

Supplemental Material

Brain Anatomy in Latino Farmworkers Exposed to Pesticides and Nicotine

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Cholinesterase Assay

Blood samples were shipped on dry ice to Oklahoma State University. Total cholinesterase, acetylcholinesterase and butyrylcholinesterase activities were assayed with a modification of the radiometric method of Johnson and Russell (1975) as reported previously (Quandt et al. 2015). To minimize reactivation of cholinesterases, whole blood samples were thawed on ice and assayed individually using a minimal tissue dilution (1:5 in the final reaction). Each sample was assayed in duplicate under two conditions. Immediately after thawing, 20 μ l aliquots of whole blood were added to four separate 7-ml glass scintillation vials. In two of the vials, 60 μ l of 50 mM potassium phosphate buffer, pH 7 (phosphate buffer) was added while in the other two, 60 μ l of the specific acetylcholinesterase inhibitor BW 284C51 in phosphate buffer was added (final concentration 0.5 μ M; acetylcholinesterase activity was completely inhibited under these conditions). Samples were vortexed, and then placed into a water bath at 26^oC for 60 seconds before adding 20 μ l of substrate ([³H]acetylcholine iodide, 1 mM final concentration) in phosphate buffer. The reactions were allowed to incubate a further 30 seconds and then terminated by addition of 100 μ l of acidified buffer (pH 2.5; (Johnson and Russell 1975)). Five ml of an organic scintillation cocktail was then added. Vials were vortexed and allowed to equilibrate overnight before counting. A time-course study was used to estimate total substrate hydrolysis using the nonlinear fit, one phase association program in GraphPad Prism version 5. Activity in unknown samples was then algebraically compared to total substrate hydrolysis (with cpm values of Ymax representing the 100 nmol acetylcholine in each reaction). Total cholinesterase activity was defined as the amount of activity in the samples without BW 284C51, butyrylcholinesterase activity was defined as the amount of activity in the samples incubated with BW 284C51, and acetylcholinesterase activity was defined as the difference in activity between those two conditions.

Cotinine Assay

The samples were all stored at -80^oC following collection and were shipped to Salimetrics LLC for the assay on dry ice. Samples collected near the end of the farming season on a work day were used in this analysis. All samples were tested for urinary cotinine in duplicate using a highly-sensitive enzyme immunoassay (Salimetrics LLC, State College, PA, USA). Six (6) standard calibrators ranging from 0.8 ng/mL to 200 ng/mL and two sets of high and low controls with established ranges were run on each test plate were. The test used 20 μ l of urine sample per determination, and had a lower limit of sensitivity of 0.15 ng/mL, a range of standard curve from 0.8 to 200 ng/mL, an average intra-assay coefficient of variation of 6.4%, and an average inter-assay coefficient of variation of 6.6%. Method accuracy determined by spike recovery averaged 99.6%, and linearity determined by serial dilution averaged 98%.

Assay results for each subject were acceptable when the coefficient of variation (%CV) between the duplicate results (result 1 and result 2) was <15%. In instances where the %CV between duplicates was >15%, results were accepted if the absolute value between result 1 and result 2 was <1.0 ng/mL. Data <0.05 ng/mL were reported as “below lower limit of assay”. Results with low values (based on Optical Density reading) but not returning a number value were reported as “none detected” (ND). Cotinine data were compiled by the testing manager and checked for accuracy by the technical supervisor before final reports were delivered.

References

- Johnson CD, Russell RL (1975) A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations *Analytical biochemistry* 64:229-238 doi:10.1016/0003-2697(75)90423-6
- Quandt SA, Pope CN, Chen H, Summers P, Arcury TA (2015) Longitudinal Assessment of Blood Cholinesterase Activities Over 2 Consecutive Years Among Latino Nonfarmworkers and Pesticide-Exposed Farmworkers in North Carolina *J Occup Environ Med* 57:851-857 doi:10.1097/JOM.0000000000000496

Assay	Group	N	Mean	Std Dev	Minimum	Median	Maximum	p-value
Blood Total Cholinesterase ^a								0.5611
	Non-farmworker	26	2.05	0.39	1.34	2.15	2.63	
	Farmworker	48	1.99	0.46	1.17	1.94	2.91	
Blood acetyl-Cholinesterase ^a								0.6602
	Non-farmworker	26	1.51	0.42	0.71	1.63	2.02	
	Farmworker	48	1.46	0.43	0.59	1.46	2.20	
Blood butyryl-Cholinesterase ^a								0.6210
	Non-farmworker	26	0.54	0.12	0.33	0.52	0.79	
	Farmworker	48	0.52	0.14	0.27	0.50	0.89	
Urine Cotinine ^b								< 0.0001
	Non-farmworker	26	288.14	996.31	0.55	2.73	4997.20	
	Farmworker	48	1602.48	2454.17	1.09	457.73	13718.29	

Supplemental Digital Content, Table S1. Measures of urine cotinine levels and blood cholinesterase activity.

