Modulation by magnesium of *N*-methyl-D-aspartate receptors in developing human brain

H Chahal, S W D'Souza, A J Barson, P Slater

Abstract

***Aim*—To investigate age related alterations in glutamate *N*-methyl-D-aspartate (NMDA) receptor binding produced by the modulatory compounds glutamate, glycine, and magnesium (Mg^{2+}) sulphate.**

***Methods*—The effects produced by glutamate plus glycine, and Mg^{2+} on the binding of [^{3}H]MK-801, a ligand for the *N*-methyl-D-aspartate ion channel phencyclidine site, were measured in membrane preparations made from prefrontal cortex from human neonate (n = 5), infant (n = 6), and adult (n = 6) necropsy brains.**

***Results*—Neonatal brains had the least [^{3}H]MK-801 binding, suggesting either a low density of NMDA receptors or a more restricted access of [^{3}H]MK-801 to cation channel sites. Infant brains had the most [^{3}H]MK-801 binding which was stimulated to a greater extent by L-glutamate (100 μM) and glycine (10 μM) than in neonatal and adult brains. Mg^{2+} invariably inhibited [^{3}H]MK-801 binding. However, the Mg^{2+} IC_{50} value was higher in neonatal brain (3.6 mM) than infant (1.4 mM) and adult (0.87 mM) brains.**

***Conclusion*—Infant brain may have excess NMDA receptors which are hyper responsive to glutamate and glycine. The lower potency of Mg^{2+} to inhibit [^{3}H]MK-801 binding in neonatal cortex may be because newborn babies have NMDA receptors without the normal complement of Mg^{2+} sites. The findings suggest that therapeutic NMDA receptor block in neonates requires higher concentrations of magnesium sulphate in brain tissue.**

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School of Biological Sciences, University of Manchester
H Chahal
P Slater

Department of Child Health
S W D'Souza

School of Pathological Sciences
A J Barson

Correspondence to:
Dr P Slater,
School of Biological Sciences,
1.124 Stopford Building,
University of Manchester,
Oxford Road,
Manchester M13 9PT.

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Magnesium (Mg^{2+}) sulphate has several actions one of which may be to protect the developing brain from the damage to neurons that is caused by the excitotoxic effects of glutamate, the main excitatory amino acid (EAA) neurotransmitter. Thus magnesium sulphate is proposed for clinical use to combat glutamate excitotoxicity associated with birth asphyxia[1] which causes glutamate to be released with increased concentrations in the extracellular compartment. Mg^{2+} may reduce injury by preventing excess glutamate from overactivating the several classes of EAA receptors, especially the *N*-methyl-D-aspartate (NMDA) receptor. Epidemiological studies have described how magnesium sulphate administered to pregnant mothers with pre-eclampsia or in preterm labour reduces the incidence of cerebral palsy in low birthweight infants.[2 3] In these clinical conditions Mg^{2+} may have several actions, including blocking NMDA receptors.

The NMDA receptor controls a calcium permeable cation channel.[4] Because calcium ion (Ca^{2+}) has a vital role as an intracellular second messenger, NMDA receptors are involved in numerous physiological processes, including coordination of synaptogenesis and synaptic plasticity in the developing brain.[5] Pathologically, overactivation of NMDA receptors may promote calcium mediated excitotoxicity such as follows periods of hypoxia–ischaemia and hypoglycaemia, conditions where excess glutamate is released.[6]

The NMDA receptor cation channel complex is unique in having several receptor and modulatory sites which recognise individual regulators found in the brain.[7] These modulatory sites include an agonist binding site that binds L-glutamate and L-aspartate,[8] a glycine co-agonist site,[9 10] a voltage-dependent Mg^{2+} site,[11 12] a voltage independent inhibitory Zn^{2+} site[13 14] and a site which is affected by the endogenous polyamines spermine and spermidine.[15] The Mg^{2+} site is within the cation channel and is occupied at normal membrane potentials which inactivates the NMDA receptor. Depolarisation of neurons, which may be achieved by activation of other glutamate receptors such as the AMPA receptor, is sufficient to overcome the Mg^{2+} block and allow ion flow through the cation channel. Another cation channel site recognises phencyclidine (PCP) and other compounds, such as the dissociative anaesthetic ketamine and the anticonvulsant MK-801 (Dizocilpine). PCP-like compounds and MK-801 are non-competitive antagonists at the PCP site.[16 17] The binding of [^{3}H]MK-801, which is determined by the open state of the cation channel and is increased by glutamate and glycine acting via their sites, is used to measure channel opening and access to PCP sites in vitro.[14]

