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14. ABSTRACT

The proposed studies aim to test the efficacy and mechanism of action of TRPV4 antagonists for the treatment of hydrocephalus. 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 suggest that TRPV4 antagonists represent such a potential drug treatment. The proposed studies will characterize and use unique rodent models of hydrocephalus to study the efficacy of drug treatment. In addition, cultured choroid plexus (CP) cells will be used to study the mechanisms of action of the drug. In the second year we have made progress in all the proposed second year experiments listed in the SOW. Specifically we have completed the MRI studies to characterizeTRPV4 antagonist treatment in pre-weaning animals of the rat model. We have performed behavioral studies in an adult rat model of the disease. We have done pilot MRI studies in the mice models and are in the process of backcrossing the mice models onto different genetic background to lessen the severity of the disease. The choroid plexus cell line has been used to characterize inflammatory proteins involved in CSF production and these data have been accepted for publication. All of these studies will provide a deeper understanding of the function of the CP and will advance the study of potential drug treatment for hydrocephalus.

15. SUBJECT TERMS
Hydrocephalus; choroid plexus cell line; in vivo animal studies; drug development

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TABLE OF CONTENTS

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 fourth (no cost extension) 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. One of the biggest problems in this year was obtaining necessary supplies to conduct the experiments required for cultured cells and electrophysiology. For this reason alone we were unable to complete the remaining experiments. We have applied for a no-cost extension in order to complete our proposed studies in Aim 4 and 5.

A major success in this year was the successful backcrossing of two mouse models of hydrocephalus (one was originally planned) onto three alternative backgrounds to lessen the disease severity so that these models can be used for both drug testing and behavioral studies. The results of these studies will be published in the near future and will provide the research community with additional hydrocephalic models. Not part of the original proposal, but an important addition to this work, is that we have obtained 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 - 9/30/21

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

PI: Blazer-Yost

Key:
Yellow completed
Pink started
Blue – Unanticipated research results indicate alternative experiments necessary. Experiments conducted.

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	27	Drs. Territo/Blazer-

rats completed and data summarized for publication		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 transporters involved in the response to TRPV4 stimulation in PCP-R cell line	Month	
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?

The data presented below are correlated with the schedule provided in the SOW above and represent work done in year 3-4 of funding. The work completed in years 1, 2 and 3 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 .

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.

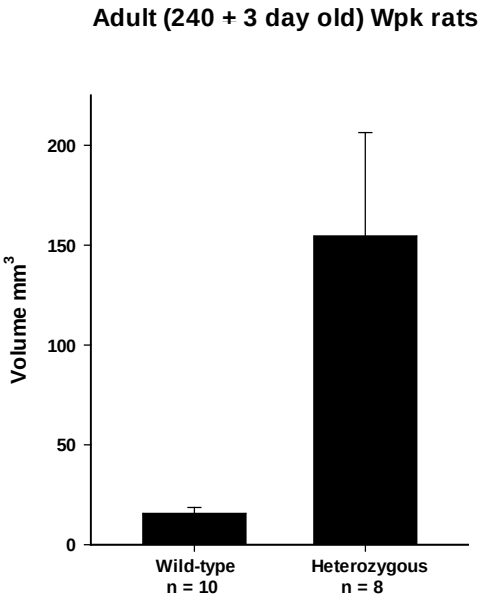

Figure 1: MRI quantitation of lateral ventricle volumes of wild type and heterozygous and TMEM67 rats at post natal day 240 (+/- 3 days). The number of animals used in each genotype is indicated by the n number at the base of the columns. The values are averages +/- SEM.

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 used in the proposed behavioral studies and were

scanned by MRI as proposed 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.

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.

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.

