

Increase in retinal vasoactive intestinal polypeptide after eyelid fusion in primates

(retina/myopia)

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ABSTRACT Lids were fused in six neonatal and one adult macaque monkey (*Macaca mulatta* and *Macaca arctoides*) and were kept fused for 1 to 18.5 months. The juvenile macaques, but not the adult one, developed myopia due to excessive elongation of the eye. In all animals, the immunohistochemical reactivity of the retina for vasoactive intestinal polypeptide (VIP) was much higher in the closed than in the open eyes. The neuropeptide was localized to the perikaryon and dendrites of amacrine cells. No difference was observed in substance P immunoreactivity between open and closed eyes, suggesting that the observed effect is selective. The change in VIP immunoreactivity could be the result of an increase in peptide synthesis, a decrease in peptide release, or a combination of the two. These results indicate that VIP may play a part in the regulation of postnatal ocular growth.

Myopia or nearsightedness is a very common error of refraction in which the image of distant objects is focused by the dioptric media in front of the photoreceptive layer of the retina. Commonly, it results from excessive, anteroposterior growth of the eye during postnatal development. Both heredity and long-lasting near work may play a part in the genesis of myopia; but the essential etiology and pathogenesis of this refractive error are unknown (1). Experiments in which the lids of macaque monkeys were sutured at birth demonstrate that axial elongation and concomitant myopia develop in primates when the retina is presented with blurred images throughout the period of postnatal eye growth (2-4). In a similar fashion, children develop myopia when their lids interfere with vision (5-8) or the dioptric media of the eye become clouded (5, 9, 10). The mechanism of lid-suture myopia seems to differ in the stump-tailed (*Macaca arctoides*) and rhesus macaque (*Macaca mulatta*): in the stump-tailed macaque, axial elongation is prevented when accommodation is suppressed by atropine or when the optic nerve is sectioned, whereas the rhesus macaque still develops myopia when accommodation is paralyzed or the visual pathways are interrupted. Therefore, the central nervous system plays a dominant role in the genesis of the refractive error in the stump-tailed macaque; on the other hand, the retina of the rhesus macaque may directly influence eye growth by releasing regulatory molecules (11).

The present study is an attempt to investigate the chemical changes induced in the retina by lid fusion. A simple hypothesis for the retinal mechanism leading to eye elongation is that the distortion of the visual input caused by lid fusion modifies the output of neurotransmitters or neuro-modulators by retinal neurons: after all, the retina is exquisitely tuned to discriminate contrast. In turn, altered amounts of these molecules may influence the growth of the eye. Among the large number of transmitters synthesized by the

retina, neuropeptides can be detected readily by immunohistochemistry. Therefore, we directed our efforts to the visualization by the fluorescent antibody technique of two neuropeptides, the vasoactive intestinal polypeptide (VIP) and substance P, in the retina of the open and closed eyes of lid-sutured macaque monkeys. We report here that lid fusion causes a striking increase in VIP-like immunoreactivity in a class of retinal amacrine cells.

MATERIALS AND METHODS

Six rhesus macaques and one stump-tailed macaque were used in this study, and Table 1 illustrates the details of the experiments carried out in each of them. In summary, the lids were sutured in six animals (five rhesus and one stump-tailed macaque) within their first year and kept closed for up to 18.5 months. All monkeys developed myopia behind the sutured lid because of excessive elongation of the eye, but the severity of the refractive error varied in different subjects. One adult rhesus macaque had unilateral lid suture for 1 month; in keeping with previous observations (2), this animal did not develop myopia. The purpose of this experiment was to assess whether changes in neuropeptide-like immunoreactivity would also occur in a mature retina, when myopia could no longer be induced because the eye had completed its postnatal development.

Of the six juvenile monkeys, one was intact and five were subjected before lid fusion to experimental procedures aimed at exploring the neural determinants of eye elongation. Two had prechiasmatic optic nerve section; two had the roots of the trigeminal ganglion cut intracranially on the same side of the lid fusion; one had bilateral fusion and unilateral removal of the superior cervical ganglion. None of the denervation procedures prevented the development of myopia.

