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Inhibition of morphine tolerance and dependence by diazepam and its relation to opioid peptides

Sribanditmongkol, Pongruk, Ph.D.

The Ohio State University, 1994
INHIBITION OF MORPHINE TOLERANCE AND DEPENDENCE BY DIAZEPAM AND ITS RELATION TO OPIOID PEPTIDES

DISSERTATION

Presented in Partial Fulfillment of The Requirements For The Degree Doctor of Philosophy in The Graduate School of The Ohio State University

By

Pongruk Sribanditmongkol, M.D.

*****

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1994

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Department of Pharmacology
To My Country
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SUMMARY

The effect of diazepam on the development of morphine tolerance and dependence was investigated. Male Sprague-Dawley rats were rendered tolerant and dependent by subcutaneous implantation of six morphine pellets. Diazepam (0.025, 0.25 or 2.5 mg/kg body weight) was injected intraperitoneally once daily into rats starting on the first day of the implantation. Antinociception was measured by tail-flick (TF) and hot plate (HP) tests, and the extent of sedation determined by a rotarod test before and one hour after diazepam injections everyday for five days. Physical dependence on morphine was assessed by an antagonist-precipitated abstinence syndrome on the fifth day of treatment by injecting naloxone 10 mg/kg subcutaneously. Diazepam (0.025-2.5 mg/kg body weight) did not produce significant antinociception or sedation (sensorimotor impairment) in rats implanted with placebo pellets. Diazepam (0.25 and 2.5 mg/kg) inhibited tolerance to TF antinociception in rats implanted with morphine pellets. Sedation as evidenced by sensorimotor impairment induced by morphine pellet implantation was not influenced by diazepam (0.025-2.5 mg/kg). Diazepam administration (0.25 mg/kg) also decreased the degree of jumping behavior observed following naloxone injection in morphine implanted rats. The serum morphine concentration in morphine-diazepam treated rats was not significantly different from that in morphine-saline treated rats. Chronic morphine treatment caused a decrease in the level of
met-enkephalin in the hypothalamus and spinal cord of morphine tolerant-dependent rats and in the hypothalamus, hippocampus, cortex and spinal cord of abstinent rats. The levels of β-endorphin and dynorphin were also altered by morphine treatment in certain brain regions. Diazepam treatment reversed the changes in the CNS opioid peptide levels, especially met-enkephalin, in both tolerant-dependent and abstinent animals. Diazepam treatment also enhanced the levels of β-endorphin in the hypothalamus and spinal cord of morphine abstinent rats, and the level of dynorphin in the pons-medulla of abstinent animals. Since the opioid peptides have been implicated in opioid tolerance and dependence, these effects of diazepam on the opioid peptide levels might account for the inhibition of morphine tolerance and dependence by this benzodiazepine.
CHAPTER I

INTRODUCTION

History of opiates

Opium, an extract from the milky exudate of the unripe poppy seeds, has been known for more than 4000 years. It has been used to suppress pain, to induce sedation and hypnosis and to produce euphoria. It was not until 1803 that Friedrich W.A. Sertturner isolated an active alkaloid component from opium and later named it morphine (Morpheus: the Greek god of dream) (1). Since then the more powerful morphine has rapidly replaced the use of opium as an analgesic and hypnotic. The chemical structure of morphine which is a phenanthrene derivative was first determined in 1902 (2). Other alkaloid compounds have also been identified from opium and referred to as opiates. Despite the long history of opium and opiates, the mechanism of action of opiates was not clear until the last two decades. The discovery of endogenous opiate-like substances (named opioids) as well as its receptors has enabled scientists to understand more about the opioid system. Following the International Narcotic Research Conference (INRC) 1983, a committee on nomenclature endorsed the term 'opioid receptor' in place of 'opiate receptor' which had been used before (3). Opiates refer to products derived from the juice of opium poppy
including morphine derivatives. The term 'opioids' includes any compound the effects of which are stereospecifically antagonized by naloxone. Opioids can be either peptides or non-peptides (4). In this dissertation, opioids refer to both opiates and opioids.

Opioid receptors

The first demonstration of stereospecific opioid binding to brain membranes was reported by Goldstein and coworkers (5). The decisive demonstrations of opioid receptors by ligand binding were first reported by three different laboratories (6,7,8). The concept of multiple opioid receptors was first postulated before the discovery of opioid binding sites (9). However, the heterogeneity of opioid receptors was widely accepted after the work performed by Martin and his colleagues (10,11). Based on pharmacological profiles studied in chronic spinal dogs, three different types of opioid receptors were proposed and named following the prototypic drugs used in the experiment; $\mu$ (morphine), $\kappa$ (ketocyclazocine), and $\sigma$ (SKF10047: N-allylnormetazocin). After the discovery of opioid peptides, enkephalins (12), Kosterlitz and his colleagues proposed another type of opioid receptor and named it the $\delta$ receptor after the bioassay system, vas deferens, that they used (13). Since then, the multiplicity of opioid receptors has been extensively investigated. The subtypes of these opioid receptors (e.g. $\mu_1$, $\mu_2$, $\kappa_1$, $\kappa_2$, $\kappa_3$ ) and new types of opioid receptors (e.g. $\varepsilon$, $\lambda$) have also been identified. Nevertheless, the significance of some of these subtypes and new types of opioid receptors is not fully understood. The three most studied and best established opioid receptors are $\mu$, $\delta$, and $\kappa$. 
types. The σ-receptor, is no longer considered as an opioid binding site, since its effects are not reversed by the opioid antagonist, naloxone. It is now classified as a phencyclidine (PCP) binding site (14,15). Table 1 summarizes the classification of opioid receptors, prototypic drugs and proposed actions.

There are evidences that opioid receptors belong to the family of G-protein coupled receptors (16). Opioid receptors (μ, and δ, and possible κ types) inhibit adenylyl cyclase activity in various tissues resulting in decreased cAMP levels in the cells. This inhibition is mediated by GTP binding protein, G_i (17). Opioid receptors also couple to ion channels via pertussis toxin sensitive G-protein. An activation of μ- or δ-opioid receptors opens K^+ channels and increases K^+ efflux, resulting in membrane hyperpolarization, conductance increase, and inhibition of firing. An activation of κ-opioid receptors inhibits K^+ -stimulated Ca^{2+} channels, leading to decreased in Ca^{2+} currents (17).
**TABLE 1** Classification of opioid receptors, prototypic drugs and proposed actions

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<th>δ-receptors</th>
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<td>Enkephalins</td>
<td>Dynorphins</td>
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<td>DPDPE</td>
<td>EKC, U50488, U69593, Bremazocine, Tifluadom</td>
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<td>Naloxone ICI-174864, Naltrindole, FIT &amp; SUPERFIT</td>
<td>Naloxone MR-2266, Nornbinaltorphimine, WIN 444413</td>
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<td>Opening K⁺ channel, Reduce cAMP production</td>
<td>Close Ca²⁺ channel, Decrease Ca²⁺ influx</td>
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<td>Positive reinforcement, Inhibition of smooth muscle contraction, Spinal analgesia</td>
<td>Spinal analgesia, Dysphoria, Sedation, Miosis, Hypothermia</td>
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Summarized from Ref. 16-19
Anatomical distribution of opioid receptors in mammals

The distribution of different types of opioid receptors have their own patterns. In some brain regions, there are species differences in the distribution of opioid receptors (20). The \( \mu \)-opioid receptors are widely distributed throughout the CNS except in the cerebellum. They are very dense in the basal ganglia, thalamus, amygdala, caudate-putamen, cerebral cortex, pyramidal cell layer of the hippocampus, periaqueductal gray region, and dorsal horn of the spinal cord. The \( \delta \)-receptors are most dense in the frontal cortex, caudate-putamen, and amygdala but sparse to nonexistent in thalamus and hypothalamus. The \( \kappa \)-receptors are very prominent in nucleus accumbens, preoptic area, caudate-putamen and hypothalamic regions of the rat, but very low in the thalamus.

Opioid peptides

After the discovery of opioid receptors, presence of endogenous opiate-like substances were proposed to exist. The search for endogenous opioid peptides was successful in the early 1970's. The first two endogenous opioid peptides were pentapeptides extracted from porcine brain (12). These two enkephalins (meaning: inside the head) were called methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) according to the amino acid at the C-terminal. The second class of endogenous opioid peptides was reported by Cox et al. (21). They observed that a fragment of lipotropin (amino acid 61-91) showed an opiate-like activity. This 31 amino acid peptide named as \( \beta \)-endorphin
(meaning: endogenous morphine), contained the same N-terminal structure as met-enkephalin. However, accumulating data has confirmed that it is not the precursor of met-enkephalin (22). Another family of endogenous opioid peptides is dynorphin (dyn: strength, power) isolated from porcine pituitary (23).

To date there are three families of endogenous opioid peptides which are derived from different precursor proteins as summarized in Table 2. These precursor proteins are derived from three distinct genes as shown in Fig. 1. Generally, opioid peptides function as neurotransmitters or neuromodulators affecting many central nervous system (CNS) functions.
TABLE 2  Opioid, nonopioid and opioid antagonist peptides derived from the three endogenous opioid precursors.

<table>
<thead>
<tr>
<th>Proenkephalin</th>
<th>Proopiomelanocortin</th>
<th>Prodynorphin</th>
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</thead>
<tbody>
<tr>
<td><strong>Opioid</strong></td>
<td><strong>Opioid</strong></td>
<td><strong>Opioid</strong></td>
</tr>
<tr>
<td>Met-Enk</td>
<td>β-End 1-31</td>
<td>Dyn A 1-17</td>
</tr>
<tr>
<td>Leu-Enk</td>
<td>α-End</td>
<td>Dyn A 1-8</td>
</tr>
<tr>
<td>Met-Enk-Arg-Gly-Leu</td>
<td>γ-End</td>
<td>Dyn B</td>
</tr>
<tr>
<td>Met-Enk-Arg-Phe</td>
<td></td>
<td>Leumorphin</td>
</tr>
<tr>
<td>Peptide F</td>
<td></td>
<td>α-Neo End</td>
</tr>
<tr>
<td>Peptide E</td>
<td></td>
<td>β-Neo End</td>
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<tr>
<td>Bam 22</td>
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<td>Leu Enk</td>
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<td>Bam 20</td>
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<td>Bam 12</td>
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<tr>
<td><strong>Nonopioid</strong></td>
<td><strong>Nonopioid</strong></td>
<td><strong>Nonopioid</strong></td>
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<tr>
<td>Synenkephalin</td>
<td>ACTH</td>
<td>Bridge peptide</td>
</tr>
<tr>
<td>Peptide I</td>
<td>α-MSH</td>
<td>C-terminal peptide</td>
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<tr>
<td>Peptide B</td>
<td>β-MSH</td>
<td>des-Tyr-ProDyn peptides</td>
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<td></td>
<td>γ-MSH</td>
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<td></td>
<td>N-acetyl-β-End</td>
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<td>des-Tyr-α-End</td>
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<td></td>
<td>des-Tyr-γ-End</td>
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<tr>
<td>Opioid antagonist(^a)</td>
<td>β-End 1-27</td>
<td>Opioid antagonist(^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>κ-selective ProDyn peptides</td>
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<tr>
<td></td>
<td></td>
<td>des-Tyr-ProDyn peptides</td>
</tr>
</tbody>
</table>

\(^a\) β-End 1-27 appears to be a competitive antagonist of opioid receptors. In contrast, des-Tyr-ProDyn peptides and κ receptor antagonists appear to be physiological antagonists of opioid actions.

