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Intracellular dynamics of sigma-1 receptors (σ_1 binding sites) in NG108-15 cells[a]

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Running title page

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d) Abbreviation: ADRP, Adipocyte differentiation-related protein; c-LD, cytosolic lipid droplets; CYP450R, NADPH-cytochrome P450 reductase; FRAP, fluorescence recovery after photobleaching; ER-LD, endoplasmic reticulum-associated lipid droplets; EYFP, enhanced yellow fluorescent protein; IP3, inositol 1,3,5-trisphosphate; PVDF, polyvinylidene difluoride; Sig-1R-EYFP, C-terminally EYFP-tagged sigma-1 receptors.

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Abstract

Sigma-1 receptors bind diverse kinds of psychoactive compounds including cocaine, and translocate upon stimulation by these compounds. However, the exact intracellular localization and dynamics of sigma-1 receptors have been unclear. We recently found that sigma-1 receptors specifically localize on cholesterol-enriched *loci* on the endoplasmic reticulum membrane that function as neutral lipid storage sites (i.e., ER lipid droplets or ER-LD) from which neutral lipids bud out to form cytosolic lipid droplets (c-LD). By combining immunocytochemistry and real-time monitoring of enhanced yellow fluorescent protein (EYFP)-tagged sigma-1 receptors (Sig-1R-EYFP) in living cells, we characterized the sigma-1 receptor translocation in this study. (+)Pentazocine, a selective sigma-1 receptor agonist, causes a significant decrease of sigma-1 receptors in ER-LD and a diffused distribution of sigma-1 receptors over the entire endoplasmic reticulum reticular network in NG108-15 cells. In the presence of sigma-1 receptor agonists, Sig-1R-EYFP move out from ER-LD and slide along the endoplasmic reticulum network toward nuclear envelope and the tip of neurites. Fluorescence recovery after photobleaching (FRAP) analysis demonstrates that Sig-1R-EYFP on endoplasmic reticulum reticular network are highly mobile compared to those in ER-LD. A sucrose gradient fractionation study shows that (+)pentazocine shifts sigma-1 receptors from ER-LD membranes to higher-density membranes. These results indicate that sigma-1 receptors localize on ER-LD and upon stimulation translocate on continuous endoplasmic reticulum reticular network toward peripheries of cells. Because sigma-1 receptors specifically target ER lipid storage sites and compartmentalize neutral lipids therein, these results suggest that sigma-1 receptors' dynamic translocation might affect lipid

transport and distribution in neuronal cells.

The brain sigma receptors are unique non-opioid, non-phencyclidine receptors which consist of two subtypes: sigma-1 and sigma-2 receptors (Quirion et al., 1992). Sigma-1 receptors were originally implicated in schizophrenia, but recent studies suggest an involvement of Sig-1R in learning and memory, depression, and drug dependence (Snyder and Largent, 1989; Maurice and Lockhart, 1997; Matsumoto et al., 2001; van Broekhoven and Verkes, 2003). Sigma-1 receptor ligands have been proposed to represent a new class of therapeutic agents for psychiatric disorders.

Sigma-1 receptors have been cloned (Hanner et al., 1996; Seth et al., 1997). The sequence of sigma-1 receptors exhibits no homology to any of other mammalian proteins, but has a 30.3% identity to the sequence of a fungal sterol C8-C7 isomerase (Hanner et al., 1996). Sigma-1 receptors, however, lack the sterol isomerase activity (Labit-Le Bouteiller et al., 1998). The exact biological action of sigma-1 receptors is still not totally clarified at present.

Sigma-1 receptors bind diverse classes of compounds including psychotherapeutics agents (Su et al., 1982, Narita et al., 1996), cocaine (Sharkey et al., 1988; Matsumoto et al., 2001), and steroid hormones such as progesterone (Su et al., 1988). Haloperidol, a clinically used neuroleptic, functions as a sigma-1 receptor antagonist (Okuyama and Nakazato, 1996). Certain antidepressants, in addition to cocaine, however, act as agonists (Hayashi and Su, 2001; Matsumoto et al., 2001; Takebayashi et al., 2002). Sigma-1 receptors and their ligands show modulatory actions *in vivo* and *in vitro*. For example, sigma-1 receptors modulate Kv 1.4 potassium channels in nerve terminals (Aydar et al., 2002), inositol 1,3,5-trisphosphate (IP_3) receptor-mediated Ca^{2+} signaling at the ER (Hayashi et al, 2000; Hayashi and Su, 2001), and the

N-methyl-D-aspartate-induced neuronal firing or dopamine release in the brain (Monnet et al., 1990; Nuwayhid and Werling, 2003). Notably, in most studies, sigma-1 receptor agonists showed no effect by themselves, but exerted modulatory actions on signal transductions related to ion channels or neurotransmitters.

Morin-Surun et al. (1999) and we (Hayashi et al, 2000; Hayashi and Su, 2001) reported that sigma-1 receptors translocate inside cells. Sigma-1 receptor agonists can cause translocation of sigma-1 receptors from light-density microsomal fractions to other subcellular fractions in a period of 10 min (Hayashi et al, 2000). Translocation of sigma-1 receptors might ensue important biological functions afforded sigma-1 receptors. In fact, we demonstrated that in NG108 cells, a portion of sigma-1 receptors are coupled to IP_3 receptors on the endoplasmic reticulum membrane and that sigma-1 receptors amplify IP_3 receptor-medicated Ca^{2+} signaling at the endoplasmic reticulum vis-à-vis their translocation away from the endoplasmic reticulum (Hayashi et al., 2000; Hayashi and Su, 2001). Furthermore, we recently found that Sig-1R specifically target neutral lipid-enriched subdomains on the endoplasmic reticulum membrane [i.e., lipid droplets on the ER (ER-LD) (Hayashi and Su, 2003)]. Specifically, studies with functionally negative sigma-1 receptors in that report strongly suggested that sigma-1 receptors at ER-LD are crucial in regulating lipid compartmentalization at the endoplasmic reticulum (Hayashi and Su, 2003). However, the temporal and spatial characteristics of sigma-1 receptor translocation in cells are still unclear. In this study, we explored the intracellular dynamics of sigma-1 receptors and the associated effects exerted by Sig-1R ligands by using immunocytochemistry, real-time monitoring of C-terminally enhanced yellow fluorescent protein-tagged Sig-1R (Sig-1R-EYFP), as well as sucrose gradient subcellular

fractionation in NG108-15 cells.

