

Experimental Modelling of Spinal Anaesthesia in Rats

PhD Thesis

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- I. **Dobos I**, Toth K, Kekesi G, Joo G, Csullog E, Klimscha W, Benedek G, Horvath G, The significance of intrathecal catheter location in rats. *Anesth. Analg.* 96: 487-492, 2003.
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- II. Klimscha W, Horvath G, Szikszay M, **Dobos I**, Benedek G, Antinociceptive effect of the S(+)-enantiomer of ketamine on carrageenan hyperalgesia after intrathecal administration in rats. *Anesth. Analg.* 56: 561-565, 1998.
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Abbreviations

AMPA – α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

CDI – calcium-dependent inactivation

CGRP – calcitonin gene-related-peptide

CSF – cerebrospinal fluid

DH– dorsal horn

DRG – dorsal root ganglia

EPSP – excitatory postsynaptic potential

FRAP – fluoride-resistant acid phosphatase

GDNF– glial cell line-derived neurotrophic factor

HP – hot plate

5-HT – 5-hydroxytryptamine

mGluR – metabotropic glutamate receptor

% MPE – maximal possible effect %

NGF – nerve growth factor

NO – nitric oxide

NMDA – N-methyl-D-aspartate

NT-3 – neurotrophin-3

NR1 – NMDA receptor subunit 1

NR2 – NMDA receptor subunit 2

NS – nociceptive specific

PAG – periaqueductal grey

PCP– phencyclidine

PWD – paw withdrawal

SP – substance P

TF – tail flick

TRPV1R – transient receptor potential vanilloid 1 receptor

VPL – ventral posterior lateral nucleus

VPM – ventral posterior medial nucleus

WDR – wide-dynamic range

1. INTRODUCTION

One of the main purposes of medicine is to relieve pain. Several examinations have been made to reveal the processes induced by painful stimuli. Different animal models are used to characterise physiological and behavioural changes after noxious stimuli (14, 75, 84, 91, 102).

The sensation of pain is fundamental in the maintenance of the biological integrity of the body, but chronic pain status cause severe somatic and psychic impairment, which give rise to considerable social and economic burden not only on the individual but also on the society.

The International Association for the Study of Pain (IASP) defines pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’ (6). This definition takes into consideration the subjectivity of the painful phenomenon and permits the understanding of important concepts concerning this subject. A noxious stimulus is capable of provoking a real or potential injury, not necessarily causing pain. In this context, pain experienced by virtue of this type of stimulus is characterized as nociceptive pain. There is a difference between the terms nociception and pain. Nociception refers to the neurophysiologic manifestations generated by noxious stimulus, while the pain requires the capacity of abstraction and the elaboration of sensory impulses (2).

Pain represents a complex sensory modality accompanied by affective, motivational and cognitive aspects, and associated with neurovegetative responses (2). In most cases, pain is initiated by events that occur on the skin, ligaments and articular capsules, or in deep tissues such as connective tissues of visceral organs, periosteum, tendon, or muscles. Specialized primary afferent neurons, called nociceptors, respond to noxious stimuli that can produce tissue damage. There are two major classes of nociceptors:

Unimodal nociceptors ($A\delta$ afferent) are thermosensitive or mechanosensitive, and respond to sharp mechanical pressure or tissue temperatures of 45 °C or more respectively.

Polymodal nociceptors (C fibers) react to mechanical, thermal or chemical stimulation (6, 12, 27). Both types of nociceptors are primary sensory neurons, whose cell bodies are located in dorsal root ganglia (DRG) or in a cranial nerve ganglion (V, VII, IX, X).

The first-order neurons transmitting pain impulses have two types. There are (1) small-diameter myelinated fibres of the A δ group that are responsible for 'fast' or 'first' pain and (2) non-myelinated C fibres (dorsal root C fibres) that give rise to a 'slow' or 'second' duller, more diffuse type of pain. Both types of first-order pain fibres terminate in the superficial layers of the dorsal horn (DH) of the spinal cord or in cervical nerve of trigeminal nucleus (6, 12, 27). The further processing of nociceptive information in different central structure may lead to pain sensation and adequate behavioural responses.

1.1. Cerebrospinal fluid (CSF)

The best way to examine the action of a drug at the spinal level in an awake animal is to introduce the drug into the spinal subarachnoid fluid and thereby bathe the spinal cord in the pharmacologically active solution.

In the region of the medulla oblongata, the central canal of the spinal cord enlarges to form the fourth ventricle. The ventricles are lined with a ciliated columnar ependyma. In the roof of the third and fourth ventricles and the medial wall of the lateral ventricle, the ependyma covering the capillary loops of the plexus choroideus becomes cuboidal (31). They secrete the cerebrospinal fluid (CSF). CSF is located within the ventricles, spinal canal, and subarachnoid spaces. CSF flows from the lateral ventricles through the interventricular foramina of Monro into the third ventricle. From there it flows into the fourth ventricle through the cerebral aqueduct of Sylvius and then through the foramina of Magendie and Luschka into the subarachnoid space. The subarachnoid space lies between the arachnoid and the pia mater, which, together with the dura mater, form the three meningeal layers that cover the brain. The CSF circulates through the freely communicating subarachnoid cisterns at the base of the brain. From the cisterns, most of the CSF is directed upward over the cerebral hemispheres and smaller amounts pass downward spinal canal and around the spinal cord. CSF is reabsorbed over the convexity of the brain into the venous sinuses (46). The volume of CSF varies between 10-20 % of brain weight, it is estimated to be approximately 140-150 ml in humans and it ranges from 300 to 400 μ l in adult rats (7, 19, 72). The lateral and third ventricles contain approximately 12 ml, and the spinal subarachnoid space about 30 ml with a production rate of 21 ml/hr in human. The subarachnoid space and major cisterns of the brain

contain most of the CSF. The turnover rate of total CSF is species dependent and varies between approximately 1 hr for rat and 5 hr for human (18, 46).

The spinal cord and the roots of the spinal nerves are closely surrounded by dura mater, a glassy, tough membrane. Only in the lumbar region is it pushed off the surface of the spinal cord by the dorsal and ventral roots. Usually the roots penetrate the tube of dura mater through several closely fitting openings and each is accompanied by a dural sheath, which is attached to the spinal ganglion as well as to the periosteum of the intervertebral foramen. Sparse narrow blood vessels traverse the epidural space. Especially in the dorsal part of the thoracic and sacral region, it contains a 0.3-0.5 mm layer of fat. In the cervical and lumbar region it is less than 0.3 mm thick. The arachnoid, a clear membrane, is loosely but not displaceable connected with the thin pia mater.

1.2. Pain transmission

1.2.1. Dorsal root ganglia

The DRG and some cranial nerves (V, VII, IX, X) ganglion contain most of the cell bodies of many of the sensory neurons that project to the spinal cord and to the periphery via the dorsal roots. The trigeminal ganglion (TRG) is a sensory ganglion of the face whereas the DRG associate with the rest of the body.

The cell bodies of primary afferents in the DRG or TRG can be divided into various subpopulations based on their neurochemistry, anatomy and physiology. First, large- and medium-sized cells (60-120 μm) have high levels of neurofilament and giving rise to large myelinated ($A\alpha/\beta$) fibres and receive input from peripheral mechanoreceptors (62). The second major group of DRG cells are small cells (14-30 μm), which have mainly unmyelinated (C) axons and are predominantly nociceptors, comprise two main populations: one part of the cells, which is referred to as CGRP/IB4- cells, because they constitutively synthesize the neuropeptide (calcitonin gene-related-peptide (CGRP) and substance P (SP), do not bind isolectin B4 *Griffonia simplicifolia* (IB4). These cells express transient receptor potential vanilloid 1 (TRPV1) and neurotrophic tyrosine kinase receptor type A receptors thus this population responds to the nerve growth factor (NGF) (22). The other part of the cells bind IB4 and express a non-lysosomal fluoride-resistant acid phosphatase (FRAP). This

group, which is referred to as IB4+, do not contain neuropeptides or only in low levels. The population of IB4+ cells express TRPV1 receptors also and selectively express P2X3 ATP-gated cation channels. In contrast to the CGRP/IB4- cells, the IB4+ population of nociceptors do not normally respond to NGF but they respond to glial cells line-derived neurotrophic factor (GDNF). These cells express GDNF family receptor α -1 (GFR α 1), GFR α 2 and/or GFR α 3 receptors (62). The central terminals of the two groups project to distinct regions of the dorsal spinal cord. IB4+ C-fibres project principally to inner lamina II (substantia gelatinosa) whereas the IB4- peptide rich C-fibres terminate more superficially in lamina I and outer lamina II (22).

An intermediate group between these two major populations comprises CGRP containing cell that are medium sized with finely myelinated A δ axons and terminate in lamina I and V of the spinal cord. Most of these cells are nociceptors of the high threshold mechanoreceptor type (62). This population of cells express TRPV2 receptors and respond to NGF and neurotrophin-3 (NT-3) (62). A small part of intermediate group cells provide mechanothermal receptors for high temperatures (45-50 °C) and some receptors for intense cold (-15 °C) and sensitized to vigorous mechanical stimuli at nonnoxious thresholds (2).

As the primary nociceptive neurons enter the spinal cord run up and down for one or two spinal cord segments, before they penetrate into the grey matter of the DH. The tract that ascends/descends before synapsing is known as Lissauer's tract (77). Generally speaking C fibres appear to travel in the most lateral part of the dorsal white matter, including Lissauer's tract, and A fibres most medially. Lissauer's tract is the bundle of fine fibres situated just lateral to the entering dorsal rootlets with a poorly defined border towards the dorsolateral funiculus. More than two thirds of axons, in the rat Lissauer's tract at thoracic, and lumbosacral levels are primary afferents, and majority are unmyelinated (67, 96).

1.2.2. Spinal cord

The spinal cord is the most caudal part of the central nervous system (CNS) and in many respects, the simplest part.

The weight of the rat spinal cord increases with the changing of the body weight as follows: 100 g of animal has near 0.43 g, 200 g of body weight it is 0.58 g and it approaches 0.7 g in animal of 300 g body weight (31). In females, spinal cord weight averages 2% higher than it

does in males. The length in adult male rats varies from 113 to 125 mm. It extends from the base of the skull to the fourth lumbar vertebra. It is divided into four major regions: cervical, thoracic, lumbar and sacral (31).

The first thoracic segment ends at the last cervical vertebra; the first lumbar segment begins at the caudal end of the 10th thoracic vertebra, and from there on the coccygeal part runs to the cranial third of the 4th lumbar vertebra.

The cord is divided into grey matter and surrounding white matter. The grey matter, which contains nerve cell bodies, is shaped like letter H, with each side subdivided into dorsal (or posterior) and ventral (or anterior) horns.

The DH is larger where sensory nerves from the limbs enter the cord because the limbs have a greater density of sensory receptors and thus send more fibres to the cord. These regions of the cord are known as lumbosacral and cervical enlargements.

The ventral horn contains interneurons in addition to motor neurons, whose axons exit the spinal cord and innervate skeletal muscles.

In the thoracic and upper lumbar regions of the spinal cord, the lateral horn interposes between the dorsal and ventral horns. The lateral horn contains preganglionic visceral motor neurons of the sympathetic nervous system. These neurons send their axons to synapse on sympathetic ganglia that innervate autonomic organs (45).

Various types of interneurons in the grey matter modulate information flowing from the sensory neurons toward the brain or/and the motor neurons, as well as information passed between groups of motor neurons.

The white matter surrounding the grey matter is divided into dorsal, lateral, and ventral columns. The dorsal columns, which lie between the two DHs of the grey matter, contain ascending axons that carry somatosensory information to the brainstem. The lateral columns include both ascending axons and fibres descending from the brainstem and neocortex that innervate interneurons and motor neurons in the spinal cord. The ventral columns also include ascending and descending axons (46).

1.2.3. Dorsal horn

The DH contains four different neuronal components (94):

1. central terminals of primary afferents

2. projecting neurons with long ascending axons synapsing to the brain
3. interneuron (intrinsic spinal neurons)
4. descending axons from various parts of the brain

The primary sensory afferent fibres terminate predominantly in the DH of the spinal cord. The DH can subdivide into six distinct layers based on the cytological features (11). Classes of primary afferent neurons that convey distinct modalities terminate in distinct laminae of the DH. Thus, there is a close correspondence between the functional and anatomical organization of neurons in the DH of the spinal cord (46).

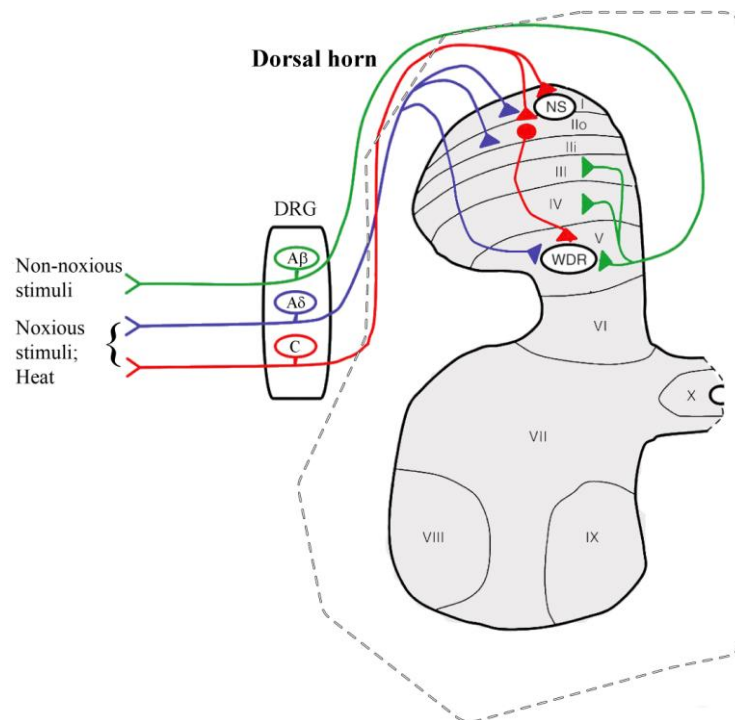


Fig.1 Schematic representation of dorsal horn

(source: <http://www.fastbleep.com/biology-notes/39/142/888>)

Lamina I receives particular attention in relation to nociception because it is a specialised region for the termination of nociceptive afferents and contains nociceptive specific (NS) cells which respond only to noxious stimuli and project to higher brain centres (Fig. 1) (96). The majority of these neurons receive direct synaptic input from Aδ and C polymodal fibres (CGRP/IB4- small diameter cells) (27, 46). The other group of cells in this lamina are the wide dynamic range (WDR) neurons which respond to mechanical, thermal and chemical stimuli coming from the Aδ, C and Aβ fibres. Because of convergence of noxious and nonnoxious fibres, this group plays a fundamental role in the mechanisms of segmental

suppression of pain involved in the gate control theory (2, 66). They are found in deeper laminae (IV, V, VI, X) too (2).

