Regeneration of vestibular horizontal semicircular canal afferents in pigeons

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ABSTRACT

Spontaneous regeneration of vestibular and auditory receptors and their innervating afferents in birds, reptiles and amphibians is well known. Here, we produced a complete vestibular receptor loss and epithelial denervation using an ototoxic agent (streptomycin), then we quantitatively characterized the afferent innervation of the horizontal semicircular canals following completed regeneration. We found that calyx, dimorph, and bouton afferents all regenerate in a manner the recapitulates the epithelial topography of normal birds, but over a slow time course. Similar to a previous findings in the vestibular otolith maculae, regeneration occurs according to a three stage temporal sequence. Bouton afferents regenerate during the first month of regeneration, followed by calyceal bearing afferents in the second and third months. Calyx afferents were the last to regenerate in the final stage of recovery after 3 months. We also found that regenerated afferents exhibited terminal morphologies that are significantly smaller, less complex, and innervate fewer receptor cells over smaller epithelial areas than those that develop through normative morphogenesis. These structural fiber changes in afferent innervation correlate to alterations in gaze responses during regeneration, although the exact underlying mechanisms responsible for behavioral changes remain unknown. Plasticity in central vestibular neurons processing motion information seem to be required to explain the observed morphologic and response adaptations observed in regenerating vestibular systems.
INTRODUCTION

Vestibular receptor loss through disease or trauma immediately results in compromised oculomotor and postural responses, as well as in dysfunctions in more complex orientation and navigation behaviors (Carey et al. 2002; Crane and Demer 1998; Dickman and Lim 2004; Haque et al. 2006; Newlands et al. 2001; Stackman and Taube 1997). For example, it has been long known that certain aminoglycoside antibiotics are ototoxic to both vestibular and auditory receptors and produce receptor cell loss and afferent denervation (Berg 1951; Wersäll and Hawkins 1962). Remarkably, in many vertebrates, including amphibians, reptiles, and birds, spontaneous regeneration of hair cells and re-innervation of the receptor epithelium occurs (Corwin and Cotanche 1988; Masetto and Correia 1997a, Weisleder and Rubel 1992; Zakir and Dickman 2006). In addition, hair cell function (Masetto and Correia 1997b), afferent responses to motion (Boyle et al. 2002; Li and Correia, 1998), and vestibular mediated behavioral responses (Carey et al. 1996; Dickman and Lim 2004; Goode et al. 1999; Haque et al. 2008) have all been shown to recover during regeneration.

In order to understand the regeneration process, it was first necessary to examine the normal topography and innervation patterns of vestibular receptors. As in all amniotes, birds have two hair cell types (Lindeman 1969; Rüsch et al. 1998; Wersäll 1956) and three classes of vestibular afferent innervation (Brichta and Peterson, 1994; Cajal, 1909; Fernandez et al., 1988; 1990; 1995; Haque et al. 2006; Lysakowski and Goldberg 1997; Schessel, et al., 1991; Si et al., 2003; Zakir et al., 2003). Bouton afferents exclusively innervate type II hair cells with bouton terminals. Calyx units innervate type I hair cells with a calyceal terminal that encloses one or multiple hair cells. Dimorph afferents contain calyceal and bouton terminals to innervate both type I and type II hair cells. Both hair cell types and afferent innervation classes exhibit regional topography in bird vestibular epithelia (Haque et al. 2006; Si et al., 2003; Zakir et al., 2003). In pigeon horizontal semicircular canals, calyceal bearing
afferents (calyx and dimorph fibers) are centrally located in the crista, while bouton afferents populate the receptor periphery (Haque et al. 2006).

In pigeons, we recently established that complete regeneration of vestibular macular receptors and their afferent innervation following a total receptor loss occurs over many months, according to a specific temporal sequence (Zakir and Dickman 2006). Three stages of regenerative recovery were observed, with different receptor cell types and afferent terminal morphologies developing sequentially. Stage 1 regeneration lasted through the first month following the initial lesion (post-streptomycin treatment: PST) and was characterized by the exclusive low density development of type II hair cells and bouton afferents (Masetto and Correia 1997a; b; Zakir and Dickman 2006). No calyceal bearing afferents were present in the maculae. Stage 2 regeneration (between 6 – 12 weeks PST), was characterized by hair cells densities that had increased to 50% that of normal, bouton afferents that exhibited increasing terminal fields, and the first calyceal terminals being developed. No true calyx afferents innervating only type I hair cells were observed during most of stage 2 regeneration, although a few rudimentary calyx afferents were observed late at 12 weeks PST. Stage 3 regeneration (18 – 24 weeks PST) was marked by the completed morphologic recovery at 6 months PST. Most notable in stage 3 regeneration was the return of the calyx afferents to the central striolar regions of the maculae. Although regenerative formation reproduced much of the normal receptor phenotype, our findings showed that there were significant differences in afferent innervation following complete regeneration (Zakir and Dickman 2006). For example, regenerated otolith afferents were 20 – 35% reduced in size (smaller and less branched), innervated fewer hair cells, and covered a smaller area of the epithelium (30% reduction), than afferents innervating the normal bird maculae (Zakir and Dickman 2006).
Since our previous regeneration morphological investigations were restricted to examination of the otolith macular receptors, we wondered whether regeneration of the semicircular canal receptors followed a similar temporal pattern and resulted in significant differences in innervation phenotype. In the investigation reported here, we produced a total receptor loss and denervation of the horizontal semicircular canal crista in pigeons using an applied single dose of intra-labyrinthine ototoxic antibiotic. We then examined afferent re-innervation of the crista sensory epithelium through a six month time course. Quantitative comparisons between afferent terminal morphologies in normal birds and regeneration birds were made. Our present findings show that similar to macular afferents, semicircular canal fibers regenerate slowly over months, with final innervation patterns that exhibit significant differences in their terminal morphologies.
METHODS

Animals:

Adult pigeons (*Columba livia*) ranging in age between 1 – 2 years before introduction into experiments were used. The birds ranged in weight from 400g to 700g. Two groups of birds were included for study. Regeneration pigeons were given an ototoxic antibiotic treatment (see below) to produce a complete vestibular receptor lesion, then allowed to recover fully before study. Normal birds were maintained in identical conditions, however they received no ototoxic treatment. All animals were used in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Animals in Research and those approved by the Institutional Animal Studies Committee. Animals were housed and cared for in the Laboratory Animal Facilities under veterinary supervision.

