Recovery of Gaze Stability During Vestibular Regeneration

Asim Haque,1,2 Mridha Zakir,1 and J. David Dickman1
1Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri; and 2School of Medicine, University of Mississippi Medical Center, Jackson, Mississippi

Submitted 18 September 2007; accepted in final form 26 November 2007


INTRODUCTION

Gaze stability is known to be fully compensatory to head motion and is dependent on vestibular system function (Carey et al. 2002; Crane and Demer 1998, 2000; Gioanni 1988b,b). Gaze responses are composed of contributions from the vestibuloocular reflex (VOR), vestibulocollic reflex (VCR), cervicocollic reflex (CCR), and head mechanics (Keshner and Peterson 1995; Peterson et al. 1981). Not surprisingly, vestibular signal loss typically results in gaze performance dysfunction (Crane and Demer 1998; Foster et al. 1997; Newlands et al. 2001). We recently reported that gaze stability in head unrestrained birds is composed primarily of head responses with much smaller eye-movement components (Haque and Dickman 2005), similar to other nonmammalian species (Dieringer et al. 1983; Gioanni 1988a,b). In contrast, large VOR eye movements stabilize gaze in birds during head-fixed motion (Dickman and Angelaki 1999; Dickman et al. 2000; Haque and Dickman 2005).

Many ototoxic agents are known to produce vestibular and auditory receptor damage through hair cell death and afferent denervation (Berg 1951; Wersäll and Hawkins 1962). In birds, reptiles, and amphibians, the occurrence of spontaneous regeneration of vestibular receptors following inner ear damage is now well established (Corwin and Cotanche 1988; Cruz et al. 1987; Weisleder and Rubel 1993). Although much of the inner ear morphology returns to normal phenotypic patterns (reviewed in Matsui et al. 2005), significant differences persist in vestibular afferent innervation following complete regeneration (Zakir and Dickman 2006). The effects of vestibular receptor loss on the central neuronal processing of motion information that govern gaze and postural stability are not yet fully understood. In mammals with little or no spontaneous regenerative capacity, central neural compensation (Johnston et al. 2002; Newlands and Perachio 1990a,b; Zennou-Azogi et al. 1994) and adaptation (Anastasio 1992; Lisberger et al. 1994b; Serafin et al. 1999) are key mechanisms. However, in animals undergoing prominent regeneration, signals from developing vestibular hair cells and afferents will change over time the type of motion information being presented to central neurons involved with recovering gaze stability (Boyle et al. 2002; Correia et al. 2001; Jones and Nelson 1992; Li and Correia 1998; Masetto and Correia 1997a,b). In addition, VOR and VCR responses that contribute to gaze stabilization have been examined during regeneration in birds with functional recovery described to be complete in weeks to a few months (Boyle et al. 2002; Carey et al. 1996; Goode et al. 1999, 2001). However, these studies used lesion methods that typically resulted in only a partial receptor loss (Carey et al. 1996; Goode et al. 1999; Masetto and Correia 1997b; Matsui et al. 2003). Thus the functional return observed was likely due to a synthesis of factors other than regeneration alone, such as residual hair cell function and nonlethal repair (Baird et al. 2002; Carey et al. 1996; Goode et al. 1999, 2001). We found that complete vestibular loss produced profound head instability and postural ataxia that slowly recovered according to a temporal sequence of events, including increased head stability, head-tremor reduction, return of postural stability, and finally recovery of orientation and navigational abilities (Dickman and Lim 2004). Based on these findings, we hypothesized that gaze stability must be established before postural and orientation deficits could recover. We further hypothesized that head-in-space stability required low-frequency gaze stabilization that would be established first during regenerative recovery, followed later by high-frequency gaze function.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
In the present work, we tested these hypotheses by first producing a verified complete bilateral vestibular receptor loss at a discrete point in time (Frank et al. 1999). We then directly characterized the functional recovery of gaze stability during vestibular regeneration based on our previously established morphologic landmarks of receptor recovery (Dickman and Lim 2004; Dye et al. 1999; Zakir and Dickman 2006). Finally, we demonstrate links between component mechanisms of gaze stability and vestibular receptor function in birds.

METHODS

Animals

Adult pigeons (Columbia livia), ranging in weight from 400 to 600 g, were used in the present study. All experimental procedures 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.

Eye search coils

Gaze, defined as the eye-in-space, is composed of the complex sum of eye-in-head and head-in-space (Guitton et al. 1984; Newlands et al. 2001; Phillips et al. 1996). To measure the eye and head components of gaze, we utilized methodologies that had been previously developed for birds (Dickman and Angelaki 1999; Dickman et al. 2000; Haque and Dickman 2005; Matsui et al. 2003). Each animal was initially given an injection of atropine (0.25 mg/kg), then anesthetized with isoflurane gas (3% in O2). During all surgical procedures, the heart rate (ECG) was constantly monitored and body temperature (40°C) maintained. An incision along the midline of the skull was made, and the underlying peristium was removed from the bone. A Delrin head stud was attached to the skull via titanium self-tapping screws and dental acrylic mixed with ampicillin powder. The head stud was positioned so that when secured to the motion delivery system, the horizontal semicircular canals of the bird would be parallel to the yaw rotation plane (Dickman et al. 2000; Haque and Dickman 2005). In some birds (n = 5), dual scleral search coils (direction and torsion) were chronically implanted in the subconjunctival space of the eye. Using aseptic technique, a circumferential incision was made through the conjunctiva, 2 mm distal to the cornea, to reveal the underlying sclera. A modified three-dimensional (3D) search coil (Hess 1990) was sutured (8-0 prolene) to the sclera and the conjunctiva proximated. Coil leads were led through the bony orbit and a miniature connector (Omnemics) was secured to the skull with dental acrylic. After surgery, butorphanol (10 mg/kg) was administered for postoperative pain, an injection of ampicillin was given as a prophylactic antibiotic, and the animal was returned to its home cage for recovery. In other birds (n = 15), an acute contact lens search coil was applied to the eye for each experiment to measure 3D eye movements (Dickman et al. 2000; Haque and Dickman 2005; Hess 1990). In all birds, head movements were measured using a second 3D search coil that was either glued to the skull with dental acrylic immediately behind the head post or attached to the head post itself. Following the gaze experiments, all animals were subsequently utilized for acute extracellular neural recording experiments (a different report), then their labyrinths were harvested for verification of the extent of receptor recovery using scanning electron microscopy (SEM).