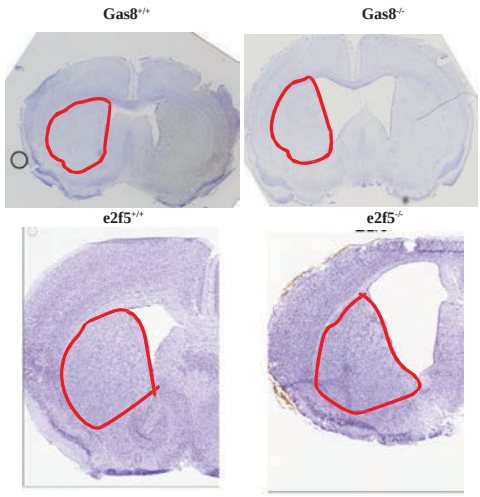

Figure 2. Nissl stain of hydrocephalic mouse lines. WT or Gas8$^{-/-}$ mice on BALB/cJ background (Top) or WT or e2f5$^{-/-}$ mice on 129S/1J background (bottom) euthanized at 6 or 4 weeks of age, respectively, sliced on a cryostat, and stained with a Nissl stain, mounted, and imaged. The striatum from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

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 were initiated in the third year and completed in the fourth year. 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.

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

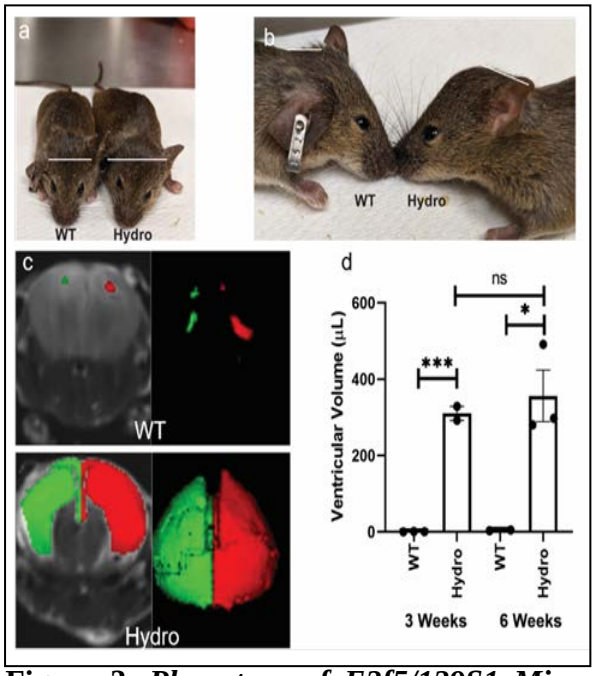

Figure 3. ***Phenotype of E2f5/129S1 Mice with Hydrocephalus.*** (a), (b): Macrocephaly and phenotype of cranial doming in 6-week old *E2f5/129S1* mice. (c) 3D rendering of lateral ventricle volume as assessed by MR in 6 week old *E2f5/129S1* mice compared to a wild-type (WT) littermate. The red and green coloring denote the right and left ventricles. (d) Quantitation of lateral ventricular volume at age 3 weeks, which persists until 6 weeks of age.

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, in year 4, finished backcrossing onto the same three genetic backgrounds as the *Gas8* model above. An example of the *E2f5* mouse models on a 129S1 background is shown in figure 3. Table 1 contains a summary of the models which we have produced and characterized as part of the funding.

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)

We have tested TRPV4 antagonists for varying periods of times during development in the less severe *Gas8* and *E2f5* mouse models of hydrocephalus. Unfortunately, none of the models responded to the treatment so this aim could not be completed as proposed. Therefore, it was incumbent on us to determine whether TRPV4 is important in the mouse models. A *Trpv4* null mouse model was acquired and correct genotyping method delineated. This colony is now maintained utilizing heterozygous breeders (as opposed to historically inbreeding mutants/nulls) and this colony is being bred with *Gas8*$^{+/-}$/B6 and *E2f5*$^{+/-}$/B6 to generate double knockouts. On a B6 background, *E2f5*$^{-/-}$ mice generally survive 2-3 weeks of age. Currently, an *E2f5*$^{-/-}$|*Trpv4*$^{-/-}$ has lived for 12 weeks, and another *E2f5*$^{-/-}$|*Trpv4*$^{+/-}$ was just weaned and appears to be normal body weight though with noticeable cranial doming. These preliminary studies indicate that although the TRPV4 antagonist treatment was not effective in the mouse models, TRPV4 is an important component of the hydrocephalic development in the models.

