The potential role of spinal ketamine in multi-component antinociception

Ph.D. Thesis

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To my Dad...

„Physiological experiment on animals is justifiable for real investigation, but not for mere damnable and detestable curiosity."

Charles Robert Darwin
(1809-1882)

Publications

Papers involved in the thesis:

Joo G., Horvath G., Klimscha W., Kekesi G., Dobos I., Szikszay M., Benedek G. The effects of ketamine and its enantiomers on the morphine- or dexmedetomidine-induced antinociception after intrathecal administration in rats. *Anaesthesiology* 2000; 93, 231-241
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Papers associated, but not involved in the thesis:

Csullog E., **Joo G.**, Toth G., Dobos I., Benedek G., Horvath G. Antinociceptive effect of continuous intrathecal administration of endomorphin-1. *Pain* 94: 31-38. 2001.
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Kekesi G., **Joo G.**, Csullog E., Dobos I., Klimscha W., Toth K., Benedek G., Horvath, G.. The antinociceptive effect of intrathecal kynurenic acid and its interaction with endomorphin-1 in rats. *Eur.J.Pharmacol.* 445:93-96. 2002
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Horvath G., **Joo G.**, Klimscha W., Dobos I., Szikszay M., Benedek G. The interaction of S(+)-ketamine with dexmedetomidine after intrathecal administration in rats. *8th Annual Meeting of European Society of Anaesthesiologists 1-4 April 2000. Vienna (*Oral presentation*)*
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List of Abbreviations

AMPA	α-amino-hydroxy-5-methyl-4-isoxazoleproprionic acid
CGRP	calcitonin-gene-related peptide
CNS	central nervous system
DRG	dorsal root ganglion
EAA	excitatory amino acid
EEG	electroencephalography
ED_{50}	effective dose 50 (the dose that yields 50% of the maximum possible effect)
Glu	glutamate
mGluR	metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
PAG	midbrain periaqueductal gray
PCP	phencyclidine
SP	substance-P
VIP	vasoactive intestinal peptide
WDR	wide dynamic range neuron
%MPE	percentage of maximum possible effec

1. Introduction

The spinal cord is an important site for modulation of pain sensations, where several mechanisms, such as interconnections between nociceptive and nonnociceptive afferent pathways, different type of interneurons, descending fibers by the released neurotransmitters and neuromudulators can control the transmission of nociceptive information to higher centers in the brain.

Glutamate (Glu) is a major excitatory neurotransmitter in the central nervous system (CNS). The laminar localization of the ionotropic glutamate receptors concentrates in the superficial dorsal horn of the spinal cord (1). Excitation of the the N-methyl-D-aspartate - type (NMDA) glutamate receptor system plays an important role in the sensitization processes (2). Therefore blocking the NMDA receptor produces only weak or no antinociception against acute thermal or mechanical stimuli in uninjured rats (3), but causes significant antinociception in persistent pain (4).

Ketamine, a non-competitive antagonist at the NMDA glutamate receptors has been used as an anaesthetic drug in clinical use for almost 40 years (5). The effect of ketamine, the so called „dissociative anaesthesia" has been proven to be advantegous in several cases, although undesired phychomimetic effects (hallucinations, vivid dreams) may accompany the recovery phase in some patients (6). Similarly to other NMDA antagonists, intrathecal racemic ketamine produces weak (7) or no (8) antinociception against acute thermal or mechanical stimuli in uninjured rats, but causes antinociception in inflammation-induced persistent pain (4,9) and after nerve damage (10). Clinical studies on the analgesia after spinal or epidural racemic ketamine as a sole agent are controversial (11,12,13). Racemic ketamine is a mixture of two optical isomers: the S(+)-, and the R(-)-enantiomers. Recent data has proved that S(+)-ketamine has four times the anaesthetic potency of the R(-)-enantiomer and may have significant clinical advantages in comparison with the racemic drug (14,15). The incidence of psychotomimetic phenomena appeared to be less common after S(+)-ketamine in comparison to racemic ketamine, besides their quality was described as far less unpleasant. Moreover, increasing experimental evidence supports a remarkable neuroprotective effect of S(+)-ketamine, which may become a promising drug for new therapeutic approaches to neuroprotection (16,17).

There is a body of evidence that suggests advantageous antinociceptive interaction by the combination of opioid or α_2-adrenoceptor agonists with NMDA antagonists after different routes of administration (18,19,20,21). The spinal administration of these drug combinations has not been examined thoroughly yet, but since both the μ opioid receptor and the α_2-adrenoceptor systems modulate greatly the transmission of pain at the spinal level their choice seemed to be obvious.

Opioid receptors – mu (μ), delta (δ) and kappa (κ) - are widely distributed throughout the CNS, including the spinal dorsal horn, especially laminae I and II (1). At spinal sites, ligands for each of the three opioid receptor can produce analgesic effect. Morphine administered via either intrathecal or epidural route are widely used to treat chronic, postoperative and labor pain (22), however due to its several side effects the limitations of spinal opioids are considerable. Endomorphin-1 is a novel endogenous μ-opioid receptor ligand with high affinity and selectivity for the receptor (23). It is a potent antinociceptive agent acting spinally-, supraspinally- and

peripherally (24), therefore it might have potential clinical significance, especially in combination with different drugs.

Noradrenergic descending inhibitory control is mediated at the spinal level by the α_2-adrenoceptors (25). Dexmedetomidine is a new, highly selective and potent α_2-adrenoceptor agonist (26). Low subsedative intrathecal doses of dexmedetomidine resulted in significant behavioral antinociception in rats (27,28). Dexmedetomidine were found to reduce the need for general anaesthetics and analgesics in patients undergoing minor surgery (29). Dexmedetomidine has been recently licensed (in 2000) in the USA for postoperative sedation in intensive care units (30).

Following the concept of the pharmacologically more advantegous combination spinal analgesic chemotherapy (31), the thesis examines the interactions of ketamine and its enantiomers with opioids – morphine and endomorphin-1 - and the α_2-adrenoceptor agonist – dexmedetomidine – after intrathecal administration in rats, using the tail flick test and investigates the possible, advantegous combinations which may have relevant importance in pain therapy in the future.

2. General background

2.1. Spinal anaesthesia in acute pain management

Despite substantial advances in the knowledge of acute pain mechanisms and in their treatment, all forms of acute pain are likely to be poorly managed: postoperative, post-traumatic, burn, acute medical diseases (e.g. pancreatitis, myocardial infarction) and obstetric pain (32). There are a variety of reasons for poor, ineffective management of acute pain such as:

- the fear of the potential for addiction to opioids,
- concerns about respiratory depression and other opioid-related side effects, like nausea, vomitting,
- lack of understanding the pharmacokinetics of various agents.

The elucidation of the different pre- and postsynaptic receptors around nociceptive transmission neurons in the dorsal horn has led to the use of spinal drug administration as a pain management technique. Spinal anaesthesia may be defined as the temporary interruption of transmission of nerve impulses produced by the injection of a locally acting anaesthetic/antinociceptive drug into the subarachnoid space. The application of relatively low doses of agents acting at specific receptor types within the spinal cord with the relative avoidance of side effects has been a major advance in the management of some pain problems.

2.2. The role of spinal cord in pain transmission

There is a close correspondence between the functional and anatomical organization of the neurons in the dorsal horn of the spinal cord. The grey matter of the spinal cord can be subdivided into ten layers (after Rexed) on the basis of cytological features of its resident neurons. The neurons of the spinal dorsal horn are classically organised into six laminae. The axons of nociceptive-specific and wide dynamic range (WDR) neurons in laminae I and II (substantia gelatinosa) and V of the dorsal horn are associated with perception of pain and temperature sensation, while neurons of laminae III, IV and VI respond mainly to nonnoxious stimuli (33).

Small-diameter primary afferent terminals in the dorsal horn contain both small electron-translucent synaptic vesicles that store glutamate, somatostatin (34) and large, dense-core vesicles that store neuropeptides e.g. substance-P (SP), calcitonin gene-related peptide (CGRP) [which appear to enhance and prolong the actions of glutamate; 90% of substance-P containing fibers also contained glutamate (35)] and galanin (34).

Glutamate and these neuropeptides are released together from primary afferent terminals and act coordinately to regulate the firing properties of postsynaptic neurons. The actions of glutamate released from sensory terminals are confined to pre-, and postsynaptic neurons in the immediate vicinity of the synaptic terminal. In contrast, neuropeptides released from sensory terminals can diffuse considerable distances from their site of release. Thus the released neuropeptides seem to influence both the primary afferents and the postsynaptic dorsal horn neurons. This feature, along with the fact that peptide levels are significantly changed in persistent pain conditions – SP and CGRP are downregulated whereas vasoactive intestinal

peptide (VIP) and galanin are upregulated (36,37)-, suggest that peptide actions contribute both to the excitability of dorsal horn neurons and to unlocalized character of many pain conditions.

2.3. Glutamate receptors

The excitatory amino acid (EAA) glutamate has major importance in the synaptic transmission of this type of impulses throughout the CNS. Glutamate receptors are divided into two categories: ionotropic and metabotropic receptors.

There are at least eight types of metabotropic glutamate receptors (mGluR), but few have been anatomically localized yet in the spinal cord. The mGluR1 and mGluR5 metabotropic receptors are concentrated in lamina II (1). Metabotropic glutamate receptors are expressed during the increased spinal excitability associated with peripheral hyperalgesia (38).

The ionotropic glutamate receptors are associated with ion channels and can be divided into 3 subcategories:

- N-methyl-D-aspartate (NMDA) receptors,
- α-amino-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors
- kainate receptors.

The last two is often called together, as non-NMDA receptors.

Laminar localization of the ionotropic glutamate receptors concentrate in the superficial dorsal horn of the spinal cord (1). There is constantly higher binding in lumbar and sacral segments than in thoracic and cervical segments. The majority of the labelling is stated to be in the superficial dorsal horn with emphasis on lamina II. Glu receptors in lamina II are seemed to be involved in sensory input, particularly C fiber input into the cord. Ionotopic Glu receptors found in lamina I may be involved in Aδ primary afferent transmission since these afferents end preferentially here. Glu receptors in lamina III might modulate the large-diameter Aβ afferent fiber input. Although it must be remembered, that there is some binding in all dorsal horn laminae and may have important functions unrelated to direct primary afferent input.

Excessive stimulation of Glu receptors can produce neurotoxic and excitotoxic effects thus the inhibition of Glu receptors improves neurologic outcome after such mechanism and reduces or even blocks the development of excitotoxic injury (9,39).

2.3.1. Basic characteristics of the NMDA receptors

NMDA-sensitive ionotropic glutamate receptors consist of tetrameric, heteromeric, subunit assemblies that have different physiological and pharmacological properties. The NMDA receptors are comprised of two classes of subunits: one subunit of NR1 and four subunits of NR2 [NR2A, NR2B, NR2C, NR2D (NR2D-1, NR2D-2)] (40). It is generally accepted that functional receptors in the mammalian central nervous system are only formed by combination of NR1 and NR2 subunits, which express the glycine and glutamate recognition sites, respectively.

The NMDA receptors are channels that are permeable to Na^+, K^+ and Ca^{2+} ions (Figure 1). NMDA elicits the entry of calcium ions into the cell (41) and the calcium that enters the cell in response to glutamate results primarily from NMDA receptors (42). The NMDA receptor is positively mediated by glycine (43) [which binds to a specific, strychnine-insensitive, $glycine_B$ site], by polyamines (spermine, spermidine), Zn^{2+}, protons and redox agents (44). Under

physiological conditions – i.e. normal membrane potential – the ion channel is blocked by a Mg^{2+} ion, held in the channel by virtue of the relative electronegativity of the cell interior (45).

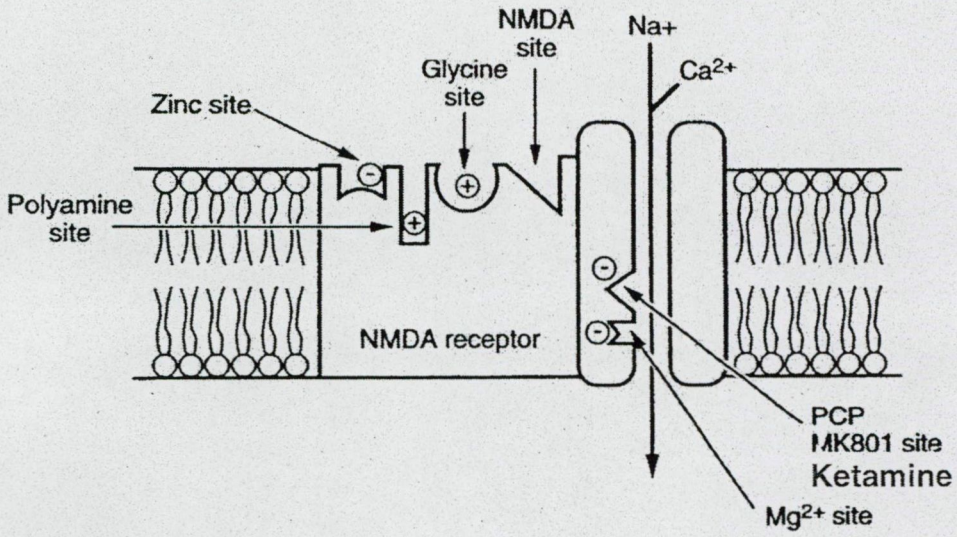


Figure 1. Structure of the NMDA receptor

Due to the use-, and voltage-dependent Mg^{2+} block, the NMDA receptors are activated only after depolarization of the postsynaptic membrane by - for example after an AMPA receptor activation -, which relieves the Mg^{2+} blockade. Uncompetitive NMDA receptor antagonists such as ketamine, dextromethorphan, phencyclidine (PCP) or MK-801 block the channel in the open state, although the blocking kinetics and voltage dependence of this effect depend on the antagonist.

