Lipid raft integrity affects GABA_A receptor, but not NMDA receptor modulation by psychopharmacological compounds

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Abstract

Lipid rafts have been shown to play an important role for G-protein mediated signal transduction and the function of ligand-gated ion channels including their modulation by psychopharmacological compounds. In this study, we investigated the functional significance of the membrane distribution of NMDA and GABA_A receptor subunits in relation to the accumulation of the tricyclic antidepressant desipramine (DMI) and the benzodiazepine diazepam (Diaz). In the presence of Triton X-100, which allowed proper separation of the lipid raft marker proteins caveolin-1 and flotillin-1 from the transferrin receptor, all receptor subunits were shifted to the non-raft fractions. In contrast, under detergent-free conditions, NMDA and GABA_A receptor subunits were detected both in raft and non-raft fractions. Diaz was enriched in non-raft fractions without Triton X-100 in contrast to DMI, which preferentially accumulated in lipid rafts. Impairment of lipid raft integrity by methyl-β-cyclodextrine (MβCD)-induced cholesterol depletion did not change the inhibitory effect of DMI at the NMDA receptor, whereas it enhanced the potentiating effect of Diaz at the GABA_A receptor at non-saturating concentrations of GABA. These results support the hypothesis that the interaction of benzodiazepines with the GABA_A receptor likely occurs outside of lipid rafts while the antidepressant DMI acts on ionotropic receptors both within and outside these membrane microdomains.

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Introduction

Lipid rafts are distinct microdomains within the cell membrane which are enriched in cholesterol and glycosphingolipids (Pike, 2004). These heterogeneous and dynamic structures float within the membrane bilayer allowing lipids and proteins to move in and out of these membrane microdomains (Kusumi and Suzuki, 2005). The concept of lipid rafts has attracted considerable interest because these structures may affect membrane trafficking and signal transduction processes (Tsiu-Pierchala et al., 2002; Patel et al., 2008).

There is increasing evidence that lipid raft association can also influence neurotransmitter receptor function by affecting transmitter binding, receptor trafficking and clustering (Allen et al., 2007). In post-mortem brain tissue of patients with major depression, G-protein subunit (Gs)α was more likely associated with lipid rafts, where it turned out to be less likely coupled to adenylyl cyclase (Donati et al., 2008). Moreover, Gsα-mediated signalling was enhanced by chronic treatment with antidepressants, which prevented the accumulation of Gsα in lipid rafts (Donati and Rasenick, 2005). With regard to ligand-gated ion channels, the serotonin (5-HT)3 receptor has been detected in raft-like domains (Eisensamer et al., 2003, 2005; Rammes et al., 2004). Likewise the GABA_A receptor subunit α1 has been reported to be lipid raft associated (Dalskov et al., 2005), whereas a differential distribution of synaptic and extrasynaptic GABA_A receptor subunits has recently been demonstrated (Li et al., 2007). Also the localization of NMDA receptors within the cell membrane appears to depend on the respective subunit composition (Hering et al., 2003; Guirland et al., 2004).

A variety of antidepressants and antipsychotics accumulate in raft-like domains, which exert their functional antagonistic effects at the 5-HT3 receptor (Eisensamer et al., 2003, 2005; Rammes et al., 2004). More
recently, we showed that 5-HT3 receptor function depends on lipid raft integrity, whereas the ability of antidepressants to modulate this receptor is retained after lipid raft impairment (Nothdurfter et al., 2010).

So far, no data are available on the significance of lipid rafts for the modulation of NMDA and GABA<sub>A</sub> receptors – crucial members of the most abundant excitatory and inhibitory neurotransmitter systems in the central nervous system – by antidepressants and benzodiazepines. We therefore investigated the effect of lipid raft integrity on the allosteric modulation of GABA<sub>A</sub> receptor function by diazepam (Diaz) and on the modulation of NMDA receptor function by the tricyclic antidepressant desipramine (DMI). Moreover, the membrane domain distribution of the receptor subunits and compounds was investigated using different conditions of lipid raft isolating procedures.

**Method and materials**

**Chemicals and drugs**

For stock solutions DMI (10 mM; Sigma-Aldrich, Germany) was dissolved in H<sub>2</sub>O and Diaz (5 mM; Sigma-Aldrich) in 80% ethanol.

**Cell cultures**

HEK 293 cells (European Collection of Cell Cultures, UK), WSS-1 cells (ATCC LGC Promochem, Germany) and HEK 293 cells stably expressing the human NMDA receptor NR1A/2A, (NMDA-HEK; Merck, Germany), were cultured in Dulbecco’s modified eagle medium (Invitrogen, Germany) supplemented with 10% foetal calf serum (FCS), 1% sodium pyruvate and antibiotics. Cell cultures

Aldrich) in 80% ethanol.

