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Differentiation-linked changes in the biosynthesis and turnover of sphingomyelins in rat male germ cells: Genes involved and effects of testosterone

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In rodents, sphingomyelins (SMs) species with very-long-chain polyunsaturated fatty acid (VLCPUFA) are required for normal spermatogenesis. Data on the expression of enzymes with roles in their biosynthesis and turnover during germ cell differentiation and on possible effects on such expression of testosterone (Tes), known to promote this biological process, were lacking. Here we quantified, in isolated pachytene spermatocytes (PtS), round spermatids (RS), and later spermatids (LS), the mRNA levels from genes encoding ceramide (Cer), glucosylceramide (GlcCer), and SM synthases (Cers3, Gcs, Sms1, and Sms2) and sphingomyelinases (aSmase, nSmase) and assessed products of their activity in cells in culture using nitrobenzoxadiazole (NBD)-labeled substrates and [^{3}H]palmitate as precursor. Transcript levels from *Cers3* and *Gcs* were maximal in PtS. While mRNA levels from *Sms1* increased with differentiation in the direction PtS→RS→LS, those from *Sms2* increased between PtS and RS but decreased in LS. In turn, the *nSmase* transcript increased in the PtS→RS→LS order. During incubations with NBD-Cer, spermatocytes produced more GlcCer and SM than did spermatids. In total germ cells cultured for up to 25 h with NBD-SM, not only abundant NBD-Cer but also NBD-GlcCer were formed, demonstrating SM→Cer turnover and Cer recycling. After 20 h with [^{3}H] palmitate, PtS produced [^{3}H]SM and RS formed [^{3}H]SM and [^{3}H]Cer, all containing VLCPUFA, and Tes increased their labeling. In total germ cells, Tes augmented in 5 h the expression of genes with roles in VLCPUFA synthesis, decreased the mRNA from *Sms2*, and increased that from *nSmase*. Thus, Tes enhanced or accelerated the metabolic changes occurring to VLCPUFA-SM during germ cell differentiation.

The biochemical steps involved in the biosynthesis, interconversions, and catabolism of sphingolipids (SLs) in mammalian cells occur in separate cell compartments, and most of the enzymes and auxiliary proteins involved have been identified and cloned to date, reviewed in refs. (1–5). Briefly, ceramide (Cer), the common precursor of glycosphingolipids (GSLs) and sphingomyelin (SM), is synthesized in the endoplasmic reticulum (ER), in part *via de novo* and in part *via* the salvage pathways. The *de novo* synthesis occurs by the condensation of palmitoyl-CoA and L-serine by serine palmitoyltransferase (SPT) to form 3-ketosphinganine, which is then reduced to dihydrosphingosine (sphinganine). The latter and an acyl-CoA are then combined to produce (dihydro) ceramide by ceramide synthase (Cers). Next, a (dihydro)Cer desaturase introduces the 4,5-*trans*-double bond that gives rise to sphingosine (Sph)-containing Cer molecules. Cer can also be formed by the reutilization of the sphingoid bases (SBs) (mainly Sph) that result from the normal catabolism of GSL and SM in acidic cell compartments (early endosomes, lysosomes). In the case of SM, acid sphingomyelinase (aSmase) followed by acid ceramidase actions separate the Sph and the fatty acid. Once in the cytosol, Sph is phosphorylated (to S1P), and the fatty acids are converted to acyl-CoAs, facilitating their solubility and transport through the cell. In the ER, S1P is dephosphorylated to recover the Sph, which is acylated by Cers to produce the salvaged Cer. A third contributor to the total Cer of a cell is the normal turnover undergone by membrane-residing SLs, for example, in plasma membranes, the conversion of SM into Cer by a membrane-located neutral sphingomyelinase, such as nSmase.

The Cer molecules synthesized in the ER are transported (6) to the Golgi apparatus, where they can be glycosylated, phosphorylated, or converted into SM. Glucosylceramide (GlcCer) is mainly formed by GlcCer synthase (Ugcg, here abbreviated as Gcs) mainly on the cytosolic face of the Golgi, where UDP-glucose is transferred to Cer. Then, the β1,4-galactosyltransferases 5 and 6 convert this GlcCer into lactosylceramide, the common precursor of most neutral and acidic GSLs other than those of the galactosylceramide series. In turn, the cell SM is synthesized within the Golgi apparatus, in different areas according to the cell (7), by an SM synthase (Sgms, here abbreviated as Sms) activity.

The Sms enzyme occurs in two main isoforms, Sms1 and Sms2, which are co-expressed in a variety of mammalian cell types, the former mainly located to the Golgi and the later mostly to the plasma membrane (8, 9). The Sms1 is primarily responsible for generating the bulk of the cellular SM, which cycles (in vesicles) between the Golgi and the plasma

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membrane, reviewed in (10). As previously shown for total Sms (*e.g.*, in refs. (11, 12)), Sms1 and Sms2 are both capable of converting phosphatidylcholine (PC) and Cer into SM and diacylglycerol (DAG) as well catalyzing the reverse reaction (PC+Cer ⇌ SM+DAG) (8). A schematic overview of the reactions described, the mentioned enzymes, and their main cellular localization is depicted in Figure 1*A*.

In rodent spermatogenic cells, Cer, SM, GlcCer, and a subset of complex GSL (13–16) are unique in that they are exceedingly rich in molecular species with very-long-chain (C28-C32) polyunsaturated fatty acids (VLCPUFAs). These fatty acids, primarily of the n-6 series in rats and of both the n-6 and n-3 series in mice, occur in nonhydroxy (n-V) and 2-hydroxy (h-V) forms (SM species containing 32:5n-6 or h-32:5n-6 are shown as examples in Fig. 1*B*). Not surprisingly, the SM species with these fatty acids substantially differ from "canonical" SM species having comparatively shorter and saturated fatty acids (*e.g.*, 16:0 SM) in biophysical properties (17, 18) as well as in their lateral distribution within the plasma membrane of germ cells (19).

In rodents, species of Cer and SM with h-V increase over those with n-V during differentiation of germ cells, as previously shown in isolated pachytene spermatocytes (PtS), round spermatids (RS), spermatids at later stages of their

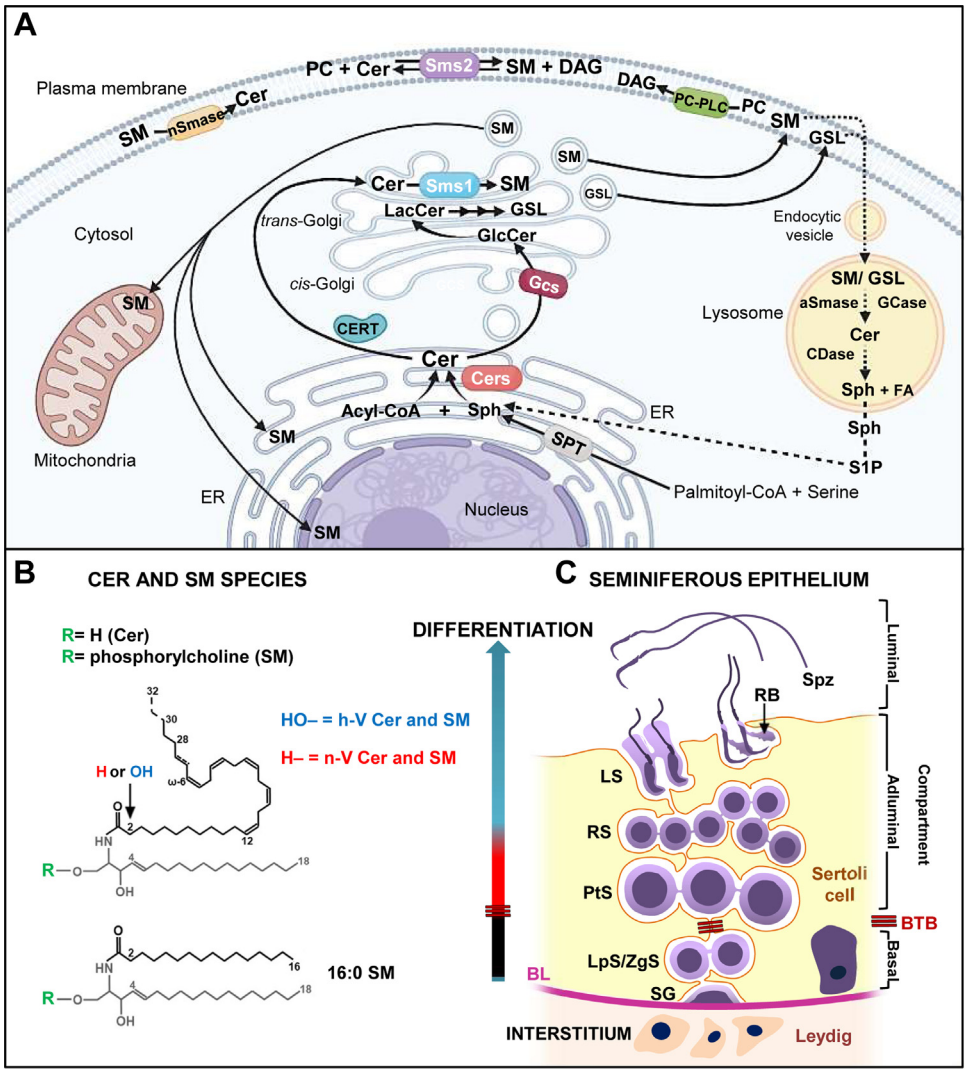

Figure 1. Schematic representations of general aspects of compartmentalization and pathways involved in mammalian cell sphingolipid metabolism; typical Cer and SM molecular species of rat germ cells, and stages of germ cell differentiation in rodents. *A*, sphingolipids: Cer, GlcCer, GSL, SM, Sph, and S1P: ceramide, glucosylceramide, glycosphingolipids, sphingomyelin, sphingosine, and sphingosine-1-phosphate, respectively. Synthases and transport proteins: SPT, Cers, CERT, Gcs, Sms1 and Sms2, serine palmitoyltransferase, Cer synthase, Cer transfer protein, GlcCer synthase, SM synthase1 and SM synthase 2, respectively. Hydrolases: PC-PLC, GCase, CDase, aSmase and nSmase, phosphatidylcholine-specific phospholipase C, acid glycosidases, ceramidase, acid Smase, and neutral Smase, respectively. Panel *A* was created with BioRender.com. *B*, the n-V 32:5 and h-V 32:5 Cer and SM species are represented in the plane, with headgroups indicated in green and with the C2 of the fatty acid containing an H (*red*) or a hydroxyl group (*blue*), respectively. A 16:0 SM species is also included. *C*, a simplified drawing representing spermatogenic cells in successive stages of their differentiation and some of the somatic cells and structures present in an adult rodent testis. BL, basal lamina; BTB, blood-testis barrier; Cer, ceramide; Cers, ceramide synthase; CERT, ceramide transfer protein; GlcCer, glucosylceramide; GSL, glycosphingolipids; LpS, ZgS, and PtS, leptotene, zygotene, and pachytene spermatocytes, respectively; RB, residual bodies; RS and LS, round and late spermatids (includes elongating and elongated spermatids), respectively; SG, spermatogonia; SM, sphingomyelin; Smase, sphingomyelinase; SPT, serine palmitoyltransferase; Spz, spermatozoa.

differentiation (LS), and spermatozoa (16) (cells graphically depicted in Fig. 1*C*). Using high-resolution-mass spectrometric imaging, Rabionet *et al.* (20) showed this change *in situ* in the mouse seminiferous epithelium, by identifying the molecular species n-28:4 SM and h-30:5 SM in spermatocytes and spermatids, respectively. Among GSL, a hallmark of rodent germ cell differentiation, described in 2005 by Sandhoff *et al.* (13), is the formation of neutral and acidic fucosylated GSLs (FGSLs) containing VLCPUFA, mostly h-V (*e.g.*, h-30:5n-6), which coincides with *Fa2h*, the gene involved in the 2-hydroxylation of fatty acids, increasing its expression (mRNA, protein) with rat germ cell differentiation (21).

Working sequentially and in concert with position-specific fatty acid desaturases, the PUFA elongases known as elongation of very-long-chain fatty acids proteins (Elovl)5 and Elovl2 are known to synthesize the C18-C22 and C20-C24 PUFA of the n-3 and n-6 series that give rise to the major (C20 and C22) PUFA of glycerophospholipids in mammalian cells (22, 23). In rat seminiferous tubules (24), and more recently in isolated germ cells (21), we showed that the C24 PUFA generated by this group of enzymes can be elongated up to C32 n-V (Fig. S1), which was linked to the expression and activity of the elongase Elovl4 (21). The importance of Elovl2 as mediator in the synthesis of the PUFA and VLCPUFA of germ cell lipids was evidenced by the finding (25) that *Elovl2*$^{-/-}$ mice are sterile, with only spermatogonia and primary spermatocytes populating their testes.

The biosynthesis of SLs with VLCPUFA in germ cells depends on the expression of the *Cers3* gene (15). The germ cell–specific deletion of *Cers3* in mice, with the consequent lack of VLCPUFA-containing Cer, SM, GlcCer, and complex GSL, leads to spermatogenic arrest due to cell instability and apoptosis during meiosis (20). After conditional deletion of *Gcs* in germ cells, Cer and SM are produced, but the lack of GlcCer leads to absence of lactosylceramide and complex GSL with VLCPUFA in the testis, also leading to compromised spermatogenesis (20). Thus, simple and complex SLs with VLCPUFA, as well as the VLCPUFA themselves (25), are required for normal spermatogenesis and rodent male fertility.

