Neuroactive Steroid Interactions with Voltage-Dependent Anion Channels:
Lack of Relationship to GABA<sub>A</sub> Receptor Modulation and Anesthesia

Ramin Darbandi-Tonkabon, Brad D. Manion, William R. Hastings, William J. Craigen, Gustav Akk, John R. Bracamontes, Yejun He, Tatiana V. Sheiko, Joseph H. Steinbach, Steven J. Mennerick, Douglas F. Covey and Alex S. Evers

Departments of Anesthesiology (RD, BDM, WRH, GA, JRB, JHS, ASE) and Molecular Biology and Pharmacology (DFC, ASE) and Psychiatry (YH, SJM)3 Washington University School of Medicine, St. Louis, Missouri; Departments of Pediatrics and Genetics (TVS, WJC), Baylor College of Medicine, Houston Texas.
Running Title: Neuroactive Steroid Interactions in VDAC deficient mouse

Corresponding Author:
Alex S. Evers, M.D.
Department of Anesthesiology,
Washington University School of Medicine
660 S. Euclid Ave, Campus Box 8054
St. Louis, MO 63110
Tel. (314) 454-8702  Fax (314) 454-5572
Email: eversa@notes.wustl.edu

Text pages: 37
Tables: 0
Figures: 7
References: 31
Abstract: 250 words
Introduction: 436 words
Discussion: 928 words
The abbreviations and trivial names that are used: 2DE gel, 2-Dimensional Electrophoresis; 3α5αP, (3α,5α)-3-Hydroxy pregnan-20-one; 3α5βP, (3α,5β)-3-hydroxy pregnan-20-one; 6-AziP, (3α,5β)-6-azi-3-hydroxy pregnan-20-one; ACN, (3α,5α)-3-Hydroxyandrost an-20-carbonitrile; Buffer EC; Enhancer and DNA-condensation buffer; CHAPS, (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate; CTβ, closing time-β; DMEM; Dulbecco’s Modified Eagle Medium; DTT, DL-Dithiothreitol; ESI, electrospray ionization; GABA, γ-amino butyric acid; GABA_{A} receptor, GABA receptor type A; HEPES, N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IPG, immobilized pH gradient; MEF; Mouse Embryonic Fibroblast; PMSF, Phenylmethylsulfonylfluoride; TBST; Trisbuffer-saline-Tween-20; TBPS, t-butylbicyclophosphorothionate, VDAC, voltage-dependent anion channel; Vhr, Volt hour;

Recommended section: Cellular & Molecular
ABSTRACT

Neuroactive steroids modulate the function of gamma aminobutyric acid type A (GABA_A) receptors in brain; this is the presumed basis of their action as anesthetics. In a previous study using the neuroactive steroid analogue, 6-AziP ((3α,5β)-6-azi-3-hydroxypregnan-20-one), as a photoaffinity-labeling reagent, we showed that voltage-dependent anion channel-1 (VDAC-1) was the predominant protein labeled in brain. Antisera to VDAC-1 were shown to co-immunoprecipitate GABA_A receptors, suggesting a functional relationship between steroid binding to VDAC-1 and modulation of GABA_A receptor function. This study examines the contribution of steroid binding to VDAC proteins to modulation of GABA_A receptor function and anesthesia. Photolabeling of 35-kDa protein with [3H]6-AziP was reduced 85% in brain membranes prepared from VDAC-1 deficient mice, but was unaffected by deficiency of VDAC-3. The photolabeled 35-kDa protein in membranes from VDAC-1 deficient mice was identified by 2-dimensional electrophoresis and ESI-tandem mass spectrometry as VDAC-2. The absence of VDAC-1 or VDAC-3 had no effect on the ability of neuroactive steroids to modulate GABA_A receptor function as evidenced by radioligand ([35S]TBPS) binding or by electrophysiological studies. Electrophysiological studies also showed that neuroactive steroids modulate GABA_A receptor function normally in VDAC-2 deficient fibroblasts transfected with α₁β₂γ₂ GABA_A receptor subunits. Finally the neuroactive steroid pregnanolone ((3α,5β)-3-hydroxypregnan-20-one) produced anesthesia (loss of righting reflex) in VDAC-1 and VDAC-3 deficient mice and there was no difference in
the recovery time between the VDAC deficient mice and wild type controls. These data indicate that neuroactive steroid binding to VDAC-1,2 or 3 is unlikely to mediate GABA\textsubscript{A} receptor modulation or anesthesia.
Certain endogenous pregnane steroids and their structural analogues are potent anesthetics in vertebrates (Selye, 1941; Atkinson et al., 1965). These neuroactive steroids are thought to produce anesthesia by modulating the function of gamma amino-butyric acid type A (GABA$_A$) receptors in the central nervous system (Harrison and Simmonds, 1984; Majewska et al., 1986, Harrison et al., 1987). The strong correlation between the ability of neuroactive steroid analogues to modulate GABA$_A$ receptors and their ability to produce anesthesia strongly supports this hypothesis (Harrison et al., 1987). The actions of neuroactive steroids on GABA$_A$ receptors are likely to be mediated via binding to specific recognition sites on the GABA$_A$ receptor protein complex. This concept is supported by the enantioselectivity of neuroactive steroids both in their modulation of GABA$_A$ receptor function and in their actions as anesthetics (Wittmer et al., 1996; Covey, 2000).

Extensive molecular biological studies (generation of chimeric receptors, site-directed mutagenesis) have been unsuccessful in identifying candidate regions or binding sites for neuroactive steroids on the GABA$_A$ receptor protein. The failure of molecular biological approaches to identify neuroactive steroid binding sites could be explained in several ways: (1) A binding site may involve multiple non-contiguous portions of the protein, foiling chimeric strategies. (2) There may be multiple binding sites mediating discrete but functionally additive effects. The functional assays used to screen mutated receptors may thus lack sensitivity. (3) The binding sites may be on an accessory protein rather than on a GABA$_A$ receptor subunit. To avoid the aforementioned problems and to provide a direct and unbiased search for neuroactive steroid binding sites, we have previously used the neuroactive steroid analogue, 6-AziP ($\alpha_3,5\beta$)-6-azi-3-
hydroxypregnan-20-one), as a photolabeling reagent. Several brain proteins were shown to be specifically labeled by 6-AziP. The most prominently labeled protein in brain was identified as voltage-dependent anion channel-1 (VDAC-1) (Darbandi-Tonkabon et al., 2003).

