

Spontaneous Locomotor Hyperactivity in a Mouse Mutant with a Deletion Including the *Snap* Gene on Chromosome 2

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The gene encoding the synaptosomal-associated protein-25 kDa (SNAP-25) was mapped by analysis of somatic cell hybrids and an intersubspecies backcross to mouse Chromosome 2. To identify potential mutants for SNAP-25, mice bearing mutations mapping to this region of Chromosome 2 were screened for *Snap* gene abnormalities. Mice heterozygous for the semidominant mutation coloboma (*Cm/+*) were identified that carried a deletion of *Snap* gene sequence. Analysis of genomic DNA revealed that the *Snap* gene dosage in *Cm/+* mice was 50% lower than control littermates. Additionally, SNAP-25 mRNA and protein expression were 50% lower in coloboma mice than control littermates. The coloboma mouse phenotype is characterized by small eyes and head bobbing; in addition, we observed that these mice were extremely hyperactive with spontaneous locomotor activity exceeding three times control mouse activity. The localization of the genetic abnormality in coloboma mice using the *Snap* gene marker will provide a powerful tool for studying the biologic basis of locomotor hyperactivity.

Pathologic states of hyperactivity are manifested in a variety of neurological conditions, including manic depressive disorder, some forms of viral encephalopathy, attention deficit hyperactivity disorder (ADHD), and several other organic brain diseases. Animal models of hyperactive behavior are of considerable interest as they provide experimental paradigms for assessing the neurologic mechanisms underlying hyperactivity syndromes. Synthetic neurotoxins such as 6-hydroxydopamine, as well as naturally occurring substances such as lead, can produce hyperactivity when administered to young animals (Silbergeld and Goldberg, 1974; Shaywitz et al., 1976). Other studies with animals have demonstrated a genetic predisposition for locomotor hyperactivity. For example, the spontaneously hypertensive rat (SHR) and the inbred mutant mouse weaver dis-

play spontaneous hyperactive behavior (Knardahl and Sagvolden, 1979; Schmidt et al., 1982; Roffler-Tarlov and Graybiel, 1984; Wultz et al., 1990). However, the genes responsible for this behavior have not been identified. Clearly, characterization of a specifically defined genetic locus would provide a more accessible model for investigating hyperactivity at the molecular level.

Here, we describe the identification of a spontaneously hyperactive mouse mutant coloboma bearing a deletion within mouse Chromosome 2 (Chr 2). This deletion spans the gene for SNAP-25 (synaptosomal-associated protein), a 25 kDa protein differentially expressed in subsets of neuronal populations, including those of the neocortex, hippocampus, anterior thalamic nuclei, and cerebellar granule cells (Oyler et al., 1989). Ultrastructural analysis has localized this neuron-specific protein to presynaptic nerve terminals, and more recently, SNAP-25 has been identified as "super protein," a major protein conveyed by fast axonal transport (Loewy et al., 1991; D. T. Hess, T. M. Slater, M. C. Wilson, and J. H. P. Skene, unpublished observations). During development, induction of SNAP-25 expression correlates precisely with the onset of synaptogenesis (Catsicas et al., 1991; Oyler et al., 1991), and in the adult, SNAP-25 expression is induced during synaptic remodeling (Geddes et al., 1990). While the remarkably high conservation of SNAP-25 across species (Catsicas et al., 1991) suggests that it plays an important role in synaptic structure and/or function, the function of SNAP-25 at the synaptic terminal is unknown.

One method for determining the function of a protein is through the identification of a mutant. To this end, we have mapped the *Snap* gene, encoding the SNAP-25 protein, to a mouse chromosome and determined that the mouse mutant coloboma bears a chromosomal deletion that spans at least the entire *Snap* gene. We have found that the phenotypic expression of this deletion includes profound spontaneous locomotor hyperactivity that may provide a novel animal model for examining the neurobiologic basis of hyperactivity syndromes.

Materials and Methods

Mouse mutants. Eight- to ten-week-old coloboma (*Cm/+*) mice and control (+/+) littermates were purchased from The Jackson Laboratory (Bar Harbor, ME). The coloboma mutation that first arose on a C3H/HeH × 101/H F1 male had been bred onto the C57BL/6By background for 32 generations. The mutation has since been maintained by backcrosses onto the C3H/HeSnJ strain for 10 generations by M. Davison at The Jackson Laboratory. After backcrossing a mutation into a new strain for 10 generations, 99.8% of unlinked genes in the mutant genome originate from the backcross strain (E. L. Green, 1981a). In all experiments, coloboma mouse mutants were compared to their normal

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control littermates derived from backcrossing *Cm/+* mice with wild-type C3H/HeSnJ mice. All other mouse mutants tested for *Snap* mutations including wellhaarig (*we*), mahogany (*mg*), lethal milk (*lm*), undulated (*un*), blind-sterile (*bs*), and brachypodism (*bp*) were also purchased from The Jackson Laboratory.

Genetic mapping. Twenty-four Chinese hamster × mouse somatic cell hybrids were selected from a larger panel of 76 hybrids described and characterized previously (Hoggan et al., 1988). Thirteen hybrids were typed for mouse chromosomes by karyology; the rest were typed for markers on specific chromosomes.

Two genetic crosses were used for mapping. For the first cross, NFS/N females were mated with *Mus m. musculus* (Skive) males and the F1 progeny were mated with *M. m. musculus* as previously described (Kozak et al., 1990). DNAs extracted from individual backcross progeny were typed by Southern analysis with SNAP-25 probe following digestion with *ScaI* as well as for other genetic markers including *Hdc* (histidine decarboxylase) typed using a rat HDC cDNA probe (Joseph et al., 1990) following digestion with *XbaI*, and *Emv-15* (endogenous ecotropic leukemia virus-15) typed following digestion with *HindIII*. The *Emv-15* probe was kindly provided by Dr. G. Barsh (University of California, San Francisco) and represents a 1.1 kilobase (kb) *EcoRI* fragment (Lovett et al., 1987). For the second cross, NFS/N females were mated with *M. spretus* males and the F1 females were mated with *M. spretus* or C58/J (Adamson et al., 1991). DNAs from individual backcross progeny were typed for *Snap* using *BamHI*, for *Hdc* using *EcoRI*, and for *Emv-15* using *HindIII*.

Interspecific and interstrain crosses. *M. spretus* males purchased from The Jackson Laboratory (Bar Harbor, ME) were mated with *M. m. musculus Cm/+* C3H/HeSnJ females. The progeny of this interspecific cross were identified as *+/+* or *Cm/+* by eye morphology; the F1 *Cm/+* mice were easily identified by their small eyes. DNAs extracted from both parent mice and a *+/+* and *Cm/+* F1 mouse were examined by Southern analysis with a SNAP-25 probe following digestion with *EcoRI* and with a BMP-2a (bone morphogenetic protein-2a) probe synthesized from the murine BMP-2a cDNA (Lyons et al., 1989) provided by B. Hogan (Vanderbilt University) following digestion with *XbaI*. For the interstrain cross, a C3H/HeSnJ *Cm/+* male was crossed with a C57BL/6J female and the progeny of the cross identified as *+/+* or *Cm/+* as described above. DNAs extracted from both parent mice and a *+/+* and *Cm/+* F1 mouse were examined by Southern analysis with a murine PAX-1 cDNA probe (Deutsch et al., 1988) kindly provided by R. Balling (Max Planck Institute of Biophysical Chemistry) following digestion with *PstI*.

Genomic Southern analysis and slot blots. The probe for *Snap* hybridizations was prepared from an 881 base pair (bp) SNAP-25 cDNA comprised of the entire 618 bp open reading frame plus 5' untranslated sequence. Control and coloboma mouse genomic DNA was prepared by digesting 2 cm clips of mouse tail in 500 µg/ml buffered [50 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% SDS] proteinase K for 16 hr at 56°C. Digests were extracted with phenol and chloroform, precipitated in ethanol, and resuspended in sterile water. Each tail clip produced ~100 µg of genomic DNA.

