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Glen Kisby
Western University of Health Sciences

Valerie Palmer
Oregon Health & Science University

Mike Lasarev
Oregon Health & Science University

Rebecca Fry
University of North Carolina

Mihail Iordanov
Oregon Health & Science University, miordanov@cu-portland.edu

See next page for additional authors

Authors
Glen Kisby, Valerie Palmer, Mike Lasarev, Rebecca Fry, Mihail Iordanov, Eli Magun, Leona Samson, and Peter Spencer

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Does the cycad genotoxin MAM implicated in Guam ALS-PDC induce disease-relevant latent changes in mouse brain that include olfaction?

Glen Kisby,[1] Valerie Palmer,[2] Mike Lasarev,[3] Rebecca Fry,[4] Mihail Iordanov,[5] Eli Magun,[5] Leona Samson[6] and Peter Spencer[2,3,7,*]

[1]Western University of Health Sciences; College of Osteopathic Medicine of the Pacific Northwest; Lebanon; [2]Global Health Center; [3]Center for Research on Occupational and Environmental Toxicology; [5]Department of Cell and Developmental Biology; School of Medicine; [7]Department of Neurology; Oregon Health & Science University; Portland, OR; [4]Department of Environmental Sciences and Engineering; Gillings School of Global Public Health; University of North Carolina; Chapel Hill, NC; [6]Center for Environmental Health Sciences and Biological Engineering Division; Massachusetts Institute of Technology; Cambridge, MA USA

Western Pacific amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC), a prototypical neurodegenerative disease (polyproteinopathy) affecting distinct genetic groups with common exposure to neurotoxic chemicals in cycad seed, has many features of Parkinson and Alzheimer diseases (AD), including early olfactory dysfunction. Guam ALS-PDC incidence correlates with cycad flour content of cycasin and its aglycone methylazoxymethanol (MAM), which produces persistent DNA damage (O^6-methylguanine) in the brains of mice lacking O^6-methylguanine methyltransferase (*Mgmt*$^{-/-}$). We showed in *Mgmt*$^{-/-}$ mice up to 7 d post-MAM treatment that brain DNA damage was linked to brain gene expression changes found in human neurological disease, cancer and skin and hair development. This addendum reports 6 mo post-MAM treatment-related brain transcriptional changes as well as elevated mitogen activated protein kinases and increased caspase-3 activity, both of which are involved in tau aggregation and neurofibrillary tangle formation typical of ALS-PDC and AD, plus transcriptional changes in olfactory receptors. Does cycasin act as a "slow (geno)toxin" in ALS-PDC?

Key words: amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, methylazoxymethanol, tauopathy

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*Correspondence to: Peter Spencer; Email: spencer@ohsu.edu

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We showed that methylazoxymethanol (MAM), the genotoxic metabolite of the cycad plant carcinogen cycasin (MAM-β-D-glucoside), induced in young adult mice lacking O^6-methylguanine (O^6-mG) methyltransferase (*Mgmt*$^{-/-}$)—the enzyme that repairs O^6-mG DNA lesions—a O^6-mG-linked brain transcriptional response associated with human neurological disease.[1] This supports an etiologic role for the azoxyglycoside cycasin in the genesis of a disappearing degenerative brain disease (amyotrophic lateral sclerosis and parkinsonism-dementia complex, ALS-PDC) found among the genetically distinct island populations of Guam and Rota (Chamorros), Honshu (Japanese) and New Guinea (Papuan New Guinean), which used cycad seed as medicine applied orally (Kii Peninsula, Honshu) or topically (West Papua), or for both topical medicine and food (Guam and Rota). In all three disease foci, periods of years or decades intervene between exposure to cycad seed and the development of ALS-PDC, suggesting the operation of a "slow toxin" able to trigger a progressive neuronal disease reminiscent of look-a-like disorders of old age (e.g., Alzheimer disease, AD) elsewhere in the world.[2] Both AD and ALS-PDC have neurofibrillary tangles containing the microtubule tau protein in a hyperphosphorylated state,[3-5] which has been linked to both activation of serine-threonine kinases (Erk-1/2, p38, c-Jun NH_2-terminal kinase) in the mitogen-activated protein kinase (MAPK) pathway and to phosphorylation of the C-terminal fragment of amyloid precursor protein (APP).[6] More recent studies suggest the activation of non-apoptotic caspases may

