AWARD NUMBER: W81XWH-17-1-0537

TITLE: Development of Pharmacotherapies for the Treatment of Hydrocephalus

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CONTRACTING ORGANIZATION: Indiana University

REPORT DATE: Sept 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Sept 2020	**Annual Progress Report**	**09/01/2019- 08/31/2020**

4. TITLE AND SUBTITLE

Development of Pharmacotherapies for the Treatment of Hydrocephalus

5a. CONTRACT NUMBER
W81XWH-17-1-0537

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Indianapolis, IN 46202-5130

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The proposed studies aim to test the efficacy and mechanism of action of TRPV4 antagonists for the treatment of hydrocephalus in rodent models. Whether the cause of hydrocephalus is brain hemorrhage as in pre-term infants, idiopathic normal pressure hydrocephalus of the elderly or post-traumatic hydrocephalus of any age, reducing the production of cerebrospinal fluid (CSF) with a pharmaceutical agent is a promising, novel treatment with the potential to revolutionize clinical outcomes. Preliminary data suggested that TRPV4 antagonists represent such a potential drug treatment. The proposed studies are characterizing and using unique rodent models of hydrocephalus to study the efficacy of drug treatment. In addition, cultured choroid plexus (CP) cells are being used to study the mechanisms of action of the drug. In the third year we have made progress in all the proposed third year experiments listed in the SOW. Unfortunately, like many research programs, our progress has been impeded by the restrictions necessary to maintain personnel safety during the COVID epidemic. However, we have made some progress and have requested, and been granted, a no-cost extension for one year in order to complete our proposed studies.

15. SUBJECT TERMS

Hydrocephalus, TRPV4 antagonists, choroid plexus, cerebrospinal fluid production, drug development, behavioral studies

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	**32**	19b. TELEPHONE NUMBER *(include area code)* 317-213-9612

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Please note: In the document, some of the outline boxes after each question have been removed because it was difficult to fill these in with the required tables, figures etc.

1. INTRODUCTION:

The proposed studies aim to test the efficacy and mechanism of action of TRPV4 antagonists for the treatment of hydrocephalus in rodent models. Whether the cause of hydrocephalus is brain hemorrhage as in pre-term infants, idiopathic normal pressure hydrocephalus of the elderly or post-traumatic hydrocephalus of any age, reducing the production of cerebrospinal fluid (CSF) with a pharmaceutical agent is a promising, novel treatment with the potential to revolutionize clinical outcomes. Preliminary data suggested that TRPV4 antagonists represent such a potential drug treatment. The proposed studies are characterizing and using unique rodent models of hydrocephalus to study the efficacy of drug treatment. In addition, cultured choroid plexus (CP) cells are being used to study the mechanisms of action of the drug. In the third year we have made progress in all the proposed third year experiments listed in the SOW. Unfortunately, like many research programs, our progress has been impeded by the restrictions necessary to maintain personnel safety during the COVID epidemic. However, we have made some progress and have requested, and been granted, a no-cost extension for one year in order to complete our proposed studies.

The most notable accomplishment of this year has been the publication of the pre-clinical study showing efficacy of a TRPV4 antagonist in a rat model of hydrocephalus in Journal of Clinical Investigation: Insight. Another on-going success is that within the next two months we will complete the backcrossing studies that were necessary in order to move the mice onto different genetic backgrounds in order to reduce disease severity. This back-crossing has taken over a year but has been very successful in generating animals with less severe disease that can be used for both drug testing and behavioral studies. Not part of the original proposal but an important addition to this work is that we are in the process of obtaining TRPV4 knock-out mice to breed with our hydrocephalic mice for proof-of-principle experiments. Another aspect that will contribute to the success of these studies is the publication (by others) of a genetic screening technique to identify the TRPV4 KO animals – strangely something that has eluded scientists using these animals making it difficult to do the important experiments that require identifying double mutants during the screen. This allows us, for the first time, to use the TRPV4 knock-out animals to determine the necessity of TRPV4 for the development of hydrocephalus.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Hydrocephalus; TRPV4 antagonists; choroid plexus; cerebrospinal fluid production; drug development; behavioral studies

ACCOMPLISHMENTS:

What were the major goals of the project?

STATEMENT OF WORK – approved - 10/19/16
Accomplishments color-coded below on the approved SOW - 9/17/20

Site: Indiana University
School of Science, 723 West
Michigan Street, Indianapolis

PI: Blazer-Yost

Key:
Yellow completed
Pink started
Blue – results of proposed experiments indicate need to conduct alternative studies

Specific Aim 1 (specified in proposal)	Time-line	Investigator
Major Task 1: Start and maintain the breeding colony for the TMEM67(-/-) and TMEM67(+/-) rats	Month	
Subtask 1 Paired matings of TMEM67(+/-) animals in the colony currently approved by local IACUC – to be used for all experiments	3-36	Dr. Blazer-Yost
Local IRB/IACUC Approval for all three animal models	1	Dr. Blazer-Yost
Milestone Achieved: HRPO/ACURO Approval for all three animal models	4	Dr. Blazer-Yost
Milestone(s) Achieved: First animals ready to start MRI analyses as soon as possible after HRPO/ACURO Approval granted	6	Dr. Blazer-Yost
Major Task 2: MRI experiments – TMEM67(-/-) rat pups- days 7 and 15 (48 pups; 6 each wt males and females, 6 each TMEM(-/-) males and females with and without drug treatment)	Month	
Subtask 1 – conduct the MRI studies on rat pups as outlined in S.A. #1	6-14	Dr. Territo
Subtask 2 – calculate ventricular volumes and summarize data	15-16	Dr. Territo
Milestone(s) Achieved: All experiments using TMEM67(-/-) completed and data summarized for publication	17	Drs. Territo/Blazer-Yost
Major Task 3: MRI experiments – TMEM67(+/-) rats - day 240 + 270 (48 adults; 6 each wt males and females, 6 each TMEM67(+/-) males and females with and without drug treatment	Month	
Subtask 1 – conduct the MRI studies on adult rats as outlined in S.A. #1	16 -24	Dr. Territo
Subtask 2 – calculate ventricular volumes and summarize data	24-26	Dr. Territo
Milestone(s) Achieved: All experiments using TMEM67(+/-) adult rats completed and data summarized for publication	27	Drs. Territo/Blazer-Yost

Major Task 4: Start the breeding colony for the Gas8^{GT} mice		
Subtask 1 Paired matings of Gas8^{GT} animals in the colony approved by local IACUC	22-32	Dr. Berbari
Milestone(s) Achieved: First Gas8^{GT} ready to start MRI analyses	24	Dr. Berbari
Major Task 5: : MRI experiments – Gas8^{GT} mice (48 pups; 6 each wt males and females, 6 each Gas8^{GT} males and females with and without drug treatment)	Month	
Subtask 1 – conduct the MRI studies on mice as outlined in S.A. #1	24-30	Dr. Territo
Subtask 2 – calculate ventricular volumes and summarize data	30-32	Dr. Territo
Milestone(s) Achieved: All experiments using Gas8 mice completed and data summarized for publication	34	Drs. Territo/Berbari/ Blazer-Yost
Specific Aim 2		
Major Task 1: Neurohistology & neuronal counting of TMEM67(+/-) rat model of slowly progressing hydrocephalus (8 months old; baseline data; 24 rats)	Month	
Subtask 1: Perfusion, brain sectioning	8-9	Dr. Goodlett
Subtask 2: Immunohistochemical processing of brain sections and analysis with confocal & light microscopy	8-11	Dr. Goodlett / Dr. Lamb
Subtask 3: Nissl staining and stereological counting of neocortical neurons	8-12	Dr. Goodlett
Subtask 4: Data summary + statistical analysis of neurohistological data	11-12	Dr. Goodlett
Milestone(s) Achieved: All baseline neurohistological experiments using TMEM67(+/-) rats completed and data summarized for publication	12	Drs. Goodlett / Lamb / Blazer-Yost
Major Task 2: Treatment & Behavioral Testing of TMEM67(+/-) rat model of slowly progressing hydrocephalus; 1st cohort (n=48) given MRI in Aim 1; 2nd cohort without MRI (n=48)	Month	
Subtask 1: 30-day treatment with TRPV4 antagonist or vehicle; ~20 rats approximately every 2 months (treatment spans both cohorts)	16-32	Dr. Blazer-Yost/Dr. Goodlett
Subtask 2a: Behavioral testing of rats of cohort 1 (given MRIs); Subtask 2b: Behavioral testing of rats of cohort 2 (no MRIs)	16-24 24-32	Dr. Goodlett
Subtask 3a: Data summary / analysis of behavioral data of cohort 1 Subtask 3b: Data summary / analysis of behavioral data of cohort 2	24-26 32-34	Dr. Goodlett
Milestone(s) Achieved: Behavioral TMEM67(+/-) adult rat experiments completed, data summarized, integrated with MRI/neurohistology	34	Drs. Goodlett / Territo / Blazer-Yost
Major Task 3: Neurohistology & neuronal counting of TMEM67(+/-) rat model of slowly progressing hydrocephalus (96 rats, 48 from cohort 1 and 48 from cohort 2)	Month	
Subtask 1: Perfusion, brain sectioning (across both cohorts)	16-32	Dr. Goodlett
Subtask 2: Immunohistochemical processing of brain sections and analysis with confocal & light microscopy (across both cohorts)	16-34	Dr. Goodlett / Dr. Lamb

