Glucagon- and Secretin-Related Peptides Differentially Alter Ocular Growth and the Development of Form-Deprivation Myopia in Chicks

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PURPOSE. Exogenous glucagon inhibits the induction of myopia in chicks, but the endogenous peptide and receptor that regulate eye growth are unknown. The purpose of this study was to determine which peptides and receptors in the glucagon-secretin family play a role in the control of ocular growth.

METHODS. The effect of intravitreally injected peptides on the development of form-deprivation (FD) myopia and on the growth of eyes with unrestricted vision was determined by refraction and A-scan ultrasonography. Chicks received three injections, one every 48 hours, of secretin-related peptides (porcine secretin, human peptide histidine-isoleucine-amide-27, vasoactive intestinal peptide [VIP], VIP fragment 6-28, or pituitary adenylate cyclase-activating polypeptide; 10^{-8}–10^{-4} M in 20 μL) or five injections of proglucagon-derived peptides (human glucagon, oxyntomodulin, miniglucagon, or glucagon-like peptide [GLP]-2 or chicken GLP-1). Immunohistochemistry was used to detect proglucagon-derived peptides in the eye.

RESULTS. Secretin-related peptides had no effect on FD myopia, whereas some proglucagon-derived peptides did. Both glucagon and oxyntomodulin dose-dependently inhibited development of myopia, primarily by inhibition of vitreous chamber elongation ($EC_{50} = 10^{-4}$ M and $10^{-5.5}$ M, respectively). GLP-1 increased deprivation-induced myopic refractive error by altering anterior chamber development. None of the peptides significantly affected refractive error in eyes with unrestricted vision, although changes in anterior and posterior eye growth were observed in response to glucagon, oxyntomodulin, GLP-1, and miniglucagon. Immunoreactivity for miniglucagon and GLP-1 was colocalized in glucagon-immunoreactive amacrine cells.

CONCLUSIONS. Prevention of experimental myopia by exogenous glucagon is mediated by receptors selective for glucagon and oxyntomodulin, indicating that glucagon-like peptides and receptors are probable endogenous retinal regulators of the development of myopia. (*Invest Ophthalmol Vis Sci.* 2005;46: 3932–3942) DOI:10.1167/iovs.04-1027

Postnatal ocular growth is visually regulated so that, in the ideal circumstance, the eye grows to reach a balance between physical length and refractive power, allowing light from distant objects to be sharply focused on the retinal photoreceptors.[1] In the chick, glucagonergic amacrine cells are one of the most promising candidates for a direct role in the regulation of ocular growth by neural processing in the retina.[2–4] Visual conditions that prevent development of myopia stimulate expression of the immediate-early gene ZENK (zif/268, Egr-1) in glucagon-synthesizing amacrine cells, whereas conditions that promote excessive ocular growth stimulate little or no expression of ZENK.[2,3] There is also a concordant increase in the expression of mRNA for the glucagon precursor, proglucagon, during treatment with positive lenses.[5] This implies that an increase in the rate of synthesis and release of one or more proglucagon-derived peptides acts as a "stop" signal for ocular growth. Similarly, administration of exogenous glucagon or the synthetic glucagon agonist $Lys^{17,18}, Glu^{21}$-glucagon-NH_2 has been found to inhibit dose-dependently the axial elongation of the eye that would otherwise result from form deprivation (FD) or negative lens wear.[4,6] However, the concentration of glucagon necessary to inhibit ocular growth in response to FD (10^{-5} M in 20 μL in the syringe; see Vessey et al.[7]), although not taking into account dilution and distribution in the vitreous, is high compared with the affinity of the peptide for its receptors ($K_d = 10^{-9}$ M; see Ref. 8). Furthermore, the effects of glucagon receptor antagonists on ocular growth and refractive error development are weak and inconsistent.[4,6] Thus, although the activity of glucagon-containing amacrine cells is increased by visual stimuli that restrain eye growth, it is possible that glucagon is not an endogenous mediator of this signal or that glucagon (exogenous or endogenous) restrains growth via a related receptor rather than through the glucagon receptor itself.

Glucagon is a 29-amino-acid peptide, originally isolated as a pancreatic hormone released in response to hypoglycemia. More recently, however, glucagon has also been proposed as a neurotransmitter in the central nervous system.[9,10] It is produced by proteolytic processing of the precursor peptide, proglucagon, which can also be processed to produce many other biologically active peptides.[10,11] Proglucagon belongs to the secretin-glucagon superfamily of peptides, which although related, exert their actions through unique and specific G protein-coupled receptors.[10] Some other peptides in the related family include the proglucagon-derived peptides, vasoactive intestinal peptide (VIP), secretin, peptide histidine-isoleucine (PHI), peptide histidine-methionine (PHM), and pituitary adenylyl cyclase activating peptide (PACAP).[12,13] Although receptors for most of these peptides are unlikely to mediate the actions of glucagon, some cross-reactivity at high concentrations has been reported.[12]

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TABLE 1. Amino Acid Sequences of Human and Chick Proglucagon-Derived Peptides

Peptide Name	Human Sequence	Chick Sequence	Homology (%)
Glucagon	HSQGTFTSDY SKYLDSRRAQ DFVQWLM**N**T	HSQGTFTSDY SKYLDSRRAQ DFVQWLM**S**T	97
Miniglucagon	AQDFVQWLM**N** T	AQDFVQWLM**S** T	91
Oxyntomodulin	HSQGTFTSDY SKYLDSRRAQ DFVQWLM**N**TK RN**RNNIA**	HSQGTFTSDY SKYLDSRRAQ DFVQWLM**S**TK RN**GQQGQ**	84
GLP-1	HAEGTFTSD**V S**SYLEGQAAK EFIAWLV**K**GR	HAEGT**Y**TSDI **T**SYLEGQAAK EFIAWLV**N**GR	87
GLP-2	HADGS**F**S**DEM N**TILD**N**L**AAR D**FINWLIQTK I**T**D	HADG**TFTSDI** NKILD**DMAAK** EFL**K**WLINTK VTQ	55

Sequence differences, in bold, were used to calculate the homology between the human and the chick peptide sequences. Except for chicken glucagon-like peptide (GLP)-1, peptides with the human sequences were used in the present study.

The bioactive peptides that can be derived from chick proglucagon are similar to those in the human proglucagon sequence: the 29-amino-acid peptide glucagon itself (chick proglucagon 55-83), which differs from the human form only in the second-from-last amino acid; the sequence for the undecapeptide, miniglucagon (miniG; glucagon 19-29), which also differs at residue 28; a C terminally extended (by eight amino acids) version of glucagon, oxyntomodulin (oxy; glucagon 1-37); and two homologous peptides, glucagon-like peptide (GLP)-1 (chick proglucagon 118-147) and GLP-2 (chick proglucagon 166-198); see Table 1 and Ref. 13). However, whether these proglucagon-derived peptides are produced in the chick retina, and if so, whether their receptors play a role in the regulation of ocular growth, has not been reported.

The purpose of this study was to determine which peptide in the secretin-glucagon family is most likely to be produced and released in response to visual stimuli that inhibit development of myopia, and therefore which receptor is most likely to mediate the action of exogenous glucagon in preventing excessive axial elongation of the eye. As the actions of secretin-glucagon–related peptides are matched with high specificity to unique receptors, the premise of this study was that the receptor responsible for the ocular "stop" growth signal may be identified by determining the exogenous peptide having the greatest capacity to inhibit the development of FD myopia. Thus, the first purpose of this study was to determine which of this family of secretin-glucagon–related peptides inhibits the development of FD myopia in chicks with the greatest efficacy. The second purpose was to use immunohistochemistry to determine whether glucagon-synthesizing amacrine cells in the chick retina store, and therefore may release, other proglucagon-derived peptides as well as glucagon.

