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Investigation of the involvement of the N-methyl-D-aspartate receptor macrocomplex in the development of spermine-induced CNS excitation *in vivo*

[1]K.M. Doyle & [2]G.G. Shaw

Department of Pharmacology, School of Pharmacy, Trinity College, 18 Shrewsbury Road, Dublin 4, Ireland

1 The involvement of the N-methyl-D-asparate (NMDA) receptor macrocomplex in the development of spermine-induced CNS excitation *in vivo* was investigated.

2 Injection of 100 μg of spermine into the left lateral cerebral ventricle of female *Laca* mice (20–25 g) resulted in the development of two distinct phases of CNS excitatory effects which were quantified by a scoring system.

3 The first phase effects occurred within minutes of injection and generally lasted for about 1 h. Most mice showed scratching of the upper body, frequent face washing and some mice developed clonic convulsions. By about 2 h after injection, the second phase of effects began to develop in the form of body tremor which worsened with time and culminated in fatal tonic convulsions, generally within 8 h of injection.

4 Pretreatment of the mice with dizocilpine (0.3 mg kg^{-1}, i.p.) resulted in antagonism of the first phase of spermine-induced effects, but a higher dose (0.3 mg kg^{-1}, (×2), i.p.) was necessary to inhibit the second phase effects.

5 Whereas the glutamate antagonist, 3-((**R**)-2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (D-CPP) (10, 20 mg kg^{-1}, i.p.), the glycine antagonist 7-chlorokynurenate (10, 30, 50 nmol, i.c.v.), or the polyamine antagonist ifenprodil (30, 60 mg kg^{-1}, i.p.) antagonized the first phase of effects produced by spermine, these agents given as monotherapy, were ineffective against the development of the second phase of effects.

6 Co-administration of ifenprodil with either D-CPP or 7-chlorokynurenate resulted in a dose-dependent antagonism of the development of the second phase of spermine-induced effects.

7 It is concluded that the development of the two temporally distinct phases of spermine-induced effects may be mediated by pharmacologically distinct mechanisms, although the results suggest that the NMDA receptor macrocomplex may be involved in both phases of effects. Furthermore, a moderate dose of D-CPP or 7-chlorokynurenate appears to enhance the inhibitory potential of ifenprodil *in vivo*.

Keywords: Spermine; polyamine; CNS excitation; convulsion; N-methyl-D-asparate receptor; ifenprodil; dizocilpine; D-CPP; 7-chlorokynurenate

Introduction

Injection of spermine (100 μg) directly into the cerebral ventricles of female *Laca* mice leads to the development of CNS excitation and convulsions (Anderson *et al.*, 1975). The mechanism underlying these effects is not known. Recently, a specific modulatory binding site for the polyamines has been found on the NMDA receptor macrocomplex (Ransom & Stec, 1988). Agonists acting at various receptor sites on the NMDA receptor macrocomplex have been shown to induce convulsions in rodents (Turski *et al.*, 1990; Chapman & Meldrum, 1993). Moreover, competitive and non-competitive antagonists acting at the NMDA receptor macrocomplex have consistently shown good anticonvulsant activity in a wide range of experimental seizure models (Witkin & Tortella, 1991; De Sarro & De Sarro, 1992; Rogawski, 1992; Chapman & Meldrum, 1993). This evidence suggests the possibility that proconvulsive effects of spermine may be mediated through NMDA receptor macrocomplex modulation. In support of this suggestion, recent evidence has demonstrated that co-application of dizocilpine (10 μM) with spermine (250 μM) can inhibit the neuronal loss produced by spermine in primary cortical neurones (Fahey *et al.*, 1993). Activation of the NMDA receptor macrocomplex can be inhibited by various compounds which act as antagonists at specific binding sites on the complex. In the present study, the effect of the open channel blocker, dizocilpine, the glutamate receptor antagonist, D-CPP, the glycine receptor site antagonist, 7-chlorokynurenate and the non-competitive putative polyamine antagonist, ifenprodil on the development of spermine-induced CNS excitation and convulsions was investigated.

