

Identification of a Novel Class of Allosteric Modulators of the NMDA Receptor

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ABSTRACT

Introduction: N-methyl-D-aspartate (NMDA) receptors are glutamate-gated ion channels that play key roles in processes underlying learning and memory. NMDA receptor dysfunction is thought to contribute to virtually every major neurological disorder, including Alzheimer's, Parkinson's, and Huntington's disease, stroke, epilepsy, neuropathic pain, schizophrenia, ADHD, chronic depression and ageing-related memory deficits. Despite the enormous potential of NMDA receptor-based therapeutics, drugs targeting this receptor have been mostly disappointing in clinical trials, due to a combination of intolerable side effects and poor efficacy. The aim of our present work is to develop a new class of NMDA receptor drugs for treating neurological disorders, which are expected to be efficacious, selective and safe.

Methods: The approach is based on our recent observation suggesting that allosteric modulators bind to the heteromeric dimer interface formed by the NMDA receptor subunit NR1 and NR2 ligand binding domains. The 3D structures for 7,000 compounds from a virtual library were docked into the NR1-NR2A dimer interface and the binding affinity was estimated for each optimal pose, using the eHiTS software (www.simbiosys.com). The top-ranking compounds were characterized electro-physiologically using 2-electrode voltage clamp in *Xenopus* oocytes.

Results & Conclusion: Out of 10 top-ranking compounds tested, 3 were negative allosteric modulators, decreasing NMDA current by 17–25%. Six compounds enhanced NMDA receptor function by 17–73%, while 1 drug was ineffective. Our preliminary data suggest that this approach is feasible. Because only 0.1% of the available compounds have been screened in silico, we expect to identify a wide range of positive and negative allosteric modulators for the NMDA receptor.

Keywords: drug screening, modulators, neurological disorders, NMDA receptor

INTRODUCTION

According to a report from the World Health Organization in 2007, neurological disorders affect up to one billion people worldwide and contribute to 12% of global deaths. That number is destined to rise as the population continues to age. Studies performed over the past 2 decades have implicated the N-methyl-D-aspartate (NMDA) receptor as a positive or negative factor in virtually every major neurological disease. The NMDA receptor is a member of the ionotropic glutamate receptor family with many critical functions in the normal central nervous system¹. NMDA receptors assemble from 4 subunits, 2 NR1

subunits and 2 NR2 subunits (Fig.1, overleaf). The NMDA receptor channel is permeable to monovalent cations, including Na⁺ and K⁺, and divalent cations, most notably Ca²⁺. Opening of the channel requires binding of 2 different agonists, glutamate and glycine, along with simultaneous depolarisation of the cell membrane². The binding sites for glutamate and glycine are located on NR2 and NR1, respectively. Another unique property of NMDA receptor is the presence of a binding site for Mg²⁺ ion within the channel pore, which prevents ion flux at resting membrane potentials. When a neuron is depolarised, due to opening of synaptic AMPA receptors,

* Presented at the SGH Scientist Award session during the SGH 18th Annual Scientific Meeting held from 17 – 18 April 2009.

Please refer to the glossary.

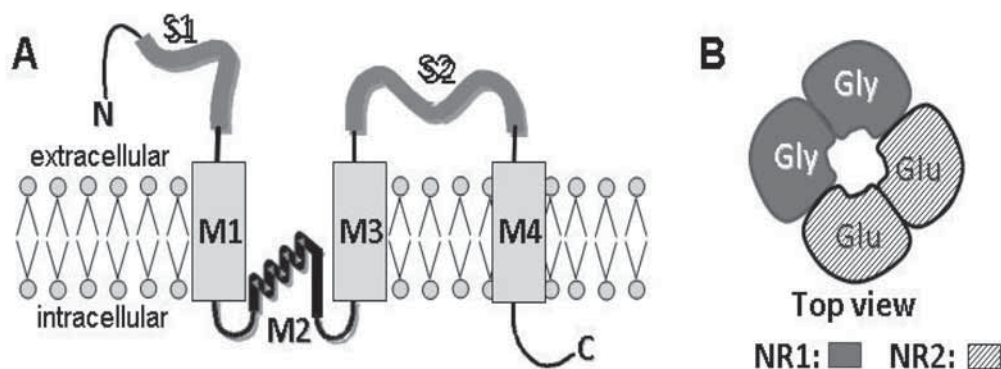


Fig 1. Schematic membrane topology of NMDA receptors subunits. A. transmembrane topology of an NR1 subunit, where the N-terminal region is extracellular. M1, M2, M3 and M4 are the membrane domains with M2 forming a re-entrant hairpin loop. S1 and S2 form the ligand binding domain. B. Top view of the subunit arrangement of the NMDA receptor.

