Research Article

Glycine Receptor Deficiency and Its Effect on the Horizontal Vestibulo-ocular Reflex: a Study on the SPD1J Mouse

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ABSTRACT

Inhibition is critical in the pathways controlling the vestibulo-ocular reflex (VOR) and plays a central role in the precision, accuracy and speed of this important vestibular-mediated compensatory eye movement. While γ -aminobutyric acid is the common fast inhibitory neurotransmitter in most of the VOR microcircuits, glycine is also found in key elements. For example, the omnidirectional pause neurons (OPNs) and inhibitory burst neurons in the horizontal VOR both use glycine as their preferred inhibitory neurotransmitter. Determining the precise contribution of glycine to the VOR pathway has been difficult due to the lack of selective tools; however, we used spasmodic mice that have a naturally occurring defect in the glycine receptor (GlyR) that reduces glycinergic transmission. Using this animal model, we compared the horizontal VOR in affected animals with unaffected controls. Our data showed that initial latency and initial peak velocity as well as slow-phase eye movements were unaffected by reduced glycinergic transmission. Importantly however, there were significant effects on quick-phase activity, substantially reducing their number (30–70 %), amplitude (~55 %) and peak velocity (~38 %). We suggest that the OPNs were primarily responsible for the reduced quick-phase properties, since they are part of an unmodifiable, or more ‘hard-wired’, microcircuit. In contrast, the effects of reduced glycinergic transmission on slow-

phases were likely ameliorated by the intrinsically modifiable nature of this pathway. Our results also suggested there is a ‘threshold’ in GlyR-affected animals, below which the effects of reduced glycinergic transmission were undetected.

Keywords: glycine receptor, glycinergic pathways, vestibulo-ocular reflex, slow-phase, quick-phase mechanisms

INTRODUCTION

In the vertebrate central nervous system, there are two fast inhibitory amino acid neurotransmitters: γ -aminobutyric acid (GABA) and glycine. Inhibitory neurons in the brain release GABA at their terminals, whereas in the spinal cord, glycine is more prominent (Moss and Smart 2001). In the brain stem, particularly in circuits used to control reflex eye movements such as the vestibulo-ocular reflex (VOR), both inhibitory neurotransmitters are known to operate. However, the distribution of GABA and glycine neurons, even within similar VOR circuits, can be different. For example, GABA is used almost exclusively as the fast inhibitory neurotransmitter in the vertical VOR, while glycine is important in mediating the horizontal VOR (hVOR) (Spencer et al. 1989; Horn et al. 1994). Within the hVOR circuit, glycine is the predominant inhibitory transmitter released by premotor medial vestibular nucleus (MVN) neurons that project to the ipsilateral abducens nucleus and inhibit motor neu-

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rons supplying the lateral rectus (Spencer et al. 1989; Horn et al. 1994). Glycine is also used by important omnidirectional pause neurons (OPNs) found in the brain stem reticular formation and are part of critical premotor networks that comprise the saccade or burst generator (Cartwright et al. 2003; Leigh and Zee 2006; Optican 2008; Otero-Millan et al. 2011). In addition, there is a newly described glycinergic projection from the fastigial nucleus of the cerebellum to vestibular and reticular neurons in the ipsilateral brain stem (Bagnall et al. 2009). As a result of these varied connections and lack of selective tools, determining the functional importance of glycine in reflexive eye movements has been difficult to unravel. To address this drawback, we used spasmodic mice with a naturally occurring glycine receptor (GlyR) mutation that provides us with a model for studying the effects of chronically reduced glycinergic transmission. Our results suggest that glycine plays a specific and critical part in generating horizontal quick-phase reflexive eye movements and that impaired quick-phase activity significantly interferes with slow-phase eye movements. As previously suggested (Camp et al. 2010), GlyR mutant mice appear to have a functional threshold below which eye movements and VOR are relatively normal. Beyond this threshold, however, the effects of diminished glycinergic transmission become evident with pathological effects on hVOR performance due in part to impairment of OPN glycinergic transmission.

METHODS

Subjects

We measured vestibular-evoked eye movements in affected homozygous (*spd/spd*) and control unaffected heterozygous (*spd+*) *spasmodic* mice (both sexes, aged 13–15 weeks, $n=6$ per group, affected male/female 2/4, control male/female 1/5). Homozygous *spasmodic* mice have a missense mutation in the $\alpha 1$ subunit gene that reduces the sensitivity, but minimally affects the number, of glycine receptors (Ryan et al. 1994; Saul et al. 1994; Graham et al. 2006). The phenotype of affected homozygous mice includes light tremor at rest and increased tremor during movements, clenching of rear legs when suspended by the tail, an impaired righting reflex when placed on their backs and a tendency to walk with an arched back and extended wrist and ankle joints. In addition to phenotype identification, all mice were genotyped after testing using tail tissue samples.