When the lids were opened, the eyes were refracted by retinoscopy, and the anteroposterior diameter of the vitreous chamber was measured by A-scan ultrasonography. Fixation of the retina by vascular perfusion was carried out at variable time intervals after opening the lids. In some animals, the newly established palpebral rima remained open; in others, the lids partially fused again, leaving a small, irregular window. For vascular perfusion, the monkeys were anesthetized first by intramuscular injection of 10 mg of ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, NY) per kg of body weight and subsequently by intravenous infusion of sodium pentobarbital (Nembutal; Abbott). The vascular tree was flushed with saline through the left ventricle, followed by perfusion with an ice-cold mixture of 2% formaldehyde/0.3% picric acid in 0.2 M Sörensen phosphate buffer (pH 7.4) (12). The eyes were removed, opened, and immersed in the fixative fluid for an additional 48 hr at 4°C; they were subsequently washed overnight at 4°C in 0.1 M

Table 1. Refractive error and retinal peptide immunoreactivity after eyelid suture

Monkey species	Sutured eye(s)	Age at lid fusion, mo	Lid opening and measurement, mo	Age at time of fixation, mo	Depth of vitreous chamber, mm	Refractive error, diopters	Retinal VIP ^a	Retinal SP ^a
<i>M. mulatta</i> ^b	O.D.	8	24.5	31	13.81	O.D. = -9.0 ^c	O.D. = 4+	O.D. = 1+
					11.61	O.S. = +1.0	O.S. = trace	O.S. = 1+
<i>M. arctoides</i> ^d	O.D.	1	13	23	10.53	O.D. = -4.0 ^c	O.D. = 4+	O.D. = trace
					9.80	O.S. = +0.5	O.S. = 1+	O.S. = trace
<i>M. mulatta</i> ^e	O.D.	4.5	23	32	12.83	O.D. = -7.0 ^c	O.D. = 2+	O.D. = 1+
					12.73	O.S. = -6.0	O.S. = 2+	O.S. = 1+
<i>M. mulatta</i> ^f	O.D.	4	15.5	20	12.24	O.D. = -4.0 ^g	O.D. = 4+	O.D. = 2+
					10.62	O.S. = +3.0	O.S. = 1+	O.S. = 2+
<i>M. mulatta</i> ^h	O.S.	2.5	16	21	10.33	O.D. = -0.5 ^g	O.D. = trace	O.D. = 1+
					12.96	O.S. = -9.5	O.S. = 4+	O.S. = 1+
<i>M. mulatta</i>	O.D.	0.5	16	19	14.19	O.D. = -12.0 ⁱ	O.D. = 4+	O.D. = 1+
					11.29	O.S. = 0.0	O.S. = 2+	O.S. = 1+
<i>M. mulatta</i>	O.D.	≈120	121	121	11.87 ^j	O.D. = +0.5 ^{i-j}	O.D. = 3+	O.D. = 1+
					12.05	O.S. = -0.5	O.S. = 1+	O.S. = 1+

O.D., right eye; O.S., left eye.

^aImmunoreactivity judged by two independent observers in a masked fashion, arbitrary scale of 0 to 4+. SP, substance P.

^bRight preganglionic trigeminal nerve section at 8 months.

^cSeven to 10 months before fixation, fused eyelids were opened for measurements and remained open.

^dRight preganglionic trigeminal nerve section at 1 month.

^eLeft superior cervical ganglion removed at 4 months.

^fBilateral optic nerve section at 4 months.

^gFive months before fixation, fused eyelids were opened for measurements but afterwards they partially fused again.

^hBilateral optic nerve section at 2.5 months.

ⁱThe retina was fixed by perfusion shortly after opening the lids.

^jDifferences in depth of the vitreous chamber and refraction were already present before lid fusion.

phosphate-buffered saline (pH 7.4) containing 30% sucrose. Fifteen to 20 micrometer-thick cryostat tissue sections were assayed for VIP or substance P by the indirect immunofluorescence method. Two primary antisera to VIP were used. One was raised in rabbits against porcine VIP (Immuno Nuclear, Stillwater, MN; lot 8608028); the other was raised in rabbits against a conjugate of porcine VIP to keyhole limpet hemocyanin (Ortho Diagnostics; lot 125036). In double-antibody-precipitation radioimmunoassay, neither antibody showed cross-reactivity to peptide histidine-isoleucine, α -melanocyte-stimulating hormone, or atrial natriuretic peptide-(1-28); each showed 0.08% cross-reactivity to secretin. A monoclonal antibody to substance P (Pel-Freez; lot B3K35) was raised in rats against synthetic substance P conjugated with carbodiimide to bovine serum albumin; this antibody recognizes the carboxyl-terminal part of substance P (13).

