(12) STANDARD PATENT (11) Application No. **AU 2009236078 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Methods and compositions for treating age-related macular degeneration

(51) International Patent Classification(s)
C07K 14/47 (2006.01) ***C07K 14/705*** (2006.01)
A61K 31/00 (2006.01)

(21) Application No: **2009236078** (22) Date of Filing: **2009.04.17**

(87) WIPO No: **WO09/129497**

(30) Priority Data

(31) Number	(32) Date	(33) Country
61/124,624	**2008.04.18**	**US**

(43) Publication Date: **2009.10.22**
(44) Accepted Journal Date: **2014.03.06**

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(56) Related Art
WO 1999/043286 A

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 October 2009 (22.10.2009)

PCT

(10) International Publication Number
WO 2009/129497 A3

(51) International Patent Classification:
A61K 31/00 (2006.01)

(21) International Application Number:
PCT/US2009/041021

(22) International Filing Date:
17 April 2009 (17.04.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/124,624 18 April 2008 (18.04.2008) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report:
3 December 2009

WO 2009/129497 A3

(54) Title: METHODS AND COMPOSITIONS FOR TREATING AGE-RELATED MACULAR DEGENERATION

(57) Abstract: The present invention provides methods for treating or limiting development of age-related macular degeneration, as well as methods for identifying compound suitable for such use.

Methods and Compositions for Treating and Identifying Compounds to Treat Age-Related Macular Degeneration

Related Applications

This application claims priority to U.S. Provisional Patent Application Serial No. 61/124,624, filed April 18, 2008, which is incorporated by reference herein in its entirety.

Statement of Government Rights

This invention was made with government support under National Institutes of Health, Grant Number R03 EY014403. The government has certain rights in the invention.

Background

Age-related macular degeneration ("AMD") is an aging-associated disease resulting in the loss of vision in the macula (the center of the visual field) because of damage to the retina. AMD is a prevalent disorder of the aged, with approximately 10% of patients 66 to 74 years and 30% of patients 75 to 85 years of age having some level of macular degeneration. Currently there is no effective treatment available for most patients with AMD, and no early stage intervention.

Summary of the Invention

In one aspect, the present invention provides methods for treating age-related macular degeneration (AMD), comprising administering to a subject with AMD an amount effective for treating AMD of an agonist of the OA1 receptor. In a second aspect, the present invention provides methods for limiting development of AMD, comprising administering to a subject at risk of developing AMD an amount effective for limiting development of AMD of an agonist of the OA1 receptor. In one preferred embodiment of either of these aspects of the invention, the agonist of the OA1 receptor is selected from the group consisting of L-DOPA and L-DOPA analogues.

In another aspect, the present invention provides methods for identifying compounds to treat AMD, comprising contacting cells with a test compound, wherein the cells comprise:

(a) a first cell population expressing OA1; and, optionally,

(b) a second cell population not expressing OA1; and

(c) identifying as positive test compounds those test compounds that increase one or both of

(i) pigment epithelium-derived factor (PEDF) expression in the first cell population relative to one or both (A) PEDF expression in the first population of cells not contacted with the test compound, and (B) the second cell population, and

(ii) intracellular calcium concentration in the first cell population relative to one or both (A) intracellular calcium concentration in the first population of cells not contacted with the test compound, and (B) the second cell population;

wherein the positive test compounds are candidate compounds for treating and/or limiting development of AMD.

In a further aspect, the present invention provides methods for identifying compounds to treat AMD, comprising

(a) administering a test compound to a tyrosinase deficient pregnant female non-human mammal, wherein the test compound is administered during embryonic photoreceptor and/or retinal ganglion development; and

(b) comparing an effect of the test compound on photoreceptor and/or retinal ganglion development in the embryo or post-natal non-human mammal, to photoreceptor and/or retinal ganglion development in an embryo or post-natal non-human mammal not administered the test compound, wherein those test compounds that increase photoreceptor and/or retinal ganglion development are candidate compounds for treating and/or limiting development of AMD.

In a still further aspect, the invention provides compositions comprising:

(a) an amount effective of L-DOPA or an L-DOPA analogue for treating or limiting development of AMD; and

(b) an amount effective for treating or limiting development of AMD of a composition comprising a source of vitamin C, a source of vitamin E, a source of vitamin A, a source of zinc, and a source of copper.

Brief Description of the Figures

Figure 1(a-c) Western blot analysis of proteins bound (B) or unbound (U) to strepavidin-conjugated beads after biotinylation of RPE *in situ*, cultured RPE (b), or COS cells transfected to express OA1-GFP (c). Blots were probed to visualize OA1 and actin after cell surface biotinylation and fractionation using streptavidin-conjugated beads. For cultured cells (b, c) cells were either maintained in 500 μM (normal DMEM) or 1 μM tyrosine for 3 days prior to analysis.

Figure 1(d) Quantification of western blot analysis by densitometry. OA1 densitometry is shown as the % of the control for paired cell cultures, transfected then split into 2 equal groups, one of which was the control, maintained in normal DMEM (control). The other group was maintained in 1 μM tyrosine DMEM (LT) until harvest. Paired t-test analysis was used to test whether the difference was significant, and * denotes $p<0.001$. Actin, analyzed the same way showed no differences, and $p=0.724$.

Figure 1(e-f) Composite confocal microscopy of pigmenting RPE cells maintained in normal DMEM (e) or 1 μM tyrosine (f) then stained with anti-OA1 antibodies and imaged at 20x. Bar = 25 μm.

Figure 2(a) Representative traces of $[Ca^{2+}]i$ during the time course of the standard experimental protocol in transfected and untransfected CHO cells. After establishment of a stable baseline for 3 minutes, the test agent was added at 1 μM. At 5 minutes, KCl was added to serve as a control that the cells were Fura-2 loaded and patent. Identical protocols were performed for both transfected cells and paired untransfected cells.

Figure 2(b) Summary data for $[Ca^{2+}]i$ in response to tyrosine, dopamine, and L-DOPA in transfected and untransfected CHO cells. Untransfected cells are shown with L-DOPA treatment. The experimental control of membrane depolarization with KCl is also shown. Each bar represents data collected from at least 10 experiments and is presented as the mean change from baseline $[Ca^{2+}]i$ after test agent addition. Error bars represent S.D., and t-test analyses were used to test for significant differences, * denotes $p<0.01$.

Analysis of pertussis toxin sensitivity of $[Ca^{2+}]i$ increase in cells transfected to express OA1 or RPE that express the natural protein. Data represent mean of at least 6 experiments.

Figure 2(c) Analysis of pertussis toxin sensitivity of $[Ca^{2+}]i$ increase in cells transfected to express OA1 or RPE that express the natural protein. Data represent mean of at least 6 experiments for each group of transfected cells and 20 individual experiments for each the treated and untreated RPE with endogenous OA1 expression. T-tests analyses were used to test for significant differences, and * denotes $p<0.01$.

Figure 2 (d) cAMP was measured in CHO transfected to express OA1. The control group represents transfected but untreated CHO cells and the basal level of cAMP in those cells. Cells were treated with 1.0 μM L-DOPA, 0.1 μM forskolin, L-DOPA + 0.1 μM forskolin, and as a positive control 1 μM forskolin. Results represent the mean cAMP levels observed in at least 6 experiments in which all experimental groups were analyzed in a paired fashion using replicate monolayers in the same culture plate. Error bars represent the S.D. of each group, and the only significant difference observed was the increase in cAMP levels after forskolin treatment.

Figure 3(a) Binding kinetics between OA1 and L-DOPA were determined using radiolabeled ligand binding assays. Results represent data collected from 5 such experiments and are presented as mean specific binding +/- SEM. The hyperbolic curve fit exhibited an R^2 value of 0.994, Kd was determined to be $9.34x10^{-6}M$ +/ $1.14x10^{-6}M$.

Figure 3(b) Comparative binding of 5μM $[H^3]$ L-DOPA to OA1 transfected CHO cells was compared in the presence of 1.0 mM dopamine, tyrosine, or L-DOPA. The data represent mean total binding +/- S.D. for each group. * denotes $p<0.05$ when comparing the results between the control group to the binding in the presence of the potential competitive ligands.

Figure 3(c) Competitive interaction between 5μM $[H^3]$ L-DOPA and dopamine were assessed to determine whether dopamine functions as an antagonist of OA1 activity. Results indicate that dopamine and L-DOPA compete for the same OA1 binding site, and the data fits the binding model with an r^2 value of 0.95. The Ki for dopamine was 2.388 +/- 0.266 μM (mean +/- SEM), similar to the Kd for L-DOPA.

Figure 3(d) Dose-dependent OA1 signaling through OA1. Data represent mean increase in $[Ca^{2+}]_i$ elicited by L-DOPA treatment of the cells at the concentrations given (n=6 for each dose). T-test analyzes were used to compare between the responses achieved at each dose, and * denotes $p<0.01$ for the comparison at 1 and 10 μM.

Figure 3(e) Scatchard plot illustrating the kinetics of a single site binding relationship based on Figure 3(a).

Figure 4(a-h) All images represent 2 μm thick confocal sections of CHO cells transfected to express OA1-GFP. β-arrestin was visualized using immuno fluorescence methods. Prior to addition of L-DOPA (a-c) and after treatment with 1 μM L-DOPA (d-f), and the merged images (c, f) illustrate regions where the two proteins co-localize, at the resolution of white light imaging. (g,h) are low magnification of field of transfected CHO cells, with two transfected cells visible (arrows) (g). The remainder of the cell population is visualized using antibodies to β-arrestin (h) to illustrate that β-arrestin recruitment to the membrane only occurred in the OA1 expressing cells (arrows).

Figure 5 (a) PEDF concentrations were determined by ELISA of cell conditioned medium. RPE cells were control cells, without L-DOPA treatment, or OA1 stimulated cells that were treated with 1 μM L-DOPA prior to being maintained for 3 days in normal DMEM. Data are presented as the mean of 3 experiments conducted in triplicate, error bars represent S.D, and * denotes $P<0.01$ using a paired t-test.

Figure 5(b) PEDF concentrations in conditioned medium from pigmenting RPE determined by ELISA. Cells were either control pigmenting RPE cultures or paired cultures treated with phenylthiourea (PTU) at 200 μM. Data are presented as the mean of 3 experiments conducted in triplicate, error bars represent S.D, and * denotes $P<0.01$ using a paired t-test.

Figure 5(c) PEDF concentrations in conditioned medium of pigmented RPE cells treated with PTU then treated with L-DOPA to stimulate OA1 signaling. ELISA assays were conducted prior to PTU treatment, then after PTU treatment, and then from the same cultures after L-DOPA stimulation. Results are presented as mean +/- S.D. of the value achieved related to that culture of cells. * denotes $p<0.01$ when comparing PTU to the control (same culture tested prior to PTU), and L-DOPA/PTU compared to the PTU sample from that same culture.

Figure 6(a) Data represents mean +/- SEM bound [3H]-L-DOPA in all fractions, total, specific and non-specific. Non-specific binding was determined by measuring radiolabeled-L-DOPA bound in the presence of excess unlabeled L-DOPA (1 mM). Specific binding at each given concentration is determined by subtracting the measured non-specific binding from the measured total binding.

Figure 6(b) The figure illustrates competitive interaction between tyrosine and L-DOPA, measured using increasing concentrations of tyrosine and 5μM [H^3] L-DOPA. Each data point represents the mean data from 5 replicate wells, and the error bars are S.D. Data illustrate that tyrosine competes for binding with L-DOPA, but with a low affinity. The results suggest tyrosine has a Ki of 52.9 μM, and fits the single site binding model with an r^2 value of 0.85. Saturation could not be achieved because of the limited solubility of tyrosine.

Figure 7 Western blot and graphical representation of PEDF secretion in wild-type vs OA deficient mice.

Figure 8(a) is a graphical representation of data demonstrating that L-DOPA supplementation increases retinal ganglion cell numbers compared to what is expected in a normal wild-type mouse.

Figure 8(b) is a graphical representation of data demonstrating that L-DOPA supplementation increases photoreceptor numbers compared to what is expected in a normal wild-type mouse.

Figure 8(c) is a Western blot showing PEDF detection in 2 wild-type and 2 OA1 -/y mice.

Detailed Description of the Invention

All references cited are herein incorporated by reference in their entirety.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

In a first aspect, the present invention provides methods for treating age-related macular degeneration (AMD), comprising administering to a subject with AMD an amount effective for treating AMD of an agonist of the OA1 receptor. \

In a second aspect, the present invention provides methods for limiting development of AMD, comprising administering to a subject at risk of developing AMD an amount effective for limiting development of AMD of an agonist of the OA1 receptor.

The human *Oa1* gene, is found on the X chromosome, and has been shown to encode a 404 amino acid protein OA1 (**SEQ ID NO:2**), likely to be a G-protein coupled receptor (GPCR) [12,13] based upon sequence analysis [14]. As disclosed in detail herein, the inventors have identified the OAl signaling pathway as a critical determinant of neurosensory retina survival, such that stimulation of this pathway will provide treatment for AMD as well as a means to limit AMD development for those at potential risk. While not being bound by any mechanism, the inventors believe that OA1 and tyrosinase participate in an autocrine loop through L-DOPA that regulates the secretion of at least one potent neurotrophic factor, PEDF. Thus administration of L-DOPA can be used to stimulate OA1 activity and thus upregulate PEDF expression, making it a valuable therapeutic to treat and limit development of AMD.

As discussed in detail below, such OA1 agonists can be identified, for example, using the drug discovery methods of the third and fourth aspects of the invention. Exemplary OA1 agonists are discussed in detail below.

The subject preferably is a human.

As used herein for all aspects and embodiments of the invention, "AMD" means an aging-associated disease resulting in the loss of vision in the macula (the center of the visual field) because of damage to the retina know as Age-related Macular Degeneration. As used herein, AMD encompasses both wet and dry AMD, described in more detail below.

AMD begins with characteristic drusen (yellow deposits) in the macula between the retinal pigment epithelium and the underlying choroid. Most people with these early changes (referred to as age-related maculopathy) have good vision. People with drusen can go on to develop advanced AMD. The risk is considerably higher when the drusen are large and numerous and associated with disturbance in the pigmented cell layer under the macula.

Subjects with age-related maculopathy may progress to either of the two main forms of advanced AMD, each of which can be treated or be limited in its development using the methods of the invention. 'Wet" AMD causes vision loss due to abnormal blood vessel growth in the choriocapillaries, through Bruch's membrane, ultimately leading to blood and protein leakage below the macula. Bleeding, leaking, and scarring from these blood vessels eventually causes irreversible damage to the photoreceptors and rapid vision loss if left untreated. "Dry" AMD occurs when light-sensitive cells in the macula slowly break down, gradually causing vision loss in the affected eye. Blurring in AMD is probably due to the accumulation of drusen under the retinal pigment epithelium (RPE) which alters to focal properties of the photoreceptors by moving them out of the plane of focus.

Dry AMD may occur in one or both eyes, and can advance from age-related maculopathy into intermediate or advanced stages of dry AMD.

Intermediate Dry AMD: Either many medium-sized drusen or one or more large drusen. Some people see a blurred spot in the center of their vision. More light may be needed for reading and other tasks.

Advanced Dry AMD: In addition to drusen, a breakdown of light-sensitive cells and supporting tissue in the central retinal area. This breakdown can cause a blurred spot in the center of vision. Over time, the blurred spot may get bigger and darker, taking more of the central vision; may have difficulty reading or recognizing faces until they are very close to you.

AMD symptoms include, but are not limited to blurred/reduced central vision, central scotomas (shadows or missing areas of vision), trouble discerning one dark color from another dark color and/or one light color from another light color; slow recovery of visual function after exposure to bright light, a loss in contrast sensitivity, so that contours, shadows and color vision are less vivid, retinal pigment epithelial (RPE) disturbance (including pigment clumping and/or dropout), RPE detachment, geographic atrophy, subretinal neovascularization, and disciform scar, and distorted vision (metamorphopsia), such that a grid of straight lines appears wavy and parts of the grid may appear blank Symptoms of dry AMD and wet AMD are generally similar early during disease progression, and thus it may not be possible to determine which early-stage patients will develop dry vs. wet forms of AMD. Dry AMD develops as

'geographic atrophy', and early AMD become 'wet' AMD when new blood vessels sprout.

As used herein, "treat" or "treating" AMD means accomplishing one or more of the following: (a) reducing the severity of AMD; (b) limiting or preventing development of one or more symptoms characteristic of AMD, as described above; (c) inhibiting worsening of one or more symptoms characteristic of AMD, as described above; (d) limiting or preventing recurrence of AMD in patients that have previously had the disorder(s); and (e) limiting or preventing recurrence of one or more symptoms in patients that were previously symptomatic for AMD. Such treating includes treating of wet AMD and dry AMD.

As used herein, the term "limiting development of" AMD means to prevent or to minimize development of AMD in individuals at risk of developing AMD, as well as limiting progression of age-related maculopathy to AMD (wet or dry), or intermediate dry AMD to advanced dry or 'wet' AMD. In one preferred embodiment, the methods comprise treating a subject with drusen accumulation (ie: age-related maculopathy), to limit development of AMD. In another preferred embodiment, the methods comprise treating a subject with an amount effective of the OA1 agonist to decrease the rate of lines of loss of vision relative to a non-treated AMD subject, or subject at risk of AMD. In another preferred embodiment, the methods comprise treating a subject with wet AMD, or at risk of developing wet AMD, an amount effective of the OA1 agonist to decrease the rate and number of new blood vessel formation. As discussed in more detail below, OA1 stimulation causes the RPE to increase PEDF secretion, and PEDF is a potent anti-angiogenic factor. Thus, OA1 stimulation strategies may stop new blood vessel development in 'wet' AMD, in addition to its effects on retinal development discussed herein.

