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Effects of Progesterone and Other Gonadal Hormones on Glutamatergic Circuits in the Retina

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Background: Gonadal hormones function in the retina; however, their targets have not yet been identified. Therefore, the present study examined the effects of progesterone and other gonadal hormones on glutamatergic circuits in the retina.

Methods: Extracellular glutamate concentrations, which correspond to the amount of glutamate released, were examined using an enzyme-linked fluorescent assay system. The activity of glutamatergic synapses between bipolar cells and ganglion cells was investigated using a patch clamp technique. Changes in retinal thickness during pregnancy were assessed using optical coherence tomography (OCT) images.

Results: Progesterone and pregnenolone sulfate increased extracellular glutamate concentrations, whereas estrogen and testosterone did not. Progesterone increased the activity of glutamatergic synapses between bipolar cells and ganglion cells. A temporal decrease in the thickness of the peripheral retina was observed in the 1st trimester.

Conclusions: Progesterone, but not estrogen or testosterone, activated glutamate release in the mouse retina. Increases in the concentration of progesterone during pregnancy did not induce any detectable change in retinal thickness. (J Nippon Med Sch 2023; 90: 333–345)

Key words: retina, glutamate, progesterone

Introduction

The effects of progesterone in the retina have been investigated at multiple levels. The presence of progesterone receptors and the localization of enzymes for the synthesis of progesterone in the retina have been demonstrated with immunohistochemical methods[1–4] and RT-PCR methods[5]. In addition, synthesis of progesterone and its derivatives has been detected in the rat retina by using high-performance liquid chromatography[6]. Therefore, accumulating evidence indicates that progesterone can modify retinal function.

Progesterone and pregnenolone sulfate are active members of the C21-steroid family in the retina. Although they are both synthesized from pregnenolone by an enzymatic reaction[7], they exert distinct effects in the retina. Progesterone was previously reported to be neuroprotective[8–13], while pregnenolone sulfate has been shown to exert positive allosteric modulatory effects on NMDA receptors, resulting in excitotoxicity in the rat retina[14]. The excitatory effects of progesterone on glutamate receptors

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have not yet been demonstrated.

The level of progesterone in blood is dependent on the estrus cycle and increases in pregnancy. Previous studies reported sex-related differences in retinal function and diseases[1,15–17] as well as estrus cycle-dependent changes in ocular function[18–22]. Visual changes, such as refractive changes[19,20] and changes in the mean threshold sensitivity of the visual field[21], commonly occur in pregnancy[23,24]. Furthermore, differences have been reported in contrast sensitivity between premenopausal and postmenopausal women[22]. Central serous chorioretinopathy, which accompanies a decrease in visual acuity, is a well-known pathological change in pregnancy[23,24].

We previously showed that an enzyme-linked fluorescent assay system was useful for monitoring the dynamics of whole glutamatergic circuits in the retina[25]. Therefore, we investigated whether progesterone modulated the activity of glutamatergic circuits in the retina by using an enzyme-linked fluorescent assay system[25] and patch clamp technique. The findings revealed that progesterone and pregnenolone sulfate increased the activity of glutamatergic circuits, whereas the other gonadal hormones tested, estrogen and testosterone, did not. Because blood progesterone concentrations gradually increase throughout pregnancy, we also attempted to clarify whether continuous progesterone-induced glutamate release induced any detectable morphological change in the human retina, which may result in a subtle change in ocular function in pregnancy[19–21,23,24], by monitoring retinal thickness in the same women throughout pregnancy. Taken together, the present results showed that progesterone activated glutamatergic circuits without any detectable morphological change when its concentration was elevated in pregnancy. Therefore, progesterone-induced glutamate release may functionally affect visual acuity in pregnant women.

Materials and Methods

Ethical Approval

The experimental procedure for enzyme-linked fluorescent assays using animals was approved by the Institutional Animal Care and Use Committee of Fujita Health University (No. AP16007 and APU19018). The experimental procedure for electrophysiological recordings of animals was approved by the Animal Experiments Ethical Review Committee of Nippon Medical School (No. 2020-012). The experimental procedure for the clinical study was approved by the Ethics Committee at the coordinating center of the University of Tokyo and the Institutional Review Board of the University of Tokyo (No. 11924-(5)).

All studies were performed in accordance with the relevant guidelines and regulations (for animal studies) and the Declaration of Helsinki. All animal studies were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Experimental Procedure for Animals

Two techniques were used in the present study to monitor glutamate release from presynaptic terminals in the animal model: an enzyme-linked fluorescent assay system and electrophysiological recordings. We previously demonstrated that the enzyme-linked fluorescent assay system was useful for visualizing extracellular glutamate concentrations at individual layers of the retina[25]. Glutamate release from presynaptic terminals was monitored as an increase in extracellular glutamate concentrations using this method. Regarding electrophysiological recordings, we assessed the activity of presynaptic terminals and the biophysical properties of postsynaptic glutamate receptors to monitor excitatory postsynaptic currents (EPSCs).

Enzyme-Linked Fluorescent Assay

Details on the method used are described in our previous study[25]. Extracellular glutamate concentrations were monitored as the fluorescent signal intensity of nicotinamide adenine dinucleotide (reduced form) (NADH), which is a product of the catalytic effects of glutamate dehydrogenase (GDH) between glutamate and nicotinamide adenine dinucleotide (oxidized form) (NAD^+).

Slice preparation

Eyeballs were enucleated from 8-week-old mice (C57 BL/6J) anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). Eyes were hemisected and the retinae were isolated from the sclera. Retinae were placed on a PTFE filter (H100A013A, Advantec Toyo) and sliced in 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)-buffered solution. Sliced retinae (thickness, 200 μm) were maintained in HEPES-buffered solution bubbled with 100% O_2 at room temperature until used. The composition of HEPES-buffered solution (in mM) was 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH was adjusted to 7.4 with KOH). The composition of high K solution (in mM) was 135 NaCl, 60 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH was adjusted to 7.4 with KOH). Samples were kept under room light until used. Since photopigments in the outer segments of photoreceptors were bleached during this incubation period, retinal slices did not respond to light stim-

uli during experiments. Therefore, we observed the direct effects of gonadal hormones on retinal neurons using the fluorescent system.

