The Lipid-associated Conformation of the Low Density Lipoprotein Receptor Binding Domain of Human Apolipoprotein E*

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Apolipoprotein E (apoE) is a 34-kDa exchangeable apolipoprotein that regulates metabolism of plasma lipoproteins by functioning as a ligand for members of the LDL receptor family. The receptor-binding region localizes to the vicinity of residues 130–150 within its independently folded 22-kDa N-terminal domain. In the absence of lipid, this domain exists as a receptor-inactive, globular four-helix bundle. Receptor recognition properties of this domain are manifest upon lipid association, which is accompanied by a conformational change in the protein. Fluorescence resonance energy transfer has been used to monitor helix repositioning, which accompanies lipid association of the apoE N-terminal domain. Site-directed mutagenesis was used to replace naturally occurring Trp residues with phenylalanine, creating a Trp-null apoE3 N-terminal domain (residues 1–183). Subsequently, tyrosine residues in helix 2, helix 3, or helix 4 were converted to Trp, generating single Trp mutant proteins. The lone cysteine at position 112 was covalently modified with N-iodoacetyl-(5-sulfo-1-naphthyl)ethylenediamine, which serves as an energy acceptor from excited tryptophan residues. Fluorescence resonance energy transfer analysis of apoE N-terminal domain variants in phospholipid disc complexes suggests that the helix bundle opens to adopt a partially extended conformation. A model is presented that depicts a tandem arrangement of the receptor-binding region of the protein in the disc complex, corresponding to its low density lipoprotein receptor-active conformation.

Apolipoprotein E (apoE) plays a critical role in lipoprotein metabolism via its ability to mediate clearance of remnant particles from circulation (1). In addition, apoE has been implicated in other biological processes including neuronal plasticity (2) and dysfunction (3), steroidogenesis (4), lipase activation (5), and platelet aggregation (6). ApoE is a 299-amino acid, 34-kDa glycoprotein existing as one of three major isoforms that differ in the residues at positions 112 and 158. ApoE2 contains a cysteine at both sites; apoE3 (the most common isoform) has an arginine replacing the cysteine at 158; and apoE4 contains arginine at both 112 and 158. The intact protein is composed of two domains, a 10-kDa C-terminal domain with high lipid binding affinity and a 22-kDa N-terminal domain that is responsible for high affinity low density lipoprotein receptor binding (7). The x-ray crystal structure of the N-terminal domain (amino acids 1–191) in the absence of lipid reveals a globular bundle of four antiparallel, amphipathic α-helices (8). This structure presents a hydrophilic exterior comprised of polar and charged amino acids that sequesters a hydrophobic core of apolar residues.

Studies of the isolated N-terminal domain revealed that the helix bundle conformation is receptor-inactive, while lipid association confers low density lipoprotein receptor binding activity (9). ApoE-N-terminal domain forms discoidal complexes with phospholipid, and these receptor-active particles provide a model system for studying the lipid-associated state (9). Phospholipid binding is accompanied by little change in the global α-helix content of the protein (10–12), suggesting that a tertiary structural alteration occurs in a manner such that helical boundaries that define the bundle are maintained. Studies of the surface properties of apoE N-terminal domain at the air/water interface indicate that the helix bundle can undergo a pronounced conformational change resulting in exposure of its hydrophobic interior (13). Fourier transformed infrared spectroscopy of recombinant apoE3 (N-terminal residues 1–183) showed that the protein orients around the periphery of phospholipid bilayer discs with its α-helical axes perpendicular to the fatty acid chains (12). Recent FRET studies of apoE3-(1–183) employing an extrinsic energy acceptor covalently bound to Cys112 revealed that, upon phospholipid binding, helix 1 repositions away from helix 3 (14). This finding is consistent with a model in which the four-helix bundle opens about a “hinge” located between helices 2 and 3 such that helices 1 and 2 move away from helices 3 and 4 (7). The net effect of this putative conformational alteration is exposure of a continuous hydrophobic surface that is available for lipid interaction. In the present study, FRET analysis of apoE3-(1–183) variants containing single Trp residues introduced into different helical segments has permitted more refined mapping of the relative position of helices in apoE3-(1–183)dimyristoylphosphatidylcholine (DMPC) disc complexes.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis was performed using the Altered Sites II in vitro mutagenesis kit (Promega, Madison, WI). Wild type apoE3-(1–183) contains four Trp residues, each located within or adjacent to helix 1. To generate single Trp proteins, residues at positions 20, 26, and 34 were mutated to Phe, generating apoE3-(1–183)W20F, apoE3-(1–183)W26F, and apoE3-(1–183)W34F. Subsequently, Trp74 was replaced with a Phe to create apoE3-(1–183)W74F. Other single Trp mutants were created by replacing naturally occurring Tyr residues with Trp at positions 74,
Lipid-bound Conformation of ApoE3 N-terminal Domain