Several reports describe how the ability of Mg^{2+} to block NMDA mediated responses changes as the brain develops, and further suggest that these changes may be regionally dependent. The Mg^{2+} block of NMDA receptors often increases with postnatal age.[18–21] However, in contrast, NMDA receptors expressed from hippocampal mRNA from 14–15 day old rats were about twofold less sensitive to Mg^{2+} than 1 to 2 day old rats.[22] It was also

Table 1 Details of subjects studied

Age	*Gender*	*PMI (hours)*	*Cause of death*
Newborn (weeks)			
31	F	48	Arthrogryposis multiplex congenita
39	M	48	Sepsis/pleural effusion/bronchopneumonia/surgical repair of gastroschisis
40	F	96	Congential laryngeal atresia/pneumothorax
41	M	80	Right tension pneumothorax
41	M	28	Intrapartum stillbirth
Infants (postnatal weeks)			
13	M	48	Acute bronchopneumonia
16	F	24	SIDS
20	F	72	SIDS
21	M	96	SIDS
23	F	24	SIDS
32	F	24	Myocarditis
Adult (years)			
43	F	15	Carcinoma stomach
52	M	48	Heart failure/ruptured aorta
69	M	12	Heart failure, coronary thrombosis
74	M	8	Left ventricular failure
76	M	62	Heart failure/myocardial infarct
81	M	26	Myocardial infarct

PMI, postmortem interval; SIDS, sudden infant death syndrome.

reported that in hippocampal CA3 pyramidal neurons, NMDA channels do not change sensitivity to Mg^{2+} induced block during development, whereas the kinetic properties of the NMDA receptor channels seem to alter.[23] It is by no means certain, therefore, how the voltage dependent Mg^{2+} block of NMDA receptor channels alters during development. In relation to the potential clinical uses of magnesium sulphate in man, it is important to know how NMDA receptors in neonatal brain are affected by Mg^{2+}.

This study investigates the age related properties of NMDA receptors in relation both to [^{3}H]MK-801 binding and Mg^{2+} inhibition of [^{3}H]MK-801 binding in human brain.

Methods

Brains were removed at necropsy from 17 subjects whose ages ranged from 31 weeks of gestation to 83 years (table 1). Strict inclusion criteria were applied in the use of brain tissue in the study, including death to post mortem examination interval (PMI) of 96 hours or less, death from an acute event without antemortem coma (agonal state), no history of central nervous system disease and no recognisable brain pathology. Post mortem examinations were made by a consultant pathologist whose findings confirmed the absence of brain pathology on both gross and histological examination. Each brain was cut into coronal slices which were rapidly frozen in isopentane at −70°C and stored below −70°C. Similar methods of brain collection and storage do not significantly alter [^{3}H]MK-801 binding in developing and adult brains.[24]

Samples of dorsolateral prefrontal cortex (Brodmann area 9) were subdissected from the frozen brain tissue. Extensively washed membranes were prepared with modifications to established procedures.[25] About 100 mg of grey matter was homogenised in 50 volumes of 50 mM TRIS–acetate buffer (pH 7.4) using an Ultra Turrax homogeniser and centrifuged (19 000 × *g* for 10 minutes at 4°C). The supernatant fluid was discarded and the pellet resuspended in 50 volumes of 50 mM TRIS–acetate (pH 7.4) and centrifuged as before. This washing procedure was repeated with 50 volumes of 5 mM TRIS–acetate (pH 7.4) and three times with deionised water and the pellets stored at −70°C for at least 24 hours. On the day of the assay, pellets were thawed at room temperature and resuspended in 50 volumes of 5 mM TRIS–acetate and centrifuged (19 000 × *g*, for 10 minutes at 4°C), followed by two further cycles of washing. Binding assays were performed in triplicate in flat bottomed 96-well microplates in a total volume of 200 µl.

