Capacitation of mouse sperm is modulated by gamma-aminobutyric acid (GABA) concentration

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Abstract. In mammals, ejaculated sperm acquire their fertilizing ability during migration through the female reproductive tract toward the area of fertilization [1]. During migration, sperm are exposed to various factors such as hormones, signal transducing molecules, enzymes, ions, and lipids secreted from female tissues [2]. These factors accelerate sperm tyrosine phosphorylation and remove cholesterol from the sperm plasma membrane [3, 4]. Many factors secreted from the female reproductive tract are involved in this complex process.

Several reports have provided evidence for the presence of neurotransmitter receptors in mammalian sperm, including those for dopamine [5], serotonin [6], and neurotensin [7, 8]. Some of these receptors can be stimulated by the corresponding ligand to regulate sperm capacitation or the acrosome reaction [5–8].

Among the neurotransmitters, gamma-aminobutyric acid (GABA) has emerged as a putative modulator of sperm function. GABA is the most widely distributed inhibitory neurotransmitter in the vertebrate central nervous system, and it is estimated that 20–50% of all synapses in the mammalian brain are GABAergic [9–11].

GABAergic signals are transduced into the cell via receptors located in the membranes of neuronal cells. In mammals, at least three types of GABA receptors have been identified, namely, types A, B, and C [12]. The GABA A receptor is a supramolecular receptor complex linked to a Cl− channel, the activation of which produces a transmembrane Cl− ion flux that is antagonized by bicuculline [13]. The GABA B receptor belongs to the G protein-coupled receptor superfamily [14]. While inhibition of the GABA B receptor is mediated by the indirect gating of either potassium or calcium channels, the GABA B receptor is activated by baclofen and antagonized by phaclofen [15]. Like GABA A, the GABA C receptor, the third member of the GABA receptor family [16], is also a ligand-gated chloride channel. However, pharmacologically, the GABA C receptor is insensitive to bicuculline and baclofen and is not regulated by many GABA A modulators, including benzodiazepines and barbiturates [17].

The expression of GABA and the components of GABAergic systems has been identified in many peripheral tissues, including the gastrointestinal tract and kidney [18–20]. GABA is generated from L-glutamate by glutamate decarboxylase (GAD). GAD has two isoforms, GAD67 (GAD1) and GAD65 (GAD2). GAD67 is present in the testes and GAD65 in the oviduct [21, 22]. GABA expression has also been investigated in the genital tracts of male and female rats, as well as in human sperm and seminal fluid [23–25]. Immunohistochemical studies indicate that GABA is localized to the mucosal layer of the rat oviduct [24].

There is some evidence that the GABA A receptor is expressed in...
human sperm and that GABA is secreted from the rat reproductive tract [24]. Furthermore, GABA has been shown to enhance the sperm acrosome reaction in both cow and rat [26, 27]. Although GABA concentration in the rat oviduct is regulated during the estrus cycle [28], the effect of GABA concentration on sperm remains unknown. Therefore, we investigated the expression of GABA receptors and examined the effects of GABA concentration on mouse sperm. Additionally, we also analyzed GABA expression and regulation in the reproductive organs of female mice.

Materials and Methods

Mouse sperm preparation

C57BL/6N mice were purchased from Japan SLC (Shizuoka, Japan). Cauda epididymal sperm were collected from male mice (aged between 12 and 52 weeks) euthanized in accordance with the Guide for the Care and Use of Laboratory Animals published by Tohoku University. Dissected cauda epididymis was punctured with needle and exposed sperm cells were transferred into 500 µl of human tubal fluid (HTF) medium [23], consisting of 101.6 mM NaCl, 4.7 mM KCl, 0.37 mM K2PO4, 0.2 mM MgSO4, 7H2O, 2 mM CaCl2, 25 mM NaHCO3, 2.78 mM glucose, 0.33 mM pyruvate, 21.4 mM sodium lactate, 286 mg/l penicillin G, 228 mg/l streptomycin, and 5 mg/ml fatty acid-free bovine serum albumin (BSA). For capacitation, the medium containing sperm cells was incubated at 37°C for 90 min for 10 min, the sperm concentration was adjusted as necessary for subsequent experiments. All the experiments were performed using pooled semen samples from three mice.