118, and 162). These mutations introduce Trp residues into helices 2, 3, and 4, respectively. DNA sequencing was performed to confirm introduction of the intended mutations and the lack of undesired mutations.

**Protein Expression, Purification, and Labeling—Recombinant apoE3 (1–183) variants were overexpressed in *E. coli* BL21(DE3), purified as described previously (15), lyophilized, and stored at −20 °C. For labeling, 500 nmol of protein was solubilized in 50 mM Tris-HCl, pH 8.0, 0.1 mM dithiothreitol and incubated with 1 mM Cys112, (5-sulfo-1-naphthyl)ethylenediamine (AEDANS; Sigma) at 37 °C for 1 h in the dark. Unreacted AEDANS was removed by Sepharose CL-6B gel filtration chromatography. Covalent labeling of Cys112 in the apoE3-(1–183) variants was stoichiometric, as determined by electrospray mass spectrometry (15) and absorption spectroscopy using a Hewlett Packard 8453 spectrophotometer. Protein concentrations were determined using the bicinchoninic acid assay (Pierce).

**Phospholipid Disc Complexes—** Protein dissolved in phosphate-buffered saline (150 mM NaCl, 3.4 mM EDTA, 100 mM sodium phosphate, pH 7.0) was added to a thin film of DMPC at a mass ratio of 1:3. Following incubation at 42 °C, the mixture was bath-brought to room temperature and further incubated for 16 h at 24 °C. Discoidal complexes were separated from unbound protein by Sepharose CL-6B size exclusion chromatography, and disc-containing fractions were pooled and concentrated to 1 ml. The lipid/protein molar ratio of the product particles was ~150:1. Native PAGE analysis of DMPC discoidal complexes composed of wild type or Trp mutant apoE3-(1–183) revealed the complexes were of similar size, consistent with earlier flotation equilibrium and electron microscopy data (15). Prior to spectroscopic analysis, sample concentrations were adjusted to ~0.05 OD at 295 nm.

**Fluorescence Measurements—** Fluorescence spectra were recorded on a Perkin-Elmer LS50 Luminescence Spectrometer using 3–5 nm for both excitation and emission slit widths at room temperature. For emission spectra, samples were excited at 295 nm. Excitation spectra were recorded from 265 to 400 nm using an emission wavelength of 490 nm.

**Calculation of Donor-Acceptor Separation—** The efficiency of energy transfer (E) was calculated from changes in donor fluorescence as follows,

\[
E = 1 - \frac{Q_{DA}}{Q_D} \quad \text{(Eq. 1)}
\]

where \(Q_D\) and \(Q_{DA}\) are the quantum yield of the donor in the absence and presence of acceptor, respectively. The distance between energy donor and acceptor (R) is given by the following,

\[
R = R_0 \left( \frac{1}{E} - 1 \right)^{\frac{3}{5}} \quad \text{(Eq. 2)}
\]

where \(R_0\) is the distance at which the transfer efficiency is 50% such that

\[
R_0 = 9.765 \times 10^4 k^2 J_{DA} J_{\lambda} \lambda_0^{-1} \quad \text{(Eq. 3)}
\]

where \(k^2\) is the orientation factor of the donor and acceptor. The value of \(\frac{1}{2} \pi\) for \(k^2\) was used based on the assumption of random rotation of the fluorophores (16); \(n\) is the refractive index of the medium between donor and acceptor (taken to be 1.4; Ref. 17); and \(J\) is the spectral overlap integral, given by the following,

\[
J = \sum_{\lambda} I_D \varepsilon_D \lambda J_{DA} \quad \text{(Eq. 4)}
\]

where \(I_D\) is the fluorescence intensity of the donor, \(\varepsilon_D\) is the molar extinction coefficient of the acceptor, and \(\lambda\) is the wavelength in cm. For distance determinations of lipid-bound protein, \(R_0\) was derived from discs that did not contain Trp-null protein, since the number of molecules present on the discs that were contributing to energy transfer could not be precisely determined.

