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Blockade by *sigma* site ligands of *N*-methyl-D-aspartate-evoked responses in rat and mouse cultured hippocampal pyramidal neurones

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1 The effects of a range of structurally-dissimilar compounds which possess affinity for σ binding sites were examined on the responses of cultured hippocampal pyramidal neurones to the excitatory amino acid analogues *N*-methyl-D-aspartate (NMDA), kainate and (RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA).

2 In mouse hippocampal neurones under whole-cell voltage-clamp, the compounds tested reversibly attenuated NMDA-, but not kainate- or AMPA-, evoked currents with a rank order potency (IC_{50} values in μM): ifenprodil (0.8) > (+)-N-allylnormetazocine (1.1) > dextromethorphan (1.8) = haloperidol (1.9) > (+)-pentazocine (7.2) > 1*S*,2*R*-(−)-*cis*-N-methyl-*N*-[2-(3, 4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl)cyclohexylamine (17) = rimcazole (18) > 1,3-di(2-tolyl)guanidine (37) > opipramol (96) > caramiphen (110) = carbetapentane (112) > > (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine (485).

3 The attenuation of NMDA-evoked responses was not mediated through interactions with the agonist, glycine (except haloperidol) or polyamine (except ifenprodil) binding sites on the NMDA receptor-channel complex but, in the light of the voltage- and, in some cases, use-dependent nature of their antagonism, an interaction with the ion channel appears to be a likely mechanism of action for many of the compounds.

4 Micromolar concentrations of selected σ site ligands also reduced NMDA-evoked rises in intracellular free calcium concentration in Fura-2-loaded cultured hippocampal neurones of the rat with the same rank order potency as observed in the electrophysiological studies.

5 The data indicate that, at micromolar concentrations, the σ site ligands tested act as NMDA receptor antagonists, an action which does not appear to be mediated by high-affinity σ binding site(s). The functional effects of micromolar concentrations of σ site ligands cannot, therefore, be attributed exclusively to interactions with high-affinity σ binding sites.

Keywords: Cultured hippocampal pyramidal neurones; σ receptors; *N*-methyl-D-aspartate

Introduction

On the basis of the characteristic behavioural changes elicited in chronic spinal dogs by N-allylnormetazocine (NANM), Martin and colleagues (1976) proposed the existence of a *sigma* (σ) opioid receptor. The term 'opioid' is now deemed obsolete due to the naloxone insensitivity of the site and its reverse stereoselectivity for benzomorphans compared to that of classical opioid receptors, and the σ binding site is now defined operationally as a unique naloxone-insensitive, haloperidol-sensitive site distinguished both autoradiographically and pharmacologically from the phencyclidine (PCP) receptor on the *N*-methyl-D-aspartate (NMDA) receptor-channel complex (Walker *et al.*, 1990; Quirion *et al.*, 1992). Compounds such as haloperidol, (+)-pentazocine, 1,3-di(2-tolyl)guanidine (DTG) and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-3-PPP) possess high (nanomolar) affinity for σ binding sites and their use in radioligand binding studies has suggested the existence of more than one sub-type of σ site (Walker *et al.*, 1990; Quirion *et al.*, 1992). Non-opioid antitussive agents, such as caramiphen, carbetapentane and dextromethorphan (DXM) also bind with high affinity to σ sites (Musacchio *et al.*, 1989) and, in turn, σ site ligands displace high-affinity [^{3}H]-DXM binding (Klein & Musacchio, 1989), findings which suggest an overlap between high-affinity σ and DXM binding sites in the central nervous system.

Although the functional role(s) of σ binding sites remain unclear, it has been proposed that they may mediate, at least in part, the antiepileptiform (*e.g.* Aram *et al.*, 1989; Apland & Braitman, 1990; Pontecorvo *et al.*, 1991) and neuroprotective activities (*e.g.* Rao *et al.*, 1990; Pontecorvo *et al.*, 1991; DeCoster *et al.*, 1995; Yamamoto *et al.*, 1995) of σ site ligands. In many cases, however, high (micromolar) concentrations of σ ligands are required to elicit these therapeutically-useful actions (see Discussion), suggesting in turn that they may reflect interactions of σ ligands with sites other than high-affinity σ binding sites. In particular, selective antagonists of the NMDA sub-type of glutamate receptor and blockers of neuronal voltage-activated Ca^{2+} channels also possess antiepileptiform and neuroprotective properties (Heinemann & Hamon, 1986; Dingledine *et al.*, 1990; Meldrum & Garthwaite, 1990; Siesjö, 1992), raising the possibility that interactions with either (or both) of the aforementioned sites may contribute to the therapeutically useful actions of micromolar concentrations of σ ligands. Accordingly, in the present study we have examined the effects of a series of structurally diverse σ site ligands on excitatory amino acid-evoked currents and rises in intracellular free calcium concentration ($[Ca^{2+}]_i$) in two preparations of cultured hippocampal neurones. The actions of σ site ligands on voltage-activated Ca^{2+} channels are described in the companion paper (Church & Fletcher, 1995).

Methods

Electrophysiological studies

Cultured hippocampal pyramidal neurones of the mouse were used for whole cell recordings by conventional voltage-clamp

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techniques. Hippocampi were dissected from 18-day-old foetal Swiss white mice and mechanically dissociated (MacDonald *et al.*, 1991). Neurones were plated at densities below 1×10^3 cells cm^{-2} on collagen-coated plates and used 10–17 days following plating. Dishes were thoroughly rinsed before the start of the experiment with an extracellular solution containing (mM): NaCl 140, $CaCl_2$ 1.3, KCl 5.4, N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) 25 and glucose 33 with 0.1 μM tetrodotoxin (TTX) and 3 μM glycine (or as indicated in the Results) added before the pH was adjusted to 7.35–7.40 with 1 M NaOH.

Agonists and antagonists were applied to neurones from a 3-barrelled perfusion system allowing for rapid (<50 ms) application of compounds to the entire neurone under voltage-clamp. Agonists were applied for 5 s to ensure that steady-state responses had been achieved. Antagonists were superfused over a neurone and agonist applications repeated until they had stabilized at a new level. In a number of experiments, where the compound tested was in limited supply, a single pulse of antagonist was applied during a more prolonged agonist application until steady state block was attained. There was no apparent difference in antagonist potency between the two protocols.

Patch electrodes were constructed from thin-walled borosilicate glass (TW150F-4, WPI Inc., Sarasota, FL). Electrodes of 1–2 μm tip diameter were filled with solution containing (mM): CsF 110, CsCl 10, HEPES 10, ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) 10 and adjusted to a final pH of 7.35–7.40 with 2 M CsOH. Alternatively, an intracellular solution containing (mM): $CsMeSO_3$ 125, CsCl 15, HEPES 10, EGTA 5, $CaCl_2$ 0.5 and $MgCl_2$ 3 with a final pH of 7.4 was sometimes used. Recordings were made at room temperature (20–24°C). The membrane potential of the neurone was held at −60 mV, unless otherwise indicated, using a patch clamp amplifier (Axopatch 1B, Axon Instruments, Inc., Foster City, CA, U.S.A.).

