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A novel null allele of mouse *Dscam* survives to adulthood on an inbred C3H background with reduced phenotypic variability

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Abstract

DSCAMs are cell adhesion molecules that play several important roles in neurodevelopment. Mouse alleles of *Dscam* identified to date do not survive on an inbred C57BL/6 background, complicating analysis of DSCAM-dependent developmental processes because of phenotypic variability related to the segregating backgrounds needed for postnatal survival. A novel spontaneous allele of *Dscam*, hereafter referred to as *Dscam*^{2J}, has been identified. This allele contains a four base pair duplication in exon 19, leading to a frameshift and truncation of the open reading frame. Mice homozygous for the *Dscam*^{2J} mutant allele survive into adulthood on the C3H/HeJ background on which the mutation was identified. Using the *Dscam*^{2J} allele, retinal phenotypes that have variable severity on a segregating background were examined. A neurite lamination defect similar to that described in chick was discovered in mice. These results indicate that in the retina, additional DSCAM-dependent processes can be found by analysis of mutations on different genetic backgrounds.

Keywords

mosaic; adhesion; arborization; development; lamination; Down syndrome

Introduction

Development of the nervous system requires cues by which neurons integrate into functional circuits. The identification of such cues and their mechanism of activity is a long-standing goal of neurogenetics. The Down Syndrome Cell Adhesion Molecule (DSCAM) gene family encodes proteins that facilitate development of neural circuits. DSCAMs mediate at least four aspects of neural development. In the chick retina DSCAMs provide adhesive cues by which different types of neurons recognize their synaptic partners and are required for synaptic lamination in the retina (Yamagata and Sanes, 2008). *Drosophila* and mouse DSCAMs mediate self-avoidance, preventing the adhesion of homotypic cells and neurites (Fuerst et al., 2009; Fuerst et al., 2008; Hughes et al., 2007; Neves et al., 2004; Wang et al., 2002). In addition, *Aplysia* DSCAM facilitates clustering of neurotransmitter receptors and *Drosophila*, zebrafish and mouse DSCAMs also act as receptors for axon guidance molecules (Li et al., 2009; Liu et al., 2009; Yimlamai et al., 2005).

Many of these functions are described in different species and it is unclear if these DSCAM functions are conserved across taxa. Determining the degree to which DSCAM functions are

conserved across taxa is complicated by the postnatal lethality of mouse *Dscam* mutations on the commonly used C57BL/6 inbred background. Both of the previously studied null alleles of *Dscam*, the spontaneously arising *Dscam^{del17}* and the targeted *Dscam^{tm1.1Kzy}* suffer from this limitation. The perinatal lethality of the homozygous *Dscam^{tm1.1Kzy}* mice was explained by strain specific defects in the brainstem respiratory rhythmicity center (Amano et al., 2009). Both alleles survive on outbred genetic backgrounds, but studies on the *Dscam^{del17}* mice demonstrate phenotypic variability that is likely the result of genetic variability, complicating the characterization of phenotypes.

Here we report an additional null allele of *Dscam*, *Dscam^{2J}*, which was identified at The Jackson Laboratory. Mice with this mutant allele survive into adulthood on the inbred genetic background on which the mutation arose and allow the reproducible analysis of the *Dscam*-dependency of neural patterning. The *Dscam^{2J}* mutation reproduces many phenotypes previously observed in the *Dscam^{del17}* retina. In addition to these phenotypes, a defect in retinal neurite lamination similar to what has been reported in chick was observed, confirming that the DSCAM function during neurite lamination that has been reported in the chick retina is conserved in mammals. We also report analysis of hearing and vestibular function, as well as systematic histological analysis of the *Dscam^{2J}* mice conducted during their history at The Jackson Laboratory.

