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Research article

GABA EXISTS AS A NEGATIVE REGULATOR OF CELL PROLIFERATION IN SPERMAOGONIAL STEM CELLS

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Abstract: γ-amino butyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system. GABA is also found in many peripheral tissues, where it has important functions during development. Here, we identified the existence of the GABA system in spermatogonial stem cells (SSCs) and found that GABA negatively regulates SSC proliferation. First, we demonstrated that GABA and its synthesizing enzymes were abundant in the testes 6 days postpartum (dpp), suggesting that GABA signaling regulates SSCs function *in vivo*. In order to directly examine the effect of GABA on SSC proliferation, we then established an *in vitro* culture system for long-term expansion of SSCs. We showed that $GABA_A$ receptor subunits, including α1, α5, β1, β2, β3 and γ3, the synthesizing enzyme GAD67, and the transporter GAT-1, are expressed in SSCs. Using phosphorylated histone H3 (pH3) staining, we demonstrated that GABA or the $GABA_AR$-specific agonist muscimol reduced the proliferation of SSCs. This GABA regulation of SSC proliferation was shown to be independent of apoptosis using the TUNEL assay. These results suggest that GABA acts as a negative regulator of SSC proliferation to maintain the homeostasis of spermatogenesis in the testes.

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Abbreviations used: bFGF – basic fibroblast growth factor; CSF-1 – colony-stimulating factor 1; dpp – days postpartum; GABA – γ-amino butyric acid; GDNF – lial cell line-derived neurotrophic factor; MEF – mouse embryonic fibroblasts; pH3 – phosphorylated histone H3; SSCs – spermatogonial stem cells; TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling

Key words: GABA, GABAA receptor, Spermatogonial stem cells, Proliferation, Muscimol, Testes

INTRODUCTION

Spermatogonial stem cells (SSCs) reside along the basement membrane of the seminiferous tubules in the testes and self-renew to produce sperm throughout the lifespan. The rarity of SSC in the testes (0.03% of all germ cells in the adult mouse testes [1]) and the lack of definitive markers make the *in vivo* study of SSCs challenging. Nevertheless, results of long-term culture of SSCs and experiments on their functional transplantation, in which they have been shown to reestablish spermatogenesis, have greatly enhanced our understanding of their characteristics and regulation [2, 3]. A culture of mouse THY1+ germ cells in serum-free medium supplemented with glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) supports the expansion of SSCs for extended periods of time [4], facilitating molecular and biochemical analyses of SSC self-renewal and division. GDNF, secreted by Sertoli cells, is a crucial factor for SSC proliferation through its involvement in the activation of both the phosphoinositide-3 kinase-Akt pathway and Sarcoma family kinase signaling [5, 6]. In addition, colony-stimulating factor 1 (CSF-1) from peritubular myoid cells, Nodal autocrine signaling, and Wnt5a have been shown to contribute to SSC maintenance and proliferation [7-9]. In a situation that is similar to that found with other cycling adult tissues, continual spermatogenesis is dependent on maintaining a certain balance in a cell pool; in this case, the balance of the SSC pool. Therefore, besides the factors essential for SSC self-renewal and expansion, there must be other extrinsic signals that negatively control SSC proliferation in their associated niche microenvironment. However, the extrinsic factors limiting SSC proliferation remain an intriguing puzzle.

γ-amino butyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system, exerts its action through three classes of GABA receptors, termed the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic G protein-coupled $GABA_B$ receptor. It has now become clear that GABA signaling also exists in peripheral organs. The testes have well-described components of the GABA system, including the three types of GABA receptor, GABA synthetic enzymes (GAD65 and GAD67), and a GABA transporter (GAT-1) [10-14]. However, very little is known about the precise roles and underlying mechanisms of GABA signaling during spermatogenesis. Aside from traditional neurotransmission, GABA is considered to act as a trophic factor during both embryonic and adult neurogenesis, regulating key developmental steps, such as proliferation, differentiation and migration [15-17]. For example, within the subventricular zone, the largest neurogenic region in the adult brain, non-synaptic GABA released by neuroblasts reduces the proliferation of GFAP-positive neural progenitors [18]. The inhibitory effect of GABA on cell proliferation is not restricted to the nervous system. GABA has recently been

shown to limit the proliferation of pluripotent embryonic stem cells and adult neural stem cells [19, 20].