Responses evoked on amino acid application were recorded using pClamp acquisition and analysis software (Axon Instruments Inc.). Each data point was the average of 3–6 responses and drug effect was expressed as a percentage reduction of the control steady-state (or peak, as indicated in the Results) agonist response. Final values of EC_{50} (the concentration of NMDA that evoked a current 50% of that maximally achieved), IC_{50} (the concentration of test compound that gave a 50% inhibition of the control agonist response), percentage reductions and n_H (the Hill coefficient, as described below) are expressed as mean ± s.e.mean, where *n* refers to the total number of neurones tested. For analysis of EC_{50} (or IC_{50}) values, data points were fitted to the logistic equation $R = R_{max}[\text{concentration}^{n_H}/(\text{concentration}^{n_H} + EC_{50}^{n_H})]$ where *R* is the agonist-evoked current (or the % reduction achieved by antagonist, as appropriate), R_{max} is the maximum response achieved and concentration refers to that of the applied agonist (or antagonist, as appropriate).

Fluorescent dye studies

Hippocampal pyramidal neurones were obtained from 18-day-old foetal Wistar rats and plated on glass coverslips at a density of $1-3 \times 10^5$ cells cm^{-2}. Experiments were performed 7–21 days after plating. Following loading with Fura-2 (see below), coverslips were placed in a chamber at 20–23°C and continuously superfused at a rate of 1.5 ml min^{-1} with a nominally Mg^{2+}-free solution containing (mM): NaCl 136.5, KCl 3, NaH_2PO_4 1.5, D-glucose 10, $CaCl_2$ 2 and HEPES 10; TTX (0.3–0.5 μM) and glycine (2 μM) were added and the pH was adjusted to 7.35–7.40 with 10 M NaOH. The amino acid excitant NMDA (20 μM) was administered in 1 ml aliquots into the inflow of the perfusion chamber and allowed to remain in contact with the neurones for 20 s before wash-out. Test compounds were applied by superfusion; their effects were expressed as the percentage change of the peak response to NMDA during their administration, in relation to the peaks of the control and recovery responses obtained before and after their administration, respectively.

Cytoplasmic free calcium concentrations were measured using the calcium sensitive fluorophore, Fura-2 (Grynkiewicz *et al.*, 1985). Neurones were incubated at 33–35°C for 60 min in 7.5 μM Fura-2 AM (Molecular Probes Inc., Eugene, OR, U.S.A.). Cells were then washed and left for 30 min before use to ensure complete hydrolysis of the acetomethoxy form of Fura-2. $[Ca^{2+}]_i$ was measured by the dual excitation fluorescence ratio method on an Attofluor Digital Fluorescence Microscopy System (Atto Instruments Inc., Rockville, MD; Carl Zeiss Canada Ltd., Don Mills, ON, Canada). Employing excitation wavelengths of 334 and 380 nm, fluorescence intensities (at 510 nm) were obtained from multiple neurone somata simultaneously. The raw intensity data at each excitation wavelength were corrected for background prior to calculation of the ratio. The *in situ* calibration method was used to convert fluorescence ratios into $[Ca^{2+}]_i$. During exposure to 10 μM Br-A23187, calibration parameters (R_{min}, R_{max} and β) were obtained in the presence (2 mM) and absence (nominally zero-Ca^{2+} solution in the presence of 100 μM EGTA) of Ca^{2+}. The published K_D value of 135 nM (at 20°C) was employed (Grynkiewicz *et al.*, 1985).

Statistical results are reported as mean ± s.e.mean, where *n* refers to the total number of neurones from which observations were made under each experimental condition. The effects of each concentration of each test compound were examined on at least three different neuronal cultures. IC_{50} values were obtained by use of the same logistic equation as in the electrophysiological studies (see above).

Sources of compounds

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), with the exceptions of (**RS**)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), (+)-3-PPP, DTG, haloperidol, rimcazole, (+)-pentazocine, (+)-NANM, ifenprodil (Research Biochemical Inc., Natick, MA, U.S.A.) and a second source of AMPA (Tocris Neuramin, Bristol, U.K.). Ketamine and opipramol were gifts from Parke-Davis Inc. (Don Mills, ON, Canada) and CIBA-Geigy (Summit, NJ, U.S.A.), respectively. 1***R***,2***S***- and 1***S***,2***R***-*cis*-(−)-N-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-(2-naphthyl)acetamide (respectively, (+)- and (−)-29) and 1*S*,2*R*-(−)-*cis*-N-methyl-*N*- [2- (3, 4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl) cyclohexylamine ((−)-44) were generous gifts from Dr B.R. de Costa (National Institutes of Health, Bethesda, MD, U.S.A.).

Results

Sigma site ligands attenuate NMDA-evoked responses

Electrophysiological studies Applications of 60 μM NMDA, 60 or 100 μM kainate and 3 or 30 μM AMPA evoked inward currents in mouse hippocampal neurones voltage-clamped at a membrane potential (V(h)) of −60 mV. The agonist concentrations employed evoke near-maximal responses in this preparation (data not shown) thus allowing for maximal sensitivity to the potential antagonist action of the compounds tested. On no occasion did the application of a σ site ligand alone evoke a detectable current in the cultured neurones even at the highest concentrations tested (*i.e.* at concentrations which produced a >90% block of the control NMDA-evoked current).

Currents evoked by NMDA were reversibly attenuated by all the compounds tested at micromolar concentrations. The IC_{50} values for the σ ligands as antagonists of NMDA-evoked currents are shown in Table 1. Due to limited supplies, IC_{50} values for the stereoisomers (+)- and (−)-29 (de Costa *et al.*, 1990) could not be estimated; rather, the potency and selectivity of a single concentration (100 μM) was examined

(Table 1). The Hill coefficients (Table 1) were not significantly different from 1 with the exceptions of ifenprodil, (+)-NANM, haloperidol and rimcazole, suggesting that a single site of action for these compounds is unlikely to explain adequately their antagonist characteristics. The potency and mechanism of block of NMDA-evoked responses by ifenprodil (Legendre & Westbrook, 1991; Church *et al.*, 1994), haloperidol (Fletcher & MacDonald, 1993), ketamine (MacDonald *et al.*, 1991) and DTG (Fletcher *et al.*, 1993) have been described previously and are only discussed here as controls for the various parameters tested.

A concentration of each compound which substantially reduced NMDA-evoked currents was tested against kainate- and AMPA-evoked responses (Table 1, Figure 1). With the exception of ifenprodil (see Church *et al.*, 1994), the compounds reversibly attenuated kainate- and AMPA-evoked currents to a limited extent (≤20% reduction of the control response; Table 1). On some of the neurones tested, short co-applications (see Figure 1 for protocol) of the highest concentrations of caramiphen, carbetapentane, (+)-pentazocine and DXM tested produced a small, fully-reversible enhancement of kainate- and AMPA-, but not NMDA-, evoked responses. This potentiation, also observed with DTG (Fletcher *et al.*, 1993), was not evident when the antagonist was applied prior to agonist application. As the phenomenon was not consistently observed, the underlying mechanism(s) were not investigated.

