ANALYSIS OF PHARMACODYNAMICS OF TOLPERISONE-TYPE CENTRALLY ACTING MUSCLE RELAXANTS IN NON-CLINICAL STUDIES AND RESEARCH INTO PRECLINICAL MODELLING OF MIGRAINE

Dissertation for the degree of Doctor of Philosophy (PhD)

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List of Abbreviations

ACSF	artificial cerebrospinal fluid
AFP	afferent fibre potential
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
C_1	cervical segment 1
cf.	*confer* (compare)
cGMP	cyclic guanosine-monophosphate
CGRP	calcitonin gene related peptide
CMR	centrally acting muscle relaxant
CNS	central nervous system
CP	creatine phosphate
CPK	creatine phosphokinase
C-T interval	conditioning – test interval
DBPCT	double-blind placebo controlled clinical trial(s)
DMEM	Dulbecco's Modified Eagle's Medium
DR	dorsal root
DRG	dorsal root ganglion (ganglia)
DRP	dorsal root potential
DRR	dorsal root reflex
DR-VRP	dorsal root stimulation induced ventral root potential
DSR	disynaptic reflex
EGTA	ethylene glycol tetraacetic acid
EMG	electromyographic (or electromyogram)
EPSP	excitatory postsynaptic potential
ES	extracellular solution
GABA	gamma-aminobutyric acid
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hRAMP1	human receptor activity modifying protein 1
i.d.	intraduodenal
IC	independent citations
IC_{50}	fifty percent inhibitory concentration
IF	impact factor
i.p.	intraperitoneal
i.v.	intravenous
ID_{50}	fifty percent inhibitory dose
IPSP	inhibitory postsynaptic potential
IS	intracellular solution
LO	locomotor activity (test)
L_X	lumbar segment X (e.g. L_5 = lumbar segment 5)
MN	motoneurone – direct excitation
MS	monosynaptic excitation
MSR	monosynaptic reflex
NMDA	N-methyl-D-aspartate

nNOS	neuronal nitric oxide synthase
NO	nitrogen monoxide
NTG	nitroglycerin
p.o.	*per os*
PAF	primary afferent excitation
PBS	phosphate-buffered saline
PSR	polysynaptic reflex
RR	rotarod (test)
S_1	sacral segment 1
s.c.	subcutaneous
S.D.	standard deviation
S.E.M.	standard error of mean
sGC	soluble guanylate cyclase
SK channel	Small conductance calcium-activated potassium channel
ST	Straub tail (test)
TI	therapeutic index
TNC	trigeminal nucleus caudalis
TR	tremor (test)
TRG	trigeminal ganglion
TRP	transient receptor potential
TTX	tetrodotoxin
TTX-R	tetrodotoxin resistant
TTX-S	tetrodotoxin sensitive
$V_{1/2}$	holding potential resulting in half-maximal current
V_H	Holding potential
VR	ventral root
VRR	ventral root reflex
vs.	*versus*
WL	weight lifting (test)

1 Preface

This dissertation presents a part of the research which was carried out by the Ph.D. applicant who has been participating in the individual instructional program of the Doctoral School of University of Pécs. Majority of the work covered by this dissertation relates to the research on centrally acting muscle relaxant drugs and was performed at Gedeon Richter Plc. This research, by focussing on spinal reflexes, headed the applicant to other associated research fields of neuropharmacology, such as pharmacology of glutamate receptors, bradykinin receptors, various pain states and migraine. All this research is beyond the volume and scope of the dissertation. However, the research on preclinical modelling of migraine, which covered the results of a good collaboration between the University of Pécs and Gedeon Richter Plc, is also presented here. The two different topics, i.e. centrally acing muscle relaxants and migraine, required organising this dissertation in two main chapters.

2 Chapter 1: Pharmacodynamics of tolperisone-type centrally acting muscle relaxants

2.1 Introduction

2.1.1 General objectives

Centrally acting muscle relaxant (CMR) drugs (antispastics) are used in the clinical practice to relieve abnormally increased muscle tone in patients suffering from certain orthopaedic, rheumatologic or neurological disorders. There are numerous clinically active drugs in use. However, usefulness of these drugs is limited for various reasons.

Mephenesin (Berger and Bradley 1946) was the first of this type of drugs found to have clinical value. However, its duration of action was rather short and it caused hemolysis as a side effect. These properties restricted the clinical use of mephenesin. Following mephenesin, a large number of propane-diol derivatives proved to have central muscle relaxant action. Among them carisoprodol and its main metabolite meprobamate (Berger *et al.* 1959) are drugs used worldwide (mainly as anxiolytics), but their moderate activity and sedative effect hindered their use as antispastics. Among the structural analogues of mephenesin, chlorphenesin carbamate has been the most widely used one, mainly in Japan. Although it has a bit longer duration of action and no hemolytic effect, it has not gained very broad use due to other safety issues.

A number of benzazol derivatives (e.g. **zoxazolamine**, chlorzoxazon) have also proved to have central muscle relaxant properties and had longer duration of action compared to mephenesin. However, some of them have been withdrawn from the clinical use due to a hepatotoxic side effect.

Since diazepam (and several other benzodiazepines, particularly tetrazepam) has a strong central muscle relaxant activity, it was widely used for several years in the clinical practice, particularly in Western Europe and the U.S.A., for relieving serious cases of spasticity, despite its obvious, strong and well known sedative effect. However, due to its side effects, also including addiction, it is rarely used as a central muscle relaxant at present.

Tolperisone (Porszasz *et al.* 1960), which was originally developed by the Chemical Works of Gedeon Richter, was found to be a CMR of clinical value with low incidence of side effects. Therefore, it had become the most widely used CMR in Japan for a long time. These early observations were confirmed also by double-blind placebo controlled clinical trials (DBPCT) indicating effectiveness of tolperisone in the treatment of post-stroke spasticity (Stamenova *et al.* 2005) and painful muscle spasms (Pratzel *et al.* 1996). Moreover, tolperisone was devoid of any sedative side effects at effective doses according to a DBPCT using an objective psychomotoric test battery as well as subjective measures (Dulin *et al.* 1998). However, tolperisone is extensively metabolised limiting its oral bioavailability via first-pass metabolism (Miskolczi *et al.* 1987) and its pharmacokinetic half-life is short, resulting in short duration of action and limited effectiveness. Due to its success and limitations an intense research has begun in Japan (Ito *et al.* 1985; Morikawa *et al.* 1987) to find tolperisone-like compounds having greater potency.

Eperisone (Tanaka *et al.* 1981) is one result of this research. It proved to have a little bit longer duration of action and better oral activity in animal experiments as well as in clinical studies.

Further tolperisone-type CMRs – inaperisone (HSR-770) (Morikawa *et al.* 1987) and lanperisone (NK433) (Sakitama *et al.* 1995) had also been under clinical development. Especially the latter one was aimed at improving oral effectiveness and duration of action. However, finally these drug candidates have not been registered probably due to some developability issues.

Afloqualone, a GABA-A receptor modulator central muscle relaxant developed in Japan (Ochiai and Ishida 1981) had also been reported to have clinical value similar to that of tolperisone (Inoue *et al.* 1981). It has had some clinical use, although it causes photosensitization as a side effect that can cause skin problems such as dermatitis (Ishikawa *et al.* 1994).

Baclofen (Bein 1972), a selective agonist at GABA-B receptors (Bowery *et al.* 1980), has a strong and long-lasting central muscle relaxant effect in experimental studies. It has proven to be very useful in the clinics. However, since GABA-B receptors are present at many sites in the brain, it may cause sometimes variable forms of unexpected side effects by acting on the CNS. In addition, only a proportion of cases of spasticity is relieved by baclofen, in particular those of spinal origin. In rehabilitation of patients suffering from some neurological disorders (e.g. paraparesis) exaggerated

reduction of the muscle tone (which is often resulted in by baclofen) is not desirable, because it may hinder normal voluntary activity. Baclofen has been reported to have no more clinical value than tolperisone (Feher *et al.* 1985) in the course of the rehabilitation of spastic patients.

Tizanidine, (Sayers *et al.* 1980) which is an agonist at α_2 adrenergic receptors, has not only muscle relaxant properties but possesses central analgesic activity, too (Davies and Johnston 1984; Davies *et al.* 1984). Probably due to this feature, it has achieved significant success in the treatment of pain-evoked spasms of orthopaedic and rheumatologic patients. However, higher doses necessary for relieving spasticity result in sedative effects, too.

There are a few other CMR drugs which have been proposed to have clinical utility in alleviating painful muscle spams or spasticity but are not discussed here (e.g cyclobenzaprine, gabapentine and orphenadrine). However, their benefit-risk ratio or clinical utility probably does not exceed that of those mentioned here. Nevertheless, the real value of the various CMR drugs cannot reliably be compared due to scarcity of comparative non-clinical and clinical studies; and majority of the controlled clinical trials provided negative results (Chou *et al.* 2004; Nair and Marsden 2014; Shakespeare *et al.* 2003; Taricco *et al.* 2006). Even if some drugs proved to provide reduction in spasticity, they did not provide functional improvement in daily living. In addition to sedation, muscle weakness and impairment of voluntary motor control are strong limiting factors.

Hence, a great unmet medical need exists for skeletal muscle relaxants with improved efficacy / side effect ratio and greater effectiveness. The assumption of utilising virtues of tolperisone and improving against its weaknesses was the main clue of our research aimed at a new drug

1) producing less side effects particularly on the CNS;
2) have less influence on the normal voluntary motor control;
3) have satisfactory duration of action and oral efficiency.

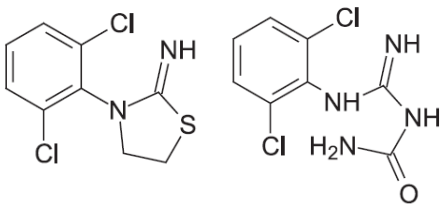
In support of this goal, a methodological toolbox was built for pharmacodynamic characterisation of CMR drugs and to assess (select and detect) improved (or worsened) profile of compounds. Studies aimed at understanding the mechanism of action of tolperisone-type drugs were also serving this ultimate goal.

With the aid of the established toolbox we selected a tolperisone-type drug candidate (RGH-5002; silperisone) that showed a favourable profile compared to both tolperisone and other CMR drugs. The selection process is beyond the scope of this dissertation. However, the profile of silperisone and its comparison to tolperisone-type drugs and other CMRs is the main topic.

2.1.2 Neuropharmacological studies on efficacy and side effects in mice

2.1.2.1 Studies of the therapeutic index

In order to identify possible therapeutic agents with advantageous side effect profile, these side effects should be compared to the genuine antispastic activity of the compounds. For this purpose, it is crucial to select appropriate models suitable for assessing pathologic increase of the muscle tone, or the underlying hyperexcitability of the motor system. Novack suggested the use of the "morphine-induced Straub tail" (Ellis and Carpenter 1974) and the "rotarod" methods for measuring CMR activity and motor side effect, respectively (Novack 1982). A high ratio between effective doses in the second *vs.* first method was proposed as an indicator of potential usefulness of CMR drugs. Nevertheless, more reliable conclusions can be drawn by using more than one method for characterising both types of effect. We observed that the tremor induced by a tremorogenic compound, 3-(2,6-dichlorophenyl)-2-iminothiazolidine (GYKI 20039) (Sineger *et al.* 1982), was dose-dependently reduced by several CMR drugs at non-toxic doses. Studies with GYKI 20039 and a chemically similar compound (LON-954; Fig. 1) suggested that they probably act on the descending extrapyramidal systems somewhere rostral to the colliculi but caudal to the cortex; and the tremor is accompanied by an increased muscle tone and signs resembling spasticity. For more details, see our paper and included references (Farkas *et al.* 2005). Therefore, in addition to the morphine-induced Straub tail test we applied the GYKI 20039-induced tremor test for the pharmacodynamic measurement of CMR activity of drugs and test substances.



GYKI 20039 **LON-954**

Fig. 1. Chemical structures of GYKI 20039 and LON-954.

For assessment of side effects four tests were used. Although each of these tests measures net results of changes in complex CNS functions, by simplification, these tests were aimed for detection of impairment in different main functions. The four tests were: measurement of rotarod performance, spontaneous locomotor activity, weight lifting and thiopental sleeping time used for assessment of dynamic voluntary motor control (co-ordination), sedation, muscle weakness and sedation (hypnotic potentiation), respectively. In order to gain reliable and comparable results for assessment of therapeutic index, uniform pre-treatment times were used for each compound over the different tests with relatively short test duration and the drugs were administered

intraperitoneally (i.p.). The measurement of the GYKI 20039 tremor and the weight lifting test were invented in our laboratory. Therefore, they are described here in more details. However, some details of the method development are beyond the scope of this dissertation and the reader is referred to our paper (Farkas *et al.* 2005). Potentiation of barbiturate hypnosis is useful for assessment of CNS depressant effects (Fujimori 1965; Vogel 2002). After i.v. injection, the redistribution of thiopental is far more important than metabolism rate in initial decline of plasma and brain concentrations and in termination of the anaesthetic effect (Burch and Stanski 1983). This feature, together with the short sleeping time makes the thiopental sleeping time test a good and quick assay for assessing CNS depressant effects.

2.1.2.2 Study on oral effectiveness and duration of action

For lead optimisation in drug research, there is often a need for pharmacodynamic tests performed in mice, particularly because test substance requirement of mouse tests is much less than that of tests on larger animals. As the GYKI 20039 tremor test appeared to be a useful method representing pathological muscle hyperactivity and being sensitive to CMR drugs, in addition to the above mentioned therapeutic index related studies, we investigated utility of the test in assessing oral efficiency and duration of action of tolperisone-type drugs: tolperisone, eperisone, lanperisone and silperisone. For this purpose, we assessed time-courses of drugs by changing the pre-treatment time with the test drugs before administration of the tremorogen.

2.1.3 Instrumental neurophysiological studies in unconscious cats and rats

Spasticity is a result of the impaired balance between the descending supraspinal facilitatory and inhibitory control to the spinal reflex activity, which is accomplished mainly through the descending reticulospinal tracts (Brooks 1986). Spastic states are often characterised by exaggerated reflexes, flexor spasms (particularly spinal spasticities), and appearance of abnormal (exteroceptive polysynaptic) reflexes (e.g. Babinsky's symptom) (Nielsen *et al.* 2007). Spinal reflexes are also obviously involved in pain induced muscle spasms. Hence, depression of exteroceptive reflexes is a possible mode of action to alleviate symptoms in both indication fields of CMRs, i.e. to relieve painful muscle spasms as well as spasticity.

Studying the effects on the descending reticulospinal pathway is also an important approach to investigate the effects of CMR drugs on the motor control (King and Unna 1954; Ochiai and Ishida 1982). Effects on the reticulospinal control can be studied using the measurement of the patellar reflex.

Myotatic reflexes play a basic role in the normal motor control, namely in maintenance of static positions as well as in controlling appropriate muscle tension during the movements. As a

consequence, in clinical cases of spasticity reduction of abnormally exaggerated myotatic reflexes is fruitful but excess inhibition or attenuation of the normal myotatic reflexes is not desirable. Therefore, we measured the patellar reflex, an easily accessible representative of these reflexes, to determine the effects of drugs on this type of reflex.

Although, there are clear-cut differences between human spasticities and the animal models of decerebrate rigidity (e.g. differences in the involvement of gamma-motoneurones, in the involvement of lateral vestibulo-spinal tract; for details see Brooks 1986), and thus decerebrate rigidity cannot be regarded as a fully relevant model of spasticity, it is a widely used method for testing CMR drugs (Fukuda *et al.* 1974; Tanabe *et al.* 1992; Sakitama *et al.* 1995). Decerebration is carried out either by removal of the cerebral hemispheres (Sherrington 1898) or by transection of the midbrain at the level between superior and inferior colliculi ("intercollicular decerebration") (Boes *et al.* 1968) or by ischemia due to occlusion of carotids and the basilar artery ("anaemic decerebration") (Pollock and Davis 1930). The anaemic decerebrate rigidity is associated with primary hyperactivity of alpha motoneurones, whereas intercollicular rigidity is thought to be associated primarily with hyperactivity of gamma motoneurones, and alpha motoneurones are activated secondarily via muscle spindle afferents (Rushworth 1960).

Tolperisone was reported to effectively diminish exteroceptive polysynaptic spinal reflexes, e.g. the flexor- and crossed extensor reflexes, in cats (Porszasz *et al.* 1960) and rats (Morikawa *et al.* 1987). It slightly suppressed the patellar reflex (Tanaka *et al.* 1981) but potently suppressed reticulospinal facilitation and inhibition of the patellar reflex (Ochiai and Ishida 1981). Tolperisone also effectively alleviated decerebrate rigidities (Tanaka *et al.* 1981; Sakitama *et al.* 1995; Ochiai and Ishida 1982; Morikawa *et al.* 1987; Ito *et al.* 1985).

We performed investigations with all the mentioned tests in order to select the most suitable one for comparative pharmacodynamic studies between tolperisone-type and other CMR drugs. Hence, the following tests were tried and applied:

- Electromyographic (EMG) recording of the flexor reflex in intact cats
- Mechanographic recording of the patellar reflex in cats
- Measurement of reticulospinal facilitation and inhibition by measurement of the patellar reflex in cats
- Measurement of the intercollicular decerebrate rigidity in cats and rats

In selecting the most appropriate method for comparative pharmacodynamic studies such practical factors were considered as:

- As simple and quick preparation as possible;
- Good success rate of experiments (e.g. achieving stable baseline recording);

- Reliability in assessment of effect sizes (apparently good dose response effects, high sensitivity to the test drugs, low individual variability of drug effects);
- Good long-term stability of the recorded parameter (outcome measure) for several hours (creditable time-course delineation);
- Sensitive to all CMR drugs of different type.

As EMG recording of the flexor reflex in cats proved to be the most suitable test fulfilling the above criteria, this test was used for pharmacodynamic characterisation and comparison of different CMRs. This model provided sufficiently stable reflex activity for following time-courses of effects of CMR drugs administered i.v. or intraduodenally. Measurements were done in spinal as well as in intact animals to assess the possible involvement of spinal and supraspinal factors in the drug effects.

With regards to *in vivo* potency, effectiveness and duration of action, silperisone was compared to several CMRs. Nevertheless, to identify pharmacodynamic similarities as well as differences, effects of silperisone were compared to those of tolperisone in a broader comprehensive set of animal models *in vitro* and *in vivo*.

2.1.4 Studies on the molecular mechanism of action of tolperisone-type drugs

The first reports by Pórszász and co-workers already described depression of multisynaptic reflexes as a salient effect of tolperisone (Porszasz *et al.* 1960; Porszasz *et al.* 1961). However, the molecular mechanism of action remained unknown. They emphasised anti-nicotinic action of tolperisone, based on inhibition of nicotine-induced seizures and lethality. However, an antinicotinic action does not really explain inhibition of spinal reflexes. Moreover, tolperisone had broader anticonvulsant effects, as it inhibited strychnine and electroshock induced convulsions, as well as harmine induced tremor.

Ono and co-workers, classifying tolperisone as a “mephenesin-type” muscle relaxant and comparing its effects to mephenesin and lidocaine, suggested participation of a “membrane-stabilising” effect in the inhibitory actions on the spinal reflexes for all these three drugs (Ono *et al.* 1984). This suggestion was based on similar concentration- or dose-dependent depressant effects on sciatic nerve conduction *in vitro*, proprioceptive afferent nerve discharges *in vivo*, as well as studying depressant effects on ventral root reflexes and on electrical excitabilities of motoneurones and primary afferents. Nevertheless, they noted that depressant effect of tolperisone on the spinal reflex was stronger than predicted from effects on nerve conduction and afferent firing.

Following the clue provided by Ono, we aimed at analysing mechanisms in the spinal reflex pathway of rats to specify the “membrane-stabilising” action more precisely. For this analysis, we carried out spinal reflex studies both *in vitro* and *in vivo*. Taking into consideration the structural and suggested pharmacodynamic similarity between tolperisone and lidocaine, in addition to comparisons between

tolperisone-type drugs, we compared tolperisone-type drugs to lidocaine as a key reference drug in several studies. However, the main focus was on the mechanism of action of silperisone.

The purpose of *in vitro* studies was to reveal the presence of spinal reflex depressant effects of tolperisone and related compounds and to establish the relevant concentrations that provide effects similar to those observed *in vivo*. Effects of tolperisone-type compounds on spinal reflexes *in vitro* had not been demonstrated previously. We choose for this purpose the method of "isolated hemisected spinal cord preparation from newborn rats" invented by Otsuka and co-workers (Otsuka and Konishi 1974; Otsuka and Yanagisawa 1980). Having established suppressant effects on the monosynaptic spinal reflex transmission, the *in vitro* preparation enabled getting further insights into the underlying mechanisms and the quantitative relationship between excitation of primary afferents and motoneurones. There are obvious limitations of this immature *in vitro* spinal cord preparation, such as possibly different receptor and ion channel composition compared to mature animals. Furthermore, these spinal cords are unable to fire action potentials at physiological temperature but only at room temperature. Nevertheless, there are obvious advantages, such as investigation of some functions in isolation, and better opportunities for pharmacological or environmental manipulations. It is also an insurmountable advantage that well sealed suction electrodes on ventral roots can yield a quasi-intracellular recording from the motoneurone pool providing an EPSP related field potential. This opportunity provides not only an easier technique with much better long-term stability but the compound field potential assures a kind of averaging over individual responses of individual neurones as compared to intracellular recording. This feature is highly favourable for quantitative pharmacological studies.

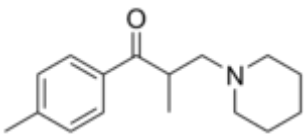
Reproducing in rats the former described effects of tolperisone-type drugs on ventral root reflexes was the starting point of further analysis of underlying mechanisms *in vivo*. In further *in vivo* experiments we also intended to look at the relationship between excitation of primary afferents and firing of motoneurones. Furthermore, we investigated potential interactions with pre- and postsynaptic inhibitory mechanisms.

Both *in vitro* and *in vivo* studies pointed to a difference between lidocane and tolperisone in terms of potency for local anaesthetic like effects on primary afferents and suppression of monosynaptic reflex transmission, respectively. Preferential reflex depressant effect compared to local anaesthetic like effect was even further discordant with silperisone. As the results suggested possibly different degree of effects on ion channels participating in membrane excitabilities and presynaptic transmitter release, we studied the effects of silperisone compared with lidocaine and tolperisone on the cation channels of sensory neurones using whole cell patch-clamp method.

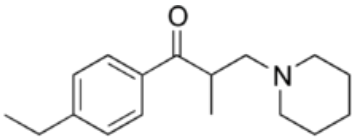
2.1.5 Drugs used in comparative pharmacological studies

In order to validate the novel methods and the whole test battery and to gain benchmarking data, we investigated ten known CMR drugs in those tests that were found to be suitable for reliable pharmacodynamic characterisation; and compared their effects to those of silperisone. These drugs included: mephenesin, zoxazolamine, carisoprodol, diazepam, tolperisone, eperisone, baclofen, tizanidine, memantine and afloqualon. In some specific tests, silperisone was compared only to tolperisone or the studies involved other tolperisone-type compounds, like eperisone, inaperisone and lanperisone. In some studies on mechanism of action, comparisons to lidocaine gained also some importance. The structures of used drugs and their claimed leading mechanism of action are summarised below in Fig. 2.

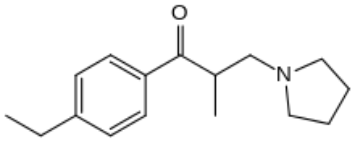
Tolperisone-type drugs (and lidocaine):



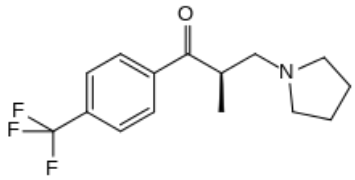
Tolperisone



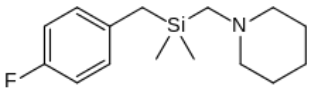
Eperisone



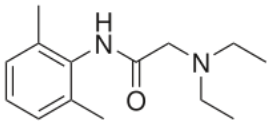
Inaperisone



Lanperisone



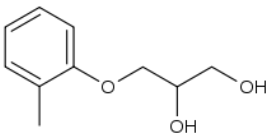
Silperisone



Lidocaine

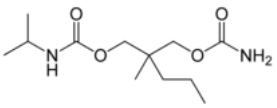
Na^+ channel block

Other CMR drugs:



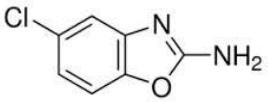
Mephenesin

„membrane stabilizer”



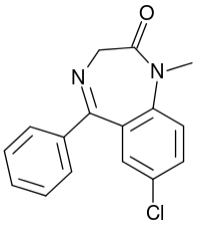
Carisoprodol

unknown, complex



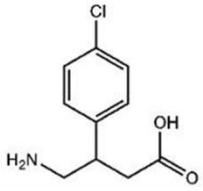
Zoxazolamine

SK channel activator



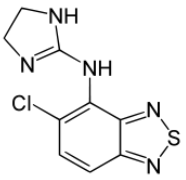
diazepam

GABA-A positive modulator



baclofen

GABA-B agonist



tizanidine

α_2 agonist

Fig. 2. Structures and postulated mechanisms of the reference drugs used

2.1.6 Summary of specific objectives

1. To assess the utility of the GYKI-20039 tremor test in estimating antispastic effectiveness of CMR drugs, including oral effectiveness and duration of action.
2. To assess the suitability of the test battery comprising GYKI-20039 tremor and morphine-induced Straub-tail tests for assessing main effects, and locomotor activity, rotarod, thiopental sleeping time and the newly invented weight lifting tests for assessing sedative and motor function impairing side effects for determining critical therapeutic indices of CMR drugs in mice.
3. To comparatively estimate therapeutic indices of clinically used CMR drugs and the novel tolperisone-type drug candidate silperisone in mice.
4. To select one particularly good neurophysiological test for comparative pharmacodynamic analysis of CMR drugs in terms of *in vivo* potency and duration of action in unconscious cats.
5. To characterise pharmacodynamics of silperisone in comparison with other CMRs and particularly with tolperisone-type compounds in the selected neurophysiological test with regards to *in vivo* potency, duration of action, oral (intraduodenal) effectiveness or functional bioavailability.
6. To compare *in vivo* potencies of CMR drugs in terms of reflex depressant effects in spinal animals as compared to those with intact neuraxis and higher CNS, which comparison might contribute to drawing conclusions on the primary sites of actions.
7. To investigate similarities and dissimilarities in the pattern of actions of various CMR drugs with different mechanism of action on spinal ventral- and dorsal root reflex potentials (in cats) and to draw conclusions on how this information might be used to indicate mode of action of (new) drugs.
8. To compare the effects of tolperisone and silperisone on the flexor reflex, patellar reflex, reticulospinal descending control of patellar reflex and decerebrate rigidity.
9. To investigate if the spinal reflex inhibitory effects of tolperisone-type CMRs appear also in an isolated spinal cord preparation *in vitro*.
10. To compare the profiles of tolperisone-type compounds in terms of the spinal segmental reflex activity (effectiveness on different components) both *in vitro* and *in vivo* (in rats) and to complement this comparison with lidocain.
11. To compare the profiles of silperisone, tolperisone and lidocaine in terms of effectiveness on excitability of primary afferents, excitability of motoneurones and monosynaptic excitation of motoneurones *in vivo*.

12. To compare the profiles of silperisone, tolperisone and lidocaine in terms of effectiveness on excitabilities of primary afferents and the EPSP of motoneurones *in vitro*.
13. Since the previous investigations suggested a depressant effect of silperisone on EPSP of motoneurones beyond that expectable from an effect on excitability of primary afferents, we also analysed potential effects that could affect the magnitude of EPSP (*in vitro*) either by presynaptic inhibition via GABA-B receptors or by postsynaptically suppressing glutamatergic excitatory transmission (via antagonism of AMPA- or NMDA-type glutamate receptors).
14. For analysis of molecular mechanism of action, we also investigated if silperisone affected GABA and/or glycine receptor mediated inhibitory pathways, such as adjacent feed-forward and recurrent (Renshaw) inhibition of monosynaptically elicited motoneurone firing *in vivo*.
15. To specify the "membrane stabilising" effects of silperisone and tolperisone in comparison with lidocaine, we investigated their effects on voltage gated sodium-, calcium- and delayed rectifier potassium currents of dorsal root ganglion (DRG) neurones by whole-cell patch-clamp and compared the effective concentrations with those in the in vitro spinal reflex experiments.
16. Along with these patch-clamp studies we made also some attempts for pharmacological dissection of Na^+ and Ca^{2+} channel subtypes affected by silperisone.

2.2 Methods

2.2.1 Neuropharmacological studies on efficacy and side effects in mice

2.2.1.1 GYKI 20039-induced tremor test

Male CFLP mice (19–21 g) were treated intraperitoneally with the tremorogenic compound, GYKI 20039 (2.5–20 mg/kg, i.p.; 10 mg/kg in CMR studies). Then the animals were placed into a light-weight plastic box (assemble of two yogurt cups, see Fig. 3) which was attached to an isometric force–displacement transducer (HSE, K 300). The transducer converted the vibration of the box induced by the tremor into a voltage signal, which was fed into an analog integrator (AC coupling; time-constant: 20 s) after half-wave rectification. Integration over periods of 1 min provided values termed "average force fluctuation" (in the following simply: "tremor value"). The measuring system was calibrated with a weight (20 g = 196 mN) loaded on the transducer and integration at DC coupling.

In experiments for studying inhibitory effects of drugs, the animals were injected with GYKI 20039 (10 mg/kg, i.p.) after intraperitoneal (-15 min; -25 min in experiments with baclofen) administration of the test compounds. The means of individual tremor values in the interval of 4–8 min following the treatment with the tremorogen were calculated, and compared with that of an independent

control (tremorogen-treated) group. Tremor values were also corrected for the spontaneous motility of the animals. Thus, drug effects were expressed as % inhibition according to the following formula:

$$inhibition = \left(1 - \frac{D - C}{T - C}\right) \bullet 100$$

where D=mean of tremor values of drug plus tremorogen treated animals, T=mean tremor value of tremorogen-treated animals, C=mean tremor value (in fact: force fluctuation) of untreated control animals. This latter value represents spontaneous motility of untreated animals. Fifty percent inhibitory doses (ID_{50}) were calculated by linear regression based on log dose–response curves. Tremor inhibitory action of CMR drugs was determined in ten mice per dose.

For assessment of oral effectiveness and duration of action, all the test substances were dissolved in physiological saline and administered at a dosing volume of 10 ml/kg. Pre-treatment times were varied for each drug between 5 and 60 minutes. Groups of ten mice were used for each test substance and pre-treatment time. Mice were fasted overnight before the oral administration, which is a deviation from the above described studies by i.p. administration. Changes were determined by comparison to a fasted control group of 10 mice.

Fig. 3. Tremor measurement of mice encaged in yogurt cups hanged on a force displacement transducer.

2.2.1.2 Morphine-induced Straub tail test

The method of Novack (1982) was used with slight modification. Male OF-1 mice (19–21 g) were injected with morphine (60 mg/kg) subcutaneously 5 min (15 min in experiments with baclofen) after intraperitoneal administration of the test compounds (N=10 per dose). Fifteen minutes after

administration of morphine, mice were scored for the presence of Straub tail, defined as an elevation of the tail steeper than 45° from the horizontal. ID_{50}s were calculated by using probit analysis.

2.2.1.3 Rotarod test

The experiments were performed on male CFLP mice weighing 20–25 g. The animals were trained one day before the experiment for the ability to remain for 120 s on a 25 mm diameter rod rotating at 15 rpm. Two or three trials were usually enough for the animals to learn this task. Drugs were tested only in those mice that were able to reproduce this performance also next morning before drug testing. Test compounds were administered intraperitoneally 20 min (for baclofen 30 min) before testing. Ability of the animals (N=10–12 per dose) to remain on the rotarod for 120 s was evaluated. The ID_{50} values (i.e. the dose causing failure in 50% of the animals) were calculated by probit analysis.

2.2.1.4 Locomotor activity

The experiments were performed on groups of 12 male OF-1 mice weighing 20–25 g. Three identical Animex-type motimeter cages (with electromagnetic sensors) having a basic area of 40x25 cm^2 were used. Three animals were placed into one cage and the motimeter summarised the activity of the three animals. Each drug dose was tested in 6–8 groups of animals. The animals were injected intraperitoneally with the test compound 15 min (for baclofen 25 min) before the beginning of the test period of 10 min. A vehicle-treated control group was always tested simultaneously with two treated groups; however, finally the control groups were pooled. Inhibition was expressed as percentage of the pooled control and ID_{50}s were calculated by linear regression based on log dose–response curves.

2.2.1.5 Weight-lifting test

The weight lifting test was invented to enable detection of moderate muscle weakness, which is achieved by compelling the animals to exert maximal efforts. We tried other methods known from the literature, like the traction test or the inclined screen test, suggested for testing muscle relaxant effects (Boissier and Simon 1960; Adeyemi *et al.* 2006). However, these tests could detect considerable effects only at seriously paralytic doses of CMR drugs.

The experiments were performed on male OF-1 mice weighing 19–21 g. A weight (mouse dumbbell) of 45 g with a 15 cm long vertical shaft (outer diameter: 6 mm) made of aluminium and covered with adhesive tape (Leucoplast) was used for testing. The experimenter hanged the animal upside-down, by its tail, and allowed it to grasp the shaft with all the four extremities. Then the animal (and the weight as well) was slowly lifted up (see Fig. 4). The weight was near the maximum that all untreated animals were able to lift up and keep for at least 3 s (test exercise). The animals (N=10 per dose)

were tested for ability to perform the test exercise 20 min (for baclofen 30 min) after intraperitoneal administration of the test compounds. The ID_{50} values (i.e. the dose causing failure in 50% of the animals) were calculated by probit analysis. Comparison for significance between the control and drug-treated groups was performed using the Fischer's exact test.

Fig. 4. A mouse held by tail is holding the dumbbell in the weight-lifting test

2.2.1.6 Thiopental sleeping time

Male CFLP mice weighing 20-21 g were treated intravenously with thiopental sodium (30 mg/kg) after intraperitoneal (-15 min) administration of the test compounds. Time between loss and recovery of the righting reflex was measured. Animals (N=10 per dose) were observed for 30 min following thiopental injection. If no recovery was seen, the sleeping time was taken as 30 min for calculation purposes (cut-off time). A saline-treated control group was also tested each day. Statistical analysis was performed by using the Kruskal–Wallis test, followed by paired comparisons using the Mann–Whitney U-test.

2.2.2 Instrumental neurophysiological studies in unconscious cats

2.2.2.1 Animals

Mongrel cats purchased from LATI Ltd. (Hungary) and Hannover Wistar rats bred at Gedeon Richter Co. were used.

2.2.2.2 Standard surgery in cats

In acute experiments in cats there was a common procedure referred to as "standard surgery": After introduction of anaesthesia trachea was exposed and cannulated to assure free spontaneous or artificial ventilation. One of the common carotid arteries was prepared and cannulated for measurement of the blood pressure (by a pressure transducer Statham P23Db). Blood pressure and

heart rate of the animals were continuously monitored and recorded throughout the experiment. One or both external jugular veins were prepared for intravenous drug administrations. A temperature sensor was introduced in the esophagus or rectum and the core temperature was regulated to 37±0.2°C using a temperature regulator (HSE) and an infrared heating lamp or a heating pad.

2.2.2.3 A special technique for quantitative evaluation of triggered electrophysiological events

For quantification of some electrophysiological signals, like EMG or electroneurogram, analog or digital integration, i.e. calculation of area under the curve is an often used method. However, at the time of starting our experiments in the "analog era" there were no commercial solutions to perform such integration for evoked (triggered) events that are lasting for a few milliseconds and are separated by several tens of seconds or even minutes of pause. Since analog integrators calculate area under the signal also during the pause, a few microvolts of deviation from zero voltage (due to offset bias or thermal noise) can yield more rubbish signal (integrated value) than the signal of interest. Furthermore, the analog integrator quantifying the signal must be very precise with extremely low drift and accurate offset setting. Moreover, in evoked reflex potentials there are often different components that are differentiated based on post-stimulus time. To solve all these issues and to separate and accurately quantify different reflex components the author of this assay has designed a signal processing system consisting of high quality (low offset drift and low bias current) differential amplifiers with broad range high- and low-pass filters, time-window discriminators (own design) enabling separation of signals of interest and also separating different reflex components by transmitting only in selected post-stimulus time. These discriminators working together with custom made high precision integrators enabled fully eliminating the offset- and noise-related rubbish signals and preserving and conditioning (only processing) for quantification the signals (components) of interest. The first stage of the analog integrator performed half-wave rectification of the signal before being fed into the analog integrating circuit. The original and time-window selected evoked potentials were displayed on digital oscilloscopes that enabled also charting display hard-copies. The outputs of integrators generated voltages proportional with the integrated signals and these voltages were visualised as deflections in various multi-chart recorders (mostly Graphtec Mark VII). Output voltage of the integrator was reset to zero periodically, which was harmonised with periodicity of the stimulations. A simplified diagram of one channel of such signal processing system is shown in Fig. 5 and was also described in our paper (Farkas *et al.* 1989) and the photo of a prototype of a two channel integrated equipment is shown in Fig. 6.

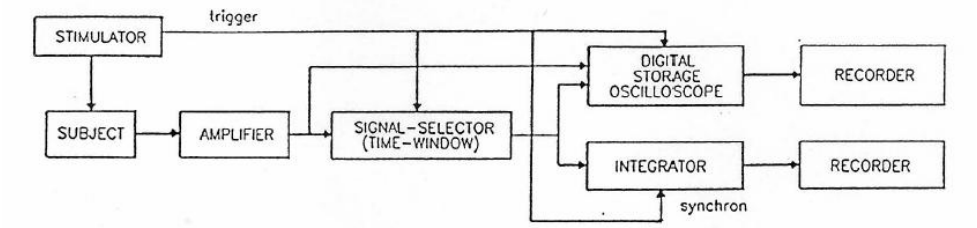


Fig. 5. Block diagram of one channel of the signal processing system for analog integration of triggered electrophysiological events.

Fig. 6. Photograph of a two-channel evoked response analysis system (built by Sándor Farkas), each channel comprising a signal conditioning amplifier, a time-window unit and an integrator.

It is worth to note that the concept of this analog signal processing system was furthered into two computerised data acquisition softwares of two generations: the "Stimulat" programmed by Peter Molnár (DOS era) and EvokeWave (Windows software by Unisip Ltd. still in use – http://www.unisip.hu/evokewave/evokewave.htm)

2.2.2.4 Flexor reflex in intact cats

The experiments were performed in chloralose anaesthetised (75 mg/kg i.p. + 4 mg/kg/h continuous i.v. infusion) intact cats of both sexes weighing from 2.2-4.5 kg. A series of 5 monophasic square-wave pulses (20 V, 4 ms, 0.4 Hz) was delivered to the hind-paw of the animal in every 30 second by means of a pair of needle electrodes inserted close to the plantar nerves. EMG reflex responses were recorded from the ipsilateral anterior tibial muscle by a concentric needle electrode, and were electronically integrated using an analog system. For more details on this signal processing see Farkas *et al.* (1989) and section 2.2.2.3 above. In a portion of the experiments after AD conversion at 10 kHz a PC based signal-processing system working on the same principles as the above analog system was used for the evaluation.