**Sucrose density gradient centrifugation**

Two confluent Petri dishes of cultured cells were washed in ice-cold PBS before resuspension in 800 µl high-salt Hapes buffer (20 mM Hapes, 5 mM EDTA, 1 M NaCl, pH 7.4) supplemented with protease-inhibitor cocktail. For solubilization 1% Triton X-100 was added directly to the saline buffer as described previously (Nothdurfter et al., 2007). Following homogenization and sonication, the suspension was diluted 1:1 with 80% sucrose solution prepared in high-salt Hapes buffer. The solution was then exchanged of solutions superfusing all receptors expressed on the cell surface within milliseconds. Currents were recorded using an EPC-9 amplifier (HEKA, Germany). Patch clamp pipettes were pulled from borosilicate glass using a horizontal puller (P-97 Puller; Sutter Instruments, USA), with resistances of 3–6 MΩ when filled with intracellular solution. Intracellular solution for NMDA-HEK: 120 mM CsCl; 10 mM EGTA; 1 mM MgCl<sub>2</sub>; 0.2 mM CaCl<sub>2</sub>; 10 mM glucose; 22 mM TEACl; 2 mM ATP; 0.2 mM cAMP; pH 7.35. Corresponding extracellular bath solution: 140 mM NaCl; 3 mM KCl; 10 mM glucose; 10 mM Hapes, 0.2 mM CaCl<sub>2</sub>; 4.5 mM sucrose; pH 7.35. D-Serine (5 µM) was present in all solutions sufficient to cause 80–85% activation of glycine B receptors. Intracellular solution for GABA<sub>A</sub> receptor mediated currents from WSS-1 cells: 140 mM KCl; 11 mM EGTA; 10 mM Hepes-KOH; 2 mM MgCl<sub>2</sub>; 0.1 mM ATP; pH 7.35. Corresponding extracellular bath solution: 162 mM NaCl; 5.3 mM KCl; 0.67 mM NaH<sub>2</sub>PO<sub>4</sub>; 0.22 mM KH<sub>2</sub>PO<sub>4</sub>; 5.6 mM glucose; 15 mM Hepes; 2 mM CaCl<sub>2</sub>; pH 7.4. Antagonism of NMDA receptor-mediated currents was measured in terms of the magnitude of the steady-state blocked current as percentage of the control current. Five cumulatively increasing concentrations of the respective compound were applied in the continuous presence of agonists and the control current for each substance concentration was calculated by a linear projection from the steady-state current before and after substance application, i.e. for the first concentration current = 0.85 x current before antagonist plus 0.15 x recovery current. For the second substance concentration, these same currents were multiplied by 0.7 and 0.3 respectively and so on. The potency of the compound was assessed by plotting the mean percentage current magnitude against the antagonist concentration and then a curve was fit using the logistic equation for which the variable parameters IC<sub>50</sub> and Hill coefficient were free and the range and background values were normally set to 100 and 0, respectively. For analysis of GABA<sub>B</sub>-induced currents in WSS-1 cells only results of stable cells showing at least 50% recovery of responses to GABA following the removal of drugs entered the final analysis. To compensate for this effect the % antagonism at each concentration was based on both the control and the recovery current by assuming a linear time-course for the rundown.

**Electrophysiological recordings**

Voltage clamp recordings were made in the whole-cell configuration at a holding potential of −70 mV. Compounds were applied using a fast superfusion device with a stepper motor-driven (Warner Instruments LLC, USA) application pipette, made from modified double-barrelled theta glass. This arrangement allows complete exchange of solutions superfusing all receptors expressed on the cell surface within milliseconds. Currents were recorded using an EPC-9 amplifier (HEKA, Germany). Patch clamp pipettes were pulled from borosilicate glass using a horizontal puller (P-97 Puller; Sutter Instruments, USA), with resistances of 3–6 MΩ when filled with intracellular solution. Intracellular solution for NMDA-HEK: 120 mM CsCl; 10 mM EGTA; 1 mM MgCl<sub>2</sub>; 0.2 mM CaCl<sub>2</sub>; 10 mM glucose; 22 mM TEACl; 2 mM ATP; 0.2 mM cAMP; pH 7.35. Corresponding extracellular bath solution: 140 mM NaCl; 3 mM KCl; 10 mM glucose; 10 mM Hapes, 0.2 mM CaCl<sub>2</sub>; 4.5 mM sucrose; pH 7.35. D-Serine (5 µM) was present in all solutions sufficient to cause 80–85% activation of glycine B receptors. Intracellular solution for GABA<sub>A</sub> receptor mediated currents from WSS-1 cells: 140 mM KCl; 11 mM EGTA; 10 mM Hepes-KOH; 2 mM MgCl<sub>2</sub>; 0.1 mM ATP; pH 7.35. Corresponding extracellular bath solution: 162 mM NaCl; 5.3 mM KCl; 0.67 mM NaH<sub>2</sub>PO<sub>4</sub>; 0.22 mM KH<sub>2</sub>PO<sub>4</sub>; 5.6 mM glucose; 15 mM Hepes; 2 mM CaCl<sub>2</sub>; pH 7.4. Antagonism of NMDA receptor-mediated currents was measured in terms of the magnitude of the steady-state blocked current as percentage of the control current. Five cumulatively increasing concentrations of the respective compound were applied in the continuous presence of agonists and the control current for each substance concentration was calculated by a linear projection from the steady-state current before and after substance application, i.e. for the first concentration current = 0.85 x current before antagonist plus 0.15 x recovery current. For the second substance concentration, these same currents were multiplied by 0.7 and 0.3 respectively and so on. The potency of the compound was assessed by plotting the mean percentage current magnitude against the antagonist concentration and then a curve was fit using the logistic equation for which the variable parameters IC<sub>50</sub> and Hill coefficient were free and the range and background values were normally set to 100 and 0, respectively. For analysis of GABA<sub>B</sub>-induced currents in WSS-1 cells only results of stable cells showing at least 50% recovery of responses to GABA following the removal of drugs entered the final analysis. To compensate for this effect the % antagonism at each concentration was based on both the control and the recovery current by assuming a linear time-course for the rundown.