Microspectrofluorimetric studies Selected compounds were examined for their ability to attenuate NMDA-evoked rises in $[Ca^{2+}]_i$ in rat hippocampal neurones loaded with Fura-2. None of the compounds tested, with the exception of rimcazole (which proved toxic to neurones when exposed to u.v. light; see Figure 2), had effects on resting $[Ca^{2+}]_i$. The σ site ligands tested in this experimental paradigm reversibly attenuated NMDA-evoked rises in $[Ca^{2+}]_i$ with a rank order potency (mean±s.e.mean IC_{50} values in μM): ifenprodil (0.7±0.1) (Church *et al.*, 1994)>DXM (3.8±0.2)=haloperidol (4.1±0.6)>rimcazole (10±0.4)> DTG (34±3) (Fletcher *et al.*, 1993)=opipramol (35±2) > carami-phen (43±2)=carbetapentane (44±2). Examples of the effects of the antidepressant, opipramol (Rao *et al.*, 1990) and the atypical antipsychotic, rimcazole (Ferris *et al.*, 1986), and the antitussive agents, caramiphen and carbetapentane, on NMDA-evoked rises in $[Ca^{2+}]_i$ can be seen in Figures 2 and 3, respectively. The rank order of potency for NMDA antagonism for the selection of σ ligands examined in these experiments was the same as that found in the electrophysiological studies, although the absolute IC_{50} values for rimcazole, opipramol, caramiphen and carbetapentane were lower than those obtained in the voltage-clamp experiments (see Table 1). The probable reason for these discrepancies is the fact that the latter compounds are more potent antagonists of voltage-activated Ca^{2+} channels than of NMDA-evoked responses, as described in the companion paper (Church & Fletcher, 1995). Under non-voltage-clamped conditions, application of NMDA will evoke rises in $[Ca^{2+}]_i$ mediated, at least in part, through voltage-activated Ca^{2+} channels consequent upon membrane depolarization. In contrast, ifenprodil (Church *et al.*, 1994), DXM (Netzer *et al.*, 1993), haloperidol (Fletcher & MacDonald, 1993; Fletcher *et al.*, 1994) and DTG (Fletcher *et al.*, 1993) are more potent NMDA antagonists than Ca^{2+} channel blockers.

Due to these difficulties, all subsequent experiments were performed in mouse hippocampal neurones under voltage-clamped conditions.

Sigma ligands did not produce a competitive antagonism of NMDA-evoked currents

In order to investigate whether σ ligands attenuate NMDA-evoked currents through direct competition at the agonist binding site, dose-response plots for NMDA were constructed in the absence and presence of the test compounds at concentrations that approximated their IC_{50} values obtained under voltage-clamp. In all cases, the compounds shifted the dose-response plots for NMDA to the right in a non-parallel fashion with an approximately 50% reduction of the maximum NMDA-evoked response (R_{max}) but without change in the EC_{50} values for NMDA. In addition, the attenuation of control NMDA-evoked responses by the test compounds was unaltered on elevation of the agonist concentration, indicating

Table 1 Potency and selectivity of σ site ligands as antagonists of NMDA-evoked currents in voltage-clamped mouse hippocampal neurones

				% reduction of control response to:		
Compound	*IC_{50} (μM)*	*n_H*	*Conc (μM)*	*NMDA*	*AMPA*	*Kainate*
Ifenprodil[a]	0.80±0.19 (7)	0.5±0.1*	100	91±2 (7)	44±6 (5)	20±7 (6)
(+)-NANM	1.06±0.19 (6)	1.5±0.2*	6	81±7 (6)	NE	NE
DXM	1.76±0.15 (7)	1.0±0.1	100	96±1 (6)	3±6 (7)	0.2±4 (6)
Haloperidol[b]	1.86±0.18 (7)	1.4±0.1*	50	81±2 (7)	8±3 (8)	12±3 (8)
Ketamine	3.54±0.05 (3)	1.2±0.1	30	92±1 (3)	NE	NE
(+)-Pentazocine	7.19±1.44 (7)	1.1±0.1	60	96±1 (5)	5±7 (7)	4±3 (12)
(-)-44	16.8±2.2 (7)	1.2±0.1	100	91±2 (7)	2±5 (3)	1±3 (4)
Rimcazole	18.3±2.1 (6)	1.5±0.1*	100	90±2 (5)	20±5 (8)	11±2 (16)
DTG[c]	36.6±5.4 (10)	1.1±0.2	300	92±2 (10)	9±2 (11)	5±1 (11)
Opipramol	95.7±9.4 (6)	1.3±0.1	300	78±5 (6)	18±12 (2)	12 (2)
Caramiphen	110±12 (9)	1.2±0.1	600	92±1 (6)	3±8 (8)	4±5 (8)
Carbetapentane	112±13 (8)	1.1±0.1	600	91±2 (5)	14±6 (6)	5±4 (7)
(+)-3-PPP	485±74 (10)	1.2±0.1	600	50±4 (11)	11±3 (6)	12±2 (12)
(-)-29	NE	NE	100	33±4 (14)	3±5 (5)	1±7 (7)
(+)-29	NE	NE	100	22±5 (6)	NE	NE

Table indicates the mean±s.e.mean of the IC_{50} values (μM) and Hill coefficients (n_H) for the test compounds, indicated to the left, as antagonists of NMDA-evoked currents in voltage-clamped neurones at V(h)=−60 mV. Asterisk against n_H value indicates a Hill coefficient significantly different from 1 (Student's *t* test, $P<0.05$). The selectivity of the test compounds is indicated to the right where values indicate the mean±s.e.mean % reduction of the control responses to NMDA (60 μM), kainate (60 μM) and AMPA (3–30 μM) in the presence of the given concentration (centre column, μM) of σ ligand. The number of neurones tested is indicated in parentheses. NE indicates value was not estimated. [a]Values taken from Church *et al.* (1994); [b]values taken from Fletcher & MacDonald (1993); [c]values taken from Fletcher *et al.* (1993).

that the agonist did not compete for the antagonist binding site. Examples of the non-competitive NMDA antagonist actions of two σ site ligands can be seen in Figure 4.

We observed variations in the EC_{50} values for NMDA between neuronal cultures and therefore it was felt more informative to utilize the ratios of the EC_{50} values for NMDA in the presence, relative to absence, of test drug (*i.e.* EC_{50} [NMDA + drug]/EC_{50} [NMDA control]). An EC_{50} ratio significantly greater than 1 would indicate that the test compound interacts competitively with the agonist binding site, resulting in an apparent increase in the EC_{50} value for NMDA. Thus, in 4 neurones tested caramiphen (100 μM) produced a 47±9% reduction of R_{max} with an EC_{50} ratio of 0.9±0.1 whilst for rimcazole (20 μM; $n=8$) the respective values were 59±4% and 1.1±0.1; for (+)-3-PPP (600 μM; $n=5$) they were 54±6% and 1.2±0.1; and for carbetapentane (200 μM; $n=4$) they were 54±8% and 1.3±0.04. For these and the other compounds tested (for $n \geq 3$ neurones), the ratio of the EC_{50} values was not significantly different from 1 (Student's paired t test, $P<0.05$).