Although spermatogenesis requires SM with VLCPUFA in germ cells, a comparison of the expression of the genes encoding for the SM synthases and phophohydrolases responsible for their synthesis and turnover during germ cell differentiation was still lacking. Also absent were reports on possible effects on such expression of testosterone (Tes), the hormone on which many aspects of growth and differentiation of germ cells depend at specific stages of the spermatogenic cycle, reviewed in (26–28).

With the hypothesis that germ cell differentiation should modify the expression of genes involved in SL formation, here we first quantified, in isolated PtS, RS, and LS, the mRNA levels from genes encoding four synthases (Cers3, Gcs, Sms1, and Sms2) and two Smase (Smpd1 and Smpd2, commonly known as aSmase and nSmase, respectively) and assessed products of their activity in germ cells in culture using NBD-labeled Cer and SM as fluorescent substrates and [^{3}H] palmitate ([^{3}H]16:0) as precursor. Samples containing total germ cells (TCs)—and in some cases samples of isolated Sertoli cells (SCs)—were included for comparison.

We also tested the hypothesis that, because the presence of Tes is naturally favorable for the advancement of germ cell differentiation *in vivo*, its presence in the medium of germ cells in culture should recapitulate at least some of the effects of differentiation on the expression of genes that participate in the formation of SLs with VLCPUFA, especially of SM. The effects of Tes on the formation of [^{3}H]Cer and [^{3}H]SM from [^{3}H] palmitate in PtS, RS, and TC, were assayed in the presence of only the hormone and in the presence of the hormone supplemented with Sertoli cell-conditioned medium (SCM), on the assumption that this medium contains several factors that are in general favorable to support homeostasis, growth, and differentiation of germ cells (29).

Finally, using preparations of TC incubated in the absence *versus* presence of the hormone, and SCM alone *versus* hormone+SCM, we determined the expression of the six genes above mentioned, and added four genes with roles in the biosynthesis of VLCPUFA, including *Elovl4* and *Fa2h*. Our results showed that differentiating rat germ cells in culture, *i.e.*, independently of the somatic cells normally present in their *in vivo* environment, can synthesize their own SLs with VLCPUFA, with biosynthetic features distinctive to each differentiation stage, and that Tes has positive influences on such biosynthesis.

Results

Expression of Cers3, Sgms1 (Sms1), Sgms2 (Sms2), and Ugcg (Gcs) in germ cells

The transcript levels from these four synthases in germ cells in three differentiation stages, measured by real-time quantitative PCR, is compared to those in SCs in Figure 2. The mRNA level from *Cers3* was the highest in PtS, *i.e.*, nearly 7-fold and 16-fold higher than in RS and LS, respectively. In comparison, SCs, whose Cer and SM are devoid of n-V (Fig. S2), did not express the *Cers3* gene. This pattern of mRNA distribution was consistent with that previously reported for the Cers3 protein in the rodent seminiferous epithelium (20) and with the observation that isolated PtS contain the highest levels per cell of Cer species with n-V, followed by RS and LS (16).

The *Gcs* mRNA level was similar between PtS and RS and displayed a moderate but significant trend to decrease between RS and LS (Fig. 2). SCs showed, on average, 4-fold less *Gcs* mRNA than germ cells.

Regarding SM synthases, the mRNA levels from *Sms1* slightly but continuously increased in the order PtS→RS→LS. In turn, those from *Sms2*, were as high as those of *Sms1* in PtS, but as much as nearly 3-fold higher than those of *Sms1* in RS (Fig. 2). Compared with germ cells, in SCs, the transcript levels from both *Sms* genes were about 100-fold lower.

Notably, the expression of *Sms2* increased significantly between PtS and RS and decreased again between RS and LS. The *Sms2/Sms1* mRNA ratios were nearly 1.0 in PtS, 2.0 in RS,

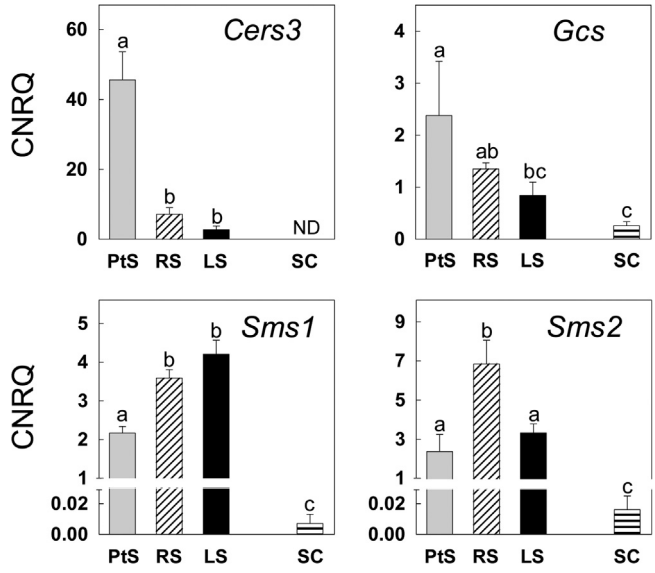

Figure 2. Expression of Cer synthase 3 (*Cers3*), the two major SM synthases (*Sms1* and *Sms2*), and GlcCer synthase (*Gcs*) genes in differentiating rat spermatogenic cells. Comparison with Sertoli cells. After isolation of pachytene spermatocytes (PtS), round spermatids (RS), late spermatids (LS), and Sertoli cells (SC), the mRNAs from the indicated genes were quantified by RT-qPCR. Data are shown as calibrated normalized relative quantities (CNRQ) of each transcript (± SEM), calculated as detailed in ref. (21). *Different letters* on the bars indicate significant differences among cell types ($p < 0.05$, ANOVA); ND, not detected. Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin.

and 0.8 in LS, pointing to the net predominance of *Sms2* mRNA in RS.

Formation of SM, GlcCer, and Cer from NBD-labeled substrates

To initially compare the biosynthetic abilities of PtS, RS, and LS to generate SM and GlcCer, the cells were incubated for 1, 3, and 5 h in the presence of nitrobenzoxadiazole (NBD)-C6-Cer in the medium we often refer to for brevity as the "basal" culture medium of germ cells (low-glucose Dulbecco's modified Eagle's medium [DMEM] supplemented with lactate and fetal bovine serum) at 33 °C. Within this interval, NBD-C6-GlcCer was accumulated more actively than NBD-C6-SM in the three cell types (Fig. 3*A*) and in TCs (Fig. 3*B*). The pattern of changes with time in TC was compatible with the biosynthesis of NBD-GlcCer and NBD-SM tending to reach their maxima at an incubation time of around 5 h. The levels of both products decreased thereafter to a somewhat lower level, which was kept with little change for about 25 h.

The amounts of NBD-C6-GlcCer and NBD-C6-SM formed per 10^8 cells after 1 h incubation were similar in PtS, RS, and LS, with differences appearing later on. Between 1 and 3 h, the amount of GlcCer predominated over that of SM by around 5-fold in PtS and twice in spermatids. Between 3 and 5 h, the formation of NBD-GlcCer tended to reach a plateau in PtS while that of NBD-SM was increasing in the three cells. At the time point of 5 h, the amounts of both NBD-lipid products were the highest in PtS (PtS > RS ≥ LS) (Fig. 3*A*).

The rates of GlcCer and SM formation from t = 0 to each of the three time points expressed as pmol per mg protein per

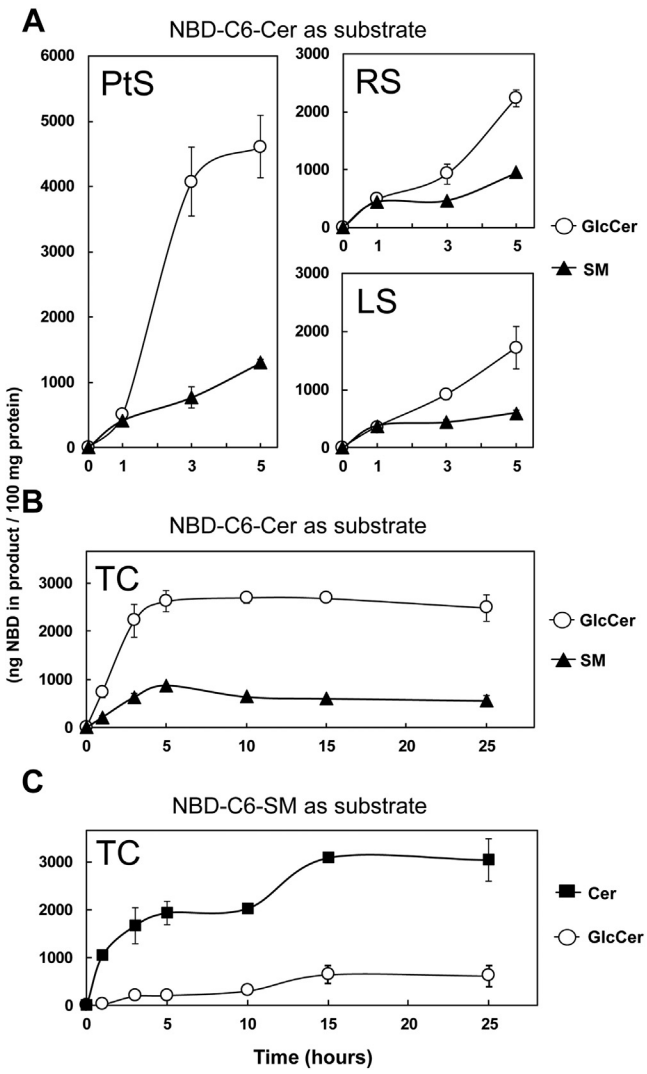

Figure 3. Formation of SM, GlcCer, and Cer from NBD-labeled substrates in rat spermatogenic cells in primary culture. *A*, PtS, RS, and LS were incubated up to 5 h in the presence of NBD-C6-Cer. *B* and *C*, total spermatogenic cells (TC) were incubated up to 25 h in the presence of NBD-C6-Cer and NBD-C6-SM, respectively. The fluorescent lipid products were extracted, separated by HPTLC, and their fluorescence was expressed on the basis of cell protein content (mean values ± SD, n = 3). Cer, ceramide; GlcCer, glucosylceramide; LS, late spermatids; PtS, pachytene spermatocytes; RS, round spermatids; SM, sphingomyelin.

hour (Fig. S3), showed that GlcCer synthesis was maximal at t = 3 h in PtS and at t = 5 h in RS and LS. In contrast, that of NBD-SM was maximal during the first 60 min in the three cell populations. The synthesis of SM maintained a constant rate of increase between 0 and 5 h in PtS, whereas in spermatids and in (spermatid-rich) TC samples, the amount of SM accumulated per hour appeared to decrease with time.

That the amounts of SM formed from Cer decreased after 5 h in TC (Fig. 3*B*) could have arisen from a decreased synthesis or from an increased turnover of SM molecules previously synthesized. To assess the contribution of SM turnover, we replicated the experiment with TC using NBD-C6-SM as substrate (Fig. 3*C*). The results not only showed that an important proportion of NBD-C6-SM was indeed actively converted into NBD-C6-Cer, but also that a nonnegligible part

of the generated Cer was used, with time, to produce NBD-C6-GlcCer. Taken together, the results in Figure 3, *B* and *C* indicated that at relatively long incubation times (*e.g.*, 10–20 h), the metabolism of SM in germ cells in the present culture conditions had reached a steady-state of equilibrium between biosynthesis, trafficking, turnover, and re-synthesis as complex as that graphically represented in Figure 1*A*.

Expression of acid and neutral Smases in rat germ cells

The activity of germ cell Smases evidenced in Figure 3*C* from the SM→Cer generation prompted us to measure the mRNA levels encoding for acid (a) and neutral (n) Smases as a function of differentiation stages (Fig. 4). Compared to germ cells, the mRNA from aSmase (*Smpd1*) predominated in SCs, where the expression of nSmase (*Smpd2*) was very low. Both genes were expressed in germ cells, where the mRNA levels from *nSmase* were 10-, 5-, and 11-fold higher than those from *aSmase* in PtS, RS, and LS, respectively. In addition, the activity of transcription of *nSmase* significantly increased in the order, PtS→RS→LS, being considerably high in spermatids.

Effect of Tes on the formation of NBD-labeled lipids

To test possible effects of this androgen on the formation of SLs, germ cells in different maturation stages were preincubated in the presence of the hormone (10^{-7} M) or its vehicle (ethanol) for 5 h, after which the NBD-C6-Cer was added as substrate, and incubations continued for 3 h (Fig. 5). In TC, the presence of Tes significantly stimulated (145% over controls) the synthesis of NBD-C6-SM from NBD-C6-Cer (Fig. 5*A*, left panel). In contrast, the levels of the lipids that could have arisen from NBD-C6-SM (Cer or GlcCer) were not affected significantly (Fig. 5*A*, right panel), suggesting that at least after this incubation time, the hormone did not modify the SM→Cer reaction.

Evidence that Tes enhanced the synthesis of SM was obtained in TC by using D609, an inhibitor of the activity of SM synthase(s) (Fig. 5*B*). Its presence not only tended to decrease (a 15%) the formation of NBD-SM from NBD-Cer in cells not exposed to the androgen but blocked completely the hormone-induced increase of NBD-SM levels.