In light of these recent findings, we investigated whether GABA is present in SSCs and if it influences their proliferation. First, we demonstrated the abundance of GABA and its synthesizing enzymes in 6-day postpartum (dpp) testes containing undifferentiated SSCs and progenitors. Then we established an *in vitro* culture system for long-term expansion of SSCs and showed for the first time that the molecular components of the GABA signaling pathway were expressed in SSCs. Further analysis revealed that GABA reduced SSC proliferation independently of apoptosis *in vitro*. The results of this study suggest that GABA, as an extrinsic signal for proliferation inhibition, is involved in the maintenance of SSC pool size.

MATERIALS AND METHODS

Animal

For these studies, we used DBA/2J mice of different ages, which were purchased from the Shanghai Laboratory Animal Center (China). The animals were treated according to local and national guidelines. All of the animal experiments were approved by the Biomedical Research Ethics Committee of the Shanghai Institute of Biological Sciences.

Isolation, culture and transplantation of SSCs

SSCs were isolated essentially according to the methods described previously [4, 21]. Briefly, decapsulated testes from 6- to 8-day old mice were digested with 1 mg/ml collagenase (Type IV, Sigma) at 37ºC with gentle agitation for 12 min. The seminiferous tubules were then washed with Hanks' balanced salt solution without calcium or magnesium (HBSS), followed by incubation at 37ºC for 8 min in HBSS containing 0.25% trypsin and 0.5 mg/ml DNase (Sigma). After neutralization with a 20% volume of fetal bovine serum, the cells were collected and filtered through a 40-μm cell strainer to remove large clumps of cells. SSC enrichment was done using magnetic-activated cell sorting with magnetic microbeads conjugated to anti-Thy-1 antibody (BD Biosciences) according to the manufacturer's instructions. Finally, Thy-1+ cells were plated at a density of ~0.5-1 $\times 10^5$ per cm^2 on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) in 24-well plates. The culture medium for the SSCs consisted of Minimum Essential Medium Alpha (MEM-α; Invitrogen), MEM Non-Essential Amino Acids (Invitrogen), MEM vitamin solution (Invitrogen), penicillin/streptomycin (Invitrogen), 10% fetal bovine serum, 2 mM L-Glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 μg/ml transferrin (Sigma), 25 μg/ml insulin (Invitrogen), 60 μM putrescine (Sigma), 60 ng/ml progesterone (Sigma), 40 ng/ml GDNF (PeproTech), 1 ng/ml bFGF (PeproTech), and 10^3 U/ml murine leukemia inhibitory factor (Chemicon). Cultures were maintained at 37ºC in a 5% CO_2 atmosphere and subcultured at 1:2 or 1:3 ratios every 4-6 days.

For testicular transplantation, the cultured SSCs were infected with GFP lentivirus. 40 mg/kg busulfan-treated male mice (aged 6-8 weeks, DBA/2J) were used as the recipient mice. Approximately 10 μl of single cell suspension containing 1×10^5 cells were injected using a 60-μm micropipette into the seminiferous tubules through the efferent duct. Two months after transplantation, the seminiferous tubules were dissociated and examined under a fluorescence microscope to detect the GFP expression.

Immunofluorescence

Testes from 6-dpp mice were dissected and fixed in 4% PFA for 12 h at 4ºC. The specimens were immersed in 20% sucrose overnight at 4ºC, then embedded in OCT compound and sectioned at 10 μm on a cryostat (Leica CM3050S). For the cultured SSCs, the collected clumps were adhered to glass slides and fixed in 4% PFA for 30 min. After a wash with PBS, these slides were permeabilized with 0.1% Triton for 5 min and blocked in 3% BSA for 1 h. Then, the slides were incubated with primary antibody overnight at 4ºC. The following antibodies were used: rabbit anti-GABA (1:1000, A2052, Sigma), rabbit anti-GAD65/67 (1:1000, G5163, sigma), rabbit anti-GFRα1 (1:100, SC-10716, Santa Cruz), rabbit anti-$GABA_AR$ β3 (1:100, NB300-199, Novus), and normal rabbit IgG (1:500, I5006, Sigma). Slides were then rinsed in PBS and incubated with Cy3 affinity-purified goat anti-rabbit IgG (1:1000, 111-165-045, Jackson ImmunoResearch) for 1 h at room temperature. The nuclei were counterstained with DAPI (Sigma). Specimens with immunofluorescence labeling were examined under a confocal microscope (Leica TCS SP5).

