Selective reduction of N-methyl-D-aspartate-evoked responses by 1,3-di(2-tolyl)guanidine in mouse and rat cultured hippocampal pyramidal neurones

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1 The effects of 1,3-di(2-tolyl)guanidine (DTG) were examined on the responses of cultured hippocampal neurones to the excitatory amino acid analogues N-methyl-D-aspartate (NMDA), kainate, quisqualate and (RS)- α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA).

2 In rat hippocampal neurones loaded with the Ca^{2+} -sensitive dye Fura-2, DTG (10–100 μM) produced a concentration-dependent depression of the NMDA-evoked rises in intracellular free calcium ($[\text{Ca}^{2+}]_i$), an effect that was not modified by changes in the extracellular glycine concentration. DTG (at 50 and 100 μM) also attenuated, although to a lesser extent, the rises in $[\text{Ca}^{2+}]_i$ evoked by naturally-derived quisqualate. In contrast, 50 and 100 μM DTG did not depress responses evoked by kainate, AMPA and synthetic, glutamate-free (+)-quisqualate although on occasions DTG enhanced kainate- and AMPA-evoked rises in $[\text{Ca}^{2+}]_i$.

3 DTG attenuated NMDA-evoked currents recorded from mouse hippocampal neurones under whole-cell voltage-clamp with an IC_{50} (mean $\pm$ s.e.mean) of $37 \pm 5 \mu\text{M}$ at a holding potential of -60 mV . The DTG block of NMDA-evoked responses was not competitive in nature and was not dependent on the extracellular glycine or spermine concentration. The block did, however, exhibit both voltage-, and use-, dependency. The steady-state current evoked by naturally-derived quisqualate was also attenuated by DTG whereas those evoked by kainate and AMPA were not.

4 We conclude that DTG, applied at micromolar concentrations, is a selective NMDA antagonist in cultured hippocampal neurones, the block exhibiting both Mg^{2+} - and phencyclidine-like characteristics. Given the nanomolar affinity of DTG for σ binding sites it is unlikely that the antagonism observed here is mediated by σ -receptors, but the data emphasize the potential danger of ascribing the functional consequences of DTG administration solely to σ receptor-mediated events.

Keywords: Glutamate receptors; N-methyl-D-aspartate (NMDA); kainate; quisqualate; (RS)- α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA); 1,3-di(2-tolyl)guanidine (DTG); σ -receptors; cultured hippocampal pyramidal neurones

Introduction

Originally phencyclidine (PCP) and 6,7-benzomorphans such as N-allylnormetazocine, cyclazocine and pentazocine were believed to produce their shared behavioural effects through interaction with a common site, the σ -opiate receptor (Martin *et al.*, 1976; Zukin & Zukin, 1981). Further studies determined, however, that these compounds bind to two non-opioid receptor populations distinct both pharmacologically and autoradiographically (Largent *et al.*, 1986; Sircar *et al.*, 1986; Quirion *et al.*, 1987). PCP, related arylcyclohexylamines such as 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP; Vignon *et al.*, 1983) and the compound, dizocilpine maleate (MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo hepten-5,10-imine-maleate; Wong *et al.*, 1986) bind preferentially to a site, the PCP site, located within the ion channel linked to the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Conversely (+)-6,7-benzomorphans bind preferentially to the σ site, but also possess some affinity for the PCP site and attenuate NMDA-evoked responses in central mammalian neurones (Berry *et al.*, 1984; Church & Lodge, 1990).

Only with the identification of the selective σ receptor ligands 1,3-di(2-tolyl)guanidine (DTG; Weber *et al.*, 1986) and (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3-PPP; Largent *et al.*, 1984) did a more rigorous assessment of the functional correlates of σ receptor occupancy become

possible. DTG in particular has been widely employed for this purpose both *in vivo* and *in vitro*, although recent observations have suggested that whilst DTG is a selective σ receptor ligand at low (nM) concentrations (see Quirion *et al.*, 1992) it may, at higher (μM) concentrations, also interact with excitatory amino acid receptors in the mammalian CNS. For example, rats trained to discriminate DTG (3 mg kg^{-1}) from saline generalize to PCP whereas DTG substitutes completely for PCP when the latter compound is used as the training stimulus (Holtzman, 1989), and DTG has been shown to depress NMDA-induced currents in cultured hippocampal neurones (Keana *et al.*, 1989). In contrast, DTG was observed to be inactive as an NMDA antagonist in the cortical slice preparation (Aram *et al.*, 1988) and in *in vivo* recordings from the central tegmental area (French & Ceci, 1990) but, variously, inactive (Malouf *et al.*, 1988) or active (Connick *et al.*, 1992) on CA1 neurones excited by iontophoretic application of NMDA in hippocampal slices. Furthermore the selectivity of DTG as an excitatory amino acid antagonist remains in doubt, as a recent study suggested that DTG non-selectively depresses both NMDA and quisqualate responses (Connick *et al.*, 1992).

The present experiments were undertaken to investigate the selectivity and potency of DTG as an excitatory amino acid antagonist in cultured hippocampal neurones. Initially, we utilized Fura-2-loaded, rat hippocampal neurones to establish the selectivity of DTG as an excitatory amino acid antagonist. Whole-cell, voltage-clamp techniques were then employed in mouse hippocampal neurones to both confirm

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the findings of the fluorescence study and to examine in greater detail the mechanisms whereby DTG depresses NMDA-evoked responses.

Methods

Fluorescent dye studies

Hippocampal neurones were obtained from 18-day-old foetal Wistar rats according to Banker & Cowan (1977) and plated on glass coverslips at a density of $1-3 \times 10^5$ cells cm^{-2} . Cultures were used at 9–28 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, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10. Tetrodotoxin 0.5 μM was added and the pH adjusted to 7.35–7.40 with 10 M NaOH. The amino acid excitants (NMDA, 20 μM ; kainate, 80 μM ; naturally-derived quisqualate, 40–80 μM ; synthetic, glutamate-free (+)-quisqualate, 80 μM ; and (RS)- α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), 40 μM were administered in 1 ml aliquots into the inflow of the perfusion chamber and allowed to remain in contact with the neurones for 25 s before wash-out. High- K^+ solutions (50 mM, by substitution for NaCl) were introduced in a similar fashion. Once reproducible responses were established, DTG was applied by superfusion. Results were expressed as the percentage change of the peak response to each excitant during the administration of the test compound, in relation to the peaks of the control and recovery responses obtained before and after its administration, respectively.