For slot blots, control or coloboma mouse genomic DNA was denatured for 30 min at 67°C in 200 µl of 10 mM Tris (pH 8.0), 1 mM EDTA, 0.3 M NaOH, cooled on ice, and neutralized with an equal volume of 2 M ammonium acetate (pH 7.0). DNA masses for all samples were equalized to 4 µg of DNA per slot with the addition of lambda phage DNA. Additionally, a control sample containing 4 µg of lambda DNA only was prepared. Samples from individual mice were slot blotted onto nitrocellulose, and the filter was air dried and baked at 80°C for 2 hr. This experiment was repeated four times using DNA from four individual control and coloboma mice.

For mouse genomic Southern blots, 10 µg of control or coloboma mouse genomic DNA were digested with the appropriate restriction endonuclease and resolved on a 0.7% agarose gel. For mouse × hamster somatic cell hybrid Southern analysis, 10 µg of DNA from 24 mouse × hamster cell lines containing various mouse chromosomes were digested with *EcoRI* and size fractionated on a 0.7% agarose gel. After hydrolysis, denaturation, and neutralization, the DNA was transferred to nylon membrane (Zetabind, CUNO Inc., Meriden, CT) and baked at 80°C for 2 hr.

Slot blot and Southern blot filters were prehybridized for 16 hr at 67°C in 3 × TESS (0.45 M NaCl, 15 mM TES [2-(tris-[hydroxymethyl]-methyl)-amino ethanesulfonic acid], 15 mM EDTA), 5 × Denhardt's solution, 0.2% SDS, 2 mM sodium pyrophosphate, and 100 µg/ml salmon sperm DNA. Filters were hybridized at 67°C for 20 hr with random-

primed ³²P-labeled probe to SNAP-25 in prehybridization solution plus 10% dextran sulfate. Blots were washed to a final stringency of 0.1 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 67°C and apposed to film for 3–7 d. For slot blots only, after SNAP-25 hybridization, probe was boiled off and the same blots were hybridized with random primed ³²P-labeled probe prepared from a cDNA comprising 740 bp of the murine β-amyloid precursor protein open reading frame (G. A. Higgins, G. A. Oyler, and M. C. Wilson, unpublished observations) as an internal control for mass of genomic DNA blotted. Film optical density was quantitated on a laser densitometer (Pharmacia-LKB).

Ribonuclease protection assay. Control and coloboma mice were killed by decapitation, the brains removed, and total cellular RNA isolated using the guanidinium thiocyanate/CsCl centrifugation procedure (Chirgwin et al., 1979). Appropriate masses of total RNA were hybridized overnight at 42°C in 80% formamide, 40 mM PIPES (piperazine-*N,N'*-bis-[2-ethane sulfonic acid]; pH 6.4), 400 mM sodium acetate, and 1 mM EDTA with 5 × 10⁴ cpm of a 400 bp SNAP-25 cRNA and 5 × 10⁴ cpm of a 250 bp β-actin cRNA. Samples were then digested with an RNase A/RNase T1 mix for 30 min at 37°C, digested with Proteinase K for 15 min at 37°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and ethanol precipitated. Samples were resuspended and size fractionated on a denaturing 5% polyacrylamide gel, and the gel was apposed to x-ray film (XAR, Kodak) for 2–5 hr.

In situ hybridization. The *in situ* hybridization procedure has been described in detail (Wilson and Higgins, 1989). ³⁵S-labeled single-stranded antisense RNA probes were generated from cDNAs of murine SNAP-25 and β-amyloid precursor protein subcloned in pBluescript as described above. After *in vitro* transcription, the DNA template was removed by digestion with RNase-free DNase (Promega) and the transcripts were sized reduced to 150–250 nucleotides by alkali treatment.

Pre-treatment of the slide-mounted sections included fixation in buffered 4% formaldehyde for 5 min at room temperature, followed by treatment with 25 µg/ml proteinase K in 5 × TE (50 mM Tris, pH 8.0, 5 mM EDTA) for 7.5 min at 37°C, treatment in 0.5N HCl for 7.5 min at room temperature, and a final 5 min postfixation in buffered 4% formaldehyde. Slides were rinsed 2 × 2 min in PBS between each of the aforementioned steps. Sections were then dehydrated in graded ethanols and air dried.

Slides were prehybridized at 52°C for 2–3 hr in approximately 750 µl of a solution containing 50% formamide, 0.75 M NaCl, 20 mM PIPES, pH 6.8, 10 mM EDTA, 10% dextran sulfate, 5 × Denhardt's solution (0.02% BSA, 0.02% ficoll, 0.02% polyvinylpyrrolidone), 50 mM dithiothreitol, 0.2% SDS, and 100 µg/ml each salmon sperm DNA and yeast tRNA. Prehybridization buffer was removed from the slides, and 75 µl of hybridization solution consisting of prehybridization buffer plus 10 ng probe were applied to each slide. Slides were coverslipped and hybridized for 16 hr at 52°C.

Following hybridization, coverslips were removed in 4 × SSC (0.15 M NaCl, 0.015 M sodium citrate) plus 300 mM 2-mercaptoethanol at room temperature, incubated in this solution for 15 min, and incubated in 4 × SSC without 2-mercaptoethanol for 15 min at room temperature. Sections were treated with 50 µg/ml pancreatic RNase A in 0.5 M NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA for 30 min at 37°C, rinsed for 30 min at 37°C in this same buffer, washed in 2 × SSC at 56°C for 15 min, and air dried. Sections were exposed to x-ray film (Du Pont Cronex) for 2–4 d.

Protein slot blots. Control and coloboma mice were killed by decapitation and the brains removed. Brain proteins were extracted from whole brain in 1% NP-40, methanol precipitated, and resuspended in 1% SDS. Protein concentrations were determined using the Pierce Micro BCA protein assay, which accommodates the presence of SDS. Proteins were diluted to appropriate concentrations in PBS, and 100 µl of each dilution were slot blotted onto nitrocellulose. Nitrocellulose blots were incubated for 30 min in 10% acetic acid, rinsed briefly in PBS, and blocked overnight in Blotto [0.16 M NaCl, 10 mM sodium phosphate (pH 7.5), 0.01% Thimerosal, 5% (w/v) nonfat dry milk, 0.006% (v/v) antifoam A]. Blots were then incubated in a 1:400 dilution of peptide-directed SNAP-25 antisera (described in Oyler et al., 1989) in Blotto for 3 hr at room temperature, washed three times in Blotto at room temperature, and incubated for 1 hr at room temperature in Blotto with ¹²⁵I-labeled *Staphylococcus* protein A. Blots were rinsed in water and apposed to film for 16 hr. Film optical density was quantitated on a laser densitometer (Pharmacia-LKB).

Behavioral testing. All mice used in the behavioral tests were obtained from The Jackson Laboratories where they were identified as *Cm/+* or

Table 1. Segregation of the *Snap* hybridizing restriction fragment with alleles of *Hdc* and *Emv-15* in progeny of an interspecies and interspecies backcross

Mice	Inheritance of alleles from the F1 parent			Number of mice	
	<i>Hdc</i>	<i>Snap</i>	<i>Emv-15</i>	Cross 1	Cross 2
Nonrecombinants	+	+	+	20	38
	-	-	-	21	31
Single recombinants	+	+	-	4	2
	-	-	+	2	5
	+	-	-	4	3
	-	+	+	<u>1</u>	<u>2</u>
			52	81	

Table 2. Recombination

Locus pair	<i>r/n</i>	Recombination (% ± SE)
<i>Hdc, Snap</i>	10/133	7.5 ± 2.3
<i>Snap, Emv-15</i>	13/133	9.8 ± 2.6

Percentage recombination between restriction fragments and SE were calculated according to E. L. Green (1981b) from the number of recombinants (*r*) in a sample size of *n*.