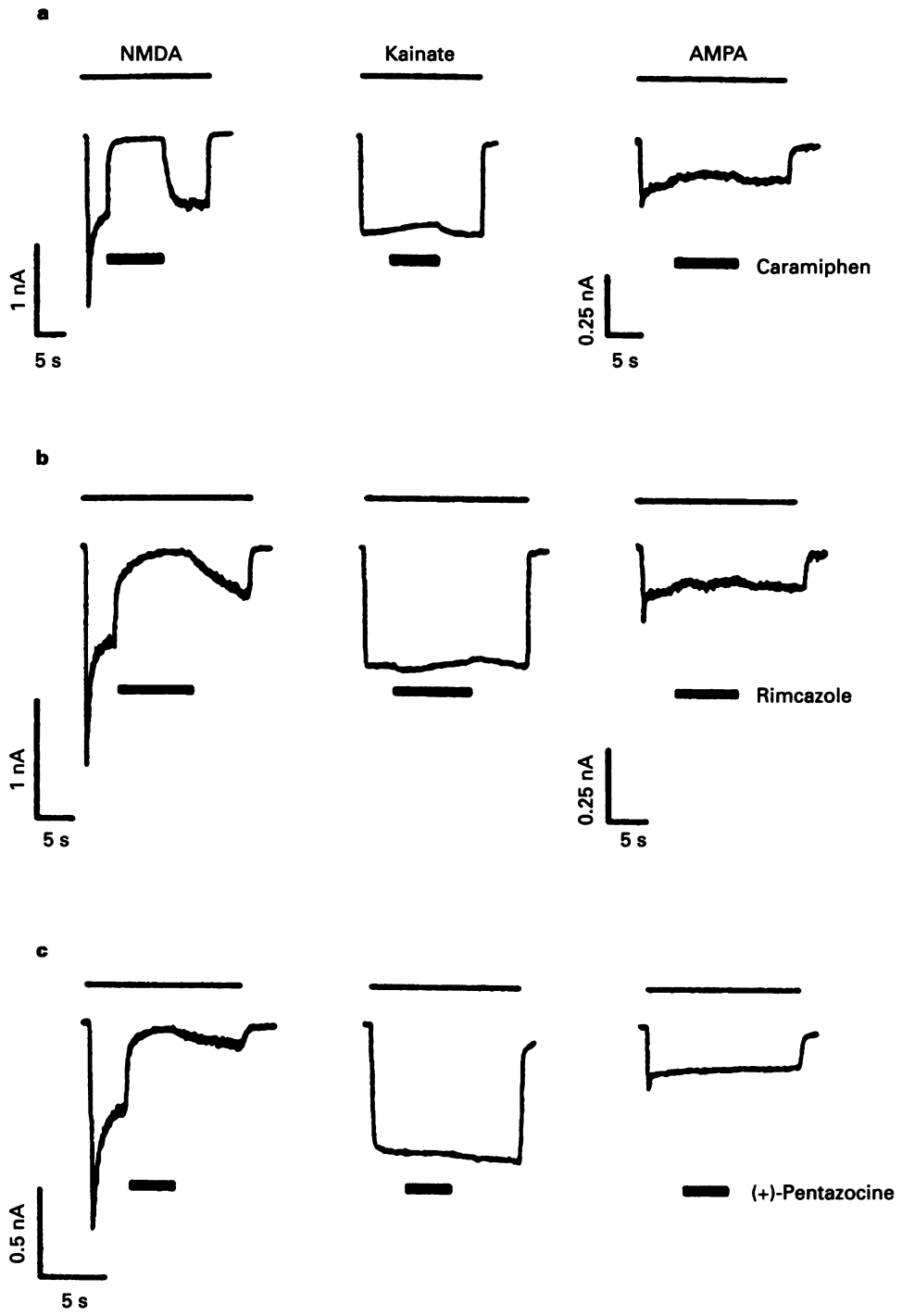

Figure 1 Selectivity of the block of excitatory amino acid-evoked currents by σ site ligands. The selectivity of the action of (a) 600 μM caramiphen, (b) 100 μM rimcazole and (c) 60 μM (+)-pentazocine on amino acid-evoked responses in 3 different hippocampal neurones held under voltage-clamp. NMDA (60 μM), kainate (60 μM) and AMPA (3 μM) were applied for the periods indicated by the thin bars above the traces. Antagonists were applied, for periods indicated by the thick bars below the traces, until the block of the NMDA-evoked responses had reached steady-state. Calibration bars: time in s, current in nA. Note that in (a) and (b) the scaling for the AMPA responses differs from those for NMDA and kainate.

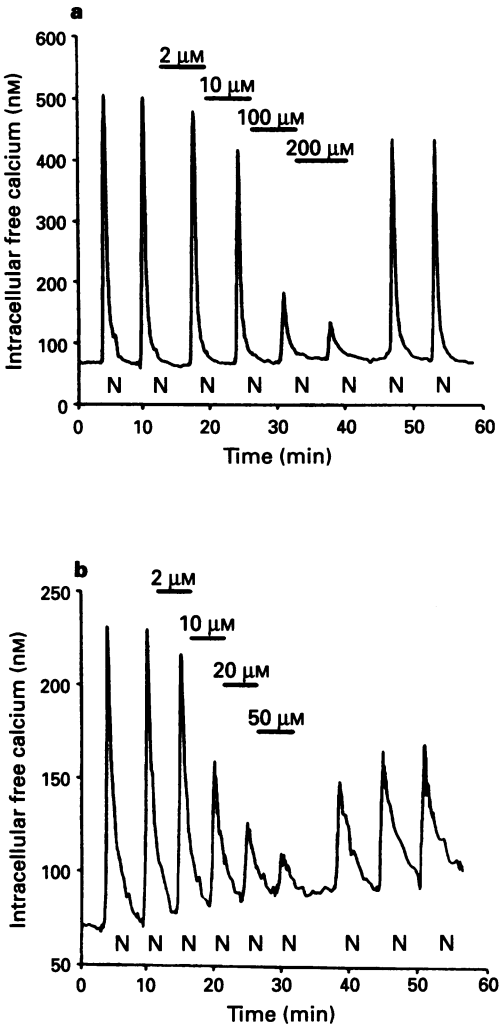

Figure 2 The effects of opipramol and rimcazole on NMDA-evoked rises in $[Ca^{2+}]_i$. (a) Opipramol 2–200 μM, applied for the periods indicated by the bars above the trace, reversibly attenuated NMDA-(N) evoked rises in $[Ca^{2+}]_i$. The trace is the mean of data obtained from 12 neurones simultaneously. (b) Rimcazole 2–50 μM, applied for the periods indicated by the bars above the trace, attenuated NMDA- (N) evoked rises in $[Ca^{2+}]_i$. The trace is a mean of data obtained from 5 neurones simultaneously. Note the gradual increase in baseline $[Ca^{2+}]_i$ during and after exposure to rimcazole and the incomplete recovery of the NMDA response. Following the final NMDA response after washout of rimcazole, an adjacent region of the same coverslip was imaged and neurones in this region, which had not been exposed to u.v. light, had normal baseline $[Ca^{2+}]_i$ and exhibited robust responses to NMDA, equivalent to the pre-rimcazole control responses shown in the figure. Interestingly, this phenomenon was observed only during perfusion with Mg^{2+}-free medium (compare with Figure 4a in the companion paper; Church & Fletcher, 1995).

