal rate of the catabolic reaction. Increasing the concentration of the competitive inhibitor further decreases the effective affinity of substrate for enzyme thus further reducing the disappearance of substrate. It is proposed that by increasing the concentration of enkephalinase inhibitor the catabolism of enkephalin is further diminished, and an increased amount of enkephalin is present to activate opiate receptors. Therefore a direct relationship between dose of enkephalinase inhibitor and response will determine whether the potentiation of SPA by enkephalinase inhibitor is due specifically to an increase in enkephalin concentration at the receptor site and a decreased rate of enkephalin breakdown. Similarly a direct dose response

relationship between analgesia and dose of enkephalinase inhibitor alone will demonstrate that antinociception is occurring by catabolic inhibition rather than a non-specific mechanism. This experiment attempts to define the specificity in the potentiating effect on SPA of enkephalinase inhibitor, by determining the relationship between dose and response. It is hypothesized that the concentration of enkephalinase inhibitor is directly related to the degree of antinociception produced by PAG stimulation.

METHOD

Subjects

Thirty male albino rats of the Sprague Dawley strain obtained from the Canadian Breeding Laboratories served as subjects. Subjects were housed and maintained as in Study I.

Surgery

Subjects weighing 180-200 g were implanted with stimulation electrodes and guide cannulae using the procedures described in Study I.

Analgesic Testing: Procedure and Apparatus

Tail flick tests using the equipment and procedures described in Study I were used to test analgesia.

Median Analgesic Current Determination

During recovery from surgery subjects were habituated to the restraint box in three ten-minute sessions. After recovery from surgery median current intensities were determined for each subject using the parameters described in Study I. Subjects demonstrating aversive responses or no analgesia at current intensities of 100 microamps were

removed from the experiment. Six subjects failing to demonstrate SPA were eliminated from the experiment.

Enkephalinase Inhibitor Test Procedure

Subjects were randomly assigned to one of four dose groups prior to the test session, and remained in that dose group throughout Study II. Test procedures of Study II were identical to those of Study I with the exception of the dosage of enkephalinase inhibitor administered intracerebrally. Half of the subjects of each dose group received saline and half received naloxone 15 minutes prior to baseline testing. In the appropriate condition enkephalinase inhibitor (Gly-Gly-Phe-Met) was administered to the PAG as previously described. Dosages of enkephalinase inhibitor administered were 1, 3, 9 or 25 micrograms in 0.5 microliters saline (pH 7.2). The dose received by each subject was constant for each application of enkephalinase throughout Study II. Seven days after the first test session (A) naloxone and saline pretreatments were reversed and tail flick response was again tested under the five conditions (Session B). Subjects received identical dosages of enkephalinase inhibitor in Session A as in Session B. Enkephalinase inhibitor peptide Gly-Gly-Phe-Met purchased from Peninsula Laboratories was used in Studies II and III.

Histology

Subjects used in Study II were also used in Study III therefore histology was performed only after Study III. Histology of the Ss will be discussed in the Results section of Study III.

Analysis of Data

The dose response experiment was designed as a Between (4 doses) by Within (4 conditions) by Within (2 pretreatments) study. Since it had been shown in Study I that intensity and duration of stimulation produced analgesia are affected by enkephalinase inhibitor a dependent measure incorporating both of these parameters was chosen. Yaksh and Wilson (1979) describe the use of the term 'Antinociceptive Index' (AI) for this purpose. Antinociceptive index is calculated from the area under a time effect curve resulting in units of MPE. Minutes. In this experiment the MPE of each tail flick test under each condition and pretreatment was calculated for each subject from the individual baseline. From these transformed data the AI for each S under each condition and pretreatment was calculated as the area under a MPE vs. Duration curve. The AI was then used as the dependent measure for all statistical analyses except for an initial comparison of baselines.

Independent t-tests were used to determine differences across test sessions within all test conditions. In the cases where these differences were nonsignificant, data from test Session A were pooled with data collected in test Session B. Since the remainder of the data were transformed, a separate analysis was performed to determine differences in baseline tail flick latency.

Under the assumptions of the analysis of variance, subjects must be randomly distributed amongst treatments. Throughout this study subjects are restricted to one dosage group. Therefore, following drug administration subjects

are not equivalent and are not randomly assigned to the subsequent treatments. Thus in this study the saline + stimulation and enkephalinase inhibitor conditions which follow drug administration are analysed separately.

The stimulation and enkephalinase + stimulation data were analysed by simple effects comparisons of means using a MSW calculated from an analysis of variance. Similar comparisons were performed as in Study I with the addition of comparisons aimed at determining a dose effect. The differences between means of AI in the saline + stimulation condition were also determined by a comparison of means using the MSW calculated from analysis of variance. A correlated t-test was used to determine differences between saline + stimulation and enkephalinase inhibitor + stimulation and stimulation condition in the 1 microgram dose group following saline pretreatment. Finally a Newman-Keuls test was performed on the enkephalinase inhibitor data.

RESULTS

Threshold: The mean baseline tail flick latency in the threshold session was 3.55 seconds for the 30 Ss. Twenty-four of the thirty subjects demonstrated maximal analgesia following stimulation at currents less than 100 microamps without adverse side effects. The mean of the median current which produced analgesia in these 24 Ss was 23.3 (Se 2.72) microamps. Two of the 6 Ss excluded from the test session demonstrated no analgesia at 100 microamps; the other 4 Ss showed signs of adverse side effects.

Enkephalinase Inhibitor Test

Replication Factor: Individual t-tests were used to determine differences between tests performed in Session A and in Session B. No differences within condition, dose group and pretreatment were significant; therefore scores within a cell were collapsed over the test sessions.

Baseline: The mean tail flick latencies observed in the test procedure are presented in Table 6. An analysis of variance demonstrated that baseline latency differences between dose groups and between pretreatment groups were not significantly different ($F(3,20)$ 1.24, $F(1,20)$ 3.06, respectively). Similarly the interaction between dose group and pretreatment effects on baseline was not significant ($F(3,20)$ 0.160). The mean baseline following saline pretreatment collapsed across dose groups was 3.54 s while the latency following naloxone injection was 3.47 s.

Stimulation and Enkephalinase Inhibitor + Stimulation: The mean antinociceptive index (AI) for stimulation and enkephalinase inhibitor + stimulation conditions for each dose group under saline and naloxone pretreatment is presented in Table 7. Using the MSW calculated from an analysis of variance the significance of differences between means were tested. Following saline pretreatment in the stimulation condition, the mean AI of each dose group did not significantly differ from any other dose group. The mean AI across dosage group in this pretreatment and condition was 24.9 MPE. Min. Similarly following naloxone injection no significant differences between mean AI of any dose group

TABLE 6

Study II: Baseline Tail Flick Latency in Enkephalinase Inhibitor Test
Mean Latency ± Standard Error (seconds)

Dose Group	Saline	Naloxone
1 μg	3.45 ± 0.034	3.425 ± 0.080
3 μg	3.583 ± 0.052	3.492 ± 0.040
9 μg	3.45 ± 0.072	3.383 ± 0.082
25 μg	3.683 ± 0.044	3.592 ± 0.056

were observed and a mean AI of 22.5 MPE. Min was produced by stimulation. The differences between saline and naloxone pretreatment at each dose group in the stimulation condition were found to be non-significant.

Following saline pretreatment enkephalinase inhibitor + stimulation produced dose dependent increases in AI. The mean AI of the 1, 3, 9 and 25 µg dose group were 204.2, 402.42, 897.0 and 1927.0 MPE. Min respectively. Mean AI was increased significantly between each dose group, hence the F ratio of comparisons between means of dosage groups 1 to 3, 3 to 9 and 9 to 25 were ($F(1,20)$ 10.92, 68.0, 294.9 respectively, $p < .01$ all). Following naloxone pretreatment the differences in AI between dose groups were not significant and the mean AI across dose group was 20.4 MPE. Min. In the enkephalinase inhibitor + stimulation condition, differences in AI at each dose level between naloxone and saline pretreatment were significant ($F(1,20)$ 5.29, 13.03, 92.5 and 580.5 at 1, 3, 9 and 25 µg dose groups respectively).

