Cellular and Molecular Neurobiology (2019) 39:1039–1049
https://doi.org/10.1007/s10571-019-00701-8

ORIGINAL RESEARCH

Regulation of the Serotonergic System by Kainate in the Avian Retina

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Received: 5 February 2019 / Accepted: 7 June 2019 / Published online: 13 June 2019

Abstract

Serotonin (5-HT) has been recognized as a neurotransmitter in the vertebrate retina, restricted mainly to amacrine and bipolar cells. It is involved with synaptic processing and possibly as a mitogenic factor. We confirm that chick retina amacrine and bipolar cells are, respectively, heavily and faintly immunolabeled for 5-HT. Amacrine serotonergic cells also co-express tyrosine hydroxylase (TH), a marker of dopaminergic cells in the retina. Previous reports demonstrated that serotonin transport can be modulated by neurotransmitter receptor activation. As 5-HT is diffusely released as a neuromodulator and co-localized with other transmitters, we evaluated if 5-HT uptake or release is modulated by several mediators in the avian retina. The role of different glutamate receptors on serotonin transport and release in vitro and in vivo was also studied. We show that L-glutamate induces an inhibitory effect on [^{3}H]5-HT uptake and this effect was specific to kainate receptor activation. Kainate-induced decrease in [^{3}H]5-HT uptake was blocked by CNQX, an AMPA/kainate receptor antagonist, but not by MK-801, a NMDA receptor antagonist. [^{3}H]5-HT uptake was not observed in the presence of AMPA, thus suggesting that the decrease in serotonin uptake is mediated by kainate. 5-HT (10–50 µM) had no intrinsic activity in raising intracellular Ca^{2+}, but addition of 10 µM 5-HT decreased Ca^{2+} shifts induced by KCl in retinal neurons. Moreover, kainate decreased the number of bipolar and amacrine cells labeled to serotonin in chick retina. In conclusion, our data suggest a highly selective effect of kainate receptors in the regulation of serotonin functions in the retinal cells.

Keywords Serotonin · Glutamate receptors · Retina · Kainate

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is widely recognized as a neurotransmitter in the vertebrate central nervous system (CNS) with multiple pre- and postsynaptic receptors involved in physiological responses and disease conditions, with implications in mood, behavior, depression, and appetite regulation (Beart 2016). 5-HT also appears to be involved in amacrine signal processing, intraocular pressure elevation, ocular blood vessel constriction, mitogenic signaling (George et al. 2005), and regulation of movement (Willis and Freelance 2017). In the vertebrate retina, it has been well described in a subpopulation of amacrine cells in the retina (Lima and Urbina 1994; Millar et al. 1988; Osborne et al. 1982; Wilhelm et al. 1993) with a high-affinity uptake system (Ghai et al. 2009; Osborne et al. 1982). A single 5-HT transporter (SERT) is responsible for extracellular 5-HT clearance (Blakely et al. 1991), a mechanism coupled to the

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co-transport of Na^+ and Cl^- down a concentration gradient. The activity of this transporter determines the amount of available serotonin for the activation of both pre- and postsynaptic serotonin receptors (Amara and Kuhar 1993; Horschitz et al. 2001). Immunohistochemical procedures revealed the development of serotonergic chick retinal neurons, as serotonin-accumulating amacrine and bipolar neurons (Rios et al. 1997). SERT expression is modulated by neurotransmitter receptors, second messengers, and protein kinases activation (Haase et al. 2001). Evidence exists that SERT can be modulated by γ-aminobutyric acid (GABA) (Tao et al. 1996) and excitatory amino acids (EAA) in midbrain and forebrain (Tao et al. 1997). However, information regarding regulation of SERT in the retina by neurotransmitters has been very limited and mechanisms responsible for this regulation remain unknown.

In the retina, glutamate is the main excitatory neurotransmitter and its uptake and release mechanisms are regulated by other neurotransmitters (Calaza et al. 2001). Two classes of receptors mediate glutamatergic synapses: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors are subdivided into N-methyl-D-aspartate (NMDA) receptors, kainate receptors, and α–amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors based on the relative selectivity of agonists and antagonists (Connaughton 1995). Kainate are ionotropic glutamate receptors that mediate fast excitatory synaptic transmission. In the vertebrate retina, they are involved with sign-conserving OFF bipolar cell synapse (Brandstatter et al. 1997). Since 5-HT is diffusely released as a neuromodulator in many brain areas and co-localized with other transmitters (Ciranna 2006), here we investigated if 5-HT uptake or release is modulated in the avian retina cell cultures. We further evaluate the role of glutamate receptors in the 5-HT uptake and release in the chick retina in vitro and in vivo. Our data suggest a highly selective effect of kainate receptors regulating alterations in serotonin functions in the retinal cells.

Materials and Methods

Experiments with animals were permitted by and carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Federal University of Pará (permit number 85/15) and Federal University of Rio de Janeiro (permit number IBCCF-035) and in accordance with the Brazilian animal protection law (law 11794/08).

Retinal Cell Cultures

Retinal cells cultures were obtained from chick embryos at 8–9 days of development as previously described (Do Nascimento et al. 1998). Briefly, retinae were dissected and dissociated using 0.05% trypsin saline solution. After tissue dissociation, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) added with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL glutamine. An estimated number of 5×10^6 retinal cells were grown in 35 mm culture dishes for 7–8 days in 5% CO_2 and 95% air.

