

AWARD NUMBER: W81XWH-17-1-0537

TITLE: Development of Pharmacotherapies for the Treatment of Hydrocephalus

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REPORT DATE: Sept 2019

TYPE OF REPORT: Annual

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

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE Sept. 2019		2. REPORT TYPE Annual		3. DATES COVERED 1 Sep 2018 - 31 Aug 2019	
4. TITLE AND SUBTITLE Development of Pharmacotherapies for the Treatment of Hydrocephalus				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-17-1-0537	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bonnie L. Blazer-Yost E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Indiana University Trustees of Indiana University 980 Indiana Ave. RM2232 Indianapolis, IN 46202				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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. 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 characterize TRPV4 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					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER (include area code)

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Please note: In the rest of the document, some of the 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 suggest 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 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 characterize TRPV4 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 for more effective drug testing. 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.

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

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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

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

PI: Blazer-Yost

Key:

Yellow completed

Pink started

Green – 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 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 <i>in vivo</i> and <i>in vitro</i>	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 2 of funding. The work completed in year 1 was summarized in the first year progress report.

Specific Aim #1

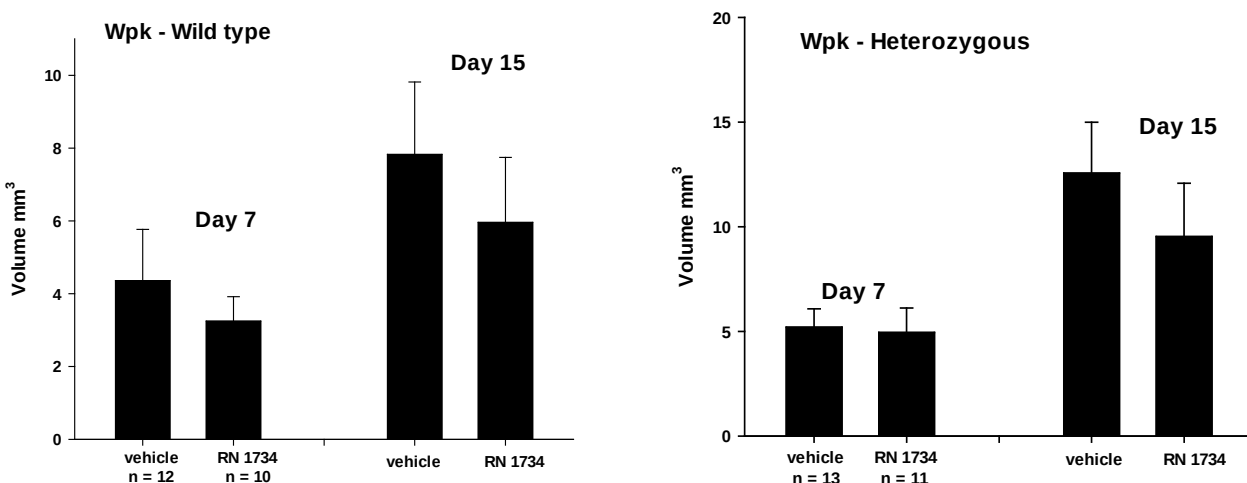
Major Task #1: Start and maintain the breeding colony for the TMEM(-/-) and TMEM (+/-) rats.

All milestones of obtaining approvals from the local IACUC as well as ACURO have been met. 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. A new 3-year IACUC protocol approval for the mice studies has been completed and approved by the IACUC and will be submitted to ACURO in the month of October, 2019.

The paired matings are on-going and will continue for all 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)

The studies outlined for subtask 1 and 2 have been 95% completed – the treatments for the last MRI studies are underway and will be completed by the end of September 2019. The slight delay was caused by major down-time of the MRI equipment for repair and update. In Figure 1 below, we summarize all of the current MRI data that is being prepared for publication. In the subsequent figures (Figures 3 and 4), we summarize associated data that will be part of the publication.



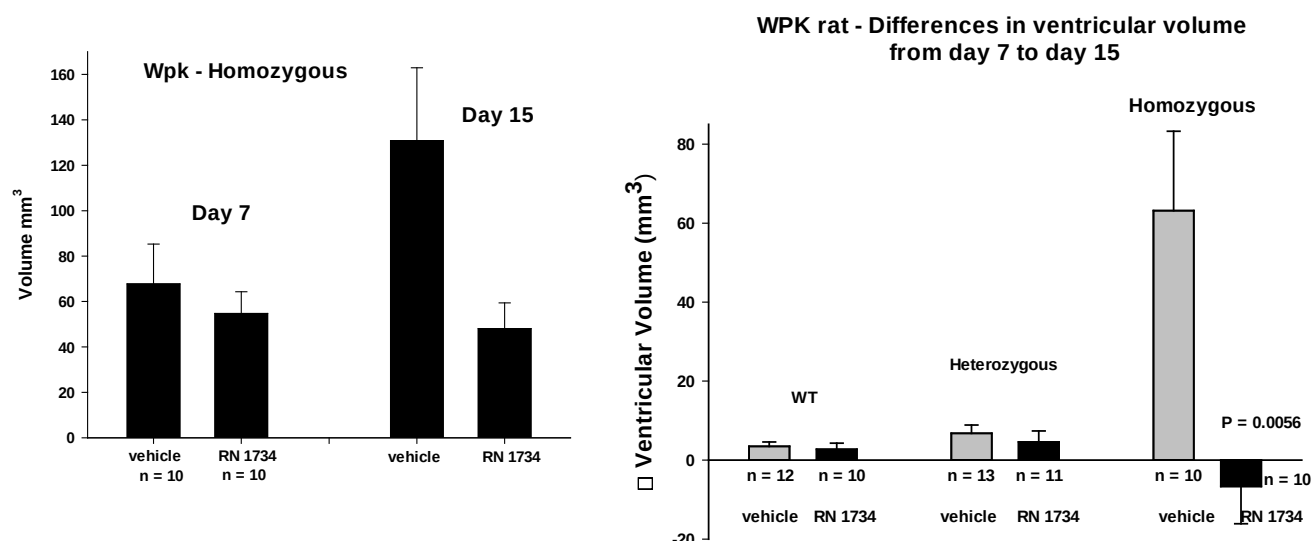


Figure 1: MRI quantitation of lateral ventricle volumes of wild type, heterozygous and homozygous TMEM67 rats at post natal days 7 and 15. The first three bar graphs represent the average values for ventricular volumes of each of the genotypes at the indicated post-natal days. RN1734 is a TRPV4 antagonist that was injected i.p. daily at a concentration of 4 mg/kg body weight from post-natal day 7 to 14. The number of animals used in each treatment group is indicated by the n number at the base of the first set of columns. The fourth graph shows the difference in ventricular volume between day 7 and day 15 for each of the genotypes. In the homozygous animals, treatment with the TRPV4 antagonist significantly reduced the increase in ventricular volume as measured a Students T test. P values of the statistically different values are indicated on the graphs.

As per our proposed studies, we have performed sufficient experiments to examine potential differences in drug responses with respect to sex of the animals (Figure 2). The final experiments (to be completed September 2019) will add to the number of homozygous animals, particularly the drug-treated females. Even with the additional animals we do not anticipate that there will be a difference in the response to drugs that can be attributed to the sex of the animals.

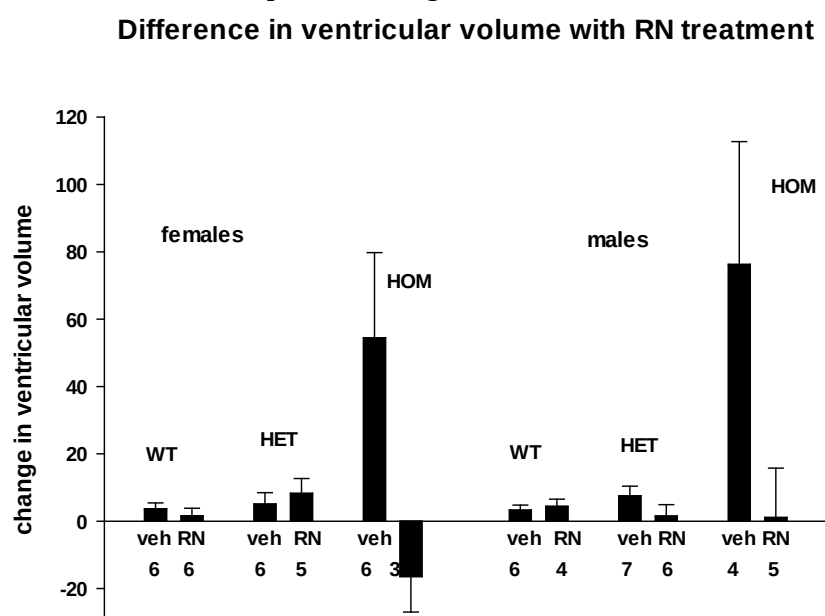


Figure 2: MRI quantitation of lateral ventricle volumes of female and male wild type, heterozygous and homozygous TMEM67 rats. RN1734 was injected i.p. daily at a concentration of 4 mg/kg body weight from post-natal day 7 to 14. The number of animals used in each treatment group is indicated by the n number at the base of the columns. The graph shows the difference in ventricular volume between day 7 and day 15 for each of the genotypes and sexes.

Adverse reactions to chronic (7 day) drug treatment are often manifested as a loss in body weight, therefore, all of the animals were weighted at the end of the MRI study shown in Figures 1 and 2 (Figure 3). As expected the homozygous animals has a lower body weight than the other two genotypes at the beginning and end of the treatment period because of the severity of their disease. The results indicate that there were no adverse reactions to the drug based on genotype or sex.

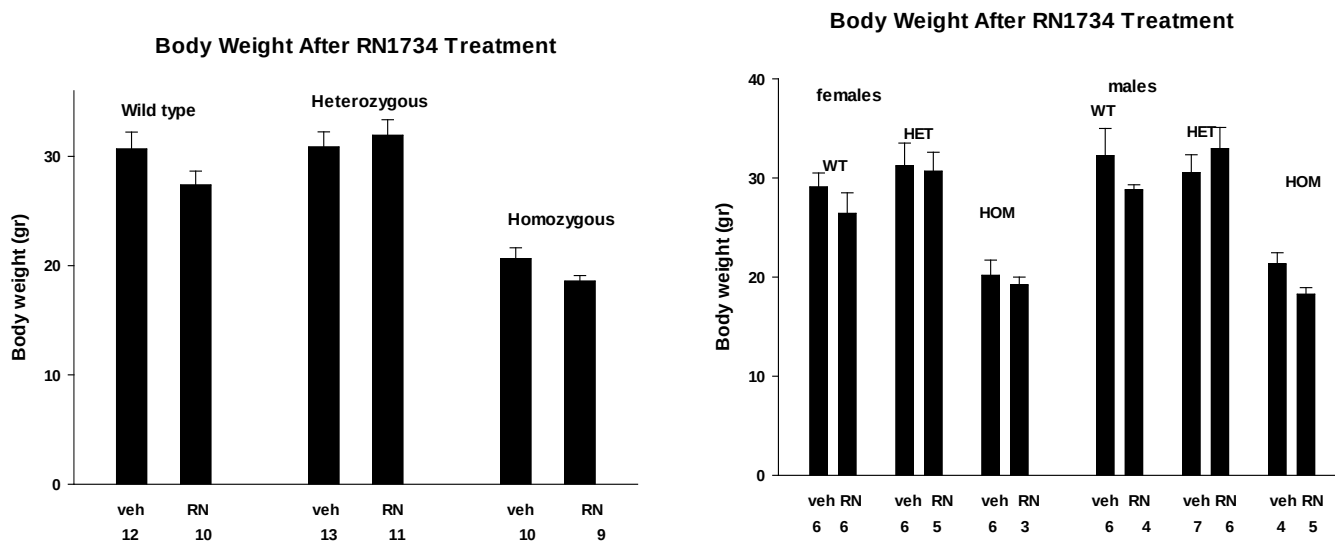


Figure 3. Body weights of female and male wild type, heterozygous and homozygous TMEM67 rats which were part of the MRI study shown in figures 1 and 2. RN1734 was injected i.p. daily at a concentration of 4 mg/kg body weight from post-natal day 7 to 14. The number of animals used in each treatment group is indicated by the n number at the base of the columns. The graph shows the difference in body weight between day 7 and day 15 for each of the genotypes (first figure) and each of the genotypes separated by the sex of the animals (second figure).

Kidney and brain weights were obtained for the animals in the majority of the studies (Figure 4). Interestingly, when considering all the treated animals, the RN1734 drug treatment substantially lowered the brain weights in the homozygous animals. In this rat model, the homozygous animals have polycystic kidney disease as evidenced by the severe increase in kidney weight. This is quite evident in the homozygous animals and was not statistically changed by treatment with the TRPV4 antagonist.

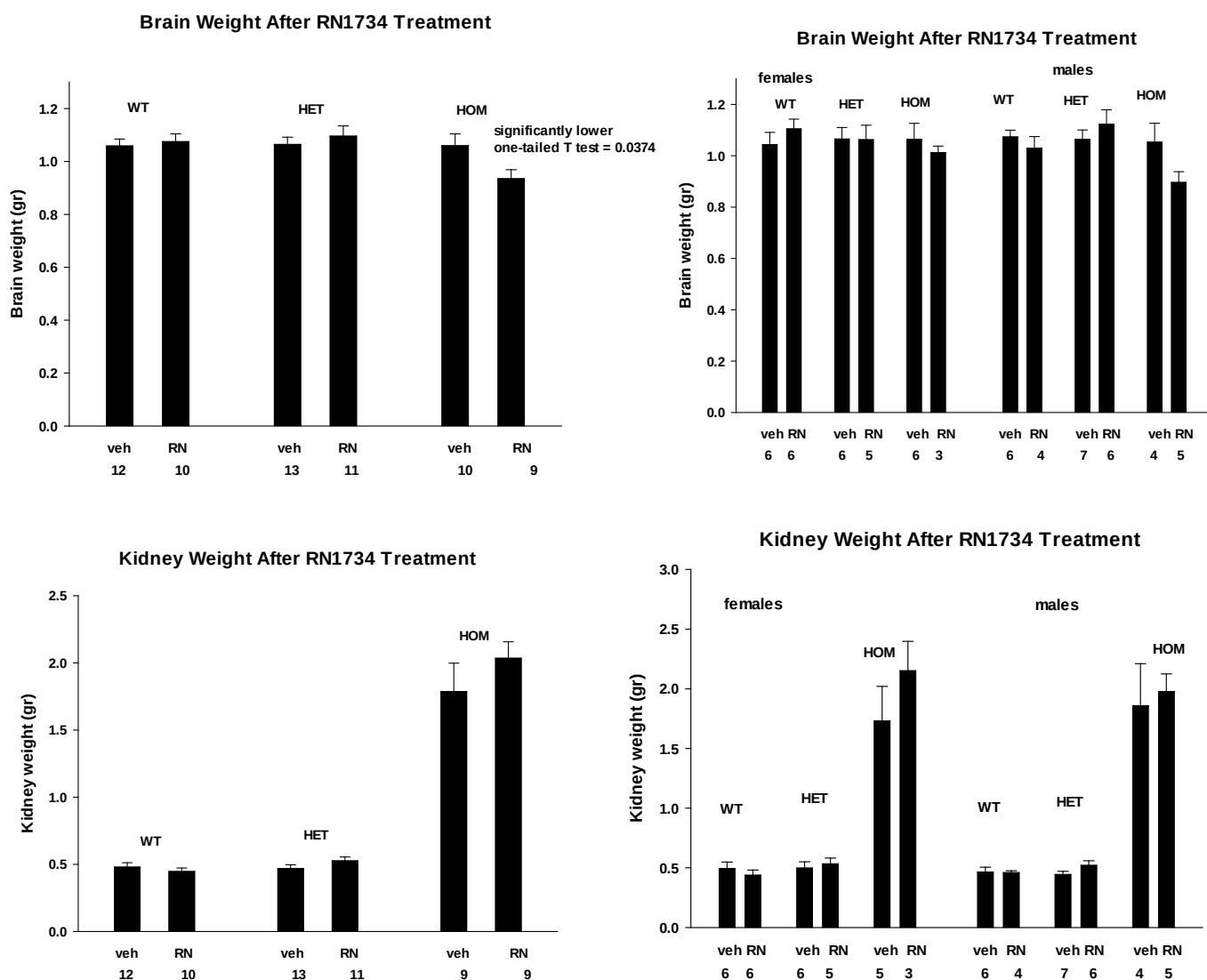


Figure 4. Brain and kidney weights of female and male wild type, heterozygous and homozygous TMEM67 rats which were part of the MRI study shown in figures 1 and 2. RN1734 was injected i.p. daily at a concentration of 4 mg/kg body weight from post-natal day 7 to 14. The number of animals used in each treatment group is indicated by the n number at the base of the columns. The graph shows the difference in both brain and body weight between day 7 and day 15 for each of the genotypes (first figure) and each of the genotypes separated by the sex of the animals (second figure).

Significance: These results further underscore and extend the preliminary data that was presented in the initial grant application and represent the most exciting findings to date. The MRIs provide a quantitative measurement of changes in ventricular volume. The results show that in this model of hydrocephalus, the TRV4 antagonist mitigates the hydrocephalic development. The accompanying data are useful for the manuscript which is being prepared for publication.

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 5 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.

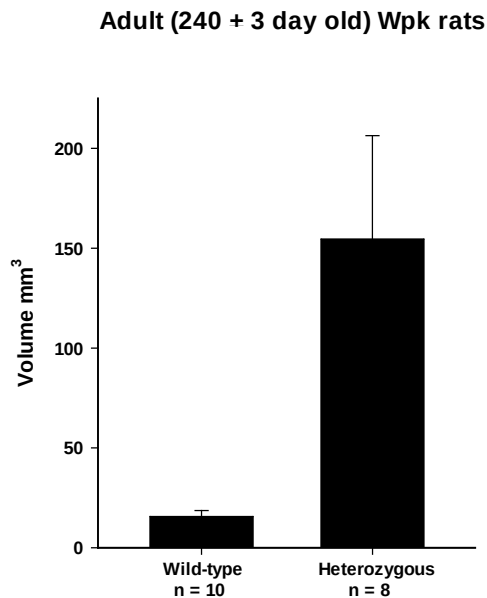


Figure 5: MRI quantitation of lateral ventricle volumes of wild type and heterozygous and TMEM67 rats at post natal day 240 (+/- 3 days). The number of animals used in each genotype is indicated by the n number at the base of the columns. The values are averages +/- SEM.

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 6). 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. Please see Specific Aim #2 for more

data and interpretation of the behavioral studies

To correct this serious problem we are re-deriving 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. This will take some time and has delayed this aim as well as the behavioral studies in Specific Aim 2.

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.

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 have decided to cross the *GAS8* mouse onto a 129S1/SvImJ (Stock #002448) background (their original background) and also to two other backgrounds (BALB/cJ (Stock #000651) and FVB/NJ (Stock #001800)). These studies are underway and some are currently in generation 6 (of 9).

Thus far we have the following results from preliminary sibling matings at intermediate generations:

Mouse crossing	Generation for testing of sibling pairs	Age at sacrifice due to severe illness
<i>GAS8/ BALB/cJ</i>	5th	6 weeks
<i>GAS8/129S1/SvImJ</i>	5th	3 weeks
<i>GAS8/ FVB/NJ</i>	4th	5 weeks

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 Ferretti (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 cytoplasmically, 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 have 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/SvImJ*) and 5 (*E2F5/FVB/NJ*) indicate that the survival times are going to be very similar to the *GAS8* mice on these backgrounds.

Because these studies have been initiated early, we anticipate that we have sufficient time perform the back-crossings and still finish the proposed experiments before the end of the third year 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)

It is our plan to do some preliminary MRIs on generation 6 crossings to determine if the hydrocephalic phenotype is manifested in all the crossings with a high fidelity. 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 third year of funding.

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 now limits 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 6). 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 6, there is substantial variability in the results. For this reason, Major Task 1 will be suspended until the results of backcrossing to attempt to recreate the original genotype/phenotype has been completed.

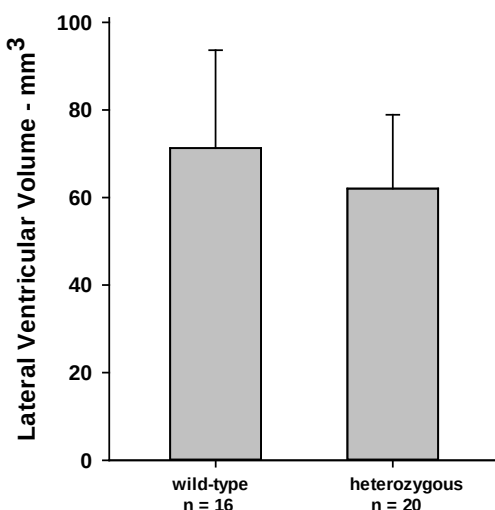


Figure 6: MRI quantitation of lateral ventricle volumes of wild type and heterozygous and TMEM67 rats at post natal day 330 (+/- 3 days). The number of animals used in each genotype is indicated by the n number at the base of the columns. The values are averages +/- SEM.

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. Those findings with adult rats in the TMEM67(+/-) heterozygous model of slowly developing hydrocephalus indicated that the emergence of a stable hydrocephalic phenotype was not evident until at least 10 months of age. Consequently, the studies of Aim 2 started with animals at 10 months of age (rather than 8 as initially planned).

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 6), 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 ($<45\text{mm}^3$) or large ($>45\text{mm}^3$) ventricles. The ventriculomegaly 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 7 shows the effect of drug treatment on the change in ventricular volume in adult animals with small ($<45\text{mm}^3$) and large ($>45\text{mm}^3$) ventricles (Figure 7). As can be seen from the figure, RN1734 did not decrease ventricular size in either cohort.

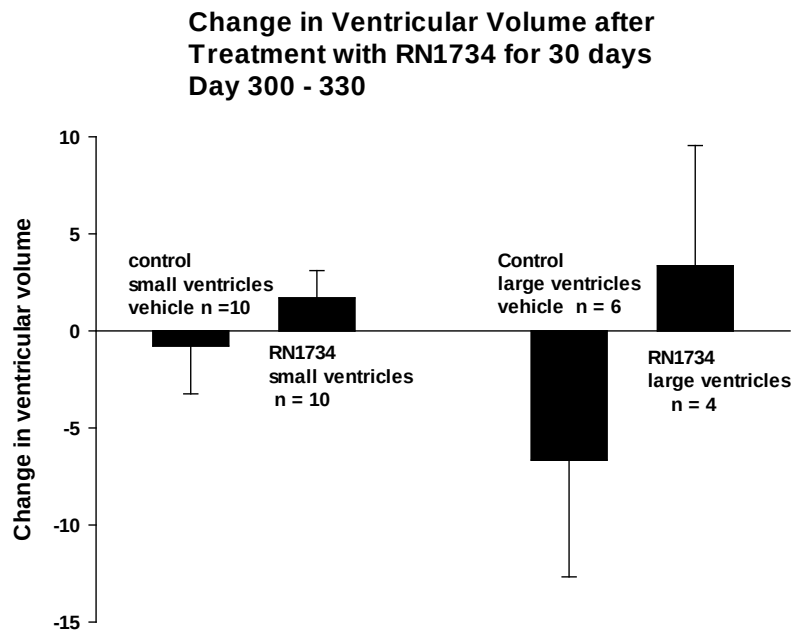


Figure 7: MRI quantitation of lateral ventricle volumes of adult wild type and heterozygous TMEM67 rats. RN1734 (4 mg/kg body weight) or vehicle (DMSO) were injected i.p. daily from post-natal day 300 to 330. The number of animals used in each treatment group is indicated by the n number at the base 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 ($<45\text{mm}^3$) and large ($>45\text{mm}^3$) 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 8 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.

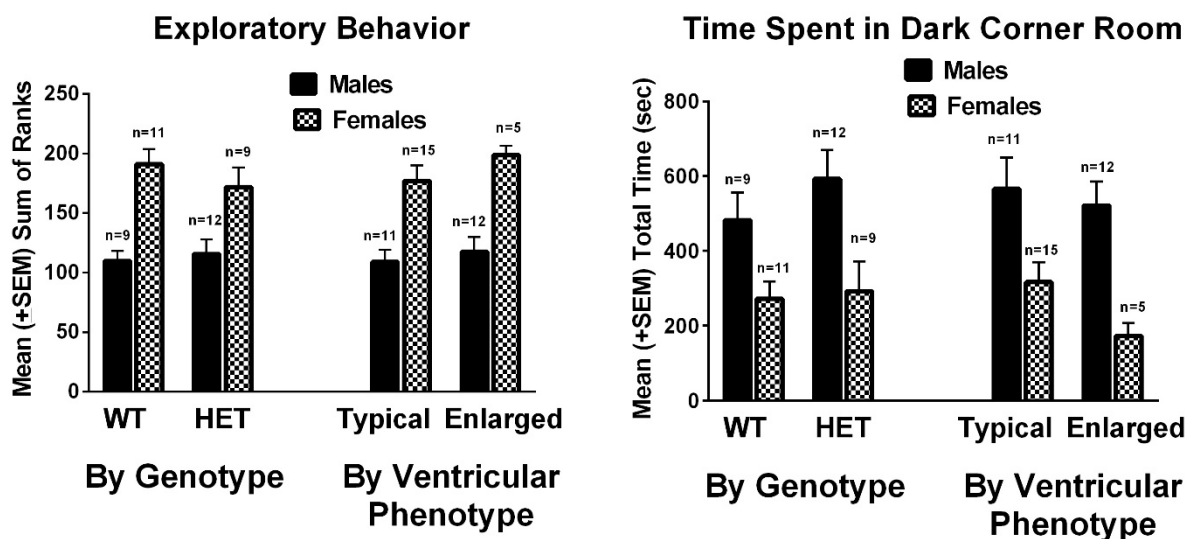


Figure 8: Analysis of Exploratory Behavior and Time Spent in Dark Corner Room in adult TMEM67 rats. The data for these two behavioral studies are expressed as differences as a function of genotype (wild-type or heterozygous) and as ventricular phenotype: typical ($<45\text{mm}^3$) and enlarged ($>45\text{mm}^3$) 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.

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.

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 ($DI=0.0827 \pm .1007$), but WT and HETs did not differ from each other.

For the Morris Water maze, Figure 9 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.

