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Mice Deficient for Spermatid Perinuclear RNA-Binding Protein Show Neurologic, Spermatogenic, and Sperm Morphological Abnormalities

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Spermatid perinuclear RNA-binding protein (SPNR) is a microtubule-associated RNA-binding protein that localizes to the manchette in developing spermatids. The *Spnr* mRNA is expressed at high levels in testis, ovary, and brain and is present in these tissues in multiple forms. We have generated a gene trap allele of the murine *Spnr,* named $Spnr^{+/GT}$. $Spnr^{GT/GT}$ mutants show a high rate of mortality, reduced weight, and an abnormal clutching reflex. In addition to minor anatomical abnormalities in the brain, males exhibit defects in spermatogenesis that include a thin seminiferous epithelium and disorganization of spermatogenesis. Most of the sperm from mutant males display defects in the flagellum and consequently show decreased motility and transport within the oviducts. Furthermore, sperm from mutant males achieve *in vitro* fertilization less frequently. Our findings suggest that SPNR plays an important role in normal spermatogenesis and sperm function. Thus, the $Spnr^{GT/GT}$ mutant male mouse provides a unique model for some human male infertility cases. **© 2001 Academic Press**

Key Words: Spnr; **gene trap; sperm; flagellum; brain; nervous system; mouse; microtubule-associated protein.**

INTRODUCTION

A high proportion of sperm with normal morphology and motility is required for successful fertilization. Gene-targeted disruption in mice provided an important framework to study the genetic basis of male infertility. Although much progress has been made in the identification of mutants with azoospermia or oligospermia, very few genes were linked to sperm malformations.

Spermatogenesis is a process characterized by radical structural changes of the diploid spermatogonial precursor, resulting in the spermatozoon. Some of these changes include the reduction to half of the chromosome set during meiosis, the replacement of histones in the DNA by transition proteins and subsequently by protamines, the condensation of the nucleus, and the formation of a flagellum. These morphological events require the synthesis of proteins involved in gene regulation, such as RNA-binding proteins (for review, see Hecht, 1998). Many RNA-binding proteins are required during midspermiogenesis, when most of the transcription terminates. During this step of spermatid differentiation the mRNAs are stored in the cytoplasm as ribonucleoprotein complexes until they are recruited for translation some days later. This tight translational repression is necessary for normal spermatid differentiation. Premature protamine1 (*Prm1*) synthesis, for instance, results in sterility due to arrested spermatid differentiation (Lee *et al.*, 1996).

Spermatid perinuclear RNA-binding protein (*Spnr*) has been identified by screening for proteins with an ability to bind to the 3′ untranslated region (UTR) of *Prm1* mRNA *in vitro*. *Spnr* contains two stretches coding for double-

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stranded RNA-binding domains (dsRBDs). Database searches have identified similar domains in several other proteins known or supposed to bind dsRNA, including the *Drosophila staufen,* the human dsRNA-activated inhibitor (DAI), and the *Escherichia coli* RNase III. Based on binding studies and computer analysis, a consensus sequence was derived that defines a 65- to 68-amino-acid dsRNA-binding domain (St Johnston *et al.,* 1992). However, SPNR expression suggests that it is not involved in *Prm1* translational regulation (Schumacher *et al.,* 1995).

Recent *in vitro* binding experiments showed that SPNR directly binds to the microtubules, acting as a microtubule-associated protein (Schumacher *et al.,* 1998). In order to test this hypothesis *in vivo* and to assess the function of *Spnr* in other tissues, we took advantage of a gene trap integration in the *Spnr* locus. *Spnr* mutant mice homozygous for the gene trap allele ($Spnr^{GT/GT}$) mice are smaller, show neurological defects, and have a high mortality rate. Furthermore, $Spnr^{GT/GT}$ showed reduced fertility, most probably due to a reduced mating drive and morphological alterations in the sperm flagellum, resulting in abnormal sperm motility.

MATERIALS AND METHODS

Embryonic stem (ES) cell transfection. R1 ES cells were transfected with the vector pGT1.8 geo containing the splice acceptor of engrailed-1 upstream of the reporter gene β-galactosidase and the selectable marker neomycin (Gossler *et al.,* 1989). Electroporation of ES cells and aggregation were performed as previously described (Stoykova *et al.,* 1998).

Genotyping. PCR genotyping was performed using SSLP primers that are polymorphic between 129/Sv and C57/Bl6. They are tightly linked to the *Spnr* locus (0.2 cM, chromosome 2). The primers used were obtained from the Web site of the Whitehead Institute (http://www-genome.wi.mit.edu/), marker name D2Mit369. Primer 1 sequence was 5′-GCC TCC ATC AAA GGA AGA CA-3′, primer 2 was 5′-TTC CTT CCC TGT CTA TGT GAT AAG G-3′. The PCR conditions were as follows: 94°C/1 min, 55°C/1 min, 72°C/1 min for 35 cycles performed in 50-μl volumes containing 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 25 pmol each primer, 2.5 U *Taq* polymerase (Boehringer), and 100 ng genomic DNA. Ten microliters of each PCR was loaded on a 6% agarose gel and visualized under ultraviolet light. The bands of 110 and 129 bp correspond to the amplification of the 129/Sv and C57/Bl6 polymorphic markers, respectively.