Obtained from Ref. 24
Figure 1  Structural organization of the genes, mRNA, and propeptides of the three opioid peptide systems. CAP, transcription initiation site; Ex, exon; I, intron; MSH, melanocyte-stimulating hormone; β-END, β-endorphin; M-E, met-enkephalin; L-E, leu-enkephalin; OCT, octapeptide (met-enkephalin-arg<sup>6</sup>-gly<sup>7</sup>-leu<sup>8</sup>); HEP, heptapeptide (met-enkephalin-arg<sup>6</sup>-phe<sup>7</sup>).

Obtained from Ref. 25
Proenkephalin peptide family

Proenkephalin containing neurons are widely distributed throughout the CNS and have been found to consist of local circuits and long tract projections (Fig. 2). The pre-proenkephalin mRNA is derived from the proenkephalin gene and then is translated to the pre-proenkephalin peptide and subsequently cleaved to proenkephalin (Fig. 1). In mammals, this enkephalin precursor contains six copies of met-enkephalin and one copy of leu-enkephalin (22). These proenkephalin-derived peptides can produce analgesia when administered intracerebroventricularly or intrathecally. Enkephalins are involved in many physical and pathological conditions (26) including drug reward processes (27, 28), tolerance, and dependence (24, 29). The preferred opioid receptor for enkephalins is the δ-type, however, they also bind quite well to μ-receptors.
Figure 2. Structure of bovine proenkephalin and distribution of proenkephalin-containing cell bodies in the rat brain.

Obtained from Ref. 22
Proopiomelanocortin (POMC) peptide family.

β-endorphin peptide is generated from the POMC gene (Fig. 1). The products of this POMC are several biologically active peptides, e.g. adrenocorticotropic hormone, melanocyte-stimulating hormone, and β-endorphin (Fig 3). POMC containing neurons are localized in the pituitary gland, arcuate nucleus of the hypothalamus, and nucleus tractus solitarius (NTS) of the caudal medulla. The neurons in the arcuate nucleus have widespread projections throughout the brain, while the neurons in the NTS appear to primarily project within the medulla and possibly into the spinal cord (30). β-endorphin has been shown to be involved in an endogenous antinociception, opioid tolerance and/or dependence (30), and self-administration processes (31). β-endorphin has similar affinity for the μ- and δ-binding sites, but much less activity for the κ-binding site (32).
Figure 3. Structure of bovine proopiomelanocortin (POMC) and distribution of POMC-containing cell bodies (stippled area) and projections (tridents) in the rat brain.

Obtained from Ref. 22
Prodynorphin peptide family

Prodynorphin containing neurons are widely distributed throughout the brain and spinal cord and form both short and long projections (Fig 4). The prodynorphin gene is encoded for prodynorphin peptides (Fig. 1). The products of the prodynorphin precursor are dynorphin A1-17, dynorphin A1-8, dynorphin B1-13, α- and β-neo-endorphin, and leu-enkephalin (Table 2). Dynorphins interact with a high affinity to the κ-receptors, of which an activation is involved in many CNS functions including sedation, aversion and dysphoria. Dynorphins also have affinity for the μ-receptors but less affinity for the δ-receptors (32).
Figure 4. Structure of porcine prodynorphin and distribution of prodynorphin-containing cell bodies in the rat brain.

Obtained from Ref. 22
Endogenous opiate alkaloids in mammals

It has been speculated that morphine or related alkaloid compounds, might be an endogenous substance in mammalian tissues. Although β-endorphin can bind to the μ-receptors quite well, the prototypic agonist for the μ-type receptors is still morphine (33). By using morphine antibodies, morphine and related alkaloids have been found in rat and rabbit skin as well as beef brain and adrenals (34), also in beef hypothalamus and adrenals (35). In human cerebrospinal fluid, morphine, codeine and their conjugates have also been identified as endogenous compounds (36). The functional role of these endogenous opiate alkaloids is not clear. They might be involved in endogenous analgesia, and adaptation processes.

Opioid tolerance and dependence

A factor that limits long term use of morphine and other opioids is the development of tolerance and physical dependence (37-39). Tolerance is usually referred to as a reduction in the magnitude and duration of an effect produced by a given dose of drug. It may develop after a single administration or after continuous treatment with morphine which is referred to as an acute tolerance (40). It may also develop after long term exposure to opioids which is defined as chronic tolerance. Dependence is an adaptive process which is a consequence of sustained exposure to morphine or other opioids and leads to a requirement of morphine and/or related substances to avoid an abstinence syndrome. Analgesic effects produced by morphine are reduced when tolerance develops, leading the need for increasing doses of
morphine to obtain the same therapeutic effect. Any combination of drugs that can reduce the degree of tolerance and dependence, as well as maintain the therapeutic effects of opioids would be very useful in the treatment of chronic pain (38).

Many investigators have examined the mechanisms of opioid tolerance and dependence and have attempted to control this phenomenon in experimental animals. Tolerance to and dependence on morphine and other opioid agonists are complex phenomena (41,42). However, a decrease in pharmacological response during tolerance cannot be explained by a change in the pharmacokinetics of opioid drugs (43,44).

After chronic treatment with morphine, receptor down-regulation was observed in the central nervous system (45-47). The μ-opioid receptors seem to be responsible for the development of morphine tolerance and dependence. Werling et al. (46) and Bhargava and Gulati (47) demonstrated down-regulation of μ-receptors, but not that of δ- or κ-receptors in certain brain regions of animals treated with morphine. Aceto and co-workers (48) observed that the μ-receptors played a major role in morphine tolerance and dependence in rats and monkeys. Using a striatal slice method, Abdelhamid and Takemori (49) demonstrated that down-regulation of both μ- and δ-receptors was responsible for morphine tolerance and dependence. Steece et al. (50) also showed that the δ-receptors were down regulated after chronic exposure to an opioid peptide, met-enkephalin. In contrast, some investigators reported an up-regulation of opioid receptors in animals during opioid tolerance and dependence (51-53). Lufty and Yoburn (52) and
Rothman et al. (53) reported an up-regulation of opioid receptors in the whole brain homogenate of morphine tolerant-dependent animals. Rothman et al., (53) also reported an increase in $K_d$ in this membrane preparation. Intrathecal administration of morphine (54) or subcutaneous administration of morphine (55) also increased the $\mu$-binding sites in the spinal cord of rats.

There is no consensus about changes in opioid receptors (either up- or down-regulation) after chronic opioid treatment. Recently, it has been suggested that changes in the opioid receptor number may not be a critical mechanism underlying the process of tolerance and dependence (56). The up-regulation of opioid receptors may reflect an adaptive process to overcome tolerance and dependence (52) and might be a biochemical marker in tolerance and dependence as proposed by Rothman et al. (53).

In an in vitro study, it has been shown that tolerance develops before changes in the opioid receptors are apparent, suggesting that other mechanisms are involved (57). Morphine tolerance was demonstrated in cell cultures that contained predominantly $\delta$-receptors (Neuroblastoma X Glioma NG-108-15 Hybrid cells) or $\mu$-receptors (7315c rat pituitary tumor cells). Acute exposure to opioids produced an inhibition of adenylyl cyclase activity in these cells. During a continuous period of exposure to morphine, these cell lines resumed an increase in adenylyl cyclase activity and cAMP production (57-59). It is postulated that increased adenylyl cyclase activity in these cells after chronic opioid exposure occurs because agonist-receptor
complexes could not couple to G-protein, especially the G\textsubscript{i}-subunit, to mediate their inhibition of adenylyl cyclase activity (60,61).

In animals treated with chronic morphine, adenylyl cyclase activity was also increased in the locus coeruleus, nucleus accumbens, amygdala and thalamus (62,63). Effects of chronic morphine treatment on G-protein levels varied in different locations of brain (63,64). G\textsubscript{i}-protein was increased in the locus coeruleus and amygdala but decreased in the nucleus accumbens and dorsal root ganglion of spinal cord.

Opioid peptides may also play a role in opioid tolerance and dependence (24,30). Opioid peptides, enkephalins and \(\beta\)-endorphin have been shown to mediate reinforcement for self-administration and electrical stimulation (27,31). Chronic administration of morphine and other opioids affect the levels of opioid peptides. The \(\beta\)-endorphin and met-enkephalin levels are deceased in the striatum (65), midbrain, pons-medulla, hippocampus and amygdala (66) after morphine pellet implantation. Uhl and coworkers (67) reported a decrease in the striatal pre-proenkephalin mRNA after chronic morphine treatment, but some reports showed no change in the pre-proenkephalin mRNA (68,69). Chronic morphine treatment decreased the pre-POMC mRNA in the hypothalamus (69,70). The biosynthesis of these two opioid peptides is decreased after chronic morphine treatment. Alterations in these endogenous opioid peptides together with other changes resulting from chronic stimulation of opioid receptors are proposed to be responsible for many neural and behavioral differences observed during opioid tolerance and dependence (24,30,67).
As mentioned before, tolerance to and dependence on morphine are complex phenomena. The actual mechanism is not yet clear. In addition to previously cited underlying mechanisms, antiopiate peptides, for instance F8Fa (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide), may play a role in the development of opiate tolerance and dependence (71).

Effect of benzodiazepines on opioid system

Benzodiazepines, which are classified as anxiolytic drugs, have been shown to modulate many opioid effects. In 1982 Zeugner and coworkers (72) reported that a benzodiazepine, tifluadom, had a high affinity for \( \kappa \) opioid receptors and exhibited an antinociceptive effect in the tail-flick test. This effect was partially antagonized by an opiate antagonist, naloxone. Bergman et al. (73) showed that diazepam enhanced fentanyl-induced antinociception in both the mouse hot plate test and the rabbit tooth pulp test, and prolonged the duration of antinociceptive effect of morphine after a single morphine injection. In contrast, other studies showed that benzodiazepines attenuated the antinociceptive effect of opiates (74,75). A dual effect of benzodiazepine on morphine-induced antinociception was reported (76). A low dose of midazolam enhanced the antinociceptive effect of morphine when both drugs were given intrathecally, but a high dose of midazolam had an opposite effect.

Not only analgesic, but sedative-hypnotic effects induced by morphine have also been shown to be potentiated by benzodiazepines. Kissin et al. (77,78) reported that diazepam and midazolam showed a
synergistic interaction on the sedative and hypnotic effect induced by morphine.

From a clinical point of view, Singh and colleagues (79) studied analgesic effects of morphine, diazepam or their combination in patients suffering from postoperative pain. The authors concluded that the combination of morphine and diazepam should be considered as the treatment of choice. Benzodiazepines also modulated other effects of morphine in patients. McDonald et al. (80) reported that lorazepam antagonized the respiratory depressant effect of morphine in intensive care patients.

