









Biophysical and Ion Channel Functional Characterization of the *Torpedo californica* Nicotinic Acetylcholine Receptor in Varying Detergent–Lipid Environments


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Abstract The nicotinic acetylcholine receptor (nAChR) cholamidopropyl]-1-propane sulfonate}, FC-12 (n-dodecylphosphocholine) and sodium cholate (3,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) structural studies have been hampered by the lack of maintaining stability and supporting ion channel function, mechanistic molecular models that describe how detergent and non-lipid-analogue detergents Cymal-6 (6-cyclohexyl-*l*-lysine), DDM (1,3-bis(2-dodecyl-D-maltopyr-thermore, elucidation of the dynamic detergent–lipid interactions of solubilized membrane proteins is a largely unexplored research field. This study examines the effects of nine detergents on: (1) nAChR-lipid composition channel function. Anapoe-9 (polyoxyethylene-[9]-dodecyl ether) and BigCHAPN, (2) stability and aggregation state (analytical size exclusion chromatography; A-SEC and electron microscopy; EM) and (3) ion channel function (planar lipid bilayers). Detergent solubilization of nAChR-enriched membranes did not result in significant native lipid depletion or destabilization. Upon purification, native lipid depletion occurred in all detergents, with lipid-analogue detergents CHAPS (3-[(3-

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Introduction

The nicotinic acetylcholine receptor (nAChR) is an essential component in the cholinergic pathways that regulate and control synaptic transmission in brain and muscle tissues. It is a member of the cysteine loop (Cys-loop) or ligand-gated ion channel (LGIC) family of receptors, which includes the neuronal and muscle-type nicotinic receptors, glycine receptor (Gly-R), α -aminobutyric acid type-A and type-C receptors (GABA_A and GABA_C) receptors, 5-hydroxytryptamine type-3 (5-HT₃) as well as the invertebrate glutamate and histidine receptors (Karlin

2002). nAChRs from Torpedo electric rays are β -subunit to support nAChR function is 45 moles of lipid per-mole integral membrane protein complexes with a stoichiometry of receptor, and that complete loss of ion channel function of $\alpha_2\beta\gamma\delta$ (two α -subunits, one β -subunit, one γ -subunit and one δ -subunit) in a pentameric arrangement, and an intermole of receptor (Jones et al. 1988). Previous analysis of the effects of detergents on nAChR has four transmembrane segments (M1 Δ M4), and this ion channel function showed that sodium cholate and amino- and carboxy-terminal domains are located extracellularly (Karlin and Akabas 1995). In vertebrate neuromuscular junctions, binding of the endogenous neurotransmitter acetylcholine (ACh) initiates membrane depolarization, generating an action potential that travels across the membrane and ultimately results in the transmission of nerve impulses.

Despite extensive study over the past 30 years, there have been no successful high-resolution structures of the complete nAChR. Recent studies have yielded a three-dimensional nAChR EM structure using native Torpedo marmorata membranes (Miyazawa et al. 2003), and an X-ray crystal structure of the recombinant mouse α_1 nAChR extracellular domain (Dellisanti et al. 2007). More recently, the crystal structure of a prokaryotic LGIC from *Erwinia chrysanthemi* at 3.3 Å resolution has been reported (Hilf and Dutzler 2008). Similar high-resolution structural data of the receptor in the detergent-solubilized state has remained elusive, partly due to a lack of understanding about dynamic detergent-protein-lipid interactions. Previously published Torpedo nAChR structural studies have generally not analyzed the effects of detergent solubilization prior to crystallization trials (Hertling-Jaweed et al. 1988), and how lipid depletion could affect nAChR function and stability. Most nAChR detergent solubilization and purification data have been obtained from receptors prepared from native or sodium cholate-solubilized Torpedo membranes that were either: (1) reconstituted into native lipid vesicles through detergent dialysis, or (2) reconstituted into lipid vesicles at defined compositions, by exchanging native lipids for specific exogenous lipids during affinity chromatography purification, followed by detergent dialysis.

Given the many variations in receptor preparation protocols in these experiments, the type of detergent, whether the receptor is detergent-solubilized or affinity-purified, and the type of lipid the receptor is reconstituted into, it is difficult to develop a clear understanding of the most favorable detergent-lipid combination and purification methodology for preserving nAChR ion channel function.

In this study, we report systematic attempts to understand how detergent type, affinity purification and associated lipid composition affect nAChR stability and function. We use a battery of already proven techniques to assay nAChR lipid composition, ion channel function and stability as well as aggregation state, including planar lipid bilayers, EM, GC-FID, GC-MSD and A-SEC. We identify desirable detergent families for nAChR solubilization and purification in order to routinely obtain stable and functional purified nAChR for future biophysical and structural studies seeking to advance our current understanding of the nAChR.

Materials and Methods

Crude Membrane Isolation, Detergent Solubilization and nAChR Ligand-Affinity Purification

Sucrose gradient isolation of nAChR-enriched crude membrane fractions was performed as described (Ochoa et al. 1983), fractioning the gradient from the bottom up for immediate use, or freezing at 20 °C. Membranes were diluted in a 1:3 ratio with dialysis buffer-1 (DB-1): 10 mM MOPS (3-(N-morpholino)propanesulfonic acid, pH 7.4), 100 mM NaCl, 0.1 mM EDTA and 0.02% NaN₃. Membrane solubilization was carried out for 1 h at 4 °C with a 1% final concentration of each detergent (Table 1) followed by high-speed ultracentrifugation for 1 h and application of the supernatant to a 1.5 × 15 cm Econo-column (Bio-Rad Laboratories, Hercules, CA) containing ~12.5 ml bromoacetylcholine affinity resin, prepared as described (Bhushan and McNamee 2000). The column was washed with ~10 times the undiluted crude membrane volume that was originally applied to the column (5 ml of crude membranes + 50 ml of wash buffer), using DB-1 buffer supplemented with the detergent of choice at 1.5 times the critical micelle concentration (CMC) (Table 1). The nAChR was eluted with ~50 ml of wash buffer supplemented with 90 mg of carbamylcholine chloride (Carb; Sigma, St. Louis, MO) and 292 mg of NaCl, concentrated to ~2.5 ml and applied to a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) to remove the Carb and ligand. The receptor was eluted from the desalting column with ~5 ml of wash buffer and concentrated to 250 µl before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and BCA protein concentration assay (Pierce Biotechnology, Rockford, IL).