Recent evidence suggests that there are reciprocal allosteric interactions between the binding sites for glutamate, glycine and the polyamines on the NMDA receptor macrocomplex (Ransom & Stec, 1988; Sacaan & Johnson, 1989; Nussenzveig *et al.*, 1991; Carter *et al.*, 1992; Schoemaker, 1992). It has been suggested that the effects induced by polyamine agonists and antagonists may depend upon the tonicity of these three systems (Carter *et al.*, 1992). In the light of this evidence it was of interest to investigate the effect of co-administering moderate concentrations of D-CPP or 7-chlorokynurenate with ifenprodil. Preliminary accounts of these studies have been presented (Doyle & Shaw, 1992; 1994).

Methods

Female *Laca* mice (20–25 g) were obtained from the Bioresources Unit, Trinity College and were housed in groups of 4–6 under a 12 h light/dark cycle with food and water *ad libitum*.

[1]Present address: Division of Biosciences, School of Natural Sciences, University of Hertfordshire, Hatfield Campus, College Lane, Hatfield, Hertfordshire, AL10 9AB
[2]Author for correspondence.

Behavioural changes produced

Mice given 100 μg of spermine directly into the left cerebral ventricle by the method described by Brittain (1966) displayed a distinct behavioural profile of effects which could be divided into two main stages. Immediately after injection they appeared sedated and hypothermic. They also showed signs of short-lived asphyxia. Within minutes after injection, the first phase of CNS excitation effects were seen. Most mice showed scratching of the upper body and frequent face-washing. Some developed clonic convulsions, which generally occurred during bouts of frenzied face-washing and scratching and often involved a temporary loss of righting reflex. These effects could last for 1 h or more, with much individual variation. Behaviour could then be quite normal for a time but by about 2 h after injection, the second phase of spermine-induced effects began to develop in the form of body tremor which increased with time. Initially the tremor was best assessed by lifting the mouse by the tail and feeling the degree of vibration, but eventually it progressed so as to be readily observable without handling. The CNS excitation culminated in the onset of tonic convulsions, which were ultimately fatal and generally occurred within 8 h of injection.

Assessment profile

The feasibility of using the development of the first phase of hyper-excitability and the influence of drugs on this as cut-off point for our experiments was first considered. However, although some 70% of mice injected with spermine (100 μg, i.c.v.) developed scratching and face-washing behaviour, only 11% of mice developed clonic convulsions. Virtually all of the animals treated with this dose of spermine developed second phase effects including tremor and tonic convulsions. Therefore, it was felt that it was not feasible to rely only on early changes and that the second phase of effects was an important component. In view of the temporal separation of the two phases which raised the possibility of distinct pharmacological mechanisms, it was resolved to build a behavioural profile covering both phases.

The mice were studied during the first 40 min period after spermine i.c.v. injection for signs of the phase one effects associated with the administration of spermine. Observations were made 10, 20, 30 and 40 min after i.c.v. injection over 2 min periods for the occurrence of clonic convulsive episodes.

For assessment of the second phase of spermine-induced CNS excitation the mice were observed at 30 min intervals over a period of up to 7.5 h after injection. A simple behavioural profile was devised to score the development of body tremor, and subsequent tonic convulsions.

Scoring system

The scoring system was as follows: (1) slight tremor; (2) moderate tremor; (3) severe tremor; (4) tonic convulsion – survived; (5) fatal tonic convulsion. The extent of body tremor was assessed by lifting the mouse by the tail and feeling the degree of tremor. The scoring system only recognises three different grades of body tremor in order to reduce scope for assessment error.

