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Cholesterol modulates ligand binding and G-protein coupling to serotonin$_{1A}$ receptors from bovine hippocampus

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Abstract

The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important member of the superfamily of seven-transmembrane domain G-protein-coupled receptors. We have examined the modulatory role of cholesterol on the ligand binding activity and G-protein coupling of the bovine hippocampal 5-HT$_{1A}$ receptor by depleting cholesterol from native membranes using methyl-β-cyclodextrin (MβCD). Removal of cholesterol from bovine hippocampal membranes using varying concentrations of MβCD results in a concentration-dependent reduction in specific binding of the agonist 8-OH-DPAT to 5-HT$_{1A}$ receptors. This is accompanied by alterations in binding affinity and sites obtained from analysis of binding data. Importantly, cholesterol depletion affected G-protein-coupling of the receptor as monitored by the GTP-γ-S assay. The concomitant changes in membrane order were reported by changes in fluorescence polarization of membrane probes such as DPH and TMA-DPH, which are incorporated at different locations (depths) in the membrane. Replenishment of membranes with cholesterol led to recovery of ligand binding activity as well as membrane order to a considerable extent. Our results provide evidence, for the first time, that cholesterol is necessary for ligand binding and G-protein coupling of this important neurotransmitter receptor. These results could have significant implications in understanding the influence of the membrane lipid environment on the activity and signal transduction of other G-protein-coupled transmembrane receptors.

Keywords: 5-HT$_{1A}$ receptor; Cholesterol; G-protein coupling; Fluorescence polarization; Methyl-β-cyclodextrin

1. Introduction

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting [1,2]. It is often found distributed non-randomly in domains or pools in biological and model membranes [1–7]. Many of these domains are believed to be important for the maintenance of membrane structure and function. In addition, cholesterol has been reported to be distributed heterogeneously among various intracellular membranes. The lowest cholesterol concentration is found in the membranes of the endoplasmic reticulum, which, interestingly, is the site of cholesterol biosynthesis [8]. The highest concentration (~90% of the total cellular cholesterol) is found in the plasma membrane [9].

A strong asymmetry exists even in the manner cholesterol is distributed among various organs in the body of higher eukaryotes. Thus, the central nervous system, which accounts for only 2% of the body mass, contains ~25% of free cholesterol present in the whole body [10]. Although the brain is an organ that is highly enriched in cholesterol, the organization and dynamics of brain cholesterol is still poorly understood [11]. Brain cholesterol is synthesized in situ [12] and is developmentally regulated [13]. Cholesterol organization, traffic, and dynamics in the brain is stringently controlled since the input of cholesterol into the central

Abbreviations: BCA, bicinchoninic acid; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GTP-γ-S, guanosine-5′ -*O*-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; MβCD, methyl-β-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene

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nervous system is almost exclusively from in situ synthesis as there is no available evidence for the transfer of cholesterol from blood plasma to brain [10]. As a result of this, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain. In the Smith–Lemli–Opitz syndrome, for example, the marked abnormalities in brain development and function leading to serious neurological and mental dysfunctions have their origin in the fact that the major input of brain cholesterol comes from the in situ synthesis, and such synthesis is defective in this syndrome [14]. The interaction between cholesterol and other molecular components (such as receptors) in the brain therefore assumes relevance for a comprehensive understanding of brain function.

Lipid–protein interactions play a crucial role in maintaining the structure and function of biological membranes [15]. A possible role of lipids in a variety of neurological disorders is well documented [14]. Effects on membrane function are presumed to be mediated by membrane proteins and, for this reason, monitoring lipid–protein interactions assume significance. A large portion of any given transmembrane receptor remains in contact with the membrane lipid environment. This raises the obvious possibility that the membrane could be an important modulator of receptor structure and function [16]. A study of such lipid–receptor interactions is of particular importance because a cell has the ability of varying the lipid composition of its membrane in response to a variety of stress and stimuli, thus changing the environment and the activity of the receptors in its membrane. In view of the importance of cholesterol in relation to membrane domains, the interaction of cholesterol with membrane proteins [17] and receptors [16] represents an important determinant in functional studies of such proteins and receptors, especially in the nervous system.

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent [18], biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous system [19]. Serotonergic signaling appears to play a key role in the generation and modulation of various cognitive and behavioral functions such as sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression and learning [20–23]. Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive compulsive disorder [21,24–26].

Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups [27,28]. Serotonin receptors are members of a superfamily of seven-transmembrane domain receptors [29] that couple to GTP-binding regulatory proteins (G-proteins) [30]. Among the 14 subtypes of serotonin receptors, the G-protein-coupled 5-HT_{1A} receptor subtype is the most extensively studied for a number of reasons. One of the main reasons for this is the availability of a selective ligand (8-OH-DPAT) that allows extensive biochemical, physiological, and pharmacological characterization of the receptor [31,32]. The 5-HT_{1A} receptor was the first among all the serotonin receptors to be cloned and sequenced [33–35]. The human, rat and mouse 5-HT_{1A} receptors have been cloned, and their amino acid sequences deduced [34–36]. The cloning of the 5-HT_{1A} receptor gene has shown that it belongs to the superfamily of G-protein-coupled receptors, with 50% amino acid homology with the β_2-adrenergic receptor in the transmembrane domain. Furthermore, it was the first serotonin receptor for which polyclonal antibodies were obtained [34,37–39] allowing their visualization at the subcellular level in various regions of the brain. The 5-HT_{1A} receptor has recently been shown to have a role in neural development [40] and protection of stressed neuronal cells undergoing degeneration and apoptosis [41]. The 5-HT_{1A} receptor antagonists represent a major class of molecules with potential therapeutic effects in anxiety- or stress-related disorders [42]. Furthermore, 5-HT_{1A} receptor levels have been shown to be increased in schizophrenia [43,44]. The 5-HT_{1A} receptor gene has also been implicated in Tourette's syndrome, a common hereditary motor and vocal tic disorder [45]. We have earlier solubilized and partially purified the 5-HT_{1A} receptor from bovine hippocampus [46,47] and other sources [48] in a functionally active form. In addition, we have shown modulation of ligand binding to the bovine hippocampal 5-HT_{1A} receptor by metal ions, guanine nucleotides, alcohols, local anesthetics, and covalent modifications of the disulfides and sulfhydryl groups [49–56].

In this paper, we have explored the modulatory role of cholesterol on the function (ligand binding) and G-protein coupling of the bovine hippocampal 5-HT_{1A} receptor by cholesterol depletion from native membranes using methyl-β-cyclodextrin (MβCD), which selectively extracts cholesterol from membranes by including it in a central nonpolar cavity. The resulting changes in membrane dynamics were monitored by measurement of fluorescence polarization of fluorescent probes DPH and TMA-DPH. Our results provide evidence, for the first time, that cholesterol is necessary for ligand binding and G-protein coupling of this important neurotransmitter receptor isolated from its native source.

2. Materials and methods

2.1. Materials

BCA, cholesterol, MβCD, DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, TMA-DPH, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). GTP-γ-S was from Roche Applied Science (Mannheim, Germany). BCA reagent kit for protein estimation was from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). [^{3}H]8-OH-DPAT

(sp. activity=123.0 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, UK). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −70 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [49]. Briefly, bovine hippocampal tissue (~100 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900×*g* for 10 min at 4 °C. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 50,000×*g* for 20 min at 4 °C. The pellet obtained was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000×*g* for 20 min at 4 °C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was suspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70 °C. Protein concentration was assayed using the BCA assay kit [57].

2.2.2. Radioligand binding assays

Receptor binding assays were carried out as described earlier with some modifications [49]. Briefly, tubes in duplicate with 0.5 mg protein in a total volume of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4) were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at room temperature (25 °C). Nonspecific binding was determined by performing the assay in the presence of 10 μM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Brandel cell harvester (Gaithersburg, MD) or a Millipore multiport filtration apparatus through Whatman GF/B 2.5-cm-diameter glass microfiber filters (1.0 μm pore size) which were presoaked in 0.15% polyethylenimine for 1 h [58]. Filters were then washed three times with 3 ml of cold water (4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

2.2.3. GTP-γ-S sensitivity assay

In experiments with GTP-γ-S, agonist binding assays were carried out as described above in the presence of varying concentrations of GTP-γ-S as described previously [51]. The concentrations of GTP-γ-S leading to 50% inhibition of specific agonist binding (IC_{50}) were calculated by nonlinear regression fitting of the data to a four-parameter logistic function [59]:

$$B = \frac{a}{1 + (x/I)^s} + b \quad (1)$$

where B is the specific binding of the agonist normalized to control binding (in absence of GTP-γ-S), x denotes concentration of GTP-γ-S, a is the range ($y_{max} - y_{min}$) of the fitted curve on the ordinate (y-axis), I is the IC_{50} concentration, b is the background of the fitted curve (y_{min}) and S is the slope factor.