**RESULTS AND DISCUSSION**

The N-terminal domain of wild type apoE3, with four Trp residues (energy donors) located in the region of helix 1, is amenable to FRET analysis. The single Cys at position 112 (helix 3) provides a convenient site for covalent attachment of an extrinsic energy acceptor. Problems associated with the use of wild type apoE3-(1–183), however, include the presence of multiple energy donors and the unknown contribution of intermolecular energy transfer in disc complexes that possess multiple copies of apoE3-(1–183). To circumvent these complications and to gain insight into the movement of other helices relative to the modified Cys in helix 3, naturally occurring Trp residues in apoE3-(1–183) were changed to Phe by site-directed mutagenesis. Subsequently, we created a Trp-null apoE3-(1–183) and single Tyr → Trp apoE3-(1–183) mutants for FRET studies (Table I). Far UV circular dichroism spectroscopy of apoE3-(1–183)W@162 (containing five substitution mutations) revealed an α-helix content characteristic of N-terminal apoE3, indicating that the mutations introduced did not affect the α-helix secondary structure content of the protein.

The fluorescence emission spectrum (excitation 295 nm) of unlabeled apoE3-(1–183)W@39 revealed a single peak with a maximum at 351 nm (Fig. 1). Similar spectra were obtained for each of the single Trp mutants (Table I), whereas Trp-null apoE3-(1–183) did not give rise to significant fluorescence emission upon excitation at 295 nm. Wild type apoE3-(1–183) displayed a fluorescence emission maximum of 344 nm, in agreement with previously determined values (10, 11, 15). The wavelength of maximum fluorescence emission observed for lipid-free apoE3-(1–183)W@39 was red-shifted compared with the other single Trp variants, indicating that naturally occurring Trp39 is more solvent-exposed than the single Trp variants created by replacement of Tyr residues in the protein. Consistent with predictions based on the x-ray crystal structure of the N-terminal fragment of apoE3, Trp residues introduced at position 74 (helix 2), 118 (helix 3), or 162 (helix 4) displayed blue-shifted λmax values. There was significant variability in Trp quantum yield (Q), ranging from 0.18 for apoE3-(1–183)W@162 to 0.05 for apoE3-(1–183)W@39. This variability may be attributed to fluorescence quenching as a function of the relative solvent exposure of the different Trp residues. Lipid association resulted in an increase in Q and a 3–4 nm blue shift in the Trp emission wavelength maximum for apoE3-(1–183)W@39 and apoE3-(1–183)W@118.

Following characterization of the fluorescence properties of the different apoE3-(1–183) variants, the proteins were labeled on cysteine 112 with AEDANS. Guanidine HCl denaturation experiments, monitored by fluorescence spectroscopy, using AEDANS-labeled apoE3-(1–183)W@118 and apoE3-(1–183)W@39 revealed denaturation midpoints between 2.3 and 2.5 M, similar to that reported for WT apoE N-terminal domain (18). Thus, we conclude that neither modification of Cys112 with AEDANS nor the substitution mutations introduced into the protein adversely affect the four-helix bundle structure or stability properties. In the case of AEDANS-apoE3-(1–183)W@39, excitation at 295 nm induced a large decrease in Trp fluorescence quantum yield accompanied by the appearance of a new fluorescence peak centered around 480 nm (Fig. 1). This peak corresponds to AEDANS emission following nonradiative energy transfer from excited Trp39. The relative peak intensities

| Table I | Fluorescence properties of apoE3-(1–183) variants |
|---------|-----------------------------------------------|
|        | Lipid-free | DMPC-bound |
|        | λmax | Q | λmax | Q |
| WT     | 344 | 0.19 | 344 | 0.28 |
| W@39   | 351 | 0.05 | 348 | 0.09 |
| W@74   | 341 | 0.15 | 337 | 0.18 |
| W@118  | 336 | 0.09 | ND | ND |
| W@162  | 339 | 0.18 | 339 | 0.29 |

a W@ refers to a single Trp mutant apoE3-(1–183), and the number indicates the amino acid position of the lone Trp.

