Degree project

GABA-B1 Identification by Immunolabeling in Neat and Capacitated Human Spermatozoa

Master Degree Project in Biomedicine

Second Cycle 30 credits

Autumn Semester 2025

Student: Annalise Hansson

Supervisor: José Luis Girela López

Examiner: Homa Tajsharghi

Abstract

Spermatozoa are ideal research subjects for the development of medical interventions in infertility treatment and contraceptive technologies - matters of relevance extending beyond borders or the nuclear familial unit. Despite their clinical relevance, the full functionality of the human spermatozoon and its surface receptors has not been fully elucidated, and the role of the ɣ-aminobutyric acid, B-type (GABA-B) receptor in this context is particularly understudied. The localization of this structure in human spermatozoa may provide insight into the sperm's metabolic processes as they pertain to its capacity for fertilization, as well as novel knowledge of the receptor itself. Assuming active receptor presence, their role may mirror their functions in inhibitory neurons by means of mediating cell-cell signaling and intracellular metabolism. This study aims to identify the GABA-B receptor in neat and capacitated human spermatozoa, where capacitation is a complex post-ejaculatory maturation process that readies the cell for oocyte interaction. Immunolabeling procedures were used to investigate the localization and density of subunit 1 of the GABA-B heterodimer in healthy human spermatozoa in neat and capacitated states. Treated spermatozoa were compared against untreated spermatozoa and neuroblastoma cells prepared in parallel as controls. Fluorochrome immunolabeling viewed under laser confocal and standard fluorescence microscopy localized the GABA-B1 receptor subunit predominantly along the principal piece in both neat and capacitated spermatozoa, with capacitated samples exhibiting higher labeling tendency than neat samples. The increased labeling of GABA-B1 in capacitated spermatozoa suggests a possible correlation with the cells' preparatory processes for oocyte interaction.

Keywords: GABA-B, GABA-B1, Receptor, Sperm, Spermatozoa

Abbreviations

cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
dPBS	Dulbecco's phosphate buffered saline
FE-SEM	Field emission- scanning electron microscope
FRT	Female reproductive tract
GABA	Gamma aminobutyric acid
GABA-B1	Gamma aminobutyric acid receptor type B-1
GIRK	G-protein-gated inwardly rectifying potassium [channel]
LSC	Laser scanning confocal [microscope]
PKA	Protein kinase A
RT-PCR	Reverse transcription-polymerase chain reaction
ZP	Zona pellucida

The following terms are referred to:

Normozoospermia: Condition of sperm samples that have been analysed to be within normal ranges for all three major fitness qualifications: concentration, morphology (shape), and motility (movement).

Oligoasthenoteratozoospermia: Condition of sperm samples analysed as having sub-optimal measurements of concentration, morphology, and motility.

Teratozoospermia: Condition of sperm samples analysed as having sub-optimal morphology.

Popular Scientific Summary

Given the accessibility and non-invasive nature of their sample collection, sperm cells are some of the more readily obtained human cells for study, which makes them ideal subjects for research in reproductive medicine. Yet, gaps still remain in men's reproductive healthcare: developed nations have raised concern over decreasing birth rates and male infertility, while developing countries continue to see a paradoxical concern over widespread, uncontrolled population growth. In light of these issues, discussions of and concerns around reproduction continue to be propagated as women's prerogative. This appropriation of such matters imposes disproportionate responsibility and blame onto women and girls, promoting their marginalization in effect. The absurdity of this dissonance is underscored by the gap in capacity for reproduction between men and women: women are generally limited to bearing one child per year, while men do not see any such limitations. In this sense, it is illogical for birth control to remain a female-oriented market. Identifying target areas within the male reproductive tract for the development of both male birth control and infertility treatments would help to enlist men as equal and active participants in the interventional efforts of reproductive healthcare. This would, in turn, work to ameliorate the pressure placed on women and aid the establishment of more balanced social and cultural dynamics.

The research in this study sought to improve understanding of the factors of fertility by investigating mechanisms of cell signaling. Generally, cell signaling is gatekept by structures referred to as surface receptors, which allow for information to be conveyed between the cell and its environment, and the other way around. The GABA-B receptor is one of these gate-keeping structures, classically known for its ability to reduce the effect of external signals on its host cell in other systems of the body, most namely the nervous system. As contemporary understanding of this structure is based on research in these other bodily systems, its role within human sperm cells has not yet been uncovered, and little evidence for its function here is able to be found. During its journey toward the egg, the sperm cell undergoes a process called capacitation, which prepares the sperm to interact with the egg. This study investigated the presence of the GABA-B receptor on healthy human sperm cells before and after they underwent capacitation ('neat' versus 'capacitated' cells). The goal of this was to uncover the possibility of the GABA-B receptor's involvement in the sperm's preparation to fertilize the egg, which may further inform understanding of the functions of both sperm cells and the receptor. To do this, sperm samples were collected from healthy donors, and the GABA-B receptor was tagged with visual markers that allowed for their observation across sperm cell surfaces using microscopes. Visual observation confirmed the presence of the receptor on both neat and capacitated sample groups, which were predominantly found along the main part of the sperm's tail. Analyses of the results found a higher occurrence, and a slightly greater density, of these structures in sperm cells that had undergone transformation in comparison to the cells that were not capacitated. The identification of these structures on sperm cells may complement current understanding of the mechanisms required for successful fertilization and may further guide future research in reproductive medicine.

Table of Contents

Abstract 2
Abbreviations 3
Popular Scientific Summary 4
Table of Contents 5
1 Introduction 1
1.1 Background 1
1.2 GABA-B receptors 3
1.3 Aim 4
2 Materials and Methods 5
2.1 Reagents 5
2.2 Semen samples 5
2.3 Sample fixation 6
2.4 Fluorochrome conjugation 6
2.5 Gold conjugation 6
2.6 Controls 7
2.7 Observational methods 7
2.8 Statistical methods 8
3 Results 9
3.1 Quantitative data and analyses 9
3.2 Observation of neat and capacitated spermatozoa 9
4 Discussion 15
4.1 Methodology and controls 16
4.2 Limitations and considerations 17
4.3 Biological interpretations & literature synthesis 18
5 Ethics and Social Impact 20
6 Declaration of AI Technologies 20
7 Conclusion 21
8 References 22

1 Introduction

Sperm cells (spermatozoa) are highly specialized and regionalized cells; as such, the molecular machinery found within a region provides insight as to the functions of both the machinery and its respective region (reviewed by Turner, 2006). The cell's surface receptors are essential in the sperm's quest toward the oocyte, bearing the responsibility of moderating environmental influences and other control mechanisms in post-ejaculatory modifications and sperm-egg interactions (de Lamirande, 1997; Yanagimachi, 1994). A particular neuroreceptor- the GABA-B receptor - is of interest for further study, as current literature has yet to fully characterize their purpose and localization in human spermatozoa. While researchers have examined the two GABA-B subunits- GABA-B1 and GABA-B2- in sperm and spermatids of non-human species (He et al., 2001; Kanbara et al., 2005; Kovarikova et al., 2023), reviews of current literature failed to identify substantial research on the GABA-B receptor or its subunits within healthy human spermatozoa. Given contemporary knowledge of the receptor's broader mechanisms, it is suggested that the receptor is involved in the moderation of cell signaling and ion balance within reproductive tissues (Calogero et al., 1996; Calogero et al., 1999). The localization of the receptor across different metabolic stages of ejaculate spermatozoa may inform this understanding of their function.

1.1 Background

The human spermatozoon has a relatively simple macro architecture; the head contains genetic information within the nucleus alongside the enzyme-rich acrosome on the cell's most rostral portion. The cell's midpiece- a specialized structure classified as part of the flagellum- houses the mitochondrial sheath and serves as the cell's source of sustained energy (Turner, 2006). The flagellum is responsible for locomotion within the female reproductive tract (FRT), as well as providing mechanical force during oocyte penetration (Herrick et al., 2004).

Three major markers of sperm fitness have been agreed upon by researchers: motility, concentration, and morphology (World Health Organization, 2021). Normal levels of all three are required to constitute normozoospermia in a given sample, and deficits in these areas can be detrimental to reproductive potential. Sperm motility is sustained by the flagellum, which is constructed of four distinct subdivisions: the connecting piece, the midpiece, the principal piece, and the end piece (Turner, 2006). Constituting the length of the flagellar core is the axoneme, a hub-and-spoke arrangement of microtubules (alpha/beta tubulins) and associated proteins- namely dyneins- which generate the flagellum's motive force (Turner, 2006). The structures surrounding the microtubules denote each flagellar region. The phosphorylation of dynein proteins, and their resulting association with adjacent microtubules, generates the power stroke that creates the sliding force of microtubules to bend the flagellum (Brokaw, 1972; Tash, 1989). These functions are largely powered by glycolysis occurring along the length of the flagellum (Miki et al., 2004). The cAMP/PKA and calcium signaling pathways are believed to be the major mediators of such processes (Finkelstein et al.,

2020), both of which are influenced by mechanisms of small and heterotrimeric classes of G-proteins, and changes in intracellular pH (Turner, 2006).

Ejaculatory spermatozoa are not yet fit to fertilize an egg; first, they must undergo a series of physiological transformations driven by chemical and mechanical signals of the FRT (Austin, 1952). This dynamic nature of the cell allows it to adapt and respond to environmental cues to achieve its end goal of fertilization (de Lamirande, 1997; Yanagimachi, 1994). Notably, sperm fail to penetrate the zona pellucida (ZP) in the absence of calcium, potassium, or glucose (Hoshi et al., 1991). In Ca^{2+}-free and K^{+}-free mediums, researchers attributed the failure to penetrate the ZP, in part, to the inhibition of the acrosome reaction; in glucose-free mediums, they attributed it, in part, to the inhibition of hyperactivation (Hoshi et al., 1991).