RT-PCR

Total RNA from the SSCs, testes and brain was extracted using Trizol according to the manufacturer's procedure. The first strand of cDNA was generated from 2 μg of total RNA with oligo-dT primer and M-MLV Reverse Transcriptase. The primer sequences used for PLZF, Stra8, Oct-4, Piwil2, GFRα1, $GABA_AR$ (α1, α5, β1, β2, β3, γ3), $GABA_BR$ (R1, R2), GAD67, GAD65, GAT-1 and β-actin are listed in Supplemental Table 1 at http://dx.doi.org/10.2478/s11658-013-0081-4). For the GABA receptor subunits GAD and GAT-1, PCR was carried out with 35 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 30 s, and extension at 72ºC for 60 s. For the SSC markers, reactions were carried out with 30 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s, and extension at 72ºC for 60 s.

Cell proliferation studies

The SSC clumps were removed from MEF feeders by gentle pipetting, which yields more than 90% pure germ cell suspensions [22]. Single cell suspensions were obtained by digestion with Trypsin and 5×10^4 cells were seeded in 48-well plates containing SSC medium and new MEF. The cells were treated for 6 days with GABA (500 μM, Sigma) or the $GABA_A$ receptor agonist muscimol (100 μM, Sigma). The control cultures received a vehicle. The SSC medium was replaced

every 24 h, and fresh GABA and muscimol were added at the specified concentrations. Cell proliferation was determined by evaluating the mitotic activity with anti-phospho-histone H3 (1:500, 06-570, Millpore). The number of pH3-positive cells in the SSC clumps was counted using a fluorescence microscope. The data were collected from three experiments. Student's unpaired t test was performed for statistical analysis with $P < 0.05$ as the level of significance.

TUNEL assay

The dissociated cells from SSC clumps were seeded with SSC medium and MEF feeders at a density of 5×10^4 cells/well in a 48-well plate. GABA (500 μM) or muscimol (100 μM) was added to the medium, followed by incubation at 37ºC for 4 days. Cisplatin (25 μM) served as the positive control for apoptosis. The TUNEL assay was performed using a One-Step TUNEL Apoptosis Assay Kit (Beyotime Biotech, China). Briefly, the SSC clusters were fixed with 4% paraformaldehyde for 45 min, and then treated with 0.1% TritonX-100 for 2 min on ice. The TUNEL reaction mixture was prepared as indicated in the manufacturer's instructions and applied to the cell monolayer for 60 min at 37ºC in the dark. The cells were counterstained with DAPI and analyzed under a confocal microscope (Leica TCS SP5).

RESULTS

GABA and its synthesizing enzymes are present in 6-dpp mouse testes

The transformation of gonocytes into SSCs is complete 6 dpp in mice [23]. At this young age, undifferentiated spermatogonia are the only germ cells present in the seminiferous tubules. We first assessed the production of GABA and the expression of its synthesizing enzymes (GADs) in the 6-dpp testes using anti-GABA and anti-GAD65/67 antibody. Immunohistochemistry revealed that GABA was specifically confined to the seminiferous tubules and not visible in interstitial cells and peritubular myoid cells (Fig. 1A). Higher-magnification images confirmed that GABA was present in the cytoplasm of undifferentiated spermatogonia and the lumen (Fig. 1C and D). GAD65/67 was found to be widely expressed in germ cells and the interstitial tissues (Fig. 1E and F). Rabbit IgG, which served as a negative control, showed no specific staining (Fig. 1B). The distribution of GABA and GADs in the neonatal testes suggests that GABA signaling might control SSC functions.

