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

1.1 Introduction

1.1.1 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.

Tolperisone was found to be a CMR of clinical value with low incidence of side effects. Its effectiveness was 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 and its pharmacokinetic half-life is short, resulting in short duration of action and limited effectiveness (Miskolczi *et al.* 1987).

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 new 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.

1.1.2 Specific objectives of the studies

1. To find and select simple and quick tests, which are suitable for assessing desired effectiveness and expected side effects of CMR drugs in mice
2. To assess the utility of the GYKI-20039 tremor test in estimating antispastic effectiveness of CMR drugs, including oral effectiveness and duration of action.
3. 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.
4. To comparatively estimate therapeutic indices of clinically used CMR drugs and the novel tolperisone-type drug candidate silperisone in mice.
5. 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.

6. 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.
7. To compare *in vivo* potencies of CMR drugs in terms of reflex depressant effects in spinal cats as compared to those with intact neuraxis and higher CNS, which comparison might contribute to drawing conclusions on the primary sites of actions.
8. 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.
9. To compare the effects of tolperisone and silperisone on the flexor reflex, patellar reflex, reticulospinal descending control of patellar reflex and decerebrate rigidity.
10. To investigate if the spinal reflex inhibitory effects of tolperisone-type CMRs appear also in an isolated spinal cord preparation *in vitro*.
11. 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.
12. 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*.
13. 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*.
14. 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) *in vitro*.
15. 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*.
16. 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.
17. Along with these patch-clamp studies we made also some attempts for pharmacological dissection of Na^+ and Ca^{2+} channel subtypes affected by silperisone.

1.2 Methods

1.2.1 Neuropharmacological studies on efficacy and side effects in mice

1.2.1.1 GYKI 20039-induced tremor test

Male CFLP mice (19–21 g) were treated intraperitoneally with the tremorogenic compound, GYKI 20039. Then the animals were placed into a light-weight plastic box (assemble of two yogurt cups) which was attached to an isometric force–displacement transducer. The transducer converted the

vibration of the box induced by the tremor into a voltage signal, which was fed into an analog integrator for quantification of intensity of the tremor.

1.2.1.2 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 (50% inhibitory doses) were calculated by using probit analysis.

1.2.1.3 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 were calculated by probit analysis.

1.2.1.4 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.

1.2.1.5 Weight-lifting test

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. 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 were calculated by probit analysis. Comparison for significance between the control and drug-treated groups was performed using the Fischer's exact test.

1.2.1.6 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.

1.2.2 Instrumental neurophysiological studies in unconscious cats

1.2.2.1 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 recording system. Drug administrations were performed only after having stable reflex activity for at least 30 minutes.

1.2.2.2 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.

1.2.2.3 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 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.

1.2.2.4 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 two controlling areas were alternately stimulated in every 5 minute for periods of 40 s.

1.2.2.5 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.

1.2.2.6 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. 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. Both patellar reflex responses and continuous EMG activities from the two above-mentioned muscles were integrated using analog integrators and integrated activities were recorded.

1.2.3 Instrumental neurophysiological studies in unconscious rats

1.2.3.1 Intercollicular decerebrate rigidity in rats

Under ether anaesthesia and artificial ventilation via a polyethylene cannula inserted previously into the trachea, both common carotid arteries were ligated. The brainstem was intercollicularly transected. 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. Animals were placed on an operating table in a supine position. EMG recording was taken through a concentric needle electrode from one of the gastrocnemius muscles and recorded the EMG spike rate. Drugs were only administered after stabilization of EMG activity, at least 30 minutes after cessation of anaesthesia.

1.2.3.2 Studies in anaesthetised spinal rats in vivo

1.2.3.2.1 Standard surgery

Rats, anaesthetised with a mixture of chloralose (25 mg/kg, i.p.) and urethane (1 g/kg, i.p.), were used. 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 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.

1.2.3.2.2 Investigation of the ventral root reflex

Ventral and dorsal roots below L4 (inclusive) 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 using a differential amplifier and displayed and analysed via computerised data acquisition.

1.2.3.2.3 Neuronal excitability test

Excitabilities of the motoneurone somata and of the primary afferent fibres were measured. 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.

1.2.3.2.4 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.

1.2.3.2.5 Study of recurrent (Renshaw) inhibition

The L_5 ventral root was dissected into two rootlets. One of them was placed on a stimulating electrode and was used for delivering 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.

1.2.3.2.6 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.

1.2.4 Studies in the isolated hemisected rat spinal cord *in vitro*

1.2.4.1 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 hemisected spinal cords was transferred into a recording chamber and perfused at 10 ml/min with standard ACSF. A glass suction electrode was used to stimulate the L_5 dorsal root. In those experiments where the compound action potentials of afferent fibres invading the spinal cord were the matter of interest, 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.

1.2.4.2 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. 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. The D.C. potential between the two compartments was continuously recorded via Ag/AgCl electrodes.

Depolarisations were evoked by repeated superfusion with glutamate receptor agonists, 80 µM NMDA or 40 µM AMPA 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 and the agonist applications were repeated.

1.2.5 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.

1.2.5.1 Preparation of sensory neurones

Neurones were acutely dissociated from rat dorsal root ganglia (DRG) of six-day-old Wistar rats, 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.

1.2.5.2 Whole-cell patch-clamp recording and analysis

Coverslips with the attached neurones were transferred into the recording chamber mounted on the stage of an inverted microscope and continuously perfused at room temperature with extracellular solution (ES). Patch pipettes 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, 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 ("anti-rundown") IS was applied, which included a nucleotide regeneration system and GTP and prevented the rundown (Forscher and Oxford 1985). The modified 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.

