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Agrin elicits membrane lipid condensation at sites of acetylcholine receptor clusters in C2C12 myotubes

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Short title : Acetylcholine receptor clustering and membrane condensation

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Abbreviations

AChR, nicotinic acetylcholine receptor; α-Bgtx, α-bungarotoxin; CTX, cholera toxin B; DRM, detergent-resistant membranes ; ESA, epidermal surface antigen ; GP, global polarization; NMJ, neuromuscular junction; MuSK, Muscle-Specific Kinase; MβCD, methyl-β-cyclodextrin; FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate.

Abstract

The formation of the neuromuscular junction is characterized by the progressive accumulation of nicotinic acetylcholine receptors (AChRs) in the postsynaptic membrane facing the nerve terminal, induced prominently through the agrin/MuSK signaling cascade. However, the cellular mechanisms linking MuSK activation to AChR clustering are still poorly understood. Here, we investigate whether lipid rafts are involved in agrin-elicited AChR clustering in a mouse C2C12 cell line. We observed that in C2C12 myotubes both AChR clustering and cluster stability were dependent on cholesterol since depletion by methyl-β-cyclodextrin inhibited cluster formation or dispersed established ones. Importantly, AChR clusters resided in ordered membrane domains - a biophysical property of rafts - as probed by Laurdan two-photon fluorescence microscopy. We isolated detergent-resistant membranes (DRMs) by three different biochemical procedures, which all generate membranes with similar cholesterol/GM1 ganglioside contents and were enriched in several postsynaptic components, notably AChR, syntrophin and raft markers flotillin-2 and caveolin-3. Agrin did not recruit AChRs into DRMs, suggesting that they are present in rafts independently of agrin activation. Consequently, in C2C12 myotubes, agrin likely triggers AChR clustering or maintain clusters through the coalescence of lipid rafts. These data led us to propose a model in which lipid rafts play a pivotal role in the assembly of the postsynaptic membrane at the neuromuscular junction upon agrin signaling.

KeyWords : agrin, C2C12 myotubes, lipid rafts, nicotinic acetylcholine receptor, neuromuscular junction.

Introduction

During synapse differentiation at the neuromuscular junction, motor innervation recruits nicotinic acetylcholine receptors (AChRs) to the sites of nerve-muscle contact. AChR clustering is stimulated by the neuronally released, extracellular matrix associated, heparan sulfate proteoglycan agrin (reviews in 1-3). The activity of agrin in AChR clustering is mediated by a signaling machinery that includes the agrin receptor MuSK (muscle-specific tyrosine kinase receptor; 4), the AChR-associated peripheral protein rapsyn (review in 2) as well as several intracellular enzymes (review in 5). However, the mechanisms linking MuSK activation to AChR clustering at the cell surface are still poorly understood. Once activated, MuSK recruits several effectors that are known to reorganize actin cytoskeleton (review in 5). Yet, interaction of AChRs with the actin membrane cytoskeleton provides both aggregation and restricted diffusion within the lipid bilayer (6-9). However, other mechanisms could also provide AChR segregation such as partitioning within specific lipid domains.

Cholesterol-sphingolipid-enriched lipid rafts form dynamic liquid ordered microdomains that float freely within the fluid, liquid disordered, cellular membranes. By the nature of their structure, these domains selectively incorporate or exclude proteins and therefore have been proposed to function as membrane platforms for the assembly of signaling complexes and for membrane sorting (10). Due to their highly dynamic structure these submicroscopic assemblies are able to coalesce upon clustering of their components, in physiological conditions by protein-driven interactions involving for example caveolins (11) or reorganization of actin cytoskeleton (12, 13), and artificially by crosslinking with antibodies or cholera toxin B subunit (CTX), a specific ligand of GM1 ganglioside (14, 15). For AChR aggregation, raft clustering might be required to generate synaptic clusters of neurotransmitter receptors characteristic of postsynaptic sites.

Insolubility in cold non-ionic detergent and perturbation of cellular process by cholesterol depletion with methyl-β-cyclodextrin (MβCD) have been used as tools to study the role of rafts in cell signaling and function, in particular in the maintenance and/or formation of central synapses (16). In the central nervous system, several postsynaptic proteins including neuronal α7-AChR, $GABA_B$ receptor, NMDA and AMPA receptors, voltage gated K^+ channel Kv2.1, postsynaptic density (PSD)-95 and glutamate receptor-interacting protein (GRIP) are partially associated with Triton X-100-resistant membranes

(17-21). Lipid rafts exist abundantly in dendrites of cultured hippocampal neurons in which postsynaptic proteins including AMPA receptors are enriched. Depletion of cholesterol/sphingolipids leads to instability of these receptors and gradual loss of synapses and dendritic spines (19). Similarly, in ciliary ganglion neurons and in PC12 cells, lipid rafts are necessary for the specific localization and stabilization of α7 nicotinic AChR within the plasma membrane (18, 20). Interestingly, Oshikawa et al, (20) showed that the fractionation of PC12 cells resulted in the differential distribution of various AChR subunits : α7, which forms postsynaptic homopentameric AChRs, was recovered in DRM fraction, whereas non synaptic α5 and β2 subunits were not. Thus, lipid rafts may be required to localize specific receptors to synaptic sites. A similar approach applied to transiently transfected COS-7 cells allowed us to demonstrate that muscle nicotinic AChR and rapsyn are cotransported in the exocytic pathway from the Golgi apparatus to the cell surface within lipid rafts (22). More recently, Campagna and Fallon (23) reported that in C2C12 myotubes CTX favors agrin-induced AChR clustering and that depletion of cholesterol disrupts AChR aggregates, supporting the notion that lipid rafts contribute to synaptic organization at the NMJ.

As cold detergent extraction may cause artefactual lipid aggregation and cholesterol depletion may not be specific to rafts (24, 25), alternative microscopic techniques such as FRET, Laurdan 2-photon microscopy and single particle tracking as well as detergent-free purification procedures have been developed to study raft properties. Here we combine a number of these approaches to study the agrin-induced AChR clusters of C2C12 myotube plasma membrane. We use immunofluorescence to quantify AChR clustering and the fluorescent lipid dye Laurdan in conjunction with 2-photon laser scanning microscopy for the biophysical characterization of AChR clusters. Laurdan's fluorescence emission spectrum depends on the fluidity of its membrane environment (26) and this approach was used to verify the cholesterol dependency of AChR clustering. Further, we analyzed DRMs from C2C12 myotubes and compared them to two alternative detergent-free extraction procedures. The biochemical data showed that AChR fractionated within light cholesterol-enriched DRMs independently of agrin treatment. Taken together, these data indicate that in muscle fibers lipid raft coalescence may serve as signaling platforms to concentrate AChRs at synaptic sites following agrin/MuSK activation.

Materials and methods

Antibodies and reagents

Mouse monoclonal (mAb) anti-syntrophin (mAb 1351E; 27) anti-rapsyn (mAb 1234; 28) antibodies were provided by Pr. S. Froehner (University of Washington, Seattle, WA) or purchased from Abcam, UK. mAb anti-transferrin receptor (H 68.4) was a gift from Pr. J.M. Trowbridge (UCSD, San Diego, CA). mAb anti-AChR α-subunit (clone 26), anti-flotillin-2/epidermal-surface antigen (ESA), and anti-caveolin-3 antibodies were obtained from Transduction Laboratories (Lexington, KY). Cholera toxin B, anti-cholera toxin B, anti-α-tubulin, methyl-β-cyclodextrin (MβCD) and Triton X-100 were purchased from Sigma (St Louis, MO). The following reagents and antibodies were used for immunofluorescence: fluorescein isothiocyanate (FITC) and Alexa 555-conjugated α-bungarotoxin (α-Bgtx) and Laurdan (6-dodecanoyl-2-dimethylaminonapthalene) were from Molecular Probes Inc. (Eugene, OR, USA), RITC-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell cultures

The culture of C2C12 cell line (adult C3H mouse leg muscle, ATCC Catalog No CRL-1772, Manassas, VA) was initiated with 10% fetal bovine serum in DMEM medium (Gibco, Eggenheim, Germany) complemented with 100 units/ml penicillin and streptomycin, 0.5% glutamine (v/v) for 3 to 4 days. Myotube differentiation was achieved in 3% horse serum (HS) in DMEM medium supplemented with 100 units/ml penicillin and streptomycin, 0.5% glutamine (v/v) during 5 to 7 days. AChR clustering was elicited by overnight incubation with 5 ng/ml recombinant 90 kDa C-terminal rat agrin (C-Ag $_{3,4,8}$) purchased from R&D Systems (Minneapolis, MN). For immunofluorescence studies, cells were plated on glass coverslips covered with a thin layer of Matrigel® (Becton Dickinson, Bedford, MA) into a 6-well plate. For biochemical studies, cells were grown in 75 cm^2 flasks. All cell cultures were maintained at 37°C in a humidified air atmosphere containing 5% CO_2.