Effect of benzodiazepines on the development of morphine tolerance and dependence

It has been suggested that there is an interaction between benzodiazepines and the opioid system (75, 76, 78, 81). Nevertheless, the effect of benzodiazepine receptor agonists on opioid tolerance and dependence has not been extensively investigated. Recently, midazolam has been shown to inhibit the development of tolerance to antinociception induced by chronic morphine injections (82). The mechanism underlying this midazolam effect is not clear. Midazolam administration in healthy volunteers was also found to attenuate the development of tolerance to analgesic effects of fentanyl (83). Other benzodiazepines affect the expression of the morphine abstinence syndrome (84). Biochemically, the benzodiazepine, diazepam, has been shown to increase the rat hypothalamic
met-enkephalin level (85). Diazepam also inhibited met-enkephalin efflux during depolarization in perfused striatal slices (86). On the basis of these observations, we speculated that diazepam might influence opioid tolerance and dependence.
Hypothesis and specific aims

The primary objective of this study is to investigate effect(s) of diazepam on the development of morphine tolerance and dependence in a rat model. The hypothesis is that concomitant administration of a low dose of diazepam can attenuate the development of tolerance to antinociceptive effect and physical dependence induced by morphine pellet implantation. In order to substantiate this hypothesis, the following specific aims are addressed:

1. To determine doses of diazepam which by themselves do not affect antinociceptive and sedative tests in control rats.

2. To investigate whether concomitant diazepam administration can attenuate the development of tolerance to antinociception induced by morphine pellets in rats.

3. To determine the effect of concomitant diazepam administration on tolerance to sedation induced by morphine pellet implantation in rats.

4. To study whether diazepam administration can decrease the severity of physical dependence induced by morphine pellet implantation in rats.

5. To investigate the effect of diazepam treatment on the opioid peptide levels in morphine tolerant and dependent rats. Since opioid peptides, especially met-enkephalin and β-endorphin, have been implicated in the development of opioid tolerance and dependence, changes in the content of these peptides in the central nervous system may be observed in rats treated with diazepam and morphine.
CHAPTER II

MATERIALS AND METHODS

Subjects

This study was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Adult male Sprague-Dawley rats, weighing 190-230 g at the beginning of the experiments, were obtained from Zivic Miller Laboratories, Inc. (Allison Park, PA). Animals were housed in a group of 3-5 animals per cage. They were kept in a room with controlled temperature (22±1°C), humidity (60%), and 12-h on-and-off lighting schedule for 5 days before being used. The animals were given laboratory rat chow and water *ad libitum*. They were brought to the experimental room and acclimatized to the test equipment (tail-flick, hot plate and rotarod) 4 days before starting any experiment.

Drugs

Morphine and placebo pellets were kindly provided by the Research Triangle Institute through the National Institute on Drug Abuse. Each morphine pellet contained 75 mg morphine base; 69.27 mg of Avicel PH-102; 0.75 mg of colloidal silicon dioxide, NF; and 1.5 mg of magnesium stearate, NF. Placebo pellets contained the same excipient except morphine
and additional 150 mg Avicel PH-102. Diazepam was purchased from the Schien Pharmaceutical, Inc. Naloxone hydrochloride was obtained from Sigma. Diazepam and naloxone solutions were prepared in 0.9% NaCl. All drug solutions were prepared in such a way that a volume of 1 ml/kg of body weight was injected into rats to deliver a desired dose of drugs.

**Reagents for opioid peptide radioimmunoassay (RIA)**

RIA buffer contains (in 2000 ml) 100 mM Sodium phosphate buffer, pH 6.0 (200 ml) prepared by adding 1 M of sodium diphosphate (Sigma) into 1 M sodium monophosphate (Sigma); 50 mM NaCl (5850 mg, Sigma); 5 mM EDTA (3720 mg, GFS, disodium salt); 0.1% gelatin (200 mg, Sigma 300 Blum); 0.1% (v/v) Triton X-100 (2 ml, Sigma) and 0.025% Thimerosal (500 mg, Sigma, sodium salt).

30% Polyethylene glycol (MW: 8000, Sigma)

Charcoal-Dextran suspension (in 100 ml of RIA buffer) contains 1.6% Norit A charcoal (1.6 g Carbon decolorizing alkaline Norit A, Fisher) and 0.16% dextran T-70 (160 mg, Pharmacia). The suspension was stirred at least 15 min before and during use.

1 M acetic acid was prepared from glacial acetic acid (Corco); 1 N NaOH was prepared from concentrated NaOH.

[^125I]-met-enkephalin and[^125I]-β-endorphin were purchased from Dupont Company, NEN Research Products (Cat #NEX-149 and 143, respectively).[^125I]-dynorphin A1-13 (porcine) was purchased from
Peninsula Laboratories (Belmont, CA), Cat #Y8676. Antiserum to met-enkephalin (RB-4) was kindly provided by Dr. S. Sabol (Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). This antiserum had 3% cross reactivity with leucine-enkephalin, 1% with Arg6-met-enkephalin and less than 0.001% Tyr-Gly-Gly-Phe and α- and β-endorphin (87,88). Antiserum to β-endorphin was produced in rabbits in this laboratory and was very sensitive, cross-reacting 100% with β-h-endorphin and β-c-endorphin, 36% with β-h-lipotropin and <0.01% with α-h-endorphin, α-MSH, met-enkephalin and leu-enkephalin (87,88). Antiserum to dynorphin 1-13 was a generous gift from A. Goldstein (Addiction Research Foundation, Palo Alto, CA). This antiserum showed less 0.001% cross reactivity with leu-enkephalin, met-enkephalin, α-neo-endorphin and β-endorphin (89,90).

**Reagents for serum morphine determination**

The radioimmunoassay kit 'Coat-a-Count Serum Morphine' was purchased from Diagnostic Products Corporation (Los Angeles, CA). The sensitivity of the assay was 0.8 ng/ml of morphine with only 0.2% cross reactivity with glucuronic acid conjugates and no cross reactivity with naloxone.

**Reagent for protein determination**

BCA Protein Assay Reagent A which was purchased from Pierce contains sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1 N sodium hydroxide.
BCA Protein Assay Reagent B purchased from Pierce contains 4% CuSO₄·5H₂O. The working reagent is 1 part of Reagent B in 50 parts of Reagent A. Albumin standard was obtained from Pierce (2 mg/ml).

**Induction of morphine tolerance and dependence**

Rats were rendered tolerant to and dependent on morphine by subcutaneous implantation of six morphine pellets during a 5-day period following the protocol described by Rothman et al. (53). Two morphine or placebo pellets were implanted in the subcutaneous tissue of the back of the rat on the first day, and another four pellets on the second day. The implantation was performed under light ether anesthesia following the technique described earlier (91). The pellets remained in the body throughout the experiment.

**Behavioral tests.**

Antinociceptive and sedative tests were conducted during 09:00 to 14:00 h. Animals were tested in the following order: tail-flick, hot plate and rotarod tests.

**Measurement of antinociception.** Tail-flick and hot plate tests were used for measuring antinociceptive responses.

1. Tail-flick (TF) test. A rat was kept in a cylindrical plexiglas tube 20 cm in length and 6 cm in diameter. The tail of the animal was extended through a slit in the rear of the tube for performing the TF test. The test
was conducted with a Model 33 Tail-flick Analgesia Meter (IITC, Woodland Hills, CA). The rheostat-controlled light (sensitivity 5, beam 90%) was directed at approximately 2.5 cm from the tip of the tail. The time interval from onset of heat stimulus to flick of the tail was recorded automatically by the instrument. Base-line latencies were 2-4 sec in naive rats. The cutoff latency was 10 sec in order to avoid tissue damage caused by intense heat (82).

2. Hot plate (HP) test. The test was performed using a model 38D Analgesia Meter (IITC, Woodland Hills, CA). The metal plate surface surrounded by plexiglas walls (28 x 28 cm and 35 cm high) was maintained at 52 ± 0.5°C. Each rat was kept in the plexiglas on the metal plate. The end point was either licking of either one hind-paw or jumping off the plate, whichever came first in response to thermal stimuli. When a rat failed to respond within 60 sec (cutoff time), the rat was removed from the equipment to avoid tissue damage (82).

Both tail-flick and hot plate response latencies are expressed as the percentage of maximum possible effect (%MPE) (92).

\[
{\text{%MPE} = \frac{(\text{Test latency} - \text{Baseline latency})}{(\text{Cutoff time} - \text{Baseline latency})} \times 100}
\]  

Measurement of sedation. A rotarod test which determines a sensorimotor performance was used as an index for sedation following the method described by Rosland and Hole (75) with some modifications. Control animals were trained to balance themselves for 60 sec on the rod that rotated
with a speed of 4 cycles per min. The interval that each rat was able to
balance on a moving rod after treatment was recorded (82).

_Precipitation of an abstinence syndrome_

On the last day of treatment (day 5), rats were injected with sc
naloxone 10 mg/kg to precipitate an abstinence syndrome. Before naloxone
injection, rats were kept in an observation glass box (a fish tank, size
25x50x30 cm) for 5 min for acclimatization. After naloxone injection, two
rats were placed at a time in the box and observed for withdrawal behaviors
for 30 min. Criteria for observing withdrawal signs were the same as
described by Blasig et al. (93). The following behavioral parameters were
counted in each rat: jumping (leaping on the edge of the box with all four
feet off the surface at the same time), whole body shaking (brief episodes of
rapid repetitive shaking of the entire trunk while standing on their hind legs)
and teeth chattering.
Experimental designs.

Experiment 1: Determination of doses of diazepam that did not produce antinociception and sedation.

Diazepam, at a high dose, can increase nociceptive response latencies in mice (75) and produce sedation in rats (77). In this experiment, we determined the highest dose of diazepam that did not produce both effects.

Male Sprague-Dawley rats were randomized into 5 groups, each group containing 4 animals. Animals in each group were injected with saline 1 ml/kg body weight or diazepam 0.025, 0.25, 2.5 or 5 mg/kg body weight ip. Tail-flick, hot plate and rotarod tests were performed 15, 30, 60, 120, and 240 min after injection of diazepam.

Experiment 2: Development of tolerance to antinociception and sedation after morphine pellet implantation.

To demonstrate a time-course response of the development of tolerance to antinociception and sedation induced by morphine pellet implantation, sixteen rats were randomly divided into 2 groups (n=8) and implanted with morphine pellets in one group and with placebo pellets in the other group. Antinociception and sedation were determined 1, 2, 4, 6 and 24 h after pellet implantation on day 1 and day 2, and then only once on day 3, day 4 and day 5 after the treatment. Then naloxone was injected to precipitate an abstinence syndrome for determining physical dependence on morphine.
**Experiment 3:** Effect of diazepam on the development of tolerance to antinociceptive and sedative effects and on the severity of physical dependence induced by morphine.

Rats were randomly divided into 8 groups, each containing 7-9 rats, and they received the following treatment:

- **Group 1**  Morphine pellets + Saline (1 ml/kg body weight ip)
- **Group 2**  Placebo pellets + Saline (1 ml/kg body weight ip)
- **Group 3**  Morphine pellets + Diazepam (0.025 mg/kg body weight ip)
- **Group 4**  Placebo pellets + Diazepam (0.025 mg/kg body weight ip)
- **Group 5**  Morphine pellets + Diazepam (0.25 mg/kg body weight ip)
- **Group 6**  Placebo pellets + Diazepam (0.25 mg/kg body weight ip)
- **Group 7**  Morphine pellets + Diazepam (2.5 mg/kg body weight ip)
- **Group 8**  Placebo pellets + Diazepam (2.5 mg/kg body weight ip)

Rats were implanted with morphine or placebo pellets and injected daily ip with saline or different doses of diazepam. Results obtained in Experiment 1 showed that these doses of diazepam by themselves did not induce any significant antinociception or sedation. Antinociception and sedation were measured every day before saline or diazepam injection as well as one hour after injections.

