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Interaction between the serotoninergic and GABAergic systems in frog retina as revealed by electroretinogram

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Functional interactions between serotoninergic and GABAergic systems in the vertebrate retina are largely unknown. In this study, the effects of isolated or combined stimulation of the serotonin receptors (with 100 μM serotonin) and ionotropic $GABA_A$ and $GABA_C$ receptors (with 5 mM TACA) on the electroretinographic (ERG) ON (b-wave) and OFF (d-wave) responses were investigated in frog eyecup preparations. It was found that serotonin alone produced a significant enhancement of the b- and d-wave amplitude, while TACA alone caused its marked diminution. The relative amplitude diminution, caused by the TACA treatment, was significantly smaller when TACA was applied on the background of the fully developed serotonin effect. This result suggests that the retinal serotoninergic system could diminish the effects of ionotropic GABA receptor activation on the ERG wave generator mechanisms. In order to separately evaluate the effects of the $GABA_C$ receptor activation, in a subset of experiments the effects of TACA or TACA + serotonin were tested during $GABA_A$ receptor blockade with 100 μM bicuculline. Bicuculline alone caused a marked increase of the b- and d-wave amplitude. The stimulation of $GABA_C$ receptors (with TACA) during bicuculline action produced a strong diminution of the b- and d-wave amplitudes. Similar relative decrease of the b-wave amplitude was produced when TACA was applied in combination with serotonin, while the relative decrease of the d-wave amplitude was less pronounced during treatment with serotonin + TACA than TACA alone. Our results demonstrate that there is an ON/OFF asymmetry in the receptors involved in the presumed interactions between serotoninergic and GABAergic systems. Serotonin may decrease the effects of $GABA_A$ receptor activation in the ON pathway, while it may decrease the effects of both $GABA_A$ and $GABA_C$ receptor activation in the OFF pathway.

Key words: electroretinogram, serotonin, GABA receptors, retina

INTRODUCTION

GABA is the major inhibitory neurotransmitter in the vertebrate retina. It is released by a large population of GABAergic neurons identified as amacrine, interflexiform and horizontal cells (for review: Popova 2014). The actions of GABA are mediated by 2 types of receptors: (1) ionotropic $GABA_A$ and $GABA_C$ ($GABA_\rho$) receptors, which are ligand gated chloride channels and (2) metabotropic G protein-coupled $GABA_B$ receptors that regulate adenylyl cyclase, voltage-gated Ca^{2+} channels or inwardly rectifying K^+ channels (reviews: Olsen and Sieghart 2009, Padgett and Slesinger 2010). Serotonin (5-hydroxytryptamine; 5-HT) is one of the monoamine neurotransmitters in the retina, which is better represented in lower than higher vertebrate retinas (Osborne et al. 1982). It is released by serotoninergic amacrine cells obtained in the retinas of many species (Ehinger 1983, Zhu et al. 1992, Hurd and Eldred 1993, Gábriel 2000, Vigh et al. 2000). Some serotonin-accumulating neurons are found among horizontal and bipolar cells, but it is thought that they are only able to transport and metabolize, but not synthesize serotonin (Marc et al. 1988, Zhu et al. 1992, Wilhelm et al. 1993, Schutte 1994, Ghai et al. 2009). Serotonin is also released by retinopetal axons that originate in dorsal raphe in fishes, rodents and primates (Villar et al. 1987, Shen and Semba 1994, Lima and Urbina 1998, Gastinger et al. 2005, 2006). The actions of serotonin are mediated by 14 receptor types divided into 7 families from 5-HT_1 to 5-HT_7. 5-HT_3 is an ionotropic receptor, while all the others are metabotropic G-protein-coupled receptors (reviews: Nichols and Nichols 2008, Berumen et al. 2012). One unanswered question in retinal physiology is how the

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GABAergic and serotoninergic systems interact and what are the consequences of these interactions for visual information processing?

One of the easiest ways to investigate electrophysiologically the global retinal function *in vivo* without perturbing any neuronal connections is by recording electroretinogram (ERG). The ERG consists of many components, but two of them are most prominent in response to long lasting stimuli: the b-wave (in response to stimulus onset) and the d-wave (in response to stimulus offset). There is general consensus that the neuronal generator of the b-wave is primarily the depolarizing (ON) bipolar cells, while the d-wave generation depends mainly on the activity of hyperpolarizing (OFF) bipolar cells with minor contribution of the photoreceptor response at stimulus offset and activity of proximal retinal neurons (reviews: Frishman 2006, 2013). Therefore, recording of the ERG b- and d-waves gives valuable information about the functioning of both the ON and OFF channels in the distal retina. The significance of the serotonin - GABA system interactions for the global retinal function could be revealed by investigating the ERG changes during manipulation (pharmacological or genetic) of the two systems. However, we could not find any ERG data concerning serotonin - GABA interaction in the vertebrate retina. It has only been shown that serotonin via protein kinase C activation reduces the currents through $GABA_C$ receptors in cultured rat rod bipolar cells (Feigenspan and Bormann 1994). On the other hand, many data indicate that serotonin can modulate GABAergic neurotransmission both at presynaptic and postsynaptic site in the central nervous system. Such modulation has been observed in structures involved in learning and memory, sensory processing, nociception and motor control (for review: Ciranna 2006). It has been shown that serotonin modulates both metabotropic $GABA_B$ receptors and ionotropic $GABA_A$ receptors in CNS. However, there is no information concerning the interaction between serotonin and $GABA_C$ receptors, probably because the latter are not abundant in CNS.

In this study, we investigated the interactions between serotonin and ionotropic $GABA_A$ and $GABA_C$ receptors in frog retina as revealed by ERG. We compared the changes in the ERG b- and d-waves during isolated stimulation of ionotropic GABA receptors (with TACA) or serotonin receptors (with serotonin) to those obtained during their combined stimulation. For a separate evaluation of the interaction between serotonin and $GABA_C$ receptors, in a subset of experiments, we repeated the treatment with TACA alone and serotonin + TACA on the background of the $GABA_A$ receptor blockade with bicuculline.

