FINAL ANNUAL REPORT

Development and Function of the Mouse Vestibular System in the Absence of Gravity Perception

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Summary of Research

I. Goals

The hypothesis that was tested in this research was that the absence of gravity perception, such as would occur in space, would affect the development and function of the vestibular and central nervous systems. Further, we postulated that these effects would be more significant at specific stages of post-natal development of the animal. We also proposed the use of molecular genetic approaches that would provide important information as to the hierarchy of gene function during the development and subsequent function of the vestibular system. The *tilted* (*tlt*) mutant mouse has been characterized as lacking the ability to provide sensory input to the gravity receptors. The *tlt/tlt* mutant mice were a particularly attractive model for the study of vestibular function since the primary defect was limited to the receptor part of the vestibular system, and there were no detectable abnormal phenotypes in other organ systems. The goal of the proposed studies was to assess immediate and delayed effects of the lack of gravity perception on the vestibular system. Particular attention was paid to characterizing primarily affected periods of vestibular morphogenesis, and to identifying downstream genetic pathways that are altered in the CNS of the *tlt/tlt* mutant mouse. The specific aims were: (1) to characterize the postnatal morphogenesis of the CNS in the *tlt* mutant mouse, using detailed morphometric analysis of isolated vestibular ganglia and brain tissue at different stages of postnatal development and assessment of apoptotic cell death; (2) to examine the expression of selected genes implicated by mutational analysis to be important in vestibular development or function by in situ hybridization or immunohistochemistry in the mutant mice; and (3) to identify other genes involved in vestibular development and function, using differential cloning strategies to isolate genes whose expression is changed in the mutant versus normal vestibular system.

II. Progress and outcome

We established colonies of both *head tilt* (*het*) and *tilted* (*tlt*) mice, which we obtained from The Jackson Labs. In lieu of a molecular probe for genotyping (the *tlt* gene has not yet been cloned), we used four criteria to genotype the mutant mice: (1) when held by tail, they tend to flex ventrally and retract their limbs rather than the normal behavior of dorsal flexion with extended forelimbs; (2) in response to linear downward acceleration (quickly lowering them by the tail), they generally fail to display the stereotypic protraction of forelimbs characteristic of normal animals; (3) when dropped from a height of several inches, they have some difficulty righting themselves; and (4) they cannot swim due to an inability to sense correct orientation.

Vestibular ganglia from both mutant and wild-type control mice at various ages of post-natal development from PN day 0 to young adults were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), paraffin-embedded, sectioned at 8-μm intervals, mounted on glass microscope slides, then stained with toluidine blue and permanently mounted. Morphometric analysis was carried out by first imaging the slides with a Spot digital camera (Diagnostic Instruments, Inc.) mounted on an Olympus IMT-2 inverted microscope, then analyzing the images with the ImageJ analysis program (National Institutes of Health, Bethesda, MD). The entire microscope-camera-computer system was calibrated using a hemocytometer as reference. With the 10x objective used in these experiments, 0.93 pixel = 1.0μm. Ganglion volume was measured by calculating the average area for each two adjacent sections, then

multiplying by the distance between sections, 8μm. The total of all such sections was recorded as the ganglion volume. An empirically determined counting window of 25 - 2000μm^2 was used to exclude cellular debris, and immature cells. The typical cell size was determined for each post-natal development stage examined by averaging the size of six free-standing cells for each ganglion at that time point. Cell number and cell size were used to calculate total cellular volume. Cell death by apoptic pathways was assessed by terminal deoxynucleotidyl transferase (TdT) – mediated dUTP biotin nick end labeling (TUNEL) according to our standard procedures.

We observed that development of vestibular ganglion volume and total cellular volume increased smoothly in the wild-type mouse from post-natal day 0 through day 10, reaching a peak on day 12, then remained constant throughout the rest of the study. In contrast, these factors in the mutant mouse changed little from day 0 through day 6, then increased rapidly on day 10, reaching a peak on day 12, and continued at a reduced level thereafter.

Specifically, the ganglion volume of control mice increased from $1.4x10^7\mu m^3$ ±0.04 SE on day 0 to $2.9x10^7\mu m^3$ ±0.04 on day 12, and remained constant through day 56 with a slightly reduced average volume of $2.5x10^7\mu m^3$ ±0.04 (Fig. 1A). In the *tlt* mutant mice, ganglion volume remained constant at ~$1.3x10^7\mu m^3$ ±0.10 from day 0 through day 6, then increased rapidly to $2.1x10^7\mu m^3$ ±0.02 on day 10 and $2.4x10^7\mu m^3$ ±0.10 on day 12, after which ganglion volume remained constant at $2.1x10^7\mu m^3$ ±0.03 from day 15, through day 56. Significant differences between the *tlt* mutant and control mice ($p<0.05$) were found on days 4, 6, 10, 12, 22, and 28, using Student's t-test.

