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# Structural Modifications to an *N*-Methyl-D-aspartate Receptor Antagonist Result in Large Differences in Trapping Block

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## ABSTRACT

Differences in the degree of trapping of initial block by *N*-methyl-D-aspartate (NMDA) receptor antagonists may affect their safety and, hence, suitability for clinical trials. In this comparative study, 23 compounds structurally related to the low-affinity, use-dependent NMDA receptor antagonist (*S*)-$\alpha$-phenyl-2-pyridineethanamine dihydrochloride (AR-R15896AR) were examined to determine the degree of trapping block they exhibit. Compounds were tested at concentrations that produced a comparable initial 80% block of NMDA-mediated whole-cell current in rat cortical cultures. A wide range of values of trapping block was found, indicating that trapping is not an all-or-none event. Fifteen of the compounds trapped significantly more than the 54 ± 3% of initial block trapped by AR-R15896AR. The off-rates of these compounds were slower than that of AR-R15896AR. Only 2 of the 23 compounds trapped significantly less than AR-R15896AR. AR-R15808, the piperidine analog of AR-R15896AR, appeared to trap only 8 ± 3% of its initial block, although its fast off-rate confounded accurate quantification of trapping. AR-R26952, which, like AR-R15896AR, contains a pyridine in place of a phenyl group, trapped 40 ± 5% of its initial block and exhibited kinetics comparable with AR-R15896AR. Structure-activity analysis suggested that the presence of two basic nitrogen atoms and decreased hydrophobicity led to decreased trapping. There was no correlation between trapping and lipophilicity as would be expected if closed-channel egress was due to escape through the lipid bilayer. However, there was a positive correlation between off-rate and degree of trapping. Models that can account for partial trapping are presented.

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Some neuropathological states, like epilepsy, are characterized by excessive activation of receptors and cells, in terms of frequency or duration. Periods of excessive activation, prolonged ischemic depolarization, or multiple spreading depression-like periods also characterize ischemia. Some of the most useful anticonvulsants, like phenytoin, are uncompetitive, or use-dependent antagonists (Meldrum, 1993). Use-dependent compounds can only access their site of action when the receptor/channel is in an active state, resulting in a delay between receptor activation and the antagonist-induced blockade. Therefore, they are less likely to block short periods of activation, which typify normal activity. This characteristic of use-dependent compounds probably accounts for their clinical utility to block convulsant episodes without severely affecting normal function.

Antagonists of the NMDA type of excitatory amino acid receptors are neuroprotective in the in vitro and in vivo models of ischemia (Kemp et al., 1987; Olney et al., 1989). In addition, cerebral ischemia has been associated with an elevation of extracellular concentrations of excitatory amino acids (Benveniste et al., 1984). However, the clinical use of NMDA antagonists has been hampered by the occurrence of side effects that appear to be a class effect (Small and Buchan, 1997). Hallucinations and dysphoria of the type produced by PCP appear to be due to blockade of normal activity mediated by NMDA receptor activation.

NMDA receptor antagonists that act at the PCP site produce a use-dependent block, acting only after the receptor enters the open state (Huettner and Bean, 1988). Unexpectedly, many of these use-dependent antagonists, like MK-801, also have no therapeutic value due to PCP-like neurobehavioral side effects (Leppik et al., 1988). Many of these compounds have high affinity for the receptor, whereas some lower affinity derivatives have reduced side effects. It was hypothesized that the lack of safety of high-affinity compounds was due to the fact that the concentrations needed to reach steady-state block rapidly enough to be therapeutic (via mass action) are much higher than those that already completely block receptor activity (Rogawski, 1993). For the

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; MK-801, dizocilpine; ADCI, 5-aminocarbonyl-10,11-dihydro-5*H*-dibenzo-[*a,d*]cyclohepten-5,10-imine; AR-R15896AR, (*S*)-$\alpha$-phenyl-2-pyridineethanamine dihydrochloride; MEM, Eagle's minimal essential medium; 9-AA, 9-aminoacridine.

lower affinity compounds, the concentrations that only partially modulate receptor activity are similar to those needed to achieve rapid block. In support of this hypothesis, memantine (Muller et al., 1995; Parsons et al., 1995), amantadine (Parsons et al., 1995), and ADCI (Rogawski et al., 1991, 1995), all of which have relatively low affinity (Chen et al., 1992; Parsons et al., 1993, 1995), lack serious side effects.

However, a comparison of a series of uncompetitive NMDA receptor antagonists for efficacy as antiepileptic drugs demonstrated that some lower affinity antagonists actually had worse therapeutic indices (Parsons et al., 1995), suggesting that other factors are also important. Trapping, a feature of many use-dependent NMDA receptor antagonists, may account for these neurobehavioral side effects. Antagonists such as MK-801, ADCI, memantine, amantadine, ketamine, and AR-R15896AR all exhibit trapping (Jones and Rogawski, 1992; Blanpied et al., 1997; Chen and Lipton, 1997; Mealing et al., 1997, 1999; Lanthorn et al., 2000; Sobolevsky and Yelshansky, 2000). Trapping occurs when agonist is removed while antagonist is present, and the antagonist remains bound to the receptor. During subsequent receptor activation, the trapped antagonist is already present, blocking activity immediately. Thus, a trapped antagonist functionally loses its use-dependence and produces a tonic block. With compounds like MK-801 and ADCI, trapping appears to be complete (Jones and Rogawski, 1992). However, in the case of the clinically safer compound, memantine, about one sixth of the blocked channels release, rather than trap, the blocker (Blanpied et al., 1997). We have recently reported significant differences in the degree of trapping among three low-affinity, use-dependent NMDA receptor antagonists that possess intermediate kinetics (Mealing et al., 1999). Ketamine, which induces psychotomimetic side effects (Ginski and Witkin, 1994; Krystal et al., 1994), showed 86% trapping, whereas memantine and AR-R15896AR, which have improved therapeutic safety profiles (Parsons et al., 1995; Palmer et al., 1996, 1997), trapped only 71% and 54%, respectively. This reduction in trapping, from ketamine to memantine to AR-R15896AR, is in the same order as that for reduction of severity of side effects as measured by behavioral and cognitive tests in rodents. Therefore, use-dependent NMDA receptor antagonists that exhibit less trapping may provide safer compounds for therapeutic use.

The purpose of this study was to examine a series of structurally related compounds (analogs of AR-R15896AR) to see if differences could be found in the degree of trapping block. We then looked for correlations between these differences and the physical and structural characteristics of the compounds.