Drugs

Spermine (Sigma, Dorset, England) was administered as a hydrochloride salt dissolved in 0.9% sterile saline in a dose volume of 20 μl. Dizocilpine (Semat (RBI), Herts, England) was dissolved in 0.9% sterile saline and administered i.p. in a dose volume of 0.1 ml 10 g^{-1}, 30 min before spermine injection. Animals treated with two doses of dizocilpine received doses 30 min before and 30 min after spermine injection. D-CPP (3-((**R**)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid) (Tocris Neuramin, Bristol, England) and 7-chlorokynurenate (Tocris Neuramin, Bristol, England) were dissolved in 0.9% sterile saline containing the minimum quantity of 1 M NaOH necessary for dissolution. D-CPP was administered i.p. in a dose volume of 0.1 ml 10 g^{-1}, 30 min before spermine injection. 7-Chlorokynurenate does not readily cross the blood brain barrier and was therefore co-administered with spermine into the left cerebral ventricle. Ifenprodil was a gift from Synthelabo, Paris, France. Ifenprodil was dissolved in 0.9% sterile saline containing the minimal quantity of Tween 80 to produce solubilization and administered i.p. in a dose volume of 0.1 ml 10 g^{-1}, 30 min before i.c.v injection of spermine.

Data analysis

The effect of NMDA antagonists on the mean number of clonic convulsions observed over the total observation time window per animal in the first phase of spermine-induced effects was assessed. Statistical significance of differences between controls and drug treated groups was calculated with a proportionality test (MultiStat, Biosoft, 1988).

The median second phase CNS excitation scores and interquartile ranges (IQR) of the spermine control group and test groups were calculated. Results were expressed in graph form as plots of median CNS excitation score versus time (h). Statistical significance of the difference between test and control subjects was calculated by the Mann-Whitney U-test. The effect of the drugs on the latency to the development of tonic convulsions in the second phase was also assessed.

Results

First phase effects

As a single dose (0.3 mg kg^{-1}, i.p.) of dizocilpine has been shown to be effective against NMDA-induced lethality (Palmer *et al.*, 1992) and produces a tolerable level of behavioural ef-

Table 1 The effect of NMDA receptor macrocomplex antagonists on the development of clonic convulsions in spermine treated mice

Drug treatment	n	*Mean number of clonic episodes per animal*
Spermine control (Spm)	35	0.80
Spm + dizocilpine 0.3 mg kg^{-1}, i.p.	10	0**
Spm + dizocilpine 0.3 mg kg^{-1}, i.p. (×2) (−30, +30)	12	1.33**
Spm + 7-chlorokynurenate 10 nmol i.c.v.	11	0**
Spm + 7-chlorokynurenate 30 nmol i.c.v.	12	0.17**
Spm + 7-chlorokynurenate 50 nmol i.c.v.	10	0.10**
Spm + D-CPP 10 mg kg^{-1}, i.p.	10	0**
Spm + D-CPP 20 mg kg^{-1}, i.p.	11	0.45*
Spm + ifenprodil 30 mg kg^{-1}, i.p.	11	0.09**
Spm + ifenprodil 60 mg kg^{-1}, i.p.	11	0**

fects, the effect of this dose against the development of spermine-induced CNS excitation and convulsions was initially assessed. Dizocilpine 0.3 mg kg^{-1}, i.p. reduced the occurrence of scratching and face-washing (data not shown) and abolished the occurrence of clonic episodes in response to spermine (Table 1).

7-Chlorokynurenate a dose of 10 nmol i.c.v. was well tolerated in mice, producing only a short-lasting sedation and slight ataxia. However, 30 or 50 nmol i.c.v. of 7-chlorokynurenate produced face-washing, head scratching and uncontrolled limb extension within the first 10 min period after injection. These effects were more pronounced with the 50 nmol dose. Despite the initial CNS behavioural effects observed after the i.c.v. injection of the higher doses of 7-chlorokynurenate, all three doses studied significantly reduced the mean number of clonic convulsion episodes per animal induced by spermine (Table 1) and the occurrence of scratching and face-washing (data not shown).

Preliminary experiments indicated that doses of D-CPP as high as 20 mg kg^{-1}, i.p. were well tolerated in mice. D-CPP (10, 20 mg kg^{-1}, i.p.) significantly reduced the mean number of clonic convulsions observed per animal (Table 1) and the occurrence of scratching and face-washing (data not shown).