2.2.4. Saturation binding assays

Saturation binding assays were carried out with varying concentrations (0.1–7.5 nM) of the radiolabeled agonist ([^{3}H]8-OH-DPAT) under conditions as described above. Nonspecific binding was measured in the presence of 10 μM serotonin. Binding data were analyzed as described previously [52]. The concentration of the bound radioligand (RL*) was calculated from the equation:

$$RL^* = 10^{-9} \times B/(V \times SA \times 2220)\ M \quad (2)$$

where B is the bound radioactivity in disintegrations per minute (dpm) (i.e., total dpm−nonspecific dpm), V is the assay volume in milliliters, and SA is the specific activity of the radioligand. The data could be fitted best to a one-site ligand binding equation. The dissociation constant (K_d) and maximum binding sites (B_{max}) were calculated by nonlinear regression analysis of binding data using the HOT module of the LIGAND program (Biosoft, Cambridge, UK) [60,61]. Data obtained after regression analysis were used to plot graphs using the GRAFIT program version 3.09b (Erithacus Software, Surrey, UK). The B_{max} values reported in Table 1 have been normalized with respect to the amount of native membrane protein used.

2.2.5. Cholesterol depletion of native membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously [62,63] with some modifications. Briefly, membranes resuspended at a protein concentration of 2 mg/ml were treated with different

Table 1
Effect of cholesterol depletion using MβCD on specific [^{3}H]8-OH-DPAT binding parameters[a]

Treatment	K_d (nM)	B_{max} (fmol/mg)
Control	0.39±0.08	124±13
40 mM MβCD	0.53±0.06	88±5

[a] Binding parameters were calculated by analyzing saturation binding isotherms with a range (0.1–7.5 nM) of radiolabeled [^{3}H]8-OH-DPAT using the LIGAND program. The data represent the means±S.E. of at least three independent experiments. See Materials and methods for other details.

concentrations of MβCD in buffer C at room temperature (25 °C) with constant shaking for 1 h. Membranes were then spun down at 50,000×*g* for 5 min, washed once with buffer C and resuspended in the same buffer. Cholesterol was estimated using the Amplex Red cholesterol assay kit [64].

2.2.6. Cholesterol replenishment of cholesterol-depleted membranes

Cholesterol-depleted hippocampal membranes were replenished with cholesterol using a water-soluble cholesterol–MβCD complex. The complex was prepared by dissolving the required amounts of cholesterol and MβCD in a ratio of 1:10 (mol/mol), respectively, in buffer C by constant shaking at room temperature (25 °C). Stock solutions (typically 4:40 mM of cholesterol–MβCD) of this complex were freshly prepared before each experiment. Cholesterol replenishment was carried out at a protein concentration of 2 mg/ml by incubating the cholesterol-depleted membranes with various concentrations of the cholesterol–MβCD complex for 1 h in buffer C at room temperature (25 °C) under constant shaking. Membranes were then spun down at 50,000×*g* for 5 min, washed once with buffer C and resuspended in the same buffer. Cholesterol was estimated using the Amplex Red cholesterol assay kit.

2.2.7. Estimation of inorganic phosphate

Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [65] using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.8. Fluorescence polarization measurements

Stock solutions of the fluorescent probes (DPH and TMA-DPH) were prepared in methanol. Membranes containing 50 nmol of total phospholipids were suspended in 1.5 ml of buffer C and used for fluorescence polarization experiments. The amount of probe added was such that the final probe concentration was 1 mol% with respect to the total phospholipid content. This ensures optimal fluorescence intensity with negligible membrane perturbation. Membranes were vortexed for 1 min after addition of the probe and kept in the dark for 1 h before measurements. Background samples were prepared the same way except that the probe was omitted. The final probe concentration was 0.33 μM in all cases and the methanol content was always low (0.03%, v/v). Control experiments showed that at this concentration of methanol, the ligand binding properties of the receptor are not altered.

Steady-state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1-cm path length quartz cuvettes at room temperature (23 °C). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal bandpasses of 1.5 and 20 nm were used. The excitation slit was kept low to avoid any photoisomerization of DPH. In addition, fluorescence was measured with a 30-s interval between successive openings of the excitation shutter (when the sample was in the dark in the fluorimeter) to reverse any photoisomerization of DPH and TMA-DPH [66]. The optical density of the samples measured at 358 nm was 0.15±0.01. The polarization values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [67]. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation [68]:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (5)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples and average values of fluorescence polarization are shown in Figs. 5 and 7.

3. Results

3.1. Concentration-dependent cholesterol depletion from hippocampal membranes by MβCD

The water-soluble compound MβCD has previously been shown to selectively and efficiently extract cholesterol from membranes by including it in a central nonpolar cavity [69–71]. Fig. 1 shows that upon treatment with increasing concentrations of MβCD, the cholesterol content of bovine

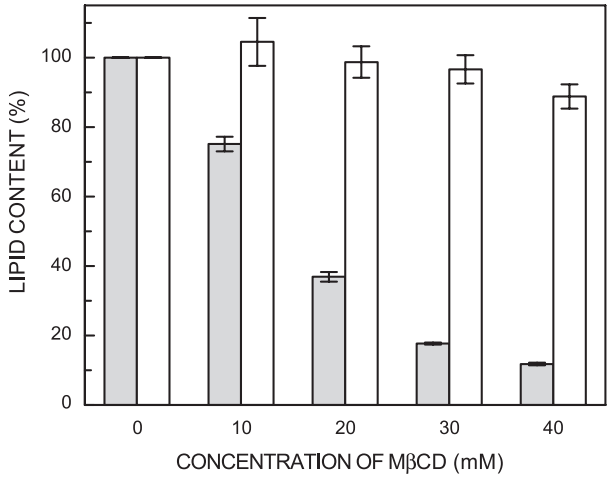

Fig. 1. Effect of increasing concentrations of MβCD on lipid composition of bovine hippocampal membranes. Cholesterol (shaded bars) and phospholipid (white bars) contents were assayed as described in Materials and methods. Values are expressed as percentages of the respective lipid content in native membranes without MβCD treatment. Data for phospholipid content represent the means±S.E. of six independent experiments. Data for cholesterol content represent the means±S.E. of three independent experiments. See Materials and methods for other details.

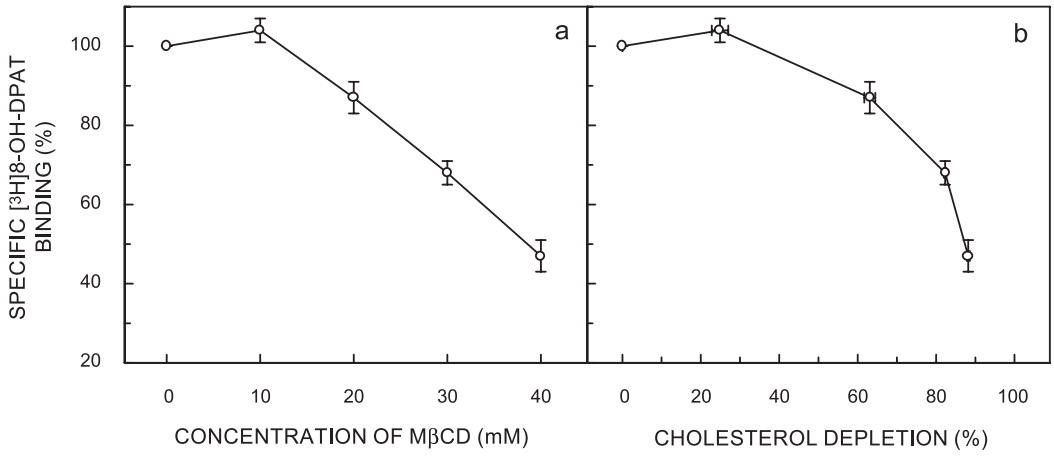

Fig. 2. Effect of (a) increasing concentrations of MβCD and (b) accompanying cholesterol depletion on specific [^{3}H]8-OH-DPAT binding to the 5-HT$_{1A}$ receptor in bovine hippocampal membranes. Values are expressed as percentage of specific binding for native membranes without MβCD treatment. Data for the extent of cholesterol depletion with increasing concentrations of MβCD are taken from Fig. 1. The values are the means±S.E. of at least three independent experiments. See Materials and methods for other details.

hippocampal membranes shows progressive depletion. Thus, when membranes are treated with 10 mM MβCD, the cholesterol content is reduced to 75% of control value. This effect levels off with increasing concentrations of MβCD, with the cholesterol content of hippocampal membranes being reduced to 12% of the original value when the membranes are treated with 40 mM MβCD (Fig. 1). Importantly, the change in phospholipid content under identical conditions is negligible even when 40 mM MβCD CD is used and, in all cases, 90% or more of phospholipids remained in the membrane after MβCD treatment.