Sample Preparation for A-SEC

Approximately 150 µl of purified nAChR was reacted with α-bungarotoxin (α-BTX)-Alexa Fluor 488 antagonist (Invitrogen-Molecular Probes, Eugene, OR) in a 2.5:1 BTX:nAChR molar ratio for ~2 h at 4 °C. 50 µl of the complex were injected into an Ultimate-3000 chromatography system (Dionex, Sunnyvale, CA) with a KN-803 chamber and the front plastic holder cavity were filled with analytical column (Shodex, Kawasaki, Japan) in a mobile phase of 20 mM HEPES [4-(2-Hydroxyethyl)piperazine-1-1 mM MgCl₂ and 150 mM NaI. Salt bridges filled with ethanesulfonic acid, pH 7.5], 200 mM NaCl and 0.09% DDM.

Sample Preparation for EM

Affinity-purified nAChR (10 µl) was applied to the surface of glow-discharged, carbon-coated 400-mesh copper grids. After 1 min, the grids were sequentially washed

through ten (10) × 200 µl distilled, deionized water drops sitting on the surface of a sheet of parafilm. A 0.5 µl drop of 1% uranyl acetate solution was then applied to the grid and allowed to sit for 1 min, before removing the excess liquid by applying filter paper to the edge of the grid. Grids were imaged using a Tecnai F20 Twin transmission EM (FEI, Hillsboro, OR) equipped with a 4 K × 4 K charge-coupled device camera, operating at an accelerating voltage of 120 keV. Grid loading and unloading were performed manually or using a robotic system (Potter et al. 2004), and images were acquired using the Legikon software (Suloway et al. 2005). Low magnification imaging was used to identify up to nine (9) intact grid squares, either by manual inspection or using automated procedures (Cheng et al. 2007). After automated focusing, two (2) to four (4) images per square were acquired at a magnification of 50,000× with the camera binned by four (4; pixel size 0.652 nm), using an electron dose of 30–50 electrons/A.

Sample Preparation, Analysis and Data Processing for Planar Lipid Bilayer Assays

Affinity-purified nAChR detergent micelles were coated with PMAL-C8 [poly(maleic anhydride-alt-1-decene substituted with 3-(dimethylamino)propylamine; Anatrace, Maumee, OH] as described (Maretz et al. 2002). A BC525D bilayer clamp amplifier (Warner Instruments, Hamden, CT), connected to an 8-pole low-pass Bessel filter and a computer through a Digidata 1322-A interface (Molecular Devices, Sunnyvale, CA), was used to probe ion channel function using the following procedure. A mixture of brain L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) and cholesterol (Anatrace) on a 3:1:1 ratio at 75 mg/ml in chloroform was prepared, evaporated under nitrogen and resuspended in decane at the same concentration. A 1:1 dilution of this mixture was applied to a 200 µm aperture in a cup-shaped Teflon chamber and allowed to air dry before reopening the aperture and fitting into the rear cavity of a rectangular plastic holder, containing two electrode apertures, which were filled with 1 M KCl. Both the Teflon chamber and the front plastic holder cavity were filled with ~1 ml of bilayer buffer solution: 10 mM HEPES (pH 7.2), 1 mM MgCl₂ and 150 mM NaI. Salt bridges filled with 1 M KCl and 2% agarose were used to connect the electrodes to both chambers in the plastic holder, and the Teflon chamber aperture was re-coated with the lipid mixture until a seal was detected (membrane capacitance = 90–170 pA). Approximately 10–20 µl of PMAL-coated nAChR vesicles were added to the front plastic chamber and stirred for ~15 min, verifying protein incorporation through a ~5–10 pA change in membrane

Table 1 Physical properties of detergents used for nAChR biophysical characterization

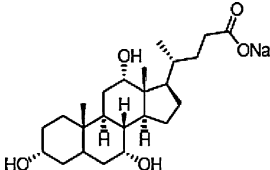
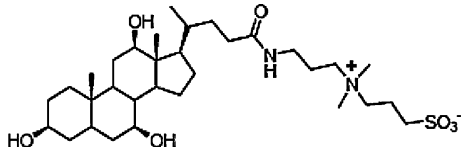
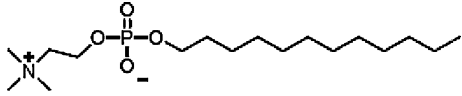
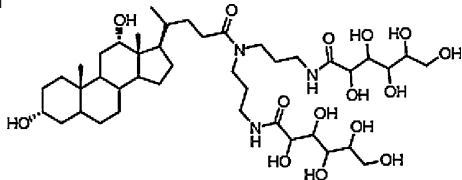
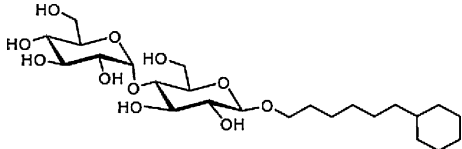
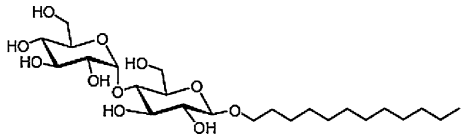
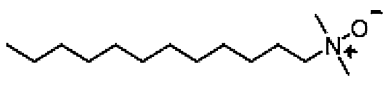
Name	CMC (mM)	Aggregation number	Solubilization concentration (mM)	Affinity column DB-1 wash buffer concentration ($1.5 \times$ CMC [mM])	Classification based on experimental results	Structure
Sodium cholate	14	~ 20	46.4 (2%)	21	Lipid-analog	
CHAPS	8	10	32.5 (2%)	12	Lipid-analog	
FC-12	1.5	∞	28.4 (1%)	2.25	Lipid-analog	
Anapoe-C ₁₂ E ₉	0.05	∞	17.2 (1%)	0.075	Retained residual amounts of native lipids	
BigCHAP	2.9	10	45.6 (4%)	4.35	Retained residual amounts of native lipids	
Cymal-6	0.56	63	19.7 (1%)	0.84	Non-lipid-analog	
DDM	0.17	~ 78	19.6 (1%)	0.26	Non-lipid-analog	
LDAO	10^2	∞	43.6 (1%)	3	Non-lipid-analog	