Table 1. KEGG pathways from DAVID based on 407/443 (DAVID recognized/submitted)

KEGG Pathway	MAM_{early}	MAM_{late}
Pathways in cancer	13	4
Insulin signaling pathway	9	
Wnt signaling pathway	10	
Purine metabolism	9	
MAPK signaling pathway	7	4
Prostate cancer	8	
Acute myeloid leukemia	5	
Chronic myeloid leukemia	5	
Neurotrophin signaling pathway	6	
Huntington's disease	5	
Focal adhesion	6	4
Neuroactive ligand-receptor interaction	5	4
Nucleotide excision repair	4	
Steroid hormone biosynthesis	4	4
Endometrial cancer	5	
Glioma	4	
Long-term potentiation	4	
Long-term depression	5	
Small cell lung cancer	4	
Colorectal cancer	5	
Apoptosis	4	
ErbB signaling pathway	5	
Melanogenesis	6	
Axon guidance	5	
Calcium signaling pathway	4	4
Endocytosis	4	
Regulation of actin cytoskeleton	5	
Chemokine signaling pathway	5	4
Basal cell carcinoma	4	
Alzheimer disease	4	
Olfactory transduction		28
ECM-receptor interaction		5
Cytokine-cytokine receptor interaction		5

Affymetrix probe IDs (MAM_{early}) or 291/355 GENBANK accession numbers (MAM_{late}). Both sets restricted to pathways containing at least four probes/genes.

be one of the earliest events that triggers tau aggregation and the accumulation of neurofibrillary tangles in tauopathies.[7-9] Here, we supplement data on the short-term actions (up to 7 d) of MAM on brain gene expression in O^6-mG-deficient mice (MAM_{early}) with preliminary findings on caspase activity and the transcription and protein expression of brain cell signaling proteins 6 mo post-treatment (MAM_{late}).

Seven 11-week-old male *Mgmt*$^{-/-}$ mice were treated with a single intraperitoneal dose of MAM (20 mg/kg body weight, n = 4) or a comparable volume of vehicle consisting of 0.5% acetic acid in saline (n = 3). Animals were housed singly, fed rodent chow ad libitum for 6 mo, during which all animals grew and maintained apparent health, and then decapitated by guillotine.

The right half of the MAM_{late} brain was employed for gene expression analysis using mouse oligo microarrays (~21,000 features) by Agilent (Santa Clara, CA). As in the previously published MAM_{early} study,[1] brain cellular networks putatively perturbed in MAM_{late} *Mgmt*$^{-/-}$ mice were identified by integrating the transcripts with their gene products and overlaying these with known molecular interactions using Ingenuity™ Pathway Analysis (IPA, Redwood City, CA). The three top-ranked IPA Biofunction Diseases and Disorders included: inflammatory response, cancer and genetic disorders. We identified 355 genes that were differentially expressed between the brains of MAM_{late}- and vehicle-treated *Mgmt*$^{-/-}$ mice. IPA analysis retained 85% of these genes (302 of the 355) in the construction of 176 networks. The three top-scoring IPA networks contained 23 (#1), 25 (#2) and 21 (#3) focus molecules, including hubs for Akt, transforming growth factor β (Tgfβ) and histone h3 (#1), calcineurin and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB complex) (#2) and Erk1/2, MAPK and collagen(s) (#3). The most significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were determined with DAVID (the Database for Annotation, Visualization and Integrated Discovery) bioinformatics software. Based on 291 genes, DAVID identified six pathways (n = 4 or more genes per pathway) common to MAM_{early} and MAM_{late} and three unique to MAM_{late} (**Table 1**): the latter included genes coding for olfactory receptors that were both upregulated (n = 25) and downregulated (n = 3). Parkinson disease was one of 23 additional KEGG pathways (n = 2 or 3 genes per pathway). Brain KEGG pathways common to both MAM_{late} and MAM_{early} animals included: Pathways in Cancer, MAPK, Focal Adhesion Pathway, Neuroactive Receptor Interaction Pathway, Steroid Hormone Biosynthesis and the Calcium Signaling Pathway (**Table 1**). The MAPK signaling pathway involved 3 upregulated genes [*activin A receptor, type 1B (Acvr1b)*], a member of the transforming-growth factorβ family linked to skin epithelial cell proliferation and hair development,[10] *calcium channel voltage-dependent* α*2/delta subunit 4*; *serine/threonine kinase-3)* (*CACNA2D3*), a neuroblastoma marker gene,[11] and 1 downregulated gene, *raffinose permease* (*RafB*), a MAPK protein pathway gene.[12]