Subtask 3: Nissl staining and stereological counting of neocortical neurons (across both cohorts)	16-34	Dr. Goodlett
Subtask 4: Statistical analysis of neurohistological data	32-34	Dr. Goodlett
Milestone(s) Achieved: Neurohistology TMEM67(+/-) experiments completed, data summarized and integrated with MRI & behavior	34	Drs. Goodlett / Territo / Lamb/ Blazer-Yost
Publication		
Major Task 1: 1-3 publications ready for submission	Month	
Subtask 1: Prepare the data regarding the effect of TRPV4 antagonist treatment on ventricular size, behavior & histology, write publications	20-34	Drs. Blazer-Yost/ Territo/Goodlett/Lamb Berbari/Fulkerson
Specific Aim 3		
Major Task 1: TRPV4 immunohistochemical staining of TMEM67(-/-) rat pups for developmental changes in expression 160 animals (2 genders x 5 time points x 4 animals per time point x 2 (wt or hydrocephalic) x 2 (drug or vehicle)	Month	
Subtask 1: Treatment of animals as per protocol; preservation of brain	14-20	Drs. Blazer-Yost/ Berbari
Subtask 2: Cryosectioning, immunostaining, confocal analysis (brain)	16-22	Drs. Blazer-Yost/ Berbari
Milestone(s) Achieved: Determination of developmental changes in TRPV4 expression in severely hydrocephalic rats	23	Drs. Blazer-Yost/ Berbari/Goodlett
Major Task 2: TRPV4 immunohistochemical staining of TMEM67(+/-) adult rats to determine developmental changes in expression of TRPV4 (64 animals: 2 sexes x 2 time points x 4 rats per time point x 2 (wt or hydrocephalic) x 2 (drug or vehicle)	Month	
Subtask 1: Treatment of animals as per protocol; preservation of brain	22-28	Drs. Blazer-Yost/ Berbari
Subtask 2: Cryosectioning, immunostaining, confocal analysis (brain)	24-30	Drs. Blazer-Yost/ Berbari
Milestone(s) Achieved: Determination of developmental changes – TRPV4 in slowly developing, chronically hydrocephalic rats	31	Drs. Blazer-Yost/ Berbari/Goodlett
Major Task 3: Immunohistochemical staining of $Gas8^{GT}$ mice pups to determine developmental changes in expression of TRPV4. 160 animals (2 sexes x 5 time points x 4 mice per time point x 2 (wt or hydrocephalic) x 2 (drug or vehicle)	Month	
Subtask 1: Treatment of animals as per protocol; preservation of brain	28-33	Drs. Blazer-Yost/ Berbari
Subtask 2: Cryosectioning, immunostaining, confocal analysis (brain)	32-34	Drs. Blazer-Yost/ Berbari
Milestone(s) Achieved: Determination of developmental changes – TRPV4 in severely hydrocephalic mice	35	Drs. Blazer-Yost/ Berbari/Goodlett
Specific Aim 4		
Major Task 1: Electrophysiological analyses of ion	Month	

transporters involved in the response to TRPV4 stimulation in PCP-R cell line		
Subtask 1: Analysis of Ca^{2+}-activated Cl^- channels in the PCP-R (porcine) cell line	1-3	Dr. Blazer-Yost
Subtask 2 Analysis of Ca^{2+}-activated K^+ channels in the PCP-R cell line	3-6	Dr. Blazer-Yost
Major Task 2: Electrophysiological analyses of the ion transporters involved in the response to a TRPV4 agonist in the HIBCPP cell line		
Subtask 1: Characterization of the response to TRPV4 activation in the HIBCPP (human; no identifiable information on source) cell line	6-9	Dr. Blazer-Yost
Subtask 2: Analysis of Ca^{2+}-activated Cl^- channels—HIBCPP cell line	9-12	Dr. Blazer-Yost
Subtask 3: Analysis of Ca^{2+}-activated K^+ channels—HIBCPP cell line	12-15	Dr. Blazer-Yost
Milestone(s) Achieved: Identification of ion channel activated in response to TRPV4-induced changes in intracellular calcium	15	Dr. Blazer-Yost
Specific Aim 5		
Major Task 1: Obtain tissue from all three animal models, section and identify the presence and polarization of identified transport proteins. No new animals – tissue used from Specific Aim 3	Month	
Subtask 1: Obtain tissue from animals – obtained from the same brains as those prepared in Specific Aim 3	14-34	Dr. Blazer-Yost
Major Task 2: Obtain tissue from two tissue culture models, stain and identify the presence and polarization of transport proteins	Month	
Subtask 2: Grow and fix cells from the PCP-R cell line	14-18	Dr. Blazer-Yost
Subtask 2: Grow and fix cells from the HIBCPP cell line	18-22	Dr. Blazer-Yost
Subtask 3: Conduct immunohistochemical localization in cells and animal tissues and visualize by confocal imaging	24-30	Dr. Blazer-Yost
Milestone(s) Achieved: Comparison of expression of transport proteins in *vivo* and *in vitro*	30	Drs. Blazer-Yost/ Berbari/Goodlett
Publication		
Major Task 1: 2-3 publications ready for submission	Month	
Subtask 1: Prepare the data regarding the effects of TRPV4 agonists in cultured cells	16-18	Drs. Blazer-Yost
Subtask 1: Prepare the data for comparison of transporters in native choroid plexus with those found in cultured cell lines	30-36	Drs. Blazer-Yost Berbari/Fulkerson
Milestone: Publish high impact papers and present the data obtained in these studies at national meetings	12-36	Drs. Blazer-Yost/ Territo/Goodlett Berbari/Fulkerson

What was accomplished under these goals?

All of the data presented below are correlated with the schedule provided in the SOW above and represent work done in year 3 of funding. The work completed in years 1 and 2 were summarized in the previous annual progress reports.

Specific Aim #1

Major Task #1: Start and maintain the breeding colony for the TMEM(-/-) and TMEM (+/-) rats.

All milestones of obtaining approvals from the local IACUC as well as ACURO were met early in the first year. As the experiments progressed, it was necessary to obtain protocol amendments. The protocol amendments were first approved by the local IACUC and then submitted to ACURO. No studies funded by the grant were initiated until ACURO approval was granted. We have completed a new 3-year IACUC protocol for the rat studies and this has been approved by the IACUC and subsequently by ACURO. In addition, in the previous year, a new 3-year IACUC protocol for the mice studies was completed and approved by the IACUC and ACURO .
The paired matings are on-going and will continue for remaining years of funding.

Major Task 2: MRI experiments – TMEM67(-/-) rat pups- days 7 and 15 (48 pups; 6 each wt males and females, 6 each TMEM(-/-) males and females with and without drug treatment).

These studies have been completed and the manuscript has been published Sept. 17, 2020 in Journal of Clinical Investigation: Insight 2020:5(18): e137646. https://doi.org/10.1172/jci.insight.137646

Major Task 3: MRI experiments – TMEM67(+/-) rats - day 240 + 270 (48 adults; 6 each wt males and females, 6 each TMEM67(+/-) males and females with and without drug treatment.
Major task 3 was initiated early and the preliminary results indicated a change in the timing of drug treatment in the experimental protocol of the adult animals. In figure 1 the volumes of the lateral ventricles of wild-type and heterozygous animals are shown as measured at day 240 (+/- 3 days). While the heterozygous animals show ventricular volumes that are statistically greater than the wild-type, the variability of the volumes in the heterozygous animals may make drug treatment data difficult to interpret if the treatment results in a partial decrease in the development of the hydrocephalus. For this reason it has been decided to shift the time of initiation of drug treatment to start at post-natal day 300 and continue to post-natal day 330. This will provide the same number of days of drug treatment but in older animals.

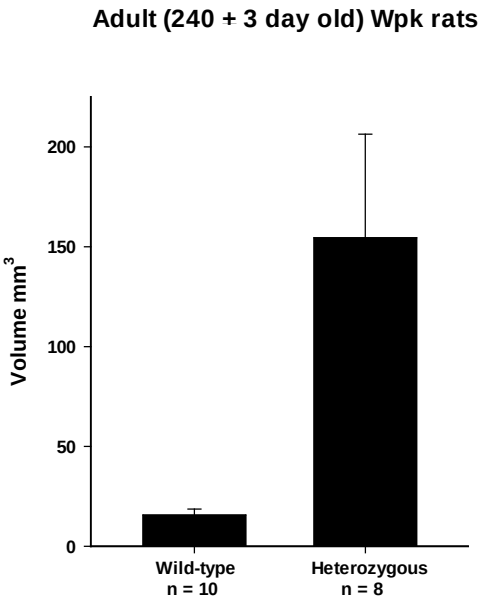


Figure 1: MRI quantitation of lateral ventricle volumes of wild type and heterozygous and TMEM67 rats at post natal day 240 (+/- 3 days). The number of animals used in each genotype is indicated by the n number at the base of the columns. The values are averages +/- SEM.

In the second year of the funding cycle, we tested animals from post-natal day 300 to 330. This was done in a blinded fashion and as part of the behavioral studies (Specific Aim #2). Unfortunately, due to the blinded nature of the studies, a large number of animals were scanned before the study was un-blinded. When the data were analyzed it was discovered that the colony no longer showed a phenotype in the adult heterozygous animals (Figure 4). We do not know what caused this change in the colony and it is rather surprising since our initial studies showed a tight genotype-phenotype correlation.

To correct this serious problem, in the third year of funding, we re-derived the colony by backcrossing some of the adult heterozygous females with clear hydrocephalus to wild-type males and then followed the fidelity of the off-spring. Unfortunately, these time-intensive experiments did not yield the anticipated outcome. The heterozygous animals produced by these matings are completely devoid of a phenotype. We will not, therefore be able to obtain a manuscript from the exact proposed studies.

Alternative Experiments

Although not originally proposed, this turn of events in the research has stimulated us to conduct whole genome sequencing in the final year of funding in order to identify the modifier genes that appear to influence the expression of the heterozygous phenotype. We anticipate that these studies may lead to a manuscript describing the findings of the whole genome sequencing.