Drug administrations were performed only after having stable reflex activity for at least 30 minutes. At the i.v. studies, the cumulative dose-response relationship was determined by successive i.v. administrations of the studied drug with appropriate intervals (3-10 min) to reach maximum effects, and then the time-course of the effect was monitored for at least 180 minutes. However, for baclofen, due to the slow onset of its effect, the dose-response relationship was determined using single doses in different animals. The ID_{50} values were determined by linear-regression based on the logarithmic dose-response relationship.

For intraduodenal drug administrations, a cannula was inserted through the stomach into the duodenum and the pylorus was ligated to prevent reflux. Effects of single doses were investigated in each animal and the drug effect was followed up to 4 hours.

2.2.2.5 Flexor reflex in spinal cats

The experiments were performed in C_1 spinal cats of both sexes weighing from 2.5-4.1 kg. Following standard surgery and ligature of both carotid arteries, which were performed under ether anaesthesia, artificial ventilation was commenced using a Starling pump. Then the atlanto-occipital membrane was exposed and incised, spinal cord was infiltrated with lidocaine and transected at C_1 level. The brainstem was then destroyed using a rod through the foramen magnum. After completing the surgery, inhalation of ether was discontinued and the flexor reflex was measured in the same way as in intact cats. Drugs were studied only after stabilisation of the reflex and at least two hours after cessation of the inhalation of ether.

2.2.2.6 Patellar reflex in cats

The experiments were performed in chloralose anaesthetised (100 mg/kg i.p.) intact cats of both sexes weighing from 2.3-3.8 kg. Following standard surgery the animals were placed in a supine position. The right hind-leg of the animal was gently fixed in a manner that femur was in a vertical and the shank in a nearly horizontal position. The popliteal region was supported from below by a transversal rod and the ankle was suspended on a spring. Patellar reflex was elicited in every 10 seconds using an electro-magnetically operated hammer. An isometric force-displacement transducer (Grass FT-10) was attached by a thread to the ankle through a pulley. Tension of the thread, and thus the patellar reflex responses were recorded on an oscillographic thermorecorder (Graphtec Mark VII).

2.2.2.7 Study on the reticulospinal control of the patellar reflex in cats

The experiments were performed in 12 cats of both sexes weighing 2.5-4.1 kg, under chloralose (100 mg/kg i.p.; 8 cases) or urethane (500-550 mg/kg i.p.; 4 cases) anaesthesia. Following the standard surgery, head of the animal was fixed in a stereotaxic frame (David Kopf) lying in a prone position.

One of the femurs was firmly fixed vertically by two sharp screws. The shank was in horizontal position hanged on an isometric force-displacement transducer (Grass FT-10) attached to the ankle. Patellar reflex responses were elicited in every 5 seconds and recorded as described above. Through small holes in the cranium, stimulating concentric needle electrodes (Rhodes NEX-100) were inserted into the pontine facilitatory and the bulbar inhibitory areas, ipsilaterally to the measurement of the reflex. The stereotaxic coordinates were determined with an inclined (45° to the horizontal) interauricular-infraorbital plane. The following coordinates were used relative to the interauricular origo: facilitatory: A:0-4, L2-4, V:5.5-8; inhibitory: P:4, L:1; V:5-8.5 (mm). Intermittent stimulation of the facilitatory and inhibitory areas was applied in every 5 minutes for 40 seconds using monophasic square-wave impulses of 1-10 V, 1 ms, 30-50 Hz. Drug administration was only performed after stabilization of responses.

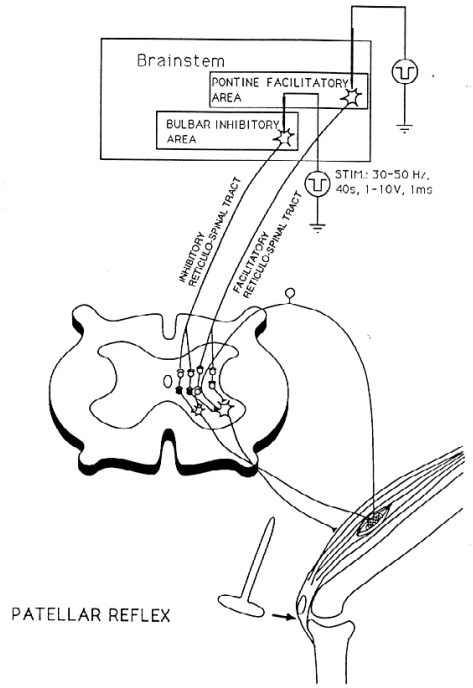


Fig. 7. Schematic illustration of the experimental arrangement for recording of reticular stimulation-induced facilitation and inhibition of the patellar reflex.

2.2.2.8 Spinal root potentials evoked by tibial nerve stimulation in cats

The experiments were performed on male cats, weighing 2.5-4.2 kg. Under ether anaesthesia, the standard surgery was carried out. After spinalisation at C_1 level (see above), the anaesthesia was stopped. Dorsal laminectomy was performed on vertebrae L_{4-7}. The ventral roots of L_6, L_7 and S_1 segments were transected on the left side. Tibial nerve of the left hind-leg was stimulated and the ventral root reflex (VRR; L_7, S_1) as well as the dorsal root potential and reflex (DRP and DRR; L_6) were recorded using bipolar Ag-AgCl hook electrodes. The rectal temperature and that of the oil pool covering spinal surface were maintained at 37±0.2 °C and 36.5±0.2 °C, respectively. The end tidal CO_2

level was kept at 4% v/v by artificial ventilation. Different reflex components were quantified and recorded using an analog integrating method described in 2.2.2.3. For more details see our paper (Farkas *et al.* 1989).

2.2.2.9 Intercollicular decerebrate rigidity in cats

The experiments were performed in cats of both sexes (2.5-5 kg, bw). Following standard surgery and ligature of both common carotid arteries under ether anaesthesia and artificial ventilation, the animals were fixed in a stereotaxic apparatus. After removing the bone of the skull above the appropriate areas, the brainstem was intercollicularly transected using a thermocauter and ether inhalation was discontinued. Drugs were tested only in the animals that showed stable rigidity. Animals were lying in supine position. Two guiding metal rods along the side of the body supported the rigid animal. An electromagnetic operated hammer tapped the patellar tendon in every 10 seconds. Electromyographic recording via a concentric needle electrode was taken from the quadriceps femoris muscle and in the majority of experiments from the m. triceps brachii as well. Patellar reflex responses were separated in the EMG of the quadriceps muscle using a signal selector (time-window discriminator) triggered from the reflex-hammer driver. Both patellar reflex responses (from response to response) and continuous EMG activities (for every minute) from the two above-mentioned muscles were integrated using analog integrators and integrated activities were recorded on an oscillographic thermorecorder (Graphtec Mark VII).

2.2.3 Instrumental neurophysiological studies in unconscious rats

2.2.3.1 Intercollicular decerebrate rigidity in rats

The method of Fukuda was used with slight modifications (Fukuda *et al.* 1974). Experiments were performed in male Wistar rats weighing 200-300 g. Under ether anaesthesia and artificial ventilation via a polyethylene cannula inserted previously into the trachea, both common carotid arteries were ligated. A winged needle (with a flexible cannula) was inserted into the tail vein for drug administrations. Head of the animal was fixed in a stereotaxic apparatus (Grastyán-type) and the brainstem was intercollicularly transected using a spatel. Following transection, anaesthesia and artificial ventilation was discontinued and animals were warmed up to 37°C by an infrared heating lamp under the control of the rectal temperature. Drugs were tested on rats displaying stable rigidity (about 20% of the animals). Animals were placed on an operating table in a supine position. Pads of both hind-foots were fixed to vertical rods (6 mm in diameter) by sticking plaster (Leucoplast), while the shanks were nearly horizontal. Thus, recordings were made under nearly isometric conditions. EMG recording was taken through a concentric needle electrode from one of the gastrocnemius muscles. Spike rate of the EMG was counted (Winston RAD-II-A discriminator and counter) at a

discrimination level of 50 µV and with a resolution of 1 s and recorded on a compensographic recorder (MTA Kutesz). Drugs were only administered after stabilization of EMG activity, at least 30 minutes after cessation of anaesthesia. Only one dose of a drug was tested in each animal and recording was maintained for 30 minutes after each drug administration. All drug administrations were performed at the volume of 1 ml/kg body weight, and the drugs or stock solutions were diluted with saline accordingly. ID_{50} values (i.e. the dose causing 50% inhibition in the EMG spike rate) were calculated by linear regression based on the linear logarithmic dose-response relationship.

2.2.3.2 Studies in anaesthetised spinal rats in vivo

2.2.3.2.1 Standard surgery

The method was essentially similar to that described earlier in more details (Farkas and Ono 1995). Male RG Wistar rats, weighing 250-350 g, anaesthetised with a mixture of chloralose (25 mg/kg, i.p.) and urethane (1 g/kg, i.p.), were used. The vagus nerves were severed and the common carotid arteries ligated bilaterally at the cervical region. Blood pressure was monitored via a cannula in the carotid artery. The femoral vein was also cannulated to allow intravenous injections. A tracheal cannula was inserted and the animals were artificially ventilated throughout the experiment. The spinal cord was infiltrated with lidocaine (1%, 50 µl) and transected at the C_1 level. The animals were fixed in a spinal stereotaxic frame and a dorsal laminectomy was performed on vertebrae L_1-L_6. A pool was formed from the skin of the back and filled with warm paraffin oil. Rectal and oil pool temperatures were maintained at 36±0.2 °C using two heating lamps. During the experiment Rindex solution was infused (10-20 $ml \cdot kg^{-1} \cdot h^{-1}$) in order to maintain the mean arterial blood pressure of spinal animals at around 60 mmHg.

2.2.3.2.2 Data acquisition and analysis

The amplified responses were fed into an IBM PC compatible computer via an analog-to-digital converter (Lab Master TM-100 with sampling rate of 25 kHz). The data acquisition software (Stimulat 2.0 by Péter Molnár) controlled the stimulator, acquired the potentials and performed on-line calculations (averaging, integration). The averaged shape of evoked responses (groups of 10), as well as on-line calculated parameters, were stored for later evaluation. Using the computerised data acquisition system different components of the evoked responses were separated according to their post-stimulus latencies and durations, and both on-line and off-line analyses were performed.

2.2.3.2.3 Investigation of the ventral root reflex

Ventral and dorsal roots below L_4 were cut bilaterally, L_5 dorsal and ventral roots on both sides were isolated and an ipsilateral pair of them was placed on bipolar silver wire hook electrodes. The dorsal root was stimulated by single impulses (stimulus strength: supramaximal voltage; pulse width: 0.05

ms; frequency: 10/min). The ventral root reflex was recorded from the L_5 ventral root using a differential amplifier and displayed using a computer.

2.2.3.2.4 Neuronal excitability test

Excitabilities of the motoneurone somata and of the primary afferent fibres were measured according to a technique essentially similar to that described by Ono and co-workers (Ono *et al.* 1979; Farkas and Ono 1995). Anaesthetised animals were paralysed with pipecuronium bromide (100 µg/kg starting dose + 50 $\mu g \cdot kg^{-1}h^{-1}$ in infusion). A tungsten microelectrode, insulated except its tip, was inserted into the motoneurone pool, which was stimulated by negative pulses (stimulus strength: 0.2-0.5 mA; pulse width: 0.05 ms; frequency: 10/min). The compound action potential evoked by direct stimulation of motoneurones (MN, first peak) and the one caused by (mono)synaptic activation of motoneurones (MS, second peak), were recorded from the L_5 ventral root. The antidromic action potential, which reflects excitability of the primary afferent fibres (PAF), was recorded from the L_5 dorsal root (see Fig. 8). Exact tip position and stimulus strength were adjusted to yield similar amplitudes for MN and MS.

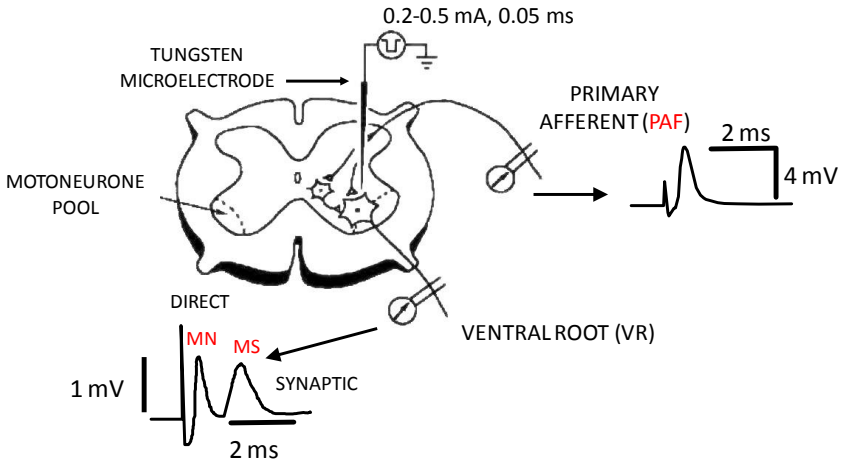


Fig. 8. Schematic illustration of the experimental arrangement for the neuronal excitability test with a stimulating electrode inserted in the L_5 motoneurone pool.

2.2.3.2.5 Study of feed-forward inhibition

The ventral root reflex was investigated as described above. The L_4 dorsal root was also placed on a bipolar silver wire hook electrode and was used for delivering conditioning pulses at different intervals before delivery of the test pulse on the L_5 dorsal root. The recording was organised in cycles of three unconditioned tests followed by 9 conditioned tests with 5, 10, 20, 40, 80, 160, 320, 640 and 1280 ms conditioning – test (C-T) intervals. The integrated monosynaptic reflex (MSR) was evaluated and the responses were normalized to the mean of the three unconditioned responses. The results of five cycles were averaged before the drug administration, and at the time of the maximum effect

in terms of depression of MSR. The conditioning stimulus strength was routinely 0.02 mA, 0.1 ms, however, occasionally experiments were performed with higher stimulus intensities (0.05 or 1.0 mA) as well.

2.2.3.2.6 Study of recurrent inhibition

The method was similar to that described by (Kaneko *et al.* 1987). The L_5 ventral root was dissected into two rootlets. One of them was placed on a bipolar silver wire hook electrode and was used for delivering supramaximal (0.5 mA, 0.2 ms) conditioning pulses at varying intervals before the test pulse applied on the L_5 dorsal root. The ventral root reflex from the other ventral rootlet was recorded. The stimulation and recording was organized in cycles of three unconditioned tests followed by conditioned tests with 4, 6, 8, 10, 15, 20, 25, 30, 40, 50 ms C-T intervals. The integrated monosynaptic reflex (MSR) was evaluated as described at testing of the feed-forward inhibition. The conditioning stimulus strength was supramaximal (5 V, 0.1 ms).

2.2.3.2.7 Study of afferent nerve conduction

In addition to the standard surgery, the sciatic nerve was exposed in the femoral-popliteal region, and placed on a bipolar silver wire electrode. A pool was formed from the skin of the back and of the leg and filled with warm paraffin oil. The sciatic nerve was stimulated by single square wave impulses (stimulus strength: supramaximal for all A fibres; pulse width: 0.5 ms; frequency: 10/min). The L_5 dorsal root, transected at the dorsal root entry zone, was placed on a silver wire bipolar electrode and crushed between the two poles for monophasic recording of the arriving compound action potential of the afferent nerve fibres. This experimental arrangement is shown in Fig. 9.

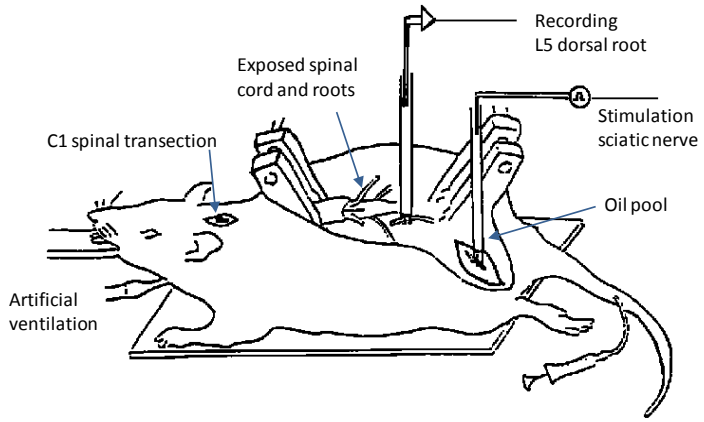


Fig. 9. Experimental arrangement for the afferent nerve conduction study.

2.2.4 Studies in the isolated hemisected rat spinal cord in vitro

2.2.4.1 Spinal root potential studies (in vitro)

Six-day-old male Wistar rat pups weighing 13-16 g were anaesthetised with ether, and then placed on crushed ice to cool down the spinal cord until the respiration of the animal stopped. The spinal cord was then removed and hemisected. The half spinal cords were transferred into a storage chamber, where incubated at room temperature (23-26ºC) in standard artificial cerebro-spinal fluid (ACSF) bubbled with carbogen for at least half an hour. One of them was placed into the recording chamber and perfused at 10 ml/min with standard ACSF (in mM: NaCl 124, KCl 3.5, NaH2PO4 1.23, CaCl2 2, MgCl2 2, NaHCO3 26, glucose 10), if otherwise not stated, at 26 °C (regulated). A glass suction electrode was used to stimulate the L_5 dorsal root with constant current stimuli. In those experiments where the compound action potentials of afferent fibres invading the spinal cord were the matter of interest, care was taken to place the stimulating electrode at a distance of at least 5 mm from the dorsal root entry zone, and a suction electrode was placed over the adjacent (L_4) dorsal root to record the afferent fibre potentials (AFP) arriving to the spinal cord surface. The ventral root potential evoked by stimulation of the dorsal root (DR-VRP) was recorded from the L_5 ventral root via a snug fitting suction electrode. In the experiments for investigating DR-VRP, routinely the stimulation intensity of 0.2 mA x 0.1 ms was applied in every 30 s. However, before and at appropriate times after drug applications the input-output relationship (I-O curve) was also determined using current intensities of 0.01; 0.016; 0.026; 0.041; 0.066; 0.105; 0.168 and 0.2 mA. A schematic drawing of the experimental arrangement and the recorded potentials are shown in Fig. 10.

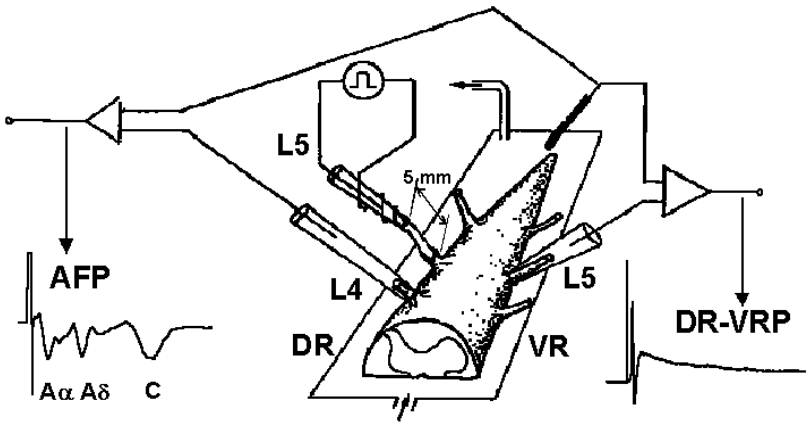


Fig. 10. The isolated hemisected rat spinal cord preparation. Schematic diagram of the experimental arrangement (modified from a figure of Ishida and Shinozaki): the perfused, hemisected spinal cord, dorsal roots (DR) on the left side, ventral roots (VR) on the right side, stimulation (L_5 DR) and recording (afferent fibre potential – AFP – from L_4 DR, ventral root potential – DR-VRP – from L_5 VR) via suction electrodes. The pure AFP ($A_{\alpha\beta}$, A_{δ} and C fibre mediated potentials) on the left side was recorded in Ca^{++}-free medium, DR-VRP shown on the right side was recorded in Ca^{++}-containing medium.

2.2.4.2 Spinal cord grease gap method (in vitro)

The spinal cords were removed and hemisected as described above from 6-day-old rats. About 2-mm-thick transversal slices of the L_5 segment were prepared, using a pair of scissors, keeping the connection with the L_5 ventral root. The L_5 dorsal root was removed. The slices were transferred into a storage chamber, and incubated at room temperature in the standard ACSF for 1 hour. Then slices were transferred into two-compartment recording chambers, so that spinal cord slice was contained in one compartment and the ventral root was in the other. A high resistance seal between the two compartments was achieved by using a perspex barrier well greased with silicone grease. The two compartments were perfused independently with ACSF containing 0.1 µM tetrodotoxin (TTX-ACSF) at a rate of 2 ml/min at room temperature. The D.C. potential between the two compartments was continuously recorded via Ag/AgCl electrodes.

Depolarisations were evoked by repeated superfusion with TTX-ACSF containing 80 µM NMDA or 40 µM AMPA (ionototropic glutamate receptor agonists) for 1 min. Thirty-minute intervals were kept between consecutive agonist applications. After stabilisation of control responses, the test compound was added to the perfusion solution 10 minutes after an agonist application, and the agonist application was repeated 20 and 50 minutes after addition of the test compound. In some experiments, after stabilisation of the control responses (to 40 µM AMPA) the test compound was added and increasing doses of AMPA (5 µM, 10 µM, 20 µM, 40 µM, 80 µM and 160 µM) were administered in 30 min intervals commencing at 50 minutes after addition of the test compound. The amplitudes of depolarisations were expressed as percentages of the control response. The results were compared (unpaired t-test) to those obtained in control experiments executed according to a similar protocol except for addition of a test compound.

2.2.4.3 Data acquisition and analysis in the in vitro hemisected spinal cord studies

The amplified recorded potentials were fed into an IBM PC AT compatible computer via an analog-to-digital converter (Axon Instruments' Digidata 1200 with sampling rate of 10 kHz. The data acquisition program (Stimulat 2.0 by Péter Molnár) controlled the stimulator, acquired the potentials, performed on-line calculations (averaging, integration, amplitude calculation). The averaged shapes of evoked responses, as well as on-line calculated parameters were stored for later evaluation. Using the computerised data acquisition system different components of the evoked responses were separated based on their post-stimulus latency and duration, and both on-line and off-line analyses were performed.

2.2.5 Patch-clamp analysis of effects on voltage-gated channels

We studied the effects of tolperisone-type CMRs and lidocaine on voltage-gated sodium-, calcium- and delayed rectifying potassium channels.

2.2.5.1 Preparation of sensory neurones

Neurones were acutely dissociated from rat dorsal root ganglia (DRG). Six-day-old Wistar rats were anaesthetised with ether, and then cooled in iced water. The thoracolumbar vertebral column was removed, fixed on a Sylgard surface and perfused with ice-cold ACSF. Then the spinal cord was exposed by ventral laminectomy, dorsal root ganglia were rapidly excised and placed into Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). Pooled ganglia from L_{1-5} were incubated with gentle agitation in Ca^{2+}- and Mg^{2+}-free PBS containing 2 mg/ml trypsin (type XI - SIGMA) for 20-30 minutes at 37 °C, rinsed twice with Dulbecco's Modified Eagle's Medium (DMEM - GIBCO) supplemented with 2% foetal bovin serum (FBS - GIBCO) and 0.5mg/ml gentamicin (Sigma), and triturated using a Pasteur pipette. Cells were then plated on sterilised glass coverslips previously coated with poly-d-lysine and kept in serum supplemented DMEM overnight at 37 °C, 5% CO_2 and 100% humidity.

2.2.5.2 Whole-cell patch-clamp recording and analysis

Coverslips with the attached neurones (20-30 hours in culture) were transferred into the recording chamber mounted on the stage of an inverted microscope (Nikon Diaphot 200) and continuously perfused at room temperature (22-25 °C) with extracellular solution (ES). Patch pipettes (resistance 1-2.5 MΩ) pulled from borosilicate capillary glass were filled with intracellular solution (IS), attached to selected DRG cells and whole-cell membrane currents were recorded.

In measurements on sodium currents ES contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, Hepes 5, HEPES-Na 5, glucose 10, saccharose 10, pH = 7.35. In a second set of experiments, mainly to reduce the magnitude of too large sodium currents often observed in DRG cells, a modified (optimised) ES was used with the following composition (in mM): NaCl 70, choline cloride 70, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 5, HEPES-Na 5, $CdCl_2$ 0.01, glucose 20; pH=7.35. The IS for sodium current measurements contained (in mM): CsF 130, NaCl 15, tetraethylammonium chloride (TEA-Cl) 10, $CaCl_2$ 0.1, $MgCl_2$ 2, ATP-Na_2 2, HEPES 10, EGTA 1; pH=7.25.

In measurements on calcium currents ES contained (in mM) choline cloride 143, $CaCl_2$ 5, $MgCl_2$ 1, HEPES 10, glucose 20; pH=7.3. The IS for calcium current measurements contained (in mM): CsCl 110, MgCl2 4.5, HEPES 9, EGTA 9, ATP-Na_2 4. Since in initial experiments an inherent rundown of calcium currents was observed, in later experiments a modified IS was applied, which included a nucleotide regeneration system and GTP and prevented the rundown (Forscher and Oxford 1985). The modified

("CPK supplemented") IS had the following composition (in mM): CsCl 110, $MgCl_2$ 4.5, HEPES 9, EGTA 9, ATP-Na_2 4, GTP 0.3, creatine phosphate (CP) 14, creatine phosphokinase (CPK) 50 U/ml.

In measurements on potassium currents ES contained (in mM): choline cloride 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $CdCl_2$ 0.1, HEPES 10, glucose 20. The IS for potassium current measurements contained (in mM): KCl 145, $MgCl_2$ 1, HEPES 10, EGTA 10.

Osmolarities of ES and IS were 310 mOsm and 290 mOsm, respectively.

Membrane currents were recorded with an Axoclamp 200A amplifier using the pClamp 6.0-8.0 software (Axon Instruments). Current signals were pre-filtered at a bandwidth of 5-10 kHz and digitized at a sampling rate of 20 kHz (Digidata 1200 interface, Axon Instruments). Capacitive transients and series resistance errors were reasonably compensated by whole-cell parameter settings, supercharging ("prediction") and "correction" controls of the amplifier. Leak currents were subtracted by the on-line P/6 protocol of the software. Apart from testing voltage dependent activation/inactivation or other complex voltage protocols, sodium currents were routinely tested by applying voltage steps (8 ms duration) to 0 mV from different holding potentials (V_H; specified in "Results") in every 10 s. Calcium and potassium currents were routinely tested by 20 ms- and 100 ms-long depolarising voltage steps, respectively, from -80 mV (holding potential) to 0 mV, unless otherwise stated. Cells were perfused directly with ES via a gravity perfusion system. Test substances were dissolved in ES and applied by rapid solution exchange.

Concentration-inhibition relationships were determined in 4-10 cells for each test substance and channel type by administering increasing concentration arrays. Mean±S.E.M. of percent inhibition was calculated for each concentration and IC_{50} was calculated by sigmoidal curve fitting (Origin 6.0 software) with asymptotes fixed at 0 and 100%.

2.2.6 Materials

The drugs used were silperisone-HCl, tolperisone-HCl, eperisone-HCl, diazepam and pipecuronium bromide all synthesised at Gedeon Richter, Hungary. GYKI 20039 (synthesized at Institute for Drug Research, Hungary), baclofen (Ciba-Geigy, Switzerland), tizanidine (Wander, Switzerland), afloqualone (Tanabe Seiyaku, Japan), mephenesin, zoxazolamine (Aldrich, Europe), memantine (Merz and Co., Germany), carisoprodol (Egis, Hungary), morphine-HCl (Alkaloida, Hungary), midazolam (inj. Dormicum, La Roche, Switzerland), and thiopental sodium (SPOFA, Czech republic) were also used. Most solutions for *in vivo* dosing were prepared with physiological saline. In the case of poorly water-soluble compounds, stock solutions were used and diluted in physiological saline as necessary for administration *in vivo*. In mice, the dosing volume was 10 ml/kg. Composition of stock solutions for mice: zoxazolamine and afloqualone (10 mg/ml in 2.5% Tween 80); carisoprodol (20 mg/ml in 2.5%

Tween 80); diazepam (10 mg/ml in a 1:2 mixture of cellosolv and glycofurol); GYKI 20039 (1 mg/ml in 20% dimethylsulphoxide).

Composition of stock solutions for cats: Afloqualone (3.33 mg/ml) was dissolved in 0.1 N HCl, zoxazolamine and carisoprodol (40 mg/ml) were dissolved in propylene glycol. A stock solution of 2 mg/ml of diazepam dissolved in a mixture of cellosolv (33%) and glycofurol (67%, v/v) was further diluted at least ten-fold with warm saline immediately before administration. Solution of 5% (w/v) of α-chloralose (Aldrich, Europe) was prepared by heating and it was stabilized by adding of 3% Na-perborate (Aldrich). Urethane (Mitsui, Japan) was used as a solution of 50% (w/v) in water. Lidocaine injection 2% (Egis, Hungary) was also used.

For *in vivo* studies in rats, the anaesthetic solution contained 0.25% α-chloralose (Aldrich) and 10% urethane (Aldrich) dissolved in distilled water. The cannula for blood pressure monitoring was filled with saline containing 200 IU/ml heparin (Richter). Rindex 10 solution (purchased from Human – Budapest, Hungary) for i.v. infusion contained in mM: NaCl-68, KCl-3.5, $CaCl_2$-1.25, $MgCl_2$-0.5, glucose-555.

For *in vitro* studies, components of ACSF were purchased from Reanal (Hungary). α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA; SIGMA), N-methyl-D-aspartate (NMDA; RBI), SCH-50911 (GABA-B antagonist; Tocris) and tetrodotoxin (TTX; Latoxan) were also used.

2.3 Results

2.3.1 Neuropharmacological studies in mice

2.3.1.1 GYKI 20039-induced tremor test

Following i.p. administration of GYKI 20039 an intensive tremor develops very quickly, peaking at 4-8 minutes post dose and gradually fading by 30 min (Fig. 11A). Dose-response studies indicated that maximum tremor can be induced by administration of 10 mg/kg (Fig. 11B). Therefore, for studying anti-tremor activity of drugs, this dose of GYKI-20039 was used as provoking agent.

All the tested CMRs attenuated the tremor in a dose dependent manner (see Fig. 12; ID_{50} values are listed in the summary table – Table 2).

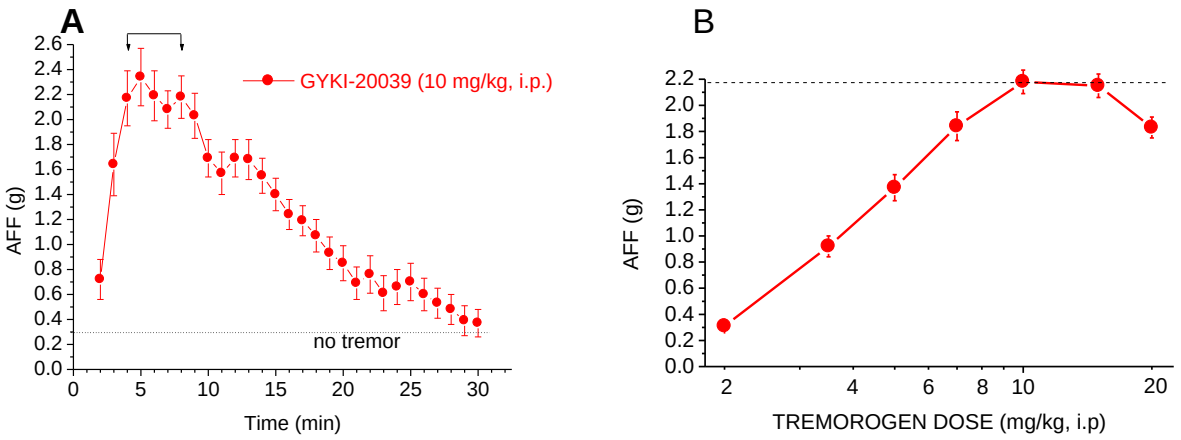


Fig. 11. Characterisation of the GYKI 20039 induced tremor. Ordinates: average force fluctuation (AFF; tremor value) mean with S.E.M. (A) Time-course of the tremor after i.p. injection of 10 mg/kg GYKI 20039 at 0 min (n=20); the arrows indicate the period of measurements in the subsequent studies; (B) dose-response relationship for GYKI 20039. The mean tremor value 4-8 min post dose was evaluated and the data were pooled from experiments carried out on different days (n=50-50).

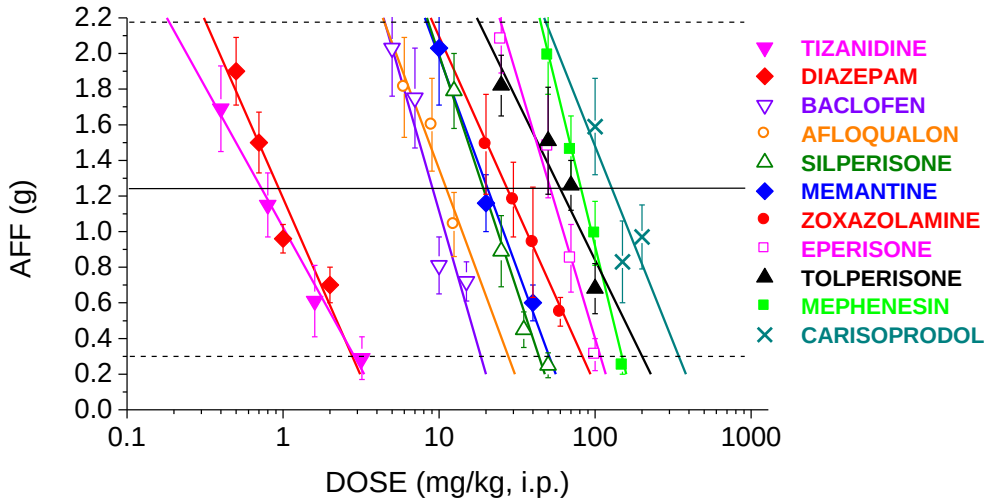


Fig. 12. Dose-response relationships of various CMRs (n=10 per group) against the tremor induced by GYKI 20039 (10 mg/kg i.p.). Note that all CMR drugs suppressed the tremor in a dose-dependent fashion. Upper and lower horizontal dashed lines indicate mean AFF of tremorogen treated and untreated control groups, respectively; the solid line indicates the value corresponding to 50% inhibition.

The results obtained by oral administration are presented in Fig. 13. All the tested drugs had a rapid onset of action, reaching a peak effect at 5 minutes post-dose. In terms of maximal effect, approximately equiactive doses were used, though the peak effect of lanperisone was slightly greater than that of the other three. Of note, the applied equiactive dose of silperisone was half of that of the others. In terms of duration of action, the apparent rank order (from shortest to longest) was: tolperisone < eperisone < lanperisone < silperisone.

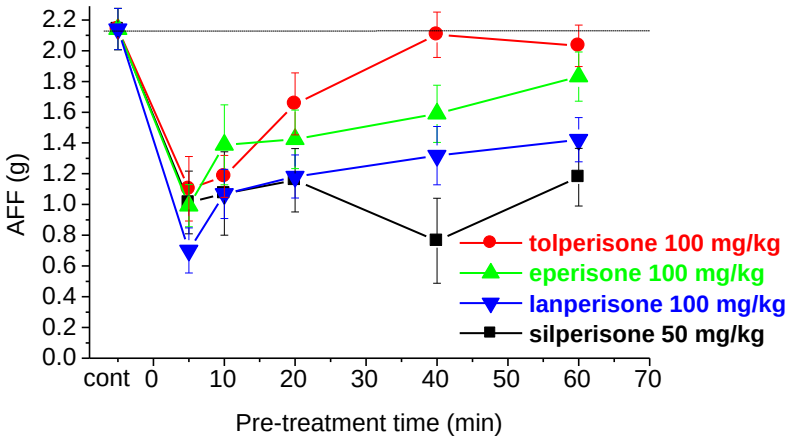


Fig. 13. Time course of effects of tolperisone-type drugs based on inhibition of GYKI 20039-induced tremor after administration of the test compounds with different pre-treatment times. Average force fluctuation (AFF; mean with S.E.M.; n=10/point) 4-8 min after dosing with the tremorogen is plotted against the pre-treatment time. The results of a temorogen treated control group (CONT) are also plotted to delineate an apparent time-course of effect.

2.3.1.2 Straub tail, rotarod, locomotor and weight-lifting tests and therapeutic indices

The detailed data obtained from these investigations are not presented here. Only some of the results from the weight lifting test are shown as a representative example (Table 1). It was a common finding in all these studies that CMR drugs exerted dose dependent inhibitory effects with the only exception of memantine in the locomotor activity test, which exhibited a stimulating effect. As in Table 1, we calculated ID_{50} values for each drug in all these studies to determine their *in vivo* potencies.

In vivo potencies (ID_{50} values) of all tested drugs in each test together with the calculated therapeutic indices (ID_{50} ratios; right part) are summarised in Table 2. For calculation of therapeutic indices (TIs) high *in vivo* potency (low ID_{50}) in the tremor (TR) and Straub tail (ST) test were considered favourable relaxant effects, whereas activity in the rotarod, locomotor and weight lifting tests were considered as side effects. Hence, the higher TI suggested lower propensity for side effects at effective therapeutic doses.

Overall, it could be concluded that there was no big separation between the favourable and unfavourable effects for all the CMR drugs, as large TIs (e.g. 5-10-fold or higher) were not seen with either drug. Instead, most of the drugs, with the exception of zoxazolamine, mephenesin and silperisone, had some TI values well below 1. Whereas tolperisone was relatively good in terms of not impairing strongly the dynamic and static voluntary motor functions (RR and WL), its effect on locomotor activity at low doses suggested some sedative effect. Silperisone was relatively outstanding among all the tested compounds having approximately 2-3-fold TIs in all aspects.

2.3.1.3 Thiopental sleeping time

Intravenous injection of thiopental resulted in a short anaesthesia of 2.35±1.3 min (mean±S.D., n=90). There were slight statistically significant differences between the daily control groups of 10 mice (group means varied between 1.55 and 3.16 min), therefore all the treated groups were compared to the respective daily control groups. However, in Table 3 only the mean of the pooled control group is shown. All the tested drugs prolonged the thiopental sleeping time at doses which yielded TIs quite similar to those in the locomotor activity test, further confirming the conclusions drawn on sedative activity of the compounds (Table 3). Silperisone exhibited the greatest TI with only mild sleep prolonging effect at 2-fold of effective doses in the tremor and Straub tail tests. In contrast, the other CMR drugs had not only narrower therapeutic index but their sleep prolonging effects were also much more pronounced.

Table 1. Effects of intraperitoneally administered muscle relaxants on the weight-lifting performance in mice.

Drugs	Dose (mg/kg,i.p.)	Observed activity No of successes/tests	Calculated ID_{50} (mg/kg)
Control	0	10/10	-
Silperisone	25	9/10	44.2
	40	5/10 *	
	60	3/10**	
Tolperisone	50	9/10	90.9
	80	7/10	
	120	3/10**	
	180	0/10**	
Eperisone	40	10/10	107
	70	9/10	
	100	7/10	
	150	0/10**	
Tizanidine	0.6	9/10	2.8
	1	6/10 *	
	1.5	5/10 *	
	2.2	6/10 *	
	3.3	5/10 *	
	6	2/10**	
	9	3/10**	
	13	3/10**	
Baclofen	6	10/10	8.2
	8	4/10**	
	12	1/10**	
	18	0/10**	

* and **: Significantly different from control, P<0.05 and P<0.01, respectively (Fischer's exact test).