Establishment and characterization of mouse SSCs

To directly examine the effect of GABA on SSC activities, we developed an *in vitro* culture system that allowed the long-term maintenance and expansion of mouse SSCs. SSCs were first enriched by MACS with anti-Thy-1 antibody after two-step enzymatic digestion of samples from 6-dpp testes. Thy-1+ germ cells

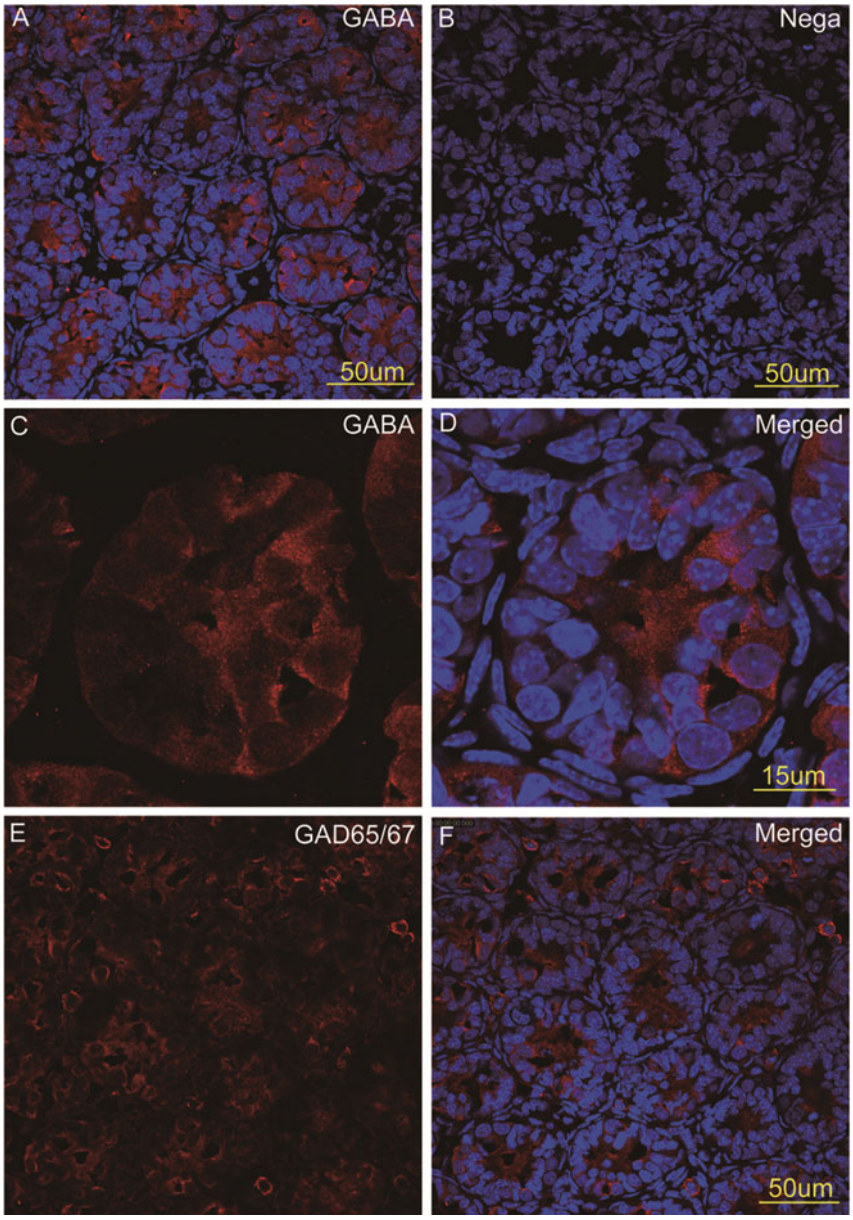

Fig. 1. Immunohistochemistry shows GABA and GAD65/67 localization in the testes of mice 6 days postpartum. A – GABA is specifically present in the seminiferous tubules, but not in the interstitial cells and peritubular myoid cells. B – Replacement of the primary antibody with normal rabbit IgG served as a negative control and no staining was observed. C and D – High magnification images indicate that GABA is localized in the cytoplasm of undifferentiated spermatogonia and the lumen. E and F – GAD65/67 is expressed in germ cells and the interstitial tissues. Scale bars: 50 μm (A, B, and F) and 15 μm (D).