Membrane currents were recorded with an Axoclamp 200A amplifier using the pClamp 6.0-8.0 software (Axon Instruments). 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 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. Test substances were dissolved in ES and applied onto investigated cell 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.

1.3 Results

1.3.1 Neuropharmacological studies in mice

1.3.1.1 GYKI 20039-induced tremor test

Dose-response relationships of various CMRs after i.p. administrations are summarised in Fig. 1

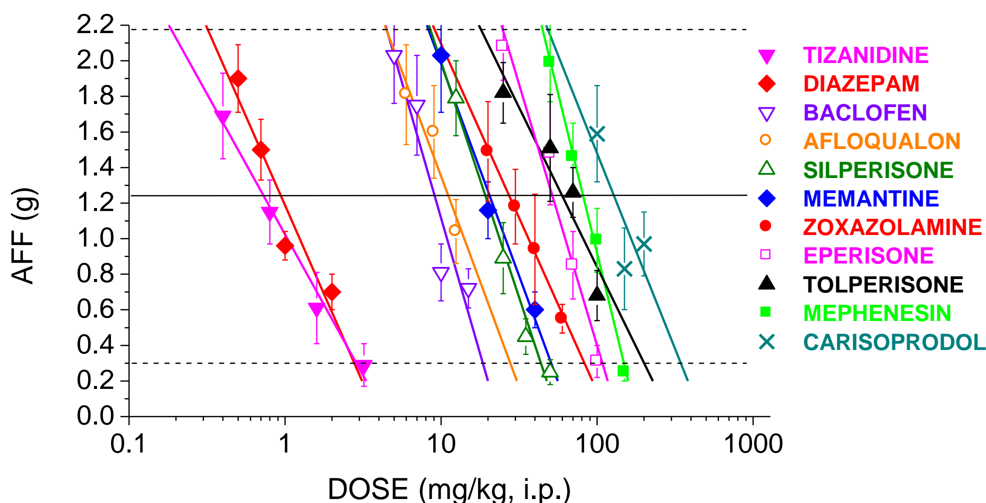

Fig. 1. Dose-response relationships of various CMRs (n=10 per group) against the tremor induced by GYKI 20039 (10 mg/kg i.p.). Note that all CMR drugs suppressed the tremor in a dose-dependent fashion. Upper and lower horizontal dashed lines indicate mean AFF of tremorogen treated and untreated control groups, respectively; the solid line indicates the value corresponding to 50% inhibition.

After oral administration, all the tested tolperisone-type CMRs had rapid onset of action, reaching a peak effect at 5 minutes post-dose. Silperisone was twice as potent as the others. In terms of duration of action, the apparent rank order (from shortest to longest) was: tolperisone < eperisone < lanperisone < silperisone.

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

The results are summarised in **Table 1.**

Table 1. Summary of results obtained in conscious mice

	ID_{50} values (mg/kg, i.p.)							Ratios (Therapeutic indices)					
	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.

1.3.2 Instrumental neurophysiological studies in unconscious cats

1.3.2.1 Flexor reflex in cats

The time-courses of effects of different CMRs are shown in Fig. 2.