Table 2 Phospholipids and cholesterol associated with the nAChR prior to- and following affinity purification

Detergent	Detergent classification based on experimental results	nAChR prior to affinity purification		nAChR following affinity purification		Molar ratio of lipids per mole of nAChR ^{a,b}
		Total phospholipid (nmoles) ^a	Total cholesterol (nmoles) ^a	Total phospholipid (nmoles) ^a	Total cholesterol (nmoles) ^a	
Sodium cholate	Lipid analog	1398	1036	33	14	18
CHAPS	Lipid analog	1718	334	25	20	16
FC-12	Lipid analog	1836	183	53	34	28
BigCHAP	Retained residual amounts of native lipid	2320	263	53	90	40
Anapoe-C ₁₂ E ₉	Retained residual amounts of native lipid	3001	1478	166	180	53
Cymal-6	Non-lipid analog	1261	150	22	28	9
DDM	Non-lipid analog	1780	814	25	41	20
LDAO	Non-lipid analog	2557	1610	5	34	17
OG	Non-lipid analog	1875	659	6	17	9

Lipid analysis results for detergent-solubilized crude membranes and affinity-purified nAChR in each individual detergent, including detergent classification, residual native lipid amounts and molar ratio of lipids per-mole of nAChR

^a Total phospholipid and cholesterol amounts for both sucrose gradient membranes and affinity column samples were normalized using protein concentration values in mg/mL obtained from BCA assay measurements

^b Molar ratio = $\frac{\text{Total phospholipid} + \text{Total cholesterol following ligand-affinity column purification (nmoles)}}{\text{nmoles of affinity-purified nAChR}}$

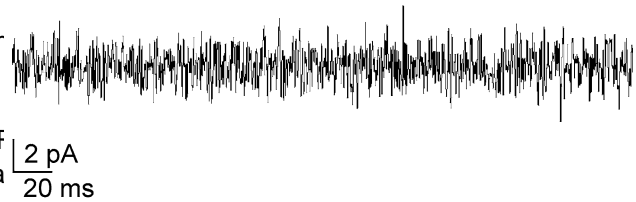
Detergent Effects on nAChR Function and Stability

In order to assess how the use of different detergents during affinity purification affects nAChR function and stability, we solubilized nAChR-enriched *D. californica* membranes, affinity-purified the receptor and assayed: (1) nAChR function by measuring ion channel conductance in planar lipid bilayers and (2) nAChR stability and aggregation by A-SEC. We found that the detergents displayed similar biophysical behaviors depending on whether they were lipid-analogue detergents, including sodium cholate, CHAPS and FC-12; detergents retaining residual amount of native lipids, including Anapoe-C₁₂E₉ and BigCHAP; or non-lipid-analogue detergents, including Cymal-6, DDM, LDAO and OG (Table 1).

Planar Bilayer Experimental Controls: Establishment of Baseline Measurements and α -BTX Blocking of Ion Channel Currents

A baseline for nAChR ion channel function measurements was established with affinity-purified nAChR in each detergent prior to each experiment, showing no signal in the absence of Carb at 70 mV membrane potential (Fig. 1a shows an example for sodium cholate). Incubation of affinity-purified nAChR in sodium cholate with α -BTX completely blocked ion channel currents in the presence of 0.5 μ M Carb (Fig. 1b).

(a) nAChR, no Carb.



(b) α -BTX, 0.5 μ M Carb.

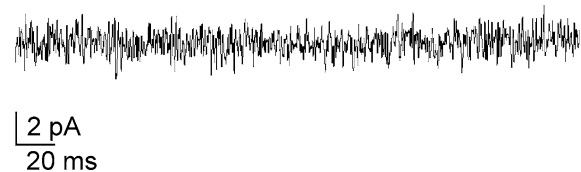


Fig. 1 Planar lipid bilayer experimental controls using affinity-purified nAChR in sodium cholate. Planar lipid bilayer ion channel function assay controls at 70 mV membrane potential: (a) affinity-purified nAChR with no ligand added and (b) nAChR incubated with α -BTX following affinity column purification in the presence of 0.5 mM Carb

Lipid-Analogue Detergents: Sodium Cholate, CHAPS and FC-12

Despite their structural differences (Table 1), these three detergents showed remarkably similar results in all categories. Figure 2a, c and e shows planar bilayer ion channel traces for sodium cholate, FC-12 and CHAPS, with clearly

defined opening/closing events; electrophysiological data. Detergents in which the nAChR retained higher amounts of residual native lipid following affinity purification: Anapoe-C₁₂E₉ and BigCHAP. Table 3. The overall data compares favorably to previous studies of Con-A purified nAChR in sodium cholate, reconstituted into soybean lipid vesicles prior to planar lipid bilayer experiments (Nelson et al 1980). These results suggest that despite significant lipid depletion upon affinity purification (Table 2), the receptor is fully functional in lipid-analogue detergents.

A-SEC stability data for these detergents showed some aggregation upon affinity column purification, indicating that the receptor was slightly destabilized, which is consistent with the observed lipid depletion. Detergent-dependent variations in the monomer-dimer ratios were also observed after purification when compared to detergent-solubilized, nAChR-enriched membranes. Sodium cholate showed ~13% aggregate and decreases ~7% in both monomer and dimer (Fig. 2b). FC-12 showed the lowest aggregation of any detergent in this study ~7%, with ~3% increase in the dimer and ~10% decrease in the monomer (Fig. 2d). CHAPS, which is structurally similar to sodium cholate (Table 1), showed a comparable amount of aggregate (15%), with ~20% decrease in the dimer and ~6% increase in the monomer (Fig. 2f).

EM data were partially consistent with A-SEC, with clearly visible nAChR molecules in both sodium cholate and FC-12 (Fig. 3a and c), while considerable aggregation was apparent for CHAPS (Fig. 3b, d and f show that the nAChR is mostly pure in all three detergents, especially sodium cholate, showing the characteristic four-band pattern with minimal contaminants.

