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Research Article

Glutamate can act as a signaling molecule in mouse preimplantation embryos†

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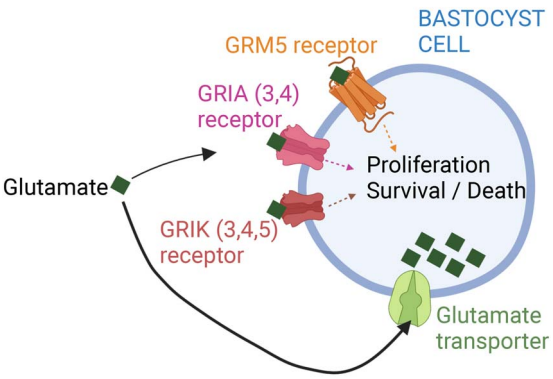
Abstract

Free amino acids are present in the natural environment of the preimplantation embryo, and their availability can influence early embryo development. Glutamic acid is one of the amino acids with the highest concentrations in female reproductive fluids, and we investigated whether glutamic acid/glutamate can affect preimplantation embryo development by acting through cell membrane receptors. Using reverse transcription-polymerase chain reaction, we detected 15 ionotropic glutamate receptor transcripts and 8 metabotropic glutamate receptor transcripts in mouse ovulated oocytes and/or in vivo developed blastocysts. Using immunohistochemistry, we detected the expression of two α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits, three kainate receptor subunits, and member 5 metabotropic glutamate receptor protein in blastocysts. Extracellular concentrations of glutamic acid starting at 5 mM impaired mouse blastocyst development, and this fact may be of great practical importance since glutamic acid and its salts (mainly monosodium glutamate) are widely used as food additives. Experiments with glutamate receptor agonists (in combination with gene expression analysis) revealed that specific AMPA receptors (formed from glutamate receptor, ionotropic, AMPA3 [GRIA3] and/or glutamate receptor, ionotropic, AMPA4 [GRIA4] subunits), kainate receptors (formed from glutamate receptor, ionotropic, kainate 3 [GRIK3] and glutamate receptor, ionotropic, kainate 4 [GRIK4] or glutamate receptor, ionotropic, kainate 5 [GRIK5] subunits), and member 5 metabotropic glutamate receptor (GRM5) were involved in this effect. The glutamic acid-induced effects were prevented or reduced by pretreatment of blastocysts with AMPA, kainate, and GRM5 receptor antagonists, further confirming the involvement of these receptor types. Our results show that glutamic acid can act as a signaling molecule in preimplantation embryos, exerting its effects through the activation of cell membrane receptors.

Summary Sentence
Several types of glutamate receptors are expressed in mouse oocytes and blastocysts, and extracellular concentrations of glutamic acid at 5 mM can inhibit blastocyst development by activating glutamate receptors.

Graphical Abstract



Keywords: blastocyst, oocyte, ionotropic glutamate receptors, metabotropic glutamate receptors, glutamic acid

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Introduction

Free amino acids are present in the natural environment of the preimplantation embryo, and several transport systems are used to deliver amino acids into embryonic cells [1–3]. Glutamic acid is one of the amino acids with the highest concentrations in female reproductive fluids, and its concentration has been shown to be in the range of 0.05–5.5 mM in the oviductal fluid of various mammalian species, including humans [4–11]. Glutamic acid (L-Glu) is a "nonessential" amino acid (classified according to nutritional requirements of several cell lines [12]) and exists in its ionic form under physiological conditions. Since glutamate salts (including monosodium glutamate, a widely used food additive) dissociate in aqueous solutions, the term glutamate can be used for both glutamate salts and glutamic acid [13].

In addition to their role as substrates for proteosynthesis, amino acids may have many other functions in cells of preimplantation embryos [1, 2]. Specifically, glutamic acid can serve as an energy source, an intermediate for transamination reactions, or as a precursor for the synthesis of several important molecules (such as glutathione, the main intracellular antioxidant [14–16]). Moreover, glutamic acid can act as a signaling molecule interacting with several types of cell membrane receptors in adult tissues. Glutamic acid is the main excitatory neurotransmitter in the central nervous system of mammals, and can also activate specific receptors in non-neural cell types [16, 17]. Glutamate-binding receptors are divided into two families: ionotropic (iGluR) and metabotropic (mGluR). The ionotropic receptor family includes the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptor subfamilies (so named according to selective synthetic agonists). Ionotropic glutamate receptors are ligand-gated ion channels composed of four subunits, and allow cation influx upon glutamate binding. Seven NMDA receptor subunits, four AMPA receptor subunits, and five kainate receptor subunits have been identified in mammals (see Figure 1). Metabotropic glutamate receptors are heptahelical membrane proteins that are further categorized into group I (with two members), group II (with two members), and group III (with four members, see Figure 2). Metabotropic glutamate receptors initiate signaling through interaction with guanosine-5′-triphosphate-binding proteins and activate various signaling cascades or cation influx. Glutamate signaling in the cell can be very complex because several types of glutamate receptors with interacting signaling pathways may be involved [18–21].

Several studies have demonstrated the beneficial effects of supplementing preimplantation embryo culture media with mixtures containing "nonessential" amino acids (including glutamic acid) at relatively low doses [1, 22]. It is generally believed that the effects of amino acids in the external environment on preimplantation embryo development are mediated by the transport of amino acids into embryonic cells [23]. In our study, we examined whether activation of cell membrane receptors may be involved in the effects of glutamate on preimplantation embryos. We firstly analyzed the expression of glutamate receptors in mouse ovulated oocytes and in vivo developed blastocysts, and then exposed blastocysts to natural and synthetic ligands of glutamate receptors. We analyzed the exposed embryos and, using specific agonist and antagonist molecules, identified receptor types involved in the observed cell responses.

Materials and methods

Animals, collection of in vivo developed oocytes, and blastocysts

All animal experiments were performed in accordance with the ethical principles under the supervision of the Ethics Committee for Animal Experimentation at the Institute of Animal Physiology and approved by the State Veterinary and Food Administration of the Slovak Republic in strict accordance with Slovak legislation based on the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes.

All experiments were performed with outbred ICR (CD-1 IGS) mice (Velaz, Prague, Czech Republic). The animals were housed in the animal facility at the Institute of Animal Physiology, Kosice, Slovakia (authorization No. SK UCH 01018) in plexiglass cages, kept under standard conditions (temperature 22 ± 2°C, humidity 65 ± 5%, 12:12-h light-dark cycle with lights on 06:00 a.m.) with free access to a standard pellet diet and water. Adult female mice (5–6 weeks old) were synchronized with eCG (pregnant mare's serum gonadotropin, 5 IU ip; Folligon, Intervet International, Boxmeer, Holland) and hCG (human chorionic gonadotropin, 4 IU ip; Pregnyl, Organon, Oss, Holland; 47 h later). Twelve to fourteen hours after hCG administration, the mice were killed by cervical dislocation, and unfertilized oocytes were isolated by flushing the oviduct using an in-house flushing-holding medium (FHM [24]) containing 1% bovine serum albumin (BSA; Sigma-Aldrich). To obtain preimplantation embryos at the blastocyst stage, females treated with eCG and hCG were mated with males of the same strain overnight. Successful mating was confirmed by the identification of a vaginal plug the next morning. Fertilized dams were killed by cervical dislocation and subjected to embryo isolation by flushing the uterus using the FHM with BSA at 96 h post-hCG and to morphological classification using stereomicroscopy (Nikon SMZ 745T, Nikon, Tokyo, Japan). Oocytes and blastocysts were washed in several drops of FHM with BSA and pooled. Cumulus cells were removed with 0.1% hyaluronidase (Sevac, Prague, Czech Republic).