VDAC-2 and VDAC-3 were also identified as proteins that may be labeled by 6-AziP. Furthermore, it was found that antisera to VDAC-1 co-immunoprecipitated GABA$_A$ receptor subunits from a detergent lysate of rat brain, indicating a strong physical association between the VDAC-1 and GABA$_A$ receptors. This is consistent with the earlier observation that VDAC-1 and/or VDAC-2 co-purified with GABA$_A$ receptors in a multi-step purification procedure from detergent-solubilized mammalian brain (Bureau et al., 1992).

These data suggested the possibility that neuroactive steroid modulation of GABA$_A$ receptor function is mediated by binding of the steroids to a voltage-dependent anion channel that is directly associated with the receptor protein. To test this hypothesis we have examined the effects of neuroactive steroids in mice lacking either VDAC-1 or VDAC-3 and in VDAC-2 deficient cells transfected with GABA$_A$ receptor subunits.
EXPERIMENTAL PROCEDURES

Membrane preparation. Mouse brains were prepared freshly after sacrificing the mice under deep halothane anesthesia. Cerebella and brain stem were trimmed from the brains and the cerebral hemispheres were used to prepare membranes with minor modification of previously described methods (Hawkinson et al., 1996). Briefly, brains were immersed in ice cold 0.32 M sucrose (10 ml/gm) and homogenized using a Teflon pestle in a motor-driven homogenizer. The homogenate was centrifuged for 10 min at 1,500 X G and the pellet was discarded. The supernatant was centrifuged for 30 min at 10,000 X G to obtain the P2 pellet, which was washed 3 times with 50 mM K-Phosphate/200 mM NaCl, pH 7.4. The pellet was re-suspended in 50 mM K-Phosphate/200 mM NaCl, pH 7.4 and recollected by centrifugation for 20 min at 10,000 X G. The final pellet was re-suspended using a teflon homogenizer and stored at -80° C.

Transfection of mouse embryonic fibroblasts with α_{1}FLAGβ_{2γ2} subunits of the GABA_{A} receptor. Mouse embryonic fibroblasts (MEF) derived from E7.5 day chimeric embryos were treated with 0.2 mg/ml G418 (GIBCO) immediately following plating of the dispersed embryos, and selected for 5 days to isolate VDAC-2 -/- cells (Cheng et al., 2003). Deficiency of VDAC-2 in MEFs following G418 selection was confirmed by PCR genotyping and immunoblotting with a previously generated isoform-specific antibody (data not shown) Wild-type (VDAC-2 +/-) and VDAC-2 -/- embryonic mouse fibroblasts were maintained in DMEM high glucose plus pyruvate (Invitrogen, Carlsbad, CA) containing 10 % fetal bovine serum (Hyclone, Logan, UT), and penicillin (100 U/ml) plus streptomycin (100 U/ml) in a humidified atmosphere containing 5% CO_{2}. 

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 19, 2023
Transient transfections were performed using Effectene Transfection Reagent (Qiagen, Valencia, CA), 300 µl buffer EC, 24 µl enhancer, 50 µl Effectene. The day before transfection, cells from nearly confluent cultures were split into 10 cm dishes at approximately 10^5 cells per dish. 3 µg of DNA was used for transfection into each 10 cm dish (1 µg of rat α1FLAG, β2 and γ2, each). Cells were cultured in complete media with the addition of 2 mM sodium butyrate (Sigma, St Louis, MO), after transfection. Transfected cells were panned using the anti-FLAG M2 antibody (Sigma, St. Louis, MO) as described previously (Chen et al., 1995). Anti-FLAG antibody was adsorbed to Dynal dynabeads (Dynal, Oslo, Norway) conjugated with goat anti-mouse antibody and applied to dishes containing panned cells (Ueno et al., 1997). Cells expressing the FLAG epitope were visualized by attached beads. Single-channel currents were recorded in the bead-identified cells and analyzed as described below. The analysis was carried out on single-channel clusters which were defined and isolated as described previously (Steinbach and Akk, 2001).

**Chemical Synthesis.** [^3H]6-Azi-pregnanolone ([^3H]6-AziP) was prepared by a multistep synthesis from commercially available progesterone as previously reported. (Darbandi-Tonkabon et al., 2003).

[^3S]TBPS Binding. [^3S]TBPS binding assays were performed using previously described methods (Hawkinson et al., 1994; Covey, 2000) with modification. Briefly, aliquots of membrane suspension (0.05 mg/ml final protein concentration in assay) were incubated with 5 µM GABA (Sigma Chemical, St. Louis, MO), 4 nM [^3S]TBPS (60-
100Ci/mmol, Perkin Elmer Life Science, Boston, MA) and 5 µl- aliquots of steroid in Me₂SO₄ solution (final steroid concentrations ranged from 1 nM to 10 µM), in a total volume of 1 ml 200 mM NaCl, 50 mM potassium phosphate buffer, pH 7.4. Control binding was defined as binding observed in the presence of 0.5% Me₂SO₄ and the absence of steroid; all assays contained 0.5% Me₂SO₄. Nonspecific binding was defined as binding observed in the presence of 200 µM picrotoxin and ranged from 12.4 to 32.6% of total binding. Assay tubes were incubated for 2 hours at room temperature. A Brandel (Gaithersburg, MD) cell harvester was used for filtration of the assay tubes through Whatman glass fiber (GF/C) filter paper. Filter paper was rinsed with 4 ml of ice-cold buffer three times and dissolved in 4 ml ScintiVerse II (Fisher Scientific, Pittsburgh, PA). Radioactivity bound to the filters was measured by liquid scintillation spectrometry and data were fit to the Hill equation using Sigma Plot

\[
B = \frac{100}{1 + \left(\frac{[C]}{IC_{50}}\right)^n}
\]

where binding was normalized to control binding, B is steroid bound, [C] is steroid concentration, IC₅₀ is the half-maximal inhibitor concentration, and n is the Hill coefficient. Each data point was determined in triplicate.

