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Gamma-aminobutyric acid (GABA) can affect physiological processes in preimplantation embryos via $GABA_A$ and $GABA_B$ receptors

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Abstract

Purpose: Several widely used substances (e.g., some therapeutics or food supplements) can act on gamma-aminobutyric acid (GABA) receptors, and we investigated whether the activation of these receptors could affect the preimplantation embryo.

Methods: Transcripts of all GABA receptor subunits and selected proteins were examined using quantitative RT-PCR and immunohistochemistry. To analyze the effects of receptor activation, in vitro culture of mouse preimplantation embryos with natural and synthetic GABA receptor ligands was used.

Results: We detected nine GABA receptor transcripts in mouse blastocysts and 14 GABA receptor transcripts in ovulated oocytes. The results of this study indicate that ionotropic $GABA_A$ receptors can be formed from α5, β3, and γ3 (or δ, π) subunits, $GABA_{A\text{-}\rho}$ receptors can be formed from ρ2 subunits and metabotropic GABA receptors can be formed from $GABA_{B1b}$ and $GABA_{B2}$ subunits in mouse blastocysts. Supplementing the culture medium with GABA at concentrations of 2–10 mM or with specific $GABA_A$ and $GABA_B$ receptor agonists (at concentrations of 10–100 μM) significantly increased the proportion of dead cells in blastocysts. The GABA-induced effects were prevented by pretreatment of embryos with $GABA_A$ and $GABA_B$ receptor antagonists.

Conclusion: The results of this study indicate that GABA and synthetic GABA receptor ligands can negatively affect preimplantation embryos via $GABA_A$ and $GABA_B$ receptors.

KEYWORDS
early pregnancy, GABA receptors, oocytes, preimplantation embryos

Veronika Kovaříková and Alexandra Špirková contributed equally to this work and should be regarded as joint first authors.

1 | INTRODUCTION

GABA (gamma-aminobutyric acid) is the main inhibitory neurotransmitter in the adult mammalian central nervous system. In addition, GABA plays an important role in neuronal development during mammalian ontogenesis, and considerable evidence indicates that GABA also mediates diverse responses in nonneural cell types, regulating cell proliferation, survival, differentiation, and migration.[1–4] GABA exerts its action in cells via ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are chloride channels that are assembled from 19 subunits (encoded by separate genes) and form numerous, mostly hetero-oligomeric, pentamers. Sixteen subunits (α1–6, β1–3, γ1–3, δ, ε, θ, and π) combine to form $GABA_A$ receptors, and three ρ subunits combine to form $GABA_{A\text{-}\rho}$ receptors (previously designated $GABA_C$ due to their unique pharmacology). The $GABA_B$ receptor belongs to the G-protein-coupled receptor (GPCR) superfamily (characterized by seven transmembrane domains) and can trigger several signaling pathways in cells. The receptor is a heterodimer composed of the $GABA_{B1}$ and $GABA_{B2}$ subunits (encoded by separate genes). Several GABA receptor subunits are alternatively spliced or transcribed from alternative promoters, and different protein isoforms can be produced from these transcripts. The two protein isoforms of the $GABA_{B1}$ receptor subunit, $GABA_{B1a}$ and $GABA_{B1b}$, are among the best described and are highly conserved among different species.[1,2,5]

There are data suggesting that a nonneuronal GABAergic system may exist in various tissues, including those of the reproductive tract. GABA-synthesizing/metabolizing enzymes, GABA itself, and GABA-binding receptors have been found in the ovarian follicles and in the oviduct of various mammalian species.[6–9] Although the physiological significance of GABA in the female reproductive tract has not yet been fully determined, the influence of GABA on the fertilizing ability of sperm has been clearly demonstrated. Stimulation of sperm capacitation and/or the acrosome reaction has been demonstrated in several mammalian species, including humans, with most results suggesting that $GABA_A$ receptors are involved in these effects.[10–13] On the other hand, there are data indicating that both $GABA_A$ and $GABA_B$ receptors may be involved in the effect of GABA on the acrosome reaction of human spermatozoa.[14,15] Expression of GABA receptor subunits has been shown in mouse spermatogenic cells and mouse, rat, and human spermatozoa, further supporting the idea that GABA acts as a modulator of sperm capacitation and the acrosome reaction in the female reproductive tract.[10,11,16,17] Little information is available on the potential role of GABA in oocytes and early embryos. Sensitivity to GABA has been demonstrated in mouse and human oocytes using electrophysiological techniques, and a $GABA_A$ receptor antagonist blocked the GABA-induced change in cell membrane potential.[18] Extremely high concentrations of GABA impaired blastocyst formation, an effect suggested to be mediated by the $GABA_B$ receptor.[19] The results of another study showed that administration of a $GABA_A$ agonist to pregnant female mice reduced BrdU incorporation into blastocysts, and the effects of $GABA_A$ receptor activation could be mediated by a receptor containing beta 3 subunit. However, the expression of GABA receptors in the embryos was not examined in this study.[20] Overall, the expression of GABA receptors in preimplantation embryos has not been systematically investigated, and contradictory data are available from studies using high-throughput genomic techniques (such as DNA microarrays[21,22]).

Excessive intake of GABA could increase the concentration of GABA in the embryo environment and disrupt the GABAergic network in the oviduct. Several substances acting on GABA receptors (such as benzodiazepines and barbiturates) are widely used as therapeutics for several neurological and psychiatric disorders, including anxiety, depression, epilepsy, and alcohol addiction.[23,24] In addition, GABA has become widely available as a "food supplement" and as a "health care product" in recent years, and can also be consumed by pregnant women or women who intend to become pregnant.[25] Thus, questions have arisen about the possible effects of GABA and GABA receptor ligands on fertility and on the developing embryo. In this study, we investigated whether the activation of GABA receptors can affect the early embryo at the stage before its implantation in the uterus. We analyzed the expression of GABA receptor subunits in in vivo–developed mouse blastocysts and ovulated oocytes. This approach allowed us to differentiate the expression of maternal and embryonic transcripts. To confirm the functionality of the identified receptors, we exposed mouse preimplantation embryos to natural and synthetic GABA receptor ligands and analyzed their effects.