Reverse transcription-polymerase chain reaction and transcript relative quantification

Total ribonucleic acid (RNA) was extracted from batches of 590–610 unfertilized mouse oocytes or blastocysts (the number of oocytes/blastocysts in each pool was exactly determined), and from mouse brain (positive tissue control). TRIzol Reagent (Invitrogen Life Technologies, Karlsruhe, Germany) was used for the extraction (according to the manufacturer's instructions). Complementary DNA was synthesized (after the genomic DNA elimination step) using the RT2 First Strand Kit (Qiagen, Valencia, CA). For both oocytes and blastocysts, three independent RNA isolates were used to prepare cDNA samples. To check for the presence of genomic DNA contamination in the RNA preparations, reverse transcriptase (RT) negative controls (no RT in the cDNA synthesis reaction) were carried out in parallel using half of each RNA sample (thus two cDNA preparations, "RT+" and "RT−," were prepared from each RNA sample). The cDNA preparations were diluted in an appropriate amount of 10 mM Tris (pH 8.3) so that 1 μl of the cDNA corresponded theoretically to 2.5 embryo/oocyte equivalents.

IONOTROPIC GLUTAMATE RECEPTORS

Receptor subunits		Amplicon size (bp)	Fold regulation (blastoc./ooc.)	P value
AMPA receptors				
Gria1	(GluR1, GluRA)	129		
Gria2	(GluR2, GluRB)	180		
Gria3	(GluR3, GluRC)	135		
Gria4	(GluR4, GluRD)	67	-241.1	0.00046
Kainate receptors				
Grik1	(GluR5)	53		
Grik2	(GluR6)	130		
Grik3*	(GluR7)	90	+38.94	0.0070
Grik4	(KA1)	168	-3.69	0.066
Grik5	(KA2)	161	-1.48	0.95
NMDA receptors				
Grin1	(NMDAR1, NR1)	91		
Grin2a	(NMDAR2A, NR2A)	57		
Grin2b	(NMDAR2B, NR2B)	156		
Grin2c	(NMDAR2C, NR2C)	87	+2.46	0.19
Grin2d*	(NMDAR2D, NR2D)	163	-1.07	0.80
Grin3a*	(NR3A)	146	+5.84	0.25
Grin3b*	(NR3B)	178	+24.48	0.0041

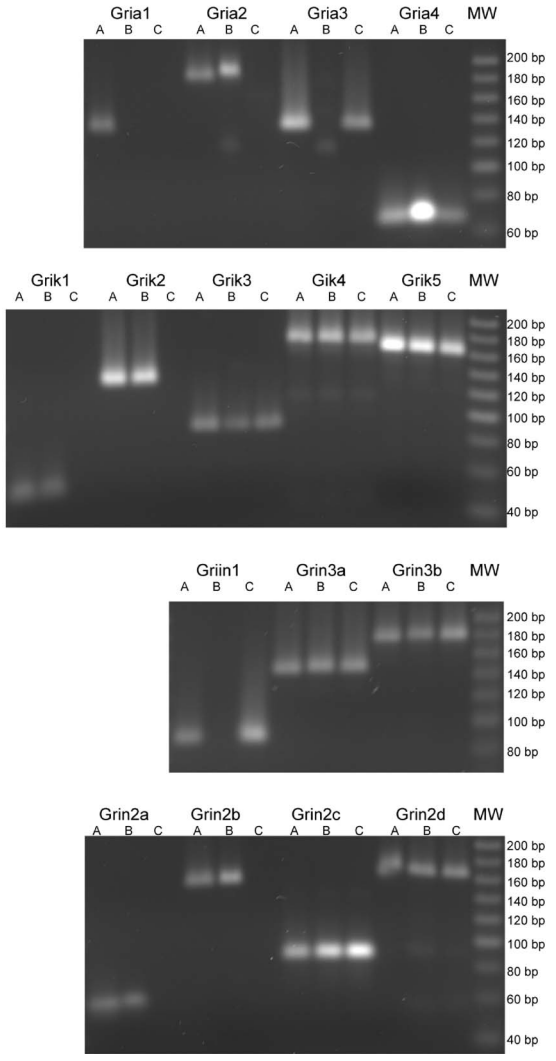

Figure 1. Transcripts encoding ionotropic glutamate receptors are expressed in mouse blastocysts and oocytes. Subunits of ionotropic glutamate receptors are listed in the table on the left. The most frequently used common names of subunits are given in parentheses. Mouse gene symbols are used (human gene symbols are the same but written with all letters capitalized, e.g., *GRIA1*). In transcripts which were consistently expressed in both blastocysts and oocytes, fold regulation values ("+" means upregulation and "-" means downregulation in blastocysts compared to oocytes) and corresponding P-values are shown. Transcripts were detected by reverse transcription-polymerase chain reaction (RT-PCR) and representative agarose gels with separated PCR products are shown in the panels on the right. Lanes: *Gria1*, Glutamate receptor, ionotropic, AMPA1; *Gria2*, Glutamate receptor, ionotropic, AMPA2; *Gria3*, Glutamate receptor, ionotropic, AMPA3; *Gria4*, Glutamate receptor, ionotropic, AMPA4; *Grik1*, Glutamate receptor, ionotropic, kainate 1; *Grik2*, Glutamate receptor, ionotropic, kainate 2; *Grik3*, Glutamate receptor, ionotropic, kainate 3; *Grik4*, Glutamate receptor, ionotropic, kainate 4; *Grik5*, Glutamate receptor, ionotropic, kainate 5; *Grin1*, Glutamate receptor, ionotropic, NMDA1; *Grin3a*, Glutamate receptor, ionotropic, NMDA3A; *Grin3b*, Glutamate receptor, ionotropic, NMDA3B; *Grin2a*, Glutamate receptor, ionotropic, NMDA2A; *Grin2b*, Glutamate receptor, ionotropic, NMDA2B; *Grin2c*, Glutamate receptor, ionotropic, NMDA2C; *Grin2d*, Glutamate receptor, ionotropic, NMDA2D; MW, molecular weight markers; (A) positive control tissue; (B) ovulated oocytes; (C) blastocysts. The MWs in base pairs (bp) are indicated to the right of the panels. *Primers for *Grik3*, *Grin2d*, *Grin3a, and Grin3b* subunits were not included in the Mouse GABA & Glutamate RT2 profiler PCR array, and commercial primer sets from Qiagen were used (see Materials and methods). Delta receptors (formed from delta 1 and delta 2 subunits), classified by sequence homology as ionotropic glutamate receptors, do not bind glutamate, and were not investigated in this study.