Mg^{2+} is expelled from the NMDA receptor pore, thereby allowing ion flow.

Glutamate is the major excitatory neurotransmitter and is essential in neuronal development, synaptic plasticity, learning and memory^{3,4}. Among the glutamate receptor subtypes, the NMDA receptor has been a major target for drug development in neurological disease, because the excitotoxicity that leads to neuronal injury and death in a wide range of both acute and chronic neurological disorders results predominantly from excessive NMDA receptor activity^{5,6}. Excitotoxicity is a pathological process caused by an excessive amount or a prolonged period of exposure to glutamate. As a consequence of Ca^{2+} accumulation, the cell produces damaging free radicals and other enzymatic processes contributing to cell death⁷. Glutamate excitotoxicity contributes to neuronal loss in chronic neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's diseases, multiple sclerosis and HIV-associated dementia. Acute disorders, such as stroke, head trauma, seizures and neuropathic pain also show a component of excitotoxicity⁸. On the other hand, hypo-function of NMDA receptor is associated with neuro-developmental disorders, such as schizophrenia, attention deficit hyperactivity disorder (ADHD), chronic depression and memory deficits developing during ageing^{9,10}. Hence, directly targeting the NMDA receptor could potentially be beneficial in a number of acute and

chronic neurological disorders. The early development of NMDA receptor-targeting drugs focused on 3 major classes of antagonists: (1) competitive antagonists that act at the glutamate or glycine-binding site; (2) non-competitive modulators that act at other extracellular sites; and (3) channel blockers that bind to sites within the channel pore (Fig. 2).

However, selective NMDA receptor antagonists have not produced positive results in clinical trials for ischaemic stroke and traumatic brain injury. The antagonists targeting the glutamate site (selfotel), the glycine site (gavestinel), and channel pore (MK-801), Dizocilpine were found to have intolerable side effects, such as agitation, confusion, reduced level of consciousness, hallucinations and hypertension^{11,12}. One possible reason behind this is that NMDA receptor induced excitotoxicity only occurs immediately after ischaemic or traumatic injury but glutamate may then resume its normal physiological functions, including facilitation of neuron survival. Therefore, the use of NMDA receptor antagonists as neuroprotective agents in stroke and traumatic brain injury could be limited by a short therapeutic time window¹³. Despite several setbacks in the development of NMDA receptor-based drugs, the endeavour to search for new therapies continues. Recent findings showed that some "old drugs" have potential to be used in "new treatment". For example, the non-competitive NMDA antagonists, ketamine and dextromethorphan, have been used in

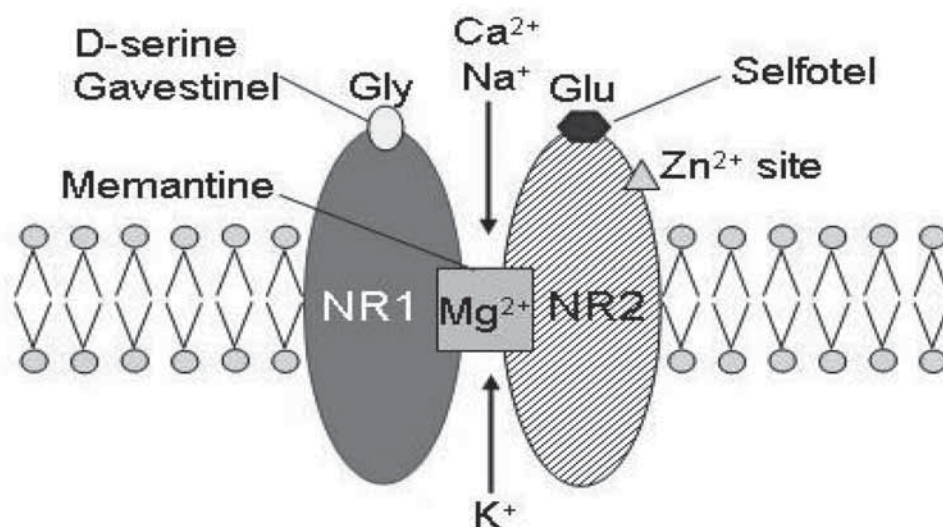


Fig. 2. Potential sites for drug action within the NMDA receptor channel. Extracellular sites include the glycine binding site on NR1 subunits, the glutamate binding site on NR2 subunits and the binding sites within the channel pore which overlap with the site for magnesium binding. NR2 subunits contain a binding site for zinc.