Results

A novel allele of *Dscam*

An autosomal recessive mutation was found in the C3H/HeDiSnJ inbred strain at The Jackson Laboratory. The mutant allele was given the allele designation “scaredy cat” because the mutant mice have an arched back and appear to walk on their toes (Figure 1 A). Mutant mice are identifiable as early as postnatal day two (P2) because they rest on their backs and struggle to maintain balance, unlike their wild type litter mates, which rest on their stomachs. The overt kyphosis and balance phenotypes are recessive and mutant mice were born at the expected Mendelian ratio (83 mutants out of 326 born or 25.5%). All adult mutant mice display a pronounced kyphosis (misshapen curvature of the spine) in the thoracic region and have a domed head. The homozygous mutants fail a swim test as they try to hold their noses to the surface but never swim in a straight line and will sink to the bottom after curling their bodies up (data not shown). In contrast, their control littermates swim in a straight line to the opposite end of the swim box. Such failures in a swim test often indicate vestibular defects and an inability to sense and maintain equilibrium. However, the scaredy cat mutant mice have a normal auditory brain stem response (ABR), indicating that cochlear hair cell function and connectivity is normal (Table 1).

In 2006 a mutation that closely resembled the scaredy cat mutation was identified at The Jackson Laboratory and mapped to an interval on Chromosome 16 that includes the *Dscam* gene. The mutation was identified as a 38 base pair deletion in *Dscam* exon 17, approximately in the middle of the open reading frame, and the allele was designated *Dscam^{del17}* to reflect this. This mutation causes a frame shift and premature stop codon, and no protein product can be detected using an N-terminal antibody, making it a likely null allele (Fuerst et al., 2008). A complementation test was performed by breeding unaffected, known heterozygotes for *scaredy cat* and *Dscam^{del17}*. Four pups with an overt phenotype similar to that of both the *Dscam^{del17}* and scaredy cat mutation were identified out of a total of 31 pups, indicating that the mutations were unable to complement each other and were therefore allelic. The scaredy cat *Dscam* gene was sequenced in the scaredy cat mice and found to contain a four base pair duplication in exon 19, and mice with a mutant phenotype from the previously performed complementation test were found to be heterozygous for both the *Dscam^{del17}* and scaredy cat mutations (Figure 1 B). The scaredy cat allele is hereafter

referred to as *Dscam*^{2J}, because it is the second mutation in *Dscam* identified at The Jackson Laboratory.

Both previously reported mutations in *Dscam*, *Dscam*^{del17} and *Dscam*^{tm1.1Kzy}, die shortly after birth on a C57Bl/6 background but can survive when outcrossed to Balb or 129 strains (Amano et al., 2009; Fuerst et al., 2008). The number of outcrossed *Dscam*^{del17} mutant mice observed after postnatal day 2 (based on kyphosis and vestibular phenotypes) from heterozygote intercrosses averaged 10.5%, of which 5.7% survived to weaning age. A subset of successfully weaned mutant mice showed a mild overt phenotype and when male homozygote mice were bred to female heterozygotes, a higher proportion of mutant pups survived until weaning age, which could be the result of fewer unaffected wild type and heterozygote pups competing for maternal resources, accumulation of beneficial modifier loci, or both. One out of 457 genotyped mice was homozygous for the *Dscam*^{del17} mutation but lacked an overt kyphosis or vestibular phenotype, indicating that in rare cases homozygous *Dscam*^{del17} mice cannot be identified based on overt phenotypes in a mixed genetic background. Several homozygote *Dscam*^{del17} mutant pairs successfully produced and weaned pups, although most homozygous female mice fail to successfully deliver and clean pups, which are often found dead within an intact amniotic sac. No overt differences were observed when comparing wild type and heterozygous *Dscam*^{del17} or *Dscam*^{2J} mice, although dosage dependent phenotypes have been observed by histological and physiological analysis (Amano et al., 2009; Fuerst et al., 2008).

