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Effects of carbonic anhydrase VIII deficiency on cerebellar gene expression profiles in the *wdl* mouse

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Abstract

Recently, the waddles (*wdl*) mouse was identified as a carbonic anhydrase VIII (*Car8*) mutant. The mutation is associated with marked deficiency of Car8, an inositol triphosphate receptor 1-binding protein expressed at high levels in cerebellar Purkinje cells. To help unravel the molecular aberrations contributing to motor dysfunction in *wdl* mice, cerebellar gene expression profiles were examined in the mutants and their wild-type littermates. Genes involved in signaling, cell division, zinc ion-binding, synapse integrity and plasticity were downregulated in *wdl* mice. Several of the upregulated genes encode proteins that function in the Golgi apparatus which suggests that Car8 deficiency has important effects on synaptic vesicle formation and transport.

Keywords: Carbonic anhydrase VIII; Dystonia; Ataxia; Cerebellum; Calcium signaling; Inositol triphosphate

The waddles (*wdl*) mouse is an autosomal recessive mutant that exhibits ataxia and dystonia. Like several other murine models of ataxia and dystonia, the *wdl* mouse exhibits no gross morphological or histological abnormalities of the nervous system [10,21,26,44]. The *wdl* mouse harbors a loss-of-function mutation in the carbonic anhydrase VIII (Car8) gene (*Car8*). Car8 is an acatalytic member of carbonate dehydratase family. The sole known function of Car8 is to inhibit inositol 1,4,5-trisphosphate (IP3) binding to IP3 receptor 1 (Ip3r1) [17], which plays a critical role in the modulation of intracellular calcium (Ca^{2+}) signaling [19]. It is, therefore, quite possible that altered Ca^{2+} signaling may, at least partially, underlie the motor phenotype observed in the *wdl* mice. Ca^{2+} signaling is involved in the regulation of a wide variety of cellular activities including synapse recycling, proliferation, fertilization, learning and memory, long term potentiation, long term depression, apoptosis, contraction, metabolism, and modulation of other signaling systems. To investigate the molecular abnormalities associated with the unique motor syndrome of *wdl* mice, we performed genome-wide cerebellar gene expression profiling using Affymetrix oligonucleotide microarrays. We found that many genes involved in synaptogenesis, synaptic vesicle formation and transport, cellular proliferation and differentiation, and signal transduction were dysregulated in *wdl* mice.

Mice were housed in animal care facilities at the University of Tennessee Health Science Center under conditions of 14 h light/10 h darkness at an ambient temperature of $20 \pm 2\,^{\circ}C$ with relative humidity of 30–60%. Experimental animal procedures and mouse husbandry were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the UTHSC Institutional Animal Care and Use Committee. For the Affymetrix microarray experiments, individual cerebella from 2-week female homozygous *wdl* mice ($N = 3$) and age-matched, wide-type (+/+)

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Table 1
Real-time PCR confirmation of some microarray data

Gene	Primer sequence	Microarray-based ratio[a]	Real-time PCR-based ratio[a]
Car8	Forward: CTGTTCATTTTAAAGAAACACAGTGTGT Reverse: GCAACAAGCAGTATGTGCAAAAG	−19.4	−16.6
Cbln3	Forward: TCCCACCAACATCAGAAACTCTT Reverse: GTTGTTCTAGGTAGGTCCGTTCATG	−5.1	−2.1
Gabra2	Forward: CTATGCCCCGAATCTTTCCA Reverse: TCTGGCGTCGTTGCACTTT	2.6	1.9
Gabra6	Forward: GCAATACTGTTGCTATTTCCCACTAAA Reverse: GTCATAGTATAGTCCACCAAGTGACCTT	−2.6	−2.9
18S	ABI Part No. 4308329		Internal control

[a] Represents a relative ratio of signal intensity of mutant vs. wild-type mice.