clinical practice for many years but for non-neurological indications. Ketamine is commonly used as a dissociative anaesthetic but recently discovered as therapeutic drugs in chronic neuropathic pain, including post surgery and cancer pain^{14,15,16}. Dextromethorphan is commonly used cough suppressant and its metabolite, dextrophan, binds to a site within NMDA channel pore^{17,18}. Dextromethorphan was recently registered in a clinical trial for the treatment of Rett's syndrome (Table 1, overleaf).

The challenge facing NMDA receptor-based drug design is that a minimal level of receptor function is required for normal neuronal function, whereas excessive activity of the receptor leads to excitotoxicity. To be clinically tolerable, a drug must block excessive activation of the NMDA receptor while leaving normal function relatively intact in order to avoid side-effects. This idea is supported by the clinical finding that a low-affinity, reversible non-competitive NMDA receptor antagonist, Memantine, is much better tolerated than MK-801 and is now approved for use in advanced Alzheimer's disease²³ (Table 1, overleaf). The objective of our present study is to develop a wide range of drugs targeting the NMDA receptor that do not suffer from the above mentioned drawbacks. This will be accomplished by designing drugs that work through a unique mechanism

of action, allosteric modulation, which endows them with superior properties. An allosteric modulator has no effect on NMDA receptor function by itself, but either enhances or attenuates the physiological function of the receptor. This means that positive allosteric modulators are expected to be safer than drugs that are agonists or partial agonists, which may display excitotoxicity at high doses. Negative allosteric modulators should be superior to either competitive or non-competitive antagonists, because their effect will be intrinsically dose-limiting: they reduce NMDA receptor function to a well-defined extent, resulting in fewer side effects.

METHODS AND MATERIALS

Xenopus Oocyte Preparation and Injection

Xenopus laevis frogs were first anaesthetised using 10% tricaine methanesulfonate. The oocytes were then surgically removed and manually defolliculated with treatment of collagenase type I (1 mg/ml)²⁴. Stage V-VI oocytes were chosen and injected with a 1:1 ratio of NR1 and NR2A cRNA (10–100ng/ μ l solution) using the Roboocyte injection system (Multichannel Systems, Reutlingen, Germany). The injected oocytes were transferred to SOS buffer and incubated at 19°C, using a chilling incubator (Torrey Pines Scientific, San Marcos, CA). SOS buffer consisted of 100 mM NaCl, 2 mM KCl, 1.8

Table 1. NMDA receptor-targeted therapies in neurological diseases.

Name of Drugs	Activities	Clinical Status	References*
D-serine	Glycine-binding site agonist	Ongoing clinical trials on schizophrenia	NCT00237848 NCT00816894 Ref. 21, 22, 33
D-cycloserine	Partial glycine-binding site agonist	On going clinical trials on schizophrenia, autism and chronic low back pain	NCT00963924 NCT00198120 NCT00125528 Ref. 19, 20
Gavestinel	Competitive glycine-binding site antagonist	The clinical trials in human with stroke or traumatic brain injury had been terminated	Ref. 12
Selfotel	Competitive glutamate-binding site antagonist	The clinical trials in human with stroke or traumatic brain injury had been terminated	Ref. 11, 12
Memantine	Open-channel blocker with low affinity	Ongoing clinical trials on Alzheimer's diseases, Parkinson diseases, traumatic brain injury, dementia	NCT00462228 NCT00235716 NCT00646204 NCT00594737 Ref. 23
Ketamine	Non-selective channel blocker	Ongoing clinical trails in neuropathic pain, including chronic post-operative pain and cancer pain	NCT00618423 NCT00961194 Ref. 14, 15, 16
Dextro-methorphan	Its metabolite, dextrorphan, bind to channel pore	On going clinical trails on Rett's syndrome	NCT00593957

*The numbers in References showing as NCTxxxxxxx are the Identifiers from ClinicalTrials.gov.

mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH-adjusted to 7.6 with NaOH and supplemented with 50 µg/ml gentamicin. All the animal experiments were approved by the Institutional Animal Care and Use Committee of National University of Singapore. All efforts were made to minimise animal suffering and to reduce the number of animals used.