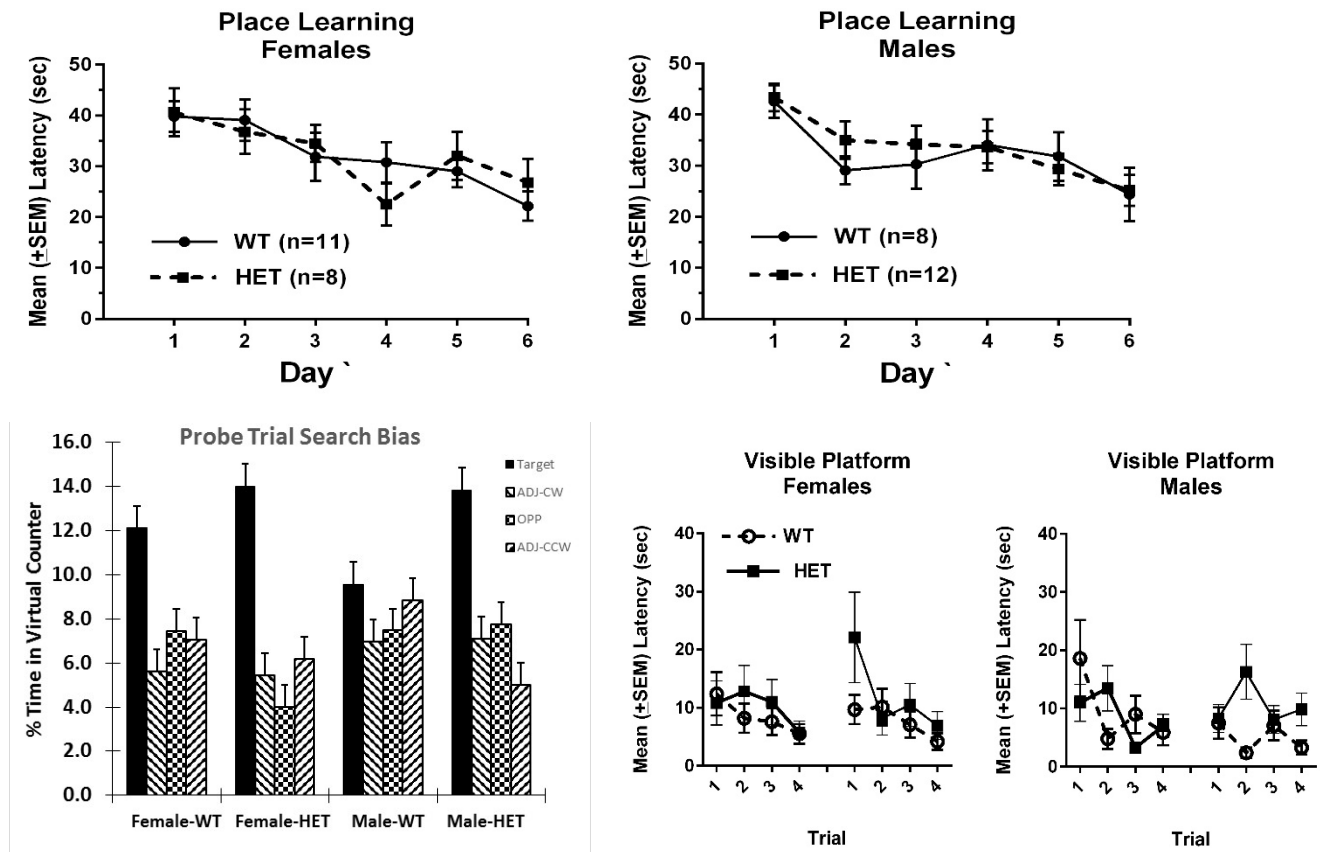


Figure 9. Morris Water Maze Performance.

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 difficulty switching from use of place strategies to visually-cued strategies, or that visual impairment associated 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 pending further testing of the genotype-phenotype correlation in the animals that are being backcrossed to recreate the original observations.

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 be completed as originally proposed so the personnel were reassigned to broaden the developmental studies.

Our preliminary immunohistochemical studies (grant application) indicated that there is 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 10. 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 10 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.

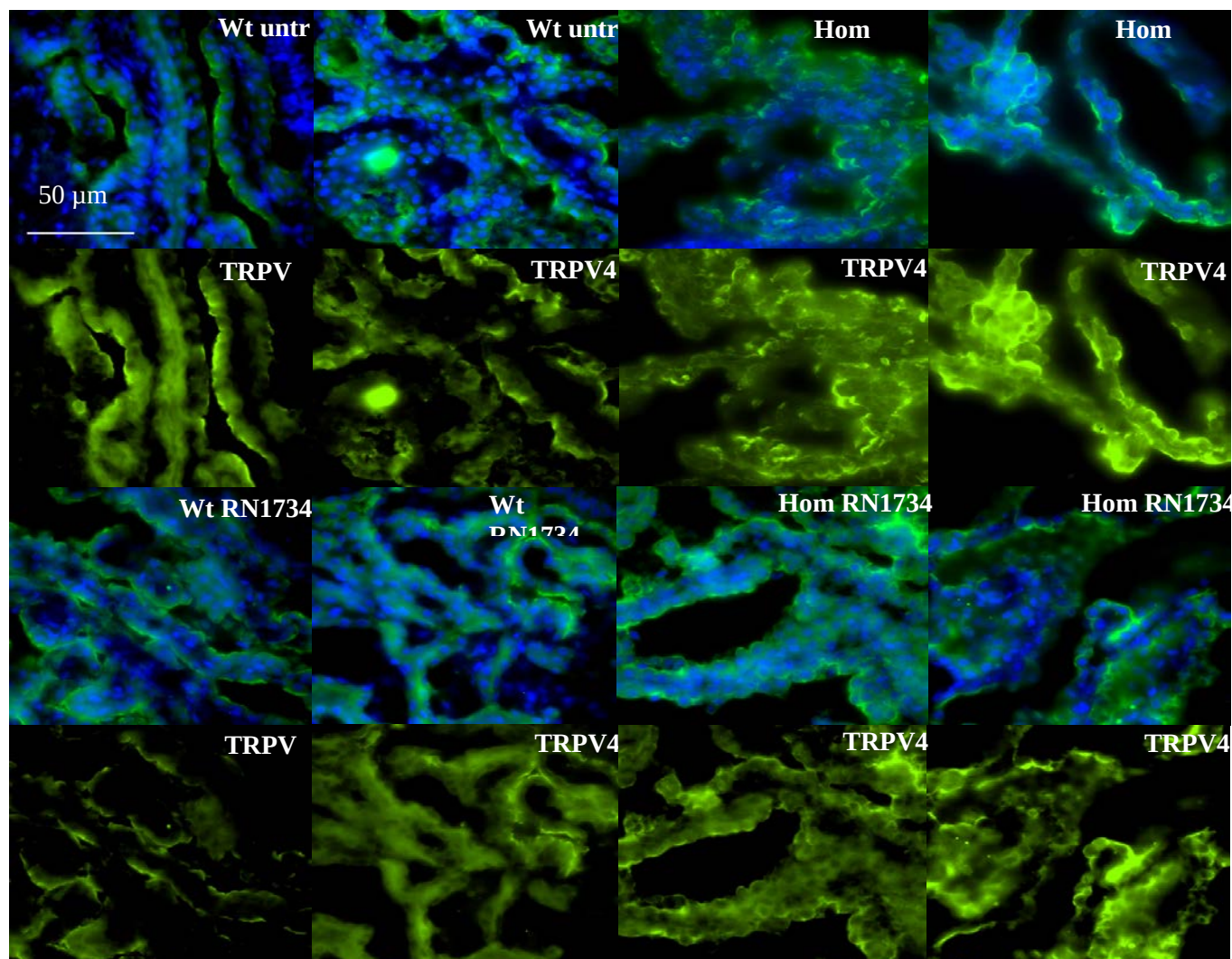


Figure 10. Immunohistochemistry of TRPV4 in choroid plexus tissues from post-natal day 15 wild type and hydrocephalic rats. All of the panels represent immunostaining that was conducted in tandem on the same day with the same vial of primary antibody (Alomone, anti TRPV4 antibody, 1:200). The images 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).

Given the difficulty in accurately quantifying the amount of TRPV4 in the tissues 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 (Figure 11).

For qPCR, choroid plexus tissue was dissected immediately following euthanization under a dissecting microscope and flash frozen in liquid nitrogen. Total RNA was isolated using the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. Purified RNA concentration was measured using an ND2000 NanoDrop (Fisher Scientific). Approximately 100 ng of total RNA was reverse transcribed into cDNA using the Monarch LunaScript RT SuperMix Kit (New England Biolabs). qPCR was carried out on a LightCycler 480 Instrument II real-time PCR system (Roche LifeScience), using LightCycler 480 SYBR Green I Master Mix (Roche LifeScience). Results are presented as relative expression fold change using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) relative to the calibrator housekeeping genes GAPDH and Rps18.

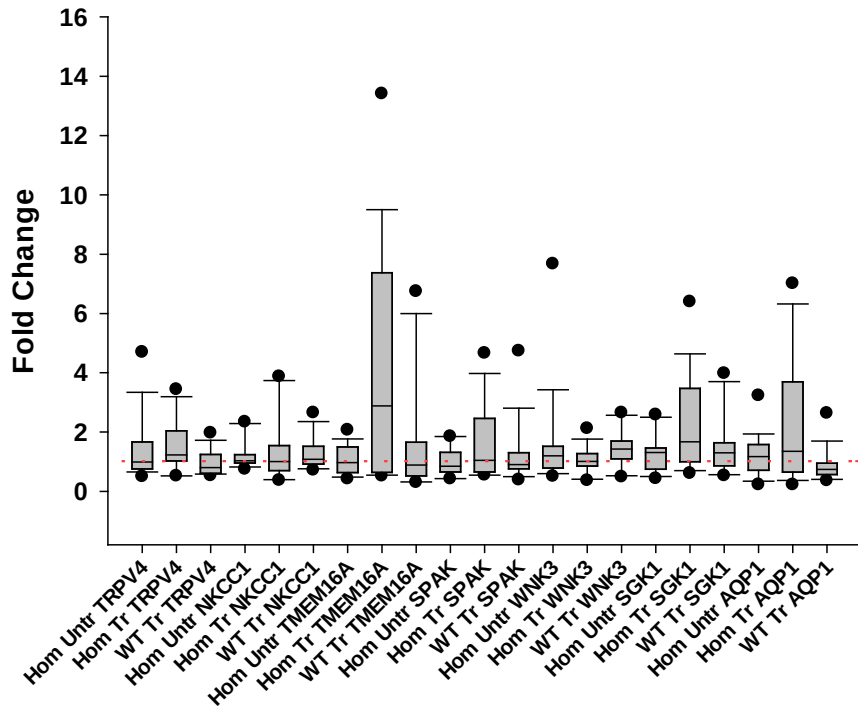


Figure 11: qPCR of rat choroid plexus taken from 15 day old animals treated with vehicle or the TRPV4 antagonist, RN1734 (i.p., 4 mg/kg body weight) from post-natal day 7 to 15. The fold change in TRPV4, NKCC1 (Na⁺K⁺2Cl⁻ cotransporter 1), TMEM16A (a Ca²⁺-activated Cl⁻ transporter), SPAK (STE20/SPS1-related proline/alanine-rich kinase), WNK3 (with no lysine kinase), SGK1 (serum glucocorticoid kinase 1) and AQP1(aquaporin 1) are shown relative to normalized controls (n=6 for each group). Control animals in

each instance are wild type, vehicle treated. GAPDH and RPS18 were used as housekeeping genes to calculate the $2^{-\Delta\Delta CT}$ fold change for each gene. Individual points shown are the minimum and maximum fold change values. Boxes represent +/- SEM for the experiments indicated, and the line within each box represents the mean value for those experiments.

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 (Figure 12). 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).

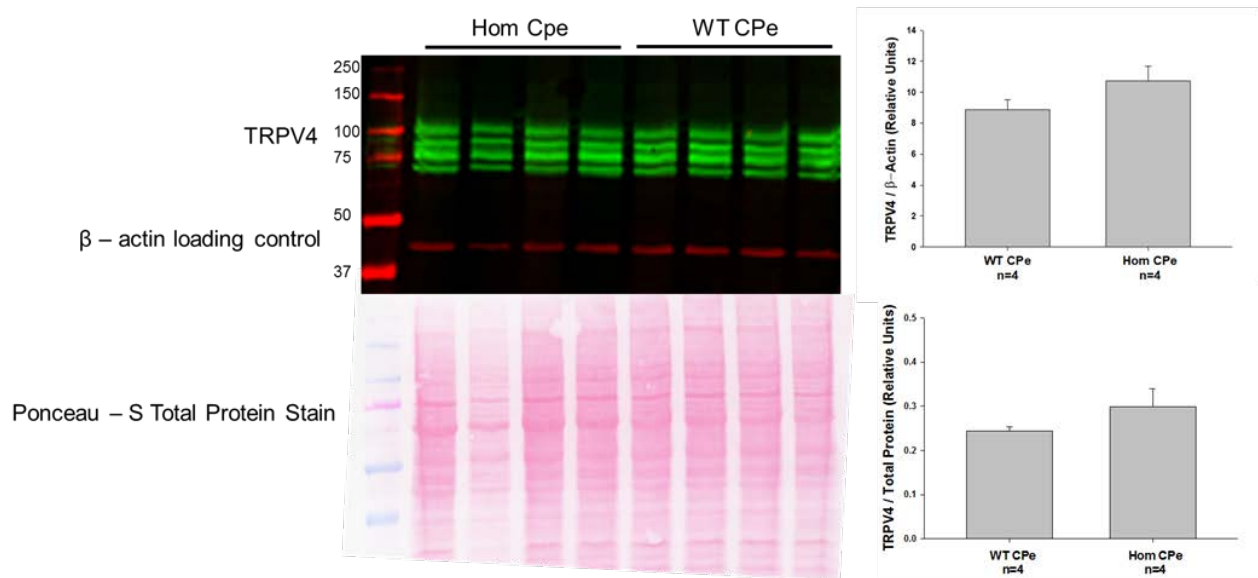


Figure 12: Relative quantification of TRPV4 in wild-type and homozygous rat pups at P15. Choroid plexus tissue was isolated from 4 wild type (WT) and 4 homozygous, hydrocephalic (Hom) P15 rat pups. The samples were solubilized and the protein was separated by gel electrophoresis. The gel was blotted onto nitrocellulose, stained with Ponceau protein stain and subsequently exposed to an anti TRPV4 antibody (Invitrogen PA5-77319 rabbit, 1:500) and an anti β -actin antibody (JacksonImmuno 1:1000). The Ponceau-stained gel was imaged using a flatbed scanner. The immunoblots were imaged with a LiCor Odyssey machine. The image intensity values were normalized using Image Studio or Image J. The bar graphs represent the average relative intensity of the TRPV4 bands as a function of the intensity of β -actin (top) or total protein (bottom). The bars on the top of the columns represent the SEM of the four values for each genotype. There were no statistically significant differences between the genotypes.

Taken together, the current results indicate that TRPV4 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.

Because of these interesting findings, we have decided to broaden our studies 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. Figure 13 illustrates some preliminary studies looking at protein expression in this area of the brain.

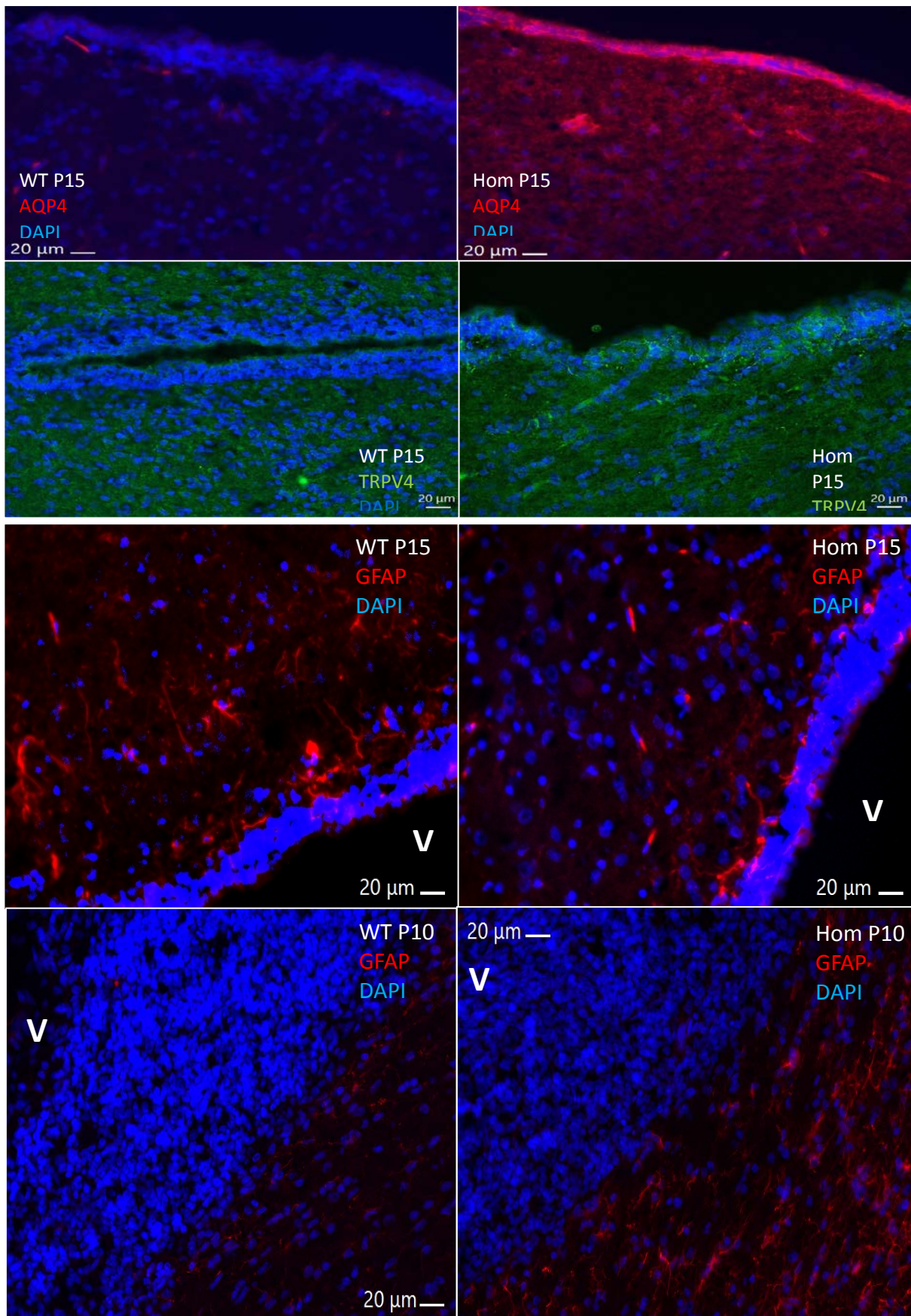


Figure 13: Immunohistochemical analysis of the subventricular zone of wild-type and homozygous TMEM67 rats at post-natal days (P) 10 and 15. Brain slices containing the subventricular zone were stained using antibodies directed against AQP4 (top red), TRPV4 (green), and GFAP (bottom 2 panels, red). In all images the nuclei are stained with DAPI (blue). The images were obtained using a 40X Keyence microscope with a 40X objective. Size bars are indicated on the figures. V indicates the area of the brain ventricles. The first three rows of images indicate tissue from P15 wild type (left) and homozygous (right) TMEM67 rat pups. The last row of images indicates tissue from P10 pups.

Figure 13 is an example of a preliminary study which has illuminated interesting differences between the wild-type pups and their hydrocephalic counterparts. This figure also indicates changes that occur during development. In particular, the expression of the AQP4 water channel is substantially over-expressed in subventricular zone of the hydrocephalic P15 pups and this will be confirmed and then examined in a developmental study encompassing several developmental time points between birth and day 15. The effect of the TRPV4 antagonist treatment on this substantial over-expression will also be documented. With regard to TRPV4 per se, there are not striking differences between the P15 pups of the two genotypes. However, for completeness, tissue from this area of the brain will be dissected and subjected to qPCR and western blotting as per the choroid plexus experiments shown in Figures 11 and 12.

Comparing P10 and P15, it is clear that the ependymal and immediate sub-ependymal cell layers are very different. This is not surprising due to the rapid developmental changes that are happening in this area of the brain in the neonatal period. What is remarkable, however, are the differences in astrocyte expression (GFAP labeling) between the two genotypes, at P10. This will be another area of study in the third year.

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 (Figures 1 and 2). It was our hypothesis that the expression of TRPV4 would be increased in the hydrocephalic state. 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 is scheduled predominately for the third year and will be dependent on a resolution of the genotype-phenotype mismatch 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 third 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.

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 for subtasks 1 and 2 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 $\Omega \cdot \text{cm}^2$

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 and it is anticipated that it will be submitted in December 2019 to American Journal of Physiology: Cell Physiology. These data will 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 choroid plexus epithelium. 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 accepted for publication in American Journal of Physiology: Cell Physiology and the manuscript can be found in the Appendix. 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-1 β , and TGF- β 1, 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- κ B, 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 are accepted for publication, 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 $\Omega \cdot \text{cm}^2$. 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 ($\sim 400 \Omega \text{cm}^2$) that they can accurately be used in electrophysiological experiments (Figure 14).

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 result 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.

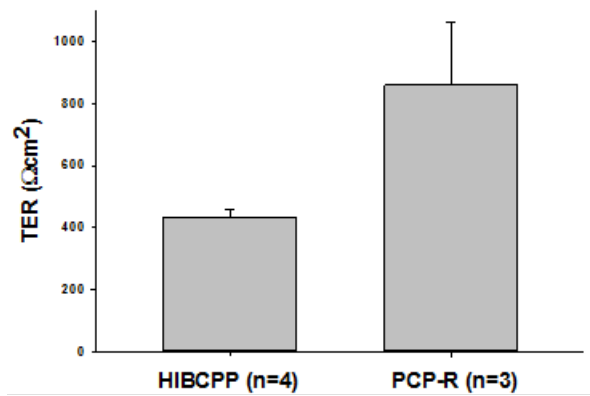
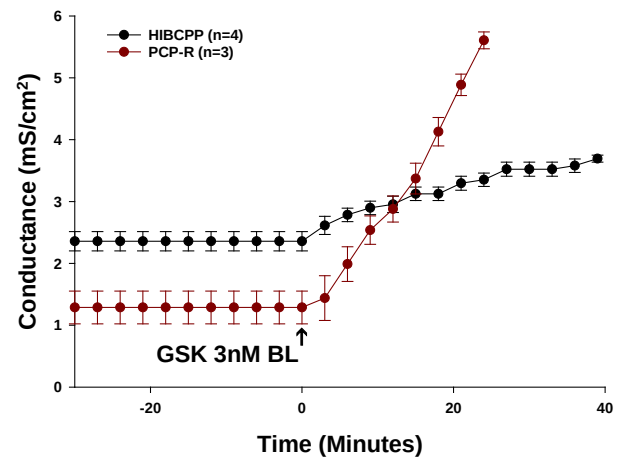
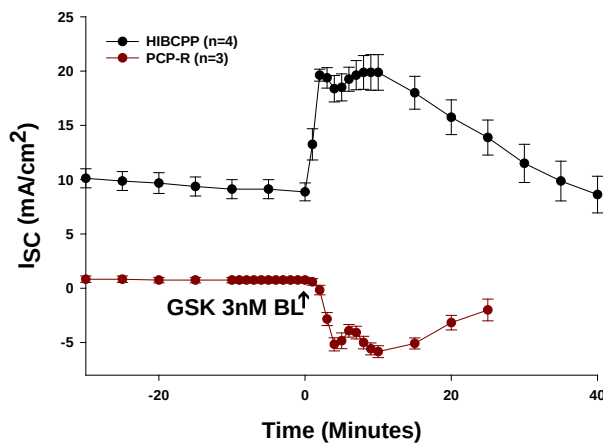


Figure 14: Comparison of the PCP-R and HIBCPP cell lines. The first panel compares the average transepithelial electrical resistances of the human HIBCPP cells with the porcine PCP-R cell line measured after mounting in Ussing chamber electrophysiological apparatuses. The lower two panels show comparisons of the short circuit current responses (I_{SC}), a measure of net transepithelial electrogenic ion flux and transepithelial conductance, a measure of permeability across the epithelial monolayer in the two cultures.



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 according to the scheduled timeframe listed in the SOW. It is worth noting that preliminary data have also been collected regarding expression of key effectors at the mRNA level (Figure 11)

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 year 3 as per SOW.

Publications

Accepted publications

1. Simpson, S., D. Preston, C. Schwerk, H. Schrotten, B. L. Blazer-Yost. Cytokine and inflammatory mediator effects on TRPV4 function in choroid plexus epithelial cells. In press

These are studies describing the role of inflammation in the PCP-R cells that have been accepted for publication in the American Journal of Physiology: Cell Physiology and a copy of the accepted manuscript can be found in the appendix.

2. 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. *Accepted for publication*

This chapter is in a book commissioned by the American Physiological Society. The authors are Dr. Blazer-Yost and two of the graduate students who are supported by the federal funding.

Publications to be submitted by December 2019:

1. Preston, D., S. Simpson, M. Reed, K. Gafunderi, B. Blazer-Yost. SPAK inhibition blocks TRPV4 mediated transepithelial ion flux in choroid plexus epithelial cells.

These are the studies examining the role of intracellular mediators in the regulation of transepithelial transport in the PCP-R cell line. The paper has been written by the first author in the style of American Journal of Physiology: Cell Physiology and is awaiting proof-reading and editing by Dr. Blazer-Yost before submission. All experiments are finalized.

2. Efficacy of TRPV4 antagonists for the treatment of hydrocephalus in a rodent model of the disease.

The important results that will be part of this paper were provided in the research summaries above. Dr. Blazer-Yost will be writing this manuscript in October and November 2019. The author list will include the Co-Investigators and the graduate students who participated in these experiments. We will target a high impact journal such as Nature Medicine for the initial submission.

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 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 students are co-authors on accepted and submitted publications. These activities are documented elsewhere in this report.

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 did a webinar for the Hydrocephalus Association on Nov. 14th, 2018. This was specifically tailored to hydrocephalic patients and their caregivers. The webinar included a question and answer period moderated by the Chief Research Officer of the Hydrocephalus Association.

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, we are closely following the original SOW with good success in the first two years. This contention has been well documented above. It is our intent to continue to do the proposed experiments and to provide deliverables in the form of presentations and publications in year 3.

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 Hydrocephalus Association is supporting the travel of one graduate student, Makenna Reed, and her mentor, Dr. Blazer-Yost, to attend an invitation-only workshop entitled “Finding Common Pathways: Extending Insights from Posthemorrhagic Hydrocephalus” Nov. 4-5, 2019. Ms. Reed will represent the Blazer-Yost laboratory with an oral presentation summarizing the results of our on-going

4. **IMPACT:** Describe 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).

