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Exogenous gamma-aminobutyric acid addition enhances porcine sperm acrosome reaction

Shouhei Kurata[1] | Kohei Umezu[2] | Hironori Takamori[3] | Yuuki Hiradate[4] | Kenshiro Hara[1] | Kentaro Tanemura[1]

[1]Laboratory of Animal Reproduction and Development, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan

[2]Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas, USA

[3]Miyagi Prefectural Livestock Experimental Station, Osaki, Miyagi, Japan

[4]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Correspondence
Kentaro Tanemura, Laboratory of Animal Reproduction and Development, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 980-0845, Japan.
Email: kentaro.tanemura.e4@tohoku.ac.jp

Abstract

The widely used porcine artificial insemination procedure involves the use of liquid-stored semen because it is difficult to control the quality of frozen–thawed porcine sperm. Therefore, there is a high demand for porcine semen. The control and enhancement of sperm function are required for the efficient reproduction of pigs. We previously reported that gamma-aminobutyric acid (GABA) enhanced sperm capacitation and acrosome reaction in mice. In this study, we demonstrated the presence of $GABA_A$ receptors in porcine sperm acrosome. Furthermore, we investigated the GABA effects on porcine sperm function. We did not detect any marked effect of GABA on sperm motility and tyrosine phosphorylation of sperm proteins. However, GABA promoted acrosome reaction, which was suppressed by a selective $GABA_A$ receptor antagonist. GABA binds to $GABA_A$ receptors, resulting in chloride ion influx. We found that treatment with 1 μM GABA increased the intracellular concentration of chloride ion in the sperm. In addition, the GABA concentration effective in the acrosome reaction was correlated with the porcine sperm concentration. These results indicate that GABA and its receptors can act as modulators of acrosome reaction. This study is the first to report the effects of GABA on porcine sperm function.

KEYWORDS
acrosome reaction, capacitation, GABA (gamma-aminobutyric acid), porcine, sperm

1 | INTRODUCTION

The demand for pig meat is continuously increasing worldwide (Roca et al., 2016). Recently, the use of artificial insemination (AI) in pigs has also increased (Rath, 2002). Thus, it is imperative to enhance fertilization and conception rates after AI. The most widely used AI procedure involves the use of liquid-stored semen, as it is difficult to control the quality of frozen–thawed porcine sperm. Consequently, the demand for porcine semen for AI is relatively high (Rath, 2002; Roca et al., 2016). Therefore, the efficient reproduction of pigs requires control and enhancement of sperm function.

Mammalian sperm cannot fertilize an egg immediately after ejaculation; thus, they must first acquire fertilizing ability by undergoing capacitation when migrating through the female reproductive tract towards the area of fertilization (Yanagimachi & Chang, 1963). During migration, sperm are exposed to various factors, such as hormones, signal-transducing molecules, enzymes, ions, and lipids

secreted by female tissues (Ghersevich et al., 2015). These factors accelerate sperm protein tyrosine phosphorylation and remove cholesterol from the sperm plasma membrane (Visconti, Moore, et al., 1995; Visconti, Ning, et al., 1999). Various factors secreted from the female reproductive tract are involved in this complex process. Previous studies have reported the presence of neurotransmitter receptors in mammalian sperm, including glutamate (Hu et al., 2004), serotonin (Jimenez-Trejo et al., 2012), dopamine (Ramirez et al., 2009), and neurotensin (Hiradate et al., 2014; Umezu et al., 2016). Some of these receptors can be stimulated by the corresponding ligand to regulate sperm capacitation or the acrosome reaction (Hiradate et al., 2014; Meizel & Turner, 1983; Momeni et al., 2021; Ramirez et al., 2009; Umezu et al., 2016). Some of these neurotransmitters have been reported to be cytotoxic (Clement et al., 2002; Kritis et al., 2015) or are too expensive for use in animal production. Among the neurotransmitters, gamma-aminobutyric acid (GABA) is known for its high safety (Oketch-Rabah et al., 2021) and is quite cheap, such that it can be easily used for livestock. We focused on GABA as a regulator of porcine sperm function.

GABA is the most widely distributed inhibitory neurotransmitter in the vertebrate central nervous system (Roberts & Frankel, 1950; Sivilotti & Nistri, 1991). GABAergic signals are transduced into cells via receptors located in the membranes of neuronal cells. To date, three types of GABA receptors have been identified in mammals: types A, B, and C (Bormann, 2000). The expression of GABA and components of GABAergic systems have been identified in many peripheral tissues (Erdo & Wolff, 1990). GABA expression has also been investigated in the genital tract. GABAergic systems have been reported in rodent and human testis (Geigerseder et al., 2003). GABA was detected in human seminal fluid (Ritta et al., 1998) and measured in the rat oviduct (Delrio, 1981).

Our previous study reported that GABA enhanced sperm capacitation and acrosome reaction in mice (Kurata et al., 2019). GABA has been shown to regulate the function of mammalian sperm in cattle and rams (delas Heras et al., 1997; Ritta et al., 2004). GABA receptor expression has also been investigated in human sperm (Wistrom & Meizel, 1993). GABA has been shown to enhance the sperm acrosome reaction in humans, cattle, and rats (Jin et al., 2009; Puente et al., 2011; Shi et al., 1997). In addition, we previously reported a correlation between GABA and sperm concentrations (Kurata et al., 2019).

GABA is an extracellular ligand that can be exogenously added. Because agonists and antagonists have already been used as drugs (Calcaterra & Barrow, 2014; Ertzgaard et al., 2017; Posternak & Mueller, 2001), we believe that modulation of GABA signaling is a highly convenient strategy considering the function of GABA signaling in AI. However, the effect of GABA concentration on porcine sperm remains unknown. Therefore, to assess whether GABA affects porcine sperm function, we investigated the expression of GABA receptors and examined the effects of GABA concentration on porcine sperm.

2 | MATERIAL AND METHODS

2.1 | Porcine sperm preparation

Semen samples (Duroc, aged 1 to 4 years, from 15 different boars) provided from the Miyagi Prefectural Livestock Experiment Station (Miyagi, Japan) were used in this study. Each semen sample was of known fertility and contained at least 60% progressively motile sperm. Each sample was added to 5 ml of 0.1% fatty acid-free bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 137 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4, 2.68 mmol/L KCl, 1.47 mmol/L KH_2PO_4, pH 7.4) and centrifuged at $430 \times g$ for 5 min at 4°C. After centrifugation, the supernatant was discarded, and the pellet was resuspended in porcine fertilization medium (PFM; IFP1010P: Research Institute for the Functional Peptides Co., Yamagata, Japan) at a concentration of 10^7 cells/ml. Each sperm suspension was used for each experiment.