Cytoplasmic free calcium concentrations ($[\text{Ca}^{2+}]_i$) were measured by the dual excitation fluorescence ratio method on an Attotluor Digital Fluorescence Microscopy System (Atto Instruments Inc.; Carl Zeiss Canada Ltd.) employing the calcium sensitive fluorophore, Fura-2 (Grynkiewicz *et al.*, 1985). Neurones were incubated at 33–35°C for 60–75 min in 5–7.5 μM Fura-2 AM (Molecular Probes Inc.) and were then washed and left for 30 min before use to ensure complete hydrolysis of the acetomethoxy form of Fura-2. With excitation wavelengths of 334 and 380 nm, fluorescence intensities (at 510 nm) were obtained from multiple neurone somata simultaneously. Raw intensity data at each excitation wavelength were corrected for background prior to calculation of the ratio. During exposure to excitants, a ratio was acquired every 2 s; much lower rates (e.g. one ratio every 30 s) were employed between excitant application to minimize photobleaching and ultraviolet-mediated cytotoxicity. The *in situ* calibration method was used to convert fluorescence ratios into $[\text{Ca}^{2+}]_i$. During exposure to 10 μM Br-A23187, calibration parameters (R_{max} , R_{min} and β) were obtained in the presence (2 mM) and absence (nominally zero- Ca^{2+} solution in the presence of 100 μM ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)) of Ca^{2+} . The published K_d value for Fura-2 of 135 nM (at 20°C) was employed (Grynkiewicz *et al.*, 1985).

Statistical results are reported as mean $\pm$ s.e.mean, where n refers to the total number of neurones from which observations were made at each concentration of DTG. The IC_{50} value for DTG (the concentration of DTG resulting in a 50% inhibition of the control NMDA response) was derived by use of the same logistic equation and fitting routine as in the electrophysiological studies (see below). For each concentration of DTG tested, at least three different neuronal cultures were employed. No differences were noted with respect to the extent of the reduction of natural quisqualate-evoked responses by 50 μM DTG when either 40 or 80 μM quisqualate was employed as the excitant and these data have therefore been pooled.

Electrophysiological studies

Dissociated mouse hippocampal neurones grown in culture were used for whole cell recordings with conventional voltage-clamp techniques. The method of culture preparation has been described in detail previously (MacDonald *et al.*, 1989). In brief, hippocampi were dissected from 18-day-old foetal Swiss White mice, mechanically dissociated and neurones plated at densities below 1×10^3 cells cm^{-2} on collagen-coated plates. Cultures were used at 10–21 days following plating.

Dishes were thoroughly rinsed before the experiment was started with an extracellular solution containing (mM): NaCl 140, CaCl_2 1.3, KCl 5.4, HEPES 25, and glucose 33. Tetrodotoxin was added to solutions at 10 nM–1 μM and glycine at 1 or 3 μM before the pH of solution was adjusted to 7.35–7.40 with 1 M NaOH. DTG was made up as a 100 mM stock in HCl and after appropriate dilution the pH adjusted. Agonists and antagonists were superfused over an entire neurone from a multibarrelled perfusion system allowing for rapid (< 50 ms) exchange between compounds (Johnson & Ascher, 1987).

Patch electrodes were constructed from thin-walled borosilicate glass (o.d. 1.5 mm). Electrodes of 1–3 μm tip diameter were pulled with a Narashige PP88 vertical puller and were filled with a solution containing (mM): CsF 110, CsCl 10, HEPES 10 and EGTA 10, pH 7.35–7.40 (adjusted with 2 M CsOH). Occasionally an intracellular solution containing (mM): CsMeSO₃ 125, CsCl 15, HEPES 10, EGTA 5, CaCl_2 0.5, MgCl_2 3 with a final pH of 7.2 was employed. Adenosine tri-phosphate (2 mM, Mg^{2+} salt) was added routinely to retard the wash-out of amino acid-evoked currents (MacDonald *et al.*, 1989). Recordings were performed at room temperature (20–24°C). The membrane of the neurone was maintained at -60 mV, unless otherwise indicated, by use of a patch clamp amplifier (Axopatch 1B, Axon Instruments Inc.).

Responses evoked on amino acid application were recorded and averaged with pClamp acquisition and analysis software (Axon Instruments Inc.). Drug effect was expressed as the percentage reduction of the control agonist response at steady state (unless otherwise indicated). Averaged data points were fitted to a standard logistic equation using Sigma Plot (Jandel Scientific) to compute the IC_{50} (the concentration of DTG resulting in a 50% inhibition of the control NMDA response) or the EC_{50} (the concentration of NMDA required for 50% maximum response (R_{max})) where appropriate. Final values indicate mean $\pm$ s.e.mean and n the number of neurones tested.

Source of compounds

All chemicals were obtained from Sigma Chemical Co., with the exceptions of, in the Fura-2 study, AMPA, synthetic (+)-quisqualate and DTG (Research Biochemicals Inc.) and, in the electrophysiological study, DTG (Research Biochemicals Inc.) and AMPA and a second sample of naturally-derived quisqualate (Tocris Neuramin).

Results

Fluorescent dye studies

DTG (10–100 μM) produced a concentration-dependent reduction of rises in $[\text{Ca}^{2+}]_i$ evoked by 20 μM NMDA (Table 1, Figure 1). The IC_{50} value for DTG against NMDA was 33.5 ± 3.3 μM , a value similar to that obtained in the electrophysiological studies (see below). Varying the NMDA concentration from 5 to 20 μM did not alter the degree of inhibition produced by either 50 or 100 μM DTG (not shown). Responses to kainate and AMPA were, in general, attenuated slightly by 50 and 100 μM DTG (Table 1, Figure

Table 1 The effects of 1,3-di(2-tolyl)guanidine (DTG), at the concentrations indicated, on high-[K⁺]_o-, N-methyl-D-aspartate (NMDA)-, kainate-, natural quisqualate- and (RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-evoked increases in [Ca²⁺]_i

DTG concentration (μM)	K ⁺	NMDA	% reduction in responses to		
			Kainate	Quisqualate	AMPA
10	2 ± 2 n = 45	17 ± 2 n = 45	NT	NT	NT
20	7 ± 2 n = 67	35 ± 3 n = 67	NT	NT	NT
50	12 ± 2 n = 19	62 ± 3 n = 69	8 ± 2 n = 19	14 ± 3 n = 26	9 ± 3 n = 45
100	43 ± 1 n = 75	85 ± 2 n = 92	10 ± 1 n = 38	28 ± 4 n = 27	11 ± 4 n = 51

Data shown are % reductions (mean ± s.e.mean) compared with control values. *n* = total number of neurones from which observations were made at each concentration of DTG. NT: not tested. Neurones in which DTG produced an increase in kainate- or AMPA-evoked responses (see text) are not included.

