8-2012

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Recommended Citation

Guha, S., Baltazar, G. C., Tu, L., Liu, J., Lim, J. C., Lu, W., Argall, A., Boesze-Battaglia, K., Laties, A. M., & Mitchell, C. H. (2012). Stimulation of the D5 Dopamine Receptor Acidifies the Lysosomal pH of Retinal Pigmented Epithelial Cells and Decreases Accumulation of Autofluorescent Photoreceptor Debris. *Journal of Neurochemistry, 122* (4), 823-833. http://dx.doi.org/10.1111/j.1471-4159.2012.07804.x

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Abstract

Optimal neuronal activity requires that supporting cells provide both efficient nutrient delivery and waste disposal. The incomplete processing of engulfed waste by their lysosomes can lead to accumulation of residual material and compromise their support of neurons. As most degradative lysosomal enzymes function best at an acidic pH, lysosomal alkalinization can impede enzyme activity and increase lipofuscin accumulation. We hypothesize that treatment to reacidify compromised lysosomes can enhance degradation. Here, we demonstrate that degradation of ingested photoreceptor outer segments by retinal pigmented epithelial cells is increased by stimulation of D5 dopamine receptors. D1/D5 receptor agonists reacidified lysosomes in cells alkalinized by chloroquine or tamoxifen, with acidification dependent on protein kinase A. Knockdown with siRNA confirmed acidification was mediated by the D5 receptor. Exposure of cells to outer segments increased lipofuscin-like autofluorescence, but SKF 81297 reduced autofluorescence. Likewise, SKF 81297 increased the activity of lysosomal protease cathepsin D in situ. D5DR stimulation also acidified lysosomes of retinal pigmented epithelial cells from elderly ABCA4$^{-/-}$ mice, a model of recessive Stargardt's retinal degeneration. In conclusion, D5 receptor stimulation lowers compromised lysosomal pH, enhancing degradation. The reduced accumulation of lipofuscin-like autofluorescence implies the D5 receptor stimulation may enable cells to better support adjacent neurons. © 2012 The Authors. Journal of Neurochemistry © 2012 International Society for Neurochemistry.

Keywords

autophagy; dopamine; lipofuscin; lysosomal pH; retina

Disciplines

Dentistry

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Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 122 | 823–833

doi: 10.1111/j.1471-4159.2012.07804.x

ORIGINAL ARTICLE

Stimulation of the D5 dopamine receptor acidifies the lysosomal pH of retinal pigmented epithelial cells and decreases accumulation of autofluorescent photoreceptor debris

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Abstract:

Optimal neuronal activity requires that supporting cells provide both efficient nutrient delivery and waste disposal. The incomplete processing of engulfed waste by their lysosomes can lead to accumulation of residual material and compromise their support of neurons. As most degradative lysosomal enzymes function best at an acidic pH, lysosomal alkalinization can impede enzyme activity and increase lipofuscin accumulation. We hypothesize that treatment to reacidify compromised lysosomes can enhance degradation. Here, we demonstrate that degradation of ingested photoreceptor outer segments by retinal pigmented epithelial cells is increased by stimulation of D5 dopamine receptors. D1/D5 receptor agonists reacidified lysosomes in cells alkalinized by chloroquine or tamoxifen, with acidification dependent on protein kinase A. Knockdown with siRNA confirmed acidification was mediated by the D5 receptor. Exposure of cells to outer segments increased lipofuscin-like autofluorescence, but SKF 81297 reduced autofluorescence. Likewise, SKF 81297 increased the activity of lysosomal protease cathepsin D *in situ*. D5DR stimulation also acidified lysosomes of retinal pigmented epithelial cells from elderly ABCA4$^{-/-}$ mice, a model of recessive Stargardt's retinal degeneration. In conclusion, D5 receptor stimulation lowers compromised lysosomal pH, enhancing degradation. The reduced accumulation of lipofuscin-like autofluorescence implies the D5 receptor stimulation may enable cells to better support adjacent neurons.

Keywords: autophagy, dopamine, lipofuscin, lysosomal pH, retina.

J. Neurochem. (2012) **122**, 823–833.

Proper neuronal function depends on the ongoing ability of adjacent cells to provide nutrients and process extruded material. In this respect, sustained photoreceptor function requires the regular delivery of visual cycle components and degradation of phagocytosed photoreceptor outer segments (POSs) by the retinal pigmented epithelium (Strauss 2005). Lysosomes of the retinal pigmented epithelial (RPE) cells are key organelles in this degradative pathway, and proper maintenance of the lysosomal environment is essential for the breakdown of material introduced through both the

Received February 24, 2012; revised manuscript received April 27, 2012; accepted May 10, 2012.

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Abbreviations used: AKAP, A-kinase anchoring proteins; CHQ, chloroquine; D5DR, D5-type dopamine receptor; FACS, fluorescence-activated cell sorter; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine; pH_L – lysosomal pH, POS, photoreceptor outer segments; PKA, protein kinase A; PKI, protein kinase inhibitor; PVDF, Polyvinylidene fluoride; RPE, retinal pigmented epithelial; TMX, tamoxifen.

ORIGINAL ARTICLE

autophagy and phagocytic pathways (Kon and Cuervo 2010). Most lysosomal enzymes are pH sensitive with optimal activity between 4.0 and 5.0 (Hayase and Tappel 1970; Mego 1984). The sharp pH dependence of key lysosomal enzymes predicts that even a moderate elevation of pH will lower enzyme efficiency and reduce the clearance of material. The control of lysosomal pH (pH_L) is complex, requiring balanced activities of vHATPase pumps, counter anion channels and pumps, and calcium transporters (Ohkuma *et al.* 1982; Jentsch 2007; Patel and Docampo 2010). While lysosomogenesis requires the appropriate delivery of these transporters to the lysosomal membrane, it is likely that their activity, and thus the pH_L, can be regulated on a rapid timescale in response to environmental changes.

We have previously determined that elevation of cytoplasmic cAMP restores the pH_L of compromised RPE cells to more acidic levels (Liu *et al.* 2008). The reacidification is inhibited by membrane-permeable myristoylated PKI amide which specifically binds to the catalytic site of cAMP-activated protein kinase A (PKA), strongly implicating PKA. Importantly, cAMP-triggered reacidification was proven functionally relevant, as it increased the degradation of outer segments by cultured RPE cells (Liu *et al.* 2008).

