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The ataxic mouse as a model for studying downbeat nystagmus

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Abstract

Downbeat nystagmus (DBN) is a common eye movement complication of cerebellar disease. Use of mice to study pathophysiology of vestibulocerebellar disease is increasing, but it is unclear if mice can be used to study DBN; it has not been reported in this species. We determined whether DBN occurs in the ataxic mutant *tottering*, which carries a mutation in the *Cacna1a* gene for P/Q calcium channels. Spontaneous DBN occurred only rarely, and its magnitude did not exhibit the relationship to head tilt seen in human patients. DBN during yaw rotation was more common and shares some properties with the tilt-independent, gaze-independent component of human DBN, but differs in its dependence on vision. Hyperactivity of otolith circuits responding to pitch tilts is hypothesized to contribute to the gaze-independent component of human DBN. Mutants exhibited hyperactivity of the tilt maculo-ocular reflex (tiltMOR) in pitch. The hyperactivity may serve as a surrogate for DBN in mouse studies. TiltMOR hyperactivity correlates with hyperdeviation of the eyes and upward deviation of the head during ambulation; these may be alternative surrogates. Muscimol inactivation of the cerebellar flocculus suggests a floccular role in the tiltMOR hyperactivity and provides insight into the rarity of frank DBN in ataxic mice.

Keywords

Nystagmus; cerebellum; ataxia; animal models; channelopathy

1. Introduction

Recent years have witnessed a dramatic increase in the use of the laboratory mouse in ocular motor physiology. There are a variety of motivations for this growth, but a strong factor is the ability to use genetic tools in this species to create animal models for ocular motor disorders that affect humans. An inherent problem with the approach, however, is that the mouse lacks a fovea (or equivalent retinal specialization subserving high-acuity vision) and consequently lacks the repertoire of eye movements devoted to positioning the fovea with respect to visual space. The mouse cannot be used, for instance, to study smooth pursuit, the behavior that enables “foveates” to track a visual target moving through a visual scene.

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Along these lines, lesions of homologous anatomical regions in mice and foveate mammals cannot be relied on to generate identical phenomena, even when the lesions cause identical deficits within mechanisms that *are* shared between the species: In the foveates the lesions may damage fovea-directing mechanisms located in the same region, or fovea-directing mechanisms outside the lesion may be brought into play as an indirect lesion effect, masking any direct effects of the lesion on the more primitive afoveate ocular motor mechanisms. The current study addresses a specific instance of this problem, i.e., whether the mouse can be used to study mechanisms and treatment of a specific eye movement disorder, downbeat nystagmus (DBN).

DBN is a pathological pattern of spontaneous eye movements in which upward drifts of the eyes (the nystagmus slow phases) alternate with rapid downward resetting movements (the nystagmus fast phases, or “beats”). The spontaneous oscillation disturbs vision and attempts to treat it have sparked considerable interest in the field [8]. DBN is a common finding in cerebellar disorders of many etiologies [20,29,50,53] and can be produced experimentally by lesioning the cerebellar flocculus [56] or uvula-nodulus [51]. DBN consists of several distinguishable components, including a component dependent on the position of the eye in the orbit, an eye position-independent velocity bias that is modulated by gravity, and another bias that is both eye position- and gravity-independent [31]. The etiology of the gaze-evoked component is dysfunction of the neural integrator, which is responsible for maintaining eccentric gaze. The origins of the bias components are controversial. Proposed contributors to the bias components include: 1) disinhibition of a pathway from the macular organs (utricle and saccule) mediating the effects of gravity on eye movements in the pitch plane [20,31]; 2) loss of a cerebellar signal that normally cancels a tone imbalance inherent in the brainstem circuits underlying the vestibulo-ocular reflex (VOR) or the maculo-ocular reflex (MOR) [5,7,19]; 3) imbalances within the vertical smooth pursuit pathways [14, 16,26,55]; 4) imbalances in the gains of the rotational VOR for upward and downward head pitch [19]; 5) mismatches between the desired eye positions specified by the gaze-holding neural integrator and the mechanisms that maintain Listing’s law during saccades [15]. These putative mechanisms are not mutually exclusive, and a mixture of processes may be present in any one patient [15,29].

The laboratory mouse has already figured indirectly in the clinical literature on DBN, as studies in the ataxic mutant mouse *tottering* [1,52] are routinely cited to explain the mechanism by which the potassium channel blocker 4-aminopyridine ameliorates DBN in humans (for instance, see [27,28,45]). Despite this interest in applying mouse studies to the clinical arena, DBN has not been reported as a typical eye movement finding in *tottering* or any other ataxic mouse. Instead, a static hyperdeviation of the eyes has been described in ataxic mice, and it was speculated that this upward *position* bias could be the murine equivalent of the upward *velocity* bias in DBN [42]. While the etiology of the hyperdeviation in the mice is unclear, a disinhibition of otolith circuits similar to that proposed to contribute to human DBN [20,31] is an intriguing possibility. The MOR in mice is robust [2,36]; a hyperdeviation of the eyes could easily be the result of hyperactivity in the system that adjusts eye position in response to maintained head tilt. If the MOR could be shown to be hyperactive in ataxic mice, there would be some support for using eye

hyperdeviation or MOR hyperactivity as surrogates for actual DBN in mouse studies of DBN therapy. To date, MOR has never been investigated in ataxic mice.

In the current study we assessed the MOR in *tottering*, an ataxic mouse strain whose cerebellar dysfunction stems from a point mutation in *Cacna1a*, the gene for the ionophore of the P/Q calcium channel [13]. The P/Q channel, in addition to its role in synaptic transmission, determines many of the critical electrical properties of the cerebellar Purkinje cell dendrite [30]. The selection of this mutant was motivated by its use in previous studies of aminopyridines noted above, by the availability of extensive data on ocular motor function in this and other *Cacna1a* mutants, by the parallels between their ocular motor deficits and those of ataxic humans [21,40,42], and by the fact that humans with a broad range of mutations of the homologous gene *CACNA1A* often suffer DBN [46]. In addition to comparing MOR function in *tottering* and normal animals, we also performed a systematic assessment for the presence of actual DBN, and we assessed whether inactivation of the cerebellar flocculus – which produces DBN in monkeys [56] – generates nystagmus or replicates any of the characteristics of the MOR of *tottering*. Preliminary results have been reported [43].

2. Methods

2.1. Animals and animal preparation

Animal experiments were approved by the Institutional Animal Care and Use Committees at the Louis Stokes Cleveland Dept. of Veterans Affairs Medical Center and Case Western Reserve University, and followed the guidelines of the National Institutes of Health on the use and care of laboratory animals. C57BL/6 control animals were obtained from The Jackson Laboratory (Bar Harbor, ME) and *tottering* mutants were bred locally. Studied animals were of either sex.

Animals were prepared for recording by surgical implantation of an acrylic head-fixation pedestal as previously described [36]. During implantation the animals were held in a stereotactic frame so that the top of the completed pedestal accurately paralleled the lambda-bregma axis. For the two animals used in the flocculus inactivation experiments, a craniotomy was fashioned in the interparietal bone and surrounded by a built-up acrylic chamber joined to the fixation pedestal.

2.2. Recording apparatus

During recording sessions the animal's head pedestal was bolted to a support armature and its body was loosely restrained in an acrylic tube. The support armature was angled so that when the restraint apparatus was held in an earth-horizontal attitude in either the tilt or rotation systems, the lambda-bregma axis was pitched 20° nose-down. Responses to static tilt positions were assessed using a manually positioned, 2-axis gimbal (the “tilt system”) that supported a light-tight box enclosing the restraint platform, video oculography camera, and infrared light source. For recording eye position in the light, the interior of the box was illuminated by white light emitting diodes. A 3DM-G orientation sensor (Microstrain, Burlington, VT) mounted to the gimbal and aligned with the rostral-caudal axis of the mouse

displayed pitch and roll angles in real-time on a laptop computer. Responses to dynamic rotational vestibular stimuli were assessed on a separate apparatus (the “rotation system”) incorporating a servo-controlled turntable. In both the tilt and rotation systems, the video oculography camera could be yawed $\pm 10^\circ$ about a vertical axis passing through the recorded eye as required for the video calibration procedure [44]. Note however, that the rotating camera feature was added to the tilt system after the tilt experiments were completed for the first eight *tottering* and ten C57BL/6 animals, results of which have been reported in part [43]. For those tilt experiments, calibration data were transferred from the rotation system as previously described [36].