+/+ mice using eye morphology as the phenotypic criterion for genotype determination. Mice were maintained in group cages on ad lib food and water with a 12 hr light/12 hr dark cycle (6 A.M., 6 P.M.). Male *Cm/+* mice (*n* = 5) and control littermates (*n* = 5) on a C3H/HeSnJ background were placed in individual Plexiglas open field photocell activity cages (60 × 60 × 40 cm) with four 2-cm-high infrared beam detectors per side. Recordings started at 10:00 A.M. and continued for 24 hr with beak breaks accumulated every 15 min. Mice were provided with food and water during the entire testing session. Activity data were analyzed by two-factor repeated-measures ANOVA, and time spent quiescent was analyzed by a Student's *t* test.

Results

Genetic mapping of *Snap*. To map the *Snap* gene to a mouse chromosome, a panel of 24 mouse × hamster hybrid cell lines containing different mouse chromosomes was screened by Southern analysis using a SNAP-25 cDNA. All cell lines were positive for hamster *Snap*, and 13 of the hybrids were positive for murine *Snap*; the mouse chromosome content of these cell lines suggested that *Snap* was located on mouse Chr 2.

The *Snap* gene was more precisely localized on mouse Chr 2 by Southern blot analysis of the progeny from two genetic crosses. In the first cross, DNA from progeny of the matings (NFS/N × *M. m. musculus*) × *M. m. musculus* was digested with *ScaI* and hybridized with a SNAP-25 cDNA probe, distinguishing *M. m. musculus* *Snap* fragments of 12.8, 6.0, and 2.9 kb from 18.5 and 8.2 kb NFS/N fragments. In the second cross, DNA from progeny of the matings (NFS/N × *M. spretus*) × *M. spretus* or C58/J was digested with *BamHI* and hybridized with a SNAP-25 cDNA probe, distinguishing an *M. spretus* *Snap* fragment of 23 kb from a 28 kb NFS/N or C58/J fragment. Analysis of the backcross mice identified 67 of 133 progeny bearing the F1 parental *Snap* allele consistent with the 1:1 segregation ratio expected for a single gene. When *Snap* segregation was compared with the Chr 2 markers *Hdc* and *Emv-15*, 10 recombination

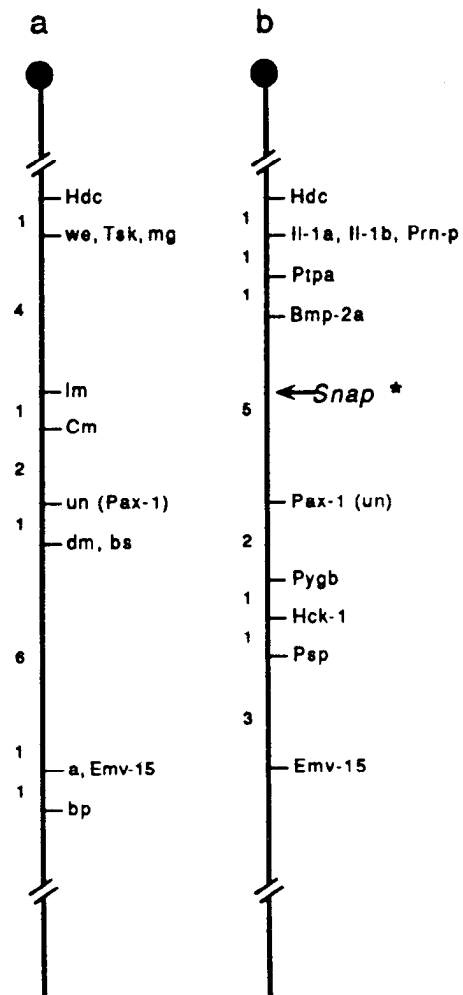


Figure 1. Genetic (*a*) and molecular (*b*) maps of the medial portion of mouse Chr 2. Mouse mutants mapping between the markers *Hdc* and *Emv-15* are illustrated on the genetic map (*a*) constructed by Lyon and Kirby (1991). All genes between *Hdc* and *Emv-15* that have been mapped using molecular probes are located on the map (*b*) constructed by Siracusa and Abbott (1991). *Snap* was located on the molecular map using relative distances between *Hdc* and *Emv-15* established from the mapping data.

events were observed between *Snap* and *Hdc* and 13 were observed between *Snap* and *Emv-15* (Tables 1 and 2). Recombination frequencies together with the known map location of *Hdc* and *Emv-15* established the gene order: *Hdc*—7.5 ± 2.3 cM—*Snap*—9.8 ± 2.6 cM—*Emv-15*.

After the chromosome location had been determined, several mouse mutants previously mapped to this region (Fig. 1*a*), including *we*, *mg*, *lm*, *Cm*, *un*, *bs*, and *bp* (Hillyard et al., 1991; Lyon and Kirby, 1991), were screened for *Snap* restriction fragment length polymorphisms (RFLPs) by Southern analysis of *EcoRI*-digested genomic DNA. No differences were observed in the *Snap* restriction fragments in any mouse mutant tested (data not shown). However, reduced *Snap* hybridization was noted in mice heterozygous for the semidominant mutation coloboma (*Cm/+*).

Coloboma mouse genomic DNA. Because weak *Snap* hybridization was observed in *Cm/+* mouse genomic DNA, coloboma mutants were examined more rigorously. As homozygous *Cm/Cm* mice have been reported to die early in embryogenesis

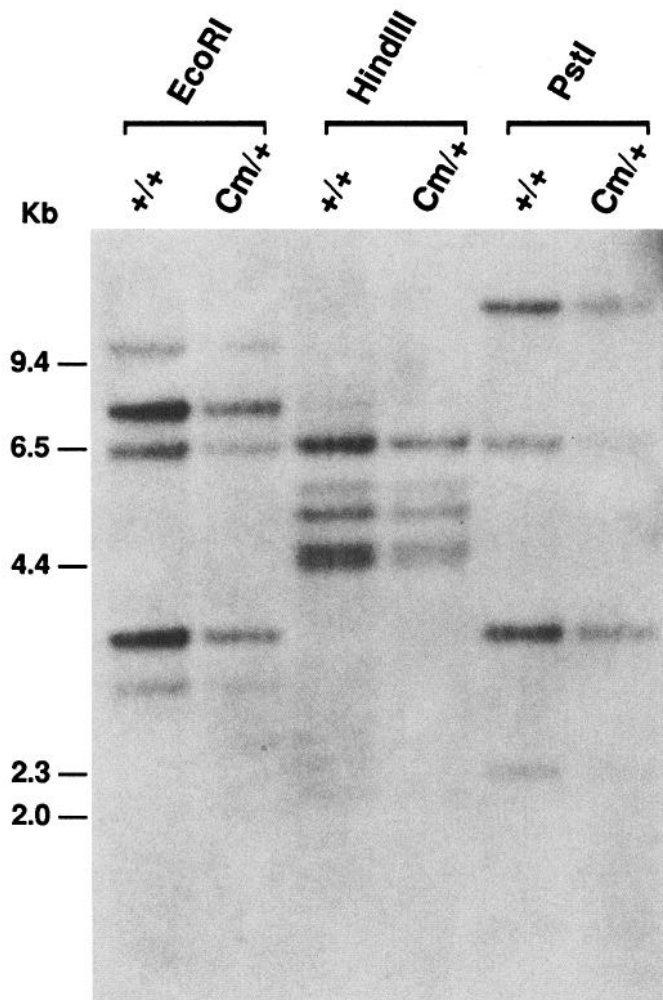


Figure 2. Genomic Southern analysis of SNAP-25 hybridization in control (+/+) and coloboma (*Cm/+*) mice. Genomic DNA (10 μ g/lane) was digested with EcoRI, HindIII, or PstI, electrophoresed, and transferred to nylon membrane. Filters were hybridized with a 32 P-labeled random-primed probe to a SNAP-25 cDNA comprised of the entire open reading frame and most of the 5' untranslated sequence.