## Materials and Methods

**Compound Selection.** Starting from a library of 131 compounds, a diverse subset of clusters of compounds structurally related to the NMDA receptor antagonist, AR-R15896AR (formerly called ARL 15896AR or FPL 15896AR), were generated. The diversity metrics used for creating these clusters were two-dimensional fingerprints, molecular weight, and dipole total. These parameters were calculated from the compounds' chemical structures using SYBYL 6.7 software (Tripos, Inc., St. Louis, MO). LogP and p$K_a$ values, computed using ACD Software (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada), were also included in the cluster creation criteria. Clusters were generated using Wards clustering algorithm (JMP software; SAS Institute, Inc., Cary, NC). Twenty-one compounds, each one selected arbitrarily from a different cluster, were tested to determine the degree of trapping they exhibited. Compounds were not selected from all clusters generated because of their unsuitability (for example, their weak antagonism). Two additional compounds, AR-R15808 and AR-R26952, were subsequently chosen to examine the impact of specific structural features (the presence of two basic nitrogen atoms) on trapping. All compounds tested were previously synthesized during the development of AR-R15896AR.

ACD_logP is the calculated LogP (octanol/water partition coefficient) for the neutral form of a molecule, accurate to ±0.3 U or better. The calculation, made using ACD software, is based on an algorithm that uses a database containing one or more experimental LogP values for over 3600 structures with 500 different functional groups. ACD_p$K_a$ is the calculated acid-base ionization constant (p$K_a$ value) for an organic structure at 25°C and zero ionic strength in an aqueous solution. The accuracy of the calculation is usually better than ±0.2 units. This calculation is also based on ACD's database and algorithms. ACD_LogD is the calculated apparent LogP value at pH 7.4.

**Chemicals and Reagents.** Dulbecco's phosphate-buffered saline, HEPES, Eagle's minimal essential medium (MEM), poly-L-lysine, tetrodotoxin, and strychnine hydrochloride were purchased from Sigma Chemical (St. Louis, MO). Heat-inactivated fetal bovine serum was purchased from Life Technologies (Gaithersburg, MD), and heat-inactivated horse serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). NMDA, (±)-2-amino-5-phosphonopentanoic acid, and ketamine were purchased from RBI/Sigma (Natick, MA). EGTA was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). NMDA receptor antagonists were synthesized by AstraZeneca USA (Worcester, MA).

**Cell Culture.** Rat cortical neurons isolated from E18 fetuses were grown in primary culture as previously described (Black et al., 1995). Timed-pregnant Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, PQ, Canada). After killing the mother by cervical dislocation under halothane anesthesia, the fetuses were removed from the uterus on day E18, their brains removed and placed in ice-cold phosphate-buffered saline, and the cortices isolated. The cortical neurons were dispersed by triturating with a 10-ml pipette, and the cells were centrifuged at 250*g* for 5 min at 4°C. The cells were gently resuspended in plating medium and viable cells, as determined by trypan blue exclusion, were counted. The cells were then plated at $10^5$ cells/cm$^2$ on poly-L-lysine-coated 35 mm culture dishes (Nunc, Roskilde, Denmark) in 2 ml of plating medium at 37°C in an atmosphere of 5% $CO_2$/95% air. Plating medium consisted of 80% MEM, with 20 mM glucose, 10% heat-inactivated fetal bovine serum, and 10% heat-inactivated horse serum containing 2 mM L-glutamine. Since the cultures contained both neurons and glial cells, they were treated with 15 mg/ml of 5-fluoro-2′-deoxyuridine and 35 mg/ml on day 5 to minimize glial cell proliferation. On day 6, half of the medium was removed and replaced with growth medium consisting of 90% MEM and 10% horse serum. Experiments were performed on cultures after 14 to 19 days in vitro.

**Whole-Cell Recording.** Cortical neurons grown on 35-mm culture dishes were mounted on the stage of an inverted Zeiss microscope equipped with Hoffman modulation optics in a perfusion system flowing continuously at 1 ml/min at 22°C. The bathing solution contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, and 3 mM glucose at pH 7.4. Bathing solutions also contained 1 $\mu$M tetrodotoxin to block $Na^+$ currents, 10 $\mu$M glycine to saturate the glycine site on NMDA receptors, and 1 $\mu$M strychnine to block glycine-activated $Cl^-$ currents.

Patch pipettes (2–4 MΩ resistance) were constructed from 1.5 mm o.d./1.0 mm i.d. Pyrex 7740 glass (Corning, Big Flats, MN) using a Brown-Flaming P80 micropipette puller (Sutter Instrument Co., San Francisco, CA). The pipette solution contained 140 mM CsCl, 1.1 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP at pH 7.2.

Whole-cell currents were acquired using an Axopatch 1-D ampli-

fier equipped with a CV-4 headstage with a 1 GΩ feedback resistor (Axon Instruments, Foster City, CA). Voltage command and current acquisition were accomplished using a personal computer equipped with a Digidata 1200 interface and pClamp 6.0 software (Axon Instruments). Data were filtered at 1 kHz and sampled at 2 kHz.

Rapid agonist or agonist-antagonist application was accomplished using a modified DAD-12 perfusion system (ALA Scientific Instruments, Westbury, NY). The system consisted of a custom-made manifold of five quartz tubes, 100 $\mu$m in diameter, that converged into a common 100-$\mu$m tip with minimal dead volume. The tubes were fed from pressurized reservoirs equipped with miniature switching valves controlled by a computer, such that solution only flowed from a single reservoir at one time. The tip of the manifold was placed less than 100 $\mu$m from the patch-clamped cell under study. Solutions were degassed prior to use, and reservoirs were pressurized at 200 to 400 mm Hg. Switching between solutions took 12 ± 1 ms ($n$ = 12), as determined by junction potential measurements using a 10% $Cl^-$ solution in one reservoir and normal $Cl^-$ in the others. However, whole-cell current responses to agonist were slower, such that maximum agonist-induced current developed at 200 ± 25 ms. All drugs were prepared fresh daily.

**Data Analysis.** The block of NMDA-evoked currents was calculated according to the formula $B = [(I - I_B) / I] \times 100$, where $I$ was determined by curve fitting the decay of the NMDA-evoked current during the NMDA application and extrapolating the fit to the end of the NMDA antagonist coapplication, and $I_B$ was the current measured at the end of NMDA/blocker coapplication. Current decays were fit to first-order exponential curves using a Chebyshev fit method and pClamp software.

The residual block of NMDA-evoked currents was calculated according to the formula $B_R = [(I_{1st} - I_{2nd}) / I_{1st}] \times 100$, where $I_{1st}$ was the maximal current measured 200 ± 25 ms after onset of the first NMDA exposure and $I_{2nd}$ was the maximal current measured 200 ± 25 ms after onset of the delayed second NMDA exposure after washout of blocker from the bath. The delay between onset of agonist application and current measurement was to allow development of maximal $I_{1st}$ and to avoid possible corruption of data measurement by artifacts.

The block trapped ($B_T$), or the amount of block remaining at the beginning of the second NMDA application as a percent of the initial block produced at the end of the previous NMDA/antagonist coapplication, was calculated according to the formula $B_T = (B_R / B) \times 100$, where $B$ and $B_R$ are defined as above.

