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Research Article

Isoflurane Enhances the Moonlighting Activity of GAPDH: Implications for $GABA_A$ Receptor Trafficking

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$GABA_A$ receptor activity is directly modulated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a protein with many nonglycolytic moonlighting functions. In addition to playing a role in the phosphorylation of the receptor, GAPDH may also participate in proper receptor trafficking to the plasma membrane. We previously showed that volatile anesthetics affect GAPDH structure and function that may contribute to the manner by which GAPDH modulates the $GABA_A$ receptor. In the current study, GAPDH interacted with engineered phospholipid-containing vesicles, preferring association with phosphatidylserine over phosphatidylcholine. Phosphatidyl-serine is known to participate in membrane trafficking of transport proteins and to play a role in $GABA_A$ receptor stability and function. We observed that GAPDH promoted the self-association and fusion of phosphatidyl-serine-rich vesicles as well as decreased membrane fluidity. Isoflurane enhanced each of these GAPDH-mediated events. Isoflurane also increased the binding of GAPDH to the cytoplasmic loop of the $GABA_A$ receptor. These observations are consistent with the working model of isoflurane playing a role in the trafficking of membrane proteins. This study is the first to implicate GAPDH and isoflurane in the regulation of $GABA_A$ receptor localization, providing insight into the mechanism of action of anesthesia.

1. Introduction

The activity of the $GABA_A$ receptor is modulated directly by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [1], which participates in the local production of ATP followed by phosphotransferase activity involving the phosphorylation of the cytoplasmic loop of the α-subunit of the $GABA_A$ receptor. There is an increased recognition of GAPDH's multifunctionality, particularly with regard to neuronal function [2]. We think that in addition to this role (i.e., kinase-dependent phosphorylation of the receptor [1]), GAPDH may also participate in the proper receptor trafficking to the plasma membrane, and perhaps the recycling event.

We previously showed that volatile anesthetics affect GAPDH structure and function [3, 4]. Sevoflurane, for example, alters the kinetic properties of GAPDH, and isoflurane affects the oligomeric configuration of the protein. Others have also showed that volatile agents influence the oxidoreductase activity of GAPDH [5, 6]. These observations support the concept that volatile anesthetic agents bind to GAPDH. We propose that the binding of anesthetic agents to GAPDH may contribute to the manner by which GAPDH modulates the $GABA_A$ receptor.

The functional diversity of GAPDH includes the ability of the protein to interact with biomembranes [7, 8]. Curiously, GAPDH was shown to have a binding site for the phospholipid, phosphatidylserine [9]. Additionally, GAPDH exhibits fusogenic properties that are associated with diverse cellular processes [10–12]. We were interested in this particular property of GAPDH and its relationship to the modulation of $GABA_A$ receptor dynamics, including assembly and trafficking.

The $GABA_A$ receptor is composed of five subunits, consisting predominantly of two pairs of α- and β-subunits and

an additional γ-subunit [13], acting as a chloride channel and mediating neuronal inhibition. Each of the subunits has four transmembrane helices. The subunits originate at the endoplasmic reticulum, bundle together as dimers and trimmers, and are trafficked to the distal synaptic sites. Connolly and coworkers [14] observed that the molecular chaperone, HSPA5, which is also called immunoglobulin heavy chain binding protein (or BiP), aids in the assembly and transportation of the $GABA_A$ receptor. In addition to HSPA5, two other molecular chaperones (i.e., calnexin and protein disulfide isomerase) participate in the assembly of $GABA_A$ receptor subunit dimers and trimers [15].

Interestingly, GAPDH expression is correlated with that of HSPA5 [16, 17]. Furthermore, GAPDH interacts with protein disulfide isomerase [18]. These observations suggest that GAPDH may play a role in the assembly/trafficking of $GABA_A$ receptor via these proteins. We previously observed that neuroblastoma cells, briefly exposed to physiologically-relevant concentrations of isoflurane, promoted the upregulation of HSPA5 [19], suggesting that volatile anesthetic agents may affect the assembly and trafficking of the $GABA_A$ receptor via the proteins associated with these processes. Furthermore, support for GAPDH being a pivotal player in this process involves observations demonstrating the interaction of GAPDH with dynein [20], which binds gephyrin [21] securing the assembled $GABA_A$ receptor to the synaptic membrane [13]. Our focus is on the role of the GAPDH protein, particularly in regard to its ability to bind biomembranes in the presence and absence of volatile anesthetics.