3.2. Agonist binding activity is altered upon cholesterol depletion

Fig. 2 shows the reduction in the specific [^{3}H]8-OH-DPAT binding with increasing concentrations of MβCD (Fig. 2a), which results in progressive cholesterol depletion (Fig. 2b). This shows that removal of cholesterol from hippocampal membranes results in loss of specific binding of the agonist [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor. The agonist binding, for example, is reduced to 47% of the original binding observed (in the absence of MβCD treatment) when 40 mM MβCD is used for cholesterol depletion. Although such dependence of ligand binding activity on membrane cholesterol content has previously been reported for other receptors [62,72,73], this result represents the first observation of such dependence for the hippocampal 5-HT$_{1A}$ receptor. As is evident from Fig. 1, the reduction in cholesterol content upon treating hippocampal membranes with increasing concentrations of MβCD is not linear. For a comprehensive understanding of the effect of cholesterol depletion on specific binding of the agonist [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor, we plotted the specific agonist binding data as a function of the extent of cholesterol depletion with increasing MβCD concentration (see Fig. 2b). It is interesting to note that although the decrease in specific agonist binding appears to be linear (for MβCD concentrations>10 mM) when plotted against MβCD concentration (Fig. 2a), it is not so when viewed as a function of cholesterol depletion (Fig. 2b). As stated above, the reason for this difference is that the reduction in cholesterol content is not linear with the concentrations of MβCD used.

Saturation binding analysis, shown in Fig. 3 and Table 1, with the agonist [^{3}H]8-OH-DPAT binding carried out with native and cholesterol-depleted hippocampal membranes reveals that the reduction in ligand binding can primarily be attributed to a reduction in the number of total binding sites with a marginal reduction in the affinity of ligand

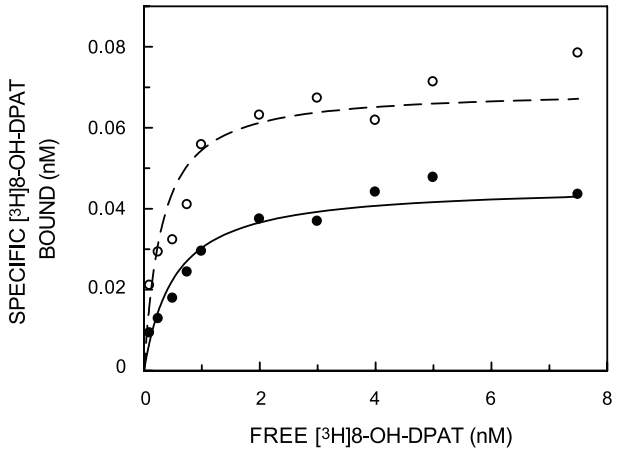

Fig. 3. Saturation binding analysis of specific [^{3}H]8-OH-DPAT binding to the 5-HT$_{1A}$ receptor in bovine hippocampal membranes treated with MβCD. Representative plots are shown for specific [^{3}H]8-OH-DPAT binding with increasing concentrations of free [^{3}H]8-OH-DPAT to control (- - -○- - -) and 40 mM (—●—) MβCD treated membranes. The curves are nonlinear regression fits to the experimental data using the LIGAND program. See Materials and methods and Table 1 for other details.

binding. There is ~26% reduction in the maximum number of binding sites (B_{max}) when 40 mM MβCD is used for cholesterol depletion.

3.3. Guanine nucleotide sensitivity of specific agonist binding in cholesterol-depleted membranes

Most of the seven-transmembrane domain receptors are coupled to G-proteins [30], and guanine nucleotides are known to regulate agonist binding. The 5-HT_{1A} receptor is negatively coupled to the adenylate cyclase system through pertussis toxin-sensitive G-proteins (G_i/G_o) [74]. We previously showed that the specific binding of the agonist [^{3}H]8-OH-DPAT to bovine hippocampal 5-HT_{1A} receptors is sensitive to guanine nucleotides and is inhibited with increasing concentrations of GTP-γ-S, a non-hydrolyzable GTP analogue [51]. Fig. 4 shows the inhibition of specific [^{3}H]8-OH-DPAT binding to the 5-HT_{1A} receptor in the presence of GTP-γ-S in a characteristic concentration-dependent manner in control and cholesterol-depleted membranes (using 40 mM MβCD). We have earlier shown that GTP-γ-S induces transition of the receptor from a high affinity to a low affinity state [51]. Table 2 shows that the half maximal inhibition concentration (IC_{50}) value for inhibition of specific [^{3}H]8-OH-DPAT binding by GTP-γ-S is 83 nM for native membranes, similar to what we reported earlier [51]. The inhibition curve for the cholesterol-depleted membranes, however, exhibits a significant ~2.5-fold shift toward higher concentration of GTP-γ-S with an IC_{50} value of 206 nM. This implies that the agonist binding to the 5-HT_{1A} receptor in cholesterol-depleted membranes is less sensitive to GTP-γ-S than otherwise indicating that G-protein coupling of the receptor is drastically reduced. This points to a possible perturbation of receptor–G-protein interaction upon cholesterol depletion. Interestingly, the cholesterol content in membranes has previously been reported to modulate receptor-independent function of G-proteins [75,76]. It is therefore possible that a similar effect could operate here and thus affect the interaction between G-proteins and 5-HT_{1A} receptors.

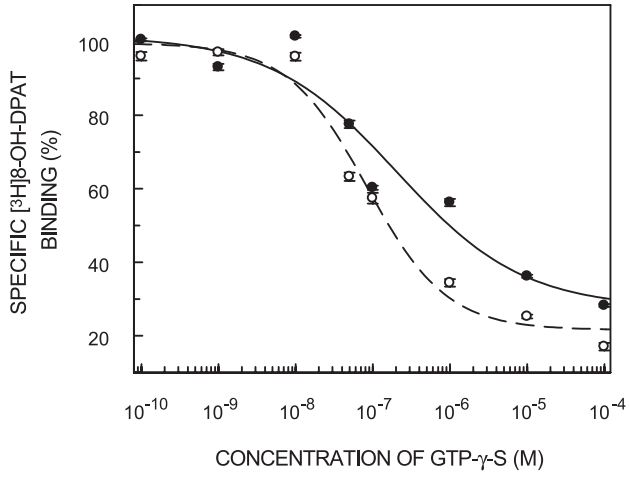

Fig. 4. Sensitivity of specific [^{3}H]8-OH-DPAT binding to GTP-γ-S in native and cholesterol-depleted membranes. Values are expressed as percentage of specific binding for membranes in the absence of GTP-γ-S. Plots show the effect of increasing concentrations of GTP-γ-S on the percentage specific [^{3}H]8-OH-DPAT binding to control (- - -○- - -) and 40 mM MβCD treated (—●—) membranes. The curves are nonlinear regression fits to the experimental data using the four-parameter logistic function [59]. The values represent the means±S.E. of four independent experiments. See Materials and methods and Table 2 for other details.

Table 2
Effect of cholesterol depletion using MβCD on specific [^{3}H]8-OH-DPAT binding sensitivity to GTP-γ-S[a]

Treatment	IC_{50} (nM)
Control	83±4
40 mM MβCD	206±17

[a] Sensitivity of specific [^{3}H]8-OH-DPAT binding to GTP-γ-S in the assay was measured by calculating the IC_{50} for inhibition of [^{3}H]8-OH-DPAT binding in the presence of a range of concentrations of GTP-γ-S. Inhibition curves were analyzed using the four-parameter logistic function. The data represent the means ± S.E. of four independent experiments. See Materials and methods for other details.