As elevation of cytoplasmic cAMP can reacidify lysosomes and improve outer segment degradation, agonists that stimulate receptors coupled to the $G_{\alpha s}$ protein, which stimulates adenylyl cyclase to produce cAMP, could prove beneficial in the treatment of conditions characterized by excessive accumulation of partially degraded material such as lipofuscin. Dopamine acts at two families of receptors, D1-like (D1 and D5) and D2-like (D2, D3, and D4) (Beaulieu and Gainetdinov 2011) with the D1-like receptors coupled to $G_{\alpha s/olf}$ (Konig and Gratzel 1994; Sidhu *et al.* 1998) and D2-like receptors couples to $G_{\alpha i}$. As dopamine receptors were identified on RPE cells (Versaux-Botteri *et al.* 1997) and D1-like receptor agonists are being tested for treatment of a variety of neural disorders (Cadet *et al.* 2010), we examined the role of D1-like dopamine receptors in the acidification of lysosomes in RPE cells.

Methods

ARPE-19 cells

ARPE-19 cells (ATCC) were grown to confluence in 25 cm^2 culture flasks in a 1 : 1 mixture of Dulbecco's modified Eagle medium and Ham's F12 medium with 3 mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 mg/mL fungizone and/or 50 μg/mL gentamicin, plus 10% fetal bovine serum (all Invitrogen Corp).

Measurement of lysosomal pH from ARPE-19 cells

ARPE-19 cells were grown in black 96-well plates, rinsed three times with isotonic solution [(IS; (in mM) NaCl 105, KCl 5, HEPES Acid 6, Na HEPES 4, $NaHCO_3$ 5, mannitol 60, glucose 5, $MgCl_2$ 0.5, $CaCl_2$ 1.3)] and incubated with 5 μM LysoSensor Yellow/Blue (Invitrogen Corp.) diluted with IS. Extensive trials have found the optimal response is obtained with 3-min dye loading and recorded within a 15-min post-incubation (Liu *et al.* 2008). Fluorescence was measured with a Fluoroskan 96-well plate reader (ThermoFisher Scientific, Waltham, MA, USA). Lysosomal pH was determined from the ratio of light excited at 340 nm versus 380 nm (> 520 nM em) and calibrated by exposing cells to 10 μM H^+/Na^+ ionophore monensin and 20 μM H^+/K^+ ionophore nigericin in 20 MES, 110 KCl, and 20 NaCl at pH 4.0–6.0 for 15 min.

siRNA silencing of D1 or D5 receptors

DRD1 and DRD5 expression was silenced using manufacturer's protocols. ARPE-19 cells were transfected with siRNAs specific for DRD1 receptor (s4283) or DRD5 receptor (s4291) purchased from Ambion, and 70–80% confluent ARPE-19 grown in 25 cm^2 flasks were transfected with siRNA using Amaxa Cell Line Nucleofector Kit V (VCA 1003, Lonza, NJ, USA). 1×10^6 cells were used per condition. Cells transfected with scrambled siRNA (Silencer negative control 1, catalog number 4611; Ambion, Austin, TX, USA) served as a negative control. As an additional control, cells were mock-transfected using transfection reagent alone. The D1, D5, or scrambled siRNA was used at a final concentration of 300 nM. Lysosomal pH was determined 72 h after transfection.

Western blots

ARPE-19 cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% Na-Deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and protease inhibitor cocktail) and centrifuged at 13000 *g* for 10 min at 4°C. Protein concentrations were determined using the BCA kit (Pierce, Rockford, IL, USA). Protein lysates were loaded in each lane in sample buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, and 0.05 M Tris-HCl, pH 6.8), separated on SDS–PAGE (Biorad, Hercules, CA, USA) and transferred to polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). For identification of the dopamine receptors, 35 μg protein was run on a 10% gel, blots were blocked with 5% non-fat milk in PBS and incubated overnight with rabbit anti-D5DR (1 : 2000) or mouse anti-D1DR (1 : 1000; both Santa Cruz Biotechnology, CA, USA). Mouse anti-β-actin was used as a control for normalizing (1 : 1000; Sigma, St. Louis, MO, USA). Visualization of the primary antibody was performed by incubating membranes with the corresponding peroxidase-conjugated secondary antibody (1 : 3000; GE Healthcare, Waukesha, WI, USA) for 1 h at 25°C. Finally, the blots were developed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, now GE Healthcare, Piscataway, NJ, USA) and captured on an ImageQuant LAS 400 image reader (GE Healthcare). Bands were quantified using the Alphaimager HP gel documentation system (ProteinSimple, Santa Clara, CA, USA).

POS membrane preparation

Fresh bovine retinas were isolated in the light under sterile conditions as previously described (Boesze-Battaglia and Yeagle 1992). Thawed retinas were agitated in 30% (w/w) buffered sucrose solution (containing 5 mM HEPES pH 7.4, 65 mM NaCl, 2 mM $MgCl_2$) followed by centrifugation in a Sorvall SS-34 rotor (7 min, 700 rpm, 4°C). The supernatant was diluted in two volumes of 10 mM HEPES pH 7.4 and further centrifuged (Sorvall SS-34 rotor,

20 min, 3600 *g*, 4°C). The resulting pellet was then homogenized and layered on top of a discontinuous sucrose density gradient. Density gradient solutions of 36, 32, and 26% sucrose (w/w) were employed, and POS membranes were harvested from the 26%/32% sucrose solution interface (Papermaster and Dreyer 1974). POS prepared this way was washed in three volumes of 0.02 M Tris buffer, pH 7.4 (Sorvall SS-34 rotor, 10 min, 20 000 *g*, 4°C). The pellet was resuspended in 2.5% (w/w) buffered sucrose solution and POS stored at −80°C.

Visualization of cellular autofluorescence

ARPE-19 cells were plated to confluence on 12 mm cover slips. The cells were then incubated without or with POS (10^6/mL) for 7 days. Culture medium and POS were renewed every alternate day during this time. After the final incubation, cells were washed to remove the non-internalized POS, and after waiting for a 2 h ''chase'' period for remaining material to be internalized, cells were fixed with paraformaldehyde and stained with DAPI for 1 min to visualize the nuclei. For localization of POS-associated autofluorescence, cells exposed to the outer segments for 7 days were incubated in 5 μM LysoTracker Red DND-99 (Invitrogen Corp) in cell culture medium for 15 min. Cells were washed again before imaging with a Nikon A1 inverted confocal microscope. Images were acquired and processed with NIS-Elements software (Nikon Instruments Inc., Melville, NY, USA).

Flow cytometry

ARPE-19 cells were grown to confluence in 6-well plates and incubated with POS (10^6/mL) for 2 h (pulse); the cells were washed thoroughly to remove non-internalized POS followed by a 2-h chase. Subsequently, the cells were incubated with and without 10 μM SKF 81297. Culture medium and POS were renewed every alternate day for 7 days. For flow cytometric quantification of lipofuscin-like autofluorescence, cells were repeatedly washed, detached with trypsin, and analyzed on one of two flow cytometers (FACS Calibur, BD Biosciences, Heidelberg, Germany or LRSII, BD Biosciences, Franklin Lakes, NJ, USA) using the FITC channel (excitation laser wavelength, 488 nm; detection filter wavelength, 530/30 nm). Cell debris and cell clusters were identified and excluded from the run analysis using FTC and SSC. Over 10 000 gated events were recorded.

