European Journal of Pharmacology 674 (2012) 13–19

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

The inhibitory potency of local anesthetics on NMDA receptor signalling depends on their structural features

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ARTICLE INFO

Article history:
Received 15 July 2011
Received in revised form 13 October 2011
Accepted 27 October 2011
Available online 4 November 2011

Keywords:
Anesthetics local
Pain
Neuropathic
Potency
Analgesic
Theories
Ion
Ion channel
Pharmacology
NMDA receptor signaling

ABSTRACT

Development of postoperative hyperalgesia depends on N-methyl-D-aspartate (NMDA) receptor activation. Local anesthetics protect against those hyperalgesic pain states and inhibit NMDA receptor activation. To outline what structural features of local anesthetics are responsible for NMDA receptor inhibition we evaluated a series of experimental lidocaine analogs (carbanilic derivates). Human GluN1/GluN2A NMDA receptors were expressed recombinantly in *Xenopus laevis* oocytes. Peak currents were measured by voltage clamp technique. Oocytes were stimulated with glutamate/glycine (EC_{50}). The responses following a 10 min incubation with in total 13 experimental derivates of local anesthetics (10^{-3} M–10^{-7} M) were measured to obtain the IC_{50}. Furthermore the *C*omprehensive *D*escriptors for *S*tructural and *S*tatistical *A*nalysis CODESSA software was used to design a *Q*uantitative *S*tructure–*A*ctivity *R*elationship (QSAR)-model for all substances. The IC_{50} values were in the range of 2.74×10^{-5} M–2.26×10^{-3} M, strongly affected by the position and the length of the aliphatic side chain in the aromatic part of the local anesthetic molecule. Substance with no substituent on the aromatic ring showed the highest inhibitory activity. The obtained QSAR model predicted that lidocaine derivatives with free positions 2 and 6 on the aromatic ring had a higher efficacy than clinically used local anesthetics for inhibition of NMDA receptor signaling. Structural changes of local anesthetic molecules can alter the potency to inhibit NMDA receptor signaling and are independent of the local anesthetic (sodium-channel blocking) potency. The development of novel drugs based on local anesthetic like structures may be a new approach for the protection or treatment of NMDA receptor mediated hyperalgesia and may be associated with a low side effect profile.

1. Introduction

The treatment of hyperalgesic pain states due to tissue or nerve injury after surgery remains a clinical challenge. The mechanisms of the development of hyperalgesic pain states are not completely understood, but the involvement of N-methyl-D-aspartate (NMDA) receptor signaling in the brain and the spinal cord leading to central changes in synaptic excitability is strongly suggested (Kaneko et al., 2011; Sandkuhler et al., 2000; Sang, 2000). NMDA receptors are widely distributed in the central nervous system and are protein complexes composed of two classes of subunits, the essential subunit GluN1 and the modulating GluN2 subunit (of which four different types exist: A–D). The GluN2 subunits alone cannot form functional channels, but they amplify GluN1 activity and induce functional variability in NMDA receptor signaling. These subunits co-assemble in various combinations to form functionally distinct NMDA receptors (Cull-Candy and Leszkiewicz, 2004). In the present study, the GluN1 subunit was co-expressed with the GluN2A subunit. This combination is widely distributed in the brain and the dorsal horn of the spinal cord and is believed to play a relevant physiological role in the development of hyperalgesia and acute opioid tolerance (Kaneko et al., 2011; Sandkuhler et al., 2000).

Inhibition of NMDA receptors may minimize the development of these neuropathic pain states and therefore is a potential therapeutical target. Currently, most agents initiate several side effects like memory impairment and ataxia (Parsons, 2001).

Studies in volunteers provide evidence that local anesthetics minimize hyperalgesia by a central mode of action which makes an interaction between local anesthetics and NMDA receptors likely (Koppert et al., 2000). In addition inhibition of NMDA receptor function by local anesthetics was demonstrated repeatedly in several in-vitro models

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doi:10.1016/j.ejphar.2011.10.035

(Furutani et al., 2010; Hahnenkamp et al., 2006; Nishizawa et al., 2002; Sugimoto et al., 2003; Zhang et al., 2000).

However, no information is available concerning the relationship between the chemical structure of the local anesthetics and their inhibitory effects on NMDA receptors. Previous studies on interactions between local anesthetics and the NMDA receptor have focused exclusively on clinically used substances representing only an insufficient structural variation.

The NMDA subunit combination GluN1/GluN2A was recombinantly expressed in the *Xenopus laevis* model and we set out A) to evaluate the structural determinants of local anesthetics necessary for the inhibition of NMDA receptor signaling based on a systematic analysis of a homologous series of experimental carbanilic local anesthetics B) to predict the structural determinants of lidocaine like local anesthetics on inhibition of NMDA receptor signaling (Vegh et al., 2006).