Major Task 4: Start the breeding colony for the Gas8^{GT} mice

This major task was scheduled to begin at the end of the second year of funding. However, we were forced to move the scheduled time ahead because the laboratory that agreed to provide this mouse model (Dr. Brad Yoder, University of Alabama, Birmingham) decided to phase out the colony at their institution. The decision was made that it was safer to start the experiments early rather than risk the time and expense of deriving the colony from frozen gametes. In hindsight this turned out to be a very fortuitous decision.

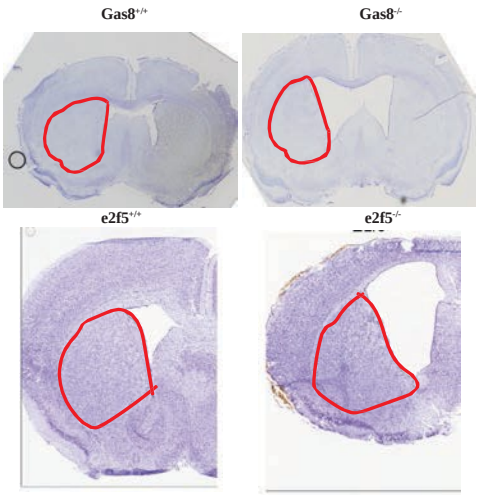


Figure 2. Nissl stain of hydrocephalic mouse lines. WT or Gas8$^{-/-}$ mice on BALB/cJ background (Top) or WT or e2f5$^{-/-}$ mice on 129S/1J background (bottom) euthanized at 6 or 4 weeks of age, respectively, sliced on a cryostat, and stained with a Nissl stain, mounted, and imaged. The striatum from one hemisphere in each animal is highlighted in red.

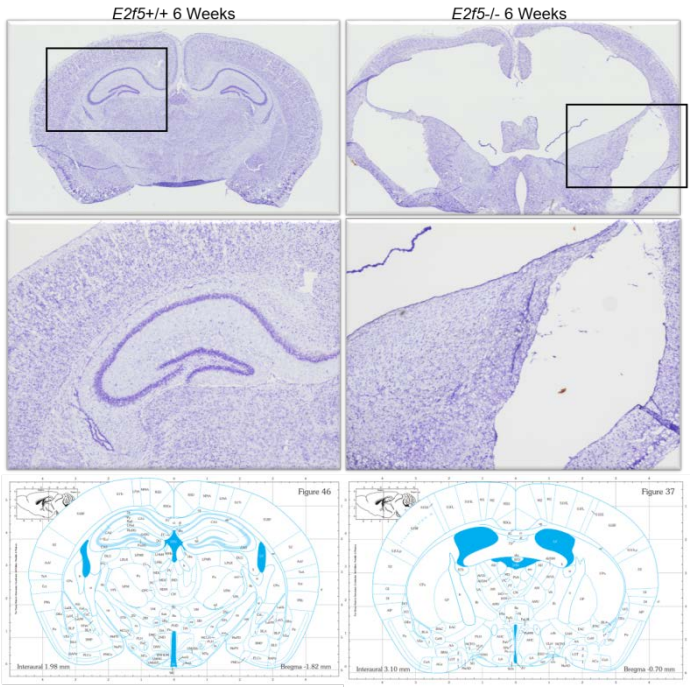
The mice were obtained and the colony was established in our facility. However, we discovered that while at Birmingham this model had been crossed onto a black 6 (B6) background. The animals were found to have very severe and rapidly progressing hydrocephalus. Due to the severity of the disease, many of the pups had to be sacrificed by post-natal day 6-8. This model would, therefore, not be compatible with drug testing (major Task #5) and the animals would be too young to obtain accurate MRI images during treatment.

Alternative Experiments

Like our rat model, the *GAS8* mouse also develops hydrocephalus as a result of cilia dysfunction however, the *GAS8* mouse does not have further confounding conditions like PKD and, therefore, we regard this as a very useful model that should be pursued. Given the predisposition of B6 mice to develop hydrocephalus, and information regarding greater disease penetrance on this background (Danielian et al 2016, Lewis et al 2016), we decided to cross the *GAS8* mouse onto a 129S1/SvlmJ (Stock #002448) background (their original background) and also to two other backgrounds (BALB/cJ (Stock #000651) and FVB/NJ (Stock #001800)). These studies are currently in generation 6 (of 9) with some very positive results. Despite hydrocephalus, the animals on all the new backgrounds are living longer than the animals originally obtained from the Yoder

laboratory. Figure 2 illustrates animals on the BALB/cJ background at 6 weeks. Despite the relatively severe hydrocephalus at 6 weeks, the animals are still behaving and eating normally. These preliminary results are very encouraging. The animals on the *BALB/cJ* and *FVB/NJ* backgrounds are likely to be the most useful for the proposed studies. If the animals live for 5-6 weeks, we can accurately treat them for 1 week and do MRIs on the first and last day of treatment.

In addition to the proposed studies, we have added another mouse model to our repertoire. The *E2f5* mouse develops hydrocephalus not as a result of ciliary dysfunction, but, rather, as a result of abnormal choroid plexus development leading to an intense secretory phenotype at the level of the choroid plexus (Lindeman GJ, Dagnino L, Gaubatz S, et al. Genes Devel. 12:1092-8). This phenotype was corroborated by Swetloff and Ferreti (Swetloff A, Ferretti P: Intern. J. Devel. Biol. 49: 859-865) and these authors found that E2f5 is responsible for normal choroid plexus development in both humans and mice. E2f5 occurs with high mRNA abundance in early gestational stages and is correlated with nuclear protein localization. Upon maturation, E2f5 is found mostly in the cytoplasm, and with reduced mRNA abundance. This suggests a role for E2f5 in normal neuroepithelial development. *E2f5* knockout mice develop severe hydrocephalus after approximately 3-4 weeks of age on an albino background, and after approximately 2-3 weeks of age on a mixed 129S1/B6 background. We obtained this model and are currently backcrossing it onto the same three genetic backgrounds as the *GAS8* model above. Preliminary studies in generation 6 (*E2F5/129S1/SvlmJ*) and 5 (*E2F5/FVB/NJ)* indicate that the survival times are going to be very similar to the GAS8 mice on these backgrounds.



At 6 weeks, these animals appear quite healthy, moving around the cages and feeding normally despite considerable cortical thinning and other brain tissue loss (Fig. 3). Earlier ages show a less severe phenotype which is more amenable to studying early brain damage. These models will allow us to co-ordinate structural and functional therapeutic potential of drug treatment.

Figure 3: Representative Nissl stains demonstrating extreme ventriculomegaly in a 6 week old mutant E2f5$^{-/-}$ mouse compared its E2f5$^{+/+}$ wild-type litter-mate. The mice were backcrossed for seven generations onto a 129S1 background in order to decrease hydrocephalic severity and prolong longevity. Since the sections are not exactly matched, corresponding mouse brain atlas coronal sections are included to reference normal ventricle sizes given the position along the rostral-caudal axis. Similar results were obtained with the *Gas8*$^{-/-}$ model.

Major Task 5: MRI experiments – Gas8^{GT} mice (48 pups; 6 each wt males and females, 6 each Gas8^{GT} males and females with and without drug treatment)

One genetic cross will be selected for the completion of Major Task 5. This major task will start as soon as the selected animals are back-crossed for 9 generations. Adding a second mouse model as listed above will supplement our proposed studies and will also provide a non-ciliopathy alternative model as proof-of-principle testing for drug efficacy before the end of the no-cost extension.

Specific Aim #2

Major Task 1: Neurohistology & neuronal counting of TMEM67(+/-) rat model of slowly progressing hydrocephalus (8 months old; baseline data; 24 rats).

There has been a change in the scope and timing of this major task. As per Specific Aim 1, Major Task 3 above, we decided to use the adult animals starting at 10 months rather than 8 months. We have aged the animals, prepared perfused brains and initiated the immunohistochemical processing. In addition, the added costs of maintenance and production of the animals limited the number of rats available to complete separate groups originally planned for the baseline neurohistological characterization, so the revised plan omitted those studies to assure that sufficient numbers will be available for the complete analysis of the pharmacological treatment effects.

Unfortunately we experienced a major experimental set-back when it was discovered that for an unknown reason, the genotype/phenotype (mild hydrocephalus in the heterozygous animals) was no longer observed (Figure 4). We are certain of the genotyping. Genotyping of the animals has been performed using two different techniques with the same results. These results are even more confounding because the lateral ventricular volumes of the wild-type animals are higher than expected while the volumes of the heterozygous animals are lower than expected. As can be seen by the error bars in Figure 4, there is substantial variability in the results.

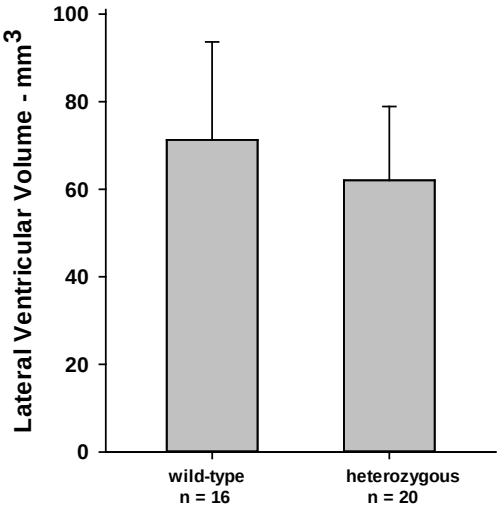


Figure 4: MRI quantitation of lateral ventricle volumes of wild type and heterozygous and TMEM67 rats at post natal day 330 (+/- 3 days). The number of animals used in each genotype is indicated by the n number at the base of the columns. The values are averages +/- SEM.

