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Short Communication

Redneck, a new mutant of the axolotl (*Ambystoma mexicanum*) likely affects the development of cranial neural crest

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ABSTRACT A novel developmental mutant in the Mexican axolotl is described. Designated *redneck* (*rn*), the mutant gene is inherited as a simple Mendelian recessive. In homozygotes, *rn* causes massive haemorrhage in the posterior head, rostrocaudal compression of the craniovisceral skeleton, abnormal differentiation of vertebral cartilage, micrognathia, aglossia, microphthalmia and abnormal hepatic development. Affected larvae become evident at the onset of feeding, and eventually die of starvation. Based on the tissues affected, we propose that *rn* affects later developmental events in the differentiation and morphogenesis of a subset of cranial neural crest cells. Thus, *rn* may prove a valuable model system for examining the role of neural crest cells in the development of cranial and endodermal derivatives.

KEY WORDS: *developmental mutation, axolotl, neural crest, cranial development, angiogenesis*

Classically, urodele amphibians were considered organisms of choice for experimental embryology, due to their large ova, external development, large clutch size, and slow developmental rate. Although not yet considered "model" organisms for molecular developmental studies due to their large genomes and long generation times, their advantages still make urodeles excellent candidates for developmental studies at the cellular, tissue, and organ systems levels.

Various urodele species have been used for developmental studies, with the organism of choice usually being what was locally available. However, only the Mexican axolotl (*Ambystoma mexicanum*) has transcended local availability. This is due to the historical accident of early importation of axolotls into Europe, and two significant advantages: they are easily bred in captivity year round, overcoming seasonal availability, and most significantly, they have many naturally-occurring developmental mutations (Armstrong and Malacinski, 1989).

Here, we describe a new lethal developmental mutation (*redneck*; *rn*), which affects cranial angiogenesis, the morphogenesis of craniofacial cartilages, and ophthalmic, glossal, and hepatic development. We hypothesize that *rn* may affect development of cranial neural crest, and thus prove an excellent model system for studying the role of neural crest in cranial development.

Redneck (*rn*) was first noted in a spawning (Fig. 1A) between full sibs that were progeny from a cross between a male, D-1, obtained from H.C. Dalton, formerly of Pennsylvania State University, and a female, I-22, from spawning number 3711 at the Indiana University Axolotl Colony. The male died shortly thereafter, so we were unable to repeat the cross. However, the female was crossed with two unrelated males, and various combinations of their progeny were subsequently crossed. Unfortunately, the mutation did not segregate in any of these crosses, but reappeared in the third generation (Fig. 1 B,C,D), and later. The mutation was expressed in 25.9% of embryos (159/614, from five separate spawnings), and thus is inherited as a simple Mendelian recessive with full penetrance.

The first recognisable characteristics of the mutants are a failure to feed and absence of circulation. Swimming movements appeared normal. Closer examination revealed that all had marked red areas at the base of the gills. Based on this characteristic, the mutation was designated *redneck*.

Mutant larvae were anaesthetised with 0.005% benzocaine for observation. Absence of food in the gut, and red spots at the bases of the gills were, the most obvious characteristics. Appearance varied among individuals, however. In some, equal-sized spots were present on either side of the head. In others, only one spot (either left or right) was present. Closer observation revealed that the spots were caused by blood pooling in the operculum. The heart beat vigorously, but had little or no blood passing through it;

Abbreviations used in this paper: rn, redneck gene.