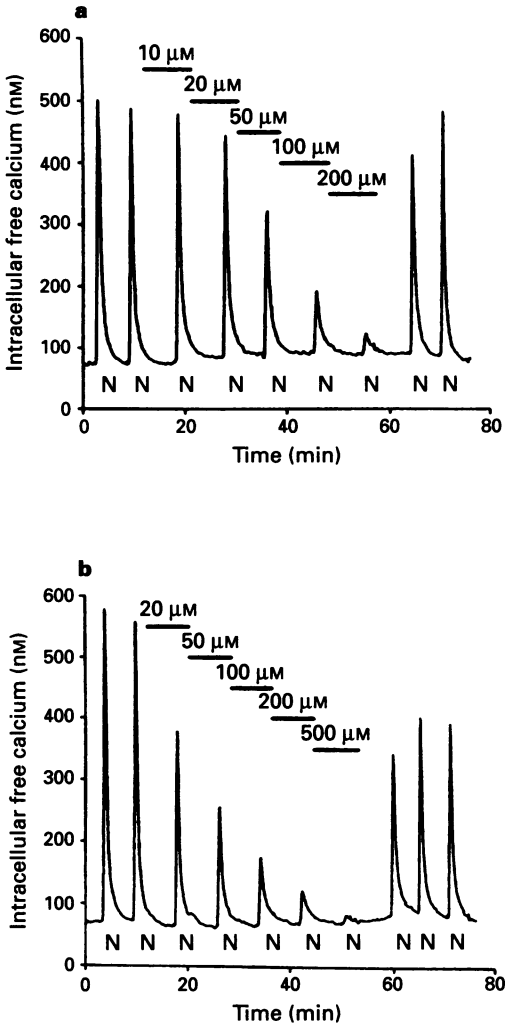

Figure 3 The effects of (a) caramiphen 10–200 μM and (b) carbetapentane 20–500 μM on NMDA-(N) evoked rises in $[Ca^{2+}]_i$. The traces are means of data obtained simultaneously from 6 and 7 neurones, respectively.

The NMDA antagonist action of σ ligands is not reversed by spermine or glycine

We explored the possibility that σ ligands may attenuate NMDA-evoked currents indirectly through interactions with allosteric modulatory sites on the NMDA receptor-channel complex. One candidate is the polyamine site at which compounds such as spermine positively modulate NMDA receptor-mediated events (Williams *et al.*, 1991). Test compounds were applied (at concentrations approximating their respective IC_{50} values) in the presence and absence of 100 μM spermine. Superfusion with spermine (in the continued presence of 3 μM glycine) potentiated, albeit not robustly, NMDA-evoked responses (in 43 neurones, a 25±3% potentiation of control response at V(h) −60 mV; range 5 to 70%). Higher concentrations of spermine were not applied as they weakly attenuate NMDA-evoked responses under the conditions employed here (see Church *et al.*, 1994). The presence of spermine did not reverse the inhibitory action of any of the compounds tested (Figure 5a). Paradoxically, spermine produced a small but reproducible increase in the inhibitory action of ifenprodil (see Church *et al.*, 1994).

The interaction of σ ligands with the strychnine-insensitive glycine site was also investigated. With the exception of haloperidol, used here as a positive control (Fletcher & MacDonald, 1993), there was no significant difference (Student's paired *t* test, $P<0.05$) between the percentage reductions of NMDA-evoked responses by any of the compounds tested in the presence of either 1 or 10 μM glycine (Figure 5b). Due to limited drug supplies, only 3 cells were tested with 100 μM (−)-29, but elevation of the glycine concentration from 1 to 10 μM did not reduce its NMDA antagonist potency (respectively, 29±3 and 30±4% reductions from control).

Voltage-dependence of the block of NMDA-evoked currents by σ ligands

Because the NMDA antagonist actions of the test compounds could not be attributed to activity at the agonist or coagonist binding sites, we assessed the interaction of σ site ligands with the receptor-associated ion channel. The percentage reductions

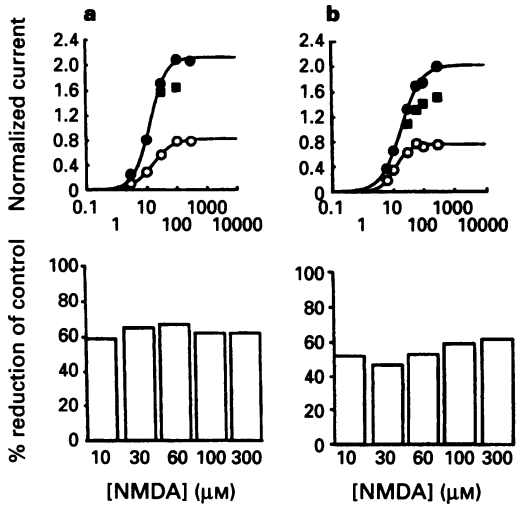

Figure 4 σ Site ligands non-competitively block NMDA-evoked currents. Plots (top) indicate concentration-response relationship for NMDA in the absence (●) and presence (○) of (a) 10 μM (+)-pentazocine and (b) 40 μM opipramol. Ordinate scale indicates agonist-evoked current amplitude normalized to the EC_{50} value for the control NMDA response. In (a) the EC_{50} values for NMDA were 12 and 14 μM in, respectively, the absence and presence of (+)-pentazocine, whilst the R_{max} was reduced from 2.12 to 0.83. In (b) the EC_{50} values for NMDA were similarly unaltered (32 and 33 μM in the absence and presence, respectively, of opipramol) whilst the R_{max} was reduced from 2.0 to 0.77. On wash (■) NMDA responses recovered to values close to those of controls. Abscissa scale: NMDA concentration in μM. Histograms (below) indicate the absence of agonist dependency to the block in the same two neurones as above. Ordinates: % reduction of control NMDA-evoked response. Abscissae: NMDA concentration in μM.

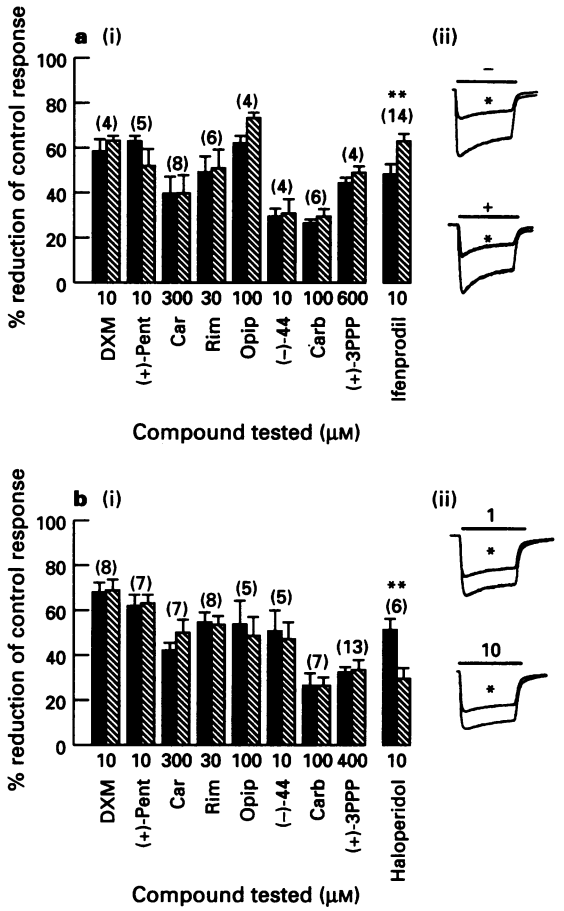

Figure 5 Spermine and glycine insensitivity of the NMDA antagonist actions of σ site ligands. Histograms indicate the % reduction (mean ± s.e.mean) of control NMDA (60 μM) responses in (a,i) the absence (solid column) and presence (hatched column) of 100 μM spermine or (b,i) the presence of 1 μM (solid column) or 10 μM (hatched column) glycine. The compounds tested together with the concentrations (μM) applied are indicated below the abscissa scale. The number of neurones tested is indicated in parentheses above the columns. A double asterisk indicates a significant difference between values (Student's paired *t*-test, $P<0.05$). Abbreviations: (+)-Pent, (+)-pentazocine; Car, caramiphen; Rim, rimcazole; Opip, opipramol; Carb, carbetapentane. Paired traces to the right indicate the attenuation of NMDA- (60 μM) evoked currents by (a,ii) 300 μM caramiphen (*) in the absence (−) or presence (+) of 100 μM spermine and by (b,ii) 30 μM rimcazole (*) in the presence of 1 or 10 μM glycine as indicated. NMDA was applied for 5 s, indicated by the bars above the traces. Control currents are normalized for ease of comparison.