Drug treatments

The depletion of cholesterol from C2C12 myotube membranes was performed by incubation of cultures with MβCD (10 mM) for 30 min at 37°C (14, 18). After washing, the

cells were processed either for AChR and Laurdan labeling or for AChR quantification as indicated (see below).

Preparation of DRM fractions from C2C12 myotubes

Three different methods were used to purify DRM fractions from differentiated myotubes: the traditional non-ionic detergent (Triton X-100) at 4°C (29), detergent-free at pH 11 (30) or shearing in an isotonic buffer containing Ca^{++}/Mg^{++} (31).

Triton X-100: fully differentiated C2C12 myotubes were washed in PBS, and with buffer A (25 mM N-morpholino-ethane-sulfonic acid pH 7.4 containing 150 mM NaCl and 2 mM EDTA). The cells were then suspended in ice-cold 1% Triton X-100 (protein/detergent ratio of ≥1/1 (wt/wt)) in 2 ml buffer A supplemented with a protease inhibitor cocktail (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidin, 3 μg/ml aprotinin, 2.5 μM phenylmethylsulfonyl fluoride (PMSF)), homogeneized with 12 to 15 strokes of a Dounce homogenizer and incubated 1 h on ice.

Alkaline extraction: C2C12 myotubes were homogenized in 0.5 M sodium carbonate pH 11 (30). Following sodium carbonate extraction, cells were sonicated 3 × 2 min on ice in a bath sonicator.

For both extraction protocols, an equal volume of sucrose 80% (w/v) in buffer A was added to the cell lysates (2 ml) and placed in the bottom of an ultracentrifugation tube. Cell lysates were overlayed successively with 4 ml 30% sucrose and 4 ml 5% sucrose in buffer A (supplemented 250 mM sodium carbonate for detergent-free extraction protocol). After centrifugation at 39, 000 rpm in a Beckman SW41 Ti rotor for 18 h at 4°C, 1 ml fractions were collected from top to bottom (designated 1 to 12).

A third simplified method adapted from Macdonald and Pike (31) was used: myotubes were washed in buffer P (20 mM Tris, pH 7.8, 1 mM $CaCl_2$, 1 mM $MgCl_2$) containing 250 mM sucrose and homogenized with 20 strokes of a Dounce homogenizer. Cells were then lysed in P buffer supplemented with anti-proteases by 5 passages through a 18G x 1 1/2'' needle followed by 15 passages through a 21G x 2'' needle. Lysates were centrifuged at 1,000 g for 10 min. The resulting post nuclear supernatants were collected and saved. The pellet was again lysed and centrifuged. Both supernatants were combined and mixed with an equal volume of sucrose 80% (w/v) in buffer P and placed in the bottom of an ultracentrifugation tube. Cell lysates were overlayed successively with 2 ml 35%, 2 ml 30 %, 2 ml 20% and 2 ml 5% sucrose in buffer P free of sucrose and gradients were centrifuged at 39, 000 rpm in a Beckman SW41 Ti rotor for 18 h at 4°C.

Western and dot blot analyses

Proteins from gradient fractions were precipitated with 10% trichloracetic acid, washed with 80% cold acetone, dissolved in Laemmli buffer (32), separated on 10% SDS-PAGE (Mini Protean II, Bio Rad, Richmond CA, USA), and electrotransferred onto nitrocellulose paper (Schleicher and Schuell, Dassel, Germany). Western blots were probed using various primary antibodies: anti-AChR α–subunit (1:300); anti-rapsyn (1:400), anti-caveolin-3 (1:15, 000); anti-flotillin-2/ESA (1:7, 500); anti-syntrophin (1:10, 000); anti-transferrin receptor (1: 3000) and anti-α tubulin (1: 30, 000), and appropriate HRP-conjugated secondary antibodies with enhanced chemoluminescent detection (ECL, Amersham Pharmacia Biotech) and exposure to Fuji X-ray films. To reveal the distribution of GM1 in the gradients, 50 μl of each fraction before TCA precipitation were spotted on nitrocellulose membrane using a BioRad Dot Blot apparatus. GM1 was detected with Cholera toxin B and anti-cholera toxin B antibodies, followed by ECL detection.

Immunofluorescence Microscopy

C2C12 myotubes were rinsed with PBS and fixed at 4°C for 15 min with 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Fixed cells were rinsed with PBS, and where needed (for immunodetection of flotillin-2 or caveolin-3) permealized with 0.1% Triton X-100 in PBS for 5 min at room temperature (or with methanol 95% for 5 min at -20°C) and washed. After preincubation for 30 min at room temperature in PBS containing 1% bovine serum albumin (BSA) and 5% goat serum (GS), C2C12 myotubes were incubated for 1 h at room temperature with primary antibodies: anti-caveolin-3 (1:400) or anti-flotillin-2/ESA (1:100) in PBS supplemented with 0.1% BSA and 0.5% GS, then rinsed 3x with PBS for 5 min and incubated with the secondary antibodies for 30 min at room temperature (RITC-conjugated goat anti-mouse, 1:400). In all experiments, AChR detection was achieved by FITC-conjugated α-Bgtx (3 μg/ml). Before observation, samples were mounted in Citifluor (UKC. Chemlab). Micrographs were taken with a Leica DMR microscope (Leica Systems) equipped with PL Fluotar x40 or Plan Apo x63 oil immersion optics and a Micro Max cooled CCD camera (Princeton Instruments, Inc.) operating at full resolution. Digital images were recorded using Meta View Imaging System (Universal Imaging Corp.) and prepared for publication using Adobe Photoshop.

Laurdan 2-Photon microscopy

Differentiated C2C12 myotubes were labeled with Laurdan by incubating washed cells (1% HS in DMEM) with 5 μM Laurdan in 1% HS in DMEM for 30 min at 18°C or 37°C. Myotubes were washed in PBS, fixed with 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After washing, cells were labeled with Alexa 555-conjugated α-Bgtx (3 μg/ml, Molecular Probes), washed and mounted in CityFluor. Multi-photon excitation of Laurdan fluorescence was at 800 nm with a Verdi/Mira 900 laser system and intensity images were recorded simultaneously in the range of 400-450 nm and 450-530 nm with a Biorad microscope. The relative sensitivity of the two channels was calibrated with 5 μM Laurdan in DMSO for each experiment. For confocal microscopy a helium-neon laser was used to excite Alexa 555 with appropriate cut-off filters and pinhole widths. For all images, a 100x oil objective, N_A=1.4 was used. Alexa IF stain was imaged first and then Laurdan at the same focal depth. For Laurdan image analysis, all calculations were carried out in floating point format and images converted into 8-bit unsigned images for presentation using the imaging software WIT. The Generalized Polarization (GP), defined as:

$$GP = \frac{I_{(400\text{-}450)} - I_{(450\text{-}530)}}{I_{(400\text{-}450)} + I_{(450\text{-}530)}}$$

was calculated for each pixel using the two Laurdan intensity images. GP images were pseudo colored in Adobe Photoshop. To determine GP values at α-Bgtx clusters, confocal images were used to mask the GP images; the confocal images defined the regions of interest (ROI) and the mean GP value of the ROI was determined for each image. GP values were corrected using the G-factor obtained for Laurdan in DMSO for each experiment (33).

Image quantification

To determine the line profiles of the AChR clusters, a line was drawn along the main axis of myotubes and the fluorescence intensity was plotted versus relative distance using Image J software 1.26t (NIH, Bethesda, MD). To estimate the magnitude of the effect of drug treatments on AChR clusters, the number and the average size of clusters were measured using a calibrated mesh. The average length of AChR aggregates l was estimated according to:

$l = \pi . n . d / 4N$,

where *n* is the number of intersections between clusters and the mesh , *d* the size of the mesh, and N the total number of clusters. For each experiment 5 optical fields selected at random were analyzed at a magnification of 1600x.

