THE INTERACTIONS OF PUTATIVE NEUROPROTECTANT COMPOUNDS WITH NMDA ION CHANNELS

by

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ABSTRACT

The NMDA (N-methyl-D-aspartate) subtype of the receptors for the excitatory amino acid L-glutamate has been implicated as a mediator of anoxic neuronal death following periods of cerebrovascular ischaemia. Laboratory observations have implicated it in neuronal death occurring in other situations, including hypoglycaemia and neurodegenerative disorders. Inhibiting the NMDA receptor could, therefore, represent a useful therapeutic intervention where CNS damage is a potential outcome resulting from conditions such as those given above. However, since NMDA receptors are involved in a number of vital CNS functions, such as synaptic transmission, simple all-or-none antagonism would present an unacceptable risk of toxicity and side effects. Determination of specific characteristics of a given agent's interaction with the NMDA receptor-ion channel complex would aid considerably in assessing that agent's clinical utility as a safe and efficacious neuroprotectant.

The ion channel associated with the NMDA receptor is the target of a number of uncompetitive NMDA antagonists. Four such agents have been chosen for study: (-)- and (+)-β-cyclazocine are members of the benzomorphan class of compounds, several others of which are known to act as NMDA antagonists; dextromethorphan (DM) is an antitussive morphinan closely related in chemical structure to the benzomorphans; and L-687,384, which is, like a number of other uncompetitive NMDA antagonists, a ligand for sigma receptors. These agents were tested for their actions against responses to NMDA. As an estimation of the potential for producing side effects, (-)-β-cyclazocine and DM were also studied for their effects on responses to the non-NMDA glutamate receptor agonists kainate and α-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazole-
propanoic acid (AMPA), as well as for their actions against voltage-gated Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) currents. Finally, (-)-\(\beta\)-cyclazocine was tested in a cell viability assay to confirm its efficacy as a neuroprotective agent.

Unitary currents through single NMDA-activated ion channels were studied using outside-out patches isolated from cultured rat hippocampal or cortical neurons. Currents recorded in the presence of NMDA were compared to those recorded following the addition of one of the agents. None of the compounds studied altered the amplitudes of the unitary currents activated by NMDA and hence did not affect the channel conductance. The compound (-)-\(\beta\)-cyclazocine concentration-dependently reduced the open state probability (\(P_0\)) of NMDA ion channels with IC\(_{50}\) values of 84 nM and 680 nM in hippocampal and cortical neurons, respectively. The reduction in \(P_0\) by (-)-\(\beta\)-cyclazocine was attributable to decreases in mean channel open time and mean channel opening frequency. In hippocampal neurons, (+)-\(\beta\)-cyclazocine was 170x less potent in reducing \(P_0\) than the (-)-enantiomer (IC\(_{50}\) = 14 \(\mu\)M for (+)-\(\beta\)-cyclazocine). DM also reduced \(P_0\), with IC\(_{50}\) values of 4.4 \(\mu\)M and 3.8 \(\mu\)M in hippocampal and cortical neurons, respectively. Again, decreases in mean open time and mean frequency were associated with the reduction in \(P_0\) by DM. L-687,384 decreased the mean open time of NMDA-activated unitary currents, but did not diminish their frequency. Consequently, the action of L-687-384 against \(P_0\) was relatively weak (IC\(_{50}\) = 61 \(\mu\)M).

The reductions in mean open time and mean frequency by (-)-\(\beta\)-cyclazocine and DM, without effects on channel conductance, were consistent with an open-channel block model for inhibition of NMDA activity. Analysis of the results assuming an open-channel blockade model gave estimations of the on-rate constants (\(k_2\)) and off-rate
constants \((k_2)\) for the interactions of the compounds with NMDA ion channels in hippocampal and cortical neurons. The \(k_2\) values in both neuronal types were similar for \((-)\-\beta\)-cyclazocine, DM, and L-687,384, all being near \(10^7 \text{ M}^{-1}\text{s}^{-1}\). The closeness of the \(k_2\) values suggests that the on-rate for a given open channel blocker is not a determinant of its potency as an NMDA activity inhibitor. The frequency reductions by \((-)\-\beta\)-cyclazocine and DM allowed for estimations of \(k_2\), an apparent unblocking, or “off”, rate constant, from the single-channel data; this was \(2.5 - 5.0 \text{ s}^{-1}\) and \(2.5 \text{ s}^{-1}\) for \((-)\-\beta\)-cyclazocine in hippocampal and cortical neurons, respectively, and \(10 - 13 \text{ s}^{-1}\) for DM in hippocampal neurons.

Optical recording of intracellular free calcium \([\text{Ca}^{2+}]_i\) responses using hippocampal or cortical neurons loaded with the \(\text{Ca}^{2+}\)-sensitive fluorescent dye fura-2 allowed further elucidation of the actions of \((-)\-\beta\)-cyclazocine and DM on responses to NMDA. Both agents depressed NMDA responses with \(\text{IC}_{50}\) values of 270 nM and 220 nM for \((-)\-\beta\)-cyclazocine in hippocampal and cortical neurons, respectively, and 4.1 \(\mu\text{M}\) and 5.4 \(\mu\text{M}\) for DM in hippocampal and cortical neurons, respectively. The action of \((-)\-\beta\)-cyclazocine was use-dependent, a property consistent with open-channel block. No effect on NMDA-evoked \([\text{Ca}^{2+}]_i\) responses was observed with \((+)\-\beta\)-cyclazocine in either neuronal type.

In either hippocampal or cortical neurons loaded with fura-2, \((-)\-\beta\)-cyclazocine at 5 \(\mu\text{M}\) was found to have no action against \([\text{Ca}^{2+}]_i\) responses to the non-NMDA agonists kainate and AMPA. Responses evoked by exposure of the neurons to high \([K^+]_o\)-containing medium were also unaffected, indicating that this agent did not interact with voltage-activated \(\text{Ca}^{2+}\) channels. DM, at a concentration of 50 \(\mu\text{M}\), reduced high \([K^+]_o\)-
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Cell viability assays showed enantioselective neuroprotection with (-)- and (+)-\textbeta-cyclazocine. Hippocampal or cortical neurons were exposed to 1 mM NMDA for 24 hr and the number of surviving neurons following this treatment were compared to the number of neurons before the toxic NMDA exposure. When present during the NMDA insult, (-)-\textbeta-cyclazocine protected both hippocampal and cortical neurons, with 50% neuroprotection being achieved near 1 \textmu M. The (+)-enantiomer was weaker as a neuroprotectant, with < 50 % protection at a concentration of 10 \textmu M.

The specificities of (-)-\textbeta-cyclazocine and DM were further assessed by studying voltage-activated Na\textsuperscript{+} and K\textsuperscript{+} currents in DRG neurons and cardiac myocytes. There was no detectable effect of either (-)-\textbeta-cyclazocine (5 \textmu M) or DM (50 \textmu M) to alter Na\textsuperscript{+} or K\textsuperscript{+} currents in neurons; (-)-\textbeta-cyclazocine (5 \textmu M) had no effect on Na\textsuperscript{+} or K\textsuperscript{+} currents in cardiac myocytes. However, DM (50 \textmu M) inhibited both Na\textsuperscript{+} and K\textsuperscript{+} currents in cardiac myocytes, with use-dependent Na\textsuperscript{+} current reduction suggesting DM block of open Na\textsuperscript{+} channels. The results indicate that (-)-\textbeta-cyclazocine is a highly potent and selective blocker of NMDA ion channels, with a concomitant neuroprotective capacity. This compound is hence suggested as a possible therapeutic agent for conditions requiring CNS neuroprotection. While DM is an uncompetitive NMDA antagonist, its relative non-selectivity, as demonstrated by actions against neuronal Ca\textsuperscript{2+} channels and cardiac Na\textsuperscript{+} and K\textsuperscript{+} channels, warrants caution with regard to its use at doses above those required for its antitussive effect and hence may limit its clinical application as a neuroprotectant.
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DEDICATION

This thesis is dedicated to my mother, Lynn, my father, Ronald, and my brother, Michael, without whose love, encouragement, and support none of my studies would have been possible.
1. INTRODUCTION

1.1. Excitotoxicity and glutamate

The involvement of glutamate receptors in the pathogenesis of neuronal injury following cerebral ischaemia/hypoxia has been acknowledged for a number of years. Observations of neuronal necrosis following systemic administration of acidic amino acids, including L-glutamate (or its salt monosodium glutamate, or MSG), L-aspartate, and L-cysteine, were the first controlled experiments to demonstrate the debilitating effects of acidic amino acids on the CNS (Olney, 1969; Olney and Sharpe, 1969; Olney et al., 1971). Examination of electron microscope data showed the most sensitive areas to be those most exposed to the systemic route of administration, specifically, the arcuate layer of the hypothalamus and the inner layer of the retina. This suggested that the damage was a result of a direct effect of the administered amino acids on the tissue.

1.1.1. Glutamate release in ischaemic brain

Evidence has accumulated to implicate the involvement of L-glutamate in neuronal brain damage in vivo, particularly where there is a brief interruption of oxygen supply to the damaged area, such as in stroke, head trauma, or hypoxia (Rothman, 1984). Under these conditions, extracellular L-glutamate concentrations rise dramatically to supraphysiologic levels (Benveniste et al., 1984). When oxygen supply fails to meet demand, cellular energy metabolism is compromised, and processes which depend on cellular ATP stores are thus impaired. For instance, the loss of
energy required to maintain cell resting potential results in neuronal depolarization, which in turn releases L-glutamate into the synapse; energy-requiring glutamate transport from the synapse is also affected. This constitutes part of a "feed-forward" cycle, in which glutamate released into the synapse (and not taken up) would therefore produce depolarization and further glutamate release from the post-synaptic cell (see Choi, 1988a,b). The net result is a dramatic increase in the extracellular concentration of L-glutamate (Benveniste et al., 1984; Rothman and Olney, 1986). Neurons that lyse upon death and release their cellular contents into the neuropil also contribute to the increased glutamate concentration in such conditions; the concentration of glutamate in neuronal tissue is in the millimolar range (Curtis and Johnston, 1974; Choi, 1988a) and is thus a large pool of glutamate that would result in a significant rise in glutamate concentration in the neuropil upon cell lysis. As well, there is evidence that astrocytes, which also contain high levels of glutamate, are also possible sources of L-glutamate, either as physiologically released L-glutamate or that arising from lysing cells (Dawson et al., 1995; Kimelberg et al., 1995; Jeftinija et al., 1996).

1.1.2. Glutamate receptors

Glutamate has long been recognized as the principal excitatory neurotransmitter in the mammalian brain. Re-evaluation of a series of experiments performed in the early 1960's (Curtis and Watkins, 1962) showed that not only was L-glutamate (or its analog L-aspartate) acting on specific receptors, but that (although not realized at the time) there were several subtypes of glutamate receptor, each signalling the neuron in different ways. There are 5 subtypes of glutamate receptor currently accepted: 3 ionotropic receptors (NMDA, kainate, and AMPA (α-amino-2,3-dihydro-5-methyl-3-oxo-
4-isoxazole-propanoic acid) receptors), a presynaptic receptor, and a metabotropic receptor. The following section is a brief description of the properties of the known glutamate receptor subtypes. For more extensive reviews concerning glutamate receptors, see Mayer and Westbrook (1987b), Monaghan et al. (1989), and Collingridge and Lester (1989).

1.1.2.1. Kainate and AMPA receptors

Two subtypes of the glutamate receptor are coupled to ion channels that are non-selectively permeable to Na⁺ and K⁺ and their activation results in neuronal depolarization (Ascher and Nowak, 1988a). These are the kainate receptors and the AMPA receptors, both named for the compounds which preferentially activate that subtype. The latter (AMPA) subtype was originally known as the quisqualate receptor but was renamed due to AMPA's higher selectivity for that subtype. The chief differences between AMPA and kainate receptors are their distribution in the CNS, the selectivity of the two subtypes for different agonists, and the membrane conductance changes mediated by either subtype (Ascher and Nowak, 1988a). Both of the above receptor subtypes are mediators of fast synaptic transmission in the mammalian CNS (Lambert et al., 1989).

1.1.2.2. L-AP4 receptors

The L-AP4 (L-2-amino-4-phosphonobutanoate) receptor subtype is an autoreceptor that controls glutamate release through a negative feedback mechanism. This receptor subtype is believed to be located presynaptically on glutamatergic terminal boutons (Monaghan et al., 1989). Because of its presynaptic location, little is
known about the mechanism through which the L-AP4 receptor limits glutamatergic synaptic activity. Additionally, progress into characterizing the L-AP4 receptor has been hampered by the fact that L-AP4 itself can activate certain metabotropic glutamate receptors (Mattson and Mark, 1996; see below).

### 1.1.2.3. Metabotropic glutamate receptors

The metabotropic glutamate receptor is unique among glutamate receptor subtypes in that its activation results not in ion channel opening but rather the triggering of second messenger systems that alter the internal biochemical environment of the cell. These include generation of intracellular second messengers, such as inositol 1,4,5-triphosphate and diacylglycerol, that in turn intracellularly regulate the excitability of the neuron (Sugiyama et al., 1987; Challiss et al., 1994). Molecular sequencing work has revealed the existence of at least seven metabotropic receptor subtypes, termed mgluR1 to 7, and this family of metabotropic glutamate receptors has now emerged as important regulators of cell behaviour (Bruno et al., 1996; Mattson and Mark, 1996). Metabotropic glutamate receptors are activated by glutamate, quisqualate, and synthetic amino acid analogs such as 1,3-aminocyclopentanedicarboxylic acid (trans-ACPD) (Bruno et al., 1996; Monaghan et al., 1989).

### 1.1.2.4. NMDA receptors

The remaining subtype, the NMDA receptor, is perhaps the most extensively studied subtype of the glutamate receptor family. A major characterizing feature of the NMDA receptor is that it is coupled to an ion channel that is highly permeable to
calcium (Dingledine, 1983; MacDermott et al., 1986) while exhibiting voltage-dependent block by Mg$^{2+}$ (Nowak et al., 1984; Jahr and Stevens, 1990). The receptor itself is thought to be a heteroligomeric complex consisting of a subunit termed NR1 in association with one of another set of subunits, termed NR2A - E (for reviews, see Mattson and Mark, 1996; McBain and Mayer, 1994). The unique properties of NMDA receptors mentioned above contribute to the unique physiology of the NMDA receptor and allow it to participate centrally in a variety of functions, including long-term potentiation (the process believed to be the cellular correlate of long-term memory; Bliss and Collingridge, 1993), synaptic development and plasticity (Constantine-Paton et al., 1990), and the generation of rhythmic motor activity (Headley and Grillner, 1990). NMDA receptors have been implicated in a number of glutamate-related pathologies, such as epilepsy (Dingledine et al. 1990), neurodegenerative disorders (Choi, 1988b; Meldrum and Garthwaite, 1990; Danysz et al., 1995), and excitotoxicity (see below), and it appears that all these conditions have as a common factor the high intracellular calcium levels induced by excessive NMDA receptor activation.

1.1.3. Regulatory sites of the NMDA receptor-ion channel complex

The NMDA receptor-ion channel complex is a complicated entity, with several sites at which ligands can act to modify the behaviour of the channel. It has regulatory sites than any known receptor, with new sites still being discovered. Investigators hope to take advantage of this multi-faceted regulation, in that many avenues to pharmacological manipulation of the activity of the NMDA system are thus potentially available.
1.1.3.1. Agonist recognition site

Compounds that occupy the receptor site where L-glutamate or NMDA attach and which do not possess intrinsic excitatory activity are by definition competitive antagonists. The majority of compounds in this class have sufficient charge on the molecule to prevent entry into the central nervous system (CNS) through the blood-brain barrier. Clinical trials of some agents that are sufficiently lipophilic to pass through the blood-brain barrier are currently under way (Lipton, 1993; Koroshetz and Moskowitz, 1996). However, CNS toxicity is a risk, since simple competitive inhibition of NMDA activity may impair synaptic transmission to an unacceptable degree (Grotta, 1994). Furthermore, it should be noted that the effects of competitive inhibition can be overcome by increasing concentrations of agonist.

1.1.3.2. Glycine coagonist site

The requirement of ambient levels of glycine, at the mid-nanomolar level, in the extracellular medium for expression of NMDA activity has led to the discovery of a coagonist site for glycine on the NMDA receptor-ion channel complex (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Reynolds and Miller, 1988). The identity of this site was initially cloaked by the ubiquitous presence of glycine, since this amino acid can be present at micromolar levels even in “purified” distilled water. The subsequent development of antagonists and partial agonists for this site, such as HA-966 (R-(+)-3-amino-1-hydroxypyrrolid-2-one; Kemp et al., 1988; Henderson et al., 1990), showed that NMDA activity could be blocked by antagonizing the effects of glycine at this site. It is believed that the recognition site for glycine on the NMDA
receptor is located near the recognition site for glutamate on the extracellular loop of the NR1 subunit (Hirai et al., 1996), and that glycine acts by regulating the level of desensitisation of the NMDA receptor-ion channel complex (Mayer et al., 1989). The receptor for glycine on the NMDA receptor-ion channel complex is different from that associated with the synaptic glycine receptor coupled to a chloride channel. First, the glycine site on the NMDA receptor has a far greater affinity for glycine than does the inhibitory receptor, with the $K_0$ of the former being near 100 nM (134 nM; Manallack et al., 1990) and the $K_0$ of the latter being close to 20 μM (24 μM; Graham et al., 1985). Second, the pharmacology of the two sites is not the same. The most notable pharmacological difference between the two types of glycine receptor is the lack of sensitivity of the NMDA-associated site to strychnine (Johnson and Ascher, 1987), which potently antagonizes the inhibitory glycine receptor (Curtis et al. 1968; Graham et al. 1985); for this reason, the NMDA-associated glycine coagonist site is commonly referred to as the strychnine-insensitive glycine site. Modulation of NMDA activity through this site can provide neuroprotection (Newell et al., 1995), and currently a number of strychnine-insensitive glycine receptor antagonists are being tried in the clinical setting for use in stroke therapy (Danysz et al., 1995).

1.1.3.3 Magnesium

The blockade of the NMDA channel by $\text{Mg}^{2+}$ confers voltage-dependent properties on the NMDA-activated membrane conductance (Nowak et al., 1984; Mayer et al., 1984; Mayer and Westbrook, 1987a). The normal concentration of $\text{Mg}^{2+}$ in the extracellular fluid is sufficient to prevent ion flux through NMDA channels at resting
potential, whereas at depolarized potentials the block by Mg$^{2+}$ is relieved and current flow through NMDA ion channels proceeds (Ault et al., 1980). In this sense, NMDA activity serves to enhance the effects of other forms of excitation, such as activation of non-NMDA glutamate channels. Alternatively, NMDA activity is engineered to "kick in" only when there is sufficient excitatory input from other sources to relieve the Mg$^{2+}$ block. The site at which Mg$^{2+}$ exerts its blocking action is deep within the ion channel; voltage-dependence occurs because the charged Mg$^{2+}$ ion must traverse a significant portion of the membrane electric field in order to reach its site (Ascher and Nowak, 1988b). Attempts to exploit the inhibitory effects of Mg$^{2+}$ on NMDA activity for clinical use in conditions such as epilepsy should be approached with caution, since in most pathological states in the CNS, neurons are depolarized to potentials where Mg$^{2+}$ has little effect. As well, the non-specificity of Mg$^{2+}$, particularly with respect to its blockade of Ca$^{2+}$ channels (Hess and Tsien, 1984; Hess et al., 1986; Lansman et al., 1986), complicates its effects. However, preliminary clinical trials dealing with the clinical application of Mg$^{2+}$ in epilepsy have shown promise in the lack of adverse effects, and phase 3 trials are currently being planned (Muir and Lees, 1995). To date, there are no known agents besides Mg$^{2+}$ that act at the magnesium site and which can be exploited for use in either the laboratory or clinical settings.

1.1.3.4. Zinc

Zn$^{2+}$ ions non-competitively inhibit NMDA activity, possibly by preventing the ability of agonists to gate open the channel (Westbrook and Mayer, 1987; Reynolds and Miller, 1988; Forsythe et al., 1988). The lack of voltage-dependence of Zn$^{2+}$
actions suggests that its binding site is located on the extracellular surface of the channel protein. Ionic zinc is present in the nerve terminals of central excitatory neurons (Frederickson et al., 1982; Danscher et al., 1985) and is co-released with glutamate from the presynaptic neuron into the synaptic cleft (Assaf and Chung, 1984; Howell et al., 1984; Charton et al., 1985). It is believed that Zn$^{2+}$ serves as a regulator of endogenous glutamate-related NMDA activity. Another endogenous compound, histamine, has recently been shown to potentiate responses to NMDA at concentrations < 0.5 mM; since this action is voltage-independent, the possibility exists that histamine acts through the Zn$^{2+}$ site, or close to it (McBain and Mayer, 1994). The Zn$^{2+}$ site on the NMDA receptor-ion channel complex has yet to be exploited as an in vivo mitigator of NMDA activity; however, further research, particularly with respect to histamine’s effects at this site (see above), may reveal any clinical significance associated with manipulation of Zn$^{2+}$ actions on NMDA activity.

1.1.3.5. Polyamine Site(s)

Another important area concerns the regulation of NMDA activity by polyamines such as spermine and spermidine. Binding experiments have shown that, in the presence of glutamate, spermine and spermidine enhance the binding of radiolabelled uncompetitive NMDA antagonists such as [$^3$H]TCP and [$^3$H]MK-801, but not [$^3$H]glycine or the competitive antagonist [$^3$H]CPP, suggesting these compounds promote channel opening via sites distinct from the recognition sites for either glutamate or glycine (Ransom and Stec, 1988; Carter et al., 1990). In whole-cell electrophysiological recordings, these compounds showed a complex mode of action, with an enhancement
of NMDA responses being observed at low (< 1 mM for spermine) concentrations and antagonism being the effect of higher concentrations (Benveniste and Mayer, 1993). The two effects may be mediated by separate sites on the NMDA ion channel, as other polyamines are capable only of the block at higher concentrations without producing potentiation at the lower concentrations (McBain and Mayer, 1994). The voltage-dependence of the NMDA-modulating properties of polyamines suggests the sites of attachment are located within the channel pore. It has been suggested that some aspects of histamine modulation of NMDA receptor activity are mediated via a polyamine binding site, including the antagonism produced by high (> 0.5 mM) concentrations of histamine (Bekkers, 1993; Vorobjev et al., 1993).

Redox sites

Redox modulation of the NMDA receptor-ion channel complex is a recently discovered phenomenon that promises to reveal much about how NMDA activity is endogenously regulated from the outside of the neuron. The NMDA complex is similar to many other transmitter-gated ion channels in this respect. Reduction of NMDA receptors with agents such as dithiothreitol (DTT) enhances their activity whereas oxidation, as with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), inhibits their activity (Gozlan and Ben-Ari, 1995). Both the former and latter agents are nonpermeant through cell membranes and are therefore affecting redox sites located on the extracellular side of the receptor. The fact that either reducing or oxidizing the receptor changes its activity suggests the resting native NMDA receptor is in an equilibrium state between a fully reduced form and a fully oxidized form (Aizenman et al., 1989).
Possible endogenous compounds that may be active in affecting NMDA receptors in this way are thiol derivatives such as cysteine, homocysteine, glutathione, lipoic acid, and dihydrolipoic acid, ascorbate, and free radicals such as nitric oxide (NO) (Lipton et al., 1993; Tang and Aizenman, 1993; Gozlan and Ben-Ari, 1995).

1.1.3.7. Steroids

Steroids such as pregnenolone have been shown to directly enhance NMDA activity (Wu et al., 1991; Irwin et al., 1992) in a manner not related to steroid effects on gene expression (Bowlby, 1993). The site through which this effect is mediated is most likely accessed via the lipid bilayer, as suggested by both the kinetics of steroid action and the observation that external application of pregnenolone can affect NMDA channel behaviour as recorded in both cell-attached and outside-out patches (Bowlby, 1993).

1.1.3.8. Inhibition by decreasing pH

Increasing the acidity of the extracellular solution inhibits NMDA responses (Traynelis and Cull-Candy, 1990) while increasing the alkalinity increases NMDA activity (Giffard et al., 1992). This action is voltage-independent and non-competitive. The modulation of NMDA activity by acidity may represent a compensatory negative feedback mechanism that would come into play during the fall in pH observed after periods of excitatory synaptic activity. Furthermore, since the apparent pKₐ of the NMDA receptor-ion channel complex is quite close to physiological pH (i.e., near pH 7.0; Traynelis and Cull-Candy, 1990, 1991), NMDA responses are necessarily quite sensitive to small changes in extracellular pH which would generally be occurring along
the steep portion of the acid-base curve. In contrast, AMPA and kainate receptors show IC₅₀'s for acid-mediated inhibition to be near pH 6.0, outside the range of normal physiological pH (Traynelis and Cull-Candy, 1991).

1.1.3.9. Intracellular phosphorylation

Intracellular factors, such as kinases and phosphatases, play an important role in the overall activity of NMDA receptor-ion channels. The phosphorylation states of certain amino acids on the channel protein (or on cytoskeletal elements associated with and possibly playing a regulatory role on NMDA receptors; see Rosenmund and Westbrook, 1993) have a profound effect on the behaviour of NMDA ion channels (Kaczmarek, 1987; Huganir and Greengard, 1990), as well as on the distribution of NMDA receptor subunits between the membrane and intracellular locations (Tingley et al., 1997). In this way, the state of the NMDA receptor may be controlled distally. For instance, generation of secondary messengers by metabotropic glutamate receptors will in turn affect the excitability of the NMDA ion channel by activating protein kinases that phosphorylate certain sites on the cytoplasmic face of the channel (Ben-Ari et al., 1992; Krieger et al., 1996). These kinases include protein kinase C (PKC; Tingley et al., 1997), cAMP-dependent protein kinase (protein kinase A, or PKA; Tingley et al., 1997), tyrosine kinases (Wang and Salter, 1994), and serine/threonine kinases (Tingley et al., 1993). Serine/threonine phosphatases, such as Ca²⁺/calmodulin-dependent phosphatase 2B (calcineurin; Lieberman and Mody, 1994) or protein phosphatases 1 and 2A (Wang et al., 1994), have also been shown to play a role in the regulation of NMDA receptor-ion channel complexes, particularly through sites on the
NR2A and NR2B subunits (Menegoz et al., 1995), which also contain the sites for regulation by tyrosine kinases (e.g., PTK pp60^src; Yu et al., 1997). Sites for PKC and PKA phosphorylation have been found on the NR1 subunit (Tingley et al., 1993; Tingley et al., 1997), and these sites have been suggested to regulate the sensitivity of NMDA receptors to Mg^{2+} (Chen and Huang, 1992), as well as NR1 subcellular distribution (see above). PKA has been shown to increase the opening frequencies and mean open times of NMDA-activated ion channels (Greengard et al., 1991). The metabolic state of the neuron can also influence NMDA activity, as kinase and phosphatase activities depend on the availability of ATP as a source of organic phosphorous. In this regard, prolonged whole-cell recording of NMDA receptor-mediated currents requires the inclusion of an ATP-generating system in the recording pipette, in order to prevent washout or rundown of these currents that would otherwise occur (MacDonald et al., 1989; Wang et al. 1993). The intracellular enzyme protein kinase C (PKC) is thought to be heavily involved in NMDA receptor-ion channel regulation, for a variety of reasons: rises in intracellular free calcium ([Ca^{2+}]), such as that produced by NMDA receptor activation, stimulate PKC activity (Gerber et al., 1989; Ben-Ari et al., 1992; Krieger et al., 1996), a self-enhancing property that may be relevant to plastic processes such as LTP; activation of metabotropic glutamate receptors has been shown to enhance NMDA receptor-mediated responses through a PKC-dependent mechanism (Kelso et al., 1992); and direct activation of PKC activity by phorbol esters potentiates NMDA responses (Gerber et al., 1989).
1.1.3.10. The PCP site

An important class of NMDA antagonist, the uncompetitive inhibitors, act through a binding site not associated with the recognition sites for glutamate or glycine. Certain features of the antagonism by the uncompetitive inhibitors and that produced by Mg$^{2+}$ suggest the existence of separate and distinct binding sites, with the latter showing properties more consistent with a location deep within the channel pore (MacDonald and Nowak, 1990; MacDonald et al., 1991). The classical ligand for the uncompetitive antagonist binding site is the dissociative anaesthetic phencyclidine, or PCP (Anis et al., 1983; Honey et al., 1985), and the binding locus is hence often referred to as the PCP site. Ketamine is another well-known example in this class of open-channel NMDA blockers (Martin and Lodge, 1985; Honey et al., 1985). Access to this site requires the ion channel to be in the open conformational state; consequently, the presence of agonist is a requirement for the action of these agents. Unlike competitive antagonists, the uncompetitive NMDA antagonists show an increasing level of blockade with increasing agonist activity (Parsons et al., 1992). The property of use-dependence, in which the level of blockade increases with subsequent agonist applications, is a characteristic of agents in this class (MacDonald et al., 1987; MacDonald and Nowak, 1990). Uncompetitive NMDA antagonists hold great promise for therapeutic application, since these agents are selective for highly active ion channels and exert little effect on low, basal levels of activity. The processes in which the NMDA receptor-ion channel complex participate (e.g. synaptic transmission, LTP) would be left relatively intact, while pathophysiological actions mediated by increased levels of agonist, and hence increased NMDA activity, would be selectively targeted.
Unlike Mg$^{2+}$, these agents interact with the blocking site relatively slowly; dissociation from the channel site occurs on a longer time scale and the channel may even close with the antagonist molecule(s) still trapped within the ion channel (Javitt and Zukin, 1989). It is indeed possible for a given agent to dissociate so slowly from the site that it may, for all intents and purposes, resemble an irreversible blocker. This situation is most clearly demonstrated by MK-801 (Kemp et al., 1986; Wong and Woodruff, 1986; Foster and Wong, 1987), an anticonvulsant that is not used in humans because of its lengthy duration of action (Lipton, 1993; Rogawski, 1993).

1.2. Mechanism of excitotoxicity

The early experiments by Olney and colleagues demonstrated a correlation between the excitatory potency of a series of glutamate and aspartate analogues and their potency in producing neuronal injury, although the latter effect required much higher concentrations than what would be expected for simple excitation of central neurons (Olney et al., 1971). This observation led to the conclusion that glutamate receptors are important mediators of the excitotoxic process.

1.2.1. Initial phase

The neuronal damage that results from exposure to high concentrations of an excitatory agent, such as L-glutamate or L-aspartate, can be separated into two phases. The first, deemed the initial phase, occurs immediately after exposure to an excitotoxic insult, and has been shown to depend on activation of non-NMDA ion channel-coupled receptors, i.e., kainate or AMPA receptors (Choi, 1987a, 1988a,b; Meldrum and Garthwaite, 1990). The sustained depolarization resulting from overactivation of these receptors produces an excessive influx of Na$^+$, Cl$^-$, and H$_2$O (Rothman, 1985; Choi,
1987a; Takahashi et al., 1995). Associated with this is an expansion of intracellular volume, seen under the light microscope as swelling of neuronal somata. The ionic influx thus depends on the compromised resting potential of the neuron; this is corroborated by the ability of high (depolarizing) concentrations of K⁺ to mimic the acute phase of excitotoxicity (Choi, 1987a). If the excitatory stimulus is withdrawn, cells can recover from this phase. However, this recovery depends on the severity of the insult (as cells may burst if the inner volume expands too greatly) and to what degree the second phase (see below) occurs.