Receptor lesion:

In order to produce a complete lesion of all horizontal semicircular canal vestibular hair cells and denervation of the receptor cristae, a bilateral intra-labyrinthine application of streptomycin was administered (Dye et al., 1999; Frank et al., 1999). Each bird was anesthetized using isoflurane gas and an incision was made over the mastoid bone directly posterior to the external ear canal. Next, a rectangular shaped bone flap of mastoid cortex was resected, exposing the underlying bony labyrinth. A small diameter (0.35mm) hole was trephined into the vestibule promontory bone immediately posterior to the oval window, using a diamond burr. A single dose of streptomycin (2mg in 1µl saline; frozen pellet) was carefully placed through the burr hole into the perilymphatic space. A muscle plug was then used to seal the opening, being overlaid with gel foam and bone wax. The mastoid bone flap was approximated, and the incision sutured closed. This procedure was then repeated on the
contralateral side. Buprenorphine (0.08mg/kg, i.m.) was given for analgesia and the animal was
returned to its home cage once standing and alert. Each bird was monitored for behavioral symptoms
of vestibular lesion and for weight loss. All birds were hand-fed twice daily (Kay-tee formula) for
approximately 2 weeks post-operatively until spontaneous feeding resumed and weight loss stabilized.

Neural tracer application:

All regeneration birds were allowed to recover for 6 to 12 months following streptomycin
treatment. Previous studies in our laboratory using identical ototoxic lesion methods have established
that regenerative recovery is complete in the otolith maculae by 6 months PST (Dye et al., 1999; Zakir
and Dickman, 2006). Normal birds were composed of appropriately age matched controls. For each
animal, biotinylated dextran amine (BDA) was injected into the vestibular nuclei and allowed to
transport retrograde along horizontal semicircular canal (HCC) afferents for 12 days (Si et al. 2003;
Zakir et al. 2003; Zakir and Dickman 2006). The animal was anesthetized with isoflurane gas,
intubated, then placed into a stereotaxic device (Kopf Instruments). An incision over the parietal bone
was made and a bone flap resected. A glass micropipette filled with BDA (10,000 MW; Molecular
Probes; 10% in saline) was lowered into the brain using predetermined coordinates for the vestibular
nuclei (Dickman and Fang, 1996). The injection site was purposely varied among animals to provide a
complete sampling of HCC afferents due to regional differences in their central projections (Dickman
and Fang, 1996). BDA was iontophoretically passed into the brain with positive current (8 μA; 50%
duty cycle at 7 sec for 20 min). Following this, the electrode was allowed to remain in position for a
few minutes, then a small negative backing current (0.02μA) was applied and the electrode retracted.
The bone flap was approximated and the skin sutured closed. Buprenorphine (0.08mg/kg, i.m.) was
given for analgesia and the animal was returned to its home cage once standing and alert.
Histology:

After 12 days of post-BDA injection survival, the birds were re-anesthetized with pentobarbital sodium (20 mg/kg, IV). The bony labyrinths were quickly exposed and 2mm openings made into the horizontal and posterior canal walls. A bilateral intra-labyrinthine perfusion was performed using 3% glutaraldehyde, 2% paraformaldehyde, and 1% acrolein in 0.1 M phosphate buffer (PBS). Following this, a transcardial perfusion was performed using a sodium nitrite (1% in saline) wash, followed by a 2% glutaraldehyde, 1% paraformaldehyde in 0.1M sodium phosphate buffer (500 ml). Next, the skull was opened and the whole head was placed in the same aldehyde solution overnight, then rinsed and kept in buffer (PBS). The membranous labyrinths were then excised and the horizontal semicircular canal cristae dissected free. The brain was excised, cryoprotected in 30% sucrose solution overnight, then frozen and serially sectioned (50µm) in the transverse plane. Next, the cristae and brain sections were blocked from endogenous peroxidase reactions by incubation in 1% H₂O₂ in 90% methanol for 15 min. These tissues were then processed for BDA histochemistry using a modified diaminobenzidine (DAB) procedure (Brandt and Apkarian 1992). Briefly, the tissues were incubated for 16 h in a solution of 10µg/ml avidin D-HRP (Vector A-2004) and 0.3% Triton-X100 in PBS. The tissue was then rinsed and reacted using the chromogen diamobenzydine (DAB, 0.5 mg/ml), with 0.005% nickel chloride and cobalt acetate as enhancers. The reaction was initiated with 0.05% H₂O₂ until a dark product was visualized, then stopped with PBS rinsing. The reacted maculae were first photographed in whole mount using a Nikon dissecting light microscope. Next, the cristae were embedded into plastic (Durcupan) and serially sectioned (10 µm thickness) using a rotary microtome. Brain sections were mounted on glass slides and counterstained (Richardson et al. 1960).
SEM preparation:

In several normal and regeneration birds, the horizontal crista on one side was not used for afferent neural tracing, but was instead prepared for examination by scanning electron microscopy (SEM). The cristae were first rinsed in distilled water, and dehydrated using a series of graded acetones (10 min each in 70, 90 and 95% acetone) followed by three washings in 100% acetone for 15 minutes each. The tissue was then incubated in increasing ratios of a mixture of tetramethylsilane (TMS) and acetone (1:1 and 3:1) for 45 min each, followed by a 2X incubation in 100% TMS for 1hr each. Finally, the tissue was dried at 60º C for 30 min, allowing the TMS to sublimate. The dried maculae were mounted on aluminum studs and gold sputter coated. Tissues were imaged using a Hitachi 2600 SEM (20kV).