RT-PCR and 5′-RACE-PCR. RT-PCR was performed from testis RNA with the First cDNA Synthesis Kit (Pharmacia), according to the manufacturer's instructions. To amplify wild-type *Spnr* message, the following primers were used: 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GADPH), a typical "housekeeping gene" was used as an internal control; the GADPH 5′ primer sequence was 5′-ACC ACA GTC CAT GCC ATC AC-3′, positions 566–585, GADPH 3′ primer was 5′-TCC ACC ACC CTG TTG CTG TA-3′, positions 998–1017. The DNA fragments produced after 25 or 30 amplification cycles with an annealing temperature of 60°C were 316 and 451 bp, for *Spnr* and GADPH, respectively. The PCR conditions were the same as described for the genotyping. Total RNA from ES cell clone VII-28 was used for 5′ RACE using the Gibco BRL kit, using the primers described in Torres *et al.* (1997). The sequence obtained is identical to that of *Spnr* (Accession No. X84692), between positions 565 and 1149.

Northern blot. RNA from testis was extracted with Trizol (Gibco) according to the manufacturer's instructions. Thirty micrograms of total RNA was heated to 65°C for 10 min in the loading buffer [50% (v/v) formamide, 1× morpholinepropane sulfonic acid (Mops) buffer, 6.5% formaldehyde] and were size fractionated in a formaldehyde 0.8% agarose gel. The formaldehyde was added to a concentration of 0.12% after the agarose was heated and cooled down to 60°C. Gel electrophoresis was performed in 1× Mops buffer (0.005 M sodium acetate, 0.001 M EDTA, 0.02 M Mops), pH 7.0 at 5 V/cm, rinsed four times, 5 min each, with water to remove formaldehyde, and blotted with 10× SSC solution (Sambrook *et al.,* 1989). The blot was hybridized with an α-^{32}P-labeled probe corresponding to the entire 3.0-kb *Spnr* cDNA.

RNA in situ hybridization probes. The plasmid containing 640 bp of *dendrin* cDNA (Herb *et al.,* 1997) was cut with *Bam*HI and antisense RNA synthesized with T7 polymerase. MAP-2c antisense RNA was synthesized with T7 polymerase from a plasmid containing 1600 bp of cDNA sequence cut with *Hin*dIII (Garner *et al.,* 1988). Calbindin and IRPR antisense fragments cloned in pBluescript SK were linearized and synthesized as described (Bian *et al.,* 1996). To synthesize antisense *Spnr,* the plasmid containing the 3-kb cDNA was cut with *Bgl*II and incubated with the T3 polymerase. For the *Spnr* sense probe, the T7 promoter was used with the *Xho*I-linearized plasmid. Probe synthesis, preparation of paraffin sections, and hybridization were carried out as previously described (Kessel and Gruss, 1991).

Histological and morphological analyses. X-Gal staining was performed as described (Stoykova *et al.,* 1998). For sperm morphological analysis, sperm smears directly isolated from cauda epididymis or from uterus of mated females were fixed in 2% glutaraldehyde or 3.7% formaldehyde and stained with hematoxylin–eosin and analyzed with a Zeiss microscope. For scanning electron microscopy, testes fixed in Bouin's fixative were dehydrated in a graded ethanol series. Subsequently, testes were dried to the critical point using ethanol as the transitional and CO_2 as the exchange fluid. The dried specimens were mounted with conducting silver and spattered with gold palladium to a layer of about 40 nm. Specimens were examined and photographed in a DSM 960 scanning electron microscope (Carl Zeiss, New York).

Analysis of fertility. Reproductive capacity of $Spnr^{GT/GT}$ and $Spnr^{+/GT}$ males was assayed by breeding with wild-type females. Sets of 10 males of each genotype were mated with various wild-type females for 6 months. During this time the mating pairs were observed for sexual activity. Each day females with positive vaginal plugs were isolated for 21 days in order to allow them to deliver any pups they were carrying. To analyze the results, the total number of vaginal plugs and sired litters during the entire mating period was counted.

Examination of sperm functions. Sperm were directly isolated either from cauda epididymis or from females mated with males. For determination of sperm number and motility, epididymal sperm were diluted in modified Tyrode's medium and counted. For acrosome reaction, epididymal sperm were incubated for 5 min at 37°C in 5% CO_2 in modified Tyrode's medium plus the calcium ionophore A23187 (20 nm; Sigma) (Bleil, 1993). Sperm were fixed and stained with Coomassie Brilliant Blue R250 as previously described (Thaler and Cardullo, 1995). At least 200 sperm from each male were assayed for presence or absence of the characteris-

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tic dark blue acrosomal crescent. To examine sperm transport in the female reproductive tract, males were mated with superovulated females 13–17 h after human chorionic gonadotropin injection. Four hours after mating, sperm from uterus and oviduct were flushed and counted.

Fertilization assay. Sexually mature male mice from each genotype were used for the *in vitro* fertilization experiments (Adham *et al.,* 1997). Female mice were superovulated and oocytes were collected 13 h after hCG administration. Cumulus cells were removed. After 1.5 h capacitation, epididymal sperm (10^6 from each genotype) were added to the oocytes in 400-μl drops of fertilization medium and incubated for 1 h (for binding assay) and 6 h (for *in vitro* fertilization) at 37°C in 5% CO_2. Two to six hours after *in vitro* fertilization, the oocytes were examined for the presence of male and female pronuclei. The oocytes were then cultured in M16 covered with mineral oil to check them for progressive development over the next 3 days.