2.2 | Detection of GABA receptor by western blotting

Sperm suspended in PFM (10^7 cells/ml) were collected by centrifugation ($8000 \times g$, 5 min, 4°C). Thereafter, radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 1% protease inhibitor) (Nacalai Tesque, Kyoto, Japan) was added to the sperm pellet, and proteins were extracted by sonication. Each sample was then resuspended in the same volume of $2\times$ sample buffer (Nacalai Tesque), and the solution was boiled for 5 min. Samples of the porcine brain were used as positive controls for the GABA receptor (KOJ 12990106; Cosmo Bio Co., Ltd. Tokyo, Japan). The proteins were separated using 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). We also applied Protein Ladder One Plus, Triple-color for SDS–PAGE (Nacalai Tesque) to calculate molecular weight of the band. Membranes were blocked with Blocking One (Nacalai Tesque) for 60 min. After blocking, the membranes were incubated overnight at 4°C with a primary antibody anti-GABA$_A$R α1–6 (H-300) (#sc-14005, 1:2000; Santa Cruz Biotechnology, Dallas, Texas, USA) or anti-GABA B receptor 2 (ab68426; 1:2000; Abcam, Cambridge, UK). Thereafter, the membranes were washed thrice with PBS containing 0.1% Tween 20 (PBS-T) and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:2,000; Promega, Madison, WI, USA) for 2 h at 25°C. After three washes, the membrane was reacted with Chemilumi One (Nacalai Tesque), and images were obtained using the LAS-3000-mini Lumino Image Analyzer (Fujifilm, Tokyo, Japan). After image analysis, three additional washes with PBS-T, the membranes were incubated in WB Stripping Solution (Nacalai Tesque) for 30 min at 25°C to remove antibodies. The membranes were washed thrice with PBS-T and blocked with Blocking One (Nacalai Tesque) for 60 min. After blocking, the membranes were incubated overnight at 4°C with a rabbit monoclonal anti-alpha-tubulin antibody (ab52866, 1:2000; Abcam) as an internal control. After three washes

with PBS-T, the membranes were incubated with HRP-conjugated anti-rabbit IgG antibody (1:2000; Promega) for 2 h at 25°C. After three washes, the membrane was reacted with Chemilumi One (Nacalai Tesque), and images were obtained using the LAS-3000-mini Lumino Image Analyzer.

2.3 | Detection of GABA$_A$ receptor by immunocytochemistry

Immunocytochemical analysis was performed to investigate the localization of GABA$_A$ receptors in the sperm. Sperm were suspended in PFM (10^7 cells/ml) and collected by centrifugation at 2000 × g for 5 min. For immunostaining, the sperm were fixed with 2% paraformaldehyde and permeabilized with 1% Triton-X 100 (Nacalai Tesque) in PBS for 15 min at 25°C, simultaneously. The cells were washed twice with PBS. The samples were smeared onto glass slides, air-dried, and blocked with Blocking One for 60 min at 25°C. The samples were incubated overnight at 4°C with a primary antibody (anti-GABA$_A$Rα1–6: #sc-14005, 1:200; Santa Cruz Biotechnology). Normal rabbit serum (Nichirei Biosciences Inc., Tokyo, Japan) was used as the negative control (at the same concentration of the primary antibody). The slides were washed three times with PBS and then incubated for 2 h at 25°C with anti-rabbit IgG-Alexa Fluor 555 (1:500; Thermo Fisher Scientific, Waltham, MA, USA), fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) (1:500; J Oil Mills, Tokyo, Japan), and Hoechst 33342 (1:2000; Thermo Fisher Scientific). Finally, the slides were again washed three times with PBS and covered with a glass coverslip. Stained cells were visualized using a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan) using the BZ-X viewer software (Keyence) and counted visually. Duplicate counting of at least 100 sperm cells was performed.

2.4 | Sperm motility assay

GABA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in PBS (10, 100 μM and 1.10 mM) to obtain a final concentration of 0 (PBS), 0.1, 1, 10, and 100 μM. PBS (control) or GABA solutions were then added to the samples (10^7 cells/ml). After 0, 10, 30, 60, and 120 min, 3 μl samples were placed onto four-chamber slides with a depth of 20 μm (SC-20-01-04-B; Leja, Nieuw-Vennep, Netherlands). Sperm cells in three fields of a chamber were divided into motile and dead sperm, and both the percentage of motile sperm and sperm motility parameters were evaluated using a computer-assisted sperm analysis (CASA) system (SMAS, DITECT, Tokyo, Japan) (Tracking ver 7.7.0.416, analysis ver 1.13.0.47, binarized brightness: 95, area [pix]: 15–89, exploration range [pix]: $X = 20$, $Y = 20$). Films were recorded for 1 s, with images captured at intervals of 1/60 s. The sperm motility parameters evaluated were straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), linearity (LIN = VSL/VCL × 100, %), the amplitude of lateral head displacement (ALH, μm), and beat-cross frequency (BCF, Hz).