Type of Hydrocephalus	**Model and species**	**Optimal Age for Treatment**	**Highlights of the Model**
Ciliary transport mutant (*Genetic*)	***Tmem67*** $^{-/-}$ **postnatal rat**	**Post-natal days (P) 7 to 15**	**TRPV4 antagonists are effective in ameliorating hydrocephalus (published)**
CSF hypersecretion *(Genetic)*	***E2f5***$^{-/-}$**; mouse**	**P14 – P21 FVB and 129S1 backgrounds P21-P42+ Balbc background**	**<u>Phenotype varies with background strain</u>: Moderate: survival >8 weeks of age – long-term treatment Severe: survival up to 4- 6 weeks of age – short-term treatment studies Very severe: survival ≤14 days of age – use for genetic studies**
Ciliary transport mutant *(Genetic)*	***Gas 8***$^{-/-}$ **mouse**		

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.

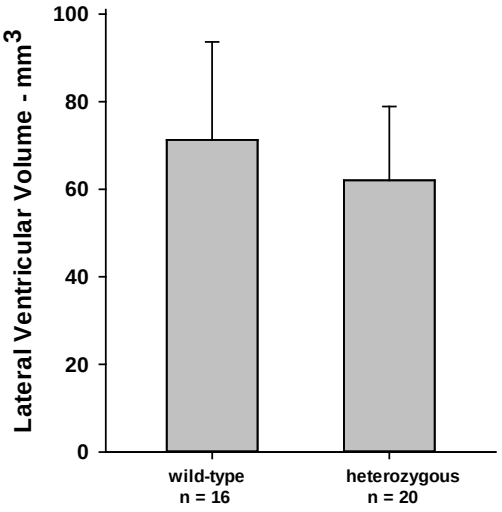

Figure 4: MRI quantitation of lateral ventricle volumes of wild type and heterozygous and TMEM67 rats at post natal day 330 (+/- 3 days). The number of animals used in each genotype is indicated by the n number at the base of the columns. The values are averages +/- SEM.

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.

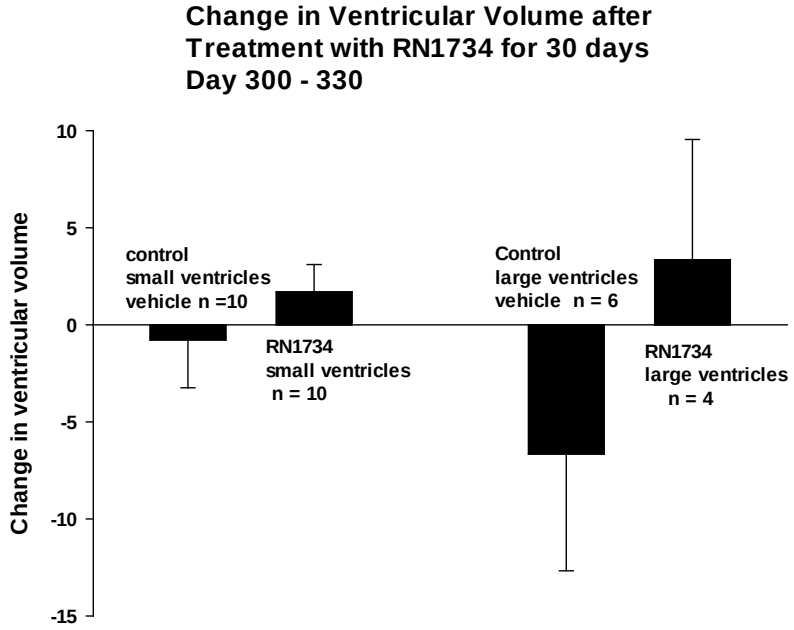

Figure 5: MRI quantitation of lateral ventricle volumes of adult wild type and heterozygous TMEM67 rats. RN1734 (4 mg/kg body weight) or vehicle (DMSO) were injected i.p. daily from post-natal day 300 to 330. The number of animals used in each treatment group is indicated by the n number at the base 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.