NMDA receptor has been implicated in a number of long-term events in the CNS such as in long-term potentiation in the hippocampus, in synaptic plasticity, in epileptic activity and in sustained motor activity (46,47,48). NMDA receptors are also involved in all types of persistent clinically important pain states, namely: inflammatory pain, neuropathic pain, allodynia and ischemic pain. A number of recent human studies with ketamine have confirmed the clinical efficacy of this NMDA antagonist in the previously mentioned pain states (49,50,51,52,53).

The currently available NMDA receptor antagonists can be divided into 4 groups according to the site and mechanism of their action:

- competitive NMDA antagoninsts,
- non-competitive NMDA antagonists,
- allosteric glycine site modulators,
- antagonists at the polyamine site.

NMDA antagonist substances are summarized in Table 1. Most of the agents are used for experimental purposes, only a few of them are applied in human practice, like ketamine (anesthetic agent) and dextrorphan (anti-tussive agent).

Table 1. NMDA-receptor antagonists

Competitive NMDA antagonists	APV (3) AP-5 (8) AP-7 (8) CPP (3,8) ASP-AMP (8) GLU-AMP (8) alfa-aminoadipic acid (8)
Non – competitive NMDA antagonists	ketamine (10) MK-801 (dizolcipin) (3) dextrorphan (8) phencyclidine (8)
Allosteric glycin-site modulators	7-Cl-kyn (3) FICA (3) HA-966 (8) 5Cl-I2CA (10)
Antagonists acting at the polyamine site	ARCA (3) ifenprodil tartrate (3)

APV = D-2-amino-5-phosphovalerate
AP-5 = [(±) 2-amino-5-phosphonopentanoilic acid]
AP-7 = [(±) 2-amino-7-phosphonopentanoil acid]
CPP = [(±) 3-(2-carboxipiperazine-4-il)-propil-1-phosphonic acid]
ASP-AMP = beta-D-aspartilaminomethyl-phosphonic acid
GLU-AMP = gamma-D-glutamilaminomethyl-phosphonic acid
7-Cl-kyn = 7-chloro-kynurenic acid
FICA = 5-fluoroindole-2-carboxilic acid
HA-966 = [(±) 3-amino-1-hydroxil-2-pirrolidine
5Cl-I2CA = 5-chloro-indole-2-carboxilic acid,

2.3.2. Localization of the NMDA receptor in the spinal dorsal horn

As it has already been mentioned, functional NMDA receptors in the mammalian CNS are only formed by combination of NR1 and NR2 subunits, which express both the glycine and glutamate recognition sites (40). At the electronmicroscopic level, most NR1 staining is localized to small patches of the cell body and dendritic membrane and in particular to the postsynaptic densities. Antibodies for NR2A and NR2B stain cells and neuropil in laminae I-III with fair intensity and dorsal root ganglion (DRG) cells with relatively high intensity (1). Enhanced phosphorylation of NR1 subunits in the dorsal horn spinothalamic tract neurons after strong noxious stimulation (intradermal capsaicin injection) support the idea that NMDA receptors in spinothalamic tract neurons play a role in the transmission of nociceptive information and that phosphorylation of these receptors contributes to the development of central sensitization of spinothalamic tract cells (54).

Some labeling is also found in presynaptic terminals, next to the presynaptic densities. Many of the labeled presynaptic terminals are central scalloped endings in glomeruli. Accordingly many of the labeled presynaptic terminals arise from primary afferents. Labelling appears in DRG cells of all sizes and there is a build-up of receptor next to a ligature of the sciatic nerve (55). Approximately 70% of the NR1 immunolabeled presynaptic profiles contain high concentrations of glutamate. This presumably indicates autoreceptors, implying that release of glutamate by a terminal depolarizes the same terminal. Such autoreceptors might underlie the phenomenon, e.g. central sensitization and wind-up.

2.4. Ketamine

Ketamine, as an anaesthetic agent was introduced into the clinical practice almost 40 years ago (5), with the hope that it would function as a "monoanaesthetic" drug: inducing analgesia, amnesia, loss of consciousness and immobility. This dream was not fulfilled because significant side effects were soon reported. However it is still used clinically for indications such as (11,52,56,57,58):

- induction of anaesthesia in patients in hemodynamic shock,
- in patients with active asthmatic disease,
- sedation of uncooperative patients - particularly children,
- supplementation of incomplete regional and local anaesthesia,
- sedation in the intensive care setting,
- the management of pain.

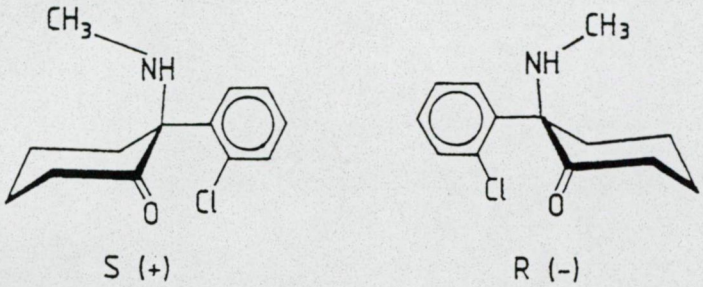


Figure 2. Structural isomers of ketamine

Ketamine is chemically related to phencyclidine and cyclohexamine. It has a molecular weight of 238 and pKa of 7.5. The molecular structure of ketamine contains a chiral centre at the C-2 carbon of the cyclohexanone ring so that two optical isomers of the ketamine molecule exist: the left-handed, S(+)-ketamine and the right-handed, R(-)-ketamine (Figure 2), which has differences between their pharmacological (59,60) and pharmacokinetic properties (61,62). Commercially available racemic ketamine preparations contain equal concentrations of the two enantiomers.

2.4.1. Pharmacologic properties

Racemic ketamine has been used in the anesthetic clinical practice from 1965 (5). The initial experinence with ketamine as a sole agents led to the recognition of unpleasant emergence

reactions and cardiovascular stimulant properties, which can be reduced by supplementation with other drugs, like benzodiazepines (11). Clinical application of racemic ketamine –besides the intravenous and intramuscular administrations - have emerged recently, there are several other ways, such as the use of oral, rectal, intranasal, epidural and intrathecal preparations for the purposes of analgesia, sedation and anesthetic induction (11, 56).

Marietta *et al.* (59) reported differences between the pharmacological potencies of the two enantiomers of ketamine in rats, saying that S(+)-ketamine has a higher therapeutic index, three- to fourfold greater than the racemate or the pure R(-)-ketamine. Both the racemic-, and the S(+)-ketamine caused a similar activation of the endocrine stress response and a comparable stimulation of the sympathoadrenergic system (63). However, application of S(+)-ketamine was associated with a remarkably smoother emergence period, a profound postoperative analgesia, a more rapid recovery of cerebral functions, and a greater preference by the study persons (64).

Results with the pure S(+)-ketamine suggest that its use may be associated with fewer side effects than the racemic mixture (65). The pure S(+)-ketamine has been approved for clinical use in 1997 in Europe (Germany, Austria) and the number of additional clinical experience is increasing since then, focusing mainly on the systemic administration. According to these studies S(+)-ketamine seems to be an ideal prehospital analgesic drug in emergency medicine (66) and in intensive care medicine (67). Monoanesthesia with S(+)-ketamine is said to be a remarkable sympathomimetic form of general anesthesia; it may offer advantages for anesthesia induction in patients with shock or bronchial asthma. The combination of S(+)-ketamine with midazolam can be used for analgosedation in unstable cardiovascular and exogenous catecholamine-demanding patients due to the opportunity for sympathomimetic and endocrine-stimulating effects (68).

A few studies have examined the effect of spinal S(+)-ketamine in humans, so far. One has found that in patients undergoing orthopedic surgery, epidural S(+)-ketamine resulted in analgesia and this was enhanced by transdermal nitroglycerin (69). Others reported that continuous long-term intrathecal S(+)-ketamine led to a significant pain reduction and reduced the doses of intrathecal morphine (70). In children, caudal S(+)-ketamine and clonidine combination has been provided excellent perioperative analgesia (21).

2.4.2. Pharmacokinetic properties

The distribution of ketamine after intravenous administration is widespread and rapid, which is reflected by a high value of the rapid disposition constant (α) (6.79±3.59 h^{-1}) and the slow disposition phase constant (β) of ketamine is 0.30±0.10 h^{-1} (62). Epidural administration showed good access of ketamine to the systemic circulation from the epidural space, with a mean bioavailability value of 77±22%. The apparent incorporation constant from epidural space to the plasma compartment (5.54±2.33 h^{-1}) implies a rapid increase in the plasma levels of the drug. The compound is metabolised extensively by hepatic cytochrome P450 system: at therapeutic concentrations CYP2B6 mediates mainly the N-demethylation of R(-)- and S(+)-ketamine (71). The human liver metabolism of ketamine demonstrates a relative isomeric selectivity, which accounts for observed differences in the clinical pharmacokinetics of racemic ketamine and its enantiomers (61). Its primary metabolit norketamine is only one-third to one-fifth as potent as the original compound but may be involved in the prolonged analgesic actions of ketamine (72). Norketamine and dehydronorketamine are excreted by the kidneys.

2.4.3. Mechanism of action

Ketamine binds to the specific, phencyclidine site in the NMDA receptor mainly when the channel is in open, activated state (73). A second NMDA receptor binding site for ketamine has also been reported that is associated with the hydrophobic domain of the receptor protein. Binding in the pore of the channel decreases its open time, whereas binding to the latter site decreases the frequency of channel opening.

Ketamine, in high dose has been shown to interact with a number of other receptor systems such as the opioid receptors (74), monoaminergic and muscarinic receptors (56,75) and with the voltage-sensitive sodium channels (76). Though in low doses it seems to result in a selective, non-competitive and stereoselective NMDA blockade (11,77).

2.4.4. Effects of ketamine on the function of the CNS

The classic anaesthetic effects of ketamine are best described as a dose-dependent central nervous system depression that leads to a so-called "dissociative" state, characterized by profound analgesia and amnesia but not necessarily loss of consciousness (78). During ketamine anesthesia the patients seem to be completely unaware of the environment, although they are not asleep. This form of catalepsy include electrophysiologic inhibition of thalamocortical pathways (65) and stimulation of the limbic system (79), which both seem to be stereospecific to the effect of S(+)-, and R(-)-ketamine.

The frequent complication of ketamine anaesthesia is the occurrence of disturbing hallucinations upon awakening („emergence reactions") (80). The drug produces effects similar to those of PCP but with a much shorter duration of action. At higher doses and plasma levels of ketamine (200ng/ml) incidence of cognitive and memory impairments, psychiatric symptoms, illusory experiences and other adverse effects increase (52).

One study suggests that inhibition of central and peripheral cholinergic transmission may contribute to the induction of ketamine-caused hallucinations, besides the preferred anaesthetic effect (81). On the other hand, S(+)-ketamine increases the dopamine efflux in the nucleus accumbens by mobilization of the dopamine storage pool to releasable sites, which can be another reason for the side effects (79).

In humans, characteristic electroencephalographic (EEG) changes were noted after administration of ketamine: dose-dependent increases in delta, theta and beta power (82). In equipotent doses, S(+)-ketamine induces similar EEG changes than the racemate.

Although both ketamine enantiomers induce less tiredness and cognitive impairment than equianalgesic small-dose racemic ketamine, S(+)-ketamine causes less decline in concentration capacity and primary memory (64). Anterograde amnesia was observed more frequently after racemic ketamine application than after the S(+)-enantiomer. Moreover the degree of worsened concentration capacity was significantly less after S(+)-ketamine than after the racemate (63).

Ketamine has been shown to protect neurons from NMDA induced damage *in vivo* - after ischemia (83) and seizures (84), as well as *in vitro* (83). Studies showed that S(+)-ketamine has a higher neuroprotective potency *in vitro* against NMDA-induced injury compared with the racemate or the R(-)-enantiomer and can induce regeneration after axonal transection (17).

2.4.5. Analgesic effects of ketamine after spinal administration

Animal studies evaluating the effect of intrathecal racemic ketamine agree that it has no (8) or only weak (7) analgetic effect on acute pain states, but significantly decreases hyperalgesia associated with carrageenan-induced inflammation (4), and pain occurring after nerve damage (10).

A series of clinical studies has suggested potent analgesia after spinal or epidural administration of racemic ketamine (11), although others found that intrathecally administered racemic ketamine as a sole agent did not provide effective analgesia (13). Clinical reports showed that racemic ketamine has beneficial effect when combined with other analgesics, like opioids (85,86,87,88), local anaesthetics (89), α_2 agonists (21) or even in combination of all of these groups (90). Although it must be stated that there are a few studies which found no advantegous interaction when combining ketamine with opioids or local anaesthetics (91,92).

