Image Defocus Modulates Activity of Bipolar and Amacrine Cells in Macaque Retina

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Purpose. To demonstrate regulation of ocular growth and refractive development by image quality and processing in the retina in higher primates. Focus-sensitive retinal neurons were labeled with immunocytochemical markers after briefly altering image quality in infant monkeys.

Methods. Six rhesus monkeys (*Macaca mulatta*) 20- to 30-days-old were fitted with goggles after 6 hours in the light. Three wore a +3 D lens and three a diffuser on the treated eye; contralateral control eyes wore plano lenses matched in transmission to the goggles on treated eyes. After 30, 60, or 240 minutes exposure, the animals were killed, the eyes opened and fixed in 4% formaldehyde, and cryosections labeled with antibodies to inducible activity markers (transcription factors Egr-1 and Fra-2) and type-specific amacrine cell markers. Labeled cells were identified and counted in a fluorescence microscope, and the spatial density of activity-labeled nuclei and the frequency of activity-labeling of specific amacrine cells were determined, without knowing treated eye or duration.

Results. Focus-sensitive immunoreactivity was demonstrated for Egr-1 and Fra-2 in a GAD65-immunoreactive (IR) subpopulation of GABAergic amacrine cells, and for Egr-1 alone in PKCalpha-, 115A10-, and CD15-IR ON-bipolar cells. Activity of ON-bipolar and GABAergic amacrine cells, as indicated by Egr-1 induction, was stimulated more by in-focus or myopically-defocused images than by hyperopically-defocused or diffusely blurred images, regardless of exposure duration.

Conclusions. This was the first evidence of focus-dependent activation of bipolar as well as amacrine cells in a primate retina. Focus-sensitive neurons are candidates for roles in vision-dependent regulation of eye growth. (*Invest Ophthalmol Vis Sci.* 2004;45:2065–2074) DOI:10.1167/iovs.03-1046

The development of optical power and axial length of the eye is normally coordinated so that the images of distant objects are focused on the retina without accommodation.[1] This condition is called emmetropia, and the developmental process by which it is achieved is called emmetropization. Emmetropization must be controlled very precisely. One of the most common refractive errors, myopia (near-sightedness), is due primarily to excessive axial elongation, and even a 2% excess in axial length causes a refractive error (about −0.5 D) that requires correction for distance vision.[2,3] Since myopia afflicts ~25%-75% of the adult population[4] and can cause blindness, it is important to understand what causes it and learn how to prevent it.

The causes of myopia remain unknown. Epidemiologic studies implicate both genetic and environmental factors.[5–9] Animal studies clearly show that myopia can be caused by inappropriate visual experience.[1,10,11] In rhesus monkeys,[12] as in chickens,[13] growth and refraction are regulated by local visual processing and signaling within the eye, which detect defocus and alter eye growth to compensate for it.[14] Better understanding of retinal control mechanisms in primate models could lead to new strategies and agents for preventing human myopia.[15]

It is likely that amacrine cells are critical for the control of eye growth. These are the earliest neurons in the visual pathway to show sufficient functional complexity for focus-defocus discrimination[16,17]; they synthesize and release a variety of intercellular messengers and have neuroregulatory functions[18]; the pharmacological agents that influence eye growth are targeted to transmitter systems known to be used by amacrine cells[19–22]; and toxins that deregulate eye growth, target amacrine cells.[23–29] It remains uncertain which amacrine cells control eye growth, and for technical reasons it would be nearly impossible to identify them by traditional neurophysiological methods. However, alternative methods are available for identifying amacrine cells that respond to growth-regulating stimuli. Stimulation can induce the transcription of immediate-early genes such as *Egr-1*[30] (also known as *ZENK*, *zif268*, and *krox-24*[31]), and *c-Fos* and other *Fos* family members including *FosB* and *Fos*-related antigens 1 and 2 (*Fra-1* and *Fra-2*),[32] whose protein products accumulate over time and can be localized immunocytochemically to specific neurons.[33] In the chick retina, switching from diffuse blur (which induces myopia) to focused, textured images (which prevent myopia) induces c-Fos and Fra-2 in an amacrine cell subpopulation,[34,35] and suppression of Fra-2 synthesis by antisense oligodeoxynucleotides causes myopia in otherwise untreated eyes (McGuire JJ and Stell WK. *IOVS* 1999;40:ARVO Abstract 4468). It is particularly interesting that Egr-1 is induced in cone ON-bipolar cells by light, and in glucagon-synthesizing amacrine cells by myopic defocus, in chicks.[34,36]

Inducible markers were used to identify amacrine cells responsive to emmetropization-modifying visual conditions in young rhesus monkeys, the preferred animal model for the human eye and vision.[37] Preliminary studies showed that diffuse blur or defocus induced rapid, sustained changes in activity in specific primate amacrine and bipolar cells, implicating them as candidates for roles in human emmetropization and myopia.

Methods

Animals

Six healthy infant rhesus monkeys (*Macaca mulatta*) were used as subjects in this study. All animals were reared in the animal care facilities of Sun Yat-Sen University of Medical Sciences, where they

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TABLE 1. Antibodies and Antisera

Antigen	Ab/Serum ID	Source	Reference
Egr-1	Krox-24 (rab poly)	R. Bravo, Squibb	40
Fra-2	#sc-604 (Q-20) (rab affin)	Santa Cruz	mfr. data sheet
Calbindin (CaBP-28K)	#C9848 (clone CB-955)	Sigma Aldrich	50; mfr. data sheet
Calretinin	#NCL-Calret. (clone 5A5)	Novocastra	52; mfr. data sheet
CRABP	Clone C1	J. Saari, U. Wash.	53, 54
GABA	#A0310 (clone GB-69)	Sigma Aldrich	42; mfr. data sheet
$GABA_A$-Rα	#MAB 339 (clone bd24)	Chemicon	55, 56; mfr. data sheet
	#1 381 440 (clone bd24)	Boehringer Mannheim	
GAT-1	#G0157 (rab affin)	Sigma Aldrich	57
GAD-65	Clone GAD-6	DSHB	43, 44
N-Acetyl-lactosamine	CD15 (supernatant)	J. Mai, Düsseldorf	50, 51
nNOS	#N2280 (clone B1)	Sigma Aldrich	58; mfr. data sheet
Olfactory epithelial Ag	115A10 (supernatant)	S. C. Fujita, Tokyo	47, 49, 50
Parvalbumin	#P3088 (clone PARV-19)	Sigma Aldrich	59; mfr. data sheet
PKCα	#sc-80 (clone MC5)	Santa Cruz	45–48; mfr. data sheet
	#P5704	Sigma Aldrich	
	#RPN.536	Amersham	
Tyrosine hydroxylase	#1022 (clone TOH A1.1)	Medicorp	60–61

were maintained on a 12-hour light/12-hour dark lighting cycle (lights on at 8:00 AM and off at 8:00 PM). The temperature was maintained at 25° ± 2°C and humidity at 45% ± 5%. Use of the animals in this study was approved by The Sun Yat-Sen University of Medical Sciences Institutional Animal Care and Use Committee and was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optical Preparation of Animals