The *Dscam*^{2J} allele shares some retinal phenotypes with the *Dscam*^{del17} allele

During its history at The Jackson Laboratory, extensive histological analysis of the *Dscam*^{2J} allele was performed. No gross pathologies were observed in the somatic organs (liver, spleen, pancreas, stomach, small intestine, colon, cecum, lungs, thymus and heart) in the course of full necropsies performed by a veterinary pathologist. Young mutant mice had no spinal column lesions and had normal muscles. At older ages up to 1 year, mutant mice had degeneration of the spinal joints, dystrophic axons in the lumbar spinal cord and small areas of degenerating epaxial skeletal muscle (data not shown). Retinal histology was not initially performed because C3H mouse strains carry the recessive *Pde6b* retinal degeneration mutation *rd1*; however, after identifying a mutation in the *Dscam* gene, which is required for normal retinal development, histological examination of the retina was performed (Keeler, 1966; Pittler et al., 1993). The *Dscam*^{2J} allele has a reduced complement of photoreceptors, as expected for a strain carrying the *Pde6b* allele *rd1*; however, the inner nuclear and retinal ganglion layers were disorganized and strongly resembled the *Dscam*^{del17} retina (Figure 2) (Fuerst et al., 2009; Fuerst et al., 2008).

Dopaminergic amacrine cells fail to form mosaics and have fasciculated neurites in the *Dscam*^{del17} retina (Fuerst et al., 2008). Similar fasciculation of DA cell neurites and clumping of soma was observed in the *Dscam*^{2J} retina (Figure 3). DSCAM immunoreactivity was detected in wild type and *Dscaml1*^{-/-} DA cells (DSCAML1 is a closely related protein, but it is not expressed in DA cells) but absent from *Dscam*^{del17} and *Dscam*^{2J} DA cells (Figure 3).

The *Dscam*^{2J} allele reveals DSCAM requirement for synaptic lamination in mouse retina

The retinal degeneration allele *rd1* was bred out of the *Dscam*^{2J} background using a C3H/HeSnJ strain that carries a wild type allele of *Pde6b* introduced from C57Bl/6J (Costa et al., 2010; Sakamoto et al., 2009). In the chick retina, adhesion molecules including DSCAMs are instructive for where in the synaptic inner plexiform layer (IPL) the dendrites of retinal ganglion cells and their synaptic partners in the inner nuclear layer arborize and stratify. Variable defects in neurite lamination had been observed in the *Dscam*^{del17} mice. These

defects were re-examined in the *Dscam*^{2J} allele in the anticipation that the inbred C3H background without the *rd1* mutation would give more consistent results.

Retinal sections from wild type mice, *Dscam*^{2J} heterozygotes, *Dscam*^{2J} and *Dscam*^{del17} homozygous mutants were stained with antibodies to label horizontal cells, bipolar cells, subpopulations of amacrine cells, and retinal ganglion cells. No differences were observed in the organization of bipolar or horizontal cells, in which expression of *Dscam* has not been reported. However, lamination of the processes of cholinergic, calbindin-positive, and bNOS-positive amacrine cells was abnormal in the homozygous *Dscam*^{2J} retina. The lamination of calbindin-positive amacrine cells was also different when comparing the *Dscam*^{del17} and *Dscam*^{2J} retinas. The central calbindin-positive amacrine cell band in the retinal inner plexiform layer maintains a tight lamination in the *Dscam*^{del17} retina, despite an increase in cell number, while this cell population loses organized lamination in the *Dscam*^{2J} retina such that three distinct calbindin positive bands are no longer observed. Dopaminergic amacrine cells maintained the stratification of their processes immediately below the inner nuclear layer even in the *Dscam*^{2J} retina (Figure 4).

