Loss of surface N-methyl-D-aspartate receptor proteins in mouse cortical neurones during anaesthesia induced by chloral hydrate in vivo

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Background. Anaesthetics may target ionotropic glutamate receptors in brain cells to produce their biological actions. Membrane-bound ionotropic glutamate receptors undergo dynamic trafficking between the surface membrane and intracellular organelles. Their subcellular distribution is subject to modulation by changing synaptic inputs and determines the efficacy and strength of excitatory synapses. It has not been explored whether anaesthesia has any impact on surface glutamate receptor expression. In this study, the effect of general anaesthesia on expression of N-methyl-D-aspartate (NMDA) receptors in the surface and intracellular pools of cortical neurones was investigated in vivo.

Methods. General anaesthesia was induced by intraperitoneal injection of chloral hydrate in adult male mice. Surface protein cross-linking assays were performed to detect changes in distribution of NMDA receptor subunits (NR1, NR2A, and NR2B) in the surface and intracellular compartments of cerebral cortical neurones.

Results. Chloral hydrate did not alter the total amounts of NR1, NR2A, and NR2B proteins in cortical neurones. However, the drug reduced NR1 proteins in the surface pool of these neurones, and induced a proportional increase in NR1 in the intracellular pool. Similar redistribution of NR2B subunits was observed between the two distinct pools. The changes in NR1 and NR2B were rapid and remained throughout the duration of anaesthesia. NR2A proteins were not altered in the surface or intracellular pool in response to chloral hydrate.

Conclusions. These data demonstrate that subcellular expression of NR1 and NR2B in cortical neurones is sensitive to anaesthesia. Chloral hydrate reduces surface-expressed NMDA receptors (specifically NR2B-containing NMDA receptors) in these neurones in vivo.


Keywords: anaesthetics; cerebral cortex; glutamate receptor; NR1; NR2A; NR2B

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Glutamatergic transmission is central to the modulation of normal neuronal and synaptic activity in the central nervous system. Glutamate exerts its pleiotropic roles by interacting with two classes of receptors: ionotropic and metabotropic glutamate receptors.1 The former includes three subtypes: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, and kainate receptors. The NMDA receptor is broadly distributed in the brain.2 3 Upon activation of this ligand- and voltage-gated receptor, small cation ions (Ca2+ and Na+) enter cells through opened receptor channels to induce the excitatory post-synaptic current and modulate Ca2+-sensitive intracellular signaling.1 4 Each NMDA receptor is a heteromeric assembly of multiple subunits. A functional NMDA receptor is assembled of obligatory NR1 subunits and modulatory NR2 subunits, principally NR2A and NR2B.5 6 A tetrameric complex assembled of two NR1 and two NR2 subunits seems to be a prototypic model of NMDA receptors. As such, three major subtypes of NMDA receptors, that is, NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B, are expressed in the central nervous system.
The property of NMDA receptors is determined by their subcellular localization. In brain cells, NMDA receptors are expressed exclusively in a membrane-bound form. They are bound to either the surface membrane or the membrane of intracellular organelles, including endoplasmic reticulum, Golgi apparatus, and vesicles. The amounts of receptors in surface and intracellular membrane compartments are determined by receptor trafficking. Under basal conditions, synthesized NMDA receptors are transported from the intracellular organelles to the surface membrane (externalization trafficking). Meanwhile, surface-expressed NMDA receptors are transported to the intracellular sites (internalization trafficking). Both externalization and internalization are integrated to control the number of NMDA receptors at synaptic sites and thereby determine the efficacy and strength of excitatory synapses. It is clear that these trafficking steps are highly sensitive to changing synaptic inputs, and are critical molecular sites for the modulation of synaptic glutamatergic transmission by a variety of extracellular stimuli.

Increasing evidence indicates that the NMDA receptor is a target of anaesthetic agents in the central nervous system. By affecting NMDA receptors, anaesthetics produce some of their biological actions. However, whether anaesthetics affect NMDA receptor trafficking has not been explored. In this study, we therefore investigated the potential impact of general anaesthesia on NMDA receptor trafficking in vivo. We selected chloral hydrate as a general anaesthetic because it is the most commonly used anaesthetic for in vivo biochemical and physiological experiments in animal studies.