In the second year of funding, 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 are preparing the first manuscript describing the pre-clinical studies in rodents for submission to a high impact journal late in 2019. We have published a paper 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.

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 6). 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 are re-deriving 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. This will take 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 have 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.

The second change in direction is not a problem but the resolution to a problem. In the year 1 progress report we noted that the human choroid plexus cell line, HIBCPP, could not be used for electrophysiological studies and proposed alternative experiments to be done in the porcine choroid plexus cell line. The alternative experiments were completed and have been accepted for publication. However, another graduate student has taken on the challenge of the human cell line and has progressed to the point that we feel the human cell line can be used for the originally proposed experiments. Those studies have been reinstituted and will progress in the third year of funding.

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, 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 will be submitted to ACURO in the month of October, 2019.

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).*

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.* Accepted, in press. Federal support was 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. Accepted. Federal support was acknowledged.

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.*

Oral Communications (no abstracts available)

Invited talks given by Dr. Blazer-Yost – Federal Funding Acknowledged:

The TRPV4 Ion Channel in Choroid Plexus: Implications for the Treatment of Hydrocephalus. Department of Physiology, Aarhus University, Denmark. (9/20/18)

Preclinical Testing of Pharmaceutical Treatments for Hydrocephalus. Hydrocephalus 2018, Meeting of the International Society for Hydrocephalus and Cerebrospinal Fluid Disorders. Bologna, Italy, 10/20/18

TRPV4 as a Hub Protein Controlling Ion Flux in Choroid Plexus Epithelial Cells. Scandinavian Physiological Society Annual Meeting. Reykjavik, Iceland. 8/9/19

Talks given by federally funded students and laboratory members at the Lake Cumberland Biological Transport Meeting, Lake Cumberland, KT June 16-18, 2019. Federal funding acknowledged.

Preclinical testing of TRPV4 antagonists in the treatment of hydrocephalus. Given by Hillary Smith

The role of water channels and the ventricular zone in a ciliopathy model of hydrocephalus. Given by Makenna Reed

Studies of TRPV4 in hydrocephalic development: mouse models and choroid plexus epithelial cells. Given by Alexandra Hochstetler

TRPV4 in the choroid plexus epithelium: pathway analysis and implications for cerebrospinal fluid production. Given by Daniel Preston. Note this presentation received an award from the American Physiological Society for the best student presentation at the meeting.

Poster Presentations

Published:

Experimental Biology 2019 – Orlando Florida – April

Smith, H.M., A. Hochstetler, D. Preston, B. Blazer-Yost. Preclinical Testing of TRPV4 Antagonists for the Treatment of Hydrocephalus FASEB Journal: Published Online: 1 Apr 2019 Abstract Number:708.4
https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.708.4

Reed, M. M., H. Smith, V. McConnell, A. Hammond, C. Goodlett, B. Blazer-Yost. Sex Differences in Behavioral Testing in an Adult Rat Model of Hydrocephalus. FASEB J. Published Online: 1 Apr 2019 Abstract Number:708.2
https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.708.2

Hochstetler, A.E., H. M. Smith, N. F. Berbari, B. L. Blazer-Yost. Functional studies of TRPV4 in Choroid Plexus Epithelial Cells. Published Online: 1 Apr 2019 Abstract Number:708.3
https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.708.3

Not Published:

TRPV4 Activation - Implications in Hydrocephalic Neurodegenerative Disease

Stefanie Simpson, Daniel Preston and Bonnie L. Blazer-Yost

Abstract for the Clinical and Translational Science Institute of Indiana (CTSI) 10th Annual Meeting, September 14th, 2018

TRPV4 Antagonist Treatment of Hydrocephalus in a Genetic Rat Model

Hillary Smith, Alexandra Hochstetler, Daniel Preston, Bonnie Blazer-Yost

Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting March 2, 2019, Wabash College, Indiana

Novel Object Recognition in TRPV4 Antagonist Treated Genetic Rat Model of Hydrocephalus

Alex Hammond, Hillary Smith, Makenna Reed, Victoria McConnell, Charles Goodlett, Bonnie Blazer-Yost

Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting March 2, 2019, Wabash College, Indiana

Effect of TRPV4 Antagonist on the Cognitive Abilities of the TMEM+/- Rat Model of Hydrocephalus

Victoria McConnell, Makenna Reed, Hillary Smith, Charles Goodlett, Bonnie Blazer-Yost

Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting March 2, 2019, Wabash College, Indiana

Sex Differences in Behavioral Testing in an Adult Rat Model of Hydrocephalus

Makenna Reed, Hillary Smith, Tori McConnell, Alex Hammond, C. Goodlett, Bonnie Blazer-Yost

Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting March 2, 2019, Wabash College, Indiana

- **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: Charley Goodlett

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

No change

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

Hillary was added to the technician line in the last half of the year.

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: Charles Goodlett

None to report

New awards: Karl Balsara

None to Report

New Awards: Nick Berbari

None to report

New Awards: Paul Territo

R13AG060708 (Rizzo) (Site PI: Territo)	9/1/18-8/31/21	0.3 calendar
University of Pittsburgh – NIH Federal Pass Through	\$5,233 (sub only)	
Improving Preclinical Translation in Alzheimer’s Disease Research		
Provide an in-depth discussion of the techniques, pitfall, and considerations that would be required for testing pharmaceuticals in animal models of Alzheimer’s disease.		

R01 DK116963 (Fogel and Tirkes)	9/1/18-8/31/21	1.2 calendar
NIH	\$432,625	
Magnetic Resonance Imaging as a Non-Invasive Method for Assessment of Pancreatic Fibrosis (MINIMAP): A Pilot Study		
We hypothesize that MRI can serve as a valuable non- invasive tool to detect Chronic Pancreatitis (CP), even in the early stages of the disease.		

U54 AG054345-03S2 (Lamb and Territo)	9/15/18-8/31/21	0.24 calendar
NIH-NIA	\$122,048	
The IU/JAX Alzheimer’s Disease Precision Models Center (Supplement: Drug Selection Criteria)		
Indiana University/Jackson Laboratory Alzheimer’s		

Disease Precision Models Center (IU/JAX ADPMC) seeks to generate and characterize novel animal models of AD, assess the relevance of these to model to human disease and to develop a preclinical testing pipeline through which novel therapies can be tested to greatly accelerate the process by which therapies are successfully moved forward to human AD clinical trials.

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.*

Appendix:

Copies of Abstracts Presented at Regional and National Meetings

Copies of Publications

Appendix

I. Abstracts - Venues and dates provided on abstracts:

Published abstracts – 3

Unpublished abstract presented as posters – 5

II. Publications

Published abstracts – 2018-2019

Smith, H.M., A. Hochstetler, D. Preston, B. Blazer-Yost. Preclinical Testing of TRPV4 Antagonists for the Treatment of Hydrocephalus FASEB Journal: Published Online:1 Apr 2019 Abstract Number:708.4

https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.708.4

Reed, M. M., H. Smith, V. McConnell, A. Hammond, C. Goodlett, B. Blazer-Yost. Sex Differences in Behavioral Testing in an Adult Rat Model of Hydrocephalus. FASEB J. Published Online:1 Apr 2019 Abstract Number:708.2

https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.708.2

Hochstetler, A.E., H. M. Smith, N. F. Berbari, B. L. Blazer-Yost. Functional studies of TRPV4 in Choroid Plexus Epithelial Cells. Published Online:1 Apr 2019 Abstract Number:708.3

https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.708.3

Copies of these abstracts are provided on the next three pages.

Preclinical Testing of TRPV4 Antagonists for the Treatment of Hydrocephalus

Hillary Smith, Alexandra Hochstetler, Daniel Preston, Bonnie Blazer-Yost

Department of Biology, Indiana University-Purdue University at Indianapolis

Cerebrospinal fluid (CSF) is a crucial component of the central nervous system that acts both mechanically and immunologically to support and protect the brain and spinal cord. Absorption and secretion of CSF must be properly maintained to achieve homeostasis. Some diseases, particularly hydrocephalus, experience an accumulation of CSF leading to an excess due to either overproduction or inadequate reabsorption. A small number of treatments are available to overcome this accumulation of CSF, all of which involve invasive surgical procedures that come with high risk and low success rates. The need for alternative treatments leads to a closer look at the choroid plexus, which consists of epithelial cells surrounding a fenestrated capillary network and produces a large portion of the CSF. This research looks specifically at the Transient Receptor Potential Vanilloid 4 (TRPV4), a non-specific cation channel found in many secretory epithelia. When activated, TRPV4 allows an influx of Ca^{2+} which leads to downstream intracellular signaling and activation of Ca^{2+} -activated ion channels. The Wpk rat model, which contains a single point mutation in the TMEM67 gene coding for Mecklin, one of the proteins responsible for the Meckel-Gruber Syndrome, was used to study possible treatments. Rats that are homozygous recessive for the TMEM67 gene develop a severe form of hydrocephalus that if, left untreated, will result in death by postnatal day 21 (P21). This rat model is analogous to pediatric hydrocephalus in humans. We have used the model to test a TRPV4 antagonist, RN1734, as a possible treatment of hydrocephalus. At P7, the animals received an MRI to measure the volume of the brain ventricles. Subsequently, the animals received daily intraperitoneal injections of the RN1734 through P14. At P15, the animals received a second MRI to observe changes over the course of the treatment. The animals were sacrificed on P15, and their brains, kidneys, and weights were collected. Upon sacrifice, homozygous animals are significantly smaller in body weight than their wild-type counterparts. The brain weights of the vehicle treated homozygous and the wild-type animals were not significantly different, however, the homozygous animals treated with the RN compound weighed statistically less than the wild-type animals. Ventricular volume measurements by MRI revealed that the RN compound caused no effects on normal ventricular growth that occurred from day 7 through 14 in wild-type and heterozygous animals, but it stopped the substantial ventricular enlargement that occurred over this time period in the homozygous animals. The successful results of the RN1734 treatment in the homozygous animals suggest TRPV4 antagonists for a potential pharmaceutical development for treating hydrocephalus.

Funding: Hydrocephalus Association and Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP).

Sex Differences in Behavioral Testing in an Adult Rat Model of Hydrocephalus

Makenna Reed, Hillary Smith, Tori McConnell, Alex Hammond, Charles Goodlett, Bonnie

Blazer-Yost

Hydrocephalus is a developmental disorder that affects children worldwide with an incidence of approximately 1:1000 births. Multiple causes have been implicated, including a dysfunction of the homeostatic mechanisms of cerebrospinal fluid (CSF) secretion from the choroid plexus, a decreased reabsorption from the subarachnoid granules, or a blockage of CSF circulation, leading to excessive accumulation of CSF within the brain ventricles. One animal model of developmental hydrocephalus involves rats carrying a point mutation on the TMEM67 gene. The homozygous form of this mutation causes a ciliopathy that was originally found in rats with polycystic kidney disease (PKD). Rats homozygous for the TMEM67 mutation show severe hydrocephalus and PKD and do not survive beyond postnatal day (P) 18. Recently we identified a TRPV4 antagonist that appears to protect against the ventriculomegaly in preweaning homozygous rats. We propose that this drug has effects directly on the choroid plexus, and since it is administered intraperitoneally, must gain access to the choroid epithelial cells. Animals heterozygous for the TMEM67 mutation develop a milder degree of hydrocephalus, no renal disease, and live past 1 year. The current study tested the hypothesis that treatment of heterozygous adult animals with the TRPV4 antagonist, RN1734, can limit the extent of hydrocephalus thereby protecting against the associated adverse effects on neurobehavioral function. In the current experiments, the behavioral studies assessed exploration of a complex novel environment called the multivariate concentric square field (MCSF), a 100 x 100 cm arena containing multiple defined zones with a large center area, three dimly-illuminated perimeter corridors, one brightly illuminated slope and bridge, an enclosed dark corner room (DCR), and a corner hurdle. Video recordings of the single 20-minute session of each rat are scored by two observers blind to group membership, and quantitative measures of behavioral categories (locomotor activity, exploratory activity, shelter seeking, risk assessment, risk taking) are derived from scores of the type, number and duration of activities in the various zones. MRI's were performed on P300 and P331, and the rats were assigned to either RN1734 or vehicle treatment on P301-P330, and testing on the MCSF occurred on P315. Several interesting and unexpected results emerged from these studies. Regardless of genotype, the proportion of males that developed hydrocephalus was significantly higher than that of females. Regardless of genotype and drug treatment, the females were more active and engaged in less shelter seeking

than males. Because of these sex differences the current studies were underpowered to determine effects of genotype and drug treatment on the behavioral activities.

Funding: Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP) and the Hydrocephalus Association.

Functional studies of TRPV4 in Choroid Plexus Epithelial Cells

Alexandra E. Hochstetler¹, Hillary M. Smith¹, Nicolas F. Berbari¹, Bonnie L. Blazer-Yost¹

¹Department of Biology, Indiana University-Purdue University at Indianapolis

Cerebrospinal fluid (CSF) is produced by the choroid plexus epithelial (CPe) cells, and is moved throughout the brain ventricular system in coordinated waves that are generated by ependymal cilia lining the ventricles and subsequently absorbed in the subarachnoid granulations. CSF is moved in a pulsatile motion which is thought to be influenced by multiple factors like blood pressure and circadian rhythms. Hydrocephalus is a blanket disease description which covers clinical manifestations from CSF over-production to decreased absorption and can occur at any age. Patients with pediatric hydrocephalus have enlarged ventricles, failure to thrive, sleepiness, sunsetting of the eyes, and vomiting. The current treatment paradigm for hydrocephalus is exclusively surgical and includes CSF diversion by shunt or endoscopic third ventriculostomy. These surgeries have known morbidity and mortality rates, and variable patient outcomes. In order to study pediatric hydrocephalus, the lab has two mouse lines which develop hydrocephalus postnatally. These models have been backcrossed to different genetic backgrounds in order to characterize disease severity, and to select the models with the most viability to undergo further studies. Disease severity is characterized by ventricular area as a function of total brain area in Nissl stained brain slices at specific points from the bregma. Additional characterization is being performed by utilizing intracranial pressure (ICP) monitoring using a small probe inserted into the brain parenchyma. The models that are the most stable and have disease severity similar to pediatric hydrocephalus will be used to test a class of drugs known as Transient Receptor Potential Vanilloid 4 (TRPV4) antagonists. TRPV4 is a widely expressed, widely translated protein which functions in many epithelial tissues as well as in the vasculature, and several neuronal cell types. TRPV4 can function as an osmosensor, mechanosensor, or thermosensor, and can be activated by a number of different metabolites. Activation of TRPV4 results in cation influx into the epithelial cells which can secondarily affect other transporters. TRPV4 antagonists have been shown to ameliorate hydrocephalus in a rat model of pediatric hydrocephalus, the *WPK* rat. To further understand the *in vivo* mechanism of TRPV4, a CPe line (Porcine Choroid Plexus – Riems) was used to characterize the effects of pressure (both chronic and acute), and shear force on the TRPV4-mediated ion flux and conductance. TRPV4-mediated ion flux is potentiated by acute pressure on the apical side of the CPe cells. In addition, both chronic and acute pressure and shear stress increase TRPV4-mediated transepithelial conductance. Successfully completing drug studies in the mouse models of hydrocephalus, as well as characterizing the mechanism of action of the TRPV4 antagonist drugs *in vivo* will provide a solid basis for further preclinical pharmacology studies.

Funding: Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP) and the Hydrocephalus Association.

Unpublished abstract presented as Posters.

Meetings and Dates provided on each page

TRPV4 Activation - Implications in Hydrocephalic Neurodegenerative Disease

Stefanie Simpson, Daniel Preston and Bonnie L. Blazer-Yost

Hydrocephalus is a disease state found in neurodegenerative diseases characterized by an imbalance in cerebrospinal fluid (CSF) production and/or reabsorption or by a blockage in the CSF circulation resulting in enlarged ventricles and an increase in brain hydrostatic pressure leading to neuronal destruction. The choroid plexus (CP) is the main producer of CSF and is composed of a high resistance barrier epithelium that surrounds a network of capillaries. The CP epithelium regulates the transport of ions and water between the ventricles and capillaries, thus controlling the production of CSF. Transient Receptor Potential Vanilloid-4 (TRPV4) is a mechano- and osmo-sensitive, cation channel present in CP epithelia. TRPV4 activation leads to an influx of calcium, which can stimulate calcium activated ion channels leading to the potential for significant transepithelial ion flux. If TRPV4 is overstimulated, it is possible that excess ion movement can cause an increase in CSF volume, resulting in hydrocephalus. Current research in our laboratory has shown that treatment with a TRPV4 antagonist ameliorates hydrocephalus in the Wpk rodent model. We have also shown that TRPV4 expression is increased in the late-stage hydrocephalic animal compared to normal. TRPV4 can be activated through a number of stimuli, including physical means osmotic changes, increases in temperature and chemical activators, such as cytokines and inflammatory mediators. In particular, the latter activators can be seen in the symptomologies of neurodegenerative diseases exhibiting hydrocephalus. Utilizing a high resistance porcine choroid plexus (PCP-R) cell line, we investigated the effects of various cytokines, inflammatory mediators, and inflammation pathways on TRPV4 activity using immunohistochemistry and electrophysiology techniques. We found that while pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF)- α and interleukin (IL)-1 β , had no effect on TRPV4 stimulated ion flux in vitro; pro-inflammatory mediators, arachidonic acid and its metabolite 5,6-epoxyeicosatrienoic acid (EET), had inhibitory effects. Furthermore, the hormone epinephrine, used to combat inflammatory anaphylactic reactions, potentiated TRPV4 activity. Inhibitors of NF-kB altered TRPV4-mediated ion flux. Together these data imply that selected components of an inflammatory response can play a role in TRPV4 activation and can potentially contribute to the subsequent development of hydrocephalus.

Funding: Hydrocephalus Association and Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP)

**Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting
March 2, 2019, Wabash College, Indiana**

TRPV4 Antagonist Treatment of Hydrocephalus in a Genetic Rat Model

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Due to its importance, the central nervous system must be supported both chemically and mechanically. This support is provided by cerebrospinal fluid (CSF), a fluid that fills spaces within the meninges of the brain and spinal cord. Homeostasis is achieved when CSF absorption and secretion are properly maintained, however, many diseases can arise when CSF is overproduced or inadequately absorbed. Hydrocephalus is one of these diseases that develops when the CSF accumulates within the brain. There are currently few treatments available to alleviate this accumulation, all of which require invasive brain surgery. The search for alternative treatments leads to the observation of the choroid plexus epithelial cells that surround a fenestrated capillary network and produce majority of the body's CSF. The major focus of this research is the Transient Receptor Potential Vanilloid 4 (TRPV4), a non-specific cation channel found in many secretory epithelial that, when activated, allows an influx of Ca^{2+} . This influx leads to downstream signaling within the cell, particularly on Ca^{2+} -activated ion channels. The WPK rat model used in this research contains a single C to T change in the TMEM67 gene, a gene that codes for Mecklin protein, resulting in Meckel-Gruber Syndrome. Homozygous recessive rats for TMEM67, form hydrocephalus so severe that death occurs by post-natal day (P) 21. Due to its analogy to human pediatric hydrocephalus, this model was used to test a TRPV4 antagonist, RN1734, to determine its potential to treat hydrocephalus. The animals were treated from P7 to P14 daily with intraperitoneal (IP) injections of RN1734. MRIs were administered on P7 and P15 to allow assessment of volume changes during the treatment. On P15 the animals were sacrificed and brains, kidneys, and weights were collected. Results show that the homozygous animals are significantly smaller in size and weight than their wild-type counterparts. Ventricular volume measurements by MRI revealed no effects of the RN compound on the normal growth and development in wild-type animals, but in the homozygous animals, substantial ventricular enlargement was halted by the treatment. Subsequent immunofluorescent staining and imaging of the collected brains revealed in both the wild-type and homozygous animals, use of the RN compound resulted in intracellular localization of TRPV4, which differs from the untreated animals' apical membrane localization of TRPV4. The success of the RN compound to reduce the ventricular volume of homozygous animals shows a potential pharmaceutical means for treating hydrocephalus.

Funding: Hydrocephalus Association and Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP).

Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting
March 2, 2019, Wabash College, Indiana

Novel Object Recognition in TRPV4 Antagonist Treated Genetic Rat Model of Hydrocephalus

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Hydrocephalus, a buildup of cerebrospinal fluid (CSF) in the brain, is a neurodegenerative disease that affects one in 1000 live births, but can occur at any age and bears a variety of complications. Current treatment options are strictly surgical, such as a CSF shunt, and are characterized by a low long-term success rate. Pharmaceutical options do not currently exist. The transient receptor potential vanilloid 4 (TRPV4) ion channel is highly expressed in the choroid plexus, the central producer of CSF. Preliminary data in the TMEM67 rat model of hydrocephalus, indicates that TMEM67(-/-) rat pups develop severe hydrocephalus with enlarged horizontal and vertical head dimensions that can be significantly reduced by treatment with a TRPV4 antagonist, RN 1734. These data led us to hypothesize that mildly hydrocephalic TMEM67(+/-) adult rats will express deficits in behavioral and cognitive functions mediated by forebrain systems that could be restored by treatment with RN 1734. To assess recognition memory and exploratory behavior, TMEM67(+/-) and Wild Type rats aged 300 ± 3 days were administered daily IP injections of either RN 1734 or Vehicle for 30 days. On day 19 of treatment, the rats were assessed on two Object Recognition Tasks, a measure of memory typically expressed as a preference to explore a novel object over a familiar object (novel object recognition), or preference for an object encountered more remotely in the past than one more recently encountered (temporal order memory). In rats with a hydrocephalic phenotype, treatment with RN 1734 improved temporal order recognition memory but had no beneficial effect on novel object recognition memory. Analyzing by genotype and treatment, however, indicated no significant effects on temporal order or long-term recognition after treatment. A reliable sex difference was evident in locomotor activity in the testing sessions, with the females significantly more active than their male counterparts. Due to overlapping phenotypes in wild-type and TMEM67(+/-) rats, RN 1734's efficacy in improving deficits associated with impaired forebrain memory systems was inconclusive.

Funding: Hydrocephalus Association and Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP).

**Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting
March 2, 2019, Wabash College, Indiana**

Effect of TRPV4 Antagonist on the Cognitive Abilities of the TMEM+/- Rat Model of Hydrocephalus

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Hydrocephalus is a neurodegenerative disease characterised by an accumulation of cerebrospinal fluid (CSF) in the ventricles of the brain, leading to an increased pressure. It may develop because of trauma, infection, intracranial haemorrhage or as a result of a congenital birth defect. Currently, the only treatments available are surgical, for example a CSF shunt. CSF is produced by the choroid plexus which contains transient receptor potential vanilloid 4 (TRPV4) channels. TRPV4 is believed to play a part in the build-up of CSF in hydrocephalus. It may be possible to reduce the production of CSF, and therefore the volume of the ventricles, using a TRPV4 antagonist in a heterozygous TMEM67(+/-) rat model of slowly progressing hydrocephalus. The TRPV4 antagonist, RN 1734, could have a positive affect on the animals' cognitive abilities and even reverse the cognitive decline associated with the progressing hydrocephalus. However, data on this rat model suggests that the heterozygous animals do not uniformly express hydrocephalus. Therefore, the behaviour and cognitive affects can be assessed by looking at hydrocephalic phenotype, which has been defined as a combined lateral ventricular volume of 45mm³ and over at age p300. Heterozygous and wild type animals were injected daily with the antagonist or a control vehicle for 30 days. On day 21 of treatments, the animals began Morris Water Maze (MWM) trials to test their behaviour, memory and use of visual cues. Overall, it was expected that the wild type animals and those treated with the antagonist will perform better and have shorter latencies in terms of finding the submerged platform. However, results show that overall the animal's latencies decreased at the same rate as days went on regardless of their genotype or phenotype. There were no significant differences in latency or path length between treated and untreated animals. These studies suggest that the testing may be been conducted before the hydrocephalic disease had progressed to the point of altering the cognitive abilities of the animals and suggest that the studies should be conducted on older animals.

Funding: Hydrocephalus Association and Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP)

Presented as a poster at the 9th Annual Meeting of the Indiana Physiological Society Meeting
March 2, 2019, Wabash College, Indiana

Sex Differences in Behavioral Testing in an Adult Rat Model of Hydrocephalus

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Hydrocephalus is a developmental disorder that affects children worldwide with an incidence of approximately 1:1000 births. Multiple causes have been implicated, including a dysfunction of the homeostatic mechanisms of cerebrospinal fluid (CSF) secretion from the choroid plexus, a decreased reabsorption from the subarachnoid granules, or a blockage of CSF circulation, leading to excessive accumulation of CSF within the brain ventricles. One animal model of developmental hydrocephalus involves rats carrying a point mutation on the TMEM67 gene. The homozygous form of this mutation causes a ciliopathy that was originally found in rats with polycystic kidney disease (PKD). Rats homozygous for the TMEM67 mutation show severe hydrocephalus and PKD and do not survive beyond postnatal day (P) 18. Recently we identified a TRPV4 antagonist that appears to protect against the ventriculomegaly in preweaning homozygous rats. We propose that this drug has effects directly on the choroid plexus, and since it is administered intraperitoneally, must gain access to the choroid epithelial cells. Animals heterozygous for the TMEM67 mutation develop a milder degree of hydrocephalus, no renal disease, and live past 1 year. The current study tested the hypothesis that treatment of heterozygous adult animals with the TRPV4 antagonist, RN1734, can limit the extent of hydrocephalus thereby protecting against the associated adverse effects on neurobehavioral function. In the current experiments, the behavioral studies assessed exploration of a complex novel environment called the multivariate concentric square field (MCSF), a 100 x 100 cm arena containing multiple defined zones with a large center area, three dimly-illuminated perimeter corridors, one brightly illuminated slope and bridge, an enclosed dark corner room (DCR), and a corner hurdle. Video recordings of the single 20-minute session of each rat are scored by two observers blind to group membership, and quantitative measures of behavioral categories (locomotor activity, exploratory activity, shelter seeking, risk assessment, risk taking) are derived from scores of the type, number and duration of activities in the various zones. MRI's were performed on P300 and P331, and the rats were assigned to either RN1734 or vehicle treatment on P301-P330, and testing on the MCSF occurred on P315. Several interesting and unexpected results emerged from these studies. Regardless of genotype, the proportion of males that developed hydrocephalus was significantly higher than that of females. Regardless of genotype and drug treatment, the females were more active in regard to exploratory behavior, risk assessment, and risk taking. Although there was no difference in shelter seeking behavior, females spent less time in the DCR than males. Because of these sex differences the current studies were underpowered to determine effects of genotype and drug treatment on the behavioral activities.