METHODS

Subjects and drug application

Experiments were carried out on dark adapted eyecup preparations of the frog (*Rana ridibunda*). Thirty frogs (balanced sex) were used in the study. We chose frogs because they are our traditional object of study and thus we can compare present results with previous ones. The advantages of frogs over mice and rats are their mixed (rod-cone) type of retina and well developed OFF response. Frogs were first anesthetized in water containing 500 mg/l Tricaine methanesulfonate (Sigma-Aldrich). They were then decapitated and pithed. The experimental procedure has been approved by the Committee for ethics in scientific research of Medical University of Sofia, Bulgaria.

Prepared eyecups were placed in a small chamber containing Ringer solution (NaCl 110 mM, KCl 2.6 mM, $NaHCO_3$ 10 mM, $CaCl_2$ 1.6 mM, $MgCl_2$ 0.8 mM, Glucose 2 mM; HCl 0.5 mM to adjust pH to 7.8) at 16 - 18° C and supplied with moistened O_2. Drugs were applied in the eyecup preparations by replacing a specified volume of the fluid (Ringer solution) in the chamber, containing the preparation, with the same volume of Ringer solution, containing the drug or the combination of the drugs tested. Final drug concentration was calculated taking into account the degree of dilution. Serotonin receptors were stimulated by using 100 μM 5-hydroxytryptamine hydrochloride (Sigma-Aldrich Chemie GmbH), while ionotropic $GABA_A$ and $GABA_c$ receptors were stimulated by using 5 mM trans-4-Aminocrotonic acid (TACA) (Tocris). The concentrations used were chosen on the basis of pilot experiments where different concentrations of the agonist were tested (see Results). Ionotropic $GABA_A$ receptors were blocked by using 100 μM bicuculline methiodide (Sigma-Aldrich). This concentration was selected on the basis of our previous study showing that concentration of 100 μM, but not 50 μM, prevents the inhibitory action of flurazepam on the b-wave amplitude, although the both concentrations have similar enhancing effect on the b- and d-wave amplitude (Popova 2003).

Experimental procedure and groups

Frogs were dark adapted for 24 h and then the eyecups were prepared under dim red light. The rhythmic light stimulation began after additional period of dark adaptation for 10 minutes. Diffuse white light stimuli (150 W tungsten halogen lamp) with 5 s duration were presented repeatedly at in-

terstimulus interval of 25 s. The light intensity was 6×10^2 quanta s^{-1} μm^{-2} falling at the plane of the retina. The stimulus intensity was chosen on the basis of our previous experiments showing that it generates responses in the steepest part of the intensity - response function for both the b- and d-waves in dark adapted frog eyecups (Popova 2000, Kupenova et al. 2008, Popova and Kupenova 2011). During the first 10 minutes of the period of light stimulation (control period), all the eyecups stayed in Ringer solution. Then the substances used for pharmacological treatment were applied.

The results are based on 60 experiments, divided into 7 experimental groups according to the substances applied: 1) *Control group* (n=11). The eyecups were treated with Ringer solution throughout the whole investigated period. At the 10th minute from the beginning of the experiments, a small volume of Ringer solution from the chamber (50 µl) was replaced with the same volume of new Ringer solution. This was done in order to account for the non-specific changes in the ERG records due to fluid replacement. 2) *Serotonin group* (n=7). The eyecups were treated with 100 µM serotonin for 15 minutes. 3) *TACA group* (n=8). The eyecups were treated with 5 mM TACA for 15 minutes. 4) *Serotonin + TACA group* (n=7). The eyecups were consecutively treated with 100 µM serotonin (for 3 minutes) and 5 mM TACA (for 12 minutes). 5) *Bicuculline group* (n=6). The eyecups were treated with 100 µM bicuculline for 28 minutes. 6) *Bicuculline + TACA group* (n=10). The eyecups were consecutively treated with 100 µM bicuculline (for 7 minutes) and 5 mM TACA (for 15 minutes). 7) *Bicuculline + serotonin + TACA group* (n=11). The eyecups were consecutively treated with 100 µM bicuculline (for 7 minutes) and 100 µM serotonin + 5 mM TACA (for 15 minutes).

ERG recording and data analysis

ERG were recorded by means of non-polarized Ag/AgCl electrodes at bandpass of 0.1–1000 Hz (DC/AC differential amplifier model 3000; A-M Systems) and digitized at 1000 Hz. The amplitude of the b-wave was measured from the peak of the a-wave to the peak of the b-wave, while that of the d-wave was measured from the baseline to the peak of the wave. Then the amplitudes were normalized to the values obtained in the 10th minute from the beginning of the experiments because it was the last minute of the control (pretreatment) period. The implicit time of the ERG waves was measured from the stimulus onset (for the b-wave) or offset (for the d-wave) to the peak of the wave. It was first measured during the control period (10th minute from the beginning of the experiment) and then during the isolated or combined drug application when the stable effect of the drug treatment was achieved (for particular treatments – see Table I).

Two-way ANOVA (OriginPro 8 software, OriginLab Corporation, Northhampton, MA) was used for statistical evaluation of the differences in the normalized amplitude values between different groups. Paired t-test was used for statistical evaluation of the implicit time changes in each group. A p value of <0.05 was considered significant. The normality of the data distribution was proved by Shapiro-Wilk test.

RESULTS

Pilot experiments for dose-effect relationship testing

These experiments were performed in order to choose the optical concentration for serotonin and TACA application in the main experimental groups. We tested four concentrations of serotonin - 25, 50, 100 and 200 µM and chose 100 µM, because this concentration was saturating one for both the b- and d-wave amplitude changes (Fig. 1 a). Six concentrations of TACA - 0.8, 2, 3, 4, 5 and 6 mM were tested and, as it can be seen from Fig. 1 b, the concentration of 5 mM was saturating for the TACA effect on the b-wave amplitude and nearly saturating for the effect on the d-wave amplitude. That is why we used this concentration in the main groups of experiments.