(Theiler and Varnum, 1981), only heterozygous *Cm/+* mice could be screened. Genomic DNA from *Cm/+* mice or their normal littermates (+/+) was digested with several restriction endonucleases, blotted, and probed with a SNAP-25 cDNA that included the 5' untranslated region and entire open reading frame. No RFLPs were detected between mutant and control mouse DNA digested with EcoRI, HindIII, or PstI (Fig. 2); however, the intensity of the *Snap* signal from the coloboma DNA appeared consistently lower than that of control mice. To quantitate more accurately possible differences in *Snap* gene dosage between control and coloboma mice, slot blots of genomic DNA from +/+ mice and *Cm/+* mice were compared. Densitometric units quantitated by laser densitometry from the slot blot autoradiographs plotted against mass of DNA/slot revealed that SNAP-25 hybridization to coloboma genomic DNA was approximately 50% that of control mouse DNA (Fig. 3). In contrast, when the same blot was reprobed with a β -amyloid precursor protein cDNA, the gene which maps to Chr 16, hybridization was comparable between mutant and control mice. The nearly identical slopes of mutant and control mice in the

β -amyloid precursor protein plot verified that equal masses of mutant and control DNA were blotted. The lack of observable RFLPs in the Southern analysis coupled with the 50% reduction in *Snap* hybridization observed in the slot blots suggests that most, if not all, of one copy of the *Snap* gene is deleted in the *Cm/+* mouse.

The gene dosage determined from the slot blot genomic analysis was verified using the progeny of the interspecific *M. m. musculus Cm/+* \times *M. spretus +/+* cross. The genetic variation distinguished by restriction endonuclease cleavage sites between these two mouse species made it likely that Southern analysis would reveal specific parental *M. m. musculus* (C3H/HeSnJ) and *M. spretus* alleles. Indeed, Southern analysis of EcoRI-digested DNA probed with SNAP-25 distinguished a 3.2 kb *M. m. musculus*-specific RFLP that was not detected in *M. spretus* (Fig. 4a, arrow). In the phenotypically normal (+/+) mice of this cross, the *M. m. musculus Snap* allele was present along with the *M. spretus* allele (Fig. 4a). However, the *M. m. musculus*-specific 3.2 kb band was not detected in the DNA from the *Cm/+* interspecific mouse, identified by ocular deformation, indicating that the *M. m. musculus Snap* allele is deleted in the coloboma interspecific mouse (Fig. 4a). These data confirmed the results of the genomic slot blot analysis, which indicate that the entire *Snap* gene is deleted from one *Cm/+* Chr 2.

Deletion mapping. Based on the estimated position of *Snap* relative to *Emv-15* and *Hdc* and the previously mapped location of *Cm*, we examined two genes within this same region (*Pax-1* and *Bmp-2a*; Fig. 1b) as molecular markers to define the outer limits of the coloboma deletion. The integrity of these genes in the coloboma mouse mutant was assessed using RFLPs exhibited in an interspecific (+/+ *M. spretus* \times *Cm/+ M. m. musculus*) cross for *Bmp-2a* or an interstrain cross (*Cm/+* C3H/HeSnJ \times +/+ C57BL/6J) for *Pax-1*. Southern analysis of XbaI-digested DNA from the interspecies cross probed with BMP-2a cDNA (Lyons et al., 1989) revealed a 4.6 kb *M. m. musculus*-specific RFLP that was not detected in *M. spretus* (Fig. 4b, arrow). The *M. m. musculus*-specific band was also present in the DNA from both the +/+ and *Cm/+* interspecific mice, indicating that the *Bmp-2a* gene is not included in the coloboma deletion. To determine whether *Pax-1* was deleted in the coloboma mutation, DNA from the interstrain cross *Cm/+* C3H/HeSnJ \times +/+ C57BL/6J was digested with PstI and probed with the PAX-1 cDNA (Deutsch et al., 1988). A 7.5 kb band present in the C3H/HeSnJ mouse DNA was distinguished from the 1.5 kb band observed in the C57BL/6J mouse DNA. The 7.5 kb C3H/HeSnJ-specific band was also detected in the DNA of both the +/+ and *Cm/+* mice produced from the interstrain cross (Fig. 4c, arrow), suggesting that *Pax-1*, like *Bmp-2a*, is not deleted in the coloboma mutation.

SNAP-25 mRNA expression in coloboma mice. Quantitative ribonuclease protection assays were performed to determine if the half gene dose of *Snap* in coloboma mice affects SNAP-25 mRNA expression. For ribonuclease protection assays, total RNA from mutant and control brains was assayed with probes to SNAP-25 and β -actin, and the resulting autoradiograph was quantitated by laser densitometry (Fig. 5). SNAP-25 mRNA expression was approximately 50% lower in the mutant than in its control littermate, while β -actin expression was comparable in mutant and control mice. These results suggest that, in coloboma mice, the loss of one *Snap* allele results in a 50% reduction in the SNAP-25 transcript without affecting other messages such as β -actin.