Assessment of degradative enzyme activity using BODIPY FL-pepstatin A probe

Cathepsin D activity was measured with the fluorescent probe BODIPY FL-pepstatin A (Invitrogen). The probe itself is synthesized by covalently conjugating the BODIPY (Boron dipyrromethene difluoride) fluorophore to pepstatin A, a potent and selective inhibitor of cathepsin D. As the probe binds to the active site of cathepsin D, fluorescence intensity provides a measure of the activity of cathepsin D. To quantify cathepsin D activity, cells were grown to confluence on black-walled, clear-bottomed 96-well plates until confluent, and then incubated for 48 h in either control culture medium, 10 μM CHQ in medium, or 10 μM CHQ + 10 μM SKF 81297. Cells were then incubated in 1 μM BODIPY probe at 37°C in the dark. After washing, fluorescence was quantified using a Fluoroskan plate reader at 485 nm/527 nm (ex/em). Background fluorescence was subtracted from the plates.

Isolation and measurement of lysosomal pH from fresh ABCA4$^{-/-}$ mouse RPE cells

ABCA4$^{-/-}$ mice were a kind gift from Dr. Gabriel Travis of the Jules Stein Eye Institute, UCLA. All mice were treated in accordance with University of Pennsylvania IACUC. Mice were reared at 5–15 lux and killed with a CO_2 overdose. Mouse eyes were isolated and processed as described (Liu *et al.* 2008). In brief, after enucleation, intact eyes were incubated in 2% dispase and 0.4 mg/mL collagenase IV for 45 min, rinsed and incubated in growth medium for 20 min (containing DMEM with 1 × MEM + non-essential amino acids, 3 mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 mg/mL Fungizone and/or 50 μg/mL gentamicin, plus 10% fetal bovine serum; all Invitrogen Corp). In some experiments, the anterior segments and retinas were removed and the eyecup was rinsed with Versene (Dow Chemical Corp., Midland, MI, USA) and incubated in 0.25% trypsin for 45 min. Sheets of RPE cells were separated from the choroid and triturated into single cells. Cells from two to six eyes were pooled, loaded with 2–5 μM LysoSensor Yellow/Blue for 5 min at RT, rinsed and distributed into wells of 384 well UV Star plates (Greiner Bio-One, Monroe, NC, USA) and measured as described above. Although eyes from ABCA4$^{-/-}$ mice were slightly autofluorescent, the signal from the dye was 100-fold greater, validating the measurements (Liu *et al.* 2008). Dopamine agonists were added to the bath 20 min before measurements were taken. Lysosomal pH was measured within 3-h post-mortem. Because of the reduced number of cells, measurements from fresh RPE cells were not calibrated and are expressed as ratio of fluorescence excited at 340 versus 380 nm and emitted > 527 nm.

Materials

SKF 81297, A68930, and A77636 were obtained from Tocris Bioscience (#s 1447, 1534, and 1701, respectively). All other reagents were obtained from Sigma Chemical Corp. unless otherwise indicated.

Data Analysis

Data are reported as mean ± SEM. Statistical analysis used a one-way ANOVA with appropriate post hoc test. Results with $p < 0.05$ were considered significant.

Results

D1/D5 receptor agonists acidify compromised lysosomes

Initial experiments examined the ability of D1-like receptor agonists to lower lysosomal pH in challenged ARPE-19 cells. Baseline pH_L levels were typically in the range of 4.5–4.8. Tamoxifen increases lysosomal pH rapidly in various cell types, presumably through its actions as both a tertiary amine and by increasing proton permeability (Altan *et al.* 1999; Chen *et al.* 1999). Previous work has demonstrated that lysosomal alkalinization of RPE cells by tamoxifen is independent of the estrogen receptor (Liu *et al.* 2008). The pH_L of cells exposed to 10 μM tamoxifen for 5 min rose significantly, while the absolute magnitude of the alkalin-

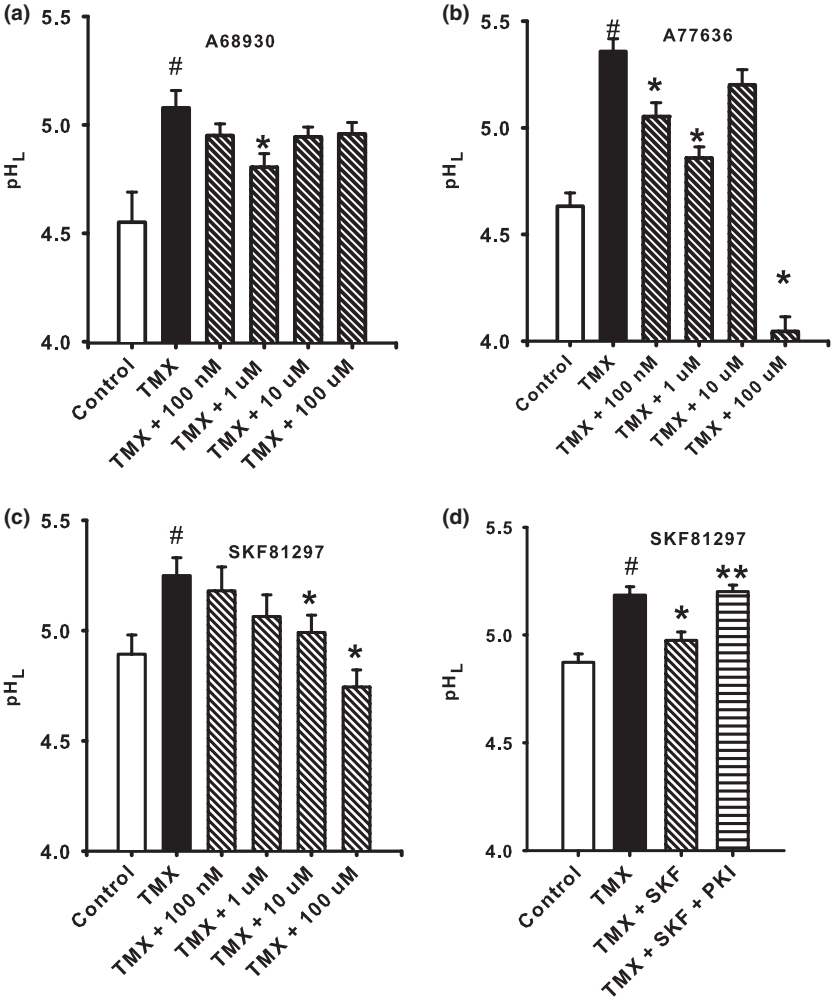

Fig. 1 D1/D5 receptor agonists lower lysosomal pH (pH_L) in challenged ARPE-19. (a) The agonist A68930 helped restore pH_L in ARPE-19 cells challenged by 10 μM of the lysotropic agent tamoxifen (TMX) (n = 14–40). (b) The D1/D5 agonist A77636 also reduced pH_L in cells exposed to 10 μM TMX (n = 44). (c) A third D1/D5 agonist SKF 81297 also acidified the lysosomes of cells treated with 10 μM TMX (n = 20). (d) The myristoylated protein kinase inhibitor (14–22) amide (100 μM), the cell-permeant inhibitor of protein kinase A, blocked the reacidifying effects of SKF 81297 (10 μM) on cells treated with TMX (10 μM), implying a role for protein kinase A in restoring pH_L (n = 94) ($^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$ vs. TMX, $^{**}p < 0.05$ vs. SKF 81297).

ization varied, the pH_L was usually in the range of 5.1–5.3. Chloroquine likewise alkalinized lysosomal pH.