Afferent Reconstruction:

The injection sites for the BDA within the vestibular nuclei were confirmed from the brain sections and only HCCs from animals without labeled somas in the vestibular efferent nuclei were included for further study. Thus, terminal morphologies examined were of primary HCC afferents. First, a contour of the epithelial surface was drawn on every other HCC section (100µm resolution) using video microscopy (Nikon E600, 60x oil) and a 3D image analysis reconstruction program (Neurolucida; MicroBrightfield, Inc.). Next, a 3D map of the epithelial surface was plotted using the traced contours. Then, labeled afferents were initially quantified as to terminal type and measured locations within the crista sections. Afferent fibers that were darkly stained and sufficiently isolated for accurate reconstruction were traced to establish the complete morphological structure of the terminal innervation within the crista epithelium. Afferents with light staining (ghost fibers), or those
that severely overlapped other afferents were not traced. For each traced afferent, a number of morphological parameters were quantified:

a) Axonal diameter: the overall average diameter of the last 5µm before penetration through the basement membrane.

b) Terminal fiber length: summation of all branching segments within the neural epithelium.

c) Terminal branches: number of branch segments per fiber.

d) Branch order: defined as the number of branching levels past the initial segment, which was assigned a branch order of 1 (see Fig. 4I).

e) Bouton terminals: number of identified en-passant and boutons terminaux per fiber.

g) Calyx hair cells: total number of type I hair cells contained in calyceal terminals per fiber.

h) Calyceal volume: measured 3D volume of all contours drawn at 1µm focal plane intervals through the calyceal terminal.

i) Innervation area: calculated by first rotating the reconstructed fiber image in 3D virtual space until an apical view was obtained. Next, the perimeter of the image was drawn using a contoured polygon planimeter (Neurolucida) in the plane of the macular surface. Since the crista surface was curved, the rotation angle differed depending upon location within the epithelium. However, each area was calculated from a planar view orthogonal to the epithelial surface. The area of the polygon was then utilized as the innervation area in µm².

j) Terminal density: calculated by dividing the total terminals (number of type I hair cells + number of bouton terminals) by the innervation area. The result was multiplied by a factor of 100, to give a product of terminal density/100µm² of epithelial surface area.
Statistical analyses:

All afferents were first divided into three main groups based upon morphological innervation patterns, including calyx, dimorph and bouton afferent classes. Comparisons of afferents between classes, as well as within classes between normal and regenerated HCC cristae were made using analyses of variance (ANOVA) with commercial software (Statistica; Statsoft, Tulsa Ok). All post hoc comparisons were made using the Sheffé follow-up test.
RESULTS

Horizontal semicircular canal hair cell density:

In order to initially characterize the extent of receptor recovery following complete regeneration, we examined the density of hair cells in two areas of the crista epithelium. SEM photomicrographs were obtained from five normal birds (aged between 1.5 and 3 years) and five regeneration birds (6 – 12 months PST). As shown in Figure 1A, two 100 x 120 micron counting frames were placed over the apical surface of both the central apex and torus regions (see Figure 2 for descriptions) of the basal HC crista. These frames did not include hair cells in the planum region (Flock and Orman, 1983; Haque et al. 2006; Masetto and Correia 1997a; Figure 2). All hair cells within the counting frames were quantified by their presence of stereocilia bundles. No attempt was made to broaden the counting frame down the peripheral crista slopes, where difficulties in identification of distinct cell borders arose due to parallax. As shown in Figure 1, hair cells containing extremely long stereocilia were observed in the torus region while hair cells located in the central apex region had decreased lengths. This was true for both normal (Fig. 1 A, C) and regenerated (Fig. 1 B, D) HC cristae (see Haque et al. 2006 for a more detailed description). In normal birds, the number of hair cells in the apex frame ranged between 208 and 242 receptors, with a mean value of 232 (SD±14) hair cells. The mean hair cell density was calculated to be 1.94 (SD±0.12) receptors/100µm². Although the range of hair cells observed (225 – 276), mean number of hair cells (245 (SD±22) hair cells) and hair cell density (2.04 (SD±0.18) hair cells/100µm²) values were slightly higher for the torus region in normal HC cristae, these measures were not significantly different from those of the apex region (ANOVA P>0.05). Similar to our previous findings (Dye et al. 1999; Frank et al. 1999; Zakir and Dickman 2006), at 4 days PST the epithelium was completely denuded of hair cells due to streptomycin ototoxicity (data not shown). Complete hair cell loss was verified in 3 birds by direct
histological examination of receptor epithelia (serial cross sections) for one lesioned side and correlated with SEM observations of total stereocilia absence for receptors on the contralateral side. We previously reported a more thorough examination of the complete lesion effects upon vestibular receptor epithelia by streptomycin application in pigeons (Dye et al. 1999; Frank et al. 1999; Zakir and Dickman 2006).

At 6 months PST, receptor regeneration was complete. Based upon our previous findings for vestibular regeneration, no significant additional hair cell proliferation or afferent innervation occurs with longer recovery periods (Frank et al. 1999; Haque et al. 2006; Zakir and Dickman 2006). As shown in Figures 1B and D, following 6 months of regeneration, the regional differences in hair cell density, stereocilia formation, and hair cell polarity were similar to that of normal animals. Although lower cell numbers and densities were sometimes observed in individual bird regenerated HC cristae, the overall mean differences for the five normal and regeneration animals were only marginally different (ANOVA, P=.074). The number of regenerated hair cells ranged between 175 and 228 for the apex and between 101 and 308 for the torus, with mean values of 204.8 (±26.5) and 225.2 (±82.5) hair cells, respectively. Accordingly, the cell density was also marginally reduced (ANOVA, P=0.75) in regeneration birds, with mean values of 1.7 (±0.22) and 1.9 (±0.69) hair cells/100µm² for the apex and torus regions, respectively.

Afferent type and regional location
Neural tracer injections into the vestibular nuclei complex produced identifiable retrograde labeled afferents in eight normal and seven regenerated HC cristae at 6 months PST. As shown in the whole organ view of Figure 2, the regenerated HC cristae exhibited a regional topography of afferent types. Figure 2A shows a postero-lateral view of the crista, with the torus on the left and central apex
to the right. Afferents in the planum were hidden from view. Therefore, the epithelium was cut through the isthus of the crista that joins the apex and planum regions (dashed line in 2A); leaving the central apex and torus regions in one tissue piece (Fig. 2B) and the planum (Fig. 2C) in a second piece laid flat for better visualization. After the whole tissue pieces were photographed, they were sectioned in order to quantify the three dimensional morphological structure. Calyceal bearing afferents were observed only in the apex and central planum regions of the crista. These fibers constituted a mixed population of both calyx (black arrowheads) and dimorph (open arrowheads) afferents, with no clear topographic separation. Bouton afferents (black arrows) were the only fiber type observed in the torus. Bouton fibers were also located in a small portion of the central apex and the peripheral region of the planum. A few scattered bouton fibers were additionally observed in the extreme periphery of the central zone, at the edges of the epithelium. These regional distributions of regenerated calyceal bearing and bouton type afferents were largely similar to that observed in normal animals, except that some expansion in the calyceal bearing fibers was noted (see below: Haque et al. 2006).