Polymerase chain reaction (PCR) analysis of glutamate receptor transcripts was performed using the Mouse GABA & Glutamate RT2 profiler PCR array (Qiagen, Cat. No. PAMM-152ZF) containing oligonucleotide primers for amplification of 20 glutamate receptor subunits/types. Four glutamate receptor subunits that were not included in the PCR array were analyzed in separate PCR reactions using commercial primer sets from Qiagen (product numbers: PPM04259A, PPM04892A, PPM34762E, and PPM34342A, respectively). PCR amplifications were performed in a Light Cycler 480 real-time PCR system (Roche Diagnostics, Rotkreuz, Switzerland). The reactions were carried out in 25 μl volumes containing 1 μl of the cDNA (corresponding theoretically to 2.5 embryo/oocyte equivalents) and SYBR

METABOTROPIC GLUTAMATE RECEPTORS

Receptor types	Amplicon size (bp)	Fold regulation (blastoc./ooc.)	P value
Group I receptors			
Grm1	107		
Grm5*	124		
Group II receptors			
Grm2	95	-333.9	0.00037
Grm3	113	-4.46	0.015
Group III receptors			
Grm4	102	-45.15	0.000001
Grm6	145		
Grm7	99		
Grm8	191	-33.2	0.00048

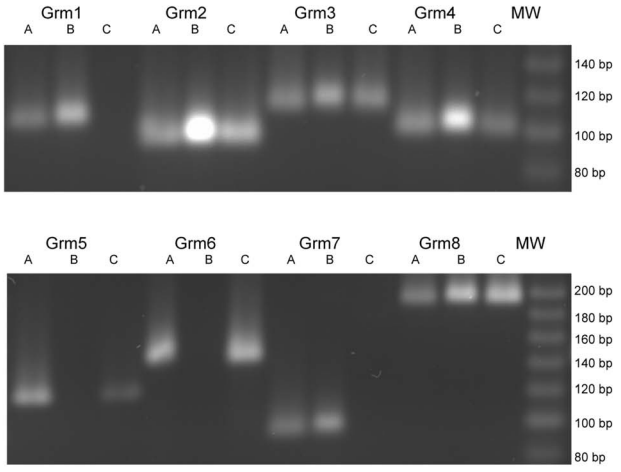

Figure 2. Transcripts encoding metabotropic glutamate receptors are expressed in mouse blastocysts and oocytes. Metabotropic glutamate receptor types are listed in the tables on the left. In transcripts which were consistently expressed in both blastocysts and oocytes, fold regulation values ("+" means upregulation and "−" means downregulation in blastocysts compared to oocytes) and corresponding *P*-values are shown. Transcripts were detected by RT-PCR and representative agarose gels with separated PCR products are shown in the panels on the right. Lanes: *Grm1*, Glutamate receptor, metabotropic 1; *Grm2*, Glutamate receptor, metabotropic 2; *Grm3*, Glutamate receptor, metabotropic 3; *Grm4*, Glutamate receptor, metabotropic 4; *Grm5*, Glutamate receptor, metabotropic 5; *Grm6*, Glutamate receptor, metabotropic 6; *Grm7*, Glutamate receptor, metabotropic 7; *Grm8*, Glutamate receptor, metabotropic 8; MW, molecular weight markers; A, positive control tissue; B, ovulated oocytes; C, blastocysts. The MWs in base pairs (bp) are indicated to the right of the panels. *Primers for *Grm5* receptor type were designed in this study.

Green qPCR mastermix (Qiagen). An initial step at 95°C for 10 min was followed by 45 cycles at 95°C for 15 s and 60°C for 60 s. Amplification specificity was assessed with melting curve analysis and agarose gel electrophoresis (see later). The experiment was performed thrice and the results were analyzed by comparative ΔΔCt method using the web-based data analysis software provided by the PCR array manufacturer (Qiagen; software available at https://dataanalysis2.qiagen.com/pcr). The fold change in gene expression (transcript up- or downregulation) in blastocysts compared with oocytes was calculated. Since the primers for the *Grm5* receptor type included in the PCR array did not work consistently, we designed our own primers. Amplification reactions contained 0.5 μM of each Grm5 primer (5′- TTCTTTCCTTCCCTGGTCCCTC-3′ and 5′-ACACAACACTCACTACCCGTTT-3′), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM MgCl2, 0.2 mM dNTPs, and 0.02 U/ml platinum Taq DNA polymerase (Invitrogen Life Technologies).

PCR products were analyzed using electrophoresis on 3% agarose gels stained with GelGreen (Biotium, Hayward, CA, USA). A 20 bp DNA ladder (Jena Bioscience, Jena, Germany) was used as marker. PCR products were visualized with a Fusion FX7 imaging system (Vilber Lourmat, France), and the size of DNA bands (PCR products) was determined with Bio1D analysis software (Vilber Lourmat).

Immunostaining

The zona pellucida of the blastocysts was removed with 0.5% pronase in FHM at 37°C. Zona-free embryos were fixed in 4% paraformaldehyde. Free aldehyde groups were blocked with 0.3 M glycine (Merck, Darmstadt, Germany), and embryos were permeabilized in phosphate-buffered saline (PBS)/BSA/SAP (PBS containing BSA and 0.5% saponin; Sigma-Aldrich, Munich, Germany). Tris buffer (9.0 pH) was used for antigen retrieval. Nonspecific immunoreactions were blocked with blocking buffer (0.05% saponin in PBS containing 10% normal goat serum (Santa Cruz Biotechnology), 0.3 M glycine (Merck, Darmstadt, Germany), and 1% BSA (Sigma-Aldrich, Munich, Germany). Embryos were incubated with primary rabbit polyclonal antibodies against selected glutamate receptors in the blocking buffer at 4°C overnight. A secondary antibody coupled with Alexa Fluor 488 (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen Life Technologies) was used to visualize the primary antibody. Cell nuclei were stained with Hoechst 33342 in PBS/BSA (10 μg/ml; Sigma-Aldrich, Germany). Afterward, embryos were mounted in Vectashield antifade reagent (Vector Laboratories, Burlingame, CA) on glass slides, sealed with coverslips, and observed using a confocal microscope (Leica TCS SPE, Leica, Wetzlar, Germany). Negative control groups of oocytes and embryos were incubated without the primary antibody or without the secondary antibody, or with rabbit gamma globulin (Rabbit Gamma globulin Control, Invitrogen, Cat# 31887).

Primary antibodies used in the study

Anti-mGluR5 Antibody (Alomone Labs, Cat# AGC-007, dilution 1:50), Anti-GRIK3 (GluK3) Antibody (Alomone Labs, Cat# AGC-040, dilution 1:100), Anti-GRIK4 (KA1) Antibody (Alomone Labs, Cat# AGC-041, dilution 1:50), Anti-GRIK5 (GluK5) Antibody (Alomone Labs, Cat# AGC-042, dilution 1:100), GRIA3 Antibody (LifeSpan Biosciences, Cat# LS-C331307, dilution 1:50), and Anti-GluR4 (GluA4) Antibody (Alomone Labs, Cat# AGC-019, dilution 1:50).