2.5 | Tyrosine phosphorylation of sperm proteins

The sperm concentration was adjusted to 10^7 cells/ml for each treatment group, and equal volumes of suspension were divided into 1.5 ml microtubes. GABA solutions was added to each suspension at a final concentration 0 (PBS), 0.1, 1, 10, and 100 μM. The suspensions were cultured for 4 h under 5% CO_2 in air at 38.5°C. Following incubation, the suspensions were centrifuged at 8000 × g for 5 min at 4°C, and the sperm pellets were collected. The supernatants were discarded, and the obtained pellets were washed with PBS, and RIPA buffer and 2× sample buffer were added to extract the proteins. The obtained proteins were separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. After blocking for 60 min with 1% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.5–7.8), the membranes were treated overnight at 4°C either with a mouse monoclonal anti-phosphotyrosine 4G10 antibody (#05-321, 1:20,000; Merck Millipore). After three washes with TBS-T, the membranes were treated with HRP-conjugated anti-mouse IgG antibodies (1:2000) for 2 h at 25°C. Detection was performed with Chemilumi One (Nacalai Tesque), and images were obtained using the LAS-3000-mini Lumino Image Analyzer (Fujifilm). After image analysis, three additional washes with PBS-T, the membranes were incubated in WB Stripping Solution (Nacalai Tesque) for 30 min at 25°C to remove antibodies. The membranes were washed thrice with PBS-T and blocked with Blocking One (Nacalai Tesque) for 60 min. After blocking, the membranes were incubated overnight at 4°C with a rabbit monoclonal anti-alpha-tubulin antibody (ab52866, 1:2000) as an internal control. After three washes with PBS-T, the membranes were incubated with HRP-conjugated anti-rabbit IgG antibody (1:2000; Promega) for 2 h at 25°C. After three washes, the membrane was reacted with Chemilumi One (Nacalai Tesque), and images were obtained using the LAS-3000-mini Lumino Image Analyzer (Fujifilm). Densitometric analyses (alpha-tubulin and the tyrosine phosphorylated sperm proteins at 32 kDa) were performed using Image Gauge v4.22 analysis software (Fujifilm).

2.6 | Acrosome reaction assay

Sperm were capacitated in PFM for 4 h, and GABA solution was added to each suspension at the final concentrations of 0 (PBS), 0.1, 1, 10, and 100 μM, and the samples (10^7 cells/ml) were incubated for 30 min at 38.5°C under 5% CO_2 in the air. Thereafter, each sample was smeared onto glass slides and air-dried. After 60 min of blocking using Blocking One, the acrosome reaction was assessed by staining with FITC-PNA (1:500) and Hoechst 33342 (1:2000) in a light-shielded humidity chamber. Thereafter, the slides were washed with PBS and covered with mounting medium and glass coverslips. Bicuculline (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan), a GABA$_A$ receptor antagonist, was dissolved in DMSO and added to sperm suspensions in the presence of PBS or GABA (1 μM). All the groups contained 0.1% DMSO. Sperm acrosomal disappearance rates were evaluated by calculating the number of PNA-negative sperm among total sperm. We regarded sperm that lost more than 80% of their

acrosomes to be acrosome reaction sperm. The acrosome-reacted sperm were counted under a fluorescence microscope (BZ-X710; Keyence). Duplicate counting of at least 100 sperm cells was performed. The percentage of sperm with no fluorescence over the acrosomal region was calculated as the number of PNA-negative sperm cells divided by the total counted sperm.

2.7 | The $[Cl^-]_i$ measurements in porcine sperm

The intracellular concentration of Cl^- ($[Cl^-]_i$) was measured in sperm populations using N-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide (MQAE) Cl^--sensitive fluorescent dye (Dojindo Laboratories, Kumamoto, Japan). Porcine sperm (10^7 cells/ml) were incubated with 5 mM MQAE for 4 h at 38.5°C in PFM. Excess MQAE dye was removed by centrifugation for 3 min at $2000 \times g$. Thereafter, the sperm pellet was resuspended in PFM to a concentration of 10^7 sperm/ml. The influence of different GABA concentrations on $[Cl^-]_i$ was determined using sperm suspensions loaded with MQAE; following the addition of GABA, the fluorescence was measured for a further 0–20 min at 360/510 nm: excitation/emission. The $[Cl^-]_i$ was calculated by measuring sperm suspensions containing different Cl^- concentrations (1–100 mM) using nigericin (5 μM), valinomycin (10 μM), and tributyltin (10 μM).

2.8 | GABA effects in high and low sperm concentrations

Porcine sperm were resuspended in PFM at a concentration of 10^6, 10^7, or 10^8 cells/ml. Sperm were capacitated for 4 h, GABA solution was added to each suspension at the final concentrations of 0 (PBS), 0.1, 1, 10, and 100 μM, and the samples were incubated for 30 min at 38.5°C under 5% CO_2 in the air. After smearing onto glass slides and air-drying, an acrosome reaction assay was conducted.

2.9 | Statistical analysis

All experiments were performed at least thrice by using individual pig. Data are presented as mean ± deviation error (SD). Statistical analyses were performed using analysis of variance (ANOVA), Dunnett test, and the Tukey–Kramer test with Statcel3 software (OMS Publishing, Tokyo, Japan). Differences were considered statistically significant at $P < 0.05$ (*$P < 0.05$).

3 | RESULTS

3.1 | Expression of GABA receptor in sperm

Western blot analysis and immunostaining were performed to determine the expression and localization of GABA receptors in sperm. In porcine sperm and brain protein, the bands were detected around the molecular weight of the GABA$_A$ receptor alpha 1 subunit by western blot analysis using GABA$_A$ receptor antibody (Figure 1a). We also used antibodies against GABA$_B$ receptors, but these could not be detected in the sperm (Figure 1a).

We observed immunoreactivity of the GABA$_A$ receptor in the acrosomal region of sperm (Figure 1b). No positive staining was observed in the negative control (Figure 1b). We observed sperm acrosomal staining patterns by double staining with FITC-PNA and GABA$_A$ receptor antibodies by immunocytochemistry (Figure 1c). The proportion of sperm showing a positive signal for the GABA$_A$ receptor before and after capacitation is shown (Figure 1d). Among the observed sperm, none expressed GABA$_A$ receptor α1 without retaining the acrosome. Furthermore, capacitation increased the number of sperm that underwent the acrosome reaction. The proportion of GABA$_A$ receptor expression among the sperm harboring the acrosome (±SD) was 98.42% ± 1.53% and 93.49% ± 3.20% before and after capacitation, respectively.

3.2 | Effect of GABA on sperm motility

We evaluated the effects of GABA treatment on porcine sperm motility, which is a critical factor for fertilization. Sperm were cultured with GABA at various concentrations (0–100 μM), and sperm motility parameters (VSL, VCL, LIN, ALH, and BCF) were analyzed using the CASA system (Table 1). No significant differences were recorded for any GABA concentration at any time point. These results suggest that GABA does not affect porcine sperm motility.

