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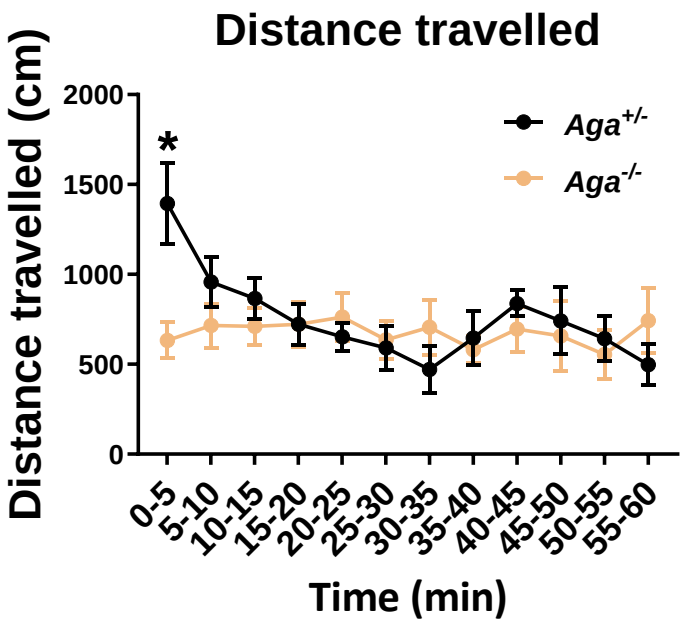
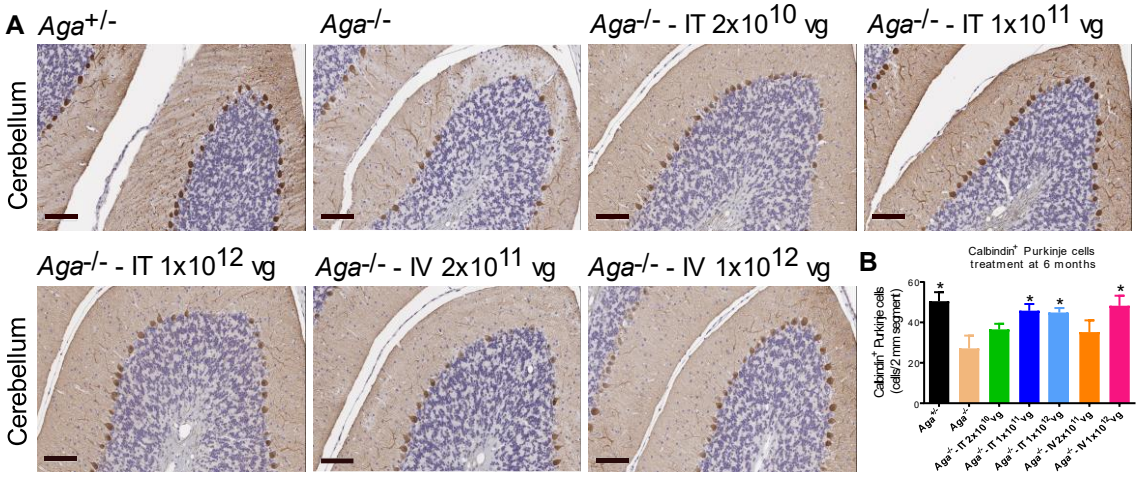
Supplemental Information

Pre-clinical Gene Therapy with AAV9/*AGA* in Aspartylglucosaminuria Mice Provides Evidence for Clinical Translation

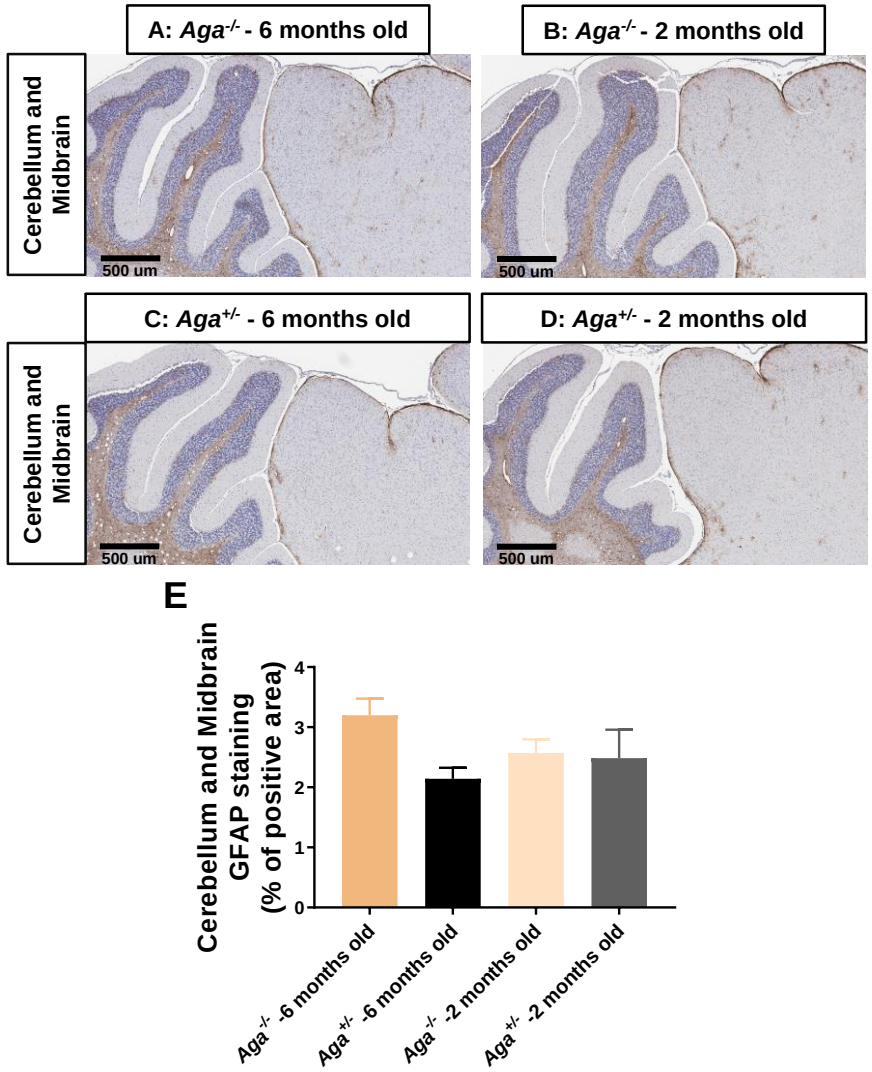
Xin Chen, Sarah Snanoudj-Verber, Laura Pollard, Yuhui Hu, Sara S. Cathey, Ritva Tikkanen, and Steven J. Gray