Lamina II (substantia gelatinosa) has been subdivided based on physiological and anatomical data, into inner and outer regions (IIi and IIo). The ventral region (IIi) contains synaptic contacts of cells that responding only to light brush and additionally the small-diameter IB4+ non peptide-expressing nociceptive cells afferent axons enter here. While the more dorsal part (IIo) has the largest number of cells afferentation that responding to light and heavy mechanical stimuli and to noxious heat and chemicals (small-diameter CGRP/IB4- cells) (46, 96).

Lamina III is located ventral to the substantia gelatinosa and contains neurons that receive monosynaptic input from A β fibres. These neurons, respond predominantly to non-noxious stimuli, have quite restricted receptive fields that are organized topographically (46).

Lamina IV contains large cells with dorsally rising dendrites and small stellate cells. They excite only by light mechanical stimuli to the skin and the response of these cells does not increase if the intensity of the pressure stimulus is increased (96).

Lamina V contains primarily wide-dynamic-range (WDR) neurons that project to the brainstem and to regions of the thalamus and respond to a variety of inputs from low to high threshold mechanical, thermal and chemical stimuli by way of the full range of diameters of afferent fibres (96). These neurons receive monosynaptic input from A β and A δ fibres. They also receive input from C fibres, either directly on their dendrites, which extend dorsally into the superficial DH, or indirectly via excitatory interneurons. Many neurons in lamina V also receive nociceptive input from visceral structures (46).

Neurons in lamina VI make up the ventral boundary of the DH (96). They receive inputs from large-diameter (A α) afferents from muscles and joints and respond to non-noxious manipulation of joints (46). However, they also respond to low and high-threshold cutaneous afferents. This convergent input from both specialized muscle receptors, cutaneous and visceral inputs is under selective modality control by way of descending pathway from the CNS (96).

Neurons in ventral horn laminae VII and VIII, many of which respond to noxious stimuli, have more complex response properties because the nociceptive inputs to lamina VII neurons are polysynaptic. Furthermore, although most DH neurons receive input from only one side of the body, many neurons in lamina VII respond to stimulation of either side. Thus, neurons of

lamina VII, through their connections with the brainstem reticular formation, may contribute to the diffuse nature of many pain conditions (46, 96).

A specialized group of cells gathered around the central canal make up lamina X. They may be related to pain mechanisms too, since some respond to high intensity bilateral stimuli and contain the same array of peptides (CGRP, SP) which characterize laminae I and II, and it could be one of the polysynaptic chains linking the entire length of cord and brainstem (96).

1.2.4. Ascending nociceptive pathways

As in other sensory systems in the mammals, the somatosensory system includes a number of parallel ascending pathways. Nociceptive information is transmitted from the spinal cord to the brain over several different ways, including the spinothalamic, spinoreticular, spinomesencephalic, spinocervical and second-order dorsal column tracts (12). The functions of the ascending nociceptive systems include signalling of the sensory discriminative aspects of pain, and they trigger motor and autonomic adjustments, as well as activating antinociceptive systems (12, 31).

1.2.4.1. Spinothalamic tract

The spinothalamic tract is the most prominent ascending nociceptive pathway in the spinal cord (12, 31). It comprises the axons of nociceptive and wide-dynamic-range neurons in laminae I and V-VII of the DH. The axons decussate within the spinal cord and they ascend within the anterolateral white matter (46, 78, 96). The nociceptive information from the body terminates in the ventral posterior lateral (VPL) nucleus and from the face — through the trigeminothalamic tract — terminates in the ventral posterior medial (VPM) nucleus (A δ fibres) and intralaminar nuclei (C fibres) of the thalamus (78). These nuclei send their axons to primary and secondary somatosensory cortex. The nociceptive information transmitted to these cortical areas is thought to be responsible for the discriminative component of pain: identifying the location, the intensity and quality of the stimuli (78).

1.2.4.2. Spinoreticular tract

The spinoreticular tract comprises the axons of neurons in laminae VII and VIII. It ascends in the anterolateral quadrant of the spinal cord and terminates in both the reticular formation (in particular the parabrachial nucleus) and intralaminar nuclei of the thalamus. In contrast to the spinothalamic tract, many of the axons of the spinoreticular tract do not cross the midline

(46). Neurons in the parabrachial nucleus project to the hypothalamus and the amygdala, thus providing nociceptive information to circuits known to be concerned with motivation and affection. These parabrachial targets are also the source of projections to the periaqueductal grey (PAG) matter of the midbrain, a structure that plays an important role in the descending control of activity in the pain pathway and it is likely to trigger somatic and autonomic motor reflexes (78, 96). The intralaminar nuclei of the thalamus provide nociceptive signals to the insula and to the cingulate cortex.

1.2.4.3. Spinomesencephalic tract

The spinomesencephalic tract comprises the axons of neurons in laminae I and V. It projects in the anterolateral quadrant of the spinal cord to the mesencephalic reticular formation and PAG matter via the spinoparabrachial tract and project to the amygdala, a major component of the limbic system which is involved in the emotion (46, 78). Thus, the spinomesencephalic tract is thought to contribute to the affective component of pain (46). Because of stimulation in the PAG results in analgesia, this structure plays an important role in the descending control of pain pathway (2, 96). Stimulation of the PAG activates enkephalin-releasing neurons that project to the 5-HT (serotonin) releasing raphe nuclei in the brainstem and descends to the DH of the spinal cord connecting with the "inhibitory interneurons" in Laminae II. When this pathway activated, these interneurons in the DH release either enkephalin or dynorphin (endogenous opioid neuromodulators), which bind to μ - and κ -opioid receptors on the axons of incoming C and A δ fibres. The activation of the μ -opioid receptors inhibit the release of SP from these incoming first-order neurons and, in turn, inhibit the activation of the second-order neuron that is responsible for transmitting the pain signal in the spinothalamic tract to the VPL of the thalamus. Thus, the nociceptive signal can be inhibited before it reaches the cortical areas that interpret the signal as "pain".

1.3. Role of glutamate receptors in nociception at spinal level

Tissue injury triggers the production and liberation of a diverse array of ions, nucleotides, lipids, peptides, amino acid derivatives and proteins at the periphery capable of activating nociceptors or augmenting nociceptor responses to different stimuli. Electrophysiological and pharmacological studies of cultured, dissociated nociceptive neurons have revealed that certain agents (e.g. protons and capsaicin) directly depolarize nociceptive neurons by triggering the opening of cation channels permeable to Na^+ and/or Ca^{2+} . In contrast, agents such as bradykinin and NGF act on G protein-coupled receptors or TRP, respectively, to trigger intracellular signalling cascades that in turn sensitize depolarizing cation channels to their respective physical or chemical regulators. Still other agents (e.g. glutamate, acetylcholine and adenosine triphosphate) activate both ion channels and G protein coupled receptors to produce a spectrum of direct and indirect effects on nociceptor membrane potential (12, 61, 101).

Glutamate is one of the main excitatory neurotransmitters of somatosensory transmission pathways. Glutamate acts on several ionotropic and metabotropic glutamate receptors, which are widely distributed in spinal nociceptive circuits (44). Glutamate also exerts a crucial role in the developing and the mature nervous system, since it regulates neurogenesis, axonal sprouting, synaptogenesis and programmed cell death (apoptosis) (93). The overactivation of glutamate receptors is a major cause of Ca^{2+} overload in cells, potentially leading to cell damage and death (92).

Glutamate level is high in the DRG and it is colocalized with neuropeptides like CGRP and SP, in peptidergic primary afferents and there is a high density of glutamatergic binding sites in the DH on interneurons and in descending nociceptive neurons (20, 23).

1.3.1. Glutamate receptors

Glutamate receptors are divided into the two categories: metabotropic (mGlu) which linked to G-proteins that modulate intracellular second messengers such as inositol phosphates and cyclic nucleotides and ionotropic, which are associated with ion channels.

Metabotropic Glu receptors can be separated into three groups based on their amino acid sequence homology:

Group I mGlu receptors include mGlu1 and mGlu5 receptor subtypes and their associated alternatively spliced variants. This receptor subclass predominantly couples to Gq/G11 G-proteins to stimulate phospholipase C (8, 39).

Group II subclass consists of mGlu2 and mGlu3 receptors and couples to Gi/Go G-proteins to inhibit adenylyl cyclase (8, 39).

Group III subclass is comprised of mGlu4, mGlu6, mGlu7 and mGlu8 receptors and isoforms of mGlu7 and mGlu8. This receptor subclass couples to Gi/Go G-proteins also (8, 39).

The ionotropic receptors can be divided into 3 subcategories according to their activation by different agonists:

1. NMDA receptors are activated by N-methyl-D-aspartate
2. AMPA receptors are activated by α -amino-hydroxy-5-methyl-4-isoxazole propionic acid
3. kainate receptors are activated by kainic acid.

All of the mammalian ionotropic glutamate receptors are multimeric ligand-gated nonselective cation channels allowing the passage of Na^+ , K^+ or Ca^{2+} (69). Activation of AMPA, kainate and NMDA receptors always produces excitatory postsynaptic potentials (EPSP) (77).

1.3.2. NMDA receptor

High levels of NMDA receptor-mediated Ca^{2+} influx during high frequency electrical stimulation enhance synaptic transmission (e.g. long-term potentiation of hippocampal neurons). In contrast, lower levels of NMDA receptor-mediated Ca^{2+} influx during low frequency stimulation result in a decrease of synaptic strength (e.g. hippocampal long-term depression). Excessive Ca^{2+} influx via NMDA receptor ion channels leads to excitotoxic cell death. The Ca^{2+} act as a second messenger to activate intracellular signalling cascades and may lead ultimately to the generation of nitric oxide, reactive oxygen species and thus to cell death (92, 107).

Traditionally, NMDA receptors are best known for their role in excitotoxicity, when overactivation of NMDA receptors causes intracellular Ca^{2+} accumulation in brain trauma and in neurodegenerative disorders such as Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis and Alzheimer's disease (105). Overactivation of excitatory pathways is also

observed in epilepsy (24, 50, 77). Because of their involvement in numerous neurological disorders, NMDA receptors are also targets of therapeutic interest. Activation of NMDA receptors by glutamate is involved in the local circuit nociceptive transmission underlying the behavioural expression of hyperalgesia and allodynia (16, 83) and plays an important role in the development and maintenance of central sensitization, a state of long-lasting increase in synaptic transmission, and neuropathic pain at several levels (54). Consequently, the blockade of spinal NMDA recognition sites elicits antinociception in various models of persistent pain (16, 83).

Several subunits of the NMDA receptor are known, in particular N(MDA)R1, N(MDA)R2 and N(MDA)R3 (32, 71, 105). The stoichiometry of NMDA receptors have not yet been established definitely, but the consensus is that NMDA receptors are tetramer that most often incorporate two NR1 and two NR2 subunits of the same or different subtypes (Fig. 2) (71). The subunit composition of NMDA receptors determines their biophysical and pharmacological properties.

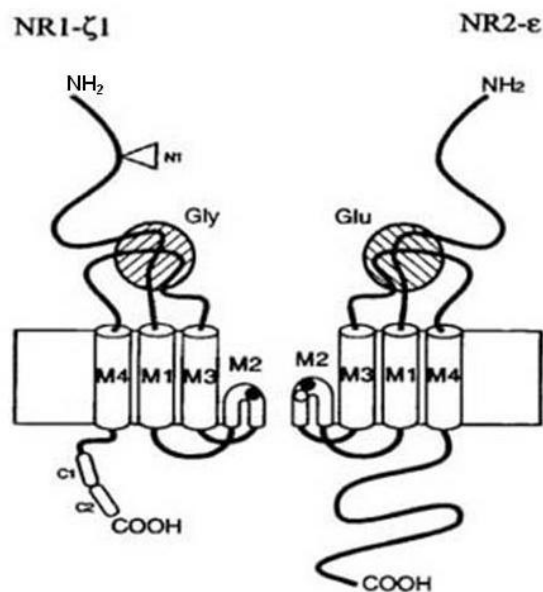


Fig. 2 Schematic representation of heteromeric NMDA receptor structure (105)

NMDA receptor subunits all share a common membrane topology characterized by

- a large extracellular N-terminus
- a membrane region comprising three transmembrane segments (M1, M3 and M4)

- a re-entrant pore loop (M2)
- an extracellular loop between M3-M4
- a cytoplasmic C-terminus (21, 71, 105).

Amino-terminal domain plays an important role in subunit assembly. The two extracellular domains, namely the ligand-binding site and the amino-terminal domain, have many similarities (32). The structure of the re-entrant second membrane loop influences the permeation properties of the ion channel (21, 71). In the heteromeric NMDA receptor complex, the quaternary arrangement of the subunits around the central symmetry axis will govern the dimerization possibilities (71).