Materials and methods

Cell culture, antibodies and chemicals

Procedures for cell culture were described previously (Hayashi et al., 2000). Antibody/sources are: Caveolin-2, VLA-2α, GM130, Lamp-1 or EEA-1/Transduction Laboratory (San Diego, CA); Src/Santa Cruz Biotechnology (Santa Cruz, CA); NADPH-cytochrome P450 reductase (CYP450R), bcl-2, Lamp-1 or synapsin II/StressGen (Victria BC, Canada); Alexa Fluoro-conjugated secondary antibodies/Molecular Probes (Eugene, OR). Polyclonal rabbit anti-guinea pig sigma-1 receptor-A and -B were raised against guinea-pig sigma-1 receptor amino acid sequence 144-165. Chemicals not specified here are all from Sigma (St. Louis, MO).

Immunostaining and the semi-quantification of sigma-1 receptor translocation

Cells grown on 12-mm poly-D-lysine/laminin-coated coverslips were fixed by 4% paraformardehyde for 30 min. Paraformardehyde was quenched by 100 mM glycine in HBSS (pH 8.5). Cells were permeabilized (0.1% Triton X-100 for 10 min) and blocked (10% non-fat milk for 60 min). In immunocytochemistry for sigma-1 receptors, fixed cells were treated with 0.05% SDS for 10 min for antigen retrieval (Brown et al., 1996). Cells were incubated with appropriate primary (4% BSA+0.5% NP-40) and secondary antibodies. Coverslips were mounted in the ProLong Antifade solution (Molecular Probe). For the counting of the population of sigma-1 receptor-translocated cells, images of NG108 cells stained with anti-guinea pig sigma-1 receptor antibody-B were captured randomly as a field which contains at least 4 cells. In non-transfected cells, sigma-1 receptor-containing ring structures were found to surround the nucleus (usually >40 ring structures/cell). For the semi-quantification of the sigma-1 receptor translocation, cells

displaying ring structures fully surrounding or covering at least 25% of the nucleus were counted as non-translocated; otherwise as translocated. In other words, sigma-1 receptors "translocated" cells, as defined in this study, have less than a quarter of the peri-nuclear area surrounded by sigma-1 receptor-contining ring structures. The performance of the semi-quantification was done by a person blind to experimental conditions.

Construction and expression of EYFP-tagged sigma-1 receptors

Procedures were described in elsewhere (Hayashi and Su, 2003). Briefly, PCR amplifications of the mouse sigma-1 receptor cDNA (Genbank accession #; AF030198) from pSPORT1-Sig-1R (Seth et al., 1997) were subcloned into the pcDNA3.1/His cloning vector (Invitrogen, Carlsbad, CA). Sigma-1 receptor cDNA was digested by EcoR1 and Bam H1 and ligated in pEYFP-C1 vector (Clontech, Palo Alto, CA) for expression of C-terminally EYFP-tagged sigma-1 receptors (Sig-1R-EYFP). Vectors were transfected by using Lipofectamine-2000 (GIBCO, Grand Island, NY).

Nile red fluorescence stainings

For Nile red staining, fixed cells were mounted in 50% glycerol/PBS containing 0.001% Nile red. For dual capturing of both Nile red and EYFP images in fixed cells, Nile red image was captured first (no crossover of EYFP to a red channel), and then the EYFP image captured following the Nile red photobleach.

Sucrose gradient fractionation for ER-LD and c-LD

NG108 cells from 2 confluent 15-cm dishes were incubated at 4 ^{o}C for 10 min in the hypotonic TME buffer (10 mM Tris, 5mM $MgCl_2$, 0.5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin; pH=7.2). TME buffer containing 68.4% sucrose was then added to obtain a final sucrose concentration at 8.6%. Cells in suspension were homogenized by a

Dounce homogenizer (20 strokes). Homogenates were centrifuged at 900xg and resultant supernatants collected. Pellets were homogenized again (10 strokes) and centrifuged (900xg). The supernatants were overlaid (2 ml) on the top of a sucrose gradient [22 ml; consisting 11 layers from 68.4% (bottom) to 15.0% (top) sucrose]. Finally, the TME buffer with 0% sucrose (2 ml) was placed as the top layer and samples were centrifuged at 120,000xg for 16 hrs. Under this condition, cytosolic LD (c-LD) float to the top layer (0% sucrose), cytosolic soluble proteins and synaptic vesicle remain in the original layer (8.6%), and other membranes move to lower layers according to their densities (see Fig. 5 in results). Thirteen fractions were collected from the top. Differential centrifugation for P3H and P3L were described elsewhere (Hayashi and Su, 2001).

Western blotting

NG108 cell lysates were prepared in sodium dodecyl sulfate sample buffer and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (%C=1.5). Proteins were electrophoretically transferred on PVDF membranes at 30V for over night (4 ^{o}C) in Towbin buffer without methanol. We found that methanol severely disturbs sigma-1 receptor transfer onto PVDF membranes. PVDF membranes were blocked with 10% non-fat milk for 6 hrs at 4 ^{o}C and incubated over night at 4 ^{o}C with anti-guinea-pig sigma-1 receptor antibody-A (1:1500) in TBST containing 1.0% Nonidet P40. Protein bands were visualized by an ECL kit (Amersham, Piscataway, NJ). For Western blotting of extracellular sigma-1 receptors, NG108 cells on 10-cm dishes were washed with pre-warmed HBSS twice and incubated in HBSS at 37 ^{o}C in the presence of cocaine. Cell supernatants were collected and centrifuged at 3000xg for 5 min to pellet cell debris. Proteins in supernatants were subjected to SDS-PAGE. Nuclei were purified

by the detergent-based nucleus purification system (Sigma). Proteins in cell supernatants and sucrose fractions were concentrated by trichloroacetic acid precipitation.

Time-lapse fluorescence microscopy on living cells

NG108 cells expressing EYFP-tagged sigma-1 receptors were grown on 15-mm poly-D-lysine/laminin-coated glass coverslips. A coverslip was placed on a closed-bath imaging chamber (chamber volume=36 μl) and Heater Platform (Warner Instrument Co. Hamden, CT). Cells were perfused by HBSS at 32°C (0.3 ml/min) and examined using an inverted Axiovert 135 microscopy (Carl Zeiss, Oberkochen, Germany). Images were collected digitally every 10 or 15 s with the Zeiss Image Series software. A water-immersion 63x C-Apochromat objective lens (NA=1.2, working distance=0.24 mm, cover slip thickness=0.14-0.18 mm; Carl Zeiss) was employed. Fluorescence recovery after photobleaching (FRAP) analysis was performed as described elsewhere (Reits and Neefjes, 2001; Nehls et al., 2000). Briefly, living cells on a coverslip were continuously perfused as mentioned above. Sig-1R-EYFP in the defined region (2.0x2.0 μm square) was photobleached at a full laser power (2 sec), and, 1 sec after photobleaching, the recovery of fluorescence was monitored by scanning the whole cell at a minimal power output. Operation of the confocal microscope and the data collection were carried out by using the Carl Zeiss FRAP software. Mobility fraction (Mf) and diffusion time (τ_D) were calculated according to the method described elsewhere (Reits and Neefjes, 2001).