3.3 | Effects of GABA on sperm protein tyrosine phosphorylation

We investigated whether GABA affected the tyrosine phosphorylation of sperm proteins, a major indicator of sperm capacitation. Sperm were cultured with GABA at various concentrations (0–100 μM) and analyzed by western blotting (Figure 2a). In the tyrosine phosphorylation of the sperm protein at approximately 32 kDa, no significant differences were observed with GABA treatment when compared with the control (Figure 2b).

3.4 | Effect of GABA on the acrosome reaction

We also evaluated the effect of GABA on the acrosome reaction after incubation for 4 h. Sperm were capacitated and further cultured in the presence of various concentrations of GABA. We observed sperm with no fluorescence over the acrosomal region with FITC-PNA staining (Figure 3a). Furthermore, at 1 μM, GABA treatment significantly enhanced the acrosome reaction (Figure 3b). When Bicuculline, a GABA$_A$ receptor-specific antagonist, was added, the GABA-induced acrosome reaction was blocked (Figure 3c). No significant effect was
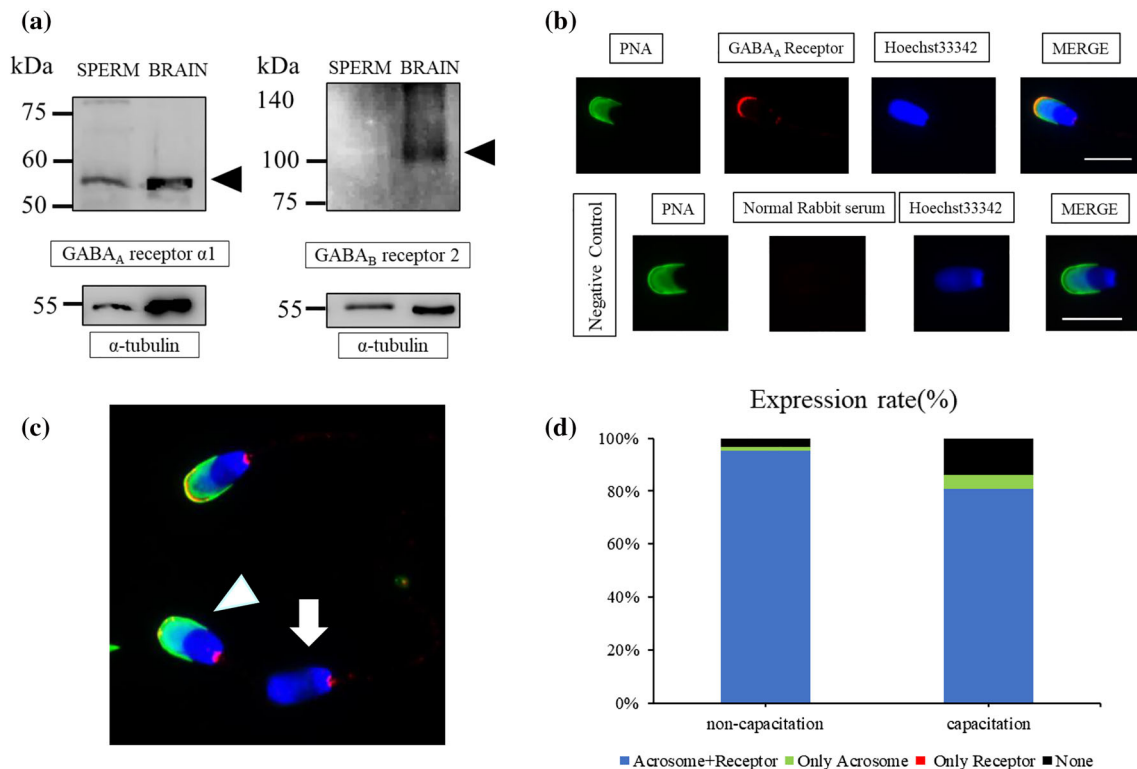
FIGURE 1 Expression of GABA receptor in porcine sperm. (a) GABA receptor expression was analyzed by western blot. Protein extracts were separated by 10% SDS–PAGE followed by GABA receptor detection with specific antibodies. Arrowheads indicate the specific bands corresponding to GABA receptor subunits at the predicted size ($n = 3$). (b) Representative immunocytochemical image showing GABA$_A$ receptor alpha 1 subunit localization in porcine sperm. Blue: nucleus (Hoechst 33342). Green: acrosome (FITC-PNA). Red: GABA$_A$ receptor alpha 1 subunit or normal rabbit serum. Magnification ($\times 100$). Scale bars $= 10\ \mu m$ ($n = 4$). (c) Representative immunocytochemical image showing GABA$_A$ receptor and acrosome expression patterns in porcine sperm. Acrosome staining patterns are visible with double staining. White arrow head indicated only acrome expression pattern in porcine sperm. White arrow indicated neither acrome nor GABA$_A$ receptor expression pattern in porcine sperm. Blue: nucleus (Hoechst 33342). Green: acrosome (FITC-PNA). Red: GABA$_A$ receptor alpha 1 subunit. Magnification ($\times 100$) ($n = 4$). (d) The graphs show the proportion of the GABA$_A$ receptor and acrosome in porcine sperm before and after capacitation ($n = 4$).

observed between the control group and the GABA + Bicuculline addition group.

3.5 | GABA increased $[Cl^-]_i$ in capacitated porcine sperm

We investigated whether GABA affected the $[Cl^-]_i$ of sperm. In the GABA-free control, the $[Cl^-]_i$ of sperm did not increase (Figure 4a). However, we observed that the $[Cl^-]_i$ of sperm was sufficiently elevated by the addition of 1 μM GABA (Figure 4a). Furthermore, the $[Cl^-]_i$ of sperm with 1 μM GABA was significantly higher than that of the other groups at 1 min after the addition (Figure 4b).

3.6 | Acrosome reaction rates in high and low sperm concentrations

GABA was added to different sperm concentrations to determine the relationship between GABA and sperm concentrations (Figure 5).

When the sperm concentration was low (10^6 cells/ml), the effective GABA concentration was 0.1 μM. However, when the sperm concentration increased (10^8 cells/ml), the effective GABA concentration was relatively high (10 μM). Notably, GABA enhanced the sperm acrosome reaction at a specific concentration; however, higher and lower GABA concentrations did not have any significant effect on the sperm acrosome reaction.

