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The γ-aminobutyric acid receptor B, but not the metabotropic glutamate receptor type-1, associates with lipid rafts in the rat cerebellum

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Abstract

Recent evidence suggests that specialized microdomains, called lipid rafts, exist within plasma membranes. These domains are enriched in cholesterol and sphingolipids and are resistant to non-ionic detergent-extraction at 4°C. They contain specific populations of membrane proteins, and can change their size and composition in response to cellular signals, resulting in activation of signalling cascades. Here, we demonstrate that both the metabotropic γ-aminobutyric acid receptor B ($GABA_B$ receptor) and the metabotropic glutamate receptor-1 from rat cerebellum are insoluble in the non-ionic detergent Triton X-100. However, only the $GABA_B$ receptor associates with raft fractions isolated from rat brain by sucrose gradient centrifugation. Moreover, increasing the stringency of isolation by decreasing the protein : detergent ratio caused an enrichment of the $GABA_B$ receptor in raft fractions. In contrast, depletion of cholesterol from cerebellar membranes by either saponin or methyl-β-cyclodextrin treatment, which solubilize known raft markers, also increased the solubility of the $GABA_B$ receptor. These properties are all consistent with an association of the $GABA_B$ receptor with lipid raft microdomains.

Keywords: brain, detergent-insoluble, lipid rafts, metabotropic GABA receptors, metabotropic glutamate receptors.

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Neurotransmitter receptors are highly concentrated and precisely localized in the plasma membrane. The correct targeting and clustering of receptors is critical for neurotransmission. Several proteins have been identified that interact with neurotransmitter receptors and link them to the cytoskeleton and to specific signal transduction pathways. These include the PSD-95/SAP90 family of synaptic proteins for NMDA receptors, GRIP for AMPA receptors and the Homer family of proteins for the metabotropic glutamate receptors (mGluRs; for a review see Fanning and Anderson 1998; Garner *et al*. 2000; Kennedy 2000; Scannevin and Huganir 2000; Sheng and Pak 2000). The inhibitory neurotransmitter receptors for glycine and GABA also interact with specific proteins, namely gephyrin and GABARAP, respectively (for a review see Kirsch 1999). One feature of these receptor-associated proteins is that they can cluster their associated receptors in both heterologously transfected cells and neurones, and they have the potential to interact with additional proteins other than their associated receptors, thereby enabling large signalling complexes to be established. Indeed, the existence of such large postsynaptic protein complexes has been elegantly demonstrated using SDS-gel electrophoresis and mass spectrometry following the isolation of NMDA receptors combined with synaptic components (Husi *et al*. 2000).

In cultured neurones postsynaptic neurotransmitter receptors and their interacting proteins form clusters, often independently, before synapse formation in culture (Richmond *et al*. 1996; Rao *et al*. 1998). Interestingly, some neurotransmitter receptors and their associated interacting proteins seem to be insoluble in Triton X-100 even when the cytoskeleton is disrupted by latrunculin-A (Allison

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Abbreviations used: DIGs, detergent-insoluble glycolipid fractions; $GABA_BR$, metabotropic GABA receptor; G protein, guanine nucleotide binding protein; MBC, methyl-β-cyclodextrin; mGluR, metabotropic glutamate receptor; PSD, postsynaptic density.

et al. 1998; Allison *et al*. 2000). Since Triton X-100 insolubility and the formation of clusters are properties of lipid rafts (Brown and London 1998; Hooper 1999; Simons and Toomre 2000), this behaviour of certain receptors suggests that perhaps they can associate with these specialized lipid domains.

Lipid rafts, also termed detergent-insoluble glycolipid fractions (DIGs) or detergent resistant membranes (DRMs), are enriched for cholesterol and sphingolipids and form a distinct, liquid-ordered phase in the lipid bilayer of membranes (Simons and Ikonen 1997; London and Brown 2000). In addition, they contain specific subpopulations of membrane proteins often being enriched in glycophosphoinositol-linked proteins (GPI-proteins) and specific tyrosine kinases. Lipid rafts also appear to concentrate lipid-modified proteins such as Src family kinases, heterotrimeric G-proteins, cholesterol-linked and palmitoylated proteins such as Hedgehog, as well as palmitoylated transmembrane proteins (for reviews see Brown and London 1998; Simons *et al*. 1999).