RESULTS

The cell line VII-28 with a gene trap integration in the *Spnr* locus was derived from a large-scale screening for genes involved in nervous system development (Chowdhury *et al.,* 1997; Stoykova *et al.,* 1998). R1 mouse ES cells were electroporated with the pGT1.8geo vector, which contains a splice acceptor upstream of the promoterless β-geo (Fig. 1A) (Skarnes *et al.,* 1995). After the generation of chimeric animals and checking for germ-line transmission, $Spnr^{+/GT}$ mice were crossed for five generations into the C57/Bl6 inbred background. Mice were genotyped either by quantitative Southern (data not shown) (Stoykova *et al.,* 1998) or by using SSLP markers (Fig. 1B). In order to check whether the gene trap vector integrated into more than one locus, Southern blot analysis was performed. Different restriction enzymes that recognize only one site of the vector were chosen. As seen in Fig. 1C, a unique band hybridized with the *lacZ* probe, indicating that there is only one gene trap integration.

In order to determine whether the X-Gal staining reflects the transcriptional activity of the endogenous promoter, *Spnr* expression was examined using an antisense *in situ* probe. Figure 1D shows that the signal distribution is basically identical at E18.5 $Spnr^{+/GT}$ brain, trigeminal ganglia, and nasal epithelium when using a *Spnr* and a *lacZ* probe. No signal was observed with the sense probe. Brains at P13 stage were also used for comparison and the expression pattern was identical in X-Gal and *in situ* hybridization histological sections. The expression of *Spnr* in the brain is very widespread. Some regions show stronger expression, such as cortex, dentate gyrus, and Purkinje cell layer and granule cells of the cerebellum (Fig. 1F). In the testis, *Spnr* expression was detected in spermatocytes (Figs. 1G and 1H).

Northern blot analysis showed that the gene trap insertion gives rise to a *Spnr* hypomorphic allele. The ~4.2-, 1.9-, and 1.4-kb bands are still found in testis of $Spnr^{GT/GT}$ mice. The ~3.2-kb band, however, could no longer be detected. The fusion transcript of the gene trap vector plus the 5′ sequence of *Spnr* results in an ~7.0-kb transcript found in $Spnr^{+/GT}$ and $Spnr^{GT/GT}$ mice. To confirm the Northern blot results, we performed RT-PCR from testis cDNA. A very light band is detected by RT-PCR after 35 cycles (Fig. 1E) with the primers shown in Fig. 1A. Thus, a reduced amount of wild-type RNA is still present in the testis of $Spnr^{GT/GT}$ mutant.

Abnormalities in mortality, weight, and neurological phenotypes in mice lacking Spnr. Genotype analysis of offspring from $Spnr^{+/GT}$ intercrosses approached the expected Mendelian ratio 1:2:1 (47 $Spnr^{+/+}$:107 $Spnr^{+/GT}$:63 $Spnr^{GT/GT}$). However, about one-third of the $Spnr^{GT/GT}$ mice died within 1 week after birth. This indicates that, whereas *Spnr* is not necessary for embryonic development, it is mandatory for postnatal development. The presence of milk in the stomach and the fact that the $Spnr^{GT/GT}$ mice died even when given to a foster mother indicates that they are not dying due to competition with their littermates. During adulthood 43% ($n = 28$) of the $Spnr^{GT/GT}$ die prematurely compared to the littermates. Necropsies did not reveal any obvious abnormalities in the gastrointestinal tract or cardiovascular or respiratory system (data not shown). $Spnr^{GT/GT}$ mice weighed less than their littermates (average weight 63.8% of the control littermates at P21; $n = 10$ for each genotype) and showed an abnormal clutching reflex when lifted by the tail. This reflex is characterized by a contraction and clenching together of the hindlimbs toward the ventral midline when the animals are being suspended by the tail. Such a reflex is a diagnostic feature of animals that show defects in brain, spinal cord, or dorsal root ganglia (Klein *et al.,* 1994; Mangiarini *et al.,* 1996). Mutant mice responded to painful stimuli in their whisker pad and were able to respond to a pinching of the tail and foot. This indicates that the trigeminal and peripheral sensory nervous systems are normal. The only anatomical alteration observed was the exposure of the superior colliculi in the $Spnr^{GT/GT}$ mutant brains ($n = 20$) (Fig. 2). No other major abnormalities were observed by histological methods (data not shown).

Spnr is not involved in the transport of specific RNAs to the dendrites. The *Drosophila staufen* gene codes for an RNA-binding protein that is involved in the transport of *bicoid* mRNA to the anterior pole of the oocyte (St Johnston *et al.,* 1991). Since SPNR has the same kind of RNA-binding domain as the STAUFEN protein, we investigated the possibility whether it regulates the transport of RNAs into specific intracellular compartments. The fact that $Spnr^{GT/GT}$ mice have neurological defects suggests that SPNR might be involved in this process. The neuron is the best example of a polarized cell and the kind of cell about which we know the most as far as RNA transport is concerned (Prochiantz, 1995).

In order to test whether *Spnr* mediates the RNA transport to the dendrites, *in situ* hybridization was performed to localize specific transcripts in these subcellular compartments. It has been suggested that the transport of RNAs into dendrites may ensure rapid local protein synthesis