Discussion

In this study a newly identified allele of the mouse *Dscam* gene, *Dscam*^{2J}, is described. The mutation is a four base pair duplication in exon eighteen, which introduces a frameshift and stop codon. Vertebrate *Dscams* are not alternatively spliced like *Drosophila Dscam* and no protein product was observed in DA cells, suggesting that this is a null allele. Mice with previously studied alleles of *Dscam* die perinatally when moved onto an inbred C57BL/6 background; however the *Dscam*^{2J} mutation is viable on the inbred C3H background, the strain on which it arose. The *Dscam*^{del17} and *Dscam*^{2J} mutations are in similar regions of the gene, both introduce frameshifts followed by a stop codon, and neither produces a detectable protein product. These similarities indicate that the difference in viability is related to the inbred C3H background of the 2J allele, suggesting that this background may contain modifier loci that promote survival of *Dscam* deficient mice. Similar increases in survival were seen with the introduction of BALB/c alleles, possibly indicating that C57BL/6 is a strain that is sensitive to the loss of *Dscam*. Other neurodevelopmental mutations, such as *Unc5c*, show a similar sensitivity and perinatal lethality in C57BL/6 backgrounds, and overt neurological, behavioral and other phenotypes in mice are often sensitive to background genetic effects (Burgess et al., 2006; Doetschman, 2009; Tarantino et al., 2000). Retinal development is also prone to strain dependent background effects (Haider et al., 2002). For example, ocular malformations in C57BL/6 are observed in between four and ten percent of mice, and a C57BL/6 background is required for ocular defects resulting from deletion of p53 or overexpression of agrin (Fuerst et al., 2007; Hettmann et al., 2000; Smith et al., 1994). Further investigation of strain differences that promote survival of *Dscam* deficient mice should be helpful in identifying these modifier loci and determining why *Dscam* mutants are viable on some inbred backgrounds and not others. Understanding these modifier loci may also help shed light on the clinical variability of neurodevelopmental disorders such as autism.

The availability of a viable mutant *Dscam* allele on an inbred background will facilitate discovery of how this gene mediates neural development. Many of the mutant phenotypes observed in *Dscam* deficient mice are variable when comparing mice on an outbred background. For example, the severity of soma lamination defects observed in the retina *Dscam*^{del17} mice on an outbred background can be quite variable (data not shown). Generation of a conditional *Dscam* allele would also be a useful way to circumvent postnatal lethality of *Dscam* null alleles on a C57BL/6 background; however, the benefits of conditional alleles are best utilized if null mutations exist to complement some of the

limitations of conditional alleles, such as incomplete gene deletion and limited transgenic Cre lines.

In addition to identifying a new genetic resource, this *Dscam* allele was used to identify a novel *Dscam*-dependent retinal phenotype in mouse. Lamination of retinal neurites was examined in *Dscam*^{2J} mutant mice and some populations of retinal neurons that require *Dscam* for spacing were found to have normal lamination, while others were found to have disorganized lamination, as predicted by studies in *Dscam*-depleted chick retina. One population of neurons, calbindin-positive amacrine cells, maintains a tight lamination within the *Dscam*^{del17} background (N>5), but has a very disorganized and loose lamination phenotype in the *Dscam*^{2J} C3H background (N>5), indicating that neurite lamination is sensitive to background effects and may be a good candidate phenotype to follow in attempting to link *Dscam*-deficiency related phenotypes to modifier loci.

DSCAMs mediate multiple neurological events, including homotypic self-avoidance, adhesion, and axon guidance. The availability of a mutation on a genetic background that promotes the survival of *Dscam* mutant mice will facilitate characterization of additional neurodevelopmental phenotypes resulting from *Dscam*-deficiency.

Materials and Methods

Animal care and handling

Mice were used in accordance with protocols approved by the Animal Care and Use Committee at Washington State University and The Jackson Laboratory. Animals were housed in PIV caging and given food and water ad libitum.

Immunohistochemistry

Enucleated eyes were fixed in 4% paraformaldehyde overnight. Tissue was sunk in 30% sucrose/PBS and frozen in OCT media. 8 μ m sections were cut using a cryostat and used immediately or stored up to two weeks at -20°C . Sections were blocked in 3% normal horse serum in 1 $\times$ PBS and 0.1% triton. Primary antibodies were incubated overnight at 4°C and subsequently washed 2 times for ten minutes in PBS. Sections were incubated at room temperature with secondary antibodies for one hour, and then washed four times in PBS for ten minutes. The last wash contained 2 μ l of 1 mg/ml Dapi per 40 ml PBS. Sections were coverslipped with 80% glycerol and stored at 4°C until imaging.