METHODS

Animals

Day-old (P1) male White Leghorn chicks (*Gallus gallus domesticus*) were obtained from the supplier Clark Hy-Line, Inc. (Brandon, Manitoba, Canada) and reared in a temperature-controlled environment (26°C) in a 12-hour light–dark cycle with a light level of 250 lux on the floor of the cage provided by standard fluorescent tubes. Food and water were available ad libitum. The care and use of animals were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the Animal Care Committee of the University of Calgary Faculty of Medicine.

Induction of Myopia and Intraocular Drug Administration

Experiment 1: The Effects of Glucagon- and Secretin-Related Peptides on Development of Myopia. To determine whether the effects of glucagon on FD myopia were due to action via glucagon- or secretin-related receptors, we investigated the effects of glucagon- and secretin-related peptides on development of FD myopia. At posthatching day (P)6, chicks were monocularly deprived of pattern vision (FD) by attachment of a translucent diffuser to the feathers surrounding one eye with contact cement (Pres-tite; LePage, Brampton, Ontario, Canada) 2 to 3 hours after the beginning of the light phase. The contralateral eye remained unoccluded as a genetically identical and functionally independent control. On P7, 2 to 3 hours after the beginning of the light phase, chicks were anesthetized with 2.0% halothane in 50% O_2 and 50% NO_2. The vitreous of the FD eye was injected through the posterodorsal side of the eye under aseptic conditions with 20 μL of either peptide or saline (treated eye, n = 5–6/dose), and the open contralateral eye was injected similarly with saline (control eye), with a 25-μL syringe (26-gauge needle; Hamilton, Reno, NV). Chicks received a total of three injections, one every 48 hours for 6 days, and at age P12 ocular measurements were taken.

The peptides that were tested included: porcine glucagon (1-29), which is the same as the human peptide and is hereafter referred to as glucagon (70%–80% glucagon, from porcine pancreas extract), porcine secretin, human peptide histidine-isoleucine-amide-27 (PHI 27), human vasoactive intestinal peptide (VIP) and VIP fragment 6-28 (VIP frag), and human pituitary adenylate cyclase-activating polypeptide (PACAP). All drugs were diluted for use in a vehicle of sterile saline (0.9%) to a concentration of 10^{-4} M. The total vitreous volume in 7- to 14-day-old chicks is ~300 to 350 μL, of which a constant 150 to 175 μL is gel (Rushforth DA, Stell WK, unpublished data, 2003). Because diffusion, uptake, destruction, or binding of the injected peptide can greatly affect its effective concentration in the vitreous and retina, for convenience, the concentrations of substances in the vitreous were assumed to be approximately 20:200×, or 1:10× those in the injected solutions. However, in the Results section and figures, doses are given as the drug concentration in 20 μL in the syringe, so that the reader can make an independent estimate of the concentration presented to membrane receptors in the tissues that line the vitreous cavity. All peptides were obtained from either Sigma-Aldrich (St. Louis, MO) or Bachem (Philadelphia, PA).

Experiment 2: The Effect of Proglucagon-Derived Peptides on Development of Myopia and Growth in Eyes with Unrestricted Vision. Because glucagon was found to be the most potent inhibitor of myopia development in Experiment 1, further experiments were conducted to determine the effects of each of the proglucagon-derived peptides on ocular growth. At age P6, chicks were monocularly deprived of pattern vision (FD; as in experiment 1).

The contralateral eye remained unoccluded as a control. On P7, the vitreous of the FD eye was injected with 20 μL of either peptide or saline (treated eye, $n = 6$–8/dose), whereas the open contralateral eye was injected with saline (control eye). Chicks received a total of five injections, one every 48 hours for 10 days, and at age P16, ocular measurements were made. To determine the effects of proglucagon-derived peptides on open-eye development, other groups of chicks ($n = 6$–8/drug) were reared with no diffusers and treated with the highest dose of peptide, on the same dose regimen as FD animals.

The following proglucagon-derived peptides were tested: human glucagon (10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$, or 10^{-4} M in 20 μL in the syringe); human oxy (10^{-8}, 10^{-6}, or 10^{-4} M); human miniG (10^{-6} or 10^{-4} M); human GLP-2 (10^{-6} or 10^{-4} M); and chicken GLP-1 (7-36) amide (10^{-6} or 10^{-4} M). Chicken GLP-1 was used instead of the human form, because it was commercially available (Table 1), whereas glucagon, oxy, miniG, and GLP-2 were not. All were diluted for use in sterile saline (0.9%). Drug doses stated are the stock concentration in 20 μL in the syringe. These synthetic peptides were obtained from Bachem, with the exception of oxy, which was generously donated by Dominique Bataille (Institut National de la Santé et de la Recherche Médicale [INSERM] U376, Montpellier, France), and biosynthetic recombinant glucagon, which was purchased from the local hospital pharmacy as 1 mg pure glucagon plus 49 mg of lactose (Eli Lilly & Co., Indianapolis, IN) and was subsequently purified by reversed-phase HPLC to remove the lactose.

Ocular Measurements

The refractive errors of peptide- or saline-treated FD and open eyes were measured by streak retinoscopy without cycloplegia. Refractive error measurements were consistently performed at a distance of 30 cm, and no correction was made for working distance or the small-eye artifact.[14] Because of the limited availability of negative lenses, a refractive error of less than -20 D could not be accurately estimated and was recorded as -20 D. The axial dimensions of the eyes were measured in chicks under halothane anesthesia, by high-resolution A-scan ultrasonography, as described in detail elsewhere,[15] and estimates of corneal thickness, anterior chamber depth, lens thickness, vitreous chamber depth, retinal thickness, choroidal thickness, and scleral thickness were obtained with an estimated resolution of ±21 μm. Total axial length was calculated by summing the A-scan dimensions of all the ocular components from the front of the cornea to the back of the sclera. While still under halothane anesthesia, chicks were euthanatized by cervical dislocation, and the eyes were enucleated, cleaned of extraneous orbital tissue, and weighed before fixation for histology. The eyes of a group of chicks ($n = 4$) that had not been treated in any way were also prepared as the untreated control for immunohistochemistry.

Fixation and Sectioning

Eyes were hemisected equatorially with a razor blade, and the anterior portions discarded along with the vitreous gel. The posterior eyecups were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 20 minutes in the case of eyes to be treated with the miniG antibody, but for 1 hour for all other primary antibodies. The eyecups were then washed three times for 15 minutes each in PBS, before cryoprotection in 30% sucrose in PBS overnight at 4°C, and were stored at -20°C. Eyecups were mounted in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA) at -20°C, and transverse sections, 12 μm thick, were cut on a cryostat (CM1900; Leica, Deerfield, IL) at -20°C; collected on coated microscope slides; air dried at room temperature; and stored at -20°C.

Histologic Staining and Immunohistochemistry

To test for nonspecific effects of the injected agents on the structural integrity of the retina, slides were washed in PBS for 30 minutes and then incubated with 0.1% toluidine blue for 2 minutes at room temperature. They were washed three times for 15 minutes each in PBS, coverslipped with 4:1 glycerol and dH$_2$O as the mounting medium, and viewed by bright-field microscopy.