**In vivo anesthesia studies.** The ability of pregnanolone and allopregnanolone to produce anesthesia was measured in 8 week old male mice weighing approximately 20 g.
Steroids were made up as a stock solution of 100 mg/dl in 4.4% ethanol and 8% Cremophor EL (Sigma Chemical, St. Louis, MO). The solution was administered at a dose of 5 mg/kg by tail vein injection. Ten seconds after injection, the mice were positioned in a supine position on a heating pad at 37 °C. Sleep time was defined as the time from the moment mice displayed loss of righting reflex (LRR) until they were able to right themselves. Mice were only included in results if they recovered fully without observable neurological deficits.

Electrophysiology. The hippocampal cultures were prepared from wild-type and VDAC-1 -/- mice, age P1-3, as described previously (Mennerick et al., 1995), with slight modifications. Mouse culture medium was supplemented with insulin/transferrin/selenium (Sigma Chemical Co, St Louis, MO). At day in vitro 4, cultures were fed with a half medium exchange using Neurobasal medium plus B27 supplement (Invitrogen, Carlsbad, CA). The electrophysiological experiments were carried out 4-6 days after plating. The single-channel data were analyzed as described previously (Steinbach and Akk, 2001). In brief, the currents were recorded in the cell-attached and inside-out patch configurations at pipette potentials of +50 to +100 mV. The receptors were activated by 10 µM GABA in the absence and presence of 1 µM ACN. The channel open times were determined from the single-channel currents using the QuB suite (http://www.qub.buffalo.edu) (Qin et al., 1996).

Whole-cell, patch-clamp recordings were obtained at a holding potential of -70 mV. The extracellular bath contained (in mM): NaCl (140), KCl (4), CaCl₂ (2), MgCl₂ (1), glucose (10), HEPES (10), pH 7.25. NBQX (1 µM), D-APV (50 µM) and
tetrodotoxin (0.5) were also included in the bath to block spontaneous excitation. The whole-cell pipette solution contained (in mM): CsCl (130), NaCl (4) CaCl₂ (0.5), EGTA (5), pH 7.25. Drug applications were achieved with a gravity-fed multi-barrel pipette with a common tip placed within 500 µm of the recorded cell. For data analysis, peak GABA responses were measured in the presence and absence of steroid. Statistical comparisons between genotypes were made by unpaired, two-tailed t-test.

**Electrophoresis, Western blot, Gel slicing.** Polyacrylamide gel electrophoresis was performed using 10% polyacrylamide gels, under reducing conditions (Laemmli, 1970). After electrophoresis, the gels were either stained, sliced or used for Western blot. Gels were silver stained (Hatzimanikatis, 1999) using modified ammoniacal silver stain (Amersham), or stained with Coomassie blue using a Novex Colloidal Coomassie G-250 Kit (Invitrogen, Carlsbad, CA).

For gel slicing, the gels were cut in vertical columns and sliced in 1 mm horizontal slices using a DE 113 Manual Gel Slicer (Hoeffer Scientific Instruments, San Francisco, CA). Slices were digested with 4 ml of tissue solubilizer consisting of 3a20™ and TS-2 (ratio 9:1) for 24 hours, and the radioactivity in each slice was determined by scintillation spectrometry.

For western blotting, proteins from the SDS-PAGE gels were transferred onto multistack membranes (Kodak Biomax Multi-Blot kit, Cat # 193 4439) using a BioRad wet transfer system. For immunoblotting with the anti- VDAC-1 monoclonal antibody (Oncogene Research Products), the membranes were blocked with 10% dried milk for 15 minutes at room temperature, and incubated for 30 minutes with anti- VDAC-1 antibody
(2 µg/ml) diluted in 10% dried milk. The membranes were washed three times with TBST then incubated for 30 minutes with peroxidase conjugated anti-mouse IgG (1:1000). Immunoreactive bands were visualized using the ECL-plus western blotting detection system (Amersham Bioscience). Immunoblotting with the anti-VDAC-2 and 3 monoclonal antibody (generated in the Craigen Laboratory, Baylor College of Medicine, Houston, TX) was performed as described above, with the exception that peroxidase conjugated anti-chicken IgG (1:1000) was used as secondary antibody.

Photolabeling. Photolabeling was performed as previously described (Darbandi-Tonkabon et al., 2003). Briefly, mouse brain membranes were placed in a quartz cuvette in buffer (50 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 5 µM GABA) at a concentration of 400 µg of membrane protein/ml and pre-incubated with [3H]6-AziP for 90 minutes at 4º C in the dark. The cuvette was then placed in a photoreactor at a distance of 8 cm from the source. (The photoreactor uses a 450 W Hanovia medium pressure mercury lamp as the light source. The lamp is filtered through a 1.5 cm thick saturated copper sulfate solution. This filter absorbs all light of wavelength <315 nM (Katzenellenbogen et al., 1974)). The samples were routinely irradiated for 3 minutes, while continuously cooled to 4º C. Following irradiation the membranes were harvested by centrifugation and solubilized in SDS-sample buffer (312.5 mM Tris HCl, 5% SDS, 0.5 M DTT, 50% glycerol and 0.1 % Bromophenol Blue) and analyzed by electrophoresis on a 10% SDS-PAGE gel. The gels were sliced and radioactivity measured in each slice.
 Autoradiography. For autoradiography, SDS gels were fixed for 30 minutes in isopropanol : water : acetic acid (25:65:10) at room temperature and then dried under vacuum. The dried gels were placed in cassettes and exposed to $[^3]H$-sensitive ultra-film (Kodak Biomax light film) at -70° C for periods ranging from five days to two weeks.