On the fifth day, one hour after saline or diazepam ip injection, an abstinence syndrome was precipitated by naloxone injection (10 mg/kg sc)
and pairs of rats were observed for a 30-min withdrawal period. Animals were then sacrificed one hour after naloxone injection. The brain was dissected into various regions which were stored at -85°C for subsequent opioid peptide analysis (Experiment 6).

**Experiment 4:** Effect of reversed diazepam and saline treatment on naloxone-precipitated jumping behavior in morphine dependent rats.

Rats were randomized into 2 groups (n=16) and were implanted with morphine pellets as described above. Animals in one group were injected ip with saline (Mor+Sal) and in the other group were injected with diazepam 0.25 mg/kg (Mor+DZP) once a day for 4 days. On the fifth day, the treatment was reversed. Animals in the Mor+Sal treated group were divided into two groups (n=8). Rats in one group received saline but in the other group were challenged with diazepam 0.25 mg/kg ip. Animals in the Mor+DZP treated group were also divided into two groups (n=8). Rats in one group were injected with diazepam (0.25 mg/kg ip) while in the other group were given saline by ip injection. Naloxone-precipitated jumping behavior was induced on the fifth day and monitored for 30 min as described in Experiment 3.
**Experiment 5:** Effect of diazepam treatment on the levels of opioid peptides met-enkephalin, β-endorphin and dynorphin in discrete brain regions of morphine tolerant and dependent animals.

Male Sprague-Dawley rats were randomly divided into 8 groups, each group containing 8-11 animals, and received treatment as described in Experiment 3. On the fifth day of the experiment, rats were sacrificed without naloxone-precipitated abstinence syndrome. These animals were considered to be morphine tolerant and dependent. The brain regions, namely, pituitary gland, hypothalamus, hippocampus, striatum, midbrain, pons-medulla, cerebral cortex, cerebellum and spinal cord were dissected and used for met-enkephalin, β-endorphin, and dynorphin analysis.

**Brain tissue preparation for radioimmunoassay (RIA)**

Each brain region from individual rat was transferred into a polypropylene tube containing 2 ml of 1 M acetic acid. The samples were heated at 95°C for 15 min, then homogenized with a sonicator at 4°C. An aliquot of the homogenate was saved in 1 N NaOH (1:2 and 1:10 dilution for pituitary and other brain regions, respectively) for protein estimation. The homogenate was centrifuged at 1500xg for 20 min. The supernatant was transferred to a new tube and then lyophilized. The lyophilized powder was reconstituted in 2 ml of RIA buffer. The reconstituted sample then was centrifuged at 1500xg for 15 min. The supernatant was used for met-enkephalin, β-endorphin and dynorphin RIA studies.
RIA for Met-enkephalin: The RIA mixture for met-enkephalin contained 100-μl of tissue extracts or standards (1-1000 pg), 100-μl of the antiserum (1:8000 dilution), 100-μl of [125I]-met-enkephalin (1:250 dilution, ≈6000 cpm) and 50-μl of 0.5% bovine γ-globulin. The mixture was vortexed, then incubated overnight at 4°C. Antibody-bound met-enkephalin was precipitated by adding 500 μl of 30% polyethylene glycol 8000 into each tube and incubated at 4°C for 20 min. The sample was centrifuged at 1500xg for 15 min. The supernatant was decanted and the pellet was counted for radioactivity in a gamma counter (Beckman) for 2 min. The amount of met-enkephalin was calculated from a met-enkephalin standard curve (using IBM personal computer with the software Graphpad Inplot).

RIA for β-endorphin: The assay mixture contained 100-μl of β-endorphin standards (1-1000 pg) in RIA buffer or 100-μl of unknown samples, 100-μl of β-endorphin antiserum (1:4000 dilution) in polypropylene tube (12x75 mm), and was incubated overnight at 4°C. On the next day, 100-μl of [125I]-β-endorphin (1:250 dilution, ≈5000 cpm) was added and incubated for another night. Separation of free and antibody-bound endorphin was performed by adding 500-μl of Charcoal-Dextran suspension into β-endorphin standards and unknown samples. These samples were centrifuged at 1500xg for 20 min. The supernatant (500 μl) was transferred to a new tube and then counted in a gamma counter for 2 min. The amount of β-endorphin was calculated from a β-endorphin
standard curve (using IBM personal computer with the software Graphpad Inplot).

**RIA for dynorphin 1-13**: The RIA mixture for dynorphin contained 100-μl of standards (1-1000 pg) or samples, 100-μl of dynorphin antiserum (1:10,000 dilution), 100-μl of [125I]-dynorphin (≈4000 cpm). The mixture was vortexed and incubated overnight at 4°C. Free dynorphin was separated from antibody-bound dynorphin by adding 500-μl of Charcoal-Dextran suspension into each tube. After 20 min incubation, the mixture was centrifuged at 1,500xg for 20 min, then the supernatant (500 μl) was transferred to a new tube and counted for 2 min in a gamma counter. The amount of dynorphin in the samples was calculated from a dynorphin standard curve (using IBM personal computer with the software Graphpad Inplot).

**Experiment 6**: Effect of diazepam treatment on the levels of opioid peptides met-enkephalin, β-endorphin and dynorphin in discrete brain regions of naloxone-precipitated abstinence animals.

Rats from different treatment groups in the behavioral study (Experiment 3) were sacrificed by decapitation. The pituitary gland, hypothalamus, hippocampus, striatum, midbrain, pons-medulla, cerebral cortex, cerebellum and spinal cord were dissected and subjected to met-enkephalin, β-endorphin and dynorphin analysis. Opioid peptides in discrete brain regions were determined by RIA as described in Experiment 5.
Experiment 7: Estimation of serum morphine concentration in morphine pellet-implanted-rats receiving daily saline or diazepam (0.25 mg/kg) injections.

Serum morphine concentration was measured by using the RIA kit 'Coat-a-Count Serum Morphine'. Trunk blood was collected, on the fifth day, from rats implanted with morphine and receiving daily saline or diazepam (0.25 mg/kg) treatment. In a separate experiment, trunk blood was collected from rats 30 min after a naloxone-precipitated abstinence syndrome. Blood was centrifuged at 15,000xg for 10 min. Serum samples were saved for morphine determination.

Protein determination: Protein concentrations in the brain homogenates were estimated using the bicinchoninic acid method with bovine serum albumin as a standard (94).
Statistical analysis.

Antinociceptive response latencies were converted to %MPE and analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni test for multiple comparison. Two-tailed, unpaired t-test was performed to compare the mean difference between morphine and placebo treated groups. Statistical analysis for sedation and frequency of abstinence behaviors was analyzed by a nonparametric test. The Mann-Whitney U-test was used to analyze the mean difference between two groups. Multiple comparison was performed by using the Kruskal-Wallis Nonparametric ANOVA test followed by Dunn's multiple comparison test. Fisher's exact test was used to assess the significance between the proportion of rats showing an abstinence syndrome. The levels of met-enkephalin, β-endorphin and dynorphin in each brain region were compared between different groups by ANOVA followed by post-hoc Newman-Keuls t-test. The serum morphine concentration of animals in morphine-saline and morphine-diazepam (0.25 mg/kg) groups was compared by using a two-tailed, unpaired Student t-test. A value of p < 0.05 was considered to be significant. Analysis was performed using an IBM personal computer with the Graphpad Instat software.
CHAPTER III

RESULTS

Doses of diazepam that did not produce antinociception and sedation.

Effect of diazepam treatment on TF and HP antinociception and sedation was determined. In the TF test, 0.025-5 mg/kg of diazepam did not induce significant antinociception as shown in Fig. 5. On the other hand, a single injection of diazepam (5 mg/kg) induced antinociception in the HP test 30 min after the injection (Fig. 6). In the rotarod test which measured sensorimotor coordination and was used as an index for sedation, diazepam 5 mg/kg impaired rotarod performance 15 min after a single injection (Fig. 7). Diazepam, at the dose of 0.025-2.5 mg/kg, by itself did not produce significant antinociception in either TF or HP tests, or sedation as determined by a rotarod test. These three doses of diazepam, therefore, were used in the following experiments. We also have extended this experiment to determine the effect of repeated diazepam injections (once a day for 5 days) on antinociception and sedation. There is no effect of repeated diazepam injections on either antinociception or sedation.
Figure 5. Effect of a single diazepam injection on TF antinociception. Rats were injected with saline or diazepam (0.025-5 mg/kg body weight) ip. The TF antinociception was determined 15, 30, 60, 120 and 240 min after the injection. On the ordinate, antinociception is depicted as mean %MPE ± SEM of each group.
Figure 6. Effect of a single diazepam injection on HP antinociception. The HP test was performed after the TF test 15, 30, 60, 120 and 240 min after the injection. Only 5 mg/kg diazepam induced HP antinociception and the peak was 30 min after the injection.
Figure 7. Effect of diazepam (5 mg/kg) on sedation. Diazepam (5 mg/kg) caused sedation as evidenced by a decrease in rotarod performance. Other doses of diazepam (0.025-2.5 mg/kg, data not shown) did not impair the rotarod test. The ordinate indicates % of animals which can balance on the rotarod for 60 sec. The abscissa represents time after diazepam injection.
Development of tolerance to antinociception and sedation after morphine pellet implantation.

A time-course response to antinociception and sedation was determined after morphine pellet implantation. Morphine pellet implantation produced antinociception as determined by TF (Fig. 8) and HP (Fig. 9) tests as early as 1 hour after two pellet implantation on the first day and four pellet implantation on the second day. There was a decline in antinociceptive effect after pellet implantation reflecting a development of tolerance to morphine, on day 3 in both TF (Fig. 8) and HP (Fig. 9) tests. The antinociceptive responses returned to control level on day 5 of treatment. Morphine impaired sensorimotor performance as determined by a rotarod test only after implantation of four pellets on the second day of treatment (Fig. 10). A return of rotarod performance to the control level indicated tolerance to sedative effect induced by morphine which gradually developed from day 3 to day 5 (Fig. 10). The rats implanted with morphine pellets exhibited physical dependence on morphine as evidenced by a naloxone-precipitated abstinence syndrome (Fig. 11-12). The signs of an abstinence syndrome (teeth chattering, jumping and whole body shakes) were not observed in placebo implanted rats injected with naloxone. These results suggest that the model and protocol of morphine treatment used in this study efficiently induces tolerance to and dependence on morphine.
Figure 8. Time course of TF antinociception after morphine pellet implantation. Rats were implanted with two pellets of morphine (●) or placebo (○) on the first day and another four pellets on the second day. Antinociception was measured 1, 2, 4, 6 and 24 h after the first and second day of the implantation and then once a day until day 5 and depicted as mean percent maximum possible effect (%MPE ± SEM) of n=8 on the ordinate. The abscissa indicates time (day) after the first pellet implantation. Statistical comparisons of antinociception in morphine and placebo pellet-implanted groups on the corresponding time were performed by the Student t-test (#p<0.0001, @ p < 0.01).
Figure 9. Time course of HP antinociception after morphine pellet implantation. Animals were treated as described in Fig. 8. The HP test was measured after the TF test. @ p<0.01, *p<0.05 compared to placebo treated group for the corresponding time period.
Figure 10. Time course of sedative effect of morphine pellet implantation. Rats were implanted with morphine (*) or placebo (o) pellets following the protocol as described in Fig 8. The rotarod test was performed 1, 2, 4, 6 and 24 h after the first and second day of the pellet implantation and then once a day until day 5. On the ordinate, rotarod performance is depicted as mean latency in sec ± SEM from 8 rats. The abscissa indicates time (day) after the first pellet implantation. Statistical analysis was performed by the nonparametric Mann-Whitney test. A value of ***p<0.001, **p<0.01, or *p<0.05 was considered to be significantly different between morphine and placebo treated groups for the corresponding time period.
Figure 11. Frequency of naloxone precipitated withdrawal signs in morphine tolerant-dependent rats. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets as described in Fig. 8. On the last day (day 5) of the experiment, rats were injected with 10 mg/kg sc naloxone and observed for a 30-min withdrawal period. Mean frequency ± SEM of each withdrawal sign; teeth chattering, jumping and whole body shakes (WBS), was counted. Comparison of each abstinence sign between morphine and placebo treated groups was conducted by the nonparametric Mann-Whitney test. ***p<0.001 compared to placebo treated (control) group.
Figure 12. Proportion of morphine tolerant-dependent rats showing naloxone-precipitated withdrawal signs. Animals received treatment as described in Fig. 11. Proportion of rats exhibiting an individual sign was determined. Statistical analysis was performed by the Fisher's exact test. ***p < 0.001, *p < 0.05 compared to control group.
Effect of diazepam on the development of tolerance to morphine induced antinociception.