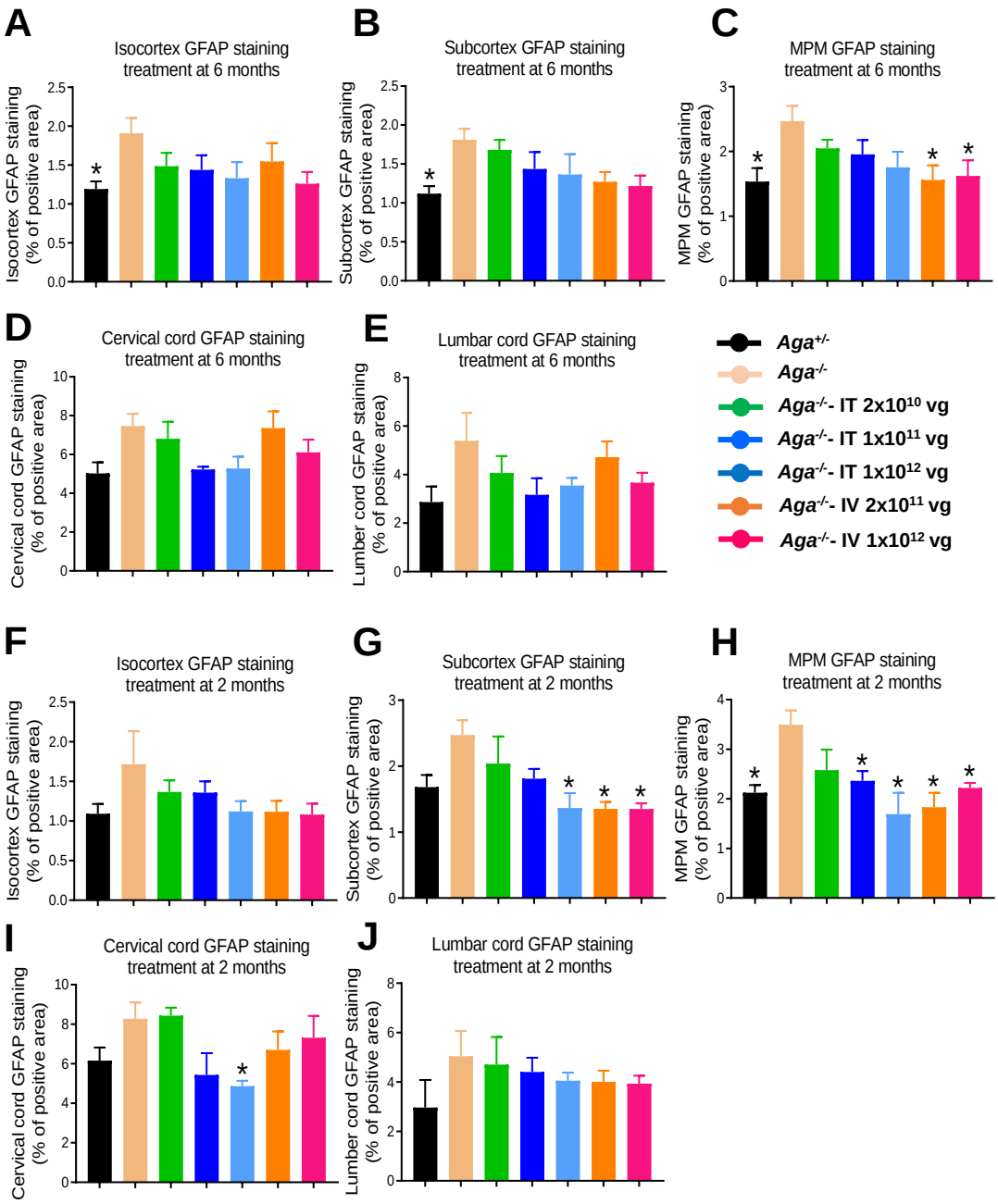
Supplemental Figure 1. *Aga*$^{-/-}$ mice at 14 months old travels significantly shorter distance during the first 5-minutes of open field tests than their *Aga*$^{+/-}$ littermates. Open field tests were performed on mice (n=12) when they reached 14 months old and distance travelled was calculated. All data are presented as mean ± SEM. * depicts significant difference ($p<0.05$) by unpaired t-test compared to the untreated *Aga*$^{-/-}$ control.