Quantification of proteins and cholesterol

The total amount of proteins in the C2C12 cells as well in the various fractions collected from the gradients was determined according to the Pierce protocol of the 'BCA™ Protein Assay Reagent kit' (Rockford, IL). Cholesterol quantification was based upon cholesterol oxidase method for cholesterol determination by spectrophotometry using Thermo Trace assay (Thermo Electron Corporation, Melbourne, Australia). Calibration was done with aqueous standard solutions of cholesterol (Thermo Electron Corporation, Melbourne, Australia). For total cholesterol determination in C2C12 myotubes, cells were previously washed in lipid-free synthetic culture medium (DMEM) and lysed by Triton X-100.

Quantification of AChR in C2C12 myotubes

Quantification of AChR (34, 35) expressed at the cell surface was determined by incubating fixed (3% paraformaldehyde, 10 min at 4°C) differentiated C2C12 myotubes with ^{125}I-α-Bgtx. For intracellular AChR quantification, fixed C2C12 myotubes were incubated with cold α-Bgtx (5 nM) for 1 h at 4°C to block the α-Bgtx binding sites of the cell surface, then permealized with Triton 0.1% for 5 min. Total pool of AChR was carried out on cells also permealized with Triton 0.1%. For cell surface, intracellular and total AChR quantification, cells were incubated for 2 h on ice with 5 nM ^{125}I-α-Bgtx (Amersham Biosciences ; 150 Ci/mmol) in PBS containing 1 mg/ml BSA, followed by three washings on ice with PBS/BSA. Cells were lysed in PBS containing 1% SDS at 37°C for 1 h. Cells lysates were collected and ^{125}I radioactivity was counted for 1 min on a MR480 gamma counter (Kontron Electronik, Eching, Germany). Non-specific binding determined after competition of ^{125}I-α-Bgtx binding with a large excess of cold α-Bgtx (5μM) was generally less than 5-10%. Each experiment was done in triplicate. In all experimental conditions, we verified that the same amount of total protein was present and expressed the data as percent of total AChR in control myotubes.

Negative staining electron microscopy

Samples of DRM fractions directly taken from the gradients were supplemented with 10 μg/ml bacitracine (Sigma). Ten μl drops were then deposited onto carbon-coated EM grids. Negative staining was achieved by washing the grids with 100 μl of 1% (wt/vol) uranyl acetate in water and dried with filter paper. Grids were observed in a Philips (Eindhoven, the Netherlands) EM12 electron microscope fitted with a LaB6 filament and operation at 80 keV. Micrographs were taken on Kodak electron microscope films.

Results

Cholesterol is required for AChR clustering in C2C12 myotubes

As lipid rafts are enriched in cholesterol, the removal of cholesterol from cell membranes causes the dispersion of lipid rafts within the fluid phase of the cell membrane and perturbs signaling efficiency (14, 18, 36). To determine whether cholesterol-enriched lipid domains are involved in AChR clustering, we used MβCD, a useful tool that has been shown to extract cholesterol from biological membranes with high preference over other lipid species, to selectively deplete cholesterol from the cell membrane in C2C12 myotubes. Similar to what is reported with other cells, MβCD treatment (10 mM, 30 min) of C2C12 myotubes released approximately 60% of total cellular cholesterol. We thus addressed whether MβCD treatment perturbs already established AChR aggregates, which were previously induced by agrin at the surface of myotubes. In myotubes incubated overnight with agrin, numerous AChR clusters measuring up to 10-20 μm (mean length 4.0±1.0 μm) were detected at the surface of myotubes by fluorescence microscopy using FITC-conjugated α-Bgtx as a marker for AChRs (Fig. 1A). In cholesterol-depleted cells small dispersed AChR aggregates with a mean size of 1.3±0.2 μm were observed instead of the large AChR clusters (Fig. 1B). Plots of line profiles along the main axis of myotubes showing the fluorescence intensity versus distance illustrated the dispersion of AChR clusters into smaller patches following MβCD treatment (Fig. 1C and D corresponding to Fig. 1A and B, respectively), which does not result in a larger number of smaller clusters. For control cultures we found 47 clusters/field ± 8, n=5 and 58 clusters/field ± 10, n=3 after MβCD treatment. In a second series of experiments we asked whether the presence of cholesterol is required for agrin-induced AChR clustering. We thus deplete cholesterol from myotubes using MβCD before induction of AChR clustering by agrin. In these conditions, no large AChR clusters (≥4 μm) were observed (Fig. 1 F, E= control). Cholesterol-depleted myotubes were thus no longer able to form typical AChR clusters as recently reported by Campagna and Fallon (23).

In previous works by us and others (22, 37, 38), raft lipids have been shown to be required for intracellular trafficking and surface transport of AChRs. To ascertain the implication of lipid rafts on agrin-elicited AChR clustering, rather than the effect of the depletion in cholesterol on AChR biosynthesis or/and surface expression, we quantified surface, intracellular and total AChRs in control and MβCD-treated myotubes by means of

^{125}I-α-Bgtx binding. We observed that MβCD-treated myotubes exhibit similar total AChR content (93±13% versus 100±19%, n=6 for MßCD-treated myotubes and control, respectively), cell surface (49±14% versus 53±14%, n=3) and intracellular (47±21% versus 60±15%, n=3) AChR, as compared to controls (all data were expressed as percent of total AChR in control myotubes =100%). These data show that in our experimental conditions (10 mM MβCD, 30 min) cholesterol depletion of myotubes has no major effect on AChR biosynthesis, surface transport or internalization. We thus concluded that cholesterol is required for the agrin-elicited AChR clustering.

AChR clusters correspond to condensed membrane domains of the myotube surface

So far, our data in agreement with other reports (23) suggest a role of cholesterol in AChR clustering. Lipid rafts, as entity, constitute a highly ordered membrane domain that is distinct from the more fluid surroundings. To demonstrate that AChR-rich microdomains indeed correspond to ordered domains, we applied Laurdan 2-photon fluorescence microscopy, which allows the direct visualization of membrane order (26). To assess the degree of lipid condensation at AChR clusters, C2C12 myotubes were labeled with Laurdan before or after agrin induction, imaged and the intensity images converted into Generalized Polarization (GP) images as previously reported (33, 39). Regions of high GP values (0.25 to 0.55) corresponded to ordered domains (colored yellow to red) while low GP (-0.05 to 0.25) corresponded to fluid domains (colored green). Figure 2 showed control (A-C) and agrin-treated myotubes (D-F) labeled with Laurdan and α-Bgtx. In control cells (A, C), no regions of high GP were detected (mean GP= 0.234 ± 0.028 ; n=24, see Table). In agrin-treated myotubes (D, F), GP values of α-Bgtx labeled AChR clusters were significantly increased (mean 0.303 ± 0.088 n=38, P<0.001) with peak values reaching 0.6 to 0.8 (see line plot in F). The line plot (F) shows the profile of GP values and α-Bgtx fluorescence intensities across three AChR clusters (E) indicating that high GP regions were associated with most AChR clusters (open arrows). However, some AChR clusters did not correspond to particularly condensed membrane domains (filled arrow in Fig. 2F). Labeling myotubes at 18˚C to block endocytosis (see Table) yielded the same results with mean GP values of agrin-induced AChR clusters of 0.312 ± 0.065 indicating that AChR clusters reside in ordered membrane domains at the cell surface. Furthermore, cholesterol depletion with MßCD (10 mM, 30 min) before or after agrin treatment (G-I) significantly decreased the mean GP value of toxin-stained membranes (J-L) when compared to control, agrin-treated myotubes (see Table). Importantly,

MßCD treatment did not globally reduce membrane structure below control membranes (compare average GP value in I and L to C), suggesting that MßCD treatment was specific to cholesterol-enriched domains. We conclude that agrin-induced AChR clusters resemble lipid rafts biophysically and that the formation, size and structure of membrane domains after agrin signaling are dependent on cholesterol.