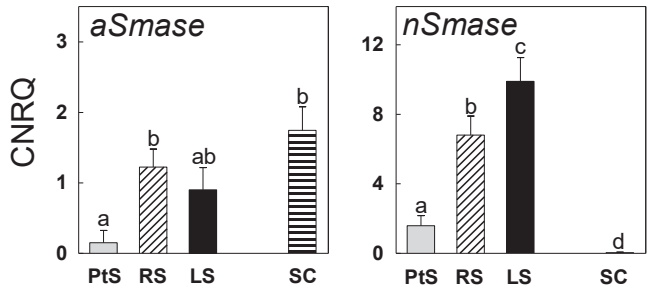

Figure 4. Expression of genes encoding acid and neutral Smases (*aSmase, nSmase*) in differentiating rat spermatogenic cells. Comparison with Sertoli cells. After isolation of PtS, RS, LS, and SC the mRNAs from *aSmase* and *nSmase* were quantified by RT-qPCR as described in Figure 2. *Different letters* on bars indicate significant differences among cell types ($p < 0.05$, ANOVA). LS, late spermatids; PtS, pachytene spermatocytes; RS, round spermatids; SC, Sertoli cells.

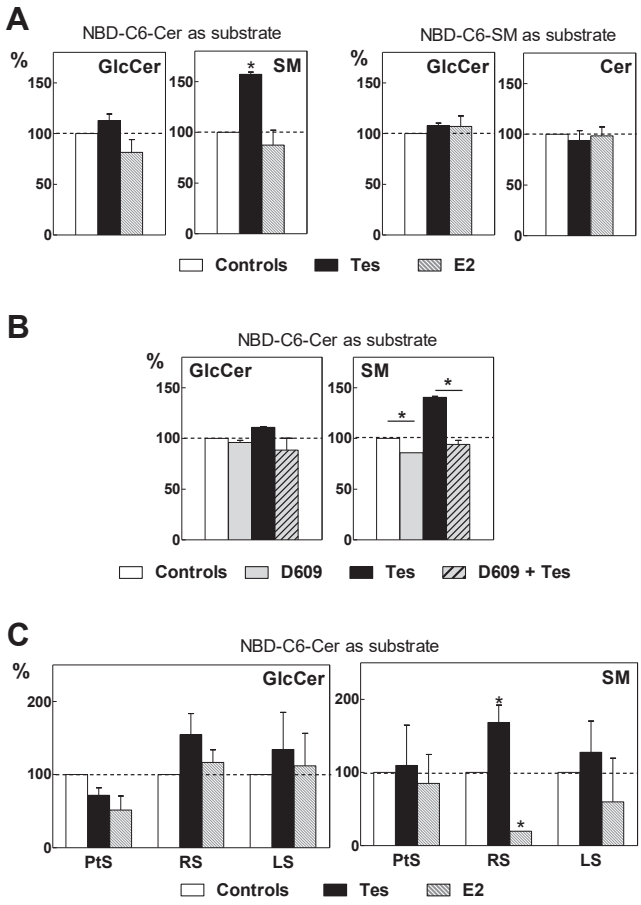

Figure 5. Formation of Cer, SM, and GlcCer from NBD-labeled substrates by rat germ cells in the presence of testosterone. *A*, formation of NBD-C6-SM from NBD-C6-Cer (*left panel*) and of NBD-C6-Cer from NBD-C6-SM (*right panel*) in total spermatogenic cells (TCs) in the absence and presence of testosterone (Tes) or estradiol (E2). The cells were incubated during 5 h in the medium containing either the vehicle ethanol (controls) or a 10^{-7} M concentration of each hormone. Then, the NBD-labeled substrates were added, and incubations continued for 3 h. *B*, effect of D609, inhibitor of SM synthase(s), on the amounts of NBD-C6-SM formed from NBD-C6-Cer in the absence and presence of testosterone. TCs were preincubated for 5.5 h without (controls) or with 15 μM D609. At t = 30 min during this period, testosterone or its vehicle were added, and incubations proceeded as before for 5 h, followed by NBD-C6-Cer addition and incubation for 3 h. *C*, formation of NBD-C6-SM and NBD-C6-GlcCer from NBD-C6-Cer in isolated PtS, RS, and LS, treated as described for TC in part *A*, *i.e.*, preincubated for 5 h with the vehicle or the hormone (Tes or E2), followed by addition of NBD-C6-Cer and incubation for 3 h. The products in *A*, *B*, and *C* were isolated by TLC their fluorescence was quantified, and the data are presented as percentages with respect to the corresponding controls (mean values ± SD, n = 3). Significant differences with controls ($p < 0.01$) are indicated by *asterisks* (*). Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin.

Of the three germ cell stages under study, the enhancement in the formation of SM by Tes did not reach significance in PtS or LS and was found to be maximal in RS (169% over controls). It must be taken into account that the RS preparations contain spermatids at the 1 to 7 stages of the seminiferous epithelium cycle, for the hormone may have not affected the whole of, but specific components within, the RS population.

Because spermatogenic cells in all stages hold aromatase activity, which increases between PtS and RS, reviewed in (30), samples of the same cell populations were separately incubated with (10^{-7} M) 17-β estradiol (Fig. 5, *A* and *C*) to test the

possibility that the hormone effects could in part be contributed by aromatization of the latter from the Tes added. Remarkably, while the amounts of NBD-C6-SM formed from NBD-C6-Cer increased in RS after (a total of 8 h) exposure to Tes, its levels fell to as little as 20% of controls in RS treated with 10^{-7} M estradiol (Fig. 5*C*).

Lipid labeling from [^{3}H]16:0 in germ cells

That Tes stimulated the formation of NBD-SM from NBD-Cer in germ cells prompted us to search for possible effects of the hormone on the synthesis of SM from Cer and their species. Using [^{3}H]16:0 as precursor, we needed to determine the ability of germ cells in culture to synthesize Cer, SM, and GlcCer from this fatty acid and possible variations between differentiation stages. Preparations of PtS, RS, LS, and TC, kept in their basal medium, received an equal volume of the same medium containing [^{3}H]16:0 and incubations continued, initially for 5 h as in Figure 3. However, most of the label was in glycerophospholipids, and no label in SLs resulted other than a few counts in [^{3}H]Cer. Given the ER-localization of SPT and Cer synthases to generate Cer from palmitic acid and the time required for Cer transport from ER to Golgi, this could have been expected, as [^{3}H]16:0 was a relatively more "remote" substrate for the formation of [^{3}H]SM than NBD-C6-Cer. Thus, we chose an incubation time of 20 h, admitting that at this time not only synthesis but a metabolic balance state of the same nature as that reached with NBD-labeled substrates had been reached.

The tendency of Cer and SM from rodent germ cells to separate into *groups* of molecular species by TLC (Fig. 6*A*) was taken to advantage to assess the synthesis of the n-V Cer and n-V SM groups of species, as these tend to run ahead, apart from the rest of species. This rest in turn consists of two groups, one that contains fatty acids also present in other cells (predominantly 16:0 and small amounts of other saturated and monoenoic (s+m) fatty acids) and another one that contains 2-hydroxy VLCPUFA (h-V). In the case of Cer, the latter two groups were also resolved, for we had three sets of species (h-V Cer, (s+m) Cer, and n-V Cer). In the case of the highly polar SM, because two-dimension TLC was used to separate SM from other polar lipids including GlcCer, the SM species with h-V and (s+m) fatty acids tended to partially overlap, for we removed them together from the plates (here named (s+m+h) SM species for brevity).

The distribution of label from [^{3}H]16:0 among lipid classes of PtS, RS, and TC after 20 h in culture is compared to that of SCs in Figure 6*B*. The label was massively incorporated in glycerophospholipids, followed by neutral lipids in all cell types. The percentage that remained as [^{3}H] label in the free fatty acid pool was 7% in PtS, 13.6% in RS, 8% in TC, and 3% in SCs.

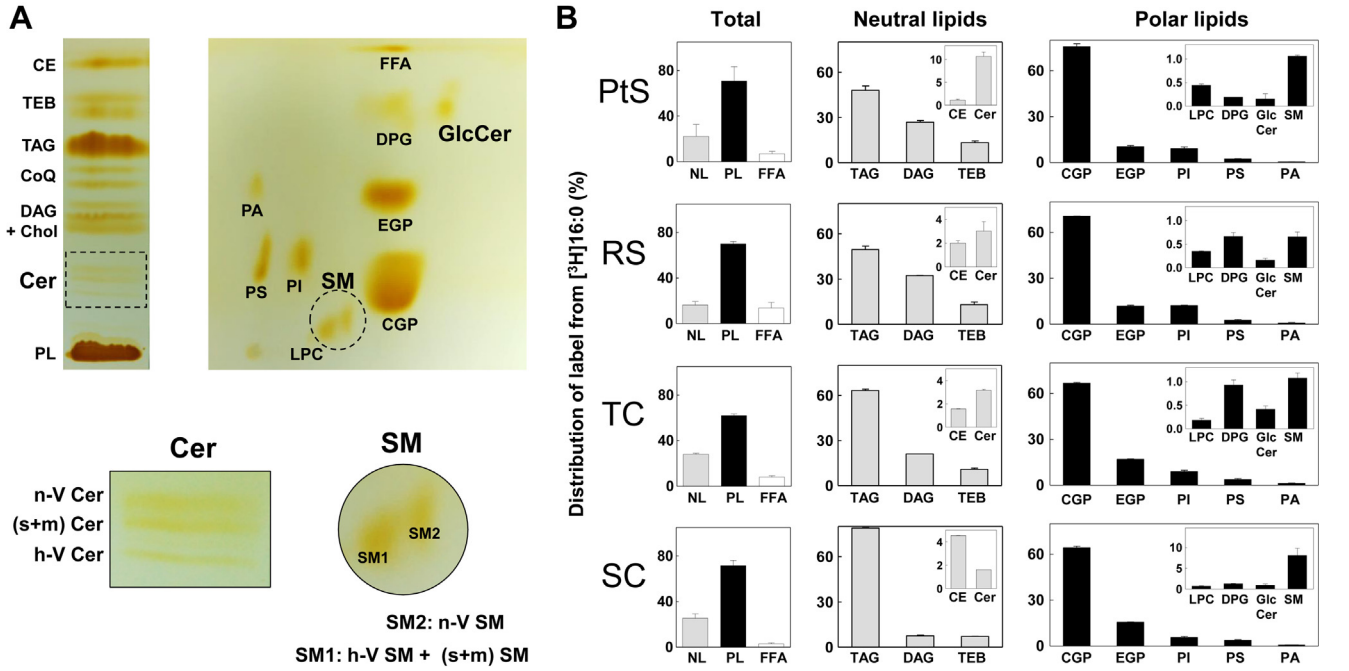

Figure 6. Separation of lipid classes from adult rat spermatogenic cells and their labeling from [^{3}H]16:0. *A*, typical TLC separations of neutral and polar lipids from TC preparations showing the spontaneous separation of Cer and SM into groups of species (amplified images shown below). The groups are named h-V Cer, (s+m) Cer, and n-V Cer, because of the fatty acids they contain (2-hydroxy VLCPUFA, C16-C18 saturates and monoenes, and nonhydroxy VLCPUFA, respectively). In the case of SM, using 2 D TLC, the group containing the n-V SM species separated from the rest, containing the h-V SM and (s+m) SM groups of species. As the latter two groups partially overlapped, we in general took them together (referred to as the "(s+m+h) SM" group). *B*, distribution of the label from [^{3}H]16:0 among lipid classes of PtS, RS, TC, and SC after 20 h in culture. Lipids were separated as in (*A*) and their [^{3}H] label was measured by liquid scintillation counting (LSC). CGP, EGP, PI, PS, and PA, choline-glycerophospholipids and ethanolamine-glycerophospholipids, phosphatidylinositol, phosphatidylserine, and phosphatidic acid; LPC, DPG, GlcCer, and SM, lysophosphatidylcholine, diphosphatidylglycerol, glucosylceramide, and sphingomyelin, respectively; NL, PL, and FFA, neutral lipids, polar lipids, and free fatty acids; (s+m), saturated and monoenoic fatty acids; (s+m+h), saturated, monoenoic, and 2-hydroxy fatty acids; TAG, DAG, TEB, CE, and Cer, triacylglycerols, diacylglycerols, triglycerides with an ether bond, cholesteryl esters, and ceramides; TC, total spermatogenic cell; VLCPUFA, very-long-chain polyunsaturated fatty acids.

Label from [^{3}H]16:0 in total Cer, SM, and GlcCer

After 20 h in primary culture, [^{3}H]Cer concentrated most of the label from [^{3}H]16:0 in the germ cells under study (Fig. 7*A*). Comparing cells, PtS generated the highest amounts of [^{3}H] SM and RS the highest of [^{3}H]GlcCer, as well as those of total [^{3}H]Cer. In the same period, SCs synthesized far more [^{3}H]SM and much less [^{3}H]Cer and [^{3}H]GlcCer per mg of protein than did germ cells. The [^{3}H]Cer/[^{3}H]SM ratio at the end of incubations approached 1.0 in PtS, 2.0 in RS, and 4.0 in TC but was as small as 0.07 in SCs.

Label from [^{3}H]16:0 in Cer and SM species

Most of the [^{3}H] label incorporated after 20 h in the [^{3}H]Cer and [^{3}H]SM of germ cells was in species that contained (s+m) fatty acids (Fig. 7*B*). This had been expected, as [^{3}H]16:0 can get into the [^{3}H] SB by the activity of SPT, and as an N-acyl group by the activity of Cer synthases. In SCs, all of the label from [^{3}H]16:0 in Cer and SM was in their (s+m) group of species, which concurs with both SLs containing almost exclusively (s+m) fatty acids from C16 to C24 (Fig. S2). Remarkably, in germ cells, an important proportion of the label from [^{3}H]16:0 was in the Cer and SM species with VLCPUFA.

In PtS, where similar amounts of total [^{3}H]Cer and total [^{3}H]SM had been formed, as much as 34% of the label in Cer, and notably even more, 40% of that in SM were contributed by the [^{3}H] n-V Cer and [^{3}H] n-V SM groups of species, respectively (Fig. 7*B*). A similar trend was followed in PtS by the [^{3}H] h-V Cer and [^{3}H] h-V SM groups, which accounted for a 14% and a 20% of the label in Cer and SM respectively.