of control NMDA-evoked responses by the test compounds were examined at a variety of potentials (V(h) from −100 to +60 mV), allowing 60–90 s intervals between 10 mV increments in V(h) to limit possible errors resulting from non-equilibration of the membrane potential. The degree of attenuation of NMDA-evoked responses by (−)-44 (10 μM; $n=3$; Figure 6a), caramiphen (100 μM; $n=9$; Figure 6c), carbetapentane (100 μM; $n=8$), (+)-3-PPP (200 μM; $n=11$), (+)-pentazocine (10 μM; $n=5$), DXM (10 μM; $n=3$) and DTG (Fletcher *et al.*, 1993) was negligible at positive holding potentials but increased with membrane hyperpolarization, a characteristic of the voltage-dependent block observed with Mg^{2+} and compounds such as ketamine and PCP (Nowak *et al.*, 1984; MacDonald *et al.*, 1991). No attempt was made to quantify differences in the degree of voltage-dependency between the individual compounds; they are classified here simply as possessing a voltage-dependent antagonist action. The characteristics of the NMDA block produced by the aforementioned compounds contrasted clearly, however, with the apparent absence of voltage-dependence to the antagonist actions of haloperidol (Fletcher & MacDonald, 1993), ifenprodil (Church *et al.*, 1994), opipramol (40 μM; $n=5$) and (+)- and (−)-29 (30 μM; $n=4$ in each case). Both voltage-dependent and -independent components to the block by rimcazole (30 μM; $n=5$; Figure 6b) suggest that this compound might interact with more than a single site on the NMDA receptor-channel complex.

Use-dependence of the NMDA block by σ ligands

Attenuation of NMDA-evoked responses by ketamine, PCP and dizocilpine requires activation of the NMDA receptor and channel opening prior to the compounds gaining access to their binding site (Huettner & Bean, 1988; MacDonald *et al.*, 1991). We therefore investigated whether block of NMDA-evoked currents by the compounds tested here also exhibited this property of use- (or agonist-) dependency. Only antagonism of NMDA-evoked responses by DXM, (+)-pentazocine and ketamine (applied at concentrations close to their estimated IC_{50} values) was fully use-dependent with little attenuation of the first NMDA-evoked response following the start of drug superfusion but increasing block on subsequent agonist applications (Figure 7a,b). A similar slow agonist-dependent recovery of the block was also observed (not shown). With the exception of DTG, which possessed both use-dependent and -independent components (Fletcher *et al.*, 1993), the NMDA antagonist actions of the other compounds tested showed little, if any, signs of a slow agonist-dependent block (for examples, see Figure 7c,d).

Sigma ligands differentially affect distinct states of the NMDA receptor-channel complex

Closer inspection of the antagonist actions of DXM, rimcazole and (+)-3-PPP revealed that they produced a greater inhibition of NMDA-evoked responses at steady-state (4.5–5 s following the peak) compared to that of the initial peak.

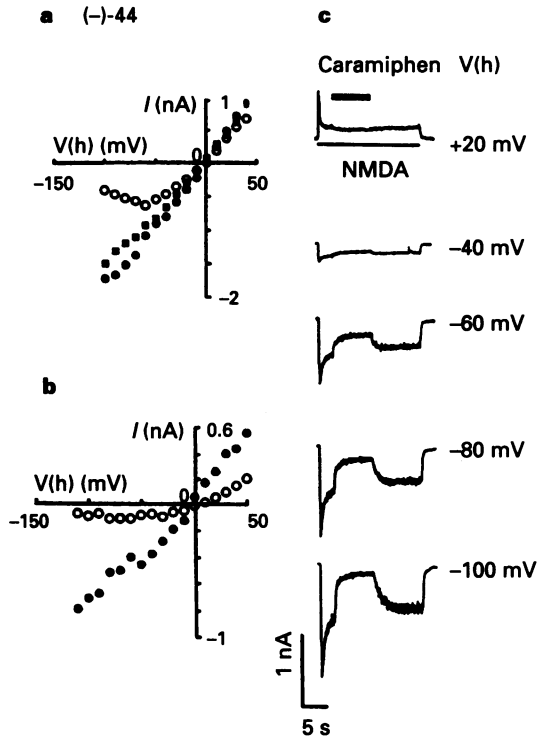

Figure 6 Voltage-dependence of the block of NMDA- (60 μM) evoked currents by σ site ligands. Current-voltage relationship for NMDA is plotted over a range of membrane potentials (V(h)) in the absence (●) and presence (○) of (a) 10 μM (−)-44 and (b) 30 μM rimcazole. Recovery of control responses was achieved on wash in control solution ((■); not shown in (b)). (c) Traces typifying the voltage-dependent block of NMDA-evoked currents (NMDA applied for the period indicated by the thin bar below the top trace) by caramiphen (100 μM, applied for the period indicated by the thick bar above the top trace) at V(h) indicated to the right of the traces. Calibration bars: current amplitude in nA; time in s.

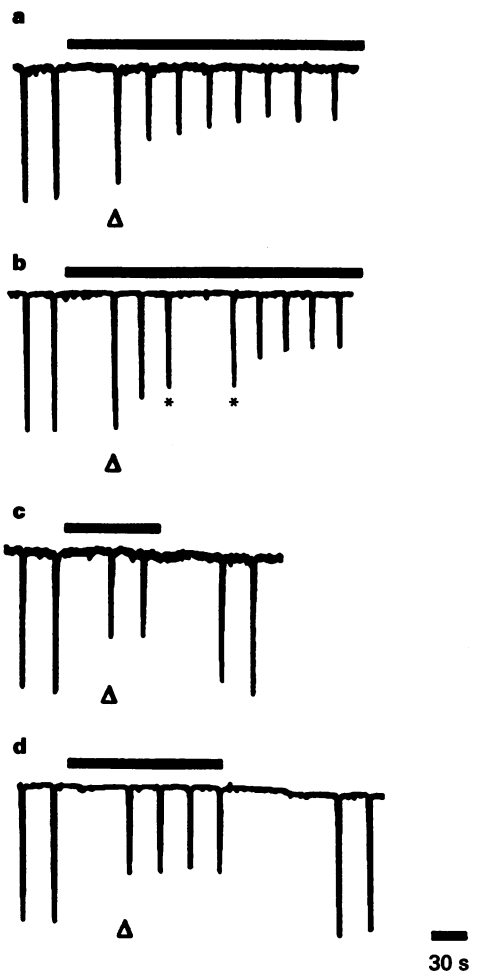

Figure 7 Use-dependent block of NMDA-evoked responses by σ ligands. Attenuation of NMDA-evoked responses by (a) 10 μM DXM and (b) 10 μM (+)-pentazocine was strongly use-dependent, whilst a use-dependent component was not seen with either (c) 100 μM carbetapentane or (d) 400 μM (+)-3-PPP. Two control NMDA (60 μM) responses are shown to the left of each trace. Antagonist application is indicated by the thick bar above the individual traces. The first NMDA application in the presence of antagonist is indicated by an open triangle. Recovery from block is indicated in traces (c) and (d) only. Note that in (b) NMDA applications were interrupted for the interval between the responses marked by an asterisk highlighting the agonist-dependence of the block by (+)-pentazocine. Data shown in (b) and (d) came from the same neurone, whilst those from (a) and (c) are from separate neurones. Control NMDA-evoked currents have been normalized for ease of comparison. Calibration, time in s.