2 | MATERIALS AND METHODS

2.1 | Animals, collection of in vivo–developed oocytes and blastocysts

All experiments were performed with outbred ICR (CD-1 IGS) mice (Velaz). The animals were housed in the animal facility at the Institute of Animal Physiology, Kosice, Slovakia (authorization no. SK UCH 01018) in plexiglass cages and kept under standard conditions (temperature 22 ± 2°C, humidity 65 ± 5%, 12:12-h light–dark cycle with lights on at 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) and hCG (human chorionic gonadotropin, 4 IU ip; Pregnyl, Organon; 47 h later). Twelve to 14 h after hCG administration, the mice were euthanized by cervical dislocation, and unfertilized oocytes were isolated by flushing the oviduct using an in-house flushing-holding medium (FHM[26]) 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 identification of a vaginal plug the next morning. Fertilized dams were euthanized by cervical dislocation and subjected to embryo isolation by flushing the uterus using FHM with BSA at 96 h post-hCG and to morphological classification using stereomicroscopy (Nikon SMZ 745T, Nikon). Oocytes and blastocysts

were washed in several drops of FHM with BSA and pooled. Cumulus cells were removed with 0.1% hyaluronidase (Sevac), and oocytes and blastocysts were analyzed by RT-PCR or immunohistochemistry.

2.2 | RT-PCR and transcript relative quantification

Total RNA was extracted from batches of 590 to 610 unfertilized mouse oocytes or blastocysts (the number of oocytes/blastocysts in each pool was precisely determined) and from the mouse brain (positive tissue control). TRIzol Reagent (Invitrogen Life Technologies) 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). 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 negative controls (no reverse transcriptase in the cDNA synthesis reaction, "RT-") 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) such that 1 µL of the cDNA corresponded theoretically to 2.5 embryo/oocyte equivalents.

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 17 GABA receptor subunits. Four GABA receptor subunits that were not included in the PCR array were analyzed in separate PCRs using commercial primer sets from Qiagen (product numbers: PPM04240A, PPM04233F, PPM05200G, and PPM57751A). PCR amplifications were performed in a LightCycler 480 real-time PCR system (Roche Diagnostics). The reactions were carried out in 25 µL volumes containing 1 µL of cDNA (corresponding theoretically to 2.5 embryo/oocyte equivalents) and SYBR 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 below). The experiment was performed three times, and the results were analyzed by the 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.

To distinguish between the mouse GABA receptor B1 transcript variants $GABA_{B1a}$ and $GABA_{B1b,}$ we designed primers located in transcript variant–specific sequences (Table 1). For $GABA_{B1a}$ amplification, the forward primer was located in exon 4 ($GABA_{B1a}$-specific sequence), and the reverse primer was located in exon 6 (NM_019439, GenBank reference sequence for GABA-B receptor 1). For $GABA_{B1b}$ amplification, the forward primer was located in coding exon 1 (GenBank sequence AF120255, $GABA_{B1b}$-specific sequence: the intron sequence in the $GABA_{B1a}$ splice variant that becomes the exon sequence in the $GABA_{B1b}$ splice variant), and the reverse primer was located in coding exon 2 (GenBank sequence AF120255) that corresponds to exon 7 in the reference sequence NM_019439 (Figure 2). Amplification reactions contained 0.5 µM of each primer, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.02 U/mL platinum Taq DNA polymerase (Invitrogen Life Technologies). An initial step at 95°C for 10 min was followed by 45 cycles at 95°C for 20 s, 65°C for 30 s, and 72°C for 20 s.

PCR products were analyzed using electrophoresis on 3% agarose gels stained with GelGreen (Biotium). A 20-bp DNA ladder (Jena Bioscience) was used as a marker. PCR products were visualized with a Fusion FX7 imaging system (Vilber Lourmat), and the size of DNA bands (PCR products) was determined with Bio1D analysis software (Vilber Lourmat). PCR products amplified with $GABA_{B1b}$ primers were sequenced (SEQme) and compared with known $GABA_{B1}$ receptor sequences (using CLUSTAL W and BLAST 2 algorithms).

2.3 | 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), and embryos were permeabilized in phosphate-buffered

TABLE 1 Primers for mouse GABA receptor B1transcript variants $GABA_{B1a}$ and $GABA_{B1b}$.

Receptor	Primer sequence (5′–3′)	GenBank Accession no.	Amplicon Size (bp)	Separating intron (bp)
$GABA_{B1a}$	FP: AGCATCTGTAGTCA GGGCCAGT	NM_019439	232	1170 + 1201
	RP: ACTTGCTGTCGTGG TGGATAAG			
$GABA_{B1b}$	FP: GCCTCTCACTCCCCTCA TCTC	AF120255	277	2971
	RP: TGGGGTCGTTGTAG AGTAGTTCA			

Abbreviations: bp, base pairs; FP, forward primer; RP, reverse primer.

saline (PBS) containing 1% BSA and 0.5% saponin (Sigma–Aldrich). Nonspecific immunoreactions were blocked with blocking buffer (PBS containing 0.05% saponin, 10% normal goat serum [Santa Cruz Biotechnology], 0.3 M glycine [Merck], and 1% BSA [Sigma–Aldrich]). Embryos were incubated with primary rabbit polyclonal antibodies against selected GABA receptor subunits in blocking buffer at 4°C overnight. Primary antibodies used in the study: GABA-A receptor alpha5 (Synaptic Systems, Cat. # 224503, dilution 1:100), GABA-A receptor beta3 (Synaptic Systems, Cat. # 224403, dilution 1:100), anti-GABRG3 (Ray Biotech, Cat. # 102–15 765, dilution 1:100), Anti-GABA (A) δ Receptor (extracellular) Antibody (Alomone labs Cat. # AGA-014, dilution 1:100), GABRR2 GABA Receptor, rho 2 (Online antibodies, Cat. # ABIN1841968, dilution 1:100), GABBR1 (Invitrogen, Cat. # PA5-27725, dilution 1:100), Anti-GABA (B) R2 (Alomone labs, Cat. #AGB-002, dilution 1:100), GABRP Antibody–N-terminal region (Aviva Systems Biology, Cat. # AVARP 13034-P050, dilution 1:50), and Anti-GABA (A) ε (Alomone labs, Cat. # AGA-015, dilution 1:50). 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). Afterward, embryos were mounted in Vectashield antifade reagent (Vector Laboratories) on glass slides, sealed with coverslips, and observed using a confocal microscope (Leica TCS SPE, Leica). Negative control groups of embryos were incubated without the primary antibody or without the secondary antibody or with rabbit gamma globulin (Rabbit Gamma globulin Control, Invitrogen, Cat# 31887).