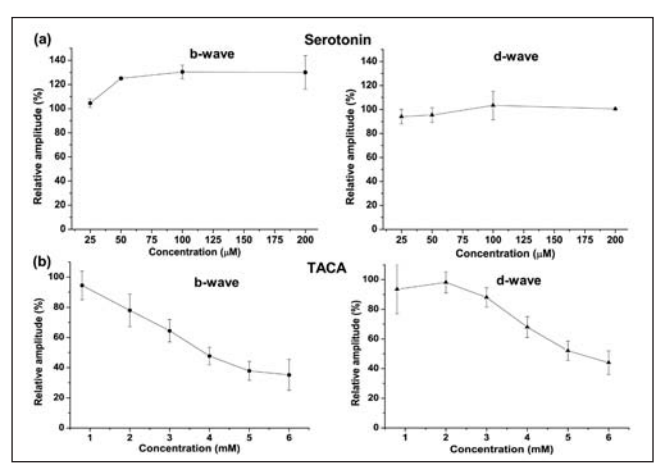

Fig. 1. (a) Dose-response relationship for serotonin effects on the b- and d-wave amplitude. The amplitude of the ERG waves during treatment with 4 different concentrations of serotonin (25 µM, 50 µM, 100 µM and 200 µM) are normalized to the values obtained in the control period. Mean values±SEM are shown (n=3) (b) Dose-response relationship for TACA effects on the b- and d-wave amplitude. The amplitude of the ERG waves during treatment with 6 different concentrations of TACA (0.8 mM, 2 mM, 3 mM, 4 mM, 5 mM and 6 mM) are normalized to the values obtained in the control period. Mean values ±SEM are shown (n=3).

Control group

The amplitude of the b- and d-waves remained relatively stable during the entire course of the control experiments. This is demonstrated in Fig. 2a and 2b, where the amplitude of the b- and d-waves was normalized to the value obtained in the 10th minute from the beginning of the experiments. A small decrease of the b-wave amplitude and a small increase of the d-wave amplitude were seen during the first two minutes after solution substitution, which was probably due to the dim red light illumination used during the procedure of fluid replacement. To be sure that this nonspecific change in the b/d amplitude ratio did not persist in latter time periods, we compared its values during the last 5 minutes of the control period (6th–10th minute) with those obtained in a later time period (15th – 19th minute from the beginning of the experiment). Two-way ANOVA revealed no statistic significant difference between the b/d amplitude values during the two periods, indicating that the relative sensitivity of the ON *vs.* OFF response was not changed. The time characteristics of both ERG responses were also unaltered. The implicit time of the ERG waves underwent no significant changes during the treatment with Ringer solution (Table I).

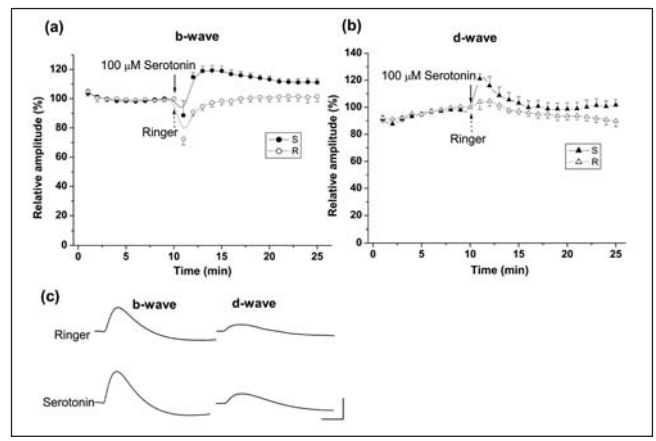

Fig. 2. Effects of serotonin on the ERG waves. (a) (b) Time course of the effects of serotonin on the amplitudes of the ERG b- and d-waves. Results of both control experiments (R, open symbols) and test experiments (S, filled symbols) are represented. The amplitudes of the ERG waves were normalized to the values obtained in the 10th minute from the beginning of the experiments. The time, when the solution containing 100 μM serotonin (resp. Ringer in control experiments) was applied, is indicated by arrows. Mean values ±SEM are shown. (c) Original ERG records (b- and d-waves) obtained during treatment with Ringer solution in the control period (10th minute from the beginning of the experiment – upper row) and 100 μM serotonin (13th minute from the beginning of the experiment – bottom row) Calibration: time – 0.500 s; amplitude – 400 μV.

Serotonin group

Application of 100 μM serotonin produced an increase in the amplitude of both the b- and d-waves with respect to their initial values (Fig. 2 a, b). The effect developed very rapidly and reached its maximum expression at the 3rd minute for the b-wave (13th min from the beginning of the experiment) and even sooner (at the 1st minute) for the d-wave (Fig. 2a, b). The amplitude of the d-wave showed a tendency for diminution in the next 2-3 minutes and afterward a stable effect of serotonin on both the b- and d-wave amplitude was observed till the end of the experiment. During this stable period, the amplitudes of the b- and d-waves were significantly higher than the corresponding values obtained in the control group (two-way ANOVA, $F_{1,179=}$177.7, P<0.0001 for the b-wave; $F_{1,179=}$12.53, P<0.0006 for the d-wave). The b-wave amplitude increased to a greater extent than that of the d-wave, which resulted in a significantly higher b/d amplitude ratio compared to that obtained in the control period (from 4.89±0.15 it diminished to 4.28±0.17; two-way ANOVA, $F_{1,69}$=5.57, P<0.022). This result indicates that serotonin has stronger stimulating effect upon the ON compared to the OFF channel activity. The implicit time of the both ERG waves was unaltered during treatment with serotonin (Table I; Fig. 2 c).