Capacitation is the major state incurred following ejaculation, once the sperm's environment shifts to that of the FRT (Baker et al., 2012). Core features of this phase include a restructuring of the cell's architecture and acquisition of hyperactivated motility (Martinez & Morros, 1996; Ooi et al., 2014). Major processes involved in this include a) the influx of calcium and carbonate ions, b) activation of the cAMP/PKA pathway, and c) protein residue phosphorylation (Davis, 1981; Visconti et al., 1999; Visconti, 2009). Capacitation is essential to the sperm's reproductive ability and allows for the subsequent acrosome reaction to take place (Austin, 1952; Chang, 1951).

The acrosome reaction is an exocytotic, catabolic process that the sperm must undertake to be able to fuse with the egg, with the core functionality being the release of enzymes specialized for the digestion of the ZP and the exposure of new domains on the plasma membrane (Davis, 1981; Martinez & Morros, 1996). The activation of upstream calcium channels is believed to be triggered by components of the acrosome associating with proteins of the oocyte's ZP upon reaching its vicinity (Yanagimachi, 1994), although other effectors have also been documented: prostaglandins, progesterone, and other components of follicular fluid (Calogero et al., 1999; Li et al., 2020; Ralt et al., 1991; Shimizu et al., 1998). Sufficient levels of intracellular calcium trigger a signal cascade featuring the activation of the phospholipase C-inositol triphosphate pathway, which in turn activates the downstream calcium channels of the acrosomal membrane (Li et al., 2020). The fusion of the acrosomal membrane with the plasma membrane allows for fenestration and vesiculation (Meizel, 1984). The action of the released enzymes, in conjunction with the mechanical force of the flagellum, allow the sperm to break through protective layers to fuse with the oocyte (Meizel, 1984; Yanagimachi, 1994).

The initiation of both the capacitation and acrosome reaction processes depend on extrinsic factors, as spermatozoa lack the intrinsic hardwiring to do so on their own (Baker et al., 2012). This property underscores the significance of cell-to-cell communication in the fertilization process, and the role of the surface receptor as the mediator of such interactions.

1.2 GABA-B receptors

GABA (ɣ-aminobutyric acid) receptors, named after the major inhibitory neurotransmitter of the CNS, are a class of transmembrane neuroreceptors known to mediate inhibitory signaling (Bettler et al., 2004). Contemporary understanding of these receptors is confounded by the fact that 1) non-inhibitory iterations have been documented (Chen & Sharma, 2023; Furukawa & Fukuda, 2023), and 2) the receptor class is not restricted to neural tissues (Geigerseder et al., 2003). The GABA-B receptor is distinguished from other types by its denomination as a metabotropic, G-protein-coupled receptor (Jewett & Sharma, 2020), which may also be the reason it remains poorly understood in relation to the well-studied GABA-A type.

Dense concentrations of the GABA-B receptor can be found throughout vital regions of the CNS (Kondziella, 2016; Princivalle et al., 2000; Young & Chu, 1990) and are also notably present in autonomic nerves and Schwann cells of the peripheral nervous system (Faroni et al., 2011; Procacci et al., 2013). They have further been identified in cardiovascular, endocrine, gastrointestinal, and reproductive tissues (Erdö & Wolff, 1990; Gladkevich et al., 2006). Evidently, the task of the GABA-B receptor is vast and varied. They serve as autoreceptors by regulating GABA release, and act as effectors of other channels to regulate non-GABA signaling (Chalifoux & Carter, 2011; Thompson et al., 1993). In neural contexts, they are found both pre- and post-synaptically, regulating voltage-gated calcium channels to moderate neurotransmitter release, and mediating potassium efflux to induce hyperpolarization, respectively (Kerr & Ong, 1995; Marshall et al., 1999).

The GABA-B receptor operates as an obligate heterodimer of the GABA-B1 and GABA-B2 subunits (Marshall et al., 1999). Ligand-binding of the first unit triggers conformational changes in the second unit, which activates inhibitory G-proteins that go on to affect further changes in the cell through a collection of signaling cascades (Marshall et al., 1999). Most chiefly, the receptor has been implicated in the direct regulation of ion balance and the cAMP-PKA pathway (Couve et al., 2000). The former is largely due its association with GIRK channels which drive potassium efflux, and the resulting prolonged state of hyperpolarization (Krapivinsky et al., 1998), which is also complemented by the ability of the G-protein to limit calcium influx by binding voltage-gated calcium channels (Couve et al., 2000). The latter is a result of the inhibition of adenylyl cyclase by G-proteins, reducing intracellular cAMP production and PKA activation, and stunting the subsequent phosphorylation of target proteins (Couve et al., 2000). The most notable effectors of these mechanisms are G$\beta\gamma$ subunits of inhibitory $G_{i/o}$ proteins (Zhang et al., 2015). This current understanding of the general mechanisms of GABA-B receptors may help elucidate its functions within non-neural contexts.

The broad responsibilities of the GABA-B receptor, as have been discussed so far, mirror a number of the principal metabolic processes that the post-ejaculatory spermatozoa must undertake to attain reproductive ability. Thus, the presence of the receptor in the human spermatozoon could indicate that

the structure is not merely a remnant of the cell's earlier developmental processes, and the localization of the receptor should contextualize its presence in this context. Still, existing research has struggled to definitively detail the structure and its exact functions in such contexts as human spermatozoa. The most recent study came from Kaewman et al. in 2021, where RT-PCR was used to examine the presence of GABA-B transcripts in human mRNA. The study analyzed levels of the GABBR2 subunit expression in both healthy and pathogenic samples of human sperm. It was found that expression was significantly higher in oligoasthenoteratozoospermic samples compared to normozoospermic samples (Kaewman et al., 2021). However, they did not find a significant difference in expression between teratozoospermic and normozoospermic samples. This discrepancy indicates a possible association between R2 expression, motility, and concentration, but not in abnormal morphology alone.

In another marked study, GABA receptors were examined via implementation of exogenous GABA-actors on human spermatozoa, which were found to stimulate the acrosome reaction in a dose-dependent manner (Calogero et al., 1999). The researchers attributed this effect to both GABA-A and GABA-B receptors, but they were able to confirm the role of the GABA-B receptor via agonistic Baclofen and antagonistic Saclofen in the presence of GABA. The former stimulated the acrosome reaction, while the latter impaired the reaction (Calogero et al., 1999). Prior to such study, the same researchers also observed the effect of Baclofen on sperm motility and hyperactivation. It was found that the selective GABA-B agonist increased both the percentage of active sperm in a sample as well as the degree of hyperactivation within the sperm (Calogero et al., 1996). In the absence of additional research, the presence of the GABA-B1 subunit in the human spermatozoon remains elusive.

As has been detailed, GABA-B receptors are linked to ion flux, the electrochemical potential of the plasma membrane, cell signaling, and the mediation of intracellular metabolic processes. Previous evidence points to an association of the receptor type with the acrosome reaction but, given the overlap between the mechanisms of post-ejaculatory sperm metabolism and the manner of operation of the GABA-B receptor, it is proposed that an additional function of the receptor lies in the regulation of motility, hyperactivation, and the broader attainment of capacity for fertilization (as the collection of requisite processes tend to be co-occurring and interdependent). Uncovering the receptor's localization may inform understanding of its function in the spermatozoa.

1.3 Aim

This research aims to identify and characterize the presence of the GABA-B1 neuroreceptor on the surface of neat and capacitated human spermatozoa using immunolabeling techniques. Healthy human spermatozoa are inspected for the presence and density of GABA-B1 receptors along the head, midpiece, and tail of the cell, and are compared at neat and capacitated states.

2 Materials and Methods

2.1 Reagents

Fetal calf serum (FCS) (Sigma Aldrich)

Bovine serum albumin (BSA) (Sigma Aldrich)

Anti-GABA-B Receptor 1 Rabbit IgG (Ab-75239, Abcam)

Alexa Fluor 488 Anti-Rabbit Goat IgG (Ab-143165, Invitrogen)

Anti-TUBA4A (α-Tubulin) Mouse IgG (T5168, Sigma Aldrich)

Alexa Fluor 488 Anti-Mouse Donkey IgG (Ab-715545150, Jackson ImmunoResearch)

12 nm Colloidal Gold AffiniPure Anti-Rabbit Donkey IgG (Ab-2340610, Jackson ImmunoResearch)

Triton X-100 (Sigma Aldrich)

Paraformaldehyde, 4% (Sigma Aldrich)

Vectashield V-1000 mounting media with 15 µg/ml DAPI (Vector Laboratories)

2.2 Semen samples

Human spermatozoa used in this study were provided by the Department of Biotechnology at the University of Alicante, obtained from healthy donors (n=5, age = 18-35) who had been asked to remain abstinent for 3 days prior to collection. Donors provided informed consent for the samples to be used in research. Following collection, samples underwent computer-assisted seminal analysis to filter for pathologies and ensure all samples were normozoospermic by World Health Organization (2021) guidelines. The parameters evaluated were volume, concentration, morphology, liquefaction, pH, motility, and viability. An aliquot of samples from each donor was transformed to induce capacitation via the swim-up method* prior to storage. Further information on the selected control groups is detailed in section 2.6.