Receptor lesion

To produce a complete lesion of all vestibular hair cells and denervation of the receptor epithelia, intralabyrinthine streptomycin was administered bilaterally (Dye et al. 1999; Frank et al. 1999). Each bird was anesthetized using isoflurane gas. An incision was made over the mastoid bone behind the external auditory meatus, and the skin was retracted. Next a rectangular-shaped bone flap of mastoid cortex was resected, exposing the underlying bony labyrinth. A small opening (0.35 mm diam) into the vestibule promontory was made immediately posterior to the oval window. A single 2 mg dose of streptomycin (1 μl total volume in a frozen pellet) was carefully placed through the opening into the perilymphatic space. The opening was then sealed with a muscle plug and reinforced with gel foam and bone wax. The mastoid bone flap was replaced, and the wound was sutured closed. This procedure was then repeated on the contralateral side. Buprenorphine (0.08 mg/kg im) was given to alleviate postoperative pain, and the animal was returned to its home cage. Each bird was monitored for behavioral symptoms of vestibular lesion and for weight loss. All birds were hand-fed twice daily (Kay-tee formula) to prevent dehydration and malnutrition for ~2 wk postoperatively until spontaneous feeding resumed.

Experimental protocols

A three-field magnetic coil system (CNC Engineering) was used to monitor rotational eye and head movements. The coil system provided a 5-in homogenous magnetic field cube centered about the pigeon’s head. The field coils were securely mounted to a servo-controlled rotator/sled system (Neurokinetics), that was driven using a program- mable interface (CED Model 1401plus, Cambridge Electronic Design) and custom-scripted software for stimulus control and data acquisition (Spike2, CED). Stimulus waveforms were monitored using a three-axis linear accelerometer and rate sensor mounted near the animal’s head.

For experimentation, each animal was placed in a padded body holder and secured in the motion stimulus device. A field-coil centered (head-fixed) reference frame (see Haque and Dickman 2005) was adopted for quantifying eye and head movements. Eye- and head-movement responses were obtained using both head-fixed (VOR) and head-free (gaze) conditions. Sinusoidal rotation motions were delivered along either an earth-vertical axis (EVA; yaw, 0.01–2 Hz, 20°/s) or an earth-horizontal axis (EHA, roll, 0.02–4 Hz, 20°/s) in complete darkness to eliminate visual influences.

To monitor the recovery of gaze stabilization reflexes, animals were tested at different stages of regenerative recovery (Zakir and Dickman 2006). The first poststreptomycin treatment time (PST 0) was assigned to the fourth day after application of streptomycin when a complete lesion of all vestibular hair cells and innervating afferent fibers had occurred (Dye et al. 1999; Zakir and Dickman 2006). All recovery stages of measurement were based on the PST 0 point, such that 1 wk PST (n = 4 birds) actually refers to the 11th day after streptomycin application. Gaze responses were also examined at 3–4 wk PST (n = 3 birds), 6–8 wk PST (n = 5 birds), 12 wk PST (n = 3 birds), and 6 mo PST (n = 5 birds). For experiments conducted during the earliest time point (1 wk PST), an abbreviated frequency bandwidth protocol was used due to the inability of the animals to maintain their heads upright during head-free conditions. Subsequent time points involved a complete stimulus frequency series. For each bird, three to four repeated measurements were obtained on subsequent days during the recovery stage. Repeated measurements were then averaged for individual birds for each time point of recovery. Most birds were tested only during a specific recovery stage (contact lens coil animals); however, five birds (chronic eye coil animals) were tested at several stages (≤3) before being used for terminal afferent recording experiments. Each recording session lasted for periods between 2 and 3 h. All animals were gradually adapted to the passive restraint for several sessions prior to collecting data. No animal exhibited signs of discomfort or stress during the experiments. All birds tolerated the body holder and motion stimulation well.
Data analysis

Procedures for analyses used in this study were the same as those utilized in our previous gaze study for normal pigeons and quails (Haque and Dickman 2005) and similar to ones described in further detail elsewhere (Angelaki et al. 2000, 2003; Dickman and Angelaki 1999; Dickman et al. 2000). Horizontal (hor), vertical (ver), and torsional (tor) eye and head movement components were defined as rotations about the animal’s dorsal-ventral (z), intra-aural (y), and naso-occipital (x) axes, respectively (Fig. 1A). Positive rotations were defined as leftward, nose downward, and right-ear downward, respectively. It should be noted that these coordinates apply to a head-fixed reference frame as opposed to rotations directed about the bird’s primary visual axis (Fig. 1; 66° away from the bill tip) that corresponds to an approximate line of sight during lateral viewing conditions (Martin and Young 1983; Martinoya et al. 1984). Prior to each experiment, spontaneous eye movements (head-fixed) and head movements (head-free) to orienting stimuli were recorded for 60 s. From these spontaneous movements, mean primary eye and head positions were calculated. This calibration procedure has often been used successfully for three-field systems to determine eye coil sensitivity and primary position in several species. It provides a good approximation as long as DC offsets are negligible (Angelaki et al. 2003; Haque and Dickman 2005; Hess et al. 1992; Tweed et al. 1990).