Methods

Animals

C57BL/6J male mice (2–3 months old) were used (Charles River, New York, NY, USA) and were individually housed in a controlled environment at a constant temperature of 23°C and humidity of 50% with food and water available ad libitum. The animal room was on a 12/12 h light/dark cycle. Mice were allowed 6–7 days of habituation to the animal colony. All procedures performed were approved by the Institutional Animal Care and Use Committee (Kansas City, MO, USA) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Anaesthesia

General anaesthesia was induced by intraperitoneal (i.p.) injections of chloral hydrate (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) at a dose of 400–500 mg kg$^{-1}$. Anaesthesia was assessed by loss of righting reflex (LORR), and time to induce LORR was usually between 3 and 6 min. Mice were kept at room temperature for 10 min before sacrifice. In the study investigating the role of body temperature, body temperature of mice was maintained with a rectal probe and a heating plate (TCAT-2 controller, Harvard Apparatus, Holliston, MA, USA). PBS was used for control injections.

Western blot analysis

Western blot was performed as described previously. Briefly, mice were killed by cervical dislocation and brains were immediately removed. The cerebral cortex was quickly dissected on ice. Brain tissues were sonicated in a sample buffer (RIPA) containing 50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 4% ionic detergent sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 5 μg ml$^{-1}$ each of apro tinin, leupeptin, and pepstatin, 1 mM Na$_2$VO$_4$, and 1 mM NaF. Protein concentrations were determined with a Pierce BCA Assay Kit (Rockford, IL, USA). Equal amounts of protein (20 μg 20 μl$^{-1}$ per lane) were separated on NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer (5% non-fat dried milk and 0.1% Tween 20) for 1 h. The blots were incubated in primary rabbit polyclonal antibodies against NR1 (Upstate, Charlottsville, VA, USA), NR2A (Upstate), NR2B (Upstate), α-actinin (Upstate), or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) usually at 1:1000 overnight at 4°C. This was followed by 1 h incubation in goat anti-rabbit horseradish peroxidase-linked secondary antibodies (Jackson Immunoresearch Laboratory, West Grove, PA, USA) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and captured into Kodak Image Station 2000R. Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA, USA) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software, and all bands were normalized to total actin amount and then to basal values. Values are expressed as percentages of basal values.

Surface receptor cross-linking assays

Surface receptor expression was assayed using a membrane-impermeable cross-linking agent bis(sulfosuccinimidyl)suberate (BS$^3$), which only cross-links proteins on the surface of cells. As described previously, brains were removed after cervical dislocation. The dissected cortex was chopped into small pieces with a pair of scissors and added to eppendorf tubes containing ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing (mM) 119 NaCl, 3.5 KCl, 1.3 MgSO$_4$, 2.5 CaCl$_2$, 1 NaH$_2$PO$_4$, 26.2 NaHCO$_3$, and 11 glucose, bubbled continuously with 95% O$_2$/5% CO$_2$ (pH 7.4). In some experiments, coronal slices (350 μm) were prepared using a
Anaesthesia regulates glutamate receptor trafficking

vibratome. The cerebral cortex was dissected from the slices and added to Eppendorf tubes containing cold oxygenated ACSF. BS³ (Pierce, Rockford, IL, USA) was added (2 mM) and incubated with gentle agitation for 30 min at 4°C. The cross-linking reaction was terminated by quenching with 20 mM glycine (10 min, 4°C). The tissues were then washed four times in cold ACSF (10 min each), homogenized to obtain total protein homogenate, and analysed directly by SDS–PAGE (4–12% Tris–glycine gels, Invitrogen).

**Behavioural assessment**

Righting reflex was assessed after administration of chloral hydrate. Righting reflex scores were evaluated according to the rating scale: a score of 0 indicated a normal right reflex; +1 indicated that the mouse righted itself within 2 s on all three trials (slightly impaired righting reflex); +2 indicated that the latency to righting was >2 s, but <10 s at the best response in three trials (moderately or severely impaired righting reflex); and +3 corresponded to the absence of righting reflex (no righting within 10 s on all three trials).¹⁸

**Statistics**

The results are presented as means (SEM) and were evaluated using a one- or two-way analysis of variance, as appropriate, followed by a Bonferroni (Dunn) comparison of groups using least squares adjusted means. Probability levels of <0.05 were considered statistically significant.