The D1-like receptor agonist A68930 led to a substantial acidification of lysosomes in cells exposed to tamoxifen (Fig. 1a). The effect was rapid, with stable pH levels observed within 10 min of drug application. A reduction in lysosomal pH was observed with 1 μM, but did not increase with concentration, perhaps because of the ability of increasing levels to stimulate D2-like receptors (DeNinno *et al.* 1991). Other D1-like receptor agonists A77636 (Fig. 1b) and SKF 81297 (Fig. 1c) were also effective at rapidly acidifying lysosomes (Fig. 1c).

While all three D1/D5 receptor agonists displayed at least some efficacy in restoring lysosomal pH, additional experiments were performed using SKF 81297 as it displayed a relatively high selectivity for D1/D5 receptors over D2-like receptors (Andersen and Jansen 1990), and it gave the most consistent results in our trials. The ability of SKF 81297 to acidify compromised lysosomes was inhibited by myristoylated protein kinase inhibitor PKI (14–22) amide (100 μM), the cell-permeant inhibitor of protein kinase A (PKA) (Fig. 1d). PKI blocked the effects of SKF 81297 by 78% (n = 53), strongly implicating PKA in the acidification of lysosomes by SKF 81297. This is consistent with the ability of cell-permeant cAMP to acidify compromised lysosomes, and with the involvement of PKA in this general activation (Liu *et al.* 2008). In addition to its effects on tamoxifen-treated cells, SKF 81297 was also effective at reversing the alkalinization produced by chloroquine, reducing lysosomal pH from 5.60 ± 0.14 to 5.11 ± 0.09 (n = 24, $p < 0.005$). However, SKF 81297 had no effect on the baseline lysosomal pH of cells treated with neither tamoxifen nor chloroquine (n = 10; p = 0.99).

Acidifying effect of single dose of SKF 81297 is sustained

Although the experiments above strongly suggested that stimulation of D1-like receptors restored the lysosomal pH in compromised RPE cells, they were all conducted over the course of several hours. To determine whether D1-like receptor stimulation could induce a sustained restoration of lysosomal pH in compromised RPE cells, agonist SKF 81297 was added to chloroquine-treated cells, as chloro-

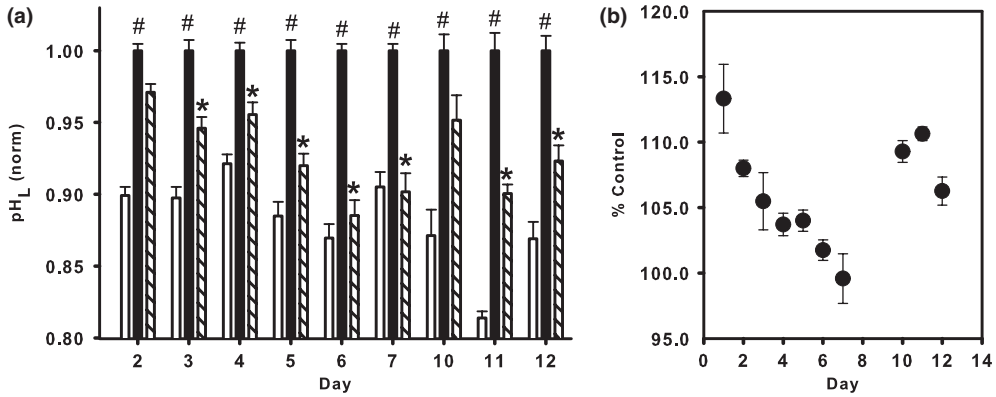

Fig. 2 Long-term restoration of lysosomal pH (pH_L). (a) The D1/D5 agonist SKF 81297 (10 μM) restored pH_L for multiple days in compromised ARPE-19 cells. Cells were treated with 10 μM chloroquine in the presence (hash bar) or absence (black bar) of 10 μM SKF 81297 on day 0; solution remained on cells but was not refreshed. pH levels were normalized to the mean value in chloroquine for each day's measurements to compensate for variation across trials. # CHQ versus control, $p < 0.05$, *$p < 0.05$ SKF 81297 versus CHQ; n = 16–40 from two to five trials. (b) The relative effectiveness of SKF 81297 to restore pH_L. Lysosomal pH in the presence of 10 μM SKF 81297 expressed as a percentage of the control pH in the same plate on the same day. The effectiveness of a single dose of SKF 81297 peaked 7 days after treatment, producing a near-complete restoration of pH_L. Calculated from mean of two to five trials derived from 16 to 40 measurements.

quine has been reported to induce prolonged effects in RPE cells *in vivo* (Peters *et al.* 2006). Confluent cells were treated with chloroquine in the presence and absence of SKF 81297 (both at 10 μM) on day 0, and the lysosomal pH was measured over the next 12 days. Medium was not changed for control or treatment wells. Measurements were performed in seven trials, each measuring lysosomal pH on different combination of days. While absolute levels varied somewhat with both plating and measurement day, trends were clearly evident. Extended exposure to 10 μM chloroquine induced a relatively constant elevation in lysosomal pH. In contrast, it was apparent that the acidifying effect of SKF 81297 changed with exposure duration (Fig. 2a). SKF 81297 lowered pH_L more effectively with increased exposure time. Remarkably, exposure of compromised cells to SKF 81297 completely restored the lysosomal pH to baseline levels at day 7 (Fig. 2b). Although the magnitude of the acidification was reduced, SKF 81297 still produced a significant acidification up to 12 days, the last day examined. No difference between treated and control cells could be discerned visually. Thus, a single dose of SKF 81297 produced a cumulative reacidification of compromised cells.