Whole retina staining

Mice were pericardially perfused with PBS and 4% paraformaldehyde. Eyes were enucleated and placed in a dish containing PBS. A small hole was made at the junction between the ciliary body and retina with a 30 gauge needle. The eye was hemisected and the retina was gently teased away from the sclera. Retinas were fixed a second time in 4% paraformaldehyde overnight, followed by a rinse in PBS. Retinas were incubated with primary antibodies in PBS, supplemented with 3% normal horse serum and 0.5% triton-x 100, for 3–5 days at 4°C , with gently rocking. Primary antibodies were washed off overnight in PBS and the retinas were incubated with secondary antibodies in PBS, 3% normal horse serum and 0.5% triton for twenty four hours. Retinas were washed for 2–4 hours in PBS and flat mounted in 80% glycerol. All fluorescent images were acquired on a Leica SP5 confocal microscope.

Antibodies

The following primary antibodies were used in this study: rabbit anti-tyrosine hydroxylase (Millipore, AB152 at 1:500), goat anti-ChAT (Millipore, AB144P at 1:500), goat anti-

CHX10 (Santa Cruz Biotechnology, 21692 at 1:500), rabbit anti-PKC α (Sigma-Aldrich at 1:1,000), goat anti-BRN3b (Santa Cruz Biotechnology, sc-6026 at 1:400), rabbit anti-calbindin (Swant, D-28K, 1:1,000) and rabbit anti-bNOS (Sigma-Aldrich at 1:10,000). The following secondary antibodies were used in this study donkey anti-rabbit-dylight405 and donkey anti-goat-cy3, donkey anti-rabbit-dylight488 (Jackson Immunological Research, at 1:500).

Strains and Genotyping

Dscam^{2J} mice were genotyped using the primers *Dscam*^{2J}F (GCG AGA TTA AGA ACG AAC) and *Dscam*^{2J}R (TCC TCC TTG GTA CGG GTA). The following PCR cycle parameters were used: 94°C 2' 35 cycles of 94°C 30", 58°C 30" 72°C 50", followed by a final incubation at 72°C for 4 minutes. DNA prepared from mice carrying the *Dscam*^{2J} mutation will yield a 152 base pair product, which is not observed in DNA samples prepared from wild type mice. Production and genotyping of *Dscam*^{del17} and *Dscaml1* null mice has been previously described (Fuerst et al., 2009; Fuerst et al., 2008).

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Figure 1. Scaredy cat mutant *Dscam* contains a four base pair duplication in exon 19

A, The Scaredy cat mouse has a dome shaped head and a marked curvature of the spine. **B**, Genomic *Dscam* DNA was amplified and sequenced. A four base pair duplication results in a frame shift, 84 novel amino acids and a premature stop codon, truncating DSCAM approximately half way through the extracellular domain.

Figure 2. Anatomy of wild type, *Dscam*^{del17} and *Dscam*^{2J} retinas

Sections of wild type, *Dscam*^{del17} and *Dscam*^{2J} retinas were stained with hematoxylin and eosin. **A**, The wild type retina is organized in three laminae, the outer nuclear layer (ONL) of photoreceptors, inner nuclear layer (INL) of horizontal, bipolar, and amacrine cells, and the retinal ganglion layer (RGL). Two synaptic laminae, the outer plexiform layer (OPL) and inner plexiform layer (IPL) separate the nuclear layers. **B**, The *Dscam*^{del17} retina has a disorganized INL, IPL and RGL. **C** and **D**, C3H strains carry a recessive allele of *Pde6b*, *rd1*, which results in rapid degeneration of the photoreceptors in the ONL. **D**, The INL, IPL and RGL of the *Dscam*^{2J} retina are disorganized. The scale bar in (**D**) is equivalent to 106 μ m.

Figure 3. DA cells are disorganized in the *Dscam*^{2J} retina

Whole wild type, *Dscam11*^{-/-}, *Dscam*^{del17} and *Dscam*^{2J} retina were stained with antibodies to tyrosine hydroxylase (TH) to label dopaminergic amacrine (DA) cells (green) and DSCAM (red). **A** and **B**, DA cells are rarely located next to other DA cells in the wild type and *Dscam11*^{-/-} retina, have arborized neurites, and in adults show a distinct puncta of DSCAM immunoreactivity at the base of the neurites (arrows). **C** and **D**, DA cells in the *Dscam*^{del17} and *Dscam*^{2J} retina have fasciculated neurites, are organized in clumps, and lack puncta of DSCAM immunoreactivity. The scale bar in (**D**) is equivalent to 132 μ m.