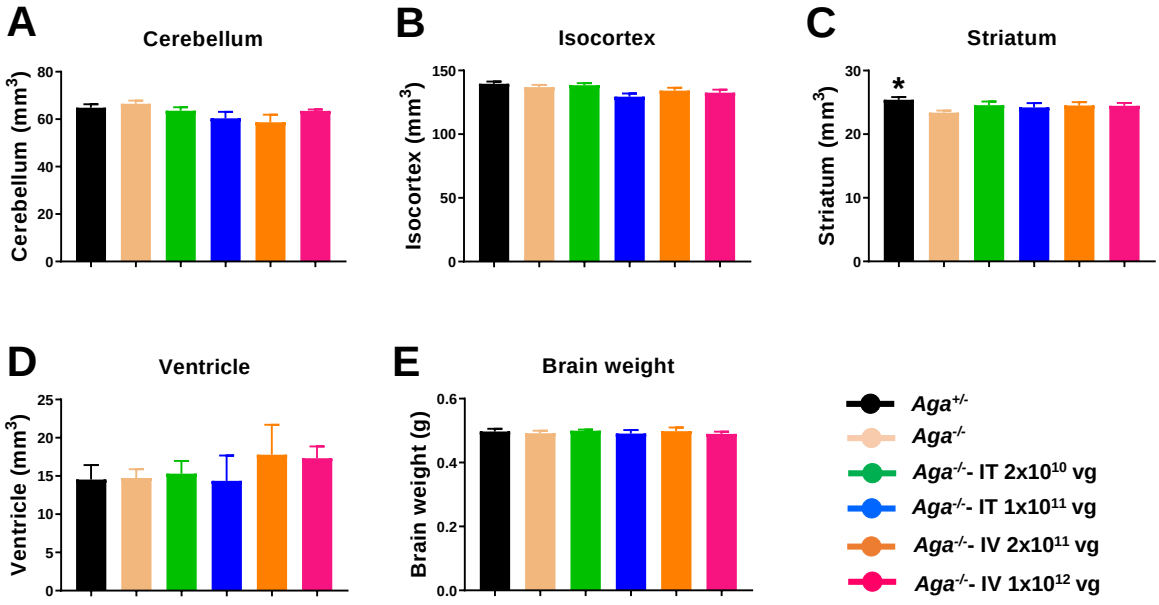
Supplemental Figure 2. AAV9/*AGA* GT significantly preserves Calbindin+ Purkinje cells in the cerebellum in *Aga*. Various doses of AAV9/*AGA* vector were administered either IT or IV to *Aga*$^{-/-}$ mice treated at 6 months old. At 18 months old, mouse brain was harvested for IHC staining using an antibody against Calbindin. Scale bars in panel A represent 100 µm. Data in panel B are presented as mean±SEM. *depicts significant difference ($p<0.05$) by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test compared to the untreated *Aga*$^{-/-}$ control. n=5-6 in (B).



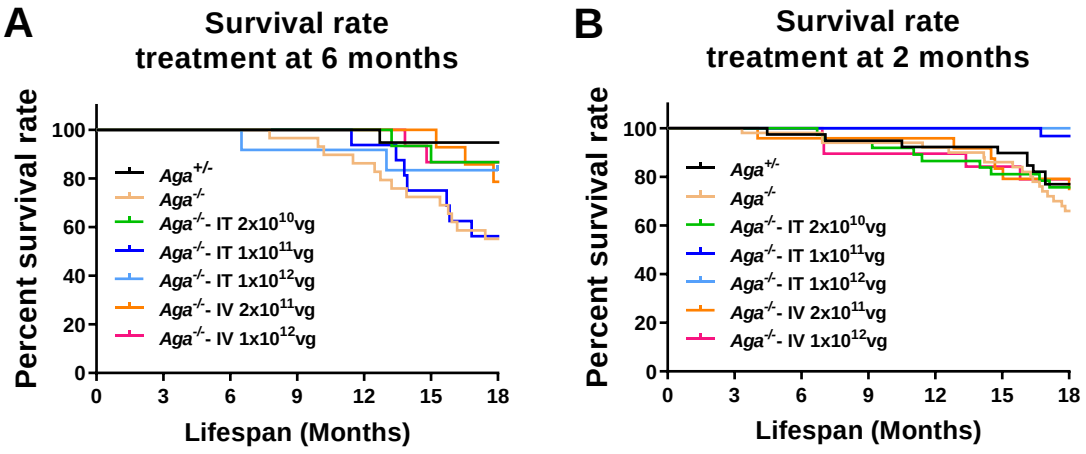
Supplemental Figure 3. *Aga*$^{-/-}$ mouse at 6 months old (A), but not at 2 months old (B), shows moderate but not significantly more gliosis than its *Aga*$^{+/-}$ littermates (C, D, and E). Mouse brain was harvested at 6 months old (**A and C**) or 2 months old (**B and D**) for glial fibrillary acidic protein (GFAP) staining. Significant difference were analyzed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test compared to the untreated *Aga*$^{-/-}$ control. No significant differences were found between any cohorts. Scale bars in all panels (**A-D**) represent 500 µm. All data in panel **E** (n=3-6) are presented as mean ± SEM.