Western blotting

Sperm suspended in HTF medium were collected by centrifugation (8000 × g, 5 min, 4°C). RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% protease inhibitor) (Nacalai Tesque, Kyoto, Japan) was added to the sperm pellet followed by sonication to extract proteins. Samples of mouse brain or retina were used as positive controls. The samples were centrifuged (8000 × g, 5 min, 4°C), and the supernatants collected. The supernatant was resuspended in the same volume of 2 × sample buffer (Nacalai Tesque) and boiled for 5 min. Proteins were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with Blocking One (Nacalai Tesque) for 60 min at room temperature. After three washes with PBS-T, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG antibodies (1:5,000; Abcam) as a internal control. After washing three times with PBS, the membranes were reacted with Chemilumi One (Nacalai Tesque), and Hoechst 33342 (1:5,000; Thermo Fisher Scientific). Finally, treated samples were washed with PBS, mounted on glass slides, and covered with a glass coverslip. Stained cells were visualized using a fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan) and counted.

Immunocytochemistry

Immunocytochemistry was performed to investigate GABA A receptor localization in mouse sperm. Cauda epididymal sperm were suspended in HTF medium and collected by centrifugation at 2,000 × g for 5 min. Sperm were then fixed with 2% paraformaldehyde (PFA) and permeabilized with 1% Triton X-100 in PBS for 15 min at room temperature. The sperm were then washed twice with PBS and blocked with Blocking One for 60 min at room temperature. The suspensions were incubated with a primary antibody (anti-GABA A receptor alpha 1; 1:100) overnight at 4°C. After being washed three times with PBS, the suspensions were incubated for 2 h at room temperature with anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific, Waltham, MA, USA), fluorescein isothiocyanate-conjugated peanut agglutinin (FITC–PNA) (J Oil Mills, Tokyo, Japan) [29], and Hoechst 33342 (1:5,000; Thermo Fisher Scientific). Finally, treated samples were washed with PBS, mounted on glass slides, and covered with a glass coverslip. Stained cells were visualized using a fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan) and counted.

Acrosome reaction assay

Sperm were capacitated in HTF medium for 90 min and equal volumes of the suspension were then divided into microtubes. GABA stock solution was added to each suspension at final concentrations of 0.1, 1, 10, and 100 µM and the samples incubated for 30 min at 37°C under a humidified atmosphere containing 5% CO2. Subsequently,
Effects of GABA on tyrosine phosphorylation of sperm proteins

We investigated whether GABA affected the tyrosine phosphorylation of sperm proteins, the major indicator of sperm capacitation. Sperm were cultured with GABA at various concentrations (0–100 µM). After 0, 10, 30, and 60 min, 4-µl samples were placed onto 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 [29] diluted 1:500 in a light-shielded humidity chamber. After washing with PBS, the slides were covered with mounting medium and glass coverslips. Bicuculline, a GABA A receptor antagonist, was dissolved in DMSO and cultured with sperm suspensions in the presence of either saline 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 acrosome-reacted sperm were counted using a confocal laser scanning fluorescence microscope (LSM700; Carl Zeiss, Jena, Germany). Duplicate counting of at least 100 sperm cells was performed. The percentage of sperm with no fluorescence in the acrosomal region was calculated as the number of PNA-negative sperm cells per total counted sperm.

Sperm motility assay

After incubation for 90 min to allow capacitation, GABA was added to each suspension at the final concentrations of 1, 10, and 100 µM. After 0, 10, 30, and 60 min, 4-µl samples were placed onto four-chamber slides, 12 µm deep (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). Films were recorded for 1 sec, with images captured at intervals of 1/60 sec. The sperm motility parameters evaluated were straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), linearity (LIN = VSL/VCL × 100, %), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz). Sperm trajectories were automatically extracted from the movie and overlaid on the last frame using the CASA system.