In the present study, we examined the interaction of GAPDH with unilamellar vesicles (ULVs) prepared with different amounts of phosphatidylserine, which is the most abundant phospholipid in human cell membranes with many functional implications [22]. The interactions between GAPDH and ULVs were studied with and without physiologically-relevant concentrations of isoflurane. Our findings along with our previous observations [19] further suggest that isoflurane may act in part through GAPDH in regulating $GABA_A$ receptor dynamics. Our working model proposes that GAPDH plays a pivotal role in $GABA_A$ receptor assembly, trafficking, and recycling, whereby volatile anesthetic may enhance this GAPDH-mediated capacity.

2. Materials and Methods

2.1. Preparation of ULVs. Unilamellar vesicles (ULVs) were prepared using a Mini-Extruder from Avanti (Alabaster, AL, USA) as described by the vendor. Phospholipid composition of the ULVs was either 90% dioleoyl phosphatidylcholine (DOPC) and 10% dioleoyl phosphatidylserine (DOPS) or 90% DOPS and 10% DOPC. Stock solutions of DOPC and DOPS, which were dissolved in chloroform, were purchased from Avanti and a mixture was dried under a stream of nitrogen gas followed by resuspension in a buffer containing 100 mM NaCl, 200 mM HEPES (pH 7.5), and 0.02% sodium azide. Chemicals were of reagent grade. The suspension was processed by several freeze-thaw cycles prior to final extrusion resulting in isolation of clear suspensions of ULVs with approximate diameters of 100 nm. Argon was layered over the samples of ULVs, which were kept shielded from light in screw-capped vials at 4°C until used.

2.2. Exposure to Isoflurane. We developed a novel procedure for exposing membranes to isoflurane that allowed us to examine the effects of the agent on protein-membrane interaction without incidental exposure of membranes to high concentrations of agent that would be detrimental to membrane integrity. Human serum albumin (HSA), which was obtained from Sigma-Aldrich (St. Louis, MO, USA), was loaded with isoflurane that was purchased from Baxter Healthcare (Deerfield, IL, USA). Isoflurane (500 μL), which is immiscible in water, was incubated with an aqueous solution of HSA (1.2 mL of 5 mg/mL) by gentle rocking prior to transfer of the upper aqueous phase (250 μL) into a sealed dialysis bag that was inserted in a 2 mL screw-capped vial containing GAPDH and ULVs. The final isoflurane concentration of the GAPDH/ULV samples was 55 μM. The method used for determining isoflurane concentration was described previously [23]. Alternately, GAPDH was directly incubated with isoflurane prior to addition of isoflurane-bound GAPDH to ULVs. In all experiments, GAPDH, which was obtained from Sigma-Aldrich (i.e., G2267; rabbit muscle), was prepared in a buffer containing 50 mM sodium phosphate (pH 7.4) and 0.6 mM EDTA and passed through a previously washed 0.2 μm Millex-GN (Millipore; Billerica, MA, USA) nylon filter. Protein concentration was determined using the molar absorption coefficient 149 $mM^{-1} \cdot cm^{-1}$ [24].

2.3. GAPDH Activity. Enzyme activity was measured as described previously [4]. The reaction was monitored by an increase in 340 nm absorbance due to formation of NADH using D-glyceraldehyde (10 mM) as the substrate in a buffer containing 40 mM triethanolamine (pH 8.6), 50 mM sodium phosphate, 5 mM EDTA, 1 mM NAD^+, and 20 mM D/L-glyceraldehyde. Addition of GAPDH (7–13 μg) initiated the reaction, which was tracked at 24°C using a Thermo Scientific (Waltham, MA, USA) Evolution 600 UV/Vis spectrophotometer. The specific activity was determined using the NADH absorption coefficient (i.e., 6.22 mM^{-1} per 1 cm light path) and given as nmols/min per mg GAPDH.