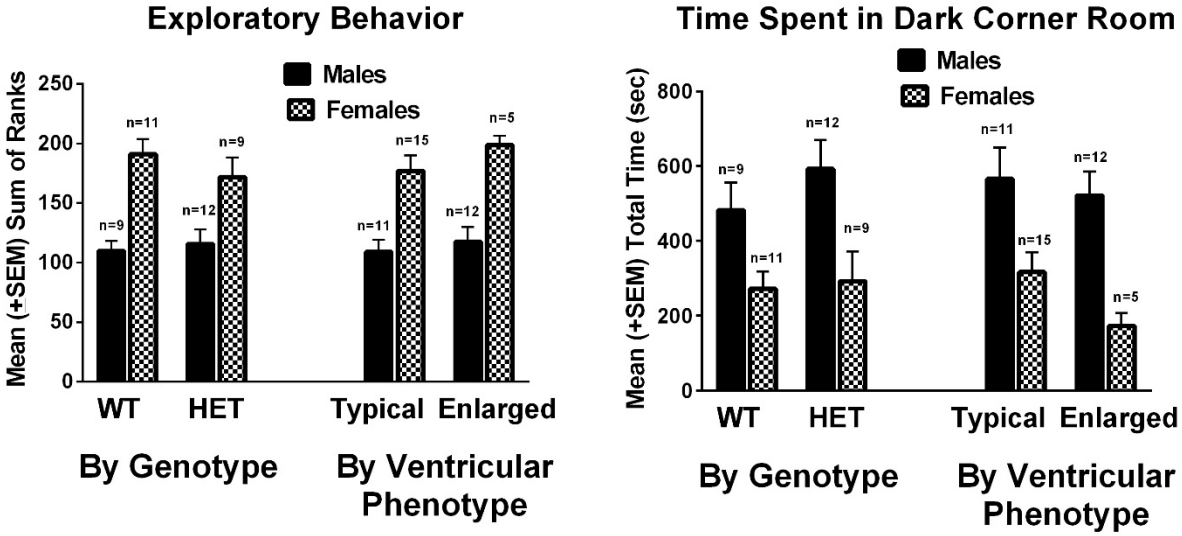

Figure 6: Analysis of Exploratory Behavior and Time Spent in Dark Corner Room in adult TMEM67 rats. The data for these two behavioral studies are expressed as differences as a function of genotype (wild-type or heterozygous) and as ventricular phenotype: typical (<45mm^3) and enlarged (>45mm^3) ventricle 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.