At 20 to 30 days of age, the monkeys were anesthetized by i.m. ketamine hydrochloride (15 mg/kg), and cycloplegia was induced with 3 drops of 0.5% tropicamide applied topically 10 minutes apart. Refraction, corneal refractive power, and vitreous chamber depth were measured 45 minutes later by cycloplegic retinoscopy, autorefraction, corneal topography, and A-scan ultrasonography, as described elsewhere.[38] Refractions in the diffuser group were +3.25 ± 0.77 diopters (D; mean ± SD), whereas refractions in the plus-lens group were +3.00 ± 0.36 D. There were no apparent differences between refractive errors in the two groups (unpaired *t*-test, $P > 0.05$), and the refractive errors of the two eyes of each animal differed by <0.5 D. Thus all eyes were moderately hyperopic at the beginning of the optical treatments described below, and a +3 D lens would correct them approximately to emmetropia.

After 72 hours for complete recovery from cycloplegia, the six monkeys were divided randomly into Group A (monkeys numbered arbitrarily 336, 338, and 340) and Group B (monkeys 342, 344, and 346). All six animals were kept in their normal environment for the first 6 hours of the daily light phase, and then fitted with light-weight helmets[38] to hold spectacles in front of the eyes. A +3.00 D spectacle lens (Group A) or a diffuser (Group B) was placed over the right eye, and a clear, zero-powered (plano) lens was placed over the left eye (both groups). Previous studies indicated that young monkeys choose to accommodate for the eye having the +3 D lens, making this eye functionally emmetropic and its plano-treated fellow-eye hyperopic; and that the plano-treated fellow-eyes of monocular diffuser animals may have clear images for greater parts of the day than normal monkeys.[14,39] Because the diffuser absorbed approximately 0.1 $\log_{10}$ of the incoming light (ND = 0.1), a clear neutral density filter of the same density was added to the plano lens worn by the Group B monkeys. It was not necessary to do this in plus-lens experiments, since the optical density of the plus-lenses was negligible. Thus, in diffuser-treated as well as plus-lens–treated animals, the average light intensity reaching the retinas of treated and control eyes of a given animal was the same.

Fixation, Sectioning, and Immunocytochemistry

Animals wore the diffusers or plus lenses for 30, 60, or 240 minutes (one per treatment duration in each group). At the end of the treatment period, each animal was killed by an overdose of barbiturates. The eyes were removed, hemisected under sterile conditions, and the vitreous removed. Each eye was given a code number, so that duration of treatment and the identity of treated and control eyes were unknown until results had been obtained. The eyecups were fixed in 4% paraformaldehyde and 3% sucrose in 0.1 M phosphate buffer, pH 7.4, for 2 hours at 4°C. This was followed by three 5-minute washes in phosphate-buffered saline (PBS, pH 7.4) and cryoprotection in PBS plus 30% sucrose.

For immunocytochemistry, eyes were thawed, embedded in O.C.T. compound (Tiss Tek; Sakura Finetek Inc., Torrance, CA), snap-frozen in liquid nitrogen, and mounted onto sectioning blocks. Sections nominally 12 to 15 μm thick were cut transversely to retina and sclera, thaw-mounted onto gelatin-coated slides, air-dried, ringed with rubber cement, and stored at −20°C until ready for antibody-labeling.[26,27,34]

Slides were washed three times for 15 minutes each in PBS, then incubated in the primary antibody solution (150 μL diluted antiserum, 5% normal goat serum, 0.3% Triton X-100) overnight at room temperature (~20°C) in a humidified incubation chamber. The slides were washed three times again for 15 minutes each in PBS and incubated with the secondary antibody (150 μL diluted in PBS) for 5 hours at room temperature. The slides were then washed as before, the sections covered with 4:1 glycerol:distilled water and coverslipped. Labeling was observed with an epi-illumination fluorescence microscope and images were recorded digitally (details below).

For double immunocytochemical labeling, slides were incubated in a mixture of two primary antibodies: one to an immediate-early gene product, Egr-1 or Fra-2 (polyclonal antiserum raised in rabbit); and one to a specific amacrine cell marker (polyclonal antiserum or monoclonal antibody raised in a species other than rabbit; Table 1). Anti-Fra-2 rather than anti-c-Fos antibodies were used because the labeling was stronger and more reliable. After incubation and washing, the slides were covered in a mixture of Cy3- and ALEXA-588-coupled second antibodies, directed to the IgG species represented by the two primary antibodies. Controls for cross-detection by the optical system were not necessary, because the antisera to Egr-1 and Fra-2 labeled only cell nuclei whereas the antibodies to amacrine cells labeled the neuronal perikarya around them.