4 | DISCUSSION

In this study, we presented for the first time that GABA$_A$ receptor was expressed in the acrosome of porcine sperm by the immunocytochemical analysis (Figure 1a,b) and that a correlation exists between the sperm acrosome and GABA$_A$ receptor localization (Figure 1d). Previous studies have indicated GABA$_A$ receptor localization in both human and mouse sperm (Kurata et al., 2019; Wistrom & Meizel, 1993). However, GABA$_A$ receptor localization in sperm differs between humans and mice. In human sperm, the GABA$_A$ receptor was detected in the equatorial region, whereas in mouse sperm, the

TABLE 1 CASA measurements of the effects of GABA on porcine sperm motility

Sperm motility parameters		Control	GABA			
			0.1 μM	1 μM	10 μM	100 μM
0 min	Motile (%)	49.94 ± 9.75	55.93 ± 15.89	54.85 ± 8.99	55.46 ± 13.59	56.12 ± 20.24
	VSL (μm/s)	21.21 ± 5.11	19.95 ± 4.02	21.11 ± 6.50	18.99 ± 3.62	21.32 ± 7.10
	VCL (μm/s)	149.15 ± 4.48	153.49 ± 39.72	137.59 ± 24.91	129.38 ± 26.71	137.03 ± 15.87
	LIN (%)	17.20 ± 5.31	17.20 ± 5.59	20.00 ± 7.18	17.40 ± 6.43	20.00 ± 5.39
	ALH (μm)	4.56 ± 0.48	4.48 ± 1.06	4.26 ± 0.77	3.83 ± 0.42	4.11 ± 0.92
	BCF (Hz)	14.18 ± 0.89	14.04 ± 0.86	14.37 ± 1.10	14.49 ± 1.27	14.08 ± 0.90
10 min	Motile (%)	47.13 ± 16.28	50.30 ± 8.97	59.28 ± 13.23	53.50 ± 13.09	49.47 ± 25.41
	VSL (μm/s)	28.10 ± 8.46	22.79 ± 2.21	18.88 ± 6.26	24.53 ± 6.89	27.03 ± 7.85
	VCL (μm/s)	134.76 ± 46.19	138.72 ± 41.77	132.10 ± 39.99	131.67 ± 49.10	128.02 ± 38.62
	LIN (%)	25.80 ± 9.42	21.00 ± 8.94	21.80 ± 4.82	23.20 ± 7.85	23.60 ± 6.11
	ALH (μm)	3.84 ± 1.35	3.99 ± 1.54	4.14 ± 1.09	3.96 ± 1.29	3.79 ± 1.17
	BCF (Hz)	16.01 ± 1.79	16.05 ± 1.20	15.39 ± 0.88	15.71 ± 0.67	15.97 ± 1.78
30 min	Motile (%)	48.67 ± 18.37	51.23 ± 14.48	48.46 ± 21.78	48.12 ± 19.91	44.29 ± 16.92
	VSL (μm/s)	26.01 ± 12.46	24.55 ± 4.85	24.09 ± 4.41	23.18 ± 5.68	20.38 ± 6.98
	VCL (μm/s)	130.56 ± 38.69	134.24 ± 38.17	123.87 ± 27.62	127.74 ± 30.04	132.18 ± 25.05
	LIN (%)	23.40 ± 6.58	21.60 ± 3.78	24.40 ± 7.30	21.00 ± 2.24	19.20 ± 5.81
	ALH (μm)	3.86 ± 1.25	3.82 ± 0.92	3.45 ± 1.12	3.82 ± 0.86	4.10 ± 0.75
	BCF (Hz)	16.33 ± 1.11	16.26 ± 0.81	16.54 ± 1.53	16.26 ± 1.82	16.39 ± 1.75
60 min	Motile (%)	33.24 ± 12.90	22.77 ± 15.31	28.48 ± 15.64	25.68 ± 15.13	23.87 ± 13.98
	VSL (μm/s)	19.44 ± 8.20	18.71 ± 10.69	13.67 ± 2.55	20.75 ± 10.03	19.16 ± 6.06
	VCL (μm/s)	110.65 ± 15.41	119.87 ± 28.24	99.32 ± 33.91	97.27 ± 19.42	111.68 ± 51.04
	LIN (%)	25.25 ± 13.23	21.50 ± 5.20	21.25 ± 5.32	26.00 ± 3.74	24.50 ± 7.72
	ALH (μm)	3.45 ± 0.66	3.48 ± 0.85	3.09 ± 0.95	3.43 ± 1.19	3.47 ± 1.50
	BCF (Hz)	17.44 ± 0.85	17.33 ± 2.20	16.28 ± 1.29	17.43 ± 1.68	17.53 ± 1.99
120 min	Motile (%)	19.81 ± 7.99	27.43 ± 8.88	23.80 ± 10.76	19.06 ± 14.07	23.07 ± 13.03
	VSL (μm/s)	25.98 ± 12.16	13.62 ± 4.88	9.99 ± 4.36	17.84 ± 11.05	19.07 ± 9.10
	VCL (μm/s)	113.52 ± 16.54	110.75 ± 24.84	92.75 ± 24.45	126.83 ± 22.35	147.07 ± 23.12
	LIN (%)	27.25 ± 10.24	18.75 ± 5.91	19.50 ± 3.51	19.25 ± 11.59	18.50 ± 6.95
	ALH (μm)	3.42 ± 1.23	3.80 ± 1.29	2.64 ± 0.67	3.90 ± 0.53	4.04 ± 0.64
	BCF (Hz)	17.30 ± 1.18	17.16 ± 0.45	17.00 ± 2.45	17.56 ± 2.29	18.67 ± 1.90

Notes: Porcine sperm were incubated with various GABA concentrations (final concentration: 0 [Control], 0.1, 1, 10, and 100 μM) for 0, 10, 30, 60, and 120 min. Data are shown as mean ± SD. Motile, sperm motility (%); VSL, straight-line velocity (μm/s); VCL, curvilinear velocity (μm/s); LIN, linearity (%); ALH, amplitude of lateral head displacement (μm); BCF, beat-cross frequency (Hz) ($n = 4$).