2.4 | Embryo culture and morphological evaluation

The 2-cell embryos were isolated by flushing the oviduct with FHM medium 40 h after hCG administration. They were then cultured in 400 μL drops of synthetic oviductal medium (EmbryoMax® KSOM [potassium simplex optimized medium] with amino acids and D-glucose [Millipore]) supplemented with GABA receptor agonists GABA (gamma-aminobutyric acid/4-aminobutanoic acid, Abcam, Cat. No. ab120359) or muscimol (5-aminomethyl-3-hydroxyisoxazole, Tocris Cat. No. 0289) or (R)-baclofen ([R]-4-amino-3-[4-chlorophenyl] butanoic acid, Tocris Cat. No. 0796). Three final concentrations of muscimol and (R)-baclofen were used: 10 μM, 50 μM, and 100 μM (these concentrations were chosen according to the information in published experiments[20,27,28]). In some experiments, a mixture of three GABA receptor antagonists containing bicuculline [(−)-bicucculine methochloride, Abcam, Cat. No. ab120110], CGP 35348 [(3-aminopropyl)(diethoxymethyl) phosphinic acid, Abcam, Cat. No. ab120167], and TPMPA [(1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid, Tocris Cat. No. 1040] at final concentration of 50 μM was used. Embryos were incubated in this antagonist mixture for 20 min prior to the addition of GABA (to a final concentration of 10 mM). Stock solutions of agonists and antagonists were prepared by dissolving in water. The control groups of embryos were cultured in the presence of equivalent amounts of solvent (water) added to the KSOM. All groups of embryos were cultured in a humidified atmosphere with 5.0% CO_2 at 37°C for 72 h. After 72 h of incubation, blastocysts were fixed in 4% paraformaldehyde (Merck) and permeabilized with 0.5% Triton X-100 solution in PBS (v/w). Embryos were then incubated with TUNEL assay reagents (TUNEL, In Situ Cell Death Detection Kit; Roche) for 60 min at 37°C in the dark. Finally, the blastocysts were counterstained with Hoechst 33342 (10 μg/mL in PBS; Sigma–Aldrich), mounted on glass slides using VECTASHIELD (Vector Laboratories) and observed using a fluorescence microscope at 400x magnification (BX51; Olympus). Microphotographs of three to five 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) upgraded with the Point Picker plugin, allowing us to pick, stack, and save nuclei located at specific coordinates in an image. The number of cell nuclei was determined in each in vitro–derived embryo, and each embryo with a blastocoel cavity was scored as a blastocyst. 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 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.

2.5 | Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). One-way ANOVA followed by the Tukey post hoc test was used to compare the blastocyst cell number and the proportion of dead cells in the blastocysts. Differences with $p < 0.05$ were considered significant.

3 | RESULTS

3.1 | Transcripts encoding GABA receptors are expressed in mouse blastocysts and oocytes

We detected transcripts of 14 ionotropic GABA receptor subunits and 2 metabotropic GABA receptor subunits in mouse ovulated oocytes and/or in vivo–developed blastocysts (Table 2). *Gabrb2*, *Gabrg2*, *Gabrq*, and *Gabrr3* transcripts were detected in oocytes but not in blastocysts. In contrast, *Gabrd* and *Gabbr2* transcripts were detected in blastocysts but not in oocytes (*Gabbr2* was detected inconsistently in oocytes: it was detected in one but not in two other oocyte samples). Other transcripts (*Gabra1*, *Gabra3*, *Gabra5*, *Gabrb3*, *Gabrg1*, *Gabrg3*, *Gabre*, *Gabrp*, *Gabrr2*, and *Gabbr1*) were detected in both oocytes and blastocysts, and *Gabra5* and *Gabrp* transcripts

TABLE 2 Results of RT–PCR analysis of GABA receptor transcripts in mouse blastocysts and ovulated oocytes.

Receptor subunit	Detection of oocytes and blastocysts		Fold regulation (Blastoc./Ooc.)	*p* Value
Gabbr1 ($GABA_{B1}$)	Y	Y	+145.7	0.000002
Gabbr2 ($GABA_{B2}$)	N/Y	Y		
Gabra1 (α1)	Y	Y/N		
Gabra2 (α2)	N	N		
Gabra3 (α3)	Y	Y/N		
Gabra4 (α4)	N	N		
Gabra5 (α5)	Y	Y	−35.2	0.00011
Gabra6 (α6)	N	N		
Gabrb1 (β1)	N	N		
Gabrb2 (β2)	Y	N		
Gabrb3 (β3)	Y	Y	+1.1	0.58
Gabrg1 (γ1)	Y	Y/N		
Gabrg2 (γ2)	Y	N		
Gabrg3 (γ3)	Y	Y	−2.89	0.096
Gabrd (δ)	N	Y		
Gabre (ε)	Y	Y	+1.77	0.19
Gabrp (π)	Y	Y	−12.76	0.0017
Gabrq (θ)	Y	N		
Gabrr1 (ρ1)	N	N		
Gabrr2 (ρ2)	Y	Y	+2.09	0.19
Gabrr3 (ρ3)	Y	N		

Note: Commonly used names/symbols of GABA receptor subunits are given in parentheses. Transcripts were detected by reverse transcription–polymerase chain reaction (RT–PCR). "Y" indicates consistent detection (specific PCR product in all three oocyte or blastocyst samples), "N" indicates nondetection (absence of specific PCR product in all three oocyte or blastocyst samples), and "N/Y" and "Y/N" indicate inconsistent detection (N/Y, detection of a specific PCR product only in one of the three samples; Y/N, detection of a specific PCR product in two of the three samples). In transcripts that were consistently expressed in both blastocysts and oocytes, fold regulation values ("+" indicates upregulation, and "−" indicates downregulation in blastocysts compared to oocytes) and corresponding *p* Values are shown.