littermates ($N=3$) were employed for each array. For relative quantitative real-time RT-PCR (QRT-PCR) validation of a small subset of differentially expressed transcripts, another panel of individual cerebella ($N=9$) from either wild-type or homozygous *wdl* mice were pooled to yield three replicates for each group. Total RNA was extracted using Trizol reagent (Life Technologies). RNA quality was checked with an Eppendorf BioPhotometer. Microarray hybridization and data generation were performed according to Affymetrix guidelines using GeneChip® Mouse Genome 430 2.0 arrays, related reagents, and GCOS 1.4 software (www.affymetrix.com). Comparative analysis was carried out between each individual wild-type and mutant sample, which generated nine comparisons. Genes showing ≥1.5 fold change on ≥7/9 comparisons were chosen for further statistical analysis using Significance Analysis of Microarrays (SAM, http://www-stat.stanford.edu/~tibs/SAM/) with a false discovery rate and q value of <0.01%. Data clustering was performed with Cluster and TreeView (Eisen Laboratory, http://rana.lbl.gov/EisenSoftware.htm). QRT-PCR was performed with SYBR Green (Applied Biosystems, ABI) and the ABI PRISM 7900 HT Sequence Detection System according to ABI protocol. To be brief, 100 ng of total RNA was reverse transcribed into cDNA. Two-step PCR cycling was carried out as follows: 10 min at 95 °C × 1 cycle followed by 15 s at 95 °C and 1 min at 60 °C × 40 cycles. The housekeeping gene 18S rRNA was used as an endogenous control. The comparative C_T method was used to determine relative expression levels. QRT-PCR primer pairs and assay results are presented in Table 1. Gene ontology functional analysis of differentially expressed genes was performed using GenMAPP software (www.genmapp.org). Lastly, literature-based biological profiling of altered gene expression patterns was carried out with Ingenuity Pathways Analysis (www.ingenuity.com).

In this study, 192 transcripts were found to be significantly dysregulated in *wdl* mice (upregulated 90, downregulated 102). Among this set, 79 (41%) were expressed sequence tags (ESTs) of unknown function (Supplementary Table 1). Hierarchical clustering analysis, a standard method of identifying coexpressed or coregulated genes, detected 6 major clusters of transcripts (Supplementary Table 2). Strikingly, many of the dysregulated transcripts with the largest fold differences were parceled into two major clusters: upregulated genes in Cluster 3 (*Glp1r*, *H2-D1*, *Paip1* and *Scn2b*) and downregulated genes in Cluster 6 (*Car8*, IMAGE cDNA clone 6309338 and *C030014A21Rik*). Consistent with previous Northern blotting results [21], the expression level of *Car8* transcript as determined with QRT-PCR was markedly depressed in *wdl* mice. Many transcripts in Cluster 6 are involved in synapse vesicle recycling, transcriptional regulation, and signal transduction regulation. In Cluster 3, upregulated genes are related to synapse vesicle transport, protein biosynthesis, immune response, and cell adhesion and migration. Of note, three genes known to be involved in cerebellar function and implicated in the pathophysiology of ataxia (*Cbln3*, *Chn2*, *and Etv1*) were found in Cluster 4 [2,24,32]. Microarray analysis and QRT-PCR yielded similar results for the four genes assessed with both techniques: *Car8*, the cerebellar-enriched *Cbln3*, and the inhibitory receptors *Gabra2* and *Gabra6* (Supplementary Table 2).

Gene ontology functional analysis showed a remarkable increase in the activities involved in cellular structure and function of the Golgi apparatus and decreased functionalities associated with intracellular signaling, cell division, and metal ion (mainly calcium and zinc) binding in *wdl* mice (Table 2). Literature-based pathway analysis with Ingenuity Pathway Analysis provided additional insight into those biological processes altered by the absence of Car8. The most significantly affected categories were cellular maintenance, growth and proliferation, organismal survival, signaling, development, and morphology (Table 3).

Although Car8 is known to be a negative regulator of intracellular Ca^{2+} signaling [17], its exact role in the IP3 signaling and cytoplasmic calcium homeostasis is unclear. Calcium plays an important role in the regulation of numerous neuronal processes including the formation and adaptive modification of neural circuits [27], neurotransmitter release, and gene transcription [5]. Calcium-binding proteins play critical roles in mediating many of the various activities of calcium ions [4]. In *wdl* mice, there were no significant changes in the expression of several major common calcium-binding protein genes including *Calb1*, *Calb2*, and *Pvalb*. However, *wdl* mice did show cerebellar upregulation of the Ca^{2+}-binding protein encoding genes *Hpcal1* and *Slc8a1* (Table 3), which are predominantly expressed in Purkinje cells