Temporal sequence of regeneration

Recently, we reported that morphological regeneration of the vestibular receptors in the otolith maculae occurs along a three stage temporal sequence (Zakir and Dickman, 2006). In order to determine if the cristae follow a similar recovery time course, we qualitatively examined hair cell and afferent innervation in birds at specific regeneration windows, including 1, 2, 4, 6, 12, and 24 weeks PST (26 cristae total). As shown in Figure 3, three stages of regeneration could be identified. During the first 4 weeks (Stage 1) of PST regenerative development, BDA labeled afferents were observed to innervate the entire cristae. However, these fibers only contained bouton and en passant type terminals. No calyceal terminals were ever observed. As shown in Figure 3a early
regeneration fibers penetrated the basement membrane with growth cones and began to collateralize.

For the first 2 weeks PST, most fibers observed consisted of a single axonal segment with terminal growth cones. Some fibers (Fig. 3a) initiated simple collateralizations with a few branch segments, each of which terminated in growth cones. Growth cones took various forms, including large canonical or elliptical structures (Fig. 3a-d), digitiform appendages (Fig. 3c-d), or rarely, fibril outgrowths (Ramon y Cajal, 1980; Fink and Morest, 1977; Tosney and Landmesser, 1985 Skaliora et al., 2000). Often, fiber segments contained large prominent swellings (Fig. 3a-e). These appeared to be nodes for budding new branch segments, as ramifying branches were often observed to emanate from prominences (Fig. 3b). By 4 weeks PST (Fig. 3b), a number of fibers had increased arborizations, but many simple fibers were also observed, including single branch fibers just penetrating the basement membrane. A number of developing fibers contained both branches with growth cones and slender branches that terminated in typical appear bouton terminals (Fig. 3b). Many of these branches appeared to target and even terminate near developing hair cells.

During the second stage of regeneration, extending from 6 – 12 weeks PST, a significant change in innervation was observed by the first appearance of calyceal terminals. Although new initial segment fibers could still be observed to enter the epithelium at 6 weeks PST, many other existing fibers exhibited increased terminal arborization complexity. As shown for a bouton afferent in the torus region in Figure 3c, newly developed branches were present, some with growth cones and some with terminal boutons. In addition, regenerating calyceal terminals and identifiable mature type I hair cells were also observed for the first time at 6 weeks PST (Fig. 3d). Although often difficult to discriminate type I and type II hair cells during development or regeneration without high resolution electron microscopy (Rüsch et al. 1988), we conservatively identified mature type I hair cells by morphologic phenotype indicators including: 1) amphora shape, 2) apical location of nucleus, 3)
constricted neck, and 4) calyceal terminal. Many calyceal terminals were incomplete, with partial encapsulations that extended from the basal toward the apical portion of the type I hair cell. Whether partial or complete, all of the observed calyceal terminals were contained on dimorph afferents, since these fibers all exhibited additional branches with either growth cones or terminal boutons. In the latter part of stage 2 regeneration (12 weeks PST), the epithelium became more densely populated by afferent fibers. There were fewer entering initial fibers observed, and most of these were located in the central regions of the apex and planum regions of the crista. Most of the observed bouton fibers continued to mature, with few growth cones and increased numbers of en passant and boutons terminaux. As shown in Figure 3e, many simple dimorph fibers were present, most with one or several small branch segments. Other more complex dimorph afferents were also observed. Type I hair cells were increasing in concentration in the central regions of the crista (Fig. 3e). In addition, at 12 weeks PST, calyx afferents were observed for the first time, as illustrated in Figure 3f. Calyx afferents were confined to the central apex region and were not very numerous. Of the observed calyx afferents, all had simple structures; innervating 1 or 2 hair cells and small innervation areas. A count of all BDA filled fibers in 3 12 week PST HC cristae resulted in the following distribution: 56% bouton, 38% dimorph, and 6% calyx afferents (n = 288).

At 6 months PST, afferent re-innervation of the epithelium was complete, the terminal point of Stage 3 regeneration. Here we observed that bouton, dimorph, and calyx afferents all densely innervated all regions of the receptor epithelium. For all afferents, branch segments ended with either calyceal or bouton terminals and no new growth cones were observed. In addition, no new initial fibers were observed to enter the crista epithelium. Based on these criteria, we concluded that vestibular afferent regeneration was essentially complete, similar to our previous observations for regeneration of the otolith maculae (Zakir and Dickman 2006). Unlike the earlier stages of
regeneration in the HC cristae, we quantified afferent innervation at the end of Stage 3 regeneration. In Figure 3, three sections (dotted lines) taken from the HC crista of Figure 2, including the torus (3g), apex (3h), and planum (3i) regions are illustrated, respectively. Only type II hair cells were located in the torus, while in the apex and planum regions a mixture of type II and type I hair cells were observed. In the torus, regenerated bouton fibers quickly ramified into multiple branches and exhibited en passant and bouton terminals (Fig. 3g-i, black arrows). These fibers were exclusive to the torus and peripheral planum regions. A few boutons were also observed in the extreme peripheral edges of the apex (3h). In contrast, calyx (black arrowheads) and dimorph afferents (open arrowheads) were heterogeneously distributed through the apex of the crista, with no clear topographic organization (3h).

In the planum, a high concentration of dimorph afferents was observed, while only a few calyx fibers were noted (Fig. 3i). As shown in the sectioned tissue (Fig. 3g-i), the HC cristae are not flat. In the torus region, the crista contains a slight central enlargement. However, in the apex, the crista is steeply curved into a U-shaped epithelium with afferents innervating the entire sensory surface. The planum is bisected by the stromal wall of the crista, with only the left half being shown in Figure 3i. The planum sensory epithelium is flattened, with only a slight inward curvature.