Embryo culture and morphological evaluation

Mouse embryos at the blastocyst stage were randomly divided into several subgroups and cultured in vitro under standard

conditions (humidified atmosphere with 5% CO_2 and 37°C) in 400 μl of EmbryoMax KSOM powdered mouse embryo medium (Millipore, UK, Cat# MR-020P) for 24 h with/without the presence of

1. L-Glutamic acid (Sigma-Aldrich) at 10 mM, 5 mM, and 2 mM concentrations
2. Specific ionotropic glutamate receptor agonists: NMDA (N-Methyl-D-aspartic acid, ab120052; Abcam UK) at 5 mM concentration, AMPA [(S)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ab120005, Abcam UK] at 5 mM and 0.3 mM concentrations and kainic acid (KA) [(2S,3S,4S)-3-(carboxymethyl)-4-(prop-1-en-2-yl)pyrrolidine-2-carboxylic acid; ab120100; Abcam UK] at 5 mM, 2 mM, 1 mM, 0.5 mM, and 0.3 mM concentrations
3. Specific metabotropic glutamate receptor (mGluR) agonists: (S)-3,5-DHPG [(S)-3,5-Dihydroxyphenylglycine, Group I mGluR agonist; ab120007, Abcam UK] at 5 mM and 0.1 mM concentrations, LY 379268 [(1R,4R,5S,6R)-4-amino-2-oxabicyclo(3.1.0)hexane-4,6-dicarboxylic acid, Group II mGluR agonist; ab120196, Abcam UK] at 5 mM concentration and L-AP4 [L-(+)-2-Amino-4-phosphonobutyric acid, Group III mGluR agonist; ab120002, Abcam UK] at 5 mM concentration
4. A mixture of AMPA and kainate receptor antagonist CNQX (disodium salt; 1,2,3,4-tetrahydro-7-nitro-2,3-dioxoquinoxaline-6-carbonitrile disodium; ab120044, Abcam UK) and GRM5 receptor antagonist MPEP [hydrochloride; 2-Methyl-6-(phenylethynyl)pyridine hydrochloride; ab120008, Abcam UK] at 0.3 mM and 0.01 mM final concentrations, respectively; blastocysts were incubated in this antagonist mixture for 20 min prior to the addition of L-glutamic acid (to 5 mM final concentration).

All compounds used in this study were dissolved to the required concentration in EmbryoMax KSOM culture medium. The control subgroup of blastocysts was cultured in EmbryoMax KSOM culture medium alone.

After 24 h of incubation, embryos were fixed in 4% paraformaldehyde (Merck) and permeabilized with 0.5% Triton X-100 (Sigma Aldrich). Blastocysts were then incubated with TUNEL assay reagents (terminal deoxynucleotidyl transferase dUTP nick end labeling) using the DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, USA) for 1 h at 37°C in the dark, to evaluate cell death incidence. To distinguish between trophectoderm (TE) and inner cell mass (ICM) cell lineages, CDX2 (caudal type homeobox 2) staining was performed. Nonspecific immunoreactions were blocked using 10% normal goat serum (Santa Cruz Biotechnology, USA) for 2 h at room temperature. After blocking, the mouse blastocysts were incubated with primary antibody (rabbit anti-mouse CDX2 polyclonal antibody; Cell Signaling Technology, Danvers, MA, USA) diluted in blocking solution at 4°C overnight. The next day, the blastocysts were washed in PBS/BSA and incubated with a secondary antibody (Cy 3-conjugated goat anti-rabbit IgG, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Finally, to evaluate the total number of nuclei and the nuclear morphology, the blastocysts were counterstained with Hoechst 33342 DNA staining (Sigma Aldrich) for 5 min at room temperature, mounted on glass slides using Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed at magnification X400 using a fluorescence microscope (BX51; Olympus, Tokyo, Japan). Microphotographs of 3–5 optical sections of each blastocyst (depending on embryo size) were obtained using a CCD camera (DP72; Olympus) and respective software (QuickPHOTO MICRO 2.3). The total number of blastomeres in the blastocyst was counted manually using ImageJ 1.23y software (National Institutes of Health, USA) upgraded with the Point Picker plugin allowing to pick, stack, and save nuclei located at specific coordinates in an image.

According to the nuclear morphology and the presence of specific DNA fragmentation in the nucleoplasm, embryonic cells were classified as normal (without morphological changes in nuclei, without TUNEL labeling) or dead (showing at least one of the following features: fragmented or condensed nucleus, positive TUNEL labeling). In each blastocyst, the percentage of dead cells was calculated as the number of dead cells relative to the total number of blastomeres in the blastocyst.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance followed by the Tukey post hoc test (analysis of 3–5 groups) and unpaired Student *t*-test (analysis of 2 groups) were used to compare the blastocyst cell number and the proportion of dead cells in the blastocysts. Differences with $P < 0.05$ were considered significant.

Results

Transcripts encoding glutamate receptors are expressed in mouse blastocysts and oocytes

We detected 15 ionotropic glutamate receptor transcripts and 8 metabotropic glutamate receptor transcripts in mouse ovulated oocytes and/or in vivo developed blastocysts (Figures 1 and 2). *Gria2*, *Grik2, Grin2a*, *Grin2b*, *Grm1, and Grm7* transcripts were detected in oocytes but not in blastocysts. In contrast, *Gria3*, *Grin1*, *Grm5, and Grm6* transcripts were detected in blastocysts but not in oocytes. *Grik1* transcript was detected in all three oocyte samples but only in one blastocyst sample. Other transcripts (*Gria4*, *Grik3*, *Grik4*, *Grik5*, *Grin2c*, *Grin2d*, *Grin3a*, *Grin3b*, *Grm2*, *Grm3*, *Grm4, and Grm8*) were consistently detected in both oocytes and blastocysts, and *Gria4*, *Grm2*, *Grm4, and Grm8* transcripts were detected in much larger quantities in oocytes than in blastocysts (241-, 334-, 45-, and 33-fold differences were found, respectively). In contrast, the amount of *Grik3* transcript was about 39 times higher in blastocysts than in oocytes. No specific PCR products were detected in blank reactions. RT-control reactions for three receptors (*Grik5* and *Grm4* in oocytes and *Grm6* in blastocysts) produced specific PCR products, however, their amounts were minimal compared to the corresponding RT+ reactions. No specific PCR products were detected in other RT-control reactions (data not shown).