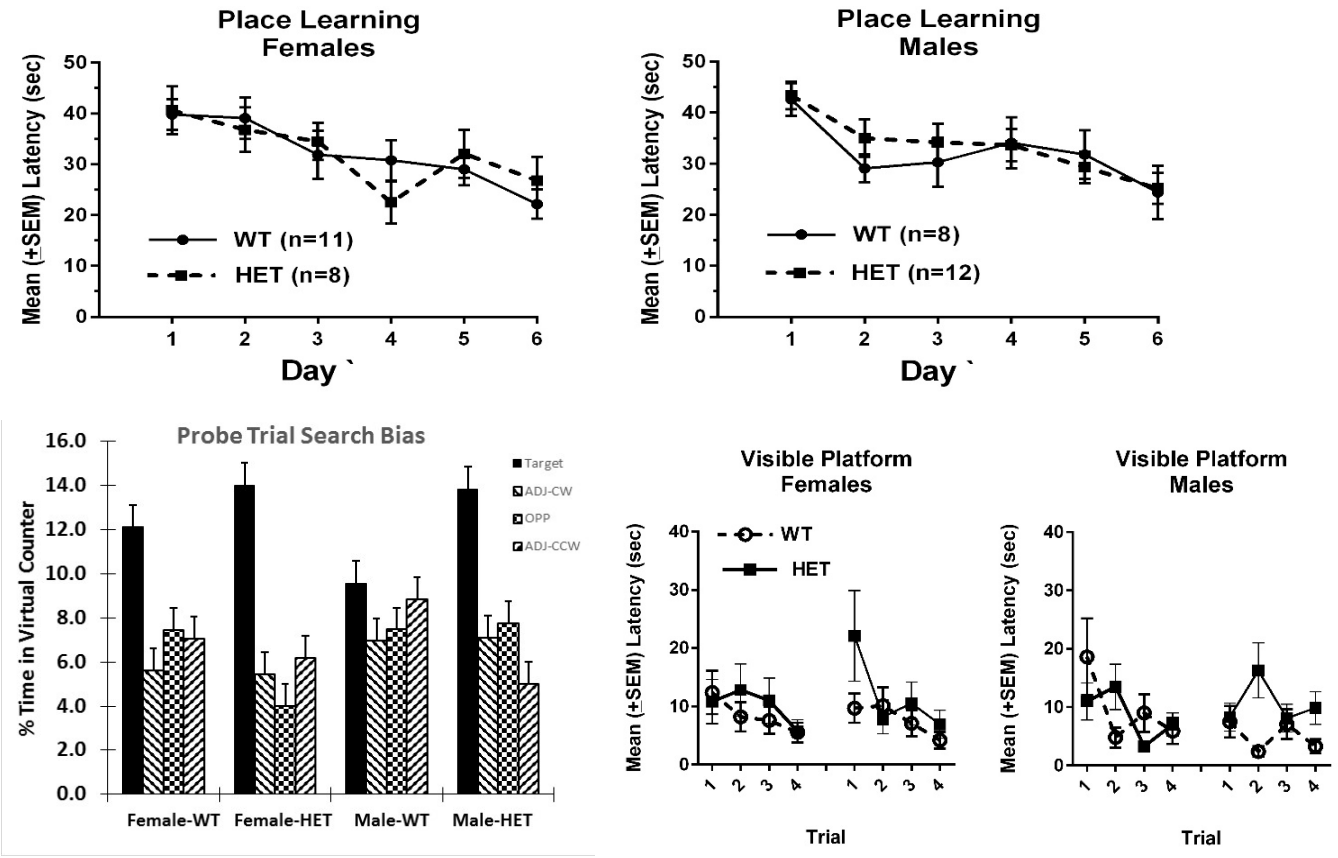

Figure 7. Morris Water Maze Performance.

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 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. 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 P15 animals (wt or homozygous) were stained and imaged in tandem. In the choroid plexus epithelial cells, the subcellular distribution of TRPV4 switches from an intracellular localization to the apical membrane in hydrocephalus. Interestingly this is accompanied by a re-localization and/or over expression of AQP1. One thing that is clear from Figure 8 is that regardless of genotype, 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.