Immunocytochemistry

Retinal cell cultures were fixed with 4% (w/v) paraformaldehyde in 0.16 M phosphate buffer for 30 min. Cultures were washed with PBS containing 0.3% Triton X-100 and pre-incubated during 1 h at room temperature in PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.3% Triton X-100 to block non-specific binding sites. Primary rabbit 5-HT-antibody (1:200 dilution; ImmunoStar) in PBS containing 1% (w/v) BSA and 0.3% Triton X-100 was incubated overnight. Following the removal of the primary antibody, cells were rinsed for 30 min with three changes of PBS at 10-min intervals. Secondary antibodies (1:200 dilution; Sigma) conjugated anti-rabbit immunoglobulin biotin for 2 h in PBS containing 1% (w/v) BSA. Subsequent to the removal of secondary antibodies, cells were exposed to rabbit ExtrAvidin peroxidase staining kit according to the manufacturer's protocol and reacted with 0.025% 3.3′-diaminobenzidine in PBS for 5 min. Finally, cells were washed with PBS, mounted on the glass slides with N-propyl-gallate, and observed with a microscope Olympus BX41.

[^{3}H]5-HT Uptake

Retinal cells in culture at stage C8 (8 days in vitro) were incubated for 10 min at 37 °C with 3 µCi of [^{3}H]5-HT (300 nM) in Ringer Locke solution (5.6 mM KCl, 148 mM NaCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, and 2.3 mM $CaCl_2$). Then, cells were washed twice with 500 µl of Ringer Locke solution. To measure [^{3}H]5-HT present in the cells, cultures were lysed with 3% TCA and the radioactivity was determined by liquid scintillation.

[^{3}H]5-HT Release

Retinal cell cultures E8C8 were exposed for 10 min to 1 µCi of [^{3}H]5-HT (100 nM) diluted in Hank´s solution (128 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 20 mM $CaCl_2$, 12 mM Glucose, 20 mM HEPES). After the incubation period, cells were washed with 500 µl µl Hank´s solution to remove the radioactivity not incorporated by the cells. Basal release was evaluated and the effects of different agents on the release were determined by liquid scintillation. Cells were then lysed with 1 ml of water followed by three freeze–thaw

cycles for at least 1 h. The remaining intracellular radioactivity was determined. The radioactivity present in each fraction was expressed in fentomoles of [^{3}H] 5-HT released or as the fractional release, which corresponded to the percentage of radioactivity released at each time point, as compared to the total radioactivity present in the cells.

Single-Cell Calcium Imaging (SCCI)

Free intracellular calcium levels ([Ca^{2+}]i) were assessed in cultured cells described initially for mice (De Melo Reis et al. 2011) and later for avian retina (Freitas et al. 2016). Cells were loaded for 40 min with 5 µM Fura-2/AM (Molecular Probes), 0.1% fatty acid-free BSA, and 0.02% pluronic F-127 (Molecular Probes) in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4), in an incubator with 5% CO_2 and 95% atmospheric air at 37 °C. To observe complete hydrolysis of the probe, cells were washed at room temperature in Krebs solution after a 10-min post-loading period. Then, 15 mm coverslip (Marienbad, Germany) with cells was mounted on a chamber in a PH3 platform (Warner Instruments, Hamden, CT) on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss). Cells were perfused with Krebs solution and stimulated with 50 mM KCl or 1 mM ATP. A fast-transition system (4 s) was used to load solutions. [Ca^{2+}]i variations were evaluated by quantifying the ratio of the fluorescence emitted at 510 nm following alternate excitation (750 ms) at 340 and 380 nm, using a Lambda DG4 apparatus (Sutter Instrument, Novato, CA) and a 510-nm long-pass filter (Carl Zeiss) before fluorescence acquisition with a 20X objective and a Cool SNAP digital camera (Roper Scientific, Trenton, NJ). Data were processed using the MetaFluor software (Universal Imaging Corp., West Chester, PA). Values for Fura-2 fluorescence ratio were calculated based on a cut-off of 15% increase in the [Ca^{2+}]i level induced by the stimulus. Cell cultures after SCCI were fixed in 4% PFA as described above.

Drugs Treatment

To analyze the effect of different neurotransmitter systems on the [^{3}H]5-HT uptake and release in retinal cell cultures, pre-incubation with histamine (50 µM and 100 µM); 2-chloroadenosine (50 µM and 100 µM); methacholine (50 µM and 100 µM); GABA (50 µM and 100 µM); and L-glutamate (50 µM) was performed 10 min before [^{3}H]5-HT uptake. Glutamatergic receptor agonists were also evaluated, adding L-glutamate (50 µM), NMDA (50 µM), AMPA (100 µM), kainate (1 µM), or Trans-ACPD (100 µM) for 10 min. Antagonists such as MK-801 (NMDA), CNQX (AMPA/Kainate), or NBQX (AMPA/kainate) were added 5 min before agonists treatment were also performed. For [^{3}H]5-HT release experiments, drugs were added after [^{3}H]5-HT uptake.

Ex vivo Retinal Stimulation of Endogenous 5-HT Release

Chicks were humanely sacrificed, and retinal pieces were obtained as previously described (Kubrusly et al. 2018). Briefly, after enucleation, the anterior half of the eye was discarded and the posterior half containing the retina was equally divided in four pieces. These retinal pieces were randomly allocated as control or each experimental condition. The Locke's solution (157 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM Na_2HCO_3, 5.0 mM HEPES, and 5.6 mM glucose), used to incubate the tissue, was balanced with 95% O_2/5% CO_2, and pH was adjusted to 7.2–7.4 with HCl 1 N and NaOH 1 N. During the 30-min incubation period, retinas were kept in a bath solution at 37 °C which was constantly perfused with 95% O_2/5% de CO_2, to maintain the tissue oxygenation.

Then, the tissue was incubated in Locke's solution (control) or in the same solution containing 10 µM kainate for 30 min. To confirm the participation of the kainate receptor, retinal pieces were pre-incubated for 20 min with 100 µM DNQX, kainate/AMPA receptor antagonist, and then incubated together for more 30 min with kainate. During this pre-incubation period, the other experimental groups remained in Ringer solution only, without the drugs.