Funding: Hydrocephalus Association and Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP)

Publications

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.* *In press*

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. *Accepted for publication*

**Cytokine and inflammatory mediator effects on TRPV4 function in choroid plexus
epithelial cells**

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Running Title: Inflammation and TRPV4 in the Choroid Plexus

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Keywords: arachidonic acid, blood-choroid plexus barrier, epoxyeicosatrienoic acid, NF- κ B

Abbreviations:

CP – Choroid Plexus

CSF – Cerebrospinal Fluid

PCP-R – Porcine Choroid Plexus-Reims

TRPV4 – Transient Receptor Potential Vanilloid 4

IL – Interleukin

TNF – Tumor Necrosis Factor

TGF – Transforming Growth Factor

AA – Arachidonic Acid

EET – Epoxyeicosatrienoic acids

29 PKD – Polycystic Kidney Disease
30 CYP – Cytochrome P450 Epoxygenase
31 ALOX – Lipoxygenase
32 COX – Cyclooxygenase
33 SCC – Short Circuit Current

34

35 S. S. and B. B. Y. designed the experiments. C.S. and H.S. provided the PCP-R cell line. S. S. and D. P.
36 conducted the experiments, analyzed data, and prepared figures. S. S., D. P., and B. B. Y. interpreted the
37 results of experiments. S.S. drafted the manuscript. S. S., D. P., C.S., H.S., and B. B. Y. edited, revised, and
38 approved final manuscript.

39 **Abstract**

40 The choroid plexus (CP), composed of capillaries surrounded by a barrier epithelium, is the main
41 producer of cerebrospinal fluid (CSF). The CP epithelium regulates the transport of ions and water
42 between the blood and the ventricles, contributing to CSF production and composition. Several studies
43 suggest a connection between the cation channel Transient Receptor Potential Vanilloid-4 (TRPV4) and
44 transepithelial ion movement. TRPV4 is a non-selective, calcium permeable cation channel present in CP
45 epithelia reported to be activated by cytokines and inflammatory mediators. Utilizing the PCP-R (porcine
46 choroid plexus- Riems) cell line, we investigated the effects of various cytokines and inflammatory
47 mediators on TRPV4-mediated activity. Select pro-inflammatory cytokines (TNF- α , IL-1 β , TGF- β 1) had
48 inhibitory effects on TRPV4-stimulated transepithelial ion flux and permeability changes whereas anti-
49 inflammatory cytokines (IL-10, IL-4, IL-6) had none. Quantitative mRNA analysis showed that these
50 cytokines had no effect on TRPV4 transcription levels. Inhibition of the transcription factor NF- κ B,
51 involved in the production and regulation of several inflammatory cytokines, inhibited TRPV4-mediated
52 activity, suggesting a link between TRPV4 and cytokine production. Contrary to published studies, the
53 pro-inflammatory mediator arachidonic acid (AA) had inhibitory rather than stimulatory effects on

54 TRPV4-mediated responses. However, inhibition of AA metabolism also caused inhibitory effects on
55 TRPV4, suggesting a complex interaction of AA and its metabolites in the regulation of TRPV4 activity.
56 Together these data imply that TRPV4 activity is involved in the inflammatory response; it is negatively
57 affected by pro-inflammatory mediators. Furthermore, arachidonic acid metabolites, but not
58 arachidonic acid itself, are positive regulators of TRPV4.

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Introduction

Neurological and neurodegenerative conditions in the brain can result in an increase in the release of inflammatory mediators into the injured areas. These conditions can include head trauma, intracranial hemorrhage, infection, brain tumor, genetic defect or neurodegenerative disease (11, 13, 16, 18, 20, 21, 27, 29, 45, 46). In many of these cases, the development of transient or chronic enlargement of the brain ventricles and an increase in pressure due to the accumulation of cerebrospinal fluid (CSF) occurs. This accrual of fluid could be caused by increases in inflammation affecting the production and/or absorption of the CSF.

The main production of CSF comes from the choroid plexus (CP), present in the lateral, third, and fourth ventricles (10). Composed of a barrier epithelium that surrounds a network of capillaries, the CP epithelial cells regulate the transport of ions and water between the ventricles and capillaries via electrolyte transporters, thus controlling the production and composition of CSF. Aberrant regulation of these transporters could be a causative agent leading to the emergence of fluid/electrolyte imbalances in the brain.

Previous data suggest a link between transepithelial ion movement and the cation channel, Transient Receptor Potential Vanilloid-4 (TRPV4) (29, 37, 44). This ion channel is found in choroid plexus epithelium (10, 37). TRPV4 is a mechano-, osmo-, and temperature-sensitive, non-selective, calcium-permeable cation channel that can serve as a hub protein for the activation of other transporters (26, 32, 37, 42). For example, in the porcine choroid plexus cell line – Riems (PCP-R), TRPV4 activation can lead to an influx of calcium into the cells, which subsequently stimulates calcium activated ion channels, such as the intermediate conductance potassium channels (37). Once these channels are activated, there is the potential for significant ion flux across the epithelia, resulting in compensatory water movement and a change in CSF production. TRPV4 can also be modulated through a number of stimuli, including chemical activators, such as cytokines and inflammatory mediators (26, 32, 37, 42). Indeed, it

has been established that the inflammatory lipid endocannabinoid anandamide (AEA) and its metabolites, including arachidonic acid (AA), are capable of activating the channel (32). In addition, cytochrome P450 epoxygenases, cyclooxygenases, and lipoxygenases can metabolize AA into both inflammatory and anti-inflammatory factors epoxyeicosatrienoic acids (EETs), prostaglandins, and hydroperoxides, respectively, which are also believed to stimulate TRPV4 activity (32, 33).

Microglia, the macrophage-like cells of the central nervous system (CNS), can be triggered to release pro-inflammatory cytokines in response to abnormal protein accumulation or neuronal injury from chemical or physical means (7, 50). It is possible that such inflammation is associated with increases in CSF production associated with neurological disease (4, 17, 18, 35, 46). Summarized in Table 1, pro-inflammatory cytokines found upregulated in neurodegenerative states include interleukin (IL)-1 β and tumor necrosis factor (TNF)- α . IL-10 and IL-4 are well-documented anti-inflammatory cytokines also found in various neurological diseases. TGF- β and IL-6 have both been implicated in pro- as well as anti-inflammatory effects (2, 7, 8, 14, 15, 24, 38, 41, 48). These cytokines can, therefore, potentially play a role in altering TRPV4 activity.

As an *in vitro* model, the PCP-R cell line can be utilized as a surrogate for *in vivo* CP tissue. This cell line exhibits many of the characteristics of the native epithelium including the formation of a barrier epithelial monolayer, expression of tight junctional proteins claudins 1 and 3, zona occludins-1 and occludin as well as the polarization of transporters and channels similar to those found *in vivo* (37, 39). A previous study has used this line to monitor changes in transepithelial ion flux and barrier permeability in response to factors that modulate choroid plexus electrolyte transporters *in vivo* (37). The polarity and barrier function exhibited by this cell line allows for experimentation modeling changes in the barrier function of choroid plexus *in vivo* (37, 39). Using a TRPV4 agonist, GSK1016790A, we are able to stimulate TRPV4 activation and observe changes in ion flux and transepithelial conductance in the presence of various cytokines and inflammatory mediators.

109 In this paper, we show that specific cytokines and inflammatory mediators can alter the function
110 of TRPV4 in PCP-R cell epithelia. This provides more insight into the role of the cation channel in neuro-
111 inflammatory states and suggesting this hub protein as a potential effector in CSF production.

Methods and Materials

Cell Culture

PCP-R cells were grown on 0.4 μm pore diameter filters inserts (MilliporeSigma, Burlington, MA; #PIHP03050) until the cultures developed a high transepithelial electrical resistance (TER) (10-12 days) (37). Only cultures with resistances above $500\ \Omega\text{cm}^2$ were utilized. During experimental protocols that result in a change in resistance, cells that drop below $100\ \Omega\text{cm}^2$ were also removed from analysis as their junctional complexes appear irreversibly altered. Cells were fed three times weekly with DMEM media (Gibco, Gaithersburg, MD; #12100-046) containing 4.5 g/L glucose, 3.7 g/L NaHCO_3 , 5.71 g/L HEPES, 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 100 U/mL penicillin, 100 mg/mL streptomycin, and 5 $\mu\text{g}/\text{ml}$ insulin. Each plate contained 6 filters in 6 different wells. Filtered inserts were bathed in 2 mL feeding media on the basolateral (bottom) side of the membrane and 1.5 mL feeding media on the apical (top) side of the membrane.

Reverse Transcriptase (RT)-PCR

For the RT-PCR, cells were grown in $75\ \text{cm}^2$ flasks until confluent. The monolayers were trypsinized, collected, and RNA from the cells extracted following the manufacturer's instructions for the New Monarch Total RNA Miniprep Kit (New England BioLabs Inc., Ipswich, MA; #T2010S). No template controls and cDNA were prepared by following manufacturer's protocol for the New England BioLabs LunaScript RT SuperMix Kit (#E3010S). Three primer sets for each gene were generated using Primer3Plus with mRNA sequences and tested on a temperature gradient. Only those primers used in the final images were included in Table 4.1. *Sus scrofa* mRNA sequences were obtained from Ensembl and additionally confirmed with sequences from the NCBI database. Forward and reverse primers (IDT) were combined with cDNA (approximately 500 ng) and GoTaq Green Master Mix (Promega Corporation, Madison, WI; #M7122) to perform gradients of PCR to determine the optimal annealing temperature. PCR products were then run on 1.5% agarose gels with ethidium bromide and 100bp flanking ladders.

Gels were imaged using a ChemiDoc XRS imager (Bio-Rad, Hercules, CA). Following confirmation of single band amplification, PCR products were Sanger sequenced by Eton Bioscience (Union, NJ). Amplicons were confirmed to be the predicted transcript for each gene, validated using NCBI BLAST.

Quantitative (q) PCR

Choroid plexus cells were grown on 0.4 μ M polycarbonate filtered inserts (MilliporeSigma) until confluent (10-12 days). 24 hours prior to use, experimental cells were treated with specific cytokines and allowed to incubate overnight. Cells were washed with cold 1X PBS twice, and total RNA was isolated using the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions for cultured cells. Purified RNA concentration was measured using an ND2000 NanoDrop (Fisher Scientific, Waltham, MA). Approximately 100 ng of total RNA was reverse transcribed into cDNA using the Monarch LunaScript RT SuperMix Kit (New England Biolabs). cDNA was diluted 1:10 with Nuclease-Free water (New England Biolabs). qPCR was carried out on a LightCycler 480 Instrument II real-time PCR system (Roche LifeScience, Penzberg, Germany), using LightCycler 480 SYBR Green I Master Mix (Roche LifeScience, #04707516001). qPCR cycle conditions were 95°C for 5 minutes; followed by 45 cycles of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. Data are presented as relative expression fold change using the $2^{-\Delta\Delta CT}$ method (28) relative to the calibrator housekeeping genes GAPDH and Rps18. Data are shown as fold change of TRPV4 in treated cells relative to the normalized controls.

Electrophysiology

Electrophysiological measurements were conducted by excising and mounting confluent (10-12 days) PCP-R cells grown on filter inserts in Ussing chambers which are attached to a DVC-1000 Voltage/Current Clamp (World Precision Instruments, Sarasota, FL) using voltage and current electrodes. To each side of the chamber, 10 mL of 37°C serum-free media was added. The chambers were water jacketed to allow the cells and media to be kept at a consistent physiological temperature (37°C).

Furthermore, the media was continuously oxygenated and circulated by bubbling 5% CO₂/95% O₂ directly into the media. Following clamping of the spontaneous transepithelial potential difference to zero for an equilibration period of at least 30 minutes, experimental compounds were added to the apical and/or basolateral membranes by means of the serum-free media and the cultures were monitored for changes in short circuit current (SCC) and transepithelial resistance (TER). SCC is a measure of net transepithelial ion flux; a positive deflection indicates either anion absorption or cation secretion and a negative deflection indicates the reverse. TER is a measure of changes in epithelial barrier function and was measured every 200 seconds through a 2mV pulse. The instantaneous change in SCC from the pulse was used to calculate TERs by means of Ohm's Law. These resistance calculations were converted to conductance by taking the inverse of the resistances. Conductance is a measure of transepithelial permeability. These parameters provided real time information about transepithelial ion flux in response to modulators of ion channels or intracellular signaling molecules. In each experiment, the cells were pre-incubated on both basolateral and apical sides with the experimental compounds for either 10 or 20 minutes or 24 hours prior to addition of TRPV4 agonist GSK1016790A as indicated on the figures. In all experiments, control and effector-treated samples were grown and analyzed in parallel cultures.

Statistics

Statistics were performed using Two-tailed Students t-test in Sigma Plot 13. $p < 0.05$ is considered significant. Students t-test was used to compare experimental groups to the control group as well as the experimental groups to each other as indicated by the symbols defined in the figure legends.

Results

To determine the ability of the PCP-R cell line to respond to and/or produce various cytokines and inflammatory mediators, RT-PCR was performed to identify specific genes of interest. Firstly, we focused on the receptors for the various cytokines tested in the cell line, of which only the mRNA for the second isoform of the IL-1 receptor (*Il1r2*) and the receptor for IL-10 (*Il10ra*) were absent (Figure 1A). Toll-like receptors (TLRs) are the major membrane-bound proteins necessary for the detection of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These receptors can also play a role in the activation of pathways responsible for cytokine production (1, 34). Within the pig genome, ten different *Tlr* genes have been described (*Tlr1-Tlr10*) (6). Somewhat surprisingly, all of these receptors were found to be present in the PCP-R cell line (Figure 1B).

Arachidonic acid (AA) is a substrate for the production of various inflammatory mediators, some of which have been reported to stimulate TRPV4 (5, 32, 47). Therefore, we investigated which of the enzymes necessary to metabolize AA were present in the cells (Figure 1C). Figure 1D provides a simplified summary of AA metabolism. Cytochrome P450 epoxygenases (CYP) are responsible for the production of EETs from AA. EETs have been reported as being activators of TRPV4 (47). While there are many known isoforms of *Cyp* genes, only two of these have currently been identified in the pig: *Cyp2c42* and *Cyp2e1*. Neither of these isoforms were present in the porcine CP cells (Figure 1C). Another pathway of AA metabolism utilizes lipoxygenases to produce hydroperoxides. In pigs, there are currently four lipoxygenase (*Alox*) gene sequences available: *Alox5*, *Alox12b*, *Alox15*, and *Alox15b*. Of these, mRNA from *Alox15* and *Alox15b* were present in the PCP-R cells. Finally, prostaglandins are formed when AA is metabolized by cyclooxygenases (COX) 1 or 2. Both *Cox1* and *Cox2* mRNA were found in the cell line (Figure 1C).

Utilizing electrophysiology to measure changes in transepithelial ion flux and epithelial barrier permeability, several pro- and anti-inflammatory cytokines and mediators that are hypothesized to be

important in inflammation-related neurological diseases were tested to assess how they would affect the basal characteristics of the PCP-R cells and subsequently their effect on the activation of TRPV4 via its agonist GSK1016790A. In each figure, controls were conducted by adding a vehicle treatment either 10 minutes or 24 hours prior to the addition of the TRPV4 agonist (GSK1016790A). Vehicle treatments consisted of only the solvent by which each cytokine was solubilized and administered. Concentrations for each cytokine were determined by either by the manufacturer's instruction or by the specific IC_{50} . All cytokines were initially added 10 minutes prior to adding the agonist. No effects were seen with any of the tested cytokines with this short pre-incubation (data not shown). In immune cells, cytokine stimulation has been reported to activate innate immune genes after 24 hours post-infection (43). Therefore, incubations were lengthened to 24 hours. Interleukin (IL)-1 β and Tumor Necrosis Factor (TNF)- α are two of the most prominent pro-inflammatory cytokines seen in neurodegenerative diseases (2, 7, 15, 41, 48). After 24 hour pre-incubation of the PCP-R cells with either cytokine, both effectors had significant effects on TRPV4-mediated ion flux, measured as short-circuit current (SCC), albeit on different phases of the ion transport response. IL-1 β caused an attenuation of the agonist response measured as SCC in the early (0-15 minute) timeframe while TNF- α caused an expedited increase in SCC toward baseline in the late response (15-30 minute) to the agonist. Interestingly, these two pro-inflammatory cytokines had opposite effects on transepithelial permeability, measured as conductance. TNF- α pre-incubated cells showed ion transport and conductances that were statistically the same as controls after a 24-hour incubation. IL-1 β caused a significant attenuation in the TRPV4 agonist-mediated change in conductance which paralleled the effect on transepithelial ion flux measured as SCC (Figures 2 & 3).

Interestingly, 24 hour pre-incubation with TGF- β 1, which has been reported to have both pro- and anti-inflammatory effects, showed changes in SCC and conductance that were remarkably similar to IL-1 β (Figure 4). However, the same treatment with IL-6, another dual pro- and anti-inflammatory

cytokine, yielded no significant change in the response to the TRPV4 agonist (Figure 5). Finally, the cells were pre-incubated with the cytokines IL-10 and IL-4. Neither of these anti-inflammatory cytokines had any long-term effects on either transepithelial ion flux or conductance changes in response to agonist stimulation (Figures 6 & 7). The receptor for IL-6 is present; however, there is no apparent receptor for IL-10 (Figure 1A).

NF- κ B is a transcription factor often associated with pro-inflammatory signaling pathways. Pro-inflammatory cytokines, mainly IL-1 β and TNF- α , are considered activators of NF- κ B, thereby mediating downstream effects on the activation of inflammation and further production of cytokines (23). As such, the PCP-R cells were pre-incubated with a selective inhibitor for NF- κ B, PDTC (52). Inhibiting NF- κ B 10 minutes prior to adding the TRPV4 agonist caused a potentiation of the intermediate (5-10 min) phase of TRPV4-mediated ion flux. There was also a corresponding increase in permeability compared to the control that is entirely consistent with the time-course of the SCC response. However, this change did not persist; permeability returned to control levels 30 minutes after agonist addition (Figure 8). As NF- κ B is a transcription factor, it might take longer for its true effects to become evident. Therefore, we also inhibited the pro-inflammatory mediator 24 hours prior to adding the TRPV4 agonist (Figure 9). In this instance a very different result occurred. TRPV4 agonist-stimulated transepithelial ion flux was significantly reduced compared to the control. Furthermore, the 24 hr incubation resulted in a substantial increase in basal conductance prior to agonist addition. In contrast to the control, the cellular permeability did not increase substantially after agonist addition in the PDTC pretreated cells.

To determine if the observed changes in TRPV4 activity may be due to changes in transporter abundance, the quantitative effect of specific cytokines on the mRNA expression of TRPV4 in PCP-R cells was examined (Figure 10). The cells were treated with specific cytokines for 24 hours and qPCR performed. There were no significant changes in TRPV4 expression levels when incubated for 24 hours with the pro-inflammatory cytokines TNF- α and IL-1 β . Similarly, cells treated with the anti-inflammatory

cytokines IL-4 and IL-10 showed no statistically significant perturbations in TRPV4 expression at the mRNA level. Finally, neither TGF- β 1 nor IL-6, shown to be both pro- and anti-inflammatory, had marked effects on TRPV4 expression.

AA and its metabolite, 5,6-EET, are often cited as being activators of TRPV4 (5, 7, 31, 32, 47). Specifically, AA has been reported to be able to activate TRPV4 at concentrations between 3-10 μ M (5, 31, 47). However, when AA was added to the PCP-R cells at 10 μ M, we did not see an increase in TRPV4-mediated ion flux or permeability changes (Figure 11). Therefore, we performed a dose response with AA in the PCP-R cells in relation to TRPV4 activation via its agonist GSK1016790A. With increasing concentrations of AA, the change in TRPV4-mediated ion flux decreased significantly (Figure 12). At the highest concentration tested (100 μ M), we saw a complete inhibition of both TRPV4-mediated ion flux and change in permeability (Figure 11).

In order to further elucidate the role of the AA pathway in the function of TRPV4, we utilized various inhibitors for the enzymes involved in AA metabolism to determine which of the pathways is involved in TRPV4 activity. Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) able to prevent the metabolism of AA into the pro-inflammatory prostaglandins by non-selectively inhibiting both cyclooxygenases (22, 25, 32, 33, 51). However, pre-incubation with indomethacin did not change the TRPV4-mediated ion movement or permeability responses (data not shown). Eicosatetraynoic acid (ETYA) is an inhibitor of all cyclooxygenases and lipoxygenases, which produce prostaglandins and leukotrienes, respectively. When added to the cells 20 minutes prior to the TRPV4 agonist, this inhibitor caused a significant attenuation to both the SCC and conductance changes. Finally, we pre-incubated the cells with SKF-525A, a non-specific inhibitor of cytochrome P450, responsible for the production of EETs. This inhibitor caused a complete inhibition of both transepithelial ion flux and changes in cellular permeability caused by TRPV4 activity (Figure 13).

As previously mentioned, the anti-inflammatory AA metabolite EET is largely considered to be the most probable metabolite involved in TRPV4 activation (5, 7, 32, 47). However, when the PCP-R cells were incubated with the isoform 5,6-EET for 10 minutes prior to adding the agonist, we did not see significant evidence of a change in TRPV4-mediated activity. While the 10-30 minute phase post-agonist addition appeared inhibited with both SCC and conductance measurements, this difference was not statistically different (Figure 14). The conductance change is similar to the change seen with higher doses of AA.

Discussion

TLRs are found on microglia, astrocytes, and other immune cells and are well known for playing a key role in the initiation of innate immunity in response to PAMPs and DAMPs. The TLRs not only play a major role in a cell's ability to respond to inflammatory reactions, but they also enable the cells to contribute to the inflammation. When the TLRs detect PAMPs or DAMPs, this can cause a cascade of events leading to activation of transcription factors NF- κ B and AP-1 and subsequent cytokine production (6, 34). In particular, TLR4 is best known for producing key pro- and anti-inflammatory cytokines, such as TNF- α , IL-6, and IL-10, and is most often associated with inflammation (6, 34). It is also of interest that expression levels of TLRs have been found to change during aging (34). Specifically, pro-inflammatory cytokine production increases while secretion of anti-inflammatory cytokines decreases. TLRs have been associated with age-related inflammatory neurodegenerative diseases, such as ischemic stroke, Alzheimer's disease, and multiple sclerosis (34).

The PCP-R cell line contains mRNA for the receptors of most cytokines tested, except for IL-10, and all 10 of the known porcine TLRs. This indicates that CP cells have the potential to respond to cytokines and inflammatory signals as well as produce several different cytokines, which would be secreted into the CSF. While we did not explore cytokine production in these studies, it is likely that autocrine or paracrine cytokines will have effects on CSF production by the CP cells.

In studies using lung and endothelial tissue, TRPV4 antagonists have been found to reduce pulmonary edema and protect against sepsis, respectively. In both studies, TRPV4 inhibition was also found to decrease inflammation and cytokine production (3, 9). This would suggest that TRPV4 acts in a pro-inflammatory manner. To confirm this in the brain, we looked at various types of cytokines, including pro-inflammatory, anti-inflammatory, and paradoxical pro- and anti-inflammatory cytokines. Indeed, long-term (24 hours) incubation with the pro-inflammatory cytokines had significant effects on TRPV4 activity. However, the responses seen with the two prominent pro-inflammatory cytokines were not the same. Furthermore, the paradoxical cytokines, TGF- β 1 and IL-6, had varying effects with TGF- β 1 causing strikingly similar results as IL-1 β while IL-6 had no significant effect.

The exact nature of the electrophysiological response to TRPV4 agonists is undoubtedly complex. We have previously shown that a component of the response is due to potassium secretion via the intermediate conductance (IK) channel (37). However, the complex nature of the SCC response, as well as the differential effects of cytokines on this response, strongly suggests a multitude of intersecting ion fluxes. SCC is a measure of net transepithelial ion movements. Although the current returns to “baseline” about 30 minutes after treatment with a TRPV4 agonist, the sustained high conductance suggests a continued transepithelial ion flux with anions and cations moving in opposite directions, resulting in an electroneutral short circuit current, or net zero electrogenic flux. The cytokines that have an effect on net ion fluxes appear to be doing so by altering different components of the complex response.

While it cannot be assumed that all pro-inflammatory pathways cause an attenuation or inhibition of the response, it is most likely, based upon our observations, that TGF- β 1 is acting in a pro-inflammatory manner while IL-6 is acting in an anti-inflammatory manner in the CP cells. Together, these data imply that pro-inflammatory cytokines diminish TRPV4 function. While upstream activators, TNF- α and IL-1 β , required 24 hours to mediate a measurable effect on the PCP-R cells, the finding that short-

term inhibition of the pro-inflammatory transcription factor NF- κ B caused a potentiation of TRPV4-mediated ion flux and permeability changes cannot be easily explained. This may indicate a preexisting tone of NF- κ B activation in the PCP-R cells. The inhibition of this baseline activity may cause an increase in barrier permeability as ions are more easily able to move across the membrane. After 24 hours of inhibition, the basal conductance is substantially higher than the control, which further suggests underlying NF- κ B activity. However, the attenuated response upon TRPV4 stimulation suggests that NF- κ B activation, and possibly its downstream inflammatory effectors, can be considered contributors to TRPV4-mediated ion flux and permeability changes.

Effects seen after long-term cytokine incubations raised the question of whether the changes in TRPV4-mediated activity were due to an alteration of the transcription of TRPV4. However there were no statistical differences in the quantitative PCR of TRPV4 in response to 24-hour cytokine incubations indicating that the cytokines are having an effect on the downstream targets of the ion channel.

Several publications have reported that AA and its metabolites are able to cause a substantial elevation of $[Ca^{2+}]_i$ by activating TRPV4 with concentrations as low as 3 μ M AA (5, 31, 47). Specifically, these studies also cite EETs as the predominant activator of the cation channel (5, 7, 32, 47). However, it has also been reported that endogenous levels of AA can differ depending on the tissue type, with physiological concentrations varying from 2 to 16 μ M. In pancreatic islet cells, the concentration was found to be as high as 75 μ M (31). Based on previous studies, we hypothesized that TRPV4 activity would be potentiated after pre-incubation with either physiological concentrations of AA or its metabolites. Surprisingly, concentrations of AA comparable to previous publications (10 μ M) failed to potentiate TRPV4-mediated activity. Rather, AA (up to 100 μ M) caused an inhibition of the TRPV4-mediated response to transepithelial ion flux and cell permeability in a dose-dependent manner. AA inhibition of TRPV4-mediated ion flux in the PCP-R cells suggests a cell-type specific effect on TRPV4.

Taken together with our cytokine data, in which selective pro-inflammatory cytokines down regulate TRPV4-mediated activity, this would imply that inflammation acts to decrease TRPV4 function.