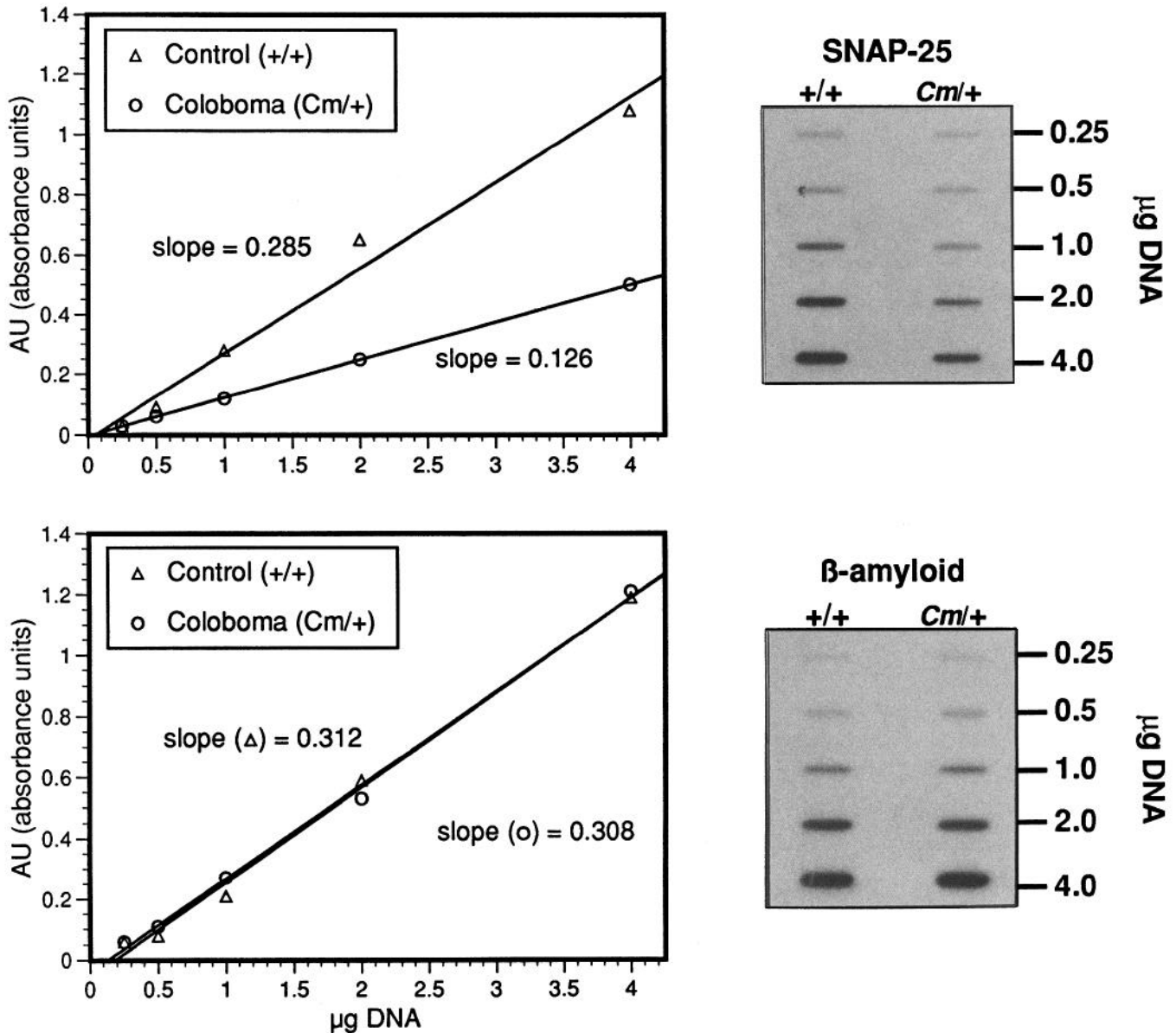


Figure 3. Slot blots of genomic DNA from a control (+/+) and a coloboma mouse. Increasing masses of genomic DNA were blotted to nitrocellulose, hybridized with a ^{32}P -labeled probe to SNAP-25 or β -amyloid precursor protein, and apposed to x-ray film. Autoradiographs were quantitated using a laser densitometer, and the arbitrary absorbance units were plotted against the mass of genomic DNA loaded in the slot. In the *top panel*, SNAP-25 hybridization in the *Cm/+* mouse is 44% of the +/+ mouse; in the *bottom panel*, where the same blot was rehybridized with β -amyloid precursor protein, hybridization between the +/+ and the *Cm/+* mouse is equal. On average, *Cm/+* hybridization was $45 \pm 4\%$ of the +/+ SNAP-25 hybridization ($n = 4$).

Neuroanatomical SNAP-25 localization. Consistent with the ribonuclease protection assay, *in situ* hybridization also indicated that SNAP-25 mRNA expression was reduced in coloboma mouse mutants (Fig. 6), while β -amyloid precursor protein mRNA expression was comparable between mutant and control mice. Although SNAP-25 expression was reduced in coloboma mice, the neuron-specific pattern of SNAP-25 expression was identical in *Cm/+* and +/+ mice. Both controls and mutants expressed high levels of SNAP-25 in the hippocampal formation, particularly in CA3 pyramidal cells, cortex, anterior thalamus, and septal nuclei. Moderate SNAP-25 expression was observed in the ventral posterior and ventral lateral thalamic nuclei, while low levels of SNAP-25 mRNA were observed in the basal ganglia. SNAP-25 hybridization was not

observed in white matter, consistent with the neuron-specific expression of this mRNA. More detailed descriptions of the distribution of SNAP-25 mRNA by *in situ* hybridization have been reported previously for both brain (Oyler et al., 1989) and retina (Catsicas et al., 1991). Additionally, neither the SNAP-25 nor β -amyloid precursor protein pattern of hybridization revealed any obvious neuroanatomic abnormalities in the coloboma mouse brain such as cell loss or ectopias observed in other neurological mouse mutants including weaver, reeler, and leaner (M. C. Green, 1981). Thus, the coloboma mutation results in a 50% reduction specifically in SNAP-25 mRNA without affecting its normal tissue distribution.

SNAP-25 protein expression in coloboma mice. It was important to determine whether the 50% reduction in SNAP-25

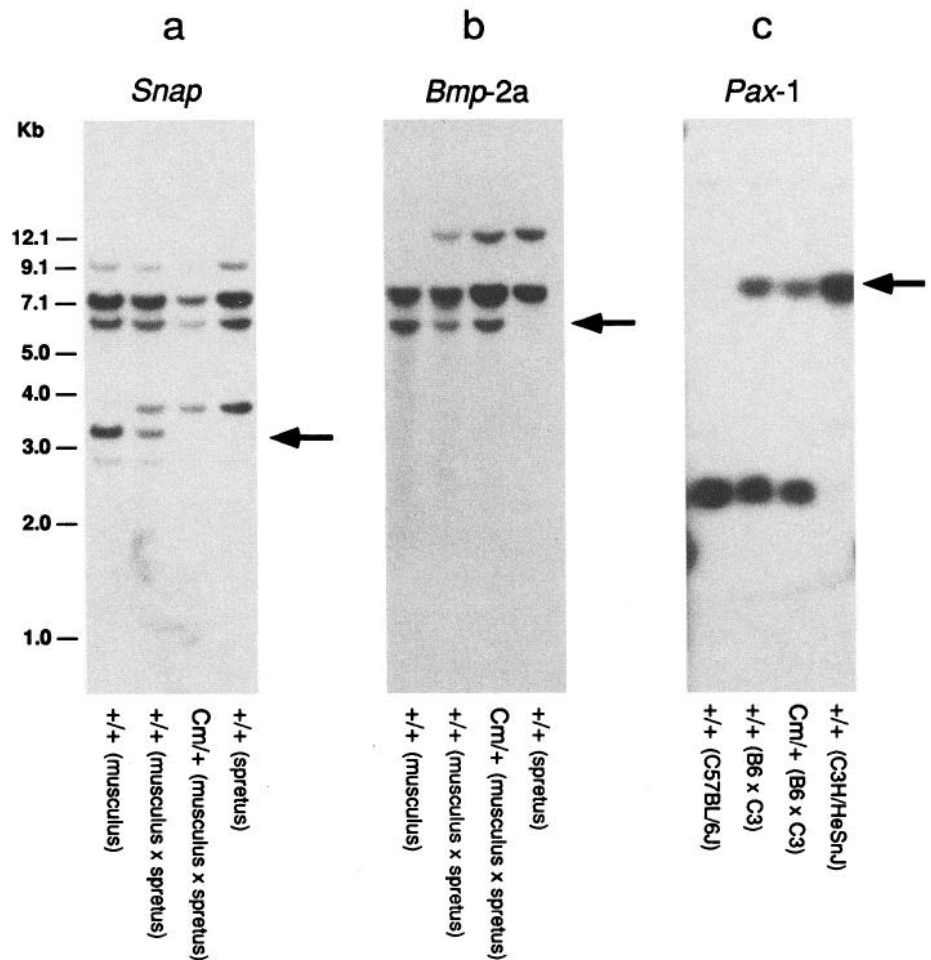


Figure 4. Southern analysis of inter-species or interstrain crosses of coloboma mice. Southern analysis of the two mouse species tested distinguished a 3.2 kb *M. m. musculus*-specific *Snap* RFLP that was not detected in *M. spretus* (a, arrow). This *Snap* *M. m. musculus*-specific band was not present in the DNA from the *Cm/+* interspecific mouse, indicating that the *M. m. musculus* *Snap* allele is deleted in the coloboma interspecific mouse. The integrity of genes mapping near *Snap* in the coloboma mouse mutant was assessed by Southern analyses of interspecific (+/+ *M. spretus* × *Cm/+* *M. m. musculus*) or interstrain crosses. The interspecies cross revealed that *Bmp-2a* is not deleted in the coloboma mutation (b) as the *M. musculus*-specific RFLP (indicated by an arrow) is present in the *Cm/+* *M. musculus* × *M. spretus* mouse. The interstrain cross (*Cm/+* C3H/HeSnJ × +/+ C57BL/6J) demonstrated that *Pax-1* is also intact in coloboma mice (c), as the C3H/HeSnJ RFLP (arrow) is present in the *Cm/+* hybrid mouse.

mRNA expression resulted in lower SNAP-25 protein expression. To quantitate the difference in SNAP-25 protein expression between *Cm/+* mice and +/+ littermates, increasing masses of brain proteins from control and mutant mice were blotted onto nitrocellulose, reacted with antibody directed against the SNAP-25 carboxy terminus (Oyler et al., 1989), and visualized with iodinated protein A. Comparison of the autoradiographs as arbitrary film densitometric units against protein mass revealed that SNAP-25 protein in brain extracts of the *Cm/+* mouse mutants was half that of the +/+ mice, suggesting that coloboma mice express half the normal levels of SNAP-25 protein (Fig. 7). Thus, the half *Snap* gene dose in coloboma mice results in 50% SNAP-25 protein expression.