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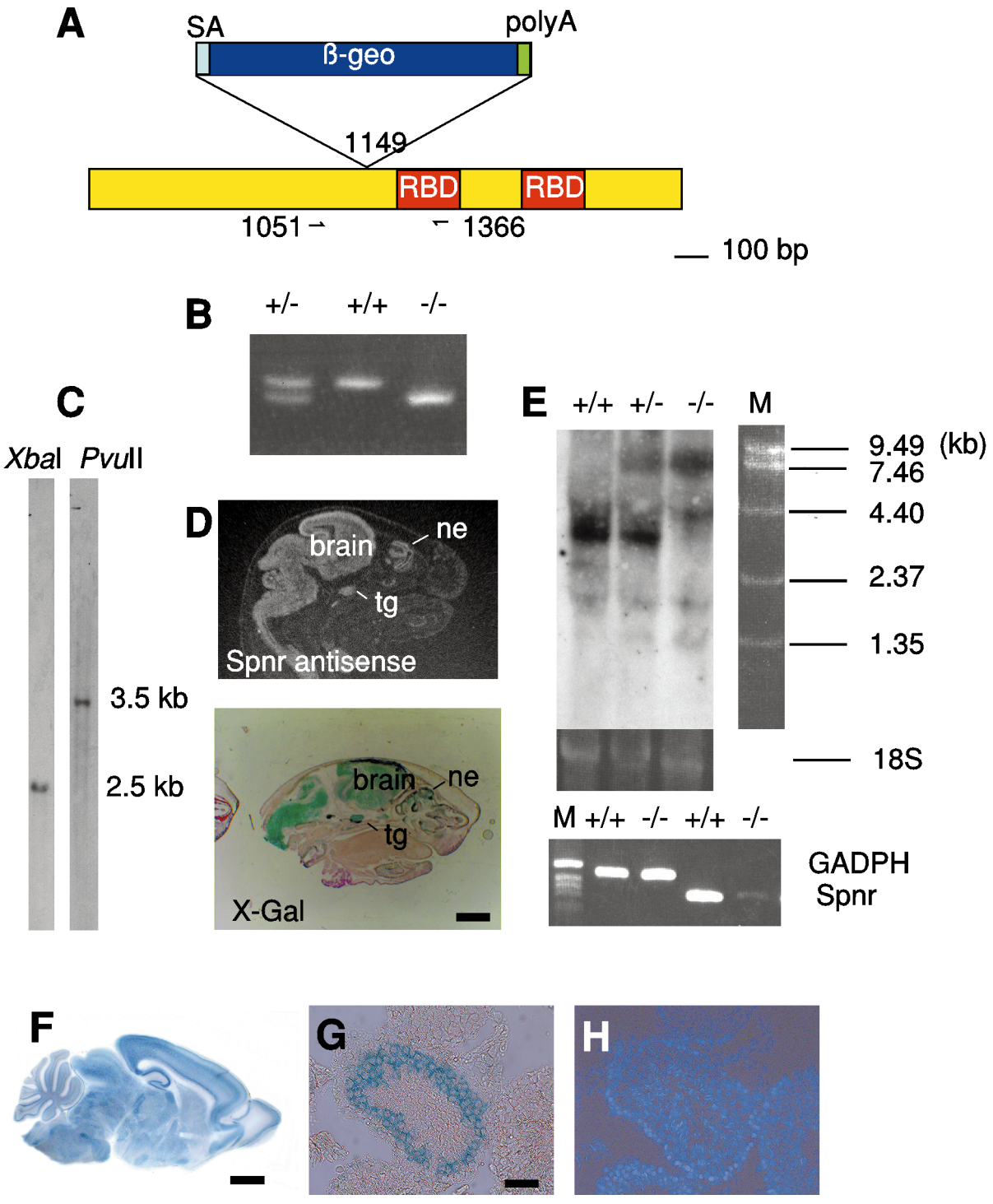

FIG. 1. Molecular characterization of the VII-28 line. (A) The coding sequence of the *Spnr* cDNA gene is schematically shown here. The protein is coded in about 2 kb of the cDNA sequence, which contains two regions of 180 bp each that predict double-stranded RNA-binding domains (RBD). The vector, which contains a splice acceptor (SA), the β-galactosidase marker fused to neomycin (β-geo), and a polyadenylation sequence (polyA), integrated at position 1149 of the *Spnr* cDNA; the primers for the RT-PCR were designed to bind before (positions 1051–1071) and after (positions 1366–1386) the integration point. The scale bar refers only to the *Spnr* cDNA, not to the vector (the coding sequence of the vector is 5.3 kb). (B) Genotypic analysis by PCR using SSLP markers. (C) DNA of $Spnr^{+/GT}$ animals digested with *Xba*I and *Pvu*I probed with *lacZ*. Only one band for each digestion could be detected, indicating that there is an integration of the vector in only one locus. (D) Top, head of an E18.5 embryo hybridized with the *Spnr* probe, showing silver staining in the entire brain, nasal epithelium (ne), and trigeminal ganglia (tg). Bottom, $Spnr^{+/GT}$ embryo at E18.5 showing X-Gal signal distribution identical to the silver staining shown above. Bar, 1.5 mm. (E) Northern blot of wild-type, $Spnr^{+/GT}$, and $Spnr^{GT/GT}$. As loading control, the agarose gel with 18S RNA is shown below. On the right is the RNA marker (M, Life Technologies) with the sizes indicated. Bottom, RT-PCR of testis cDNAs derived from a wild-type (+/+) and a $Spnr^{GT/GT}$ (−/−) animal. As control for the amount of material amplified, primers for glyceraldehyde-3-phosphate dehydrogenase (GADPH) were used. (F) Expression pattern of *Spnr* in a P13 mouse brain is widespread. Bar, 1.2 mm. (G) *Spnr* fusion transcript localization within the testis assayed by X-Gal staining in a cryostat section. (H) The same section stained with the Hoechst nuclear dye. Bar, 80 μm.