 2-Dimensional Electrophoresis. 2-Dimensional electrophoresis was performed as previously described (Darbandi-Tonkabon et al., 2003). The final gels were either processed for autoradiography, or stained with silver stain or Coomassie blue. The non-radioactive gels were stained with Coomassie blue. The gels with tritiated samples were silver stained and all stained spots in the area identified in the original autoradiography were excised, digested and analyzed by scintillation spectrometry. The spots on the Coomassie blue stained gels corresponding to spots containing maximum radioactivity were manually excised. The excised samples were prepared for mass spectrometry by in-gel proteolytic digestion with Promega sequencing grade modified trypsin (Jimenez, 2000). The recovered tryptic peptides were analyzed on a Micromass Q-TOF Micro electrospray mass spectrometer utilizing a Waters Capillary HPLC with a nano-spray emitter. Data was searched using Mascot software by Matrix Science. Analyses were performed at the Protein and Nucleic Acid Chemistry Laboratories (PNACL) at Washington University School of Medicine.
RESULTS

Photolabeling. Photolabeling with \(^{3}\text{H}\)6-AziP was performed in brain membranes from VDAC-1 and -3 deficient (VDAC -/-) and strain controlled (VDAC +/+ ) mice to confirm that VDAC-1 was the major photolabeled protein in brain and to determine if other VDAC isoforms were also photolabeled. Brain membranes from the mice were photolabeled with 10 µM \(^{3}\text{H}\)6-AziP and analyzed by SDS-PAGE with gel slicing. In VDAC-3 -/- and VDAC-3 +/+ (C57 Black), radioactivity was incorporated into two major protein bands, one at approximately 35-kDa and one at approximately 60-kDa. There was no discernible difference in the labeling of either peak between the VDAC-3 -/- and the VDAC-3 +/+ membranes, indicating that VDAC-3 protein is either not labeled by \(^{3}\text{H}\)6-AziP or represents a negligible component of the labeled protein (Figure 1A). In contrast, brain membranes from VDAC-1 +/+ (CD 1) mice showed a single major peak of radiolabel incorporation at 35-kDa (Figure 1B). A minor peak of radiolabel incorporation was consistently observed at 60-kDa; the size of this peak varied between experiments and the apparent difference in the size of the 60-kDa peak between Figures 1A (C57 Black) and 1B (CD 1) does not represent a consistent finding. The 35-kDa peak was reduced by 85% (area under the curve) in VDAC-1 -/- membranes, with a small residual peak of radiolabel incorporation remaining at 35-kDa. These data confirm that VDAC-1 is the major protein labeled by \(^{3}\text{H}\)6-AziP in brain. The data also show that a 35-kDa protein other than VDAC-1 is photolabeled.

Identification of radiolabeled proteins in VDAC-1 deficient mice: Membranes prepared from the brains of VDAC-1 -/- and VDAC-1 +/+ mice were labeled with 10 µM \(^{3}\text{H}\)6-AziP; labeled proteins were separated by two-dimensional electrophoresis and
visualized by autoradiography. The resultant autoradiograms (Figure 2) show labeled spots at 35-kDa, 60-kDa and approximately 18-kDa in the VDAC +/+ membranes. Several of the 35-kDa spots and the 18-kDa spots are absent in the VDAC -/- membranes indicating that these spots represent VDAC-1 (with various post-translational modifications) and proteolytic fragments of VDAC-1. The spots at 18-kDa are only present in the VDAC +/+ gels and are therefore likely to represent proteolytic fragments of VDAC-1 that contain a 6-AziP binding site. This is consistent with the earlier observation that VDAC-1 antisera immunoprecipitate a $[^3]H$6-AziP labeled protein band of 18-kDa from rat brain (Darbandi-Tonkabon et al., 2003).

Three prominent radiolabeled spots were observed at 35-kDa in membranes from VDAC-1 -/- mice; two of these spots were more prominently labeled than the third. Silver staining of two-dimensional gels from the VDAC-1 -/- animals showed a single silver-stained spot (circled in Figure 2) that corresponded to one of these radiolabeled spots. This spot was excised and analyzed by ESI-tandem mass spectrometry. Three peptides were identified (LTLSALVDGK, YQLDPTASISAK and LTFDTTFSPNTGK) which showed sequence identity with VDAC-2 (gi|10720224). This provides a definitive identification of the protein as VDAC-2. Mass spectrometric analysis did not identify any other proteins in the excised spot.

**Immunoblotting:** To confirm the identity of the radiolabeled 35-kDa spots in two-dimensional gels from photolabeled VDAC-1 -/- brain, immunoblots were performed using antisera to VDAC-1,-2 and -3 on two dimensional gels of brain membranes from VDAC-1 +/+ and VDAC-1 -/- mice. As shown in Figure 3, VDAC-1 and VDAC-2 both
have molecular weights of 35-kDa and both are represented by multiple spots. The VDAC-2 spots have distinctive pI values from the VDAC-1 spots. The VDAC-2 immunostained spots correspond well to the radiolabeled spots observed in the autoradiograms of [$^3$H]6-AziP labeled VDAC-1 -/- brain (Figure 2); this is consistent with the identification of VDAC-2 as the other photolabeled 35-kDa protein in brain. Immunoblotting with VDAC-3 antisera indicates that VDAC-3 is minimally expressed in mouse brain. Immunoblots prepared from VDAC-1 -/- animals confirm the absence of VDAC-1 in brain membranes.