Finally the means at each dose level were compared between conditions. Following saline injection AI scores were significantly greater in enkephalinase inhibitor + stimulation conditions as compared with the stimulation condition for each dose group. F values for these comparisons were $F(1,20)$ 5.39, 12.72, 89.77 and 577.3 for dose groups 1, 3, 9 and 25 µg respectively. No such differences between conditions were observed under naloxone pretreatment.

TABLE 7

Study II: Effect of Stimulation and Enkephalinase Inhibitor + Stimulation on Nociceptive Response

STIMULATION

Antinociceptive Index ± Standard Error (MPE.Min)

Pretreatment	Saline	Naloxone
Dose Group		
1 μg	20.103 ± 3.37	18.692 ± 4.23
3 μg	28.348 ± 6.41	25.868 ± 8.038
9 μg	28.203 ± 5.1	18.033 ± 3.48
25 μg	23.017 ± 4.71	27.533 ± 5.75

ENKEPHALINASE INHIBITOR + STIMULATION

Antinociceptive Index + Standard Error (MPE . Min)

Pretreatment	Saline	Naloxone
Dose Group		
1 μg	204.163 ± 23.77	21.925 ± 5.48
3 μg	402.422 ± 69.7	24.842 ± 6.4
9 μg	897.01 ± 43.3	17.10 ± 4.19
25 μg	1926.97 ± 115.60	17.767 ± 4.03

Saline + Stimulation: Antinociceptive Index Means for each dose group following saline and naloxone pretreatment in the saline + stimulation condition are presented in Table 8. An analysis of variance was performed to assess the significance of differences between appropriate means. No significant differences between dose group means within pretreatment in this condition were observed. The mean AI across dose groups for the saline pretreatment was 33.14, whereas the mean AI was 25.37 following naloxone pretreatment. Similarly differences between pretreatments at each dose level were also non-significant.

Saline + Stimulation, 1 microgram Dose Group: A comparison of the analgesia produced by saline + stimulation and by enkephalinase inhibitor + stimulation under saline pretreatment was performed using a correlated t-test. Since it was predicted that the smallest dose of enkephalinase inhibitor would produce the minimum effect, the one microgram dose group was selected for the comparison. The differences in AI between the two conditions was significant (t obs (5) 7.1) implying that the smallest dose of enkephalinase inhibitor + stimulation produced an effect greater than saline + stimulation. A similar test was performed between saline stimulation and stimulation conditions under saline pretreatment within the 1 microgram dose group. This difference in mean AI was not significant.

Enkephalinase Inhibitor: Statistical analysis of the enkephalinase inhibitor condition was performed using Newman-Keuls tests (Winer, 1962). Mean AI for each dose group

TABLE 8

Study II: Effect of Saline + Stimulation on Nociceptive Response

Antinociceptive Index ± Standard Error (MPE.Min)

Pretreatment Dose Group	Saline	Naloxone
1 μg	22.531 ± 3.81	21.828 ± 5.87
3 μg	45.527 ± 9.01	21.765 ± 6.03
9 μg	29.44 ± 5.15	20.572 ± 3.88
25 μg	35.068 ± 6.86	37.833 ± 6.57

under each pretreatment is presented in Table 9. Statistical tests demonstrated that the mean AI of the 25 microgram dose group following saline pretreatment was significantly greater than all other levels of enkephalinase inhibitor ($p < .01$). The 9 microgram dose group demonstrated analgesia significantly greater than the 3 microgram dose group under saline pretreatment.

SUMMARY OF RESULTS

Figure 4 presents the relation of intensity in MPE with duration of analgesic effect for each dose group of the enkephalinase inhibitor + stimulation condition. The MPE values at each test trial were pooled for saline + stimulation and stimulation under saline (as differences were not significant) and presented in Figure 4. Similarly, pooled naloxone results were also presented in Figure 4, since no differences in MPE were observed for naloxone pretreatment data.

Figure 5 presents the relation of enkephalinase inhibitor dose in the enkephalinase inhibitor + stimulation condition (saline pretreatment) to the resulting antinociceptive index and regression line formed by the least mean of squares. The relation between EI dose and AI as presented in Figure 5 appears linear.

TABLE 9

Study II: Effect of Enkephalinase Inhibitor + Stimulation on Nociceptive Response

Antinociceptive Index ± Standard Error (MPE.Min)

Pretreatment Dose Group	Saline	Naloxone
1 μg	2.767 ± 3.6	0.167 ± 2.79
3 μg	4.667 ± 7.8	4.333 ± 3.59
9 μg	45.567 ± 19.7	2.167 ± 3.06
25 μg	101.522 ± 28.14	3.650 ± 2.59

Figure 4. Maximum percent effect (MPE) of four enkephalinase inhibitor doses at 2 min. intervals for duration of the analgesic effect.