Much work on rafts has been done in the haematopoietic system, where they were shown to be important for signalling processes in basophils and mast cells (Baird *et al*. 1999) and for T cell activation (Moran and Miceli 1998; Viola *et al*. 1999). As a result lipid rafts have been suggested to serve as assembly and sorting platforms for signalling complexes. Less is known about rafts in the central nervous system, although some proteins have been identified that associate with rafts in neurones. These include GAP-43 and NAP-22 (Maekawa *et al*. 1999; Laux *et al*. 2000) the cell adhesion molecules Kilon (Funatsu *et al*. 1999) and F3 (Olive *et al*. 1995) and some tyrosine kinase receptors (Wu *et al*. 1997). However, relatively little is known about the neurotransmitter receptor content of lipid rafts in the brain. Of the neurotransmitter receptors, the α7-subunit nicotinic acetylcholine receptor (Bruses *et al*. 2001) and the ionotropic AMPA-type glutamate receptor (Suzuki *et al*. 2001) have been shown to associate with lipid rafts. In addition, the voltage-gated K^+ channel $K_v2.1$, but not $K_v4.2$, is associated with rafts (Martens *et al*. 2000).

The $GABA_B$ receptors ($GABA_BRs$) and the metabotropic glutamate receptors (mGluRs) belong to class C of the seven-transmembrane-domain receptors. The $GABA_B$ receptor is a heterodimer composed of the homologous $GABA_BR1$ and $GABA_BR2$ subunits, neither of which is fully functional when expressed alone. It is thought that subunit dimerisation promotes proper post-translational processing and membrane targeting (Kaupmann *et al*. 1997; Kaupmann *et al*. 1998; White *et al*. 1998). Since the initial cloning of $GABA_BR1a$ and $GABA_BR1b$, several other splice variants have been identified both for $GABA_BR1$ and $GABA_BR2$ (Blein *et al*. 2000; Couve *et al*. 2000). The $GABA_BR$ couples through the heterotrimeric guanine-nucleotide binding (G) proteins Gi and Go to adenyl cyclase and Ca^{2+} and K^+ channels (Blein *et al*. 2000; Couve *et al*. 2000). It also interacts with members of the 14-3-3 family of signalling molecules (Couve *et al*. 2001), and the transcription factors ATFx and ATF4 (Bruckner *et al*. 1999; White *et al*. 2000). However, no interacting molecules have yet been identified that target or cluster the receptor.

$GABA_BRs$ share sequence similarity with the mGluRs which can be categorised into three subgroups on the basis of their pharmacology and sequence (Pin and Duvoisin 1995). mGluR1 and its splice variants belong to the group-1 mGluRs which are predominantly postsynaptic, and interact with the EVH-domain-containing protein Homer (Brakeman *et al*. 1997). This interaction may be important for receptor trafficking, clustering and the formation and stabilization of signalling complexes at the plasma membrane (Tu *et al*. 1998, 1999; Xiao *et al*. 1998; Ciruela *et al*. 1999b, 2000). The group 1 mGluRs activate the phospholipase C signalling pathway and can also couple to calcium and potassium channels (Pin and Duvoisin 1995). Both the mGluRs and the $GABA_BRs$ modulate central nervous system neurotransmission, albeit in different directions, and have been implicated in a number of pathologies. These include neurodegenerative disorders for the mGluRs (Pin and Duvoisin 1995; Conn and Pin 1997;), and drug addiction, epilepsy and depression for the $GABA_BRs$ (Blein *et al*. 2000; Billinton *et al*. 2001;). This together with the fact that lipid rafts can concentrate specific G proteins and other proteins into signalling complexes prompted us to investigate the association of these neurotransmitter receptors with lipid rafts in the rat brain. Here we show that, while both the $GABA_BR$ and mGluR1 are insoluble in Triton X-100, only the $GABA_B$ receptor associates with lipid raft fractions.

Materials and methods

Materials

Monoclonal anti-Thy-1 antibody was a kind gift from Prof. N. Barclay (Molecular Immunology Group, University of Oxford, UK), and polyclonal anti $G_{\alpha o}$ and $G_{\alpha i}$ antibodies were kindly donated by Prof. G. Milligan (Division of Biochemistry and Molecular Biology, University of Glasgow, U.K.). Anti-mGluR1 polyclonal antibodies F1 (pan-mGluR1) and F2 (anti-mGluR1a) (Ciruela *et al*. 1999a) and polyclonal antibodies against $GABA_BR1a/b$ and $GABA_BR2$ (White *et al*. 2000) have been described previously. Ganglioside GM1 was detected with cholera toxin subunit β conjugated to horseradish peroxidase (Sigma). Protein was quantified using the Bradford (Bio-Rad) or Bicinchoninic Acid assay (Pierce) with BSA as standard.