**Effect of 6-AziP on [$^{35}$S]TBPS binding:** Neuroactive steroids are known to allosterically inhibit binding of the caged convulsant [$^{35}$S]-butyrbicyclophosphorothionate ([$^{35}$S]TBPS) to the picrotoxin binding site of GABA$_A$ receptors (Majewska et al., 1986). Figure 4A shows that pregnanolone completely inhibits [$^{35}$S]TBPS binding in both VDAC-1 -/- and VDAC-1 +/- mouse brain membranes; the pregnanolone inhibition curves for the VDAC-1 -/- and +/- membranes were virtually identical. Allopregnanolone ((3α,5α)-3-hydroxypregnan-20-one) also completely inhibited [$^{35}$S]TBPS binding in VDAC-1 -/- and +/- mouse membranes (Figure 4C). The allopregnanolone inhibition curves were virtually identical in VDAC-1 -/- and VDAC-1 +/- mouse brain membranes. Pregnanolone (Figure 4B) and allopregnanolone (Figure 4D) also completely inhibited [$^{35}$S]TBPS binding in VDAC-3 -/- and VDAC-3 +/- mouse brain membranes. The presence or absence of VDAC-3 had no discernable influence on the steroid inhibition curves. These results suggest that neither VDAC-1 nor VDAC-3 is required for neuroactive steroid (pregnanolone or allopregnanolone) modulation of GABA$_A$ receptors in mouse brain.
Electrophysiology. We examined the effect of ACN ((3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile) on single-channel currents elicited by 10 µM GABA. Sample currents from wild type and VDAC-1 -/- mice, recorded in the absence and presence of 1 µM steroid, are shown in Figure 5A. At 10 µM GABA, the currents consisted of isolated openings and gating bursts. No single-channel clusters were evident. The mean open durations were 3.6±1.3 ms (n=5) in wild-type neurons, and 5.4±1.2 ms (n=4) in VDAC-1 deficient mice. Exposure to ACN resulted in an increase in the mean open duration of the channel as seen in our previous observations with recombinant GABA<sub>A</sub> receptors (Akk and Steinbach, 2003). The mean open duration in the presence of 1 µM ACN was 7.2±1.5 ms (n=4) in wild type and 10.2±3.5 ms (n=3) in VDAC-1 -/- neurons.

The similarity in the effect of steroid on the channel mean open time strongly suggests that GABA responses in VDAC-1 deficient cells can be modulated by neuroactive steroids. However, it is possible that components of single-channel behavior not explicitly examined in our open time analysis are differentially affected by steroids in the two genotypes and are reflected in whole-cell current potentiation. We thus examined steroid potentiation of whole-cell currents from cultured hippocampal neurons. At low concentrations of GABA (2 µM) and steroid (200 nM) to avoid saturation effects, we found no significant difference between wild-type and VDAC-1 -/- neurons with regard to potentiation by either 5α-reduced or 5β-reduced steroids (Figures 5C and 5D). These results thus confirm the lack of difference in steroid effect on channel activity by ACN and extend the results to other 5α-reduced steroids and to 5β-reduced steroids.
Electrophysiological studies in VDAC-2 deficient fibroblasts. We next examined the effect of ACN on rat α1FLAGβ2γ2 GABA_A receptors expressed in VDAC-2-deficient mouse fibroblasts. Sample single-channel clusters from cells lacking VDAC-2, and from control, wild-type fibroblasts are shown in Figure 6A. The clusters were elicited by 50 µM GABA. This concentration corresponds to approximately EC_{50} of the dose-response curve for such receptors expressed in HEK 293 cells (Steinbach and Akk, 2001). The co-application of ACN along with GABA resulted in significant changes in the receptor activity and cluster kinetic properties. First, the mean open duration increased in the presence of GABA and 1 µM ACN (Figure 6B). It has been shown previously that the increase in the channel mean open duration, in the presence of ACN, is caused predominantly by an increase in the duration of the longest-lived component of the three intracluster open time classes (Akk and Steinbach, 2003). These data agree with the results obtained from the VDAC-2 -/- and wild-type fibroblasts expressing the α1FLAGβ2γ2 GABA_A receptor (Figure 6C). In addition to changes in the channel open times, the intracluster closed times are affected in the presence of ACN. The rates of entry into all intracluster closed states are reduced as a result of the increase in the mean open duration. However, the rate of entry into an activation-related component, CTβ, is selectively reduced to a much greater extent in the presence of ACN (Figure 6D). The occurrence of CTβ reflects the channel closing rate which is reduced by ACN. The changes seen in the relative frequency of the CTβ component of the intracluster closed times, as well as changes in the open time durations observed upon exposure to ACN, are similar to ones described previously for α1FLAGβ2γ2 GABA_A receptors expressed in HEK 293 cells (Akk and Steinbach, 2003), or for receptors expressed in wild-type mouse...
fibroblasts (Figure 6). Hence the data suggest that the presence of VDAC-2 is not a requirement for positive modulation of GABA$_A$ receptor currents by ACN.

**In vivo anesthesia studies:** The ability of pregnanolone to produce anesthesia was studied in VDAC-1 and VDAC-3 deficient mice and in litter mate strain-controlled animals. There was no significant difference in the ability of pregnanolone (5 mg/kg) to cause anesthesia (LRR) or in the duration of its anesthetic effect between VDAC-1 -/- and VDAC-1 +/+ mice (Figure 7A$_1$) or between VDAC-3 -/- and VDAC-3 +/+ mice (Figure 7B). The ability of allopregnanolone to produce anesthesia was also tested in VDAC-1 -/- and +/+ mice. There was no significant difference in the ability of allopregnanolone to produce anesthesia or in the duration of the anesthetic effect between VDAC-1 -/- and +/+ mice (Figure 7A$_2$). These data indicate that neither VDAC-1 nor VDAC-3 is necessary for the anesthetic actions of neuroactive steroids.
DISCUSSION