Table 2. Summary of results obtained in conscious mice

	ID_{50} values (mg/kg, i.p.)						Ratios (TIs)					
	Muscle relaxant effect			Side effect			ID_{50} side effect/ID_{50} relaxant effect					
	TR	ST		RR	LO	WL	RR/ TR	LO/ TR	WL/ TR	RR/ ST	LO/ ST	WL/ ST
Silperisone	19.3	21.8		36.9	64.0	44.2	1.91	3.32	2.29	1.69	2.94	2.03
Tolperisone	60.0	63		116	31.1	90.9	1.93	0.52	1.52	1.84	0.49	1.44
Eperisone	51.8	63.6		72.1	55.7	107	1.39	1.08	2.07	1.13	0.88	1.68
Tizanidine	0.73	1.4		1.2	0.10	2.8	1.64	0.14	3.84	0.86	0.07	2.00
Baclofen	9.1	2.8		7.9	3.1	8.2	0.87	0.34	0.90	2.82	1.11	2.93
Mephenesin	81.5	121		145	120	255	1.78	1.47	3.13	1.20	0.99	2.11
Zoxazolamine	27.4	32.2		50.1	60.5	43.7	1.83	2.21	1.59	1.56	1.88	1.36
Diazepam	0.94	1.0		0.6	3.1	0.8	0.64	3.30	0.85	0.60	3.10	0.80
Afloqualon	11.1	7.8		11.4	10.5	12.6	1.03	0.95	1.14	1.46	1.35	1.62
Carisoprodol	128.8	71.2		130	156	107	1.01	1.21	0.83	1.83	2.18	1.50
Memantine	20.5	34.9		23	>100	29	1.12	>4.90	1.41	0.66	>2.90	0.83

Values lower than 1.5 (i.e. not remarkably higher than 1) are marked by shading.

Table 3. Effects of drugs on thiopental sleeping time in mice

Drugs	Dose (mg/kg, i.p.)	Mean sleeping time (min)	Died[a]	Dose 2X[b] (mg/kg)	Therapeutic index	
					vs. Tremor ID_{50}	*vs.* Straub ID_{50}
Control[c]	0	2.35	0	-	-	-
Silperisone	20	1.70	0	40	2.07	1.83
	30	1.77	0			
	40	5.13*	0			
Tolperisone	60	8.25	1	≤60	≤1.00	≤0.95
	90	6.61**	0			
	120	21.1**	1			
Eperisone	52	3.48	0	78	1.51	1.23
	78	10.6**	0			
	104	19.7**	0			
Tizanidine	0.1	14.1**	0	≤0.1	≤0.14	≤0.07
	0.2	22.0**	0			
	0.8	>30.0 *	2			
Baclofen	1.4	3.04	0	2.8	0.31	1.00
	2.8	25.7**	1			
	5.6	>30.0**	1			

a: Number of animals died within 30 min from thiopental injection. b: Lowest dose causing >2-fold prolongation *vs.* pooled control. c: Pooled from daily control groups (n=90). * and **: Significantly different from concomitant control, $p<0.05$ and $p<0.01$, respectively (Mann-Whitney U-test).

2.3.2 Instrumental neurophysiological studies in unconscious cats

2.3.2.1 Flexor reflex in intact cats

Experimenting with different anaesthesia protocols, we ultimately found that the best long-term stability could be achieved by an initial dose of 75 mg/kg chloralose i.p. followed by 4 mg/kg/h continuous i.v. infusion throughout the experimental session. Compared to mechanographic recording of muscle contractions and stimulation of exposed nerves (classical methods) the most convenient and minimally invasive method for stable long-term recording of flexor reflex proved to

be simply inserting two needle electrodes into the plantar surface of hind paw of cats for bipolar stimulation and inserting a concentric needle electrode for EMG recording into the anterior tibial muscle. In this way, stable recording of the reflex could be obtained for at least 4 hours (see "Vehicle" curve in Fig. 18).

As a representative example of an experiment, the effects of tolperisone on the EMG reflex response and the derived integrated recording is shown in Fig. 14.

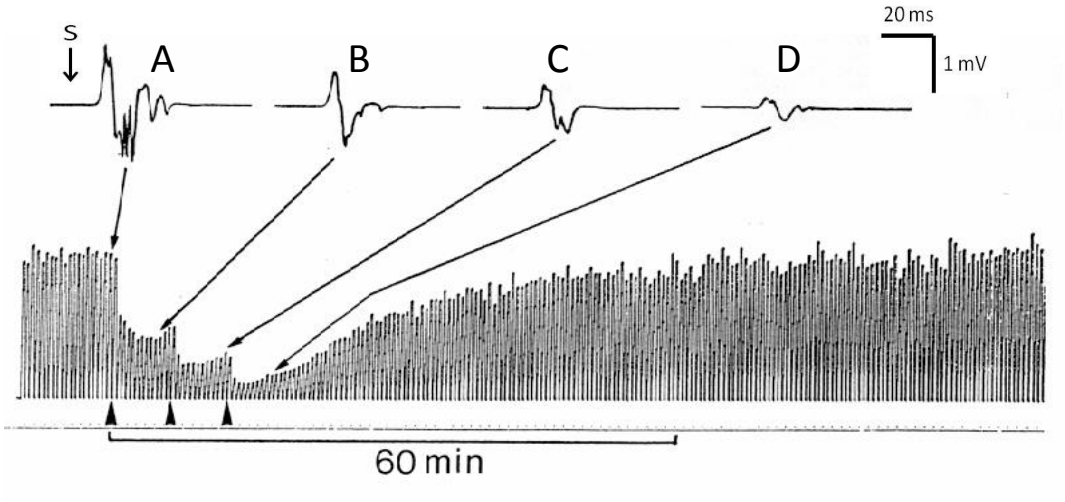


Fig. 14. Recordings from a typical flexor reflex experiment with cumulative administration of tolperisone. In the upper panel oscilloscopic charts of EMG responses recorded from the anterior tibial muscle of a cat are shown, before (A) and after successive administration of 2.5 mg/kg (B), 2.5 mg/kg (C) and 5 mg/kg (D) tolperisone i.v. at approximately 5 min intervals. The lower panel shows chart recording of the output voltage of an analog integrator. Each deflection accumulates the integral of 5 responses, and then the integrator is reset to zero in every 30 second. As seen, tolperisone depresses the flexor reflex immediately after the administrations and reaches a maximum within 5 minutes. The reflex starts to recover also relatively quickly with almost complete recovery within 60 minutes. Dose-response and time-course charts presented in Fig. 15 and Fig. 16, respectively, were generated by evaluation and averaging such integrated records.

The EMG reflex response recorded from the anterior tibial muscle consisted of two characteristic components. The first one appeared in the post-stimulus time-window of 10-25 ms, the second one between 25-45 ms. In some cases the two components appeared well separated (e.g. bottom right insert of Fig. 15), in others their separation was not evident (e.g. top left insert of Fig. 15). All the studied drugs exerted a more pronounced depressant effect on the second component, which component was sometimes missing. However, only two drugs, i.e. carisoprodol and afloqualon (see the insert in Fig. 15), possessed a selective action regarding the two components. Namely, they hardly attenuated the first component but abolished the second one in their low dose-range. Nevertheless, for quantitative evaluation we used the summed integration of both components for practical reasons. Dose-response curves based on the integrated EMG reflex response, and the calculated ID_{50} values of silperisone and 9 other CMR drugs administered i.v. are presented in Fig. 15.

Their time-courses of effects are shown in Fig. 16. In terms of peak effect, silperisone was slightly less potent than tolperisone and eperisone. However, its effect was much longer lasting than those of tolperisone and eperisone (Fig. 16). Except for mephenesin, which had relatively short duration of action like tolperisone and eperisone, the other drugs showed similarly long-lasting actions to that of silperisone. Baclofen was the one, which possessed particularly long lasting effects. Namely, its inhibitory effect showed no tendency to recover within 180 min even at its lowest dose. In contrast with the other CMRs, tizanidine displayed inconsistent effects. In 6 experiments it elicited practically 6 different reactions from the strong reflex depression to the marked potentiation (Fig. 17). Only its short duration of action, i.e. complete recovery within 120-180 min, was a consistent finding.

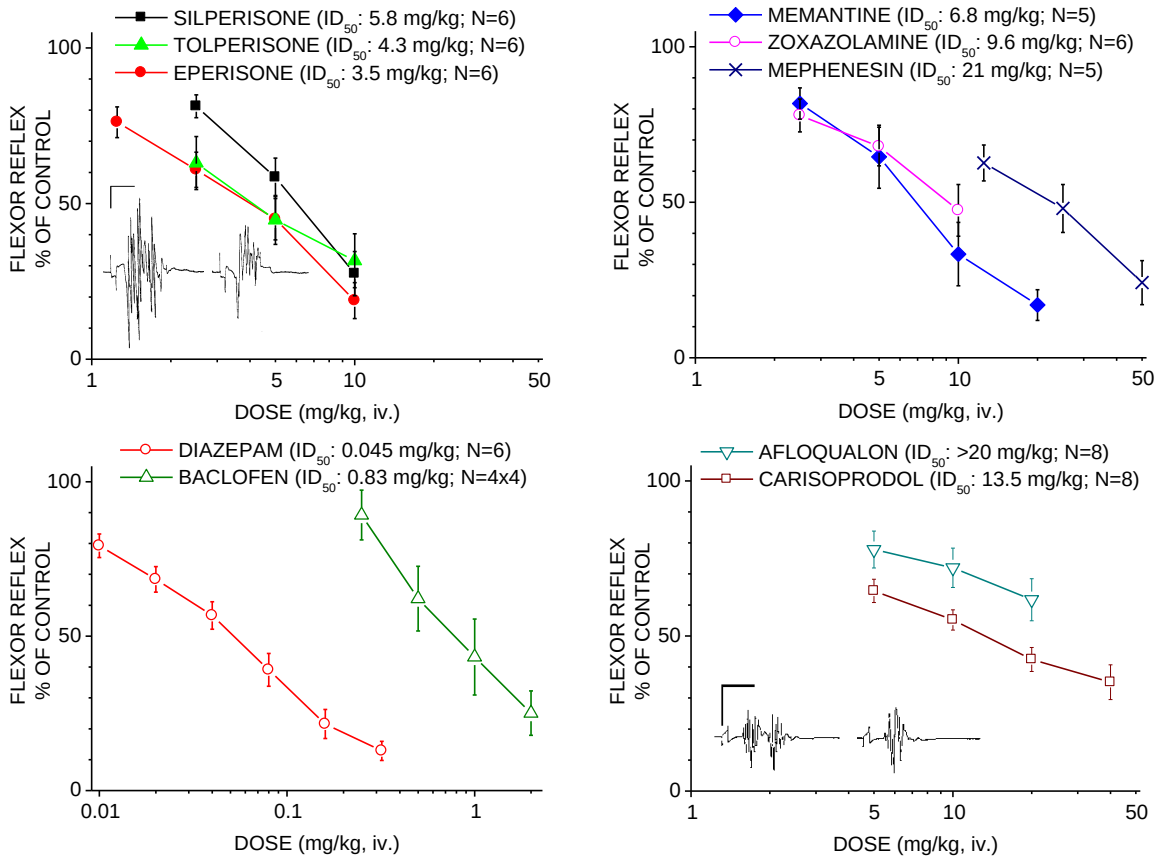


Fig. 15. Dose-response curves and ID_{50} values of silperisone and various CMR drugs in the flexor reflex test in intact anaesthetised cats. Ordinates: integrated EMG expressed as percent of the value prior to drug administrations. Except for baclofen, the results were obtained by cumulative intravenous administration of different doses of a drug to the same animal. Depending on the onset and recovery rates an appropriate interval (3-15 min) was kept between subsequent drug administrations to reach a maximum effect but to achieve real cumulating. In the case of baclofen, different doses were tested in separate animals (4 animals per dose). Data are presented as mean±S.E.M. The insert in the top left graph shows a control EMG record (left; calibration: 1 mV/20 ms) and that after administration of 5 mg/kg silperisone (right). Note that the first and second burst of EMG activity was not well separated and silperisone depressed both. The insert in the bottom right graph shows EMG records before (left; calibration: 0.4 mV/20 ms) and after 5 mg/kg afloqualon (right). Note that the first and second burst of EMG activity was well separated in this case, and afloqualon selectively depressed the second burst.

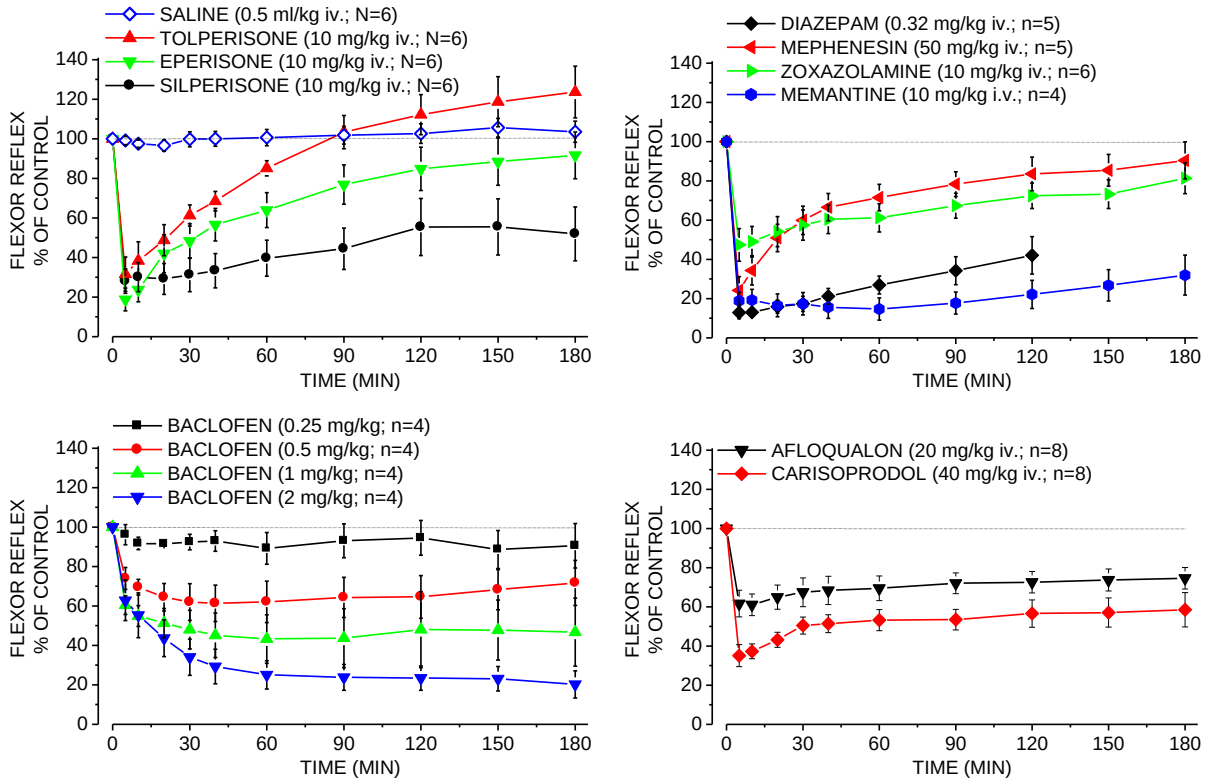


Fig. 16. Time-course of the effect of silperisone and of various CMR drugs in the flexor reflex test in intact anaesthetised cats after intravenous administration. Except for baclofen the curves show the recovery time-courses of the effects after cumulative administration of the test substances as described at Fig. 15. Ordinates: integrated EMG expressed as percent of the value just prior to drug administrations. Abscissas: time elapsed after the last dosing. All doses of baclofen were administered as single doses and the time course was followed afterwards. Data are presented as mean±S.E.M. Note that recovery rate of silperisone was much slower than those of tolperisone and eperisone.

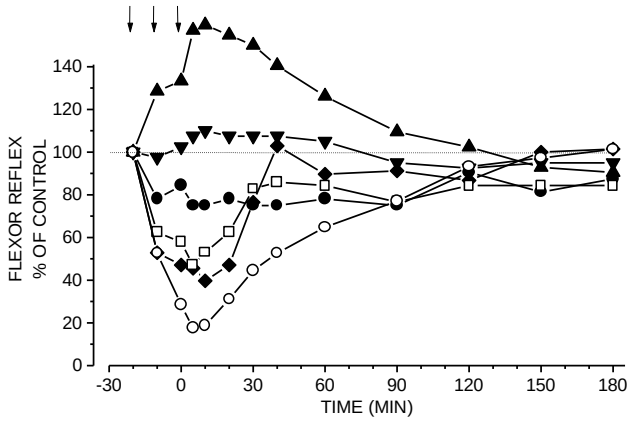


Fig. 17. Effects of tizanidine on the flexor reflex in intact anaesthetised cats. The 6 curves show the changes in the flexor reflex in 6 different cats after cumulative intravenous administrations of 0.025, 0.025 and 0.05 mg/kg tizanidine as indicated by the arrows. Ordinate: integrated EMG expressed as percent of the value just prior to drug administrations. Abscissa: time elapsed relative to the last dosing. The big individual variations in the reaction did not allow meaningful averaging of the data, however the results clearly show the short duration of action of tizanidine.

Silperisone was compared to tolperisone and eperisone by intraduodenal administration as well to estimate functionally the relative oral bioavailability. Consistent with its long-lasting effect after i.v. dosing, intraduodenally it proved to be more potent and its effect was much longer lasting than those of tolperisone and eperisone (Fig. 18). Whereas 25 mg/kg tolperisone and eperisone produced moderate (<40%) and short lasting (<120 min) inhibitory effects, 12.5 mg/kg silperisone exerted a greater effect with little tendency to recover within 4 hours. Even its low dose of 2 mg/kg displayed a slight but clear-cut suppressant effect on the flexor reflex.

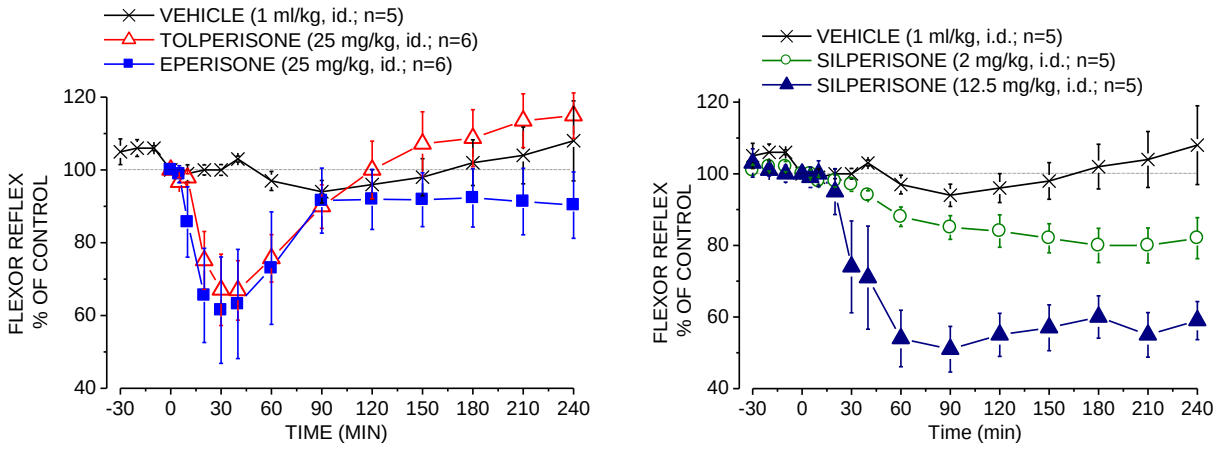


Fig. 18. The effects of CMRs administered intraduodenally on the flexor reflex in chloralose anaesthetised intact cats. Data are presented as means±S.E.M. Note the difference in the doses used and that silperisone even at 2 mg/kg had a distinct reflex depressant effect.

2.3.2.2 Flexor reflex in spinal cats.

The EMG reflex response in spinal cats was similar to that in the intact ones, except that the second component could be observed only in a few (10 out of 52) animals. Dose-response curves and the calculated ID_{50} values of silperisone and 8 other CMR drugs administered i.v. are presented in Fig. 19. The potency of silperisone was similar to that of tolperisone and eperisone and also similar to its potency observed in intact cats. The time courses of effects were similar to those observed in intact cats. The effect of silperisone again was much longer lasting than that of tolperisone or eperisone (not shown). *In vivo* potencies of the studied drugs in spinal cats were not substantially different from those in intact cats (see Table 4) except for diazepam, which was about 5-times less potent, and for carisoprodol, which selectively depressed the second component of the reflex that was mostly missing in spinal cats. Therefore, carisoprodol afforded negligible suppressant effect in average. Tizanidine, which was studied in three spinal cats, enhanced the flexor reflex by 25-125% at maximum in the dose range of 0.025-0.8 mg/kg i.v. without displaying a consistent dose-response pattern (not shown).

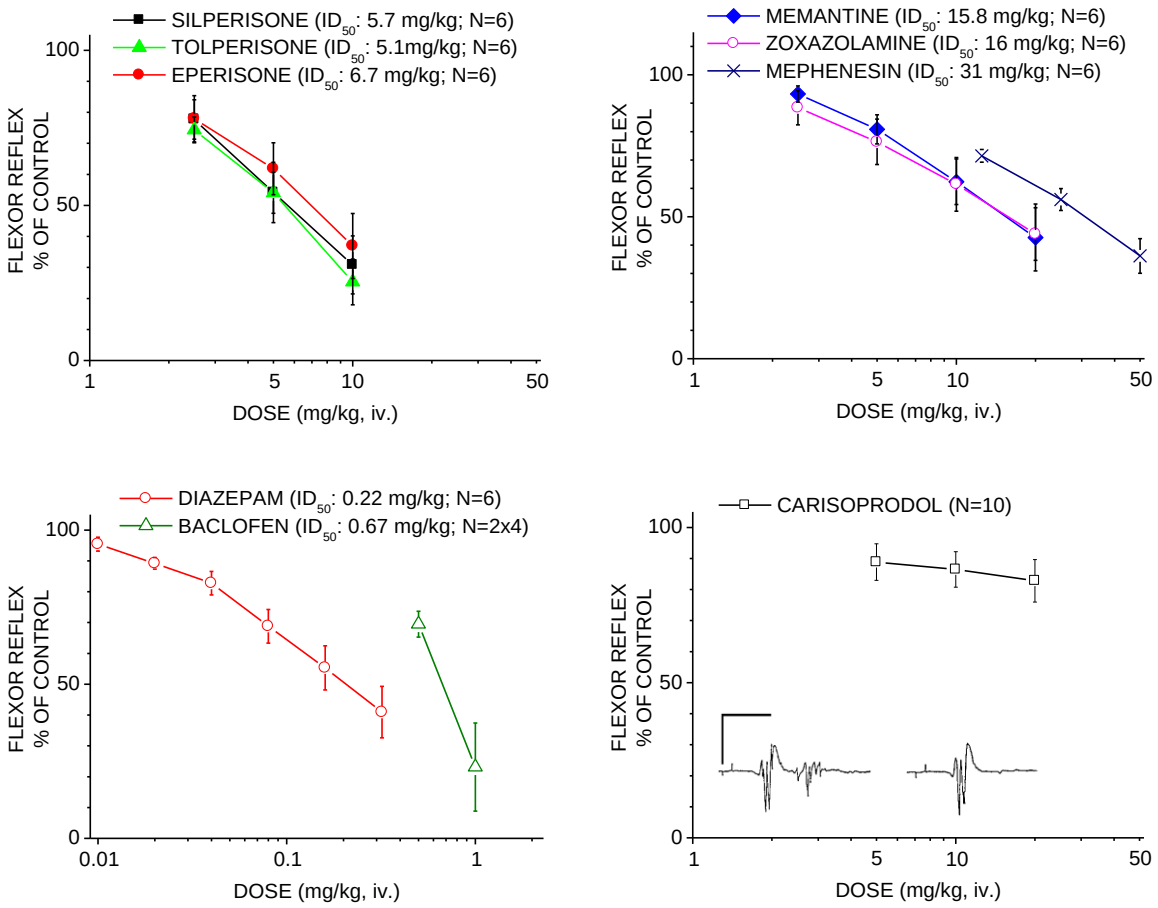


Fig. 19. Dose-response curves and ID_{50} values (i.e. doses causing 50% inhibition) of silperisone and various CMR drugs in the flexor reflex test in spinal cats. Ordinates: integrated EMG expressed as percentage of the value just prior to drug administrations. Except for baclofen, the results were obtained by cumulative intravenous administration of different doses of a drug to the same animal. Depending on the onset and recovery rates an appropriate interval (3-15 min) was kept between subsequent drug administrations to reach a maximum effect, but to achieve real cumulating. In the case of baclofen, different doses were tested in separate animals (4 animals per dose). Data are presented as mean±S.E.M. The insert in the bottom right graph shows EMG records before (left; calibration: 2 mV/20 ms) and after 5 mg/kg carisoprodol (right). Note that carisoprodol selectively depressed the second group of EMG discharges.

Table 4. *In vivo* potencies (ID_{50}s mg/kg., i.v.) of CMRs in the flexor reflex test in intact and spinal cats

Drugs	ID_{50} (mg/kg) in cats	
	intact	spinal
Silperisone	5.8	5.7
Tolperisone	4.3	5.1
Eperisone	3.5	6.7
Memantine	6.8	15.8
Mephenesin	21.0	31.0
Zoxazolamine	9.6	16.0
Baclofen	0.83	0.67
Diazepam	0.045	0.22
Carisoprodol	13.5	>>20

2.3.2.3 Patellar reflex in cats

The effects of tolperisone and silperisone (10 mg/kg i.v. each) on the patellar reflex are presented in Fig. 20. Tolperisone exerted mild and short lasting depressant effects on the patellar reflex, amounting maximum 15% in average. In some cases silperisone slightly decreased, while in other cases did not influence the amplitude of the patellar reflex at all. However, it stabilised the response to response variation, which was considerable in some cases, even when it had no effect on the average amplitude of the responses. Compared to tolperisone, the inhibitory action of silperisone on the patellar reflex was weaker. This result was also confirmed in comparative experiments when these drugs were applied successively to the same subject with keeping appropriate intervals between the drug applications.

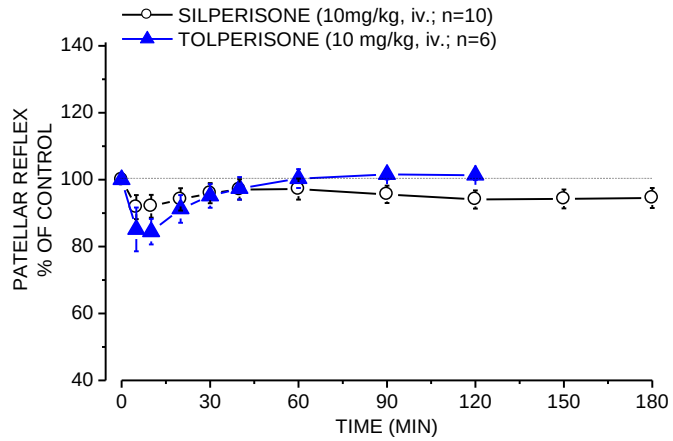


Fig. 20. Effects of tolperisone and silperisone on the patellar reflex in intact anaesthetised cats. Ordinate: amplitude of the reflex contractions expressed as percent of the value just prior to drug administrations. Data are presented as mean±S.E.M. Abscissa: time elapsed after dosing. Note that for sake of clarity and resolution the ordinate starts from 40%, as the effects are small.

2.3.2.4 Study on the reticulospinal control of the patellar reflex in cats

Electrical stimulation of the pontine facilitatory and bulbar motor inhibitory areas, as expected from previous descriptions (King and Unna 1954; Ochiai and Ishida 1981), enhanced and suppressed respectively the patellar reflex. Tolperisone, as known from an earlier report (Ochiai and Ishida 1981), inhibited both the stimulus-induced facilitation and the inhibition, and moderately suppressed also the "resting" patellar reflex (i.e. between two brain stem stimulations; see Fig. 21). Complete recovery was observed from the effect of tolperisone within 60-90 minutes in most cases.

Experiments were performed also with silperisone (5 or 10 mg/kg) administered intravenously. The records of two representative experiments are shown in Fig. 22. The results of individual experiments displayed some variations:

1.) Significant diminution of the "resting" patellar reflex responses was often observed. This finding was in contrast with the results of experiments where effect on the patellar reflex activity was studied alone (section 2.3.2.3); and was probably attributable to some persistent excitement of the stimulated facilitatory area between stimulations.

2.) The amplitude of the patellar reflex seen during the facilitatory stimulation was always decreased by the drug administration. However, the extent of this decrease was strongly variable from animal to animal. Complete abolishment of facilitation or reversal of facilitation into inhibition (e.g. bottom trace of Fig. 22) was seen in several cases.

3.) Patellar reflex depressing effect of stimulation of the inhibitory area was found to be enhanced in some cases (e.g. top trace in Fig. 22) or was not influenced by silperisone.

Only partial recovery was seen within 60 minutes, which is in line with long duration of action of silperisone.

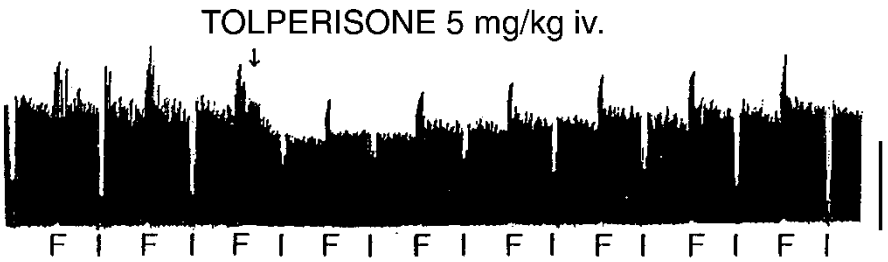


Fig. 21. Effect of tolperisone on the reticulospinal facilitation and inhibition of the patellar reflex of a cat. The traces show isometric recording of the patellar reflex contractions (upward deflections) on a chart recorder. Brainstem facilitatory (F) and inhibitory (I) areas were stimulated for 40 second periods in an alternating manner. The same type of stimulus was applied in every 10 minutes. Vertical calibration bar: 100 g. Timescale: 10 minutes between stimuli of the same type. Note that tolperisone suppressed both the facilitated responses (peaks and duration) and the inhibitions (smaller white valleys in the chart).

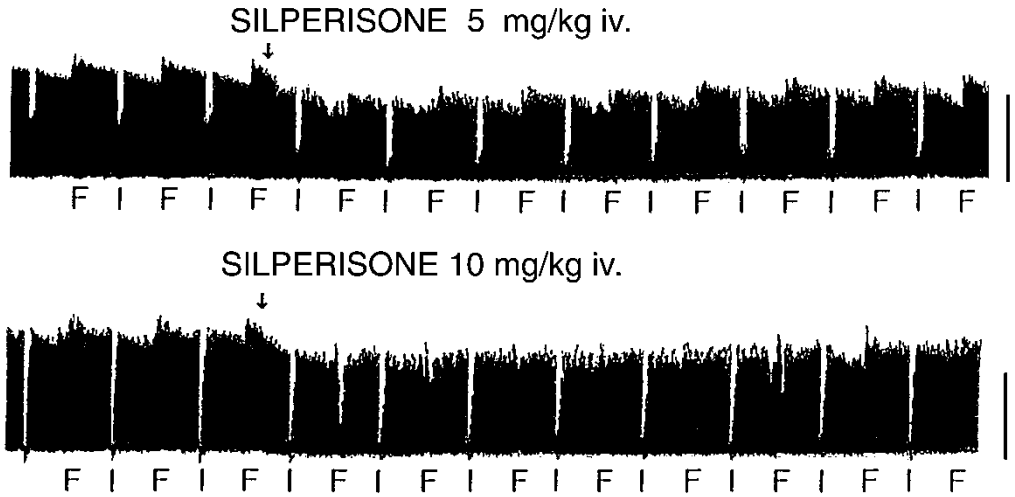


Fig. 22. Effect of silperisone on the reticulospinal facilitation and inhibition of the patellar reflex. The traces show isometric recording of the patellar reflex contractions (upward deflections) from two cats. Brainstem facilitatory (F) and inhibitory (I) areas were stimulated for 40 second periods in an alternating manner. The same type of stimulus was applied in every 10 minutes. Vertical calibration bar: 100 g. Timescale: 10 minutes between stimuli of the same type. Note that silperisone, although suppressed the facilitated responses, it either facilitated (upper trace) or at least did not inhibit the brainstem stimulation-induced inhibition (lower trace), which was a striking difference compared to tolperisone.

2.3.2.5 Spinal root potentials evoked by tibial nerve stimulation in cats

The recorded spinal root potentials and their separated components are shown in Fig. 23. These include (1) the ventral root reflex (VRR of L_7 or S_1 segments), which represents firing of motoneurones; (2) the dorsal root potential (DRP of L_6 or L_7 segments), which reflects slow and long lasting GABA-A receptor mediated depolarisation of primary afferents and (3) the dorsal root reflex (DRR), which represents (antidromically conducted) firing of the primary afferents emerging in ascending phase of the primary afferent depolarisation. From VRR the mono- (MSR) and polysynaptic reflexes (PSR) were separated for recording.

The effects of silperisone were compared to those of tolperisone, baclofen, mephenesin, diazepam and midazolam (a water soluble benzodiazepine). The dose-response relationships were determined in a cumulative manner (except for baclofen tested only at 0.5 mg/kg, i.v.), and then the time-course of the effect was observed for at least two hours. We have previously published the results with the reference compounds (Farkas *et al.* 1989) and for more details on the reference compounds the reader is referred to that paper. Here we present detailed data only on tolperisone and silperisone (Fig. 24, Fig. 25 and Fig. 26) but summarise different profiles of the different CMR drugs in a summary table (Table 5). The changes caused by silperisone were qualitatively similar to those induced by tolperisone. The monosynaptic reflex (MSR), the polysynaptic reflex (PSR) and the dorsal root reflex (DRR) were significantly reduced, while the DRP was minimally affected. In some cases (e.g. the one

in Fig. 25) a slight enhancement in the integrated DRP and sometimes in the DRP amplitude was observed. Nevertheless, experiments were inconsistent in this respect resulting in no significant change in average. The dose-response curves of silperisone for MSR and PSR, when based on the maximum inhibitions, were almost overlapping with the corresponding curves of tolperisone. Attenuation of MSR was the most pronounced effect with both compounds. However, the depression of DRR caused by silperisone was weaker than that caused by tolperisone, suggesting some minor differences in their presynaptic effects. On the other hand, silperisone exerted a much longer lasting effect as compared to tolperisone, which is in line with results in other tests presented above.

As discussed in our paper in details (Farkas *et al.* 1989), the two benzodiazepines – diazepam and midazolam –, as expected, potentiated the GABA-A mediated DRP and DRR, did not have a significant effect on MSR but depressed PSR. Baclofen exerted very strong (complete abolishing) effect on MSR, less pronounced but still strong reducing effect on PSR and much weaker effects on DRP and DRR. Mephenesin, which had been termed as an interneurone depressant (King and Unna 1954), selectively suppressed PSR without affecting MSR. It had small effect on DRP but markedly reduced DRR (Table 5). Dissociation between effects on DRP and DRR were even more characteristic for tolperisone and silperisone. These dissociations may reflect inhibitory effects on the spike generation, and in this respect the effect of tolperisone seemed to be relatively stronger than that of silperisone.

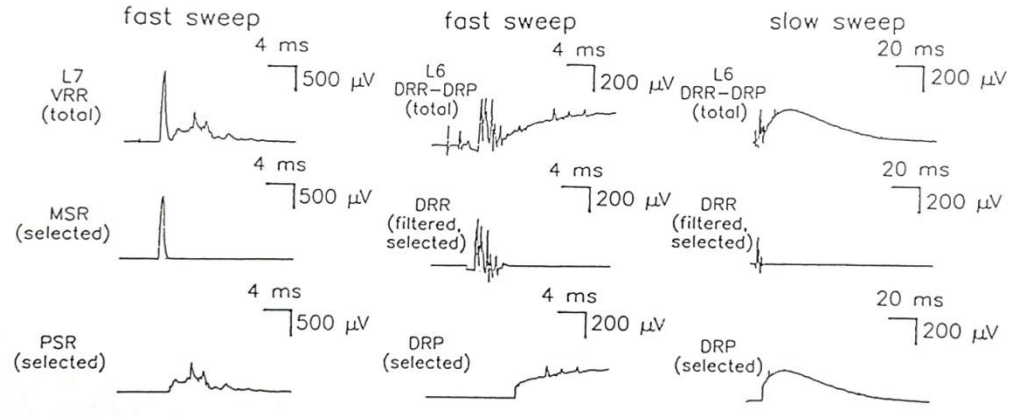


Fig. 23. Representative recordings (oscilloscopic charts) of ventral and dorsal spinal root potentials elicited by tibial nerve stimulation in a cat. Charts in the upper row show the total potentials (VRR: ventral root reflex; DRR-DRP dorsal root reflex and potential). The lower two rows show the different separated components that were fed into the integrators for quantification.

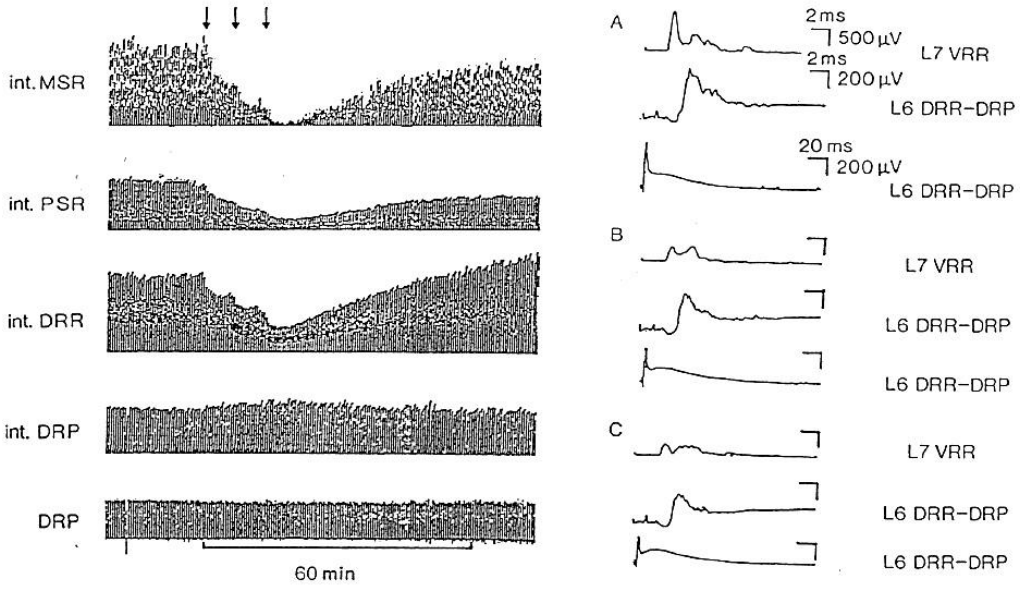


Fig. 24. Effect of tolperisone on the spinal root potentials in a cat. On the left side: integrated records of the monosynaptic (MSRint) and polysynaptic (PSRint) ventral root reflex (VRR), dorsal root reflex (DRRint) and dorsal root potential (DRPint). The bottom trace is direct recording of the upward (negative) deflections of the dorsal root potential (DRP). Arrows indicate successive intravenous administrations of 2.5, 2.5 and 5 mg/kg tolperisone. On the right side: Individual responses A: before drug administrations; B: 5 minutes after the 1st administration; C: 5 minutes after the 2nd administration.