2. Material and methods

2.1. Oocyte harvesting and preparation

Procedures for *X. laevis* oocyte isolation, synthesis of NMDA receptor mRNA and microinjection technique were published previously. (Hahnenkamp et al., 2006) In brief, after approval by the local Animal Care and Use Committee, female *X. laevis* frogs were anesthetized by immersion in cold 0.2% 3-aminobenzoic-methyl-ester until unresponsive to a painful stimulus (toe pinching). Approximately 200 oocytes were surgically removed by an abdominal mini cross-laparotomy. After finished operation the frog was observed in a separate tank for 2 h for complete recovery. The oocytes were defolliculated by 1 h treatment with collagenase type 1A (1 mg ml^{-1}) diluted in Oocyte Ringer's (OR2) solution (containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES, pH adjusted to 7.4). Microscopic observation confirmed that the follicle coats had been removed and only oocytes with development stage VI (Dumont) were selected.

2.2. NMDA receptor expression

The NMDA receptor combinations tested consist of GluN1 and GluN2A subunits. The human GluN1 (~3000 bp) and GluN2A (~5500 bp) subunits were obtained from P.J. Whiting M.D. (Merck Sharp & Dohme Research Laboratories, Harlow, UK) as a complementary DNA in pcDNAI/Amp vectors. These constructs were linearized by either the restriction endonuclease XbaI for GluN1 or EcoRV for GluN2A (New England Biolabs GmbH, Frankfurt am Main, Germany). Transcription was done in the presence of capping analogue by bacteriophage RNA polymerase T7, using a commercial RNA preparation kit (mMESSAGE mMACHINE®, T7 Kit, Ambion Inc., Austin, TX, USA). Oocytes were injected, using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA, USA), with 6 ng of GluN1/GluN2A subunits in a 1:5 weight ratio in 30 nl RNase-free sterile water. Correct injection was confirmed by a slight increase in cell size. Then oocytes were stored for 48–72 h in ND96 solution (containing 96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH adjusted to 7.4, (additionally 5 mM Sodium Pyruvate, 10 μg ml^{-1} Gentamicin and 100 IU ml^{-1} Benzylpenicillin were added)) at 16 °C in a laboratory refrigerator (LABO-100, Kirsch, Offenburg, Germany) for receptor expression.

2.3. Electrophysiology

A single oocyte was positioned in a continuous-flow chamber containing a volume of 0.5 ml and superfused (5 ml/min) with modified Mg^{2+}/Ca^{2+}-free Tyrode's solution containing Ba^{2+} (containing 150 mM NaCl, 5 mM KCl, 1.8 mM $BaCl_2$, 10 mM dextrose and 10 mM HEPES, pH adjusted to 7.4). To avoid effects of secondary activated Ca^{2+}-dependent Cl^- currents modified Tyrode's solution was used. Microelectrodes were pulled in one stage from capillary glass on a vertical micropipette puller (P-30, Sutter Instrument Company, Novato, CA, USA). Electrode tips were broken to a diameter of ~10 μm, providing a resistance of 3–10 MΩ, and filled with 3 M KCl. The two electrode tips were introduced carefully into a single cell. The oocytes were voltage clamped using a two-electrode voltage clamp amplifier (OC725C, Warner Instruments Corp., New Haven, CT, USA) connected to a data recorder (IX 118, iworx™, Dover, NH, USA) and a personal computer. For data acquisition and analysis the software LabScribe™ (iworx™, Dover, NH, USA) was used. All measurements were performed at a holding potential of −70 mV; data were recorded for 120 s. All electrophysiological experiments were performed at room temperature.

2.4. Study protocols and data analysis

The physiological agonist glutamate (Glu) in combination (as determined in previous studies by this group) (Hahnenkamp et al., 2006) with the obligatory co-agonist glycine (Gly) were diluted in modified Tyrode's solution to the required EC_{50} concentrations (Glu 10 μM/Gly 10 μM) and were delivered into the bath for 20 s. Electrophysiological responses were quantified by measurement of peak currents and were reported in μA. Substances used for incubation were diluted in modified Tyrode's solution to the required concentrations. All substances were tested in the concentrations of 0.1 μM, 1 μM, 10 μM, 100 μM, and 1 mM, except for the substances XX and XXI, by which only the concentrations from 0.1 μM to 100 μM were tested because of the limited water solubility (Table 1). Following the initial control measurement of the oocyte's response to Glu/Gly (10 μM), the oocyte was perfused for 5 s with the solution containing tested substances at the desirable concentration in the flow chamber. Oocytes were then incubated in this solution for 10 min. After that the second response to the application of Glu/Gly (10 μM) was measured. Each oocyte provides in this way its own baseline response.

Oocytes in the control group remained in the modified Tyrode's solution for 10 min after the initial control measurement and subsequently the second response was measured to eliminate the effect of the incubation period.

The results were reported in change in % of control group response. For each concentration of local anesthetic inhibition curves were generated (percentage inhibition of control response) and 50% inhibition concentrations (IC_{50}) were calculated. In addition to that, a third application was done for exemplary measurements of reversibility following 10 min wash-out.