Data sampling

Quartz glass (22 × 40 mm, Matsunami) coated with GDH was used in the experiments. GDH-coated quartz glass was maintained in HEPES-buffered solution at 4°C and used within 24 hours.

Sliced retinae were mounted onto GDH-coated quartz glass placed on an inverted microscope (Nikon, TMD-300). Slices were covered with a PTFE filter (H100A013A, Advantec Toyo) and fixed with an anchor placed on the filter. The fluorescent signals of NADH excited by LED (emission wavelength 365 nm) were sampled through a barrier filter (>470 nm) by using the ARGUS/AC system (Hamamatsu Photonics) to an offline computer every 2 minutes. The focus of the objective lens was adjusted to the surface of quartz glass.

To reduce possible contamination from background signals, slices were superfused with HEPES-buffered solution for 7.5-10 min before the superfusion of HEPES-buffered solution containing 5 mM NAD^+ and 50 μM DL-*threo*-β-benzyloxyaspartic acid (TBOA), an antagonist of glutamate transporters[26], for 5-10 min. Slices were then exposed to HEPES-buffered solution containing one of the gonadal hormones (progesterone, estrogen, or testosterone) or their derivative (pregnenolone sulfate), 5 mM NAD^+, and 50 μM TBOA. All solutions were bubbled with 100% O_2 and perfused at a rate of 1.5 mL/min. The high K solution was applied as a control stimulation to assess the viability of samples. All experiments were performed at room temperature. Gonadal hormones or their derivatives (purchased from Sigma) were dissolved in HEPES-buffered solution containing 0.1% dimethyl sulfoxide.

We previously reported the dynamics of fluorescent signals at the individual layer in detail[25]. In brief, elevated fluorescent signals in the outer plexiform layer (OPL) and the inner plexiform layer (IPL) reflected glutamate release from presynaptic terminals. Elevated fluorescent signals in the neighboring layers reflected the lateral diffusion of glutamate from OPL or IPL, since glutamate uptake into presynaptic terminals was inhibited by TBOA under experimental conditions. A high signal intensity in the outer segment reflected the contamination of the intrinsic fluorescent signal of NADH. Therefore, we monitored and analyzed fluorescent signals in OPL and IPL to assess glutamate release from presynaptic terminals.

Data Analysis

Data were selected using the same criteria reported in our previous study, to avoid contamination by artifacts, and selected data were used in the analyses[25]. To assess the effects of progesterone, estrogen, testosterone, and pregnenolone sulfate at the layer level, percent change in signal intensity (dF/F) at individual regions of interest was calculated using our previously described method (**Fig. 1A**). All statistical tests were performed using Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

Electrophysiological Recordings

We followed the method described in our previous studies[27,28].

Procedure for Whole-Mount Preparations

In brief, mice (2 months old, C57BL/6J) were killed by cervical dislocation, both eyes were enucleated and hemisected, and the retinae were isolated from the sclera. The detached retina was maintained in Ringer's solution (which contained [in mM]: 115 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.1 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose; pH 7.4, bubbled with 95% O_2 and 5% CO_2) until used. Recordings were performed in Ringer's solution at room temperature.

Recordings of spontaneous EPSCs (sEPSCs) from retinal ganglion cells

A whole-mount preparation was placed on the chamber, vitreous side up, and viewed under a fluorescent microscope (BX50WI, Olympus, Tokyo, Japan). The input resistance of patch pipettes was 8-12 MΩ when filled with Cs^+-based intracellular solution ([in mM] 115 CsCl, 5 QX-314, 0.5 $CaCl_2$, 5 HEPES, 5 EGTA, 5 ATP-3Na, and 1 GTP-1Na; pH was adjusted to 7.3 with CsOH). Retinal ganglion cells were used for recordings. To avoid contaminated recordings from displaced amacrine cells, cells with a membrane capacitance of <14 pF were excluded from the analysis. The average membrane capacitance and input resistance of recorded cells were 26 ± 8 pF and 43 ± 9 MΩ (mean ± SD), respectively. sEPSCs were recorded using a patch clamp amplifier (Axopatch-200B; Axon Instruments, Foster City, CA, USA) at a holding potential of −70 mV after blocking IPSCs in Ringer's solution containing 1 μM strychnine and 100 μM picrotoxin. Data were sampled at 10 kHz after passing through a low-pass filter at 5 kHz, using a commercially available program (pCLAMP9; Axon Instruments, Foster City, CA, USA). Progesterone was dissolved in dimethyl sulfoxide (final concentration in Ringer's solution of 0.1%).