Eye movement recording in two dimensions was accomplished using a commercial pupil-tracking video oculography system (ETL-200, ISCAN, Inc., Burlington, MA), operating under infrared illumination at a sampling rate of 120 Hz [39,40,44]. The recorded eye was treated with 0.5% physostigmine salicylate to limit pupil dilation in the dark. The horizontal and vertical positions of the pupil, horizontal and vertical positions of a reference corneal reflection, and pupil diameter were output from the oculography system as analog signals. These signals as well as turntable position were passed through four-pole Bessel low-pass filters (corner frequency 200 Hz) and then stored to computer via a digital acquisition system (System 3, Tucker-Davis Technologies, Gainesville, FL) at a rate of 500 samples/s. Raw eye position signals were converted by the trigonometric procedure detailed previously [36] to horizontal angle with respect to the rostral-caudal axis of the mouse (0° = aligned with nose), and vertical elevation angle with respect to the animal's horizontal plane. (The animal's horizontal plane is defined as a plane paralleling earth-horizontal when the restraint platform is held in the 0° pitch, 0° roll attitude.) Since the camera moves with the animal, the horizontal/vertical reference frame for eye movements remains locked to the animal as it is tilted in space. Note that due to the orientations of the eyes in the mouse head, both pitch and roll head tilts generate eye movements with significant torsion components. The torsion components are not captured in the two-dimensional recording, but neglecting them does not affect the goals of the current experiments, which include only the comparison of tilt responses in different mouse strains, and qualitative detection of nystagmus. Estimation of true MOR gains from two-dimensional recordings has been treated elsewhere [36].

2.3. Measurement of static tilt maculo-ocular reflex

Equilibrium eye positions were assessed over a range of $\pm 90^\circ$ in 15° steps (where 0° is defined as the lambda-bregma axis being parallel to earth horizontal). We included in this pitch sequence the neutral gimbal position, which places lambda-bregma at 20° pitch-down. Since this position did not fit with the equally spaced pitch steps about the lambda-bregma 0° angle, it was not included in the plots of eye position vs. pitch angle. However, it was included, along with measures at the neutral position taken during the roll sequence, in the measure of vertical eye position at gimbal zero ($E_V @ \text{Gimbal}_0$). Static responses to roll tilt were assessed similarly over a $\pm 90^\circ$ roll angle in 15° steps, with the lambda-bregma axis maintained at 20° pitch-down with respect to the roll axis. All pitch or roll tilts were performed in single blocks, within which the sequence of tilt angles was randomized. For each animal, we performed 2–4 sessions of recording (no more than one session/day), and for each set of two sessions, the order of the pitch and roll blocks was also randomized. Each

tilt position was achieved by rotating the animal in the dark at a slow rate to the desired angle. Once the eye position had stabilized, several seconds of pupil position were captured, both with the lights off and on. The goal of these recordings was to capture the equilibrium position of the eye, and the state of illumination was controlled so as to encourage the animal to come to that equilibrium as quickly as possible. Specifically, the approximate equilibrium position was fairly obvious at any given tilt angle. On occasions when the animal executed saccades that took the eye far from that equilibrium, we often extinguished the lights (if the saccade occurred in the light) to hasten the return of the eye to the equilibrium position. This intervention was less often necessary in *tottering*, which tended to return toward its central position more rapidly than control animals whether in darkness or light, likely owing to both its shorter neural integrator time constant (which speeds the centripetal drift) and weaker optokinetic reflex (which reduces the ability of the animal to use the optokinetic reflex when in the light to retard the centripetal drift) [42].

The equilibrium horizontal and vertical eye positions were quantified in offline analysis. After excluding sections of each record where eye tracking was lost, we selected all portions of the record in which the eye was stable, and calculated from them the average eye position. As noted above, on occasions animals executed saccades away from the region we had tentatively identified, during data collection, as the equilibrium eye position for that particular tilt angle. When such saccades occurred in the light, the eye might then hold the new position relatively stably until the lights were extinguished, particularly in the case of control animals. When analyzing such records, we selected for averaging only the region of the record after the eye had returned from the eccentric position. In cases in which there were linear drifts without an identifiable equilibrium position, the entire drifting record was used. Likewise, on occasions where there were repeated saccades and it was unclear which stable eye positions represented the equilibrium region, we averaged all of the stable eye positions. Each 30-second record yielded a result set consisting of a single average horizontal and vertical eye position for light and a corresponding pair of measures for dark. Where more than one record was obtained in a session at a given tilt angle, the results were averaged across the records to obtain a single result for the tilt angle in the session, and finally those results were averaged across multiple sessions to generate a single set of results for each animal.

2.4. Measurement of tilt and rotational responses with flocculus inactivation

In animals whose tilt and rotational responses were to be studied before and after flocculus inactivation, several preliminary recording sessions were conducted to identify the coordinates of the approximate center of the left flocculus. The flocculus was identified by the presence of Purkinje cells whose complex spikes were modulated in response to rotatory optokinetic stimuli about either the vertical (yaw) axis, or about a horizontal axis deviated 135° from the nose toward the left side [21]. The subsequent data acquisition sessions were conducted using a double-barreled micropipette with one channel filled with 2M NaCl and 4% Fast Green, and the other barrel filled with a 1 $\mu\text{g}/\mu\text{L}$ solution of muscimol in 0.9% saline. The muscimol channel was coupled to a pressure injection unit (Picospritzer II, Parker Hannifin, Pine Brook, NJ). The animal was mounted in the tilt apparatus and the electrode was advanced to a depth of approximately 1500 μm , well short of the depth at

which any optokinetically sensitive Purkinje cells were identified in the preliminary sessions. With the electrode at this position, a set of pre-treatment responses to pitch and roll were collected as described above, with the modification that the pitch sequence was restricted to a lambda-bregma pitch range of -30° to $+45^{\circ}$, and the roll sequence was restricted to 30° ipsilateral ear-down to 45° contralateral ear-down. The range was restricted primarily so that the post-muscimol data collection could be completed quickly enough to make any decay of the muscimol effect a minor influence on the form of the tilt response curve, and also to keep the length of the entire recording session within the tolerance of the animal. The range was asymmetrical to reflect the fact that the muscimol treatment would elevate the eye, and very high eye positions were more difficult to track and also might be influenced by limitations in eye-in-orbit range. Thus the nose-down and ipsilateral ear-down end of the stimulus range – both directions that elevate the eye – were made shorter than the nose-up and contralateral ear-down ranges.

After collecting the pre-inactivation data, we advanced the electrode to the mapped location of the flocculus. Single brief (100-300 ms) pressure pulses were applied to the muscimol channel, each followed by a minimum 2-minute observation period, until a strong upward deviation of the eye was produced. In practice, the effect was *highly* potent; a single, properly located ejection was sufficient to deviate the eye, and the deviation became apparent within a few seconds and then gradually increased over the course of several minutes. In cases where the first ejection attempt was ineffective, the electrode ultimately proved to have been superficial to the flocculus (as demonstrated by success being achieved promptly after driving the electrode deeper) or the ejection channel had become plugged (as demonstrated by success being achieved by increasing ejection pressure or lengthening the ejection pulse, or because the drug could not be ejected after the electrode was removed and examined under a microscope). Our goal was to eject only enough drug to produce an unambiguous vertical offset of the eye. We aimed for the minimum unambiguous effect because a larger ejection could drive the eye so high that pupil tracking was difficult, and a large injection increased the risk that the muscimol would spread outside the flocculus to the adjacent brainstem. A minimum of ten minutes was allowed to elapse after the successful ejection to allow the drug effect to stabilize, after which we collected a post-inactivation set of responses to pitch and roll.

In 6 sessions, upon conclusion of the post-muscimol tilt series we immediately transferred the animal to the rotation apparatus to determine if the muscimol engendered a downbeat nystagmus revealed only in the context of rotational eye movements, a phenomenon that had been observed in pilot experiments. For this purpose, eye movements were tested during $0.8 \text{ Hz} \pm 4.6^{\circ}$ sinusoidal rotation in dark (vestibulo-ocular reflex, VOR) and light (vision/vestibulo-ocular reflex, VVOR). The resultant eye movements were examined for DBN as described below.