All procedures for animal care and experimental protocols described below were approved by the Animal Care and Ethics Committee of the University of New South Wales.

Animal preparation

As previously described (Migliaccio et al. 2011), a bolt was mounted onto the skull under general (isoflurane, 2–3 %) and local (lidocaine, 1 %, ≤ 0.2 cc) anaesthesia to facilitate head restraint during eye movement recordings. Briefly, the dorsal surface of the skull was exposed and the periosteum was removed. Three stainless steel screws (#0 $\times$ 1/8, Micro Fasteners Pty. Ltd., Thomastown, VIC, Australia) were implanted: two screws in the anterior parietal bone, one on each side of the sagittal suture, and one screw on the posterior parietal bone, adjacent to the posterior suture. A lightweight countersunk metal bolt was placed upside down with flat head positioned in the centre of the three-screw formation and embedded in a dental acrylic (Protemp IV, 3 M) cap engulfing the three implanted screws.

Eyelashes and vibrissae were shortened to minimise irritation during the experiment. The upper and lower eyelids were anaesthetised with 0.5 % Alcaine and retracted using ophthalmic sutures (10-0, BSL5, Nylon monofilament, Sharpoint, Reading, PA, USA). Saline was regularly applied to keep the eyes moist. The animal was restrained in a close-fitting plastic capsule, and both the capsule and animal were placed onto a Fick gimbal superstructure mounted atop a servo-controlled motor. The head post was pitched $\sim 30^\circ$ nose down so that the horizontal semicircular canals were approximately ($\sim \pm 10^\circ$) perpendicular to the Earth-vertical axis of rotation (Calabrese and Hullar 2006). Thus, rotation of the servo motor maximally stimulated the horizontal semicircular canals (hSCCs). The animals were allowed to completely recover from surgery and anaesthesia before rotational testing. Response to ambient sounds, light or touch of the paw and tail was used to determine animal well-being.

Rotational testing

Each animal's angular VOR response was measured during (horizontal) rotations in the plane of the hSCC. The experimental protocol included whole-body sinusoidal rotations at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz at peak velocities of 20, 50 and 100 $^\circ/\text{s}$, and transient acceleration stimuli at 3,000 and 6,000 $^\circ/\text{s}^2$ reaching a velocity plateau of 150 $^\circ/\text{s}$ (for 3,000 $^\circ/\text{s}^2$) and 300 and 600 $^\circ/\text{s}$ (for 6,000 $^\circ/\text{s}^2$).

Eye movement recording

A high-speed video-oculography system was used to measure 3-D binocular eye movements. This technique has been used previously in mice and larger

rodents and was described in detail elsewhere (Migliaccio et al. 2005, 2011). Eye movements were calculated using point tracking of marker arrays that were affixed to the eyes. Each marker array was opaque except for three fluorescent dye-soaked $200 \times 200 \mu\text{m}^2$ squares separated by $200 \mu\text{m}$ and arranged in a 45° right triangle. Before applying the marker array, each cornea was treated with topical anaesthetic (0.5 % Alcaine) and a miotic agent (1 % pilocarpine) to ensure pupil constriction. Cyanoacrylate was used to attach the marker array directly above the constricted pupil so as to occlude vision.

Two high-speed CCD colour cameras (DX-COL-CS, Point-Grey, Canada), each one operating at 200 fps, were used to track the fluorescence marker arrays. The camera's optical axis was aligned with the centre of corneal curvature to within $72 \mu\text{m}$ using a technique described previously (Migliaccio et al. 2005). We assumed that in this position, the optical axis aligned with the centre of rotation of the eye (and marker array). The video system had a spatial resolution of 204 pixels/mm, capturing an area of $2.5 \times 1.7 \text{ mm}^2$. The shutter speed was set to $1/1,250$ of a second to ensure sharp images during fast eye movements. We assumed an eye radius of $1.67 \pm 0.18 \text{ mm}$ (the eye radius of a 100-day-old C57BL6 mice) (Schmucker and Schaeffel 2004), with resulting eye rotation measurement resolution $<0.3^\circ/\text{pixel}$.

A Data-Acquisition Card (NI PCI-6034E, National Instruments) was used to record analogue position and velocity of the servo motor (GOLDLINE DDR D083, Danaher). Both position and velocity were calculated from the servo motor two million counts/revolution sine encoder and converted into a $\pm 10 \text{ V}$ analogue signal on the real-time Motion Controller Card (NI PCI-7340, National Instruments) system. A shared timing signal set at 200 Hz was used to trigger both camera shutters and to sample motor position and velocity signals. We verified synchronisation of motor and video data acquisition by tracking an Earth-fixed marker array (placed at approximately where the mouse eye would be located) during 5-Hz (peak velocity $100^\circ/\text{s}$) sinusoidal rotation of the gimbal superstructure and cameras. The motor and video data were synchronised to within $0.25 \pm 0.4 \text{ ms}$.