**Innervation patterns of the regenerated HC crista**

In a recent study from our laboratory, we characterized the regional distribution and terminal morphology of vestibular afferents innervating the horizontal semicircular canal crista in adult normal pigeons (Haque et al. 2006). The data from that study (91 afferents) were combined with new additional normative data (65 afferents) currently obtained for comparisons against the innervation patterns of regenerated fibers. A total of 156 normal and 132 regenerated Stage 3 HC afferents were quantified through anatomical reconstructions. Figure 4 shows photomicrographs and anatomical
reconstructions of the terminal innervation patterns for representative calyx, dimorph, and bouton
regenerated afferents. Each photomicrograph was taken at the point of axonal penetration through the
basement membrane. Below the photomicrograph are two reconstruction views. The first
reconstruction view is in a transverse plane equal to that of the section and the second is a 90° rotated
apical surface view illustrating the terminal field around which the innervation area contour was
drawn. For each type of afferent, two general categorizations could be made based on terminal
morphology. First, a subjective examination of the apical surface view revealed that each terminal
innervation pattern appeared to be arranged in either a “flower” or a “linear” profile. This was similar
to pigeon afferents innervating both the semicircular canals (Haque et al. 2006) and otolith maculae
(Si et al., 2003; Zakir et al., 2003). Flower profiles (Figs. 4A-F) consisted of terminals (calyceal
and/or boutons) arranged in a rounded or petal formation. Linear profiles (Figs. 4G-I) consisted of
elongated, narrow terminal fields. Second, each afferent type could be classified as being either simple
or complex, depending on the number of calyceal terminals, hair cells per calyx, and/or branch order.
The locations of all regenerated afferents that were quantified through anatomical reconstructions are
shown in Figure 5.

Calyx afferents. A total of 43 regenerated calyx units were reconstructed. Calyx afferents were
located in the central portions of the apex, isthmus, and planum regions of the HC crista (Fig. 5).
Similar to normal birds, calyx units exclusively innervated one or more type I hair cells with calyceal
terminals (Fig. 4A – C). The size, structure, and complexity of the calyx afferent innervation patterns,
from the simple to the more complex, are illustrated in Figure 6. Also shown is the epithelial location
for each. Table 1 shows the range and mean values for the regenerated afferent terminal innervation
parameters. Generally, regenerated calyx fibers had short, un-branched fiber segments that terminated
adjacent to the epithelial penetration. The simplest calyx fiber observed had a single branch fiber and
one calyceal terminal that contained 1 hair cell. These very simple innervations were extremely rare (2/43 reconstructed), however. More typical were afferents with a single calyceal terminal innervating 2 to 4 hair cells (Fig. 6A, B). These fibers had smaller calyceal volumes, with small terminal fields and innervation areas. Since the terminal density was defined by the number of hair cells within the terminal area, these values were characteristically low also. The more complex calyx afferents had a single large calyceal terminal that innervated 5 or 6 type I hair cells (Fig. 6C). Accordingly, the calyceal volumes, innervation area, and terminal density also increased. Although in our previous study of normal HC crista, complex calyces were defined as contacting $\geq 7$ type I hair cells, only one such afferent was observed in regenerated epithelia and it had a single calyceal terminal that innervated 9 hair cells. Finally, several regenerated calyx fibers (6/43) exhibited multiple branches and 2 – 3 calyceal terminals with 2 – 3 hair cells each (Fig. 6D). These units had smaller calyces, but large innervation areas.

Dimorph Afferents. A total of 41 regenerated dimorph afferents were reconstructed. Similar to normal fibers, each dimorph unit contained both calyceal and bouton terminals (Fig. 4 D-F). The dimorph units were more widely distributed than were calyx afferents, with fibers being observed throughout the planum, isthmus, and apex regions of the crista (Fig. 5). Unlike normal birds, a few regenerated dimorph units were also observed at the edge of the torus and in the peripheral planum regions (Fig. 5). Apparently, some regenerated dimorph units had expanded beyond the range of epithelial locations normally observed (Haque et al. 2006). Similar to normal dimorph afferents, regenerated terminal innervation patterns varied in complexity (Table 1). Simple dimorph patterns included a single calyceal terminal that contained 1 – 3 hair cells and very few branch segments with terminal boutons (Fig. 6E). These units accounted for approximately 1/3 of the reconstructed fibers. Similar to the calyx units, dimorph calyceal terminals containing only a single hair cell were rarely
observed (3/41). More typical were regenerated dimorphs with 1 or 2 calyceal terminals innervating a
total of 3 – 4 hair cells. These units also contained a number of branch fibers with multiple (3 – 11)
bouton terminals (Fig. 6F, G). The most complex dimorph afferents (5/41) had innervation patterns
that could contain a combination of either several calyceal terminals (3 – 4) and/or many branch
segments and numerous (11 -25) bouton terminals (Fig. 6H). Statistical comparisons between the
innervation patterns of regenerated dimorph and calyx units showed that there were differences
between the two classes of fibers that were characteristic to those of normally developed units (Table
2). Dimorph afferents had significantly longer fibers with more branch segments, complex
arborization patterns (branch order), larger terminal fields, and higher terminal densities. In contrast,
calyx afferents innervated significantly more hair cells with larger calyceal terminals. These
differences in terminal morphological characteristics of regenerated calyx and dimorph afferents are
graphically represented in Figure 7.

Bouton Afferents. A total of 48 bouton afferents were reconstructed. There appeared to be
fewer bouton afferents than any other fiber type. These fibers exclusively innervated type II hair cells
with bouton terminals over larger terminal fields (Fig. 4 G-I). Most bouton innervations exhibited
rounded or flower patterns (Fig. 2B), with fewer units exhibiting terminal fields that were arranged in a
linear fashion (Fig. 2A, B, 4I). Bouton afferents predominantly innervated the torus and peripheral
planum regions. Very few bouton afferents were observed in the peripheral margins of the apex. This
area was dominated by calyx units (Fig. 5). The innervation patterns of regenerated bouton fibers
exhibited differing degrees of complexity (Table 1). Nearly a third of the regenerated fibers were
simple bouton units (15/48) that contained a few branch segments with low numbers (5-13) of bouton
terminals (Fig. 6I). Consequently, these units covered a small area of the epithelium with low terminal
densities. Nearly half of the regenerated bouton fibers (25/48) had innervation patterns with multiple
branches (15–30) and moderate numbers (20–40) of bouton terminals (Fig. 4C, 6J-K). The majority of these fibers had innervation areas of several hundred microns$^2$ and moderate terminal densities (3–7 terminals/100µm$^2$). A small number of bouton fibers (8/48) had the most complex bouton innervation patterns noted, which consisted of numerous branch segments and many (>40) bouton terminals (Fig. 6L). These complex fibers had large innervation areas (>500 µm$^2$) with high terminal densities (>6 terminals/100µm$^2$).

Comparisons between all three fiber types were performed. As shown in Figure 7 and statistically verified (Table 2), regenerated bouton afferents were characterized by having the longest fibers with the most branches, the highest branch order, and the densest innervations. Bouton units also had significantly more bouton terminals than dimorph units (Table 2), but had similar terminal field sizes. Interestingly, bouton units had longer fibers with more branches than dimorph afferents, but similar volumes. This indicates that bouton fibers were generally of smaller diameter than dimorphs throughout their length.

