Proposition 1.1. *Let $\mathcal{A}$ be a k-subspace arrangement of $\mathbb{C}^n$. Set $d = n - k$.*

(1) *$S_{\mathcal{A}}$ is a (strong) deformation retract of $M_{\mathcal{A}}$. In particular, the inclusion $i : S_{\mathcal{A}} \to M_{\mathcal{A}}$ induces isomorphisms*

$$H_*(S_{\mathcal{A}}; R) \cong H_*(M_{\mathcal{A}}; R) \quad \text{and} \quad \pi_*(S_{\mathcal{A}}) \cong \pi_*(M_{\mathcal{A}}).$$

(2) *$M_{\mathcal{A}}$ and $S_{\mathcal{A}}$ are $(2d-2)$-connected.*

(3) *There is the isomorphism*

$$\Psi = (\Psi_U) : H_{2d-1}(M_{\mathcal{A}}; R) \to \bigoplus_{U \in \mathcal{A}} H_{2d-1}(\mathbb{C}^n \setminus U; R) \cong \bigoplus_{U \in \mathcal{A}} R$$

induced by the inclusions $i_U : M_{\mathcal{A}} \to \mathbb{C}^n \setminus U$, where R is an arbitrary abelian group.

(4) *If $d \geq 2$, then $M_{\mathcal{A}}$ and $S_{\mathcal{A}}$ are simple spaces and*

$$\pi_{2d-1}(M_{\mathcal{A}}) \cong \pi_{2d-1}(S_{\mathcal{A}}) \cong \bigoplus_{U \in \mathcal{A}} \mathbb{Z}.$$

Here $H_q(-; R)$ means the q-th singular homology group with coefficients in R, and $\pi_q(-)$ means the q-th homotopy group.

Proof. (1) The map $r : M_{\mathcal{A}} \to S_{\mathcal{A}}$ defined by $r(x) = x/\|x\|$ gives a retraction and a homotopy $H : M_{\mathcal{A}} \times I \to M_{\mathcal{A}}$ between id and r is given by $H(x,t) = x/\|x\|^t$.

(2) Let $\alpha : \mathbb{S}^i \to M_{\mathcal{A}}$ be any continuous map for $0 \leq i \leq 2d-2$. Since α is null homotopic in $\mathbb{C}^n$, we take a homotopy $H : \mathbb{S}^i \times I \to \mathbb{C}^n$ between α and c a constant map into $M_{\mathcal{A}}$. Since

$$\dim \mathbb{S}^i \times I + \dim N_{\mathcal{A}} = i + 1 + 2k \leq 2n - 1 < \dim \mathbb{C}^n,$$

we can deform H such that the image of H is in $M_{\mathcal{A}}$ relative to $\mathbb{S}^i \times \{0,1\}$. Therefore α is null homotopic in $M_{\mathcal{A}}$, and so $S_{\mathcal{A}}$ and $M_{\mathcal{A}}$ are $(2d-2)$-connected.

(3) We prove it by induction on $|\mathcal{A}|$ the number of elements in $\mathcal{A}$. When $|\mathcal{A}| = 1$, we see $M_{\mathcal{A}} = \mathbb{C}^n \setminus U \cong \mathbb{C}^k \times (\mathbb{C}^d \setminus \{0\})$, which is homotopy equivalent to $\mathbb{S}^{2d-1}$. Hence $H_{2d-1}(M_{\mathcal{A}}; R) \cong R$. Now let $\mathcal{A} = \{U_1, \ldots, U_r\}$, $r \geq 2$. Set $\mathcal{B} = \{U_1, \ldots, U_{r-1}\}$ and $M = \mathbb{C}^n \setminus \bigcup \mathcal{B}$. Suppose $H_{2d-1}(M; R) \cong \bigoplus_{U \in \mathcal{B}} R$. Set $N = \mathbb{C}^n \setminus U_r$; then $H_{2d-1}(N; R) \cong R$. Since $M_{\mathcal{A}} = M \cap N$, by the Mayer-Vietoris exact sequence, we have

$$\to H_{2d}(M \cup N) \to H_{2d-1}(M_{\mathcal{A}}) \to H_{2d-1}(M) \oplus H_{2d-1}(N) \to H_{2d-1}(M \cup N) \to$$

where the coefficient group is R. Since $M \cup N = \mathbb{C}^n \setminus \bigcap_{U \in \mathcal{A}} U$ and $\dim \bigcap_{U \in \mathcal{A}} U \leq 2(k-1)$, $M \cap N$ is at least $2d$-connected by a similar argument as (2). By the Hurewicz

theorem, we have $H_{2d}(M \cup N) = H_{2d-1}(M \cup N) = 0$. Thus one can see that

$$H_{2d-1}(M_{\mathcal{A}}; R) \cong H_{2d-1}(M) \oplus H_{2d-1}(N)$$
$$\cong \bigoplus_{i=1}^{r} H_{2d-1}(\mathbb{C}^n \setminus U_i; R) \cong \bigoplus_{U \in \mathcal{A}} R.$$

(4) If $d \geq 2$, then M is 1-connected by (2), and hence $M_{\mathcal{A}}$ is simple. By the Hurewicz theorem, we have

$$\pi_{2d-1}(M_{\mathcal{A}}) \cong H_{2d-1}(M_{\mathcal{A}}; \mathbb{Z}) \cong \bigoplus_{U \in \mathcal{A}} \mathbb{Z}.$$

□

Remark. If $d = 1$, $M_{\mathcal{A}}$ is not necessarily 1-simple; namely, $\pi_1(M_{\mathcal{A}})$ is not necessarily abelian. For example, let $\mathcal{A} = \{(z_1, z_2) \in \mathbb{C}^2 \mid z_1 z_2 (z_1 + z_2) = 0\}$. Then $M_{\mathcal{A}}$ is homotopy equivalent to $(S^1 \vee S^1) \times S^1$, see [5]; so $\pi_1(M_{\mathcal{A}}) \cong F_2 \times \mathbb{Z}$, where F_2 is the free group of rank 2.

We introduce the multidegree of a map from a $(2d-1)$-dimensional closed manifold N to $M_{\mathcal{A}}$ the complement of a k-subspace arrangement in $\mathbb{C}^n$, where $d = n - k$. Let $f : N \to M_{\mathcal{A}}$ be any continuous map. Assume that N is orientable and N is given an orientation by the fundamental class $[N]$ of N. On the other hand, $M_{\mathcal{A}}$ has an orientation coming from the standard orientation of $\mathbb{C}^n$. Thus we define the multidegree $\mathrm{mDeg}\, f$ of f by

$$\mathrm{mDeg}\, f = \Psi(f_*([N])) \in \bigoplus_{U \in \mathcal{A}} \mathbb{Z}.$$

Here $\Psi = (\Psi_U) : H_{2d-1}(M_{\mathcal{A}}; \mathbb{Z}) \to \bigoplus_{U \in \mathcal{A}} H_{2d-1}(\mathbb{C}^n \setminus U; \mathbb{Z}) = \bigoplus_{U \in \mathcal{A}} \mathbb{Z}$ is the isomorphism induced from the inclusions $i_U : M_{\mathcal{A}} \to \mathbb{C}^n \setminus U$.

Next we consider the case where X is non-orientable. Let $[N]_2 \in H_{2d-1}(X; \mathbb{Z}/2)$ denote the mod 2 fundamental class of N. We define the mod 2 multidegree $\mathrm{mDeg}_2 f$ of f by

$$\mathrm{mDeg}_2\, f = \Psi_2(f_*([N]_2)) \in \bigoplus_{U \in \mathcal{A}} \mathbb{Z}/2.$$

Here $\Psi_2 = (\Psi_{2,U}) : H_{2d-1}(M_{\mathcal{A}}; \mathbb{Z}/2) \to \bigoplus_{U \in \mathcal{A}} H_{2d-1}(\mathbb{C}^n \setminus U; \mathbb{Z}/2) = \bigoplus_{U \in \mathcal{A}} \mathbb{Z}/2$ is the isomorphism induced from the inclusions $i_U : M_{\mathcal{A}} \to \mathbb{C}^n \setminus U$. These multidegrees are homotopy invariants for maps.

Example 1.2. Let $\mathcal{A} = \{(z_1, z_2) \in \mathbb{C}^2 \mid z_1 z_2 = 0\}$. We define maps $f_{p,q} : \mathbb{S}^1 \to M_{\mathcal{A}}$ by $f_{p,q}(z) = (z^p, z^q)$. Then $\mathrm{mDeg}\, f_{p,q} = (p, q)$.

2. A Hopf type theorem

Let X and Y are topological spaces. We denote by $[X, Y]$ the set of (unbased) homotopy classes of continuous maps from X to Y.

Classification of homotopy classes of maps or determination of the set $[X, Y]$ is a crucial problem in algebraic topology. Let X be a finite CW complex of dimension m and Y be an n-sphere $\mathbb{S}^n$. If $m < n$, then every continuous map $f : X \to \mathbb{S}^n$ is null homotopic; namely, $[X, \mathbb{S}^n]$ consists of one element. In our setting, we generalize this result as follows.

Theorem 2.1. *Let $M_{\mathcal{A}}$ be the complement of a k-subspace arrangement $\mathcal{A}$ in $\mathbb{C}^n$ and X a connected finite CW complex of dimension m less than $2d-1$, where $d = n-k$. Then every continuous map $f : X \to M_{\mathcal{A}}$ is null homotopic; namely, $[X, M_{\mathcal{A}}]$ consists of one element.*

Proof. Let $Z_{(i)}$ denote the i-skeleton of a relative CW complex $(X \times I, X \times \partial I)$ for $0 \leq i \leq m+1$. Set

$$H_{-1} := f \coprod c : Z_{(-1)} := X \times \partial I \to M_{\mathcal{A}},$$

where c is a constant map. We construct a map $H_i : Z_{(i)} \to M_{\mathcal{A}}$ extending H_{-1} inductively for $i \geq 0$. Suppose that there is a map $H_i : Z_{(i)} \to M_{\mathcal{A}}$. Note that $Z_{(i+1)}$ has a form $Z_{(i+1)} = Z_{(i)} \cup_j D_j^{i+1}$, where D_j^{i+1} are $(i+1)$-dimensional cells. Since $M_{\mathcal{A}}$ is $(2d-2)$-connected, $H_{ij} = H_i|_{\partial D_j^{i+1}} : \mathbb{S}^i \to M_{\mathcal{A}}$ extends to $\bar{H}_{ij} : D^{i+1} \to M_{\mathcal{A}}$ for $0 \leq i \leq m$. Thus we have a map

$$H_{i+1} = H_i \cup_j \bar{H}_{ij} : Y_{(i+1)} \to M_{\mathcal{A}}.$$

Consequently we have a homotopy $H : X \times I \to M_{\mathcal{A}}$ between f and c. □

H. Hopf [2] considered continuous maps from an n-dimensional closed manifold N to an n-sphere $\mathbb{S}^n$ and showed the so-called Hopf classification theorem below.

Theorem 2.2 (Hopf classification theorem)**.** *Let N be an n-dimensional closed manifold, where $n \geq 1$.*

(1) *If N is orientable, then the degree* $\deg f$ *of maps gives a bijection*

$$\deg : [N, \mathbb{S}^n] \to \mathbb{Z}.$$

(2) *If N is non-orientable, then the mod 2 degree* $\deg_2 f$ *of maps gives a bijection*

$$\deg_2 : [N, \mathbb{S}^n] \to \mathbb{Z}/2.$$

We generalize this result as follows.

Theorem 2.3. *Let $M_{\mathcal{A}}$ be the complement of a k-subspace arrangement $\mathcal{A}$ of $\mathbb{C}^n$ and N a closed manifold of dimension $2d-1$, where $d=n-k$. If $d=1$, then we assume that $\pi_1(M_{\mathcal{A}})$ is abelian.*

(1) *If N is orientable, then the multidegree* $\mathrm{mDeg}\, f$ *of maps gives a bijection*

$$\mathrm{mDeg} : [N, M_{\mathcal{A}}] \to \bigoplus_{U \in \mathcal{A}} \mathbb{Z}.$$

(2) *If N is non-orientable, then the mod 2 multidegree* $\mathrm{mDeg}_2\, f$ *of maps gives a bijection*

$$\mathrm{mDeg}_2 : [N, M_{\mathcal{A}}] \to \bigoplus_{U \in \mathcal{A}} \mathbb{Z}/2.$$

Remark. By Proposition 1.1 (1), the inclusion $i : S_{\mathcal{A}} \to M_{\mathcal{A}}$ induces a bijection $[N, S_{\mathcal{A}}] \cong [N, M_{\mathcal{A}}]$. In particular, if $k=0$, then it follows that $S_{\mathcal{A}} = \mathbb{S}^{2n-1}$ and the multidegree coincides with the ordinary degree $\deg f$. Therefore we obtain the Hopf classification theorem in the case where n is odd.