TLC for lipid analysis

After fractionation of NG108 cell membranes, lipids in each obtained fraction were extracted according to methods described elsewhere (Hayashi and Su, 2003). Total protein content in each fraction was measured by BCA kit (Pierce, Rockford, IL). Lipid

extracts were dissolved in chloroform/methanol (2:1) and separated on Silica Gel TLC plates (Merck, Billerica, MA) with hexane/ether/acetic acid (80:20:1). Plates were sprayed with the H_2SO_4 solution followed by charring at 110 ^{o}C for 40 min. Spots were analyzed by NIH Image software.

Statistical analyses

One-way ANOVA followed by Fisher's PLSD post-hoc test was used (significance level at $p<0.05$).

Results

Translocation of endogenously expressed sigma-1 receptors by (+)pentazocine in NG108 cells.

Endogenously expressed sigma-1 receptors and Sig-1R-EYFP both localized in peri-nuclear areas as dense clusters (Fig. 1a-d). Higher magnification indicated that sigma-1 receptors and Sig-1R-EYFP were on “ring-like” ER-LD structures and accompanying endoplasmic reticulum tubular elements (Fig. 1b, inset) (see also Hayashi and Su, 2003). Sig-1R-EYFP-positive ER-LD contained neutral lipids such as cholesteryl esters (CE) and triglycerides (TG) that are stained with Nile red (Fig. 1c). Enlargement of ER-LD was seen in some Sig-1R-EYFP-overexpressing cells (Fig. 1d). (+)Pentazocine decreased densities of sigma-1 receptors in the perinuclear ER-LD and concomitantly caused an even distribution of sigma-1 receptors over the endoplasmic reticulum reticular structure (Fig. 1e-f). We attempted to semi-quantify the sigma-1 receptor translocation caused by (+)pentazocine (see Methods). Results show that sigma-1 receptor translocation was seen in about 40% of cells even without any exogenous stimulation. This suggests a possibility that certain endogenous sigma-1 receptor ligand(s) mediate the sigma-1 receptor translocation. In the presence of (+)pentazocine, sigma-1 receptor translocation was seen in 70-90% of cells (Fig. 1g).

Intracellular dynamics of sigma-1 receptors in living NG108 cells

Movements of EYFP-tagged Sig-1R in living NG108 cells were monitored. Not all Sig-1R-EYFP moved. Some Sig-1R-EYFP, especially those clustered in ER-LD, did not move. However, a portion of Sig-1R-EYFP apparently moved. They moved out from ER-LD and slid on the endoplasmic reticulum reticular network even in the absence of

sigma-1 receptor ligands. Under confocal microscopy, only the movement from ER-LD to endoplasmic reticulum tubular elements was observed (Fig. 2a). The reverse movement, if any, from tubular elements to ER-LD was not seen, at least under the microscopic observation. Sigma-1 receptor agonists (+)pentazocine and cocaine increased the mobility of Sig-1R-EYFP in the following fashion: (1) Clustered Sig-1R-EYFP moved anterogradely on a neurite toward the tip or varicosities (Fig. 2b); (2) Sig-1R-EYFP moved along endoplasmic reticulum tubular elements toward the nuclear envelope (Fig. 2c). Interestingly, Sig-1R-EYFP reached at varicosities did not stay and accumulate at these *loci* (See arrow #1 in Fig. 2b). Because in NG108 cells vesicles (e.g., synaptic vesicles) are known to be exocytosed from varicosities and tips of neurites (Fried and Han, 1995), this result suggested a possibility that sigma-1 receptors at these *loci* could be released to extracellular space and/or transported back toward cell body. To test this possibility, we examined the content of sigma-1 receptors in the extracellular space after sigma-1 receptor agonist stimulations (see Materials and Methods). Cocaine dose- and time- dependently caused increases in sigma-1 receptors in the extracellular space as well as in the nucleus (Fig. 2d). These results are in agreement with the dynamic patterns of sigma-1 receptors shown in Fig. 2 a-b, and are supportive of a possibility that at least a portion of sigma-1 receptors could pass the plasma membrane and be exocytosed.

FRAP analysis

To further characterize dynamics of sigma-1 receptors, FRAP analysis was performed. When fluorescent molecules are irreversibly photobleached in a small area of the cell by a high-powered focused laser beam, subsequent diffusion of surrounding non-bleached

fluorescent molecules into the bleached area leads to a recovery of fluorescence. Thus, FRAP enables one to measure the mobility of fluorescent molecules on continuous membranes by monitoring fluorescence recovery in a photobleached area. When Sig-1R-EYFP on a single ER-LD were photobleached, no significant recovery of fluorescence was seen until at least 30 min after photobleaching (mobile fraction Mf=9.0±0.7% at 32 ^{o}C, N=4) (Fig. 3a, c). Thus, the movement of sigma-1 receptors from endoplasmic reticulum tubular elements into ER-LD is highly restricted, consistent with the result in time-lapse monitoring that shows the movement of sigma-1 receptors only from ER-LD to endoplasmic reticulum tubular elements. Sig-1R-EYFP on endoplasmic reticulum tubular elements were however highly mobile (Mf=76.3±5.2%, diffusion time τ_D=15.5±1.4 s at 32 ^{o}C, N=4) (Fig. 3b, c). These results suggest that mobility of sigma-1 receptors in the ER-LD is different from that at the endoplasmic reticulum tubular element and that certain mechanism may exist in regulating the lateral diffusion of proteins between these two endoplasmic reticulum subcompartments.