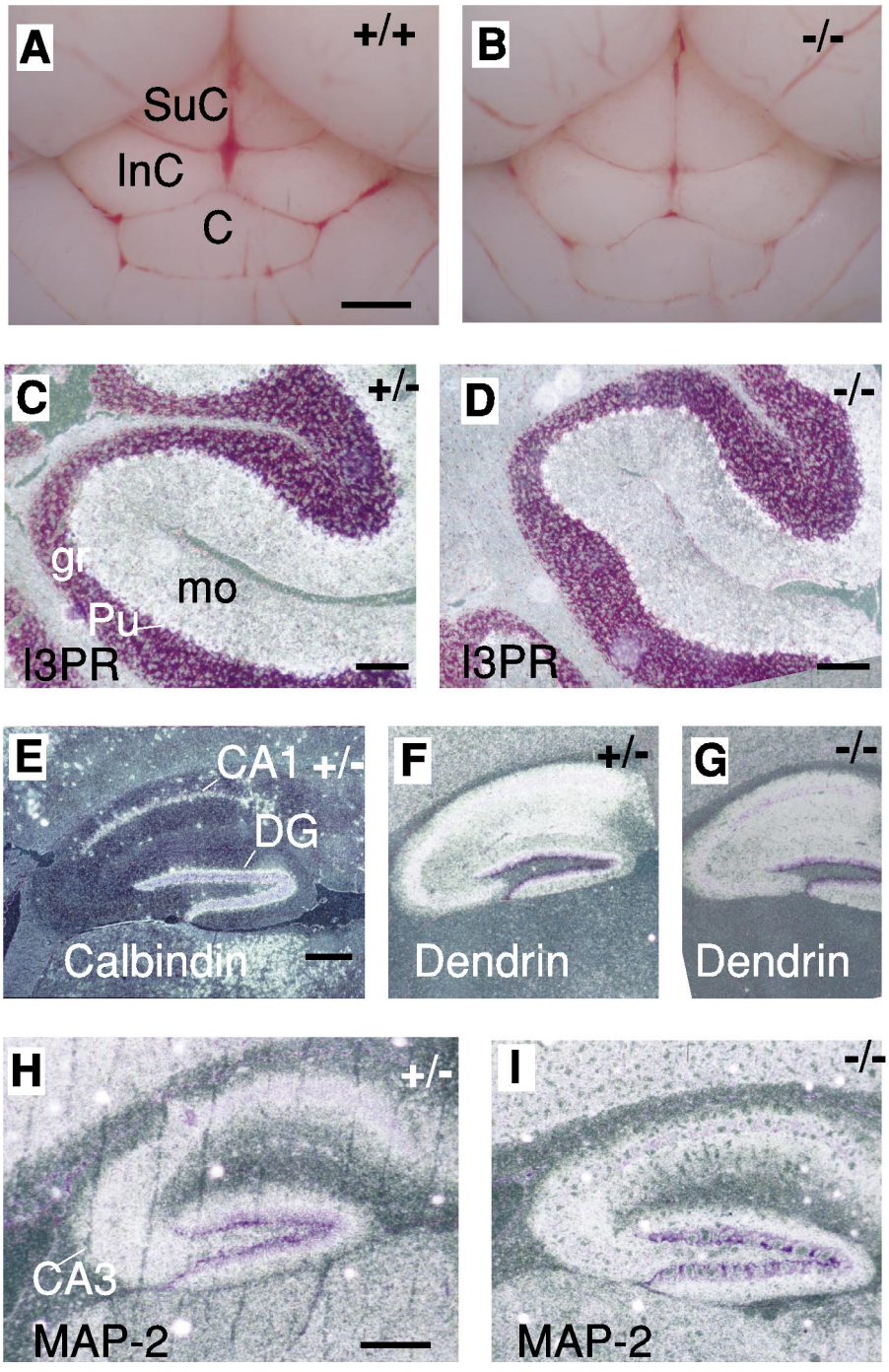

FIG. 2. Characterization of the brain phenotype. (A) Higher magnification of the mesencephalon of a wild-type brain, showing the superior colliculi (SuC), inferior colliculi (InC), and culmen (C). (B) *Spnr*$^{GT/GT}$ mesencephalon has a more exposed superior colliculi compared to the littermate brain. (C) *Spnr*$^{+/GT}$ cerebellum hybridized with the I3PR probe showing signal in the Purkinje cell body (Pu) and in the dendritic-rich molecular layer (mo). No silver staining is observed in the granular layer (gr). (D) I3PR has a normal dendritic distribution in the *Spnr*$^{GT/GT}$ cerebellum. (E) Hippocampus of a *Spnr*$^{+/GT}$ showing silver staining in the soma of neurons in the dentate gyrus (DG) and in CA1 as revealed by a calbindin probe. (F) The *dendrin* probe reveals signal distribution over the stratum lucidum and stratum radians of the hippocampus of a *Spnr*$^{+/GT}$ brain, indicating dendritic labeling. (G) *Spnr*$^{GT/GT}$ hippocampus has normal *dendrin* distribution. (H) Dendritic distribution of MAP-2 RNA in the CA3 neurons of a *Spnr*$^{+/GT}$ hippocampus. (I) The distribution of MAP-2 RNA is normal in *Spnr*$^{GT/GT}$ hippocampus. Bars, (A and B) 1 mm and (C–I) 0.2 mm.