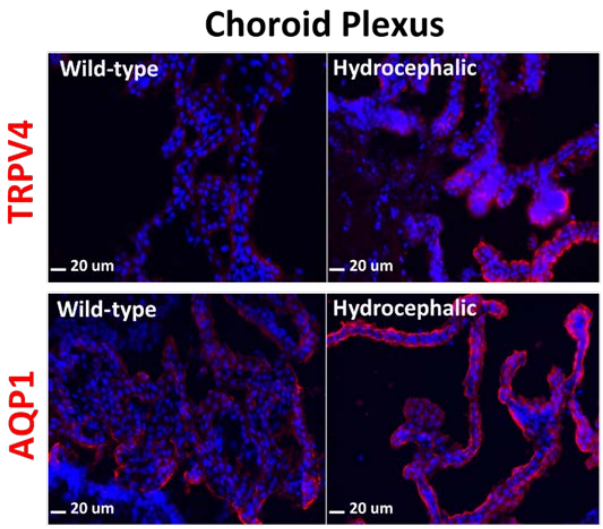

Figure 8: **Immunohistochemistry of TRPV4 and AQP1 in choroid plexus.** Images from postnatal day 15 wildtype and *Tmem*67 homozygous (hydrocephalic) rats with TRPV4 (top) and AQP1 (bottom). Immunostaining shown in red; nuclei are stained with DAPI (blue).

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. 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).

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

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 cell 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. In addition, 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).. Recent reports in the literature suggest a functional interaction between TRPV4 and Aquaporin (AQP) water channels. It is likely that TRPV4 will have effects in the glymphatic system via interactions with these important transport proteins. Our preliminary data indicates an upregulation of both TRPV4 and AQP4 in the brain parenchyma of the *Tmem67* hydrocephalus model compared to wild-type littermates (Figure 9).

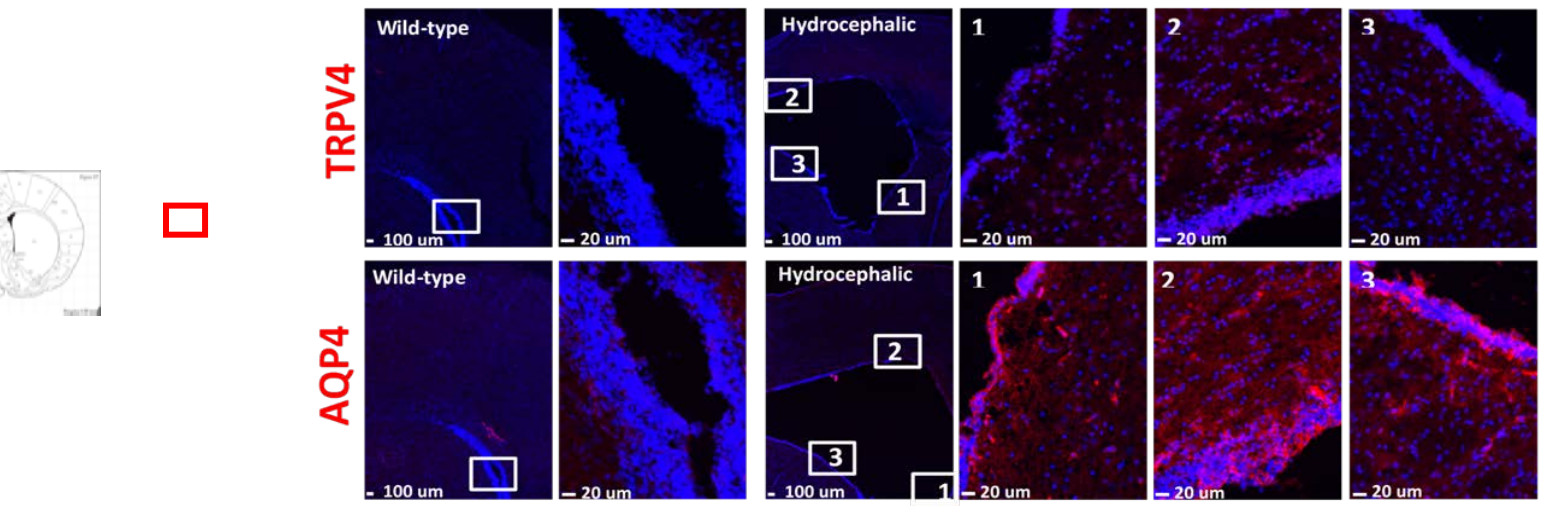

Figure 9. Sections from postnatal day 15 wildtype and *Tmem*67-/- (Hydrocephalic) rats immunostained with TRPV4 (top) and AQP4 (bottom). Ventricular surface images at 4X (columns 1 and 3) and 40X (columns 2,4,5,6) magnification. The approximate area of the images is shown by the red box in the brain atlas image of the ventricular system on the left. All images were taken at the same exposure using a Keyence microscope. Antibodies against AQP4 and TRPV4 are in red, and nuclei are DAPI stained (blue).