The sequences of the regions lining the pore are highly conserved in the NR1 and NR2 subunits and accordingly, permeation properties as well as affinity for the pore blocker Mg^{2+} . By contrast, incorporating the NR3 subunit markedly decreases single-channel conductance, Ca^{2+} permeability and Mg^{2+} block (71).

1.3.3. NMDA receptor modulation

1.3.3.1. Endogenous modulators

NMDA receptor subunits and splice variant compositions determine the sensitivity of receptors to modulation by a wide variety of compounds. Thus, several endogenous agents modulate NMDA receptors within physiological ranges and in a subunit-specific manner. These modulatory agents include glycine, kynurenic acid, polyamines, Zn^{2+} , Mg^{2+} , protons, (Fig. 3) (32). That the binding sites for these modulators are found in the amino terminal domain of their receptors and these modulatory sites allow considerable potential for pharmacological manipulations too (21, 32, 40, 71, 105).

Glycine is an essential co-agonist that facilitates the actions of glutamate at submicromolar levels (39, 40, 74, 77). Thus, the maximal activation of NMDA receptor channels requires binding of both 2 L-glutamate and 2 glycine molecules (21, 32, 71, 105). Site-directed mutagenesis has identified that the glycine binding site is formed by the region preceding segment M1 of the NR1 and NR3 subunits and the glutamate binding site was shown to reside in the homologous regions of the NR2A and NR2B subunits (21, 105) (Fig. 2). In low extracellular glycine concentration (<100nM) NMDA receptor currents are potentiated by dynorphin peptides that contain glycine residues, perhaps as a result of

proteolytic release of glycine from the parent peptide by the tissue. But dynorphin A(1-13) can also inhibit NMDA receptor activation in a voltage-independent manner that is non-competitive with either NMDA or glycine but is dependent on the NR2 subunit, being greatest for NR2A. Positively charged peptides may have multiple effects on NMDA receptors, best illustrated by the effects of the dynorphin peptide (21).

In the resting state, the NMDA receptor channel is blocked by Mg^{2+} ions at a site deep within the channel itself and binding of glutamate to the agonist-binding site, even in the presence of glycine, does not result in Ca^{2+} entry through the postsynaptic membrane. Depolarization of the membrane removes the Mg^{2+} -mediated channel blockade and Ca^{2+} flux through the channel can then occur (Fig. 3) (24, 39, 40, 77).

Since AMPA and NMDA receptors are, in many cases co-localized at central synapses, the rapid activation and brief open time of AMPA facilitates the unblock of NMDA receptors by Mg^{2+} and thus the more slowly activating NMDA receptors participate in the long-lasting maintenance of synaptic currents (21).

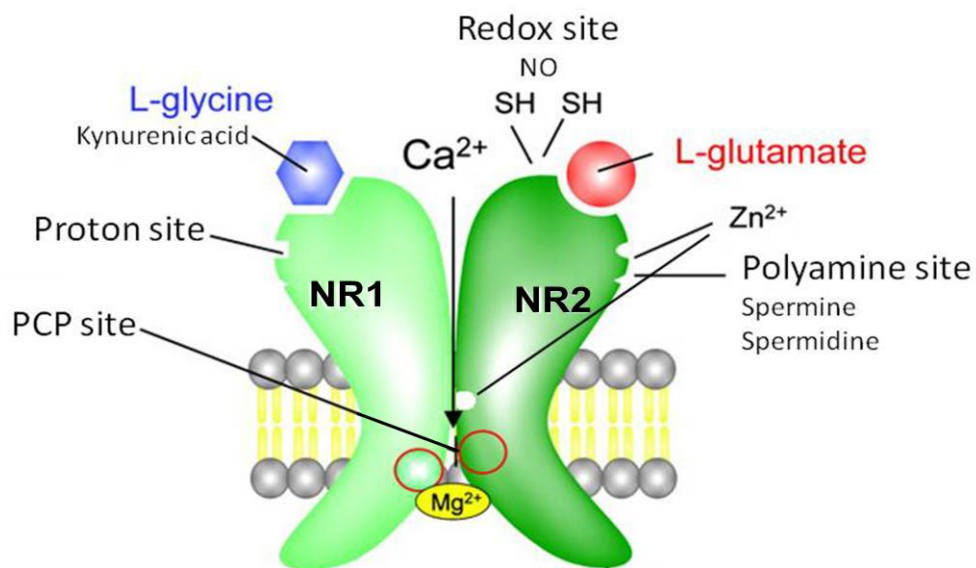


Fig. 3 Modulation sites and some endogenous ligands of the NMDA receptor

(source: www.neurology.org)

In addition, Mg^{2+} ions are known to enter and block open glutamate receptor channels (Fig. 3). Thus, the rate of the onset of blockade is use-dependent and is accelerated by increases in open channel probability. Once bound, however, the blocker can be trapped by

channel closure (21). Channel block by trapping blockers is slow to reverse and requires channel reactivation by agonists before the blocker can dissociate (95).

NMDA receptor channels at physiological pH are tonically inhibited by proton (21). The inhibition of NMDA receptors by protons is controlled by the NR2 subunit as well as alternative exon splicing in the NR1 subunit. Mutations that influence pH sensitivity are broad ranging in both NR1 and NR2 subunits and include amino-terminal acidic residues, cysteine residues that may participate in disulfide bond formation residues in the extracellular M3-M4 loop as well as residues that are thought to comprise the channel pore-forming region. This latter association between pore-forming residues and pH sensitivity might suggest that the proton sensor is tightly coupled to the movement of the gate.

Zn^{2+} is concentrated at many glutamatergic synapses, and it is likely to be an endogenous allosteric modulator of NMDA receptors (71). Two types of Zn^{2+} -binding sites are located on separate modules and inhibit NMDA receptor currents through distinct mechanisms (Fig. 3). Each NMDA receptor subunit consists of an N-terminal domain that binds allosterically active ligands including Zn^{2+} . Voltage-independent inhibition is mediated by N-terminal domain of NR2 subunits. Zn^{2+} binding changes the receptor's gating kinetics but not its conductance. At high concentrations ($>10\mu M$) extracellular Zn^{2+} reduces opening frequency and decreases open duration (3, 21, 39). However, at submicromolar ($EC_{50} = 0.5\mu M$) concentrations, Zn^{2+} potentiates homomeric NR1 channels lacking the N1 insert in a voltage-independent manner, whereas little or no potentiation is observed for NR1 channels containing the N1 insert. It follows that the sensitivity of the heteromeric NMDA receptor channel to Zn^{2+} inhibition is determined by NR1 subunit splice variants (21, 32, 71, 105). NR2A subunit is much more sensitive to Zn^{2+} , being inhibited in the nanomolar range ($IC_{50}=5-30\text{ nM}$) by as much as 70 to 80 % and NR2B subunit having low sensitivity ($\sim 1\mu M$), while Zn^{2+} does not bind NR2C or NR2D (3). Zinc inhibition of NR1/NR2A receptors is proton dependent. Thus, protonation of its binding residue may be critical for zinc inhibition, and/or the zinc binding domains may interact functionally and/or structurally with proton sensitive elements (32). Voltage-dependent Zn^{2+} inhibition of NMDA receptors is also presented at all NMDA receptor subtypes and is likely to occur within the ion channel pore and may involve the conserved asparagine residue in segment M2 of the NR1 subunit. Asparagine residues in the second transmembrane helix contribute to the narrowest portion of the permeation pathway and are critical for voltage-dependent. Glycine substitutions at these critical positions increase pore diameter and reduce channel sensitivity to voltage-dependent

block by orders of magnitude. This asparagine residue is also involved in the Ca^{2+} permeability and the Mg^{2+} block of NMDA receptor channel (3, 32, 105).

The redox modulatory site of NMDA receptor channel exists in equilibrium between fully reduced (thiol, R-SH) and fully oxidized (disulphide, R-S-S-R) states (105). This site contains two labile cysteine residues on the NR1 and NR2 subunits where sulphhydryl groups of subunits may interact with nitric oxide (NO) derivatives to modify channel function (21, 39) (Fig. 3). Lei et al. demonstrated that NO can form a disulfide bond(s), which is thought to constitute the redox modulatory site of the receptor, resulting in a relatively persistent blockade of NMDA responses (52). These reactions with NO can afford protection from NMDA receptor-mediated neurotoxicity. This is a new pathway for NO regulation of physiological function. It is not via cGMP, but instead involves reactions with membrane-bound thiol groups on the NMDA receptor-channel complex. The redox site has physiological and pathological importance. Among the most prominent of these are free radicals, superoxide and hydroxyl radicals, which suppress NMDA receptor activation.

Effects of polyamines on NMDA receptors were first reported in 1988 (80). The polyamines putrescine, spermidine and spermine are present in almost all cells and have important roles in protein synthesis, cell division and cell growth. Spermine and spermidine are present in high concentrations in the nervous system, and uptake and depolarization induced release of polyamines from brain slices has been reported (30, 85, 100). They are organic polycations that are protonated at physiological pH and can potentially interact with a variety of cellular targets including nucleic acids and proteins (100). Polyamines can stimulate NMDA receptor function to a greater degree at low glycine concentrations than at saturating glycine concentrations. This stimulation reflects approximately a 3-fold increase in glycine affinity. Both glycine-independent and glycine-dependent forms of potentiation of NMDA receptor function are influenced by the NR2 subunit (Fig. 3). However, whereas glycine-dependent potentiation occurs at NR2A- and NR2B-containing receptors, glycine-independent potentiation is observed exclusively at receptors that incorporate the NR2B subunit. The glycine-independent form of potentiation has been suggested to arise from the relief of tonic proton inhibition at physiological pH. That is, polyamines shift the pKa of the proton sensor to acidic values, reducing the degree of tonic inhibition at physiological pH, which appears as a potentiation of function (21, 100). Spermine was also reported to relieve voltage-independent Zn^{2+} inhibition of the heteromeric NMDA receptor channel (80, 85). Polyamines also have inhibitory effects. The inhibitory effects can be divided into a decrease

in the affinity for glutamate and strongly voltage-dependent inhibition (21, 100, 105). Extracellular spermine can block NMDA channels at negative membrane potentials, and at extreme negative potentials, spermine can permeate the NMDA channel to pass into the cell (100). Single-channel studies indicated that the enhancement of NMDA receptor current by spermine at low concentration (10 μ M) was due to an increase in opening frequency of NMDA receptor single channels, whereas at higher concentrations (1 mM) spermine decreased average open duration and conductance of the NMDA single-channel currents at negative holding potentials (85, 86).

Kynurenic acid is a metabolite of the tryptophan metabolism with a complex pharmacology (92). It is able to antagonise non-NMDA as well as NMDA receptor activation, because it had an especially high affinity at the glycine site and may even distinguish subpopulations of kainite receptors therefore, it can possess neuroprotective activity (Fig. 3) (92). The kynurenine pathway is present primarily in the glial cells population in the CNS such as microglia. When these cells activated by immunogenic stimuli such as bacterial lipopolysaccharide or interferon- γ , they are able to generate up to 1000 times the basal levels of kynurenines. Because of activating the pathway, there is an increased generation of excitotoxicity compounds such as quinolinic acid, an endogenous agonist at NMDA receptors and 3-hydroxykynurenine. An alternative strategy to the direct blockade of glutamate receptors is to modify activity in the kynurenine pathway, changing the balance between the generation of quinolinic acid and kynurenic acid. Inhibition of the enzymes kynurenine hydroxylase and kynureninase by nicotinylalanine results in a decrease in the levels of endogenous quinolinic acid and an increase of kynurenic acid. Quinolinic acid acts selectively at NMDA receptors containing NR2A and NR2B subunits, with massive calcium entry into neurons and astrocytes (76). The *in vivo* overstimulation of NMDA receptors by quinolinic acid causes an early impairment of the sarco/endoplasmic reticulum Ca^{2+} -ATPase pump which may result in important disturbances in intracellular Ca^{2+} signalling (56).

NMDA receptor-induced Ca^{2+} influx has been shown to promote rapid inactivation of NMDA receptor (63). This calcium-dependent inactivation (CDI) serves as a negative feedback control system to regulate Ca^{2+} influx. CDI is unaffected by ATP, phosphatase, inhibitors, and other agents that affect protein phosphorylation (87). CDI is regulated by the actin cytoskeleton. Actin destabilizing agents such as the cytochalasins specifically eliminate CDI, suggesting that the NMDA receptors may be associated with the actin. Zhang et al. showed that, the calcium binding protein, calmodulin, binds to two sites (CBS1 and CBS2) on

the C terminus of the NR1 subunit of the NMDA receptor (25). Calmodulin binding to the C terminus of the NR1 subunit decreases the open probability on NMDA receptor channels (25). CB1 region of the NR1 subunit also interacts with α -actinin2, an actin-associated protein and that calmodulin can compete for α -actinin2 binding to the NR1 subunit. Zhang et al. demonstrated that deletions or point mutations of CB1 in the NR1 C terminus substantially block CDI (107). Deletion of CB2 has no effect on CDI. In addition, intracellular infusion of a calmodulin inhibitory peptide blocks CDI of both recombinant and native NMDA receptors in hippocampal neurons. Intact actin cytoskeleton is required for CDI of NMDA receptors because the NMDA receptor might directly interact with a cytoskeletal protein (α -actinin2) that dissociates from the channel in a Ca^{2+} -dependent manner, leading to inactivation. Moreover, α -actinin2 co-localizes with NMDA receptors at excitatory synapses (107).

1.3.3.2. Exogenous modulators

Several drugs acting on NMDA receptors may be suitable for the treatment of neuropathology, others cause severe cognitive and behavioural disturbances. NMDA receptor antagonists can divide four categories: *competitive antagonists* bind to and block the glutamate binding site (e.g. selfotel); *glycine antagonists* bind to and block the glycine site (e.g. kynurenic acid); *non-competitive antagonists* block the ion channel by binding to a site within it (e.g. MK-801, memantine, ketamine) (Fig. 4).