Analysis of the concentration-inhibition plots for DXM, rimcazole and (+)-3-PPP indicated that the IC_{50} values for the peak and steady-state components differed approximately two fold; a four fold difference has previously been reported for haloperidol (Fletcher & MacDonald, 1993) and ifenprodil (Church *et al.*, 1994). None of the other compounds tested showed similar discrepancies suggesting that they did not result from a systematic error in the experimental design such as incomplete acquisition of the initial peak (which is likely, however, to occur and contribute to an underestimate of the % reduction of this component). A difference in IC_{50} values for the peak and steady-state components of the NMDA response with DXM was also observed by Netzer and colleagues (1993) who suggested that the slow kinetics of block by this relatively potent compound might underlie the phenomenon. However, the latter hypothesis does not adequately explain the differences seen with (+)-3-PPP and rimcazole, which are relatively low affinity blockers with fast kinetics of block, and, conversely, the absence of such differences with the high affinity, use-dependent blockers (+)-pentazocine and ketamine. An example of the difference in the action of (+)-3-PPP, but not ketamine, on the early and late components of the NMDA-evoked current can be seen in Figure 8.

Discussion

The data presented form the first quantitative study of the relative potency and selectivity of a range of structurally-dissimilar σ site ligands as excitatory amino acid antagonists at the single cell level. The compounds tested attenuated NMDA-evoked currents and NMDA-evoked rises in $[Ca^{2+}]_i$ in cultured hippocampal neurones at micromolar concentrations with minimal activity on kainate- and AMPA-evoked currents, suggesting that they are both relatively potent and selective NMDA antagonists.

It is clear that both the absolute and the rank order potencies of the σ ligands tested as NMDA antagonists (Table 1) do not parallel their respective affinities for high-affinity σ binding sites. For example, although haloperidol and (+)-3-PPP have comparably high (nanomolar) affinities for the σ site (Largent *et al.*, 1986; Rothman *et al.*, 1991), they differ more than 200 fold in their NMDA antagonist potencies, which are observed at micromolar concentrations. Similarly rimcazole and DXM, σ ligands with 10 fold lower affinities for the σ site than DTG (Ferris *et al.*, 1986; Rothman *et al.*, 1991), were more potent NMDA antagonists than the latter compound, and caramiphen and carbetapentane, although equipotent as NMDA antagonists, differ more than 10 fold in their affinity for σ binding sites (Rothman *et al.*, 1991). The N-substituted

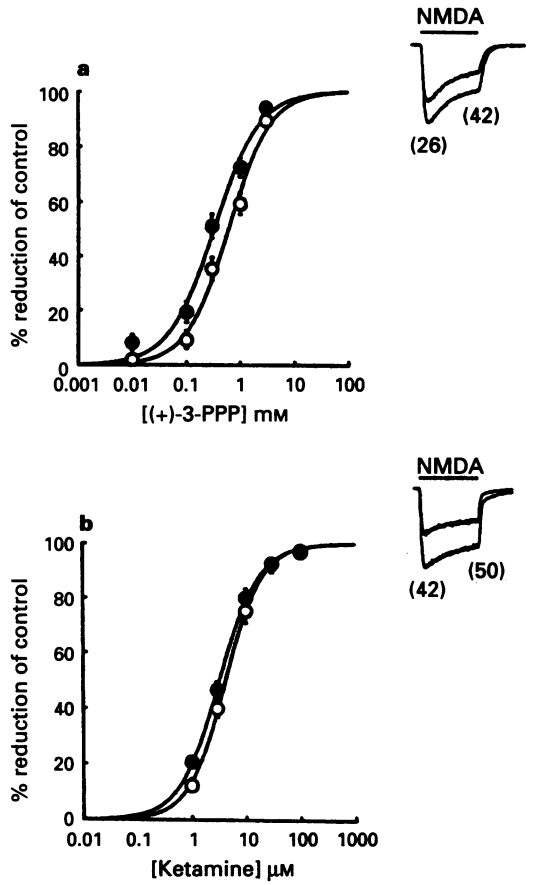

Figure 8 (+)-3-PPP, but not ketamine, differentially blocks early (peak) and late (steady-state) components of the NMDA-evoked response. Concentration-inhibition plots for (a) (+)-3-PPP and (b) ketamine on the steady-state (●) and peak (○) components of control NMDA-evoked responses. Values indicate mean±s.e.mean % reduction of control NMDA-evoked current by antagonist at concentrations indicated on abscissa scale. IC_{50} values for (+)-3-PPP on steady-state and peak responses were 322±49 and 602±89 μM, respectively ($n=5$; significantly different, Student's paired t test, $P<0.05$) whilst for ketamine the respective values were 3.3±0.1 and 4.4±0.2 μM ($n=3$; not significantly different). Paired sample traces to the right indicate % attenuation (in parentheses) of both components of a 5 s NMDA- (60 μM) evoked response by (top) 300 μM (+)-3-PPP and (bottom) 3 μM ketamine.

cis-N-methyl-2-(1-pyrrolidinyl)cyclohexylamines (+)- and (−)-29 and (−)-44, novel high affinity probes for σ sites with K_i values against [^{3}H]-(+)-3-PPP binding of 1372, 8.7 and 1.3 nM, respectively (de Costa *et al.*, 1990), were only effective NMDA receptor antagonists at concentrations some 10,000 times greater than their reported affinities for the σ site. No stereoselectivity was observed in the NMDA antagonist potencies of the (−)- and (+)-enantiomers of compound 29 yet they differ 160 fold in their affinity for σ sites. In summary, as no correlation exists between the potencies of the compounds tested as NMDA antagonists and their reported affinities for, or stereoselectivity at, σ binding sites, their observed NMDA antagonist actions are unlikely to be mediated through high-affinity σ binding sites.