TACA group

Treatment of the eyecups with 5 mM TACA caused a marked diminution of the b- and d-wave amplitude. The suppressing effect developed very rapidly and it was fully expressed on the 2nd minute after the drug application. The effect was very stable in time and remained maximal till the end of the experiments (Fig. 3 a, b). Statistically significant differences were obtained between the ERG amplitude values in the test and control groups during this period (two-way ANOVA, $F_{1,189}$=3532, P<0.0001 for b-wave; $F_{1,189}$=851, P<0.0001 for d-wave). The b-wave amplitude was diminished more strongly than that of the d-wave and as a consequence, the b/d amplitude ratio showed significantly lower values during TACA treatment (from 2.71±0.11 it decreased to 1.54±0.05, Two way ANOVA $F_{1,79}$=82.09, P<0.0001). The result presented shows that the ionotropic GABA receptor activation has greater inhibitory effect upon the ON than the OFF channel activity. The implicit time of the b-wave was significantly delayed during TACA treatment, while that of the d-wave was not significantly changed, although a tendency for its shortening emerged (Table I, Fig. 3 c). The effects of TACA were partially reversible. The amplitude and time charac-

teristics of the ERG waves showed a tendency for recovery in an eyecup, where part of the solution containing TACA was substituted with Ringer solution only (Fig. 3 c).

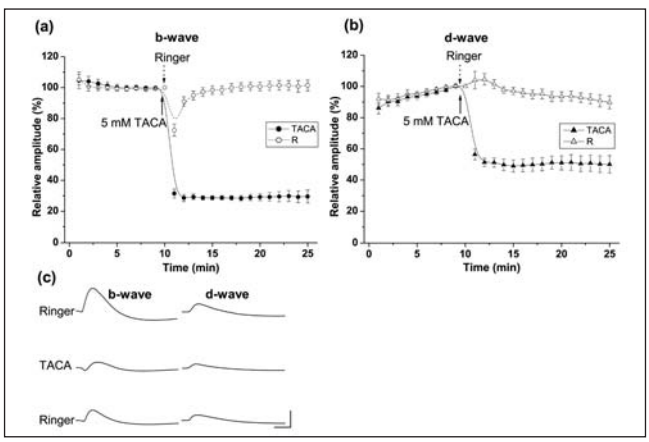

Fig. 3. Effects of TACA on the ERG waves. (a) (b) Time course of the effects of TACA on the amplitudes of the ERG b- and d-waves. Results of both control experiments (R, open symbols) and test experiments (TACA, filled symbols) are represented. The amplitudes of the ERG waves were normalized to the values obtained in the 10th minute from the beginning of the experiments. The time, when the solution containing 5 mM TACA (resp. Ringer in control experiments) was applied, is indicated by arrows. Mean values ±SEM are shown. (c) Original ERG records (b- and d-waves) obtained during treatment with Ringer solution in the control period (10th minute from the beginning of the experiment - upper row), 5 mM TACA (13th minute from the beginning of the experiment - middle row) and Ringer solution in the recovery period (32nd minute from the beginning of the experiment – bottom row) Calibration: time – 0.3 s; amplitude – 500 μV.

Serotonin + TACA group

To investigate the interaction between serotoninergic and GABAregic systems, in this group of experiments we applied 100 μM serotonin first (for 3 minutes) and 5 mM TACA afterwards. As would be expected, serotonin caused an enhancement of the b- and d-wave amplitude (Fig. 4 a, b). When TACA was applied afterward, it caused a great diminution of the amplitude of both the ON and OFF responses. However, the decrease of the b- and d-wave amplitude was significantly smaller than that obtained in TACA group (two-way ANOVA, $F_{1,149}$=163.1, P<0.0001 for b-wave; $F_{1,149}$=129.03, P<0.001 for d-wave) (Fig. 4 a, b). To be sure that this difference was not due to the different amplitude values before TACA application in the two groups, we normalized the b- and d-wave amplitude to its value just before TACA application. As could be seen from Fig. 4 c, d, the relative decrease of the ERG wave amplitude was again smaller in the serotonin + TACA group compared to that in the TACA group (two-way ANOVA, $F_{1,149}$=85.96, P<0.001 for b-wave; $F_{1,149}$=38.06, P<0.0001 for d-wave). In addition, the effect of TACA on the implicit time of the b-wave was fully abolished during serotonin receptor activation (Table I, Fig. 4 e). Thus, it appears that serotonin receptor activation decreases the effects of ionotropic GABA receptor activation on the mechanisms responsible for ERG b- and d-wave generation.

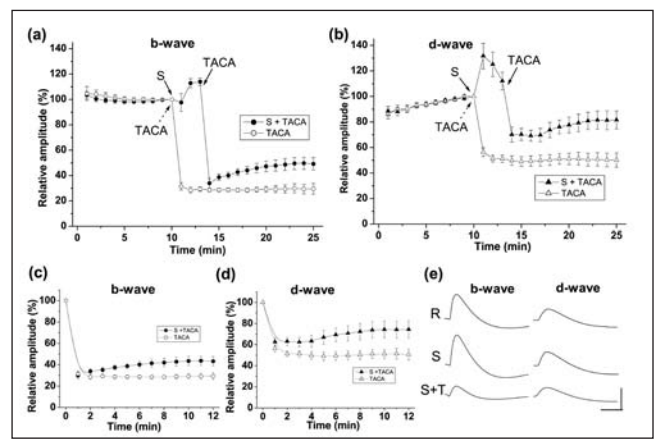

Fig. 4. Effects of combined serotonin plus TACA action on the ERG waves. (a) (b) Time course of the effects of serotonin + TACA on the amplitudes of the ERG b- and d-waves. Results of the experiments with combined serononin + TACA application (S + TACA, filled symbols) are compared to the results obtained in TACA group (TACA, open symbols) The amplitudes of the ERG waves were normalized to the values obtained in the 10th minute from the beginning of the experiments. The times, when the solutions containing 100 μM serotonin (S) or 5 mM TACA were applied, are indicated by arrows. Mean values ±SEM are shown. (c) (d) Relative amplitude changes of the b- and d-waves in serotonin + TACA group and TACA group. The amplitudes of the ERG waves were normalized to the values obtained just before TACA application (at 0 minute) (e) Original ERG records (b- and d-wave) obtained during treatment with Ringer solution in the control period (10th minute from the beginning of the experiment - upper row), 100 μM serotonin (13th minute from the beginning of the experiment – middle row) and 100 μM serotonin + 5 mM TACA (16th minute from the beginning of the experiment – bottom row) Calibration: time – 0.5 s; amplitude – 500 μV.