Immunohistochemistry

Retinas from 14-day chick embryos or retinal pieces, after the ex vivo experiment, were fixed by immersion in 4% paraformaldehyde in 0.16 M sodium phosphate buffer (pH 7.2) for 1 h and washed in sodium phosphate buffer. For cryoprotection, retinas were submitted to sucrose gradient (15% and 30%) overnight. Then, radial retinal sections (12 µm) were obtained in cryostat (CM1850, Leica Microsystems, Wetzlar, Germany). Retinal sections from all groups of the same experiment, control and treated retinas, were collected in the same slide, to guarantee the submission to the same histological procedure.

Sections were incubated with a blocking solution composed of 5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) for 1 h, and then with 1:500 rabbit 5-HT-antibody (ImmunoStar) overnight. Thereafter, retinal sections were incubated with Alexa 488-labeled secondary anti-rabbit antibody (1:500) for 2 h and mounted with Glycergel (Dako). All incubation steps were followed by washes with phosphate-buffered saline (PBS, pH 7.4). BSA, primary, and secondary antibodies were

diluted in PBS plus 0.25% Triton X-100. The absence of primary antibody produced no specific labeling.

Images were acquired with DFC310 FX camera with epifluorescence microscope DM2500 (Leica Microsystems, Wetzlar, Germany).

Optical Densitometry

We applied a similar procedure as previously described (Calaza et al. 2001; Guimaraes-Souza and Calaza 2012). The intensity of serotonin immunoreactivity in the IPL was analyzed using digitized images captured with DFC310 FX camera (Leica Microsystems, Wetzlar, Germany) with epifluorescence microscope DM2500 (Leica Microsystems, Wetzlar, Germany). The digitized images were processed with ImageJ software (version 1.38, NIH, USA). Using the freehand selection tool, the area of interest (IPL, amacrine cells, or bipolar cells) was surrounded and the fluorescence intensity within the selected area was acquired. In each experiment, each group had triplicates in the same slide and at least three pictures of each retinal section were taken for the fluorescence intensity analyzes.

Statistical Analysis

All experiments were performed utilizing at least five individual retinal cell cultures preparations. Statistical tests and analysis of variance (ANOVA–Turkey test) were performed using BIOSTAT version 3.0 for Windows.

Results

5-HT Neurons in the Chick Retinal Cultures

Immunocytochemistry was performed in order to investigate the presence of serotonergic neurons in retinal cell cultures. Cells were labeled primarily in cell bodies (Fig. 1a, arrows), although weak staining in some cell processes was also observed (Fig. 1a, arrowheads in the inset). We also performed immunohistochemistry for serotonin in the retina at 14-day-old chick embryos. We confirm the previously published data that serotonin is found in amacrine and bipolar cells, as well as in the inner plexiform layer (Fig. 1b). In addition, we show that serotonergic cells are co-immunolabeled to tyrosine hydroxylase (TH), the main marker of dopaminergic amacrine cells (Fig. 1b), known to be co-localized with cannabinoid CB1 receptors (da Silva Sampaio et al. 2018). This superposition is pertinent to the previous evidence that CB1 receptor activation inhibits 5-HT release from cortical (Nakazi et al. 2000) and brainstem (dorsal raphe) neurons (Haj-Dahmane and Shen 2011).

Regulation of [^{3}H]5-HT Uptake

We investigated the regulation of serotonin uptake by different transmitter systems in the chick retinal cell cultures: histaminergic, purinergic, cholinergic, GABAergic, and glutamatergic. As shown in Fig. 2, L-glutamate is the only transmitter that affected serotonin transport. Cultures treated with 50 µM of glutamate showed a reduction in serotonin uptake to approximately 70% of basal levels ($p < 0.01$). Furthermore, fluoxetine, a well-known SERT inhibitor, was used as

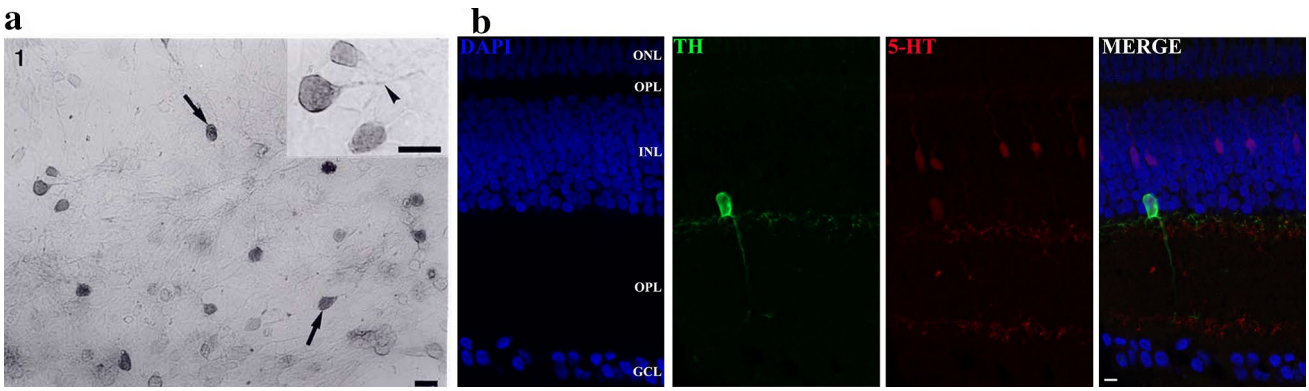

Fig. 1 Immunodetection of serotonin in cells in both retinal cell cultures and embryonic retina. **a** In culture at E8C6, staining was concentrated primarily in somata (arrows), although some labeling was also noticed in the cellular processes (arrowheads, see insert). Bar = 10 µm. **b** In the embryonic avian retina (E14), amacrine and bipolar cells are, respectively, heavily and weakly labeled by anti-serotonin antibody (TH). Amacrine tyrosine hydroxylase positive cells (TH) also show serotonin immunolabeling. The labeling with DAPI reveals all nuclear layers of the retina. Bar = 20 µm