Sigma-1 receptor translocation assessed by sucrose gradient fractionation

In a subcellular fractionation study using differential centrifugation, we found that endogenously expressed sigma-1 receptors in NG108 cells are enriched in the light-density microsomal fraction (P3L) (Hayashi and Su, 2001). (+)Pentazocine (100 nM, for 10 min) caused a reduction of sigma-1 receptors in P3L whereas an increase in P1, P2 and heavy-density microsomal (P3H) fractions (Fig. 4). Here, we examined sigma-1 receptor translocation more extensively by fractionating NG108 cell membranes in sucrose gradients. Furthermore, because sigma-1 receptors localize specifically on ER-LD (Hayashi and Su, 2003), but not on c-LD which are formed by neutral lipids budding

from ER-LD into cytosol (Murphy and Vance, 1999; van Meer, 2001; Brown, 2001), we wanted to successfully separate ER-LD, c-LD, and other ER membrane into different fractions. Therefore, sucrose gradients consisting of 13 fractions (0-53.4% sucrose) were employed (see Materials and Methods). Results show that sigma-1 receptors and caveolin-2 (Cav-2), both shown to localize on ER-LD in our previous study (Hayashi and Su, 2003), were enriched in 15 to 25% sucrose fractions (Fig. 5a). Adipocyte differentiation-related protein (ADRP), a c-LD protein (Brasaemle et al., 1997; Murphy and Vance, 1999), existed in the 0% sucrose fraction (Fig. 5a, third panel). The sigma-1 receptor-enriched fractions did not contain any organelle marker proteins except a very low amount of CYP450R (Fig. 5b). Most of CYP450R (~90%) were in 46.2-53.4% sucrose fractions indicating that most endoplasmic reticulum tubular elements were in these heavy fractions (Fig. 5b). Sigma-1 receptor-enriched fractions (15-25% sucrose) contained moderate levels of neutral lipids and free cholesterol (FC; Fig. 5c). The top fraction (ADRP-positive c-LD fraction) contained high levels of neutral lipids and free cholesterol (Fig. 5c). Sig-1R-EYFP-transfected NG108 cells were accordingly fractionated, and the resultant fractions observed under fluorescence confocal microscopy. In the 20.5%-sucrose fraction, vesicular particles varied in size and shape, but most of them contained Sig-1R-EYFP (Fig. 5d). No Sig-1R-EYFP was seen in the c-LD fraction. On the other hand, the top fraction (0% sucrose) contained round lipid droplets that are uniformed in size and shape (Fig. 5e). Taken together, these results confirmed a successful separation of ER-LD, c-LD, and other suborganells using this 13-sucrose layer fractionation method.

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With this method successfully established as shown above, we examined effects of (+)pentazocine on the sigma-1 receptor translocation. The treatment of NG108 cells with (+)pentazocine (1 μM, for 30 min at 37 ^{o}C) caused a significant decrease of sigma-1 receptors in the ER-LD fraction, but an increase of sigma-1 receptors in heavier fractions containing endoplasmic reticulum tubular elements (Fig. 6). Importantly, (+)pentazocine did not cause any significant change of sigma-1 receptors in the c-LD-containing fractions, suggesting that sigma-1 receptors on ER-LD translocate to endoplasmic reticulum tubular network but not to c-LD.

Discussion

We reported previously (Hayashi and Su, 2003) and now that endogenous sigma-1 receptors as well as transfected Sig-1R-EYFP localize mainly on the endoplasmic reticulum. Although several other studies suggest the existence of sigma-1 receptors on both endoplasmic reticulum and plasma membrane (McCann and Su, 1990; Ramamoorthy et al., 1995; Alonso et al., 2000), we could not detect significant levels of sigma-1 receptors on the plasma membrane. It is also reported that sigma-1 receptors localize on the plasma membrane when expressed by gene transfection in oocytes (Aydar et al., 2002). A plausible explanation may be that the subcellular localization is different between cell types and/or stages of cell differentiation. Alternatively, sigma-1 receptors might move between endoplasmic reticulum and plasma membrane, but cannot stay and accumulate on the plasma membrane in NG108 cells employed in this study. Our results showing that sigma-1 receptors could be detected in the extracellular space after an agonist-stimulation (Fig. 2d) suggest that at least a portion of sigma-1 receptors can reach plasma membranes.

Sigma-1 receptors localized on both ER-LD and endoplasmic reticulum tubular network, but are predominantly abundant on ER-LD. The real-time monitoring of Sig-1R-EYFP in living cells indicates that sigma-1 receptors translocate from ER-LD to the endoplasmic reticulum tubular network. Because sigma-1 receptors are significantly decreased in low-density microsomes by the treatment with (+)pentazocine, and because sigma-1 receptors are membrane proteins, we previously speculated that a vesicle transport might be involved in the sigma-1 receptor translocation (Hayashi and Su, 2001). However, our present data show that sigma-1 receptor translocate on continuous endoplasmic reticulum structures through lateral movements and not *via* vesicular translocation following budding processes. But, on the other hand, we also observed an accumulation of Sig-1R-EYFP caused by (+)pentazocine on the plasmalemmal cortices

which consist of cytoskeleton lattice (data not shown). Further, Sig-1R-EYFP disappeared from the original *loci* when fixed cells were permeabilized with a detergent (0.5-0.2% Triton X-100) in immunocytochemical studies. Interestingly, the unique cytosolic transport vesicle "argosomes", that are exocytosed, presumably for cell-to-cell communication, have also been shown to disappear after a similar membrane permeabilization procedure (Greco et al., 2001). Therefore, it remains possible that, because of their close proximity to the plasma membrane, sigma-1 receptors may be separated from endoplasmic reticulum membrane as vesicles that could be exocytosed (Fig. 2d).

The translocation of sigma-1 receptors on the endoplasmic reticulum structure may not be simply due to a lateral diffusion of sigma-1 receptors on phospholipid bilayers. Translocation of sigma-1 receptors is regulated by specific ligands and temperature (data not shown), and the translocation directions are vectorial (i.e., one-way toward plasma membrane and nuclear membrane). Because sigma-1 receptors are associated with ankyrin (Hayashi and Su, 2001), a cytoskeleton adaptor protein, it is tempting to speculate that cytoskeletal filaments might be involved in directing the intracellular translocation of sigma-1 receptors.