2.5. Assessment of downbeat nystagmus during static tilt and rotation

The tilt data used to determine equilibrium eye positions were also systematically reviewed for the presence or absence of downbeat nystagmus. The order of the records to be reviewed was randomized, and the reviewers were blinded to the genotype of the animal, tilt angle,

and (where relevant) whether a record was obtained before or after intrafloccular injection of muscimol. Downbeat nystagmus was classified as present if any period of continuous light or darkness within a 30-second record contained at least one downward saccade flanked by two periods of upward drift, or two downward saccades embracing one period of upward drift. Furthermore, the size of the saccade(s) had to be at least 1° , and had to be in most beats larger than the amplitude of any concomitant horizontal saccades.

Five of the C57BL/6 and ten of the *tottering* mice used in the tilt experiments had also been used for a different experiment on the rotation system, providing us with responses to 0.8 Hz, $\pm 4.6^\circ$ sinusoidal yaw rotation in light and dark. These records were reviewed and scored for the presence of DBN in a fashion identical to the tilt data, with the exception that because the downward beats frequently occurred in the context of large, horizontal fast phases of the vestibular-induced eye movement, we omitted the criterion that the vertical movement had to be larger than the horizontal component.

2.6. Head pitch during ambulation

In all of the animals used in the tilt experiments we also determined pitch of the head during natural ambulation, using the method previously described [36]. Briefly, a lightweight plate bearing visible markers was screwed to the acrylic head pedestal. A video recording was made of each mouse as it ambulated repeatedly along a narrow platform. Off-line, the angle of the marker plate with respect to the horizontal (indicating the pitch of the lambda-bregma axis) was determined using a commercial motion analysis system (Peak Motus, Vicon, Los Angeles, CA). The regions of the record in which the centerpoint of the marker plate was in forward motion were identified and the marker angles for these periods were averaged.

Throughout, values are reported as mean $\pm$ SD except where noted. All statistical tests are 2-tailed.

3. Results

3.1. Eye hyperdeviation in tottering corresponds to a hyperactive pitch MOR

The eye position:tilt relationship was determined for 15 C57BL/6 control animals (aged 93–359 days) and a similarly aged cohort of 15 *tottering* (aged 87–359 days). A human study has observed different responsiveness to treatment when DBN appears in the presence of, versus the absence of, radiologically demonstrable cerebellar atrophy [38]. Elderly *tottering* do develop progressive atrophy [23,24], and several of the ocular motor abnormalities in mouse *Cacna1a* mutants tend to increase with age [40,42]. Therefore, we also tested 5 older *tottering* (aged 432–680 days) to control for the possibility that DBN or a surrogate sign might appear only after the development of the age-related atrophy.

The current experiments replicated the previous finding that while at a neutral head position, eye elevation in the main group of *tottering* (referred to henceforth as “young” *tottering*) exceeded that of C57BL/6, as well as the wide scatter of the results and the extensive overlap between the two genotypes [42]. Taking into account all data acquired in the light when the gimbal was in the 0° roll, 0° pitch position (equivalent to a lambda-bregma angle of -20°), *tottering* eye position averaged $25.4 \pm 7.5^\circ$ vs. $18.8 \pm 4.0^\circ$ for controls. The corresponding

$E_V@Gimbal_0$ values from the similarly aged animals in the previous study (i.e., the union of young and middle-aged groups of that study) were $26.2 \pm 4.9^\circ$ for *tottering* ($n = 24$) and $18.7 \pm 4.8^\circ$ for controls ($n = 39$). The older age group in the current experiments failed to demonstrate the elevation, but this may reflect the wide variability of this measure in conjunction with the very small sample size.

Figure 1 depicts the average equilibrium eye position:tilt angle relationships for the pitch tilts in young *tottering* and controls, and Fig. 2 depicts the relationship during roll tilts. In both figures, the top panels are for the light and the bottom panels for dark, while the left panels depict horizontal eye position and the right panels depict vertical eye position. A vertical cursor in all plots of Fig. 1 marks the average lambda-bregma pitch observed as control animals ambulate naturally (see below). The general forms of the control curves are identical to those reported previously for the C57BL/6 genotype [36]. For pitch, as the animal is held at progressively steeper nose-down attitudes, the rest eye position shifts progressively up and temporal. For roll, the eye shifts up and nasal as the animal is rolled toward the recorded eye. During contralateral roll, the eye moves down and temporal, but the horizontal relationship plateaus once the eye reaches an eye position of approximately 55° with respect to the rostro-caudal axis. The rest positions are essentially identical in light and dark, and consequently we will focus most of the ensuing analysis on the relationship in the light.

Qualitative inspection of the graphs reveals that the mutants exhibit several differences from controls of varying degrees of robustness. For pitch tilts, the mutants exhibit a steeper vertical eye response over most of the tested range, as well as slightly steeper horizontal responses over much of the pitch-down half of the curve. In the roll relationship, *tottering* exhibited a shallower degree of elevation as the ipsilateral ear was rolled down and a retreat from the maximal abducted position as the head was rolled toward the opposite extreme.

Figure 3 compares the tilt:eye position relationships for the old and young groups of *tottering*. Pitch and roll are combined on the same figure, and only the responses in the light are shown. The pitch response of old *tottering* was similar to the younger animals, excepting that the curve was offset, a finding of uncertain significance, given the small number of animals ($n = 5$) and the wide animal-to-animal variability of vertical eye position commented on above. The roll relationship also shows the vertical offset in eye position. The curves for horizontal eye position of the two *tottering* groups separate about the 0° roll position, but this separation derives from just two of the five older animals; inspection of a plot of horizontal eye position vs. roll angle (not shown) for all animals in the study did not reveal any consistent change in horizontal eye position at 0° roll as a function of age.

Of the qualitative differences in the eye position:tilt relationships of mutants and controls, the one most relevant to the original observation of elevated eye positions in *Cacna1a* mutants is the increased slope of vertical eye position in the pitch MOR in the vicinity of the neutral head position. We assessed this difference quantitatively in terms of the slope of the vertical position vs. pitch curve in each animal, where the slope was measured by linear regression over the range of lambda-bregma pitch from -30° to $+30^\circ$. We again restricted our attention to the response in light, and did not include the older *tottering*. This pitch MOR

slope averaged -0.337 ± 0.063 for *tottering* vs. 0.241 ± 0.038 for controls, and the difference was statistically significant ($p < 0.0001$). Figure 4 explores the extent to which animal-to-animal variations in pitch MOR slope determined the variations in rest position of the eye in the neutral head position (again, in the light). There was a mild but statistically significant negative correlation for both controls ($r^2 = 0.28$, $p = 0.044$) and *tottering* ($r^2 = 0.25$, $p = 0.023$), indicating that a stronger pitch MOR may contribute to higher resting eye positions. The fact that *tottering* and controls lie on nearly the same regression relationships (note similarity of the regression lines; also, regression on both groups together yields $r^2 = 0.47$, $p < 0.0001$) is also consistent with differences in pitch MOR gain being responsible for the genotype-specific differences in $E_V@Gimbal_0$. In contrast, there was no relationship between resting eye position at the neutral tilt position and the slope of the roll MOR calculated over the -30° to $+30^\circ$ range of roll tilts (all coefficients of determination 0.06 and below, plots not shown).

3.2. Head pitch is elevated during ambulation in tottering

The pitch of the lambda-bregma axis during natural ambulation was determined in the same 15 control and 20 *tottering* animals used to determine the relationships between eye position and tilt angle described above. We quantified only head pitch because of the increased gain of the pitch MOR described above. (In contrast, the roll MOR was normal in the vicinity of normal head position, and in any case, an abnormality of the roll MOR gain would presumably manifest equally on the two sides of the head, and thus would not be expected to generate an abnormal roll head posture.) Omitting the five older *tottering*, head pitch measured $-27.4 \pm 2.6^\circ$ in *tottering* vs. $-30.5 \pm 4.4^\circ$ in controls, a significant difference ($p = 0.039$). Figure 5 explores the relationship between the MOR data and the spontaneously maintained head attitude. The left panel plots pitch MOR gain (in light) vs. ambulation pitch, and the right panel plots $E_V@Gimbal_0$ vs. ambulation pitch. Older animals are distinguished as open triangles. For the younger *tottering* but not the control animals, higher pitch MOR gain was associated with shallower ambulation head pitches ($r^2 = 0.30$, $p = 0.034$; including the older animals reduced the values to $r^2 = 0.23$, $p = 0.032$). Likewise, higher values of $E_V@Gimbal_0$ were associated with shallower ambulation head pitches ($r^2 = 0.35$, $p = 0.019$; including the older animals reduced the values to $r^2 = 0.25$, $p = 0.026$). These results are consistent with the idea that hyperactive pitch MOR in *tottering* results in the mutants maintaining their heads at shallower pitch angles. It also suggests that the abnormality in static pitch reflexes in *tottering* affects vestibulocollic and maculo-ocular reflexes in a similar fashion.