Immunohistochemical staining of oviduct and uterus

Sexually matured 7- or 8-week-old female mice were primed with 5 IU of equine chorionic gonadotropin (PMSG) for 48 h and 5 IU of human chorionic gonadotropin (hCG) for 14 h. After cervical dislocation, the oviduct and uterus were dissected and fixed in 4% PFA in PBS overnight at 4°C. The fixed tissues were dehydrated and embedded in paraffin, following which 4-µm sections were cut and mounted on glass slides. After deparaffinization and rehydration, antigens were retrieved in HistoVT One (Nacalai Tesque) for 30 min at 90°C. The slides were then blocked with 1% BSA-PBS for 60 min and incubated overnight at 4°C with a rabbit polyclonal primary antibody against GABA (Ab8891; 1:100; Abcam). For an antibody preabsorption test, the anti-GABA antibody was preincubated with a 50-fold excess of antigen-blocking peptide (GABA) overnight at 4°C and used as a negative control. After washing in TBS-T, the slides were covered with glass coverslips. Stained tissues were visualized using a confocal laser scanning fluorescence microscope (LSM700; Carl Zeiss).
μM) and analyzed by western blotting. As shown in Fig. 2A, tyrosine phosphorylation of sperm proteins of around 30, 50, and 70 kDa increased following incubation with 1 μM GABA for 90 min. The protein at 110 kDa was not affected by capacitation [3, 30]. The total amount of tyrosine phosphorylated sperm proteins incubated in HTF medium with BSA (Fig. 2A) and without BSA (Fig. 2B) was increased by GABA application. These results suggest that exposing sperm to GABA enhances capacitation, likely through interaction between GABA and the GABA A receptor.

Effect of GABA on the acrosome reaction

We also evaluated the effect of GABA on the acrosome reaction after incubation for 90 min. Sperm were capacitated and further cultured in the presence of various concentrations of GABA. We observed sperm with no fluorescence over the acrosomal region (arrowhead). Blue: nucleus (Hoechst 33342); green: GABA A receptor alpha 1 subunit; red: GABA A receptor antibody (red). The panel shows the proportion of the GABA A receptor and acrosome in mouse sperm. Blue: nucleus (Hoechst 33342); green: acrosome (FITC–PNA); red: GABA A receptor alpha 1 subunit. Scale bar = 10 μm.

Effect of GABA on sperm motility

We evaluated the effects of GABA treatment on sperm motility, which is critical for fertilization. When sperm movement shifts to hyperactivated motility, the flagellar bend and beat patterns change to asymmetric and acquire a higher amplitude; this is reflected in the motility parameters, namely, increased VCL and ALH, and decreased LIN [8]. Using the CASA system, we observed increasing tendencies in VSL and VCL with 1 μM GABA treatment for 60 min. However, no significant difference was recorded with any GABA concentration at any time point (Table 1). These results suggest that GABA does not affect sperm motility.

Acrosome reaction rates for high and low sperm concentrations

To determine the relationship between GABA and sperm concentrations, GABA solutions were added to different sperm concentrations (Fig. 4). When the sperm concentration was low (5 × 10^5 cells/ml), the effective GABA concentration was 0.1 μM. When the sperm concentrations increased, the effective GABA concentration was shifted higher. While GABA enhanced the sperm acrosome reaction...
at a specific concentration, higher concentrations had no effect. These results suggest that there is an optimal concentration range for the induction of the acrosome reaction in sperm cells (Fig. 4).

**GABA expression in the uterus and oviduct**

GABA immunoreactivity was detected in luminal epithelial cells of the oviduct. This reactivity was observed throughout the epithelium (Fig. 5A), suggesting that this was the source of GABA secretion. In the uterus, immunoreactivity was also detected in epithelial cells (Fig. 5B). Negative control sections incubated with GABA antibody and epitope-blocking peptide exhibited no positive staining.