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 assess 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 was 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).

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 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 (Figure 10).

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.

The proposed experiments will now be conducted. However, it was not possible to complete these experiments during year 4 because we were unable to obtain the Transwell supports that are necessary to grow in cells for electrophysiological experiments. The Transwells have been ordered from several companies and all are back-ordered for over 6 months. We assume this is a supply chain issue stemming from the COVID pandemic. Therefore, we have requested a second no-cost extension to complete those experiments when the Transwells become available.

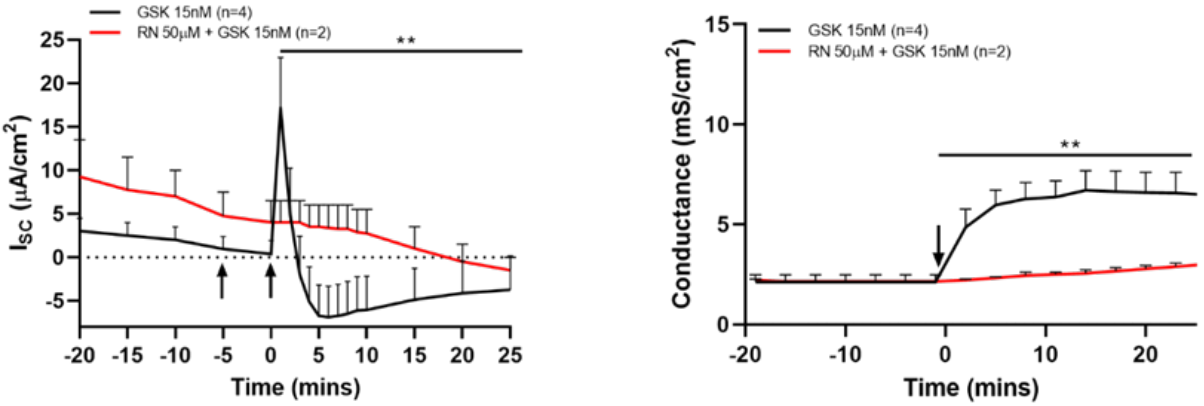

Figure 10. Effect of a TRPV4 agonist on net transepithelial ion flux and conductance in HICBPP, a human choroid plexus cell-line. Addition of GSK1016790A, a TRPV4 agonist (added at t=0), resulted in a change in net transepithelial ion flux (short circuit current, Isc, left), and an increase in transepithelial conductance, a measure of permeability across the cell monolayer (right). Pre-incubation for 10 minutes with RN1734, a TRPV4 specific antagonist (red line), inhibited this response, suggesting that the response is specific to TRPV4. *Statistically significant differences in the slope of the line between condition and GSK control, as measured by Student's *t*-test, paired data.

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

The material from the animals has been collected.

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 when the Transwells are available and we can grow the human cells for comparison.

Publications

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.

Submitted Publications

Reed, MM, BL Blazer-Yost Channels and Transporters in Astrocyte Volume Regulation in Health and Disease. In revision. J. Cell. Physiol. Biochem. Special Issue on "*Mechanisms and Functional Significance of Cell Volume Regulation"*

Anticipated Publications

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 was asked to give the Keynote address at the annual meeting of the Indiana Physiological Society meeting, March 7, 2020. Both Dr. Blazer-Yost and her graduate students had multiple oral presentations and posters at local, national and international meetings and these are listed in a separate section below.

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 complete 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.

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 published the first manuscript describing the pre-clinical studies 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 and we will be unable to complete the experiments.