b Excitation wavelength was 295 nm.

c Quantum yield is based on a value of 0.20 for 30 nm tryptophan in butanol.

d ND, not determined.
are dependent upon the distance between the donor and accep-
tor chromophores in the folded protein as reflected by the
efficiency (E) of energy transfer. Table II gives E values derived
from donor (Trp) emission peak intensity for lipid-free and
phospholipid disc particle-bound AEDANS-labeled apoE3-(1–
183) proteins. For each mutant studied, DMPC interaction
resulted in a decrease in the efficiency of energy transfer. Table
III lists other parameters employed in FRET-derived distance
determinations.

When AEDANS-apoE3-(1–183)/W@39 was bound to DMPC, a
significant reduction in the efficiency of energy transfer oc-
curred, as indicated by the increase in Trp emission peak
intensity (340 nm) together with a pronounced decrease in
AEDANS emission intensity (Fig. 2). On the other hand, fluo-
rescence spectra of discs prepared with Trp-null apoE3-(1–
183)/AEDANS-labeled Trp-null apoE3-(1–183) verified the lack
of Trp in this variant and demonstrated that direct excitation
of AEDANS at this wavelength is minimal. In the case of single
Trp variants, it is conceivable that energy transfer occurs be-
tween chromophores located on different disc complexes (inter-
disc energy transfer). To determine if interdisc energy transfer
contributes to the observed results, disc complexes prepared
using apoE3-(1–183)/W@39 were mixed with a second popula-
tion of discs prepared using AEDANS-Trp-null apoE3-(1–183).
In this case, no energy transfer was detected, indicating that
interdisc energy transfer in this system is negligible (data not
shown). Likewise, since multiple copies of apoE3-(1–183) are
bound per disc, it is conceivable that intermolecular energy
transfer could complicate interpretation of FRET data and
subsequent distance measurements. To control for this possi-
bility, Trp-null apoE3-(1–183) was used to dilute AEDANS-
labeled single Trp apoE3-(1–183) variants (4:1 weight ratio).

The spectrum of DMPC-bound WT AEDANS-apoE3-(1–
183) or AEDANS-apoE3-(1–183)/W@74 revealed decreases in
AEDANS fluorescence intensity that were similar to those
observed for apoE3-(1–183)/W@39. Furthermore, these spectra
were largely unaffected by dilution with Trp-null apoE3-(1–
183), consistent with conclusions drawn earlier regarding lipid
binding-induced repositioning of helix 1 and helix 3 (14). In the
case of AEDANS-apoE3-(1–183)/W@74, the data fit the open
conformation model wherein helix 1 and 2 reposition relative to
helix 3.

The fluorescence spectrum of AEDANS-apoE3-(1–183)
/W@162 in the lipid-free state revealed efficient energy transfer
(Fig. 3; Table II). In contrast to results obtained with WT
apoE3-(1–183) or mutants with single Trp residues at positions
39 or 74, interaction of AEDANS-apoE3-(1–183)/W@162 with
DMPC to form discoidal lipid particles did not induce a sig-
ificant change in Trp or AEDANS emission intensity. To assess
the contribution of intermolecular energy transfer to the spec-
trum of DMPC-bound AEDANS-apoE3-(1–183)/W@162, dilu-
tion experiments were performed using Trp-null apoE3-(1–
183). In this case, a dramatic increase in Trp fluorescence
intensity was observed together with a corresponding decrease
in AEDANS emission. Thus, unlike the other single Trp vari-
ant apoE3-(1–183) proteins, it appears that DMPC binding

FIG. 1. Effect of AEDANS labeling
on the fluorescence properties of
apoE3-(1–183)/W@39. Fluorescence emis-
sion spectra were recorded for a 1 μm
protein sample in 20 mM phosphate
buffer, pH 7.0 (295-nm excitation) at room
temperature. Dashed line, apoE3-(1–
183)/W@39; solid line, AEDANS-apoE3-
(1–183)/W@39.