3.4. Changes in hippocampal membrane dynamics due to cholesterol depletion

A large portion of any given transmembrane protein, the 5-HT_{1A} receptor in this case, remains in contact with the membrane lipid environment. This raises the obvious possibility that the overall membrane order and dynamics could be an important modulator of receptor structure and function [15,16]. In order to understand the changes in membrane order induced by cholesterol depletion, we monitored the steady-state fluorescence polarization of two membrane probes, DPH and TMA-DPH. DPH and its derivatives represent popular membrane probes for monitoring organization and dynamics in membranes [77]. Fluorescence polarization is correlated to the rotational diffusion [68] of membrane-embedded probes, which is sensitive to the packing of fatty acyl chains and cholesterol. Since the membrane is considered to be a two-dimensional anisotropic fluid, any possible change in membrane order may not be uniform and restricted to a unique location in the membrane. It is therefore important to monitor the change in membrane order at multiple regions in the membrane to obtain a comprehensive understanding of any change in membrane (lipid) dynamics induced by cholesterol depletion. One of us has previously shown that stress such as heat shock can induce anisotropic changes in membrane fluidity, i.e., the change in membrane fluidity was different when monitored at different regions in adult rat liver cell plasma membranes [78]. It is worth mentioning here that such depth-dependent modulation of membrane order induced by alcohols [79] and local anesthetics [80] has also been reported. It is for this reason that we employed two fluorescent probes, DPH and TMA-DPH, to assess any change in membrane order and dynamics induced by cholesterol depletion. These

probes differ in their orientation and location in the membrane. DPH, which is a rod-like molecule, partitions into the interior of the bilayer. However, its precise orientation in the membrane interior is not known. TMA-DPH is a derivative of DPH with a cationic moiety attached to the para position of one of the phenyl rings [81]. While DPH is known to partition into the hydrophobic core of the membrane, the amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid–water interface [82]. Its DPH moiety is localized at ~11 Å from the center of the bilayer and reports the interfacial region of the membrane [83]. In contrast to this, the average location of DPH has been shown to be ~8 Å from the center of the bilayer [83].

Fig. 5 shows the change in fluorescence polarization of DPH and TMA-DPH incorporated in hippocampal membranes treated with increasing concentrations of MβCD (Fig. 5a), which results in progressive cholesterol depletion (Fig. 5b). The fluorescence polarization of DPH in hippocampal membranes (without any MβCD treatment) is found to be 0.334. This is very close to the value found in the detergent-resistant fraction of liposomes composed of bovine phosphatidylcholine, sphingomyelin, cerebroside, along with cholesterol [84]. The corresponding value of fluorescence polarization for TMA-DPH is 0.363. The higher polarization of TMA-DPH compared to DPH is indicative of the shallower location of TMA-DPH in the membrane, as has been observed earlier for anthroyloxy-labeled fluorescent membrane probes [85]. The fluorescence polarization of both the probes shows a continuous decrease with increasing concentrations of MβCD. Interestingly, the fluorescence polarization of both the probes shows an almost linear dependence when plotted against increasing extents of cholesterol depletion (see Fig. 5b). More importantly, the percentage change in fluorescence polarization upon cholesterol depletion is higher for DPH (16%) compared to TMA-DPH (9%) when 40 mM MβCD is used for cholesterol depletion. The polarization values remained identical even after dilution of membrane samples indicating the absence of any scattering artifact [67]. Thus, the asymmetric reduction in membrane order in different regions upon cholesterol depletion is accompanied with a reduction in the agonist binding activity of the 5-HT_{1A} receptor.

3.5. Cholesterol replenishment restores agonist binding activity of the 5-HT_{1A} receptor and membrane order in hippocampal membranes

In order to check the reversibility of the changes induced by cholesterol depletion, cholesterol replenishment of depleted membranes (achieved using 40 mM MβCD) was carried out by incubating the depleted membranes with cholesterol–MβCD complex as described in Materials and methods. As shown in Fig. 6a, this procedure is able to replenish bulk of the cholesterol to the depleted membranes as shown by the cholesterol content of the replenished membranes. Interestingly, the efficiency of the cholesterol replenishment process (and also effect on ligand binding activity, see Fig. 6b) appears to be influenced by the concentration of the cholesterol–MβCD complex used even while maintaining the same ratio (1:10, mol/mol) of cholesterol to MβCD. The optimum efficiency of loading cholesterol back to depleted membranes is found with 1 mM cholesterol complexed with 10 mM MβCD. Use of higher concentrations of the complex with the same molar ratio failed to increase the cholesterol replenishment efficiency (not shown). Thus, ~72% of the original cholesterol content could be loaded back when 1 mM cholesterol complexed with 10 mM MβCD is used. The corresponding recovery in ligand binding activity is shown in Fig. 6b with

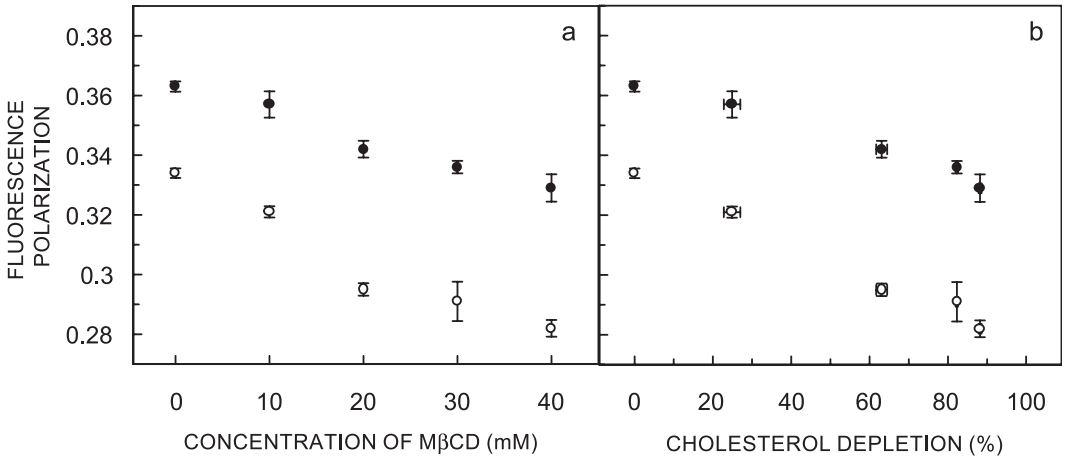

Fig. 5. Effect of (a) increasing concentrations of MβCD and (b) accompanying cholesterol depletion in bovine hippocampal membranes on fluorescence polarization of membrane probes DPH (○) and TMA-DPH (●). Fluorescence polarization experiments were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23 °C). Data for the extent of cholesterol depletion with increasing concentration of MβCD are taken from Fig. 1. The values represent the means±S.E. of three independent experiments. See Materials and methods for other details.

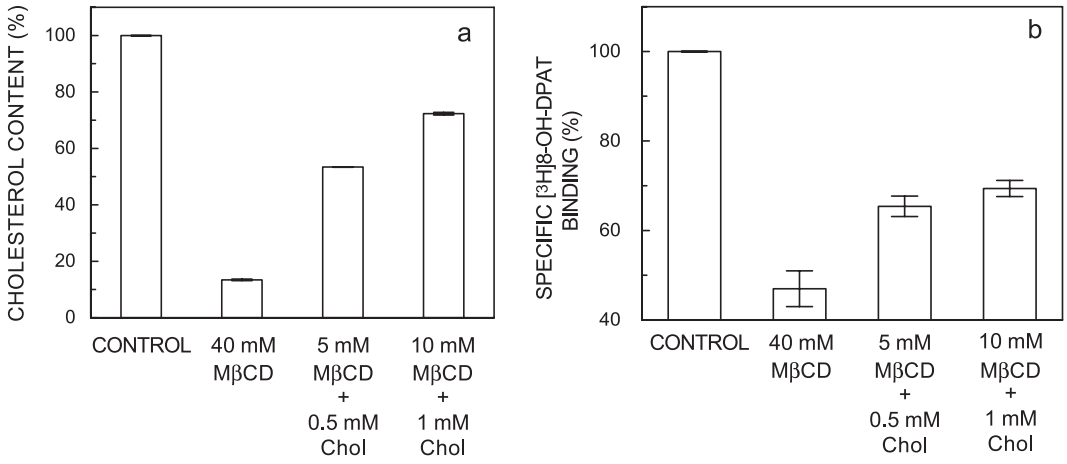

Fig. 6. Cholesterol replenishment into bovine hippocampal membranes treated with MβCD and its correlation with (a) cholesterol content and (b) specific [^{3}H]8-OH-DPAT binding activity of the hippocampal 5-HT$_{1A}$ receptor. Cholesterol depletion was carried out using 40 mM MβCD as described in Materials and methods. Panel (a) shows the final cholesterol content when cholesterol-depleted membranes were treated with cholesterol–MβCD complex at a final concentration of 0.5:5 mM and 1:10 mM (mol/mol of cholesterol–MβCD). Values are expressed as percentage of cholesterol content in native membrane without any treatment. Panel (b) shows the accompanying effect of cholesterol replenishment on the specific [^{3}H]8-OH-DPAT binding to membranes. Values are expressed as percentage of specific radiolabeled agonist binding in native membranes without any treatment. The data represent means±S.E. of three independent experiments. See Materials and methods for other details.

the agonist binding recovering to 69% of that seen in native membranes.