1.2.2. Delayed phase

The second, or late, phase is separated from the initial phase by a length of time exceeding several hours. It is characterized by a marked degeneration of the neuronal plasma membrane, visible under the light microscope as a "beading" of the cell surface. This phase is dependent on the presence of extracellular calcium (Choi, 1987a; Takahashi, 1995), and the calcium ionophore A23187 can produce a similar pattern of neurodegeneration by allowing a supraphysiological influx of Ca²⁺ into the cell (Choi, 1987a). Indeed, it has been shown that the greatest accumulation of Ca²⁺ into neurons in vivo following ischaemic insult occurs in the CNS areas that are most vulnerable to excitotoxic damage (Baumgarten and Zimmerman, 1992). While the initial and the delayed phases can both lead to neuronal death, evidence indicates the latter phase is more important at lower levels of toxic excitatory amino acid exposure and may predominate under many pathological conditions (Choi, 1988a).
1.2.2.1. Role of calcium

Entry of supraphysiologic levels of Ca\textsuperscript{2+} serves as a trigger for processes that eventually lead to disintegration of structural components and loss of control of ionic disposition via leak through the compromised plasma membrane. Ca\textsuperscript{2+} serves as an activator of many intracellular enzyme systems (reviewed in Choi 1988a,b; Danysz et al., 1995), for example, (a) the degradative protein calpain 1 (Siman and Noszek, 1988), (b) phospholipases that liberate arachidonic acid, which is in turn acted on by intracellular oxidases to generate superoxide radicals (Chan and Fishman, 1980), and (c) a Ca\textsuperscript{2+}-activated protease that converts xanthine dehydrogenase to xanthine oxidase, which in turn produces toxic oxygen-containing free radicals (Dykens et al., 1987). Mitochondrial oxidative phosphorylation can also be impaired by excessively high intracellular Ca\textsuperscript{2+} levels, leading to decreased efficiency of energy-dependent Ca\textsuperscript{2+} transporters (Siesjö, 1988; Choi, 1988b). Additionally, elevated intracellular calcium can itself stimulate further increases in its own concentration, by (i) reversing the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, allowing Ca\textsuperscript{2+} to enter the cell via this pump (Nachsen et al., 1986), (ii) generation of inositol 1,4,5-triphosphate, which can liberate Ca\textsuperscript{2+} from intracellular stores (Berridge, 1993), (iii) activation of endoplasmic reticulum (ER)-based ryanodine-sensitive receptors causing Ca\textsuperscript{2+} release from the ER (Mody and MacDonald, 1995), and (iv) acting with diacylglycerol to activate PKC, which enhances the activity of voltage-gated calcium channels, NMDA channels, and other channels (Kaczmarek, 1987; Connor et al., 1988).
1.2.3. Involvement of NMDA receptors

The observation that the late phase of glutamate-induced neurotoxicity is antagonized by APV (Choi, 1988a) has revealed the central role that NMDA receptors play in excitotoxicity. Exposure of neurons in vitro to high levels of glutamate in the presence of APV abolishes the late excitotoxic phase while leaving the initial phase intact (Choi et al., 1988). During periods of excessive glutamate activation, Ca\(^{2+}\) enters the neuron through NMDA ion channels, especially when Mg\(^{2+}\) block is relieved by depolarization via activation of non-NMDA receptors. Concurrently, intracellular free calcium ([Ca\(^{2+}\)]) rises dramatically, some from direct entry via NMDA ion channels, and some from other sources, such as Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular organelles (Mody and MacDonald, 1995). It is not known what proportion of the increase in [Ca\(^{2+}\)] is directly from [Ca\(^{2+}\)] entry through NMDA ion channels, and how much is derived from other sources, such as voltage-operated calcium channels (Tsien et al., 1988) activated upon glutamate-induced membrane depolarization (Dingledine, 1983), non-specific membrane "leak" due to compromised integrity of the plasma membrane (Choi, 1988b), and [Ca\(^{2+}\)]- or other messenger- induced release from intracellular organelles such as the endoplasmic reticulum (Mody and MacDonald, 1995). What is known from the delayed phase block by APV (Choi et al., 1988) is that the [Ca\(^{2+}\)] influx via NMDA receptor-coupled ion channels is a critical initial step in signalling the changes that the neuron undergoes during excitotoxicity.

1.3. Pharmacology of excitotoxicity

Interventions anywhere along the chain of events occurring under excitotoxic conditions would be expected to protect neurons from excitotoxicity-related damage.
The pharmacology of NMDA receptors is particularly well described and hence at this stage is a reasonable initial approach in the management of glutamate-induced CNS injury. Experiments in vivo involving conditions of hypoxia, ischaemia, or hypoglycaemia have demonstrated the efficacy of NMDA antagonists in attenuating the CNS damage observed in such situations (Foster et al., 1988; Gill et al., 1988; McDonald et al., 1989; Kochhar et al., 1991; Lippert et al., 1994). Excitotoxicity experiments using high levels of NMDA or glutamate to induce damage in cultured neuronal preparations have verified the efficacy of NMDA antagonist drugs in protecting neurons from excitotoxic injury (Rothman et al., 1987; Goldberg et al., 1988; Choi et al., 1988; Tortella et al., 1994) and suggest that this type of experiment may serve as a convenient screen for neuroprotectant compounds.

1.3.1.1. The concept of "delayed rescue"

For optimal treatment of excitotoxicity-related events, an intervening agent would have to be present during the time of the injurious episode. If a set of conditions or a previous incident is present that would produce a raised probability of the occurrence of an excitotoxicity-related episode, such as a previous stroke, then prophylactic treatment, if appropriate (but see Section 1.4.1.2), could be applied. However, many situations, such as head trauma or a first stroke, strike suddenly and without warning. In these cases, the most beneficial type of therapy could be effective even if applied sometime after the injury. There is a substantial amount of evidence to indicate that the chain of events that results in excitotoxicity-induced neuronal necrosis is a time-dependent phenomenon (e.g., see Choi, 1987a; Choi et al., 1988) and that there is a
"therapeutic window" during which treatment is expected to be successful. The "delayed rescue" of CNS neurons, both in vitro and in vivo, has been demonstrated in a variety of situations (Rothman et al., 1987; Steinberg et al., 1988; Gill et al., 1988; Hartley and Choi, 1989; Bakker and Foster, 1991; Kochhar et al., 1991). The antagonism of EAA-induced toxicity is therefore a rational form of therapy for stroke and CNS trauma, as it affords the clinician a certain amount of time to apply treatment before irreversible damage occurs.

1.3.2. Uncompetitive NMDA antagonists

One class of NMDA inhibitors, the uncompetitive antagonists, acts by decreasing current flow through the NMDA ion channel subsequent to receptor activation. Compounds from this class, including MK-801 (Wong et al., 1986) and the dissociative anaesthetic ketamine (Martin and Lodge, 1985; MacDonald et al., 1987), bind to the PCP (phencyclidine) site within the channel pore (Collingridge and Lester, 1989; MacDonald et al., 1991). The ability of uncompetitive NMDA antagonists to protect neurons from excitotoxic challenge has been well-documented (Weiss et al., 1986; Lysko et al., 1989; Hartley and Choi, 1989; Grigg and Anderson, 1990; Lippert et al., 1994). Channel blockers acting uncompetitively may offer some advantage over competitive NMDA antagonists. For example, due to the nature of uncompetitive block, drug actions on open channels are enhanced during excessive NMDA activation; thus, the effects of uncompetitive blockade, unlike competitive antagonism, would not be overcome by increased levels of agonist (Lipton, 1993; Baukrowitz and Yellen, 1996). At present, however, the clinical utility of uncompetitive drugs has been limited by their
neurobehavioural side effects, including motor impairment (Willets et al., 1990; Rogawski et al., 1991).

1.3.2.1. Opiates

Agents from the opiate class of drugs have been found to protect neurons against challenge by high levels of NMDA (Choi and Viseskul, 1988). This protective effect occurred despite an NMDA ion channel-stimulating effect induced by opiate-mediated stimulation of intracellular PKC activity (Chen and Huang, 1991; see section 1.1.3.10). Opiates that protected neurons from NMDA-induced injury included methadone, morphine, dextrorphan, and levorphanol; others that did so but much more weakly (i.e. with higher IC₅₀'s) were fentanyl, dextropropoxyphene, naloxone, and nalorphine. The high concentrations employed (millimolar or high micromolar range), the reversed stereoselectivity compared with classical opioid effects, and the effectiveness of the opioid antagonists naloxone and nalorphine in this model suggest that the protective effects did not occur through classical μ, δ, or κ receptors. On the other hand, the κ-opiates U50,488H and U62,066E (spiradoline mesylate) protected hippocampal neurons in an in vivo gerbil ischaemic model while the close structural relative but non-κ compound U54494A did not have any effect (Hall and Pazara, 1988; Gomez-Pinilla et al., 1989). It is unknown whether or not the endogenous κ-opioid dynorphin A exerts its modulatory effects on NMDA receptor activity (Shukla and Lemaire, 1994) through mechanisms similar to those through which opiates produce anti-excitotoxic neuroprotection, although it should be noted that the selective κ-opiate
ethylketocyclazocine has been shown to have no effect on NMDA-evoked responses in cat spinal cord (Berry et al., 1984).

1.3.2.2. Antitussives

One class of drugs that has shown considerable promise for clinical management of excessive NMDA activity, such as might occur in stroke or epilepsy, is the antitussives. Many of these agents were originally thought to behave as opiates in their antitussive actions, similar to the μ-opioid receptor-mediated respiratory depressive effects produced by compounds from the opiate class. Indeed, many antitussives, such as the morphinan dextrophan and its derivative dextromethorphan, are simply optical enantiomers of classical opiates, levorphanol and methyllevorphanol, respectively. However, since many compounds that behave similarly in their antitussive effects, e.g. caramiphen and carbetapentane, are not related to opiates, it is now accepted that this class of compounds exerts its antitussive effect through a CNS mechanism distinct from the opioid system. Another important feature of DM's pharmacological profile was demonstrated by the discovery of a distinct high-affinity dextromethorphan (DM) binding site in the brain (Craviso and Musacchio, 1983a,b). Non-opiate antitussives have been shown to reduce NMDA responses (Fletcher et al., 1995). The stereoselectivity for NMDA antagonist action (dextrophan > levorphanol) is the opposite to that seen with opioid effects (levorphanol > dextrophan) (Church et al., 1985). Binding data have shown dextrophan to compete for \(^{3}H\)PCP binding in rat brain cortex and to share a binding locus with many other PCP site ligands (Mendelsohn et al., 1984). The observation of selective NMDA blockade by
dextromethorphan and dextrorphan, with levorphanol being considerably weaker, in cat and rat spinal neurons confirmed their putative actions on the PCP site in or near the NMDA ion channel (Church et al., 1991b). A neuroprotective effect against NMDA challenge that paralleled the results of Church et al. (1991b) has been demonstrated (Choi, 1987b). It should be noted that all of the above effects occur at concentrations somewhat (~10X) above those that are believed to occur in the CNS for remediation of cough, suggesting the antitussive and neuroprotective effects are mediated by separate systems. The demonstration of DM's neuroprotective efficacy in vivo have led to recent clinical trials for dextromethorphan and dextrorphan as adjuncts in stroke therapy.

1.3.2.3. Sigma ligands

Many agents that bind to the PCP site in the NMDA ion channel are also ligands of the sigma (σ) receptor, an entity once thought to be a subtype of the opioid receptor but now considered to be separate from the other opioid receptor subtypes, μ, δ, and κ. For a review of σ receptor pharmacology and function, see Walker et al. (1990). Sigma agents have been found to modulate the NMDA receptor-ion channel complex in a biphasic manner, with low (nanomolar) concentrations potentiating NMDA activity (Monnet et al., 1990; Monnet et al., 1992) and high (micromolar) concentrations inhibiting NMDA activity (Keana et al., 1989; Monnet et al., 1992; Fletcher et al., 1993). While it is possible that the depression of NMDA receptor-mediated events by σ ligands represents a direct action of these agents on NMDA ion channels, little information regarding this issue is available.
1.3.3. (-)-β-cyclazocine

Inhibition of NMDA responses by 5,9-dialkylbenzomorphan "analgetic antagonists" (Gordon et al., 1961) such as pentazocine and α-cyclazocine shows many of the properties expected of uncompetitive channel blockers acting at the PCP site (Thomson and Lodge, 1985; Sagratella et al., 1985; Church and Lodge, 1990); compounds from this class also serve as neuroprotectants (Olney et al., 1986; Lysko et al., 1992). The related compound (-)-β-cyclazocine (the trans isomer of α-cyclazocine; Lasagna et al., 1964) accesses the CNS (Lasagna et al., 1964) and has been found to interact with the PCP site of the NMDA channel (Todd et al., 1990). In a rat cortical wedge preparation, (-)-β-cyclazocine inhibited depolarizations induced by NMDA; in rat spinal cord neurons studied in vivo, the agent attenuated the increase in firing rate produced by iontophoretic application of NMDA (Church et al., 1991a). Both of the above experiments, employing extracellular macroscopic recording techniques, showed that (-)-β-cyclazocine has an IC$_{50}$ against NMDA responses in the submicromolar range. The high potency of (-)-β-cyclazocine suggests detailed studies with this compound to elucidate its molecular mechanism of action are warranted. Furthermore, the NMDA antagonist properties of this agent earmark it as a candidate for neuroprotection; therefore, assessment of (-)-β-cyclazocine's ability to protect neurons from excitotoxic challenge would have the utility both of providing information about a novel neuroprotective compound and of demonstrating the predictive power of NMDA antagonism data for screening potential neuroprotectants.
1.3.4. Dextromethorphan

As discussed in section 1.3.1.2, dextromethorphan (the in vivo effects of dextromethorphan are no different from those of dextrorphan: methylesterifying the free hydroxyl group of dextrorphan produces dextromethorphan, and this process is reversed in vivo to the extent that much of the former is available to the site of action; Tortella et al., 1994) has been shown to protect neurons from NMDA challenge (Choi, 1987b), to produce neuroprotection in animal ischaemic models (Steinberg et al., 1988; George et al., 1988), and is currently undergoing clinical trials for use against stroke-induced brain damage. While its neuroprotective property (Choi, 1987b) may be related to its action against NMDA responses (Church et al., 1985), little is known about its interaction with single NMDA receptor-ion channels. The assumption that it acts uncompetitively to block NMDA-induced currents by blocking open NMDA ion channels has never been tested; this information is crucial to understanding how blockade of NMDA ion channels proceeds towards neuroprotection, and might aid in the development of dextromethorphan-related agents that would be useful in clinical therapy.

1.3.5. L-687,384

L-687,384 (1-benzylspiro[1,2,3,4-tetrahydro-1,4-naphthalene-1,4-piperidine]) is a high affinity ligand for haloperidol- and ditolylguanidine (DTG)-sensitive sigma sites. In an in vitro radioligand binding assay, [3H]DTG was displaced by L-687,384 from crude guinea pig brain membranes; another σ ligand, [3H]SKF-10,047, was potently displaced by L-687,384 in an in vivo radioligand binding assay (Middlemiss et al., 1991). In this study, L-687,384 had minimal affinity for D2 dopamine receptors, α-adrenoceptors, and
5-HT$_{1A}$ serotonin receptors. *In vitro* displacement of [$^3$H]haloperidol by L-687,384 from rat and human cerebellum was subsequently demonstrated (Barnes *et al.*, 1992); in these authors' hands, L-687,384 was the most potent and selective agent for σ receptors compared to a number (> 20) of other σ agents. Like other σ ligands, L-687,384 produced a bell-shaped concentration-response curve when tested on NMDA-evoked excitations *in vivo*, showing NMDA antagonism at high concentrations (Bergeron *et al.*, 1992).

1.4. Experimental approach

1.4.1. Rationale

For an agent to be appropriate for the clinical management for conditions of hypoxia and ischaemia, such as in thrombotic or aneurytic stroke, hypoglycaemia, or CNS trauma, certain conditions must be met. For example, the agent in question must be stable enough to survive in storage, and to survive the route of administration; the body's metabolic processes must allow the agent to be present in sufficient quantities to be effective at the site(s) of action. Other considerations are outlined in the sections below.

1.4.1.1. Blood-brain barrier

To be useful as a CNS agent in clinical practice, a systemically administered compound must be sufficiently lipophilic to traverse the blood-brain barrier. Many compounds can be shown to be effective as neuroprotectants in *in vitro* models but would not offer any utility as clinical agents because of low blood-brain barrier permeation. This is illustrated by the competitive antagonist APV, which can protect
neurons from the damaging effects of anoxia *in vitro* (e.g., Grigg and Anderson, 1990) but whose molecules contain sufficient charge that blood-brain barrier penetration is negligible.

1.4.1.2. Lack of toxicity

A suitable agent would be minimally toxic, both within and outside the CNS. A striking example of the toxicity problem arises in the case of the anticonvulsant uncompetitive NMDA antagonist MK-801, which is an effective neuroprotectant and anticonvulsant *in vitro* (Wong et al., 1986; Huettner and Bean, 1988; Lippert et al., 1994), yet produces far too many untoward CNS effects, such as motor impairment, to be useful in the clinic (Willetts et al., 1990). The toxicity of MK-801 has been attributed to its length of action, which in turn is manifested by its prolonged interaction with its binding site in the NMDA ion channel (Chen et al., 1992; Lipton, 1993). Analysis of the kinetic parameters associated with the drug-channel site interaction revealed slow rates for both association with and dissociation from the channel site (Chen et al., 1992), whereas better tolerated compounds exhibited faster kinetics of interaction. The identification of compounds that interact with the binding site quickly, rather than slowly, would be a powerful tool in screening for effective yet safe neuroprotective agents (Rogawski, 1993; Lipton, 1993). Therefore, when assessing the suitability of a given agent as a neuroprotectant, kinetic parameters are of great initial importance.

1.4.1.3. Selectivity

An agent is most useful if it affects only the specific process(es) involved in the pathology in question. In the present case, the central importance of NMDA receptors
in mediating the effects of excitotoxicity makes it a rational target for the amelioration of the damaging effects of excitotoxicity. Activity of an otherwise suitable compound against other, non-excitotoxicity-related, systems would be undesirable and could contribute significantly to unwanted side effects. Therefore, studies of a potential neuroprotectant’s selectivity for the NMDA responses, compared with responses mediated by other systems, such as non-NMDA receptors and voltage-gated ion channels, would be a crucial step in assessing its clinical suitability.

1.4.1.4. Functional efficacy

The final step in assessing a candidate drug's therapeutic potential as a neuroprotectant in vitro is to apply the drug to whole neurons that have been challenged with excitotoxic conditions. NMDA, at a concentration of 1 mM, is sufficient to induce delayed-phase neurotoxicity in vitro and is roughly analogous to conditions present during in vivo excitotoxic stress. The potency and efficacy of a candidate neuroprotectant can then be studied by exposing the stressed cells to varying concentrations of the blocking agent and assessing its effect on the NMDA-induced damage.

1.4.2. Hypothesis

Desirable characteristics of neuroprotectant drugs can be predicted by analysis of a given agent’s kinetic parameters of interaction with the NMDA receptor-ion-channel complex, as well as that agent’s selectivity for NMDA responses.
1.4.3. Tissues employed

1.4.3.1. Cultured neurons

Cultured neurons from the rat were chosen for study for a number of reasons. A great deal of work has been done on this type of neuron, in this laboratory as well as other laboratories, and their characteristics are well-described. Prior demonstration of NMDA receptor-ion channel activity indicates that these neurons are suitable for studies in which NMDA activity is a necessary component. The ease of manipulation of cultured neurons allows a variety of treatments to be performed on the same type of preparation; the neurons can be maintained in culture for up to 3 weeks and cells can be available at any given time for experimental use. Lastly, the large number of cells available from cultures greatly facilitates experiments in which large quantities of cells are required.

1.4.3.2. CNS areas used

Both hippocampal and cortical neurons were selected for use. Different areas of the CNS are differentially sensitive to excitotoxic damage (Baumgarten and Zimmerman, 1992); therefore, it was necessary to study 2 cell types so that the results would be applicable to a wider range of CNS cell types than if only one type were used. The characteristics of neurons from the hippocampus and cortex are well-described (Mattson and Mark, 1996) and the use of cells from these two areas would facilitate comparisons with previously published data. Pyramidal neurons of the hippocampus in vivo are exquisitely sensitive to hypoxic/ischaemic damage (Kirino, 1982; Suzuki et al., 1983). While it can be argued that the hippocampus may be in a position to be the
most affected by an interruption in blood flow, as in hypoxia-induced vasospasm, the observation that granule cells in the dentate gyrus of the hippocampus are strikingly resistant to this treatment suggests pyramidal neurons possess a susceptibility to excitotoxic damage not necessarily shared by all central neurons (Smith et al., 1984; Siesjö, 1986). Neurons of the frontal cortex also show vulnerability to ischaemic damage, but less so than hippocampal neurons (Pulsinelli et al., 1982; Pulsinelli and Duffy, 1983; Smith et al., 1984; Siesjö, 1986). Nevertheless, neocortical neurons are important because of their proclivity to excitotoxic damage following epileptic seizures or certain types of stroke. Additionally, conditions which involve degeneration of frontal cortex neurons, such as Alzheimer's disease, may involve excitotoxic mechanisms, and it is therefore useful to examine the properties of this cell type that are relevant to excitotoxicity and the amelioration of this process (Baumgarten and Zimmerman, 1992).

A series of experiments were carried out in neurons to ascertain effects of DM and (-)-β-cyclazocine on voltage-gated ion currents. The purpose of the experiments was to determine if the compounds could have significant effects to alter electrical transmission in neurons. In particular, interactions of these compounds with voltage-gated currents involved in action potential generation, such as sodium ($I_{Na}$) and potassium ($I_{K}$) currents, might compromise their use as neuroprotectants. The cultured neurons used in the NMDA experiments (either hippocampal or cortical) were not suitable for these studies since their relatively small size ($\sim 10 \times \sim 4 \mu m$) yielded small macroscopic currents. Thus, although $I_{Na}$ and $I_{K}$ were visible, they were sufficiently small to preclude pharmacological analysis. Instead, a cell line from mouse dorsal root ganglion developed by Dr. S. U. Kim has been employed in the macroscopic current
experiments. The cells from this line were larger than either the hippocampal or cortical neurons, with lengths up to 20 μm, and were found to express robust $I_{Na}$ and $I_K$.

1.4.3.3. Cardiac myocytes

Care must be taken in the clinical application of agents that affect ion channel function because they may not affect only neuronal tissue, but non-neuronal excitable tissue as well. The heart is an example of an organ consisting of the latter type of tissue, and particular attention must be paid to an agent's possible toxic effects on cardiac ion currents if it is to be tried for clinical practice, for example. Therefore, cardiac ventricular myocytes acutely isolated from adult Wistar rats were also employed in this series of experiments in order to check for side effects of (-)-β-cyclazocine and DM on cardiac function. These cells expressed robust $I_{Na}$ and two types of $K^+$ current: $I_o$ (the transient outward $K^+$ current) and the delayed-rectifier current. It should be noted that the $I_{Na}$ in myocytes has some different properties from $I_{Na}$ in neurons; for example, the concentration of TTX required for block of $I_{Na}$ in myocytes is much higher, by at least 10-fold, than that required to block $I_{Na}$ in neurons. The $I_o$ is the primary repolarizing current in ventricular myocytes and has similar pharmacology to $I_A$ in neurons, an example being the block of both currents by 4-aminopyridine (4-AP). The delayed rectifier $I_K$ has similar properties in heart and in neurons, as exemplified by the inhibition of both currents by tetraethylammonium (TEA).
1.4.4. Strategy

A multi-tiered experimental strategy has been utilised in order to provide a rational approach for identifying candidate neuroprotectant drugs. Agents were initially screened for NMDA antagonist activity using outside-out patches from both hippocampal and cortical neurons. Selectivity of these agents for NMDA vs. non-NMDA responses and NMDA ion channels vs. voltage-gated calcium channels was then investigated using microspectrofluorimetric measurements of intracellular calcium responses to excitants specific for the above systems. Use-dependence of the agents was also determined with this technique, thus yielding information about the mechanism of neuroprotectant action. Functional assays on whole hippocampal and cortical neurons in culture were then performed as a final gauge of neuroprotectant activity. Finally, additional experiments were performed to further elucidate the biophysical mechanism of action and the selectivity of candidate neuroprotectants, the former involving temperature manipulation of outside-out patches exposed to NMDA and/or putative neuroprotective drugs, and the latter consisting of whole-cell experiments measuring effects of the drugs on sodium and potassium currents, using acutely isolated rat cardiac myocytes.
2. MATERIALS AND METHODS

2.1. Tissue culture

Day-18 Wistar rat hippocampal or cortical cultures were prepared according to the method of Banker and Cowan (1977). Hippocampi or cortices of day-18 foetal Wistar rats were dissected, cells were isolated by trituration of the tissue through Pasteur pipettes, and suspended in separate Hank's Balanced Salt Solution (HBSS; Gibco), modified with (mM; source) NaHCO₃ (4.5; Fisher), glucose (27.8; Sigma), hydroxyethylpiperazine ethanesulphonate (HEPES) (15; Sigma), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), and adjusted to pH 7.14 - 7.18 with NaOH (Fisher), for counting in a hemocytometer. Once counted, the cell suspension was diluted to 10⁵ cells/ml, with Dulbecco's Modified Eagle's Medium (DMEM; Gibco), to which was added (final concentration in mM) NaHCO₃ (45), glucose (8.33), HEPES buffer (10), penicillin (100 U/ml), streptomycin (100 μg/ml), horse serum (20%; Gibco), and, if prevention of yeast contamination was necessary, fungizone (Gibco; if added, this would result in 135 nM amphotericin B and 247 nM sodium deoxycholate in the DMEM) and plated onto 18 mm circular glass coverslips, or, in the case of hippocampal neurons destined for the neuroprotection experiments, onto poly-D-lysine/laminin (Sigma)-coated 12 mm circular glass coverslips from a suspension of 3 x 10⁵ cells/ml. Coverslips containing neurons were inserted into Falcon 6-well plates (Becton Dickinson) and incubated in 2 ml modified DMEM (as above) in a 5% CO₂/95% air atmosphere at 35.5 - 36.0°C until use. Hippocampal neurons were placed into the wells upside-down (i.e., cells on the underside of the coverslip as in Brewer and
Cotman, 1989) while cortical neurons were inserted face-up. For cortical neurons intended for use in the neuroprotection experiments, cells were plated directly onto the floor of 12-well tissue culture plates (Falcon) and incubated in modified DMEM. Cells survived under these conditions for up to 8 weeks. In these neurons, \([\text{Ca}^{2+}]_i\) and unitary current responses to NMDA are fully expressed one week post-isolation and remain stable throughout the second week; cells that had been between 7 and 17 days in culture were used for the electrophysiological and microspectrofluorimetric experiments. In the neuroprotection experiments, cells were used 13 - 14 days following isolation, as this age showed maximal susceptibility to the degenerative effects of NMDA.

2.2. Electrophysiology

The advent of patch clamp techniques for measurement of cell membrane currents has allowed investigators to study the behaviour of single ion channels. Additionally, using the patch clamp technique, current through the entire cell membrane can be measured with minimal disruption of the membrane, thus averting leakage of cell contents into the extracellular medium and allowing the cell to maintain its own membrane potential. This compares favourably with conventional intracellular microelectrode recording where the cell is punctured, resulting in leakage of cellular contents into the extracellular medium and loss of membrane potential. The ability of the patch clamp technique to detect tiny currents in cell membranes is due to two advances that occurred in the field of electrophysiological recording in the late 1970's, the first being the inclusion of a high resistance feedback resistor in the recording headstage, and the second being the development of a procedure that allowed the
formation of a high-resistance, gigohm seal between the membrane and a solution-filled glass recording electrode (Hamill et al., 1981) so that current leak between the electrode and the membrane patch was eliminated.

2.2.1. Patch clamp configurations

The details of patch-clamp recording are too extensive to completely describe in this thesis; for a thorough treatment of the technique, consult Hamill et al. (1981) and Sakmann and Neher (1984). Briefly, there are four configurations that one can use to record membrane activity. The first, and most basic, is the "cell-attached" configuration, in which unitary currents are measured while the recording electrode is positioned on an intact cell. Excising the patch by pulling the electrode away from the cell produces a membrane patch on the tip of the electrode in the "inside-out" configuration. In this mode, the inner surface of the membrane patch is exposed to the contents of the external recording medium. Alternatively, once the cell-attached configuration is achieved, the patch of membrane enclosed by the recording pipette can be broken, using either high suction or high-voltage pulses. This makes the interior of the cell continuous with the contents of the pipette so that currents through the entire cell membrane can be detected; this is the "whole-cell" mode of recording and was used in the present work to study macroscopic currents in cultured mouse DRG neurons and isolated rat ventricular myocytes. The final configuration involves removing the electrode from the cell, as is done to achieve the inside-out configuration. However, if this manoeuvre is performed while starting from the whole-cell configuration, instead of the cell-attached mode, the patch spontaneously forms the "outside-out" configuration.
2.2.1.1. Outside-out patches

The outside-out patch configuration is the most informative single-channel mode in terms of investigating drug potency and mechanisms. In this preparation, the exterior surface of the membrane is exposed to the extracellular medium, while the contents of the recording electrode replace the internal environment of the cell. One is thus enabled, through exchange of the surrounding bath medium, to apply several treatments, such as several drug concentrations, along with control and recovery, to a single patch. A drawback to the outside-out configuration is that it is the most difficult patch clamp preparation to achieve and maintain. It is necessary to attempt many trials before a reasonably stable and noise-free patch can be obtained. As well, the period when the patch signal is "clean" enough to discern single-channel activity is often too brief to perform an entire experiment; indeed, many patches last only seconds before the patch seal deteriorates to an unacceptable level. Furthermore, a patch that is suitable for study ideally contains only one active ion channel. Many patches exhibit no channel activity. Others have more than one active channel; the records from these patches are too complex to interpret and thus, as with silent patches, must be discarded. As for the presence of other active ion channel species in a patch, these must be suppressed either by pharmacological means or by using an ionic environment appropriate to the system being studied. For example, when studying NMDA ion channels (which are permeant to Na⁺ and K⁺), voltage-dependent Na⁺ and K⁺ channel activity must be inhibited by the addition of tetrodotoxin to the bath and by the use of caesium to replace potassium in both the bath and the pipette. Another potential drawback is the extent to which the manipulations performed to achieve the outside-out
configuration disturb the normal functioning of the ion channels in the patch. To this end, work in our laboratory has shown the unitary properties of NMDA ion channels in outside-out patches are comparable to those observed in cell-attached patches, despite loss of intracellular factors upon excision to the outside-out configuration.

2.2.2. Single-channel patch clamp procedure

2.2.2.1. Pipette preparation

Patch clamp electrodes were fabricated from pipette glass (A-M Systems) made from Corning #7052 glass without microfilaments, with an outer diameter of either 1.2 mm (0.68 mm inner diameter), or 1.65 mm (inner diameter of 1.2 mm). The pipettes were prepared with a standard two-pull technique using a Narishige PP-83 glass microelectrode puller, producing pipette electrodes with tip diameters of approximately 1-2 µm corresponding to a resistance of 4 - 8 MΩ once filled with bath solution (see below). The pulled pipettes were then fire-polished on a microforge consisting of a glass-coated heating filament, whose temperature and heating area were controlled by a jet of air, positioned on the stage of an ordinary laboratory ocular microscope. The polished electrodes were filled with pipette solution, consisting of (mM; all from Sigma) CsCH₃SO₃ (140), CaCl₂ (0.5), ethylene glycol-bis(β-aminoethyl ether)-N,N',N'′,N'′-tetraacetic acid (EGTA; 5) and HEPES (10), immediately before use. Previous to filling, all pipette solutions were filtered twice using Millipore Type GS circle filters with a 0.22 µm pore size.
2.2.2.2. The recording bath

Coverslips containing neurons were placed in a 5 ml Perspex circular recording chamber to which was added, to a level just above the top of the coverslip, bath solution consisting of (mM; source): NaCl (140; BDH), CsCl (5; BDH), CaCl$_2$ (1.8; Sigma), HEPES (10; Sigma), and TTX (0.001; Sigma); the pH was adjusted to 7.2 with NaOH (Fisher). Caesium was used in place of potassium as described in section 2.2.1.1. No Mg$^{2+}$ was added to any of the solutions, as this divalent cation has profound effects on the kinetics of the NMDA channel (Jahr and Stevens, 1990; Mayer et al., 1984; Nowak et al., 1984), but the possibility of contaminating Mg$^{2+}$ being present in the solutions necessitated an analysis of these solutions for residual magnesium; the Mg$^{2+}$ level in the bath solution, as determined by flame analysis on a Jarrell-Ash 280 atomic absorption spectrometer, was established to be 0.4±0.2·μM. The recording chamber was affixed to the stage of a Nikon TMS inverted microscope with attached phase-contrast optics. In this system, viable neurons were easily identified by a characteristic "glow" and the pipette tip could be clearly seen during the patch formation procedure. The bath was exchanged three times with bath solution after the cover slip was inserted so that any culture medium remaining on the coverslip was completely rinsed away. An agar-filled bridge was submerged in the bath and a wire connected to ground was inserted into the bridge so that the recording circuit was complete. Experiments were performed at room temperature (24°C).
2.2.2.3. Pipette mounting

Filled pipettes were loaded into the pipette holder which was mounted onto the headstage (Axon model CV-3 with 1/100 gain). These were mounted onto a Newport 360-90 lab jack fitted with a Newport 423 series actuator powered by a Newport model 861 handheld motion controller for manipulation of the pipette position; the microscope and lab jack were placed on a nitrogen-suspended floating table (Newport) so that the cells in the bath were in a vibration-free environment. After mounting into the pipette holder, a slight positive pressure was applied to the pipette to prevent buildup of debris around the tip area, and the pipette was rapidly lowered into the recording bath and manoeuvred into position just above a suitable neuron. Only smooth, healthy appearing cells with a bipolar shape and an average length of approximately 10-12 \( \mu \)m along the long axis were chosen for recording.

