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Effects of Dietary Emulsifiers on Vasopressin and Oxytocin in Mice

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EFFECTS OF DIETARY EMULSIFIERS ON VASOPRESSIN AND OXYTOCIN IN MICE

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by

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KRISHNA DINESH MEHTA

Under the Direction of Geert J. de Vries, Ph.D

ABSTRACT

Microbiota are micro-organisms that colonize the internal and external surfaces of the body and contribute to developmental processes and the microbiota-gut-brain axis, thereby to the high comorbidity between mental and gastrointestinal diseases. Recent evidence suggests that the gut microbiota influence the expression of vasopressin (AVP), a neuropeptide that mediates anxiety, stress and sickness behaviors and oxytocin (OXT), a neuropeptide that modulates social behavior and energy homeostasis. One way that the gut microbiota can be altered is through diet. Dietary emulsifiers, ambiphillic molecules used to stabilize emulsions of oil and water, alter the gut microbiome and increase intestinal inflammation. Recently, we have demonstrated that the emulsifiers carboxymethylcellulose (CMC) and polysorbate 80 (P80) increase anxiety-like behavior and reduce social behavior. Here, we investigate the roles of AVP and OXT as potential neural mechanisms by which emulsifiers alter anxiety and social behaviors. To test, male and female mice were weaned at 3 weeks of age, started in water (control), CMC or P80 treatment, and remained on treatment throughout the duration of the experiment. A standard battery of anxiety and social behavior tests was conducted starting at 10 weeks of age. A week following the completion of the last behavioral task, mice were euthanized, and brains were harvested and processed immunoreactivity (IR) of AVP and OXT in brain areas associated with stress and

metabolism, such as the paraventricular nucleus of the hypothalamus (PVN) and paraventricular nucleus of the thalamus (PVT). There was no main effect of treatment in AVP-IR in the PVN or PVT or OXT-IR in the PVN. There was a main effect of sex in AVP-IR in the PVN. Therefore, the results do not support the hypothesis regarding vasopressin and oxytocin expression in the PVN and PVT of mice with emulsifier-induced intestinal inflammation. Altered expression of AVP and OXT may not be a mechanism by which dietary emulsifiers cause changes in anxiety-like and social behavior.

INDEX WORDS: Microbiota-gut-brain axis, emulsifiers, vasopressin, oxytocin, paraventricular nucleus of the hypothalamus, paraventricular nucleus of the thalamus

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INTRODUCTION

Microbiota are all the micro-organisms that colonize the internal and external surfaces of the body (Montiel-Castro et al., 2013). The microbiota species that reside in the intestines or gut play critical roles in digestion and absorption of food as well as in development of the immune system. In addition, the gut microbiota has been shown to communicate bi-directionally with the brain, via the microbiota-gut-brain axis. For example, there is a high comorbidity between neurological and gastrointestinal disorders, which may be explained by this communication pathway (Foster & Neufeld, 2013). Furthermore, patients with autism spectrum disorder (ASD) show increased frequency of functional GI disorders, which are caused by altered microbiome-gut-brain signals, specifically in the form of decreased tryptophan levels, increased serotonin metabolite levels, and increased cytokine levels correlating with differences in microbiome composition between patients with and without ASD (Luna et al., 2016).

Disruptions of the microbiota-gut-brain axis lead to significant changes in behavior. Mice raised without microbiota, germ free (GF) mice, have an immature immune system, decreased hypothalamic-pituitary axis stress response, and decreased anxiety-like behavior (De Palma et al., 2014). GF mice also display reduced social behaviors (Desbonnet et al., 2014). In contrast, mice with an increase in pro-inflammatory microbiota show increased anxiety-like behaviors (Neufeld et al., 2011).

Dietary emulsifiers are detergent-like, ambiphillic molecules commonly added in processed foods, such as ice cream, mayonnaise, margarine, and peanut butter, that prevent the separation of the lipid and aqueous layers. It has been shown that dietary emulsifiers lead to the proliferation of the pro-inflammatory microbiota, leading to symptoms of low-grade inflammation and metabolic syndrome in mice, such as increased adiposity and dysglycemia

(Chassaing et al., 2015). Two commonly found dietary emulsifiers are carboxymethylcellulose (CMC) and polysorbate 80 (P80). These two emulsifiers also lead to robust effects in preliminary behavior tests, such as in the open field test, elevated plus maze, and three-chambered sociability test (Holder et al., 2016).