AChR partitions within DRMs in C2C12 cells

The biochemical characterization of lipid rafts from various cell types has been mainly facilitated by isolation procedures based on their relative insolubility in cold, non-ionic detergents (typically 1% Triton X-100). However, it has become clear that detergent treatment itself can induce the formation of lipid domains, which do not exist in the native membranes prior to detergent extraction. To circumvent a possible artefactual association of AChR with DRMs, alternative detergent-free extraction procedures have been developed (30, 31). Here we used three different extraction protocols to characterize synaptic components in DRM fractions purified from cultured muscle cells. In a first approach, we applied the standard Triton X-100 extraction protocol on C2C12 myotubes to isolate DRMs in a 5/30/40% discontinuous sucrose density flotation gradient. Fractions were analyzed for total protein and raft lipids (cholesterol and GM1 ganglioside, Fig. 3A). Relatively little protein was present in the light/DRM fractions (fractions 4-6 corresponding to the 5/30% interface), the bulk of the proteins being found in the dense fractions, as generally reported. Conversely, cholesterol and GM1 levels were high in the DRM fractions (about 150 μg cholesterol/mg protein) compared to high-density fractions (10-20 μg cholesterol/mg protein). These properties are typical characteristics of rafts/DRMs. We further characterize these fractions by immunoblotting using antibodies against known raft proteins such as caveolin-3, the muscle isoform of caveolin (40), and flotillin-2/epidermal surface antigen (ESA), the flotillin isoform upregulated during the differentiation of skeletal myoblasts (41). As shown in Figure 3 B, these two markers are enriched in fractions 4-6, as expected for DRMs. Further, light fractions, which displayed canonical biochemical properties of lipid rafts also contained robust amounts of AChR. Interestingly, the signaling/adapter protein syntrophin, a postsynaptic protein concentrated at AChR-rich sites in skeletal muscles (27, 42) was also recovered in DRM fractions. Small amounts of rapsyn were also detected in these fractions. Conversely, the non-raft proteins tubulin and transferrin receptors are present almost exclusively in the heavier fractions of the gradient. Consistent with other reports (43, 44), we also observed that the protein/detergent (Triton X-100) ratio was critical for AChR

partitioning in DRMs. A minimal protein/detergent ratio of 1/1 (wt/wt) was required to recover AChRs within light fractions of the gradients as DRMs. The present data are in agreement with a previous work carried out in transiently transfected COS-7 cells, in which we showed that the majority of AChR was recovered in TritonX-100-resistant membranes (22*)*.

Because DRMs were isolated from post-lysis membranes, which might either induce the formation of lipid domains or exclude proteins that are weakly associated with rafts, we used alternative detergent-free methods for DRM isolation: extraction of myotubes by sodium carbonate, pH 11/ sonication (30) shown in Fig. 4 or a simplified extraction method using shearing of cells in a divalent cations buffer (calcium/magnesium buffer; 31) shown in Fig.5. In alkaline extracted myotubes, the distributions of cholesterol, GM1 and total protein along the gradients as well as the cholesterol/protein ratio of the light fractions (5/30% interface, Fig. 4) were similar to those obtained after Triton X-100 extraction (Fig. 3A). AChR was almost totally recovered in low buoyancy fractions (Fig. 4B) together with raft markers flotillin-2 or caveolin-3. Again, part of rapsyn immunoreactivity was recovered in DRMs. Extraction in Ca^{++}/Mg^{++} buffer, according to Macdonald and Pike (31) resulted in the isolation of a cholesterol-rich fraction at the 30% to 35% interface of the sucrose gradient in which flotillin-2 and AChR are totally recovered (Fig. 5B). Compared to the two other purification procedures, a slight shift towards the light fractions was observed for GM1 ganglioside compared to cholesterol (Fig. 5A). Note that the highest intensities of AChR and flottilin immunoreactivities were more coincident with cholesterol-enriched fractions than with GM1-enriched ones. This shift of GM1 could result from the more analytical sucrose gradient which allows a fine separation of subclasses of DRMs with different cholesterol/GM1 ratios. Taken together, the biochemical analysis of DRMs obtained from three extraction procedures based on different biochemical principles, demonstrated that AChR, syntrophin, rapsyn and possibly other synaptic components such as MuSK (data not shown) are associated with raft-like membrane domains.

Electron microscopic examination of the DRM samples derived from the three extraction procedures (Fig.6) disclosed vesicular profiles with diameters ranging from 0.1 to 0.5 μm. Such structures are similar to those of PSD-70-enriched dendritic raft fraction isolated from rat forebrain (44). The Ca^{++}/Mg^{++} fraction was more homogenous than the two others and differed in vesicle mean diameter (0.1 to 0.2 μm). Given the small size attributed to native rafts in situ (46), these large DRM vesicles likely correspond to aggregates of raft domains.

To assess whether agrin recruits AChR into rafts, we analyzed the DRM fractions obtained from control and agrin-treated myotubes. In all extraction conditions (Triton X-100, pH 11 and $Ca^{++}Mg^{++}$ buffer), we were not able to detect any significant difference in the distribution patterns of AChRs along the gradients (Fig. 7) between control and agrin-treated myotubes. We conclude that AChRs are constitutively associated with cholesterol-rich membrane domains and that agrin did not significantly contribute to translocation of AChR into lipid rafts.

AChRs, caveolae and flotillin-enriched rafts segregated as distinct microdomains at the cell surface of myotubes upon agrin engagement

In mammalian cells several classes of rafts have been described, most prominently caveolin-containing membrane invaginations (caveolae) or non-caveolin-containing rafts (47). Owing to their specific biochemical composition, all DRMs display similar buoyancy and therefore cannot be separated by current gradient flotation centrifugation. It is likely that all proteins enriched in the light fractions from C2C12 myotubes resulted from the accumulation of the various raft subclasses. In order to determine whether caveolin, flotillin and AChR are present in specialized subclasses of rafts, in situ, we studied the localizations of AChR clusters, caveolin-3, and flotillin-2 by immunofluorescence microscopy in C2C12 myotubes following agrin treatment. Figure 8 shows that caveolin-3 (A, B) as well as flotillin-2 (C) were both excluded from agrin-elicited AChR clusters. A similar mutual exclusion between AChR and caveolin-1-rich membrane domains has previously been observed in transfected COS-7 cells (22). We thus conclude that upon agrin engagement, AChR-enriched rafts become clustered at the cell surface independently of caveolin-3 or flotillin-2-enriched raft subclasses. The present data suggest that several functionally specialized subsets of rafts coexist at the surface of myotubes, and that AChR clustering results from the regulated coalescence of one (or a few) distinct raft subclass (es), enriched in postsynaptic components.

Discussion

Cholesterol- and sphingolipid-enriched microdomains or lipid rafts have been added to the bilayer model to explain lateral segregation within cellular membranes. As such, lipid rafts would regulate local concentrations of membrane proteins and lipids. Accordingly rafts have been implicated in protein and lipid sorting and trafficking, vesicle formation and docking, entry and exit of pathogens as well as in receptor-mediated cell signaling (48, 49). Because lipid rafts can move laterally and cluster into larger patches (39), they have been proposed to play a role in the redistribution of specific molecules to specialized cellular structures. Indeed, rafts have been shown to favor formation and maintenance of synaptic receptor clusters in neurons of the central nervous system (17-20) and in myogenic cells (23).

Here we showed that in C2C12 myotubes, agrin-elicited AChR clusters correlated with condensed membrane domains - the biophysical hallmark of lipid rafts. Further, we demonstrated that the formation of AChR clusters and their size, as well as the structure of the membrane domains they reside in, depend on cholesterol. We also report that three different extraction procedures generated DRMs with high cholesterol/GM1 ganglioside content, which are enriched in several postsynaptic components, notably AChR, syntrophin and raft markers flotillin-2 and caveolin-3. Interestingly, agrin did not recruit AChR into DRMs suggesting that AChR is present in rafts independently of agrin activation. Taken together, the present data suggest that lipid rafts represent platforms for AChR clustering at the neuromuscular junction.