Receptor solubilization

Cerebellum from adult Wistar rats was homogenized as outlined below, and incubated with 1% Triton X-100 for 30 min on ice followed by centrifugation at 14,000 g for 30 min. The detergent-insoluble pellets and their corresponding supernatants were

analyzed by SDS–PAGE and immunoblotting as previously described (Ciruela *et al*. 1999a).

Lipid raft isolation

Detergent-insoluble glycolipid fractions (DIGs) were prepared following a protocol adapted from Hooper and co-workers (Parkin *et al*. 1999). All steps were carried out on ice or at 4°C. Cerebellum from adult Wistar rats was homogenized in 10 volumes (w/v) of MES-buffered saline (MBS; 25 mM MES, pH 6.5, and 0.15 M NaCl) and a mixture of protease inhibitors. Homogenization was performed with 20 passes of a Dounce homogenizer followed by five passages through a 23-gauge needle. Samples were assayed for protein and adjusted to 3 mg/mL unless otherwise indicated, and Triton X-100 was added to a final concentration of 1% (w/v). The samples were then sonicated at 25% maximum power for 5 × 20 s with a 60-s cooling period between each round of sonication using a Soniprep Sonifier. After dilution with an equal volume of 80% (w/v) sucrose in MBS, 1.7 mL of sample was injected under a 10.3-mL 5–30% (w/v) sucrose gradient in MBS. Following centrifugation at 140,000 g for 18 h at 4°C in a SW 40.1 rotor (Beckman Instruments), 1 mL fractions were harvested from the top to the bottom of the gradient, and the detergent-insoluble pellet at the base of the gradient was resuspended in 1 mL of MBS. Samples were analyzed using SDS–PAGE and immunoblotting. Ganglioside GM1 was detected subsequent to sample electrophoresis, transfer to Immobilon-*p* membrane (Sigma) and blocking the membrane with skimmed milk (5% in PBS with 0.05% Tween-20) by incubation with cholera toxin subunit β conjugated to horseradish peroxidase (Sigma) 1 : 10,000 overnight at 4°C and visualization using enhanced chemiluminescence (Pierce). Cholera toxin subunit β binds specifically to the ganglioside GM1 (Schon and Freire 1989) and is used routinely to visualize GM1 (Wu *et al*. 1997; Kawabuchi *et al*. 2000).

Alkaline phosphatase assay

Samples were assayed for the GPI-anchored enzyme, alkaline phosphatase, using p-nitrophenyl phosphate as substrate, and the product was quantified spectrophotometrically (Parkin *et al*. 1999). Enzyme activity was calculated as a percentage of the total activity.

Comparison of DIGs and total membranes

Total membranes were collected from cerebellar postnuclear supernatants by centrifugation at 100,000 g for 2 h. DIGs were concentrated by dilution of the relevant sucrose gradient fractions fivefold with MBS followed by ultracentrifugation at 100,000 g for 2 h. Equal amounts of total protein were analyzed by SDS–PAGE and immunoblotting.

Cholesterol extraction with methyl-β-cyclodextrin

Cerebellar homogenates were incubated either with 50 mM methyl-β-cyclodextrin or without for 30 min at 4°C prior to detergent lysis and sucrose gradient floatation.

Saponin-treatment of membranes

Total membrane fractions from cerebellum were incubated with or without 0.5% (w/v) saponin for 30 min on ice prior to extraction with 1% Triton X-100 and centrifugation at 14 000 g for 30 min. Following resuspension of the pellets to the starting volume, equal volumes of the fractions were analyzed by SDS–PAGE and immunoblotting.

Results

Solubilization of $GABA_BR$ and mGluR1

Initial studies were performed on the detergent solubility of the $GABA_BR$ and mGluR1 by incubating rat cerebellar homogenates with 1% Triton X-100 prior to centrifugation at 14,000 g. Following centrifugation, almost all of the $GABA_BR1/2$ and mGluR1a/b were localised in the pellet (Fig. 1, pellet), indicating that the receptors did not solubilize in Triton X-100 under these conditions. Both Thy-1 and GM1, which are commonly used raft markers (Brown and London 1998; Hooper 1999), were also found almost exclusively in the precipitate. In contrast, the integral membrane protein Na^+/K^+ ATPase, which is not raft associated (Martens et al. 2000), was found almost exclusively in the supernatant. Since lipid raft components are insoluble in Triton X-100 because of their high cholesterol and sphingolipid content, these results could indicate raft association of the receptors. Alternatively their insolubility could be due to cytoskeletal association. To distinguish between these possibilities raft fractions were isolated by sucrose gradient centrifugation.