Figure 4. *Dscam* is required for lamination of some amacrine cell neurites

A–D, Sections of wild type, *Dscam*^{2J/+}, *Dscam*^{2J/2J} and *Dscam*^{del17} retina (N=3) were stained with antibodies to TH (blue), to label dopaminergic amacrine (DA) cells and ChAT (red), to label cholinergic amacrine cells. DA cell neurites laminated proximal to the inner nuclear layer in all backgrounds. Cholinergic amacrine cell neurites, particularly the inner nuclear layer-proximal OFF band, were diffuse in the *Dscam*^{2J/2J} and *Dscam*^{del17} retinas compared to wild type and heterozygous controls. **E–H**, Sections of wild type, *Dscam*^{2J/+}, *Dscam*^{2J/2J} and *Dscam*^{del17} retina were stained with antibodies to PKC-α (green) and CHX10, to label bipolar cells. Bipolar cells laminated in a similar fashion in the mutant retina, and rod bipolar cell axons terminated in the retinal ganglion cell layer proximal portion of the inner plexiform layer in all examined genotypes. An increase in the number of rod bipolar cell axon varicosities was apparent in the *Dscam*^{2J/2J} and *Dscam*^{del17} retina compared to wild type and heterozygous controls. **I–L**, Sections of wild type, *Dscam*^{2J/+}, *Dscam*^{2J/2J} and *Dscam*^{del17} retina were stained with antibodies to BRN3b (red), to label retinal ganglion cells (RGCs) and calbindin (green), to label a subset of amacrine cells and horizontal cells. No difference in the organization of horizontal cells was observed when comparing *Dscam*^{del17}, *Dscam*^{2J/2J}, *Dscam*^{2J/+} and wild type retinas. An increase in the number of RGCs was apparent in the *Dscam*^{2J/2J} and *Dscam*^{del17} retina compared to the *Dscam*^{2J/+} and wild type retina. Calbindin labels three neurite bands in the wild type and *Dscam*^{2J/+} retina. An increase in the number of calbindin positive amacrine cells was observed in the *Dscam*^{2J/2J} and *Dscam*^{del17} retinas compared to controls. A marked disorganization of calbindin-positive neurite bands is apparent in the *Dscam*^{2J/2J} retina compared to *Dscam*^{del17}, heterozygous and wild type controls. **M–P**, Sections of wild type, *Dscam*^{2J/+}, *Dscam*^{2J/2J} and *Dscam*^{del17} retina were stained with antibodies to bNOS (green), to label bNOS-positive amacrine cells and ChAT (red), to label cholinergic amacrine cells. bNOS-positive amacrine cell neurites laminate proximal to the retinal ganglion cell layer, proximal to the inner nuclear layer and between cholinergic bands in the wild type and *Dscam*^{2J/+} retina. bNOS-positive amacrine cell banding in the *Dscam*^{2J/2J} and *Dscam*^{del17} retina is absent proximal to the retinal ganglion cell layer and spread diffusely across the inner plexiform layer between the inner nuclear layer and ON cholinergic band. The scale bar in (**P**) is equivalent to 116.25 μm.

Table 1
Auditory Brainstem Response (ABR) threshold for *Dscam*^{2J/2J} and control mice

No significant difference in ABR response thresholds were observed when comparing control and *Dscam*^{2J/2J} mice. Ages are in weeks, values are decibel levels necessary for a suprathreshold signal in ABR at with stimuli of the frequencies indicated.

Genotype	Age	Click	8kHz	16kHz	32kHz
+/?	22	40	40	20	40
+/?	22	40	40	20	40
+/?	23	40	40	20	40
2J/2J	22	40	40	20	40
2J/2J	23	50	50	20	50