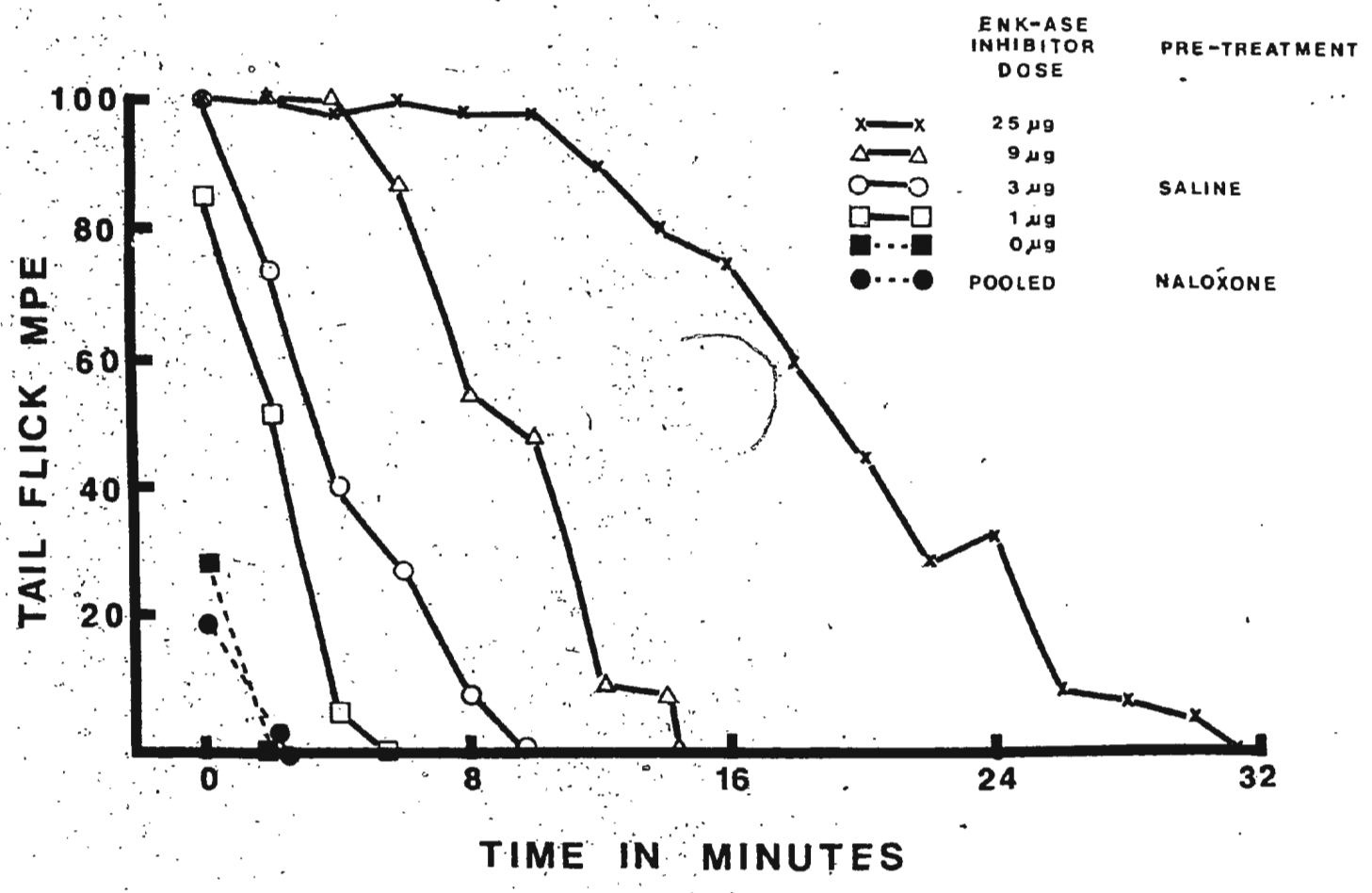
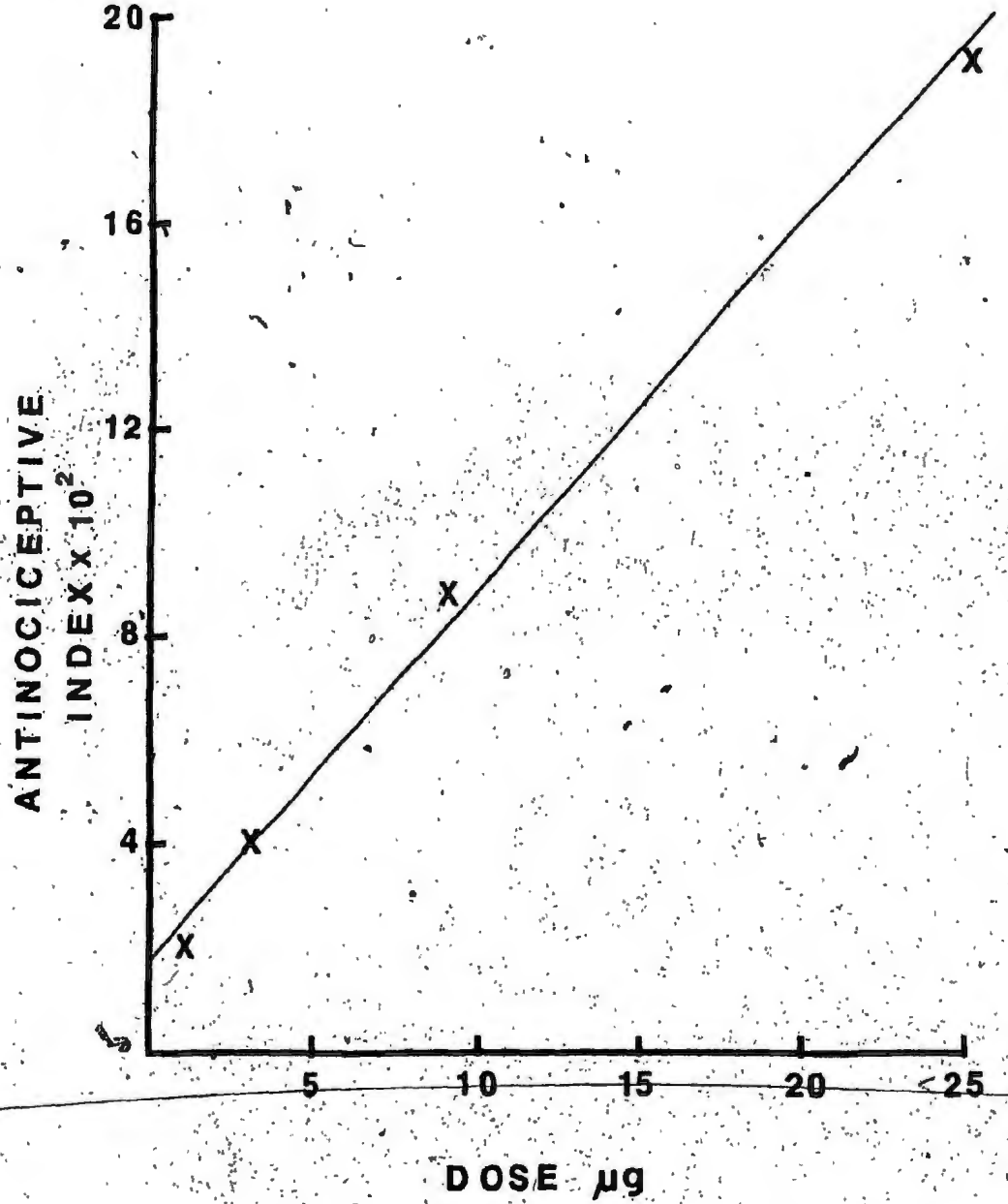

ENK-ASE INHIBITOR DOSE
PRE-TREATMENT
25 µg
9 µg
3 µg
1 µg
0 µg
POOLED
SALINE
NALOXONE
TAIL FLICK MPE
100
80
60
40
20
0
8
16
24
32
TIME IN MINUTES

Figure 5. Dose response curve demonstrating the relationship between enkephalinase inhibitor does in µg and the antinociceptive index in PAG stimulated rats. Regression line plotted from least mean squares.


20
16
12
8
4
0
ANTINOCICEPTIVE INDEX X 10^2
X
X
X
X
5
10
15
20
<25
DOSE µg

DISCUSSION

Figure 4 clearly demonstrates the effect of enkephalinase inhibitor on analgesia produced by PAG stimulation. The area under the intensity vs. duration curves is directly related to the dose of enkephalinase inhibitor administered. It was demonstrated that the intensity of each dose over 1 microgram in the first two analgesia tests is limited by the cutoff point and therefore assumed maximal or 100% MPE. This ceiling effect is difficult to eliminate without inducing tissue damage. It is difficult to differentiate antinociceptive effects at a point where extremely limited degrees of pain are perceived. However, from the trend of the intensity duration curve it may be postulated that the intensities of analgesia in the initial period after administration was greater for Ss receiving larger doses of enkephalinase inhibitor. The problem of ceiling effect in analgesic intensity is circumvented by assessing the intensity of analgesia with respect to duration, allowing an estimate of the total analgesic effect of a drug. Figure 4 demonstrates that the intensity of analgesia at each test following saline pretreatment is directly related to dose of enkephalinase inhibitor. The curves for the pooled naloxone treated, and the curves for the stimulation pooled with saline + stimulation following saline pretreatment are also presented in Figure 4. Both control procedures demonstrate significantly reduced areas of antinociceptive index as compared with the

curves of enkephalinase inhibitor + stimulation administered and saline pretreated subjects. Therefore it has been demonstrated that enkephalinase inhibitor potentiates SPA in a dose dependent naloxone reversible manner. Figure 5 demonstrates the relation between enkephalinase inhibitor dose and antinociceptive index. This relationship is linear. Since enkephalinase inhibitor acts competitively it is postulated that enzyme active site blockage is linearly related to enzyme inhibition of enkephalin catabolism. Therefore the linear enkephalinase inhibitor dose to response relation suggests that antinociception may be linearly related to the concentration of enkephalin in the PAG and presumably, as enkephalin effects are receptor mediated, the number of molecules at the receptor site.

It was also demonstrated that enkephalinase inhibitor administration alone is directly related to antinociceptive index in a naloxone reversible manner. The reduced analgesic effect in this condition as compared to the enkephalinase inhibitor + stimulation condition supports the contention that SPA releases enkephalin into the PAG. The linearity of the enkephalinase inhibitor alone to antinociceptive relationship however suggests again that enkephalin concentration in the PAG is inversely related to nociception. It is concluded that enkephalin within the PAG tonically (in this test situation) modulates the transmission of noxious information and therefore the perception of pain.

Study II has demonstrated that the potentiation of the antinociceptive effect of SPA is dependent on the dose of

enkephalinase inhibitor. It appears that a substantial portion of the analgesic effect of PAG stimulation is directly and linearly related to the concentration of enkephalin at the receptor site. Therefore it is proposed that enkephalin released in the PAG is responsible for analgesia and the concentration of enkephalin is linearly related to the antinociceptive index.