Isolation of lipid rafts from rat cerebellum

A widely used approach for isolating lipid rafts is to treat membranes with detergent followed by floatation of the detergent-insoluble glycolipid-enriched domains (DIGs) on sucrose gradients (Brown and London 1998; Hooper 1999). When Triton X-100 solubilized membranes are loaded at the bottom of a sucrose gradient and centrifuged at high speed,

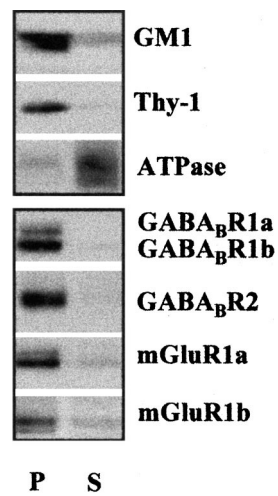

Fig. 1 $GABA_B$ receptor and mGluR1 are insoluble in Triton X-100. Cerebellar homogenates were incubated with 1% Triton X-100 for 30 min and subsequently centrifuged. Equal volumes of detergent-insoluble pellets (P) and corresponding supernatants (S) were analysed by SDS–PAGE and immunoblotting for the raft markers GM1 and Thy-1, the non-raft Na^+/K^+ ATPase and the metabotropic neurotransmitter receptors $GABA_BR1/2$, mGluR1a and mGluR1b.

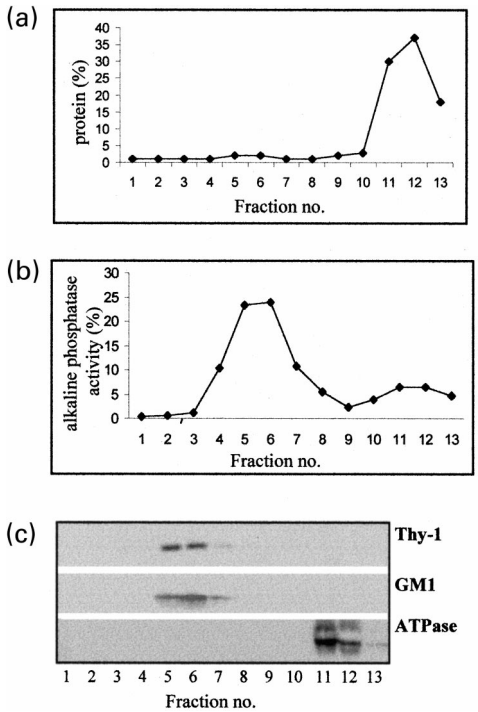

Fig. 2 Analysis of sucrose floatation gradients of rat cerebellum. Detergent-insoluble glycolipid fractions were prepared as described in Materials and methods using 1% Triton X-100. Sucrose gradients were harvested in 1 mL fractions (fraction 1, top of gradient; fraction 12, base of gradient; fraction 13, insoluble pellet). (a) Distribution of protein in the sucrose gradient (given as the percentage of total protein). (b) Distribution of alkaline phosphatase activity in the sucrose gradient (given as the percentage of total enzyme activity). (c) Equal volumes of sucrose gradient fractions were analysed by SDS–PAGE and immunoblotting. The immunoblots were probed for the raft markers GM1 and Thy-1, and the non-raft associated enzyme Na^+/K^+ ATPase. Comparable results were obtained in five separate experiments.

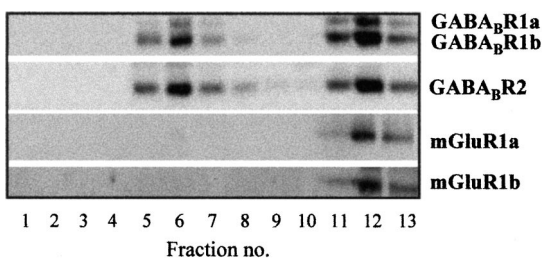

Fig. 3 $GABA_BR$, but not mGluR1, is present in detergent-insoluble, glycolipid-enriched domains isolated from rat cerebellum. Following solubilization in 1% Triton X-100 at a protein to detergent ration of 3 : 1 the lysate was centrifuged on a sucrose gradient as described in Materials and methods. Equal volumes of the sucrose gradient fractions were analysed by SDS–PAGE and immunoblotting. The immunoblots were probed for the metabotropic neurotransmitter receptors $GABA_BR1/2$, mGluR1a and mGluR1b. Comparable results were obtained in five separate experiments.