Fig. 7 shows the increase in fluorescence polarization of DPH and TMA-DPH following cholesterol replenishment with increasing concentrations of cholesterol–MβCD complex (Fig. 7a) which results in progressive increase in cholesterol content (Fig. 7b). This increase in fluorescence polarization again displays all characteristic features as were observed with data shown in Fig. 5, i.e., the linearity with increasing cholesterol content (see Fig. 7b) and DPH fluorescence polarization being more sensitive to membrane cholesterol content compared to TMA-DPH. Importantly, the polarization values obtained with membranes replenished with 1 mM cholesterol complexed with 10 mM MβCD are 95–98% of the polarization values obtained in control membranes for DPH and TMA-DPH. The recovery of fluorescence polarization values upon cholesterol replenishment to nearly those found with control membranes further ensures, over and above the cholesterol content analysis, that membrane physical properties (such as membrane order) are restored to a large extent. As stated earlier, cholesterol is often found non-randomly

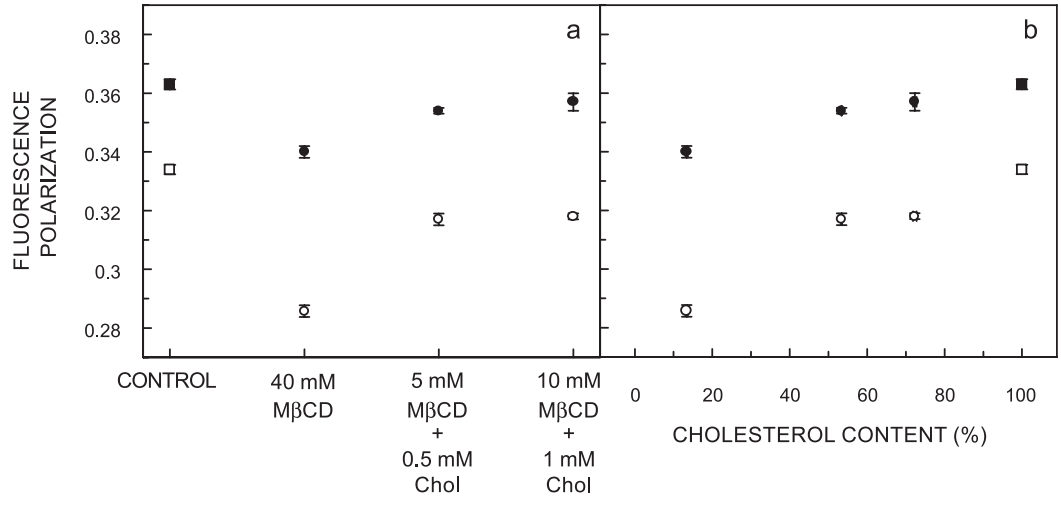

Fig. 7. Effect of cholesterol replenishment on the fluorescence polarization of membrane probes DPH (○) and TMA-DPH (●). Fluorescence polarization experiments were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23 °C). Panel (a) shows the effect of increasing concentrations of MβCD–cholesterol complex on fluorescence polarization of DPH and TMA-DPH in membranes. Panel (b) shows the effect of increasing extent of cholesterol replenishment (expressed as percentage of cholesterol content in native membranes without MβCD treatment) on fluorescence polarization in membranes. Data for the extent of cholesterol replenishment are taken from Fig. 6a. Data for fluorescence polarization of DPH (□) and TMA-DPH (■) for control membranes (considered as 100% cholesterol content) are provided for comparison and are taken from Fig. 5. The values are the means±S.E. of three independent experiments. See Materials and methods for other details.

distributed in domains or pools in membranes. Cholesterol depletion by MβCD is reported to be asymmetric with respect to the amount extracted from these pools [86,87]. Replenishment of cholesterol therefore may not bring the membrane back to its original state as far as distribution of cholesterol in different pools is concerned. Thus, although the overall level of cholesterol after replenishment may be nearly equal to the original level of cholesterol, it does not necessarily guarantee recovery of all functional attributes (ligand binding) since it may require a much more stringent criterion such as comparable distribution of cholesterol among various pools and proper conformation of membrane receptors which are influenced by neighboring lipids molecules.

4. Discussion

The 5-HT_{1A} receptor belongs to the family of G-protein-coupled receptors. These receptors constitute a superfamily of proteins whose function is to transmit information across a cell membrane from the extracellular environment to the interior of the cell, thus providing a mechanism of communication between the exterior and the interior of the cell [29]. G-protein-coupled receptors represent the single largest family of cell surface receptors involved in signal transduction. This receptor superfamily includes over 2000 receptors which respond to a variety of molecules such as neurotransmitters, hormones, taste and odorant molecules, and even photons, thus mediating a multitude of functions. According to current estimates, about 1% of the mammalian genome codes for this type of receptors [88]. These transmembrane receptors act as key players in diverse physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation and growth, and inflammatory and immune responses. G-protein-coupled receptors therefore represent major targets for the development of novel drug candidates in all clinical areas [89]. It is estimated that 30% of clinically prescribed drugs function as either agonists or antagonists of G-protein-coupled receptors, which points to their immense therapeutic potential [90].

The membrane organization of G-protein-coupled receptors assumes significance in the light of their role in health and disease. Recent evidence has indicated that the receptors and G-proteins are less dynamic than previously appreciated. A spatiotemporally organized system rather than a freely diffusible system has been suggested to be responsible for rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [88,91]. Moreover, the specificity of signaling in intact cells appears to be significantly greater than that observed in reconstituted systems. Based on this and other types of evidence, it has been proposed that the G-protein-coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains [88,91–93]. It has been shown that some of these domains are enriched in cholesterol. For example, it has recently been reported that 5-HT_{2A} receptors in smooth muscle cells are localized in membrane microdomains (caveolae) and serotonergic signaling in these cells depends on cholesterol content [94]. Localization of G-protein-coupled receptors into domains has given rise to new challenges and complexities in receptor signaling since signaling has to be understood in context of the three-dimensional organization of various signaling components, which include receptors and G-proteins [92].

We show here that removal of cholesterol from bovine hippocampal membranes using varying concentrations of MβCD results in a concentration-dependent decrease in specific binding of the agonist 8-OH-DPAT to 5-HT_{1A} receptors. This is accompanied by alterations in binding affinity and sites obtained from analysis of binding data. Importantly, cholesterol depletion affected G-protein-coupling of the receptor as monitored by the GTP-γ-S assay. The concomitant changes in membrane order were reported by changes in fluorescence polarization of membrane probes, such as DPH and TMA-DPH, which are incorporated at different locations (depths) in the membrane. Replenishment of membranes with cholesterol led to recovery of ligand binding activity as well as membrane order to a considerable extent. The clinical significance of membrane cholesterol levels resulting in receptor dysfunction has been aptly exemplified in the case of cholecystokinin (CCK) receptors [95,96]. Thus, agonist binding is reduced and G-protein coupling affected for CCK receptors isolated from muscle tissues in human gallbladders with cholesterol stones. These effects are reversed upon treatment with cholesterol-free liposomes.

Since cholesterol is an essential component in eukaryotic cell membranes [97], one would be tempted to consider that the function of several G-protein-coupled receptors would be affected in a similar fashion upon cholesterol depletion. A careful study of the existing literature reveals this to be not true and the real effect observed depends on the specific receptor type and the membrane environment the receptor is present in. For instance, the 5-HT_{1A} receptor belongs to the subset of G-protein-coupled receptors that bind to biogenic amines. This family also includes the β_2-adrenoceptor and rhodopsin. The 5-HT_{1A} receptor shows ~50% homology in the transmembrane region to the β_2-adrenoceptor whereas most structural simulations of the G-protein-coupled receptors, including the 5-HT_{1A} receptor [98], make use of the rhodopsin crystal structure as a template. This highlights the similarities between these proteins. Yet, work with purified and reconstituted receptor preparations has revealed that while the β_2-adrenoceptor requires membrane cholesterol to promote efficient ligand binding, G-protein coupling and downstream signal transduction [99,100], analogous functions of rhodopsin, are inhibited in the presence of membrane cholesterol [101–103]. As the above example shows, even among the members of the G-protein-coupled receptor

family, that the 5-HT_{1A} receptor belongs to, previous literature already shows a lack of consensus in the manner in which cholesterol modulates receptor function.