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placed at the bottom of a centrifuge tube (Beckman Coulter, USA) and overlaid with 1400 µl 35% sucrose and finally with 1000 µl 5% sucrose (both prepared in low-salt Hepes buffer: 20 mM Hepes; 5 mM EDTA; 0.5 M NaCl; pH 7.4). Samples were ultracentrifuged at 40,000 rpm (210,000 g) in a Beckman SW60 rotor for 4 h at 4 °C. Subsequently, 10 fractions (400 µl each) were taken from the top to the bottom of the gradient.

The experimental protocols for mouse brain tissue fractionation were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany. Brains of male C57Bl6 mice aged 28–42 d were put into ice-cold artificial cerebrospinal fluid; sagittal slices of the medial prefrontal cortex (350 µm) were put into ice-cold artificial cerebrospinal fluid; sagittal slices of the medial prefrontal cortex (350 µm) were homogenized/sonified in 1 ml high-salt Hepes buffer supplemented with protease-inhibitor cocktail. The suspension was centrifuged at 1000 g, 10 min, 4 °C. The supernatant was again centrifuged at 8000 g, 10 min, 4 °C. For solubilization the supernatant of the second centrifugation step was supplemented with 0.4% Triton X-100 and incubated for 30 min on ice. The solution (1850 µl) was then diluted 1:1 with 80% sucrose, overlaid with 3200 µl 35% sucrose and 2300 µl 5% sucrose. After ultracentrifugation in an SW41 rotor at 40,000 rpm (250,000 g) for 4 h at 4 °C, 13 fractions (710 µl each) were collected.

**Cholesterol depletion**

Cholesterol depletion using low doses of methyl-β-cyclodextrin (MβCD) to maintain cell viability for electrophysiological recordings was performed as described previously (Nothdurfter et al., 2010). Briefly, cells were incubated with 0.25 or 0.5 mM MβCD for 12 h at 37 °C in serum-free DMEM prior to the electrophysiological recordings.

**Western blotting**

Equal volumes of sucrose gradient fractions were adjusted 1:5 with Laemmli sample buffer. For the detection of GABAA receptor subunit γ2, samples were heated at 70 °C, 10 min; all other samples were denatured at 95 °C, 5 min. SDS-PAGE was performed on 12% gels. After transfer to a nitrocellulose membrane and blocking (TBS-T/5% milk powder), blots were probed with specific antibodies: transferrin receptor (mouse monoclonal, Zymed, USA, dilution 1:1000); caveolin-1 (mouse monoclonal, BD Biosciences, USA, dilution 1:3000); flotillin-1 (mouse monoclonal, BD Biosciences, dilution 1:1000); GABAA receptor subunit α1 (goat polyclonal, Santa Cruz Biotechnologies, USA, dilution 1:500); GABAA receptor subunit β3 (rabbit polyclonal, Phosphosolutions, Germany, dilution 1:200); GABAA receptor subunit γ2 (Centre of Brain Research of the University of Vienna, Austria, dilution 1:1000); NMDA receptor subunit NR1A (goat polyclonal, Santa Cruz Biotechnologies, dilution 1:300). After incubation with horseradish peroxidase-coupled secondary antibodies, specific antibody binding was visualized by ECL chemiluminescence (Amersham Biosciences, Germany).