To show the theorem, we recall obstruction theory, see [1] for the detail. Let X be an n-dimensional finite CW complex, and let $K(\pi, n)$, π is abelian, denote an Eilenberg-MacLane space; i. e., $\pi_n(K(\pi,n)) \cong \pi$ and $\pi_q(K(\pi,n)) = 0$ for $q \neq n$. By the universal coefficient theorem and the Hurewicz theorem, there are natural isomorphisms

$$H^n(K(\pi,n);\pi) \cong \mathrm{Hom}_{\mathbb{Z}}(H_n(K(\pi,n);\mathbb{Z}),\pi) \cong \mathrm{Hom}_{\mathbb{Z}}(\pi,\pi).$$

Let $\iota \in H^n(K(\pi,n);\pi)$ be the element, called the fundamental class of $K(\pi,n)$, corresponding to the identity of π. Then one can define a map

$$\bar{\phi} : [X, K(\pi,n)] \to H^n(X;\pi)$$

by $\bar{\phi}([f]) = f^*(\iota)$. On the other hand, using the obstruction class $\gamma(f,c)$ between f and c a constant map, one can define a map

$$\bar{\psi} : [X, K(\pi,n)] \to H^n(X;\pi)$$

by $\bar{\psi}([f]) = \gamma(f,c)$. A fundamental result of obstruction theory is the following.

Proposition 2.4 ([1, chapter 7])**.** *Both $\bar{\phi}$ and $\bar{\psi}$ are bijections and they coincide.*

Now we give the proof of Theorem 2.3. Since $M_{\mathcal{A}}$ is a smooth manifold, it has a CW structure. Attaching cells of dimension greater than $2d$ to $M_{\mathcal{A}}$, one can kill the

homotopy groups of $M_{\mathcal{A}}$ greater than $(2d-1)$-dimension and so one obtains a space which is $K(\pi, 2d-1)$. Consequently one may assume that $K(\pi, 2d-1)$ is a CW complex including $M_{\mathcal{A}}$ as a subcomplex and the relative dimension of a pair $(K(\pi, 2d-1), M_{\mathcal{A}})$ is $2d+1$. By the cellular approximation theorem, there is an isomorphism

$$i_* : \pi_{2d-1}(M_{\mathcal{A}}) \to \pi_{2d-1}(K(\pi, 2d-1)),$$

where i is the inclusion. We identify these homotopy groups and set

$$\pi = \pi_{2d-1}(M_{\mathcal{A}}) = \pi_{2d-1}(K(\pi, 2d-1)) \cong \bigoplus_{U \in \mathcal{A}} \mathbb{Z}.$$

Then there is a bijection

$$\bar{\phi} = \bar{\psi} : [N, K(\pi, 2d-1)] \to H^{2d-1}(N; \pi).$$

By the cellular approximation theorem, we also see that there is a bijection

$$i_* : [N, M_{\mathcal{A}}] \to [N, K(\pi, 2d-1)],$$

where $i : M_{\mathcal{A}} \to K(\pi, 2d-1)$ is the inclusion. In fact, for any map $\alpha : N \to K(\pi, 2d-1)$, there exists a map $\alpha' : N \to K(\pi, 2d-1)$ such that α' and α are homotopic and $\alpha'(N) \subset K(\pi, 2d-1)_{(2d-1)} = M_{\mathcal{A}}$. Hence $[\alpha'] \in [N, M_{\mathcal{A}}]$ and $i_*([\alpha']) = [\alpha]$. Next if α, $\beta : N \to M_{\mathcal{A}}$ are homotopic in $K(\pi, 2d-1)$, then the homotopy H between α and β is homotopic to a homotopy

$$H' : N \times I \to K(\pi, 2d-1)_{(2d)} = M_{\mathcal{A}} \subset K(\pi, 2d-1)$$

relative to $N \times \partial I$. Hence i_* is injective. Thus we obtain a bijection

$$\phi := i_* \circ \bar{\phi} : [N, M_{\mathcal{A}}] \to H^{2d-1}(N; \pi),$$

where $\pi = \pi_{2d-1}(M_{\mathcal{A}})$. In this case, there are natural isomorphisms

$$H^{2d-1}(M_{\mathcal{A}}; \pi) \cong \mathrm{Hom}_{\mathbb{Z}}(H_{2d-1}(M_{\mathcal{A}}; \mathbb{Z}), \pi) \cong \mathrm{Hom}_{\mathbb{Z}}(\pi, \pi).$$

Let $\nu \in H^{2d-1}(M_{\mathcal{A}}; \pi)$ be the element corresponding to the identity of π. Then we have

$$\phi([f]) = f^*(\nu) = \gamma(f, c) \in H^{2d-1}(N; \pi).$$

2.1. **The orientable case.** We first consider the orientable case. Then there is the isomorphism

$$\kappa : H^{2d-1}(N;\pi) \to \pi$$

defined by

$$\kappa(x) = \langle x, [N]\rangle,$$

where $[N] \in H_{2d-1}(N;\mathbb{Z})$ is the fundamental class of N. Thus we have

$$\kappa(f^*(\nu)) = \langle f^*(\nu), [N]\rangle = \langle \nu, f_*([N])\rangle = h^{-1}(f_*([N])) \in \pi,$$

where $h : \pi = \pi_{2d-1}(M_{\mathcal{A}}) \to H_{2d-1}(M_{\mathcal{A}};\mathbb{Z})$ is the Hurewicz isomorphism. Identifying π with $H_{2d-1}(M_{\mathcal{A}};\mathbb{Z}) = \bigoplus_{U\in\mathcal{A}} \mathbb{Z}$ via the Hurewicz isomorphism, we conclude

$$h(h^{-1}(f_*([N]))) = f_*([N]) = \mathrm{mDeg}\, f.$$

Thus $\mathrm{mDeg} : [N, M_{\mathcal{A}}] \to \bigoplus_{U\in\mathcal{A}} \mathbb{Z}$ is a bijection.

2.2. **The non-orientable case.** There are isomorphisms

$$r^* : H^{2d-1}(N;\pi) \to H^{2d-1}(N;\pi/2\pi)$$

and

$$\kappa_2 : H^{2d-1}(N;\pi/2\pi) \to \pi/2\pi,$$

where r is the mod 2 reduction, and κ_2 is the homomorphism defined by

$$\kappa_2(x) = \langle x, [N]_2\rangle$$

and $[N]_2 \in H_{2d-1}(N;\mathbb{Z}/2)$ is the mod 2 fundamental class of N. Indeed the reduction $r : \pi \to \pi/2\pi$ induces a surjection $r^{\#} : C^{2d-1}(N;\pi) \to C^{2d-1}(N;\pi/2\pi)$ and since the coboundary maps on $C^{2d-1}(N\,\pi)$ and $C^{2d-1}(N;\pi/2\pi)$ are zero, $C^{2d-1}(N;\pi)$ and $C^{2d-1}(N;\pi/2\pi)$ are cocycles. Therefore r^* is a surjection. On the other hand, $H^{2d-1}(N;\mathbb{Z})$ and $H^{2d-1}(N;\mathbb{Z}/2)$ are isomorphic to $\bigoplus_{U\in\mathcal{A}} \mathbb{Z}/2$ respectively. Hence r^* must be an isomorphism. By the universal coefficient theorem over $\mathbb{Z}/2$, we see that κ_2 is an isomorphism. Thus we have

$$\kappa_2(r(f^*(\nu))) = \langle r^*(f^*(\nu)), [N]_2\rangle = \langle r^*(\nu), f_*([N]_2)\rangle = h_2^{-1}(f_*([N])) \in \pi/2\pi,$$

where $h_2 : \pi = \pi_{2d-1}(M_{\mathcal{A}}) \otimes \mathbb{Z}/2 \to H_{2d-1}(M_{\mathcal{A}};\mathbb{Z}/2)$ is the mod 2 Hurewicz homomorphism. Identifying $\pi/2\pi$ with $H_{2d-1}(M_{\mathcal{A}};\mathbb{Z}/2) = \bigoplus_{U\in\mathcal{A}} \mathbb{Z}/2$ via the mod 2 Hurewicz isomorphism, we conclude

$$h_2(h_2^{-1}(f_*([N]_2))) = f_*([N]_2) = \mathrm{mDeg}_2\, f.$$

Thus $\mathrm{mDeg}_2 : [N, M_{\mathcal{A}}] \to \bigoplus_{U\in\mathcal{A}} \mathbb{Z}/2$ is a bijection. Thus the proof is complete. □

Example 2.5. Let $\mathcal{A} = \{ (z_1, z_2) \in \mathbb{C}^2 \mid z_1 z_2 = 0\}$. Then we have

$$[\mathbb{S}^1, M_{\mathcal{A}}] \cong \mathbb{Z} \oplus \mathbb{Z}$$

and the homotopy classes are represented by the maps $f_{p,q}$ defined in Example 1.2 for $(p, q) \in \mathbb{Z} \oplus \mathbb{Z}$.

3. Remarks on 1-dimensional case

If $d = 1$, then $\pi_1(M_{\mathcal{A}})$ is not necessarily abelian. In this section, we consider the case where $\pi_1(M_{\mathcal{A}})$ is non-abelian. Since $d = 1$, then the 1-dimensional closed manifold N must be a circle $\mathbb{S}^1$. We want to know the homotopy set $[\mathbb{S}^1, M_{\mathcal{A}}]$. As is well known, $[\mathbb{S}^1, M_{\mathcal{A}}]$ coincides with the orbit set of the $\pi_1(M_{\mathcal{A}})$-action on $[\mathbb{S}^1, M_{\mathcal{A}}]_0$ $(= \pi_1(M_{\mathcal{A}}))$; see for example [1]. Since the $\pi_1(M_{\mathcal{A}})$-action is the conjugate action on $\pi_1(M_{\mathcal{A}})$, we have $[\mathbb{S}^1, M_{\mathcal{A}}] \cong C(\pi_1(M_{\mathcal{A}}))$, where $C(\pi_1(M_{\mathcal{A}}))$ is the set of conjugacy classes of elements in $\pi_1(M_{\mathcal{A}})$.

Example 3.1. Let $\mathcal{A} = \{ (z_1, z_2) \in \mathbb{C}^2 \mid z_1 z_2 (z_1 + z_2) = 0\}$. Then $\pi_1(M_{\mathcal{A}}) \cong F_2 \times \mathbb{Z}$ as mentioned in section 1. In this case, $C(F_2 \times \mathbb{Z}) \cong C(F_2) \times \mathbb{Z}$ and hence $[\mathbb{S}^1, M_{\mathcal{A}}] \cong C(F_2) \times \mathbb{Z}$.

Finally we give a remark in the case where $M_{\mathcal{A}}$ is $K(\pi, 1)$ and N is an n-dimensional closed manifold N. If π is abelian, then we have

$$[N, M_{\mathcal{A}}] \cong H^1(N; \pi)$$

by obstruction theory. For example, let

$$\mathcal{A} = \{(z_1, \ldots, z_n) \in \mathbb{C}^n \mid z_1 \cdots z_n = 0\}.$$

Then

$$M_{\mathcal{A}} \cong (\mathbb{C} \setminus \{0\}) \times \cdots \times (\mathbb{C} \setminus \{0\}) \quad (n \text{ times}).$$

Hence $M_{\mathcal{A}}$ is $K(\mathbb{Z}^n, 1)$; in particular, we conclude

$$[N, M_{\mathcal{A}}] \cong H^1(N; \mathbb{Z}^n) \cong \bigoplus^n H^1(N; \mathbb{Z}).$$

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A Potential Neuroprotective Role of Apolipoprotein E-containing Lipoproteins through Low Density Lipoprotein Receptor-related Protein 1 in Normal Tension Glaucoma*

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Background: No effective treatment exists for normal tension glaucoma (NTG), which induces a significant loss of retinal ganglion cells (RGCs).
Results: Apolipoprotein E-containing lipoproteins (E-LPs) blocked Ca^{2+}-dependent apoptosis induced by glutamate in RGCs.
Conclusion: Administration of E-LPs protects RGCs from glutamate-induced degeneration *in vitro* and *in vivo*.
Significance: Protection from neuron death by E-LPs provides a novel strategy of treatment for NTG.