STUDY III

THE SPINAL MECHANISM OF ENKEPHALINASE INHIBITOR

Stimulation produced analgesia and morphine analgesia induced from the PAG are proposed to share a common mechanism of action. This mechanism has been demonstrated as an activation of serotonergic containing cells of the nucleus raphe magnus which project to lamina I and V of the spinal cord leading to an inhibition of noxious input by the release of serotonin (Yaksh and Wilson, 1979; Yaksh and Tyce, 1979).

It has been demonstrated in Study II that enkephalinase inhibitor potentiates SPA in a dose dependent manner. The conclusion that SPA is mediated by enkephalin release and therefore that endogenous enkephalin release in the PAG modulates pain threshold can be tested by demonstrating that the potentiation effect utilizes an identical mechanism to stimulation, and to opiate induced analgesia. The present study attempts to define the mechanism by which enkephalinase inhibitor potentiates SPA. It is hypothesized that as SPA and opiate analgesia utilize descending serotonergic fibers, a specific lesion of these fibers will eliminate any effects that involve a common mechanism. The demonstration that potentiation of SPA by enkephalinase inhibitor is attenuated by destruction of these descending serotonergic fibers is conclusive evidence that stimulation produced analgesia is mediated by a release of enkephalin and that endogenous release of enkephalin in the PAG acts to modulate the transmission of noxious input by a serotonergic mechanism.

An experimental design similar to that used in Studies I and II is used to determine the effects of enkephalinase inhibitor on SPA in animals to which were spinally administered selective neurotoxins. Selective lesion of 5HT and NE fibers is compared to saline treated controls. It is hypothesized that the potentiation effect of enkephalinase inhibitor on SPA will be attenuated in 5HT lesioned animals, identifying the mechanism of PAG enkephalin modulation of nociception and further demonstrating that enkephalin modulates pain perception.

METHOD

Subjects: Eighteen of the twenty-four subjects used in Study II and randomly assigned to one of three neurotoxin treatment groups were used as subjects in Study III.

Surgery: Subjects in the serotonin depletion (5, 7 DHT) treatment group were administered dimethylimipramine 25 mg/kg IP, while Ss in the norepinephrine depletion treatment group were administered imipramine 25 mg/kg IP 30 mins prior to surgery. All subjects were anaesthetized with Nembutal (45 mg/kg), and were placed in a stereotaxic instrument with the nose bar set at -10mm. The spinal cord was placed in traction by extending the hindlimbs. Following a small incision, the first layer of neck muscle was removed from the occipital bone. The remaining neck muscle was separated revealing the spinal vertebrate. The edge of a 20 gauge needle was used to break the membrane between the occipital bone and the vertebra of C_1. The appropriate neurotoxin (5, 7 dihydroxytryptamine, 6 micrograms, in 2 microliters of 2% ascorbic acid

saline solution or 6 hydroxydopamine, 4 micrograms in 2 microliters of 2% ascorbic acid saline) or physiological saline was administered bilaterally to the exposed cord at co-ordinates L ± 0.7, DV -1.5 (from the surface of the cord) through a 26 gauge cannula connected to a 5 microliter Hamilton syringe by a plastic tubing. Each injection solution was administered over a period of 5 minutes to minimize nonspecific tissue damage. Following the injection, the muscle and incision was sutured, and Ss were allowed 5 days of post surgery recovery.

5, 7 dihydroxytryptamine (creatinesulfate) and 6, hydroxydopamine (HBr) were obtained from Sigma Laboratories. Dimethylimipramine was obtained from Geigy Laboratories. Imipramine was obtained from Mount Royal Chemicals.

Procedure and Apparatus

All analgesic test equipment and procedures were identical to those described in Study I. Stimulation currents for each subject were identical to those determined in Median Analgesic Current tests in Study II. Only one dose of enkephalinase inhibitor (6 micrograms /0.5 microliters saline) was administered in Study III. All subjects received saline fifteen minutes prior to baseline response latency tests. Only one session of the enkephalinase inhibitor test was performed.

Histology and Monoamine Assay

Subjects not participating in Study III were sacrificed, perfused, and their brains prepared for histological examination as in Study I.

On the two days following the test session of Study III, all subjects were sacrificed by decapitation. Brains were removed and placed in sucrose formalin overnight. Histological sections of brains were taken for identification of electrode and cannula placements as described in Study I. Spinal cords of subjects were extracted on ice, weighed and homogenized in Butanol (5 ml). Norepinephrine and serotonin levels in each spinal cord were determined using the fluorometric technique of Jacobowitz and Richardson, (1978). Briefly norepinephrine was extracted in phosphate buffer and reacted with Versene, and serotonin was extracted in HCl and reacted with orthophthaldialdehyde to form active fluorophores. The fluorescence was then measured in a Turner Model 430 spectrophotofluorometer at excitation 385/emission 485 and 360/470 for NE and serotonin level determination respectively. Fluorescence reading was then converted into nanogram of monoamine per gram tissue by comparison with the appropriate standard (0.1 microgram of monoamine) similarly extracted.

Data Analysis

A single dependent measure, antinociceptive index, was calculated for each subject in each condition as described in Study II. Treatment differences in baseline and test conditions were determined by comparisons of means using a MSW calculated in a (1 x 3) and (3 x 4) analysis of variances respectively. When differences between two treatment groups were not significant, the combined mean AI of the two treatment groups was compared to the third group. Analyses of variance were also used to calculate MSWs used in a determination of differences between spinal norepinephrine and serotonin

levels.

RESULTS

Enkephalinase Inhibitor Test

Baseline: The mean baseline tail flick latency for each treatment group is presented in Table 10. It was found that baseline responses of 5, 7 DHT ($\bar{X}$ = 3.3 s) and 6-OHDA (3.0 s) treated groups were not significantly different, but the combined mean baseline latency of neurotoxin treated groups (3.2 s) was significantly smaller than the latency observed in saline treated subjects (3.8 s) ($F(1, 15) = 16.52$, $p < .01$).

Between Treatment: The mean antinociceptive index for each condition of the treatment groups is presented in Table 11. The mean AI scores for saline treated Ss within stimulation ($\bar{X}$ = 30.55), enkephalinase inhibitor + stimulation (574.85), saline + stimulation (29.4) and enkephalinase inhibitor (20.85) condition were not found to be significantly different from the scores of 6-OHDA pretreated subjects in the identical condition ($\bar{X}$ = 27, 591.1, 37.4, and 17.53) respectively. Therefore combined saline and 6-OHDA means were tested against mean AI scores of 5, 7 DHT treated subjects in each condition. The mean antinociceptive index scores of 5, 7 DHT treated Ss were (7.1, 5.5, 6.9, 3.2) for stimulation, enkephalinase inhibitor + stimulation, saline + stimulation and enkephalinase inhibitor conditions respectively. Differences between pooled saline and 6-OHDA groups, and 5, 7 DHT treated groups were significant only within the enkephalinase

TABLE 10

Study III: Baseline Tail Flick Latency in Enkephalinase Inhibitor Test

Treatment Group	Mean Latency (sec) ± Standard Error
5, 7-DHT	3.325 ± 0.10
6-OHDA	3.025 ± 0.09
Saline	3.758 ± 0.07

TABLE 11

Study III: Effects of Spinal Treatment on Antinociceptive Response in the Enkephalinase Inhibitor Test

Mean Antinociceptive Index ± Standard Error (MPE.Min)

CONDITION	TREATMENT GROUP		
	Saline	6-OHDA	5,7-DHT
Stimulation	30.55 ± 4.18	27.07 ± 5.60	7.05 ± 2.98
Enkephalinase Inhibitor + Stimulation	574.85 ± 20.5	591.08 ± 10.20	5.53 ± 1.82
Saline + Stimulation	29.37 ± 6.20	37.367 ± 4.87	6.85 ± 1.91
Enkephalinase Inhibitor	20.85 ± 6.44	17.53 ± 4.01	3.23 ± 3.51

inhibitor + stimulation condition (F (1,30) 1778.4, $p < .01$).