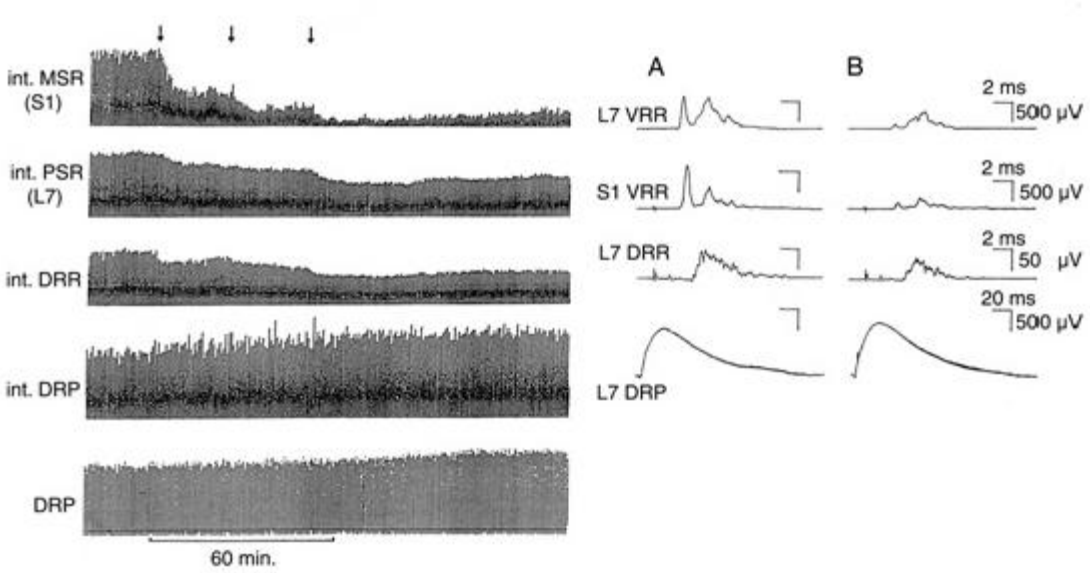


Fig. 25. Effect of silperisone on the spinal root potentials in a cat. On the left side: integrated records of the monosynaptic (MSRint) and polysynaptic (PSRint) ventral root reflex (VRR), dorsal root reflex (DRRint) and dorsal root potential (DRPint). The bottom trace is direct recording of the upward (negative) deflections of the dorsal root potential (DRP). Arrows indicate successive intravenous administrations of 2.5, 2.5 and 5 mg/kg silperisone. On the right side: Individual responses, (A) before drug administrations; (B) 20 minutes after the last administration.

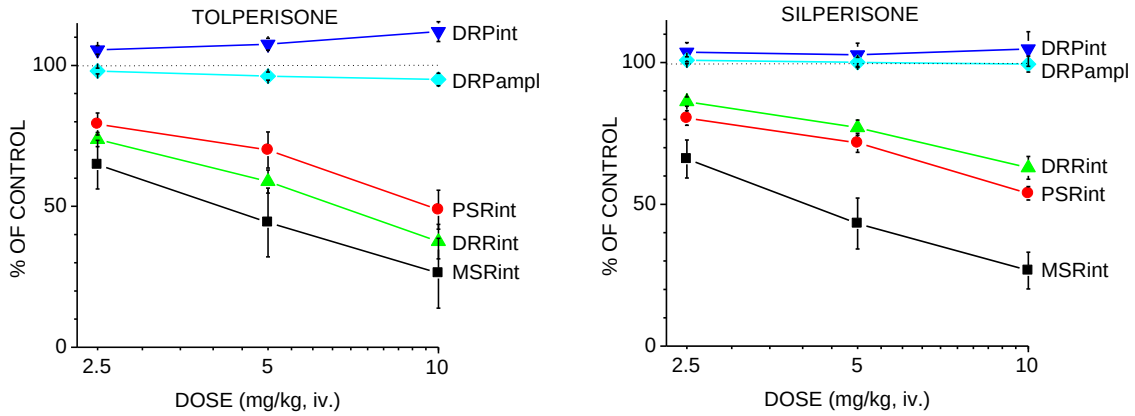


Fig. 26. Cumulative dose-response curves of silperisone and tolperisone based on the effects on spinal root potentials in cats. Results are presented as mean±S.E.M. in 6 animals. Note that effects of both drugs on ventral root reflexes were practically of the same magnitude, only the weaker effect of silperisone on DRR was a notable difference.

Table 5. Summary of profiles of different CMR drugs in the cat spinal root potential test

Drug (top dose*, i.v.)	MSR	PSR	DRR	DRPint	DRPampl
Mephenesin (50 mg/kg)	0	↓↓	↓↓	↓	↓
Baclofen (0.5 mg/kg)	↓↓↓↓	↓↓↓	↓↓	↓	↓
Diazepam (3.2 mg/kg)	0	↓↓	↑↑	↑↑	↑
Midazolam (3.2 mg/kg)	0	↓↓	↑↑	↑↑	↑
Tolperisone (10 mg/kg)	↓↓↓	↓↓	↓↓↓	0↑	0
Silperisone (10 mg/kg)	↓↓↓	↓↓	↓↓	0	0

Notations:
Ranges of % change: 0 [<±10]; ↓ [-15-40]; ↓↓ [-40-60]; ↓↓↓ [-60-80]; ↓↓↓↓ [-80-100]; 0↑ [+12]; ↑ [+15-40]; ↑↑ [>50]
*cumulatated dose
MSR: monosynaptic reflex; **PSR**: polysynaptic reflex; **DRR**: dorsal root reflex; **DRPint**: dorsal root potential integral (area under curve); **DRPampl**: dorsal root potential amplitude; MSR, PSR and DRR were quantified also by area under curve (integral).

2.3.2.6 Intercollicular decerebrate rigidity in cats

The effect of silperisone (10 mg/kg, i.v.) was studied in five cats. Representative records from an experiment are shown in Fig. 27. Tolperisone (5 mg/kg, i.v.) was also tested in 2 of these animals after a 3-hour interval, and in one case 2 hours before the treatment with silperisone. The effect of 10 mg/kg tolperisone was compared to the effect of 10 mg/kg silperisone in one experiment. The comparison between the two drugs is presented in Table 6. The method we used allowed simultaneous detection of the patellar reflex, post-activation enhancement in the muscle-tone ("afterdischarge") and "resting" EMG activity. "Afterdischarge" was defined as a transiently increased EMG activity for 2-5 seconds after a short (ca. 40-80 ms) inhibitory period following the huge peak corresponding to synchronized EMG discharge of the patellar reflex (see right traces in Fig. 27). In the experiment shown in the figure, 10 mg/kg silperisone nearly completely abolished the "resting" activity and markedly suppressed the "afterdischarge". For quantitative evaluation we used the

integrated EMG records which summed these two types of activities. On the other hand, silperisone did not attenuate the patellar reflex at all. It even slightly potentiated the reflex sometimes, such as in the case presented in Fig. 27, where the fluctuations in the continuous EMG activity (afterdischarge+resting) and in the magnitude of the patellar reflex were anti-coincident. We assume that this may have been due to recurrent inhibition of the patellar reflex that was promoted by the fluctuating tonic efferent activity.

Compared to tolperisone, the effects were qualitatively similar. However, silperisone was less potent in attenuating the decerebrate rigidity. Namely, 10 mg/kg provided quantitatively comparable effects to 5 mg/kg tolperisone. When the effects of 10 mg/kg of the two drugs were compared in the same cat, the effect of tolperisone was greater (inhibition of 97% *vs*. 80%). Unfortunately, the decerebrate rigidity was not stable over a long period of time. Therefore these experiments did not allow drawing conclusions on the time-courses of drug effects and did not prove to be reliably good for pharmacodynamic characterisation of CMR drugs.

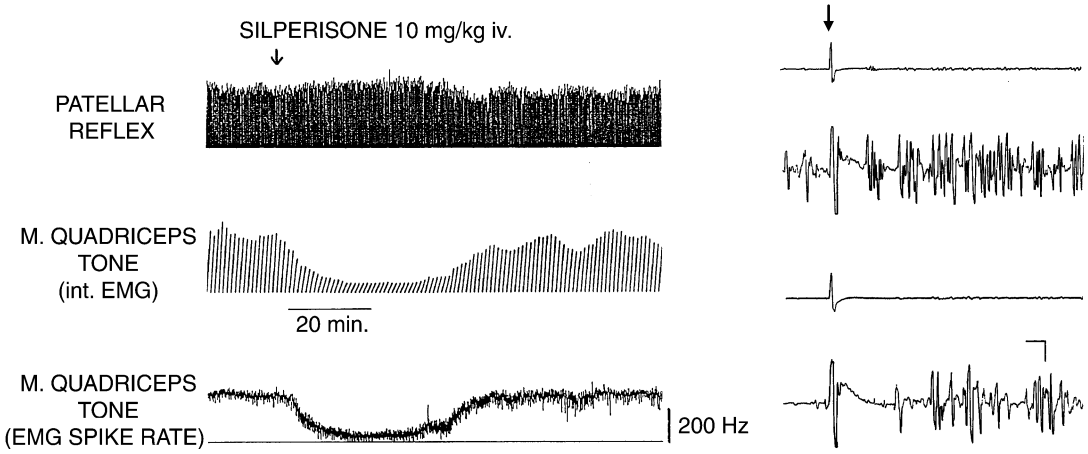


Fig. 27. Effect of silperisone on intercollicular decerebrate rigidity in a cat. The right patellar tendon was tapped every 10 seconds. Traces on the left were derived by analog signal processing from the EMG activity recorded from the right quadriceps femoral muscle. Top trace: height of each deflection represents the integrated patellar reflex related EMG potential. Middle trace: continuous integral of the EMG activity reset every second. Bottom trace: ratemeter record of the EMG spike rate. The latter two records represent mainly the "resting" activity and "afterdischarges" between stimulations. Right panel traces: EMG activity. The patellar reflex was elicited at the arrow and recorded at two different amplifications. Top two traces: before silperisone; bottom two traces: after silperisone. Vertical calibration bar: 5 mV and 100 µV for low and high amplification records, respectively. Horizontal bar: 20 ms. Please, note that high-pass filtering was also different for the low and high amplification records (1 Hz and 200 Hz, respectively) and that in the latter one the deflections of the patellar reflex were truncated. The short segment before the patellar reflex related deflections shows the "resting" activity, whereas the activity following a short post-stimulus inhibitory period is the "afterdischarge".

Table 6. Comparison of the effect of silperisone (n=5) and tolperisone (n=3) on the intercollicular decerebrate decerebrate rigidity in cats. In 2 cases tolperisone was administered at least 3 hours after, in 1 case 2 hours before silperisone to the same cats.

	Inhibition of integrated EMG expressed as % of control (mean±S.E.M.)	
	M. quadriceps femoris	M. triceps brachii
Silperisone 10 mg/kg i.v.	70±14.9	66±8.3
Tolperisone 5 mg/kg i.v.	63±1.7	75±14.4

2.3.3 Instrumental neurophysiological studies in unconscious rats

2.3.3.1 Investigation of the ventral root reflex potentials

We differentiated three characteristic components of the the dorsal root-evoked ventral root reflex (VRR) potential of rats: mono-, di- and polysynaptic reflexes (MSR, DSR and PSR, respectively, see Fig. 28), as described previously (Farkas and Ono 1995).

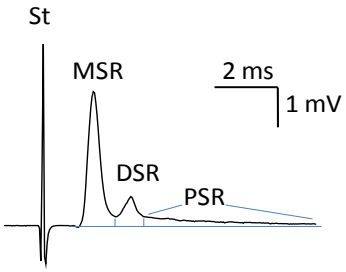


Fig. 28. The ventral root reflex recorded from the L_5 segment and its three distinguished components, which were quantified by integration (calculation of area under curve): monosynaptic reflex (MSR), disynaptic reflex (PSR) and polysynaptic reflex (PSR). "St" denotes the stimulation artefact.

A typical ventral root reflex response to stimulation of the dorsal root, and its attenuation by silperisone are shown in Fig. 29A-B. Silperisone (10 mg/kg, iv.) attenuated all the three components with the following sensitivity order for the various components: MSR>DSR>PSR. The onset of its effect was interestingly slower for DSR and PSR than for MSR, which feature was unique among the studied drugs. Its duration of action was longer than that of tolperisone, eperisone and lidocaine (Fig. 29C). The profiles of actions of tolperisone, eperisone and silperisone in terms of relative effect on the three different VRR components were very similar. However, peak effects of tolperisone were greatest and its duration of action was the shortest among the tolperisone-type compounds. The profile of lidocaine was substantially different, as it exerted relatively much weaker depressant effect on MSR (Fig. 29D).

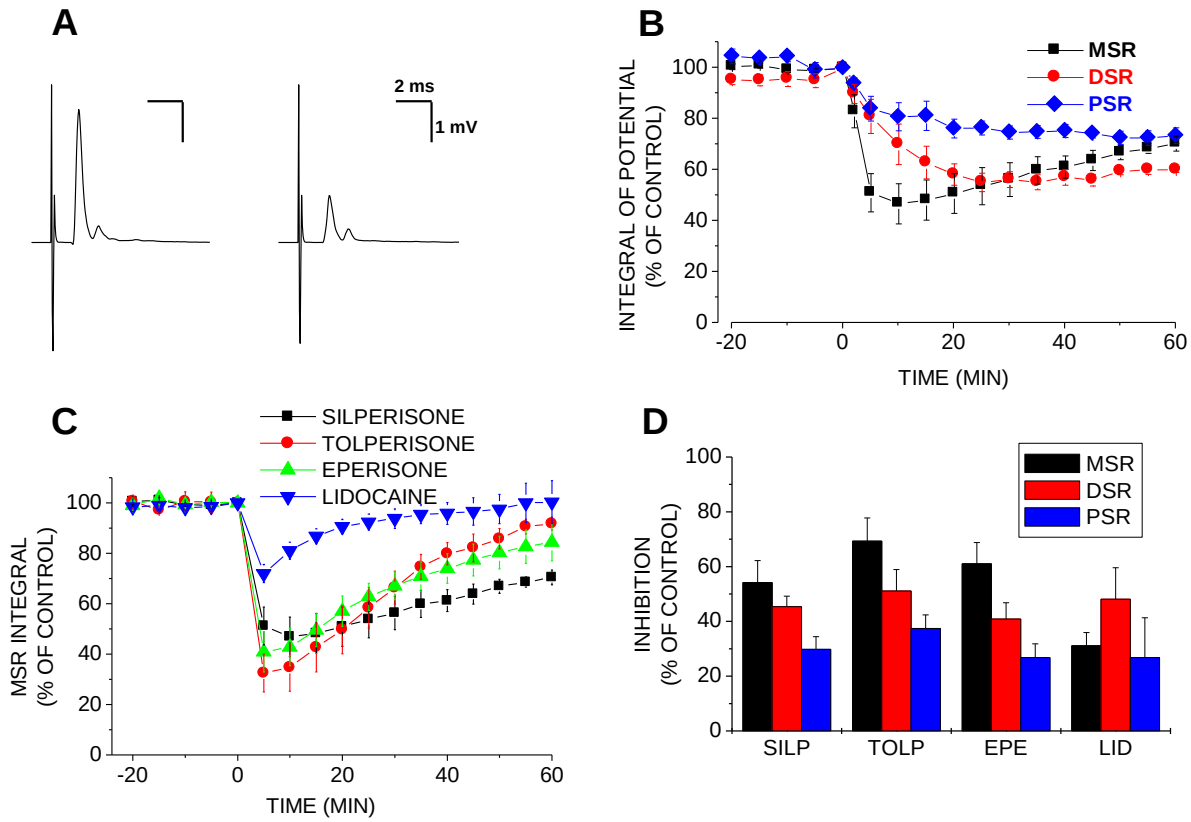


Fig. 29. Effects of drugs on the ventral root reflex evoked by dorsal root stimulation *in vivo* in spinal rats. A: representative averaged potential before (left) and 10 min after (right) administration of silperisone (10 mg/kg i.v.). Calibration bars: 1 mV, 2 ms. B: time course of effect of silperisone on the VRR components: monosynaptic reflex (MSR), disynaptic reflex (DSR), polysynaptic reflex (PSR). C: Time course of effect of four drugs (10 mg/kg i.v. each) on MSR. D: Maximum inhibitory effects of drugs (10 mg/kg i.v.) on different reflex components expressed as percentage of the pre-dose control responses. All the data (B-D) are presented as mean±S.E.M. from five experiments.

2.3.3.2 Neuronal excitability test

The effect of silperisone on excitability of motoneurones and primary afferents was compared to that of tolperisone and lidocaine. For comparative purposes we usually investigated the effect of two or three drugs in the same experiment with at least one-hour interval between drug administrations. However, only the first drug administrations were included in the statistical evaluation. Silperisone, tolperisone and lidocaine were administered as first test compound in 5, 3 and 3 experiments, respectively.

Characteristic potentials recorded from the dorsal and ventral roots following intraspinal electrical stimulation applied into the motoneurone pool are shown in Fig. 30A. The compound action potential recorded from the dorsal root reflects excitability of primary afferents (PAF). From the ventral root two successive potentials can be recorded: the first one representing direct excitability of the motoneurones (MN), and the second one, which is due to monosynaptic (MS) activation of motoneurones (Ono *et al.* 1984; Farkas and Ono 1995).

Silperisone (10 mg/kg i.v.) depressed direct excitability (MN response) of motoneurones by 15%, while the synaptic (MS) response was decreased by 68% in average (Fig. 30A-B). It exerted no effect on excitability of primary afferents (PAF). The onset of the effect of silperisone was relatively slow, reaching the maximum in 15 min. Tolperisone (10 mg/kg i.v.) reached a maximum effect of 24, 80 and 8% inhibition of MN, MS and PAF, respectively, in 6-8 min (Fig. 30C). Lidocaine (10 mg/kg i.v.) produced more marked depression of MN and PAF (30% and 14%, respectively; see Fig. 30D) than tolperisone or silperisone. On the other hand its depressant effect on MS (67%) was similar to that of silperisone and less pronounced compared with tolperisone. Although the differences in the average results and profiles of actions may seem to be small, they were confirmed when the different compounds were administered successively in the same experiment.

In summary, lidocaine had significantly greater depressant effect on the direct electrical excitability of motoneurones and primary afferents than silperisone, whereas their efficacies to inhibit the synaptic transmission were similar. The profile of tolperisone was in between these drugs, as it had a small effect on PAF.

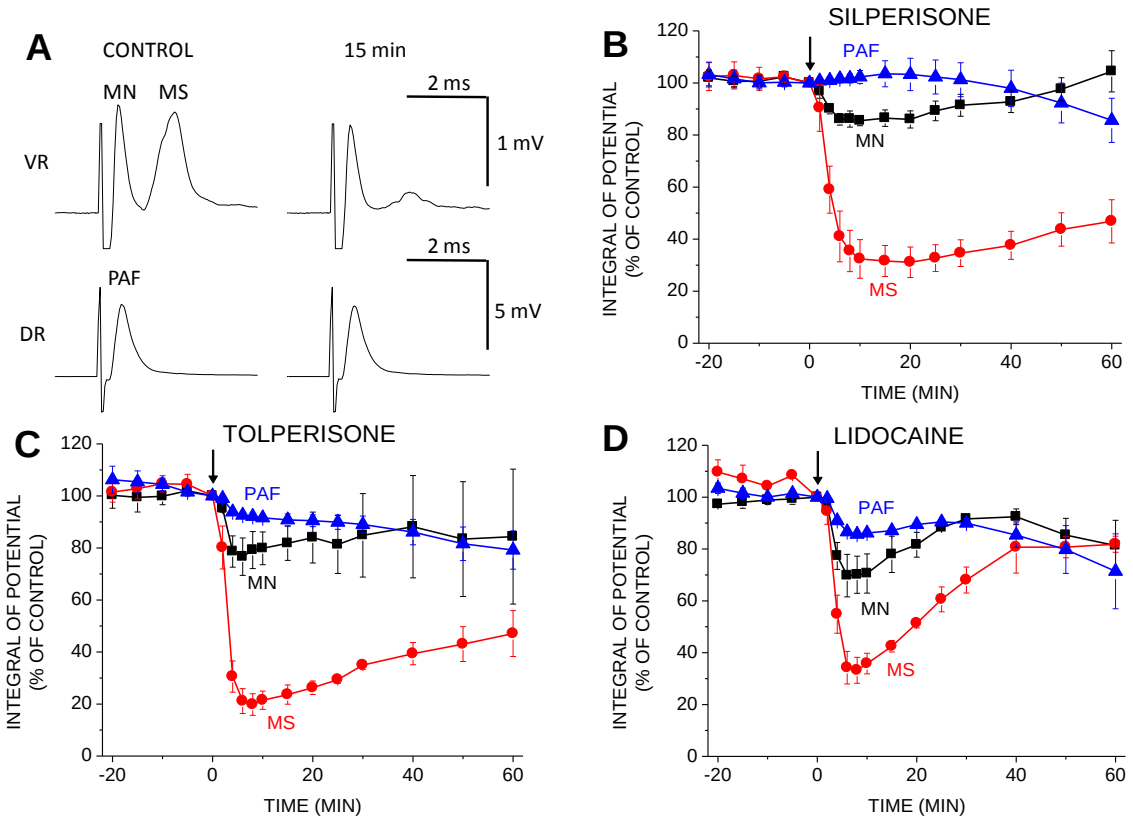


Fig. 30. Effects of drugs on direct electrical excitability of motoneurones (MN), their monosynaptic excitation (MS) and excitability of primary afferents (PAF). A: averaged potentials recorded from L_5 ventral root (top – VR) and from L_5 dorsal root (bottom – DR), before (left) and 15 minutes after (right) administration of silperisone (10 mg/kg i.v.). Effect of silperisone (B; N=5) tolperisone (C; N=3) and lidocaine (D; N=3) on MN (■), MS (●) and PAF (▲), 10 mg/kg i.v. for all. Data are presented as percentage of pre-dose (time zero) control mean±S.E.M. Arrows indicate the time of drug administrations.

2.3.3.3 *Study of feed-forward inhibition*

The effect of silperisone on feed-forward inhibition was investigated in 7 experiments. The characteristic inhibition curve, i.e. evoked responses plotted against the C-T interval under control condition, is shown in Fig. 31A. The evaluation was based on recording of the area under the curve of MSR. We deliberately applied low-intensity conditioning stimuli which produced moderate degree of presynaptic inhibition, thereby enabling detection of either facilitating or depressant effects on the inhibition. The inhibition curve showed a first peak at C-T intervals of 5-10 ms, which may be attributed to a short latency feed-forward postsynaptic inhibition on motoneurones, such as the one involved in reciprocal inhibition (Eccless 1964). A second peak at 80 ms and a prolonged long-lasting slow decline in the inhibition curve represents the presynaptic inhibition of MSR, which is associated with GABA-A receptor mediated primary afferent depolarisation. Silperisone (5 mg/kg, i.v.) depressed MSR by 26.8±4.9% (mean±S.E.M.). However, the unconditioned and conditioned responses decreased to the same extent. Thus the inhibitory curve scaled up by normalising to the unconditioned response remained unchanged. Similar results, i.e. no change in the normalized inhibitory curve by silperisone, were observed in experiments where higher conditioning stimulus intensities were used (not shown).

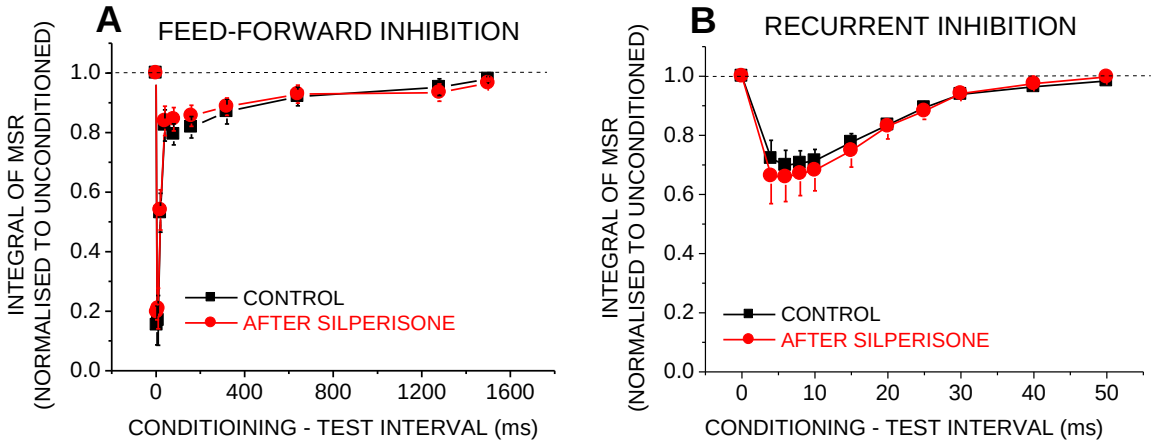


Fig. 31. Lack of effect of silperisone on two inhibitory pathways of the spinal cord. A: Effect of 5 mg/kg silperisone on feed-forward inhibition (N=7). B: Effect of 5 mg/kg i.v. silperisone on recurrent inhibition (N= 7). The integrated MSR is plotted against the interval between conditioning and test stimuli. All data were normalized to the value recorded without conditioning stimulation both before (■) and after (●) the drug administration. Data are presented as mean±S.E.M. Note that the decrease in unconditioned response caused by silperisone is masked by this kind of plotting.

2.3.3.4 *Study of recurrent inhibition*

The effect of silperisone on the recurrent inhibition was investigated in 7 experiments. The results are shown in Fig. 31B. Testing was also based on recording of the area under the curve of MSR. The inhibitory response shown in the figure was elicited by supramaximal conditioning stimulation. It was

maximal at C-T intervals of 6-10 ms and after a gradual decline of the inhibition a nearly complete recovery occurred within 50 ms. Silperisone (5 mg/kg iv.) possessed no effect on the recurrent inhibition, although attenuated the unconditioned MSR by 30.9±6.6% (mean±S.E.M.).

2.3.3.5 Study of afferent nerve conduction

Possible contribution of a local anaesthetic-type inhibition of the afferent nerve conduction to the reflex inhibition following systemic administration of silperisone, tolperisone and lidocaine (10 mg/kg iv. each) was investigated. Typical compound AFP reflecting action potentials of A fibres recorded from the L_4 dorsal root following stimulation of the sciatic nerve before and after administration of the drugs are shown in Fig. 32. Silperisone and tolperisone left AFP practically unchanged Fig. 32A-B). Lidocaine exerted some very slight but noticeable inhibitory effect manifested in both decreased potential amplitude and increased conduction time (Fig. 32C). However, this slight depression seemed to be negligible compared to the depression of VRR afforded by the same dose of lidocaine (*cf.* Fig. 29).

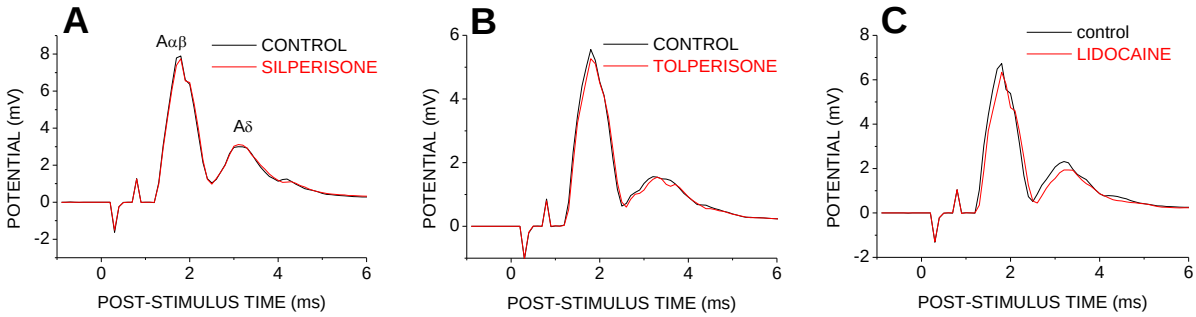


Fig. 32. Drug effects on the afferent nerve conduction *in vivo*. Panels show the compound afferent fibre potentials (average of ten traces) recorded from the L_4 dorsal root in three representative experiments. Black line: before, red line: after the drug administration. Effects of 10 mg/kg i.v. silperisone (A), tolperisone (B) and lidocaine (C) are shown. A very small depressant (and delaying) effect is discernible after lidocaine, perhaps can be noticed after tolperisone and is unnoticeable after silperisone.

2.3.3.6 Intercollicular decerebrate rigidity in rats

The effect of silperisone was studied at doses of 2.5, 5, 10 mg/kg (i.v.), that of tolperisone at doses of 1.25, 2.5, 5 mg/kg (i.v.; 5 experiments for each dose; Fig. 33). Both silperisone and tolperisone dose-dependently inhibited the decerebrate rigidity. Regarding the maximum effects, tolperisone was 1.5 times more potent than silperisone. Concerning the time-courses of the effects at various doses both compounds yielded somewhat contradictory results. Namely, high doses seemed to have shorter duration of action than medium ones. However, the lack of long-term stability of rigidity again made it difficult to draw reliable conclusions about the time-course of effects based on this method. Taking into account also the low success rate of these experiments, we did not propose the decerebrate rigidity as a routine pharmacodynamic test for comparative studies between CMR drugs.

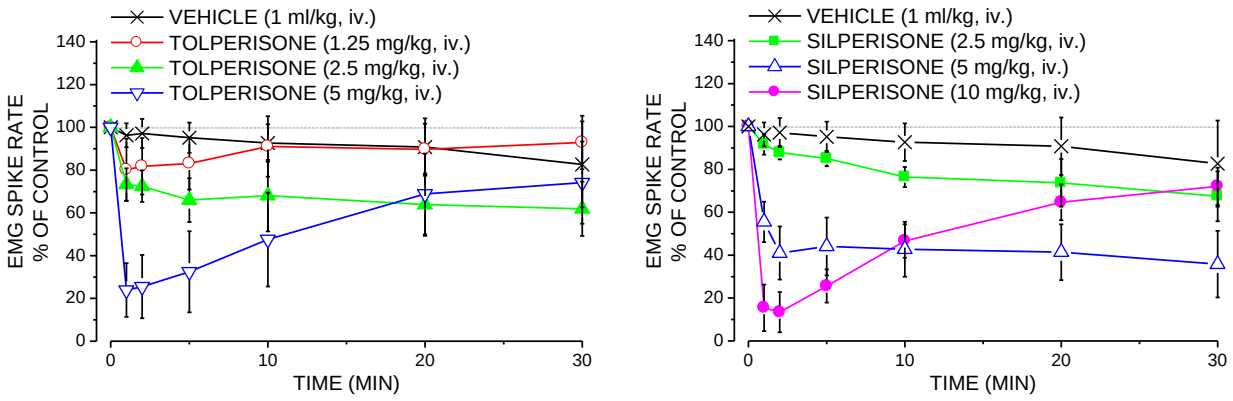


Fig. 33. Effects of different intravenous doses of silperisone and tolperisone on the intercollicular decerebrate rigidity in rats. Ordinate: spike rate of the EMG activity from gastrocnemius muscle expressed as percent of the value just prior to drug administrations. Data are presented as mean±S.E.M from 5 experiments each. Abscissa: time elapsed after dosing.

2.3.4 Studies in the isolated hemisected rat spinal cord in vitro

2.3.4.1 Spinal root potential studies

A characteristic afferent fibre potential (AFP) arriving at the spinal cord surface and a dorsal root stimulation induced ventral root potential (DR-VRP) are shown in Fig. 10. When normal ACSF is applied, the afferent fibre potential (AFP) recorded from the dorsal root adjacent to the stimulated one is superimposed on the DRP (primary afferent depolarisation; see Fig. 40A). Therefore, when we intended to study the pure AFP, Ca^{2+}-free ACSF was applied (left trace in Fig. 10), which abolished the synaptic transmission and DRP. A typical DR-VRP (Fig. 34A) under routine conditions consisted of an initial biphasic population spike (monosynaptic reflex; MSR) superimposed on a tonic long-lasting potential shift reaching a maximum at 10-15 ms following the stimulation of the dorsal root. This latter potential basically represents population excitatory postsynaptic potential (EPSP) of motoneurones (Siarey *et al.* 1992). Nevertheless, small waves attributable to asynchronous firing of motoneurones were often discernible superimposed on this tonic potential. Elevation of the temperature of the bathing solution depressed MSR and allowed the study of an almost pure EPSP related potential (Fig. 34B).

In the present study we investigated DR-VRP components only within the post-stimulus time-window of 0-180 ms. Consistent with previous findings (Thompson *et al.* 1992; Kocsis *et al.* 2003) these components, including the highly NMDA antagonist sensitive “tail part” of EPSP (80-180 ms), were apparently dependent on stimulation of A-type afferent fibres and reached a maximum at a stimulus strength where the stimulation of Aδ fibres became supramaximal (Fig. 35).

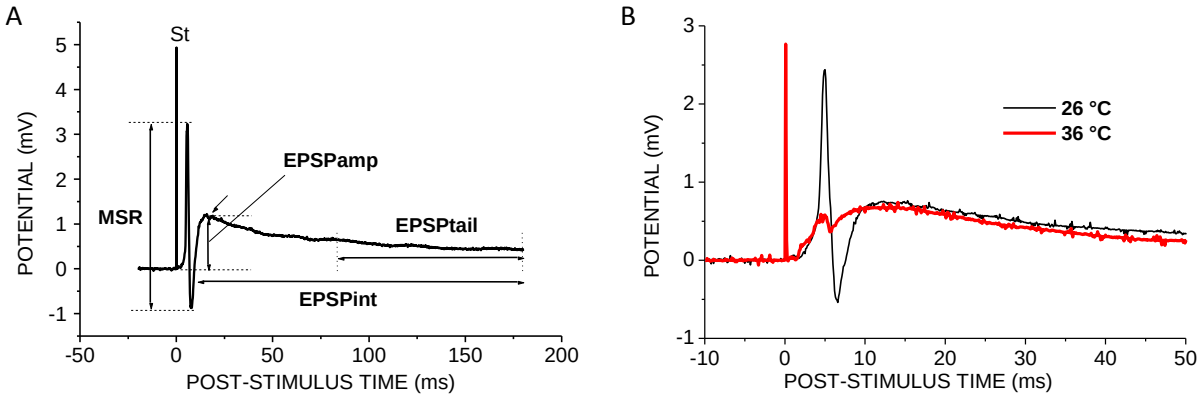


Fig. 34. A: Components of a typical DR-VRP (recorded at 26 °C). It consists of a monosynaptic compound action potential superimposed on a prolonged potential shift representing population EPSP of motoneurones. Occasionally polysynaptic reflex discharges (see arrow) could also be observed superimposed on top of the population EPSP. St: stimulus artefact. The routinely distinguished and evaluated components: peak to peak amplitude of the monosynaptic reflex (MSR), amplitude of EPSP (EPSPamp) integral (area under the curve) of EPSP (10-180 ms) (EPSPint) and integral of the "tail" (80-180 ms) of EPSP (EPSPtail). B: Elevation of the temperature attenuated the reflex potential but hardly influenced the EPSP related potentials.

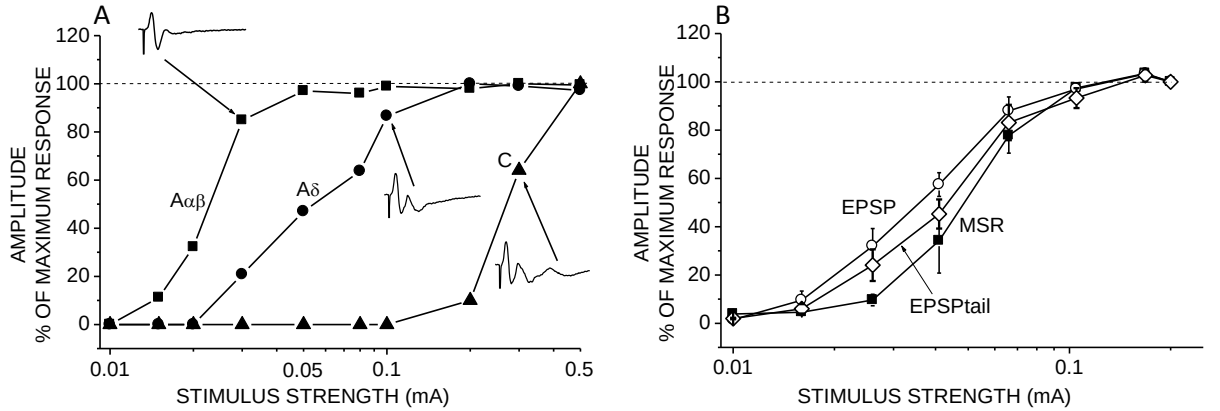


Fig. 35. Dependence of the recorded potentials on the stimulus strength. Pulse width was 0.1 ms in all cases. A: different fibre related components of the afferent fibre potential (AFP) in Ca^{++} free medium. The inserts show examples of AFP at different stimulus intensities from which the corresponding points of the curves were derived. Peak-to-peak amplitudes of the three components (as shown by the arrows) are plotted against the stimulus strength. Presented data are from a representative experiment. B: Different components of the dorsal root evoked ventral root potential (recorded in normal ACSF). Amplitude of the monosynaptic reflex (MSR – peak-to-peak), EPSP (zero-to-peak) and integral of the late part of EPSP (tail – 80-180 ms) are plotted against the stimulus strength. Data are presented as mean±S.E.M. from 4 experiments.

Silperisone caused concentration dependent depression of all the studied components of DR-VRP. MSR and tail of EPSP were the most sensitive parameters, whereas early part of EPSP was apparently less attenuated (Fig. 36A-C). Apart from responses just above the threshold, the depression afforded by different doses of silperisone was not dependent on the stimulus strength. Namely, maximum responses of I-V curves were attenuated, rather than a leftward shift of the curve occurred (Fig. 36D).

Hence, the depressant effect was not attributable to an increase in the excitability threshold of primary afferents.