2.2.2.4. Patch formation

The pipette was slowly lowered into physical contact with the soma of the cell; pipette contact with the cell was viewed through the microscope and was also monitored using a 0.2 mV test pulse with an audio signal being applied during the lowering procedure. Unitary currents were detected with the Axopatch 1B patch clamp amplifier (Axon Instruments, Foster City, CA), which also sent out the test pulses, and the Axopatch output signal was visualized on a Kikusui 5020 20 Mhz digital oscilloscope. When the pipette was touching the cell, a gigohm (>10 G\( \Omega \)) seal was made by applying a small negative pressure through a mouth suction tube which was hooked up to the pipette holder. The large increase in pipette tip resistance associated
with seal formation was monitored by the disappearance of the 0.2 mV test pulse on the oscilloscope screen and was confirmed by the audio feedback signal characteristic of a tight seal. Attempts to form a gigohm seal were not always successful, and if only a partial seal was attainable the pipette was discarded. A greater suction, and, if necessary, a 0.2 V pulse of between 1 and 10 ms duration, was then applied in order to enter the whole-cell configuration. Following this manoeuvre, the patch was excised to obtain the outside-out configuration.

2.2.2.5. Patch clamp recording, data acquisition, and storage

The patch-clamp data were acquired using an Axopatch DigiData 1200 interface and recorded onto a 40 MHz 486 PC loaded with Microsoft MS-DOS 3.2 and pCLAMP version 6.0.2 (Axon Instruments Inc.). For backup recording, an Instrutech VR-10 digital data recorder was connected to a Panasonic Omnivision Hi-Fi MTS VHS, model PV-4760-K. Patch-clamp recordings were sampled at 5 kHz and filtered at 2 kHz with a low-pass -3 dB filter. In some cases, especially when the frequency of channel openings was low, an Al 2020 event detector was used so that computer recording commenced only when an event had occurred. Data from outside-out patches were recorded in 10-second segments, during which a hyperpolarizing potential, corresponding to a negative pipette potential ($V_p$), from the range -20 to -100 mV and in steps of 20 mV, was applied so that data were collected over a range of membrane voltages (as in Antonov and Johnson, 1996). Several recording segments were made at each $V_p$, and the sequence of pipette potentials applied for recording was constantly varied in order to eliminate any interactive effects with previously applied voltages. No
currents were apparent when patches were held at 0 mV, consistent with a reversal
potential of 0 mV for $[\text{Na}^+]_e = [\text{Cs}^+]_i = 140 \text{ mM}$.

2.2.2.6. Perfusion of solutions

Bath solutions, as in section 2.2.1.3, containing agonists (10 μM NMDA and 1
μM glycine; both from Sigma) or agonists + antagonists were stored in inverted syringe
tubes suspended above the level of the bath and allowed to flow into the bath by
gravity via polyethylene connecting tubes at a rate of approximately 2 ml/min. Solution
volume in the bath was kept at just over 2 ml by removing solution by vacuum suction.
After forming a stable outside-out patch and any ambient activity recorded, agonist
solution consisting of bath solution plus 10 μM NMDA and 1 μM glycine was added to
the bath. Providing unitary currents with characteristics corresponding to those of
NMDA ion channel activity (generally, the most observable criterion was the value for
the channel conductance, which for NMDA ion channels in outside-out patches is in the
range 40-50 pS; Ascher and Nowak, 1988; Bertolino et al., 1988) were apparent in the
patch, recordings were then made at various patch potentials. After this, the solution in
the bath was switched to the same solution with the addition of a putative antagonist.
The antagonists studied were dextromethorphan (5 - 50 μM), L-687,384 (5 - 40 μM; a
gift from Dr. D. N. Middlemiss, Merck, Sharp, and Dohme Research Laboratories,
Harlow, U.K.), (-)-β-cyclazocine (0.05 - 4 μM; Sterling-Winthrop, U.K.), and (+)-β-
cyclazocine (1 - 10 μM; Sterling-Winthrop, U.K.). Generally, for addition of agonist
solution, and for addition of agonist + antagonist solution, a total of 10 ml was allowed
to flow out of the syringe tube; this was more than sufficient for complete exchange of
the bath solution. For recovery conditions, where solution containing only the agonists was used to wash out antagonist, a greater amount of solution was necessary for complete removal of antagonist from the patch; in some cases this required up to 30 ml.

2.2.2.7. Data analysis

Unitary currents were analyzed using the Fetchan module from pCLAMP. Using this module, single-channel events in the data records were selected manually according to the criteria outlined below, and the amplitude, open time, and duration of the previous closed interval for each selected event were stored in an idealized record. Events selected for the idealized record were constrained to conform to minimum amplitude criteria (see Section 2.2.2.8); these criteria were unique for each pipette voltage and were established such that selecting events conforming to these criteria would produce monotonic, normally-distributed amplitude histograms. With the filter settings used and the seal resistances obtained in this study, only openings of 400 μs or greater were properly resolved; openings briefer than this were not selected for the idealized records. The minimum resolvable closed time duration was set at 100 μs. The pSTAT subroutine of pCLAMP was then used to calculate the mean amplitudes, mean open times, mean event frequencies, and closed time constants from this idealized record. This module was also used to generate histograms. The amplitude, open time, and closed time histograms, such as those shown in section 3.2, were plotted using a Hewlett Packard Color Pro graphics printer. A minimum of 200 events was included in each open and closed time histogram for kinetic analysis, except where high concentrations of potent antagonists were applied, necessitating a lower minimum
of 100 events per histogram because of the strong effect of some drugs to lower channel opening frequency. The open and closed time histograms were curve fitted by pSTAT, which uses a non-linear least-squares curve-fitting routine to fit 1 - or 2-exponential functions by the Levenberg-Marquardt (LMQ) technique with Simplex optimization. In this routine, the data were weighted by their $\chi^2$ values and, starting from seed values that were either manually entered or automatically determined by pSTAT, the fit proceeded until convergence, defined either when improvements in $\chi^2$ were less than $2.5 \times 10^{-7}$, or if all of the parameters converged when parameter change vectors went to zero. In order to determine if a particular histogram could be fitted with more than two exponentials (as in the case of the closed duration histograms), the two fast time components would first be fit to the left-most portion of the histogram; then, the curve fit would be performed again with the data range now modified to include the portion of the curve fitted with the second component plus the data to be fit with a third component, and so on. Errors are expressed as ±S.E.M., where $n$ refers to the number of patches studied; experiments were performed at room temperature (24°C).

2.2.2.8. Identification of unitary currents through NMDA ion channels

A patch may contain ion channels other than the NMDA channel under investigation. The following is a list of criteria used to identify unitary currents carried by NMDA ion channels. (i) Channel conductance. The NMDA ion channel is characterized by a conductance state with a conductance (G) value of 40 - 50 pS (Ascher et al., 1988; Ascher and Nowak, 1988). At a given $V_p$, unitary currents with amplitudes that did not conform to within 10% of this conductance range were not
included for analysis. It has been suggested that NMDA ion channels may themselves have multiple conductance states (Jahr and Stevens, 1987), with other states having conductance values lower than the primary 40 - 50 pS conductance state. When smaller currents were intermittently observed in the present electrophysiological experiments (see Section 3.2.1.1), they were not included in the analysis since the aim of the study was to investigate the primary high-conductance state of the NMDA-activated ion channel. (ii) Requirement for glycine. Unitary currents conforming to the 40 - 50 pS conductance criterion were observed at a vanishingly low frequency (< 0.1 Hz) when NMDA, without glycine, was perfused onto outside-out patches; perfusing NMDA with 1 µM glycine resulted in the activation of unitary currents at frequencies in the expected range (see Sections 3.2.3.4 and 3.2.9.4). Correlation of the frequency of NMDA-activated unitary currents and occupancy of the strychnine-insensitive glycine site has been previously documented (Johnson and Ascher, 1987). (iii) Sensitivity to 2-amino-5-phosphonovalerate (APV). Currents observed following application of NMDA and glycine to outside-out patches that conformed to the 40 - 50 pS conductance requirement were not observed if 100 µM APV was included in the perfusion solution (n = 4). Removal of APV from the perfusion solution resulted in the reappearance of unitary currents. APV is a competitive antagonist of NMDA receptors which acts by displacing agonist from its binding site (Peet et al., 1986; Olverman et al., 1988). (iv) Sensitivity to Mg²⁺. Application of Mg²⁺ to the external surface of outside-out patches exposed to NMDA and glycine produced an alteration in the appearance of unitary currents that was characterized by numerous fast transitions between an open level and a non-conducting level ("fast flickering"; cf. Figure 1). This action of Mg²⁺ is well-
known and represents fast-kinetics block of open NMDA ion channels (Nowak et al., 1984; Jahr and Stevens, 1990). The single-channel patch clamp studies in the present work are based on the behaviour, and the response of that behaviour, to a set of compounds, of unitary currents fulfilling the above criteria.

2.2.2.9. Statistics

As patches tested with the compounds served as their own control (i.e. control and drug values were non-independent), data were generally expressed as the mean of the percents of the value obtained in the presence of the compounds tested to that obtained in the control for each patch, ± the standard error of the mean. Channel parameters (e.g. mean open time, channel open probability) were assumed to be normally distributed and therefore parametric t-tests utilizing the ratios were performed as outlined in Howell (1987); parametric statistics were obtained only for values where \( n \geq 3 \) (Antonov and Johnson, 1996). The \( P \) values obtained in the tests, representing the probabilities of Type I errors (i.e. the probabilities that the claimed treatment differences could have occurred by chance), have been included in the Results section.

2.3. Microspectrofluorimetry

2.3.1. Fura-2 experiments

A digital fluorescence microscopy system (Attofluor), set up for the dual-wavelength excitation ratio method (Gryniewicz et al., 1986), was used with hippocampal or cortical neurons, prepared as in section 2.1, loaded with fura-2, a fluorescent free-calcium indicator dye (Gryniewicz et al., 1986) to assess the effects of dextromethorphan, L-687,384, and \((-\)- and \((+)-\beta\)-cyclazocine on NMDA-induced
increases in free intracellular calcium, according to the procedure outlined below. In addition, this technique was used to determine the selectivity of (-)-β-cyclazocine for inhibiting NMDA activity relative to its effects on responses elicited via α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, kainate receptors, or high-voltage-gated calcium channels.

2.3.1.1. Fura-2 loading

Medium containing the acetomethoxy ester of fura-2 (fura-2-AM) was prepared by dissolving 2 μl stock fura-2-AM (Molecular Probes, Eugene, OR) in 25 μl dimethylsulphoxide (DMSO) and vortexing. Stock fura-2-AM was previously mixed with the dispersing agent Pluronic F-127 (Molecular Probes; see Poenie et al., 1986) before storing at -70°C and was thawed immediately before DMSO addition. This solution was slowly added, while vortexing, to 3 ml of loading medium, consisting of (mM) NaCl (136.5), KCl (3.0), NaH₂PO₄ (1.5), glucose (10), HEPES (10) with pH adjusted to 7.40 with NaOH, to which was added 10 μl bovine serum albumin (BSA; thawed by vortexing from stock previously frozen at -70°C). Each 18 mm cover slip containing foetal rat hippocampal or cortical neurons at a density of 10⁵ cm⁻² was loaded, using forceps sterilized in 75% ethanol, into 1 ml of this medium + 1 ml loading medium, without fura-2AM/DMSO/BSA, for a final fura-2 concentration of 7.1 μM, for 80 min at 35°C in 5% CO₂/95% air and then washed by incubating at room temperature (20°C) in 3 ml loading medium, without fura-2AM/DMSO/BSA, for at least 30 minutes. This last wash step was used to ensure complete hydrolysis of the esterified dye so that leakage of fura-2
both from the cells to the extracellular medium and into intracellular organelles was minimized (Puil et al., 1990).

2.3.1.2. Measurement of fura-2 fluorescence

A coverslip containing fura-2-loaded cells was inserted into a Perspex laminar flow-through chamber which in turn was inserted into a stainless steel bath; a rubber ring lined with silicone gel was used to seal the coverslip into the holder and prevent leakage of solution into the spectrofluorimetric apparatus. The chamber was locked into position on the stage of a Zeiss inverted microscope with phase-contrast optics and equipped with an imaging camera. A peristaltic pump was used to deliver solution from holding reservoirs into the bath chamber, at 2 - 3 ml/min, via an inlet port and suction was used to keep the solution level just above the top of the cells. The preparation was perfused with the same loading medium used above (without fura-2-AM/DMSO/BSA) to which 0.3 μM TTX and 2 μM glycine had been added. Rises in \([Ca^{2+}]_i\) were evoked by 20 s bolus applications of loading medium to which either 20 μM NMDA, 80 μM kainate, 40 μM (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), or 50 mM K⁺ (by substitution for NaCl) were added. Dextromethorphan, (5 - 100 μM), L-687,384 (10 - 100 μM), (-)-β-cyclazocine (0.02 - 5 μM), or (+)-β-cyclazocine (5 μM) were added through superfusion. A DC-powered 200 W Hg arc lamp was used to illuminate the cells for \([Ca^{2+}]_i\) measurement; a stepmotor-driven turret operated by computer was used to switch between two 10 nm bandpass differential interference filters to give first 334 nm and then 380 nm light for determination of bound \([Ca^{2+}]_i\) and free \([Ca^{2+}]_i\), respectively. The ratio of these two intensities gives a measure
of \([\text{Ca}^{2+}]_i\) which is independent of fura-2 concentration (Gryniewicz et al., 1985). During measurement of responses to excitant applications, ratios were acquired every 2 s; this rate was decreased to one every 20 s between excitant applications in order to minimize photobleaching and ultraviolet light-mediated cytotoxicity. Fluorescence intensities were detected at 510 nm from multiple neuronal somata simultaneously. The fluorescence signals were corrected for background before calculation of the ratio by measuring fluorescence intensity emitted from a portion of the coverslip where no cells were present. All fura-2 measurements were taken at 20°C.

2.3.1.3. Calculation of \([\text{Ca}^{2+}]_i\)

The concentration of cytosolic free calcium was calculated from the ratio of the emitted fluorescence at 334 nm to 380 nm (i.e., bound to free calcium) according to the method of Gryniewicz et al., (1985). Briefly, 

\[
[\text{Ca}^{2+}]_i = K_d \times \beta \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)},
\]

where \(K_d\) is the equilibrium dissociation constant for the association of fura-2 with cytosolic free calcium, \(\beta\) is the ratio of 380 nm light with zero \([\text{Ca}^{2+}]_i\), to 380 nm light with infinite \([\text{Ca}^{2+}]_i\), \(R\) is the experimentally determined ratio of 334 nm to 380 nm light, \(R_{\text{min}}\) is the ratio of 334 nm to 380 nm light with zero external calcium (\([\text{Ca}^{2+}]_j\)), and \(R_{\text{max}}\) is the ratio of 334 nm to 380 nm light with infinite external calcium. The values for \(\beta\), \(R_{\text{min}}\), and \(R_{\text{max}}\) were determined from calibration procedures using 10 \(\mu\)M of the calcium ionophore Br-A23187 either in the absence of \([\text{Ca}^{2+}]_j\), with 100 \(\mu\)M EGTA, or with excess (2 mM) \([\text{Ca}^{2+}]_j\). The published \(K_d\) value for fura-2 of 135 nM (at 20°C) was employed (Gryniewicz et al., 1985). Responses were measured as the difference between \([\text{Ca}^{2+}]_i\) at the peak of the response and \([\text{Ca}^{2+}]_i\) at the baseline immediately
previous to the excitant challenge. In this way drift of the baseline is accounted for. Results are expressed as mean±S.E.M., where \( n \) refers to the total number of neurons from which data were obtained under each experimental condition. Each experiment was performed on at least three different neuronal cultures.

2.4. Neuroprotection experiments

The properties of (-)- and (+)-\( \beta \)-cyclazocine as neuroprotectants were investigated by assessment of cell viability with the application of high concentrations of NMDA to cultured hippocampal or cortical neurons. Viability was assessed using a quantitative morphometric technique described below. Neurons were kept in maintenance medium (DMEM (Gibco) plus (mM; source), NaHCO\(_3\) (22; Sigma), HEPES (10; Sigma), 10% heat-inactivated horse serum (Gibco), and 6.0 g/L glucose (Sigma); pH was adjusted to 7.40 with NaOH (Fisher). Cultures were prepared in exactly the same manner as for the electrophysiological and microspectrofluorimetric experiments, with the exception of the modifications mentioned in section 2.1, and maintained in a 37°C incubator with a 5%CO\(_2\)/95% air atmosphere prior to use. All manipulations were performed in a laminar-flow fume hood with all surfaces sterilized by wiping with 95% ethanol. Solutions were added to and removed from the culture wells containing the coverslips by a pipet-aid automatic pipettor (Drummond); all media were filter-sterilized using a Falcon 7107 0.22 Micron Easy Flow filter (Becton Dickinson) and pH-adjusted to 7.40 with NaOH (Fisher) or HCl (Fisher) immediately before use. (-)- or (+)-\( \beta \)-cyclazocine were added using an Eppendorf micropipettor with Eppendorf disposable tips. Initially, the cultures were stained with trypan blue (Sigma; see Paul, 1975), which stains dead cells dark while leaving viable cells unstained, by adding 2 ml/well 0.4%
trypan blue in 0.81% NaCl (BDH) and 0.06% K$_2$HPO$_4$ (Sigma) for 10 minutes, at which time the stain was replaced with maintenance medium. The neurons were then counted, using a Nikon TMS microscope, by totalling the number of cells present in seven non-overlapping fields situated at pre-selected locations in the field of cells. Repeated counts of the same culture well confirmed the cell totals to be highly reproducible (within 20%). Only smooth, healthy-appearing cells that were not trypan blue-stained and that conformed to previous descriptions of primary hippocampal neuronal cultures (Olney et al., 1971; Banker and Cowan, 1977; Choi, 1987a; Rothman et al., 1987; Choi et al., 1988; Mattson et al., 1993) were chosen for counting. The culture wells were required to yield at least 100 cells in total when counted in this manner to be considered for later use in the experiment; however, most culture wells used yielded between 300-400 cells by this counting technique. Ten minutes prior to amino acid excitant challenge, the appropriate amount of (-)- or (+)-β-cyclazocine was added to each of the culture wells, except for those where only NMDA was to be added or those where neither NMDA nor β-cyclazocine were to be added. After 10 minutes, the maintenance medium in the wells was replaced with the same medium except for the addition of 1 µM glycine (Sigma), and, where appropriate, 1 mM NMDA (Sigma) and a given concentration of either (-)- or (+)-β-cyclazocine; all replacement media were adjusted to pH 7.40 with NaOH. After 24 hours, the cover slips were once again stained with trypan blue for 10 minutes, as above, and counted in exactly the same manner as before the amino acid challenge. Again, cells that had taken up trypan blue were excluded from the count. Results are expressed as the percentage of neurons remaining viable at the 24-hour count relative to those present in the pre-challenge
count; this is termed the survival rate for a given culture well. For results reported as percent neuroprotection, mean survival rates for (-)- and (+)-β-cyclazocine-treated culture wells within a given culture batch were first corrected for procedural cell loss (for example, suction of cells into the pipette during solution exchange), by dividing by the survival rate obtained in cultures where only 1 μM glycine was added, and then normalized to the survival rate obtained for control culture wells where 1 mM NMDA + 1 μM glycine (with no (-)- or (+)-β-cyclazocine) having been added. Results for control cultures (1 mM NMDA + 1 μM glycine) were also corrected for procedural cell loss as assessed by culture wells to which only 1 μM glycine had been added. The number of separate cultures (i.e., those derived from different mother rats) on which the experiment was performed was eight, which is referred to in section 3 as n. Within a given culture, each treatment was assigned to a minimum of 3 cover slips.

2.4.1.1. Statistics

The counts for the cover slips within a given culture were averaged and the results were expressed as the mean of the n = 8 such values for a given treatment, ± the standard error of the mean. Cell counts were assumed to be normally distributed across treatments and a one-way analysis of variance, followed by a Tukey post hoc test, were employed to determine significant changes in viability.

2.5. Temperature studies

Outside-out patches exposed to NMDA in the presence and/or absence of antagonist drugs were varied from 24°C to 10°C by a proportional temperature control system. The bath temperature was detected by Peltier devices, sampled, and
deviations away from a previously determined set point were corrected by a thermistor
device coupled to the bath setup. The setup used was similar to that described in
Chabala et al., 1985. After formation of an outside-out patch, NMDA and glycine were
added to the bath and control data collected. The bath containing the patch was then
cooled until a previously determined set point, usually 10°C below ambient room
temperature, had been reached, whereupon data were again collected. Recordings
were also made during the cooling run. When sufficient data had been obtained at the
lower temperature, the solution in the bath was replaced with a new solution containing
NMDA, glycine, and drug, and recooled to the original set point. Data were again
collected before allowing the patch to warm up to room temperature; recordings were
made during this warming phase, as well as upon reaching room temperature. When
possible, control solution containing NMDA and glycine, but no drug, was used to wash
out the drug and recovery data were taken. The cooling rate was set so that over a
ten-second data recording interval, the temperature changed by no more than 0.4°C.
The temperature response of the system was calibrated using a digital thermometer
inserted into the bath containing standard bath solution. The dependence of a certain
quantity on temperature was expressed as a $Q_{10}$±S.E.M., which is the ratio of the
values for that quantity over a 10°C change in temperature, expressed so that $Q_{10}>1$. It
should be noted that $Q_{10}$ depends on the specific temperature range used for the
calculation; therefore, all $Q_{10}$ values refer to measurements taken at 24.0±0.2°C and
14.0±0.2°C.
2.6. Experiments with voltage-gated Na\(^+\) and K\(^+\) currents

2.6.1. Preparation and use of DRG neurons

The neurons employed in the whole-cell experiments were from an immortalized cell line derived from rat dorsal root ganglion neurons and were developed in the laboratory of Dr. Seung U. Kim of the Department of Neurology at the University Hospital site of the University of British Columbia. Further details concerning the preparation and maintenance of this cell line should be directed to the above laboratory. The procedures employed in recording macroscopic currents from these neurons were identical to those used for the rat ventricular myocytes and are described below (Section 2.6.3).

2.6.2. Isolation of rat ventricular myocytes

The rat ventricular myocytes were dissociated and isolated from male rats (Sprague-Dawley) by slight modifications of procedures described previously (Mitra and Morad, 1985; McLarnon and Xu, 1995). Briefly, hearts were removed rapidly from animals anaesthetized with pentobarbitone and were perfused using a constant-flow Langendorff system with a zero-Ca\(^{2+}\) Tyrode's solution containing (mM; source): NaCl (133.5; BDH), KCl (4; BDH), MgCl\(_2\) (1.2; Sigma), NaH\(_2\)PO\(_4\) (1.2; Sigma), TES (10; Sigma), and glucose (11; Sigma); the pH was adjusted to 7.4 with NaOH (Fisher). The perfusate was oxygenated and maintained at 37°C. After 4 min, the perfusate was exchanged for another solution containing 0.07% collagenase (Type II, Worthington Biochemical), 25 µM CaCl\(_2\) (Sigma), and 200 mg of BSA (Boehringer Mannheim). After 15 to 20 minutes, the Tyrode's solution (containing 25 µM CaCl\(_2\)) was re-applied with gentle agitation of the digested tissue. The myocytes were stored at room temperature
between intervals of washing with successively increasing concentrations (up to 100 μM). Cells were plated on coverslips precoated with laminin (4 μg per coverslip) and used for electrophysiological study. Light microscopic examination revealed the cells had a mean length of 72±6 μm (n=22) and a mean diameter of 7.8±0.5 μm (n=22).

2.6.3. Whole-cell electrophysiology

2.6.3.1. Macroscopic recording

Currents were recorded from rat cardiac myocytes using the whole-cell patch-clamp configuration. Macroscopic currents, specifically transient outward K⁺ currents (Iₒ), and inward sodium currents (IₐNa), were recorded from isolated rat ventricular myocytes for periods up to 8 hours after plating. The details of the whole-cell patch clamp configuration are given in Section 2.2.1, and the procedure followed to obtain the whole-cell configuration was nearly identical to the procedure to obtain outside-out patches (Section 2.2.2); the exceptions to the procedure are herewith given. The micropipettes used were prepared in exactly the same fashion, except that lower resistances (in the range 2 to 4 MΩ) were used in order to improve access to the interior of the cell. The solution used to fill the micropipettes was different from that used for outside-out patch recording and consisted of (mM; source): NaCl (10; BDH), KCl (140; BDH), MgCl₂ (1; Sigma), EGTA (10; Sigma), Mg-ATP (5; Sigma), and HEPES (10; Sigma); the pH was adjusted to 7.4 with KOH (Fisher). The bath set-up was also identical to that described in Section 2.2.2, except that, since voltage-activated macroscopic currents were being studied, no agonists (e.g., NMDA) were included in the perfusion tubes. An Axopatch amplifier (200A, Axon Instruments) was used to
record currents with the low-pass filter set at 1 or 2 kHz; recording of currents began at the establishment of the whole-cell configuration without any attempt to excise a patch of membrane. Capacitative currents and series resistance were compensated by use of analog circuitry and leak subtraction was also used in some experiments; P/N subtraction was not routinely done and when carried out had no effect on macroscopic currents. The mean value of seal resistance was 36±5 GΩ (n=12); with series resistance compensation set at 80-85%, the mean series resistance was 4.2±1 MΩ (n=10) and the mean cell capacitance was 92±6 pF (n=6). The voltage protocols were generated by the CLAMPEX subroutine of pCLAMP (Axon Instruments) and data were recorded on disk for subsequent analysis. Descriptions of the voltage protocols have been included in the Results (Section 3.5.2). All experiments were carried out at room temperature (20-24°C).

2.6.3.2 Data analysis

The data analysis was performed using the CLAMPFIT subroutine of the pCLAMP software (Axon Instruments). This program was used to fit the inactivation time-course of \( I_o \) with single exponential functions, with the time-course denoted by \( \tau \); using dual- or multi-exponential functions did not improve the goodness of fit to the data. Peak current responses for \( I_o \) and \( I_{Na} \) were also measured using CLAMPFIT. Comparison was made of values obtained in this manner and those recorded in the absence and presence of either (-)-β-cyclazocine or DM, and were expressed as either the mean of the percentages or the ratios of the latter to the former, ± the standard error of the mean.
3. RESULTS

3.1. Organization

The organization of this part of the thesis is first considered. Firstly, four compounds have been studied in detail. These were: L-687,384, a ligand for the putative sigma (σ) receptor (Middlemiss et al., 1991); dextromethorphan (DM), a non-opioid antitussive; and the two optical isomers of β-cyclazocine (the geometric isomer of the 6,7-benzomorphan α-cyclazocine). All of these compounds have potential use as neuroprotectants. Since similar experimental strategies and protocols have been applied to characterize drug interactions for all four compounds, the results are considered on the basis of actions on various electrophysiological, microspectrofluorescent, and neuroprotective properties rather than entirely separate sections for each of the compounds. This organization allows a summary of overall salient points to be included in sections labelled as Conclusions; details specific to particular compounds are indicated separately. Thus, the results are organized as follows:

Electrophysiology

(A) Drug actions on amplitudes and conductance of unitary NMDA currents

(B) Drug actions on kinetics of unitary NMDA currents

(C) Data consistency with open channel blockade

(D) Analysis of data using open channel block scheme and conclusions

(E) Use of biophysical probes for mechanisms of channel block
Microspectrofluorescence

(F) Drug actions on NMDA-evoked \([Ca^{2+}]_i\) responses

(G) Drug selectivity for NMDA-evoked \([Ca^{2+}]_i\) responses vs. responses mediated via non-NMDA receptors and voltage-gated Ca\(^{2+}\) channels

Neuroprotection

(H) Neuroprotection assays

Actions of the compounds on voltage-gated ion channels

(I) Studies of drug actions on Na\(^+\) and K\(^+\) currents in neurons and cardiac myocytes

Conclusions
3.2. Electrophysiology

The results presented in this section place emphasis on (-)-β-cyclazocine, which was found to exhibit the most utility as a putative neuroprotectant. Thus, for clarity, the figures shown in this section will largely use this compound as a demonstration of drug actions on the NMDA ion channel, along with comparisons to its enantiomer, (+)-β-cyclazocine. Figures showing the actions of the other agents, dextromethorphan (DM) and L-687,384, are included either to illustrate particular effects unique to these compounds, or where the particular properties of these compounds afforded analysis superior to that which could be performed with (-)-β-cyclazocine. An example of the latter case was the determination of the voltage-dependence of drug actions with L-687,384. This compound did not reduce frequencies of channel openings, unlike the other compounds; hence, a large number of unitary events could be collected over a range of patch potentials from -100 mV to -20 mV (pipette potential, or $V_p$).

**HIPPOCAMPAL NEURONS**

3.2.1. Drug actions on amplitudes of unitary NMDA currents and channel conductance in hippocampal neurons

3.2.1.1. Amplitudes of unitary NMDA currents and channel conductance in control solution

Figure 1A shows typical single channel NMDA currents recorded from an outside-out patch isolated from a rat hippocampal neuron. The currents were recorded following application of 10 μM NMDA plus 1 μM glycine to the Mg$^{2+}$-free bath solution. A current-voltage (I/V) plot for the same patch is shown in Figure 2, along with a linear
Figure 1. Unitary NMDA currents in hippocampal neurons. (A) Typical unitary currents from an outside-out patch, taken from a hippocampal neuron exposed to 10 μM NMDA and 1 μM glycine (control solution) at the indicated $V_p$. (B) A record from the same patch after exposure to control solution with the addition of 20 μM Mg$^{2+}$. Channel openings are represented as downward deflections from a non-conducting baseline level.
Figure 1
least-squares fit to the data points. The slope conductance \( G \) was 40 pS. Overall \( (n = 18 \text{ patches}) \) the channel conductance was 44.4±4.1 pS at room temperature. Previous studies of outside-out patches from mouse central neurons have revealed the primary conductance state \( G \) for the NMDA channel of 40 - 50 pS (Ascher et al., 1988), as have investigations of cultured rat cortical neurons (Bertolino et al., 1988), and of cultured rat hippocampal neurons (Lester et al., 1990). It should be noted that the present experiments employed a higher external \( \text{Ca}^{2+} \) concentration (1.8 mM) than used for the earlier estimates (1 mM); it has been shown that \( G \) is diminished by increased external \( \text{Ca}^{2+} \) (Mayer and Westbrook, 1987). The reversal potential \( (V_{\text{rev}}) \), i.e., the voltage at which there was no observed current, was extrapolated to +4.3±0.7 mV for \( n = 18 \) patches. This value for reversal potential is close to that documented in previous studies (Ascher et al., 1988; Gibb and Colquhoun, 1991) and is consistent with the channel being non-selective for \( \text{Na}^+ \) and \( \text{K}^+ \) (or \( \text{Cs}^+ \)). Further identification of the channel was done by adding 20 pM \( \text{Mg}^{2+} \) to the perfusion (bath) solution (Figure 1B). The addition of \( \text{Mg}^{2+} \) resulted in rapid transitions between the open state and a non-conducting, or blocked, state. The action of \( \text{Mg}^{2+} \) to cause a fast channel block of the NMDA channel at hyperpolarizing potentials has been previously described (Mayer et al., 1984; Nowak et al., 1984). As well, addition of 100 pM 2-amino-5-phosphonovalerate (APV) to the external solutions eliminated the unitary currents (data not shown). Previous work has shown that APV acts as a competitive NMDA antagonist (Davies et al., 1981; Evans et al., 1982).

Occasionally, events of lower amplitude, i.e. with a conductance smaller than the primary conductance state of the NMDA channel, were observed in some patches, in both control conditions and in the presence of added agents. These
Figure 2. Current-voltage relation for the patch shown in Figure 1 in control solution. The slope of the linear regression fit to the data points is the channel conductance (G), which was 40 pS for this patch; the intercept of the fitted line with the abscissa is the zero-current, or reversal, potential ($V_{rev}$), which was 4 mV for this patch.
openings (generally about 50% of the size of the larger openings, with a voltage-independent mean open time near 2.5 ms) likely represented a subconductance state of the NMDA ion channel. Although the low conductance events were not common, the use of single-channel recording allowed for isolation of the large conductance events and rejection of the relatively infrequent small conductance events. In the latter case, the macroscopic currents are a sum from all states of the system and would include contributions from states other than the primary conductance level. It should be noted that previous studies have also documented lower conductance NMDA substates in hippocampal (Jahr and Stevens, 1987) and cerebellar (Cull-Candy and Usowicz, 1987) neurons. In the present study, only events belonging to the primary large conductance state were included for analysis.