Immunohistochemistry was used to detect and localize proglucagon-derived peptides in the retina of untreated chicks (antibodies described later). Slides were thawed at room temperature for 30 minutes, and the sections were encircled with a hydrophobic barrier (rubber contact cement). The slides were washed once for 10 minutes in PBS at room temperature, then twice for 10 minutes each in PBS containing 50 mM NH$_4$Cl, and then for 5 minutes in PBS containing 0.1% Triton X-100. Finally, the slides were washed twice for 10 minutes each in PBS, incubated for 20 minutes in a blocking solution of PBS containing 2% bovine serum albumin (BSA), and washed a final time for 10 minutes in PBS. The slides were incubated in the primary antibody overnight at room temperature in a humidified chamber and then washed four times for 5 minutes each at room temperature in PBS to remove unbound primary antibody. The bound primary antibody was then visualized by a fluorescent-labeled secondary antibody, for 1 hour at room temperature while protected from light. Unbound secondary antibody was removed by washing the slide four times for 15 minutes each in PBS and coverslipped using 4:1 glycerol and dH$_2$O as the mounting medium. Slides were viewed on a fluorescence microscope (Carl Zeiss Meditec, Jena, Germany) with a water-immersion 25× objective lens (Neofluar; Carl Zeiss Meditec) connected to a digital imaging camera (Spot RT; Diagnostic Instruments, Inc., Sterling Heights, MI) and visualized on a computer monitor with the accompanying imaging program (Spot, ver. 3.2.6). Images were archived digitally, and selected frames representative of findings for each retina were stored in JPEG format. Each JPEG image was adjusted for contrast and linear remapping for ease of discerning labeled structures, and final images were prepared (PhotoShop, ver. 7.0; Adobe Systems, Mountain View, CA).

Antibodies

Primary antibodies and antisera to proglucagon-derived peptides included human glucagon (mouse monoclonal antibody, N-terminal specific, dilution 1:50; donated by Michael Gregor, University of Tübingen, via Center for Ulcer Research and Education [CURE], University of California, Los Angeles [UCLA]), human miniG (rabbit polyclonal, dilution 1:100; donated by Dominique Bataille, Institut National de la Santé et de la Recherche Médicale, Montpellier, France), human oxy (rabbit polyclonal, dilution 1:20; donated by CURE, UCLA), and human GLP-1 (rabbit polyclonal 1167, dilution 1:500; donated by Julia M. Polak, Hammersmith Hospital, London, UK). Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugate (1:1000; Molecular Probes, Eugene, OR), the Alexa Fluor 488 goat anti-rabbit IgG (H+L) conjugate (1:1000; Molecular Probes), and the Cy-3 goat anti-rabbit IgG (H+L) conjugate (1:1000). All antibodies were diluted for use in PBS containing 0.2% BSA.

Antibodies/antisera were preabsorbed overnight with the corresponding peptide to control for nonspecific binding. Glucagon immunoreactivity was inhibited by preabsorption with human glucagon (10^{-5} M). Immunoreactivity for GLP-1 was inhibited by preabsorption with chick GLP-1 (10^{-5} M), consistent with the specificity summary provided by Hammersmith Hospital, which also indicated that the GLP-1 antibody should not cross-react with either glucagon or GLP-2. A similar test could not be completed with the miniG antiserum because only a limited amount of antiserum was available; however, specificity tests performed previously by the laboratory of Dominique Bataille showed that immunoreactivity for miniG could be inhibited by preabsorption with miniG (10^{-5} M) but not by glucagon itself. This was expected, as the antiserum is directed to the N terminus of miniG, which is not exposed in glucagon.[16] These tests and references indicated that the antibodies are specific for their respective proglucagon-derived peptides and do not cross-react with other peptides. In colocalization studies, bleedthrough of fluorescence from the inappro-
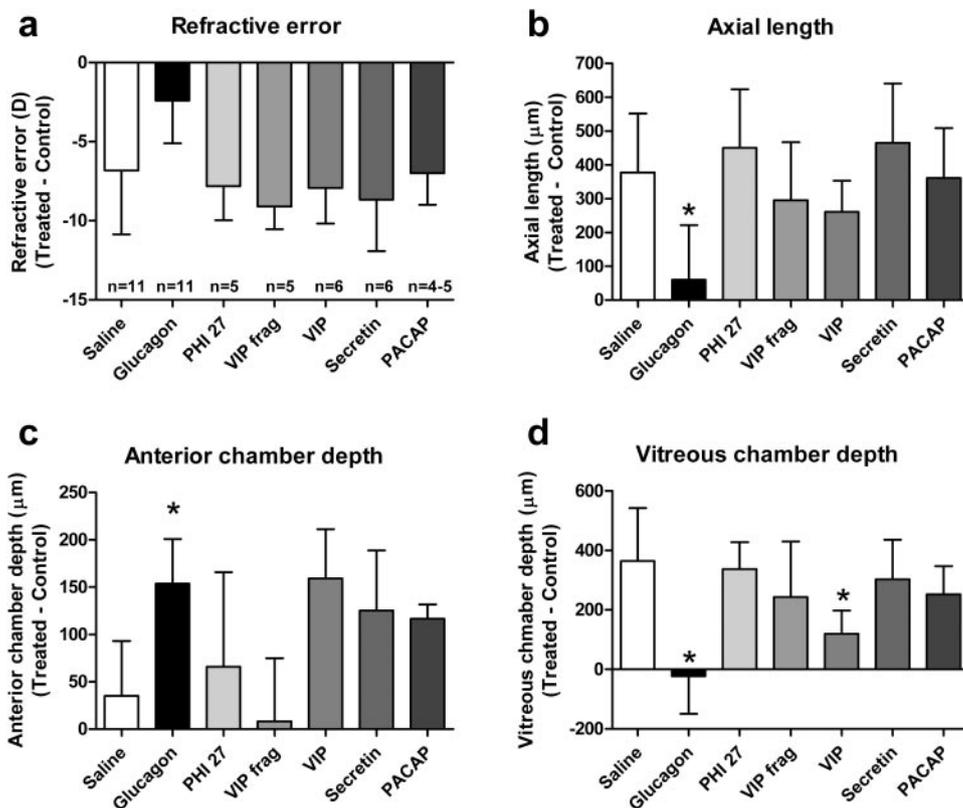
FIGURE 1. The effects of the glucagon- and secretin-related peptides (10^{-4} M in 20 μL in the syringe) glucagon, (PHI 27), human VIP, VIP frag, porcine secretin, and human PACAP, on the development of FD myopia in chicks. Data are expressed as the interocular differences between the treated and contralateral control eyes for (**a**) refractive error, (**b**) axial length, (**c**) anterior chamber depth, and (**d**) vitreous chamber depth. Bars represent the mean $\pm$ SD for (n) different animals. *The effects of a concentration of a given peptide on FD myopia were significantly different from the effects of saline (ANOVA, Tukey post hoc, $P < 0.05$).

priate fluorophore was ruled out by confirming that each label was not visible except with the appropriate filter set.

Statistical Analysis

All experimental groups (agent and dose) comprised five to eight chicks, or five to eight pairs of treated and control eyes. Differences between data for the treated and control eyes were compared on computer (Prism v. 4.0; GraphPad Software Inc., San Diego, CA). Screening of the raw data for treated and control eyes separately using a multiple analysis of variance (ANOVA) with a Newman-Keuls posttest showed that the treatments in the experimental (left) eye had no significant effect on the contralateral control (right) eye, regardless of whether the left eye was treated with saline or a glucagon-related agent—that is, within any experiment, the data for control eyes in different treatment groups were statistically indistinguishable from one another. Therefore, for simplicity and clarity, results are expressed as the mean of the differences between data for experimental (treated) versus contralateral (control) eyes (i.e., the treated minus the control value for each animal). For each treatment, the (treated − control) data were averaged and are expressed in the text, tables, and graphs as the mean $\pm$ SD.

For statistical analysis of the difference (treated − control) data, parametric statistical analyses were completed, after an initial test to confirm that the data were distributed in a Gaussian fashion. An ANOVA was used to compare three or more treatment groups for a given response parameter, and the Tukey post hoc test was used to identify which pairs of treatment-group data were significantly different. When the difference between a treated and control eye in the same animal was assessed, a paired *t*-test was used. Differences were considered significant at $P < 0.05$. By nonlinear regression (Prism 4; GraphPad Software, Inc.), a sigmoidal dose–response curve was fitted to the refractive error data for both glucagon and oxy, to determine the 50% effective concentration for inhibiting the development of myopia (EC$_{50}$); curve fits are not shown.