Tissue sections were incubated at 37°C for 1 hr either with anti-VIP antiserum or with anti-substance P antibody, each diluted 1:250 (vol/vol) in 0.05 M phosphate-buffered saline with 0.3% Triton X-100. For the secondary antiserum, tissue sections were incubated for 45 min at 37°C with either goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) or goat anti-rat IgG conjugated to FITC (Cappel, Organon Teknika, Malvern, PA), depending on the primary antibody. Both secondary antibodies were diluted 1:300 (vol/vol) with 0.05 M phosphate-buffered saline containing 0.3% Triton X-100.

The following controls were carried out. (i) Anti-VIP antisera diluted 1:250 were incubated overnight at 4°C with 1.0 μ M synthetic VIP (Peninsula Laboratories, San Carlos, CA); the substance P antibody diluted 1:250 was incubated overnight at 4°C with 1.0 μ M synthetic substance P (Peninsula Laboratories). The preabsorbed antisera were then substituted for the primary antisera in the immunohistochemical procedure. (ii) Tissue sections were incubated with the FITC-conjugated secondary antisera alone. Neither the preabsorbed primary antisera nor FITC-conjugated secondary antisera stained the retina in the closed or open eyes.

RESULTS

In all animals with unilateral lid fusion, the staining of the retina for VIP-like epitopes was much more intense in the closed than in the open eye (Table 1 and Fig. 1). This difference in fluorescence intensity was observed in both juvenile *M. mulatta* and *M. arctoides* and in the adult rhesus macaque, whose right lids were kept closed for 1 month. In the single rhesus macaque with bilateral lid fusion, the staining of the retina was equally intense in both eyes. Finally, VIP-like immunoreactivity did not have any correlation with the denervation procedures that had been carried out on five of the animals prior to lid fusion (see Table 1).

VIP-like immunoreactivity was localized chiefly in a tangential plexus situated in the middle of the IPL. A few processes were also visualized in both the vitreal and scleral regions of the IPL. In places, a dense cluster of fluorescent processes surrounded a nonfluorescent perikaryon in the most vitreal cell row of the inner nuclear layer. The cell bodies that gave rise to the immunoreactive processes were about 12 μ m in diameter and appeared either round or ovoid. Ovoid cells had their long axis perpendicular to the retinal surface and gave rise to a stout process that branched at a right angle within the IPL. Although most fluorescent cells were located in the vitreal portion of the inner nuclear layer, a few were placed among the ganglion cell bodies. Because of the position of the cell body, distribution of their processes, and lack of a stained axon, the immunoreactive neurons most likely represent amacrine cells. Their precise subtype(s) could not be determined from the immunohistochemical specimens.

In the closed eye, the perikarya of the stained amacrine cells were more frequent in the central area, decreasing in number toward the periphery. Similarly, the density and/or staining intensity of the plexus formed by their dendrites decreased toward the retinal periphery. In the open eye, the stained perikarya were fewer, the fluorescence intensity was weaker, and the number of visible dendrites was significantly less. Furthermore, reactive cells and processes were not found as far toward the retinal periphery as in the closed eye.