Animals administered ifenprodil (30 or 60 mg kg^{-1}, i.p.) became moderately sedated for approximately 1 h after injection. Ifenprodil produced a statistically significant reduction in the mean number of clonic convulsions per animal at both doses tested (Table 1) and also the occurrence of scratching and face-washing (data not shown).

Second phase effects

In contrast to the effective inhibition of clonic convulsions produced by 0.3 mg kg^{-1}, i.p. of dizocilpine, the development of body tremor and tonic convulsions was largely unaffected by this dose of dizocilpine (data not shown).

The ataxia, hyperactivity and circling behaviour produced by a higher dose of dizocilpine (1 mg kg^{-1}, i.p.) interfered with the scoring process. Since the behavioural effects elicited by dizocilpine generally lasted for only about 1 h, the effect of administering two doses of dizocilpine (0.3 mg kg^{-1}, i.p.) 1 h apart was investigated. Whereas this dosage exacerbated the mean number of clonic convulsions observed per animal (Table 1) (which may reflect the CNS stimulatory effect of dizocilpine itself), it significantly inhibited the development of the second phase of spermine-induced effects. There was a statistically significant reduction in the median CNS excitation score

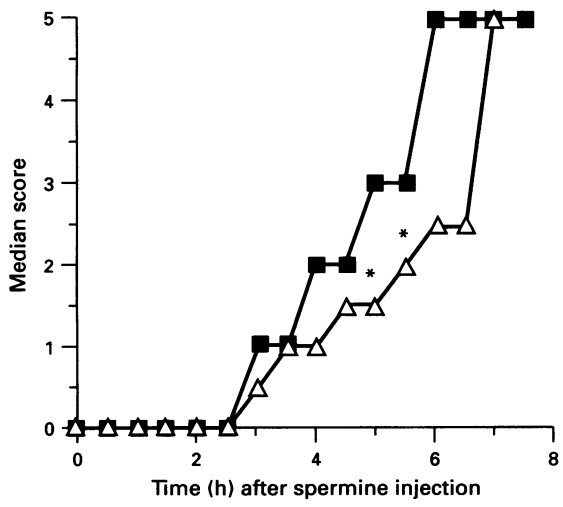

Figure 1 The effect of the administration of two doses of dizocilpine 0.3 mg kg^{-1}, i.p. 1 h apart ((△; $n=12$) on median CNS excitation score after injection of 100 μg of spermine i.c.v. (■, $n = 36$), (*$P<0.05$, Mann-Whitney, U-test).

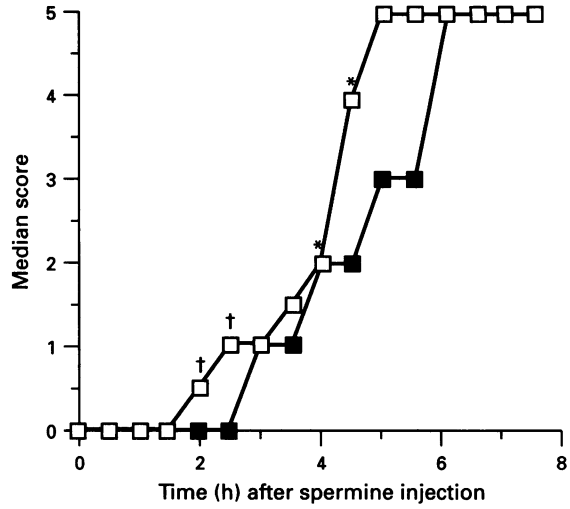

Figure 2 The effect of the administration of 7-chlorokynurenate 50 nmol, i.c.v. (□; $n=10$) on median CNS excitation score after injection of 100 μg of spermine i.c.v. (■; $n=36$), (*$P<0.05$; †$P<0.01$, Mann-Whitney U-test).

at the 5.0 and 5.5 h time points and a statistically significant delay in the median time to onset of first tonic convulsion ($P<0.05$; Figure 1).

In contrast, 7-chlorokynurenate potentiated the development of the behavioural signs of the second phase of CNS excitation. This effect was dose-dependent. A significant increase in CNS excitation score was observed with all three doses tested (50 μg dose effect shown in Figure 2). 7-Chlorokynurenate also produced a statistically significant reduction in the median time to onset of first tonic convulsion at all doses tested ($P<0.05$; Figure 2).