There is controversy concerning which isoform of EET is responsible for activating TRPV4 (12). It is possible that different isoforms of EET affect TRPV4 in a cell-specific manner. While 5,6-EET is most often considered to be the activating isoform in other cell types, this does not appear to be the case when added exogenously in CP cells. It is possible that the 5,6-EET is only effective when produced inside the cells. However, it is unknown whether the PCP-R cell line is able to produce EETs. The production of EETs occurs when AA is metabolized by the enzymes cytochrome P450 epoxygenase, of which there are multiple isoforms (40). Currently, only two of these enzymes have been sequenced in pigs, *Cyp2c42* and *Cyp2e1*, neither of which were present in the cells. However, there are several other isoforms still untested, including those most associated with EET production, *Cyp2c8*, *Cyp2c9*, *Cyp2c19* and *Cyp2j2* (40). Therefore, these data cannot lead to the conclusion that there are no cytochrome P450 epoxygenases present. Furthermore, the dramatic inhibitory effects of the SKF-525A CYP450 inhibitor imply that endogenous EET production may activate TRPV4 in these cells. It is possible that a different EET isoform other than 5,6-EET is affecting TRPV4 in the CP or that the production occurs within a membrane limited compartment that is closely linked to the channel.

We determined the potential effects of the other AA metabolites in the CP cells. Both isoforms of cyclooxygenase were present in the PCP-R cells, therefore the cells are able to produce prostaglandins, prostacyclin, and thromboxane A₂ (36). However, inhibition of both COX1 and COX2 with the NSAID Indomethacin did not alter the effects of TRPV4 activation in the cells. Thus, these metabolites likely do not play a role in TRPV4-mediated pathways.

The lipoxygenase (LOX) pathway contains seven known gene isomers: *Aloxe3*, *-5*, *-5ap*, *-12*, *-12b*, *-15*, and *-15b* (30). Of the enzymes, ALOX12B, *-E3*, and *-15B* are found in the skin and other epithelial cells and ALOX5, *-12*, and *-15* are located primarily in immune cells. Many of the isomers have been

reported in different forms of cancers, but all of them can be involved in inflammation (30). Of the four sequenced porcine isomers, only ALOX15 and ALOX15B were found in our cell line. Interestingly, ALOX15 is considered one of the isoforms that can be partially controlled by cytokines. Anti-inflammatory cytokines, such as IL-4 and IL-13, have the potential to increase expression of this gene, which can then decrease production of pro-inflammatory cytokines, such as IL-12 (30). The isoform has also been associated with maintaining dermal integrity through anti-inflammatory suppression of inflammation in the skin (19). On the other hand, ALOX15 is also capable of increasing pro-inflammatory matrix metalloproteinase (MMP) expression and contributing to arthritic disease progression (49, 53). Once again, this leads to the question of whether TRPV4 is part of a pro- or anti-inflammatory process. Less is known about the ALOX15B isoform, but it has been implicated in decreasing epithelial barrier permeability (30). Thus, increases in this isoform could contribute to increases in transepithelial ion flux and conductance in the CP tissue.

As previously stated, inhibiting cyclooxygenases alone does not affect the cellular response to TRPV4 agonists. However, when both cyclooxygenases and lipoxygenases are inhibited, there is a partial inhibition of the TRPV4-mediated response. As shown above, inhibiting the CYP450s and subsequent EET production results in complete inhibition of the TRPV4-mediated response. Thus, the interaction between the AA metabolites and TRPV4 is complicated and may involve both positive and negative effectors produced in a stimulus dependent manner. Future experiments will investigate how these metabolites interact with the channel.

In summary, TRPV4-mediated transepithelial ion flux and increases in conductance are negatively affected by pro-inflammatory cytokines and mediators. We also found that, in contrast to previously reported studies, arachidonic acid does not increase TRPV4-related activity. Rather, when added to choroid plexus cells, arachidonic acid diminished TRPV4-mediated transepithelial ion flux and changes in permeability. Finally, we confirmed that in the choroid plexus, inhibition of EET production

394 causes a complete inhibition of TRPV4-mediated transepithelial ion flux and cellular permeability
395 changes. Therefore, in the choroid plexus, TRPV4 regulation by inflammatory mediators is complex,
396 consistent with the role of this channel as a hub protein which can integrate multiple extracellular
397 signals and subsequently mediate a multiphasic change in transepithelial ion transport.

398

399 **Acknowledgements**

400 We would like to thank Nicolas Berbari and Patrick Antonellis for their input in optimizing and
401 troubleshooting our qPCR assay methods.

402

403 **Grant Support**

404 This research was supported by the Indiana Clinical and Translational Sciences Institute Predoctoral
405 Award UL1TR001108 (SS) and the United States Department of Defense Investigator Initiated Research
406 Award W81XWH-16-PRMRP-IIRA (BBY).

407

408 **Disclosures**

409 None.

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Figure Legends

Figure 1: RT-PCR in PCP-R cells. A) mRNA for the various cytokine receptors. All were present except for the second isoform of the IL-1 receptors (*Il1r2*) and the IL-10 receptor (*Il10ra*). B) mRNA for porcine Toll-like receptors (TLR) 1-10 is present in the PCP-R cell line. C) RT-PCR results for various enzymes capable of arachidonic acid (AA) metabolism. Cytochrome P(CYP)450 epoxygenases, *Cyp2c42* and *Cyp2e1*, are both absent. mRNA for lipoxygenase (*Alox*)15 and *Alox15b* are present whereas *Alox5* and *Alox12* are absent. Cyclooxygenase (*Cox*) 1 and 2 are both present. *Gapdh* was utilized as positive controls for each gel. Band product sizes can be found in Table 2. Ladder = 100 bp. T = Template. Lanes denoted (+) or (-) "T" signifies addition or no addition of template control. D) Diagram of AA metabolism via the three different pathways, pathway inhibitors, and subsequent metabolites.

Figure 2: Pre-treatment of PCP-R cells with pro-inflammatory cytokine IL-1 β for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. IL-1 β (10 ng/mL in PBS) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with IL-1 β is signified by white circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current. * = $p < 0.05$ against control.

Figure 3: Pre-treatment of PCP-R cells with pro-inflammatory cytokine TNF- α for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. TNF- α (0.15 ng/mL in deionized water) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with TNF- α is signified by white circles.

GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current. * = $p < 0.05$ against control.

Figure 4: Pre-treatment of PCP-R cells with pro- and anti-inflammatory cytokine TGF- β 1 for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. TGF- β 1 (2 ng/mL in 4 mM HCl) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with TGF- β 1 is signified by white circles.

GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current. * = $p < 0.05$ against control.

Figure 5: Pre-treatment of PCP-R cells with anti-inflammatory cytokine IL-6 for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. IL-6 (10 ng/mL in 4 mM HCl) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with IL-6 is signified by white circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current.

Figure 6: Pre-treatment of PCP-R cells with anti-inflammatory cytokine IL-10 for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. IL-10 (5 ng/mL in PBS) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with IL-10 is signified by white circles. GSK1016790A control is

signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current.

Figure 7: Pre-treatment of PCP-R cells with pro- and anti-inflammatory cytokine IL-4 for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. IL-4 (20 ng/mL in PBS) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with IL-4 is signified by white circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current.

Figure 8: Pre-treatment of PCP-R cells with NF- κ B inhibitor PDTC for 10 minutes prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. PDTC (10 μ M in DMSO) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -10 minutes. TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 10-minute incubation with PDTC is signified by white circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current. * = $p < 0.05$ against control.

Figure 9: Pre-treatment of PCP-R cells with NF- κ B inhibitor PDTC for 24-hours prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. PDTC (10 μ M in DMSO) was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -24 hours (not shown). TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 24-hour incubation with PDTC is signified by white circles. GSK1016790A control is signified by

black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated.

SCC = short circuit current. * = $p < 0.05$ against control.

Figure 10: Relative fold change of TRPV4 mRNA in PCP-R cells 24 hours post-incubation with specific cytokines known to be involved in inflammatory responses. TNF- α (0.15 ng/ml), IL-1 β (10 ng/ml), TGF- β 1 (2 ng/ml), IL-4 (10 ng/ml), IL-6 (20 ng/ml), or IL-10 (5 ng/ml) were added both apically and basolaterally in individual wells (n=5 for each cytokine). The fold change of TRPV4 in treated wells is presented relative to the normalized controls (n=6). GAPDH and RPS18 were used as housekeeping genes to determine the $2^{-\Delta\Delta CT}$ fold change in TRPV4. The minimum and maximum fold-change values are shown as individual points on the graph. Circles signify the maximum and minimum values. Lines within the boxes signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated.

Figure 11: Effect of a range of concentrations of arachidonic acid (AA) on TRPV4-mediated transepithelial ion flux and cellular permeability. AA solubilized in 100% EtOH was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -10 minutes. TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. AA pre-treatment at 10 μ M is signified by white circles. AA pre-treatment at 100 μ M is signified by gray circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current. * = $p < 0.05$ against control. τ = $p < 0.05$ against 10 μ M AA pre-treatment.

Figure 12: Dose effect of arachidonic acid (AA) on the change in TRPV4-mediated transepithelial ion flux in response to TRPV4-agonist GSK1016790A. PCP-R cells were pre-treated with arachidonic acid (AA) solubilized in 100% EtOH at various concentrations 10 minutes prior to TRPV4 agonist addition. AA was

added to the PCP-R media on both basolateral and apical sides of the membrane. The change in transepithelial ion flux was measured 5 minutes after TRPV4 agonist addition (Δ SCC). Error bars signify the S.E.M. for number of experiments indicated. Δ SCC = change in short circuit current. * = significant against 0 μ M AA control with p values given.

Figure 13: Pre-treatment of PCP-R cells with arachidonic acid (AA) metabolism inhibitors 20-minutes prior to TRPV4 agonist addition. Effect of inhibitor pre-incubation on TRPV4-stimulated transepithelial ion flux and conductance was measured. All inhibitors were solubilized in 100% EtOH. Cyclooxygenase and lipoxygenase inhibitor ETYA was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -20 minutes. Cytochrome P450 inhibitor SKF-525A was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -20 minutes. TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. ETYA pre-treatment is signified by white circles. SKF-525A pre-treatment is signified by grey circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current. * = $p < 0.05$ against control. τ = $p < 0.05$ against ETYA pre-treatment.

Figure 14: Pre-treatment of PCP-R cells with arachidonic acid metabolite 5,6-EET at 10-minutes prior to TRPV4 agonist addition. Effect on transepithelial ion flux and conductance was measured. 5,6-EET solubilized in 100% EtOH was added to the PCP-R media on both basolateral and apical sides of the membrane at T = -10 minutes. TRPV4 agonist GSK1016790A was added to basolateral side of the membrane only at T = 0 minutes. 10-minute incubation is signified by white circles. GSK1016790A control is signified by black circles. Circles signify mean values and bars signify $\pm$ S.E.M. for number of experiments indicated. SCC = short circuit current.

660 **Table 1.**

Inflammation Pathway	Cytokines	References
Pro-inflammatory	IL-1 β , TNF- α	2, 7, 15, 41, 48
Anti-inflammatory	IL-10, IL-4	15, 41, 48
Pro- and Anti-Inflammatory	TGF- β , IL-6	2, 7, 8, 14, 15, 24, 38, 41, 48

661

662 Table 1: Reported cytokines function in the inflammatory pathway.

663

<i>Sus</i> <i>Scrofa</i> gene	Protein	Primer Sequences 5' - 3'		Product Size (bp)
		Forward Primer	Reverse Primer	
<i>Il1r1</i>	IL1R1	TTACACAGAGACTACCGGTTGC	CAGTTTTATCACCCGGGAGACA	263
<i>Il1r2</i>	IL1R2	AAATGATTCCACGACCATCCCA	TCCCAAACAGGATGATGAGG	874
<i>Tnfrsf1a</i>	TNFRSF1A	GAAACAGGACACCATCTGCAAC	AAGCTAAGCCAACGAAGAGGAA	218
<i>Tgfb1</i>	TGFBR1	GAGACAGGCCATTTGTATGTGC	TAAATCTCTGCCTCACGGAACC	520
<i>Tgfb2</i>	TGFBR2	GGAAGCTGATGGAGTTCAGTGA	TCTCTGTCTCCAGGAGGCATA	252
<i>Tgfb3</i>	TGFBR3	ATATTCACCACAAGCCTGTCGT	ATACTCCTTCGGGCCCAATTT	224
<i>Il6r</i>	IL6R	AAGATTCTGCGTCGGTACCATT	TGTTGCTGACATCATAAGGGCT	312
<i>Il10ra</i>	IL10RA	GGATGAAGTGACTCTGACGGTT	GCTTCACCCTGACACACAATTC	257
<i>Il4r</i>	IL4R	AGAGCTACCTGTACTCGGAAC	CCCTTTGTCTCCTCCTCTTCC	695
<i>Tlr1</i>	TLR1	TGTCCCACAACAAGTTGGAGAA	TTGTGGGAAACTGAACACCTCA	650
<i>Tlr2</i>	TLR2	TCCCAAATCTGCGAATCCTGAA	ATGCAACCTCCGGACTGTTAAT	512
<i>Tlr3</i>	TLR3	CGATGACCTCCCGGCAAATATA	GAGATTTTCCAGTTGGAGCTGC	382
<i>Tlr4</i>	TLR4	TTCTCTCCTGCCTGAGATCTGA	ACTCCAGGTAGGTATTCCTGCT	555
<i>Tlr5</i>	TLR5	TCCTGTGGTCTCTCTGATGCTA	GGGTCATACACTTCCCCCAAT	284
<i>Tlr6</i>	TLR6	AGACAATCTTGTGCCATCCCAT	GGCCCTTGAGTGAGTTCCAATA	409
<i>Tlr7</i>	TLR7	GACACTAAAGACCCAGCAGTGA	CTGAAGGGGCTTCTCAAGGAAT	297
<i>Tlr8</i>	TLR8	CTGAGGCAGAACAGGATTCCT	TTCATCACCCAGTCTGTGACAG	542
<i>Tlr9</i>	TLR9	GCCTACGAACTCTCAACCTCAA	GGAAGTTCTCACTCAGGTCCAG	696

<i>Tlr10</i>	TLR10	TCAGGTGCTTGCCCAGAAATAT	TCTTGCCAGGATCAGAGTTTCC	717
<i>Cyp2c42</i>	CYP2C42	TCCTGTCTGCTTCTCCTTTAC	GGGAGCACAGTCCAGGATAAAA	489
<i>Cyp2e1</i>	CYP2E1	TCGAGATTTCACTGACACCCTG	GTAAAGTGCTGCAAGATGGCA	592
<i>Alox5</i>	5-LOX	TGACAGTGGATGAAGAACTGGG	TGTTTTTGCCGTGTTTCCAGTT	259
<i>Alox12b</i>	12R-LOX	ACACCATCCAGATCAACAGCAT	CCAGAGGACCAATAGGACGATG	602
<i>Alox15</i>	12/15-LOX	CAGGAGGATGAACTCTTTGGCT	TCGAATATACCTCCGTCCGAGA	578
<i>Alox15b</i>	15-LOX2	GCGAAATGCTGAGTTCTCCATC	CAGGGTGGAATAGTTCAGCTGT	283
<i>Cox1</i>	COX1	TCACCCGCAATACTATGAGCTC	TGTGTGATAGGGAGGAGGACAT	510
<i>Cox2</i>	COX2	CCCTTTCCAAGTAGGCTTCCAA	TAGTCGTCCTGGGATAGCATCT	529
<i>Gapdh</i>	GAPDH	TTTAACTCTGGCAAAGTGGAC	CATTGTCGTACCAGGAAATGAG	884

Table 2: *Sus scrofa* forward and reverse primer sequences used for RT-PCR with corresponding product sizes (bp). Three different primer sets were generated and tested for each gene. Primers included in this table were utilized for Figure 1A-C. GAPDH was used as a positive control.

Figure 1A.

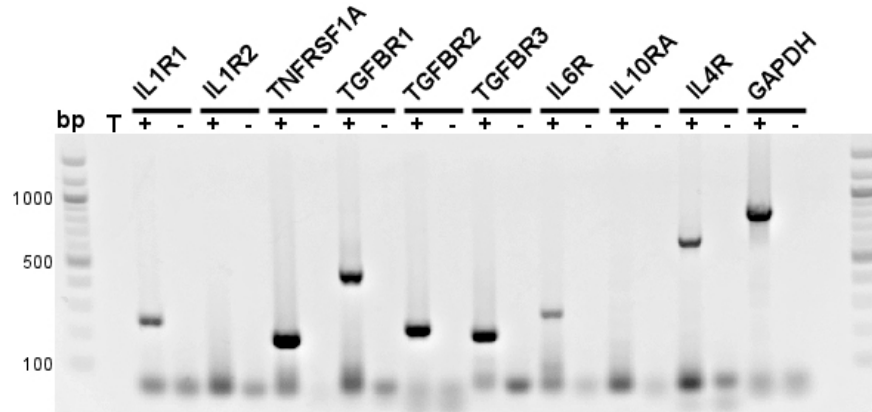


Figure 1B.

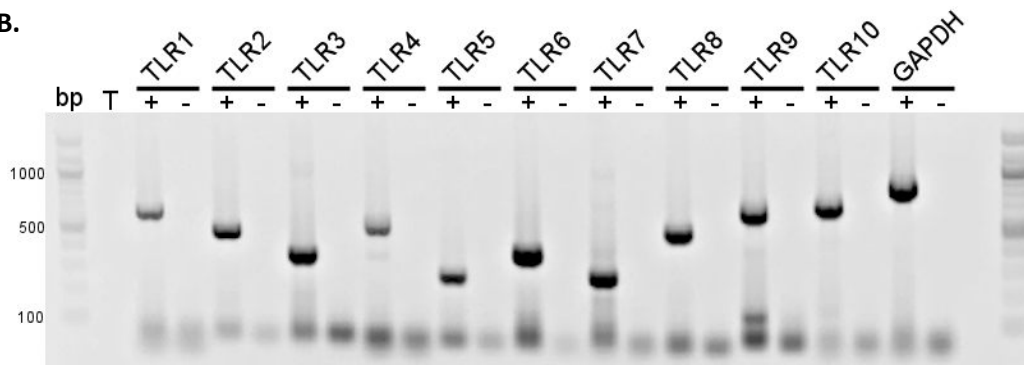


Figure 1C.

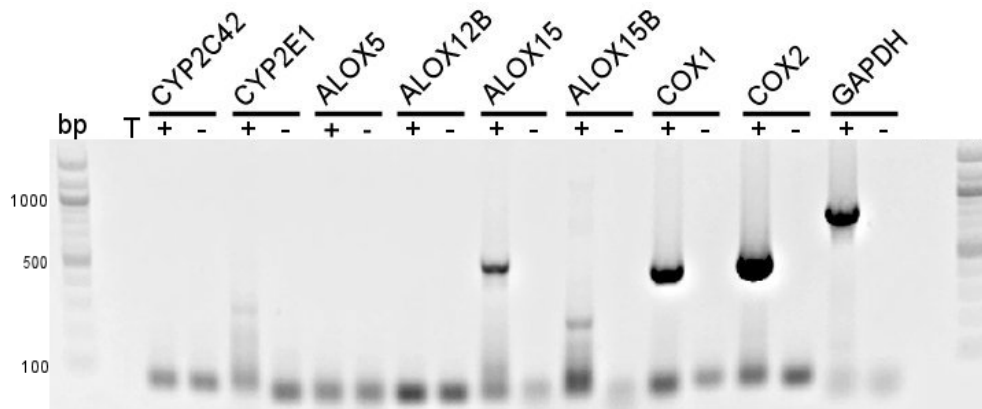


Figure 1D.

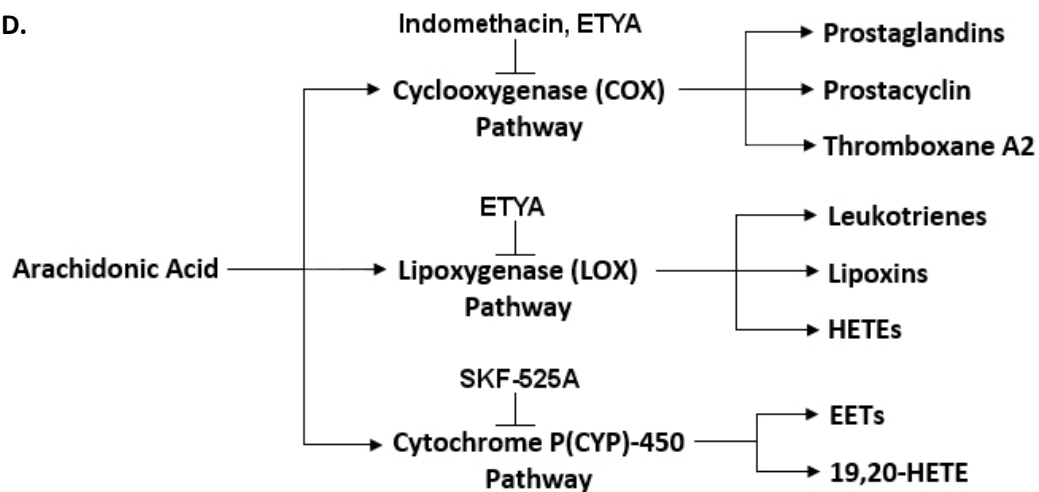


Figure 2.

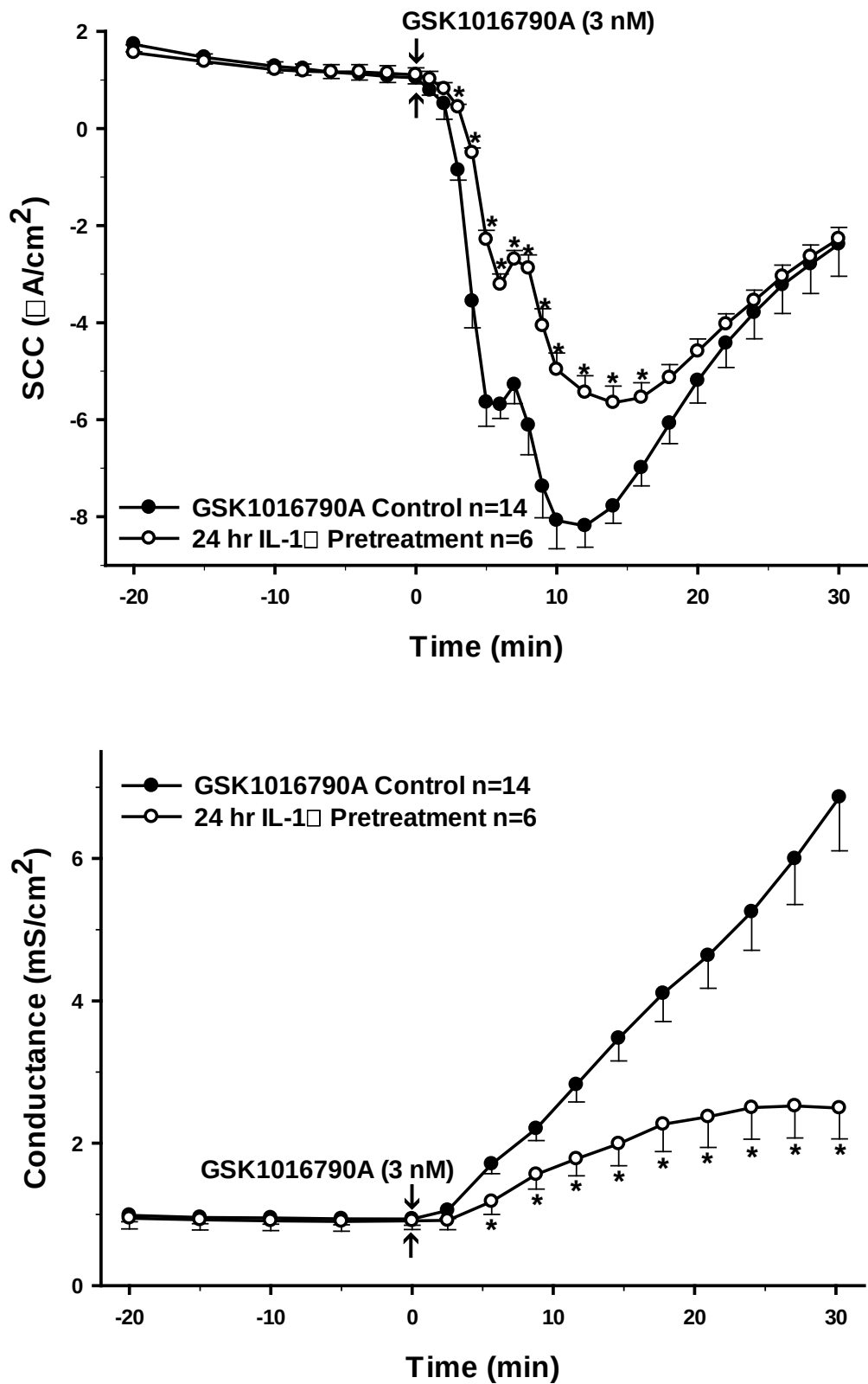


Figure 3.