The results obtained in control solution were then used as a basis for investigation of the properties of the potential neuroprotectant compounds L-687,384, DM, and (+)- and (-)-β-cyclazocine. The procedure was to apply 10 µM NMDA, along with 1 µM glycine, to the external face of outside-out patches, and, after recording at various patch potentials, to add one of the test compounds to the NMDA/glycine-containing external solution and again to record at various patch potentials. As noted above, reduction of the frequency of events by compounds other than L-687,384 often prevented the recording of data over a wide range of voltages. In some cases, more than one concentration of the agents was applied. The test compound was then washed out, and recovery recordings, in the presence of NMDA and glycine, were made. Data obtained in the presence of drug were compared with control and recovery
data in order to determine drug effects on the unitary properties of the NMDA ion channel.

3.2.1.2. Effects of the compounds on unitary NMDA current amplitudes and channel conductance

Typical unitary currents recorded from an outside-out patch in (A) control solution, (B) 1 µM (-)-β-cyclazocine and (C) 1 µM (+)-β-cyclazocine are shown in Figure 3. The $V_p$ was -40 mV and between (B) and (C), recovery data (wash-off of (-)-β-cyclazocine) was obtained (not shown). The amplitudes of the unitary currents in control (A) and in the presence of the applied agents (B) and (C), as well as in the recovery between (B) and (C), were near 2.0 pA. From the traces in Figure 3 (A-C) it is evident that neither enantiomer of β-cyclazocine altered the heights of the unitary currents induced by NMDA.

In order to quantitate the actions of (-)- and (+)-β-cyclazocine on the unitary conductance of the NMDA channel, distributions for channel amplitudes were constructed for currents recorded in the absence and presence of drug. Typical amplitude distributions are shown for control (Figure 4A), in the presence of 1 µM (-)-β-cyclazocine (Figure 4B), and after exposure to 1 µM (+)-β-cyclazocine (Figure 4C). As in Figure 3, current heights recorded with either drug present in the bath solution (up to 4 µM for (-)-β-cyclazocine and up to 10 µM for (+)-β-cyclazocine) were unchanged from control values over the voltage range studied (-100 mV $\leq V_p \leq$ -20 mV). Hence, neither enantiomer of β-cyclazocine altered the amplitudes of unitary currents relative to control values.
Figure 3. Unitary NMDA currents before and after exposure to (-)- or (+)-β-cyclazocine in hippocampal neurons. (A) Typical unitary currents from an outside-out patch, taken from a hippocampal neuron, in control solution. (B) Openings from the same patch exposed to control solution with the addition of 1 μM (-)-β-cyclazocine. (C) Openings with the control solution containing 1 μM (+)-β-cyclazocine. All solutions contained 10 μM NMDA and 1 μM glycine; $V_p = -40$ mV. Channel openings are represented as downward deflections from a non-conducting baseline level.
Figure 3

- Control

1 μM (-)-β-cyclazocine

1 μM (+)-β-cyclazocine
Figure 4. Amplitude distributions in the absence and presence of (-)- or (+)-β-cyclazocine in hippocampal neurons. Unitary current amplitude distributions ($V_p = -40$ mV) are shown for the same patch as in Figure 4: (A) in control solution, (B) with 1 μM (-)-β-cyclazocine, and (C) with 1 μM (+)-β-cyclazocine. All solutions contained 10 μM NMDA and 1 μM glycine. Amplitude distributions were fit with Gaussian functions with means (A) 1.88 pA, (B) 1.86 pA, and (C) 1.92 pA. The number of events included in each histogram was 587 in (A), 112 in (B), and 253 in (C). The relatively small number of events in (B) is due to the effect of (-)-β-cyclazocine to lower opening frequency.
Figure 4

(A) Graph showing the amplitude distribution with peak at 0 pA and two smaller peaks at -2 pA.

(B) Graph with a single peak at 0 pA.

(C) Similar to (B) with a peak at 0 pA.

Number of observations
Amplitude (pA)
Figure 5. Current-voltage relation for the patch shown in Figures 3 and 4 exposed to 1 μM (-)-β-cyclazocine. The slope of the linear regression fit to the data points is $G$, 39 pS for this patch. The intercept of the fitted line with the abscissa is $V_{\text{rev}}$, 6 mV for this patch.
Figure 5
Figure 5 shows an I/V plot for currents recorded from the patch shown in Figure 3 in the presence of 1 µM (-)-β-cyclazocine. For (-)-β-cyclazocine, as well as for (+)-β-cyclazocine (data not shown), the slopes of the I/V relations, as determined by linear least-squares fits to the points, corresponded to 40 pS conductances. The conductance (G) values for all patches were G = 40.6±2.5 pS for (-)-β-cyclazocine (n = 4) and G = 40.2±4.4 pS for (+)-β-cyclazocine (n = 5). A comparison with control data showed that neither enantiomer altered the conductance of the NMDA ion channel (see Section 3.2.1.1, Figure 2). The extrapolated reversal potential (V_{rev}) for the patch shown in Figure 5 was 4.0 mV, while for all patches V_{rev} was 5.6±0.4 mV for (-)-β-cyclazocine (n = 4) and 4.0±0.5 mV for (+)-β-cyclazocine (n = 5), respectively, and unchanged from the value found in control (4.3 mV). Thus, channel selectivity, which in these experiments embraced inward Na⁺ and Ca²⁺ and outward Cs⁺ flows, was not altered by either enantiomer of β-cyclazocine. The data for current amplitudes, G, and V_{rev} in control and in the presence of (-)- or (+)-β-cyclazocine are shown in Table 1.

Table 1 summarizes the values for unitary NMDA properties in outside-out patches from hippocampal neurons, for control solution and in the presence of all of the compounds studied. The amplitude measurements are the mean values of amplitude distribution means, while G and V_{rev} are from linear least-squares fits to I/V plots constructed for all the patches exposed to a given drug. The number of patches tested with a given drug is given by n; means are ±S.E.M.
Table 1. Unitary NMDA current amplitudes ($I_{\text{NMDA}}$), conductances ($G$), and reversal potentials ($V_{\text{rev}}$) for control solution and in the presence of the compounds indicated, as measured in outside-out patches from hippocampal neurons.

<table>
<thead>
<tr>
<th>compound</th>
<th>$V_p = 20 \text{ mV}$</th>
<th>$V_p = 40 \text{ mV}$</th>
<th>$V_p = 60 \text{ mV}$</th>
<th>$V_p = 80 \text{ mV}$</th>
<th>$V_p = 100 \text{ mV}$</th>
<th>$G (\mu\text{S})$</th>
<th>$V_{\text{rev}} (\text{mV})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-1.12±0.09</td>
<td>-2.02±0.06</td>
<td>-2.74±0.07</td>
<td>-3.64±0.11</td>
<td>-4.75±0.22</td>
<td>44.4±4.1</td>
<td>+4.3±0.7</td>
</tr>
<tr>
<td>(-)-β-cyc</td>
<td>-0.93±0.17</td>
<td>-1.96±0.06</td>
<td>-2.84±0.27</td>
<td>-3.45±0.10</td>
<td>ND</td>
<td>42.2±2.5</td>
<td>+4.4±0.4</td>
</tr>
<tr>
<td>(+)-β-cyc</td>
<td>-1.08±0.01</td>
<td>-1.98±0.04</td>
<td>-2.67±0.17</td>
<td>ND</td>
<td>ND</td>
<td>43.3±4.4</td>
<td>+5.2±0.5</td>
</tr>
<tr>
<td>DM</td>
<td>ND</td>
<td>-2.07±0.04</td>
<td>-2.80±0.09</td>
<td>-3.64±0.20</td>
<td>ND</td>
<td>39.3±2.2</td>
<td>+12.3±0.7</td>
</tr>
<tr>
<td>L-687,384</td>
<td>ND</td>
<td>-1.93±0.11</td>
<td>-2.71±0.24</td>
<td>-3.56±0.34</td>
<td>-4.26±0.91</td>
<td>39.2±8.1</td>
<td>+9.5±2.1</td>
</tr>
</tbody>
</table>

All solutions contained 10 μM NMDA and 1 μM glycine. (-)-β-cyc = 1 μM (-)-β-cyclazocine, (+)-β-cyc = 1 μM (+)-β-cyclazocine, DM = 5 μM dextromethorphan, and L-687,384 = 20 μM L-687,384. Values are reported as ±S.E.M. ND = not determined.
3.2.2. Summary of drug effects on unitary NMDA current amplitudes and channel conductance in hippocampal neurons

None of the agents altered the conductance properties of the NMDA ion channel measured in Mg\textsuperscript{2+}-free solutions. In control solution, the I/V relation for the NMDA ion channel in the presence of 1.8 mM external Ca\textsuperscript{2+} and in the absence of Mg\textsuperscript{2+} was linear in the range \(-100 \leq V_p \leq -20 \text{ mV}\) (Figure 2). This result is consistent with previous measurements of the conductance of NMDA single-channel behaviour in mouse cortical and cerebellar neurons (Ascher et al., 1988; Ascher and Nowak, 1988) and in rat hippocampal and cerebellar neurons (Jahr and Stevens, 1987; Gibb and Colquhoun, 1991). In the presence of (-)-\textbeta-cyclazocine (Figure 5), or in the presence of the other compounds, the I/V relation was also found to be linear, showing no voltage-dependent action of the compounds on unitary NMDA conductance at the potentials studied. It should be noted that the potentials used in the study lie near the physiological resting potential and encompass potentials encountered during normal synaptic transmission; as well, these voltages are likely representative of the depolarized potentials that would be commonly encountered during pathological situations where neuronal homeostasis is compromised (Parsons et al., 1993, 1995; Danysz et al., 1995). Other aspects of unitary NMDA conductance were also unchanged by the compounds. The ionic selectivity filter for NMDA ion channels is non-selective for Na\textsuperscript{+} and Cs\textsuperscript{+} (or K\textsuperscript{+}) (Mayer and Westbrook, 1987b; Ascher et al., 1988; Ascher and Nowak, 1988). The NMDA ion channel is unusual among ligand-gated ion channels in that it also has high permeability to Ca\textsuperscript{2+} (Mayer and Westbrook, 1985; Ascher and Nowak, 1986; MacDermott et al., 1986), with the magnitude of the
Ca\(^{2+}\) permeability relative to the permeability for Na\(^{+}\) (P\(_{Ca}/P_{Na}\)) having a value of 10.4 (Mayer and Westbrook, 1987b). However, since [Na\(^{+}\)] was over 100 x [Ca\(^{2+}\)] at the external face of the patch in these experiments ([Na\(^{+}\])\(_{o}\) = 140 mM, [Ca\(^{2+}\])\(_{o}\) = 1.8 mM), the conductance properties of the NMDA ion channel were largely determined by the inward flow of Na\(^{+}\). In the present experiments, the concentrations of Na\(^{+}\) and Cs\(^{+}\) were identical on either side of the patch ([Na\(^{+}\])\(_{o}\) = 140 mM = [Cs\(^{+}\)]) and thus the zero-current potential found from I/V plots was close to 0 mV (Figure 3). The values obtained for G and \(V_{rev}\) were unchanged by any of the compounds, showing that these agents did not alter the properties of the channel elements responsible for determining ionic selectivity or channel conductance.

3.2.3. Drug actions on the kinetics of unitary NMDA currents in hippocampal neurons

The principal aim of the electrophysiological studies was to characterize the kinetic interactions of the putative uncompetitive NMDA antagonists \(\beta\)-cyclazocine, dextromethorphan (DM) and L-687,384 with NMDA ion channels. The study of the kinetic behaviour of unitary currents requires the analysis of open and closed time distributions following the collation of a large number of events. This analysis would yield information on the effects of agents on mean open and closed times, frequency of channel opening, and channel open probability (P\(_{o}\)).

3.2.3.1. Mean open time of single NMDA ion channels in control solution

The mean open time (t\(_{op}\)) is the arithmetic mean of the time each channel event dwelt at the open state level in the data records. The mean open time for the NMDA
ion channel in hippocampal neurons was measured from analysis of recordings from outside-out patches, such as the patch shown in Figure 1, and was found to be 6.2±1.0 ms at $V_p = -40$ mV ($n = 17$). Previous studies of NMDA ion channel kinetic behaviour have shown the mean open time to be near 6 ms at $V_p = -60$ mV in outside-out patches from mouse central neurons (Ascher and Nowak, 1987) and from rat cortical neurons (Bertolino et al., 1988).

3.2.3.2. Effects of the compounds on mean open time

As shown in Figure 3, the effects of (-)-p-cyclazocine on channel kinetics included a decrease in the mean open time ($t_{op}$) of the ion channel. This effect could be reversed upon wash-out of the compound (not shown). (+)-p-cyclazocine had little effect on $t_{op}$, although a small decrease in mean open time was observed at the highest concentration employed (10 μM). It can be noted that channel events in the presence of (-)-p-cyclazocine did not evince any rapid flickerings from open levels to a nonconducting state such as was found with Mg$^{2+}$ block of the NMDA channel (see Figure 1B).

Typical channel open time distributions from the same patch as shown in Figure 3 are shown in Figure 6 for NMDA-induced channel openings in the absence of drug (A), in the presence of 1 μM (-)-p-cyclazocine (B), and in the presence of 1 μM (+)-p-cyclazocine (C) ($V_p = -40$ mV). A single peak in each log open time distribution allowed each distribution to be fit with a single-exponential function. As exemplified in the patch in Figure 3, the mean open time with 1 μM (-)-p-cyclazocine was reduced relative to control. The mean of the control distribution in Figure 6A was 4.6 ms, while the mean of the (-)-p-cyclazocine distribution in Figure 6B was 3.2 ms. At this same
Figure 6. Open time distributions in the absence and presence of (-)- or (+)-β-cyclazocine in hippocampal neurons. Open dwell-time histograms are shown for the same patch as in Figures 3 - 5 ($V_p = -40$ mV) (A) in control solution, (B) with 1 μM (-)-β-cyclazocine, and (C) with 1 μM (+)-β-cyclazocine; all bath solutions contained 10 μM NMDA and 1 μM glycine. Open time distributions were fit with single-exponential functions. The time constants of the fitted curves were 4.62 ms in (A), 3.23 ms in (B), and 4.37 ms in (C), with the same number of events being included in each histogram as in Figure 4.
Figure 6
concentration, the (+)-enantiomer caused only a small decrease in mean channel open time in this patch; the mean of the (+)-β-cyclazocine distribution in Figure 6C was 4.4 ms. Overall, at $V_p = -40$ mV, mean open times were reduced to 74±15% of control by 200 nM (-)-β-cyclazocine ($n = 2$), 65±6% of control by 1 μM (-)-β-cyclazocine ($n = 4$; significant at the $P < 0.05$ level), and 50±8% of control by 4 μM (-)-β-cyclazocine ($n = 2$). The (+)-enantiomer did not reduce mean open time at concentrations of 1 μM ($n = 5$; $P = 0.17$) or 4 μM ($n = 3$; $P > 0.20$); applying 10 μM (+)-β-cyclazocine produced a mean open time that was 81±17% of control ($n = 2$) at $V_p = -40$ mV.

The effects of DM and L-687,384 also included reductions in NMDA ion channel mean open times in outside-out patches from hippocampal neurons. For the former compound, the mean open time was reduced to 84±4% of control with 5 μM DM ($n = 4$), 70±6% of control with 20 μM DM ($n = 4$), and 68±18% with 50 μM DM ($n = 2$), all at $V_p = -40$ mV. The reduction produced by 20 μM DM was significant ($P = 0.02$), while that produced by 5 μM DM was not ($P = 0.17$). For the latter compound, at the same potential, mean open times were reduced to 96±9% of control by 5 μM L-687,384 ($n = 4$) and 71±6% of control by 20 μM L-687,384 ($n = 4$); a single patch with 40 μM L-687,384 exhibited a mean open time that was 64% of control. The reduction produced by 20 μM L-687,384 was significant ($P = 0.02$) while that produced by 5 μM L-687,384 was not ($P > 0.20$). The effects of both agents on mean open time could be fully reversed by washing out of the agents. Fast transitions to the baseline (non-conducting) level as evidenced with block of the NMDA ion channel by Mg$^{2+}$ (see Figure 1) were not observed for DM or L-687,384.
Figure 7. Opening frequencies of NMDA ion channels in the presence of (-)- or (+)-β-cyclazocine in hippocampal neurons. The frequency of opening events relative to control, ±S.E.M., is shown for (-)-β-cyclazocine (solid bars; n = 2, 4, and 2 for 0.2, 1, and 4 μM (-)-β-cyclazocine, respectively) and (+)-β-cyclazocine (striped bars; n = 5, 3, and 2 for 1, 4, and 10 μM (+)-β-cyclazocine, respectively) in outside-out patches from hippocampal neurons; $V_p = -40$ mV. All solutions contained 10 μM NMDA and 1 μM glycine. * significant difference (P < 0.01) between control and drug.
Figure 7
3.2.3.3. NMDA ion channel opening frequency in control solution

The effects of agents on the frequency of openings was determined. The procedure was to divide the total number of events by this recording period to obtain a value for mean frequency. In n = 17 patches, the mean channel opening frequency was 13.8±2.4 s⁻¹ at Vₚ = -40 mV.

3.2.3.4. Effects of the compounds on channel opening frequency

The (-)-enantiomer, and to a lesser extent, the (+)-enantiomer, of β-cyclazocine diminished the frequency of NMDA ion channel opening in a concentration-dependent manner (e.g. see Figure 3). This action was reversed by washing out the agents (not shown). The effects of β-cyclazocine on opening frequency are summarized in Figure 7 at Vₚ = -40 mV for both enantiomers at concentrations up to 4 μM. The decrease in opening frequency by these agents paralleled their action to decrease mean open time, although the effect of decreasing frequency was more pronounced than reduction of open time at any concentration tested.

DM, like (-)-β-cyclazocine, concentration-dependently diminished opening frequency, an effect that could be reversed by washing out. Channel opening frequencies, at Vₚ = -40 mV, were 81±18% (n = 4), 65±46% (n = 4), and 32±10% (n = 2) of control in the presence of 5, 20, and 50 μM DM, respectively. The effect of DM on frequency was significant at 20 μM (P < 0.02) but not at 5 μM (P > 0.20). In contrast to (-)- and (+)-β-cyclazocine and DM, L-687,384 had no effect on frequency at concentrations up to 40 μM. At 5 and 20 μM L-687,384 (Vₚ = -40
Figure 8. Open state probabilities for NMDA ion channels in the presence of (-)- or (+)-β-cyclazocine in hippocampal neurons. Log concentration-response curves, where the response is the open state probability ($P_0$), at $V_p = -40$ mV, normalized to the open state probability in control solution, in outside-out patches taken from hippocampal neurons and in the presence of (A) (-)-β-cyclazocine and (B) (+)-β-cyclazocine. The n values for each concentration were the same as those given for mean open time (Section 3.2.3.2) and frequency (Section 3.2.3.4). The lines are least-squares logistic fits to the data. The IC$_{50}$'s, where $P_0 = 0.5$, were 84 nM for (-)-β-cyclazocine and 14 µM for (+)-β-cyclazocine. *$P < 0.02$. 

- 83 -
Figure 8
mV), the frequency was 114±69% of control (n = 4) and 109±7% of control (n = 4); neither were significantly changed from control (P > 0.20 in both cases).

3.2.3.5. NMDA ion channel open state probability in control solution

The channel open state probability (P₀), calculated from the product of mean open time and mean opening frequency is an index of the kinetic behaviour of a channel. The mean control value for P₀ in n = 17 patches was 0.082±0.013 for Vₚ = -40 mV, indicating the channel was in a conducting state approximately 8% of the time. At other Vₚ, open channel probabilities were calculated as 0.103±0.078 for Vₚ = -20 mV (n = 2), 0.094±0.025 for Vₚ = -60 mV (n = 14), 0.087±0.016 for Vₚ = -80 mV (n = 9), and 0.105±0.010 for Vₚ = -100 mV (n = 2). P₀ was not found to vary with Vₚ (P > 0.20 by one-way analysis of variance).

3.2.3.6. Effects of the compounds on open state probability

Figure 8 shows semi-log plots of open channel probability calculated at Vₚ = -40 mV for increasing concentrations of (-)-β-cyclazocine (A) and (+)-β-cyclazocine (B). P₀ values shown were calculated as the product of mean open time and channel opening frequency. To illustrate the actions of the agents on open state probability, the P₀ value for each patch has been normalized to its open state probability in control solution. Logistic fits to the data for each enantiomer are shown superimposed on the points. The plots in Figure 8 were then used to extrapolate IC₅₀ values for β-cyclazocine effects on P₀ and the results showed an IC₅₀ of 84 nM for (-)-β-cyclazocine (Vₚ = -40 mV) and an IC₅₀ of 14 μM for (+)-β-cyclazocine (Vₚ = -40 mV).
The relationship between macroscopic and unitary (microscopic) currents is
\[ I_{\text{MACRO}} = I_{\text{UNIT}} \cdot N \cdot P_0 \]
where \( I_{\text{MACRO}} \) is the macroscopic whole-cell current, \( I_{\text{UNIT}} \) is the unitary current size, \( N \) is the number of conducting ion channels in the cell membrane, and \( P_0 \) is the mean channel open state probability. An effect of a given agent on one of the parameters on the right side of the equation would produce a proportional effect on \( I_{\text{MACRO}} \). Since neither (-)- nor (+)-\( \beta \)-cyclazocine were found to alter the size of unitary NMDA currents (see Section 3.2.1.2), then, assuming no change in the number of conducting channels, the results for \( P_0 \) yield IC\(_{50}\) values for inhibition of macroscopic NMDA currents. The values also show the (-)-enantiomer to be about 170X more potent than the (+)-enantiomer against NMDA ion channel open probability in hippocampal neurons at \( V_p = -40 \text{ mV} \).

The effects of DM or L-687,384 were to concentration-dependently reduce \( P_0 \). As with (-)-\( \beta \)-cyclazocine, DM's effect on \( P_0 \) was more potent than its effect on either \( t_{\text{op}} \) or frequency. This was expected, since \( P_0 \) is the product of \( t_{\text{op}} \) and frequency, which were both lowered by this agent. On the other hand, the action of L-687,384 was relatively weak and similar in potency to its effect on \( t_{\text{op}} \); this was due to its lack of effect on opening frequency. The \( P_0 \) values for DM and L-687,384 were used to determine IC\(_{50}\) values for inhibition of NMDA channel open probability by these compounds. These values were 4.4 \( \mu \text{M} \) for DM (\( V_p = -60 \text{ mV} \)) and 61 \( \mu \text{M} \) for L-687,384 (\( V_p = -60 \text{ mV} \)).
3.2.4. Summary of drug effects on unitary NMDA kinetics in hippocampal neurons

DM, (-)- and (+)-β-cyclazocine, and L-687,384 reduced the mean open time and mean opening frequency of the NMDA ion channel in a concentration-dependent and reversible manner. The rank order of potency for this effect was (-)-β-cyclazocine > DM > (+)-β-cyclazocine > L-687,384. The open state probability was also reduced with the same rank order of potency; this was due to the effects of the agents on mean open time and an additional action, with the exception of L-687,384, of decreasing channel opening frequency. L-687,384 did not reduce the rate of channel opening and therefore had a relatively weak effect on $P_0$. For each agent, the reduction of $P_0$ was found to increase as $V_p$ became more negative. The voltage-dependent actions of the four agents on single NMDA ion channel $P_0$ suggest that these compounds would exhibit voltage-dependent inhibition of NMDA activity at the whole-cell level, similar to what has been found for PCP (Honey et al., 1985; Bertolino et al., 1988), ketamine (Honey et al., 1985; MacDonald et al., 1987), MK-801 (MacDonald et al., 1991), and memantine (Chen et al., 1992; Parsons et al., 1993). The present results with (-)- and (+)-β-cyclazocine are the first to demonstrate voltage-dependence of benzomorphan actions against NMDA activity; similarly, this is the first demonstration of DM's voltage-dependent blocking action, although voltage-dependence of the DM metabolite, dextrorphan, has been previously observed (Parsons et al., 1993).

3.2.5. Consideration of mechanism of uncompetitive antagonist action

The overall results obtained with the 4 agents on single NMDA ion channels are now considered in order to arrive at a mechanism of action for inhibition of currents
through NMDA ion channels. The rationale for this approach is that determination of the on- and off-rates of drug interactions with NMDA ion channels would enable predictions concerning their utility as putative neuroprotectants in vivo.

3.2.5.1. Lack of drug effects on unitary NMDA ion channel conductance

The results presented above are consistent with open channel block by the agents (-)- and (+)-β-cyclazocine, DM, and L-687,384. For example, the size of the unitary currents activated by NMDA and the conductance of the ion channel were unchanged by any of the agents, suggesting the channel was open prior to block. No fast transitions between open and non-conducting levels were observed; hence, the compounds did not exhibit a Mg$^{2+}$-like open channel blockade. The reduced frequency of openings, as evident with all agents but L-687,384, would indicate relatively long interaction times with the NMDA receptor-ion channel complex. The results also showed the agents did not act to block and unblock the ion channel with extremely fast kinetics (i.e. numerous open-blocked transitions occurring within the 0.3 ms resolution time), since such rapid kinetics would appear as diminished heights of the unitary currents. In this regard, it should be noted that no apparent increase in baseline noise was evident upon application of any of the 4 agents; observation of increased baseline noise between otherwise typical channel openings could represent opening events that were blocked so fast as to have their full amplitude poorly resolved. Also, the agents had no effect on the ionic selectivity of the channel, since there was no difference in the reversal potential in the presence or absence of any of the compounds.
3.2.5.2. Decrease of mean open time and mean opening frequency by the compounds

The most salient indicator of open channel blockade was the concentration-dependent reductions in mean open time found with all four compounds. The decrease in the mean open time of the NMDA-activated unitary events by the compounds is in contrast to agents that exert inhibitory effects on receptor-coupled ion channels strictly through competition with the agonist at the receptor site (competitive antagonists); with the latter type of antagonist, no decrease in mean open time is associated with attenuation of channel activity (Katz and Miledi, 1971; Ascher et al., 1988).

In a model in which the agent exhibits a bimolecular interaction with its site and thus produces ion channel blockade, the reduction in frequency corresponds to the presence in the single-channel records of non-conducting periods when the agent occupies the site and blocks current flow. Longer blocking periods would produce greater reductions in frequency; hence, the potency of a given agent as a reducer of channel opening frequency is related to the length of time the agent occupies the blocker site. This feature of the compounds' interaction with NMDA ion channels is an important determinant of their actions to reduce $P_e$. It should be noted that it is possible that the channel may close with the agent trapped inside, as has been demonstrated for MK-801 (Huettner and Bean, 1987) and memantine (Blanpied et al., 1997). The extent to which this occurs with the present agents was not tested.

3.2.5.3. Applicability of open channel blockade model

An open channel blockade scheme can be represented by the following diagram
where C is the ion channel in its closed state, O is the channel in the open state, and B is the channel in the blocked state. The rate constants are defined as $k_1 = \text{the rate constant for isomerization of the ion channel from the open to closed state}$, $k_{-1} = \text{the rate constant for isomerization of the ion channel from the closed to open state}$ (the reverse of $k_1$), $k_2 = \text{the on-rate constant for the blocking agent}$, and $k_{-2} = \text{the off-rate constant for the blocking agent}$. Since the lifetime of a given state is equal to the inverse of the sum of the rate constants leading away from that state, the following equation is applicable:

$$(t_{op})^{-1} = k_1 + k_2[D].$$

Here $(t_{op})^{-1}$ is the inverse of the mean open time in the presence of a given concentration $([D])$ of the agent. Thus, a plot of $(t_{op})^{-1}$ vs. $[D]$ would be linear with slope $k_2$ and ordinate intercept $k_1$ (Neher and Steinbach, 1978; Ascher and Nowak, 1988). This analysis was applied to the 4 agents studied and the results are given below.

3.2.6. Analysis of data using an open channel blockade scheme

3.2.6.1. Determination of on-rate constants

A plot of the inverse of mean open time vs. drug concentration at $V_p = -40 \text{ mV}$ is presented in Figure 9 for (-)-$\beta$-cyclazocine up to 4 $\mu$M (A) and for (+)-$\beta$-cyclazocine up to 10 $\mu$M (B). Both plots were well-fit with linear relationships over the concentrations studied, indicating the applicability of the open channel blockade model with the two enantiomers of $\beta$-cyclazocine. The values for the onward (blocking) rate constant $k_2$ were found to be $7.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ ($n = 4$) for (-)-$\beta$-cyclazocine, $6.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for (+)-$\beta$-
Figure 9. Determination of onward rate constants for (-)- and (+)-β-cyclazocine in hippocampal neurons. Plots of $1/t_{op}$ vs. concentration are shown for (A) (-)-β-cyclazocine ($n = 4$) and (B) (+)-β-cyclazocine ($n = 5$) in hippocampal neurons; $V_p = -40$ mV. The lines shown are fits using linear regression analyses. Values for $k_2$ are given in the text.
Figure 9
Figure 10. Determination of onward rate constants for DM in hippocampal neurons. A plot of $1/t_{op}$ vs. concentration is shown for DM in a hippocampal neuron at the indicated $V_p$. The lines are fits using linear regression analyses. The values for $k_2$ are given in the text.
Figure 10
Figure 11. Determination of onward rate constants for L-687,384 in hippocampal neurons. A plot of $1/t_{op}$ vs. concentration is shown for L-687,384 in a hippocampal neuron at the indicated $V_p$. The lines are fits using linear regression analyses. The values for $k_2$ are given in the text.
Figure 11
cyclazocine (n = 5). The mean open time in the absence of the drug could be found from extrapolation of the plots to the ordinate and inverting the intercept. This procedure yielded mean open times near 5 ms (V_p = -40 mV), close to those found from analysis of open times in the current records for control (Section 3.2.3.1).

A similar open channel blockade analysis was applied to the actions of DM and L-687,384. The results are shown in Figure 10 for DM and Figure 11 for L-687,384; the plots were constructed using data from n = 6 patches for each compound. At V_p = -40 mV, the respective blocking rate constants k_2 were 5.0 x 10^6 M^{-1}s^{-1} and 2.1 x 10^7 M^{-1}s^{-1}. Thus, at a patch hyperpolarization of -40 mV the rank order for k_2 values was (-)-β-cyclazocine > (+)-β-cyclazocine > L-687,384 > DM. As evident in Figures 10 and 11, the results include V_p other than -40 mV. With DM, the plots also include V_p = -60 mV and -80 mV, and with L-687,384, plots are shown for -100 mV ≤ V_p ≤ -40 mV. Such plots were not possible with (-)-β-cyclazocine since the marked effect of this agent to reduce frequency did not allow a sufficient number of openings to be recorded at the different V_p. With DM, the k_2 values were 8.5 x 10^6 M^{-1}s^{-1} at V_p = -60 mV, and 1.0 x 10^7 M^{-1}s^{-1} at V_p = -80 mV. A similar trend was found for L-687,384, where the k_2 values were 3.6 x 10^7 M^{-1}s^{-1} at V_p = -60 mV, 5.9 x 10^7 M^{-1}s^{-1} at V_p = -80 mV, and 8.9 x 10^7 M^{-1}s^{-1} at V_p = -100 mV.

3.2.6.2 Determination of off-rate constants

A determination of the magnitudes of k_2 (the off- or unblocking rate constant) is not as straightforward as the quantitation of k_2. In principle, two procedures can be used to evaluate k_2. One method is to attribute fast channel flickering in single channel
Figure 12. Determination off-rate constant $k_2$ in hippocampal neurons. Closed dwell-time histograms are shown for a single outside-out patch isolated from a hippocampal neuron ($V_p = -40$ mV) (A) in control solution and (B) in the presence of 1 µM (-)-β-cyclazocine; all bath solutions contained 10 µM NMDA and 1 µM glycine. Closed time distributions required 3 exponential terms for a satisfactory curve-fit. In (A) the closed time components were 0.41 ms, 4.0 ms, and 60.8 ms, while in (B), closed time components were 0.63 ms, 18.2 ms, and 388 ms, with the same number of events being included in each histogram as in Figures 4 and 6.
Figure 12
records to drug actions and determine mean values of times during the flickering episodes. Since none of the compounds evinced this type of behaviour, an analysis of this sort was not possible. A second procedure is to compare closed time distributions between controls and drug and relate any differences in time components to the unblocking effect of the agent. This second approach was used in this work.

One complication in using closed-time distributions for the analysis of $k_2$ is that in controls such distributions exhibited several time components. In these experiments, closed time distributions were best fit with 3 components. Previous studies have also reported multiple closed time component distributions in hippocampal neurons (Gibb and Colquhoun, 1992; Jahr and Stevens, 1990; Gibb, 1989) and in cerebellar granule cells (Howe et al., 1988, 1991).