Glaucoma is an optic neuropathy and the second major cause of blindness worldwide next to cataracts. The protection from retinal ganglion cell (RGC) loss, one of the main characteristics of glaucoma, would be a straightforward treatment for this disorder. However, the clinical application of neuroprotection has not, so far, been successful. Here, we report that apolipoprotein E-containing lipoproteins (E-LPs) protect primary cultured RGCs from Ca^{2+}-dependent, and mitochondrion-mediated, apoptosis induced by glutamate. Binding of E-LPs to the low density lipoprotein receptor-related protein 1 recruited the *N*-methyl-D-aspartate receptor, blocked intracellular Ca^{2+} elevation, and inactivated glycogen synthase kinase 3β, thereby inhibiting apoptosis. When compared with contralateral eyes treated with phosphate-buffered saline, intravitreal administration of E-LPs protected against RGC loss in glutamate aspartate transporter-deficient mice, a model of normal tension glaucoma that causes glaucomatous optic neuropathy without elevation of intraocular pressure. Although the presence of α2-macroglobulin, another ligand of the low density lipoprotein receptor-related protein 1, interfered with the neuroprotective effect of E-LPs against glutamate-induced neurotoxicity, the addition of E-LPs overcame the inhibitory effect of α2-macroglobulin. These findings may provide a potential therapeutic strategy for normal tension glaucoma by an LRP1-mediated pathway.

From global surveys, the second leading cause of blindness next to cataracts is glaucoma (1). Glaucoma is the major optic neuropathy and is characterized by significant death of retinal ganglion cells (RGCs)[2] (2). Although an elevated intraocular pressure greater than 21 mm Hg increases the risk of developing primary open-angle glaucoma, many glaucoma patients suffer from normal tension glaucoma (NTG), a subset of primary open-angle glaucoma, with a normal range of intraocular pressure (10–21 mm Hg). The occurrence of NTG varies worldwide. However, of Japanese adult patients with primary open-angle glaucoma, 92% were classified as having NTG (3). In addition, the proportion of glaucoma patients who had NTG (70%) was 4-fold higher than those with high intraocular pressure (17%) in a Japanese American clinical population (4). Moreover, it was also reported that all American Indian and Alaska Native patients with glaucoma had normal eye pressure (5). Although several factors appear to be associated with the development of this disorder (6), the cause of NTG has not been identified. Current clinical treatments for NTG, mostly controlling intraocular pressure, are very limited and unsatisfactory. Although the strategies for providing neuroprotection by Ca^{2+} channel blockers, neurotrophins, and inhibitors of the *N*-methyl-D-aspartate (NMDA) receptor (7–9) against RGC

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[2] The abbreviations used are: RGC, retinal ganglion cell; apo, apolipoprotein; E-LP, apolipoprotein E-containing lipoprotein; GLAST, glutamate aspartate transporter; GSK, glycogen synthase kinase; HBSS, Hanks' balanced salt solution; LRP1, low density lipoprotein receptor-related protein 1; NMDAR, NMDA receptor; NTG, normal tension glaucoma; Z, benzyloxycarbonyl; fluoromethyl ketone; EGFP, enhanced green fluorescent protein; Glu, glutamate; a2M, α2-macroglobulin.

degeneration have been recently considered, these treatments have not been clinically successful. Thus, a novel strategy of treatment for NTG is urgently needed.

It has been reported that nerve injury promotes the secretion of significant amounts of apolipoprotein E-containing lipoproteins (E-LPs) (10, 11). This response of glia to nerve injury has been suggested to provide support for the repair of neurons by supplying materials for the cells. We have reported that glia-derived E-LPs promote axon extension of RGCs mediated by receptor(s) of the low density lipoprotein (LDL) receptor family after axon injury (12). One multifunctional endocytotic and signaling receptor of this family is the LDL receptor-related protein 1 (LRP1). We have also demonstrated that E-LPs strongly protect RGCs from neurodegeneration elicited by withdrawal of trophic additives (brain-derived neurotrophic factor, ciliary neurotrophic factor, basic fibroblast growth factor, and other supplements) (13). This neuroprotection was initiated upon binding of E-LP to LRP1, which induced an intracellular signal involving phospholipase Cγ1, protein kinase Cδ, and glycogen synthase kinase 3β (GSK3β), without endocytosis of the E-LPs (14). Thus, we propose that E-LPs not only supply lipids but also can function as an endogenous neuroprotective factor for suppressing neurodegeneration by inducing intracellular signaling.

Here we provide a potential therapeutic strategy for NTG by intravitreal administration of E-LPs. E-LPs bind to LRP1, recruit NMDA receptors, and inhibit intracellular Ca^{2+} elevation in RGCs. The inhibition of Ca^{2+} elevation by E-LPs suppresses mitochondrion-mediated and caspase-dependent apoptosis in RGCs. In addition, treatment of E-LPs inactivates the proapoptotic kinase GSK3β *in vitro* and *in vivo*. A deficiency of the glutamate aspartate transporter (GLAST), a major glutamate transporter in the retina, in mice induces optic neuropathy without affecting intraocular pressure and exhibits many features similar to human NTG (15). Thus, GLAST-deficient mice are utilized as an animal model for NTG. In this study, intravitreal administration of E-LPs prevented RGC loss induced in GLAST-deficient mice. Although an increase of α2-macroglobulin, another endogenous ligand of LRP1, in vitreous humor of GLAST-deficient mice may interfere with the neuroprotective effect of E-LPs, exogenous administration of E-LPs overcomes this inhibition.

EXPERIMENTAL PROCEDURES

Materials—A rabbit polyclonal anti-LRP1 antibody (R2629) was generously provided by Dr. D. K. Strickland (University of Maryland School of Medicine, Baltimore, MD) (16). A colony of GLAST-deficient mice was established at Kumamoto University from mice obtained from Tokyo Medical and Dental University. All experimental procedures were approved by the Animal Care Committee of Kumamoto University.

Primary Culture of Retinal Ganglion Cells—Sprague-Dawley rats (2 days old) were used for primary culture of RGCs according to Barres *et al.* (17) with minor modifications (13, 14). Briefly, retinae were digested with papain (16.5 units/ml) for 30 min at 37 °C and then triturated in minimum essential medium (Invitrogen) with rabbit anti-rat macrophage antiserum (Accurate Chemical, Westbury, NY). The cell suspension was first incubated on a panning plate (150-mm Petri dish) coated with goat anti-rabbit IgG (Pierce Biotechnology) for 20 min at room temperature. Nonadherent cells were incubated for 35 min on a second panning plate (100-mm Petri dish) coated with goat anti-mouse IgMμ (Pierce) and mouse anti-Thy1.1 antibodies secreted from T11D7e2 cells (American Type Culture Collection, Manassas, VA). The plate was washed with phosphate-buffered saline (PBS), and then adherent RGCs were released by treatment with 0.125% trypsin for 10 min at 37 °C. Isolated RGCs were suspended in medium containing 1 mM glutamine, 5 μg/ml insulin, 60 μg/ml *N*-acetylcysteine, 62 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 0.1 mg/ml bovine serum albumin, 40 ng/ml triiodothyronine, 0.1 mg/ml transferrin, 1 mM sodium pyruvate, 2% B-27 supplement (Invitrogen), 10 μM forskolin, 50 ng/ml brain-derived neurotrophic factor (PeproTech, Rocky Hill, NJ), 50 ng/ml ciliary neurotrophic factor (PeproTech), and 50 ng/ml basic fibroblast growth factor (PeproTech) in Neurobasal medium. Culture plates (96 wells) were coated with poly-D-lysine (Sigma) and laminin (Sigma). RGCs were plated at a density of 5,000 cells/well in 96-well plates, 5,000 cells/culture insert for μ-dishes (ibidi, Munich, Germany), or 15,000 cells/dish for compartmented cultures and were cultured for at least 10 days before experiments.

Isolation of Glia-derived E-LPs, Plasma High Density Lipoproteins, and Reconstituted E-LPs—Glia were isolated from the cerebral cortex of 2-day-old Sprague-Dawley rats, digested with 0.25% trypsin, and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The glial cultures were enriched in astrocytes (>80%) (14). Glia were cultured for 3 days in the same medium used for RGCs but without forskolin, brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. This culture medium was centrifuged for 10 min at 1,000 × *g*, and the supernatant is defined as glia-conditioned medium. Mouse or rat plasma high density lipoproteins (HDL) were isolated from blood collected from the abdominal aorta of C57BL/6J mice, apoE-deficient mice or Sprague-Dawley rats, as indicated. Reconstituted E-LPs were prepared as described previously (13) and contained 1-palmitoyl-2-oleoyl-glycerophosphocholine (Sigma), cholesterol (Sigma), and recombinant human apoE (Wako, Osaka, Japan) at a molar ratio of 100:10:1 or 100:0:1 as indicated. Briefly, 2.17 mg of 1-palmitoyl-2-oleoyl-glycerophosphocholine, with or without 0.11 mg of cholesterol, were dissolved in chloroform and then evaporated under nitrogen gas. Four hundred μl of 10 mM Tris-HCl (pH 7.4) containing 0.9% NaCl were added and incubated for 1 h on ice. One hundred μl of 15 mg/ml sodium cholate were added. The mixture was incubated for 2 h on ice, mixed with 1 mg of recombinant apoE3 or apoE4, and incubated for 1 h on ice. Bio-Beads (100 mg; Bio-Rad) were added to the mixture, rotated for 3 h at 4 °C, and filtered to remove beads. The mixture contained reconstituted lipoproteins. Glia-conditioned medium, plasma, or reconstituted lipoproteins were centrifuged in a SRP28SA1 rotor (Hitachi, Tokyo, Japan) at 100,000 × *g* for 72 h at 4 °C on a discontinuous sucrose gradient consisting of the following solutions: 3 ml of density 1.30 g/ml, 3 ml of density 1.2 g/ml, 3 ml of density 1.1 g/ml, and 6 ml of density 1.006 g/ml. Ten fractions (1.5 ml) were collected from the top of the gradient and immunoblotted for

apoE as described below. Fractions containing apoE were concentrated using an Amicon Ultra filter (UFC905008, Millipore, Bedford, MA). The amount of lipoproteins was adjusted for cholesterol concentration (2 μg/ml) for glia-derived E-LPs and HDL or for protein concentration (100 ng/ml) for reconstituted lipoproteins. The cholesterol and protein concentrations of lipoproteins were measured by a LabAssay cholesterol kit (Wako) and BCA protein assay kit (Thermo Fisher Scientific), respectively. α2-Macroglobulin (Sigma) was activated by treatment with 100 mM methylamine for 1 h at room temperature (18).

Immunoblotting—Immunoblotting was performed as described previously (13, 14). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with primary and peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized with SuperSignal West Pico or Dura (Thermo Fisher Scientific). The following primary antibodies were used: mouse anti-β-actin (a5441, Sigma), goat anti-albumin (A90-134A, Bethyl Laboratories, Montgomery, TX), goat anti-α2-macroglobulin (M5649, Sigma), goat anti-human apoE (k74190g, Meridian Life Science, Inc., Cincinnati, OH), goat anti-mouse apoE (sc-6384, Santa Cruz Biotechnology), rabbit anti-protein kinase Cδ (2058, Cell Signaling Technology, Danvers, MA), rabbit anti-GSK3β and phospho-Ser-9-GSK3β (9315 and 9336S, Cell Signaling Technology), goat anti-Brn-3a (sc-31984, Santa Cruz Biotechnology), rabbit anti-LRP1 (2703-1, Epitomics, Burlingame, CA), mouse anti-LRP1 (545503, R&D Systems, Minneapolis, MN), rabbit anti-phospholipase Cγ1 (sc-81, Santa Cruz Biotechnology), rabbit anti-NMDAR2A (AB1555P, Millipore), and mouse anti-NMDAR2B (610416, BD Biosciences).