Neither D-CPP nor ifenprodil had any statistically significant effect on the development of CNS excitation score or tonic convulsions (data not shown) at any dose tested.

Effect of the co-administration of ifenprodil with NMDA receptor macrocomplex antagonists

First phase effects As expected from the findings of the experiments using monotherapy, co-administration of ifenprodil with 7-chlorokynurenate or D-CPP completely abolished the development of clonic convulsions in response to spermine.

Second phase effects In contrast to the finding when ifenprodil was administered alone, co-administration of ifenprodil with a moderate dose (30 nmol i.c.v.) of 7-chlorokynurenate caused a dose-dependent inhibition of the development of the second phase of spermine-induced effects. A statistically significant reduction in the CNS excitation score and increase in the median latency to the development of first tonic convulsion was observed with the higher dose of ifenprodil (Figure 3).

Similarly, co-administration of ifenprodil with D-CPP produced a statistically significant reduction in the development of the second phase of spermine-induced effects. Co-administration of ifenprodil and D-CPP produced a dose-dependent reduction in the development of CNS excitation and a statistically significant increase in the delay to onset of first tonic convulsion (60 mg kg^{-1} dose effect shown in Figure 4).

Discussion

The NMDA receptor antagonist studied, when given as monotherapy, effectively antagonized the development of the first phase of spermine-induced effects, which suggests that the

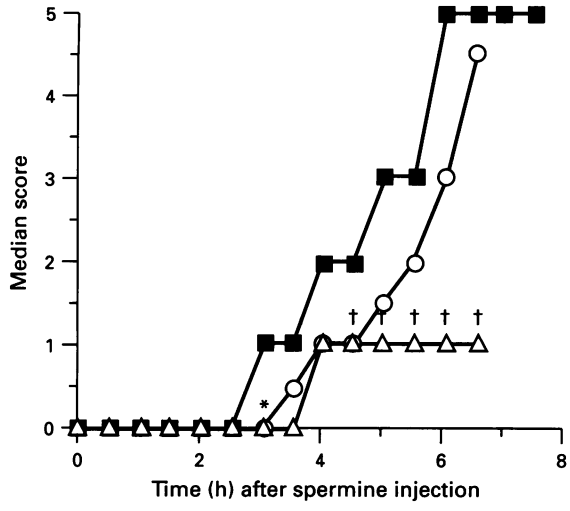

Figure 3 The effect of the coadministration of 7-chlorokynurenate 30 nmol, i.c.v. and ifenprodil 30 mg kg^{-1}, i.p. (○; $n=10$) or 60 mg kg^{-1}, i.p. (△; $n=16$) on median CNS excitation score after injection of 100 μg of spermine i.c.v. (■; $n=36$), (*$P<0.05$; †$P<0.01$, Mann-Whitney U-test).

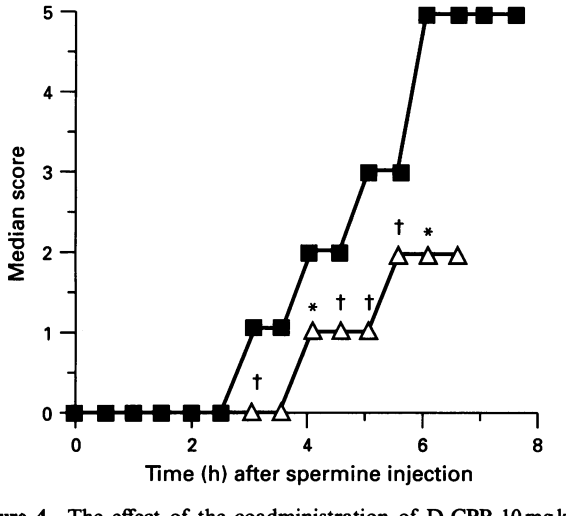

Figure 4 The effect of the coadministration of D-CPP 10 mg kg^{-1}, i.p. and ifenprodil 60 mg kg^{-1}, i.p. (△; $n=13$) on median CNS excitation score after injection of 100 μg of spermine i.c.v. (■; $n=36$), (*$P<0.05$; †$P<0.01$, Mann-Whitney U-test).