In RS, where more total [^{3}H]Cer and much less total [^{3}H] SM than in PtS had been formed, [^{3}H] n-V Cer concentrated a 22% of the label in Cer and [^{3}H] n-V SM as much as 39% of the label in SM. At the same time, [^{3}H] h-V Cer and [^{3}H] h-V SM accounted for a 22% and nearly a 30% of the label in RS Cer and SM, respectively.

After separating the SBs from the fatty acids in germ cell [^{3}H]Cer and [^{3}H]SM (Fig. 7*C*), an important proportion of the label from [^{3}H]16:0 was in their SB. The percentages of the total label were similarly high in the SB of Cer from PtS and RS (61% and 65%, respectively, the difference with 100% corresponding to the label in [^{3}H] fatty acids). In contrast, the percentages differed between the SB of SM from PtS and RS and were lower than those in Cer (44% and 32%, respectively). Therefore, most of the label in the [^{3}H]SM of PtS and RS, especially in the latter (56% and 68%, respectively), corresponded to [^{3}H] N-bound acyl chains, part of which were VLCPUFA.

The finding of considerable amounts of [^{3}H] n-V and [^{3}H] h-V in [^{3}H] Cer and [^{3}H]SM having started from [^{3}H]16:0 as substrate indicates that products from [^{3}H]16:0 metabolism

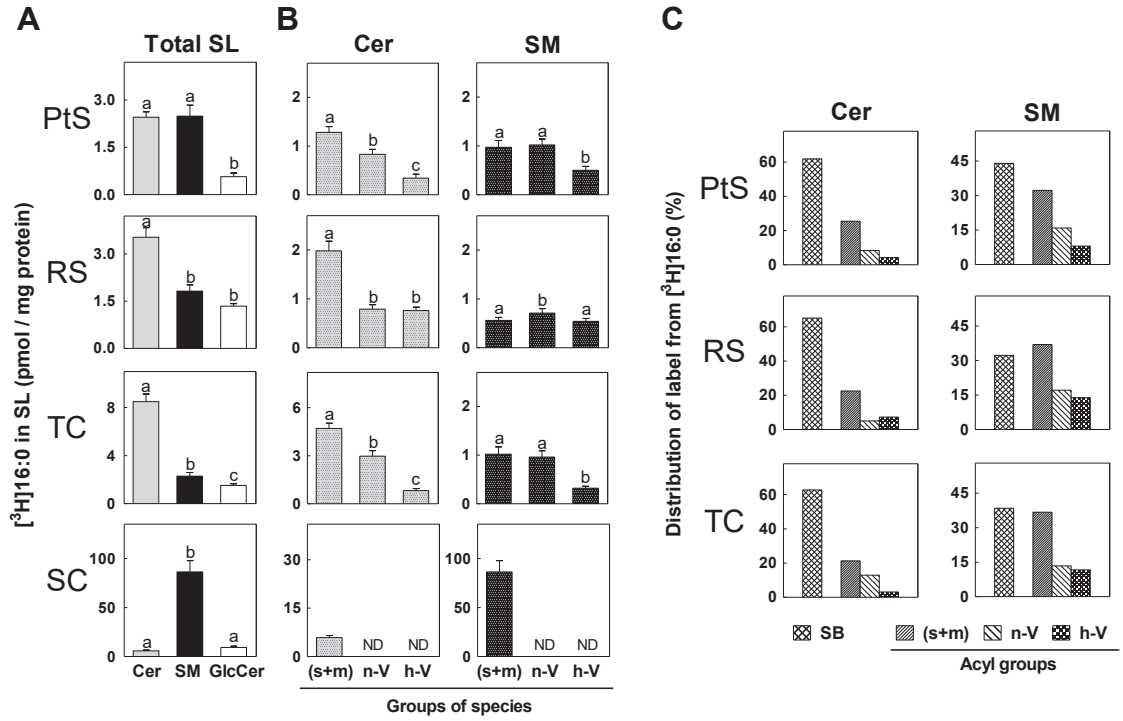

Figure 7. Changes associated with differentiation in the formation of Cer, SM, and GlcCer from [^{3}H]16:0 in rat germ cells. Comparison with Sertoli cells. PtS, RS, TC, and SC were cultured in the presence of [^{3}H]16:0 for 20 h followed by lipid extraction and TLC as illustrated in Figure 6 to separate the sphingolipids (*A*), and groups of species of Cer and SM (*B*). [^{3}H] was measured by LSC. In the case of SM, the group of species containing (s+m+h) SM was eluted from the plates and resolved into (s+m) SM and h-V SM groups by argentation TLC. *Different letters* indicate significant differences among lipids or species ($p < 0.05$, ANOVA); ND, not detected. *C*, distribution of the label from [^{3}H]16:0 among the sphingoid bases (SBs) and the amide-bound fatty acids (FAs) of Cer and SM from germ cells. After isolation, [^{3}H]Cer and [^{3}H]SM were subjected to methanolysis, their SB and FA (as methyl esters) were separated by TLC, and their [^{3}H] label was recorded. The results show the percentages of the label in the SB and in the three groups of fatty acids (s+m), n-V and h-V from Cer and SM. Cer, ceramide; GlcCer, glucosylceramide; LS, late spermatids; PtS, pachytene spermatocytes; RS, round spermatids; (s+m), saturated and monoenoic fatty acids; SC, Sertoli cells; SM, sphingomyelin; TC, total spermatogenic cells.

(*e.g.*, [^{3}H]acetate and/or [^{3}H]malonate), generated during the 20 h in culture, were utilized by germ cells to elongate endogenous (initially unlabeled) C20 to C24 long-chain PUFA to n-V. This implied the activity of PUFA desaturases and elongases to synthesize the n-V and the 2-hydroxylation of the latter to form the h-V (Fig. S1).

Notably, a 24% and as much as a 30% of the label in the total fatty acids of the SM from PtS and RS, respectively, were contributed by [^{3}H]VLCPUFA. Between PtS and RS, the proportion of [^{3}H] n-V acyl chains in SM was similar, while that of [^{3}H] h-V SM was twice higher in RS than in PtS.

Germ cell responses to inhibitors of SL synthesis

L-cycloserine (CS) and fumonisin B1 (FB1), inhibitors of the enzymes SPT and Cer synthase(s), respectively, were used to evaluate the contribution of *de novo* and salvage pathways to Cer synthesis, as the former decreases only the Cer synthesized *via* the first route while the latter reduces the Cer formed by both pathways (Fig. S4).

Pachytene spermatocytes

In PtS treated with CS, the label in total [^{3}H]Cer dropped to as little as 28% of the control value, while the amounts of total [^{3}H]SM and [^{3}H]GlcCer still produced accounted to as much as nearly 60% of the controls (Fig. 8*A*). These results are compatible with CS inhibiting quite strongly the formation of [^{3}H]sphinganine and thereby reducing the formation of [^{3}H]Cer *via de novo* but not the formation of [^{3}H]Cer *via* the salvage pathway. The bulk of the salvaged SBs are expected to derive (i) in part from SLs containing the newly formed (labeled) [^{3}H]SBs, which in this case were heavily reduced by the exposure to CS and (ii) in part from the turnover of membrane SLs preexisting in the cells (and their recycling) contributing unlabeled SBs. Thus, although decreased with respect to controls, the SBs available in the ER were N-acylated by the Cer synthases using the [^{3}H-labeled fatty acids also available there, giving rise to [^{3}H]Cer molecules mostly labeled in their [^{3}H] acyl chains, able to be taken up as substrates by the SM and GlcCer synthases.

Treatment of PtS with FB1 resulted in a reduction of total [^{3}H]Cer, [^{3}H]SM, and [^{3}H]GlcCer with respect to controls, as expected from FB1 interfering with the ability of Cer synthases to use the newly formed as well as the salvaged SBs to produce [^{3}H]Cer from [^{3}H]16:0. The FB1 treatment reduced the amounts of total [^{3}H]Cer significantly *less* and those of total [^{3}H]SM and [^{3}H]GlcCer significantly *more*, than did CS (Fig. 8*A*).

Round spermatids

In the RS population, the changes after FB1 were similar as in PtS (less total Cer → less total GlcCer and SM). However, the changes after CS differed with the cell stage, as the amount of total [^{3}H]Cer was much *less* decreased, while those of [^{3}H] SM and [^{3}H]GlcCer were much *more* decreased, in RS than in

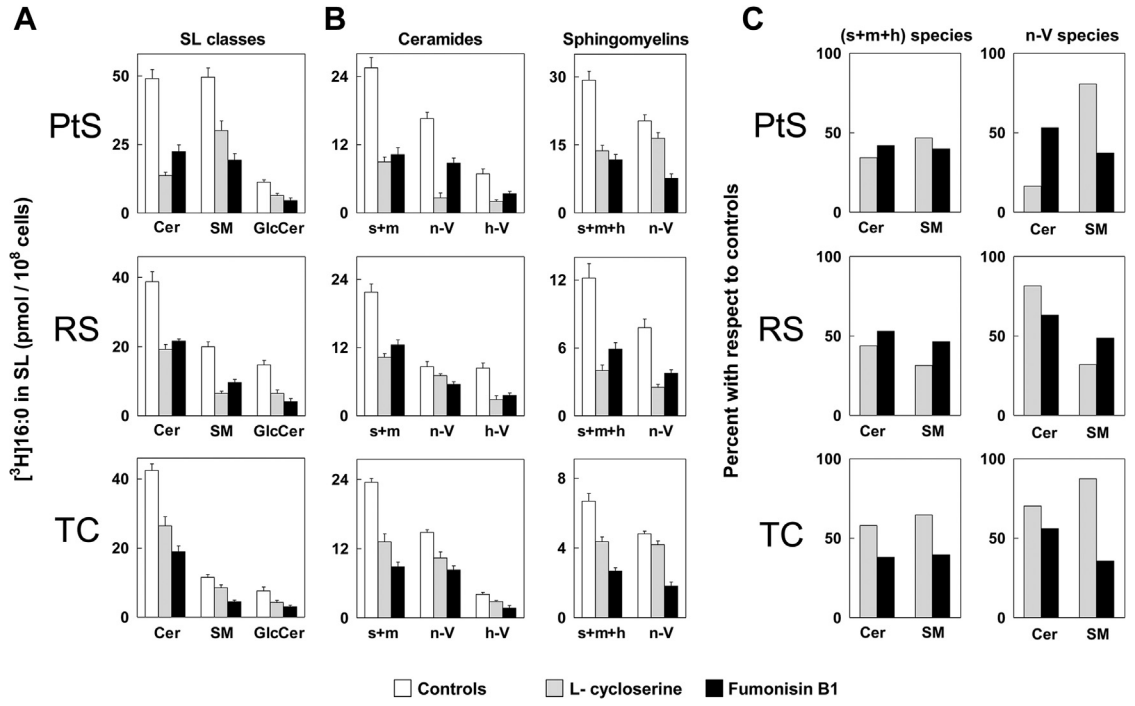

Figure 8. Differential responses of spermatocytes and spermatids to inhibitors of the *de novo* and the salvage pathways of Cer synthesis. Preparations of PtS, RS, and TC were preincubated for 5 h in their usual medium in the presence of L-cycloserine, fumonisin B1, (inhibitors of SPT and Cer synthase(s), respectively), or their vehicles (controls), after which the solution containing [^{3}H]16:0 was added, and incubations continued for 20 h. The amounts of [^{3}H] label from [^{3}H]16:0 incorporated per 10^8 cells in the three SL classes under study and in groups of Cer and SM species are shown in *A* and *B*, respectively. The decreases exerted by both inhibitors were in all cases significant ($p < 0.01$, Student's *t* test). *C*, percent changes with respect to the values in controls, taken as 100%, in Cer and SM species, estimated from the mean values shown in *B*. In the case of Cer, the amounts of [^{3}H] label in (s+m) and h-V species were added up to create an (s+m+h) Cer comparable with (s+m+h) SM. Cer, ceramide; PtS, pachytene spermatocytes; RS, round spermatids; (s+m), saturated and monoenoic fatty acids; (s+m+h), saturated, monoenoic, and 2-hydroxy fatty acids; SL, sphingolipid; SM, sphingomyelin; SPT, serine palmitoyltransferase; TC, total spermatogenic cells.

PtS (Fig. 8*A*). These differences suggest (i) that despite SM having undergone a reduced *de novo* synthesis because CS reduced total Cer levels, [^{3}H]SM continued to be synthesized from [^{3}H]Cer molecules derived from the salvage pathway in cells in both stages; (ii) that once synthesized, part of the CS-decreased [^{3}H]SM continued with its "usual" [^{3}H]SM→[^{3}H] Cer conversion (evidenced at t = 20 h in Fig. 3*C* with NBD-C6-SM as substrate), a reaction not affected by CS or FB1; and (iii) that such conversion was an important contributor to the high total [^{3}H]Cer levels in germ cells, especially in RS.

CS, FB1, and the n-V containing Cer and SM species

An important proportion of the changes induced by both inhibitors in PtS and RS corresponded to the [^{3}H] labeled n-V group of species of Cer and SM (Fig. 8*B*). With FB1, in PtS and RS, the amounts of [^{3}H] n-V Cer were reduced to 53% and 60% and those of [^{3}H] n-V SM to 38% and 48%, respectively (Fig. 8*C*). Notably, after CS, the n-V species of Cer and SM also decreased, albeit quite differently between these two cell stages, evidently contributing to the changes in total Cer and SM just described.