Molecular identification of D5 receptor subtype

As available pharmacological tools are currently unable to distinguish between the D1 and D5 receptors with reasonable specificity, molecular approaches were used to determine which receptor was responsible for the lysosomal acidification by SKF 81297. Western blots confirmed that an antibody against the D1DR detected a band at expected size of 74 kD. The intensity of the band was reduced by siRNA against the D1DR (Fig. 3a). Likewise, an antibody against the D5DR detected a band at the expected size of 45 kD, with the intensity of the band reduced by siRNA against the D5DR. When normalized to actin and quantified to levels in scrambled siRNA, the band intensity of D1DR was decreased to 57% by siRNA against D1DR while siRNA against the D5DR increased expression to 162% of scrambled levels. Levels of D5DR were decreased by siRNA against D5DR to 75%, with siRNA against the D1DR leading to 106% of scrambled levels.

As these siRNA probes were able to selectively reduce expression of the receptor target protein, their effect on the ability of SKF 81297 to restore acidity was tested. The baseline pH did not differ between cells transfected with scrambled siRNA, D1DR siRNA, D5DR siRNA, or transcription controls in seven separate transfection experiments (p 0.74, 0.68 and 0.53 vs. scrambled, respectively; transfection itself had a slight alkalinizing effect). To control for variations that occurred between trials, pH values were normalized to the mean value for scrambled control for each experiment, but still there was no difference in baseline levels ($p > 0.22$). However, significant differences were observed when the ability of SKF 81297 to acidify the lysosomes of compromised cells was examined. Tamoxifen produced a similar alkalinization of lysosomes in all cells. SKF 81297 acidified the lysosomes of cells transfected with scrambled siRNA or exposed to transfection medium. While SKF 81297 likewise acidified the lysosomes of cells transfected with D1DR siRNA, the drug had little effect on lysosomal pH in cells exposed to D5DR siRNA (Fig. 3b). When the % reacidification of the effect of tamoxifen was calculated, SKF 81297 blocked 100.4 ± 9.1% of the alkalinizing effects of tamoxifen in the presence of D1DR siRNA, while it blocked only 10.4 ± 19.1% of the alkalin-

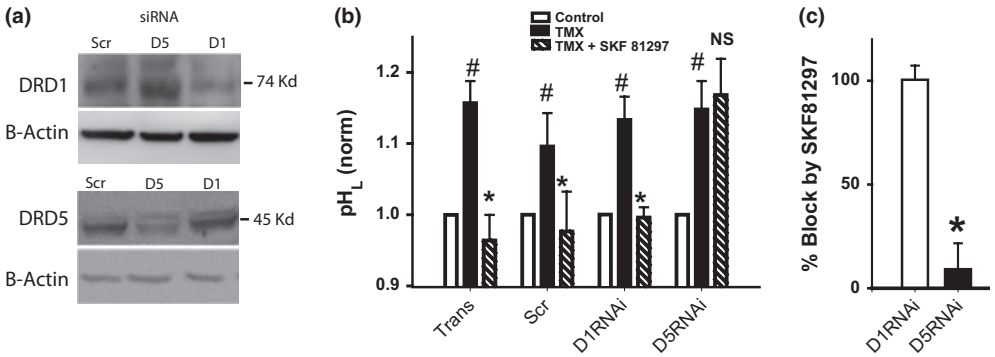

Fig. 3 Stimulation of D5 receptor restores lysosomal acidity. (a) Western blots confirm specificity of the gene knockdown, as siRNA against the D1 receptor reduced expression of the D1 receptor (DRD1) but not the D5 receptor (D5DR, top panel). Likewise siRNA against D5 receptor reduced expression of the DRD5 but not the D1DR. Scr, scrambled RNA. β-actin staining was used to normalize levels 72 h post transfection. (b) RNAi knockdown of D5 receptor but not D1 receptor removed acidification by 10 μM D1/D5 agonist SKF 81297. TransCon, transfection control; Scramb, scrambled RNAi; D1RNAi, RNAi against D1 receptor; D5RNAi, RNAi against D5 receptor. Paired *t*-test $^{\#}p < 0.05$ TMX versus Control $^{*}p < 0.05$, Tmx versus Tmx + SKF 81297, $n = 6$ plates, 2–4 wells each. Data were normalized to the mean control in each set to account for variation between each separate set of transfections. (c) Quantification of effect of receptor knockdown. The magnitude of the acidification by 10 μM SKF 81297 was defined as % reacidification = 100 × (TMX − (TMX + SKF))/(TMX − Control). The % reacidification was unaffected when cells were transfected with siRNA against D1DR. However, siRNA against the D5DR reduced the % reacidification to only 10%. This strongly implicates the D5 receptor in the reacidification by SKF 81297. Paired *t*-test, $^{*}p = 0.006$ versus D1RNAi, $n = 4$.

ization in the presence of D5DR siRNA ($p = 0.006$, Fig. 3c). This strongly suggested that the response was mediated by the D5 dopamine receptor.

Of note, while the immunoblots suggest an increase in D1DR expression with D5DR siRNA knockdown, there was no evidence of an effect on a physiological level as baseline lysosomal pH did not differ significantly between cells treated with D1DR siRNA or D5DR siRNA, and as mentioned, the effect of SKF 81297 was decreased, not increased, by D5DR siRNA. This further supports the role for the D5DR in lysosomal acidification.

D5 stimulation enhances degradative activity of RPE lysosomes

Degradative lysosomal enzymes are pH sensitive, acting optimally over a relatively narrow range of acidic values. As such, conditions which elevate lysosomal pH are predicted to reduce rates of degradation, whereas treatments to reacidify lysosomes are predicted to enhance degradation. RPE lysosomes are required to degrade photoreceptor outer segments phagocytosed daily (Kevany and Palczewski 2010). As such, the effect of lysosomal pH manipulation of outer segment degradation was tested.

Initial experiments were designed to confirm that outer segments were internalized to the lysosomes. Unlabeled photoreceptor outer segments were fed to confluent ARPE-19 cells for 2 h and then medium was returned. This procedure was repeated every other day for 7 days. On the final day, cells were maintained in outer segment-free medium for 2 h to ensure sufficient time for binding, phagocytosis, and trafficking. While cells not exposed to POS displayed little autofluorescence, cells exposed to POS displayed clear spots of autofluorescence when excited at 488 nm (Fig. 4a i, ii). As this pattern of autofluorescence suggests organelle staining, costaining with LysoTracker Red was examined. The punctate pattern of autofluorescent staining from outer segments overlapped with the pattern for LysoTracker Red, indicating that most of the autofluorescence was restricted to lysosome-like organelles at this point (Fig. 4a iii–vi).

Having established that photoreceptor outer segments were delivered to lysosomes within 2 h, the autofluorescence was quantified and the ability of SKF 81297 to alter this autofluorescence was calculated. The cells were fed photoreceptor outer segments for 2 h, kept in outer segment-free medium for 2 h to allow for internalization. After 2 h, cells were fed 10 μM SKF 81297 for 19 h, at which point the outer segment feeding was resumed. This complex ''pulse-chase'' protocol was followed to ensure that drug treatment did not interfere with POS binding or internalization.