As noted above, the back-crossing has been completed and we obtained the unanticipated result of no phenotype in the heterozygous animals. Therefore, the experiments listed in this aim will not be possible and will be replaced by alternative experiments listed under various Major Tasks.

Major Task 2: Treatment & Behavioral Testing of TMEM67(+/-) rat model of slowly progressing hydrocephalus; 1st cohort (n=48) given MRI in Aim 1; 2nd cohort without MRI (n=48)

As indicated in the last annual report on progress, it was necessary to change the scope and timing of Specific Aim 2 based on initial MRI outcomes of Specific Aim 1, Major Task 3. Based on our preliminary data and in agreement with accepted protocols, the analyses in this series were done in a blinded fashion and the MRIs were not analyzed until the code was broken. This blinded cohort was used not only for drug testing but, at the same time for behavioral studies.

A total of 41 rats completed behavioral testing (19 WT; 22 HET), and 36 of those completed both the pre-testing and post-testing MRI, so all analyses are limited by small group sizes (4-6 rats for each combination of genotype, treatment, and sex). There were no significant differences in ventricular volume between WT and HET rats either at 300 days or 330 days of age (Figure 4), and there were no significant effects of RN treatment on ventricular volume (data not shown). However, there was wide variability of ventricular volumes within each group, and mutually exclusive subgroups within each genotype and sex combination could be classified as having small ($<45mm^3$) or large ($>45mm^3$) ventricles. The ventricluomegaly did not correlate with genotype, but the presence of categorically different ventricular

phenotypes prompted us to perform secondary analyses of the behavioral outcomes based on classification of ventricle size derived from the MRI volumes (small or large), in addition to the planned primary analysis based on genotype. Figure 5 shows the effect of drug treatment on the change in ventricular volume in adult animals with small (<45mm^3) and large (>45mm^3) ventricles (Figure 5). As can be seen from the figure, RN1734 did not decrease ventricular size in either cohort.

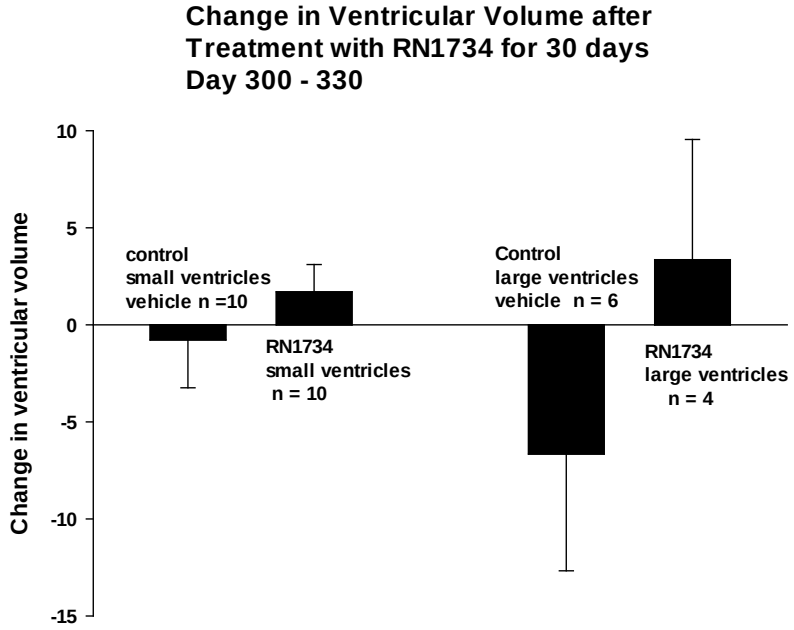


Figure 5: MRI quantitation of lateral ventricle volumes of adult wild type and heterozygous TMEM67 rats. RN1734 (4 mg/kg body weight) or vehicle (DMSO) were injected i.p. daily from post-natal day 300 to 330. The number of animals used in each treatment group is indicated by the n number at the base of the columns. The graph shows the difference in ventricular volume between day 300 and day 330 for each of the groups of animals segregated by small(<45mm^3) and large (>45mm^3) ventricles.

For the behavioral studies, drug treatment began on postnatal day 300, the day after MRI scanning was performed. After two weeks of treatment, the rats were tested sequentially on a series of three behavioral tasks: 1) the Multivariate Concentric Square Field (MCSF) that assessed activity, exploration, risk-taking, risk assessment, shelter-seeking, and stress-related behaviors in a complex arena in a 20-minute session; 2) Novel Object Recognition (NOR) that assessed recognition memory as measured by the memory-based preference to explore a novel (unfamiliar) object when two objects—one familiar and one novel—are encountered; and 3) learning in a Morris water maze to assess spatial learning (6 days of training to swim to a same place in a large tank to find a submerged invisible escape platform, including a probe trial with the platform removed to assess memory for the location), reversal learning (3 days in the opposite location), and visually guided navigation (with a dark-rimmed platform visible just above the surface, placed in different locations each trial).

For the MCSF, there were significant and robust sex differences on many of the measures. Compared to males, females were more active [$F(1,33)=38.41$, $p<.001$], engaged in more exploratory behavior [$F(1,33)=32.49$, $p<.001$], risk-taking behavior [$F(1,33)=12.27$, $p=.001$] and risk-assessment behavior [$F(1,33)=8.55$, $p=.006$] and spent less time in the dark corner room (DCR) [$F(1,33)=13.84$, $p=.001$]. Figure 6 depicts exploratory behavior and for time in the DCR and shows that the robust sex differences were evident whether plotted as a function of genotype or ventricular phenotype. The secondary analysis using ventricular phenotype rather than genotype as a grouping factor obtained the same pattern of significant sex differences on the same measures.

In addition to the large sex differences, a significant interaction between genotype and drug treatment was found for activity, exploratory behavior [$F(1.33)=4.143$, $p<.0499$], and shelter-seeking [$F(1,33)=5.170$, $p=.03$]. For those measures, the RN treatment had opposite effects on WT compared to HET rats: it increased activity and exploration and decreased shelter-seeking in WT rats, whereas it decreased activity and exploration and increased shelter seeking in HET rats, again regardless of the ventricular phenotype.

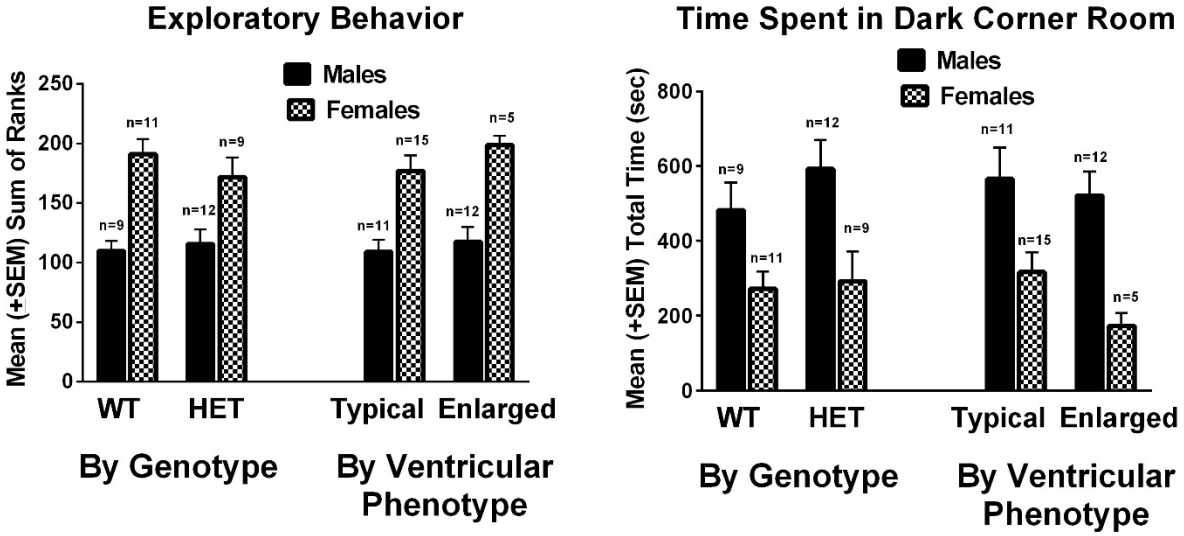


Figure 6: Analysis of Exploratory Behavior and Time Spent in Dark Corner Room in adult TMEM67 rats. The data for these two behavioral studies are expressed as differences as a function of genotype (wild-type or heterozygous) and as ventricular phenotype: typical (<45mm^3) and enlarged (>45mm^3) ventricle sizes. The results of and analyses are also divided by sex of the animals. The number of animals in each group is provided by the n number at the top of each column.

For novel object recognition (NOR), there were no statistically significant group differences for discrimination index (DI), the measure of preferential attention to the novel object over the familiar object in the final trial. Notably, the DI for the WT rats was significantly greater than 0 (DI=0.237$\pm$ 0.055, t(19)=4.270, p<.001), confirming a detectable preference for the novel object. In contrast, the HET group's DI was not significantly different from 0 (DI=0.0827 $\pm$ .1007); WT and HETs did not differ from each other.

For the Morris Water maze, Figure 7 summarizes the outcomes on place learning, reversal, and visually-cued navigation. No significant effects of genotype were found in the primary analysis of place learning and reversal, nor of ventricular phenotype in the secondary analysis. However, the HET rats were significantly impaired on the visible platform task performed on the last two days (see panel C). This outcome was unexpected, and might be a function of retinopathy that our group has found to occur in the rat model. Ongoing studies will evaluate the status of retinal structure in the eyes obtained from these rats. Alternatively, the subtle but significant deficits in visual-cue navigation in the HET rats may be a function of an impaired ability to shift strategies from place cues to visual cues after the extended place training; this can be identified by counterbalanced training orders in future studies.