In PtS, the [^{3}H] n-V Cer group was *the most* reduced (to as little as 16% of controls) after CS, whereas the [^{3}H] n-V SM group was *the less* decreased (an 81% of the amount in controls continued to be formed). This indicated that, as time elapsed, most of the [^{3}H] n-V Cer that had been used as substrate by SM synthases to make such [^{3}H] n-V SM had not been formed *de novo*, mostly deriving from the salvage pathway.

In contrast to the effect in PtS, in RS the [^{3}H] n-V Cer group was the *less* decreased by CS (an 81% of the amount formed in controls was still formed), while the [^{3}H] n-V SM group was the *most* decreased (just a 32% of the amount formed in controls remained) (Fig. 8*C*).

The opposite trends between CS-treated PtS and RS suggested that in germ cells, the total [^{3}H] n-V Cer species were contributed (i) by those formed by synthesis (*via de novo* and salvage pathways) and used as *bona fide* substrates by SM synthases to generate [^{3}H] n-V SM in both cells and (ii) by those formed, after [^{3}H] n-V SM synthesis, *via* the [^{3}H] n-V SM → [^{3}H] n-V Cer conversion. While the former predominated over the latter in PtS, the opposite occurred in RS, thus explaining the overabundance of [^{3}H] n-V Cer in the presence of CS in RS.

Effect of Tes on the formation of [^{3}H]SM from [^{3}H]16:0

Possible effects of Tes on the formation of Cer, SM, and their species in germ cells were investigated in cell samples preincubated with either the vehicle or the hormone (10^{-7} M) for 5 h, after which [^{3}H]16:0 was added, and incubations proceeded for 20 h (Fig. 9). In agreement with the data obtained from NBD-C6-Cer experiments (Fig. 5), the amount of total [^{3}H]SM formed from [^{3}H]16:0 after 25 h, apparently unchanged by the presence of the hormone in PtS, was increased in RS and TC (Fig. 9).

In PtS, an unchanged label in total [^{3}H]Cer and total [^{3}H] SM concurred with Tes increasing the label in [^{3}H]GlcCer. Regarding groups of molecular species, none of those of PtS [^{3}H]Cer was affected by the hormone. Interestingly, the apparently unchanged label in total [^{3}H]SM compared to controls was made up by a combination of decreased label in the [^{3}H] (s+m+h) SM group and increased label in the [^{3}H] n-V SM group (Fig. 9).

In RS, in contrast to PtS, the exposure to the hormone increased significantly the label in both, [^{3}H]Cer and [^{3}H]SM, while reducing that in [^{3}H]GlcCer. In the case of [^{3}H]Cer, its increase involved the (s+m) Cer and the n-V Cer groups of species, and in the case of [^{3}H]SM, its increase involved the (s+m+h) SM and the n-V SM groups.

Tes and SCM

The SCM was prepared by culturing SCs during 5 to 6 days in serum-free DMEM/F-12 medium, followed by centrifugation and filtration. Germ cells incubated in their basal DMEM medium in the presence of either vehicle or Tes (10^{-7} M) for 5 h were supplemented with an equal volume of the SCM and cell cultures continued for 20 h (Fig. 10). The SCM had previously been prepared to contain the [^{3}H]16:0 used as precursor and either vehicle or hormone to maintain their concentrations, for the composition of the culture medium differed from that in Figure 9 in only the presence of the SCM.

In PtS cultured in the SCM-containing medium, the formation of total [^{3}H]SM was significantly stimulated by the presence of Tes (SCM *versus* Tes+SCM in Fig. 10). Remarkably, this hormone-dependent stimulus was again molecular

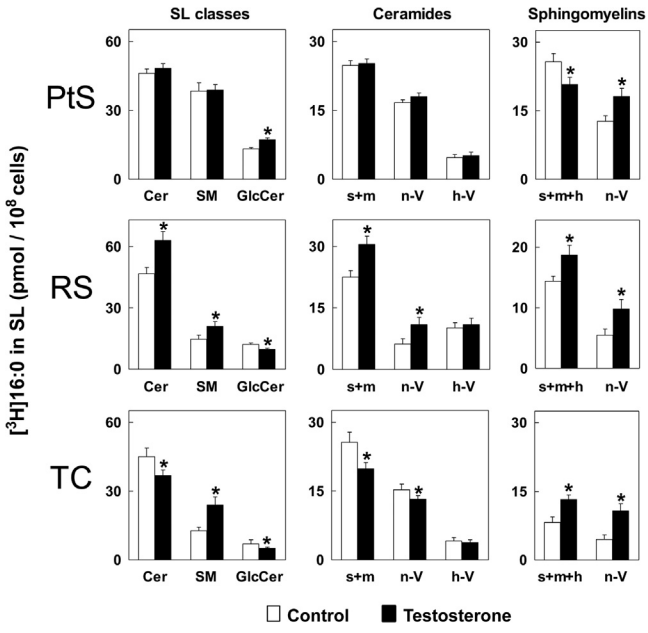

Figure 9. Labeling from [^{3}H]16:0 of germ cell Cer, SM, and GlcCer in the presence of testosterone. Preparations of PtS, RS, and TC were incubated for 5 h in their usual medium, in the presence of testosterone (10^{-7} M) or its vehicle (controls). The solution containing [^{3}H]16:0 was then added, and incubations proceeded for 20 h. The SL classes and groups of Cer and SM species were separated, and the [^{3}H] label was quantified as in previous figures. *Asterisks* (*) indicate significant differences with respect to controls ($p < 0.01$). Cer, ceramide; GlcCer, glucosylceramide; PtS, pachytene spermatocyte; RS, round spermatid; SM, sphingomyelin; TC, total spermatogenic cell.

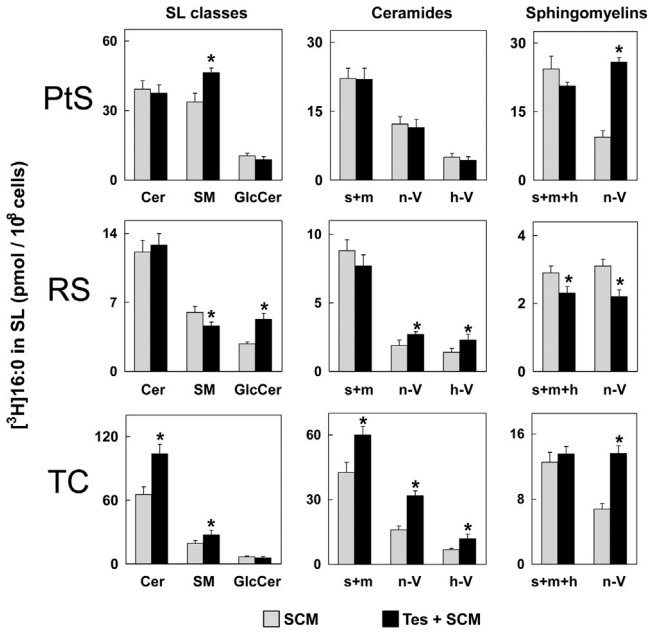

Figure 10. Labeling from [^{3}H]16:0 of germ cell Cer, SM, and GlcCer in the presence of testosterone when the cell medium was supplemented with a Sertoli cell-conditioned medium (SCM). The SCM had been prepared previously by culturing Sertoli cells during 5 to 6 days in DMEM/F-12. After collecting and filtering the supernatants, this medium was divided into two groups, one containing the hormone vehicle ethanol and the other one 10^{-7} M testosterone (here named SCM and Tes+SCM, respectively), both of which received the same amount of [^{3}H]16:0. Separately, samples of PtS, RS, and TC were preincubated in their habitual basal medium as in Figure 9 with either vehicle or 10^{-7} M testosterone for 5 h. Next, each of these cell preparations was supplemented with an equal volume of the ([^{3}H]16:0-containing) SCM or Tes+SCM previously prepared, and incubations proceeded for 20 h. Lipids were then extracted, separated, and analyzed as in previous figures. *Asterisks* (*) indicate significant differences between SCM and Tes+SCM ($p < 0.01$). Cer, ceramide; GlcCer, glucosylceramide; PtS, pachytene spermatocyte; RS, round spermatid; SM, sphingomyelin; TC, total spermatogenic cell.

species-selective, as the amount of (s+m+h) SM did not change significantly while that of n-V SM increased more than twice.

With or without the presence of the medium containing SCM, the "lipid target" of the hormone in PtS was SM, in both cases involving the formation of additional amounts of n-V SM species. Along with these lipid changes, in PtS samples cultured with Tes+SCM, occasional spindle-shaped cells appeared, some of which showing thin projections (Fig. S5). This was consistent with the possibility that the more advanced PtS that composed this population were undergoing an incipient advance into the next stage of their differentiation.

In RS, over the medium that contained SCM alone, the one combining Tes+SCM resulted in increased label of the relatively minor [^{3}H]GlcCer and of the already abundant [^{3}H]Cer, while decreasing the label in [^{3}H]SM (Fig. 10). Taken together with the data in Figure 3, *A* and *C*, this decrease suggests that after initially favoring [^{3}H]SM synthesis, the hormone influenced the [^{3}H]SM→[^{3}H]Cer conversion that was ongoing at longer incubation times.

Among the groups of [^{3}H]Cer species in RS, those with n-V and h-V increased significantly with Tes+SCM against SCM alone (around +42% and +64%, respectively), while those with (s+m) fatty acids did not change (−14%, ns). Among the groups of [^{3}H]SM species of RS, those with n-V and (s+m+h) acyl chains had both decreased, suggesting that the former and the h-V SM that composes the latter group could have been the source of the [^{3}H]-labeled n-V Cer and h-V Cer that increased in RS.

In TC, the labeling profiles after incubation with SCM alone and Tes+SCM evidenced, as in PtS, an increase in the amount of [^{3}H]SM formed, involving almost exclusively the [^{3}H] n-V SM group of species, and as in RS, a significant increase in the generation of [^{3}H]Cer, in this case including its three groups of species.

Taken together, at the end of experiments, the actions of Tes on the lipids under study tended to go in the same direction when the hormone was added to cells in the basal DMEM medium (Fig. 9) and in the DMEM:SCM combination (Fig. 10), albeit in the latter case, the effects were somewhat more intense in PtS than in RS.

Tes and genes with roles in germ cell SL levels

The effects of Tes on the mRNA levels from four genes encoding enzymes with different roles in the generation of the VLCPUFA that compose germ cell SLs, on the four synthase genes presented in Figure 2, and on the two Smase genes presented in Figure 4, were surveyed in TC samples (Fig. 11). The cell suspensions were incubated for 5 h with either the vehicle or the hormone (i) in the habitual basal DMEM medium used in Figure 9 and (ii) in the DMEM:SCM medium used in Figure 10.

Among the genes with roles in PUFA transformations leading to n-V and h-V, the presence of Tes increased highly the mRNA levels of *Elovl5* and moderately those of *Elovl4*, not affecting the expression of *Elovl2*, in germ cells cultured in DMEM (Fig. 11*A*), as well as in the medium containing the combination DMEM:SCM (Fig. 11*B*). Remarkably, within this group of genes, the one whose expression was the most intensely stimulated by the hormone in both media was *Fa2h*.

Regarding SL synthases, the presence of Tes did not modify the mRNA from *Cers3*, *Sms1*, or *Gcs* in TC cultured for 5 h in their habitual DMEM but reduced the *Cers3* transcript (to a 70%) when Tes was added in combination with the SCM (Fig. 11*B*).

An initially surprising finding was that Tes reduced dramatically the transcript level from *Sms2* (Fig. 11*A*), to a meager 10 to 12% of that in controls. Interestingly, the addition to the same cells of SCM, either containing the vehicle or Tes, resulted in mRNA levels from *Sms2* that were both similarly low as the latter (Fig. 11*B*). This suggests that at least one of the (multiple) components present in the SCM was able by itself to directly or indirectly replicate the effect of Tes on *Sms2* expression.

On the catabolism side, the presence of Tes did not modify the expression of *aSmase* or *nSmase* after 5 h in cells in DMEM. However, the hormone resulted in significantly increased mRNA from the *nSmase* gene when it was in the presence of the SCM (Fig. 11*C*).