3.3. Flocculus inactivation affects MOR but does not replicate tottering's deficits

We studied the effect of pharmacologically inactivating the cerebellar flocculus ipsilateral to the recorded eye in multiple sessions in two C57BL/6 mice, with the goal of determining the extent to which reduced flocculus output mimics the MOR phenotype of *tottering*. An additional goal was to determine whether unilateral flocculus activation engenders DBN. Flocculus dysfunction has been argued to be the cause of several of the abnormalities of compensatory eye movements in *Cacna1a* mutants [21,40,42], and anatomical destruction of one flocculus in control animals replicates some of the *tottering* abnormalities [21].

Flocculus dysfunction has also been demonstrated to contribute to DBN in humans [16], and flocculus lesions produce DBN in monkeys [56].

Pitch and roll relationships were determined in 11 sessions in the two animals. Figure 6 plots the eye position vs. gimbal angle in the lighted condition. (The curves for positions recorded in darkness were essentially identical to those in the light.) Top panels depict the horizontal and vertical eye position response to pitch tilts, while bottom panels plot the relationships for roll. The primary effect of the injection was to offset all the eye positions upward and nasally. Both effects are easily predicted based on the influence of floccular Purkinje cells on the semicircular canal brainstem pathways. The Purkinje cells inhibit the pathway from the ipsilateral anterior semicircular canal to the superior rectus of the ipsilateral eye, and from the horizontal canal to the ipsilateral medial rectus [25]. Reduced flocculus output would ultimately activate the superior and medial recti. (Conversely, *stimulation* of the flocculus outflow tracts causes eye *depression* and *abduction* [35,48].) The unilateral muscimol injection did not replicate the horizontal eye movement peculiarities of *tottering* described above, including its temporally deviated eye positions relative to controls when pitched nose-down, or its relatively nasal positions when rolled contralateral ear down. The slope of the vertical response to pitch (calculated, as before, from linear regression of E_V on lambda-bregma pitch over the -30° to $+30^\circ$ range) did increase, from -0.281 ± 0.084 to -0.376 ± 0.080 . The difference in slopes was statistically significant (paired *t*-test, $p = 0.013$), but it is clear that the large separation of pre- and post-muscimol curves is largely due to the appearance of an offset, and this differs from the *tottering* and control curves in Fig. 1, in which the vertical separation is entirely a consequence of the differences in slope. In sum, the increase in pitch MOR gain that we engendered indicates an influence of the flocculus on MOR gain, and leaves open the possibility that abnormalities of flocculus output in *tottering* do contribute in some way to its tilt MOR phenotype. However, the strong vertical offset in eye position, which is predictable from the known connections between the flocculus and the semicircular canal pathways, differs from the effects of the mutation. The extent to which flocculus inactivation engendered DBN is treated below.

3.4. Spontaneous downbeat nystagmus is occasionally seen in tottering, but differs from human DBN

In the majority of animals and tilt angles, the eye rapidly achieved a stable equilibrium, i.e., no sustained nystagmus was found. However, as was the case in a previous study limited to resting eye position and rotational responses of *Cacna1a* mutants [42], there were occasions in which we observed spontaneous downbeating nystagmus of varying persistence, frequency, amplitude, and waveform (the lattermost varying from linear to exponential). We will refer to this motion as DBN for convenience, although we will argue in Discussion that its equivalence to human DBN is doubtful. Figure 7 provides examples of records that illustrate the spectrum of DBN behavior we observed. In each, darkened portions of the traces indicate data segments that were acquired in darkness. Panels A and B are from a *tottering* animal that was particularly prone to developing nystagmus. Panel A was acquired at a lambda-bregma angle of $+30^\circ$ (nose up), and panel B was at $+75^\circ$. Panels C and D were obtained from a C57BL/6 at pitch $+75^\circ$ and $+90^\circ$, respectively. In both animals the nystagmus intensified as the animal was pitched upward.

We systematically evaluated the DBN probability, waveform character, tilt sensitivity, and relationship to pitch MOR gain. We reviewed all the raw tilt records ($n = 15$ C57BL/6, 1920 records; $n = 15$ young *tottering*, 1834 records; $n = 5$ old *tottering*, 579 records) for the presence of DBN, scoring according to the criteria described in Methods. Figure 8 shows the probabilities of finding DBN in a record as a function of tilt angle, with each animal weighted equally. To generate the probabilities, we scored records as 1.0 for DBN present and 0 for DBN absent, and then calculated the mean and standard error of the scores at each tilt angle. Here the standard error is equivalent to SD for a binomial quantity [54]. Top plots are for pitch and bottom plots are for roll, while the left side is for light and the right side is for darkness. The functions are noisy and the error bars are wide because DBN was rare, so the vertical scale is very magnified. Also, it should be recalled that the status of the illumination was controlled so as to hasten the determination of equilibrium eye position. As such, the periods of light and dark were rarely of equal length, and it was possible that a record with DBN could be scored as a '0' in the light (or dark) because the nystagmus period was too long to meet the criteria within any single period of constant illumination. Despite these limitations, several firm conclusions can be drawn from the histograms. First, DBN does occur in mice, although it is an infrequent phenomenon. Second, *tottering* appeared more likely to exhibit DBN than control animals, and old *tottering*, in which many eye movement deficits become more pronounced [42], was more likely to exhibit DBN than young *tottering*, consistent with this DBN being an accompaniment to cerebellar dysfunction, as is DBN in humans. Third, the mouse DBN did not exhibit the dependence on inclination seen in humans, in which DBN increases as the head is pitched forward [31]. If anything, DBN became most apparent during the roll series with the animal upright or rolled slightly toward the contralateral ear.

These same records were also assessed for the presence of upbeat nystagmus, applying the same criteria for its presence as were used for DBN. Only 4 of the 4333 records exhibited upbeat; three records were from young *tottering* and one record was from an old *tottering*. Upbeat only occurred in darkness, and never in control animals. There was no consistent waveform or position of the eye at which upbeat was seen, i.e., it occurred in records in which the eye was above, at, or below the elevation maintained during the neutral head position.

To summarize the histograms of DBN probability during tilt, and also to circumvent some of their inherent noisiness, we determined the average probability of DBN per tilt angle within the five angles spanning the central $\pm 30^\circ$ of pitch or roll: Probability of DBN was first averaged over all the records of one animal at a specific attitude, then those averages were averaged across the five positions within animals, and then these five-position averages were averaged across animals with in each animal group. These average probabilities are summarized in Table 1. The tabulated values illustrate that in young *tottering* DBN was present in 2–4% of the records at the attitudes at or near the upright position. In contrast, it was exceedingly rare in the C57BL/6 mice, for which it was never seen during roll in light, and probability was less than 1% in the other conditions. While this summary statistic supports the impression from the histograms that DBN was more common in *tottering*, statistical comparison between control animals and the similarly aged *tottering* reached significance only for the roll series data obtained in the light. The lack of significance re-

enforces the impression that the presence of DBN cannot be considered a typical distinction between the eye movements of *tottering* vs. those of control animals. Along similar lines, DBN probability (as measured by the average probability per tilt angle within the $\pm 30^\circ$ range) exhibited no significant correlation with the measures of MOR function, including $E_V@Gimbal_0$ and the MOR slopes for pitch and roll in light and dark (p values for r ranging 0.31– 1.0). Thus the relatively rare phenomenon of DBN was not linked to *tottering*'s more general abnormalities of static MOR.