In another preferred embodiment, the methods comprise treating a subject that has blurred or reduced central vision with an amount of OA1 agonist effective to increase the lines of visual acuity in one or both eyes. In this embodiment, the lines of visual acuity are as measured by the standard Snellen test, where the increase or decrease in 'lines' of visual acuity are based on which smallest 'line' on a Snellen chart a patient can read clearly.

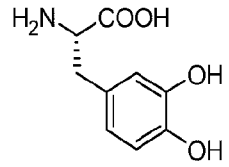
"Subjects at risk of developing AMD" mean anyone with any risk factor for development of AMD, including but not limited to being over 50 years old (in various preferred embodiments, over 60 years old, over 65 years old, over 70 years old, or over 75 years old), presence of drusen deposits, Caucasian race, having a blood relative that has or had AMD, a mutation in the complement factor H gene (CFH) of (Tyr402His), Arg80Gly variant of the complement protein C3 gene, hypertension, high cholesterol

levels, obesity, smoking, a high fat intake, and mutations in the fibulin 5 gene. Thus, in a preferred embodiment, the subject to be treated has one or more of these risk factors, particularly in methods for limiting development of AMD.

The phrase "therapeutically effective amount," as used herein, refers to an amount that is sufficient or effective to limit development of or treat (prevent the progression of or reverse) AMD. The appropriate dosage range depends on the choice of the compound, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

In a preferred embodiment, the OA1 receptor agonist comprises a compound selected from the group consisting of L-DOPA and L-DOPA analogues.

L-DOPA is [2-amino-3-(3,4-dihydroxyphenyl)propanoic acid] known for use in treating Parkinson's, and has the following structure.

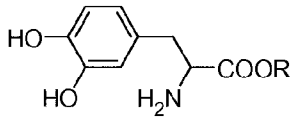


L-DOPA is commercially available and methods for its synthesis are known to those of skill in the art.

As used herein, "L-DOPA analogues" are those L-DOPA variants that retain OA1-stimulatory activity, including L-DOPA prodrugs, of which many are known in the art; exemplary such analogues are disclosed below. While not being bound by a specific mechanism of action, the inventor believes that L-DOPA binding to OA1 involves two sites of binding, one involving one or both hydroxyl groups, and one involving the carboxylic acid group. In one embodiment, the L-DOPA analogues are L-DOPA prodrugs that are metabolized to L-DOPA after administration (and generally prior to binding to OA1 on the cell surface), and thus are expected to retain OA1-stimulatory activity. In another embodiment, one or both hydroxyl group and/or the carboxyl group

can be substituted to produce various analogues (prodrug or otherwise) for use in the methods of the invention.

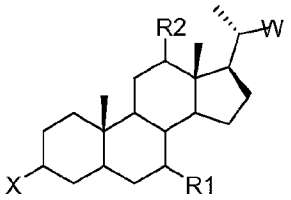
In another embodiment, the L-DOPA analogues comprise L-DOPA esters Exemplary L-DOPA esters, and methods for preparing them, are disclosed in WO/1997/016181; US 4663349; US 4873263; US 4873263; US 5345885, and US 4771073. In various preferred embodiments, the L-DOPA ester is selected from the group consisting of L-DOPA methyl ester, L-DOPA butyl ester, L-DOPA pentyl ester, L-DOPA cyclohexyl ester, L-DOPA benzyl ester, and L-DOPA ethyl ester. In various further preferred embodiments, the L-DOPA esters are selected from the alkyl, aryl and substituted and unsubstituted aralkyl esters of L-DOPA. In a further preferred embodiment, the L-DOPA esters are represented by the following formula:



wherein R is a straight or branched chain alkyl (C_1 –C_{20}) such as methyl, ethyl, propyl, butyl, myristyl, palmityl, pentyl, tetradecyl, hexadecyl and the like; aryl(C_6 –C_9) such as phenyl, tolyl and the like; substituted and unsubstituted mono, di or polyhydroxyalkyl(C_1 –C_{20}) such as benzyl, alkoxybenzyl, 4-hydroxybutyl, 2-hydroxypropyl, 2,3-dihydroxypropyl, 1,3-dihydroxypropyl, 6-hydroxyhexyl and 5-hydroxypentyl and the like optionally having a substituent such as alkoxy(C_{1-5}) [methoxy, ethoxy, butoxy and the like]; carbalkoxy (C_{1-5}) [methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl butoxycarbonyl and the like]; amino; mono or dialkylamino(C_{1-10}) [methylamino, methylethylamino, diethylamino and the like]; acylamino(C_{1-5}) [acetamido, butyramido and the like]; ketoalkyl (C_{1-5}) [methylketo, ethylketo, butylketo and the like]; halo [chloro, bromo and the like] or carboxamide; substituted and unsubstituted aralkyl(C_{7-20}) such as benzyl, alkoxybenzyl C_{8-14}) [methoxy, ethoxy, isobutoxy and the like]; phenylethyl; phenylpropyl; phenylbutyl; phenylhexyl; phenyloctyl and the like; and pharmaceutically acceptable organic or inorganic counterion salts.

Synthetic processes for preparing the esters of L-DOPA and the salts thereof are known in the art, for example, in U.S. Pat. Nos. 3,891,696; 4,035,507; and 5,354,885; and Journal of Pharmaceutical Sciences, 62, p. 510 (1973), each incorporated by reference herein in their entirety.

In another embodiment, the L-DOPA analogues comprise bile acid conjugates as are known in the art. Exemplary L-DOPA bile acid conjugates, and methods for preparing them, are disclosed in WO/2002/028882 and US20020151526. Upon oral administration, these prodrugs are cleaved within the enterohepatic system to release the parent drug and/or an active metabolite from the bile acid into the systemic circulation. Significantly, only a fraction (typically<50%) <50%) of the prodrug is cleaved during each pass through the enterohepatic cycle. Thus, the enterohepatic circulation serves as a reservoir of the drug enabling sustained systemic drug levels to be achieved. Naturally occurring bile acids such as cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, ursocholic acid and lithocholic acid are particularly preferred. The site of conjugation of these bile acids to L-DOPA or other L-DOPA analogue is preferably via the 3-hydroxy group or the C-24 carboxyl moiety. Optionally, cleavable linker functionality may be introduced between the drug and the bile acid and this linker may be selected. In a preferred embodiment, such L-DOPA bile acid conjugates are represented by the following formula



wherein R1 is selected from the group consisting of hydrogen and OH;
R2 is selected from the group consisting of hydrogen and OH;
X is selected from the group consisting of OH and D-Y-, where Y is selected from the group consisting of a covalent bond and a cleavable linker group covalently connecting D to the steroid;
D is a member selected from the group consisting of L-DOPA and other L-DOPA analogues;
W is selected from the group consisting of (a) a substituted alkyl group containing a moiety which is negatively charged at physiological pH, which moiety is selected from the group consisting of—COOH, --SO_3H, --SO_2H, --P(O)(OR6)(OH),

--OP(O)(OR6)(OH), --OSO_3H and the like and pharmaceutically acceptable salts thereof, where R6 is selected from the group consisting of alkyl, substituted alkyl, aryl and substituted aryl; and (b) a group of the formula -M-Y'-D'

where M is selected from the group consisting of—$CH_2OC(O)$-- and—$CH_2CH_2C(O)$--;

Y' is a covalent bond or a cleavable linker group covalently connecting D' to M;

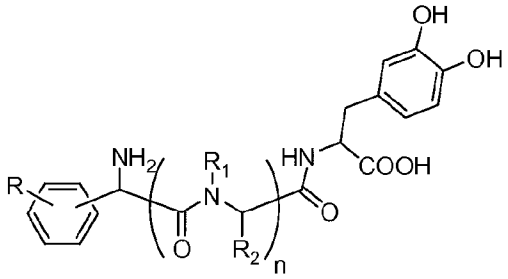
D' is a member selected from the group consisting of L-DOPA and other L-DOPA analogues;

with the proviso that either X is -Y-D and/or W is -M-Y'-D'

wherein the compound of formula (I) above is a substrate for an intestinal bile acid transporter;

or a pharmaceutically acceptable salt thereof.

In another embodiment, the L-DOPA analogues comprise di or tri-peptide derivatives. Exemplary L-DOPA di- or tri-peptide analogues, and methods for preparing them, are disclosed in US 3803120 and US5686423. Oral absorption of the di- and tri-peptide L-DOPA prodrugs show high oral bioavailability with some compounds having the plasma concentration 60-100 fold higher than that of L-dopa. In a preferred embodiment, such L-DOPA prodrugs are represented by the following formula



wherein n is 0 or 1; R is hydrogen or hydroxyl, preferably R is hydroxyl;

R1 is hydrogen; and

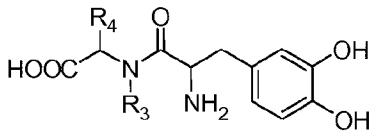
R2 is hydrogen, alkyl of from one to four carbon atoms, alkyl of from one to four carbon atoms substituted with one --OH, --SH, --SCH_3, --NH_2, --$NHC(=NH)NH_2$, --COOH, phenyl, hydroxyphenyl, indolyl or imidazolyl group, alkyl from one to four carbon atoms substituted with one carboalkoxyl group of from one to six carbon atoms, preferably R2 is

hydrogen, methyl or hydroxymethyl; or

R1 and R2 together are trimethylene.

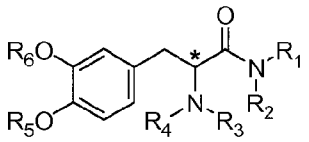
Preferably, R1 and R2 of the di- or tri-peptide derivative of L-DOPA (2-amino-3-(3,4-dihydroxyphenyl-)propanoic acid) of the formula (I) together is trimethylene.

In another embodiment, di-peptide derivatives of L-DOPA [2-amino-3-(3,4-dihydroxyphenyl)propanoic acid] are represented by the following formula



wherein R3 is hydrogen; and R4 is phenyl or hydroxyphenyl; or R3 and R4 together is trimethylene.

In another embodiment, the L-DOPA analogues comprise amine prodrugs as are known in the art. Exemplary L-DOPA amine analogues, and methods for preparing them, are disclosed in US20060025385 and WO/2004/069146. In one preferred embodiment, such L-DOPA amine analogues are represented by



wherein *C denotes a chiral carbon;

R1, R2, R3 and R4 are each independently selected from the group consisting of hydrogen, alkyl having 1-30 carbon atoms, alkenyl having 1-30 carbon atoms, alkynyl having 1-30 carbon atoms, cycloalkyl, aryl, O-carboxy, C-carboxy, carbonyl, thiocarbonyl, O-carbamyl, O-thiocarbamyl and a fatty acid acyl, or, alternatively, R1 and R2 and/or R3 and R4 form a five- or six-membered ring; and

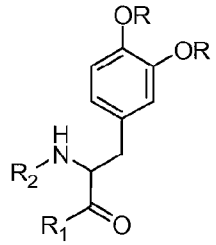
R5 and R6 are each independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl and phosphonyl,

or a pharmaceutically acceptable salt thereof.

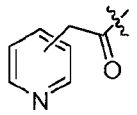
Preferred L-DOPA amine analogues include: compounds wherein R5 and R6 are each hydrogen; compounds wherein R1 and R2 are each hydrogen; compounds wherein

R3 and R4 are each hydrogen; compounds wherein at least one of R1, R2, R3 and R4, preferably R3 and/or R4 is carbonyl, e.g., acetyl. Additional preferred compounds according to the present embodiments include compounds wherein at least one of R1, R2, R3 and R4 is an alkyl, alkenyl or alkynyl having 1-30 carbon atoms, or, alternatively, at least one of R1, R2, R3 and R4 is a fatty acid acyl, derived from, for example, myristic acid, lauric acid, palmitic acid, stearic acid, oleic acid, arachidonic acid, linoleic acid or linolenic acid. Further preferred examples of L-DOPA amine analogues according to the present embodiments include α-amino-3,4-dihydroxy-benzenepropanamide, α-N-acetyl-3,4-dihydroxy-benzenepropanamide and pharmaceutically acceptable salts thereof.

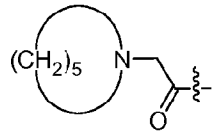
In a further preferred embodiment, L-DOPA prodrugs for use in the present invention, and methods for their synthesis, are disclosed in US 4065566 and 4035507 and are represented by the formula



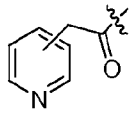
wherein each R is independently selected from the group consisting of a hydrogen atom, an acyl group, a



group, a --CO-pyridyl group, and a --CO--R3 group, wherein R3 represents the residue of any N,N-C1 -C2 dialkylamino acid or a C4 -C6 cycloalkylamino acid

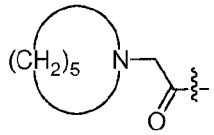


wherein R1 represents a member selected from the group consisting of a hydroxyl group and a --OM group, wherein M is an alkali metal (Na, K, etc.) or an ammonium ion; and wherein R2 represents a member selected from the group consisting of a

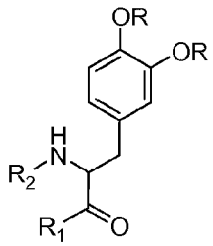


group,

a --CO-pyridyl group, and a --CO--R3 group, wherein R3 represents the residue of any N,N-(C1 -C2)-dialkylamino acid or a C4 -C6 -cycloalkylamino acid



Further L-DOPA prodrugs for use in the present invention, and methods for their synthesis, disclosed in US 4065566 and 4035507 are represented by the formula



wherein R represents an acyl group; wherein R2 represents a hydrogen atom; and wherein R1 represents a -NHCH(R4)COOR5 group, wherein R4 represents the residue of any naturally occurring amino acid, and wherein R5 represents a member selected from the group consisting of a hydrogen atom, a C1 -C5 alkyl group (e.g., methyl, ethyl, propyl, butyl, pentyl), and a C1 -C5 alkylaryl group (e.g., $--CH_2-C_6H_5$, $--CH_2--CH_2-C_6H_5$, etc.), and the HX salts thereof, wherein X is a conventional pharmaceutically acceptable acid addition salt anion (e.g., chloride, bromide, perchlorate, methanesulfonate, succinate, etc.);

Preferred exemplary L-DOPA prodrugs disclosed in US 4065566 and 4035507 include the following:

1. Glycyl-3,4-diacetyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

2. Glycyl-3,4-diacetyloxy-L-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

3. 3,4-diacetyloxy-L-phenylalanyl-glycine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

4. N-nicotinoyl-3,4-dihydroxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

5. N-nicotinoyl-3,4-diacetyloxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

6. N-nicotinoyl-3,4-dipivalyloxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

7. 3,4-diacetyloxy-L-phenylalanyl-glycine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

8. 3,4-diacetyloxy-L-phenylalanyl-glycine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

9. 3,4-diacetyloxy-L-phenylalanyl-glycine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

10. 3,4-diacetyloxy-L-phenylalanyl-glycine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

11. 3,4-diacetyloxy-L-phenylalanyl-L-leucine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

12. 3,4-diacetyloxy-L-phenylalanyl-L-leucine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

13. 3,4-diacetyloxy-L-phenylalanyl-L-leucine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

14. 3,4-diacetyloxy-L-phenylalanyl-L-leucine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

15. 3,4-diacetyloxy-L-phenylalanyl-L-isoleucine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

16. 3,4-diacetyloxy-L-phenylalanyl-L-isoleucine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

17. 3,4-diacetyloxy-L-phenylalanyl-L-isoleucine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

18. 3,4-diacetyloxy-L-phenylalanyl-L-isoleucine-benzyl ester and its HX salt,

wherein X represents a pharmaceutically acceptable anion.

19. 3,4-diacetyloxy-L-phenylalanyl-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

20. 3,4-diacetyloxy-L-phenylalanyl-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

21. 3,4-diacetyloxy-L-phenylalanyl-phenylalanine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

22. 3,4-diacetyloxy-L-phenylalanyl-phenylalanine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

23. Glycyl-3,4-diacetyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

24. Glycyl-3,4-dipivalyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

25. Glycyl-3,4-diacetyloxy-L-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

26. Glycyl-3,4-diacetyloxy-L-phenylalanine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

27. Glycyl-3,4-diacetyloxy-L-phenylalanine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

28. L-leucyl-3,4-diacetyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

29. L-leucyl-3,4-diacetyloxy-L-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

30. L-leucyl-3,4-diacetyloxy-L-phenylalanine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

31. L-leucyl-3,4-diacetyloxy-L-phenylalanine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

32. L-isoleucyl-3,4-diacetyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

33. L-isoleucyl-3,4-diacetyloxy-L-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

34. L-isoleucyl-3,4-diacetyloxy-L-phenylalanine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

35. L-isoleucyl-3,4-diacetyloxy-L-phenylalanine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

36. Phenylalanyl-3,4-diacetyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

37. Phenylalanyl-3,4-diacetyloxy-L-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

38. Phenylalanyl-3,4-diacetyloxy-L-phenylalanine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

39. Phenylalanyl-3,4-diacetyloxy-L-phenylalanine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

40. 3,4-diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine and its HX salt, wherein X represents a pharmaceutically acceptable anion.