Dynamics of sigma-1 receptors at the ER-LD and at the endoplasmic reticulum reticular network are apparently different. The FRAP data suggest that mobility of sigma-1 receptors from the endoplasmic reticulum reticular network to ER-LD is highly restricted. After a complete photobleaching of Sig-1R-EYFP fluorescence in a single ER-LD, no significant recovery of Sig-1R-EYFP fluorescence could be seen until at least 30 min thereafter, indicating that sigma-1 receptors on endoplasmic reticulum reticular network cannot move back into ER-LD, or very slowly, if any. This result is consistent with our previous observation that once sigma-1 receptor translocate, it takes more than 1 hr, after removal of sigma-1 receptor agonist, for sigma-1 receptors to return to normal levels at the original *loci* (Su and Hayashi, 2001). At present, we do not know what

regulates protein movements between ER-LD and other endoplasmic reticulum structures. However, in a recent study, we found that sigma-1 receptors in ER-LD form raft-like microdomains enriched in cholesterol (Hayashi and Su, 2003). Lipid membrane fluidity on ER-LD should be lower than other ER membranes due to an enrichment of cholesterol on the ER-LD (Barenholz, 2002). Therefore, the mobility of sigma-1 receptors might be highly restricted in the so-called “liquid-ordered” phase of lipid raft membranes (Simons and Toomre, 2000) whereas sigma-1 receptor mobility in other areas of the endoplasmic reticulum membrane is not.

Sigma-1 receptors have been shown in several reports to be present in microsomes, suggesting that they are on the endoplasmic reticulum (McCann and Su, 1990; Hayashi and Su, 2001). However, McCann and Su (1990) demonstrated that when brain membranes are further fractionated by a sucrose density gradient, sigma-1 receptors are present in a unique fraction different from those containing either plasma membrane marker or endoplasmic reticulum marker. Our present results from immunocytochemistry and sucrose fractionation studies confirmed that sigma-1 receptors locate on the specialized area of endoplasmic reticulum membranes (see also Hayashi and Su, 2003). It is very likely that sigma-1 receptor-containing membranes have a lower density than that of other endoplasmic reticulum membranes due to the enrichment of neutral lipids and cholesterol. Therefore, sigma-1 receptor-containing membranes can be separated from other endoplasmic reticulum membranes in the sucrose gradient centrifugation.

Newly synthesized neutral lipids (e.g., cholesteryl esters and triglycerides) are stored at ER-LD and eventually bud out to form c-LD (Murphy and Vance, 1999; van Meer, 2001). It is known that in adipocytes and steroidogenic cells the neutral lipid mass in matured c-LD is regulated by specific c-LD proteins and other receptor-mediated signal transductions (Londos et al., 1999). The activation of adrenaline or insulin receptors causes the protein kinase A activation and a subsequent phosphorylation of perilipin and hormone sensitive lipase (Londos et al., 1999). The phosphorylation causes

translocation of perilipin and HSL, resulting in the facilitation of lipolytic reaction and changes in the neutral lipid mass in c-LD. However, mechanisms that regulate the export of neutral lipids from ER-LD to c-LD and the formation of c-LD are totally unknown. In this study, we found that a selective sigma-1 receptor agonist (+)pentazocine apparently causes translocation of sigma-1 receptors from ER-LD. It is plausible that the level of sigma-1 receptors on ER-LD might affect the ER-LD membrane environment that in turn affects compartmentalization of neutral lipids and their export at the endoplasmic reticulum (Hayashi and Su, 2003). Translocation of sigma-1 receptors, thus, may have a significant impact on biological functions at the endoplasmic reticulum. In fact, in our recent study, we found that transfection of a functional negative sigma-1 receptors alters the homeostasis of lipids and the morphology of the endoplasmic reticulum in NG108 cells (Hayashi and Su, 2003).

In summary, our present study demonstrates that sigma-1 receptors target unique lipid-enriched sites on the endoplasmic reticulum (ER-LD) and translocate to the endoplasmic reticulum-associated reticular network upon stimulation by psychoactive drugs. Further, this translocation of sigma-1 receptors utilizes lateral movement on the continuous endoplasmic reticulum network to reach peripheries of cells, apparently not involving vesicle buddings from ER-LD. Our data, on the other hand, raise some questions that should be addressed in the future: (1) Why do sigma-1 receptors translocate even in the absence of exogenous sigma-1 receptor ligands (endogenous activators?)? and (2) What are the translocated sigma-1 receptors doing at the periphery of cells? Because sigma-1 receptors have a high homology to a fungal sterol C8-C7 isomerase (Hanner et al., 1996), some reports speculate that sigma-1 recepors act as an enzyme in cholesterol metabolism (Moebius et al., 1997; Labit-Le Bouteiller et al., 1998). Indeed, a number of enzymes involved in lipid metabolisms are located on the smooth endoplasmic reticulum similar to sigma-1 receptors (Khelef et al., 1998; Koning et al., 1996). However, most well-characterized cholesterol-metabolizing enzymes are on

the endoplasmic reticulum reticular network (Khelef et al., 1998; Koning et al., 1996), suggesting that sigma-1 receptors may not be cholesterol-metabolizing enzymes. Biological roles of ER-LD may be intimately related to the molecular function of sigma-1 receptors. Because sigma-1 receptors specifically target lipid storage sites on the endoplasmic reticulum, our results with the dynamic translocation of sigma-1 receptors indicate that in neuronal cells sigma-1 receptors might affect lipid transport on the endoplasmic reticulum as well as on the Ca^{2+} signaling as previously reported by us (Hayashi and Su, 2001). Sig-1R ligands such as neurosteroids and cocaine may thus modulate plasma membrane remodeling including promotion of raft formation and synaptogenesis by affecting lipid mobilization at the endoplasmic reticulum. Thus, a demonstration of sigma-1 receptors, especially in the form of a distinct class of sigma-1 receptor/cholesterol/sphingolipid-associated rafts, in other neuronal systems such as primary cultures, would be critical in advancing understanding on the role of sigma-1 receptors in the central nervous system and perhaps other central nervous system-related behaviors or diseases such as addiction and cholesterol/lipid-related psychiatric or neurological disorders.

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Foot notes

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Figure legends

Figure 1. Translocation of endogenously expressed sigma-1 receptors in NG108 cells.

NG108 cells were fixed, and antigen-retrieved, followed by staining with polyclonal anti-guinea-pig sigma-1 receptor antibody-B. **a-b**: Perinuclear distribution of sigma-1 receptors in cells without treatment (inset in **b**: Sigma-1 receptor-positive ring structures with tubular elements at a higher magnification). **c-d**: Cellular localization of Sig-1R-EYFP similar to that of endogenous sigma-1 receptors in NG108 cells. A single Sig-1R-EYFP-positive ER-LD stained with Nile red in **c**. Note: enlargement of ER-LD by overexpression of Sig-1R-EYFP in **d**. **e-f**: (+)Pentazocine ((+)PTZ; 100 nM, 10 min)-treated cells. Arrows in **e** indicate cells after sigma-1 receptor translocation from ring structures (reticular diffused pattern instead of clustered bright spots). Scale=10 μm. **g.** Percents of cells showing translocation of sigma-1 receptors from the ring structures. (+)Pentazocine was present in the culture medium (37 ^{o}C) over the indicated period of experiment. Bars represent mean±SE. (N=10-22). Translocation of sigma-1 receptors (%) are significantly higher in all (+)pentazocine-treated groups (at all concentrations and time-points) when compared to non-treated groups ($p<0.01$).