Recorded eye movement signals were first converted to rotation vectors for the eye in head-fixed (E_h, E_v, E_t) or for the eye (gaze) in head-free (G_h, G_v, G_t) conditions along with the head movement components (H_h, H_v, H_t) using Matlab (MathWorks). These signals were then manually desaccaded (Dickman and Angelaki 1999) and differentiated into rotational velocity vectors corresponding to eye (E_h, E_v, E_t) conditions along with the head movement components (H_h, H_v, H_t) using Matlab (MathWorks). These signals were then manually desaccaded (Dickman and Angelaki 1999) and differentiated into rotational velocity vectors corresponding to eye (E_h, E_v, E_t) and head (H_h, H_v, H_t) conditions along with the head movements (H_h, H_v, H_t) (Haque and Dickman 2005). Because each subject made occasional volitional gaze saccades during head-free trials, data were accepted only when the head was held within 30° of reference position during the stimulus. Several cycles were averaged and fit with a sine curve using a least-squares algorithm where the mean eye position was the sum of a DC offset position and a modulation term. The fitted mean sine curves were used to calculate gain and phase values in each of the three planes. Gain was expressed as the ratio of peak eye/head/gaze velocity to peak rotation velocity. The gain and phase of the eye-in-head responses were computed from the complex vectorial equation \[ E = (G - H) \], where \( E \), \( G \), and \( H \) represent the eye, gaze, and head response components, respectively. Average responses for gain and phase were also computed in the complex plane (Haque and Dickman 2005; Wei and Angelaki 2001). Finally, SD for gain and phase were calculated using the error propagation formula (Bevington and Robinson 1992).

To test equivalence in eye movement measurement techniques, three animals with implanted eye search coils were also given an acute contact lens coil applied to the same eye during some experimental protocols. Comparisons between the response gains for the two coils measuring identical eye movements were made. Figure 1B shows an example VOR (head-fixed) response from an animal that was rotated about the EVA axis at 1 Hz (20°/s peak velocity) with both implanted and contact lens coils attached to the same eye. The desaccaded averaged eye velocity traces were then overlaid for comparison. The gain values for the implanted and contact lens coils were 0.48 and 0.46, respectively. Clearly, the results from the two different coil measurement techniques yielded equivalent measures. Comparable findings were obtained in the other birds examined for all the stimulus frequencies used here; providing us the confidence to utilize the combination of results from the two eye coil methods for subsequent analyses.

Electron microscopy

To correlate the physiological responses with anatomical changes, SEM was used to examine the density and distribution of regenerated hair cells. Each pigeon was anesthetized, and the bony labyrinth was bilaterally exposed. Both the horizontal and posterior canals were opened and an intralabyrinthine perfusion was performed with a 5 ml volume of 3% glutaraldehyde, 2% paraformaldehyde, and 1% Acrolein in 0.1 M phosphate buffer (PB). The animal was subsequently perfused transcardially with a 2% glutaraldehyde, 1.5% paraformaldehyde solution in 0.1 M PB, and the whole head was placed in the aldehyde fixative for 24 h. The membranous labyrinth was then excised, and each of the canal cristae and otolith maculae were dissected free. The tissues were rinsed (dH2O), then dehydrated using a series of graded acetones (70, 90, 95, and 100%). Next, the tissues were incubated in a 1:1 mixture of tetramethylsilane (TMS) and acetone for 45 min and then transferred to a 100% TMS solution for 1 h. The tissues were subsequently sublimated by desiccation at 60°C in an open container. The dried cristae were mounted on aluminum...
studs, gold-coated, and photographed using a Hitachi 2600 SEM (20 kV).

**Statistical analyses**

Most response values at different regeneration stages were compared using repeated-measures ANOVA with commercial software (Statistica, Tulsa, OK). Main effects were collapsed across responses to all frequencies to examine mean effects due to regenerative recovery. Interactions were used to compare the response profile (gain or phase) across frequencies by a factor of regenerative recovery. Post hoc comparisons were performed using the Scheffé procedure.

**RESULTS**

**Gaze response in normal animals**

Previously, we established that the VOR response (eye in head-fixed) in pigeons is compensatory in direction but under-compensatory in amplitude for rotational stimulation along all axes of motion (Dickman et al. 2000; Haque and Dickman 2005). Similar findings were also observed in the present study, as shown by the responses for one pigeon in Fig. 2 and by the averaged responses for six normal birds in Fig. 3. During EVA rotational motion with the head fixed in the dark, a large horizontal eye movement component was observed with occasional small secondary vertical and torsional components that were typically an order of magnitude less than the primary response. However, the normal head-fixed VOR was under-compensatory and could only maintain a partial visual fixation during passive EVA rotational motion (Figs. 2 and 3). For example, the highest mean eye velocity gain values (eye velocity/head velocity) observed were only 0.64 during 1.0-Hz EVA rotation (Fig. 3). However, when the head was freed to assist in stabilization during rotational motion, an improved gaze (eye-in-space) response was observed (Fig. 2). In fact, the averaged gains approached unity at mid to high frequencies (i.e., 0.5–2 Hz), as shown in Fig. 3. The increase in gaze stability was achieved primarily through an augmented head movement response (Fig. 2). Head component gains alone were higher than the corresponding head-fixed VOR gains at 0.05 Hz and remained so during all higher frequencies of motion (Fig. 3). In contrast, the eye-in-head component gains during head-free motion were actually attenuated below the values obtained for the head-fixed VOR response (Figs. 2 and 3). These results show that the compensatory head response served as a significant contributor toward gaze stabilization during head-free motion in normal pigeons. Further, gaze values approached unity for frequencies >0.5 Hz. This indicates that a near complete compensatory response for head stabilization occurs at higher rotational frequencies, even in the dark. Similar gain profiles as a function of rotation frequency were observed for the head component and VOR responses (Fig. 3). In contrast, the eye component gains during head-free motion remained flat across the frequency bandwidth examined, except for low responses at 0.01 and 0.02 Hz. During EVA motion, the phase values were similar for all gaze and VOR components. At low head motion frequencies, all com-