Preabsorption controls for antibody specificity were not performed, because in most cases the antibodies have been well characterized and patterns of amacrine cell labeling for these markers are well documented. Rabbit antiserum to KROX-24 (Egr-1), produced by Rodrigo Bravo (Bristol Myers Squibb, Princeton, NJ), was kindly provided by Avi Chaudhuri (McGill University, Montreal, Canada). It has been characterized as highly specific for this factor in mammals[40] and has been applied successfully for activity-dependent labeling in primate visual cortex.[41] Affinity-purified rabbit antiserum to an N-terminal pep-

tide of human Fra-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) has been characterized by immunocytochemistry, Western blot analysis, and immunoprecipitation as cross-reactive with Fra-2 from mammals including humans, but not with c-Fos, Fra-1, or FosB (Santa Cruz data sheet). Mouse monoclonal antibody to γ-aminobutyric acid (GABA) coupled to bovine serum albumin has been found by dot-blot immunoassay to be specific for GABA, having no cross-reactivity with other amino acids including glycine, L-glutamate, L-glutamine, L-aspartate, and L-alanine (Sigma data sheet; Sigma-Aldrich Canada, Oakville, ON). GABA labeling similar to ours has been reported in macaque retina.[42] Mouse monoclonal antibody to the GABA-synthesizing enzyme isoform, glutamic acid decarboxylase–65 kDa (GAD65; Developmental Studies Hybridoma Bank [DSHB], Ames, IA) was raised against GAD purified from rat brain.[43] It recognizes GAD65, but not the alternative isoform GAD67, in rat brain homogenate[43] and selectively labels GABAergic neurons and pancreatic islet cells (DSHB data sheet). Similar GAD65 labeling has been reported in primate retina.[44] Mouse monoclonal antibody to protein kinase C (PKC) from bovine brain cross-reacts with the hinge region (residues 292 to 317) of the activated enzyme, which is highly conserved in isoforms alpha, beta, and gamma from mammals including humans.[45] This antibody has been widely used in retinal studies, and is known to recognize primarily the alpha isoform, strongly in rod bipolar cells and weakly in cone ON-bipolar cells, in retinas of mammals including monkeys.[46–48] Monoclonal antibody 115A10, which was raised to a homogenate of mammalian olfactory epithelium,[49] also recognizes both rod- and cone ON-bipolars in primates,[47,48,50] and monoclonal antibody CD15, which binds to N-acetyl lactosamine-containing glycoproteins,[51] labels type DB6 diffuse cone ON-bipolars in macaque retina.[50] References to the characteristics of other antibodies, which were less crucial for the results of this study, are indicated in Table 1.

Microscopy

Slides were viewed by epifluorescence, using a Zeiss Universal microscope with 25x water-immersion objective (Zeiss Plan-Neofluar 25/0.8; Carl Zeiss Canada Ltd., Toronto, ON), Optovar setting at 1.6, and 8x high-eyepoint eyepieces. The relative density of labeled nuclei was determined visually: Nuclei were rated as immunopositive if they were subjectively brighter than background, and the numbers of immunopositive nuclei were counted in each microscope field (approximately 480 μm of retinal width per field). Comparable areas of the fundus in experimental and control eyes were studied. In most cases all fields were counted in ten sections, for a total of thirty fields per retina per marker combination. Microscope images were captured with a Spot RT black-white cooled-CCD digital camera, operating under Spot software (Diagnostic Instruments Inc., Sterling Heights, MI), set for automatic exposure. The CCD chip array was 1520 × 1080 pixels and the length of the field captured under these conditions was ~480 μm; therefore the spatial resolution was ~0.3 μm/pixel. Images were captured at 8 or 12 bits of gray-level and transferred into Adobe Photoshop (Adobe Systems Inc., San Jose, CA) at 8 bits of gray-level for final representation and superposition of double labels. A slide micrometer was imaged for calibration of magnification.

Elimination of Bias in Data Acquisition

The observer knew only a code number for each eye, and therefore was "blind" to the experimental conditions until after all data had been obtained.

Results

Treatment Conditions and Effects of Treatment

Monkeys were treated either one of two ways, in addition to varying treatment duration: (1) *Diffuser versus Plano.* In one set of three animals, one eye was covered with a diffuser that disrupted image formation and reduced the available light intensity by approximately 20%, while the other eye was covered with a plano (0 D) lens plus a Wratten neutral density filter (N.D. = 0.1) that equalized the light levels for the two eyes. During the treatment period, the retina of the diffuser-covered eye received only diffuse blur, while that of the plano-covered eye received images of the same intensity as the diffuser retina, but presumably well-focused. Since the plano eye was the only one providing unobstructed vision, it could be assumed to control its own accommodation. Given that these eyes had low hyperopic refractive errors (+3.0 D), the plano eye should have been able to focus accurately for most viewing distances. Therefore, while the distance at which these animals were fixating and accommodating was not determined, it is logical that the retinas of their plano-covered eyes were receiving a blend of in-focus and hyperopically-defocused images, and a paucity of myopically defocused images, during the treatment period. For convenience these eyes were termed "emmetropic", because the net exposure to in-focus images was far greater in these than in the contralateral diffuser-covered ("deprived") eyes. (2) *Plano versus Plus-Lens*. In the other set of three animals, one eye was covered with a plano lens, and the other with a +3 D lens of equal transmission (near 100%) which compensated perfectly for the hyperopic (+3 D) refractive error of the eye. During the treatment period, fixation would be controlled by the now functionally emmetropic plus-lens eye. Since accommodation of the two eyes is yoked in primates, regardless of fixation distance the plus-lens eye would determine the accommodative state of the plano eye as well,[14] making the plano eye net hyperopic by 3 D with respect to the plus-lens eye. While in this situation, too, the retinas of both eyes receive a blend of myopically and hyperopically defocused as well as in-focus images. Again it is logical that the retinas of the plano-covered eyes were receiving a net excess of hyperopically-defocused images, and a net paucity of myopically defocused images, compared to the retinas of plus-lens eyes. Recognizing this, for convenience the plus-lens eyes were called "emmetropic", and the fellow plano eyes "hyperopic". There was no apparent difference in mean labeling frequency, for either Fra-2 or Egr-1, in the plano-lens eyes of diffuser-reared monkeys versus the plus-lens eyes of the monkeys treated with +3.0 D lenses (see below). Therefore, it seems unlikely that the small difference in luminance ($0.1 \log_{10} = \sim 20\%$) could have caused the difference in labeling between these two paradigms.

Focus-Dependent Labeling of Retinal Neurons

Egr-1. The nuclei of many cells in the inner nuclear layer were Egr-1 immunoreactive (Egr-1-IR), in open control as well as treated eyes (Fig. 1). Most of the labeled nuclei were located near the middle of the inner nuclear layer (INL), suggesting that they were nuclei of bipolar cells. Closer to the border of the inner plexiform layer were the less abundant labeled nuclei of other neurons, presumably amacrine cells. The identities of some Egr-1–immunoreactive cells were confirmed by immunocytochemical double-labeling in representative sections (below, and Fig. 1). Without double-labeling every single section, however, it would be impossible to determine reliably the identity of every Egr-1-IR cell. Therefore, the counts of total cells labeled, presented here, represent the sum of labeled bipolar and amacrine cells, but ≥90% of them were bipolar cells.