A major goal of the present study was to establish whether condensation of membrane domains or ordered membrane phases, by definition lipid rafts, correlate to AChR aggregation in myotubes upon agrin engagement. Laurdan fluorescence microscopy has been instrumental in quantifying the formation membrane order and defining cellular requirements of condensed membrane domains (26). By visualizing lipid structure and raft domains in living cells, Laurdan 2-photon microscopy provided strong support for the lipid raft hypothesis. Generally, live or fixed cells displayed two distinct plasma membrane regions. Those with a mean GP of 0.10-0.25 correspond to liquid-disordered phases and regions with a mean GP of 0.35 to 0.55 correspond to liquid-ordered phases (33, 39, 50). Our experiments reveal that AChR clusters in agrin-treated myotubes are associated with mean GP values (0.303 ± 0.088 at 37°C and 0.312 ± 0.065 at 18°C) statistically higher than the membrane of untreated myotubes (0.234 ±

0.028). Some discrete regions have a much higher GP up to 0.6/ 0.8, matching with Alexa-conjugated α-Bgtx staining (see Fig. 2 F). However, some well-defined AChR clusters, although showing a lower α-Bgtx staining, exhibited a lower GP. These regions probably accounted for the lower mean GP value of AChR clusters. These data are in agreement with early studies by the Barrantes group on the high lipid order of AChR-rich postsynaptic membranes from Torpedo fish as studied by Laurdan spectroscopy (51).

This technique also allowed us to assess the effect of cholesterol depletion on lipid structure in myotubes. In good agreement with dispersion of AChR cluster or inhibition of AChR clustering upon MβCD treatments, GP values also significantly decreased to quasi-disordered membrane phases. Because of its limited resolution (183 nm at 800 nm excitation wavelength), the 2-photon technique is not presently capable of visualizing individual rafts of estimated diameter 30 to 50 nm. Consequently, we cannot discriminate between a disappearance of AChR rafts from the cell surface and a dispersion of AChR clusters into unresolved small rafts upon cholesterol depletion. The data strengthen our conclusion that AChR-enriched membrane domains are cholesterol dependent. This is in agreement with other reports (23) but in contrast to raft coalescence in T cells where MßCD treatment diminished membrane order but did not abolished it (39).

Although raft biochemical extraction have raised controversies (52, 53), the demonstration by Gaus et al. (44) that THP-1 macrophages treated either by 0.2% Triton or sonication yield membranes of similar lipid structure, prompted us to biochemically investigate rafts from C2C12 myotubes. We show here that three different biochemical preparations of DRM fractions using either cold Triton X-100 or detergent-free extraction procedures (Fig. 3 to 6) yielded vesicular light fractions showing a remarkable similarity as they are enriched in raft lipids (cholesterol and ganglioside GM-1), in the raft markers caveolin-3 and flotillin-2, and in AChR. These DRMs also contained syntrophin as well as a small part of rapsyn and MuSK (data not shown), pointing to the possibility that the regulation of AChR clustering at synaptic sites by the agrin/MuSK/rapsyn machinery is dependent on rafts. These observations are in agreement with our previous study showing that AChR and rapsyn expressed transiently in COS-7 cells also partitioned in DRMs (22).

Our finding that AChR as well as other synaptic proteins are included within rafts is consistent with the accumulation of cholesterol and GM1 ganglioside in AChR-rich postsynaptic membranes purified from Torpedo electric tissue (54, 55), and with freeze-fracture data of filipin-treated rat myotubes in which spontaneous AChR clusters appear

enriched in cholesterol (56). Cholesterol is also important for AChR function as it preserves agonist-induced affinity state transitions (57). Moreover, the physical state of the AChR-rich postsynaptic membrane from Torpedo as studied by Electron Spin Resonance (58, 59) showed that the protein-vicinal lipids are immobile relatively to the rest of the membrane lipids. Our findings are also in keeping with recent reports showing that several neurotransmitter receptors and ion channels are biochemically located in lipid DRMs and that cholesterol is required for their maintenance at postsynaptic sites or dendritic spines (16).

In a recent publication (23) it was reported that agrin treatment results in an increase in AChR association with lipid rafts. However, this assertion was based on fluorescence experiments, the resolution of which (~200 nm) is far below the estimated size of individual rafts (10 to 50 nm). As such, it is not possible to deduce from these experiments whether AChRs reside in rafts before clustering. Yet, DRM fractions obtained from untreated myotubes in various extraction conditions (Triton X-100, pH 11 and $Ca^{++}Mg^{++}$ buffer) contained robust amounts of AChR and we were not able to detect any difference in the distribution patterns of AChRs along the gradients between control and agrin-treated myotubes. These findings suggest that the association of AChRs with DRM is not dependent on agrin. This observation was not surprising since rafts participate in intracellular trafficking and surface targeting of AChR (22). In addition, cells defective in sphingolipid biosynthesis express low amounts of cell surface AChRs (60). Since intracellular trafficking and surface targeting of AChR are not likely to be controlled by agrin, the AChRs delivered to the cell surface are very likely constitutively embedded in rafts. Collectively, the present data lead to the notion that agrin activation regulates the coalescence of AChR-containing rafts rather than the recruitment of AChR to rafts.

Considered as platforms for concentrating individual receptors activated by ligand binding, rafts are believed to play important roles at the cell surface through signal transduction (48, 61). Besides the formation of the immunological synapse (62, 63, 64), many other signaling pathways including EGF, GNDF and Ras signaling (48), tyrosine phosphorylation (12), and signaling through phosphatidylinositol-4, 5-bisphosphate (65, 66) rely on lipid rafts. In this view, the occurrence of several effectors of the agrin signaling pathway leading to AChR clustering such as MuSK, rapsyn and syntrophin in DRMs is of interest. Accumulating evidence indicate that rafts are too small to engage in membrane function. To trigger signaling transduction processes, they should usually have to cluster. An attractive hypothesis from the present work is that raft coalescence may be an important step of the agrin-induced AChR clustering, through activation of MuSK. Given the small size of

individual rafts, it is possible that each raft contains a different subset of proteins i.e. AChR, MuSK or rapsyn. Upon agrin engagement, AChR clustering could occur when many rafts cluster together forming a large platform where the proteins can meet. Activated platforms could then recruit in a reversible and dynamic manner scaffolding and/or cytoskeletal proteins that promote large raft assembly (67, 68). Along this line, it is worth noting that agrin-induced AChR clustering depends on an actin-driven movement of AChR protein within the sarcolemma (8) and that MuSK activation also triggers actin cytoskeleton reorganization via activation of Rac/Cdc 42 (9, 69, 70). Yet, lipid rafts, as preferred platforms for membrane-linked actin polymerization (68), might serve as nucleation sites for AChR clustering upon MuSK signaling (see Figure 9). Raft-regulated AChR clustering process may also be important for synapse specification by keeping raft-borne synaptic proteins in an "off" state, to prevent undue AChR clustering outside synaptic sites.

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Legends to figures

Figure 1. Cholesterol is required to induce and maintain AChR clusters in C2C12 myotubes. Agrin-elicited AChR clusters were detected by FITC-conjugated α-Bgtx labeling in C2C12 myotubes (A, E). Cholesterol depletion by MβCD post-agrin (10 mM, 30 min at 37°C, see Materials and Methods) induced the dispersion of agrin-induced AChR clusters into patches of smaller size (B). Fluorescence intensity for AChR aggregates were plotted versus relative distances along the main axis of myotubes corresponding to fig A and B, respectively (C, D). Depletion of cholesterol before agrin treatment prevented the formation of AChR aggregates at the surface of myotubes (F). Internal controls (A, F) were provided for each set of experiments. Bar =10 μm.

Figure 2. Membrane condensation at agrin-induced AChR clusters in C2C12 myotubes. Laurdan-labeled C2C12 myotubes were simultaneously imaged for the Laurdan intensity in two channels (400-450 nm and 450-530 nm) using 2-photon laser scanning microscopy and for Alexa 555-conjugated α-Bgtx. Intensity images were converted to GP images as described in Materials and Methods. A,D, G and J Global Polarization images were pseudo-coloured for low and high GP from blue to yellow, respectively (scale in A). B, E, H and K show the confocal images of Alexa 555-α-Bgtx staining of AChRs of the same fields and focal depth as the corresponding GP images. C, F, I, L show plot profiles of the fluorescence intensity of the toxin (blue, left y axis) and the GP values (red, right GP axis) along the blue line indicated in B, E, H, K, respectively. A-C: control myotubes, D-F: agrin treated myotubes, G-I: MßCD pre-agrin , J-L: MßCD post-agrin. The plot profile in F disclosed high GP corresponding to two AChR clusters (open arrows) and an AChR cluster with low GP (filled arrow). Note that GP values of MßCD-treated myotubes fall down to control values (compare red plots in I, L with C).