**Results**

**Compartment-specific distribution of NMDA receptors in cortical neurones**

We first analysed the compartment-specific distribution of NMDA receptors in cortical neurones under normal conditions. We used a biochemical technique to isolate native NMDA receptor subunits from surface and intracellular pools in live neurones.¹⁶¹⁹ As shown in Figure 1A, BS³-linked and -unlinked receptor proteins are readily distinguished. In BS³-treated cortical tissue, each NR1, NR2A, and NR2B subunit displayed an additional high molecular weight band. This band is much higher than a normal molecular weight band. This band is much higher than a normal molecular weight band from BS³-treated tissue (115 kDa), NR2A (175 kDa), and NR2B (180 kDa) monomers. This is due to the fact that it contained BS³-linked high molecular weight receptors from the surface pool. Whereas a single strong monomeric molecular weight band from control tissue contained receptors from both surface and intracellular compartments, the normal molecular weight band from BS³-treated tissue was reduced in density because it contained receptors only from the intracellular pool. The selectivity of BS³ in cross-linking surface receptors was confirmed by the lack of its cross-linking with the intracellular proteins, α-actinin (Fig. 1A) and synapsin (data not shown). Moreover, treatment of cortical homogenates with BS³ completely eliminated the monomeric bands of NR1 (Fig. 1B). This elimination remained unchanged after co-incubation of chloral hydrate (5 mM) with BS³ (Fig. 1C), indicating that chloral hydrate itself has no impact on the cross-linking capacity of BS³.

The percentage of the three subunit proteins in the intracellular pool was determined by comparing the amount of intracellular subunits in BS³-treated tissue with the total subunit protein abundance in control tissue. Under normal conditions, about 28–39% of each subunit is intracellular [NR1, 38.6 (4.5)%; NR2A, 34.1 (3.8)%; and NR2B, 28.4 (5.9)%; Fig. 1D]. This appears to indicate that the majority of NMDA receptor proteins of cortical neurones are expressed in the surface pool under basal conditions.

We also tested surface expression of NR1 in BS³-treated cortical slices. The results were similar to those observed in the chopped small pieces of cortical tissue. This indicates that BS³ diffuses equally well in these two types of preparations.

**Anaesthesia did not affect total NMDA receptor expression**

We then investigated whether anaesthesia alters total protein abundance of NMDA receptors in cortical neurones in vivo. We found that total NR1 protein levels were not altered by anaesthesia. The amount of NR1 proteins in anaesthetized mice did not differ from that in conscious control mice [96.7 (8.3)% of control, P>0.05]. Similarly, total NR2A expression was insensitive to anaesthesia as it remained at 97.3 (5.1)% of control (P>0.05) after anaesthesia. Like both NR1 and NR2A, NR2B was not altered in its total protein abundance by anaesthesia. The total NR2B level in anaesthetized mice was 94.6 (6.2)% which is comparable with its level in control mice (P>0.05). These data demonstrate that anaesthesia induces no significant change in total NMDA receptor subunit proteins in cortical neurones.

**Anaesthesia causes redistribution of specific NMDA receptor subunits**

Although anaesthesia did not alter the total amount of three NMDA receptor subunits based on the above data, we next investigated whether anaesthesia affects subcellular expression of these proteins. We found that the amount of BS³-linked NR1 proteins was reduced in anaesthetized mice when compared with control mice treated with PBS [81.7 (1.7)% of PBS control, P<0.05; Fig. 2A]. As opposed to this loss of NR1 in the surface membrane, the level of BS³-unlinked NR1 proteins (intracellular NR1) in anaesthetized mice was increased to 134.8 (5.9)% of control (P<0.05; Fig. 2A). Owing to the parallel decrease and increase of NR1 in the surface and intracellular pool, respectively, the ratio of surface to intracellular NR1 was markedly reduced after anaesthesia (Fig. 2A). The intracellular protein
α-actinin did not exhibit any change in abundance after anaesthesia (Fig. 2A). These data reveal a significant redistribution of NR1 in cortical neurones after anaesthesia.

Unlike NR1, NR2A showed no change in its abundance in either surface or intracellular pool in response to anaesthesia. As shown in Figure 2C, the NR2A level in the two compartments of anaesthetized mice did not differ from that of PBS-treated mice. As a result, the ratio of surface to intracellular NR2A remained unchanged after anaesthesia (Fig. 2D).