Supplemental Figure 4. AAV9/*AGA* GT reduces gliosis in various part of the CNS in *Aga*$^{-/-}$ mice. Various doses of AAV9/*AGA* vector were administered either IT or IV to *Aga*$^{-/-}$ mice at 6 months old (**A-E**) or 2 months old (**F-J**). At 18 months old, mouse brain was harvested for GFAP staining. All data are presented as mean ± SEM. * depicts significant difference ($p<0.05$) by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test compared to the untreated *Aga*$^{-/-}$ control. Subcortex, hippocampus + thalamus + hypothalamus; MPM, Midbrain + Pons + Medulla. n=5-7 in (**A-E**) and n=4-7 in (**F-J**).



Supplemental Figure 5. There is no significant difference between any groups in terms of brain size (A-D) or brain weight (E). MRI was performed on mice at 16 months old and brain size (**A-D**) was calculated. Brain weight (**E**) was obtained at 18 months old during necropsy. All data are presented as mean ± SEM. Significant difference was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test compared to the untreated *Aga*$^{-/-}$ control. No significant differences were found between any cohorts. n=3-8 in (**A-D**) and n=4-11 in (**E**).



Supplemental Figure 6. AAV9/*AGA* GT does not decrease survival rate in *Aga*$^{-/-}$ mice. Various doses of AAV9/*AGA* vector were administered either IT or IV to *Aga*$^{-/-}$ mice at 6 months old (**A**) or 2 months old (**B**). Significant difference was analyzed via log-rank [Mantel Cox] test. No significant differences were found between any cohorts. n=12-29 in (**A**) and n=15-50 in (**B**).

Supplemental Quality Control Summary

UNIVERSITY OF NORTH CAROLINA
VECTOR CORE

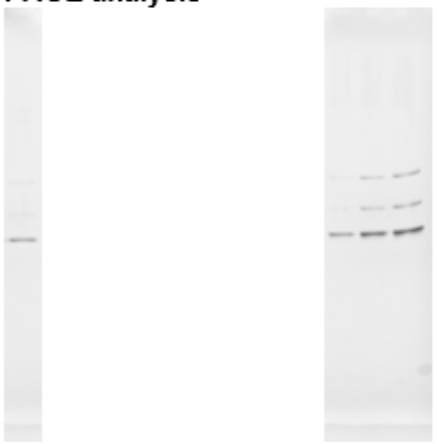
Quality Control Summary

Lot #		LAV9

Test by qPCR

Test #	Titer, vg/mL	Analyst	Date	File
1	1.11E+13	PZ	08/9/2016	20160809-1634-gh-pz

PAGE analysis



Loaded 5.00E+09 vg PV021 std 2e9vg 5e9vg 1e10vg
Calculated 2.50E+09 vg

Analyst	Ali Hernandez
Date	08/16/2016
Reference #	20160816-silver

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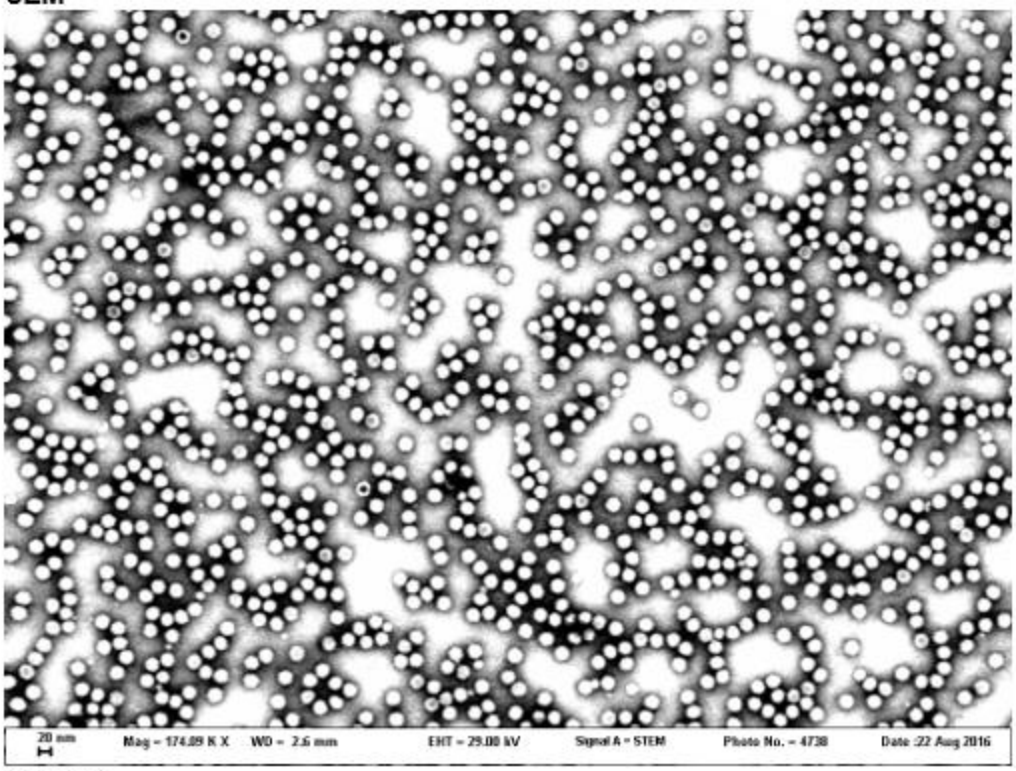
VECTOR CORE