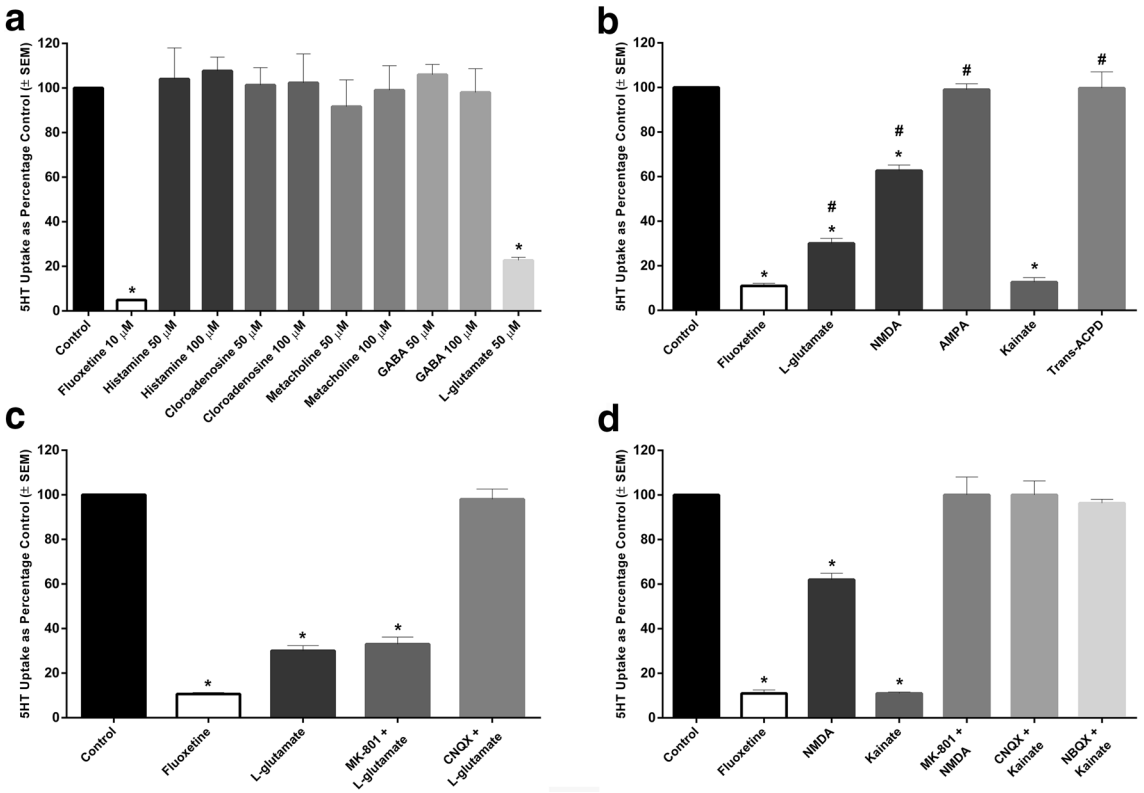

Fig. 2 [^{3}H]5-HT uptake in retinal cell cultures is decreased by L-glutamate through kainate receptors. **a** The effect of several neurotransmitter agonists in the 5-HT uptake was evaluated. Cells were pre-treated with histamine (50 μM and 100 μM), 2-chloroadenosine (50 μM and 100 μM), methacholine (50 μM and 100 μM), GABA (50 μM and 100 μM), and L-glutamate (50 μM) for 10 min prior to the [^{3}H]5-HT uptake assay. Among all agonists only L-glutamate showed an effect. n = 5. **b** Effect of glutamate receptor agonists on the 5-HT uptake. Cells were pre-incubated with L-glutamate (50 μM), NMDA (50 μM), AMPA (100 μM), kainate (1 μM), and Trans-ACPD (100 μM) by 10 min prior to the [^{3}H]-HT uptake assay. n = 5. **c** The inhibition of [^{3}H]-5-HT uptake by L-glutamate in retinal cell cultures is only prevented by antagonist of kainate/AMPA receptor (CNQX), but not by NMDA receptor antagonist (MK-801). MK-801 (10 μM) and CNQX (50 μM) were pre-incubated before L-glutamate treatment. n = 5. **d** The effect of iGluRs agonists, kainate or NMDA, depends on selective receptor activation. NMDA receptor antagonist, MK-801 (10 μM), and kainate/AMPA receptor antagonist, CNQX (50 μM), were pre-incubated before 10 μM agonist treatment and blocked the respective agonist action. n = 5. Fluoxetine (10 μM) was used to evaluate the specific [^{3}H]5-HT uptake through serotonin transporter. Data are expressed as percent control (mean ± S.E.M.). ANOVA *t* test *p < 0.01 vs. control, #p < 0.01 vs. fluoxetine treatment

a positive control to determine specific serotonin uptake in all experiments (fluoxetine-sensitive uptake) (Fig. 2a).

To further investigate the modulation of serotonin uptake by the glutamatergic system, selective glutamatergic receptor agonists were used. Our results demonstrated that while 100 μM AMPA or 100 μM Trans-ACPD (metabotropic glutamate receptor agonist) had no effect in the [^{3}H]5-HT uptake, 50 μM NMDA or 1 μM kainate was inhibited by 40% or 90% [^{3}H]5-HT uptake, respectively ($p < 0.01$) (Fig. 2b).

In order to confirm which ionotropic glutamate receptors were involved in the inhibition of [^{3}H]5-HT uptake mediated by L-glutamate, retinal cell cultures were co-treated with L-glutamate in the presence of glutamate receptor antagonists. Addition of 50 μM CNQX, an antagonist of kainate/AMPA receptors, completely inhibited the L-glutamate effect on the [^{3}H]5-HT uptake (Fig. 2c). On the other hand, MK-801, non-competitive NMDA antagonist, had no effect on the decrease of [^{3}H]5-HT uptake induced by L-glutamate (Fig. 2c). As expected, the addition of 50 μM CNQX or 50 μM NBQX, kainate/AMPA receptor antagonists, fully blocked the kainate effect on the [^{3}H]5-HT uptake. Alternatively, the addition of 10 μM MK-801 inhibited the effect of NMDA (Fig. 2d), but not of L-glutamate.