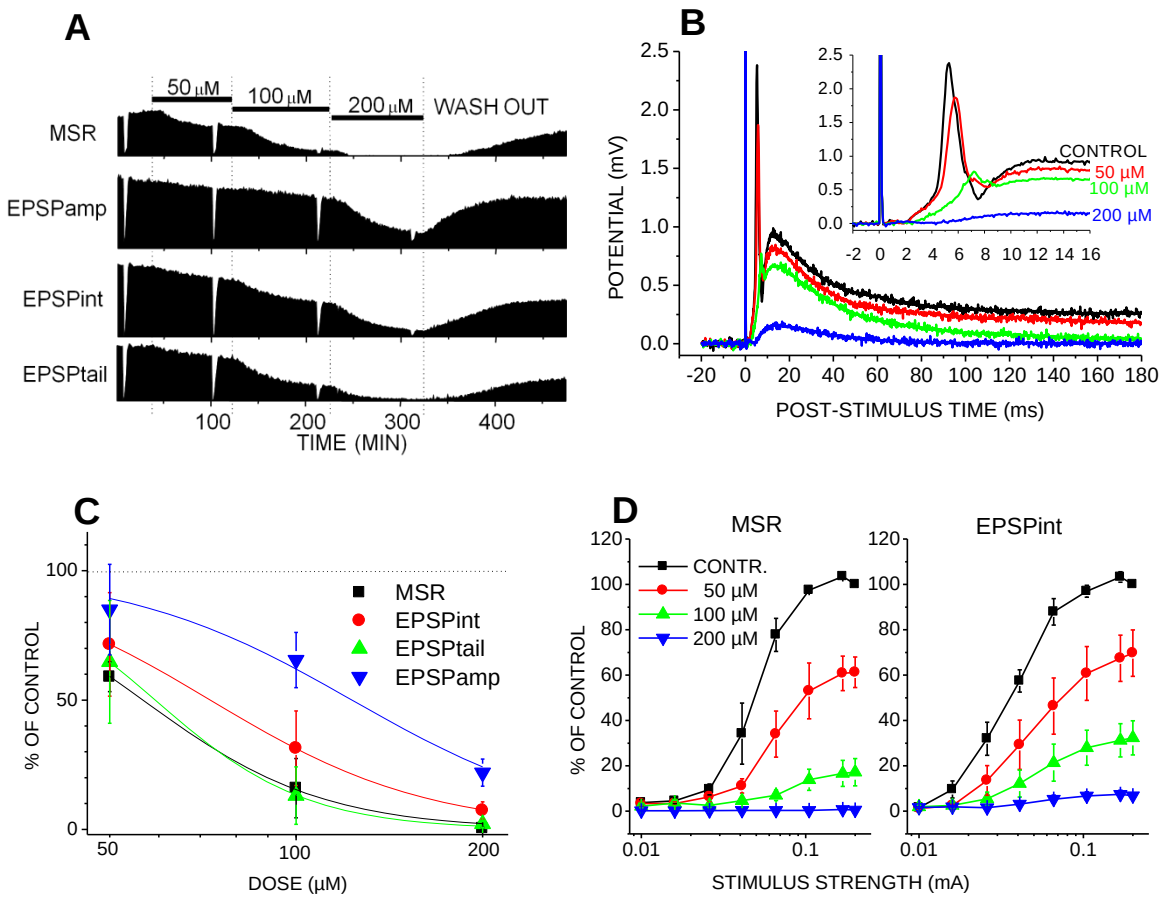


Fig. 36. Effects of silperisone on dorsal root stimulation evoked ventral root potential (DR VRP) components *in vitro*. A: Response-time plots from a typical experiment. Heights of the graphs are proportional to the calculated parameters: peak-to-peak amplitude of monosynaptic compound action potential (MSR), integral of EPSP (EPSPint), amplitude of EPSP (EPSPamp), integral of late part of EPSP (EPSPtail), see also Fig. 34A. Increasing concentrations of silperisone (50-200 µM) were applied via the bathing solution, and finally washed out with drug-free ACSF. The valleys before each dosing and wash out represent testing of the input-output relationship shown in D. B: Potentials from the same experiment. The insert shows the same potentials on a more extended time-scale to allow the observation of the effect on the monosynaptic reflex. C: The dose-response curves of silperisone based on effects on the four calculated parameters. D: Effect of silperisone on the stimulus-strength dependence of two calculated parameters of the DR-VRP. Data are presented as mean±S.E.M. from 4 experiments. Note that efficacy of silperisone was not dependent on the stimulus intensity.

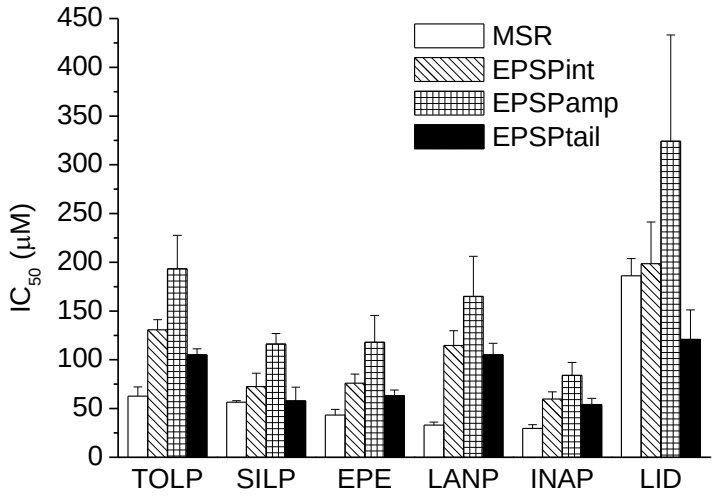


Fig. 37. Profiles of actions of different tolperisone type CMR drugs (tolperisone-TOLP, silperisone-SILP, eperisone-EPE, lanperisone-LANP, inaperisone-INAP) and lidocaine (LID) on dorsal root stimulation evoked ventral root potential (DR-VRP) components *in vitro*. IC_{50} values of the drugs studied are based on inhibition of different reflex components – peak-to-peak amplitude of monosynaptic compound action potential (MSR), integral of EPSP (EPSPint), amplitude of EPSP (EPSPamp), integral of late part of EPSP (80-180 ms post-stimulus time; EPSPtail). All columns represent mean±S.E.M. from 4 experiments.

Comparing the profiles of different tolperisone-type compounds including tolperisone, silperisone, eperisone, lanperisone and inaperisone, as well as of lidocaine, with regards to rank order of potencies on different DR-VRP components, quite similar patterns of effects were observed with all the tolperisone-type compounds and somewhat different with lidocaine (Fig. 37). Inaperisone was the most potent among the tolperisone-type compounds; eperisone and silperisone were approximately equally potent, whereas lanperisone had the strongest effect on MSR but relatively weaker effects on the EPSP components. Lidocaine was the least potent among the tested drugs especially in depressing MSR and early part of EPSP. However, it was relatively potent in attenuating the "tail" and in this respect it was almost equally potent with tolperisone and lanperisone. Hence, its profile was different from the tolperisone-type CMR drugs.

The spinal reflex inhibitory effect of silperisone was not reversed by the GABA-B antagonist SCH-50911. On the contrary the effect of the GABA-B agonist baclofen was concentration dependently reversed by SCH-50911 (Fig. 38).

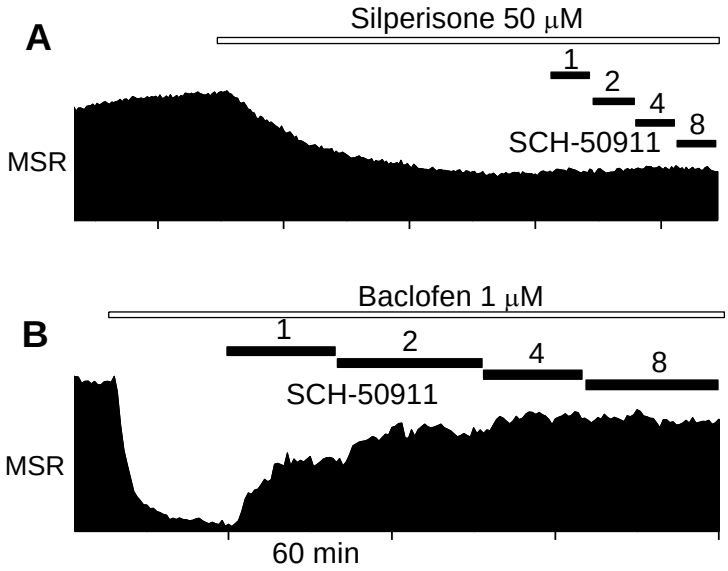


Fig. 38. A: The lack of effect of the GABA-B antagonist SCH-50911 on inhibition of DR-VRP by silperisone in a representative experiment. B: In contrast, effect of baclofen is reversed by SCH-50911. Response-time plots from two experiments. Heights of the graphs represent MSR peak-to-peak amplitude. Time scales are shown by regular 60 min tickmarks. Open horizontal bars show administration of silperisone and baclofen in A and B, respectively. Solid horizontal bars show administration of SCH-50911. Numbers above the bars indicate concentrations in μM.

In order to assess the contribution of the local anaesthetic effect on the afferent nerve conduction to the spinal reflex depression, afferent fibre potentials (AFP) were investigated and the isolated spinal cords were perfused with silperisone, tolperisone or lidocaine at concentrations corresponding to their approximate IC_{50} values for depression of MSR. (The applied concentrations of 50 μM, 75 μM and 150 μM were based on preliminary assessment of potencies on MSR, therefore slightly deviate from the final IC_{50} values of 56 μM, 63 μM and 168 μM, respectively, plotted in Fig. 37.) A typical AFP recorded in Ca^{2+}-free medium showed three major biphasic potential waves attributable to excitation of Aαβ-, Aδ- and C-fibres (Fig. 39). All the three drugs resulted in some depression of Aαβ-, Aδ- and C-fibre related potentials. However, even more conspicuous sign of the local anaesthetic action was the prolongation of the delays of the potentials. The effects of all the three drugs were most prominent on the C-fibre conduction. Lidocaine exerted the greatest effect on afferent nerve conduction, whereas silperisone hardly influenced the A-fibre mediated conduction. In terms of nerve conduction inhibitory effect, tolperisone was in between the two others.

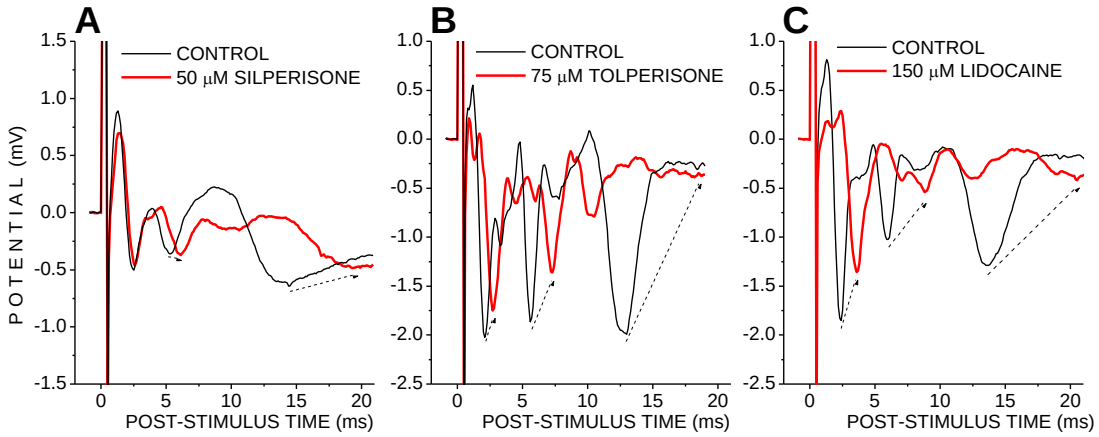


Fig. 39. The effect of silperisone (A), tolperisone (B) and lidocaine (C) on the afferent fibre potential recorded from the L_4 dorsal root in Ca^{++}-free medium in three separate experiments *in vitro*. The L_5 dorsal root was stimulated (0.2 mA; 0.1 ms). The first, second and third major biphasic, dominantly negative waves represent the compound action potentials conducted by Aαβ, Aδ and C fibres, respectively. The control potentials were recorded before the drug application, while the potentials in presence of the drugs were recorded at their maximum effects, i.e. about 10 minutes after start of drug perfusion. Since the applied drugs increased the latencies of components it might be difficult to identify them in the potentials under treatment. However, continuous observation of the gradual change allowed us to identify the corresponding components, which are indicated by the dotted arrows. The three drugs were applied at their approximate monosynaptic reflex inhibitory IC_{50} doses.

To further analyse the relationship between depression of the afferent nerve conduction and of the synaptic transmission, dose-response studies were performed with simultaneous recording of AFP and DR-VRP. In order to record the EPSP related component without the initial population spike, these experiments were carried out at higher temperature (31-33 °C), at which excitability of motoneurones in the hemisected spinal cord preparation is depressed. In these experiments, due to the faster conduction, Aαβ-fibre related potential was not clearly separated from the stimulus artefact but the Aδ potential was clear and evaluable (Fig. 40A). Although all the three studied drugs attenuated both AFP and EPSP amplitude, silperisone preferentially depressed EPSP, whereas lidocaine depressed AFP more strongly. Profile of tolperisone was again between the two others, as it equally inhibited both AFP and EPSP (Fig. 40).

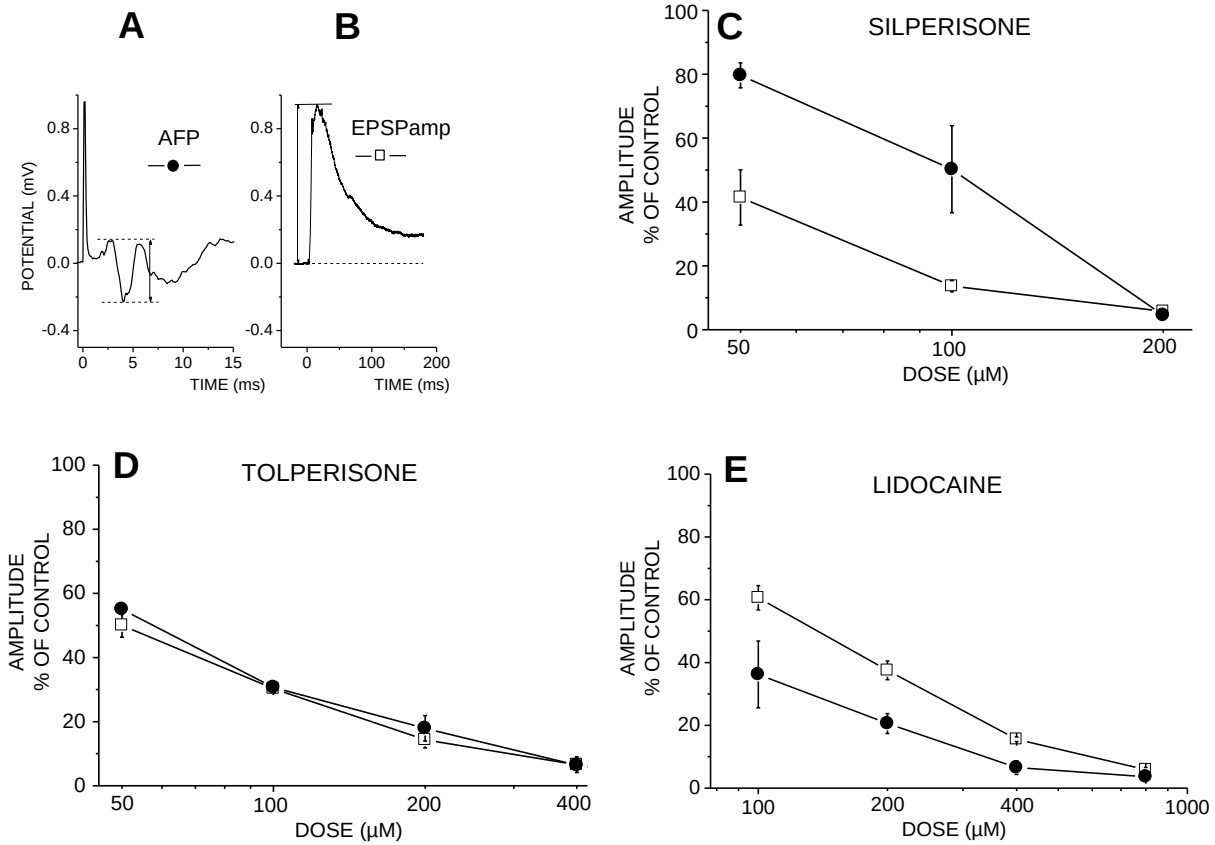


Fig. 40. Effects of silperisone, tolperisone and lidocaine on population EPSP and on afferent fibre potential AFP. These experiments were performed at bath temperature of 31-33 °C, to eliminate the monosynaptic reflex. The L_5 dorsal root was stimulated (0.2 mA; 0.1 ms) A: afferent fibre potential recorded from the L_4 dorsal root. B: population EPSP recorded from the L_5 ventral root. C-E: dose-response curves of the three drugs based on the peak-to-peak amplitude of AFP (●) and zero-to-peak amplitude of EPSP (□). Data are presented as mean±S.E.M. from 3-3 experiments.

2.3.4.2 Spinal cord grease gap study

The depressant effect on EPSP could be due either to a presynaptic action on the excitatory transmitter release or to a postsynaptic antagonism of ionotropic glutamate receptors. In order to test this latter possibility, using the grease gap method, we tested if silperisone exerts any antagonist effect on AMPA- or NMDA-induced depolarisations of motoneurones. Silperisone (100 µM) apparently did not affect the baseline DC potential. Depolarisations of the spinal cord slices evoked by NMDA (80 µM) were obviously unaffected by 100 µM silperisone. Those evoked by AMPA (40 µM) were slightly reduced by 18% in average (Fig. 41A), which change, however, was not statistically significant. Since this result equivocally raised the possibility of a slight depressant effect of silperisone on AMPA mediated events, a second study was performed by testing the effect of silperisone on the concentration-response curve of AMPA. In this study, the response to 40 µM AMPA was exactly equal (i.e 100% in average) to the first test response with the same dose (Fig. 41B) and the concentration-response curves in presence and absence of silperisone were essentially

identical. Hence, potential effect of silperisone on AMPA or NMDA receptor mediated events was ruled out.

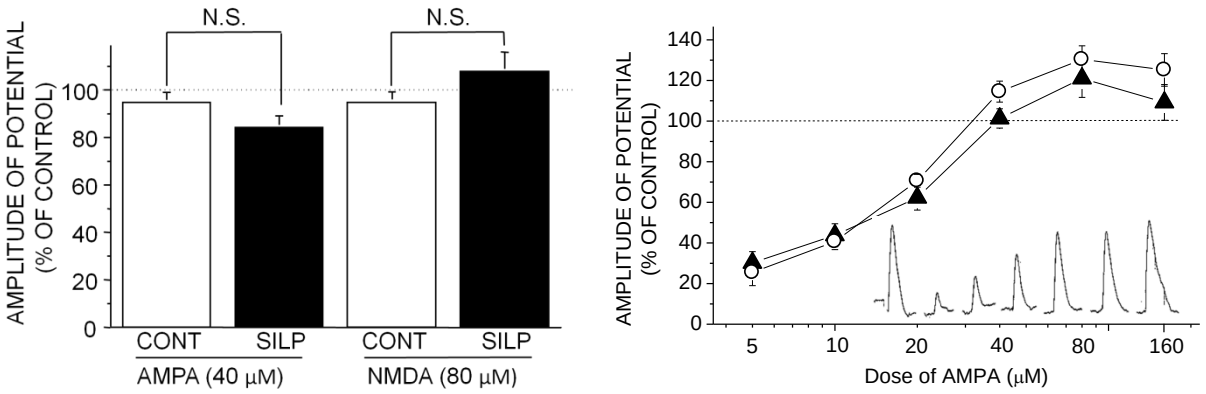


Fig. 41. Effect of 100 μM silperisone on the depolarisations evoked by ionotrop glutamate receptor agonists recorded by the spinal cord grease gap method. A: effect of silperisone on depolarisation evoked by 40 μM AMPA or 80 μM NMDA. Solid and open columns represent amplitudes of depolarisations (mean, S.E.M.) after 50 min perfusion with silperisone (N=7) or control (N=5 with ACSF only) respectively, expressed as percentage of pre-treatment control response (N.S. - no significant difference, $p>0.05$; Student's unpaired t-test). B: effect of 100 μM silperisone (▲) on the dose response curve for depolarisation evoked by AMPA compared to control (○). Data are presented as percentage (mean±S.E.M. of 6 and 9 experiments, control and silperisone treated, respectively) of pre-treatment control response to 40 μM AMPA. Inserts show representative records of depolarisations evoked by 1-minute administration of different doses of AMPA, namely 40 (control), 5, 10, 20, 40, 80, 160 μM. The experiments were performed in ACSF containing tetrodotoxin (0.1 μM).

2.3.5 Patch-clamp analysis of effects on voltage-gated channels

2.3.5.1 Effects on voltage-gated sodium channels

In accordance with the literature (Kostyuk *et al.* 1981b; Roy and Narahashi 1992; Elliott and Elliott 1993) we found that at least two distinct sodium-channel subtypes are present in the DRG cells, i.e. a tetrodotoxin sensitive (TTX-S) and a tetrodotoxin resistant (TTX-R) subpopulation (see Fig. 42). They are different not only in their pharmacological sensitivities but also in activation-inactivation kinetics and steady-state current availability (voltage dependence of inactivation). The two subtypes were often present in the same cells: "mixed cells". Among large cells (i.e. diameter above 30 μm) we could often find cells which almost exclusively expressed TTX-S channels, while among relatively small cells (i.e. diameter below 25 μm) we often found fully TTX-R currents. In cells expressing predominantly TTX-R channels, a small portion of TTX-S current could clearly be differentiated in the steady-state inactivation curve at high negative holding potentials ($V_h < -80$ mV; see Fig. 42D). Concomitant application of TTX in the experiments on TTX-R currents removed this TTX-S "contamination" (Fig. 42D) but apparently did not affect the effects of test drugs on the TTX-R current (pilot experiments not shown).

In order to classify the cells for Na^+ current subtype composition at the beginning of an experiment 0.5 μM TTX was applied and the sodium current tested with voltage steps to zero from a holding potential of -100 mV. Initially, cells which were either almost fully sensitive (i.e. amplitude of the remaining current during perfusion of TTX was less than 15% of the control) or resistant (i.e. remaining current during perfusion with TTX ≥ 85%) were included in the study. However, later the criteria for accepting cells for testing TTX-R Na^+ current testing were relieved to >40% as the presence of TTX during the experiment assured homogeneity of the studied channel population. Hence, these experiments were further conducted in continuous presence of 0.5 μM TTX. The fast activation-inactivation kinetics of a TTX-S and the slow kinetics of a TTX-R current are shown in Fig. 42A and Fig. 42B, respectively. Fig. 42C shows steady state current availability relation plotted as a function of holding potential (in the followings briefly: "steady-state inactivation curve") recorded from a TTX-S, from a TTX-R as well as from a "mixed" cell with a ratio of about 50-50%. TTX-S currents become inactivated at relatively high membrane potential as compared to TTX-R ones.

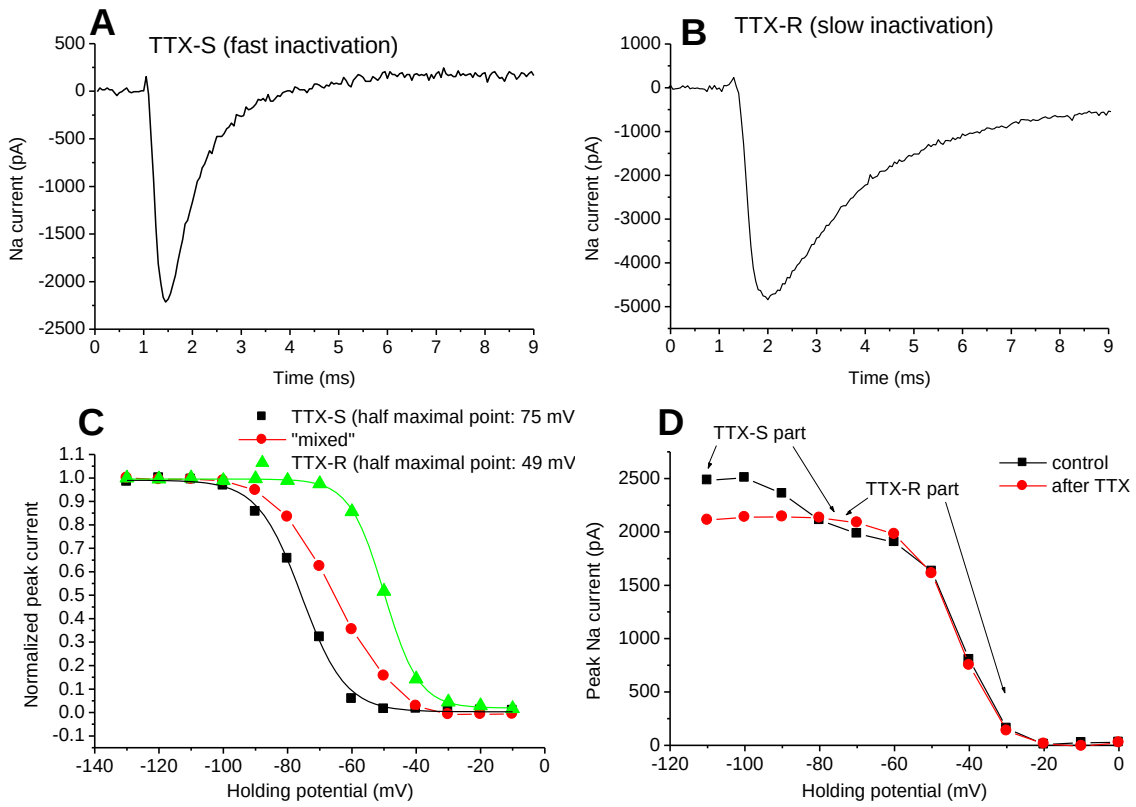


Fig. 42. Characteristics of the whole-cell currents of DRG neurons. A: activation-inactivation time-course of the Na^+ current in a cell with TTX-sensitive (TTX-S) channels - "fast" kinetics. B: time-course of the Na^+ current in a cell with TTX-resistant (TTX-R) current – "slow" kinetics. Currents of A and B were activated by step commands (time: 1-9 ms) to zero from V_H of -80 mV. C: Normalised steady-state inactivation (current availability) relationship of a TTX-S (■), of a TTX-R (▲) and of a "mixed" cell (●, ≈ 50-50%). D: steady-state inactivation curve from a mainly TTX-R cell. Note that a small TTX-S component, i.e. some depression caused by TTX (0.5 μM), can be detected only at relatively high negative holding potentials.

Silperisone attenuated both the TTX-S and the TTX-R currents. It decreased the maximal current measured at high negative holding potential but depression of the current was more prominent at the declining part of the steady-state inactivation curve (Fig. 43 A and B). Thus, silperisone shifted the normalised steady-state inactivation curve to hyperpolarised direction. Under such circumstances the inhibitory potency of a drug is dependent on the relation of the holding potential during testing to the transitory phase ($V_{1/2}$) of the steady-state inactivation curve. Therefore, we determined the potency of the drug at different holding potentials

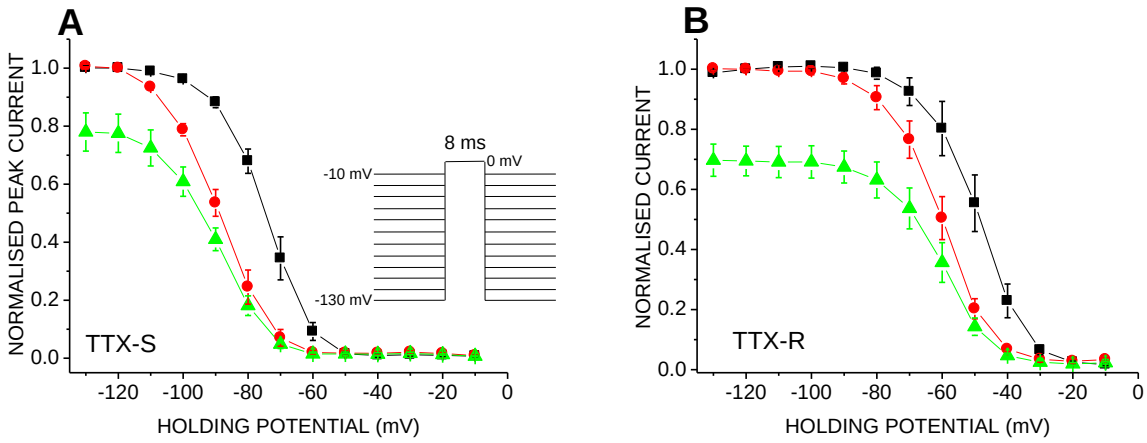


Fig. 43. Effect of silperisone (80 µM) on the steady-state inactivation curves in cells with TTX-S (A) and TTX-R Na^+ channels (B). Averaged normalized steady-state inactivation curves in the control solution (■), during perfusion with silperisone 80 µM (▲, normalized to the control peak current at V_H of -120 mV) and the latter curve scaled up (●, normalized to the peak current during silperisone at V_H of –120 mV). Note that the maximum available currents – at high V_H values – were also depressed but depression in the transitory phase was more pronounced. Data are presented as mean±S.E.M. in 5-6 experiments. The insert in A shows the command voltage protocol with varying the holding potential (V_H) used for obtaining these curves.

Activation of Na^+ currents occurred at more hyperpolarised level for TTX-S than for TTX-R channels. However, the step-voltage dependence of Na^+ channel activation was not affected by silperisone, as could be concluded from the current-voltage relationship (I-V) curves and the activation curves (normalised conductance – voltage: G-V curves) derived from the I-V curves (Fig. 44). Although the normalised (and upscaled) activation curves showed some apparent difference at -40 mV and -20 mV on TTX-S and TTX-R channels, respectively, between control and silperisone treated state, we attributed this difference to the too large control Na^+ current, which resulted in a pipette series resistance error that could not be fully compensated *cf.* (Marty and Neher 1995). Having recognised this error, we modified the extracellular solution to record smaller Na^+ currents at which series resistance error could be properly compensated. Repeating the experiments with silperisone by recording smaller currents and proper series resistance compensation confirmed the lack of effect of silperisone on voltage dependence of activation (Fig. 48 A and B).

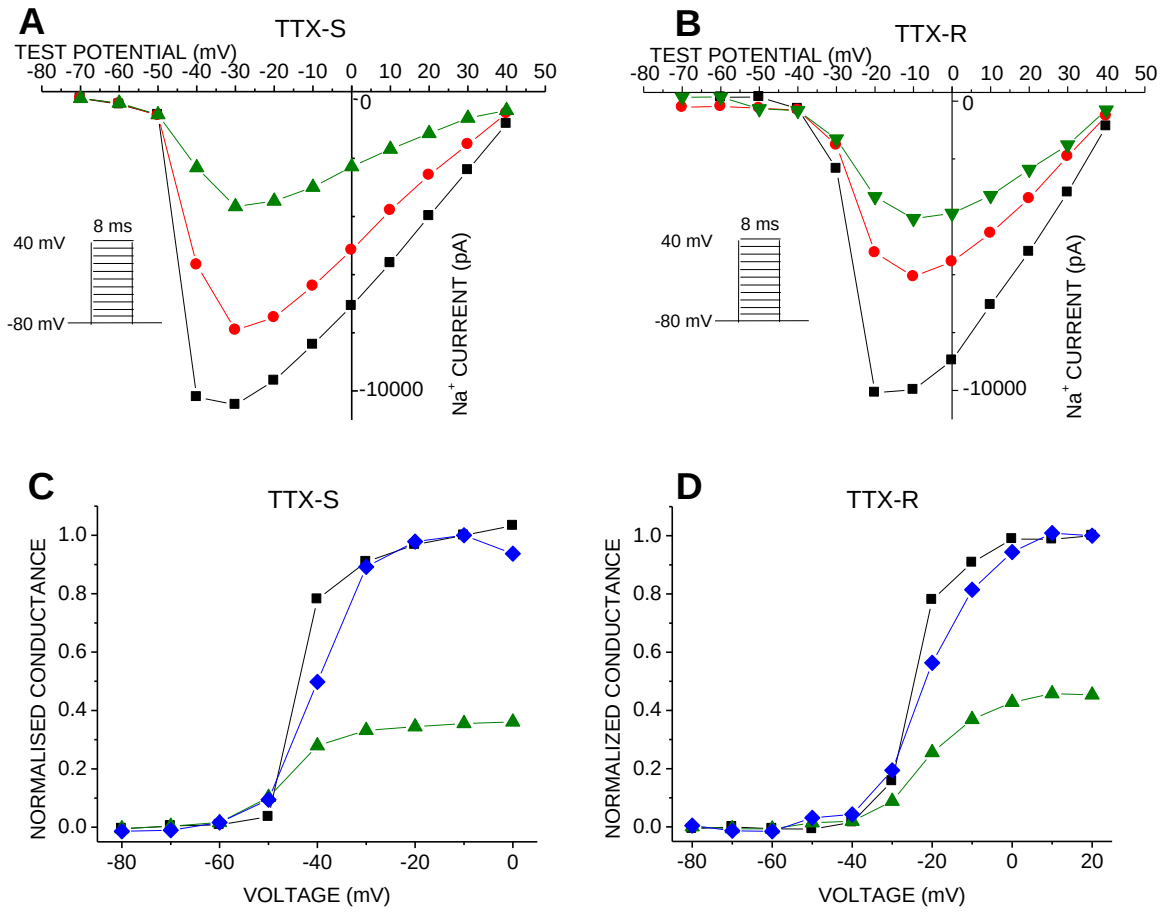


Fig. 44. Effect of silperisone on step-voltage-current relationship of sodium currents (A and B), and on activation curves (C and D; normalised conductance – step-voltage plots derived from the step-voltage-current plots). Data obtained from a cell with TTX-S (A and C) and one with a TTX-R (B and D) channel. Silperisone 40 µM (●) and 80 µM (▲) suppressed the sodium currents as compared to control (■) and the effect was independent of the amplitude of the applied voltage step. C and D: Control activation curve (■), that during perfusion with 80 µM silperisone (▲) and the same curve scaled up to maximum current of the control (◆). Note that apart from an apparent difference in the rising phases, between ■ and ◆, which is probably due to an imperfect compensation of series resistance error (artefact) at such large currents (≈10 nA), inhibitory effect of the drug was independent of the voltage steps. Hence, silperisone did not shift the activation curves in either Na^+ channel type.

We tested the potency of silperisone on Na^+ currents elicited by voltage steps to 0 mV from holding potentials of -80 mV and -70 mV on TTX-S; and at -80 mV and -60 mV on TTX-R currents. Representative records of individual experiments on concentration-response relationship are shown in Fig. 45, whereas Fig. 46 presents the average results of dose-response studies at the different channel subtypes and holding potentials. Silperisone suppressed the Na^+ currents in a concentration dependent manner of both TTX-S and TTX-R channels. The onset of effect was rapid, nearly maximal effect was achieved within 20 seconds (2 episodes). As expected, it was apparently more effective at relatively more depolarised holding potentials, where a concentration of 10 µM had considerable Na^+ channel blocking effect. The IC_{50}s ranged between 23 µM and 106 µM depending on the holding

potential and channel type. For comparison, tolperisone was tested in TTX-S channels at V_H -80 mV and proved to be less potent than silperisone (Fig. 46B).

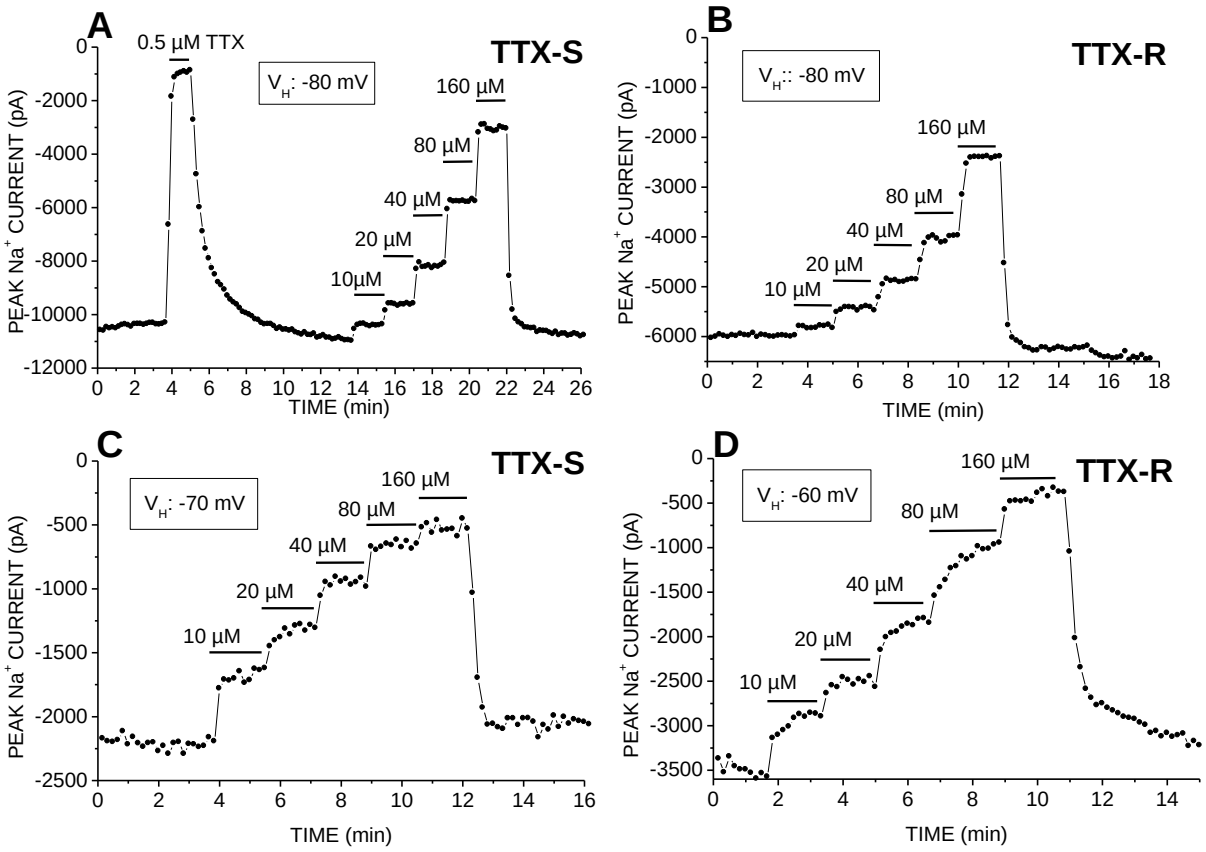


Fig. 45. Representative peak Na^+ current *vs.* time plots obtained during concentration-response studies with silperisone for sodium current inhibition. Dose-dependent inhibition by silperisone in DRG cells with TTX-S (A and C) and TTX-R (B and D) channels at different holding potentials (V_H). TTX sensitivity was tested by short administration of TTX (0.5 µM) as shown at panel A then silperisone was applied at various concentrations. Note that silperisone had more pronounced effects at more depolarised membrane potentials.

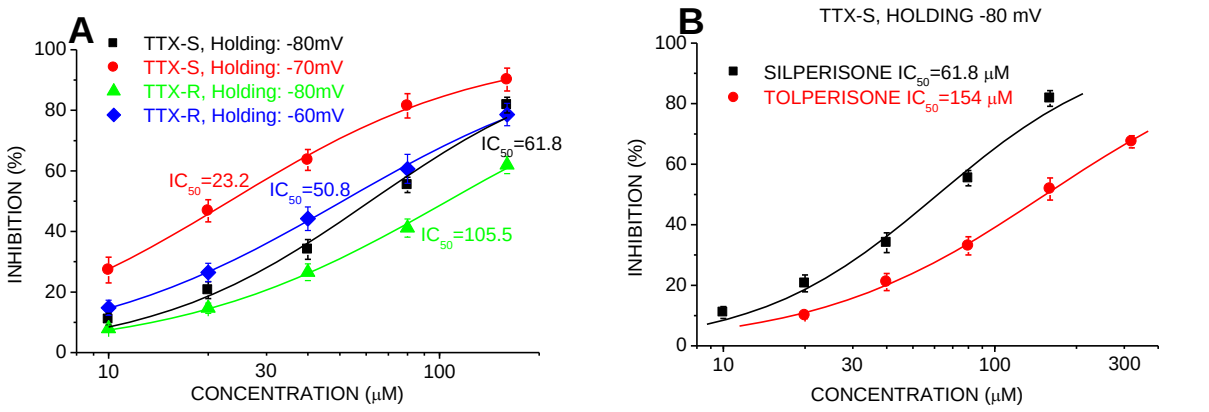


Fig. 46. A: Average concentration-response curves for silperisone taken on TTX-S and TTX-R Na^+ currents elicited from different holding potentials; a summary from experiments like those shown in Fig. 45. **B:** Comparison of effects of silperisone and tolperisone on TTX-S Na^+ currents at V_H -80 mV. Data are presented as mean±S.E.M. from 4-5 experiments. Calculated IC_{50} values (in µM) are also presented.