SEM



94% full

Analyst	Ali Hernandez
Date	08/22/2016
Reference #	20160822-LAV9-T17-02

University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75390
Phone 214-648-1402
Mary.Wight-Carter@UTSouthwestern.edu

FINAL REPORT

Safety Study of scAAV9/AGA Vectors in AGA Knock-out and Heterozygous mice

Report No. 18-088

Sponsor Name:	Rare Trait Hope Fund
Study Director:	Steven Gray, PhD
Contract Pathologist:	Mary Wight-Carter, DVM, DACVP
Date of Final Report:	April 15, 2019

Mary Wight-Carter, DVM, DACVP Date

University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75390
Phone 214-648-1402
Mary.Wight-Carter@UTSouthwestern.edu

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University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75390
Phone 214-648-1402
Mary.Wight-Carter@UTSouthwestern.edu

1. OBJECTIVE

The objective for this study was to evaluate the safety of long-term high-levels of systemic expression of scAAV9/AGA Vectors in AGA Knock-out mice. These mice were injected intrathecally (IT) at 6 or 2 months old with $1x10^{12}$ vg/mouse of scAAV/AGA vectors. Age- and sex-matched mice were injected with vehicle and maintained as part of the study cohort for comparison.

2. ABBREVIATIONS

AAV	Adeno-Associated Virus
AGU	Aspartylglucosaminuria
AGA KO	AGA Knock-out mouse, animal model for AGU disease
Het	Heterozygous, mice that carry the mutation in one allele but exhibit normal phenotype
AGA	Aspartylglucosaminidase
vg	Vector genomes

3. MATERIALS AND METHODS

Experimental Design: In the current study, AGA Knock-out mice were injected intrathecally (IT) at 6 or 2 months old with $1x10^{12}$ vg/mouse of scAAV/AGA vectors. Age- and sex-matched mice were injected with vehicle and maintained as part of the study cohort for comparison.

The in-life portion of the study was performed in the laboratory of Dr. Gray at the University of North Carolina at Chapel Hill. Postmortem analysis was conducted at the University of Texas Southwestern Medical Center. The work was not conducted in full compliance with the Good Laboratory Practice (GLP) regulations for nonclinical studies (21 CFR Part 58). Animals were grouped and analyzed as detailed in the table below.

University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75390
Phone 214-648-1402
Mary.Wight-Carter@UTSouthwestern.edu

Table 1. Study design for safety of scAAV9/AGA vectors in AGA Knock-out mice.

Genotype	Route	Treatment Age (months)	Volume injected (uL)	Dose (vg/mouse)	Viral Source	Number of mice injected	Body weight /survival	Endpoint[b]
AGA Het	-	6	-	-	N/A	4	Yes	18 months old
AGA KO	IT[a]	6	5	Vehicle	UNC-VC	4	Yes	18 months old
AGA KO	IT	6	5	$1x10^{12}$	UNC-VC	4	Yes	18 months old
AGA Het	-	2	-	-	N/A	4	Yes	18 months old
AGA KO	IT	2	5	Vehicle	UNC-VC	4	Yes	18 months old
AGA KO	IT	2	5	$1x10^{12}$	UNC-VC	4	Yes	18 months old

[a] IT: Intrathecal injection through lumber spinal cord. [b] Mice were sacrificed at 18 months of age for histopathology and compared to 4 age- and sex-matched mice.

Tissues were stored in 70% ETOH, trimmed into tissue cassettes and sent for processing to IDEXX laboratories. Hematoxylin and Eosin stained slides were produced from the cassettes. Tissues and the corresponding slides were labeled with the following ID's: 254.19, 264.80, 264.81, 264.86, 222.23, 222.26, 223.30, 223.31, 224.34, 224.35, 224.36, 224.39, 232.81, 232.82, 232.88, 234.97, 254.18, 255.29, 261.58, 261.60, 261.63, 267.00, 268.10, and 273.44. Brain, heart, liver, lung, gonad, spleen, kidney, sciatic nerve, cervical and lumbar spinal cord were submitted for all animals except for the following instances. The sciatic nerve was not present for animal ID 222.26, 224.35, 222.23, 223.30, 224.34, 232.81, 223.31, 234.97, 268.10, 273.44, 261.58, and 264.80. The gonad was not present for animal ID 224.35, 222.23, and 223.30. The lungs, kidney and spleen were also missing for animal ID 224.35 and 223.30.