Figure 3. DRM association of AChR, syntrophin and rapsyn in non-ionic detergent extracts of C2C12 myotubes. (A) Distributions of cholesterol, proteins and GM1 across the sucrose step gradient. Protein and cholesterol content of each fraction were determined as indicated in Materials and Methods, and normalized to 1, according to their respective

maximal values. For GM1 distribution along the gradient, dot blots were revealed out using CTX and anti-CTX antibodies followed by ECL detection. (B) Western blots showing the distribution of caveolin-3, flotillin-2/ESA, AChR (α-subunit), syntrophin, rapsyn, transferrin receptor and tubulin along the sucrose density gradients performed after extraction of myotubes on ice with Triton X-100 (1/1 Triton/protein ratio : wt/wt). AChR and syntrophin comigrated with the two raft markers in light fractions (4 to 6 =5/30% interface), well separated from non-raft transferrin receptor and tubulin recovered in the heavy fractions (9-12) at the bottom of gradient. Small amounts of rapsyn were also recovered in the DRM fractions.

Figure 4. Alkaline extraction of DRMs from C2C12 myotubes. (A) Distributions of cholesterol, proteins and GM1 across the sucrose step gradient after carbonate extraction of C2C12 myotubes. Cholesterol, GM1 and total protein profiles appear similar to those obtained following Triton X-100 extraction. (B) AChR partitioned in the light fractions (4 to 6) as did flotillin-2 and caveolin-3. Rapsyn was partially recovered in the DRM fractions.

Figure 5. Isotonic Ca^{++}/Mg^{++} *extraction of DRMs from C2C12 myotubes.* (A) Distributions of cholesterol, proteins and GM1 across an expanded sucrose step gradient after isotonic Ca^{++}/Mg^{++} extraction. Cholesterol-enriched fractions were recovered in the 30% and 35% layers together with AChR and raft marker flotillin-2 (B). Note that in this expanded gradient, GM1 ganglioside-enriched membranes float with a slight shift towards light fractions.

Figure 6. Electron micrographs of DRM fractions from C2C12 myotubes. Negatively stained AChR-rich DRM fractions from the three different purification protocols (A= Triton X100, B= isotonic cation buffer, C= pH 11).

Figure 7. Absence of effect of agrin on DRM association of AChR. The effect of agrin on the distribution of AChR in step gradients following Triton X-100, pH 11, and $Ca^{++}Mg^{++}$ buffer was monitored by Western blotting. To minimize individual culture or extraction variations, control and agrin treated myotubes were strictly processed in the same conditions. In the Triton X-100 extraction conditions, we selected an experiment in which AChR was recovered both in light and heavy fractions to optimize the detection of a possible differential

AChR partitioning between DRM and non-DRM. The stars indicated the position of the AChR α-subunit (42 kDa).

Figure 8. AChR aggregates independently of caveolin-3 and flotillin-2/ESA-enriched microdomains in agrin-treated C2C12 myotubes. FITC-α-Bgtx labeling of AChR clusters (green fluorescence) did not coincide with anti-caveolin-3 (A, B) or anti-flotillin-2/ESA (C) labelings (red fluorescence). Merged images are shown. Bar = 10μm.

Figure 9. Model of postsynaptic apparatus formation by agrin –elicited raft coalescence at the neuromuscular junction. Rafts containing various components of the postsynaptic membrane (AChR, MuSK, rapsyn, syntrophin, etc…) were assembled and sorted in the exocytic compartments (1), transported within vesicles (2), and targeted to the cell surface, via microtubules (3). Tyrosine phosphorylation of MuSK by nerve-secreted agrin would induce actin-driven coalescence of AChR-containing rafts through reorganization of the actin cytoskeleton (4). Alternatively, it is possible that each raft accommodates a different subset of proteins i.e. AChR, MuSK or rapsyn. Upon agrin engagement, AChR clustering could occur when many rafts cluster together forming a large platform where the proteins can meet. Abbreviations: DG= dystroglycan, MT= microtubule, PM= postsynaptic membrane, PY= phosphotyrosine, TV= transport vesicle.

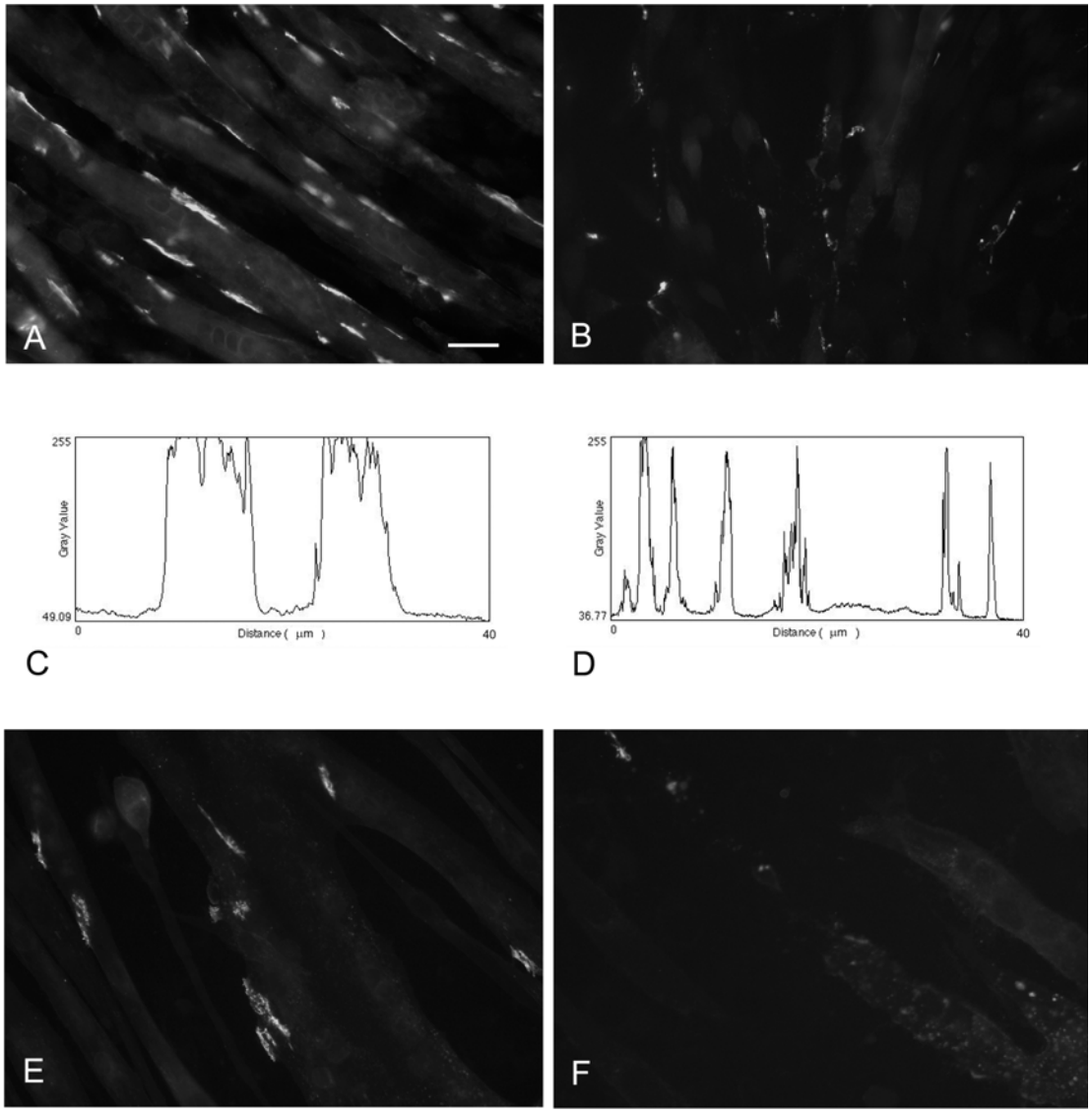
Table

Mean GP values ± standard deviations at AChR cluster membranes in agrin-treated and after cholesterol depletion of C2C12 myotubes.