were then cultured on MEF feeders in MEMα medium supplemented with GDNF, bFGF and LIF. Cultured Thy-1+ cells divided and formed typical SSC colonies, showing grape-like clumps of densely packed germ cells with tightly adhering membranes (Fig. 2A). RT-PCR analysis showed that these clump-forming cells expressed markers for SSCs, GFRα1, PLZF, Stra8, Oct-4 and Piwil2 (Fig. 2B). GFRα1 was shown to have primary expression on the membrane and minor expression in the cytoplasm of cultured SSCs (Fig. 2C). These results indicate that the cultured Thy-1+ cells represent an undifferentiated SSC phenotype [24].

To further confirm the functionality of cultured SSCs, we transplanted SSCs infected with GFP lentivirus into the seminiferous tubules of busulfan-treated mice

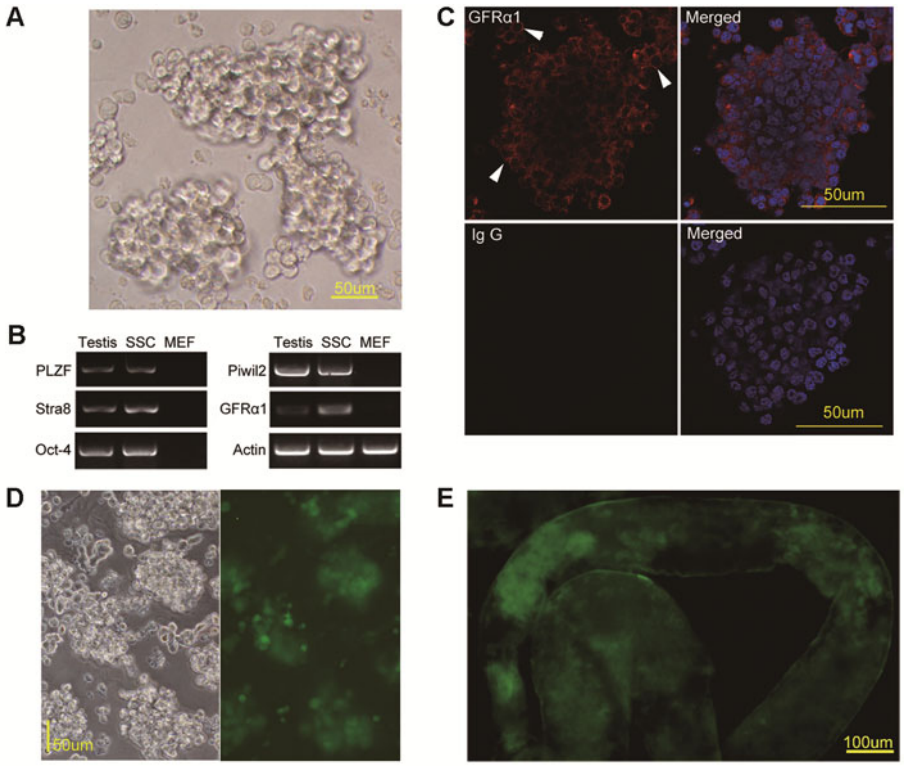

Fig. 2. Establishment of a long-term culture system for mouse SSCs. A – Phase-contrast image of SSCs after 4 months proliferation *in vitro*. The SSCs form grape-like clumps of stem cells with tightly adhering membranes. B – RT-PCR analysis of marker gene expression in the testes, SSCs and MEF. Cultured SSCs expressed PLZF, Stra8, Oct-4, Piwil2 and GFRα1. C – Immunocytochemistry of GFRα1 (red) in cultured SSCs. The staining indicates GFRα1 had primary expression on the membrane (arrow head) and minor expression in the cytoplasm of cultured SSCs. D – Donor SSCs infected with GFP lentivirus show green fluorescence. E – GFP-positive colonies within the seminiferous tubule of the recipient testes 2 months after transplantation. Scale bars: 50 μm (A, C and D) and 100 μm (E).

in which endogenous germ cells were depleted. Two months later, colonies of GFP-positive germ cells were observed in the recipient testes (Fig. 2D and E). These results demonstrate that cultured SSCs are still capable of restoring spermatogenesis in recipient testes. In this study, the established mouse SSC lines could proliferate continuously for more than 4 months without losing stem cell activity.