In previous work we have shown that the neuroactive steroid analogue photolabeling reagent, 6-AziP, modulates GABA<sub>A</sub> receptor function, and that the brain protein most prominently photolabeled by [<sup>3</sup>H]6-AziP is VDAC-1 (Darbandi-Tonkabon et al., 2003). Provocatively, VDAC-1 has also been shown to associate with GABA<sub>A</sub> receptors, as evidenced by co-purification (Bureau et al., 1992), and by co-immunoprecipitation (Darbandi-Tonkabon et al., 2003) of the two proteins. In the current study, VDAC-1 and -3 deficient mice were used to confirm that VDAC-1 is the major VDAC isoform photolabeled by [<sup>3</sup>H]6-AziP and to show that VDAC-2, but not VDAC-3, is also labeled. The observed labeling pattern reflects the relative abundance of the three isoforms in mouse brain (Figure 3), and is not indicative of isoform-specific labeling. The data presented in this manuscript provide strong evidence that VDAC isoforms do not mediate the GABAergic or anesthetic effects of the neuroactive steroids. The electrophysiological, binding and behavioral data in VDAC-deficient mice show that neither VDAC-1 nor VDAC-3 is responsible for steroid modulation of GABA<sub>A</sub> receptors or anesthesia. Similarly, electrophysiological data in VDAC-2 deficient fibroblasts indicate that VDAC-2 is not required for neuroactive steroid modulation of GABA-A receptor currents (Figure 6). It is, however, conceivable that VDAC-1 and VDAC-2 can both mediate neuroactive steroid modulation of GABA<sub>A</sub> receptors and can substitute one for the other. The absence of VDAC-2 deficient animals precludes looking at the double knockout and definitively eliminating this possibility.

The current data does not identify the functional significance of specific neuroactive steroid interactions with VDAC isoforms. In addition to their actions as
GABAA receptor modulators and anesthetics, neuroactive steroids are thought to have other biological activity. At the behavioral level, neuroactive steroids have neuroprotective effects (Claudio and Stefano, 2000). At the cellular level, the steroid have been shown to influence the growth and differentiation of myelin-specific proteins in oligodendrocytes and to promote myelination of neurites in tissue culture (Jung-Testas et al., 1999); they are also thought to modulate apoptosis via a caspase-3-dependent pathway (Cascio et al., 2002). The VDAC family of proteins are pore-forming proteins (Colombini, 1996) which enable permeability to the outer mitochondrial membrane (Rostovtseva, 1996), and are thought to be important in various aspects of mitochondrial function (Rostovtseva, 1997). There is also substantial evidence that one or more VDAC isoforms may play a central role in apoptosis (Shimizu, 2000; Shimizu, 2001). Finally, VDAC-1 deficient mice have been shown to have a deficit in both spatial learning and hippocampal long-term potentiation (Weeber et al., 2002). Interactions of neuroactive steroids with VDAC isoforms could thus be modulators of mitochondrial function, synaptic plasticity, or regulators of apoptosis. A role in preventing apoptosis could, in part, explain the neuroprotective actions of the neuroactive steroids. With regard to all of these possibilities, it will be important to examine the actions of neuroactive steroids on the electrophysiological properties of VDAC-1 and 2 as well as on other VDAC actions.

An additional unresolved question is why VDAC-1, and possibly VDAC-2, associates with the GABAA receptor. This may be an interaction that occurs in a detergent lysate, but may not happen in an intact cell. VDAC-1 is a very abundant protein which may non-specifically adhere to detergent-solubilized hydrophobic proteins such as the GABAA receptor. Alternatively, interactions of VDAC-1 with ligand-gated ion
channels may serve a role in localizing mitochondria to energy-requiring post-synaptic nerve endings.

Finally, the protein that binds neuroactive steroids and mediates their actions on GABA_A receptors remains to be identified. In addition to the VDAC isoforms, 6-AziP labels a number of other proteins in a lysate of brain membrane (Figure 2) (Darbandi-Tonkabon et al., 2003). The VDAC isoforms were studied first, because they are most prominently labeled; this is largely a reflection of their abundance in brain. There are also prominently labeled proteins at 58-kDa (pI = 4-5) that could mediate the anesthetic and GABAergic actions of the steroids. These proteins are being identified and studied and will be the subject of a subsequent report. It is also important to note that 2-D autoradiograms of [3H]6-AziP labeled mouse brain (Figure 2) show a single faintly labeled spot at pI=8-9 and molecular weight of 50-60-kDa. This is the predicted pI and mass of most GABA_A receptor subunits and suggests the possibility that 6-AziP also labels a specific GABA_A receptor subunit. This spot is not visualized by silver staining of the 2-D gels and has thus not been identified by mass spectrometry; we are pursing isolation and identification of this spot. It is important to reiterate that quantitative immunoprecipitation of [3H]6-AziP photolabeled, epitope-tagged GABA_A receptors (α_1β_1FLAG) from HEK 293 cells does not yield any radiolabeled GABA_A receptor; GABA-elicited currents in these cells are, however, potentiated by neuroactive steroids (data not shown).

This presents the possibility that there is selectivity to labeling such that 6-AziP binds to a site on the GABA_A receptor but does not covalently photolabel the site. To address this possibility we are in the formative stages of preparing alternative neuroactive steroid...
analogue photolabeling reagents in which the photolabeling moiety is located in different regions of the molecule or in which there is a different chemical mechanism of labeling. Finally, there are several faintly labeled proteins on autoradiograms of 2D-gels (best seen after prolonged exposure of the film to the gel). If the efficiency of photolabeling is modest (<10%) and the protein of interest interacts with GABA_\text{A} receptors at 1:1 stoichiometry, faint labeling would be predicted. These proteins also need to be identified.
Acknowledgement

We thank Zong-Jin Cai for technical assistance and Dr. Charles Zorumski for helpful discussions.
REFERENCES


Footnotes

This work was supported in part by National Institutes of Health Grants PO1-GM47969 (to J.H.S., D.F.C. and A.S.E.), AA12952 (to S.J.M.), RO1 GM055713 (to W.J.C), by a grant from the Klingenstein Foundation (to S.J.M.) and the Alcoholic Beverage Medical Research Foundation (to G.A).