A comparison of closed time distributions in control solution and in the presence of (-)-β-cyclazocine or DM indicated that the primary effect of the compounds was to produce a prolonged third time component. This result was used to estimate values for $k_2$ for the two compounds. Figure 12 shows the closed time distributions for a typical patch under control conditions (A) and following the addition of 1 μM (-)-β-cyclazocine. The distribution in Figure 12A was fit using 3 components with values 0.41 ms, 4.0 ms, and 60.8 ms. Overall, in the control, the smallest time component was in the range 0.4 - 1.0 ms, with two other components, an intermediate component in the 2 - 10 ms range and a slow component in the 50 - 80 ms range, also being evident. The effects of (-)-β-cyclazocine on closed time distributions are indicated in Figure 12B ($V_p = -40$ mV); here, the 3 closed time components were 0.63 ms, 18.2 ms, and 388 ms. The slow time component in closed time distributions for (-)-β-cyclazocine was in the 200 - 400
ms range. For simple open-channel blockade, this time component represents the introduction of non-conducting states, by the agent, of mean length 200 - 400 ms and served to obscure the slow time component observed in control closed time distributions. The enhancement of this later component was reflected in the lower event frequency observed in the presence of (-)-β-cyclazocine, and is presumably a reflection of the time the agent associated with its blocking site. The value of this time component is the inverse of the off-rate, or unblocking, constant for the agent. Thus, \( k_2 \) was 2.5 \(-\) 5.0 s\(^{-1}\) for (-)-β-cyclazocine at \( V_p = -40 \text{ mV} \) (\( n = 4 \)). The dissociation constant \( K_D \) is calculated as the ratio \( k_2/k_2 \). For (-)-β-cyclazocine, this quantity was 3 - 7 nM (\( n = 4 \)). While this value is lower than the IC\(_{50}\) for (-)-β-cyclazocine reduction of \( P_o \) (84 nM), it is worthwhile noting that the former calculation is subject to greater error than the latter, since the \( k_2 \) term itself has limitations in reliability due to the complexity of closed time distributions.

It should be noted that an effect of (-)-β-cyclazocine addition in some patches, such as that shown in Figure 12, resulted in a prolongation of the intermediate time component. This change was not as consistent as the effect of the agent on the late component, nor was the change generally as large as that for the third time component. The view taken with regard to this observation was that the introduction of a large component by addition of the agent tended to artefactually increase the value of the intermediate time component determined by the fit. However, the possibility cannot be excluded that (-)-β-cyclazocine may have an effect on the closed time distributions in addition to the action of introducing a long time component as described above.
The effect of DM of altering closed time distributions was smaller than for \((-\)-\(\beta\)-cyclazocine, \(i.e.,\) the change in the long time component was not as great with the former agent compared to the latter. A slow time component in the range 80 - 100 ms was evident in closed time distributions for DM, which allows \(k_2\) for DM to be estimated at 10 - 13 s\(^{-1}\) \((V_p = -60 \, \text{mV}; \, n = 6)\). Using the rate constants \(k_2\) and \(k_2\), a \(K_o\) of 1.2 - 1.5 \(\mu\text{M}\) was estimated for DM interaction with NMDA ion channels \((n = 6)\). Conversely, distributions for L-687,384 were not changed from control, a reflection of this compound's lack of effect on opening frequency. It was assumed that the \(k_2\) for L-687,384 was sufficiently high that the association time for this agent with the NMDA ion channel was of similar value to one of the closed time components observed in control distributions. This last point was reflected in the lower potency of this agent as a reducer of NMDA ion channel \(P_0\).
CORTICAL NEURONS

3.2.7. Drug actions on unitary NMDA current amplitude and channel conductance in cortical neurons

Cortical neurons were also studied in the present work since the differential distribution of NMDA receptor subunits in the CNS, as well as variations in tissue susceptibility to excitotoxic damage, suggests that there may exist differences in NMDA receptor-ion channel properties between tissues (Baumgarten and Zimmerman, 1992; McBain and Mayer, 1984; Danysz et al., 1995). For example, the stoichiometry of NMDA receptor subunit expression exhibits some degree of variation in both recombinant and wild type receptors (McBain and Mayer, 1994), producing varying sensitivity to both agonist and antagonist actions (McBain and Mayer, 1994). NMDA receptors from different areas of the CNS may have varying subunit compositions (McBain and Mayer, 1994); thus, the properties of active NMDA receptors as well as their antagonist sensitivity may depend on their location within the CNS. Since L-687,384 and the (+)-enantiomer of β-cyclazocine were found to exhibit low potency as NMDA antagonists in hippocampal neurons, only (-)-β-cyclazocine and DM were studied in cortical neurons. In this section, the results with (-)-β-cyclazocine are presented for illustrative purposes while the results with DM are summarized in tabular form. The organization of this section follows that outlined above in the analysis of hippocampal neurons.
3.2.7.1. Amplitudes of unitary NMDA currents and channel conductance in control solution

Typical unitary currents recorded from rat cortical outside-out patches exposed to NMDA and glycine are presented in Figure 13 \((V_p = -60 \text{ mV})\) in the presence of 10 \(\mu\text{M}\) NMDA and 1 \(\mu\text{M}\) glycine. An \(I/V\) curve was constructed from this patch and is shown in Figure 14. The slope conductance was 44 pS and the zero-current potential was +12 mV, values similar to those obtained in the hippocampal neurons and to those obtained in previous studies of NMDA receptor-ion channel properties (Ascher et al., 1988; Bertolino et al., 1988; Gibb and Colquhoun, 1991). The values for \(G\) and \(V_{rev}\) (extrapolated) for all patches in control solution were 44.2±3.9 pS and +11.9±1.1 mV \((n = 7)\).

3.2.7.2. Effects of (-)-\(\beta\)-cyclazocine and DM on unitary NMDA amplitudes and channel conductance

Typical unitary currents recorded in the presence of 200 nM (-)-\(\beta\)-cyclazocine are shown in Figure 15; the data are from the same patch as shown in Figure 14. The amplitude distributions from this patch are shown for control (Figure 16A) and for 200 nM (-)-\(\beta\)-cyclazocine (Figure 16B), both at \(V_p = -80 \text{ mV}\). Here, the distribution means were 4.6 pA in control and 4.7 pA in the presence of (-)-\(\beta\)-cyclazocine. The \(I/V\) plot for this patch in the presence of 200 nM (-)-\(\beta\)-cyclazocine is shown in Figure 17. (-)-\(\beta\)-cyclazocine had no effect to alter the conductance of the NMDA channel (conductance in the presence of 1 \(\mu\text{M}\) (-)-\(\beta\)-cyclazocine = 40 pS or the ionic selectivity (zero-current potential in the presence of 200 nM (-)-\(\beta\)-cyclazocine = +14 mV). The values for \(G\) and
Figure 13. Unitary NMDA currents in cortical neurons. Shown are typical unitary currents from an outside-out patch, taken from a cortical neuron, exposed to 10 μM NMDA and 1 μM glycine (control solution) at the indicated $V_p$. Channel openings are represented as downward deflections from a non-conducting baseline level.
Figure 13
Figure 14. Current-voltage relation for the patch shown in Figure 13 in control solution. The slope of the linear regression fit to the data points is $G$, which was $44 \, \text{pS}$ for this patch; the intercept of the fitted line with the abscissa is $V_{\text{rev}}$, which was $12 \, \text{mV}$ for this patch.
Figure 14
Figure 15. Unitary NMDA currents after exposure to (-)-β-cyclazocine in cortical neurons. Shown are openings from the same patch as in Figure 13 exposed to control solution with the addition of 200 nM (-)-β-cyclazocine at the indicated $V_p$. Channel openings are represented as downward deflections from a non-conducting baseline level.
Figure 15
Figure 16. Amplitude distributions in the absence and presence of (-)-β-cyclazocine in cortical neurons. Unitary current amplitude distributions ($V_p = -80\ mV$) are shown for the same patch as in Figures 13 - 15: (A) in control solution, and (B) during exposure to 200 nM (-)-β-cyclazocine. All solutions contained 10 μM NMDA and 1 μM glycine. Amplitude distributions were fit with Gaussian functions with means (A) 4.59 pA and (B) 4.71 pA. The number of events included in each histogram was 362 in (A) and 267 in (B).
Figure 16
Figure 17. Current-voltage relation for the patch shown in Figures 13 - 16 during exposure to 200 nM (-)-β-cyclazocine. The slope of the linear regression fit to the data points is $G$, which was 40 pS for this patch; the intercept of the fitted line with the abscissa is $V_{rev}$, which was 14 mV for this patch.
\( V_{\text{rev}} \) for all patches were 39.5±6.1 pS and +14.7±1.9 mV (\( n = 4 \)), respectively. The amplitudes at various \( V_p \), conductance, and zero-current potential is shown for control solution and in the presence of either (-)-\( \beta \)-cyclazocine or DM in Table 2 for cortical neurons. No significant difference in the amplitudes at a given potential was found between control and either of the two compounds (\( P > 0.05 \) by two-way analysis of variance).

3.2.8. Summary of drug effects on unitary NMDA amplitudes and channel conductance in cortical neurons

I/V relations for unitary currents activated by NMDA in cortical neurons in 1.8 mM external \( \text{Ca}^{2+} \) and in the absence of \( \text{Mg}^{2+} \) were found to be linear, consistent with previous descriptions of NMDA single-channel behaviour in mouse cortical and cerebellar neurons (Ascher et al., 1988; Ascher and Nowak, 1988) and in rat hippocampal and cerebellar neurons (Jahr and Stevens, 1987; Gibb and Colquhoun, 1991). Neither (-)-\( \beta \)-cyclazocine, at concentrations up to 4 \( \mu \text{M} \), nor DM, at concentrations up to 20 \( \mu \text{M} \), altered the conducting properties of the NMDA ion channel in the range -80 mV \( \leq V_p \leq -40 \) mV, where I/V relations maintained linearity in the presence of either compound. The mean amplitude of the unitary currents measured at a given \( V_p \) were the same in the presence of either agent as in the control; values for slope conductance and \( V_p \) (Figure 14, Table 2) were also unchanged from the control. These results showed that, as for hippocampal neurons, DM and (-)-\( \beta \)-cyclazocine had no effect on the channel elements responsible for ionic selectivity and conductance.
Table 2. Unitary NMDA current amplitudes ($I_{\text{NMDA}}$), conductances ($G$), and reversal potentials ($V_{\text{rev}}$) for control solution and in the presence of the compounds indicated, as measured in outside-out patches from cortical neurons.

<table>
<thead>
<tr>
<th></th>
<th>$V_p = -20$ mV</th>
<th>$V_p = -40$ mV</th>
<th>$V_p = -60$ mV</th>
<th>$V_p = -80$ mV</th>
<th>$G$ (pS)</th>
<th>$V_{\text{rev}}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-1.39±0.14</td>
<td>-2.36±0.09</td>
<td>-3.27±0.17</td>
<td>-3.97±0.35</td>
<td>44.2±3.9</td>
<td>+11.9±1.1</td>
</tr>
<tr>
<td>(-)-β-cyc</td>
<td>-1.52±0.02</td>
<td>-2.27±0.21</td>
<td>-3.35±0.41</td>
<td>-4.47±0.33</td>
<td>49.1±9.5</td>
<td>+9.0±1.7</td>
</tr>
<tr>
<td>DM</td>
<td>ND</td>
<td>-2.28±0.24</td>
<td>-3.10±0.40</td>
<td>-3.97±0.64</td>
<td>42.3±7.7</td>
<td>+13.8±2.5</td>
</tr>
</tbody>
</table>

All solutions contained 10 μM NMDA and 1 μM glycine. (-)-β-cyc = 200 nM (-)-β-cyclazocine, and DM = 5 μM dextromethorphan. The data for control are the means of 7 patches, while data for (-)-β-cyclazocine and DM are each the means of 3 patches. Values are reported as ±S.E.M.
3.2.9. Drug effects on channel kinetics in cortical neurons

3.2.9.1. Effects of (−)-β-cyclazocine and DM on unitary NMDA mean open time

The mean open time for \( n = 7 \) patches from cortical neurons was 6.1 ± 0.4 ms at \( V_p = -60 \text{ mV} \). Figure 15 shows that (−)-β-cyclazocine decreased the open-state dwell time of NMDA-activated unitary currents. This effect was concentration-dependent and reversible (not shown). Fast transitions (flickering) between the open level and a nonconducting state, as found with Mg\(^{2+}\) block of the NMDA channel (Mayer et al., 1984; Nowak et al., 1984), were not observed with (−)-β-cyclazocine. Figure 18 shows typical channel open time distributions (from the same patch as shown in Figures 13 and 15) for channel openings in the absence (A) and in the presence of 200 nM (−)-β-cyclazocine (B) for \( V_p = -80 \text{ mV} \). Open time distributions were fit with single-exponential functions. The mean of the control distribution in Figure 18A was 2.2 ms, while the mean of the (−)-β-cyclazocine distribution in Figure 18B was 1.8 ms. Overall, mean open times were reduced to 78 ± 1% with 200 nM (−)-β-cyclazocine (n = 3; significant with \( P = 0.04 \)) and to 59 ± 18% of control with 1 μM (−)-β-cyclazocine (n = 2) at \( V_p = -80 \text{ mV} \).

DM was also found to reduce the mean open dwell times of NMDA-activated unitary currents in cortical neurons. Mean open time was 68 ± 18% of control at \( V_p = -80 \text{ mV} \) (n = 3; significant at \( P = 0.05 \)); at the same \( V_p \), a single patch exposed to 20 μM DM had an observed mean open time that was 35% of control. No flickering between open and closed states, such as found with fast block by Mg\(^{2+}\), was observed.
Figure 18. Open time distributions in the absence and presence of (-)-β-cyclazocine in cortical neurons. Open dwell-time histograms are shown for the same patch as in Figures 13 - 17 (\(V_p = -80\) mV): (A) in the control and (B) with 200 nM (-)-β-cyclazocine. All bath solutions contained 10 μM NMDA and 1 μM glycine. Open time distributions were fit with single-exponential functions. The time constants of the fitted curves were 2.17 ms in (A) and 1.78 ms in (B), with the same number of events being included in each histogram as in Figure 16.
Figure 18

A

B

Number of observations

Open time (ms)

Figure 18
3.2.9.2. Effects of (-)-β-cyclazocine and DM on unitary NMDA opening frequency

The effects of (-)-β-cyclazocine on the mean frequency of channel opening was studied for cortical neurons. The procedures were the same as outlined in Section 3.2.3.4 for hippocampal neurons where the total number of events was divided by total recording time (at least 10 s) to obtain a mean frequency. In n = 7 patches exposed to control solution, the mean frequency of channel opening was 9.2±3.0 s⁻¹ at \( V_p = -60 \) mV.

(-)-β-cyclazocine reversibly and in a concentration-dependent manner diminished the mean frequency of opening in cortical neurons, although this effect was not found to be significant (\( P = 0.08 \) at 200 nM (-)-β-cyclazocine). The reduction of the mean frequency of unitary current events by 200 nM (-)-β-cyclazocine is illustrated in Figures 13 and 15. Figure 19 shows the effect of (-)-β-cyclazocine to reduce frequency over the concentration range 0.05 - 4 μM (\( V_p = -60 \) mV).

The reduction of channel opening frequency by DM was also observed in cortical neurons. In n = 3 patches (\( V_p = -60 \) mV) exposed to 5 μM DM, the frequency was 74±23% (significant at \( P = 0.05 \)) of that observed in control solution; in a single patch exposed to 20 μM DM, the opening frequency was 57% of that observed in the same patch exposed to control solution.
Figure 19. Opening frequencies of NMDA ion channels in the presence of (-)-β-cyclazocine in cortical neurons. The frequency of opening events, normalized to control, is shown for 0.05, 0.2, 1, and 4 μM (-)-β-cyclazocine (n = 1, 3, 2, and 1, respectively) in outside-out patches from cortical neurons; V_p = -60 mV. All solutions contained 10 μM NMDA and 1 μM glycine. *P = 0.08.
Figure 19
3.2.9.3. Effects of (-)-β-cyclazocine and DM on unitary NMDA channel open probability

The mean values of $P_0$, the product of mean opening frequency and mean open time, for NMDA-activated ion channels in cortical neurons were $0.035 \pm 0.023$ ($n = 2$), $0.054 \pm 0.010$ ($n = 7$), $0.033 \pm 0.017$ ($n = 6$), and $0.064 \pm 0.029$ ($n = 3$) at $V_p = -20$ mV, -40 mV, -60 mV, and -80 mV, respectively. $P_0$ was not dependent on $V_p$ in cortical neurons. The $P_0$ values determined in cortical neurons were lower than the corresponding values determined for hippocampal neurons (see Section 3.2.2.7), although this difference was significant only for $V_p = -40$ mV and $V_p = -60$ mV.

The actions of (-)-β-cyclazocine and DM on $P_0$ were assessed by determination of changes in the product of mean open time and mean opening frequency relative to control. Figure 20 shows a semi-log plot of open channel probability ($P_0$) at $V_p = -40$ mV for increasing concentrations of (-)-β-cyclazocine, with a logistic fit to the data shown superimposed on the points. The $P_0$ values have been normalized to the control open state probability calculated for each patch. The plot in Figure 20 was used to determine an $IC_{50}$, which in this case was 680 nM for (-)-β-cyclazocine inhibition of $P_0$ in outside-out patches from cortical neurons. Assuming that (-)-β-cyclazocine did not alter the effective number of conducting channels in the cell membrane, and using the result that amplitudes of unitary currents were not changed by the agent, then the single channel results predict that a (-)-β-cyclazocine concentration of 680 nM would attenuate macroscopic NMDA responses by a factor of 50% at a potential of -40 mV.

Reduction in $P_0$ by DM was also calculated, and it was found that, in $n = 3$ patches, 5 μM DM produced a $P_0$ that was $47 \pm 17\%$ of that in control solution ($V_p = -60$ mV).
Figure 20. Open state probability of NMDA ion channels in the presence of (-)-β-cyclazocine in cortical neurons. Log concentration-response curves, where the response is the open state probability ($P_o$), at $V_p = -60$ mV, normalized to the open state probability in control, in outside-out patches taken from cortical neurons in the presence of (-)-β-cyclazocine. The $n$ values for each concentration are the same as those given for mean open time (Section 3.2.9.1) and frequency (Section 3.2.9.2). The lines are least-squares logistic fits to the data. The IC$_{50}$, where $P_o = 0.5$, was 305 nM for (-)-β-cyclazocine. *$P < 0.05$. 


Figure 20

Po

[(-)-β-cyclazocine] (µM)
mV); the single patch tested with 20 μM DM exhibited a \( P_0 \) that was 41% of that observed in control solution for that patch (\( V_p = -60 \) mV). As only two concentrations of DM were tested on cortical neurons, it was not possible to construct a full concentration-response curve for \( P_0 \) vs. [DM]. However, using the results quoted above, an IC\(_{50}\) for DM inhibition of NMDA \( P_0 \) of 3.8 μM (\( V_p = -60 \) mV) was estimated.

3.2.10. Summary of drug effects on kinetics of unitary NMDA currents in cortical neurons

The actions of (−)-β-cyclazocine and DM on unitary NMDA current kinetics were to reduce both the open time and the frequency of opening of the ion channel. These changes resulted in a concentration-dependent decrease in channel open state probability, which would predict a similar reduction in macroscopic currents mediated through NMDA receptor-ion channels. (−)-β-cyclazocine was found to be ∼10X more potent than DM in reducing \( P_0 \). The actions of the two agents on channel kinetics in cortical neurons were similar to those observed in hippocampal neurons.

3.2.11. Applicability of open channel block model in cortical neurons

The results of the study on cortical neurons can be interpreted in the same manner as described previously for the agents' action on hippocampal neurons: that is, open channel blockade underlies the effects of the compounds on NMDA-activated unitary currents. The lowering of \( P_0 \) by the agents was due to decreases in both \( t_{op} \) and frequency. The decrease in \( t_{op} \) is characteristic of uncompetitive antagonism via open channel blockade, as competitive antagonism is not associated with alterations in mean open time (Katz and Miledi, 1972; Ascher and Nowak, 1988). As noted in Section 3.2.5.3, the relationship between concentration and mean channel open time for an
open-channel blocker is described by the equation \((t_{op})^{-1} = k_1 + k_2[D]\) (Neher and Steinbach, 1978). An analysis for the rate constants \(k_2\) and \(k_{-2}\) is presented below and follows the procedures outlined above (see Section 3.2.6).

3.2.12 Analysis of cortical neuron data using an open-channel block scheme

3.2.12.1 Determination of on-rate constants

Plots of \((t_{op})^{-1}\) vs. \([D]\) are shown in Figure 21 with (-)-\(\beta\)-cyclazocine and in Figure 22 with DM. The values for \(k_2\) found from the slopes of these plots were \(12.4 \times 10^6\) M\(^{-1}\)s\(^{-1}\) for (-)-\(\beta\)-cyclazocine (Figure 21; \(V_p = -40\) mV) and \(7.9 \times 10^6\) M\(^{-1}\)s\(^{-1}\) for DM (Figure 22; \(V_p = -60\) mV). The patch with (-)-\(\beta\)-cyclazocine was sufficiently stable to obtain data at several \(V_p\) and produced \(k_2\) values of \(7.7 \times 10^6\) M\(^{-1}\)s\(^{-1}\) for \(V_p = -20\) mV, \(13.5 \times 10^6\) M\(^{-1}\)s\(^{-1}\) for \(V_p = -60\) mV, and \(31.2 \times 10^6\) M\(^{-1}\)s\(^{-1}\) for \(V_p = -80\) mV (not shown). The increase in \(k_2\) with patch hyperpolarization suggests the site of action of these agents to be located within the electrical field of the membrane, such as within the channel pore. An interesting result is that despite a large difference in potency between (-)-\(\beta\)-cyclazocine and DM (in excess of tenfold) there was little difference between the onward (blocking) rate constants \(k_2\). This finding would suggest that the potency for open channel blockade is not mediated by differences in the blocking rate constant.

3.2.12.2 Determination of off-rate constants

Closed time distributions were analyzed in order to estimate the unblocking rate constant \(k_2\) for (-)-\(\beta\)-cyclazocine. While the effect of (-)-\(\beta\)-cyclazocine to reduce
Figure 21. Determination of the onward rate constant for (-)-β-cyclazocine in cortical neurons. A plot of $1/t_p$ vs. concentration is shown for (-)-β-cyclazocine ($n = 4$) in cortical neurons; $V_p = -40$ mV. The lines shown are fits using linear regression analyses. Values for $k_2$ are given in the text.
Figure 21
Figure 22. Determination of the onward rate constant for DM in cortical neurons. A plot of $1/t_{op}$ vs. concentration is shown for DM ($n = 5$) in cortical neurons; $V_p = -60$ mV. The line is a fit using linear regression analyses. Values for $k_2$ are given in the text.
Figure 22
Figure 23. Determination of off-rate constant $k_2$ in cortical neurons. Closed time histograms are shown for a single outside-out patch isolated from a cortical neuron ($V_p = -80$ mV) (A) in control solution and (B) with 200 nM (-)-β-cyclazocine; all bath solutions contained 10 μM NMDA and 1 μM glycine. Closed time distributions required 3 exponential terms for a satisfactory curve-fit. In (A), the closed time components were 0.96 ms, 7.9 ms, and 147 ms, while in (B), closed time components were 0.45 ms, 15.9 ms, and 448 ms, with the same number of events being included in each histogram as in Figures 16 and 18.
Figure 23

A

B

Figure 23
frequency in most cases made the analysis of closed time distributions in the presence of the agent impossible, one patch was sufficiently stable to derive distributions with enough events to provide a reliable analysis. Figure 23 shows typical closed time histograms from the same outside-out patch, taken from a cortical neuron, as is shown in Figures 13 - 18 in control solution (A), and in the presence of 200 nM (-)-β-cyclazocine (B). Closed time distributions in both the presence and the absence of drug were fit with three-component exponential equations. A longer slow time component was evident in distributions for (-)-β-cyclazocine when compared to control, while the other two time components were unaffected. The value of this time component were near 400 ms, which presumably represents the mean time the agent interrupted ion flow through the channel; this corresponds to a k_2 of 2.5 s^{-1} at V_p = -80 mV. The dissociation constant K_D, calculated as the ratio k_2/k_0, was 80 µM for (-)-β-cyclazocine at this V_p.

3.2.13. Conclusions regarding open-channel block analysis in both neuronal types

Several observations concerning the actions of the tested agents on NMDA-activated unitary currents have been applied to generate a model for the antagonism of NMDA receptors by the compounds: (i) the lack of effect of either agent on NMDA channel conductance, (ii) concentration-dependent inhibition of NMDA open state probability by the agents, with the action of reducing the mean dwell time spent in the open state and of introducing non-conducting level "gaps" in the current records (and thus decreasing frequency) being the primary determinants of the agents' effect of reducing P_0, (iii) the presence of a prolonged exponential component in closed time
distributions for the applied agents that was not present in control, leading to a decreased frequency of opening, and (iv) the linear relationship between $(t_{op})^{-1}$ and concentration of the applied agent. All of these results are consistent with and support the conclusion that the compounds (-)-β-cyclazocine and DM inhibit current through NMDA ion channels via an open channel block mechanism. The basis for drug actions is not likely due to differences in $k_2$ since, especially for cortical neurons, onward rate constants were similar in magnitude. Instead, the IC$_{50}$ values for inhibition of $P_o$ were close to the $K_o$ values found by dividing $k_2$ by $k_2$ and it is suggested that $k_2$ is a major determinant of the potency for a given agent.

3.2.14. Biophysical probes: (i) studies with Mg$^{2+}$ in hippocampal neurons

3.2.14.1. Interaction of DM with the Mg$^{2+}$ site

A single experiment using Mg$^{2+}$ was used to yield information as to the channel site for binding of DM. As noted previously, the divalent cation Mg$^{2+}$ interacts in a voltage-dependent manner with the NMDA ion channel (Nowak et al., 1984; Mayer et al., 1984). Experiments in which 20 μM Mg$^{2+}$ was added to the bath solution containing NMDA and glycine resulted in fast transitions between the open level and a blocked state such that the channel mean open time was reduced by 60% at $V_p = -80$ mV (Figure 1B). This pattern of activity is consistent with previous observations of Mg$^{2+}$ actions on NMDA ion channels (Nowak et al. 1984; Ascher and Nowak, 1988). In an outside-out patch from a hippocampal neuron, channel kinetics were studied in control conditions, in the presence of 20 μM added Mg$^{2+}$, in 20 μM DM, and, finally, in 20 μM DM and 20 μM Mg$^{2+}$. The concentrations of Mg$^{2+}$ and DM chosen were those which,
when applied separately, would decrease mean open times to approximately one-half of control magnitude at $V_p = -60 \text{ mV}$; the concentration of the former agent was also well below those which produce other (enhancing) effects on NMDA ion channel activity, such as an increase in glycine affinity (Wang and MacDonald, 1995), a reduction of unitary current amplitude (Wang and MacDonald, 1995), and an increase in open channel probability (Paoletti et al., 1995). When $Mg^{2+}$ was added (Figure 1B), the mean open time was reduced by 49% ($V_p = -60 \text{ mV}$) and by 64% ($V_p = -80 \text{ mV}$). With DM added to the control solution, the mean open time was reduced by 52% ($V_p = -60 \text{ mV}$) and by 50% ($V_p = -80 \text{ mV}$). DM further reduced mean open time when added to the solution containing $Mg^{2+}$; at $V_p = -60 \text{ mV}$ the decrease was 66% and at $V_p = -80 \text{ mV}$ the decrease was 72%. In addition, the frequency of openings was considerably reduced when DM was added to the control solution with $Mg^{2+}$. If the decrease in mean open time produced by each agent were mediated through independent sites, one would expect the fractional inhibitions to be additive, i.e. values of 76% for $V_p = -60 \text{ mV}$ and 82% for $V_p = -80 \text{ mV}$ in the presence of the two agents should have been observed. However, the values obtained show the effects of the agents to be less than additive, suggesting that the inhibition of mean open time by both agents was mediated by the same site, or a common locus was involved in the action of both agents. However, the deviation from additivity was not great (< 10%), making a definite conclusion from the available data impossible. Further experiments regarding this matter are required in order to ascertain whether a common site is involved in the actions of DM and $Mg^{2+}$ on NMDA ion channels.
3.2.15. Biophysical probes: (ii) temperature studies with outside-out patches from cortical neurons

In order to further investigate the biophysical interaction of uncompetitive NMDA antagonists with their site of action, variable temperature studies were carried out on outside-out patches derived from cortical neurons. The use of temperature as a biophysical probe has not often been used and it was reasoned that thermodynamic analysis could be used to help elucidate some of the mechanisms involved in uncompetitive NMDA blockade.

3.2.15.1. Dependence of unitary NMDA current amplitudes on temperature in control solution

Preliminary experiments were carried out in order to investigate the effects of changing temperature on unitary NMDA currents. Decreasing the bath temperature of a cortical outside-out patch preparation from 24°C to 14°C diminished the amplitude of unitary currents activated by 10 μM NMDA (Figure 24). The use of 24°C was chosen as this was close to room temperature and thus the data acquired at this temperature is comparable to all the other data in this work; cooling by 10°C was chosen in order to minimize unresolvable fast transitions. The $Q_{10}$ values for temperature dependence of current size were found by taking the ratio of current amplitudes at 24°C to current amplitudes at 14°C. The $Q_{10}$ values were found to be 1.4±0.1 at $V_p = -60$ mV ($n = 6$); refer to Table 3 for $Q_{10}$'s at other $V_p$. $Q_{10}$ values of this size indicate that NMDA unitary current heights were not strongly dependent on temperature. The value for conductance ($G$) in cortical patches held at 24°C was 47±4 pS ($n = 6$), while $G$ at 10°C
Figure 24. Dependence of unitary NMDA currents on temperature. Shown are unitary currents in an outside-out patch taken from a cortical neuron at 24°C (A) and 14°C (B) at $V_p = -60$ mV. The external bathing solution contained 10 μM NMDA and 1 μM glycine.
Figure 24

A  24°C

B  14°C
Table 3. Q_{10} values for channel amplitudes in the absence and presence of DM.

<table>
<thead>
<tr>
<th>V_p (mV)</th>
<th>-40 mV</th>
<th>-60 mV</th>
<th>-80 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.42±0.05</td>
<td>1.41±0.06</td>
<td>1.73±0.01</td>
</tr>
<tr>
<td>DM</td>
<td>1.37±0.11</td>
<td>1.60±0.05</td>
<td>1.99</td>
</tr>
</tbody>
</table>

Q_{10} values shown are the ratio of channel amplitude at 24°C to that at 14°C as determined in outside-out patches from cortical neurons. The concentration of DM was 5 μM. The control data were from n = 6 patches; the DM data were from n = 3 patches, except the value at V_p = -80 mV, which was a single observation. Means are ±S.E.M.
was 35±6 pS (n = 6), corresponding to a $Q_{10}$ of 1.4. A $Q_{10}$ of 1.4 strongly suggests that rate of ion flux through the channel is determined chiefly by free diffusion and is not affected by non-diffusible, non-ionic elements such as protein gates. Previous work on measurements of conductance in frog node of Ranvier has shown this quantity to generally have low sensitivity to temperature, with $Q_{10}$'s in the range 1.3 - 1.5 (Hille, 1975). The present results are also similar to findings for calcium-activated potassium channels in inside-out patches from cardiac myocytes (McLarnon and Wang, 1991) and ATP-dependent potassium channels, also in inside-out patches from cardiac myocytes (McLarnon et al., 1993), where respective $Q_{10}$ values for conductance were 1.4 and 1.5.

3.2.15.2. Dependence of NMDA ion channel kinetics on temperature in control solution

Cooling outside-out patches from 24°C to 14°C increased the time spent in the open state of unitary currents activated by 10 μM NMDA. The ratio of the mean open time at 14°C to that at 24°C was taken as the $Q_{10}$ for NMDA ion channel mean open time. The $Q_{10}$ for this effect was 2.7±0.3 ($V_p = -60$ mV; n = 6), indicating that the channel spent, on the average, 2.7 times longer in the open state at 14°C than at 24°C (Table 4). A clear conclusion from these $Q_{10}$ measurements for amplitude and mean open time is that the behaviour of these two parameters is governed by different rate-limiting processes.

The frequency of single-channel events activated by 10 μM NMDA was diminished upon cooling the outside-out patches from 24°C to 14°C (Figure 24). The mean event frequency of outside-out patches exposed to 10 μM NMDA was 8.7±2.8 s$^{-1}$
Table 4. $Q_{10}$ values for channel mean open times in the absence and presence of DM.

