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Contribution of vestibular efferent system alpha-9 nicotinic receptors to vestibulo-oculomotor interaction and short-term vestibular compensation after unilateral labyrinthectomy in mice

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Abstract

Sudden unilateral loss of vestibular afferent input causes nystagmus, ocular misalignment, postural instability and vertigo, all of which improve significantly over the first few days after injury through a process called vestibular compensation (VC). Efferent neuronal signals to the labyrinth are thought to be required for VC. To better understand efferent contributions to VC, we compared the time course of VC in wild-type (WT) mice and $\alpha 9$ knockout ($\alpha 9^{-/-}$) mice, the latter lacking the $\alpha 9$ subunit of nicotinic acetylcholine receptors (nAChRs), which is thought to represent one signaling arm activated by the efferent vestibular system (EVS). Specifically, we investigated the time course of changes in the fast/direct and slow/indirect components of the angular vestibulo-ocular reflex (VOR) before and after unilateral labyrinthectomy (UL). Eye movements were recorded using infrared video oculography in darkness with the animal stationary and during sinusoidal (50 and 100°/s, 0.5–5 Hz) and velocity step (150°/s for 7–10 s, peak acceleration 3000°/s²) passive whole-body rotations about an Earth-vertical axis. Eye movements were measured before and 0.5, 2, 4, 6 and 9 days after UL. Before UL, we found frequency- and velocity-dependent differences between WT and $\alpha 9^{-/-}$ mice in generation of VOR quick phases. The VOR slow phase time constant (TC) during velocity steps, which quantifies contributions of the indirect component of the VOR, was longer in $\alpha 9^{-/-}$ mutants relative to WT mice. After UL, spontaneous nystagmus (SN) was suppressed significantly earlier in WT mice than in $\alpha 9^{-/-}$ mice, but mutants achieved greater recovery of TC symmetry and VOR quick phases. These data suggest (1) there are significant differences in vestibular and oculomotor functions between these two types of mice, and (2) efferent signals mediated by $\alpha 9$ nicotinic AChRs play a role during VC after UL.

Keywords

Vestibular compensation; VOR; Vestibular efferent system

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1. Introduction

Unilateral afferent vestibular loss causes oculomotor, postural and motion symptoms and signs including vertigo, spontaneous nystagmus, ocular counter-roll and skew deviation, roll and yaw head tilt, contralateral limb extension and ipsilateral limb flexion, barrel rolling and circular walking [1]. Most symptoms and signs of a large difference in vestibular input from the two ears following unilateral labyrinthectomy (UL) decrease markedly over the first few days to weeks after injury, through a process of central nervous system adaptation commonly called vestibular compensation (VC). Immediately after UL, asymmetry in activity of neurons in the vestibular nuclei (VN) elicits oculomotor and postural symptoms because VNs neurons provide the major premotor drive to motoneurons mediating the vestibulo-ocular and vestibulo-spinal reflexes [2]. VC mechanisms rebalance activity between ipsilateral and contralateral VN via inhibitory commissural GABAergic and glycinergic connections [3,4]. Suppression of oculomotor and postural symptoms over the first 48 h after UL corresponds to a marked increase of GABA release in the ipsilateral VN and a subsequent return to near-normal levels by 96 h after UL [3]. Following these rapid compensatory changes, ipsilateral VN gliosis, neurogenesis and plasticity mediated in part by changes in extracellular matrix also contribute to long-term VC [4].

The importance of cerebellar contributions to VC is demonstrated by the fact that compensatory recovery of VOR symmetry and suppression of spontaneous nystagmus (SN) after UL is severely delayed or abolished by prior ipsilateral flocculectomy [5] or genetic cerebellar deficiency [6].

The efferent vestibular system (EVS) is also thought to play a role in VC [7]. Vestibular efferent neurons synapse upon both hair cells and primary vestibular afferents [8,9]. In reptiles, birds, and mammals the EVS has strong synaptic inputs to calyx afferents, type II hair cells, and bouton afferents innervating type II hair cells [10]. Vestibular efferent neurons use acetylcholine receptors (AChRs) for synaptic transmission, in particular, an efferent-mediated inhibition is provided by activation of $\alpha 9/\alpha 10$ nicotinic AChRs on only the type II hair cells, while the $\alpha 4\beta 2^*$ nicotinic AChRs and muscarinic AChRs contribute to fast and slow efferent-mediated excitation in type II hair cells and calyx afferents [11].