GABA$_A$ receptor was detected in the acrosomal cap. We demonstrated the GABA$_A$ receptor expression in porcine sperm was observed in acrosome, similar to mice (Figure 1b). GABA$_A$ receptors were expressed in almost all sperms harboring acrosomes, before and after capacitation, respectively (Figure 1d). In addition, we observed none of the sperms expressed GABA$_A$ receptor α1 without retaining the acrosome. This indicates that the GABA signal is transduced into sperm via the GABA$_A$ receptor on the acrosome. Furthermore, GABA and its receptor-mediated signal transduction system are thought to contribute to sperm function.

GABA concentrations have been reported in the female reproductive tract, in rats and humans (Delrio, 1981; Erdo et al., 1989; Louzan

et al., 1986). GABA (around 100–300 μM) was measured in fluid in the ovarian bursa (Louzan et al., 1986). GABA concentrations in human seminal plasma were also detected, incubation with a high μM concentration of GABA and low μM concentrations of the GABA$_A$ receptor agonist were sufficient to significantly increase sperm hyperactivity (Ritta et al., 1998). By these reports, we inferred the physiological concentration of GABA in the mammalian reproductive fluid was 0.1 μM to 1 mM. GABA concentration in the female reproductive tract changes during the estrous cycle (Forray et al., 1993; Louzan et al., 1986). So we thought changes in the GABA concentrations may affect sperm function.

Tyrosine phosphorylation of several sperm proteins is induced during capacitation (Visconti, Bailey, et al., 1995). In porcine sperm, it

FIGURE 2 Effect of GABA on tyrosine phosphorylation of sperm proteins. Protein tyrosine phosphorylation in porcine sperm incubated with various GABA concentrations (final concentration: 0 [Control], 0.1, 1, 10, and 100 μM) was determined by western blotting. (a) The panel shows a representative blot image. (b) Quantification and comparison of a tyrosine phosphorylated protein band of approximately 32 kDa between control and GABA-treated sperm. α-Tubulin was used as an internal control. We applied the Dunnett test. Data are shown as the mean ± SD (n = 4).
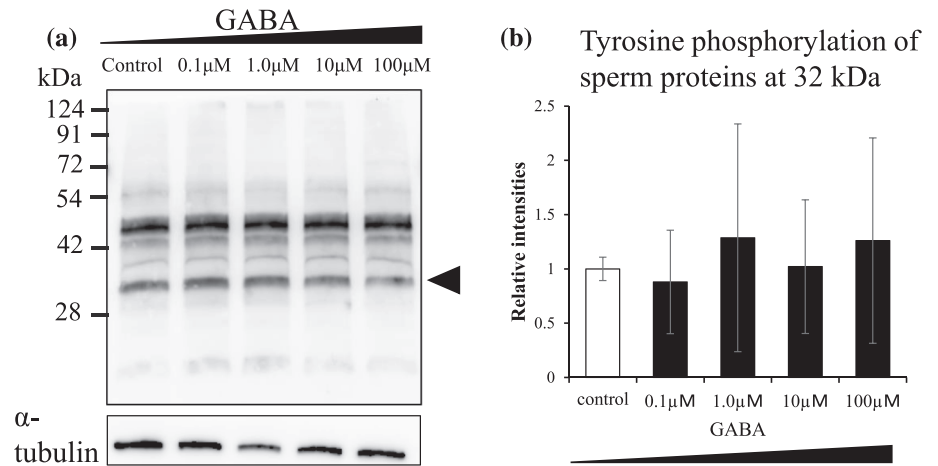
FIGURE 3 Effect of GABA on the acrosome reaction. (a) Representative image of acrosome-reacted (PNA-negative) sperm and not reacted (PNA-positive) sperm. Blue: nucleus (Hoechst 33342). Green: acrosome (FITC-PNA). Magnification (×20). (b) GABA administration enhanced the acrosome reaction. Porcine sperm were capacitated in PFM for 4 h. PBS or various concentrations of GABA dissolved in PBS were added (final concentration: 0 [Control], 0.1, 1, 10, and 100 μM) to the suspension (1000 μl: 10^7 cells/ml) and cultured for 30 min. We applied the Dunnett test. Data are shown as the mean ± SD (*P < 0.05) (n = 4). (c) The effect of Bicuculline, a GABA$_A$ receptor-specific antagonist. A sperm suspension was cultured with DMSO or Bicuculline (1 μM) dissolved in DMSO in the presence of PBS or GABA (1 μM). All the groups contained 0.1% DMSO. Sperm acrosomal disappearance rates were evaluated by calculating the number of PNA-negative sperm among total sperm. The Tukey–Kramer test was applied. Data are shown as the mean ± SD (*P < 0.05) (n = 4).
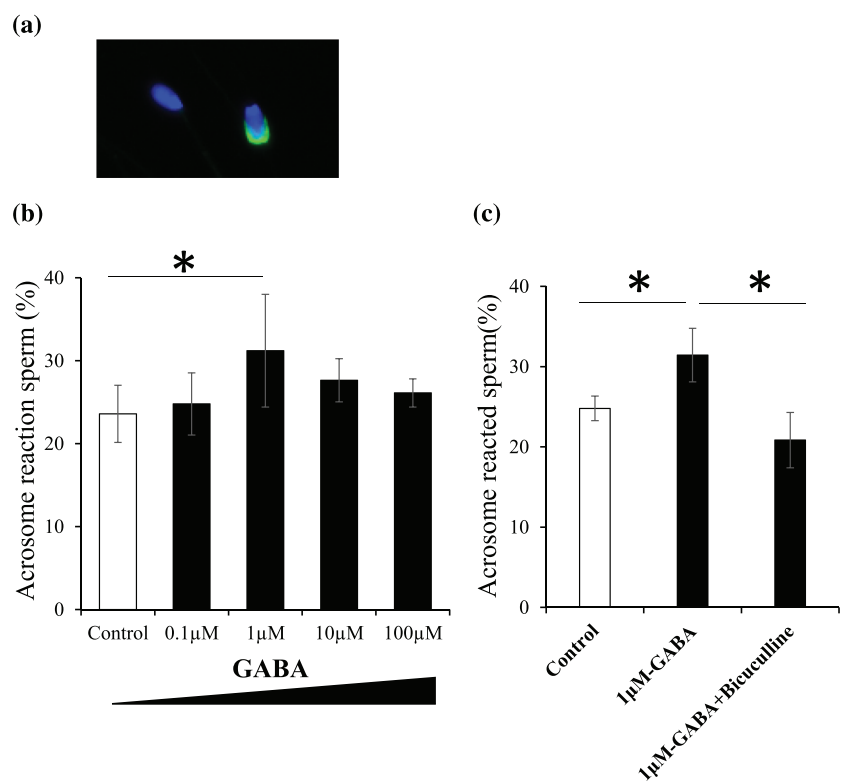
was reported that the tyrosine phosphorylation of acrosin-binding protein (at 32 kDa) was related to capacitation (Kerns et al., 2018). Our results showed that tyrosine phosphorylation of sperm proteins at 32 kDa was not significantly different following treatment with GABA (Figure 2). Furthermore, GABA did not exert any significant effect on sperm motility (Table 1). We showed that the acrosome reaction in capacitated sperm was facilitated by treatment with 1 μM GABA (Figure 3b), which was blocked by Bicuculline, a GABA$_A$ receptor-specific antagonist (Figure 3c). These results suggest that GABA and GABA$_A$ receptors influence the regulation of sperm function.