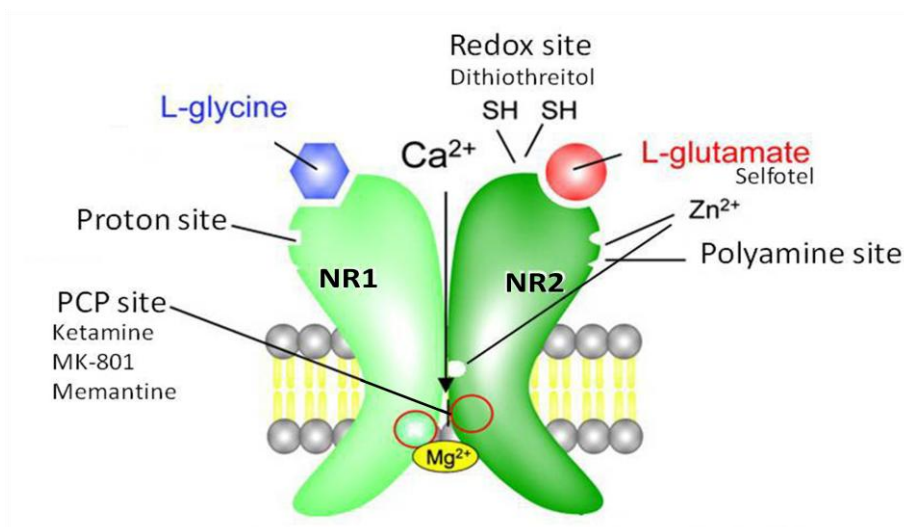


Fig. 4 Modulation sites and exogenous ligands of the NMDA receptor

(source: www.neurology.org)

Reducing agents such as dithiothreitol can potentiate NMDA receptor channels, while oxidizing agents such as 5-5'-dithiobis-2-nitrobenzoic acid are inhibitory (92, 105) (Fig. 4).

At intoxicating concentrations, ethanol is another non-competitive NMDA receptor antagonist, and trichloroethanol, the active metabolite of the hypnotic chloral hydrate, also blocks NMDA receptors noncompetitively at anaesthetic concentrations. Ethanol molecules accommodate into a physical cavity in NMDA receptors, which is reachable from lipophilic milieu favours entry and affinity/efficacy of longer-chain alcohols on the receptor (68). Block by ethanol was more potent with the NR2A or NR2B than the NR2C subunit (21, 68), but the degree of block was not affected by pH, Zn^{2+} or redox state of the receptor (21). Elevating extracellular Mg^{2+} from close to zero Mg^{2+} to more than physiological concentrations, increases ethanol sensitivity of NMDA receptors (60). Ethanol inhibition is stronger if calcium and calmodulin-dependent inactivation of NMDA receptors are allowed to function properly (4). Hoffman et al. found that ethanol had a potent and selective effect on the NMDA receptor in the primary cultures of cerebellar granule cells (36). In these cells, glutamate acting at kainite and NMDA receptors increases the cGMP level. This effect might be mediated via an action on agonist-stimulated calcium influx. These biochemical data were corroborated by electrophysiological experiment showing that ethanol was a very potent inhibitor of NMDA-gated currents in hippocampal cell culture (36).

Selfotel (CGS 19755) is a competitive NMDA antagonist, it has proved to be neuroprotective in different animal models of global ischemia, focal ischemia and brain trauma (73) (Fig. 4).

Phencyclidine (PCP) is a dissociative anaesthetic, antagonizes NMDA-evoked responses non-competitively, and has no effects on the binding of glutamate to the NMDA receptor recognition site (26). PCP-like drugs (phencyclidine, ketamine or tiletamine) block NMDA receptor responses by binding to a site, which is downstream from the transmitter recognition site possibly an activated or opened configuration of the channel complex (26) (Fig.4).

MK-801 (dizocilpine) binds at PCP's binding site of NMDA receptors and blocks it in a use- and voltage-dependent manner. Thus, the channel must open for the drug to bind inside it, preventing the flow of ions, including an influx of Ca^{2+} , through the channel (Fig. 4). MK-801 is a well-known neuroprotectant in models of stroke, trauma, Parkinsonism and organophosphate-induced seizures (50). Despite this, MK-801, like other PCP receptor

ligands, such as phencyclidine, ketamine or tiletamine, induces psychotic behaviour and neuronal degeneration. MK-801 exposure causes neuronal degeneration in axon terminals, increases glucose metabolism, induces cytoplasmic vacuoles, heat shock protein and necrotic cell death in neurons of the rodent in many brain areas (38).

Memantine is an uncompetitive NMDA receptor antagonist, which has been used clinically for years for the treatment of Parkinson's disease, spasticity, and dementia (55). Memantine is able to block the excitotoxic effects of NMDA receptor overactivation without altering normal synaptic transmission because it has a low-affinity to the NMDA receptors and has a relatively rapid off-rate from the channel (51). Memantine has been shown to be less voltage-dependent than Mg^{2+} , which allows memantine to continue to block the channels under relatively depolarized conditions. In addition, studies have found that memantine effectively blocks NMDA receptor activity in the presence of prolonged elevations of glutamate concentrations (13) (Fig. 4).

1.3.3.3. Ketamine

Ketamine [2-(O-chlorophenyl)-2-methylamino cyclohexanone] (MW: 238), a non-competitive NMDA receptor antagonist. Its effects are mediated primarily by non-competitive antagonism at the NMDA receptor Ca^{2+} channel pore and these effects appear to be the primary mechanisms of the anaesthetic and analgesic action of ketamine in rodents and humans {139, 140, 141, 168, 171}. Ketamine acting on the phencyclidine site of NMDA receptor and binds to opioid and acetylcholine receptors also, but the affinity of ketamine for these receptors is lower than that for the NMDA receptors (43, 82). Ketamine produces a so-called 'dissociative' anaesthetic state, which has been described as a functional and electrophysiological inhibition of thalamocortical pathways and stimulation of the limbic system (49, 64, 98).

It was synthesized as Ketalar in 1962, first used on American soldiers during the Vietnam War (70). The racemic mixture was approved for general clinical use in 1970, but its clinical usefulness has been limited because of its cardiovascular-stimulating properties and high incidence of disturbing emergence reactions (98). The molecular structure contains a chiral center at C-2 of the cyclohexanone ring so that there are two optical isomers (enantiomers) of

the ketamine molecule: S(+)-ketamine and R(-)-ketamine (Fig. 5). The S(+) ketamine has a three- to four-fold greater affinity compared the R(-) form on the NMDA receptor (49,70,97,98). Commercially available racemic ketamine preparations contain equal concentrations of the two enantiomers (42,43,49,70,98).

Ketamine is metabolized extensively by hepatic drug-metabolizing enzyme systems. The major pathway of biotransformation involves N-demethylation of ketamine to form norketamine that can then be hydroxylated to form hydroxyl-norketamine compounds, which in turn can be conjugated to more water-soluble glucuronide derivatives (42,49,98). The primary metabolite norketamine is only one-third to one-fifth as potent as the original compound, but it may be involved in the prolonged actions of ketamine (49,70,98). The metabolites of norketamine are excreted by the kidneys (49).

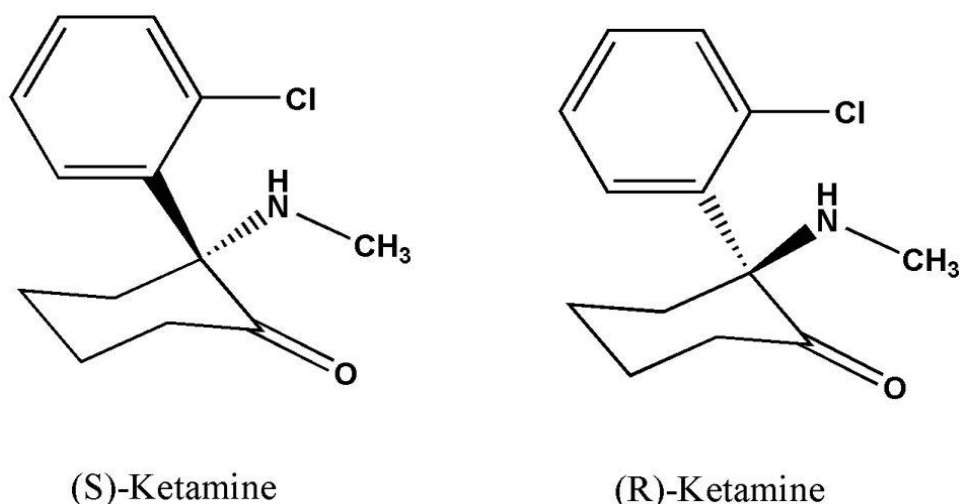


Fig. 5 Stereoisomers of ketamine

Ketamine has high lipid solubility and crosses the blood-brain barrier easily. It acts in the CNS but it also has local anaesthetic properties (34,70,98). Smidt et al. in 1985 were the first who examined the effect of the systemic treatment of ketamine in acute pain test in rodents (90). They found that the systemic ketamine (160 mg/kg intraperitoneal) significantly increased tail flick (TF) latency and this effect was prevented by naloxone (3 mg/kg intraperitoneal). Intraperitoneal ketamine treatment attenuates established neuropathic nociceptive behaviours including mechanical allodynia and hyperalgesia, cold allodynia, spontaneous pain and cold stress-induced pain in rats (79). Ryder et al. showed that S(+)

enantiomer is three times more potent than R(-) enantiomer in phenylquinone writhing test in mouse (88) . The intrathecal administration of racemic ketamine produces only weak or no analgesic effects against acute pain, such as that in the TF test (57) , although it reduces the hyperalgesic effect of a hind paw carrageenan injection (83) and of nerve injury (106) . There have been only a few published reports on the analgesic properties of the individual optical isomers of ketamine after systemic administration (59,82) , but no reports were available on their analgesic properties after intrathecal administration.

2. AIMS

Experiment I.

The spinal cord is an important neuronal structure for pain transmission, and it is one of the pharmacologic sites of action for the antinociceptive effects of different drugs. Subarachnoid administration of local anaesthetics is widely used for providing surgical anesthesia, postoperative analgesia and treatment of chronic pain. A single injection of local anaesthetics into the epidural or intrathecal space has a limited duration of action. Catheterization techniques have been developed to prolong anaesthetic effect. In our previous studies we observed side differences after intrathecally administered drugs in pain tests of rats and only few authors have noted that the position of the catheter tip in the transverse plane is one of the important factors governing the successful subarachnoid spread of a solution (1, 33, 99). The particular aims of the work were:

1. To observe the side differences in the motor and sensory disturbances after intrathecal administration of different doses of the local anaesthetic lidocaine in rats.
2. To determine the distribution of intrathecal catheter tips at longitudinal and transversal plane.
3. To investigate the correlation between the location of the catheter tip, the motor and sensory disturbances after intrathecal administration of a small dose of lidocaine to a large number of animals. Such a correlation might be very important, especially if small doses of administered drugs and their effects are investigated on both sides.

Experiment II.

More than 50 % currently used drugs are chiral compounds and about 90 % of them have been marketed and clinically administered as racemates, which contain an equal mixture of two enantiomers. Enantiomers are well known to show the differences in pharmacodynamics, which originate from the stereostructure-specific actions. One enantiomer may be preferred over its enantiomeric counterpart or a racemic mixture because it may increase beneficial activity and may decrease adverse toxicity, indicating the clinical advantage of using a single enantiomer. Such enantiomeric drugs include general and local anaesthetics, sedatives,

hypnotics, anti-inflammatory drugs, analgesics and β -adrenergic antagonists. The particular aims of our work were:

4. To investigate the antinociceptive effect of intrathecal administration racemic ketamine on carrageenan-induced thermal hyperalgesia in paw withdrawal test (PWD).

5. To investigate the antinociceptive effect of intrathecal administration S(+)- and R(-) ketamine enantiomer on carrageenan-induced thermal hyperalgesia.

6. To investigate the antinociceptive effect of intrathecal administration S(+)- and R(-) ketamine enantiomer on acute heat pain sensitivity in TF test.

7. To investigate the antinociceptive effect of intrathecal administration S(+)- and R(-) ketamine enantiomer on acute heat pain sensitivity in hot-plate (HP) test.

3. MATERIALS AND METHODS

3.1. Animals

After institutional approval had been obtained from the Animal Care Committee of the University of Szeged, Faculty of Medicine, male Wistar rats weighing (160–330 g) were studied (n = 448).

All experiments were performed in freely moving animals during the same period of the day (8:00 AM to 1:00 PM) to exclude diurnal variations in pharmacological effects. The animals were randomly assigned to treatment groups, and the observer was blinded to the treatment administered. In order to reduce the stress of animals, they were habituated to the hand before experiments.

3.2. Intrathecal catheterization

For spinal drug administration, the rats were surgically prepared under ketamine and xylazine anaesthesia (72 and 8 mg/kg intraperitoneally, respectively). Polyethylene tubing was sterilized with absolute alcohol than was rinsed with sterile distilled water before use. An intrathecal catheter (PE-10: I.D. 0.28 mm; O.D. 0.61 mm) was inserted through a small opening in the cisterna magna and passed 8.5 cm caudally into the intrathecal space, as described previously (104). After surgery, the rats housed individually, had free access to food and water and were allowed to recover for at least 3 days before use. Rats exhibiting postoperative neurologic deficits (approximately 10%) were excluded.

3.3. Drugs

The drugs used were ketamine hydrochloride (Ketalar; Pfizer Med-Inform, Vienna, Austria), xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany), phenobarbital sodium (Hunгарopharma Rt., Budapest, Hungary), lidocaine 2% and 10% (Egis, Budapest, Hungary) carrageenan- λ (Sigma-Aldrich Kft, Budapest, Hungary), racemic ketamine [SR(+)-ketamine], S(+)- ketamine, and R(-)-ketamine (all ketamines were Parke-Davis; a generous gift from Gödecke Ltd., Vienna, Austria).