<table>
<thead>
<tr>
<th>$V_p$ (mV)</th>
<th>-40 mV</th>
<th>-60 mV</th>
<th>-80 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.82±0.26</td>
<td>2.71±0.30</td>
<td>3.98±0.75</td>
</tr>
<tr>
<td>DM</td>
<td>2.33±0.34</td>
<td>2.89±0.64</td>
<td>2.77</td>
</tr>
</tbody>
</table>

$Q_{10}$ values shown are the ratio of channel mean open time at 14°C to that at 24°C as determined in outside-out patches from cortical neurons. The concentration of DM was 5 μM. The control data were from $n = 6$ patches; the DM data were from $n = 3$ patches, except the value at $V_p = -80$ mV, which was a single observation. Means are ±S.E.M.
at $V_p = -60$ mV. Cooling from 24°C to 14°C reduced the event frequency to $3.5 \pm 1.9$ s$^{-1}$ at the same $V_p$.

### 3.2.15.3. Effects of DM on temperature modulation of channel behaviour

In 3 cases, the viability of the outside-out patches was preserved for long times and allowed a measurement of the temperature dependence of the actions of DM on the same patches as studied in the controls. At 14°C, the amplitude of NMDA-activated unitary currents and channel conductance were not affected by DM, whereas mean open time and event frequency were diminished by DM. It was found that the $Q_{10}$'s for mean open time in the presence of DM were similar to the $Q_{10}$'s obtained in control solution (Table 4). Therefore, it may be stated that the actions of DM on channel conductance and mean open time were independent of temperature. However, these data must be interpreted with some degree of caution, as the low opening frequency at low temperature meant that distributions of amplitude and mean open time were less than adequate in terms of containing sufficient events for reliable data analysis.

### 3.2.16. Summary of the use of biophysical probes to elucidate drug actions

The decrease in mean open time produced by DM or Mg$^{2+}$ are each mediated by separate sites. If the two agents were indeed acting at the same intrachannel site, the decrease in mean open time expected upon addition of one agent to control solution would have the same value as the decrease expected upon addition to a solution containing the other agent. Exposing an outside-out patch to the agents together produced an effect that was less than the addition of the effects of the two agents
alone. This infra-additivity is also suggestive of an inhibition of one of the agents' actions by the other, as if DM occludes access by Mg\(^{2+}\) to its site, or vice versa.

The low Q\(_{10}\)'s for unitary current amplitudes and conductance found in the present study indicate that NMDA ion channel conductance was not strongly dependent on temperature. The values obtained are suggestive of a process rate-limited by aqueous diffusion of ions and not by, for example, a process involving changes in protein conformation such as a gating protein. On the other hand, the higher Q\(_{10}\)'s obtained for mean open time provide evidence that the channel elements responsible for channel gating are indeed rate-limited by non-diffusible species, e.g. protein-based gating structures. While the results with DM are somewhat difficult to interpret, the fact that addition of this agent did not change the Q\(_{10}\)'s for channel amplitude and mean open time suggests that uncompetitive antagonist action did not involve an additional process involving channel proteins or other non-solubilized structures. The conclusion derived from these experiments is that the mechanism of NMDA channel blockers is rate-limited by diffusion of these agents to their site of action.

3.3. **Microspectrofluorimetric studies**

Microspectrofluorimetric studies using hippocampal and cortical neurons loaded with the Ca\(^{2+}\)-sensitive dye fura-2 were carried out: (1) to assess drug effects on NMDA-induced increases in [Ca\(^{2+}\)], this last quantity being particularly important in terms of neurotoxicity mediated through NMDA receptors (Choi, 1988; Meldrum and Garthwaite, 1990); and (2) to assess drug specificity in terms of actions on other glutamate receptor types, i.e. NMDA vs. kainate or AMPA receptors, and on other Ca\(^{2+}\)-permeable ion channels, i.e. NMDA vs. voltage-activated Ca\(^{2+}\) channels. Following
identification of potential neuroprotectants using electrophysiological means, further information regarding their actions is necessary in order to elucidate how their application affects neuronal functioning. The technique of optical recording from cells loaded with the Ca\(^{2+}\)-sensitive dye would thus provide a useful supplement to the electrophysiological experiments in this regard. Note that depolarization mediated via non-NMDA receptors, as with AMPA and kainate receptor activation, would also result in inward Ca\(^{2+}\) flux through voltage-activated Ca\(^{2+}\) channels; however, studies have shown that some forms of the AMPA receptor can also conduct Ca\(^{2+}\) into the cell (McBain and Mayer, 1994). As in the electrophysiological work, two tissue types, specifically hippocampal and cortical neurons, were employed, due to recent evidence that the subunit composition and thus properties of NMDA receptors may vary throughout the CNS (McBain and Mayer, 1994; Danysz et al., 1995).

3.3.1. Uncompetitive inhibition of NMDA responses in hippocampal neurons

3.3.1.1. β-cyclazocine blockade of NMDA-evoked [Ca\(^{2+}\)] responses: use-dependence

[Ca\(^{2+}\)] responses to 20 μM NMDA were tested in the absence and presence of (-)-β-cyclazocine, from 0.2 - 5 μM, or (+)-β-cyclazocine, 5 μM. In these experiments, 20 μM NMDA evoked [Ca\(^{2+}\)] rises in hippocampal neurons of 481±46 nM from a baseline of 74±2 nM (n = 452). The NMDA-induced rise in [Ca\(^{2+}\)] began approximately 10 s after injection of NMDA into the chamber and persisted for about 30 s following washout. The return to baseline [Ca\(^{2+}\)] was complete about 3 min following washout; the time course of [Ca\(^{2+}\)] was similar in all cultures. (-)-β-cyclazocine reduced the
Figure 25. Attenuation of NMDA-evoked rises in $[\text{Ca}^{2+}]_i$ by $\beta$-cyclazocine in hippocampal neurons. Transient increases in intracellular calcium were evoked by injections of 20 µM NMDA (N). Superfusion of 5 µM (-)-$\beta$-cyclazocine (right-hand solid horizontal bar) use-dependently attenuated the calcium response to the NMDA injections while superfusion of 5 µM (+)-$\beta$-cyclazocine (left-hand screened horizontal bar) had relatively little effect. The inhibition of the NMDA response by (-)-$\beta$-cyclazocine in this experiment was 92.8% at the 2nd NMDA application and the steady-state inhibition produced by (+)-$\beta$-cyclazocine was 20.5% at the 4th NMDA application. This record is a mean of data taken from 28 neurons simultaneously. The use of 5 µM as a concentration allows comparison between the two enantiomers, as this concentration with (-)-$\beta$-cyclazocine nearly abolishes the NMDA responses while with (+)-$\beta$-cyclazocine the NMDA response are almost unaffected at this concentration. Inset: (-)-$\beta$-cyclazocine concentration-dependently inhibited $[\text{Ca}^{2+}]_i$ rises evoked by NMDA (data ±S.E.M. from 3 culture batches at each concentration). The logistic fit to the data produced an IC$_{50}$ value for (-)-$\beta$-cyclazocine of 272 nM.
Figure 25
NMDA-evoked rises in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner (Figure 25, left and centre). The action of the drug on NMDA responses was use-dependent, so that several (usually 5 or 6) agonist applications were necessary in order to observe a steady-state drug block. Similarly, several NMDA applications after drug washout were also required before the response stabilized again (Figure 25, right). The development of drug blockade was not dependent on time: initial responses to NMDA following superfusion of hippocampal neuronal cultures with (-)-β-cyclazocine for periods of > 1 hr, without agonist applications, were the same as the initial response in cultures in which the superfusion had lasted only 5 minutes (not shown). The number of agonist applications in the presence of (-)-β-cyclazocine required to reach a steady-state response level was inversely dependent on the drug concentration; that is, the higher the concentration of (-)-β-cyclazocine, the fewer the applications of NMDA that were necessary to achieve steady-state blockade. The mean number of applications necessary to achieve equilibrium block is given in Table 5, along with the degree of reduction of NMDA responses measured at equilibrium blockade. An IC$_{50}$ value of 272 nM for (-)-β-cyclazocine blockade of NMDA-evoked $[\text{Ca}^{2+}]_i$ increases in hippocampal neurons was calculated from these results. Due to the low potency of (+)-β-cyclazocine determined electrophysiologically in hippocampal neurons, only one concentration (5 µM) was tested. Neither enantiomer of β-cyclazocine altered resting $[\text{Ca}^{2+}]_i$ in hippocampal neurons.
Table 5. Reduction of responses to NMDA by β-cyclazocine in hippocampal neurons.

<table>
<thead>
<tr>
<th>[(−)-β-cyclazocine] (µM)</th>
<th>n</th>
<th>mean # to ss</th>
<th>% blockade ± S.E.M.</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>110</td>
<td>6.3</td>
<td>37.3±4.5</td>
<td>6</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>6.0</td>
<td>66.2±8.4</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>4.3</td>
<td>86.3±0.5</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>115</td>
<td>2.0</td>
<td>92.9±0.1</td>
<td>2</td>
</tr>
</tbody>
</table>

5 µM (+)-β-cyclazocine 41 1.0 15.9±4.7 1

The percentage difference in steady-state blockade between control and during superfusion with the indicated concentration of (−)-β-cyclazocine, as measured from the #th NMDA application, is given as % blockade. The results with 5 µM (+)-β-cyclazocine are given for comparison. The mean number of NMDA applications required to reach steady state blockade is given as mean # to ss. The total number of neurons tested with each drug concentration is given as n. Means are ±S.E.M. of 3 culture batches.
3.3.2. DM effects on NMDA-evoked \([Ca^{2+}]_i\) responses

The effect of DM on NMDA-evoked rises in \([Ca^{2+}]_i\) was also studied in hippocampal neurons. In all hippocampal neurons tested, DM (1 - 50 \(\mu\)M; \(n > 34\) at each concentration) reduced NMDA-evoked rises in \([Ca^{2+}]_i\) in a concentration-dependent manner, with 5, 20 and 50 \(\mu\)M DM reducing NMDA-evoked responses by 55±1% (\(n = 127\)), 88±1% (\(n = 74\)) and 97±1% (\(n = 145\)), respectively. An IC\(_{50}\) value of 4.1±0.2 \(\mu\)M was calculated from these results. In contrast to (-)-\(\beta\)-cyclazocine, there was no use-dependence observed in DM blockade of NMDA responses: the second response to NMDA in the presence of a given concentration of DM was always the same size as the first. Therefore, the first NMDA response following commencement of DM superfusion was used as the measurement of drug action. DM had no effect to alter resting \([Ca^{2+}]_i\) in hippocampal neurons.

3.3.3. L-687,384 effects on NMDA-evoked \([Ca^{2+}]_i\) responses

The effect of L-687,384 on \([Ca^{2+}]_i\) responses to boli of NMDA was tested in cultures of hippocampal neurons loaded with the calcium-sensitive fluorophore fura-2. L-687,384 (1-100 \(\mu\)M) produced a concentration-dependent and reversible reduction in the rise in \([Ca^{2+}]_i\) evoked by NMDA with an apparent IC\(_{50}\) value of 49±8 \(\mu\)M (\(n > 40\) at each concentration tested), with no effect on resting \([Ca^{2+}]_i\). At the highest concentration tested (100 \(\mu\)M), L-687,384 produced a 67±1% reduction of NMDA-evoked rises in \([Ca^{2+}]_i\) (\(n = 81\)). The potency of L-687,384 as an antagonist of NMDA-evoked rises in \([Ca^{2+}]_i\) is in good agreement with the electrophysiological data, in which
Figure 26. Selectivity of (-)-β-cyclazocine for NMDA responses in hippocampal neurons. Hippocampal neurons were challenged with either 50 mM K⁺ (K⁺), 20 µM NMDA (N), 80 µM kainate (K), or 40 µM AMPA (A). The horizontal bar indicates the presence of 5 µM (-)-β-cyclazocine in the external perfusion medium. NMDA-evoked [Ca²⁺]i rises were significantly affected by the agent (P < 0.001; analysis of variance followed by Tukey test) while high-K⁺, kainate, and AMPA responses were minimally affected. This record is a mean of data obtained from 17 neurons simultaneously.
Figure 26.
channel open probability was reduced by approximately 50% by L-687,384 at 60 µM. Since this potency was low, no further experiments were performed with L-687,384.

3.3.2. Drug effects on calcium responses to raised K⁺ in hippocampal neurons

3.3.2.1. Effects of (-)-β-cyclazocine on [Ca²⁺]ᵢ responses to raised K⁺

Neuronal depolarization, such as that induced by raising [K⁺]ₑ, has been shown to raise [Ca²⁺]ᵢ in neurons by allowing entry through voltage-activated, L-type calcium channels (Fletcher et al., 1993; Church et al., 1994a). Application of 50 mM K⁺ in the β-cyclazocine experiments produced a [Ca²⁺]ᵢ rise of 612±31 nM in hippocampal neurons. As with NMDA applications, the [Ca²⁺]ᵢ response began approximately 10 s after 50 mM K⁺ injection into the chamber and persisted for about 30 s following washout. However, the relaxation of [Ca²⁺]ᵢ to baseline was faster following K⁺ washout than following NMDA washout (Figure 26). Responses to raised K⁺ in the presence of 5 µM (-)-β-cyclazocine were 96.9±8.5% of control in hippocampal neurons. Thus, (-)-β-cyclazocine had no effect to inhibit L-type calcium channel activity in hippocampal neurons at a concentration that abolished responses to NMDA.

3.3.2.2. Effect of DM on [Ca²⁺]ᵢ responses to raised K⁺

Superfusion of 50 µM DM attenuated the increase in [Ca²⁺]ᵢ produced by application of 50 mM K⁺ by 40% in hippocampal neurons (Figure 27). In other experiments, where more than one application of 50 mM K⁺ was tried following commencement of DM superfusion, it was found that steady-state antagonism was achieved at the first 50 mM K⁺ application (not shown); therefore, the action of DM to
Figure 27. Selectivity of DM for NMDA responses in hippocampal neurons. The effect of DM applied for the period indicated by the bar above the trace, on $[\text{Ca}^{2+}]_i$, rises evoked by 50 mM K$^+$ (K$^+$), 20 µM NMDA (N), 80 µM kainate (K), or 40 µM AMPA (A) is shown. The response to NMDA was almost abolished, whereas responses evoked by K$^+$, kainate, and AMPA were each reduced by approximately 45%. This record is a mean of data obtained from 5 neurons simultaneously.
Figure 27
block voltage-gated Ca\(^{2+}\) channels was not use-dependent. The action of DM to
antagonize voltage-gated calcium channels was in contrast to (-)-\(\beta\)-cyclazocine, which
was ineffective against [Ca\(^{2+}\)]\(_i\) responses to raised K\(^+\). The action of DM to inhibit
responses mediated by voltage-activated calcium channels was not unexpected, as this
action has been previously observed electrophysiologically in cultured cortical neurons
(Netzer et al., 1993) and in DM antagonism of K\(^+\)-stimulated \(^{45}\)Ca\(^{2+}\) uptake into brain
synaptosomes (Carpenter et al., 1988).

### 3.3.3. Drug effects on non-NMDA responses in hippocampal neurons

### 3.3.3.1 Effects of (-)-\(\beta\)-cyclazocine on [Ca\(^{2+}\)]\(_i\) responses to non-NMDA
agonists

The effects of (-)-\(\beta\)-cyclazocine on [Ca\(^{2+}\)]\(_i\) responses to 80 \(\mu\)M kainate and 40 \(\mu\)M
AMPA were examined in cultured neurons. Both excitants activate separate non-
NMDA ion channel subtypes and produce much of their [Ca\(^{2+}\)]\(_i\) response by
depolarizing the neurons and allowing Ca\(^{2+}\) flux through voltage-activated Ca\(^{2+}\)
channels, although there is evidence that some forms of AMPA receptor-ion channels
are permeable to Ca\(^{2+}\) (Danysz et al., 1995). Application of 80 \(\mu\)M kainate produced
[Ca\(^{2+}\)]\(_i\) rises of 270±67 nM in hippocampal neurons, whereas 40 \(\mu\)M AMPA produced
[Ca\(^{2+}\)]\(_i\) rises of 253±11 nM in hippocampal neurons. Figure 26 shows that, as with
raised K\(^+\), 5 \(\mu\)M (-)-\(\beta\)-cyclazocine had no effect to attenuate [Ca\(^{2+}\)]\(_i\) responses to non-
NMDA agonists; responses to kainate and AMPA were 114.1±12.8% and 99.1±9.6%,
respectively, of control in hippocampal neurons. These results show (-)-\(\beta\)-cyclazocine
to be selective for the NMDA subtype of glutamate receptor in hippocampal neurons.
3.3.3.2. Effects of DM on [Ca\textsuperscript{2+}]\textsubscript{i} responses to non-NMDA agonists

The actions of DM to affect [Ca\textsuperscript{2+}]\textsubscript{i} rises evoked by the non-NMDA excitants AMPA and kainate were tested in hippocampal neurons. Reductions in 80 μM AMPA- and 40 μM kainate-evoked responses by DM were, respectively, 4±2% and 0±3% (5 μM), 16±2% and 16±4% (20 μM), and 41±2% and 44±3% (50 μM) in hippocampal neurons (Figure 27). The reductions of AMPA- and kainate-evoked responses by DM paralleled the reduction of rises in [Ca\textsuperscript{2+}]\textsubscript{i} evoked by 50 mM K\textsuperscript{+} and thus reflect blockade by DM of high voltage-activated Ca\textsuperscript{2+} channels rather than non-NMDA receptor antagonist activity (see Netzer et al., 1993; Church et al., 1994), although contributions from calcium-permeable states of the AMPA receptor (Danysz et al., 1995) cannot be definitely ruled out. It is therefore likely that, at the concentrations employed in the single channel studies (5, 20 and 50 μM), the action of DM was predominantly selective for the NMDA subtype of glutamate receptor.

3.3.4. Drug blockade of NMDA ion channels in cortical neurons

3.3.4.1. β-cyclazocine blockade of NMDA-evoked [Ca\textsuperscript{2+}]\textsubscript{i} responses

Cortical neurons were also used to study the time course of the development of NMDA antagonism by (-)-β-cyclazocine. [Ca\textsuperscript{2+}]\textsubscript{i} responses to NMDA in cortical neurons were similar to those observed in hippocampal neurons. In cortical neurons, NMDA at 20 μM induced mean rises in [Ca\textsuperscript{2+}]\textsubscript{i} of 360±60 nM from a baseline of 75±6 nM (n = 531). This rise in [Ca\textsuperscript{2+}]\textsubscript{i} began seconds after injection of NMDA into the chamber and persisted for about 30 s following washout. Return to baseline [Ca\textsuperscript{2+}]\textsubscript{i} was generally complete approximately 3 min following washout. (-)-β-cyclazocine effects on NMDA
Figure 28. Reduction of 20 μM NMDA-evoked [Ca$^{2+}$]$_j$ responses by β-cyclazocine in cortical neurons. Values shown are the steady-state means of measurements taken from three different cortical culture batches for each concentration tested, corrected for baseline [Ca$^{2+}$]$_j$ (see Section 2.3.1.3) and normalized to concentration responses with 20 μM NMDA only; error bars refer to the S.E.M. for the normalized values of 3 culture batches. The total number of neurons tested under each treatment (grouping together the three culture batches) is indicated in parentheses above each bar.
Figure 28

![Graph showing the effect of different concentrations of (-)-β-cyclazocine on the % control Ca\textsuperscript{2+} response.](image-url)
Table 6. Reduction of NMDA responses by β-cyclazocine in cortical neurons

<table>
<thead>
<tr>
<th>[(-)-β-cyclazocine] (μM)</th>
<th>n</th>
<th>mean # to ss</th>
<th>% blockade</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02</td>
<td>43</td>
<td>1.0</td>
<td>13.4±1.4</td>
</tr>
<tr>
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<td>92.2±2.1</td>
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<tr>
<td>5 μM (+)-β-cyclazocine</td>
<td>46</td>
<td>1.0</td>
<td>21.5±2.4</td>
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The percentage difference in steady-state blockade between control and during superfusion with the indicated concentration of (-)-β-cyclazocine, as measured from the #th NMDA application, is given as % blockade. The results with 5 μM (+)-β-cyclazocine are given for comparison. The mean number of NMDA applications required to reach steady state blockade is given as mean # to ss. The total number of neurons tested with each drug concentration is given as n. Means are ±S.E.M.
responses in cortical neurons were similar to those observed in hippocampal neurons, in terms of use-dependence and in level of blockade at equilibrium. Figure 28 shows the concentration-response curve for β-cyclazocine inhibition of NMDA responses in fura-2-loaded cortical neurons, while the values obtained at each concentration are shown in Table 6, along with information pertaining to the use-dependence of (-)-β-cyclazocine. Low concentrations reached equilibrium immediately following commencement of (-)-β-cyclazocine superfusion; mean # to ss (steady state) is hence labelled 1.0. The IC₅₀ value for (-)-β-cyclazocine inhibition of NMDA-induced [Ca²⁺]ᵢ increases in cortical neurons was 220 nM. 5 µM (+)-β-cyclazocine decreased NMDA responses by 21.5±2.4% in cortical neurons (not shown). Neither enantiomer of β-cyclazocine affected baseline [Ca²⁺]ᵢ in cortical neurons.

3.3.4.2 DM blockade of NMDA-evoked [Ca²⁺]ᵢ responses

As shown in Figure 29, DM acted to decrease [Ca²⁺]ᵢ responses to NMDA in cortical neurons. No use-dependence was observed with DM blockade of NMDA-evoked responses in cortical neurons. NMDA responses were reduced to 72±6%, 46±7%, 19±5%, and 4.4±0.2% of control by 2, 5, 20, and 50 µM DM, respectively (n = 75). An IC₅₀ value of 5.4 µM was obtained for inhibition of NMDA-evoked [Ca²⁺]ᵢ rises in fura-2-loaded cortical neurons, which is similar to the value obtained for DM inhibition of NMDA-evoked rises in [Ca²⁺]ᵢ in hippocampal neurons, 4 µM. DM had no effect to alter resting [Ca²⁺]ᵢ in cortical neurons.
Figure 29. Reduction of 20 μM NMDA-evoked \([\text{Ca}^{2+}]_j\) responses by DM in fura-2-loaded cortical neurons. DM was applied for the period indicated by the bar above the trace at the concentrations shown. The n for each treatment is indicated in parentheses above each bar. This record is a mean of data obtained from 22 neurons simultaneously.
Figure 29
3.3.5. Drug effects on calcium responses to raised \( K^+ \) in cortical neurons

3.3.5.1. Effect of \((-\)-\(\beta\)-cyclazocine on \([Ca^{2+}]_i\), responses to raised \( K^+ \)

Application of 50 mM \( K^+ \) evoked \([Ca^{2+}]_i\) rises of 302±18 nM in cortical neurons (\( n = 71 \)). Figure 30 shows the effect of 5 \( \mu M \) \((-\)-\(\beta\)-cyclazocine on \([Ca^{2+}]_i\), responses to 50 mM \( K^+ \) in cortical neurons. Here, the responses to 50 mM \( K^+ \) were not affected by this concentration of \((-\)-\(\beta\)-cyclazocine, whereas in contrast the NMDA-evoked response was eliminated by the second application of agonist. Responses to raised \( K^+ \) in the presence of 5 \( \mu M \) \((-\)-\(\beta\)-cyclazocine were 102.0±2.2% of control in cortical neurons, indicating that, as with hippocampal neurons, \((-\)-\(\beta\)-cyclazocine has no effect on voltage-sensitive \( Ca^{2+} \) channels in cortical neurons.

3.3.5.2. Effect of DM on \([Ca^{2+}]_i\), responses to raised \( K^+ \)

\([Ca^{2+}]_i\), responses to raised \( K^+ \) (50 mM) in the absence and presence of DM are shown in Figure 31. In contrast to the lack of effect produced by \((-\)-\(\beta\)-cyclazocine against voltage-activated \( Ca^{2+} \) channels, 50 \( \mu M \) DM inhibited \( K^+ \)-evoked rises in \([Ca^{2+}]_i\), to 63.7±6.5% of control in cortical neurons. DM inhibition of neuronal \( Ca^{2+} \) channels has been documented previously (Carpenter et al., 1988; Netzer et al. 1993).

3.3.6. Drug effects on non-NMDA responses in cortical neurons

3.3.6.1. Effects of \((-\)-\(\beta\)-cyclazocine on \([Ca^{2+}]_i\), responses to non-NMDA agonists

Figure 30 also shows kainate- and AMPA-evoked \([Ca^{2+}]_i\), rises in the absence and presence of \((-\)-\(\beta\)-cyclazocine. Injection of 80 \( \mu M \) kainate into the chamber
Figure 30. Selectivity of 5 μM (-)-p-cyclazocine in cortical neurons. [Ca^{2+}]_i responses to 50 mM potassium (K⁺), 20 μM NMDA (N), 80 μM kainate (K), and 40 μM AMPA (A) are shown for control, during superfusion of 5 μM (-)-p-cyclazocine (solid bar), and after washout of (-)-p-cyclazocine. Responses in the presence of (-)-p-cyclazocine were 86.8%, 30.8%, 8.2%, 109.6%, and 106.9% of control for K⁺, N, N, K, and A, respectively. This trace is the mean of responses from 27 neurons measured simultaneously.
Figure 30
Figure 31. Responses to raised K$^+$ and non-NMDA agonists in the absence and presence of DM in cortical neurons. [Ca$^{2+}$]$_j$ responses to 50 mM potassium (K$^+$), 20 µM NMDA (N), 80 µM kainate (K), and 40 µM AMPA (A) are shown for control, during superfusion of 50 µM DM (solid bar), and after washout of DM. Responses in the presence of DM were 57.2%, 15.9%, 103.2%, and 84.7% of control for K$^+$, N, K, and A, respectively. This trace is the mean of responses from 19 neurons measured simultaneously.
produced \([\text{Ca}^{2+}]\), rises of 146±23 nM in cortical neurons. 40 µM AMPA produced \([\text{Ca}^{2+}]\), rises of 83±7 nM in cortical neurons. Superfusion of (-)-β-cyclazocine, at 5 µM, had little effect to alter these rises. Responses to kainate and AMPA were 94.5±2.2% and 99.4±0.3% of control, respectively. These results are in agreement with those obtained in hippocampal neurons in that they show (-)-β-cyclazocine to be selective for the NMDA subtype of glutamate receptor.

3.3.6.2. Effects of DM on \([\text{Ca}^{2+}]\), responses to non-NMDA agonists

Following the same procedure as with the hippocampal neurons, DM was tested for its actions on \([\text{Ca}^{2+}]\), responses to the non-NMDA agonists kainate and AMPA. Responses to 80 µM kainate or 40 µM AMPA were reduced to 96.0±7.3% and 89.2±4.5% by 20 µM DM in cortical neurons (Figure 31). The reductions in responses to kainate and AMPA were smaller than the reductions produced by DM against responses to 50 mM K⁺, suggesting that the \([\text{Ca}^{2+}]\), responses produced by kainate and AMPA in cortical neurons, unlike hippocampal neurons, were not mediated directly through DM-sensitive Ca²⁺ channels. In any case, DM's actions against glutamate receptors were restricted to the NMDA subtype.

3.4. Neuroprotection studies

3.4.1. (-)-β-cyclazocine as a candidate neuroprotectant

The action of (-)-β-cyclazocine to block the NMDA ion channel, as found in the electrophysiological and microspectrofluorimetric experiments, would favour the possibility of its use as a clinical neuroprotective agent. Selectivity for NMDA vs. calcium channels was much more pronounced with (-)-β-cyclazocine than with DM, an
agent that has been previously shown to have neuroprotective properties \textit{in vitro} (Choi, 1987) and is currently undergoing clinical trials against stroke (Parsons \textit{et al.}, 1995; Danysz \textit{et al.}, 1996). Therefore, it was appropriate to test the ability of (-)-\(\beta\)-cyclazocine to protect neurons \textit{in vitro} in order to support its identification as a candidate neuroprotectant. Cultured rat hippocampal and cortical neurons, prepared in the same manner as those employed in the electrophysiological and microspectrofluorimetric studies, (see Methods) were used to assess the neuroprotective properties of (-)-\(\beta\)-cyclazocine against NMDA excitotoxicity.

\textbf{3.4.2. Neuroprotection studies with hippocampal neurons}

\textbf{3.4.2.1. Excitotoxicity produced by NMDA \textit{in vitro}}

In the absence of drug, hippocampal cultures exposed to 1 mM NMDA + 1 mM glycine exhibited a marked decrease in the number of cells extruding trypan blue dye. Neurons that took up the dye following excitotoxic challenge displayed signs of severe neurotoxicity. For example, the plasma membranes appeared rough and granular, axonal processes were either discontinuous or not present at all, and vacuole formation could clearly be observed in the cytoplasm. As well, the overall morphology of the cells most affected by the treatment was severely distorted. These neuronal somata lost their characteristic bipolar or pyramidal shape, and instead were rounded and swollen to greater than twice the average volume observed prior to treatment. The survival rate of cultured hippocampal neurons following 24 hr exposure to 1 mM NMDA was 29.7\(\pm\)5.2\% (Figure 32). Addition of 1 \(\mu\)M glycine only, with no NMDA, produced cultures that 24 hr later appeared healthy and morphologically similar to those
Figure 32. Protection against NMDA excitotoxicity by β-cyclazocine in hippocampal neurons. Cultured hippocampal neurons were exposed to culture medium containing 1 mM NMDA plus 1 μM glycine, with or without either (-)-β-cyclazocine (filled bars) or (+)-β-cyclazocine (grey bars). Cell survival was assessed 24 hours later. Bars indicate percentage of surviving neurons present at 24 hours relative to the amount present immediately preceding NMDA exposure. The mean values (±S.E.M., n = 8) for cultures containing (-)-β-cyclazocine are represented by the solid bars, while the striped bars represent the means (±S.E.M., n = 8) obtained from cultures to which had been added (+)-β-cyclazocine. *P < 0.05; **P < 0.001 as determined by one-way analysis of variance followed by the Tukey test.
Figure 32

-172-
observed before the treatment. Experiments where no NMDA was used showed a survival rate of 72.7±4.4% for hippocampal cultures over the 24 hr period.

3.4.2.2. Neuroprotection by (-)-β-cyclazocine in hippocampal neurons

Addition of (-)-β-cyclazocine concentration-dependently protected cultured rat hippocampal neurons from morphological damage induced by 1 mM NMDA (Figure 32). 67.3±2.1% (n = 8) of cells survived following NMDA + 1 μM (-)-β-cyclazocine challenge, compared to 29.7±5.2% surviving after challenge with NMDA alone. 10 μM (-)-β-cyclazocine almost completely protected the culture from NMDA-induced excitotoxicity (73.8±4.3% cells surviving; n = 8). The majority of neurons observed following treatment with NMDA + 10 μM (-)-β-cyclazocine appeared healthy and morphologically similar to those observed before treatment; these cells did not take up the trypan blue stain. No obvious vacuolization in the cells extruding the stain was observed, indicating (-)-β-cyclazocine itself was not toxic to the neurons at the concentrations used (Olney et al., 1989). The extrapolated IC₅₀ for (-)-β-cyclazocine inhibition of NMDA-induced excitotoxicity in hippocampal neurons was approximately 0.9 μM. Raising the glycine concentration to 10 μM did not alter the effectiveness of (-)-β-cyclazocine in hippocampal neurons (data not shown).

3.4.2.3. Effect of (+)-β-cyclazocine in hippocampal neurons

The neuroprotective properties of the (+)-enantiomer were also studied. While the (+) enantiomer was considerably less active than the (-)-enantiomer, some degree of protection was seen with 10 μM (+)-β-cyclazocine. In hippocampal neurons, survival rate with 10 μM (+)-β-cyclazocine was 48.5±6.2% while survival with 1 μM (+)-β-
Figure 33. Stereospecific neuroprotection by β-cyclazocine in cortical neurons. Shown is the percentage of neurons protected from 1 mM NMDA + 1 µM glycine-induced neuronal degeneration by either (-)-β-cyclazocine or (+)-β-cyclazocine, measured at 24 hours. Values were corrected for procedural cell losses (as estimated from control cultures where no NMDA or β-cyclazocine was added) and normalized to the mean values obtained from cultures where only 1 mM NMDA + 1 µM glycine was added. All treatments were performed in parallel in 7 different culture batches; each treatment was replicated 3 times within a given culture batch. Error bars indicate S.E.M. for the culture batches. Statistics were performed as a one-way Analysis of Variance followed by a Tukey test. *P < 0.05; **P < 0.005; ***P < 0.001.
Figure 33

![Bar graph showing neuroprotection percentages for different concentrations of (+)-β-cyclodextrin (β-cyc) and (-)-β-cyclodextrin (β-cyc). The bars represent the mean ± SEM.](image-url)

- 1 μM (+)-β-cyc
- 10 μM (+)-β-cyc
- 1 μM (-)-β-cyc
- 10 μM (-)-β-cyc

The graph indicates a significant increase in neuroprotection with increasing concentration of (-)-β-cyclodextrin compared to (+)-β-cyclodextrin.
Cyclazocine was 37.5±5.1% (Figure 32). Note that 1 μM (+)-β-cyclazocine afforded almost no protection against NMDA-induced neurotoxicity. The low potency of (+)-β-cyclazocine in the neuroprotection assay paralleled the potency difference between (-)- and (+)-β-cyclazocine observed in the electrophysiological experiments.