Coloboma mouse behavioral phenotype. The *Cm* mutation is semidominant wherein the heterozygous state (*Cm/+*) results in a mutant phenotype, while homozygous (*Cm/Cm*) mice are not viable and die during embryogenesis. It has been suggested that this lethality occurs prior to embryonic day 6 (Theiler and Varnum, 1981). Heterozygous coloboma mouse mutants were readily identified at postnatal day 14 by their small eyes, which result from the failure of the cornea and lens epithelium to separate during development (Theiler and Varnum, 1981). These mice also displayed head bobbing as described previously by Searle (1966). Additionally, we noted that these mice were extremely active.

To quantitate better the hyperactivity exhibited by coloboma mice, the spontaneous locomotor activity of +/+ and *Cm/+*

littermates on a C3H/HeSnJ background was recorded in automated open field activity cages over 24 hr starting at 10:00 A.M. All mice used in the behavioral tests were obtained from The Jackson Laboratory where they were identified as *Cm/+* or +/+ mice using eye morphology as the phenotypic criterion for genotype determination. Both mutant and control littermates habituated to the cages within the first 150 min of the testing session (Fig. 8). Coloboma mice appeared to have a normal circadian rhythm that paralleled control littermate activity, with increased activity at night and occasional bursts of daytime activity associated with eating. *Cm/+* mice were significantly more active than their +/+ littermates (two-factor ANOVA, $p < 0.005$), with activity counts at night more than three times control activity. In fact, the nocturnal locomotor activity of some mutants exceeded 10 times that of their control littermates. Although coloboma mice exhibited enhanced locomotor activity, no difference was observed between controls and mutants in time spent quiescent (Student's *t* test, $p > 0.05$), as assessed by number of 15 min intervals per mouse during which no photocell beams were broken. Thus, these mutants are extraordinarily active while maintaining normal sleep/wake patterns.

Discussion

The genetic analysis presented here mapped *Snap* to the same region of Chr 2 as the *Cm* mutation in coloboma mice. *Cm* was previously mapped to Chr 2 using agouti (*a*) and wellhaairig (*we*)

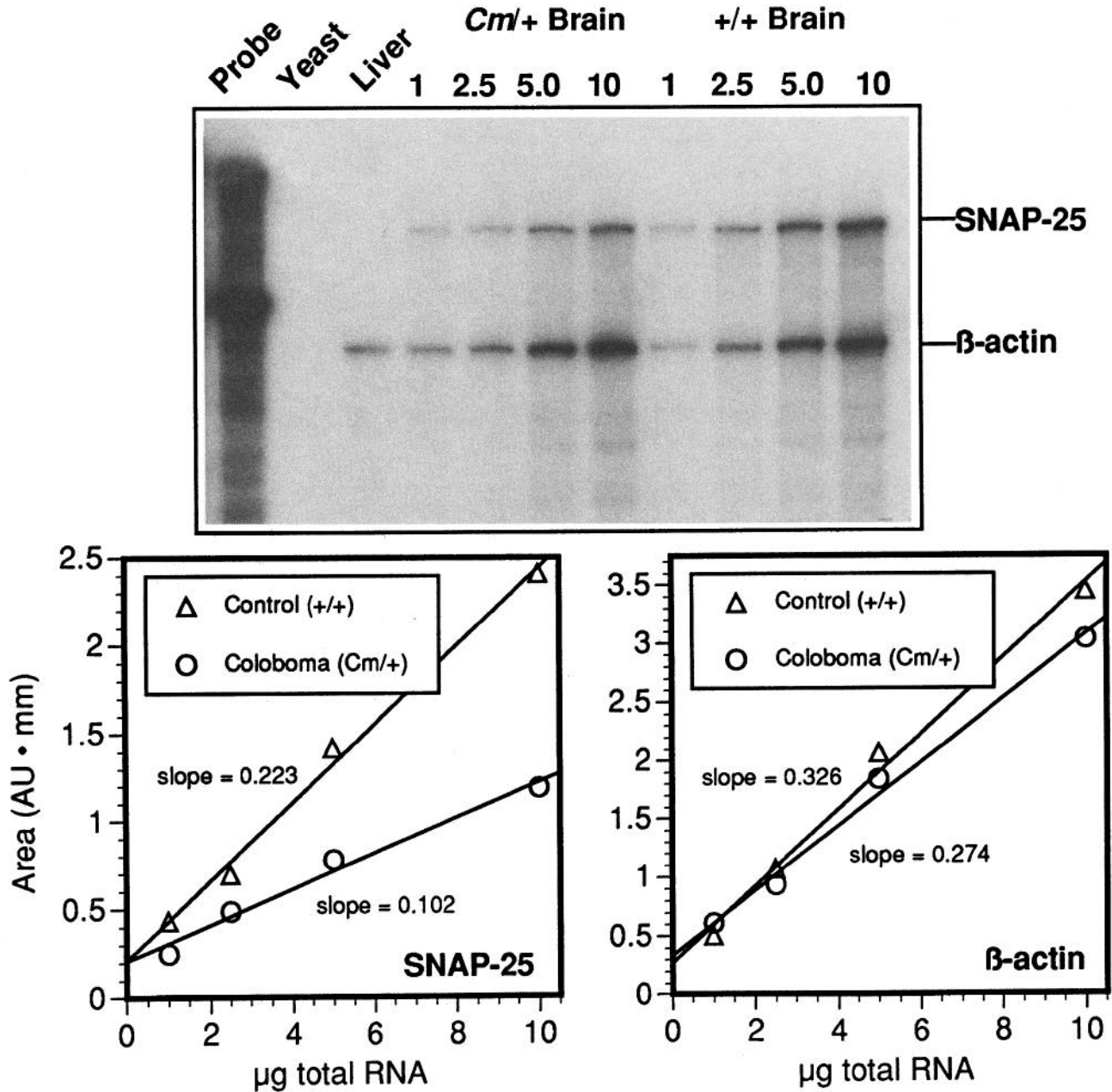


Figure 5. Ribonuclease protection assay of SNAP-25 mRNA expression. Increasing masses of total RNA extracted from the brains of a control (+/+) mouse and coloboma (*Cm/+*) littermate were assayed with 32 P-labeled cRNA probes to SNAP-25 or β -actin. SNAP-25 RNA expression in *Cm/+* brain is approximately 50% lower than in +/+ brain, while β -actin expression is comparable between control and mutant. Yeast and liver RNAs were used as negative controls.

as markers (Snell and Bunker, 1967; H. Bunker in The Jackson Laboratory GBASE data base). Recombination frequencies between *Cm*, *a*, and *we* placed the *Cm* locus between these two loci (Fig. 1*a*). In this study, we have mapped the *Snap* gene to this same region approximately equidistant between *Hdc* and *Emv-15* and demonstrated that *Snap* is deleted in the *Cm* mutation.