2.4. Light Microscopy of ULVs. GAPDH was first exposed to isoflurane (0.1 mM) prior to incubation with ULVs (phosphatidylserine-rich: 10% DOPC and 90% DOPS), which were kept unstirred at 4°C for 11 days after being purged with argon. Samples were placed on slides with sealed coverslips, were kept for 45 min at room temperature, and subsequently analyzed by light microscopy using an Olympus IX81 motorized inverted microscope (Center Valley, PA, USA). Images were collected at a magnification of 400X.

2.5. Right-Angle Light Scattering. GAPDH (17 μM) was exposed to isoflurane (0.12 mM) by gentle tumbling for 10 min at room temperature. The samples were briefly

centrifuged and an aliquot of GADPH (500 μL) was added to ULVs (130 μg/mL), mixed, and read immediately using a Perkin Elmer LS50B fluorometer. The device was set to read intensity with the excitation and emission set to 480 nm (slits at 10 nm) and the cutoff filter set at 515 nm with integration time at 5s acquiring readings for 3 min. Comparisons with control values were made using one-way ANOVA followed by Holm-Sidak posthoc tests.

2.6. Measurement of Membrane Fluidity. The interaction of GAPDH with ULVs was examined using the lipid membrane probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Membrane fluidity was measured as previously described [25] with DPH (1 μM) and ULVs (72 μg lipid/mL) in the presence and absence of GAPDH (2 μM). Anisotropies (r) were calculated using the following formula: $r = (\text{Ivv} - \text{Ivv}_\phi) - [\text{GF}(\text{Ivh} - \text{Ivh}_\phi)]/(\text{Ivv} - \text{Ivv}_\phi) - [2\text{GF}(\text{Ivh} - \text{Ivh}_\phi)]$, where GF was set at 1.27, and intensities (Ivv_ϕ and Ivh_ϕ) and (Ivv and Ivh) were measured before and after addition of DPH, respectively, using a Perkin-Elmer LS50B fluorometer (ex: 360 nm; em: 430 nm; slits 7.5 nm; integration 5s). We also determined the degree of change of r as a function of temperature at a rate of 0.6°C per min.

2.7. Interaction with $GABA_A$ Receptor Cytoplasmic Loop. A plasmid construct coding for the second intracellular loop region (specifically, amino acid residues 334–420, which we designated as GCL for $GABA_A$ receptor cytoplasmic loop) of the human α1 subunit was a kind gift from Dr. Jacques J. Laschet (INSERM, Université Paris Descartes). This DNA construct produces a YFP- and 6X histidine-tagged GCL [1]. GCL was expressed transiently by transfecting 293T cells (gift from Dr. Asma Zaidi) with the DNA construct using Lipofectamine 2000 from Life Technologies (Grand Island, NY, USA). GCL was purified using HIS-Select nickel affinity gel from Sigma-Aldrich (St. Louis, MO, USA). The lysate volume required for saturation of the nickel resin with GCL was determined by YFP fluorescence. Cell lysate (500 μL) was diluted 1 : 1 with 500 μL binding buffer containing 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH 8.0. Diluted cell lysate (1 mL) was incubated with nickel resin (50 uL), rotated overnight at 4°C, washed 4X with wash buffer containing 60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH 8.0 and then washed once with binding buffer. Untreated GAPDH (5 μg) and GAPDH treated for 10 min with isoflurane (0.12 mM) was diluted in 1 mL binding buffer and incubated with GCL-saturated resin and rotated for 1 hr at 4°C. Resin was then washed 1X with binding buffer containing 0.05% Tween-20, washed 2X with binding buffer and then protein was eluted in 50 μL elution buffer containing 0.05 M glycine-HCl pH 1.5. Eluted protein (10 μL) was pipetted onto dry nitrocellulose membrane and allowed to evaporate at 45°C for 30 min. The membrane was then blocked in 5% nonfat dry milk for blotting using a HRP-conjugated mouse anti-GAPDH antibody from Sigma-Aldrich (St. Louis, MO, USA). GAPDH protein was detected using SuperSignal West Pico chemiluminescent substrate from Thermo Scientific (Waltham, MA, USA).