As noted above, the slow phases of DBN varied from linear to exponential. We determined the waveform in records previously assessed as containing DBN, visually classifying the waveform as predominantly linear, predominantly exponential, or mixed/indeterminate. Table 2 summarizes the results of the qualitative assessment. In both dark and light, *tottering* tended to exhibit more linear slow phases than did controls, in which the drifts were more frequently exponential. The table also includes the p values for the Pearson's χ^2 , comparing the different animal groups in terms of the proportions of the records with DBN that exhibited linear waveforms. When assessed in darkness, young *tottering* exhibited a higher proportion of linear DBN than controls, and old *tottering* exhibited more linear DBN than did young *tottering*. The predisposition to linear waveforms is reminiscent of the case in human DBN, in which the waveform is more often (but not exclusively) linear [20,53]. Thus there may be some shared etiology between DBN in human patients with cerebellar disorders and the DBN during static tilt in *tottering*, but the rarity, the different relationship to tilt, and the non-relationship to MOR indices suggest they are not homologous abnormalities.

3.5. Rotation-induced DBN was more common than spontaneous DBN

We also reviewed records from controls and *tottering* acquired during sinusoidal rotation in dark and light to determine whether DBN appeared when the eye was set in motion about the yaw axis, respectively, by the VOR or by the visually enhanced VOR (VVOR). The motivation was that a latent upward drift might be revealed during yaw rotation, because the horizontal fast phases induced by the rotation could unleash a vertical component intended to bring the eyes back toward a “desired” vertical position, and this resetting motion would be permissive for a subsequent upward drift. Figure 9 shows one example in a *tottering* animal in which the eye was stable in the stationary condition, but developed DBN during rotation. Rotation data were available for 10 *tottering* (5 young, 5 old) and 5 control animals used to develop the MOR tuning curves of Figs 1, 2. The probabilities of observing DBN during rotation are summarized in Table 3. Note that these values are larger than what was observed during the static conditions of the pitch and roll series, with DBN reaching 49% of records for young *tottering* rotated in the light. In the lighted condition, probability of DBN in *tottering* was significantly greater than in controls. Table 4 shows the breakdown of the records with DBN into the linear, exponential, and indeterminate categories. Control animals were more likely to exhibit exponential slow phases of nystagmus in darkness, which is reminiscent of the tendency of the control animals' nystagmus to be exponential during the tilt series. However, in other respects there was little similarity between the DBN in the rotation and tilt series. For instance, whereas in the tilt series the most significant difference between controls and mutants in DBN occurred in darkness (see Table 1), during rotation the

only significant difference occurred in light (see Table 3), and in light, *tottering* and control animals exhibited similar proportions of linear and exponential waveforms (see Table 4).

3.6. Flocculus inactivation slightly increased the occurrence of DBN in static tilt and rotation conditions

Figure 10 shows the histograms of DBN probability vs. static pitch angle from the muscimol experiments. Note that the range of tilt angles was reduced in the muscimol experiments, but the abscissa scaling of Fig. 8 (which assesses the relationship between probability of DBN and genotype) was retained to facilitate the comparison between Figs 8 and 10. No DBN was observed during the pre-muscimol period of the tilt testing. Muscimol inactivation of the flocculus did produce DBN in some instances, particularly in the pitch-up attitude, where probability of DBN reached as high as 30% of records. It is clear from comparison of Figs 8 and 10 that the tilt-dependency of DBN following flocculus inactivation exhibited an important difference from the DBN of *tottering*; whereas DBN could be encountered in *tottering* at any pitch or roll angle and did not obviously correlate with angle, DBN after flocculus inactivation primarily appeared during nose-up attitudes, and the probability scaled roughly with the pitch angle. The observation that DBN after flocculus inactivation occurred predominantly at off-neutral head attitudes might suggest that it was produced by failure of the brainstem neural integrator, and thus manifest only when the static MOR drove the eye toward eccentric eye positions (“eccentric,” as defined by any eye position away from the equilibrium position observed in the neutral head attitude). However, this interpretation is contradicted by the fact that the downbeat was seen in both positive and negative roll angles, i.e., when the eye was both elevated and depressed compared to its neutral position. Simple integrator failure would have predicted downbeat during depressed eye positions and upbeat for elevated equilibrium positions, but upbeat was never observed. Furthermore, DBN was much more probable for nose-up positions than for contralateral ear-down positions, even though consultation of the curves in Figs 1, 2 will show that the resultant vertical eye positions were comparable. This suggests that the DBN was a function of the response to head attitude, not the ability to maintain specific eye positions. Finally, of the records exhibiting DBN in the lighted condition, about half exhibited a linear pattern (see Table 5), whereas integrator failure would produce exclusively exponential patterns. The fact that identical depressed eye positions produced by pitch and roll tilts did not engender identical increases in DBN can also be used to argue that the increase is not a reflection of Alexander's Law (the tendency of nystagmus to increase in intensity when the eye deviates in the direction of the nystagmus fast phase, see [29]).

Rotation data were also available after muscimol infusion in 6 sessions, and were compared to baseline stability of the same animals determined in 4 sessions. These data are summarized in Table 6. DBN occurred in 14% of dark and light baseline records, not greatly dissimilar to its rates (25% in dark and 15% in light) in the larger sample of control animals contributing to Table 3. After muscimol, the probability increased fourfold in the light, although this change just missed statistical significance ($p = 0.062$), likely due to the small size of the baseline sample. (The fact that the rates of DBN pre/post-muscimol were identical in darkness, and there was a significant difference between the rates post-muscimol

in light vs. dark, supports the difference between pre- and post-muscimol in light representing a real effect, albeit one obscured by the small number of baseline records.)

Table 7 summarizes the waveforms of rotation-induced DBN at baseline and following muscimol. Most instances of DBN following muscimol (particularly in the lighted condition, where the effect of muscimol on DBN occurrence was most dramatic) were of exponential form. This contrasts with the DBN found in *tottering* during rotation in the light, where linear waveforms were more common. On the other hand, both *tottering* and the muscimol-treated animals exhibited more DBN during rotation in the light (as opposed to the dark), which suggests that both vertical instabilities reflect abnormal processing of optokinetic data.

4. Discussion

Tottering mice exhibited hyperactivity of the MOR, suggesting a homology with humans with DBN, in which hyperactivity of otolith circuits has been hypothesized to contribute to the vertical instability. As in the humans with DBN, the abnormal sensitivity to tilt was restricted to the pitch plane. This hyperactivity is at least partially responsible for the previous observations of abnormally high resting eye positions in *tottering* and other ataxic mouse mutants, supporting the possibility that hyperdeviation of the eyes could be used as a surrogate for DBN when using mice to study pathomechanisms and treatment of the human disorder. The abnormality in MOR gain in the mutants was actually somewhat stronger than the hyperdeviation itself; the mutant vs. control contrast function for pitch MOR slope measured 0.165, vs. 0.149 for the contrast function of vertical eye position, and the mutant vs. control unpaired *t*-tests for pitch MOR slope and vertical eye position were respectively $p < 0.0001$ vs. $p = 0.006$. Thus pitch MOR slope may actually be a more consistent surrogate for DBN than vertical eye position, which exhibits a strong degree of moment-to-moment, day-to-day, and animal-to-animal variability. On the other hand, determining MOR slope requires considerably more time and complex apparatus than does determining resting eye position at the natural head position (or at any other, single inclination). The small group of older *tottering* exhibited pitch MOR hyperactivity almost identical to younger animals, suggesting that pitch MOR hyperactivity is not one of the *tottering* abnormalities that progresses with age [42].

In addition to the connection between MOR hyperactivity and abnormally high eye positions, the MOR gain also associated with an abnormally elevated head pitch during ambulation. While the abnormality was quantitatively modest (mutant vs. control contrast function 0.054, mutant vs. control *t*-test $p = 0.039$), it correlated with both the pitch MOR gain and resting eye position. These connections raise the possibility that ambulation head pitch could be another experimental surrogate for DBN – an intriguing possibility, because measurement of head pitch is potentially amenable to use in high-throughput behavioral assays. Whereas our method of determining head pitch made use of an implanted head fixation pedestal and automated marker tracking software, the assay could potentially be performed with still photos of the mouse without artificial visual markers, or of a mouse bearing markers attached temporarily with adhesive. The association between hyperdeviation of the eye and relative elevation of the head during ambulation is also

important because it suggests that the distinct maculo-ocular and maculo-colic pathways are similarly affected by the cerebellar abnormalities, or that the abnormal cerebellar signals enter the otolith pathway at a point proximal to the divergence of the ocular and neck pathways. This commonality may prove useful in determining how the cerebellar dysfunction generates the otolith circuit hyperactivity; any hypothesis must explain the effects on both the eye and the neck. The weakness of the effects and correlations is inconvenient for any experiment designed to use eye or head position as a marker for vestibulocerebellar dysfunction. However, the weakness of the “signals” is not entirely unexpected, as it seems likely that many factors besides the gain of the pitch MOR collaborate in governing the resting positions of head and eyes, thereby depressing correlations between any single pair of measures.