Treatment with photoreceptor outer segments substantially increased cellular autofluorescence, whereas treatment with SKF 81297 clearly decreased autofluorescence (Fig. 4b). In five trials, treatment with outer segments raised autofluorescence over 3-fold, while exposure to SKF 81297 reduced autofluorescence by 73 ± 12% (Fig. 4c). This implies that stimulation of the D5 receptor can enhance digestion of photoreceptor outer segments.

To provide additional evidence that stimulation of the D5 receptor increased lysosomal activity, the binding of the

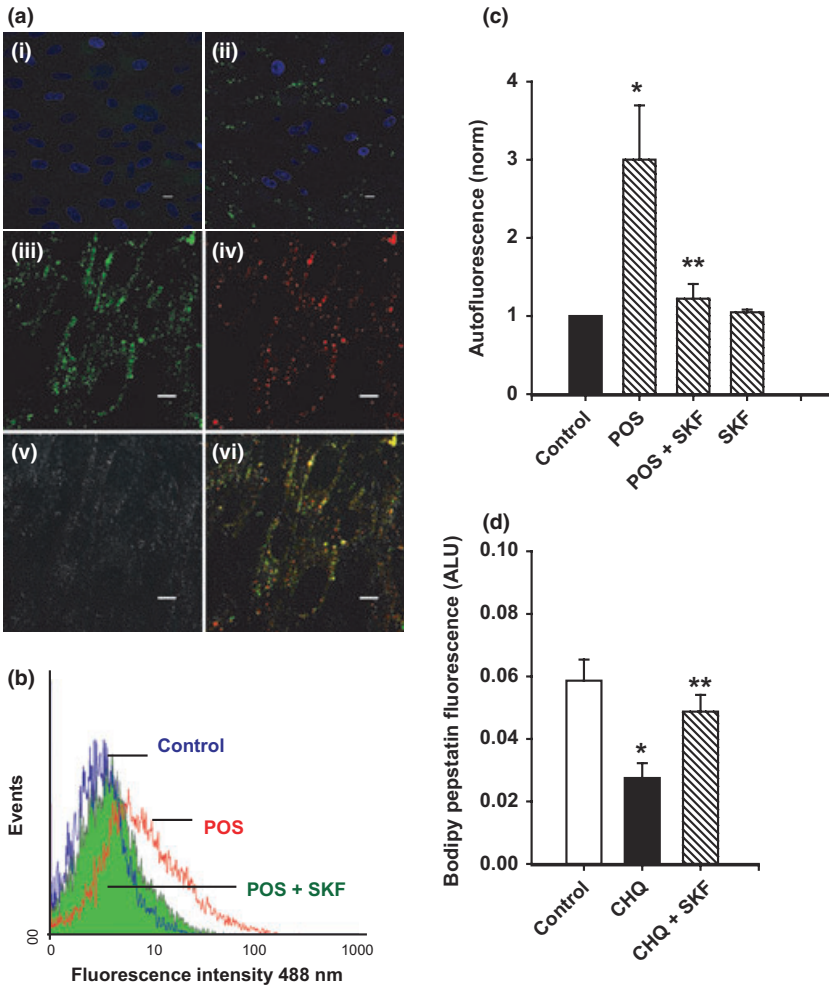

Fig. 4 D5 agonists reduce levels of photoreceptor outer segment autofluorescence. (a) Cultured ARPE-19 cells were examined by confocal fluorescence microscopy following 7 days of incubation without (i) or with (ii) unlabeled photoreceptor outer segments (POS). Lipofuscin-like cellular autofluorescence (green) was detected in (ii) using a fluorescein filter set (ex 480 nm, em 535 nm). Nuclei were visualized by DAPI staining blue. Scale bar = 10 μm. Autofluorescence associated with POS incubation (iii) pseudocolored green and the signal from LysoTracker Red (ex 540 nm, em > 570) showed considerable overlap (vi) implying the majority of POS were in acidic organelles 2 h after outer segments were removed from the bath. (v) DIC image. Scale bar = 10 μm. (b) SKF 81297 reduced the autofluorescence from internalized POS. Cells were fed POS for 3 h, washed, and 2-h chase period were allowed for outer segment delivery to the lysosomes. At this point, 10 μM SKF 81297 was added to the cells (adding the drug after the 2-h interval ensured effects were restricted to outer segment digestion and did not alter binding or phagocytosis). This two-stage treatment was repeated every 1–2 days for 1 week, with a total of three treatments. Cells were dissociated and the autofluorescence excited at 488 nm was determined using flow cytometry. Compared with control cells (blue), exposure to POS shifted the fluorescence to the right (red), indicating an increased fluorescence. Treatment with SKF 81297 shifted the curve back to the left (green) as autofluorescence was reduced. (c) Quantification of autofluorescence reduction by SKF 81297. The mean autofluorescence was increased by incubation with POS but restored to low levels by SKF 81297. SKF 81297 alone did not alter autofluorescence levels. Bars represent the mean ± SEM fluorescence in each sample and are representative of results in three separate experiments. Data were normalized to peak levels in untreated cells to control for variation between trials. *$p < 0.05$ versus control; **$p < 0.05$ versus POS. (d) Bodipy-pepstatin A binding is improved by SKF 81297. While binding of the probe was reduced by treating cells with 10 μM chloroquine for 48 h, concurrent exposure to 10 μM SKF 81297 restored fluorescence. These results are consistent with chloroquine decreasing activity of pH sensitive lysosomal enzyme cathepsin D, and of SKF 81297 restoring enzyme activity. *$p < 0.05$ vs. control; **$p < 0.05$ CHQ vs. CHQ + SKF, $n = 13$.

fluorescent Bodipy-pepstatin A to cells was assessed. Pepstatin A inhibits the lysosomal protease cathepsin D, and thus, fluorescence is indicative of cathepsin D activity *in situ*. Incubation of cells with 10 μM of chloroquine significantly decreased the fluorescence, as expected. However, coincubation of SKF 81297 with the chloroquine

substantially increased the pepstatin A fluorescence (Fig. 4d). These results are consistent with the ability of lysosomal alkalinization by chloroquine to decrease the activity of cathepsin D and the reacidification of lysosomes by SKF 81297 to restore activity. Together with the ability of SKF 81297 to reduce the autofluorescence associated with photoreceptor outer segments, these findings strongly suggest that SKF 81297 can increase the activity of degradative lysosomal enzymes in compromised cells.