![Fig. 2. Pigeon 3-dimensional gaze, head, eye, head-fixed eye (VOR) responses to EVA yaw 1.0 Hz (20°/s) rotational motion. \( \Omega_{\text{tor}}, \Omega_{\text{ver}}, \Omega_{\text{hor}} \) refer to the torsional, vertical, and horizontal positional components of gaze, head, and eye. \( \dot{\Omega}_{\text{tor}}, \dot{\Omega}_{\text{ver}}, \dot{\Omega}_{\text{hor}} \) represent the torsional, vertical, and horizontal velocity components with only the slow phases shown and fast phases removed. Bottom traces: stimulus velocity, \( H_{\text{vel}} \). For all traces, positive is upward and corresponds to leftward, downward, and counterclockwise movements with respect to the animal.](https://www.jn.org/jn/article-pdf/99/2/856/285104/856.pdf)
ponents led head velocity by 60–90°. These advanced leads declined to values of near 30° by 0.1 Hz and 5–10° at 0.5–2 Hz.

Gaze responses were also examined using EHA rotational motion in normal birds (n = 6). As shown in Fig. 3, during sinusoidal roll rotations, the mean primary eye, head, and gaze components were spatially compensatory in direction about the animal’s head axis (Fig. 1). However, the gaze response dynamics were different from those observed with yaw rotations. The gaze and head component gains during EHA motion were augmented at the lower frequencies as compared with similar responses during EVA motion. In addition, the response phases for all components were not greatly advanced at the lower frequencies, but in fact were flat across the frequency bandwidth examined with values remaining close to head velocity. The increased low-frequency gains and decreased phase leads at low rotational frequencies for EHA versus EVA motion have previously been attributed to the contribution of otolith signals providing an additional reference of head velocity (Angelaki and Hess 1996; Barmack 1981; Dickman et al. 2000; Rude and Baker 1988).

Receptor recovery

To characterize the extent of receptor recovery for each of the regenerative periods examined behaviorally, SEMs of the semicircular canals were obtained. Figure 4 shows representative canal neuroepithelia for the horizontal (HC), anterior (AC), or posterior (PC) semicircular canals under normal, 1 wk PST, 4 wk PST, 8 wk PST, 12 wk PST, and 6 mo PST. In normal birds (Fig. 4A), hair cells densely populated the receptor epithelia. Hair cells with the longest stereocilia were observed in the torus and apex regions as well as along the edges of the epithelium (see Haque et al. 2006 for a more detailed description). At 1 wk PST, the epithelium was completely denuded of hair cells due to streptomycin ototoxicity as shown in Fig. 4B where no stereocilia are visible. We previously have verified histologically that a complete lesion of the vestibular receptor epithelia is present by 4 days PST (Dye et al. 1999; Frank et al. 1999; Zakir and Dickman 2006). By 4 wk PST (Fig. 4C), stereocilia begin to appear in the torus and peripheral regions of the canal cristae, but the central apex and isthmus regions were sparsely populated. Few of the stereocilia present at this regeneration stage were of similar lengths as those observed under normal conditions (Fig. 4A), suggesting that mostly immature hair cells were present. At 8 wk PST (Fig. 4D), hair cell density was increased in all regions but remained proportionally lower in the central crista. Numerous hair cells with long stereocilia were present in the torus and peripheral regions. Although not quantified in the present semicircular canal tissues, previously, we found that hair cell density had returned to ~60% of normal by 8 wk PST in vestibular otolith organs (Dye et al. 1999). Hair cell density and stereocilia lengths were greatly increased in central regions by 12 wk PST (Fig. 4E), and by 6 mo PST, the neuroepithelium exhibited hair cell densities that were comparable to those observed in normal cristae (Fig. 4F) (Dye et al. 1999).

Gaze response during regeneration

After streptomycin surgery (PST 2–4 days), all of the birds utilized in the present study (n = 20) exhibited markedly different behavior from that observed during normal conditions, primarily characterized by pronounced ataxia. Evident among all of these animals were postural instabilities and a
total lack of head control, including difficulty in standing or perching (Dickman and Lim 2004). At 1 wk PST, all 20 animals were unable to maintain head stability with continuous head oscillations being observed even when standing stationary. These animals could not orient their head position relative to gravity, and most exhibited circling and/or staggered gaits.

Consistent with the severity of dysfunction observed, all gaze stabilization response components were abolished (near zero gains; \( n = 4 \) birds) during rotational testing at 1 wk PST and significantly differed from that in normal (\( n = 6 \) birds) [e.g., gaze: \( F(1,8) = 358.4, \ P < 0.001 \)] as illustrated in Fig. 5. With all gaze component gains being near noise levels, the response phase values were quite varied and exhibited large SDs. A similar total loss in VOR response was also observed during head-fixed rotational motion.