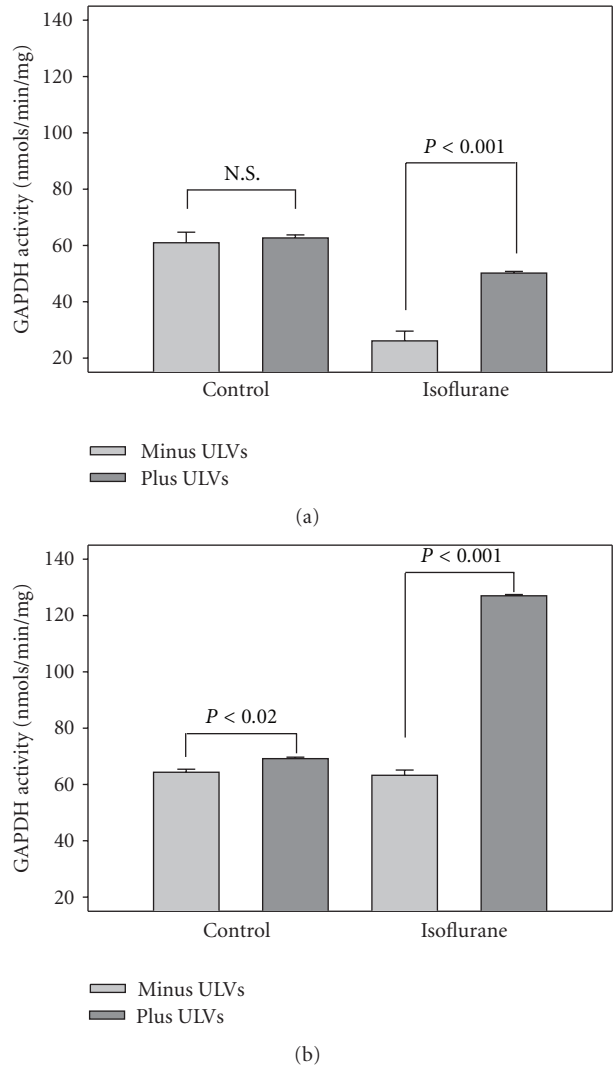

Figure 1: Effects of isoflurane on GAPDH activity with and without ULVs. GAPDH was incubated with ULVs, which were either enriched with phosphatidyl-choline (a) or phosphatidylserine (b), prior to measurement of enzyme activity. Samples were exposed to isoflurane (55 μM) and compared with those without agent. Data are presented as means of duplicate measurements from a representative experiment. Error bars indicate standard deviation.

3. Results

We examined the interaction of GAPDH with ULVs by incubating GAPDH (5 μM) and ULVs (240 μg/mL) briefly (2 min) prior to determining enzyme activity. In the presence of ULVs made from 90% DOPC and 10% DOPS, designated as PC-rich vesicles, there was no change in GAPDH enzyme activity with and without ULVs (Figure 1(a)).

In contrast, PS-rich vesicles made from 10% DOPC and 90% DOPS promoted an increase in GAPDH enzyme activity (Figure 1(b)), suggesting that the presence of PS increases GAPDH activity and presumably increases the binding of GAPDH to the vesicles. The addition of isoflurane resulted in an increase in GAPDH activity above control