**Quantification of DMI and Diaz**

For the quantification of DMI, 250 µl sucrose gradient fractions, 750 µl H2O, 0.1 ml internal standard solution and 1 ml 2 M sodium hydrogencarbonate buffer (adjusted to pH 10.5) were mixed and supplemented with 5 ml n-hexane/1.5% isoamyl-alcohol. After centrifugation (4000 g, 15 min), the organic layer was transferred to a tube containing 0.25 ml 0.18 M phosphoric acid, mixed and centrifuged at 4000 g for 10 min. The organic layer was discarded and an aliquot of the aqueous phase was injected for chromatographic separation. For HPLC analysis, a Beckman Coulter (USA) 166 variable-wavelength UV detector and a Beckman Coulter gradient pump 126 Solvent Module equipped with a Beckman Coulter autoinjector 508 autosampler were used. Separations were made on four coupled Chromolith performance RP-18e 100 × 4.6 mm columns (Merck KGa A). The mobile phases (A, 0.22% orthophosphoric acid adjusted to pH 3.5; B, acetonitrile) were degassed in an ultrasonic bath immediately before use. The column temperature was 30 °C, the flow of the mobile phase was 1.5 ml/min. A mobile phase gradient was used for the chromatographic analysis. DMI was determined by UV absorption 214 nm wavelength. Samples were calibrated using spiked samples at different concentrations. The quantification was performed by calculating the internal standard peak:area ratio of the analyte and a regression model was fitted to the peak:area ratio of the compound to internal standard vs. concentration.

Diaz concentrations were measured using liquid chromatography/mass spectrometry, which consisted of an Agilent 1100 HPLC system (Agilent technologies, Germany) and an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Germany) equipped with a turbo ion spray ionization interface in positive MRM mode. Chromatographic separation was performed on a GEMINI C 18, 50 mm × 2.0 mm (Phenomenex, USA) with particle size 5 µm. The mobile phase consisted of methanol-H2O (85:15, v/v) containing 0.1% formic acid and 10 mM ammonium formate. Isocratic separation was performed with a flow rate of 0.2 ml/min and a run time of 4 min. Column oven temperature was adjusted to 40 °C. Protriptyline was selected as the internal standard. The column effluent was monitored at the following transitions: Diaz m/z 285.1→154.0 and the internal standard protriptyline 264.3→155.1. Diaz was extracted with a modified liquid/liquid extraction procedure with acetonitrile.
Lowry protein assay

Protein concentration was determined by a modified method of Lowry et al. (1951).

Cholesterol assay

Cholesterol concentrations were determined colorimetrically with a commercial assay kit (Boehringer, Germany).

Statistical analysis

Differences in electrophysiological parameters were compared using t tests for paired samples. As a nominal level of significance, \( \alpha = 0.05 \) was accepted.

Results

Isolation of lipid rafts

In view of the controversial discussion about lipid raft association of proteins in relation to the use of detergents, we analysed the membrane distribution of the lipid raft marker proteins flotillin-1/caveolin-1 and the transferrin receptor, a bona fide non-raft protein, in the absence and presence of detergent. HEK 293 cells (Fig. 1a) and mouse neocortex (Fig. 1b) were fractionated by sucrose gradient density centrifugation. In the absence of detergent flotillin-1 and caveolin-1 as well as the transferrin receptor were detected both in low buoyant density (LBD) and in high buoyant density (HBD) fractions of sucrose gradients of HEK 293 cells and mouse neocortex. In contrast, the addition of Triton X-100 shifted the transferrin receptor to the HBD fractions, whereas flotillin-1 and caveolin-1 were retained in the LBD fractions, but lost in the HBD fractions. With regard to the distribution of cholesterol within the cell membrane, cholesterol concentrations were enriched predominantly in the LBD fractions of mouse neocortex (Fig. 1c, upper panel). Interestingly, the use of detergent did not affect the cholesterol distribution within membrane fractions (Fig. 1c, lower panel). Thus, methodological issues such as the use of detergent are crucial for the designation of proteins to be raft-associated or not, while cholesterol distribution in the respective membrane fractions remains unaffected. Consequently, we studied the membrane distribution of ionotropic receptor subunits and psychopharmacological compounds both under detergent-free and detergent-containing conditions in the following experiments.

Effect of lipid raft integrity for NMDA receptor modulation by DMI

The antidepressant DMI reduced NMDA-induced cation currents through human NMDA receptor subunits NR1A/2A in a dose-dependent fashion with an IC\(_{50}\) value of 2.7 ± 0.3 \( \mu \)M (Fig. 2a, b). The control NMDA currents and NMDA currents in the presence of different concentrations of DMI reached steady-state after approximately 5 s, thereby assuring correct current analysis after receptor desensitization has been accomplished.