Data analysis

Binocular 3-D eye rotation data were displayed in real time in eye coordinates during acquisition and converted to rotation vectors in head coordinates during subsequent analysis. We report the horizontal components of eye and head data with the head coordinate system centred on the skull stereotactic origin with the positive yaw axis (positive indicating leftward)

dorsal and perpendicular to the hSCC plane. The methods of analysis are similar to those that have been described previously (Migliaccio et al. 2004, 2005, 2010, 2011). A second-order zero-phase Savitzky–Golay filter with five side points was used to reduce noise prior to slow-phase eye component analysis. Unfiltered eye velocity data were used to analyse the quick-phase component. Eye velocity data were inverted prior to calculation of gains and phases, so that an ideal VOR would yield a gain of +1 and phase of 0° . Positive phase lead denotes eye velocity leading head velocity.

Quick-phase removal and extraction

Quick-phases were either removed or extracted using a semi-automatic de-saccading technique that we optimised based on several published algorithms (Migliaccio et al. 2006; Fauchoux et al. 2007). In brief, we calculated the square of the raw and smoothed (zero-phase Savitzky–Golay filter) eye acceleration signals. Thus, both signals were always positive and were more sensitive to acceleration. For slow-phase analysis, we manually determined the acceleration threshold for optimal quick-phase detection. The quick-phase start was counted as valid only when there was a threshold crossing in both raw and smoothed signals, ensuring that quick-phases detected were not due to transient noise in the acceleration signal. Once a valid quick-phase start was found, a pattern recognition algorithm detected the quick-phase end. The algorithm checked quick-phase duration, peak velocity and standard deviation to exclude false positives and outliers. To avoid bias in slow-phase analyses, the values of data points between quick-phase starts and ends were not interpolated or included in the mean response analysis (see below).

Analysis of transient acceleration steps

Data from >10 transient acceleration stimuli per direction were superimposed and quick-phases were removed. VOR performance was measured using the following three parameters: acceleration gain (G_A), constant-velocity gain (G_V) and latency. Least-square linear regressions were fit to the head and eye velocity traces during the constant-acceleration part of the stimulus for G_A calculation and the constant-velocity part for G_V calculation (Fig. 1A). G_A was calculated as the mean ratio of horizontal eye/head *acceleration* (using the slopes of the fitted lines) during the 10 to 30 ms interval after stimulus onset. G_V was calculated as the mean ratio of horizontal eye/head *velocity* during the 200 to 400 ms interval after stimulus onset. Respective line fits to the mean eye and head velocity traces (Fig. 1A) were used to determine the stimulus

FIG. 1. **A** VOR eye movements (inverted) in response to transient steps of acceleration for one control and affected mouse (head velocity, black; slow-phase, dark grey; quick-phase, light grey). Regression fits (dashed lines) to the grey highlighted part of the signal were used to calculate acceleration gain (G_A), velocity gain (G_V) and latency of the slow-phase VOR response. **B** Comparison of start and end eye positions (in eye coordinates) during transient rotations for one control and one affected mouse. The affected mouse data show how eye position drifts

to the edge of the oculomotor range in the absence of quick-phases, whereas eye movements in control animals are recentered due to quick-phases. This resetting of eye position results in ~50 % more cumulative angular travel of the eyes in control compared to affected mice. **C** Comparison of G_A , G_V and latency for control and affected mice ($n=6$ per group). Affected mice displayed significantly reduced G_V for both tested stimuli, likely due to the absence of resetting quick-phases (star in **A**).

and response onsets, i.e., the times at which each of these line fits intercept with the zero velocity axis. The latency of the VOR was defined as the time difference between stimulus (head rotation) and response (eye rotation) onset. We measured the change in horizontal eye position in eye coordinates (i.e., primary position was defined as the zero reference) during transient stimuli. Start and end eye positions were calculated by averaging eye position during the 200 ms period immediately prior to the stimulus acceleration (onset) and immediately prior to stimulus de-acceleration, respectively (see Fig. 1B).

In addition to slow-phase velocity, we analysed the slow-phase cumulative eye position (SCEP) during transient acceleration step stimuli. SCEP gives a measure of the total angular distance the eye travels during a given head rotation and was calculated by extracting quick-phases from the eye position data and summing the changes in eye position during each slow-phase during the transient stimulus.