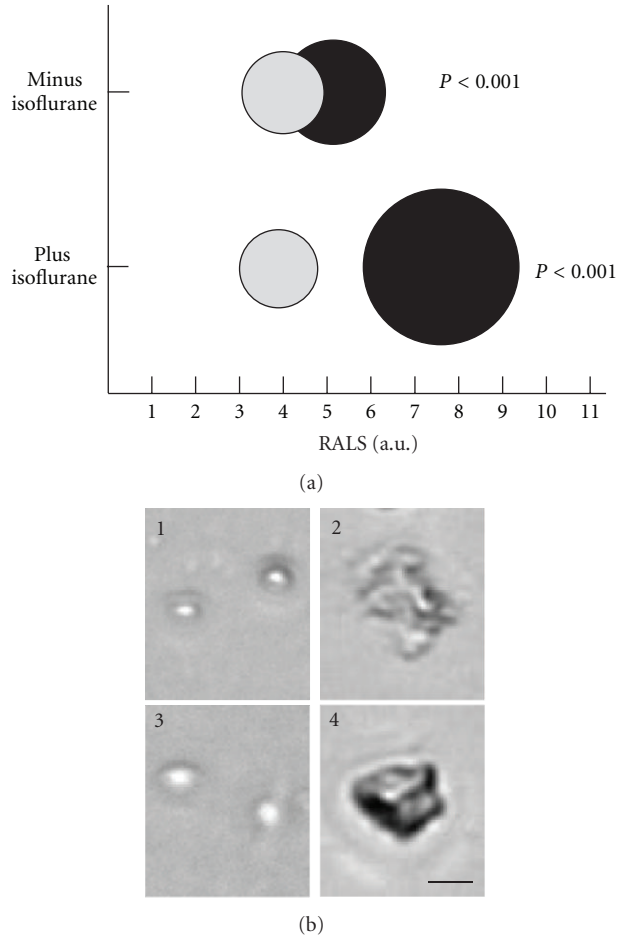

Figure 2: Isoflurane enhances GAPDH-induced ULV aggregation/fusion. (a) GAPDH was first exposed to isoflurane (0.1 mM) prior to incubation with ULVs and subsequent measurement of right-angle light scattering (RALS). Conditions are given on the y-axis. Data are presented visually to indicate size, which is given on the x-axis. The vesicles sizes are scaled relative to the plus isoflurane control. Data are presented as means of triplicate measurements from a representative experiment. Error bars are omitted for clarification, and the within-group significance is shown to the right of the shapes. (b) GAPDH was first exposed to isoflurane (0.1 mM) prior to incubation with ULVs (phosphatidylserine-rich) and subsequent analysis by light microscopy. Panels: (1) ULVs only; (2) ULVs with GAPDH minus isoflurane; (3) ULVs without GAPDH plus isoflurane; (4) ULVs with GAPDH plus isoflurane. Bar, 5 μm. Magnification, 400X.

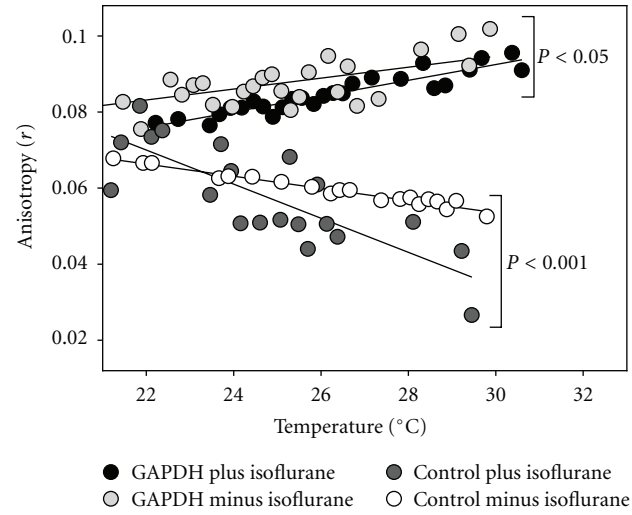

Figure 3: Effects of GAPDH and isoflurane on membrane fluidity. GAPDH was first exposed to isoflurane (0.12 mM) prior to brief incubation with phosphatidylserine-rich ULVs and subsequent measurement of membrane fluidity using a fluorescent lipid probe. The linear regression lines represent changes in membrane fluidity as a function of increasing temperature.

in the samples with PS-rich vesicles, but not with the PC-rich vesicles. The isoflurane-stimulated activity was most pronounced with the PS-rich vesicles.

We further examined the role of isoflurane on the binding of GAPDH to PS-rich vesicles by incubating GAPDH (14 μM) with ULVs (130 μM) prior to measuring vesicles size by right-angle light scattering. Both untreated GAPDH and isoflurane-treated GAPDH increased the values for right-angle light scattering (Figure 2(a)).