The number of detectable Egr-1-IR nuclei per 480 μm field of view (hereafter called "Egr-1-IR cell density" for convenience) was apparently altered by treatment with a plus-lens or diffuser for 30, 60, or 240 minutes (Fig. 2). The mean Egr-1-IR cell densities were relatively high, and similar in value (~60 to 65 cells/field), in plus-lens–treated eyes contralateral to plano-treated eyes, and in plano-treated eyes contralateral to diffusers [both "emmetropic"]; but lower, and similar in magnitude

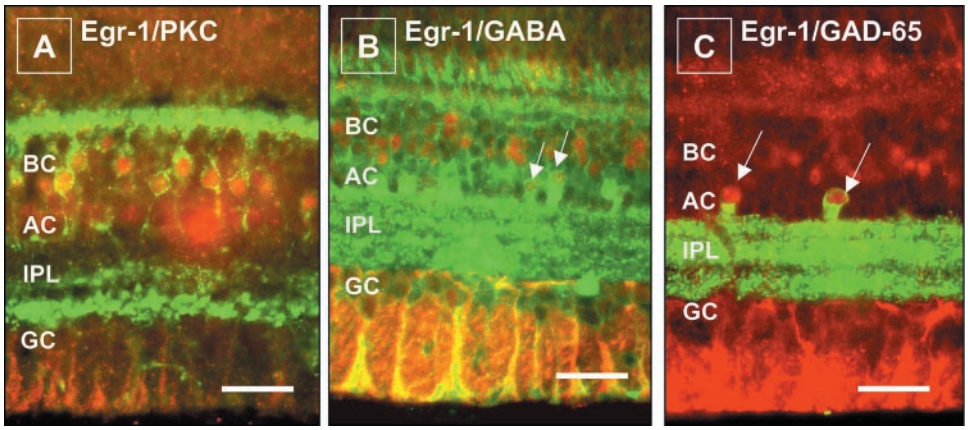

FIGURE 1. Blended images of double-labeling for induced Egr-1 (*red*), and constitutive markers (*green*) for: (**A**) ON-bipolar cells: PKCa/b; and GABAergic amacrine cells, (**B**) GABA, and (**C**) GAD-65. Monkey was treated for 240 minutes with a plus-lens on one eye (illustrated), a plano lens on the other. *Arrows* indicate Egr-1 in GABAergic amacrine cells. BC, bipolar cells; AC, amacrine cells; IPL, inner plexiform layer; GC, ganglion cell layer. Scale bar: 30 μm.

(~45 to 50 cells/field), in diffuser-treated eyes ["deprived"] contralateral to plano-treated eyes, or in plano-treated eyes contralateral to plus-lenses ["hyperopic"] (Figs. 2A, 2B). Only one animal was examined for each combination of treatment and duration, and the effects of treatment duration on Egr-1-IR cell density were not apparently different. Treating the three animals observed for different durations as a single sample, the substantial difference in aggregate means suggested that cells in the INL are stimulated more to produce Egr-1 when retinal stimuli are enriched in in-focus and myopically-defocused images, than when retinal stimuli are dominated by hyperopic or diffusely blurred images (Figs. 2A, 2B).