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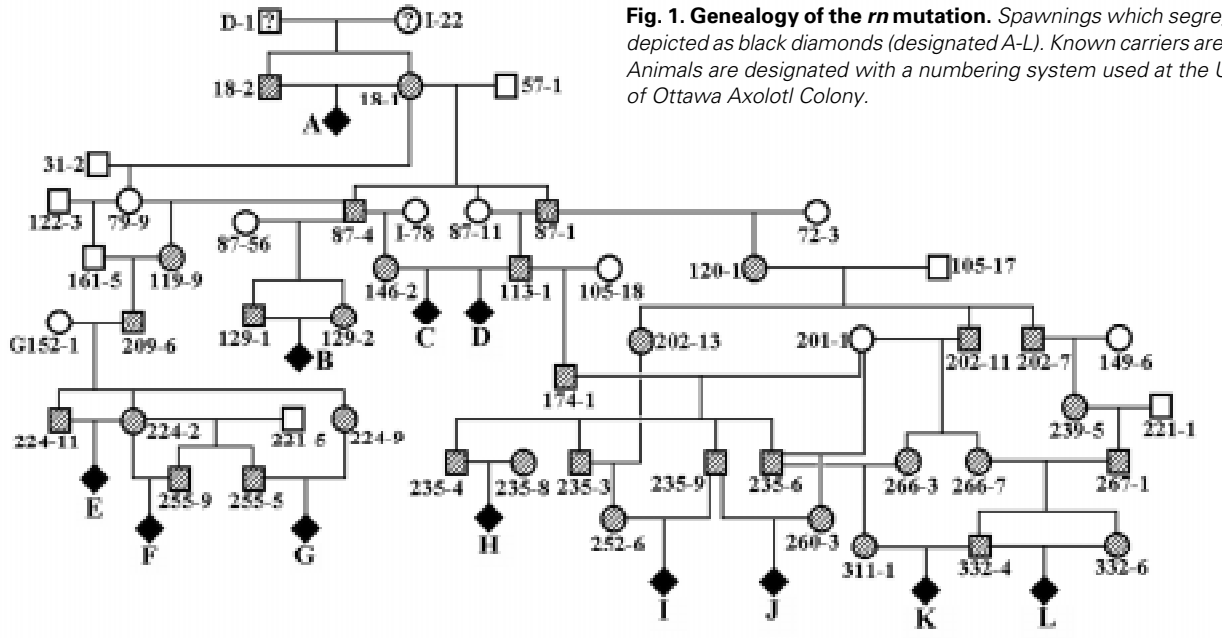

Fig. 1. Genealogy of the *rn* mutation. *Spawnings which segregated are depicted as black diamonds (designated A-L). Known carriers are hatched. Animals are designated with a numbering system used at the University of Ottawa Axolotl Colony.*

spleen and liver were colourless. Circulation in the gills and other areas of the body mostly or completely ceased, suggesting massive cranial haemorrhage. Heads of affected larvae were shorter and noticeably broader than wild-type sibs, and the opercula were flared laterally and ventrally. The opercula had small, fleshy projections ventrally, near the base of the gills. As well, affected individuals were micrognathic and microphthalmic. The body was not severely edematous, despite the absence of circulation. Pigmentation and body shape were both normal. All *rn* larvae died well after hatching, apparently of starvation.

Whole-mount skeletal preparations were made to observe the craniovisceral skeleton (Fig. 2). The head skeleton of *rn* larvae is compressed rostrocaudally, giving a laterally flared appearance. The ceratobranchial cartilages were shorter, and more perpendicular to the body axis. The size of the hyoid apparatus is reduced, and the hypohyal and hypobranchial cartilages form less acute angles with the first basibranchial (Fig. 2 C,D). The second basibranchial is very much ventrally angled (Fig. 2 E,F). The otic capsules are well developed in mutant larvae as is the dentition. Vertebral cartilages are poorly developed.

Internal morphology was examined through serial cross sections of *rn* hatchlings and wild-type siblings. The tongue was absent from *rn* larvae. The olfactory organ was slightly reduced in size, but otherwise normal. Optic vesicles were smaller, and lenses appeared extruded, and of a similar size to wild-type lenses (Fig. 3 C,D). The brain was less well developed, and trabecular cartilages were dorsoventrally shorter. In the posterior head/anterior trunk, *rn* embryos had a more areolar arrangement of soft tissues, especially in the operculum (Fig. 4 A,B), possibly due to incipient edema. No vasculature was apparent at the base of the gills; tissues were flooded with blood. Notochord and neural tube were normal. In the trunk (Fig. 4 C,D), *rn* hearts were slightly distended, but otherwise normal, the gut had more glandular tissue, and the liver was poorly developed, with very large sinuses and little hepatic tissue. Pronephric tubules were normal.