The arbitrary units of RALS are proportional to vesicle size, with ULVs in the presence of untreated GAPDH being larger than that of ULVs alone (5.3 ± 0.01 versus 4.1 ± 0.01, resp.). Likewise, ULVs in the presence of isoflurane-treated GAPDH were also significantly larger than that of vesicles incubated with isoflurane-treated buffer (7.9 ± 0.11 versus 4.0 ± 0.01, resp.). Interestingly, ULVs incubated with isoflurane-treated GAPDH were significantly larger than the ULVs that contained untreated GAPDH, suggesting that isoflurane enhanced the effects of GAPDH. In order to confirm the right-angle light scattering data, we examined the size and shape of PS-rich ULVs under phase-contrast light microcopy. In the presence of GAPDH, the vesicles appeared clustered and fused, appearing visibly larger (Figure 2(b)). In the absence of GAPDH, the vesicles appeared evenly and sparely distributed with uniform shape. Furthermore, clusters were not observed in the minus GAPDH samples. With untreated GAPDH, the ULV aggregates appeared as grape-like clusters. While the vesicles incubated with isoflurane-treated GAPDH also showed some clusters, they predominantly exhibited a more fusiform structure.

We looked at the effects of GAPDH on membrane fluidity by measuring the anisotropy using a lipid bilayer probe, DPH. Control ULVs exhibited the expected pattern, whereby the anisotropy values decreased with increasing temperature (Figure 3). In the presence of isoflurane, control vesicles also exhibited a decrease in anisotropy with increased temperature. The response of ULVs to temperature (as measured by the slope) in the presence of isoflurane was greater than that of untreated ULVs. Curiously, the anisotropy of the ULVs that were incubated with GAPDH showed higher anisotropy values, which actually increased with temperature rather than decreasing with temperature. In the presence of isoflurane, the ULVs incubated with GAPDH showed even higher slopes (i.e., anisotropy as a function of temperature) than

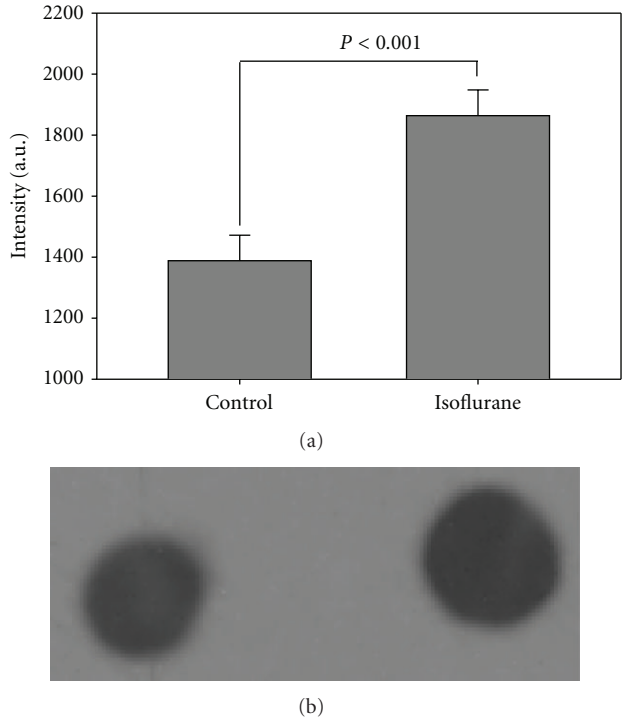

Figure 4: Isoflurane enhances the association of GAPDH and $GABA_A$ receptor cytoplasmic loop. Dot blot analysis (b) revealed the relative binding of GAPDH to the $GABA_A$ receptor C-loop, using anti-GAPDH antibody for detection. Densitometric analysis was performed and results were compared (a).

the samples without isoflurane, suggesting that the effects of GAPDH on ULVs are further enhanced by the anesthetic agent.