development of clonic convulsions may be due to an interaction of spermine with a polyamine binding site on the NMDA receptor macrocomplex. Conversely, the development of body tremor and tonic convulsions were not antagonized by administration of D-CPP, 7-chlorokynurenate or ifenprodil alone, yet were antagonized by dizocilpine (0.3 mg kg^{-1} (×2), i.p.). The ability of dizocilpine to antagonize these effects of spermine, albeit at a high dose, also raises the possibility that the second phase of spermine-induced effects may be mediated, at least in part, through an action at the NMDA receptor macrocomplex. However, the lack of activity of the other NMDA receptor antagonists when used as monotherapy, indicates that this later phase of spermine action is pharmacologically distinct from the earlier events.

Recent evidence has shown that spermine acts at multiple receptor sites on the NMDA receptor macrocomplex. At present, it is generally accepted that there are at least two distinct binding sites through which polyamines may directly modulate activation of the macrocomplex, the stimulatory polyamine site and the inhibitory polyamine site (for review, see Romano & Williams, 1994). Spermine and spermidine can also indirectly modulate activation of the NMDA receptor macrocomplex by altering the affinity of the glutamate and glycine receptor sites for agonists and antagonists (Romano & Williams, 1994). Recent data from whole-cell voltage-clamp recordings in rat hippocampal neurones has shown that spermine potentiates the effects of NMDA through an increase in apparent affinity for glycine as well as potentiation through a mechanism unrelated to glycine (Benveniste & Mayer, 1993). Whether these effects on receptor affinity are mediated through the stimulatory polyamine site, the inhibitory polyamine site, or a further polyamine site is not yet clear. However, a recent study investigating the effect of N-(3-aminopropyl)-1,10-diaminodecane (APDA10), a synthetic mixed agonist/partial agonist at the polyamine receptor site on the NMDA receptor, has demonstrated that the effects of the polyamines on glutamate and glycine binding may be mediated through two distinct binding sites (Williams *et al.*, 1992). APDA10 increased the affinity of [^{3}H]-CPP for the NMDA receptor, but had no effect on the binding of [^{3}H]-glycine (Williams *et al.*, 1992).

The reverse also seems to be true. Ligands which bind to the glutamate or glycine sites can modulate binding at the polyamine site (Ransom & Stec, 1988; Nussenzveig *et al.*, 1991). As previously mentioned, recent evidence has suggested that the inhibitory potential of polyamine antagonists may be dependent on the tonicity of the glutamate, glycine and polyamine receptors on the NMDA receptor macrocomplex. Glycine has been found to reduce and 7-chlorokynurenate to increase the proportion of [^{3}H]-TCP displaced with high affinity by ifenprodil, presumably through an action at the polyamine binding site (Carter *et al.*, 1992). These results suggest that glycine may indirectly reduce the effectiveness of polyamine antagonists. In support of this, under normal conditions in the hemisected rat spinal cord, ifenprodil was largely ineffective in blocking the effects of NMDA (Carter *et al.*, 1992). However, in the presence of sub-maximal concentrations of 7-chlorokynurenate, ifenprodil was an effective antagonist (Carter *et al.*, 1992). Synergism between glycine antagonists and polyamine antagonists has also recently been demonstrated *in vivo* in a model investigating the stimulatory effects of intrastriatally administered NMDA on spermine or spermidine release (Voltz *et al.*, 1994). Co-administration of ifenprodil (30 mg kg^{-1}, i.p.) and 7-chlorokynurenate (3 μmol) blocked the effects of NMDA on polyamine release, although monotherapy of either drug was ineffective (Voltz *et al.*, 1994).

The results of the present study concur with the above findings. In our *in vivo* model, synergism between the glycine and polyamine binding sites was demonstrated by the facilitation of inhibition of the second phase of spermine-induced effects by ifenprodil when a moderate concentration of 7-chlorokynurenate was administered. Furthermore, in the present study, a similar synergism between the glutamate and polyamine binding sites was also demonstrated.