Analysis of sinusoidal rotations

Quick-phases were removed for low-frequency stimuli ≤ 1 Hz. For stimulus frequencies >1 Hz, quick-

phases were rare and often distorted the whole cycle. In those cases, the affected cycle was excluded from the analysis. Horizontal head and eye velocity cycles (10–100, depending on frequency) were superimposed. Least-square pure sine waves (frequency was a fixed variable, amplitude and phase were free variables) were fit to each head and eye velocity cycle to compute gain and phase (Fig. 2). Gain was defined as the ratio of eye/head velocity amplitude of the least-square sinusoidal fits. Mean and standard deviation of gain and phase across cycles is reported.

Analysis of quick-phases

Quick-phase eye movements, like saccades, are notable for their consistent and stable characteristics (Fig. 3A, B). Therefore, duration and peak velocity can be characterised by their stereotypical relation with respect to amplitude, and this relation is referred to as the ‘main sequence’ (Stahl et al. 2006). We analysed quick-phases for sinusoidal stimuli at 0.1, 0.2 and 0.5 Hz at 100 °/s and transient acceleration steps of 3,000 °/s² reaching a peak velocity of 150 °/s (3k150). The technique used to remove quick-phases from slow-phase analysis was now used to extract quick-phases (see above). However, instead of manually adjusting the acceleration threshold for each individual trace, we automated the process by fixing the acceleration threshold parameter to 5,000 °/s². Extracted quick-phases were separated by stimulus direction (leftward or rightward) and eye (left or right). Amplitude was defined as the difference in horizontal eye position between the quick-phase start and end. Peak velocity was defined as the maxima of the horizontal eye velocity trace during the quick-phase. Because quick-phase peak velocity and amplitude histograms were Weibull distributions, we reported the median, rather than mean, quick-phase peak velocity and amplitude. The relationship between peak velocity and amplitude was determined by a least-square linear fit (Fig. 3A). The relationship between amplitude and duration was determined by a non-linear regression fit of the form:

$$D = K(1 - e^{-A/\tau})$$

where D stands for quick-phase duration and A for quick-phase amplitude. K and τ are fit constants for the exponential equation and represent the saturated duration and non-linear component of the fit, respectively (Sakatani and Isa 2007).

Statistical analysis

Statistical analysis was performed using Minitab. We used a general linear model with two-factor interac-

tions. Independent factors included mouse type (affected or control), eye measured (left or right) and direction of rotation (positive or negative). For sinusoidal stimulus analysis, the additional independent factors were peak velocity and frequency, and the dependent factors were gain and phase. For transient stimulus analysis, an additional independent factor was stimulus (3k150, 6k300, 6k600), and dependent factors were G_A , G_V and latency. For quick-phase analysis, an additional independent factor was stimulus (0.1 Hz, 0.2 Hz, 0.5 Hz, 3k150), and dependent factors were amplitude, peak velocity, duration and number. All variables were included in the model initially and those found insignificant were subsequently removed. Post hoc pairwise comparisons were performed using the Tukey’s honestly significant difference (HSD) method. Mean and SD were reported for normal distributed factors.

RESULTS

VOR response to transient steps of acceleration

Figure 1A shows an example of typical VOR eye movements for a control and an affected mouse in response to transient acceleration step stimuli (leftward 3,000 °/s² constant acceleration reaching 150 °/s velocity plateau, i.e., 3k150). Acceleration gains (G_A) did not differ significantly between test groups ($F_{(1, 212)}=2.95$, $P=0.087$; Fig. 1C) with a gain difference between control and affected mice of 0.07 ± 0.04 at 3,000 °/s² ($T_{HSD}=-1.780$, $P=0.286$) and 0.02 ± 0.03 at 6,000 °/s² (pooled data from 6k300 and 6k600) ($T_{HSD}=-0.493$, $P=0.961$). Similarly, VOR latency showed no significant difference between control and affected mice ($F_{(1, 203)}=2.02$, $P=0.156$) with a difference of 0.86 ± 0.51 ms at 3k150 ($T_{HSD}=1.708$, $P=0.528$), 0.07 ± 0.53 ms at 6k300 ($T_{HSD}=-0.140$, $P=1.000$) and 0.52 ± 0.55 ms at 6k600 ($T_{HSD}=0.948$, $P=0.934$).

Figure 1B shows that the final eccentricity of the eye is closer to the centre of the oculomotor range in control compared to affected mice, even though the starting eye positions are similar. For control mice, the mean difference between *start* and *end* eye positions during transient stimuli was 0.105 ± 0.072 (rotation vector units, corresponding to $\sim 12^\circ$; $P<0.001$), whereas for affected mice, the mean difference was larger at 0.149 ± 0.102 ($\sim 17^\circ$; $P<0.001$). The difference between these two means of $\sim 5^\circ$ was significant ($P<0.05$) (see Fig. 1B). The total angular travel of the eyes (i.e., SCEP) during 3k150 transient acceleration step stimuli of 180° was calculated for each mouse type. During these rotations, the average SCEP of control mice was 0.817 ± 0.350 (rotation vector units, corresponding to $\sim 79^\circ$), while the average SCEP of affected mice was 0.523 ± 0.189 (rotation vector units,