* For both the transformation of cells and quality verification, the "swim-up" method of capacitation was performed prior to the commencement of this thesis work in order to ensure the condition and fitness of the sperm being used for research. This process involves the incubation of cells in specialized, capacitation-inducing media to isolate the candidate (morphologically healthy and motile) cells that swim into the upper layer of fluid, while leaving behind the unfit sperm cells which are unable to swim up.

2.3 Sample fixation

Spermatozoa samples were fixed on poly-L-lysine-treated (1%) glass coverslips and left to air dry before being bathed in blocking solution (5% BSA and 2% FCS in dPBS) for 20 min. to block non-specific binding sites.

For positive controls, neuroblastoma cells fixed on glass coverslips were treated with 4% paraformaldehyde in dPBS for 10 min. before being treated with blocking solution for 20 min. Sperm samples used for control via α-tubulin labeling were permeabilized with Triton X-100 (1% in dPBS) for 10 min. and briefly rinsed with dPBS before being treated with blocking solution for 20 min. See section 2.6 for further details of division of control groups.

2.4 Fluorochrome conjugation

Coverslips fixed with neat spermatozoa, capacitated spermatozoa, and neuroblastoma cells were put through a series of antibody incubations and washing stages. The treatments were done in a humid chamber on hydrophobic film using an array of drops holding the glass coverslips. Antibodies were prepared via suspension in blocking solution. Cells were incubated in Anti-GABA-B1 Rabbit IgG (1:100 dilution) or Anti-α-Tubulin Mouse IgG (1:100 dilution) for 1 hour, then washed with dPBS for 5 minutes twice (2 x 5 min.). The two fluorochrome-conjugated secondary antibodies, Alexa Fluor 488 Anti-Rabbit (A488) and Alexa Fluor 488 Anti-Mouse (A488), respectively, were used at 1:300 dilutions. The coverslips were left in the secondary antibody for 1 hour and then washed for five minutes in dPBS twice (2 x 5 min.). Paraformaldehyde (4% for 5 min.) was used to preserve the junction between the antibody and antigen as the final step before mounting with Vectashield-DAPI media to prevent fluorescent fading and mark DNA.

Fluorochrome conjugation viewed using LSC microscopy provides a relatively accessible means of observing the surfaces of cells, serving as a quicker and more cost-effective option compared to methods like FE-SEM. Due to this, along with the LSC's efficacy in obtaining data for large cell counts, this method was selected for data collection purposes in this study.

2.5 Gold conjugation

Gold was used in addition to fluorochrome as a supplemental means of labeling and observing the receptor along the surface of the spermatozoa, as each method corresponds with a different means of observation. The FE-SEM used to observe gold-conjugated antibodies allows for viewing at a considerably higher resolution than LSC observation, lending a much more precise visualization of the location and density of the bound receptors.

Following sample fixation and blocking, neat and capacitated cells were incubated in Anti-GABA-B1 IgG (1:100 dilution) for 1 hour, then washed twice with fresh dPBS (2 x 5 min.). The secondary antibody used was 12 nm

Colloidal Gold AffiniPure IgG (1:200 dilution) and cells were incubated for 1 hour. Coverslips were again washed twice with dPBS (2 x 5 mins). Coverslips were washed in ultrapure water for 5 min. and then briefly rinsed in absolute ethanol to clarify the samples of excess salts that would impede observation. Prepared coverslips were mounted and sent to be treated with carbon coating by a third party prior to observation.

2.6 Controls

Prior to the day of experiment, neuroblastoma cells had been stored in cell growth medium at 4°C on glass coverslips.

Neat and capacitated spermatozoa treated with GABA-B1 antibodies were prepared alongside four additional groups for comparison: 1) neat and capacitated spermatozoa treated with blocking solution in place of the primary GABA-B1 IgG treatment, 2) neat and capacitated spermatozoa treated with blocking solution in place of the secondary IgG treatment, 3) neuroblastoma cells that were fully incubated in both the GABA-B1 and A488 antibodies, and 4) neat and capacitated sperm cells treated with anti-α-tubulin and A488 fluorochrome antibodies, to validate the fluorescence observation.

The first and second control groups function to validate the selected primary and secondary antibodies, ensuring observation of the intended target. The involvement of the third control group, the neuroblastoma cells, provided a known baseline in which test groups could be compared against, as the presence of the target GABA-B1 structure is already well-studied in these cells. Finally, by substituting the primary antibody in the fourth control group for anti-α-tubulin, the use of the secondary antibody - necessary for the selected observational method - was able to be evaluated in a context independent of anti-GABA-B1 in order to test optimal concentration and incubation time of the selected antibodies.

2.7 Observational methods

Laser scanning confocal (LSC) microscopy (LSM-800, Zeiss) and standard fluorescence/phase contrast microscopy (Leitz DMRB, Leica) were used to observe the fluorochrome-conjugated samples. Field emission-scanning electron microscopy (FE-SEM) (Merlin Compact, Zeiss) was used to observe the gold-conjugated samples; this protocol was included due to it offering higher resolution than LSC and the DMRB.

Infinity Capture software (version 6.3.1) was used to obtain images during microscopy. GIMP software (version 3.0.4) was used for image processing to adjust curves for optimized visibility of targets. All images included in later sections of this report were equally processed, i.e., images of the controls, with unlabeled targets, also had their levels adjusted so as to avoid bias in viewing. The images were not modified beyond the mentioned adjustment of the curves. Canva (web access) was used to compile images into single figures.

2.8 Statistical methods

Several analyses, named in the following paragraphs, were selected to understand the relationship between the metabolic stage of the sperm (neat or capacitated), and the rate of GABA-B1 labeling in sperm samples. Data collection consisted of the quantification of the number of cells in each sample which showed the presence of GABA-B1 labeling, analyzed as a proportion of total cells counted in a sample. Ten separate groups (five donors x two treatments each) were analyzed, with the goal of categorizing 200 cells within each of the 10 groups as either positive or negative for fluorescent marking. The data collection process was conducted using standard fluorescence microscopy.

To analyze the significance of the difference in GABA-B1 labeling between neat and capacitated spermatozoa, a binary logistical regression was selected to illustrate effect size and odds. A Pearson's chi-squared test of independence was selected to further detail the likelihood of dependence between the state of capacitation and the rate of GABA-B1 labeling in the spermatozoa. Yate's continuity correction was added to this test as a protective factor, making estimates more conservative.

Finally, in order to understand the significance of donor-level variance between treatment groups, a Fisher's exact test was conducted for each donor. All analyses were carried out through RStudio (web access via Posit Cloud). P-values were used to determine significance of results, with differences considered significant when $p < 0.05$.

3 Results

3.1 Quantitative data and analyses

Fluorochrome labeling of GABA-B1 differed between neat and capacitated spermatozoa, shown in Table 1. The proportion of positively labeled neat cells (75.40%) was lower than capacitated cells (97.60%), yielding a prevalence ratio of 1.29. Pearson's chi-squared test with Yates' continuity correction highlights the significance of the variation between conditions ($X2 = 180.21$, $df = 1$, $p = 4.36e-41$).

The relationship between the two conditions was further supported by a logistical regression: the odds of labeling in the neat group was 3.06:1 (95% CI: 2.66 - 3.55), and the odds of labeling for cells in the capacitated group was 40.95:1 (95% CI: 27.05 – 66.00). Effect sizes were further derived from the odds: cells in the neat group had 7.48% (95% CI: 4.60% - 11.60%, $p < .001$) the likelihood of the capacitated group to be labeled. Inversely, spermatozoa in the capacitated group had 13.36 times (95% CI: 8.60 - 21.96, $p < .001$) the odds of labeling compared to neat cells.

Within-donor variance was also examined using Fisher's exact test, which showed a significant difference ($p < .05$) between sample conditions for each donor (Table 1).

Table 1. Dataset with Fisher's p-value of donor-level variance

Donor	Neat labeled	Neat Total	Neat %	Cap. Labeled	Cap. Total	Cap. %	Fisher's P-value
1	177	200	88.5%	197	200	98.5%	5.26e-05
2	101	200	50.5%	200	200	100.0%	2.41e-37
3	120	200	60.0%	197	200	98.5%	1.78e-24
4	169	200	84.5%	196	200	98.0%	1.31e-06
5	187	200	93.5%	29	39	74.4%	1.04e-03
Total	754	1000	75.4%	819	839	97.6%	-

Table 1. Individual cell counts and occurrence proportions are shown for all tested sample groups. P-values from Fisher's exact test are also included to indicate the significance of the differences between the neat and capacitated conditions for each donor. The discrepancy in counts for donor #5 is further discussed in section 4.2.

3.2 Observation of neat and capacitated spermatozoa

Bright yellow regions revealed GABA-B1 binding on sperm and neuroblastoma cells under fluorescent LSC microscopy. Areas of positive GABA-B1 labeling in neat spermatozoa are shown in Figure 1, and the marked regions were largely localized to the flagellum. Capacitated spermatozoa similarly showed distinct regions of labeling under LSC microscopy (Figure 2), which were also largely localized to the flagellum. Instances of labeling in the heads was observed at times (Figure 3). Figure 3a shows labeling of the midpiece, which was generally labeled at a lower rate than the more distal portions of the flagellum.