Significance: The lack of a genotype-phenotype association with hydrocephalus or with functional behavioral deficits may indicate the heterozygous adult TMEM67(+/-) rats are not an optimal model to pursue treatments for brain pathology resulting from slowly developing hydrocephalus. However, several important conclusions do emerge from the data set, despite the limitations imposed by the small sample size. The place learning of these 10-11 month-old rats was notably slower than typically observed in younger rats, indicating that the water maze training likely needs to extend for several more days to allow these older groups to achieve asymptotic performance. Subsequent transfer tests (e.g., reversal) would then be more likely to yield more interpretable outcomes. It is important to determine whether the deficit found for the visible platform task is associated with a genotype-dependent retinopathy. Future studies likely should incorporate more sensitive measures of visual acuity and function. The dramatic sex differences seen in the MCSF emphasizes the importance of including both sexes in preclinical models of therapeutics that extend across the lifespan. The MCSF test did provide one outcome that suggests that HET rats may respond in an

opposite fashion to TRPV4 antagonists compared to WT rats; it caused the HETs to reduce active exploration and increase shelter seeking but caused the WTs to increase activity and reduce shelter seeking. This suggests that the drug may stimulate behavioral arousal in the WT rats, but may be anxiogenic or reduce arousal in the HET rats.

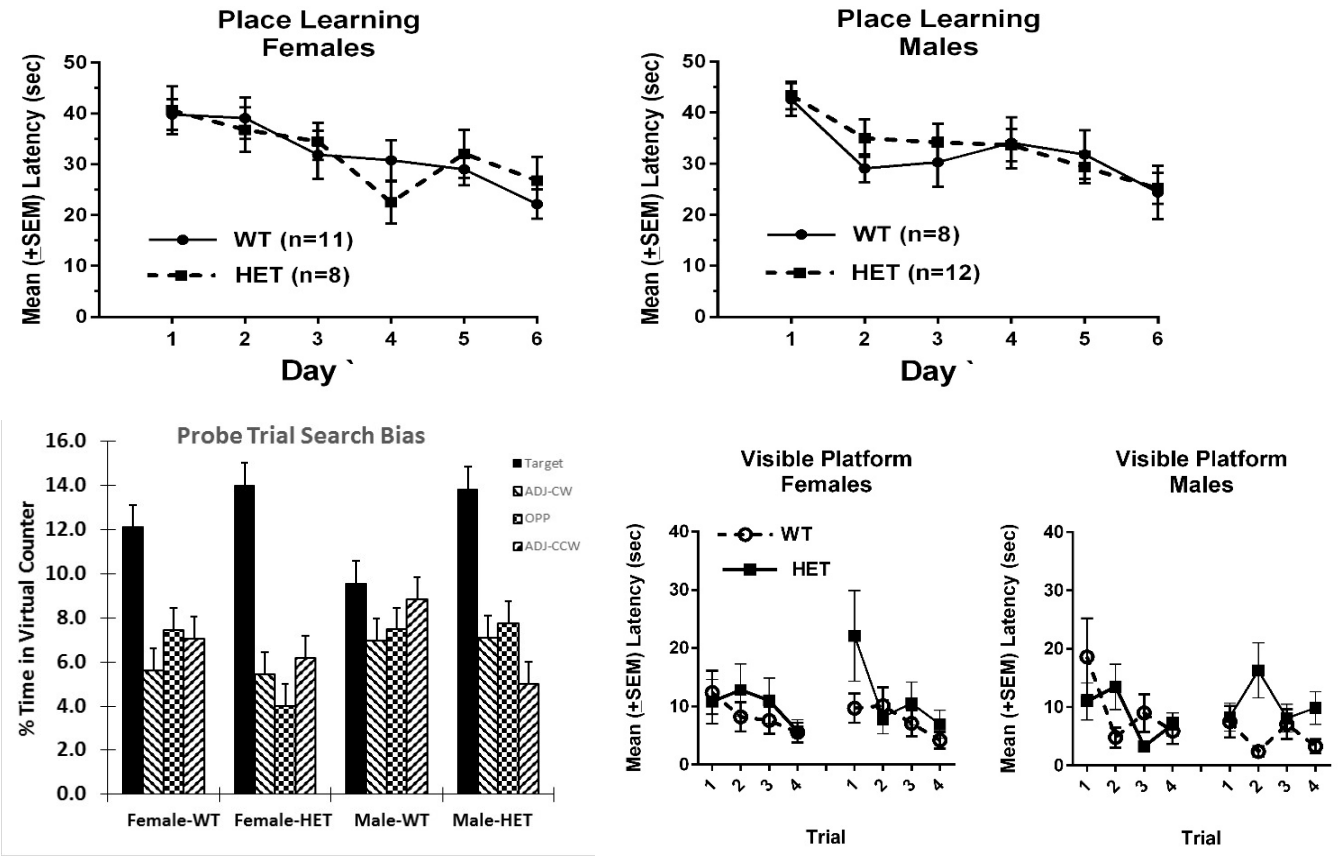


Figure 7. Morris Water Maze Performance.

Acquisition (top panels). Heterozygous (HET) and wild type (WT) rats showed comparable acquisition of place learning in the Morris water maze task, though these 10-month-old rats were notably less proficient at place learning than young-adult rats.

Probe Trial (bar graph). The HET rats and the female WT rats showed significant place biases on the probe trial and no significant main or interactive effects of genotype or treatment were found on the probe trial; the only significant effect was quadrant due to the greater time spent searching in the target quadrant.

Reversal learning. No group significant group differences were present during reversal training (data not shown).

Visible platform (last panels). The HET rats of both sexes showed a significant deficit on the visible platform task [main effect of genotype, $F(1,30)=8.032$, $p=.008$], particularly evident on the first (female) or second (male) trial of the second day. The unexpected deficit in visually-cued navigation suggests either that the HET rats had difficultly switching from use of place strategies to visually-cued strategies, or that visual impairment associate with emerging retinopathy may have impaired use of the local visual cue on rim of the platform. Notably, analysis of the water maze data using ventricle size as the categorical grouping factor (rather than genotype) failed to reveal any significant effects on any stage of water maze testing, suggesting the deficits in visually-cued navigation in HETs was not directly related to enlarged ventricles.

Major Task 3: Neurohistology & neuronal counting of TMEM67(+/-) rat model of slowly progressing hydrocephalus (96 rats, 48 from cohort 1 and 48 from cohort 2).

The heterozygous genotype did not produce significant group differences in ventricular volume nor did it produce strong evidence of group differences in behavioral outcomes. This lack of genotype-phenotype correlation undermined the original rationale for performing extensive neurohistological analyses comparing HET and WT brains to pursue the structural correlates associated with hydrocephalus expected in the HETs. A scaled-down set of studies was performed on a subset of brains chosen on the basis of ventricular phenotype, regardless of the animals' genotypes using immunohistochemistry to evaluate effects on astrocytes (labeled with GFAP) and microglia (labeled with Iba-1). There were no differences noted in any of the groups and, therefore, these studies were suspended.

Specific Aim #3

Major Task 1: TRPV4 immunohistochemical staining of TMEM67(-/-) rat pups for developmental changes in expression.

We have extended this Specific Aim to include biochemical analyses as well as immunohistochemical staining for aquaporin 1 and 4 (AQP1; AQP4) as well as TRPV4. In addition, we have further extended the studies to look at other brain regions, specifically the ependymal subventricular zone. The reasons for these changes are 1) emerging data suggesting roles for AQP1 and 4 in the hydrocephalic state; 2) data showing that, in contrast to our preliminary studies, the expression of TRPV4 did not increase during neonatal development; 3) emerging data to indicate the importance of other electrolyte transporters and intracellular mediators in hydrocephalic development; and 4) that the adult animals studies could not completed as originally proposed so the personnel were reassigned to broaden the developmental studies.

Our preliminary immunohistochemical studies (grant application) indicated that there an increase in TRPV4 expression in hydrocephalic animals. However more extensive studies looking at developmental time, degree of hydrocephalus, and drug treatment failed to confirm these observations. We therefore sought to examine the expression levels via other methods including qPCR and western blotting. It is worth noting that these studies also overlap with studies outlined in Specific Aim #5.

An example of the most recent immunohistochemical data is provided in Figure 8. In this figure, choroid plexus from each of two P15 animals per condition (wt or homozygous; vehicle or drug treated) were stained and imaged in tandem. The figure provides a good example of the variety of intensity of antibody staining that is independent of genotype or treatment. It is controlled studies such as this example that lead us to question whether and amount and/or expression of the TRPV4 was changing. One thing that is clear from Figure 8 is that regardless of genotype or treatment, the TRPV4 is found on the apical membrane (facing the cerebrospinal fluid) and that there is also cytoplasmic labeling indicating a substantial intracellular pool of the TRPV4.

Given the difficulty in accurately quantifying the amount of TRPV4 in the choroid plexus with immunohistochemistry, two alternate methods, qPCR and Western blotting, were employed to more directly examine mRNA and protein production. qPCR studies were performed to compare mRNA expression of a variety of transporters and intracellular signaling components in wild-type animals with their hydrocephalic counterparts. For this Specific Aim the analysis of TRPV4 mRNA in hydrocephalic and treated animals relative to wild-type vehicle is of most interest. The qPCR data show that there is no difference between the wild-type and homozygous animals and also no difference during treatment with the TRPV4 antagonist These data have been published in JCI:Insight (https://doi.org/10.1172/jci.insight.137646).