Comparison of regenerated and normal HC afferents

For purposes of quantitative comparisons, the terminal innervation patterns of 51 calyx, 55 dimorph, and 50 bouton afferents were reconstructed from normal pigeons (92 of these normal fibers were obtained from Haque et al. 2006). As noted above, much of the topographic organization of afferent type across the epithelium was reproduced in regenerated cristae similar to that observed in normal birds. However, quantitative comparisons of the afferent innervation patterns for individual fibers found significant differences between the terminal fields of regenerated afferents and those developed normally. Figure 8 shows the mean comparisons for the three classes of HC afferents, including calyx, dimorph, and bouton fibers. Regenerated calyx fibers had significantly (asterisks)
smaller diameter axons, smaller calyceal terminals, fewer type I hair cells, and smaller innervation areas than afferents innervating normally developed cristae (Table 1). Since calyx afferents typically consisted of unbranched short segments, no differences in the arborization, length, or fiber volume were noted. Dimorph afferents were the most numerous fiber type found in the HC crista. As also shown in Figure 8, regenerated dimorph afferents had significantly smaller diameter axons, shorter length, lower volume, and smaller innervation areas than normally developed afferents (Table 1). In addition, similar to calyx afferents, regenerated dimorph fibers exhibited smaller calyceal terminals that contained fewer type I hair cells than normal (Fig. 8). Lastly, the number of bouton terminals per regenerated dimorph afferent was marginally less than that observed in normal animals (Table 1).

Regenerated bouton afferents showed similar significant reductions in number of fiber branches, fiber length, fiber volume, and terminal field innervation area as compared to normally developed afferents (Table 1). In addition, regenerated bouton fibers contained fewer bouton terminals. Since all three fiber types in regenerated birds exhibited fewer terminal contacts (bouton or type I hair cells) and smaller terminal field size, there was no net change in mean terminal density, as compared to fibers in normal birds (Fig. 8, Table 1).

**DISCUSSION**

Our findings present the first comprehensive examination of afferent innervation of the vestibular semicircular canals during regeneration in any species. We found that regeneration occurred slowly over months, according to a temporal staged sequence, similar to that previously observed for pigeon otolith maculae (Zakir and Dickman 2006). Regeneration of the HC crista was complete after 6 months of recovery, where our quantitative comparisons between normal and regenerated afferents
were performed. We found that much of the normally observed regional topography for hair cell
distribution and afferent type was reproduced during regeneration. However, small increases in the
extent of the areas covered by calyceal bearing dimorph afferents were also noted in regenerated
cristae. We also found that following complete regenerative recovery, the terminal morphologies of
the calyceal bearing and bouton afferents were significantly different from the innervation patterns
exhibited in normal cristae.

Temporal pattern of regeneration

Recently, we reported that morphological regeneration of the vestibular receptors in the otolith
maculae occurs along a three stage temporal sequence (Zakir and Dickman, 2006). Our present
findings confirm a corresponding pattern of recovery in the HC crista. We previously proposed (Zakir
et al. 2006), that vestibular afferents may regenerate through transdifferentiation, and our current data
support that view as illustrated in Figure 9. One possible mechanism that could guide the innervation
pattern decision process would be the presence/absence of as yet unknown trophic signaling cues
released by developing hair cells. In support of that idea are the following observations. First, during
Stage 1 regeneration, exclusive formation of bouton afferents and hair cells exhibiting a type II
morphologic phenotype were observed. Coincident to the afferent innervation development, we
previously found that hair cells regenerated primarily in the peripheral regions of the canal cristae and
otolith maculae, with fewer cells present in the central apex or striolar regions (Haque et al. 2008; Dye
et al. 1999; Zakir et al. 2006). Still, some afferents and hair cells did occupy these central regions and
when present, were always observed to exhibit a bouton innervation pattern. This was true even
though these central regions would later be devoid of bouton fibers when regeneration was complete,
and would be populated by dimorph and calyx afferents. Either these central regenerated bouton fibers
died, or they transdifferentiated into dimorph afferents (Fig. 9). Further, bouton fibers exclusively develop in the torus and peripheral regions of the planum. Here only type II hair cells are observed, again consistent with the suggestion that cell type and afferent morphology have common, possibly symbiotic determinants. Second, as presently observed and reported previously for the otolith receptors (Zakir et al. 2006), the distinguishing characteristic of Stage 2 regeneration was the appearance of the calyceal terminal and identifiable Type I hair cell morphologic phenotype. Yet, throughout most of Stage 2 recovery, the regenerated afferents were dimorph fibers, innervating both type II and type I hair cells. In fact, often partial calyces were observed developing on established afferents with several or many branches. This suggests that calyceal terminal formation occurs on existing bouton fibers when type I hair cells begin to develop nearby (Fig. 9). Third, calyx afferents develop last, mostly during Stage 3 regeneration. Coincident with calyx afferent formation is an increase in the density of type I hair cells in the central regions of the cristae and maculae. We propose that as type II hair cells diminish, so too do the available cues to maintain bouton terminals. These signaling cascades elicit simple dimorph afferents to shed their small bouton branch segments producing the birth of calyx afferents, largely due to the higher concentration of neighboring type I hair cells (Fig. 9). Simultaneously, trophic cues from the more abundant type I hair cells induce calyceal formation, including larger more complex calyceal structures incorporating several or a number of type I hair cells. In essence, the “decision” to differentiate into either a dimorph or calyx afferent would depend primarily on the concentration of neighboring cell type. Whether afferent transdifferentiation actually occurs is unknown, but the proposed regeneration scheme is consistent with our observations for both cristae and macular recovery.

Comparison between regeneration and normal afferent innervation
Our present findings of completed regeneration in the HC crista also observed differences in the terminal afferent innervation patterns. In pigeons, similar to other vertebrates, calyceal bearing afferents populate the central regions of the cristae while bouton afferents are located in the periphery (Brichta and Peterson 1994; Desai et al. 2005; Haque et al. 2006). This highly conserved regional topography was largely reproduced during regeneration of the HC crista. However, small increases in the range of regenerated dimorph afferents was noted, as was also true for regenerated macular fibers (Zakir et al. 2006). In birds, like mammals, calyceal bearing afferents comprise the majority of the fibers innervating the crista (Fernandez et al. 1988; 1995; Haque et al. 2006). Together, calyx and dimorph afferents are distributed over a larger region of the epithelial surface than are bouton afferents.