Two-Electrode Voltage-Clamp Electrophysiology

Functional expression of NMDA receptors was evaluated 2 to 6 days after injection using a 2-electrode voltage-clamp amplifier (OC-725; Warner Instrument, Hamden, CT). The extracellular recording solution consisted of low-Barium Ringer's solution (Lobar: 100 mM NaCl, 5 mM KCl, 0.5 mM BaCl₂, 10 mM HEPES, and 200 µM EDTA), pH-adjusted to 7.35 with Tris base and maintained at room temperature. Barium was used as the divalent cation to minimise secondary activation of Ca²⁺-activated Cl⁻ currents²⁵. EDTA was included to chelate trace amounts of the soft metal divalent cations Cd²⁺ and Zn²⁺, which have been reported to contaminate buffer solutions and inhibit the NMDA receptor by binding to a high-affinity site^{26,27}. EDTA also removes a zinc-dependent component of desensitisation²⁸. Maximal current response was elicited

by co-application of 10 µM glycine and 100 µM L-glutamate, unless stated otherwise. Oocytes were impaled with low-resistance glass microelectrodes (1.0–5.0 MΩ) filled with 3 M KCl and maintained at a holding potential of -60 mV. Voltage-clamp recordings were performed in a perfusion chamber (Warner Instruments) optimized for laminar flow, and solution exchanges were accomplished using a gravity-fed, computer-controlled perfusion system, at a flow rate of ~15 ml/min. Data acquisition and voltage control were performed with pClamp hardware and software (Molecular Devices, Sunnyvale, CA). The peak currents were measured by Clampfit software (Molecular Devices, Sunnyvale, CA). All data are presented as mean ± SEM. from 3 to 7 oocytes.

High Throughput Screening

The eHiTS docking software (www.simbiosys.com) was utilised to virtually screen a library of 3D structures of commercially available compounds. The library consisted of a subset of 7,500 compounds available from TimTec (www.timtec.com). Each compound was optimally docked into the NR1 and NR2A dimer interface and the binding affinity of the optimal pose was estimated by eHiTS. This resulted in a ranking of the 7,500

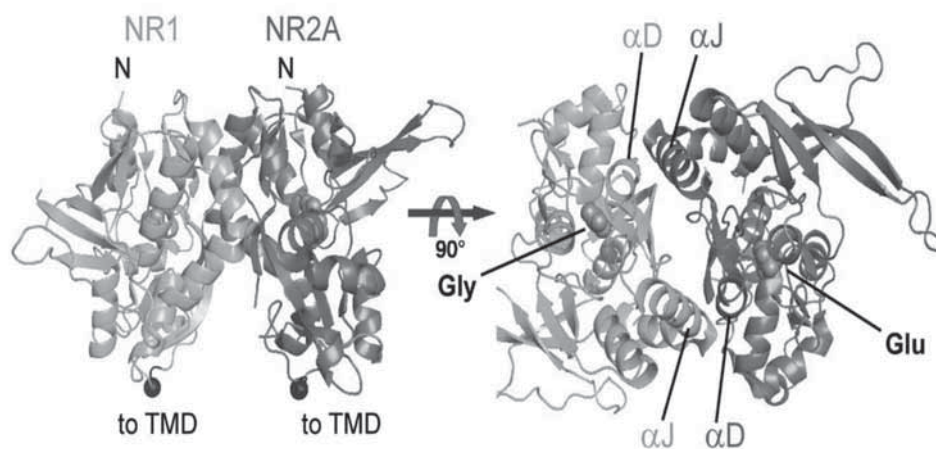


Fig. 3. The structure of the dimer formed by the NR1 and NR2 ligand binding domains. Modified from Gielen *et al.*²² with permission from Dr Pierre Paoletti. TMD: Transmembrane domain.

compounds. The high ranking compounds were selected and their effects on NMDA receptor maximal currents were tested.

Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO). The stock solutions of all the drugs were initially made in dimethyl sulfoxide and diluted to their final concentrations in Lobar solution. For those experiments, dimethyl sulfoxide was added to all recording solutions to maintain a standard concentration.

RESULTS & DISCUSSION

In order to design drugs which have an allosteric mechanism of action, we need to find compounds that should not affect the agonist binding sites (for both glutamate and glycine), nor should they act as open channel blockers (like MK-801). Recent unpublished results from our laboratory point towards a structural domain — the NR1 and NR2A dimer interface — that can be targeted for designing allosteric modulators.

Rational of Experimental Design

Functional NMDA receptors are hetero-tetramers consisting of 2 NR1 and 2 NR2 subunits. The extracellular ligand binding domains (LBDs) of the NR1 and NR2A subunits, which bind glycine

and glutamate respectively, have been crystallized and high-resolution X-ray structures have been published^{29, 30}. The NR1 and NR2A LBDs form a hetero-dimer complex, illustrated in Fig. 3. As can be seen from this figure, the dimer interface displays several complex cavities, which could act as binding sites for small molecules. The situation is similar to the LBD dimer interface of the GluR2 AMPA receptor, which has been shown to form binding sites for cyclothiazide, a positive allosteric modulator for the AMPA receptor³¹. Whereas the AMPA receptor LBD dimer is formed by 2 identical subunits, resulting in a two-fold symmetry, the NMDA receptor dimer is heteromeric, and has a unique complex interface. Recent unpublished results from our laboratory have shown that point mutations spanning the NR1 and NR2 ligand binding cleft had profound effects on the maximum open probability induced by agonist activation, and altered the efficacy of the positive allosteric modulator pregnenolone sulfate. This suggests that the NR1-NR2A dimer interface of the NMDA receptor could be the target of endogenous allosteric modulators. It should therefore be possible to design small molecules that bind in the cavities formed by the interface (Fig. 3) and either stabilise or destabilise it. This was investigated by (virtually) screening a library of 3D structures of commercially available compounds using the eHiTS docking software (www.simbiosys.com).

Table 2. Summary of the effects of ten top-ranking compounds on NMDA receptor responses.

Names	% Changes	Number
Drug 001	+17.6 ± 1.4	3
Drug 002	-25.1 ± 2.5	4
Drug 003	-17.7 ± 2.1	6
Drug 004	+ 23.6 ± 3.5	5
Drug 005	-6.5 ± 6.0	4
Drug 006	+19.5 ± 7.0	6
Drug 007	-22.8 ± 2.8	7
Drug 008	+73.1 ± 11.9	3
Drug 009	+51.0 ± 11.5	5
Drug 010	+60.9 ± 6.7	2

Identification of Allosteric Modulators for NMDA Receptors

The library consisted of a subset of 7,500 compounds from TimTec (www.timtec.com), which were used to identify compounds that potentially modulate NMDA receptors allosterically. As mentioned above, the NR1 and NR2A dimer interface is our target for designing allosteric modulators. Each compound was optimally docked into the interface and the binding affinity of the optimal pose was estimated by eHiTS. A ranking of the binding affinity for the 7,500 compounds was obtained. The top-ranking compounds have several interaction points with both the NR1 and NR2A subunits, suggesting they may affect interface stability.

To test if the top-ranking compounds interact with NR1-NR2A dimer interface, the top 10 compounds of the initial screen were evaluated by measuring their effect on NMDA currents induced by supra-maximal agonist concentrations. The concentrations of glycine and glutamate to elicit maximal currents are 10 μ M and 100 μ M, respectively. Fig. 4 demonstrates representative currents traces showing the effect of 10 compounds. Drug 3 and 4 affected maximal NMDA currents when applied in the channel closed state, whereas Drug 1, 2, 6, 7, 8, 9 10 changed maximal NMDA currents when applied in the channel open state. Drug 5 has no effect on maximal NMDA currents. The averaged results are summarized in Table 2. Out of 10 top-ranking compounds tested, three decreased maximal NMDA current by 17–25%, whereas 6 compounds increased maximal current by 17–73%, while 1 drug was ineffective. These compounds have no effect on

NMDA current when applied alone and they only exert their action in the presence of both agonists (glycine and glutamate), indicating they modulate NMDA receptor function by an allosteric mechanism. Interestingly, although the chemical structures of these compounds are all different, all of them fit closely in the NR1-NR2 dimer interface. Our data suggested that our strategy for designing of NMDA receptor-specific therapeutic agents is feasible.