Rats administered diazepam together with morphine pellet implantation developed less tolerance to antinociception than rats treated with saline during morphine implantation (Fig. 13). On the third day of treatment, morphine pellet implanted rats receiving daily diazepam (2.5 mg/kg) injections showed significantly higher antinociception than those in the morphine-saline treated animals (p<0.05). On the fifth day of treatment, morphine treated rats receiving daily diazepam (0.25 mg/kg) injections showed significantly less tolerance to antinociception than rats in the morphine-saline treated group (p<0.01). This modulation of tolerance to antinociception in rats by diazepam was observed in the tail-flick test, but not in the hot plate test. Diazepam by itself at different doses did not produce any significant antinociception after the first injection (acute treatment) or repeated injections (chronic treatment) in rats implanted with placebo pellets (Fig. 14). The inhibition of tolerance to tail-flick antinociception by diazepam is unlikely to be due to an acute synergistic effect of diazepam on morphine effect. As shown in Fig. 15, %MPE of TF antinociception before diazepam injection was not significantly different from that observed one hour after the injection of diazepam in rats implanted with morphine pellets, suggesting that these doses of diazepam, when given in acute treatment, did not acutely enhance the antinociceptive effect induced by morphine.
Figure 13. Effect of diazepam on the development of tolerance to morphine induced antinociception. Rats were implanted with morphine and injected once daily ip with saline (1 ml/kg) or diazepam (DZP) 0.025, 0.25 or 2.5 mg/kg. Each bar represents mean percent maximum possible effect (%MPE ± SEM) of TF antinociception measured one hour after the ip injections (n=7-9 rats). The abscissa represents day (1, 3, and 5) after the first morphine pellet implantation. Statistical comparisons were performed by one-way ANOVA followed by post hoc Bonferroni test. A value of **p<0.01 and *p<0.05 was considered to be significantly different from morphine-saline treated group on the corresponding day.
Figure 14. Effect of diazepam on antinociception in placebo implanted animals. Rats were implanted with placebo pellets and injected ip with saline (1 ml/kg) or diazepam (DZP) 0.025, 0.25 or 2.5 mg/kg. Each bar represents TF antinociception one hour after injections on day 1, 3, and 5. No statistically significant difference was observed between groups as performed by one-way ANOVA test.
Figure 15. Antinociceptive responses induced by morphine pellet implantation before and one hour after diazepam injection on each day of treatment. On the ordinate, mean %MPE ± SEM of TF antinociception depicted from 7-9 rats per group. The abscissa represents time (day) after the first morphine pellet implantation. The arrow indicates %MPE measured one hour after saline or diazepam (0.25mg/kg) injections. **p<0.01 and *p<0.05 compared to morphine-saline treated group on the corresponding time (one-way ANOVA followed by post-hoc Bonferroni test).
Effect of diazepam on the development of tolerance to morphine induced sedation.

Diazepam has been shown to impair sensorimotor performance in mice (75). A high dose of diazepam (5 mg/kg) caused sedation as shown in Fig. 7. Morphine itself also produced sedation (Fig. 10). We demonstrated in this experiment that diazepam did not enhance or antagonize sedative effects induced by morphine pellet implantation. As shown in Fig. 16, there is no significant difference in rotarod latencies between animals treated with morphine-saline and morphine-diazepam on day 1 of the treatment. Diazepam also did not significantly affect the development of tolerance to sedation induced by morphine pellet implantation (Fig. 16, day 3 and 5). Diazepam (0.025-2.5 mg/kg) by itself did not significantly impair sensorimotor performance either in acute or chronic treatment in placebo pellet implanted rats (Fig. 17).
Figure 16. Effect of diazepam on tolerance to sedative effect induced by morphine pellet implantation. Rats were implanted with morphine pellets and received saline or diazepam injections (0.025-2.5 mg/kg). A rotarod test was performed after antinociceptive test. Each bar represents mean latency in sec ± SEM (n=7-9) of rats that can balance on the rotarod for 60 sec, one hour after ip treatment. The abscissa indicates day (1, 3 and 5) after the first pellet implantation. There is no statistical difference between groups on the same days of treatment as determined by the Kruskal-Wallis Nonparametric ANOVA test.
Figure 17. Effect of diazepam on a rotarod test in placebo implanted rats. Animals were implanted with placebo pellets and received different doses of diazepam (0.025-2.5 mg/kg). Each bar represents rotarod latency (± SEM) one hour after diazepam injection.
Effect of diazepam on physical dependence induced by morphine

Morphine dependent rats displayed a stereotyped jumping behavior (93) after naloxone injection (10 mg/kg sc) compared to placebo treated rats (p < 0.01, Fig. 18, 19). Repeated diazepam injections in placebo pellet implanted rats did not induce any naloxone-precipitated withdrawal sign. Rats implanted with morphine along with daily concomitant diazepam injections showed lesser degree of physical dependence as determined by naloxone-precipitated jumping than that exhibited by animals treated with morphine alone (Fig. 18, 19). The frequency and the proportion of rats showing jumping behavior were less in rats treated with morphine-diazepam (0.25 mg/kg) than in rats treated with morphine-saline (p < 0.05). Although morphine treated rats receiving daily injection of diazepam (0.025 or 2.5 mg/kg) displayed less naloxone-precipitated jumping than morphine-saline treated rats, the results were not statistically significant (Fig. 18). Other morphine withdrawal behaviors (whole body shakes and teeth chattering) were not affected by diazepam treatment.
Figure 18. Effect of diazepam on naloxone-precipitated jumping behavior (frequency). Rats were implanted with morphine (Mor) or placebo (Plc) pellets and once daily injected ip with saline (Sal) or different doses of diazepam (DZP 0.025, 0.25 and 2.5 mg/kg body weight) for 5 days. On the fifth day, one hour after DZP injection, an abstinence syndrome was precipitated in rats by naloxone injection (10 mg/kg sc) and observed for a 30-min withdrawal period. Mean frequency of jumping was determined from 7-9 rats per group. Statistical analysis was conducted by the nonparametric Mann-Whitney test. **p<0.01, *p<0.05 compared to placebo-saline treated group. #p<0.05 compared to morphine-saline treated group.
Figure 19. Effect of diazepam on naloxone-precipitated jumping behavior (proportion of rats showing jumping). Statistical comparison between groups was performed by the Fisher's exact test. **p<0.01 compared to placebo-saline treated group. #p<0.05 compared to morphine-saline treated group.
Effect of reversed diazepam and saline treatment on naloxone-precipitated jumping behavior in morphine dependent animals

Morphine-saline treated rats receiving a first time diazepam injection on the last day prior to naloxone injection did not show a significant decrease in jumping behavior compared to morphine-saline treated rats receiving saline treatment prior to naloxone-precipitated withdrawal (Fig. 20). Morphine-diazepam treated rats receiving daily diazepam injections including one on the last day, showed significantly less jumping behavior than morphine-saline treated rats (p<0.05, Fig. 20). There was no significant difference in jumping behavior in morphine-diazepam treated rats receiving either diazepam or saline injection on the fifth day prior to a naloxone injection (Fig. 20). Morphine-diazepam treated rats receiving saline injection on the fifth day also exhibited less jumping than morphine-saline treated rats (p<0.01, Fig. 20). These results suggest that diazepam induces an increase in antinociception and a reduction of naloxone-precipitated jumping behavior which is due to the effect of diazepam on the development of morphine tolerance and physical dependence rather than an acute effect of diazepam on these parameters.
Figure 20. Effect of reversed diazepam and saline treatment on naloxone-precipitated jumping behavior in morphine tolerant-dependent rats. Morphine-saline treated rats were challenged with diazepam (0.25 mg/kg) on the last day of treatment before naloxone-precipitated withdrawal. Animals implanted with morphine and daily injected with diazepam for 4 days were challenged with saline (Sal) on the last day of treatment one hour before naloxone injection. The label under each bar indicates the treatment from day 1 through day 4. The label on the top of each bar refers to the treatment animals received on the fifth day before precipitated withdrawal. *p<0.05, @p<0.01 compared to the group treated with morphine-saline receiving a saline injection on the fifth day.
Effect of diazepam treatment on the levels of met-enkephalin in discrete brain regions of morphine tolerant and dependent animals and of morphine abstinent animals