NR2B is another subunit sensitive to anaesthesia. After anaesthesia, the BS3-linked NR2B level in the surface pool was reduced to 74.7 (2.3)% of control (P<0.05; Fig. 2E). In contrast, BS3-unlinked NR2B proteins in the intracellular pool was enhanced to 154.9 (6.3)% of control (P<0.05; Fig. 2E). These comparable changes in opposite directions resulted in a reduction of the ratio of surface to intracellular NR2B (Fig. 2F). The data here indicate that NR2B, like NR1, underwent a significant redistribution of its abundance during anaesthesia.

Effects of a subanaesthetic dose of chloral hydrate were also tested. Injection of chloral hydrate at 100 mg kg⁻¹ did not produce deep anaesthesia, and animals showed spontaneous body and limb movements. Similarly, no significant change in BS3-linked and -unlinked NR1, NR2A, and NR2B proteins was observed in animals injected with chloral hydrate when compared with those injected with PBS (data not shown).

**Time-dependent effects of anaesthesia on NR1 and NR2B redistribution**

Time-course studies were carried out to examine the temporal property of anaesthesia-induced changes in NR1 and
NR2B proteins in the different pools. In behavioural experiments, chloral hydrate at a dose of 500 mg kg$^{-1}$ induced a relatively short-lived anaesthesia as measured by the LORR over different time points (Fig. 3A). A state of deep anaesthesia was rapidly induced 10 min after drug injection as no righting reflex was observed at this time point. Animals started to recover from anaesthesia 90–110 min after injection (Fig. 3A). In separate biochemical experiments, changes in NR1 levels as manifested by a decrease in the surface pool and an increase in the intracellular pool were evident 10 min after chloral hydrate injection and remained at 1 h (Fig. 3B). These changes were insignificant between anaesthetized mice and control mice at the 2 h time point (Fig. 3B). NR2A in both pools showed no significant changes at all time points surveyed (Fig. 3C). NR2B, however, showed the same temporal pattern of changes as NR1 in both surface and intracellular pools (Fig. 3D). These data reveal the closely related changes in behavioural and NR1/NR2B responses to anaesthesia.

Redistribution of NMDA receptors is independent of hypothermia
Anaesthesia is known to reduce body temperature.$^{20}$ We next evaluated the role of body temperature in the redistribution of NR1 and NR2B during anaesthesia. Body temperature of animals was maintained at 37°C during anaesthesia. Animals were killed 10 min after injection of 500 mg kg$^{-1}$ chloral hydrate for monitoring changes in NR1 and NR2B proteins in the surface and intracellular pools with BS$^3$-cross-linking assays. We found that the BS$^3$-linked NR1 and NR2B levels in the surface pool were reduced, whereas the BS$^3$-unlinked NR1 and NR2B levels
in the intracellular pool were elevated (Fig. 4). These data demonstrate that the effect of anaesthesia on the redistribution of NR1 and NR2B is not contributed by hypothermia.

**Discussion**

The present study investigated the effects of anaesthesia on protein levels of all three NMDA receptor subunits in different subcellular compartments of mouse cortical neurones in vivo. We found that anaesthesia led to a significant reduction of NR1 protein abundance in the surface pool. Anaesthesia also increased NR1 protein levels in the intracellular pool. In parallel with NR1, NR2B proteins were reduced in the surface pool and enhanced in the intracellular pool. In contrast to NR1 and NR2B, NR2A was stable in both compartments in response to anaesthesia. In addition, anaesthesia did not alter total protein levels of the three subunits in cortical neurones. These results for the first time provide evidence that the subcellular location of NMDA receptors, specifically NR2B-containing NMDA receptors, in cortical neurones is subject to modulation by anaesthesia, and general anaesthesia causes a significant redistribution of the receptor from the surface pool to the intracellular pool. Of note, this study was conducted on cerebral cortical neurones. It is currently unclear if the redistribution of NMDA receptors observed in cortical neurones also occurs to other brain regions during anaesthesia. In addition, we have determined that the redistribution of NR1/NR2B was independent of the effect of hypothermia. However, the potentially confounding effects

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**Fig 3** Time-dependent effects of anaesthesia on the righting reflex behaviour and compartment-specific expression of NMDA receptor subunits in mouse cortical neurones. (A) Effects of anaesthesia on righting reflex scores in mice. Effects of anaesthesia on NR1 (B), NR2A (C), and NR2B (D) expression in the two different compartments at different time points. Anaesthesia was induced by chloral hydrate (500 mg kg⁻¹, i.p.). Mice were scored in righting reflex assessments at indicated times (A) or killed at different time points for protein expression assays (B–D). Control mice received a PBS injection. The data are expressed in means (SEM) (n=4–6 per group). *P<0.05 vs PBS.