41. 3,4-diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine-methyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

42. 3,4-diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine-ethyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

43. 3,4-diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine-benzyl ester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

44. N-[N,N-dimethylamino]-glycyl-3,4-diacetyloxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

45. N-nicotinoyl-3,4-dinicotinoyloxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

46. N-3-pyridylacetyl-3,4-dihydroxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

47. N-3-pyridylacetyl-3,4-diacetyloxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

48. 3,4-N,N-dimethylaminoglycyl-L-phenylalanine methylester and its HX salt, wherein X represents a pharmaceutically acceptable anion.

49. N-[N,N-dimethylamino]glycyl-3,4-[N,N-dimethylaminoglycyl]-L-phenylalanine and its M salt, wherein M represents an alkali metal.

50. N-[N,N-diethylaminoglycyl]-3,4-diacetyloxy-L-phenylalanine and its M salt, wherein M represents an alkali metal.

As used herein, the term "alkyl" refers to a saturated aliphatic hydrocarbon including straight chain and branched chain groups. The alkyl group preferably has between 1 and 30 carbon atoms, more preferably between 1 and 20 carbon atoms. While lower alkyls, e.g., of between 1 and 6 carbon atoms may facilitate the formulation of the compounds, higher alkyls provides for enhanced permeability thereof through the BBB.

The alkyl group, according to the present invention, may be substituted or non-substituted. When substituted, the substituent group can be, for example, cycloalkyl, alkenyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, halo, carboxy, alkoxycarbonyl, thiocarboxy, carbamyl, and amino, as these terms are defined herein.

As used herein, the term "cycloalkyl" refers to an all-carbon monocyclic or fused ring (i.e., rings which share an adjacent pair of carbon atoms) group wherein one of more of the rings does not have a completely conjugated pi-electron system. Examples, without limitation, of cycloalkyl groups are cyclopropane, cyclobutane, cyclopentane, cyclopentene, cyclohexane, cyclohexadiene, cycloheptane, cycloheptatriene and adamantane. The cycloalkyl group, according to the present invention, may be substituted or non-substituted. When substituted, the substituent group can be, for example, alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, halo, carboxy, alkoxycarbonyl, thiocarboxy, carbamyl, and amino, as these terms are defined herein.

The term "alkenyl" refers to an alkyl group which consists of at least two carbon atoms and at least one carbon-carbon double bond.

The term "alkynyl" refers to an alkyl group which consists of at least two carbon atoms and at least one carbon-carbon triple bond.

As is discussed above, both the alkenyl and the alkynyl groups preferably have between 1 and 30 carbon atoms.

An "aryl" group refers to an all-carbon monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) group having a completely conjugated pi-electron system. Examples, without limitation, of aryl groups are phenyl, naphthalenyl and anthracenyl. The aryl group, according to the present invention, may be substituted or non-substituted. When substituted, the substituent group can be, for example, alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy,

thiohydroxy, thioalkoxy, thioaryloxy, halo, carboxy, alkoxycarbonyl, thiocarboxy, carbamyl, and amino, as these terms are defined herein.

The term "C-carboxy" refers to a +C(=O)-OR' group, where R' is hydrogen, alkyl, cycloalkyl, alkenyl, aryl, heteroaryl (bonded through a ring carbon) or heteroalicyclic (bonded through a ring carbon) as defined herein.

The term "O-carboxy" refers to a R'--C(=O)-O- group, where R' is hydrogen, alkyl, cycloalkyl, alkenyl, aryl, heteroaryl (bonded through a ring carbon) or heteroalicyclic (bonded through a ring carbon) as defined herein.

The term "carbonyl" refers to a -C(=O)-R' group, where R' is as defined hereinabove.

The term "thiocarbonyl" refers to a -C(=S)-R' group, where R' is as defined hereinabove.

An "O-carbamyl" group refers to an -OC(=O)-NR'R" group, where R' is as defined hereinabove and R" is as defined for R'.

An "O-thiocarbamyl" group refers to an -OC(=S)-NR'R" group, where R' is and R" are as defined hereinabove.

A "fatty acid acyl" refers to a R'"C(=O)-O- group, where R'" is a saturated or unsaturated hydrocarbon chain having at least 10 carbon atoms.

The term "alkoxy" refers to both an -O-alkyl and an -O-cycloalkyl group, as defined hereinabove. Representative examples of alkoxy groups include methoxy, ethoxy, propoxy and tert-butoxy.

The -O-alkyl and the O-cycloalkyl groups, according to the present invention, may be substituted or non-substituted. When substituted, the substituent group can be, for example, cycloalkyl, alkenyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, halo, carboxy, alkoxycarbonyl, thiocarboxy, carbamyl, and amino, as these terms are defined herein.

The term "thioalkoxy" refers to both an -S-alkyl group, and an -S-cycloalkyl group, as defined herein.

The term "hydroxy" refers to an -OH group.

The term "thiohydroxy" refers to an -SH group.

An "aryloxy" group refers to both an -O-aryl and an -O-heteroaryl group, as defined herein.

A "thioaryloxy" group refers to both an -S-aryl and an -S-heteroaryl group, as defined herein.

The term "amino" refers to a -NR'R" group, with R' and R" as defined hereinabove.

The term "alkoxycarbonyl", which is also referred to herein interchangeably as "carbalkoxy", refers to a carboxy group, as defined hereinabove, where R' is not hydrogen.

The term "heteroaryl" group includes a monocyclic or fused ring (i.e., rings which share an adjacent pair of atoms) group having in the ring(s) one or more atoms, such as, for example, nitrogen, oxygen and sulfur and, in addition, having a completely conjugated pi-electron system. Examples, without limitation, of heteroaryl groups include pyrrole, furane, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrimidine, quinoline, isoquinoline and purine.

A "heteroalicyclic" group refers to a monocyclic or fused ring group having in the ring(s) one or more atoms such as nitrogen, oxygen and sulfur. The rings may also have one or more double bonds. However, the rings do not have a completely conjugated pi-electron system.

The term "halo" refers to a fluorine, chlorine, bromine or iodine atom.

The term "phosphonyl" describes an $-P(=O)(OR')_2$ group, with R' as defined hereinabove.

In any embodiment of the first or second aspect of the invention, the methods may comprise administering two or more compounds selected from the group consisting of L-DOPA and L-DOPA analogues. In another preferred embodiment, the methods may further comprise administering a further therapeutic compound to the subject, including but not limited to an L-amino acid decarboxylase inhibitor, such as carbidopa or benserazide. Such L-amino acid decarboxylase inhibitors can be used, for example, to increase plasma half-life of L-DOPA and reduce conversion of L-DOPA to dopamine peripherally, which reduces side effects of L-DOPA treatment. In another embodiment, the methods may further comprise administering one or more other compounds useful for treating or limiting development of AMD, including but not limited to anti-angiogenic therapeutics, such as anti-vascular endothelial growth factor (VEGF) agents, including but not limited to VEGF antibodies (or fragments thereof) such as ranibizumab or bevacizumab, or VEGF aptamers, such as pegaptanib. In another embodiment, the L-

DOPA or L-DOPA analogues may be present in a more complex mixture, such as in a nutritional supplement containing L-DOPA or L-DOPA analogues.

In a preferred embodiment, any one or more of the L-DOPA and/or L-DOPA analogues described herein may be used in the form of a dietary supplement. Such a supplement may combine any one or more further components that might be beneficial in treating or limiting development of AMD. In one preferred embodiment, L-DOPA and/or an L-DOPA analogue are combined with a combination of vitamin C source, vitamin E source, Vitamin A source, zinc source, and, and copper source, disclosed in US Patent No. 6,660,297 as useful in treating AMD; US 6,660,297 is incorporated by reference herein in its entirety. Any suitable amount of each of these additional components can be used in combination with L-DOPA and/or L-DOPA analogues in carrying out the methods of the invention. In a further preferred embodiment, this combination may further comprise lutein and/or zeaxanthin in an amount suitable to provide further protective retinal effects, preferably between 1 mg and 100 mg; between 1 mg and 50 mg, between 2 mg and 25 mg, or between 2 mg and 10 mg per day. In a further preferred embodiment of any of the above preferred embodiments, this combination may further comprise docosahexaenoic acid (DHA) and/or eicosapentaenoic acid (EPA) in an amount suitable to provide further protective retinal effects, preferably between 250 mg and 1000 mg; between 300 mg and 750 mg, between 350 mg and 750 mg, or between 350 mg and 650 mg per day. The use of such compositions for treating AMD patients is discussed, for example, at web site www.areds2.org/ and links therein.

Ascorbic acid is the preferred source of vitamin C, although other sources such as for example sodium ascorbate could alternatively be used.

Dl-alpha tocopheryl acetate is the preferred source of vitamin E, although other sources of vitamin E, such as for example trimethyl tocopheryl acetate and/or vitamin E succinate, may be used in the alternative.

Beta-carotene is preferred in the subject composition due to its ready commercial availability although alternative carotenoid proforms of vitamin A could likewise be used.

Zinc is preferred in the form of zinc oxide in subject tablets due to the fact zinc oxide provides the most concentrated form for elemental zinc and is well tolerated in the digestive system. However, other forms of zinc such as for example zinc gluconate may alternatively be used or be used in combination with zinc oxide in the subject composition.

Copper in the form of cupric oxide is preferred in the subject tablets to help prevent zinc induced copper deficiency anemia, although other forms of copper such as for example copper gluconate may alternatively be used or used in combination with cupric oxide in the subject composition.

In a preferred embodiment, the amounts of each of these other components (on a per day basis) is as follows:

between 450 mg and 600 mg vitamin C (approximately 7-10 times the recommended daily allowance (RDA))

between 400 IU and 540 IU vitamin E (approximately 13-18 times the RDA);

between 17.2 mg and 28 mg beta carotene (approximately 6-10 times the RDA of vitamin A; beta carotene is a prodrug of vitamin A);

between 68 mg and 100 mg zinc (approximately 4-7 times the RDA for zinc); and

between 1.6 mg and 2.4 mg copper.

In a further preferred embodiment, the amounts of each of these other components (on a per day basis) is as follows:

500 mg Vitamin C;

400 IU Vitamin E;

0 mg or 15 mg beta carotene;

25 mg or 80 mg zinc oxide; and

2 mg cupric oxide.

In a further preferred embodiment, that may be combined with any other embodiments herein, other ingredients believed to be of benefit in maintaining eye health may likewise be combined with L-DOPA and/or L-DOPA analogues, including but not limited to lutein and/or zeaxanthin in an amount suitable to provide further protective retinal effects, preferably between 1 mg and 100 mg; between 1 mg and 50 mg, between 2 mg and 25 mg, or between 2 mg and 10 mg per day; and/or docosahexaenoic acid (DHA) and/or eicosapentaenoic acid (EPA) in an amount suitable to provide further protective retinal effects, preferably between 250 mg and 1000 mg; between 300 mg and 750 mg, between 350 mg and 750 mg, or between 350 mg and 650 mg per day. Further examples of additional compounds that may optionally be used include but are not limited to alpha-lipoic acid and, phenolic compounds such as for example but not limited to oligomeric proanthocyanidins, anthocyanosides and combinations thereof.

L-DOPA and/or L-DOPA analogues can be administered individually or in combination, usually in the form of a pharmaceutical composition. Such compositions are prepared in a manner well known in the pharmaceutical art. L-DOPA and/or L-DOPA analogues can be administered as the sole active pharmaceutical agent, or they can be used in combination with one or more other compounds useful for carrying out the methods of the invention, including but not limited to an anti-angiogenic therapeutics such as VEG-F, and L-amino acid decarboxylase inhibitors, such as carbidopa and benserazide. When administered as a combination, combination can be formulated as separate compositions that are given at the same time or different times, or can be given as a single composition.

The L-DOPA and/or L-DOPA analogues may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The L-DOPA and/or L-DOPA analogues may be applied in a variety of solutions and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

The L-DOPA and/or L-DOPA analogues may be administered by any suitable route, including but not limited to oral, topical (including but not limited to eye drops and ophthalmic ointments), parenteral, intranasal, pulmonary, or rectal in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a compound of the invention and a pharmaceutically acceptable carrier. L-DOPA and/or L-DOPA analogues may be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing L-DOPA and/or L-DOPA analogues may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Eye drops can be prepared using any technique in the art, including but not limited to using a tonicity agent such as sodium chloride or concentrated glycerin, a buffer such as sodium phosphate or sodium acetate, a surfactant such as polyoxyethylene sorbitan monooleate, polyoxyl 40 stearate or polyoxyethylene hydrogenated castor oil, a stabilizer such as sodium citrate or sodium edetate, a preservative such as benzalkonium chloride or paraben as needed. The pH of the eye drops is preferably in the range of from 4 to 8.

Ophthalmic ointments can be prepared with a generally used base such as white soft paraffin or liquid paraffin.

L-DOPA and/or L-DOPA analogues intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring agents and preservative agents in order to provide palatable preparations. Tablets contain the L-DOPA and/or L-DOPA analogues in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques. In some cases such coatings may be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

Formulations for oral use may also be presented as hard gelatin capsules wherein the L-DOPA and/or L-DOPA analogue is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the L-DOPA and/or L-DOPA analogues in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the L-DOPA and/or L-DOPA analogues in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents may be added to provide palatable oral preparations. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

Pharmaceutical compositions for use in the methods of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening and flavoring agents.

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations may also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Specific methods for intranasal administration of L-DOPA and L-DOPA analogues are known in the art; see, for example, Kao et al., Pharmaceutical Research 17(8):978-984 (2000).

The dosage range depends on the choice of the compound, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art. In certain embodiments, L-DOPA and/or L-DOPAS analogues can be administered at dosages of between 10 mg/day and 1500 mg/day; in various preferred embodiments administration can be between 20 mg and 1200 mg/day, 50 mg and 1000 mg/day, 100 mg and 500 mg/day, and 200 mg and 400 mg/day.

Pharmaceutical compositions containing the compounds described herein are administered to an individual in need thereof. In a preferred embodiment, the subject is a mammal; in a more preferred embodiment, the subject is a human. In therapeutic applications, compositions are administered in an amount sufficient to carry out the methods of the invention. Amounts effective for these uses depend on factors including, but not limited to, the nature of the compound (specific activity, etc.), the route of administration, the stage and severity of the disorder, the weight and general state of health of the subject, and the judgment of the prescribing physician. The active compounds are effective over a wide dosage range. However, it will be understood that the amount of the compound actually administered will be determined by a physician, in the light of the above relevant circumstances. Therefore, the above dosage ranges are not intended to limit the scope of the invention in any way.

In a third aspect, the present invention provides compositions comprising:

(a) an amount effective of L-DOPA or an L-DOPA analogue for treating or limiting development of AMD; and

(b) an amount effective for treating or limiting development of AMD of a composition comprising a source of vitamin C, a source of vitamin E, a source of vitamin A, a source of zinc, and a source of copper.

The amount of L-DOPA and/or L-DOPAS analogues in the compositions is suitable to provide for administration at dosages of between 10 mg/day and 1500 mg/day; in various preferred embodiments administration can be between 20 mg and 1200 mg/day, 50 mg and 1000 mg/day, 100 mg and 500 mg/day, and 200 mg and 400 mg/day.

Ascorbic acid is the preferred source of vitamin C in the subject tablets, although other sources such as for example sodium ascorbate could alternatively be used. Dl-alpha tocopheryl acetate is the preferred source of vitamin E in the subject tablets although other sources of vitamin E, such as for example trimethyl tocopheryl acetate and/or vitamin E succinate, may be used in the alternative. Beta-carotene is preferred in the subject composition due to its ready commercial availability although alternative carotenoid proforms of vitamin A could likewise be used. Zinc is preferred in the form of zinc oxide in subject tablets due to the fact zinc oxide provides the most concentrated form for elemental zinc and is well tolerated in the digestive system. However, other forms of zinc such as for example zinc gluconate may alternatively be used or be used in combination with zinc oxide in the subject composition. Copper in the form of cupric oxide is preferred in the subject tablets to help prevent zinc induced copper deficiency anemia, although other forms of copper such as for example copper gluconate may alternatively be used or used in combination with cupric oxide in the subject composition.

In one preferred embodiment of this third aspect of the invention, composition "b" provides a formulation suitable to permit ingestion of the following amounts of each component:

Ascorbic acid: at least 450 mg;

dI-alpha tocopheryl acetate: 400 IU;

beta carotene: 17.2 mg;

zinc oxide: 68 mg; and

cupric oxide: 1.6 mg.

In one preferred embodiment of this third aspect of the invention, composition "b" provides a formulation suitable to permit ingestion of the following amounts of each component:

500 mg Vitamin C;

400 IU Vitamin E;

0 mg or 15 mg beta carotene;

25 mg or 80 mg zinc oxide; and

2 mg cupric oxide.

The preferred daily dosage of the subject composition as specified above may be administered in the form of 1, 2, 3, 4, or more dosage forms according to any suitable route of administration as disclosed above. In preferred embodiments, the dosage form is

an oral or topical dosage form, according to any embodiment of such dosage forms described herein. In another preferred embodiment the daily dosage of the subject composition is provided in the form of one dosage form taken twice daily, for a total of two dosage forms a day, or in the form of two dosage forms taken twice daily, for a total of four dosage forms a day. Compared to taking the total daily dose once a day, twice daily dosing of half the total daily dose in one or more dosage forms per dose provides improved absorption and better maintenance of blood levels of the essential ingredients. Accordingly, if two dosage forms of the preferred formulation of the subject composition are to be ingested each day, each dosage form is formulated to preferably provide not less than approximately 225 mg ascorbic acid, approximately 200 IU dl-alpha tocopheryl acetate, approximately 8.6 mg beta-carotene, approximately 34 mg zinc oxide and approximately 0.8 mg cupric oxide upon oral administration. If four tablets of the preferred formulation of the subject composition are to be ingested each day, each tablet is formulated to preferably provide not less than approximately 112.5 mg ascorbic acid, approximately 100 IU dl-alpha tocopheryl acetate, approximately 4.3 mg beta-carotene, approximately 17 mg zinc oxide, approximately 0.4 mg cupric oxide, and between 5 mg and 750 mg or L-DOPA and/or L-DOPA analogues.