Immunocytochemistry—Cultured RGCs were fixed in acetone for 10 min at −20 °C and then blocked with 1% bovine serum albumin and 5% goat serum in PBS for 1 h at room temperature. The cells were incubated with rabbit anti-LRP1 (Epitomics), mouse anti-NMDAR2B (32-0700, Invitrogen), or mouse anti-cytochrome *c* (556432, BD Biosciences) in PBS containing 5% goat serum for 1 h at room temperature, washed three times with PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen), Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), or Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen) for 1 h at room temperature. For staining of mitochondria, RGCs were incubated with 2 nM MitoTracker Red CMXRos (Invitrogen) for 30 min 1 day before the experiment. Fluorescence images were taken with an Olympus IX71 microscope or FV500 confocal microscope.

Apoptosis of RGCs—Primary cultured RGCs were washed twice (15-min incubation at 37 °C) with Hanks' balanced salt solution (HBSS; Invitrogen) containing 2.4 mM $CaCl_2$, 20 mM HEPES without magnesium. Magnesium was omitted from the washing solution to avoid blocking the NMDA receptor (19). Subsequently, RGCs were incubated ± 300 μM glutamate and 10 μM glycine, a co-activator of the NMDA receptor, in HBSS containing 2.4 mM $CaCl_2$, 20 mM HEPES without magnesium for 2 h at 37 °C. After control (HBSS containing 2.4 mM $CaCl_2$, 20 mM HEPES without magnesium) or glutamate treatment, RGCs were cultured in the same medium without forskolin, brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor for 22 h at 37 °C. For detection of apoptosis with Hoechst 33342 (Dojindo, Kumamoto, Japan), RGCs were incubated with 1 μg/ml Hoechst 33342 for 15 min. Fluorescent images (six images/well) were randomly taken using an IX71 fluorescence microscope. For each treatment, at least 12 images/two wells in 96-well plates were obtained. Fragmented or shrunken nuclei stained with Hoechst dye were counted as apoptotic neurons, and round/smooth nuclei were counted as healthy neurons. More than 300 neurons were blindly counted for each treatment. For detection of apoptosis with annexin V-EGFP apoptosis detection kit containing annexin V-EGFP, propidium iodide, and binding buffer, the manufacturer's instructions (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) were followed. Annexin V-EGFP and propidium iodide are membrane-impermeable reagents. During early stages of apoptosis, phosphatidylserine becomes exposed on the outer leaflet of the plasma membrane and is accessible to annexin V. Propidium iodide stains nuclei of necrotic cells and also end stage apoptotic cells. Healthy cells are not stained with either reagent.

Intracellular Ca^{2+}—RGCs were incubated with 3 μM Fluo-8 acetoxymethyl ester (AAT Bioquest, Sunnyvale, CA) for 30 min at 37 °C. The cells were washed twice (15-min incubation each) with HBSS containing 2.4 mM $CaCl_2$, 20 mM HEPES without magnesium and then administered 300 μM glutamate and 10 μM glycine. Fluorescent images were acquired every 500 ms using an ORCA-R2 digital CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed by the MetaFluor fluorescence ratio imaging software (Molecular Devices, Sunnyvale, CA).

RNA Silencing of Protein Kinase Cδ—Negative control small interfering RNA (siRNA) (300 nM) (Accell non-targeting pool, Thermo Fisher Scientific) or siRNA specific for protein kinase Cδ (Accell SMARTpool, E-080142-00-0005, Thermo Fisher Scientific) was added to culture medium as indicated by the manufacturer and then incubated with RGCs for 6 days. The knockdown by negative control or protein kinase Cδ siRNA was confirmed by immunoblotting.

Compartmented Culture of RGCs—RGCs in compartmented cultures were prepared as described previously (12, 20). Distal axons and cell bodies/dendrites/proximal axons of the primary neurons can be separately maintained with different media in compartmented cultures. A Teflon divider, which creates three compartments, was applied to the μ-dish (ibidi) with silicone grease. RGCs were plated in the center compartment in RGC culture medium to which were added 25 ng/ml brain-derived neurotrophic factor and 25 ng/ml ciliary neurotrophic factor. The side compartments were supplied with the same medium that also contained 75 ng/ml brain-derived neurotrophic factor, 25 ng/ml ciliary neurotrophic factor, and 50 ng/ml basic fibroblast growth factor. Axons of RGCs crossed under the silicone grease into the side compartments within 5 days. Prior to the experiments, RGCs were cultured for at least 14 days.

Intravitreal Injection of E-LPs and Collection of Vitreous Humor—*Glast*$^{+/+}$, *Glast*$^{+/-}$, or *Glast*$^{-/-}$ mice (3 weeks old) were anesthetized by intraperitoneal injection of 50 mg/kg of

sodium pentobarbital. For intravitreal injection, the vitreous of one eye was injected with 1 μl of E-LPs (1.5 μg of protein/ml) or HDL (30 μg of cholesterol/ml), and the other eye was injected with the same volume of PBS through a 33-gauge needle (Terumo, Tokyo, Japan) connected to a Hamilton syringe (Bonaduz, Switzerland). This procedure was performed under a stereomicroscope (SZX7, Olympus) so that the lens and retina were not injured. For collection of vitreous humor, the same set of needles and syringes was used as for intravitreal injection. Each sample of vitreous humor for immunoblotting was combined from 10 eyes.

Histological Studies of Retinae—Eyes from 3- and 6-week-old mice were enucleated and fixed with Super Fix (KY-500, Kurabo, Osaka) overnight at 4 °C, and the cornea and lens were removed. Retinae with sclera were embedded in paraffin. Sequentially, 4-μm paraffin sections of retina were cut through the optic nerve and stained with hematoxylin and eosin. The number of cells in the ganglion cell layer was counted from one end through the optic nerve to the other end on the retinal section. Ten sections, more than 2,000 cells, were counted in each retina.

Co-immunoprecipitation—Co-immunoprecipitation was performed according to May *et al.* (21). RGC lysates were prepared in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1% Triton X-100 with Complete EDTA-free protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and PhosSTOP phosphatase inhibitor (Roche Diagnostics). The lysate was passed 15 times through a 22-gauge needle and centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant was precleared with 40 μl of 50% equilibrated protein G-Sepharose (GE Healthcare, Buckinghamshire, UK) for 1 h at 4 °C, and then the Sepharose beads were removed by centrifugation. Rabbit anti-LRP1 (Epitomics) or rabbit anti-NR2B antibody (AB1557, Millipore) was added, and the lysate was rotated for 12 h at 4 °C. Forty μl of 50% equilibrated protein G-Sepharose were added to the lysate and rotated for 1 h at 4 °C. The Sepharose beads were washed three times with lysis buffer containing 0.1% Triton X-100. For immunoblotting, 30 μl of sample buffer were added, and the beads were boiled for 5 min. The supernatant was subjected to SDS-PAGE and immunoblotting.

Statistical Analysis—Statistical analyses were performed using one-way analysis of variance followed by Bonferroni's multiple comparison. A confidence level of >95% was considered significant ($p < 0.05$).

RESULTS

Glutamate-induced Apoptosis in Retinal Ganglion Cells—Glutamate is a major excitatory neurotransmitter and also acts as an excitatory neurotoxin in acute and chronic central nervous system disorders such as cerebral ischemia, amyotrophic lateral sclerosis, Alzheimer disease and glaucoma (15, 22, 23). However, Ullian *et al.* (24) reported that RGCs were not vulnerable to NMDA excitotoxicity. Thus, we determined whether or not glutamate induced neurotoxicity in RGCs. As shown in Fig. 1*A*, 300 μM glutamate induced fragmentation or shrinkage of nuclei stained with Hoechst dye (a marker of apoptosis) in RGCs in the presence of 10 μM glycine (*Glu*: glutamate plus

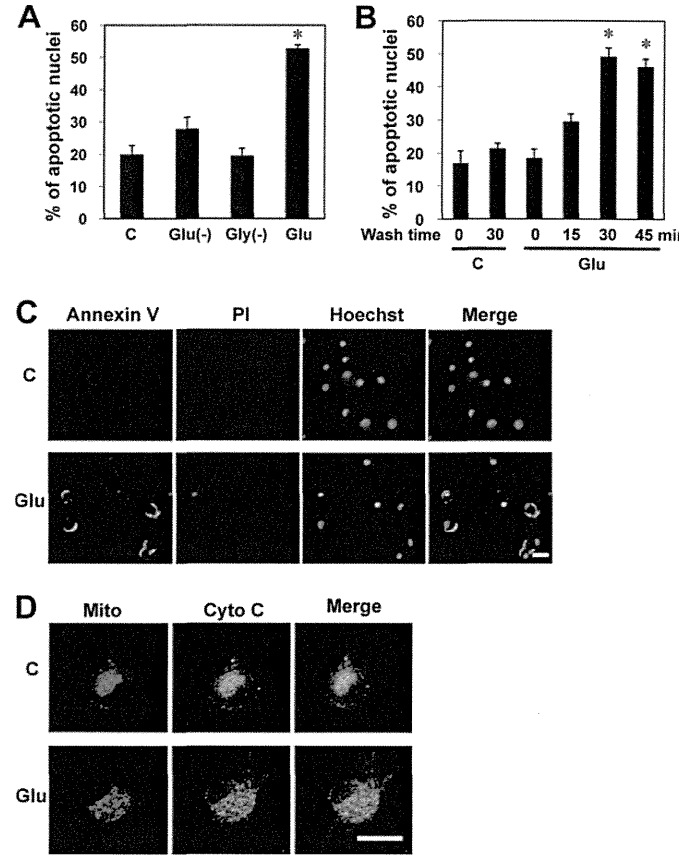

FIGURE 1. **Glutamate with glycine induces apoptosis in RGCs.** *A*, fragmented or shrunken nuclei in RGCs were detected by Hoechst staining 24 h after control (*C*; HBSS) or treatment with glutamate alone (*Glu(−)*; 300 μM glutamate), glycine alone (*Gly(−)*; 10 μM glycine), or glutamate + glycine (*Glu*; 300 μM glutamate + 10 μM glycine). Data are means ± S.E. from 4 independent experiments. *, $p < 0.001$ for control *versus* Glu. *B*, fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control (HBSS) or glutamate + glycine treatment (*Glu*; 300 μM glutamate plus 10 μM glycine) with 0, 15 (one 15-min wash), 30 (two 15-min washes), or 45 min (three 15-min washes) of washing by HBSS. *C*, fluorescence images of RGCs stained with annexin V-EGFP, propidium iodide (*PI*), and Hoechst 12 h after control (HBSS) or Glu treatment (300 μM glutamate + 10 μM glycine). *Scale bar*, 20 μm. *D*, RGCs stained with 2 nM MitoTracker Red (*Mito*) were immunostained with anti-cytochrome *c* (*Cyto C*) 12 h after control or Glu treatment. *Scale bar*, 20 μm.

glycine), but neither component alone (*Glu(−)*, glutamate alone; *Gly(−)*, glycine alone) induced neurotoxicity. Thus, to induce neurotoxicity, glycine was added with glutamate as co-activator in the following experiments. Washing of the RGCs for 30 min (two 15-min washes) was required for induction of glutamate-induced neurotoxicity, but a longer washing time (45 min: three 15-min washes) did not cause further neurotoxicity (Fig. 1*B*), nor did a higher concentration of glutamate (1 mM) (data not shown). Exposure of phosphatidylserine on the cell surface after glutamate treatment was monitored as another marker of apoptosis. Annexin V-EGFP-positive and propidium iodide-negative RGCs (*i.e.* apoptotic RGCs) were observed 12 h after glutamate treatment but not without glutamate (Fig. 1*C*). Moreover, glutamate induced cytochrome *c* release from mitochondria (an additional marker of apoptosis), as indicated by less overlap of cytochrome *c* and MitoTracker Red (*i.e.* mitochondria) in fluorescence images in the presence of glutamate when compared with control (Fig. 1*D*). These