The neuraxial use of racemic ketamine raised questions about potential toxicity (93), since some neurologic lesions were reported after its intrathecal administration (7,94). One case report (95) speaks about some neurological damage after a multi-drug treatment that included racemic ketamine also, but in that case it is not certain that this component was the cause of the lesion. Preservatives (benzethonium chloride or chlorobutanol) used in racemic ketamine solution in some cases were thought to cause neurological damage in animals, but the pure enantiomers of ketamine do not induce this kind of lesion themselves (93). The preparation that contains the pure S(+)-ketamine is prepared in a preservative-free solution, which makes it more preferable, besides its other pharmacological and pharmacokinetic advantegeous properties, to be used in clinical practice.

2.5. Endogenous pain controlling mechanisms at the spinal level

Transmission of nociceptive information is subject to modulation at several levels of the neuraxis including the dorsal horn of the spinal cord (32). Afferent impulses arriving in the dorsal horn initiate both inhibitory and excitatory mechanisms, which are greatly influenced by the descending modulatory control systems.

The midbrain periaqueductal gray (PAG) is part of the CNS cirquit that controls nociceptive information at the level of the spinal cord (Figure 3). PAG integrates inputs from the limbic forebrain, diencephalon, the medial prefrontal and insular cortex, the hypothalamus and the amygdala. It also receives inputs from the brainstem (locus coeruleus, pontomedullary reticular formation, nucleur cuneiformis and other catecholaminergic neurons) and significant projection from the spinal lamina I nociceptive neurons. The PAG projects only minimally to the spinal dorsal horn and the pain-modulating action of the PAG upon the spinal cord is relayed largely through the rostral ventromedial medulla (RVM).

The PAG and the adjacent nucleus cuneiformis are the major source of inputs to the RVM: it receives input from the PAG via enkephalin, substance P and γ-amino-butyric acid (GABA) containing neurons, from the dorsal raphe through serotonin (5-HT)-containing neurons and there is also a neurotensinergic connection between the PAG and the RVM. Direct spinal projections to the RVM are sparse but it may receive spinal input indirectly from the PAG and nucleus cuneiformis. Another indirect route for spinal input is from the adjacent medullary

nucleus reticularis which has a large projection from nociceptive spinoreticular neurons and projects massively to the RVM. The RVM also receives noradrenergic input from the A5 and A7 cell groups of the dorsolateral pontomesencephalic tegmentum (DLPT).

The DLPT also plays a critical role in the pain-modulating actions of the PAG and RVM. This strusture includes all of the noradrenergic neurons that project to the RVM and spinal cord.

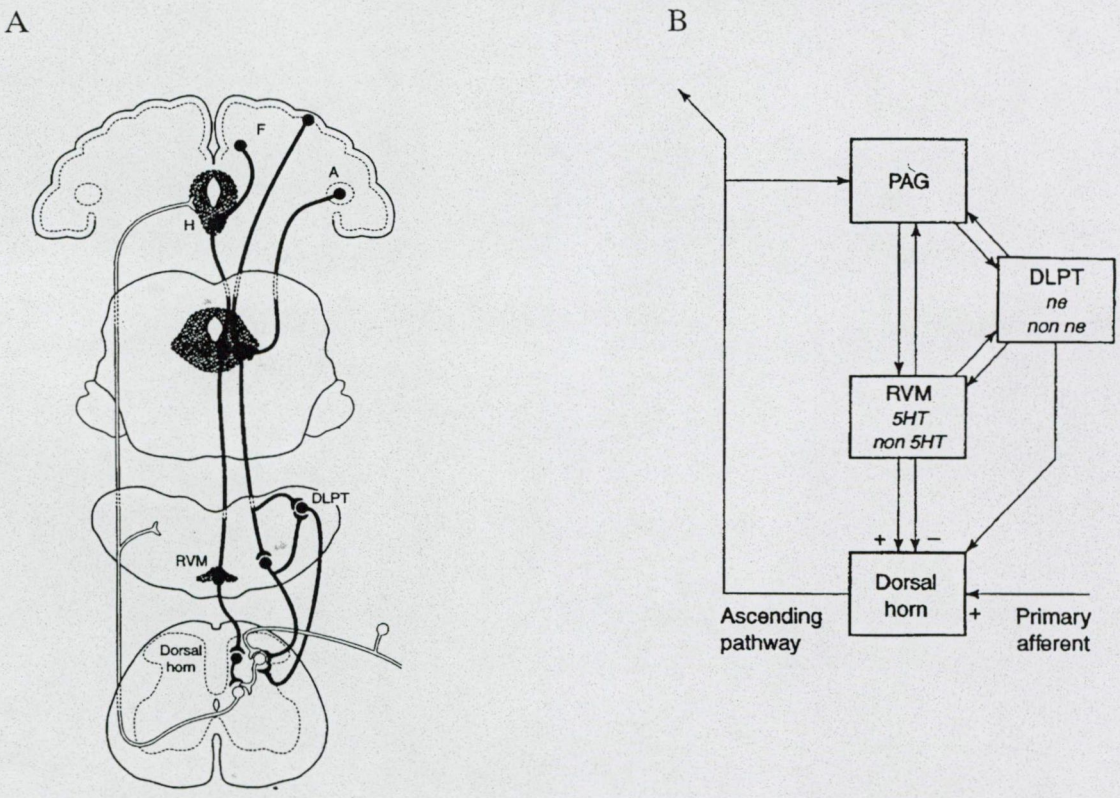


Figure 3. A: Diagram of the major pain modulating pathways. (PAG = midbrain periaqueductal gray, RVM = rostral ventromedial medulla, F = frontal lobe, A = amygdala, H = hypothalamus, DLPT = dorsolateral pontine tegmentum, • = inhibitory-, o = excitatory interneurons). **B**: Brainstem connections relevant to pain modulating system. (5HT = serotonin, ne = noradrenergic innervation).

Diagrams are taken from Wall, Melzack: Textbook of pain (32).

Descending inhibition from these neuronal systems occurs through the effect of local interneurons, primary afferent fibres and descending pathways at the spinal level. In the dorsal horn, incoming nociceptive messages are modulated by endogenous ligands acting on different receptors located pre- and postsynaptically. These receptors are possible targets of different pharmacological active agents.

2.5.1. Opioid receptors and endogenous opioid peptides

Opioid receptors in the dorsal horn congregate in the superficial laminae, lamina I and particularly in lamina II (1). Opioid peptides also concentrate in these laminae. Four family of endogenous opioid peptides have been discovered so far:

- leucine (leu)- and methionine (met)-enkephalin,

- β-endorphin,
- dynorphin (α-neoendorphin),
- endomorphin-1 and endomorphin-2.

The distribution of these endogenous ligands and their receptors are dense in the spinal dorsal horn. Enkephalin and dynorphin containing neuronal cell bodies and axon terminals are found in laminae I and II (1). β-Endorphin in the spinal cord mainly derives from supraspinal origin, but it is also present at the spinal level (96). Endomorphin-1-like immunoreactivity is slight, whereas endomorphin-2-like immunoreactivity is more pronounced in the spinal cord (97). Endomorphin-2 is co-localized with SP, CGRP and μ-opioid receptors in the primary afferent terminals (98), thus it can inhibit the release of these transmitters and consequently inhibit the development of painful stimuli.

The distribution of opioid receptors in the rat spinal cord is as follows: 70% μ, 24% and δ 6% κ of the total opioid sites (99). There is a combined pre- and postsynaptic action of opioids whithin the spinal cord, since δ receptors are mainly restricted to axons (99,100) (many of which are primary afferents), whereas μ and κ receptors are present on primary afferent axons, the cell bodies and dendrites of a population of neurons in the superficial dorsal horn (99,100,101). This suggests that δ receptors are involved in the pre-synaptic component, while μ and κ receptors mediate both pre- and postsynaptic effects.

At spinal sites ligands for each of the three receptors can produce analgesic effect. Opioids inhibit the release of neurotransmitters from primary afferents by hyperpolarising the C fibres (102) and they have been shown to directly inhibit lamina II interneurons (1). *In vitro* studies indicate that μ-opioid receptor agonists block the release of glutamate and substance-P from primary afferents (103). Intrathecal or epidural morphine is widely used to treat several pain states (22), although the side effects of spinal opioids - pruritus, nausea, urinary retention, hypotension – should always be taken into consideration.

The endomorphin family includes two tetrapeptides that differ in one amino acid: endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). These endogenous substances differ from conventional endogenous opioid receptor ligands in their N-terminal sequence, peptide length and C-terminal amidation (24). They exhibit the highest affinity and specificity for the μ-opioid receptors in the mammalian nervous system. Both endomorphin-1 and endomorphin-2 are active both supraspinally and spinally (104), although the results comparing the antinociceptive potencies of the two endomorphins are inconclusive. Several authors found that endomorphin-1 was more active than endomorphin-2 in inhibiting the pain responses after intracerebroventricular administration on acute pain tests, in mechanical pain tests and in the model of formalin-induced inflammatory pain (23,105,106). Data also suggest endomorphin-1 to be significantly more potent spinally than supraspinally – in mice - and at the spinal level it seems to be significantly more potent than endomorphin-2 (104). In contrast, intrathecal results - performed in rats - showed that both compound had similar potencies in increasing the tail flick latency, decreasing the carrageenan-induced hyperalgesia and the allodynia caused by a sciatic nerve injury (106,107). Moreover, continuous intrathecal administration of endomorphin-1 effectively inhibited thermal hyperalgesia in rats (108).

Some pharmacological differences from morphine were observed, i.e. they are more potent than morphine in neurophatic pain (106), though in contrast to morphine, the effect of endomorphins is short lasting and the data are indicative of the development of acute tolerance (or tachyphylaxis) (107,109). Furthermore, endomorphin-induced antinociception exhibited a steady plateau at about 40% of the maximum possible effect (%MPE) in acute pain tests (110). It has been suggested that the different patterns of G-protein activation observed for the agonists at μ-opioid receptors might account for this low efficacy exhibited by endomorphins in the production of μ-opioid receptor-mediated antinociception. One possibility to overcome the development of plateau effect and the previously mentioned problems might be by combining them with different drugs.

2.5.2. Effect of spinally acting noradrenergic inhibitory control

The locus coeruleus or the so called A6 cell group along with the A5 and A7 group of noradrenaline containing neurons are the major source of noradrenergic projections to the dorsal horn, where they end on the α_2-adrenoceptors (25). The α_2-adrenergic ligands preferentially bound to laminae I and II in the spinal cord (1). α_2-Adrenoceptor mediated spinal analgesia has been extensively investigated both in animal (111,112,113) and human studies (21,114).

Some α_2-adrenoceptor agonists are already applied in human therapy, namely: clonidine (114,115,116,117,118), dexmedetomidine (119) and guanfacine (120) - which is used as an anti-hypertensive agent. α_2-adrenoceptor agonists have several beneficial effects as adjuncts to anesthesia and surgery, such as reduced anaesthetic requirements and improved cardiovasular and adrenergic stability during surgery. The greater relative potency as well as the higher selectivity and specificity of dexmedetomidine compared to clonidine raised great expectations in the past years.

Dexmedetomidine is the dextro isomer of medetomidine and has shown high affinity towards α_2-binding sites while having only low affinity towards α_1-adrenoceptors (26). Dexmedetomidine has an α_1:α_2 –adrenoceptor ratio of 1600:1, more than 7 times greater than that of clonidine (116). Its elimination half-life is approximately 2 h, whereas that of clonidine is more than 8 h. In animal studies it has been shown to possess potent anaesthetic properties (121). In humans, intravenous dexmedetomidine had no effect on pain threshold (electric stimulation of tooth pulp), but significantly attenuated the unpleasantless of torniquet-induced ischeamic pain (122). The approved sedative indication of dexmedetomidine is for the intensive care treatment of postoperative surgical patients (30). Systemic dexmedetomidine increased the mechanical and thermal thresholds in a dose-dependent manner in normal rats, while after nerve injury its analgesic potency was enhanced (123). Intrathecal administration of dexmedetomidine results in a potent and long-lasting antinociception on the tail flick test and this effect can be completely abolished by atipamezole, the selective α_2-antagonist (27,111). This finding is supported by electrophysiological experiments in which both systemic and intrathecal administration of dexmedetomidine and medetomidine inhibited nociceptive responses in dorsal horn neurons (124). Because of its sympatholytic and vagomimetic actions, dexmedetomidine is approved with a warning about hypotension, bradycardia and sinus arrest and can be used only in a monitored situation.

3. Goals of the study

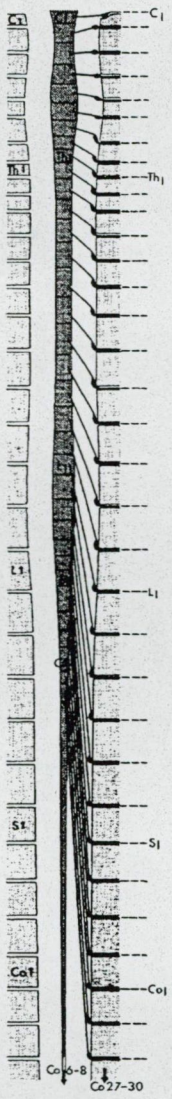
The thesis examines the spinal antinociceptive effect of different drugs (ketamine, morphine, endomorphin-1, dexmedetomidine) and their combinations applying an acute pain test in rats.