Membranes (17–33 µg protein) were incubated in the presence of 5 mM TRIS–acetate (pH 7.4), 4 nM [^{3}H]MK-801 (22 Ci/mmol) for 90 minutes at 25°C. Non-specific binding was defined by 100 µM unlabelled MK-801. Magnesium inhibition curves were performed in the presence of 100 µM glutamate and 10 µM glycine to enhance [^{3}H]MK-801 binding. Assays were terminated by vacuum filtration through glass fibre filters (pre-soaked in 0.1% polyethylanimine) using a cell harvester. Wells were washed for 5 seconds with ice cold 5 mM TRIS–acetate (pH 7.4) and filters dispersed in 2 ml of scintillant. Radioactivity was determined after 12 hours using a Packard Tricarb liquid scintillation analyser at 50% counting efficiency. Protein concentrations were measured using a dye method,[26] using bovine serum albumin as standard.

Inhibition of binding was quantified and IC_{50} values were calculated using PRISM (GraphPad Software). Statistical analyses were performed using Spearman rank correlation, Student's *t* test and one way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison post hoc test, using INSTAT (GraphPad Software).

Compounds used and the sources were: MK-801 ([+]-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate) from RBI; [^{3}H]MK-801 from NEN-DuPont; L-glutamate and glycine from Sigma; magnesium sulphate from BDH.

Results

The brains available for the study were subdivided into three groups according to the ages of the subjects (table 1). Two groups had subjects of both sexes whereas the adult subjects were nearly all male. The causes of death, which were many and varied, were typical of subjects of these ages. The only group with a near consistent diagnosis was the infant group (sudden infant death syndrome; SIDS). In our previous work on binding to the NMDA receptor PCP site we have not found that gender influences SIDS. Pathologically, brains from SIDS cases are normal. The post mortem examination interval (PMI) was different for each group and shortest for the adult subjects. A longer PMI for neonates reflects the inevitable necropsy delays.

Some measurements of [^{3}H]MK-801 binding were made without the exogenous amino acids L-glutamate and glycine. The non-stimulated [^{3}H]MK-801 binding was higher in brains from the group of 13–32 week old infants compared with neonatal brain tissue

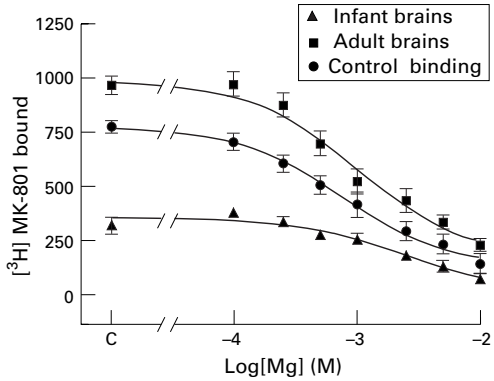

Figure 1 Reduction by Mg^{2+} of specific [^{3}H]MK-801 binding (mean fmol/mg protein ± SEM) in human brain cortex membranes prepared from five neonatal brains, six infant brains, and six adult brains. Control binding (C) in the absence of Mg^{2+} is also shown. Assays were performed in triplicate using 4 nM [^{3}H]MK-801 in the presence of L-glutamate (100 μM) and glycine (10 μM).

(table 2), which implies that the infant brains have a greater number of NMDA receptors. Adding L-glutamate (100 μM) and glycine (10 μM) to binding assays invariably increased the amounts of bound [^{3}H]MK-801, with the least increase being recorded in the neonatal brains. L-glutamate and glycine produced the largest stimulation of [^{3}H]MK-801 binding in the infant brains. A postnatal increase in NMDA receptor numbers may be accompanied by increased sensitivity to agonists of the specific glutamate or glycine receptor sites.

Mg^{2+} inhibited glutamate/glycine-stimulated [^{3}H]MK-801 binding in all three groups of brains, according to the concentration (fig 1). Mg^{2+} IC_{50} values were calculated for each of the subject age groups. A significantly higher IC_{50} value shows that Mg^{2+} was much less potent at inhibiting [^{3}H]MK-801 binding in neonatal brains than infant and adult brains (table 2).

The possible influence of PMI on the [^{3}H]MK-801 binding data was examined using statistical testing. There was no significant correlation between all 17 PMI times and the individual brain IC_{50} values for Mg^{2+} inhibition of [^{3}H]MK-801 binding. When the Mg^{2+} IC_{50} values for each of the three brain groups were tested with ANOVA, using PMI as covariate, a significant difference ($p < 0.05$) between IC_{50} values remained, probably due to the difference between the neonate and adult groups as shown by the outcome of Tukey-Kramer tests.