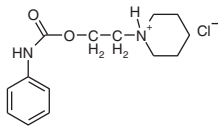
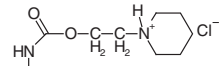
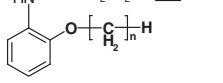
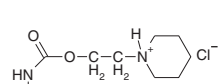
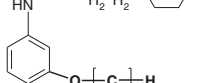
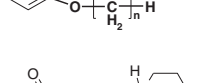
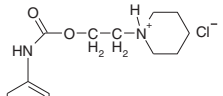
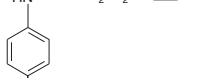
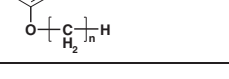
Because the variability between batches of oocytes is common, at least 12 oocytes from at least 3 frogs were studied for each data point and responses were normalized to control values obtained on the same day in oocytes from the same batch. Results are reported as mean ± S.E.M. unless stated otherwise. Differences between treatment groups were analyzed using one-way ANOVA, corrected for multiple comparisons (Dunnet's test). $P<0.05$ was considered significant. IC_{50} and Hill coefficients for tested local anesthetics were calculated using SigmaPlot 2001 software (GraphPad Inc., San Diego, CA) by fitting the data to a sigmoidal dose–response (variable slope) equation (Hill − 3 parameters).

$$(\%I) = (\%I_0) \times [1-(\%I_{max})] \times C(\text{local anesthetic})^n / [IC50^n + C(\text{local anesthetic})^n].$$

2.5. Materials

Tested substances were provided by Prof. RNDr. Jozef Cizmarik, PhD, Department of Pharmaceutical Chemistry, Comenius University, Bratislava, Slovakia (Cizmarik et al., 1978). All other chemicals were from Sigma Aldrich Chemie GmbH, Steinheim, Germany.

Table 1
Overview of tested local anesthetic derivates, basic physico-chemical properties, measured relative local anesthetic potency (topical: cocaine = 1, infiltration: procaine = 1), measured half maximal inhibition concentrations (IC_{50}) for the inhibition of glutamate/glycine in half maximal effective concentration (EC_{50}) induced currents in NMDA receptors (GluN1/GluN2A) recombinantly expressed in *Xenopus laevis* oocytes, Hill coefficients of the obtained inhibition curves are almost the same, suggesting the same mode of inhibition. All measured values presented as mean of value and S.D. (standard deviation).

Name	Structural formula	n	MW	Log P	pKa	Relative local anesthetic potency: topical/infiltration	Measured IC_{50} (M), SD	Hill coefficient, SD	R	R^2
IR-1		0	284.80	2.81	8.88	0.1/1.2	2.74×10^{-5} 1.35×10^{-5}	−0.5735 0.1673	0.9718	0.9718
I		1	314.82	2.71	8.86	–	3.16×10^{-4} 3.56×10^{-5}	−0.6386 0.0040	0.9974	0.9949
VII		3	342.88	3.81	8.88	1/33	7.21×10^{-4} 2.21×10^{-4}	−0.4137 0.1929	0.9778	0.9561
XIII		5	370.94	4.97	8.86	28.7/58	6.30×10^{-4} 1.22×10^{-4}	−0.6692 0.1156	0.9971	0.9943
XIX		7	399.00	5.62	8.90	100/171	–	–	–	–
II		1	314.82	2.92	8.86	–	3.73×10^{-4} 0.7×10^{-4}	−0.8383 0.1929	0.9893	0.9788
VIII		3	342.88	4.06	8.88	10.9/35.3	2.91×10^{-4} 0.54×10^{-4}	−0.6632 0.2265	0.9834	0.9671
XIV		5	370.94	5.08	8.88	80/135.6	1.71×10^{-4} 0.36×10^{-4}	−0.7957 0.3609	0.9703	0.9415
XX		7	399.00	5.53	8.89	67.1/60.6	1.44×10^{-4} 0.48×10^{-4}	−0.5445 0.2156	0.9797	0.9598
III		1	314.82	2.55	8.87	–	3.65×10^{-4} 0.85×10^{-4}	−0.8593 0.2564		
IX		3	342.88	3.78	8.89	1/3.9	1.71×10^{-4} 0.41×10^{-4}	−0.6763 0.1274	0.9941	0.9882
XV		5	370.94	4.9	8.90	25.1/31.6	1.33×10^{-4} 0.18×10^{-4}	−0.7467 0.1261	0.9959	0.9918
XXI		7	399.00	5.87	8.91	19.3/40	3.16×10^{-5} 1.16×10^{-5}	−0.4431 0.1356	0.9858	0.9718

2.6. Computational Analysis and QSAR study

In short, to transfer observed results to other types of local anesthetics, we have searched for the most appropriate *Q*uantitative *S*tructure–*A*ctivity *R*elationship (QSAR) models describing the structural features of tested local anesthetics. The values of the IC_{50} were reduced to $-\log(IC_{50})$ and assigned to appropriate optimized geometries of studied compounds. The structures of the local anesthetics were constructed from the Cambridge Structural Database (Cambridge Crystallographic Data, Cambridge, UK) (Sivý et al., 2000). The best regression equation obtained was then used to predict the IC_{50} of lidocaine derivates. Details for the computational analysis are provided in the supplementary online information.