4. RESULTS AND DISCUSSION

The cerebrum and olfactory bulb of all the mice were microscopically normal. There were no abnormalities found in the cervical or lumbar cord of the mice. There were no abnormalities found in the sciatic nerves in any of the mice.

There was a mild to moderate decrease in the number of Purkinje cells in at least one lobe and occasionally multiple lobes of the cerebellum from mouse numbers 222.23, 223.30, 223.31, 224.34, 224.36, 232.81, 234.97, 267.00, and 273.44.

The seminiferous tubules of animal ID 224.36, 254.18 and 261.58 had multiple variably sized vacuoles that replaced various levels of the seminiferous epithelium in a few tubules. There was no evidence of accompanying germ cell degeneration. Since there were a very few tubules affected and there was no accompanying degeneration, it suggests that this was an incidental finding. No other lesions were present in the mouse testicles.

All of the ovaries that were present were normal and the structures within the ovaries were consistent with various points in the estrus cycle.

The hearts of animal ID 254.18, 261.58, 261.63, 267.00, 224.36, 232.88 and 261.60 had multifocal areas with separation of cardiomyocytes by increased collagen fibers (fibrosis). The areas of fibrosis affected 5-15% of the heart in all instances except for 261.58 and 261.63. 261.58 and 261.63 had approximately 40% of the heart affected. The remainder of the hearts were normal. There was no evidence of heart failure in the other organs, so these degenerative changes were not clinically significant.

The kidneys of 232.81 and 254.19 showed multifocal mild to moderate thickening of the glomerular tufts, multifocal tubular regeneration, mild multifocal interstitial fibrosis, mild to moderate multifocal interstitial and perivascular infiltrates with mononuclear cells (glomerulonephropathy). The multiple glomerular tufts were thickened with eosinophilic proteinaceous material in kidneys of 264.80 (mild glomerulopathy). The kidneys of 264.80 and 261.63 had mild multifocal interstitial fibrosis, with tubular regeneration and mild perivascular infiltrates with small to moderate numbers of mononuclear cells.

There was mild to moderate perivascular infiltrates with small to moderate numbers of mononuclear cells in kidneys of 261.60, 264.86, 254.18, 273.44, 223.31, 224.34, 261.58, and 267.00. There were mild multifocal peripelvic infiltrates with small to moderate numbers of mononuclear cells in kidneys of 224.36, 234.97, 264.80, 222.26, 224.39, 255.29, 268.10, and 232.88.

The tubules of kidneys of 224.36 had a few small areas of mineralization. The renal pelvis had mild to moderate dilation of a few tubules of kidneys of 264.80, 264.86, 224.34, 232.82, 232.88, and 268.10. The above described lesions are not uncommon in adult or aged mice and typically are more frequent in male mice.

The renal pelvis of 255.29 contained a focal area of tubular hyperplasia. This is an incidental finding with no evidence of cellular atypia as would be expected with a neoplastic process.

The livers of the following mice had mild to moderate peribiliary infiltrates with mononuclear cells with no corresponding fibrosis or hepatocellular necrosis: 264.80, 267.00, and 273.44. Minimal to moderate peribiliary and perivascular infiltrates are a common finding in mice and increase in incidence as the mice age.

The livers of the following mice contained multifocal infiltrates with small numbers of mixed inflammatory cell infiltrates with hepatocellular necrosis (micro-abscess): 264.81, 224.34, 232.82, 232.88, 254.18, 261.63, and 267.00. Areas with 1-2 cell hepatocyte necrosis accompanied by inflammatory cells can occur spontaneously in the mouse liver with increased incidence as the mice age.

Animal numbers 223.31 and 232.81 had multifocal areas of extramedullary hematopoiesis present in the liver parenchyma. This is less common in rodents as they age and typically occurs in response to increased hematopoietic demand.

Animal 232.88, 254.18, and 261.63 had a liver nodule grossly which microscopically was morphologically consistent with a hepatocellular adenoma which expanded the parenchyma and compressed the adjacent normal tissue. Adenomas are common findings in adult B6 mice.

Multifocal hepatocytes throughout the livers from 264.80, 264.86, 222.26, 223.31, 224.34, 232.88, 254.18, 255.29, and 261.60 had round variably sized intracytoplasmic vacuoles that are morphologically consistent with lipidosis.

The liver of mouse 254.19 was diffusely infiltrated with a histiocytic sarcoma. Mouse 261.60 had a focal island of tumor tissue that was morphologically consistent with histiocytic sarcoma. This is not an uncommon tumor of older mice on a B6 background.