By 3–4 wk PST, gaze stabilization responses began to slowly recover as shown in Fig. 5. Recovery was incomplete, however, as the main effect for mean (\( n = 3 \)) gaze \[ F(1,7) = 24.8, \ P < 0.001 \], head \[ F(1,7) = 14.4, \ P < 0.01 \], eye \[ 0.01–0.1 \text{ Hz}: F(1,7) = 9.0, \ P < 0.05 \], and VOR \[ F(1,7) = 13.4, \ P < 0.01 \] component gains were all significantly reduced below that observed in normal animals. Strikingly, the initial return of function was manifested only during the higher head movement frequencies. In fact, low-frequency gaze responses were absent with no significant gaze component gain observed to rotational motions <0.05 Hz. Thus the frequency response functions were tuned to only the faster head motions at this recovery stage as evidenced by significant interactions between recovery time and frequency for all mean gaze components [gaze: \( F(6,42) = 11.3, \ P < 0.001 \); head: \( F(6,42) = 8.5, \ P < 0.001 \); eye \[ 0.01–0.1 \text{ Hz}: F(6,42) = 3.2, \ P < 0.01 \]; VOR: \( F(6,42) = 3.3, \ P < 0.01 \)] as compared with normal (\( n = 6 \)) birds. In terms of the specific gaze mechanisms, it was the head response component that was the initial major contributor to gaze stabilization in head-free conditions, whereas the eye movement component remained low. This was particularly true for the low to mid frequencies of rotational motion (<0.1 Hz), where the head component was the only mechanism contributing to gaze with no eye response significantly above noise level (\( P > 0.05 \)). In fact, for all frequencies <0.5 Hz, the head component gain was equivalent to the gaze gain (\( P > 0.05 \)). However, during faster head motions (i.e., ≥0.5 Hz), the eye was contributing to gaze stabilization, at levels equal to that observed prelesion. In terms of phase, significantly increased leads with large variances were observed for all gaze components during slow head motions [e.g., main effect gaze: \( F(1,7) = 26.9, \ P < 0.01 \)]. At higher rotational frequencies, phase values were less variable. Still the response phase values remained significantly advanced for the mid-frequency rotations and approached normal measures (near head velocity) at only 1 and 2 Hz. During head-fixed rotations, the VOR gain also recovered early for frequencies >0.5 Hz but remained significantly less than normal levels \[ F(1,7) = 13.4, \ P < 0.01 \]. Correspondingly during early regenerative recovery, animals regained some postural stability as circling behavior subsided. Generally, head stability during stationary or walking behaviors improved; however, frequent head oscillations were still observed (see Dickman and Lim 2004).

At 6–8 wk PST, the majority of birds did not exhibit ataxic behavior and were able to maintain stable head posture at rest. During rotational motion, increasing gains in all gaze response
components were observed, particularly in the low- to mid-frequency range (0.05–0.1 Hz). However, the complete gaze response profile \( (n = 5\) birds) remained significantly lower than normal \( [\text{main effect: } F(1,9) = 14.3, P < 0.01] \) with gains being only 80% of the magnitude seen prelesion. The relative contributions of each component to the total gaze response also exhibited a change during head-free motion. The head component gain response actually diminished, whereas a corresponding rise in the eye movement component significantly increased above normal values at the mid-high frequencies \( [0.1–5\) Hz; \( F(1,9) = 5.2, P < 0.05] \) but not at low frequencies \( (<0.1\) Hz). Furthermore, the VOR response continued to improve toward preinjury levels. Of note was the close correspondence in the head-fixed VOR gain and head component gain during head-free conditions. This trend continued throughout recovery \( (\text{see following text})\). With respect to the response phase, variability diminished as gain values increased. The phase leads were more aligned with preinjury values than at 3–4 wk PST but were still significantly more advanced than normal bird values \( [\text{e.g., gaze main effect: } F(1,9) = 23.4, P < 0.001] \).

Continuing the trend seen during the 6–8 wk postlesion responses, by 12 wk PST \( (n = 3\) birds), substantial increases across the tested bandwidth of head motion were observed, including the low-frequency range. In fact, there was no significant difference between the 12 wk PST gaze gains and those observed in normal birds for either the main effect or the interaction comparisons. Essentially, by 12 wk PST, overall gaze stability during rotational motion had recovered to prelesion levels. However, as the overall gaze gain increased, the relative contributions by the head and eye component gains appeared to change. The head component was attenuated while the eye component was augmented compared with the previous recovery time points, particularly at the mid- to high-frequency response levels \( (\text{Fig. 5})\). Thus the head gain was significantly reduced \( [\text{main effect: } F(1,7) = 7.8, P < 0.05] \) and the eye component gain increased marginally for the mid to high frequencies \( [\text{main effect: } F(1,7) = 2.1, P < 0.17] \) for the 12-wk PST group \( (n = 3)\), as compared with normal birds. The large variance seen in the 12-wk values were primarily due to one of the three tested birds that maintained low gain components more similar to the 6- to 8-wk recovery stage animals. Still, the values for this bird were included in all statistical comparisons. During rotational motion when the head was fixed, the VOR response remained comparable to normal values. Phase values were slightly increased for all gaze components relative to normal values, but these differences were only marginally significant \( (i.e., P < 0.1)\).

At 6 mo PST \( (n = 5\) birds), the last regeneration time point tested, no significant change in the overall gaze stability above that observed for the 12-wk PST responses was present. Gaze gain and phase values were not significantly different \( (\text{main effect or interaction})\) from normal bird responses. These findings show that for gaze stability, recovery of the compensatory response as well as the frequency bandwidth for rotational motion in regenerated birds were no different from those of normal birds. However, as observed for the 12-wk PST animals, a marked difference in the relative contributions between the head and eye components toward the overall gaze response had occurred in regenerated animals. This was true for four of the five birds tested, whereas the remaining bird exhibited large head and small eye component contributions to gaze; more similar to the pattern exhibited by normal animals. This observation points to an increase in response variability in regenerated recovery animals as compared with that of normally developed birds. Nonetheless, the mean head gains for all five of the 6-mo PST animals were significantly lower \( [\text{main effect: } F(1,7) = 7.8, P < 0.05] \) than normal \( [\text{main effect: } F(1,7) = 7.8, P < 0.05] \).