TABLE II
Efficiency of energy transfer for apoE3-(1–183) single Trp variants
Values are the average of three independent experiments and were
determined as described under “Experimental Procedures.” apoE3-
protein-DMPC discs were prepared using Trp-null-apoE3-(1–183) and a
given AEDANS-labeled single Trp apoE3-(1–183) at a 4:1 ratio (w/w).

| ApoE3-(1–183) | Lipid-free | DMPC-bound |
|---------------|------------|------------|
| WT            | 0.46 ± 0.09| 0.13 ± 0.08|
| W@39          | 0.52 ± 0.2 | 0.22 ± 5E-3|
| W@74          | 0.44 ± 0.01| 0.066 ± 0.02|
| W@162         | 0.54 ± 0.06| 0.28 ± 0.1 |

TABLE III
Parameters of resonance energy transfer
Values are the average of three independent experiments.

| ApoE3-(1–183) | Lipid-free | DMPC-bound |
|---------------|------------|------------|
|               | R          |            |
|               | A cm²/mol  | A cm²/mol  |
| WT            | 22 5.9 x 10⁻¹⁵| 21.1 6.0 x 10⁻¹⁵|
| W@39          | 19 7.9 x 10⁻¹⁵| 21.7 1.2 x 10⁻¹⁵|
| W@74          | 23 1.0 x 10⁻¹⁴| 22.9 1.9 x 10⁻¹⁴|
| W@162         | 23 8.3 x 10⁻¹⁵| 20.7 9.5 x 10⁻¹⁵|

* Determined as described under “Experimental Procedures.” Values
are the average of three independent experiments.
results in repositioning of Trp\textsuperscript{162} to a location where it is available to excite an AEDANS moiety located on a neighboring apoE3-(1–183) in the disc complex. The residual energy transfer observed upon dilution of AEDANS apoE3-(1–183)/apoE3-(1–183)W@162 with Trp-null apoE3-(1–183) probably reflects the newly established intramolecular distance between Cys\textsuperscript{112} and Trp\textsuperscript{162}.

As shown in Table IV, FRET-derived distances from lipid-free AEDANS-labeled apoE3-(1–183) single Trp variants are in good agreement with values obtained from the x-ray crystal structure of this protein. For DMPC-bound protein, however, all FRET-derived distances do not agree with values inferred from a simple conformational opening of the four-helix bundle\textsuperscript{7}. The value for WT apoE3-(1–183) was lower (36 ± 3 Å versus 44 ± 4 Å) than that previously reported (14). Since energy transfer observed with this protein represents the average contribution of the four Trp residues in the vicinity of helix 1, it is difficult to draw firm conclusions, although it is clear that interaction with DMPC results in an increased distance between helix 1 and helix 3. When three of the four Trp residues in WT apoE3-(1–183) were replaced by Phe to generate apoE3-(1–183)W@39, the measured distance between AEDANS and Trp\textsuperscript{39} also increased upon interaction with DMPC. This increase, however, is somewhat less than that expected on the

| ApoE3-(1–183) | Lipid-free | DMPC-bound |
|---------------|------------|------------|
|               | Measured\textsuperscript{a} | Calculated\textsuperscript{b} | measured\textsuperscript{c} |
| WT            | 23.1 ± 0.4 | 21         | 36.0 ± 3.0 |
| W@39          | 19.4 ± 2.0 | 17         | 28.3 ± 2.0 |
| W@74          | 24.5 ± 0.9 | 24         | 40.1 ± 0.1 |
| W@162         | 23.1 ± 0.8 | 24         | 32.4 ± 0.1 |

\textsuperscript{a} From FRET measurements.
\textsuperscript{b} Distance calculations determined using the program Insight II (Biosym, San Diego).
\textsuperscript{c} DMPC discs were made as per Fisher et al. (15). $R_0$ used in distance measurements was obtained from discs made in the absence of Trp-null protein.
basis of simple conformational opening of the helix bundle. Because other fluorescence data (Fig. 1) indicate that Trp39 is relatively exposed to solvent, its orientation relative to the AEDANS acceptor group may be unique. Thus, it will be important to compare distance values derived from other variants with single Trp residues in helix 1. In the case of apoE3-(1–183)W@74, DMPC binding resulted in increased distance between Trp74 and AEDANS-Cys112, consistent with reorganization of the helix bundle. The observed change in distance between Trp162 and the AEDANS moiety on Cys112 upon interaction with phospholipid (and dilution with Trp-null apoE3-(1–183)) suggests that helix 4 repositions relative to helix 3 upon interaction with DMPC.