In another preferred embodiment, the compositions comprise

(a) between 5 mg and 1500 mg L-DOPA or L-DOPA analogue;

(b) between 450 mg and 600 mg vitamin C (approximately 7-10 times the recommended daily allowance (RDA))

(c) between 400 IU and 540 IU vitamin E (approximately 13-18 times the RDA);

(d) between 17.2 mg and 28 mg beta carotene (approximately 6-10 times the RDA of vitamin A; beta carotene is a prodrug of vitamin A);

(e) between 68 mg and 100 mg of zinc (approximately 4-7 times the RDA for zinc); and

(f) at least 1.6 mg of copper.

In various preferred embodiments, the composition may comprise between 10 mg and 1200 mg; between 25 mg and 1000 mg; between 50 mg and 500 mg, or between 100 mg and 400 mg L-DOPA or L-DOPA analogue.

In a further preferred embodiment, that may be combined with any other embodiments herein, other ingredients believed to be of benefit in maintaining eye health may likewise be combined with L-DOPA and/or L-DOPA analogues, including but not limited to lutein and/or zeaxanthin in an amount suitable to provide further protective retinal effects, preferably between 1 mg and 100 mg; between 1 mg and 50 mg, between 2 mg and 25 mg, or between 2 mg and 10 mg per day; and/or docosahexaenoic acid (DHA) and/or eicosapentaenoic acid (EPA) in an amount suitable to provide further protective retinal effects, preferably between 250 mg and 1000 mg; between 300 mg and 750 mg, between 350 mg and 750 mg, or between 350 mg and 650 mg per day. The amounts necessary in any particular dosage form to provide the recited amounts can be determined by one of skill in the art based on the teachings herein and the number of dosage forms to be administered per day.

In a fourth aspect, the present invention provides in vitro methods for identifying compounds to treat AMD, comprising contacting cells with a test compound, wherein the cells comprise:

(a) a first cell population expressing OA1; and, optionally,

(b) a second cell population not expressing OA1; and

(c) identifying as positive test compounds those test compounds that increase one or both of

(i) pigment epithelium-derived factor (PEDF) expression in the first cell population relative to one or both (A) PEDF expression in the first population of cells not contacted with the test compound, and (B) the second cell population, and

(ii) intracellular calcium concentration in the first cell population relative to one or both (A) intracellular calcium concentration in the first population of cells not contacted with the test compound, and (B) the second cell population

wherein the positive test compounds are candidate compounds for treating and/or limiting development of AMD.

As described above, human OA1 (**SEQ ID NO:1-2** NP 000264.1) is a G-protein coupled receptor and the inventors have herein identified L-DOPA as an OA1 ligand. As disclosed in more detail below, the inventor has discovered the existence of an autocrine

loop between OA1 and tyrosinase linked through L-DOPA, and this loop includes the secretion of at least one very potent retinal neurotrophic factor (PEDF) as well as an increase in intracellular calcium concentration. OA1 is a selective L-DOPA receptor whose downstream effects govern spatial patterning of the developing retina. Thus, test compounds that selectively up-regulate PEDF expression and/or intracellular calcium concentration via stimulation of the OA1 pathway are candidate compounds for treating and/or limiting development of AMD. The methods of this aspect of the invention can be carried out with any OA1 homologue of, including but not limited to:

Mouse: SEQ ID NO:3-4 (NM_010951);

Xenopus tropicalis: SEQ ID NOS:5-6 (NM_001011018);

Cow: SEQ ID NOS:7-8 (XM_001506318);

Rat: SEQ ID NOS: 9-10 (NM_001106958);

Platypus: SEQ ID NOS: 11-12 (XM_001506318);

Xenopus laevis: SEQ ID NOS: 13-14 (NM_001096842)

Chicken: SEQ ID NOS:15-16 (XM_416848);

Zebrafish: SEQ ID NOS: 17-18 (NM_200822);

Chimpanzee: SEQ ID NO: 19 (XR_025625);

Rhesus monkey: SEQ ID NOS:21-22 (XM_001090139; and

Macaque: SEQ ID NO: 23 (BV209253).

PEDF is pigment epithelium-derived factor (Exp Eye Res 53: 411-414), and is a known neurotrophic factor with the potential to alter neurosensory retina development, and to inhibit blood vessel growth. The methods of this aspect of the invention can be carried out with any PEDF homologue of, including but not limited to:

Human: SEQ ID NOS:25-26 (NM_002615);

Rat: SEQ ID NOS:27-28 (NM_031356);

Zebra finch: SEQ ID NOS: 29-30 (XM_002197419);

Horse: SEQ ID NOS:31-32 (NM_001143954);

Xenpous tropicalis: SEQ ID NOS:33-34 (NM_203755);

Mouse: SEQ ID NOS:35-36 (NM_011340);

Atlantic salmon: SEQ ID NOS:37-38 (NM_001140334);

Sheep: SEQ ID NOS:39-40 (NM_001139447);

Guinea pig: SEQ ID NOS:41-42 (EF679792);

Cow: SEQ ID NOS:43-44 (NM_174140);
Wild boar: SEQ ID NOS:45-46 (NM_001078662);
Platypus: SEQ ID NOS:47-48 (XM_001507128);
Wolf: SEQ ID NOS: 49-50 (NM_001077588);
Macaque: SEQ ID NOS: 51-52 (AB174277);
Chimpanzee: SEQ ID NOS: 53-54 (XM_001154665);
Rhesus monkey: SEQ ID NOS: 55-56 (XM_001117361); and
Flounder: SEQ ID NOS: 57-58 (DQ115406).

The first and second population of cells can be any suitable eukaryotic cell types, where the first population of cells is capable of expressing OA1 as a cell surface receptor protein. In one preferred embodiment, the first and second populations of cells are of mammalian origin, such as mouse, rat, hamster, or human cells. All eukaryotic cells tested to date have been found suitable for carrying out the methods of the invention, particularly when used with embodiments involving analysis of intracellular calcium concentration. Cell types tested to date for conservation of the OA1 signaling pathway disclosed herein with respect to one or both of intracellular calcium signaling and/or PEDF secretion include MCF7 (breast cancer epithelial cells), COS cells (kidney fibroblasts), MDCK cells (kidney epithelial), CHO (Chinese hamster ovary), Mouse RPE, and 3T3 (mouse fibroblast), as well as those disclosed in the examples below. Such cells are commercially available from a variety of sources (LifeLine Cell Technology, Walkersville, MD; ATCC (American Type Culture Collection)), or can be isolated using methods known in the art and described below.

In one embodiment, a first portion of the first population of cells expressing OA1 as a cell surface receptor protein are contacted with the test compound, and a second portion of the first population of cells are not contacted with the test compound, and those compounds that increase expression of PEDF and/or increased intracellular calcium concentration in the first portion relative to the second are candidate compounds for treating and/or limiting development of AMD.

Alternatively, the method may comprise use of a second population of cells not expressing OA1 as a cell surface receptor protein, and those compounds that increase expression of PEDF and/or increased intracellular calcium concentration in the first cell population relative to the second cell population are candidate compounds for treating

and/or limiting development of AMD. In a preferred embodiment, the first and second populations of cells are the same cell type, with the first being engineered to recombinantly express OA1, while the second population of cells is not. In this embodiment, the second population of cells may be transfected with a similar expression vector as the first population of cells; such transfection may comprise transfection with an empty expression vector (ie: no expressed protein driven from the vector in the transfected cells), or an expression vector capable of expressing a truncated or mutated OA1 that does not insert appropriately into the cell membrane. Alternatively, cells can be transfected with an expression vector encoding an OA1 mutant known to be inactive for OA1 signaling, or an engineered form of OA1 that can signal through a different GPCR pathway (eg:cAMP).

For example, one could fuse the 7 transmembrane domains of OA1 with a different intracellular c-terminal tail to change its activity without changing the ligand binding.

As used herein, an "increase in PEDF expression" or "increase in intracellular calcium concentration" is any increases in PEDF expression or intracellular calcium concentration in the first population of cells during the course of the assay above that seen in the second population of cells (or the first portion of the first population relative to the second portion). The method does not require a specific amount of increase in PEDF expression or intracellular calcium concentration over control, so long as the compound(s) promotes an increase in PEDF expression or intracellular calcium concentration above that seen in the control. In a preferred embodiment, the increase is a statistically significant increase as measured by standard statistical measurements.

Determining intracellular calcium concentrations is well known in the art and exemplary methods using Fura-2 cell loading and ratiometric imaging are described in the examples below. However, intracellular calcium concentration can be measured using any method known to those of skill in the art, including but not limited to Fura™ I (see below), or high throughput methods using FLIPer™.

Determining expression levels of PEDF in the cell populations can be performed using any technique in the art such as those described below, including but not limited to, mRNA hybridization (Northern blot, slot blot, etc.), reverse transcription-polymerase chain reaction techniques using any suitable primer sets, fluorescence-in situ hybridization, and antibody detection in conditioned cell medium expressing/secreting

PEDF (Western blot, immunocytochemistry, ELISA). PEDF antibodies are commercially available (for example, from Abcam, Cambridge, MA). Protein analysis can be on conditioned cell medium (since PEDF is an expressed protein); all assays can also be conducted at intracellular PEDF protein/mRNA production. In another embodiment, recombinant cells can be generated that include an expression vector driving expression of a detectable signal (GFP, luciferase, etc.) from the PEDF promoter; such cells can be used as the first cell population where "PEDF expression" is measured via measuring the detectable fluorescent intensity or other signal driven by the PEDF promoter.

As used in this fourth aspect, the term "contacting" means in vitro under suitable conditions to promote binding of OA1 ligands to OA1 expressed on the cell surface of the first population of cells. As used herein the "contacting" can occur at the time of initiating the culturing, or any time subsequent to initiating the culturing of the cell populations. PEDF expression and/or intracellular calcium concentration can be measured at any time after contacting with the test compound as determined appropriate for a given assay. In one embodiment, a time course is carried out, measuring levels pre-contacting and at various times post-contact. In various embodiments, such measurements of calcium signaling after contacting are made between 5 seconds and 60 minutes; more preferably 10 second and 30 minutes, 10 seconds and 10 minutes, and 10 seconds and 5 minutes. 10 seconds and 1 minutes, and 10 seconds and 30 seconds. In various embodiments, measurement of PEDF expression can range between 1 minute and 72 hours, with analysis of PEDF secretion requiring later measurements than analysis of PEDF mRNA expression, PEDF intracellular protein expression, or expression of detectable signals driven by the PEDF promoter.

Any suitable cell culture conditions can be used as appropriate for a given assay. In one preferred embodiment, the contacting occurs in cell culture medium that has either a very low concentration of tyrosine (for example, between 0.1 um and 10 um tyrosine) or no tyrosine, to reduce its production of endogenous L-DOPA in the cells, and to maintain the amount of OA1 present at the cell surface (since OA1 internalizes to the endosomes upon ligand binding). In one preferred embodiment, cells are cultured prior to test compound contacting in low tyrosine medium to maximize OA1 expression and localization at the cell surface, followed by plating into tyrosine-free media for contacting with the test compounds. In another preferred embodiment, contacting occurs in low tyrosine medium. In another preferred embodiment, which can be combined with other

embodiments disclosed above, the culture media includes a tyrosinase inhibitor, including but not limited to phenylthiourea, to limit cell production of L-DOPA from tyrosine. This embodiment is particularly preferred when using pigmented cells.

In another preferred embodiment, the method may further comprise use of one or more of L-DOPA, tyrosine, and dopamine as competitors for binding to OA1. This embodiment may be carried out after identifying a test compound as an OA1 ligand, or it may be carried out in an initial screen of test compounds for binding to OA1. As shown in the examples below, at concentrations of 1 mM and above, tyrosine and dopamine can compete with L-DOPA for binding to OA1. Thus, competitive assays using tyrosine and/or dopamine at concentrations between 1 mM and 100 mM, preferably between 1 mM and 50 mM or between 1 mM and 25 mM, can be used to further verify that the test compounds are operating via the OA1 pathway, and to measure the ability of tyrosine and dopamine to displace positive test compound binding to OA1 as compared to displacement of L-DOPA. Similarly, competitive binding compared to L-DOPA (at similar molarity to the test compounds being tested) can help identify those compounds with increased avidity for OA1 compared to L-DOPA.

Any suitable test compounds can be assessed using the methods of the fourth and fifth aspects (see below) of the invention, including small molecules, polypeptides, and nucleic acids. When the test compounds comprise polypeptide sequences, such polypeptides may be chemically synthesized or recombinantly expressed. Recombinant expression can be accomplished using standard methods in the art, as disclosed above. Such expression vectors can comprise bacterial or viral expression vectors, and such host cells can be prokaryotic or eukaryotic. Synthetic polypeptides, prepared using the well-known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc (Nα-amino protected Nα-t-butyloxycarbonyl) amino acid resin with standard deprotecting, neutralization, coupling and wash protocols, or standard base-labile Nα-amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids. Both Fmoc and Boc Nα-amino protected amino acids can be obtained from Sigma, Cambridge Research Biochemical, or other chemical companies familiar to those skilled in the art. In addition, the polypeptides can be synthesized with other Nα-protecting groups that are familiar to those skilled in this art. Solid phase peptide synthesis may be

accomplished by techniques familiar to those in the art and provided, such as by using automated synthesizers.

When the test compounds comprise antibodies, such antibodies can be polyclonal or monoclonal. The antibodies can be humanized, fully human, or murine forms of the antibodies. Such antibodies can be made by well-known methods, such as described in Harlow and Lane, Antibodies; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1988).

When the test compounds comprise nucleic acid sequences, such nucleic acids may be chemically synthesized or recombinantly expressed as well. Recombinant expression techniques are well known to those in the art (See, for example, Sambrook, et al., 1989, *supra*). The nucleic acids may be DNA or RNA, and may be single stranded or double. Similarly, such nucleic acids can be chemically or enzymatically synthesized by manual or automated reactions, using standard techniques in the art. If synthesized chemically or by in vitro enzymatic synthesis, the nucleic acid may be purified prior to introduction into the cell. For example, the nucleic acids can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the nucleic acids may be used with no or a minimum of purification to avoid losses due to sample processing.

When the test compounds comprise compounds other then polypeptides, antibodies, or nucleic acids, such compounds can be made by any of the variety of methods in the art for conducting organic chemical synthesis.

Test compounds identified as increasing the expression of PEDF and/or intracellular calcium concentration in the first cell population relative to the second cell population, can be further assessed for use as a candidate compound for treating or limiting development of AMD using any further technique, including but not limited to the in vivo methods of the fourth aspect of the invention, described below. In one preferred embodiment, the method may further comprise re-testing the positive test compounds in the assay in the presence of competitive amounts of tyrosine and/or dopamine, as described above.

In a fifth aspect, the present invention provides methods for identifying compounds to treat AMD, comprising

(a) administering a test compound to a tyrosinase deficient pregnant female non-human mammal, wherein the test compound is administered during embryonic photoreceptor and/or retinal ganglion development; and

(b) comparing an effect of the test compound on photoreceptor and/or retinal ganglion development in the embryo or post-natal non-human mammal, to photoreceptor and/or retinal ganglion development in an embryo or post-natal non-human mammal not administered the test compound, wherein those test compounds that increase photoreceptor and/or retinal ganglion development are candidate compounds for treating and/or limiting development of AMD.

The inventor has determined that OA1 signaling can be used to rescue photoreceptor and ganglion cell development in tyrosinase-deficient animals, and in the process establish the neurotrophic effect of OA1 signaling. Thus, compounds that rescue neurosensory retinal development through OA1 signaling are good candidates for AMD treatment. The present invention provides the first establishment of such an animal model for AMD drug screening.

As described in more detail herein, tyrosinase acts on tyrosine to create L-DOPA. Thus, a tyrosinase deficient mammal does not produce L-DOPA, permitting the use of such mammals to identify activators of OA1 (via rescue of retinal development and/or increased PEDF expression) in the absence of endogenous L-DOPA. As used herein, a "tyrosinase deficient" means that the pregnant female non-human mammal does not produce adequate amounts of tyrosinase to create L-DOPA in amounts adequate for normal pigment formation. In one preferred embodiment, the pregnant non-human mammal is a knockout animal (deleted for portion or all of the tyrosinase gene, or have naturally occurring mutations in the tyrosinse gene or accessory genes that control, activate, or traffic tyrosinase to the melanosome) with no ability to express or traffic functional tyrosinase. Such tyrosinase knockouts are known in the art and are commercially available (Lexicon Pharmaceuticals, Jackson Laboratories, Taconic Farms. In other embodiments, the tyrosinase deficiency may be transiently induced by methods known in the art including, but not limited to, administering siRNAs targeting tyrosinase, tyrosinase antibody/aptamer treatment, etc.

The non-human mammal can be any in which tyrosinase-deficient (retinal albino) females can be obtained, which includes all mammals. In various preferred embodiments, the non-human mammal is mouse, pig, apes, and rat.