Data are expressed as means ± S.E.M. ($n$ = number of cells). Statistical analysis was performed using Microcal Origin (Northampton, MA) software. Comparisons were made by analysis of variance, with $p < 0.01$ considered as significant.

## Results

**The Physiological Relevance of Trapping.** In general, trapping experiments were conducted with antagonist removed between the initial agonist/antagonist coapplication and the later agonist application. However, in a therapeutic situation, antagonist would be present continuously, making it important to demonstrate that antagonist could still escape from the channel in this situation. As is shown in Fig. 1, AR-R15896AR does escape from the channel whether or not it is present in the bath between applications of NMDA. There was no significant difference in trapping whether antagonist was continually present (61 ± 2%, $n$ = 3) or not (54 ± 3%, $n$ = 9). This demonstrates that the partial trapping of an antagonist is a physiologically relevant phenomenon.

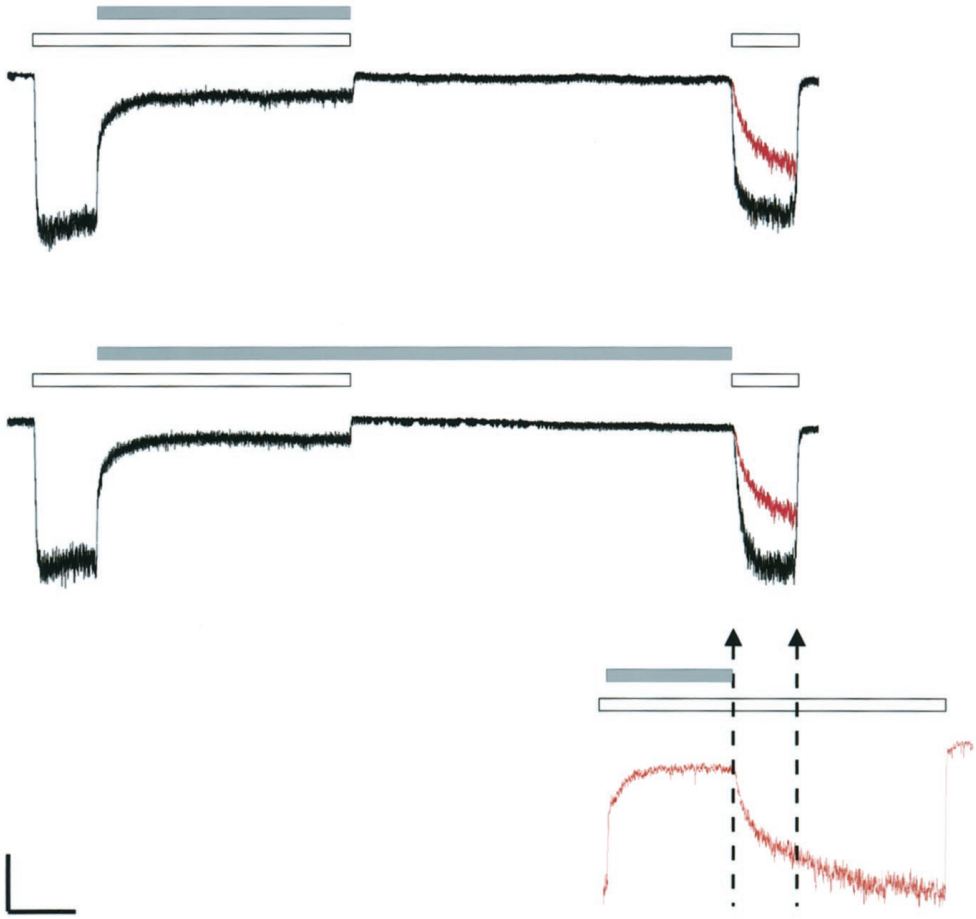


**Fig. 1.** Partial trapping occurs regardless of whether the antagonist is continuously present during the 30 s between agonist applications (middle trace) or not (top trace). The bottom current trace (in red) shows the onset and immediate relief of block using the same concentration of antagonist used in the top traces. The section of this trace between the dashed lines corresponds to the antagonist off-rate and has been superimposed (in red) where agonist was reapplied in the upper traces. This illustrates the difference between the similar partial trapping observed in the top two traces and their appearance if trapping were complete. Open bars indicate application of 10 $\mu$M NMDA, and shaded bars indicate application of 50 $\mu$M AR-R15896AR. Scale, 100 pA; 5 s.

**On-/Off-Rates and Trapping of Compounds Structurally Related to AR-R15896AR.** The concentration of each compound that was required to produce a block of NMDA-induced whole-cell current, not significantly different from the block of 81 ± 1% produced by 50 $\mu$M AR-R15896AR, was determined. Then on-/off-rates and degree of trapping of the initial block were measured using the appropriate concentration of antagonist. Representative current traces for selected compounds that trap significantly less than, equivalent to, or more than AR-R15896AR are shown in Fig. 2, top, middle, and bottom, respectively. On-/off-rates were measured by curve fitting the development of block, or relief from it, as neurons were exposed for 5 s to 10 $\mu$M NMDA, followed by a 30-s coapplication of NMDA + antagonist and a 50-s reexposure to NMDA. Trapping was determined by exposing cells to 10 $\mu$M NMDA for 5 s, followed by a 30-s coapplication of NMDA + antagonist (in black), and then 120 s later reapplying 10 $\mu$M NMDA for 20 s (shown superimposed in red upon the initial response). The degree of trapping and the on-/off-rates for each compound at the appropriate concentration are summarized in Table 1.

Two compounds trapped significantly less than AR-R15896AR. We measured a trapping block of only 8 ± 3% with AR-R15808 (Fig. 2, top). However, this value may be an underestimation of trapping, because the off-rate of AR-R15808 exceeded the resolution of our system. A minor, slower component of its off-rate ($\tau_{off}$ = 6.4 ± 1.1 s) was also observed in 6 of 11 cells tested. The structure of AR-R15808 resulted from changing the pyridine group of AR-R15896AR to a piperidine ring, thus increasing the basicity of this nitrogen. The second compound that trapped significantly less than AR-R15896AR was AR-R26952. Although AR-R26952