GABA may stimulate acrosome reactions in capacitated sperm through its interaction with GABA$_A$ receptors. The Cl$^-$ channel and

GABA$_A$ receptor co-exist as a GABA$_A$ receptor-chloride ion channel complex. GABA binding to GABA$_A$ receptors opens chloride ion channel, resulting in Cl$^-$ influx and an increase in [Cl$^-$]$_i$ in sperm. We showed that certain concentrations of GABA increased the concentration of [Cl$^-$]$_i$ in porcine sperm (Figure 4). It was reported that GABA addition immediately induced an increase in bull sperm [Ca^{2+}]$_i$ (Ritta et al., 2004). Db-cAMP/IBMX addition immediately induced an increase in mouse sperm [Cl$^-$]$_i$ (Chavez et al., 2012). The membrane potential of mouse sperm changed immediately after valinomycin addition and returned within a few minutes (Chavez et al., 2012). Therefore, we think that porcine sperm [Cl$^-$]$_i$ increased immediately (1 min) and returned to normal within a few minutes (after 2 min) for only the effective GABA concentration (Figure 4).
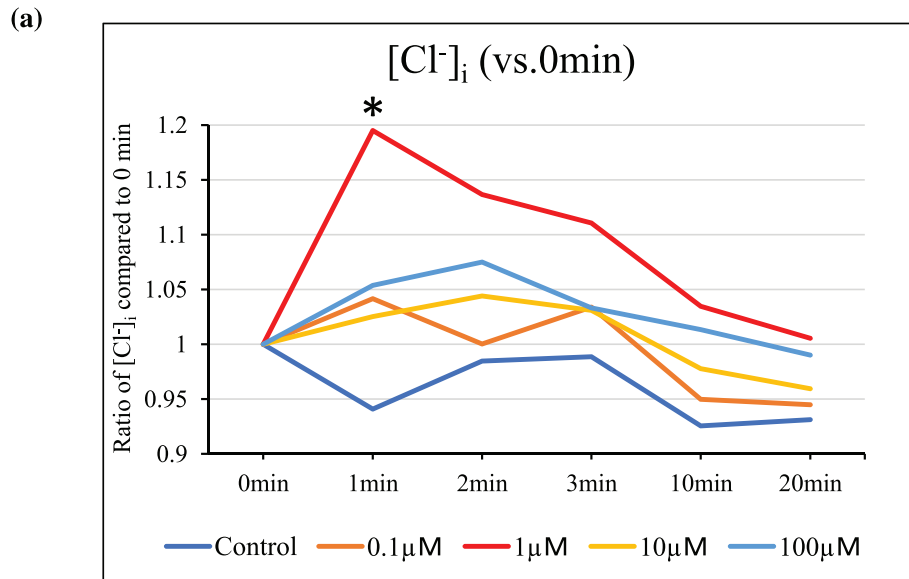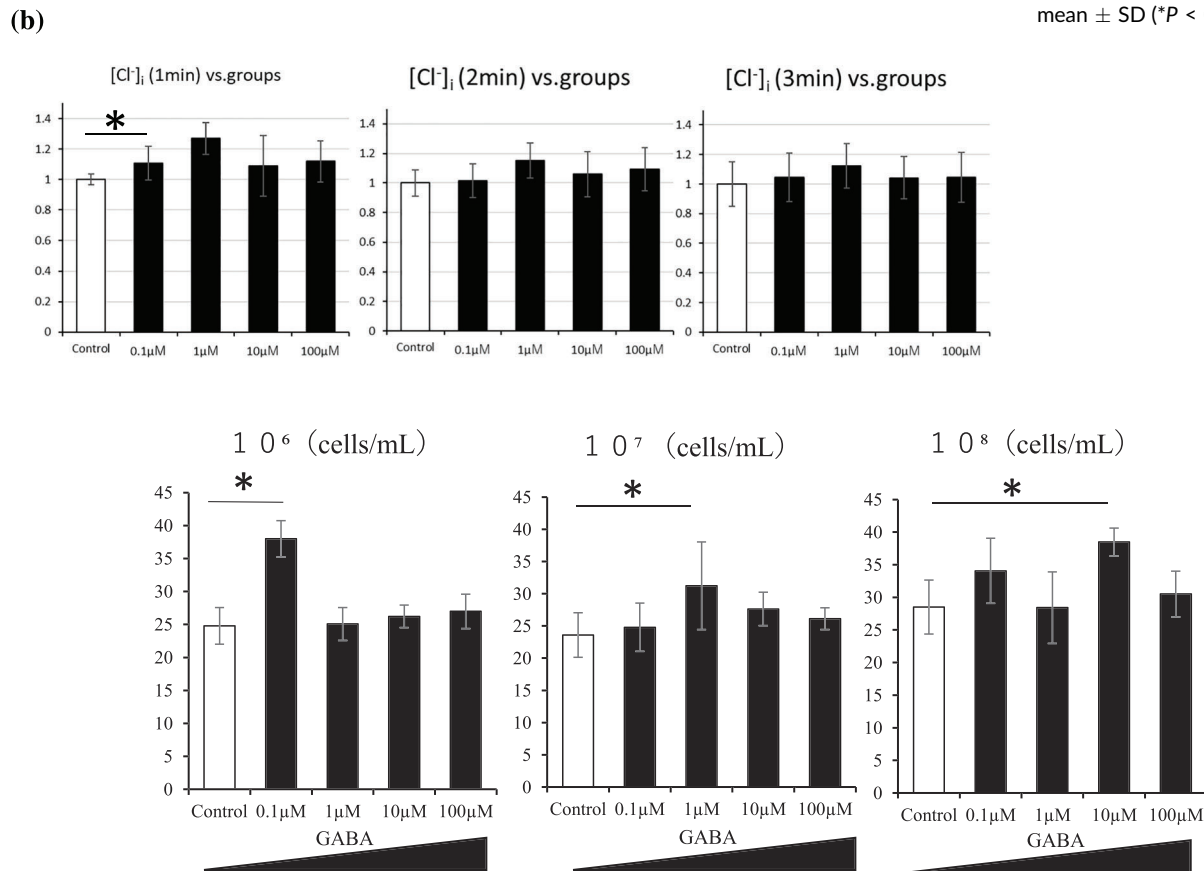
(a)