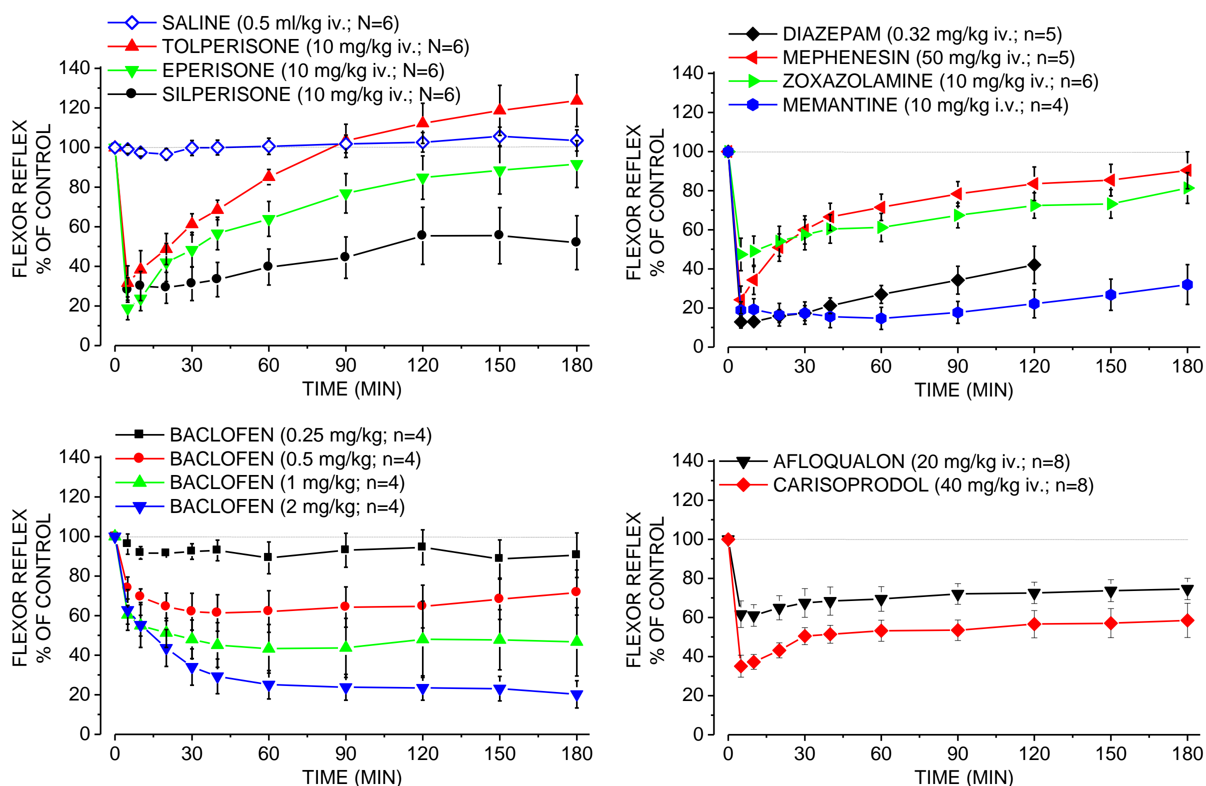

Fig. 2. Time-course of the effect of silperisone and of various CMR drugs in the flexor reflex test in intact anaesthetised cats after intravenous administration. Data are presented as mean±S.E.M. Note that recovery rate of silperisone was much slower than those of tolperisone and eperisone.

In vivo potencies of the various CMR drugs tested are summarised in Table 2

Table 2. *In vivo* potencies (ID_{50}s, 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

1.3.2.2 Patellar reflex in cats

Tolperisone (10 mg/kg, i.v.) exerted mild and short lasting depressant effects on the patellar reflex, amounting maximum 15% in average. In some cases silperisone (10 mg/kg, i.v) 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.

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

Tolperisone, as expected, inhibited both the brainstem stimulus-induced facilitation and the inhibition, and moderately suppressed also the “resting” patellar reflex. Complete recovery was observed from the effect of tolperisone within 60-90 minutes in most cases.

Experiments with silperisone displayed some variations:

1.) Significant diminution of the “resting” patellar reflex responses was often observed.

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 was seen in several cases.

3.) Patellar reflex depressing effect of stimulation of the inhibitory area was found to be enhanced in some cases 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.

1.3.2.4 Spinal root potentials evoked by tibial nerve stimulation in cats

The effects of silperisone were compared to those of tolperisone, baclofen, mephenesin, diazepam and midazolam. 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 (Fig. 3 and Fig. 4.). Quantitatively, whereas silperisone exhibited similar effectiveness on MSR and PSR, its effect on the DRR was considerably weaker. Other CMRs exhibited different unique and mechanism specific profiles of actions (Table 3).

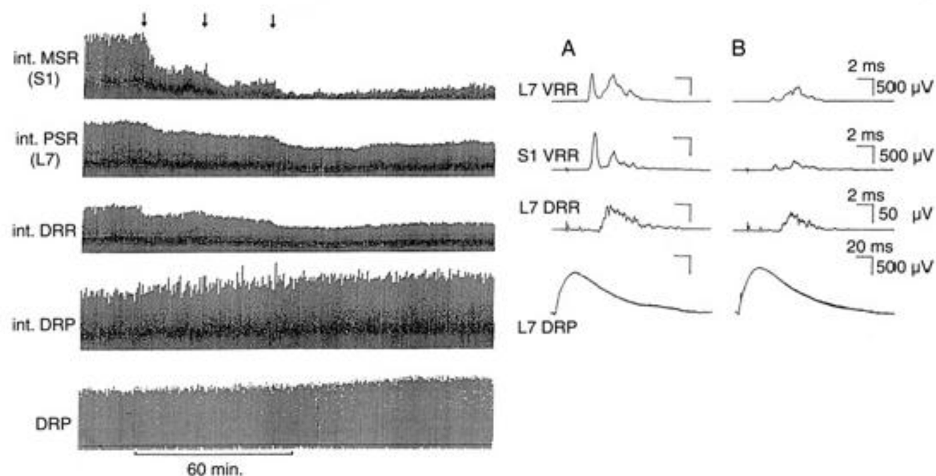

Fig. 3. Effect of silperisone on the spinal root potentials in a cat. On the left side: integrated records of the monosynaptic (MSRint) and polysynaptic (PSRint) ventral root reflex (VRR), dorsal root reflex (DRRint) and dorsal root potential (DRPint). The bottom trace is direct recording of the upward (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.

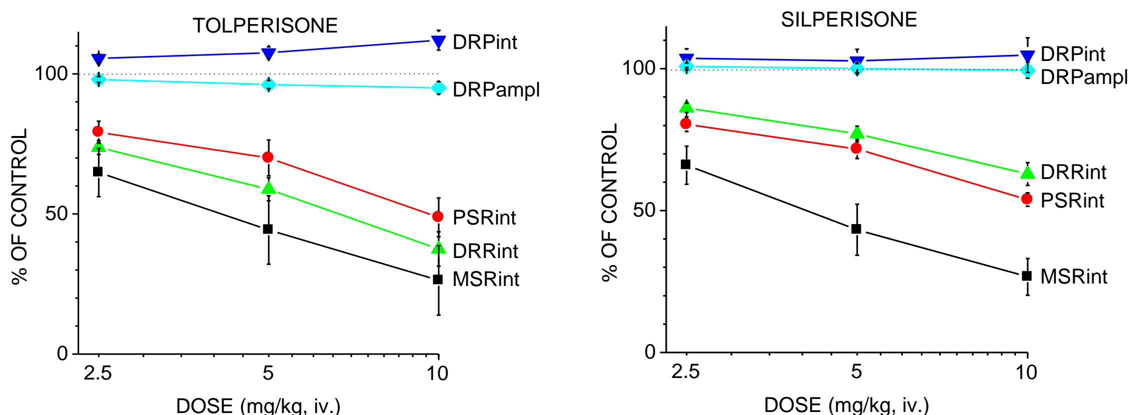

Fig. 4. Cumulative dose-response curves of silperisone and tolperisone based on the effects on spinal root potentials in cats. Results are presented as mean±S.E.M. in 6 animals. Note that effects of both drugs on ventral root reflexes were practically of the same magnitude, only the weaker effect of silperisone on DRR was a notable difference.