FIG. 2. **A** VOR eye movements (inverted) in response to sinusoidal head rotation for one control and affected mouse (head velocity, *black*; slow-phase, *dark grey*; quick-phase, *light grey*). Sine waves were fit to head velocity and slow-phase data (*dashed lines*) to calculate gain and phase of the VOR response. **B** Comparison of gain (column one) and phase

(column two) for control and affected mice ($n=6$ per group). Each row represents a peak velocity of 20, 50 or 100 %/s respectively. The VOR eye movements of affected mice were significantly affected during low-frequency, high-velocity stimuli (0.1–1 Hz at 100 %/s).

FIG. 3. **A** The main sequence represents the relationship between quick-phase amplitude, peak velocity and duration. Adjacent histograms aid in comparing the distribution of quick-phase eye movements. Affected mice (grey, $m=1,553$ quick-phases from $n=6$ mice) showed reduced amplitudes ($\sim 55\%$) and peak velocities ($\sim 38\%$) compared to controls (black, $m=3,061$

quick-phases from $n=6$ mice). **B** Quick-phase eye movements from one control and one affected mouse superimposed. **C** Comparison of the number of quick-phases per stimulus cycle for four different stimuli. The number of quick-phases in affected mice was substantially reduced (30–70 %).

corresponding to $\sim 55^\circ$), showing a significant difference ($T_{42}=3.690$, $P<0.001$) (see Fig. 1B).

The differences in final eccentric eye position and SCEP between mouse types could be due to altered quick-phases (that recentre the eye, see below) and G_V (that affects the total travel of the eye). ANOVA analysis of the constant velocity response (G_V) identified a significant difference between control and affected mice ($F_{(1, 188)}=64.18$, $P<0.001$). Affected mice had a significantly lower G_V compared to control mice during both 3k150 and 6k300 stimuli: affected mice were lower by 0.35 ± 0.03 ($T_{\text{HSD}}=-10.230$, $P<0.001$) and 0.15 ± 0.04 ($T_{\text{HSD}}=-4.172$, $P<0.001$), respectively (see Fig. 1C). G_V for 6k600 stimuli was not calculated, because both control and affected mice did not have slow-phase eye movements at this velocity.

VOR response to sinusoidal rotation

The VOR was tested for sinusoidal whole-body rotations in darkness at frequencies of 0.1–10 Hz at peak velocity 20, 50 and 100 °/s. Figure 2A shows an example of typical low-frequency (0.2 Hz–100 °/s) stimuli and eye-movement responses for a control and an affected mouse. The affected mouse quick-phase response shown here was asymmetrical, with small and infrequent leftward quick-phases, but no detectable rightward quick-phases. The lack of rightward quick-phases (during rightward head rotations) brought the eyes to the limit of the left oculomotor range, which impeded leftward slow-phases and distorted the leftward sinusoidal response.

The VOR gain was significantly different between control and affected mice ($F_{(1, 796)}=24.22$, $P<0.001$). Post hoc analysis using Tukey's HSD test showed no difference in VOR gain between controls and affected mice during 20 and 50 °/s stimuli ($T_{\text{HSD}}=-1.334$, $P=0.766$ and $T_{\text{HSD}}=-2.183$, $P=0.246$, respectively). However, a significant difference was detected during 100 °/s stimuli ($T_{\text{HSD}}=-5.120$, $P<0.001$). We analysed the 100 °/s sinusoidal data using pairwise comparison at each frequency between mouse types.

At frequencies between 0.1 and 0.5 Hz, the affected mouse VOR gain was significantly lower (~50 % on average) than the control mouse gain (see Fig. 2B) by 0.22 ± 0.05 at 0.1 Hz ($T_{\text{HSD}}=-4.398$, $P<0.01$), 0.24 ± 0.05 at 0.2 Hz ($T_{\text{HSD}}=-4.771$, $P<0.001$) and 0.21 ± 0.05 at 0.5 Hz ($T_{\text{HSD}}=-4.145$, $P<0.01$). There was no difference in VOR gain for frequencies between 1 and 10 Hz; the differences were 0.12 ± 0.05 at 1 Hz ($T_{\text{HSD}}=-2.257$, $P=0.656$), 0.02 ± 0.05 at 2 Hz ($T_{\text{HSD}}=-0.480$, $P=1.000$), 0.13 ± 0.05 at 5 Hz ($T_{\text{HSD}}=-0.225$, $P=0.498$) and 0.01 ± 0.05 at 10 Hz ($T_{\text{HSD}}=-0.225$, $P=1.000$).