Morphine treatment decreased the level of met-enkephalin in the spinal cord (65%, p<0.001) and hypothalamus (28%, p<0.0553) of tolerant-dependent animals (Fig. 21) and in the spinal cord (46%, p<0.01), cortex (60%, p<0.05), hippocampus (43%, p<0.01) and hypothalamus (33%, p<0.01) of morphine tolerant-dependent animals undergoing naloxone-precipitated abstinence (Fig. 28). Daily diazepam treatment for five days altered the levels of met-enkephalin in placebo implanted rats compared to placebo-saline treated group. In the midbrain of placebo implanted animals, diazepam 0.025 mg/kg increased the level of met-enkephalin significantly different from the level in placebo-saline treated group (p<0.01, Fig. 24). In the pons-medulla, daily diazepam (2.5 mg/kg) treatment enhanced the met-enkephalin level in placebo implanted rats significantly different from the level in placebo-saline treated animals (p<0.05, Fig. 25). In the spinal cord of placebo implanted rats, daily diazepam injections significantly decreased the level of met-enkephalin compared to the level in placebo-saline treated group (Fig. 27 and Fig. 32). Effects of diazepam treatment on the level of met-enkephalin in morphine implanted animals have been observed in the cortex and spinal cord of tolerant-dependent rats (Fig. 26-27), and in the hypothalamus, hippocampus, cortex and spinal cord of naloxone-precipitated withdrawal rats (Fig 29-32). In the cortex of tolerant-dependent animals, the level of met-enkephalin in
morphine-diazepam (2.5 mg/kg) treated animals was significantly higher than the level in morphine-saline treated group (p<0.001, Fig. 26). In the spinal cord, diazepam (0.25 mg/kg) increased the level of met-enkephalin in morphine implanted rats significantly higher than was observed in morphine-saline treated animals (p<0.01, Fig. 27). During abstinence, doses of diazepam (0.025, 0.25, and 2.5 mg/kg) significantly increased the level of met-enkephalin in the spinal cord of morphine implanted animals compared to morphine-saline treated rats (p<0.05, p<0.001 and p<0.001, respectively, Fig. 32). In the hypothalamus and hippocampus, diazepam also reversed the decrease in met-enkephalin levels observed in morphine withdrawal rats. The hypothalamic met-enkephalin level in the morphine-diazepam treated group was higher than the level in morphine-saline treated group (Fig. 29). In the hippocampus, the level of met-enkephalin in morphine-diazepam (0.25, 2.5 mg/kg) treated rats was significantly higher than the level in the morphine-saline treated group (p<0.05, p<0.01, Fig. 30). These results suggest that concomitant diazepam injections reverse the decrease in met-enkephalin levels induced by morphine pellet implantation in a tolerant-dependent state and in an abstinence state.
Figure 21. Met-enkephalin levels in discrete brain regions and spinal cord of morphine tolerant-dependent rats. Animals received morphine or placebo pellet implantation and injected daily with saline for five days. Rats were sacrificed on the fifth day of treatment. Discrete brain regions and spinal cord were dissected and subjected to met-enkephalin radioimmunoassay. ***p<0.001 and @p=0.0553 compared to the placebo treated group.
Figure 22. Effect of diazepam treatment on the met-enkephalin level in the hypothalamus of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Met-enkephalin was determined by RIA.
Figure 23. Effect of diazepam treatment on the met-enkephalin level in the hippocampus of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo pellets (open bar) and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Met-enkephalin was determined by RIA.
Figure 24. Effect of diazepam treatment on the met-enkephalin level in the midbrain of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Met-enkephalin was determined by RIA. ## p<0.01 compared to placebo-saline treated group.
Figure 25. Effect of diazepam treatment on the met-enkephalin level in the pons-medulla of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Met-enkephalin was determined by RIA. **p<0.01 compared to morphine-saline treated group. #p<0.05 compared to placebo-saline treated group.
Figure 26. Effect of diazepam treatment on the met-enkephalin level in the cortex of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Met-enkephalin was determined by RIA. ***p < 0.001 compared to morphine-saline treated group. ###p < 0.001 compared to placebo-saline treated group.
Figure 27. Effect of diazepam treatment on the met-enkephalin level in the spinal cord of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Met-enkephalin was determined by RIA. **p < 0.01 compared to morphine-saline treated group. ###p < 0.001, #p < 0.05 compared to placebo-saline treated group.
Figure 28. Met-enkephalin levels in discrete brain regions and spinal cord of morphine pellet implanted rats during an abstinence syndrome. Animals received morphine or placebo pellet implantation and were injected daily with saline for five days. Rats were sacrificed one hour after naloxone injection on the last day of treatment. Discrete brain regions and spinal cord were dissected and subjected to met-enkephalin radioimmunoassay. **p<0.01 and *p<0.05 compared to the placebo treated group.
Figure 29. Effect of diazepam treatment on the met-enkephalin level in the hypothalamus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. Brain tissues were collected and analyzed for met-enkephalin level. ***p<0.001, *p<0.05 compared to morphine-saline treated group. ###p<0.001, #p<0.05 compared to placebo-saline treated group.
Figure 30. Effect of diazepam treatment on the met-enkephalin level in the hippocampus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and injected daily with saline or diazepam (0.025-2.5 mg/kg) and were sacrificed on the last day of the experiment. Met-enkephalin content was determined by RIA. **p<0.01, *p<0.05 compared to morphine-saline treated group. ##p<0.01 compared to placebo-saline treated group.
Figure 31. Effect of diazepam treatment on the met-enkephalin level in the cortex of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and injected daily with saline or diazepam (0.025-2.5 mg/kg) and were sacrificed on the last day of the experiment. Met-enkephalin content was determined by RIA. #$p<0.05$ compared to placebo-saline treated group.
Figure 32. Effect of diazepam treatment on the met-enkephalin level in the spinal cord of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and injected daily with saline or diazepam (0.025-2.5 mg/kg) and were sacrificed on the last day of the experiment. Met-enkephalin level was measured by RIA. ***p<0.001, *p<0.05 compared to morphine-saline treated group. ###p<0.001, ##p<0.01 compared to placebo-saline treated group.
Effect of diazepam treatment on the levels of $\beta$-endorphin in discrete brain regions of morphine tolerant and dependent animals and of morphine abstinent animals

Morphine pellet implantation for 5 days caused a significant decrease in the $\beta$-endorphin levels in the hypothalamus (70%, $p<0.01$), hippocampus (69%, $p<0.05$), striatum (87%, $p<0.01$), midbrain (52%, $p<0.01$) and pons-medulla (100%, $p<0.01$) compared to control rats (Fig. 33-34, and 39). In morphine tolerant-dependent rats undergoing naloxone-precipitated abstinence, the levels of $\beta$-endorphin were significantly decreased in the pituitary (30%, $p<0.05$) and hippocampus (50%, $p<0.05$) compared to placebo treated group (Fig. 40-41). Diazepam treatment also affected $\beta$-endorphin levels in control animals. In the hypothalamus and hippocampus, the levels of $\beta$-endorphin in placebo implanted rats receiving different doses of diazepam were significantly lower than the levels in placebo-saline treated group (Fig. 35-36). In the striatum, diazepam (0.025 and 0.25 mg/kg) reduced the level of $\beta$-endorphin in placebo implanted animals compared to control group. ($p<0.05$, Fig. 37). In the midbrain, diazepam (0.25 and 2.5 mg/kg) showed a decrease in the $\beta$-endorphin level in placebo implanted rats compared to control animals injected with saline ($p<0.01$, Fig. 38). The levels of $\beta$-endorphin in the pons-medulla of placebo-diazepam (0.025 and 2.5 mg/kg) treated groups were significantly lower than the level in placebo-saline treated group ($p<0.05$, Fig. 39). When given together, concomitant diazepam injections did not increase the levels of $\beta$-endorphin in morphine tolerant-dependent animals. The levels of $\beta$-
endorphin in the hypothalamus (Fig. 35), hippocampus (Fig. 36), striatum (Fig. 37), midbrain (Fig. 38), and pons-medulla (Fig. 39) of morphine-diazepam (0.025-2.5 mg/kg) treated rats were not significantly different from the levels in morphine-saline treated group. In morphine tolerant-dependent rats undergoing naloxone-precipitated abstinence, diazepam treatment increased the levels of β-endorphin. The hypothalamic β-endorphin levels in morphine-diazepam (0.25 and 2.5 mg/kg) treated animals were higher than the level in morphine-saline treated group (p<0.001 and p<0.05, respectively, Fig. 42). In the spinal cord of morphine withdrawal rats, only diazepam 0.25 mg/kg increased the β-endorphin level compared to morphine-saline group (p<0.05, Fig. 44).
**Figure 33.** β-endorphin levels in the pituitary and hypothalamus of morphine tolerant-dependent rats. Animals received morphine or placebo pellet implantation and were injected daily with saline for five days. Rats were sacrificed on the fifth day of treatment. The pituitary and hypothalamus were dissected and subjected to β-endorphin radioimmunoassay. ***p<0.001 compared to the placebo treated group.
Figure 34. β-endorphin levels in discrete brain regions and spinal cord of morphine tolerant-dependent rats. Animals received morphine or placebo pellet implantation and were injected daily with saline for five days. Rats were sacrificed on the fifth day of treatment. Discrete brain regions and spinal cord were dissected and subjected to β-endorphin radioimmunoassay. **p < 0.01 and *p < 0.05 compared to the placebo treated group.
Figure 35. Effect of diazepam treatment on the β-endorphin level in the hypothalamus of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. β-endorphin in the hypothalamus was determined by RIA. ###p < 0.001 compared to placebo-saline treated group.
Figure 36. Effect of diazepam treatment on the β-endorphin level in the hippocampus of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. β-endorphin in the hippocampus was determined by RIA. ###p<0.001, ##p<0.01, and #p<0.05 compared to placebo-saline treated group.
Figure 37. Effect of diazepam treatment on the β-endorphin level in the striatum of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. β-endorphin in the striatum was determined by RIA. ###p<0.001, ##p<0.01, #p<0.05 compared to placebo-saline treated group.
Figure 38. Effect of diazepam treatment on the β-endorphin level in the midbrain of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. β-endorphin in the midbrain was determined by RIA. ##p<0.01 compared to placebo-saline treated group.
Figure 39. Effect of diazepam treatment on the β-endorphin level in the pons-medulla of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. β-endorphin in the pons-medulla was determined by RIA. ###p<0.01, #p<0.05 compared to placebo-saline treated group.
Figure 40. β-endorphin levels in the pituitary and hypothalamus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals received morphine (filled bar) or placebo (open bar) pellet implantation and daily injections with saline for five days. Rats were sacrificed one hour after naloxone injection on the last day of treatment. The pituitary and hypothalamus were dissected and subjected to β-endorphin RIA. *p<0.05 compared to the placebo treated group.
Figure 41. β-endorphin levels in various brain regions of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals received morphine (filled bar) or placebo (open bar) pellet implantation and daily injections with saline for five days. Rats were sacrificed one hour after naloxone injection on the last day of treatment. Discrete brain regions were dissected and subjected to β-endorphin RIA. *p<0.05 compared to the placebo treated group.
Figure 42. Effect of diazepam treatment on the β-endorphin level in the hypothalamus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine pellets and received daily saline (open bar) or diazepam (DZP, filled bar) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The hypothalamus was collected and analyzed for β-endorphin level by RIA. ***p<0.001, and *p<0.05 compared to morphine-saline treated group.
Figure 43. Effect of diazepam treatment on the β-endorphin level in the hippocampus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine pellets and received daily saline (open bar) or diazepam (DZP, filled bar) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The hippocampus was collected and analyzed for β-endorphin level by RIA.
Figure 44. Effect of diazepam treatment on the β-endorphin level in the spinal cord of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine pellets and received daily saline (open bar) or diazepam (DZP, filled bar) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The spinal cord was collected and analyzed for β-endorphin level by RIA. *p<0.05 compared to morphine-saline treated group.
Effect of diazepam treatment on the levels of dynorphin in discrete brain regions of morphine tolerant and dependent animals and of morphine abstinent animals

Morphine pellet implantation significantly decreased the dynorphin levels in the hypothalamus (30%, \( p < 0.05 \)) and spinal cord (42%, \( p < 0.05 \)), but increased the levels in the midbrain (24%, \( p < 0.05 \)) and cerebellum (43%, \( p < 0.05 \)) of tolerant-dependent rats compared to the levels in the placebo implanted animals (Fig. 45-46). In morphine tolerant-dependent rats undergoing abstinence, the levels of dynorphin were increased in the hypothalamus (104%, \( p < 0.05 \)), hippocampus (68%, \( p < 0.05 \)) and striatum (66%, \( p < 0.01 \)) compared to control rats (Fig. 50-51). In the hypothalamus, placebo implanted rats treated with diazepam (0.025 mg/kg) showed lower dynorphin levels than placebo implanted rats injected with saline (\( p < 0.05 \), Fig. 47). In the spinal cord, diazepam (0.25 and 2.5 mg/kg) decreased the dynorphin levels in placebo implanted rats compared to control animals (\( p < 0.01 \) and \( p < 0.05 \), Fig. 48). On the other hand, diazepam (2.5 mg/kg) treatment in placebo implanted animals increased the level of dynorphin in the cerebellum (\( p < 0.01 \), Fig. 49). When given together with morphine pellets, diazepam did not significantly change the level of dynorphin compared to the level in morphine-saline treated group (Fig. 47-49).