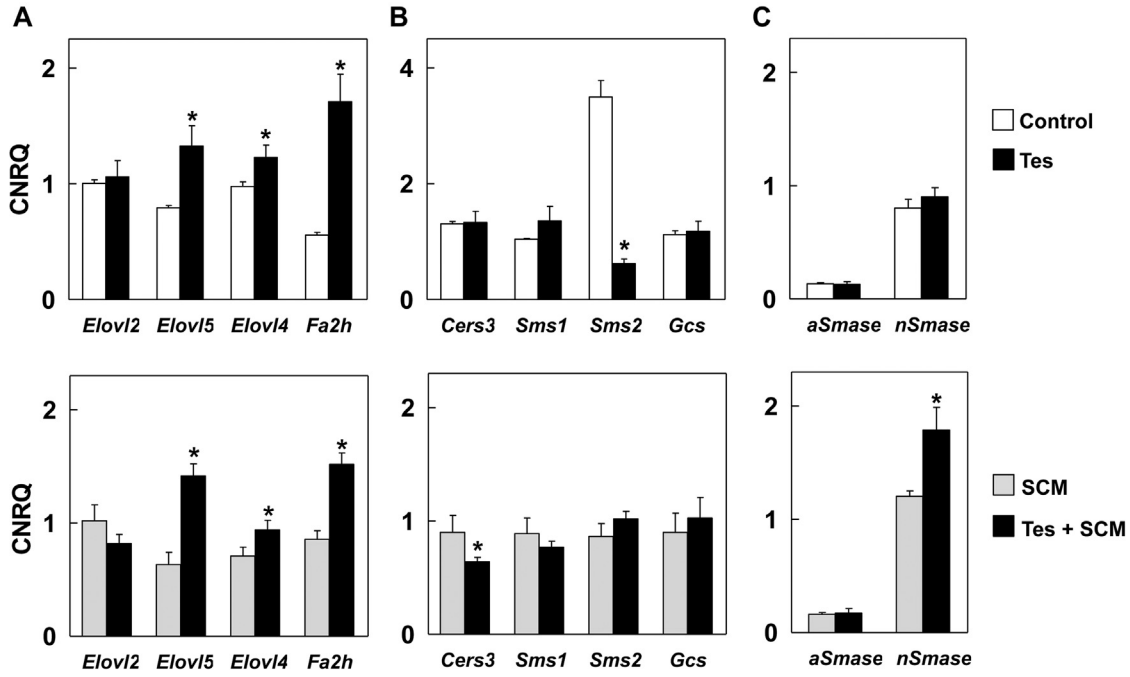

Figure 11. Influence of testosterone, alone or in the presence of the SCM, on the expression of genes with a role in germ cell sphingolipid metabolism. The mRNA levels were measured by RT-qPCR in preparations of TC that had been cultured during 5 h in the conditions described in Figure 9, cells in their basal medium with vehicle or testosterone and in Figure 10, cells in the same medium with vehicle or with Tes, to both of which the SCM had been added (controls *versus* Tes and SCM *versus* Tes+SCM, respectively). The ten genes expressed as mRNA are presented as follows: in *A*, the PUFA elongases *Elovl2*, *Elovl5*, *Elovl4*, and the fatty acid 2-hydroxylase *Fa2h*; in *B*, the sphingolipid synthases *Cers3*, *Sms1*, *Sms2*, and *Gcs*, and in *C*, the sphingomyelinases *aSmase* and *nSmase*. The data represent normalized relative expression values (calibrated normalized relative quantity, CNRQ ± SEM). The *asterisks* (*) indicate significant differences between controls and testosterone ($p < 0.01$). SCM, Sertoli cell-conditioned medium; TC, total spermatogenic cell.

Discussion

Expression of genes with roles in SM synthesis and turnover in germ cells

The present results showed that genes with key roles in the biosynthesis and turnover of SM changed their expression in spermatogenic cells not only as they differentiate between spermatocytes and spermatids but also between spermatids at different stages of their maturation. Regarding SM synthesis, notably distinctive was the pattern of expression during germ cell differentiation of the *Sms1* (*Sgms1*) and *Sms2* (*Sgms2*) genes, which encode for the SM synthases primarily located to the Golgi and plasma membranes in mammalian cells, Sms1 and Sms2, respectively (9). That the mRNA from *Sms1* was maintained with gradual increases along differentiation stages agrees with the role of Sms1 as responsible for generating the bulk of the total SM in cells, which cycles (in vesicles) between the Golgi and the rest of cell membranes (10). The finding that *Sms2* gene expression was upregulated between PtS and RS but then decreased between RS and LS (PtS<RS>LS, Fig. 2) was consistent with the changes in the expression of the Sms2 protein in the rat seminiferous epithelium reported by Lee *et al.* (31). Thus, from a low representation in spermatocytes, the protein Sms2 intensely collects in RS, with its highest expression detected in late RS and also but with diminished intensity in elongating spermatids, to become no longer detectable in elongated spermatids in their latest steps. The significant decrease of the *Sms2* mRNA level here observed between RS and LS may reflect the normal breakdown of the *Sms2* transcript following its translation into Sms2. Alternatively, a differentiation-associated downregulation or silencing of the gene might be involved, aimed at decreasing the production of Sms2, and thereby the amount of SM. The decrease in SM was previously shown to take place in rat germ cell plasma membranes with the progression of differentiation from RS to LS (19).

Concerning SM turnover, also distinctive were the amounts and differentiation-associated patterns of expression between the *aSmase (Smpd1)* and *nSmase (Smpd2)* genes, which encode the SM phosphohydrolases primarily located to lysosomes and plasma membranes (32), respectively. That the *aSmase* mRNA levels were upregulated between PtS and RS was consistent with aSmase performing an active catabolism of preexisting SLs in RS, thus contributing Sph and fatty acids utilizable by Cer synthases *via* the salvage pathway, apparently more active in RS than in PtS. The decrease of this transcript between RS and LS in turn concurs with the total amount of the main substrate of aSmase, SM, strongly decreasing between isolated RS and LS (16). Besides being several fold higher than those from *aSmase*, the transcript levels from *nSmase* were found to increase continuously in the PtS→RS→LS order of differentiation, indicating that the plasma membrane-located nSmase was a major contributor to the decreased amounts of total SM in the presence of increased total Cer we previously showed to take place in the same order of cells, especially concentrated in their plasma membranes (19).

Focusing on spermatids, the concomitant and inverse change taking place between RS and LS in the mRNA levels from *Sms2* (decrease, Fig. 2) and *nSmase* (increase, Fig. 4), when both encode plasma membrane proteins with opposite effects on the amounts of SM, are at the basis of the metabolic transformations of SM and Cer with differentiation we report here using NBD-labeled and [^{3}H]16:0 as substrates. Taken together, the expression of these two genes indicates that from round to elongating and elongated spermatids, the need of germ cells to generate and maintain their initial SM levels decrease, while the formation of Cer as an endogenous (not transient) component of spermatid membranes increases. The dynamic changes in the Cer/SM ratios during rat spermatid differentiation are consistent with the final cellular products of this process, spermatozoa, containing SM in the plasma membrane of their minute heads, and Cer being a component of their conspicuous tails (33).

SM synthesis and turnover in germ cells

Using NBD-C6-Cer as substrate, our observation that NBD-C6-SM synthesis was faster during the first hour incubation than the amounts formed per hour during subsequent periods, especially in RS and LS, suggested that the rapidly formed SM molecules may represent those synthesized by Sms2 in the cell plasma membrane from Cer molecules available in the immediate environment of such plasma membrane, and the SM progressively accumulating in cells over this amount at later time points (*e.g.*, at t = 5 h) the SM generated at the Golgi *via* the activity of Sms1.

The significant time-dependent NBD-SM→NBD-Cer conversion that could be observed using TC (Fig. 3*C*) evidenced that in addition to SM synthesis, SM turnover was highly active in germ cells. Notably, the enzyme Gcs used part of this "secondhand" NBD-Cer to generate NBD-GlcCer, the experiment demonstrating that SM-derived Cer is recycled by germ cells. Therefore, the SM synthases may have also used part of this Cer to re-synthesize SM. The SM→Cer conversion is potentially able to be iterated from the re-synthesized SM, at short incubation times locally in the plasma membrane or endosomal structures nearby by the activity of Sms2, and at later times in the Golgi itself *via* the activity of Sms1. Considering that Sms2 and Sms1 are both able (8) to generate SM and Cer, given the reversibility of the reaction they catalyze (PC+Cer⇌SM+DAG), they could in part have played a role in the increased formation of NBD-Cer from NBD-SM here observed. However, the human plasma membrane–associated nSMase2 was shown to traffic from the Golgi to the plasma membrane as a membrane-bound protein to the cell surface and to recycle back to the Golgi through the endosomal/recycling compartment (34), so it has the potential ability to generate Cer from SM in the same subcellular sites where SM synthases generate SM from Cer. In our case, in all rat germ cell types, the transcript levels from *nSmase* (Fig. 4) were similar or higher than those from *Sms1* or *Sms2* (Fig. 2), particularly in spermatids (*nSmase*/*Sms2* ≈ 3 in LS), suggesting that nSmase played a major role in Cer recycling in the last stages of spermatogenesis.

When our germ cells used [^{3}H]16:0 as an initial substrate, the formation of total [^{3}H]SM after 20 h was far more active in PtS than in RS, while the opposite occurred with the formation of total [^{3}H]Cer, most active in spermatids. That the [^{3}H]Cer/[^{3}H]SM ratio was twice higher in RS than in PtS, while the sum of label in [^{3}H]Cer+[^{3}H]SM was similar in both (Fig. 7) supported the view that an important part of the [^{3}H]Cer in RS had been generated at the expense of [^{3}H]SM synthesized early on by an SM synthase. Taken together with the changes taking place in TC at t = 20 h with NBD-Cer and NBD-SM as substrates (Fig. 3), we conclude that besides synthesis by Cer synthases (generating Cer in part *via* the *de novo* and in part *via* the salvage pathways), the third principal contributor to the total Cer levels of germ cells, especially of spermatids, was the Cer generated from the turnover of SM, mainly *via* the action of their nSmase.

In the case of [^{3}H]-labeled Cer, SM, and GlcCer, the data at t = 20 h were compatible with the following scenario, shared by PtS and RS albeit with a bias toward SM synthesis (Cer→SM) in PtS and toward Cer generation, mostly from SM (SM→Cer), in RS. The [^{3}H]Cer molecules formed by ER-located Cer synthases including Cers3 were used by Golgi- and plasma membrane-located SM synthases to produce [^{3}H]SM, which was distributed among cell membranes. Of the newly formed [^{3}H]SM species, particularly those localized in plasma membranes, part functioned as substrates of nSmase to produce [^{3}H]Cer. The SM-turnover-derived [^{3}H]Cer molecules produced by nSmase were in part recycled in the proximate endosomes and Golgi to resynthesize GlcCer and SM, and the rest (*via* aSmase and ceramidases) contributed their Sph to the pool of SBs that ER-located Cer synthases utilized to make Cer molecules, thereby closing a complete cycle. This cycle was iterated many times as hours elapsed in our cells in culture. At early incubation times (*e.g.*, at t = 5 h), we had negligible label in total (*de novo* and salvage-derived) [^{3}H]Cer because SPT requires some time to produce [^{3}H]SBs from [^{3}H]16:0, and the SBs available (*via* aSmase) were mostly unlabeled, as they derived from the recycling of (unlabeled) SLs (mainly SM) pre-existing in the cells. At a later incubation time (*e.g.*, t = 20 h), the cells appear to have reached a nearly steady state of SM metabolism, with SM synthesis and SM hydrolysis occurring in both, but the former predominating in PtS and the latter in spermatids. Given the plasma membrane localization of Sms2 and nSmase, our results on their mRNA changes with differentiation, together with the dynamic changes their respective products [^{3}H]SM and [^{3}H]Cer showed, correlated well with the differentiation-linked decreases in the amounts of SM and increases in those of Cer, we previously reported to take place between PtS, RS, and LS (16), mainly concentrated in their plasma membranes (19).

Tes and the expression of Sms2 and nSmase

In vivo, this androgen is physiologically required for PtS to complete meiosis and for RS to complete spermiogenesis, reviewed in (26–28). In spermatids, a principal action of Tes is to facilitate the maturation of round to elongating spermatids

(*in vivo*, steps 1–7 and 8–14 during the first round of spermiogenesis, respectively), the latter followed by the formation of elongated spermatids (steps 15–19) during the second round. Tes specifically promotes the maturation of spermatids between stages VII and VIII of the spermatogenic cycle (35), *i.e.*, in spermatids between late step 7 and step 8 of their maturation.

A novel finding of the present study was that the exposure of TCs to 10^{-7} M Tes during 5 h resulted in a dramatic *decrease* in the mRNA levels from *Sms2* (Fig. 11*B*), not affecting significantly those from *Sms1* (or *Gcs*). Because round, elongating, and elongated spermatids, together, outnumber the PtS present in TC samples, and taking into account the increased levels displayed by *Sms2* in differentiating RS (Fig. 2), Tes may have promoted the increase in the transcript and the protein Sms2 in late RS, considering that *in vivo* this protein increases in spermatids precisely when they are in their (Tes-responsive) late RS steps (31). This would respond for the increased amount of NBD-SM formed from NBD-Cer in TC (Fig. 5).

Another known role of Tes is to stimulate the release of mature (step 19) spermatids into the lumen of the seminiferous tubules, a process known as spermiation, reviewed in (26–28). The cumulative increase that the amounts of the germ cell *nSmase* transcript exhibited in the PtS→RS→LS differentiation order (Fig. 4), and the increased [^{3}H]Cer with decreased [^{3}H]SM labeling from [^{3}H]16:0 displayed by RS (and TC) in comparison with PtS (Fig. 7), suggested the possible intervention of the hormone in upregulating the expression of the enzyme nSmase in the last steps of spermiogenesis. However, the mRNA level from *nSmase* did not change significantly in TC incubated for 5 h with 10^{-7} M Tes (Fig. 11*C*) in the same conditions that had led to *Sms2* mRNA decrease (Fig. 11*B* upper panel). Moreover, this lack of change of the *nSmase* transcript change was reflected in the lack of a Tes-related increase of NBD-Cer formation from NBD-SM as substrate in TC (Fig. 5*A*, right panel). Perhaps 5 h was too short an incubation time for the whole process between gene expression and enzymatic activity to be completed in the case of nSmase. Nevertheless, that Tes enhanced significantly the expression of *nSmase* in germ cells cannot be ruled out, as it evidently occurred when the hormone was in the presence of the SCM (Fig. 11*C*).

Taken together, our results suggest that Tes exerted its effects on SM metabolism during the differentiation of spermatids (i) by promoting the Cer→SM synthesis in late RS and (ii) by promoting the SM→Cer conversion in LS and more advanced spermatids, *via* its effects on the expression of the *Sms2* and *nSmase* genes, respectively, in both cases over a background level of expression that both transcripts displayed all over spermatogenesis.