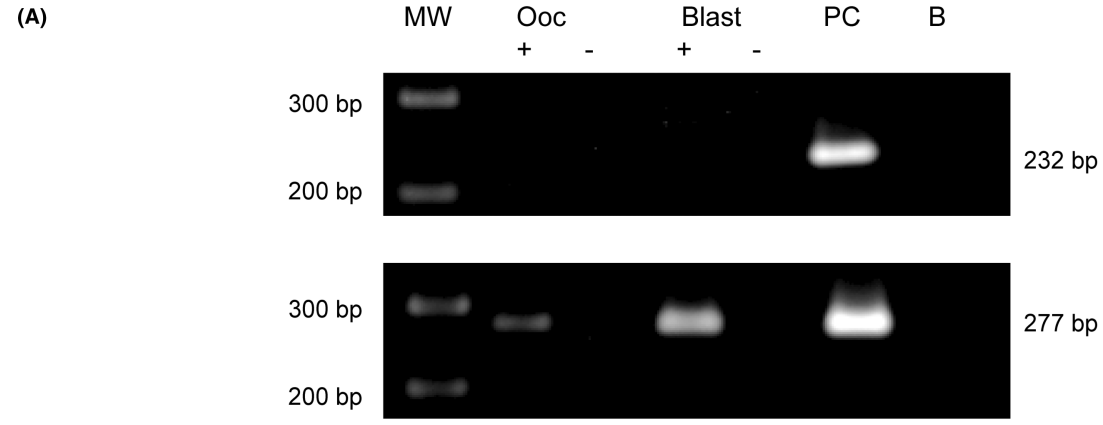
Abbreviations: *Gabbr1*, Gamma-aminobutyric acid (GABA) B receptor, 1; *Gabbr2*, Gamma-aminobutyric acid (GABA) B receptor, 2; *Gabra1*, Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1; *Gabra2*, Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2; *Gabra3*, Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3; *Gabra4*, Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4; *Gabra5*, Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5; *Gabra6*, Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6; *Gabrb1*, Gamma-aminobutyric acid (GABA) A receptor, subunit beta 1; *Gabrb2*, Gamma-aminobutyric acid (GABA) A receptor, subunit beta 2; *Gabrb3*, Gamma-aminobutyric acid (GABA) A receptor, subunit beta 3; *Gabrd*, Gamma-aminobutyric acid (GABA) A receptor, subunit delta; *Gabre*, Gamma-aminobutyric acid (GABA) A receptor, subunit epsilon; *Gabrg1*, Gamma-aminobutyric acid (GABA) A receptor, subunit gamma 1; *Gabrg2*, Gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2; *Gabrg3*, Gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3; *Gabrp*, Gamma-aminobutyric acid (GABA) A receptor, subunit pi; *Gabrq*, Gamma-aminobutyric acid (GABA) A receptor, subunit theta; *Gabrr1*, Gamma-aminobutyric acid (GABA) C receptor, subunit rho 1; *Gabrr2*, Gamma-aminobutyric acid (GABA) C receptor, subunit rho 2; *Gabrr3*, Gamma-aminobutyric acid (GABA) C receptor, subunit rho 3.

were detected in much larger quantities in oocytes than in blastocysts (35- and 12-fold differences were found, respectively). In contrast, the amount of *Gabbr1* transcript was approximately 145 times higher in blastocysts than in oocytes. No specific PCR products were detected in the blank reactions. RT-control reactions for two subunits (*Gabra1* and *Gabrp* 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).

To determine which of the two conserved isoforms of $GABA_B$ receptor 1 ($GABA_{B1a}$ or $GABA_{B1b}$ or both) is expressed in mouse oocytes and preimplantation embryos, we designed specific primers that distinguish between the two transcript variants. We detected a PCR product corresponding to the $GABA_{B1a}$ transcript (232 bp) in positive control tissue (brain) but not in ovulated oocytes or blastocysts (only faint products of size other than the expected 232 bp were produced). The PCR product corresponding to the $GABA_{B1b}$ transcript (277 bp) was detected in oocytes, blastocysts, and positive control tissue. No PCR products were detected in the reactions where reverse transcriptase or cDNA was omitted (Figure 1A). To confirm the $GABA_{B1b}$ transcript identity, the 277-bp PCR product amplified with $GABA_{B1b}$ primers was sequenced. We found 100% identity between the nucleotide sequence of the 277-bp PCR product and the corresponding regions of the published mouse $GABA_{B1b}$ mRNA and mouse $GABA_{B1}$gene sequences (Figure 1B, Figure S1). Comparison of the predicted amino acid sequence of the mouse $GABA_{B1b}$ isoform with the reference amino acid sequence of the human $GABA_{B1b}$ isoform showed 98% identity (Figure S2), and this high amino acid sequence conservation indicates the biological importance of the GABAB1b isoform.

3.2 | GABA receptor proteins are expressed in mouse blastocysts

Transcripts that were consistently expressed in blastocysts were examined for protein translation. We detected proteins of the alpha-5 (GABRA5), beta-3 (GABRB3), gamma-3 (GABRG3), pi (GABRP), and delta (GABRD) subunits, which can form $GABA_A$ ionotropic receptors in blastocysts. Although the *Gabre* transcript was detected in all blastocyst samples, we did not detect the epsilon subunit protein (data not shown). We detected rho-2 subunits (GABRR2), which can form $GABA_{A\text{-}\rho}$ (formerly called $GABA_C$) receptors (Figure 2). Translation of the three transcripts that were detected in most but not all blastocyst samples (*Gabra1*, *Gabra3* and *Gabrg1*, Table 2) is also feasible but we did not investigate this possibility. We also detected $GABA_{B1}$ (GABBR1) and $GABA_{B2}$ (GABBR2) subunits, which can form $GABA_B$ metabotropic receptors in blastocysts. All examined proteins were detected in both ICM and TE cells. The fluorescence signal was strongest at the cell periphery in most cases, suggesting localization of receptors to 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



(B)

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277bpPCR        ------------------------------------------------------------
AF120255        ATGGGCCCGGGGGGACCCTGTACCCCGGTGGGGTGGCCGCTGCCTCTTCTGCTGGTGATG   CDS-Ex1