Stimulation of D5 receptors acidifies lysosomes from RPE cells of ABCA4$^{-/-}$ mice

While the ability of D5 receptor stimulation to restore lysosomal pH in cells exposed to chloroquine or tamoxifen suggests general relevance, exposure to *N*-retinylidene-*N*-retinylethanolamine (A2E) may be a more direct challenge to RPE cells. A2E elevates the lysosomal pH of cultured RPE cells (Holz *et al.* 1999; Liu *et al.* 2008). The ABCA4$^{-/-}$ mouse model of recessive Stargardt's disease is characterized by excessive accumulation of A2E (Mata *et al.* 2001), and the lysosomal pH of these mice is elevated as compared with age-matched controls (Liu *et al.* 2008). Given the potential importance for RPE pathophysiology, the ability of D5 receptor stimulation to lower lysosomal pH in RPE cells from ABCA4$^{-/-}$ mice was examined.

RPE cells were freshly isolated from ABCA4$^{-/-}$ mice and lysosomal pH was measured *in vitro*. Exposure of RPE cells from 11-month-old mice to 1 μM A68930 or 1 μM A77636 decreased lysosomal pH (Fig. 5a). Interestingly, these drugs had no significant effect on the lysosomal pH of 5-month-old ABCA4$^{-/-}$ mice. Additional experiments demonstrated that SKF81297 also reduced the signal from 12-month-old ABCA4$^{-/-}$ mice (Fig. 5b).

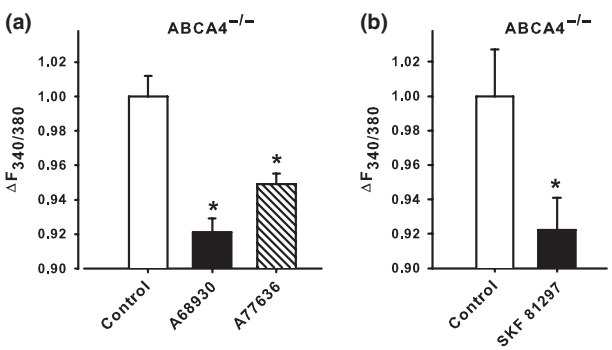

Fig. 5 Acidification of retinal pigmented epithelial (RPE) lysosomes from ABCA4$^{-/-}$ mice. (a) Simulation of dopamine D1-like receptors by A68930 (1 μM) and A77636 (1 μM) decreased pH_L of RPE cells freshly isolated from 11-month-old ABCA4$^{-/-}$ mice. *$p < 0.01$ versus untreated ABCA4$^{-/-}$. $n = 8$ measurements. (b) In a separate set of experiments, SKF 81297 (50 μM) also reduced the lysosomal pH in RPE cells freshly isolated from 12-month-old ABCA4$^{-/-}$ mice. *$p < 0.05$ versus untreated ABCA4$^{-/-}$. $n = 3$.

Discussion

Changes in lysosomal pH have direct and indirect actions on activity of degradative lysosomal enzymes. This study has demonstrated that in RPE cells, stimulation of the D5 dopamine receptor can enhance degradation and may be of benefit in conditions where lysosomal pH is increased. Several lines of evidence support this conclusion. Three different D1/D5 receptor agonists, A68930, A77636, and SKF 81297, all acidified compromised lysosomes in RPE cells (Fig. 1). Such reacidification occurred in lysosomes alkalinized by either tamoxifen or chloroquine. The acidification was dependent on the actions of PKA, consistent with pathways identified previously (Liu *et al.* 2008). Of note, a single dose of agonist SKF 81297 was sufficient to acidify lysosomes for 12 days, with complete restoration found maximally 5–7 days after treatment (Fig. 2). Knockdown of the D5 receptor reduced the acidification by SKF 81297, whereas knockdown of the D1 receptor did not (Fig. 3), implying that the D5 receptor was responsible. SKF 81297 increased the degradation of photoreceptor outer segments and reduced their lipofuscin, like autofluorescence and the activity of cathepsin D (Fig. 4), supporting a link between lysosomal acidification and increased activity of degradative enzymes. Finally, stimulation of the receptor lowered lysosomal pH of RPE cells from aged ABCA4$^{-/-}$ mice (Fig. 5), indicating that the pathways linking the D5 receptor to lysosomal acidification were maintained even in compromised RPE cells from ''middle aged'' mice. Overall, these findings demonstrate a clear link between stimulation of the D5 receptor, reduction of lysosomal pH, and improved degradation by lysosomal enzymes.

Receptor pharmacology

Analysis of individual dopamine receptors is complicated by the lack of specificity demonstrated by many of the pharmacological tools, and D1 and D5 (D1b) receptors share over 80% homology (Beaulieu and Gainetdinov 2011). Selective reduction of the D1 and D5 receptors using molecular approaches demonstrated that the acidification of lysosomes in RPE cells was mediated by D5 receptors. Although cultured bovine RPE cells were reported to contain predominantly D5 receptors (Versaux-Botteri *et al.* 1997), the presence of bands in the present study suggests cultured human ARPE-19 cells contain both D1 and D5 receptors. Although receptor expression may be coordinated, it is clear that only the D5 receptor mediated lysosomal reacidification in these cells.

The agonists A77636 and A68930 are generally characterized as D1-like receptor agonists. A68930 acts at D1 receptors with an EC_{50} of 2.9 nM, and at D2 receptors with an EC_{50} of 3.8 μM (DeNinno *et al.* 1991). A77636 acts at D1-like receptors with a Ki = 39.8 nM and at D2-like

receptors with a Ki > 10 μM, however (Kebabian *et al.* 1992). Enhanced stimulation of D2 receptors at higher concentrations may complicate effects of A68930 on lysosomal acidification; as D2 receptors are coupled to G_i proteins stimulation would work against an acidification. However, the relative selectivity of A68930 at the D1 versus D5 receptor may also contribute to the response (Nergårdh *et al.* 2005). Although SKF 81297 is reported to act more selectively at D1 receptors (Beaulieu and Gainetdinov 2011), present results argue that it is also an effective agonist at D5 receptors. It should be noted that although the majority of experiments in this study were performed with SKF 81297, this does not rule out possible beneficial effects from other D1/D5 agonists. The oral availability of A77636 may be of interest in this regard (Kebabian *et al.* 1992).

Mechanisms of action

The ability of D5 receptor stimulation to lower lysosomal pH is most likely related to an elevation of cAMP levels. We have previously demonstrated that increasing cytoplasmic cAMP, either directly or via G-protein-coupled receptors, lowers lysosomal pH in RPE cells (Liu *et al.* 2008). Figure 1d demonstrates that the acidifying actions of the agonist SKF 81297 are inhibited by PKI (14–22) amide, strongly suggesting that PKA is required for lysosomal acidification. Preliminary data suggest that the PKA-activated Cl^- channel CFTR contributes to the PKA-dependent acidification of RPE lysosomes (Mitchell *et al.* 2008). Phosphorylation by PKA was recently demonstrated to enhance insertion of the vHATPase into the plasma membrane of proton-secreting kidney cells, enhancing secretion (Alzamora *et al.* 2010). It is possible that an analogous mechanism occurs on lysosomal membranes, and PKA also acts by enhancing expression of proton pumps in lysosomal membranes, although this remains to be determined. The inability of SKF 81279 to decrease baseline lysosomal pH is consistent with data indicating cAMP exhorts an acidification of greater magnitude from cells with alkalinized lysosomes than from baseline (Liu *et al.* 2012). This suggests a model where the cAMP increase following D5DR stimulation affects the regulation of lysosomal pH but not its baseline maintenance.