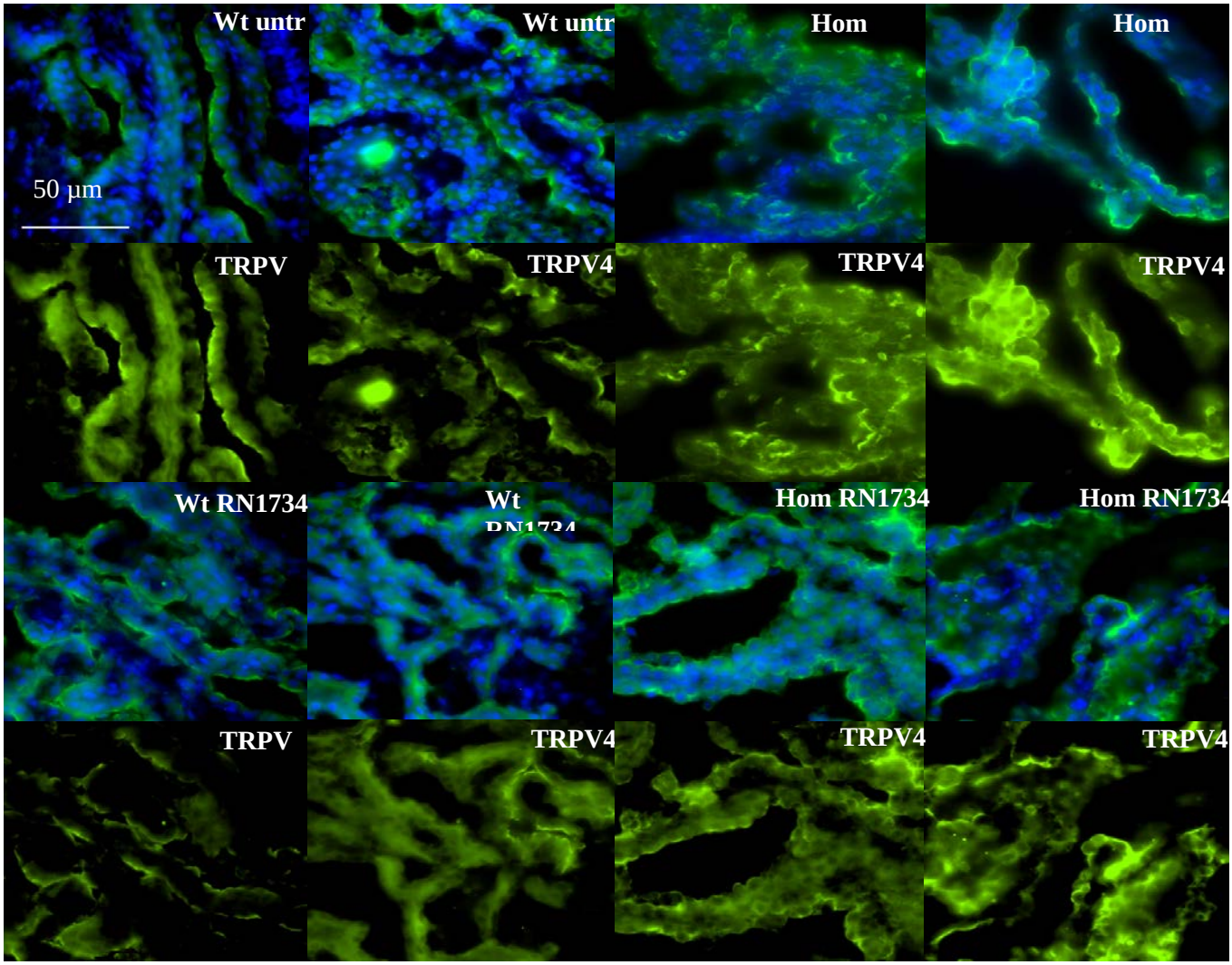


Figure 8. Immunohistochemistry of TRPV4 in choroid plexus tissues from post-natal day 15 wild type and hydrocephalic rats. All of the panels represent immunostaining that was conducted in tandem on the same day with the same vial of primary antibody (Alomone, anti TRPV4 antibody, 1:200). The images were collected using identical microscope settings. Abbreviations: Wt = wild-type; Hom = homozygous; RN1734 – treated with the TRPV4 antagonist RN1734 (i.p., 4 mg/kg body weight) from post-natal day 7 to 15. If no treatment is indicated, the animals were injected with DMSO in place of RN1734. The panels represent tissues from two different animals of each genotype and treatment. The first and third rows show concurrent staining for TRPV4 (green) and nuclei (DAPI, blue). The second and third rows show the TRPV4 stain without the DAPI counterstain. Negative controls showed no non-specific labeling (data not shown).

In agreement with the qPCR results, a Western blot study indicated a lack of change in the expressed protein level of the TRPV4 in choroid plexi isolated from 15 day old rat pups. In this study 4 different animals were used for each genotype and the results were quantified using two different standards (Ponceau total protein and internal actin staining). These data have also been published (https://doi.org/10.1172/jci.insight.137646).

Taken together, the current results indicate that TRPV4 in the choroid plexus is likely not upregulated at the mRNA or protein level in day 15 homozygous pups compared to the wild-type controls. It is possible that the membrane expression of TRPV4 may be altered in the homozygous animals and in the third year of funding we will explore this using membrane surface–specific labeling techniques.

Alternative Experiments

Because we cannot perform the experiments that were proposed in the adult animals, we broadened our studies in the hydrocephalic pups to include other areas of the brain and other cells types in a larger developmental study than originally proposed. The subventricular zone is a specific area of the brain just below the ventricular lining that is a site of neurogenesis during development and, although controversial, may also be an important site for new neuron formation after injury in adults. Astrocytes are an important element in the neurogenic process and we will explore changes in TRPV in astrocytes during hydrocephalic development.

We are adding studies to examine the interactions between TRPV4 and AQP4. AQP4 and TRPV4 are both found in astrocytes as well as the ependymal cells lining the ventricle. AQP4 has been proposed to be important in osmotic regulation in the brain. In certain cases of neurological disease, AQP4 is crucial in brain waste clearance (Xu et al., 2015). AQP4 and TRPV4 interact in astrocytes to perform the RVD mechanism. RVD is important in osmotic regulation in astrocytes. We hypothesize that RVD decrease in astrocytes may play a role in the dysfunction during hydrocephalus. Preliminary data shows increased cortical expression of both TRPV4 and AQP4 in *TMEM*67 homozygous animals in comparison to wild-type (WT) (Figure 9). We hypothesize that we will see alterations in the expression of AQP4, AQP1 and TRPV4 in the *TMEM*67, *Gas8*, and other hydrocephalic models. Brain sections will be stained and co-stained for AQP4, AQP1 and TRPV4 antibodies. Images will be taken using the Keyence fluorescence microscope or upright Leica SP8 confocal microscope. Developmental stages (P0, P5, P10, P15) will be immuno-stained to observe the timeline of the hypothesized channel expression changes.

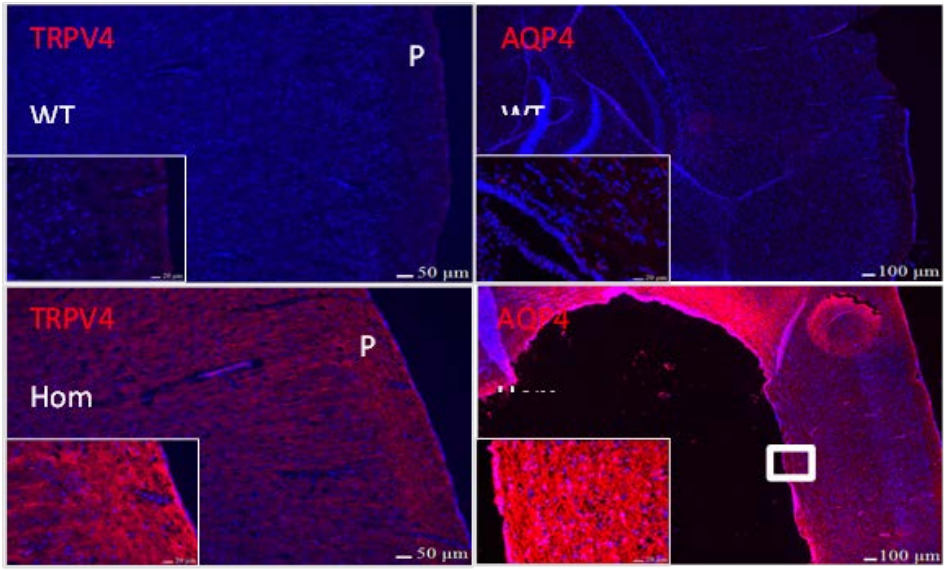


Figure 9. TRPV4 (left) and AQP4 (right) in untreated TMEM67 WT and Hom brains (15 µm) at P15. All WT and Hom upper/lower pairs were imaged at the same time and exposure. V= ventricle, P= pial surface.

Significance: The understanding of the development of hydrocephalus is key to developing drug targets. The TRPV4 antagonist treatment clearly ameliorated the hydrocephalic development in the rat model (https://doi.org/10.1172/jci.insight.137646). It was our hypothesis that the expression of TRPV4 would be increased in the hydrocephalic state in the choroid plexus epithelial cells. This does not appear to be the case in the TMEM67 rat model, despite the efficacy of the TRPV4 antagonist. Therefore, our experimental approach was altered to accommodate the new findings. We are exploring potential biochemical pathways that have been shown to alter TRPV4 activity in other tissue and, simultaneously examining other areas of the brain that express TRPV4 to asses changes in TRPV4 activity and/or expression outside of the choroid plexus.