Table 3. 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).

1.3.2.5 Intercollicular decerebrate rigidity in cats

Compared to tolperisone, the effects of silperisone 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 (Table 4).

Table 4. 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

1.3.3 Instrumental neurophysiological studies in unconscious rats

1.3.3.1 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).

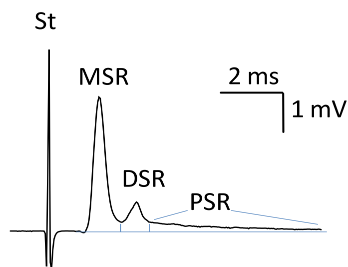

Fig. 5. The dorsal root-evoked ventral root reflex recorded from the L_5 segment in rats and its three distinguished components.

Silperisone (10 mg/kg, iv.) attenuated all the three components with the following sensitivity order for the various components: MSR>DSR>PSR. 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 much weaker depressant effect on MSR, whereas inhibited the later components to similar extent.

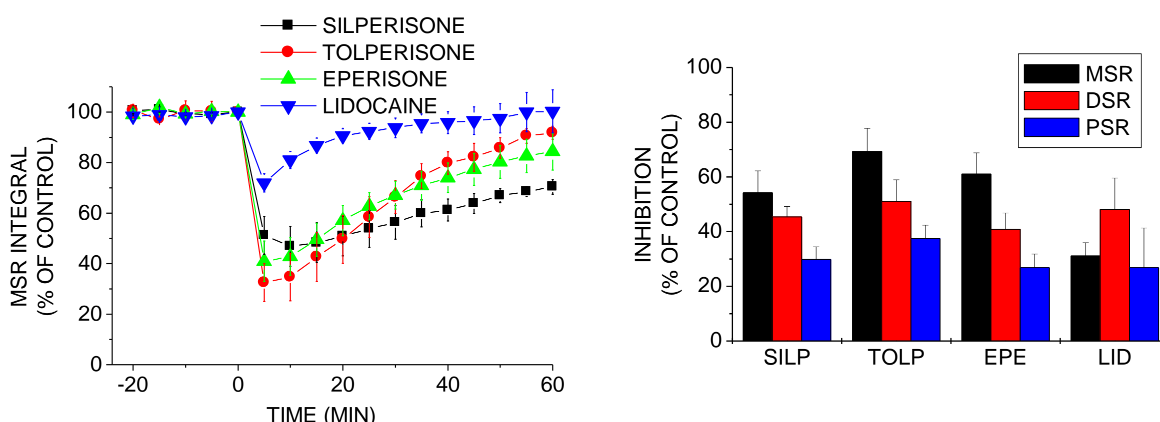

Fig. 6. Effects of investigated compounds on the ventral root reflex evoked by dorsal root stimulation *in vivo* in spinal rats. Left: Time course of effects of the four compounds (10 mg/kg i.v. each) on MSR. Right: Maximum inhibitory effects of compounds (10 mg/kg i.v.) on different reflex components expressed as percentage of the pre-dose control responses. All the data are presented as mean±S.E.M. from five experiments.

1.3.3.2 Neuronal excitability test

Lidocaine had significantly greater depressant effect on the direct electrical excitability of motoneurones (MN) and primary afferents (PAF) than silperisone, whereas their efficacies to inhibit the monosynaptic transmission (MS) were similar. The profile of tolperisone was in between these drugs, as it had a small effect on PAF (Fig. 7).