To investigate the membrane localization of the NMDA receptor, we fractionated NMDA-HEK cells by sucrose gradient density centrifugation and probed the respective fractions with an antibody against the NMDA receptor subunit NR1A (Fig. 2c). When no detergent was used, immunoreactivity was detected both in the low buoyant density fractions (LBD, at the top of the gradient) and in the high buoyant density fractions (HBD, at the bottom of the gradient). The use of Triton X-100 completely shifted the NMDA receptor subunit NR1A to the HBD fractions. To affect lipid raft integrity, we partially depleted cholesterol from cell membranes by means of low-dose Mj/CD treatment thereby retaining cell viability for subsequent electrophysiological recordings as described previously (Nothdurfter et al., 2010). Pre-treatment of primary hippocampal neurons with 0.1 mM Mj/CD for 12 h did not alter the inhibitory effect of DMI on NMDA/D-serine-evoked cation currents (Fig. 2d, e).
configuration. 200 neurons were recorded in a whole-cell voltage-clamp density fractions. (X-100. LBD, low buoyant density fractions; HBD, high buoyant without detergent, the other part was solubilized with Triton receptor subunit NR1. One part of the cells was prepared immunoblotted with a polyclonal antibody against the NMDA and were analysed by SDS-PAGE. Replicate gels were and represent the means ¡ S.E.M. of five independent experiments. (c) Membrane fractions of NMDA-HEK cells were prepared by means of sucrose gradient density centrifugation and were analysed by SDS-PAGE. Replicate gels were immunoblotted with a polyclonal antibody against the NMDA receptor subunit NR1. One part of the cells was prepared without detergent, the other part was solubilized with Triton X-100. LBD, low buoyant density fractions; HBD, high buoyant density fractions. (d) Cation currents of primary hippocampal neurons were recorded in a whole-cell voltage-clamp configuration. 200 µM NMDA/5 µM D-serine was applied for 2 s in the absence or presence of 3 µM DMI. One portion of cells was pre-treated with 0.1 mM methyl-β-cyclodextrine (M/βCD) for 12 h prior to the recordings (lower panel), the other portion of cells remained untreated (control; upper panel). The upper bar indicates the presence of NMDA/ D-serine, the lower bar indicates the presence of DMI. One representative recording is shown, respectively. (e) Peak amplitude and plateau of untreated cells and after pre-treatment with 0.1 mM M/βCD for 12 h of seven independent experiments are shown in the presence of 3 µM DMI in relation to values in the absence of DMI, which are set as 100% (control). Data are shown as means ± S.E.M. % of control. † Tests

Fig. 2. Inhibitory effect of desipramine (DMI) on NMDA-evoked cation currents and membrane distribution of the NMDA receptor subunit NR1A. (a) Cation currents of HEK 293 cells stably expressing NR1/NR2A composed NMDA receptors (NMDA-HEK) were recorded in a whole-cell voltage-clamp configuration. Currents were recorded under a continuous stimulation with 200 µM NMDA in the presence of 10 µM glycine. Cells were exposed to increasing concentrations of DMI (10 to 100 µM). The panel shows representative NR1/2A receptor currents. The upper bar indicates increasing concentrations of DMI; the lower bar indicates the presence of NMDA/glycine. (b) Dose–response relationship of DMI against the current amplitude. Results are expressed in % of the control NMDA-induced currents obtained without DMI and represent the means ± S.E.M. of five independent experiments. (c) Membrane fractions of NMDA-HEK cells were prepared by means of sucrose gradient density centrifugation and were analysed by SDS-PAGE. Replicate gels were immunoblotted with a polyclonal antibody against the NMDA receptor subunit NR1. One part of the cells was prepared without detergent, the other part was solubilized with Triton X-100. LBD, low buoyant density fractions; HBD, high buoyant density fractions. (d) Cation currents of primary hippocampal neurons were recorded in a whole-cell voltage-clamp configuration. 200 µM NMDA/5 µM D-serine was applied for 2 s in the absence or presence of 3 µM DMI. One portion of cells was pre-treated with 0.1 mM methyl-β-cyclodextrine (M/βCD) for 12 h prior to the recordings (lower panel), the other portion of cells remained untreated (control; upper panel). The upper bar indicates the presence of NMDA/ D-serine, the lower bar indicates the presence of DMI. One representative recording is shown, respectively. (e) Peak amplitude and plateau of untreated cells and after pre-treatment with 0.1 mM M/βCD for 12 h of seven independent experiments are shown in the presence of 3 µM DMI in relation to values in the absence of DMI, which are set as 100% (control). Data are shown as means ± S.E.M. % of control. † Tests

Membrane distribution of desipramine and diazepam and colocalization with the NMDA and GABA A receptor

To determine whether the addition of detergent also affects the differential accumulation of psychopharmacological compounds, we quantified the concentrations of DMI in sucrose gradient fractions of HEK 293 cells after incubation with 10 µM DMI. As shown previously (Eisensamer et al., 2005), DMI was enriched in the LBD fractions using a detergent-free preparation procedure. However, the addition of Triton X-100 caused a shift of DMI to the HBD fractions (Fig. 3). Thus, the membrane distribution of both proteins and antidepressive compounds appears to be sensitive to the use of detergent.