The lungs from the following mice had mild to moderate perivascular infiltrates with mononuclear cells: 254.19, 264.81, 224.36, 234.97, 254.18, 261.58, 267.00, and 268.10. The lungs from the following mice had mild to moderate peribronchiolar infiltrates with mononuclear cells: 222.23, 222.26, 223.31, 232.81, 232.82, 232.88, and 273.44. These infiltrates are commonly seen in the lungs of adult mice.

Mouse 264.80 had multifocal airways with multiple eosinophilic crystals. Mouse 224.36 and 267.00 had a focal area of acidophilic macrophage pneumonia in which the eosinophilic crystals are within macrophages in the alveoli. This lesion is a common idiopathic finding disease in mice on a B6 background.

Mouse 224.34 had a focal bronchoalveolar adenoma. The alveoli in mouse 232.82 contained multifocal islands of neoplastic small lymphocytes (Lymphoma).

The spleen of all mice had variable amounts of extramedullary hematopoiesis and hemosiderin within the macrophages of the red pulp. This is considered normal in older mice. Mouse 232.82 had enlarged spleens grossly and the white pulp was expanded with neoplastic small lymphocytes. (Lymphoma). The spleen of 223.31 was moderately enlarged grossly and the white pulp was diffusely hyperplastic.

5. CONCLUSIONS

The tumors, increased number of inflammatory cell infiltrates and degenerative lesions within multiple organs that were seen are considered to be common background lesions in aged mice. None of the microscopic findings are suggestive of adverse effects related to vector administration in these mice.

University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75390
Phone 214-648-1402
Mary.Wight-Carter@UTSouthwestern.edu

6. APPENDIX – HISTOPATHOLOGIC FINDINGS IN INDIVIDUAL MICE

Tissues were reviewed by a pathologist that was blinded to the study groups. The tumors and increased number of inflammatory cell infiltrates and degenerative lesions seen in these mice are expected in mice as they age.

Genotype	AGA Het				AGA KO					AGA KO				
Treatment Age (months)	6				6					6				
Dose (vg/mouse)	N/A				Vehicle					$1x10^{12}$				
Mouse ID	222.26	224.35	224.39	232.82	222.23	223.30	224.34	232.81	224.36	232.88	223.31	234.97		
Gender	M	M	F	F	M	M	F	F	M	M	F	F		
Gross finding				Splenomegaly	enlarged bladder	enlarged bladder		enlarged fallopian tube		liver nodule	splenomegaly			
cerebrum	normal	normal	normal	normal		normal	normal	normal	normal	normal	normal	normal		
cerebellum: mild to moderate decrease in the number of Purkinje cells in at least one lobe and occasionally multiple lobes of the cerebellum	normal	normal	normal	normal	x	x	x	x	x	normal	x	x		
olfactory bulb	normal	normal	normal	normal		normal	normal	normal	normal	normal	normal	normal		
cervical cord	normal	normal	normal	normal		normal	normal	normal	normal	normal	normal	normal		
lumbar cord	normal	normal	normal	normal		normal	normal	normal	normal	normal	normal	normal		
sciatic nerve	no data	no data	normal	normal	no data	no data	no data	no data	normal	normal	no data	no data		
ovaries			normal	normal			normal	normal			normal	normal		
testicles: multiple variably sized vacuoles, which is an incidental finding		no data			no data	no data			x					
heart: multifocal areas with separation of cardiomyocytes by increased collagen fibers (fibrosis), which were not clinically significant.									x	x				
kidney: multifocal mild to moderate thickening of the glomerular tufts, multifocal tubular regeneration, mild multifocal interstitial fibrosis, mild to moderate multifocal interstitial and perivascular infiltrates with mononuclear cells (glomerulonephropathy), which is not uncommon in adult or aged mice								x						
kidney: multiple glomerular tufts were thickened with eosinophilic proteinaceous material (mild glomerulopathy), which is not uncommon in adult or aged mice														
kidney: mild multifocal interstitial fibrosis, with tubular regeneration and mild perivascular infiltrates with small to moderate numbers of mononuclear cells														
kidney: moderate perivascular infiltrates with small to moderate numbers of mononuclear cells, which is not uncommon in adult or aged mice							x				x			
kidney: mild multifocal peripelvic infiltrates with small to moderate numbers of mononuclear cells, which is not uncommon in adult or aged mice	x		x						x	x		x		
Kidney: a few small areas of mineralization in the tubules, which is not uncommon in adult or aged mice									x					
kidney: mild to moderate dilation of a few tubules, which is not uncommon in adult or aged mice				x			x			x				
kidney: a focal area of tubular hyperplasia in renal pelvis, an incidential finading														
liver: mild to moderate peribiliary infiltrates with mononuclear cells with no corresponding fibrosis or hepatocellular necrosis, which is a common finding in mice and increase in incidence as the mice age														
liver: multifocal infiltrates with small numbers of mixed inflammatory cell infiltrates with hepatocellular necrosis (micro-abscess), which occurs spontaneously in the mouse liver with increased incidence as the				x			x			x				
liver: multifocal areas of extramedullary hematopoiesis present in the liver parenchyma, which is less common in rodents as they age and typically occurs in response to increased hematopoietic demand								x			x			
liver: hepatocellular adenoma which expanded the parenchyma and compressed the adjacent normal tissue. Adenomas are common findings in adult B6 mice										x				
liver: round variably sized intracytoplasmic vacuoles that are morphologically consistent with lipidosis	x						x			x	x	x		
liver: histiocytic sarcoma, which is not an uncommon tumor of older mice on a B6 background														
lung: moderate perivascular infiltrates with mononuclear cells, which are commonly seen in the lungs of adult mice									x			x		
lung: mild to moderate peribronchiolar infiltrates with mononuclear cells, which are commonly seen in the lungs of adult mice	x			x	x			x		x	x			
lung: multifocal airways with multiple eosinophilic crystals, a common idiopathic finding disease in B6 mice									x					
lung: a focal bronchoalveolar adenoma							x							
lung: multifocal islands of neoplastic small lymphocytes (Lymphoma) in the alveoli				x										
spleen: white pulp was expanded with neoplastic small lymphocytes. (Lymphoma).				x										
spleen: white pulp was diffusely hyperplastic												x		