The role of the commissural inhibitory system in short-term VC has been extensively investigated [3,4]; however, few published data are available to describe the contributions of the EVS, particularly efferent-mediated inhibition, to vestibulo-oculomotor function during VC after partial vestibular loss. Release of ACh due to EVS stimulation does act via $\alpha 9/\alpha 10$ nAChRs on type II hair cells, leading to Ca^{2+} influx, activation of Ca^{2+} -dependent potassium channels, and a rise of potassium efflux, which in turn causes hyperpolarization of type II hair cells. This hyperpolarization subsequently reduces Ca^{2+} influx, mainly through action on voltage-dependent calcium channels, thereby reducing depolarization of hair cells [10,12].

To investigate contributions of the efferent-mediated inhibitory system, we examined VOR responses during VC in alpha-9 knockout ($\alpha 9^{-/-}$) mice lacking the $\alpha 9$ subunit of the nAChR. Specifically, we sought to quantify differences between WT mice and $\alpha 9^{-/-}$ mice

with regard to the action of two conceptually distinct (but likely overlapping and interrelated) pathways of the VOR: the direct/fast pathway, thought to be predominantly mediated by second order vestibular eye movement related neurons [2], and the indirect/slow pathway, which is thought to be mediated mainly by pure vestibular related neurons in VN [13]. These two different pathways of the commissural system, which have been localized to the caudal and rostra-medial parts, respectively, of medial vestibular nuclei, have been studied extensively in primates [14]. However, compensation of the fast and slow components of the VOR after UL has not yet been fully described in mice undergoing VC. We therefore investigated the early time course of VC by quantifying changes in fast and slow components of VOR over the first 9 days after UL.

2. Methods

2.1. Subjects and surgery

Breeding of CBACaJ;129S-*Chrna9*^{tm1Bedy}/J and C57BL/6 (The Jackson Laboratory) was performed to obtain F2 homozygous $\alpha 9^{-/-}$ knockout mice and homozygous $\alpha 9^{+/+}$ wild-type (WT) mice. Six WT mice and three $\alpha 9^{-/-}$ mice of same mixed genetic background were used. We performed all experiments on adult male mice at 3 months of age with fully matured vestibular system [15]. All protocol procedures were approved by the Johns Hopkins Animal Care and Use Committee consistent with European Community Directive 86/609/EEC.

Isoflurane anesthesia (3–5% in O₂) was used for all surgical procedures. A head holder was implanted on the skull using steel screws and dental cement. Two WT and three $\alpha 9^{-/-}$ mice underwent UL on the right side without violation of bone over the paraflocculus. Each animal fully recovered from anesthesia prior to eye movement measurements. Apart from VOR testing sessions, animals resided under normal diurnal lighting and feeding conditions. Second left-side labyrinthectomy was performed 10 days post-UL.

2.2. Eye movement recording

Horizontal and vertical eye angular positions were recorded in darkness using a video-oculography technique based on a system previously described in detail [16] but adapted to perform pupil tracking under infrared illumination without application of corneal markers that might otherwise interfere with vision in between VOR measurements (Fig. 1). To constrict the pupil during video eye tracking, each eye was topically treated with pilocarpine 1%.

To confirm calibration of video system, one deeply anesthetized mouse with 0.3 mm marker affixed to the cornea (red square, Fig. 1Bd) was positioned in a gimbal so that the mouse could be rotated about an Earth-vertical axis through the eye with respect to an Earth-fixed camera. After visual inspection confirmed absence of VOR responses, the animal was reoriented at different angles over a $\pm 40^\circ$ range in 5° increments (Fig. 1Ad and C), while the recording system identified the pixel centroid of the constricted pupil (Fig. 1Bc). Horizontal angle of eye rotation was calculated:

$$H_{deg} = \arcsin \left(\frac{H_{pix} \times 0.3mm}{1.69mm \times M_{pix}} \right) \times \frac{180}{\pi} \quad (1)$$

where H_{deg} , H_{pix} = horizontal position deviation from starting center position, measured in degrees and in pixels, respectively; M_{pix} = size of side of square marker in pixels; 1.69 mm is the mean radius of an adult mouse eye [17]. Although the mouse eye is not spherical and refractive effects of the cornea result in small differences between true angular vs. measured angle of the eye positions, errors due to this approximation were very small and therefore neglected. There is perfect correlation of recorded and actual eye angles using this recording system (Fig. 1C). A similar procedure was performed with a marker placed briefly on the eye at the end of each experiment, to measure a marker's size in pixels (Fig. 1Bd). To minimize blinks during imaging of marker, the cornea was anesthetized with proparacaine (0.5% topical), which was not used for eye movement recording.