Further research in our lab has found that emulsifier administration increases anxiety-like behaviors in both male and female mice and decreases social behaviors in female mice. These sexually dimorphic behavior changes were seen with chronic low grade inflammation, increased adiposity, and increased drive for feeding induced by dietary emulsifiers (Holder et al., 2016). Although these physiological and behavior effects of dietary emulsifiers have been established, it is unclear how administration of emulsifiers leads these effects. Perturbations in the gut microbiome may induce these effects in several ways, from altered neurotransmitter or neuropeptide levels, autonomic nervous system signaling, or changes in components of the innate immune system (Li and Zhou, 2016).

A potential mechanism by which dietary emulsifiers may induce pathophysiological and behavioral changes is via the vasopressin and oxytocin systems of the brain. Vasopressin (AVP), or anti-diuretic hormone is expressed in a sexually dimorphic manner in the brain and has numerous functions in the body including water and solute balance and stress response via the hypothalamic-pituitary-adrenal (HPA) axis. Chronic stress can alter HPA axis activity, inducing gut dysbiosis and changing the composition and function of the gut microbiota (Bienenstock et al., 2015). Vasopressin is also released during chronic stress and may be a method by which changes in the gut microbiome induce changes the brain. Oxytocin is a similar neuropeptide with various functions, such as uterine contraction, milk letdown, energy homeostasis, and modulation of social behaviors. Both neurohormones are synthesized in and released from cell

bodies of neurons located in the paraventricular nucleus of the hypothalamus (PVN), a prominent brain area involved in the stress response. Vasopressin neurons in the suprachiasmatic nucleus (SCN) project to the paraventricular nucleus of the thalamus (PVT), an essential brain area in the HPA axis that integrates multiple neural and humoral inputs to control food intake, energy expenditure, and hormone levels (Fodor et al., 2013). Examining the AVP and OXT systems in these specific brain areas would be beneficial to determine a possible mechanism of action of dietary emulsifiers' effects on behavior and the body.

Therefore, the overall research question is how does administration of two commonly used dietary emulsifiers, CMC and P80, influence vasopressin and oxytocin, two neuropeptides implemented in anxiety-like and social behavior? We predict that because vasopressin is a neuropeptide released during stress and emulsifier-induced intestinal inflammation is a type of stressor, vasopressin immunoreactivity in both the PVN and PVT will be increased in mice treated with emulsifies. We also predicted that because oxytocin modulates social behaviors and social behaviors are decreased during stress and following emulsifier treatment, oxytocin immunoreactivity in the PVN will be decreased in mice treated with emulsifiers.

METHODS

Animals

C57Bl/6 dams with litters (3 male and 3 female 14-day-old pups) were purchased from Charles River Laboratories. Mice were housed in ventilated transparent OptiMouse plastic cages with Bed-O-Cobs® and AlphaDri bedding (35.6 x 48.5 x 21.8cm; at Georgia State University). Room lights were set to a 14h:10h light:dark cycle (lights off at 0900 ET), and ambient temperature was maintained at 23°C. Food (Purina rodent chow no. 5001) and water were available *ad libitum*. Mice were weaned postnatal day 21 (P21) and put into a new cage (randomized to littermates) such that each experimental group contained mice from all litters and that each litter was used for all experimental groups. All procedures were in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the Georgia State University.

Treatments

Upon weaning at age P21, male and female mice were transferred to new cages and began treatment with water or a 1% concentrated solution of either sodium carboxymethylcellulose (CMC) or Polysorbate 80 (P80) in the drinking water, forming six different experimental groups of six animals each. Animals were monitored weekly, their body weights were recorded, and they remained on treatment throughout the duration of the experiment.

Behavioral Testing

After ten weeks on treatment starting at age P70, the animals underwent a standard battery of behavior tests for anxiety-like, depression-like, and social behavior. One behavior test was conducted each week. A total of six different behavior tests were conducted, including the

open field test, elevated plus maze, light/dark box, marble burying test, three-chambered sociability test, and forced swim test.