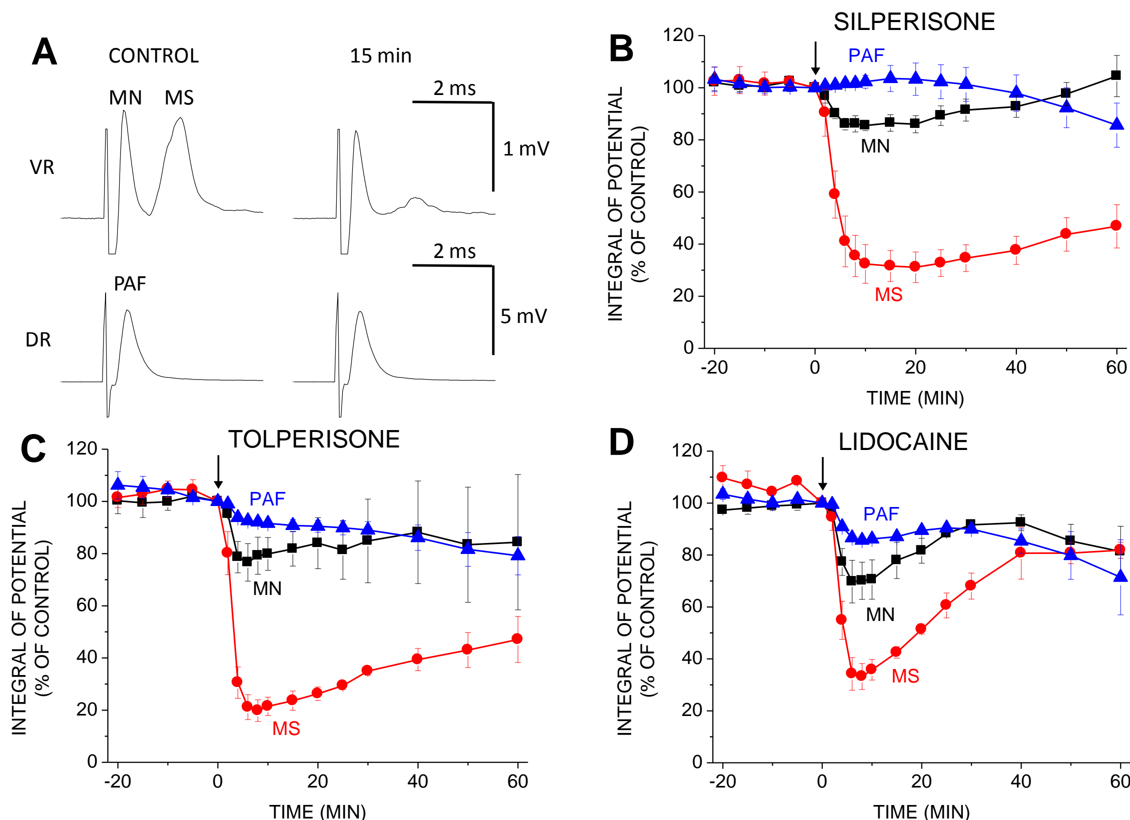

Fig. 7. Effects of drugs on direct electrical excitability of motoneurones (MN), their monosynaptic excitation (MS) and excitability of primary afferents (PAF). A: averaged potentials recorded from L_5 ventral root (top – VR) and from L_5 dorsal root (bottom – DR), before (left) and 15 minutes after (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.

1.3.3.3 Study of feed-forward inhibition

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. Hence, silperisone did not affect the feed-forward inhibition.

1.3.3.4 Study of recurrent inhibition

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.).

1.3.3.5 Study of afferent nerve conduction

Silperisone and tolperisone (10 mg/kg, i.v.) left the A-afferent fiber potential elicited by a more distant afferent nerve stimulation arriving at the spinal cord surface practically unchanged. Lidocaine (10 mg/kg, i.v.) exerted some very slight but noticeable inhibitory effect manifested in both decreased potential amplitude and increased conduction time. However, this slight depression seemed to be negligible compared to the depression of VRR afforded by the same dose of lidocaine.

1.3.3.6 Intercollicular decerebrate rigidity in rats

Regarding the maximum effects after intravenous administration, tolperisone was (1.5 times) more potent than silperisone, in line with findings obtained in cats.

1.3.4 Studies in the isolated hemisected rat spinal cord *in vitro*

1.3.4.1 Spinal root potential studies

Effects of different tolperisone-type CMRs and lidocaine were compared with regards to profile of actions on different components of the dorsal root stimulation evoked ventral root potential (DR-VRP). 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. The profiles of different tolperisone-type compounds were quite similar, however, different from that obtained with lidocaine (Fig. 8).

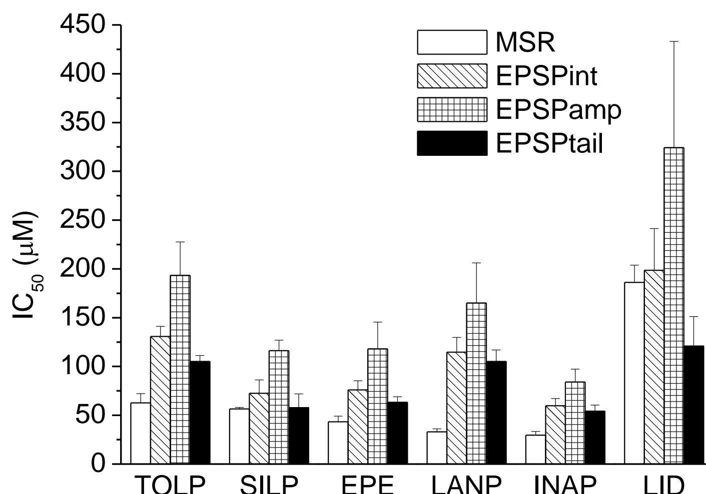

Fig. 8. Profiles of actions of different tolperisone type CMR drugs (tolperisone-TOLP, silperisone-SILP, eperisone-EPE, lanperisone-LANP, inaperisone-INAP) and lidocaine (LID) on dorsal root stimulation evoked ventral root potential (DR-VRP) components *in vitro*. IC_{50} values of the drugs studied 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.

In a pharmacological experiment on participation of GABA-B receptors in spinal reflex inhibitory effects of silperisone *in vitro*, the effect of silperisone was not reversed by the GABA-B antagonist SCH-50911.

To 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 and comparative investigation of silperisone, tolperisone and lidocaine. 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. 9).