^aBlood Cholinesterase Activity ($\mu\text{mol /min/ml}$) summer 2013

^bUrine Cotinine Levels (ng/mL of urine)

Statistical Contrast	Region	Size (# of voxels)	p-value ^a	T score	MNI coordinates (X, Y, Z) ^b
FW > NFW					
	Right putamen	874	0.006	4.49	28, 6, 1
	Left cerebellum	1170	0.002	4.20	-27, -60, -48
	Cerebellar vermis	670	0.013	3.38	-3, -39, -14
	Left putamen ^c	200	0.145	3.24	-27, 9, 3
	Right cerebellum	322	0.070	3.92	21, -58, -48
FW < NFW					
	Right ventrolateral prefrontal	645	0.015	4.47	51, 15, 19
	Right dorsolateral prefrontal	502	0.028	3.99	26, 30, 45
	Right medial prefrontal	292	0.083	3.39	-4, 48, -21 ^d
	Left medial prefrontal	1172	0.002	5.36	-9, 56, 1
	Right temporal	418	0.043	3.23	70, -28, -12
	Right inferior temporal	7	0.813	2.75	45, -64, -18

Supplemental Digital Content Table S2. Regional differences in gray matter controlling for age, total intracranial volume, and average total serum cholinesterase activity.

^a p-value for comparing farmworkers to non-farmworkers.

^b MNI (Montreal Neurological Institute) coordinates for peak voxel location.

^c Indicates that the region in the left putamen was fragmented into many smaller areas. The largest area is noted here.

^d The region in the right medial prefrontal cortex actually extended into the left hemisphere. The peak location was located on the left but the majority of the area was in the right hemisphere.

FW = farmworker NFW = non-farmworker

Statistical Contrast	Region	Size (# of voxels)	p-value ^a	T score	MNI coordinates (X, Y, Z) ^b
FW > NFW					
	Right putamen	821	0.007	4.45	28, 6, 1
	Left cerebellum	1177	0.002	4.49	-27, -60, -48
	Cerebellar vermis	877	0.006	3.53	-3, -39, -14
	Left putamen ^c	197	0.149	3.16	-26, 8, 1
	Right cerebellum	296	0.082	3.79	21, -58, -48
FW < NFW					
	Right ventrolateral prefrontal	686	0.013	4.57	52, 15, 16
	Right dorsolateral prefrontal	572	0.021	4.12	26, 30, 45
	Right medial prefrontal	354	0.060	3.34	-4, 48, -21 ^d
	Left medial prefrontal	1343	0.001	5.46	-10, 56, -2
	Right temporal	462	0.035	3.25	70, -12, -2
	Right inferior temporal	17	0.689	2.86	45, -64, -18

Supplemental Digital Content Table S3. Regional differences in gray matter controlling for age, total intracranial volume, and average butyrylcholinesterase activity.

^a p-value for comparing farmworkers to non-farmworkers.

^b MNI (Montreal Neurological Institute) coordinates for peak voxel location.

^c Indicates that the region in the left putamen was fragmented into many smaller areas. The largest area is noted here.

^d The region in the right medial prefrontal cortex actually extended into the left hemisphere. The peak location was located on the left but the majority of the area was in the right hemisphere.

FW = farmworker NFW = non-farmworker

Statistical Contrast	Region	Size (# of voxels)	p-value ^a	T score	MNI coordinates (X, Y, Z) ^b
FW > NFW					
	Right putamen	890	0.005	4.51	28, 6, 1
	Left cerebellum	1212	0.002	4.53	-27, -60, -48
	Cerebellar vermis	648	0.015	3.55	-3, -39, -14
	Left putamen ^c	210	0.136	3.20	-27, 9, 3
	Right cerebellum	320	0.071	3.91	21, -58, -48
FW < NFW					
	Right ventrolateral prefrontal	668	0.013	4.44	51, 15, 19
	Right dorsolateral prefrontal	495	0.029	3.99	26, 30, 45
	Right medial prefrontal	336	0.065	3.47	-4, 48, -21 ^d
	Left medial prefrontal	1249	0.001	5.45	-9, 56, 1
	Right temporal	448	0.037	3.12	70, -28, -12
	Right inferior temporal	8	0.798	2.77	45, -64, -18

Supplemental Digital Content Table S4. Regional differences in gray matter controlling for age, total intracranial volume, and average acetylcholinesterase activity.

^a p-value for comparing farmworkers to non-farmworkers.

^b MNI (Montreal Neurological Institute) coordinates for peak voxel location.

^c Indicates that the region in the left putamen was fragmented into many smaller areas. The largest area is noted here.

^d The region in the right medial prefrontal cortex actually extended into the left hemisphere. The peak location was located on the left but the majority of the area was in the right hemisphere.