We performed a more comprehensive comparative study between silperisone, tolperisone and lidocaine after small modifications in the methodology with regards to the extracellular solution and inclusion criteria. These experiments were performed with ES aimed at reducing whole-cell Na^+ currents, as described above. Furthermore, to get more reliable comparison, the control steady-state inactivation curve of each clamped cell was tested first, and only cells with half maximal TTX-S current between -70-90 mV or with half maximal TTX-R current between -50-70 mV were included in the study. TTX-S and TTX-R currents were measured at V_H of -80 mV and -60 mV, respectively. The results of this comparative study are presented in Fig. 47 and Table 7. Tolperisone and lidocaine appeared to be almost equally potent blockers of sodium channels and were 1.6- to 4-fold less potent than silperisone. Greater potency of silperisone was more conspicuous on TTX-R channels.

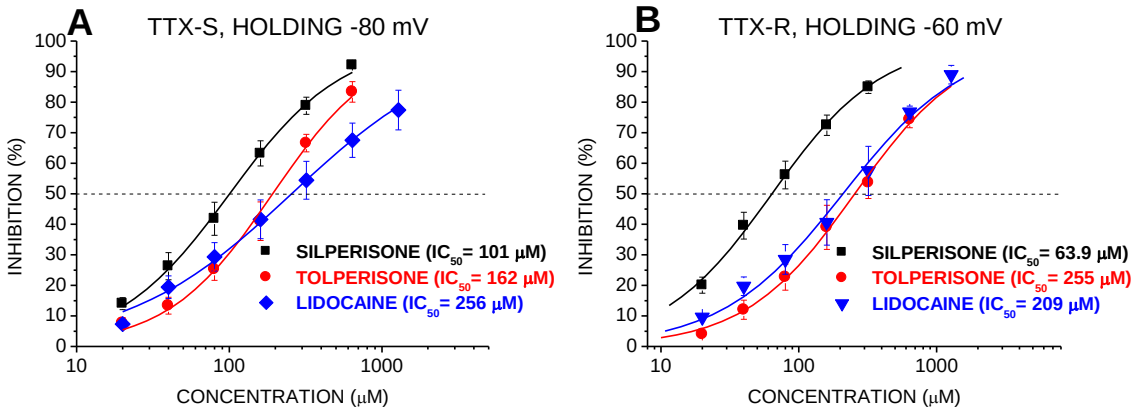


Fig. 47. Comparison of Na^+ channel blocking potencies of silperisone, tolperisone and lidocaine on TTX-S channels at V_H -80 mV (A) and TTX-R channels at V_H -60 mV (B). Data are presented as mean±S.E.M. from 6-10 experiments. Calculated IC_{50} values (in μM) are also presented.

Neither silperisone nor tolperisone nor lidocaine shifted the activation voltage curve of sodium channels Fig. 48.

In summary, both tolperisone and silperisone block TTX-S and TTX-R Na^+ channels of DRG neurones in a similar manner to lidocaine. Whereas Na^+ channel blocking potency of tolperisone is similar to that of lidocaine, that of silperisone is 2.5-3 times more potent.

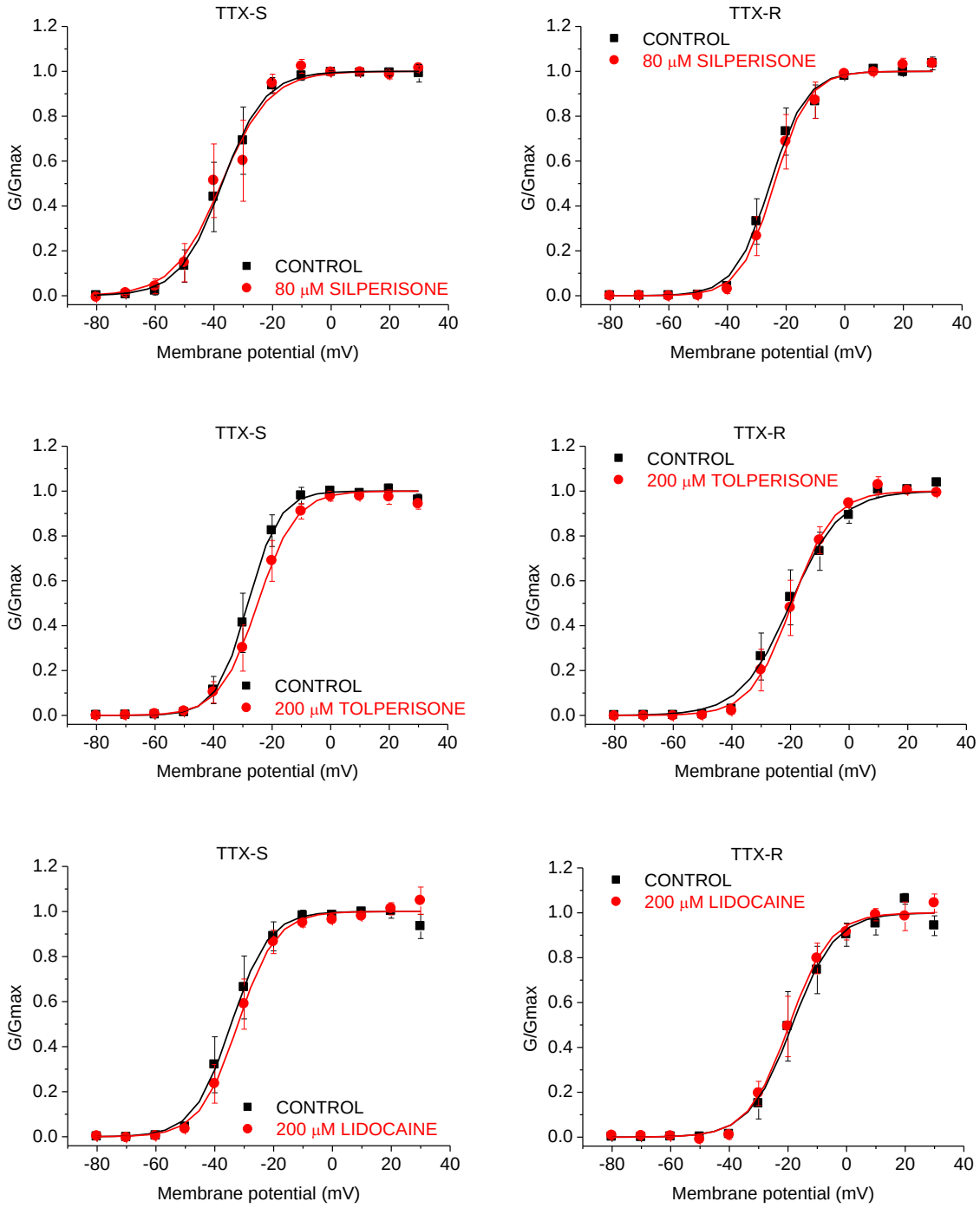


Fig. 48. Effects of silperisone, tolperisone and lidocaine on activation curves (normalised conductance [G/G_{max}] – step-voltage plots) of TTX-S and TTX-R Na^+ channels in DRG cells. Data are presented as mean±S.E.M. (N=6-7). The data indicate lack of a shift. In contrast with Fig. 44 these experiments were performed in modified ES reducing the peak Na^+ current and thus the curves were not affected by series resistance error, as the series resistance could be properly compensated.

2.3.5.2 Effects on voltage-gated calcium channels

When investigating voltage gated calcium channels, we observed rather rapid rundown of the recorded Ca^{2+} current after establishing the whole-cell configuration. This inherent decline in the

amplitude of Ca^{2+} channel currents, which occurs after changing solution at the inner side of the membrane from the normal intracellular fluid to an artificial salt solution is due to dialysis of the cell interior and has been described in the literature (Kostyuk *et al.* 1981a). Several methods had been proposed to eliminate the rundown (Hoppe *et al.* 2005) and we used the method of supplementing the intracellular solution with GTP, CP and CPK (Forscher and Oxford 1985). This approach indeed stabilised the recorded Ca^{2+} current, even more, often some increase of the recorded current could be observed after cell penetration. However, comparing experiments with and without CPK supplementation, we also found that the supplementation markedly reduced apparent potency of silperisone (see Fig. 49) and other calcium channel blocking drugs (e.g. verapamil, not shown). Therefore, we performed some experiments under both conditions. However, to obtain more reliable comparisons, the comparative experiments on potencies of drugs were performed in the "CPK supplemented" condition, accepting the caveat that drug potencies may be underestimated in this manner.

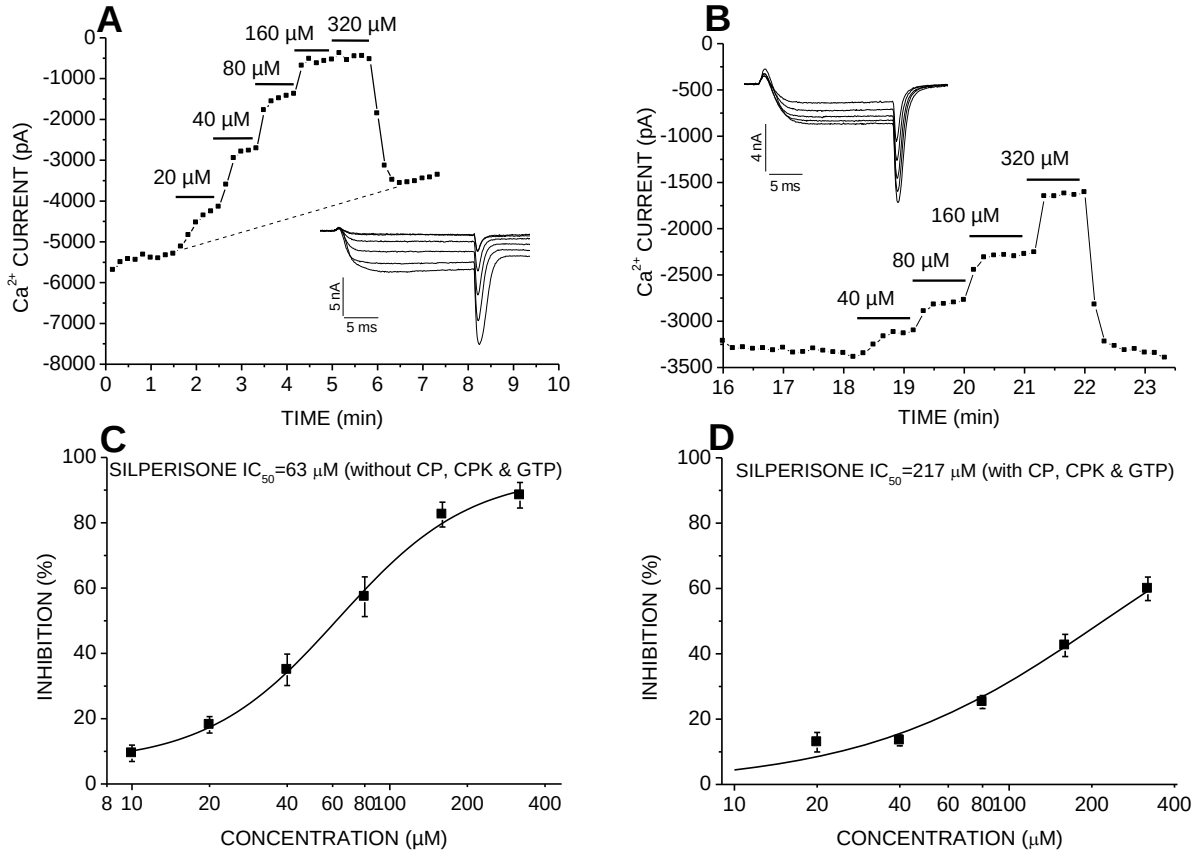


Fig. 49. Concentration-response study with silperisone at two different conditions: A and C with intracellular solution (IS) without CPK supplement; B and C with CPK supplemented IS. Note that without CPK supplement silperisone is much more potent but a substantial rundown is present, which latter is fully eliminated by the CPK supplemented IS. However, the CPK supplement reduced the apparent potency of Ca^{2+} channel blockade by 3.4-fold. Data in C and D are presented as mean±S.E.M. from 4-6 experiments. The inserts show the current time course plots obtained with 20-ms-long depolarising pulses before and after administration of increasing concentrations of silperisone.

Silperisone blocked the voltage gated calcium currents of DRG cells with IC_{50} values comparable to the sodium channel blocking IC_{50}s, particularly when we took into account the Ca^{2+} channel blocking IC_{50} without CPK supplementation (Fig. 49C). The IC_{50} with CPK supplementation was two-fold higher than the IC_{50} at V_H -80 mV in TTX-S Na^+ channels (Table 7). The strength of the Ca^{2+} channel blockade was not considerably dependent on either the preceding holding potential Fig. 50A or the activation voltage (Fig. 50B).

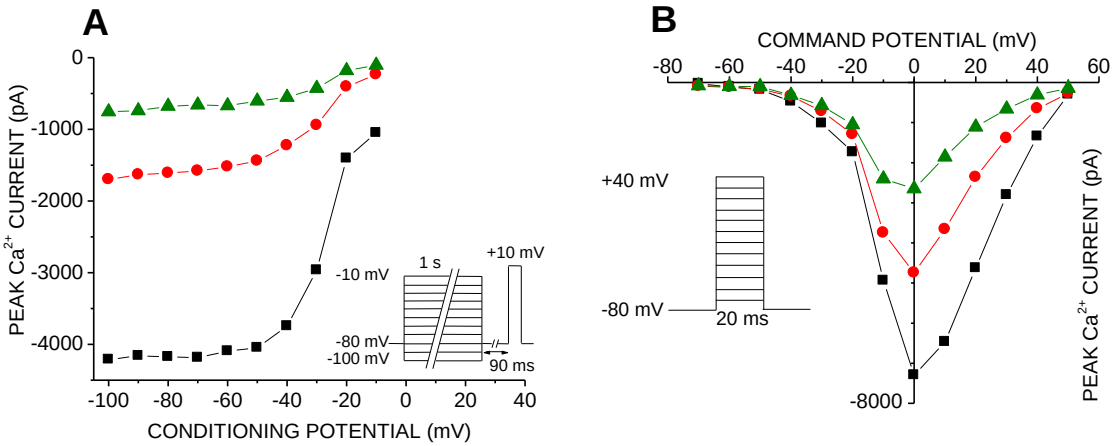


Fig. 50. Holding- and activating voltage independent depressant effect of silperisone on high voltage activated Ca^{2+} current of DRG cells. (Not CPK supplemented condition). Steady-state inactivation curves from a cell (A) and activation voltage – current relationships from another cell (B) in control solution (■) and in the presence of 80 μM (●) or 160 μM (▲) silperisone.

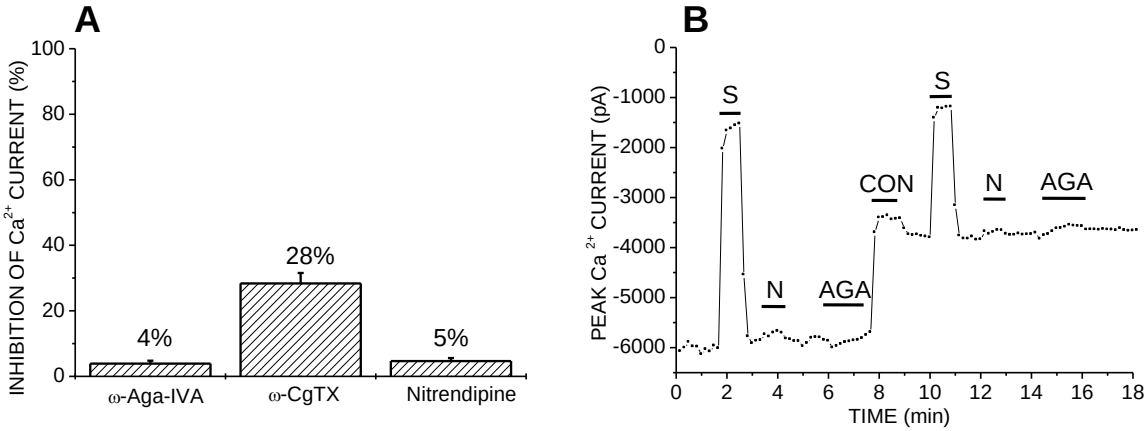


Fig. 51. Effects of various calcium channel blockers on HVA calcium currents in DRG cells (CPK supplemented condition). A: The amount of blockade provided by different subtype selective Ca^{2+} channel blockers: nitrendipine (N, 10 μM, L-type selective), ω-conotoxin-GVIA (CON, 3 μM, N-type selective) and ω-agatoxin-IVA (AGA, 0.1 μM, P-type selective). Data are mean±S.E.M. from 4-9 cells. B: Effect of silperisone (S, 320 μM) compared with those of specific blockers in a representative experiment. Silperisone blocked also drug resistant (R-type) calcium currents, which were abundant in these DRG cells.

In order to identify the Ca^{2+} channel subtypes on which our testing was carried out, and which were blocked by silperisone, we investigated the effects of specific channel blockers (Fig. 51). Under the applied circumstances, the recorded calcium current was only very mildly inhibited by either the P/Q-

type blocker ω-agatoxin IVA or the L-type selective nitrendipine, though a small distinct and reversible effect of these test substances could be noticed in most of the cases. In contrast, the N-type blocker ω-conotoxin GVIA produced a remarkable irreversible suppression of the measured Ca^{2+} current, which however still amounted only 28% in average. Silperisone (320 µM) blocked a great portion of the Ca^{2+} current, which included Ca^{2+} channels resistant to all the above three channel blockers. A representative experiment summarising pharmacological sensitivity of the tested calcium channels is presented in Fig. 51B.

Comparative concentration response studies with silperisone, tolperisone and lidocaine exhibited approximately 6.5 times lower potency of tolperisone *vs.* silperisone on Ca^{2+} channels, whereas lidocaine produced remarkable (still <20%) effect only at as high as 1000 µM concentration. Hence, taking also into account the potentially underestimated potencies associated with CPK supplementation, Na^{+} and Ca^{2+} blockade may contribute to similar extent to the pharmacodynamic effects in the case of silperisone. For tolperisone, some contribution of Ca^{2+} blockade in addition to the Na^{+} channel blockade may be assumed, whereas the Ca^{2+} blocking effect of lidocaine, *vs.* Na^{+} channel blockade may be considered negligible.

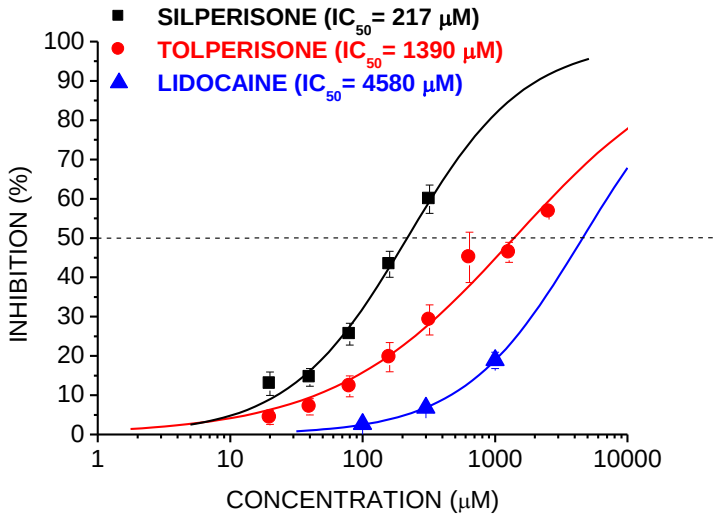


Fig. 52. Comparison of Ca^{2+} channel blocking potencies of silperisone, tolperisone and lidocaine in DRG neurones (CPK supplemented). Data are presented as mean±S.E.M from 4-7 experiments. Calculated IC_{50} values (in µM) are also presented. However for lidocaine it is only an extrapolated rough estimate.

2.3.5.3 Effects on voltage-gated potassium channels

Long-lasting depolarizing steps activate the potassium channels of DRG neurones. Concluding from the (mainly non-inactivating) time-course of the current during the activating voltage step, we observed mainly delayed rectifier K^{+} currents in the tested medium sized DRG neurones. The current voltage relationship of activation is shown in Fig. 53A and the K^{+} channel blocking effect of 10 µM silperisone. Silperisone reduced the K^{+} current and its blocking effect was independent of the

activation voltage. Comparative concentration-response studies with silperisone, tolperisone and lidocaine (Fig. 53B) indicated some clear-cut (18%) suppression of the potassium current by 40 µM silperisone and a remarkable (>50%) K^+ channel blockade at 320 µM. K^+ channel channel blocking potencies of tolperisone and lidocaine were much weaker with estimated (extrapolated) IC_{50} values in excess of 1000 µM.

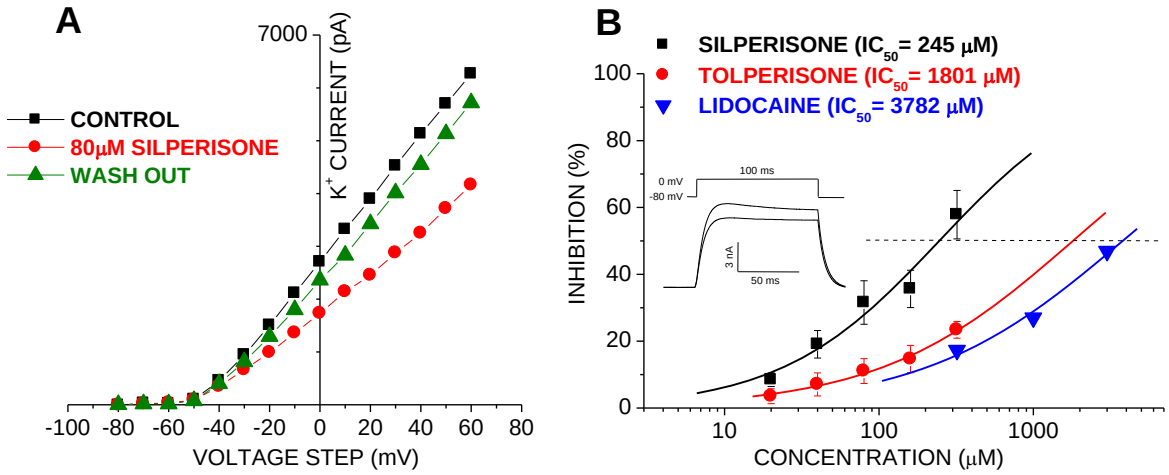


Fig. 53. Characterisation of delayed rectifier K^+ channel blocking effect of silperisone in a current-voltage relationship study in a DRG cell with administration and wash out of 80 µM silperisone (A). Comparison of K^+ channel blocking potencies of silperisone, tolperisone and lidocaine in DRG neurones. Data are presented as mean±S.E.M (B). The insert shows the elicited potassium current before and after 40 µM silperisone and the command voltage protocol used in the concentration-response studies.

Table 7. Comparative summary table of key *in vitro* potencies (IC_{50}s) of silperisone, tolperisone and lidocaine.

	Spinal reflex (*in vitro*)		Voltage gated channel blocking (patch-clamp)			
	MSR	EPSPint	TTX-S Na^+	TTX-R Na^+	Ca^{2+}	K^+
	IC_{50} (µM)					
SILPERISONE	57	73	101	64	217	245
TOLPERISONE	63	131	162	255	1390	1801*
LIDOCAINE	186	198	256	209	4580*	3782

*remote extrapolation (vague estimate)

2.4 Discussion

For assessing desired effectiveness of CMRs ("antispastic effect") we used the GYKI 20039 tremor and the morphine induced Straub tail tests. Furthermore, we used three widely used routine tests and a newly invented test (weight-lifting) for assessing side effects, such as sedation and impairment in muscle strength and motor coordination.

Morphine-induced Straub tail reaction, which is probably caused by an effect of morphine at spinal level, as proved by intrathecal administration (Hylden and Wilcox 1980; Hasegawa *et al.* 1990), has

been used by several researchers as a method for assessing muscle relaxant potential of drugs (Ellis and Carpenter 1974; Novack 1982; Simiand *et al.* 1989; Musso *et al.* 2003). However, the GYKI 20039 tremor test for testing CMR drugs was invented by the author of this dissertation (Farkas *et al.* 2005).

All the tested CMR drugs inhibited GYKI 20039 tremor dose-dependently; and in several cases at doses that did not significantly impair normal voluntary motor functions as judged from rotarod and weight lifting performance. Furthermore, CMRs except for baclofen inhibited the tremor with similar or lower potency than that for the Straub tail reaction. For tolperisone-type drugs potencies in the two antispastic methods were practically equal. These facts suggest that this tremor inhibition test may be a suitable objective tool for assessing antispastic potential of drugs.

The relatively weak effect of baclofen in the tremor test is in contrast with its high potency in the Straub tail test. Presumably, the differential sensitivity of the two models for baclofen may be explained by the involvement of supraspinal, and spinal sites in the generation of tremor activity and the induction of Straub tail, respectively. A spinal mechanism preference of baclofen is apparently consistent with the clinical experience that baclofen is more useful in spinal than in cerebral spasticity (Young and Delwaide 1981; Emre 1993). Baclofen was also reported to affect descending control pathways in animals to much smaller extent than spinal segmental mechanisms (Curtis and Malik 1985).

Our result concerning the rotarod/Straub tail effectiveness ratio of tizanidine (0.86) is significantly different from that reported by Novack (5.71). This discrepancy might be explained by the uncertainty in determining the two ID_{50} values for tizanidine due to its flat and fluctuating dose-response curves. Our results concerning baclofen and mephenesin were not significantly different from those of Novack, and the efficient muscle relaxant dose of tizanidine as assessed by the tremor method (ID_{50} 0.7 mg/kg) was also similar to that reported by him for the Straub tail test (ID_{50} 0.4 mg/kg).

Despite the uncertainty in assessing muscle relaxant potential of tizanidine by the Straub tail method our results are consistent with clinical findings that tizanidine causes relatively little undue muscle weakness (Emre 1989). Namely, its TI *vs.* weight-lifting performance (2 and 3.8) was favourable. However, its very potent depressant effect on locomotor activity and its barbiturate potentiating effect at very low doses seem to correlate with its most frequent side effects experienced in the clinical practice, i.e. tiredness and drowsiness (Emre 1989).

In terms of muscle relaxant/side effect ratio calculated with the lower of the two antispastic ID_{50} values, baclofen, tolperisone and eperisone were relatively free from side effects represented by the rotarod and weight-lifting tests. However, they produced depression of the locomotor activity at

similar or lower doses than the ones causing muscle relaxation. The classical CMRs mephenesin (Berger and Bradley 1946) and zoxazolamine (Funderburk and Woodcock 1955) showed evenly acceptable ratios, the TI calculated with the tremor ID_{50} were mostly above 1.5. Carisoprodol (Berger *et al.* 1959) and afloqualon (Ochiai and Ishida 1981; Ochiai and Ishida 1982) did not show acceptable ratios in any respect. Diazepam, despite its well known sedative effect in man, reduced the locomotor activity with relatively low potency in mice, pointing to the limitations in the predictive value of these rodent tests. Slight enhancing effect of low doses of diazepam on locomotor activity has been well known and was attributed to its anxiolytic effects. Nevertheless, potency of diazepam to cause CNS related adverse effects was well reflected by small TI calculated with the rotarod and weight-lifting test results. Memantine (Maj *et al.* 1974), a channel blocking NMDA antagonist (Parsons *et al.* 1999), did not depress locomotor activity at all but impaired the rotarod and weight-lifting performance at doses similar to those causing muscle relaxation.

Overall, our findings in mice are consistent with the clinical experience that the therapeutic indices of all currently used CMRs show a narrow safety margin. Silperisone was outstanding among the CMR drugs with all TIs of approximately 2 or higher. Nevertheless, ideally even higher TIs would be desirable and the complex test battery we used appears to be suitable for selecting more ideal CMR drugs.

The rank order of durations of actions of tolperisone-type drugs in the GYKI 20039-induced tremor test were silperisone > lanperisone > eperisone > tolperisone. This rank order was consistent with durations of actions reported by Tanaka *et al.* 1981 and Sakitama *et al.* 1997 in spinal reflex and decerebrate rigidity studies in rats and cats and our results obtained in the cat flexor reflex studies confirming the utility of this simple test carried out on mice for an initial screening assessment.

We have developed a new analog signal processing system that enabled on-line and off-line quantitative measurement of evoked reflex activities as recorded by either EMG or electroneurography.

With the aid of this system we established and validated a relatively simple method and anaesthesia protocol for stable recording of the flexor reflex in anaesthetised cats (Farkas and Karpati 1988). This EMG flexor reflex test proved to be a suitable tool for comparative pharmacodynamic characterisation of CMR drugs; and we characterised *in vivo* potencies (ID_{50} values) and time courses of actions of 10 reference CMR drugs. The information that these drugs suppress the flexor reflex is not novel, since effectiveness of these drugs in attenuating some forms of flexor reflex in cats or rats had been demonstrated (Geiger *et al.* 1958; Wand *et al.* 1977; Kamata *et al.* 1980; Sayers *et al.* 1980; Ochiai and Ishida 1982; Ito *et al.* 1985). Effective doses were in line with those in the previous

studies. However, our observation that carisoprodol and afloqualone selectively inhibited a long latency component of the reflex was new. Our finding of large variation of the effects of tizanidine between facilitation and inhibition of flexor reflex in intact chloralose anaesthetised cats was in agreement with the first report on this drug (Sayers *et al.* 1980) and could be due to presence of individual variability in descending facilitatory tone from supraspinal sites. Other studies described depression of flexor reflex in intact rats but enhancement in spinal rats (Chen *et al.* 1987). The depressant effect of tizanidine in intact animals could be explained by α_2 agonism driven removal of noradrenergic facilitation originating from locus coeruleus, as tizanidine inhibits firing of locus coeruleus neurones (Chen *et al.* 1987) but an inhibitory effect via spinal presynaptic α_2 sites has also been postulated (Sakitama 1993). The enhancement in spinal rats was attributed to stimulation of spinal α_1 receptors by tizanidine (Chen *et al.* 1987; Sakitama 1993).

Discounting tizanidine (as discussed above), comparison of *in vivo* potencies of CMR drugs in the flexor reflex test (see Table 4) in intact and spinal cats revealed remarkable (more than two-fold) difference only for two drugs: diazepam and carisoprodol. These drugs were much more potent in intact animals. For diazepam, this finding probably indicates significant involvement of supraspinal sites in its effectiveness. However, some effect at spinal level is evident from the clear-cut though moderate effects in spinal cats even in the low dose-range (0.01-0.04 mg/kg, see Fig. 19). With regards to carisoprodol, concluding on supraspinal dominance is not so straightforward, as its effect was restricted to a long latency component of the reflex, which was rarely present in spinal cats, but when it was present, it was similarly blocked by carisoprodol. For the rest of drugs (tolperisone-type drugs, zoxazolamine, mephenesin, baclofen and memantine) the intact and spinal ID_{50}s were not substantially different, though most of them were slightly more potent in intact cats.

Overall, these data indicate that our flexor reflex test in intact cats is sensitive to all CMR drugs (myorelaxants, antispastics) with broadly different mechanisms of action and is particularly suitable for quantitative pharmacodynamic evaluation of tolperisone-type drugs.

Comparison of the tolperisone-type drugs, i.e. tolperisone, eperisone and silperisone, showed little differences in their *in vivo* potencies after i.v. administration (see Table 4). The effect of eperisone was slightly longer lasting than that of tolperisone, however, both compounds had shorter duration of action compared to other reference drugs, except mephenesin, which is also a short acting drug. In contrast, the effect of silperisone was much longer lasting, suggesting its slow elimination compared to tolperisone and eperisone. Intraduodenal administration mimics oral administration in anaesthetised animals. It is a preferable mode of administration instead of intragastric injection via a cannula, since gastric emptying is markedly suppressed in anaesthetised animals (Yuasa and Watanabe 1994). As expected from its slower elimination, the functional bioavailability of silperisone

was much higher than those of tolperisone and eperisone after intraduodenal administration, resulting in greater inhibition of the flexor reflex at half dose compared to the two reference drugs. These data indicated that silperisone fulfilled our objective of significant improvement in oral bioavailability and duration of action compared to tolperisone. These comparative results obtained in cats were in good agreement with the comparative pharmacodynamics in mice (see section 2.3.1.1; Fig. 13), suggesting that these favourable features are not restricted to a single species and might be better extrapolated to man.

The pattern of actions of silperisone on reflex activities was quite similar to that of tolperisone. It slightly suppressed the patellar reflex but strongly reduced the monosynaptic spinal root reflex and the pattern of actions on other spinal root potentials was also similar but different from other CMR drugs. However, there were also some minor but distinctive differences between silperisone and tolperisone. The most striking difference was that silperisone, in contrast with tolperisone, did not inhibit the reticular stimulation-induced suppression of the patellar reflex, while it suppressed the facilitation. Instead, it sometimes potentiated the inhibition. The effect of silperisone is quite unique in this respect. Not only tolperisone-type drugs but mephenesine-type drugs (propane-diols) and benzazols (such as zoxazolamine) have been reported to inhibit both the pontine facilitation and bulbar inhibition of patellar reflex (Ochiai and Ishida 1981; King and Unna 1954; Funderburk *et al.* 1953).

Another important finding was that silperisone (and also tolperisone) usually did not completely block the facilitation. Although responses during stimulation were diminished, the stimulation (a phasic forced activity) could partially break through the inhibitory effects of these drugs.

What are the implications of these results for clinical cases of spasticity? Spasticity (either of cerebral or spinal origin) is due to the impaired balance of facilitatory and inhibitory descending control, primarily due to a disturbance in inhibition, rather than to an increased facilitation (Brooks 1986). On the other hand, the facilitatory and inhibitory reticulospinal pathways are important outputs of the extrapyramidal motor system. The "classical" CMR drugs restore the balance by blocking the facilitatory activity, too. However, supposing some residual activity in the inhibitory pathway in paretic patients having partial ability for voluntary movements, either depression of the reticulospinal inhibition or complete blockade of the facilitation would be disadvantageous. Instead, support of the residual inhibitory activity is desirable. Results of the studies directed on the reticulospinal pathways seem to indicate that silperisone may fulfil these requirements, namely, shifting the balance towards inhibition by supporting residual inhibitory activity and reversibly diminishing but not blocking facilitation.

Another minor difference between silperisone and tolperisone was a relatively weaker effect of the former on DRR. Previously we concluded that a clear difference in the reaction of the DRR and DRP might reflect an inhibition of spike-generation (Farkas *et al.* 1989). The somewhat weaker effect of silperisone on DRR suggests that compared to effects on mono- and polysynaptic transmission, inhibition of spike generation at least at the primary afferent membranes seems to be slightly less sensitive to silperisone than to tolperisone.

Tolperisone and silperisone (10 mg/kg i.v.) strongly depressed the flexor reflex (by 70%), while apparently did not affect or only slightly suppressed the patellar reflex. Since the patellar reflex is thought to use the monosynaptic pathway (Lloyd 1943), one would have expected a strong inhibition, since these test compounds potently blocked MSR in the spinal root potential study. However, this seeming contradiction is not a unique finding. Similar results, i.e. much weaker inhibition of patellar reflex than MSR, were found with eperisone (Tanaka *et al.* 1981), tolperisone and afloqualon (Ochiai and Ishida 1982), and baclofen (our unpublished observation). The difference in the mode of stimulation implies a variety of influencing factors: While electrical stimulation yields an absolutely synchronized afferent volley, tapping of the patellar tendon results in asynchronous afferent discharge (i.e. dispersed in a few 10-msec scale), which at its beginning initiates both facilitatory and inhibitory processes. Furthermore, while at the patellar reflex the afferent source and the efferent motoneurones studied are recruited according to their physiological function (greater occlusion may be assumed), at the reflex potential study they are recruited anatomically (same nerve or dorsal root). These differences in the conditions may cause a significant difference in the safety factor in these reflex pathways resulting in a difference in their sensitivity to drugs. The greater response-to-response fluctuation of MSR potential may also reflect a weaker safety factor, indicating that at least part of the motoneurones responding must be working near the threshold. A similar difference is seen in the stability when comparing recordings of the flexor reflex and polysynaptic reflex potentials. In this case flexor reflex was more variable and also more sensitive to tolperisone and silperisone. Thus, a clear differentiation between mono- and polysynaptic pathways by tolperisone-type drugs cannot be concluded. Rather, these drugs seem to differentiate between responses evoked by mild and strong activation. A strong activation can partially break through the inhibitory effect of these drugs.

Silperisone inhibited the intercollicular decerebrate rigidity in cats as well as in rats to a similar extent, indicating that there is no remarkable difference between the sensitivities of the two species to the effects of this drug. Considering peak effects, silperisone was doubtlessly slightly weaker in inhibiting decerebrate rigidity than tolperisone both in cats and rats. However, decerebrate rigidity in some respects is different from clinical cases of spasticity (Brooks 1986). While in decerebrate rigidity

an excessive facilitatory activity bombards the motoneurones and hyperactivity of the lateral vestibular nucleus plays an important role, in spastic patients rather the impaired reticulospinal inhibition is responsible for the imbalance.

Tolperisone-type compounds suppressed the spinal segmental reflex activity both *in vitro* and *in vivo*. Their profiles of actions on various components of ventral root reflex potentials were very similar, suggesting similarity in the underlying mechanisms. In line with the findings of ventral root reflex studies in cats, tolperisone-type compounds had stronger effect on the monosynaptic reflex as compared to later components also in rats. The pattern of actions of lidocaine was more different, as it had relatively smaller effect on the monosynaptic reflex of rats both *in vitro* and *in vivo*.

In further analysis, we identified also some differences in the profiles of tolperisone and silperisone and in some respects the profile of tolperisone was between those of silperisone and lidocaine. In fact, lidocaine had relatively stronger effect than silperisone on afferent volleys as compared to effects on either the early EPSP or MSR recorded either *in vitro* or *in vivo*. This difference between silperisone and lidocaine (and tolperisone being in between) is most strikingly demonstrated by the concentration-response curves on AFP and EPSP in Fig. 40. However, the relative effects on AFP *in vitro* (Fig. 39) and *in vivo* (Fig. 32) and the study of primary afferent and motoneurone excitability (Fig. 30) also supported this notion. Relatively stronger "membrane stabilising" effect of tolperisone compared to silperisone on primary afferents was in line with its stronger effect on the dorsal root reflex observed in the pharmacodynamic studies in cats. Hence, the different studies *in vitro* and *in vivo* delineated a quite consistent picture.

Having the strongest effect on the monosynaptic reflex provided a good substrate for analysing the chain of events along this simple reflex pathway, which may give an insight into the key mechanism(s) of the spinal reflex depressant action of tolperisone-type compounds and particularly silperisone (see Fig. 54).

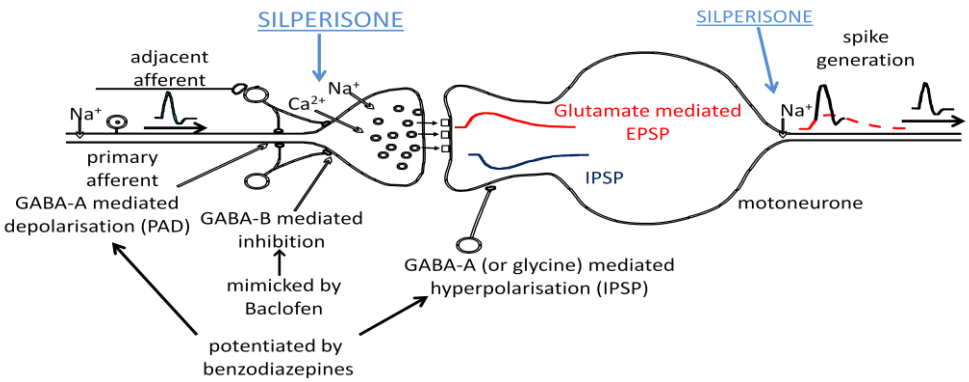


Fig. 54. Schematic diagram of the cascade of events in the monosynatic pathway and postulated actions of silperisone.