In our current study, we compared the relative terminal morphologies between regenerated calyx and dimorph fibers. We found that regenerated calyx afferents had larger calyceal terminals that contained more type I hair cells than did dimorph units; similar to normally developed HC crista fibers. In contrast, regenerated dimorph afferents exhibited larger terminal fields, with more branches, longer segments and increased innervation areas compared to calyx units; again similar to normal afferents. However, when comparisons between normal and regenerated calyx and dimorph fibers were made, significant reductions in size and complexity were observed. Regenerated calyceal bearing afferents on average had smaller diameter axons, shorter and less voluminous segments, a 30% reduction in calyceal terminal size, a 30% reduction in the number of hair cells contained, and a 25% reduction in terminal field size (area), as compared to normal afferents. In the pigeon maculae, regenerated calyceal bearing afferents were also significantly reduced, but to a lesser degree than HC fibers (Zakir and Dickman 2006). Regenerated bouton fibers exhibited the largest, most complex, terminal innervation patterns when compared to calyx and dimorph units, similar to the relative differences observed in normal animals. However, regenerated bouton fibers also exhibited significant
morphological differences in complexity and size as compared to those observed in normative
development, with a 30% reduction in length, number of branch segments, number of bouton
terminals, and innervation area, a 15% reduction in branch order, and a 50% reduction in fiber volume.

Functional considerations of regeneration afferents

In mammals, spontaneous regeneration of vestibular receptors following complete receptor loss
does not typically occur, but may vary depending upon species (Kawamoto et al. 2008; Meiteles and
Raphael 1994). Still, head and eye responses partially recover (Stapely et al. 2006), presumably using
extra-vestibular motion cues to enhance central neural compensation (Newlands and Perachio 1990a,
b; Zennou-Azogi et al. 1994) and adaptation (Anastasio 1992; Serafin et al. 1999) processes. Recently,
we demonstrated that vestibular mediated gaze stabilization during rotational motion recovers fully in
birds undergoing regeneration, with a time course lasting several months (Haque et al., 2008).
Interestingly, gaze stability during high frequency motion recovered first, at a time when only bouton
afferents innervated the vestibular epithelia (Stage 1 regeneration). Further, these early regeneration
gaze responses consisted almost entirely of a head response component, with the eye response
component contributing very little. As regeneration progressed (late Stage 2), gaze stability returned
for the mid-frequency rotations, followed lastly by recovery during slow rotational head movements.
These later recovery dynamics coincided with the regeneration of calyceal bearing afferents that
innervated the central crista regions (Haque et al. 2008). In mammals, the central regions of the cristae
contain irregularly firing high gain dimorph and low gain calyx afferents with large dynamic ranges
(Baird et al. 1988; Fernandez et al. 1988; Lysakowski et al. 1995). We previously suggested that these
central regions appear to provide the signals needed for low frequency gaze stability, as gaze responses
to the lowest frequencies of rotation only became significant at the last stage of regeneration. Our
current findings of the later development of regenerating calyx and dimorph afferents in the central
crista would support that view. In a related manner, we presently found that the regenerated calyceal
bearing HC afferents had terminal morphologies that were significantly reduced in complexity as
compared to normal fibers. Whether these differences in afferent innervation are related to the change
in functional contribution between the head and eye components to gaze stability in regenerated birds
is unknown. However, although pigeon gaze stability recovers to normal values, the relative
contributions from the head and eye components change during regenerative recovery (Haque et al.
2008). In fact, the eye component during head-free rotations increases while the head component
decreases in significance to total gaze stability, in converse to the behavior observed in normal birds
(Haque and Dickman, 2005; Haque et al. 2008). Our findings suggest that the same calyx and dimorph
afferents regenerating to contribute low-frequency dynamic information are also increasing their signal
efficacy to eye movement related central vestibular neurons in the production of gaze responses.
Plasticity in central vestibular neurons processing motion information would appear to be required to
explain the observed morphologic and response adaptations observed in regenerating vestibular
systems. If true, how central vestibular neurons compensate to the changing vestibular afferent inputs
during regeneration remains a topic of substantial interest.

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REFERENCES


FIGURE CAPTIONS

Figure 1. Scanning electron photomicrographs of horizontal semicircular canal cristae. (A) Normal HC crista with two 300 x 100µm counting frames superimposed. (B) Regeneration HC crista at 9 months PST. (C) Magnified view of central counting frame in A. (D) Magnified view of central counting frame in B. Scale bars in A, B = 200 µm and in C, D = 50 µm.

Figure 2. Stereo-photomicrographs of a single regeneration HC crista. (A) HC crista epithelium viewed from the postero-lateral surface. BDA labeled calyx (black arrowhead), dimorph (open arrowhead), and bouton (black arrows) afferents can be seen throughout the torus (left) and apex regions (middle). The planum (right) contains labeled afferents that are hidden from view. The dashed line indicates the location that a cut was made for better visualization of the apex (B) and planum (C) regions. Dotted lines g – i, indicate sections illustrated in Figure 3. Scale bars = 100µm.

Figure 3. Stages of regeneration. S1: denotes stage 1 regeneration observed between 1 – 4 weeks PST. (a) Two weeks PST, with growth cone (left) and developing bouton fiber (right). (b) More advance developing bouton fiber at four weeks PST. S2: denotes stage 2 regeneration, with mostly bouton fibers regenerating at 6 wks PST (c). Calyceal bearing dimorph afferents first regenerate at 6wks PST (d). At 12 wks PST, many dimorph afferents are developed, some with only a single calyceal terminal and branch fiber (e). Simple calyx afferents first develop at 12 wks PST (f). S3: denotes completed Stage 3 regeneration (24 wks PST). Torus (g), apex (h), and left half of planum (i) regions of the S3 crista are shown. Bouton afferents (black arrow) were the only fiber type observed in the torus region, but were also found in the peripheral apex and planum regions. Calyceal bearing
afferents, including calyx (*black arrowhead*) and dimorph (*open arrowhead*) fibers were only located in the apex and central planum regions. Scale bars = 10µm (a – f); 25µm (g – i).