Bicuculline group

Isolated blockade of $GABA_A$ receptors with 100 μM bicuculline caused marked increase of the b- and d-wave amplitude (Fig. 5 a, b). The effect developed rapidly and reached its full expression 2 - 4 minutes after the blocker application. The amplitude of the b-wave was relatively stable till the end of the experiments, while that of the d-wave showed a tendency for diminution. Bicuculline caused greater enhancement of the OFF response amplitude compared to the ON response amplitude. This resulted in a smaller value of the b/d amplitude ratio during $GABA_A$ recep-

tor blockade (from 2.5±0.08 it decreased to 1.7±0.07, two-way ANOVA $F_{1,59}$=43.43, P<0.0001). This result is consistent with our previous reports showing that $GABA_A$ receptor blockade increases to a greater extent the d-wave than the b-wave amplitude (Vitanova et al. 2001, Popova 2003, Kupenova et al. 2008). Bicuculline not only augmented the ERG waves' amplitude, but it also significantly delayed their implicit times (Table I; Fig. 5 c).

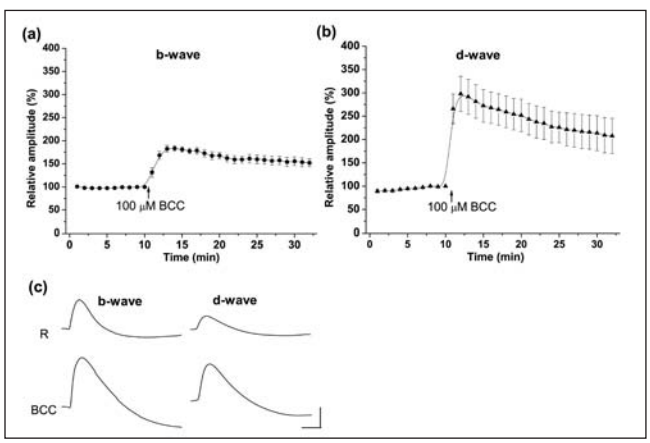

Fig. 5. Effects of bicuculline on the ERG waves. (a) (b) Time course of the effects of bicuculline on the amplitudes of the ERG b- and d-waves. The amplitudes of the ERG waves were normalized to the values obtained in the 10th minute from the beginning of the experiments. The time, when the solution containing 100 μM bicuculline (BCC) was applied, is indicated by arrow. Mean values ±SEM are shown. (c) Original ERG records (b- and d-wave) obtained during treatment with Ringer solution in the control period (10th minute from the beginning of the experiment - upper row) and 100 μM bicuculline (BCC) (17th minute from the beginning of the experiment – lower row) Calibration: time – 0.3 s; amplitude – 225 μV.

Bicuculline + TACA group

This group of experiments was undertaken in order to reveal the effects of isolated $GABA_C$ receptor stimulation on the ERG responses. To fulfill this aim, we first blocked $GABA_A$ receptors with 100 μM bicuculline and applied 5 mM TACA afterwards. Bicuculline caused a great increase of the b- and d-wave amplitude and lengthening of their implicit times (Fig. 6 a, b; Table I). When the blocker's effects were fully developed (at the 18th minute from the beginning of the experiment), 5 mM TACA was added to the solution. As could be seen from Fig. 6 (a, b), the application of TACA diminished considerably the b- and d-wave amplitude in comparison to their values during the preceding bicuculline treatment. It was interesting to compare the relative decrease of the ERG wave amplitudes caused by TACA in this group with that obtained in the TACA group (expressed as a percentage of the values obtained just before TACA application). When such normalization was done (Fig. 6 c, d), it was evident that TACA caused significantly greater diminution of the b- and d-wave amplitude in the Bicuculline + TACA group than that in the TACA group (two-way ANOVA, $F_{1,168}$=15.24, P<0.0002 for b-wave; $F_{1,168}$=27.12, P<0.0001 for d-wave). Thus, it appears that the blockade of $GABA_A$ receptors augments the depressing effects of $GABA_C$ receptor activation on the ERG wave amplitude. The diminution of the b-wave amplitude was again greater than that of the d-wave, which resulted in significantly lower values of the b/d amplitude ratio in comparison to its values obtained during the preceding bicuculline treatment (from 1.64±0.05 it decreased to 1.08±0.06; two-way ANOVA $F_{1,94}$=40.77, P<0.0001). This result indicates that isolated $GABA_C$ receptor activation has stronger inhibitory effect on the amplitude of the ON than OFF ERG response. The effects of TACA on the time characteristics of the ERG waves were also altered during bicuculline action. TACA did not change significantly the b-wave implicit time, but it significantly shortened the d-wave implicit time (Table I).

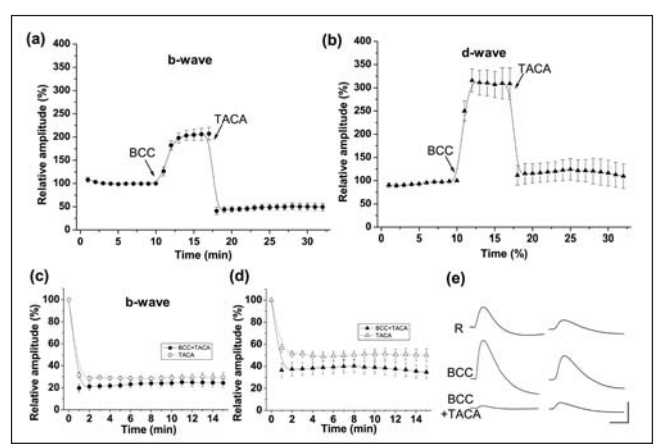

Fig. 6. Effects of combined bicuculline plus TACA action on the ERG waves. (a) (b) Time course of the effects of bicuculline + TACA on the amplitudes of the ERG b- and d-waves. The amplitudes of the ERG waves were normalized to the values obtained in the 10th minute from the beginning of the experiments. The times, when the solutions containing 100 μM bicuculline (BCC) and 5 mM TACA were applied, are indicated by arrows. Mean values ±SEM are shown. (c) (d) Relative amplitude changes of the b- and d-waves in bicuculline + TACA group and TACA group. The amplitudes of the ERG waves were normalized to the values obtained just before TACA application (at 0 minute) (e) Original ERG records (b- and d-wave) obtained during treatment with Ringer solution in the control period (10th minute from the beginning of the experiment – upper row), 100 μM bicuculline (17th minute from the beginning of the experiment – middle row) and 100 μM bicuculline + 5 mM TACA (20th minute from the beginning of the experiment – bottom row) Calibration: time – 0.5 s; amplitude – 250 μV.