It is important to note that stimulation of the D5 receptor effectively reacidified RPE cells, overriding the alkalinization caused by either tamoxifen or chloroquine. Further, receptor stimulation reacidified RPE cells from $ABCA4^{-/-}$ mice, where excess A2E is likely to increase lysosomal pH (Holz *et al.* 1999; Mata *et al.* 2000; Bergmann *et al.* 2004). Overall, this implies that the ability of D5 receptor stimulation to reacidify lysosomes is not specific for a particular type of alkalinizing insult. In other words, the lysosomal reacidification is mediated via a general mechanism that may be effective against a range of insults.

Physiological Implications

Stimulation of the D5 receptor in RPE cells by SKF 81297 induced several responses that suggest the approach may be worth further consideration. A single exposure to 10 μM SKF 81297 lowered lysosomal pH in chloroquine-treated ARPE-19 cells for 12 days. The restoration of acidity was cumulative, with the pH equal to control levels after 7 days. Importantly, the autofluorescence excited at 488 nm was substantially increased in cells fed outer segments, consistent with a lipofuscin-like accumulation. However, treatment with SKF 81297 decreased this autofluorescence by 54 ± 4%. Not only does the improved clearance by SKF 81297 reinforce the relationship between lysosomal pH and degradative enzyme activity, but it also provides crucial functional evidence that this approach can improve the clearance of outer segments by these cells. It is important to stress that the pulse-chase approach to feeding cells outer segments ensured that outer segments were predominantly within lysosomes before cells were treated with SKF 81297, implying the actions were specifically due to changes in lysosomal pH and not the binding or internalization stages. As such, the approach also applies to material delivered through autophagic pathways to the lysosomes. Experiments with Bodipy-pepstatin provide additional support for this link, and stress that the reacidification induced by SKF-81297 occurs over a relevant pH values. Like many lysosomal enzymes, the activity of Cathepsin D is sharply dependent of the pH of the surrounding milieu, with activity falling by 80% once the pH has risen to only 5.3 (Barrett 1977). These experiments demonstrate that the functional effects of SKF-81297 on compromised RPE cells are substantial and likely to improve degradation of compromised lysosomes in RPE cells.

The ability of D5 receptor stimulation to enhance outer segment degradation in RPE cells with alkalinized lysosomes may have implications for patients with macular degenerations such as Stargardt's disease, for the lysosomal pH was increased in RPE cells from the $ABCA4^{-/-}$ mouse model of the disease (Liu *et al.* 2008). As such, the ability of receptor agonists to acidify lysosomes from RPE cells taken from older $ABCA4^{-/-}$ mice is important, for it implies that the mechanisms necessary to mediate receptor-driven reacidification of lysosomes are still functioning even though the lysosomes in the cells have been distressed for an extended period. The lysosomal pH increased with age in these mice (Liu *et al.* 2008), consistent with the enhanced accumulation of A2E with age (Mata *et al.* 2000). The negligible effect of D5DR agonists in younger mice with near-normal lysosomal pH may be related to the increased magnitude of acidification induced by cAMP when given to cells with alkalinized lysosomes. This is also supported by the observation that SFK 81297 had no effect on cells that had not been treated with an alkalinizing agent. This makes the treatment of impaired tissue with D5 agonists ideally suited, as the lysosomal pH of any healthy cells should be minimally affected.

The potential therapeutic usefulness of D5 receptor stimulation will be limited by the specificity of the actions. Although dopamine contributes to numerous processes in the retina, D4 receptors mediate many of the effects of dopamine on retinal function including modulation of photoreceptor proteins (Nir *et al.* 2002; Pozdeyev *et al.* 2008). In the neural retina, as in the brain, there is a relative paucity of D5 receptors (Beaulieu and Gainetdinov 2011), with immunohistochemical identification suggesting localization is limited to a selection of amacrine cells and ganglion cells (Versaux-Botteri *et al.* 1997; Nguyen-Legros *et al.* 1999). In the RPE, the overall effects of D5 receptor stimulation of RPE are likely to be complex. Elevation of cAMP slows the rate of photoreceptor phagocytosis by RPE cells (Edwards and Flaherty 1986), and stimulation of D5 receptors may well reduce phagocytosis rates (Masri *et al.* 1996). As phagocytosis generally follows a circadian pattern, temporal control of the delivery of agonists may enable the effects on phagocytosis and lysosomal degradation to be separated *in vivo* as they were *in vitro*. In this regard, chronic treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine decreased the dopaminergic amacrine cells in the retina and significantly increased the number of highly fluorescent yellow lipofuscin granules in the RPE (Mariani *et al.* 1986). The lipofuscin associated with dopamine reduction displayed the same spectral profile as the lipofuscin in RPE cells from older animals. This suggests a model where by dopamine released from the amacrine cells normally keeps the lysosomal pH of RPE cells low and outer segment degradation running smoothly; the removal of this source of dopamine may lead to lysosomal alkalinization and accumulation of autofluorescent debris There are, of course, alternative explanations, but this model is consistent with the results in Fig. 4, whereby application of SKF 81297 substantially reduced the degree of autofluorescence in RPE cells. Overall, these findings suggest that D5 receptor stimulation may be a critical pathway to enhance degradation in RPE cells *in vivo*.

The control of lysosomal function in supportive cells may also have broader implications for neuronal–glial interactions. Recently, astrocytes were shown to actively phagocytose material extruded from the midst of axons (Nguyen *et al.* 2011). It remains to be seen whether alkalinization of astrocytic lysosomes can impede this novel function, or whether stimulation of the D5DR can enhance this process.

Acknowledgements

This work is supported by grants from the NIH EY013434 and EY015537 (CHM), EY017045 (AML), EY018705 (KBB), Vision Research Core Grant EY001583 (CHM, AML, KBB), Research to Prevent Blindness (AML), the Paul and Evanina Bell Mackall Foundation Trust (AML), and the Jody Sack fund (WL). The authors thank Michael Boulton for advice on the FACS analysis of autofluorescent cells, Bruce Shenker at the UPenn SDM Flow Cytometry facility, and Gabriel Travis for the ABCA4$^{-/-}$ mice. The authors have IP associated with these findings, but no financial assistance has been received.

Portions of this work have been previously presented in abstract form (Guha *et al.* 2009, 2010).

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