Considering the antagonistic effects of DMI at the NMDA receptor we investigated whether the GABA A receptor is also sensitive to modulation by DMI. Compared to NMDA-induced currents, GABA A receptor-mediated currents of WSS-1 cells were only slightly inhibited by DMI even at high concentrations, which are far above those achieved under clinical conditions (Glotzbach and Preskorn, 1982; Fig. 4a). Following application of 30 µM DMI, chloride currents were reduced only to 73.0 ± 5.2% of control (charge of currents expressed as the area under the curve in relation to the charge obtained in the absence of DMI, which is set as 100%). With a reduction to 86.6 ± 9.8%, the peak of receptor currents was even less affected. To investigate whether this rather moderate modulatory potency of DMI at the GABA A receptor is related to a divergent distribution of the compound and receptor subunits within the cell membrane, we studied the membrane localization of GABA A receptor subunits in WSS-1 cells

for paired samples revealed no statistically significant differences between peaks and plateaus of untreated and M/βCD pretreated cells.
Fig. 4. Effect of desipramine (DMI) on GABA-evoked chloride currents and membrane distribution of GABA_A receptor subunits. (a) 30 μM DMI was applied to WSS-1 cells containing recombinant α1γ and low levels of endogenous β2 GABA_A receptor subunits. Chloride currents were recorded in a whole-cell voltage-clamp configuration under conditions of a 500 ms stimulation with 100 μM GABA. The upper panel shows a representative original recording. The upper bar indicates the presence of GABA; the lower bar indicates the presence of DMI. The lower panel shows peak amplitude and charge expressed as the area under the curve (AUC) of the GABA-evoked currents in relation to values obtained in the absence of DMI, which is set as 100% (% of control). Data are presented as the means ± S.E.M. of ten independent experiments. For calculation of the final DMI effect, values from control and recovery traces were included into the analysis. (b) 30 μM DMI was applied to WSS-1 cells in serum-free medium (untreated) and after cholesterol depletion induced by 0.5 mM methyl-β-cyclodextrine (MJ/CD). Chloride currents were recorded in a whole-cell voltage-clamp configuration under conditions of a 500 ms stimulation with 100 μM GABA. The upper panel shows representative original recordings. The upper bar indicates the presence of GABA; the lower bar indicates the presence of DMI. The lower panel shows peak amplitude and charge expressed as the AUC of the GABA-evoked currents in relation to values obtained in the absence of DMI, which is set as 100% (% of control). Data are presented as the means ± S.E.M. of five independent experiments. For calculation of the final DMI effect, values from control and recovery traces were included into the analysis. T tests for paired samples revealed a statistically significant difference between peaks of untreated and MJ/CD pre-treated cells; *p<0.05. (c) WSS-1 cells and mouse neocortical tissue were homogenized, sonicated and fractionated by sucrose gradient density centrifugation either in the presence or absence of detergent. Fractions of the gradient were collected and analysed by SDS-PAGE. Replicate gels were immunoblotted with antibodies against the GABA_A receptor subunits α1, β2 and γ2 and mouse neocortex (α1, β2 and γ2; Fig. 4c). Similarly to the NMDA receptor, all GABA_A receptor subunits were detected both in LBD and HBD fractions of cells and tissue using a detergent-free protocol, but only in the HBD fractions when using Triton X-100. Thus, the membrane distribution of GABA_A receptor subunits does not explain the considerably weaker effect of DMI at this ligand-gated ion channel. Interestingly, the inhibitory potency of DMI on GABA_A receptor-mediated currents appeared to be partially reversed under cholesterol-depleting conditions (charge: 61.5±5.0% untreated vs. 75.5±10.7% MJ/CD pre-treated; peak: 79.2±5.6% untreated vs. 97.5±3.4% MJ/CD pre-treated; Fig. 4b).

Benzodiazepines are potent positive allosteric modulators of GABA_A receptors. We therefore investigated the modulatory properties of Diaz on GABA_A receptor function in relation to the membrane distribution of GABA_A receptor subunits and that of Diaz. As shown previously (Davies et al., 2000), 1 μM Diaz markedly potentiated GABA-evoked chloride currents of WSS-1 cells (235.7±15.2%); charge of the currents expressed as the area under the curve in relation to the charge obtained in the absence of Diaz, which is set as 100%; data represent
Impairment of lipid raft integrity by pre-treatment of WSS-1 cells with M/βCD for 12 h dose-dependently diminished GABA-evoked chloride currents (Fig. 6a–c). The shift of the GABA dose–response curve to the right after M/βCD treatment (Fig. 6d) indicates reduced agonist effectiveness when receptors are not integrated in lipid rafts. When receptors were activated at submaximal GABA concentrations (Fig. 6e) the GABA enhancing effect of Diaz i.e. increase in GABA potency was even more pronounced under M/βCD pre-treatment conditions. However, the maximal Diaz-induced potentiating effect of receptor currents did not change (Fig. 6f), i.e. maximal currents observed with saturating concentrations of GABA were the same both in the presence and absence of Diaz.