The second change in direction was also previously reported and we now have new 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 approximately 18 months backcrossing these onto three inbred genetic backgrounds: 129S1/SvlmJ, FVB/nJ, and BALB/cJ in an effort to reduce phenotype severity (Lee, 2013). Backcrossing was conducted for 9 generations. This was remarkably successful and we now have both the *Gas8* model and a second mouse model, *E2f5*, that we added to compensate for some of the experiments we could not perform. Both models on the three backgrounds live for 4+ weeks (table 1) The 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.*

Dr. Blazer-Yost and her students embraced the virtual conference format and attended, and presented at, several national and international conferences during the pandemic isolation period.

Oral Communications (no abstracts available)

Talks given by federally funded students - Federal funding acknowledged.

Underline = speaker

Hochstetler AE, L Hulme, BL Blazer-Yost. Modulation of cerebrospinal fluid production by the Transient Receptor Potential Vanilloid 4 channel: Implications for hydrocephalus and other disorders of brain fluid volume. Virtual meeting - Future Physiology (U.K.) April 21, 2021

Hochstetler AE, L Hulme, BL Blazer-Yost. Modulation of cerebrospinal fluid production by the Transient Receptor Potential Vanilloid 4 channel: Implications for hydrocephalus and other disorders of brain fluid volume. Virtual presentation Experimental Biology Annual Meeting April 27, 2021

Reed MM, BL Blazer-Yost. Changes in TRPV4, AQP1, and AQP4 in a Genetic Model of Hydrocephalus. Lake Cumberland Biological Transport Annual Meeting in person. June 22, 2021.

Hochstetler AE, BL Blazer-Yost. Serum- and Glucocorticoid- Induced Kinase 1 Modulates Cerebrospinal Fluid Secretion at the Choroid Plexus. Lake Cumberland Biological Transport Annual Meeting – in person. June 22, 2021.

Hulme L, BL Blazer-Yost. Investigation into the TRPV4-SGK1 signaling axis in *in vitro* choroid plexus models. Lake Cumberland Biological Transport Annual Meeting – in person. June 22, 2021.

Poster Presentations

Reed MM, BL Blazer-Yost. Alterations in TRPV4, AQP1 and AQP4 in a genetic model of hydrocephalus. Annual Indiana Physiological Society Conference. Virtual March 7, 2021

Hulme L, AE Hochstetler, BL Blazer-Yost. Serum and glucocorticoid-inducible kinase 1 (SGK1) is involved in transepithelial ion flux in the choroid plexus epithelium. Greater Indianapolis Society for Neuroscience, Virtual April 9, 2021

Reed MM, BL Blazer-Yost. Alterations in TRPV4, AQP1 and AQP4 in a genetic model of hydrocephalus. Greater Indianapolis Society for Neuroscience, Virtual April 9, 2021

Hochstetler AE, L. Hulme, BL Blazer-Yost. Modulation of cerebrospinal fluid production by the Transient Receptor Potential Vanilloid 4 channel: Implications for hydrocephalus and other disorders of brain fluid volume. Greater Indianapolis Society for Neuroscience, Virtual April 9, 2021

Reed MM, BL Blazer-Yost. Alterations in TRPV4, AQP1 and AQP4 in a genetic model of hydrocephalus. Future Physiology (U.K.) April 21, 2021

Hulme L, AE Hochstetler, BL Blazer-Yost. Serum and glucocorticoid-inducible kinase 1 (SGK1) is involved in transepithelial ion flux in the choroid plexus epithelium. Virtual presentation Experimental Biology Annual Meeting. April 27, 2021

Reed MM, BL Blazer-Yost. Alterations in TRPV4, AQP1 and AQP4 in a genetic model of hydrocephalus. Virtual presentation Experimental Biology Annual Meeting. April 27, 2021

- **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

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: Alexandra Hochstetler
Project Role: Graduate student
No change

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

Name: Louise Hulme
Project Role: Graduate Student
Change: Louise replaced Stefanie Simpson who graduated.

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

7.

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.*

8. 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.