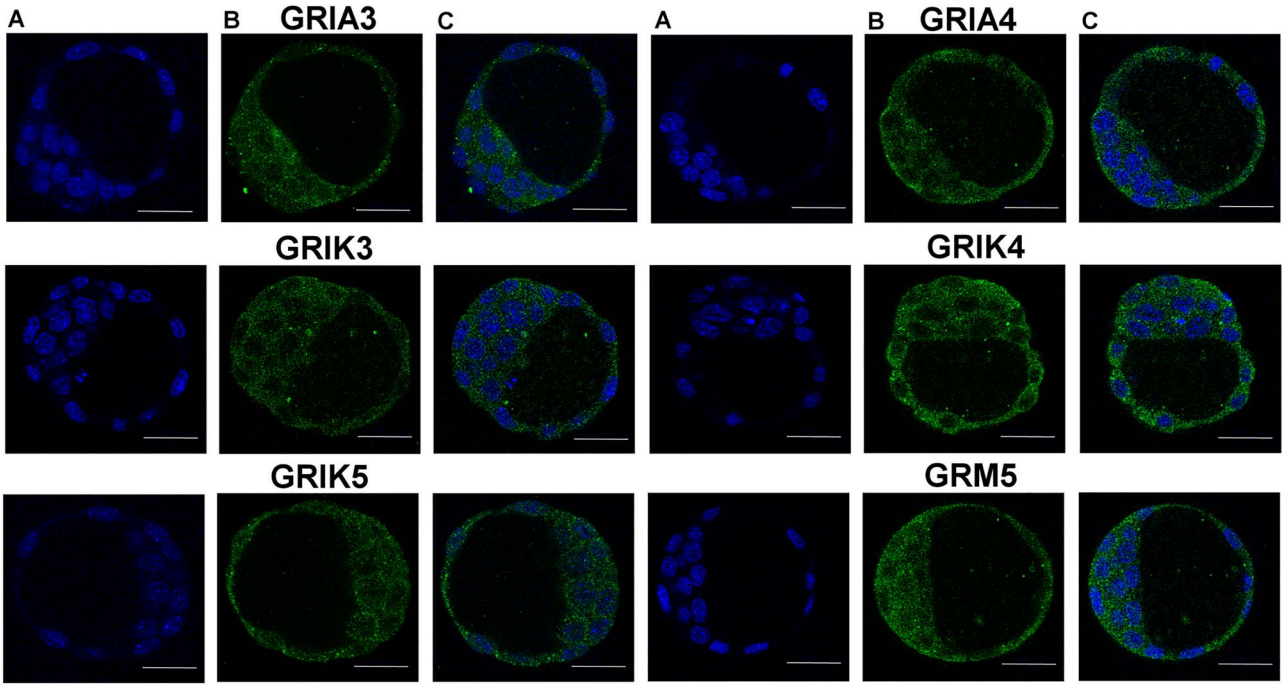

Figure 3. Glutamate receptor proteins are expressed in mouse blastocysts. Glutamate receptor subunits/types were detected by immunofluorescence. Representative images are shown. Optical sections were observed via CLSM. Cell nuclei were stained with Hoechst 33342 (blue staining, A columns). Embryos were incubated with primary antibodies against the glutamate receptor subunits/types and with a secondary antibody labeled with Alexa Fluor 488 (green staining, B columns); C columns, merged images. For negative controls, see Supplementary Figure S1. Scale bars, 10 μm.

Glutamate receptor proteins are expressed in mouse blastocysts

We examined the expression of GRIA3, GRIA4, GRIK3, GRIK4, GRIK5, and GRM5 proteins in mouse blastocysts using specific primary antibodies and fluorescently labeled secondary antibodies. The selection of proteins for immunohistochemical analysis was made on the basis of two criteria: (1) proteins had to belong to the receptor types whose agonists produced an effect in our receptor functional studies (see later) and (2) the corresponding transcripts had to be detected in blastocysts. For AMPA receptor subunits, GRIA4 protein was detected in similar amounts in TE and ICM cells but the signal for GRIA3 protein was slightly stronger in ICM than in TE cells. For kainate receptor subunits, all three examined proteins (GRIK3, GRIK4, and GRIK5) were detected in both TE and ICM cells. GRM5 protein was detected in both blastocyst cell lineages (Figure 3). The fluorescence signal was in some cases (e.g., GRIK5, GRIA3, and GRIA4) strongest at the cell periphery, suggesting localization of receptors in the cell membrane. The specificity of the signal was confirmed using several negative controls. The intensity of the immunostaining signal was significantly reduced in controls incubated with rabbit gamma globulin (instead of the primary antibody) and in controls incubated without the primary antibody or without the primary and the secondary antibody (see Supplementary Figure S1).

Glutamate can impair blastocyst development

To identify which glutamate receptor types are functional in mouse blastocysts, we stimulated the embryos with increasing concentrations of natural ligand (L-glutamic acid) and with receptor type-specific synthetic ligands in our functional studies. In the first experiment, mouse blastocysts were cultured in a medium supplemented with L-glutamic acid at 2 mM, 5 mM, and 10 mM concentrations. Blastocysts were cultured in this medium for only 24 h to reduce the accumulation of ammonium in the culture medium. No significant effects were found in blastocysts treated with 2 mM glutamate. Higher glutamate concentrations significantly decreased cell numbers in blastocysts, and both ICM and TE cells were affected (the reduction was more pronounced in ICM cells than in TE cells: the mean cell number was about 25% lower in ICM cells and about 10% lower in TE cells in glutamate-treated blastocysts in comparison with control blastocysts). Blastocysts exposed to 5 mM and 10 mM glutamate showed significantly higher proportions of dead cells than control blastocysts (Figure 4).

Specific ionotropic glutamate receptor agonists interfere with blastocyst development

Since 5 mM L-glutamic acid produced significant effects on blastocysts, we used a 5 mM concentration of specific agonists in the first set of experiments. Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA) significantly decreased cell numbers in blastocysts. A separate examination of TE and ICM cell lineages showed that both agonists influenced mainly TE cells. Analysis of dead cell incidence showed that blastocysts exposed to 5 mM AMPA or KA had significantly higher proportions of dead cells than control blastocysts. No significant effects were found in blastocysts treated with 5 mM NMDA (Figure 5A).

To verify that lower agonist concentrations were effective, we used AMPA at 300 μM and KA at 1 mM final concentrations in the second set of experiments (these concentrations were chosen according to the information in published experiments [25–28]). A relatively high concentration of KA had

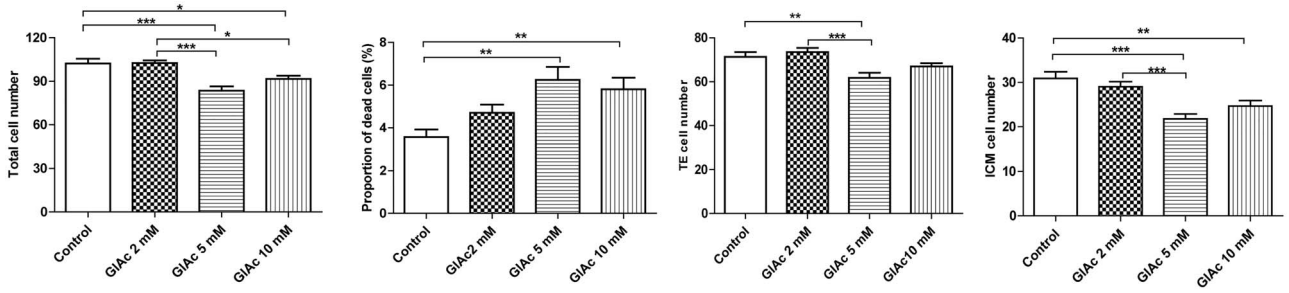

Figure 4. Glutamic acid can impair blastocyst development. Cell numbers and proportions of dead cells in blastocysts after incubation with L-glutamic acid. The blastocysts were incubated in the presence of the indicated concentrations of L-glutamic acid (GIAc) for 24 h. TE, trophectoderm, ICM, inner cell mass. Numbers of blastocysts in the groups (*n*): Control, $n = 44$; GIAc 2 mM, $n = 51$; GIAc 5 mM, $n = 48$; GIAc 10 mM, $n = 47$. The values are arithmetical mean + SEM. Statistical significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