To examine this phenomenon further, intermolecular energy transfer was evaluated using discs containing AEDANS-labeled Trp-null apoE3-(1–183) and a given unlabeled apoE3-(1–183) single Trp mutant (4:1 ratio; w/w). In this design, AEDANS can only be excited by energy transfer from a Trp donor present on a neighboring apoE3-(1–183) in the disc complex. Background AEDANS emission was established from spectra of discs containing AEDANS-labeled Trp-null apoE3-(1–183) and Trp-null apoE3-(1–183)W@69 (dotted line); apoE3-(1–183)W@162 (dashed line); apoE3-(1–183)W@74 (dotted line with triangles); apoE3-(1–183)-W@118 (dotted line with circles).
energy donor to an AEDANS moiety present on a second, neighboring apoE3-(1–183) molecule. In a similar manner, energy transfer was also detected between apoE3-(1–183)/W@118 and AEDANS-labeled Trp-null apoE3-(1–183). Thus, it appears that, in DMPC complexes, apoE3-(1–183) can orient in such a way that helix 3 and helix 4 of one molecule are positioned nearby helix 4 and helix 3 of a second apoE3-(1–183), respectively. By the same token, this does not appear to be the case for helix 1 or helix 2.

Taken together, the data presented in this study are consistent with an alternate open conformation model (Fig. 5) wherein helix 3 from one molecule aligns with helix 4 of a second molecule, each adopting a partially extended open conformation. This model is consistent with the fact that no interhelical salt bridges exist between helix 1 or 2 and helix 3 or 4. Thus, opening of the bundle about the hinged loop connecting helix 2 and helix 3 would not disrupt any interhelical salt bridges. On the other hand, five interhelical salt bridges exist between residues in helix 3 and helix 4 in the bundle conformation (7). It should be noted, however, that although the present model requires disruption of such intramolecular salt bridges, they could be replaced by similar or new intermolecular helix 3-helix 4 salt bridges between paired apoE molecules. While this conformational change does not appear to be the most energetically favorable, it is consistent with data currently available. Clearly, other possibilities exist including an antiparallel “belt” arrangement of fully extended apoE molecules. Further study, including FRET analysis of other apoE variants will be required to verify or reject the model presented in Fig. 5. The present model implies that each disc possesses an even number of apoE3-(1–183) molecules. Calculation of the number of apoE3-(1–183) per disc as described by Pitas et al. (19), based on an average particle diameter of 15 nm and a lipid/protein molar ratio of 150:1, yields a value of 3.5 apoE3-(1–183) per disc. Assuming four apoE3-(1–183) per disc, these data correspond to a particle molecular weight of 440,000. Previous hydrodynamic and electrophoretic characterization of apoE3-(1–183)-DMPC discs, however, indicated a molecular weight of approximately 600,000 (15), implying that discs may exist with up to six apoE3-(1–183). This possibility is not inconsistent with apoE3-(1–183)-DMPC disc size heterogeneity observed by electron microscopy (15).

An interesting aspect of this model is the fact that it presents a tandem arrangement of the receptor-binding region of the protein (residues 130–150 in helix 4). Given the side by side arrangement produced by this juxtaposition, this molecular organization offers an explanation for earlier results that indicate synergy with respect to apoE copy number and receptor binding activity (20). Such a concept also fits well with the presence of seven independently folded, calcium-chelating, ligand-binding repeats in the receptor molecule (21). Structural data on these repeats suggest that they may work in concert to optimize ligand binding (22, 23). Although controversy exists about the molecular determinants of receptor recognition by apoE, it is known that modification of positively charged amino acid side chains in the protein abolishes its receptor recognition property (7). Other data indicate that a tandem alignment of receptor binding regions increases the affinity of the ligand for receptor binding. For example, Pitas et al. (19) showed that the number of receptor-active apoE per DMPC disc particle is positively correlated with binding affinity. It is worth noting that the present studies were conducted with the isolated receptor-binding domain of apoE. Based on available data, it is not known how the 10-kDa lipid surface-seeking C-terminal domain of intact apoE would be accommodated in terms of the present model. The fact that N- and C-terminal domain interactions are known to exist in this protein (24) suggests that the presence of one domain can influence the structure and/or function of the other.

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