Within Treatment: The differences within treatment groups between stimulation and enkephalinase inhibitor conditions were significant in both the 6-OHDA and the saline but not in the 5, 7 DHT treated groups (F (1,30) 1696.6, 1580.13, $p < .01$) respectively. Differences in AI scores between saline stimulation and enkephalinase inhibitor were similarly significant in 6-OHDA and saline treated Ss, but not in 5, 7 DHT treated Ss (F (1,30) 1635.3; 1587.0, $p < .01$). The means of stimulation and saline + stimulation were not significantly different in any treatment group. t-tests were performed to determine the difference of mean AI of enkephalinase inhibitor condition from baseline. Within the saline and the 6-OHDA, but not the 5,7 DHT groups, enkephalinase inhibitor condition was significantly greater than baselines (T (5) 2.96 and 3.99 respectively $p < .05$ both).

Spinal Monoamine Assay

Spinal concentration of norepinephrine and serotonin as determined by fluorometric assay are presented for each treatment group in Table 12. Non-significant differences in NE level between saline and 5, 7 DHT were found, therefore the mean concentration of NE was pooled for these groups. NE levels were significantly lower in the 6-OHDA treated group as compared to the NE levels of the pooled saline and 5, 7 DHT groups (F (1,15) 66.43, $p < .01$).

Spinal concentration of serotonin was not significantly different between 6-OHDA and saline treated subjects. Serotonin levels were significantly lower in 5, 7 DHT treated groups as compared with pooled serotonin levels of 6-OHDA

TABLE 12

Study III: Action of Specific Neurotoxin on Spinal Monoamine Content

Monoamine Level (nanogram/gram tissue) ± Standard Error

Treatment	Norepinephrine	Serotonin
Saline	232.3 ± 14.3	250.8 ± 18.5
6-OHDA	*59.26 ± 6.75 (25.5%)	257 ± 18.4
5,7-DHT	233.3 ± 25.8	*46.0 ± 8.9 (18.3%)

* $p < .01$

and saline treated Ss ($F(1, 15)$ 122.16, $p < .01$).

Histology

Histological examination of brain sections demonstrated that the tips of all SPA producing electrodes were in, or adjacent to the PAG within 0.2mm from AP + 1mm. (See Figure 6). Guide cannulae tip placements were found within the PAG region 0.2 mm from AP - 1 mm. (See Figure 7). No obvious differences in electrode or cannula placements between dose groups or treatment groups were found.

DISCUSSION

Study III has clearly demonstrated that enkephalinase inhibitor potentiation of SPA is attenuated by lesion of spinal serotonergic containing neurons. Since SPA has been shown to be mediated by spinal serotonergic neurons, it must be concluded that the potentiation effect acts by an identical mechanism. Since the potentiation is induced by an inhibition of enkephalin catabolism this study has demonstrated as in vivo analgesic effect of PAG enkephalin, which is mediated by a similar mechanism to that of opiate and stimulation produced analgesia.

Interestingly, baseline tail flick response latency was decreased by spinal administration of 6-OHDA, however SPA and the potentiation of SPA by enkephalinase inhibitor was not reduced in animals spinally depleted of norepinephrine. Takagi et al (1978) and Kuraishi et al (1979) have suggested that morphine analgesia and tonic modulation of noxious input from the NRPG is mediated by spinal NE containing neurons. The demonstration of depressed pain threshold in 6-OHDA treated animals is congruent with this hypothesis of tonic influence.

Figure 6. Schematic diagram of electrode tip sites of rats demonstrating stimulation - produced analgesia in Studies II & III [Numbers refer to co-ordinates from the intra-aural line in millimeters. Taken from Pellegrine & Cushman, (1967)].

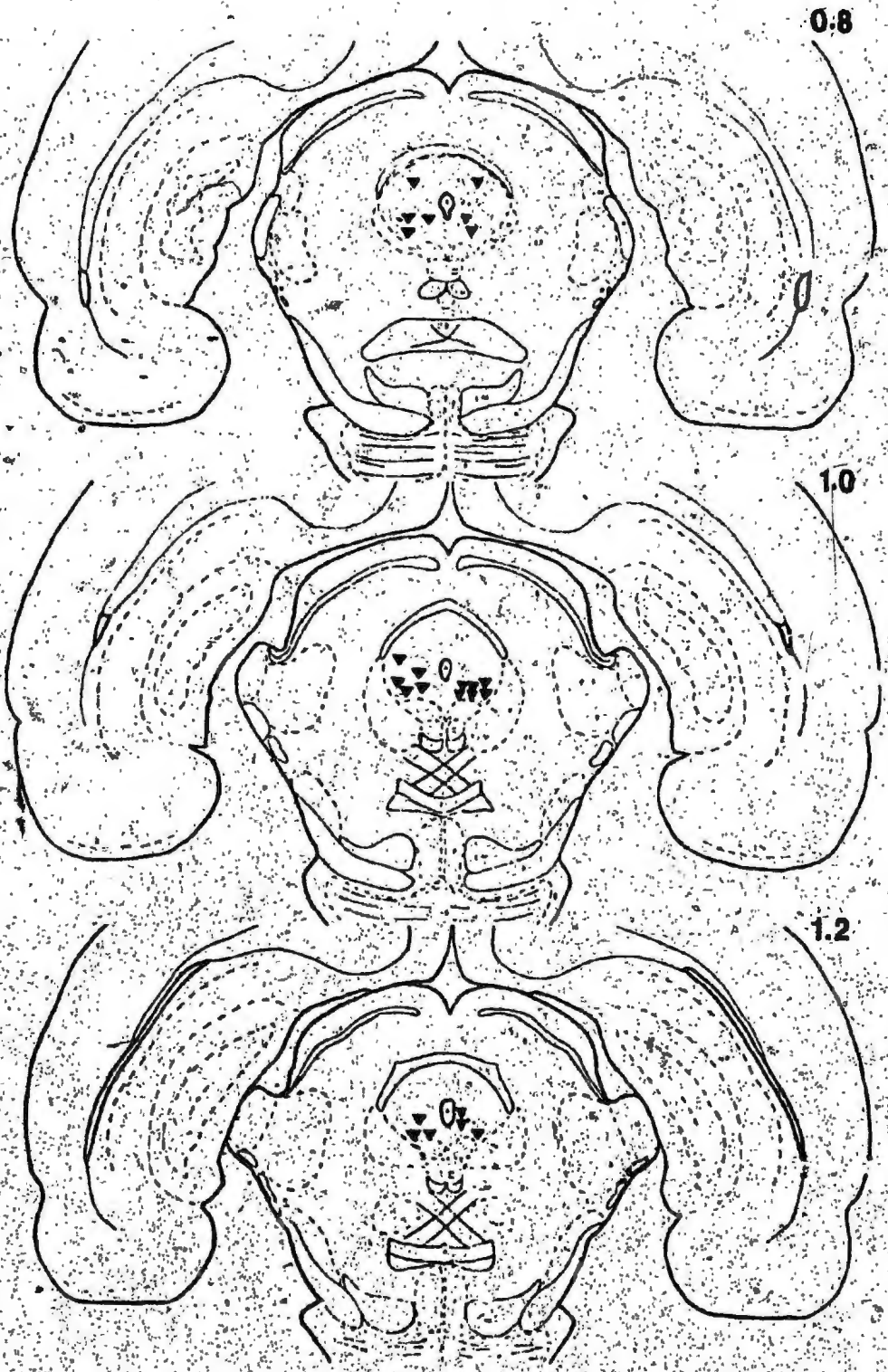
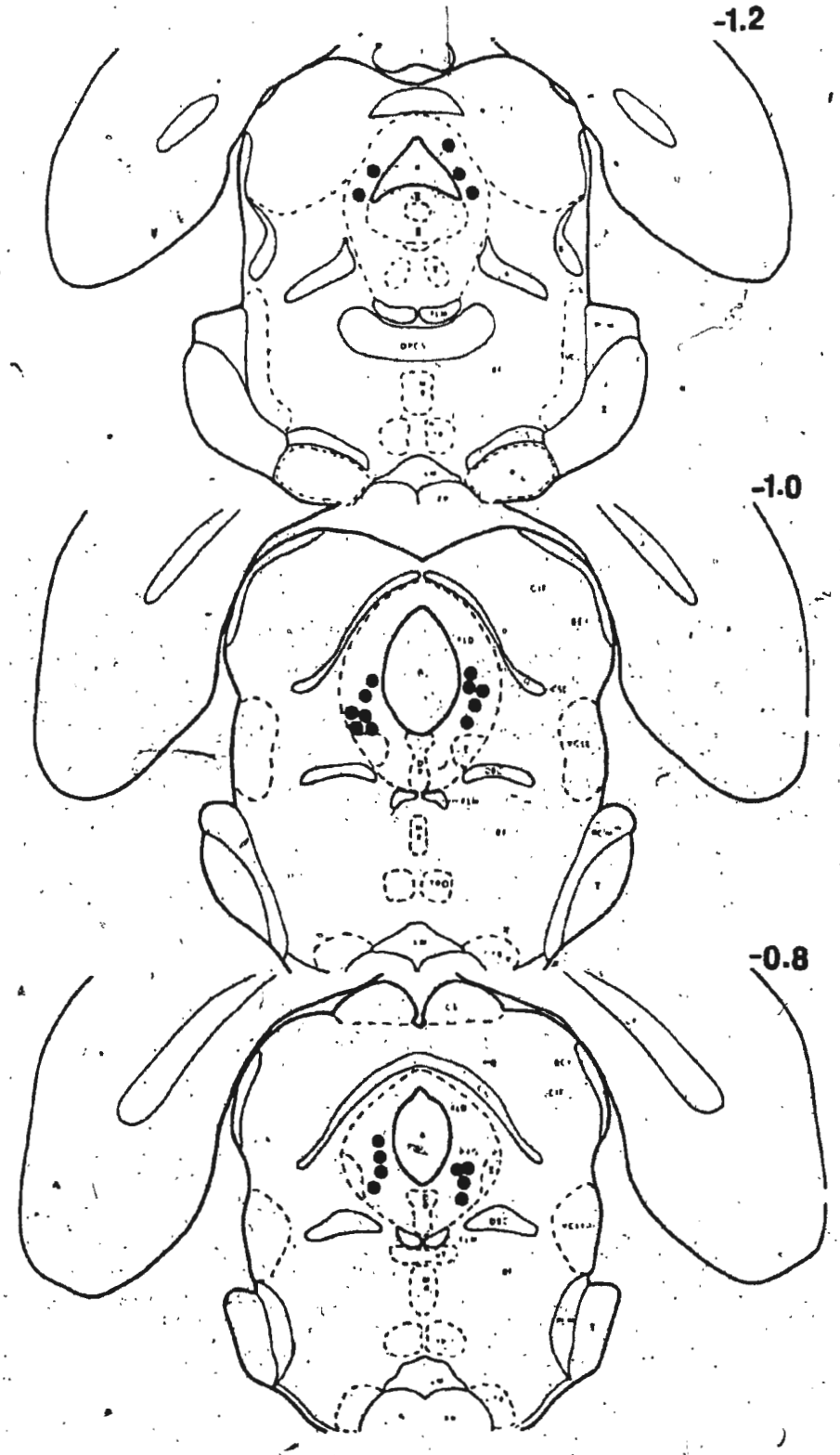