3.4.3. Neuroprotection studies with cortical neurons

3.4.3.1. Effects of NMDA only

Similar to hippocampal neurons, cortical neurons were also severely damaged by NMDA, showing a survival rate of 32.0±3.8% after 24 hr exposure to 1 mM NMDA (Figure 33). The majority of cells present 24 hr following NMDA treatment had rough, discontinuous plasma membranes, increased somatic volumes, cytoplasmic vacuoles, and a 'bath-chain' appearance in their axonal processes. In cortical neurons, adding 1 μM glycine only, with no NMDA, produced cultures that 24 hr later appeared healthy and morphologically similar to those observed before the treatment. Experiments where no NMDA was used showed a survival rate of 72.0±3.8% over the 24 hr period for cortical cultures.

3.4.3.2. Neuroprotection by (-)-β-cyclazocine in cortical neurons

Cortical neurons were also protected from NMDA-induced neurotoxicity by (-)-β-cyclazocine. This is illustrated in Figure 33, which shows a significant protecting effect by 1 μM (-)-β-cyclazocine (60.8±10.1% neuroprotection; n = 7), and nearly full neuroprotection by 10 μM (-)-β-cyclazocine (93.4±17.2% neuroprotection; n = 7). With the latter concentration, the appearance of cells closely resembled that of cells not exposed to NMDA, indicating the neurotoxicity described by Olney et al. (1989) did not
occur. Raising the glycine concentration to 10 μM did not alter the effectiveness of (-)-β-cyclazocine in cortical neurons (data not shown). These results show that the properties of (-)-β-cyclazocine to prevent neuronal damage were not restricted to the hippocampal subtype, and support a generalization to other neuronal subtypes susceptible to excitotoxic death mediated by NMDA receptors.

3.4.3.3. Effects of (+)-β-cyclazocine in cortical neurons

The (+)-enantiomer of β-cyclazocine, while affording some degree of neuroprotection at higher concentrations, was not nearly as effective a neuroprotectant \textit{in vitro} as the (-)-enantiomer in cortical neurons. Figure 33 shows the results with (+)-β-cyclazocine: 1 μM (+)-β-cyclazocine produced 2.1±6.5% neuroprotection while 10 μM produced 51.3±9.9% neuroprotection. The difference in neuroprotective potency between the two enantiomers corresponds well with the difference in IC$_{50}$'s as determined in the electrophysiological experiments. This enantiomeric selectivity was also observed in hippocampal neurons, and suggests a similar mode of interaction with NMDA receptors in both cell types.

3.5. Actions of the compounds on voltage-gated ion channels

3.5.1. Macroscopic currents in dorsal root ganglion (DRG) cell line neurons

An ideal neuroprotectant would inhibit the neuronal process(es) responsible for producing excitotoxicity, while leaving elements involved in signal transmission and maintenance of resting membrane potential intact. The latter functions are mediated by
Figure 34. TEA-sensitive delayed rectifier in DRG neurons. $I_K$ was elicited by a sequence of 1800 ms depolarizing steps from the holding potential (-60 mV) to +60 mV in increments of +10 mV. (A) $I_K$ recorded in control solution. (B) $I_K$ in the same neuron (same protocol) but following extracellular application of 5 mM TEA.
A

control

B

5 mM TEA

100 ms

0.1 nA

+60 mV

-60 mV

Figure 34
voltage-gated ion channels, chiefly Na\(^+\) and K\(^+\) channels. In order to determine possible interactions of (-)-\(\beta\)-cyclazocine and DM with voltage-gated ion channels, a study of whole-cell currents using specific voltage protocols to activate Na\(^+\) and K\(^+\) currents was carried out. For this series of experiments, a mouse DRG cell line was used since these neurons yielded sufficiently large whole-cell currents for study, whereas the cultured hippocampal and cortical neurons used in the previous studies were generally small. Robust TTX-sensitive sodium currents (\(I_{Na}\)) and TEA-sensitive potassium currents (\(I_{K}\)) were observed in the DRG neurons and were studied for their sensitivity to the presence of high concentrations of (-)-\(\beta\)-cyclazocine or DM.

3.5.1.1. Properties and pharmacology of \(I_{K}\)

In neurons, depolarizing steps from a holding potential of -60 mV activated an outward current whose size depended on the magnitude of the voltage step. Examination of the time course of this current revealed a fast approach to a non-zero steady-state level, as shown in Figure 34A. This current was sensitive to millimolar levels of externally applied TEA, as illustrated in Figure 34B. This current thus conforms to the properties of the delayed rectifier K\(^+\) current and will subsequently be referred to as \(I_{K}\).

3.5.1.2. Effects of DM and (-)-\(\beta\)-cyclazocine on \(I_{K}\)

The effects of DM and (-)-\(\beta\)-cyclazocine were tested at concentrations which completely antagonized NMDA activity in the electrophysiological and microspectrofluorimetric studies. The size of \(I_{K}\) activated by the largest depolarizing step (to +60 mV from a holding potential of -60 mV) in controls was 473±91 pA (\(n = 8\)).
Figure 35. Neuronal $I_k$ in the absence and presence of DM. (A) $I_k$ in control solution, elicited by the same protocol as in Figure 34. (B) $I_k$ in the same neuron after extracellular application of 50 µM DM.
Figure 35

A
control

B
50 μM DM

-60 mV

100 ms

0.1 nA

+60 mV
For DM (50 µM), the current activated by this step was 86±23% of control (n = 6). An example of the action of DM against $I_K$ is shown for a typical neuron in Figure 35.

As well, (-)-β-cyclazocine (5 µM) did not act to alter $I_K$; the size of the current activated by the step to +60 mV was 124±12% of control (n = 3). While it appears there was a small tendency for (-)-β-cyclazocine to actually increase the $I_K$, this was due to the behaviour of a single neuron, in which the size of $I_K$ increased by 45.7% upon superfusion of 5 µM (-)-β-cyclazocine. Factors such as changes in cell shape, affecting the voltage clamp, induced by solution flow may have contributed to this aberrant result. The above results show that neither DM nor (-)-β-cyclazocine produce any actions against neuronal $I_K$ at concentrations which fully inhibit NMDA activity and which provide strong neuroprotection against excitotoxic insult (Choi, 1987b).

3.5.1.3.Properties and pharmacology of $I_{Na}$ in neurons

From a holding potential of -60 mV, depolarizing steps to -20 mV, preceded immediately by a hyperpolarizing prepulse (-100 mV) to remove sodium channel inactivation, induced an inward current (mean amplitude 3.6±0.8 nA) that inactivated within 8 ms and was blocked by low concentrations of TTX. This is shown in Figure 36, in which a typical neuron initially passing an inward current of 6.5 pA showed almost no current response to depolarization after exposure to 0.1 µM TTX. Full recovery of this current following TTX washout was observed in this neuron (not shown). This current conforms to previous descriptions of sodium channel activity in neurons (Hodgkin and Huxley, 1952; etc.) and will subsequently be referred to as $I_{Na}$. 
Figure 36. TTX-sensitive sodium current in DRG neurons. $I_{Na}$ was elicited by a 15 ms depolarizing step to -20 mV following a 30 ms prepulse (-100 mV) from the holding potential (-60 mV). (A) $I_{Na}$ in control solution. (B) $I_{Na}$ in the same neuron (same protocol) but following extracellular application of 0.1 µM TTX.
Figure 36

A
control

B

0.1 μM TTX

-60 mV
-100 mV
-20 mV

5 ms
2 nA
Figure 37. Neuronal $I_{Na}$ in the absence and presence of DM. (A) $I_{Na}$ was elicited as in Figure 36 in the presence of control solution. (B) $I_{Na}$ in the same neuron but following extracellular application of 50 μM DM.
Figure 37
3.5.1.4. Effects of DM and (-)-β-cyclazocine on $I_{Na}$

Perfusion of the external solution with (-)-β-cyclazocine did not alter the size of $I_{Na}$. Current amplitudes at the peak were $1.04\pm0.04 \, (n = 2)$ times as large as in control when exposed to 5 μM (-)-β-cyclazocine (not shown), a concentration >30 times the $IC_{50}$ for reduction of NMDA open channel probability, and a concentration which abolished the $[Ca^{2+}]_{i}$ response to NMDA in the microspectrofluorimetric studies. This concentration of (-)-β-cyclazocine also did not alter the time course of $I_{Na}$ inactivation.

The addition of DM also did not affect the size of $I_{Na}$ measured at the peak. The $I_{Na}$ in a typical neuron in control and in the presence of 50 μM DM is illustrated in Figure 37. Current amplitudes in the presence of 50 μM DM, a concentration 10 times the $IC_{50}$ for reduction of NMDA $P_o$ and which brought the NMDA-evoked $[Ca^{2+}]_{i}$ response to less than 5% of control, were $1.02\pm0.03 \, (n = 4)$ times the amplitude in control. The time course of inactivation was also unaffected by 50 μM DM.

The lack of effects against $I_{Na}$ by either (-)-β-cyclazocine or DM at neuroprotective concentrations indicates that (-)-β-cyclazocine and DM would act as effective neuroprotective agents in neurons without compromising action potential generation or propagation.

3.5.1.5. Conclusions regarding drug effects on macroscopic currents in neurons

The addition of either DM or (-)-β-cyclazocine to the recording bath produced no changes in macroscopic $I_K$ and $I_{Na}$. The results for the compounds were determined at concentrations that were at least 10 times the $IC_{50}$'s for blockade of NMDA currents,
and that were near the concentrations required to completely protect neurons from severe NMDA-type glutamatergic insult (Choi, 1987; Section 3.4). Since $I_K$ and $I_{Na}$ are involved in both the generation and the termination of action potential spikes, the present results imply that neither agent would act to alter the time course or frequency of action potentials. It can therefore be predicted that the use of either DM or (-)-β-cyclazocine in neuroprotectant therapy would produce minimal toxic side effects with respect to neuronal transmission.

3.5.2. Macroscopic currents in ventricular myocytes

An agent used to protect the brain from excitotoxic damage would have maximal utility if it affected no other organs, especially those organs, such as the heart, involved in life-sustaining processes. Hence, voltage-gated currents in acutely dissociated rat ventricular myocytes were studied as a indicator of the potential for (-)-β-cyclazocine or DM to produce cardiac side effects. For this series of experiments, $I_{Na}$ and $I_K$ were examined for their susceptibility to alteration by the two studied compounds. As in the neurons, the agents were applied at concentrations well above the IC$_{50}$'s for inhibition of NMDA activity in hippocampal and cortical neurons, and that were near the concentrations shown to produce full neuroprotection in cultured neurons.

3.5.2.1. Properties and pharmacology of $I_{to}$ in ventricular myocytes

Depolarizing steps to potentials more positive than -30 mV from a holding potential of -70 mV activated an outward current which quickly attained a peak and then inactivated to a non-zero steady-state level, as shown in Figure 38A. This current could be pharmacologically separated into two components, the first being a transient,
Figure 38. Actions of DM on cardiac I_o. Currents were activated with a depolarizing step (+60 mV) from the holding potential (-70 mV). (A) I_o in control solution. (B) I_o in the presence of 10 μM DM. (C) I_o in the presence of 50 μM DM.
Figure 38
inactivating current sensitive to 4-AP, and the second being a slowly-activating residual current insensitive to 4-AP. These two components are therefore considered as separate entities distinguishable by their pharmacology and their kinetic behaviour (Apkon and Nerbonne, 1991; Castle and Slawsky, 1992; McLarnon and Xu, 1995), these being a rapidly inactivating component, which subsequently will be referred to as $I_{to}$, for transient outward current, and a residual delayed rectifier current. The present study concentrated on the effects of DM and (-)-$\beta$-cyclazocine on two parameters of $I_{to}$: the time course of inactivation ($\tau$) and the total charge passed by this entity. The effects of the agents on the residual steady-state portion of the current response was also studied and the results are presented below.

3.5.2.2. Effects of (-)-$\beta$-cyclazocine on $I_{to}$

In control, voltage steps to +60 mV induced an outward current that decayed with a $\tau$ of 44±3 ms ($n = 6$). The effect of (-)-$\beta$-cyclazocine on $\tau$ was small, with a concentration of 10 $\mu$M bringing $\tau$ to 80±11% ($n = 3$) of control. The integral of total charge movement, found by first subtracting the steady-state portion of the current and determining the area underneath the current amplitude vs. time curve, was also very weakly affected by the presence of (-)-$\beta$-cyclazocine. Values for total charge passed by $I_{to}$ in the presence of (-)-$\beta$-cyclazocine were 75±18% ($n = 3$) of the value in control, which was 87±14 nA ms for $n = 6$ myocytes.

The steady-state current remaining after inactivation of $I_{to}$ may represent a slowly-activating $I_{k}$, such as the delayed rectifier. The size of this current in $n = 6$ cardiac myocytes was 0.97±0.21 nA in control solution. Perfusion of 10 $\mu$M (-)-$\beta$-
cyclazocine had no effect on the size of this current, which was 102±15% (n = 3) of control following application of the agent.

3.5.2.3. Effects of DM on $I_{to}$

In contrast to (-)-$\beta$-cyclazocine, DM had actions to decrease the $I_{to}$ in cardiac myocytes. These actions were concentration-dependent, with greater reductions in $I_{to}$ being observed at 50 $\mu$M DM than at 10 $\mu$M DM. The decay of $I_{to}$ was quickened by perfusion of DM; the value for $\tau$ was 58±1% (n = 2) of control at 10 $\mu$M DM, and 38±20% (n = 2) at 50 $\mu$M DM (Figure 38B and C). The amount of charge passed was affected to an even greater degree, with 10 $\mu$M DM reducing the integral of charge movement to 27±6% (n = 2) of control, and 50 $\mu$M DM reducing it to 8±2% (n = 2) of control. These actions on $I_{to}$ would prolong the duration of the action potential in cardiac myocytes and thus would generally represent an unwanted side effect at doses only slightly higher than those therapeutically effective for neuroprotection.

The effect of DM on the delayed rectifier portion of the depolarization response was also studied. This entity was less sensitive to the presence of DM than $I_{to}$; no effect was observed at 10 $\mu$M DM (96±4% of control; n = 2), although a small but inconsistent effect was produced by 50 $\mu$M DM (63±20% of control; n = 2). The difference in susceptibility to DM between $I_{to}$ and the residual steady-state current indicates that, in the cardiac myocytes employed in the present study, the $I_{to}$ and the delayed rectifier are pharmacologically distinguishable entities.
Figure 39. Actions of DM on cardiac $I_{Na}$. Currents were elicited by a 15 ms depolarizing step to -20 mV following a 30 ms prepulse (-110 mV) from the holding potential (-70 mV); a series of 20 such activations is shown superimposed upon one another in each case. (A) $I_{Na}$ in control solution. (B) $I_{Na}$ in the presence of 10 μM DM. Note the increase in block with each pulse, seen as a series of successively smaller peaks superimposed upon one another. (C) $I_{Na}$ in the presence of 50 μM DM.
Figure 39

A
Control

B
10 μM DM

C
50 μM DM

-60 mV
-70 mV

40 ms
1 nA
3.5.2.4. Properties and pharmacology of $I_{Na}$ in ventricular myocytes

From a holding potential of -60 mV, a depolarizing step to -20 mV, delivered immediately after a hyperpolarizing prepulse (-90 mV) to remove sodium channel activation, elicited an inward current that attained a peak of $-8.3\pm0.4$ nA ($n = 6$) within 3 ms and quickly inactivated, within 6 ms, to zero net current (Figure 39A). While this pattern of activation and deactivation was similar to that observed in neurons, the pharmacology was different in that >10 times the concentration of TTX was required to block this current in cardiac myocytes compared to neurons (not shown). It was therefore of interest to study the effects of the two compounds (-)-β-cyclazocine and DM in the myocyte preparation.

3.5.2.5. Effect of (-)-β-cyclazocine on $I_{Na}$ in ventricular myocytes

Perfusion of the myocytes with 10 μM (-)-β-cyclazocine did not alter the peak size of $I_{Na}$, which was $108\pm2\%$ of control ($n = 2$). No action to alter the time course of inactivation was observed (not shown). The results of these experiments show the (-)-β-cyclazocine did not block cardiac sodium channels at concentrations greater than those required for neuroprotection.

3.5.2.6. Effect of DM on $I_{Na}$ in ventricular myocytes

Unlike (-)-β-cyclazocine, DM reduced the size of peak $I_{Na}$ in cardiac myocytes. This action was observed without any effect to alter the time course of inactivation. The $I_{Na}$ in a typical myocyte in control and in the presence of 10 and 50 μM DM is shown in Figure 39. The reduction in the peak size was use-dependent, with a steady-state level of block being achieved by the 16th pulse if the pulse protocol was delivered
at a rate of 10 s⁻¹. This can be seen in the example illustrated in Figure 39B and C, where succeeding pulses produced greater block and produced the thickening of the trace at the current peak. The use-dependent reduction of Iₘₙ suggests that DM inhibits cardiac sodium channels via an open-channel block mechanism. Overall, the size of the peak Iₘₙ was 49±13% (n = 2) of control in the presence of 10 μM DM and 25±7% (n = 2) of control in the presence of 50 μM DM. These concentrations are very near those producing similar levels of NMDA blockade in neurons and would therefore represent a potentially harmful cardiac side effect were DM to be used in neuroprotection therapy.

3.5.2.7. Conclusions regarding drug effects on macroscopic currents in ventricular myocytes

Perfusion of cardiac myocytes with a high concentration of (-)-β-cyclazocine produced no changes in Iₜ₀, the delayed rectifier, or Iₘₙ. These currents are involved in cardiac action potential generation and frequency and therefore any agent, such as (-)-β-cyclazocine, which produces no effect in these currents at therapeutically relevant concentrations, would be expected to be relatively free of cardiac side effects. The action of DM to enhance inactivation of Iₜ₀, as represented by the decrease in τ following DM perfusion, and to decrease the amplitude of the delayed rectifier, indicates that cardiac K⁺ channels are inhibited by this agent, even at a concentration of 10 μM which is close to the IC₅₀ for NMDA channel blockade (5 μM). This concentration also produced use-dependent open-channel block of cardiac Na⁺ channels. The above actions of DM would interfere with the process of action potential generation and termination and would alter spike frequency. It is therefore a legitimate
concern that concentrations of DM effective in neuroprotection could potentially produce cardiac side effects, such as arrhythmias.
4. DISCUSSION

4.1. Neuroprotection by (-)-β-cyclazocine

4.1.1. NMDA ion channel blockade by (-)-β-cyclazocine results in neuroprotection

One important finding of the work described in this thesis is that the neuroprotectant properties of (-)-β-cyclazocine are based on blockage of NMDA ion channels. This results from the agent's effects to decrease the open state probability (P_0) of single NMDA ion channels in the electrophysiological studies and its action to inhibit intracellular free calcium ([Ca^{2+}]) responses to NMDA in the microspectrofluorimetric studies. The antagonism of NMDA ion channels by this agent was a strong, if not complete, determinant of the neuroprotective effect: (-)-β-cyclazocine was, on one hand, a potent neuroprotectant while, on the other hand, acting as a potent non-competitive NMDA antagonist with little or no activity on other ion channels. In particular, the fura-2 measurements indicated little or no effects of the compound on AMPA or kainate responses or on responses mediated by voltage-gated Ca^{2+} channels; whole-cell patch clamp measurements showed the compound to have no effect on voltage-gated Na^{+} or K^{+} channels.

The potent neuroprotective properties of (-)-β-cyclazocine were mirrored in its potent blockade of responses to applied NMDA in both hippocampal and cortical neurons. In hippocampal neurons, the IC_{50} for (-)-β-cyclazocine reduction of P_o was 84 nM in patch-clamp measurements of single NMDA ion channels, whereas the IC_{50} for attenuation of NMDA-evoked increases in intracellular free calcium was 270 nM. The
three-fold difference in IC\textsubscript{50} values may represent differences in the parameters being measured by the two techniques: the microspectrofluorimetric method measures drug effects on rises in intracellular free calcium, whereas the electrophysiological measurements reflect drug actions on both sodium and calcium influxes through the NMDA-associated ion channel. In the cortical neurons, the IC\textsubscript{50} for reduction of single NMDA ion channel open state probability was 680 nM, whereas the IC\textsubscript{50} for inhibition of NMDA-evoked intracellular free calcium responses was 220 nM. Again, the differences may be referable to the methodology employed in the measurements.

4.1.2. Actions of (-)-\(\beta\)-cyclazocine relevant to neuroprotection

The study of the interaction of (-)-\(\beta\)-cyclazocine with single NMDA ion channels revealed two main effects of the compound on the kinetic behaviour of the channel. First, the mean open time of the channel was reduced, in a concentration-dependent manner, by (-)-\(\beta\)-cyclazocine. This reduction was observed in both hippocampal and cortical neurons. The action of (-)-\(\beta\)-cyclazocine to reduce channels mean open time (\(t_{o,p}\)) is a feature that would contribute to its antagonism of NMDA receptor-ion channel complexes independently of the channel opening frequency, and hence \(P_o\).

Diminution of the frequency of NMDA-activated unitary currents was observed concomitantly with the decrease in \(t_{o,p}\) following application of (-)-\(\beta\)-cyclazocine. These two effects of the compound combined to produce a decrease in the open state probability of the ion channel, such that the \(P_o\) was 50% of control at (-)-\(\beta\)-cyclazocine concentrations of 84 nM and 270 nM in hippocampal and cortical neurons, respectively. Since macroscopic current can be expressed as the product of unitary current, the number of conducting channels, and \(P_o\), it follows that changes in \(P_o\) can be used as a
comparison with other agents' effects on whole-cell currents. The IC\textsubscript{50} values obtained for (-)-\(\beta\)-cyclazocine show this compound to be one of the most potent blockers of NMDA activity known, with similar potency as PCP. Indeed, only dizocilpine (MK-801) is a more potent uncompetitive NMDA antagonist (Huettner and Bean, 1988; MacDonald et al., 1991).

4.1.2.1. Open channel block and the on-rate constant

A sequential open channel block model was used to characterize the interaction of (-)-\(\beta\)-cyclazocine with the NMDA ion channel. Plots of the inverse of mean open time versus (-)-\(\beta\)-cyclazocine concentration were linear for both hippocampal and cortical neurons, consistent with a simple open channel blockade scheme. This finding is important, since it indicates that, over the concentration range studied, (-)-\(\beta\)-cyclazocine acts only as an uncompetitive channel blocker. Such a relationship is characteristic of a bimolecular interaction between the blocking agent and a binding site on the receptor-ion channel complex. The blocking rate constants, \(k_2\), obtained from the slope of this plot, were found to be \(7.6 \times 10^8\) M\(^{-1}\)s\(^{-1}\) and \(1.2 \times 10^7\) M\(^{-1}\)s\(^{-1}\) at \(V_p = -40\) mV in hippocampal and cortical neurons, respectively. Open channel block has been used previously to characterize the interactions of a number of agents, including dizocilpine, PCP and several tricyclic antidepressants, with the NMDA channel (Huettner and Bean, 1988; Semagor et al., 1989; MacDonald et al., 1991). The results from whole-cell patch-clamp studies using ketamine, PCP, dizocilpine, and memantine have provided estimates for onward rate constants in the range \(2 \times 10^4 - 3 \times 10^5\) M\(^{-1}\)s\(^{-1}\) (MacDonald et al., 1991; Chen et al., 1992). As noted previously for dizocilpine
(Huettner and Bean, 1988), the blocker can gain access to its site only when the channels are open (or, more precisely, blockade via open channels proceeds at a much faster rate than via closed channels; Kloog et al., 1988). Therefore, whole-cell measurements of blocking rate constants must be corrected for the low open probability of the NMDA channel. Estimates of $P_0$ with recordings of unitary currents from outside-out patches have been given at 0.006 for 2 μM NMDA in outside-out patches (Bertolino et al., 1988) and 0.026 for 30 μM NMDA in cell-attached patches (McLarnon and Sawyer, 1993); these values for $P_0$ indicate the channel was open 0.6% and 2.6% of the time, respectively. In the present study, the $P_0$ was 0.08 in hippocampal neurons and 0.05 in cortical neurons for 25 μM NMDA at $V_p = -40$ mV. Thus, using a $P_0$ value of 0.05, corrected whole-cell onward rate constants would generally fall in the range $5 \times 10^6$ - $3 \times 10^7$ M$^{-1}$s$^{-1}$. The cluster of onward rate constants in the range $10^7$ - $10^8$ M$^{-1}$s$^{-1}$ suggests a similar site of action for the various agents. Furthermore, values near $10^7$ M$^{-1}$s$^{-1}$ are characteristic of blockers for other channel systems, such as with blockade of acetylcholine receptors by the lidocaine derivative QX-222, and are suggestive of a diffusion-limited process (Neher and Steinbach, 1978). The high values of the on-rate constants found for (−)-β-cyclazocine in the present study are reminiscent of the smaller amantadine derivatives investigated by Antonov and co-workers, suggesting that (−)-β-cyclazocine resembles these agents in terms of a possible deep binding site in the channel vestibule (Antonov et al., 1995; Antonov and Johnson, 1996).
4.1.2.2. The off-rate constant

Distributions for channel closed time were altered in the presence of (-)-β-cyclazocine. Sections 3.2.6.2 and 3.2.12.2 describe the presence of a long closed time component seen after (-)-β-cyclazocine addition. In theory, the unblocking rate constant can be found from comparison of closed time distributions in control solution and in the presence of the blocking compound. In the present experiments, a clear differentiation between channel blocking transitions to a non-conducting state and normal transitions to the closed state was not possible. For example, no evident rapid transitions (channel flickering) were observed in the presence of any of the studied compounds, including (-)-β-cyclazocine. However, closed time distributions with (-)-β-cyclazocine showed a longer, third time component which was generally absent, or rarely present, in control closed time distributions. The change in the longest time component most likely represents the time the drug molecule associates with the NMDA channel. The presence of a time component in the 200 - 400 ms range was the clearest effect of (-)-β-cyclazocine on closed time distributions. With the assumption that this long time component was a measure of the observed action of (-)-β-cyclazocine to significantly reduce open frequency, the unblocking rate constant was estimated to be near 5 s\(^{-1}\). Thus, channel block by (-)-β-cyclazocine was associated with a relatively slow off-rate constant, compared with Mg\(^{2+}\), for drug binding to a channel site. This was consistent with the lack of channel flickering observed in the presence of (-)-β-cyclazocine at any patch potential or drug concentration.
4.1.2.3. Potency of (-)-β-cyclazocine and comparison with other agents

The IC₅₀ values obtained for (-)-β-cyclazocine blockade of NMDA ion channels in the present study can be compared with the respective values of 1.2 μM and 10 nM obtained for memantine (Chen et al., 1992) and dizocilpine (Huettner & Bean, 1988). In the case of the latter compound, its low IC₅₀ has been attributed to a low k₂, or, in other words, its protracted association time with the channel site. Indeed, as pointed out above, our results suggest that the different magnitudes of IC₅₀ are primarily dependent on differing values of k₂ for blockade of the NMDA channel, since onward blocking rate constants are not markedly different among agents. The present results indicate that (-)-β-cyclazocine dissociates from its blocking site much faster than dizocilpine, which appears to remain associated with its site for >10 s (Huettner and Bean, 1988; Parsons et al., 1995). This point is important because the unusually slow offset time of dizocilpine is suggested to be a limiting factor in the clinical utility of this anticonvulsant (Chen et al., 1992; Lipton, 1993). For example, dizocilpine has been found to impair motor function in intact rats (Rogawski et al., 1991; Parsons et al., 1995). It would appear that the potency of a given agent is proportional to the amount of time spent by the agent in the channel blocking site; hence, the magnitude of k₂ is a major determinant in the blocking potency of uncompetitive antagonists.

4.1.2.4. Inhibition of NMDA-evoked [Ca²⁺]responses

Fluorescence microscopy of both hippocampal and cortical neurons loaded with the free calcium marker fura-2 revealed (-)-β-cyclazocine to be a potent antagonist of NMDA-evoked [Ca²⁺]responses. In these experiments, the level of (-)-β-cyclazocine
blockade increased each time the agonist was applied until an equilibrium level of blockade was achieved. Conversely, during washout, each agonist application in the absence of (-)-β-cyclazocine resulted in a greater change in $[Ca^{2+}]_i$ than with the previous application. The marked use-dependence observed with (-)-β-cyclazocine blockade has been previously noted for NMDA responses in rat neocortical wedges (Church et al., 1991) and is qualitatively similar to that seen with PCP, ketamine, and dizocilpine in macroscopic currents recorded from cultured murine hippocampal neurons following application of NMDA, L-aspartate, or L-glutamate (MacDonald et al., 1987; MacDonald et al., 1991). The degree of use-dependence (that is, how many agonist applications are required to achieve steady-state blockade or drug wash-off) is a consequence of the speed of interaction of a given agent with the blocking site, which is in turn governed by the blocking rate constant $k_2$ and the unblocking rate constant $k$. Indeed, of all the agents tested, only (-)-β-cyclazocine exhibits any degree of use-dependence, as evidenced by the fura-2 measurements. Since this compound showed the slowest off-rate (i.e., the lowest $k_2$), it is likely that the degree of use-dependence observed with a given compound is mainly dependent on the magnitude of $k_2$.

4.1.2.5. Trapping of antagonist within closed channels

Several bolus applications of NMDA were necessary in order to attain a new steady-state response following discontinuation of (-)-β-cyclazocine superfusion. This suggests the agent was trapped within the channel due to channel closure following anatagonist binding. Alternatively, this suggests that (-)-β-cyclazocine does not prevent channel closure; this aspect of the compound's action is supported by thke
similarity between the microscopic $K_D$ found in the single-channel experiments and the whole-cell $K_D$ determined in the fura-loaded neurons (Antonov et al., 1995). The size of the off-rate constant may be related to the tendency for the agent to be trapped within the ion channel. In the case of (-)-β-cyclazocine, the magnitude of $k_2$ found for (-)-β-cyclazocine in the electrophysiological studies was such that the length of time the drug molecules were associated with their site exceeded the normal opening/closing kinetics of the channel. Since (-)-β-cyclazocine exhibited the smallest $k_2$, the requirement of a number of agonist activations in order to wash away (-)-β-cyclazocine following its removal from the superfusion medium appears to be inversely correlated with the size of $k_2$. This facet of (-)-β-cyclazocine's actions on NMDA activity is consistent with a "drug-trapping" scheme in which the channel can close with the antagonist molecule(s) still remaining within the channel. Egress of the agent from closed channels proceeds at such a slow rate that it may be a requirement that the channel must be open for the blocker molecule to exit. In this sense, the model of (-)-β-cyclazocine interaction with NMDA ion channels is consistent with the "guarded receptor" hypothesis originally proposed by Starmer and colleagues (1986) for local anaesthetic block of Na⁺ channels, and modified by MacDonald et al. (1991) for uncompetitive antagonist block of NMDA channels, where access of a channel blocker to its site is controlled by the channel gating mechanism.

4.1.2.6. Potent neuroprotection with β-cyclazocine is stereospecific

The compound (-)-β-cyclazocine was found to be highly potent in the present neuroprotection model involving NMDA-induced excitotoxicity, with a high level of
neuroprotection being achieved at a concentration of 1 μM. The lack of (−)-β-cyclazocine action against neuronal Ca²⁺ channels, neuronal voltage-activated Na⁺ and K⁺ channels, and cardiac voltage-activated Na⁺ and K⁺ channels suggests that the neuroprotective effect of (−)-β-cyclazocine was chiefly due to its action to block NMDA ion channels. Whereas other agents which have been shown to be uncompetitive antagonists of the NMDA receptor-ion channel complex have been also found to possess neuroprotective properties (Olney et al., 1986; Choi, 1987b; Rogawski et al., 1991; Chen et al., 1992), to date, only MK-801 has been shown to be more potent than (−)-β-cyclazocine against NMDA-mediated neurotoxicity (Lysko et al., 1989).