3. Results

3.1. Functional expression of NMDA receptors in Xenopus oocytes

Consistent with previous reports on recombinant NMDA receptors, uninjected oocytes were unresponsive to either Glu/Gly or to NMDA (Hahnenkamp et al., 2006). In contrast, oocytes injected with GluN1/GluN2A receptor mRNA 48–72 h previously responded to Glu/Gly (EC_{50}) with inward currents (Fig. 1B). The currents consisted of a rapid initial peak current, followed by a gradual return to baseline. Peak current values were used for further analysis. Glu activation of either receptor subunit combination in the presence of Gly was concentration-dependent. The selective agonist NMDA (100 μM) in combination with Gly (10 μM) was applied to ooctytes, expressing GluN1/GluN2A receptor subunit combination. Responses were indistinguishable from those stimulated by Glu/Gly (Fig. 1A and B). Therefore, for further experiments, the physiologic agonist Glu in combination with Gly at EC_{50} concentration was used. EC_{50} concentrations as determined in a previous study from our group were employed: Glu/Gly 10 μM (Hahnenkamp et al., 2004; Hahnenkamp et al., 2006).

3.2. Local anesthetics inhibition of NMDA signaling in Xenopus oocytes

All tested local anesthetics (except XIX) concentration-dependently inhibited Glu/Gly induced NMDA receptor signaling. No significant inhibition at 100 μM was observed for XIX, therefore an IC_{50} value cannot be calculated. Compound IR-1 (Fig. 1D) was the most effective (Fig. 1B). The obtained results for each of the compounds are shown in Fig. 2 and the values are additionally indicated in Table 1.

It was worked out that increasing the size of the side substitution in both the *meta*-position (3) (Fig. 3A) and the *para*-position (4) (Fig. 3B) of the aromatic ring of the experimental local anesthetic derivates increases their effectiveness to inhibit NMDA signaling. Increasing the length of the side substitution increases the compound lipid solubility and also enhanced the potency to block NMDA signaling. However, this was not observed for the substitution in the *ortho*-position (2) (Fig. 3C). Although increasing the length of the side substitution on the ortho-position (2) increases the lipid solubility in a very similar way, the potency to block NMDA signaling is decreasing.

Thus the compound XIX with the longest side substitution, although being the most lipid soluble in the series of ortho-substituted compounds, exerts no significant inhibitory activity (Fig. 3C).

Surprisingly, IR-1, as the most potent NMDA inhibitor (Figs. 1B and 3C, Table 1), is a compound with a simple phenylring in the lipophilic part of the molecule and also the least lipid solubility. The Hill coefficients for all compounds are almost the same, suggesting the same mode of inhibition. The inhibition of NMDA receptor signaling for all tested local anesthetics was reversible (Fig. 1C).