**Fig 4** Effects of anaesthesia on NR1 and NR2B subunit expression in surface and intracellular pools of mouse cerebral cortical neurones. Anaesthesia was induced by chloral hydrate (500 mg kg⁻¹, i.p.), and body temperature was maintained at 37°C. Mice were killed 10 min after injection. Control mice received a PBS injection. The quantified data of NR1 and NR2B subunit proteins were analysed from separate experiments [means (SEM), n=4–5 per group]. *P<0.05 vs PBS.
of hypotension, hypoxia, hypercarbia, and acidosis have not been investigated in the current study, and need to be evaluated in the future.

The important finding in the present work is the redistribution of NR2B-containing NMDA receptors between the surface and the intracellular pools of cortical neurones after anaesthesia. This redistribution is characterized by the loss of the receptor in the surface pool and the accumulation of the receptor in the intracellular pool. The mechanism(s) responsible for this redistribution are unclear. Presumably, the delivery process of the receptor from the intracellular organelles to the surface membrane, that is, an externalization trafficking process, is preferentially suppressed. Alternatively, an internalization trafficking process removing surface receptors to intracellular sites is selectively accelerated. Either suppressed externalization or accelerated internalization or both could cause a redistribution phenomenon characterized by the subtraction of the receptor in the surface pool in combination with the proportional addition of the receptor in the intracellular pool, whereas total receptor proteins remain unchanged.

Future studies will be required to elucidate possible molecular mechanisms underlying the anaesthesia-induced redistribution of NMDA receptors.

This study did not explore the functional consequences of the reduction of surface NMDA receptors during anaesthesia. It is also unclear if the loss of NMDA receptors occurred at synaptic or extrasynaptic sites. The affected receptors could be extrasynaptic with little immediate impact on synaptic transmission. If it occurred at synaptic sites, the loss of surface NMDA receptors is believed to weaken NMDA receptor function and reduce the efficacy and strength of excitatory synapses containing NMDA receptors. With regard to cellular mechanisms underlying general anaesthesia, potentiation of inhibitory synaptic transmission seems to be a common pathway mediating many forms of general anaesthesia induced by a variety of general anaesthetics. Specifically, general anaesthetics, including chloral hydrate, augment GABA<sub>A</sub>-mediated inhibitory transmission to produce anaesthesia. A large number of reports have documented that chloral hydrate or trichloroethanol, an active metabolite of chloral hydrate, augmented GABA<sub>A</sub>-mediated transmission. The concentration (1–10 mM) at which trichloroethanol inhibited glutamate receptor-mediated currents is higher than those (0.2–0.5 mM) required for producing threshold effect on GABA<sub>A</sub> receptors. Thus, the depression of NMDA receptor-mediated glutamatergic transmission may not significantly contribute to anaesthesia specifically induced by chloral hydrate, even though it contributes to anaesthesia induced by the dissociative anaesthetics such as ketamine and phencyclidine.

Recently, we found that the general anaesthetic propofol inhibited phosphorylation of NR1 subunits at two specific serine sites (serine 896 and serine 897) and impaired NMDA receptor-mediated Ca<sup>2+</sup> influx in cultured rat cortical neurones. We also found that propofol affected AMPA receptor phosphorylation and inhibited activation of extracellular signal-regulated protein kinases in hippocampal neurones. In this study, we revealed that general anaesthesia caused the loss of surface-expressed NMDA receptors. These new biochemical data join a large volume of early electrophysiological results to support the profound effect of various general anaesthetics on NMDA receptors. Although the role of depressed NMDA receptors in inducing anaesthesia remains to be defined, NMDA receptors might well contribute to some specific biological actions of anaesthetics. Future studies need to elucidate the importance of NMDA receptors in processing a specific cellular response to a given anaesthetic agent.

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