In one preferred embodiment, administration of test compound is continued during the post-natal period of photoreceptor and/or retinal ganglion development. The embryonic and post-natal photoreceptor and/or retinal ganglion development pathways in various non-human mammals is well understood by those of skill in the art. In one exemplary embodiment, mouse embryonic photoreceptor and retinal ganglion development begins on embryonic day 10 (E10) and retinal development is complete by postnatal day 14 (P14) when the pups eyes are open. Thus, in various embodiments, test compounds are first administered at about day E7, E8, E9, or E10 (to facilitate its presence at the earliest stage of ocular development) and administration can continue as desired for a given assay between day P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, and day P14 or later as desired (up to one year post-natal). As will be understood by those of skill in the art, administration will be to the pregnant female mother during the embryonic phase and to the pup postnatally. In another embodiment, pigmented cell development begins in earnest at approximately day E10.5 (when OA1 and tyrosinase appear), and thus in one embodiment, administration of test compound may begin on about day E10, E10.5, or E11 and continue as desired up to about day P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14 or later as desired. In another embodiment, test compound administration may be limited to between day E7 and E10 or E11. In a further embodiment, retinal ganglion development begins in earnest at about day E12, and thus in one embodiment, administration of test compound may begin on about day E12 or E13 and continue as desired up to about day P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14 or later as desired. In another embodiment, test compound administration may be limited to between day E7 and E12 or E13. In a most preferred embodiment test compounds are first administered daily from day E7 until day P14. As will be understood by those of skill in the art, the exact timing of test compound administration will depend on the goals of the particular assay and can be determined by one of skill in the art based on the teachings herein.

The test compounds may be administered by any route suitable for use with experimental animals, including those routes of administration disclosed above for therapeutic administration of L-DOPA or L-DOPA analogues. In a preferred embodiment, the test compounds are administered in the animal's drinking water, parenterally (as discussed above) or topically (for example, in eye drops or ophthalmic ointments). Frequency of test compound administration can be as often as appropriate for

a given assay; in a preferred embodiment, test compound is administered daily throughout the desired course of treatment; in other embodiments, administration is every second, third, fourth, or fifth day during the course of treatment; the frequency of administration can be determined by one of skill in the art based on the teachings herein and the specific goals of a given assay.

As used herein, an "increase in photoreceptor and/or retinal ganglion development" is any increase in photoreceptor and/or retinal ganglion development in test-compound treated vs. non-treated embryos/animals. The method does not require a specific amount of increase in photoreceptor and/or retinal ganglion development over control, so long as the compound(s) promotes an increase in photoreceptor and/or retinal ganglion development above that seen in the control. In a preferred embodiment, the increase is a statistically significant increase as measured by standard statistical measurements. In one embodiment, animals are euthanized at the appropriate time point, and retinal ganglion cells and/or photoreceptors are counted using standard methods in the art, including but not limited to those disclosed in the examples below.

Test compounds identified as increasing photoreceptor and/or retinal ganglion development, can be further assessed for use as a candidate compound for treating or limiting development of AMD using any further technique, including but not limited to re-testing the positive test compounds using the in vitro methods disclosed in the third aspect of the invention in the presence of competitive amounts of tyrosine and/or dopamine. As shown in the examples below, at concentrations of 1 mM and above, tyrosine and dopamine can compete with L-DOPA for binding to OA1. Thus, competitive assays using tyrosine and/or dopamine at concentrations between 1 mM and 100 mM, preferably between 1 mM and 50 mM or between 1 mM and 25 mM, can be used to further verify that the test compounds are operating via the OA1 pathway, and to measure the ability of tyrosine and dopamine to displace positive test compound binding to OA1 as compared to displacement of L-DOPA.

Examples: L-DOPA is an Endogenous Ligand for OA1

Background: Albinism is a genetic defect characterized by a loss of pigmentation. The neurosensory retina, which is not pigmented, exhibits pathologic changes secondary to the loss of pigmentation in the retina pigment epithelium (RPE). How the loss of

pigmentation in the RPE causes developmental defects in the adjacent neurosensory retina has not been determined, but offers a unique opportunity to investigate the interactions between these two important tissues. One of the genes which causes albinism encodes for an orphan GPCR (OA1) expressed only in pigmented cells, including the RPE.

Methodology/Principle Findings: The function and signaling of OA1 was investigated in RPE and transfected cell lines. The results indicate that OA1 is a selective L-DOPA receptor, with no measurable second messenger activity from two closely related compounds, tyrosine and dopamine. Radiolabeled ligand binding confirmed that OA1 exhibited a single, saturable binding site for L-DOPA. Dopamine competed with L-DOPA for the single OA1 binding site suggesting it could function as an OA1 antagonist. OA1 response to L-DOPA was defined by several common measures of GPCR activation including influx of intracellular calcium and recruitment of β-arrestin. Further, inhibition of tyrosinase, the enzyme that makes L-DOPA, resulted in decreased PEDF secretion by RPE. Further, stimulation of OA1 in RPE with L-DOPA resulted in increased PEDF secretion.

Conclusions/Significance: Taken together the results illustrate an autocrine loop between OA1 and tyrosinase linked through L-DOPA, and this loop includes the secretion of at least one very potent retinal neurotrophic factor. OA1 is a selective L-DOPA receptor whose downstream effects govern spatial patterning of the developing retina. The results suggest that the retinal consequences of albinism caused by changes in melanin synthetic machinery may be treated by L-DOPA supplementation.

Introduction: Albinism is a group of inherited genetic diseases in which there is a variable loss of pigmentation in the eye, hair or skin. When the eye is affected, there are significant alterations in neurosensory retina development that lead to low vision [1-8]. There are two broad classes of albinism, ocular-cutaneous albinism (OCA) and ocular albinism (OA). OCA occurs when all pigmented tissues exhibit hypopigmentation and involves genetic mutations that result in defects in the melanin synthetic machinery [3,7-9]. OA occurs when cutaneous tissues pigment normally, but the ocular tissues are hypopigmented [10,11]. Since the same proteins produce pigment in all tissues, OA most likely results from lack of expression of the melanogenic enzymes in ocular tissue rather than an inability to synthesize melanin because the other tissues pigment normally.

OA can be linked to at least one gene, *Oa1,* which is found on the X chromosome. *Oa1* encodes a 404 amino acid protein likely to be an orphan G-protein coupled receptor (GPCR), OA1 (Genbank GPR143) [12,13] based upon sequence analysis [14]. Schiaffino *et al.* has demonstrated that OA1 associates with several G_{α} subunits as well as G_{β} adding further evidence that OA1 is a GPCR [14,15]. Indeed, Innamorati *et. al.* used a combinatorial expression strategy to illustrate GPCR-like activity from OA1, as well as β-arrestin association, even in the absence of a ligand [16]. This work suggested that OA1 could signal through a Gαq subunit through phospholipase C and inositol triphosphate second messengers. In a yeast based expression system, Staleva and Orlow have demonstrated GPCR signaling from OA1 that appeared to be activated by a component in the melanosomal compartment [17]. Despite the significant amount of circumstantial evidence that OA1 is a GPCR, confirmation is lacking because no ligand has been identified. Other data has called into question the idea that OA1 is a GPCR. For example, the localization of OA1 as a fully intracellular protein is not typical of GPCRs and suggests that it would be a unique member of the family [14]. OA1 is primarily localized to the endolysomal compartment [14,15,18-21] and melanosomes [11,14,22] rather than the cell surface.

In this study the function of OA1 as a potential GPCR was investigated, based on the hypothesis that the endosomal localization of OA1 in cultured cells was due to internalization of OA1 in response to an agent in the culture medium. Further, a ligand for OA1 was sought based on the observation that all forms OCA and OA appear to have the same retinal phenotype, indicating that tyrosinase activity and OA1 signaling are coupled upstream of retinal development. Thus, tests on whether tyrosinase activity produces the ligand for OA1 were carried out. A by-product of melanin synthesis is L-DOPA, which is released to the retina during melanin synthesis in the RPE at a critical time in retinal development [23,24]. The data suggest that OA1 is a highly selective L-DOPA receptor, and that L-DOPA causes OA1 signaling with the downstream effect of neurotrophic factor secretion by RPE. Thus, the first evidence is presented of a ligand for OA1, and provide a mechanism through which either tyrosinase or OA1 deficiency results in changes to retinal development.

Results:

Cell Surface Localization of OA1.

OA1 has previously been localized in pigment granules *in situ* [22], however, using transfected cells of various types, OA1 also has been localized to both the plasma membrane [16,17] and the endosomal fraction of cultured cells [14,16-18,20,21]. The investigation began by determining where OA1 resides in the human tissue using cell surface biotinylation/western blot strategies. In the human eye, OA1 was present on the apical cell surface of the RPE *in situ* (Fig. 1 A). Quantification of cell surface, biotinylated OA1 in five human eyes indicated that at least 3.5 +/- .7% of the total OA1 resided on the apical cell surface of RPE *in situ*. Access to the biotinylation reagent using eye cup preparations is restricted to the apical surface, so the polarity of OA1 in the epithelium cannot be determined. Further, the total cell surface OA1 is likely underestimated because of the lack of access to the basal cell surface. Blots were also probed with antibodies against actin as a control to verify that cytoplasmic proteins were not biotinylated. In each experiment actin was only found in the unbound fraction.

Others have reported that recombinant OA1 and OA1-GFP is almost exclusively localized to the endosomal compartment in cultured cells [14,15,17,18,20-22]. However, when overexpressed [16], or when endocytosis is inhibited [17], OA1 accumulates at the cell surface. The observation that OA1 protein is present on the apical surface of RPE *in situ* led us to explore the issue further.

Effects of Tyrosine on OA1 Expression and Distribution

Endosomal localization of GPCRs occurs normally after exposure to a ligand. Therefore, it was investigated whether a ligand for the receptor was present in the standard incubation medium that could drive internalization of OA1. Since the standard culture medium contains 500 μM tyrosine, and tyrosine is the starting material for pigment synthesis, the effect of tyrosine on receptor distribution was evaluated. To test whether tyrosine affected OA1 distribution in cultured cells DMEM was formulated without tyrosine, and dialyzed fetal bovine serum was used. In the presence of tyrosine-free medium, OA1 was detected on the plasma membrane of cultured RPE cells both in the absence (not shown), and in medium containing low concentrations of tyrosine (1 μM, Fig. 1 B). Averaged over five experiments, 4.5 +/- 1% of total OA1 protein was observed on the surface of cultured RPE maintained in 1 μM tyrosine, similar to what was observed for RPE *in situ*. In all experiments actin was observed in the unbound protein

fraction, demonstrating the absence of any cytoplasmic protein in the cell surface assay. Similarly, OA1-GFP expressed in COS illustrated a cell surface expression that was tyrosine sensitive (Fig. 1 C). Quantification of six such experiments indicated significant variability in the amount of OA1 found at the cell surface using transient transfections. The range of OA1 in the bound fraction of transfected cells maintained in 1 μM tyrosine ranged between 5-40%, unlike the results with the endogenous OA1 protein that were reproducibly ~5% .

Not only was the distribution of OA1 in transfected cells sensitive to tyrosine levels in the medium, total OA1-GFP expression was increased 5-fold in cells maintained in 1 μM tyrosine. To verify that this difference related to OA1 expression rather than cell number, actin expression was evaluated from the paired samples. The data (Fig. 1 D) presented as optical density units indicate no difference in actin. The amount of cell surface OA1 between the normal and low tyrosine groups was also compared. Importantly, in the five RPE experiments and six OA1-GFP in COS experiments, OA1 in the plasma membrane fraction of cells in standard medium was not reproducibly detected, similar to that found by others.

The distribution of OA1 in RPE cells also was evaluated by confocal microscopy. OA1 has previously been characterized as an endosomal protein in cultured RPE cells as shown in (Fig. 1 E). In contrast, the distribution of OA1 in low tyrosine medium was diffuse on the plasma membrane of cultured RPE cells, with little endosomal accumulation (Fig. 1 F), an observation consistent with the results obtained using biochemical methods.

L-DOPA as a Natural Agonist for OA1.

Tyrosinase function in melanogenesis begins with its activity on tyrosine to create L-DOPA, followed by a second reaction to create dopaquinone that leads to pigment formation [25]. Of the intermediates between tyrosine and melanin, L-DOPA has the greatest half-life, and L-DOPA is released into the subretinal space apical to the RPE when melanin synthesis occurs [23,24]. L-DOPA is also the precursor to dopamine, a neurotransmitter produced by dopaneurgic neurons from tyrosine. The release of calcium from intracellular stores is a common downstream effect of GPCR activation by a ligand. Since the expression of OA1 on the cell surface appears to be sensitive to tyrosine, it was examined whether tyrosine, or its metabolites L-DOPA and dopamine, could stimulate

influx of Ca^{2+} into the cytoplasm in an OA1-dependent manner. CHO cells were transfected with an OA1 expression vector then maintained in DMEM containing 1 μM tyrosine for 48 hours followed by tyrosine-free DMEM for 24 hours to facilitate cell surface expression of OA1. Intracellular Ca^{2+} was evaluated using Fura-2, and $[Ca^{2+}]i$ was determined by ratiometric imaging [26]. In the absence of any ligand, $[Ca^{2+}]i$ was not significantly different between transfected and untransfected cells (Fig 2). Tyrosine and several tyrosine metabolites were tested at 1 μM for an effect on $[Ca^{2+}]i$. As a positive control each experiment was ended by treatment with 20 mM KCl to depolarize the cell and increase $[Ca^{2+}]i$ via activation of voltage-gated channels. This maneuver served to verify the Fura-2 loading and responsiveness of the cells being tested (Fig. 2). Only L-DOPA elicited a significant increase in $[Ca^{2+}]i$ (Fig. 2 A). Tyrosine and dopamine had no positive effect on intracellular at $[Ca^{2+}]i$ concentrations up to 1 mM (not shown). The slight negative effect of 1 μM dopamine was not statistically significant, but reproducible among the 11 experiments with dopamine (Fig. 2 B).

Over expression of GPCRs in non-native cell lines can lead to false signal transduction coupling. To verify that OA1 signaling in response to L-DOPA was indeed a natural response, OA1 was expressed in RPE cells (Fig. 2 C). Results using transfected RPE cells were similar to those achieved with transfected CHO cells. RPE cells transfected to express OA1 responded to 1.0 μM L-DOPA with an increase in $[Ca^{2+}]i$. It was next determined whether RPE cells expressing the endogenous OA1 receptor, at endogenous levels exhibited L-DOPA responsiveness. Like all of the transfected cell experiments, RPE expressing OA1 demonstrated an increase in $[Ca^{2+}]i$ after treatment with 1.0 μM L-DOPA (Fig. 2 C).

To further characterize OA1 signaling activity, pertussis toxin was used to distinguish between G_q coupled $[Ca^{2+}]i$ signaling and G_i linked signaling (Fig. 2 C). In all cells studied, pertussis toxin lowered the basal level of $[Ca^{2+}]i$, indicating its activity on inhibition of the background signaling through G_i subunit activity. Pertussis toxin was used in experiments conducted in cells transfected to express OA1 including both CHO and RPE, as well as RPE expressing the endogenous OA1 protein at natural levels. In all transfected cells tested the measured $[Ca^{2+}]i$ response to L-DOPA was greater than in the absence of the toxin (Fig 2), owing largely to the lower initial $[Ca^{2+}]i$. Thus, the signaling through OA1 in response to L-DOPA that results in increase $[Ca^{2+}]i$ is not pertussis toxin

sensitive and likely G_q subunit mediated. The second messenger cAMP was also measured in CHO cells transfected to express OA1 (Fig. 2 D). Using inactive cells or a submaximal forskolin treatment, the experiments were set up to measure either an increase or decrease in cAMP in response to L-DOPA. In six such experiments, no change in cAMP was observed suggesting neither G_s nor G_i subunits are involved in OA1 signaling.

Standard methods of radiolabeled ligand binding were used to characterize the interaction between OA1 and L-DOPA (Fig. 3 A). CHO cells were transfected to express OA1, then binding of L-DOPA was quantified in a concentration-dependent manner, and the results were further characterized by Scatchard Plot analysis (Fig. 3E). Results illustrate saturable binding of L-DOPA to OA1 expressing cells with a Kd of 9.35×10^{-6}M. No specific binding was observed in untransfected CHO cells, indicating that the cells do not have an endogenous L-DOPA receptor (not shown). All binding parameters, total, specific, and nonspecific are shown as supplemental data (Figure 6A). Tyrosine exhibited the potential to interact with OA1, but neither tyrosine nor dopamine stimulated OA1 signaling (see Fig. 2). Competitive ligand binding was used to determine whether either tyrosine or dopamine competed with L-DOPA for OA1 binding. At high concentrations (1 mM), both tyrosine and dopamine competed with L-DOPA for OA1 binding (Fig. 3 B). To further characterize this the kinetics of the competition between L-DOPA and either dopamine (Figure 3 C) or tyrosine (Fig. 6B) was examined. Dopamine exhibited competitive binding to a single site with L-DOPA with a Ki of 2.33×10^{-6} +/- 0.2×10^{-6} M. Similar experiments with tyrosine demonstrated inhibition of L-DOPA binding only at high concentrations (Fig. 6B). Saturation kinetics were not possible with tyrosine because of its low affinity and insolubility at the high concentrations.

Given the relatively low affinity of OA1 for L-DOPA it was determined whether its signaling activity was dose-dependent in the range of this binding affinity. The concentrations in which binding data suggested the steepest rise in association between L-DOPA and OA1, 1.0 – 10 μM were tested, and results illustrate a concentration dependent GPCR response as measured by $[Ca^{2+}]i$ (Fig. 3 C). Thus, the activation kinetics of L-DOPA and OA1 matched the concentration range observed in radiolabeled ligand binding experiments.