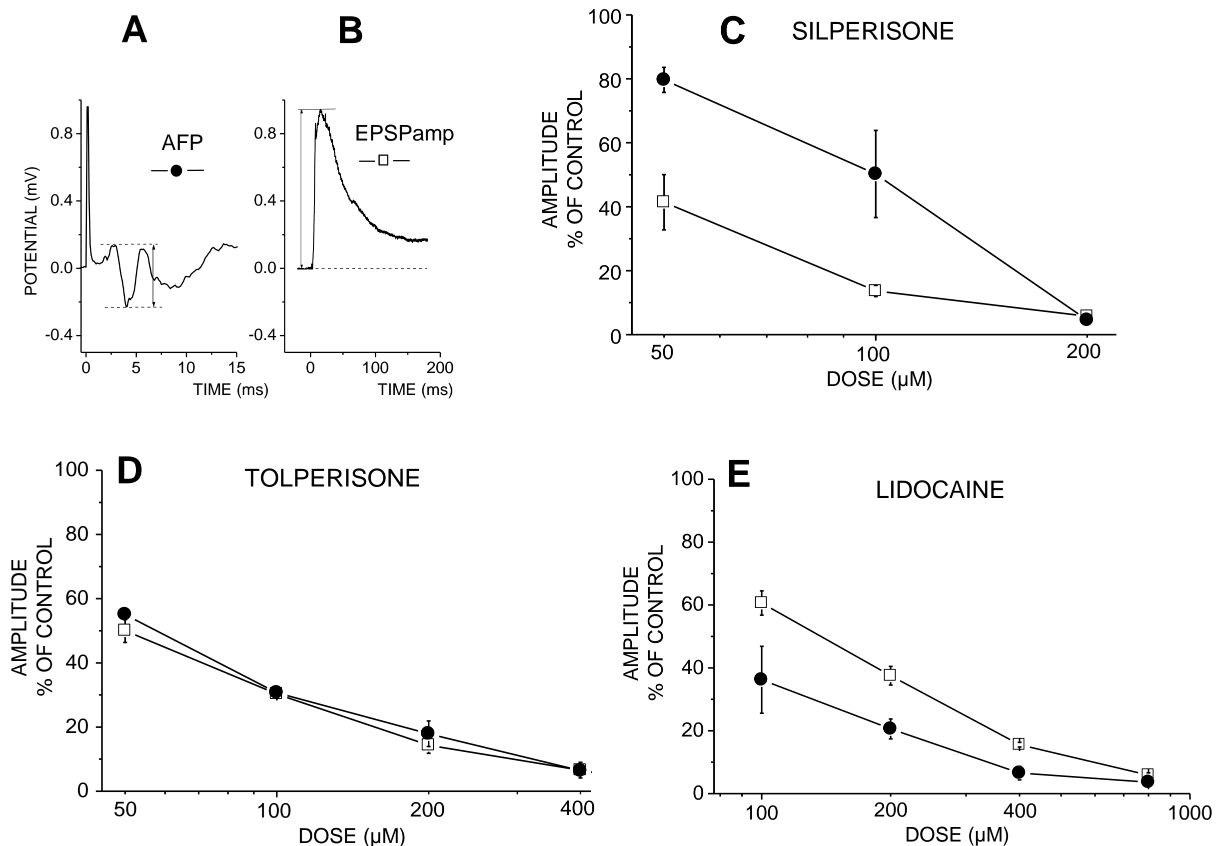

Fig. 9. Effects of silperisone, tolperisone and lidocaine on population EPSP and on afferent fibre potential AFP. These experiments were performed at bath temperature of 31-33 °C, to eliminate the monosynaptic reflex firing. The L_5 dorsal root was stimulated (0.2 mA; 0.1 ms) A: afferent fibre potential. 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.

1.3.4.2 Spinal cord grease gap study

Depolarisations of the spinal cord slices evoked by NMDA (80 µM) or AMPA (40 µM) were unaffected by 100 µM silperisone.

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

1.3.5.1 Effects on voltage-gated sodium channels

Silperisone as well as tolperisone shifted the steady state inactivation curve of both tetrodotoxin resistant (TTX-R) and tetrodotoxin sensitive (TTX-S) Na^+ channels to hyperpolarizing direction, indicating a state dependent channel blocking effect, though the maximum available current also diminished (Fig. 10).

Neither silperisone nor tolperisone nor lidocaine shifted the activation voltage curve of sodium channels Fig. 11.

Finally, we compared Na^+ channel blocking potencies of silperisone, tolperisone and lidocaine on TTX-S channels at holding potential of -80 mV and on TTX-R channels at holding potential of -60 mV. The results are summarised in Table 5. 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.