Some animals exhibited a downbeat nystagmus during static tilt. It was more common in *tottering*, and the slow phases with linear (as opposed to exponential) position profiles were also more likely in the mutants. We refer to this nystagmus as DBN for lack of a better descriptive term, although its phenomenological connections to human DBN are suspect, for multiple reasons: Unlike eye hyperdeviation and MOR hyperactivity (which are common in the ataxic mice, just as DBN is common in ataxic humans), mouse DBN during static tilt was rare. Unlike human DBN, in which the nystagmus appears or becomes more prominent with forward inclination, the mouse DBN did not correlate in any way with pitch angle. And whereas human DBN is postulated to correspond to a hyperactivity in the pitch otolith circuit [31], there was no correlation between the probability of DBN in the mice and degree of hyperactivity of the pitch MOR. We conclude that the DBN seen during static tilt in the mouse is a concomitant of cerebellar dysfunction, but is not the mouse equivalent of any component of human DBN. In contrast, DBN was a much more common phenomenon during 0.8 Hz rotation, and in that setting the nystagmus may bear some relationship to human DBN (see below). DBN during rotation in the light was significantly more common in *tottering* than in controls, and bore no relationship to the likelihood of DBN occurring in the same animal during static tilt.

In the interest of determining the role of flocculus hypofunction in the MOR abnormalities of *tottering*, we assessed MOR gains in control animals following unilateral intrafloccular injection of muscimol. Although the inactivation did produce a significant increase in pitch MOR gain, it didn't replicate the overall pattern of MOR abnormalities seen in *tottering*, suggesting the abnormalities are dictated in whole or part by dysfunction at other sites. A strong candidate locus is the nodulus and ventral uvula, which receive a heavy termination of primary and secondary vestibular afferents from the utricle and saccule and are traditionally held to be particularly involved in otolith-related reflexes [4, 9,17]. Unilateral flocculus inactivation differed particularly from the *tottering* mutation in that it produced strong vertical offsets in eye position. In contrast, in *tottering* it appeared that the hyperdeviation of the eye could be explained largely by the increase in pitch MOR gain, without the need of an additional offset. The strong elevating effects of flocculus inactivation follow naturally from the fact that normal floccular Purkinje cells tonically inhibit neurons of the anterior canal vestibulo-ocular reflex that ultimately contribute to excitation of the superior rectus muscle of the ipsilateral eye. The fact that flocculus inhibition caused a mixture of offset and enhanced pitch MOR slope, whereas the mutation seemed to affect

only MOR slope argues that any role of the flocculus in the eye elevation and MOR gain abnormality in *tottering* is not a simple reduction of flocculus tonic output on semicircular canal circuits.

In comparing the muscimol-treated animals to *tottering*, it should be recognized that some of the robustness of the vertical offset in the muscimol experiments is related to the muscimol treatment being unilateral, which differs from what is presumably bilaterally symmetrical flocculus dysfunction in the mutant. Whereas the unilateral treatment simulates rotation about the ipsilateral anterior semicircular canal, any reduction in tonic output of the flocculus in the mutant mimics the effect of rotating the animal about the vectorial sum of both anterior semicircular canal axes, i.e., about the pitch axis. The preferred axis of the ipsilateral anterior canal in C57BL/6 mice lies at approximately 130° (see [10]), and the mouse eye axis at the natural head inclination lies at approximately 50° (see our Fig. 1). As such, the difference between the anterior canal and eye axes is about 80°, whereas the difference between pitch and eye axes is only 40°. With the larger separation between stimulated canal and eye axes, the elevating effect of unilateral flocculus hypofunction is greater than that of bilateral flocculus hypofunction. Even though some of the strength of the offset stems from the unilateral nature of the muscimol injection, the apparent lack of any offset in the mutant curves in Fig. 1 suggests that hyperdeviation in the mutants is more related to effects on otolith circuitry than to disinhibition of the anterior semicircular canals.

The most striking outcome of the flocculus inactivation experiment was that it did not routinely generate nystagmus as it would in the monkey, which may point to peculiarities of the mouse that also lead to its failure to manifest routinely DBN. On occasions when flocculus inactivation did produce DBN, it differed from the rare spontaneous DBN seen in *tottering*. During static tilt it was most common during pitch-up attitudes, whereas *tottering's* DBN showed no relationship to pitch angle. Flocculus inactivation also produced primarily (89%) exponential waveforms during rotation in light (the condition in which DBN produced by flocculus inactivation became most common, occurring in 56% of records). While this was also the condition causing the most DBN in *tottering* (occurring in 49% of records), in *tottering* the DBN waveform in light was predominantly linear (72% of records). In sum, the flocculus inactivation experiments suggest that flocculus hypofunction probably does not itself account for the MOR and static elevation abnormalities seen in *tottering*, but we cannot exclude flocculus hypofunction being a contributing factor. This state of affairs is similar to the situation in human DBN, where flocculus dysfunction is felt to be critical [32], but contributions from other sites have been posited [22,51].

4.1. Origins and significance of mouse DBN

The causes of DBN in mice – on the occasions when it is observed – remain unclear. With regard to the DBN seen during static tilt, there were no consistent patterns in waveforms, effect of illumination, and effects of tilt that suggest a mechanism of origin. As argued above, there are so many dissimilarities between the DBN during static tilt and human DBN that they seem unlikely to be related, except that both may be rooted in cerebellar dysfunction.

A connection between the rotation-evoked mouse DBN and spontaneous human DBN is more possible, although the data are somewhat contradictory. The fact that the rotation-evoked DBN appeared most prominently in mutants in the lighted condition suggests a role of optokinetic inputs. It may be relevant that *tottering* exhibits a strong up/down asymmetry of the optokinetic reflex about the roll axis [42], and it is conceivable that this optokinetic imbalance reveals itself when the eye is in motion. The flocculus contributes strongly to the optokinetic reflex in afoveates [6,21, 34] which raises the possibility that flocculus-mediated optokinetic dysfunction underlies the rotation-induced DBN. This idea is supported by the fact that flocculus inactivation replicated the *tottering* mutation insofar as it produced the greatest amount of DBN during rotation in the light (as opposed to tilt, or rotation in darkness). Floccular Purkinje cells in afoveates sensitive to roll-axis optokinetic stimulation increase their simple spike firing rate during downward motion of the target on the side of recording [18,21]. Based on the connections of the flocculus, this activity would enhance firing in the pathways driving downward motion of the ipsilateral eye [25]. Thus a deficit of flocculus output would decrease the tone in circuits depressing the eye and increase the tone in circuits elevating the eye, and might accordingly engender upward drifts of the eye when optokinetic circuits are stimulated. All these observations tie to human DBN, in that a similar defect in outputs from Purkinje cells excited by downward motion of a smooth pursuit target has been invoked to explain DBN in humans [16]. Another possible connection between the rotation-induced mouse DBN and human DBN was that there was a potential relationship in our data between rotation-evoked DBN and MOR hyperactivity. As reviewed in Introduction, hyperactivity of the pitch-responsive otolith circuits has been argued to contribute to human DBN. Thus it is noteworthy that the probability of rotation-induced DBN correlated weakly with pitch MOR slope ($r = -0.513$, $p = 0.13$), indicating a trend toward the animals having higher pitch MOR gains being more likely to exhibit rotation-evoked DBN. While the correlation did not reach statistical significance, it would also be statistically incorrect to conclude that MOR gains and rotation-evoked DBN are unrelated; the issue would need to be investigated further in a more highly powered study.

Unfortunately, the parallel between the mouse's rotation-evoked DBN and human DBN breaks down in that whereas the excess DBN of the mutants and lesioned animals appeared only during rotation in the light, the human DBN is only rarely affected by vision [20,53]. The difference might still be explained by the fact that while both optokinetic and smooth pursuit circuits pass through the flocculus and have related effects on Purkinje cell firing, the two systems differ in many other respects. The other difference between human DBN and the mouse rotation-induced DBN is the fact that the human DBN appears spontaneously, i.e., without the need for rotation. However, this difference may be less consequential, for reasons discussed below.