In response to ligand binding, GPCRs recruit β-arrestin to the plasma membrane which is followed by internalization of the ligand-receptor complex [27-33]. The effect of

L-DOPA on β–arrestin localization was then tested (Fig. 4). Cells were transfected to express OA1 then cultured in 1 μM tyrosine DMEM for 48 hours prior to analysis to allow cell surface expression of the protein. Cells were then treated with 1 μM L-DOPA followed by rapid fixation on ice in cold methanol. Initially, under resting conditions in the absence of an agonist, OA1-GFP was found at the cell surface and β-arrestin was diffuse in the cytoplasm (Fig. 4 A-C), with no co-localization between the proteins. After stimulation with L-DOPA, OA1 and β-arrestin were co-localized at the plasma membrane (Fig. 4 D-F). Untransfected cells showed no response to L-DOPA treatment (Fig. 4 G,H), illustrating that the L-DOPA effect on β-arrestin distribution was OA1 dependent, similar to results obtained for $[Ca^{2+}]i$.

Effects of 1-DOPA on PEDF Secretion

Mutations in OA1 cause defects in the development of the neurosensory retina. In previous work it has been shown that pigmented RPE secrete significantly more PEDF than nonpigmented RPE [34], and PEDF is a neurotrophic factor with the potential of altering neurosensory retina development [35-41]. Mutations in OA1 cause a loss of pigmentation in the RPE, suggesting that OA1 activity governs RPE pigmentation. Thus, it was determined whether L-DOPA stimulation of pigmented RPE cells caused increased secretion of PEDF (Fig. 5). This assay is made somewhat more difficult because pigmenting RPE cells produce L-DOPA, which is the agonist for OA1, and OA1 is not readily detectable in nonpigmented cultures of RPE. Thus, pigmented RPE were used to determine whether L-DOPA stimulation increases PEDF expression/secretion. RPE cells were placed in tyrosine-free medium for 24 hours then treated with 1 μM L-DOPA for one hour. After treatment, the cells were returned to standard medium without exogenous L-DOPA for three days. Control cells were not treated with L-DOPA, but the medium was changed at the same time the experimental cells were returned to normal medium. Conditioned medium was collected after three days and PEDF was measured. Results illustrate a significant increase in the secretion of PEDF in pigmented cells treated with L-DOPA when compared to paired, control monolayers of pigmented RPE (Fig. 5 A). Importantly, this significant increase occurred in cells which were pigmenting and therefore expressed OA1 and had a basal level of PEDF expression.

To determine whether pigmented RPE cells secrete PEDF through an autocrine loop involving tyrosinase activity and OA1 signaling, a specific tyrosinase inhibitor phenylthiourea (PTU) was used to inhibit pigmentation and L-DOPA production (Fig. 5 B). In these experiments, pigmented RPE cells were either maintained in DMEM, or DMEM containing 200 μM PTU for three days, then PEDF secretion was measured. Pigmented RPE secreted substantial PEDF, but PTU caused a significant decrease in PEDF secretion indicating that tyrosinase activity is necessary for the high level of PEDF secretion observed in pigmented RPE cells. To verify that it was the lack of L-DOPA in the PTU treated cells that caused the decreased PEDF secretion, 3 different cultures of pigmented RPE were used, and exposed to PTU for 48 hours, then treated with 1.0 μM L-DOPA in the continued presence of PTU; PEDF was measured after 72 hours (Fig. 5 C). The data are presented as percent of control for this experiment because the cultures used varied in both pigmentation and PEDF expression before the experiment began. PTU treated RPE responded to the added L-DOPA by increasing PEDF secretion, indicating that the effect of PTU on PEDF secretion is caused be the lack of L-DOPA production when tyrosinase is inhibited.

Discussion:

There is a complex inter-tissue relationship between the RPE and the neurosensory retina. One aspect of this relationship is centered on RPE pigmentation, and defects in melanin synthesis which result in significant neurosensory retina alterations [8,23,42]. The data suggest that OA1 and tyrosinase participate in an autocrine loop through L-DOPA that regulates the secretion of at least one potent neurotrophic factor, PEDF. The data also suggest that the pathologic changes in retinal development that occur in albinism may result from changes in the activity of the OA1 signaling pathway. Reduced OA1 signaling activity can be caused either directly through OA1 mutations or indirectly through changes in L-DOPA production by tyrosinase activity. Thus, it is hypothesized that the similar retinal phenotypes that accompany the diverse forms of albinism can be reconciled to a single common pathway, OA1 signaling.

In the study, OA1 on the apical surface of human RPE *in situ* was observed. Previous reports have suggested that OA1 in mice is localized to the melanosome [22], and in cultured cells to the endosomal compartment [15-18,20-22,43]. The results from *in situ* RPE preparations indicate that OA1 is distributed to the apical surface of the RPE.

The limited quantities of OA1 on the surface of the RPE (~3.5% of total OA1) may account for the lack of observation of the protein in previous studies where immunogold electron microscopy was used. Like many cell surface GPCRs, OA1 is not an abundant protein.

The endosomal localization of OA1 reported in previous studies using cultured cells was reproduced in this study for both the endogenous protein and the transgenic protein. When tested in normal culture medium little detectable OA1 protein on the cell surface was found, in agreement with all previous work. However, reduction of tyrosine in the medium caused a modest increase in cell surface receptor accumulation of both the endogenous and recombinant OA1 proteins. This suggests that the distribution of OA1 to the cell surface in cultured cells is sensitive to tyrosine. A previous study has demonstrated OA1 could be localized to the cell surface when endocytosis in inhibited [17] and OA1 on the apical surface of human RPE was observed *in situ*. The data suggest OA1 is a cell surface GPCR, but is a target for endocytosis that may be stimulated by tyrosine or tyrosine metabolites. In this regard, the results differ from past reports of OA1 localization that have classified OA1 as a unique type of intracellular GPCR. Most GPCRs are cell surface proteins that are internalized by a variety of signals, and the data suggest OA1 is similar to most other GPCRs.

OA1 signaling activity was stimulated by L-DOPA, but not by either its precursor, tyrosine, or its neuronal metabolite dopamine. This result suggests an exquisitely sensitive receptor activity able to distinguish between closely related molecules, after all L-DOPA and tyrosine differ by a sole hydroxyl group. OA1 is sensitive to tyrosine, as tyrosine causes an intracellular localization of OA1 in cultured cells. However, no signaling response to tyrosine was noted, and competition binding studies suggest that tyrosine has a low affinity for OA1. The data suggest that the continuous exposure of cells to high concentrations of tyrosine present in normal medium is sufficient to result in internalization of OA1, but it is unlikely to result in measurable OA1 activation. Strong evidence of a single site competitive interaction between L-DOPA and dopamine was found. The Ki observed for dopamine was similar to the Kd observed for L-DOPA, suggesting that the affinity for the two tyrosine metabolites is similar. The results illustrated a slight, but reproducible, decrease in OA1 signaling from dopamine, suggesting that dopamine may be an effective antagonist or inverse agonist for OA1.

As an orphan GPCR, its signaling pathway has not previously been identified. In this study it was illustrated that OA1 signaling in response to L-DOPA causes an increase in $[Ca^{2+}]i$. The data illustrate that the increased $[Ca^{2+}]i$ observed in response to L-DOPA was insensitive to pertussis toxin and no effects on cAMP were found, indicating that OA1 is likely signaling through a G_q subunit. Previous work has suggested that OA1 can associate with multiple subunits in transfected cells including members of the G_o, G_i, and G_q subunit families. Innamorati *et al.* has shown that spontaneous activity of overexpressed OA1 is likely signaled through a Gq subunit [16]. The data indicate that ligand-dependent signaling from endogenous OA1 in RPE most likely occurs through a G_q mediated pathway, and no promiscuous coupling activities were observed when comparing OA1 over expression in CHO and RPE to natural OA1 expressed in RPE. Interestingly, two overactive mutant forms of Gq subunits cause hyperpigmentation in skin and hair [44], but whether they have an effect in RPE is unknown. RPE and cutaneous melanocytes use the same enzymes to produce pigmentation but differ in their control of melanogenesis. A recent report suggests that OA1 may signal through Gαi3, because the retinal phenotype of OA1 $^{-/-}$ and $G\alpha i3^{-/-}$ are similar [45]. That study provided no data regarding interaction or signaling between Gαi3 and OA1, and the results do not support OA1 signaling through Gαi3. However, both OA1 and Gαi3 could have activity in convergent pathways that govern some part of the complex system of retinal development.

The response of OA1 to L-DOPA was measured in three ways, increased $[Ca^{2+}]i$, recruitment of β-arrestin to plasma membrane OA1, and the increased secretion of PEDF. In addition, inhibiting the activity of tyrosinase in pigmented RPE inhibits L-DOPA production, and results in a decreased secretion of PEDF. Taken together, these studies present a strong argument for a productive ligand:receptor relationship between L-DOPA and OA1. Further, the data suggest selectivity among tyrosine and its metabolites, with only L-DOPA being a productive ligand for OA1. We have determined the binding kinetics between OA1 and L-DOPA, and observed a typical one site receptor:ligand relationship between the two. The binding affinity between OA1 and L-DOPA, with a Kd in the μM range, is not uncommon for an endogenous ligand:receptor relationship. Future identification of a specific, high affinity antagonist for OA1 will aid in further

biochemical characterization of the interaction between OA1 and L-DOPA, and be useful in determining whether dopamine is an inverse agonist.

This study illustrated the selective activation of OA1, an orphan GPCR, by L-DOPA, an intermediate product of melanin synthesis. This study has also illustrated that OA1 activity stimulates PEDF secretion by RPE, a molecule that has the potential to support normal retinal development [40,41]. In humans, this suggests that pharmacologic intervention through OA1 activation could be useful for albinism caused by defects in the melanogenic machinery (OCA 1-4). Unfortunately, the data also suggest that OA1 is necessary for such pharmacologic intervention, and mutations in *Oa1* are the most common cause of albinism.

Methods:

Cell Culture

RPE- Cells were isolated as described [46] and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). For experiments in which tyrosine concentrations were lowered, custom manufactured DMEM produced without tyrosine by JRH Biosciences (Lenexa, KS) was used. Dialyzed FBS was purchased from Invitrogen, (San Diego, CA).

COS-7 and **CHO-** Cells were obtained from ATCC and cultured in DMEM supplemented with 5% FBS. For analysis of OA1 distribution, cells were cultured in tyrosine-free DMEM supplemented with 1 μM tyrosine, 5% dialyzed FBS for 2-4 days, then tyrosine-free media as described for the experiment.

Cell Surface Biotinylation

Human RPE *in situ*- Human eyecups were produced by dissection ~2mm anterior to the equator and removals of the anterior segment. The vitreous and retina were removed without impairing the underlying RPE monolayer, and the retina was cut at the optic nerve head. The resulting eyecups with RPE exposed were rinsed three times with reaction buffer (100 mM NaCl, 50 mM NaHCO3, pH 8.0) then filled with Sulfo-NHS-LC-Biotin (1 mg/ml) two times for thirty minutes. The reaction was stopped with TG buffer (25 mM Tris, 192 mM Glycine, pH 8.3) then the cells were harvested in lysis buffer (2 mM EDTA, 1% Triton X and 1% Tween 20 in Tris Base Saline Buffer) containing Halt Protease Inhibitor Cocktail. Intact cells and pigment granules were

removed by centrifugation at 14,000 rpm for 20 minutes. Biotinylated proteins were captured overnight with immobilized streptavidin beads and then mixed with 4X reducing buffer (250 mM Tris, pH 6.8, 8% SDS, 40% Glycerol, 20% Beta-mercaptoethanol, 0.08% bromophenol blue). The OA1 protein was separated on a 10% SDS-PAGE gel and identified by a using a polyclonal rabbit OA1 antibody for western blot analysis. Paired western blots were probed with a monoclonal antibody directed against actin.

Cultured Cells- RPE and transfected cells were maintained in DMEM containing tyrosine concentrations described for the experiment. Cultures were rinsed three times in reaction buffer, then biotinylated as described above for the *in situ* preparation.

Cloning of *Oa1*

A cDNA library was constructed from pooled tissue from 6 human donor eyes. Total RNA was harvested using Trizol reagent, then cDNA was synthesized using Poly-T primers for the first strand synthesis, and random hexamers for the second strand. Following cDNA synthesis, RNA was removed using RNase A. The coding sequence for OA1 was obtained by PCR using terminal primers that added restriction sites to the 5' and 3' ends and removed the native stop codon. The PCR product was ligated in frame with GFP in the pEGFP N-1 vector (Clontech). The sequence was verified by automated sequencing in both directions over the entire sequence.

Immunocytochemistry

Cells on slides were fixed with 3% paraformaldehyde at RT, rinsed with 0.1% Triton X-100 in 10% milk in TBST then blocked with 10% milk in TBST. β-arrestin was visualized using a polyclonal antibody directed against β-arrestin, and incubated overnight at 4°C. Cover slips were mounted using 50% glycerol and immunostaining was analyzed by optical sectioning using a Nikon Eclipse E800 laser scanning confocal microscope powered by Compix Confocal Imaging Systems software (Simple PCI Version 4.0.6.1605). Three-dimensional analysis of OA1-GFP and β-arrestin distribution was performed in Image J 1.32.

Measurement of $[Ca^{2+}]i$

OA1-GFP expressing CHO cells plated on glass cover slips were rinsed in Ca^{2+} containing HEPES buffered Hanks Balanced Salt Solution (HBSS) (pH 7.45), then incubated with 2.5 μM Fura-2 (solubilized in anhydrous dimethylsulfoxide and 0.002% pluronic acid) for 20 minutes at 37°C, 5% CO_2.The Fura-2 loaded cells were rinsed with HBSS for 15 minutes at 37°C, 5% CO_2 to allow for full cleavage of the dye to its active form. Each cover slip was incubated in 1 ml of HBSS in a chamber held at 37°C on the stage of an inverted Olympus IX70 microscope equipped with a 40 x 1.35 NA UV-fluor objective.

Using a filter wheel, excitation light from a 200 W Xe bulb was passed alternately through 340 and 380 nm filters. A 10 nm bandpass filter, centered at 510 nm, selected for the emitted fluorescence which was passed to a CCD camera (Photometrics CH-250). For each experiment, image pairs were taken every minute for the first three minutes, which established a stable baseline. Then L-DOPA (1 μM final concentration) was added and image sets were taken every 30 seconds for the next three minutes. Finally, KCl (20 mM final concentration) was added one minute before completion of each experiment as a positive control to establish that the cells were loaded with Fura-2. The same was repeated independently for tyrosine and dopamine (both at 1 μM final concentration). Using a Silicon Graphics Personal IRIS computer, the 340/380 nm ratio was computed for each pixel within a cell, and then analyzed using Microsoft Excel version 4.0 (Microsoft, Redmond, WA). Once the 340/380 nm ratio was determined, each ratio was normalized to 1 (ratio at time zero divided by itself), then the free ion concentration was calculated using the following equation:

$$[Ca_i]\# = Kd\# * (R - R_{min}\#)/R_{max}\# - R)$$

in which R, R_{min}, and R_{max} are the measured, minimum, and maximum ratios, respectively. R_{max} represents the ratio of fluorescence intensity of ion-sensitive wavelengths under fully deprotonated conditions, whereas R_{min} is the ratio for the dye when it is fully protonated. In the case of Fura-2, R increases with increasing Ca^{2+}; hence R_{min} represents Fura-2 in the absence of Ca^{2+} (Ca^{2+} <1 nM) whereas R_{max} represents the Ca^{2+}-Fura-2 chelate as previously described [26]. R_{min}, R^{max} and Kd were determined in independent

experiments in Fura-2 loaded cells, and subsequently utilized for calculation of free Ca^{2+} for the experimental procedures.

Radiolabeled Ligand Binding

CHO cells were transfected to express OA1-GFP were plated into 24-well plates. Cells were chilled to -2C, then rinsed in cold binding buffer, 25 mM Tris, 150 mM NaCl, 5 mM EDTA, 5 μM digitonin (pH 7.45). Cells were incubated for two hours in binding buffer containing [^{3}H]-L-DOPA (Moravek Biochemicals, Brea, CA) at concentrations between 10^{-4}M to 10^{-9}M. The temperature was not allowed to exceed -2°C at any step of the assay. Controls included assays conducted on nontransfected CHO and specific binding was determined by competition with excess unlabelled L-DOPA at 10^{-3}M. Bound L-DOPA was quantified by scintillation spectroscopy.

Measurement of cAMP

Cells were pretreated with forskolin (15 minutes) then challenged with L-DOPA using an assay setup as previously described [47]. After 1 minute of ligand exposure, cells are scraped into ice-cold buffer, boiled then centrifuged. Equivalent volumes, 50 μl, of supernate and ^{3}H-cAMP (New England Nuclear) then combined with 100 μl cold PKA. After 2 hours, the solution is passed over activated charcoal, and supernates are counted in a scintillation counter. Results are compared to those achieved using a standard curve, instead of cytosol, produced using 50 μl of cAMP 0.25–32.0 pmole/50μl.

Example 2: The OA1 loop functions in vivo

PEDF secretion in OA deficient mice was compared to wild type mice, and showed that wild-type mice secreted significantly more PEDF than OA1-/y mice. The culture medium (C.M.) used contains PEDF, and it is likely that PEDF in the CM from OA1 -/y is from the medium used, not the RPE. Results (Figure 7) are quantified and summarized in the graph. The difference, even with the background PEDF in the CM for both groups is significant. T-test analysis results are presented

Tyrosinase deficient pregnant mice were maintained under normal conditions (No L-DOPA), or supplemented with 1.0mg/ml L-DOPA in there drinking water, beginning

on embryonic day 7 for their pups. Animals were maintained on supplemental until post-natal day 14, when ocular development is over and the eyes are open.