The center of oculomotor range was determined in each experiment to account for apparent changes in VOR gain as a function of ocular deviation from this center. To estimate the center of the oculomotor range, pupil position was measured during horizontal sinusoidal rotations in darkness at 1 Hz, and the average position calculated before, after and during these rotations was taken as the starting eye position (x_o , y_o), where x is horizontal and y is vertical eye angular position in the eye frame of reference. Thereafter, horizontal eye position was estimated as:

$$H_{deg} = \arcsin \left((H_{pix} - x_o) \times 0.3 / (1.69 \times M_{pix} \times \cos [\arcsin (V_{pix} - y_o) \times 0.3 / 1.69 \times M_{pix}]) \right) \times \frac{180}{\pi}$$

where x_o and y_o are the pixel coordinates of the center pupil position; V_{pix} is the eye vertical angular position measured in pixels; H_{deg} , H_{pix} are the horizontal position measured in degrees and in pixels, respectively; M_{pix} is size in pixels of side of square marker.

2.3. Stimulation paradigm and data collection

For VOR measurement, an animal was mounted atop a rotator (D083 M, Kollmorgen, Radford, VA) with head pitched 35° nose down to align the horizontal semicircular canals with Earth-horizontal [18]. This orientation was kept consistent for each mouse throughout all experiments. All animals were tested before right-side UL, while two WT and three $\alpha 9^{-/-}$ mutant mice were tested again 0.5, 2, 4, 6 and 9 days after UL and after second-side labyrinthectomy to confirm absence of vestibular input from both labyrinths.

Eye movements were recorded in darkness with the head stationary to quantify SN. To quantify performance of the VOR's direct pathway, the horizontal VOR gain was measured during sinusoidal head rotations via passive whole-body rotations about an Earth-vertical axis at 0.5, 1, 2, 4 and 5 Hz with peak velocity 50 and 100°/s. Eye velocity was computed using a discrete-time differentiation of the eye angular position data (Fig. 1D and E). Eye velocity responses to sinusoidal rotations were desaccaded and approximated by a sine function using a modified gradient descent optimization algorithm. The gain and phase of the best-fit sinusoid approximating leftward and rightward mean half-cycles were

determined separately (Fig. 1D). The presence or absence of VOR quick phases (i.e., saccades) was calculated for sinusoidal head rotations at 0.5–5 Hz with peak velocity 50 and 100°/s.

To quantify performance of the VOR's indirect pathway, the decay time constant (TC) of horizontal VOR responses was measured during velocity trapezoid stimuli comprising steps of acceleration (3000°/s²) to a plateau head velocity of 150°/s for 7–10 s (Fig. 1E).

2.4. Statistical analysis

In all WT mice eye movements of at least one eye were recorded; in WT mouse #7 eye movements of both eyes were recorded. Due to the relatively small number of animals in this study, data for each mouse are described individually. Data that can be approximated by a normal distribution are presented as mean $\pm$ SE. For non-normal data, median, lower and upper quartiles were calculated. To compare data “before” and “after” UL, the parametric Student's t-test and nonparametric Wilcoxon test were used.