2) observations which may reflect, at least in part, the ability of these concentrations of DTG to reduce rises in [Ca²⁺]_i evoked by high-[K⁺]_o medium (Table 1, Figure 1 and 2). In the neurones employed in these experiments, high-[K⁺]_o-, kainate- and AMPA-mediated responses probably reflect Ca²⁺ influx through voltage-gated Ca²⁺ channels, since they are abolished in the absence of extracellular Ca²⁺ (J.C. and K.A.-H., unpublished observations). The ability of DTG at high concentrations to attenuate Ca²⁺ influx through voltage-gated Ca²⁺ channels is shared by other dextromethorphan/σ receptor ligands (Church *et al.*, 1991). The possibility that the reduction of NMDA-evoked responses by DTG involved an interaction at the glycine binding site on the NMDA receptor-channel complex was investigated in 11 neurones. The addition of 20 μM glycine to the standard, nominally glycine-free, perfusion medium enhanced NMDA-evoked rises in [Ca²⁺]_i by 11 ± 3% but did not significantly (Student's *t* test, *P* < 0.05) alter the degree of inhibition associated with the application of 50 μM DTG (60 ± 4% and 55 ± 6% reductions respectively in the absence and presence of 20 μM glycine).

In contrast to the relative lack of antagonism of AMPA-evoked rises in [Ca²⁺]_i, 50 and 100 μM DTG consistently reduced naturally-derived quisqualate-evoked responses. The

Figure 1 DTG blocks NMDA-evoked increases in [Ca²⁺]_i. The effects of 20, 100 and 10 μM DTG, applied for the periods indicated by the bars above the trace, on high-[K⁺]_o-(K⁺) and NMDA-(N) evoked increases in [Ca²⁺]_i. The record is a mean of measurements obtained from 11 neurones simultaneously. DTG produced a concentration-dependent reduction in NMDA-evoked responses and reduced high-[K⁺]_o-evoked responses at 100 μM. For abbreviations in this and subsequent figure legends, see text.

Figure 2 The effect of 100 μM DTG, applied for the period indicated by the bar above the trace, on high-[K⁺]_o-(K⁺), NMDA-(N), kainate-(K) and AMPA-(A) evoked rises in [Ca²⁺]_i. DTG reduced responses evoked by NMDA to a greater extent than those evoked by high-[K⁺]_o, whereas kainate- and AMPA-evoked responses were little affected. The record is a mean of data obtained from 24 neurones simultaneously.

possible reasons for this finding were examined in greater detail in the electrophysiological studies (see below). However, it is noteworthy that the overall percentage reduction of the response to natural quisqualate (Sigma source; Table 1, Figure 3) was greater than that of the synthetic, glutamate-free (+)-quisqualate (14 ± 3% reduction with 100 μM DTG, *n* = 17). Interestingly, in a subset of the total population of neurones examined, 50 and 100 μM DTG enhanced responses to kainate and/or AMPA, but not to natural or synthetic quisqualate. Thus, 50 μM DTG increased kainate-evoked responses in 2 out of 21 neurones (by 8 and 10%) and AMPA-evoked responses in 6 out of 51 neurones (by 16 ± 6%; range 5–46%) whilst 100 μM DTG increased kainate-evoked responses in 2 out of 40 neurones (by 5 and 15%) and AMPA-evoked responses in 9 out of 60 neurones (by 20 ± 7%; range 6–45%). However, statistical analysis by χ^2 goodness of fit of the potentiated AMPA responses during exposure to DTG failed to demonstrate deviations from the normal distribution and therefore the possibility of the existence of two different neuronal populations could not be substantiated. An example of an enhancement of AMPA-evoked responses, in the presence of a decrease in NMDA- and natural quisqualate-evoked responses, by 100 μM DTG is shown in Figure 3.

Low (nM) concentrations of DTG have been reported to

Figure 3 DTG occasionally enhanced AMPA-evoked rises in $[Ca^{2+}]_i$. The record shows the mean response of the 4 neurones, out of a total of 17 recorded simultaneously, which displayed an augmented rise in $[Ca^{2+}]_i$ in response to AMPA (A) in the presence of 100 μM DTG (applied for the period indicated by the bar above the trace). DTG did not affect AMPA-evoked responses in the remaining 13 neurones, but all 17 neurones displayed a reduction in NMDA-(N) evoked rises in $[Ca^{2+}]_i$. Note also that 100 μM DTG reduced the natural quisqualate-(Q) evoked responses to a greater extent than the synthetic, glutamate-free (+)-quisqualate-(+)Q evoked responses.

potentiate NMDA-evoked responses (Monnet *et al.*, 1990; 1992). The effects of DTG applied at 0.5, 1, 5, 10, 20, and 50 nM were investigated on NMDA-evoked rises in $[Ca^{2+}]_i$ ($n > 15$ at each concentration of DTG tested); in no case was a potentiation of the response observed. At 10, 20 and 50 nM, DTG also failed to affect rises in $[Ca^{2+}]_i$ evoked on application of high- $[K^+]_o$, kainate or AMPA ($n > 6$ at each concentration of DTG tested).

Electrophysiological studies

Selectivity of the DTG block Submaximal, inward currents were evoked in hippocampal neurones by 3 s applications of 60 μM NMDA, 60 μM naturally-derived quisqualate, 10 μM AMPA and 60 μM kainate. Responses to 60 μM NMDA were dose-dependently reduced by DTG. The percentage reduction at each concentration of DTG was estimated only when NMDA responses had reached a stable lower value in the continued presence of DTG, often requiring up to 10 consecutive applications of NMDA (see below). Superfusion with 50 μM DTG depressed both peak and steady-state NMDA-evoked current by $60 \pm 4\%$ ($n = 8$) but produced only small reductions of control kainate- or steady-state AMPA-evoked responses (respectively $6 \pm 2\%$, $n = 10$ and $9 \pm 2\%$, $n = 6$). At 300 μM , DTG attenuated the NMDA response by $92 \pm 2\%$ ($n = 8$) but with no additional reduction of kainate- or AMPA-evoked responses (respectively $5 \pm 1\%$, $n = 11$ and $9 \pm 2\%$, $n = 11$). Due to the limitations of the perfusion system the rapid peak of the AMPA-evoked responses may be underestimated but there was no apparent inhibition of this particular component on superfusion with 300 μM DTG. The IC_{50} value for DTG against NMDA was $36.6 \pm 5.4 \mu M$ ($n = 10$) and the slope of the plot was not significantly different from 1 (Student's *t* test, $P < 0.05$, Figure 4a). An example of the activity of DTG can be readily observed in Figure 4b (using a different protocol from that described above) where DTG was applied during prolonged agonist application to highlight the selectivity and reversibility of the action of DTG on NMDA-evoked events.