RESULTS

Experiment 1: The Effect of Glucagon-Secretin–Related Peptides on FD Eyes

In accordance with previous studies,[17,18] saline-treated FD eyes exhibited a significant myopic refractive error shift of -7 ± 4 D ($n = 11$; paired *t*-test, $P < 0.001$; Fig. 1a). This resulted from a 380-μm increase in axial elongation of the globe (paired *t*-test, $P < 0.0001$; Fig. 1b), due mainly to a 365-μm increase in the vitreous chamber depth of the treated eye compared with the contralateral control eye (paired *t*-test, $P < 0.0001$; Fig. 1d). As previously reported,[6] 10^{-4} M glucagon in 20 μL in the syringe completely blocked the deprivation-induced increases in the total axial ($n = 11$; ANOVA, $P < 0.01$) and vitreous chamber elongation (ANOVA, $P < 0.01$) of the globe compared with saline-treated FD eyes (Fig. 1). Although glucagon tended to reduce the myopic refractive error, the effect was not statistically significant in this particular experiment (ANOVA, $P = 0.09$; Fig. 1a). In contrast, secretin-related peptides generally had no effect on FD myopia, including axial elongation and choroidal thickness. The only exception was that VIP significantly inhibited excessive vitreous chamber elongation in FD eyes ($n = 6$; ANOVA, $P < 0.01$; Fig. 1d). Because glucagon was the only consistently effective peptide tested in this experiment, further experiments were conducted with glucagon and other proglucagon-derived peptides, to determine which of these peptides, and therefore which receptors, were most likely to mediate the effects of glucagon on ocular growth.

Experiment 2: The Effect of Proglucagon-Derived Peptides on FD Eyes

Glucagon. Glucagon was found to inhibit the development of myopia in eyes deprived of form vision for 10 days
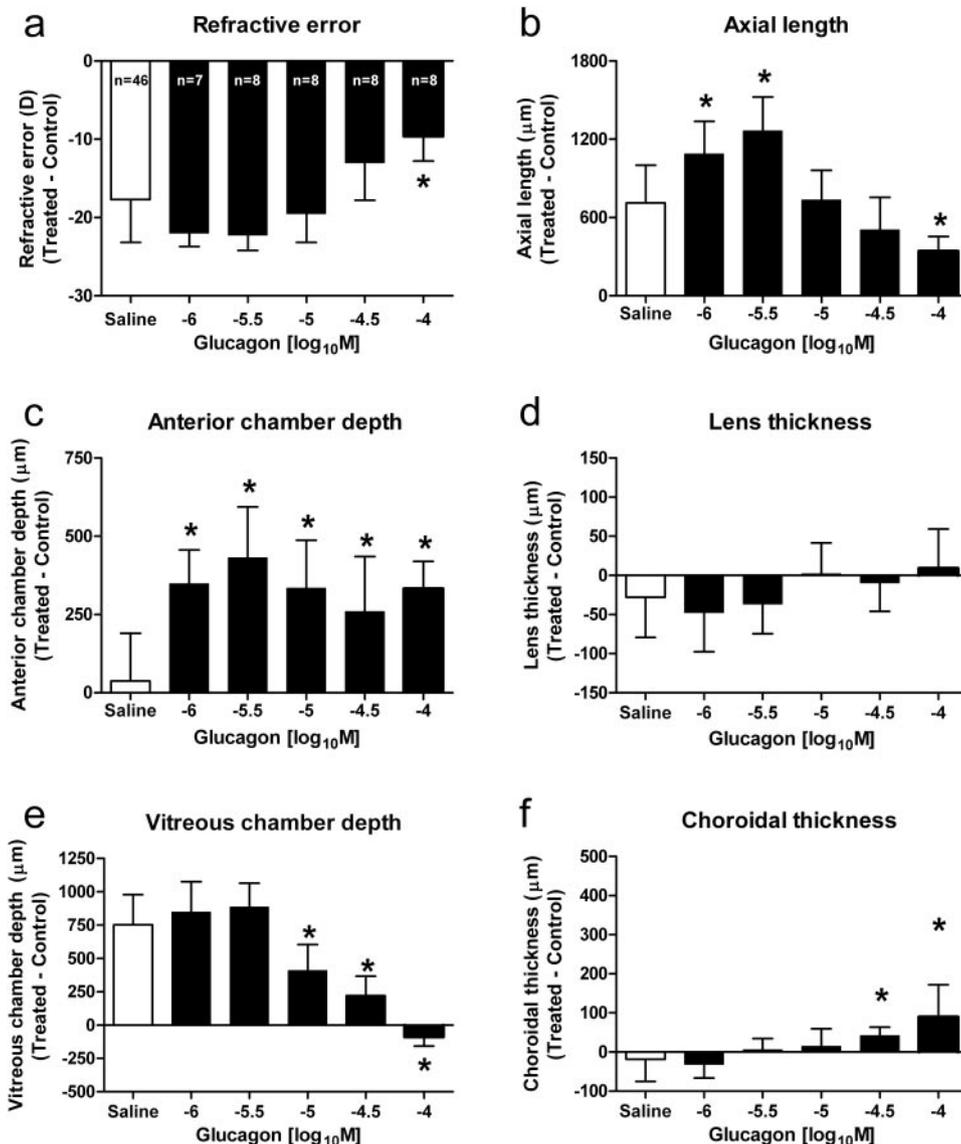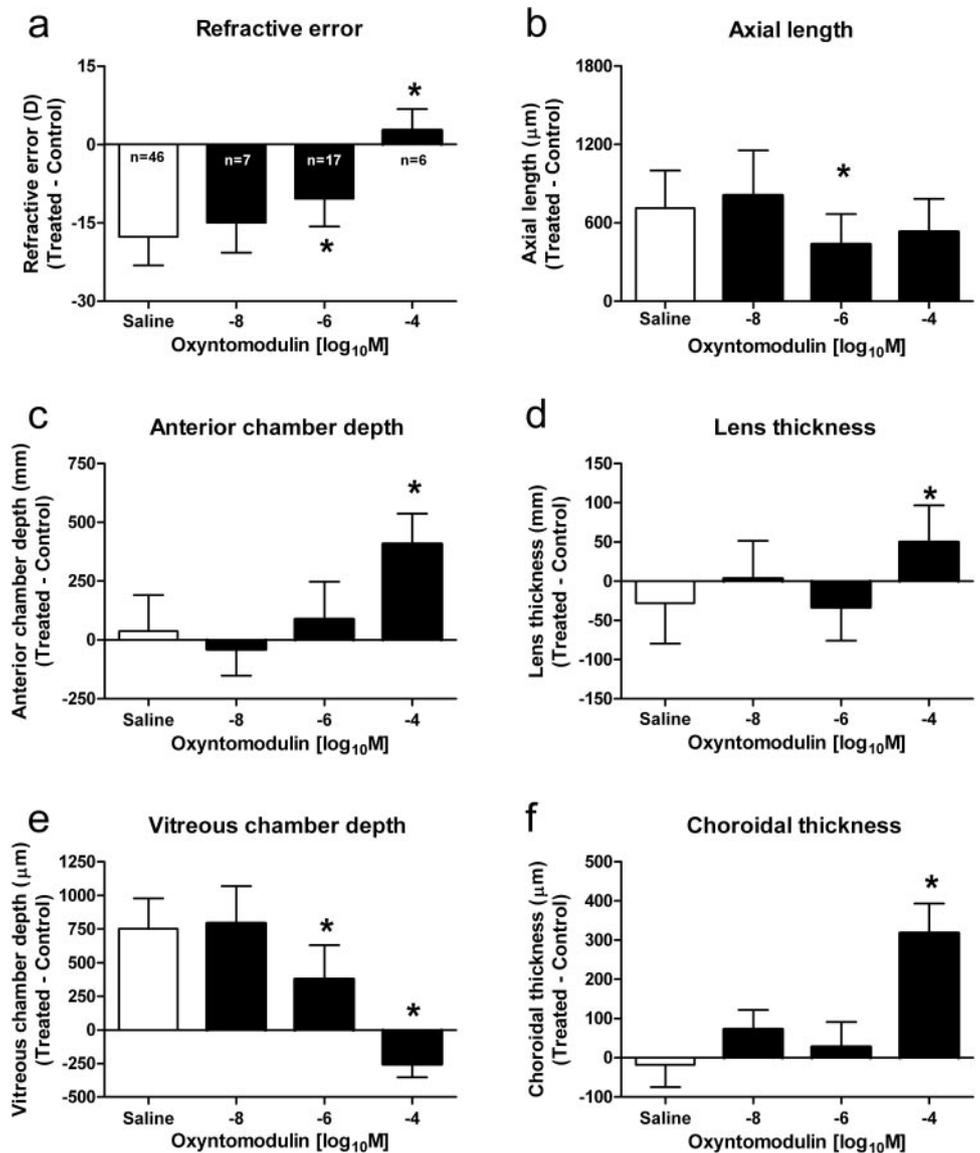
FIGURE 2. The effect of increasing concentrations of human glucagon on FD myopia in chicks. Data are expressed as the interocular differences between the treated and contralateral control eyes for (a) refractive, (b) axial length, (c) anterior chamber depth, (d) lens thickness, (e) vitreous chamber depth, and (f) choroidal thickness. Bars represent the mean ± SD for (n) different experiments. *The effects of a concentration of glucagon on FD myopia were significantly different from the effects of saline (ANOVA, Tukey post hoc, $P < 0.05$).