3. Results

3.1. Changes in quick phase of VOR and SN during 9 days post-UL

WT and $\alpha 9^{-/-}$ mice differed significantly with regard to the presence or absence of VOR quick phases during sinusoidal head rotations before and after UL (Tables 1 and 2). Before UL (Table 1), quick phases to both sides occurred only during 0.5 Hz, 50°/s sinusoidal rotations in all WT mice, while in all $\alpha 9^{-/-}$ mice quick phases were observed over the full 0.5–2 Hz range of stimuli. During 100°/s head rotation, they occurred at 0.5 and 1 Hz in all WT mice and at 2 Hz in four of them, while in all $\alpha 9^{-/-}$ mice they occurred during stimuli up to 4 Hz. After UL (Table 2), recovery of ipsilesional VOR quick phases was estimated during sinusoidal head rotations at velocity 50°/s at 0.5, 1 Hz and velocity 100°/s at 1 Hz. Before UL, the WT mice exhibited quick phases in the same direction as head rotation for rightward head movements at 100°/s, 1 Hz but not at 50°/s, 1 Hz. After UL no ipsilesional quick phases were observed for WT mice under any test condition. In contrast, all $\alpha 9^{-/-}$ mice exhibited ipsilesional quick phases by post-UL day 9 for 50°/s (0.5 and 1 Hz) and 100°/s (1 Hz) head rotations. Earlier ipsilesional quick phases were identified already by day 4 for 50°/s and by post-UL time 0.5 days for 100°/s in 2 of 3 mice. Contralateral quick phases, which include compensatory VOR quick phases and beats of SN, were generated after right-side UL, as well, but they cannot be distinguished from one another during sinusoidal rotation.

SN was absent pre-UL but was vigorous with quick phases toward the intact (left) side after right UL in all mice. The time course of suppression of post-surgical SN in WT and $\alpha 9^{-/-}$ mice was different. In WT mice, SN was dramatically decreased by day 2 and absent by day 4, while in $\alpha 9^{-/-}$ mice SN decreased more gradually over 6 days and was absent by day 9 (gray frames, Fig. 2A and B).

After UL, there were no differences in VOR gain recovery between WT and $\alpha 9^{-/-}$ mice.

3.2. Changes in TC of VOR after UL

Before UL, $\alpha 9^{-/-}$ mutants exhibited a significantly longer VOR TC and more pronounced decrease in TC with repeated stimuli (i.e., VOR habituation) than did WT mice (Fig. 2C). In WT mice the range of median values TC was from 2.1 to 4.2 s for both ipsi- and contralesional head rotations at 150°/s while in $\alpha 9^{-/-}$ mutants the range was from 3.9 to 6.3 s (Fig. 2D). The median difference for unpaired data between WT and $\alpha 9^{-/-}$ mice was 1.1 s ($p = 0.002$) for step velocity rotation at 150°/s (Fig. 2E).

After UL, changes in TC differed between WT and $\alpha 9^{-/-}$ mice (Fig. 2F and G). In WT mice after right-side UL, the TC for ipsilesional step rotations decreased dramatically to ~15% of pre-UL values at 150°/s velocity and did non-significantly recover (~18% by day 9), while TC to contralesional steps decreased to ~51% of pre-UL values after UL and recovered to 60–84% of pre-UL levels (Fig. 2F). In $\alpha 9^{-/-}$ mutants after UL the TC of ipsilesional steps also significantly dropped to 12–17% of pre-UL values and then recovered to 28–46% by day 9, while TC of contralesional step rotations decreased to ~65% of its pre-UL starting point and was to 45–71% by post-lesions day 9 (Fig. 2G). Thus, WT mice exhibited pronounced asymmetry in recovery of ipsi- and contralesional TCs, while in $\alpha 9^{-/-}$ mice these TCs were more symmetric.

4. Discussion

We observed that both before and after UL the frequency range over which VOR quick phases were generated was lower in WT mice relative to $\alpha 9^{-/-}$ mice (Table 1). Furthermore, after UL ipsilesional VOR quick phases did not recover in WT mice, whereas they did in mutants (Table 2). Thus, our data suggest that generation of the saccadic or quick phase component of VOR during sinusoidal rotations, which is position and velocity-dependent, apparently has a different frequency-related “threshold” for the two types of mice, and the threshold at which mutants generate saccades is lower than for WT mice. Absence of ipsilesional quick phases after UL in WT mice and their generation during 9 days post-UL in mutant mice indicate that signals mediated by $\alpha 9$ nAChRs either directly or indirectly affect vestibulo-oculomotor interaction during VC. Potential substrates may include the saccade burst generator, secondary VOR neurons, and the extraocular motor nuclei and muscles. Although in literature there are no data about presence of $\alpha 9$ nAChR subunits in the central VNs and neurons involved in saccade generation, sensitivity of central vestibular neurons could be changed secondary due to deactivation of $\alpha 9$ nAChR-related effects on type II hair cells.