**FIG. 5.** Recovery of gaze responses to EVA (yaw) rotational motion during regeneration at 1, 3–4, 6–8, and 12 wk and 6 mo PST. Mean gaze (black circles), head (red triangles), eye-in-head (blue squares), and VOR (open blue squares) component responses are shown, plotted as a function of rotational motion frequency. Gain = component response/peak head velocity. Phase = component response peak relative to peak head velocity (deg). Error bars = SE.
$F(1,9) = 6.2, P < 0.05$], whereas the eye component gains were significantly increased [main effect: $F(1,9) = 7.2, P < 0.05$] as compared with normal birds. The change in component contributions was most pronounced at mid to high frequencies as exhibited by the significant interaction between frequency and PST level [$F(6,54) = 6.0, P < 0.01$]). The eye, but not the head, phase values were slightly increased for the lower rotational frequencies (i.e., $<0.5$ Hz) by $5–10^\circ$ above normal [$F(1,9) = 9.9, P < 0.05$]. VOR responses were similar to those observed at 12 wk PST and were not different from those observed prelesion.

**Gaze recovery depends on frequency of rotational motion**

To examine more closely the relationship between functional recovery and speed of head motion, the mean gaze response components were examined for two different frequencies of interest. The responses at 0.05 Hz were examined to represent the low-frequency range and 0.5-Hz responses for the mid- to high-frequency range. The component gain values for gaze, head, eye-in-head-free, and VOR were plotted for both earth-vertical axis (EVA) and earth-horizontal axis (EHA, roll) rotations, as shown in Fig. 6. The comparisons between the different frequency responses readily show that gaze stability recovered more quickly to prelesion levels during 0.5-Hz rotational motion than at lower frequencies. For example, gaze gains recovered quickly to 80% of normal levels for EVA rotations by 4 wk PST for 0.5 Hz, whereas they did not return to the same level of function until 12 wk PST at the lower frequency range. A similar trend was noted for EHA rotations, although these responses exhibited a slower recovery time course. It was not until 12 wk PST for the EVA responses [$F(1,7) = 0.03, P < 0.83$] and 6 mo PST for the EHA responses [$F(1,9) = 1.02, P < 0.35$] that gaze function had recovered to prelesion levels. As noted earlier, the major response component to gaze function was the head movement particularly during early recovery. The eye-in-head component (head-free motion) increased proportionally through recovery time for both EVA and EHA stimulation to help stabilize gaze. As noted in the preceding text, the head component gain decreased and the eye component gain increased for all rotational motion responses during the latter recovery periods.

![Comparison of gaze recovery at representative low (0.05) and mid-high (0.5) frequencies of rotational motion. Mean gain gaze (black circles), head (red triangles), eye-in-head (blue squares), and VOR (open blue squares) component responses are shown, plotted as a function of regeneration time (PST). Mean gaze gain responses for EVA motion are shown in the top panels and for EHA (roll) motion in the bottom panels. Error bars = SD.](image-url)
To better quantify the relationship between head and eye responses to gaze stability during regeneration, the ratios between the head component and gaze, as well as the eye and head components, were computed. Figure 7 shows the comparison between head:gaze and eye:head ratios as a function of regenerative recovery during 0.5-Hz rotational motion. The head:gaze ratios significantly decreased \( (r^2 = 0.82) \) while the eye:head ratios significantly increased \( (r^2 = 0.8) \), compared with normal birds as indicated by the regressions slopes being different from zero. A similar relationship was observed for the responses to roll motion. For the regressions, the 1-wk recovery ratios were not used because these values were essentially zero. These findings indicate that although the initial recovery of gaze stability mostly relied on head movement components, later recovery included a more substantial eye component response than was typical in normal development responses.

Next we sought to determine if the VOR followed the recovery of either the gaze components during recovery as suggested from the dynamic responses observed previously. Thus the relationships between the eye and head gaze function components during head-free motion were examined relative to the head-fixed VOR responses using mean correlation plots for both EVA and EHA rotational motion as shown in Fig. 8. The response gains for the different components were evaluated for both a low \( (0.05 \text{ Hz}) \) and a high \( (0.5 \text{ Hz}) \) rotational frequency. For each comparison, the unity slope line (dashed) indicates equal gain contributions. For low frequencies, the early regenerating animals had eye component and VOR gains clustered near the unity axis denoting near equal values for eye movements during both EVA and EHA conditions (Fig. 8A). Albeit, these gains were relatively small. However, as regenerative recovery progressed, eye movements during head-fixed conditions increased in gain while during head-free motion, the eye component was comparatively less. The effect was more pronounced at the higher \( (0.5 \text{ Hz}) \) rotational motion frequency (Fig. 8C). Here, the head-fixed VOR increased rapidly during recovery, while the eye-in-head component gain during head-free gaze also increased but with a lesser slope. At both frequencies, the 12-wk and 6-mo PST recovery gains were similar to that seen in normal animals. A different relationship emerged when comparing head component to fixed-VOR responses (Fig. 8, B and D). Here, head component gains were nearly matched by the increasing fixed-VOR gains over regeneration time for both low- and high-frequency motion. The 12-wk and 6-mo PST recovery values for the head component gains were similar to each other but again showed a difference between regenerated and normal birds. In this case, a decreased contribution of the head to gaze stability was noted, more equivalent to that observed during VOR alone with head-fixed motion.