Figure 2. Intracellular dynamics of Sig-1R-EYFP in living NG108 cells.

a. Sig-1R-EYFP movement from a single ER-LD to an endoplasmic reticulum tubular element (arrow). **b-c.** Movement of Sig-1R-EYFP in the presence of (+)pentazocine (**b**;

100 nM) or cocaine (**c**; 10 μM). N: nucleus. Bars: 10 μm. Rectangular areas were monitored for time-lapse images in **b** and **c**. **d.** Increases of sigma-1 receptor protein levels by cocaine (10 min) in the extracellular space and nuclei (see Methods). Western blotting for sigma-1 receptors was visualized by anti-guinea pig sigma-1 receptor antibody-A.

Figure 3. FPAP analysis of Sig-1R-EYFP mobility in living NG108 cells.

a-b. Rectangular areas (**a**; single ER-LD, **b**; Endoplasmic reticulum tubular element) were first photobleached. Images were obtained before photobleaching and at the indicated time points after photobleaching. Cells were perfused continuously with HBSS at 33 ^{o}C. Fluorescence recovery was monitored every 10 sec. Scale bars=10 μm. **c.** The degree and time course of fluorescence recovery for Sig-1R-EYFP in either ER-LD or endoplasmic reticulum tubular elements (normalized to prebleach values).

Figure 4. Effect of (+)pentazocine on subcellular distribution of sigma-1 receptors in NG108 cell membranes separated by differential centrifugation.

Enrichment of sigma-1 receptors in each fraction was analyzed by Western blotting using anti-guinea pig sigma-1 receptor antibody-A. Twenty μg of total protein lysates was loaded in each lane. P3L, light-density microsome; P3H, heavy-density microsome; (+)PTZ, treatment with 100 nM (+)pentazocine for 10 min at 37 ^{o}C.

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Figure 5. Separation of sigma-1 receptor-containing ER-LD and the c-LD from NG108 cell homogenates by sucrose gradients.

a. Low-density vesicles enriched in sigma-1 receptors exist in 15-25% sucrose fractions. Note the enrichment of caveolin-2 (Cav-2), but not ADRP, in the same fractions. Sigma-1 receptors were detected by anti-guinea-pig sigma-1 receptor antibody-A. **b.** Enrichment of organelle markers in each respective fraction. PM: plasma membrane. Each protein was assessed by Western blottings using specific antibodies followed by densitometorical analysis. **c.** Moderate enrichment of neutral lipids and free cholesterol (FC) in sigma-1 receptor-containing fractions. Lipids in each fraction were extracted and separated by TLC. TLC spots visualized by H_2SO_4 spray (and baking) were densitometorically analyzed. **d.** Vesicles in the 20.5%-sucrose fraction from Sig-1R-EYFP-transfected NG108 cells. Left, Nomarski image; right, overlaid image (Sig-1R-EYFP in green). **e.** Vesicles from 0%-sucrose fraction. Inset: higher magnification. Scale bar=5 μm.

Figure 6. Translocation of sigma-1 receptors from ER-LD to high density fractions caused by (+pentazocine.

(+)Pentazocine ((+)PTZ; 1 μM, 30 min at 37 ^{o}C) caused a decrease of sigma-1 receptors in ER-LD-enriched fractions (#3-5) as shown by sucrose density gradients (see Figure 5). Note: a concomitant increase of sigma-1 receptors in fractions 11 and 12. Sigma-1 receptors were detected by Western blottings using anti-guinea pig sigma-1 receptor antibody-A.

Fig. 1

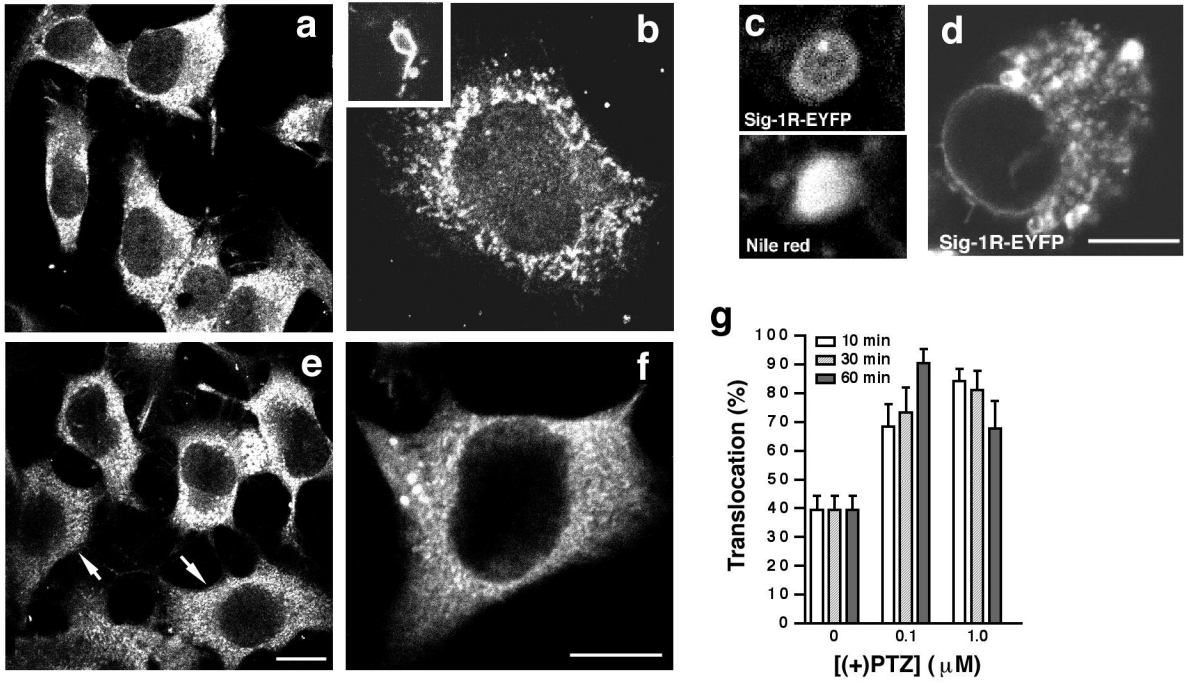
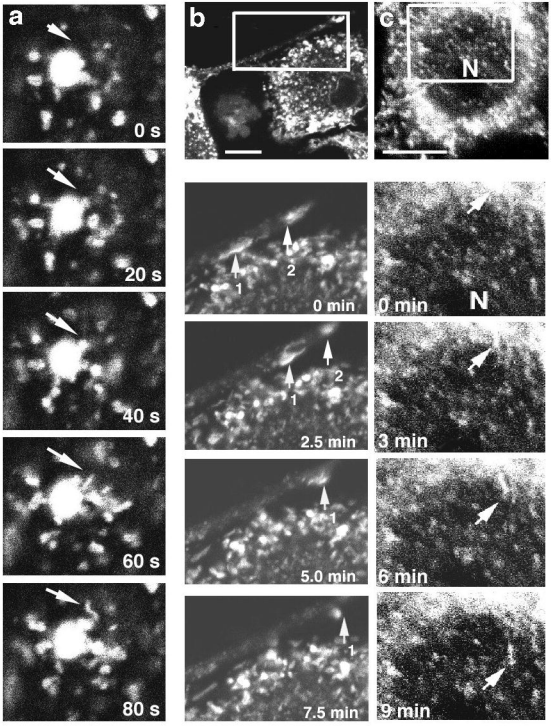