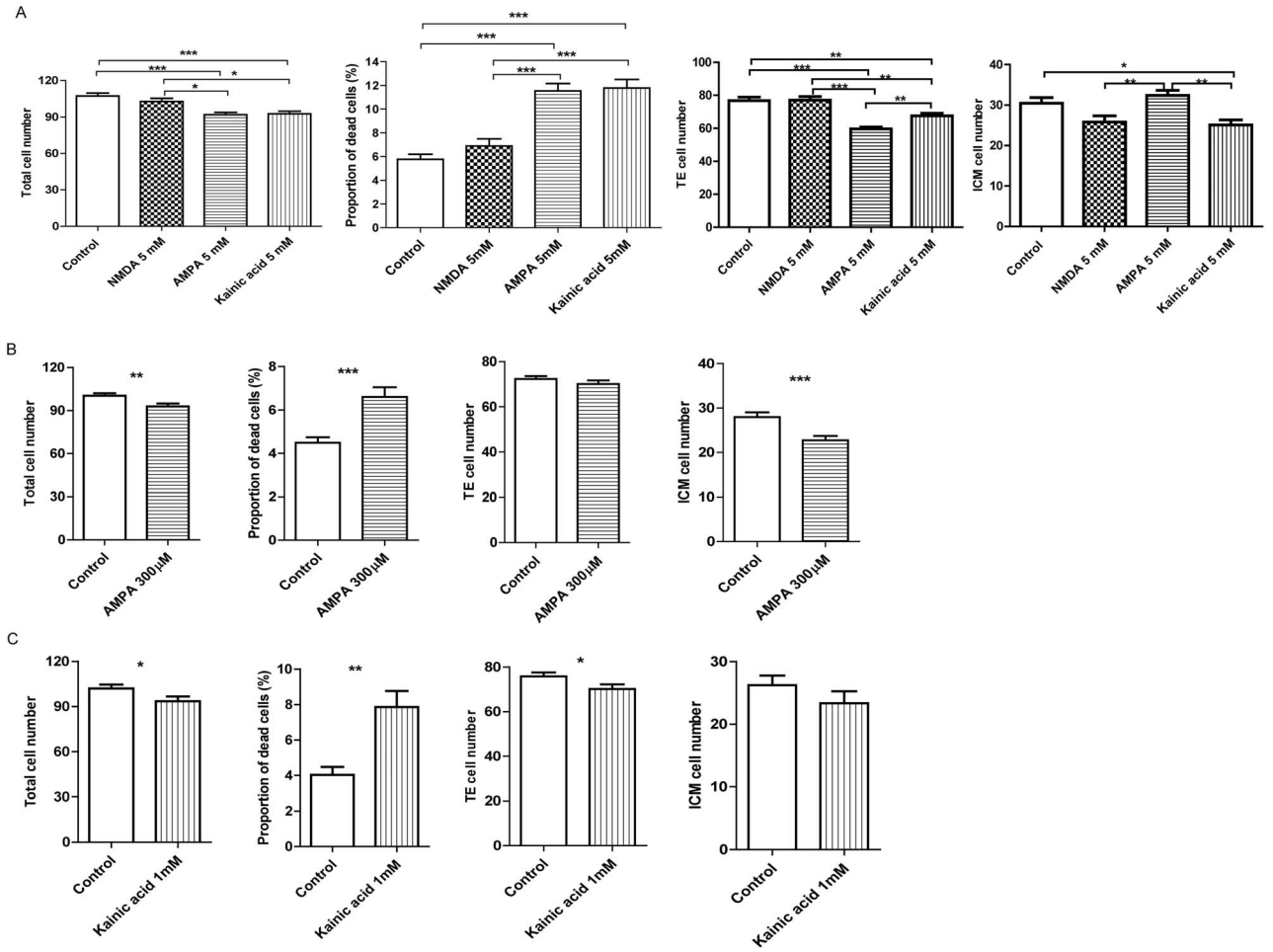

Figure 5. Specific ionotropic glutamate receptor agonists interfere with blastocyst development. (A) Cell numbers and proportions of dead cells in blastocysts after incubation with agonists of ionotropic glutamate receptors. The blastocysts were incubated in the presence of 5 mM NMDA, 5 mM AMPA, and 5 mM kainic acid for 24 h. Numbers of blastocysts in the groups (*n*): Control, $n = 61$; NMDA, $n = 35$; AMPA, $n = 78$; kainic acid, $n = 45$. (B, C) Cell numbers and proportions of dead cells in blastocysts after incubation with lowered concentrations of AMPA (B) and kainic acid (C). The blastocysts were incubated in the presence of 300 μM AMPA and 1 mM kainic acid for 24 h. Numbers of blastocysts in the groups (*n*): Control (for AMPA group), $n = 59$; AMPA, $n = 63$; control (for kainic acid group), $n = 32$; kainic acid, $n = 23$. TE, trophectoderm, ICM, inner cell mass. The values are arithmetical mean + SEM. Statistical significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

to be used since concentrations below 1 mM were not effective (see Supplementary Figure S2). Treatment with 300 μM AMPA and 1 mM KA significantly decreased cell numbers and increased proportions of dead cells (Figures 5B and C) in blastocysts. A comparison of the effect of 5 mM and 300 μM AMPA showed that 5 mM AMPA only affected TE cells, whereas 300 μM AMPA was effective mainly in ICM cells (see Figure 5 and Supplementary Figure S3).

Group I metabotropic glutamate receptor agonist interferes with blastocyst development

Mouse blastocysts were cultured for 24 h in a medium supplemented with specific metabotropic glutamate receptor agonists in 5 mM concentrations. (S)-3,5-DHPG (group I agonist), LY 379268 (group II agonist), and L-AP4 (group III agonist) were used. Treatment with 5 mM (S)-3,5-DHPG (group I agonist) induced strong blastocyst damage, with