Figure 4 Selectivity of DTG for NMDA-evoked currents. (a) Concentration-inhibition plot for DTG as an NMDA antagonist. NMDA applications (3 s, 60 μM) were repeated in the presence of DTG until steady state block achieved. Data points indicate mean $\pm$ s.e.mean and are fitted using a logistic equation. Averaged IC_{50} value for DTG against NMDA was $36.6 \pm 5.4 \mu M$ with a slope of 1.1 ± 0.2 ($n = 10$). Holding potential of neurone was -60 mV. Ordinate scale: % inhibition of control response to NMDA. Abscissa scale: DTG concentration in μM . (b) The action of 50 μM DTG (left) and 300 μM DTG (right) on amino acid-evoked currents (applied for period indicated by thin bar below trace) in a single hippocampal neurone. DTG (solid bar) produced a reversible depression of NMDA-evoked currents (53 and 93% at DTG 50 and 300 μM , respectively). Responses to AMPA and kainate were reduced by $< 10\%$ at either concentration. Calibration: current amplitude in pA; time in s.

DTG (100 and 300 μM) attenuated the steady-state, but not the peak current, evoked by naturally-derived quisqualate (Figure 5a). At 100 μM , DTG produced a $24 \pm 4\%$ reduction of natural quisqualate responses (range 10–50%; $n = 6$), whilst at 300 μM DTG the inhibition increased to $57 \pm 10\%$ (range 35–82%; $n = 4$). The degree of inhibition was not dependent on the source of natural quisqualate (Sigma or Tocris) although older stock (stored for more than 12 months at $< 4^\circ C$) had a greater DTG-sensitive component (at 100 μM DTG, $74 \pm 4\%$ reduction; $n = 4$). Superfusion with 10–20 μM DL-2-amino-5-phosphonovalerate (DL-AP5), or 1 mM Mg^{2+} , produced a similar reduction of natural quisqualate responses (for DL-AP5 a $47 \pm 5\%$ reduction

Figure 5 DTG reversibly depressed natural quisqualate-evoked responses. (a) Application of 60 μM natural quisqualate for 2 s evoked a rapidly desensitizing response with a more prolonged plateau. Superfusion with 50 μM DTG (top trace *) or 20 μM DL-AP5 (bottom trace **) depressed the plateau response leaving the rapid peak intact. This effect was fully reversible (wash superimposed on control). (b) In the same neurones as (a), DTG (thick bar) was applied for 2 s during the steady-state phase of the quisqualate response (6 s application, thin bar). The block was examined at a series of holding potentials (V(h)) from -60 mV to +60 mV (% inhibition indicated in brackets). (c) On a second neurone, a response to 10 μM AMPA (thin bar) was unaffected by co-application of 50 μM DTG (thick bar). Increasing the holding potential from -60 mV to -90 mV did not reveal an inhibition by DTG. Calibration: current amplitude in pA; time in s.

$n = 3$; see Figure 5a) confirming the contribution made by NMDA receptor activation to the natural quisqualate-evoked current. Furthermore, in the presence of 2 mM Mg^{2+} there was no additional reduction of natural quisqualate responses on co-application of 50 or 100 μM DTG ($n = 3$). The reduction of natural quisqualate-evoked currents by DTG appeared to be voltage-dependent (Figure 5b) whilst that of DL-AP5 was not (data not shown).

As hyperpolarization enhanced the DTG inhibition of quisqualate responses (Figure 5b) a similar protocol was applied to reassess the activity of DTG on AMPA-evoked responses. At neither -60 mV nor -90 mV did DTG produce an appreciable inhibition of AMPA-evoked responses (Figure 5c), confirming that DTG has low intrinsic activity at the AMPA receptor. In agreement with the fluorescent dye studies, DTG potentiated AMPA-evoked currents in 4 out of 10 neurones tested (range 5–43%), but on no occasion was there potentiation of kainate- or natural quisqualate-evoked currents.

DTG (50 μM) was applied in the presence of haloperidol; on the 3 cells tested 2 μM haloperidol did not reverse the inhibitory action of DTG on NMDA-evoked currents. In addition, DTG applied at concentrations between 0.01 μM and 100 nM, did not potentiate NMDA responses ($n = 8$).

DTG block of NMDA responses was not competitive As DTG has been reported to have non-selective actions at concentrations >100 μM (Malouf *et al.*, 1988) a concentra-

tion of DTG of 50 μM was used in the remaining studies. The latter concentration of DTG produced a $60 \pm 5\%$ ($n = 10$) reduction of the maximal response (R_m) evoked on NMDA application with no change in the estimated EC_{50} values for NMDA (respectively 30 ± 6 and $35 \pm 5 \mu\text{M}$ in the absence and presence of DTG, $n = 11$, Figure 6a). The attenuation of NMDA responses by 50 μM DTG was not dependent on agonist concentration; there was no significant difference (Student's *t* test, $P < 0.05$) in the effect of DTG on responses evoked by 10, 60, 100 or 300 μM NMDA (Figure 6b, top chart).

Increasing the concentration of glycine in the medium from nominally glycine-free to 10 μM glycine increased the amplitude of the NMDA-evoked currents but did not alter the percentage reduction of the control responses by DTG (Figure 6b, middle chart). In the presence of 3 μM glycine, co-application of the polyamine spermine (at 100 μM) produced an additional enhancement of the NMDA-evoked responses (between 10 and 100%, $n = 9$). DTG attenuated both control and polyamine-enhanced currents to a similar degree (Figure 6b, bottom chart) and dose-inhibition plots for DTG were not shifted in the presence of 100 μM spermine ($n = 2$; data not shown).

Voltage-dependent block of NMDA-evoked responses The voltage-dependence of the action of DTG was investigated on 14 neurones. Increasing depolarization of the membrane reduced the percentage inhibition of NMDA-induced cur-

Figure 6 DTG does not interact with agonist-recognition, glycine or polyamine sites. (a) Concentration-response plots for NMDA were constructed in the presence and absence of 50 μM DTG. Responses were normalized to the EC₅₀ value for NMDA for each neurone in control solution and data points indicate mean $\pm$ s.e.mean ($n = 11$). Control: EC₅₀ = 30 ± 6 μM; DTG 50 μM: EC₅₀ = 35 ± 5 μM. Ordinate scale: response normalized to the EC₅₀ value for NMDA in each neurone. Abscissa scale: NMDA concentration in μM (b) percentage reduction of control response to 60 μM NMDA by 50 μM DTG in the presence of increasing concentrations of NMDA (upper), glycine (middle) and spermine (lower). Under none of the conditions were % inhibitions significantly different from control values (Student's t test, $P < 0.05$). Bars indicate s.e.mean. Number of neurones tested indicated in parentheses above error bars.

rents produced by DTG, whilst hyperpolarization increased the inhibition. An example of the voltage-dependency of the block of NMDA-, but not kainate-, evoked currents by DTG can be seen in Figure 7a and b.