Our results show that manipulations of membrane cholesterol content can induce significant changes in the activity and G-protein coupling of the 5-HT_{1A} receptor. Whether such manipulations in membrane cholesterol content could be induced in vivo represents a challenging question. The turnover of brain cholesterol is very low, with a half-life of ~6 months [10]. As a result of this, the cerebrospinal fluid levels of cholesterol are ~40–50-fold lower than the plasma cholesterol [104]. Due to the presence of the blood brain barrier, alterations in serum levels of cholesterol are believed not to affect the total cholesterol level in the central nervous system. However, under such conditions, the neuronal plasma membrane fractions have not been studied adequately. In addition, regions in the central nervous system (such as the hypothalamic area) that are somewhat weakly protected by the blood brain barrier may be sensitive to plasma cholesterol fluctuations. Interestingly, a recent report has suggested that chronic in vivo administration of cholesterol-lowering drugs like statins specifically reduces brain cholesterol levels, leaving the serum cholesterol levels unaffected [105]. More importantly, it has been shown that treating humans with such cholesterol-lowering drugs significantly decreases the incidence of Alzheimer's disease [106,107]. It is possible that the more severe deficiency in cholesterol levels in the brain occurs on account of the lower turnover of cholesterol in this tissue. Interestingly, low serum cholesterol concentration has been correlated with an increase in the prevalence of suicide in humans [108] and is partly attributed to an altered serotonin metabolism [109]. Furthermore, a recent report describes the attenuation of 5-HT_{1A} receptor antagonist binding and signaling in brains of suicide victims [110]. In light of our present results on the requirement of membrane cholesterol to influence the 5-HT_{1A} receptor function, its role in the etiology of psychological disorders that are correlated with an altered cholesterol metabolism clearly needs to be investigated further.

In summary, our results show that cholesterol has an important role in regulation of ligand binding activity and G-protein coupling of the 5-HT_{1A} receptor. These results are relevant in understanding the role of the membrane lipid environment on the activity and signal transduction of other G-protein-coupled transmembrane receptors.

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References

[1] L. Liscum, K.W. Underwood, Intracellular cholesterol transport and compartmentation, J. Biol. Chem. 270 (1995) 15443–15446.
[2] K. Simons, E. Ikonen, How cells handle cholesterol, Science 290 (2000) 1721–1725.
[3] F. Schroeder, J.K. Woodford, J. Kavecansky, W.G. Wood, C. Joiner, Cholesterol domains in biological membranes, Mol. Membr. Biol. 12 (1995) 113–119.
[4] S. Mukherjee, A. Chattopadhyay, Membrane organization at low cholesterol concentrations: a study using 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled cholesterol, Biochemistry 35 (1996) 1311–1322.
[5] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[6] X. Xu, E. London, The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation, Biochemistry 39 (2000) 843–849.
[7] R. Rukmini, S.S. Rawat, S.C. Biswas, A. Chattopadhyay, Cholesterol organization in membranes at low concentrations: effects of curvature stress and membrane thickness, Biophys. J. 81 (2001) 2122–2134.
[8] M.S. Bretscher, S. Munro, Cholesterol and the Golgi apparatus, Science 261 (1993) 1280–1281.
[9] Y. Lange, Disposition of intracellular cholesterol in human fibroblasts, J. Lipid Res. 32 (1991) 329–339.
[10] J.M. Dietschy, S.D. Turley, Cholesterol metabolism in the brain, Curr. Opin. Lipidol. 12 (2001) 105–112.
[11] W.G. Wood, F. Schroeder, N.A. Avdulov, S.V. Chochina, U. Igbavboa, Recent advances in brain cholesterol dynamics: transport, domains, and Alzheimer's disease, Lipids 34 (1999) 225–234.
[12] J.J. Kabara, A critical review of brain cholesterol metabolism, Prog. Brain Res. 40 (1973) 363–382.
[13] S.D. Turley, D.K. Bruns, J.M. Dietschy, Preferential utilization of newly synthesized cholesterol for brain growth in neonatal lambs, Am. J. Physiol. 274 (1998) E1099–E1105.
[14] H.R. Waterham, R.J.A. Wanders, Biochemical and genetic aspects of 7-dehydrocholesterol reductase and Smith–Lemli–Opitz syndrome, Biochim. Biophys. Acta 1529 (2000) 340–356.
[15] A.G. Lee, Lipid–protein interactions in biological membranes: a structural perspective, Biochim. Biophys. Acta 1612 (2003) 1–40.
[16] K. Burger, G. Gimpl, F. Fahrenholz, Regulation of receptor function by cholesterol, Cell. Mol. Life Sci. 57 (2000) 1577–1592.
[17] R.M. Epand, S. Maekawa, C.M. Yip, R.F. Epand, Protein-induced formation of cholesterol-rich domains, Biochemistry 40 (2001) 10514–10521.
[18] A. Chattopadhyay, R. Rukmini, S. Mukherjee, Photophysics of a neurotransmitter: ionization and spectroscopic properties of serotonin, Biophys. J. 71 (1996) 1952–1960.
[19] B.L. Jacobs, E.C. Azmitia, Structure and function of the brain serotonin system, Physiol. Rev. 72 (1992) 165–229.
[20] F. Artigas, L. Romero, C. de Montigny, P. Blier, Acceleration of the effect of selected antidepressant drugs in major depression by 5-HT_{1A} antagonists, Trends Neurosci. 19 (1996) 378–383.
[21] S. Ramboz, R. Oosting, D.A. Amara, H.F. Kung, P. Blier, M. Mendelsohn, J.J. Mann, D. Brunner, R. Hen, Serotonin receptor 1A knockout: an animal model of anxiety-related disorder, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14476–14481.