in a concentration-dependent manner over the concentration range 10^{-6} to 10^{-4} M, with an estimated EC_{50} of $\sim 10^{-4}$ M (in 20 μL in the syringe; Fig. 2a). The reduction in development of myopic refractive error in FD eyes in response to glucagon treatment was associated with a dose-dependent reduction in vitreous chamber enlargement by a maximum of 750 μm (Fig. 2e). Glucagon also induced a significant, but not concentration-dependent (over the range of doses tested), increase in anterior chamber depth of ~ 300 μm in FD eyes (Fig. 2c), which resulted in a significant increase in overall axial elongation of the eye at glucagon concentrations of 10^{-6} and $10^{-5.5}$ M (Fig. 2b). Furthermore, the increase in anterior chamber depth, coupled with the decrease in vitreous chamber depth in FD eyes treated with higher doses of glucagon, resulted in little net change in axial length, except at 10^{-4} M glucagon, which reduced axial length (Fig. 2b). FD induced a significant reduction in the choroidal thickness of saline-treated eyes (-18 ± 56 μm; $n = 46$; paired t-test, $P = 0.035$), whereas glucagon induced a concentration-dependent increase in choroidal thickness that reached 120 μm at 10^{-4} M (Fig. 2f). FD also induced a significant reduction in lens thickness of saline-treated eyes (-28 ± 52 μm; $n = 46$; paired t-test, $P <$

0.001), but this change in lens thickness was not affected significantly by glucagon at any dose tested (ANOVA, $P > 0.05$; Fig. 2d).

Oxyntomodulin. In general, oxy affected the growth of the FD eye in a manner similar to the effect of glucagon. Increasing doses of oxy inhibited the development of myopia in FD eyes more potently than did glucagon, with an EC_{50} of $\sim 10^{-5.5}$ M (in 20 μL in the syringe; ANOVA, $P < 0.0001$; Fig. 3a). This reduction in myopia was accompanied by significant reductions in the FD-induced increases in vitreous chamber and overall axial length (ANOVA, $P < 0.01$; Figs. 3b, 3e). Unlike glucagon, oxy did not affect anterior chamber depth at 10^{-6} M; however, like glucagon, oxy at the highest concentration (10^{-4} M) also induced a comparable and statistically significant increase in anterior chamber depth of ~ 400 μm (ANOVA, saline vs. 10^{-4} M, $P < 0.0001$; Fig. 3c). Also like glucagon, oxy had no effect on choroidal thickness at 10^{-6} M but induced an increase in choroidal thickness in FD eyes at the highest dose tested (ANOVA, saline vs. 10^{-4} M, $P < 0.001$; Fig. 3f), although the increase in choroidal thickness induced by oxy was of a greater magnitude than that observed in response to the same concentration of glucagon. Oxy also induced a small but significant increase in lens thickness of FD

FIGURE 3. The effect of increasing concentrations of human oxy on FD myopia in chicks. Data are expressed as the interocular differences between the treated and contralateral control eyes for (**a**) refractive error, (**b**) axial length, (**c**) anterior chamber depth, (**d**) lens thickness, (**e**) vitreous chamber depth, and (**f**) choroidal thickness. Bars represent the mean ± SD for (*n*) different experiments. *The effects of a concentration of oxy on FD myopia were significantly different from the effects of saline (ANOVA, Tukey post hoc, $P < 0.05$).

eyes at the highest dose (ANOVA, $P < 0.01$; Fig. 3d). Although the increase in lens thickness of ~50 μm and the increase in choroidal thickness of ~350 μm in FD eyes treated with oxy (10^{-4} M in 20 μL in the syringe) contributed to the reduction in vitreous chamber depth, ~650 μm of the decrease in vitreous chamber depth was due to growth inhibition.

Glucagon-like Peptide-1. Unlike glucagon and oxy, GLP-1 produced a modest but significant *increase* in myopia in FD eyes, although only at the highest concentration (10^{-4} M in 20 μL in the syringe; ANOVA, saline vs. 10^{-4} M, $P < 0.01$; Fig. 4a). In fact, this concentration of GLP-1 (10^{-4} M) induced a myopic refractive error of greater than -20 D in so many of the treated eyes that, because of limited availability of trial lenses, a refractive error of -20 D was recorded, and the myopia in these eyes was underestimated. This enhancement of myopic refractive error was due to a 350-μm increase in anterior chamber depth (ANOVA, $P < 0.0001$; Fig. 4c), which probably steepened the cornea. At the highest concentration only, GLP-1 also induced a significant increase in lens thickness (ANOVA, $P < 0.01$; Fig. 4d), which could have contributed further to the myopic shift. A significant increase in overall axial length was also observed (ANOVA, $P < 0.001$; Fig. 4b), which was predominantly mediated by the increase in anterior chamber depth. In contrast to

glucagon and oxy, GLP-1 induced a modest but significant reduction in choroidal thickness (ANOVA, $P < 0.01$; Fig. 4f) and no change in vitreous chamber depth of FD eyes (ANOVA, $P > 0.05$; Fig. 4e).

GLP-2 and MiniG (Glucagon [19-29]). The effects of both GLP-2 and miniG on the development of FD myopia were tested at concentrations of 10^{-6} and 10^{-4} M (in 20 μL in the syringe). Even at the highest concentration, however, neither of these peptides had any significant effect on the development of myopia or the usual changes in ocular dimensions that occur in FD eyes (data not shown).