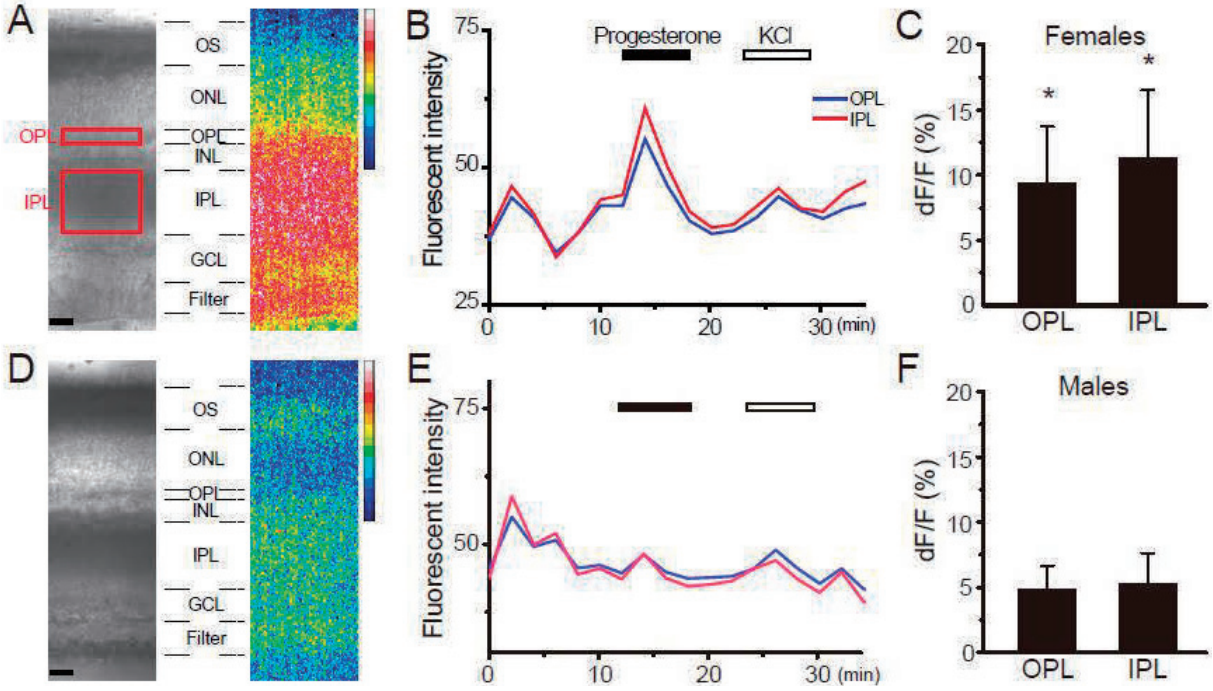

Fig. 1 Responses to progesterone stimulation under an enzyme-linked fluorescent assay system in females (A-C) and males (D-F). A and D. Differential microscopic image of a sliced retina (left) and its pseudo-color image of fluorescent signals (right). The pseudo-color image shows the difference in fluorescent signal intensities before and during application of progesterone. Red-lined rectangles shown in A are regions of interest (ROIs). ROIs correspond to the outer plexiform layer (OPL) and inner plexiform layer (IPL). B and E. Changes in signal intensity during progesterone or high K stimulation. Bars above the traces show the timing of the application of 1 μM progesterone or 60 mM KCl. C and F. Bar graph of the change in signal intensity during progesterone stimulation. The number of samples was 11 for females and 5 for males. OS: outer and inner segments of photoreceptors, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer, Filter: filter paper. The Wilcoxon signed-rank test (C) and a one-sample *t*-test (F) were performed. *P<0.05.

Data Analysis of sEPSCs

Recorded signals were analyzed offline. Because contamination of sEPSCs made calculation of mean baseline noise difficult, we were unable to identify sEPSCs by using calculated mean baseline noise. Therefore, signals with an amplitude >8 pA (approximately 5-fold that of the estimated mean baseline noise when contamination of sEPSCs was absent) were automatically detected with the commercial program Minianalysis (Synaptosoft, Decatur, GA, USA) in the present study. A whole trace was then visualized to check for the over-or-under-detection of events. The timing of sEPSC events was defined as the time of an individual sEPSC peak. We analyzed the sEPSCs of cells when the frequency of sEPSCs was >1 Hz. The average frequency, amplitude, rise and decay times, charge transfer of individual sEPSCs, and the sum of the charge transfer of sEPSCs for 30 seconds in the presence and absence of progesterone were analyzed. Statistical analyses were performed using a paired *t*-test (two-tailed).

Clinical Study

Participants and enrollment criteria

We followed-up 12 pregnant women in the present study. Informed consent was obtained from all participants, and those who did not grant authorization for the use of their medical records in research were excluded from the analysis. The medical histories of participants were reviewed at the outpatient clinic of the University of Tokyo Hospital. Inclusion criteria for pregnant women were as follows: (1) eyes with a spherical equivalent between −6 diopters and +3 diopters, and (2) eyes with clear ocular media. Exclusion criteria were the presence of other eye diseases (e.g., chorioretinal atrophy in the macula, glaucoma, and any other retinal disorders) and high myopia (−6.0 diopters or less). None of the participants were diagnosed as having central serous chorioretinopathy. All data were fully anonymized before the assessment of data.

Eye examination

Participants underwent a set of comprehensive ophthalmological examinations in the 1st, 2nd, and 3rd trimes-

ters and the postpartum period. These examinations included measurement of best-corrected visual acuity, axial length (AL) (IOL master, Tomey OA-2000, version 5.4.4.0006; Tomey, Aichi, Japan), and refractive errors (KR-8900 version 1.0.7; Topcon Corp., Tokyo, Japan). After image capturing in each study visit, exported OCT (HRA spectralis; Heidelberg Engineering GmbH, Dossenheim, Germany) images were analyzed using custom written software. An automated graph-based method was used to measure retinal thickness.

Measurement of progesterone

Progesterone concentrations were measured in blood samples collected at the 4 stages of gestation (the 1st, 2nd, and 3rd trimesters and postpartum period) according to the manufacturer's instructions (progesterone ELISA kit, Cosmo Bio).

Measurement of retinal thickness

We separated the retina into 9 regions (**Fig. 5A**) and measured the thickness of each region (for see **Table 2**) to assess possible glutamate toxicity. Changes in the thickness of the retina during pregnancy were assessed using the thicknesses of the fovea and 2 surrounding concentric regions (the parafovea and perifovea) (**Fig. 5B**). The thicknesses of the parafovea and perifovea were calculated as the average thickness of 4 regions (temporal, nasal, superior, and inferior).

Statistical analysis

All statistical analyses were conducted using the statistical programming language "R" (version 3.1.3; The R foundation for Statistical Computing, Vienna, Austria).

Results

Effects of Gonadal Hormones on Extracellular Glutamate Concentrations

In the enzyme-linked fluorescent assay system, extracellular glutamate concentrations were estimated based on the fluorescent intensity of NADH[25], which is a product of the catalytic effects of GDH between glutamate and NAD^+.

In females, application of 1 μM progesterone increased the intensity of fluorescent signals (**Fig. 1A and B**). In most samples, the intensity of fluorescent signals peaked within 2-4 min and then gradually decreased. We then applied a high K solution to the same samples to confirm the viability of samples. The increase in dF/F was significant in both OPL and IPL (**Fig. 1C**). In males, there appeared to be an elevation in fluorescent signals (**Fig. 1D and E**). However, the increase in dF/F was not significant (**Fig. 1F**). These results indicated for the first time that progesterone activated glutamatergic circuits in the retina.