Although antemortem agonal status may influence [^{3}H]MK-801 binding in necropsy human cortex,[24] it was not a factor in the present study. Prolonged coma or hypoxia was not involved in the deaths of the infants and neonates, and none of the adult patients was mechanically ventilated.

Table 2 Specific [^{3}H]MK-801 binding in prefrontal cortex membranes from brains of adults, infants and neonates, and inhibition by Mg^{2+}

	Adults	*Infants*	*Neonates*
Non-stimulated binding	292 ± 11	312 ± 17	249 ± 15[1]
Stimulated binding*	774 ± 28	933 ± 40	335 ± 38[2]
and % increase	+165	+199	+35
Mg^{2+} IC_{50} - mM*	0.87 ± 0.1	1.4 ± 0.3	3.6 ± 1.3[3]

* [^{3}H]MK-801 binding was stimulated with 100 μM L-glutamate plus 10 μM glycine. Data values are mean ± SEM obtained with 6 adult brains, 6 infant brains or 5 neonatal brains.
[1] Data from neonates are significantly different from infant data ($p < 0.05$), or [2] both infant and adult data ($p < 0.001$), or [3] adult data ($p < 0.05$).

Discussion

The main findings reported in this paper are the postnatal increase in the numbers of neocortical NMDA receptors and their response to the co-agonists glutamate and glycine, and the relatively low sensitivity to Mg^{2+} of NMDA receptors in neonatal brain. [^{3}H]MK-801 labels PCP sites which are part of the NMDA receptor complex and occur within the associated cation channels. Thus [^{3}H]MK-801 binding reflects both the numbers of NMDA receptors and access to the sites in the channels (channel opening). Various agonists and antagonists, each with an affinity for an NMDA modulatory site, will modify the channel opening to expose more (or fewer) PCP sites, and thereby alter the binding of [^{3}H]MK-801. In contrast, Mg^{2+} sites are not modulatory to channel opening. Instead Mg^{2+} sites are within or close to the cation channels. Mg^{2+} occupies the sites and blocks the channels. In a complex mutual interaction between the intrachannel sites for Mg^{2+} and PCP, Mg^{2+} may act to decrease the affinity of [^{3}H]MK-801 binding.

Although altered potency of Mg^{2+} to block NMDA channels during postnatal development has been described, it is not clear whether Mg^{2+} is more or less effective in neonatal brain preparations and young cultured cells. Most studies, including this one, describing a postnatal increase in the sensitivity to Mg^{2+} of [^{3}H]MK-801 binding in rat brain,[27] report a low sensitivity to Mg^{2+} in neonatal brain tissues.[18–21 28] However, others claim that the sensitivity to Mg^{2+} of NMDA receptor channels stays constant or decreases during development.[23 29] Because interactions may occur between the Mg^{2+} site and other modulatory sites, such as those for glycine, polyamines, and protons,[30–32] experimental conditions may not always show a consistent response to Mg^{2+}.

Molecular studies have produced new information on developmental changes in NMDA receptors. Native NMDA receptors are assembled from different proportions, depending on the region of brain, of the five molecular subunits NMDA-R1 and NMDA-R2A-D. Messenger RNA (mRNA) from neonatal rat brains expressed NMDA receptors in Xenopus oocytes which were more sensitive to blockade by Mg^{2+} than receptors expressed by 2 week old rat brains.[22] Either the NMDA subunits expressed may not have been the same as those of the native receptors, or this was an example of regional heterogeneity of NMDA receptors. Blockade by Mg^{2+} of NMDA channels is decided mainly by which NMDAR2 subunits are present. Heteromeric receptors made from NMDAR1/NMDAR2A or NMDAR1/NMDAR2B have their channels blocked more potently by Mg^{2+} than channels formed by NMDAR1/NMDAR2C or NMDAR1/NMDAR2D.[33] Recent reports sug-

gest that the expression of several NMDA receptor subunits in the brain varies both regionally and during development.[34–36] We have explained the age dependent changes in sensitivity to Mg^{2+} reported here in terms of developmentally controlled expression of different NMDA subunits, but other possible mechanisms should be considered. Although Mg^{2+} sensitivity of NMDA receptors may be modulated *in vitro* by co-activation of polyamine sites,[18] there is unlikely to be enough endogenous polyamines to explain the pronounced developmental changes in Mg^{2+} sensitivity of NMDA receptors. Millimolar concentrations of Mg^{2+} may potentiate rather than block NMDA responses, perhaps via polyamine sites.[32] The overall effects of Mg^{2+} on NMDA channel currents may be a balance between reduction via the channel Mg^{2+} sites and some potentiation which is dependent on other mechanisms. This balance may alter slightly in the developing brain which expresses immature type NMDA subunits.