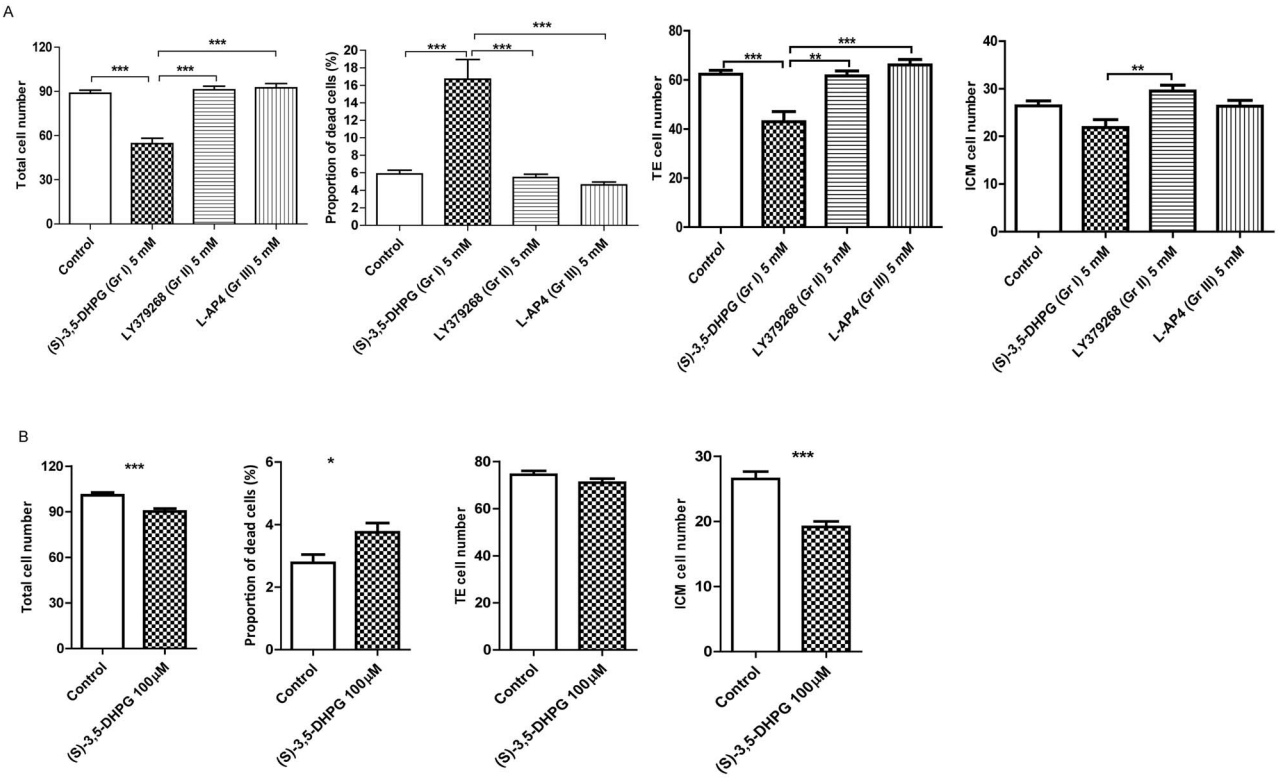

Figure 6. Group I metabotropic glutamate receptor agonist interferes with blastocyst development. (A) Cell numbers and proportions of dead cells in blastocysts after incubation with agonists of metabotropic glutamate receptors. The blastocysts were incubated in the presence of 5 mM (S)-3,5-DHPG (group I agonist, "Gr I"), 5 mM LY 379268 (group II agonist, "GR II"), and 5 mM L-AP4 (group III agonist, "Gr III") for 24 h. Numbers of blastocysts in the groups (*n*): Control, *n* = 65; (S)-3,5-DHPG, *n* = 19; LY 379268, *n* = 39; L-AP4, *n* = 47. (B) Cell numbers and proportions of dead cells in blastocysts after incubation with lowered concentration of (S)-3,5-DHPG (metabotropic group I agonist). The blastocysts were incubated in the presence of 100 μM (S)-3,5-DHPG for 24 h. Number of blastocysts in the groups (*n*): Control, *n* = 37; (S)-3,5-DHPG, *n* = 41. TE, trophectoderm, ICM, inner cell mass. The values are arithmetical mean + SEM. Statistical significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

81% of the embryos showing collapsed blastocoele cavity and massive cellular shrinkage. In the remaining blastocysts, we found significantly lower cell numbers and significantly higher proportions of dead cells than in control blastocysts (Figure 6A). We found no significant changes in blastocysts treated with 5 mM LY 379268 (group II agonist) and L-AP4 (group III agonist; Figure 6A).

To verify the effects of (S)-3,5-DHPG agonist on blastocysts at a lower concentration, we used 100μM (S)-3,5-DHPG in the following experiment (this concentration was chosen according to the information in published experiments [29–31]). No severe damage to blastocysts was found after treatment with the lower (S)-3,5-DHPG dose. We found significantly lower cell numbers and higher proportions of dead cells in blastocysts treated with 100 μM (S)-3,5-DHPG than in control blastocysts (Figure 6B). Comparison of the effect of 5 mM and 100 μM (S)-3,5-DHPG showed that 5 mM (S)-3,5-DHPG affected both cell lineages, while 100 μM (S)-3,5-DHPG was effective mainly in ICM cells (see Figure 6 and Supplementary Figure S3).

Glutamate effects are blocked with AMPA/kainate and GRM5 receptor antagonists

In the final experiment, mouse blastocysts were cultured for 24 h in a medium supplemented with L-glutamic acid (at 5 mM final concentration) and compared with blastocysts incubated in the presence of AMPA/kainate and GRM5 receptor antagonists (mix of CNQX and MPEP at 300 μM and 10 μM final concentrations, respectively) prior to L-glutamic acid exposure. Glutamic acid decreased cell numbers in blastocysts (both ICM and TE cells were affected) and this effect was blocked by 20 min of blastocyst pretreatment with AMPA/kainate and GRM5 receptor antagonists. Similarly, the increased incidence of cell death induced by glutamic acid was blocked by the antagonists (Figure 7).

Discussion

We examined messenger RNA (mRNA) for all 16 subunits of ionotropic glutamate binding receptors and 8 types of metabotropic glutamate binding receptors in mouse blastocysts and ovulated oocytes and found several expression profiles. Expression in oocytes but not in blastocysts was found in six glutamate receptor transcripts, indicating that these maternal transcripts are degraded during preimplantation development. In contrast, four transcripts were found in blastocysts but not in oocytes, indicating that transcription of these genes begins after embryonic genome activation. Several glutamate receptor transcripts were detected in both oocytes and blastocysts, and the expression levels in oocytes and blastocysts differed significantly in some transcripts. The expression of glutamate receptors has not been systematically studied in preimplantation embryos, although partial information is available from studies using high-throughput genomics techniques (such as DNA

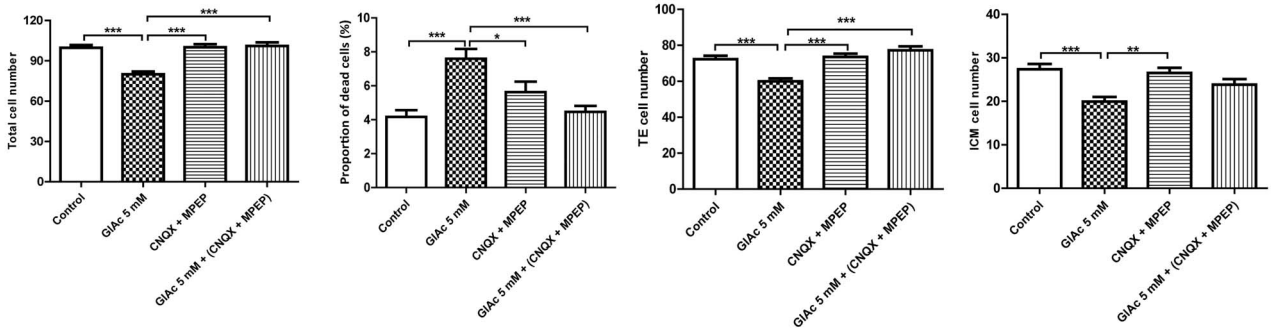

Figure 7. Glutamate effects are blocked with AMPA/kainate and GRM5 receptor antagonists. Cell numbers and proportions of dead cells in blastocysts pretreated with the mixture of AMPA, kainate, and GRM5 receptor antagonists (CNQX and MPEP) prior to L-glutamic acid exposure. GlAc 5 mM, blastocysts incubated with L-glutamic acid (at 5 mM final concentration) for 24 h; CNQX + MPEP, blastocysts incubated with CNQX and MPEP (at 300 μM and 10 μM final concentrations, respectively) for 24 h; GlAc 5 mM + (CNQX + MPEP), blastocysts incubated with CNQX + MPEP antagonists for 20 min prior to addition of L-glutamic acid (for the following 24 h incubation). TE, trophectoderm, ICM, inner cell mass. The number of blastocysts in the groups (*n*): Control, $n = 32$; GlAc 5 mM, $n = 33$; CNQX + MPEP, $n = 28$; GlAc 5 mM + (CNQX + MPEP), $n = 34$. The values are arithmetical mean + SEM. Statistical significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

microarrays and massively parallel sequencing). For instance, He et al. [32] compared gene expression in mouse and human preimplantation embryos and detected two NMDA receptor transcripts (*Grin1 and Grin2c*) in mouse embryos and one AMPA (*GRIA1*), two NMDA (*GRIN2B and GRIN3A*), and several kainate (*GRIK 1,2,4,5*) and metabotropic receptor transcripts (*GRM 2,5,6,8*) in human embryos. One AMPA (*Gria2*), two kainates (*Grik 1,2*), and three metabotropic (*Grm 3,5,7*) receptor transcripts were shown to be among the genes, which were differentially expressed between in vivo developed and intracytoplasmic sperm injection-generated mouse blastocysts [33]. One AMPA (*GRIA1*), one kainate (*GRIK4*), two NMDA (*GRIN3A, GRIN2D*), and several metabotropic (*GRM 2,6,7,8*) receptor transcripts were shown to be downregulated in blastocysts derived from oocytes obtained from young donors (women below 30 years of age) as compared with older donors (women above 42 years of age [34]). A comparison of our results with the results in the earlier discussed studies shows that we found several transcripts in mouse blastocysts (*Gria 3,4, Grik 3,4,5, Grin 2d, 3a, 3b, Grm 2,4,6,8*), which were not detected in those studies. However, we did not find the three transcripts (*Gria2, Grik2, and Grm7*) that were found in mouse blastocysts in the study by Giritharan et al. [33]. The discrepancy between these results may have been due to the different methodologies used.

