Longitudinal Studies of Neonatal Cerebellum Phenotypes Development in Gbx2-CKO Mutant Mice Using MEMRI

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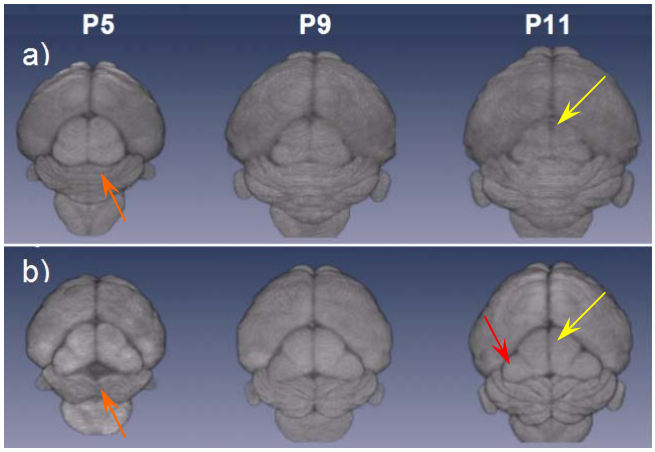
Introduction: The cerebellum is a critical brain structure involved in coordination and movement control, and there is growing evidence for additional roles in sensory processing. Abnormal development of cerebellum has been linked to a variety of neurodevelopmental diseases, including autism spectral disorder (ASD). In mice, cerebellum patterning takes place largely within the first 2 weeks after birth and a number of genes are known to control this process [1]. We previously showed that *in vivo* manganese-enhanced MRI (MEMRI) can be used to detect midline cerebellum defects in *Gbx2* conditional knockout (*Gbx2*-CKO) mice at postnatal day P11 [2]. *Gbx2*-CKO mice have variable deletion of vermis, the central cerebellum, which motivates the need for longitudinal imaging to understand the temporal evolution of the mutant phenotypes. We therefore performed longitudinal MEMERI studies of *Gbx2*-CKO mutant and wild type littermates between P3 and P11, performing volumetric analyses of the cerebellum phenotypes in these mice.

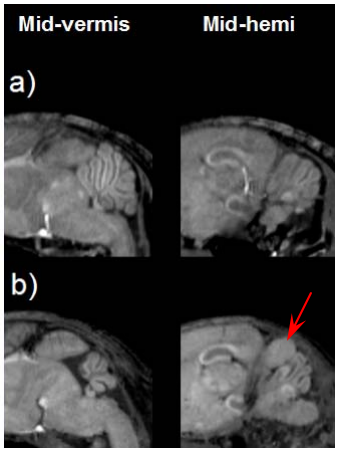
Methods: All mice used in this study were maintained under protocols approved by the Institutional Animal Care and Use Committee of New York University School of Medicine. Mn was delivered to neonates through the milk after maternal IP injection of $MnCl_2$ (40mg/kg) in isotonic saline (0.9 % NaCl in water) 5 times every other day from 3rd to 11th day after birth. MRI data was acquired on a Bruker 7T microimaging system equipped with actively shielded 750 mT/m gradients. A 25-mm (inner diameter) quadrature Litz coil (Doty Scientific) was used to acquire the MR signals. Neonates were positioned in a custom made holder. 3D T1-weighted brain images were collected using 3D gradient echo pulse sequence (TE = 3.6ms, TR = 50ms, Flip Angle = 40°, NEX = 2, FOV = 2.56x2.56x2.56cm, matrix = 256x256x256) achieving isotropic resolution of 100 μm in 1h and 49 min. Volumetric analysis and 3D rendering were performed using AMIRA.