Similar to gain, there was a significant difference in VOR phase between control and affected mice ($F_{(1, 382)}=62.18$, $P<0.001$). Post hoc pairwise comparison showed no significant difference of VOR phase between control and affected mice for 20 °/s sinusoidal stimulation ($T_{\text{HSD}}=0.070$, $P=1.000$). However, there was a significant difference between control and affected mice for 50 and 100 °/s velocity stimuli ($T_{\text{HSD}}=3.857$, $P<0.01$ and $T_{\text{HSD}}=10.75$, $P<0.001$, respectively). At 50 °/s, there was a consistent phase shift of 3.69 ± 0.96 °. However, there was no significant interaction between mouse type and frequency at this velocity ($F_{(7, 271)}=0.70$, $P=0.671$). ANOVA analysis of 100 °/s sinusoidal data identified a significant difference between mouse types ($F_{(1, 274)}=167.43$, $P<0.001$) as well as an interaction between mouse type and frequency ($F_{(7, 274)}=15.93$, $P<0.001$). Thus, we analysed the data using pairwise comparison at each frequency between mouse types. Similar to gain, the affected mouse VOR phase was significantly different for frequencies between 0.1 and 1 Hz by 25.0 ± 2.2 ° at 0.1 Hz ($T_{\text{HSD}}=11.550$, $P<0.001$), 17.9 ± 2.2 ° at 0.2 Hz ($T_{\text{HSD}}=8.280$, $P<0.001$), 14.5 ± 2.1 ° at 0.5 Hz ($T_{\text{HSD}}=6.800$, $P<0.001$) and 10.4 ± 2.1 ° at 1 Hz ($T_{\text{HSD}}=4.684$, $P<0.001$). The affected mouse VOR phase for frequencies between 2 and 10 Hz was not significantly different from control mice; however, it resembled the same trend of decreasing phase shift with increasing frequency, i.e., the difference was 7.2 ± 2.2 ° at 2 Hz ($T_{\text{HSD}}=3.275$, $P=0.079$), 0.6 ± 2.2 ° at 5 Hz ($T_{\text{HSD}}=0.273$, $P=1.000$) and 1.2 ± 2.2 ° at 10 Hz ($T_{\text{HSD}}=0.530$, $P=1.000$).

VOR quick-phases and saccadic main sequence

Quick-phase eye movements were typically observed during sinusoidal frequencies <1 Hz, as well as during the constant velocity part of the transient stimuli. The eye velocity responses to steps of accelerations (Fig. 1A) and sinusoidal rotations (Fig. 2A) show that affected mice had substantially fewer and smaller quick phases compared to control mice.

Figure 3A, B shows the quick-phase shape, amplitude, peak velocity and duration for controls and affected mice. Each data point in both graphs of the main sequence (Fig. 3A) represents 1 of 4,614 quick-phase eye movements analysed (3,061 control and 1,553 affected). Affected mice showed lower numbers of quick-phases, and their quick-phases were considerably smaller in peak velocity and amplitude compared to control mice. The median quick-phase amplitude for control mice was 9.77 °, whereas for the affected mice, it was 4.49 ° (reduced by 55 ± 5 %). Similarly, median quick-phase peak velocity for control mice across the four stimulus conditions was 462 °/s, whereas for affected mice, it was 288 °/s (attenuated by 38 ± 5 %). In control mice, the slope of the linear fit between quick-phase peak velocity and amplitude was $\sim 39.8\pm0.41$ s⁻¹, whereas in the affected mice, the slope was $\sim 42.8\pm0.55$ s⁻¹, showing a significant difference ($T_{2,122}=-56.42$, $P<0.001$).

In control mice, the K and τ terms in the non-linear fit between quick-phase duration and amplitude were 61 ± 5 ms and 1.77 ± 4.54 °, respectively. Affected mice had a significantly lower saturated quick-phase duration of $K=51\pm4$ ms ($T_{3,777}=73.54$, $P<0.001$) and a significantly different exponential constant $\tau=1.20\pm2.28$ ° ($T_{4,607}=5.68$, $P<0.001$).

Figure 3C shows the average number of quick-phases per stimulus cycle. The number of quick-phase eye movements per stimulus cycle dropped by 30–70 % in affected mice compared to controls ($F_{(1, 40)}=19.49$, $P<0.001$): 6.3 ± 2.5 in controls vs. 2.4 ± 1.5 in affected mice at 0.1 Hz ($T_{42}=3.09$, $P<0.01$), 4.2 ± 1.6 in controls vs. 2.5 ± 1.9 in affected mice at 0.2 Hz ($T_9=1.53$, $P=0.159$), 1.4 ± 0.6 in controls vs. 0.4 ± 0.5 in affected mice at 0.5 Hz ($T_9=3.05$, $P<0.05$) and 5.6 ± 1.3 in controls vs. 4.2 ± 1.4 in affected mice during 3k150 ($T_9=1.82$, $P=0.102$).