(b)

FIGURE 5 Effect of GABA on the acrosome reaction at three sperm concentrations. Rates of acrosome-reacted (PNA-negative) sperm at three sperm concentrations (10^6, 10^7, or 10^8 cells/ml) treated with various GABA concentrations (final concentration: 0 [Control], 0.1, 1, 10, and 100 μM). Sperm acrosomal disappearance rates were evaluated by calculating the number of PNA-negative sperm among total sperm. We applied the Dunnett test. Data are shown as mean $\pm$ SD (*$P < 0.05$) ($n \geqq 3$)

Chloride channels are involved in acrosome reactions in mammalian sperm (Garcia & Meizel, 1999). It was discussed that Cl^- influx may increase intracellular other ions (Wistrom & Meizel, 1993). There is a close relationship between HCO_3^- transport and activation of GABA receptor (Jin et al., 2009). It was provided evidence that Cl^- and HCO_3^- are essential for inducing rat sperm acrosome reaction by GABA through $GABA_A$ receptor/Cl^- channel (Jin et al., 2009). $GABA_A$ receptors may open voltage-activated Ca^{2+} channels and mediate

elevation of Ca^{2+} (Ritta et al., 2004; Williams et al., 2000). Both of HCO_3^- and Ca^{2+} have an important role in the acrosome reaction (Breitbart, 2002; Visconti, Stewart-Savage, et al., 1999). So these researches suggest GABA promotes the acrosome reaction in sperm by ions transport and replacement. Further, studies in mice, cattle, and rats strongly indicated that GABA promotes the acrosome reaction in sperm (Jin et al., 2009; Kurata et al., 2019; Puente et al., 2011).

The results of the present study demonstrate that GABA induces the acrosome reaction at 1 μM, compared with other GABA concentrations. Different concentrations of GABA were added to different sperm concentrations to determine the relationship between GABA and sperm concentrations (Figure 5). When the sperm concentration was low, the effective GABA concentration was relatively low. Furthermore, when sperm concentration increased, the effective GABA concentration was relatively high. Therefore, when the sperm concentration was changed, the GABA concentration that promoted the acrosome reaction rate also changed. These results suggest that there is an optimal concentration range for the induction of the acrosome reaction in sperm cells (Figure 5). We expected that when the sperm concentration (sperm amount) changed, the required effective concentration (required amount) also changed. Notably, GABA enhanced the sperm acrosome reaction at a specific concentration. Lower and higher concentrations did not affect sperm acrosome reaction. However, high concentrations of GABA at low concentrations of sperm did not significantly decrease the acrosome reaction rate compared with the control (Figure 5).

We demonstrated that the effect of GABA on porcine sperm was biphasic (Figures 3–5). Furthermore, it has been reported that GABA has a biphasic effect in human, mouse, and rat sperm models, especially in acrosome reactions (Hu et al., 2002; Kurata et al., 2019; Shi et al., 1997). There are several hypotheses about this mechanism and the biphasic effect of GABA on the enhancement of the acrosome reaction. This biphasic effect could be due to the relative effects of $GABA_A$ and $GABA_B$ receptors in rat sperm (Hu et al., 2002). However, similar to that in mice (Kurata et al., 2019), the localization of $GABA_B$ receptors could not be confirmed in porcine sperm (Figure 1b). Our study indicated porcine sperm $[Cl^-]_i$ was not increased in high concentration of GABA (Figure 4). It means high concentration of GABA compared with sperm, high GABA binding on sperm $GABA_A$ receptors, was very little effect on sperm $[Cl^-]_i$. We thought that the reason why the sperm $[Cl^-]_i$ did not increase by high GABA concentration was that the $GABA_A$ receptor-chloride ion channel complex did not work effectively. So we hypothesize that high concentration of GABA compared with sperm may suppress the $GABA_A$ receptor-chloride ion channel complex function. And sperm interaction may change effective GABA concentration. It was known porcine sperm concentrations during preincubation affected oocytes penetration rate (Nagai et al., 1984). Therefore, further studies on the molecular mechanism of GABA signaling including high concentration in porcine sperm are required.

Thus, the results of the current study demonstrate that the exogenous addition of GABA affects porcine sperm function. However, the effect of GABA on fertilization has not yet been reported.

Nevertheless, many reports show that GABA improves sperm function in many species (delas Heras et al., 1997; Hu et al., 2002; Kon et al., 2014; Kurata et al., 2019; Ritta et al., 2004; Shi et al., 1997). So addition of GABA to promote the sperm acrosome reaction at the time of ovulation may lead to improved conception rates in pigs. It is easy to add GABA artificially. Therefore, injection of GABA into the female affects sperm function and may help improve the conception rate.

GABA is known to be highly safe (Oketch-Rabah et al., 2021) and foods containing GABA are commercially available. The application of GABA in livestock and animal production is considered to be easy to understand for consumers. Therefore, improving sperm function by adding GABA may directly contribute to the improvement of livestock productivity. As described above, we consider modulation of GABA signaling to be a highly convenient strategy considering its application in AI. The results of our study might aid in the development of new AI technology, which will contribute to efficient pig production in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interests for this article.

ORCID

Shouhei Kurata https://orcid.org/0000-0002-9379-8829
Kenshiro Hara https://orcid.org/0000-0001-8531-8349

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