In morphine tolerant-dependent rats undergoing withdrawal, only rats receiving diazepam (2.5 mg/kg) treatment showed higher dynorphin level in
the pons-medulla compared to animals in morphine-saline treated group (Fig. 55). Daily diazepam injections did not affect the dynorphin level in other brain regions of morphine abstinent animals as shown in Fig. 52-54.
Figure 45. Dynorphin levels in the pituitary and hypothalamus of morphine tolerant-dependent rats. Animals received morphine or placebo pellet implantation and were injected daily with saline for five days. Rats were sacrificed on the fifth day of treatment. The pituitary and hypothalamus were dissected and subjected to dynorphin radioimmunoassay. *p < 0.05 compared to the placebo treated group.
Figure 46. Dynorphin levels in discrete brain regions and spinal cord of morphine tolerant-dependent rats. Animals received morphine or placebo pellet implantation and were injected daily with saline for five days. Rats were sacrificed on the fifth day of treatment. Discrete brain regions and spinal cord were dissected and subjected to dynorphin radioimmunoassay. *p<0.05 compared to the placebo treated group.
Figure 47. Effect of diazepam treatment on the dynorphin level in the hypothalamus of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Dynorphin in the hypothalamus was determined by RIA. #p<0.001 compared to placebo-saline group.
Figure 48. Effect of diazepam treatment on the dynorphin level in the spinal cord of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Dynorphin in the spinal cord was determined by RIA. ##p<0.01, #p<0.05 compared to placebo-saline group.
Figure 49. Effect of diazepam treatment on the dynorphin level in the cerebellum of morphine tolerant-dependent rats. Rats were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed on the fifth day of treatment. Dynorphin in the cerebellum was determined by RIA. ##p<0.01, #p<0.05 compared to placebo-saline group.
Figure 50. Dynorphin levels in the pituitary, hypothalamus and spinal cord of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals received morphine (filled bar) or placebo (open bar) pellet implantation and were injected daily with saline for five days. Rats were sacrificed one hour after naloxone injection on the last day of treatment. The pituitary, hypothalamus and spinal cord were subjected to dynorphin RIA. *p<0.05 compared to the placebo treated group.
Figure 51. Dynorphin levels in various brain regions of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals received morphine (filled bar) or placebo (open bar) pellet implantation and were injected daily with saline for five days. Rats were sacrificed one hour after naloxone injection on the last day of treatment. Discrete brain regions were dissected and subjected to dynorphin RIA. **p<0.01, *p<0.05 compared to the placebo-saline treated group.
**Figure 52.** Effect of diazepam treatment on the dynorphin level in the hypothalamus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The hypothalamus was collected and analyzed for dynorphin by RIA. **##**p<0.01, **#**p<0.05 compared to placebo-saline treated group.
Figure 53. Effect of diazepam treatment on the dynorphin level in the hippocampus of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals, implanted with morphine (filled bar) or placebo (open bar) pellets, received daily saline or diazepam (DZP) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The hippocampus was collected and analyzed for dynorphin by RIA. #p<0.05 compared to placebo-saline treated group.
Figure 54. Effect of diazepam treatment on the dynorphin level in the striatum of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The striatum was collected and analyzed for dynorphin by RIA. ##p<0.01, #p<0.05 compared to placebo-saline treated group.
Figure 55. Effect of diazepam treatment on the dynorphin level in the pons-medulla of morphine tolerant-dependent rats undergoing an abstinence syndrome. Animals were implanted with morphine (filled bar) or placebo (open bar) pellets and received daily saline or diazepam (DZP) injections. Rats were sacrificed one hour after naloxone injection on the fifth day. The pons-medulla was collected and analyzed for dynorphin by RIA. **p<0.01 compared to morphine-saline treated group. ###p<0.001, ##p<0.01, #p<0.05 compared to placebo-saline treated group.
Serum morphine concentration in morphine treated animals receiving daily saline or diazepam injections

Serum morphine concentration was determined by RIA kit 'Coat-a-Count Serum Morphine'. Morphine concentration was determined in rats implanted with morphine pellets and injected with saline 1 ml/kg or diazepam 0.25 mg/kg for five days in tolerant-dependent animals and in animals exhibiting naloxone-precipitated abstinence. As shown in Table 3, the mean serum morphine concentration in tolerant-dependent animals was 732.4 ± 54 ng/ml and 631.3 ± 75 ng/ml in the morphine-saline and the morphine-diazepam treated group, respectively. There is no statistical difference in the serum morphine between these two groups. During an abstinence syndrome the serum morphine concentration in morphine implanted rats receiving daily saline injections was 662 ± 54.5 ng/ml, and was 756 ± 159 ng/ml in animals treated with morphine and diazepam 0.25 mg/kg for five days. The serum concentrations in these two groups were not statistically different from each other when compared by the Student t-test. These results show that concomitant diazepam injections do not affect the serum morphine concentration. The effects of diazepam on morphine tolerance to antinociception and naloxone-precipitated jumping are unlikely to be due to alteration in the pharmacokinetics of morphine by diazepam treatment.
TABLE 3. Effect of chronic diazepam treatment (0.25 mg/kg) on serum morphine concentration in morphine tolerant-dependent rats and in animals exhibiting naloxone-precipitated abstinence.

<table>
<thead>
<tr>
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<th>Morphine-Saline (ng/ml ± SEM)</th>
<th>Morphine-Diazepam (ng/ml ± SEM)</th>
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<tbody>
<tr>
<td>Tolerant-dependent$^a$</td>
<td>732.4 ± 54 (n=6)</td>
<td>631.3 ± 75 (n=9)</td>
</tr>
<tr>
<td>Naloxone-precipitated$^a$</td>
<td>662 ± 54.5 (n=8)</td>
<td>756 ± 159 (n=7)</td>
</tr>
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Trunk blood was collected on the last day of treatment in tolerant-dependent rats (without naloxone injection) and in naloxone-precipitated abstinent rats. Serum was used to measure morphine concentration by RIA kit 'Coat-a-Count Serum Morphine'. There was no significant difference in the serum morphine concentrations between morphine-saline and morphine-diazepam treated groups as analyzed by the Student t-test.

$^a$: both experiments were conducted separately.
Tolerance to and dependence on morphine can be developed by many paradigms either by repeated injections, continuous infusion or pellet implantation. The advantages of using pellet implantation are that it is a convenient procedure, it rapidly induces a high degree of tolerance and dependence and avoids the problem of associative tolerance. By using morphine pellet implantation, a slow and continuous release of morphine induces the development of tolerance to antinociception as measured by TF (Fig. 8) and HP (Fig. 9) tests, and sedation as determined by a rotarod test (Fig, 10). The development of tolerance to morphine pellet implantation was not due to a decrease in morphine concentration. Yoburn et al. (43) demonstrated that after morphine pellet implantation, mean plasma concentration reached a peak at 4-6 hours, then remained at a steady-state level for at least 72 hours after implantation. A physical dependence induced by morphine also developed as evidenced by a naloxone-precipitated abstinence syndrome in these animals (Fig. 11,12). These data are in an agreement with the results reported earlier using similar morphine-pellet implantation protocols (53,95).
The pharmacological actions of benzodiazepines include anxiolytic, anticonvulsant, muscle relaxant, and sedative-hypnotic effects. In animal models, diazepam induced antinociceptive and sedative responses (75). The doses of diazepam that did not affect TF, HP and rotarod tests in rats were established as shown in Fig. 5-7 and used in the following experiments. Although diazepam is an old drug compared to other benzodiazepines, it is considered to be safe and is a commonly used drug (96,97). It is also one of the benzodiazepine prototypes (98).

Diazepam has been demonstrated to modify many opioid effects. However, effects of diazepam on the development of tolerance to and dependence on morphine have not been extensively studied. We have demonstrated in this study that diazepam inhibits the development of tolerance to morphine-induced antinociception in a TF test (Fig. 13,15), and reduces the severity of physical dependence as determined by monitoring naloxone-precipitated jumping behavior (Fig. 18-20). These results are consistent with the report that a benzodiazepine receptor agonist midazolam inhibits the development of tolerance to morphine-induced TF antinociception in rats (82). Diazepam did not show any effect on the development of tolerance to antinociception as determined by a HP test. Although both TF and HP tests determine nociceptive response to thermal noxious stimuli, it has been shown that the mechanism and neural pathway of both tests are different (99,100). A TF test measures spinal as well as central analgesia while a HP test is an indicative of central analgesia which can be observed even in spinalized animals.
Benzodiazepines have been shown to enhance the antinociceptive response induced by morphine (76,81). An increase in antinociceptive response in morphine-diazepam treated rats is illustrated in Fig. 13 and might be due to an acute synergistic effects of diazepam and morphine in increasing antinociception. Due to this morphine-diazepam synergism, the antinociception was much greater than with morphine alone. Because of the 10-sec cutoff time this enhanced antinociception could not be measured, and thus, the development of tolerance could not be detected. However, as shown in Fig. 15, antinociception measured one hour after diazepam injection is not significantly different from that measured before diazepam injection in the animals treated with morphine, suggesting that an inhibition of morphine-tolerance by diazepam is not due to an acute synergistic effect between morphine and diazepam.

A high dose of diazepam has been shown to impair sensorimotor performance in mice (75). Diazepam 5 mg/kg but not 0.025-2.5 mg/kg induced sedation in rats (Fig. 7). A synergism between morphine and diazepam has been reported (77). The doses of diazepam that were used in this study did not affect sensorimotor performance in control rats (Fig. 17) or enhance sedation induced by morphine treatment (Fig. 16) Tolerance to sedative effects of morphine developed after the third day of implantation as evidenced by a return of the rotarod performance to the control level (Fig. 10). Concomitant diazepam treatment did not influence the development of tolerance to sedation induced by morphine (Fig. 16).
The morphine pellet implantation induced a physical dependence in rats as evidenced by monitoring a naloxone-precipitated abstinence syndrome. Morphine tolerant-dependent rats exhibited significant withdrawal signs (jumping, whole body shakes and teeth chattering) compared to placebo treated rats (Fig. 11, 12). This confirms that these rats indeed were in a dependent state even though the effect of morphine was not observed on the last day of treatment. These withdrawal signs were not observed in placebo implanted rats or placebo-diazepam treated rats injected with naloxone.

The results from this study showed that concomitant diazepam treatment reduced the severity of physical dependence on morphine. Rats treated with diazepam and morphine showed a lesser degree of physical dependence as determined by naloxone-precipitated jumping than animals treated with morphine and saline (Fig. 18, 19). The frequency and proportion of rats exhibiting jumping behavior were less in rats treated with morphine and diazepam (0.25 mg/kg) than in rats treated with morphine and saline. This decrease in escape-jumping behavior could not be due to the muscle relaxant property of diazepam since doses of diazepam used in this study did not affect sensorimotor performance. Other withdrawal behaviors (teeth chattering and whole body shakes) were not affected by diazepam treatment. It has been suggested that the neuronal pathways and the receptors mediating individual signs of naloxone-precipitated abstinence are different (101-104). It is possible that diazepam may modify parts of neuronal pathways and receptors involved in the development of tolerance and dependence, resulting in
modulation only in jumping behavior but not teeth chattering or whole body shakes.