Regulation of [^{3}H]5-HT Release

In order to study the regulation of [^{3}H]5-HT release, several neurotransmitter agonists were tested on retinal cell cultures. Our results demonstrated that histamine, 2-chloroadenosine, GABA, methacholine, atropine, or trans-ACPD did not induce significant changes on the [^{3}H]5-HT release. However, L-glutamate treatment increased [^{3}H]5-HT release from retinal cell cultures (Table 1). The effects of glutamate receptor agonists and antagonists were evaluated on

Table 1 [^{3}H]-5-HT release induced by multiple neurotransmitters

Drugs/treatment (n)	[^{3}H]-5-HT released (Percent of basal)
Basal (4)	100
Histamine (5)	95 ± 7.5
2-Chloroadenosine (3)	92.5 ± 17.5
GABA (3)	97.5 ± 2.5
Methacholine (3)	105 ± 3.75
Atropine (3)	97.5 ± 2.5
Trans- ACPD (4)	92.5 ± 7.5
L-Glutamate (5)	262.5 ± 15

Results are demonstrated as percent of 5-HT basal released and values are expressed as mean ± SEM. Number of determination within parentheses

[^{3}H]5-HT release. Glutamate and kainate increased threefold the [^{3}H]5-HT release, which were completely abolished by CNQX treatment (Fig. 3a). No effect was observed in retinal cell cultures treated with AMPA or NMDA (Fig. 3a). We also evaluated if [^{3}H]5-HT release was induced by kainate in a Ca^{2+}-dependent manner. Our results demonstrated that [^{3}H]5-HT release by kainate is attenuated in the absence of Ca^{2+}. These data suggest a specific effect of kainate receptor that can selectively regulate serotonin release in retinal cells. Addition of fluoxetine had no effect on [^{3}H]5-HT release, demonstrating that kainate-induced release was not related to SERT (Fig. 3b), suggesting that an exocytotic-like mechanism is involved in the process.

Serotonin is a monoamine known to control neuronal excitability and modulate calcium transients in different models such as in *Caenorhabditis elegans* (Zahratka et al. 2015) or in the retina where RGCs respond to 5-HTR$_{2C}$ activation (Trakhtenberg et al. 2017). Therefore, we evaluated if 5-HT could alter Ca^{2+} shifts in retinal neurons or glial cell as shown under fluorescence (Fig. 4a and b). As shown, 10 µM (Fig. 4c) or 50 µM 5-HT (Fig. 4d) had no intrinsic activity in neuroglial cells. However, the addition of 10 µM 5-HT decreased the response of neurons activated by 50 mM KCl (Fig. 4e and f), but not the response of glia activated by 100 µM ATP (Fig. 4g and h).

To confirm whether the regulation of serotonin release by glutamate occurs in the retina, and it is not a feature of the culture model, experiments with ex vivo retina were performed. Therefore, retinas were stimulated with 10 µM kainate for 30 min, and serotonin release was evaluated by quantification of immunohistochemical staining to serotonin in comparison to control. The staining for serotonin in control, not stimulated, retinas showed a similar previously described pattern (Millar et al. 1988; Osborne 1982; Wilhelm et al. 1993) with faintly labeled bipolar cells, strongly labeled amacrine cells with processes extending mainly in two sublayers of the inner plexiform layer (IPL). Kainate treatment reduces the number of amacrine and bipolar serotonin-positive cell bodies (Fig. 5a), around 50% and 40%, respectively (Fig. 5b and c). Kainate stimulation also decreases almost 70% the immunostaining for serotonin in the IPL (Fig. 5a and d). The decrease in serotonin immunoreactivity, due to serotonin release, was completely prevented by DNQX, kainate/AMPA receptor antagonist, indicating that this effect depends on kainate receptors.

Discussion

Serotonergic neurons are found in the chick retina as a particular subset of amacrine and bipolar cells. This is an interesting experimental model to study the regulation of serotonin transport during retinal development. The role of

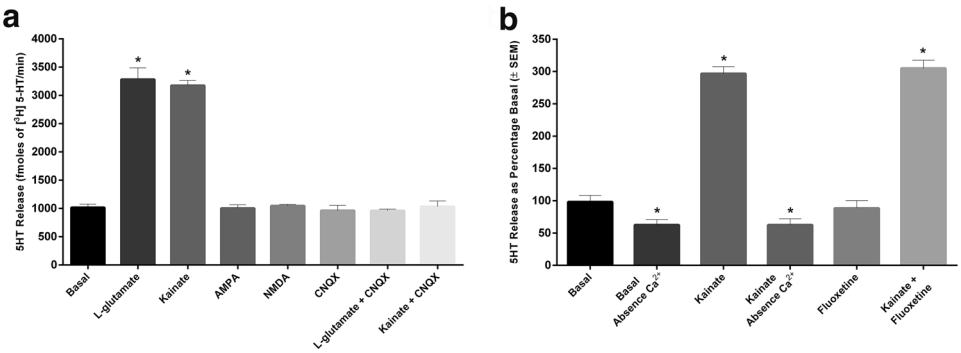

Fig. 3 Effects of glutamatergic receptor agonists and antagonists on the retinal culture 5-HT release. **a** glutamate (100 µM) and kainate (50 µM) induces 5-HT release, while AMPA (100 µM) and NMDA (100 µM) had no effect. The glutamate and kainate effects were blocked by pretreatment with kainate/AMPA receptor antagonist CNQX (100 µM). Data are expressed as [^{3}H]5-HT released as percent basal (mean ± S.E.M.). ANOVA test-t. *$p < 0.01$ vs. Basal. **b** Graph shows the effect of Ca^{++} on [^{3}H]5-HT release evoked by Kainate (50 µM) or Basal, and of fluoxetine (10 µM) treatment on [^{3}H]5-HT release evoked by Kainate or Basal. Data are expressed as [^{3}H]5-HT released as percent basal (mean ± S.E.M.). ANOVA t-test *$p < 0.01$ vs. Basal