The Effect of Proglucagon-Derived Peptides on Open Eyes

The effects of the five proglucagon-derived peptides (10^{-4} M in 20 μL in the syringe) on the development of refractive error and ocular growth were also tested in the eyes of chicks that had unrestricted vision with both eyes (Fig. 5). Although none of these peptides had a significant effect on refractive error (paired *t*-test, $P > 0.05$ in all cases; Fig. 5a), they stimulated significant changes in eye growth, similar to those observed in FD eyes treated with the same dose of
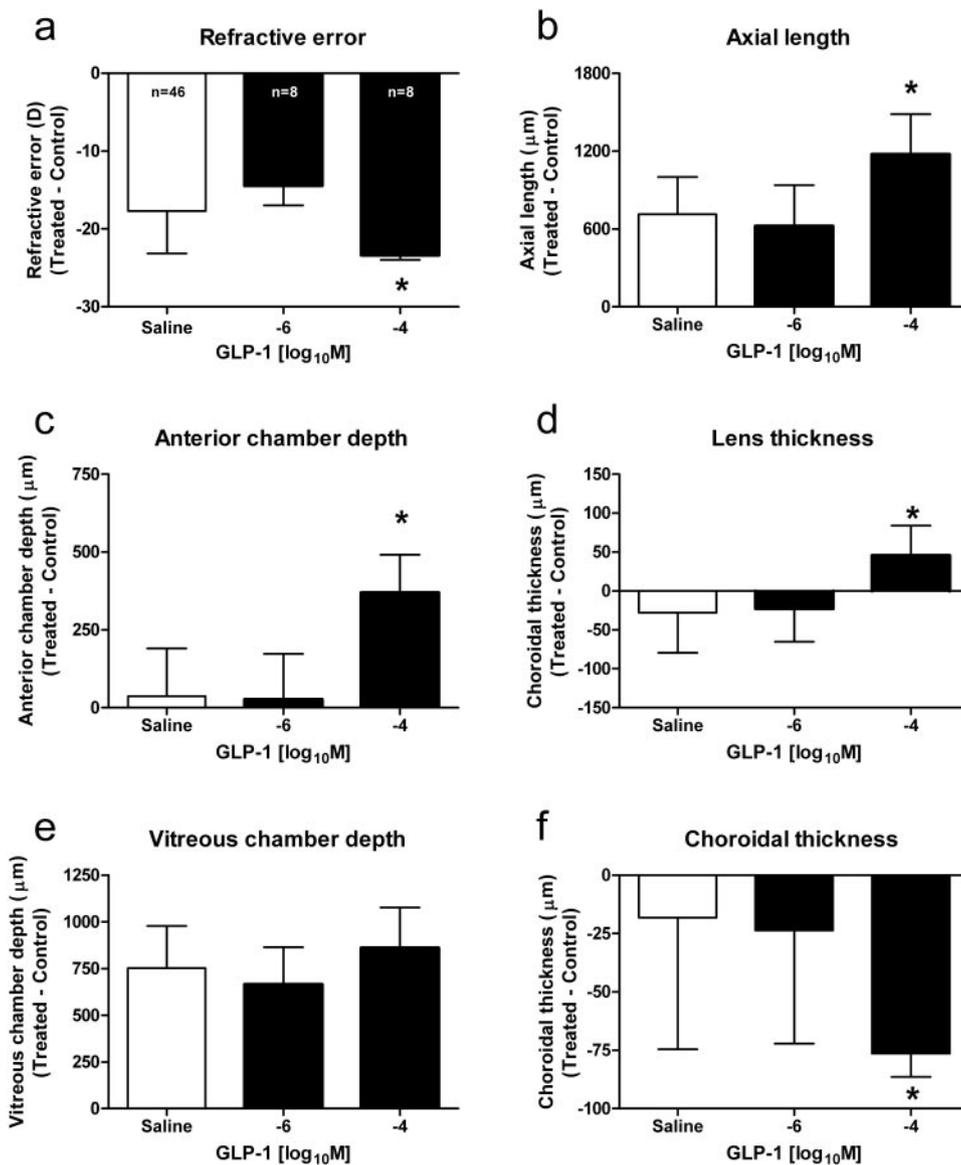
FIGURE 4. The effect of increasing concentrations of chicken GLP-1 on FD myopia in chicks. Data are expressed as the interocular differences between the treated and contralateral control eyes for (**a**) refractive error, (**b**) axial length, (**c**) anterior chamber depth, (**d**) lens thickness, (**e**) vitreous chamber depth, and (**f**) choroidal thickness. Bars represent the mean ± SD for (*n*) different experiments. *The effects of a concentration of GLP-1 on FD myopia were significantly different from the effects of saline (ANOVA, Tukey post hoc, $P < 0.05$).

drug. Glucagon, oxy, and GLP-1 all induced a significant increase in anterior chamber depth (~200 μm or greater; paired *t*-test, $P < 0.05$; Fig. 5c), as well as a significant increase in lens thickness (paired *t*-test, $P < 0.05$; Fig. 5d). Glucagon and oxy also induced a reduction in vitreous chamber depth (paired *t*-test, $P < 0.05$; Fig. 5e), which was coupled partially to a significant increase in choroidal thickness (paired *t*-test, $P < 0.05$; Fig. 5f) of a magnitude similar to that observed in FD eyes. GLP-1, in contrast to its effects in FD eyes, also induced a significant reduction in vitreous chamber depth that was accompanied by a matching increase in choroidal thickness (paired *t*-test, $P < 0.05$; Figs. 5e, 5f). Although miniG had no effect on the development of myopia in FD eyes, in eyes with unrestricted vision, miniG induced a small but significant reduction (~75 μm) in both axial length and vitreous chamber depth (paired *t*-test, $P < 0.05$; Figs. 5b, 5e).

Immunohistochemistry for Proglucagon-Derived Peptides in the Chick Eye

Immunohistochemistry was used to test whether the proglucagon-derived peptides, miniG, oxy, and GLP-1, are present in the glucagon-containing amacrine cells of the chick retina (Fig. 6). As previously reported,[19,20] glucagon immunoreactivity (IR) was expressed in a subset of amacrine cells in the innermost part of the inner nuclear layer (INL) with processes extending into two strata in the inner plexiform layer (IPL; Figs. 6a, 6c). MiniG IR was also expressed in amacrine cells of the chick retina (Fig. 6b) and colocalized with glucagon IR, as did GLP1 IR (Figs. 6c, 6d). However, the antibody to oxy did not show any specific binding in the chick retina (data not shown), probably because the antibody was raised against the oxy-specific C terminus of the human peptide which differs significantly in amino acid sequence from that of the chick peptide (Table 1).

Frozen sections of open eyes treated with the various proglucagon-derived peptides (10^{-4} M) were stained with toluidine blue for general histologic examination and compared to their contralateral control eyes ($n = 3$ eyes/peptide treatment). There did not appear to be any morphologic damage, such as distortion or loss of cells in any cellular layers in the retinas of treated eyes (data not shown).
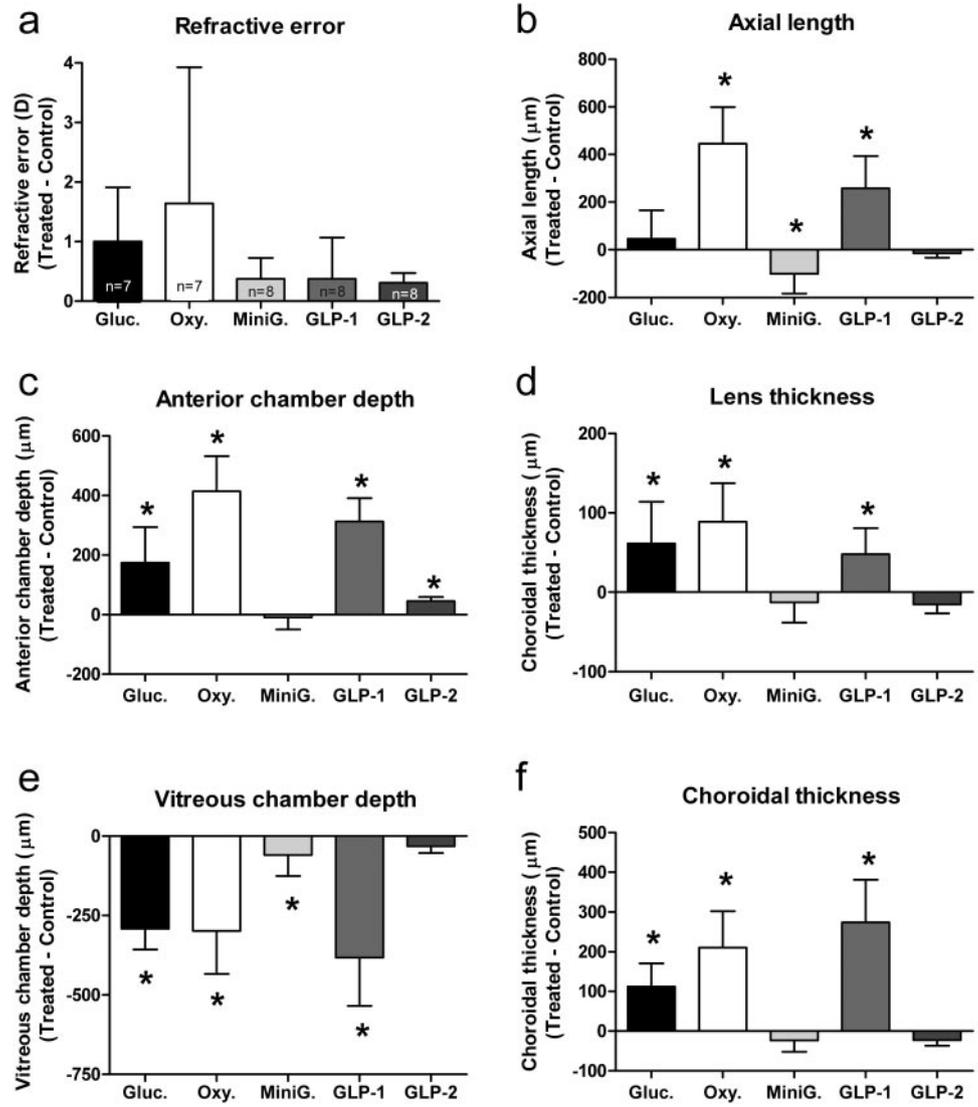
FIGURE 5. The effect of progluca-gon-derived peptides on eye growth in chicks with unrestricted vision. The proglucagon-derived peptides tested were human glucagon, oxy, miniG, and GLP-2 and chick GLP-1 (10^{-4} M in 20 μL in the syringe). Data are expressed as the interocular differences between the treated and contralateral control eye for (a) re-fractive error, (b) axial length, (c) anterior chamber depth, (d) lens thickness, (e) vitreous chamber depth, and (f) choroidal thickness. Bars represent the mean ± SD for (n) different experiments. *Significant difference between the peptide-treated eye and the saline-treated control eye (paired *t*-test, $P < 0.05$).