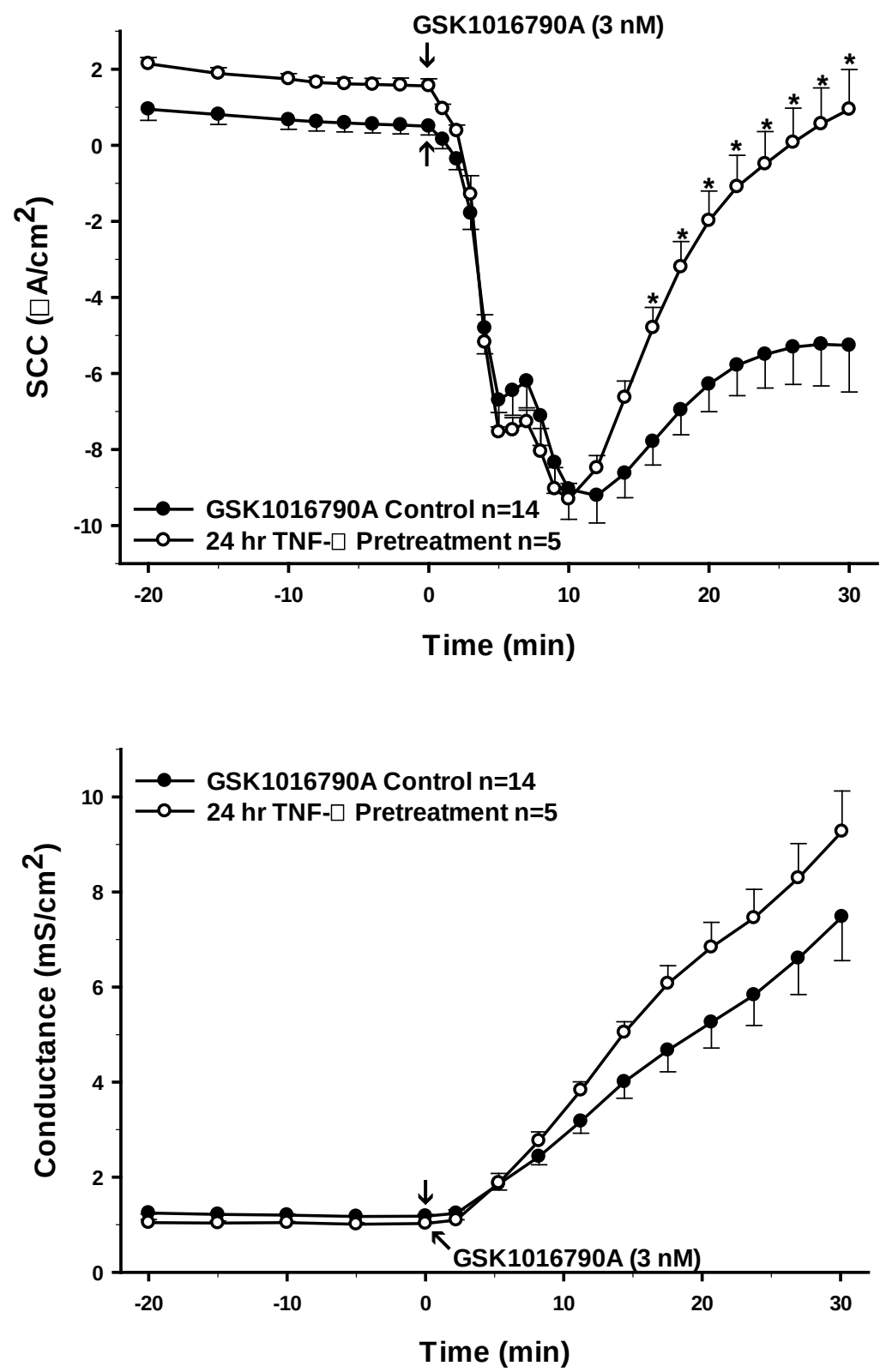


Figure 4.

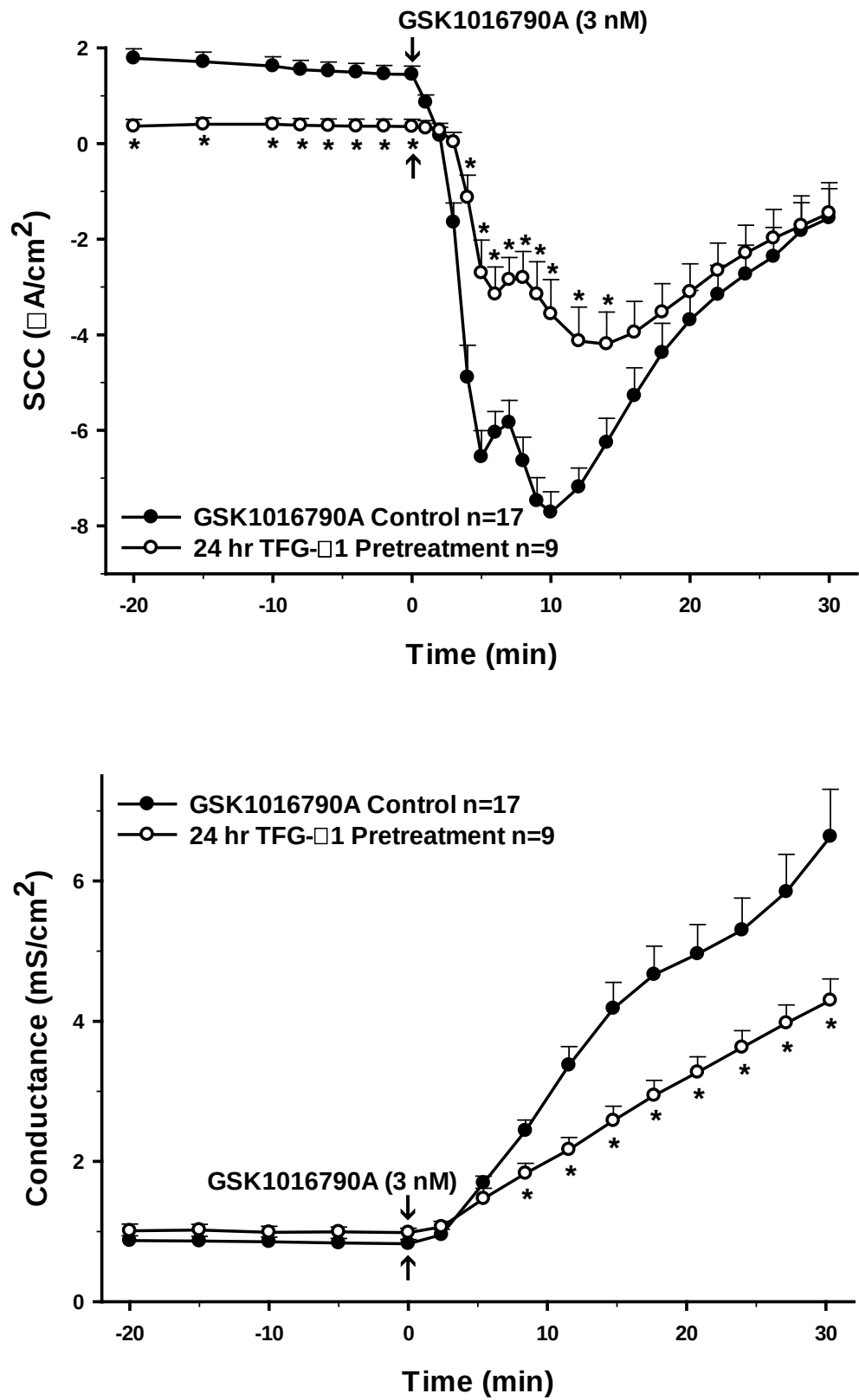


Figure 5.

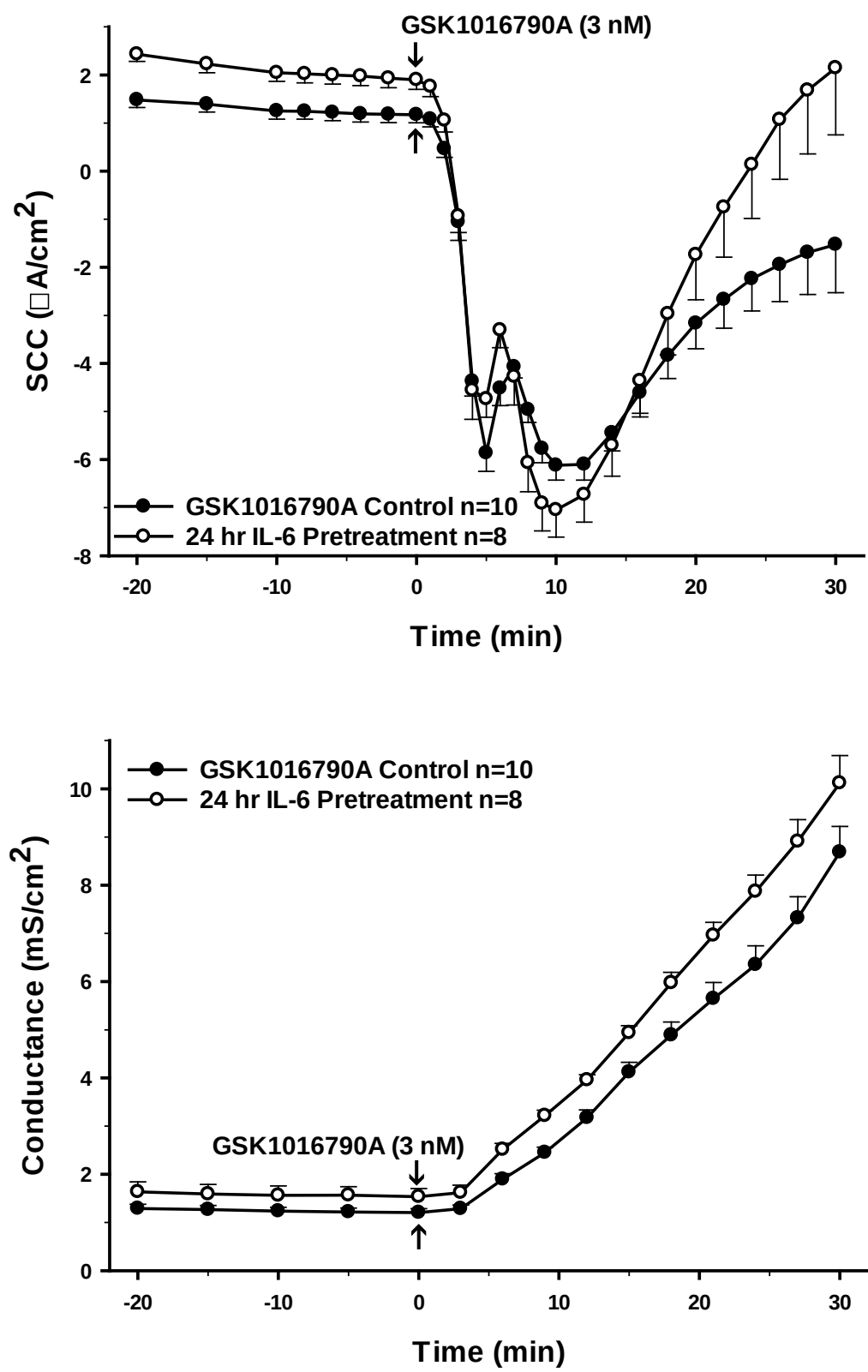


Figure 6.

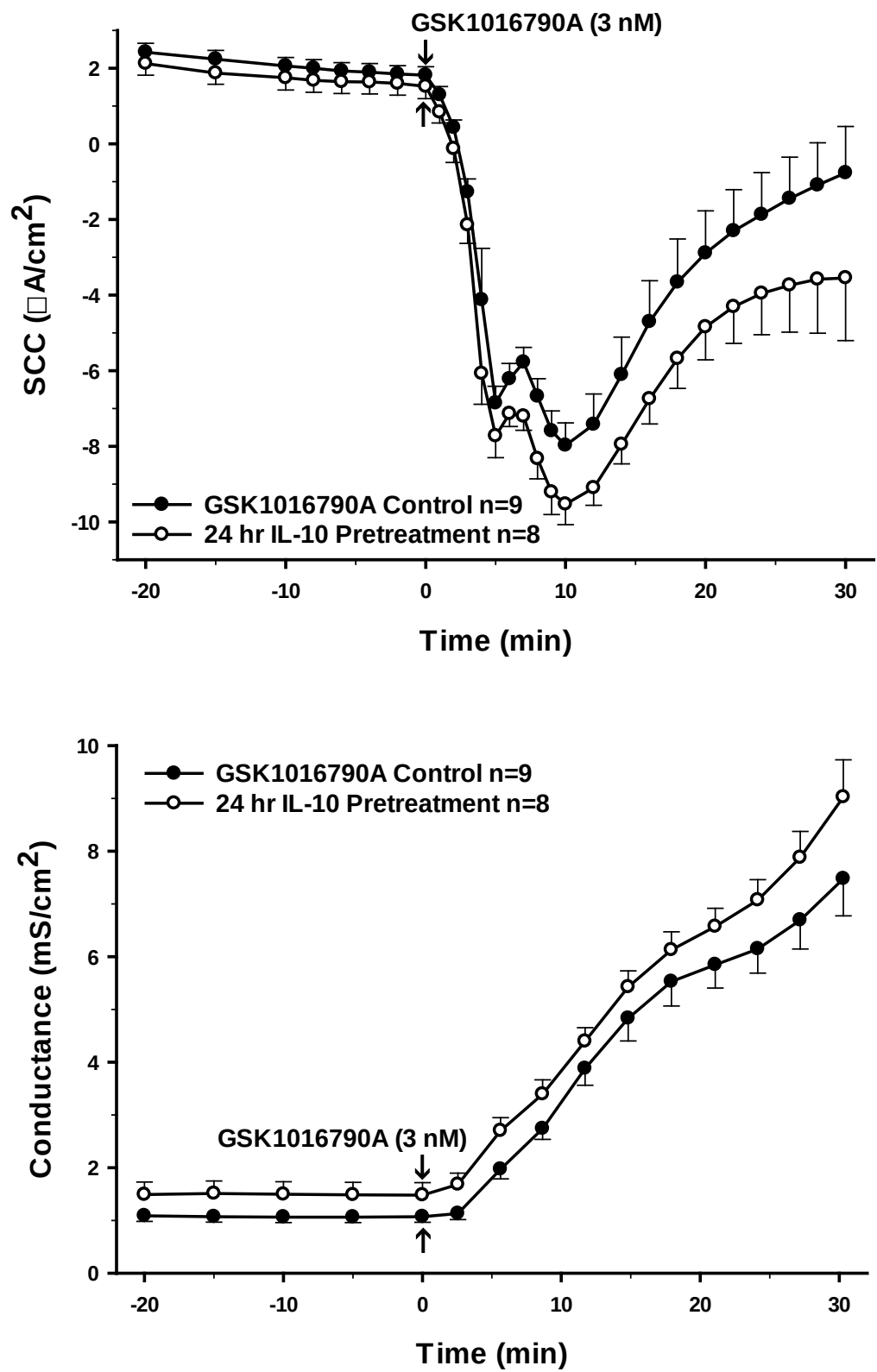


Figure 7.

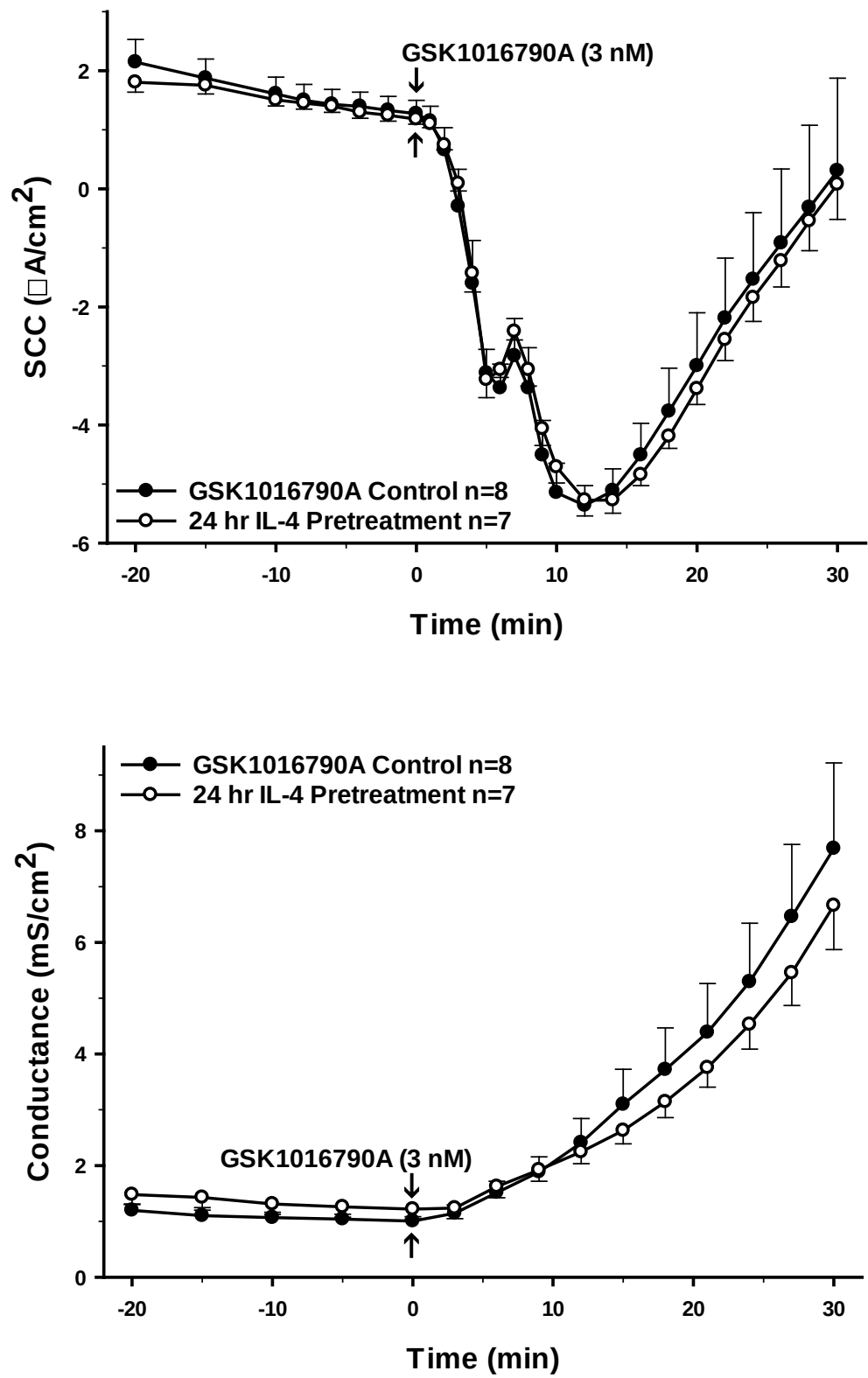


Figure 8.

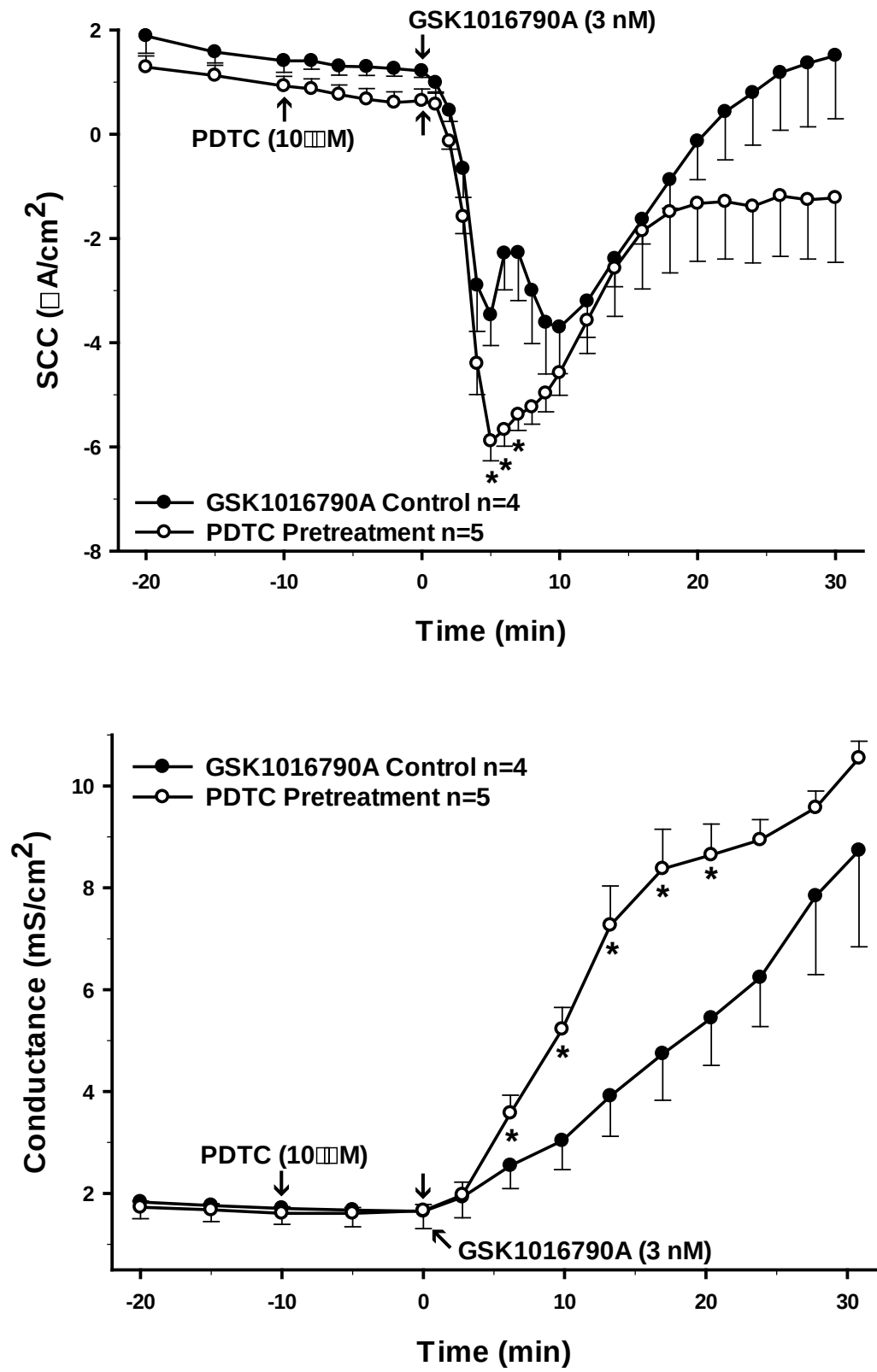


Figure 9.

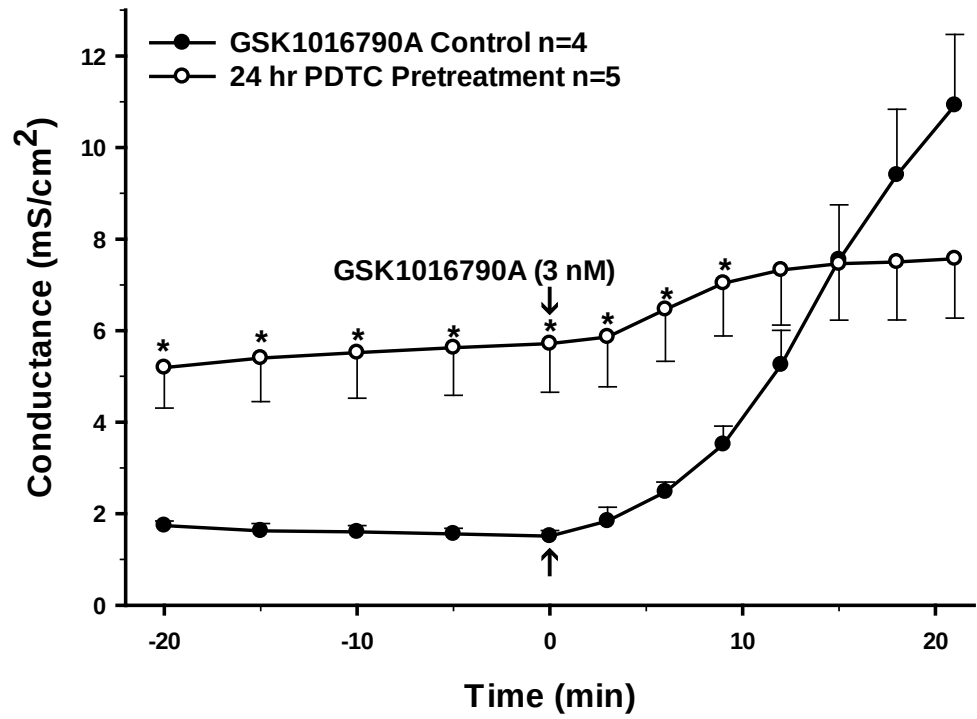
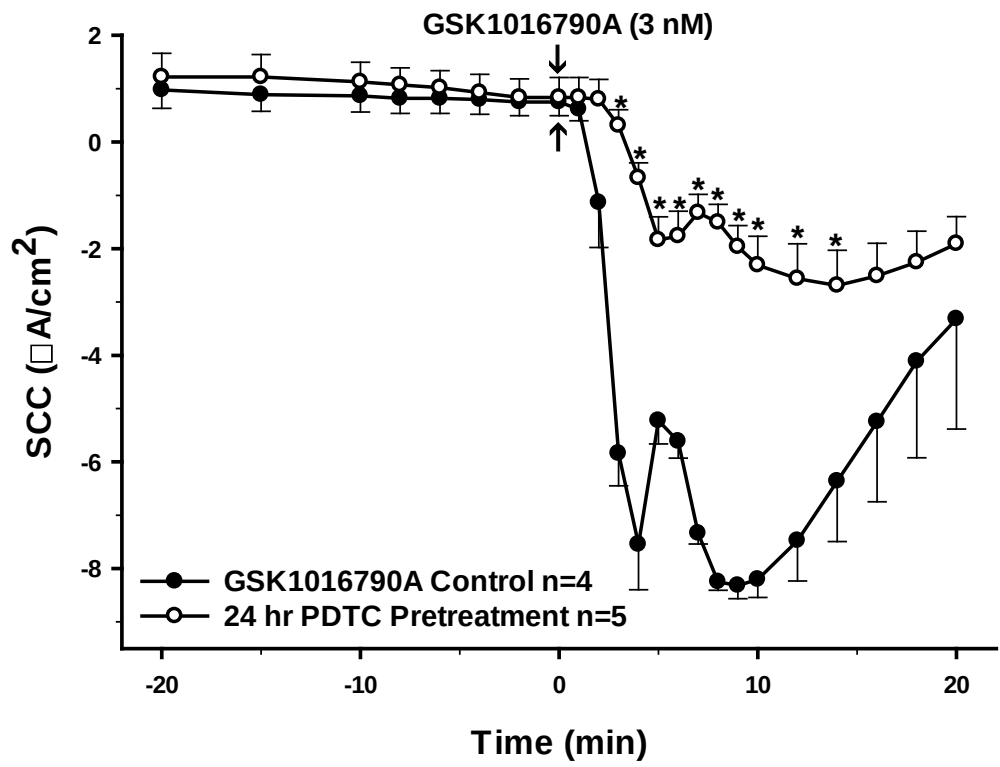


Figure 10.