Our aims were the following:

1. To examine the antinociceptive effect of racemic ketamine and its enantiomers at the spinal level.
2. To examine the effect of both ketamine enantiomers on morphine-induced antinociception.
3. To examine the effect of both ketamine enantiomers on antinociception induced by the specific α_2-adrenoceptor agonist, dexmedetomidine.
4. To examine the effect of S(+)-ketamine on endomorphin-1-induced antinociception.
5. To examine the possible interaction of endomorphin-1 and dexmedetomidine after intrathecal administration.
6. To investigate the possible, advantegous interaction of the triple combination of endomorphin-1 and dexmedetomidine with S(+)-ketamine.

4. Methods and experimental design

4.1. Anatomy of the rat's spinal cord



Since we have implanted a chronic, indwelling cannula into the spinal subarachnoid space of the rats, a few points have to be mentioned about the anatomy of the rat's spinal cord (Figure 4). The rat's vertebral column contains: 8 cervical (C), 13 thoracic (T), 6 lumbar (L), 4 sacral (S), 27-30 coccygeal (Co) vertebrae (125). The weight of the spinal cord of a 200 g rat is near 0.58 g and the length of the spinal cord in adult males varies from 113 to 125 mm. The topographic relation of the spinal cord to the vertebral column reveals a strong ancensus. The 1st thoracic segment ends at the last cervical vertebra, the 1st lumbar segment begins at the caudal end of the 10th thoracic vertebra and the last lumbar segment ends at the cranial third of the 1st lumbar vertebra. The sacral part extends from the 1st to the cranial end of the 3rd lumbar vertebra and from there on the coccygeal part runs to the cranial third of the 4th lumbar vertebra.

The afferent fibres from the tail run in the paired dorsolateral and ventrolateral tail nerves and terminate in laminae I and II of the dorsal horn of segments S3-Co3. The motor neurons of the tail muscles reside in the ventral horn of ten segments, L4-Co3 (126).

Figure 4. Spinal cord of the laboratory rat

4.2. Intrathecal catheterization

The best way to examine the biological action of a drug at the spinal level in behaving animal is to inject the drug solution into the spinal subarachnoid fluid and thereby bathe the spinal cord in the pharmacologically active solution. Yaksh and Rudy (127) developed a technique, which permits repeated injection of drug solutions into any selected level of the spinal subarachnoid space in species as small as a rat. The technique involves the insertion of a chronic indwelling polyethylene canule into the spinal subarachnoid space via a puncture in the atlanto-occipital membrane.

Accordingly, after institutional approval had been obtained from our Animal Care Committee, male Wistar rats weighing 250-350 g were studied (n=666). After the rats were

surgically prepared under ketamine-xylazine anaesthesia (87 and 13 mg/kg intraperitoneally, respectively), an intrathecal catheter (PE-10 tubing) was inserted through a small opening in the cisterna magna and passed 8.5 cm caudally into the intrathecal space. Following surgery, the rats were housed individually, had free access to food and water and were allowed to recover for at least 3 days before use. Rats exhibiting any postoperative neurological deficits - about 10% (128)- were not used. After experimental use, each rat was killed with an overdose of pentobarbital, and 1 % methylene blue was injected to confirm the position of the catheter and the probable spread of the injectate. The position of the tip of the intrathecal catheter following the above-mentioned method was always at the proper place - vertebral T13-L3 level-, corresponding to the spinal segments, which innervate the tail.

4.3. Nociceptive testing

Application of noxious heat to the rat's tail generates a quick withdrawal response that is called the tail flick reflex. This reflex has been used frequently to study pain mechanisms; it is clearly visible and occurs at a consistent latency if a constant heat intensity is used. The tail flick reflex is a flexor reflex that is organised at the spinal level, but it is under supraspinal control. This simple reflex measure permit the animal to have control over stimulus magnitude and thus ensure that the animal can control the level of pain.

Anatomical, pharmacological and physiological studies implicate that the substantia gelatinosa (where the tail afferents end) is an important site for inhibition of the tail flick reflex by different drugs.

We have followed the method of Janssen *et. al* (1963) to determine the nociceptive threshold. The reaction time in the tail flick test was determined by immersing the lower 5 cm portion of the tail in the hot water (51.5 °C water) until the typical tail-withdrawal response was observed (cut-off time: 20 s). Baseline latencies were obtained immediately before and then 10, 30, 60 and 90 min after the drug injections. All experiments were performed during the same period of the day (8:00 to 11:00 AM) to exclude diurnal variations in pharmacological effects. The animals were randomly assigned to treatment groups (n=5-13 rats/group).

4.4. Drugs

Drugs employed were the following:

- ketamine hydrochloride (Ketalar, Parke-Davis, Vienna, Austria),
- xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany),
- racemic-ketamine hydrochloride, R(-)-ketamine hydrochloride, S(+)-ketamine hydrochloride (all ketamines for intrathecal administration: Parke-Davis; generous gifts from Gödecke Ltd., Vienna, Austria),
- morphine hydrochloride (Alkaloida, Tiszavasvári, Hungary),
- dexmedetomidine (a generous gift from Orion-Farmos, Finland),
- endomorphin-1 was synthetized by a solid-state method and purified by means of high performance liquid chromatography in the Isotope Laboratory of the Biological Research Centre of the Hungarian Academy of Sciences.

Drugs were dissolved in sterile, physiological saline. The drugs were administered intrathecally over 30 s in a volume of 5 µl, followed by a 10 µl flush of physiological saline.

4.5. Series of experiments

1. Single-drug studies

The first series of experiments was planned to evaluate the time-response and dose-response effect of intrathecally administered:

a.) racemic ketamine, S(+)-ketamine and R(-)-ketamine: 30, 100 and 300 µg

b.) morphine: 0.1, 0.3, 1 and 3 µg

c.) dexmedetomidine: 0.1, 0.3, 1, 3, 6 and 10 µg

d.) endomorphin-1: 0.6, 2, 6, 18 and 50 µg

2. Drug interaction studies

The second series of experiments was planned to examine the possible interaction of the following drug combinations:

a.) 30 or 100 µg racemic, S(+)-, R(-)-ketamine with 0.1, 0.3, 1 and 3 µg morphine

b.) 30 or 100 µg racemic, S(+)-, R(-)-ketamine with 0.3, 1 and 3 µg dexmedetomidine

c.) 30 or 100 µg S(+)-ketamine with 2, 6 and 18 µg endomorphin-1

d.) endomorphin-1 and dexmedetomidine in a 4:1 fixed dose ratio (Table 2)

e.) triple combination of 100 µg S(+)-ketamine with a fixed, 4:1 dose ratio of endomorphin-1 and dexmedetomidine (Table 2)

Table 2. Applied doses for endomorphin-1, dexmedetomidine and S(+)-ketamine combinations

Group	Endomorphin-1 (µg)	Dexmedetomidine (µg)	S(+)-ketamine (µg)
Endomorphin-1: Dexmedetomidine=4:1	1	0.25	
	2	0.5	
	4	1	
	8	2	
Endomorphin-1+ Dexmedetomidine+ S(+)-ketamine	0.04	0.01	100
	0.12	0.03	100
	0.4	0.1	100
	1	0.25	100

4.6. Statistical analysis

Analgesic latencies in acute pain tests were converted to the percentage of the maximal possible effect (%MPE) by using the formula:

%MPE=([observed latency - baseline latency]/[cut off - baseline latency])*100

Data are presented as means ± SEM. Time course and dose-effect curves were constructed for each drug or drug combinations.

The ED_{50} (the dose that yielded 50% of the maximum possible effect) values with 95% confidence intervals (CI) were calculated by linear regression. In the special case of an agent lacking pharmacological effect (in this case ketamine), any statistically significant decrease in the ED_{50} of the other, active component (i.e. endomorphin-1, dexmedetomidine of morphine) denotes synergism.

Data sets were examined by one- and two-way analyses of variance. Post-hoc comparison was carried out with the Newmann-Keuls test. A *P*-value less than 0.05 was considered significant.

Isobolographic analysis is the standard approach to characterize the interaction of two drugs as additive, synergistic, or antagonistic (129). In this approach, a straight line – the line of additivity – is drawn between the dose of one drug necessary to achieve a specific effect (plotted on the y axis) and the dose of a second drug required to achieve the same effect (plotted on the x axis). If the same effect is produced by co-administration of the two drugs at dosages that, when plotted on the same graph, occur below the line of additivity, the interaction is said to be synergistic. If doses of the two drugs coadministered to produce the same effect occur above the line of additivity, it is said to be antagonistic interaction. In our experiments isobolographic analysis was performed to study the interaction between dexmedetomidine and endomorphin-1. Theoretical simple additive ED_{50} for each ratio of two drugs was then generated from the equation:

$$Z_{add} = Z_1^{\circ}/(p_1+R^*p_2)$$

where Z_{add} is the total additive dose, Z_1° is the ED_{50} of endomorphin-1, R is the potency ratio of two drugs, p_1 is the proportion of endomorphin-1 in the total dose and p_2 is that of dexmedetomidine. The confidence intervals for the drug components of the theoretical additive ED_{50} were obtained from the variances about ED_{50} for each drug administered alone. This theoretical additive point was compared with the experimentally derived values for the mixture by Student *t* test. A significant potency ratio with the experimental ED_{50} significantly less than the theoretical additive ED_{50} indicated a synergistic interaction.

5. Results

There was no significant difference in the baseline tail flick latencies between the groups (7.0 ± 0.10 sec) before any drug administration for all animals. The tail flick latencies of the control group did not change significantly during the time of the investigation.

All the significant time points compared either versus control (saline) or to the single-drug treatment are summarized in Tables 3 and 4 (in the end of this chapter). On the time course curves of the combination studies only those points are marked which were significantly different from either the single-drug treatment or from the racemic ketamine treatment.

5.1. Single-drug studies

Ketamine enantiomers alone did not have significant effect on influencing the tail flick latencies, except the racemate at the highest dose at 10th min (Figure 5/A). Racemic-ketamine and S(+)-ketamine in the 300 µg dose caused a short-lasting motor impairment, while this effect could not be seen in the case of the R(-)-enantiomer.

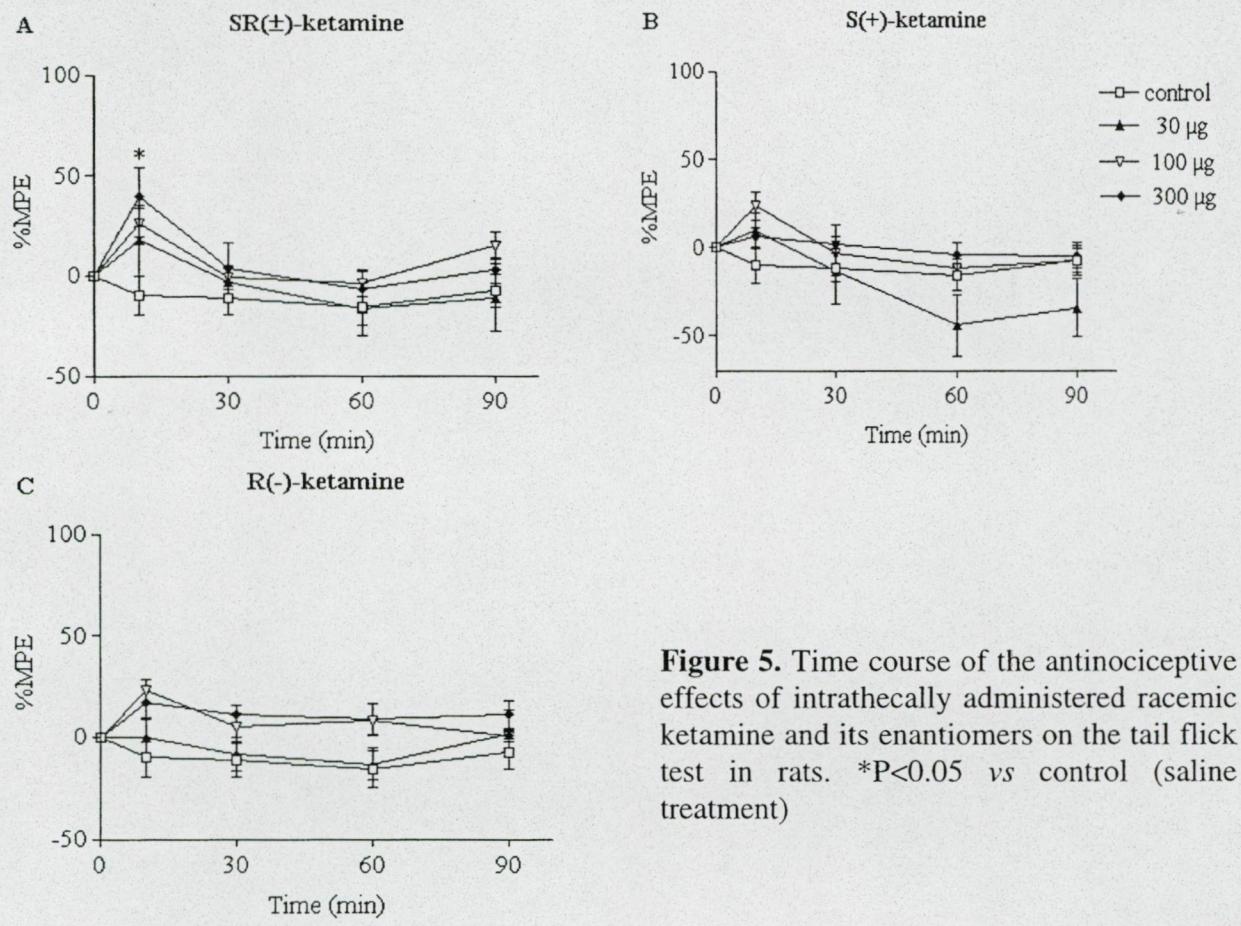


Figure 5. Time course of the antinociceptive effects of intrathecally administered racemic ketamine and its enantiomers on the tail flick test in rats. *P<0.05 *vs* control (saline treatment)

Morphine resulted in a dose-dependent increase in the thermal withdrawal latency, with the peak effect occurring at 30 min after the injection (Figure 6/A). This increase in the pain threshold was statistically significant after the higher doses (1 and 3 µg). The intrathecal injection of morphine in the dose range tested did not interfere with the gross behaviour of the animals.