	Mean GP value of 'receptor clusters'
- agrin	0.234 ± 0.028 (n=24)[a,b]
+ agrin	0.303 ± 0.088 (n=38)[a,c,d]
+ agrin, Laurdan labelling at 18˚C	0.312 ± 0.065 (n=15)[b]
mßCD post-agrin	0.255 ± 0.071 (n=20)[c]
mßCD pre-agrin	0.262 ± 0.058 (n=21)[d]

Pairs of a, b indicate P<0.001; c, d indicate P<0.05. All experiments were carried out at 37°C, except if indicated.

Figure 1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0
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C
255
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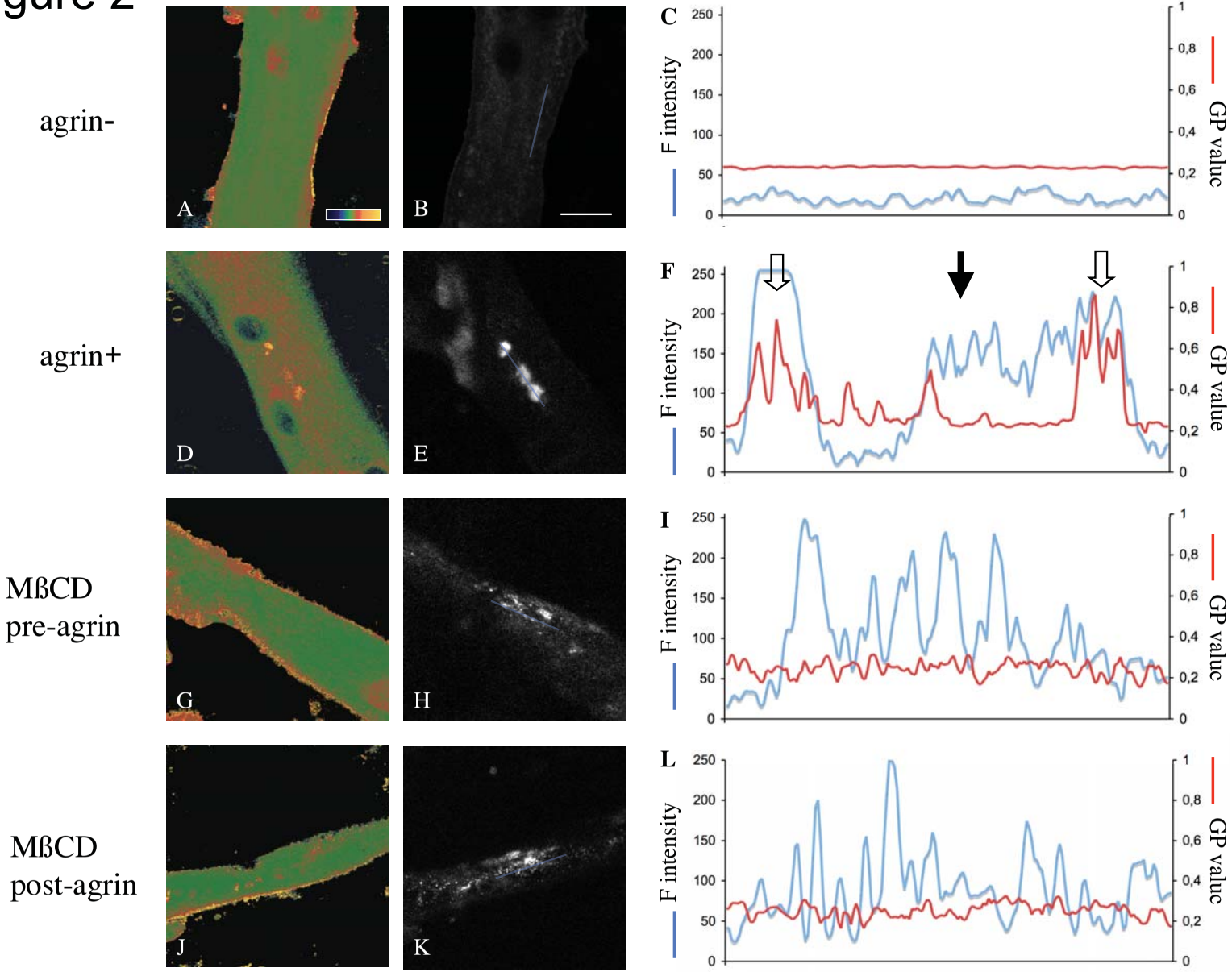
D
E
F

Figure 2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agrin+
MßCD
pre-agrin
MßCD
post-agrin
A
B
C
D
E
F
G
H
I
J
K
L
F intensity
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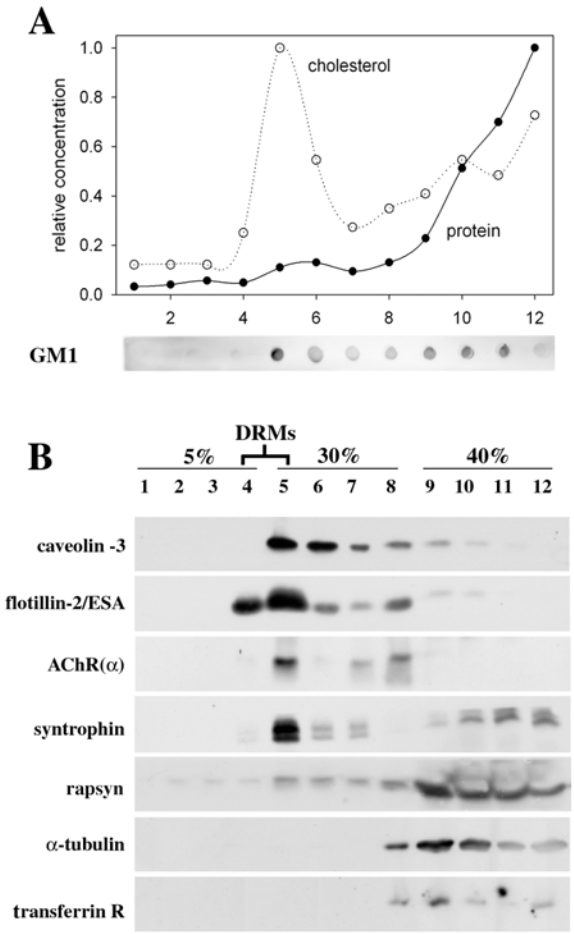
0,4
0,2
0

Figure 3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protein
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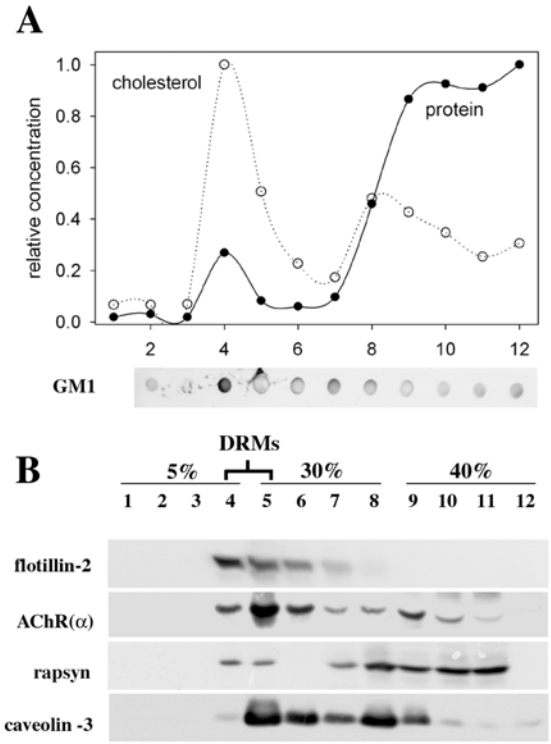
4
6
8
10
12
GM1
B
DRMs
5%
30%
40%
1 2 3 4 5 6 7 8 9 10 11 12
caveolin -3
flotillin-2/ESA
AChR(α)
syntrophin
rapsyn
α-tubulin
transferrin R