Further analysis of the voltage-dependency of the DTG block was carried out using the Woodhull model [1] (Woodhull, 1973) by which one can estimate the fraction (δ) of the transmembrane potential (V) experienced by a channel-blocking ion/molecule on the assumption, made here, that the ion/molecule does not interact with other ions either in the solution or bound to the channel. The equation derived from the model [1] is

$$[1] \quad \frac{I}{I_{dtg}} = 1 + \left(\frac{[dtg]}{Kd(0)} \right) \exp \left(\frac{-zFV\delta}{RT} \right)$$

Figure 7 Voltage-dependence of the NMDA block induced by 50 μM DTG. (a) On the left, a 6 s application of DTG (thick bar) reduced steady-state current evoked by NMDA (applied for period indicated by thin bar); those induced by kainate were not affected (right). As the holding potential of the neurone (V_h) was changed from -60 mV to +60 mV, a reduction in the % inhibition (indicated in parentheses) was observed. Calibration: current amplitude in pA; time in s. (b) On a second neurone, superfusion with 50 μM DTG (○) reduced control NMDA-evoked currents (●) in a voltage-dependent manner. The membrane potential was altered in 5 mV increments and allowed to stabilize before NMDA (60 μM) application. Action of DTG was fully reversed on wash in control solution (□). Ordinate scale: current amplitude in pA. Abscissa scale: membrane holding potential in mV.

Where I/I_{dtg} is the ratio of currents in the absence and presence of the antagonist DTG, $Kd(0)$ is the dissociation constant of the antagonist for the binding site at zero transmembrane potential, and R , F and T have their usual values. The equation can be re-arranged and natural logarithms taken to give the linear equation

$$[2] \quad \ln \left(\frac{I}{I_{dtg} - 1} \right) = \ln \left(\frac{[dtg]}{Kd(0)} \right) - \left(\frac{z\delta F}{RT} \right) \cdot V$$

Plotting $\ln(I/I_{\text{dtg}}-1)$ vs V gave a straight line for data points which when fitted by least squares gave values for δ and $K_d(0)$ of 0.76 ± 0.08 ($n = 7$) and $163 \pm 41 \mu\text{M}$, respectively. From the latter value the dissociation constant $K_d(-60 \text{ mV})$, the dissociation constant at a transmembrane potential of -60 mV , was calculated to be $27 \pm 4 \mu\text{M}$ which compares well with the IC_{50} value of $37 \pm 5 \mu\text{M}$ obtained from the dose-inhibition plot for DTG at the same holding potential.

Site of action of DTG

A series of combination experiments were carried out with DTG, Mg^{2+} and MK-801 to determine whether the binding sites for these compounds overlap. Superfusion with $300 \mu\text{M}$ DTG or 10 mM Mg^{2+} resulted in almost complete block of the NMDA-evoked response (Figure 8a and b) with immediate recovery of block by magnesium and slightly slower (within 2 to 3 NMDA applications) recovery from DTG block. By contrast prolonged ($\sim 15 \text{ s}$) applications of $3 \mu\text{M}$ MK-801 were required to block NMDA-evoked responses fully and these were not reversed appreciably within the first 5 min of wash (Figure 8c). Both Mg^{2+} and DTG, when applied prior to and during application of MK-801, prevented the development of the long duration block char-

acteristic of MK-801, suggesting that they both prevent MK-801 from binding within the ion channel.

Use-dependent block of NMDA-evoked responses

Superfusion with DTG resulted in a large reduction of the first NMDA-evoked response in a manner similar to that of DL-AP5 and Mg^{2+} . The block by DTG, but not that of DL-AP5 and Mg^{2+} was, however, further enhanced on subsequent NMDA applications. Approximately $66 \pm 3\%$ ($n = 9$) of the total inhibition was achieved in the presence of DTG on the first NMDA application, implying that the remaining 34% of the block was use-dependent.

The biphasic block of NMDA responses by DTG was further confirmed on estimating the kinetics of the onset and offset of the block with a protocol similar to that in Figure 4b. Onset of the block by DTG could be fitted by two time constants, a fast component of $0.18 \pm 0.02 \text{ s}^{-1}$ ($n = 6$) followed by a slower component of $5.8 \pm 0.7 \text{ s}^{-1}$ ($n = 5$). The recovery from block by DTG was equally well-fitted by two time constants of 0.50 ± 0.07 and $5.55 \pm 1.19 \text{ s}^{-1}$. The fast component contributed to approximately 76% of the final block, in close agreement with the percentage block (66%) of the first NMDA response in the experiments described above.

Figure 8 DTG and Mg^{2+} protect against the prolonged block induced by MK-801. The control response to $60 \mu\text{M}$ NMDA (5 s application, thin bar) was profoundly attenuated by superfusion with (a) 10 mM Mg^{2+} and (b) $300 \mu\text{M}$ DTG, as indicated by solid bars above traces. MK-801 ($3 \mu\text{M}$) was then co-applied (hatched bar below traces) in the continued presence of (a) Mg^{2+} or (b) DTG, and NMDA then applied for 12 s. Both antagonists were then washed out for 5 min before NMDA was reapplied (far right). Full recovery from block by Mg^{2+} was achieved on the first application of NMDA (a, trace far right) whilst that from block with DTG required one further NMDA application (b, last 2 traces far right taken at 40 s intervals). In the bottom trace (c) application of MK-801 alone produced a slowly developing block during a 12 s application of NMDA that showed no sign of reversing even with prolonged wash (right). Ordinate scale: 200 pA; Abscissa scale: time in s.

Discussion

DTG is one of the preferred ligands for investigating the functional roles of σ sites. In the present study, concentrations of DTG selective for the latter site (i.e. in the low nanomolar range) had no effect on excitatory amino acid-evoked currents and rises in $[Ca^{2+}]_i$ in cultured mouse and rat hippocampal neurones, respectively. However, higher (μM) concentrations of DTG selectively attenuated NMDA-evoked responses in both assays. The NMDA antagonist action of micromolar concentrations of DTG was haloperidol-insensitive, in agreement with others (Connick *et al.*, 1992), again suggesting a lack of involvement of σ receptors in the NMDA-antagonist properties of micromolar concentrations of DTG. Although high concentrations of DTG (100 μM) have been reported to have non-selective properties (Malouf *et al.*, 1988) the effects observed here cannot be attributed to non-specific effects on membrane excitability. Furthermore as the IC_{50} value for DTG as an antagonist of NMDA-evoked responses in preparations of both rat and mouse hippocampal neurones was similar, one may assume that there is no large species differences in NMDA receptor pharmacology.