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**Table 1.** CASA measurements of the effects of GABA on mouse sperm motility

| Sperm motility parameters | Control | 0.1 µM | 1 µM | 10 µM | 100 µM |
|---------------------------|---------|--------|------|-------|--------|
| 0 min Motile (%)         | 54.66 ± 4.59 | 51.77 ± 3.75 | 60.13 ± 1.20 | 47.67 ± 3.67 | 52.06 ± 3.14 |
| VSL (µm/sec)             | 28.30 ± 2.80 | 20.30 ± 2.00 | 19.33 ± 1.79 | 21.79 ± 2.21 | 22.77 ± 3.62 |
| VCL (µm/sec)             | 108.84 ± 6.18 | 71.19 ± 9.15 | 72.86 ± 4.27 | 83.98 ± 8.91 | 92.09 ± 15.86 |
| LIN (%)                  | 0.30 ± 0.04 | 0.35 ± 0.05 | 0.34 ± 0.04 | 0.31 ± 0.03 | 0.30 ± 0.03 |
| ALH (µm)                 | 3.53 ± 0.15 | 2.65 ± 0.42 | 2.46 ± 0.12 | 2.87 ± 0.20 | 2.95 ± 0.46 |
| BCF (Hz)                 | 11.90 ± 0.26 | 12.21 ± 0.24 | 12.36 ± 0.09 | 11.85 ± 0.40 | 11.99 ± 0.62 |
| 10 min Motile (%)        | 60.95 ± 4.83 | 54.63 ± 3.66 | 60.48 ± 4.65 | 54.78 ± 2.12 | 58.50 ± 3.00 |
| VSL (µm/sec)             | 24.39 ± 4.93 | 25.01 ± 1.54 | 21.80 ± 2.35 | 21.13 ± 1.31 | 25.81 ± 2.37 |
| VCL (µm/sec)             | 87.93 ± 8.25 | 105.03 ± 5.28 | 78.48 ± 8.68 | 86.16 ± 8.78 | 95.21 ± 5.20 |
| LIN (%)                  | 0.32 ± 0.05 | 0.28 ± 0.04 | 0.32 ± 0.04 | 0.31 ± 0.02 | 0.32 ± 0.04 |
| ALH (µm)                 | 3.00 ± 0.18 | 3.39 ± 0.18 | 2.80 ± 0.22 | 3.06 ± 0.35 | 3.15 ± 0.19 |
| BCF (Hz)                 | 12.74 ± 0.05 | 12.09 ± 0.32 | 12.01 ± 0.16 | 12.41 ± 0.23 | 12.03 ± 0.16 |
| 30 min Motile (%)        | 57.75 ± 5.23 | 60.12 ± 2.80 | 52.76 ± 2.11 | 54.50 ± 2.75 | 57.58 ± 2.22 |
| VSL (µm/sec)             | 21.75 ± 1.89 | 21.68 ± 1.43 | 18.76 ± 1.43 | 25.55 ± 2.80 | 28.08 ± 3.01 |
| VCL (µm/sec)             | 85.74 ± 4.72 | 81.60 ± 3.08 | 74.58 ± 6.02 | 92.06 ± 9.79 | 105.36 ± 14.60 |
| LIN (%)                  | 0.34 ± 0.04 | 0.33 ± 0.02 | 0.34 ± 0.05 | 0.29 ± 0.02 | 0.31 ± 0.05 |
| ALH (µm)                 | 2.92 ± 0.12 | 2.88 ± 0.20 | 2.59 ± 0.18 | 3.04 ± 0.27 | 3.46 ± 0.41 |
| BCF (Hz)                 | 12.83 ± 0.32 | 12.12 ± 0.18 | 12.27 ± 0.30 | 12.17 ± 0.33 | 12.11 ± 0.16 |
| 60 min Motile (%)        | 50.38 ± 3.32 | 45.40 ± 7.00 | 43.35 ± 6.32 | 50.64 ± 4.01 | 45.64 ± 6.26 |
| VSL (µm/sec)             | 25.81 ± 1.74 | 37.66 ± 13.95 | 39.46 ± 12.41 | 32.18 ± 9.38 | 29.68 ± 5.65 |
| VCL (µm/sec)             | 98.94 ± 8.35 | 125.73 ± 39.37 | 131.70 ± 34.62 | 118.41 ± 23.48 | 125.17 ± 18.43 |
| LIN (%)                  | 0.28 ± 0.03 | 0.32 ± 0.02 | 0.30 ± 0.02 | 0.28 ± 0.03 | 0.24 ± 0.02 |
| ALH (µm)                 | 3.39 ± 0.31 | 4.44 ± 1.46 | 4.86 ± 1.69 | 4.30 ± 0.89 | 4.48 ± 0.93 |
| BCF (Hz)                 | 12.16 ± 0.23 | 12.50 ± 0.11 | 11.81 ± 0.34 | 12.42 ± 0.27 | 11.69 ± 0.41 |