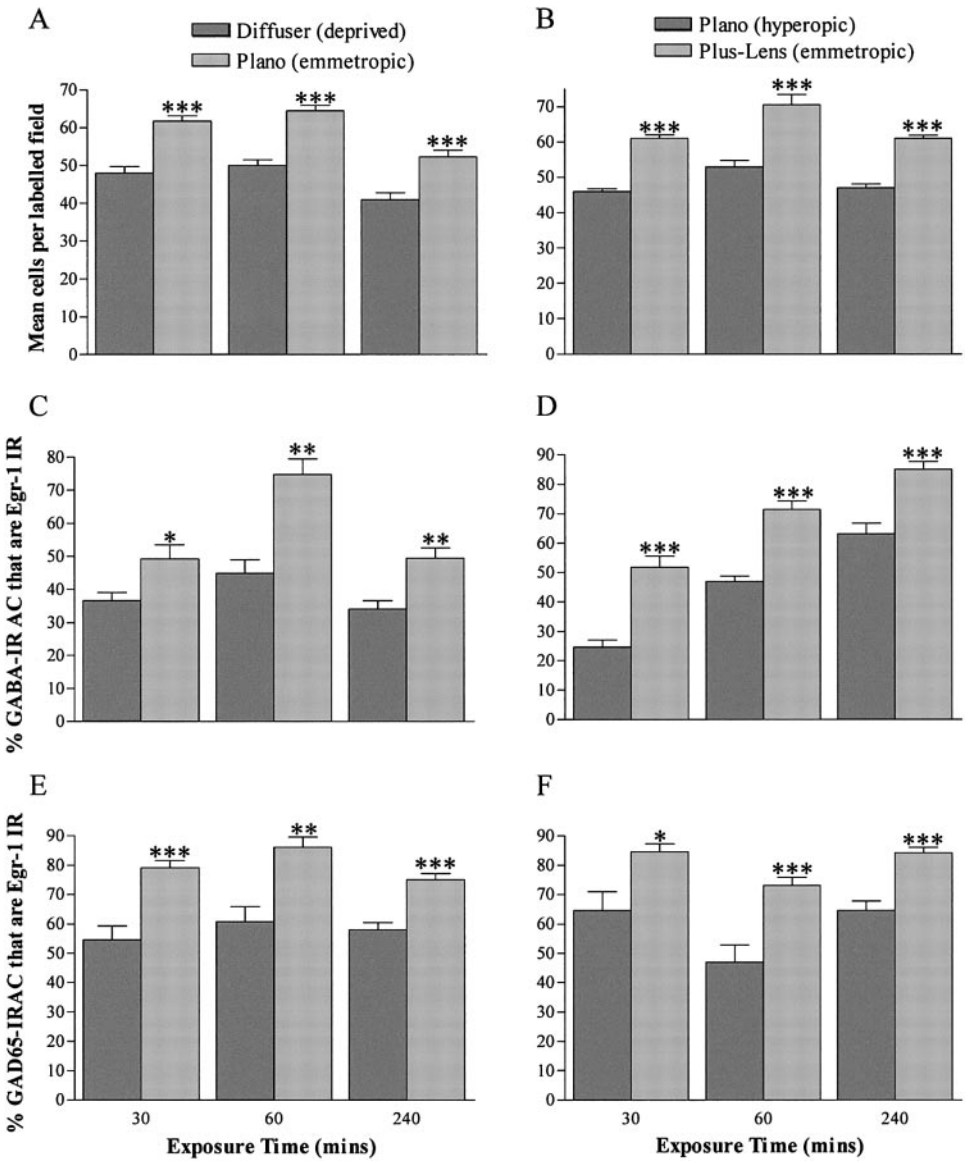

FIGURE 2. Graphs of vision-dependent Egr-1 labeling in infant monkey retinas. Monkeys were treated for 30, 60, or 240 minutes (Exposure Time), and Egr-1-IR cells were counted in 30 microscope fields per eye. Each bar represents data from one animal. Data for three key parameters are represented in separate graphs: (**A**, **B**) mean number of labeled nuclei per microscope field; (**C**, **D**) mean % of GABA-IR amacrine cells that were Egr-1-IR, and (**E**, **F**) mean % of GAD65-IR amacrine cells that were Egr-1-IR. Each parameter was measured in retinas of two experimental groups: (**A**, **C**, **E**) diffuser versus tinted plano lens, and (**B**, **D**, **F**) +3 D lens versus untinted plano lens. Bars and error bars at the three exposure times represent the mean counts per field ± SD. Since one animal was tested at each exposure time, these statistics indicate the consistency of labeling frequency over the entire area of retina in which counts were made (n = 30 fields).

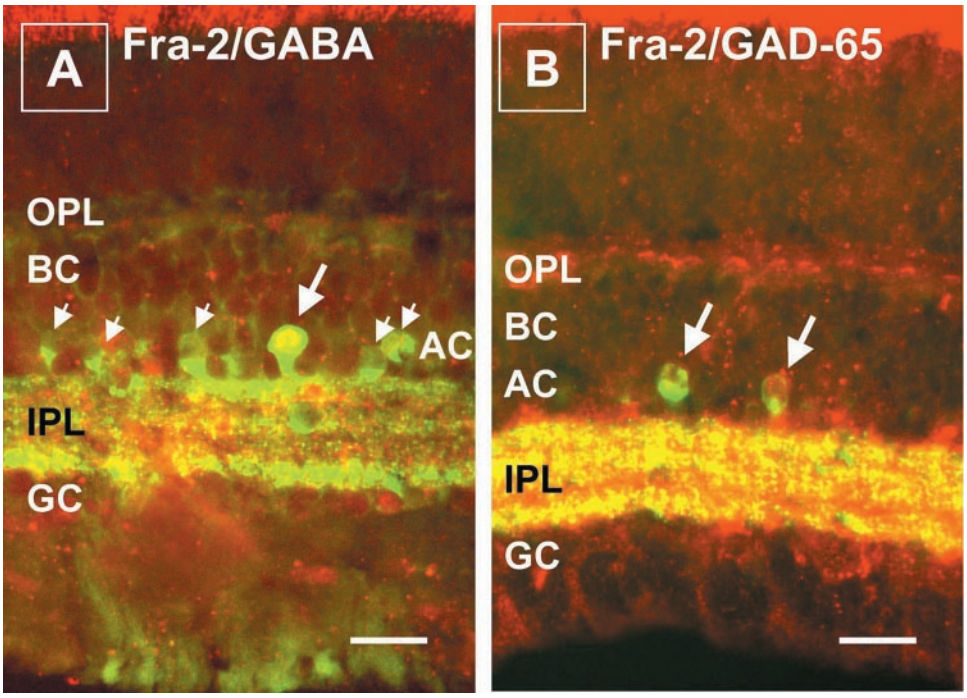

FIGURE 3. Blended images of double-labeling for induced Fra-2 (*red*), and constitutive markers (*green*) for GABAergic amacrine cells: (**A**) GABA, and (**B**) GAD-65. Same conditions and abbreviations as for Figure 1; OPL, outer plexiform layer. *Small arrows* in (**A**) indicate smaller amacrine cells that are GABA-positive but Fra-2-negative. Scale bar: 20 μ.

Fra-2. Fra-2 immunoreactivity (Fra-2-IR), like Egr-1-IR, was expressed in cells in the inner nuclear layer, in open control as well as treated eyes (Fig. 3). Unlike Egr-1-IR, however, Fra-2-IR was not seen in bipolar cells, but only in cells close to the IPL, which are presumably amacrine cells. Partly for this reason, the counts of Fra-2-IR cells were only approximately 5 to 10% of the counts of Egr-1-IR cells per unit area.

As with Egr-1-IR, the Fra-2-IR cell density was noticeably lessened by treatment with a diffuser for 30 or 60 minutes (Fig. 4A). In contrast to Egr-1-IR, however, the Fra-2-IR cell density was not apparently affected by treatment with a diffuser for 240 minutes, or a plus-lens for any duration (Figs. 4A, 4B). Again, the effects of the duration of the +3.0 D lens regimen on Fra-2-IR cell density did not appear to be different. The combined data from three animals convey the impression that amacrine cells in general showed no consistent net induction of Fra-2 under the optical conditions in these experiments (Figs. 4A, 4B).

Identification of Focus-Sensitive Neurons

Egr-1: Bipolar Cells. Putative bipolar cells, cells having Egr-1-IR nuclei in the middle of the INL, were identified positively by suitable antibody markers. Many of the Egr-1-IR nuclei were found in cells immunoreactive for protein kinase C alpha subunit (PKCα; Fig. 1A), as well as cells immunoreactive for antigen 115A10[46–48] (not illustrated), which are mainly rod-bipolar cells but also include cone ON-bipolars.[46–48] Since most Egr-1-IR cells in the INL were bipolars, the differential effect of different visual conditions on Egr-1-labeled cell density indicates that ON-bipolar cells in monkey retina were responsive to defocus. Double-labeling for Egr-1 and CD15, which labels type DB6 diffuse cone-ON-bipolar cells in the macaque,[50] confirmed that Egr-1 expression is modulated by defocus in cone- as well as rod- ON-bipolars; 50.4 ± 2.1% of CD15-IR cells were also Egr-1-IR in the "deprived" retina treated with a diffuser for 240 minutes, whereas 57.4 + 2.2% of CD15-IR bipolars were Egr-1-IR in the "emmetropic" plano control retina [P = 0.024, n = 30 fields] (not illustrated).