Suppression of SN in WT mice implicates changes in the vestibular commissural inhibitory system, which links vestibular nuclei of the two sides of the brainstem. Prior studies have shown that contralesional SN is significantly suppressed at 48 h post-UL due to the increase of GABA release in the ipsilesional VN via a commissural inhibitory system [3]. In the present study, we found that mutant mice lacking $\alpha 9$ nAChR-mediated efference exhibit significantly altered time course of SN suppression after UL (Fig. 2B), suggesting that changes in $\alpha 9$ nAChR-mediated efference normally complements, augments or gates GABA-ergic central mechanisms of VC in mice; however, whether such interactions

between $\alpha 9^{-/-}$ status and GABA-mediated commissural inhibition occur is at this point a matter of speculation.

The slow component of VOR, which is mediated by the indirect pathway and dependent on the “velocity storage” mechanism [14], has been well described in many kinds of animals, but it is not considered to be an important gaze-stabilizing mechanism in rodents. Our results show clear differences in velocity storage between WT and $\alpha 9^{-/-}$ mice (Fig. 2C and E), suggesting that normal rodents do in fact rely upon a velocity storage mechanism analogous to that defined in primates and cats; however, in WT mice this mechanism is more inhibited than in $\alpha 9^{-/-}$ mutants. Our data also reveal significant differences between WT and $\alpha 9^{-/-}$ mice during recovery of the VOR slow component during VC after UL. In WT mice, the TC during ipsilesional/rightward step rotations significantly decreased and remained low throughout 9 days of followup after lesion, so that a large asymmetry in ipsi- vs. contralesional TCs persisted during compensation (Fig. 2F). In contrast, $\alpha 9^{-/-}$ mice exhibited more robust recovery of ipsilesional TC and less pronounced recovery of contralesional TC, consequently regaining TC symmetry more rapidly than WT mice (Fig. 2G). Better recovery of the slow/indirect VOR component in type $\alpha 9^{-/-}$ mice could be explained by an increase of excitatory influences to central neurons in VNs due to inactivation of efferent-mediated hyperpolarization on type II hair cells and subsequent afferent inhibition [10,12].

The role of vestibular efference during VC has long been a subject of speculation; however, contributions of $\alpha 9/\alpha 10$ nAChR-related efferent-mediated inhibition to vestibulo-ocular-motor responses in normal life and after partial vestibular loss have yet to be fully described. In this study, we observed significant differences in vestibular and oculomotor function between WT and $\alpha 9^{-/-}$ mice, which collectively suggest inactivation of efferent-mediated inhibition effectively augments excitatory afferent input to secondary vestibular neurons in both the direct and indirect VOR pathways. Such an increase of efferent excitation to central vestibular cells might also impair the efficacy of the inhibitory commissural system.

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HIGHLIGHTS

- Saccades occur at higher frequencies of yaw head rotations in $\alpha 9^{-/-}$ mice vs. WT mice.
- After UL, no ipsilesional saccades occurred in WT mice in contrast to $\alpha 9^{-/-}$ mice.
- After UL, $\alpha 9^{-/-}$ mice and WT mice have different time course of SN suppression.
- Pre-UL, $\alpha 9^{-/-}$ mice have a longer vestibulo-ocular reflex time constant vs. WT mice.
- After UL, better recovery of time constant occurred in $\alpha 9^{-/-}$ mice than in WT mice.

Fig. 1.

Eye movements recorded in mice using video oculography (VOG) under infrared (IR) illumination. (A) With the eye in resting position, the camera's optic axis and the eye's optic axis were aligned. During experiments, the head was in the center of table rotation (a); during calibration of VOG system the eye was at the center of rotation (b). (B) View of the left eye. Four IR light-emitting diodes (940 nm IR LEDs) were mounted close to the eye to illuminate the constricted pupil and oriented to avoid LED corneal reflections overlapping the pupil (white circle, Ba). Camera had resolution 500 × 400 (horizontal × vertical) pixels image at 180 frame/s (Ba–d). (C) Recorded eye position vs. actual position in a deeply anesthetized mouse. Linear regression indicates nearly perfect correlation across the ±40° range ($R^2 = 0.992$). (D) Response during 1 Hz sinusoidal rotations at peak velocity 50°/s in IR light. Motion to right is positive. Red line is sinusoidal approximation of desaccaded eye velocity (bottom panel). (E) Horizontal eye position and velocity during constant velocity step rotations (150°/s for 7–10 s, peak acceleration 3000°/s²) in IR light, with estimate of VOR slow phase time constant (TC).