Bicuculline + serotonin + TACA group

Finally, we investigated the interaction between serotonin and isolated $GABA_C$ receptor activation, on the mechanisms responsible for ERG b- and d-wave generation. Firstly, $GABA_A$ receptors were blocked by 100 μM bicuculline and afterwards a combination of 100 μM serotonin and 5 mM TACA was applied. Similarly to previously described groups, bicuculline caused marked enhancement of the b- and d-wave's amplitude and lengthening of their implicit times (Fig. 7 a, b; Table I). When the blocker's effects were fully developed (at the 18th minute from the beginning of the experiment), serotonin + TACA were added to the solution. The simultaneous activation of serotonin and $GABA_C$ receptors produced great diminution of the ERG ON and OFF response amplitude. The effect was strongest during the first 5 minutes, it gradually decreased during the next 3-5 minutes and stabilized afterwards (Fig. 7 a, b). It was interesting to compare the relative decrease of the ERG wave amplitude (normalized to the value obtained during the last minute of the isolated bicuculline treatment) in this group with that obtained in BCC + TACA group. This comparison showed that the relative decrease of the b-wave amplitude was practically identical in the two groups (Fig. 7 c) except for the first 5 minutes, when it was greater in bicuculline + serotonin + TACA group than in bicuculline + TACA group (two-way ANOVA $F_{1,71}$=10.91, P<0.0016). This result indicates that serotonin did not diminish the depressing effect of isolated $GABA_C$ receptor activation on the b-wave amplitude. On the other hand, the relative decrease of the d-wave amplitude in bicuculline + serotonin + TACA group was significantly smaller than that in bicuculline + TACA group (two-way ANOVA $F_{1,179}$=4.58, P<0.034) except for the first 5 minutes, when no significant difference was obtained (Fig. 7 d). Thus, it appeases that serotonin diminishes the inhibitory effect of $GABA_C$ receptor activation on the d-wave amplitude, but it takes some time (~5 minutes) for effect development. The rapid effect of serotonin on the d-wave amplitude in serotonin + TACA group (Fig. 4 d) is probably due to its interaction with $GABA_A$ receptor activation. The time characteristics of the ERG waves in bicuculline + serotonin + TACA group were altered in a similar manner to that obtained in bicuculline + TACA group. The implicit time of the b-wave was not significantly changed (compared to that obtained during preceding bicuculline treatment), while the implicit time of the d-wave was significantly shortened (Table I; Fig. 7 e).

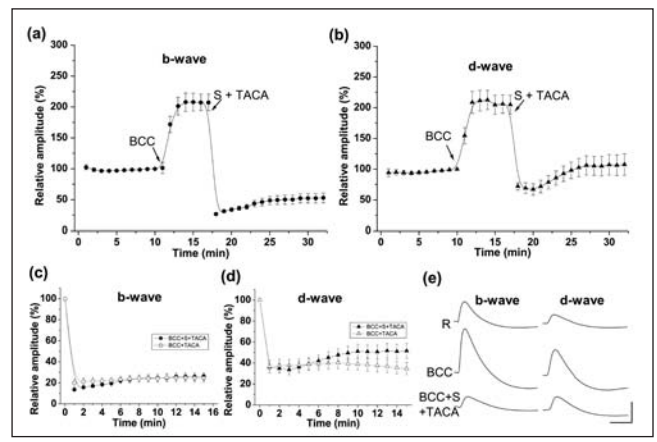

Fig. 7. Effects of combined bicuculline plus serotonin plus TACA action on the ERG waves. (a) (b) Time course of the effects of bicuculline + serotonin + TACA on the amplitudes of the ERG b- and d-waves. The amplitudes of the ERG waves were normalized to the values obtained in the 10th minute from the beginning of the experiments. The times, when the solutions containing 100 μM bicuculline (BCC) and 100 μM serotonin (S) + 5 mM TACA were applied, are indicated by arrows. Mean values ±SEM are shown. (c) (d) Relative amplitude changes of the b- and d-waves in bicuculline + serotonin + TACA group and bicuculline + TACA group. The amplitudes of the ERG waves were normalized to the values obtained just before serotonin + TACA or TACA application (at 0 minute) (e) Original ERG records (b- and d-wave) obtained during treatment with Ringer solution in the control period (10th minute from the beginning of the experiment – upper row), 100 μM bicuculline (17th minute from the beginning of the experiment – middle row) and 100 μM bicuculline + 100 μM serotonin + 5 mM TACA (20th minute from the beginning of the experiment – bottom row) Calibration: time – 0.5 s; amplitude – 750 μV.

DISCUSSION

Our results suggest that some interactions between serotoninergic and GABAergic systems exist in frog retina. To the best of our knowledge this is the first study where the effects of these interactions on the overall retinal function have been documented. We demonstrated that serotonin could diminish the effects of ionotorpic GABA receptor activation on the mechanisms responsible for the ERG b- and d-wave generation. Moreover, we showed that there is a clear ON/OFF asymmetry in the proposed interaction between serotonin and different types of ionotropic GABA receptors. While serotonin may decrease the effects of only $GABA_A$ receptor activation in the ON pathway, it may decrease the effects of both $GABA_A$ and $GABA_C$ receptor activation in the OFF pathway.