Since pregnenolone sulfate was previously shown to activate glutamatergic circuits, we investigated whether it mimicked the effects of progesterone (**Fig. 2A, B**). Application of 1 μM pregnenolone sulfate induced a significant increase in fluorescent signals in IPL, but not in OPL.

We then examined the effects of estrogen or testosterone on glutamatergic circuits in females. The application of 1 μM 17-β-estradiol did not markedly affect the intensity of fluorescent signals (**Fig. 2C, D**). The increase in dF/F was not significant. The application of 1 μM testosterone did not induce any increase in fluorescent signals (**Fig. 2E, F**).

Effects of progesterone on sEPSCs

We further investigated whether progesterone increased the release of glutamate in IPL. To assess the effects of progesterone on the activity of glutamatergic circuits, we monitored sEPSCs from retinal ganglion cells that receive glutamatergic inputs from bipolar cells (**Fig. 3A, B, and C**). We examined the following 6 parameters of sEPSCs. Frequency and total charge transfer are used to evaluate the activity of glutamatergic synapses, while the amplitude, decay time, rise time, and charge transfer of individual sEPSCs are employed to assess presynaptic vesicle sizes or the properties of postsynaptic glutamatergic receptors. In the present study, we limited our electrophysiological analysis data to distinguish sEPSCs from baseline noise (amplitude >8 pA), as described in the Methods section.

In females, progesterone increased the frequency (**Fig. 3D**, **Table 1**) and total charge transfer (**Fig. 3I**, **Table 1**) of sEPSCs. In males, slight increases were observed in both the frequency (**Fig. 3D**, **Table 1**) and total charge transfer (**Fig. 3I**, **Table 1**). Progesterone increased the charge transfer of individual sEPSCs in males (**Fig. 3H**, **Table 1**), whereas no significant differences were observed in females (**Fig. 3H**, **Table 1**). Furthermore, no significant changes were noted in amplitudes (**Fig. 3E**, **Table 1**), rise times (**Fig. 3G**, **Table 1**), or decay times (**Fig. 3F**, **Table 1**) in males or females.

The present results indicate that progesterone increased the frequency of glutamate release from bipolar cell terminals without affecting presynaptic vesicle sizes or the properties of postsynaptic glutamate receptors in females. On the other hand, the effects of progesterone on the transmission of glutamatergic signals were subtle in males. Therefore, electrophysiological recordings confirmed the results of the enzyme-linked fluorescent assay

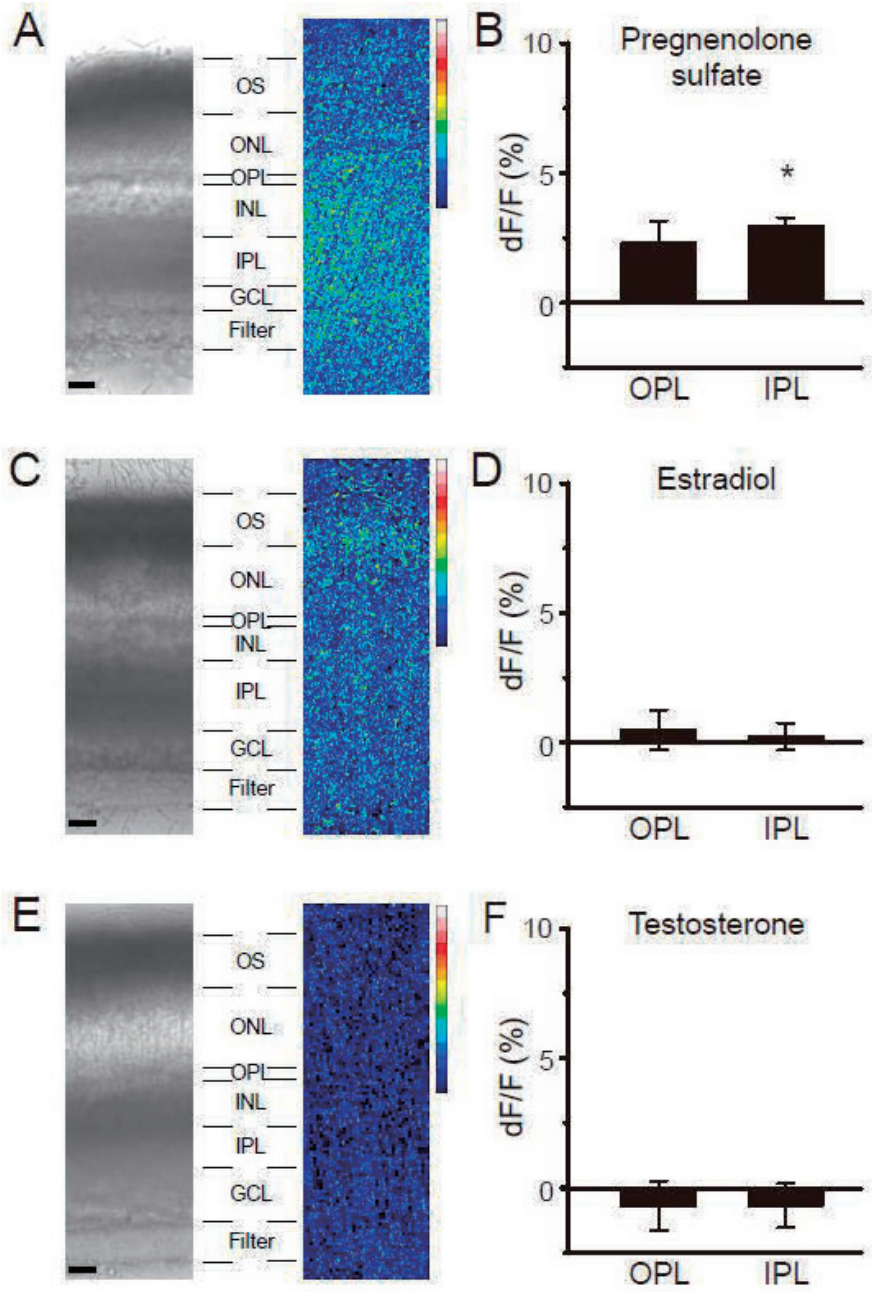

Fig. 2 Responses to pregnenolone sulfate (A, B), 17-β-estradiol (C, D), or testosterone (E, F) stimulation using the enzyme-linked fluorescent assay system in females. The experimental protocol was the same as that shown in Figure 1. Differential microscopic image of a sliced retina (left) and its pseudo-color image of fluorescent signals (right). B, D and F: Bar graph of the change in signal intensity to pregnenolone sulfate (B), 17-β-estradiol (D), or testosterone (F) stimulation. The numbers of samples were 3 (pregnenolone sulfate), 5 (estradiol), and 5 (testosterone). OS, outer and inner segments of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer, Filter: filter paper. A one-sample *t*-test was performed. *P<0.05.

system.