We reported earlier that the maximum number of [^{3}H]MK-801 binding sites in developing human brain occurred in infancy.[25] There is a widely held opinion that NMDA receptors have trophic functions in the developing brain, and a transient postnatal rise in NMDA receptor numbers[37] may reflect an increased role of the receptor in neural plasticity[5] and also a transient overproduction of glutamatergic synapses.[37 38] In rat brain[36] and probably also in man, there are early postnatal alterations in the proportions of the mRNAs which express the subunits that make functioning NMDA receptors. NMDA receptors in the brain of neonates and young infants may be an immature type of receptor with properties which are more suited to trophic functions than the mature or adult receptor type. The functions and the pharmacological properties of native heteromeric receptors are determined by the proportions of the various co-agonist and modulatory receptor sites which coordinate the opening of cation channels. They in turn are dependent on the subunit composition of the NMDA receptors. The pharmacological properties of NMDA receptors measured in brain preparations may be expected, therefore, to alter during brain development as receptors undergo molecular rearrangements. The present data provide an example of a developmental alteration in NMDA receptor properties in that glutamate and glycine produce a larger increase in [^{3}H]MK-801 binding in brains from 6 month old infants than adult brain.

Mg^{2+} sites are an important part of the NMDA receptor complex because they produce the voltage dependent block of NMDA induced ion currents which has been shown in various brain tissue preparations.[11 39 40] Analyses of the properties of the voltage gating of cation channels by Mg^{2+} suggest that within the NMDA receptor ion channel complex there are several Mg^{2+} sites which are either close to, or just inside, the channel lumen.[41] One Mg^{2+} site in cortical neurons occurs almost one third of the distance into the channel pore from the intracellular side.[12] Two distinct sites for Mg^{2+} were identified which are available to Mg^{2+} entering the NMDA channel pore from either the extracellular or the cytoplasmic sides.[42] We predict from our data that neonatal brain NMDA receptors do not express subunits with one of the sites for Mg^{2+}, and that one of the effects of postnatal NMDA subunit reorganisation is increased expression of subunits with the normal complement of Mg^{2+} sites.

The normal voltage dependent block of channels by Mg^{2+} is highly important for the sort of functions NMDA receptors have to perform. The block is only relieved by an appropriately timed depolarisation of the neuron which may be brought about by activation of other types of glutamate receptor, including the fast acting AMPA subtype. Membrane depolarisation to remove the block of channels by Mg^{2+} is a prerequisite for NMDA activation and synaptic plasticity, whereas protracted or intense NMDA activation may cause neural injury such as follows cerebral ischaemia. The age related alteration in Mg^{2+} efficacy reported here may reflect a fine tuning of NMDA receptors so that the relative ease or difficulty in removing the Mg^{2+} block with depolarisation varies as the trophic and other functions of NMDA receptors are adjusted in the developing brain. Blockade of NMDA receptors by other divalent cations such as Zn^{2+} may serve a similar purpose.

The current interest in magnesium sulphate stems from its potential use as a neuroprotective compound which, by blocking NMDA receptor channels in brain after systemic administration, may be valuable as a treatment given to neonates who have had cerebral hypoxia or perfusion arrest. These are major causes of morbidity and perinatal brain damage which may involve excitotoxic injury caused by glutamate release. In a mouse model of excitotoxic neuronal death magnesium sulphate afforded protection only after the stage of brain development at which Mg^{2+} could block NMDA channels.[43] As we report here and elsewhere, the activity and regulation of NMDA receptors in human brain changes during development and undergo substantial alterations between about 36 weeks of gestation and late infancy.[4 25] A temporary and altered sensitivity of NMDA receptors to Mg^{2+} induced blockade in the neonatal brain has obvious practical implications for treatment with magnesium sulphate. Our data show that neonatal cortex has fewer NMDA receptors than older subjects with a below normal response to Mg^{2+}. The findings imply that the plasma concentration of magnesium sulphate needed to achieve neuroprotection in neonates will be greater than that in older infants or adults.

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