Two cell types are reduced in number in albinism: retinal ganglion cells and photoreceptors. Figure 8A demonstrates that L-DOPA supplementation increases retinal ganglion cell numbers compared to what is expected in a normal wild-type mouse. Figure 8B shows the same result for photoreceptors. Photoreceptors are not counted directly as they are too dense. Rather, the area occupied by photoreceptor nuclei is measured as a measure of photoreceptor numbers. L-DOPA supplementation increased the photoreceptor nuclear area, so the number of photoreceptors were increased. Again, this appeared to restore the albino animal to normal levels.

As shown in Figure 8C, Four paired littermate animals, 2 wild-type and 2 OA1 -/y (female OA1 deficient) were euthanized and the retinas from each animal were loaded independently in a lane, then proteins were western blotted to detect PEDF, which was readily observed in the retina from wild-type mice. In contrast, PEDF is not readily detected in the retinas from the OA1 -/y mice.

In summary this data illustrate that OA1 -/y mice make less PEDF than wild type mice. L-DOPA stimulation in tyrosinase defective mice rescues the two most prominent neurosensory retina defects of albinism: a loss of photoreceptor cells and retinal ganglion cells. Finally, PEDF levels are reduced in the retinas of mice lacking OA1. Thus, it is concluded that the OA1 autocrine loop functions in vivo, and can be stimulated with oral L-DOPA.

The data together illustrate that the linkage between RPE pigmentation and AMD are likely through the signaling activity of OA1. The data illustrate that the ligand for OA1 is L-DOPA, and that OA1 signaling from L-DOPA controls the expression of PEDF. PEDF is the most potent neurotrophic factor made by RPE. Thus, the identification of L-DOPA as the ligand for OA1, which controls PEDF expression, ties together L-DOPA and neurotrophic activity in the RPE. Because L-DOPA is produced as a by-product of pigment production, this established for the first time a linkage between RPE pigmentation and neurotrophic activity. This system is defined as the OA1 autocrine loop. Tyrosinase makes pigment and releases L-DOPA. Released L-DOPA binds to and initiates signaling through OA1. OA1 signaling controls the expression of both tyrosinase and PEDF.

To date the data illustrate this model biochemically, in cultured cells, and *in vivo*. The fact that retinal development in an albino animal can be rescued using dietary L-DOPA indicates that dietary L-DOPA can be used to stimulate RPE trophic factor expression *in vivo*. AMD is clearly tied to an RPE defect somehow related to its pigmentation. Blue-eyed individuals get AMD at a much greater frequency than dark-eyed individuals, so the level of RPE pigmentation controls the AMD process. The level of RPE pigmentation is controlled by OA1 signaling and is part of the same OA1 autocrine loop described above. Thus, AMD is related to OA1 signaling in RPE. Therefore, those with lower RPE pigmentation will have lower tyrosinase, lower L-DOPA, lower OA1 signaling, and lower PEDF production. We can use dietary L-DOPA or related compounds as ligands for OA1 and stimulate that activity. The final determinant of the health of the neurosensory retina is PEDF, but we can use OA1 signaling to increase the OA1 loop activity, and increase the neurotrophic activity of the RPE. The effect of OA1 signaling will be to foster neuron survival.

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I claim

1. A method for treating age-related macular degeneration (AMD), comprising administering to a subject with AMD an amount effective for treating AMD of an agonist of the OA1 receptor.

2. A method for limiting development of age-related macular degeneration (AMD), comprising administering to a subject at risk of developing AMD an amount effective for limiting development of AMD of an agonist of the OA1 receptor.

3. The method of claim 1 or 2, wherein the agonist of the OA1 receptor is selected from the group consisting of L-DOPA and L-DOPA analogues.

4. The method of claim 3, wherein the compound comprises L-DOPA.

5. The method of claim 3, wherein the compound comprises an L-DOPA prodrug.

6. The method of claim 5, wherein the L-DOPA prodrug comprises an L-DOPA ester.

7. The method of claim 5, wherein the L-DOPA prodrug comprises a bile acid conjugate of L-DOPA.

8. The method of claim 5 wherein the L-DOPA prodrug comprises a di- or tri-peptide L-DOPA analogue.

9. The method of claim 5 wherein the L-DOPA prodrug comprises an amide L-DOPA analogue.

10. The method of any one of claims 1-9, wherein the subject is over the age of 60.

11. The method of any one of claims 1-10, wherein the method is for treating AMD, and wherein the subject has wet AMD.

12. The method of any one of claims 1-10, wherein the method is for treating AMD, and wherein the subject has dry AMD.

13. The method of any one of claims 1-10, wherein the method is for limiting development of AMD, and wherein the subject has drusen deposits.

14. The method of claim 13, wherein the subject is Caucasian.

15. A method for identifying compounds to treat AMD, comprising contacting cells with a test compound, wherein the cells comprise:

(a) a first cell population expressing OA1; and, optionally,

(b) a second cell population not expressing OA1; and

(c) identifying as positive test compounds those test compounds that increase one or both of

(i) pigment epithelium-derived factor (PEDF) expression in the first cell population relative to one or both (A) PEDF expression in the first population of cells not contacted with the test compound, and (B) the second cell population, and

(ii) intracellular calcium concentration in the first cell population relative to one or both (A) intracellular calcium concentration in the first population of cells not contacted with the test compound, and (B) the second cell population;

wherein the positive test compounds are candidate compounds for treating and/or limiting development of AMD.

16. The method of claim 15 wherein step (c) comprises identifying as positive test compounds those test compounds that increase pigment epithelium-derived factor (PEDF) expression in the first cell population relative to one or both (a) PEDF expression in the first population of cells not contacted with the test compound, and (b) the second cell population.

17. The method of claim 15 wherein step (c) comprises identifying as positive test compounds those test compounds that increase intracellular calcium concentration in the first cell population relative to one or both (a) intracellular calcium concentration in the first population of cells not contacted with the test compound, and (b) the second cell population.

18. The method of any one of claims 15-17, wherein the first cell population and the second cell population are selected from the group consisting of mouse, rat, hamster, and human cells.

19. The method of any one of claims 15-18, wherein the first cell population and the second cell population are retinal pigment epithelial cells.

20. The method of any one of claims 15-19, wherein the contacting occurs in the presence of between 0 uM and 10 uM tyrosine.

21. The method of any one of claims 15-20, wherein the contacting occurs in the presence of a tyrosinase inhibitor.

22. A method for identifying compounds to treat AMD, comprising

(a) administering a test compound to a tyrosinase deficient pregnant female non-human mammal, wherein the test compound is administered during embryonic photoreceptor and/or retinal ganglion development; and

(b) comparing an effect of the test compound on photoreceptor and/or retinal ganglion development in the embryo or post-natal non-human mammal, to photoreceptor and/or retinal ganglion development in an embryo or post-natal non-human mammal not administered the test compound, wherein those test compounds that increase photoreceptor and/or retinal ganglion development are candidate compounds for treating and/or limiting development of AMD.

23. The method of claim 22, further comprising continuing administration of the test compound postnatally.

24. The method of claim 22 or 23 wherein the mammal is a mouse.

25. The method of any one of claims 22-24, wherein administration of the test compound begins between embryonic day 7 and embryonic day 12.

26. The method of any one of claims 22-25, further comprising continuing administration of the test compound postnatally until postnatal day 1 to postnatal day 14.

27. The method of any one of claims 22-26, wherein the effect measured comprises measuring a number of retinal neurons.

28. The method of any one of claims 22-27, wherein the effect measured comprises measuring a number of retinal ganglion cell numbers.

29. The method of any one of claims 22-28, wherein the effect measured comprises measuring a number of photoreceptors.

30. The method of any one of claims 1-14, further comprising administering to the subject a combination of a vitamin C source, a vitamin E source, a beta-carotene source, a zinc source, and, and a copper source.

31. The method of claim 30, comprising administering

between 450 mg and 600 mg vitamin C;

between 400 IU and 540 IU vitamin E;

between 17.2 mg and 28 mg beta carotene;

between 68 mg and 100 mg zinc; and

between 1.6 mg and 2.4 mg copper.

32. A composition comprising:

(a) an amount effective of L-DOPA or an L-DOPA analogue for treating or limiting development of AMD; and

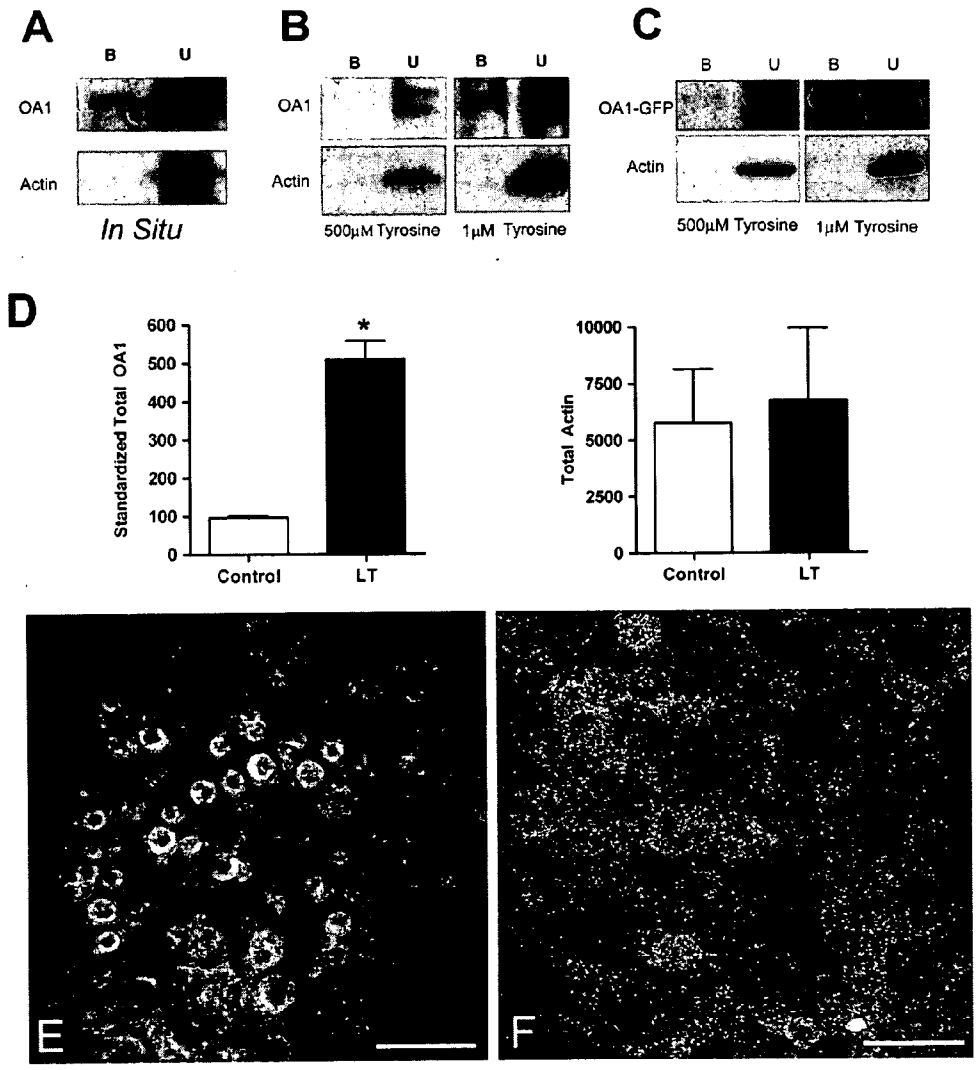
(b) an amount effective for treating or limiting development of AMD of a composition comprising a source of vitamin C, a source of vitamin E, a source of vitamin A, a source of zinc, and a source of copper.

33. The composition of claim 32 comprising between 5 mg and 1500 mg L-DOPA or L-DOPA analogue; between 450 mg and 600 mg vitamin C; between 400 IU and 540 IU vitamin E; between 17.2 mg and 28 mg beta carotene; between 68 mg and 100 mg of zinc; and at least 1.6 mg of copper.

34. A method of treating age-related macular degeneration (AMD), substantially as herein described with reference to the description of the preferred embodiments.

35. A composition for treating age-related macular degeneration (AMD), substantially as herein described with reference to the description of the preferred embodiments.

Figure 1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OA1
Actin
In Situ
B
B
U
B
U
OA1
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U
B
U
OA1-GFP
Actin
500μM Tyrosine
1μM Tyrosine
D
Standardized Total OA1
600
500
400
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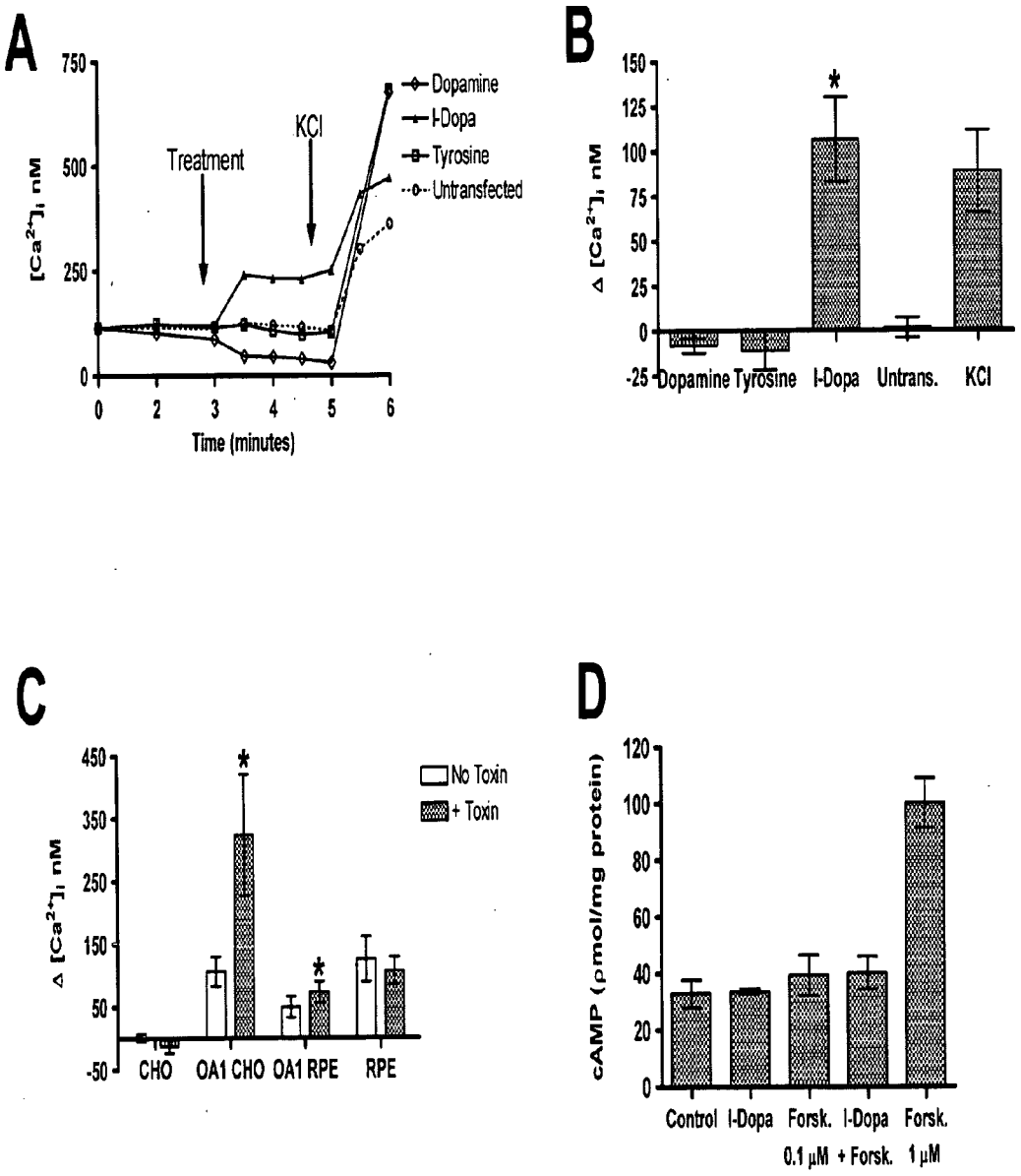
Control
LT
E
F

Figure 2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Treatment
KCl
Dopamine
l-Dopa
Tyrosine
Untransfected
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Time (minutes)
B
150
125
100
75
50
25
0
-25
Δ [Ca2+]i nM
*
Dopamine Tyrosine l-Dopa Untrans. KCl
C
450
350
250
150
50
-50
Δ [Ca2+]i nM
*
*
No Toxin
+ Toxin
CHO OA1 CHO OA1 RPE RPE
D
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100
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cAMP (pmol/mg protein)
Control l-Dopa Forsk. l-Dopa Forsk.
0.1 μM + Forsk. 1 μM