Fig. 2.

Changes of SN and VOR TC during 9 days following right-side UL. (**A** and **B**) Eye position recorded in darkness at $0^\circ/\text{s}$ velocity in WT and $\alpha 9^{-/-}$ mice. After right-side UL, all mice had SN. Gray frame shows time when SN was significantly suppressed. (**C**) TC value vs. trial number tested in pre-UL studies in one WT mouse and one $\alpha 9^{-/-}$ mutant mouse for step head rotations at $150^\circ/\text{s}$ to the right (filled symbols) and to the left (open symbols). In the mutant mouse, habituation of the TC is apparent. (**D**) TC (median, lower and upper quartiles) before UL in all mice during rightward (filled symbols) and leftward (open

symbols) horizontal step head rotation at 150°/s for 7–10 s. **(E)** The median difference values of TC for unpaired data between groups of WT mice and $\alpha 9^{-/-}$ mice. **(F and G)** Changes of TCs during 9 days following right-side UL in two WT mice and three $\alpha 9^{-/-}$ mice.

Table 1

Incidence of saccades or VOR quick phase during sinusoidal head rotations before UL.

Mouse	Stimulus velocity at 50°/s					Stimulus velocity at 100°/s				
	0.5Hz	1Hz	2Hz	4Hz	5Hz	0.5Hz	1Hz	2Hz	4Hz	5Hz
WT #3 L eye	X	-	-	-	-	X	X	X	-	-
WT #4 L eye	X	-	-	-	-	X	X	-	-	-
WT #6 R eye	X	-	-	-	-	X	X	X	-	-
WT #7 L eye	-	-	-	-	-	X	X	X	-	-
WT #7 R eye	X	-	-	-	-	X	X	X	-	-
WT #8 R eye	X	-	-	-	-	X	X	X	-	-
WT #9 R eye	X	-	-	-	-	X	X	-	-	-
Mutant #1 R eye	X	X	X	-	-	X	X	X	X	-
Mutant #3 R eye	X	X	X	-	-	X	X	X	NA	NA
Mutant #4 L eye	X	X	X	-	-	X	X	X	X	-

X and hyphen (-) denote present or absent of VOR quick phases, respectively. NA means not tested.

Table 2

Incidence of ipsilesional VOR quick phases during sinusoidal head rotations during 9 days of recovery.

Mice	Stimulus velocity 50°/s (0.5 Hz and 1 Hz)						Stimulus velocity 100°/s (1 Hz)					
	Before	Days after UL					Before	Days after UL				
		0.5	2	4	6	9		0.5	2	4	6	9
WT #3 L eye	X/-	NA	NA	NA	NA	NA	X	NA	NA	NA	NA	NA
WT #4 L eye	X/-	NA	NA	NA	NA	NA	X	NA	NA	NA	NA	NA
WT #6 R eye	X/-	-/-	NA	NA	NA	NA	X	-	NA	NA	NA	NA
WT #7 L eye	-/-	NA	-/-	-/-	-/-	-/-	X	NA	-	-	-	-
WT #7 R eye	X/-	-/-	-/-	-/-	-/-	-/-	X	-	-	-	-	-
WT #8 R eye	X/-	-/-	-/-	-/-	-/-	-/-	X	-	-	-	-	-
WT #9 R eye	X/-	-/-	NA	NA	NA	NA	X	-	NA	NA	NA	NA
Mutant #1 R eye	X/X	-/-	-/-	X/X	X/X	X/X	X	X	X	X	X	X
Mutant #3 R eye	X/X	-/-	-/-	-/-	-/-	-/-	X	-	-	-	-	X
Mutant #4 L eye	X/X	-/-	-/-	-/-	-/-	-/-	X	X	X	X	X	X

Slash symbol (/) separates data for 0.5 Hz and 1 Hz. X and hyphen (-) denote present or absent of VOR quick phases, respectively. NA means not tested.