The actions of β-cyclazocine on NMDA ion channels were stereospecific, as evident from the differential potency between the (−)- and (+)-enantiomers in their block of NMDA unitary currents, their inhibition of intracellular free calcium and their protection of cells subjected to excitotoxic insult. Stereospecificity of β-cyclazocine, with the (−)-enantiomer being more potent than the (+)-enantiomer, has previously been observed in NMDA responses of rat cortical wedge preparations in vitro and of rat spinal neurons in vivo (Church et al., 1991) and in binding experiments assaying the competition of the two enantiomers for a radiolabelled PCP receptor ligand (Todd et al., 1990). In PCP-trained rats, (−)-β-cyclazocine was 50 times more potent in substituting for PCP than (+)-β-cyclazocine (Slifer and Balster, 1988). The higher potency of the (−)-enantiomer of β-cyclazocine is consistent with the actions of other benzomorphan compounds on NMDA receptors, including pentazocine and α-cyclazocine (see Church and Lodge, 1990; an exception is SKF 10,047), and is opposite to compounds from
other classes acting on this site, including ketamine (Lodge et al., 1982) and the morphinans (Church et al., 1985).

4.1.2.7. Non-opiate mechanism

The use of outside-out patches would preclude indirect actions, such as effects via opioid receptors, of (-)-β-cyclazocine on currents through the NMDA ion channel. Since the potencies of (-)-β-cyclazocine in the microfluorescence studies and in the neuroprotection studies were similar to the potency in the electrophysiological studies, the possible contribution of opioid receptors to drug effects was likely small. For example, both morphine and naloxone have been previously found to each have no effect on NMDA-induced $[\text{Ca}^{2+}]$, in hippocampal neurons (Church et al., 1994a). While opiates have been shown to protect neurons from NMDA-mediated cell death in vitro (Choi and Viseskul, 1988), high concentrations of the agents were required for neuroprotection, including the classical μ opioid receptor agonist morphine (40 - 60% neuronal injury reduction at $[\text{morphine}] = 100 \ \mu\text{M}$); hence, the opiates were far less potent than (-)-β-cyclazocine was found to be in the present study. Finally, the anxiolytic actions of (-)-β-cyclazocine were found to be PCP-like rather than morphine-like in a punished behaviour model in rats (Porter et al., 1989). Nevertheless, the possibility that opioid actions of (-)-β-cyclazocine may have played a small role, or even may have produced a combined effect with its NMDA-blocking properties, in its neuroprotective capacity cannot be excluded and remains to be investigated in the future.
4.1.2.8. Predictive power of model

The application of an open channel block model to the results of the electrophysiological studies, subsequently confirmed by the observation of use-dependence in the microspectrofluorimetric experiments, was successfully used to predict the neuroprotective property of (-)-β-cyclazocine. Its high potency in reducing NMDA open channel probability in outside-out patches and in reducing NMDA-evoked \([\text{Ca}^{2+}] \) responses was matched by its high potency in protecting neurons from damage induced by exposure to a high concentration of NMDA. The relationship between electrophysiological potency (and potency in the microspectrofluorimetric studies) held true for two types of neurons, hippocampal and cortical. Furthermore, the rate constants associated with blockade also are of predictive value, since it was found that potency of a given agent to reduce NMDA open channel probability, and hence to protect against NMDA receptor-mediated neuronal damage, is a consequence of the magnitude of the unblocking rate constant, \(k_2\). Lipton (1993) has proposed a \(k_2\) "window" within which an NMDA antagonist is effective and safe. Agents with high \(k_2\) values, such as with the fast channel blocker Mg\(^{2+}\), interact too quickly with the ion channel and produce little overall antagonism. On the other hand, compounds with extremely low \(k_2\) values, such as MK-801, remain associated with the ion channel for long periods of time and consequently produce toxicity due to interference with the processes in which the NMDA receptor-ion channel play an important functional role, e.g., synaptic transmission. The \(k_2\) values found for (-)-β-cyclazocine with hippocampal and cortical neurons in the present study is much smaller than the \(k_2\) value for Mg\(^{2+}\) (as evidenced by the observed lack of flickering between open and
blocked states in the presence of (-)-β-cyclazocine) yet greater than the $k_2$ for MK-801 (around 0.003 s$^{-1}$; MacDonald et al., 1991), thus falling into the "window" of acceptable $k_2$ values for effective and well tolerated neuroprotectants. It is therefore suggested that the strategy described in the present work is an accurate identifier of putative neuroprotectants, and that further investigations using these techniques could be employed in order to identify other uncompetitive NMDA antagonists with neuroprotective properties.

4.1.2.9. Selectivity for NMDA channels vs. other channels

Challenges with other excitants (i.e., kainate and AMPA) revealed (-)-β-cyclazocine to be a selective blocker of NMDA ion channels vs. non-NMDA ion channels. The selectivity of (-)-β-cyclazocine for NMDA over non-NMDA ion channels agrees with the findings of Church et al. (1991) that responses to microelectrophoretically applied NMDA, but not the AMPA receptor agonist quisqualate, were inhibited when the agents were used with rat cortical wedges in vitro and with rat lumbar spinal neurons in vivo. The selective actions of (-)-β-cyclazocine on NMDA ion channels are consistent with the NMDA-selective effects of other benzomorphans, including N-allylnormetazocine (SKF-10,047; Berry et al., 1984), α-cyclazocine (Thomson and Lodge, 1985; Church and Lodge, 1990), and pentazocine (Church and Lodge, 1990).

Raised $K^+$-evoked $[Ca^{2+}]_i$ responses were unaffected by (-)-β-cyclazocine in both hippocampal and cortical neurons. This is in contrast with other uncompetitive antagonists, such as DM (see below) and PCP, which antagonize $Ca^{2+}$ channels at
concentrations only slightly above those effective in blocking NMDA responses (ffrench-Mullen and Rogawski, 1992). The responses to raised $K^+$ in the present experiments were due chiefly to $Ca^{2+}$ influx mediated through high voltage-gated calcium channels activated following neuronal membrane depolarization produced by the $K^+$ applications. Raised $K^+$-evoked $[Ca^{2+}]_i$ responses in cultured hippocampal neurons have been shown to be chiefly mediated via dihydropyridine-sensitive ion channels, or L-type $Ca^{2+}$ channels (Church et al., 1994a); however, a small part of the $[Ca^{2+}]_i$ response to raised $K^+$ was contributed by a $\omega$-conotoxin-sensitive component (most likely N-type $Ca^{2+}$ channels) and by a dihydropyridine- and $\omega$-conotoxin GVIA-resistant component. These experiments with the fura-2-loaded cells constitute the first evidence that (-)-$p$-cyclazocine is unable to block voltage-activated calcium channels, whether they be L-type, N-type, or non-L,N-type, in CNS neurons at concentrations which are completely effective in attenuating NMDA activity.

No effect of (-)-$p$-cyclazocine on voltage-gated ion channels were found in whole-cell patch clamp studies on neurons. Currents through voltage-gated $Na^+$ channels, which are responsible for the rising phase of the action potential, were not altered by the presence of (-)-$p$-cyclazocine, at a concentration sufficient to block NMDA activity by >90% (5 $\mu$M). The tetraethylammonium (TEA)-sensitive $I_K$, a delayed rectifier-type $K^+$ current which acts to repolarize neurons following action potential spikes, was also not affected by the same high concentration of (-)-$p$-cyclazocine. The lack of effects on amplitudes and kinetic behaviour of voltage-gated $Na^+$ and $K^+$ currents would suggest the compound would not impair neural transmission at concentrations which are neuroprotective due to NMDA blockade. This point does not
include the possibility that (-)-\(\beta\)-cyclazocine could act on other \(K^+\) channels which contribute to the repolarization phase of action potentials.

High concentrations of (-)-\(\beta\)-cyclazocine (5 \(\mu\)M) did not alter voltage-gated ion currents in ventricular myocytes. Since the pharmacology of \(I_{Na}\) in cardiac myocytes differs somewhat from that in neurons (the most salient example being the ten-fold difference in potencies of TTX blockade), it was reasonable to check for a differential action of (-)-\(\beta\)-cyclazocine on \(Na^+\) channels from the two tissue types. However, (-)-\(\beta\)-cyclazocine was found to be without effect on cardiac \(I_{Na}\). The two components of the \(K^+\)-carried current, the initial \(I_{Na}\) phase and the residual delayed-rectifier \(I_K\), were also unaffected by a high concentration of (-)-\(\beta\)-cyclazocine which was fully effective in blocking NMDA activity in neurons. These results show the pharmacology of (-)-\(\beta\)-cyclazocine to be similar in two types of excitable tissue, and also suggests that the use of (-)-\(\beta\)-cyclazocine as a neuroprotectant would not be expected to cause cardiac side effects, such as ventricular arrhythmias.

4.1.3. (-)-\(\beta\)-cyclazocine is a candidate neuroprotectant

The rescue of hippocampal neurons from NMDA-induced cell death by low concentrations of (-)-\(\beta\)-cyclazocine suggests further study with respect to its potential as a neuroprotectant compound. The involvement of NMDA receptors in several neuropathologies, including epilepsy, neurodegeneration following episodes of ischaemia, hypoxia, hypoglycaemia, or CNS trauma, and degenerative disorders such as Huntington's chorea, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, has been targetted as a possible focus for therapeutic intervention (Choi, 1988a; Choi and Rothman, 1990). The demonstrated uncompetitive nature of (-
-β-cyclazocine antagonism of NMDA activity in hippocampal neurons suggests that drug efficacy would be enhanced with escalating levels of glutamate. This point is important because drug effects on synaptic transmission mediated by normal levels of glutamate would be minimized, whereas NMDA receptors exposed to relatively high glutamate concentrations, the presence of which may be central in precipitating neurodegenerative disorders (Rogawski, 1993), would be selectively targeted. Furthermore, the higher magnitude of the unblocking rate constant compared to the clinically intolerable MK-801 suggests that the application of (-)-β-cyclazocine in CNS therapy would be relatively unhindered by neurological side effects. Therefore, the results obtained in the present study are indicative of (-)-β-cyclazocine's candidacy as a clinical agent appropriate in the therapeutic intervention of the pathology associated with stroke and chronic neurodegenerative disease.

4.2. NMDA antagonism by DM

4.2.1. Interactions of neuroprotectant concentrations of DM with NMDA ion channels

DM has been previously shown to act as an NMDA blocker (Church et al., 1985; Church et al., 1991b) and as a protector against glutamate insults in vitro (Choi, 1987b) and against ischaemic conditions in vivo (Steinberg et al., 1988). At present, however, the interactions of DM with the NMDA receptor-ion channel are not well documented. We have used measurements of DM actions on unitary NMDA currents and NMDA-evoked [Ca²⁺] responses to address this point.
4.2.2. Actions of DM relevant to neuroprotection

4.2.2.1. Alteration in NMDA ion channel kinetics

The actions of DM on unitary currents activated by NMDA included reversible, concentration-dependent reductions in mean open time and in opening frequency. Together, these changes resulted in decreases in the $P_0$ of the NMDA-associated ion channel. Similar alterations in unitary NMDA kinetics were seen with (-)-β-cyclazocine, suggesting that the two agents share a common mechanism of action with respect to NMDA antagonism. The observation of the reduction in $P_0$ in isolated patches shows the alteration in behaviour of NMDA ion channels to be a direct effect of DM, rather than its metabolite dextrorphan, to which DM is converted in vivo and which has a higher affinity as an NMDA antagonist (see Church et al., 1985).

4.2.2.2. Open channel block by DM and the forward rate constant

DM, at concentrations of 5 to 50 μM, had no effect on the magnitudes of unitary currents induced by activation of the 50 pS primary conductance state of the NMDA channel. This result, coupled with this agent's action to diminish channel mean open times and the frequency of openings, was suggestive of open channel block by DM. Previous studies (see below) have used various open channel block schemes to analyze drug interactions with the NMDA channel. In this work, a simple uncompetitive open channel block model appeared adequate to describe DM's actions on unitary NMDA currents, since a plot of the inverse of mean open time, in the presence of DM, vs. drug concentration was linear. The blocking rate constant $k_2$ was obtained from the slope of the relation and had magnitudes of $8.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in hippocampal neurons.
and $7.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in cortical neurons; these on-rates were similar, although slightly slower, than those for (-)-p-cyclazocine ($7.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ in hippocampal neurons and $1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ in cortical neurons) and not far removed from the blocking rate constant of $3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ that has been estimated for dizocilpine block of unitary NMDA currents (Huettner and Bean, 1988). Thus, it would seem that onward (blocking) rate constants for a range of putative uncompetitive NMDA channel blocking drugs are not markedly different.

4.2.2.3. Voltage-dependence of on-rate constant

The on-rate constant $k_2$ was found to depend on potential, using the single-channel methods employed in the present study. The values for $k_2$ increased with more negative $V_p$; however, this voltage dependence was less than that associated with Mg$^{2+}$ block, where the voltage-dependence was quite pronounced (Huettner and Bean, 1988). It should also be noted that mean open times in controls were dependent on potential and diminished by about 20% for each 20 mV increase in patch hyperpolarization. The voltage dependence associated with channel blockade is thus strongly influenced by an additional contribution from the voltage dependence of the unblocking rate constant, as evidenced by the trend for greater reduction in channel opening frequency with more negative $V_p$. Voltage-dependence for off-rate constants has been observed in previous studies with dizocilpine (Huettner and Bean, 1988); voltage-dependence in the onward rate constant has also been noted with several derivatives of adamantane (Antonov et al., 1995; Antonov and Johnson, 1996), of
which memantine and adamantane are also derivatives showing voltage-dependent on-
and off-rate constants (Blanpied et al., 1997).

4.2.2.4. High off-rate constant for DM and the implication for clinical use

It would seem evident that differences in potencies for uncompetitive NMDA
channel-blocking agents primarily reflect differences in the magnitudes of the
unblocking (off-rate) constants from a site(s) associated with the NMDA channel. In
this regard, DM was found to wash off relatively rapidly after application of control
solution to the excised patches and exhibited similar kinetic behavior of unitary currents
to that observed with memantine (Chen et al., 1992). Despite having relatively fast
kinetics of interaction with the NMDA receptor-operated channel, DM appears to
possess a narrow therapeutic window in seizure tests on rats (Löscher and Hönack,
1993), although it has been employed successfully for treating seizures in humans
(e.g., Schmitt et al., 1993) and exhibits little toxicity in clinical dose-escalation safety
studies (Steinberg and Bell, 1992). The former findings may reflect DM's rapid
metabolism in vivo to the relatively high-affinity uncompetitive NMDA antagonist
dextrorphan (see Church et al., 1989) and does not, therefore, mitigate the suggestion
that relatively low-affinity uncompetitive NMDA antagonists with fast kinetics of
interaction with the NMDA receptor-operated channel may be well-tolerated clinically.

4.2.2.5. The nature of the DM binding site: biophysical studies

Biophysical studies with DM were carried out in order to obtain further
information about the nature of the binding site for uncompetitive blockers and the
manner in which the blockers interact with the site. An outside-out path exposed
simultaneously to DM and the endogenous uncompetitive NMDA antagonist Mg$^{2+}$ behaved as if the two agents were acting at different sites, with Mg$^{2+}$ possibly reducing access of DM to its site. The results of temperature studies on cortical neurons intended to elucidate the nature of the interaction of DM with NMDA ion channels were inconclusive. While the conductance mediated by NMDA channels was associated with a $Q_{10}$ of 1.4, indicating a process rate-limited by diffusion, channel kinetics were associated with much higher $Q_{10}$'s, suggestive of a process more complicated than one governed by free diffusion of channel elements between two locations or configurations. This high temperature dependence in itself served to mask the effects of temperature on DM access to and egress from its blocking site. Therefore, because of the complex nature of NMDA ion channel kinetics, these experiments were not able to yield any definitive information regarding the manner in which DM inhibits current flow through the channel.

4.2.2.6. DM reduction of NMDA-evoked [Ca$^{2+}$]$_i$ responses

Consistent with its reduction of unitary NMDA $P_o$ in outside-out patches, DM concentration-dependently and reversibly inhibited NMDA-evoked [Ca$^{2+}$]$_i$ increases in both hippocampal and cortical neurons. With hippocampal neurons, reasonable agreement between the IC$_{50}$ value obtained in the microspectrofluorimetric studies and the DM concentration associated with a channel $P_o$ of 0.5 (4 μM in both cases) was found. Similarly, with cortical neurons, the DM concentration at which standardized NMDA $P_o$ was 0.5, 4 μM, matched closely the IC$_{50}$ value obtained from inhibition of NMDA-evoked [Ca$^{2+}$]$_i$ increases (5 μM). These values show that the sites through
which uncompetitive antagonism of NMDA activity is effected in the two neuronal subtypes is indistinguishable by DM. There was no use-dependence associated with DM blockade of NMDA-evoked $[\text{Ca}^{2+}]_i$ increases, with equilibrium blockade being reached after a single excitation. This is contrast to $(-)$-β-cyclazocine, for which up to 6 NMDA applications were necessary to achieve steady state. DM was also $\approx 20X$ less potent than $(-)$-β-cyclazocine in inhibiting NMDA responses. Together, these two findings suggest that the potency of an agent as an open channel blocker is related to its off-rate constant, with the faster approach of DM to equilibrium blockade being indicative of a higher $k_2$.

4.2.2.7. DM actions on voltage-activated $\text{Ca}^{2+}$ channels

The lack of selectivity of DM for NMDA-activated ion channels vs. voltage-activated $\text{Ca}^{2+}$ channels was observed in both hippocampal and cortical neurons. The action of DM on $\text{Ca}^{2+}$ channels is consistent with previous observations in cultured hippocampal neurons (Church et al., 1991b), in synaptosomes from whole rat brain (Carpenter et al., 1988), and in PC12 cells (Carpenter et al., 1988) and has been observed to occur concurrently with NMDA antagonism, albeit with lesser potency (Jaffe et al., 1989; Church et al., 1991b). It has been suggested that the neuroprotectant action of DM (Choi, 1987b; Steinberg et al., 1988; Tortella et al., 1989) may be related to the combination of its actions at the two sites; although $\text{Ca}^{2+}$ channel antagonism has shown promise as a neuroprotective strategy (Siesjö, 1986; Pizzi et al., 1991), problems related to efficacy have been encountered (Koroshetz and Moskowitz, 1996).
4.2.2.8. Lack of effect on non-NMDA ion channels

The actions of DM to reduce responses evoked by kainate and AMPA in hippocampal neurons was no larger than the reduction in $K^+$-evoked $Ca^{2+}$ responses observed simultaneously. Therefore, it is likely that DM acted to reduce the kainate- and AMPA-evoked $[Ca^{2+}]_i$ responses by virtue of its effect to block $Ca^{2+}$ channels that mediated the $[Ca^{2+}]_i$ increase produced by non-NMDA receptor-mediated depolarization. Interestingly, in cortical neurons, DM had no effect on kainate- and AMPA-evoked $[Ca^{2+}]_i$ responses at a concentration that resulted in $Ca^{2+}$ channel blockade. It would appear that, in cortical neurons, $[Ca^{2+}]_i$ responses to kainate and AMPA were not dependent on activation of either NMDA or $Ca^{2+}$ channels that were sensitive to DM. One tempting explanation for this phenomenon would be that these cortical neurons contained non-NMDA receptors that were permeable to calcium, as with AMPA receptors lacking the GluR2 subunit (McBain and Mayer, 1994; Mody and MacDonald, 1995); further studies are required to clarify this issue.

4.2.2.9. Lack of DM actions on voltage-gated ion channels in neurons and cardiac myocytes

Very little work has been done with regard to DM actions against voltage-activated ion channels. This is surprising, because DM has entered clinical trials for neuroprotection (Koroshetz and Moskowitz, 1996), using doses higher than required for its antitussive action. The purpose of this section was to investigate the effects of DM on voltage-gated $K^+$ and $Na^+$ currents using whole-cell studies in neurons. These experiments showed that, like (-)-$\beta$-cyclazocine, DM is ineffective against other voltage-
gated ion channels. Neither the TTX-sensitive $I_{\text{Na}}$ nor the TEA-sensitive $I_{\text{K}}$ were blocked by DM, even at a concentration of 50 $\mu$M, 10X the $IC_{50}$ for inhibition of NMDA open channel probability. Two conclusions can be made from these observations. First, effects of DM to impair intraneural transmission would not be expected. Second, together with the results with (-)-$\beta$-cyclazocine on the same neurons, the lack of effect of DM on voltage-activated $Na^+$ and $K^+$ channels shows that neither of these two channel types played a significant role in the neuronal damage produced by exposure to a high concentration of NMDA, and that the neuroprotective properties of DM (Choi, 1987; Steinberg et al., 1988; Tortella et al., 1989) are not dependent on $Na^+$ or $K^+$ channel blockade.

In contrast to the results obtained in neurons, studies into the effects of DM on whole-cell currents in ventricular myocytes revealed blockade of cardiac voltage-gated ion channels. The two distinguishable components, $I_{\text{to}}$ and the delayed $K^+$ current, of the outward $K^+$ response to depolarizing pulses were reduced relative to control in the presence of DM. Also, DM concentration- and use-dependently reduced the size of the $Na^+$ current, suggesting an open-channel block of cardiac $Na^+$ channels. It is possible that DM may have acted to shift the voltage-dependence of activation of the $Na^+$ current; more detailed experiments would be necessary in order to elucidate the nature of DM interaction with cardiac $Na^+$ channels. The observation that DM blocks voltage-gated ion channels in cardiac tissue without a parallel effect in neuronal tissue suggests that both $Na^+$ and $K^+$ channels show variation between the two tissue types in terms of pharmacological properties. The introduction of DM for use as a neuroprotective agent should, therefore, proceed with considerable caution, as effects
on cardiac voltage-gated ion channels were observed at concentrations not much higher than the IC$_{50}$ for NMDA antagonism, the effect of DM most relevant to its neuroprotective action.

4.3. NMDA antagonism by L-687,384

4.3.1. Interaction of L-687,384 with NMDA ion channels

4.3.1.1. Open-channel block of NMDA ion channels

The actions of the σ ligand L-687,384 on NMDA channel kinetics differed from the other tested agents in that no change in opening frequency was observed. However, similar to (-)-β-cyclazocine and DM, L-687,384 concentration-dependently reduced mean open time of the NMDA ion channel, and hence showed an effect to reduce channel open probability at micromolar concentrations. The increase in $t_{op}^{-1}$ was found to be linear with increasing [L-687,384], consistent with an open channel block model for this compound. Therefore, despite its lack of effect on opening frequency, L-687,384 likely antagonized NMDA activity via the same open channel block mechanism as (-)-β-cyclazocine and DM.

4.3.1.2. Fast kinetics of block

The inability of L-687,384 to alter channel opening frequency at concentrations up to 40 μM was reflected in the closed time distributions, which also were unchanged by the compound. The failure to observe an additional component in closed time distributions may have been due to the fact that any term added by the compound to the closed time distribution was similar in value to the terms present in control, i.e., the
occupation time of L-687,384 in its blocking site is smaller than the normal closed time of the channel in control. Additionally, in the microspectrofluorimetric experiments, the reduction of NMDA-evoked \([\text{Ca}^{2+}]_i\) responses by L-687,384 quickly reached equilibrium (no use-dependence was observed) and the blocking effect was quick to wash out following superfusion with control solution. A high off-rate constant for L-687,384 is thus suggested, and is consistent with its low potency to reduce \(P_o\) and NMDA-evoked \([\text{Ca}^{2+}]_i\) increases. Correlations between values for off-rate constants and IC\(_{50}\) values have been observed previously for a number of NMDA blockers, including PCP, dizocilpine, and ketamine (MacDonald \textit{et al.}, 1991) and a series of memantine derivatives (Parsons \textit{et al.}, 1995). The present results with L-687,384 are therefore consistent with descriptions of open channel blocker properties in other laboratories.

4.3.1.3. Relationship between open channel block and \(\sigma\) effects

The NMDA antagonist action of L-687,384 observed in the present study is unlikely to account for the absence of potentiation of NMDA responses reported following 'high' (500 \(\mu\)g kg\(^{-1}\) i.v.) doses of the drug \textit{in vivo} (Bergeron \textit{et al.}, 1992). In the case of DTG, a dose of 1 \(\mu\)g kg\(^{-1}\) i.v. (corresponding to a theoretical maximal brain concentration of < 5 nM; Monnet \textit{et al.}, 1990) potentiates neuronal firing induced by NMDA, whereas 5 mg kg\(^{-1}\) (corresponding to a maximal concentration in brain of 25 \(\mu\)M) fails to do so (Monnet \textit{et al.}, 1992). The IC\(_{50}\) value for DTG as an antagonist of NMDA-evoked currents under whole-cell voltage clamp is 37 \(\mu\)M (Fletcher \textit{et al.}, 1993), suggesting that functional NMDA receptor antagonism may underlie the failure of high doses of DTG to potentiate the NMDA response \textit{in vivo} (Monnet \textit{et al.}, 1990; Monnet et
Brain concentrations of L-687,384 following peripheral administration are unknown, but a dose of 500 μg kg$^{-1}$ i.v. would theoretically produce a maximal brain concentration of about 1 μM, considerably lower than the apparent IC$_{50}$ value for the reduction of NMDA-evoked rises in [Ca$^{2+}$]. Unlike DTG, the actions of L-687,384 at the NMDA channel are evident only at more than 1000-fold higher concentrations than the concentrations required for displacing [$^3$H] ligands from the σ recognition site (Middlemiss et al., 1991; Barnes et al., 1992).
5. SUMMARY

This study has incorporated a multi-faceted approach to study the interactions of four potential neuroprotectant compounds acting at the NMDA receptor-ion channel complex. The compounds chosen belong to classes of agents which encompass ones that are known to possess NMDA antagonist properties, including benzomorphans (the enantiomers (-)- and (+)-β-cyclazocine), and σ ligands (DM and L-687,384). The approach used is novel in that electrophysiological measurements have been combined with optical recordings to yield biophysical data on mechanisms of action. In turn, the elucidation of mechanisms have aided in the interpretation of the concentration-response data regarding the direct effects of the agents as neuroprotectants against excitotoxicity. Furthermore, ancillary whole-cell recordings have allowed some insight into possible side effects of the compounds through actions mediated via voltage-gated ion channels. A number of conclusions can be drawn from this work. These are summarized as follows.

1. (-)-β-cyclazocine exhibits marked neuroprotective activity against EAA-induced toxicity on cultured hippocampal and cortical neurons. The protection of neurons evinced by (-)-β-cyclazocine is strong evidence that alteration in single-channel behaviour by this agent would result in neuroprotection where NMDA overactivity is a key determinant of cellular toxicity.

2. The basis for the agents' neuroprotection is uncompetitive open channel blockade of the NMDA ion channel. The IC₅₀ values for open channel blockade by (-)- and (+)-β-cyclazocine paralleled the ED₅₀ values obtained in
the neuroprotection assays. The ED\textsubscript{50} for open channel block by DM was higher than that obtained with (-)-\textit{\beta}-cyclazocine and accounts for its lower potency in the neuroprotection assays documented by Choi (1987).

3. The magnitudes of the onward (blocking) rate constants are similar for (-)-\textit{\beta}-cyclazocine, DM, and L-687,384, yet potencies of the compounds for open channel NMDA blockade differ markedly and present in a different rank order than that for \(k_2\). Thus, the magnitude of the onward rate constant is not a critical determinant of an agents' potency to block NMDA ion channels.

4. The magnitude of the unblocking rate constant \(k_2\) for (-)-\textit{\beta}-cyclazocine and DM is important in determining the potency of open channel blockade. The \(k_2\) values for both compounds are much higher than that found for MK-801 (Huettner and Bean, 1988), showing that the former compounds dissociate much faster from their blocking site relative to the latter compound. This finding is significant because the very slow offset time of MK-801 is likely a major contributor to the serious neurological side effects produced by this agent.

5. Cellular responses with fura-2-loaded neurons showed use-dependence associated with the onset of (-)-\textit{\beta}-cyclazocine action. This feature of the antagonist profile indicates that the open NMDA channel is the target, consistent with the patch clamp data. Use-dependent activity would be expected to be advantageous for a neuroprotectant, in that the agent would be more efficacious under situations of excessive receptor activation, as in excitotoxic conditions.
6. The microspectrofluorimetric studies were also useful in determining the selectivity of the agents for NMDA ion channels. The results indicated (-)-β-cyclazocine and DM had no significant actions against non-NMDA (i.e. kainate and AMPA) ion channels. This point is important because such interactions could compromise the clinical utility of the compounds (for example, interference with fast synaptic transmission mediated by kainate and AMPA receptors). In addition, (-)-β-cyclazocine had negligible effects on voltage-gated Ca\(^{2+}\) channels. It was noted, however, that DM had an action to block Ca\(^{2+}\) channels at concentrations about 4x the IC\(_{50}\) for block of NMDA ion channels.

7. Whole-cell patch clamp experiments showed (-)-β-cyclazocine had no significant effects on voltage-gated Na\(^{+}\) or K\(^{+}\) currents in neurons and cardiac cells. The recorded K\(^{+}\) currents likely represent ones active during the repolarization phase of the action potential, including transient and delayed rectifier components. DM had actions to inhibit both Na\(^{+}\) and K\(^{+}\) currents in cardiac, but not neuronal, cells. This result would suggest the possibility that DM could produce cardiac side effects at concentrations at which it would exert neuroprotective effects.

8. The use of temperature variations is suggested to generally be a useful biophysical probe to study the interactions of agents with sites associated with ion channels. In this work, the relatively low Q\(_{10}\) for the temperature dependence of NMDA channel conductance would suggest diffusion-limited current flux. The considerable temperature dependence associated with
channel blockade kinetics \((Q_{10} \text{ near } 3)\) would suggest utility in both increasing and decreasing temperature to study channel interactions. Unfortunately, the variable temperature studies on kinetic behaviour in the present work were complicated by two findings. Firstly, the kinetic behaviour of the NMDA channel, in the absence of channel blockers, was quite strongly dependent on temperature. Secondly, low temperature caused a significant decrease in the frequency of channel events. This effect, when combined with the actions of (-)-\( \beta \)-cyclazocine and DM to reduce frequency of openings, did not allow for resolution of sufficient events for kinetic distributions.

The utility of (-)-\( \beta \)-cyclazocine as a candidate neuroprotectant is strongly indicated by the results of this work. However, the issue of drug-induced psychotomimesis that has been previously encountered with other non-competitive NMDA antagonists must be considered before proceeding with further research into this agent's clinical role in the treatment of excitotoxic brain damage. Use of the open-channel NMDA blocker PCP is associated with unpredictable psychotic symptoms emerging some time following its administration (Willetts et al., 1990). This feature of PCP action is a complication that has led to suspicion that psychotomimesis induced by compounds of this type is a function of NMDA blockade itself, and hence would present an inescapable side effect of NMDA antagonists (Willetts et al., 1990). However, the present study has shown DM, which is not psychotomimetic in humans (Tortella et al., 1989), to exhibit NMDA channel-blocking properties. Furthermore, PCP is not selective for NMDA ion channels; this agent exhibits considerable actions to inhibit voltage-gated
Ca"2+ channels (ffrench-Mullen and Rogawski, 1992), acetylcholine-gated channels (Aguayo and Albuquerque, 1986), and Na"+ and K"+ channels (Tourneur et al., 1982). These additional actions of PCP may also contribute significantly to the generation of side effects by this compound. As noted above, optical measurements showed (-)-p-cyclazocine had no action to block voltage-gated Ca"2+ channels in hippocampal or cortical neurons. In addition, whole-cell patch clamp measurements showed the compound did not modify Na"+ or K"+ currents in excitable cells (neurons or cardiac myocytes).

In conclusion, the rescue of hippocampal neurons from NMDA-induced cell death by low concentrations of (-)-β-cyclazocine suggests further study with respect to its potential as a neuroprotectant compound. The same conclusion is possible with DM with the proviso that the safety margin between neuroprotectant actions and effects on other voltage-gated channels is not large. The involvement of NMDA receptors in several neuropathologies, including epilepsy, neurodegeneration following episodes of ischaemia, hypoxia, hypoglycaemia, or CNS trauma, and degenerative disorders such as Huntington's chorea, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, has been targeted as a possible focus for therapeutic intervention (Choi, 1988a; Danysz et al., 1995). The evident uncompetitive nature of (-)-β-cyclazocine antagonism of NMDA activity in hippocampal neurons suggests that drug efficacy would be enhanced with escalating levels of glutamate. This point is important because drug effects on synaptic transmission mediated by normal levels of glutamate would be minimized, whereas NMDA receptors exposed to relatively high glutamate concentrations would be selectively targeted. Furthermore, the selectivity of (-)-β-
cyclazocine for NMDA ion channels, as well as the higher magnitude of the unblocking rate constant compared to the clinically intolerable MK-801 suggests that the application of (-)-β-cyclazocine in CNS therapy would be relatively unhindered by neurological side effects. Therefore, the results obtained in the present study are indicative of (-)-β-cyclazocine's candidacy as a clinical agent appropriate in the therapeutic intervention of the pathology associated with stroke and chronic neurodegenerative disease.
6. REFERENCES


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