Total cellular volume increased smoothly from $2.4x10^6\mu m^3$ ±0.19 SE on day 0 to $6.5x10^6\mu m^3$ ±0.28 on day 15 in the wild-type, then fell smoothly to $5.2x10^6\mu m^3$ ±0.17 on day 56. Cellular volume in the mutant changed little from day 0 through day 6 (~$2.4x10^6\mu m^3$ ±0.14), then rose sharply to $5.1x10^6\mu m^3$ ±0.18 on day 10, remaining relatively constant at $4.4x10^6\mu m^3$ ±0.17 thereafter. Significant differences between the *tlt* mutant and controls ($p<0.05$) were found on days 6, 12, and 15.

The cell number in the wild-type mice remained relatively constant throughout, rising slowly from 1770 ±135 SE cells on day 0 to 2239 ±195 cells on day 10, then falling slowly to 1472 ±47 cells on day 56. In the *tlt* mutant ganglia, cell number decreased at first, falling smoothly from 1654 ±84 cells on day 0 to 1149 ±69 on day 6, then rose sharply to 1689 ±58 cells on day 10, then fell slowly to 1376 ±65 cells on day 56. Significant differences in the number of cells in the ganglia between *tlt* and control mice ($p<0.05$) were found on days 4, 6, 12, and 15. However, the pattern of reduction in cell number between days 0 and 6 in the mutant ganglia was not found to be significant (ANOVA). Likewise, ANOVA analysis revealed no significant differences in dynamic of cell number in wild-type cell number during this period.

When the superior and inferior lobes of the vestibular ganglion were assessed separately, differences were seen in their development. In general, the cellular volume of superior lobes of both wild-type and mutant rose smoothly through day 12, then fell smoothly through day 56, with the mutant developing more slowly and with somewhat less regularity. In the superior lobe, significant differences between mutant and controls ($p<0.05$) were found on days 6, 12, and 15, and in the inferior lobe, on days 2, 6, and 15.

Wile intriguing, the trend of reduction in cell number in the mutant ganglion was not found to be statistically significant between post-natal days 0-6 in mutant mice. TUNEL assays performed on the ganglion of mutant and wild type animals on postnatal day 0, 2, 4, 6, 8, 10, 12, 15 22, 28 and 8 week did not show any dying cells at all examined time points, but did detect

dying cells in embryonic vestibular and trigeminal ganglia. Positive controls from A1 mutant mouse testes and mammary gland were included in the experiment and exhibited positive tunnel staining. Perhaps, any reduction in cell number due to atrophy resulting from the lack of gravity stimulation was below the size threshold of the counting window. However, the reduction in cell number was not seen in the wild-type control mice. The absence of gross abnormalities in the vestibular ganglion of mutant mice may be due to spontaneous activity of receptor cells which maintained tonic stimulation of the ganglionic cells. Ornitz et al. (Hear Res, 1998. 122(1-2):60-70) found that *tlt/tlt* mutant mice posses normal-appearing sensory epithelia. By all of the parameters by which the development of the vestibular ganglia was assessed, including ganglion volume, cellular volume, and cell number, wild-type control and mutant mice began with similar values on post-natal day 0, quickly diverged, then attained similar values once again by the end of the study. Delayed development seen in the vestibular ganglia of mice lacking otoconia and subsequent catch up with wild type mice by eight weeks of age could be due to low-level stimulation from intact hair cells in the maculae of the mutant animals".

We have some additional data on apoptosis in the developing vestibular system (during embryonic development) which we hope to tally and submit for publication. The colony of *het* and *tlt* mice has now been depleted; however, mice of these genotypes can be obtained from The Jackson Labs.

III. Publications

Smith M, Yuan Wang X, Wolgemuth DJ, and Murashov AK. (2003). Development of the mouse vestibular system in the absence of gravity perception. Brain Res Dev Brain Res. 140(1):133-135.

Murashov, A.K., Smith M., Wang, X.Y., and Wolgemuth, D.J. (2001). Development of the Mouse Vestibular System in Mutant Mice that Lack Otoconia. Society for Neuroscience Abstracts, Vol. 27, Program Number: 249.20.

Wolgemuth, DJ, Wang, XY, Smith, M, and Murashov, AK. (2001). Development of the Mouse Vestibular System in the Absence of Gravity Stimulation by Otoconia. Abstract, ASGSB Bulletin, Vol. 15, p. 57.

IV. Significance

The *tilted* mutant mouse, which lacks otoconia in the inner ear, was used to study development of the mouse vestibular system in the absence of gravity perception. Otoconia are dense particles composed of proteins and calcium carbonate crystals suspended in the gelatinous macular membrane. They enhance, and are largely responsible for, sensitivity to gravity. Morphometric analysis of the vestibular ganglion showed that the mutant developed more slowly than the normal controls, both in rate of development and cell number, particularly during the first week of post-natal development. The mutant ganglia also exhibited a reduction of cells during the first six days of post-natal development. Understanding the genetic and molecular basis of postnatal development of the sensory systems will provide important insights for the development of new approaches for correction and treatment of sensory deficits. At present, little is known about the molecular mechanism(s) by which sensory deprivation and vestibular deprivation in particular exerts its effect on neural development.

V. Subject inventions

There were no subject inventions that resulted from this work.

VI. Final Inventory Report of Federally Owned Property

There was no federally owned property resulting from this grant.