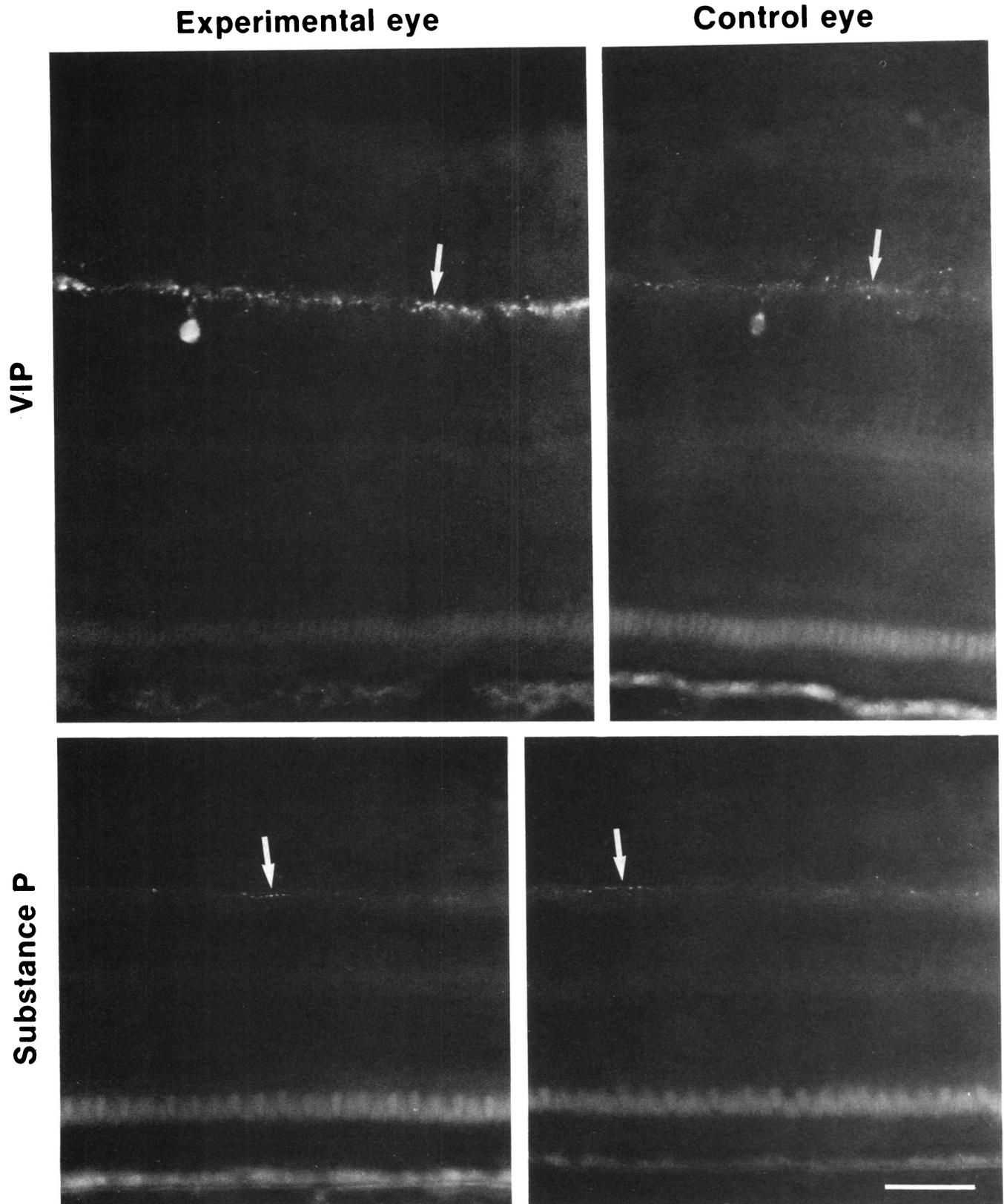


FIG. 1. Neuropeptide immunoreactivity after unilateral eyelid suture of the primate retina immunostained for VIP or substance P. Fluorescence micrographs show representative fields of the retinas from the juvenile *M. mulatta* whose lids were sutured in one eye at age 0.5 months with no preceding surgery. Enhanced immunoreactivity to VIP is evident in the inner plexiform layer (IPL) (arrows) of the closed eye (4+) as compared to the control (2+); more fibers are stained and the fluorescence intensity is greater in the experimental eye. The bodies of the amacrine cells that give rise to the fluorescent dendrites stain with greater intensity and in greater numbers in the closed eye. Both anti-VIP antisera gave the same differential staining pattern. Substance P immunoreactivity is equal (1+) in the IPL (arrows) of the two eyes. The photoreceptors are at the bottom of the micrographs. (Bar = 50 μm .)