A single diazepam injection (2.5, 5 and 10 mg/kg) has been shown to inhibit naloxone-precipitated abstinence in mice (84). It is possible that a decrease in naloxone-precipitated jumping observed in our study (Fig. 18) is simply due to the effect of the last diazepam injection before naloxone-precipitated withdrawal. We, therefore, have performed an experiment to determine whether diazepam affects the development of physical dependence or simply alters an expression of the abstinence syndrome. As shown in Fig. 20, diazepam (0.25 mg/kg) given acutely, before a naloxone injection did not significantly reduce jumping in morphine dependent rats. In contrast morphine treated rats receiving diazepam (0.25 mg/kg) on day 1 through day 4 exhibited less jumping even though they did not receive diazepam on day 5 prior to precipitating abstinence as shown in Fig. 20. The severity of jumping behavior in these rats was not different from morphine-diazepam treated animals receiving diazepam injection on the last day prior to naloxone administration. We conclude that repeated diazepam administrations are necessary for inhibition of morphine tolerance and physical dependence and diazepam affects the process of tolerance and dependence rather than just modifies an expression of withdrawal behaviors. Although psychological dependence on morphine developed as well, its severity could not be estimated in this model. It is also of interest to investigate the effect of benzodiazepines on psychological dependence on opioids.
The inhibitory effect of diazepam on development of morphine tolerance and dependence is unlikely to be due to changes in morphine levels induced by diazepam. The serum morphine concentration in morphine-diazepam treated rats did not significantly differ from the concentration in morphine-saline treated animals in either tolerant-dependent or abstinent states (Table 3). Rosland et al. (105) also reported that the level of morphine in the serum, brain and spinal cord did not change in mice treated with morphine and diazepam compared to the level in mice treated with morphine alone. This suggests that the effect of diazepam on morphine tolerance and dependence is not due to an alteration of the serum morphine concentration by diazepam.

The mechanism underlying the effect of diazepam induced inhibition of morphine tolerance and dependence is not fully understood. The effect of diazepam on the CNS opioid peptides of morphine tolerant and dependent rats may be one of the underlying mechanisms involved.

Endogenous opioid peptides have been implicated in pain suppression, drug reward, tolerance and dependence (24). It has been unveiled that opioid peptides, enkephalins and β-endorphin are involved in central reward processes and may mediate euphoric subjective states (27,106). Intraventricular injection of enkephalins (27,28) or β-endorphin (31) induces self-administration behavior in animal models. Rats will press a lever for direct delivery of enkephalins or β-endorphin into their brains. The opioid receptor antagonist naloxone blocks the reward elicited by electrical
stimulation of certain brain regions (107) as well as other facilitator effects of heroin on brain stimulation reward (108). This reward process is involved in an electrical activation of opioid-rich brain regions by animal itself. By pressing a lever, animals will activate electrical stimulation causing a release of endogenous opioids. The number of this self stimulation is reduced after pretreatment with the opioid antagonist, naloxone. These reports suggest the possibility that the reward of drug self administration may involve, at least in part, the central release of endogenous opioid peptides. If the reinforcement associated with administration of drug is a contributory factor in the development and maintenance of drug dependency, then the involvement of endogenous opioid peptides may be a unifying feature (109).

Trujillo et al. (29) have proposed a mechanism for how endogenous opioid peptides may be involved in morphine tolerance and dependence. Neurotransmission is controlled by feedback inhibition. Administration of an exogenous neurotransmitter or its agonists leads to a decrease in the biosynthesis and release of that transmitter. According to this mechanism, chronic administration of exogenous opioids such as morphine would produce feedback inhibition of endogenous opioid neurons, leading to a down regulation of opioid peptide biosynthesis and release (67,70). In this study, we observed a decrease in the level of met-enkephalin in the hypothalamus and spinal cord; β-endorphin was decreased in the hypothalamus, hippocampus, striatum and midbrain; and dynorphin was decreased in the hypothalamus and spinal cord of morphine tolerant-
dependent animals. However, we detected an increase in the dynorphin level in the midbrain and cerebellum of tolerant-dependent rats. This change in endogenous opioid activity together with other changes resulting from chronic stimulation of opioid receptors, are proposed to be responsible for many neural and behavioral changes observed during opiate tolerance and dependence (24,29,67). During naloxone-precipitated abstinence, we observed a decrease in the met-enkephalin level in the hypothalamus, hippocampus, cortex and spinal cord; β-endorphin in the pituitary and hippocampus; but an increase in the dynorphin level in the hypothalamus, hippocampus, and striatum. Naloxone injection did not affect the level of opioid peptides in control animals (68). The changes of opioid peptides observed here can reflect a withdrawal state. As mentioned before, met-enkephalin and β-endorphin are involved in reward mechanism. However, the κ-opioid system is involved in aversive behavior (106). It has been suggested that under 'normal' conditions, there is an equilibrium between rewarding and aversive opioid effects. This equilibrium is interfered with and then re-established after exogenous opioid treatment. Withdrawal of continuously applied opioids may uncover the compensatory mechanism, leading to an overexpression of aversive κ-receptor mediated system (106). Since dynorphin is relatively selective for κ-receptors, an increase in the CNS dynorphin levels observed in our study, might reflect a hypertrophy of the aversive κ-opioid system during an abstinence syndrome.

Overlaps between opioid and benzodiazepine system have been reported. Naloxone has been shown to block the anticonflict effects of
diazepam, the facilitation of lateral hypothalamic self-stimulation produced by chlordiazepoxide (110,111), analgesic effects of midazolam (76), and locomotor effects of benzodiazepines (112). On the other side, flumazenil, a benzodiazepine receptor blocker, potentiated the action of morphine on body temperature (113). Benzodiazepines have been shown to affect the analgesic effect of morphine. The inhibition of morphine induced analgesia by benzodiazepines was partially blocked by a benzodiazepine receptor antagonist, Ro 15-1788 (114) or a GABA_A receptor antagonist, bicuculine (74) suggesting that the BZD-GABA receptor complex is involved in the inhibition of morphine induced analgesia by benzodiazepines. In addition, diazepam also enhanced the rewarding effect of opiate administration in methadone-maintenance patients (115). The colocalization of opioids and BZD-GABA neurotransmitter has been demonstrated at least in the cerebellum (116) and spinal cord (117).

A direct relation between benzodiazepines and opioid peptides has been reported. Acute administration of diazepam induced enkephalin release in certain brain regions (85,86). Duka et al. (85) observed an increase in the rat hypothalamic met-enkephalin level 5 min after, but not one hour after the injection of diazepam. In our study, we examined these brain regions for met-enkephalin levels two hours after the last diazepam injections. We observed that diazepam 0.025 and 2.5 mg/kg increased the met-enkephalin level in the midbrain and pons-medulla, (Fig. 24,25). But the level was decreased in the spinal cord of animals in the placebo-diazepam treated group compared to placebo saline (Fig. 27,32). These
changes in the met-enkephalin levels might reflect the effect of repeated diazepam treatment on certain brain regions of placebo pellet implanted animals. Harsing et al. (86) reported that diazepam inhibited met-enkephalin efflux during depolarization in perfused striatal slices in the rat. It is possible that the increase in met-enkephalin levels in brain regions of morphine-diazepam treated animals compared to morphine-saline treated group as shown in Fig. 27,29,30,32, may be due to an inhibition in the efflux of met-enkephalin from neuronal cells during diazepam treatment. Harsing et al. (86) also reported that inhibition of met-enkephalin efflux by diazepam required a certain amount of GABA and the availability of GABA receptors. Morphine administration has been shown to increase GABA content and glutamate decarboxylase activity in the rat spinal cord and thalamus (118). Morphine may promote inhibition of met-enkephalin efflux by diazepam by increasing GABA contents in the brain.

Diazepam treatment may enhance the met-enkephalin biosynthesis in morphine treated animals by mediating through GABAergic system. It has been reported that an activation of GABAergic system by GABA transaminase inhibitors increases the pre-proenkephalin mRNA in the striatum of rats (119). The pre-proenkephalin mRNA has been shown to initially decrease followed by an increase after a combined administration of the GABA agonist muscimal and diazepam into mice (120). Other studies, on the other hand, reported a decrease in the pre-proenkephalin mRNA after chronic treatment with GABA transaminase inhibitors (121). A direct evidence that diazepam or other benzodiazepine receptor agonists affects the
met-enkephalin biosynthesis has not yet been demonstrated. Benzodiazepines, however, have been known to potentiate GABAergic system (122-124). It is also possible that diazepam reverses a decrease in the met-enkephalin levels induced by morphine treatment by increasing the met-enkephalin biosynthesis via GABAergic system.

Effects of GABA/BZD on the release of β-endorphin have been reported. GABA has been shown to be an inhibitory neurotransmitter in the control of β-endorphin secretion from the anterior pituitary (125). Maiewski and colleagues (126) reported that diazepam 0.25 mg/kg by itself did not affect the basal level of β-endorphin over 40 min of detection. We did not observe any change in the β-endorphin levels in the pituitary in the placebo-diazepam group. But we found a decrease in β-endorphin level in the hypothalamus, hippocampus, striatum, midbrain and pons-medulla of placebo implanted rats receiving daily diazepam injections. Maiewski et al. (126) also reported that coadministration of diazepam (5 mg/kg) and morphine (5 mg/kg) significantly attenuated the stress-induced release of β-endorphin. In our study, we observed that diazepam and morphine treatment enhanced the β-endorphin level in the hypothalamus and spinal cord of rats in morphine abstinence, but not in tolerant-dependent rats (compared to morphine-saline treatment). This effect might be the result of diazepam and morphine acting to inhibit β-endorphin release specifically in these two brain regions during abstinence.

A direct effect of diazepam treatment on dynorphin levels has not been demonstrated. The interaction between diazepam and dynorphin,
however, might be speculated. Since midazolam, a benzodiazepine drug, competed for the \( \kappa \)-binding site (76) for which dynorphin is relatively selective, diazepam might affect the dynorphin levels in the CNS. We demonstrated in this study that repeated diazepam treatment for 5 days, affected the level of dynorphin in certain brain regions of placebo implanted rats (Fig. 47-49,53,55). When given together with morphine pellets, diazepam 2.5 mg/kg significantly increased the dynorphin level in the pons-medulla of tolerant-dependent animals undergoing abstinence, compared to the morphine-saline treated group. We did not observe any effect of diazepam treatment in other brain regions of these animals, compared to morphine-saline treated group.

In summary, we have demonstrated that diazepam administration to morphine pellet implanted rats decreases tolerance to morphine-induced antinociception and reduces the degree of physical dependence on morphine. This effect of diazepam might be very useful in the treatment of pain. Levels of three opioid peptides have changed in specific brain regions of morphine treated rats compared to placebo treated animals. Diazepam treatment reverses the changes in the CNS opioid peptide levels, especially for met-enkephalin, in both tolerant-dependent and abstinent animals. Diazepam treatment also increases the levels of \( \beta \)-endorphin in the hypothalamus and spinal cord, and dynorphin in the pons-medulla of morphine abstinent animals. Since the opioid peptides have been implicated in opioid tolerance and dependence, these effects of diazepam on the opioid peptide levels might account for the inhibition of morphine tolerance and
dependence. These results also support an interaction between benzodiazepines and the opioid system. These findings might lead to further investigations of effect of novel benzodiazepines on opioid tolerance and dependence as well as effects of benzodiazepines on other parameters, for instance opioid receptors, which might be involved in the process of opioid tolerance and dependence. On the other hand, effects of morphine treatment on the GABA/benzodiazepine system is also of interest.
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