Tissue Collection

One week following the last behavior test, mice were deeply anesthetized under isoflurane (5%v/v). Blood was collected by retrobulbar intraorbital capillary plexus. Hemolysis-free serum was generated by centrifugation of blood using serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). Mice were euthanized by cervical dislocation, and the colons, spleens, livers, and adipose were collected for subsequent analysis. Brains were removed and submersed into a fixative solution of 5% acrolein in sodium phosphate buffer (0.1M, pH 7.4) at 4°C, followed by cryoprotection in 30% sucrose in phosphate buffered saline (PBS: 0.05M, ph7.4). Brains were sectioned (30µm) in the coronal plane in a cryostat and stored in a cryoprotectant solution (ethylene glycol/sucrose in sodium phosphate buffer) until immunostained.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted to visualize the immunoreactivity (IR) of vasopressin (AVP) and oxytocin (OXT). The IHC process utilizes the natural and biological function of antibodies binding to antigens to detect the presence of specific antigens, in this case AVP and OXT, in brain areas of interest.

For the process of AVP immunohistochemistry, brain tissue underwent washes in 0.05 M Tris-buffered saline (TBS) to remove all traces of the cryoprotectant, and then were incubated in 0.05 sodium citrate in TBS at 70°C, cooled, incubated in 0.1 M Glycine in TBS, and then in blocking solution containing TBS, 10% normal goat serum (NGS), 0.4% Triton-X, and 1% hydrogen peroxide. Each incubation was followed by rinses in TBS solution. Then, the tissue

was incubated in rabbit anti-AVP primary antibody (Bachem, T-4563) overnight at a dilution of 1:46,000 in TTG, a solution containing 94% of 0.05 M TBS, 4% of 10% Triton-X, and 2% of NGS. After this incubation, the series of tissue was rinsed in TTG and then incubated in Biointylated goat-anti-rabbit IgG secondary antibody (Vector BA-7000) at a dilution of 1:800 in TTG. Following rinses in TBS containing 0.4% Triton-X, the tissue was incubated in avidin-biotin solution (Vector ABC-Elite standard kit) in TBS for 1 hour at room temperature. Next, tissue underwent more TBS rinses and washes in sodium acetate solution, followed by a 30-minute incubation in DAB solution, containing sodium acetate buffer, nickel sulfate, 3, 3, diaminobenzidine, and 5% hydrogen peroxide. After visualization, sections were rinsed in sodium acetate buffer and TBS, the experimental sections were stored in TBS at 20°C until mounting.

The process of OXT immunohistochemistry was a two-day protocol similar to that of AVP IHC. Differences included no incubation in sodium citrate, and the tissue was incubated in guinea pig anti-OXT primary antibody (Peninsula Labs) at a dilution of 1:12,000 in TTG, and later in Biotinylated goat-anti guinea pig IgG secondary antibody (Vector BA-7000) at a dilution of 1:800 in TTG. Upon completion of the DAB IHC protocol for OXT, sections were stored in TBS at 20°C until mounting.

All experimental tissue was mounted rostro-caudally onto double-dipped, gelatin coated slides. Slides were labeled to ensure unbiased analysis and allowed to dry overnight at room temperature. The slides then underwent timed baths of increasing alcohol concentration to dehydrate the tissue and then washes in xylene to clear the tissue of residual alcohol and fats. They were coverslipped with glass coverslips using permount, allowed to dry overnight, then cleaned with 100% ethanol.

Threshold Image Analysis

The processed sections were imaged with a photomicroscope at anatomically-matched positions. Three to four images encompassing the PVN and PVT of the stained tissue were captured for each animal. Microscope conditions were standardized to prevent variability between images. Examples of these images for the various groups are included in the results section. Next, photomicrographs were subjected to gray-level threshold analysis using a previously-established algorithm in the Image J software (Murray et al., 2011; Rood et al., 2008). The area of AVP or OXT-immunoreactivity (IR) in pixels was recorded for each image and the average area was calculated for each subject.