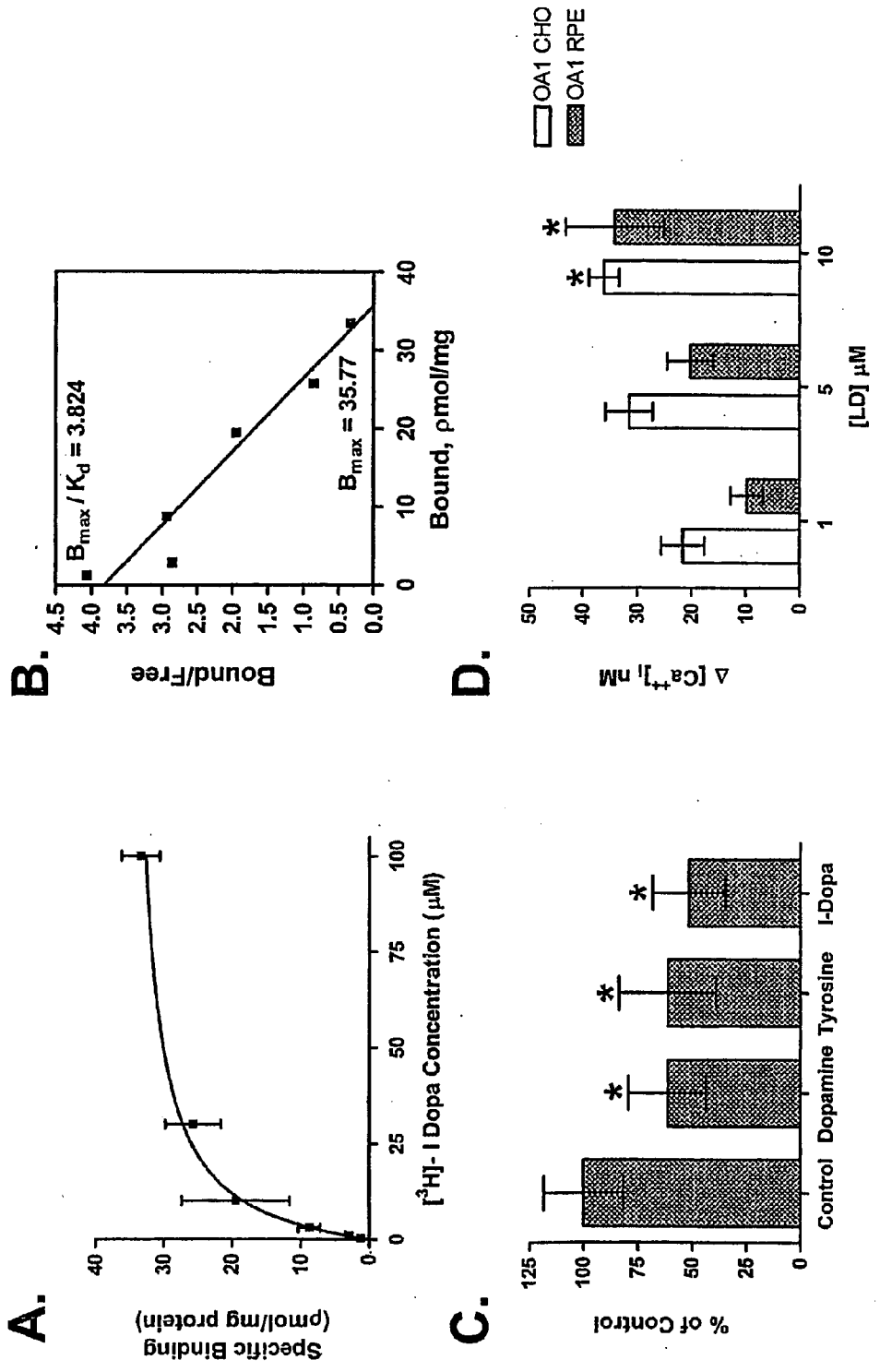
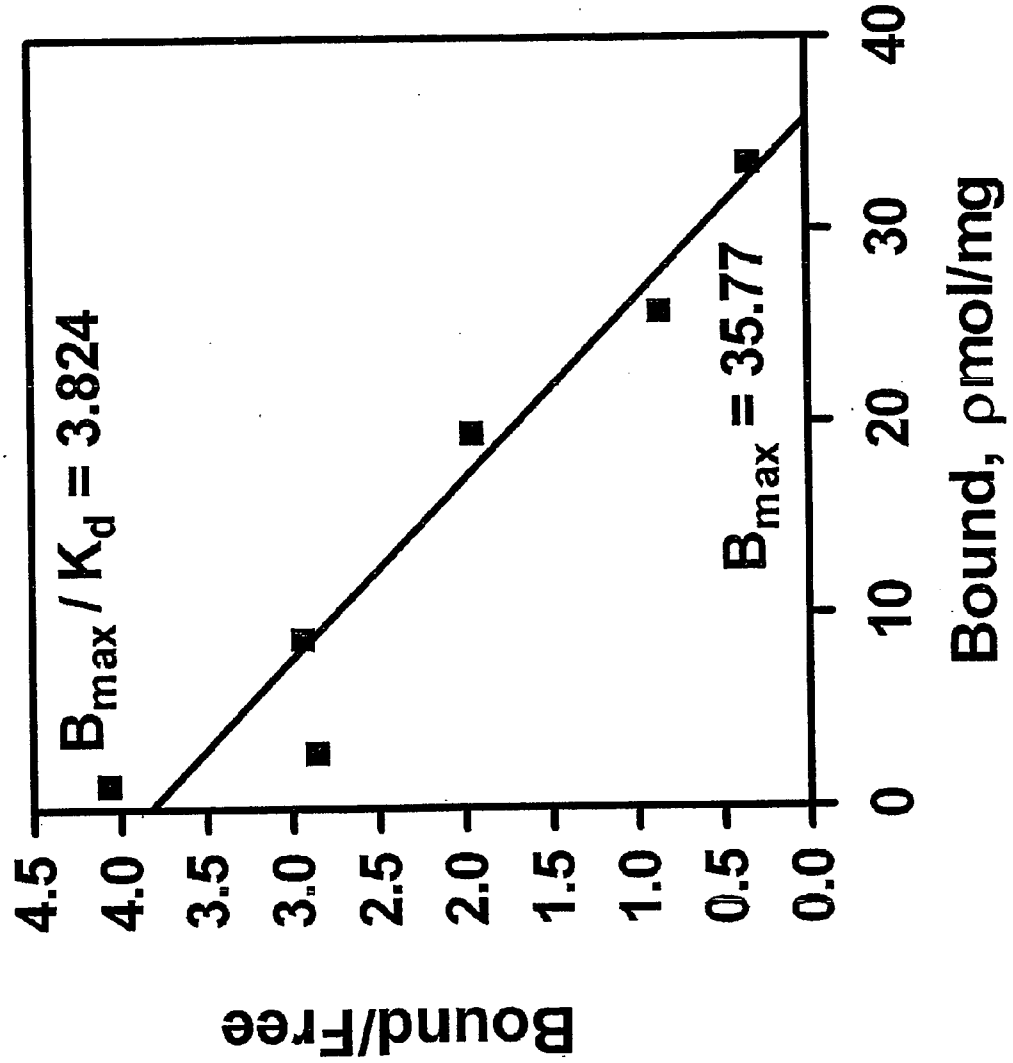

A.
Specific Binding (pmol/mg protein)
[3H]- l Dopa Concentration (μM)
B.
Bound/Free
Bound, ρmol/mg
Bmax / Kd = 3.824
Bmax = 35.77
C.
% of Control
Control
Dopamine
Tyrosine
l-Dopa
D.
Δ [Ca++]i nM
OA1 CHO
OA1 RPE
[LD] μM

FIGURE 3


Bound/Free
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3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
Bound, ρmol/mg
B_{max} / K_d = 3.824
B_{max} = 35.77

FIGURE 3E

Figure 4

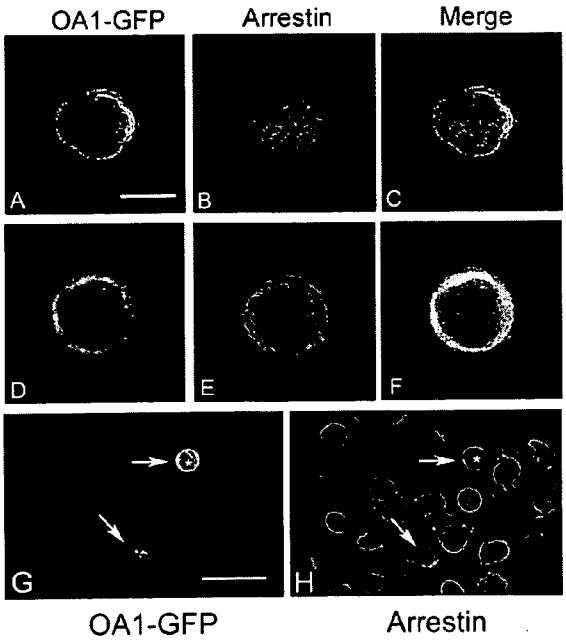
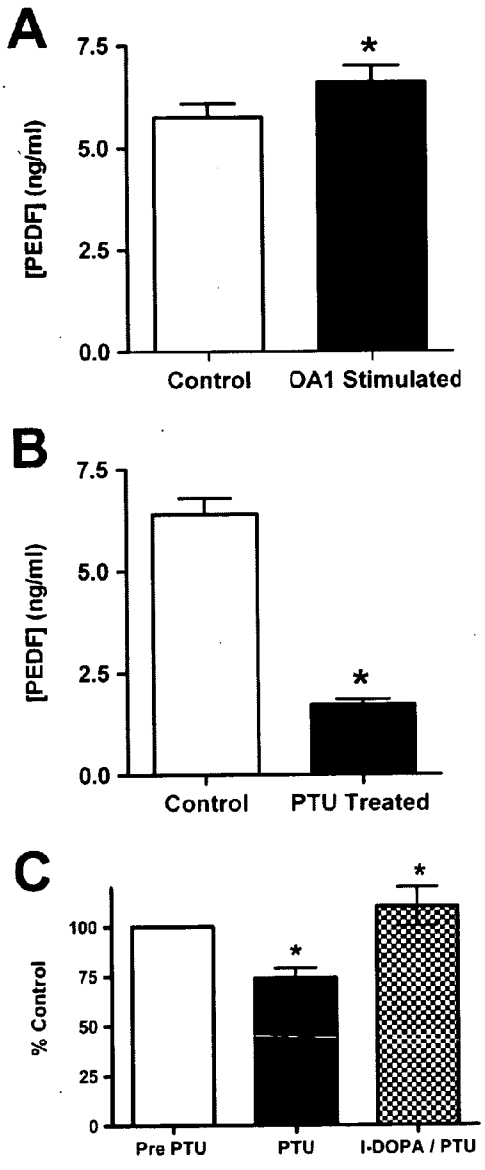


Figure 5


A
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5.0
2.5
0.0
[PEDF] (ng/ml)
*
Control
OA1 Stimulated
B
7.5
5.0
2.5
0.0
[PEDF] (ng/ml)
*
Control
PTU Treated
C
100
75
50
25
0
% Control
*
*
Pre PTU
PTU
I-DOPA / PTU

Figure 6

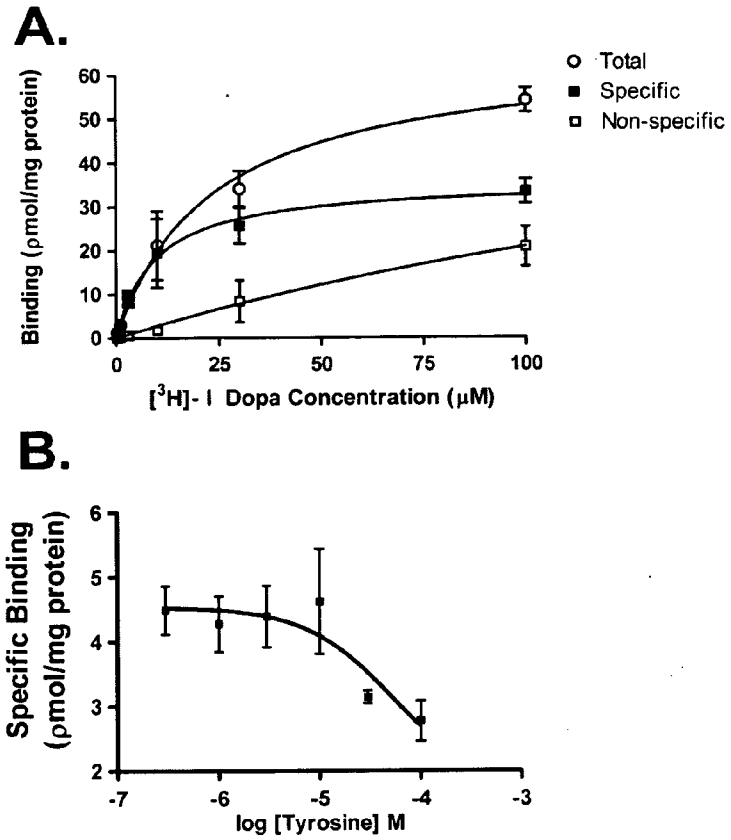

A.
Binding (pmol/mg protein)
60
50
40
30
20
10
0
0
25
50
75
100
[3H]- l Dopa Concentration (μM)
Total
Specific
Non-specific
B.
Specific Binding (pmol/mg protein)
6
5
4
3
2
-7
-6
-5
-4
-3
log [Tyrosine] M

Figure 7

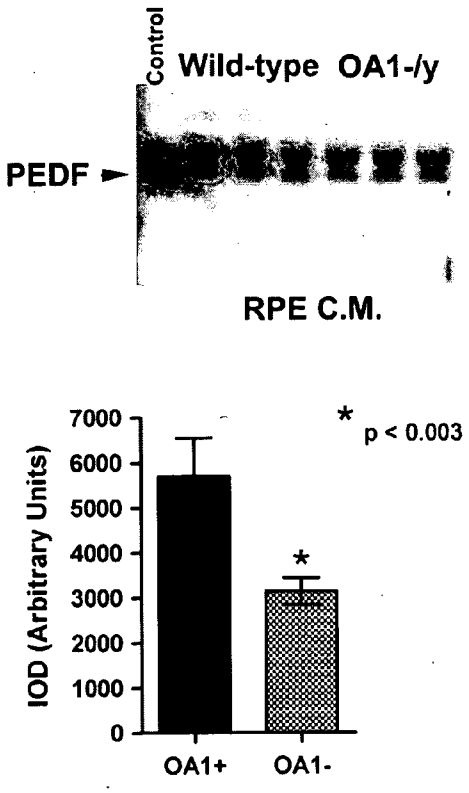
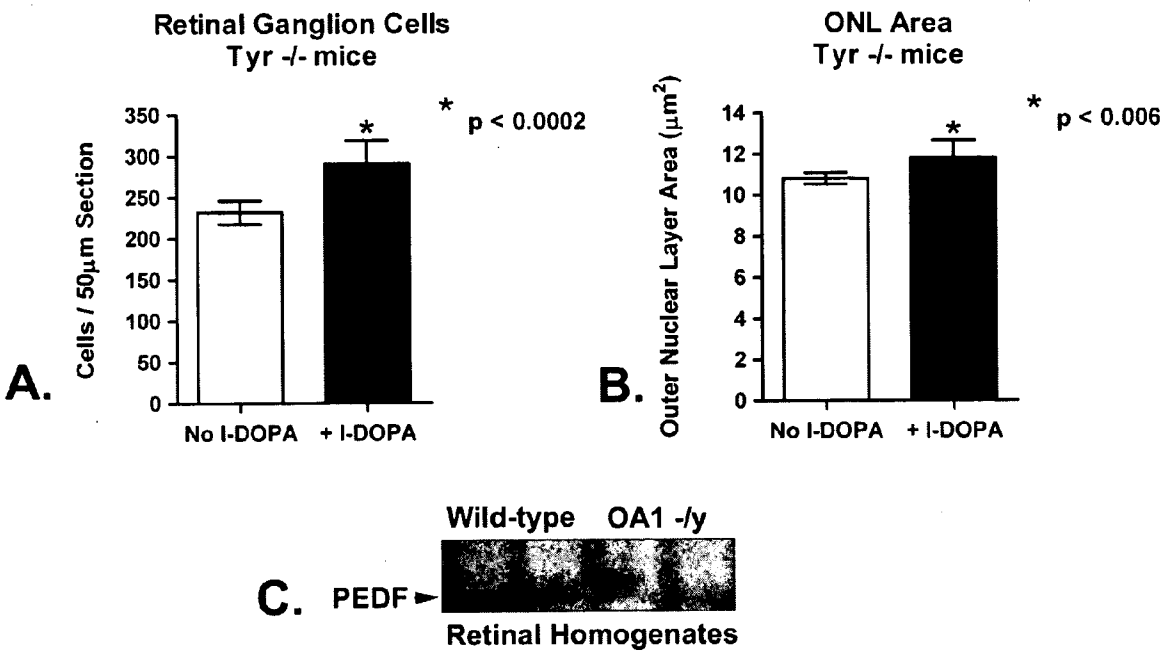

Control
Wild-type
OA1-/y
PEDF
RPE C.M.
IOD (Arbitrary Units)
7000
6000
5000
4000
3000
2000
1000
0
*
p < 0.003
*
OA1+
OA1-

Figure 8


Retinal Ganglion Cells
Tyr -/- mice
A.
Cells / 50µm Section
350
300
250
200
150
100
50
0
*
p < 0.0002
No l-DOPA
+ l-DOPA
ONL Area
Tyr -/- mice
B.
Outer Nuclear Layer Area (µm²)
14
12
10
8
6
4
2
0
*
p < 0.006
No l-DOPA
+ l-DOPA
Wild-type
OA1 -/y
C.
PEDF
Retinal Homogenates