The mechanism of the block of NMDA currents by DTG was not competitive but appeared to be both voltage- and partially use-, dependent in nature. The voltage-dependence of the DTG block is similar to that seen with Mg^{2+} (Nowak *et al.*, 1984) and many other compounds including ketamine (MacDonald *et al.*, 1991), MK-801 (Huettner & Bean, 1988), tricyclic antidepressants (Sernagor *et al.*, 1989), tetrahydroaminoacridine (Herschkowitz & Rogawski, 1991), arcaine (Donevan *et al.*, 1992) and strychnine (Bertolino & Vicini, 1988). Using the Woodhull model (Woodhull, 1973) it was possible to estimate the fraction of the electrostatic field (δ) experienced by DTG at its binding site. In this study δ was 0.76, close to that reported for Mg^{2+} (Ascher & Nowak, 1988). Furthermore, the similarity between the computed affinity of DTG at -60 mV in the kinetic experiments was similar to the IC_{50} value calculated from dose-inhibition plots (also carried out at a holding potential of -60 mV) suggesting that binding at the predicted site within the ion channel can largely account for the inhibitory action of DTG on NMDA-evoked responses. The voltage-dependence of DTG probably stems from a positive charge on the imine group of the molecule at physiological pH. Combination experiments were also suggestive of a Mg^{2+} -type block of NMDA-evoked currents by DTG (Huettner & Bean, 1988; MacDonald *et al.*, 1991). Furthermore, kinetic studies revealed that the rates of onset and offset of DTG block were similar, although not identical, to those of Mg^{2+} and both were significantly faster than those reported for ketamine (MacDonald *et al.*, 1991). In contrast to Mg^{2+} , however, a component of the DTG block was use-dependent, a characteristic of PCP-like ligands. This possibility is supported by binding studies in which DTG displaced [3H]-MK-801 and [3H]-TCP with IC_{50} values of 11 and 8 μM , respectively (Keana *et al.*, 1989). Nevertheless, the present studies are in agreement with those of Keana and colleagues (1989), who proposed that the rapid rate of recovery from the DTG block reflected its low affinity for the NMDA receptor-linked ion channel. Thus the slow rate of recovery from block with MK-801 or ketamine reflects a higher affinity, and thus slower dissociation, of these ligands from the NMDA receptor-channel complex (MacDonald *et al.*, 1991).

Both glycine and polyamines (e.g. spermine) potentiate NMDA receptor-mediated responses through activation of distinct allosteric sites on the NMDA receptor-channel complex (Johnson & Ascher, 1987; Ransom & Stec, 1988). Notably ifenprodil, a non-competitive NMDA antagonist inhibits polyamine-stimulated binding of [3H] channel ligands (see Reynolds & Miller, 1989; Beart *et al.*, 1991), and displaces [3H]-(+)-3-PPP binding (Karbon *et al.*, 1990; Benavides *et al.*, 1992) with similar nanomolar affinities, tentatively sug-

gesting an overlap between polyamine and σ binding sites. In the present experiments, DTG did not act as a polyamine or glycine site ligand as the inhibition of NMDA-evoked responses by DTG was not reversed on addition of spermine or glycine. Moreover, DTG did not potentiate NMDA-evoked responses even when an appreciable potentiation on NMDA-evoked responses by glycine and/or spermine could be achieved.

In the absence of an appreciable depression of AMPA-evoked responses by DTG it was surprising that DTG attenuated those evoked by natural quisqualate. As the NMDA receptor antagonists, Mg^{2+} and DL-AP5, produced a similar reduction of quisqualate responses and occluded quisqualate inhibition by DTG, it is likely that natural quisqualate responses are mediated, at least in part, through activation of the NMDA subtype of glutamate receptor. The latter might result from contamination of natural quisqualate with a potent NMDA receptor agonist, most likely to be glutamate and aspartate (Cha *et al.*, 1989), as suggested by our studies on synthetic, glutamate-free (+)-quisqualate. Alternatively, quisqualate may be a partial agonist at the NMDA receptor, a property that AMPA does not, from our studies, appear to possess. In this regard it is noteworthy that quisqualate displaces [3H]-D-AP5 binding from CNS membranes with micromolar affinity (Olverman *et al.*, 1988) and has been reported to evoke responses in *Xenopus* oocytes exclusively expressing an NMDA receptor clone (Moriyoshi *et al.*, 1991). We made no attempt to quantify rigorously the contribution made by each of these factors but our results emphasize once again that quisqualate is not a good choice of ligand for studying drug selectivity at subtypes of glutamate receptor. The reported lack of selectivity of DTG between NMDA- and quisqualate-evoked responses (Connick *et al.*, 1992) may reflect a lack of selectivity of the agonist used rather than that of the antagonist.

Some σ receptor ligands, applied at low concentrations, have been reported to potentiate NMDA receptor-mediated responses in both the hippocampus (Malouf *et al.*, 1988; Monnet *et al.*, 1990; 1992) and elsewhere in the CNS (Iyengar *et al.*, 1990; Rao *et al.*, 1991). We did not observe potentiation of NMDA-evoked responses by nanomolar concentrations of DTG (or (+)-3-PPP; E.J.F., unpublished observations). Although this finding may reflect the fact that our cultured neurones (predominantly derived from the hippocampal CA1 region) do not possess σ binding sites, differences in methodologies may also underlie the discrepancies in the reported NMDA modulatory effects of σ ligands. Under non voltage-clamped conditions (e.g. extracellular recordings *in vivo* or *in vitro* and neurochemical assays) σ ligands may indirectly modulate receptor function through the activation, or inhibition, of other ionic currents. DTG has been reported to block a ligand-gated K^+ current (Bobker *et al.*, 1989) and can reduce ^{86}Rb efflux through K^+ channels (Fletcher *et al.*, 1989). The occasional enhancement of AMPA- and kainate-evoked responses by micromolar concentrations of DTG observed in the present experiments may reflect the interaction of DTG with other ion channels located on, presumably, only a small percentage of our cultured neurones.

In conclusion, we have shown that DTG, at low micromolar concentrations, selectively blocks NMDA receptor-mediated responses in both rat and mouse cultured hippocampal pyramidal neurones. The block of NMDA responses by DTG is not competitive in nature and appears to be both voltage- and use-dependent suggesting that the action of DTG may have characteristics of both Mg^{2+} and PCP-like compounds. The results indicate, however, that DTG, at micromolar concentrations, cannot be employed as a selective probe for investigating the physiological, biochemical, pharmacological or behavioural characteristics of σ receptors. Given the appreciable activity of DTG as an NMDA antagonist, the actions of DTG at these concentrations *in vivo* and *in vitro* may reflect not only the interaction of DTG

with σ receptors but also with the NMDA receptor-channel complex. Indeed, the NMDA antagonist activity associated with micromolar concentrations of DTG and other σ ligands (e.g. caramiphen; see Church *et al.*, 1991) may account in large part for their PCP-like discriminative stimulus properties (Holtzman, 1989; Singh *et al.*, 1990), their neuroprotective properties (Keana *et al.*, 1989; Pontecorvo *et al.*, 1991; Long *et al.*, 1992; Shalaby *et al.*, 1992) and their antiepilep-

tiform activity in studies of Mg^{2+} -free bursting activity (J.C., unpublished observations).