0.8
1.0
1.2

Figure 7. Schematic diagram of guide cannulae tip sites of rats demonstrating stimulation - produced analgesia in Studies II & III [Numbers refer to co-ordinates from the intra-aural line in millimeters. Taken from Pellegrino & Cushman, (1967)].


-1.2
-1.0
-0.8

However the lack of reduction of SPA and potentiation supports the postulation that both effects are initiated in the PAG and are mediated by serotonergic but not norepinephrine fibers.

It was demonstrated that enkephalinase inhibitor alone induces a significant elevation of tail flick latency from baseline in saline and 6-OHDA but not 5, 7 DHT treated Ss. Therefore it may be suggested that an enkephalin modulation of nociception using a descending serotonergic pathway is active during the test situation in the PAG.

It is concluded from Study III that SPA is mediated by PAG enkephalin activation of bulbospinal serotonergic fibers as lesions of these fibers eliminate both SPA and the potentiation of analgesia by enkephalinase inhibitor. Therefore enkephalin released from the PAG has been demonstrated to induce analgesia via a serotonergic spinal mechanism.

DISCUSSION AND CONCLUSIONS

Nociception is modulated by an inhibitory bulbospinal system which can be activated from the PAG by electrical stimulation or opiate administration. The identification of opiate receptors and enkephalin containing cell bodies within the PAG leads to the hypothesis that endogenous opiate peptides inhibit nociception.

The rapid development of experiments aimed at corroborating this hypothesis has resulted in supportive but circumstantial evidence. Thus PAG administration of enkephalin which produces a short lived analgesia does not demonstrate endogenous release. Opiate antagonist and intraventricular catabolic inhibitor administration fails to localize a site of enkephalin or endorphin pain threshold modulation and requires the endogenous release of endorphins that appear to be dependent on test situation. Finally endorphins released by PAG stimulation have not been shown to be directly responsible for the analgesia observed in this paradigm. The present experiment attempted to more directly study the involvement of PAG enkephalin in the modulation of nociception.

As a result of the three studies it is concluded that enkephalin released from cells in the PAG induced analgesia. Study I demonstrated that the inhibition of enkephalin catabolism within the PAG potentiates the intensity and duration of analgesia produced by PAG stimulation, in a naloxone reversible manner. This potentiation of analgesia as measured by the increase in AI is dependent upon the dose of

enkephalinase inhibitor administered and is consistently and totally attenuated by naloxone administration as demonstrated in Study II. This result indicates that the potentiation effect is dependent upon the concentration of enkephalin in the PAG. It is concluded that a significant fraction of the analgesia produced by PAG stimulation is mediated by enkephalin release and therefore enkephalin within the PAG modulates nociception. Finally it has been demonstrated that a part of the mechanism responsible for the potentiation of SPA enkephalinase inhibition is identical to the bulbospinal serotonergic mechanism demonstrated to mediate opiate and stimulation induced analgesia. Therefore, from the results of Study III it is concluded that the antinociceptive effect of enkephalin released in the PAG is the result of an activation of a spinal serotonergic pathway.

The results of the enkephalinase inhibitor alone condition of the present experiment indicates that inhibition of enkephalin catabolism within the PAG can produce a dose dependent naloxone reversible analgesia by a serotonergic mechanism. This result supports the hypothesis that PAG enkephalin modifies nociception. The observed antinociceptive effect which relies on endogenous release of enkephalin in the test situation is of lesser intensity and duration than that observed in the stimulation + enkephalinase inhibitor condition. This discrepancy indicates that enkephalin is released by PAG stimulation, and that this release of enkephalin is responsible for the demonstrated

analgesia.

Naloxone pretreatment completely abolished the analgesia observed in the enkephalinase inhibitor alone condition, implicating opiate receptor mediation of the antinociceptive action of increased PAG enkephalin levels. Naloxone, however, did not significantly reduce baseline tail flick latency in any of the three studies. Therefore it is concluded that opiate receptor blockade does not increase the pain threshold in this test situation in agreement with Yaksh, Yeung and Rudy, (1976).

Werthe and Frederickson (1979) demonstrated that stress increases enkephalin levels and produces a naloxone reversible analgesia. The habituation trials given to Ss in the present studies may have reduced the stress and therefore the release of enkephalin in response to the test situation explaining the lack of a significant naloxone effect on baseline response. It must be proposed however, in view of the results of the enkephalinase inhibitor alone condition, that a minimal release of enkephalin is present in the PAG, either tonically or in response to intracerebral injection. Therefore, application of the enkephalinase inhibitor acts to unmask this minimal release of enkephalin, and demonstrates naloxone reversible antinociceptive action of PAG enkephalin.