[22] B.A. Rocha, K. Scearce-Levie, J.J. Lucas, N. Hiroi, N. Castanon, J. Crabbe, E.J. Nestler, R. Hen, Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor, Nature 393 (1998) 175–178.
[23] A. Meneses, 5-HT system and cognition, Neurosci. Biobehav. Rev. 23 (1999) 1111–1125.
[24] L.K. Heisler, H.-M. Chu, T.J. Brennan, J.A. Danao, P. Bajwa, L.H. Parsons, L.H. Tecott, Elevated anxiety and antidepressant-like responses in serotonin 5-HT_{1A} receptor mutant mice, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 15049–15054.
[25] C.L. Parks, P.S. Robinson, E. Sibille, T. Shenk, M. Tóth, Increased anxiety of mice lacking the serotonin$_{1A}$ receptor, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 10734–10739.
[26] Z. Sarnyai, E.L. Sibille, C. Pavlides, R.J. Fenster, B.S. McEwen, M. Tóth, Impaired hippocampal-dependent learning and functional abnormalities in the hippocampus in mice lacking serotonin$_{1A}$ receptors, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 14731–14736.
[27] N.M. Barnes, T. Sharp, A review of central 5-HT receptors and their function, Neuropharmacology 38 (1999) 1083–1152.
[28] D. Hoyer, J.P. Hannon, G.R. Martin, Molecular, pharmacological and functional diversity of 5-HT receptors, Pharmacol. Biochem. Behav. 71 (2002) 533–554.
[29] K.L. Pierce, R.T. Premont, R.J. Lefkowitz, Seven-transmembrane receptors, Nat. Rev., Mol. Cell Biol. 3 (2002) 639–650.
[30] D.E. Clapham, The G-protein nanomachine, Nature 379 (1996) 297–299.
[31] L.E. Arvidsson, U. Hacksell, J.L. Nilsson, S. Hjorth, A. Carlsson, P. Lindberg, D. Sanchez, H. Wikstrom, 8-Hydroxy-2-(di-*n*-propylamino)tetralin, a new centrally acting 5-hydroxytryptamine receptor agonist, J. Med. Chem. 24 (1981) 921–923.
[32] H. Gozlan, S. el Mestikawy, L. Pichat, J. Glowinski, M. Hamon, Identification of presynaptic serotonin autoreceptors using a new ligand: ^{3}H-PAT, Nature 305 (1983) 140–142.
[33] B.K. Kobilka, T. Frielle, S. Collins, T. Yang-Feng, T.S. Kobilka, U. Francke, R.J. Lefkowitz, M.G. Caron, An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins, Nature 329 (1987) 75–79.
[34] A. Fargin, J.R. Raymond, M.J. Lohse, B.K. Kobilka, M.G. Caron, R.J. Lefkowitz, The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT_{1A} receptor, Nature 335 (1988) 358–360.
[35] P.R. Albert, Q.-Y. Zhou, H.H.M. Van Tol, J.R. Bunzow, O. Civelli, Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine$_{1A}$ receptor gene, J. Biol. Chem. 265 (1990) 5825–5832.
[36] A. Charest, B.H. Wainer, P.R. Albert, Cloning and differentiation-induced expression of a murine serotonin1A receptor in a septal cell line, J. Neurosci. 13 (1993) 5164–5171.
[37] S. el Mestikawy, M. Riad, A.M. Laporte, D. Verge, G. Daval, H. Gozlan, M. Hamon, Production of specific anti-rat 5-HT_{1A} receptor antibodies in rabbits injected with a synthetic peptide, Neurosci. Lett. 118 (1990) 189–192.
[38] E.C. Azmitia, P.J. Gannon, N.M. Kheck, P.M. Whitaker-Azmitia, Cellular localization of the 5-HT_{1A} receptor in primate brain neurons and glial cells, Neuropsychopharmacology 14 (1996) 35–46.
[39] H.K. Kia, M.-C. Miquel, M.-J. Brisorgueil, G. Daval, M. Riad, S. el Mestikawy, M. Hamon, D. Vergé, Immunocytochemical localization of serotonin$_{1A}$ receptors in the rat central nervous system, J. Comp. Neurol. 365 (1996) 289–305.
[40] E. del Olmo, J.F. López-Giménez, M.T. Vilaró, G. Mengod, J.M. Palacios, A. Pazos, Early localization of mRNA coding for 5-HT_{1A} receptors in human brain during development, Mol. Brain Res. 60 (1998) 123–126.
[41] J.K. Singh, B.A. Chromy, M.J. Boyers, G. Dawson, P. Banerjee, Induction of the serotonin$_{1A}$ receptor in neuronal cells during prolonged stress and degeneration, J. Neurochem. 66 (1996) 2361–2372.
[42] G. Griebel, 5-HT_{1A} receptor blockers as potential drug candidates for the treatment of anxiety disorders, Drug News Perspect. 12 (1999) 484–490.
[43] T. Sumiyoshi, C.A. Stockmeier, J.C. Overholser, G.E. Dilley, H.Y. Meltzer, Serotonin$_{1A}$ receptors are increased in postmortem prefrontal cortex in schizophrenia, Brain Res. 708 (1996) 209–214.
[44] O. Fajardo, J. Galeno, M. Urbina, I. Carreira, L. Lima, Serotonin, serotonin 5-HT_{1A} receptors and dopamine in blood peripheral lymphocytes of major depression patients, Int. Immunopharmacol. 3 (2003) 1345–1352.
[45] S. Lam, Y. Shen, T. Nguyen, T.L. Messier, M. Brann, D. Comings, S.R. George, B.F. O'Dowd, A serotonin receptor gene (5HT1A) variant found in a Tourette's syndrome patient, Biochem. Biophys. Res. Commun. 219 (1996) 853–858.
[46] A. Chattopadhyay, K.G. Harikumar, Dependence of critical micelle concentration of a zwitterionic detergent on ionic strength: implications in receptor solubilization, FEBS Lett. 391 (1996) 199–202.
[47] A. Chattopadhyay, K.G. Harikumar, S. Kalipatnapu, Solubilization of high affinity G-protein-coupled serotonin$_{1A}$ receptors from bovine hippocampus using pre-micellar CHAPS at low concentration, Mol. Membr. Biol. 19 (2002) 211–220.
[48] A. Chattopadhyay, Md. Jafurulla, S. Kalipatnapu, Solubilization of serotonin$_{1A}$ receptors heterologously expressed in Chinese hamster ovary cells, Cell. Mol. Neurobiol. 24 (2004) 293–300.
[49] K.G. Harikumar, A. Chattopadhyay, Metal ion and guanine nucleotide modulations of agonist interaction in G-protein-coupled serotonin$_{1A}$ receptors from bovine hippocampus, Cell. Mol. Neurobiol. 18 (1998) 535–553.
[50] K.G. Harikumar, A. Chattopadhyay, Modulation of agonist and antagonist interactions in serotonin 1A receptors by alcohols, FEBS Lett. 438 (1998) 96–100.
[51] K.G. Harikumar, A. Chattopadhyay, Differential discrimination of G-protein coupling of serotonin$_{1A}$ receptors from bovine hippocampus by an agonist and an antagonist, FEBS Lett. 457 (1999) 389–392.
[52] K.G. Harikumar, P.T. John, A. Chattopadhyay, Role of disulfides and sulfhydryl groups in agonist and antagonist binding in serotonin$_{1A}$ receptors from bovine hippocampus, Cell. Mol. Neurobiol. 20 (2000) 665–681.
[53] K.G. Harikumar, A. Chattopadhyay, Effect of alcohols on G-protein coupling of serotonin$_{1A}$ receptors from bovine hippocampus, Brain Res. Bull. 52 (2000) 597–601.
[54] K.G. Harikumar, A. Chattopadhyay, Modulation of antagonist binding to serotonin$_{1A}$ receptors from bovine hippocampus by metal ions, Cell. Mol. Neurobiol. 21 (2001) 453–464.
[55] S. Kalipatnapu, A. Chattopadhyay, Interaction of serotonin$_{1A}$ receptors from bovine hippocampus with tertiary amine local anesthetics, Cell. Mol. Neurobiol. 24 (2004) 403–422.
[56] V. Javadekar-Subhedar, A. Chattopadhyay, Temperature-dependent interaction of the bovine hippocampal serotonin$_{1A}$ receptor with G-proteins, Mol. Membr. Biol. 21 (2004) 119–123.
[57] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[58] R.F. Bruns, K. Lawson-Wendling, T.A. Pugsley, A rapid filtration assay for soluble receptors using polyethylenimine-treated filters, Anal. Biochem. 132 (1983) 74–81.
[59] T. Higashijima, K.M. Ferguson, P.C. Sternweis, M.D. Smigel, A.G. Gilman, Effects of Mg^{2+} and the beta gamma-subunit complex on the interactions of guanine nucleotides with G proteins, J. Biol. Chem. 262 (1987) 762–766.
[60] P.J. Munson, D. Rodbard, Ligand: a versatile computerized approach for characterization of ligand-binding systems, Anal. Biochem. 107 (1980) 220–239.
[61] G.A. McPherson, Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC, J. Pharmacol. Methods 14 (1985) 213–228.