DISCUSSION

Our recent study in three strains of mice with naturally occurring GlyR mutations (spastic, spasmodic and oscillator) identified shared differences in MVN neurons related to action potential (AP) and discharge properties (Camp et al. 2010). When compared to wild-type mice, these differences in

mutant mice manifested themselves in a subset of MVN neurons as a reduction in background discharge rates, larger AP after-hyperpolarisations (AHP) and lower sensitivities to injected current. We hypothesised that these were compensatory changes in activity, due to chronically attenuated glycinergic transmission in the mutant strains. Underlying these presumed compensatory changes was an alteration in the ionic conductances responsible for the AHP leading to significantly less excitable cells (Camp et al. 2010). Based on these results, we proposed that, within a narrow range, the VOR in mutant GlyR strains would be unaffected. But beyond this range, the compensatory cellular changes, such as the reduced excitability of MVN neurons, would significantly impair VOR performance. Our tests of the hVOR in spasmodic mice support this notion but also reveal that glycinergic transmission has a more complex role in vestibular-mediated reflexive eye movements than first presumed. Our data indicate that glycine affects two distinct eye movement outcomes: one associated with the slow-phase of the hVOR and the other with the generation of quick-phases. As described below, we suggest that these two observations are inextricably linked.

Transient stimuli

In affected and unaffected mice, there were surprisingly no significant differences in the early (acceleration) portion of the transient hVOR stimulus (Fig. 1). Neither onset latencies nor acceleration gains (G_A) appeared to be disturbed by the GlyR mutation, even at the highest accelerations ($6,000^\circ/\text{s}^2$) and largest velocity plateau ($600^\circ/\text{s}$). This apparently unaltered acceleration segment was in marked contrast to the later constant velocity segment, where affected animals showed significantly reduced velocity gains (G_V) in comparison to unaffected controls. In addition, the deceleration component at the end of the constant velocity segment was also reduced, resulting in only very small counter-rotatory eye movements in affected mice. These two observations of reduced activity may be explained, in part, by a third observation. There was a significant reduction or loss of quick-phases during hVORs, seen during both transient and sinusoidal stimuli. Quick-phases ‘reset’ eye position after a slow-phase VOR response. They can be seen as rapid deflections in the control records (grey lines, Figs. 1A and 2A). Therefore, any problem associated with quick-phases will have immediate and significant impact on slow-phase responses. For example, in affected animals, after the initial eye acceleration (G_A eye; Fig. 1A, bottom panel), the eye decelerates and then oscillates about an average G_V value

~ 0 . Effectively, without a quick-phase reset, the eyes are no longer able to respond to the constant velocity segment of the stimulus, which is demonstrated by the larger final eccentricity (Fig. 1B) and smaller cumulative total travel of the eyes in affected animals (55° vs. 79°). Similarly, the absence of quick-phases may subsequently limit eye movements during the *deceleration* segment of the transient stimulus, although this appears to be less apparent (Fig. 1A).

Sinusoidal stimuli

The impaired ability to generate quick-phases in affected mice was further explored in responses to sinusoidal stimuli of increasing intensity (Fig. 2). At the highest intensity, $100^\circ/\text{s}$, the most perturbed responses were those at the lowest frequency. This might be expected since more quick-phases are required to reset the eyes during longer stimulus cycles (periods of 10, 5 and 2 s). Reduced or absent quick-phases, as seen in affected animals (Fig. 2A), limit the velocity amplitude of response and thereby reduce gain (Fig. 2B, $100^\circ/\text{s}$). At higher frequencies (1 Hz and above), lost or attenuated quick-phases become less of a factor minimising any discrepancies. At lower intensities (20 and $50^\circ/\text{s}$), however, there appears to be no difference between affected and unaffected animals. These data suggest that the effects of reduced glycinergic transmission on quick-phase activity have a ‘threshold’, below which the system appears to operate normally. This is in keeping with the known phenotype where, under quiescent conditions, motor behaviour in GlyR mutants appears to be unaffected. Only when startled or stressed do these animals express the characteristic whole-body shaking behaviour. This threshold may also represent a limit of a proposed feedforward inhibition mechanism that shapes the tuning of central vestibular neurons in non-mammalian models (Malinvaud et al. 2010; Rossert et al. 2011).

Quick-phases and main sequence

To understand the effects reduced glycinergic transmission has on quick-phases, we analysed their properties in affected and unaffected mice. Affected mice had fewer quick-phases and a markedly reduced main sequence (see Garbutt et al. 2001) with peak velocity and amplitude values approximately half that of the controls, although the duration remained the same (Fig. 3A, B). These differences were statistically significant when comparing slopes of peak velocity and duration with amplitude (Fig. 3A). Since the velocity of a saccade cannot be altered voluntarily, significant

differences are regarded as pathological and presumably the result of the GlyR mutation.