Egr-1: Amacrine Cells. Egr-1-immunoreactivity was present in many GABA-IR (Fig. 1B) and GAD65-IR (Fig. 1C) amacrine cells, particularly large cells with somata ~10 μm in diameter and thick (6 to 7 μm) stalks connecting them to the IPL. These large cells were relatively scarce, comprising a small minority of all GABA-IR amacrines but the vast majority of GAD65-IR amacrines; the large cells were Egr-1–labeled frequently, the small cells infrequently (Figs. 1B, 1C). Egr-1-IR nuclei were also localized to GAT-1–, $GABA_A$- and nNOS-IR amacrine cells (not illustrated), but because insufficient material was available, whether Egr-1–immunoreactivity in these cells is treatment-dependent could not yet be determined. Egr-1–immunoreactivity was not detected in parvalbumin-, calretinin-, calbindin-, cellular retinoic acid binding protein (CRABP)-, or tyrosine hydroxylase (TH)-IR amacrine cells.

The relative activation of putatively GABAergic amacrine cells was determined by counting the number of *GABA-IR* cells per 100 that were Egr-1-IR ("% GABA-IR cells activated" to produce Egr-1), as done previously for glucagon-IR amacrine cells in the chick.[34,36] The percentage of GABA-IR cells activated, like the overall density of Egr-1-IR cells, was apparently influenced by treatment with plus-lens or diffuser for 30, 60, or 240 minutes (Figs. 2C, 2D). The mean percents of GABA-IR cells activated to produce Egr-1 were relatively (and about equally) high in plus-lens-treated eyes and plano-treated eyes contralateral to diffusers, but relatively (and about equally) low in diffuser-treated eyes or plano-treated eyes contralateral to plus-lenses. The percent GABA-IR cells activated did not appear to be influenced by treatment duration. The mean % GABA-IR cells activated was ~50 to 85% in "emmetropic" eyes (plus-lens– or plano-treated contralateral to diffusers); but lower, ~25 to 65%, in "deprived" (diffuser-treated) or "hyperopic" eyes (plano-treated, contralateral to plus-lens; Figs. 2C, 2D). Effects of treatment for 30, 60, or 240 minutes were not discernibly different. In general, GABA-IR amacrine cells produced detectable Egr-1 approximately 50% more often when retinal stimuli were enriched in in-focus and myopically defocused images, than when retinal stimuli comprised predominantly hyperopic or diffusely blurred images (Figs. 2C, 2D).

The percentages of *GAD65-IR* amacrines activated to produce Egr-1 were similar to those for GABA-IR amacrines. Pooling of data from the three animals showed that, on average,

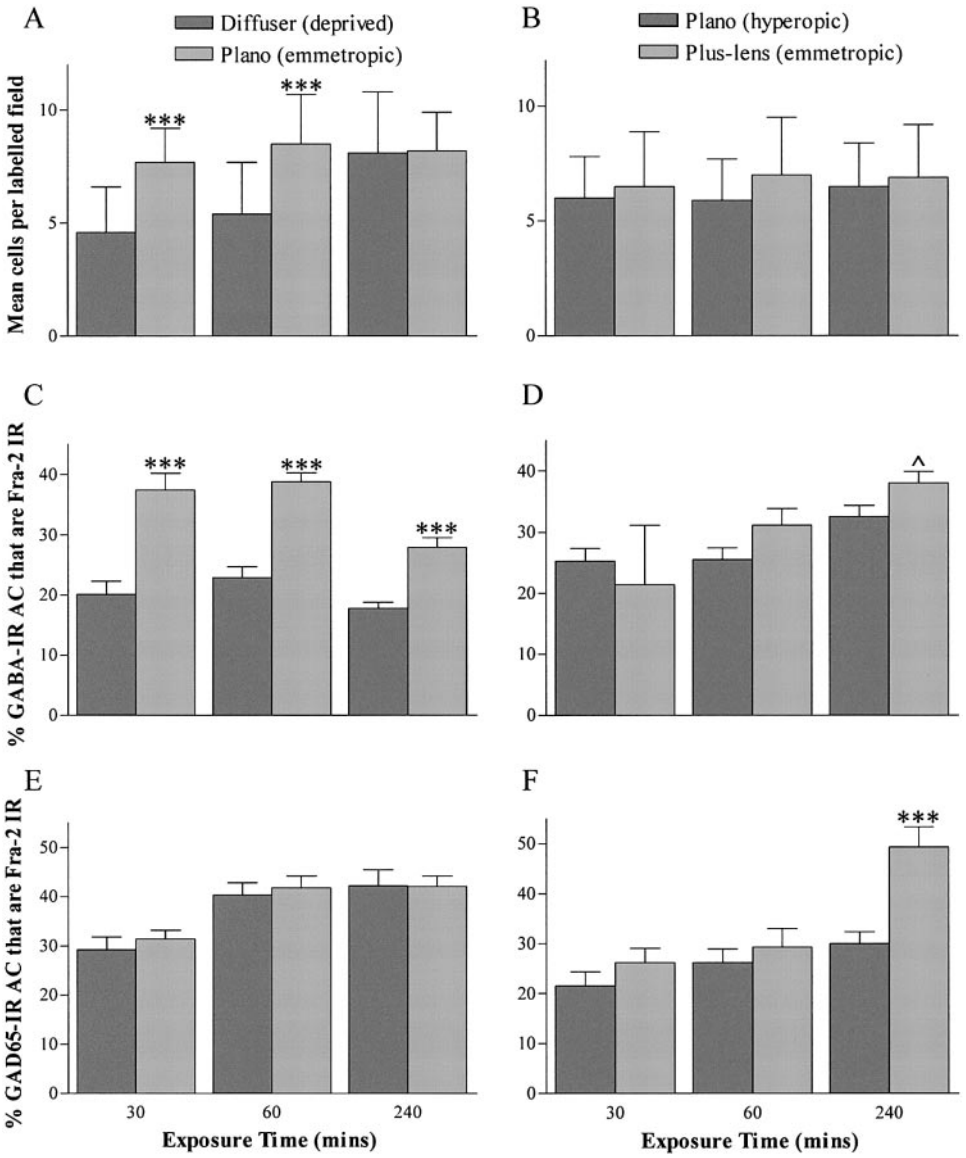

FIGURE 4. Graphs of vision-dependent Fra-2 labeling in infant monkey retinas. All conditions and labels exactly as in Figure 2, except that here Fra-2 was the activity-label.