3.4. Intrathecal administration

Intrathecal applied drugs were dissolved in sterile, physiological saline freshly prepared on the day of the experiment and given in single (experiment I.) or cumulative (experiment II.) doses. Drugs were injected intrathecally over 30 s in a volume of 5 μ l, followed by a 10 μ l flush of physiological saline.

3.5. Carrageenan-induced inflammation

Unilateral inflammation was induced by intraplantar injection of 3 mg of carrageenan in 0.1 ml of physiological saline into the right hind paw. The carrageenan solution was prepared freshly on the day of the experiment.

3.6. Pain tests

The nociceptive sensitivity was assessed by using tail flick, hot plate and paw withdrawal techniques with moderate stimulus intensities.

3.6.1. Tail flick test

Janssen et al. have described the TF technique, as a tail withdrawal response (41). The rats were covered with a towel on the measuring surface and were held at rest. The time was started by foot switch when lower 5 cm portion of their tail were immersed in hot water (51.5°C) and was stopped when the animals reacted by flicking their tails (cut off time: 20 s). The reaction time in the TF test is the elapsed time between immersion of tail and tail withdrawal response.

3.6.2. Hot-plate test

A clear Plexiglas chamber was situated on the surface of hot plate. All animals were placed individually into the chamber. The latency of licking one of the hind paws or jumping was measured. The surface temperature of the HP was maintained 52.5 °C; this temperature was verified by periodically monitoring the surface of the HP with a contact thermometer. Animals that failed to react to the thermal stimulus within 60 seconds were removed from the HP surface and assigned 60 seconds response latency. No tissue damage was apparent in subjects that failed to react prior to the 60 second cut off time.

3.6.3. Paw withdrawal test

Nociceptive sensitivity of the hind paws was determined by studying the unilateral PWD latency in response to radiant heat stimulation (29). All rats were placed individually on a glass shelf in rectangular chambers made of clear Plexiglas and were allowed to acclimatize to their surroundings for 20 min. A movable radiant heat source powered by a constant current supply was focused on the glass from below. A single switch activated both the radiant heat source and an electronic timer capable of measuring 0.1-s intervals. After the animal had settled in place on the glass shelf and it was ensured that the plantar skin was in contact with the glass, the heat source was positioned under the plantar surface of the desired hind paw. The heat source was turned on when the hind limb was withdrawn from the glass shelf. The cut off time was set at 20 s to avoid any thermal injury.

3.7. Assessment of motor impairment

The motor impairment was determined in two ways:

3.7.1 Determination of motor paralysis

The motor impairment was determined after lidocaine administration. Clubbing of the hind paw and the inability to stand on one of the hind limbs were indicative of motor block (n = 376).

3.7.2. Grip-strength test

The second method was to determine the grip strength. We used an apparatus designed for the measurement of unilateral hind limb grip strength (28). The device consisted of a horizontally mounted, 6-mm mesh stainless steel (0.6-mm diameter) screen attached to an electronic force-gauge dynamometer. A rat was positioned on a Plexiglas bench adjacent to the screen so that the two forelimbs and the one of the hind limbs rested on the bench and the other hind limb rested on the screen. For each test, the rat's tail was pulled backward in a smooth but firm manner until the rat's grip on the screen was broken. The mean of the results of three consecutive attempts (the duration of the test was approximately 20 s) was used for statistical analysis. Grip-strength testing was performed before and within 5 min after lidocaine administration (n = 23).

3.8. Localization of the catheter tip

After experimental use rats ($n = 376$) were killed with an overdose of phenobarbital sodium, and laminectomies were performed between vertebrae C7 and L5 without removing the spinal cord from the vertebrae. Before laminectomies 5 μ l of methylene blue was injected into the cannula, and the level and side positions of the catheter tip were determined. This method also helped us to visualize whether the catheter tips were placed epidural, subdural, or inside the spinal cord. Catheter tip positions in the longitudinal plane were identified by the level of the vertebral body. In the transverse plane, the subarachnoid space was subdivided into four parts — left, right, dorsal, and ventral — as shown in Figure 6. It has been postulated that, if the tip of the catheter is closer to one side, the effect of the drug should be more effective on that side. Therefore, in contrast with Asato et al. (5), we used the dorsal or ventral categories only when the tip of the catheter was very close to the midline.

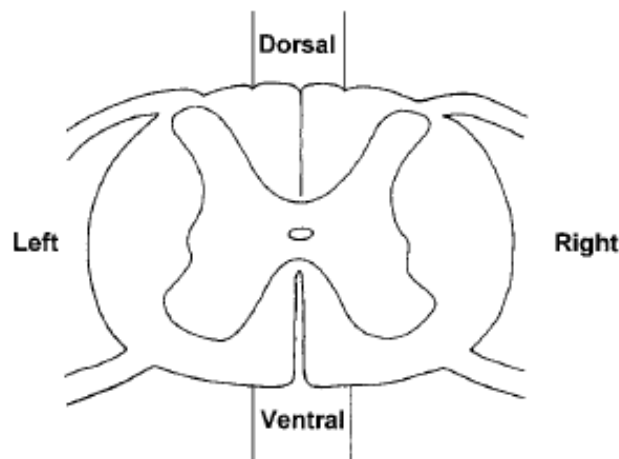


Fig. 6 Schematic diagram of the categorization of the transversal plane of the rat subarachnoid space.

3.9. Experimental protocols

The following steps were in all experiments:

1. Insertion of the intrathecal catheter,
2. One day after surgery: visual control of the motor impairment,
3. 3-5 days after surgery: intrathecal drug administration, and pain tests implementation.

Experiment I.

In the first group of animals, we determined the time response curve of 100 µg of lidocaine (n = 45). PWD testing was performed before and 1, 5, 10, and 20 min after lidocaine administration. In the second group of animals, we examined the possibility of correlation between the side position of the catheter tip and the side differences in the paralysis and PWD latencies after 100 µg (n=264) or 500 µg (n=112) of lidocaine.

The first step was the determination of the motor block, and then we measured the PWD latencies in the thermal testing apparatus within 5 min. The person conducting the behavioural test was blinded to the administered dose.

In the third group of animals (n=23), the effect of 100 µg lidocaine on the hind paw grip strength was investigated. We also studied the possibility of a correlation between the side differences in the grip strength and the side position of the cannula tip.

After all experiments, the side position of the catheter tip was determinate.

Experiment II

In the first group of animals, we examined the antinociceptive effect of cumulative doses of ketamine isomers on the PWD test. First we obtained baseline PWD latencies (precarrageenan values at - 180 min), and the next step was the intraplantar carrageenan injection into the right hind paw.

Three hours after carrageenan injection, paw withdrawal latencies were obtained again (postcarrageenan baseline values at 0 min), then 10 and 30 min after the drug injections (10, 50, and 100 µg ketamine isomers). Values from 10 and 30 min after each drug injection were averaged to obtain one value for each drug dose.

In the second group of animals, we examined the antinociceptive effect of cumulative dose of ketamine isomers on the HP and TF tests. The baseline latencies were obtained immediately before ketamine injection, then the measurement was repeated 10 and 30 min after the drug injections (10, 100, and 500 µg ketamine isomers) and 60 min after last administration of ketamine isomers (500 µg).

3.10. Data analysis

Data are given as means ± SEM. Data sets were examined with analysis of variance and correlation analysis by using STATISTICA software (StatSoft Inc., Tulsa, OK).

The statistical significance of different enantiomers (treatment) and doses was assessed by two-way analysis of variance (ANOVA). One-way ANOVA was used to compare treatment effects, with the Newman-Keuls test for post hoc comparison for differences between means. A level of $P < 0.05$ was considered significant.

4. RESULTS

Experiment I

The baseline PWD was 10.0 ± 0.20 s for the right hind paw and 9.8 ± 0.21 s for the left hind paw; the difference between the two sides was not significant. Regarding the time course of the effect of $100 \mu\text{g}$ of lidocaine, the PWD latency was the longest between 1 and 5 min, and the effect was short (Fig. 7). In further experiments, therefore, the anaesthetic and motor effects of lidocaine were determined within 5 min after the intrathecal injection.

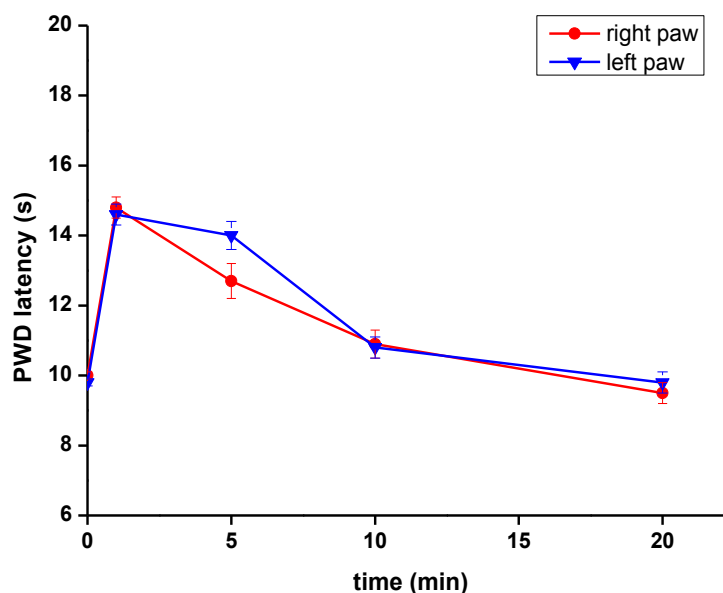


Fig. 7 Time-dependent effect of $100 \mu\text{g}$ lidocaine on the PWD test.

Both sensory and motor impairments depended on the dose of lidocaine applied (Fig. 8). Regarding the pain threshold changes, the distribution of the PWD latencies show that, in contrast with $100 \mu\text{g}$ of lidocaine, $500 \mu\text{g}$ of lidocaine caused high (18–20 s) PWD latencies in a larger percentage (Fig. 8 A–D). $100 \mu\text{g}$ lidocaine caused maximal effect on both sides in 3 (1.1 %) of 264 animals, whereas the larger dose of lidocaine did so in 45 (40.2 %) of 112 animals. Similar to the PWD results, whereas $100 \mu\text{g}$ of lidocaine caused paralysis on both sides in 4.4 % of the animals, $500 \mu\text{g}$ of lidocaine did so in 88.4 % of the animals (Fig. 8 E and F). It was also observed that the numbers of left and right side paralysees were similar.

Accordingly, to examine the correlation between the location of the catheter tip and the motor paralyses, we used 100 μg of lidocaine in further experiments.

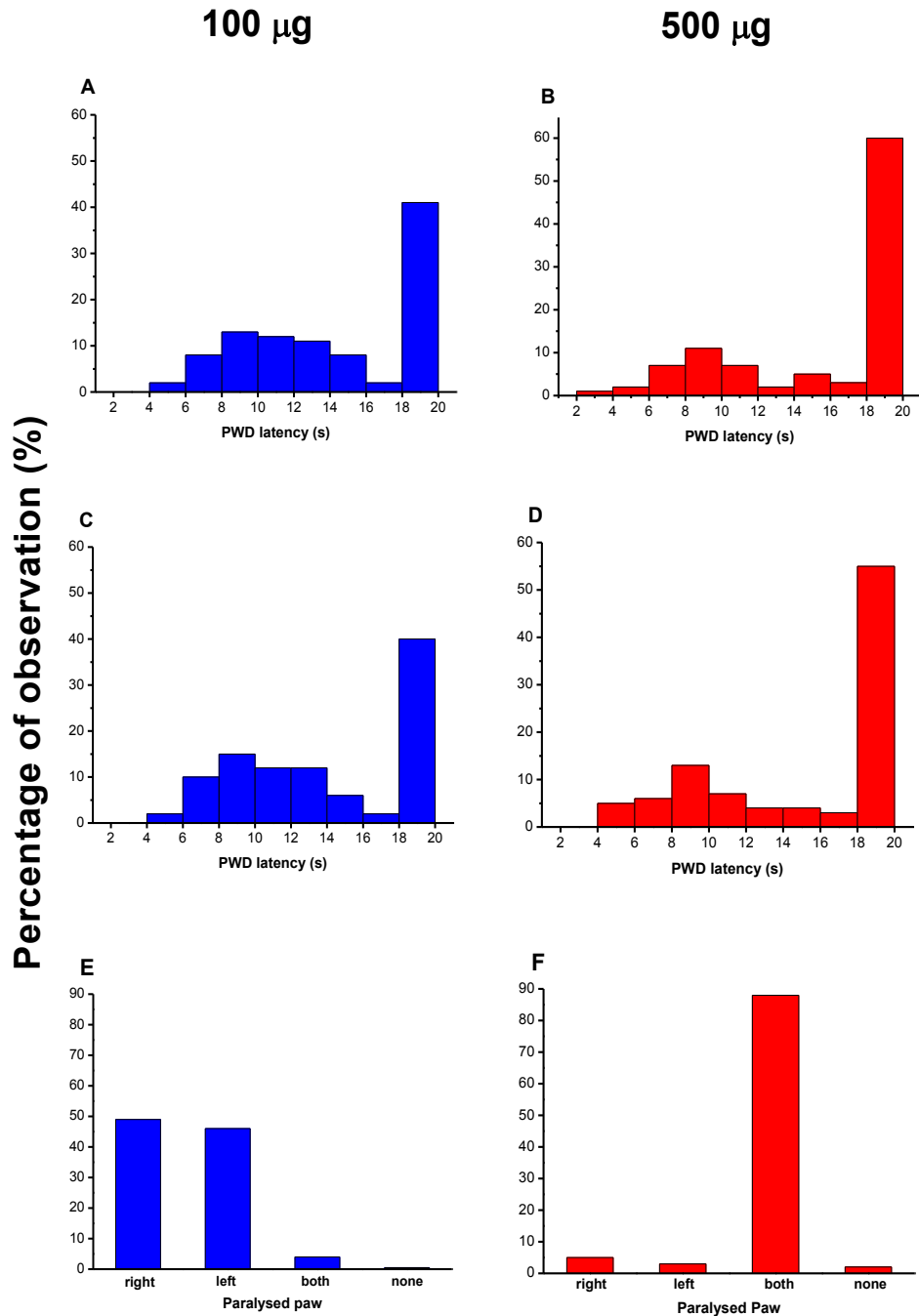


Fig. 8 Histograms showing the distribution of paw withdrawal (PWD) latencies (A and B right paw; C and D left paw) and the distribution of motor paralysis after 100 μg ($n = 264$) (E) and 500 μg of lidocaine ($n = 112$) (F).