Data are shown as the means ± SE. VSL, straight line velocity; VCL, curvilinear velocity; LIN, linearity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
To determine which reproductive tissue might regulate GABA release, we performed quantitative RT-PCR. GAD67 (Gad1) mRNA was detected in the oviduct and uterus, with higher levels in the oviduct (Fig. 6A). However, GAD65 (Gad2) mRNA was not detected in the uterus (Fig. 6B). We also compared Gad mRNA levels in the oviduct and uterus at the estrus and diestrus stages. GAD67 (Gad1) mRNA was expressed at both stages in the oviduct and uterus. Additionally, at the estrus stage, GAD67 (Gad1) mRNA levels were significantly higher than at the diestrus stage (Fig. 6C). GAD65 (Gad2) mRNA levels in the oviduct and uterus were measured using quantitative RT-PCR. The mRNA expression levels at the estrus stage were normalized to 1 (Fig. 6D). The mRNA expression levels at the diestrus stage were normalized to 1 (Fig. 6E).
higher than at the diestrus stage (Fig. 6C and 6D). However, no difference was detected in GAD65 (Gad2) mRNA levels between estrus and diestrus stages in the oviduct (Fig. 6E).

Discussion

Visconti et al. [30] showed that tyrosine phosphorylation of several proteins was induced during capacitation. Tyrosine phosphorylation of sperm proteins is induced after PKA activation following an increase in the intracytoplasmic cAMP level [3,31]. We showed that tyrosine phosphorylation of sperm proteins was enhanced by treatment with 1 µM GABA in HTF medium, both with BSA (Fig. 2A) and without BSA (Fig. 2B). Therefore, we hypothesized that GABA may enhance capacitation because tyrosine phosphorylation of sperm proteins was increased by incubation in HTF medium without BSA. We also showed that the acrosome reaction in capacitated sperm (5 × 10⁶ cells/ml) was facilitated by treatment with 1 µM GABA (Fig. 3A) and that the acrosome reaction was blocked by bicuculline, a GABA A receptor-specific antagonist (Fig. 3B). In contrast, GABA did not exert any significant effect on sperm motility (Table 1). GABA was reported to suppress progesterone-enhanced hyperactivation in hamsters, but, in the absence of progesterone, GABA did not affect motility and hyperactivation rates [32]. This suggests that other factors besides GABA may be required to affect these parameters in the mouse. Overall, both tyrosine phosphorylation and the acrosome reaction were highly induced by 1 µM GABA treatment at a sperm concentration of 5 × 10⁶ cells/ml.