In contrast with the findings obtained using VIP antisera, the retinal substance P immunoreactivity was identical in the two eyes (Table 1 and Fig. 1). Fluorescent processes were predominantly localized in the middle of the IPL; a few processes were also noted in its scleral and vitreal regions. Most fluorescent perikarya were located in the vitreal portion of the inner nuclear layer; a few were placed in the ganglion cell layer. Thus, as for VIP, the substance P immunoreactivity was associated with amacrine cells but no difference in number, intensity, or distribution of the stained perikarya or dendrites was observed between closed and open eyes. In the peripheral nerves of the corneoscleral coat or uvea, no significant difference was observed in VIP-like and substance P immunoreactivity between open and closed eyes.

DISCUSSION

Fusion of the lids has profound effects on the postnatal development of the visual cortex (14) and causes myopia because of excessive axial elongation of the eye (2, 11). The common denominator in both events is not light deprivation but the fact that inappropriate environmental cues, possibly the lack of contrast, interfere with the correct expression of the genetic program.

The present study documents another remarkable effect of lid fusion: the retina reacted much more intensely with VIP-like epitopes in the closed than in the open eyes. Although immunohistochemistry is a qualitative technique and a more rigorous biochemical quantitation of this phenomenon needs to be done, the difference in fluorescence intensity was so striking and consistent in all animals that it undoubtedly reflects real variation in the retinal content of VIP. Since substance P was not affected, we are encouraged to believe that the change in VIP represents a selective neural response to lid fusion.

An unusual aspect of this effect of lid fusion is that it can be produced in the adult, whereas the cortical and refractive effects are restricted to juvenile animals (2, 14). In a similar fashion, biochemical changes were observed in the mature visual cortex after lid fusion (15). Thus, the adult visual system retains a certain degree of plasticity, for its biochemistry can be altered when the sensory input is drastically changed.

It is well known that VIP and substance P are contained in specific retinal amacrine cell types of a number of vertebrate species (16, 17). In the present instance, the finding of a VIP plexus in the IPL of two macaque species extends a prior observation in *Macaca fascicularis* (16). It is unclear, however, whether these peptides function as classical neurotransmitters or neuromodulators. Furthermore, the role for these amacrine cells in the processing of visual information remains to be established. The increased VIP-like immunoreactivity in the retina of closed eyes is a dynamic event; it may be caused by enhanced synthesis of the peptide, decreased release and subsequent accumulation, or a combination of both. It is remarkable that, in animals raised with fused lids, this change is stable because it persists for weeks after the lids are opened. Thus, in juvenile monkeys lid fusion may cause a permanent alteration in the metabolism of VIP by a class of amacrine cells; alternatively, lid fusion may alter the connectivity of these cells in such a way that the number of release sites is reduced. It would be important to establish whether the high VIP content of the retina in the closed eye persists also in the adult after the lids are opened.

What are the events that lead to this dramatic increase of VIP-like immunoreactivity in a class of amacrine cells? Light deprivation seems an unlikely cause because distortion of the visual input, rather than reduction of the number of photons entering the eye, is responsible for the altered development of both the visual cortex and the eye after lid fusion (11, 14). An attractive idea is that VIP-containing amacrine cells respond with an increase in peptide synthesis when the retina

is exposed to a featureless visual world. In other vertebrate species, it has been shown that patterns represent a more effective stimulus than uniform illumination for amacrine cells (18, 19). A definitive answer to this problem must await studies on the receptive field properties of this particular class of amacrine cells.

In terms of regulation of eye growth, the crucial question is whether the increase in VIP-like immunoreactivity of amacrine cells is responsible for the excessive elongation of the eye and myopia caused by lid fusion. It is known that this peptide has effects on the blood vessels (20) and smooth musculature of the eye (21), and, perhaps, it is part of the regulatory mechanisms that control the growth of the eye during its postnatal development. On the other hand, three findings argue against VIP as the molecule that is responsible for eye elongation: (i) when the sutured eye is opened, VIP remains high but the progression of myopia stops (2); (ii) the peptide increases in the adult rhesus macaque, which does not develop myopia (2); and (iii) the peptide increases in the juvenile stump-tailed macaque, whose myopia is triggered by the central nervous system and mediated by excessive accommodation (11). Certainly, future studies are justified to clarify the relation between VIP and ocular growth.

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