Major Task 2: TRPV4 immunohistochemical staining of TMEM67(+/-) adult rats to determine developmental changes in expression of TRPV4 (64 animals: 2 sexes x 2 time points x 4 rats per time point x 2 (wt or hydrocephalic) x 2 (drug or vehicle).

This Major Task will not be possible because of the loss of the phenotype-genotype match in the adult animals. It will be replaced by a more in-depth analysis of other areas of the brain as discussed above.

Major Task 3: Immunohistochemical staining of Gas8^{GT} mice pups to determine developmental changes in expression of TRPV4. 160 animals (2 sexes x 5 time points x 4 mice per time point x 2 (wt or hydrocephalic) x 2 (drug or vehicle).

This is scheduled for the last year of funding. The methods will be similar to extended studies outlined above in Specific Aim 3, Subtask 1. In preparation for these studies, immunohistochemical staining of Gas8^{GT} pups has been initialized and antibodies have been validated. Initial experiments have been performed to standardize and streamline protocols. Thus far, we do not find differences in TRPV4 expression, or protein localization between the normal and hydrocephalic mice in the choroid plexus. We have not yet evaluated effect of drug treatment or other areas of the brain.

Specific Aim 4

Major Task 1: Electrophysiological analyses of ion transporters involved in the response to TRPV4 stimulation in PCP-R cell line

Explanation of electrophysiological methods used in this Specific Aim were provided in the progress report for year 1 and will not be repeated here. However, for ease of interpretation we have reiterated a few definitions:
Studies were done in a continuous porcine choroid plexus cell line, the PCP-R (porcine choroid plexus – Rheims) line and in a human choroid plexus cell line (HIBCPP)
TER = Transepithelial resistance (TER) – measured in $\Omega.cm^2$
Conductance = the inverse of the TER – widely used as a measure of transepithelial permeability.
Short-circuit current (SCC; I_{SC}) = a measurement of net transepithelial ion flux. As per convention, a positive deflection in the SCC is either anion secretion (from blood to CSF) or cation absorption (CSF to blood) and a negative deflection indicates the opposite.

Subtask 1: Analysis of Ca^{2+}-activated Cl^- channels in the PCP-R (porcine) cell line

The graphs containing the initial studies looking at the effect of inhibitors of Ca^{2+}-activated Cl^- channels were presented in the progress report for year 1 and will not be repeated here. These data have been solidified and new data added. The manuscript is being prepared for publication.. These data were also be a chapter in the Master's thesis of Daniel Preston, a named graduate student supported by the grant funding.

Significance: These data show that Ca^{2+}-activated Cl^- channels are involved in TRPV4–stimulated electrolyte flux across the porcine choroid plexus epithelial cells. In addition these channels are critical for the change in transepithelial permeability that is stimulated in response to the TRPV4 agonist.

Subtask 2 Analysis of Ca^{2+}-activated K^+ channels in the PCP-R cell line.

Between the time of submission of the grant and the beginning of the funding, we examined Ca^{2+}-activated K^+ channels in the PCP-R cell line and these findings have been published:
https://www.physiology.org/doi/abs/10.1152/ajpcell.00312.2017

Because these studies were published, we used the grant funding to extend the cell culture studies and examine the effects of inflammatory mediators on electrolyte transport in the PCP-R cell line. This was first noted in the progress report for year 1. These studies have now been completed and published https://doi.org/10.1152/ajpcell.00205.2019

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In brief, our findings have characterized the modulation of TRPV4 by various cytokines in the PCP-R cell line. The study demonstrated that select pro-inflammatory cytokines, TNF-α, IL-1β, and TFG-β1, had inhibitory effects on TRPV4-stimulated ion flux and conductance changes. Anti-inflammatory cytokines had no effect on TRPV4 activity. Contrary to published studies in other tissues, this work also demonstrated that arachidonic acid was inhibitory to TRPV4-stimulated transepithelial ion flux. Conversely, inhibition of EET production inhibited TRPV4 activity suggesting the importance of these arachidonic acid metabolites for TRPV4-mediated electrogenic ion flux. Lastly, this study showed that inhibition of transcription factor NF-kβ, through the use of an inhibitor, blocked TRPV4-stimulated activity, thereby suggesting a role for TRPV4 in cytokine production. The grant funding is acknowledged in the manuscript.

Major Task 2: Electrophysiological analyses of the ion transporters involved in the response to a TRPV4 agonist in the HIBCPP cell line

We obtained the human choroid plexus cell line (HIBCPP) cell line and established it in culture in our laboratory. In the progress report for year 1 we reported that despite considerable effort, we were unable to obtain cultures that were useable for electrophysiology and proposed doing the inflammatory studies in the PCP-R cells in place of the proposed HIBCPP cells. Although those alternative experiments were completed and published, another graduate student, Alexandra Hochstetler, decided to make additional attempts to culture the HIBCPP to the point that they could be used for electrophysiological studies. She was quite successful in this regard and has obtained preliminary data showing the effects of TRPV4 agonists in this human line.

Confluent monolayers of PCP-R cells consistently exhibit a transepithelial electrical resistance in excess of 600 $\Omega.cm^2$. In electrophysiology experiments cellular monolayers with starting TER below 500 are not used for experiments. The HIBCPP cells have a lower TER but now have a high enough starting resistance (~400 Ωcm^2) that they can accurately be used in electrophysiological experiments.

In these experiments, addition of a TRPV4 agonist causes an increase in short-circuit current in the HIBCPP cells and a decrease in the PCP-R cells. It is our hypothesis that each of these aggregate short circuit current responses results from multiple, overlapping ion transport events and this interesting difference in response suggests differences in the electrolytes that are being transported in response to TRPV4 activation in the two lines. Concurrently with the stimulation of net electrolyte flux, there is a change in permeability of the epithelial monolayer as measured by transepithelial conductance. In both cell lines, the conductance is increased although magnitude of this measured permeability change is different in the two cell lines. These studies open the way for many additional experiments aimed at characterizing the nature of the electrolyte stimulation in both tissues. These studies will continue in the next year of funding.

Specific Aim 5

Major Task 1: Obtain tissue from all three animal models, section and identify the presence and polarization of identified transport proteins. No new animals – tissue used from Specific Aim 3

We are collecting tissues during the experiments and anticipate that the studies will be conducted in the last year of funding. It is worth noting that preliminary data have also been collected regarding expression of key effectors at the mRNA levels and Western blotting.

Major Task 2: Obtain tissue from two tissue culture models, stain and identify the presence and polarization of transport proteins

These experiments will be conducted in the final year of funding.

Publications

Accepted publications

Preston, D., S. Simpson, D. Halm, A. Hochstetler, C. Schwerk, H. Schroten, B.L. Blazer-Yost, Activation of TRPV4 stimulates transepithelial ion flux in a porcine choroid plexus cell line. Am J Physiol Cell Physiol, 2018. 315(3): p. C357-C366. https://doi.org/10.1152/ajpcell.00312.2017

Simpson, S., D. Preston, C. Schwerk, H. Schroten, B.L. Blazer-Yost, Cytokine and inflammatory mediator effects on TRPV4 function in choroid plexus epithelial cells. Am J Physiol Cell Physiol, 2019. 317(5): p. C881-C893. https://doi.org/10.1152/ajpcell.00205.2019

Hochstetler, A.E., M.M. Reed, B.L. Blazer-Yost. Chapter 7: TRPV4, a Regulatory Channel in the Production of Cerebrospinal Fluid by the Choroid Plexus. In Choroid Plexus in Health and Disease. Editors: J. Praetorius, H. Damkier and B.L. Blazer-Yost, Springer. 2020

Hochstetler AE, HM Smith, DC Preston, et al., Treatment with TRPV4 Antagonists Ameliorate Ventriculomegaly in a Rat Model of Hydrocephalus. J. Clin. Invest: Insight 2020:5(18): e137646. https://doi.org/10.1172/jci.insight.137646.

Anticipated Publications

Graduate student Makenna Reed and Bonnie Blazer-Yost have been invited to write a review article on Cell Volume Regulation in Astrocytes for the Journal Cellular Physiology & Biochemistry which is publishing a Special Issue on "*Mechanisms and Functional Significance of Cell Volume Regulation*". The manuscript is due in October 2020.

Ms. Reed anticipates a first author paper, coauthored with other graduate students and Dr. Blazer-Yost describing the changes in TRPV4 and AQP4 in various brain regions during the development of hydrocephalus with and without TRPV4 antagonist treatment.

Alexandra Hochstetler, also a graduate student, anticipates a first author paper, coauthored with other graduate students and Dr. Blazer-Yost describing transepithelial ion flux in the human choroid plexus cell line in response to TRPV4 antagonists.

Alexandra Hochstetler, also anticipates a first author paper, coauthored with other graduate students and Dr. Blazer-Yost describing the backcrossing of the Gas8 mice model onto different genetic backgrounds and the resulting phenotype. She will also be treating these animals with TRPV4 antagonists and will likely submit a second paper describing these results.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

The project was not intended to provide training and professional development opportunities per se. However, it should be noted that the one-on-one training was consistently provided to the graduate students by the senior members of the experimental team and the students have excellent opportunities to present at scientific meetings. In addition, the graduate students are co-authors on all publications that have resulted from these studies.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Dr. Blazer-Yost and Ms. Reed were invited to attend a Hydrocephalus Association Workshop "Finding Common Pathways: Extending Insights from Posthemorrhagic Hydrocephalus" Nov. 4-5, 2019, in St. Louis. At this meeting Ms. Reed presented a talk about her research in the *TMEM67* rat model.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

With regard to the scientific portion of the project, it is our intent to continue to do the proposed experiments and to provide deliverables in the form of presentations and publications as outlined in this progress report and in the SOW. This also includes new research which was conceived based on evolving experimental results

With regard to plans for continued interactions with communities of interest, we will continue to work with the Hydrocephalus Association to provide information regarding our on-going research to patients and their caregivers/parents.