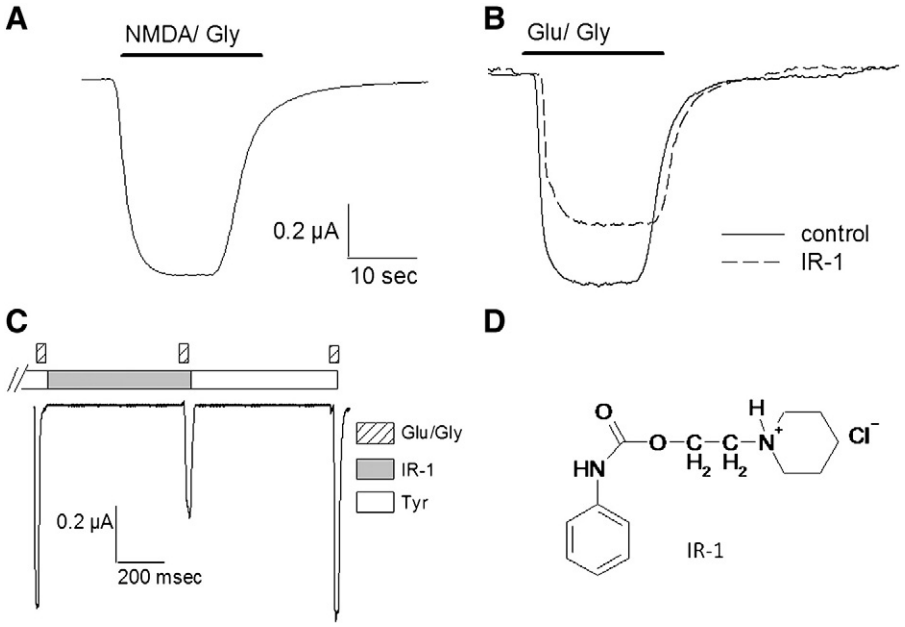


Fig. 1. A–D: Pharmacological characterization of NMDA (GluN1/GluN2A) receptors, recombinantly expressed in *Xenopus laevis* oocytes. Example traces of responses to 20 s agonist administration. 1A: In oocytes injected with NMDA (GluN1/GluN2A) mRNA 24–48 h prior to experiments, the selective agonist NMDA (100 µM) in combination with glycine (10 µM) (NMDA/Gly) evoked inward currents. Black line above the trace indicates the duration of agonist application. 1B: Glutamate/glycine (Glu/Gly) at half maximal effective concentration (EC_{50}) evoked inward currents in NMDA receptor expressing oocytes. Responses were indistinguishable from those stimulated by NMDA in combination with glycine. Superimposed dashed trace displays inward currents evoked by glutamate/glycine at EC_{50} concentration after 10 min incubation in IR-1 (100 µM). IR-1 does not affect the shape of the traces. Black line above the trace indicates the duration of agonist application. 1C example trace of EC_{50} glutamate/glycine evoked responses on a single oocyte expressing NMDA (GluN1/GluN2A) receptors. The response was inhibited to 56% after 10 min incubation IR-1 100 µM and restored after 10 min wash out phase in modified Tyrode's solution. Coarse bars indicate agonist application, gray bar indicates the incubation in IR-1, white bars indicate modified Tyrode's solution. 1D: Structure of the most effective NMDA receptor current inhibiting compound, IR-1.

3.3. Prediction of IC_{50} for selected compounds using obtained QSAR models

The best regression equation (best value of R^2) finally resulted in 3 descriptors: two of them were geometric (ZX Shadow, Moment of inertia) and one constitutional (Relative number of C atoms). The parameters of the resulting final regression equitation are shown in Table 2.

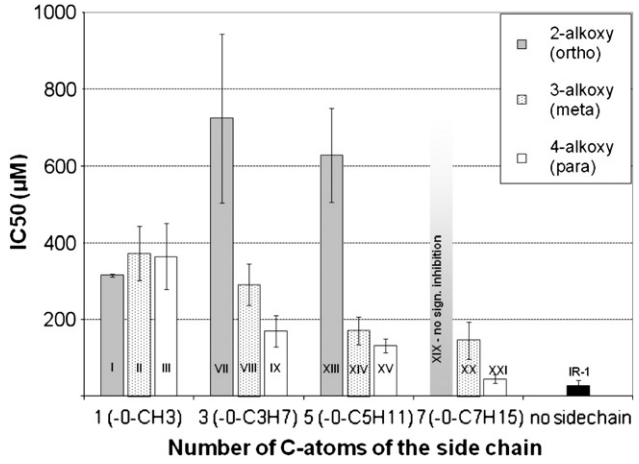


Fig. 2. Half maximal inhibition concentrations (IC_{50}) of tested local anesthetics on glutamate/glycine in half maximal effective concentration (EC_{50}) induced currents in NMDA receptors (GluN1/GluN2A) recombinantly expressed in *Xenopus laevis* oocytes. No significant inhibition at 100 µM was observed for compound XIX, IC_{50} was therefore not calculated. Increasing the size of the side substitution in both the *meta*-position (3) and the *para*-position (4) of aromatic ring of our experimental local anesthetic derivates increases the ability of local anesthetics to inhibit the NMDA signaling. For these compounds increasing the side substitution length made compounds more liposoluble and enhanced the potency to block NMDA signalling. This was not observed for the substitution in the *ortho*-position (2).

IC_{50} values for lidocaine and its selected derivates were predicted. The studied structures and the predicted values of IC_{50} are shown in Table 3. Significant correlations were found between the calculated descriptors of the tested compounds and their IC_{50} for the inhibition of Glu/Gly induced currents in NMDA receptors. Correlation of the experimental and computed values of IC_{50} is presented in Fig. 4. (for further details see supplementary online information).

4. Discussion

Almost all local anesthetics share the basic structural characteristics already described by Löfgren (Löfgren, 1948) — consisting of a lipophilic group (frequently an aromatic ring/eventually a carbocyclic ring) connected by an intermediate chain (commonly including an ester or amide) to an ionizable group (usually a tertiary amine).

4.1. Structural features of local anesthetic-induced inhibition of NMDA signaling

In this study we used experimental derivatives of local anesthetics. These compounds consist of all the above mentioned basic structural requirements for local anesthetics. Many of them may be taken as analogues of “classical” local anesthetics such as lidocaine, mepivacaine, bupivacaine and ropivacaine (Heavner, 2007). More potent local anesthetics with lower toxicity were prepared by modifications of these compounds (Akerman et al., 1971). However, only diperodon (Diothane®) (Rider, 1933) and pentacaine (trapencaine INN) (Stolc and Mai, 1993) have reached clinical usage.