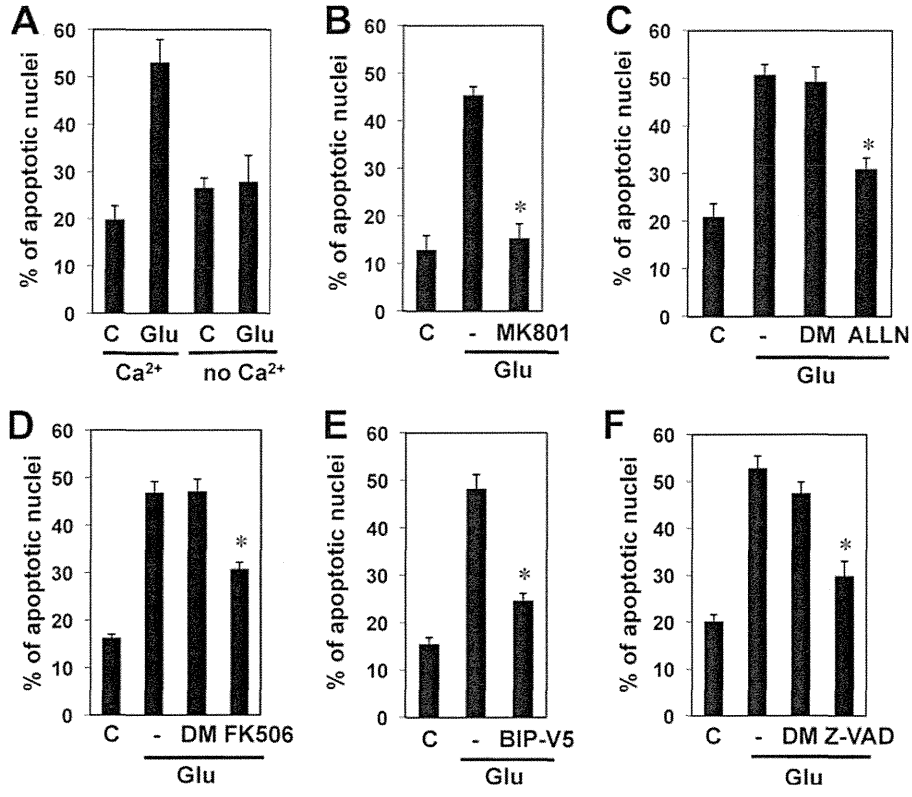

FIGURE 2. **Contributors to glutamate-induced neurotoxicity in RGCs.** Fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control (*C*; HBSS) or glutamate treatment (*Glu*; 300 μM glutamate + 10 μM glycine). *A*, Glu was incubated with the cells with or without Ca^{2+}. *B–F*, RGCs were treated with 10 μM MK801 (inhibitor of the NMDA receptor) (*B*), 1 μM *N*-acetyl-leucine leucine norleucinal (*ALLN*) (calpain inhibitor) (*C*), 1 μM FK506 (calcineurin inhibitor) (*D*), 200 μM Bax-inhibitory peptide V5 (BIP-V5; inhibitor of Bax) (*E*), 20 μM Z-VAD-fmk (Z-VAD; caspase inhibitor) (*F*), or dimethyl sulfoxide (*DM*) when Glu was added. In *B–F*, *, $p < 0.001$ for Glu *versus* Glu+inhibitor. Data are means ± S.E. from 4–6 independent experiments.

results indicate that glutamate induces apoptosis of RGCs in the presence of glycine.

We next determined mechanisms by which glutamate induces neuronal apoptosis in RGCs, as detected by Hoechst staining (Fig. 2). Glutamate induced apoptosis within 24 h in the presence, but not in the absence, of Ca^{2+} (Fig. 2*A*). Several inhibitors were used to identify molecules that contributed to the glutamate-induced neurotoxicity in RGCs. Inhibitors of the NMDA receptor (10 μM MK801), calpain (1 μM *N*-acetyl-leucine leucine norleucinal), calcineurin (1 μM FK506), Bax (200 μM BIP-V5), and caspase (20 μM Z-VAD-fmk) significantly reduced glutamate-induced neurodegeneration (Fig. 2, *B–F*).

E-LPs Are Neuroprotective for RGCs—We previously reported that apoptosis of RGCs induced by withdrawal of trophic additives was inhibited by glia-derived E-LPs (13, 14). Thus, glia-derived E-LPs (2 μg of cholesterol/ml) and rat plasma HDL (2 μg of cholesterol/ml) were applied to determine whether these lipoproteins protected RGCs from glutamate toxicity. Both types of lipoproteins containing apoE protected RGCs against glutamate neurotoxicity (Fig. 3*A*). In addition, reconstituted E-LPs containing recombinant apoE, cholesterol, and phospholipids protected RGCs in a dose-dependent manner (Fig. 3*B*). The protective effect of E-LPs was saturated at ~70% with over 100 ng of protein/ml E-LP. We next determined which components of E-LPs were necessary for the neuroprotection. The protection required the association of apoE with lipid (Fig. 3*C*). However, neither cholesterol alone (11 ng/ml) nor cholesterol combined with phosphatidylcholine (11 ng of cholesterol/ml) promoted survival, whereas apoE associated with phosphatidylcholine alone (100 ng of protein/ml) was neuroprotective. In addition, cholesterol enhanced the protective effect of apoE associated with phosphatidylcholine. In contrast to plasma HDL isolated from wild type mice, plasma HDL isolated from apoE-deficient mice failed to protect RGCs.

Human apoE3 and apoE4 isoforms differently affect neurodegeneration, particularly in Alzheimer disease (25, 26). However, we found no difference in the neuroprotective efficacy of lipoproteins containing human apoE3 and apoE4 in glutamate-treated RGCs (Fig. 3*D*).

Interaction of LRP1 and the NMDA Receptor Prevents Intracellular Ca^{2+} Elevation—We determined whether E-LPs prevented the increase in intracellular Ca^{2+} stimulated by glutamate because glutamate neurotoxicity in RGCs depends on extracellular Ca^{2+} (Fig. 2). Fluorescence ratio images with pseudocolors demonstrate that glutamate markedly increased intracellular Ca^{2+}. When compared with the absence of E-LPs, glutamate failed to increase intracellular Ca^{2+} significantly in the presence of E-LPs, as indicated by green staining of the RGCs (Fig. 4*A* and *B*). The inhibitory effect of E-LPs on Ca^{2+} elevation was similar to that of 10 μM MK801 and E-LP+MK801. Because MK801 is a selective inhibitor of the NMDA receptor, this result indicates that E-LPs suppress the intracellular Ca^{2+} elevation mediated by the NMDA receptor. To determine whether inhibition of intracellular Ca^{2+} elevation was mediated by a receptor(s) of the LDL receptor superfamily, we used a polyclonal antibody directed against multiple

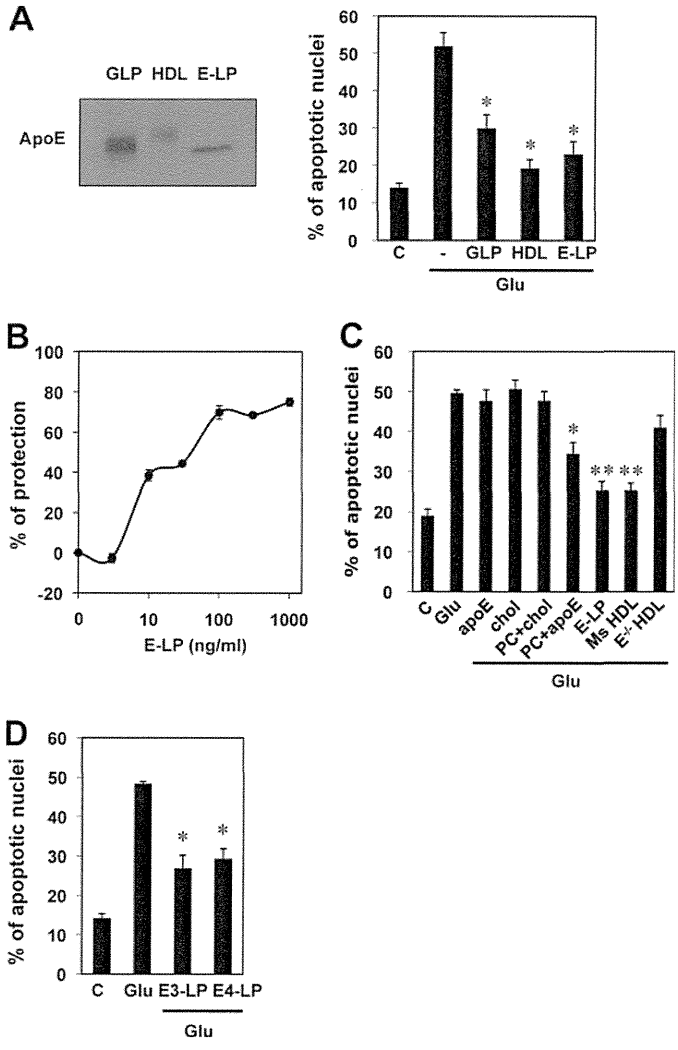

FIGURE 3. **Lipoproteins prevent apoptosis induced by glutamate.** Fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control (*C*; HBSS) or glutamate treatment (*Glu*; 300 μM glutamate + 10 μM glycine). *A*, immunoblot for apoE in lipoproteins (2 μg of cholesterol/ml) isolated from glia-conditioned medium (*GLP*, glia-derived E-LPs), HDL (2 μg of cholesterol/ml) isolated from rat serum and reconstituted human apoE-containing lipoproteins (100 ng of protein/ml) (E-LP). RGCs were incubated for 15 min with GLP, HDL, or E-LP, and then Glu was added as indicated. *, $p < 0.005$ for Glu *versus* Glu+GLP, Glu+HDL or Glu+E-LP. *B*, dose-dependent protection of E-LP against glutamate neurotoxicity. RGCs were incubated for 15 min with the indicated concentrations of E-LP, and then Glu was added. The percentage of protection by E-LPs was calculated such that control was 100% and glutamate treatment without E-LP was 0% of protection. *C*, RGCs were incubated with lipid-free apoE (100 ng of protein/ml), cholesterol (*chol*) (11 ng/ml), phosphatidylcholine+cholesterol (*PC+chol*) liposomes (11 ng of cholesterol/ml), PC+apoE lipoproteins (100 ng of protein/ml), E-LP containing apoE, cholesterol and phosphatidylcholine (100 ng of protein and 11 ng of cholesterol/ml), HDL (2 μg of cholesterol/ml) from mouse plasma (*Ms HDL*), or HDL (2 μg of cholesterol/ml) from apoE-deficient mouse plasma ($E^{-/-}$ HDL), and then Glu was added as indicated. * and **, $p < 0.001$ and 0.0001, respectively, for Glu *versus* Glu+lipoproteins. *D*, RGCs were incubated with reconstituted human apoE3- (*E3-LP*) or apoE4-containing lipoproteins (*E4-LP*) for 15 min, and then Glu was added as indicated. *, $p < 0.001$ for Glu *versus* Glu+E3-LP or Glu+E4-LP.

epitopes of LRP1 to inhibit the binding of E-LPs to this receptor (13, 16). The anti-LRP1 antibody (10 μg/ml) prevented the blocking effect of E-LPs against intracellular Ca^{2+} elevation and the neuroprotective effect of E-LPs, whereas nonspecific rabbit IgG (10 μg/ml) did not (Fig. 4, *C* and *D*). We next examined whether LRP1 interacted with the NMDA receptor and whether E-LPs modulated this interaction. The NMDA receptor subunits NR2B and NR2A were co-immunoprecipitated with LRP1 using an anti-LRP1 antibody. Treatment with E-LPs for 15 min increased the interaction among NR2B, NR2A, and LRP1 (Fig. 4*E*). In addition, LRP1 was co-immunoprecipitated with NR2B by the NR2B antibody, and this interaction was facilitated by E-LPs (Fig. 4*F*).

A Neuroprotective Signal Caused by E-LPs Inactivates GSK3β—We previously reported that glia-derived E-LPs protected RGCs from apoptosis induced by withdrawal of trophic additives (13, 14). This neuroprotection was initiated upon binding of E-LPs to LRP1 at the cell surface, which transduced an intracellular signal involving phospholipase Cγ1 and protein kinase Cδ and subsequently inactivated the proapoptotic kinase GSK3β. Thus, we tested whether this neuroprotective signal of E-LPs was also turned on against glutamate neurotoxicity. The phospholipase C inhibitor U73122 (5 μM) prevented the neuroprotection by E-LPs without inducing apoptosis by itself (Fig. 5*A*). In addition, although E-LPs protected RGCs incubated with a control siRNA, this protective effect of E-LPs was abolished in RGCs incubated with protein kinase Cδ siRNA (Fig. 5*B*). Neither negative control siRNA nor protein kinase Cδ siRNA induced apoptosis. Furthermore, the phosphorylation of GSK3β was decreased 16 h after glutamate treatment but was restored to the control level by E-LP treatment (Fig. 5*C*), suggesting that inactivation of GSK3β contributes to the protective effect of E-LPs against glutamate neurotoxicity.