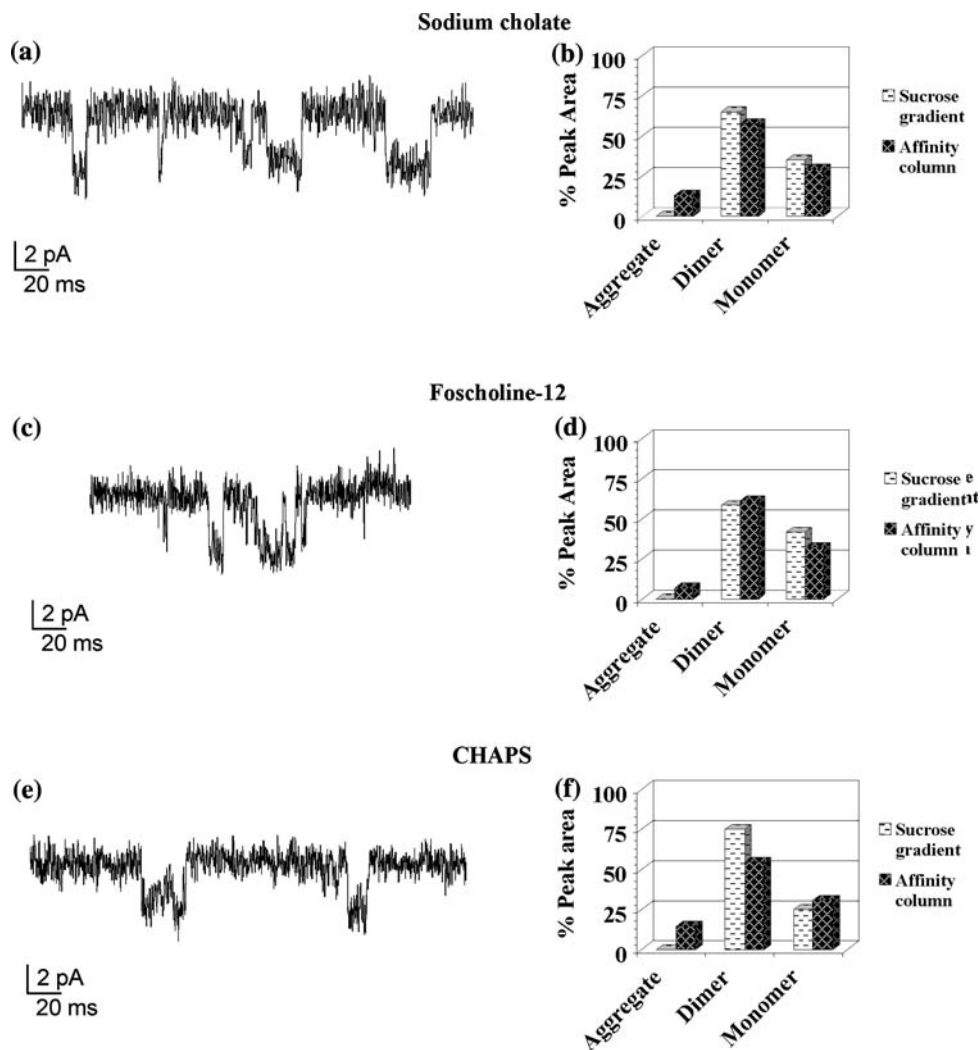
Table 3 nAChR planar bilayer assay data summarizing nAChR ion channel function parameters

Detergent	Detergent classification based on experimental results	Average number of processed events per-bilayer (N)	Channel current (mean ± SD) pA	Open channel time (mean ± SD) ms
Cholate	Lipid analog	214	-(2.48 ± 0.03)	3.0 ± 0.1
CHAPS	Lipid analog	262	-(2.69 ± 0.04)	3.51 ± 0.04
FC-12	Lipid analog	285	-(2.48 ± 0.02)	2.52 ± 0.07
Anapoe-C ₁₂ E ₉	Retained residual amounts of native lipid	274	-(1.77 ± 0.02)	2.40 ± 0.03
BigCHAP	Retained residual amounts of native lipid	235	-(1.76 ± 0.05)	2.05 ± 0.05
Cymal-6	Non-lipid analog	N/A	N/A	N/A
DDM	Non-lipid analog	N/A	N/A	N/A
LDAO	Non-lipid analog	N/A	N/A	N/A
OG	Non-lipid analog	N/A	N/A	N/A

Electrophysiological parameters for affinity-purified nAChR in each individual detergent, including detergent classification, average number of processed events per-bilayer experiment (N), mean channel current and mean open channel time

N/A, Carb-induced nAChR ion channel function was not observed. For functional detergents, an average of three (3) bilayer experiments with durations of ~30 min were performed. For detergents that did not display Carb-induced nAChR ion channel function, an average of six (6) bilayer experiments with durations of ~45 min were performed. Histograms from which mean ion channel current and mean open channel time electrophysiological parameter values were obtained are shown as supplementary materials

Fig. 2 Ion channel function and stability assays of affinity-purified nAChR in lipid-analog detergents. Planar bilayer current traces at -70 mV membrane potential in the presence of 0.5 μ M Carb. (left panels for: (a) Sodium cholate, (c) FC-12 and (e) CHAPS. A-SEC stability assay percent (%) peak areas (right panels) for sucrose gradient detergent-solubilized membranes (white background) and ligand affinity-purified nAChR (black background) in (b) Sodium cholate (sucrose gradient % peak areas: 65% dimer, 35% monomer; affinity column % peak areas: 13% aggregate, 58% dimer, 29% monomer), (d) FC-12 (sucrose gradient % peak areas: 58% dimer, 42% monomer; affinity column % peak areas: 7% aggregate, 61% dimer, 32% monomer) and (f) CHAPS (sucrose gradient % peak areas: 75% dimer, 25% monomer; affinity column % peak areas: 15% aggregate, 55% dimer, 31% monomer)



considerably more aggregation, similar to CHAPS other detergents in the study. Cymal-6 showed the lowest (Fig. 4g). SDS-PAGE gels in Fig. 4f and h show noticeably aggregation with ~23%, similar to Anapoe-CE₉ and more contaminants than what was observed for lipid-analog detergents, specially for BigCHAP, perhaps as a result of the residual native lipids, while the characteristics similar to Cymal-6 (Table 1), showed ~30% aggregation, nAChR bands are still observable.

Non-Lipid-Analogue Detergents: Cymal-6, DDM, LDAO and OG

Native lipid depletion in these detergents was comparable to that in lipid-analogue detergents (Table 2), but ion channel function was completely suppressed following nAChR purification (Table 3).