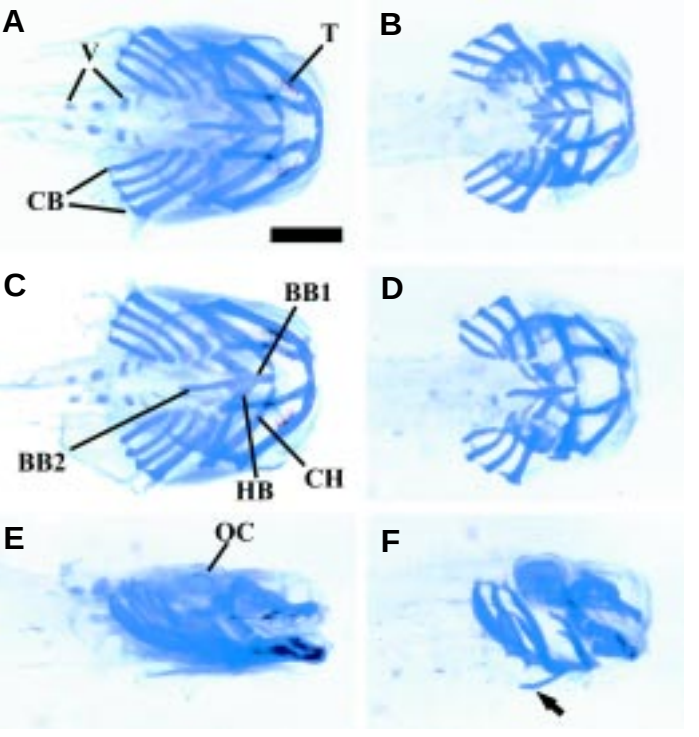

Fig. 2. Skeletal morphology of wild-type (A,C,E) and *rn/rn* (B,D,F) cleared hatchlings stained blue for cartilage and red for mineralised tissues. *Dorsal* **(A,B)**, *ventral* **(C,D)**, *and right lateral* **(E,F)** *views are shown. Note the general rostrocaudal compression of the mutant skull, the poorly differentiated vertebral cartilages and the abnormal ventral projection of the second basibranchial (arrow). However, teeth and the otic capsule appear essentially normal. Abbreviations: BB1, first basibranchial; BB2, second basibranchial; CB, ceratobranchials; CH, ceratohyal; HB, hypobranchial; OC, otic capsule; T, teeth; V, vertebral cartilage. Bar, 1mm.*