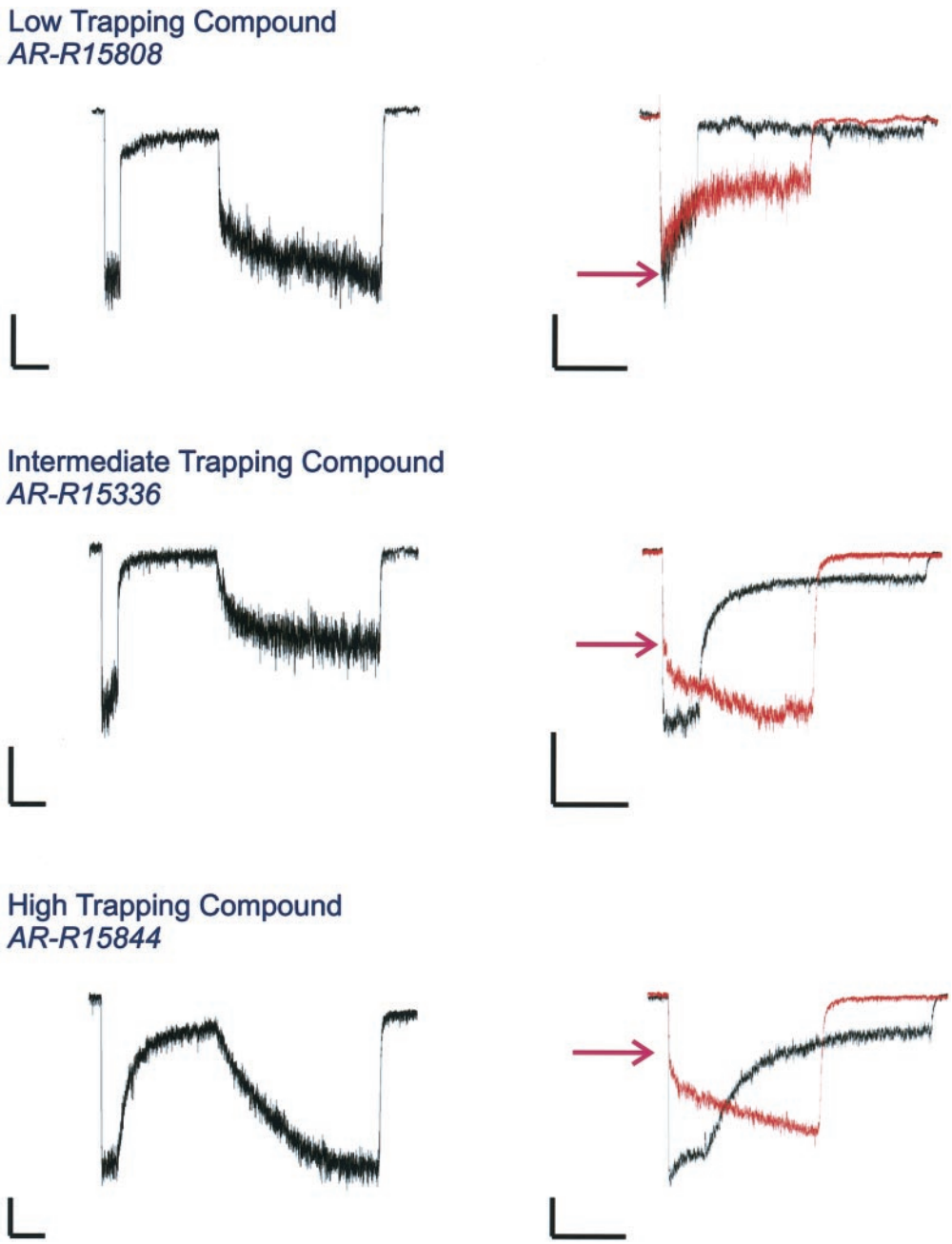
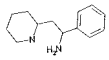
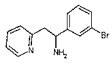
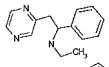
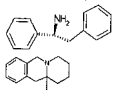
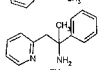
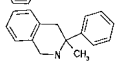
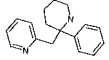



**Fig. 2.** The left current trace for each compound shows the response to a 5-s exposure to 10 $\mu$M NMDA, followed by a 30-s coapplication of NMDA + antagonist and a 50-s reexposure to NMDA. The right set of two current traces shows an initial response (in black) to a 5-s exposure to 10 $\mu$M NMDA, followed by a 30-s coapplication of NMDA + antagonist. Superimposed (in red) upon this initial response is the response to a 20-s exposure to 10 $\mu$M NMDA exactly 120 s later. Arrows indicate the initial level of current upon reapplication of agonist. Scale, 100 pA; 10 s.