Localization of Glutamate Toxicity and E-LP-induced Neuroprotection in RGCs—We used compartmented RGC cultures to investigate in which part of the neuron glutamate neurotoxicity and E-LP-induced neuroprotection occurred. Compartmented cultures maintain the cell body/proximal axons/dendrites and distal axons of the same neuron in different media (12, 20). We first determined the localization of LRP1 and NR2B in RGCs maintained in compartmented cultures. Immunocytochemistry shows that LRP1 and NR2B co-localize in cell bodies/proximal axons/dendrites of RGCs with a minor signal for LRP1 on distal axons (Fig. 6*A*). Consistent with this observation, application of glutamate to the cell body-containing compartment caused apoptosis, which was attenuated by the addition of 100 ng of protein/ml E-LPs to the cell body-containing compartment (Fig. 6*B*). In contrast, treatment of distal axons alone with glutamate did not induce RGC apoptosis, and E-LPs did not affect survival (Fig. 6*C*). Thus, glutamate toxicity and neuroprotection by E-LPs are induced in cell bodies/proximal axons/dendrites of RGCs, suggesting that administration of E-LPs to the retina, the location of cell bodies/proximal axons/dendrites of RGCs, might protect RGCs from glutamate injury *in vivo*.

Intravitreal Treatment with E-LPs Promotes Survival of RGCs in Glast$^{-/-}$ Mice—On the basis of these *in vitro* observations, we next determined whether E-LPs protected RGCs from neurodegeneration in GLAST-deficient mice, a model of NTG. In *Glast*$^{-/-}$ mice, RGC loss progresses after 3 weeks but does not worsen after 5 weeks (15). To avoid surgical injuries, and to perform accurate injections into vitreous humor of mice under the microscope, we performed intravitreal injections in

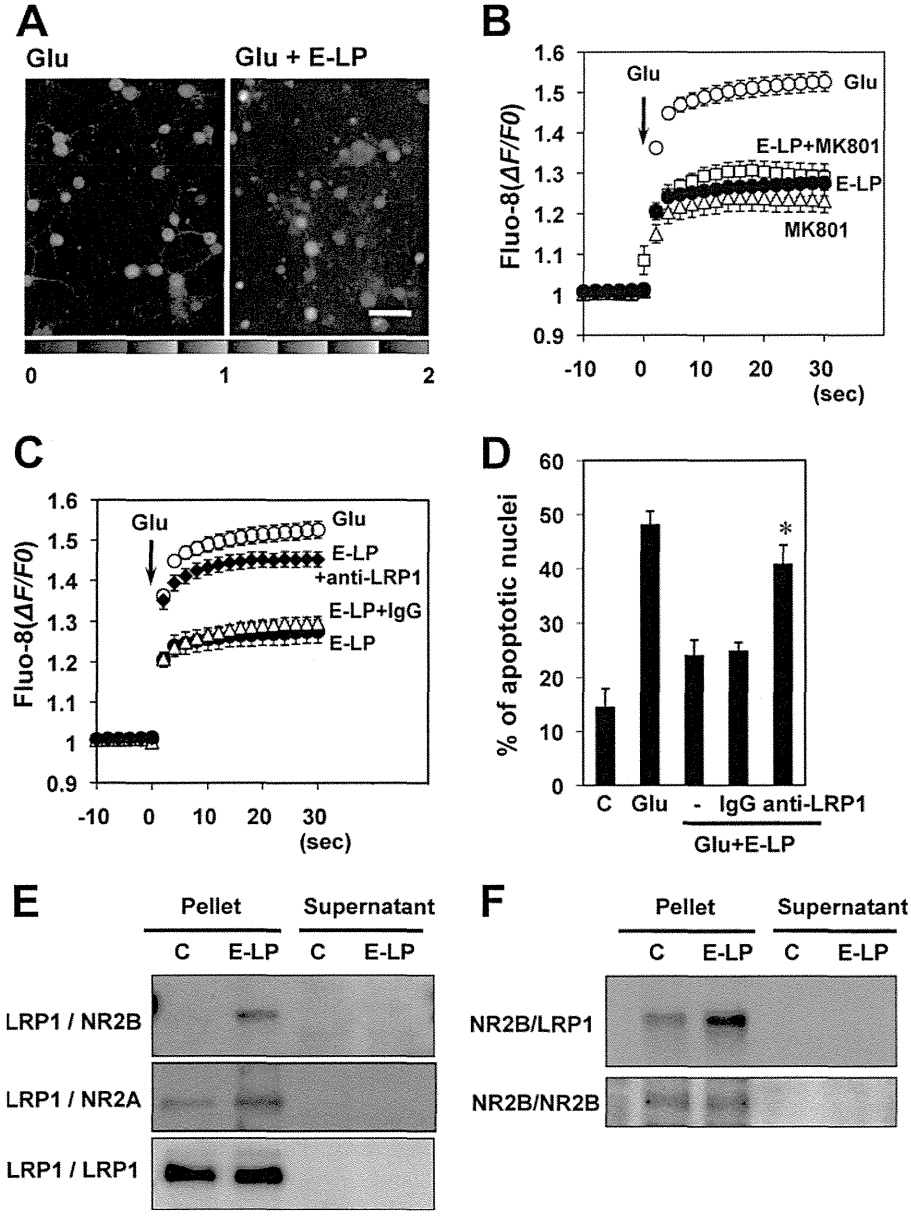

FIGURE 4. **E-LPs inhibit the elevation of intracellular Ca^{2+} by interaction of LRP1 with the NMDA receptor.** *A*, RGCs were labeled with Fluo-8 acetoxymethyl ester for 30 min, and then Glu (300 μM glutamate + 10 μM glycine) was added ± E-LP (100 ng of protein/ml). Fluorescence ratio images are displayed in pseudocolor as indicated by the *color bar* at the *bottom*. Pseudocolor represents changes in fluorescence ratios between 0 (*blue*) and 2 (*red*) corresponding to 1 (*green*), which is defined as the basal fluorescence intensity before Glu stimulation. *Left* and *right panels* show ratio images with Glu and Glu+E-LP, respectively. Data are from one experiment representative of 8 experiments with similar results. *Scale bar*, 80 μm. *B* and *C*, changes in Fluo-8 fluorescence are expressed as $\Delta F/F0$, where *F0* is basal fluorescence intensity before Glu stimulation. RGCs were incubated with 100 ng of protein/ml E-LP, 10 μM MK801, 100 ng of protein/ml E-LP+10 μM MK801, 100 ng of protein/ml E-LP+10 μg/ml anti-LRP1 antibody, or 100 ng of protein/ml E-LP+10 μg/ml IgG for 15 min, and then Glu was added as indicated. Data are means ± S.E. from 6–8 independent experiments. *D*, fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control (*C*; HBSS) or Glu treatment. E-LP (100 ng of protein/ml), E-LP+anti-LRP1 (10 μg/ml), or E-LP+IgG (10 μg/ml) was added to RGCs for 15 min, and then Glu was added. *, $p < 0.01$ for Glu+E-LP *versus* Glu+E-LP+anti-LRP1. Data are means ± S.E. from 4 independent experiments. *E* and *F*, LRP1 (*E*) or NMDA receptor subunit NR2B (*F*) was immunoprecipitated from lysates of RGCs treated ± E-LP for 15 min. Immunoprecipitates (*Pellet*) and supernatant were probed with antibodies raised against LRP1, NR2B, or NR2A. Data are from one experiment representative of 3 experiments with similar results.

3-week-old GLAST-deficient mice and collected retinae at 6 weeks. Immunoblotting of Brn-3a, a marker of RGCs, indicated that in 6-week-old $Glast^{-/-}$ mice, RGC loss in retinae was prevented by E-LP treatment (Fig. 7*A*). In addition, the phosphorylation of GSK3β was dramatically lower in retinae of 6-week-old, when compared with 3-week-old, $Glast^{-/-}$ mice, whereas phosphorylation of GSK3β was maintained by E-LP treatment (Fig. 7*B*). Histological studies demonstrated that the number of RGCs in the ganglion cell layer of retinae of 6-week-old $Glast^{+/-}$ and $Glast^{-/-}$ mice treated with PBS was lower than in RGCs from 3-week-old $Glast^{+/-}$ and $Glast^{-/-}$ mice, respectively. In contrast, in 6-week-old $Glast^{-/-}$ mice, RGCs were retained in retinae treated with E-LPs or HDL (Fig. 7*C*). Quantitative analysis reveals that when compared with PBS treatment, administration of E-LPs or HDL partially protected RGCs from degeneration in $Glast^{+/-}$ and $Glast^{-/-}$ mice (Fig. 7*D*).

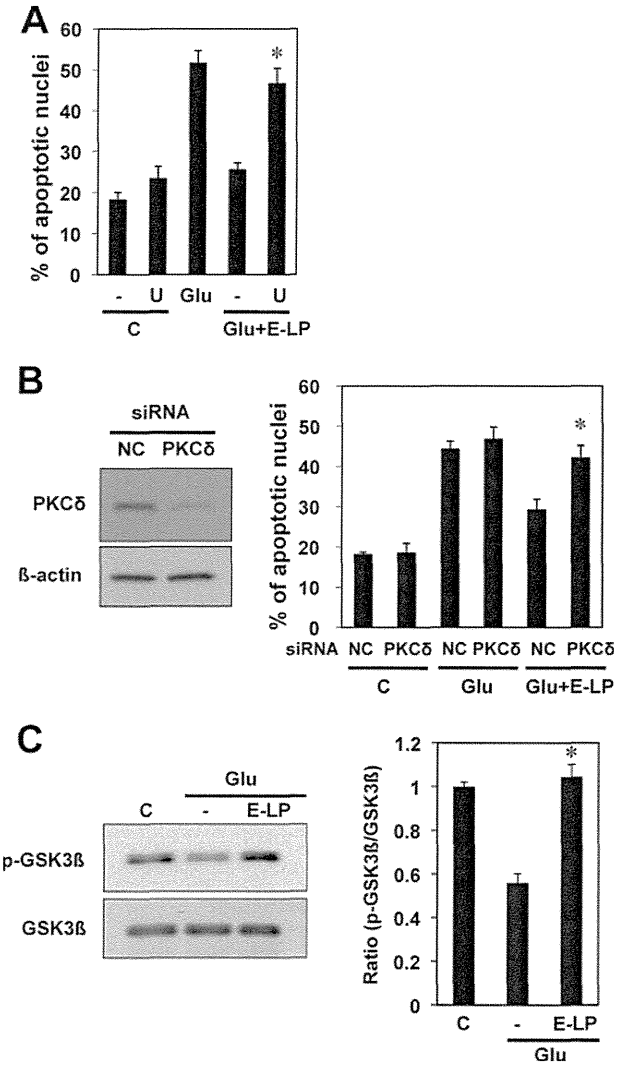

FIGURE 5. **Phospholipase C, protein kinase Cδ, and GSK3β contribute to the protective effect of E-LP against glutamate neurotoxicity.** *A*, fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control (*C*; HBSS) or glutamate (*Glu*; 300 μM glutamate + 10 μM glycine) treatment. RGCs were incubated with 100 ng of protein/ml E-LP or E-LP+U (*U*, 5 μM U73122, phospholipase C inhibitor) for 15 min, and then Glu was added. Data are means ± S.E. from 5 independent experiments. *, $p < 0.005$ for Glu+E-LP *versus* Glu+E-LP+U. *B*, knockdown of protein kinase Cδ (PKCδ) in RGCs was induced by PKCδ siRNA. RGCs were incubated with 300 nM negative control (*NC*) or PKCδ siRNA for 6 days, and then PKCδ was detected by immunoblotting. β-Actin was used as loading control. Fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control, Glu, or Glu+E-LP treatment with knockdown by negative control or PKCδ siRNA. Data are means ± S.E. from 5 independent experiments. *, $p < 0.05$ for Glu+E-LP+NC *versus* Glu+E-LP+PKCδ. *C*, RGCs were incubated for 16 h after control, Glu, or Glu+E-LP treatment. RGC proteins were immunoblotted with antibodies raised against GSK3β phosphorylated at Ser-9 (*p-GSK3β*) or total GSK3β (*GSK3β*). Quantification of Ser9 phosphorylation of GSK3β is shown from 4 independent experiments. *, $p < 0.001$ for Glu *versus* Glu+E-LP.