Neat (Figure 4a-b) and capacitated (not shown) spermatozoa which did not receive anti-GABA-B1, and neat and capacitated spermatozoa which did not receive the secondary fluorochrome-conjugated IgG (not shown) did not show any regions of fluorescent labeling. Neuroblastoma cells used as positive controls (Figure 4c-e) displayed regions of fluorescent GABA-B1 labeling, and α-tubulin labeled neat and capacitated sperm cells showed the same bright yellow shade of A488 labeling through their flagellums (not shown). Gold labeling of neat and capacitated spermatozoa observed under FE-SEM failed to produce reliable results and are not shown.

Instances of labeling in neat and capacitated groups of treated spermatozoa were identical to the areas of true signal on the neuroblastoma positive controls: defined, bright, and opaque markings denote the labeling of the GABA-B receptor subunit. The most notable region of labeling in both treatment groups was in the principal piece of the flagellum, which can be seen in Figures 1 and 2. Not only was this region the most consistently labeled across both groups, it was also consistently the most densely labeled in both neat and capacitated treatment groups. The midpiece of the sperm in both treatment groups routinely showed less signal, if any, in comparison to the principal piece of the flagellum. The exception to this seemingly occurred alongside the unusual instances of when signal was seen in the acrosome of the heads of spermatozoa (Figure 3). A difference in appearance under fluorescent observation using LSC microscopy was seen between the negative controls and both the neat and capacitated spermatozoa which were labeled with both anti-GABA-B1 and A488 fluorochrome. The controls are discussed further in section 4.1.

Figure 1. Neat spermatozoa labeled for GABA-B1 under fluorescence.

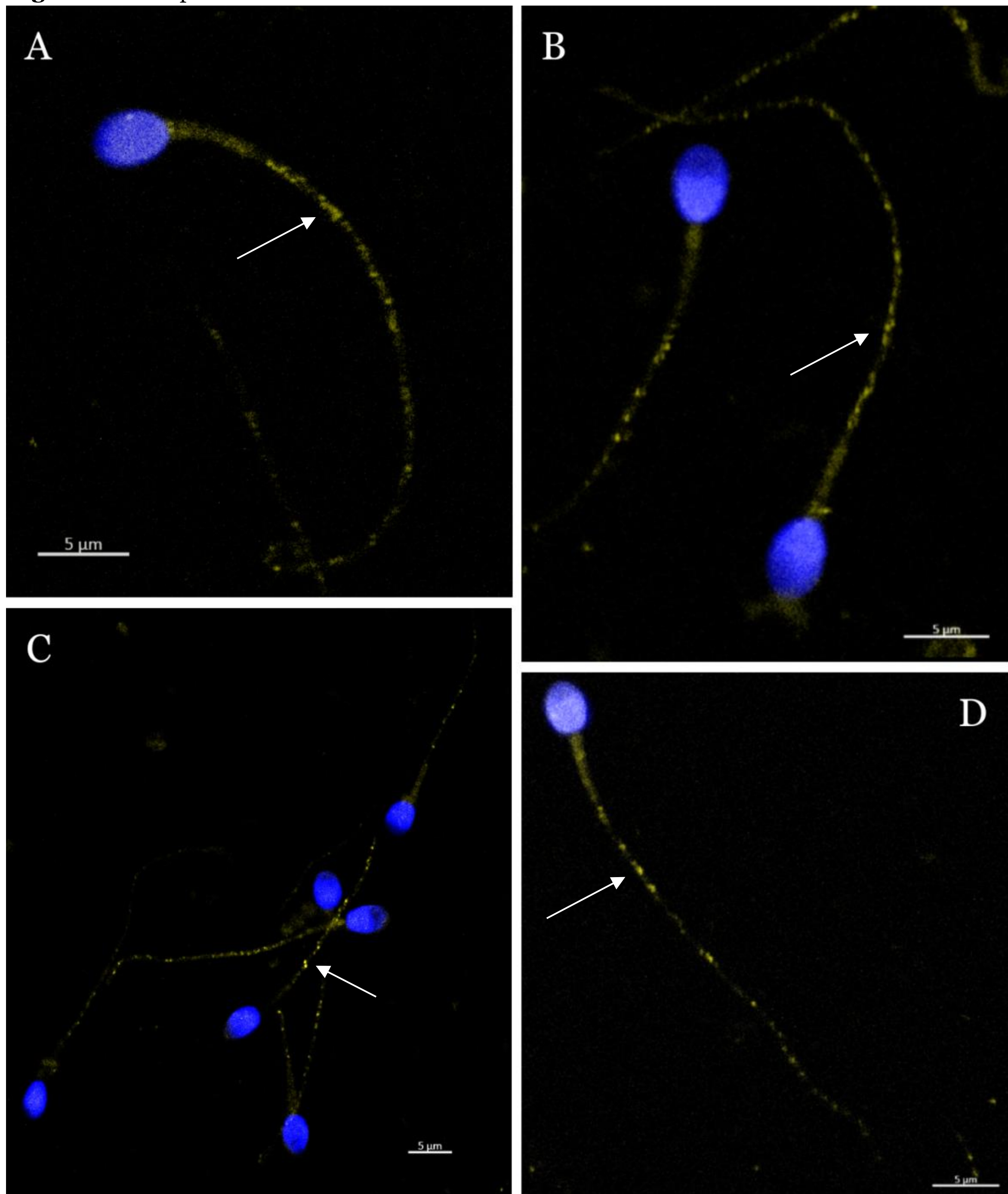

Figure 1 A-D: Several images for the same treatment group are shown to collectively demonstrate variety in appearance of cells within samples, while supporting and strengthening the reliability of the findings. Neat spermatozoa treated with anti-GABA-B1 and A488 fluorochrome-conjugated IgGs are shown under fluorescence using laser scanning confocal microscopy. Blue labeling shows regions of DNA marked by DAPI, while bright yellow-green regions show GABA-B1 labeling. Arrows are used to indicate instances of fluorescence but could not be used to show every single instance within an image.

Figure 2. Capacitated spermatozoa labeled for GABA-B1 under fluorescence.

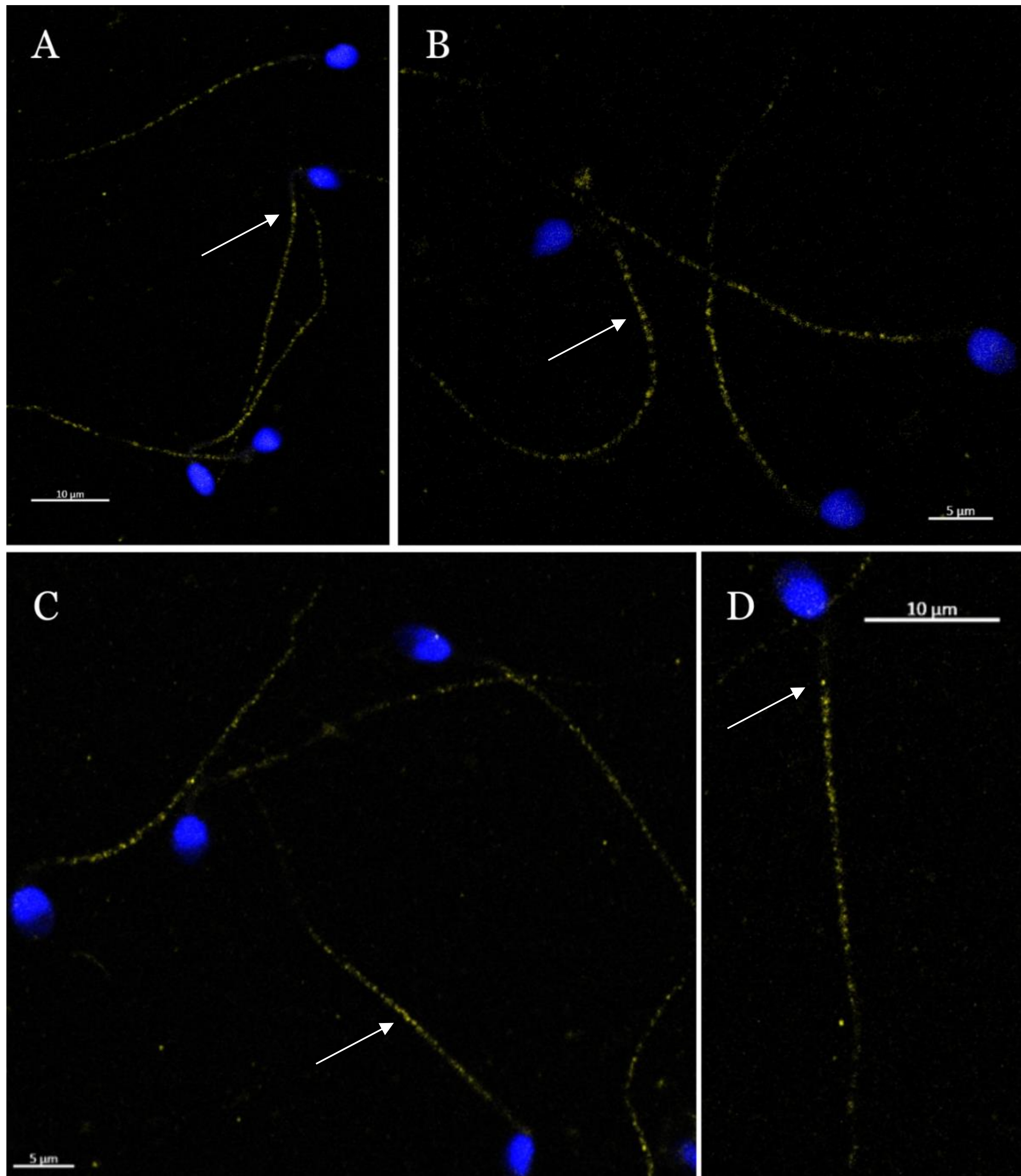

Figure 2 A-D: Several images for the same treatment group are shown to collectively demonstrate variety in appearance of cells within samples, while supporting and strengthening the reliability of the findings. Capacitated spermatozoa treated with anti-GABA-B1 and A488 fluorochrome-conjugated IgGs are shown under fluorescence using laser-scanning confocal microscopy. Blue labeling shows regions of DNA marked by DAPI, while yellow-green regions show GABA-B1 labeling. Arrows are used to indicate instances of fluorescence but could not be used to show every single instance within an image.