TABLE 1
Summary of properties of compounds structurally related to AR-R15896AR

| AR-R | Structure | FW (g) | [$IC_{80}$] ($\mu$M) % B | % $B_T$ | $\tau_2$ on (s) | $\tau_1$ on (s) | $\tau_{off}$ (s) |
|---|---|---|---|---|---|---|---|
| Low-trapping compounds[a] | | | | | | | |
| 15808 See Fig. 2 (Upper) |  | 277.2 | 150<br>84 ± 2 (22) | 8 ± 3 (8)<br>See text | 0.2 ± 0.0<br>89 ± 1% | 5.8 ± 0.6 | See text |
| 26952 | | 311.3 | 150<br>80 ± 2 (12) | 40 ± 5 (6) | 0.5 ± 0.3<br>70 ± 4% | 4.9 ± 0.5 | 6.9 ± 1.0 |
| High-trapping compounds[b] | | | | | | | |
| 16442 |  | 352.30 | 20<br>86 ± 2 (12) | 70 ± 4 (7) | 1.1 ± 0.1<br>48 ± 2% | 5.1 ± 0.2 | 10.6 ± 2.2 |
| 15278 | | 281.85 | 50<br>86 ± 2 (8) | 72 ± 2 (6) | 1.0 ± 0.1<br>52 ± 8% | 7.0 ± 1.2 | 7.5 ± 3.6 |
| 12859[c] | | 332.36 | 10<br>84 ± 1 (11) | 72 ± 3 (6) | 1.2 ± 0.2<br>49 ± 8% | 8.0 ± 1.5 | 7.1 ± 1.8 |
| 15824[c] | | 321.98 | 50<br>81 ± 2 (9) | 76 ± 3 (5) | 1.9 ± 0.3<br>49 ± 4% | 13.9 ± 2.3 | 10.5 ± 2.1 |
| 16283 |  | 264.67 | 50<br>84 ± 1 (10) | 76 ± 4 (6) | 0.5 ± 0.1<br>51 ± 5% | 2.9 ± 0.2 | 3.7 ± 0.6 |
| 15232 |  | 233.74 | 10<br>85 ± 2 (15) | 77 ± 5 (6) | 1.3 ± 0.1<br>61 ± 3% | 5.9 ± 0.9 | 10 ± 0.8 |
| 15545[c] | | 263.4 | 100<br>82 ± 2 (11) | 78 ± 2 (5) | 3.5 ± 0.3<br>60 ± 8% | 18.8 ± 3.0 | 13.3 ± 1.1 |
| 15832 | | 339.31 | 20<br>76 ± 3 (6) | 78 ± 6 (3) | 1.3 ± 0.2<br>35 ± 6% | 11.5 ± 0.2 | 15.4 ± 1.4 |
| 18526 | | 266.78 | 20<br>81 ± 1 (13) | 80 ± 3 (8) | 0.8 ± 0.1<br>49 ± 2% | 6.4 ± 0.3 | 10.7 ± 1.3 |
| 15366 | | 319.3 | 20<br>89 ± 1 (8) | 81 ± 2 (6) | 1.8 ± 0.1<br>54 ± 2% | 8.1 ± 0.1 | 23.8 ± 0.5 |
| 15859[c] |  | 285.22 | 20<br>83 ± 2 (12) | 82 ± 2 (6) | 0.9 ± 0.1<br>41 ± 3% | 7.2 ± 0.8 | 8.6 ± 2.4 |
| 15514[c] | | 253.8 | 10<br>84 ± 4 (10) | 84 ± 3 (5) | 2.4 ± 0.3<br>32 ± 6% | 10.9 ± 1.1 | 45.5 ± 0.6 |
| 15844[c] See Fig. 2 (Lower) |  | 259.77 | 20<br>83 ± 1 (14) | 84 ± 2 (8) | NA | 5.3 ± 0.2 | 21.7 ± 1.2 |
| 15492[c] | | 395.5 | 200<br>85 ± 3 (6) | 92 ± 3 (4) | 2.3 ± 0.5<br>16 ± 6% | 12.4 ± 0.2[e] | 63 ± 13 |
| 15793[c] |  | 310.39 | 20<br>86 ± 1 (13) | 93 ± 3 (8) | 1.1 ± 0.1<br>39 ± 2% | 7.6 ± 0.6 | 20.8 ± 5.3 |
| Intermediate-trapping compounds[d] | | | | | | | |
| 15896 | | 271.2 | 50<br>81 ± 1 (9) | 54 ± 3 (9) | NA | 2.4 ± 0.6 | 2.8 ± 0.9 |
| 15336 See Fig. 2 (Middle) | | 282.39 | 100<br>85 ± 3 (6) | 57 ± 6 (5) | 0.5 ± 0.2<br>65 ± 7% | 4.9 ± 0.6 | 6.6 ± 1.1 |
| 15006 | | 444.44 | 30<br>83 ± 1 (15) | 60 ± 4 (10) | 1.2 ± 0.1<br>56 ± 2% | 7.2 ± 0.6 | 15.0 ± 0.7 |
| 15614[c] | | 293.86 | 10<br>80 ± 1 (8) | 60 ± 5 (5) | 0.8 ± 0.1<br>56 ± 1% | 7.8 ± 0.9 | 13.4 ± 0.5 |
| 15209 | | 259.78 | 10<br>83 ± 1 (7) | 61 ± 4 (5) | 0.6 ± 0.1<br>52 ± 2% | 7.5 ± 0.6 | 13.1 ± 6.6 |
| 15870[c] | | 291.03 | 10<br>82 ± 1 (9) | 62 ± 7 (5) | 2.3 ± 0.2<br>55 ± 4% | 11.0 ± 0.9 | 13.3 ± 3.3 |
| 15688 | | 450.56 | 50<br>82 ± 2 (11) | 68 ± 3 (6) | 0.5 ± 0.1<br>65 ± 3% | 3.8 ± 0.8 | 3.0 ± 1.2 |

FW, formula weight; $B_T$, block trapped; NA, not applicable
[a] Significantly less trapping block than AR-R15896AR.
[b] Significantly more trapping block than AR-R15896AR.
[c] Fully saturated $\alpha$-carbon.
[d] Not significantly different trapping block than AR-R15896AR.
[e] Not steady-state at 30 s.

trapped only 40 ± 5% of its initial block, its off-rate ($\tau_{off}$ = 6.9 ± 1.0 s) was well within resolution, making a quantitative determination of trapping possible. Both AR-R26952 and AR-R15896AR contain a pyridine instead of a phenyl group.

Six compounds showed intermediate trapping characteristics, not significantly different from AR-R15896AR. Onset and relief of block and trapping of one of these compounds, AR-R15336, is shown in Fig. 2, middle. Trapping in this group of compounds ranged from 54 ± 3 to 68 ± 3%, whereas the $\tau_{off}$ of these compounds was between 2.8 ± 0.9 and 15.0 ± 0.7 s.

Fifteen NMDA receptor antagonists produced significantly more trapping block than AR-R15896AR, ranging from 70 ± 4 to 93 ± 3%. AR-R15844, which trapped 84 ± 2% of the initial block, is shown in Fig. 2, bottom. The off-rates of these compounds were slower than that of AR-R15896AR. In fact, we observed a significant positive correlation ($p < 0.0001$; $r$ = 0.85) between Log $\tau_{off}$ and the degree of trapping among all the compounds tested (Fig. 3A). The distinction between trapping and just coming off slowly could not be made for AR-R15492 ($\tau_{off}$ = 63 ± 13 s), given the time frame of the experimental protocol used. However, depolarizing the cell to +60 mV in the presence of the agonist could drive off AR-R15492. The on-rate for AR-R15492 was also sufficiently slow that a steady-state block was not reached after 30 s. It is noteworthy that the structures of the four highest trapping compounds have a fully saturated carbon atom in the $\alpha$ position. In fact, 8 of the 15 high trapping compounds have a fully saturated carbon atom in the $\alpha$ position, whereas only 2 of the 7 intermediate trapping compounds and none of the low trapping compounds have a fully saturated carbon atom in the $\alpha$ position.

**Correlation between Physical Characteristics and Trapping.** There was no significant correlation between lipophilic character and trapping, as estimated by ACD_LogP (Fig. 3B). A negative correlation would be expected if partial trapping was due to lipophilic escape from the closed channel. On the contrary, a significant positive correlation was found between trapping and ACD_LogD (Fig. 3C), a parameter that takes into account the ionic character of compounds at physiological pH. This result is mirrored by a significant negative correlation between ACD_p$K_a$ values and trapping (Fig. 3D). In addition, compounds that exhibited the least trapping contained two basic nitrogen atoms that were significantly protonated at physiological pH, thus increasing the hydrophilic nature of the molecule.

## Discussion

This study demonstrates that partial trapping of block during repetitive stimulation can occur even in the continuous presence of antagonists. Therefore, the degree of trapping of an antagonist is a relevant factor to consider in the physiological situation. It is likely that trapping of channel block contributes to the side effects that have limited the clinical development of use-dependent NMDA receptor antagonists. Our studies confirm and extend the observation that trapping is not an all-or-nothing phenomenon. Among the 24 compounds tested, we observed levels of trapping that varied over a broad range, from essentially complete (93%) to at least as low as 40%. In fact, we measured trapping of only 8% for AR-R15808. However, this value may be an underestimate, since this compound's off-rate exceeded the resolution of our system. If the factors that led to these differences in degree of trapping were amenable to drug design, compounds with increased safety could be synthesized.

