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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 down-regulated in *wdl* mice. Several of the up-regulated 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

Introduction

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 (Ca²⁺) signaling [19]. It

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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.

Materials and methods

Mice were housed in animal care facilities at the University of Tennessee Health Science Center under conditions of 14 hr light/10 hr darkness at an ambient temperature of $20 \pm 2^\circ\text{C}$ 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 (+/+) 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 3 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 9 comparisons. Genes showing a ≥ 1.5 fold change on $\geq 7/9$ comparisons were chosen for further statistical analysis using SAM (Significance Analysis of Microarrays, <http://www-stat.stanford.edu/~tibs/SAM/>) with a false discovery rate and q value of $< 0.01\%$. Data clustering were 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, 100ng of total RNA was reverse transcribed into cDNA. Two-step PCR cycling was carried out as follows: 10 min at $95^\circ\text{C} \times 1$ cycle followed by 15 s at 95°C and 1 min at $60^\circ\text{C} \times 40$ 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).

Results

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 expression of genes involved in cellular structure and function of the Golgi apparatus and decreased expression of genes 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).

Discussion

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 [30,33]. It is interesting to note that two other Ca^{2+} -binding protein genes, *Nptx1* and *Pclo*, whose protein products are enriched in presynaptic plasma membranes [9], were downregulated in the mutant mice (Table 2). Based on predominant expression of *Car8* in Purkinje cells and the *Car8* interaction with IP3R1, *Car8* deficiency is predicted to exert most of its effects via increased concentration of free cytosolic in cerebellar Purkinje cells. However, due to the effects of Ca^{2+} on multifarious neuronal processes, it is difficult to determine if *Car8* may have additional cellular roles independent of its effects on Ca^{2+} .

Hippocalcin like 1 (*Hpcal*, Table 3), a member of the family of intracellular neuronal calcium sensors involved in the calcium-dependent regulation of signal transduction cascades, may be involved in the control of clathrin-coated vesicle trafficking [20] whereas solute carrier family 8 (sodium/calcium exchanger) member 1 (*Slc8a1*, Table 3) is a primary regulator of intracellular Ca^{2+} concentration by pumping Ca^{2+} out of cells [22]. Neuronal pentraxin 1 (*Nptx1*) has been implicated in the modulation of hypoxic-ischemic injury through an interaction with glutamate receptors [18]. The neuronal pentraxins are characterized by calcium-dependent ligand binding. Piccolo or presynaptic cytomatrix protein (*Pclo*) is a novel shared component of glutaminergic and GABAergic synapses that functions as a presynaptic calcium sensor and may regulate neurotransmitter release [13]. Given the variety of cell types that contribute to olivocerebellar and cerebellar cortical local area networks, it is difficult to dissect direct versus compensatory effects of *Car8* deficiency in Purkinje cells. For instance, neuronal pentraxin 1 is expressed in both granule and Purkinje cells [37].

An important finding in this study was the significant effects of *Car8* deficiency on genes involved in the structure and function of the Golgi apparatus (GA), particularly vesicle assembly and transport. In neurons, GA is the place for the packing and transport of synaptic

vesicles containing neurotransmitters. Fragmentation of the neuronal GA has been implicated in several neurodegenerative disorders including spinocerebellar ataxia type 2 [14]. Interestingly, we found increased expression of GA genes including *Arcn1*, *Clasp2*, *Pik4cb*, *St6galnac5*, and *Tctex1* in *wdl* mice (Table 2). Archain vesicle transport protein 1 (*Arcn1*) is one of major components of synapse vesicle coat assembly [31]. CLIP-associated protein 2 (*Clasp2*) participates in the local regulation of distal microtubule dynamics [1], which is related to vesicle trafficking. Phosphatidylinositol 4-kinase (*Pik4cb*) interacts with frequenin (also known as neuronal calcium sensor 1) in the exocytosis of secretory vesicles [29]. Finally, t-complex testis-expressed 1 protein (*Tctex1*), a light-chain subunit of the dynein motor complex, interacts directly and selectively with N- and P/Q-type Ca^{2+} channels [23]. Taken together, these gene expression changes suggest that increased activity of the GA and downstream vesicle transport may contribute to the pathophysiology of ataxia and/or dystonia by virtue of increased receptor delivery to the neuronal cell surface. Since the GA has been confirmed as an important distinct IP_3 -sensitive Ca^{2+} store [35], our findings also suggest that *Car8* deficiency may exert direct rather than compensatory effects on GA activity.