GABA signaling components are expressed in mouse SSCs

We next characterized the expression profile of the molecular components of the GABA signaling pathway in cultured SSCs. RT-PCR analysis revealed that mRNAs for the $GABA_A$ receptor subunits α1, α5, β1, β2, β3 and γ3 were expressed in SSCs. $GABA_B$ receptor subunit R1 had a low expression in SSCs but R2 did not, which indicates that there is no functional $GABA_B$ receptor in SSCs. GAD67 mRNA, but not GAD65, was expressed in SSCs. GAT-1, the only membrane GABA transporter found in the testes and sperm of rodents [12, 25], was also detected in SSCs (Fig. 3A). The immunocytochemistry confirmed the positive reactivity of GAD and $GABA_A$ receptor subunit β3. Because GAD65 mRNA was not expressed in SSCs, the staining of GAD represented the localization of GAD67. It was located in the cytoplasm of SSCs. The staining of the β3 subunit revealed its expression in SSCs, especially in the cytoplasm and membrane (Fig. 3B). Collectively, these results indicate that mouse SSCs possess the complete components of GABA signaling.

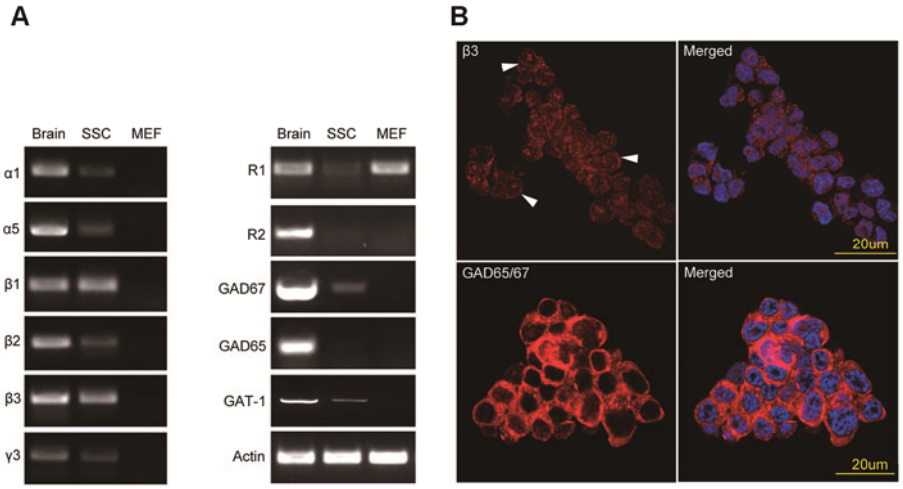

Fig. 3. Expression of GABA signaling components in SSCs. A – RT-PCR analysis of $GABA_A$ receptor subunits α1, α5, β1, β2, β3 and γ3, $GABA_B$ receptor subunits R1 and R2, GAD65, GAD67, GAT-1 and actin from the mouse brain, SSCs and MEF. B – Immunocytochemistry of β3 subunit of $GABA_A$ receptor and GAD65/67 in cultured SSCs. The staining of the β3 subunit indicated that it had an expression in SSCs, especially in the cytoplasm and membrane (arrow head). Immunoreactivity for GAD65/67 was found in the cytoplasm of SSCs. Scale bars: 20 μm (B).