277bpPCR        ----------------------GCCTCTCACTCCCCTCATCTCCCGCGGCCTCACCCG
AF120255        GCGGCTGGGGTGGCTCCGGTGTGGGCCTCTCACTCCCCTCATCTCCCGCGGCCTCACCCG
                                       **********************************

277bpPCR        AGGGTCCCCCCGCACCCCTCCTCAGAACGGCGTGCAGTATACATCGGGGCGCTGTTTCCC
AF120255        AGGGTCCCCCCGCACCCCTCCTCAGAACGGCGTGCAGTATACATCGGGGCGCTGTTTCCC
                ************************************************************

277bpPCR        ATGAGCGGGGGCTGGCCGGGGGGCCAGGCCTGCCAGCCTGCGGTGGAGATGGCGCTGGAG
AF120255        ATGAGCGGGGGCTGGCCGGGGGGCCAGGCCTGCCAGCCTGCGGTGGAGATGGCGCTGGAG
                ************************************************************

277bpPCR        GACGTTAACAGCCGCAGAGACATCCTGCCGGACTACGAGCTCAAGCTTATCCACCACGAC
AF120255        GACGTTAACAGCCGCAGAGACATCCTGCCGGACTACGAGCTCAAGCTTATCCACCACGAC
                ************************************************************

277bpPCR        AGCAAGTGCGACCCAGGGCAAGCCACCAAGTACTTGTATGAACTACTCTACAACGACCCC   CDS-Ex2
AF120255        AGCAAGTGCGACCCAGGGCAAGCCACCAAGTACTTGTATGAACTACTCTACAACGACCCC
                ************************************************************

277bpPCR        A-----------------------------------------------------------
AF120255        ATCAAGATCATCCTCATGCCCGGCTGCAGCTCTGTGTCCACACTGGTAGCCGAGGCTGCC
                *

277bpPCR        --------------------
AF120255        CGGATGTGGAACCTTATTGTG
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FIGURE 1 RT–PCR analysis of $GABA_B$ receptor 1 transcript variants in mouse blastocysts and oocytes. (A) Representative agarose gels with separated PCR products are shown. Lanes: MW, molecular weight markers; Ooc, oocytes; Blast, blastocysts; PC, positive control tissue (brain); B, blank reaction. PCR templates: +, cDNA synthesis with reverse transcriptase; −, corresponding reverse transcriptase negative controls. The MWs and the predicted sizes of the PCR products in base pairs (bp) are indicated to the left and right of the panels, respectively. (B) Alignment of the 277-bp PCR product nucleotide sequence (amplified with $GABA_{B1b}$ primers in mouse oocyte, blastocyst and brain cDNA samples) with the published mouse $GABA_{B1b}$ N-terminal nucleotide sequence (GenBank acc.no. AF120255[29]). Asterisks indicate positions with identical nucleotides in the compared sequences. CDS-Ex1, the nucleotide sequence of $GABA_{B1b}$ coding exon 1; CDS-Ex2, the nucleotide sequence of $GABA_{B1b}$ coding exon 2 (corresponding to exon 7 in the $GABA_{B1a}$ transcript variant, GenBank acc.no. NM_019439, see Figure S1). Primer sequences are shown in boxes (RP primer sequence is reverse complementary).