These results therefore support the possibility that the development of body tremor and tonic convulsions may be mediated through activation of the NMDA receptor macrocomplex. It is also clear that the first and second phase of spermine-induced effects, which were observed to be temporally distinct, are mediated by distinct pharmacological mechanisms. The inhibitory effect exerted by ifenprodil on the second phase of spermine-induced effects appears to be dependent upon the tonicity of either the glutamate or the glycine receptor sites on the NMDA receptor macrocomplex. However, the inhibitory effect of ifenprodil on the first phase of spermine-induced effects seems to be independent of this tonicity.

It is of interest to note that recent evidence has suggested that the clonic and tonic convulsions induced by a wide variety of different convulsant agents may also be mediated by different mechanisms. Dizocilpine and dextrorphan, (a non-competitive NMDA receptor antagonist), have been demonstrated to inhibit the tonic convulsions and mortality produced by NMDA, AMPA, kainate, bicuculline and pentylenetetrazol, but were ineffective against the clonic seizures produced by these agents (Akaike & Himori, 1993).

Previous evidence has suggested NMDA heterogeneity in the central nervous system, based on regional differences in the affinity of NMDA receptors for various agonists and antagonists (Reynolds & Palmer, 1991; Subramaniam & McGonigle, 1991). More recently, investigation of the properties of recombinant NMDA receptors expressed by the cloning of cDNAs encoding subunits of the NMDA receptor has suggested that the pharmacological heterogeneity observed in studies using native NMDA receptors may be explained by combinations of different subunits (Katsuwasa *et al.*, 1992; Lynch *et al.*, 1995). Furthermore, *in vitro* evidence has suggested that polyamine antagonists may be candidates for a selective action at a particular NMDA receptor subtype (Nankai *et al.*, 1995). An investigation of NMDA-induced release of acetylcholine and spermidine from rat striatal slices has shown that while dizocilpine blocked the release of acetylcholine and spermidine in response to NMDA with equal potency, compounds such as ifenprodil, eliprodil, aracaine, philanthotoxin$_{343}$ and argiotoxin$_{636}$, which interact with polyamine receptors, blocked acetylcholine release, but were ineffective against spermidine release (Nankai *et al.*, 1995). NMDA receptor heterogeneity may underlie this apparent selective action of polyamine antagonists. In a similar manner, NMDA receptor heterogeneity may also underlie the differences observed in the development of spermine-induced clonic and tonic convulsions in the present study.

Additional work in this laboratory has demonstrated that clinically used anticonvulsants which act through mechanisms distinct from blockade of the NMDA receptor macrocomplex can also affect the development of the two behavioural phases induced by spermine (data not shown). Agents which act through GABA enhancement, such as diazepam, clonazepam and phenobarbitone, were particularly effective antagonists of the second phase of spermine-induced effects (preliminary data). Whether this antagonism simply reflects a physiological antagonism, or a direct interaction between GABA and polyamine receptors remains unclear. Nisoldipine, an L-type Ca^{2+} channel blocker was also found to inhibit effectively the development of both phases of spermine-induced effects (preliminary data). Dihydropyridines, such as nisoldipine, have been found to antagonize effectively all types of convulsions in a wide range of models of epileptogenesis (Speckmann & Walden, 1993). This suggests that the ability of nisoldipine to antagonize spermine-induced convulsions in our laboratory may be a general effect reflecting the essential role of the influx of Ca^{2+} in the generation of all types of convulsions. However, more specific effects on Ca^{2+} entry or Ca^{2+} channel activation have not been excluded.

In summary, the results of this study demonstrate that the mechanisms underlying the development of the two temporally distinct phases of spermine-induced effects may be pharmacologically distinct. There is evidence that activation of the NMDA receptor macrocomplex may be involved, at least in part, in both phases. This study also demonstrates that the action of ifenprodil can be facilitated by 7-chlorokynurenate or D-CPP *in vivo*.

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