Changes in Retinal Thickness during Pregnancy

Although the effects of progesterone on glutamatergic circuits has not been examined previously, the present study showed that it activated glutamatergic circuits in the retina *in vitro*. A previous study demonstrated that when extracellular glutamate concentrations were high, glutamate had the potential to act as a toxin inducing

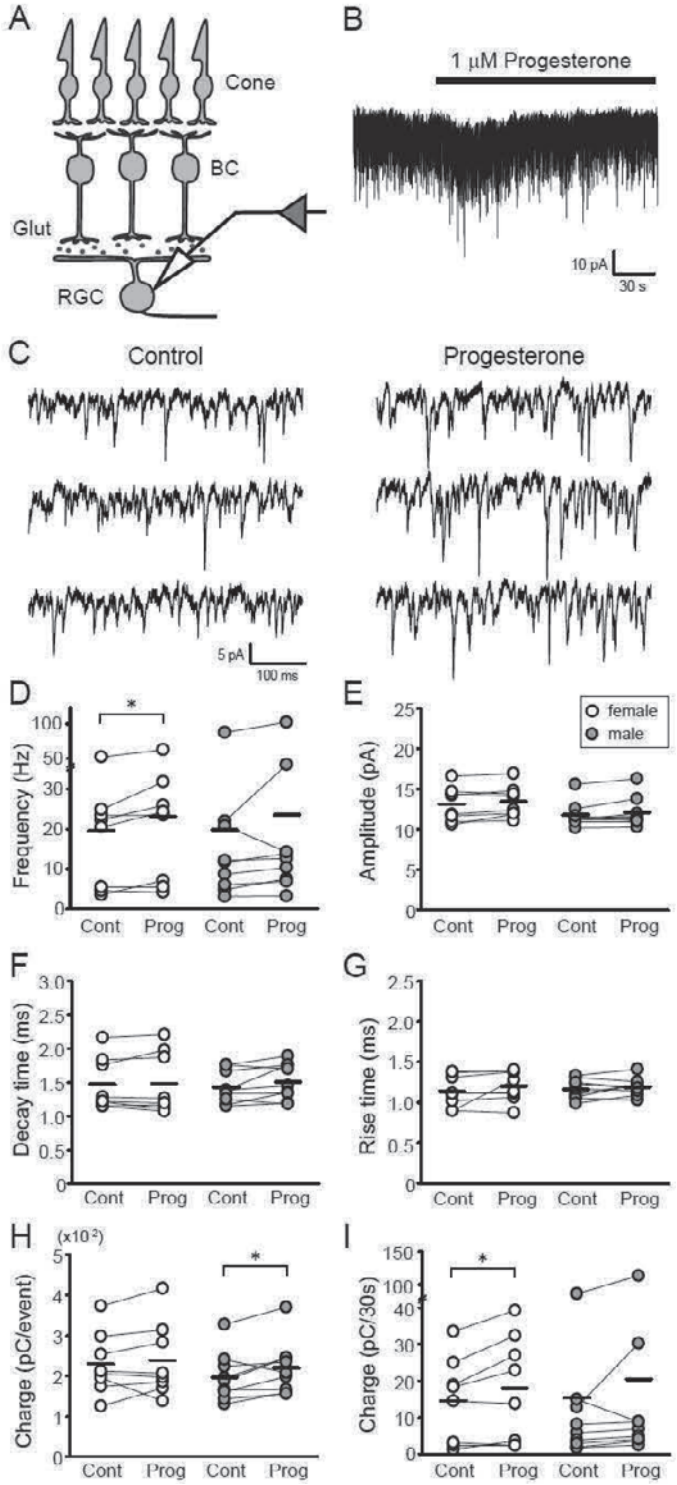

Fig. 3 Effects of progesterone on spontaneous excitatory postsynaptic currents (sEPSCs) in retinal ganglion cells (RGCs). A. Schematic drawings of sEPSC recordings. Whole-cell recordings were performed using RGCs to monitor sEPSCs. Since RGCs receive glutamate (Glut, small gray dots) released from bipolar cells (BCs), sEPSCs recorded from RGCs reflected the activity of glutamatergic synapses between BCs and RGCs. B. Example of a whole-cell recording from an RGC. An RGC was held at −70 mV. Downward sharp lines superimposed on baseline noise correspond to sEPSCs. The bar above the trace shows the timing of the application of 1 μM progesterone. C. Enlarged images of sEPSCs shown in B. Traces represent before (Control) or during (Progesterone) application of progesterone. An individual sharp downward deflection corresponds to an individual sEPSC. D-I. Effects of progesterone on the parameters of sEPSCs before (Cont) and during (Prog) the application of progesterone. D, frequency; E, amplitude; F, decay time; G, rise time; H, charge transfer of an individual sEPSC; I, total charge transfer for 30 seconds. Horizontal lines correspond to the mean. The numbers of recorded cells were 9 from 5 male mice and 8 from 4 female mice. The paired *t*-test was performed. *P<0.05.