A-SEC stability assays for these detergents showed similar effects on monomer/dimer ratios upon detergent solubilization of the native membranes as observed for other detergents. However, following affinity column purification and native lipid depletion, significant increases in aggregation were observed, higher than for all of the

Cymal-6 showed the lowest aggregation with ~23%, similar to Anapoe-CE₉ and more contaminants than what was observed for lipid-analog detergents, specially for BigCHAP, perhaps as a result of the residual native lipids, while the characteristics similar to Cymal-6 (Table 1), showed ~30% aggregation, with a similar ~10% decrease in the dimer but a nearly double decrease in the monomer of 20% (Fig. 5b). LDAO showed ~33% aggregation, slightly higher than Cymal-6 and DDM, with decreases of 20% in the dimer and ~13% in the monomer, almost the opposite results to DDM (Fig. 5c). OG showed nearly double the amount of aggregation of the other non-lipid-analogue detergents at ~60%, the highest by far of the group, with a 44% reduction of the dimer and complete depletion of the monomer (Fig. 5d).

EM data show clearly visible pentamers in both Cymal-6 and DDM (Fig. 5e and g), while significant aggregation was apparent in both LDAO and OG (Fig. 5f and k). SDS-PAGE gels in Fig. 5f, h, j and l show minimal contamination, with the characteristic nAChR band pattern

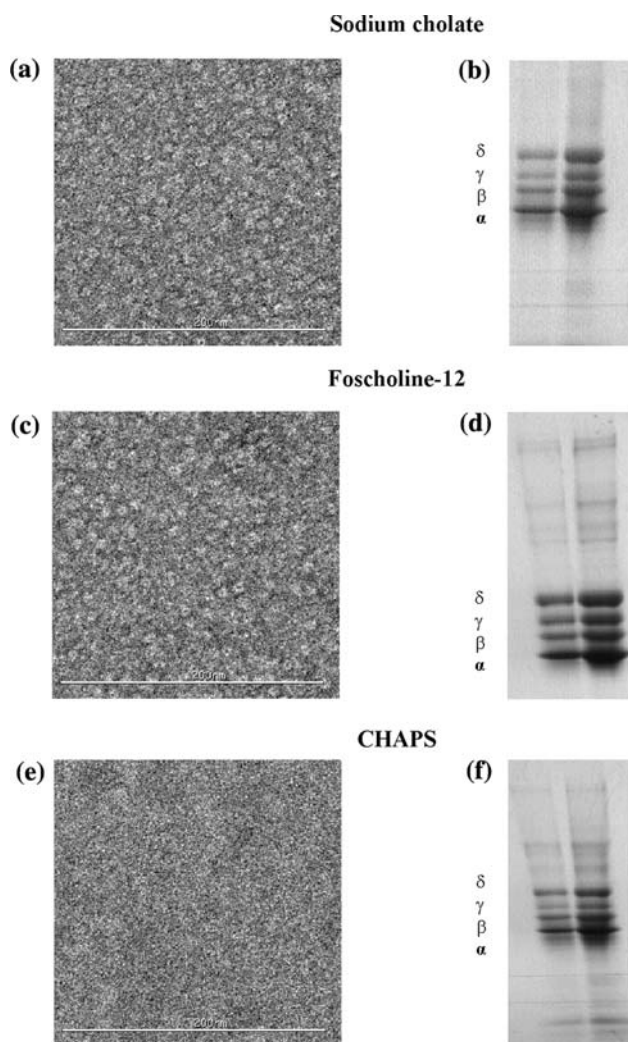


Fig. 3 EM and SDS-PAGE of affinity-purified nAChR in lipid-analog detergents. Negative-stain EM (left panels) for: (a) Sodium cholate, (c) FC-12 and (e) CHAPS; scale bar = 200 nm. SDS-PAGE gels (right panels) for: (b) Sodium cholate, (d) FC-12 and (f) CHAPS

comparable to FC-12 and CHAPS and a marked improvement over Anapoe- $C_{2}E_{9}$ and BigCHAP, but not as clean as that observed for sodium cholate.

Discussion

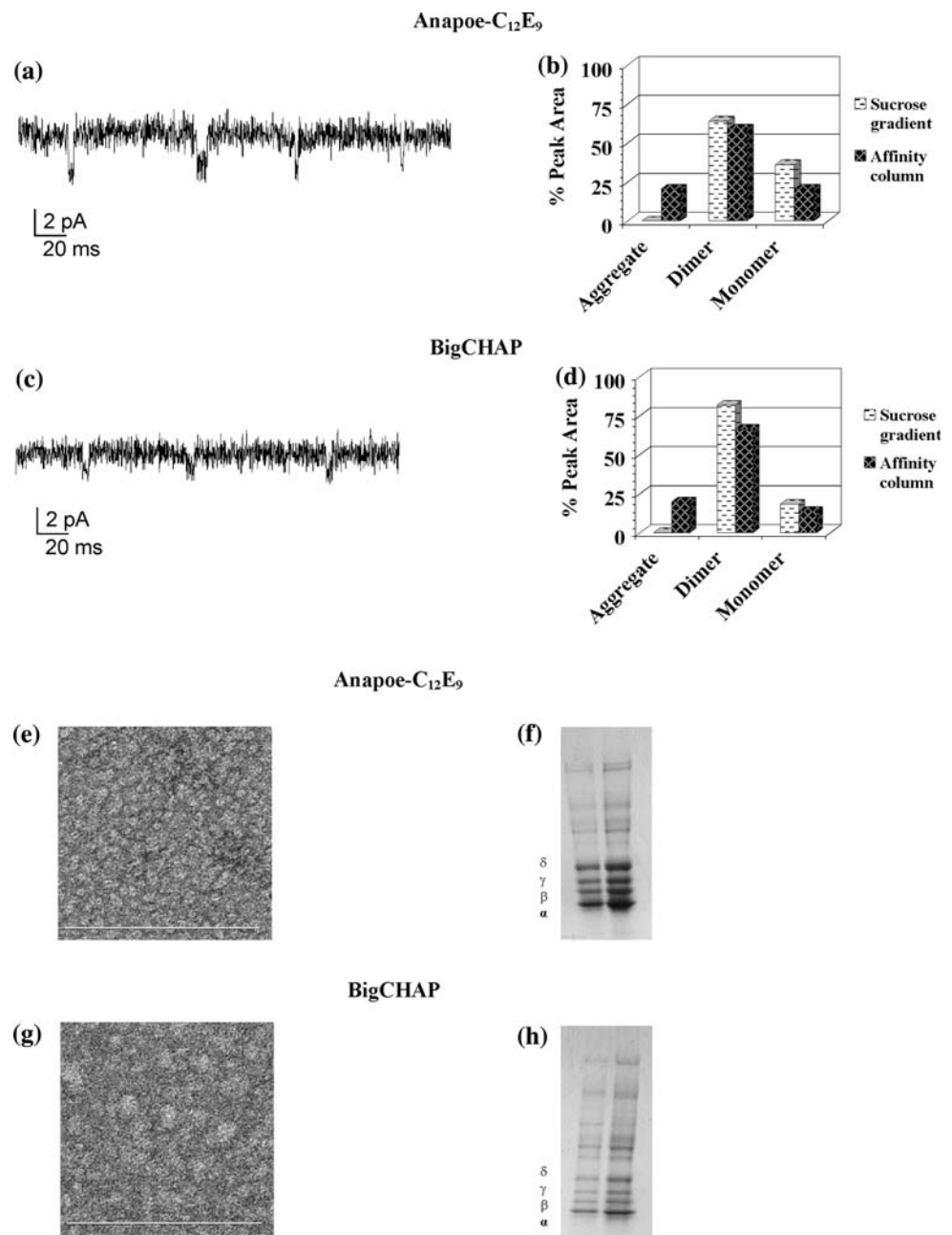
We found considerable differences in all biophysical parameters measured between detergent-solubilized sucrose gradient membranes and affinity-purified nAChR, mainly as a result of alteration of the native lipid composition. Detergent solubilization of sucrose gradient nAChR-enriched membranes did not result in significant native lipid depletion (Table 2), and nAChR stability was not substantially affected. A-SEC assays showed no aggregation upon detergent solubilization, consistent with their