Because cholesterol depletion decreased membrane stability and deteriorated patch-clamp recording conditions the generation of a concentration–response curve in the presence of Diaz was not achievable with 0.5 mM M/βCD. However, leak current and membrane resistance of HEK cells after treatment with 0.5 mM M/βCD were comparable to those cells that were untreated. Pre-treatment with 0.25 mM M/βCD reduced membrane cholesterol content to 82.10% of control (data not shown). Consequently, this concentration did not result in a sufficient shift to the right of the GABA concentration–response curve as observed at 0.5 mM M/βCD. These results indicate that substantial amounts of GABA A receptors were still incorporated into lipid rafts.

**Discussion**

In the present study we investigated the membrane distribution of NMDA and GABA A receptor subunits in WSS-1 cells. The distribution of Diaz within the cell membrane was determined in sucrose gradient fractions of HEK 293 cells and mouse neocortex after incubation with 1 mM Diaz (Fig. 5b). In contrast to DMI, which was enriched in the LBD fractions, Diaz preferentially accumulated in the HBD non-raft fractions already under detergent-free conditions. This distribution pattern was not altered when Triton X-100 was used for preparation of lipid rafts (data not shown). Thus, both ligand-gated ion channel subunits and psycho-pharmacological compounds show different distribution patterns within the cell membrane with regard to lipid raft association.
relation to their allosteric modulation by DMI and Diaz. The assignment of receptor subunits and drugs to specific membrane fractions turned out to be strongly dependent on the preparation procedure. A partial colocalization of receptor subunits and the compounds within the cell membrane was found under detergent-free conditions and a complete overlap in non-raft fractions when using detergent (Fig. 7). Impairment of lipid raft integrity by cholesterol depletion did not substantially alter the modulatory potency of DMI at the NMDA and GABA_A receptor. However, impairment of lipid raft integrity affected the positive allosteric modulation of the GABA_A receptor by benzodiazepines.

Preparation of lipid rafts heavily relies on the purification method employed. Commonly, lipid rafts are considered as membrane fractions that are resistant to non-ionic detergents such as Triton X-100 (Brown and Rose, 1992). In fact, we found that the use of detergent allowed proper separation of the lipid raft marker proteins caveolin-1 and flotilllin-1 from the transferrin receptor, which is widely considered as a non-raft protein (Nothdurfter et al., 2007). This is in line with previous findings reporting a dose-dependant effect of Triton X-100 on the separation of raft from non-raft proteins (Brady et al., 2004). On the other hand, the use of detergent-free protocols has been suggested to more closely reflect the physiological situation (Schnitzer et al., 1995a, b; Smart et al., 1995; Song et al., 1996; Luria et al., 2002; Eissensamer et al., 2005; Persaud-Sawin et al., 2009). Accordingly, different compositions and properties of lipid rafts have been reported depending on the extraction method used (Foster et al., 2003; Kim et al., 2004; Sprenger and Horrevoets, 2007; Garner et al., 2008; Pike, 2009). Consequently, we studied the membrane distribution of ligand-gated ion channels and psycho-pharmacological compounds under both detergent-free and detergent-containing conditions in the present study. The detergent-free protocol revealed a much more differential distribution pattern of ionotropic receptor subunits and compounds investigated, although the separation of prototypical markers of raft and non-raft proteins appeared more challenging.

Concerning the NMDA receptor, the NR1A subunit has been located in non-raft membrane fractions of rat forebrain when using an Optiprep gradient without detergent solubilization (Wu et al., 1997). In a study by Hering et al. (2003), NR1 was found both in raft and in non-raft fractions under detergent-containing conditions. A non raft-association has been reported for the NR1, NR2A and NR2B subunits using Triton X-100 as a detergent (Suzuki et al., 2001). Besshoh et al. (2007) found lipid raft association of the NR2A subunit under detergent-containing conditions, whereas the NR2B subunit in rat brain tissue shifted from post-synaptic densities to lipid rafts with increasing age. A study by Ponce et al. (2008) suggested an important role of lipid raft integrity for NMDA receptor function, showing that the reduction of cholesterol levels by simvastatin might protect from NMDA-induced neuronal damage by reducing the association of the NR1 subunit with lipid rafts. In our study, impairment of lipid raft integrity by cholesterol depletion did not affect the modulatory potency of DMI at the NMDA receptor, which suggests that the membrane localization of this receptor and compound plays a minor role for its modulation by this antidepressant. In some contrast to the NMDA receptor, lipid raft impairment partially reversed the antagonistic effect of DMI at the GABA_A receptor, although this effect appeared only marginally. Detergent-free lipid raft preparation yielded a clear distribution of both DMI and partially GABA_A receptor subunits in the raft fractions. Thus, cholesterol depletion might interfere with the pharmacological effects of DMI on GABA_A receptors by e.g. impairing ligand × receptor interaction, thereby weakening the antagonistic potency.