**Figure 4.** Photomicrographs and anatomical reconstructions of horizontal semicircular canal regenerated afferents. Photomicrographs and anatomical reconstructions are illustrated for 3 BDA-labeled fibers. Calyx (A-C), dimorph (D-F), and bouton (G-I) afferents with transverse section micrographs (A, D, G) and reconstructions for the transverse (B, E, H) and surface (C, F, I) views, respectively. In the surface reconstructions, calyceal contours were drawn at focal plane intervals of 1 µm (*thin black lines*) and innervation areas were measured using a digital planimeter around the apical surface view of the terminal pattern (*blue lines*). Terminal boutons are indicated by *red circles*. In (H), numbers indicate branch order of fiber segments. Scale bar = 5µm.

**Figure 5.** Wireframe surface map of the HC crista with locations of reconstructed regeneration afferents. Surface contours are separated by 100µm sections. Calyx (diamonds) and dimorph (green) afferents were primarily observed in the central regions of the crista. Bouton (blue) afferents were found in the torus and peripheral regions of the crista.

**Figure 6.** Anatomical reconstructions for Stage 3 regenerated HC crista afferents. Calyx (A-D), dimorph (E – H), and bouton (I – L) afferent reconstructions arranged in increasing order of complexity. For all reconstructions, fiber branch segments and calyceal terminals are in *black*, bouton terminals in *red*. Composite surface map (*green*) of the HC epithelium shows the relative locations of the afferents featured in the reconstructions. Scale bar = 10µm.
**Figure 7.** Comparison of afferent innervation morphological properties for Stage 3 (6 – 12 months PST) calyx, dimorph, and bouton HC fibers. Area = innervation area of receptor epithelium (μm²); Volume = sum of all fiber branch segment volumes (μm³); Length = sum of all branch segments (μm); Branches = number of branch segments/fiber; Bouton = number of bouton terminals/fiber; Branch order = number of branch levels; HC I = number of type hair cells/fiber; Diameter = axonal fiber diameter (μm).

**Figure 8.** Comparison of regeneration and normal afferent innervation morphological properties. Mean values are shown for each parameter for normal (grey) and regeneration (hatched) afferents. Diameter = average fiber diameter (μm); Branches = number of branch segments/fiber; Length = sum of all branch segments (μm); Volume = sum of all fiber branch segment volumes (μm³); Area = innervation area of receptor epithelium (μm²); HC I = number of type hair cells/fiber; Bouton = number of bouton terminals/fiber; Cal Vol = volume of calyceal terminal; Terminals = sum of bouton terminals and calyceal hair cells; Density = terminals/innervation area (#/μm²). For clarity, quantities for fiber length, innervation area, and volume are plotted as a factor of ten reduction. Error bars = standard errors. Asterisk = p<0.05 level (ANOVA).

**Figure 9.** Timeline of proposed transdifferentiation of regenerated vestibular afferents. Stage 1 afferents regenerate as bouton fibers due to the exclusive signaling cues from type II hair cells. During Stage 2 regeneration, initial developing fibers and existing fibers develop as bouton afferents. When appropriate cues arise from developing type I hair cells, calyceal terminal formation occurs. High concentrations of type II of hair cells promotes bouton afferent phenotype. The presence of mixed type I and type II hair cell signals promotes dimorph development. Stage 3 regeneration occurs...
primarily in the central regions of the cristae and macular regions. Here, dimorph fibers develop early
during regeneration and later lose their bouton terminal branches to become calyx afferents if high
concentrations of type I hair cells are present. If mixed type I and type II hair cells occupy the
neighboring epithelial space, more complex dimorph afferent phenotypes develop. Newly entering
initial fibers could also immediately form as calyx afferents depending upon the density of surrounding
type I hair cells.
<table>
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<tr>
<th>Comparison</th>
<th>Calyx</th>
<th>Dimorph</th>
<th>Bouton</th>
<th>Main Effect</th>
<th>Cal vs Dim</th>
<th>Cal vs Bout</th>
<th>Dim vs Bout</th>
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<td>2.5 (0.7)</td>
<td>2.3 (0.58)</td>
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<td>0.5 – 5.0</td>
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<td>1.3 – 3.9</td>
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<td>Branches</td>
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<td>1.1 (0.4)</td>
<td>4.1 (1.5)</td>
<td>6.1 (2.0)</td>
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<td>3 – 12</td>
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<td>Fiber length</td>
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<td>304.4 (232.8)</td>
<td>292.7 (238.6)</td>
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<td>75.6 – 1491.7</td>
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<td>Fiber volume</td>
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<td>377.3 (138.7)</td>
<td>309.6 (196.6)</td>
<td>$F(2, 129)=3.0$</td>
<td>$p = 0.08$</td>
<td>$p = 0.95$</td>
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<td>70.2 – 755.8</td>
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<td>Type I HC</td>
<td>3.7 (1.5)</td>
<td>3.0 (1.4)</td>
<td>3 - 21</td>
<td>$F(1, 82)=6.0$</td>
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<td>1 - 9</td>
<td>1 – 7</td>
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<td>Calyceal volume</td>
<td>3506 (1406)</td>
<td>2514 (1906)</td>
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<td>1133 – 7702</td>
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<td>Boutons</td>
<td>—</td>
<td>7.0 (5.0)</td>
<td>23.6 (15.4)</td>
<td>$F(1, 87)=43.7$</td>
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<td>0.6 – 4.8</td>
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<td>3 – 21</td>
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Mean (SD) = standard deviation; * denote one-way ANOVA only.
Table 2. *Statistical comparisons for pooled regenerated versus normal afferents.*

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<td>Normal</td>
<td>Regen</td>
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<td>Diameter</td>
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<td>(0.9)</td>
<td>2.5 (0.9)</td>
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<td>Branches</td>
<td>1.2</td>
<td>(0.8)</td>
<td>1.2 (0.6)</td>
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<td>Branch order</td>
<td>1.1</td>
<td>(0.5)</td>
<td>1.1 (0.4)</td>
</tr>
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<td>Length</td>
<td>28.5</td>
<td>(15.9)</td>
<td>33.3 (19.9)</td>
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<td>Fiber volume</td>
<td>245.1 (164.1)</td>
<td>241.2 (168.8)</td>
<td>555.9 (468.4)</td>
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<td>Area</td>
<td>384.5 (196.1)</td>
<td>298.8 (130.5)</td>
<td>510.0 (248.8)</td>
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<td>Type I HC</td>
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<td>3506 (1023)</td>
<td>4095 (2237)</td>
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<td>Boutons</td>
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<td>1.3</td>
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Mean (SD) – standard deviation.