observed minimal lipid depletion, and both nAChR monomer and dimer species were observed in all detergents as previously reported (Anholt et al. 1980, Figs. 2, 4 and 5). Affinity column purification caused significant lipid depletion in all detergents (Table 2), to levels at or below the previously reported functional thresholds (Jones et al. 1988). Lipid depletion destabilized the receptor, as evidenced by higher aggregation levels, altered monomer:dimer ratios and effects on ion channel function, in a detergent-dependent manner. Agonist and antagonist binding was unaffected by the identity of the detergent, since we were able to purify the nAChR in all detergents by ligand-affinity, and formation of the nAChR-Alexa Fluor 488 α -BTX antagonist complex, a necessary condition for detection in A-SEC stability assays, occurred in all detergents (Figs. 2, 4 and 5).

Based on the similarity of their behavior in the biophysical characterization assays, the detergents were grouped into three categories (Table 1): lipid-analogue detergents, including sodium cholate, CHAPS and FC-12; detergents retaining residual amounts of native lipids, including Anapoe- $C_{2}E_{9}$ and BigCHAP, and non-lipid-analogue detergents, including Cymal-6, DDM, LDAO and OG. Three experimental outcomes were observed upon nAChR purification: (1) stable and functional nAChR in lipid-analogue detergents, despite native lipid depletion, (2) moderately stable and functional nAChR in detergents that retained residual amounts of native lipids, and (3) unstable and non-functional nAChR in non-lipid-analogue detergents. We propose an equilibrium model to explain these results (Fig. 6), where k_{sg} (sucrose gradient constant) governs the monomer:dimer equilibrium for nAChR-enriched, sucrose gradient detergent-solubilized membranes. Upon affinity purification and native lipid depletion, k_a (overall affinity-purified nAChR constant) governs the monomer:dimer equilibrium, while k_{am} (affinity-purified nAChR monomer constant) and k_d (affinity-purified nAChR dimer constant) govern

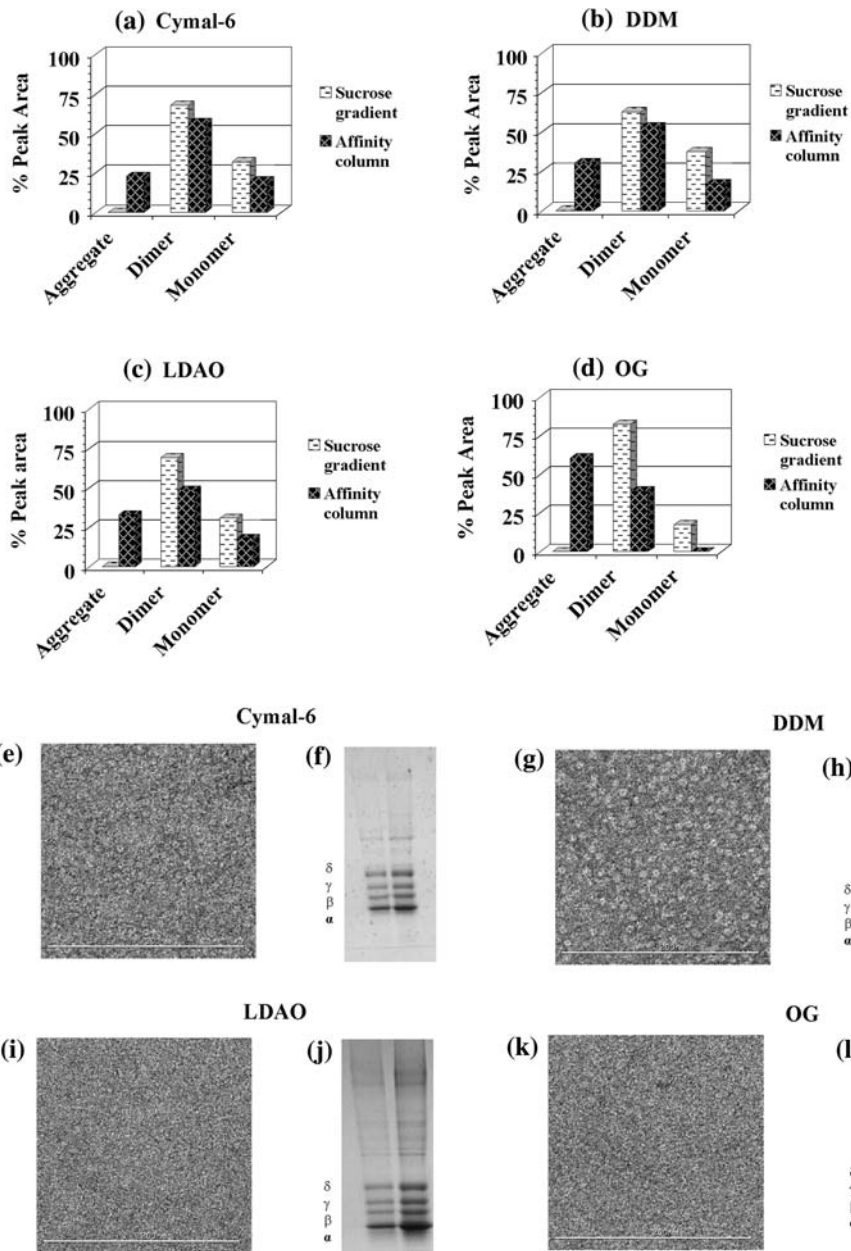
depletion of the monomer and dimer states, respectively, to form the aggregate. The reversibility of this aggregation remains to be established, but the observed depletion of the dimer and monomer species in a detergent-dependent manner suggests that both nAChR species could aggregate, perhaps by different mechanisms. It is very likely that, to some extent, aggregation could lead to partial or completely irreversible nAChR denaturation, as evidenced by the correlation between increased aggregation and loss of ion channel function. Native lipid depletion upon receptor purification, a necessary step for structural studies, could expose the nAChR transmembrane domains to the aqueous buffer environment, leading to premature aggregation and loss of ion channel function, as observed for some detergents.