To whom page proofs and reprint request should be addressed:

Alex S. Evers, M.D.
Department of Anesthesiology,
Washington University School of Medicine
660 S. Euclid Ave, Campus Box 8054
St. Louis, MO 63110
Tel.: (314) 454-8702
Fax: (314) 454-5572
Email: eversa@notes.wustl.edu
FIGURE LEGENDS

Fig. 1: Photolabeling of VDAC-1 and -3 deficient and strain controlled mouse brain membranes- A) VDAC-3 -/- (Δ) and VDAC-3 +/+ (●) mouse brain membranes were photolabeled with 10 µM [3H]6-AziP and analyzed by SDS-PAGE. Radioactivity was incorporated into two major protein bands, and the presence of VDAC-3 had no discernable effect on the labeling pattern. B) Brain membranes from VDAC-1 +/+ mice (●) showed a single major peak of radiolabel incorporation at 35-kDa; a small peak of radiolabel incorporation was consistently observed at 60-kDa. The 35-kDa peak was reduced by 85% (area under the curve) in VDAC-1 -/- membranes (Δ), with a small residual peak of radiolabel incorporation remaining at 35-kDa.

Fig. 2: Autoradiogram of 2-Dimensional Gels of [3H]6-AziP Photolabeled Mouse Brain Membranes- Brain membranes were prepared from VDAC-1 +/+ mice (top panel) and VDAC-1 -/- mice (lower panel). In the VDAC-1 +/+ membranes labeled spots are observed at 35-kDa, 60-kDa and approximately 18-kDa. In the VDAC-1 -/- membranes several of the 35-kDa spots and the 18-kDa spots are absent, indicating that these spots represent VDAC-1. The spots at 18-kDa are only present in the VDAC +/+ gels and are therefore likely to represent proteolytic fragments of VDAC-1 that contain a 6-AziP binding site. Three radiolabeled spots were observed at 35-kDa in membranes from VDAC-1 -/- mice. The circled spot was identified by ESI-MS/MS as VDAC-2. The spots at approximately 60-kDa are unaffected by VDAC-1 deficiency.
Fig. 3: VDAC-1,-2 and -3 Immunoblotting of Mouse Brain Membranes-
Immunoblots were performed using antisera to VDAC-1,-2 and -3 on two dimensional gels of brain membranes from VDAC-1 +/+ (left panels) and VDAC-1 -/- (right panels) mice. VDAC-1 and VDAC-2 both have molecular weights of 35-kDa and both are represented by multiple spots. The VDAC-2 spots have distinctive pI values from the VDAC-1 spots. The circled spot on the VDAC-2 immunoblots corresponds to the circled spot on the VDAC-1 -/- autoradiogram from Fig. 2. Immunoblotting with VDAC-3 antisera indicates that VDAC-3 is minimally expressed in mouse brain. Immunoblots prepared from VDAC-1 -/- animals show that VDAC-1 is absent and that there is not an obvious compensatory increase in either VDAC-2 or VDAC-3.

Fig. 4: VDAC-deficiency Does Not Affect Neuroactive Steroid Modulation of $[^{35}S]$TBPS binding- A) Pregnanolone ($3\alpha 5\beta P$) completely inhibits $[^{35}S]$TBPS binding in both VDAC-1 -/- (○) and VDAC-1 +/+ (●) mouse brain membranes. $3\alpha 5\beta P$ inhibits $[^{35}S]$TBPS binding in VDAC-1 deficient mouse membranes with an $IC_{50}$ of $68.3\pm32.9$ nM and a Hill Slope of $0.42\pm0.06$. VDAC-1 wild type mouse brain membranes demonstrate an $IC_{50}$ of $67.5\pm55.1$ nM and a Hill Slope of $0.41\pm0.10$. B) $3\alpha 5\beta P$ inhibits $[^{35}S]$TBPS binding in VDAC-3 deficient mouse membranes (○) with an $IC_{50}$ of $504.8\pm192.7$ nM and a Hill Slope of $0.50\pm0.08$. In VDAC-3 wild type mouse brain membranes (●) $3\alpha 5\beta P$ inhibits with an $IC_{50}$ of $962.1\pm426.3$ nM and a Hill Slope of $0.52\pm0.12$. C) $3\alpha 5\alpha P$ inhibits $[^{35}S]$TBPS binding in VDAC-1 deficient mouse membranes (○) with an $IC_{50}$ of $21.0\pm9.3$ nM and a Hill Slope of $0.67\pm0.10$. VDAC-1 wild type mouse brain membranes (●) demonstrate an $IC_{50}$ of $27.4\pm17.7$ nM and a Hill Slope of
0.80±0.25. D) 3α5αP inhibits [35S]TBPS binding in VDAC-3 deficient mouse membranes (o) with an IC50 of 23.5±8.4 nM and a Hill Slope of 0.62±0.07. VDAC-3 wild type mouse brain membranes (●) reveal an IC50 of 27.8±10.3 nM and a Hill Slope of 0.64±0.08.

Fig. 5: Electrophysiological Characterization of Steroid Potentiation in Wild Type and VDAC-1-deficient Mice- A) Single-channel currents from wild type and VDAC-1 deficient hippocampal neurons. The receptors were activated by 10 μM GABA in the absence and presence of 1 μM ACN. The openings are shown downward. B) Channel mean open durations in the absence and presence of 1 μM steroid from wild type and VDAC-1-deficient neurons. For both cell types, the increase seen in the open durations upon exposure to steroid is statistically significant (t-test, p<0.05). C) Potentiation of whole-cell currents by 200 nM ACN on wild type and VDAC-1 -/- hippocampal neurons. GABA (2 μM) was the agonist. D) Summary of effects of 200 nM steroid on responses from wild type and VDAC-1 -/- neurons. Open bars represent data from wild type neurons, shaded bars represent data from VDAC-1 -/- neurons. Responses were normalized to the peak current evoked by 2 μM GABA. There was no statistical difference between potentiation levels for any of the steroids tested (n = 12, wild-type cells and 17 VDAC-1 -/- cells for 3α5αP and 3α5βP, and n = 4, wild type and VDAC-1 -/- cells for ACN).