As regards the 8.5-cm length of the catheter, it served to place the tip between vertebrae T12 and L2. The level of the tip correlated significantly with body weight ($r = 0.4$, $P < 0.05$) (Fig. 9).

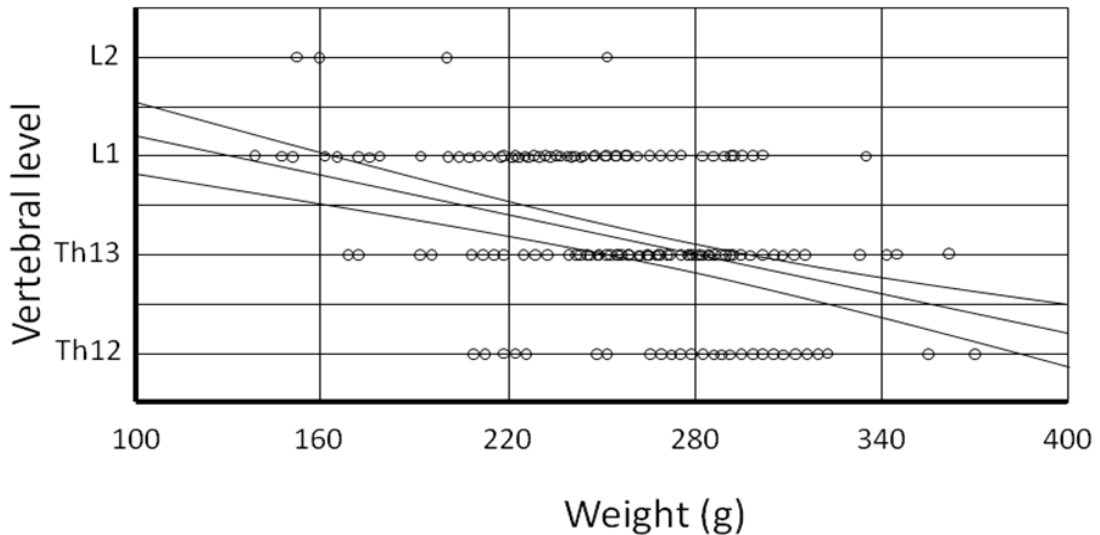


Fig. 9 Correlation between weight and the level of the catheter tip in the longitudinal plane ($n = 376$).

The catheter tips were found subdurally in most cases, but in 7 animals, without any motor impairment, the catheter penetrated the cord and the catheter tip was located inside the spinal cord. The catheter tip was located on the left side in 39.6 %, on the right side in 39.4 %, dorsally in 14.4 %, and ventrally in 4.84% of the 376 rats. Therefore, in 79 % of the cases, the catheter tip was situated on one side (left or right) (Fig. 10).

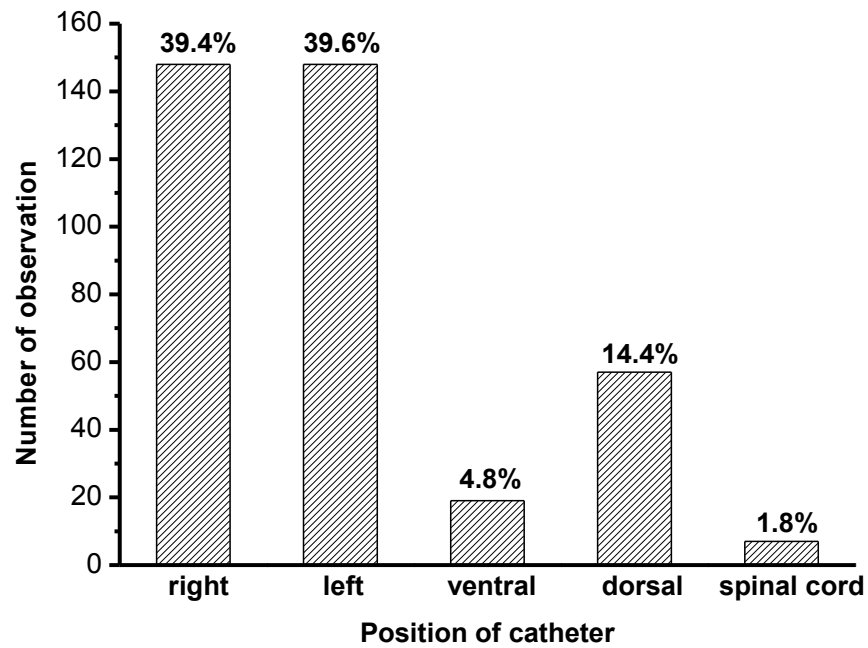


Fig. 10 Distribution of the position of the catheter tip in the transversal plane (n= 376).

It has been observed that the side on which the catheter tip was located correlated significantly with the side on which motor paralysis was observed (n = 264, $r = 0.77$) (Fig. 11 A) and also with the PWD latencies (n = 264; $r = 0.60$ and $r = 0.67$ for the right and the left side, respectively) (Fig. 11 B and C). There was also a close correlation between the motor paralysis and the PWD latencies (n = 193; $r = 0.75$ and $r = 0.82$ for the right and the left hind paw, respectively).

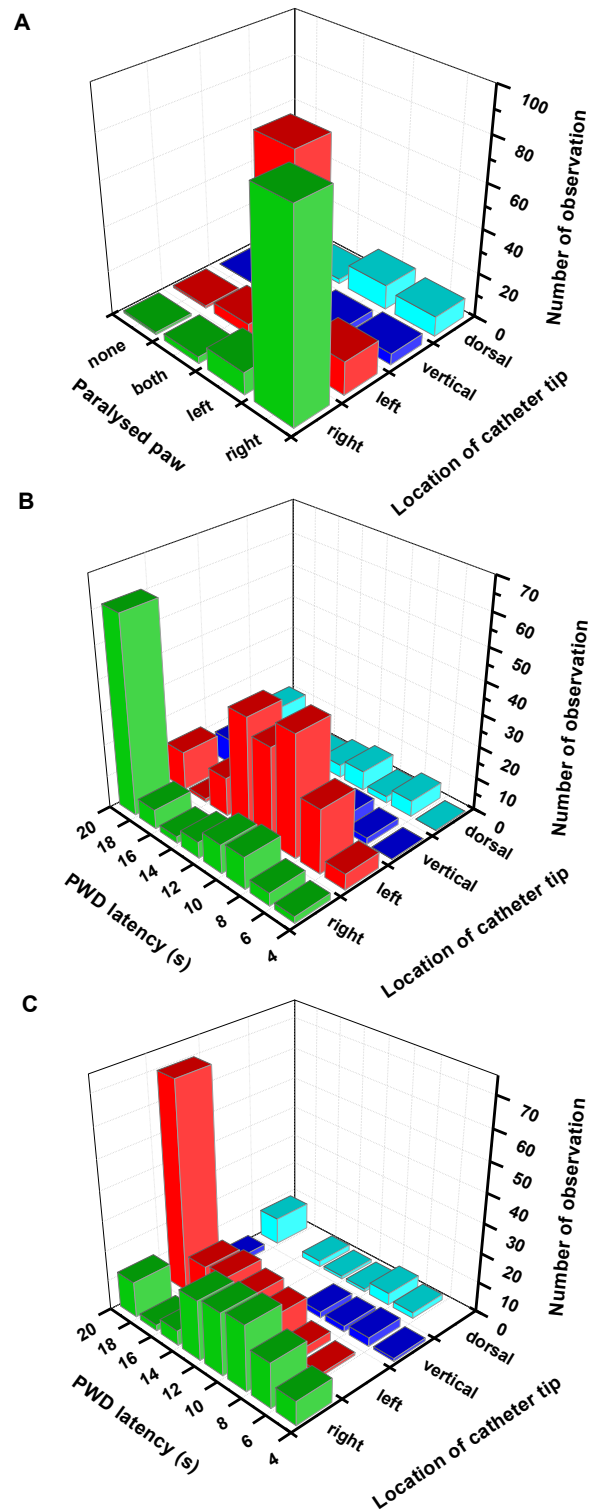


Fig. 11 Three-dimensional bivariate distribution histograms showing the correlation between the side position of the catheter tip and the side of the motor paralysis (A) and the correlation between the side position of the catheter tip and the paw withdrawal (PWD) latency at the right side (B) and left side (C) ($n = 376$).

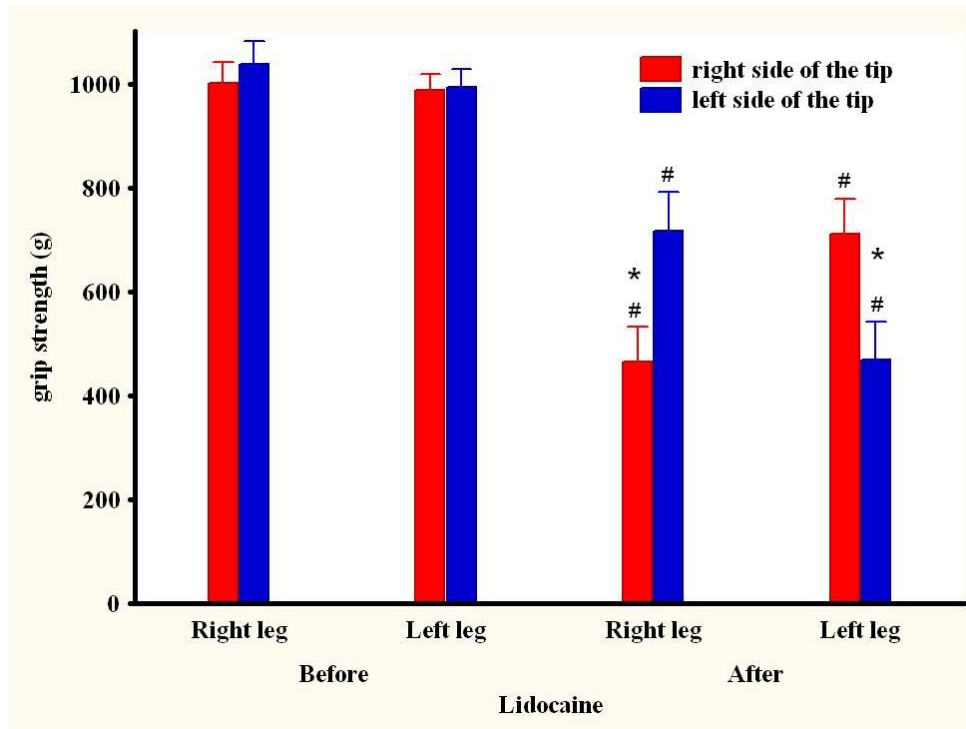


Fig. 12 Grip strength of right and left hind limb before and after 100 µg lidocaine administration.* Significantly different from baseline grip strength. # Significant differences between right and left hind limb depending on the side position of the catheter tip after lidocaine administration (n = 23) (P<0.005).

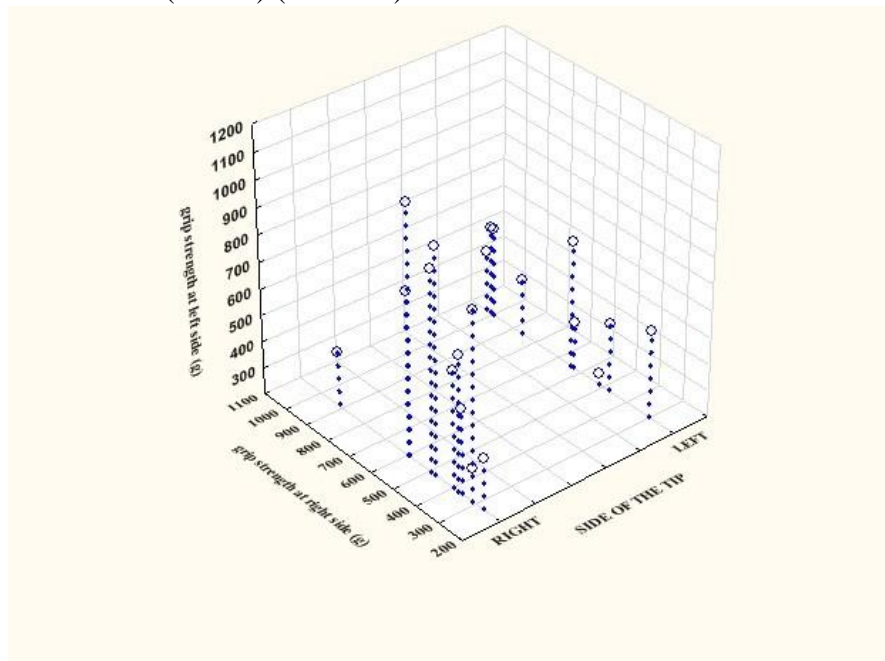


Fig. 13 Three-dimensional bivariate distribution histograms showing the correlation between the side position of the cannula tip and the grip strength at the right side and the left side after 100 µg lidocaine administration.