We also examined the effect of isoflurane on the interaction of GAPDH with the $GABA_A$ receptor cytoplasmic loop (GCL). The GCL polypeptide was immobilized on nickel resin and incubated with treated (0.12 mM isoflurane) and untreated GAPDH. Unbound GAPDH was removed by washing and bound GAPDH was detected after spotting the eluted protein on nitrocellulose. Isoflurane-treated GAPDH showed an increased association with GCL in this binding assay (Figure 4).

4. Discussion

We observed that GAPDH interacted with engineered vesicles (i.e., phospholipid bilayers) using an *in vitro* model representing biological membranes. This observation is consistent with the previously documented functional diversity of GAPDH, namely, its ability to interact with biomembranes [7, 8]. The interactions between GAPDH and vesicles that are reported in this study were more salient when ULVs were prepared with a higher percentage of phosphatidylserine. This phospholipid is highly abundant and is largely found in the cytoplasmic surface of the lipid bilayer [22]. Additionally, phosphatidylserine participates in membrane trafficking of transport proteins [27]. Phosphatidylserine also appears to play a role in $GABA_A$ receptor stability [28] and function [29]. Furthermore, GAPDH has a binding site for phosphatidylserine [9], and this protein has been shown to promote membrane fusion [10–12]. We propose that GAPDH may be involved in the membrane assembly and trafficking of the $GABA_A$ receptor and that isoflurane may enhance this process.

This study extends our previous suggestion that GAPDH plays a role in anesthesia [30]. Our current data suggests that GAPDH may be organizing the structure of the membrane (Figure 3) through its interaction with the phospholipids. This phenomenon may be mediated by attachment of GAPDH to the lipid component as well as interaction with neighboring GAPDH molecules that appears enhanced by the presence of anesthetic agent. The GAPDH-mediated fusion event may involve a coordinated ordering of the lipids during the exchange between adjacent bilayers. The decreased entropy (i.e., energy required) in this event may be compensated by the increased entropy of released water upon hydrophobic interactions. Isoflurane may enhance this process.

It is reasonable to speculate that anesthetic potency may be correlated to an agent's ability to promote GAPDH-mediated vesicle fusion that is required for the delivery/recycling of the $GABA_A$ receptor. Furthermore, this potency is proportional to its lipid partitioning properties, and thus consistent with the Meyer-Overton rule as well as the modern perspective of anesthetic-protein interactions.

We previously observed that sevoflurane inhibited the activity of GAPDH [4]. In this study, we also observed that isoflurane decreased the activity of GAPDH (Figure 1(a)) in the absence of ULVs. Although this observation exhibited inconsistency (Figure 1(b)), the significant result is that isoflurane enhanced GAPDH activity in the presence of ULVs containing both phospholipid types. Regarding the results presented in Figure 2(b), the microscopic analysis of the samples involved using ULVs that were kept at 4°C for 11 days. While the vesicle preparation was layered with argon to prevent oxidation during storage, this prolonged duration may have contributed to the susceptibility of the ULVs to aggregation. The extended storage was fortuitous in finding the right conditions for observing this phenomenon microscopically, as the initially produced ULVs are approximately 100 nm in diameter.

The interactions between GAPDH and ULVs were markedly influenced by exposure to physiologically relevant concentrations of isoflurane. We previously showed that neuroblastoma cells treated with isoflurane resulted in a significant and persistent upregulation of the gene expression of HSPA5 [19]. This gene product is a molecular chaperone, which is also known as BiP and responsible for $GABA_A$ receptor assembly and trafficking [13, 14]. Furthermore, GAPDH expression is correlated with that of HSPA5 [16] suggesting that GAPDH may play a role in $GABA_A$ receptor assembly, trafficking, and perhaps even recycling. Our working model proposes that volatile anesthetics may enhance this GAPDH-mediated process (Figure 5).