Fig. 4 Ion channel function and stability assays, EM and SDS-PAGE of affinity-purified nAChR in detergents retaining residual amounts of native lipid. Planar bilayer current traces at -70 mV membrane potential in the presence of 0.5 μ M Carb. (top left panels) for: (a) Anapoe-G₂E₉ and (c) BigCHAP and A-SEC stability assay percent (%) peak areas (top right panels) for sucrose gradient detergent-solubilized membranes (white background) and ligand affinity-purified nAChR (black background) in (b) Anapoe-G₂E₉ (sucrose gradient % peak areas: 64% dimer, 36% monomer; affinity column % peak areas: 20% aggregate, 59% dimer, 21% monomer) and (d) BigCHAP (sucrose gradient % peak areas: 82% dimer, 18% monomer; affinity column % peak areas: 20% aggregate, 66% dimer, 14% monomer). Negative-stain EM for: (e) Anapoe-G₂E₉ and (g) BigCHAP (bottom left panels); scale bar = 200 nm. SDS-PAGE gels for: (f) Anapoe-G₂E₉ and (h) BigCHAP (bottom right panels)



The structural features of lipid-analogue detergents could affect affinity purification conditions, a nearly total native lipid have allowed them to interact favorably with nAChR depletion was observed. Detergents retaining slightly higher amounts of residual ~ 20 – 45 moles of lipid/per-mole of receptor functional native lipids showed aggregation and ion channel function threshold (Jones et al. 1988), thus preserving nAChR parameters with intermediate values between those functionality in spite of native lipid depletion. The comparatively low aggregation observed in A-SEC and EM thus, there is a shift of k_{on} and k_{ad} toward aggregation for lipid-analogue detergents suggests that k_{on} and k_{ad} (Fig. 6), but not enough to cause loss of ion channel were small (Fig. 6), indicating that these detergents are function. Thus, the presence of increased amounts of clearly the best choice for maintaining both stability and residual native lipids appears to offset the apparent structural effect patterns observed for lipid- and non-lipid analog achieving a reasonably high level of purity. One of the drawbacks of lipid analogue detergents is that under these conditions required two (2) to four (4) times more detergent for

Fig. 5 Stability assays, EM and SDS-PAGE of affinity-purified nAChR in non-lipid analog detergents. A-SEC stability assay percent (%) peak areas (top of the graph) for sucrose gradient detergent-solubilized membranes (white background) and ligand affinity-purified nAChR (black background) in: (a) Cymal-6 (sucrose gradient areas % peak areas: 68% dimer, 32% monomer; affinity column % peak areas: 23% aggregate, 57% dimer, 20% monomer); (b) DDM (sucrose gradient % peak areas: 63% dimer, 37% monomer; affinity column % peak areas: 30% aggregate, 53% dimer, 17% monomer); (c) LDAO (sucrose gradient % peak areas: 69% dimer, 31% monomer; affinity column % peak areas: 33% aggregate, 49% dimer, 18% monomer) and (d) OG (sucrose gradient % peak areas: 83% dimer, 17% monomer; affinity column % peak areas: 61% aggregate, 39% dimer, 0% monomer). Negative-stain EM (bottom of the graph) for: (e) Cymal-6, (g) DDM, (i) LDAO and (k) OG; scale bar = 200 nm. SDS-PAGE gels for: (f) Cymal-6, (h) DDM, (j) LDAO and (l) OG



nAChR solubilization than the other detergents, which function (Boheim et al. 1981). Some of the ion channel could cause partial denaturation. Furthermore, its comparative opening-closing events on this study were as well-defined as those for sodium cholate (Nelson et al. 1980) while functional groups in CHAPS and sodium cholate (Table 1) others were not, which would be consistent with our own results for Anapoe-C₂E₉ and BigCHAP. The fact that both nAChR transmembrane domains. The presence of residual native lipids in Anapoe-C₂E₉ appeared to allow this non-lipid-analogue detergent to partially compensate for the nearly-total native lipid depletion observed for all other detergents, makes them viable choices for nAChR purification. However, the observed reduction in ion channel planar bilayer studies in Triton X-100-solubilized, Tween-20-solubilized, and OG-solubilized nAChR, both non-lipid analog detergents, prior to reconstitution into 1,3-SMPC (1-stearoyl-3-myristoyl-glycero-2-phosphocholine) have shown ion channel interactions.