Table 1 Summary of EPSC analyses

	Parameters	Control (Mean±SD)	Progesterone (Mean±SD)	P value
Females	Frequency (Hz)	19.7 ± 16.2	23.2 ± 19.3	0.032
	Charge transfer of an individual EPSC (pC/event)	0.023 ± 0.008	0.024 ± 0.009	0.458
	Amplitude (pA)	13.1 ± 2.1	13.4 ± 1.9	0.133
	Rise time (ms)	1.135 ± 0.195	1.203 ± 0.192	0.183
	Decay time (ms)	1.47 ± 0.39	1.48 ± 0.46	0.753
	Total charge transfer (pC/30 s)	14.7 ± 11.7	17.9 ± 14.5	0.036
Males	Frequency (Hz)	19.7 ± 26.4	23.6 ± 31.5	0.178
	Charge transfer of an individual EPSC (pC/event)	0.020 ± 0.006	0.022 ± 0.007	0.039
	Amplitude (pA)	11.8 ± 1.5	12.1 ± 1.8	0.196
	Rise time (ms)	1.165 ± 0.120	1.191 ± 0.108	0.371
	Decay time (ms)	1.43 ± 0.24	1.51 ± 0.27	0.101
	Total charge transfer (pC/30 s)	15.4 ± 27.0	20.4 ± 35.9	0.188

n = 8 (females), n = 9 (males)

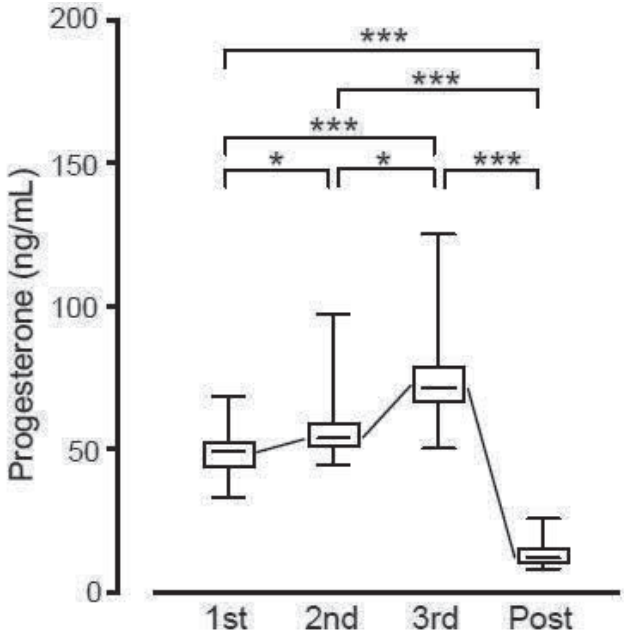

Fig. 4 Box plot of progesterone concentrations in the 1st, 2nd, and 3rd trimesters and the postpartum period in pregnant women. Whiskers show the maximum and minimum, and box boundaries show the 25th and 75th percentiles. Horizontal lines correspond to the median. *P<0.05, ***P<0.001, the Kruskal-Wallis test. Data were sampled from 12 pregnant women.

cell death[29]. Previous studies reported estrus cycle-dependent changes in ocular function[18–22]. In addition, estrogen-induced increases in the thickness of the choroid were demonstrated in pregnancy[24,30–33]. Since the retina and choroid are in close contact, an increase in the thickness of the choroid may induce morphological or functional changes in the retina, together with an elevated glutamate concentration by progesterone.

Therefore, we investigated whether the elevated concentration of progesterone in pregnancy was associated with detectable morphological changes in the human retina.

A change in foveal retinal thickness in pregnancy has been reported in age-matched studies[34]. However, another study revealed no significant changes in central macular thickness, despite a change in the vascular density of the retina[35]. Furthermore, the thickness of the retina is known to be affected by race, sex, and age[36–38]. Therefore, a follow-up study of the same women throughout their pregnancy is needed to detect any subtle changes in the retina. In the present study, we followed-up the retinal thickness of the same women from the 1st trimester to the postpartum period and, on the basis of the results obtained, we assessed morphological changes in the retina.

In the postpartum stage, progesterone concentrations (43 ± 16 nM [13 ± 5 ng/mL], mean ± SD) were within normal ranges for the regular menstrual cycle (**Fig. 4**). An elevation in progesterone concentrations during pregnancy was observed in the 1st trimester. The mean concentration of progesterone in the 1st trimester (153 ± 27 nM [48 ± 9 ng/mL], mean ± SD) was 2.5-fold that of the highest concentration under the regular menstrual cycle. Progesterone concentrations continued to gradually increase in the 2nd trimester (188 ± 51 nM [59 ± 16 ng/mL], mean ± SD) and peaked in the 3rd trimester (243 ± 61 nM [76 ± 19 ng/mL], mean ± SD).

We used the thickness of the retina in the postpartum stage as the control because the progesterone concentration returned to the level of the regular menstrual cycle. We measured the thickness of the retina in 9 regions (**Table 2**, **Fig. 5**). Retinal thickness was calculated at the

Table 2 Thickness of the retina in pregnant women

	1st trimester	2nd trimester	3rd trimester	Postpartum
F	258.0 ± 13.2	256.0 ± 12.2	257.6 ± 11.5	257.5 ± 11.3
SPa	339.5 ± 12.6	341.0 ± 11.7	340.6 ± 10.4	342.8 ± 13.0
TPa	322.5 ± 10.1	324.0 ± 10.7	322.7 ± 9.0	325.2 ± 11.0
NPa	339.3 ± 12.6	340.5 ± 10.8	340.4 ± 9.7	342.6 ± 12.5
IPa	334.0 ± 10.6	335.1 ± 11.8	334.8 ± 9.5	337.5 ± 12.1
SPe	302.3 ± 15.5	303.3 ± 15.2	303.7 ± 13.7	303.6 ± 15.6
TPe	285.0 ± 15.5	285.9 ± 15.1	287.6 ± 14.3	288.3 ± 16.6
NPe	318.3 ± 16.4	318.9 ± 16.3	318.7 ± 14.8	320.0 ± 16.1
IPe	288.5 ± 17.0	289.3 ± 17.5	290.5 ± 16.6	291.2 ± 18.0

Data are shown as the mean ± SD.
F: fovea, Spa: superior parafovea, TPa: temporal parafovea, NPa: nasal parafovea, IPa: inferior parafovea, Spe: superior perifovea, TPe: temporal perifovea, NPe: nasal perifovea, IPe: inferior perifovea