Figure 3. Uncommon instances of head labeling of GABA-B1 in neat cells under fluorescence.

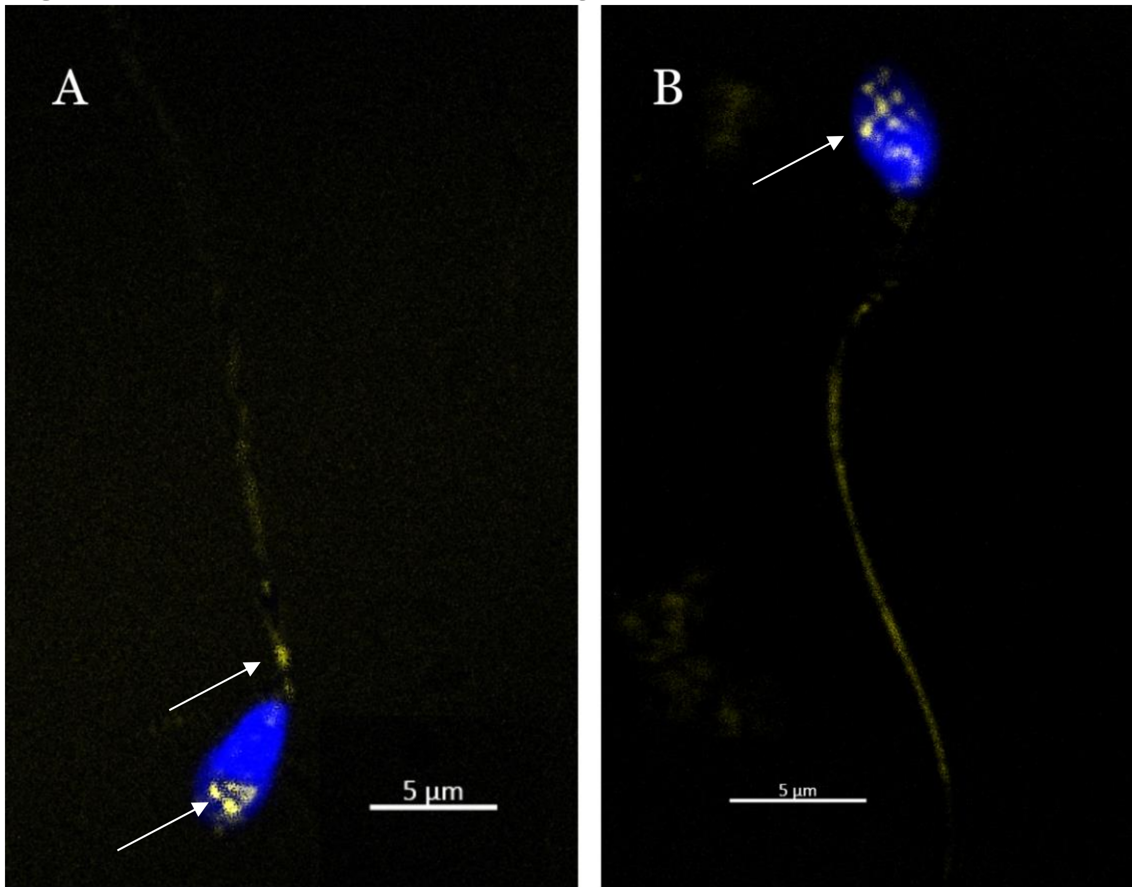

Figure 3 A & B: Images of two different cells from the same treatment group show variety in appearance of cells within samples. Neat spermatozoa labeled with GABA-B1 and A488 fluorochrome antibodies shown under laser scanning confocal microscopy. Green-yellow areas show dense GABA-B1 labeling in the heads, and blue shows DNA. Arrows point out instances of fluorescent signaling of GABA-B1 binding.

Figure 4. Control spermatozoa and neuroblastomas under fluorescence.

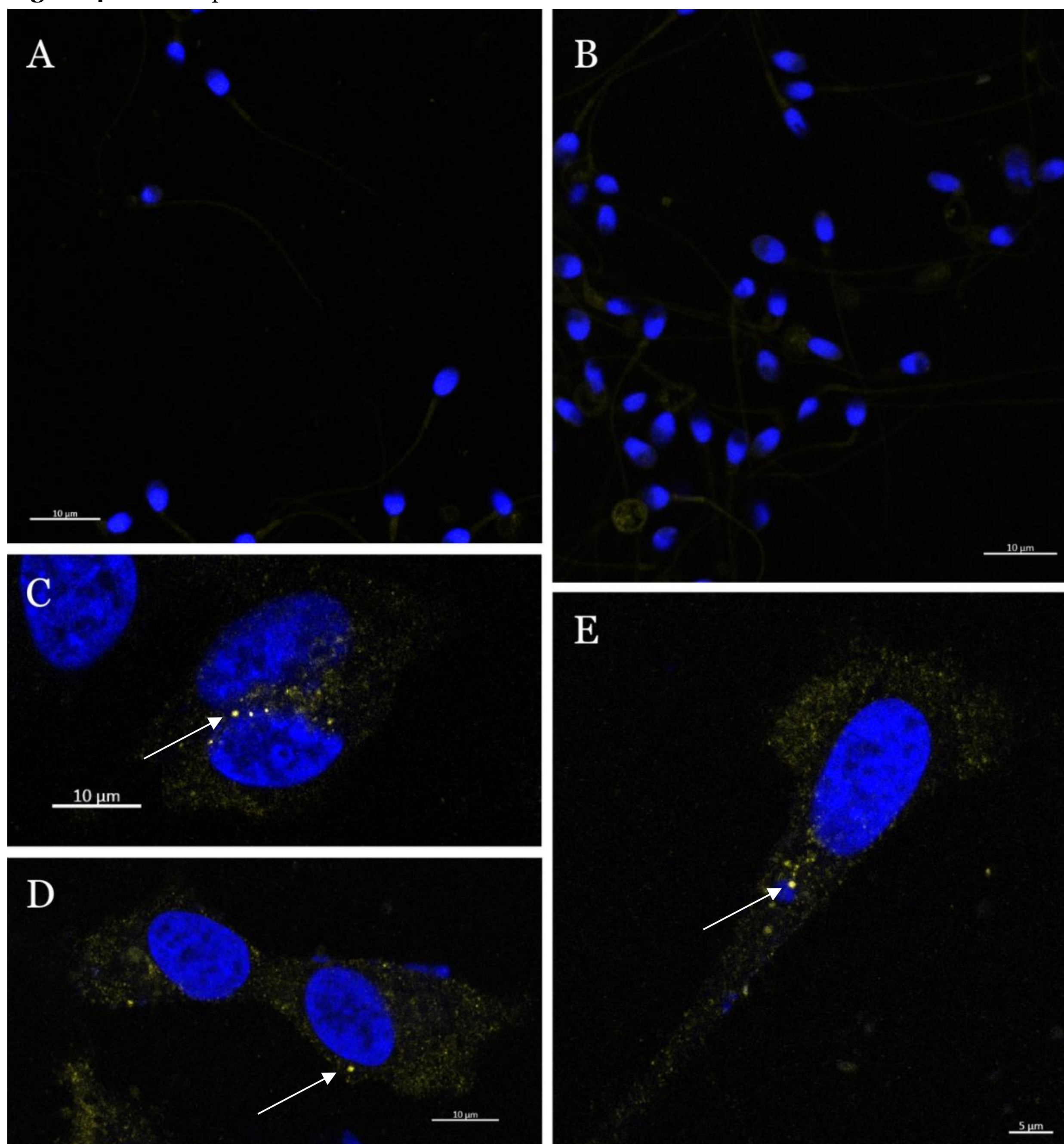

Figure 4: Control groups with fluorescent labeling are visualized using laser-scanning confocal microscopy. Blue fluorochrome shows DNA under the DAPI/UV channel. Yellow fluorochrome shows GABA-B subunit 1 receptor binding. A & B: Two images from the same treatment group show variety in appearance of cells within samples to strengthen and support findings: neat spermatozoa treated with only the A488 fluorochrome-conjugated IgG (no anti-GABA-B1). C-E: Three images from the same treatment group show variety in appearance of cells within samples to strengthen and support findings: neuroblastoma cells treated with both the anti-GABA-B1 and A488 fluorochrome-conjugated IgGs. Arrows indicate signaling by GABA-B1 receptor binding.