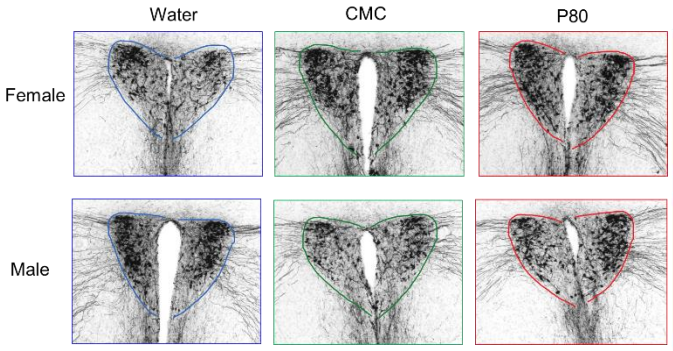
Statistical Analysis

Data are represented as means + SEM or ± SEM where appropriate. The average IR of each neuropeptide in each experimental group was determined and statistically analyzed using repeated measures 2-way analysis of variance (ANOVA) where treatment and sex were the between-subjects variables. Significance for all analyses was set at $p < 0.05$.

RESULTS

Vasopressin

We hypothesized that administration of emulsifiers will increase AVP-IR in the PVN of the brain. The average immunoreactivity area in pixels for each of the six groups was quantified. There were no significant main effects of treatment ($F(2, 29) = 1.802$; $P > 0.05$) or sex ($F(1, 29) = 0.4303$; $P > 0.05$). In addition, there was no interaction between sex and treatment ($F(2,29) = 0.06106$; $P > 0.05$).



Photomicrographs of AVP-IR in adult male and female mice.

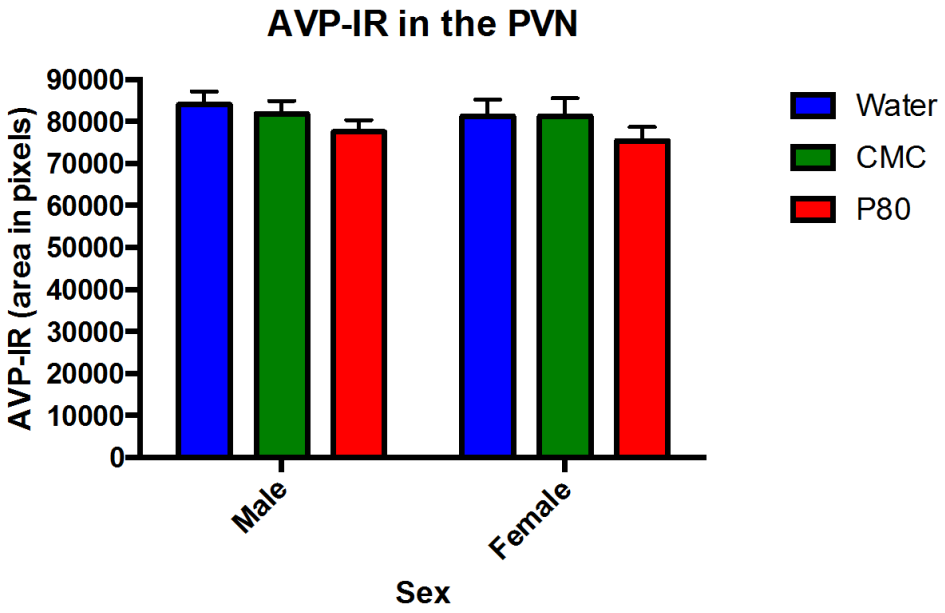
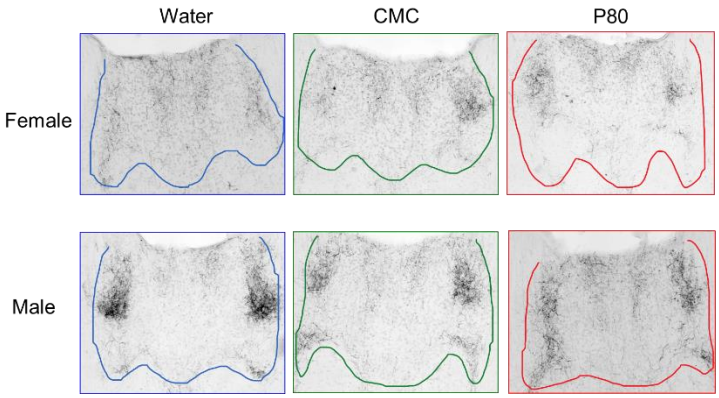