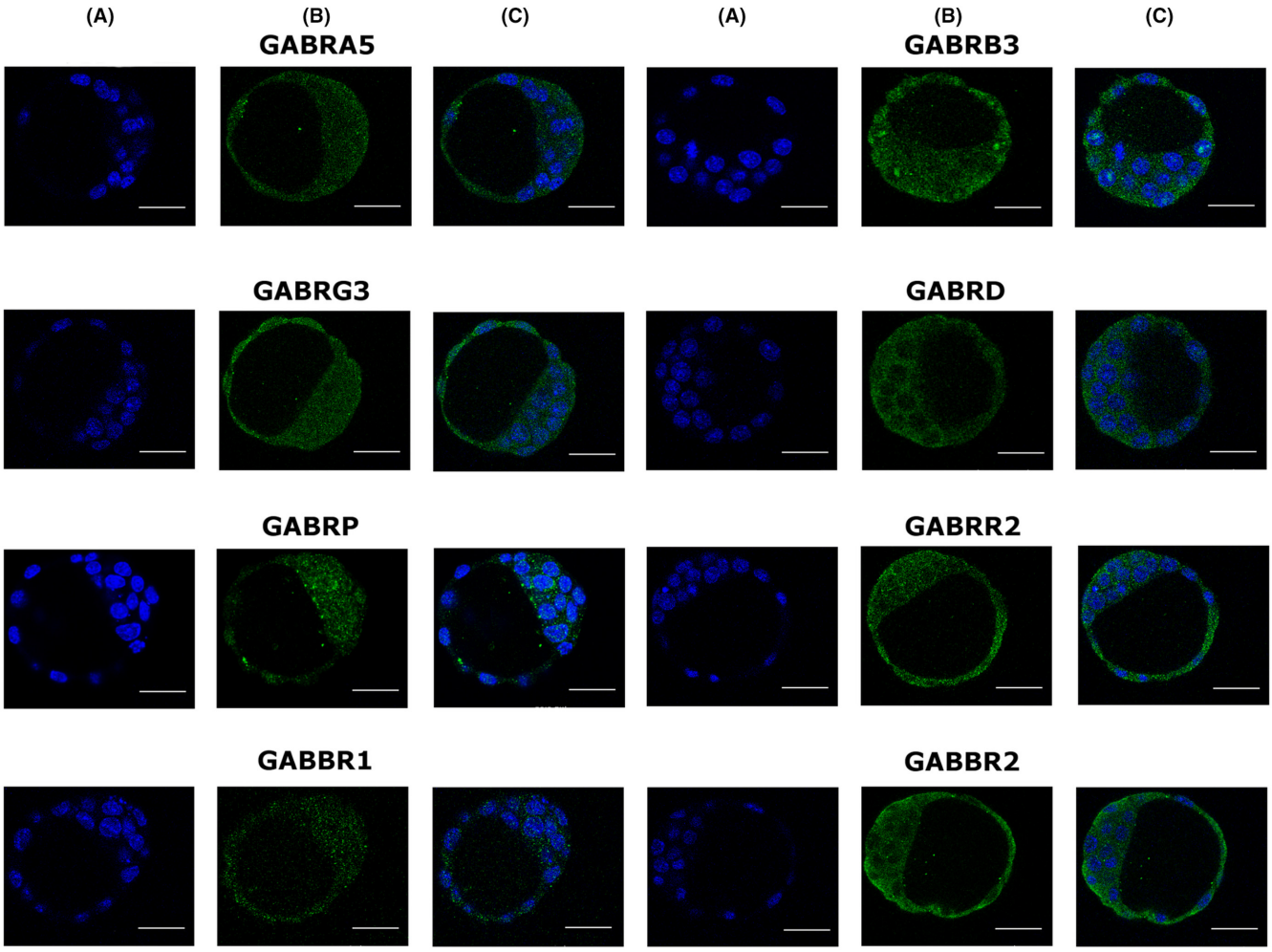

FIGURE 2 Expression of GABA receptor proteins in mouse blastocysts. GABA receptor subunits were detected by immunofluorescence. Representative images are shown. Optical sections were observed via CLSM. Cell nuclei were stained with Hoechst 33342 (blue staining, A columns). Embryos were incubated with primary antibodies against the GABA receptor subunits and with a secondary antibody labeled with Alexa Fluor 488 (green staining, B columns); C columns, merged images. For negative controls, Figure S3. Scale bars, 30 μm.

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 (Figure S3).

3.3 | GABA and specific GABA receptor agonists can impair physiological processes in blastocysts

To examine the effects of GABA and the participation of GABA receptors in these effects, we stimulated mouse preimplantation embryos with increasing concentrations of natural GABA receptor ligand (GABA, gamma-aminobutyric acid) and with receptor type–specific synthetic ligands (muscimol, [R]-baclofen) in our functional studies. In the first experiment, mouse 2-cell embryos were cultured for 72 h in medium supplemented with GABA at concentrations of 2 mM, 5 mM, and 10 mM. We found no differences in the proportion of embryos that reached the blastocyst stage (data not shown). Cell numbers in blastocysts treated with GABA tended to be lower than those in control blastocysts, but this difference did not reach statistical significance. On the other hand, we found significantly higher proportions of dead cells (showing TUNEL-positive labeling) in GABA-treated blastocysts than in control blastocysts (Figure 3A).

To determine whether both $GABA_A$ and $GABA_B$ receptors were functional in mouse preimplantation embryos, the embryos were cultured in medium supplemented with muscimol ($GABA_A$ receptor agonist) and (R)-baclofen ($GABA_B$ receptor agonist) in the second set of experiments. We found no differences in the proportion of embryos that reached the blastocyst stage (data not shown) or in cell numbers in blastocysts. Both muscimol and (R)-baclofen significantly increased the proportions of dead cells in blastocysts, and this effect was concentration-dependent (Figure 3B,C).

In the final experiment, mouse embryos were cultured in medium supplemented with GABA (at final concentration of 10 mM) and compared with embryos incubated in the presence of GABA receptor antagonists (a mixture of bicuculline, CGP 35348 and TPMPA at final concentration of 50 μM) prior to GABA exposure. Our results showed that the increased incidence of cell death induced by GABA was blocked by the antagonists (Figure 3D).