Most autosomal recessive ataxias are multisystem disorders due to loss-of-function mutations. DNA repair and maintenance are critical to the molecular pathophysiology of some types of ataxic disorders such as ataxia telangiectasia, AT-like disease, ataxia with oculomotor apraxia 1 and 2, and spinocerebellar ataxia with axonal neuropathy [40]. In our study, three genes (*Cbln3*, *Chn2*, and *Etv1*) that are either exclusively or predominantly expressed in the cerebellum were significantly downregulated in *wdl* mice (Supplementary Table 2). The peptide encoded by *Cbln3* may play an important role in the synaptic integrity and plasticity of Purkinje cells via interaction with cerebellin (*Cbln1*) [16,32]. *Chn2* encodes a GTPase-activating protein (GAP) for p21 ras-related Rac [24]. For review, Rac, a primary member of Rho GTPase family, may be a key regulator of synaptogenesis and dendritogenesis by coordinating assembly of the actin and microtubule cytoskeleton [15,43]. Lastly, *Etv1* encodes a proprioceptive marker which may control the expression of other proprioceptive neuron-specific genes through collaboration with Runx3 for their expression [25]. Interestingly, *Etv1* mutant mice exhibit a severe limb ataxia due to faulty assemblage of sensorimotor circuitry within the spinal cord [2]. These results suggest that *Car8* deficiency could result in impaired neuronal connectivity through reduced synapse formation and dendritogenesis. By extrapolation, similar development defects may underlie some recessive ataxias in humans.

Although zinc plays an important role in cell surface signaling and acts as an enzymatic cofactor, little is known about cellular mechanisms mediating zinc homeostasis. The concentration of zinc has been shown to be altered in a number of disorders of the central nervous system including Friedreich's ataxia [12]. Furthermore, mutations of several zinc ion-binding protein genes have been implicated in several ataxic disorders, such as *Aptx* in autosomal recessive ataxia [11], *Egr3* in sensory ataxia [42], and *Zic1* in knockout mice with severe ataxia [3]. In our study, transcripts for several zinc ion-binding proteins (*Pip5k3*, *Chn2*, *Zfp52*, *Ttc3*, *Rph3a*, *9830124H08Rik*, and *BC021442*) were downregulated in *wdl* mice (Table 2). Among these, *Rph3a* is implicated in synaptic transmission [38], while the granule cell-specific and ataxia-related *Chn2* has been confirmed to be involved in regulation of cell proliferation [48] and diacylglycerol signaling [8].

Purkinje cells have already finished cell division at birth whereas granule cells proliferate vigorously during the first three postnatal weeks [47] (<http://www.cdtdb.brain.riken.jp/CDT/Top.jsp>). Therefore, the down-regulation of several genes involved in cell division, including *Sept3*, *Sept4*, *Pafah1b1*, and *Ccne2* (Table 2), may reflect decreased granule cell proliferation and/or neuritogenesis in the mutants. Septin 3 (*Sept3*) is a brain-specific member of the septin family of GTPase enzymes that assemble as intracellular filamentous scaffolds and are involved in cytokinesis or exocytosis. Most

members of the septin family appear to interact with each other in order to assemble as filaments, such as the interaction of Septin 4 (Sept4) and Septin 8 (Sept8) in vesicle trafficking [6]. Since Car8 expression in cerebellar cortex is limited to Purkinje cells, any down-regulation of *Sept3* and *Sept4* transcripts that is occurring within granule cells must be some type of feedback response to abnormal Purkinje cell signaling.

Previous work has shown that the expression patterns of the $\alpha 2$ (Gabra2) and $\alpha 6$ (Gabra6) subunits of gamma-aminobutyric acid type A (GABAA) receptors are temporally associated with the early ($\alpha 2$) and late ($\alpha 6$) postnatal development of cerebellar granule cells [28,39,45]. Both granule and Purkinje cells express the $\alpha 2$ subunit whereas only granule cells express the $\alpha 6$ subunit [36]. Thus, the down-regulation of Gabra6 transcript in *wdl* mice must be an indirect effect of Purkinje cell Car8 deficiency. It is interesting to note that the $\alpha 6$ subunit is also down-regulated in stargazer mice, a motonic mutant that exhibits dystonia and ataxia [41]. In the context of cerebellar development it should be noted that the $\alpha 6$ subunit may mediate cell surface anchoring [34]. The $\alpha 6$ subunit of GABAA receptors expressed by granule cell may regulate several aspects of normal cerebellar function [6,7,46].

In summary, our gene expression profiling analysis suggests that calcium homeostasis, synaptogenesis and dendritogenesis, vesicle formation and transport, and, possibly, granule cell proliferation and/or neuritogenesis, are dysregulated in *wdl* mice. Furthermore, cell-surface signaling pathways in *wdl* mice may be distorted through changes in intracellular Ca^{2+} , alterations in the expression of zinc-binding proteins, and increased activity of the GA. These findings suggest that ultrastructural abnormalities of specific neuronal elements may be present in *wdl* cerebellar cortex. In addition, the substantial number of dysregulated genes in *wdl* mice indicates that Car8 plays an important physiological role in cerebellar cortex. Overall, the scope of our results is not surprising given that significant changes in gene expression profiles have been described in a phenotypically-normal mouse model of Friedrich's ataxia (10).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Table 1
Real-time PCR confirmation of some microarray data