FW = farmworker NFW = non-farmworker

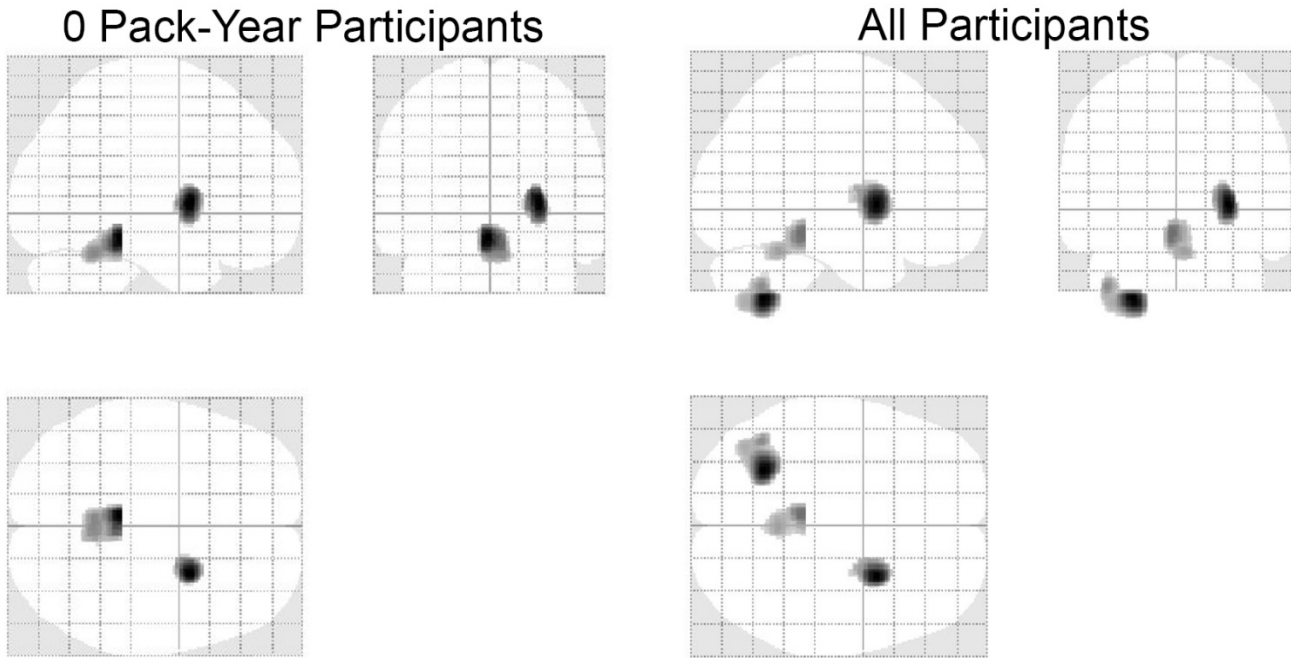
Statistical Contrast	Region	Size (# of voxels)	p-value ^a	T score	MNI coordinates (X, Y, Z) ^b
FW > NFW					
	Right putamen	772	0.009	4.47	28, 6, 3
	Left cerebellum	516	0.027	3.76	-28, -60, -48
	Cerebellar vermis	560	0.022	3.47	-4, -37, -14
	Left putamen	180	0.166	4.40	-21, -10, 4
	Right cerebellum	n/a	n/a	n/a	n/a
FW < NFW					
	Right ventrolateral prefrontal	1577	>0.001	4.42	52, 14, 16
	Right dorsolateral prefrontal	1056	0.003	4.5614	24, 32, 45
	Right medial prefrontal	854	0.006	4.13	18, 62, -5
	Left medial prefrontal	1024	0.003	5.20	-14, 56, -2
	Right temporal	31	0.574	2.91	70, -27, -11
	Right inferior temporal	384	0.051	3.39	46, -63, -18

Supplemental Digital Content Table S5. Gray matter differences controlling for age, total intracranial volume, urine cotinine, and smoking history.

^a p-value for comparing farmworkers to non-farmworkers.

^b MNI (Montreal Neurological Institute) coordinates for peak voxel location.

FW = farmworker NFW = non-farmworker



Supplemental Digital Content Figure S1. Gray matter differences between farmworkers and non-farmworkers. The left side of the image shows the comparison limited to individuals with zero pack-years smoking history (22 farmworkers and 19 non-farmworkers). For reference purposes, the right figure shows comparisons of all farmworkers ($n = 48$) to non-farmworkers ($n = 26$). Note that the main group differences in the right putamen and the cerebellar vermis were significant even in the reduced sample. The region in the left cerebellum was fragmented into many small areas that did not reach statistical significance. The data in the figure on the right are the same data as in Figure 1 and Table 2 of the main manuscript. The images are “glass brain” projections showing regions of statistical significance after correcting for multiple comparisons. Analyses controlled for age and total intracranial volume.