4.2. Why doesn't the ataxic mouse exhibit spontaneous DBN?

The current results, together with prior studies, indicate that the ataxic mouse possesses many of the pathological processes that are postulated to produce DBN in humans. *Tottering* and the allelic mutant *rocker* have been demonstrated to have shortened time constants of the gaze-holding neural integrator, at least in the horizontal plane [42]. The current study demonstrates the hyperactivity of pitch tilt circuits posited to cause the gravity-dependent,

eye position-independent component of DBN in humans. And inactivation of the normal mouse flocculus engenders DBN during rotation that can be explained in terms of the same Purkinje cell activation patterns that are invoked to explain the floccular contributions to DBN in humans and non-human primates [16]. The question then, is why the cerebellar dysfunction in the mouse does not routinely produce a spontaneous DBN similar to that of the human patients?

A number of explanations for the lack of spontaneous DBN were proposed in the pilot study [43] and most remain consonant with the results of the completed study. First, since mice lack a fovea they have lesser reason (and probably lesser ability) to exert control over absolute position of the retina with respect to the visual world. Thus a deviation of eye position would not necessarily trigger a restorative saccade; the eyes might simply remain at whatever position is dictated by mechanisms such as the MOR. In contrast, a foveate mammal would execute corrective saccades, preventing the establishment of a new equilibrium position and thereby perpetuating drifts toward the “desired” equilibrium. Second, mice may have a relatively low tendency to initiate fast phases for any reason. For instance, when mice are exposed to prolonged unidirectional optokinetic stimulation, an initial optokinetic response may be followed by a decline in the execution of fast phases and the eyes coming to rest at an eccentric position [41]. Constant gravitational stimulation may, like constant optokinetic stimulation, produce eccentric stable eye positions through this failure to sustain fast phase generation. The difference between the effects of flocculus inactivation in mice and monkeys may similarly reflect a low propensity to initiate fast phases: Flocculus lesions or pharmacological inactivation produce a vestibular imbalance which would be expected to produce nystagmus, and in fact does produce nystagmus in monkeys [37,49,56]. In contrast, our muscimol injections rarely engendered nystagmus in the upright condition. A third reason why ataxic mice may not exhibit human-like DBN may reflect a difference in the role of the MOR in foveate and afoveate mammals. The MOR in afoveate mammals is commonly regarded as acting to bring the retinas into some favorable orientation with respect to the overall visual world [11,33,36]. Consequently, its response to maintained tilt is particularly robust. If the MOR in afoveates is configured to control position, then it is quite likely that an over-active tilt-MOR would lead to an abnormal eye position, rather than an abnormal eye velocity. In contrast, the MOR in foveates is felt to be related to compensating for translational head velocities; the response to velocity is robust, while the response to maintained tilt (which produces identical displacements of the otolithic membranes) is weak [3, 11]. Accordingly, it is plausible that hyperactivity in the otolith circuits in a foveate mammal could manifest as a velocity bias rather than a position bias. A further possibility that was posited previously for the lack of DBN in mice [43] is that the weakness in the mutants of the gaze-holding neural integrator would generate centripetal drifts that would counteract the upward drift of the hyperactive otolithic circuits, resulting in an equilibrium eye position that was not so extreme as to trigger fast phases on the basis of eye-in-orbit eccentricity. The current results however reveal a defect in this explanation. The same integrator weakness that would counteract the upward drift when the eye is elevated would be expected to exacerbate the DBN when the eye is depressed. However, the current results showed that DBN was not elicited by tilt angles that depressed the eye.

Given the absence of DBN in the static condition, why does it frequently appear during the response to horizontal visual-vestibular stimulation? One possibility is that the horizontal and vertical fast phases of vestibular nystagmus lie under the control of a single population of omnipause neurons, as is the case for gaze-shifting saccades in foveate mammals [12]. The silencing of omnipause neurons during horizontal fast phases could thus release a vertical fast phase that does not otherwise occur because signals from the vertical system are themselves insufficient to trigger the pause in omnipause firing. In this speculation, it is the lack of vertical fast phases that is entirely responsible for the failure to develop a sustained nystagmus. A contrasting possibility is that the horizontal image motion that accompanies visual-vestibular stimulation (amounting in *tottering* to about 30–40% of the table velocity during oscillation at 0.8 Hz [42]) in some way renders the mouse more sensitive to a tone imbalance in its vertical optokinetic system, such as that hypothesized to arise from the loss of floccular Purkinje cells, as discussed above. In this case the DBN would appear because rotation potentiates a latent slow phase, rather than a latent fast phase. Still another possibility relates to the observation that in humans, DBN is routinely augmented during eccentric gaze [5]. DBN could appear in the mice during rotation because the rotational stimuli increase the percentage of time that the eye is in an off-center position. To appreciate this speculation, it should be recognized that in contrast to the behavior of a foveate mammal, absent a vestibular or optokinetic stimulus, the mouse will tend to maintain its eyes within a relatively narrow region, as specified by its tiltMOR curve.

4.3. Application of the mouse to studies of origin and therapy of DBN

As reviewed in the Introduction, in humans, DBN reflects varying combinations of a failure of the brainstem integrator, a gravity-dependent eye position-independent component possibly related to MOR hyperactivity, and a gravity-independent, eye position-independent component that may originate in either the smooth pursuit system or the circuitry of the rotational VOR. The current results represent the only systematic attempt, to date, to find spontaneous DBN in an ataxic mouse; the DBN we found during static tilt was rare, fitful, exhibited significant differences from human DBN, and as such is not a useful model for the human abnormality. The DBN evoked during rotation in the light shows more promise as a model, but its dependence on illumination represents a problem, and more work is required to determine its mechanism.

Another approach to selecting surrogates for DBN is by selecting mouse eye movement abnormalities that arise from the pathological mechanisms thought to contribute to DBN in humans. Using this approach, one could use direct assays of the time constant for gaze holding in mice as a surrogate for the integrator deficiency component of human DBN. Integrator time constants are relatively easy to measure in mice [42, 47]. In fact, this measurement is easier in mice than in foveate mammals because of the relative infrequency of spontaneous saccades, which reduces the potential for interruption of the integrator-mediated centripetal drift. With regard to the gravity-dependent component of DBN, the pitch MOR gain is the most obvious murine surrogate. Alternatively, one could simply assess vertical eye position at a single tilt angle; it was the hyperdeviation of the eye that led us to suspect MOR hyperactivity in the first place. However, it must be recognized that there is a wide variability of vertical eye position moment-to-moment, day-to-day, and animal-to-

animal, and the average difference between mutants and controls only measures a few degrees. Thus mouse DBN studies using measurements of eye position at a single tilt angle would have to incorporate replications of measurements across multiple sessions and/or use large numbers of animals in order to be adequately statistically powered. The head pitch during ambulation potentially requires less equipment and animal preparation than any assay of eye position, but the average difference between *tottering* mutants and controls is even smaller than is the difference in vertical eye positions.

The best murine homolog for the gravity-independent bias component of DBN is unclear, but the strongest candidate suggested by the current results is the rotation-evoked DBN. The possibility rests on several interrelated facts: 1) It could be replicated to some extent by flocculus inactivation; 2) It seemed to arise from the optokinetic system; 3) Flocculus hypofunction (either unilateral or bilateral) would create a tone imbalance in the optokinetic system that could drive the eye (s) upward; 4) DBN in humans and non-human primates has been argued to arise from flocculus hypofunction, either because such hypofunction generates a tone imbalance within the smooth pursuit system [16, 32], or because the normal flocculus cancels a VOR imbalance in the brainstem [7].

Strictly speaking, the current data only allow us to speculate on surrogates for human DBN in *tottering* mutants. The degree to which results in *tottering* apply to other ataxic mutants is unclear. However, *rocker*, another *Cacna1a* mutant, possesses very similar deficits, including the eye hyperdeviation that led us to investigate MOR gain. Very limited data from an entirely different cerebellar mutant (*lurcher*) suggests it also exhibits eye hyperdeviation [39]. Thus at present there is no reason to believe that *tottering* is unusual and ample reason to believe that its MOR hyperactivity will be the rule for ataxic mutants. Future characterizations of mouse cerebellar mutants would be enhanced by inclusion of assessments of pitch tilt MOR (or at least, the absolute vertical eye position at a specified head pitch) in addition to the more common assessments of rotational reflexes such as the VOR and OKR.

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Fig. 1.