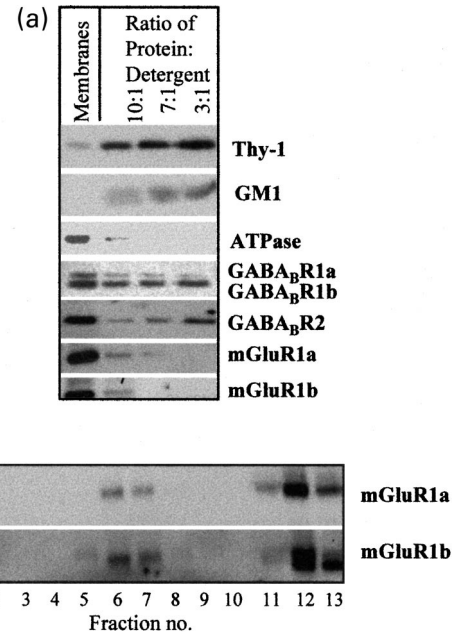

Fig. 4 $GABA_BR$, but not mGluR1, is enriched in detergent-insoluble, glycolipid-enriched domains when the protein:detergent ratio is decreased. Cerebellar homogenates were prepared at the different protein:detergent ratios indicated prior to sonication and sucrose gradient centrifugation. (a) Equal amounts of protein (2 μg) from cerebellar total membrane (TM) and from DIGs isolated at a protein to detergent ratio of 10 : 1, 7 : 1 and 3 : 1 were analysed by SDS–PAGE and immunoblotting for the raft markers GM1 and Thy-1, the non-raft Na^+/K^+ ATPase, and the metabotropic neurotransmitter receptors $GABA_BR1/2$, mGluR1a and mGluR1b. (b) Cerebellar homogenates prepared at a protein : detergent ratio of 7 : 1 were centrifuged on a sucrose gradient and analysed by SDS–PAGE and immunoblotting for mGluR1a and mGluR1b.

DIGs and their associated proteins migrate to the upper, low-density, region of the gradient, because of their high lipid content. Detergent-soluble material will remain in the lower fractions and the detergent-insoluble cytoskeletal components pellet at the bottom of the tube. Proteins that are DIG-associated after detergent extraction are presumed to have been raft-associated in the intact cell (Brown and London 1998; Hooper 1999).

Rat cerebellum was solubilized in Triton X-100 at a ratio of 3 : 1 protein to detergent and centrifuged as described in 'materials and methods'. Fractions were collected from the top of the gradient. Comparing these for protein content, 90% of the protein was found in the lower fractions with approximately 20% present in the insoluble pellet. In contrast, only about 5–8% of protein was present in the lighter DIG fractions (Fig. 2A). However, more than 80% of alkaline phosphatase activity, a raft-associated enzyme, was found in the DIG fractions (Fig. 2B). Similarly, the raft markers Thy-1 and GM1 distributed almost exclusively to

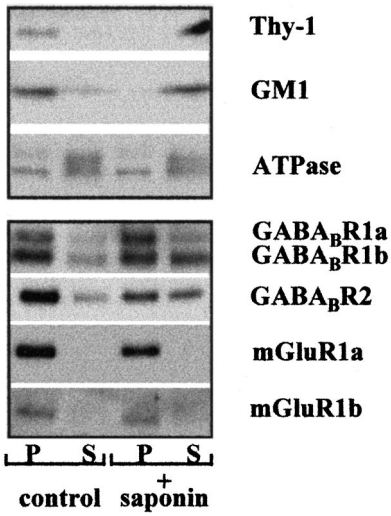

Fig. 5 Saponin-treatment increases the detergent-solubility of the $GABA_B$ receptor. Cerebellar membranes were incubated for 30 min on ice with or without 0.5% (w/v) saponin prior to extraction with 1% Triton X-100 and centrifugation at 14,000 g for 30 min The pellets (P) were resuspended to the original volume and equal volumes of these and the corresponding supernatants (S) were analysed by SDS–PAGE and immunoblotting for the raft markers GM1 and Thy-1, the non-raft Na^+/K^+ ATPase and the metabotropic neurotransmitter receptors $GABA_B$R1/2, mGluR1a and mGluR1b.

DIG fractions, whereas the non-raft associated enzyme Na^+/K^+ ATPase, was found in the lower fractions only (Fig. 2C).