Glycinergic pathways of the hVOR

We suggest that changes in the quick-phase circuitry underlie the reduced velocity gain (G_V) we observed in transient and sinusoidal stimuli. Indeed, the OPN within the reticular formation, a key glycinergic component in the quick-phase and saccadic circuits, is the prime suspect as the source of impaired quick-phase activity in affected mice. The precise role of OPNs in quick-phase and saccadic eye movements is still unclear; however, we know that they have a powerful influence on premotor burst neurons (BNs), part of the burst generation circuit, responsible for driving and inhibiting extraocular motor neurons. Recent evidence suggests that rather than acting as a passive gating mechanism and simply releasing BNs for the initiation of a saccade, OPNs actively induce post-inhibitory rebound discharge in these neurons. This rebound discharge, mediated by low-threshold calcium channels, is thought to augment the initial burst acceleration signal to ocular motor neurons (see also Rucker et al. 2011). With impaired glycinergic transmission between OPNs and BNs, the possibility exists that active release is diminished and therefore saccades and quick-phase initiation is substantially compromised. Supporting this notion of impaired OPN function are the small, high-frequency ocular oscillations seen in affected animals during the constant velocity segment of a transient stimulus (star in Fig. 1A). Similar oscillations have been used as a behavioural marker of OPN inactivation (Ramat et al. 1999; Rucker et al. 2011).

Alternatively or in addition, other possibilities exist. It has been hypothesised that OPNs share reciprocal feedback inhibition with long lead burst neurons, another member of the burst generator circuit. Changing the balance of this mutually inhibitory microcircuit, most likely by the superior colliculus, results in triggering of saccades and microsaccades (Otero-Millan et al. 2011). Conceivably, reduction of glycinergic transmission means that this trigger mechanism for saccades, and presumably quick-phase activity as well, is adversely impacted. However, the existence of the reciprocal pathway and its effects on quick-phases has yet to be verified (for network diagram, see also Curthoys 2002; Cartwright et al. 2003).

Contributory factors to aberrant quick-phase activity may also originate from less direct sources. For example, since MVN neurons initiate the quick-phase pathway (Cartwright et al. 2003), reduction in their excitability seen in GlyR mutant mice (Camp et al. 2010) could lead to significant downstream effects. In

addition, glycine is the preferred inhibitory transmitter released by premotor MVN neurons that includes floccular target neurons heavily influenced by the vestibulo-cerebellum (Lisberger and Pavelko 1988; Scudder and Fuchs 1992; Ramachandran and Lisberger 2008; Shin et al. 2011). The vestibulo-cerebellum, which plays an essential role in VOR adaptation and compensation (Robinson 1976), may play a role in compensating for the GlyR deficiency and normalising the response of these neurons. Therefore, one interpretation of our data is that unlike the quick-phase circuitry, which is relatively 'hard-wired', the slow-phase component of the hVOR is relatively unaffected by the GlyR deficiency because of the highly adaptable/compensatory nature of the slow-phase circuitry. While it is understood that slow- and quick-phase circuits are not mutually exclusive, sharing many of the same neurons, undoubtedly quick-phase-associated neurons, is preferentially affected by the GlyR mutation.

Although there are clear indications that affected animals are compromised during transients and high-velocity sinusoids, the data also support our original contention that there is a behavioural threshold associated with the VOR (Camp et al. 2010). For sinusoidal stimuli, this threshold appears to be somewhere between 50 and 100 °/s. Below the threshold, reduced glycinergic transmission in affected animals does not impact the hVOR. It is possible that compensatory mechanisms, including reduced MVN neuronal excitability (Camp et al. 2010), provide near-normal function. Beyond this threshold, however, sinusoidal stimuli that require a number of quick-phases are compromised. Although we saw no evidence of a functional threshold in our transient results, it is possible that the stimuli we used were all above this proposed functional threshold.

CONCLUSION

While quick-phases are significantly disrupted in affected animals, slow-phases were essentially unaltered. As seen in high-intensity/high-frequency sinusoids, where quick-phases were normally absent, there was no difference between affected and unaffected animals. Therefore, despite overlapping neuronal circuits, our data suggest that the 'fixed' quick-phase pathway is susceptible to glycinergic down-regulation, whereas the more adaptable slow-phase pathway is not.

Further experiments are needed to define the precise role of glycine in eye movement reflexes. One future study would be to compare hVOR and vertical VOR (vVOR) responses in spasmodic animals. The results would help us determine the

differences of glycine (hVOR) or GABA (vVOR) as the preferred inhibitory neurotransmitter in premotor inhibitory burst neurons.

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