The following chain of events was taken into consideration in this analysis: Presynaptic events: Excitability of primary afferents (A-fibres at the stimulating electrode); extraspinal conduction of the afferent volley; intraspinal conduction till the terminals; influx of sodium and calcium ions at the nerve terminals via voltage gated channels; release of the excitatory transmitter (glutamate). Postsynaptic events: EPSP generation due to stimulation of AMPA- and NMDA- type glutamate receptors, electrotonic conduction of EPSP to the initial segment of motoneurones; generation of action potential at the initial segment; conduction of the efferent volley.

Theoretically, the coupling between arrival of the afferent volley to the terminal and the transmitter release can be modulated by a variety of presynaptic mechanisms, including intraspinal blockade of conduction (attenuation of action potential amplitude) via GABA-A mediated primary afferent depolarisation and change in the membrane conductance and inhibition of Ca^{2+}-influx via activating GABA-B receptors coupled to the voltage gated Ca^{2+}-channels (Tatebayashi and Ogata 1992). The coupling between EPSP and spike-generation might be influenced by postsynaptic mechanisms, such as hyperpolarizing inhibitory postsynaptic potentials (IPSPs) mediated via glycine or GABA-A receptors as well as other ionic mechanisms leading to changes in the membrane potential or membrane conductance of motoneurones. Such changes can influence excitability of motoneurones.

The fact that silperisone suppressed excitability of motoneurones suggests that it certainly possessed some postsynaptic effect influencing spike-generation. This may be due to a lidocaine-like local anaesthetic effect, i.e. inhibition of voltage gated sodium channels, on motoneurones. However, lidocaine had relatively stronger effect on the excitability of motoneurones than tolperisone-type drugs, whereas the latter had stronger effects on the synaptic transmission. These findings suggested that the MSR depressant effects of tolperisone-type drugs and particularly of silperisone could not be solely explained by suppression of motoneurone excitability but presumably some presynaptic actions could also be involved in the effects of tolperisone-type compounds. Furthermore, the relative importance of such presynaptic effects might be somewhat more expressed with silperisone than with tolperisone.

The pathway of recurrent inhibition (Renshaw 1946; Eccles *et al.* 1954) involves both glycine- and GABA-A receptor transmission (Cullheim and Kellerth 1981; Schneider and Fyffe 1992). The lack of effect of silperisone on the recurrent inhibition rules out its interaction with postsynaptic glycine- or GABA-ergic mechanisms.

The EPSP of motoneurones was depressed by both tolperisone-type compounds and lidocaine. The early part of EPSP may be depressed either by a presynaptic effect reducing the release of the excitatory transmitter from primary afferents or by postsynaptic antagonism of ionotropic glutamate

receptors. The population EPSP can be completely abolished by co-administration of the AMPA antagonist GYKI 52466 and the NMDA antagonist APV (Kocsis *et al.* 1997). Since silperisone possessed no effect on AMPA- or NMDA-induced depolarisation of motoneurones (Fig. 41), the conclusion remains that its depressant effect on EPSP was probably due to presynaptic inhibition of the release of the excitatory transmitter glutamate. Therefore, we focused the further analysis on potential presynaptic effects.

The GABA-B agonist baclofen, just like tolperisone or silperisone, possessed a more pronounced inhibitory effect on the monosynaptic than on the polysynaptic ventral root reflex (Farkas *et al.* 1989). Moreover some researchers reported that a tolperisone-type compound, inaperisone, inhibited the micturition reflex by acting indirectly on GABA-B receptors (Morikawa *et al.* 1992). Therefore, it was plausible to investigate if stimulation of GABA-B receptors is a possible mode of action of silperisone. However, the lack of reversal of the effect of silperisone by the GABA-B antagonist SCH-50911 ruled out this possibility. Moreover, the lack of effect of silperisone on the feed-forward inhibition of MSR ruled out also its interaction with any form of GABA-ergic transmission (*cf.* Stuart and Redman 1992). The lack of effects on these inhibitory pathways indicated also that silperisone did not suppress the excitatory transmission towards the GABA- and/or glycinergic inhibitory interneurones in the spinal cord.

If silperisone and tolperisone had a lidocaine-like local anaesthetic effect on afferent nerve conduction, this action could certainly contribute to their presynaptic effect. Indeed, all the three drugs studied possessed some depressant effect on impulse conduction in the A- as well as C- fibres. However, their synaptic depressant (EPSP attenuating) effects were not proportional to their suppressing effect on AFP. Silperisone strongly depressed EPSP whereas its effect on AFP was moderate. On the other hand, the order of relative sensitivities of EPSP and AFP to lidocaine were just the opposite and tolperisone was between the two other compounds in this respect. These data suggested that even if a lidocaine-like effect contributed to the presynaptic effect of silperisone and tolperisone, some difference existed in their effects at presynaptic sites or tolperisone and particularly silperisone had some additional effects (e.g. effects on Ca^{2+}-channels), which strengthen their presynaptic inhibitory action on the transmitter release from primary afferents.

In the isolated hemisected spinal cord preparation, the concentrations surrounding the afferent nerves and spinal cord are similar. However, under *in vivo* conditions and systemic administration, the distribution of the drugs is influenced by pharmacokinetic factors such as perfusion of the tissue, penetration through the blood-brain-barrier, and differential penetration into different tissues.

According to the *in vivo* studies, the effect of systemically administered lidocaine on the extraspinal afferent nerve conduction just reached the level of detectability, whereas those of silperisone and tolperisone were negligible. This is in contrast with the *in vitro* findings, whereby all three drugs suppressed to some extent the afferent fibre potentials. However, they still possessed strong suppressant effects on the spinal reflex transmission *in vivo*. These data suggest that they probably have intraspinal presynaptic actions. Since perfusion of the neural tissue within the spinal cord gray matter is much better than that of the peripheral nerves, a lidocaine-like local anaesthetic effect on afferent fibres near their termination within the spinal cord may be a common presynaptic effect of the three drugs. However, as we pointed out, there may be some differences in their actions due to which lidocaine is relatively stronger in depressing excitabilities of motoneurones and primary afferents, whereas silperisone is a stronger depressant of spinal reflexes. In other words, lidocaine may be closer to an ideal local anaesthetic with nerve conduction blocking actions, while silperisone to an ideal CMR with reflex inhibitory actions. These small pharmacological differences may also underlie the findings of efficacy/side effect studies, which indicated that silperisone compared to several CMR drugs was relatively free from sensory-motor side effects.

Until recently the exact molecular mechanisms underlying reflex inhibitory actions of tolperisone-type compounds remained largely unexplored. The only hints were Ono's work suggesting a lidocaine-like "membrane stabilising" action (Ono *et al.* 1984) and a report on calcium channel blocking effects of eperisone and tolperisone in snail neurones (Novales-Li *et al.* 1989). Therefore, to clarify the nature of the "membrane stabilising" action and to investigate relative contribution of different voltage gated ion channels involved in excitation and transmitter release, we performed a comparative study with silperisone, tolperisone and lidocaine. Selection of lidocaine as reference drug was justified not only by pharmacodynamic similarities but also by structural similarities according to pharmacophore modelling studies (Fels 1996). Testing these effects in the presynaptic neurones of the monosynaptic spinal reflex pathway, i.e. the primary sensory neurones, was a plausible choice.

We were the first to demonstrate directly in patch-clamp experiments that a tolperisone-type compound (RGH-5002 = silperisone) indeed blocks Na^+ channels (Bielik *et al.* 1997). The effects of tolperisone-type compounds were similar to lidocaine in that they shifted the steady-state inactivation curve of Na^+ channels to hyperpolarised direction; however they also decreased the maximum available current at hyperpolarised state (Fig. 43) (Kocsis *et al.* 2005). As a consequence, the apparent potencies of these drugs are highly state- and/or use-dependent and quite different figures can be obtained, depending on the experimental conditions, first of all depending on the resting holding potential in voltage clamp studies. Large differences in the transition voltage ($V_{1/2}$ of

steady-state inactivation) between the two pharmacologically distinct subtypes of sodium channels (mean $V_{1/2}$ -75 mV for TTX-S and 52 mV for TTX-R) also further complicate the assessment. Therefore the IC_{50}s for silperisone varied between 23 µM and 106 µM depending on the applied holding potential and the tested Na^+ channel subtype. Although the functions are not clearly separated, TTX-R channels ($Na_V1.8$ and $Na_V1.9$) are predominantly expressed in small diameter sensory neurones (Aδ and C) involved in pain transmission, whereas TTX-S (particularly $Na_V1.1$ and 1.6) channels are highly expressed in large diameter sensory neurones transmitting proprioceptive information (Ho and O'Leary 2011). Both pharmacologically distinct subtypes can mediate therapeutically important effects, however, from the perspective of antispastic effects and concerning the monosynaptic reflex pathway the effects on TTX-S channels might be regarded more relevant. Concerning the relevance of the applied holding potentials, we do not know exactly how much is the resting membrane potential of sensory neurones and primary afferents. From the literature quite diverse estimates can be drawn. For example, Safronov and co-workers measured an average resting membrane potential of -80 mV by whole-cell patch-clamp method in DRG slices from newborn rats *in vitro* (Safronov *et al.* 1996). However, Djouhri and co-workers (1999) found -45--50 mV in guinea pig DRG neurones *in vivo* by sharp intracellular microelectrodes, and the true physiological values are probably encompassed by these two examples. Furthermore, these resting values are subject to depolarizing effects during pathological processes and also during GABA-A mediated primary afferent depolarisation, which is a key mechanism of physiological presynaptic inhibition. Hence, even the measurement at V_H -70 mV on TTX-S channels may have underestimated the potency (overestimated IC_{50}) compared to the *in vivo* real-life situation. Since there was in average 23 mV difference between $V_{1/2}$ of TTX-R and TTX-S channels, in the routine pharmacological comparative study we applied similar (20 mV) difference, i.e. -60 mV and -80 mV, respectively, as the holding potential. Tolperisone and lidocaine were almost equally potent and were (1.6-4-fold) less potent than silperisone on both TTX-S and TTX-R channels. Apart from small variations in relative potencies neither drug can be concluded as really preferring either channel subtype. What is important to note, Na^+ channel blocking potencies and spinal reflex inhibitory potencies were approximately similar for all the three drugs with little differences (*cf.* EPSPint and TTX-S in Table 7). These data provide a strong argument for that Na^+ channel blockade is probably a leading mechanism involved in their reflex inhibitory actions.

Assessment of the potencies of tolperisone-type drugs on Ca^{2+} channels was not as much affected by the holding potential (nor by activation voltage) as the Na^+ channels, therefore investigations at different voltage holding (and activating) voltages were not justified. However, here another experimental condition had to be taken into consideration. It is well known by patch-clampists that calcium channels are prone to relatively rapid spontaneous inactivation ("rundown") under whole-

cell patch clamp conditions due to dialysis of the cell enterior that affects energy carrier molecules and phosphorilation state of the channel (Kostyuk *et al.* 1981a; Hoppe *et al.* 2005). However, the rundown can be prevented by supplementing the intracellular solution with GTP, CP and CPK (Forscher and Oxford 1985). Nevertheless, it has not been propagated, at least we did not see in the literature, that use of this supplementing reduces apparent effectiveness of drugs. We could confirm that CPK supplementation resulted in an excellent stabilising effect and prevented (or reversed) the rundown; but it markedly reduced apparent efficacy/potency of the tested drugs, among them silperisone. Hence, the rundown strongly limits the reasonable duration of reliable experiments to a few minutes and still can lead overestimation of potency in the case of a serial escalating concentration-response study (see Fig. 49A). However, comparing recordings in Fig. 49A and Fig. 49B also clearly showed that there was really a large difference in the effectiveness of comparable concentrations of silperisone between the two conditions, i.e. without and with CPK supplementation, which is evident also if the rundown were subtracted. These data suggest that CPK supplementation can lead to underestimation of drug potencies/efficacies. In this dilemma between stability and underestimation of potency, finally we choose the stability, in order to get more reliable experiments, and keeping in mind that the potency figures we get may be underestimated.

Using subtype selective blockers, in line with data published by Wu and Pan (Wu and Pan 2004), we found that only small portions of the high voltage activated calcium channels of DRG neurones were L- or P/Q-type channels. There was a much larger ω-conotoxin-GVIA sensitive component, representing N-type channels but there remained a great portion of drug-resistant (R-type) current. Silperisone (320 µM) inhibited both the ω-conotoxin-GVIA sensitive component and also most of the R-type current. Potency of silperisone on Ca^{2+} channels without CPK supplementation was similar to Na^{+} channel blocking potencies (IC_{50} of 63 µM) and was 3.4-fold less (IC_{50} of 217 µM) with supplementation. These results suggest that in addition to Na^{+} channel blockade Ca^{2+} channel blocking effects probably significantly contribute to the pharmacodynamic profile of silperisone. Tolperisone was 7-fold less potent than silperisone. The ratio between its Na^{+} and Ca^{2+} channel blocking potencies is certainly larger than for silperisone (see Table 7). However, taking into consideration that at 160 µM, which is equivalent to its TTX-S Na^{+} channel IC_{50}, it produced 20% Ca^{2+} channel blockade and the potential underestimation of potency due to CPK supplementation, we have to assume some contribution of the Ca^{2+} channel blockade to pharmacodynamics of tolperisone, too. In contrast, lidocaine was much less potent to block Ca^{2+} channels and therefore Ca^{2+} channel blockade may play negligible role in its pharmacodynamic effects. These relative additions of Ca^{2+} channel blockade to Na^{+} channel blockade, i.e. similar, smaller and negligible for silperisone, tolperisone and lidocaine, respectively, may well explain the above described profile

differences between the three drugs with regards to preferentially inhibiting afferent fibre potential or ventral root reflex (EPSP), since addition of Ca^{2+} channel blockade can further reduce presynaptic transmitter release which leads to greater suppression of EPSP in the case of silperisone and tolperisone as compared to lidocaine.

Our simple investigation on delayed K^+ currents of DRG neurones indicated that silperisone suppressed delayed rectifier potassium current of DRG neurones with approximately 3-5-fold lower potency (higher IC_{50}) as compared to Na^+ channel blockade. However, the effective concentration range on K^+ channels at least overlapped with that on Na^+ channels suggesting some small participation of K^+ channel blockade to the pharmacological profile of silperisone. However, it is difficult to ascertain how this effect, which might slightly decelerate repolarisation after action potentials, would really modulate the main pharmacological effects of this drug and whether it contributes to relative freedom of silperisone from side effects shown in pharmacological studies in behaving rodents or to the difference between tolperisone and silperisone in affecting reticulospinal inhibition. Concluding from the small effects at 160-320 µM and the high extrapolated IC_{50} values, the contribution of delayed rectifier K^+ channel blockade to the effects tolperisone and lidocaine is probably negligible. Nevertheless, it is worth to note that low concentrations (1-100 µM) of lidocaine were reported to slightly potentiate delayed rectifier K^+ currents of spinal dorsal horn neurones (Olschewski *et al.* 2002) and lidocaine had also blocking effect on A-type (rapidly inactivating) potassium currents with IC_{50} of 163 µM (Olschewski *et al.* 1998), which effects were out of the scope of our study. Furthermore, potentiation of delayed rectifier K^+ currents and thereby late post-stimulus repolarisation might explain relatively strong effect of lidocaine on late part of EPSP (EPSPtail) in our *in vitro* spinal reflex study (see Fig. 37).

Following our first report on silperisone (Bielik *et al.* 1997), and in parallel with our further publications in this field (Kocsis *et al.* 2005; Farkas 2006), two other research groups performed investigations and published data on effects of tolperisone-type drugs on voltage gated ion channels. Koppenhofer's group investigated action potentials and membrane currents at Ranvier nodes by potential clamp (vaseline gap) method on isolated myelinated fibres from sciatic nerves of the toad *Xenopus laevis* (During and Koppenhofer 2001; Hinck and Koppenhofer 2001). They observed inhibitory effects of silperisone and tolperisone on Na^+ permabilities with dissociation constants of 110 µM and 60 µM and also inhibitory effects on K^+ currents characterised with apparent dissociation constants of 24 µM and 320 µM, respectively. Schreibmayer's group investigated the effects of tolperisone and lidocaine on 7 recombinant Na^+ channel isoforms $Na_V1.2$-1.8 expressed in *Xenopus* oocytes (Hofer *et al.* 2006; Quasthoff *et al.* 2003). They observed blocking effects of both compounds on all Na^+ channel subtypes with somewhat different potency profiles. IC_{50}s for tolperisone ranged

from 49 µM ($Na_V1.8$) to 802 µM ($Na_V1.3$), those for lidocaine from 68 ($Na_V1.2$) to 687 µM ($Na_V1.3$). Holding / activation step potentials were -70 mV / +20 mV for TTX-R ($Na_V1.5$ and 1.8) channels and -100 / 0 mV for the rest of channels. Besides similarities in their effects, e.g. shifting the steady-state inactivation curve to hyperpolarisation, they observed some kinetic differences. Perhaps one of their most interesting findings was that although tolperisone was more potent than lidocaine at most isoforms ($Na_V1.4$, 1.6, 1.7 and 1.8) it was less potent at the cardiac specific $Na_V1.5$ channels and, in contrast with lidocaine, it did not prolong fast recovery from inactivation.

The above data from other groups further supported that Na^+ channel block plays a crucial role in pharmacodynamic effects of tolperisone-type compound and that a K^+ channel blocking effect makes little (if any) contribution for tolperisone but probably gains significance in the case of silperisone.

In summary, silperisone exhibited a tolperisone-like pharmacological profile with clear-cut reflex inhibitory actions mediated primarily at the spinal level but with a much more prolonged duration of action and functionally revealed oral bioavailability. However, there were also minor differences in the pharmacological profile of silperisone as compared to tolperisone, i.e. lack of inhibition of reticulospinal inhibition and slightly weaker effect on the dorsal root reflex, direct excitability of primary afferents and on the decerebrate rigidity. This profile may be associated with a relatively weak effect of silperisone on normal voluntary motor functions, which was indicated by the pharmacological profiling studies in mice and which might be a favourable clinical feature for a new CMR drug candidate. The slight differences in the pharmacodynamic profiles might be explained by differences in effects on voltage gated cation channels.

2.5 Summary of conclusions

1. The GYKI-20039 tremor test seems suitable for testing desired effectiveness and time course of effects of antispastic drugs in mice.
2. The test battery comprising GYKI-20039 tremor and morphine-induced Straub-tail tests for assessing main effects, and locomotor activity, rotarod, thiopental sleeping time and the newly invented weight lifting tests for assessing sedative and motor function impairing side effects appears to be suitable for determining critical therapeutic indices of CMR drugs.
3. All the tested clinically used CMR drugs had narrow therapeutic indices in some respect.
4. Silperisone exhibited relatively outstanding profile in terms of therapeutic index among CMR drugs.

5. The on-line (and off-line analog and digital) data analysis system that we designed was suitable for fine quantitative investigation of triggered electrophysiological events, e.g. reflex responses, recorded either by EMG or electroneurography.

6. Out of the different tests used, the flexor reflex test in cats was particularly suitable for pharmacodynamic characterisation of CMR drugs, as it exhibited long-lasting stability and sensitivity to all known CMR drugs.

7. Most CMR drugs, including tolperisone-type compounds, had similarly potent flexor reflex depressant effects in spinal animals as compared to those with intact neuraxis and higher CNS, indicating spinal cord as primary site of action. Diazepam and carisoprodol were exceptions.

8. Investigation of spinal ventral- and dorsal root reflex potentials revealed different and mechanism-specific patterns of actions for different CMR drugs.

9. Tolperisone-type compounds suppressed the spinal segmental reflex activity both *in vitro* and *in vivo*. Their profiles of actions on various components of ventral root reflex potentials were very similar but different from that of lidocaine, which had relatively smaller effects on the monosynaptic reflex.

10. In different spinal reflex studies, silperisone had similar *in vivo* potency to tolperisone and more or less similar profile of actions after i.v. administration but its effect was much longer lasting and therefore it was much more potent after oral or intraduodenal administration, i.e. it had greater functional bioavailability.

11. However, small but clear-cut pharmacodynamic differences also existed between silperisone and tolperisone, which included: (a) less pronounced depressant effect of silperisone on dorsal root reflex (reflecting afferent fibre excitability); (b) both drugs inhibited reticulospinal reflex facilitation but only tolperisone suppressed reticulospinal inhibition; (c) less pronounced depressant effect of silperisone on decerebrate rigidity.

12. Lidocaine, tolperisone and silperisone, all had some effects on excitabilities of primary afferents, motoneurones and the EPSP of motoneurones *in vitro*.

13. Silperisone had relatively weaker effect on primary afferent- and motoneurone excitabilities and relatively stronger effects on early EPSP and monosynaptic reflex as compared to lidocaine, while the profile of tolperisone was between that of silperisone and lidocaine.

14. Silperisone apparently did not have GABA-B agonist effect or antagonist effect on AMPA and NMDA receptors in the isolated rat spinal cord, which findings suggest that its depressant effect

on EPSP is probably due to a presynaptic inhibitory effect on glutamate release, via an action other than GABA-B agonism.

15. Silperisone apparently did not affect GABA and glycine receptor mediated feed-forward and recurrent inhibition in the spinal cord.

16. Silperisone, tolperisone and lidocaine all had concentration dependent blocking effects on both tetrodotoxin sensitive and tetrodotoxin resistant Na^+ channels of DRG neurones with characteristics resembling local anaesthetics. The effective concentrations were similar to the reflex inhibitory concentrations, suggesting Na^+ channel blockade as a leading mechanism causing reflex inhibition.

17. Silperisone suppressed also N-type and R-type Ca^{2+} currents of DRG neurones, which effects were also detected at concentrations comparable to Na^+ channel blocking concentrations, suggesting that Ca^{2+} channel blockade may also be involved in shaping pharmacodynamic profile of silperisone, i.e. its relatively stronger effect on (mono)synaptic transmission and EPSP (*in vivo* and *in vitro*) as compared to afferent fibre excitability effects of lidocaine.

18. Lidocaine had negligible Ca^{2+} channel blocking effects at relevant and significant Na^+ channel blocking concentrations, whereas relative Ca^{2+} channel blocking effect of tolperisone was between those of silperisone and lidocaine, suggesting that Ca^{2+} channel blockade slightly contributes to its pharmacodynamics by suppressing excitatory transmitter release in this way as well.

19. Silperisone attenuated also delayed rectifier K^+ currents of DRG neurones with somewhat lower potency but overlap with Na^+ channel blocking concentrations. Such effects were much weaker, possibly negligible, for both tolperisone and lidocaine.

20. Overall, our results showed an improved pharmacodynamic profile for silperisone as compared to either tolperisone or other CMR drugs.

21. Besides a lot of similarities with tolperisone, the small differences in pharmacodynamic profile of silperisone might be explained by differences in their voltage gated channel blocking profiles, as silperisone was less selective for Na^+ channel blockade.

2.6 Epilogue

Silperisone underwent single- and multiple-dose Phase I clinical trials and proved to be safe and well tolerated in healthy volunteers at doses that were expected to be in therapeutic range as judged from preclinical data, pharmacokinetics and EEG. Unfortunately clinical development of silperisone had to be suspended due to adverse ophthalmologic findings in chronic toxicity studies (Farkas

2006). Nevertheless, our studies exemplified that the use of the elaborated methodology and the approach of optimising a tolperisone-type compound might yield a better antispastic drug for human therapy, which result would meet a highly unmet medical need.

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3 Chapter 2: Preclinical modelling of migraine

3.1 Introduction

Migraine is a disabling headache disorder characterised by moderate to severe, intense throbbing or pulsating pain generally occurring on one side of the head and which may be aggravated by routine physical activity; other symptoms may include light or sound sensitivity, nausea and vomiting. Various forms of migraine affect approximately 18% of women and 6% of men (Estemalik and Tepper 2013). Although acute treatment of migraine is well manageable by triptans, there are many patients who cannot tolerate triptans or would need preventive medication. However, efficacy and tolerability of the preventive armamentarium, including antiepileptics, such as topiramate and valproate, and beta-adrenergic receptor blockers are unsatisfactory (Silberstein *et al.* 2012). Therefore, a great unmet need exists for novel effective, safe and well-tolerated pharmacotherapies of migraine.

Although recent research has revealed numerous details of the mechanisms participating in migraine generation, the primary initiating phenomena, the underlying neural and vascular mechanisms and their interrelationships are not understood and are surrounded by serious debates on various proposed theories, such as the vascular, the neural and the neurogenic inflammation theories of migraine (Moskowitz 1993; Messlinger *et al.* 2011; Ashina 2012; Noseda and Burstein 2013).

In line with the lack of a well-established theoretical background, numerous models have been proposed as useful tools for testing novel antimigraine drug candidates in animals or in human pharmacodynamic studies. Out of these, the most widely studied and accepted one is the nitroglycerin (NTG)-induced model of migraine (Olesen and Jansen-Olesen 2012).

For preclinical modelling of migraine we tried and used several approaches. Out of the work in this field two studies authored by the writer of this dissertation were published in international scientific journals (Farkas *et al.* 2015a; Farkas *et al.* 2015b). One of these was dealing with electrophysiological characterisation of the inflammatory sensitisation affecting neuronal activity in the trigeminal nucleus caudalis (TNC). The other was dealing with the NTG models of migraine. Since presenting the electrophysiological study (first author: Bence Farkas) is beyond the scope and volume of this dissertation, the reader is referred to that paper (Farkas *et al.* 2015a). However, the NTG model related studies (Farkas *et al.* 2015b) are presented and discussed here.

NTG administration causes an immediate headache in healthy subjects and a delayed migraine-like headache only in migraineurs (Thomsen *et al.* 1994; Olesen *et al.* 2013). The immediate headache in healthy volunteers was significantly attenuated by sumatriptan (Iversen and Olesen 1996) and the delayed headache incidence in migraineurs was reduced by valproate (Tvedskov *et al.* 2004a).

The majority of the work for establishing NTG-induced migraine models in animals was done in rats detecting the effect by various outcome measures, such as increased cerebral- and more controversially meningeal blood flow or blood vessel diameters (Read *et al.* 1999; Srikiatkhachorn *et al.* 2002; Gozalov *et al.* 2008; Greco *et al.* 2011; Pryazhnikov *et al.* 2014), increase in c-Fos protein expression in the trigeminal nucleus caudalis (Tassorelli and Joseph 1995; Pardutz *et al.* 2000; Knyihar-Csillik *et al.* 2008; Ramachandran *et al.* 2012), increase in neuronal nitric oxide synthase (nNOS) protein expression in trigeminal ganglia (TRGs) and/or TNC (Pardutz *et al.* 2000; Srikiatkhachorn *et al.* 2002; Dieterle *et al.* 2011), electrophysiological detection of increase in neuronal activity of TNC neurons (Jones *et al.* 2001; Koulchitsky *et al.* 2009) and hypersensitivity to pain either on the paw/tail or in the facial (trigeminal) region measured by behavioural responses to chemical, mechanical or thermal stimuli (Tassorelli *et al.* 2003; Tassorelli *et al. 2*006; Di *et al.* 2015; Greco *et al.* 2015). Whereas the use of rats has several advantages, there are also reasonable considerations in favour of utilising mice, such as better availability of transgenic animals or the need to use much lower amounts of expensive substances for *in vivo* studies. However, much more limited experience exists concerning the NTG model in mice. Recently reported data indicated that increased c-Fos expression (Bates *et al.* 2010; Markovics *et al.* 2012; Goloncser and Sperlagh 2014), thermal hyperalgesia (Bates *et al.* 2010; Goloncser and Sperlagh 2014) and mechanical allodynia of the paw (Bates *et al.* 2010; Pradhan *et al.* 2014) are utilisable outcome measures of NTG-induced changes in mice. In addition, our previous study suggested that increased cranial blood flow and light aversive behaviour are also suitable endpoints to detect NTG-induced changes in mice (Markovics *et al.* 2012). The aim of the present studies was to critically assess a panel of utilisable outcome measures in mice by revisiting previous findings, as well as by adding endpoints that have not been tested in mice yet, e.g. nNOS expression in the TRG and TNC, as well as pain hypersensitivity of the face, which formally might be a more relevant indicator of migraine than paw hyperalgesia.

There was another confounding factor in previous studies which determined our goals. In various studies different formulations of NTG were used, which were composed either for infusing or for sublingual spray application in patients, and the exact compositions of these often remained elusive. Several different NTG formulations contain propylene glycol and ethanol or propylene glycol and glucose; others (e.g. Nitrolingual) have even more complex vehicle without description of the exact composition. Some relatively dilute aqueous solutions of NTG (Nitro Pohl and Nitronal) contain only 5% glucose and mild acidification. Some of the above mentioned constituents (e.g. ethanol) may be assumed not to be entirely inert in studies of central nervous system functions. Nevertheless, many previous studies compared the effects of formulated NTG to a saline group instead of using an appropriate vehicle control (Bates *et al.* 2010; Di *et al.* 2015; Srikiatkhachorn *et al.* 2002; Tassorelli *et*

al. 2003; Tassorelli *et al.* 2006) and in lack of control vehicle we did the same in a previous study (Markovics *et al.* 2012) using Nitrolingual formulation of NTG. However, in the present studies, to establish well-controlled NTG models, we intended to use appropriate vehicle controls. For this purpose we clarified the composition of Nitrolingual and composed an appropriate vehicle for control experiments. In addition, we started the studies with investigating two different formulations, Nitrolingual and Nitro Pohl, which latter we considered as the possibly most inert one from the assortment.

Six types of studies were performed. In the first two studies, which included detection of light aversion and cranial blood flow, both NTG preparations were tried. However, based on the experience gained in these studies, the use of Nitrolingual was dismissed for the rest of the studies. Instead, in the immunohistochemistry studies and in the novel mouse orofacial pain test we carried out some pharmacological validation besides detecting the effects of NTG.

3.2 Materials and methods

3.2.1 Drug treatments and control vehicles

Two NTG preparations were used: Nitrolingual aerosol and Nitro Pohl solution for infusion (both obtained from Pohl-Boshkamp GmbH, Germany). The Nitrolingual aerosol contained 7.7 mg/ml NTG and was administered at a dosing volume of 1.3 ml/kg for a dose of 10 mg/kg intraperitoneally (i.p.). The vehicle control solution for Nitrolingual was compounded at Gedeon Richter Plc. and comprised (in % w/w): Miglyol 812 77.3%, ethanol 20%, glyceryl caprylate 2% and peppermint oil 0.7%.

The Nitro Pohl solution contained 1 mg/ml NTG and was administered at a dosing volume of 10 ml/kg for a dose of 10 mg/kg i.p. In addition to NTG, the aqueous Nitro Pohl solution contained 49 mg/ml glucose monohydrate. As vehicle control for Nitro Pohl, we used Rindex 5 solution for infusion (Teva, Hungary), which contained 5% glucose in an aqueous solution, and also contained NaCl 68 mmol/l, KCl 3.5 mmol/l, MgCl2·0.5 mmol/l and CaCl2 1.25 mmol/l. Sumatriptan succinate was dissolved in physiological (0.9%) saline solution (“saline”) and administered subcutaneously (s.c.) at a dosing volume of 10 ml/kg. Topiramate 80 mg/kg was dissolved in saline and administered i.p. at a dosing volume of 10 ml/kg.

3.2.2 Light aversion test

Light-aversive behaviour was examined in mice both in the early (0–30 min) and late phases (90–120 min) following administration of NTG. Male NMRI mice weighing 25-35 g were used. The mice were individually tested in the light aversion chamber (60 cm length× 26 cm width× 29 cm height) with two equally-sized compartments: one brightly lit (1000 lux, thermal-neutral fibre optic source, Fiber-lite,

Dolan-Jenner Inc., USA), painted white and lacking a top, the other not lit, painted black and top-covered. A small opening (7×7 cm) connected the two compartments. After 1-h conditioning period in the testing room, the animals were injected i.p. with NTG or vehicle and were immediately placed into the light-dark box. Two randomised experiments were performed with the two different formulations of NTG and their respective control vehicles (2x2 groups). In the experiment with Nitrolingual a third, physiological saline, group was also included. Two observation periods, equally 30 min long, were chosen on the basis of a series of previous experiments (Markovics *et al.* 2012). Following the early observation period, the mice were put back to their home cages and placed into the light–dark box again 90 min after treatment to see how they behave in the late period. The experiments were recorded with a digital camera and evaluated later by an observer who was blinded to the treatment allocation. The time spent in the light was measured and the percent time spent in the light compartment was calculated and plotted.

3.2.3 Cranial blood flow experiments

Male CD1 mice weighing 22-28 g were anaesthetised with urethane (1.5 g/kg i.p.) and placed onto a heating pad maintained at 38 °C. A cannula was inserted into the trachea and mice were breathing spontaneously throughout the experiment. The head was fixed and the skin and connective tissues covering the cranium were carefully removed. The cranial blood perfusion was investigated using a non-invasive laser Doppler scanner (PIM II System, Perimed AB, Sweden) through the closed cranium. The scanning device was positioned 18 cm above the head and the scanned region was extended to the top surface of both hemispheres. The scanner measures an averaged total microcirculatory blood perfusion in arbitrary units, including blood flows in capillaries, arterioles, venules and shunting vessels of meninges and superficial cortex. In this way an overall mean cranial blood flow could be assessed. Perfusion changes were expressed as percentage of baseline measurement to enable comparison of results. Control baseline monitoring measurements were repeated until the mean perfusion value had stabilised. Then an NTG preparation (10 mg/kg Nitrolingual or Nitro Pohl) or corresponding vehicles were injected i.p. and the post-dose recording lasted for 4 h. The experiments were carried out in two series; one comparing Nitrolingual, the other comparing Nitro Pohl to the corresponding vehicle.

3.2.4 Immunohistochemistry study of c-Fos and nNOS in TNC and TRG

Four groups of six male CD1 mice weighing 22-28 g were injected i.p. either with NTG (3 groups with 10 mg/kg Nitro Pohl) or vehicle (10 ml/kg Rindex 5). Two groups treated with NTG received also treatment of sumatriptan (2x5 mg/kg 30 min before and after NTG) or topiramate (80 mg/kg 30 min before NTG). Two hours following injection of NTG or vehicle, the mice were anaesthetised with

2 g/kg urethan (i.p.) and perfused transcardially with 20 ml 0.1 M sodium phosphate-buffered saline (PBS; pH 7.6) followed by fixative (150 ml ice-cold 4% paraformaldehyde) solution. The whole brain with the rostral cervical spinal cord and TRGs was dissected and post-fixed for 24 h in 6% paraformaldehyde solution.

To study the caudal division of the spinal trigeminal nucleus, the medulla oblongata caudal to the obex with the rostral cervical spinal cord was embedded in 4% agar gel. Starting at the obex, 20 µm coronal sections were cut on a Lancer Vibratome and collected throughout the rostro-caudal extent of the brainstem and the spinal cord. Then, they were placed into anti-freeze solution consisting of 30% glycerol, 20% ethylene glycol and 0.1 M sodium phosphate buffer, and stored at -20 °C until further use. Two series of sections were used for c-Fos and nNOS immunohistochemistry. For details of the staining see Farkas *et al.* (2015b).

TRGs were dehydrated in 50 v/v%, 70 v/v%, 90 v/v%, and 100 v/v% ethanol solutions. Sectioning was carried out on paraffin-embedded accurately oriented and blocked samples. Two series of five 4-µm-thick longitudinal sections each interspaced by 60 µm were collected and mounted on silan-pretreated slides, dried and used for c-Fos and nNOS immunohistochemistry. For details of the staining see Farkas *et al.* (2015b).

For each type of evaluation in TNC, approximately 28 sections per animal interspaced by 60 µm and belonging to the first and second cervical segments of the spinal cord were selected according to Sidmann *et al*. (1971), then 5 good quality sections per animal were designated for quantitative evaluation. Immunoreactive neurons in laminae I-III were evaluated. Also 5 longitudinal sections interspaced by 60 µm were selected from 6-8 sections covering the whole diameter of the ganglion for each type of evaluation in TRG. Evaluated section selections and quantifications were performed by an experienced neuroanatomist blinded to group assignment of the samples. The contents of c-Fos in the TRG and TNC were determined by simple manual counting of all c-Fos-positive cell nuclei in non-edited digital images. For the TNC, the absolute number of immunoreactive neurons per section was counted. For the TRG, the number of immunoreactive neurons was expressed in percentage of total number of cell nuclei. The observed absolute and relative counts were summed and averaged in each animal.

3.2.5 Thermal hyperalgesia of the paw measured with the hot plate test

Male NMRI mice weighing 25-35 g were used. The hot plate test was performed using an electronically controlled hot plate apparatus heated to 46.5 °C (±0.3 °C). The animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placing of the animal on the hot plate and the occurrence of licking of hind paws or jumping off the surface was

recorded as response latency. The cut-off time was 60 s. The response latency was recorded 60 min before and 60, 120, 180 and 240 min following i.p. injection of NTG (10 mg/kg Nitro Pohl) or vehicle.

3.2.6 Mechanical allodynia of the paw measured with von Frey filaments

Male NMRI mice weighing 25-35 g were used. Hind paw withdrawal thresholds were tested by von Frey filament stimulation of the plantar surface of hind paw using an up-and-down paradigm (Chaplan *et al.* 1994). Mice were placed in plastic chambers with mesh metal floor and allowed a habituation period of 30 min. A series of 10 von Frey hairs (Stoelting Co., USA) with incremental stiffness (ranging from 0.008 g to 2.0 g) was applied to the mid-plantar surface of the hind paw. According to the up-and-down paradigm increasing or decreasing of the stiffness of the next filament by one step was dependent on negative or positive paw withdrawal response of the actual trial. The 50% paw withdrawal threshold (PWT expressed in grams) was determined from 5 critical test responses starting with the first positive response (Dixon 1980). NTG (10 mg/kg Nitro Pohl) or vehicle was injected and PWT was measured before and 60, 120, 180 and 240 min after the injection.

3.2.7 Orofacial pain sensitivity tested with von Frey filaments

Male NMRI mice weighing 25-35 g were used. The mice were placed into a 9-cm-long restraining glass cylinder so that only the head poked out and allowed a habituation period of 5 min. A von Frey filament of 0.4 g force was used (Stoelting Co., USA). The filament was applied to the whisker pad on right side of the snout 12 times at approximately 90° angle until bent (Krzyzanowska *et al.* 2011). The responses were recorded and scored as follows: uni- or bilateral forepaw swipes across the face (1 point), aggression/biting of the probe following stimulus (0.25 points) or clear withdrawal of the head from the stimulus (0.25 points). The points (accumulated in the 12 trials) were summed to give the overall “response score”. The animals were assigned to five groups and NTG (10 mg/kg Nitro Pohl, in four groups) or vehicle (one group) was injected i.p. Thirty minutes later various doses of sumatriptan (0.3-3 mg/kg) or saline (control) solution were injected subcutaneously to the NTG-treated animals and saline also to the vehicle-treated animals. Then von Frey testing was repeated at 60, 90 and 120 min post-NTG. Mice assigned to different groups were treated and tested in parallel in a randomised arrangement. The experimenter was blind to the treatment during testing.