[62] U. Klein, G. Gimpl, F. Fahrenholz, Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor, Biochemistry 34 (1995) 13784–13793.
[63] T.J. Pucadyil, A. Chattopadhyay, Exploring detergent insolubility in bovine hippocampal membranes: a critical assessment of the requirement for cholesterol, Biochim. Biophys. Acta 1661 (2004) 9–17.
[64] D.M. Amundson, M. Zhou, Fluorometric method for the enzymatic determination of cholesterol, J. Biochem. Biophys. Methods 38 (1999) 43–52.
[65] C.W.F. McClare, An accurate and convenient organic phosphorus assay, Anal. Biochem. 39 (1971) 527–530.
[66] A. Chattopadhyay, E. London, Fluorimetric determination of critical micelle concentration avoiding interference from detergent charge, Anal. Biochem. 139 (1984) 408–412.
[67] B.R. Lentz, B.M. Moore, D.A. Barrow, Light-scattering effects in the measurement of membrane microviscosity with diphenylhexatriene, Biophys. J. 25 (1979) 489–494.
[68] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluwer–Plenum Press, New York, 1999.
[69] A.E. Christian, M.P. Haynes, M.C. Phillips, G.H. Rothblat, Use of cyclodextrins for manipulating cellular cholesterol content, J. Lipid Res. 38 (1997) 2264–2272.
[70] E.P.C. Kilsdonk, P.G. Yancey, G.W. Stoudt, F.W. Bangerter, W.J. Johnson, M.C. Phillips, G.H. Rothblat, Cellular cholesterol efflux mediated by cyclodextrins, J. Biol. Chem. 270 (1995) 17250–17256.
[71] S. Hartel, H.A. Diehl, F.S. Ojeda, Methyl-beta-cyclodextrins and liposomes as water-soluble carriers for cholesterol incorporation into membranes and its evaluation by a microenzymatic fluorescence assay and membrane fluidity-sensitive dyes, Anal. Biochem. 258 (1998) 277–284.
[72] L. Pang, M. Graziano, S. Wang, Membrane cholesterol modulates galanin–GalR2 interaction, Biochemistry 38 (1999) 12003–12011.
[73] D.H. Nguyen, D. Taub, CXCR4 function requires membrane cholesterol: implications for HIV infection, J. Immunol. 168 (2002) 4121–4126.
[74] M.B. Emerit, S. el Mestikawy, H. Gozlan, B. Rouot, M. Hamon, Physical evidence of the coupling of solubilized 5-HT_{1A} binding sites with G regulatory proteins, Biochem. Pharmacol. 39 (1990) 7–18.
[75] S. Gudi, J.P. Nolan, J.A. Frangos, Modulation of GTPase activity of G proteins by fluid shear stress and phospholipid composition, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2515–2519.
[76] S. Ropero, A. Chiloeches, A. Montes, M.J. Toro-Nozal, Cholesterol cell content modulates GTPase activity of G proteins in GH4C1 cell membranes, Cell. Signal. 15 (2003) 131–138.
[77] B.R. Lentz, Membrane fluidity as detected by diphenylhexatriene probes, Chem. Phys. Lipids 50 (1989) 171–190.
[78] C.J. Revathi, A. Chattopadhyay, U.K. Srinivas, Change in membrane organization induced by heat shock, Biochem. Mol. Biol. Int. 32 (1994) 941–950.
[79] S. Kitagawa, H. Hirata, Effects of alcohols on fluorescence anisotropies of diphenylhexatriene and its derivatives in bovine blood platelets: relationships of the depth-dependent change in membrane fluidity by alcohols with their effects on platelet aggregation and adenylate cyclase activity, Biochim. Biophys. Acta 1112 (1992) 14–18.
[80] I. Yun, E.-S. Cho, H.-O. Jang, U.-K. Kim, C.-H. Choi, I.-K. Chung, I.-S. Kim, W.G. Wood, Amphiphilic effects of local anesthetics on rotational mobility in neuronal and model membranes, Biochim. Biophys. Acta 1564 (2002) 123–132.
[81] F.G. Prendergast, R.P. Haugland, P.J. Callahan, 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties, and use as a fluorescence probe of lipid bilayers, Biochemistry 20 (1981) 7333–7338.
[82] Z. Sojcic, H. Toplak, R. Zuehlke, U.E. Honegger, R. Buhlmann, U.N. Wiesmann, Cultured human skin fibroblasts modify their plasma membrane lipid composition and fluidity according to growth temperature suggesting homeoviscous adaptation at hypothermic (30 degrees C) but not at hyperthermic (40 degrees C) temperatures, Biochim. Biophys Acta 1104 (1992) 31–37.
[83] R.D. Kaiser, E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth, Biochemistry 37 (1998) 8180–8190.
[84] R. Schroeder, E. London, D. Brown, Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 12130–12134.
[85] A. Chattopadhyay, S. Mukherjee, Depth-dependent solvent relaxation in membranes: wavelength-selective fluorescence as a membrane dipstick, Langmuir 15 (1999) 2142–2148.
[86] S. Ilangumaran, D.C. Hoessli, Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane, Biochem. J. 335 (1998) 433–440.
[87] P.G. Yancey, W.V. Rodrigueza, E.P.C. Kilsdonk, G.W. Stoudt, W.J. Johnson, M.C. Phillips, G.H. Rothblat, Cellular cholesterol efflux mediated by cyclodextrins. Demonstation of kinetic pools and mechanism of efflux, J. Biol. Chem. 271 (1996) 16026–16034.
[88] E.-M. Hur, K.-T. Kim, G protein-coupled receptor signalling and cross-talk; achieving rapidity and specificity, Cell. Signal. 14 (2002) 397–405.
[89] S.W. Edwards, C.M. Tan, L.E. Limbird, Localization of G-protein-coupled receptors in health and disease, Trends Pharmacol. Sci. 21 (2000) 304–308.
[90] L. Kallal, J.L. Benovic, Using green fluorescent proteins to study G-protein-coupled receptor localization and trafficking, Trends Pharmacol. Sci. 21 (2000) 175–180.
[91] R.R. Neubig, Membrane organization in G-protein mechanisms, FASEB J. 8 (1994) 939–946.
[92] R.S. Ostrom, S.R. Post, P.A. Insel, Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G(s), J. Pharmacol. Exp. Ther. 294 (2000) 407–412.
[93] R.S. Ostrom, New determinants of receptor–effector coupling: trafficking and compartmentation in membrane microdomains, Mol. Pharmacol. 61 (2002) 473–476.
[94] K. Dreja, M. Voldstedlund, J. Vinten, J. Tranum-Jensen, P. Hellstrand, K. Swärd, Cholesterol depletion disrupts caveolae and differentially impairs agonist-induced arterial contraction, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1267–1272.
[95] Z.-L. Xiao, Q. Chen, J. Amaral, P. Biancani, R.T. Jensen, J. Behar, CCK receptor dysfunction in muscle membranes from human gallbladders with cholesterol stones, Am. J. Physiol. 276 (1999) G1401–G1407.
[96] Z.-L. Xiao, Q. Chen, J. Amaral, P. Biancani, J. Behar, Defect of receptor–G protein coupling in human gallbladder with cholesterol stones, Am. J. Physiol. Gasterointest. Liver Physiol. 278 (2000) G251–G258.
[97] P.L. Yeagle, The Membranes of Cells, Academic Press, Orlando, FL, 1987, pp. 120–138.
[98] I. Sylte, A. Bronowska, S.G. Dahl, Ligand induced conformational states of the 5-HT_{1A} receptor, Eur. J. Pharmacol. 416 (2001) 33–41.
[99] J. Kirilovsky, M. Schramm, Delipidation of a beta-adrenergic receptor preparation and reconstitution by specific lipids, J. Biol. Chem. 258 (1983) 6841–6849.
[100] N. Ben-Arie, C. Gileadi, M. Schramm, Interaction of the beta-adrenergic receptor with Gs following delipidation. Specific lipid requirements for Gs activation and GTPase function, Eur. J. Biochem. 176 (1988) 649–654.
[101] K. Boesze-Battaglia, A.D. Albert, Cholesterol modulation of photoreceptor function in bovine retinal rod outer segments, J. Biol. Chem. 265 (1990) 20727–20730.

[102] A.D. Albert, K. Boesze-Battaglia, Z. Paw, A. Watts, R.M. Epand, Effect of cholesterol on rhodopsin stability in disk membranes, Biochim. Biophys. Acta 1297 (1996) 77–82.
[103] S.L. Niu, D.C. Mitchell, B.J. Litman, Manipulation of cholesterol levels in rod disk membranes by methyl-beta-cyclodextrin: effects on receptor activation, J. Biol. Chem. 277 (2002) 20139–20145.
[104] T. Sooksawate, M.A. Simmonds, Increased membrane cholesterol reduces the potentiation of $GABA_A$ currents by neurosteroids in dissociated hippocampal neurons, Neuropharmacology 37 (1998) 1103–1110.
[105] C. Kirsch, G.P. Eckert, W.E. Mueller, Statin effects on cholesterol micro-domains in brain plasma membranes, Biochem. Pharmacol. 65 (2003) 843–856.
[106] B. Wolozin, W. Kellman, P. Ruosseau, G.G. Celesia, G. Siegel, Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors, Arch. Neurol. 57 (2000) 1439–1443.
[107] H. Jick, G.L. Zornberg, S.S. Jick, S. Seshadri, D.A. Drachman, Statins and the risk of dementia, Lancet 356 (2000) 1627–1631.
[108] P.H.A. Steegmans, D. Fekkes, A.W. Hoes, A.A.A. Bak, E. van der Does, D.E. Grobbee, Low serum cholesterol concentration and serotonin metabolism in men, Br. Med. J. 312 (1996) 221.
[109] M. Zureik, D. Courbon, P. Ducimetière, Serum cholesterol concentration and death from suicide in men: Paris prospective study I, Br. Med. J. 313 (1996) 649–651.
[110] S.-C. Hsiung, M. Adlersberg, V. Arango, J.J. Mann, H. Tamir, K.-P. Liu, Attenuated $5\text{-}HT_{1A}$ receptor signaling in brains of suicide victims: involvement of adenylyl cyclase, phosphatidylinositol 3-kinase, Akt and mitogen-activated protein kinase, J. Neurochem. 87 (2003) 182–194.