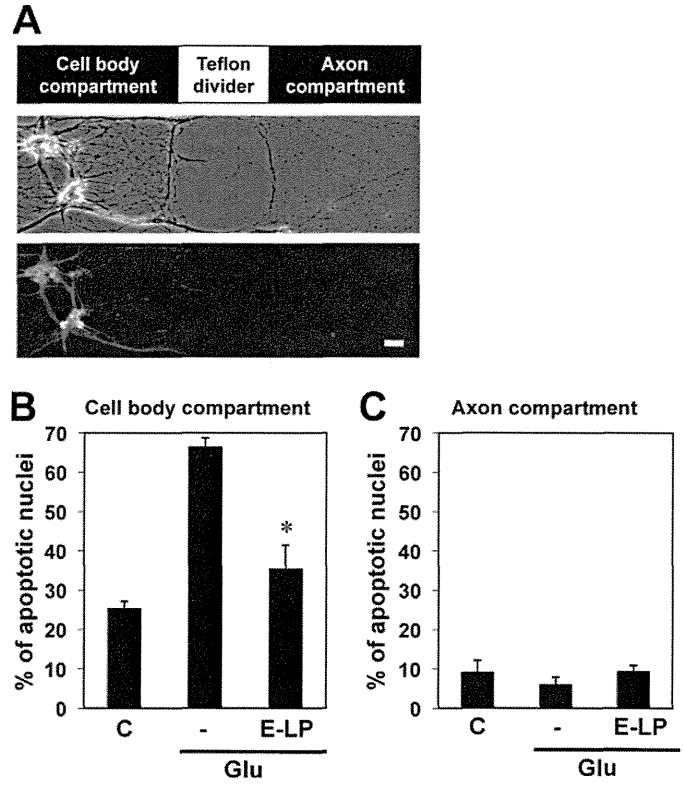

FIGURE 6. **Apoptosis is induced by glutamate in cell body, but not distal axon, compartments.** *A*, *upper panel*, phase contrast image of RGCs in one track shows that cell bodies are localized to the cell body compartment and that distal axons are present in the right-hand axon compartment. *Bottom panel*, RGCs were stained with anti-LRP1 (*green*) and anti-NR2B (*red*) antibodies. *Scale bar*, 50 μm. *B* and *C*, fragmented or shrunken nuclei were detected by Hoechst staining 24 h after control (*C*; HBSS) or glutamate (*Glu*; 300 μM glutamate + 10 μM glycine) treatment of cell bodies (*B*) or distal axons (*C*). Cell bodies and axons of RGCs were incubated with 100 ng of protein/ml E-LP for 15 min, and then Glu was added to cell bodies (*B*) and distal axons (*C*). *, $p <$ 0.005 for Glu *versus* Glu+E-LP. *B* and *C*, data are means ± S.E. from 4 independent experiments.

α2-Macroglobulin Inhibits the Protective Effect of E-LP—Because the endogenous expression of apoE in the central nervous system is increased in response to nerve injury (10, 27), we compared apoE levels in retinae and vitreous humor of $Glast^{+/+}$ and $Glast^{-/-}$ mice. The amount of apoE in the retina and vitreous humor of 3-week-old, but not 6-week-old, $Glast^{-/-}$ mice was higher than in $Glast^{+/+}$ mice (Fig. 8, *A* and *B*), indicating that RGCs were injured in 3-week-old $Glast^{-/-}$ mice. In contrast, levels of LRP1 were not significantly different between $Glast^{+/+}$ and $Glast^{-/-}$ mice at 3 and 6 weeks of age (Fig. 8*A*). Furthermore, α2-macroglobulin, another LRP1 ligand, in vitreous humor of 6-week-old $Glast^{-/-}$ mice was markedly higher than in $Glast^{+/+}$ mice (Fig. 8*B*). Although α2-macroglobulin itself did not affect RGC survival, α2-macroglobulin significantly attenuated the protective effect of E-LPs against glutamate neurotoxicity *in vitro* (Fig. 8*C*). However, this inhibitory effect of α2-macroglobulin against neuroprotection by E-LPs was overcome by a high concentration of E-LPs (Fig. 8*D*).

DISCUSSION

The present study shows that E-LPs might provide a potential therapeutic strategy for NTG. We have demonstrated that glutamate-induced apoptosis of cultured RGCs was attenuated by E-LPs and that E-LPs blocked Ca^{2+}-induced neurotoxicity through the NMDA receptor by promoting formation of an LRP1-NMDA receptor complex. In addition, E-LPs inactivated GSK3β *in vitro* and *in vivo*. Furthermore, intravitreal administration of E-LPs partially protected RGCs from neurodegeneration in GLAST-deficient mice.

The NMDA receptor is a specific type of ionotropic glutamate receptor and is one of the major components associated

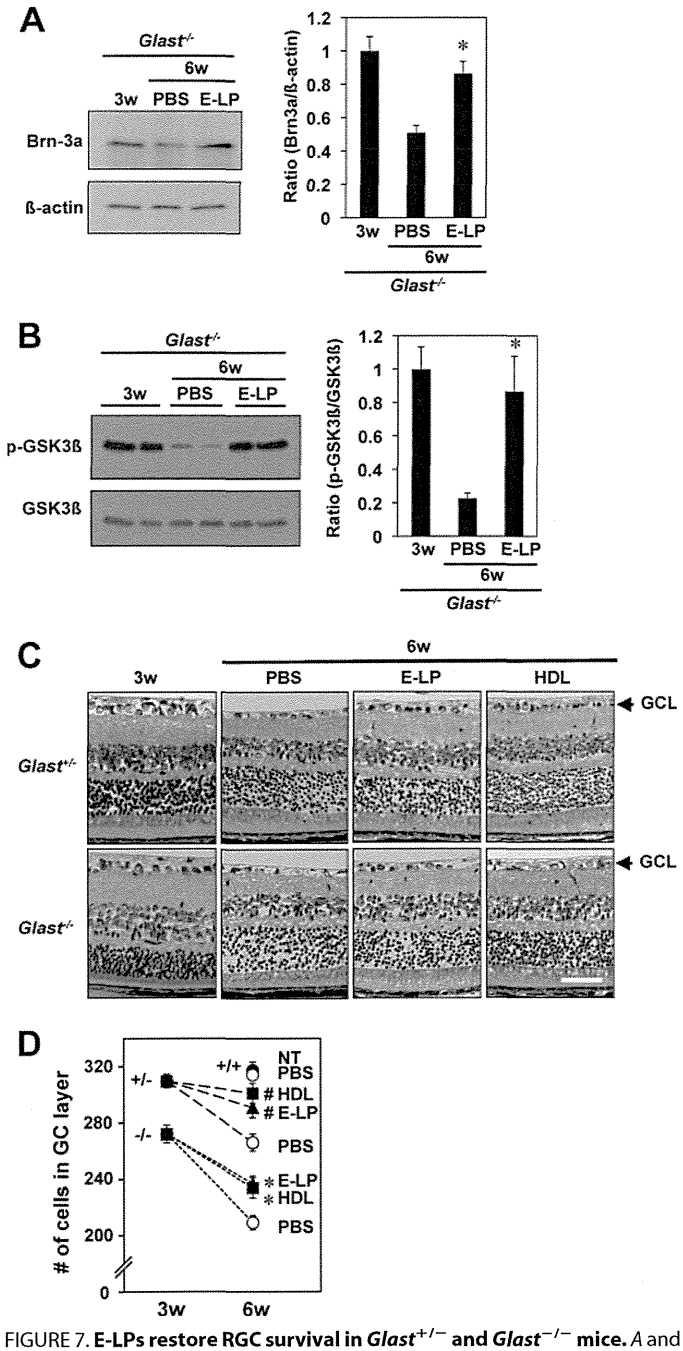

FIGURE 7. **E-LPs restore RGC survival in *Glast*$^{+/-}$ and *Glast*$^{-/-}$ mice.** *A* and *B*, retinae from 3-week-old (*3w*) and 6-week-old (*6w*) *Glast*$^{-/-}$ mice injected with 1 μl of PBS or 1 μl of 1.5 μg of protein/ml E-LPs were subjected to immunoblotting with anti-Brn-3a and anti-β-actin antibodies (*A*) or antibodies raised against GSK3β phosphorylated at Ser-9 (*p-GSK3*β) and total GSK3β (*GSK3*β) (*B*). *A* and *B*, quantification of Brn-3a relative to β-actin (*A*) and phosphorylation at Ser-9 of GSK3β relative to total GSK3β (*B*) from 5 and 6 independent experiments, respectively. *, $p < 0.05$ for PBS *versus* E-LP in 6-week-old *Glast*$^{-/-}$ mice. *C*, hematoxylin and eosin staining of a retinal section from *Glast*$^{+/-}$ and *Glast*$^{-/-}$ mice (3 or 6 weeks old) injected with 1 μl of PBS, 1 μl of 1.5 μg of protein/ml E-LPs, or 30 μg of cholesterol/ml HDL. *Arrowhead* indicates ganglion cell layer of retina (*GCL*). *Scale bar* represents 40 μm. Data are from one retinal section representative of 8 retinae with similar results. *D*, quantification of number of RGCs in 6-week-old wild type (+/+), 3- and 6-week-old *Glast*$^{+/-}$ (+/−), and *Glast*$^{-/-}$ (−/−) mice injected without (*NT*: no treatment) or with 1 μl of PBS, 1 μl of 1.5 μg of protein/ml E-LPs, or 1 μl of 30 μg of cholesterol/ml HDL. Data are from 8 independent experiments. *, $p < 0.05$ for PBS *versus* E-LP or HDL in 6-week-old *Glast*$^{-/-}$ mice. #, $p < 0.05$ for PBS *versus* E-LP or HDL in 6-week-old *Glast*$^{+/-}$ mice. *GC layer*, ganglion cell layer.

with Ca^{2+}-dependent neuronal apoptosis (28). Our data show that RGCs are invulnerable to glutamate treatment alone but that glutamate plus the co-activator glycine induce mitochondrion-dependent apoptosis (Fig. 1). These results are consistent with previous findings that the NMDA response is potentiated by glycine (29, 30). Furthermore, Fig. 2 demonstrates that Ca^{2+}, the NMDA receptor, calpain, calcineurin, Bax, and caspase contribute to glutamate-induced apoptosis in RGCs. These observations suggest that upon glutamate-induced Ca^{2+} increase in RGCs mediated by the NMDA receptor, Ca^{2+}-dependent enzymes, such as calpain and calcineurin, are activated. Calcineurin is cleaved by calpain generating a constitutively active calcineurin fragment (31). Activation of calcineurin leads to mitochondrion (cytochrome *c*)-mediated, and caspase-dependent, apoptosis (32) because Bax, a proapoptotic protein of the Bcl-2 family, is translocated to mitochondria, thereby releasing cytochrome *c* from mitochondria (33). Thus, cytochrome *c* release induces neuronal apoptosis by activation of a caspase cascade (32). These findings indicate that Ca^{2+} elevation mediated by the NMDA receptor is a key step in initiating apoptosis in RGCs (Fig. 9).

Glia-derived E-LPs, plasma HDL, and reconstituted E-LPs protected RGCs against glutamate-induced toxicity, whereas lipid-free apoE, lipid alone, or plasma HDL from apoE-deficient mouse did not (Fig. 3, *A* and *C*). It has been reported that cholesterol associated with E-LPs promotes synaptogenesis in RGCs (34). Our study shows that cholesterol alone does not increase RGC survival but enhances the protective effect of apoE associated with phosphatidylcholine, indicating that delivery of cholesterol might indirectly improve neuronal survival. ApoE4 is the strongest known genetic risk factor for development of Alzheimer disease (35). However, the association between human apoE gene polymorphisms and glaucoma in humans is controversial (36–38). We have reported that human apoE4-LPs are less neuroprotective than apoE3-LPs against apoptosis induced by withdrawal of trophic additives (13). However, in the present study, human apoE4-LPs and apoE3-LPs similarly protected RGCs from glutamate-induced neurotoxicity (Fig. 3*D*). Therefore, we speculate that the differential neuroprotective effect of apoE isoforms depends on the underlying cause of neurodegeneration.