Supported by the Medical Research Council of Canada Grants to J.C. and J.F.M. ('Nerve, Cell and Synapse' Group award) and National Centres of Excellence Grant to J.F.M. E.J.F. is a Medical Research Council of Canada Fellow and J.C. is a B.C. Health Research Foundation Scholar.

References

- ARAM, J.A., MARTIN, D., TOMCZYK, M., ZEMAN, S., MILLAR, J., POHLER, G. & LODGE, D. (1988). Neocortical epileptogenesis *in vitro*: studies with N-methyl-D-aspartate, phencyclidine, sigma and dextromethorphan receptor ligands. *J. Pharmacol. Exp. Ther.*, **248**, 320–328.
- ASCHER, P. & NOWAK, L. (1988). The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurones in culture. *J. Physiol.*, **399**, 247–266.
- BANKER, G.A. & COWAN, W.M. (1977). Rat hippocampal neurons in dispersed culture. *Brain Res.*, **126**, 397–425.
- BEART, P.M., MERCER, L.D. & JARROTT, B. (1991). [125 I]ifenprodil: a convenient radioligand for binding and autoradiographic studies of the polyamine-sensitive site of the NMDA receptor. *Neurosci. Lett.*, **124**, 187–189.
- BENAVIDES, J., PENY, B., ALLEN, J. & SCATTON, B. (1992). Pharmacological characterization of *in vivo* [3 H]ifenprodil binding sites in the mouse brain. *J. Pharmacol. Exp. Ther.*, **260**, 896–901.
- BERRY, S.C., DAWKINS, S.L. & LODGE, D. (1984). Comparison of sigma- and kappa-opiate receptor ligands as excitatory amino acid antagonists. *Br. J. Pharmacol.*, **83**, 179–185.
- BERTOLINO, M. & VICINI, S. (1988). Voltage-dependent block by strychnine of N-methyl-D-aspartate cationic channels in rat cortical neurons in culture. *Mol. Pharmacol.*, **34**, 98–103.
- BOBKER, D.H., SHEN, K.-Z., SUPRENANT, A. & WILLIAMS, J.T. (1989). DTG and (+)-3-PPP inhibit a ligand-activated hyperpolarization in mammalian neurons. *J. Pharmacol. Exp. Ther.*, **251**, 840–845.
- CHA, J.-H.J., HOLLINGSWORTH, S.R., GREENAMYRE, J.T. & YOUNG, A.B. (1989). Contamination of commercially available quisqualic acid by glutamate-like and aspartate-like substances. *J. Neurosci. Methods*, **27**, 143–148.
- CHURCH, J. & LODGE, D. (1990). Cyclazocaine and pentazocine as N-methylaspartate antagonists on cat and rat spinal neurons *in vivo*. *J. Pharmacol. Exp. Ther.*, **253**, 636–645.
- CHURCH, J., SHACKLOCK, J.A. & BAIMBRIDGE, K.G. (1991). Dextromethorphan and phencyclidine receptor ligands: differential effects on K^{+} - and NMDA-evoked increases in cytosolic free Ca^{2+} concentration. *Neurosci. Lett.*, **124**, 232–234.
- CONNICK, J.H., ADDAE, J.I., NICHOLSON, C.D. & STONE, T.W. (1992). The σ ligand 1,3-di-o-tolylguanidine depresses amino acid-induced excitation non-selectively in rat brain. *Eur. J. Pharmacol.*, **214**, 169–173.
- DONEVAN, S.D., JONES, S.M. & ROGAWSKI, M.A. (1992). Arcaine blocks N-methyl-D-aspartate receptor responses by an open channel mechanism: whole-cell and single-channel recording studies in cultured hippocampal neurons. *Mol. Pharmacol.*, **41**, 727–730.
- FLETCHER, E.J., DREW, C., LODGE, D. & O'SHAUGHNESSY, C.T. (1989). Efflux of rubidium in rat cortical synaptosomes is blocked by sigma and dextromethorphan binding site ligands. *Neuropharmacology*, **28**, 661–666.
- FRENCH, E.D. & CECI, A. (1990). Non-competitive N-methyl-D-aspartate antagonists are potent activators of ventral tegmental A10 dopamine neurons. *Neurosci. Lett.*, **199**, 159–162.
- GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{++} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.
- HERSHKOWITZ, N. & ROGAWSKI, M.A. (1991). Tetrahydroamino-acridine block of N-methyl-D-aspartate-activated cation channels in cultured hippocampal neurons. *Mol. Pharmacol.*, **39**, 592–598.
- HOLTZMAN, S.G. (1989). Opioid- and phencyclidine-like discriminative effects of ditolylguanidine, a selective sigma ligand. *J. Pharmacol. Exp. Ther.*, **248**, 1054–1062.
- HUETTNER, J.E. & BEAN, B.P. (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1307–1311.
- IYENGAR, S., MICK, S., DILWORTH, V., MICHEL, J., RAO, T.S., FARAH, J.M. & WOOD, P.L. (1990). Sigma receptors modulate the hypothalamic-pituitary-adrenal (HPA) axis centrally: evidence for a functional interaction with NMDA receptors, *in vivo*. *Neuropharmacology*, **29**, 299–303.
- JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurones. *Nature*, **325**, 522–525.
- KARBON, E.W., PATCH, R.J., PONTECORVO, M.J. & FERKANY, J.W. (1990). Ifenprodil potently interacts with [3 H](+)-3-PPP-labeled σ binding sites in guinea pig brain membranes. *Eur. J. Pharmacol.*, **176**, 247–248.
- KEANA, J.F.W., MCBURNEY, R.N., SCHERZ, M.W., FISCHER, J.B., HAMILTON, P.N., SMITH, S.M., SERVER, A.C., FINKBEINER, S., STEVENS, C.F., JAHR, C. & WEBER, E. (1989). Synthesis and characterization of a series of diarylguanidines that are non-competitive N-methyl-D-aspartate receptor antagonists with neuroprotective properties. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5631–5635.
- LARGENT, B.L., GUNDLACH, A.L. & SNYDER, S.H. (1984). Psychotomimetic opiate receptors labeled and visualized with (+)-[3 H]-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 4983–4987.
- LARGENT, B.L., GUNDLACH, A.L. & SNYDER, S.H. (1986). Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[3 H]-SKF 10,047, (+)-[3 H]-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine and [3 H]-1-[1-(2-thienyl)cyclohexyl]piperidine. *J. Pharmacol. Exp. Ther.*, **238**, 739–748.
- LONG, J.B., GASPARI, F.J., DECOSTER, M.A. & DE COSTA, B.R. (1992). Sigma ligands protect cultured rat spinal cord neurons from NMDA-induced toxicity. *Soc. Neurosci. Abstr.*, **18**, 482.10.
- MACDONALD, J.F., MODY, I. & SALTER, M.W. (1989). Regulation of N-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurones in culture. *J. Physiol.*, **414**, 17–34.
- MACDONALD, J.F., BARTLETT, M.C., MODY, I., PAHAPHILL, P., REYNOLDS, J.N., SALTER, M.W., SCHNEIDERMAN, J.H. & PENNEFATHER, P.S. (1991). Actions of ketamine, phencyclidine and MK-801 and NMDA receptor currents in mouse hippocampal neurones. *J. Physiol.*, **432**, 483–508.
- MALOUF, A.T., SWEARENGEN, E. & CHAVKIN, C. (1988). Comparison of the actions of phencyclidine and sigma ligands on CA1 hippocampal pyramidal neurons in the rat. *Neuropharmacology*, **27**, 1161–1170.
- MARTIN, W.R., EADES, C.G., THOMPSON, J.A., HUPPLER, R.E. & GILBERT, P.E. (1976). The effects of morphine- and nalorphine-like drugs in the non-dependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.*, **197**, 517–532.
- MONNET, F.P., DEBONNEL, G. & DEMONTIGNY, C. (1992). *In vivo* electrophysiological evidence for a selective modulation of N-methyl-D-aspartate-induced neuronal activation in rat CA3 dorsal hippocampus by sigma ligands. *J. Pharmacol. Exp. Ther.*, **261**, 123–130.
- MONNET, F.P., DEBONNEL, G., JUNIEN, J.-L. & DEMONTIGNY, C. (1990). N-methyl-D-aspartate-induced neuronal activation is selectively modulated by σ receptors. *Eur. J. Pharmacol.*, **179**, 441–445.
- MORIYOSHI, K., MASU, M., ISHII, T., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1991). Molecular cloning and characterization of the rat NMDA receptor. *Nature*, **354**, 31–37.
- NOWAK, L., BREGESTOVSKI, P., ASCHER, P., HERBERT, A. & PROCHIANTZ, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, **307**, 462–465.
- OLVERMAN, H.J., JONES, A.W. & WATKINS, J.C. (1988). [3 H]D-2-Amino-5-phosphonopentanoate as a ligand for N-methyl-D-aspartate receptors in the mammalian central nervous system. *Neuroscience*, **26**, 1–15.