Laschet and coworkers [1] showed that the $GABA_A$ receptor is modulated directly by the kinase activity of

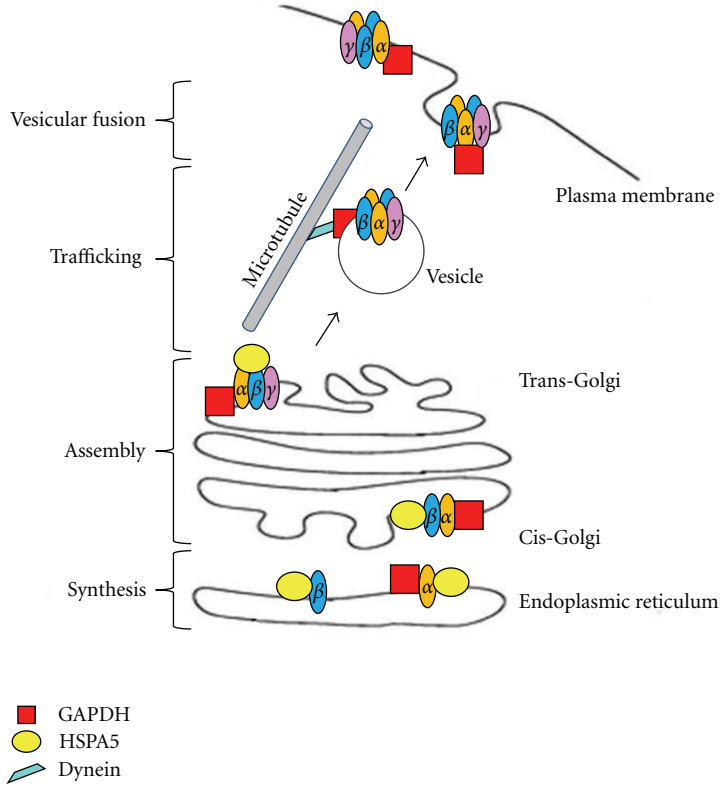

Figure 5: Proposed mechanism of $GABA_A$ receptor trafficking. The scheme illustrates the fundamental events associated with trafficking of this receptor that is known to require HSPA5 [13, 14] and dynein [20]. We include in our working model that GAPDH may be playing a role in some aspects of this process due to its known affinity to dynein, the receptor α-subunit, and biomembranes. This mechanism is consistent with the observation that isoflurane promotes trafficking of another neuronal membrane transport protein [26]. The current study supports this model, including isoflurane-induced enhancement of GAPDH-mediated processes (i.e., membrane fusion and $GABA_A$ cytoplasmic loop binding).

GAPDH. This multifunctional protein directly phosphorylates the cytoplasmic loop of the α-subunit of the $GABA_A$ receptor. There is growing recognition of GAPDH's nonglycolytic functions [7, 8], particularly membrane-associated processes, such as autophagy and nuclear membrane reassembly. GAPDH's binding to the cytoplasmic domain of the α-subunit of the $GABA_A$ receptor may be involved in the assembly and trafficking of the receptor.

We previously demonstrated that volatile anesthetics affect GAPDH structure and function [3, 4], findings that are consistent with those from other studies [5, 6]. We propose that the binding of anesthetic agents to GAPDH may contribute to the effect of GAPDH on the $GABA_A$ receptor, particularly those involving membrane dynamics.

In addition to HSPA5 being involved in the trafficking of the $GABA_A$ receptor [14], protein disulfide isomerase also participates in the assembly of $GABA_A$ receptor subunit dimers and trimers [15]. Curiously, GAPDH interacts with protein disulfide isomerase [18], further supporting the contention that GAPDH may play a role in the assembly/ trafficking of $GABA_A$ receptor via these proteins. Additionally, GAPDH interacts with dynein [20], which binds gephyrin [21], securing the assembled $GABA_A$ receptor to the synaptic membrane [13].

In this study, isoflurane enhanced every GAPDH-mediated event. Isoflurane has also been shown to have an effect on the trafficking of a neuronal glutamate transporter [26]. Interestingly, in addition to stimulating the activity of this glutamate transporter, isoflurane increases its trafficking. These authors suggested that isoflurane-stimulated trafficking of this glutamate receptor may contribute to the phenomenon of anesthesia. The trafficking of $GABA_A$ receptor, whose activity is modulated by volatile anesthetics, may also contribute to anesthesia. Our data is consistent with this model and proposes a novel molecular mechanism of action of volatile anesthetics in the CNS.

Acknowledgments

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