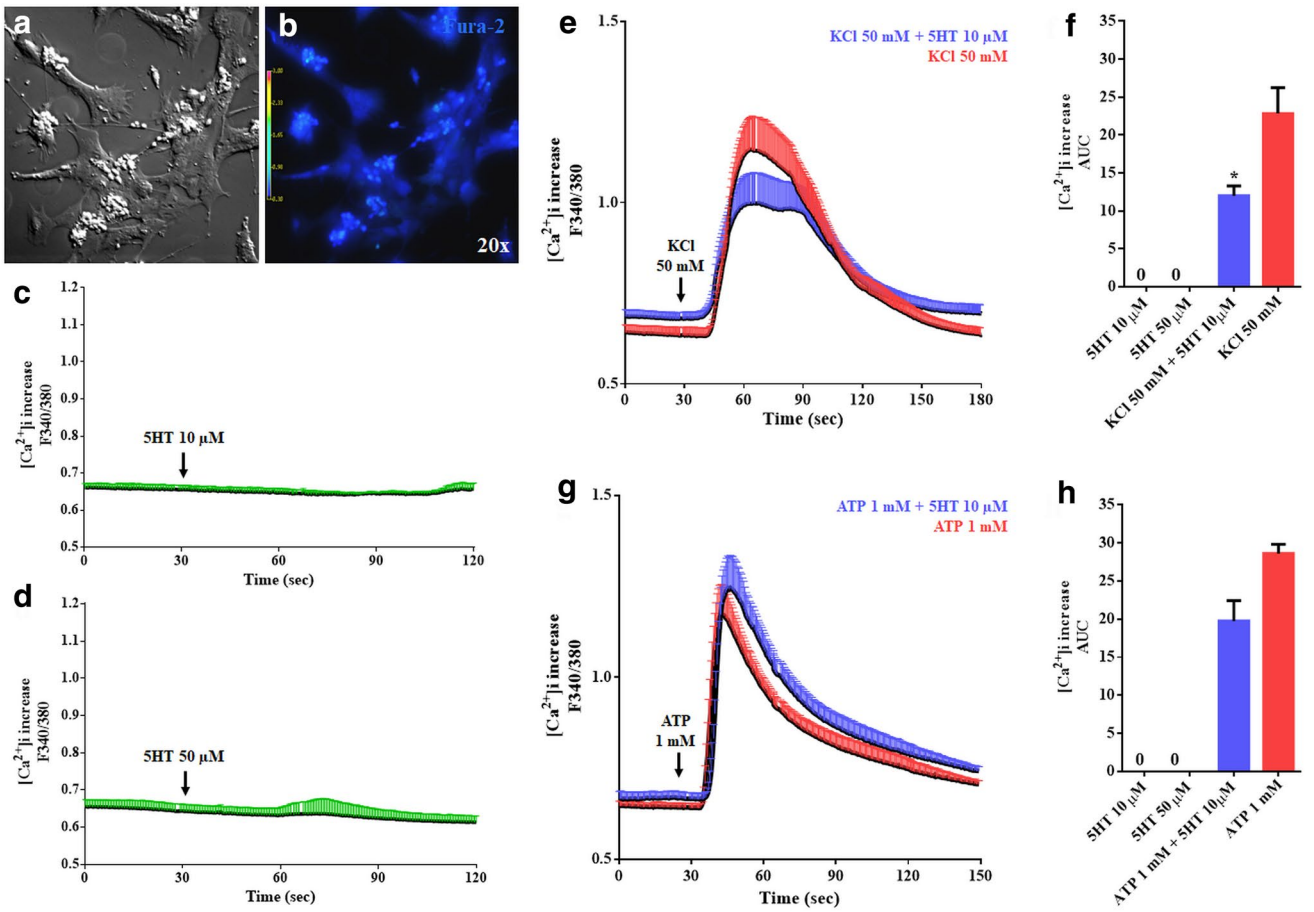

Fig. 4 Intracellular calcium ([Ca^{2+}]i) transients in the presence of 5-HT, potassium chloride (KCl), or adenosine triphosphate (ATP) in mixed retina neuroglial cells. **a** Bright field and **b** Fura-2 fluorescence images reveal neurons and glia from embryonic retinal cell cultures. **c** There was no [Ca^{2+}]i increase induced by 10 µM 5-HT or **d** 50 µM 5-HT in neuroglia cells in culture. **e** Addition of 50 µM 5-HT in the presence of 50 mM KCl decreases [Ca^{2+}]i shifts in neurons compared to activation promoted by 50 mM KCl (red) as quantified in (**f**). This difference is significant when the respective areas under the curve (AUC) are compared ($p < 0.05$). **g** However, addition of 1 mM ATP + 10 µM 5-HT-induced Ca^{2+} shifts similar to 1 mM ATP (red), with no significant changes between [Ca^{2+}]i curves, quantified in H. as comparison of AUC ($p > 0.05$). N = 4 for all conditions, at least 1000 cells analyzed

retinal serotonin is still unclear but evidence shows involvement with a variety of effects, acting as a mitogenic agent, regulating amacrine cells, altering intraocular pressure, and blood vessel constriction (George et al. 2005).