The metabotropic $GABA_B$R containing the R2 subunit together with R1a or R1b showed a mixed distribution, with 30% to 40% present in the DIG fractions and the remainder being found mainly in the detergent-soluble fractions (Fig. 3; comparable results obtained in five separate experiments). This distribution was consistently observed for all three subunits analysed, suggesting that similar ratios of $GABA_B$R1a/R2 and $GABA_B$R1b/R2 associate with lipid rafts in cerebellar membranes. In contrast, mGluR1a or mGluR1b were found exclusively in the soluble fractions and the insoluble pellet. Similar results were obtained when using synaptosomes, rather than homogenates, as starting material (data not shown). Together these results indicate that a significant fraction of the $GABA_B$R is found in the raft domains in the cerebellum.

$GABA_B$R is enriched in detergent-insoluble, glycolipid-enriched domains when the protein : detergent ratio is decreased

Proteins that are almost exclusively raft-associated should be enriched in DIGs when compared to total membrane fractions. This is indeed the case for Thy-1 as shown in Fig. 4(A) where equal amounts of protein from differently extracted cerebellum are compared. The ganglioside GM1 is similarly enriched in these fractions. However, $GABA_B$R, which is only partially raft-associated, was not enriched to the same degree compared to Thy-1 and GM1 (Fig. 4A) at all the different protein to detergent ratios tested. However, as the ratio of protein to detergent was decreased, thereby increasing the stringency of the raft isolation procedure, the amount of $GABA_B$R present in the DIG fractions became a greater proportion of the total DIG fraction (Fig. 4A). This indicates that, while only a fraction of the receptor associates with lipid rafts, this association is specific.

Interestingly, at less stringent protein to detergent ratios we found some mGluR1a and b in the upper light fractions of the sucrose floatation gradients (Fig. 4B, where a protein:detergent ratio of 7 : 1 was used, and Fig. 4A). This probably reflects contamination of DIG fractions with non-raft proteins at the high protein to detergent ratios. This is consistent with detection of traces of Na^+/K^+ ATPase in the lighter fractions at these protein:detergent ratios (Fig. 4A and not shown).

Cholesterol-depletion increases the detergent-solubility of $GABA_B$R

Saponin is a detergent that complexes with membrane cholesterol and can increase the solubility of the raft associated proteins (Cerneus *et al.* 1993). Cerebellar membranes were therefore incubated with 0.5% saponin for 30 min on ice prior to treatment with 1% Triton X-100 and centrifugation at 14,000 g for 30 min. In the samples not treated with saponin, most of the raft markers Thy-1 and GM1 were pelleted by centrifugation, confirming that they were not soluble in Triton X-100 (Fig. 5, 'control' lanes). However, in the saponin-treated samples, almost all of the Thy-1 and GM1 was found in the supernatants (Fig. 5, ' + saponin' lanes). Therefore, reducing the cholesterol content in membranes solubilized these raft-associated proteins. In contrast, most of the Na^+/K^+ ATPase was soluble in 1% Triton X-100. The amount of soluble $GABA_B$R increased significantly after saponin treatment indicating that indeed a proportion of the receptor is in the raft fraction. The fact that not all of the receptor was solubilized meant that the remaining $GABA_B$R behaved like the mGluRs which were not solubilized following saponin treatment (Fig. 5). The relative difficulty of solubilizing the mGluRs and a proportion of the $GABA_B$R probably reflects their association with cytoskeletal elements, in the case of the mGluR1a via an association with Homer proteins and/or tubulin (Brakeman *et al.* 1997; Ciruela *et al.* 1999a).

To investigate the effect of cholesterol depletion on raft-association of $GABA_B$R, cerebellar homogenates were treated with methyl-β-cyclodextrin (MBC) prior to Triton X-100 solubilization and centrifugation. MBC removes cholesterol from membranes without binding to or inserting into the membrane (Klein *et al.* 1995). In lymphocytes, MBC has been shown to release substantial amounts of

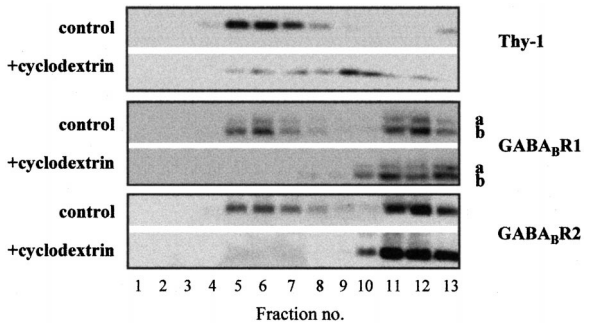

Fig. 6 Cyclodextrin-treatment abolishes floatation of the $GABA_B$ receptor on sucrose gradients. Cerebellar homogenates were incubated with 50 mM methyl-β-cyclodextrin for 30 min at 4°C prior to detergent lysis and sucrose gradient floatation. Equal volumes of the gradient fractions were analysed by SDS–PAGE and immunoblotting for the raft marker Thy-1, and for the metabotropic neurotransmitter receptor $GABA_BR1/2$.