Endomorphin-1 resulted in a dose-dependent increase in the thermal withdrawal latency, with the peak effect occurring at 10 min and all of the results at this time point were statistically significant compared to the control group values (Figure 6/B). The antinociceptive effect of lower doses of endomorphin-1 (0.6, 2, 6, 18 μg) was short lasting: it developed at 10th min and disappeared afterwards. The highest dose of endomorphin-1 (50 μg) performed a longer-lasting effect, being significant throughout the whole experiment, besides it caused close to 100% MPE and also some temporary motor dysfunction (rigidity).

Dexmedetomidine at lower doses (0.1-3 μg) produced slight and short-lasting increase in %MPE (23-26%); the two higher dose (6 and 10 μg) caused very effective (50-100% MPE), long-lasting antinociception which were statistically significant (Figure 6/C). Dexmedetomidine in higher doses (3-10 μg) was associated with substantial diuresis and sedation (a decreased spontaneous exploring activity, but the animals were still responsive to acoustic or tactile stimuli).

The rank order of potency was morphine > dexmedetomidine > endomorphin-1, from the calculated ED_{50} values: 1.66 (CI: 1.04-2.32) μg, 4.9 (CI: 3.81-6.14) μg and 14.2 (9.1-19.3) μg respectively (Table 5).

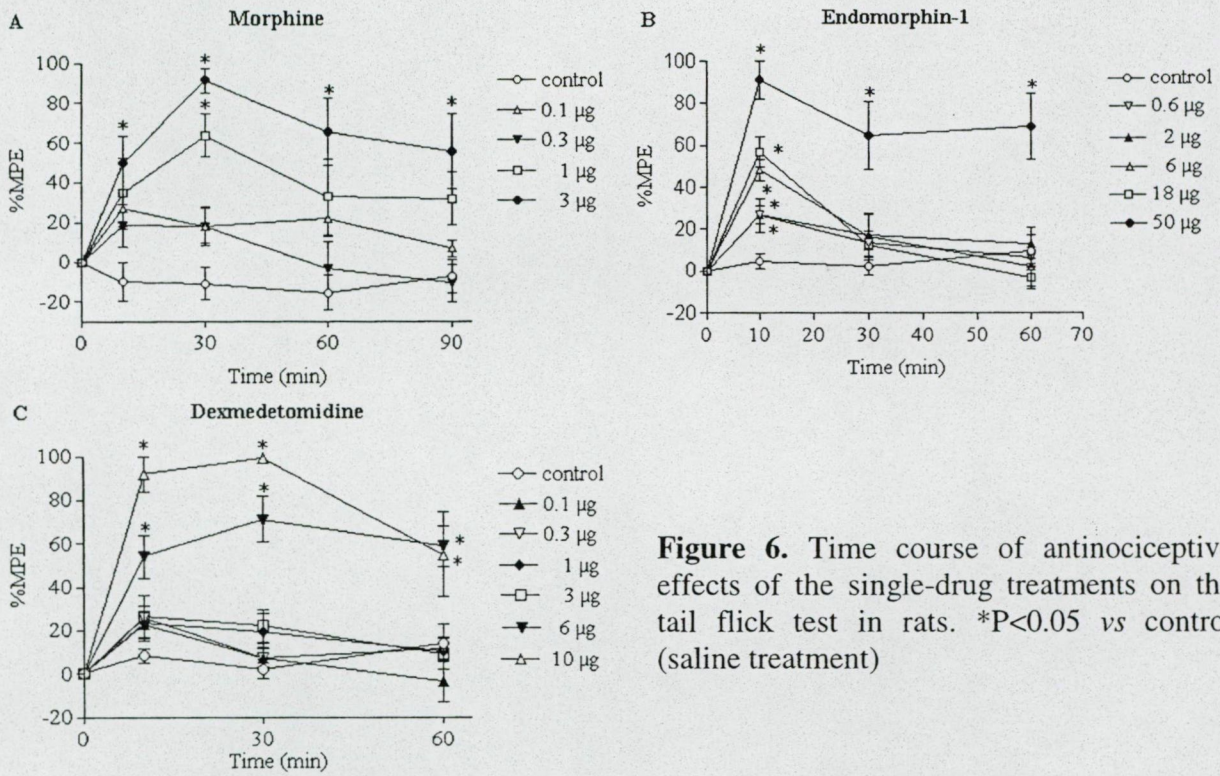


Figure 6. Time course of antinociceptive effects of the single-drug treatments on the tail flick test in rats. *P<0.05 *vs* control (saline treatment)

5.2. Drug combination studies

Since in the single-drug studies with ketamine we found that 300 μg caused some temporary motor impairment in the case of the racemic-, and S(+)-ketamine, therefore we applied 30 and 100 μg racemic ketamine or its enantiomers in the combination experiments, which did not cause any side effects.

In the case of endomorphin-1 and dexmedetomidine we applied the drugs in a fixed 4:1 dose ratio for the following reason. The ratio of the observed ED_{50} values (Table 5) for dexmedetomidine and endomorphin-1, as single-drug treatments were approximately 3:1. The reason why we did not use this ratio was, that as the single-drug studies showed, dexmedetomidine in high dose (3 μg) already caused sedation, so the possible applied doses could be 2 μg and lower.

Animals receiving any of the following drug combinations didn't exhibit any unusual behaviour.

5.2.1. Ketamine + morphine

Results with the low dose of ketamine

Compared to the saline treated control group, 30 μg racemic ketamine and its enantiomers significantly potentiated the morphine-induced antinociception (Table 4/1). In contrast to the single morphine treatment (Figure 6/A), this low dose ketamine combinations significantly enhanced its antinociceptive effect - in the case of 0.3 μg morphine in almost all the cases until the 30th min and with the 1 μg morphine with all the three ketamine compounds up to the 60th min (Table 4/1).

Compared to the morphine treatment alone, the combination of 30 μg racemic-ketamine and its enantiomers with different morphine doses (0.1, 0.3, 1 μg) did not cause any significant changes in the tail flick latencies (Figure 7/A, B, C). The decrease of the ED_{50} value of morphine (Table 5) after this dose of either ketamine compound was not significant.

Results with the high dose of ketamine

The 100 μg S(+)-ketamine and racemic ketamine, synergistically enhanced the antinociception caused by intrathecal 0.1 and 0.3 μg morphine (Figure 7/D, E). This enhancement of the effect was statistically significant compared to the single morphine treatment after 0.3 μg morphine plus 100 μg S(+)-ketamine at all time points, while in the case of 0.1 μg morphine only at 10 min. Both the racemic and the S(+)-ketamine reduced the ED_{50} value of morphine (Table 5), while the R(-)-enantiomer had no such effect. The dose-response curves showed a leftward shift after either racemic-ketamine or S(+)-ketamine (100 μg) co-administration, but this change could not be observed with the R(-)-enantiomer (Figure 9/B).

The antinociceptive effect of intrathecal morphine alone in the applied doses peaked in the first 30 min and gradually decreased afterwards. In the cases of the 0.3 or 1.0 μg morphine plus ketamine combinations the effect stayed on almost without any major decrease for 60 min. Analysis of the time course curves revealed that the S(+)-ketamine in this dose not only potentiated, but also prolonged the antinociceptive effect of 0.3 and 1 μg morphine (Figure 7/E, F), while the racemic ketamine did so only in the case of 1 μg morphine (Figure 7/F). Longer and statistically significant antinociception could be observed at this dose of S(+)-ketamine with 0.3 μg morphine than with the racemic ketamine combination (Figure 7/E), which suggests that S(+)-ketamine was more potent than the racemate in prolongating the effect of morphine.

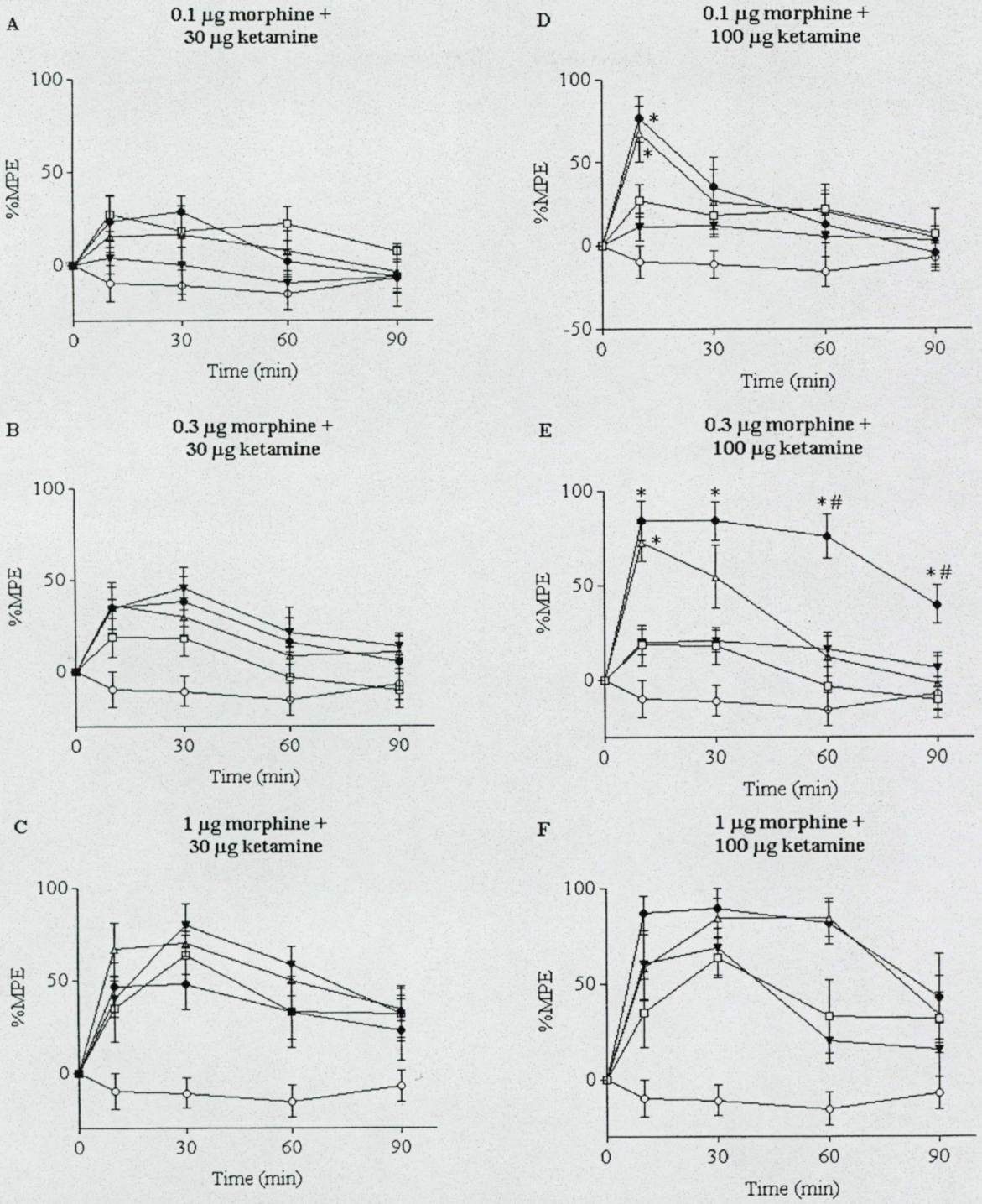


Figure 7. Time course of antinociceptive effects of various mixtures of racemic ketamine and its enantiomers with morphine on the tail flick test in rats. Each point represents the mean ± SEM of the data from 5-14 animals. *P<0.05 *vs* morphine treatment, #P<0.05 *vs* SR-ketamine + morphine treatment

o control

□ morphine

Δ morphine + SR(±)-ketamine

• morphine + S(+)-ketamine

▼ morphine + R(-)-ketamine

5.2.2. Ketamine + dexmedetomidine

Results with the low dose of ketamine

In contrast to the single dexmedetomidine treatment (Figure 6/C), its combination with 30 μg racemic ketamine or the enantiomers caused significant antinociception compared to the saline treated control group (Table 4/1).