4 Discussion

This study intended to examine a total of 200 cells from each test group, 400 cells per donor (n=5), and 1000 cells for each treatment group (the short count for donor five is discussed in section 4.2). Among donors one through four, individual Fisher's Exact tests showed a significant difference between the neat and capacitated groups for each donor (exact values shown in Table 1). As illustrated by the statistical analyses - further discussed below - the marked increase in GABA-B1 labeling in capacitated cells demonstrates a positive, dependent relationship between the two factors. The findings of the Fisher's Exact test are reinforced by those of the logistic regression and Pearson's Chi-square test of independence - all together failing to preclude the possibility of a causal relationship between fertility, sperm fitness, and GABA-B1 surface receptor labeling. This provides a basis for further investigation of the GABA-B1 structure, its coordinating ligands, and their manner of operation within the environment of the FRT throughout reproductive processes.

The chi-square test indicated it is more likely than not that the factors are not independent of one another, or, that there is correlation between a spermatozoon's metabolic state and GABA-B1 labeling. The logistic regression supported this relationship, clarifying the direction of the relationship between the two variables and denoting possible activity of GABA-B1 through the pre-fertilization processes. Dividing the odds of labeling in neat cells by that of the capacitated cells produces the odds ratio of .0749 (7.49%), which shows that the odds of labeling in the neat group is much lower than the odds for cells in the capacitated group. While the difference from the variance in true odds (75.4%, 97.6%) produced by the odds ratio appears to be an overestimation, exaggerated effect size tends to be a feature of logistic regressions when the outcome is already quite likely for both groups. Thus, both proportions and odds should be considered in interpretations of the degree to which the cell's metabolic state may be useful in predicting its binding of GABA-B1 antibodies to the cell surface. Given the importance of capacitation-related metabolic shifts in spermatozoa to oocyte interaction and fertilization, an increase in GABA-B1 labeling following this process may reflect the reproductive utility of this structure. Although the findings are unable to show a definitive cause-effect relationship, it is likely that capacitation of spermatozoa allows for the increased accessibility of the GABA-B1 binding site as a result of the restructuring of the plasma membrane. The findings of this study also support the possibility that this structural shift plays a role in the cell's successful journey and/or interaction with the oocyte, which could occur as a result of the receptor's known ability to moderate ion flux and cell signaling. Collectively, the relationship elucidated by the statistical analyses helps to inform understanding of the availability and function of GABA-B1 binding sites in post-ejaculate sperm, and indicates possible involvement of the structure in the cell's transformation needed to penetrate the oocyte. Discussion of these biological implications are continued in section 4.3.

The observed results were not surprising overall, with the lowest proportion of GABA-B1 labeling seen in the neat sample of donor #2 (50.5%), followed by the neat sample of donor #3 (60%). Yet, compared to other groups, these still stand out as outliers with lower labeling rates than expected; in observations prior to data collection, the vast majority of spermatozoa samples did show labeling surpassing the 50-60% range. This variation in samples can result from several different factors, including individual donor differences, errors in sample preparation, and/or observational limitations organic to any microscope-based data collection. Nonetheless, the total proportion of 75.4% for the neat group seems to be a much more reliable estimation of the true prevalence of GABA-B1 labeling in neat spermatozoa, even possibly an underestimation. Moreover, as great variation exists across (and even within) donor samples, ascertaining a true average sperm profile can prove difficult, and future studies would benefit from a higher sample size to minimize the effect of outliers as seen from donor #5 in this study. All in all, the findings of the statistical analyses from Fisher's Exact test, the chi-square test, and the logistical regression show a correlation between the metabolic state of spermatozoa and labeling of GABA-B1 binding sites. Given an increase in the labeling of these receptors in capacitated sperm cells, GABA and GABA-B receptors may be more involved in mechanisms of capacitation and oocyte-interaction than previously thought. To better gauge which parts of the pre-fertilization pathway would be most closely linked to GABA-B1 activity, continued investigation would be needed; future directions are further discussed in section 4.3 and 7.

4.1 Methodology and controls

The use of FE-SEM was chosen to complement fluorescence microscopy since the method offered superior clarity and precision compared to the other options due to its higher resolution. Unfortunately, the initial runs of gold-tagged cells for FE-SEM failed to yield expected results, as the markers could not be identified throughout the sample when examining the cells in this setting. Therefore, images from this procedure were not shown in the results as they would not have provided any relevant insight. The protocol sensitivity for the FE-SEM preparation process is likely to be higher than that of the fluorochrome-conjugation immunolabeling due to the 100% failure rate across all samples. Even in the suboptimal runs of fluorochrome immunolabeling, the presence of the conjugate was still evident via residual particles in the surrounding environment, which contrasts the complete absence of the conjugate under FE-SEM. Additionally, the gold-conjugated immunolabeling was conducted in parallel to fluorochrome labeling, which showed expected results. Therefore, the failure to obtain results from FE-SEM observation does not imply the absence of the binding site, but more so points to a procedural error which constituted the difference between methodologies.

As briefly discussed in section 2.6, groups of neat and capacitated spermatozoa treated with GABA-B1 antibodies were compared against four additional groups throughout the course of the experiment: 1) neat and capacitated

spermatozoa which did not receive the anti-GABA-B1 IgG treatment to rule out secondary antibody-driven fluorescence, 2) neat and capacitated spermatozoa which did not receive the fluorochrome/secondary IgG treatment, for fluorescent observation comparison, 3) neuroblastoma cells, which were selected as positive controls due to their known GABA-B surface receptor sites, and received complete treatments of anti-GABA-B1 and secondary antibody incubations, and 4) neat and capacitated spermatozoa treated with anti-α-tubulin and A488 fluorochrome to assess fluorescence observation independent of the anti-GABA-B1. One of the negative control groups was run to rule out secondary antibody-driven fluorescence, using only the secondary fluorochrome-conjugated antibody in the absence of the primary anti-GABA-B1 antibody. Neat and capacitated spermatozoa which did not receive anti-GABA-B1 displayed no fluorescence in any region upon observation, indicating that when fluorescence could be seen, there was binding of the biomarker to GABA-B1 sites. This outcome provided support for the validity and efficacy of the methods and results of the study. Neuroblastoma cells which were fully treated with both anti-GABA-B1 and fluorochrome incubations showed labeling in their cytoplasms, along with the anti-α-tubulin-labeled cells, showed fluorescence where expected; this functioned effectively to assess and validate the specificity and reliability of the selected methods of immunolabeling and observation. The absence of labeling in negative controls and presence of labeling in positive controls showed that anti-GABA-B1 and the secondary fluorochrome-conjugated antibodies were able to identify the regions of interest and that the LSC microscopy was an effective method of observing and analysing the samples.

Quality assurance of semen samples used computer-assisted seminal analysis to measure specific parameters, but this was conducted out-of-lab, prior to and independently from this study. For capacitated samples, validation was both intrinsic to the methods used to capacitate the sperm (swim-up methodology, described in section 2.2) and was verified before beginning experiments via capacitation-specific biomarkers, such as the qualitative analysis of tyrosine phosphorylation. Spontaneous capacitation is also a possibility within neat samples.

4.2 Limitations and considerations

The results of the study were obfuscated by an inability to obtain complete counts for the capacitated cells from donor #5. Data from this donor was limited by low sperm concentration upon observation, resulting in this group showing the lowest success rate of GABA-B1 labeling within the capacitated treatment group. A possible explanation could be errors during the immunolabeling procedure- likely a handling error with the coverslip that would hinder the incubation process. During observation, the coverslip that was analyzed from this donor had a marked increase of off-target material and debris relative to the other samples, all of which were prepared concurrently. The divergence of this sample data from the rest makes it an outlier, as the only donor group which showed an inverse relationship between capacitation

and GABA-B1 labeling. These factors limit the reliability of Fisher's p-value for this donor-level data, which designated the difference as significant. An additional improvement for a study of this sort would be the incorporation of a multi-level logistical regression, as cells were treated as independent rather than as belonging within a certain group, and this may have skewed statistical power. Still, the Fisher’s exact test helps to balance the effects of this. Finally, data analyses regarding this type of study should also account for observer bias. Individual cells had to be categorized as either labeled or unlabeled, which disregarded nuance for cells that could not be cleanly categorized due to observational limitations inherent to fluorescence microscopy. To keep results on the conservative side, spermatozoa which were not clearly demarcated were categorized as negative. This may have resulted in an underestimation of the percentage of positively labeled cells.

Instances of background fluorescence are apparent in some of the images in the results. Still, these occurrences remain visually distinct enough to differentiate background fluorescence from receptor labeling; the true signal is much brighter and definitively opaque in comparison. This background signal was especially apparent in the labeling of the neuroblastoma cells used as positive controls. Although labeling on the neuroblastoma cells was still clear, they also showed less true signal in comparison to the spermatozoa. A possible reason for this can be due to their size, as the mature neuroblastoma cell is much larger than both neat and capacitated spermatozoa. This provided a clearer depiction of labeling when viewed under the same resolution, while the labeling in the smaller spermatozoon was more compact, in comparison.

External constraints limited time spent in confocal microscopy and delayed the timeline of the research and results by several weeks; this shift also limited the scope of the research able to be carried out. The most noteworthy preclusions were the incorporation of acrosome reacted cells and the obtainment of useable results with FE-SEM. The failed FE-SEM observations were not able to be amended with secondary attempts, and the optimal dilution for the colloidal gold-conjugated IgG was not able to be confirmed after the initial failure for this method. All of the workflows were tested numerous times with different antibody dilutions and incubation periods; the times and dilutions detailed in the methods reflect the values that produced reliable and usable results. Finally, as the primary antibody used was specific to the GABA-B1 subunit, labeling could not definitely demarcate the presence of the complete GABA-B heterodimer. Nevertheless, the primary antibody used was of high specificity, and blocking solution was used liberally to prevent binding of unwanted sites.