The present results demonstrated that GABA induces the acrosome reaction in capacitated mice sperm via the GABA A receptor, as previously described for cows [26]. In addition, results from previous studies indicate that the effects of GABA on sperm are linked to Ca²⁺, Cl⁻, and HCO₃⁻ influx via GABA A receptors [27]. However, in mammals, GABA A receptor localization in sperm has been shown only for humans. In this study, immunocytochemistry analysis revealed that GABA A receptor expression is restricted to the acrosomal cap of mouse sperm (Fig. 1B), and that a correlation exists between the sperm acrosome and GABA A receptor localization (Fig. 1C). This indicates that the GABA signal is transduced into the sperm via the GABA A receptor on the acrosome. GABA and its receptor-mediated signal transduction system is thought to contribute to sperm capacitation. In human sperm, the GABA A receptor was detected in the equatorial region [33], while in this study the GABA A receptor was detected in the acrosomal region of mouse sperm. The expression patterns of the dopamine type 2 receptor (DRD2) reportedly also differ between human and mouse sperm [34], suggesting that interspecies differences in sperm morphology may be responsible for the different receptor expression patterns. However, GABA also induces the acrosome reaction in human sperm [35], indicating that the influence of GABA on sperm function may be partly shared between human and mouse sperm, as the GABA A receptor was also identified in the head region.

GABA is known to be produced in epididymal epithelial cells and the GABA A receptor has been detected in mouse epididymal sperm [36]. However, the epididymal GABA concentration is not known. In this study, we revealed that sperm capacitation was affected by GABA concentration, implying that GABA concentration in the epididymis may not be effective for sperm capacitation.

We found that the effective GABA concentration for the acrosome reaction changed according to the sperm concentration. At a sperm concentration of 5 × 10⁶ cells/ml, the acrosome reaction rate was highest with 0.1 µM GABA, while at a sperm concentration of 5 × 10⁷ cells/ml, the acrosome reaction rate was highest with 10 µM GABA. This shows that a low GABA concentration is needed to enhance the acrosome reaction rate at low sperm concentrations and vice versa. Therefore, we consider that GABA concentration to enhance the acrosome reaction may correspond to sperm concentration, and we also suggest that GABA does not promote sperm function in a dose-dependent manner. Instead, GABA may promote sperm function only within a specific concentration range. However, the relationship between sperm concentration and the effective GABA concentration must be investigated for in vitro fertilization applications.

GABA and its receptor-mediated signal transduction are thought to contribute to fertility at the sites where GABA is secreted. Therefore, we investigated the localization of GABA in the uterus and oviduct, important points in the sperm route during the fertilization process. Immunoreactivity against GABA was detected in the endometrium epithelium of the uterus and epithelial cells of the oviduct, indicative of GABA secretion (Fig. 5A and 5B). In addition, we also found that the GAD67 (Gad1) mRNA level in the oviduct was significantly higher than that in the uterus (Fig. 6A) and that the GAD67 (Gad1) mRNA level at estrus was higher than that at diestrus (Fig. 6C and 6D). Interestingly, GAD65 (Gad2) mRNA was not detected in the uterus (Fig. 6B) and no significant difference in GAD65 (Gad2) mRNA levels was detected between the estrus and diestrus stages in the oviduct (Fig. 6E). These results indicate that GABA secreted from the female reproductive tract may enhance fertility at the estrus stage. Therefore, because the acrosome reaction occurs in the oviduct, we consider that the high GAD expression in the oviduct strictly regulates GABA concentrations. Moreover, the effective GABA concentration for sperm capacitation may correspond to sperm concentration. Further studies are needed to clarify the working molecular mechanism for GABA signaling in mouse sperm.

Collectively, our results showing an enhanced acrosome reaction are consistent with previous reports in rat and cow [26, 27]. However, this study is the first to report a correlation between GABA and sperm concentrations to understand the potential usefulness of GABA. There are various anatomical and physiological differences between rat and cow, such as oviduct length and the time required for fertilization after ejaculation. Consequently, further elucidation of the physiology of GABA secretion in females will help in the understanding of these interspecies differences. GABA-mediated signaling may have great potential for the control of sperm functions.

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