GAD65-IR amacrine cells were stimulated to produce Egr-1 almost 50% more when retinal stimuli were enriched in in-focus and myopically-defocused images, than when retinal stimuli comprised predominantly hyperopic or diffusely blurred images However, the frequency of Egr-1 labeling was consistently higher in the GAD65-IR subset of GABAergic amacrines than in the larger set of all GABA-IR cells for all treatment durations and conditions (Figs. 2E, 2F).

Fra-2: Specific Amacrine Cells. Fra-2 immunoreactivity was present in many GABA-IR and GAD65-IR amacrine cells (Fig. 3), in which the effect of different optical treatments on activity was determined. The cells most frequently labeled were the large cells that comprised a small fraction of GABA-IR cells (Fig. 3A) but a large fraction of GAD65-IR cells (Fig. 3B) in the amacrine cell layer. The Fra-2-labeled amacrine cells appeared identical with those labeled most frequently by Egr-1 (see above). Fra-2-IR nuclei were also localized to GAT-1–, $GABA_A$- and nNOS-IR amacrine cells (not illustrated), but because insufficient material was available for study, it was not yet determined whether Fra-2-immunoreactivity in these cells is treatment-dependent. No Fra-2-immunoreactivity was detected in parvalbumin-, calretinin-, calbindin-, cellular retinoic acid binding protein (CRABP)-, or tyrosine hydroxylase (TH)-IR amacrine cells (not illustrated).

The number of *GABA-IR* cells per 100 that were Fra-2-IR ("% GABA-IR cells activated" to produce Fra-2) was apparently decreased by diffuser treatment for all durations (Fig. 4C), whereas the % GAD65-IR cells activated was not (Fig. 4E). The percentage of GABA-IR cells activated was not apparently affected by plus-lens treatment for 30 minutes or 60 minutes, and was only slightly increased by plus-lens treatment for 240 minutes (Fig. 4D). The same was true of the % GAD65-IR cells activated, although the increase at 240 minutes was stronger statistically (Fig. 4F). The % GAD-IR cells activated did not appear to be influenced consistently by treatment duration. In sum, Fra-2-immunoreactivity was induced in many putatively GABAergic amacrine cells in the plano-lens eyes of the diffuser-reared monkeys, but not influenced by the plus-lens treatment; in contrast, the GAD65-IR subpopulation of GABAergic amacrine cells was ~20 to 40% activated under all conditions and not apparently responsive to diffusers or plus-lenses, except for the anomalous result at 240 minutes of plus-lens treatment.

Summary of Treatment Effects

(1) In infant macaques, transcription factor Egr-1 was expressed more strongly under predominantly emmetropic

viewing conditions than under predominantly hyperopic viewing conditions or diffuse blur (plano lens vs. contralateral diffuser, corrective +3 D lens vs. contralateral plano lens[14]). (2) This visual modulation of Egr-1 was seen in ON-bipolar cells, as well as in putatively GABAergic amacrine cells including the GAD65-IR subpopulation. (3) The Fos-related transcription factor, Fra-2, was expressed in GABA amacrine cells but not bipolar cells. Like Egr-1, Fra-2 was expressed more strongly under emmetropic conditions in comparison to form-deprivation, but at lower basal levels. (4) Expression of Egr-1 was about equally high in retinas treated by a plano lens contralateral to a diffuser, or by a plus-lens contralateral to a plano lens; and about equally low in retinas treated by a plano lens opposite a plus-lens, or by a diffuser opposite a plano lens. These effects were not due to differences in light intensity, although the light levels were approximately 20% lower in eyes covered with plano-lens/neutral-density filter combinations than in eyes covered with unattenuated plus and plano lenses. (5) The expression of Egr-1, especially in the GAD65-IR subpopulation of amacrine cells, was higher under conditions producing consistently well-focused retinal images than under conditions of form-deprivation or consistently hyperopic defocus. The expression of immunoreactive Fra-2, in contrast, was lessened by form-deprivation in GABA-IR but not GAD65-IR amacrine cells. Since Fra-2 expression was affected differentially by defocus in GAD65-IR amacrine cells in only one animal, this anomalous result may not represent a true functional effect.

Discussion

Inducible markers have given retinal neurobiologists a powerful new tool for identifying the visual tuning properties of nonspiking interneurons without microelectrode recording. The modulation of *fos* gene expression by turning light on and off was first reported by Sagar and colleagues.[62–64] Subsequently this approach was extended to more complex stimuli, moving and textured displays,[65] as well as the induction of other markers including Egr-1.[33, 66, 67] Recently it has been shown that the synthesis of c-Fos, Fra-2, and Egr-1 is induced or modulated by focus and defocus in chick retina.[34,36] Neurons and circuits tuned to focus and defocus are logical candidates for key roles in the visual control of human ocular growth, emmetropization, and myopia development. The present studies are the first to apply this strategy to the retina of a higher primate, and to provide evidence suggesting that conditions of focus and defocus selectively affect the activity of specific types of primate retinal neurons, and therefore are important as a first step toward understanding the retinal circuitry for emmetropization in primates. Unfortunately, these results must be regarded as preliminary, because no statistical test is meaningful for such small data sets. The best conclusion, therefore, is that the differential counts in each experimental animal are reliable, and generally consistent with an explanation based on sensitivity to defocus.

Dependency of Transcription Factor Induction on Treatment Duration

The expression of immediate-early gene products such as c-Fos and Egr-1, especially slowly-induced Fos isoforms such as Fra-2, is generally time-dependent, reaching a maximum one to several hours after a new stimulus begins and then declining in its continued presence.[30,32] In the chick retina, the expression of c-Fos, Fra-2, and Egr-1 showed this sort of time-dependency.[34,68] Therefore similar behavior was anticipated in the monkey retina. While some data did suggest an increase in content with treatment duration (e.g., Egr-1 in GABA-IR cells, Figs. 2C, 2D; or Fra-2 in GAD65-IR cells, Figs. 4D, 4F), such findings were either inconsistent or minor; they might represent peculiarities of individual animals, or differences in responses of individual eyes to enucleation and fixation. Studies with larger numbers of animals will be needed to determine whether the visual induction of Fra-2 and Egr-1 in the monkey retina is time-dependent as expected.