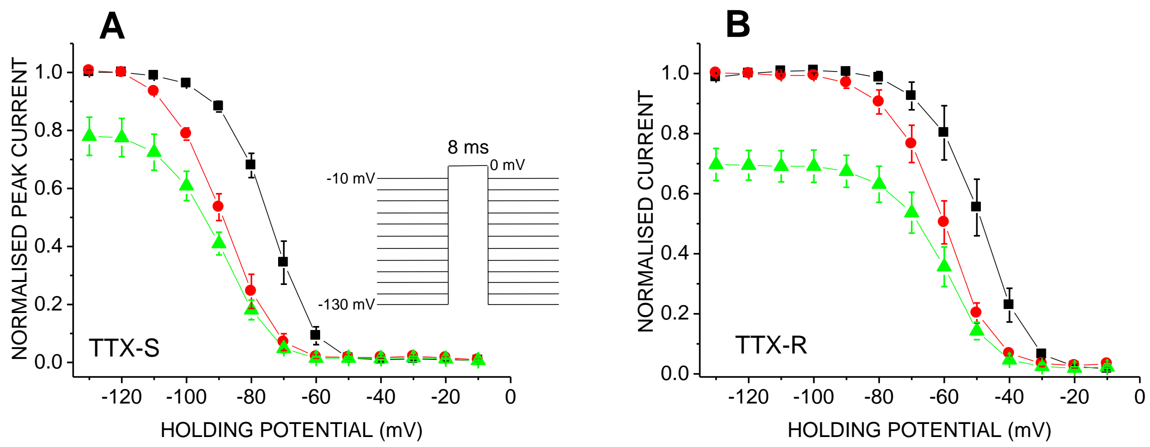

Fig. 10. Effect of silperisone (80 µM) on the steady-state inactivation curves in cells with TTX-S (A) and TTX-R Na^+ channels (B). Averaged normalized steady-state inactivation curves in the control solution (■), during perfusion with silperisone 80 µM (▲, normalized to the control peak current at V_H of -120 mV) and the latter curve scaled up (●, normalized to the peak current during silperisone at V_H of –120 mV).

Fig. 11. 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).

1.3.5.2 Effects on voltage-gated calcium channels

Silperisone blocked the voltage gated calcium currents of DRG cells with IC_{50} values comparable to the sodium channel blocking IC_{50}s at conditions without anti-rundown supplementation of the intracellular solution. The IC_{50} with anti-rundown supplementation was two-fold higher than the IC_{50} at V_H -80 mV in TTX-S Na^+ channels (Table 5). The strength of the Ca^{2+} channel blockade was not considerably dependent on either the preceding holding potential or the activation voltage.

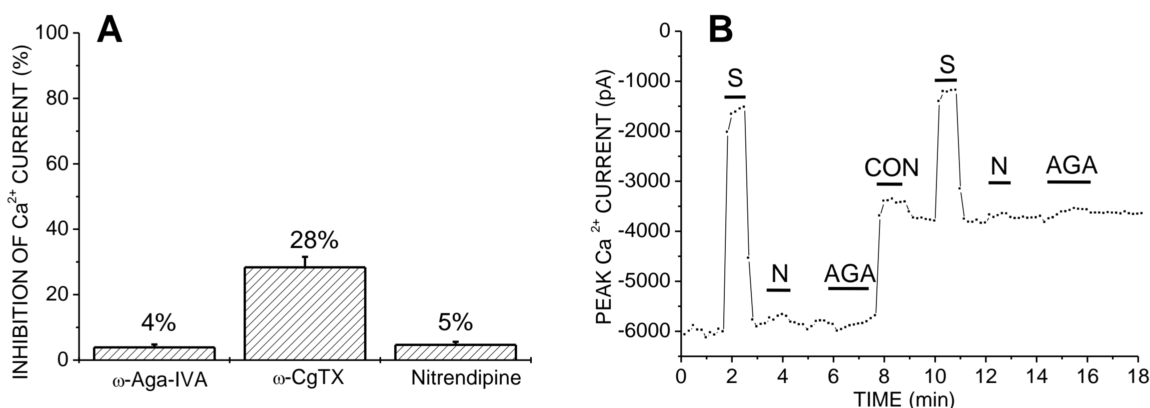

Fig. 12. Effects of various calcium channel blockers on HVA calcium currents in DRG cells (anti-rundown condition). A: The amount of blockade provided by different subtype selective Ca^{2+} channel blockers: nitrendipine (N, 10 μM, L-type selective), ω-conotoxin-GVIA (CON, 3 μM, N-type selective) and 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.

Silperisone (320 μM) blocked a great portion of the Ca^{2+} current of DRG cells, which included both N- and R-type Ca^{2+} currents whereas the measured currents were not mediated by remarkable amount of L- or P/Q-type Ca^{2+} channels (Fig. 12).

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. suggesting that 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.

1.3.5.3 Effects on voltage-gated potassium channels

We observed mainly delayed rectifier K^+ currents in the tested medium sized DRG neurones. Silperisone reduced the K^+ current and its blocking effect was independent of the activation voltage. Comparative concentration-response studies with silperisone, tolperisone and lidocaine 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.

Table 5. 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)

1.4 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. Out of the different neurophysiological 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.
6. 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.
7. Investigation of spinal ventral- and dorsal root reflex potentials revealed different and mechanism-specific patterns of actions for different CMR drugs.
8. 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.
9. 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.
10. 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.
11. Lidocaine, tolperisone and silperisone, all had some effects on excitabilities of primary afferents, motoneurones and the EPSP of motoneurones in vitro.
12. 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.
13. 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.

14. Silperisone apparently did not affect GABA and glycine receptor mediated feed-forward and recurrent inhibition in the spinal cord.
15. 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.
16. 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 or in comparison with lidocaine.
17. 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 presynaptic excitatory transmitter release in this way as well.
18. 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.
19. Overall, our results showed an improved pharmacodynamic profile for silperisone as compared to either tolperisone or other CMR drugs.
20. 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.

1.5 References

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2 Chapter 2: Preclinical modelling of migraine

2.1 Introduction

Migraine is a disabling headache disorder characterised by moderate to severe, intense throbbing or pulsating pain.

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).

The present study was dealing with the NTG models of migraine. NTG administration causes an immediate headache in healthy subjects and a delayed migraine-like headache only in migraineurs (Thomsen *et al.* 1994; Olesen 2008, Ashina *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. However, much more limited experience exists concerning the NTG model in mice. 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 trigeminal ganglia (TRG) and trigeminal *nucleus caudalis* (TNC), as well as pain hypersensitivity of the face, which formally might be a more relevant indicator of migraine than paw hyperalgesia.