Thy-1 and GM1 from raft membranes (Ilangumaran and Hoessli 1998). Following MBC treatment, Thy-1 distributed to lower fractions on the sucrose density gradients when compared to controls (Fig. 6, top), showing its raft association was disrupted by the cholesterol depletion. Similarly, $GABA_BR$ no longer floated to the upper regions of the gradient following MBC treatment when compared to controls (Fig. 6, centre and bottom)

Thus, as with the saponin treatment, we find that cholesterol depletion by MBC solubilizes $GABA_BR$. All these results confirm that $GABA_BR$ is indeed raft-associated.

G protein subunits $G_{\alpha o}$ and $G_{\alpha i}$ are present in detergent-insoluble, glycolipid-enriched domains

What could be the functional implication of the raft-association of the $GABA_BR$? Lipid rafts are thought to function as signalling platforms and have been shown to cluster some receptors with their downstream signalling pathways in the haematopoietic system. To test whether lipid rafts may also function in clustering the $GABA_BR$ to its downstream signalling pathway, we tested the gradient fractions for presence of the G proteins $G_{\alpha o}$ and $G_{\alpha i}$.

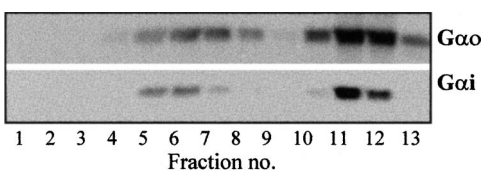

Fig. 7 G protein $G_{\alpha o}$ and $G_{\alpha i}$ subunits are present in detergent-insoluble, glycolipid-enriched domains isolated from rat cerebellum. Following solubilization in 1% Triton X-100 at a protein to detergent ration of 3 : 1 the lysate was centrifuged on a sucrose gradient as described in Materials and methods. Equal volumes of the sucrose gradient fractions were analysed by SDS–PAGE and immunoblotting.

$GABA_BRs$ modulate their effector systems by coupling to these inhibitory G proteins.

We found that approximately 30% of proteins $G_{\alpha o}$ and $G_{\alpha i}$ localize to DIG fractions (Fig. 7). These results may indicate that lipid rafts in rat cerebellum function to cluster the $GABA_BR$ and its immediate downstream signalling molecules.

Discussion

Together all of the data presented in this report provide compelling evidence for the association of a significant fraction (30% to 40%) of the $GABA_BR$ with lipid raft domains in rat cerebellum. The stringency of the isolation procedure was such that no detectable Na^+/K^+ ATPase was found in the isolated DIG fractions nor were the related seven transmembrane spanning receptors mGluR1a and b. In addition, treatment of the cerebellar membranes with saponin or MBC reduced the association of the $GABA_BR$ and known raft associated proteins with the DIGs. Thus all of the results are consistent with the isolated DIGs corresponding to raft domains in the tissue, and support the presence of a significant fraction of the $GABA_BR$ in a DIG fraction in the rat cerebellum. We also find approximately 30% of the G protein subunits $G_{\alpha o}$ and $G_{\alpha i}$ in the DIG fractions. The $GABA_BR$ couples through these inhibitory G proteins to downstream effector systems (Couve *et al.* 2000), and the presence of both the receptor and the G proteins in lipid rafts may indicate a possible functional role for the receptor's raft association.