DISCUSSION

In the chick, glucagonergic amacrine cells are among the most promising candidates for a direct role in the regulation of ocular growth at the retinal level.[4–6,20] Results from the present study confirm that glucagon inhibits the development of FD myopia[6] in a dose-dependent manner, with an estimated EC_{50} of 10^{-4} M (in 20 μL in the syringe). Furthermore, of all the peptides tested in the secretin-glucagon family, glucagon and the proglucagon-derived peptides, oxy and GLP-1, ap-peared to have the most significant effects on the development of FD myopia and on the growth of eyes with unrestricted vision. Oxy, like glucagon, was found to have a potent inhibi-tory effect on the induction of FD myopia, mainly by inhibiting excessive elongation of the vitreous chamber, with an EC_{50} of $\sim10^{-5.5}$ M (in 20 μL in the syringe). In contrast, chick GLP-1 was found to increase the development of myopic refractive error in FD eyes mainly by promoting excessive deepening of the anterior chamber, while miniG and GLP-2 were ineffective. Thus, the prevention of experimental myopia by exogenous glucagon is mediated by receptors sensitive to glucagon or oxy, indicating that one of these peptides, acting through such receptors, is the most probable endogenous mediator of myo-pia inhibition.

A role for a glucagon-like receptor in the inhibition of experimental myopia has also been suggested by previous findings. Glucagon (10^{-9}–10^{-5} M in 20 μL in the syringe) was found to inhibit FD myopia in a dose-dependent manner when administered every day for 5 days.[7] A 10-fold higher dose of glucagon was required in the present study, to mediate the same effect as previously; however, this difference may be attributable to the fact that, in the present study, the peptide was administered only every second day, and in a different strain of chicks. Furthermore, as opposed to the earlier find-ings,[7] in the present study lower doses of glucagon actually significantly increased axial length, indicating a more complex effect on ocular growth than was initially perceived; however, it is possible that this change was only discernible with the more sensitive technique of A-scan ultrasonography as op-posed to the wet weight and digital caliper measurements used previously. Regardless of these small discrepancies, the current findings confirm that glucagon is consistently capable of inhib-iting myopia progression in the chick.

The synthetic glucagon receptor agonist, Lys[17,18],Glu[21]-glu-cagon-NH$_2$, also inhibits induction of myopia in response to negative lens wear[4] and FD[6] in the chick eye. The results of the present study more specifically indicate the importance of the N-terminal region of glucagon for receptor binding and activa-
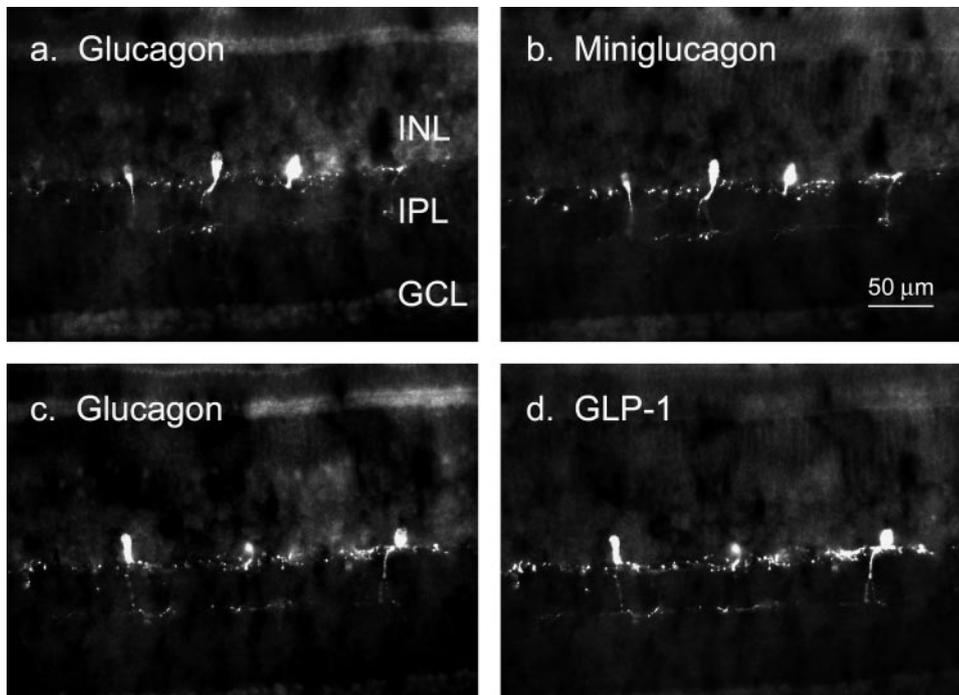
FIGURE 6. Immunohistochemistry for proglucagon-derived peptides in the chick retina at P10. Chick retina labeled for (a) glucagon immunoreactivity (glucagon IR; 1:50 dilution of N-terminal-specific mouse anti-human glucagon antibody) in the same section of retina as (b) miniG IR (1:100 dilution of rabbit anti-human miniG antibody) and for (c) glucagon IR in the same section of retina as (d) GLP-1 IR (1:100 dilution of rabbit anti-human GLP-1 antibody).

tion, and the inhibition of myopia. MiniG (glucagon 19-29), produced by enzymatic cleavage at residues 17-18 and removal of the N-terminal 18 amino acids of glucagon, was ineffective in regulating the development of FD myopia, even though its presence in glucagonergic amacrine cells was indicated by immunohistochemistry. In contrast, human glucagon, oxy, and $Lys^{17,18},Glu^{21}$-glucagon-NH_2, all of which contain the same N-terminal amino acids (1-16), have been found to inhibit the development of experimental myopia. This interpretation is consistent with the results of studies using chimeric proglucagon-derived peptide combinations, which have shown that the N-terminal portion of glucagon is required for binding to and activation of the glucagon receptor, whereas the C-terminal region contributes only modestly to peptide affinity.[21,22] Furthermore, although the proglucagon-derived peptides generally mediate their actions through unique and specific G protein-coupled receptors,[10,11] oxy is able to bind to and activate the glucagon receptor with high affinity.[23] Thus, although an independent receptor and unique action of oxy cannot be excluded, it is likely that the glucagon receptor (or a glucagon-like receptor with high affinity for glucagon, oxy, and $Lys^{17,18},Glu^{21}$-glucagon-NH_2) mediates the effects of these peptides on ocular growth.