Relationship of horizontal (left panels) and vertical (right panels) eye position to pitch tilt angle in controls and young *tottering*. Top panels are for eye position in light and bottom panels are for position in darkness. Error bars are ± 2 SEM. Pitch angle is with respect to the lambda-bregma axis (positive angles are nose-up), and vertical cursor indicates the average lambda-bregma angle measured in the control animals during ambulation.

Fig. 2. Relationship of horizontal (left panels) and vertical (right panels) eye position to roll tilt angle in controls and young *tottering*. Top panels are for eye position in light and bottom panels are for position in darkness. Positive roll angles are ipsilateral ear up. Error bars are ± 2 SEM.

Fig. 3. Relationship of horizontal (left panels) and vertical (right panels) eye position to pitch tilt (top panels) and roll tilt (bottom panels) in young *tottering* and old *tottering*. Only results in light are shown as results in darkness were similar. Error bars for young *tottering* are ± 2 SEM. The large error bars for the small group of old *tottering* are omitted to permit use of the vertical scale from Figs 1, 2. Vertical cursor in top panels are ambulation head pitch in controls (copied from Fig. 1).

Fig. 4. Relationship between vertical eye position at the neutral head position (0° pitch and roll angles of the tilt apparatus) and pitch MOR slope. A weak but significant correlation supports a role for pitch MOR hyperactivity in the abnormal vertical eye positions of *tottering*. Regression lines for young *tottering* (heavy line) and controls (thin line) are superimposed.

Fig. 5. Relationship of eye position indices and ambulation head pitch. Left panel: pitch MOR gain vs. ambulation pitch. Right panel: $E_v@Gimbal_0$ vs. ambulation pitch. Regression lines for young *tottering* (heavy line) and controls (thin line) are superimposed.

Fig. 6. Relationship of horizontal (left panels) and vertical (right panels) eye position to pitch tilt (top panels) and roll tilt (bottom panels) before and after injection of muscimol in the flocculus ipsilateral to the recorded eye. Results reflect averaging the curves obtained in 11 sessions. All data obtained in the lighted condition. Error bars are ± 2 SEM.

Fig. 7.

4 examples of spontaneous downbeat nystagmus during static tilt in *tottering* (panels A, B) and controls (panels C, D). Traces are darkened to indicate periods when the animal was in darkness. See text for pitch angles at which records were captured. Ordinate and abscissa labels are shared across panels.

Fig. 8.

Probability of DBN being observed as a function of tilt angle. Top panels are for pitch tilts and bottom panels are for roll tilts; left panels are for lighted condition and right panels are for darkness. Error bars are ± 1 SD.

Fig. 9.

Example of DBN appearing during yaw rotation in a *tottering* animal. Top traces show a record obtained with the animal stationary; no nystagmus was present. Darkened traces denote periods of darkness. Bottom traces show a record obtained while the animal was oscillated in the light at 0.8 Hz. During oscillation a DBN with predominantly linear slow phases appeared.

Fig. 10. Probability of DBN being observed before and after muscimol injection, plotted as a function of tilt angle. Top panels are for pitch tilts and bottom panels are for roll tilts; left panels are for lighted condition and right panels are for darkness. Error bars are $\pm 1SD$.

Table 1

Probability of DBN near neutral position

	Dark	Light	Dark vs. Light
Pitch series			
C57	0.009 ± 0.026	0.009 ± 0.026	$p = 1$
Young <i>tottering</i>	0.018 ± 0.033	0.028 ± 0.057	$p = 0.566$
Old <i>tottering</i>	0.090 ± 0.134	0.050 ± 0.061	$p = 0.561$
C57 vs. young <i>tottering</i>	$p = 0.391$	$p = 0.239$	
Young vs. old <i>tottering</i>	$p = 0.062$	$p = 0.472$	
Roll series			
C57	0.008 ± 0.026	0.000 ± 0.000	$p = 0.228$
Young <i>tottering</i>	0.045 ± 0.062	0.035 ± 0.090	$p = 0.716$
Old <i>tottering</i>	0.119 ± 0.153	0.086 ± 0.089	$p = 0.688$
C57 vs. young <i>tottering</i>	$p = 0.042$	$p = 0.141$	
Young vs. old <i>tottering</i>	$p = 0.133$	$p = 0.284$	

Probability of observing DBN in a record taken at any one pitch or roll angle within $\pm 30^\circ$ of the neutral tilt position. Table includes p values for the indicated comparisons, obtained from the t -test.

Table 2

Breakdown by waveform of the records exhibiting DBN during static tilt

	Linear	Exponential	Indeterminate
Dark			
C57, $n = 20$	35.0%	60.0%	5.0%
Young <i>tottering</i> , $n = 40$	57.5%	32.5%	10.0%
Old <i>tottering</i> , $n = 40$	72.5%	22.5%	5.0%
C57 vs. young <i>tottering</i>	$p = 0.100$		
Young vs. old <i>tottering</i>	$p = 0.160$		
Light			
C57, $n = 11$	0.0%	90.9%	9.1%
Young <i>tottering</i> , $n = 30$	43.3%	53.3%	3.3%
Old <i>tottering</i> , $n = 29$	86.2%	10.3%	3.4%
C57 vs. young <i>tottering</i>	$p = 0.008$		
Young vs. old <i>tottering</i>	$p = 0.0006$		

The number of records is listed separately by animal group. Table includes p values (from Pearson's χ^2) comparing the different animal groups for the proportion of their nystagmus records that exhibited a linear configuration.

Table 3

Probability of DBN during 0.8 Hz rotation in light and dark

	Dark	Light	Dark vs. Light
C57	0.25 ± 0.056	0.15 ± 0.046	$p = 0.160$
Young <i>tottering</i>	0.25 ± 0.056	0.49 ± 0.066	$p = 0.006$
Old <i>tottering</i>	0.33 ± 0.060	0.39 ± 0.062	$p = 0.502$
C57 vs. young <i>tottering</i>	$p = 1$	$p < 0.0001$	
Young vs. old <i>tottering</i>	$p = 0.310$	$p = 0.260$	

Values are mean ± standard error, where the standard error is the error of the estimate for a binomial measure. Table includes p values for the unpaired t -tests along the rows and columns.

Table 4

Breakdown by waveform of the records exhibiting DBN during 0.8 Hz rotation

	Linear	Exponential	Indeterminate
Dark			
C57, $n = 15$	13.3%	86.7%	0.0%
Young <i>tottering</i> , $n = 15$	46.7%	46.7%	6.7%
Old <i>tottering</i> , $n = 22$	50.0%	36.4%	13.6%
C57 vs. young <i>tottering</i>	$p = 0.046$		
Young vs. old <i>tottering</i>	$p = 0.842$		
Light			
C57, $n = 9$	66.7%	22.2%	11.1%
Young <i>tottering</i> , $n = 29$	72.4%	27.6%	0.0%
Old <i>tottering</i> , $n = 25$	68.0%	28.0%	4.0%
C57 vs. young <i>tottering</i>	$p = 0.740$		
Young vs. old <i>tottering</i>	$p = 0.723$		

The number of records is listed separately by animal group. Table includes p values (from Pearson's χ^2) comparing between the different animal groups the proportion of the records that exhibited a linear configuration.

Table 5

Breakdown by waveform of the records exhibiting DBN during post-muscimol static tilt

	Linear	Exponential	Indeterminate
Dark, $n = 14$ records	50%	50%	0%
Light, $n = 10$ records	40%	60%	0%

Table 6

Probability of DBN during pre- and post-muscimol 0.8 Hz rotation

	Dark	Light	Dark vs. Light
Pre-muscimol	0.14 ± 0.14	0.14 ± 0.14	$p = 1$
Post-muscimol	0.19 ± 0.10	0.56 ± 0.13	$p = 0.028$
Pre- vs. post-muscimol	$p = 0.80$	$p = 0.062$	

Table includes p values (from Pearson's χ^2) and compares the proportions of total records exhibiting DBN across rows and columns.

Table 7

Breakdown by waveform of the records exhibiting DBN pre- and post- muscimol during 0.8 Hz rotation

	Linear	Exponential	Indeterminate
Dark			
Pre-muscimol, $n = 1$	0%	100%	0%
Post-muscimol, $n = 3$	33.3%	66.6%	0%
Light			
Pre-muscimol, $n = 1$	0%	100%	0%
Post-muscimol, $n = 9$	11.1%	88.8%	0%