a
b
c
Sig-1R-EYFP
Nile red
d
Sig-1R-EYFP
e
f
g
10 min
30 min
60 min
Translocation (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1.0
[(+)PTZ] (μM)

Fig. 2


a
0 s
20 s
40 s
60 s
80 s
b
c
N
1
2
0 min
2.5 min
5.0 min
7.5 min
0 min
N
3 min
6 min
9 min

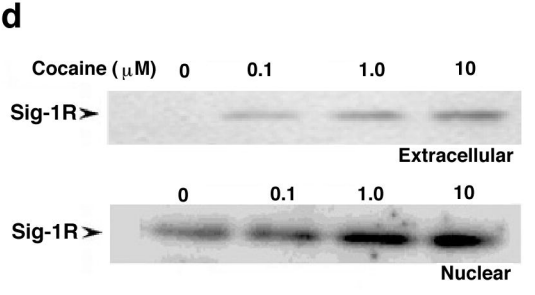
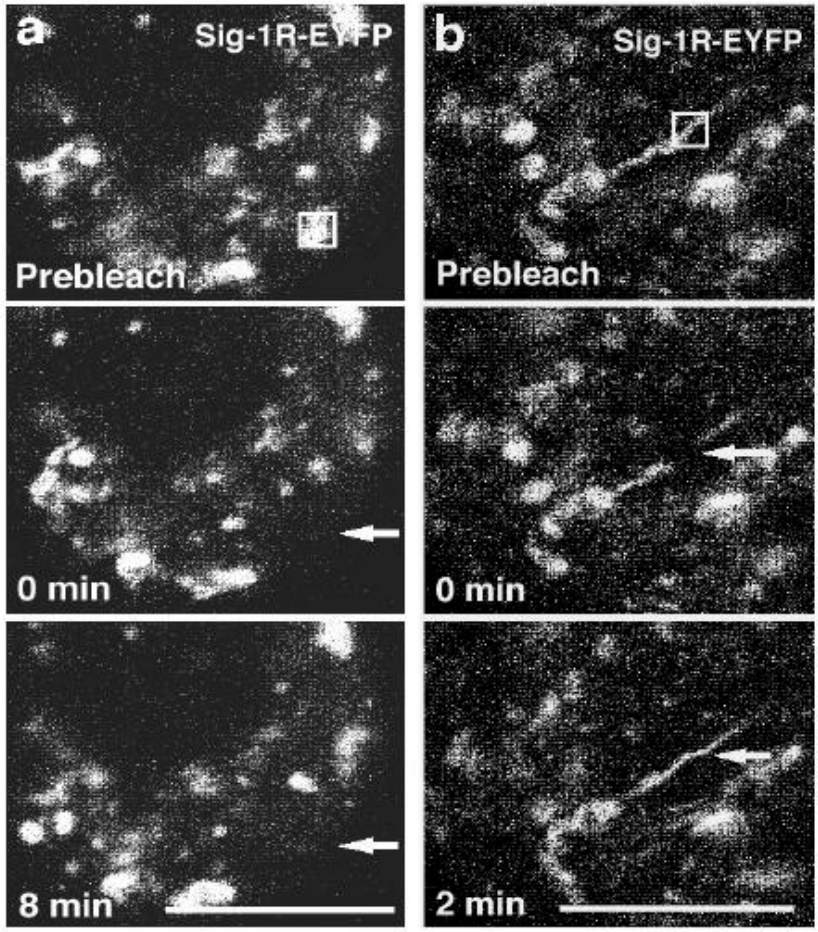