Gene	Primer Sequence	Microarray-based ratio ¹	Real-time PCR-based ratio ¹
Car8	Forward: CTGTTCAATTTTAAAGAAACACAGTGTGT Reverse: GCAACAAGCAGTATGTGCAAAAG	-19.4	-16.6
Cbln3	Forward: TCCACCAACATCAGAACTCTT Reverse: GTTGTCTAGGTAGGTCCGTTTCATG	-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	

Note: 1. represents a relative ratio of signal intensity of mutant vs wild-type mice

Table 2

Biological profiling of *Car8* mutant mice identified using MAPPFinder¹

Change	GOID	GO Name	GO Type	Number Changed	Number Measured	Number in GO	Percent Changed	Z Score	PermutP	Affected genes
Increase	5794	Golgi apparatus	C	6	6	382	100	2.4	0.03	Arcn1, C530046L02Rik, Clasp2, Plk4cb, Stogalnac5, Tetex1
	5198	structural molecule activity	F	5	5	774	100	2.2	0.05	C530046L02Rik, Rpl14, Rpl15, Ning1, Tubb5
	7242	intracellular signaling cascade	P	7	8	793	87.5	2.4	0.04	Car8, Chn2, Pclo, Pip5k3, Snx6, Sprk2, Tulp4
Decrease	51301	cell division	P	4	4	136	100	2.2	0.04	Ccne2, Pafah1b1, Sept3, Sept4
	46872	metal ion binding	F	14	21	2864	66.7	2.1	0.05	6330416L07Rik, 9830124H08Rik, BC021442.Car8, Chn2, Npx1, Pclo, Pip5k3, Rora, Rph3a, Sprk2, Syt11, Ttc3, Zfp52

¹ **Note:** 1. Detailed explanation of columns GOID, GO name, GO type, Number changes, Number measured, Number in GO, Percent changed, Z score and PermutP can be found in GenMAPP website (<http://www.genmapp.com>). GO represents gene ontology; C stands for component; F stands for function; P stands for process.

Table 3

Network	Genes ¹	Score ²	Focus genes ³	Top functions
1	DNCH1, DNCH2, DNCI2, DNCL1, DNCL2B, DNCL11, DNCLIC2, EIF4A1, FLJ30092, FMRI1, GNB1, GNG4, HOXA9, MBNL1, MEIS1, NDE1, NDE11, NFYB, PABPC1, PAFAH1B1, PAIP1, PBX2, PKNOX1, RABEP1, RAD50, RAPGEF6, REC8L1, RPH3A, SMC1L1, SNX6, TCTEL1, TGFB2, TTC3, VPS52, YWHAG	22	15	Cellular Function and Maintenance, Cancer, Hematological Disease
2	ADM, ANXA1, ANXA2, CD200, CD200R1, CEBPB, CHN2, CLEC11A, CTSC, CYB5, DOK1, DOK2, EFN2, EGFR, FADS2, FASN, GLO1, GPX3, H2-D1, HPCAL1, IL13, IL8RB, INSR, KIDINS220, KLF7, MT1A, PRKD1, RPL14, RPL15, RPS6KA2, SC5DL, SRC, SREBF1, SREBF2, TNF	18	13	Organismal Survival, Cancer, Cellular Growth and Proliferation
3	ATPIA1, ATP1A2, ATP1B1, CAPI, CCNE2, CDC42, EGFR, FXYP7, GABRA2, GABRA6, GABRB1, GABRB2, GABRG2, GABRG3, IL4, INS1, INSR, ITPR1, JAK2, KIF3B, PIK4CB, PIP5K3, PRKCB1, RAB4A, RNPS1, RUFY1, SCN3A, SLC1A3, SLC8A1, STS-1, STX1A, SYT11, TGFB1, TGFB2	18	13	Cell Signaling, Cellular Development, Cell Morphology
4	APC, APP, ARCN1, BUB1, CLASP2, CSNK1D, CSNK1E, DCTN2, DUSP1, EGFR, FKBP1B, GAS, HSPA8, KLF4, MAPK1, MAPRE1, MYOD1, NPTX1, PACRG, PARK2, PBP, POLB, RPS6KA2, RPS6KA3, RYR1, SCHIP1, SEPT4, SNCA, SORL1, STIPI, TCF7L2, TCPI, TFE3, TP53, VPS33A, ACAAI, ACTA1, ACTG1, CAMK4, CEBPB, CRABP1, CREB1, EGFR, ERBB2, ERG, ETV1, FASN, FOS, GCG, GHI, GLP1R, HK3, INPP5F, INSR, LEP, NIPSNAP1, PCLO, PFN1, PFN2, PIB5PA, PRKAR2B, PTGDS, RPS6KA3, RPS6KA4, SLC19A1, SRPK2, TMSB4X, TUBA3, TUBB, TUBB4	16	12	Cellular Compromise, DNA Replication, Recombination, and Repair, Cell Cycle
5		16	12	and Function, Lipid Metabolism, Gene Expression Connective Tissue Development

Notes: 1, the collection of related genes in the network; 2, the “score” is derived from P values and indicates the likelihood that the “Focus genes” in a particular network are not grouped together by chance alone; and 3, the number of “Focus genes” identified in this study.