There are a few studies where the effects of the serotoninergic system on the ERG waves have been investigated. Contradictory results have been obtained in experiments where the retinal serotoninergic neurons were selectively destruct with 5,7-dihydroxy-

Table I. Drug effects on the implicit time of the ERG b- and d-waves

Group	Implicit time (ms)				
ERG wave	Before (10 min)		After		
Ringer (*n=11*)					
b-wave	333±9.33		•333±10.26		
d-wave	376±14.94		•391±13.04		
Serotonin (*n=7*)					
b-wave	360±16.51		•356±10.81		
d-wave	377±9.48		•378±46.91		
TACA (*n=8*)					
b-wave	334±10.29		•379±13.80		
		p<0.004			
d-wave	355±16.24		•340±22.53		
Serotonin + TACA (*n=7*)					
b-wave	320±9.37		•316±10.40		*324±14.80
d-wave	356±7.45		•347±10.58		*336±17.54
Bicuculline (*n=6*)					
b-wave	299±10.37		°322±16.03		
		p<0.027			
d-wave	307±14.10		°345±17.57		
		p<0.030			
Bicuculline + TACA (*n=10*)					
b-wave	329±12.24		°354±9.80		∇350±10.62
		p<0.019			
d-wave	366±9.82		°381±10.58		∇335±18.73
		p<0.036		p<0.018	
BCC + S + TACA (*n=11*)					
b-wave	297±26.97		°351±35.73		∇337±23.74
		p<0.005			
d-wave	304±25.56		°379±37.09		∇317±22.50
		p<0.0011		p<0.043	

The implicit time was measured at the following minutes after the beginning of the experiment: • – 13 minute; ∗ – 16 minute; ° – 17 minute; ∇ – 20 minute. Means ±SEM are presented. The statistical significance of the differences between the values in adjacent columns is evaluated using paired t-test.

tryptamine (5,7-DHT). While Portiatti et al. (1989) have demonstrated that 5,7-DHT treatment increases the b-wave amplitude without affecting its peak latency in pigeon ERG, Nakatsuka and Hamasaki (1985) reported that 5,7-DHT causes diminution of the b-wave amplitude with prolongation of its duration without changing the off response in rabbit ERG. It appears that endogenous serotonin has inhibitory role on the b-wave generating mechanisms in pigeon retina, but excitatory role on the same mechanisms in rabbit retina. It has been found that application of exogenous serotonin increases the amplitude of the b-wave in cat (Skrandies and Wässle 1988) and rabbit (Bragadottir et al. 1997) retina, which supports the suggestion that serotoninergic system has excitatory role on the b-wave generating mechanism in mammalian retina. Our finding that serotonin increases the amplitude of the b-wave in frog retina indicates that serotoninergic system has similar role in the amphibian retina as well. We demonstrated also that serotonin enhances the amplitude of the ERG OFF response, which has been not previously reported in any type of retina. Thus, we may state that serotonin has enhancing effect on the amplitude of both the ERG ON and OFF responses without altering their time characteristics. This effect may be due to direct action of serotonin on the ON and OFF bipolar cells, or it may originate in altered activity of photoreceptors and/or retinal interneurons (horizontal and amacrine cells) connected to bipolar cells. Serotonin receptors have been localized on photoreceptors (Pootanakit and Brunken 2001, Pennesi et al. 2012), Muller glial cells (Han et al. 2007) and all types of retinal neurons (Mangel and Brunken 1992,

Pootanakit et al. 1999, Haverkamp et al. 2009, Hidaka 2009, Pennesi et al. 2012). Thus, exogenous serotonin could affect the activity of the ON and OFF bipolar cells via action on serotonin receptors localized on all these cell types. We could not localize the exact site of its action, but we may state that the final effect of serotonin action is an enhancement of the activity of both types of bipolar cells.

In this study, we demonstrated that the simultaneous stimulation of $GABA_A$ and $GABA_C$ receptors by TACA caused marked diminution of the ERG b- and d-wave amplitude. This result is consistent with our previous findings showing that application of exogenous GABA has inhibitory effect on the frog ERG ON and OFF responses (Vitanova et al. 2001, Popova 2003), while simultaneous blockade of these receptors (with picrotoxin) enhances the b- and d-wave amplitude in all conditions of light stimulation and adaptation (for review see: Popova 2014). In the present study, we found that TACA has greater inhibitory effect upon the b- than d-wave amplitude suggesting that the ionotropic GABA receptor activation has stronger depressing effect upon the activity of frog ON than OFF bipolar cells. However, we have previously shown that picrotoxin has greater enhancing effect upon the d- than b-wave amplitude under the same conditions of light stimulation and adaptation (Popova 2000, Popova et al. 2016). The discrepancy between our present and previous results may be due to the different potency of TACA to stimulate $GABA_C$ and $GABA_A$ receptors. Some data indicate that TACA is at least 100 times more potent as agonist at $GABA_C$ than at $GABA_A$ receptors (Kusama et al. 1993, Johnston 1996) and therefore TACA should activate to a greater degree $GABA_C$ than $GABA_A$ receptors. If the ON bipolar cells have greater proportion of $GABA_C$ receptors than the OFF bipolar cells, it could explain the stronger depressing effect of TACA on the b- than d-wave amplitude. It has been well documented that in mammalian retina the $GABA_C$ receptors mediate most of the response to GABA in both rod and cone ON bipolar cells, while in cone OFF bipolar cells there is about equal contributions of $GABA_A$ and $GABA_C$ receptors or the $GABA_A$ receptor component is even greater than the $GABA_C$ receptor component (for review see: Popova 2014). The relative contribution of each receptor type to the overall GABA current elicited in the ON and OFF bipolar cells in nonmammalian retina is not well evaluated. We may suggest that in frog retina the $GABA_A$ receptors have greater contribution to the OFF bipolar cell response, while the $GABA_C$ receptors have greater contribution to the ON bipolar cell response. This suggestion is supported by the following results obtained in the present study. (1) Blockade of $GABA_A$ receptors with bicuculline had greater enhancing effect on the amplitude of the ERG OFF than ON response, which is consistent with our previous findings in frog retina (Popova 2003, Kupenova et al. 2008). This means that endogenous GABA acting on $GABA_A$ receptors suppresses to a greater extent the activity of OFF than ON bipolar cells. (2). When $GABA_A$ receptors were blocked by bicuculline, isolated $GABA_C$ receptor stimulation with TACA produced a greater suppression of the ERG ON than OFF response. This means that $GABA_C$ receptor activation has stronger depressing effect upon the ON than OFF bipolar cell activity and this effect predominates in the overall TACA action.