University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard
Dallas, Texas 75390
Phone 214-648-1402
Mary.Wight-Carter@UTSouthwestern.edu

Genotype	AGA Het				AGA KO				AGA KO			
Treatment Age (months)	2				2				2			
Dose (vg/mouse)	N/A				Vehicle				$1x10^{12}$			
Mouse ID	254.18	261.60	254.19	264.80	261.63	267.00	268.10	273.44	255.29	261.58	264.81	264.86
Gender	M	M	F	F	M	M	F	F	M	M	F	F
Gross finding	liver nodule		heptosplenomegaly		liver nodule		spleen lesion					
cerebrum	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
cerebellum: mild to moderate decrease in the number of Purkinje cells in at least one lobe and occasionally multiple lobes of the cerebellum	normal	normal	normal	normal	normal	x	normal	x	normal	normal	normal	normal
olfactory bulb	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
cervical cord	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
lumbar cord	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
sciatic nerve	normal	normal	normal	no data	normal	normal	no data	no data	normal	no data	normal	normal
ovaries			normal	normal			normal	normal			normal	normal
testicles: multiple variably sized vacuoles, which is an incidental finding	x									x		
heart: multifocal areas with separation of cardiomyocytes by increased collagen fibers (fibrosis), which were not clinically significant.	x	x			x	x				x		
kidney: multifocal mild to moderate thickening of the glomerular tufts, multifocal tubular regeneration, mild multifocal interstitial fibrosis, mild to moderate multifocal interstitial and perivascular infiltrates with mononuclear cells (glomerulonephropathy), which is not uncommon in adult or aged mice			x									
kidney: multiple glomerular tufts were thickened with eosinophilic proteinaceous material (mild glomerulopathy), which is not uncommon in adult or aged mice				x								
kidney: mild multifocal interstitial fibrosis, with tubular regeneration and mild perivascular infiltrates with small to moderate numbers of mononuclear cells				x	x							
kidney: moderate perivascular infiltrates with small to moderate numbers of mononuclear cells, which is not uncommon in adult or aged mice	x	x				x		x		x		x
kidney: mild multifocal peripelvic infiltrates with small to moderate numbers of mononuclear cells, which is not uncommon in adult or aged mice				x			x		x			
Kidney: a few small areas of mineralization in the tubules, which is not uncommon in adult or aged mice												
Kidney: mild to moderate dilation of a few tubules, which is not uncommon in adult or aged mice				x			x					x
kidney: a focal area of tubular hyperplasia in renal pelvis, an incidential finading									x			
liver: mild to moderate peribiliary infiltrates with mononuclear cells with no corresponding fibrosis or hepatocellular necrosis, which is a common finding in mice and increase in incidence as the mice age				x		x		x				
liver: multifocal infiltrates with small numbers of mixed inflammatory cell infiltrates with hepatocellular necrosis (micro-abscess), which occurs spontaneously in the mouse liver with increased incidence as the	x				x	x					x	
liver: multifocal areas of extramedullary hematopoiesis present in the liver parenchyma, which is less common in rodents as they age and typically occurs in response to increased hematopoietic demand												
liver: hepatocellular adenoma which expanded the parenchyma and compressed the adjacent normal tissue. Adenomas are common findings in adult B6 mice	x				x							
liver: round variably sized intracytoplasmic vacuoles that are morphologically consistent with lipidosis	x	x		x					x			x
liver: histiocytic sarcoma, which is not an uncommon tumor of older mice on a B6 background		x	x									
lung: moderate perivascular infiltrates with mononuclear cells, which are commonly seen in the lungs of adult mice	x		x			x	x			x	x	
lung: mild to moderate peribronchiolar infiltrates with mononuclear cells, which are commonly seen in the lungs of adult mice								x				
lung: multifocal airways with multiple eosinophilic crystals, a common idiopathic finding disease in B6 mice				x		x						
lung: a focal bronchoalveolar adenoma												
lung: multifocal islands of neoplastic small lymphocytes (Lymphoma) in the alveoli												
spleen: white pulp was expanded with neoplastic small lymphocytes. (Lymphoma).												
spleen: white pulp was diffusely hyperplastic												