GABA reduces the proliferation of mouse SSCs

Since GABA has been previously shown to modulate the proliferation of many different cell types, including ES cells and adult neural stem cells, we sought to investigate the significance of $GABA_AR$ activation in SSC proliferation. Phosphorylation of serine 10 in histone H3, a well-characterized mitosis marker in G_2/M, could be used to evaluate the mitotic activity of rat SSC clumps *in vitro* [26]. Here, we quantified the numbers of pH3-positive cells per SSC clump after exposure to GABA or the $GABA_AR$-specific agonist muscimol. It was found that GABA or muscimol significantly reduced the numbers of pH3-positive cells without affecting the SSC clump morphology (Fig. 4A and C). The TUNEL assay revealed that apoptosis was not triggered in SSCs after GABA or muscimol treatment (Fig. 4B). These results indicate that in SSCs, GABA exerts its effect through the inhibition of proliferation instead of through apoptosis induction.

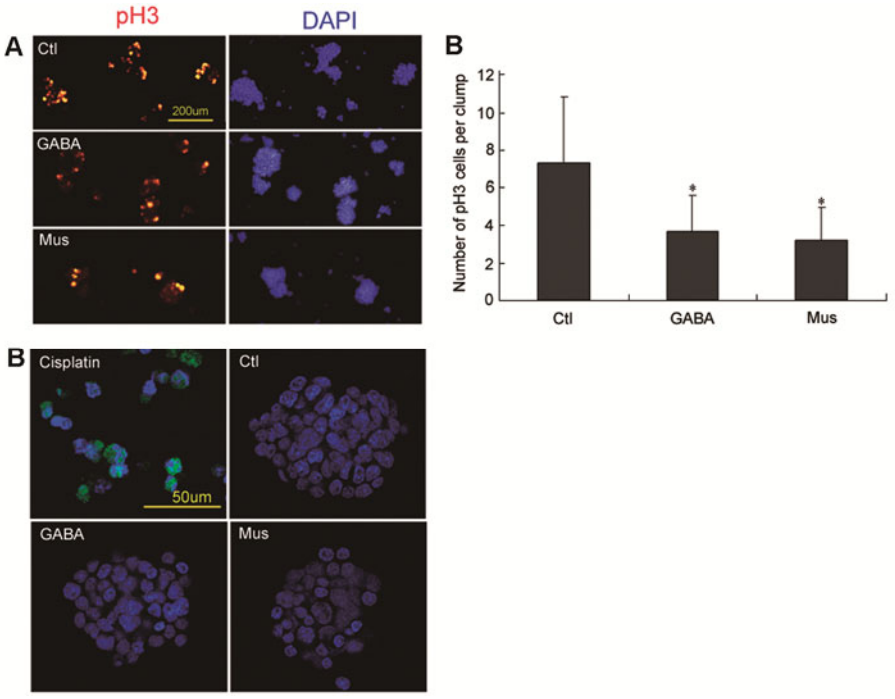

Fig. 4. GABA decreases the proliferation of SSCs. A – Representative images of pH3 staining of SSCs after a 6-day exposure to the control, GABA (500 μM) and the $GABA_AR$-specific agonist muscimol (100 μM). B – Number of pH3-positive cells per SSC clump. GABA or muscimol significantly reduced the numbers of pH3-positive cells. Data are shown as means ± SEM. *$P < 0.05$ versus control. C – TUNEL assay of SSCs treated with the control, GABA, muscimol or cisplatin (30 μM). Cisplatin was used as positive control for germ cell apoptosis. SSCs treated with cisplatin were detached and showed green TUNEL staining. GABA, muscimol and the control showed no staining. Scale bars: 200 μm (A) and 50 μm (C).

DISCUSSION

In this study, we established a long-term culture system to recapitulate SSC self-renewal *in vitro*, which was enormously valuable for the identification of extrinsic niche factors that regulated SSC functions *in vivo*. Here, we identified GABA as a novel niche factor that negatively regulated SSC proliferation. Immunohistochemistry showed that GABA and its synthesizing enzymes were abundant in mouse testes 6 days postpartum. We showed for the first time that the GABA signaling components, including $GABA_A$ receptor, synthesizing enzyme GAD67 and transporter GAT-1 were expressed in SSCs. The R1 subunit of $GABA_B$ receptor had a low expression in SSCs, and a functional $GABA_B$ receptor could not exist because of the lack of the R2 subunit. Furthermore, we found that GABA restricted SSC proliferation independently of apoptosis. These findings imply that GABA is involved in the maintenance of SSC homeostasis during spermatogenesis.