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TABLE 1
Fertility of $Spnr^{+/+}$, $Spnr^{+/GT}$, and $Spnr^{GT/GT}$ Males

Parameter	Genotype		
	+/+	±	−/−
Number of males mated	9	7	30
Number of wild-type females mated	10	10	30
Males producing plugs	9	7	6
Plugs that led to pregnancy (%)	100	90	40
Average litter size	6.6	6.7	2.0
Fertility rate[a]	100	101	30

[a] Defined as the percentage of average litter size in +/+ mice.

important for synapse-specific responses (Burgin *et al.*, 1990; Lyford *et al.*, 1995; Steward, 1995). The mRNA localizations of microtubule-associated protein-2 (MAP-2), inositol 1,4,5-trisphosphate receptor type 1 (I3PR), and *dendrin* were tested (Fig. 2) (Furuichi *et al.*, 1993; Garner *et al.*, 1988; Herb *et al.*, 1997). In order to check for the specificity of the staining, we included a control (Fig. 2E) to show that nuclear staining in the hippocampus is restricted to a very specific layer of the dentate gyrus that does not overlap with the dendritic-rich area. Since the silver grain distribution was identical in $Spnr^{+/GT}$ and $Spnr^{GT/GT}$ brains ($n = 3$ for each genotype), namely in the dendrites, we conclude that *Spnr* is not involved in the dendritic localization of the tested mRNAs (Fig. 2E).

Spermatogenesis in SPNR-deficient male mice. SPNR was originally characterized for its *in vitro* capability of binding *Prm1* mRNA, which codes for a testis protein. *Spnr* expression in the testis was confirmed by Northern blot analysis (Fig. 1E). *Spnr* transcript localization, as assayed by X-Gal staining, was detected in meiotic germ cells, the spermatocytes (Fig. 1G). Previous immunolocalization studies of the SPNR protein within the testis have shown that it is expressed exclusively in postmeiotic germ cells (Schumacher *et al.*, 1995). This is in agreement with the fact that most of the transcription ceases at midspermiogenesis in the mouse and the stored mRNAs are synthesized only several days later (Monesi, 1964). The absence of the SPNR protein in spermatocytes suggests that its expression is translationally regulated. In order to verify potential fertility abnormalities, we mated mutant males with wild-type mice and checked for the presence of a vaginal copulation plug the following morning. The neurological defects seem to affect the sexual drive of the male $Spnr^{GT/GT}$ mutant mice, since only 6 of 30 males mated with the wild-type females mice during a 6-month mating period (Table 1). Although the vaginal plug was detected, only 4 of these males produced offspring. About 40% of the plugs led to pregnancy, and very often the males were able to fertilize the females only once or twice. The litters were usually smaller, with an average of 2 pups. In contrast, wild-type or $Spnr^{+/GT}$ male matings almost always led to pregnancy and an average litter size of 6 or 7 pups was generated. Although the external genitalia and testicular descent of $Spnr^{GT/GT}$ males appeared normal, the cauda epididymis of mice at 10 weeks of age showed a reduced testis weight and about half the number of sperm found in the $Spnr^{+/+}$ and $Spnr^{+/GT}$ littermates (Table 2). In some cases no sperm were found. Sperm of all genotypes did not significantly differ in assays

TABLE 2
Parameters of Sperm and Testis Function

Parameter	E^a	Genotype		
		+/+ (n)	± (n)	−/− (n)[b]
Testis weight (mg)		112 ± 5 (10)	101 ± 7 (10)	72 ± 7 (10)
Sperm number				
Epididymal ($\times 10^6$)	3	4.1 ± 0.4 (10)	3.9 ± 0.3 (10)	2.1 ± 02 (10)
Abnormal (%)		17.6	18.0	60.8
Uterus ($\times 10^3$)	10	7.2 ± 1.7 (15)	6.8 ± 1.3 (15)	1.3 ± 0.5 (2)
Oviduct	10	320 ± 35 (15)	287 ± 25 (15)	47 ± 6 (2)
Motile sperm (%)		66 ± 4 (10)	68 ± 6 (10)	41 ± 4 (10)
Acrosome reacted (%)				
0 min incubation	3	13 ± 3 (5)	15 ± 3 (5)	12 ± 2 (5)
5 min incubation	3	64 ± 5 (5)	61 ± 4 (5)	67 ± 4 (5)
Binding to ZP[c]	3	100	106	63

Note. Male mice were 10 weeks of age. All values are means ± SD of n individual measurements indicated in parentheses.
[a] Number of experiments.
[b] Values for all parameters (except acrosome reaction) are significantly different from ± and +/+ ($P > 0.5$, Student's test).
[c] Oocytes from wild-type mice were incubated with 10^6 sperm from each genotype and the number of wild-type sperm bound to zona pellucida (ZP) was taken as 100.

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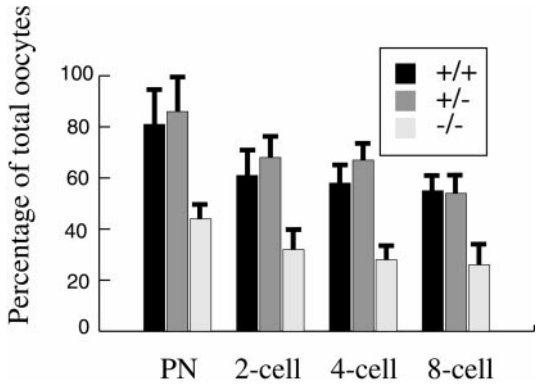

FIG. 3. Fertilization assay. For *in vitro* fertilization assay, oocytes from superovulated wild-type females were isolated and incubated for 6 h with sperm from $Spnr^{+/+}$ (+/+), $Spnr^{+/GT}$ (+/−), and $Spnr^{GT/GT}$ (−/−) males. The oocytes were assessed for the presence of male and female pronuclei (PN) and development to the 8-cell stage. $n = 3$ for each genotype, $P < 0.01$; Pearson's χ^2.

of acrosome reaction in the presence of calcium ionophore (Table 2). However, sperm motility of $Spnr^{GT/GT}$ was significantly reduced (Table 2). Two $Spnr^{GT/GT}$ mating males were used to test the oviduct sperm transport. The number of sperm isolated from uterus and oviduct 4 h after mating with those males was much lower than with $Spnr^{+/GT}$ and wild-type males (Table 2). We isolated viable swimming sperm from wild-type, $Spnr^{+/GT}$, and $Spnr^{GT/GT}$ mice and analyzed their ability to bind and fertilize wild-type eggs *in vitro*. A significant reduction in binding to zona pellucida (Table 2) and *in vitro* fertilization was observed for $Spnr^{GT/GT}$ sperm (Fig. 3).