The Advantages of Allosteric Modulators

Allosteric modulators of ion channels have been the focus in recent years in the search for therapeutic agents. These small molecules do not bind to the ligand binding site but instead act at an alternatively located binding site (allosteric site), to either potentiate or inhibit activation of the receptor by its natural ligand³³. Allosteric compounds have a number of theoretical advantages over agonists or antagonists as potential therapeutic agents. First, since the allosteric modulators do not display any agonism in the absence of endogenous ligands and only exert their effect in the presence of a released agonist, they have the potential to maintain both temporal and spatial aspects of endogenous physiological activity. Second, allosteric modulators with limited positive or negative effect would impose a “ceiling” on the magnitude of their allosteric effect. This property implied that they have a lower propensity towards target-based toxicity than the agonists or antagonists. Moreover, as allosteric modulators impose a limited effect on the receptor activity, a new level of pharmacological responsiveness would be introduced, whereby they can allow for a subtle re-setting of endogenous agonist activity.

The lesson from previous clinical trials of NMDA receptor-targeting drug appears to be: in order for NMDA receptor drugs to be safe in humans, they should not be able to inhibit receptor function completely. As indicated by our results, negative allosteric inhibitors have this desirable property, since their effect is intrinsically dose-limiting and only affect the maximal activation of NMDA receptors. Drugs that simply compete with glutamate or glycine at the agonist binding sites block normal function and therefore do not meet this requirement, and may thus cause severe and undesirable side-effects. In fact, competitive antagonists compete one for one with the agonist (glutamate or glycine) and therefore will block healthy areas of the brain before they can affect pathological areas where higher levels of agonist accumulate. Thus, such drugs would preferentially block normal activity and would most likely be displaced from the receptor