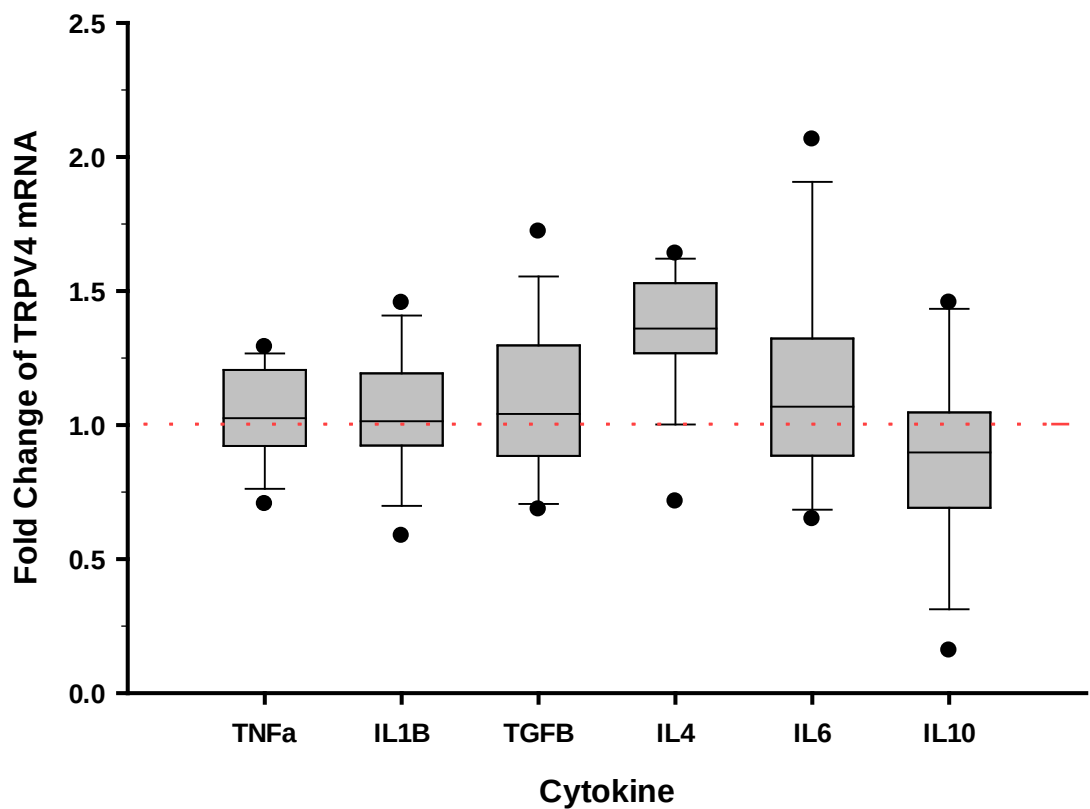


Figure 11.

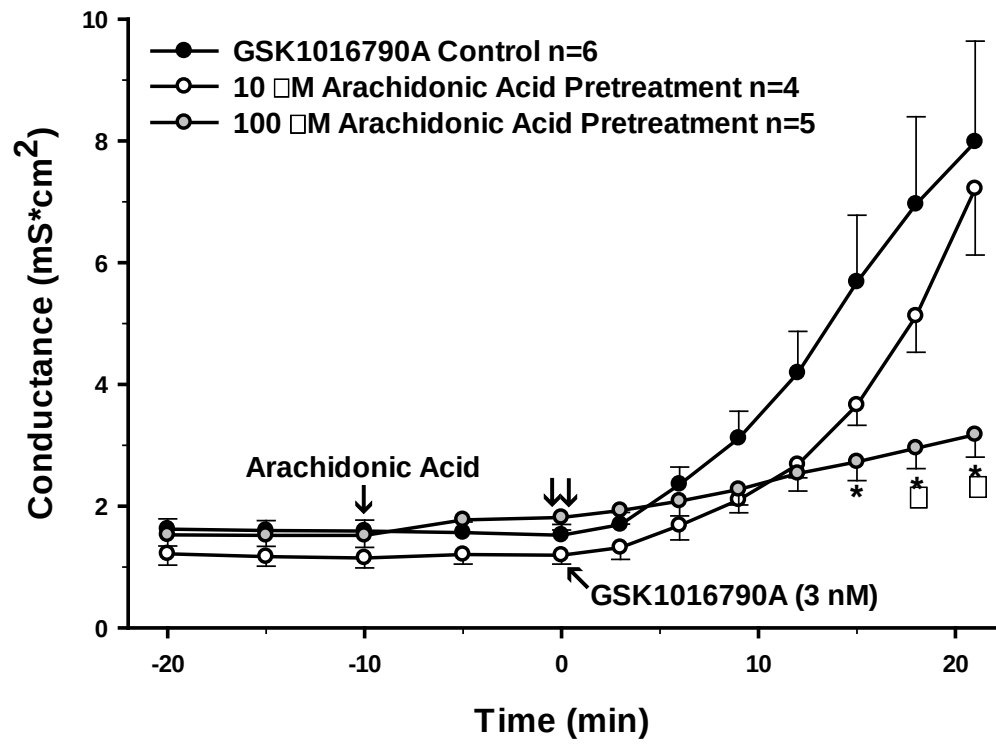
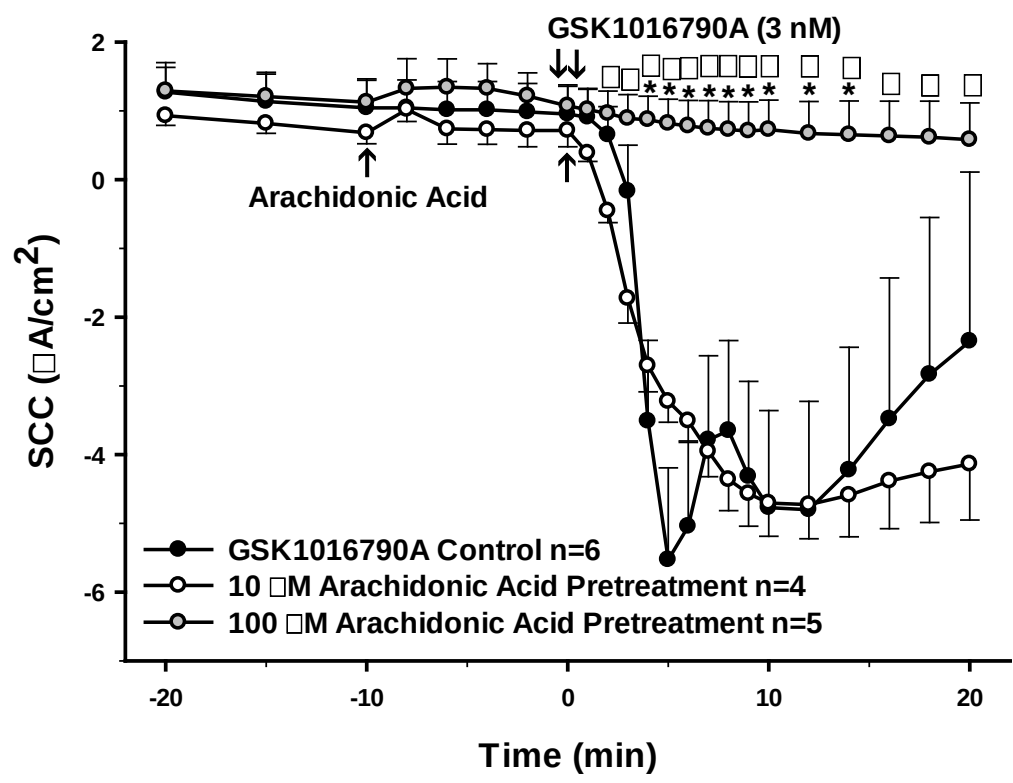


Figure 12.

Dose Effect of AA on GSK-Stimulated Transport

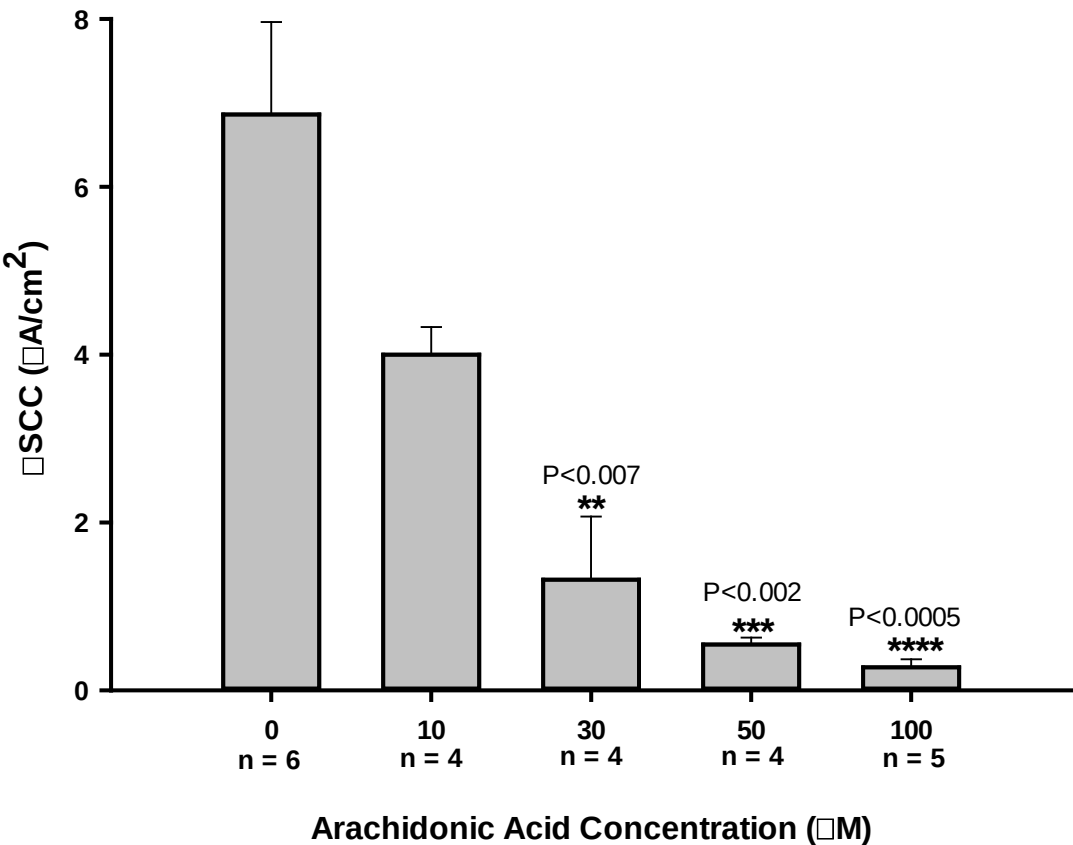


Figure 13.

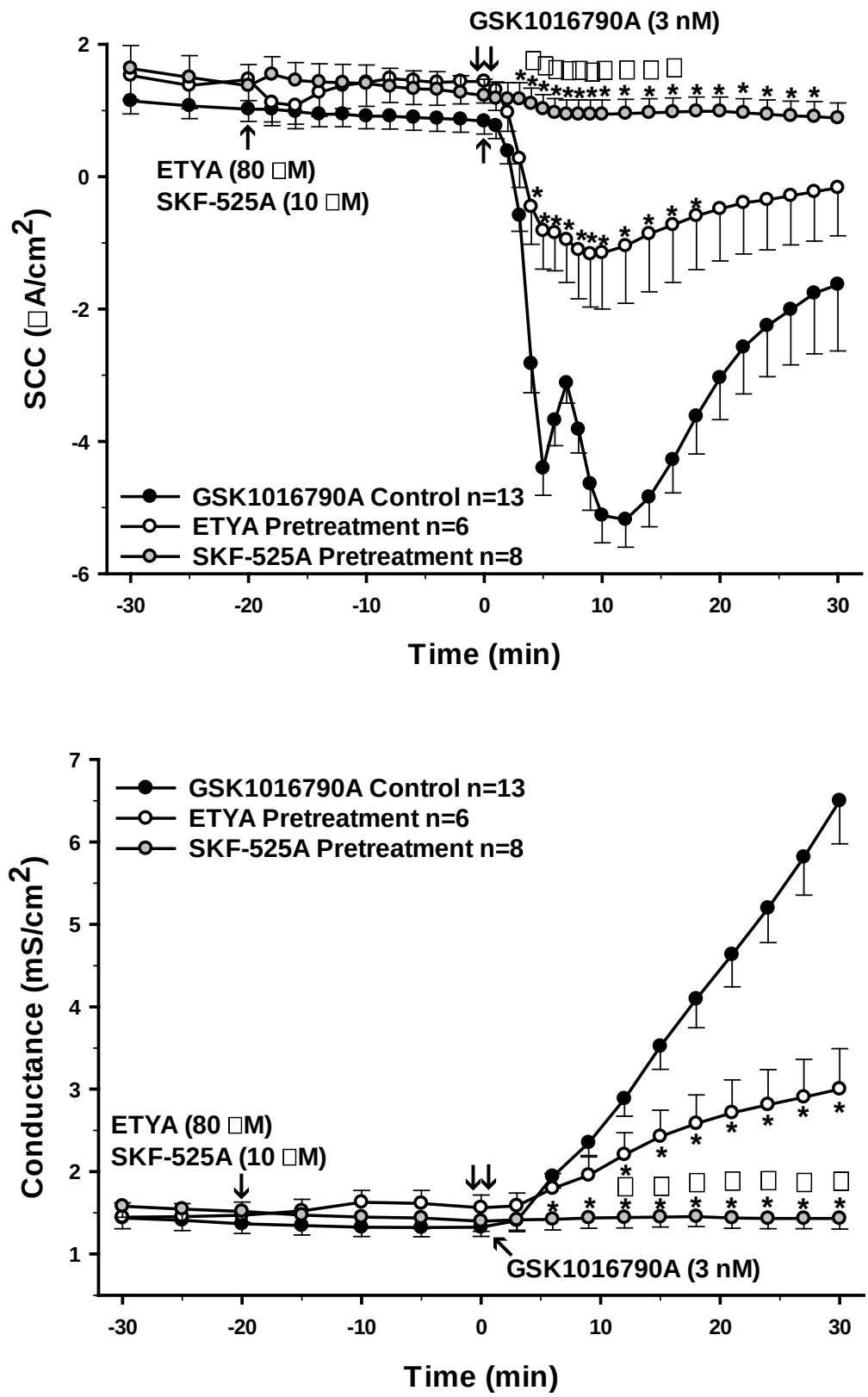
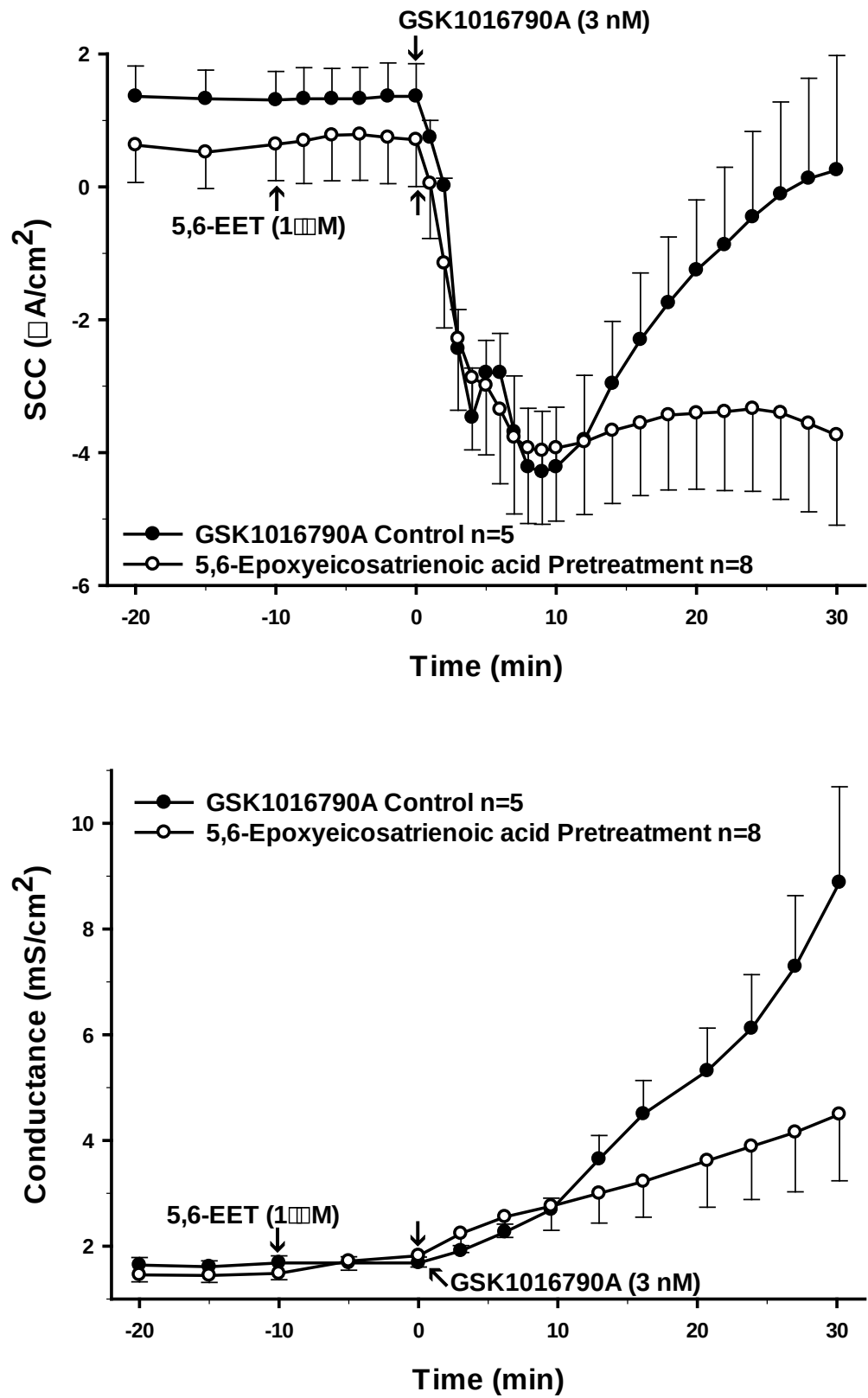


Figure 14.



Chapter : TRPV4, a Regulatory Channel in the Production of Cerebrospinal Fluid by the Choroid Plexus

Abstract:

Transient receptor potential, vanilloid 4 (TRPV4) channel is an osmo- and mechano-sensitive nonselective cation channel expressed throughout the body. TRPV4 is activated and regulated by a wide variety of endogenous and exogenous stimuli. In addition to its relatively ubiquitous distribution and regulation by diverse stimuli including pressure, pH, inflammation, cell swelling and cytokines, TRPV4 appears to be a hub protein in many signaling cascades integrating input from multiple receptors and kinases. TRPV4 is present in the apical membrane of choroid plexus epithelial cells and emerging studies have linked this channel with changes in transepithelial ion and water flux.

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Introduction

Cerebrospinal fluid (CSF) is the cushioning and nutritive fluid component of the central nervous system. The osmolyte composition of the CSF is acutely controlled by the action of many channels and transporters located in the membranes of the choroid plexus epithelial cells, as was elegantly described in Chapter 1. One of the more recently described of these transporters is the transient receptor potential, vanilloid 4 (TRPV4) channel. TRPV4 has been characterized as a relatively ubiquitous, polymodal, nonselective cation channel and emerging data suggest that regulation of this channel in the choroid plexus epithelial cells may contribute to CSF production and composition.

TRPV4 is a member of a large family of functionally diverse TRP channels. The founding member of the TRP family was characterized in *Drosophila* as being a calcium permeable channel constituting a phototransducing component (Cosens and Manning 1969). Subsequently, many other transporters have been added under the TRP family umbrella and, to date, the TRP channels can be divided into six subfamilies: TRPA (Ankyrin), TRPC (Canonical), TRPM (Melastin), TRPML (Mucolipin), TRPP (Polycystin), and TRPV (Vanilloid). The TRP family is broadly classified under the voltage-gated superfamily of ion channels inclusive of voltage-gated K^+ , Na^+ , and Ca^{2+} channels, though most TRP channels also possess ligand-gating capabilities (Yu et al. 2005). Mammalian TRP channels are expressed throughout the body and are activated and regulated by a wide variety of endogenous and exogenous stimuli. Most TRP channels share sequence homology in their six membrane spanning domains, but there are many structural and functional differences in their C- and N-termini. TRP channels normally assemble as homo- or hetero-tetramers where each subunit includes six membrane-spanning alpha helices (Gees et al. 2010; Cheng et al. 2010).

TRPV4 Channelopathies

There are a number of human diseases and disorders that have been linked to mutations in TRPV4 which are broadly divided into the categories of skeletal dysplasias and arthropathy, motor-sensory neuropathy, and motor-neuropathies. In humans, these channelopathies show remarkable phenotypic diversity. Within the last decade, numerous mutations in TRPV4 have been shown to cause varying severities in five skeletal dysplasias and FDAB (Familial Digital Arthropathy-Brachydactyly). These are characterized by abnormalities of bone and cartilage growth, causing various forms of dwarfism, short trunk, and progressive osteoarthropathy (Inda et al. 2012; Nishimura et al. 2012; Lamande et al. 2011). While many different TRPV4 mutations have been described within skeletal dysplasias and arthropathy, the majority occur in the ankyrin-repeat domain.

There have also been mutations in TRPV4 linked to two different motor-neuropathies: congenital distal and scapulo-peroneal spinal muscle atrophies (Dubowitz et al. 1995; Main et al. 2003). Additionally, several TRPV4 mutations have been shown to cause Charcot-Marie-Tooth disease type 2C which causes limb, diaphragm, and laryngeal muscle weakness (McEntagart 2012). Several pathophysiological mechanisms of disease have been proposed as the cause of these TRPV4 channelopathies in humans. These include, briefly: altered TRPV4-dependent neuritogenesis (Jang et al. 2012), altered TRPV4-dependent gene expression via SOX9, a chondrocyte differentiating transcription factor (Kornak and Mundlos 2003), dysregulated cellular trafficking of TRPV4 due to improper binding and ubiquitination (Cuajungco et al. 2006; Wang et al. 2007; Zerangue et al. 1999; Owsianik et al. 2003; Verma et al. 2010), and dysregulated protein-protein interactions such as those with Na-K-ATPase, Kir4.1, syntrophin, and aquaporin 4 (Lanciotti et al. 2012).

Interestingly, despite the heterogeneity and severity in human diseases, there is little evidence that a global knockout of TRPV4 in rodents results in any severe phenotype in the absence of stressors. Due to the mild phenotypes exhibited by these models, they have been widely used to study the physiological role of TRPV4 in multiple tissues.

Animal models of TRPV4 deletion

Liedtke and colleagues created the first *trpv4*^{-/-} mouse by flanking Exon 12 with a neo-cassette and loxP sites and excising exon 12, rendering the TRPV4 polypeptide nonfunctional and targeted for degradation. These mice are viable and fertile up to one year of age, though they exhibit reduced water intake due to altered osmosensation under a stressed hyperosmotic environment (Liedtke and Friedman 2003; Liedtke 2008). Additionally, these animals exhibit a larger bladder capacity due to the impairment of TRPV4 as a stretch sensor in the bladder wall (Gavaert et al. 2007). A second *trpv4*^{-/-} mouse was generated in the laboratory of Suzuki et al. (2003) in which Exon 4 was excised using a neo-cassette marker resulting in a lack of TRPV4 production. These mice have slightly differing, albeit mild, phenotypes. They exhibit thicker bones due to impaired osteoclast differentiation (Masuyama et al. 2008), and compromised vascular endothelial function as demonstrated by a preference for warmer temperatures and an overall inability to thermoregulate in stressed conditions (Vriens et al. 2005; Lee 2005; Saliez et al. 2008; Loot et al. 2008; Sonkusare et al. 2012). They also exhibit some sensory defects such as hearing alteration (Tabuchi et al. 2005), impaired pressure sensation (Suzuki et al. 2003), and compromised pain sensing (Cortright et al. 2009). Recently, Deruyver and colleagues (2018) successfully created a *Trpv4*^{-/-} rat and used this

model to study induced urinary bladder dysfunction. Unfortunately no data were provided regarding the overall phenotype of this rat model.

Though these models have been informative for TRPV4 functionality, they do not increase understanding of human diseases which are often due to alteration rather than elimination of TRPV4 functionality. There have been several gain-of-function mutations in mice and zebrafish which have demonstrated skeletal dysplasia-like phenotypes similar to the human diseases (Wang et al. 2007; Masuyama et al. 2012). However, these models still do not accurately reflect the variety of disease presentation found in human patients. Regardless, the findings that TRPV4 deletion causes mild phenotypes, which are unmasked under stressed conditions, is indicative that TRPV4 may be a redundant channel in rodents. The mild phenotypes in the knockout animals combined with the demonstration that TRPV4 antagonism has no aberrant physiological effects in rodent models has led to the suggestion that TRPV4 is a promising therapeutic target (Everaerts et al. 2010; Thorneloe et al. 2012).

TRPV4 Structure and Expression

The TRPV4 protein has been shown to be expressed in many epithelia as well as nervous, liver, heart, and vascular tissue (<https://www.proteinatlas.org/ENSG00000111199-TRPV4/tissue>). Emerging findings suggest tissue-specific effects, which can be modulated by the type of stimulus, the isoform present, or differences in intracellular signaling effectors. Uniprot recognizes isoforms 1, 2, 4, 5, and 6 as characterized by Arniges et al. (2006). These authors described the original TRPV4-A (98 kDa) as well as four splice variants TRPV4-B (Δ 384-444), TRPV4-C (Δ 237-284), TRPV4-D (Δ 27-61), and TRPV4-E (Δ 237-284 and Δ 384-444) cloned from human airway epithelial cells and expressed in HeLa cells. They demonstrated that TRPV4-A and D can be either homo- or hetero-multimerized in the endoplasmic reticulum (ER), and are targeted to the membrane upon stimulation. A commonality among the B, C and E splice variants is that they all have deletions in the cytoplasmic N-terminal region which affect an ankyrin domain. Consequently, these variants do not oligomerize and are retained in the ER. The first four helices of TRPV4 (S1-S4) form the voltage-sensing or voltage-sensing like domain, and the last two segments (S5, S6) form the ion-conducting pore along with the reentrant pore loop (Leidtke et al. 2000; Deng et al. 2018). TRPV4 protein is composed of 871 residues, and normally assembles as a homotetramer (Stewart et al. 2010). However, there have been reports of TRPV4 assembling as heterotetramers such as TRPC1-TRPV4 (Ma et al. 2011); TRPP2-TRPV4 (Stewart et al. 2010); and even TRPC1-TRPP2-TRPV4 (Du et al. 2014).

TRPV4 contains many well characterized binding domains within both its amino and carboxy termini (Figure 1). In the N-terminus, there is evidence that phosphorylation at Tyr110 increases the abundance of TRPV4 at the membrane (Wegierski et al, 2009). Just following Tyr110, the proline rich domain (PRD) has been shown to be an important binding site for PACSIN-3, which also increases membrane expression (Cuajungco et al. 2006; D'hoedt et al. 2007). Next to the PRD, there is an Ankyrin Repeat Domain (ARD) composed of 6 Ankyrin repeats which have been shown to be an important interaction domain for OS-9 (Everaerts et al. 2019; Inda et al. 2012). OS-9 is an endoplasmic-reticulum-resident protein involved in protein folding and ubiquitination (Wang et al. 2007). Ubiquitination can also occur in conjunction with E3 ubiquitin ligase AIP-4 (atrophin-1-interacting protein 4), which causes a reduction in membrane expression by promoting endocytosis without inducing degradation of TRPV4 (Wegierski et al. 2006). Therefore, ubiquitination is an important regulatory mechanism of TRPV4 wherein its basal activity can be directly regulated.