The COVID-19 pandemic has severely restricted our ability to attend scientific meetings in order to present our data.

We are actively seeking funding in order to continue the next phase of these pre-clinical studies with a view to advancing TRPV4 antagonists as a possible drug treatment option for multiple forms of hydrocephalus

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The team assembled for this project has made substantial progress toward proving the hypothesis that a channel protein called TRPV4 is an important regulator in the cells that produce cerebrospinal fluid. Our studies suggest that an inhibitor of this channel may be a target for drug development in the treatment of hydrocephalus in rodent models of the disease. Since there are no drugs available to treat hydrocephalus, these studies, if ultimately transferable to humans, could have a large clinical impact on disease treatment. We have prepared the first manuscript describing the pre-clinical studies in rodents and this was accepted and published in Journal of Clinical investigation: Insight. We have published two papers describing studies conducted in culture models of the cells that produce cerebrospinal fluid and a scientific review article on the subject of TRPV4 in the brain. We have been invited to submit an additional two review articles and continue to collect data for submission of multiple peer-reviewed articles from these studies.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

The changes in approach have arisen as the results of the proposed experiments were analyzed and it was determined that unanticipated findings dictate a change in experimental direction. This is usual in the normal course of scientific work and does not represent a change in the objectives or scope of the work.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

In the second year of the funding cycle, we tested animals from post-natal day 300 to 330. This was done in a blinded fashion and as part of the behavioral studies (Specific Aim #2). Unfortunately, due to the blinded nature of the studies, a large number of animals were assessed before the study was un-blinded. When the data were analyzed it was discovered that the colony no longer showed a phenotype in the adult heterozygous animals (Figure 4). We do not know what caused this change in the colony and it is rather surprising since our initial studies showed a very tight genotype-phenotype correlation.

To correct this problem we decided to re-derive the colony by backcrossing some of the adult heterozygous females with clear hydrocephalus to wild-type males and then following the fidelity of the off-spring. As estimated in the previous progress report this took approximately a year and has delayed the behavior studies (Specific Aim #2; major task 2) as well as the neurohistology studies in the adult animals (Specific Aim #2; major task 3). In the interim, we expanded our histology studies in the neonatal animals to include various parts of the brain (Specific Aim 3; major task 1) and other important protein targets.

We now have the results of the animals that were back-crossed in three separate lines. In all cases the result was the opposite from the hypothesized outcome. None of the heterozygous has a hydrocephalic phenotype. Dr. Berbari has suggested that the best course forward would be to have whole genome sequencing done on the lines that now have no phenotype compared to the original lines to determine the

modifier genes that are contributing to this change. We are in the process of colleting samples and setting up the whole genome sequencing with a core facility on our campus.

The second change in direction was also previously reported and we now have ne results to report. When we obtained the *Gas8* mouse model, we found that on the C57Bl/6J background these animals did not survive long enough to do MRIs or drug testing. Therefore we spent over a year backcrossing these onto three inbred genetic backgrounds: 129S1/SvlmJ, FVB/nJ, and BALB/cJ in an effort to reduce phenotype severity (Lee, 2013). Backcrossing is still progressing with the aim of backcrossing completely onto each background (9 generations). Preliminary data demonstrates a major improvement in the survival of the animals as well as a reduction in phenotypic severity between several backgrounds, even at only 6 generations of backcrossing (Figures 2 and 3). These data alone will serve as the foundation for a manuscript detailing the phenotypic severity of mouse models of hydrocephalus on different genetic backgrounds, and could serve as a starting point for an interesting bioinformatics project exploring the presence of various modifier genes responsible for the severity of congenital hydrocephalus in humans. In addition, these models can now be shared with other researchers in the hydrocephalus community. As part of our initial goal, we now have mice models of hydrocephalus that survive long enough for both drug and behavioral testing.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable – there are no human subjects in the proposed or conducted experiments

Significant changes in use or care of vertebrate animals

All milestones of obtaining approvals from the local IACUC as well as ACURO have been met. As the experiments progressed and changes were made as explained above, it was necessary to obtain protocol amendments. The protocol amendments were first approved by the local IACUC and then submitted to ACURO. No studies funded by the grant are initiated until ACURO approval was granted.

We have completed a new 3-year IACUC protocol for the rat studies and this has been approved by the IACUC and subsequently by ACURO. A new 3-year IACUC protocol approval for the mice work has been completed and approved by the IACUC and ACURO. This has been approved.

Significant changes in use of biohazards and/or select agents

Not applicable to these studies

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Preston, D., S. Simpson, D. Halm, A. Hochstetler, C. Schwerk, H. Schroten, B.L. Blazer-Yost, Activation of TRPV4 stimulates transepithelial ion flux in a porcine choroid plexus cell line. Am J Physiol Cell Physiol, 2018. 315(3): p. C357-C366.
  https://doi.org/10.1152/ajpcell.00312.2017

  Simpson, S., D. Preston, C. Schwerk, H. Schroten, B.L. Blazer-Yost, Cytokine and inflammatory mediator effects on TRPV4 function in choroid plexus epithelial cells. Am J Physiol Cell Physiol, 2019. 317(5): p. C881-C893.
  https://doi.org/10.1152/ajpcell.00205.2019 Federal support acknowledged.

  Hochstetler AE, HM Smith, DC Preston, et al., Treatment with TRPV4 Antagonists Ameliorate Ventriculomegaly in a Rat Model of Hydrocephalus. J. Clin. Invest: Insight 2020:5(18): e137646. https://doi.org/10.1172/jci.insight.137646. Federal support acknowledged.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Hochstetler, A.E., M.M. Reed, **B.L. Blazer-Yost.** Chapter 7: TRPV4, a Regulatory Channel in the Production of Cerebrospinal Fluid by the Choroid Plexus. In Choroid Plexus in Health and Disease. Editors: J. Praetorius, H. Damkier and B.L. Blazer-Yost.
2020. Federal support was acknowledged.

In addition, Dr. Blazer-Yost served as an editor for this book.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Unfortunately the number of presentations given by Dr. Blazer-Yost and her students was lower than in previous progress reports. We had the data but the limitations imposed by COVID-19 and the cancellation of

major conferences have limited our ability to present our work. The information below was for conferences before the COVID lockdown.

Oral Communications (no abstracts available)

Talks given by federally funded students - Federal funding acknowledged.

Underline = speaker

Reed, M., A. Hochstetler, B.L. Blazer-Yost
Role of TRPV4 in CSF Production: In Vivo and In Vitro Mechanistic Studies
Hydrocephalus Association Workshop - Driving Common Pathways, St Louis MS - Nov. 14, 2019

Hochstetler, A. B.L.Blazer-Yost.
Functional Studies of Ion Transport in Choroid Plexus Epithelial Cells. Keystone Symposia- Cerebral Fluid Flow and Function: Lymphatics, Glymphatics and the Choroid Plexus, February 17th, 2020, Santa Fe, NM, USA

Smith, H., A. Hochstetler, B.L. Blazer-Yost
SGK1 Inhibitor Treatment of Hydrocephalus in a Genetic Rat Model
10th Annual meeting of the Indiana Physiological Society, Marion University, Indianapolis, IN, March 7, 2020

Poster Presentations

Reed, M., A. Hochstetler, B.L. Blazer-Yost
Changes in Astrocytic Water Channels During Hydrocephalic Development
Keystone Symposia- Cerebral Fluid Flow and Function: Lymphatics, Glymphatics and the Choroid Plexus, Feb. 18, 2020, Santa Fe, NM

Reed, M., V. McConnell, H. Smith, B.L. Blazer-Yost, T. Belecky-Adams
Changes in Astrocytic Water Channels During Hydrocephalic Development
10th Annual meeting of the Indiana Physiological Society, Marion University, Indianapolis, IN, March 7, 2020

Hochstetler, A., D. Preston, E. Delpire, B.L. Blazer-Yost
Functional Studies of Ion Transport in Choroid Plexus Epithelial Cells
10th Annual meeting of the Indiana Physiological Society, Marion University, Indianapolis, IN, March 7, 2020

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Bonnie Blazer-Yost
Project Role: PI
No change

Name: Nick Berbari
Project Role: Co-investigator
No change

Name: Paul Territo
Project Role: Co-investigator
No change

Name: Karl Balsara
Project Role: Co-investigator
No change

Name: Amanda Riley
Project Role: Imaging tech
No change

Name: Lei Jiang
Project Role: Post-doc, imaging center
No change

Name: Scott Persohn
Project Role: Image Analyst
No change

Name: Daniel Preston
Project Role: Graduate student
Graduated during the funding year

Name: Alexandra Hochstetler
Project Role: Graduate student
No change

Name: Stefanie Simpson
Project Role: Graduate Student
Change: Ms. Simpson has graduated and is no longer on the grant

Name: Makenna Reed
Project Role: Graduate student
Change: Makenna is a new graduate student replacing Stefanie Simpson who graduated

Name: Hillary Smith
Project Role: Technician
No longer part of the project – left the end of July 2020 to go to medical school. Her salary will be used to support graduate students during the no-cost extension.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

New Awards amongst the Senior Personnel

New Awards: Bonnie Blazer-Yost
None to report

New awards: Karl Balsara
None to Report

New Awards: Nick Berbari
None to report

New Awards: Paul Territo
None to report in the current year.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

The URLs of all publications are provided above so the articles can be directly down-loaded.