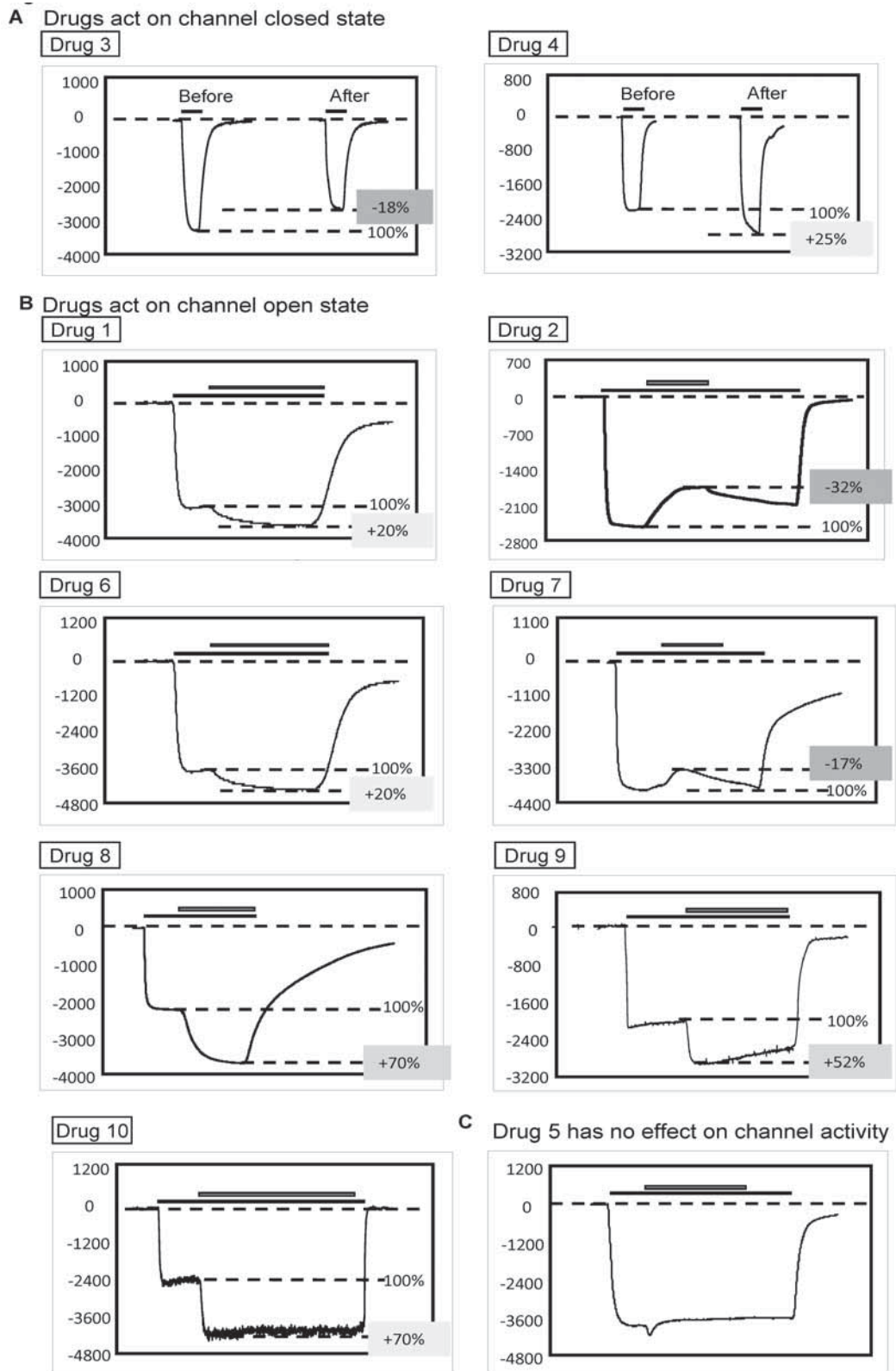


Fig. 4. Effect of 10 drugs on NMDA currents elicited by supra-maximal agonist concentrations. A. The representative current traces for Drug 3 and 4 that affect the maximal NMDA current when applied in the channel closed state. B. The representative current traces for drugs that affect the maximal NMDA current when applied in the channel open state. C. The representative current traces for Drug 5. All the lower bars above the current traces denote the co-application of 10 μ M glycine and 100 μ M L-glutamate while the upper bars denote the application of drugs.

by the high concentrations of glutamate for prolonged periods that can exist under excitotoxic conditions.

We have identified 6 positive allosteric enhancers for NMDA receptors in the present study. The positive allosteric modulators are expected to be safer than drugs that are agonists or partial agonists, which may display excitotoxicity at high doses. A classic example of positive allosteric modulators is benzodiazepines that modulates γ -aminobutyric acid (GABA)A receptors³⁴. Benzodiazepines provide an effective and safe approach to the treatment of anxiety and sleep disorders without inducing the potentially lethal effects of direct acting GABA receptor agonists.

Our present preliminary results suggested our approach is feasible for identifying possible allosteric modulators for NMDA receptors. Further experiments need to be carried out to test these compounds' bioavailability, cross reactivity, dosage and liver toxicity in animal models. We are currently exploring the mechanisms on how these compounds interact with NMDA receptors using site-directed mutagenesis and electrophysiology techniques.

CONCLUSIONS

Our present results have identified several positive and negative allosteric modulators for NMDA receptors. Because only 0.1% of the available compounds in the library have been screened in silico, we expect to identify a wide range of such modulators for the NMDA receptors. The discovery and characterisation of novel NMDA receptor modulators will provide fundamental new insights into the mechanisms of action, range of activities and keys to chemical optimization of these compounds as therapeutic agents for the treatment of a variety of neurological disorders. Allosteric potentiators of the NMDA receptor could be used in treatment of neurological disorders caused by hypo-function of the NMDA receptor system, such as schizophrenia, ADHD and chronic depression, while allosteric inhibitors are promising therapeutic drugs for both acute and chronic neurodegenerative diseases. Our report here has provided proof of concept that could fuel the discovery of next generation of drugs.

GLOSSARY

1. AMPA: amino 3-hydroxy-5-methyl-4-isoxazolepropionic acid
2. HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

3. EDTA: ethylenediaminetetraacetic acid

ACKNOWLEDGEMENTS

This work is supported by Ministry of Health and Agency for Science, Technology and Research (A*STAR) in Singapore. We would like to thank Dr Gavin Dawe for providing the *Xenopus* oocytes for our work.

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