The studied compounds create a homologous series with incrementally elongated side chain of alkoxysubstitution in every position on the aromatic ring of the lipophilic part. Increasing the number of carbon atoms (elongating the alkyl chain) of this side chain increases the lipid solubility of these compounds and increases the volume of

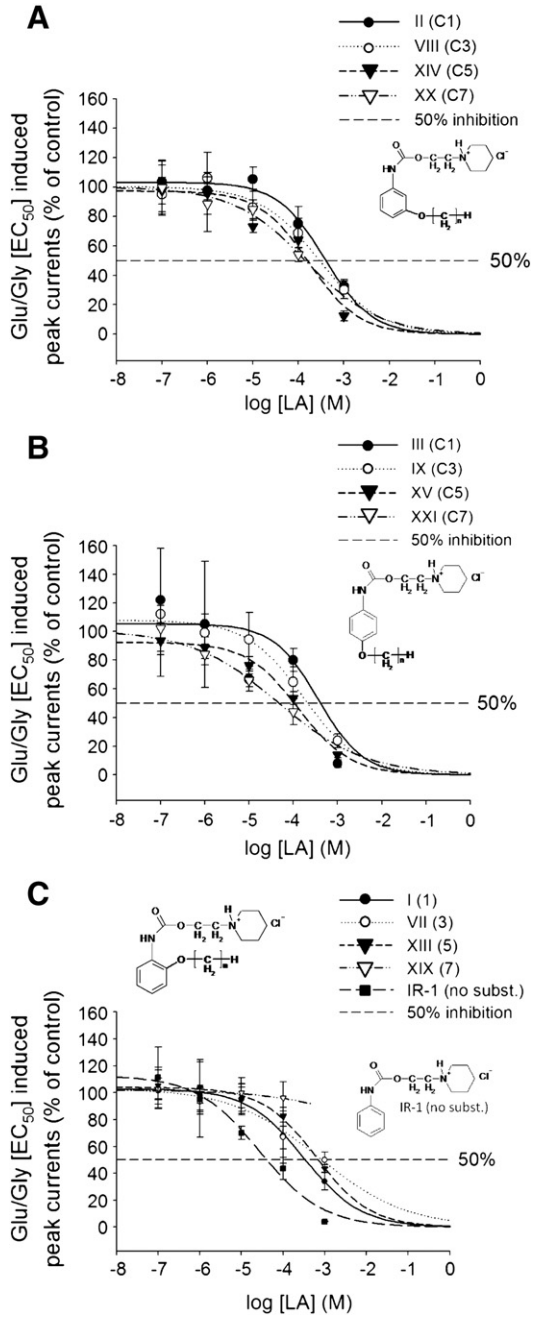


Fig. 3. A–C: Fitted inhibition curves for the tested experimental derivates of carbalinic local anesthetics on glutamate/glycine in half maximal effective concentration (EC_{50}) induced currents in NMDA regulated receptor channels (GluN1/GluN2A recombinantly expressed in *Xenopus laevis* oocytes), n = 12 for each data point, The curves were constrained to max 100% inhibition. (Negative values were not allowed, y (peak currents) > 0.) 3A: 3-Alkyloxyderivates of local anesthetics (meta-position of substituents), structure displays the differing lengths of substitutions in meta-position of the aromatic ring. 3B: 4-alkyloxyderivates of local anesthetics (para-position of substituents), structure displays the differing lengths of substitutions in para-position of the aromatic ring. 3C: 2-Alkyloxyderivates of local anesthetics (ortho-position of substituents and unsubstituted derivate IR-1), structures display the differing lengths of substitutions in ortho-position of the aromatic ring and IR-1, the most effective compound without substituents. Measured values for each concentration and S.E.M. are displayed.

the substituent. Changing the position of this chain on the aromatic ring enabled us to observe the steric effects and interactions.

Generally, increased lipid solubility results in faster membrane permeation and increased efficacy. However, the obtained results presented in Table 1 lead to interesting conclusions:

Table 2
Results of the best correlation for the local anesthetics from the CODESSA software package: 3 parameters ($R^2 = 0.9613$ F = 66.20 $S^2 = 0.0107$).

No.	X	dX	*t*-test	Name of the descriptor
0	−4.7781e+01	5.1626e+00	−9.2552	Intercept (constant)
1	4.0065e−02	3.3195e−03	12.0696	ZX Shadow (S_{ZX})
2	1.3966e+02	1.4457e+01	9.6604	Relative number of C atoms ($N_{C,r}$)
3	−9.7523e+00	3.0472e+00	−3.2004	Moment of inertia A (I)

QSAR equation: $-\log(IC_{50}) = 0.040065 \times [S_{ZX}] + 139.66 \times [N_{C,r}] - 9.752 \times [I] - 47.78$.
Legend:
S2 = standard deviation.
X = coefficient for regression parameter (obtained value).
dX = X − standard error for the coefficient.
t-test = statistical significance of the descriptor.

1. The inhibitory activity increased only in the series of derivates with the alkoxy side chain in the positions 3 and 4.
2. Despite the increased lipid solubility, the inhibitory activity of the derivatives with substituents in the position 2 of the aromatic ring decreases with elongation of the alkoxy side chain. For the side chain length of 7C-atoms no significant activity at 10^{-4} M was observed at all.
3. Substances with no substituent in the aromatic ring and the least lipophilic (IR-1) show the highest inhibitory activity.

Local anesthetics of comparable lipid solubility and pKa, only differing in the position of their alkoxy side chain, showed remarkable differences in their inhibitory effect. The pKa values for all tested substances were about 8.9 ensuring that the ratio of the charged and uncharged molecules of all tested substances is almost the same. Thus permeability of local anesthetics through the membrane appeared not to be the limiting factor. A long substituent only in the position 2 close to the connecting chain between the aromatic ring and the hydrophilic amine part prevents almost any significant inhibitory activity on NMDA receptor signaling. Therefore a significant steric interaction is suggested.