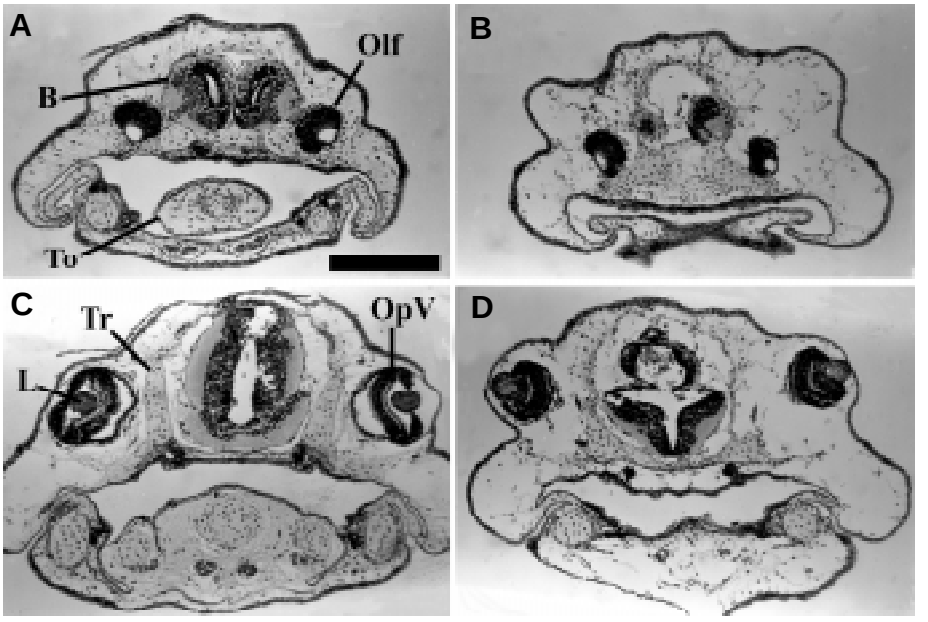

Fig. 3. Cross-sectional morphology of the heads of wild-type (A,C) and mutant (B,D) hatchlings, at the level of the nares (A,B) and eyes (C,D). *Note in particular the complete absence of a tongue (To) and the reduced size of the optic vesicle (OpV) in the mutant. However, the lens (L) appears to be approximately the same size, although more extruded from the optic vesicle. The olfactory organ (Olf) appears slightly smaller, the brain (B) appears less well developed, and the trabecular cartilages (Tr) do not extend as far dorsally in mutant larvae. Bar, 500μm.*

In this paper, we describe a spontaneous mutation in *A. mexicanum* which we designate *redneck* (*rn*). Inherited as a simple recessive gene, it is lethal in homozygotes and causes a syndrome of effects. The major characteristics are massive haemorrhage in the posterior head, abnormally compressed craniovisceral skeleton, and poor differentiation of the vertebral cartilages and liver.

It is unclear which embryonic tissue(s) are directly affected by *rn*. Based on the effects, we feel that *rn* affects late cranial neural crest development. Neural crest contributes to cranial blood vessels (Noden, 1991), which rupture in mutants, and the mutation affects the craniovisceral skeleton, which is derived from cranial neural crest (Graveson, 1993). Bashir and Armstrong (1999) have shown that cardiac neural crest in the axolotl contributes to both cardiac outflow tract and branchial arches. Whereas no obvious defects were seen in the hearts of mutants, it is possible that defective branchial arch neural crest is responsible for their abnormally compressed morphology. If cardiac neural crest also contributes to branchial vasculature, a defect in development could cause haemorrhage at the base of the gills.

Abnormal optic vesicle development may indicate a problem with either cranial neural crest, which is involved in eye development (see Brun, 1993), or *rn* may directly affect optic tissues. Oddly, though the optic vesicle is smaller in *rn*/*rn* individuals, the lens is not, even though optic vesicle and lens regulate their sizes mutually in Ambystomatids (Ballard, 1939). Placodal ectoderm is unlikely to be affected by *rn*, since olfactory and otic organs were normal in mutants.

Liver and gut development are also affected by *rn*. Little is known about the development of these organs, and neural crest cells are not believed to be involved in their development. However, the *premature death* (*p*) gene, which affects neural crest development also affects hepatic development (Graveson and Armstrong, 1990; 1996). As well, there is largely forgotten evidence that neural crest is required for endodermal organ development (Okada, 1957; Takata, 1960).

Redneck is distinct from *p*, though they affect many of the same tissues, (notably craniovisceral cartilages, liver, and eyes; Graveson and Armstrong, 1990; 1996). However, unlike *p*, *rn* acts much later in development and does not affect chondrogenic or dental differentiation, or gill, heart, or placodal development.