The baseline grip strength was 1006 ± 26.5 g for the right and 968 ± 23.6 g for the left hind limb, with no significant differences between the two sides (Fig. 12).

A significant correlation was observed between the side position of the cannula tip and the grip strength after 100 μ g lidocaine administration ($r = 0.50$ and $r = 0.51$ for the left and the right hind limb, respectively; $P < 0.05$) (Fig 13).

Experiment II

There were no significant differences in paw withdrawal responses to noxious thermal stimuli between the hind paws before the intraplantar injection of carrageenan. The overall mean paw withdrawal time for the right and left paws were 9.3 ± 0.26 and 9.9 ± 0.25 s ($n = 72$), respectively. The carrageenan injection induced an inflammation of the injected hind paw, as evidenced by oedema and erythema. The paw withdrawal latencies of the carrageenan-injected paw were significantly reduced to 3.6 ± 0.13 s ($P < 0.001$, $n = 72$). Thus, thermal hyperalgesia was consistently produced in rats with carrageenan-induced inflammation.

The effects of ketamine enantiomers injected into the subarachnoid space were evaluated during the peak hyperalgesic response 3-6 h after carrageenan. Individual ketamine enantiomers, racemic ketamine, and saline had no influence on paw withdrawal latencies of the noninflamed paws (Fig. 14-17).

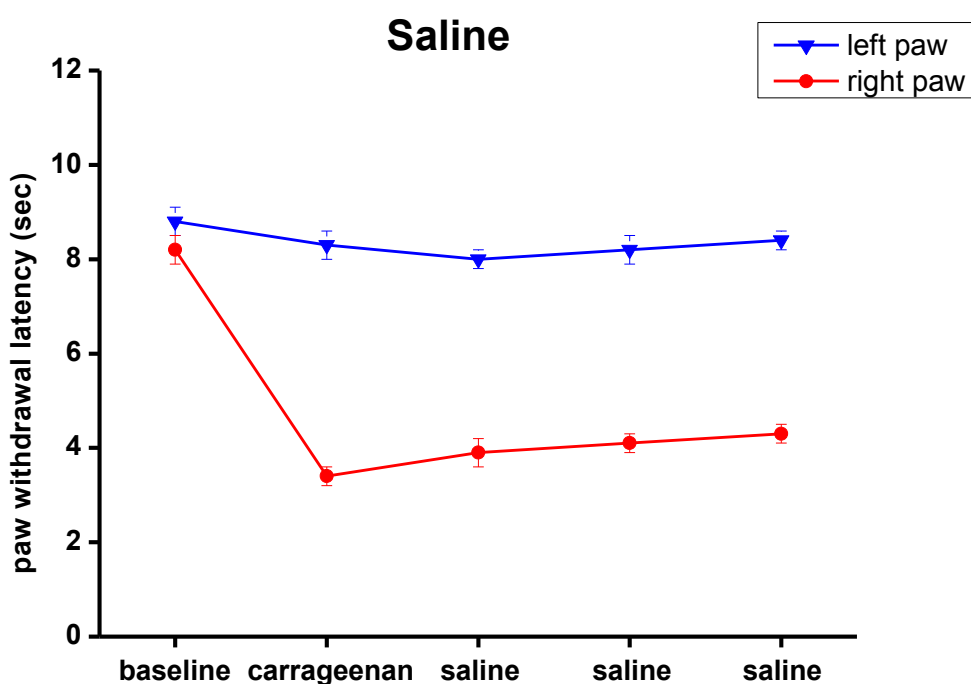


Fig. 14 Effect of saline on paw withdrawal latencies of rats receiving unilateral injection of carrageenan into the right hind paw. Withdrawal latencies from the thermal stimuli of both injected and noninjected paws are plotted against repeated saline administration ($n = 18$).

In the injected paws, two-way ANOVA on raw data revealed a significant effect for treatment and dose [F-values [df]: 4.19 [3], 38.9 [3], respectively]. ANOVA for repeated measures showed significant treatment effects for S(+)-, R(-)-, and racemic ketamine within groups [F-values [df]: 12.54 [3], 10.69 [3], 17.67 [3], respectively]. Racemic ketamine had significant effects after the second and third dose (Fig. 15), and S(+)-ketamine had significant effects only at the largest dose compared with saline (Fig. 16). Although indicating a clear trend toward a dose-dependent reversal of thermal hyperalgesia, R(-)- ketamine did not achieve statistical significance compared with saline (Fig. 17).

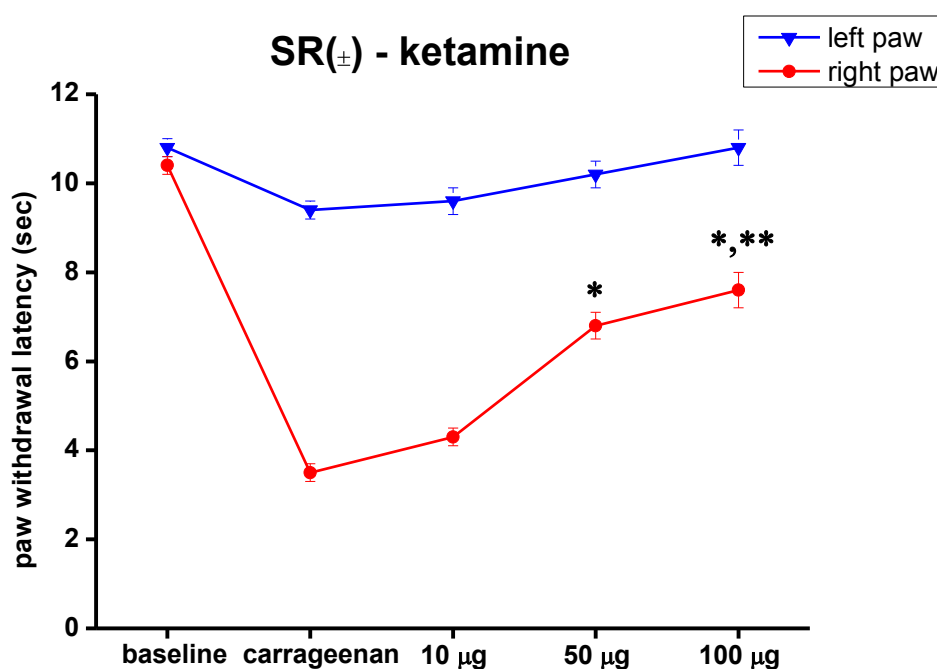


Fig. 15 Effect of racemic ketamine on paw withdrawal latencies of rats receiving unilateral injection of the carrageenan into the right hind paw. Withdrawal latencies from the thermal stimuli of both injected and noninjected paws are plotted against different drug doses. *Significantly different from saline treatment. **Significantly different from R(-) ketamine treatment ($P < 0.05$) ($n = 18$).

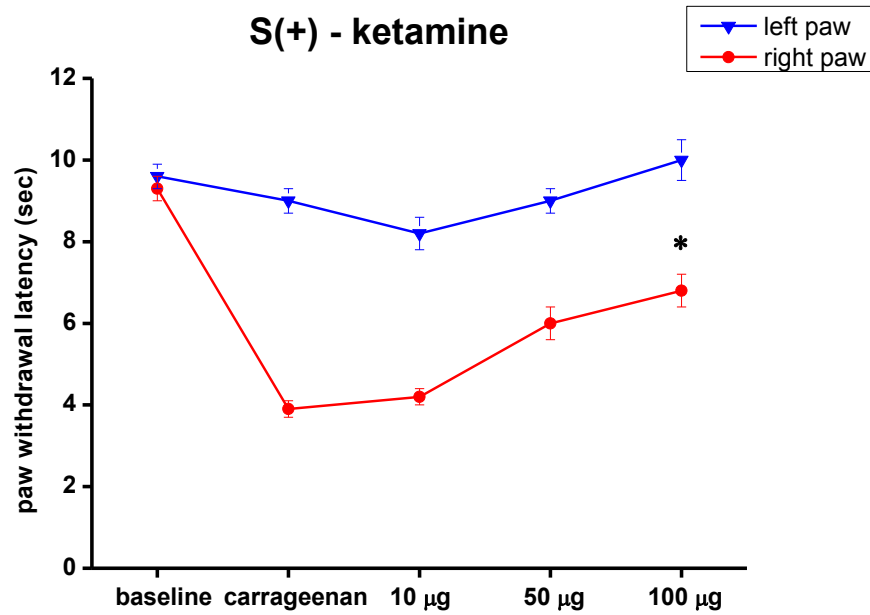


Fig. 16 Effect of S(+)-ketamine isomer on paw withdrawal latencies of rats receiving unilateral injection of the carrageenan into the right hind paw. Withdrawal latencies from the thermal stimuli of both injected and noninjected paws are plotted against different drug doses. *Significantly different from saline treatment ($P < 0.05$). (n = 18)

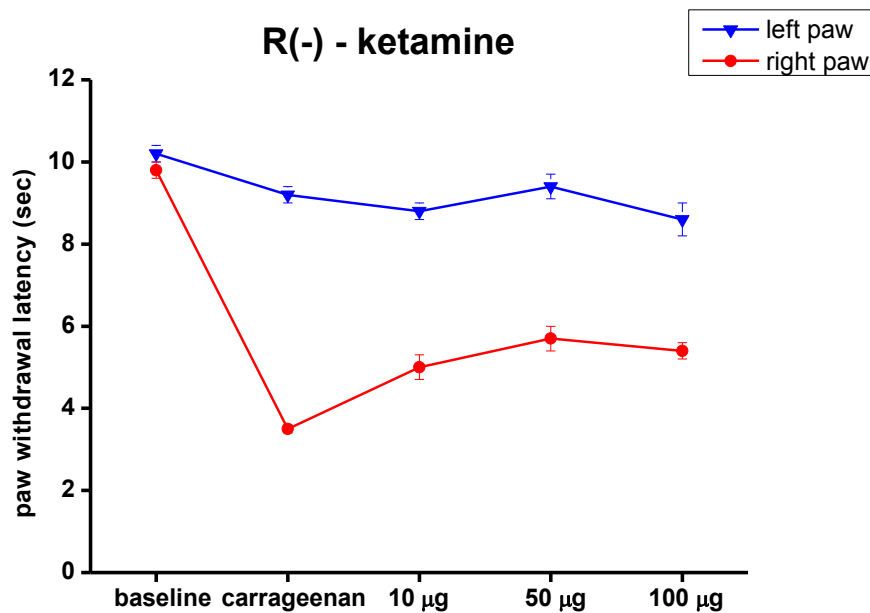


Fig. 17 Effect of R(-)-ketamine isomer on paw withdrawal latencies of rats receiving unilateral injection of the carrageenan into the right hind paw. Withdrawal latencies from the thermal stimuli of both injected and noninjected paws are plotted against different drug doses (n = 18).

In the hot plate test, the largest dose of both isomers caused a significant increase in licking latency at 10 min (Fig. 17), although this dose induced supraspinal effects, as well; for example, the animals demonstrated circling movements and head weaving.

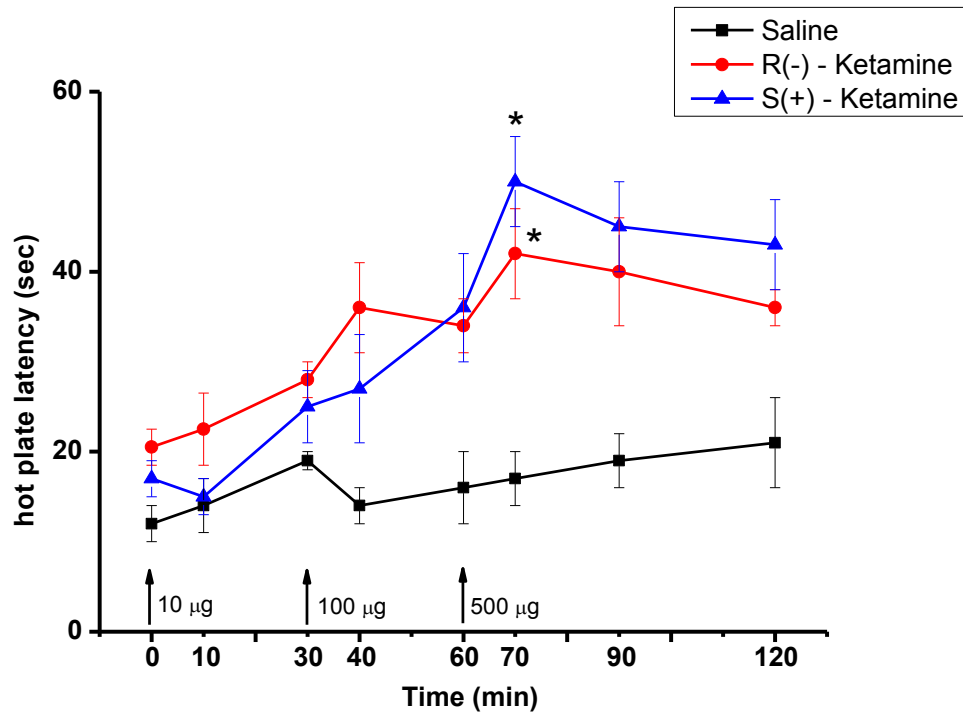


Fig. 18 Effects of ketamine isomers and saline on hot plate latencies. Arrow = drug administration. *Significant different from saline treatment ($P < 0.05$) ($n = 18$).