In the C-terminus, there is a well characterized TRP box which helps to stabilize the closed conformation and may be responsible for the interaction with the widely used agonist GSK10106790A (Teng et al. 2015). There are also binding sites preferential for calmodulin, MAP7, actin, and tubulin (Strotmann et al. 2003; Suzuki et al. 2003; Goswami et al. 2010; Garcia-Elias et al. 2008). Important phosphorylation sites for several regulatory kinases are located within these latter domains. Thus, both termini of the TRPV4 channel structure are intracellular and serve important roles in phosphorylation, activation, and vesicular trafficking of the protein.

TRPV4, like many ionotropic channels, is located in a vesicle pool just below the cell surface allowing for the regulation of expression between the membrane and cytosolic domains (Becker et al. 2008; Lei et al. 2013; Ferrandiz-Hertas et al. 2014). There are a number of proteins which are postulated to regulate this expression. Specifically, phosphorylation at Serine 824 within the calmodulin binding site by serum and glucocorticoid-induced kinase I (SGK1) has been shown to be important for the interaction with F-actin (Shin et al. 2012) and subsequently, membrane targeting. In addition to membrane expression, SGK1 phosphorylation regulates agonist sensitivity, single channel activity, and Ca^{2+} influx (Shin et al. 2012; Lee et al. 2010). Phosphorylation via the protein kinase A (PKA) pathway, also targeting Serine 824, has been shown to increase the amount of TRPV4 which is trafficked to the membrane (Mamenko et al. 2013; Fan et al. 2009). Conversely, phosphorylation via the PKC (via Serine 165, Tyrosine 175, and Serine 189) pathway doesn't appear to increase the amount of trafficked TRPV4, but it does increase the activity/sensitivity of membrane-bound TRPV4 (Mamenko et al. 2013; Fan et al. 2009) in renal cells, dorsal root ganglia (DRG) as well as when exogenously expressed in cultured cell lines. Though TRPV1 and TRPV4 co-express in the DRG, the

TRPV4 modulation was shown to be independent of TRPV1 activity as modulated by capsaicin (Cao et al 2018). Similarly, activation of the PLC pathway has been shown to up-regulate TRPV4 activity at the membrane (Garcia-Elias et al. 2013). With no lysine deficient protein kinases 1 and 4 (WNK1 and WNK4) down regulate membrane expression of TRPV4 in renal cells (Fu et al. 2006). Depletion of intracellular Ca^{2+} stores stimulates the insertion of TRPV4 into the plasma membrane thereby increasing Ca^{2+} influx. However in contrast to most other studies, the form of the Ca^{2+} -sensitive channel is a TRPV4-TRPVC1 heteromeric channel (Ma et al. 2010; 2011).

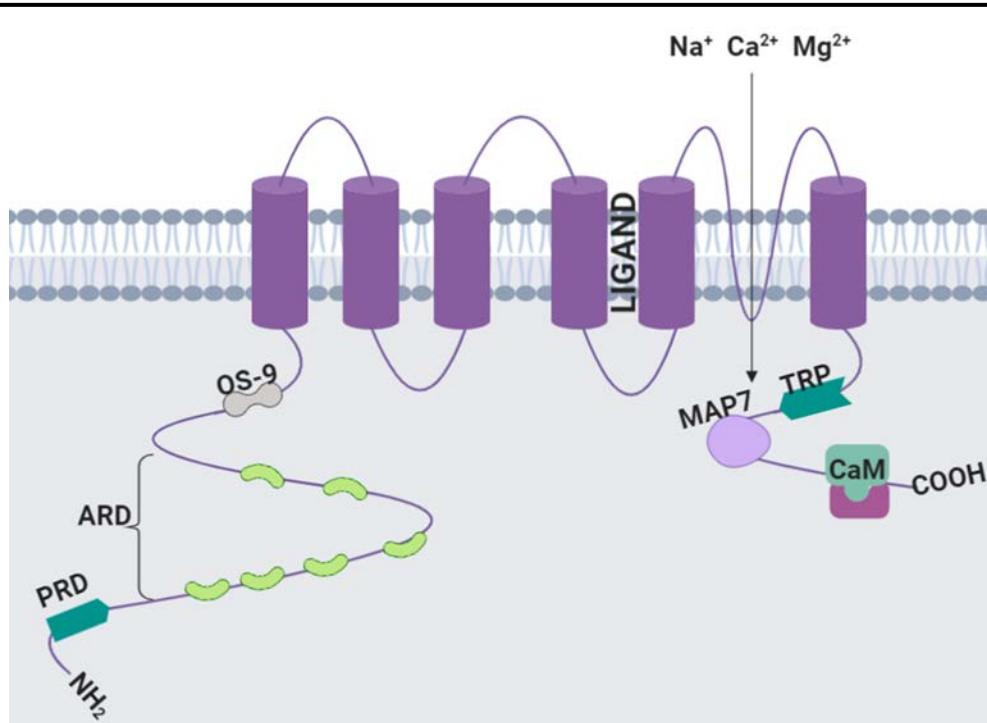


Figure 1: Structure of TRPV4 and important binding domains. PRD = Proline Rich Domain. ARD = Ankyrin Repeat Containing Domain. OS-9 = Binding site for OS-9. TRP = TRP Box. MAP7 = MAP7 binding domain. CaM = Calmodulin/Actin/Tubulin Binding Domain. LIGAND = ligand binding domain.

Activation and Modulation of TRPV4

TRPV4, originally classified as vanilloid receptor-related osmotically active channel (VR-OAC), is activated by extracellular osmolarity (Liedtke et al. 2000). It is active under isotonic conditions, shows increased activation with decreased extracellular osmolarity, and is inactivated with increased extracellular osmolarity (Strotmann et al. 2000, White et al. 2016). TRPV4 is also mechanosensitive and can respond to cell stretch in a variety of tissue types. However, the exact mechanism of mechanical activation remains a highly debated topic. One of the proposed mechanisms is activation

of the channel by direct membrane tension changes. Evidence supporting this theory includes the channels association with adherence junctions (Janssen et al. 2011, White et al. 2016), and co-expression with stretch-activated channels (Alessandri-Haber et al. 2009, Nilius et al. 2012). Additional theories include activation by direct mechanical force on cell membrane structures gating the channel (Brohawn et al. 2014, Kung 2005) or membrane deformation-stimulated release of activation products that gate TRPV4, such as cell swelling. Additionally, it has been shown that TRPV4 is activated by phospholipase A2-dependent formation of arachidonic acid metabolites by the cytochrome P-450 epoxygenase-dependent pathway (Watanabe et al. 2003; White et al. 2016).

TRPV4 can also be activated by heat, pH, and inflammation. It is constitutively active at normal body temperatures, but demonstrates temperature activation above 25° C (Watanabe et al. 2002, Güler et al. 2002, Gao et al. 2003). TRPV4 is sensitized to heat in response to hypotonic stress and reduced in hyperosmotic conditions. The exact purpose of TRPV4 activation due to heat increases remains uncertain and may be species dependent. Likewise, pH has been shown to modulate TRPV4 activity, as demonstrated by a study wherein low pH in hamster ovarian cells opened the channel (Suzuki et al. 2003). TRPV4 gene disruption also decreased acid nociception in mice (Suzuki et al. 2003). Conversely, in esophageal epithelial cells acidic conditions can inhibit TRPV4 Ca²⁺ cellular influx. Inhibition of TRPV4 with acid is thought to aggravate the condition in gastroesophageal reflux disease (GERD) (Shikano et al., 2011). TRPV4 antagonists have also been shown to reduce inflammation and pain in models of inflammatory bowel disease (Fichna et al. 2012).

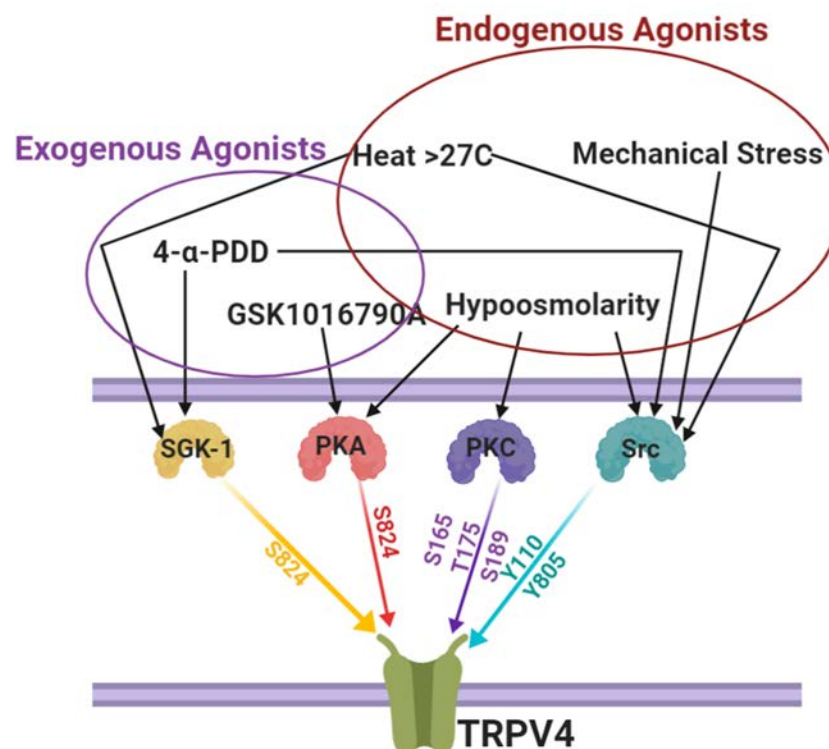


Figure 2: Exogenous and Endogenous Agonists sensitize TRPV4 through the action of different kinases. SGK-1 = Serum and Glucocorticoid-Induced Kinase 1. PKA = Protein Kinase A. PKC = Protein Kinase C. Src = Src Family Kinase.

Inflammation also can affect TRPV4 in part because it results in temperature and pH changes and inflammatory responses sensitize TRPV4 to hypotonic stimuli (Chen et al. 2007). However, activation of TRPV4 can produce proinflammatory cytokines such as TNF- α and interleukins (IL)-1 α , IL-1 β , IL-6, and IL-8 (Pairet et al. 2018, Wang et al. 2019; D'Aldebert et al. 2011). Inhibition of TRPV4 can decrease release of these cytokines (Shi et al. 2013, Wang et al. 2019) or TRPV4 activity can, in turn, be modulated by cytokines (Simpson et al 2019). Taken together, these results suggest that TRPV4 can be regulated by a variety of physiological conditions including acidic conditions, as well as heat and corresponding inflammation.

TRPV4 Modulation by GPCRs

TRPV4 has been shown to be modulated by a number of different G-protein coupled receptors via the action of various downstream kinases, as shown in Figure 3. Studies by Xia Y et al. (2013) demonstrated a role for TRPV4 in enhanced pulmonary vasoreactivity to serotonin (5-HT) in chronic hypoxic pulmonary hypertension indicating that TRPV4 contributes to 5-HT-dependent pharmacomechanical coupling in pulmonary arteries. They found that either global knockout of TRPV4 (such as in the *trpv4*^{-/-} mouse) or addition of submicromolar HC-067047, a specific TRPV4 antagonist, decreased the sensitivity of 5-HT-mediated contraction of pulmonary arteries. This effect was independent of endothelin or phenylephrine-mediated contraction. Akiyama et al. (2016) demonstrated a role for TRPV4 in serotonin-mediated itch response in the peripheral nervous system. In this study, approximately 90% of all 5-HT-sensitive DRG cells were found to contain TRPV4. In *trpv4*^{-/-} mice, they found an overall decrease in serotonin-mediated scratching bouts, and this same result was recapitulated by treating wild-type mice with a TRPV4 antagonist. Additionally, this study utilized calcium imaging of primary DRG cultures to demonstrate that there was a decrease in 5-HT-activated, TRPV4-mediated calcium influx in *trpv4*^{-/-} cultures, but not in response to other pruritogens.

Rahman et al. (2018) demonstrated that a TRPV4 agonist, 4 α -PDD triggers the release of ATP via pannexin channels in human pulmonary fibroblasts. This activation can be attenuated by P2Y purinergic receptor/channel blocker pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS).

Initial studies by Grant et al. (2007) demonstrated that the protease-activated receptor-2 (PAR2) sensitizes TRPV4 to initiate mechanical hyperalgesia in mice through sensitizing calcium signals in DRGs. In HEK cells TRPV4 expression was found to generate sustained intracellular calcium increases, which were attenuated by Src inhibition of PAR2 (Poole et al. 2013). This role for PAR2 activation of TRPV4 was found to be dependent upon the Tyr-110 residue as a binding site for

Src kinase. Additionally, PAR2 was shown to induce long term depression in the hippocampal network through the PKA-dependent, TRPV4-mediated activation of NMDAR (N-methyl-D-aspartate receptors) (Shavit-Sten et al. 2017). Lastly, TRPV4 has been shown to activate in response to PAR2 through a PLCB3-dependent mechanism in human respiratory epithelial cells (Li et al. 2011).

TRPV4 activation has been linked to muscarinic receptor mediated endothelium-dependent vasodilation, which is enhanced by the shear stress mechanism. This was demonstrated by Darby et al. (2018), where shear stress was shown to increase endothelial TRPV4 agonist (GSK1016790A) sensitivity. In shear treated arterioles, the acetylcholine response was attenuated by a TRPV4 antagonist (GSK2193874). Additionally, TRPV4 has been found to colocalize with PKC in mesenteric arteries in rats, enabling multiple TRPV4 channels to have a functional effect by opening calcium sensitive potassium channels. (Pankey et al. 2014). In summation, this large body of work indicates that GPCR activation can sensitize TRPV4 activity through either direct activity or the interaction with various kinases such as Src, PKA, and PKC.

The multiplicity of possible external stimuli combined with a panoply of intracellular signals has led to the characterization of TRPV4 as a hub protein capable of integrating multiple signals in a tissue-specific manner.

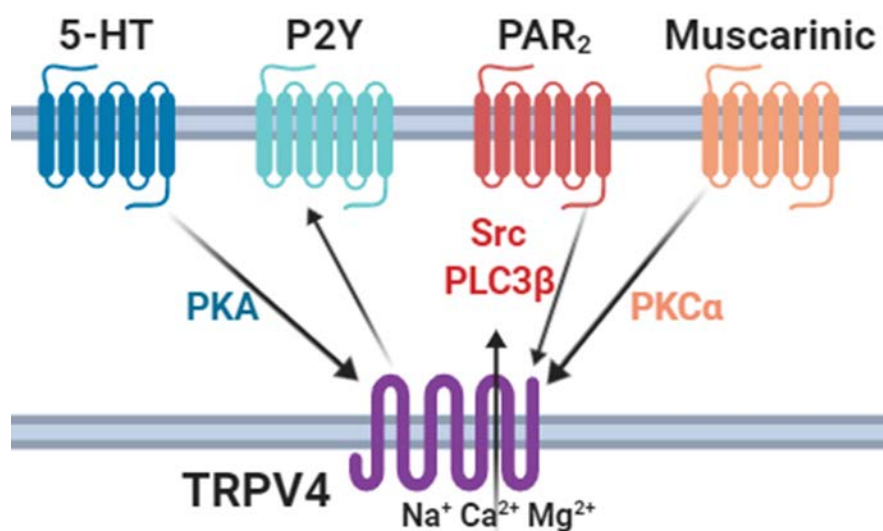


Figure 3: Activation of TRPV4 via G-protein Coupled Receptors is sensitized by various kinases.

TRPV4 in the Brain

In the nervous tissue, specifically in neurons and glia, TRPV4 has numerous roles although the mechanisms remain poorly characterized. TRPV4 plays a major role in neurovascular coupling,

excitatory glia, and sensory nerve transmission (Cao et al. 2009, Dunn et al. 2013, Shibasaki et al. 2014, Shibasaki et al. 2007, Kumar et al. 2018).

Activation of TRPV4 alters astrocyte, the support cells of the central nervous system, by changing their morphology to an activated state. In astrocytes, activation of TRPV4 causes cell hypertrophy and increases the amount of glial fibrillary activation protein (GFAP), thereby changing the morphology of the cells and increasing their aggregative properties (Wang et al. 2019). TRPV4 is localized predominately in astrocyte endfeet where it regulates vascular permeability and contributes to neurovascular coupling (Dunn et al. 2013). In cases of oxidative stress, TRPV4 can also mediate astrocyte cell death (Bai and Lipski, 2010). Furthermore, TRPV4 as an osmosensor has been implicated in regulatory volume decrease (RVD). In this mechanism, specific water channels called aquaporins (AQP) have been postulated to interact with TRPV4 either directly or indirectly. In astrocytes, the AQP4 water channel interacts with TRPV4 and this complex is considered to be crucial for RVD (Benfenati et al. 2011).

TRPV4 has been shown to be osmotically activated and expressed in the circumventricular organs (CVOs), where *Trpv4*^{-/-} mice showed reduced nociceptive responses to hypotonic and mildly hypertonic stimuli (Benfenati et al. 2007, Liedtke et al. 2003, Alessandri-Haber et al. 2003, Kumar et al. 2018). TRPV4 was also shown to significantly increase mEPSC (mini, excitatory postsynaptic current) frequency in hippocampal neurons via the PKC pathway, indicating a role for TRPV4 in excitatory neural signaling in the limbic system (Cao et al. 2009). TRPV4 increases the frequency of mEPSCs in dorsal horn neurons, indicating that TRPV4 also has a role for synaptic transmission in the periphery (Cao et al. 2009). In peripheral nociceptive neurons, TRPV4 acts as a sensor for noxious mechanical and osmotic stimuli (Alessandri-Haber et al. 2003). Furthermore, TRPV4 appears to play a role in the detection and modulation of mechanical hyperalgesia and mechanical allodynia, indicating it as a promising target for a novel class of analgesics (Alessandri-Haber et al. 2004; Alessandri-Haber et al. 2006).

TRPV4 expression was found to increase in many crucial brain and spinal cord regions which are indicated in aged individuals (Lee et al., 2014). TRPV4 expression has also been shown to be upregulated in regions associated with neurodegenerative pathologies, such as the cerebral cortex, hippocampal formation, basal ganglia, thalamus, cortical pyramidal neurons, and neurons in the spinal cord (Lee et al. 2014, Lee et al. 2012, Kumar et al. 2018). Increased TRPV4 expression has been observed in a mouse model of amyotrophic lateral sclerosis (ALS) (Lee et al., 2012). In models of stroke, TRPV4 is activated and can be followed by neuronal death (Jie et al., 2016; Li et al., 2013). While intriguing, the significance of these changes remains unknown.

TRPV4 has specific vascular functions which can impact brain tissue including roles in angiogenesis, capillary permeability, and fluid exchange (Dejana et al. 2013, Pries et al. 2014, Wu et al. 1996). Reductions in intravascular pressure as well as flow-induced “shear stress” have been shown to activate endothelial TRPV4 channels (Bagher et al. 2012, Mendoza et al. 2010) and increase TRPV4 membrane localization (Loot et al. 2008). Stimulation of TRPV4 with an agonist, GSK1016790A, causes dilation of blood vessels while antagonist-mediated inhibition of TRPV4 can prevent vasodilation (Mendoza et al. 2010, Köhler et al. 2006). Agonist activation of TRPV4 increases cerebral arteriogenesis (Schierling et al., 2011) and it has been implicated in endothelial proliferation in brain capillaries (Hatano et al. 2013). TRPV4 has been shown to be important in blood-tissue barriers. In pulmonary endothelia, agonist treatment increased permeability of the tissue (Alvarez et al. 2006, Villalta et al. 2014, Wu et al. 2009) but it is not known if this permeability effect is also manifested within the brain vasculature and if so, whether this is as a direct effect or mediated via astrocyte control of the blood brain barrier.

In summary, TRPV4 may play important roles in brain homeostasis via its effects in astrocytes and the vasculature. In addition, a myriad of roles in neuronal function may have important implications for signaling and sensation. Of particular interest in this regard is the increased expression of TRPV4 in neurological disease and during the normal ageing process.

TRPV4 in the Choroid Plexus

The presence of TRPV4 in choroid plexus epithelia was demonstrated by Leidtke and colleagues (2000) and subsequent studies have shown both an intracellular and apical membrane distribution of the channel (Willette et al. 2007; Takayama et al. 2014; Narita et al. 2015). While TRPV4 broadly localizes to the apical membrane of choroid plexus epithelium, it does not localize to the tufts of immotile cilia on the apical surface as it does in the oviduct, cortical collecting duct, mesenchymal stem cells, the eye, and the airway (Narita et al. 2015; Li et al. 2019; Saigusa et al. 2019; Corrigan et al. 2018; Luo et al. 2014; Alenmyr et al. 2013). Though TRPV4 is expressed in astrocytes and neurons, it has been noted with both *in situ* hybridization and immunofluorescence that the TRPV4 transcript and protein localization are most abundant in the choroid plexus epithelia compared to the rest of the brain tissue (Leidtke et al. 2000; Willette et al. 2007; Hochstetler et al. 2018; Smith et al. 2019).

Functional studies to elucidate the role of TRPV4 in the choroid plexus have used a variety of primary and continuous cells lines. Murine, rat, swine and primate primary cultures have been characterized and used for a range of studies (Klas et al. 2010; Narita et al. 2015; Takayama et al.

2014; Gregoridades 2018; Delery and MacLean 2019). These cultures demonstrate epithelial phenotypes, correct polarization of transporters, and complete junctional complexes, but few exhibit tight barrier function as evidenced by high transepithelial electrical resistance measurements.

TRPV4 has been studied in a subset of the described primary cultures with some interesting results. In primary cultures of swine choroid plexus, the TRPV4 agonist, GSK1016790A (10 nM), decreased barrier integrity by disrupting tight junctions as seen by loss of fluorescent signal when immunostaining for ZO-1 and Claudin-1 (Narita et al. 2015). The agonist also led to a substantial drop in transepithelial electrical resistance as well as a degradation of the filamentous actin cytoskeletal structure and the integrity of the epithelial monolayer. It was suggested that this change may facilitate the infiltration of immune cells into the CSF. Treatment with HC067047, a TRPV4 antagonist, decreased the amount of basolateral to apical transport of α -2-macroglobulin which the authors hypothesize indicates the involvement of TRPV4 in intracellular vesicular trafficking of proteins at the apical membrane. The change in transepithelial electrical resistance, an indicator of barrier function of the epithelium, is in agreement with findings in vascular endothelium where TRPV4 has been shown increase permeability of the tissue.

In terms of channel regulation in the choroid plexus, the well-documented Ca^{2+} influx mediated by TRPV4 activation has the potential to secondarily stimulate Ca^{2+} -activated electrolyte channels. In mouse primary choroid plexus cultures, activation of TRPV4 generated an outward Cl^- current (Takayama et al. 2014) via ANO1 (TMEM16A), a Ca^{2+} -sensitive Cl^- channel. This was activated by either warmth or hypo-osmolarity, and was blocked in cells that were deficient in TRPV4. Furthermore, the authors reported a physical interaction between TRPV4 and ANO1. Cell volume changes were dependent on ANO1 activation and the cell volume was decreased at negative membrane potentials by TRPV4-induced Cl^- channel activation. The authors hypothesized the formation of a complex containing TRPV4, ANO1 and AQP4 in the apical membrane.

An important addition to the research armamentarium for studying choroid plexus function was the development of two continuous high resistance choroid plexus cell lines: Human Choroid Plexus Papilloma Cells (HIBCPP) and Porcine Choroid Plexus – Reims (PCP-R) lines (Schwerk et al. 2012; Schroten et al. 2012). These lines have been well characterized and express many of the characteristics of the native tissue including the expression and polarization of key transport proteins.

In contrast to Narita et al. (2015), Preston et al. (2018) demonstrated that in PCP-R cells the TRPV4 agonist GSK1016790A (3 nM) did not affect the expression of tight junctional protein claudin-1 nor did the epithelial cells show morphological changes or loss of monolayer structure. However, the agonist did dramatically increase the conductance of the epithelial monolayer indicating a change

in transepithelial barrier function. In agreement with Narita et al. (2015), they found that an antagonist of TRPV4 (RN 1734) inhibited this change in conductance. The differences in the results between the two studies with regard to effects on the tight junctions and cell structure may be the use of a continuous, high resistance cell line (Preston et al. 2018) versus the use of medium to low resistance primary cultures (Narita et al. 2015) and/or the differences in agonist concentration.

Preston et al. (2018) identified a multiphasic change in electrogenic transepithelial ion flux in response to TRPV4 activation. A portion of the stimulated flux was consistent with cation secretion and was dependent on the Ca^{2+} -sensitive intermediate conductance channel IK (KCNN4c; $\text{K}_{\text{ca}3.1}$).

Simpson et al. (2019) characterized the modulation of TRPV4 by various cytokines in the PCP-R cell line. This study demonstrated that select pro-inflammatory cytokines TNF- α , IL-1 β , and TGF- β 1 had inhibitory effects on TRPV4-stimulated ion flux and conductance changes. Anti-inflammatory cytokines had no effect on TRPV4 activity. In opposition to published studies in other tissues, this work also demonstrated that arachidonic acid was inhibitory to TRPV4-stimulated transepithelial ion flux. However, inhibition of EET production also inhibited TRPV4 activity suggesting the importance of these arachidonic acid metabolites. Lastly, this study showed that inhibition of transcription factor NF- κ B through the use of an inhibitor blocked TRPV4-stimulated activity, thereby proposing a role for TRPV4 in cytokine production.

In a preliminary report, TRPV4 antagonists have been shown to be an effective treatment for hydrocephalus in preclinical studies in rodent models (Smith et al 2019), presumably via an effect on TRPV4 in the choroid plexus. Although its role is understudied in the choroid plexus, preliminary studies have indicated a role for TRPV4 in fluid secretion, barrier integrity, and immune surveillance within the blood-cerebrospinal fluid barrier.

Concluding Statement

TRPV4 is a polymodal, ubiquitous hub protein which acts acutely to normalize osmotic imbalances, reestablish membrane potentials, control cellular permeability, modulate immune responses, and activate downstream signaling cascades. Future studies with the potential for clinical implications in many diseases including those that involved cerebrospinal fluid production by the choroid plexus are eagerly awaited.

Acknowledgements

The authors are supported by grants from the United States Department of Defense, Investigator Initiated Research Award W81XWH-16-PRMRP-IIRA and the Mayfield Education and Research Foundation.

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