An interesting observation in the present study was the finding that the relative decrease of the b- and d-wave amplitude during isolated $GABA_C$ receptor stimulation was even greater than that obtained during simultaneous stimulation of $GABA_A$ and $GABA_C$ receptors. Some data indicate that serial inhibition mediated by $GABA_A$ receptors may limit the direct inhibition mediated by $GABA_C$ receptors on bipolar cell terminals (Eggers and Lukasiewicz 2011). It has been shown that blocking of the $GABA_A$ receptors with bicuculline increases the $GABA_C$-mediated currents in bipolar cells (Eggers and Lukasiewicz 2006). Similar results have been obtained for the ERG b-wave in mice retina. It has been demonstrated that the blockade of $GABA_C$ receptors with TPMPA has stronger effect on the b-wave amplitude when TPMPA is applied simultaneously with $GABA_A$ receptor blocker (gabazine) than when it is applied alone (Smith et al. 2015). In accordance with the cited reports, we showed that the suppressing effect of TACA on the amplitude of both the b- and d-waves was stronger in eyecups treated with bicuculline than in intact eyecups. Thus, we may suggest that blocking of $GABA_A$ receptors with bicuculline increases the effect of $GABA_C$ receptor activation (by TACA) on the ON and OFF bipolar cell activity in frog retina. It is true not only for the amplitude of the responses, but also for their time characteristics. We obtained that the isolated $GABA_C$ receptor activation prevented the implicit time lengthening of the b-wave (seen during simultaneous activation of $GABA_A$ and $GABA_C$) and significantly shortened that of the d-wave. These results implicate that isolated activation of $GABA_C$ receptors leads to speeding the ERG ON and OFF responses. Results of many authors indicate that the $GABA_C$ receptor blockade slows down the kinetics of the b-wave (for review: Popova 2014) and that of the d-wave (Vitanova et al. 2001), indicating that $GABA_C$ receptors are involved in speeding the time course of the ERG responses. Similar results have been reported for the bipolar cell responses which become more sustained during inhibition of $GABA_C$ receptors (Dong and Werblin 1998, Euler and Wassle 1998).

The most interesting and new results in our study concern the possible functional interactions between retinal serotoninergic and GABAergic systems. Our findings imply that serotonin may decrease the effects of simultaneous $GABA_A$ and $GABA_C$ receptor activation (by TACA) on the ERG b- and d-wave generating mechanisms. This effect may be due to direct action of serotonin on the $GABA_A$ and $GABA_C$ receptors that has been shown to exist on the ON and OFF bipolar cells in many species (for review see Popova 2014) or it may reflect opposite actions of serotoninegic and GABAergic systems exerted at different retinal stages. Unfortunately, we could not find any data concerning the interaction between serotonin and $GABA_A$ receptors on the retinal bipolar cells. Conflicting results exist about the interaction between serotonin and $GABA_A$ receptors in non-retinal neurons. While serotonin reduces the postsynaptic $GABA_A$ receptor currents in dissociated prefrontal pyramidal neurons (Feng et al. 2001) and in human iPS-derived neurons (Wang et al. 2016), the opposite effect has been documented in dissociated spinal cord neurons (Xu et al. 1998, Wang et al. 1999, Li et al. 2000), although the effect was mediated by the same 5-HT_2 receptor type. Other authors could not find any modulation by serotonin of the currents of cloned $GABA_A$ receptor expressed in *Xenopus laevis* oocytes (Ochoa-de la Paz et al. 2012). Still other authors argue that activation of different serotonin receptor types may cause opposite effects on the $GABA_A$ receptor mediated inhibitory postsynaptic potentials in hippocampal slice preparations (Bijak and Misgeld 1997). Our data suggest that serotonin probably decreases (directly or indirectly) the effects of $GABA_A$ receptor activation on both the ON and OFF bipolar cells. The functional interaction of serotonin with $GABA_C$ receptors is largely unknown. We could find only 2 works where this interaction was investigated. In both of them it is shown that serotonin inhibits the GABA elicited currents through $GABA_C$ receptors (Feigenspan and Bormann 1994; Ochoa-de la Paz et al. 2012), which is consistent with our finding for the ERG OFF response. Our results lead to the suggestion that serotonin may decrease (directly or indirectly) the effects of both $GABA_A$ and $GABA_C$ receptor activation on the bipolar cells activity. However, there is an ON/OFF asymmetry of GABA receptors involved in this serotonin action. It appears that serotonin diminishes the effect of both $GABA_A$ and $GABA_C$ receptor activation on the activity of the OFF bipolar cells, while it diminishes the effect of only $GABA_A$ receptor activation on the activity of ON bipolar cells. Further studies are needed to reveal the exact mechanism of the described serotonin-GABA interactions in the vertebrate retina.

CONCLUSIONS

Our results clearly show that serotonin has enhancing effect on the amplitude of both the ON and OFF response of frog ERG. Moreover, it may decrease the depressing effects of ionotropic GABA receptor activation on the mechanisms responsible for ERG b- and d-wave generation. The latter effect demonstrates an ON/OFF asymmetry according to the GABA receptors involved. While serotonin may decrease the effects of only $GABA_A$ receptor activation in the ON pathway, it may decrease the effects of both $GABA_A$ and $GABA_C$ receptor activation in the OFF pathway.

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