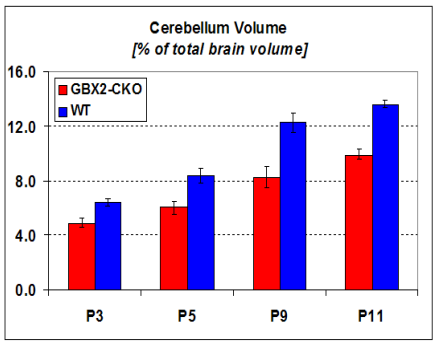
Results and Discussion: 3D volumetric analysis of MEMRI data was performed at 4 different developmental stages including: P3, P5, P9, and P11. The brain volume and cerebellum size were analyzed for 3 *Gbx2*-CKOs and 3 WT littermates at each of the developmental stages (Fig. 1). In addition to volumetric analyses, the cerebellum foliation patterns were also clearly visualized from MEMRI data, showing the deletion of vermis in *Gbx2*-CKO mutants, with a resulting replacement of the medial cerebellum by the expanded hemisphere. Paired-sample student's t-test revealed statistically significant differences ($p<0.05$) in the total brain volume, and cerebellum size after controlling for overall brain size at each of the developmental stages (Fig. 3). The variability in the overall brain and cerebellum size was greater in *Gbx2*-CKO mutants which is consistent with variable brain phenotype in these mice (Fig. 3). In addition to changes in cerebellum volume and patterning, MEMRI also revealed an enlargement of the midbrain regions, with the inferior colliculus extending more laterally toward hemispheres and superior colliculus extending towards the cerebellum (Fig. 1 and 2). Preliminary data also indicate abnormal patterning of the deep cerebellar nuclei (DCN) in the *Gbx2*-CKO mice. DCN are important relay centers projecting from the cerebellum to targets throughout the brain and spinal cord. Therefore, abnormalities in the DCN structures may reflect abnormal cerebellum circuitry and activity patterns that could be further explored with MEMRI.

To assess whether repeated administration of $MnCl_2$ at early postnatal stages affected development of cerebellum mice exposed to manganese and control mice were allowed to develop further until P26 and were sacrificed for histology and immunohistochemistry. Cerebellar morphology was examined using calbindin and fluorescent Nissl (Neurotrace) double staining, which were used to visualize Purkinje and granule cells respectively. No gross differences in cerebellar anatomy were observed between mice exposed and not exposed to Mn (data not shown). Additional studies are being performed to more carefully assess the potential toxicity of manganese on brain and animal development and efforts will be undertaken to minimize the dose while preserving contrast and SNR in the MEMRI images.

These preliminary results demonstrate the feasibility of longitudinal *in vivo* studies of mice development using MEMRI, without requiring invasive injections of $MnCl_2$ into developing pup, with Mn delivered to the neonatal brain via maternal milk, yielding sufficient contrast for effective 3D-voluemtric analyses. The ability to follow one animal during its course of development is especially valuable for characterizing models like the *Gbx2*-CKO mutants variable phenotypes.







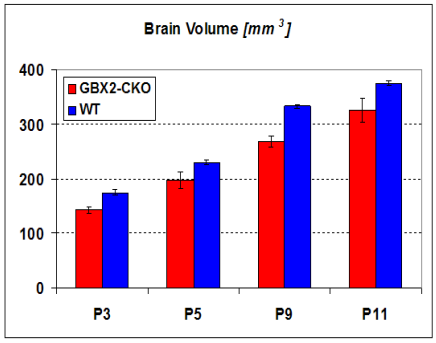


Figure 1. Whole brain 3D-rending of mice that were followed longitudinally using MEMRI between P3 and P11. 3D renderings viewed form the back of the brain are shown for a) wild type (WT) at P5, P9, and P11 and b) a *Gbx2*-CKO mutant littermate the same stages. Deletion of vermis (orange arrow) and enlarged midbrain (yellow arrow) were clearly visible from MEMRI. **Figure 2.** Sagittal sections through mid-vermis and mid-hemispheres in a) WT and b) *Gbx2*-CKO mice at P11. Note the presence of the inferior colliculus (midbrain) in the section through mid-hemisphere in *Gbx2*-CKO mutant (red arrow in Figs. 1 and 2). **Figure 3.** Bar graphs representing cerebellum size expressed as % of the total brain volume (top) and brain volume measured in mm^3 (bottom) at 4 different developmental stages (n=3 at each stage). Statistically significant difference in terms of cerebellar size between *Gbx2*-CKO and WT littermates was found at each stage.

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References: [1] Sillitoe RV and Joyner AL. *Ann. Rev. Cell Dev. Bio.*; 23:549-77 (2007), [2] Wadghiri YZ et al. *NMR in Biomed*, 17: 613-19 (2004), [3] Li JYH at al. *Neuron*; 36: 31-43 (2002)