Histological examination of adult $Spnr^{GT/GT}$ mice by transmission electron microscopy revealed extensive albeit heterogeneous pathology within the seminiferous tubules (Figs. 4A–4D). The seminiferous epithelium was abnormally thin in diameter (40 μm) and showed gaps in different places and disorganization in spermatogenesis. Numerous degenerated germ cells in different stages appeared throughout the seminiferous epithelium and a reduced number of mature spermatozoa were observed. When inspected by light and electron microscopy, a high percentage of the spermatozoa (60%) was highly atypical. Most of them showed defects in the flagellum, which were coiled around the spermatozoa head (Fig. 4F). This suggests that *Spnr* is required for normal morphological differentiation of male germ cells.

DISCUSSION

The mutagenesis by gene trap performed in the present study generated a mouse line with neurological and spermatogenic defects. By performing 5′ RACE, the gene was identified as coding for an RNA-binding protein which associates with the microtubules, the spermatid perinuclear RNA-binding protein (Chowdhury *et al.*, 1997). *Spnr* contains two stretches coding for dsRBDs. Database searches have identified similar domains in several other proteins known or supposed to bind dsRNA, including the *Xenopus* 4F.1 and 4F.2, the human DAI, and the *Drosophila staufen* (Bass *et al.*, 1994; Ferrandon *et al.*, 1997; Green and Mathews, 1992).

The growth of $Spnr^{GT/GT}$ mice was obviously disturbed at P21 and the resulting deficit in size persisted throughout postnatal life. The causes for the smaller growth rate and higher mortality of $Spnr^{GT/GT}$ mice were not determined. The neurological defects shown by the $Spnr^{GT/GT}$ mice could not be correlated with any gross histological change such as neuronal degeneration or tissue organization. Reasons for the abnormal clasping reflex are not known, although it is similar to murine models for neurodegeneration such as Huntington's disease (Mangiarini *et al.*, 1996) and for defects in dorsal root and spinal cord (Klein *et al.*, 1994). The exposure of the superior colliculi could be the result of a relative decrease in cortex size. More experiments are necessary to assess this matter. The same phenotype was also found in *Otx1*, which suggests that this gene could be involved in neuronal processes similar to *Spnr* (Acampora *et al.*, 1996). *Otx1* mutants are also smaller and show neurological defects.

Asymmetric mRNA transport within the cells is mediated by RNA-binding proteins that form stable ribonucleoprotein complexes. However, the nature of the protein components of these complexes in vertebrates is still unknown. In *Drosophila*, genetic studies have identified a number of potential genes that are necessary for localization of mRNAs in oocytes, such as *staufen*. In embryos from females with mutations in *staufen*, the localization of *bicoid* mRNA to the anterior cortex of the oocyte is disrupted. The STAUFEN protein contains five copies of the dsRBD, and a third of them bind dsRNA *in vitro*. The function of SPNR in RNA transport was tested in brain cells, because for these kinds of studies the mammalian nervous system is one of the best characterized (for review, see Gao, 1998). Although most of the mRNAs are confined to the perikaryon, an increasing number appear to be actively transported to specific cytoplasmic compartments of nerve cells. Specific regions of the brain, e.g., the molecular layer of the cerebellum and the stratum lucidum and radians of the dentate gyrus, are highly enriched in dendrites. In order to test whether SPNR plays a role in RNA transport to the dendrites, MAP-2, *dendrin*, and I3PR were tested for the subcellular localization of their mRNAs. Isotopic *in situ* hybridization experiments in brains derived from $Spnr^{GT/GT}$ mice showed that the transport of these mRNAs is not affected. However, a possible role for SPNR in the RNA localization of other genes cannot be ruled out. There are numerous transcripts that can be detected in the dendrites only by very sensitive techniques (Miyashiro *et al.*, 1994).