Germ cells as targets of Tes

In vivo, changes in gene transcripts and metabolic reactions in isolated germ cells in response to Tes imply that these cells should have a form of receptor to de hormone. However, except for spermatogonia and early spermatocytes (36, 37), the X-linked androgen receptor (AR) gene is not expressed as a protein in spermatogenic cells. This occurs because during the long-lasting PtS subphase of meiosis, the X and Y chromosomes are tightly packaged into the compact structure known as the "sex body" or "XY body" (38, 39), thereby all of the genes located on X and Y are transcriptionally silenced. This general silencing mostly continues in RS, even though some genes on X are reactivated, and a few are expressed *de novo* (40, 41). However, most somatic cells of the testis, and *also* germ cells, express (mRNA, protein, activity) the ER-located enzyme aromatase (P450arom), codified by the *Cyp19* gene, which is able to convert Tes into 17-β estradiol (42, 43). The activity of aromatase increases between PtS and RS and is greatest in elongated spermatids, reviewed in (44). Because we show here that RS→LS differentiation and decreased amounts of SM are associated, our initially intriguing observation that RS responded to *estradiol* by drastically reducing the synthesis of NBD-SM from NBD-Cer (Fig. 5) suggests that Tes could have acted (i) directly *via* ARs alternative to the AR, such as that described in ref. (45) or (ii) indirectly, *via* its aromatase-mediated conversion into estradiol in germ cells, particularly in spermatids. The latter option is not unlikely, as germ cells do express the estrogen receptors ESR1 and ESR2 (46). Preliminary observations from our lab showing that in comparable experimental conditions estradiol reduced the expression of *Sms2* in spermatids even more intensely than did Tes (unpublished work) support the latter possibility. However, the spermatogenic process is so complex that both could play specific roles in specific situations, for example in different cells at a given stage or in the same cell at different stages.

Tes and the SCM

The addition of the SCM seemed to have enhanced or facilitated the effects observed with the hormone alone on the [^{3}H]SM metabolic changes, acting as if it had improved the availability of the hormone to the cells or accelerated its effects. Among the many proteins that SCs release to the seminiferous fluid *in vivo* and to the culture medium *in vitro*, one that is capable of androgen binding, transport, and delivery to its target (spermatogenic and epididymal) cells is the androgen-binding protein (ABP in rats, sex steroid binding globulin in humans). Gérard *et al.* (47) showed that rat germ cells, from spermatogonia to elongating spermatids, possess binding sites on their plasma membranes through which ABP is internalized *via* an endocytic pathway and that it is able to reach the nucleus when provided as a [^{3}H]steroid+ABP complex. Notably, the steroid bound to this complex can be either Tes or 17-β estradiol at concentrations of a similar order as those used here. Functionally, the same teamwork had previously shown that ABP increases the synthesis of secreted proteins, more actively in PtS than in RS, some of which were identified as already produced by these germ cells and others being new ones (48). Later studies showed that highly purified ABP, *on its own*, is able to promote the differentiation of spermatids (49). These notable features of

ABP could respond for the present observations (i) that the SCM, by itself, resulted in levels of the *Sms2* transcript as low as those seen with Tes+SCM and that both, SCM and Tes+SCM, had led to *Sms2* levels as low as those caused by Tes alone (Fig. 11*B*, lower *versus* upper panel); (ii) that in the presence of the SCM, Tes induced a decrease of the *Cers3* transcript level; and (iii) that the addition of SCM to Tes-exposed cells enhanced the labeling of [^{3}H]SM species with n-V in PtS, and increased the [^{3}H]Cer/[^{3}H]SM ratio in RS, in the same direction but comparatively more intensely that did Tes alone.

Germ cell Cer and SM with VLCPUFA and Tes

In addition to its quantitative decrease, the total SM localized in germ cell plasma membranes undergoes a major qualitative change associated with the PtS→RS differentiation, as in the SM of PtS the n-V SM species predominate, while in the SM of RS, the proportion of h-V SM increases over that of n-V SM (17). Thus, after meiosis, RS "inherit" part of their plasma membrane n-V SM species from PtS but need to generate their own new h-V SM species. This seems to be a good reason for the expression of the *Sms2* gene to be intensified in germ cells at the RS over the PtS stages (Fig. 2).

In the stages following RS, spermatids do not require further Cer→SM synthesis. The process with rising importance is an intense membrane remodeling accompanied by increased turnover of SM, with Cer generation (SM→Cer), which in rat germ cell plasma membranes mainly involves their species with n-V and h-V (19). In spermatids, this appears to be linked to a decreasing expression of *Sms2* and an increasing expression of *nSmase*, as observed in this study (i) in RS differentiating into LS (Fig. 2) (a transition known to depend on Tes) and (ii) in TC incubated in the presence of Tes (Fig. 11, *B* and *C*).

The higher ability of PtS to synthesize n-V SM than h-V SM, and vice versa in RS, was confirmed here using [^{3}H]16:0 as primary substrate in isolated cell samples cultured independently during 20 h (Fig. 7). Taken together, these labeling data and the effects of inhibitors (Fig. 8) indicated that the Cers3 present in the ER of PtS and RS must have formed [^{3}H]Cer species by N-acylating SBs (made *de novo* and *via* recycling) with CoA thioesters of [^{3}H]n-V and [^{3}H]h-V and that such Cers were used as substrates by the SM synthases, in particular by plasma membrane Sms2.

The expression of *Elovl4* (mRNA, protein, activity) was previously shown to predominate in PtS over RS and this over LS, while *Fa2h* was expressed as mRNA in the three stages but as a protein mainly in spermatids, its cellular localization changing between RS and LS (21). Taking into account the lower proportions of PtS than spermatids in TC preparations, the present finding that the exposure of TC to Tes increased significantly the transcript levels from both genes (*Elovl4* < *Fa2h*) suggests that the hormone may have enhanced the expression of *Elovl4* mostly in PtS and that of *Fa2h* mostly in spermatids.

The observation that, by itself or in the presence of the SCM, Tes upregulated significantly within 5 h the expression of genes with roles in PUFA elongation (*Elovl5*, *Elovl4*) and 2-hydroxylation (*Fa2h*) (Fig. 11*A*) suggests that this hormone-dependent effect may have preceded and favorably influenced the activity of Cer synthases including Cers3 in PtS, by increasing the availability of the fatty acyl groups required as substrates, thereby facilitating Cer synthesis. Such an increase was not reflected in a changed mRNA level from *Cers3* with Tes alone in TC but was manifested in a modest but significant decrease of the *Cers3* transcript when the hormone was in the presence of the SCM (Fig. 11*B*), suggesting that the transcript may have been catabolized after being translated into the Cers3 protein, as we previously proposed for the decrease of *Sms2*.

In PtS, the exposure to Tes, by itself and especially in the presence of the SCM (Figs. 9 and 10) stimulated significantly the formation of [^{3}H] n-V SM over the rest of SM species, even when the latter group included [^{3}H]16:0 among the SM acyl groups. This implies that previously the Cers3 protein, highly expressed in PtS (20), must have previously formed the [^{3}H]Cer molecules containing n-V required as substrates by SM synthases. Tes-dependent Increases or decreases in *Cers3* gene expression or in [^{3}H] n-V Cer amounts in PtS samples may have been missed in PtS samples (i) if they occurred at incubation times earlier than t = 20 h or (ii) if the n-V Cer molecules did not change because they were readily channeled into [^{3}H] n-V SM as soon as they were formed.

In RS, the exposure to Tes increased in 20 h the formation of n-V and h-V [^{3}H]Cer and decreased those of the corresponding [^{3}H]SM species, in line with the decreased expression of *Sms2* and increased of *nSmase* (Fig. 11, *B* and *C*) in hormone-exposed TC. When Tes and the SCM were together, the RS formed even less [^{3}H]SM and even more [^{3}H]Cer, the increase in the [^{3}H]Cer/[^{3}H]SM ratio marking the tendency of cells in the RS population to progress into the next differentiation stage. In the case of TC samples, which in addition to RS contain LS (elongating and elongated spermatids), the [^{3}H]Cer/[^{3}H]SM ratio increased even more than in RS with the hormone. The increase in [^{3}H]Cer and the decrease in [^{3}H]SM in RS (Figs. 9 and 10) in the presence of Tes may originate in the hormone significantly decreasing the expression of *Sms2* while augmenting that of *nSmase*.

Concluding remarks

Taken together, the present data highlight that the Cer→SM synthesis predominates in PtS, where it contributes to cell growth, while the SM→Cer conversion, involved in membrane remodeling and Cer recycling, coexists with SM synthesis in RS, but progressively prevails in spermatids as they mature through elongating and elongated forms. We learned from this work that the almost negligible labeling of SM in the latest (elongated) forms of spermatids we had observed with fluorescent and radioactive precursors responds to the fact that SM synthesis from Cer decreases or even stops after RS

(consistent with the *Sms2* transcript decreasing from RS to LS) while SM conversion into Cer increases (consistent with the cumulative increase of the *nSmase* transcript occurring in the same direction). Both processes may take place one after the other with time in each spermatid but superpose one over the other in a whole population like RS, both contributing to minimize the amount of SM. A small amount of Sms2 in the most mature spermatids may suffice to maintain SM levels in their plasma membrane from the Cer available *via* recycling of the SM that had been formed in previous stages. The fate of the *Sms1* transcript seems to differ from that of *Sms2* and might be the same as that of many other mRNAs, *i.e.*, concentrate in the cytoplasmic lobe of spermatids to be collected in residual bodies. The stimulation of germ cell differentiation by Tes was manifested, regarding SLs, in isolated germ cells in culture by changes in the expression of specific genes: (i) in PtS, with increased expression of genes whose final products (long-chain PUFA destined to glycerophospholipids, VLCPUFA destined to SLs) resulted in increased amounts of n-V-containing SM species and (ii) in spermatids, the Tes-stimulated decrease of the *Sms2* and increase in *nSmase* transcript levels were manifested in decreased SM and increased Cer levels as main final products. Germ cell differentiation is associated with a myriad gene-encoded anabolic and catabolic processes precisely programmed to take place at specific times during spermatogenesis, among them those undergone by membrane lipids. Testosterone, by repressing, ignoring, or stimulating their expression, contributes to each fulfilling its biological functions.

Experimental procedures

Spermatogenic cells

Primary cell cultures of spermatogenic cell populations were established from testes of adult (90-day-old) rats. The protocols for animal use were approved by the institutional Committee for the Care and Use of Experimental Animals of the *Universidad Nacional del Sur, Argentina*, with guidelines in agreement to the NIH Guide for the Care and Use of Laboratory Animals. Seminiferous tubules were obtained from testes immediately after CO_2-induced euthanasia. Peritubular and interstitial cells were detached by exposure to a Krebs-Henseleit medium containing 0.5 mM $CaCl_2$, 10 mM DL (+)-lactate, type II collagenase (0.5 mg/ml), and DNase (20 μg/ml) and then removed. The spermatogenic and SCs were released after digestion of the tubules with trypsin (0.4 mg/ml) and DNase (20 μg/ml). The cell suspensions were gently passed through cotton fibers to remove spermatozoa and cell aggregates and then successively filtered through 250 μm and 60 μm nylon meshes. These filtrates, here referred to as TC, usually contained 20 to 25% spermatocytes (mainly PtS) and 70 to 75% spermatids (approx. 40% of RS and 30% of more differentiated forms of spermatids—here abbreviated as late spermatids (LS)—with the remaining 5 to 10% being made up by germ cells in other stages and some SCs (less than 0.5%).

The cell populations containing PtS, RS, and LS were obtained from the TC suspensions using the STA-PUT procedure (50). The size and the typical aspect of each cell nuclei, stained with Hoechst 33342 (51), were used to identify the cells in each preparation, which usually showed an acceptable level of purity (84–88%, 90–95%, and 76–80%, respectively, the difference with 100% being germ cells in the previous and the subsequent stages of development).

The spermatogenic cells were cultured in the medium we often refer to for brevity as the "basal" culture medium of germ cells, which consisted of a low-glucose (5.5 mM) DMEM (Gibco) supplemented with 6 mM lactate, 10% fetal bovine serum (v/v), penicillin, and streptomycin (100 U/ml each). After suspension in this medium, the cells were seeded at a density of 5 to 10 × 10^6 cells per ml in 35 mm diameter dishes and cultured at 33 °C under an air/CO_2 (95:5) atmosphere.

Sertoli cells

The SC preparations were obtained from 16-day-old rat testes as described in ref. (52) and cultured in serum-free DMEM/F-12 1:1 (v/v) medium at 37 °C under 5% CO_2 for 5 to 6 days. During that period, traces of accompanying germ cells were eliminated by the endogenous phagocytic activity of SCs, which were able to continue growing in monolayer up to confluence, reaching a purity of >90%.

Quantification of gene expression

The mRNA levels were assessed by real-time quantitative PCR. The total RNA was isolated from freshly isolated cells using the TRIzol reagent (Invitrogen) in RNase-free water according to the manufacturer's instructions. After quality and concentrations assessment using a PicoDrop spectrophotometer, it was stored at −80 °C until use. According to the 260/280 absorbance ratios, the RNA purity ranged from 1.75 to 1.95 (mean 1.85).

Total RNA (2 μg) was used to synthesize cDNA by reverse transcription in a final volume of 25 μl. The reaction vials contained 1 μg random hexanucleotide primers (Biodynamics), (1X) M-MLV reverse transcriptase reaction buffer, the dNPT mix (0.5 mM each of the four nucleotides), 25 IU RNase inhibitor (Promega), and 200 IU M-MLV reverse transcriptase (Promega). The cDNAs were amplified by (qPCR) using the SensiFAST SYBR kit (BioSystems) with a 0.40 μM concentration of each primer in a final reaction volume of 10 μl.