4.3 Biological interpretations & literature synthesis

Considering the localization of GABA-B1 predominantly to the principal piece of the spermatozoon, a function of the receptor could be tied to the cell’s motility and hyperactivation via the cAMP-PKA pathway, regulation of ion flux, and/or tyrosine phosphorylation of the flagellum. The low tendency for labeling throughout the midpiece is consistent with the anatomy of the cell, as

this region is specialized for mitochondrial oxidative phosphorylation, and might not have much use for the GABA-B receptor in such a process. Further, the general absence of labeling in the head seemingly contradicts previous research which associated the GABA-B receptor with the acrosome reaction (Calogero, 1999), although it could very well still mediate this process via other mechanisms at a distance, such as upstream modulation of calcium flux. It is also possible that receptor transport to the membrane at the head is more of a transient process than an enduring staple of the metabolic transformation. The findings of this study also contrasted earlier reports that the presence of the GABA-B receptor correlated with worse semen quality (Kaewman, 2021), since receptor density and prevalence in this study was positively correlated with markers of fitness to fertilize the oocyte (capacitation). Importantly, though, that study examined expression of the GABA-B2 subunit rather than the GABA-B1 subunit. Nonetheless, the increase in receptor labeling following capacitation suggests that the binding sites become more accessible as a result of the metabolic shifts the cell undergoes during capacitation. Therefore, it is proposed that GABA-B1 labeling is positively associated with the restructuring of the plasma membrane necessary to the cell's capacity for oocyte fertilization.

Immunolabeling studies can help guide further research by filling in some gaps and highlighting those that remain. Though, an inherent limitation of this study type is the inability to definitively inform the functioning and mechanisms of the receptor, or the regions the receptor is localized to. Functional assays would also be of use to more confidently link GABA-B1 localization with functions of human spermatozoa and would supplement the findings of this study. The first recommendation for a follow-up study would be running the same immunolabeling procedure on the GABBR2 subunit in order to understand if the GABA-B receptor is fully present on the spermatozoon, which would confirm the ability for full functionality. Future studies on the GABA-B receptor in human spermatozoa should then additionally seek to test and compare receptor activity of neat and capacitated cells against acrosome reacted cells, implement the use of higher resolution imaging options like FE-SEM, and be sure to use higher donor cohorts to improve the reproducibility of results, limit the effect of outliers, and best represent the greater population. Results would further be improved by the use of automated image analysis or AI tools that would eliminate observer bias to strengthen objectivity and reproducibility.

5 Ethics and Social Impact

The need for accessible healthcare and resources within the realm of reproductive healthcare is apparent, yet existing market options for both assisted reproductive technologies and male contraceptives are limited and/or costly, putting them out of reach for the global majority. Insight into the mechanisms of male fertility and its interactions with the female reproductive tract may aid the search for solutions to both matters of infertility and birth control. The necessity for these solutions is a matter of both gender- and class-equity, as under-resourced women and children around the world continue to bear the brunt of structural and gender-based violence that is amplified by fertility-related issues and a lack of access to critical resources.

While this research sought to improve understanding of the biological processes underpinning the mechanisms of fertility and human reproduction, issues of fertility and reproduction necessarily span across many realms of study and thus cannot be understood by examining any single area alone. Comprehensive analyses of these matters must further account for sociocultural, economic, and geopolitical infrastructures, which dictate the degree to which certain factors affect birth outcomes in a given population.

This study followed all ethical protocols relevant to research involving human subjects, as laid out by the European Code of Conduct for Research Integrity and the Declaration of Helsinki. No animal subjects were involved in this research, and all donors provided informed consent for samples to be used for scientific research purposes.

6 Declaration of AI Technologies

During this research, ChatGPT (web access) was used to generate R code for RStudio. After using the software, all input and the resulting outputs were reviewed and edited as needed, and the author takes full responsibility for the result of the work.

7 Conclusion

The localization of GABA-B1 in healthy spermatozoa suggests that they may play a role in one or some of the many processes that spermatozoa must undergo in order to fertilize the egg. The increase in labeling following capacitation of the cell raises the question of whether the receptor is actively involved in the capacitation or acrosome reactive processes- which may be by means of regulating ion flow, the intracellular pH, or the induction of signaling cascades. For functional analysis, further research on the GABA-B receptor in human spermatozoa is needed. These studies should aim to dissect the functions of these receptors in both male and female tissues with regard to their involvement in pre-fertilization and fertilization processes, as there is certainly a global need for the establishment of accessible reproductive healthcare and family planning resources. The development of a cost-effective male contraceptive would contribute greatly, and pathologies of male fertility are of additional importance to global healthcare equity as families dealing with infertility face high fees, long wait times, and extensive processes through existing treatment pathways. The improvement of the scientific understanding of mechanisms of male infertility would assist the ongoing search for solutions to such pathologies, from diagnostic methods to therapeutic treatments.

Further research in this realm should include testing of receptor specificity in different mediums, identifying alternative splice variations of the encoding mRNA transcripts, functional assays of fitness parameters in the presence of GABA and analogs, and correlation studies assessing gene expression and reproductive outcomes. Both GABA-B subunits, GABA-B1 and GABA-B2, should be investigated in concert. As mentioned earlier, modern understanding of the role of the GABA-B receptor in spermatozoa and the reproductive tracts is especially limited by the fact that most research has been done on animal models rather than human models. As mechanisms and requisite components of reproduction are generally species-dependent, it is necessary that further studies on this topic are done on human spermatozoa.

The fields of reproductive medicine and biotechnology are vast, with potential for wide-reaching influence across diverse fields. The study of neuroreceptors and neuroligands outside the nervous system, such as on and within human spermatozoa navigating the FRT, can improve understanding of auxiliary functions of these receptors. Thus, their investigation has scientific relevance that extends beyond the realm of reproductive medicine and could be especially important to neurologic and endocrinologic disciplines. Further, deeper and novel knowledge of the mechanisms of cell-to-cell communication can inform other forms of technological pursuits, as they have in the past, such as the development of neural networks in machine learning and tissue engineering in biotechnology. Evidently, the GABA-B receptor's possible involvement in intercellular processes- as a component of the metabolic and structural shifts that the spermatozoon must undergo on its journey to fertilize the oocyte- provides an interesting new perspective on the function of the metabotropic neuroreceptor, and makes it worthy of deeper scientific consideration in the realm of cell-to-cell communication.

8 References

Austin, C. R. (1952). The "Capacitation" of the Mammalian Sperm. Nature, 170(4321), 326–326. https://doi.org/10.1038/170326a0

Baker, M. A., Nixon, B., Naumovski, N., & Aitken, R. J. (2012). Proteomic insights into the maturation and capacitation of mammalian spermatozoa. Systems Biology in Reproductive Medicine, 58(4), 211–217. https://doi.org/10.3109/19396368.2011.639844

Bettler, B., Kaupmann, K., Mosbacher, J., & Gassmann, M. (2004). Molecular Structure and Physiological Functions of GABABReceptors. Physiological Reviews, 84(3), 835–867. https://doi.org/10.1152/physrev.00036.2003

Brokaw, C. J. (1972). Flagellar Movement: A Sliding Filament Model. Science, 178(4060), 455–462. https://doi.org/10.1126/science.178.4060.455

Calogero, A. E., Burrello, N., Ferrara, E., Hall, J., Fishel, S., & D'Agata, R. (1999). γ-Aminobutyric acid (GABA) a and b receptors mediate the stimulatory effects of GABA on the human sperm acrosome reaction: interaction with progesterone. Fertility and Sterility, 71(5), 930–936. https://doi.org/10.1016/s0015-0282(99)00063-1

Calogero, A. E., Hall, J., Fishel, S., Green, S., Hunter, A., & D'Agata, R. (1996). Effects of γ-aminobutyric acid on human sperm motility and hyperactivation. Molecular Human Reproduction, 2(10), 733–738. https://doi.org/10.1093/molehr/2.10.733

Chalifoux, J. R., & Carter, A. G. (2011). GABAB receptor modulation of synaptic function. Current Opinion in Neurobiology, 21(2), 339–344. https://doi.org/10.1016/j.conb.2011.02.004

Chang, M. C. (1951). Fertilizing Capacity of Spermatozoa deposited into the Fallopian Tubes. Nature, 168(4277), 697–698. https://doi.org/10.1038/168697b0

Chen, R. J., & Sharma, S. (2023). GABA receptor. PubMed; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK526124/

Couve, A., Moss, S. J., & Pangalos, M. N. (2000). GABAB receptors: a new paradigm in G protein signaling. Molecular and Cellular Neurosciences, 16(4), 296–312. https://doi.org/10.1006/mcne.2000.0908

Davis, B. J. (1981). Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. 78(12), 7560–7564. https://doi.org/10.1073/pnas.78.12.7560

De Lamirande, E. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Molecular Human Reproduction, 3(3), 175–194. https://doi.org/10.1093/molehr/3.3.175

Erdö, S. L., & Wolff, J. R. (1990). γ-Aminobutyric Acid Outside the Mammalian Brain. Journal of Neurochemistry, 54(2), 363–372. https://doi.org/10.1111/j.1471-4159.1990.tb01882.x

Faroni, A., Mantovani, C., Shawcross, S. G., Motta, M., Giorgio Terenghi, & Valerio Magnaghi. (2011). Schwann-like adult stem cells derived from bone marrow and adipose tissue express γ-aminobutyric acid type B receptors. Journal of Neuroscience Research, 89(9), 1351–1362. https://doi.org/10.1002/jnr.22652