Another important finding of this study is that, within the initial group of 21 compounds selected, none trapped significantly less than AR-R15896AR, whereas 15 of them trapped significantly more. AR-R15896AR was chosen as a clinical

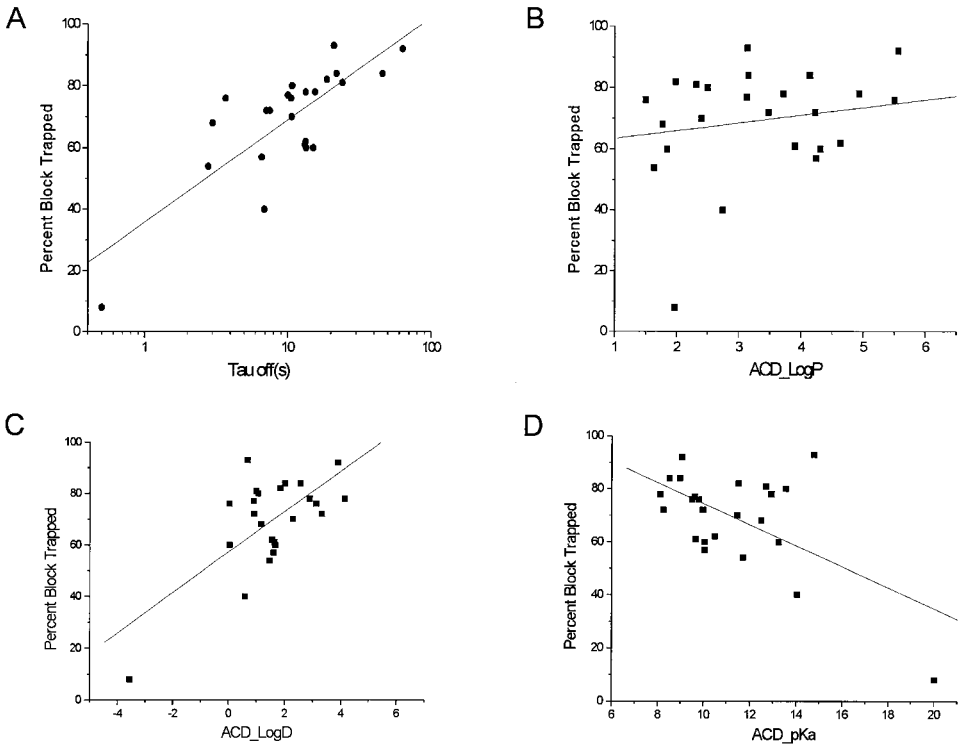



**Fig. 3.** A, plot of Log$\tau_{off}$ versus percent block trapped. Linear fit shows a significant positive correlation ($p < 0.0001$; $r$ = 0.85). B, plot of ACD_LogP versus percent block trapped. Linear fit shows no significant correlation ($p$ = 0.18; $r$ = 0.28). C, plot of ACD_LogD versus percent block trapped. Linear fit shows a significant positive correlation ($p < 0.003$; $r$ = 0.67). D, plot of ACD_Logp$K_a$ versus percent block trapped. Linear fit shows a significant negative correlation ($p < 0.003$; $r$ = 0.59).

candidate over other compounds in this group based strongly on its lack of PCP-like effects in rats (Palmer et al., 1999). Taken together, these observations are consistent with the hypothesis that trapping is an important factor in the PCP-like behaviors seen with many use-dependent NMDA receptor antagonists.

Specific structural features influence the degree of trapping. From examining structures of the 21 compounds in the initial group selected, the presence and nature of two basic nitrogen atoms appeared to be important. AR-R15896AR itself contains a pyridine and is less trapped than compounds containing a phenyl group in the analogous position. This suggested that a pyridine or other heterocyclic group might reduce trapping in other compounds. Two additional compounds, AR-R26952 and AR-R15808, were specifically chosen to test this hypothesis. Like AR-R15896AR, AR-R26952 contains a pyridine instead of a phenyl group. AR-R26952 trapped only 40% of its initial block, which was significantly less than that of AR-R15896AR. In the case of AR-R15808, changing the pyridine of AR-R15896AR to a piperidine ring increased the basicity of this nitrogen. This substitution resulted in a compound with the lowest apparent degree of trapping and the fastest off-rate of all compounds tested. At the other end of the spectrum, the four highest trapping compounds have a fully saturated carbon atom in the $\alpha$ position. In fact, 8 of the 15 high trapping compounds have a fully saturated carbon atom in the $\alpha$ position, whereas only two of the seven intermediate trapping and none of the low trapping compounds have a fully saturated carbon atom in the $\alpha$ position. Thus, changes in structural features influence the degree of trapping, suggesting that a specific binding site is responsible for differences in trapping and that partial trapping is an inherent feature of the trapping mechanism in NMDA receptors.

A simple mechanism that could account for differences in the degree of trapping is closed-channel egress via a lipophilic pathway. However, there was no significant correlation between the extent of trapping and ACD_LogP among the 24 compounds examined in this study. This observation is supported by our previous report that lipophilicity did not correlate with trapping among AR-R15896AR, memantine, and ketamine (Mealing et al., 1999). Furthermore, that study also demonstrated that trapping was stable over relatively long periods of time, which would not be predicted if lipophilic diffusion was occurring. Cumulatively, this evidence strongly argues that lipophilic diffusion through the receptor protein or lipid bilayer is not responsible for partial trapping.

Our data indicate a positive linear correlation between $\mathrm{Log}\tau_{\mathrm{off}}$ and degree of trapping, which is consistent with antagonist unbinding being an exponential process. Furthermore, among three NMDA receptor antagonists with intermediate kinetics that we previously used to investigate trapping, a similar trend is evident (Mealing et al., 1999). We also noted that the degree of trapping was the same across a range of concentrations of antagonist. This finding is consistent with the correlation between off-rate and degree of trapping, because off-rate is concentration-independent. Compounds with a fast off-rate could escape being trapped simply because they exit faster than the receptor closes. This could possibly be described by modifications to existing linear models of trapping block. However, a sequential or linear mechanism of trapping block would predict inward tail currents, developing upon removal of agonist following onset of block, since the blocked receptor must return to a closed state via an open state (Lingle, 1983). In this study, tail currents were not observed with any of the compounds tested. Using similar methodology, we have previously demonstrated tail currents using the sequential channel blocker, 9-AA (Mealing et al., 1999). However, failure to observe tail currents with 23 of the compounds tested is not compelling evidence against a sequential mechanism of block. These compounds all have a much slower off-rate than 9-AA. Consequently, tail currents spanning a longer duration with smaller amplitude could have escaped detection. However, AR-R15808, whose off-rate, like that of 9-AA, was beyond our resolution, also failed to show tail currents. Therefore, although we cannot rule out a sequential mechanism of block for compounds that exhibit partial trapping, we can offer no evidence to support it.