Naloxone pretreatment also fails to completely eliminate the analgesia induced by PAG stimulation. Although it is possible that this lack of naloxone reversibility is due to a greater affinity of enkephalin, rather than naloxone, for enkephalin receptors (Chang and Cuatracasas, 1979), this

explanation is unlikely, as administration of IP naloxone, at doses over 1 mg/kg, has been demonstrated to functionally inactivate all opiate receptors of the body within 15 minutes (Grevert and Goldstein 1977). The lack of total naloxone reversal of SPA is in agreement with the findings of Akil (1976) and Pert and Walter (1976) who accounted for the presence of SPA in naloxone pretreated Ss by suggesting that the stimulating current activates some non-enkephalinergic neurons within or adjacent to PAG, which induces analgesia. This proposal is strenghtened by the recent findings of the analgesia produced by PAG administration of Baclofen (Levey and Proudfit, 1979). Similarly, since SPA was significantly reduced by spinal 5, 7 DHT administration it is possible that naloxone non-reversible SPA is due to direct stimulation of spinal serotonergic fibers similar to the analgesia produced by NRM stimulation (Oliveras et al, 1977) which is postulated as a later stage in PAG enkephalin induced analgesia.

The results of the present experiment directly demonstrate a potentiation of the antinociceptive effect of PAG stimulation by inhibition of PAG enkephalinase. Pretreatment with naloxone completely inhibited the potentiation effect of the enkephalinase inhibitor indicating that the increase in antinociception was due to an opiate receptor action. Since the locus of pharmacological manipulation was limited to the PAG and the catabolic inhibitor was specific for enkephalinase, it must be concluded that the observed potentiation of SPA was due to a PAG enkephalin action at the opiate receptor.

The effect of spinal neurotoxin treatment on baseline tail flick latency in Study III demonstrates that both 5HT and NE spinal systems are involved in the modulation of antinociception in the test situation. However the enkephalinase inhibitor test demonstrated that spinal serotonergic, but not norepinephrine fibers, are involved in SPA, the antinociceptive effect of enkephalinase inhibitor alone, and the potentiation of SPA by enkephalinase inhibitor administration to the PAG. The antinociceptive effect of NRPG stimulation which is proposed to be mediated by spinal NE neurons (Akaike, 1978) has therefore been shown to be independent of the analgesic effect of enkephalin within the PAG. This independence of bulbospinal pathways involved in modulation of nociception is supported by the anatomical findings of Basbaum et al (1978). It was demonstrated that two separate bulbospinal pathways terminate specifically in laminae receiving noxious stimuli, one pathway originates in the NPGC, postulated to contain NE (Satoh, et al, 1979), the other originates in the NRM, which is serotonergic. Therefore the analgesic effect observed from enkephalin action within the PAG is mediated by stimulation of the serotonergic bulbospinal systems of the NRM, and appears to be distinct from the NE system originating in the NPGC, which may also modulate nociception.

Future experiments which must be performed in order to allow a lucid understanding of SPA involve the identification of the alternate pathways that induce analgesia when stimulated. These studies would require pharmacological manipulation

of the transmitter system under investigation and microstimulation techniques. For example, the physiological significance of the bulbospinal NE system originating in the NPGC has yet to be identified. Additionally the importance of β-endorphin in SPA must be evaluated. Although β-endorphin action appears important in human SPA (Akil et al, 1978) the action of the larger endorphin appears minimal in rats as implied by this experiment and by Stein (cited by Liebeskind, 1978). This may be due to greater importance of β-endorphin as compared to enkephalin in antinociception in man, the reverse being true in the rodent as implied by the relative concentrations of the two peptides in the PAG region (Gramsch et al 1979; Bloom et al, 1979). Watson et al (1978) demonstrated that rodent hindbrain β-endorphin can be significantly reduced by basal tuberal hypothalamic lesion. The relative contribution of β-endorphin and enkephalin in analgesia produced by stimulation of the PAG in rodent can therefore be studied using basal tuberal hypothalamic lesions, and enkephalinase inhibitor as experimental manipulations.

The other area in which research must now be focused is the nature of stimuli which induce the release of enkephalin from the PAG. The preliminary work in this area has been performed by Hayes et al (1978). Naloxone reversibility of analgesic tests was used to measure opiate release following the application of specific stimuli including stress. These experiments can be performed more directly by the application of enkephalinase inhibitor to the PAG.

concomitant with natural stimuli which are proposed to release enkephalin, and the induced analgesia used to indicate positive results.

The present experiment has demonstrated that the administration of enkephalinase inhibitor to the PAG produces naloxone reversible analgesia indicating that enkephalin released from the PAG in this test situation modulates pain perception. The administration of enkephalinase inhibitor to the PAG potentiates the analgesia produced by PAG stimulation in a dose dependent naloxone reversible manner.

It may be concluded that stimulation produced analgesia is mediated, at least in part, by the release of enkephalin from cells of the PAG. This released enkephalin stimulates opiate receptors, and induces analgesia via a bulbospinal serotonergic system. Therefore enkephalin in the PAG acts to modulate the perception of pain.

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APPENDIX A

TABLE 1

Study I: Intensity of Analgesia (Latency of Tail Flick Response)

Source	df	SS	MS	F
Subject	13	9.25	0.711	
Pretreatment	1	15.22	15.22	78.86
Subject x Pretreatment	13	2.512	0.193	
Condition	4	64.991	16.248	132.1
Subject x Condition	52	6.392	0.123	
Pretreatment x Condition	4	36.1	0.125	72.78
Subject x Pretreatment x Condition	52	6.462	0.124	
TOTAL	139	140.926		

TABLE 2

Study I: Duration of Analgesia

Source	df	SS	MS	F
Subject	13	30.429	2.341	
Pretreatment	1	128.571	128.571	39.39
Subject x Pretreatment	13	42.429	3.264	
Condition	3	218.572	72.857	52.2
Subject x Condition	39	54.428	1.396	
Pretreatment x Condition	3	166.571	55.52	27.6
Subject x Pretreatment x Condition	39	78.429	2.011	
TOTAL	111	719.429		

TABLE 3

Study II: Baseline Latency

Source	df	SS	MS	F
Dose Group	3	0.371	0.124	1.459
Error	20	1.697	0.085	
Pretreatment	1	0.057	0.057	3.061
Dose Group x Pretreatment	3	0.009	0.003	0.160
Error	20	0.371	0.019	
TOTAL	47	2.505		

TABLE 4

Analgesic Effect of Stimulation and Enkase Inhibitor + Stimulation (Antinociceptive Index)

Source	df	SS	MS
Dose	3	2668840	88961.0
Error	20	214478	10723.9
Condition	1	4139370	4139370
Dose X Condition	3	2633710	877903.0
Error	20	189404	9470.23
Pretreatment	1	4229760	4229760
Dose X Pretreatment	3	2667610	889203.0
Error	20	170068	8503.38
Condition X Pre-treatment	1	4181810	4181810
Dose X Condition X Pretreatment	3	2706440	902147.0
Error	20	182016	9100.8
TOTAL	95	23983500	

TABLE 5

Study II: Analgesic Effect of Saline + Stimulation (Antinociceptive Index)

Source	df	SS	MS	F
Dose	3	1669.91	556.637	2.086
Error	20	5336.62	266.831	
Pretreatment	1	700.779	700.779	2.593
Dose X Pretreatment	3	1253.43	417.81	1.546
Error	20	5405.64	270.282	
TOTAL	47	14366.400		

TABLE 6

Study II: Analgesic Effect of Enkephalinase Inhibitor (Antinociceptive Index)

Source	df	SS	MS	F
Dose	3	19908.7	6636.24	5.403
Error	20	24565.1	1228.26	
Pretreatment	1	15596.3	15596.3	14.243
Dose X Pretreatment	3	18811.6	6270.52	5.726
Error	20	21900.3	1095.02	
TOTAL	47	100782.0		

TABLE 7

Study III: Baseline Latency of Tail Flick Response

Source	df	SS	MS	F
Treatment	2	1.631	0.816	15.02
Error	15	0.815	0.054	
TOTAL	17	2.446		

TABLE 8

Study III: Test Session (Antinociceptive Index)

Source	df	SS	MS	F
Treatment	2	411959	205979.0	408.29
Error	15	7567.37	504.49	
Condition	3	1854270	618090.0	1506.64
Treatment X Condition	6	928528.0	154755.0	377.225
Error	45	18461	410.24	
TOTAL	71	3220780		