The left half of each brain was analyzed by protein gel blotting for components of

the MAPK (perturbed in both MAM_{late} and MAM_{early}) and phosphatidylinositol-3-kinase/Akt (PI3K/Akt) signaling pathways and activity of caspase-3 (the APP cleavage protein linked to AD).[8,9] In MAM_{late}, significant increases were found in Erk-1 ($p < 0.03$) and fodrin cleavage ($p < 0.01$) (**Fig. 1**). Brain fodrin cleavage, which was increased in MAM_{late} animals, indicates the activation of caspase-3, an enzyme with an important role in cleaving tau.[14] MAM_{late} transcriptional changes in extracellular-matrix-receptor interaction (4 genes upregulated, 1 downregulated gene) and cytokine-cytokine receptor interaction (3 genes upregulated, 2 downregulated genes) suggest brain inflammatory response modulation, which is consistent with early changes in tau-related neurodegeneration.[15]

The MAM_{late} transcriptional profile was dominated by the presence of 28 (of a total of ~1,300) genes involved in olfactory transduction,[16] including *chloride channel activated 6*, which suggest the presence of a MAM-induced change in olfaction status. While caution is merited when comparing rodent and human data, olfactory dysfunction is among the first signs of neurodegenerative disease.[17] Marked olfactory deficits, first reported in Guam PDC, are also similarly present in Chamorro patients with ALS, pure parkinsonism and pure dementia, and in some controls with possible sub-clinical ALS-PDC.[18] Olfactory deficits are also among the first signs of Alzheimer disease and idiopathic Parkinson disease.[19,20] The inability to distinguish the nature of olfactory dysfunction among Guam PDC, AD[21] and ALS patients[18] suggests a common neurologic substrate and underlines the close relationship between ALS-PDC and the more familiar neurodegenerative disorders seen in the West.

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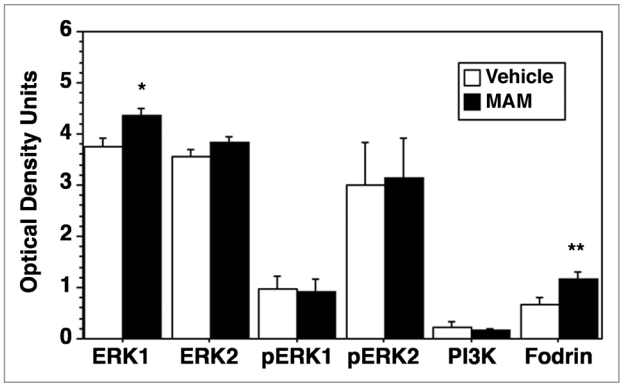

Figure 1. Effect of MAM_{late} on components of the MAPK signaling pathway and caspase activity. The left half brains of vehicle- (n = 3) and MAM- (n = 4) treated *Mgmt*$^{-/-}$ mice were flash frozen in liquid N_2, the frozen tissue subjected to ultrasonication in gel electrophoresis buffer to avoid loss of protein modifications or lysis, and the homogenate heat-denatured at 95°C for 5 min. An aliquot of the brain tissue homogenate (50 μg) was resolved on a 10% polyacrylamide gel, transferred to PVDF membranes, the blocked membranes probed with monoclonal antibodies to ERK (ERK1, ERK2), phosphorylated ERK (pERK1, pERK2), PI3K (p110γ) (Santa Cruz Biotechnology, Inc.,) and α-fodrin (Chemicon), and the bands visualized by chemiluminescence detection. α-Fodrin cleavage was determined using the 120 kDA band. Membranes were scanned on a Microtek flatbed scanner and each band quantified using Molecular Analyst software (BioRad, Inc.), with background subtraction as described previously in reference 13. Values are the mean ± standard error. Significantly different from vehicle (*$p < 0.03$ or **$p < 0.01$ by ANOVA).

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