On the other hand, cyclic models, like those of Gurney and Rang (1984) or Benveniste and Mayer (1995), explicitly allow for the possibility of the return from a closed, blocked state directly to the closed state. These models do not predict tail currents prior to channel closure. Changes in the rate constant for the escape of antagonist from the closed channel, or "closed-channel egress", could allow partial trapping to occur. However, these models give no insight into mechanisms that could mediate closed-channel egress.

A physical description of trapping channel block involves closing of the channel that is associated with trapping of the antagonist molecule. The question arises as to where the blocker is trapped. It is unlikely that these use-dependent antagonists can traverse the NMDA channel. Therefore, closing of the channel cannot prevent the antagonist from rejoining the extracellular fluid. We can visualize the channel to be a "lower barrier" through which antagonists cannot pass into the cell (Fig. 4). To account for antagonists being trapped, there must be an additional, more extracellularly located, "upper barrier", whose closure restricts the antagonist from rejoining the extracellular space. In this scheme, once the upper barrier closes, the antagonist is trapped in a "vestibule", formed by the space between these two barriers, and the receptor is in the closed, blocked state. This scheme is similar to one proposed for quaternary ammonium compounds block of potassium channels (Armstrong, 1971). Barring lipophilic diffusion, the receptor cannot return to the closed, unblocked state without going through a blocked state in which the upper barrier is open. Partial trapping could occur if the upper barrier closes more slowly than the channel. In large, structurally complex molecules like receptor channels, this is not improbable. Compounds with fast off-rates could escape trapping because they escape the vestibule faster than the upper barrier closes. Therefore, the term closed-channel egress would be more accurately defined as closed-channel/open-vestibule egress.

An interesting corollary to this model is that the antagonist should also be able to enter the vestibule during the closed-channel/open-vestibule state. A significant amount of entry would reduce apparent closed-channel egress. In our experiment to test the physiological relevance of closed-channel egress, we did not detect any significant differences suggestive of closed-channel/open-vestibule entry. However, since macroscopic on-rate is a function of concentration, it is possible that this effect would be prominent only at higher concentrations of antagonist. It is also possible that the pres-

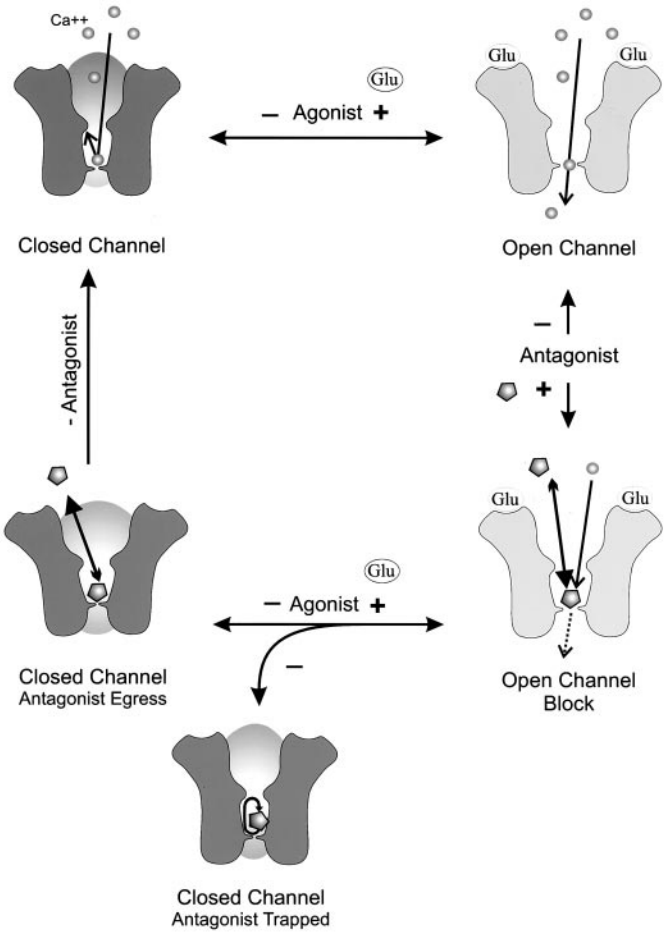



**Fig. 4.** Cartoon illustrating closed-channel trapping and egress. In the upper left-hand corner, a closed channel is shown whose conformation does not permit ion flow. Application of agonist causes channel opening (upper right). Concurrent application of agonist and antagonist allows antagonist into a "vestibule" to act at the channel pore, resulting in an open channel block (lower right). Removal of the agonist results in channel closure. If the "upper barrier" of the vestibule closes more slowly than the "lower barrier", the channel pore, the antagonist may escape, resulting in closed channel egress (lower left). However, if the upper barrier closes more rapidly than the antagonist can leave the channel pore, trapping results (bottom).

ence of antagonist in the vestibule retards closing, but it can then proceed rapidly following egress. In addition, physicochemical factors might favor antagonist egress over re-entry into the vestibule.

We have proposed that closed-channel egress provides a means to escape the problem that trapping presents to use-dependence. We noted that AR-R15896AR had the lowest trapping of the compounds chosen using objective selection criteria. Other post hoc comparisons (e.g., AR-R18526) suggest that compounds with high degrees of trapping produce significant amounts of PCP-like behaviors in rats at lower doses (G. Palmer, personal communication). Therefore, we would expect compounds with small degrees of trapping to have low liabilities for producing PCP-like behaviors. Unfortunately, behavioral studies using the two lowest trapping compounds, AR-R15808 and AR-R26952, could not be completed due to complications with bioavailability and specificity. Therefore, the correlation between degree of trapping and safety remains a plausible hypothesis, but presently untestable with the present inventory of compounds.

Rogawski (1993) proposed that low-affinity NMDA receptor antagonists would be safer because they prevent supramaximal block and the delayed recovery resulting from it. However, trapping of antagonist can override the benefits of low-affinity and use-dependence. Partial trapping permits at least some degree of use-dependence to continue. We suggest a model in which partial trapping occurs because of very fast off-rates of compounds. Since fast off-rate is one determinant of low affinity, safety in use-dependent NMDA receptor antagonists can be accounted for by a modified version of Rogawski's proposal. Parsons et al. (1995) have also noted the importance of fast off-rate kinetics to compound safety, especially during strong depolarization, as would occur during excessive receptor activation.

## References


Armstrong CM (1971) Interaction of tetraethylammonium derivatives with the potassium channels of squid giant axons. *J Gen Physiol* **58:**413–437.

Benveniste M, Drejer S, Schousboe A and Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ishaemia monitored by intracerebral microdialysis. *J Neurochem* **43:**1369–1374.