The mRNA for the glucagon receptor is expressed by the chick retina, as well as in the RPE and choroid,[5] and glucagon receptor activation has been shown to be coupled to cAMP production in cultures of both the chick retina and RPE, indicating a direct functional response to glucagon in these tissues.[24,25] Preliminary results reported recently by two laboratories, including ours (Beloukhina N, et al. IOVS 2005;46:ARVO E-Abstract 3337; Zhu X, et al. IOVS 2005;46:ARVO E-Abstract 3338), are consistent with the RPE's being the direct target for the myopia-preventing actions of glucagon. Further studies are necessary to determine conclusively the site of action of glucagon in regulating ocular growth in the chick.

Unlike glucagon and oxy, GLP-1 at the highest concentration (10^{-4} M in 20 μL) actually enhanced the myopic refractive error induced by FD by increasing anterior chamber depth and therefore most likely corneal curvature, without affecting vitreous chamber enlargement. It is also possible that GLP-1, as well as the other glucagon-related peptides, induce corneal accommodation in the chick,[26] thereby contributing to the myopic refractive error. Other drugs that have been found to affect anterior chamber development during induction of experimental myopia include TGF-β,[27] melatonin,[28] the D1-like receptor antagonist SCH 23390,[28] and the GABA$_{OR}$ receptor agonist cis-4-aminocrotonic acid (CACA).[29] In the present study, the lowest concentration of glucagon (10^{-6} M in 20 μL) induced changes in ocular growth of the FD eye similar to those induced by GLP-1, including a trend toward increased myopic refractive error and significant increases in anterior chamber depth and overall axial length, but no change in vitreous chamber depth. It is possible that GLP-1 mediated the increase in anterior chamber depth in FD eyes by acting on glucagon receptors but that a 100-fold higher GLP-1 concentration was necessary because of its lower binding affinity at this receptor. This seems unlikely, however, because a greater than 1000-fold increase of GLP-1 concentration is necessary to activate the glucagon receptor in most mammalian model systems.[21] Another possibility is that GLP-1 mediates effects on ocular growth similar to those caused by glucagon, but via a receptor other than that for glucagon or GLP-1.[10] Further research is needed to characterize fully the receptor involved in mediating the effects of GLP-1 on anterior chamber depth and enhanced development of myopia in response to FD.

Although GLP-1 (10^{-4} M) enhanced the myopia induced by FD, it did not affect refractive development in eyes with unimpaired visual input. In open eyes treated with GLP-1 the anterior chamber depth, and therefore likely corneal curvature, was increased; however, GLP-1 also caused a compensatory reduction in vitreous chamber depth due to an increase in choroidal thickness, which was not observed in FD eyes. This indicates that even though GLP-1 affected development of the anterior chamber segment at this dose, a separate mechanism to maintain emmetropia was also working in open eyes. This mechanism was mediated primarily by expansion of the choroid to reduce the length of the vitreous and push the retina forward into the plane of the image, as in recovery from FD or compensation to positive lens wear.[30]

Both glucagon and oxy exerted effects similar to those of GLP-1 on growth of eyes with unrestricted vision. Other drugs have been found to affect the growth of open eyes, some through a receptor-mediated mechanism: for example, muscimol, the $GABA_A$ receptor agonist, which has been found to induce myopia and axial lengthening in eyes with unimpaired visual input.[29] Other drugs, such as colchicine,[20] kainate,[31] quisqualic acid[31] and NMDA,[32] affect ocular growth via a toxic mechanism that disrupts some retinal pathway important in emmetropization. Routine histologic examination of toluidine blue–stained cryosections of retinas that had been treated with the proglucagon-derived peptides did not indicate any damage to cells or loss of cellular layers usually associated with retinal toxicity, such as occurs after treatment with colchicine,[20] kainate,[33] NMDA, and quisqualic acid.[32] However, further research, using immunocytochemical markers for example, is needed to determine whether the effects of glucagon and oxy at these high doses are due to a receptor-mediated mechanism or retinal toxicity.

Immunohistochemistry was also used to determine whether proglucagon derived-peptides other than glucagon[9,11,13] are generated by proglucagon processing in the glucagon-IR amacrine cells of the chick retina. The presence of miniG and GLP-1 IRs in these cells implies that they, like glucagon, may play neuromodulatory roles in the retina. In the turtle retina, glucagon is released in a calcium-dependent manner in response to depolarization of the cell and is believed to modulate information transmission by the OFF-center visual pathway.[34] Although the roles of miniG and GLP-1 in the chick retina are still unknown, the demonstration that proglucagon-derived peptides are functional neurotransmitters and modulators in the central nervous system[35–38] implies that they have physiological roles in the chick retina. In contrast to glucagon, miniG, and GLP-1, oxy IR was not detected. However, the antibody to oxy used in this study was directed to the C terminus of human oxy (Table 1), a region so different in the chick peptide that this antibody is unlikely to cross-react with it. Similarly, no antibody is available to chick GLP-2, which differs greatly from its mammalian counterpart. Therefore, the presence of GLP-2 or oxy in the chick retina could not be tested with available antibodies. It is possible, however, that these two peptides are also made and released by the glucagon-IR amacrine cells of the chick retina.

Whereas there is little direct evidence for glucagon production and signaling in the mammalian retina, high-affinity binding sites for glucagon have been characterized in the rat retina,[39] and the expression of preproglucagon and glucagon receptor messages has been reported in mouse retina.[40] The failure of previous studies to detect glucagon in mammalian retinas does not prove that glucagon is not present— only that, if present, it is in amounts or concentrations too small to detect. Furthermore, the importance of emmetropization for survival suggests that mechanisms of emmetropization may be conserved across species. In support of this concept, the same early-response transcription factor (Egr-1) that is induced by plus defocus in glucagon amacrine cells in the chick[2] has been reported to be induced by defocus in macaque[41] and tree shrew (Stell WK, et al. *IOVS* 2004;45:ARVO E-Abstract 1159). Glucagon has not been shown either to be or not to be synthesized in the amacrine cells in which Egr-1 is induced in these mammalian retinas. Further experiments are needed to determine whether glucagon plays the same role in emmetropization in mammals as it does in chicks. In the mean time, the chick remains a useful model for studying the mechanisms underlying emmetropization, which are likely to be similar in mammals.

In conclusion, glucagon, miniG, and GLP-1 were all found to be present in the chick retina, whereas the presence or ab-

sence of oxy and GLP-2 remains to be determined. The roles of miniG and GLP-1 in retinal signaling in the chick are yet to be identified. In addition, the results of this study imply that either glucagon or oxy is the most likely endogenous peptide, and the glucagon receptor is its probable mediator, for inhibition of myopia and axial elongation as well as induction of choroidal thickening under "stop"-growth conditions. In contrast, GLP-1 was found to increase the development of myopic refractive error in FD chick eyes. None of the proglucagon-derived peptides had a significant effect on refractive error of chicks with unrestricted vision, but changes in anterior and posterior eye growth were observed in response to glucagon, oxy, miniG, and GLP-1. Thus, the role of proglucagon-synthesizing amacrine cells in emmetropization may be altered by selective processing of proglucagon and release of different proglucagon-derived peptides in response to changes in visual stimulus conditions.

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