There was a confounding factor in previous studies which also determined our goals. In various studies different formulations of NTG were used. 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.

2.2 Materials and methods

2.2.1 Drug treatments and control vehicles

Two NTG preparations were used: Nitrolingual aerosol and Nitro Pohl. 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. Sumatriptan succinate and topiramate were used as anti-migraine drugs for method validation.

2.2.2 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. The mice were individually tested in the light aversion chamber with two compartments: one brightly lit and the other not lit. 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.

2.2.3 Cranial blood flow experiments

Male mice were anaesthetised with urethane 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 cranial blood perfusion was investigated using a non-invasive laser Doppler scanner (PIM II System, Perimed AB, Sweden). 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. Perfusion changes were expressed as percentage of baseline measurement to enable comparison of results. After stabilisation of baseline perfusion, 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.

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

Four groups of six male mice 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 trigeminal nucleus caudalis (TNC) and trigeminal ganglia (TRGs) were processed for c-Fos and nNOS immunohistochemistry.

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

The hot plate test was performed using mice. The animals were placed 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.

2.2.6 Mechanical allodynia of the paw measured with von Frey filaments

Hind paw withdrawal thresholds of mice were tested by von Frey filament stimulation of the plantar surface of hind paw using an up-and-down paradigm (Chaplan *et al.* 1994). 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 hind paw. The 50% paw withdrawal threshold (PWT expressed in grams) was determined. 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.

2.2.7 Orofacial pain sensitivity tested with von Frey filaments

Mice were placed into a 9-cm-long restraining glass cylinder. A von Frey filament of 0.4 g force was 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. 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.

2.3 Results

2.3.1 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. 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 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. 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. Most importantly, we could not detect any significant differences between the NTG groups and their simultaneous vehicle controls in either experiment.

2.3.2 Cranial blood perfusion

The absolute values of baseline perfusion did not differ significantly between corresponding NTG and vehicle groups. Injection of Nitrolingual, as well as its vehicle initiated a progressive increase in the cranial blood perfusion. 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. 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 and there was no significant difference between Nitro Pohl and the corresponding vehicle group. 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.

2.3.3 Immunohistochemistry for c-Fos and nNOS

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. However, a statistically significant increase could not be detected in the TRG. 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. 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. 13).

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.

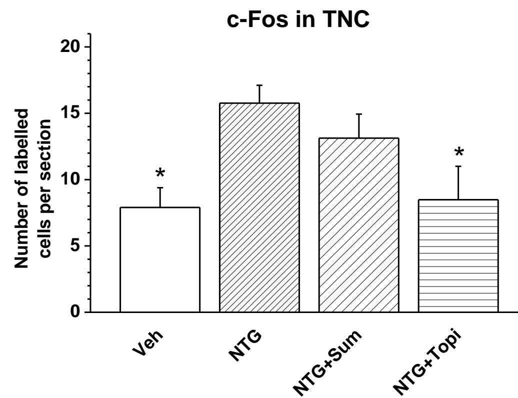

Fig. 13. Effects of nitroglycerin (NTG; 10 mg/kg i.p.) compared to vehicle (Veh) control with quantitative immunohistochemistry for c-Fos in the trigeminal nucleus caudalis (TNC); and the effects of sumatriptan (Sum; 2x5 mg/kg, s.c.) and topiramate (Topi; 80 mg/kg, i.p.) in mice injected with NTG. Data 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).

2.3.4 Thermal hyperalgesia of the paw

Hot plate latencies of vehicle-treated mice were very stable over time with repeated testing up to 240 min post dose. 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.

2.3.5 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. 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.

2.3.6 Orofacial pain sensitivity

Repeated recording of orofacial pain scores indicated no overt change in the pain-related behaviour of vehicle-treated animals (Fig. 14). 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. 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 (Fig. 14).

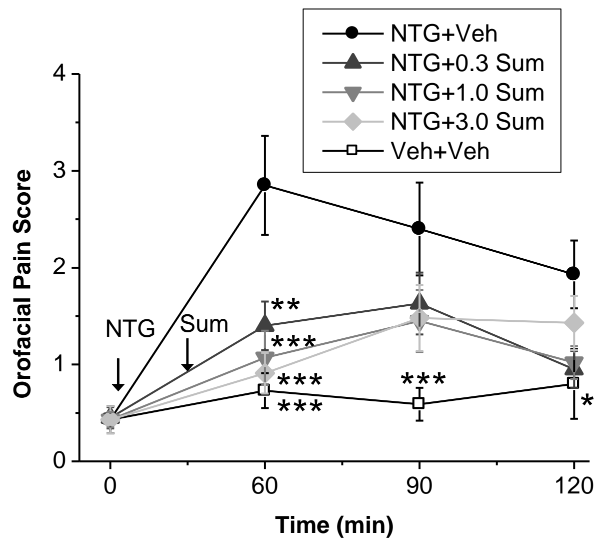

Fig. 14. Effects of nitroglycerin (NTG; 10 mg/kg, i.p.) compared to vehicle controls (Veh+Veh) on orofacial pain elicited by tactile stimuli, and alterations of the NTG-induced facial allodynia by different doses of sumatriptan (0.3-3 mg/kg, s.c.; NTG+Sum). Data are presented as mean ± SEM (N=10-11/group) 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.

2.4 Summary of conclusions

1. 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.
2. 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.
3. 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.
4. 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.
5. 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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