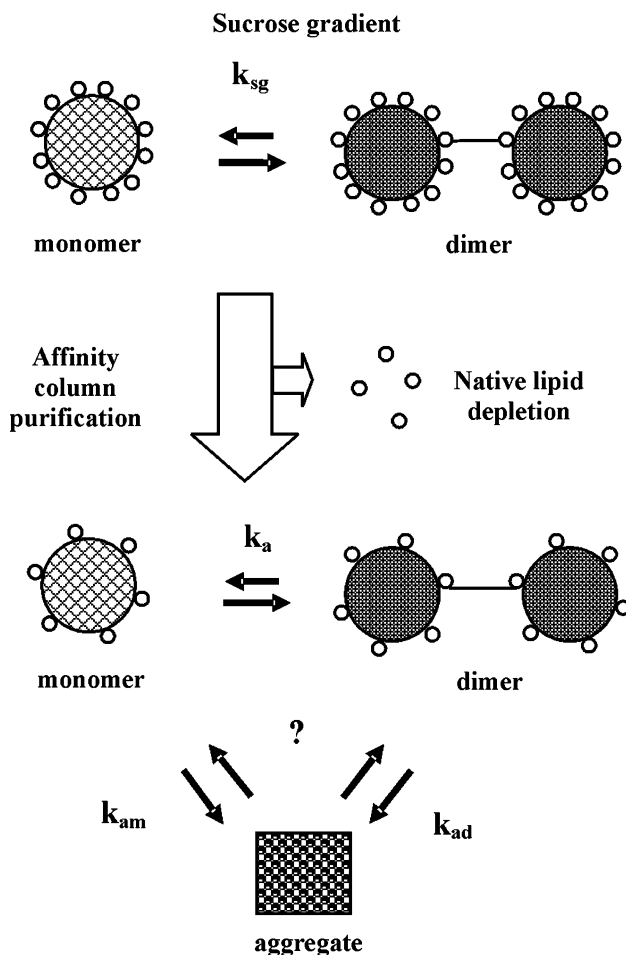


Fig. 6 Proposed nAChR monomer-dimer equilibrium scheme. The figure describes a possible mechanism to explain the observed experimental results based on how the nAChR monomer-dimer equilibrium could be affected during affinity purification as a result of native lipid depletion

Non-lipid-analogue detergents significantly increased aggregation and native lipid depletion upon affinity purification, as observed in both A-SEC (specially in LDAO and OG) and EM (except Cymal-6 and DDM), along with a total loss of ion channel function. These results are consistent with the apparent inability of these detergents to effectively interact with residual lipids and shield potentially exposed nAChR transmembrane domains from the aqueous buffer environment. These potential structural constraints would increase k_{am} and k_{ad} values (Fig. 6), shifting the equilibrium towards aggregation.

Previous studies with OG-solubilized crude membranes reconstituted in soybean lipid vesicles showed no nAChR ion channel flux (Anholt et al. 1981). Direct functional measurements of detergent-solubilized membranes were attempted but were ultimately unsuccessful in our study due to the significant amount of non-nAChR proteins incorporated into the bilayer, which almost always led to premature membrane breakage. However, our overall results suggest that since detergent-solubilized membranes did not show significant native lipid depletion or decreased stability in A-SEC, the nAChR should be stable and functional under these conditions. It is possible that non-lipid analogue detergents could interact with other critical domains to suppress ion channel function, which would be consistent with a direct negative effect of OG on the nAChR, as reported by Anholt et al. 1981.

Our results showed that upon nAChR affinity purification, native lipid depletion caused destabilization and loss of ion channel function. This finding is in disagreement with previously-published results suggesting that OG-solubilized, affinity-purified nAChR showed ion channel flux, although for these particular studies, the affinity-purified nAChR was reconstituted in native lipid vesicles (Paraschos et al. 1982). It is possible that the observed ion channel flux was produced by a small fraction of intact nAChR channels that were protected from detergent exposure by either: (1) the reduced amount of detergent used during solubilization, which at 34 mM was barely above the OG CMC value of 23.4 mM, (2) the presence of native lipids in reconstituted vesicles, or both. This discrepancy highlights the potential advantages of the methodologies presented in this study, which more closely resemble the actual detergent-solubilized environment in which most structural studies would be performed. In our study, the protein remained in contact with the detergent at all times, as opposed to the complete detergent removal necessary for reconstitution in lipid vesicles, which could reverse the potentially-detrimental effects of OG or other detergents.

It is clear that non-lipid-analogue detergents, at least the ones tested in this study, are not suitable for nAChR affinity purification in the absence of native or exogenous lipids, because of their negative effects on nAChR ion channel function and stability. Certainly, the results observed by Paraschos et al. 1982 for OG, Boheim et al. (1981) for Triton-X100/Tween-80, and our own results for Anapoloe-G₂E₉ and BigCHAP suggest that the presence of exogenous or residual native lipids could preserve or even restore ion channel function in non-lipid-analogue detergents. Perhaps addition of native or exogenous lipids during purification to non-lipid-analogue detergents could increase nAChR stability to the levels required to restore ion channel function, something that could be addressed in a future study.

The overall results of this study provide suitable biological physical methodologies to probe detergent-lipid-protein interactions prior to membrane protein structural studies, while perhaps shedding light on some of the problems that have hampered these studies over the past few decades. The fact that all of these techniques can be used on the

detergent-solubilized state, which more closely mimics the actual environment to which the protein is exposed to prior to structural studies, provides a clear advantage over previous studies performed in native membranes or reconstituted lipid vesicles. The combination of A-SEC and EM as sample stability and aggregation state prediction tools, as well as planar lipid bilayers with the PMAL protein delivery method to probe nAChR ion channel function, and the more conventional GC-FID and GC-MSD for lipid analyses, has provided valuable insight into the overall state of the protein sample in the detergent-solubilized state which was not previously available.

In conclusion, the nAChR purified under these conditions is by no means in its optimal state for structural studies, and significant challenges remain in terms of obtaining a homogenous, stable and functional sample. These problems would need to be addressed in future biophysical or structural studies, and this study provides an important starting point in that direction. Further optimization of nAChR purification conditions using biophysical techniques as probes, perhaps through the addition of native or exogenous lipids during or after affinity purification, could lead to significant improvements in nAChR stability in the detergent-solubilized state, increasing the likelihood of success in future nAChR and membrane protein structural studies.

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