Fig. 6: Electrophysiological characterization of steroid potentiation in wild type and VDAC-2-deficient fibroblasts- A) Single-channel clusters from wild-type and VDAC-2-deficient fibroblasts. The cells were transiently transfected with rat α1FLAG, β2 and γ2
subunits. The receptors were activated by 50 µM GABA in the absence and presence of 1 µM ACN. The openings are shown downward. (B-D) Results from fitting the intracluster open and closed interval duration distribution histograms. B) The channel mean open duration is increased in the presence of ACN. The mean open durations for wild-type fibroblasts are: control 2.9±0.04 ms, +ACN 6.1±0.1 ms; for VDAC-2 -/- fibroblasts, control 2.5±0.04 ms, +ACN 11.0±0.1 ms. C) The increase in the mean open duration, in the presence of ACN, is a result of an increase in the duration of the longest-lived component (OT3) in the open duration histograms. The mean durations for OT3 in wild-type fibroblasts are: control 5.1±0.4 ms, +ACN 14.7±0.6 ms; in VDAC-2 -/- fibroblasts, control 5.9±0.7 ms, +ACN 23.3±2.2 ms. D) The addition of ACN affects the closed interval distributions. The relative frequency of the component associated with the channel closing rate (CTβ) is reduced in the presence of ACN. The fractions of CTβ for wild-type fibroblasts are: control 27±1 %, +ACN 2.7±0.4 %; for VDAC-2 -/- fibroblasts, control 24±2 %, +ACN 4.9±0.4 %. The data are from one (wild-type + ACN) or two (wild-type) patches from a single transfection, and four (VDAC-2 -/-, VDAC-2 -/- + ACN) patches, two each from two transfections. The total numbers of events used in the analysis were: 10257 (wild-type), 5020 (wild-type + ACN), 10188 (VDAC-2 -/-), 9181 (VDAC-2 -/- + ACN).

Fig. 7: VDAC-1 or -3 Deficiency Does Not Affect Neuroactive Steroid Anesthesia in Intact Mice- The ability of pregnanolone (3α5βP) and allopregnanolone (3α5αP) to produce anesthesia was studied in VDAC-1 and VDAC-3 deficient mice and in litter
mate strain-controlled animals. **A1)** VDAC-1 +/+ mice (n=9 males) had sleep times of 19.0±1.0 minutes and VDAC-1 -/- (n=6 males) had sleep times of 23.5±2.5 minutes following administration of 3α5βP (5 mg/kg i.v.). **A2)** VDAC-1 +/+ mice (n=5 males) had sleep times of 8.5±0.3 minutes and VDAC-1 -/- (n=5 males) had sleep times of 8.6±0.2 minutes following administration of 3α5αP (5 mg/kg i.v.). **B)** VDAC-3 -/- mice (n=6 males) had sleep times of 29.6±3.3 minutes and VDAC-3 +/+ mice (n=4 males) had sleep times of 26.6±0.5 minutes following administration of 3α5βP (5 mg/kg i.v.).
Figure 1.

A.

B.
Figure 2.

pH 3

66-kDa

46-kDa

30-kDa

pH 10

VDAC-1 (+/+)

VDAC-1 (-/-)
Figure 3.

VDAC-1 western in VDAC-1 +/-

pH 6.5

pH 10

30 kDa

46 kDa

VDAC-1 western in VDAC-1 -/

pH 6.5

pH 10

30 kDa

46 kDa

VDAC-2 western in VDAC-1 +/-

pH 6.5

pH 10

30 kDa

46 kDa

VDAC-2 western in VDAC-1 -/

pH 6.5

pH 10

30 kDa

46 kDa

VDAC-3 western in VDAC-1 +/-

pH 6.5

pH 10

30 kDa

46 kDa

VDAC-3 western in VDAC-1 -/
A. Specific [35S]-TBPS Binding (% of control)

B. Specific [35S]-TBPS Binding (% of control)

C. Specific [35S]-TBPS Binding (% of control)

D. Specific [35S]-TBPS Binding (% of control)
Figure 5.

A

10 μM GABA

10 μM GABA, 1 μM ACN

VDAC1−/−

10 μM GABA

10 μM GABA, 1 μM ACN

WILD-TYPE

B

mean open duration (ms)

GABA +ACN  GABA +ACN

WILD-TYPE VDAC1−/−

C

WILD-TYPE

VDAC1−/−

GABA  +ACN  GABA  +ACN

D

normalized current

3x5αP  3x5βP  ACN
Figure 6.

(A) WILD-TYPE

50 μM GABA

+1 μM ACN

VDAC2 -/-

50 μM GABA

+1 μM ACN

(B) mean open duration (ms)

(C) OT3 duration (ms)

(D) C(β) (fraction of total)

GABA +ACN

WILD-TYPE

VDAC2 -/-

GABA +ACN
Figure 7.

A1.

Sleep time (Min)

<table>
<thead>
<tr>
<th></th>
<th>VDAC-1 -/-</th>
<th>VDAC-1 +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α5βP</td>
<td>Bar graph</td>
<td>Bar graph</td>
</tr>
</tbody>
</table>

A2.

Sleep time (Min)

<table>
<thead>
<tr>
<th></th>
<th>VDAC-1 -/-</th>
<th>VDAC-1 +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α5αP</td>
<td>Bar graph</td>
<td>Bar graph</td>
</tr>
</tbody>
</table>

B.

Sleep time (Min)

<table>
<thead>
<tr>
<th></th>
<th>VDAC-3 -/-</th>
<th>VDAC-3 +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α5βP</td>
<td>Bar graph</td>
<td>Bar graph</td>
</tr>
</tbody>
</table>