The time course curves revealed that 30 μg racemic ketamine or its enantiomers significantly potentiated the antinociceptive effects of dexmedetomidine – compared to the single drug treatment - in the case of 0.3 μg dexmedetomidine at 10 min, while in the case of 3 μg dexmedetomidine this potentiation stayed on during the whole experiment (Figure 8/A, B, C).

All of the ketamine treatments significantly reduced the ED50 value of dexmedetomidine (Table 5). The dose-response curves shifted to the left after co-administration of either racemic-ketamine or both enantiomers (Figure 9/C).

Results with the high dose of ketamine

Both the racemic ketamine and its enantiomers in the 100 μg dose potentiated the antinociceptive effect of dexmedetomidine. This potentiation was statistically significant compared to the dexmedetomidine treatment in the case of 0.3 μg dexmedetomidine plus S(+)-ketamine at 10-60 min of observation and in the case of racemic ketamine and R(-)-ketamine only at 10 min (Figure 8/D). In the case of 1 μg dexmedetomidine the R(-)-ketamine caused significant potentiation at 10-30 min, while the racemate only at 30th min. In the highest dexmedetomidine dose used (3 μg) S(+)-ketamine caused statistically significant increase in the tail flick latency compared to the single-drug treatment throughout the whole experiment, while the racemate caused similar effect until the 60th min (Figure 8/F).

All of the 100 μg ketamine treatments caused significant reduction in the ED_{50} value of dexmedetomidine (Table 5), although the S(+)-ketamine and racemic-ketamine was the most effective of all. The dose-response curves showed a leftward shift after co-administration of either racemic-ketamine or both enantiomers, suggesting a synergistic interaction (Figure 9/D).

This dose of S(+)-ketamine not only potentiated, but also prolonged the antinociceptive effect of dexmedetomidine in the applied doses (Figure 8/D, F), while the racemate did so in the cases of 1 and 3 μg dexmedetomidine (Figure 8/F). Longer and statistically significant antinociception could be observed at this dose of S(+)-ketamine with 0.3 μg dexmedetomidine compared to racemic combination, which lasted for 60 min (Figure 8/D). Prolonged effect could also be observed after 3 μg dexmedetomidine plus S(+)-ketamine or racemic-ketamine combinations, but in this case the difference was slight between the two treatments. (Figure 8/F).

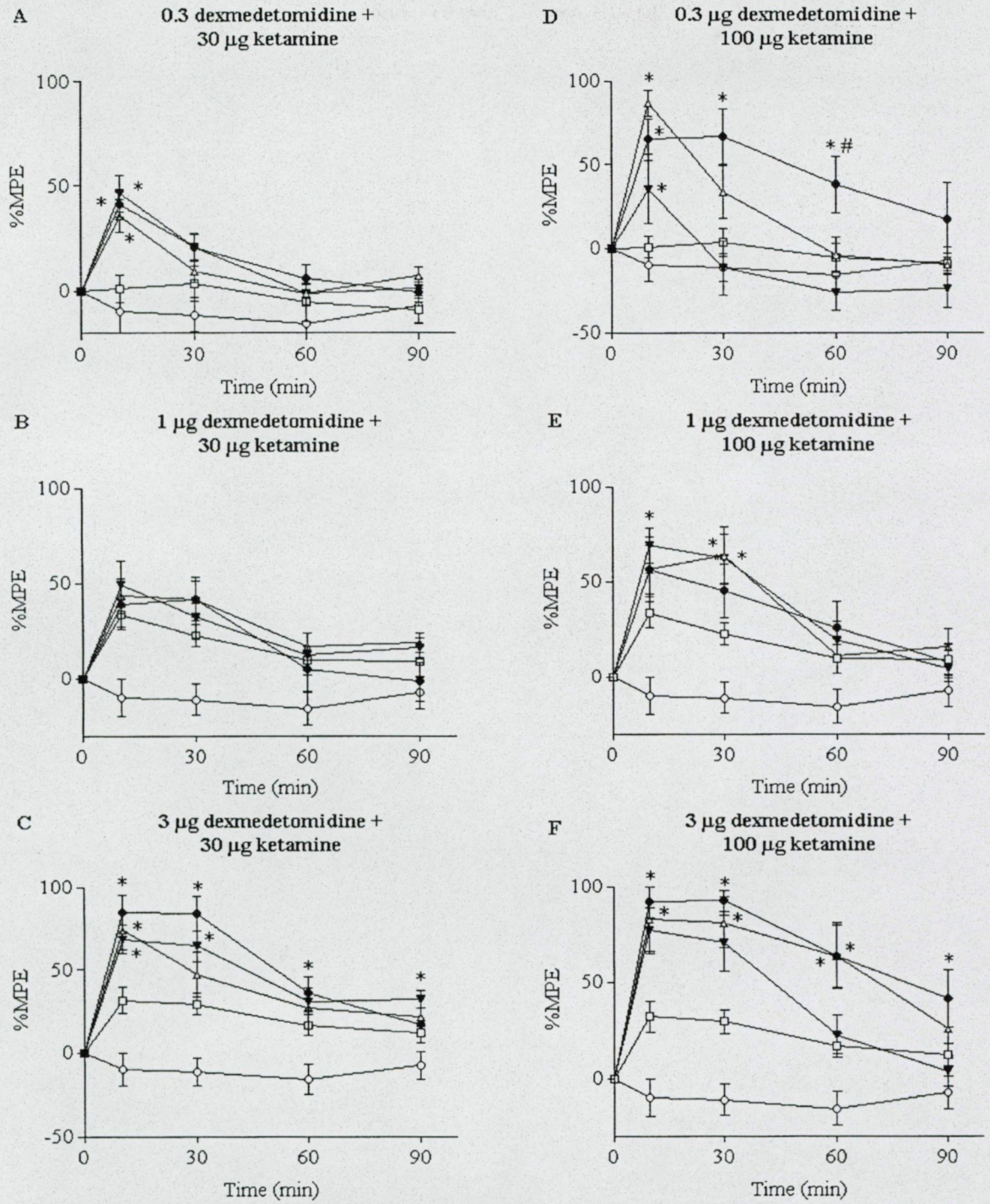


Figure 8. Time course of antinociceptive effects of various mixtures of racemic ketamine and its enantiomers with dexmedetomidine on the tail flick test in rats. Each point represents the mean ± SEM of the data from 5-14 animals. *P<0.05 *vs* DEX treatment, #P<0.05 *vs* SR-ketamine + DEX treatment

o control

□ dexmedetomidine

Δ dexmedetomidine + SR(±)-ketamine

● dexmedetomidine + S(+)-ketamine

▼ dexmedetomidine + R(-)-ketamine

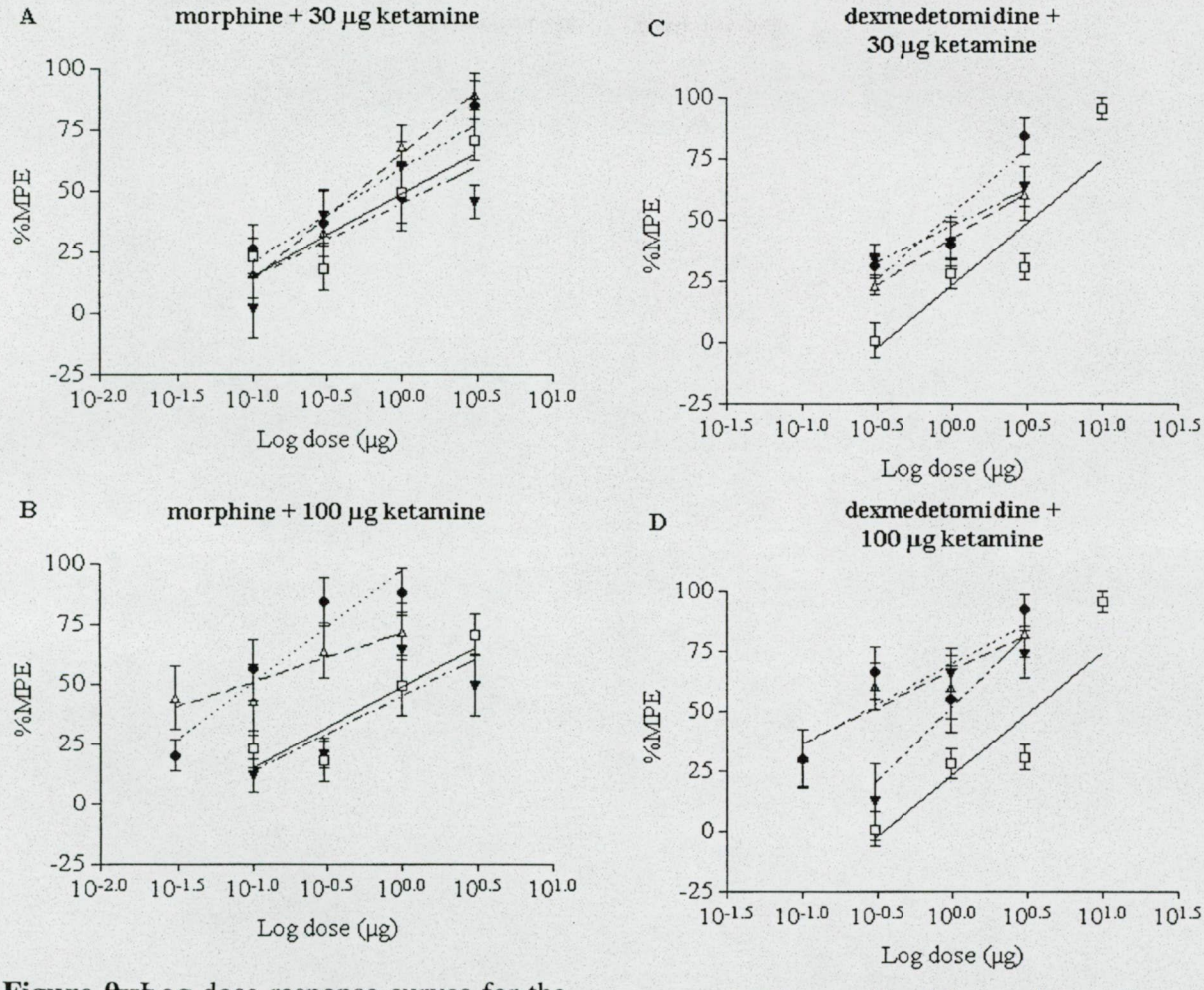


Figure 9. Log dose-response curves for the antinociceptive effects of intrathecal morphine (left) or dexmedetomidine (right) alone and in combination with racemic ketamine and its enantiomers on the tail flick test in rats. Each point represents the mean ± SEM of the data from 5-14 animals.

5.2.3. Endomorphin-1 + S(+)-ketamine

Results with the low dose of ketamine

Time course (Figure 10/A, B, C) curves for endomorphin-1 plus S(+)-ketamine co-administration revealed that 30 μg S(+)-ketamine did not influence the antinociceptive effect of endomorphin-1 at any time points. The decrease observed in the ED_{50} value of endomorphin-1, after co-administration of this dose of S(+)-ketamine was not significant (Table 5).

Results with the high dose of ketamine

Co-administration of 100 μg S(+)-ketamine potentiated (at 10 min) the antinociceptive effect of endomorphin-1, which difference was statistically significant compared to the single endomorphin-1 treatment only in the case of 6 μg endomorphin-1 combination (Figure 10/B). The ED_{50} value of the endomorphin-1 and this dose of S(+)-ketamine combination was significant (Table 5).