The previous linkage studies coupled with the molecular genetic data presented herein confirm that the morphologic and neurologic abnormalities exhibited by coloboma mice are attributable to the mutant *Cm* deletion, which includes the *Snap* gene. The complex phenotype exhibited by coloboma mice in-

cludes head bobbing, ophthalmic malformation, and hyperactivity; these traits have been found to cosegregate consistently in a Mendelian fashion with the *Cm* mutation. In breeding this mutant strain through +/+ \times *Cm/+* matings, we have observed approximately 50% of the offspring exhibiting head bobbing, ophthalmic deformation, and hyperactivity (E. J. Hess and M. C. Wilson, unpublished observations), consistent with the 1:1 segregation ratio expected for a single genetic locus. Similarly, progeny of interspecies (*M. m. musculus* \times *M. spretus*) crosses demonstrated co-inheritance of the *Snap* deletion with ophthalmic dysmorphology. Additionally, in open field tests of motor activity, *Cm/+* mutants identified solely on the basis of op-

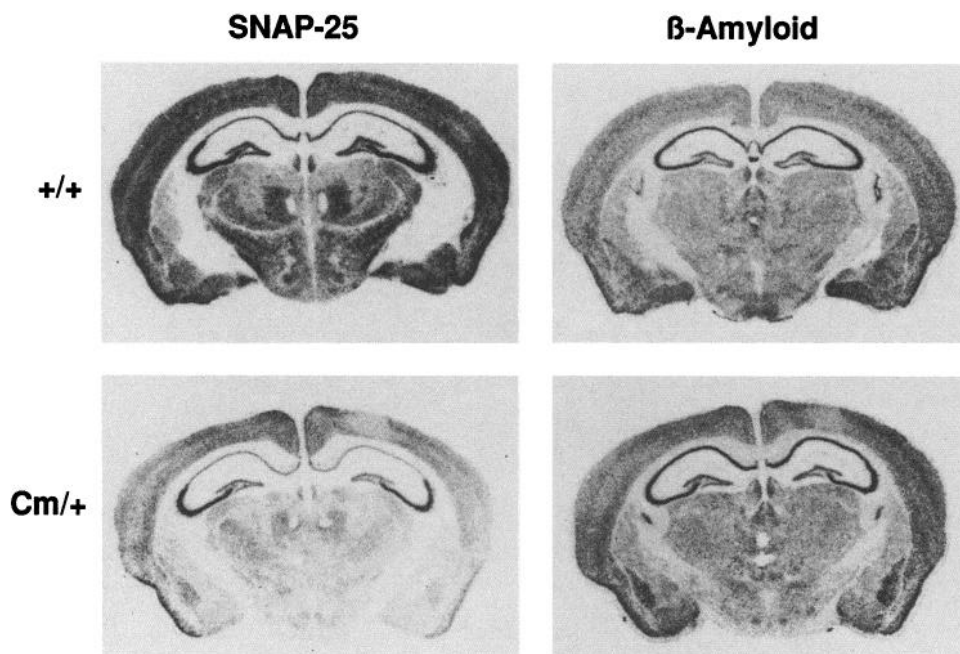


Figure 6. Localization of SNAP-25 and β -amyloid precursor protein mRNA in control (+/+) and coloboma (*Cm*/+) mouse brains. Coronal sections (20 μ m) from mutant and normal littermates were processed for *in situ* hybridization and apposed to film for 60 hr. Experiments were performed on 8–10-week-old male mouse mutants ($n = 2$); wild-type C3H/HeSnJ age- and sex-matched littermates were used as controls.

thalamic deformation were consistently hyperactive in comparison to their normal littermates. These observations suggest that the phenotypic and genotypic abnormalities in coloboma mice result from a mutation that segregates as a single genetic locus.

Consistent with *Cm*/+ mice being hemizygous for *Snap*, 50% of the normal *Snap* gene dose was detected in coloboma mice by genomic DNA slot blot analysis. Although the *Snap* gene dosage in coloboma mice was half that of controls, *Snap* RFLPs were not observed between coloboma and control mouse DNA, suggesting that the entire *Snap* gene is deleted in the coloboma mutation. Preliminary studies have suggested that the *Snap* gene consists of as many as eight or more exons dispersed over greater than 50 kb of genomic DNA (Bark et al., 1990). Although the entire *Snap* gene is deleted in the coloboma mutation, the coloboma karyotype assessed by G-banded chromosomes is normal (Davisson and Lewis, 1990), suggesting that the coloboma mutation is not a gross chromosomal abnormality.

At present, the extent of the coloboma deletion is unknown; in fact, it could encompass one or more genes in addition to *Snap*. Analysis of RFLP segregation in the mouse backcrosses localized the *Snap* gene to a region of mouse Chr 2, where several other genes have been mapped. Genes mapping in the same region as *Snap* include *Il-1a* (interleukin 1A), *Prn-p* (prion protein), *Bmp-2a* (bone morphogenetic protein-2a), and *Pax-1* (paired box gene-1), suggesting that the human *Snap* gene resides in the syntenic region of human Chr 20p (Copeland and Jenkins, 1991). Although further studies aimed at providing a physical map of this locus will be required to define precisely the extent of the *Cm* deletion, *Pax-1* and *Bmp-2a*, cloned markers mapping in the same region as *Snap*, are not deleted. These data together with the observation that the deletion is not detectable microscopically suggest that this deletion likely includes 5 cM or less of the mouse genome.

The embryonic lethality observed on the homozygous *Cm*/*Cm* mutant may result from the deletion of both *Snap* genes.

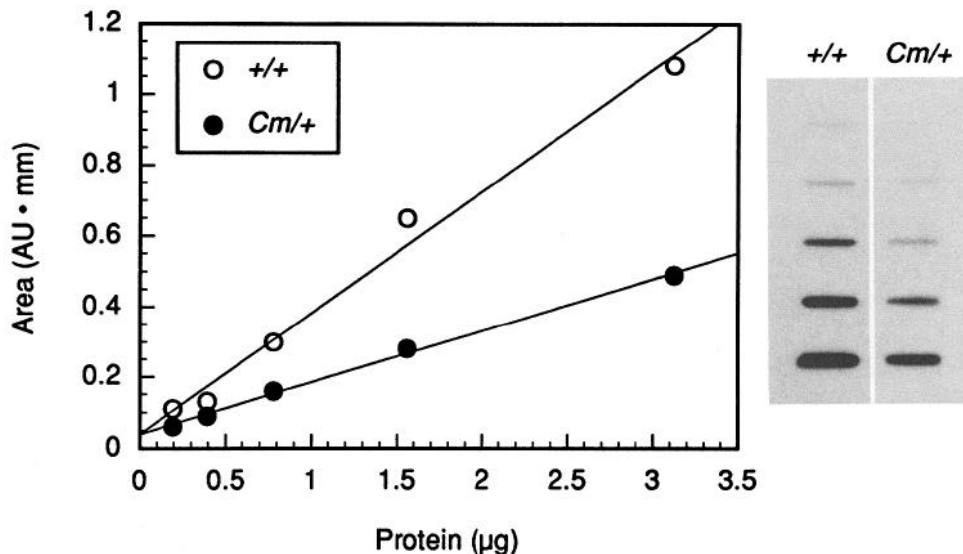


Figure 7. SNAP-25 protein expression in control (+/+) and coloboma (*Cm*/+) mice. Increasing masses of brain protein prepared from mutant and phenotypically normal littermates were blotted onto nitrocellulose, reacted with anti-SNAP-25 antibody, visualized using 125 I protein A, and apposed to x-ray film. Autoradiographs were quantitated using a laser densitometer, and the arbitrary absorbance units were plotted against the mass of brain protein loaded in the slot ($n = 2$). SNAP-25 protein expression in the coloboma (*Cm*/+) brain was approximately 50% lower (slope = 0.147) than in the normal (+/+) brain (slope = 0.343).