The gene-specific primer pairs used for amplification of rat *Cers3*, *Sms1*, *Sms2*, *Gcs*, *aSmase*, and *nSmase* (Table 1) were designed using the Primer3 designing tool software and purchased from Invitrogen/Life Technologies. For the fatty acid elongase (*Elovl5*, *Elovl2*, and *Elovl4*) and 2-hydroxylase (*Fa2h*) genes, the primer pairs used were described and validated in a previous report (21). In all cases, three reference genes (*Agps*, *Hprt*, and *Pgk1*) were used for validation. Gene expression levels and DNA melt curve analyses were done using the Rotor-Gene 6000 real-time PCR machine (Corbett Research).

The PCR conditions were as follows: 40 cycles of denaturation at 94 °C for 20 s, annealing and extension at 60 °C for 30 s, and a final extension step at 72 °C for 30 s. The procedures used for the analysis of data were described in the

Table 1
Sequences of the primer pairs used and size of the fragments produced by quantitative RT-PCR

Name	Gene ID	Primer sequences (5′-3′)	Amplicon lengths (nt)	Efficiency (%)
Cers3	499174	F: GTCTTTGTGAAAGCGTCCCAC R: ATTGGCTAGAGGTGTGGCAA	105	104
Sgms1[a]	353229	F: TGCATAGTTGGCACGCTGTA R: GCACTTGAGCTTCCCAGTCT	121	105
Sgms2[a]	310849	F: CTTCCTCTTCAGCGGCCATA R: TGGTACCACCAGAAGTGACG	87	93
Ugcg[a]	83626	F: ACGTAGCTGACAGACAAGGC R: CATCCCCGTCACACACTTGA	116	95
Smpd1[a]	308909	F: CCCCGCCTGCAAAGTCTTAT R: ACAGCTGACTGGCACACATT	138	98
Smpd2[a]	83537	F: CTAAGCGGACTGGTGCTCAA R: TGTGTGGTGGATGAACTGGG	126	103
Agps	84114	F: CCGAGTACCAATGAGTGCAA R: CCATCCATTCCATTTCATAAGTT	132	110
Hprt	24465	F: CTCATGGACTGATTATGGACAGGAC R: GCAGGTCAGCAAAGAACTTATAGCC	123	110
Pgk1	24644	F: GCAAAGACTGGCCAAGCTAC R: GCCTCAGCATATTTCTTACTGCT	95	108

[a] The abbreviations *Sms1*, *Sms2*, *Gcs*, *aSmase* and *nSmase* are used in the text to name *Sgms1*, *Sgms2*, *Ugcg*, *Smpd1* and *Smpd2*, respectively.

mentioned report (19). Results were calculated and are presented as calibrated normalized relative quantity values.

Using the described protocols, possible effects of Tes on the mRNA levels of the abovementioned genes were investigated in TC that had been incubated during 5 h in the germ cell basal medium lacking or containing the hormone, either in the absence or the presence of the SC–conditioned medium (SCM), prepared as described later on in this section.

Lipid labeling from NBD-labeled substrates

The NBD-C6-Cer and NBD-C6-SM (Molecular Probes) used as substrates were diluted to a concentration of 1 mM in ethanol, from which aliquots were taken to reach a final concentration of 2 μM when added to cell suspensions. To evaluate the conversion of NBD-C6-Cer into NBD-C6-SM and NBD-C6-GlcCer, cell suspensions containing 10×10^6 PtS, RS, and LS per ml of the germ cell basal culture medium were incubated at 33 °C with NBD-C6-Cer during 1, 3, and 5 h. Long-term incubations with either NBD-C6-Cer or NBD-C6-SM (up to 25 h) were performed similarly, in this case using TC preparations.

The effect of Tes on the synthesis of NBD-labeled lipids was assessed by preincubating germ cell preparations containing 5×10^6 cells per sample for 5 h in the germ cell basal medium in the presence of Tes (and in one case 17-β-estradiol) at a 10^{-7} M concentration, (or their vehicle, ethanol added to controls), after which the NBD-Cer or NBD-SM probes were added, and incubations continued for 3 h.

The effect of the Sms inhibitor D609 (Sigma Aldrich) on the synthesis of NBD-C6-SM from NBD-C6-Cer was assessed in TC. The cells were preincubated for 5.5 h in their basal medium in the presence of 15 μM D609 (or its vehicle water added to controls). At t = 30 min during this period Tes or its vehicle ethanol were added, and incubations proceeded as before for 5 h, followed by NBD-C6-Cer addition and incubation for 3 h.

In all cases, the fluorescent lipids produced after incubations were extracted as described for the [^{3}H] labeled lipids and separated by TLC using chloroform: methanol: water (65:25:4, v/v), located under UV light, eluted, dried, and dissolved in ethanol. Their fluorescence was measured using a model 4800 SLM spectrofluorimeter (SLM Instruments) at 466 nm and 539 nm as the excitation and emission wavelengths, respectively.

Lipid labeling from [^{3}H]16:0

Before use, appropriate amounts of [^{3}H]palmitate (57.0 Ci/mmol, PerkinElmer Life Sciences Inc) were mixed with unlabeled 16:0 in ethanol, and aliquots were added to the basal culture medium containing fatty acid–free BSA to get a final solution that included 1 μCi [^{3}H]16:0 per ml, a total free 16:0 concentration of 2.5 μM, and a BSA/16:0 mol ratio of 1:2. This mixture of (labeled+unlabeled) 16:0 was added to capsules containing 5×10^6 germ cells per ml. As quantified by gas-liquid chromatography, this amount of PtS, RS, and LS contributed an endogenous (unlabeled) amount of free 16:0 of 398, 315, and 341 ng of 16:0, respectively. When these amounts were considered together with the exogenously added (labeled+unlabeled) amount of.16:0, the specific radioactivity of the total [^{3}H]16:0 initially present in the system was, respectively, 0.15, 0.16, and 0.16 μCi/nmol 16:0, *i.e.*, virtually the same for the three germ cell preparations.

Cell suspensions in 1 ml of basal medium (cells as freshly isolated or preincubated during 5 h according to the experiment) received 1 ml of the [^{3}H]16:0-containing solution and incubations proceeded at 33 °C during 20 h. At the end of incubations, aliquots were taken from the cell suspensions to determine cellular protein content using the DC Protein assay (Bio-Rad, Life Science Group). Initial attempts to use 5 h incubation of germ cells with [^{3}H]16:0 (as we had done with NBD-Cer) failed to show a significant label in Cer, SM, and GlcCer (as glycerophospholipids and neutral glycerides had taken up most of the label). The LS preparations were initially included among the germ cells incubated with [^{3}H]16:0 but, even after 20 h, they incorporated too little [^{3}H] label in the SLs under study.

To assess the effects of the inhibitors CS and FB1 on lipid labeling from [^{3}H]16:0, PtS, RS, and TC were preincubated for 5 h in 1 ml of germ cell basal medium containing 1 mM CS or 100 μM FB1 (Sigma Aldrich) (the equivalent volume of the corresponding vehicles, water, or DMSO, was added to controls). Then, the [^{3}H]16:0/BSA-containing medium was added (1 ml, prepared to also contain the inhibitors or their vehicles to maintain their concentrations), and incubations proceeded for 20 h.

Testosterone

Appropriate aliquots were taken from stock solutions of Tes (Sigma Aldrich) in ethanol to obtain a final dilution able to give, in 2 μl ethanol, a hormone concentration of 10^{-7} M when added to culture media (0.1% of the medium volume). The same volume of its vehicle, ethanol, was added to controls. Possible effects of Tes on the labeling by [^{3}H]16:0 of the SLs under study were investigated by preincubating PtS, RS, and TC for 5 h in 1 ml of the basal medium containing either Tes (10^{-7} M) or its vehicle, after which 1 ml of the medium containing [^{3}H]16:0 was added (also including hormone or vehicle to maintain their concentrations), and incubations proceeded for 20 h. Isolated LS were also examined, but the results were erratic given the negligible label of LS SLs.

SC-conditioned medium

SCs isolated as described above were maintained in primary culture for 5 to 6 days in DMEM/F-12 medium. The medium overlying the cells was collected daily, combined, and passed through 0.22 μm syringe filters. The SCM was prepared to lack Tes (only the vehicle) or to contain the hormone (10^{-7} M), for brevity referred to as "SCM" and "Tes+SCM" media, respectively. The [^{3}H]16:0, prepared as described before, was added to both of these two media.

Samples of PtS, RS, and TC were preincubated for 5 h in their basal culture medium (1 ml) with either vehicle or Tes (10^{-7} M). Then, each of them was divided into two groups, which received an equal volume (1 ml) of the previously prepared [^{3}H]16:0-containing SCM or Tes+SCM media, and incubations continued for 20 h.

Cell viability

After incubations with [^{3}H]16:0 in the described (20–25 h) culture conditions, the germ cell samples under study were examined using a Nikon Eclipse TE 2000 microscope for possible morphological changes and to assess their viability (Fig. S5). Viability was evaluated by incubating the cells at 33 °C for 10 min in the presence of 5 μM Hoechst 33342 and 1 μM propidium iodide (Sigma Aldrich) in PBS and estimating the ratio between Hoechst-positive cells (total nuclei) and propidium iodide-positive cells (nuclei of dead cells) in this case using a Nikon Eclipse TE 2000 inverted fluorescence microscope.

Lipid extraction and analysis

After incubations with [^{3}H]16:0, cells were transferred to glass tubes, separated from [^{3}H]-containing media by centrifugation (5 min at 800*g*), and washed twice by suspensions in PBS (pH 7.4) and centrifugation. The total lipid was extracted, partitioned, washed (53), dried under N_2, and dissolved in chloroform:methanol (2:1, v/v). Neutral lipids were separated on silica gel G TLC plates by running chloroform:methanol:ammonia (9:1:0.2, v/v) up to the middle of the plates followed by hexane:ether (80:20, v/v) up to near the top. Polar lipids were resolved by two-dimension TLC, using chloroform:methanol:ammonia (65:25:5, v/v) and chloroform:acetone:methanol:acid acetic:water (30:40:10:10:3, v/v) for the first and second dimensions, respectively. Because Cer and SM were relatively minor lipid components of germ cells, adequate amounts of unlabeled Cer and SM previously isolated from TC preparations were added as carriers to facilitate manipulation and improve detection of their groups of species. In the case of Figure 7, the SM species which partially overlapped after two-dimension TLC, grouped as (s+m+h) SM, were eluted and separated into (s+m) and h-V species using an additional argentation TLC step (18). To determine the intramolecular distribution of the label from [^{3}H]16:0 in germ cell Cer and SM, both lipids were subjected to acid-catalyzed methanolysis (54). The resulting sphingoid bases (SBs) and fatty acid methyl esters (MEs) were resolved by TLC. Chloroform:methanol:ammonia (40:10:1, v/v) was run up to about 1/3 of the plates (which separated the bases and the h-V ME from the origins) and, after drying this solvent under N_2, hexane:ether (80:20, v/v) was allowed to run up to near the top (which separated the relatively "shorter" (s+m) ME from the n-V ME).

To determine the amount of [^{3}H] label in the lipid classes, species, and their moieties, the spots were localized with I_2 vapors, the silica gel was scraped off into glass vials, thoroughly mixed with water and a detergent-based scintillation counting mixture and allowed to settle down. The [^{3}H] radioactivity was measured in a liquid scintillation counter (Wallak Oy model 1214 Rackbeta).

Statistical analysis

Data are presented as mean values ± SD or SEM from at least three samples, each obtained from independent cell preparations. Statistical analysis was performed by ANOVA using the Graph Pad Prism software, version 5.0. The Student *t* test was used to compare differences between two data and the Tukey's test for multiple comparisons. p values <0.05 were considered significant.

Data availability

All the data are contained within the article and supporting information.

Supporting information—This article contains supporting information.

Author contributions—F. X. S. V., M. I. A., and G. M. O. conceptualization; F. X. S. V., M. I. A., and G. M. O. methodology; F. X. S.

V., M. I. A., and G. M. O. data curation; F. X. S. V., M. I. A., and G. M. O. writing the original draft; F. X. S. V. and G. M. O. investigation; M. I. A. and G. M. O. supervision; M. I. A. and G. M. O. project administration; M. I. A. and G. M.O. funding acquisition.

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Abbreviations—The abbreviations used are: ABP, androgen-binding protein; *Agps*, alkylglycerone phosphate synthase; AR, androgen receptor; Cer, ceramide; Cers, ceramide synthase; CERT, ceramide transfer protein; CS, L-cycloserine; E2, 17β-estradiol; Elovl, elongation of very-long-chain fatty acids protein; Fa2h, fatty acid 2-hydroxylase; FB1, fumonisin B1; GlcCer, glucosylceramide; GSL, glycosphingolipids; *Hprt*, hypoxantine guanine phosphoribosyl transferase; LS, late spermatids; *Pgk1*, phosphoglycerate kinase; PtS, pachytene spermatocytes; RS, round spermatids; (s+m), saturated and monoenoic fatty acids; (s+m+h), saturated, monoenoic, and 2-hydroxy fatty acids; SB, sphingoid base; SC, Sertoli cells; SCM, Sertoli cell-conditioned medium; Sgms, phosphatidylcholine:ceramide cholinephosphotransferase or sphingomyelin synthase (here shortened as Sms); SL, sphingolipid; SM, sphingomyelin; Smase, sphingomyelinase; Sph, sphingosine; SPT, serine palmitoyltransferase; TC, total germ cells; Tes, testosterone; Ugcg, UDP-glucose ceramide glucosyltransferase or glucosylceramide synthase (here shortened as Gcs); VLCPUFA, very-long-chain polyunsaturated fatty acids (with n-V and h-V referring to nonhydroxy and 2-hydroxy forms).

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