Although the concept that the male reproductive system contained a GABA system has gradually become accepted, most previous studies characterized the existence of GABA signaling at the level of testis histology [11-14, 27, 28]. Our results show that SSCs, the foundation for spermatogenesis, possess the GABA signaling pathway, attesting to the existing of GABA signaling at the germ cell level. Consistent with a recent study [14], we found that GABA was specifically present in the seminiferous tubules, but not in interstitial cells. This also provided the evidence for the spatial regulation of SSCs by GABA signaling. The expression of the $GABA_A$ receptor subunits α1, α5, β1, β2, β3 and γ3 in SSCs is authentic because these subunits have also been detected in the testes in other studies [14, 28]. The $GABA_A$ receptor is a heteropentameric chloride-selective channel assembled from various combinations of subunits in the brain [29]. The subunit composition of the $GABA_A$ receptor in SSCs remains undetermined. Our RT-PCR analysis shows that the β3 subunit is the most abundant subunit of the $GABA_A$ receptor in SSCs, indicating that this subunit provides a molecular target to clarify the precise mechanisms by which GABA signaling affects SSCs.

GABA is a versatile molecule with diverse functions in organisms from bacteria through plants to higher mammals [30]. In the male reproductive system, GABA has been shown to promote the acrosome reaction of spermatozoa [31, 32]. However, the precise physiological function of GABA in germ cell development remains unclear. Here, we propose that via $GABA_A$ receptors, GABA negatively regulates the proliferation of SSCs based on the following evidence. First, GABA and synthesizing enzymes are abundantly expressed in the testes 6 days postpartum, suggesting the *in vivo* production of GABA in the SSC niche. Second, cultured SSCs have all the molecular components of GABA signaling. Third, GABA or the $GABA_A$R-specific agonist muscimol reduces the mitotic activity of SSCs *in vitro*. Further studies using targeted disruption of $GABA_A$R are necessary to assess this concept.

Constant spermatogenesis is supported by a highly robust stem cell system in the testes. SSCs go through 8 to 9 mitotic divisions and 2 successive meiotic divisions before development into spermatozoa. On average, one SSC can generate 2048 spermatozoa, or sometimes even 4096 spermatozoa [33]. Therefore, the size of the SSC population, which fundamentally determines an individual's reproductive potential, is precisely coordinated by niche signals. The pool size of SSCs is maintained by self-renewal throughout life in the basement membrane of the seminiferous epithelium. Currently, some of the extrinsic factors mentioned above (GDNF, bFGF, CSF-1, Nodal and Wnt5a) and two signaling pathways (PI3K/Akt and Ras/Erk1/2) are known to promote the self-renewal of SSCs [5-9, 34]. However, the regulation of SSC pool size has not been fully elucidated. It has been observed that the overexpression of GDNF in transgenic mice leads to the development of malignant testicular cancer, similar to the situation with classic human seminoma, and the Ras-cyclin D2 activation induces the tumorigenesis of SSCs [35, 36]. Moreover, it is believed that human spermatocytic seminoma may originate from SSCs [37]. These studies strongly suggest that the excessive SSCs self-renewal results in germ cell tumors. Therefore, the *in vivo* testicular microenvironment undoubtedly provides an inhibitory signal for SSC proliferation to maintain spermatogenesis homeostasis. GABA signaling is such a proliferation inhibitor allowing the maintenance of SSC homeostasis. We propose that physiological levels of GABA synthesized by SSCs or interstitial cells through $GABA_A$ receptors result in a decreased proliferation of SSCs. GABA control over proliferation is also found in ES cells and adult neural stem cells, even in tumors where putative cancer stem cells reside, suggesting a developmentally conserved role of GABA as a negative regulator of stem cell proliferation [19, 20, 38].

In conclusion, our study provides direct evidence that the GABA system is present in mouse SSCs, and indicates that GABA inhibits SSC proliferation independently of apoptosis *in vitro*. This result suggests that GABA, as a negative regulator of SSC proliferation, maintains the homeostasis of spermatogenesis in the testes.

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