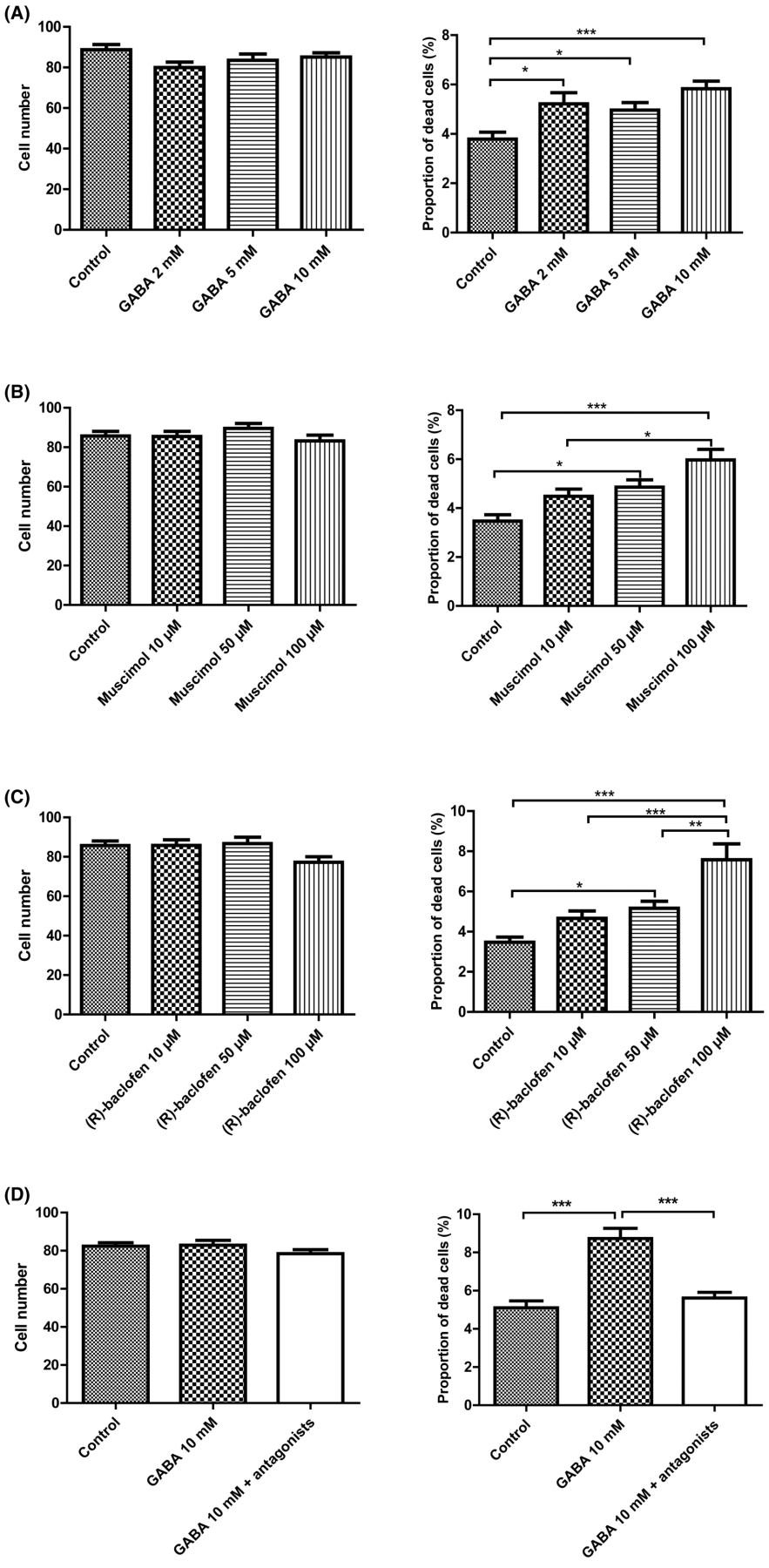

FIGURE 3 Effects of GABA and GABA receptor agonists on cell number and proportion of dead cells. (A) Cell numbers and proportions of dead cells in blastocysts incubated with the indicated concentrations of GABA. Numbers of blastocysts in the groups (*n*): Control, $n=38$; GABA 2 mM, $n=24$; GABA 5 mM, $n=35$; GABA 10 mM, $n=42$. (B) Cell numbers and proportions of dead cells in blastocysts incubated with indicated concentrations of muscimol. Numbers of blastocysts in the groups (*n*): Control, $n=42$; muscimol 10 µM, $n=36$; muscimol 50 µM, $n=43$; muscimol 100 µM, $n=37$. (C) Cell numbers and proportions of dead cells in blastocysts incubated with indicated concentrations of (R)-baclofen. Numbers of blastocysts in the groups (*n*): Control, $n=42$; (R)-baclofen 10 µM, $n=32$; (R)-baclofen 50 µM, $n=31$; (R)-baclofen 100 µM, $n=32$. (D) Cell numbers and proportions of dead cells in embryos pretreated with the mixture of GABA receptor antagonists (mixture of bicuculline, CGP 35348, and TPMPA at final concentration of 50 µM) prior to the addition of GABA. Numbers of blastocysts in the groups (*n*): Control, $n=38$; GABA 10 mM, $n=45$; GABA 10 mM + antagonists (bicuculline+CGP35348 + TPMPA), $n=48$. The values are arithmetical means + SEMs. Statistical significance of differences: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

4 | DISCUSSION

We examined mRNA of all 19 subunits of ionotropic GABA receptors (*Gabra1*, *Gabra2*, *Gabra3*, *Gabra4*, *Gabra5*, *Gabra6*, *Gabrb1*, *Gabrb2*, *Gabrb3*, *Gabrg1*, *Gabrg2*, *Gabrg3*, *Gabrd*, *Gabre*, *Gabrp*, *Gabrq*, *Gabrr1*, *Gabrr2*, and *Gabrr3*) and two subunits of metabotropic GABA receptors (*Gabbr1* and *Gabbr2*) in mouse blastocysts and ovulated oocytes and found several expression profiles. Expression in oocytes but not in blastocysts was found in four GABA receptor transcripts (*Gabrb2*, *Gabrg2*, *Gabrq*, and *Gabrr3*), indicating that these maternal transcripts are degraded during preimplantation development. In contrast, two transcripts (*Gabrd* and *Gabbr2*) were found in blastocysts but not in oocytes, indicating that transcription of these genes begins after embryonic genome activation. Of the 14 transcripts expressed in oocytes (*Gabra1*, *Gabra3*, *Gabra5*, *Gabrb2*, *Gabrb3*, *Gabrg1*, *Gabrg2*, *Gabrg3*, *Gabre*, *Gabrp*, *Gabrq*, *Gabrr2*, *Gabrr3*, and *Gabbr1*), only seven were consistently detected in blastocysts (*Gabra5*, *Gabrb3*, *Gabrg3*, *Gabre*, *Gabrp*, *Gabrr2*, and *Gabbr1*), and the expression levels in oocytes and blastocysts differed significantly in some transcripts. These data indicate that significant changes in the expression of genes encoding GABA receptor subunits occur after oocyte fertilization.

Our results indicate that multiple GABA receptor transcripts are translated into proteins in mouse blastocysts. Ionotropic $GABA_A$ receptors in blastocyst cells are probably composed of α5, β3, and γ3 subunits, with possible replacement of the γ subunit by δ or π subunits. Our results also indicate the expression of $GABA_{A\text{-}\rho}$ receptors (designated $GABA_C$ previously) composed of ρ2 subunits in blastocysts. We detected proteins of both metabotropic GABA receptor subunits ($GABA_{B1}$ and $GABA_{B2}$) in mouse blastocysts, indicating that a functional $GABA_B$ receptor dimer can be formed in the cells of the early embryo. Although the antibody used in our study did not distinguish between $GABA_{B1a}$ and $GABA_{B1b}$ isoforms (which differ in their N-terminal regions), our RT–PCR results indicated that the detection signal corresponded to the $GABA_{B1b}$ protein isoform. Numerous studies have shown differences in the spatial and temporal expression patterns of $GABA_{B1a}$ and $GABA_{B1b}$ indicating separate transcriptional regulation and possible distinct functional roles.[2] For instance, differential behavioral responses were found in mice deficient in $GABA_{B1a}$ and $GABA_{B1b}$ receptor isoform, and the stress resilience in mice lacking $GABA_{B1b}$ was coupled with increased proliferation and survival of newly born cells in the adult ventral hippocampus following early-life stress.[30,31] The possible physiological significance of $GABA_{B1b}$ but not $GABA_{B1a}$ expression in preimplantation embryos remains to be elucidated.