**DISCUSSION**

Our findings present the first comprehensive examination of gaze stabilization recovery during vestibular regeneration using both head-free and head-fixed motion. We found that gaze stability returned to normal function according to a temporal sequence lasting several months. However, regenerative gaze stability was comprised differently from that observed in intact pigeons. We also found that the dynamic recovery of gaze function was not homogeneous for all types of motion. In fact, high-frequency motion stability was first achieved, followed much later by slow movement stability. In addition, we found that initial gaze stability was established using almost exclusive head response components with little eye movement contribution. However, that trend reversed as recovery progressed to levels significantly different from normal birds.

What insights have we gained regarding how gaze stability is achieved during regenerative recovery? First, it should be noted that following bilateral vestibular loss in mammals where little or no regeneration occurs, head and eye responses to motion can partially recover (Dichgans et al. 1973; Stapley et al. 2006; Waterston et al. 1992). These functional returns are thought to be manifested largely by central vestibular mechanisms such as neural compensation (Newlands and Perachio 1990a,b; Zennou-Azogi et al. 1994) and adaptation (Anastasio 1992; Serafin et al. 1999) to any available motion cues. Most investigations have examined functional recovery following unilateral vestibular lesions (Dieringer and Precht 1979; Newlands and Perachio 1990a,b; Smith and Curthoys 1988) or partial bilateral loss (Galiana et al. 2001; Yakushin et al. 2005) where significant motion signals remain from intact vestibular...
receptors. Although very few studies have examined central recovery mechanisms following complete bilateral vestibular loss (similar to the types of lesions produced in the present study), it has been shown that central vestibular cells can recover some responsiveness to motion. The recovery is likely due to increased sensitivity to extra-vestibular cues such as vision, proprioception, somatosensory, and visceral receptors (Heimbrand et al. 1996; Jensen 1979; Mittelstaedt 1992; Yates et al. 2000). In animals that demonstrate spontaneous regeneration, a more complicated interplay is certainly involved, where a coupling occurs between immediate neural plasticity and the gradual return of dynamic vestibular receptor signals over a prolonged time. This provides for unique central neural recovery mechanisms that have yet to be investigated.

However, recent work has shed light on receptor, afferent, and behavioral recovery during regeneration in birds. Here we propose a scheme for gaze stabilization recovery using the known characteristics learned from these efforts, given the caveat that plasticity mechanisms are also likely to be involved. Immediately following the lesion, no receptor cells were present and the epithelia were completely denervated (Dye et al. 1999; Zakir and Dickman 2006). No afferent signals relating head velocity or position to central vestibular neurons were available, thus no measurable VOR or VCR response was observed consistent with the findings of others (Boyle et al. 2002; Carey et al. 1996; Goode et al. 1999; Matsui et al. 2003). This left only the CCR and head mechanics to maintain head stability. We suggest that constant inertial forces, augmented by the long articulated neck in pigeons (Chiaisson 1984), produced large head displacements. Head motion then initiated increased neck proprioceptive signals to drive the CCR in an attempt to right the head relative to the body (Goldberg and Peterson 1986; Keshner and Peterson 1995). However, without VCR compensatory feedback, the CCR alone (essentially open-loop) was insufficient to maintain head position and failure resulted. The results presented with large-amplitude head shakes, severe postural ataxia, and spatial disorientation (Dickman and Lim 2004). These unstable head shakes (1–2 every few seconds) fall within the bandwidth that head mechanics and the CCR contribute to head stability in mammals (Goldberg and Peterson 1986; Keshner and Peterson 1995).

Recently we reported that morphological regeneration of the vestibular receptors occurs along a three stage temporal sequence (Zakir and Dickman 2006). Stage 1 regeneration lasted through the first month 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). During this time, it is likely that increased sensitivity in central vestibular neurons to head/neck proprioceptive and somatosensory signals occurred through adaptive plasticity (Yates et al. 2000). Plasticity, combined with the limited regenerating vestibular afferent signals was sufficient to elicit some recovery in the head component of gaze because closed feedback mechanisms were now being provided to the VCR and CCR. Interestingly, the VCR has previously been shown to recover much before the subsequent return of the VOR during regeneration (Carey et al. 1996; Goode et al. 1999, 2001). Thus early regenerative gaze stability was dominated by the VCR and CCR with eye components and the VOR contributing far less (Figs. 5–7). The striking finding that
early recovery was restricted to only the higher frequencies of motion (>0.1 Hz) was unexpected. We originally hypothesized that low-frequency gaze stability would recover first, which our findings now refute. Because only bouton fibers and type II hair cells innervating the peripheral zones of the cristae and maculae exist at this regeneration stage, these neurons must be providing the dynamic signals necessary to control the high-frequency recovery observed (present data; Zakir and Dickman 2006). Bouton fibers in these regions have generally been reported to have a more regular discharge rate, lower gain, and lower frequency bandwidth but do respond within the dynamic range needed to supply the recovering VCR (Baird et al. 1988; Fernandez et al. 1988; Lysakowski et al. 1995; Myers and Lewis 1990). In previous studies of stage 1 regenerating birds, some vestibular afferents were reported to respond within this motion frequency range, but they did so inconsistently with low gains and variable phase values (Boyle et al. 2002; Li and Correia 1998). Our second hypothesis predicted that maintenance of a stable head in space must precede the recovery of gaze or visual stabilization mechanisms. Our previous navigation study found that the constant head shakes exhibited early postlesion had greatly subsided by 2–3 wk PST (Dickman and Lim 2004). These maladaptive head shakes occurred in a mid- to high-frequency range. As afferent head motion signals became available, central compensation more similar to that occurring following a partial vestibular loss could begin (Galiana et al. 2001; Yakushin et al. 2005), which resulted in reduced error in the VCR and CCR responses. Maintenance of a stable head position relative to gravity appears to be vital for stabilizing motor behavior in birds. Indeed when head-in-space orientation is compromised by restricting head movement relative to the body in normal birds (using a stiff collar), flight and orientation abilities are lost (Warrick et al. 2002). This suggests that substantial neural mechanisms are dedicated toward that function.