Benveniste M and Mayer M (1995) Trapping of glutamate and glycine during open channel block of rat hippocampal neuron NMDA receptors by 9-aminoacridine. *J Physiol* **483:**2:367–384.

Black MA, Tremblay R, Mealing G, Ray R, Durkin JP, Whitfield JF, Blosser J and Morley P (1995) *N*-methyl-D-aspartate- or glutamate-mediated toxicity in cultured rat cortical neurons is antagonized by FPL 15896AR. *J Neurochem* **65:**2170–2177.

Blanpied TA, Boeckman FA, Aizenman E and Johnson JW (1997) Trapping channel block of NMDA-activated responses by amantadine and memantine. *J Neurophysiol* **77:**309–323.

Chen HV and Lipton S (1997) Mechanism of memantine block of NMDA-activated channels in rat retinal ganglion cells: uncompetitive antagonism. *J Physiol* **499:** 27–46.

Chen HSV, Pellegrini JW, Aggarwal SK, Lei SZ, Warach S, Jensen FE and Lipton SA (1992) Open-channel block of *N*-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated toxicity. *J Neurosci* **12:**4427–4435.

Ginski MJ and Witkin JM (1994) Sensitive and rapid behavioural differentiation of *N*-methyl-D-aspartate receptor antagonists. *Psychopharmacology* **114:**573–582.

Gurney AM and Rang HP (1984) The channel blocking action of methonium compounds on rat submandibular ganglion cells. *Br J Pharmacol* **82:**623–642.

Huettner JE and Bean BP (1988) Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant (+)-MK-801: selective binding to open channels. *Proc Natl Acad Sci USA* **85:**1307–1311.

Jones SJ and Rogawski MA (1992) The anticonvulsant (±)-5-aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (ADCI) selectively blocks NMDA-activated current in cultured rat hippocampal neurones: kinetic analysis and comparison with dizocilpine. *Mol Neuropharmacol* **2:**303–310.

Kemp JA, Foster AC and Wong EHF (1987) Non-competitive antagonists of excitatory amino acid receptors. *Trends Neurosci* **16:**527–532.

Krystal JH, Karper LP, Seibyl JP, Freeman GK, Delany R, Bremner JD, Heninger GR, Bowers MB Jr and Charney DS (1994) Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychomimetic, perceptual, cognitive and neuroendocrine responses. *Arch Gen Psychiatry* **51:**199–214.

Lanthorn TH, Mealing GAR and Morley P (2000) Differences in rates of trapping between AR-R15896AR and other uncompetitive NMDA receptor antagonists. *Amino Acids* **17:**10.

Leppik IE, Marienau K, Graves NM and Rask CA (1988) MK-801 for epilepsy: a pilot study. *Neurology* **38:**405.

Lingle C (1983) Blockade of cholinergic channels by chlorisondamine on a crustacean muscle. *J Physiol* **339:**394–417.

Mealing GAR, Lanthorn TH, Murray CL, Small DL and Morley P (1999) Differences in degree of trapping of low-affinity uncompetitive NMDA receptor antagonists with similar kinetics of block. *J Pharmacol Exp Ther* **288:**204–210.

Mealing GAR, Lanthorn TH, Small DL, Black MA, Laferriere NB and Morley P (1997) Antagonism of *N*-methyl-D-aspartate-evoked currents in rat cortical cultures by ARL 15896AR. *J Pharmacol Exp Ther* **281:**376–383.

Meldrum BS (1993) Pharmacology and mechanism of action of lamotrigine, in *Lamotrigine: A New Advance in the Treatment of Epilepsy, International Congress and Symposium Series 204* (Reynolds EH ed) pp 5–14, Royal Society of Medicine Services Limited, New York.

Muller WE, Mutschler E and Riederer P (1995) Noncompetitive NMDA receptor antagonists with fast open-channel blocking kinetics and strong voltage-dependency as potential therapeutic agents for Alzheimer's dementia. *Pharmacopsychiatry* **28:**113–124.

Olney JW, Labruyere J and Price MT (1989) Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science (Wash DC)* **244:** 1360–1362.

Palmer GC, Cregan EF, Bialobok P, Sydserff SG, Hudzick TJ and McCarthy DJ (1999) The low-affinity, use-dependent NMDA receptor antagonist AR-R 15896AR. *Ann NY Acad Sci* **890:**406–420.

Palmer GC, Miller JA, Cregan EF, Gendron P and Peeling J (1997) Low-affinity

NMDA receptor antagonists: the neuroprotective potential of ARL 15896AR. *Ann NY Acad Sci* **825:**220–231.

Palmer GC, Murray RJ and DJ McCarthy (1996) Neuroprotective potential of the low affinity NMDA receptor antagonist ARL 15896AR, in *Advances in the Understanding of Therapy of Ischemic Stroke*, 2nd ed (Savage LM ed) pp 34–64, IBC Biomedical Library Series, Southbridge, MA.

Parsons CG, Gruner R, Rozental J, Millar J and Lodge D (1993) Patch clamp studies on the kinetics and selectivity of *N*-methyl-D-aspartate receptor antagonism by memantine (1-amino-3,5-dimethyladmantan). *Neuropharmacology* **32:**1337–1350.

Parsons CG, Quack G, Bresink I, Baran L, Przegalinski E, Kostowski W, Krzascik P, Hartmann S and Danysz W (1995) Comparison of the potency, kinetics and voltage-dependency of a series of uncompetitive NMDA receptor antagonists *in vitro* with anticonvulsive and motor impairment activity *in vivo*. *Neuropharmacology* **34:**1238–1258.

Rogawski MA (1993) Therapeutic potential of excitatory amino acid antagonists: channel blockers and 2,3-benzodiazepines. *Trends Pharmacol Sci* **14:**325–331.

Rogawski MA, Lee DQ, Uyakul D, Pannell L, Subramaniam S, Yamaguchi S and Kokate TG (1995) Anticonvulsant efficacy of ADCI (5-aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) after acute and chronic dosing in mice. *Epilepsia* **36:**566–571.

Rogawski MA, Yamaguchi S, Blake PA, Jones SM, Bieda M, De Costa BR and Thurkauf A (1991) Anticonvulsant 1-phenylcycloalkylamine analogs: uncompetitive NMDA antagonists with low motor toxicity, in *Multiple Sigma and PCP Receptor Ligands: Mechanisms for Neuromodulation and Neuroprotection?* (Kamenka J-M and Domino EF eds) pp 747–758, NPP Books, Ann Arbor, MI.

Small DL and Buchan AM (1997) NMDA antagonists: their role in neuroprotection, in *Neuroprotective Agents and Cerebral Ischaemia* (Green AR and Cross AJ eds) pp 137–171, Academic Press, New York, NY.

Sobolevsky AI and Yelshansky MV (2000) The trapping block of NMDA receptor channels in acutely isolated rat hippocampal neurones. *J Physiol* **526:**493–506.

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