However, the observed NMDA antagonist potencies of the compounds tested agrees well with their potencies in displacing [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) or [^{3}H]-dizocilpine binding from cortical membrane preparations. Ifenprodil, (+)-NANM, DXM, haloperidol, ketamine and (+)-pentazocine were the most potent NMDA antagonists with IC_{50} values <10 μM, which agree well with their K_i values against [^{3}H]-TCP binding: ifenprodil, ≈5 μM (Carter *et al.*, 1988); (+)-NANM, 0.4 μM (Largent *et al.*, 1986); DXM, 3 μM (Sills & Loo, 1989); ketamine, 5 μM (vs. dizocilpine binding; Fagg, 1987); and (+)-pentazocine, 15 μM (Tam *et al.*, 1988). The weak NMDA antagonist action of (+)-3-PPP reflects its low affinity for the [^{3}H]-TCP binding site ($IC_{50}>100$ μM; Largent *et al.*, 1986). Similarly, (+)- and (−)-29 and (−)-44 do not inhibit [^{3}H]-TCP binding tested up to 10 μM (de Costa *et al.*, 1990). The weak activity of haloperidol in [^{3}H]-TCP binding assays in membrane preparations ($K_i>50$ μM; Largent *et al.*, 1986), relative to its potency as an NMDA antagonist, may be attributed to its indirect mode of interaction with the TCP-binding site through the allosteric glycine site (Fletcher & MacDonald, 1993), although it has recently been reported that haloperidol inhibits [^{3}H]-TCP binding in intact neuronal cells with an IC_{50} value of 1.86 μM (Yamamoto *et al.*, 1995), the same as that found for antagonism of NMDA-evoked currents in the present study (Table 1). By contrast the observed discrepancies for caramiphen and DTG, which displace [^{3}H]-TCP binding with greater affinity ($K_i<20$ μM and 8–15 μM, respectively; Sills & Loo, 1989; Keana *et al.*, 1989) than predicted from their relatively weak NMDA antagonist activities under voltage-clamped conditions, are not readily explained, although 100 μM carbetapentane has been reported to be inactive in displacing [^{3}H]-TCP from rat brain membranes (Calderon *et al.*, 1994). Conversely, rimcazole appears to be a weaker displacer of [^{3}H]-TCP binding ($IC_{50}>200$ μM; Largent *et al.*, 1988) and [^{3}H]-PCP binding ($IC_{50}=43$ μM; Ferris *et al.*, 1986) than NMDA antagonist, possibly reflecting its activity at multiple sites on the NMDA receptor-channel complex (see below). Although the activity of opipramol appears to be dependent on binding conditions, it displaces [^{3}H]-TCP binding biphasically with IC_{50} values for the low affinity component ranging from 25–158 μM (Sills & Loo, 1989), concentrations associated with NMDA antagonism in the present study.

Radiolabelled ifenprodil, a putative ligand at the allosteric polyamine site (see Williams *et al.*, 1991), is displaced from cortical membrane preparations by both σ site ligands and polyamines (see Beart *et al.*, 1992). Interestingly, [^{3}H]-(+)-3-PPP binding is displaced by both ifenprodil and polyamines (Karbon *et al.*, 1990; Paul *et al.*, 1990), suggesting that σ ligands might modulate NMDA-evoked responses through interactions with the polyamine site. Our studies indicate, however, that the compounds tested here are not active at either the glycine or polyamine sites (with the exceptions of haloperidol and ifenprodil, respectively; see Fletcher & MacDonald, 1993; Church *et al.*, 1994). Rather, the non-competitive nature of the NMDA antagonism by the compounds tested is similar to that observed with dissociative anaesthetics (MacDonald *et al.*, 1991), dizocilpine (Huettner & Bean, 1988) and Mg^{2+} (Mayer & Westbrook, 1987). The voltage-dependence of the NMDA antagonist actions of DXM, (+)-pentazocine, DTG, (+)-3-PPP, caramiphen, carbetapentane and (−)-44 suggests an interaction with the receptor-linked ionophore which is confirmed, in the cases of DXM, (+)-pentazocine and DTG, by the use-dependent nature of their antagonism. An apparent absence of use-dependence to the NMDA block by (+)-3-PPP, (−)-44, caramiphen and carbetapentane need not imply lack of interaction with the receptor-linked ion channel; these compounds may, like Mg^{2+} (Nowak *et al.*, 1984), interact too rapidly with the channel for observation of this phenomenon. The latter explanation is in agreement with the low NMDA antagonist potencies of these compounds and the higher concentrations applied, factors which would enhance the kinetics of ligand binding. More detailed analysis of the voltage-dependence of the kinetics of block by the compounds might clarify this issue and help to determine whether they interact at a common locus within the channel (Woodhull, 1973). However, in the cases of opipramol and (+)- and (−)-29, which did not show voltage- or use-dependent block, the possibility exists that they may interact with novel site(s) on the NMDA receptor-channel complex, as has been suggested for compounds such as pentamidine (Reynolds & Aizenman, 1992). Finally, it cannot be discounted that at least some of the compounds tested may interact with multiple sites on the NMDA receptor-channel complex. For

example, in the light of the voltage-dependent and -independent components of the block by rimcazole and its non-unitary Hill coefficient, this compound may act at more than a single site.

We have shown that a variety of σ site ligands act as NMDA antagonists when applied at micromolar concentrations, but to what extent might this action contribute to the reported activity of these compounds in behavioural and neurochemical studies? NMDA receptor-mediated events have been implicated in the aetiology of a range of neurological disorders and NMDA receptor antagonists have therapeutic potential as neuroprotective and anticonvulsant agents (see Dingledine *et al.*, 1990; Meldrum & Garthwaite, 1990). Reports indicate that a variety of σ site ligands are both effective neuroprotective agents (*e.g.* Keana *et al.*, 1989; Rao *et al.*, 1990; Pontecorvo *et al.*, 1991; Tamura *et al.*, 1993; DeCoster *et al.*, 1995; Yamamoto *et al.*, 1995) and anticonvulsants in experimental models both *in vivo* and *in vitro* (*e.g.* Tortella & Musacchio, 1986; Leander *et al.*, 1988; Aram *et al.*, 1989; Apland & Braitman, 1990). Although, in many cases, these effects have been attributed to the activity of σ ligands at high-affinity σ binding sites, the present results indicate that non-σ site-mediated NMDA antagonism probably underlies these therapeutically-useful actions. Thus, the micromolar concentrations of ifenprodil, (+)-NANM, DXM, ketamine, (+)-pentazocine and DTG required for effective antiepileptiform or neuroprotective activity *in vitro* (Aram *et al.*, 1989; Apland & Braitman, 1990; Tamura *et al.*, 1993; De Coster *et al.*, 1995) approximate the IC_{50} values for NMDA antagonism found in the present study. For example, the rank order neuroprotective potency of some of the compounds tested here against glutamate-induced injury in rat cultured cortical neurones (EC_{50} values in μM taken from DeCoster *et al.*, 1995) is (+)-NANM (0.8) > DXM (3.1) = haloperidol (3.7) > (+)-pentazocine (8.5) > DTG (43); both the absolute potency values and the rank order potency for these compounds are remarkably similar to those found in the present study for NMDA antagonism (Table 1). The failure of both (+)-3-PPP (tested up to 150 μM; DeCoster *et al.*, 1995) and σ site-selective concentrations (*i.e.* lower than those required for appreciable NMDA antagonist activity) of σ ligands to exert neuroprotective effects (Poignet *et al.*, 1992; Lesage *et al.*, 1993) adds weight to the possibility that the NMDA antagonist effects of the compounds examined in the present study underlie their neuroprotective actions. On the other hand, the weak NMDA antagonist activities of compounds such as caramiphen and carbetapentane appear unlikely to be able to account for their relatively potent antiepileptiform (Aram *et al.*, 1989; Apland & Braitman, 1990) and neuroprotective (DeCoster *et al.*, 1995) actions which, rather, may reflect their blockade of voltage-activated Ca^{2+} channels as described in the companion paper (Church & Fletcher, 1995).

In conclusion, micromolar concentrations of σ site ligands interact selectively with the NMDA subtype of glutamate receptor at sites distinct from those of the agonist and coagonists (glycine and polyamines) but which appear, in most cases, to be associated with the receptor-linked ion channel. The NMDA antagonist effects of the compounds tested do not appear to be mediated by high affinity σ binding sites, although the data presented do not preclude the involvement of σ binding sites in various neurological disorders or the therapeutic potential of σ ligands at σ site-selective concentrations. However, the appreciable activity of a number of σ ligands as NMDA antagonists highlights the need for considerable caution in ascribing the functional effects of micromolar concentrations of σ site ligands solely to interactions with high-affinity σ binding sites.

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