The second stage of morphologic regeneration occurred between 6 and 12 wk PST. During early stage 2 regeneration, hair cell densities had increased to 50% that of normal, bouton afferents exhibited larger terminal fields, and the first calyceal terminals were beginning to develop (Masetto and Correia 1997a,b; Zakir and Dickman 2006). However, these calyceal terminals were rudimentary and were exclusively located on simple dimorph afferents (fibers that contain both calyceal and bouton terminals), as calyx afferents (containing exclusive calyceal terminals) did not yet exist (Zakir and Dickman 2006). Early stage 2 regeneration was also marked by the lack of significant numbers of hair cells or afferents in the central regions of the receptor epithelia, areas known to contain the highest density of type I hair cells and calyceal terminal-bearing afferents (Brichta and Peterson 1994; Haque et al. 2006; Lysakowski and Goldberg 1997; Si et al. 2003; Zakir et al. 2003). Here gaze responses exhibited increasing gains, particularly in the mid- to high-frequency range, although the lowest frequencies tested (0.01 and 0.02 Hz) still produced little compensatory response. The mid-range gaze responses returned, as afferent innervation grew toward the central regions of the cristae and maculae. During early stage 2 regeneration, two interesting novel trends in the composition of the gaze response were observed that continued throughout recovery. First, the eye-in-head and VOR responses were increasingly augmented, producing a concurrent reduction in the head component response (Figs. 5 and 7). Second, the head-fixed VOR gain response profile began to match that of the head component during head-free gaze. Neither of these trends was characteristic of gaze stability in normal birds (Fig. 3). As previously reported, initial recovery of head-in-space stability can be achieved independent of vision, solely through recovery of the VCR (Goode et al. 2001). However, as head stability increased, a significant reduction in the visual error signal ensued so that a manageable range for compensation of eye movements in response to motion using retinal slip and adaptive cerebellar mechanisms could follow (Broussard and Lisberger 1992). Indeed a major class of central vestibular neurons (eye-head cells) are thought to be involved in VOR learning and carry both eye and head movement signals (Lisberger et al. 1994a,b; Roy and Cullen 2003; Scudder and Fuchs 1992). Further, these neurons likely include the vestibulo-ocular collicular cells, known to receive direct vestibular afferent input and collaterally project to both vestibuloooculocerebral and vestibulospinal targets (Boyle 1983; Boyle et al. 1992; McCrea et al. 1999). It is tempting to speculate that these central vestibular neurons form a substrate during regeneration that functions in a state-dependent manner to equilibrate the gaze contributions from the head component during head-free motion and the VOR during head-fixed motion. If so, this mechanism would be a novel product of neural processing established during regeneration as the head component and the VOR responses are not equivalent in normal birds (Fig. 2) (Haque et al. 2006). These findings are consistent with our navigation study, where we observed increased postural stabilization, the initiation of directed head saccades, and improved orientation (Dickman and Lim 2004). Thus high-frequency gaze stabilization (tremor reduction) continued to improve during early stage 2 regeneration as well as the ability to maintain the head stationary in space.

During late stage 2 regeneration (12 wk PST), gaze stability finally returned for the lowest head motion frequencies, and overall gaze stability had recovered to prelesion levels (Figs. 5 and 8). Morphologically, the peripheral bouton afferents had increased in complexity and the central receptor regions were becoming innervated by dimorph and calyx afferents (Zakir and Dickman 2006; personal observation). In mammals, the central regions of the cristae and striolar regions of the maculae contain irregularly firing high gain dimorph and low gain calyx afferents with large dynamic ranges (Baird and Lewis 1986; Baird et al. 1988; Fernandez et al. 1988; Goldberg et al. 1990; Lysakowski et al. 1995). We suggest that these central regions 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. Consistent with this finding are reports that show reversible silencing of the most irregular firing afferents in primates reduced or eliminated VOR responses to low-frequency motion but not to mid- to high-frequency rotations (Angelaki et al. 1992; Minor and Goldberg 1991).

Morphologic recovery was completed by the third and final stage of regeneration, measured at 6 mo to 1 yr PST (Zakir and Dickman 2006). However, we previously found that the inner-nerve patterns of regenerated calyx, bouton, and dimorph afferents were significantly different from normal fibers. Regenerated 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). 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, at 6 mo PST, no difference in total gaze or VOR responses when compared with normal birds were found, indicating that gaze stability had fully recovered for both head-free and head-fixed motions. We also found that the behavioral components contributing to gaze stabilization shift in their weighting during regeneration, different from those of normal birds. How vestibular central neurons appropriately adapt to provide the rich gaze behavioral repertoire needed to survive remains a fascinating subject relevant to neural control and reparative processes in general.

Acknowledgments

The authors acknowledge the invaluable contributions of D. Huss and D. Angelaki.

Grants

This work was supported in part by Howard Hughes Medical Institute Grant 57003555, the American Otological Society, National Institute of Deafness and Other Communications Disorders Grants F31-DC-006574, DC-003286, DC-007618, and DC-006913, and National Aeronautics and Space Administration Grant NNA04CC52G.

References