d
Cocaine (μM)
0
0.1
1.0
10
Sig-1R
Extracellular
0
0.1
1.0
10
Sig-1R
Nuclear

Fig. 3


a
Sig-1R-EYFP
Prebleach
0 min
8 min
b
Sig-1R-EYFP
Prebleach
0 min
2 min

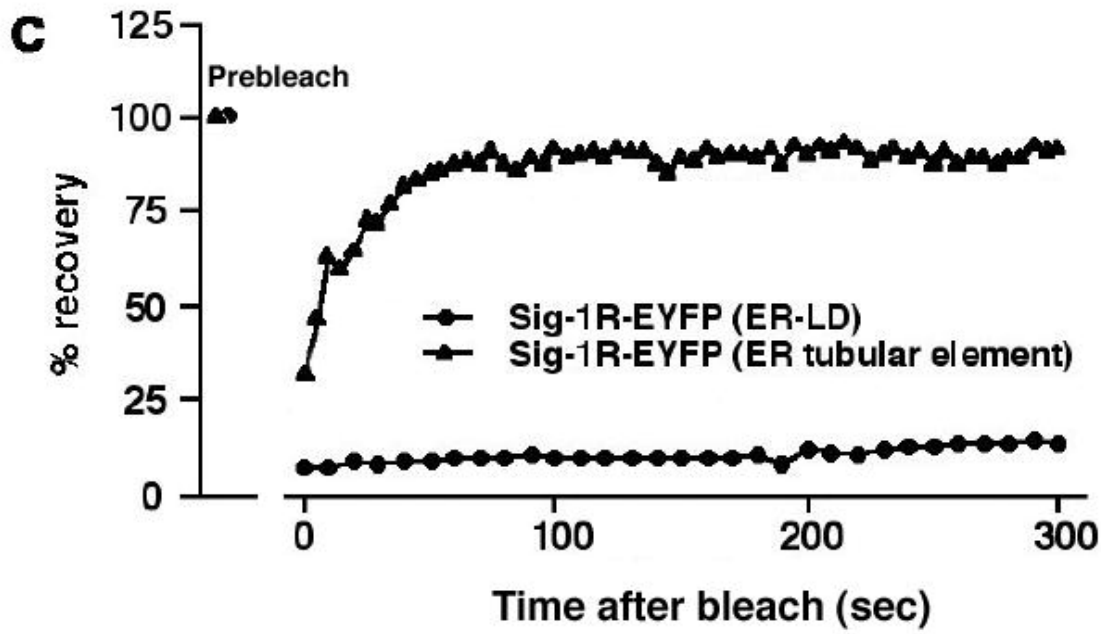

c
125
100
75
50
25
0
Prebleach
% recovery
Sig-1R-EYFP (ER-LD)
Sig-1R-EYFP (ER tubular element)
0
100
200
300
Time after bleach (sec)

Fig. 4

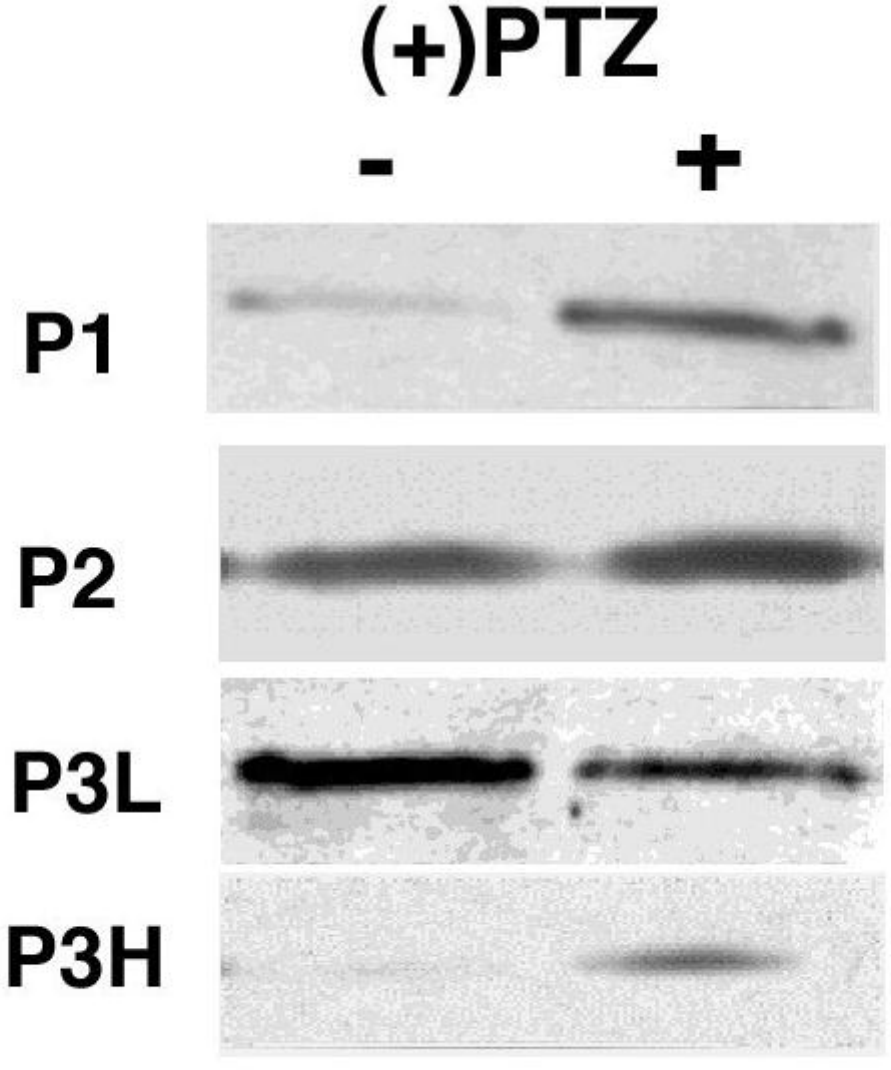
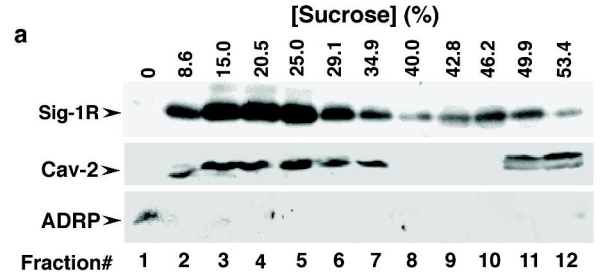
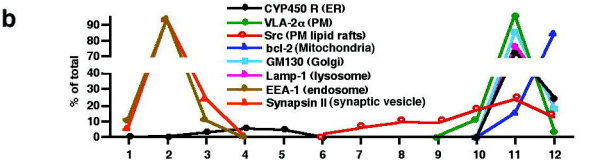
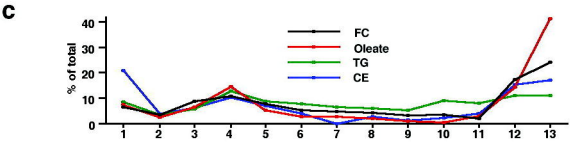


Fig. 5


a
[Sucrose] (%)
0
8.6
15.0
20.5
25.0
29.1
34.9
40.0
42.8
46.2
49.9
53.4
Sig-1R
Cav-2
ADRP
Fraction#
1 2 3 4 5 6 7 8 9 10 11 12


b
CYP450 R (ER)
VLA-2α (PM)
Src (PM lipid rafts)
bcl-2 (Mitochondria)
GM130 (Golgi)
Lamp-1 (lysosome)
EEA-1 (endosome)
Synapsin II (synaptic vesicle)
% of total
1 2 3 4 5 6 7 8 9 10 11 12


c
FC
Oleate
TG
CE
% of total
1 2 3 4 5 6 7 8 9 10 11 12 13

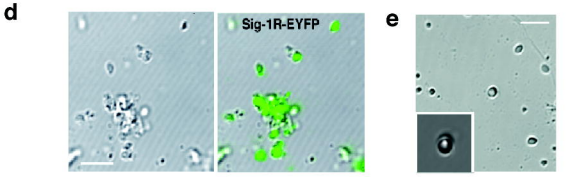

d
Sig-1R-EYFP
e

Fig. 6