Induction of Transcription Factors in Limited Subset of Retinal Interneurons

The induction of transcription factors in retinal neurons would be of limited interest if it were dependent only on global conditions such as changes in ambient light intensity or characteristic of most types of cells. In the chick retina, Egr-1 and Fra-2 were induced in different subpopulations of amacrine cells, and Egr-1 but not Fra-2 was induced in bipolar cells.[34] The present results in monkey were rather similar, in that both Egr-1 and Fra-2 were expressed by cells in the amacrine cell layer, Egr-1 but not Fra-2 was present in bipolar cells, and Fra-2 but not Egr-1 was localized to cells (perhaps displaced amacrines) in the ganglion cell layer. Although both markers were localized to similar-appearing GAD65-IR amacrine cells, whether they were co-expressed in the same subpopulation of cells could not be determined because all available antibodies to Egr-1 and Fra-2 were raised in the same species. GAD-65-IR amacrine cells in macaque retinas have been described as a minority of GABAergic amacrine cells, having somata 6 to 10 μm in diameter and processes ramifying mainly in the outer, middle, and inner levels of the IPL.[69–71] It is noteworthy that, while both Egr-1 and Fra-2 were detected in GAD-65-positive amacrine cells, only the frequency of Egr-1 detection was affected differentially by stimulus conditions (Figs. 2E, 2F versus Figs. 4E, 4F). This suggests that the expression of Egr-1 could be induced, at least in part, by synaptic mechanisms or transduction cascades different from those that induce Fra-2.

Focus-Dependency of Transcription-Factor Induction

The induction of Fra-2 and Egr-1 in monkey retina identified a small number of specific cell types, which are particularly responsive to differences in goodness of focus and sign of defocus. The focus-sensitive tuning of cells labeled by induction of Fra-2 and Egr-1 was consistent with the suggestion that sparsely distributed, wide-field amacrine cells such as the GAD65-IR amacrines in monkey may be tuned exquisitely to very specific stimuli and play predominantly neuromodulatory roles.[18,72]

Induction of Egr-1 in ON-Bipolar and Amacrine Cells is Influenced Primarily by Image Quality. The differential labeling of bipolar and amacrine cells was not due only to differences in intensity of light reaching the retina, because the diffusers and the plano lenses matched with them transmitted light equally (~80%). It follows that Fra-2 and Egr-1 expression in diffuser-treated eyes were lower than in plano-treated eyes because of visual "form" or pattern deprivation, rather than a reduction in light intensity. Light transmission by plus-lenses and the plano lenses matched with them also was equal (near 100%). Therefore, the greater induction of transcription factors by plus lenses than by plano lenses was due to differences in image quality (focus-defocus) rather than light intensity. Thus, some amacrine cells responded differentially to image quality (focus-defocus) in the monkey, as in the chick; ON-bipolar cells in the monkey retina, however, appeared to be functionally more complex than

their counterparts in the chick retina, in which Egr-1 synthesis in ON-bipolars was modulated by changes in light intensity but not focus and defocus.[34]

Induction of Fra-2-Immunoreactivity in Amacrine Cells Is Influenced by Continuous and Severe Image Degradation. In the monkey retina, Fra-2-IR was induced more strongly in eyes with unrestricted vision than in form-deprived eyes at equal luminance (plano vs. diffuser), but about equally in predominantly emmetropic and net-hyperopic eyes (plus-lens vs. plano). The same trends were seen for a percentage of GABA-IR and GAD65-IR cells labeled for Fra-2, as for total Fra-2-IR nuclei per unit retinal area. Differential labeling of GABA-IR and GAD65-IR amacrines would not be surprising, because there is evidence that GAD65 and GAD67 amacrines differ functionally.[73] In the experiments reported here, however, a difference in response of GAD65-IR versus GABA-IR amacrines was seen only with a percentage of Egr-1 labeling, in diffuser versus plano conditions (Fig. 2C versus Fig. 2E).

Identity of Amacrine Cells in which Egr-1 Synthesis is Focus-Dependent

In both chick and monkey retinas, viewing-conditions weighted toward plus-defocus were found to induce Egr-1 in a low-density subpopulation of widely-dispersed amacrine cells. In the chick, these cells were identified mainly as glucagon-containing amacrine cells.[34] In mammals, however, glucagon content is near or below the limits of detection by radioimmunoassay (monkey not tested),[74] and even with a proven antibody, glucagon-immunoreactive neurons or processes could not be detected immunocytochemically in the monkey retina (unpublished observations).

The cells in which Egr-1 expression is regulated by image clarity in monkey retina may contain a neuropeptide, because peptidergic amacrine cells are likely to mediate long-term, spatially diffuse modulatory or adaptational functions.[18,72,75] The visual modulation of growth might be a good example of such functions. While glucagon may not be present in mammalian retinas, other neuropeptides including neuropeptide Y, substance P, somatostatin, cholecystokinin, corticotrophin-releasing factor, and vasoactive intestinal polypeptide (VIP) and its relatives have been identified in wide-field amacrines in the monkey retina.[72,76] NPY amacrine cells in the rat play a key role in the spatial-frequency tuning of ganglion cells.[77] VIP is a good candidate for a role in defocus-compensation, since it is abundant in many mammalian retinas,[74] the amacrine cells that contain it in primate retinas have been well characterized,[72,76] and VIP metabolism and content are modulated by visual deprivation and recovery in monkey[78] (Raviola E, et al. *IOVS* 1991;32:ARVO Abstract 2615; Young TL, et al. *IOVS* 1994;35:ARVO Abstract 3776; Fernandes A, et al. *IOVS* 2003;44:ARVO E-Abstract) and rat.[79,80] Herbst and Thier[80] argued against a primary role for VIP peptide and VIP-releasing amacrine cells in rapid synaptic transmission and visual processing, and suggested instead a role in slow adaptational processes, a concept supported by the function of the glucagon system in compensation for plus-defocus in the chick.[81–83] It remains to be seen in the monkey whether VIP amacrines in particular are activated by focus or defocus, and whether VIP or some other intercellular messenger mediates focus-dependent changes in ocular growth and refraction.

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