Figure 4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0.8
0.6
0.4
0.2
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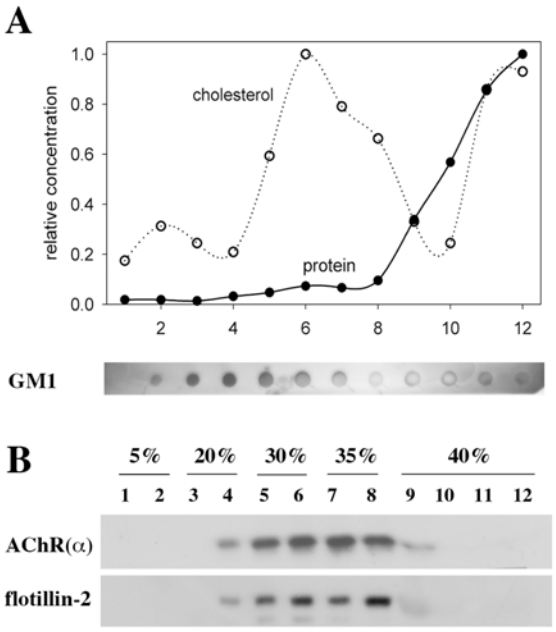
2
4
6
8
10
12
GM1
B
DRMs
5%
30%
40%
1 2 3 4 5 6 7 8 9 10 11 12
flotillin-2
AChR(α)
rapsyn
caveolin -3

Figure 5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cholesterol
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8
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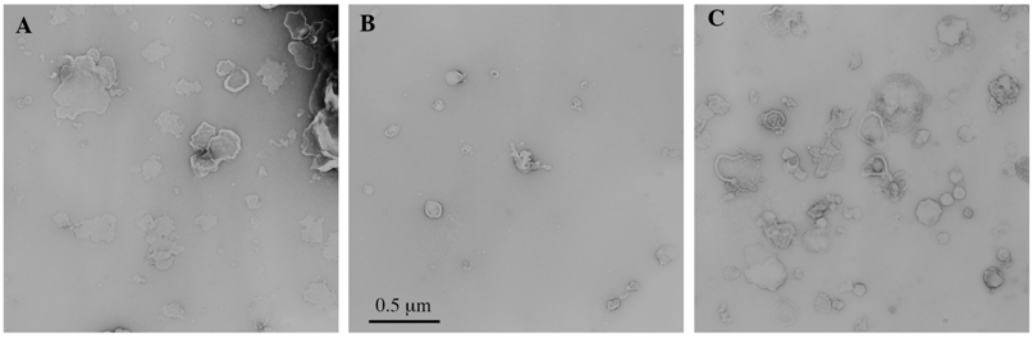
12
GM1
B
5%
20%
30%
35%
40%
1 2 3 4 5 6 7 8 9 10 11 12
AChR(α)
flotillin-2

Figure 6



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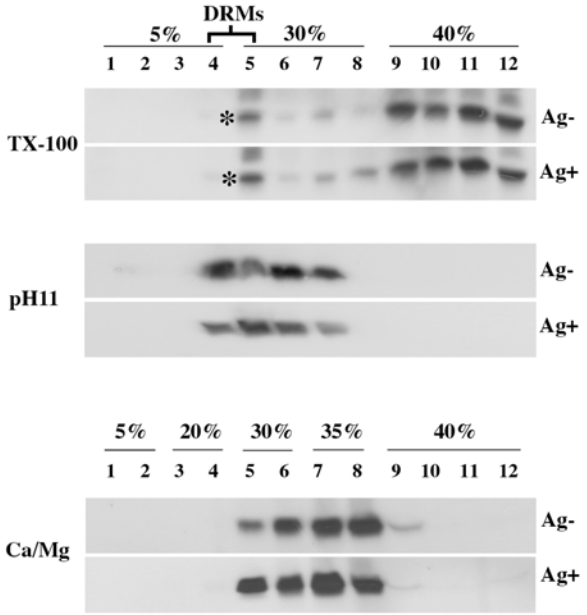
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30%
40%
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TX-100
Ag-
Ag+
pH11
Ag-
Ag+
5%
20%
30%
35%
40%
1 2 3 4 5 6 7 8 9 10 11 12
Ca/Mg
Ag-
Ag+

Figure 8

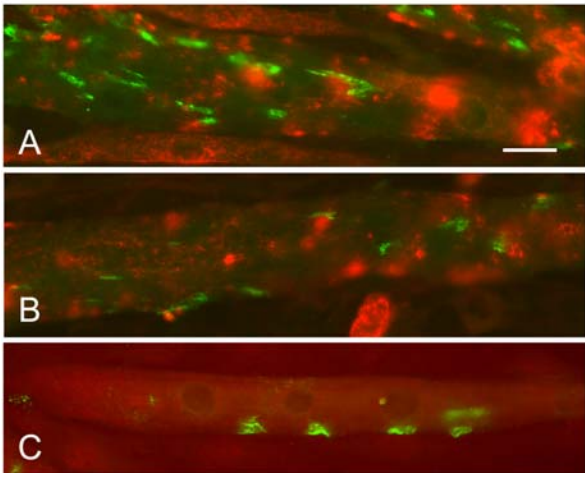
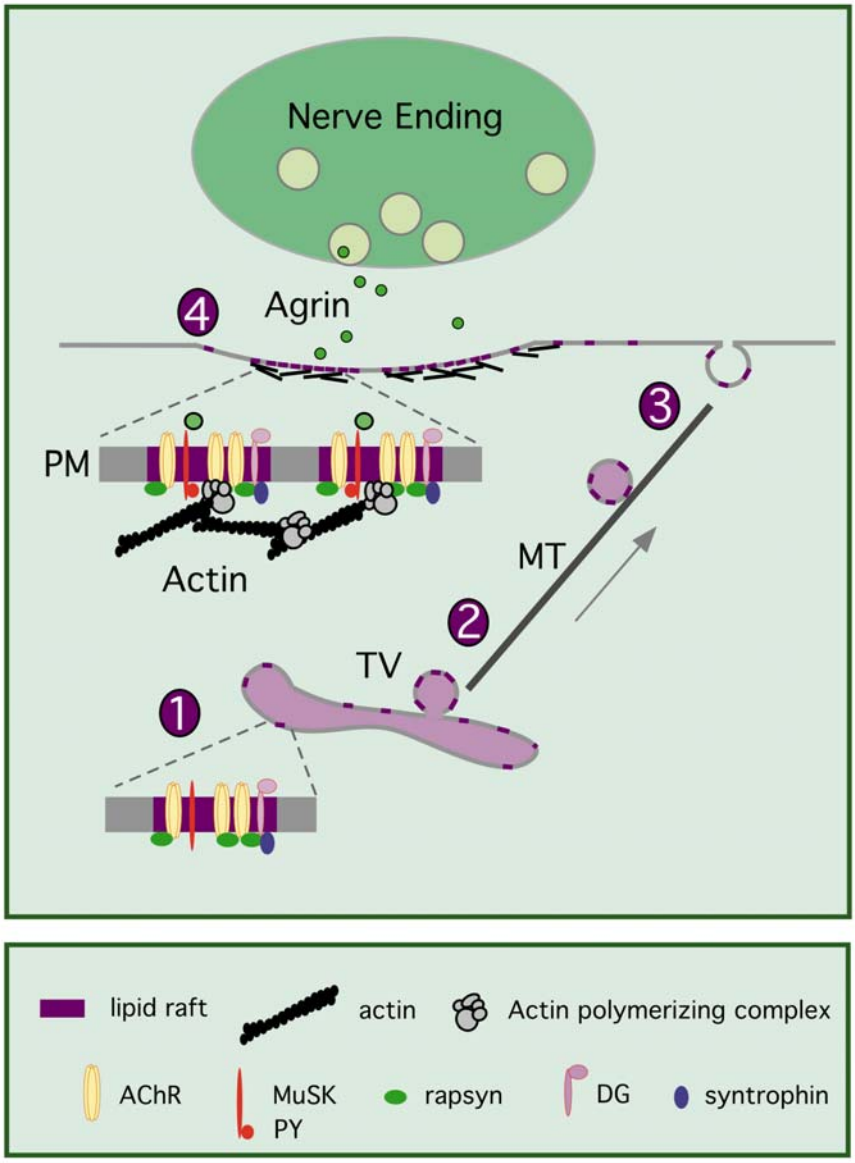


Figure 9


Nerve Ending
Agrin
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PM
Actin
3
MT
2
TV
1
lipid raft
actin
Actin polymerizing complex
AChR
MuSK
PY
rapsyn
DG
syntrophin