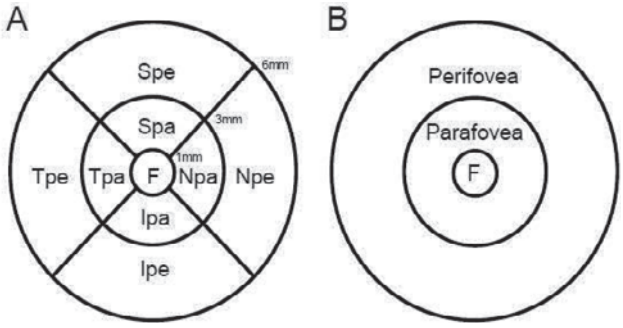

Fig. 5 Calculation of retinal thickness. A. Nine regions used for the measurement of retinal thickness. F, fovea; Spe, superior perifovea; Spa, superior parafovea; Tpe, temporal perifovea; Tpa, temporal parafovea; Npe, nasal perifovea; Npa, nasal parafovea; Ipe, inferior perifovea; Ipa, inferior parafovea. B. The central region (fovea) and two concentric regions (the parafovea and perifovea) used for assessment of retinal thickness. The thickness of the parafovea used was (Spa + Tpa + Npa + Ipa)/4. The thickness of the parafovea used was (Spe + Tpe + Npe + Ipe)/4.

parafovea and perifovea for individuals and averaged (see the Methods section). Significant decreases in thickness were observed in the parafovea and perifovea in the 1st trimester (**Fig. 6**), but not in the 2nd trimester. In the 3rd trimester, a significant decrease in thickness was detected only in the parafovea.

Discussion

The present results demonstrated that progesterone and pregnenolone sulfate increased the activity of glutamatergic circuits, whereas estrogen and testosterone did not. Many physiological and sometimes pathological changes occur in the eye during pregnancy[23,24]. Therefore, physiological changes in visual functions during pregnancy may reflect modified glutamatergic signal transmission through the excitatory effects of progesterone on glutamatergic circuits.

In the present study, we examined the effects of progesterone, pregnenolone sulfate, estrogen, and testosterone in the retina at 1 μM. At this concentration, progesterone and pregnenolone sulfate activated glutamate release, whereas estrogen and testosterone did not. This result indicated that the elevated concentration of progesterone in pregnancy increased glutamate release from glutamatergic synapses. However, the blood concentration of progesterone in pregnant women in the present study was 0.15-0.3 μM on average, even in the 3rd trimester. In women, progesterone and estrogen concentrations in blood increase and decrease during the menstrual cycle and markedly increase in pregnancy[39]. In a regular menstrual cycle, the concentrations of progesterone and estrogen (estradiol) fluctuate between 3 and 60 nM and between 0.3 and 1.2 nM, respectively. In pregnancy, the concentrations of progesterone and estrogen (estradiol) both gradually increase until delivery. At the end of the 3rd trimester, the mean concentration of both progesterone and estrogen (estradiol) was approximately 0.3 μM, 0.05 μM in singletons[40]. On the other hand, the concentration of testosterone in blood is <35 nM[39]. Therefore, the absence of the effects of estrogen and testosterone at a concentration of 1 μM strongly suggests that glutamatergic circuits are not modulated by elevated estrogen and testosterone concentrations in pregnancy. Regarding the effects of progesterone at 1 μM, a previous study showed that the progesterone concentration during a twin pregnancy may reach a maximum of 1 μM[40], suggesting that progesterone-induced glutamate release is of physiologi-

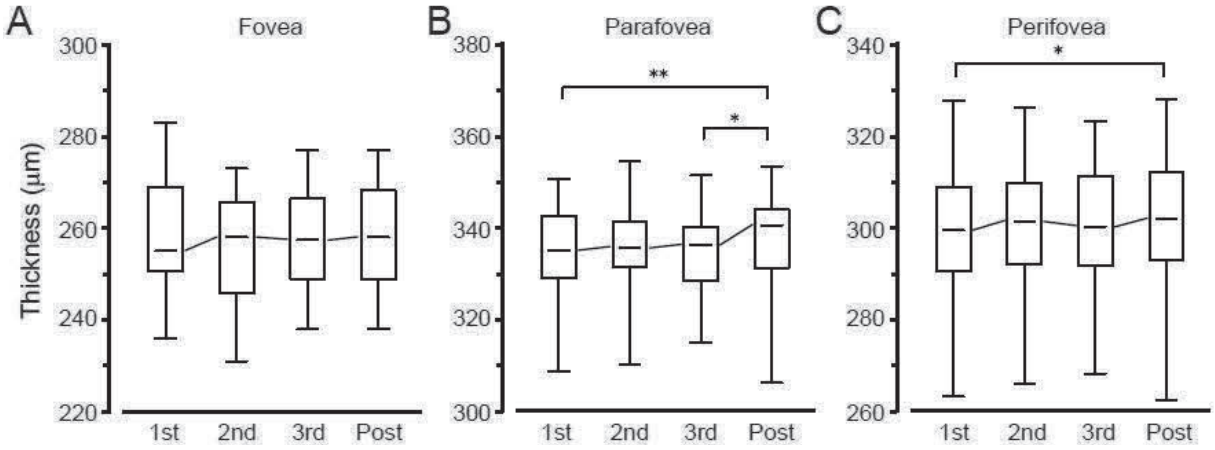

Fig. 6 Box plot of retinal thickness in the fovea (A), parafovea (B), and perifovea (C) from the 1st, 2nd, and 3rd trimesters and the postpartum period in pregnant women. Whiskers show the maximum and minimum, and box boundaries show the 25th and 75th percentiles. Horizontal lines correspond to the median. Data are the sum of both the right and left eyes of 12 pregnant women (n = 24). *P<0.05, **P<0.01. Samples were collected from the right and left eyes of 12 pregnant women (n = 24). Details of the definition of 3 regions (fovea, parafovea, and perifovea) are described in the Methods section.

cal relevance in the specific case of twins. In most pregnant women, however, because the maximum physiological concentration of progesterone is <0.3 μM, caution is needed concerning the possible off-target effects of progesterone. Therefore, further studies are warranted to establish whether progesterone activates glutamate release at the submicromolar range.