Finkelstein, M., Etkovitz, N., & Breitbart, H. (2020). Ca2+ signaling in mammalian spermatozoa. Molecular and Cellular Endocrinology, 516, 110953. https://doi.org/10.1016/j.mce.2020.110953

Furukawa, T., & Fukuda, A. (2023). Maternal taurine as a modulator of Cl− homeostasis as well as of glycine/GABAA receptors for neocortical development. Frontiers in Cellular Neuroscience, 17. https://doi.org/10.3389/fncel.2023.1221441

Geigerseder, C., Doepner, R., Thalhammer, A., Frungieri, M. B., Gamel-Didelon, K., Calandra, R. S., Köhn, F. M., & Mayerhofer, A. (2003). Evidence for a GABAergic System in Rodent and Human Testis: Local GABA Production and GABA Receptors. Neuroendocrinology, 77(5), 314–323. https://doi.org/10.1159/000070897

Gladkevich, A., Korf, J., Hakobyan, V. P., & Melkonyan, K. V. (2006). The peripheral GABAergic system as a target in endocrine disorders. Autonomic Neuroscience, 124(1-2), 1–8. https://doi.org/10.1016/j.autneu.2005.11.002

He, X. B., Hu, J. H., Wu, Q., Yan, Y. C., & Koide, S. S. (2001). Identification of GABAB Receptor in Rat Testis and Sperm. Biochemical and Biophysical Research Communications, 283(1), 243–247. https://doi.org/10.1006/bbrc.2001.4732

Hernández-González, E. O., Sosnik, J., Edwards, J., Acevedo, J. J., Mendoza-Lujambio, I., López-González, I., Demarco, I., Wertheimer, E., Darszon, A., & Visconti, P. E. (2006). Sodium and Epithelial Sodium Channels Participate in the Regulation of the Capacitation-associated Hyperpolarization in Mouse Sperm. Journal of Biological Chemistry, 281(9), 5623–5633. https://doi.org/10.1074/jbc.m508172200

Herrick, S. B., Schweissinger, D. L., Kim, S.-W., Bayan, K. R., Mann, S., & Cardullo, R. A. (2004). The acrosomal vesicle of mouse sperm is a calcium store. Journal of Cellular Physiology, 202(3), 663–671. https://doi.org/10.1002/jcp.20172

Hoshi, K., Tsukikawa, S., & Sato, A. (1991). Importance of Ca2+, K+ and glucose in the medium for sperm penetration through the human zona pellucida. The Tohoku journal of experimental medicine, 165(2), 99–104. https://doi.org/10.1620/tjem.165.99

Jewett, B. E., & Sharma, S. (2020). Physiology, GABA. PubMed; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK513311/

Kaewman, P., S. Nudmamud-Thanoi, P. Amatyakul, & S. Thanoi. (2021). High mRNA expression of GABA receptors in human sperm with oligoasthenoteratozoospermia and teratozoospermia and its association with sperm parameters and intracytoplasmic sperm injection outcomes. Clinical and Experimental Reproductive Medicine, 48(1), 50–60. https://doi.org/10.5653/cerm.2020.03972

Kerr, D. I. B., & Ong, J. (1995). GABAB receptors. Pharmacology & Therapeutics, 67(2), 187–246. https://doi.org/10.1016/0163-7258(95)00016-a

Kanbara, K. (2005). Cellular Localization of GABA and GABAB Receptor Subunit Proteins During Spermiogenesis in Rat Testis. Journal of Andrology, 26(4), 485–493. https://doi.org/10.2164/jandrol.04185

Kondziella, D. (2016). The Top 5 Neurotransmitters from a Clinical Neurologist's Perspective. Neurochemical Research, 42(6), 1767–1771. https://doi.org/10.1007/s11064-016-2101-z

Kovaříková, V., Špirková, A., Zuzana Šefčíková, Pisko, J., Kalatová, L., Koppel, J., Fabian, D., & Štefan Čikoš. (2023). Gamma-aminobutyric acid (GABA) can affect physiological processes in preimplantation embryos via GABAA and GABAB receptors. Reproductive Medicine and Biology, 22(1). https://doi.org/10.1002/rmb2.12528

Krapivinsky, G., Kennedy, M. E., Nemec, J., Medina, I., Krapivinsky, L., & Clapham, D. E. (1998). Gβγ Binding to GIRK4 Subunit Is Critical for G Protein-gated K+ Channel Activation. Journal of Biological Chemistry, 273(27), 16946–16952. https://doi.org/10.1074/jbc.273.27.16946

Li, K.-M., Li, Y.-Y., Jia, Y.-P., & Duan, L.-Y. (2020). Participation of the inositol 1,4,5-trisphosphate-gated calcium channel in the zona pellucida- and progesterone-induced acrosome reaction and calcium influx in human spermatozoa. Asian Journal of Andrology, 22(2), 192. https://doi.org/10.4103/aja.aja_44_19

Marshall, F. H., White, J., Main, M., Green, A., & Wise, A. (1999). GABAB receptors function as heterodimers. Biochemical Society Transactions, 27(4), 530–535. https://doi.org/10.1042/bst0270530

Martinez, P., & Morros, A. (1996). Membrane lipid dynamics during human sperm capacitation. Frontiers in Bioscience, 1(4), d103-117. https://doi.org/10.2741/a119

Meizel, S. (1984). The importance of hydrolytic enzymes to an exocytotic event, the mammalian sperm acrosome reaction. Biological Reviews, 59(1), 125–157. https://doi.org/10.1111/j.1469-185x.1984.tb00404.x

Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M., & O'Brien, D. A. (2004). Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proceedings of the National Academy of Sciences of the United States of America, 101(47), 16501–16506. https://doi.org/10.1073/pnas.0407708101

Ooi, E. H., Smith, D. J., Gadêlha, H., Gaffney, E. A., & Kirkman-Brown, J. (2014). The mechanics of hyperactivation in adhered human sperm. Royal Society Open Science, 1(2), 140230. https://doi.org/10.1098/rsos.140230

Princivalle, A., Regondi, M. C., Frassoni, C., Bowery, N. G., & Spreafico, R. (2000). Distribution of GABAB receptor protein in somatosensory cortex and thalamus of adult rats and during postnatal development. Brain Research Bulletin, 52(5), 397–405. https://doi.org/10.1016/S0361-9230(00)00256-2

Procacci, P., Ballabio, M., Castelnovo, L. F., Mantovani, C., & Magnaghi, V. (2013). GABA-B receptors in the PNS have a role in Schwann cells differentiation? Frontiers in Cellular Neuroscience, 6. https://doi.org/10.3389/fncel.2012.00068

Ralt, D., Goldenberg, M., Fetterolf, P., Thompson, D., Dor, J., Mashiach, S., Garbers, D. L., & Eisenbach, M. (1991). Sperm attraction to a follicular factor(s) correlates with human egg fertilizability. Proceedings of the National Academy of Sciences, 88(7), 2840–2844. https://doi.org/10.1073/pnas.88.7.2840

Shimizu, Y., Yorimitsu, A., Maruyama, Y., Kubota, T., Aso, T., & Bronson, R. A. (1998). Prostaglandins induce calcium influx in human spermatozoa. Molecular Human Reproduction, 4(6), 555–561. https://doi.org/10.1093/molehr/4.6.555

Tash, J. S. (1989). Protein phosphorylation: The second messenger signal transducer of flagellar motility. Cell Motility and the Cytoskeleton, 14(3), 332–339. https://doi.org/10.1002/cm.970140303

Thompson, S. M., Capogna, M., & Massimo Scanziani. (1993). Presynaptic inhibition in the hippocampus. Trends in Neurosciences, 16(6), 222–227. https://doi.org/10.1016/0166-2236(93)90160-n

Turner, R. M. (2006). Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, Fertility and Development, 18(2), 25. https://doi.org/10.1071/rd05120

Visconti, P. E. (2009). Understanding the molecular basis of sperm capacitation through kinase design: Fig. 1. Proceedings of the National Academy of Sciences, 106(3), 667–668. https://doi.org/10.1073/pnas.0811895106

Visconti, P. E., Ning, X., Fornés, M. W., Alvarez, J. G., Stein, P., Connors, S. A., & Kopf, G. S. (1999). Cholesterol Efflux-Mediated Signal Transduction in Mammalian Sperm: Cholesterol Release Signals an Increase in Protein Tyrosine Phosphorylation during Mouse Sperm Capacitation. Developmental Biology, 214(2), 429–443. https://doi.org/10.1006/dbio.1999.9428

World Health Organization. (2021). WHO laboratory manual for the examination and processing of human semen, 6th ed. World Health Organization. Retrieved 2025 November 20 from https://iris.who.int/handle/10665/343208

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: its development and relativity. Zygote, 2(4), 371–372. https://doi.org/10.1017/s0967199400002240

Young, A. B., & Chu, D. (1990). Distribution of GABAA and GABAB receptors in mammalian brain: Potential targets for drug development. Drug Development Research, 21(3), 161–167. https://doi.org/10.1002/ddr.430210303

Zhang, Z., Zhang, W., Huang, S., Sun, Q., Wang, Y., Hu, Y., Sun, N., Zhang, Y., Jiang, Z., Minato, N., Pin, J., Su, L., & Liu, J. (2015). GABAB receptor promotes its own surface expression by recruiting a Rap1-dependent signaling cascade. Journal of Cell Science, 128(12), 2302–2313. https://doi.org/10.1242/jcs.167056