3.2.8 Data analysis and statistics

For different outcome measures data were expressed either as absolute values or percentages of baseline values. Results are plotted as the mean ± standard error of the mean (SEM). Where repeated measurements were performed statistical significance of differences was tested by two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni’s post-test for time-matching samples. For immunohistochemistry data one-way ANOVA was performed after

confirming normality of distribution by Kolmogorov-Smirnov test. In case of significance, all other groups were compared to NTG control by Dunnett's post-test. A p value less than 0.05 was considered significant in all tests.

3.3 Results

3.3.1 Light aversion assay

Mice injected with saline did not exhibit any overt tendency of change with regards to the average time spent in the light compartment ('light-time') either within the early or the late phase following the injection, although their time spent in light was less in the late phase (Fig. 55A-B). In contrast, the time spent in the light was significantly reduced by both Nitrolingual and its vehicle in the early phase observation period, suggesting rapid development of photophobia (Fig. 55A) elicited primarily by the solvent of Nitrolingual itself. Nevertheless, by the late phase there was no apparent difference in the light avoiding behaviour between the saline, Nitrolingual and vehicle groups (Fig. 55B). In the experiment with Nitro Pohl, apart from a moderate gradual decline of the light-time, the injection caused no overt effect either in the early or the late phase (Fig. 55C-D). Most importantly, we could not detect any significant differences between the NTG groups and their simultaneous vehicle controls in either experiment.

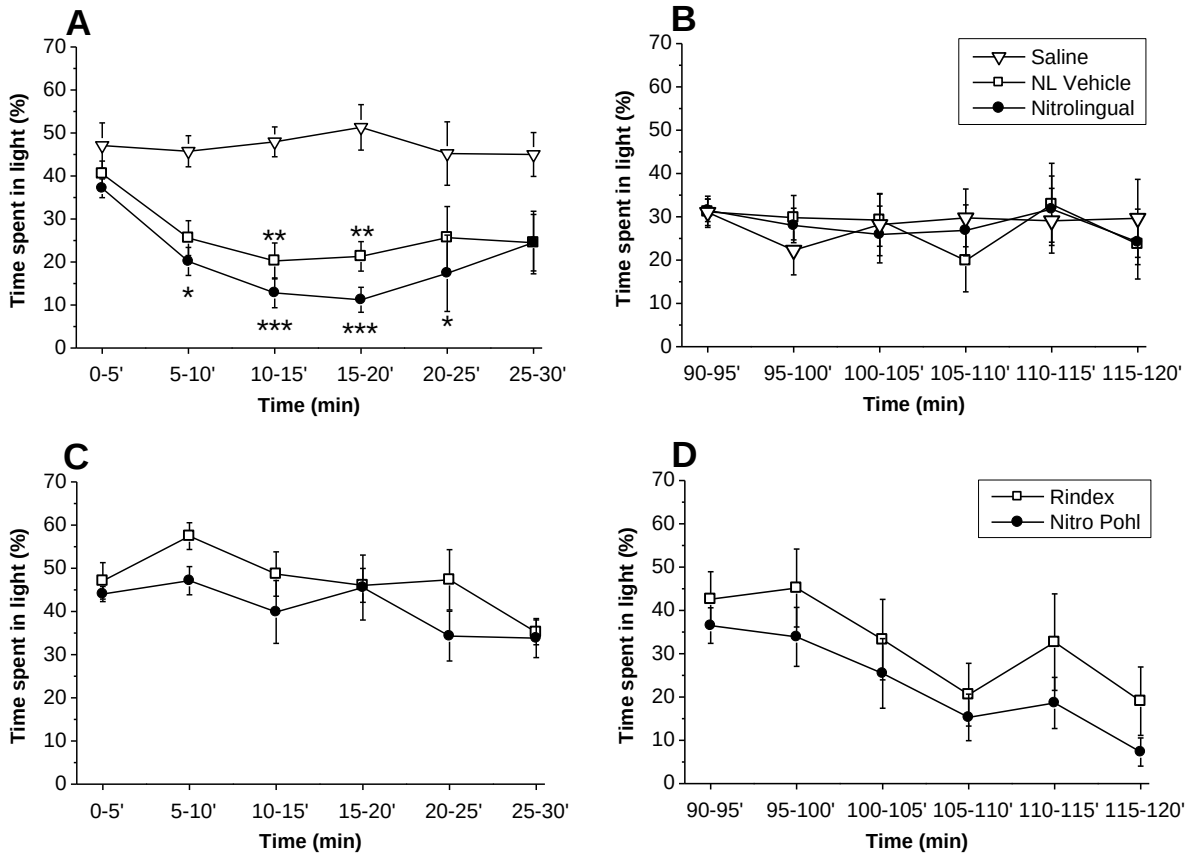

Fig. 55. Effects of two different formulations of nitroglycerin (NTG; 10 mg/kg, i.p.) compared to vehicle controls in the light aversion test. Nitrolingual (NL) was compared to both a saline group and a solvent mimicking „NL vehicle" group (A, B). Nitro Pohl was compared to its corresponding vehicle, Rindex (C, D). Data are presented as mean ± SEM (N=12-13/group in A-B and 8/group in C-D) of percent time spent in the light compartment of the light-dark box. Results were evaluated separately in the early 0-30 min (A, C) and late 90-120 min phases (B, D) following NTG injection. Abscissas show the time after NTG injection. Asterisks show statistically significant differences compared to the saline group (*p<0.05, ** p<0.01, ***p<0.001; two-way repeated measures ANOVA followed by Bonferroni's test). There were no significant differences when NTG formulations were compared to their respective vehicle controls.

3.3.2 Cranial blood perfusion

The absolute values of baseline perfusion (as expressed in arbitrary units) did not differ significantly between corresponding NTG and vehicle groups. The baseline values (mean±SD) for NTG and vehicle were respectively 4.00±0.58 and 3.67±0.7 units in the Nitrolingual series and 5.07±0.34 and 5.12±0.63 units in the Nitro Pohl series of experiments. Injection of Nitrolingual, as well as its vehicle initiated a progressive increase in the cranial blood perfusion as measured by laser Doppler scanning. The enhancement amounted approximately 20% by 120 min post dose and remained sustained till the end of the 4-hour observation period in both groups (Fig. 56A). Apart from the non-significant difference that the rise in cranial blood perfusion occurred somewhat faster in the Nitrolingual group, there was no overt difference between the changes elicited by Nitrolingual or its vehicle. In

contrast, injection of Rindex, the vehicle for Nitro Pohl, caused no remarkable change in the cranial blood perfusion, apart from <5% decrease in the first hour, and there was no significant difference between Nitro Pohl and the corresponding vehicle group (Fig. 56B). Hence, again, we could not detect any significant effect of NTG compared to the relevant control groups. Therefore, having observed a remarkable effect with its solvent, we excluded Nitrolingual from our further studies.

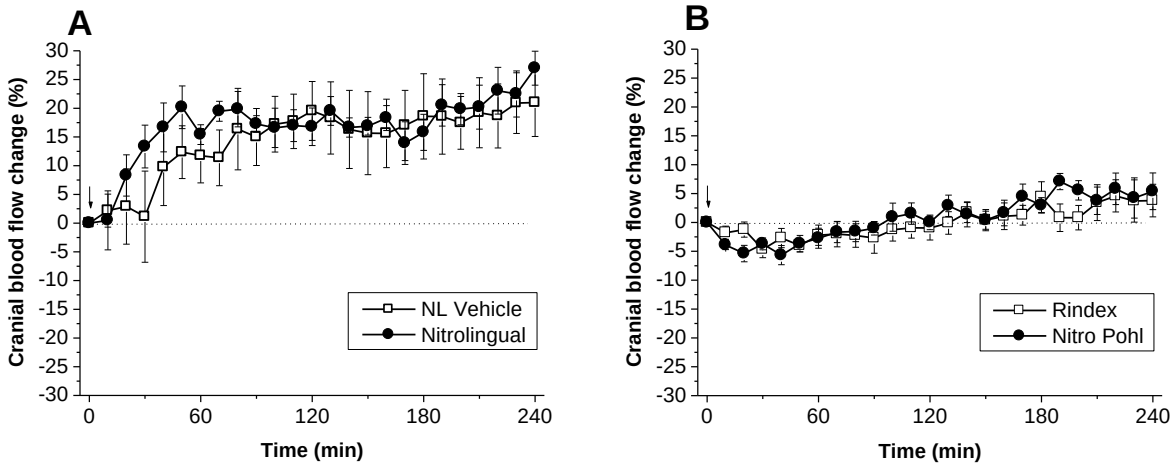

Fig. 56. Effects of two different formulations of nitroglycerin (NTG 10 mg/kg, administered i.p. at 0 min) compared to their respective vehicle controls on cranial blood perfusion measured by Laser Doppler scanning. Data are presented as mean ± SEM (N=6/group) of percentage change relative to the baseline recording. There were no significant differences when the different NTG formulations (Nitrolingual: NL and Nitro Pohl) were compared to their respective vehicle controls (two-way repeated measures ANOVA followed by Bonferroni's test).

3.3.3 Immunohistochemistry for c-Fos and nNOS

We combined verification of the effect of NTG with pharmacological validation by testing the sensitivity of this model to the abortive and preventive antimigraine drugs sumatriptan and topiramate, respectively. Quantification of c-Fos positive nuclei in the TNC indicated statistically significant two-fold increase after treatment with NTG (Nitro Pohl) compared with the vehicle control (Rindex) group (Fig. 57A). However, a statistically significant increase could not be detected in the TRG (Fig. 57B, ANOVA p=0.31). Compared to the NTG treated group, a high dose of sumatriptan (5 mg/kg, s.c.) administered before and after NTG injection did not reverse significantly the increase in c-Fos expression in TNC. Although an apparent trend towards normalisation might have been noticed, it was far from significance (Dunnett's adjusted p=0.63). In contrast, pretreatment with topiramate (80 mg/kg, i.p.) completely prevented the NTG-induced increase in c-Fos expression in TNC, which effect was significant (Fig. 57A; p=0.03).

Immunostaining for nNOS did not reveal any significant alteration induced by NTG injection or any effect of the antimigraine drugs. The number of nNOS positive cells showed rather low variability in the TNC but much higher in the TRG (Fig. 57C-D).

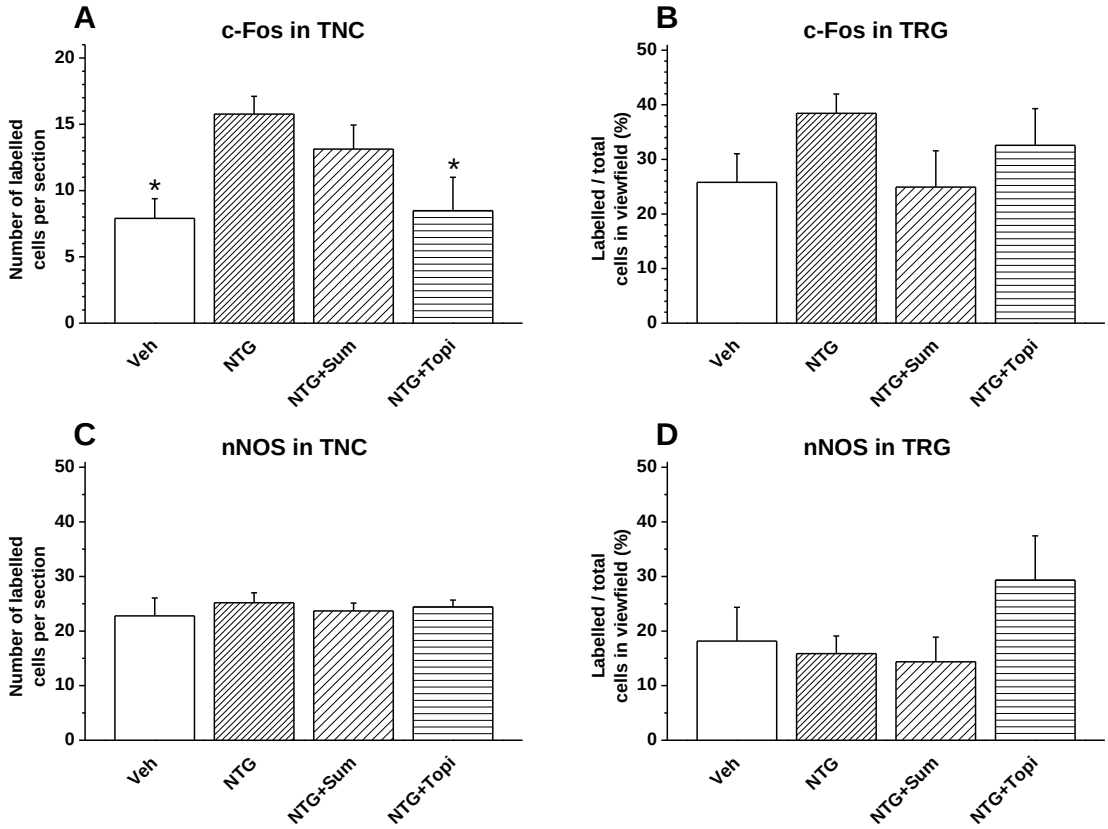

Fig. 57. Effects of nitroglycerin (NTG; 10 mg/kg i.p.) compared to vehicle (Veh) controls on quantitative immunohistochemistry for c-Fos (A-B) and nNOS (C-D) in the trigeminal nucleus caudalis (TNC) and trigeminal ganglia (TRG), and the effects of sumatriptan (Sum; 2x5 mg/kg, s.c.) and topiramate (Topi; 80 mg/kg, i.p.) in mice injected with NTG. Data are presented as mean ± SEM (N=5-6/group). Asterisks show statistically significant differences compared to the NTG group (*p<0.05; one-way ANOVA followed by Dunnett's test).

3.3.4 Thermal hyperalgesia of the paw

Intraperitoneal injection of neither the vehicle nor Nitro Pohl caused any signs of pain related behaviour suggesting peritoneal irritation. Hot plate latencies of vehicle-treated mice were very stable over time with repeated testing four times up to 240 min post dose (Fig. 58A). NTG injection caused a clear-cut hyperalgesia, manifested in reduced defensive response latency, which effect was maximal and statistically significant at 60 min post dose. Nearly complete recovery was apparent by 180 min and there was no sign of a delayed secondary wave of hyperalgesia (Fig. 58A).

3.3.5 Mechanical allodynia of the paw

Changes in tactile hind paw withdrawal thresholds showed similar time course of the effect of NTG to that seen in the hot plate test (Fig. 58B). Although the mechanical threshold data of the vehicle control group displayed larger variability (coefficient of variation) and lower stability as compared to hot plate results, the apparent effect was also larger resulting in statistically significant changes at 60 min and 120 min post dose. The thresholds at 180-240 min after injection clearly indicated a tendency towards recovery without any sign of a late phase secondary sensitisation.

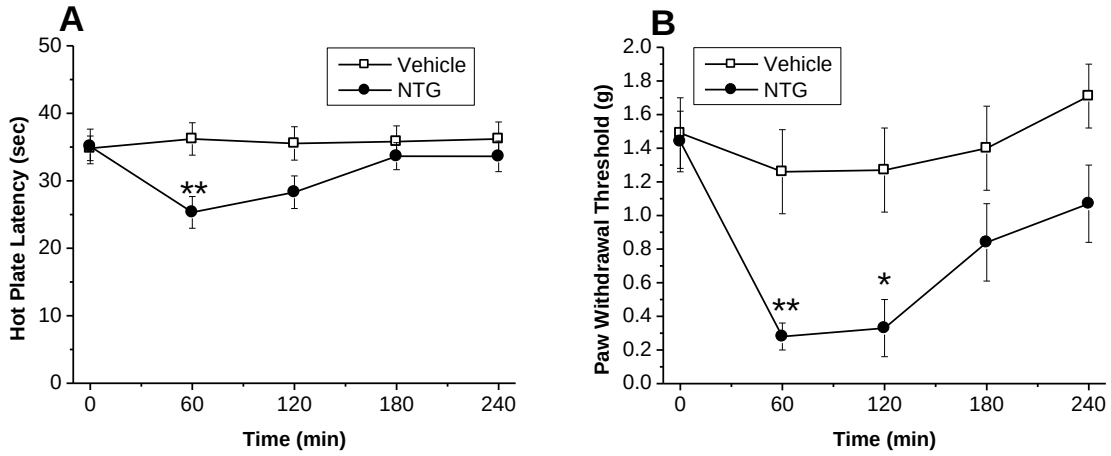

Fig. 58. Effects of nitroglycerin (NTG; 10 mg/kg i.p.) compared to vehicle controls on thermal hyperalgesia (A) and mechanical allodynia (B) of the hind paw. Data are presented as mean ± SEM (N=10/group in A and 8/group in B). Abscissas represent the time after NTG injection. Asterisks show statistically significant differences (*p<0.05; **p<0.01; two-way repeated measures ANOVA followed by Bonferroni's test).

3.3.6 Orofacial pain sensitivity

Repeated recording of orofacial pain scores indicated no overt change in the pain-related behaviour of vehicle-treated animals (Fig. 59). However, mice treated with NTG exhibited a significant increase in nocifensive behaviour, which peaked at 60 min post-NTG and was declining later. The observed time-course was apparently similar to that seen in the plantar pain tests. Having established relatively short duration of action of NTG in the paw hyperalgesia/allodynia tests, we did not extend the study duration of the orofacial allodynia testing further than 2 h after NTG. Instead, we included three further groups for pharmacological validation by a dose-response study of sumatriptan. Administration of sumatriptan 30 min before the first post-NTG testing remarkably suppressed the pain behaviour of mice. The dose-response relationship was apparent and flat at 60 min post NTG as the lowest dose of 0.3 mg/kg sumatriptan caused profound (more than 50%) inhibition. However, the effect of sumatriptan was not statistically significant at later time-points as the NTG-treated

control group started to decline and the reversing effect of sumatriptan remained only partial (Fig. 59).

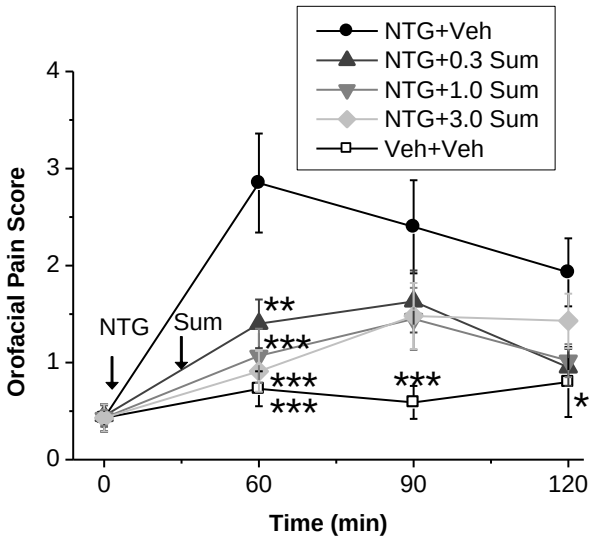

Fig. 59. Effects of nitroglycerin (NTG; 10 mg/kg, i.p.) compared to vehicle controls (Veh+Veh) on orofacial pain elicited by tactile stimuli, and alterations of the NTG-induced facial allodynia by different doses of sumatriptan (0.3-3 mg/kg, s.c.; NTG+Sum). Data are presented as mean ± SEM (N=10-11/group) Abscissa represents the time after NTG injection. Asterisks show statistically significant differences compared to the NTG+vehicle treated group (*p<0.05; **p<0.01; ***p<0.001; two-way repeated measures ANOVA followed by Bonferroni's test). Arrows indicate the time of administration of drugs.

3.4 Discussion

Altogether we investigated 9 outcome measures in 6 studies and confirmed the utility of 4 of these for detecting the effects of NTG in mice. These outcome measures are (1) c-Fos expression in the TNC, (2) thermal hyperalgesia of the paw, (3) mechanical allodynia of the paw and (4) orofacial pain sensitivity. These might be useful to predict anti-migraine effectiveness of drugs or target related genetic manipulations, provided that their sensitivity to anti-migraine drugs is proven in pharmacological validation experiments. Effectiveness of a single dose of NTG to influence the first 3 of these endpoints in mice has been proven previously (Bates *et al.* 2010; Goloncser and Sperlagh 2014). However, some pharmacological validation by using sumatriptan has been provided only for the paw allodynia and hyperalgesia (Bates *et al.* 2010). Therefore, we included some groups to perform validation of those mouse tests that had no previous pharmacological validation data at all, i.e. in the immunohistochemistry and the orofacial pain tests. Nevertheless, it is worth to note that validation for predictive power of such migraine models would require the use of several different abortive and preventive medications with clinical proof of effectiveness, such as triptans, topiramate, propranolol and CGRP antagonists (Ashina *et al.* 2013) as well as investigation of some clinically failed

negative controls. Extensive validation of these models was beyond the scope of the present investigations but might be the subject of further studies.

The protein product (c-Fos) of the proto-oncogene *c-fos* is a sensitive and widely applicable marker of neuronal activation in response to various stimuli and its enhanced expression in the TNC after NTG administration in rats has been reported by several groups (Tassorelli and Joseph 1995; Pardutz *et al.* 2000; Knyihar-Csillik *et al.* 2008; Ramachandran *et al.* 2012). NTG-induced increase of nNOS protein expression in the TNC and TRG has also been demonstrated in rats (Pardutz *et al.* 2000; Srikiatkhachorn *et al.* 2002; Dieterle *et al.* 2011). Whereas we also proved the NTG-induced increase of c-Fos in the TNC, we could not confirm NTG-induced changes of nNOS expression in mice either in the TNC or in the TRG. Since nNOS inducing effects in rats were revealed by at least three different research groups, we may assume that the discrepancy can be a species specificity issue. Concerning NTG-induced c-Fos induction in TRG, we did not observe any significant effect in mice and we did not find any preceding literature data assessing this parameter in rats. However, c-Fos also proved not to be a suitable pain marker in sensory neurons of dorsal root ganglia of rats in contrast to the phosphorylated extracellular signal-regulated kinase which was induced both in dorsal horn and sensory ganglia by painful interventions (Gao and Ji 2009). Having a significant NTG-induced increase only for c-Fos in the TNC, our attempt for pharmacological validation can be meaningfully interpreted only with regards to this outcome measure. The NTG-induced increase in c-Fos expression was sensitive to pretreatment with topiramate, which completely prevented the effect of NTG. Although preventive treatment by repeated dosing would have better mimicked the clinical setting, in this preliminary validation experiment administration of a single high dose was sufficient. On the contrary, our mouse NTG model with c-Fos expression readout was apparently insensitive or weakly sensitive to sumatriptan, administered 30 min before and after NTG, which did not produce a significant effect. In rats, Ramachandran *et al.* (2012) observed partial reversal of NTG-induced c-Fos increase in the TNC, but that model used much lower dose of NTG (80 µg/kg) by intravenous (i.v.) infusion. It remains an interesting question whether this difference in sensitivity to sumatriptan treatment is the consequence of species difference or the different provoking dose of NTG. In brief, c-Fos expression in TNC induced by NTG at the dose of 10 mg/kg i.p. in mice might be an utilisable outcome measure for detecting the effect of preventive treatments.

Our findings with regards to thermal hyperalgesia and tactile allodynia of the paw were in good concordance with those of Bates *et al.* (2010), showing maximal thermal hyperalgesia at 60 min and maximal or close to maximal tactile allodynia at 60-120 min post-NTG, and complete or partial recovery, respectively, by 4 h post dose. Our results with the hot plate method showed greater stability but smaller percent reductions in latency than their results by the Hargraves method. On the

other hand, the baseline thresholds in our tactile allodynia test in NMRI mice were more than two-fold higher than in their C57BL6 mice, which is in agreement with our unpublished experience on comparison of the two strains. However, starting from a higher control threshold, we observed also a greater NTG-induced drop in percentage terms, which provides a greater window for investigation of preventive effects. Here it is relevant to note that Pradhan *et al.* (2014) have shown the single dose (10 mg/kg i.p.) NTG-induced plantar allodynia of mice to be sensitive to both sumatriptan and topiramate, whereas the progressive sustained mechanical allodynia induced by repeated dosing was sensitive only to topiramate.

Measurement of orofacial pain sensitisation has apparently greater construct validity with regards to modelling migraine than measurements in other somatic areas. It reflects sensitisation in the innervations field of the trigeminal nerve, which is believed to play a central role in migraine generation (Messlinger *et al.* 2011). Spontaneous migraine is often accompanied by cephalic cutaneous allodynia, though extracephalic (somatic) allodynia is also prevalent (Bigal *et al.* 2008; Lipton *et al.* 2008; Bernstein and Burstein 2012). Occurrence of orofacial pain hypersensitivity after NTG injection has been demonstrated in rats (Di *et al.* 2015; Greco *et al.* 2015), but not in mice. Our results show that pain-related behaviour scoring in mice can be used to detect orofacial pain sensitising effect of NTG. This orofacial hyperalgesia had similar time course to the plantar hyperalgesia with maximum at 60 min post NTG and a tendency of recovery afterwards. Furthermore, the NTG-induced orofacial hyperalgesia was significantly alleviated by sumatriptan administration. These data suggest that measurement of orofacial pain can also be a useful outcome measure for modelling NTG-induced migraine-like headaches in mice; and this model is sensitive to triptans, like the plantar hyperalgesia/allodynia (Bates *et al.* 2010). It remains to be answered by extensive pharmacological validation experiments, whether the orofacial and the plantar (or other somatic) hyperalgesia/allodynia elicited by NTG exhibit differences in their pharmacological sensitivity, which might become an argument for favouring orofacial over plantar testing, or they prove to carry equal information in terms of modelling migraine.

We confirmed our previous finding (Markovics *et al.* 2012) that injection of Nitrolingual enhanced the cranial blood perfusion as measured by transcranial laser Doppler scanning over the hemispheres of mice. However, comparison of the results with the relevant vehicle indicated that the enhanced blood perfusion was not due to the effect of NTG but the vehicle itself caused such pronounced effect and there was no significant difference between Nitrolingual and its vehicle. Since the vehicle contains several pharmacologically active constituents, including ethanol and peppermint oil, its vasodilating effects might be attributed to these ingredients. Ethanol can cause vascular relaxation by activation of transient receptor potential (TRP) vanilloid 1 receptors and CGRP release (Nicoletti *et*

al. 2008). Peppermint oil (which contains menthol and menthone) can cause vasodilation through CGRP release caused by activation of the TRP ankyrin 1 and TRP menthol 8 receptors (Namer *et al.* 2012). More surprisingly, we could not demonstrate any remarkable effect of NTG on cranial blood perfusion when it was administered in an apparently inert vehicle (Nitro Pohl *vs.* Rindex 5), as neither the vehicle nor Nitro Pohl produced any overt change.

It is unclear if this lack of effect is due to species differences or the administration route or the inability of the technique to detect NTG-induced changes, as it averages blood flows in different depths and vessels or other experimental conditions, such as anaesthesia. In rats, typically meningeal blood vessel diameters have been assessed by imaging techniques and local cortical blood flow measurements were performed by laser Doppler flowmetry under anaesthesia. Cortical blood flow measurements after i.v. administration of small NTG doses (20-60 μg/kg) indicated increased flow (Read *et al.* 1999; Gozalov *et al.* 2008). Increased cortical laser Doppler flow was reported also after a large (10 mg/kg) i.v. dose of NTG (Srikiatkhachorn *et al.* 2002). Meningeal arterial diameters indicated strong dilations after a small i.v. NTG dose (Gozalov *et al.* 2008). Nevertheless, after an i.p. dose of 10 mg/kg in rats opposite changes, i.e. increased diameter of cortical and decreased diameter of meningeal arterioles were observed (Pryazhnikov *et al.* 2014). Hence, a possible explanation for lack of effect of NTG is that opposing vascular changes mutually neutralised in the net perfusion that was measured by the laser Doppler scanning technique. The impact of anaesthesia might also be considered. Gozalov et al., (2008) and Srikiatkhachorn et al., (2002) reported NTG-induced meningeal and cortical vasodilations in pentobarbital-anaesthetised rats, whereas Pryazhnikov *et al.* (2014) reported the opposing effects on cortical and meningeal arterioles in urethane- as well as ketamine-xylazine-anaesthetised rats. We also used urethane in our experiments, first, to reproduce the conditions of Markovics *et al.* (2012), a study comparing Nitrolingual to saline, second, to achieve a stable and long-lasting anaesthesia with relatively preserved autonomic reflexes and cardiovascular tone (Janssen *et al.* 2004).

In mice, measurements of cranial vascular effects are more difficult than in rats due to their smaller size. A study applying intravital microscopy in pentobarbital-anaesthetised mice found that, in contrast with rats, meningeal vasodilating effect of CGRP could be detected only after preconstriction with endothelin-1 (Gupta *et al.* 2006). However, we could not find any reports of NTG-induced changes in cranial circulation of mice. The transcranial laser Doppler scanning seemed to be a promising and relatively non-invasive approach in mice. Nevertheless, although this method was apparently suitable to detect probably ethanol+menthol-induced enhancement of cranial blood flow in mice, it did not reveal 10 mg/kg i.p. NTG-induced changes in our experiments.

Photophobia is a common accompanying symptom of migraine, and light-avoiding behaviour is considered as a relevant outcome measure mimicking the migraineous state in mice. Light aversion could be induced by intracerebroventricular CGRP treatment in transgenic mice sensitised to CGRP by overexpression of the human receptor activity modifying protein 1 (hRAMP1) associated with functional CGRP receptors (Recober *et al.* 2009; Recober *et al.* 2010). In a previous study we showed that Nitrolingual induced significant light aversion when compared to a saline-treated group (Markovics *et al.* 2012). Significant differences were seen at the end of the early as well as the late phase of the study. In the present study, the light aversive behaviour of saline control animals was less fluctuating and significant light aversion of Nitrolingual-treated mice was apparent only in the early phase. However, it turned out that the light aversive behaviour was attributable to the vehicle of Nitrolingual, as the genuine vehicle-treated animals exhibited a similar pattern to that with Nitrolingual. Furthermore, comparison of Nitro Pohl to its relevant vehicle control did not reveal any significant NTG-induced effect either in the early or in the late phase of the study. Hence, according to the present findings, it was not the NTG which induced light aversion in mice but the vehicle of Nitrolingual.

Doses and routes of administration of NTG varied between animal studies making the interpretation and comparison of the results difficult. We uniformly applied 10 mg/kg i.p. dose of NTG in all experiments. This dose selection was based on the report of Bates *et al.* (2010), who established in dose-response studies that this was the minimum dose essential for detecting overt hypersensitivity in both thermal hyperalgesia and mechanical allodynia tests in mice. The relevance of such large systemic NTG doses has been criticised as being 1000-fold higher than the doses provoking headache and migraine in volunteers and patients, respectively (Ramachandran *et al.* 2012; Jansen-Olesen *et al.* 2013). In the human NTG model typically 2.4-10 μg/kg (0.12-0.5 μg/kg/min for 20 min) i.v. dose (Iversen and Olesen 1996; Tvedskov *et al.* 2010) or 0.5 mg (≈8 μg/kg) sublingual dose (Juhasz *et al.* 2005) is applied. Advocates of low-dose NTG proposed 80 μg/kg (4 μg/kg/min for 20 min) i.v. infusion in rats. After this dose, increased c-Fos expression in the TNC was evident (Ramachandran *et al.* 2012); but pain hypersensitivity has not been demonstrated so far. However, oral bioavailability of NTG in rats was low (F=1.6%) and associated with fast elimination (T1/2=4 min) and strong first-pass effect (Yap and Fung 1978). Several factors may contribute to that about 100-fold higher doses are needed in mice by i.p. administration to elicit pain hypersensitivity than the suggested i.v. dose in rats provoking c-Fos increase: (1) the first-pass effect may be also significant after i.p. administration (Lukas *et al.* 1971), (2) allometric scaling between rats and mice might be an argument for higher doses in mice; and (3) the potential need for somewhat higher or longer exposures to elicit consistent pain hypersensitivity. In fact, almost all studies demonstrating NTG-induced pain

hypersensitivity in healthy mice or rats used the dose of 10 mg/kg, i.p. (Tassorelli *et al.* 2003; Tassorelli *et al.* 2006; Bates *et al.* 2010; Pradhan *et al.* 2014; Di *et al.* 2015; Greco *et al.* 2015) or 15 mg/kg, i.p. (Goloncser and Sperlagh 2014). The only exception applied 0.1 mg/kg i.p. dose and found short-lasting periorbital tactile threshold decrease in naïve rats (Oshinsky and Gomonchareonsiri 2007). The time-course of headache ratings in healthy volunteers after 20-min-long infusion of NTG exhibits only an early peak, whereas that in migraine patients an early and a late peak. The second peak, the delayed headache, particularly if elicited by 0.5 µg/kg/min infusion rate, fulfils International Headache Society criteria of migraine (Tvedskov *et al.* 2004a; Tvedskov *et al.* 2004b). Our results in mice, in line with that of others (Bates *et al.* 2010; Goloncser and Sperlagh 2014), displayed only an "early" peak at 60 min with a tendency towards recovery starting by 120 min. Assuming analogy with the human model this single peak in healthy mice may represent the early peak in healthy volunteers without a secondary headache generation. The time-course in mice appears to be shorter than in rats, where the hyperalgesic effect is sustained beyond 4 h post NTG (Tassorelli *et al.* 2003; Di *et al.* 2015).

There is a great interest in human models of migraine, which could be used as indicators of efficacy in early clinical development of anti-migraine drugs, particularly for prophylactics, for which proof-of-concept studies are very resource intensive (Tvedskov *et al.* 2004a; Tvedskov *et al.* 2004b). The human NTG model either in volunteers with detection of the early headache or in patients with the delayed migraineous headache could be a useful test. However, it is not unequivocally sensitive to all clinically proven preventive migraine drugs, probably depending on whether their targeted mechanisms are downstream or upstream from the NTG-induced effects in the migraine generation process. The NTG-induced early and late headache were both significantly reduced by valproate but were unaffected by pretreatment with clinically effective doses of propranolol (Tvedskov *et al.* 2004a; Tvedskov *et al.* 2004b). This latter finding might ruin the confidence in using the human NTG model in early clinical studies for dose finding or proof of effectiveness. If it were proven in further validation experiments that pharmacological sensitivity of the rodent NTG models with any of the feasible outcome measures predicts the sensitivity of the human model, then the rodent model can be used as a pre-screening test for drug candidates and mechanisms that are worth to investigate in the human NTG model with high confidence.

It is worth to note that the utilisable endpoints in rodents, either c-Fos in TNC or somatic and/or face allodynia/hyperalgesia, are different from the most important endpoint in volunteers or patients, which is reported (scored) headache. Although presence of allodynia has been detected in spontaneous migraine (Bigal *et al.* 2008), such measurements have not been reported from the human NTG experiments. Nevertheless, as the migraineous pain may be a manifestation of

trigeminal or more universal pain hypersensitivity (Bernstein and Burstein 2012), it is not unlikely that pharmacological sensitivity of the different endpoints used in rodents and human subject finally prove to be similar. Therefore, comparative validation by using distinguished positive and negative control drugs in the rodent and human NTG models might provide a good translational research tool supporting the development of new anti-migraine drugs with mechanisms liable to NTG-induced headache.

NTG is believed to function as a nitrogen monoxide (NO) donor via an enzymatic reaction catalysed by the mitochondrial aldehyde dehydrogenase enzyme (Chen *et al.* 2002; Ignarro 2002). In vascular smooth muscle cells, the released NO acts via the enzyme soluble guanylate cyclase (sGC) to stimulate the generation of cyclic guanosine-monophosphate (cGMP) and elicit vasodilation (Miller and Megson 2007). However, sGC stimulation without NO mediation by low concentrations of NTG has also been proposed (Kleschyov *et al.* 2003). Multiple data suggest involvement of endogenous NO production and stimulation of the NO – sGC pathway during migraine attack (Olesen 2008). Another mechanism which certainly plays a role in migraine generation is the release of CGRP in the trigeminovascular system. CGRP antagonists proved to effectively alleviate migraine (Edvinsson 2008; Karsan and Goadsby 2015). There is an intricate interplay between NTG or NO and CGRP. It has been suggested that NO causes release of CGRP (Strecker *et al.* 2002) and vice versa CGRP elicits NO release (Li *et al.* 2008), though the data from different sources are not unequivocal as reviewed by Olesen (2008). Recently, it was shown that CGRP per se is not algogenic but becomes algogenic only after NTG treatment (Capuano *et al.* 2014).

Release of CGRP and consequently increased regional or systemic plasma levels of CGRP were observed during headache phase of spontaneous migraine attacks (Goadsby *et al.* 1990) and during NTG-induced migraineous attacks in patients (Juhasz *et al.* 2003). Nevertheless, the results on CGRP plasma levels during migraine attack are controversial, and a study using intrapatient comparison design failed to find any increase in jugular and cubital vein plasma levels (Tvedskov *et al.* 2005). No overt increase in systemic CGRP levels was observed during the early headache phase after NTG either in healthy volunteers or patients (Ashina *et al.* 2001; Juhasz *et al.* 2003; Kruuse *et al.* 2010). Furthermore, while involvement of CGRP in NTG-induced hyperalgesia and c-Fos expression was proved in rats by the use of CGRP antagonists MK-8825 (Greco *et al.* 2013) and olcegepant (Ramachandran *et al.* 2014), olcegepant was ineffective in preventing the migraine attack in the patients induced by 20 min x 0.5 µg/kg/min infusion of NTG (Tvedskov *et al.* 2010). These results could be interpreted in a way that the CGRP pathway is not involved in the human NTG model but is involved in the rat model either with the low-dose (4 µg/kg/min) infusion (Ramachandran *et al.* 2014) or the high-dose (10 mg/kg) i.p. injection (Greco *et al.* 2013) as well as in the natural human

migraine. Therefore, the rat (or rodent) NTG models might be better in terms of predictive validity than the human NTG model. However, it should be noted that, in order to mimic an abortive paradigm, olcegepant was administered after the NTG infusion in the human study. Furthermore, olcegepant was effective in rats only by pre-treatment but not by post-treatment (Ramachandran *et al.* 2014). Therefore, investigating a CGRP antagonist (or antibody) in man also by pre-treatment would provide more reliable results with regards to presence or absence of involvement of CGRP in the early as well as the late headache.

In summary, we proved that c-Fos expression in the TNC, as well as somatic and facial pain sensitisation, are potentially useful endpoints in the mouse for detecting NTG-induced changes and modelling migraine. Further work is needed to confirm the predictive validity of these mouse models by extensive cross-validation using drugs and pharmacological tools by pre-treatment in the human and mouse NTG models and by comparisons with effectiveness in migraine patients. We could not confirm the utility of nNOS expression in the TNC or TRG, and also failed to show NTG-induced light avoidance in mice. For detection of NTG-induced vascular changes either different methods are needed instead of transcranial laser Doppler scanning or the mouse is not an appropriate species. Investigation of NTG effects using relatively inert vehicles and by comparison with adequate vehicle controls is highly recommended to avoid false conclusions in preclinical studies of NTG.

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4 Publications of the applicant

4.1 Publications substantiating the topics of the dissertation

1. Farkas S, Bolcskei K, Markovics A, Varga A, Kis-Varga A, Kormos V, Gaszner B, Horvath C, Tuka B, Tajti J, Helyes Z (2016) Utility of different outcome measures for the nitroglycerin model of migraine in mice. *J.Pharmacol.Toxicol.Methods* **77**, 33-44.
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