Regarding the physiological importance of functional changes in the retina, we herein demonstrated that the modulatory effects of progesterone on glutamatergic circuits were stronger in females than in males. In other words, glutamatergic circuits may be more sensitive to progesterone in females than in males. However, no significant differences in the localization or expression of progesterone receptors were reported between male and female mice[10], suggesting that the high sensitivity of glutamatergic circuits in females cannot simply be explained by the different anatomical distribution of progesterone receptors. Since the effects of progesterone are controlled by estrogen[41,42], the female-specific effects of progesterone on glutamatergic circuits may be linked to a high blood level of estrogen or the estrous cycle. Experiments in the present study were performed *in vitro* (in the absence of estrogen); therefore, further studies of the estrus cycle are needed.

We observed a detectable reduction in retinal thickness in the 1st trimester. The results of the enzyme-linked fluorescent assay system and electrophysiological recordings also showed that 1 μM progesterone increased the activity of glutamatergic circuits. However, the transient reduction in retinal thickness in the 1st trimester did not appear to be a result of glutamate toxicity induced by an increased progesterone concentration, because elevated progesterone concentrations continued throughout pregnancy[40]. The lack of glutamate toxicity may be explained as follows. The increase in sEPSCs induced by progesterone may increase the concentration of glutamate at the synaptic cleft. However, in the enzyme-linked fluorescent assay system, we monitored the extracellular concentration of glutamate in the presence of TBOA, an antagonist of glutamate transporters[26]. We previously reported that fluorescence did not increase in the absence of TBOA when a high K stimulation was performed[25], suggesting that glutamate concentrations at the synaptic cleft are tightly controlled by glutamate transporters under physiological conditions. Although pregnenolone sulfate has been reported to function as a positive allosteric modulator of NMDA receptors in the rat retina, cytotoxic effects at the histological level in the retina were detectable when the concentration of pregnenolone sulfate was >50 μM[14]. The increase observed in glutamate release in electrophysiological recordings may alter the pattern of glutamatergic signal transmission, potentially inducing the subtle functional modulation of visual function in pregnancy[19–24]. In the present study, we used retinal thickness in the postpartum period as the control because the progesterone concentration in blood is close to that in the non-pregnant stage (in other words, in the regular menstrual cycle). In a previous age-matched study, foveal thickness in the 1st trimester was similar to that in the

non-pregnant stage, whereas a significant increase in foveal thickness was detected in the 2nd and 3rd trimesters[34]. If this increase in retinal thickness persists for a few months after delivery, a decrease in retinal thickness may be reconsidered from a different viewpoint. Alternatively, a temporal decrease in retinal thickness may be explained by increased pressure from the choroid, which becomes thicker because of increased blood flow in pregnancy[24,30–33]. Further studies are warranted.

The present findings demonstrated that progesterone and pregnenolone sulfate both increased extracellular glutamate concentrations, using an enzyme-linked fluorescent assay system. We also showed that progesterone increased glutamatergic inputs from bipolar cells in retinal ganglion cells. This result indicates that bipolar cells are a target of progesterone. In the mouse retina, the distribution of immunoreactivity for progesterone receptors A and B, nuclear-localized receptors, in OPL and IPL has been reported[10]. Although there are no somas of bipolar cells in OPL or IPL, previous immunohistochemical findings support progesterone acting on bipolar cells. Alternatively, progesterone and pregnenolone sulfate may activate TRP channels (TRPM1[43], TRPM3[44–46], TRPC6[47], and TRPM8[48]). In the retina, TRPM3 is expressed in the inner retina (IPL and the ganglion cell layer)[49] and TRPM1 has been detected in the dendrites of ON bipolar cells[50]. Pregnenolone sulfate has been shown to activate TRPM3 and increase Ca influx in the mouse retina[46]. However, TRPM3 channels do not appear to be the target of progesterone because progesterone was found to inhibit TRPM3 channels via a pregnenolone-independent mechanism[45]. The activation of TRPM1 by pregnenolone sulfate has also been supported using the recombinant system[43]. The findings of this study showed that the current amplitude of homomeric TRPM1 receptors was very small, while those of the chimeric receptors of TRPM1 and TRPM3 were large. Therefore, further studies are needed to identify the actual target of progesterone.

Based on animal and human data, our findings suggest that elevated concentrations of progesterone in pregnancy modify glutamatergic transmission without significant glutamate toxicity. Our *in vitro* animal model provided a platform to examine the effects of individual gonadal hormones on glutamatergic circuits and showed that 1 μM progesterone increased the activity of glutamatergic circuits. A follow-up study of the same women throughout their pregnancy revealed that the elevated concentration of progesterone in pregnancy did not induce any significant glutamate toxicity in the retina. Changes in endocrine and metabolic conditions and the vascular supply to the eye have been reported in pregnant women[51]. Many pregnant women develop physiological changes in the eye[19–21,23,24]. Marked changes in vascular flow have been shown to induce retinal and choroidal diseases, such as central serous chorioretinopathy[52,53]. Among the pathophysiological changes reported, those that occur in the visual field[21] may be related to the present results. A change in the visual field was detected as a change in mean threshold sensitivity without subjective symptoms. Although increases in progesterone concentrations in pregnancy may induce functional changes in the retina, these changes may be compensated for by plastic changes in the visual system. Further studies are needed to confirm whether subtle subjective symptoms are present in healthy pregnant women.

Author Contributions: M. Ohkuma, E. Miyachi, and M. Kaneda conducted the experiments using the enzyme-linked fluorescent assay. T. Maruyama and T. Ishii conducted the experiments using the patch clamp technique. N. Igarashi, T. Inoue, K. Azuma, and R. Obata conducted the clinical experiments. Original clinical data were not shared with other authors. M. Ohkuma, T. Maruyama, T. Ishii, N. Igarashi, T. Inoue, and M. Kaneda prepared the figures. T. Ishii and M. Kaneda wrote the main text. The main text was checked and corrected by all authors. M. Ohkuma, T. Ishii, N. Igarashi, and M. Kaneda designed the experiments.

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