Previous studies have demonstrated that EAA and GABA modulate 5-HT transport in the CNS (Tao et al. 1996, 1997). However, only a few reports evaluated these effects on the serotonergic transport in retinal cells (Schutte and Witkovsky 1990; Weiler and Schutte 1985). Here, we show that among several neuromodulators only iGluRs, more specifically kainate receptor, activation interferes with serotonin release in retinal neurons. SERT is a member of a large family of Na^+-dependent neurotransmitter carrier proteins that regulate serotonin levels in the synaptic cleft. Regulation of this transporter suggests physiological relevance in the control of retinal circuitry since functional modulation has also been demonstrated for most members of transporter family (Beckman et al. 1999; do Nascimento and de Mello 1985; Gerson and Baldessarini 1980).

Although SERT is influenced by EAA in retinal cells, other receptor systems were not effective to regulate the serotonergic transporter. Interestingly, the main effect is mediated by kainate, but not by AMPA receptors.

Glutamate activates different AMPA and kainate receptors on OFF bipolar cells, leading to an alternative mechanism of control upon the serotonergic system. We also demonstrate that selective stimulation of kainate receptors increase [^{3}H]5-HT release. The results described in this work show that kainate and glutamate promote a rapid serotonin release two to threefold larger than control levels. This action is not due to unspecific effects or general damage of the cells in cultures since neither kainate nor glutamate had any effect on the release of [^{3}H] leucine (data not shown).

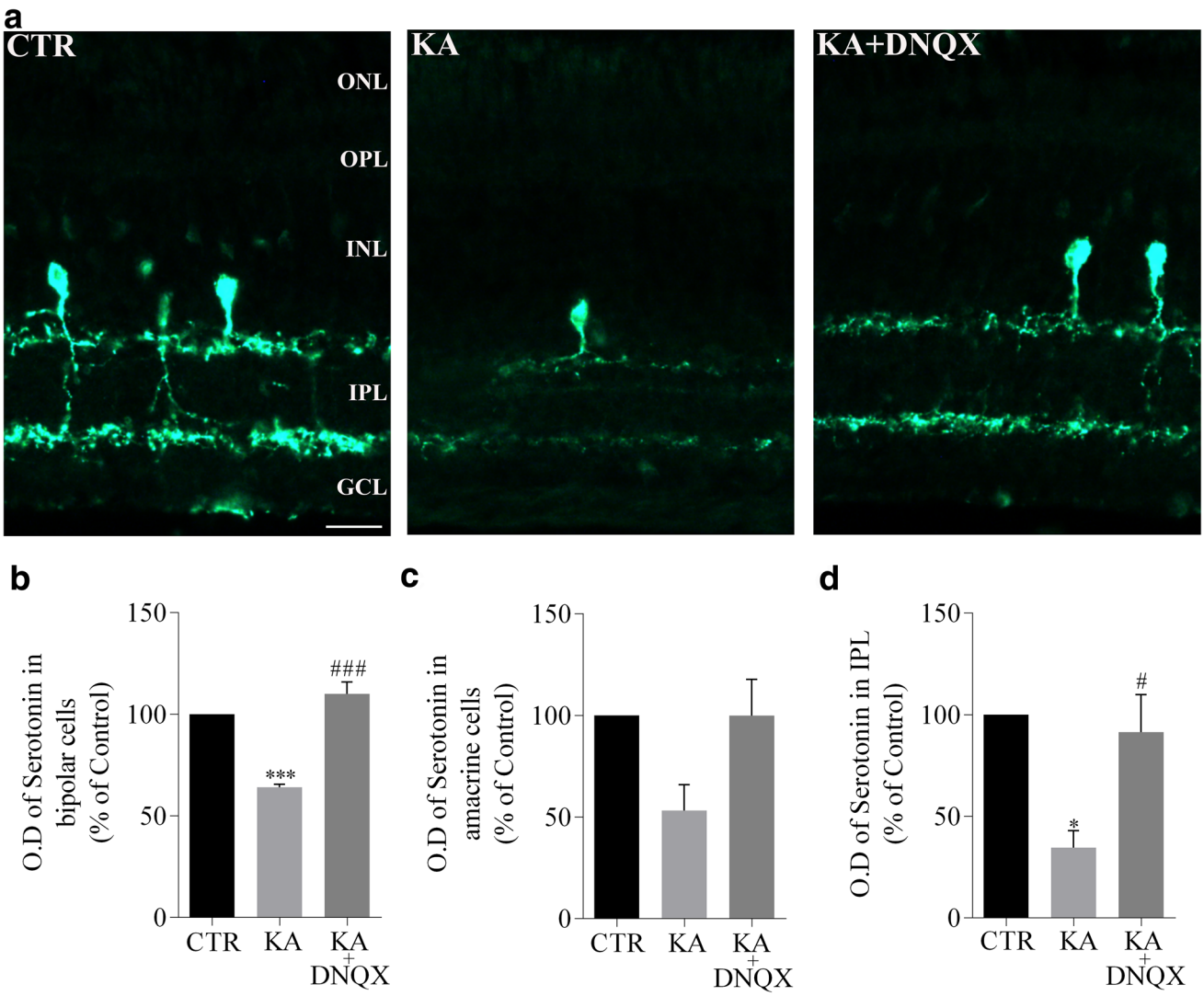

Fig. 5 Antagonism of the glutamatergic receptor prevents [^{3}H]-5-HT uptake in retinal cell cultures. **a** Photomicrographs of chick retina radial sections immunolabeled for serotonin after different treatments. Control retinas kept in Locke's solution for 50 min (Control, left panel); retinas kept in Locke's solution for 20 min plus 10 µM kainate for 30 min (KA, middle panel); retinas treated with DNQX for 20 min followed by the exposure to 10 µM kainate plus DNQX for 30 min (KA + DNQX, right panel). (**b**–**d**) Histograms show optical densitometry of bipolar cells (**b**), amacrine cells (**c**), and IPL (**d**). The values are expressed as mean ± SEM, n = 3. (*$p < 0.05$; *** $p < 0.001$, with respect to controls; #$p < 0.05$; ###$p < 0.001$, with respect to kainate). Scale bar: 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer

Kainate receptors have been shown through electrophysiological recordings to function in the mouse retinal circuitry during light stimulation in OFF cone bipolar cells producing slow/sustained while AMPA is related to fast/transient effects. This phenomenon emerges from the differential expression of these glutamatergic receptors (Borghuis et al. 2014). Indeed, data on macaque monkey retina strengthen that OFF bipolar cells interact primarily through kainate receptors; in fact, the presence of selective antagonists was shown to block inputs to the (OFF)-midget and -parasol cells (Puthussery et al. 2014). Previous studies showed that intravitreal injections of kainic acid lead to loss of amacrine cells, displaced amacrine cells and the majority of the bipolar cells orphan (De Nardis et al. 1988). This fact can lead to the loss of transmitters after injection of kainic acid and it has been used to reveal the role of amacrine cell transmitters in synaptic inputs to other amacrine and bipolar cells. On the other hand, differentiation of a population of amacrine cells in the chick retina with a serotonergic phenotype and bipolar neurons expressing a 5-HT-phenotype was also described (Rios et al. 1997). It is possible that an association of glutamatergic inputs with kainate-specific site with dendritic spines is enriched in somatodendritic serotonin. It was described in mammals that glutamate binds to kainate

receptors on OFF bipolar cell synapse and this is an important locus in temporal processing (DeVries 2000). Previous findings have demonstrated in turtle retina that kainic acid induces the serotonin release from OFF bipolar cells (Weiler and Schutte 1985). The expectation is that serotonin can regulate this flow into parallel processing pathways.

A previous paper showed that kainate injected intravitreously in the rabbit retina did not change serotonin uptake by GABA amacrine cells for 7 days (Osborne et al. 1995). The authors concluded that serotonin uptake was irreversibly damaged in the GABA/serotonin cells. In our hands, several neurotransmitter agonists had no effect on [^{3}H]5-HT release, except for glutamate ionotropic receptors activation (Table 1).

AMPA and the metabotropic agonist Trans-ACPD did not alter the serotonin uptake. The L-glutamate effect was blocked with CNQX, kainate/AMPA receptors antagonist. Since kainate, but not AMPA, shows the same effect of L-glutamate, the data suggest that serotonin transport modulation by L-glutamate occurs preferentially by kainate receptors stimulation. AMPA/kainate receptor antagonist NBQX and CNQX completely blocked the effect of kainate.

The release of serotonin induced by kainate is Ca^{2+} dependent (Fig. 3). Furthermore, the SERT inhibitor fluoxetine did not block serotonin release induced by kainate. Thus, our data show that kainate could release serotonin through a mechanism, which might involve exocytotic-like mechanism.

We observed that stimulation of retinal cells with NMDA or AMPA did not evoke serotonin release in the presence of Ca^{2+}; this effect was only evoked by glutamate or kainate. The response induced by kainate or glutamate was completely blocked by CNQX, a kainate/AMPA receptor antagonist. Thus, extracellular glutamate could lead to activation of kainate receptors that stimulates serotonin release in a Ca^{2+}-dependent manner, as shown in other species (Osborne and Patel 1984; Weiler and Schutte 1985). In the ex vivo experiments, the results show that kainate decreases serotonin immunoreactivity in both amacrine and bipolar cells, in addition to processes in the IPL. The kainate effect was totally blocked by DNQX, kainate/AMPA receptor antagonist, confirming that activation of kainate receptor induces the release of serotonin in all cell types. Kainate effect on serotonin release was a bit more potent in bipolar cells than amacrine. It has been demonstrated that chick retinal bipolar cells accumulate serotonin, but do not synthesize it (Wilhelm et al. 1993). Possibly, because of that serotonin-staining was weaker in bipolar cells compared to amacrine cells in control retinas. Since bipolar cells show fainter labeling for serotonin, it is possible that the effect of kainate, increasing the release of this neurotransmitter while blocking the uptake, would be more visible than that observed in amacrine cells. However, kainate also diminishes the immunolabeling in the amacrine cells as well as in the processes in the IPL, both ON and OFF sub-laminae. So, the results indicate that kainate induces serotonin release from both bipolar and amacrine cells.

Several novel functions have been attributed to kainate receptors in the retina (Huettner 2003). The high-affinity kainate receptor GluK5 is expressed in the outer plexiform layer in the mouse retina, almost exclusively localized to the presynaptic ribbon of photoreceptor terminals (Haumann et al. 2017). Indeed, macaque and mouse Off bipolar cells receive glutamatergic input primarily through kainate-type glutamate receptors (Gayet-Primo and Puthussery 2015). In summary, kainate receptors have an important role in the 5-HT uptake and release regulation in the avian retina.

Acknowledgments Grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia de Neurociência Translacional (INCT-INNT), Instituto Nacional de Ciência e Tecnologia de Neuroimodulação (INCT-NIM), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) supported this work. DPF thanks CAPES/Brazil for doctoral fellowships. JLMN and AMH thank CNPQ for the individual research fellowship. KCC and RAMR thank CNPq and FAPERJ for the individual research fellowship. We thank Granja Americano (Santa Izabel Alimento LTDA) for kindly providing Fertilized White Leghorn eggs (*Gallus gallus*) for our experiments.

Author Contributions Conceived and designed the experiments: LSS, HRF, KCC, RAMR, and JLMN. Performed the experiments: ACFP, SMAL, LSS, HRF, FAFR, and DPF. Analyzed the data: AMH, KRHMO, KCC, RAMR, and JLMN. Contributed reagents/materials/analysis tools: KCC, RAMR, and JLMN. Wrote the paper and financial support and administrative support: KCC, RAMR, and JLMN.

Funding This study was funded by CNPq (Grant number 552491/2011-0).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All experiments involving animals were approved and carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Federal University of Pará (permit number 85/15) and Federal University of Rio de Janeiro (permit number IBCCF-035).

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