Neither S(+)- nor R(-)-ketamine significantly influenced the tail flick latency in the applied doses (Fig. 19)

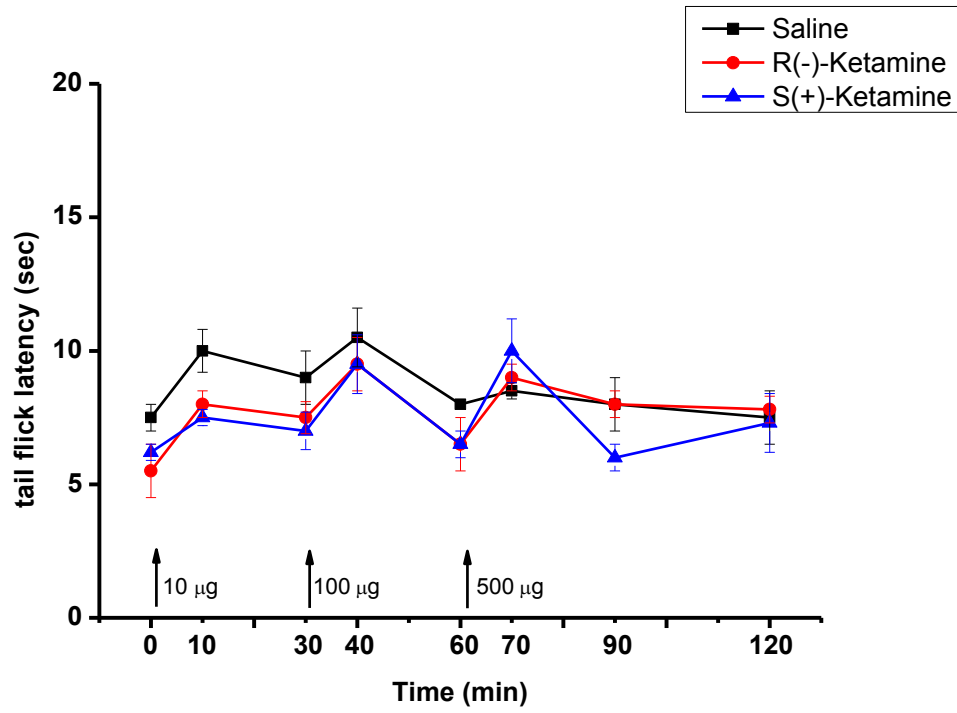


Fig. 19 Effects of ketamine isomers and saline in tail flick latencies. Arrow = drug administration (n = 18).

5. DISCUSSION

Experiment I

Our results demonstrated that the intrathecal catheter tips were always at appropriate level, corresponding to the spinal segments that innervate the hind paw. The side position of the catheter tips, however, exhibited large variability, and they influenced significantly the anaesthetic effect of the small dose of lidocaine. The anaesthetic potency of lidocaine correlated with the location of the catheter tip.

Yaksh and Rudy introduced Intrathecal catheterization in small animals (104). Concerning the site of action of a drug administered into the spinal subarachnoid space, they suggest that there was clearly an alternative: the effect could be exerted either on the nerve roots or on cell bodies and synapses that lie within the grey matter of the cord. In the latter case, there is a possibility that the ability of the drug to produce an effect is contingent upon its ability to reach the grey matter. Physicochemical properties of the drug injection, such as molecular weight and lipophilicity, should therefore be considered in assessing the efficacy of action of any drug, because these properties are likely to influence the penetration into the spinal cord, the circulation, or both. Yaksh and Rudy performed calibration experiments with bromophenol blue dye, [3H]naloxone HCl and ¹⁴C-urea in a few animals (104). These data revealed that there was little rostrocaudal diffusion of the injection along the spinal axis and that the spreading depended on the volume applied. The spread of the dye in a volume of 5 µl was limited to approximately 0.5–1.5 cm from the catheter tip. Furthermore, even for compounds such as naloxone, which can permeate neural tissues rapidly, the levels that appear in the brain after spinal subarachnoid administration of the drug were small. In contrast, our earlier results demonstrated that the supraspinal, mydriatic effect of the intrathecally administered lipophilic α_2 -adrenoceptor agonist, dexmedetomidine, appeared at a smaller dose than that required for its antinociceptive effect (37). Thus, the properties of the drug and the longitudinal position of the catheter tip clearly play major roles in the effect of the compound, whereas few authors have noted that the position of the catheter tip in the transverse plane is also important as concerns successful subarachnoid spread. The volume of the drug and the route of delivery (single injection or microinfusion) also could influence the spread and the effect of the applied drugs (99, 104).

Wiesenfeld-Hallin reported that the location of the catheter tip affected the antinociceptive effect of intrathecal morphine in rats (99). It is very important that she applied a continuous intrathecal infusion in a small dose and volume (24 µg/h and 0.5 µl/h, respectively). In that work, rats with the catheter tip situated on the dorsal lumbar enlargement of the spinal cord displayed a clear antinociceptive effect of morphine in the hot-plate test and in the reduction of autotomy, whereas rats without this placement did not. Furthermore, in 18 of 26 animals, the catheter tip was located away from the dorsum of the spinal cord, which also suggested a large variability in the position. However, the exact locations of the catheter tips were not mentioned. Using autoradiography, Cridland et al. reported that the intrathecal spread of labelled substance P was dependent on the catheter tip position (10, 17). When unilateral anaesthesia was observed after the injection of local anaesthetics, lateral subarachnoid placement of the catheter was subsequently confirmed. The authors suspected that the unilateral uptake of the label simply reflected the position of the catheter tip. Hersh et al. determined the location of the catheter tip within the subarachnoid space in 29 animals (33). They observed that the intensity of the conduction block achieved after chlorprocaine in a large volume (40 µl) was dependent on the location of the catheter tip. Akerman investigated motor and sensory anaesthesia after 20 µl of 5% lidocaine (1). Although this dose was 10 times larger than what we applied, they also observed unilateral blockade in 2 of 10 operated rats. Similar to our results, a very fast onset of anaesthesia and short-lasting effects were observed in rats and mice. It was also mentioned that the tip of the catheter was not always located dorsally in the subarachnoid space. Asato et al. reported a study of the distribution of intrathecal catheter tip positions in rats (n = 66) (5). Because they used another categorization of the subarachnoid space, our results display several differences from their observations; e.g., in their study the catheter tips were located laterally in 58% of the cases, whereas the corresponding figure in our study was 79%. Furthermore, Asato et al. found that the longitudinal position of the catheter tip was significantly different in the ventral and dorsal catheter tip groups (5). We did not find such a correlation: in contrast, the longitudinal position of the catheter tip correlated significantly with the weight of the animals. Similar to Asato et al., we found animals without a motor impairment despite that their catheter tip was situated in the spinal cord (5).

There are several methods for determining the effect of a drug on both hind paws after unilateral inflammation or nerve injury. In these cases it cannot be excluded that the effects of

small doses of such drugs might be influenced by the location of the catheter tip. Because our results revealed that the location of the catheter tip correlated with the anaesthetic potency of small-dose lidocaine, we suggest that studies of the effects of drugs might well be predicted by preliminary determination of the potency of lidocaine on both sides. If the goal of a study is to investigate the effect of a drug on the injured side, then the injury should be performed on the side where lidocaine is more effective.

Experiment II

The importance of stereochemistry in clinical pharmacology and therapeutics has been widely recognized in the last decade, particularly in the arena of new drug development (10). Most drugs are optically active and are used clinically as the racemate (an equal mixture of optical isomers). Approximately 60 % of anaesthetics are chiral drugs; some of them are administered as individual enantiomers (for an excellent review, see Reference (10)). Although the R- and S-isomers contain the same substituent groups, they occupy different positions in space. Consequently, the R- and S-enantiomers may form different three-dimensional relationships in the asymmetric environment of receptors and there may be significant differences in their pharmacodynamic and pharmacokinetic properties, i.e., individual enantiomers may differ in their receptor effects, dispositions, and toxicity.

Originally, Marietta et al. reported differences in pharmacological potencies of the optical isomers of ketamine in rats after intravenous injection (59). The (+)-enantiomer had a higher therapeutic index than the racemate or the (-)-enantiomer, and at equihypnotic doses, the (+)-isomer caused less locomotor stimulation than the (-)-isomer. Ryder et al. showed that the (+)-isomer was 3 times more potent than the (-)-isomer as an analgesic in the phenylquinone writhing test in mice (88). Stereoselective differences in ketamine affinity for NMDA receptors, and ketamine effects on NMDA receptor ion-current, catecholamine reuptake, and acetylcholinesterase activity have also been observed (48).

However, some effects of ketamine are non-stereospecific. Hirota et al. found that the two ketamine isomers produced equipotent spasmolytic effects on airway smooth muscle precontracted with histamine, but they differed in their ability to potentiate the relaxing effect of adrenaline, with S(+)-ketamine producing the greater potentiation (35). Furthermore, the optical isomers of ketamine had equipotent cardiodepressant effects in the guinea pig (89).

In human studies, White et al. reported differences in their anaesthetic potency, intraoperative effects, postoperative analgesia, and side effects (97). The S(+)-enantiomer has 4 times the potency of the R(-)- enantiomer of ketamine and may have significant clinical advantages in comparison with the racemic drug. Furthermore, S(+)-ketamine possesses superior efficacy with fewer side effects compared with the racemate. There are also differences in the pharmacokinetics of the ketamine enantiomers. Administered individually, the plasma clearance of S(+)-ketamine is greater than that of the R(-)-enantiomer. Kharasch and Labroo have demonstrated a relative enantiomeric selectivity in the human metabolism (47).

A series of clinical studies has suggested potent analgesia after epidural or spinal administration of racemic ketamine (82). Ketamine has been administered epidural to humans for pain relief without the side effects, such as respiratory depression, urinary retention, or pruritus, frequently observed after epidural opioids (81). The neuraxial use of ketamine raised questions about potential toxicity (58), although in clinical studies, neurologic lesions were not reported after the intrathecal administration of ketamine (81). Preservatives (benzethonium chloride or chlorobutanol) used in ketamine solution in some cases were thought to cause neurological damage in animals (9). The isomers of ketamine do not induce spinal cord lesions themselves, but the preservative chlorobutanol led to significant spinal cord damage in rabbits (58). For the possible future clinical use of S(+)-ketamine, it is important that it be produced as a preservative-free solution (See Ketanest-S, Pfizer).

Our results are consistent with the observation that the NMDA receptor antagonists have a particular role in mediating persistent pain and hyperalgesia (15, 16, 20, 83). In behavioural tests, NMDA receptor antagonists attenuate or reverse the thermal hyperalgesia evoked by local inflammation or peripheral neuropathy, without any analgesic effect on the response to noxious stimulation of the contralateral, non-affected limb (83, 106). Furthermore, when tested in rats without nerve injury or tissue damage, NMDA receptor antagonists usually do not produce significant behavioural analgesia until the doses used result in visible motor dysfunction (20, 103, 106). However, in contrast to results from systemic administration in humans superior efficacy was observed in racemic ketamine, rather than in S(+)-ketamine (97). This finding can hardly be interpreted based on our study. The neurochemical mechanism of ketamine-induced depression of spinal nociceptive neurons in the spinal cord is not clear. It interacts with not only NMDA receptors, but also with opioid, monoaminergic, and muscarinic receptors and voltage-sensitive calcium channels (34, 90). In addition, it has

been shown that in the spinal cord, acute NMDA produced thermal hyperalgesia is mediated by activation of the constitutive neuronal form of nitric oxide synthase and the production of nitric oxide (53, 65). Therefore, several neuronal receptor systems may be involved in the depressant action of ketamine on the excitation of WDR neurons in the DH of the spinal cord. The antinociceptive effects of subanaesthetic doses of ketamine are likely to occur at concentrations sufficient to block the NMDA channels. At larger (anaesthetic) doses, other receptors, in particular opioid δ -receptors and μ -receptor sites, may contribute to the pharmacological effects of ketamine (90). Joo et al. investigated the interaction of intrathecal ketamine and its enantiomers with morphine and the α_2 -adrenoceptor agonist, dexmedetomidine, in the TF test (43). They observed synergistic interactions between the higher dose (100 μ g) of racemic ketamine or S(+)-ketamine and morphine (3 μ g) or dexmedetomidine in rats and this potentiation was independent of the anaesthetic drug applied. Coadministration of a low dose of morphine (0.3 μ g) or dexmedetomidine with S(+)-ketamine (100 μ g), which in itself induced no changes in pain sensation or behaviour, caused a significant long-lasting antinociception, without any side-effects.

6. SUMMARY

Experiment I

Many previous studies suggest that the correct location of the catheter tip is crucial concerning the effect obtained. However, the previous studies did not suggest a reliable method for the prediction of the location of the catheter tip before experiments on living animals.

We revealed that:

1. the tips of chronic intrathecal catheters were found to be variously located in the transverse plane in the rat spinal subarachnoid space.
2. the position of the intrathecal catheter tip influences the pharmacological effects of the local anaesthetic lidocaine.
3. the paralytic and/or antinociceptive effect of a small dose of lidocaine before the experiments is a simple and reliable method for prediction of the side position of the catheter tip before rats are used in further studies of spinal systems that mediate nociception and antinociception.
4. the differences between the effects of the drug on the two sides might be very important, especially if small doses of the drug are applied and their effects are investigated on both sides.

Experiment II

This was the first study to examine the antinociceptive efficacy of the optical isomers of ketamine after intrathecal administration.

We demonstrated that:

1. Both racemic and S(+)-ketamine increased significantly paw withdrawal latencies in the inflamed paw.
2. The R(-)-ketamine was ineffective to reducing thermal hyperalgesia.
3. Conversely, acute pain tests did not reveal any differences between ketamine enantiomers; i.e. only the largest dose (500 μ g) caused a non-stereospecific, significant increase in hot plate latency, but this dose caused supraspinal effects as well. Neither S(+)-nor R(-)-ketamine did not show any significant effect in tail flick test in applied doses.

Our result suggest that, the potential advantages of using pure enantiomers rather than a racemate include a less complex and more selective pharmacodynamic profile, a higher therapeutic index, less complex pharmacokinetics, less complex drug interactions, and less complex concentration-response relationships.

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