- PONTECORVO, M.J., KARBON, E.W., GOODE, S., CLISSOLD, D.B., BOROSKY, S.A., PATCH, R.J. & FERKANY, J.W. (1991). Possible cerebroprotective and *in vivo* NMDA antagonist activities of sigma agents. *Brain Res. Bull.*, **26**, 461–465.
- QUIRION, R., BOWEN, W.D., ITZHAK, Y., JUNIEN, J.L., MUSACCHIO, J.M., ROTHMAN, R.B., SU, T.-P., TAM, S.W. & TAYLOR, D.P. (1992). A proposal for the classification of sigma binding sites. *Trends Pharmacol. Sci.*, **13**, 85–86.
- QUIRION, R., CHICHEPORTICHE, R., CONTRERAS, P.C., JOHNSON, K.M., LODGE, D., TAM, S.W., WOODS, J.H. & ZUKIN, S.R. (1987). Classification and nomenclature of phencyclidine and sigma receptor sites. *Trends Neurosci.*, **10**, 444–446.
- RANSOM, R.W. & STEC, N.L. (1988). Cooperative modulation of [³H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. *J. Neurochem.*, **51**, 830–836.
- RAO, T.S., MICK, S.J., CLER, J.A., EMMET, M.R., DILWORTH, V.M., CONTRERAS, P.C., GRAY, N.M., WOOD, P.L. & IYENGAR, S. (1991). Effects of sigma ligands on mouse cerebellar cyclic guanosine monophosphate (cGMP) levels *in vivo*: further evidence for a functional modulation of N-methyl-D-aspartate (NMDA) receptor complex-mediated events by sigma ligands. *Brain Res.*, **561**, 43–50.
- REYNOLDS, I.J. & MILLER, R.J. (1989). Ifenprodil is a novel type of N-methyl-D-aspartate receptor antagonist: interaction with polyamines. *Mol. Pharmacol.*, **36**, 758–765.
- SERNAGOR, E., KUHN, D., VYKLYCKY, L. & MAYER, M.L. (1989). Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron*, **2**, 1221–1227.
- SHALABY, I.A., CHENARD, B.L., PROCHNIAK, B.L. & BUTLER, T.W. (1992). Neuroprotective effects of the N-methyl-D-aspartate receptor antagonists ifenprodil and SL-82,0715 on hippocampal cells in culture. *J. Pharmacol. Exp. Ther.*, **260**, 925–932.
- SINGH, L., WONG, E.H.F., KESINGLAND, A.C. & TRICKLEBANK, M.D. (1990). Evidence against an involvement of the haloperidol-sensitive σ recognition site in the discriminative stimulus properties of (+)-N-allylnormetazocine ((+)-SKF 10,047). *Br. J. Pharmacol.*, **99**, 145–151.
- SIRCAR, R., NICHTENHAUSER, R., IENI, J.R. & ZUKIN, S.R. (1986). Characterization and autoradiographic visualization of (+)-SKF10,047 binding in rat and mouse brain: further evidence for phencyclidine 'sigma opiate' receptor commonality. *J. Pharmacol. Exp. Ther.*, **237**, 681–688.
- VIGNON, J., CHICHEPORTICHE, R., CHICHEPORTICHE, M., KAMENKA, J.-M., GENESTE, P. & LAZDUNSKI, M. (1983). [³H]TCP: a new tool with high affinity for the PCP receptor in rat brain. *Brain Res.*, **81**, 531–542.
- WEBER, E., SONDERS, M., QUARUM, M., MCLEAN, S., POU, S. & KEANA, J.F.W. (1986). 1,3-Di(2-[5-³H]tolyl)guanidine: a selective ligand that labels σ -type receptors for psychotomimetic opiates and antipsychotic drugs. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8784–8788.
- WONG, E.H.F., KEMP, J.A., PRIESTLEY, T., KNIGHT, A.R., WOODRUFF, G.N. & IVERSEN, L.L. (1986). The novel anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7104–7107.
- WOODHULL, A.M. (1973). Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.*, **61**, 687–708.
- ZUKIN, R.S. & ZUKIN, S.R. (1981). Multiple opiate receptors: emerging concepts. *Life Sci.*, **29**, 2681–2690.

(Received January 8, 1993

Revised March 23, 1993

Accepted April 2, 1993)