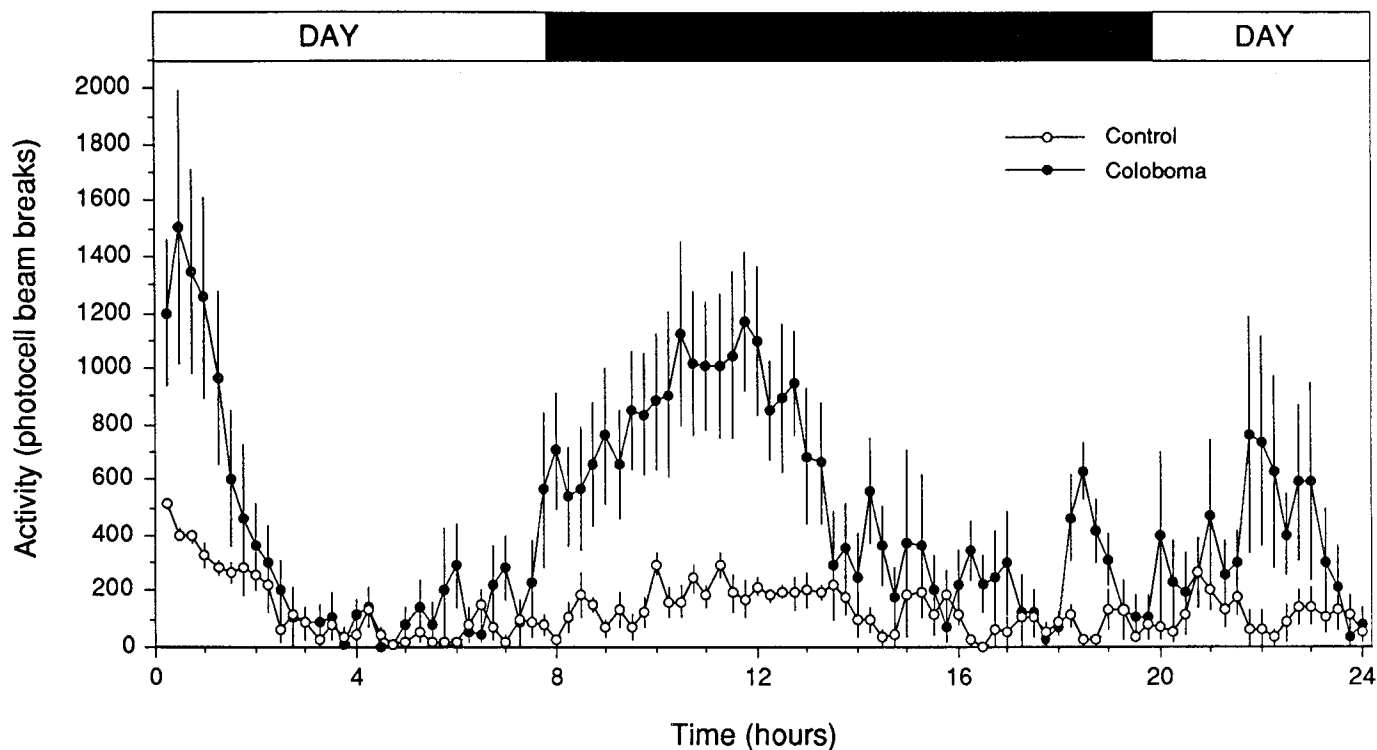


Figure 8. Spontaneous locomotor activity in $+/+$ ($n = 5$) and $Cm/+$ ($n = 5$) littermates over 24 hr. Recordings were performed in automated open field activity chambers starting at 10:00 A.M. with photocell beam breaks accumulated every 15 min. The spontaneous activity of coloboma mice was significantly greater than control littermates ($p < 0.005$, two-way repeated-measures ANOVA). However, time spent quiescent was not significantly different between control and coloboma mice ($p > 0.05$, Student's t test). Data represent mean \pm SEM.

SNAP-25 protein expression has been detected in developing rat brain as early as embryonic day 15 (Oyler et al., 1991) and may be expressed earlier in embryogenesis. In fact, SNAP-25 expression is coincident with synaptogenesis (Catsicas et al., 1991) and neurite differentiation (Sanna et al., 1991), and is a major component of presynaptic terminals. Given the close association between SNAP-25 expression and synaptogenesis, the homozygous *Snap* deletion mutation may impair the development of the nervous system and contribute to the death of the developing embryo.

The half *Snap* gene dose observed in $Cm/+$ mice was reflected in a 50% reduction in both SNAP-25 mRNA and protein expression. These data suggest that transcription and subsequent translation of the remaining *Snap* gene in coloboma mice do not compensate for the loss of the *Snap* gene on the mutant chromosome. The reduction in SNAP-25 protein expression in coloboma mice may ultimately be reflected in the $Cm/+$ phenotype. As the eye is derived from neural crest cells and the retina expresses SNAP-25 (Catsicas et al., 1991), it is possible that the abnormal ocular phenotype results from a SNAP-25 deficiency. Additionally, the neuron-specific expression of SNAP-25 may play a role in the coloboma mouse behavioral abnormalities such as head bobbing and extreme hyperactivity. The presynaptic localization of SNAP-25 in nerve terminals implicates SNAP-25 in neural transmission. Aberrant SNAP-25 protein concentrations within the nerve terminal may disrupt intercellular signaling to the extent that the corrupt synaptic function is ultimately manifested as hyperactivity. Alternatively, half gene doses of other genes possibly encompassed by the deletion may also contribute to the morphological and behavioral abnormalities observed in coloboma mice.

The profound spontaneous hyperactivity exhibited by coloboma mice exceeded three times the activity of the control littermates, with the most active mutants approaching 10 times control mouse activity. Comparable levels of hyperactivity have also been observed in mice after ingesting lead for 40 d (Silbergeld and Goldberg, 1974). In other animal models of hyperactivity, the enhanced locomotor activity is not often so obvious. Activity in weaver mice, a neurological mouse mutant with severe dopaminergic deficits and cerebellar atrophy, is approximately 1.5 times greater than control littermates (Schmidt et al., 1982) and is confounded by cerebellar-induced ataxia. Similarly, experimental models of hyperactivity in rat such as neonatal 6-hydroxydopamine treatment and the SHR exhibit locomotor activity 1.5–2 times greater than controls (Shaywitz et al., 1976; Knardahl and Sagvolden, 1979; Wultz et al., 1990). In contrast to these animal models of hyperactivity, the coloboma deletion mutation induces extreme spontaneous hyperactivity without chemical intervention or gross neuropathology.

Several characteristics of coloboma mice parallel the hyperactivity syndrome ADHD, a common pediatric disorder characterized by inattention, impulsivity, and hyperactivity. Like children with ADHD (Greenhill et al., 1983), coloboma mice are hyperactive during their active (nocturnal) phase and otherwise exhibit normal patterns of sleep. Additionally, although the etiology of ADHD is unknown, there is evidence that suggests that ADHD may have a genetic basis (Deutsch et al., 1982; Biederman et al., 1986). As psychostimulants, such as methylphenidate, are effective in ameliorating the disorders of activity in many affected children, an animal model of ADHD should appropriately exhibit similar responses to these drugs. Further investigations characterizing the behavioral response of colo-

boma mice to psychostimulants are in progress to determine whether this mouse mutant is an accurate model of ADHD.

The *Snap* gene is clearly deleted in coloboma mice, and these data suggest that the coloboma mutant may represent a heritable model of hyperactivity. It is unclear if other genes are included in this deletion and how they influence the mutant phenotype. Indeed, if several genes are involved in the coloboma mouse phenotype, it may be possible to dissect the mutant phenotypes according to gene. For example, one gene may contribute to the ocular deformation whereas another may play a role in the hyperactivity exhibited by these mice. Thus, in the future, it may be possible to engineer a mouse that exhibits only the hyperactivity without head bobbing or ocular deformation.

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