We demonstrate for the first time that E-LPs facilitate the formation of an LRP1-NMDA receptor complex, reduce the elevation of intracellular Ca^{2+} caused by glutamate, and protect RGCs from neurodegeneration. Nevertheless, it has been reported that tissue plasminogen activator, a ligand for LRP1, potentiates the NMDA-stimulated Ca^{2+} influx in primary cultured cortical neurons (39). In addition, α2-macroglobulin itself induced Ca^{2+} influx through the NMDA receptor in cortical neurons (18). Moreover, human apoE4, but not apoE3, treatment increased the intracellular Ca^{2+} signals and neurotoxicity induced by NMDA via member(s) of the LDL receptor family in hippocampal neurons (40). Thus, the interaction between LRP1 and the NMDA receptor for intracellular Ca^{2+} responses may be different in different classes of neurons.

We previously demonstrated that the proapoptotic kinase GSK3β is activated upon withdrawal of trophic additives from cultured RGCs and that glia-derived E-LPs inactivate

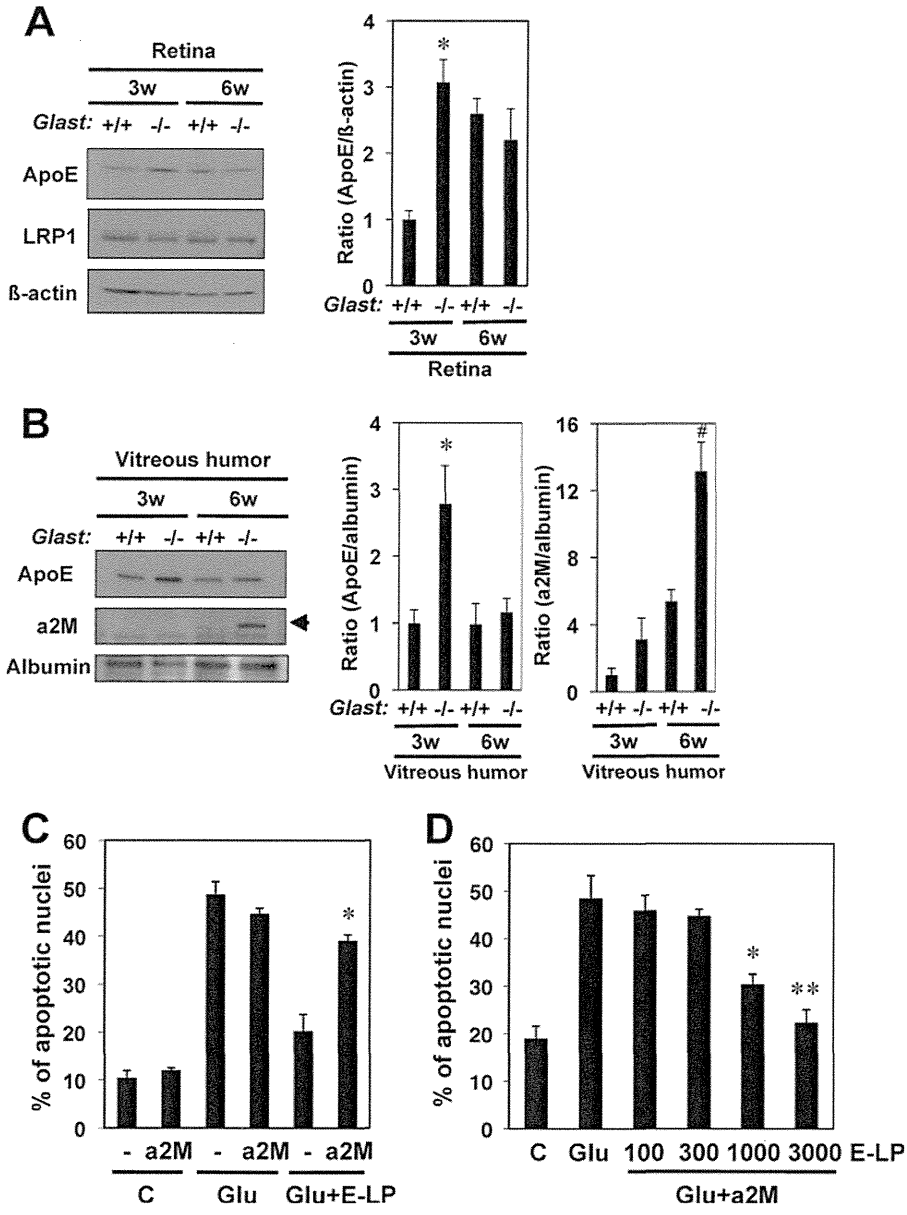

FIGURE 8. **Inhibitory effect of α2-macroglobulin is overcome by E-LPs.** *A* and *B*, retinae and vitreous humor from 3-week-old (*3w*) and 6-week-old (*6w*) *Glast*$^{+/+}$ and *Glast*$^{-/-}$ mice were immunoblotted with antibodies raised against apoE, LRP1, β-actin, α2-macroglobulin (*a2M*),and albumin. *Arrowhead* indicates a2M. *A*, quantification of apoE relative to β-actin from 4 independent experiments. *, $p < 0.05$ for 3-week-old *Glast*$^{+/+}$ *versus* 3-week-old *Glast*$^{-/-}$ retina. *B*, quantification of apoE and a2M relative to albumin from 3 independent experiments. *, $p < 0.05$ for 3-week-old *Glast*$^{+/+}$ *versus* 3-week-old *Glast*$^{-/-}$ vitreous humor. #, $p < 0.05$ for 6-week-old *Glast*$^{+/+}$ *versus* 6-week-old *Glast*$^{-/-}$ vitreous humor. *C*, fragmented or shrunken nuclei in RGCs were detected by Hoechst staining 24 h after control (*C*; HBSS), glutamate (*Glu*; 300 μM glutamate + 10 μM glycine), or Glu + 100 ng of protein/ml E-LP treatment ± 100 nM a2M. *, $p < 0.05$ for Glu + E-LP *versus* Glu + E-LP + a2M. *D*, RGCs were incubated with 100 nM a2M and E-LP (100–3000 ng of protein/ml) for 15 min, and then Glu was added. *C* and *D*, data are means ± S.E. from 4 independent experiments. * and **, $p < 0.05$ and 0.005, respectively, for Glu *versus* Glu + a2M + E-LP.

GSK3β through the LRP1-phospholipase Cγ1-protein kinase Cδ-GSK3β pathway (13, 14). As shown in Figs. 5 and 9, the neuroprotective pathway of E-LPs, involving phospholipase C, protein kinase Cδ, and GSK3β, in RGCs was also activated in response to glutamate-induced neurotoxicity.

Cell bodies of RGCs are localized in the retina, whereas their axons project into the brain. Thus, cell bodies and axons of the same RGC reside in different environments *in vivo*. We demonstrate using compartmented cultures of RGCs that glutamate toxicity and neuroprotection by E-LPs are induced in cell bodies/proximal axons/dendrites of RGCs but not in distal axons (Fig. 6). These results suggest that glutamate neurotoxicity in the retina is important for development of the optic neuropathy *in vivo* and also support the idea that intravitreal administration of E-LPs might protect RGCs from glutamate injury *in vivo*.

Glutamate transporters remove glutamate from extracellular fluid and avoid excess excitation of neurons by glutamate. GLAST is a glutamate transporter expressed in Müller cells of the retina (41). The GLAST-deficient mouse has been used as a model for NTG and exhibits RGC loss without elevation of intraocular pressure (15). As shown in Fig. 7, intravitreal administration of E-LPs or HDL partially protected RGCs from degeneration induced in *Glast*$^{+/-}$ and *Glast*$^{-/-}$ mice. In addi-

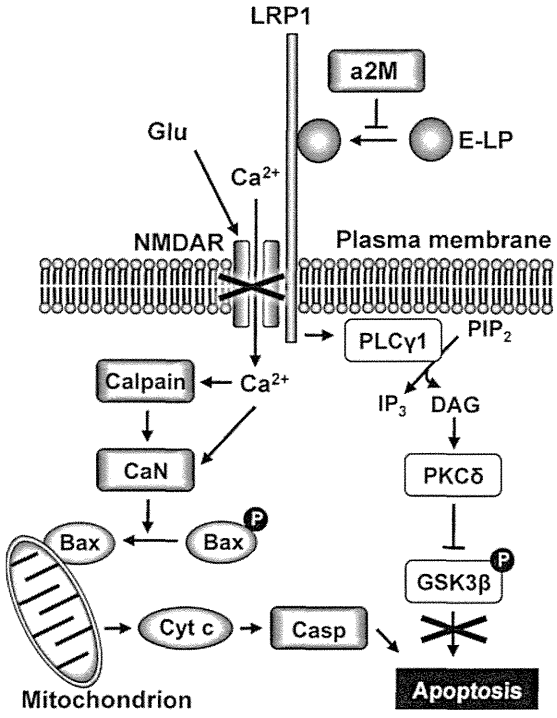

FIGURE 9. **Proposed protective pathway by E-LP against glutamate-induced apoptosis in RGCs.** E-LPs protect RGCs from glutamate-induced apoptosis via an LRP1-mediated signaling pathway. Glutamate (*Glu*) induces the elevation of intracellular Ca^{2+} and activates calpain and calcineurin (*CaN*). Calcineurin dephosphorylates phospho (*P*)-Bax and leads to mitochondrion (cytochrome *c*; *Cyt c*)-mediated and caspase (*Casp*)-dependent apoptosis. When E-LPs bind to LRP1, the formation of an LRP1-NMDA receptor (*NMDAR*) complex is promoted and attenuates the elevation of intracellular Ca^{2+} caused by glutamate. Thus, RGCs are protected from neurodegeneration. In addition, binding of E-LPs to LRP1 activates phospholipase Cγ1 (*PLCγ1*), and PKCδ then increases the phosphorylation (*P*) of the proapoptotic kinase GSK3β, thereby inactivating GSK3β. α2-Macroglobulin (*a2M*), directly or indirectly, interrupts the protective effect of E-LPs. *PIP_2*, phosphatidylinositol 4,5-bisphosphate; *IP_3*, inositol 1,4,5-trisphosphate; *DAG*, diacylglycerol.

tion, GSK3β was inactivated by E-LP treatment in the retina of 6-week-old *Glast*$^{-/-}$ mice. These results demonstrate that intravitreal administration of E-LPs attenuates nerve injury induced in the retina of GLAST-deficient mouse.

Bai *et al.* (42) recently reported that levels of α2-macroglobulin are markedly higher in vitreous humor of human glaucoma patients but not in cataract patients, consistent with our observation that the amount of α2-macroglobulin is higher in vitreous humor of 6-week-old *Glast*$^{-/-}$, than *Glast*$^{+/+}$, mice (Fig. 8*B*). Because α2-macroglobulin is a ligand for LRP1, we tested whether α2-macroglobulin reduced the neuroprotective effect of E-LPs against glutamate toxicity in primary cultures of RGCs. α2-Macroglobulin itself did not induce RGC death but attenuated the protective effect of E-LPs; this attenuation was overcome by the addition of E-LPs (Fig. 8, *C* and *D*), suggesting that the balance between E-LPs and α2-macroglobulin in the eye might be crucial for RGC survival in NTG pathology.

In summary, we demonstrate that E-LPs and HDL protect cultured RGCs from glutamate-induced apoptosis and prevent the progressive loss of RGCs *in vivo* in GLAST-deficient mice. E-LPs not only block intracellular Ca^{2+} elevation but also inactivate the proapoptotic kinase GSK3β *in vitro* and *in vivo*. Furthermore, we speculate that attenuation of the neuroprotective effect of E-LPs by α2-macroglobulin might contribute to NTG *in vivo*. The present study has important implications for treatment of NTG because the cause of this disorder has not been identified and treatment options are very limited. Our findings indicate a potential therapeutic strategy mediated by E-LPs through LRP1 for NTG.

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