With the present evidence, we feel that *rn* affects the later development of a subset of cranial neural crest cells. Thus, *rn* may be useful for studying the later specification of these cells, and for examining the tissue mechanisms responsible for craniovisceral morphogenesis and neural crest involvement in endodermal devel-

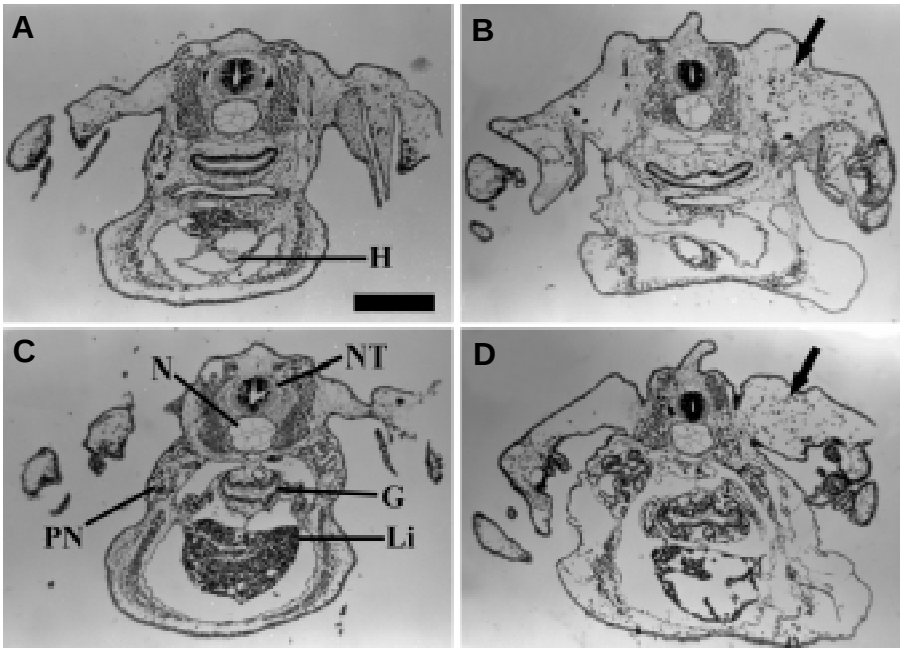

Fig. 4. Cross-sectional morphology of the posterior head and anterior trunk of wild-type (A,C) and mutant (B,D) hatchlings. *Note the pools of blood in the looser tissue of the mutant operculum (arrows). The notochord (N), neural tube (NT), heart (H) and pronephric tubules (PN) appear relatively normal in the mutant. However, the gut (G) appears to contain more glandular tissue in the mutant (D) and the liver (L) is poorly developed, with large sinuses and little tissue (C). Bar, 500μm.*

opment. Further study will be needed to define what embryonic tissue(s) are directly affected.

Experimental Procedures

Embryos

Embryos were obtained from hormonally induced spawnings at the University of Ottawa Axolotl Colony. Embryos were kept at 18°C in 25% modified Holtfreter's medium with penicillin and streptomycin. Staging was per axolotl normal tables (Armstrong and Malacinski, 1989).

Histological analysis

Mutant and normal larvae were anaesthetised in 0.005% benzocaine one week post-hatching, examined for gross defects, fixed overnight at 4°C in freshly prepared PLP fixative (Bourque *et al.,*1993), washed (2 X 30 min) in phosphate-buffered saline, dehydrated in ethanol, cleared in Histoclear®, paraffin embedded, serially sectioned at 10 µm, and stained with Hall-Brunt quadruple stain (Hall, 1986). Three mutant and two wild-type siblings were sectioned.

Whole-mount staining

Larvae were fixed in 10% neutral buffered formalin, stained with Alcian blue and Alizarin red (Hanken and Wassersug, 1981). Times were decreased to compensate for their small size. Three mutant and two wild-type siblings were examined.

Image analysis

Images were digitised using a Polaroid® SprintScan® 35/LE, and were cropped, assembled into plates and labelled using Adobe® PhotoShop® 4.0 LE.

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