Elongating the alkyl chain does not only influence the lipid solubility, but for the *ortho*-substituted compounds increase the steric hindrances on the connecting chain of local anesthetic molecules (Benes, 1986). In *ortho*-derivatives the position of the alkoxy-chain is largely restricted by the intra-molecular H-bond between the alkoxyl oxygen atom and hydrogen atom of the carbamic moiety ($C_{ar}-N-COO-$). Moreover, the alkoxy-chain winds up to the connecting chain to minimize the solvent accessible surface and might prevent or hinder interactions with the receptor site. Increasing the length of the alkoxy chain in the position 2 on the aromatic ring may thus increase the importance of this steric hindrance.

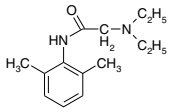
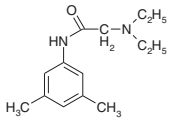
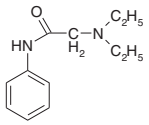
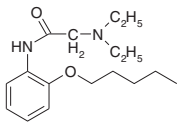
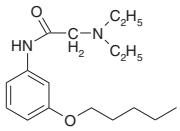
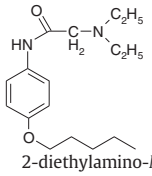
A small difference between *meta*- and *para*-alkoxysubstituted derivates can be explained by the fact that the alkoxysubstituent can reach and accommodate the presumed lipophilic interaction site with approximately the same efficacy. Elongating the side chain only increases the lipid solubility and the membrane permeation.

To transfer structure–activity observations to clinically relevant compounds, we applied quantitative structure activity relationships (QSAR) for the study of local anesthetic effects on NMDA signaling. QSAR methods are well established when studying local anesthetics and other agonists/antagonist of NMDA receptors (Buyukbingol et al., 2007; Karelson et al., 1996; Patankar and Jurs, 2002). QSAR represents a valuable computational tool for studying structure–activity relationships when the structure of the target protein is not known (for further details see supplementary online information).

4.2. Limitations of the model and the software calculation

Although NMDA receptor expression in *Xenopus* oocytes is an established model some limitations of the model should be noted. First, our

Table 3
Prediction of IC_{50} for NMDA receptor signaling inhibition for selected lidocaine derivates using the regression equations from the CODESSA software package.

Compound	Predicted IC_{50} (M) (3 parameters)
 Lidocaine 2-diethylamino-*N*-(2,6-dimethylphenyl)acetamide	8.253×10^{-4}
 2-diethylamino-*N*-(3,5-dimethylphenyl)acetamide	6.414×10^{-4}
 2-diethylamino-*N*-phenylacetamide	4.503×10^{-4}
 2-diethylamino-*N*-(2-pentoxyphenyl)acetamide	1.743×10^{-3}
 2-diethylamino-*N*-(3-pentoxyphenyl)acetamide	1.599×10^{-3}
 2-diethylamino-*N*-(4-pentoxyphenyl)acetamide	1.204×10^{-3}

experiments were performed at 18° up to 20 °C (room temperature), whereas the expressed receptor is derived from mammalian sources and normally functions at 37° up to 39.5 °C. Theoretically this could influence the behavior of the receptors. Nevertheless, we prioritized to maintain the cell membrane in its normal ambient conditions. Second, an isolated receptor model cannot simulate the complex physiologic conditions in humans. However, it may be helpful to clarify the basic or general mechanisms for receptor functions.

As the structural changes in the compounds used in our set for the QSAR model involve only the changes in the aromatic part, the predictions made by using our model are therefore limited only to lidocaine derivatives modified in the aromatic part (for the discussion of the validity of QSAR computational analysis see supplementary online information).

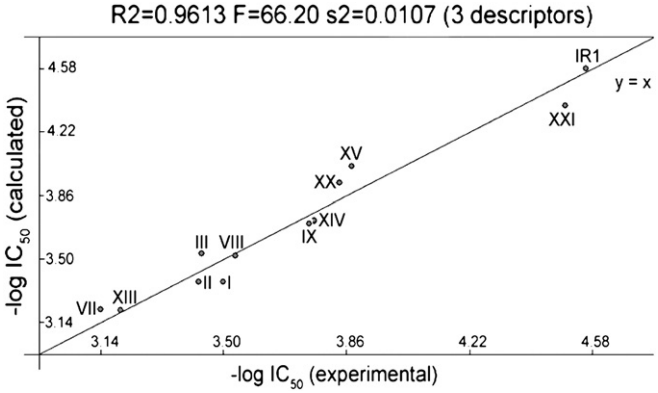


Fig. 4. Comparison of experimental and calculated values. Experimental values of IC_{50} ($-\log IC_{50}$) of local anesthetics are plotted against the abscissa, the calculated values of IC_{50} ($-\log IC_{50}$) were obtained using the 3-parametric regression equation (1) from the CODESSA software package and are plotted against the ordinate. Experimental values (dots) are close to the calculated values ($r^2=1$, $y=x$).