The lower fertility of $Spnr^{GT/GT}$ males is probably due to a

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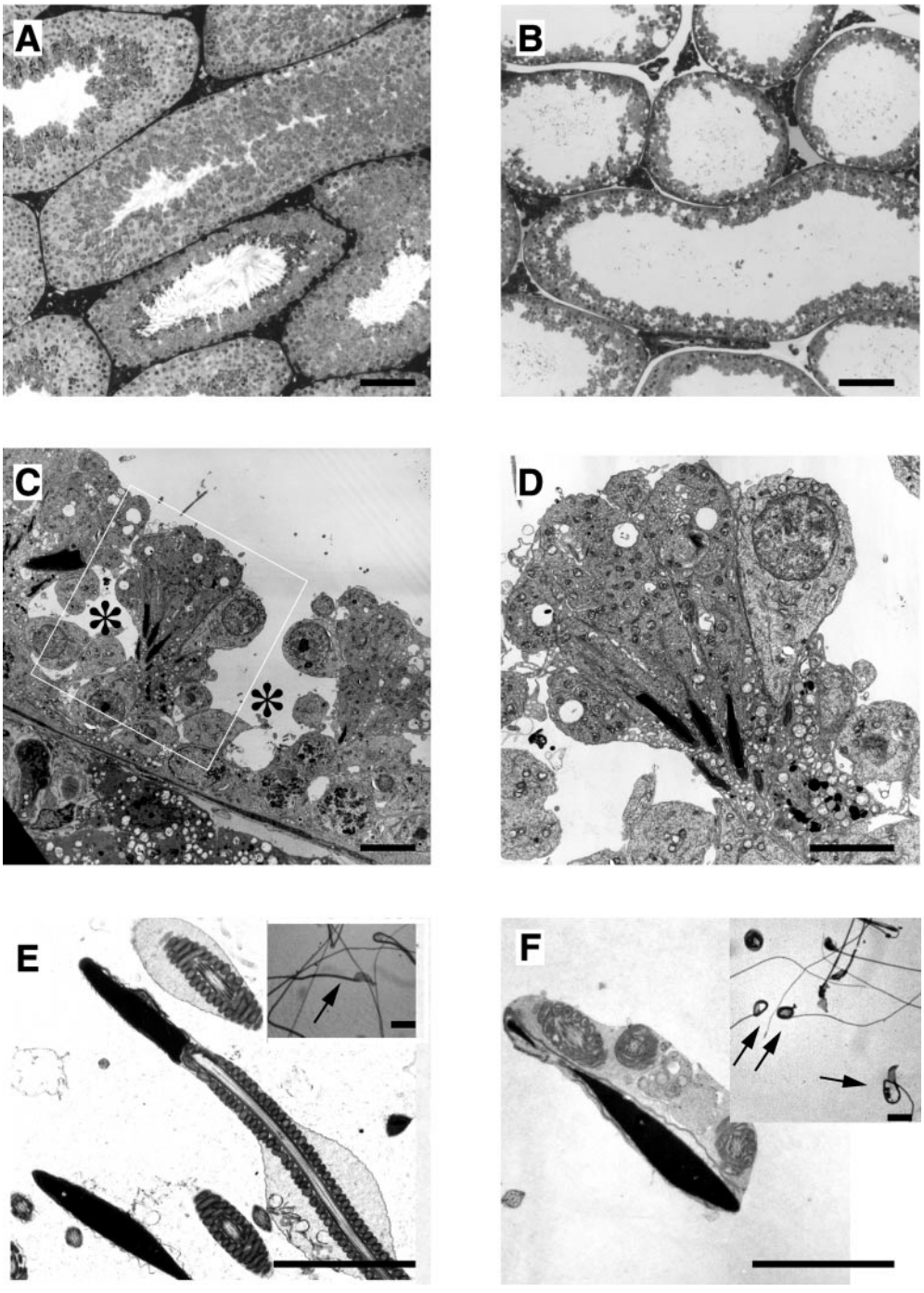

FIG. 4. Testis and sperm morphology. (A–F) Transmission electron micrographs. Insets in E and F are light micrographs. (A) Seminiferous tubules of a wild-type mouse. (B) *Spnr*$^{GT/GT}$ mutant has thinner seminiferous tubules. (C) In some parts of *Spnr*$^{GT/GT}$ seminiferous tubules, there is a lack (*) of differentiating spermatids. (D) Inset of (C) showing that some spermatids differentiate normally. (E) Normal spermatozoa. (F) Mutant spermatozoa have coiled flagellum around the head (arrows). Bars, (A and B) 70 μm, (C) 7 μm, (D) 5 μm, and (E and F) 2.5 μm.

combination of a lack of sexual drive and a malformation of spermatozoa. Although the lack of sexual drive could be explained by some indirect effect due to the smaller size or fitness of the mice, the lower fertility seems to be caused by specific defects of the sperm flagellum. The coiling of the tail morphology around the head prevented the normal

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flagellar motion. Therefore, a high proportion of the sperm showed lower motility and consequently did not reach the uterus and oviduct. Although some normal sperm were present, their number was reduced to about half of the normal number. Examination of the seminiferous tubules revealed that some spermatocytes did not fully differentiate and defective sperm underwent phagocytosis by Sertoli cells (data not shown).

The gene product of *Spnr* was isolated in a screening for proteins that bind the *Prm1* 3′ UTR (Schumacher *et al.*, 1995). Consistent with the findings that *Spnr* RNA was recovered from this screen three times from a pachytene spermatocyte library, the *Spnr-lacZ* fusion transcript was localized in the spermatocytes. The timing of SPNR protein, however, rules out its role in *Prm1* translational regulation. Since SPNR protein is detected at stage 9, a few days after *Prm1* transcription, it is unlikely that it regulates *Prm1* translation (Schumacher *et al.*, 1995). The premature translation of *Prm1* leads to sterility due to the arrest of spermatid differentiation (Lee *et al.*, 1995). Sperm of $Spnr^{GT/GT}$ mice, however, differentiated to term, suggesting that there is no premature *Prm1* translation. There are other candidate proteins that may regulate the synthesis of the*Prm1* protein. An example is *Prm1* RNA-binding protein, whose immunocytochemical localization and RNA-binding properties are consistent with such a role (Lee *et al.*, 1996).

In addition to the RNA-binding properties, SPNR was found to bind microtubules *in vitro* and to associate with manchettes *in vivo* (Schumacher *et al.*, 1998). Manchette is a rod-like structure composed of microtubules that attaches to the nucleus in step 8 spermatids and extends caudally around the developing flagellum in steps 9–16 (Russell *et al.*, 1991). The manchette is thought to shape the nuclear morphology as well as being a device that works like a "track" for cytoplasmic components to the caudal region of the cell. Consistent with this hypothesis, many of the spermatozoa derived from $Spnr^{GT/GT}$ mice have a defective head and flagellum. It can be assumed that SPNR plays an important structural role in appropriate microtubular organization and/or organelle transport.

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