The reason for an incomplete association of the $GABA_B$ receptor with the raft fraction is not clear, but may reflect a dynamic and changing distribution of the receptor in the neuronal membrane. Experiments in the haematopoietic system have suggested strongly that lipid rafts can rapidly recruit other signalling complexes into functional assemblies following appropriate stimulation of the cells (Brown and London 1998; Moran and Miceli 1998; Viola *et al.* 1999; Simons and Toomre 2000). In the central nervous system raft signalling and recruitment of proteins has been shown to occur in the regulation of axonal growth and fasciculation via ephrins and ephrin tyrosine kinases (Bruckner *et al.* 1999), and the glial derived neurotrophic growth factor GNDF appears to act through recruitment of its co-receptor GFRα1 and the tyrosine kinase RET into lipid rafts (Tansey *et al.* 2000). Similarly, cross-linking of the cerebellar neuronal adhesion molecule TAG-1 causes recruitment of the tyrosine kinase Lyn into lipid rafts whilst cross-linking of the adhesion molecules NCAM 120 and F3 in maturing oligodendrocytes similarly recruits the kinase Fyn (Kramer *et al.* 1999). In the course of this study the AMPA glutamate receptors have been shown to be raft associated, and here too only a proportion of the receptors was present in the raft fraction (Suzuki *et al.* 2001).

Alternatively, the association of a proportion of the $GABA_B$ receptors with lipid rafts may modulate the signalling of a specific set of the $GABA_B$ receptors. A modulatory function of raft-association has been shown for the voltage-gated K^+ channel $K_v2.1$, at least in stably transfected cell lines, where disruption of rafts by cyclodextrin significantly shifts the steady-state inactivation of this channel by almost 40 mV (Martens *et al*. 2000). It is noteworthy that the same study showed that the related voltage-gated potassium channel $K_v4.2$ was not associated with lipid rafts. In our study the mGluR1 receptors, which show homology with the $GABA_B$ receptors, were also not found in the cerebellar raft fractions, a finding that suggests that the association of the $GABA_B$ receptor with these membrane domains has physiological relevance.

Biochemical studies are consistent with the $GABA_B$ receptor having pre- and post synaptic components, with the R1a and R1b subunits present in synaptic plasma membranes, but with only R1a being highly enriched in the postsynaptic density preparations (Benke *et al*. 1999). In our study whilst both the $GABA_B$ R1a and R1b receptor subunits are found in the raft fractions, saponin treatment of the cerebellar membranes preferentially solubilized $GABA_B$ R1b. This could suggest that $GABA_B$ receptors formed from the R1a and R2 subunits may be in a population of lipid rafts different from those containing the R1b and R2 subunits, which could contribute to the differential targeting of the differently spliced R1 subunits. Interestingly, $GABA_B$ receptors formed from R1a and R2 subunits appear to couple differently from those formed from R1b and R2 in transfected HEK 293 cells (Leaney and Tinker 2000). In these cells, the R1a/R2 receptors utilized both $G_{i\alpha 2}$ and $G_{o\alpha a}$ for signalling, whereas R1b/R2 receptors coupled only through the former. This behaviour of the different splice forms of $GABA_B$R1 subunits could be due to their targeting into different raft populations.

It has been also suggested that axonal targeting of some proteins may be dependent on their association with lipid rafts. Thus, the targeting of Thy1 to the axon in cultured rat hippocampal neurones seemed to be affected by cholesterol depletion of the cultures (Ledesma *et al*. 1998) and the axonal targeting of the voltage gated potassium channel $K_v1.4$ was found to be dependent on its association with PSD-95. This interaction is dependent on the palmitoylation of PSD-95 which itself undergoes a redistribution to the axon in the presence of $K_v1.4$, and palmitoylation is a modification of proteins that can promote raft association (Topinka and Bredt 1998). The formation of lipid rafts and synthesis of sphingomyelin and cholesterol have also been reported to be important for the maturation of axons in cultures of rat hippocampal neurones (Ledesma *et al*. 1999).

Relatively little is known about the lipid raft association of neurotransmitter receptors in the brain. Apart from the AMPA receptor (Suzuki *et al*. 2001), only the α7-subunit nicotinic acetylcholine receptor in the chick ciliary ganglion (Bruses *et al*. 2001) has been shown to associate with lipid rafts. No function was ascribed to the AMPA receptor raft association but, for the acetylcholine receptor, clustering in the ciliary cells was suggested to be regulated by raft association. It will be interesting to see if other central nervous system neurotransmitter receptors are found to be present in these specialized membrane domains.

In summary, we find that $GABA_B$R associates with lipid raft fractions isolated from rat cerebellum, whereas the related mGluR1 does not. Furthermore, we find the inhibitory G protein $G_{\alpha o}$ and $G_{\alpha i}$ subunits in lipid raft fractions, indicating perhaps that lipid rafts in rat cerebellum function to cluster the $GABA_B$R and its immediate downstream signalling pathway. These findings increase the potential signalling complexity for this important neurotransmitter receptor. It will now be important to identify whether additional components of the $GABA_B$R signalling pathway are present in rafts and how raft association may modulate receptor function.

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