We examined the effects of GABA on mouse preimplantation embryos in vitro and found that GABA at 2–10 mM concentrations significantly increased the proportion of dead cells in blastocysts. Although there may not always be a direct correlation between the rate of apoptosis and embryo viability, significantly increased cell death is generally accepted as a marker of lower embryo quality, and a number of experiments have shown a reduced developmental potential of blastocysts containing an increased proportion of dead cells.[32–34] We found no significant changes in blastocyst formation or blastocyst cell number, suggesting that increased cell death could be one of the first responses of the early embryo to stimulation with low millimolar concentrations of GABA. A longer period of observation, including postimplantation development, would probably be necessary for the increased proportion of dead cells to be reflected in possibly altered embryo development. On the other hand, Tian et al.[19] showed that GABA at concentrations ranging from 10 to 50 μg/μL (97–485 mM) significantly decreased the rate of blastocyst formation in mouse preimplantation embryos cultured from the 2-cell stage for 72 h (they found no effect below a GABA concentration of 10 μg/μL, i.e., 97 mM). They also demonstrated that the effect of 291 mM (30 μg/μL) GABA could be prevented by pretreatment of embryos with 40 μM competitive $GABA_B$ antagonist 2-hydroxysaclofen. The authors concluded that the negative effect of GABA was mediated by $GABA_B$ receptors. However, the GABA concentrations used in the experiment were extremely high, and the effect on osmolality or other toxic effects could have contributed to the observed deterioration of embryo development. Tian et al.[19] also demonstrated negative effects of GABA on mouse preimplantation embryo development (and endometrial receptivity) in vivo. However, again, the doses they used were extremely high (6–12 mg/g body weight); for a 50-kg woman, this dose would require the consumption of approximately 500 g of GABA per day during the preimplantation period. Thus, these results probably demonstrate a general toxic effect of extremely high doses of GABA on the organism rather than specific effects on preimplantation emryo, or a combination of these effects.

To investigate whether both $GABA_A$ and $GABA_B$ receptors could be involved in the effect of GABA, we cultured embryos in medium supplemented with muscimol ($GABA_A$ receptor agonist) and (R)-baclofen ($GABA_B$ receptor agonist). We found a similar effect to that of GABA: both agonists significantly increased the proportions of dead cells in blastocysts. In addition, pretreatment of embryos with $GABA_A$ and $GABA_B$ receptor antagonists prevented the increase in the incidence of cell death induced by GABA administration. These results indicate that GABA can activate both types of GABA receptors in cells of preimplantation embryos. A possible role of $GABA_A$ receptor signaling in the regulation of early embryo development was demonstrated in a study by Andäng et al.[20]; the authors also proposed a model of GABA-mediated control of proliferation in mouse embryonic stem cells (derived from preimplantation embryos) that involves activation of $GABA_A$ receptors, hyperpolarization via Cl^- influx, and phosphorylation of histone H2X by phosphatidylinositol 3-kinases. On the other hand, Schwirtlich et al.[35] showed that both $GABA_A$ and $GABA_B$ receptors can affect the proliferation of mouse embryonic stem cells, and changes in intracellular Ca^{2+} were involved in these effects.

Our results indicate that activation of GABA receptors induces cell death in preimplantation embryos, but the underlying molecular mechanisms are unknown. The involvement of GABA receptors in the regulation of cell death has been demonstrated in several cell types, including trophoblastic cells and cancer cells. Increased

apoptosis and decreased viability were demonstrated in a human placental cell line derived from first-trimester extravillous trophoblasts transfected with a construct containing the π subunit of the $GABA_A$ receptor (this subunit was detected in human and mouse placentas). However, when related signaling pathways were examined, conflicting results were obtained when both proapoptotic and antiapoptotic Bcl-2 family members were found to be upregulated in cells overexpressing the π subunit of the $GABA_A$ receptor.[36] Stimulation of apoptosis by activation of $GABA_A$ receptors has been shown in some types of brain tumors. In human neuroblastoma cells, benzodiazepine-mediated activation of $GABA_A$ receptors induced apoptosis and inhibited mitogenic signaling through AKT and MAP kinases.[37] In medulloblastoma cells, activation of $GABA_A$ receptor (containing α5 subunit) increased apoptosis through enhanced Cl^- efflux that induced depolarization of mitochondrial membrane.[38] Stimulation of apoptosis by the $GABA_B$ receptor was shown in colorectal cancer cells, where the activated $GABA_B$ receptor induced apoptosis by inhibiting cAMP-dependent ERK-CREB phosphorylation and cIAP2 (cellular inhibitor of apoptosis protein 2) expression.[39] On the other hand, inhibition of apoptosis and stimulation of proliferation upon activation of $GABA_A$ and $GABA_B$ receptors have been demonstrated in various cancer and other cell types.[40–42] The physiological role of GABA signaling in preimplantation embryos is unknown. Several other neurotransmitter receptors are expressed in preimplantation embryos, and their activation can affect early embryonic development.[43,44] These data suggest that molecules functioning as neurotransmitters in the adult organism may be used as regulatory molecules at very early developmental stages before the nervous system is formed.

The fact that GABA and synthetic GABA receptor ligands can directly affect preimplantation embryos may be of great practical importance. Dysfunction of GABA receptors is linked to a broad variety of neurological and psychiatric disorders, and GABA receptor agonists are widely used therapeutics for these diseases.[45–47] GABA receptor signaling can also be affected by ethanol,[48] and alcohol consumption is widespread in women of reproductive age (and the trend is increasing) in western countries.[49–51] Alcohol intake has been shown to inhibit preimplantation embryo development,[52,53] and our results suggest that one of the mechanisms involved in this effect could be the interaction of ethanol with embryonic GABA receptors. In addition, GABA itself is used as a "dietary supplement" in products claiming "stress-reducing" or "sleep-improving" effects.[25] While the ability of dietary GABA to cross the blood–brain barrier (and affect brain function) is disputed, the transfer of GABA from the circulation to the oviductal fluid is highly probable, and the role of GABA in sperm capacitation and/or acrosome reaction (which takes place in the oviduct in vivo) has been demonstrated in several studies.[10–15] Although elevated GABA concentration in the oviduct alone does not necessarily have fatal effects, it may be part of a variety of negative factors acting synergistically on the embryo and may contribute to its damage.

In conclusion, we showed that both $GABA_A$ and $GABA_B$ receptors are expressed in mouse preimplantation embryos and that their activation can negatively influence the embryo. Our results suggest that excessive consumption of substances acting on GABA receptors (such as specific therapeutics, alcohol, or GABA-containig food additives) could negatively influence preimplantation embryos and thus represent a risk for the successful initiation of pregnancy.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ANIMAL STUDIES

All animal experiments were performed in accordance with the ethical principles under 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 EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes.

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