Our results indicate that multiple glutamate receptor transcripts are translated into proteins in mouse blastocysts. We detected two AMPA receptor subunits (GRIA3 and GRIA4), and GRIA3 protein seemed to be more abundantly expressed than GRIA4 protein. Proteins of three kainate receptor subunits (GRIK3, GRIK4, and GRIK5) were detected in blastocysts, and our comparison of mRNA relative amounts between oocytes and blastocyst suggests that GRIK3 expression increases after oocyte fertilization. In accordance with the detection of *Grm5* (member 5 metabotropic glutamate receptor) transcript in blastocysts, we also detected the GRM5 protein in blastocyst cells.

Numerous studies have investigated the importance of amino acids in the mammalian preimplantation embryo environment. In general, the addition of amino acids to the culture medium improves early embryo development, although different effects of "essential" and "nonessential" amino acids added at different developmental stages have been found [22, 35, 36]. Glutamic acid has usually been added as part of "nonessential" amino acid mixtures (in final concentrations of individual amino acids 0.05–0.1 mM), and these culture medium supplements have had a stimulatory effect in the cleavage stage as well as on post-compaction mouse and human embryos [37–41]. Few researchers have examined the effects of individually added glutamic acid on preimplantation embryo development. In an early study, Brinster [42] added 21 amino acids individually at 1 mM and 8 mM concentrations to the culture medium of mouse 2-cell embryos and reported a slight positive effect ("some cleavage" as assessed by the author using very simple morphological analysis) of most amino acids, including glutamic acid. No effect of glutamic acid added to the culture medium at 0.05 and 0.5 mM concentrations was found in hamster preimplantation embryos [43]. The addition of 0.4 mM glutamic acid to the pig oocyte maturing medium led to an increase in male pronuclear formation after in vitro fertilization, but no effect on subsequent embryo cleavage and blastocyst formation was observed [44].

We examined the effects of L-glutamic acid on mouse blastocysts in vitro starting at 2 mM concentration and found that L-glutamic acid at 5 mM and 10 mM concentrations significantly impaired blastocyst development. Numerous studies have demonstrated that activation of glutamate receptors can influence the viability and survival of neural cells. Glutamate-induced neuronal cell death (excitotoxicity) is a well-known phenomenon, and experimental studies using various neural cell lines have revealed several mechanisms involved in this process [45]. Moreover, physiological effects of glutamate receptor activation have also been demonstrated in some non-neural cell types [17, 27].

To find out whether glutamate receptors participate in the effect of glutamic acid, we added specific glutamate receptor agonists into the blastocyst culture medium. We found significantly impaired blastocyst development after the application of AMPA and KA (agonists binding to AMPA receptors and kainate receptors). Our results from gene expression analyses suggest that the effects of AMPA on blastocysts were mediated by receptors formed from GRIA3 and/or GRIA4 subunits. KA

could act through kainate receptors formed from GRIK 3 and GRIK 4 or GRIK 5 subunits (which we detected in blastocysts). Schiffer et al. [46] showed that GRIK3 can form functional homomeric receptors as well as functional heteromers with GRIK4 and GRIK5, and these receptors are less sensitive to KA than other kainate receptors. In accordance with this, a relatively high concentration of KA was necessary to induce that effect in blastocysts in our experiment. NMDA receptors can be activated by the binding of glutamate and glycine co-agonists (canonical NMDA receptor signaling) as well as by the binding of glutamate (or NMDA) or glycine alone [47, 48]. We examined exclusively the effects of glutamate/NMDA and our results showed that the NMDA agonist alone had no significant effect on blastocysts. Of the metabotropic glutamate receptor agonists, only the group I-specific agonists affected the evaluated parameters in blastocysts. Metabotropic glutamate receptor group I comprises two members, and we detected only GRM5 (at mRNA and protein levels), and not GRM1, in mouse blastocysts. So we suppose that the GRM5 receptor is responsible for the (S)-3,5-DHPG effect in mouse blastocysts. Finally, the effects induced by glutamic acid were prevented or reduced by pretreatment of blastocysts with AMPA, kainate, and GRM5 receptor antagonists, confirming that these receptors were involved in glutamate action.

Our results indicate that glutamate can activate specific receptors in cells of mouse blastocysts, influencing embryo development. Another amino acid, glycine, has been shown to positively affect the development of mouse preimplantation embryos via glycine receptors [49]. Results obtained in mouse embryonic stem cells (derived from blastocyst ICM cells) indicate that activation of metabotropic glutamate receptors GRM5 and GRM4 can regulate self-renewal and differentiation of these cells [50, 51]. Other amino acids have also been suggested as acting as signaling molecules in early embryonic cells, although amino acid uptake and subsequent metabolism (rather than binding to cell membrane receptors) have been identified in their action [52–56].

In the in vivo context, most glutamate ingested by the maternal organism (as protein constituent or in free form) is metabolized in the intestinal mucosa [15]. However, there are data showing that oral intake of higher glutamate doses can increase glutamate concentration in circulation [57, 58]. Glutamic acid and its salts are widely used as additives to enhance the natural flavors of foods [59], and it cannot be ruled out that excessive intake of these ingredients could increase glutamate concentration in the preimplantation embryo environment. Further research is needed to clarify whether the intake of glutamate as a food additive can interfere with the preimplantation embryo development.

In conclusion, our data show that glutamic acid present in the environment of the early embryo not only functions as a "nutritional factor," but can also affect embryo development through activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and metabotropic (GRM5) glutamate receptors. Our results show that glutamate extracellular concentrations at 5 mM can adversely affect preimplantation embryo development in vitro. The reason that relatively high concentrations of glutamic acid are needed to stimulate glutamate receptors in mouse blastocyst cells is probably related to the activity of amino acid transporters capable of delivering glutamate into cells of preimplantation embryos [23, 60–62]. These data indicate that glutamate can act in two ways in early embryos: as an intracellular metabolite and as an extracellular signaling molecule.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Authors' contributions

Š.Č. conceived the study, designed experiments, analyzed and interpreted data, and wrote the manuscript; A.Š. and V.K. designed and performed experiments, analyzed and interpreted data; Z.Š. designed and performed experiments, analyzed and interpreted data; D.F. interpreted data and critically revised the manuscript; J.P. and M.K. performed experiments; and J.K. critically revised the manuscript. All authors discussed the results and edited the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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