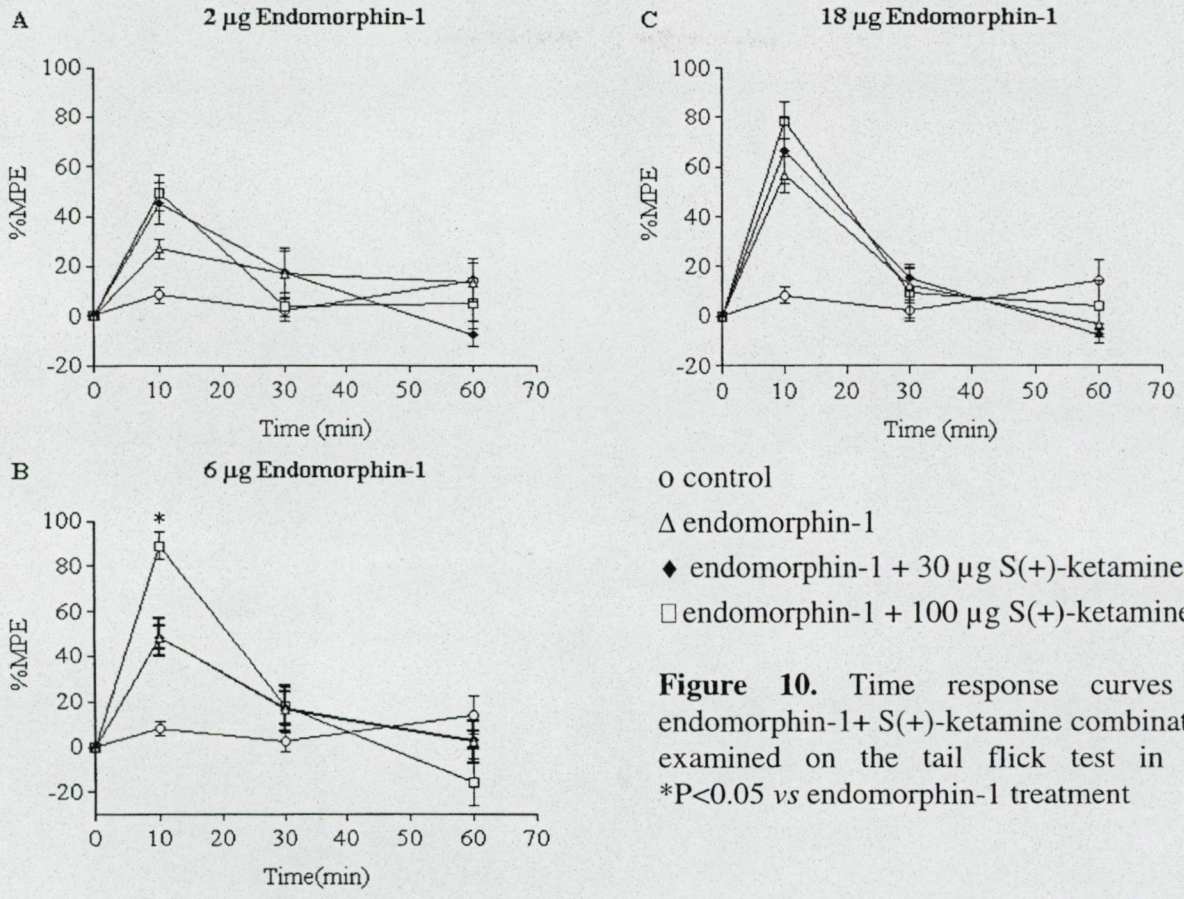


Figure 10. Time response curves for endomorphin-1+ S(+)-ketamine combinations examined on the tail flick test in rats. *P<0.05 *vs* endomorphin-1 treatment

5.2.4. Endomorphin-1 + dexmedetomidine

Intrathecal co-administration of dexmedetomidine (0.25, 0.5, 1, 2 µg) and endomorphin-1 (1, 2, 4, 8 µg) in a fixed dose ratio (4:1) resulted in a significant increase in the tail flick latency following a dose-dependent fashion (Figure 11/A).

The isobolographic analysis demonstrated that this interaction was synergistic, because the doses of dexmedetomidine and endomorphin-1 necessary to produce 50% MPE were significantly less than those calculated to be necessary for an additive interaction (Figure 11/B and Table 5). The dose-effect curve of endomorphin-1 showed a leftward shift when it was combined with dexmedetomidine (Figure 12/B).

Animals receiving the combination didn't exhibit any no unusual behaviour, except for one group (8 µg endomorphin-1 and 2 µg dexmedetomidine) they showed some strickt signs of sedation.

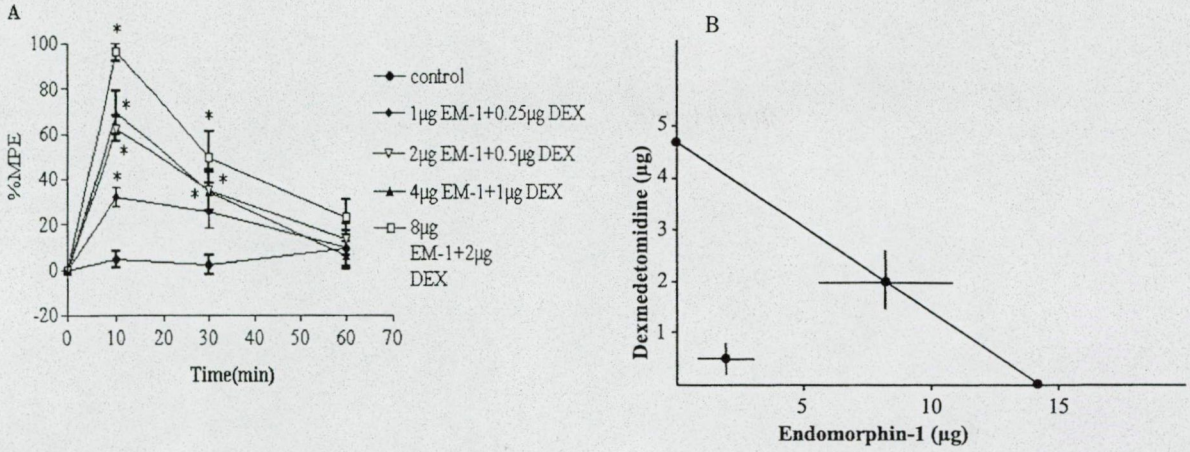


Figure 11. **A**: Time-response curve for endomorphin-1 (EM-1) and dexmedetomidine (DEX) in 4:1 fixed dose ratio. **B**: Isobologram for the interaction of EM-1 and DEX *P<0.05 *vs* endomorphin-1 treatment. The experiments were carried out on the tail flick test in rats

5.2.5. Triple combination of endomorphin-1 and dexmedetomidine with S(+)-ketamine

Co-administration of endomorphin-1 and dexmedetomidine in the previously mentioned fixed dose ratio of 4:1 along with 100 μg S(+)-ketamine caused a dose-dependent antinociception and a slightly prolonged effect could be observed at higher doses (Figure 12/A). The dose-response curve of endomorphin-1 shifted to the left when it was given in this triple combination (Figure 12/B).

The ED_{50} for this triple combination did not differ significantly from the two binary combinations [S(+)-ketamine plus dexmedetomidine or S(+)-ketamine plus endomorphin-1], although the confidence interval decreased (Table 5). The statistical significance for the differences in the degree of synergism reached the level of P<0.05 between the third binary combination (endomorphin-1 + dexmedetomidine) on one side and the triple combination on the other.

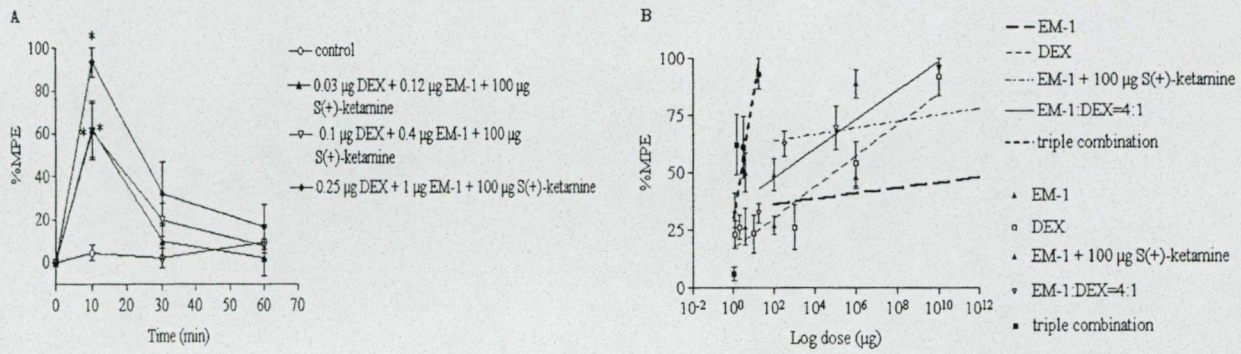


Figure 12. Time response (**A**) and dose response (**B**) curves for the triple combination of dexmedetoimidine (DEX) and endomorphin-1 (EM-1) with S(+)-ketamine examined on the tail flick test in rats. *P<0.05 *vs* control (saline treatment)

Table 3. Significant time points after single drug treatment.

Treatment	P<0.05 *vs* control (saline alone)			
	10 min	30 min	60 min	90 min
S(+)-ketamine 30 µg				
100 µg				
300 µg				
R(-)-ketamine 30µg				
100 µg				
300 µg				
SR-ketamine 30 µg				
100 µg				
300 µg	*			
Morphine 0.1 µg				
0.3 µg				
1.0 µg		*		
3.0 µg	*	*	*	*
Endomorphin-1 0.6 µg	*			
2.0 µg	*			
6.0 µg	*			
18.0 µg	*			
50.0 µg	*	*	*	
Dexmedetomidine 0.1 µg				
0.3 µg				
1.0 µg				
3.0 µg				
6.0 µg	*	*	*	
10.0 µg	*	*	*	

* significant by Newmann-Keuls test at P<0.05

Under the stripped areas the experiment was not performed

Table 4/1. Significant time points after drug combinations I.

Treatment	P<0.05 *vs* control (saline alone)				P<0.05 *vs* single-drug treatment (Mo/Dex/EM-1 alone)				P<0.05 *vs* SR-ketamine combination			
	10 min	30 min	60 min	90 min	10 min	30 min	60 min	90 min	10 min	30 min	60 min	90 min
0.1µg morphine + 30µg S(+)-ketamine												
+ 30µg R(-)-ketamine												
+ 30 µg SR-ketamine												
0.3µg morphine + 30µg S(+)-ketamine	*	*										
+ 30µg R(-)-ketamine	*	*										
+ 30µg SR-ketamine		*										
1.0µg morphine + 30µg S(+)-ketamine	*	*	*									
+ 30µg R(-)-ketamine	*	*	*									
+ 30µg SR-ketamine	*	*	*									
0.1µg morphine + 100µg S(+)-ketamine	*				*							
+ 100µg R(-)-ketamine												
+ 100 µg SR-ketamine	*				*							
0.3µg morphine + 100µg S(+)-ketamine	*	*	*	*	*	*	*	*			#	#
+ 100µg R(-)-ketamine												
+ 100µg SR-ketamine	*	*			*							
1.0µg morphine + 100µg S(+)-ketamine	*	*	*									
+ 100µg R(-)-ketamine	*	*	*									
+ 100µg SR-ketamine	*	*	*									
0.3µg dexmedetomidine + 30µg S(+)-ketamine	*				*							
+ 30µg R(-)-ketamine	*				*							
+ 30µg SR-ketamine	*				*							
1.0µg dexmedetomidine + 30µg S(+)-ketamine	*	*										
+ 30µg R(-)-ketamine	*	*										
+ 30µg SR-ketamine	*	*										
3.0µg dexmedetomidine + 30µg S(+)-ketamine	*	*	*		*	*	*					
+ 30µg R(-)-ketamine	*	*			*	*		*				
+ 30µg SR-ketamine	*	*		*	*							

* /#= significant by Newmann-Keuls test at P<0.05

Table 4/2. Significant time points after drug combinations II.

Treatment	P<0.05 *vs* control (saline alone)				P<0.05 *vs* single-drug treatment (Mo/Dex/EM-1 alone)				P<0.05 *vs* racemic ketamine combination			
	10 min	30 min	60 min	90 min	10 min	30 min	60 min	90 min	10 min	30 min	60 min	90 min
0.3µg dexmedetomidine + 100µg S(+)-ketamine	*	*	*		*	*	*				#	
+ 100µg R(-)-ketamine	*				*							
+ 100µg SR-ketamine	*	*			*							
1.0µg dexmedetomidine + 100µg S(+)-ketamine	*	*										
+ 100µg R(-)-ketamine	*	*			*							
+ 100µg SR-ketamine	*	*			*	*						
3.0µg dexmedetomidine + 100µg S(+)-ketamine	*	*	*	*	*	*	*	*				
+ 100µg R(-)-ketamine		*										
+ 100µg SR-ketamine	*	*	*		*	*	*					
2.0µg endomorphin-1 + 30µg S(+)-ketamine	*											
+ 100µg S(+)-ketamine	*											
6.0µg endomorphin-1 + 30µg S(+)-ketamine	*											
+ 100µg S(+)-ketamine	*				*							
18.0µg endomorphin-1 + 30µg S(+)-ketamine	*											
+ 100µg S(+)-ketamine	*											
4µg endomorphin-1 + 1µg dexmedetomidine	*	*										
8µg endomorphin-1 + 2µg dexmedetomidine	*	*										
1µg endomorphin-1 + 0.25µg dexmedetomidine	*	*										
2µg endomorphin-1 + 0.5µg dexmedetomidine	*	*										
0.03µg Dex + 0.12µg EM-1 + 100µg S(+)-ketamine	*											
0.1µg Dex + 0.4µg EM-1 + 100µg S(+)-ketamine	*											
0.25µg Dex + 1+A27µg EM-1 + 100µg S(+)-ketamine	*											

*/# = significant by Newmann-Keuls test at P<0.05

Under the stripped areas the experiment was not performed.