With regard to lipid raft association of GABA_A receptors, an almost exclusive lipid raft association has been claimed for the α1 subunit of rat cerebellar granule cells after purification with the detergent Brij 98 (Dalskov et al., 2005). Following multiple solubilization steps with Triton X-100 of rat forebrain tissue, distinct GABA_A receptor subunit combinations have been attributed to different cell membrane compartments (Li et al., 2007). The α and β subunits in the absence of γ subunits, which might represent extra-synaptic receptors, have been detected in detergent-resistant membranes/lipid rafts. In contrast, synaptic assemblies containing α and β subunits together with the γ2 subunit showed only negligible lipid raft association.
In our study, the use of the detergent Triton X-100 shifted NMDA and GABA<sub>A</sub> receptor subunits to the HBD fractions of sucrose gradients. However, when using a detergent-free protocol, a much more differential distribution pattern of ionotrophic receptors within the cell membrane could be observed. NMDA receptor (NR1A subunit) and GABA<sub>A</sub> receptor subunits (α<sub>1</sub>, β<sub>2</sub>, γ<sub>2</sub>) were distributed both over the LBD and the HBD fractions of the sucrose gradient. In contrast, the 5-HT<sub>1</sub>B receptor was located predominantly in the lipid raft-associated LBD fractions (Eisensamer et al., 2003; Nothdurfter et al., 2010). Presumably, physiological and pharmacological processes are more reliably reflected using detergent-free protocols.

Currently, only few data are available concerning the distribution of psychopharmacological drugs within membrane fractions. The tricyclic antidepressant DMI, which modulates the 5-HT<sub>1</sub>B receptor in a non-competitive manner (Eisensamer et al., 2003), was predominantly accumulated in raft-like domains and colocalized with the 5-HT<sub>1</sub>B receptor protein under detergent-free conditions as shown previously for various other antidepressants and antipsychotics (Eisensamer et al., 2005). However, the addition of Triton X-100 during preparation completely shifted DMI to the HBD fractions, but had no effect on the cholesterol distribution within the cell membrane. These results are in contrast to the benzodiazepine Diaz, which was enriched in the HBD fractions already under detergent-free conditions. Thus, in spite of their common lipophilic properties, various psychopharmacological compounds display a differential association with lipid rafts, which might contribute to their distinct psychopharmacological profile.

It has been shown previously that the tricyclic antidepressant DMI acts as a functional antagonist at the NMDA receptor (Sernagor et al., 1989; Watanabe et al., 1993; Szasz et al., 2007). Our experiments performed with NMDA receptors expressed in HEK cells confirm these results. Furthermore, currents showed a nearly complete recovery to the control steady-state level after removal of the antagonist. This observation and the fact that the original cytosolic content is mainly dominated by the intracellular pipette solution with no protein left, renders a DMI-mediated mechanism via receptor internalisation unlikely and rather speaks in favour of a direct antagonism at the receptor. Although the concentrations required for these modulatory effects are in the low micromolar range, they are nevertheless of clinical relevance in view of a high brain: plasma ratio of tricyclic antidepressants (Glotzbach and Preskorn, 1982). On the other hand, DMI delivered at rather high concentrations only slightly affected GABA-evoked chloride currents, thereby indicating that the effect of DMI on GABA<sub>A</sub> receptors appears to be less potent as compared to NMDA receptors and is not of clinical relevance. Thus, the colocalization of DMI with subunits of ionotropic receptors seems not to be sufficient for a potent allosteric receptor modulation in general.

The benzodiazepine Diaz markedly potentiated GABA-evoked chloride currents, which is in line with previous studies (Davies et al., 2000). Diaz was colocalized with GABA<sub>A</sub> receptor subunits in the HBD fractions already under detergent-free conditions, which supports the hypothesis that synaptic GABA<sub>A</sub> receptors, which are sensitive to benzodiazepines, are located predominantly within non-raft fractions (Li et al., 2007). To investigate the role of the membrane localization of the GABA<sub>A</sub> receptor for its modulation by benzodiazepines we impaired lipid raft integrity by MI<sub>CD</sub>-induced cholesterol depletion. MI<sub>CD</sub> treatment diminished GABA<sub>A</sub> receptors currents per se, similarly to the results previously found for the 5-HT<sub>1</sub>B receptor (Nothdurfter et al., 2010). The potentiating effect of Diaz appeared to be enhanced at lower GABA concentrations under cholesterol-depleting conditions. However, due to technical limitations related to cholesterol depletion, we could not perform Schild plot analysis which would have provided definite clarity about the effects of benzodiazepines at the GABA<sub>A</sub> receptor under conditions of cholesterol depletion. Nevertheless, our results suggest that, at submaximal GABA concentrations, benzodiazepines show an increased effectiveness at GABA<sub>A</sub> receptors after cholesterol depletion. Together with the preferential accumulation of Diaz in non-raft fractions, these results argue for the hypothesis that the interaction of benzodiazepines with the GABA<sub>A</sub> receptor likely occurs outside lipid rafts.

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Statement of Interest

None.

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