TABLE 9

Study III: Assay of Monoamine Content

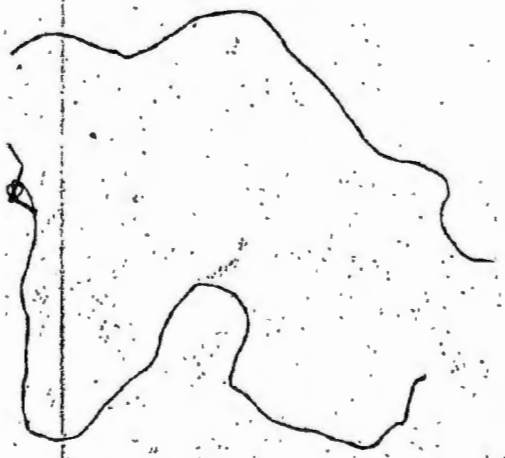
Norepinephrine

Source	df	SS	MS	F
Treatment	2	120505.0	60252.3	39.32
Error	15	22983.9	1532.26	
TOTAL	17	143488.00		

Serotonin

Source	df	SS	MS	F
Treatment	2	172976.0	86466.0	68.412
Error	15	18963.3	1264.22	
TOTAL	17	191939.0		

APPENDIX B



APPENDIX B

MPE Analgesia. Intensity of analgesia in MPE as calculated from tail flick latency is presented in Figure B1. Comparisons between the mean MPE produces identical significant differences, as when compared as, latency data, as demonstrated in Table B1. Under saline pretreatment, enkephalinase inhibitor + stimulation mean MPE (97.37%) is significantly greater than, that calculated for stimulation (34.44%), or saline stimulation (24.00%) conditions ($F(1,39)$ 230.68, $F(1,39)$ 314.09 respectively $p < .01$ both). Again stimulation produced a significantly greater effect than saline stimulation ($F(1,39)$ 6.42, $p < .05$).

Following naloxone pretreatment there were no differences between the MPE of stimulation (15.72%), saline stimulation, (20.33%) or enkephalinase inhibitor + stimulation (17.56%) conditions. In comparing naloxone to saline pretreatment, the MPE of both stimulation and enkephalinase inhibitor stimulation conditions, were significantly greater under saline injection ($F(1,39)$ 20.54, $F(1,39)$ 371.6 respectively $p < .01$). The saline + stimulation effect was not significantly different under the two pretreatments. Enkephalinase inhibitor alone, under saline, produced a MPE of 16.5%, and of -4.40% under naloxone. The pretreatment effect of this condition was significant ($F(1,39) = 25.48$). The summary table of the Anova performed on this data is presented in Table B2.

Figure BI. Mean percent effect of enkephalinase inhibitor plus stimulation on tail flick latency of rats in Study I.

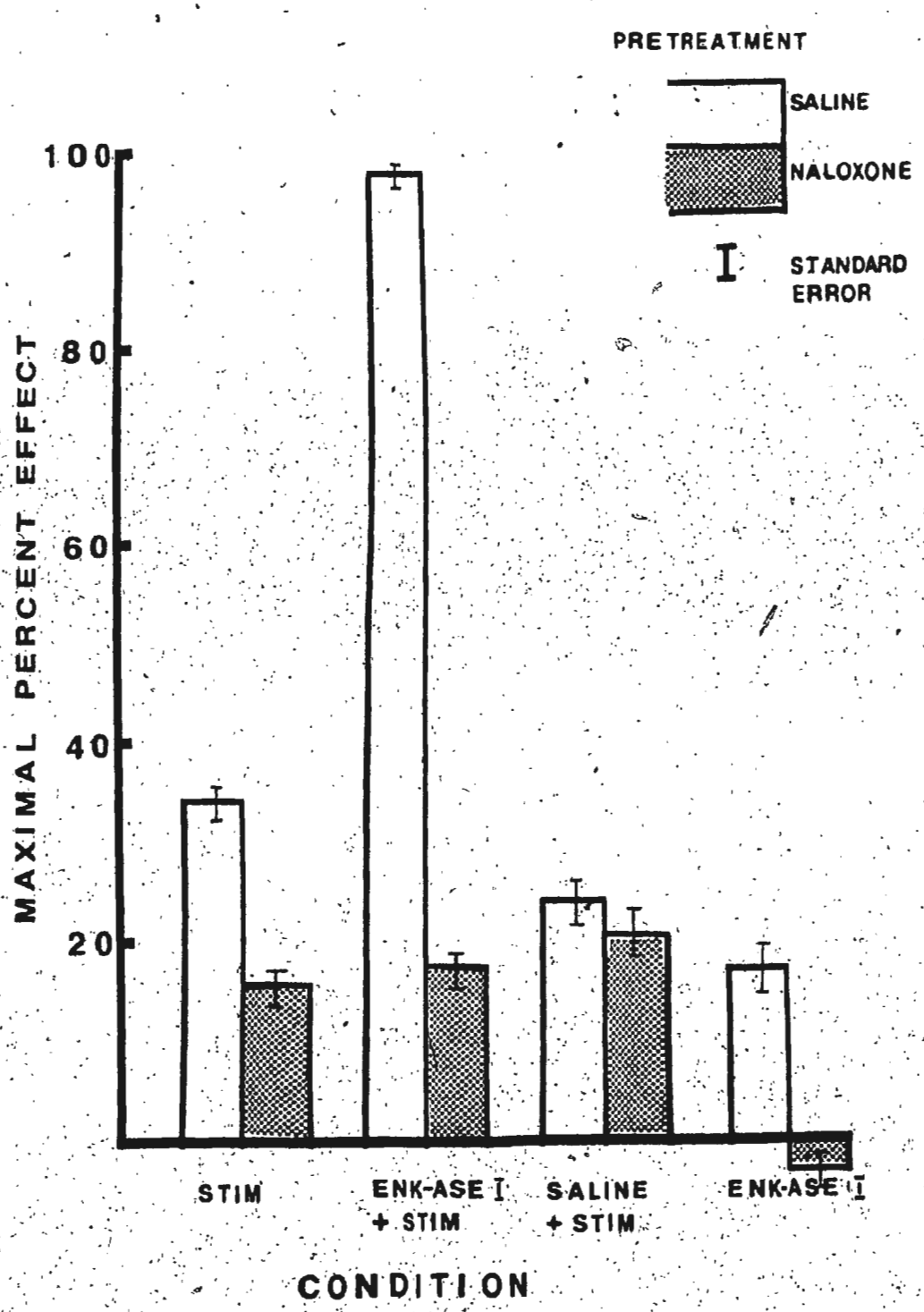

PRETREATMENT
SALINE
NALOXONE
STANDARD ERROR
MAXIMAL PERCENT EFFECT
100
80
60
40
20
STIM
ENK-ASE I + STIM
SALINE + STIM
ENK-ASE I
CONDITION

TABLE 1

Study I: Enkephalinase Inhibitor Test; F Ratio of Comparisons in Maximal Percent Effect

		SALINE				NALOXONE			
		Stimulation	Enkase I + Stim	Saline + Stim	Enkase I	Stimulation	Enkase I + Stim	Saline + Stim	Enkase I
SALINE	Stimulation	-							
	Enkase I + Stim	230.68*	-						
	Saline + Stim	6.42+	314.09*	-					
	Enkase Inhibitor				-				
NALOXONE	Stimulation	20.54*				-			
	Enkase I + Stim		371.6*			0.196	-		
	Saline + Stim			0.08		1.24	0.45	-	
	Enkase Inhibitor				25.48*				-

* $p < .01$

\+ $p < .05$

TABLE 2

Study I: Intensity of Analgesia in Maximal Percent Effect

Source	df	SS	MS	F
Subject	13	7420.7	570.823	
Pretreatment	1	26537.3	26537.3	110.4
Subject X Pretreatment	13	3124.71	240.362	
Condition	3	38977.7	12992.6	146.1
Subject X Condition	39	3467.26	88.904	
Pretreatment X Condition	3	23671.3	7890.44	65.75
Subject X Pretreatment X Condition	39	4679.97	119.999	
TOTAL	111	107879.0		