Table 5. ED_{50} values of the applied drugs and drug combinations

Agents	Predicted ED_{50} (CI)	Observed ED_{50} (CI)
Morphine		**1.66 (1.04-2.32)**
30 μg S(+)-ketamine + morphine	1.66 (1.04-2.32)	1.19 (0.55-1.81)
100 μg S(+)-ketamine + morphine	1.66 (1.04-2.32)	0.16* (0.01-0.39)
30 μg Racemic-ketamine + morphine	1.66 (1.04-2.32)	1.04 (0.64-1.44)
100 μg Racemic-ketamine + morphine	1.66 (1.04-2.32)	0.18* (0.02-0.71)
30 μg R(-)-ketamine + morphine	1.66 (1.04-2.32)	2.61 (0.24-3.55)
100 μg R(-)-ketamine + morphine	1.66 (1.04-2.32)	2.32 (0.55-3.35)
Dexmedetomidine		**4.9 (3.81-6.14)**
30 μg S(+)-ketamine + DEX	4.9 (3.81-6.14)	1.39* (0.94-1.83)
100 μg S(+)-ketamine + DEX	4.9 (3.81-6.14)	0.15* (0.01-1.54)
30 μg Racemic-ketamine + DEX	4.9 (3.81-6.14)	2.15* (1.29-2.92)
100 μg Racemic-ketamine + DEX	4.9 (3.81-6.14)	0.18* (0.01-1.88)
30 μg R(-)-ketamine + DEX	4.9 (3.81-6.14)	1.83* (0.99-2.72)
100 μg R(-)-ketamine + DEX	4.9 (3.81-6.14)	1.39* (0.5-2.33)
Endomorphin-1		**14.2 (9.1-19.3)**
Endomorphin-1 + 30 μg S(+)-ketamine	14.2 (9.1-19.3)	6.2 (1.8-14.2)
Endomorphin-1 + 100 μg S(+)-ketamine	14.2 (9.1-19.3)	0.8* (0.1-5.1)
Endomorphin-1 : DEX = 4:1	9.63 (6.2-13.1)	2.4* (1.0-3.8)
EM-1 : DEX = 4:1 + 100 μg S(+)-ketamine	9.63 (6.2-13.1)	0.41+ (0.19-0.64)

CI = confidence interval, DEX = dexmedetomidine, EM-1 = endomorphin-1.

Predicted ED_{50} value means the data received when the agent (morphine, dexmedetomidine or endomorphin-1) was administered alone.

* Significant difference from the results of single morphine/endomorphin-1 or dexmedetomidine treatments.

+ Significantly different from endomorphin-1 + dexmedetomidine combination.

Significatly lower ED_{50} value (compared to the predicted one) suggests synergistic interaction between the examined drugs.

6. Discussion

The improved knowledge of the neurotransmitters, membrane receptors and intracellular mediators involved in the dorsal horn nociceptive processing in the last 25 years - since the first controlled study on the application of spinal opioids (130) – resulted so that this mode of analgesia has become widely used. Besides, advances in preclinical pain research have led to recognition of the spinal cord as a key target for inhibition of nociception during these years.

In an effort to gain better control over pain acutely and to maintain such control over the long term, scientists have advanced from single-drug therapy to the co-administration of two or three drugs. The growing trend of applying spinal drug combinations that target multiple mechanisms of analgesia reflects multi-drug therapy, the so called „combination analgesic chemotherapy".

The main reasons for developing combination analgesics are to gain efficacy, increase the duration of analgesia, to reduce toxicity or side effects and as a consequence improve patient compliance. Combining analgesics that act at different targets along the pain pathway can do so. Analgesic combinations should satisfy two important pharmacodynamic criteria: the components should display additive or synergistic analgesia and the interaction should allow lower doses of each substance to be used in combination, resulting in an improved safety profile.

Our experiments fitted well into this progress, since our results reveal new possible drug combinations that may have major clinical importance in spinal analgesia.

Ketamine

We examined the antinociceptive effect of intrathecally administered racemic ketamine and the pure ketamine enantiomers on acute pain sensation in rats. Our results with S(+)-, and R(-)-ketamine treatment correlates well with the previous observations on the racemic mixture (7), since we didn't find any significant antinociceptive effect on the tail flick test after either ketamine enantiomers or the racemate in doses that did not cause any side effects. So this observation contributes to the previous ones that ketamine enantiomers alone also don't have antinociceptive effect in acute pain after intrathecal administration.

The potential advantages of using a pure isomer rather than a racemate include a less complex and more selective pharmacodynamic profile, a higher therapeutic index, less complex pharmacokinetics-, drug interactions- and concentration-response relationships. For financial reasons, pure isomers have so far only been used for research purposes, but the wide-ranging clinical use of pure isomers is nowadays financially feasible due to new production techniques (131). Since the first reported experimental data about (59) the pharmacological differences of the optical isomers of ketamine, human studies revealed differences in the anaesthetic potency, intraoperative effects, postoperative analgesia and side effects of the two enantiomers (65). Data show that S(+)-ketamine is four times as potent as the R(-)-ketamine as an anaesthetic agent. The reason for this might be that pharmacokinetically it can be presumed that in the racemic combination R(-)-ketamine decreases the elimination of S(+)-ketamine, as suggested by Karash *et al.* (61). Furthermore, it has been shown that R(-)-ketamine also binds to the NMDA receptors, but with lower affinity (56) and others suggest that R(-)-ketamine also has a tendency to cause significant antinociception (132).

Drug combination studies

Following the concept of the "combination spinal analgesic chemotherapy", our main goal was to determine the possible interactions between ketamine and the typical μ-opioid agonist morphine or the endogenous μ-opioid receptor agonist endomorphin-1, and the α_2-adrenergic agonist dexmedetomidine. The different pharmacological actions of these drugs suggest that there might be an advantageous interaction after their co-administration.

Ketamine plus morphine combination

Our results showed potent synergism after intrathecal administration of ketamine with morphine under the circumstances of acute pain in rats. We found that only the racemic-, and the S(+)-ketamine were effective in reducing the ED_{50} of morphine, suggesting the development of a synergistic effect. These results are in agreement with the previous ones which have already shown that the combination of threshold doses of morphine with other NMDA antagonists elicits marked inhibitions of nociceptive responses and improves morphine effectiveness, as it does in some neuropathic models (133). Various studies have already proven the beneficial antinociceptive effect of opioids and other NMDA receptor antagonists co-administered both in acute and chronic pain by systemic (134) and in chronic pain applied via the intrathecal route (135). Powerful synergism arises from combinations of threshold doses of intrathecal morphine with low doses of NMDA antagonists (135,136,137) in agreement with our results. However, two studies failed to observe any potentiation of ketamine on morphine-induced antinociception in acute pain (135,138). Although the used pain tests were similar, the different effect could be explained by the subcutaneous route of administration in one case and the much lower doses in both studies.

Ketamine plus endomorphin-1 combination

The obtained data with intrathecal endomorphin-1 and S(+)-ketamine suggests that it can also be a potent combination, since we found that S(+)-ketamine (in the high dose used) synergistically enhanced the antinociception of intrathecal endomorphin-1, although the duration of this action was short lasting. A few studies have already examined the possible interactions between endomorphins and other drugs since their discovery. Beneficial interaction has been described between endomorphin-1 and spinal nociceptin (139) (the endogenous ligand of the opioid receptor-like orphan receptor), also with lidocaine (140) and clonidine (141) after intrathecal administration. A recent study showed that the combination of low doses of kynurenic acid (endogenous antagonists on ionotropic glutamate receptors, with preferential affinity for the glycine site of the NMDA receptor) and endomorphin-1 provides effective and well-controlled antinociception without any side effects on carrageenan-induced thermal hyperalgesia in rats (142).

Ketamine plus dexmedetomidine combination

Our results with dexmedetomidine and ketamine showed synergism between the α_2-agonist and all three ketamine compounds, although the racemic-, and the S(+)-ketamine were more effective than the R(-)-enantiomer. Results after intrathecal co-administration of endomorphin-1 with dexmededetomidine in a fixed 4:1 dose ratio also showed a synergistic interaction, which suggests that these two receptor systems can cause a more effective antinociceptive effect in combination. There is also a body of evidence about the advantageous interaction between α_2-

adrenoceptor agonists and NMDA antagonists in the literature. Systemic co-administration of these two compounds is a widely used combination in veterinary practice to provide adequate analgesia, muscle relaxation and complete immobilization (143). Behavioral and electrophysiological studies in rodents indicated the importance of adrenoceptors in modulating behavior and excitation induced by the intrathecal administration of NMDA (144). Yaksh *et al.* (112) have shown that the intrathecal co-administration of clonidine with MK-801 resulted in a dose-dependent increase in the mechanical pain threshold in a peripheral neuropathic pain modell in rats.

Synergy

There are several possible explanations for the observed synergistic interactions between ketamine and morphine, endomorphin-1 or dexmedetomidine. Since opioid-, glutamate- and α_2-adrenergic receptors are all abundant in the spinal cord (1), co-activation or antagonism of these receptors could have a beneficial effect on inhibiting the pain sensation at low doses which cause minimal side effects, in spite of the differences in the mechanism of action between the applied drugs. Ketamine decreases the activation of dorsal horn neurons, by inhibiting the ionotropic glutamate receptor opening (56). While the major mechanism of spinal opioid and α_2-adrenoceptor agonist analgesia is the inhibition of transmitter release from the C-fibre primary afferent terminal - thus reduces or blocks the C fiber inputs onto dorsal horn nociceptive neurons-, besides having inhibitory effect on both interneurons and projecting neurons (99,145). A presynaptic action of these latter drugs contrasts with the postsynaptic location of the NMDA receptor. The dual actions at both pre- and postsynaptic sites allows for possible synergy.

In some combinations (ketamine plus morphine or dexmedetomidine) we have found a prolonged antinociception besides potentiation. It is possible that this augmented activity resulted from a decreased clearance of drugs from the intrathecal space, since the duration of action of the ketamine combinations was longer than that of morphine or dexmedetomidine alone. In spite of these findings S(+)-ketamine did not prolong the antinociceptive effect of endomorphin-1, which suggests a mainly pharmacodynamic interaction between these drugs. The results obtained from the triple combination between fixed dose ratios of endomorphin-1 and dexmedetomidine combined with S(+)-ketamine showed a more potent antinociceptive activity than either of the single-, or double-drug treatments, which can be explained also by the previously mentioned statements.

As a conclusion we can say, that intrathecal combination of low doses of ketamine and its pure enantiomers with morphine, endomorphin-1 or dexmedetomidine have proved to be effective antinociceptive treatments on acute pain sensations in rats. The relatively higher antinociceptive potency of S(+)-ketamine (besides its fewer side effects) in the examined drug combinations suggest that it would be worthwhile to introduce and use it more widely in human pain management. Our data contributes a lot to the pharmacology of spinal anesthesia and follows the concept of the "combination analgesic chemotherapy" that emphasises the therapeutic significance of co-applied drugs with sinergistic interaction.

7. Summary

The thesis studied the antinociceptive properties of intrathecally co-applied ketamine with morphine and dexmedetomidine in rats, laying emphasis upon the effectiveness of racemic-, S(+)-, and R(-)-ketamine separately. Furthermore, it examined the interaction between the recently discovered endogenous μ-opioid agonist peptide - endomorphin-1 -, S(+)-ketamine or dexmedetomidine.

The experiments were performed on male Wistar rats; the acute nociceptive sensitivity was assessed by the hot water immersion tail flick test.

The following observations have been made:

1. Neither the racemic-ketamine nor its enantiomers alone had significant antinociceptive effect on influencing the acute pain sensation.
2. Intrathecal administration of morphine, endomorphin-1 or dexmedetomidine alone resulted in a dose-dependent increase in the thermal withdrawal latency. The rank order of potency was morphine > dexmedetomidine > endomorphin-1.
3. The high dose of intrathecal S(+)-ketamine and racemic ketamine (100 μg) synergistically enhanced the antinociception caused by intrathecal morphine, while the R(-)-ketamine had no such effect. Both the racemic-, and the S(+)-ketamine not only potentiated, but also prolonged the antinociceptive effect of morphine.
4. All of the ketamine treatments significantly enhanced the antinociception of dexmedetomidine and reduced its ED_{50} value, although the high dose of S(+)-ketamine and racemic ketamine were the most effective. This dose of racemic-, and S(+)-ketamine not only potentiated, but also prolonged the antinociceptive effect of dexmedetomidine.
5. Co-administration of 100 μg S(+)-ketamine potentiated, but did not prolong the antinociceptive effect of endomorphin-1.
6. Co-administration of endomorphin-1 with dexmedetomidine showed synergistic interaction.
7. Co-administration of endomorphin-1 and dexmedetomidine with S(+)-ketamine caused a dose-dependent antinociception and a prolonged effect, which be observed at higher doses. The triple combination showed further potentiation of the antinociceptive effect than the binary combinations between endomorphin-1 and dexmedetomidine or endomorphin-1 and S(+)-ketamine.

The synergistic interaction between these drugs may be of therapeutic significance in the future spinal anesthesia by allowing a decrease of the dose of either drug required to achieve an acceptable level of analgesia.

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Appendix

Publications:

I. **Joo G.**, Horvath G., Klimscha W., Kekesi G., Dobos I., Szikszay M., Benedek G. The effects of ketamine and its enantiomers on the morphine- or dexmedetomidine-induced antinociception after intrathecal administration in rats. *Anaesthesiology* 2000; 93, 231-241
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