4.3. Comparison with present data

The inhibitory effects of local anesthetics on NMDA receptors were shown by many authors in the concentration range of 10^{-5} M–10^{-3} M (Hahnenkamp et al., 2006; Nishizawa et al., 2002; Sugimoto et al., 2003; Ueta et al., 2006). However, in these studies only clinically used local anesthetics have been analyzed. These were synthesized to achieve maximum local anesthetic effects by blocking Na^+-channels and their structures were optimized for these purposes, not for the inhibition of the NMDA receptor function, which uses a different pathway. Conversely, the Na^+-channels blocking properties of local anesthetics can cause severe adverse effects in higher or even toxic plasma- or cerebrospinal fluid-concentrations (Heavner, 2007).

In our study some similarities to the work of Nishizawa et al. (2002) were observed: procaine, with the unsubstituted aromatic ring, although being the least lipid soluble of the four local anesthetics examined, inhibited the NMDA receptor function the most. We have observed the same: compound IR-1, the least lipid soluble of tested local anesthetics, was the most active. It is hypothesized, that spatial effects of the bulky side substitution may hinder some important molecular interactions between the local anesthetic molecule and the target structure and thus decrease or even prevent the effect of the compounds on NMDA receptor signaling. The mechanism of the inhibitory action of local anesthetics on NMDA receptor function has been studied extensively (Hahnenkamp et al., 2006; Nishizawa et al., 2002; Sugimoto et al., 2003). The inhibitory effect of local anesthetics was not dependent on the type of GluN2 subunit co-expressed with the GluN1 subunit in *Xenopus oocytes*. Sugimoto et al. (2003) proposed different sites of action and mechanisms for the effects of local anesthetics on NMDA signaling and an extracellularly located site of action closely related to those of Mg^{2+} and ketamine for the ester-type local anesthetic procaine. However, in our own study on the mechanism of action local anesthetics on NMDA receptor function, no differences in the site of action between amide- and ester-type local anesthetics could be detected. Instead all local anesthetics inhibited NMDA receptor function from an intracellular site of action. Our results strongly suggested that local anesthetics inhibit NMDA signaling indirectly, via inhibition of a proteinkinase C dependent pathway (Hahnenkamp et al., 2006). This mechanism of action has been proposed by other groups in several models (Guan et al., 2004; Tomoda et al., 1990; Villarruel et al., 2011). For amid local anesthetics, the 2,6-dimethyl substitution of the aromatic ring seems to be essential for the inhibition of Na^+-channels (Courtney and Strichartz, 1987). Lidocaine derivatives without this substitution have significantly lower local anesthetic potency than parental lidocaine. For example, the demethylated lidocaine derivative (2-diethylamino-N-phenylacetamide), although having almost the same lipid solubility and pKa as lidocaine has almost no surface anesthetic effect and the infiltration local anesthetic potency is 10 times lower than that of lidocaine. This is also valid for adverse cardiovascular side effects: for the same extend of cardiac Na^+-channel block almost 10 times higher concentration is needed. Thus the toxicity and potential for adverse side effects of non-substituted amid compounds are lower.

Recently, systemic lidocaine administration has been found to affect the inflammation response following abdominal surgery positively (Herroeder et al., 2007). In vitro experiments suggest an interaction of local anesthetics with different receptor systems, notably the thromboxane A_2-, prostaglandine E_2- or the lisophosphatidic acid receptors (Heavner, 2007). Structural analysis of local anesthetics on these receptor systems could reveal these alternative effects of local anesthetics.

For the NMDA receptor (GluN1/GluN2A) we demonstrated that experimental carbanilic local anesthetic derivates inhibited Glu/Gly induced receptor responses in a concentration-dependent manner. The effect was strongly influenced by the size and the position of the side chain on the aromatic ring of the lipophilic part of the local anesthetic molecule. Compounds with the free positions of 2 and 6 on the aromatic ring have a higher potency and the substance with no substituent on the aromatic ring has the highest potency to block NMDA receptor signaling. These effects were independent of the local anesthetic potency and lipid solubility.

In conclusion, this study shows that structural changes of local anesthetic molecules can alter the potency to inhibit NMDA receptor system responses expressed in *X. laevis* oocytes. The development of novel drugs based on local anesthetic like structures may mean a different approach for the treatment of NMDA receptor mediated hyperalgesia and may be associated with a lower adverse effects profile.

Funding

This work was supported in part by the Department of Anaesthesiology and Intensive Care, University Hospital Münster, Münster, Germany; and the Heinrich Hertz-Stiftung, Nordrhein-Westfalen, Germany, scholarship for Vladimir Vegh PharmD.

Acknowledgments

The authors thank Paul Whiting, Ph.D. (Merck Sharp & Dohme Research Laboratories, Harlow, United Kingdom), for providing the human N-methyl-D-aspartate receptors and Prof. RNDr. Jozef Čižmárik, PhD, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia for providing the local anesthetics.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2011.10.035.

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