FIGURE 1. Effects of emulsifiers on vasopressin immunoreactivity in the PVN

We hypothesized that administration of emulsifiers will increase AVP-IR in the PVT of the brain. The average immunoreactivity area in pixels for each of the six groups was quantified. There were no significant main effects of treatment ($F(2, 29) = 0.6942$; $P > 0.05$). There was a significant main effect of sex observed ($F(1, 29) = 5.429$; $p = 0.0270$), in which females showed significantly lower AVP-IR in the PVN compared to males. There was no interaction between sex and treatment ($F(2,29) = 0.1601$; $p > 0.05$).



Photomicrographs of AVP-IR in the PVT of adult mice

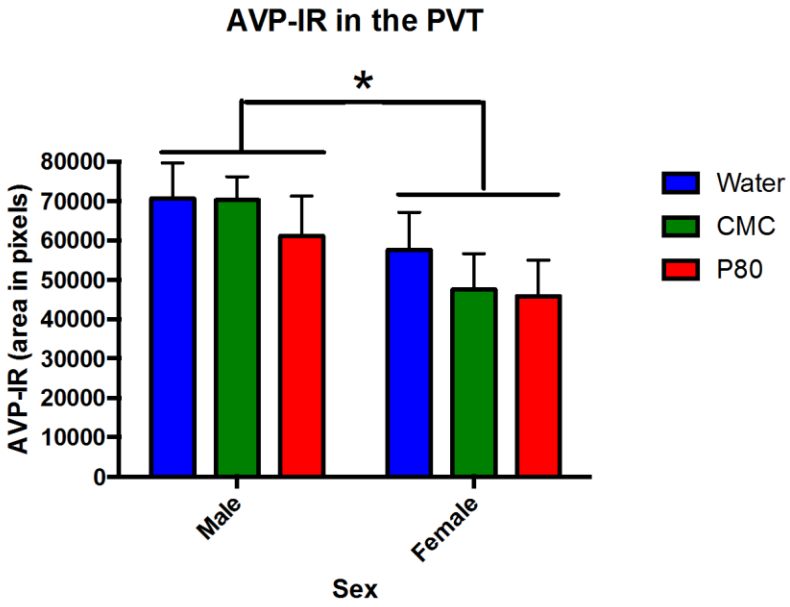
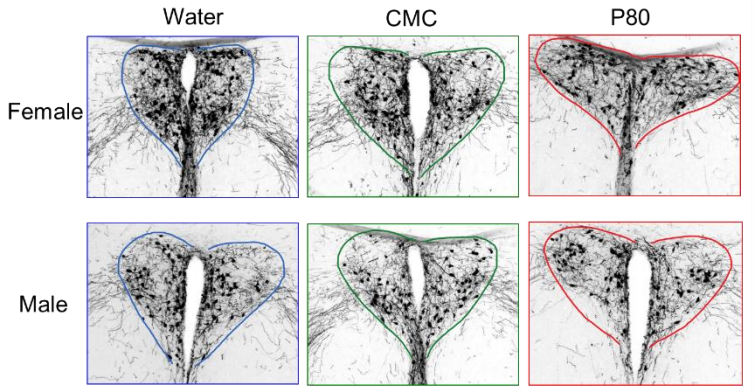


FIGURE 2. Effects of emulsifiers on vasopressin immunoreactivity in the PVT

Oxytocin

We hypothesized that administration of emulsifiers will decrease OXT-IR in the PVN of the brain. The average immunoreactivity area in pixels for each of the six groups was quantified. There were no significant main effects of treatment ($F(2, 29) = 0.8444$; $p > 0.05$) or sex ($F(1, 29) = 0.08876$; $p > 0.05$). In addition, there was no interaction between sex and treatment ($F(2,29) = 0.6430$; $p > 0.05$).



Photomicrographs of OXT-IR in the PVN of adult mice

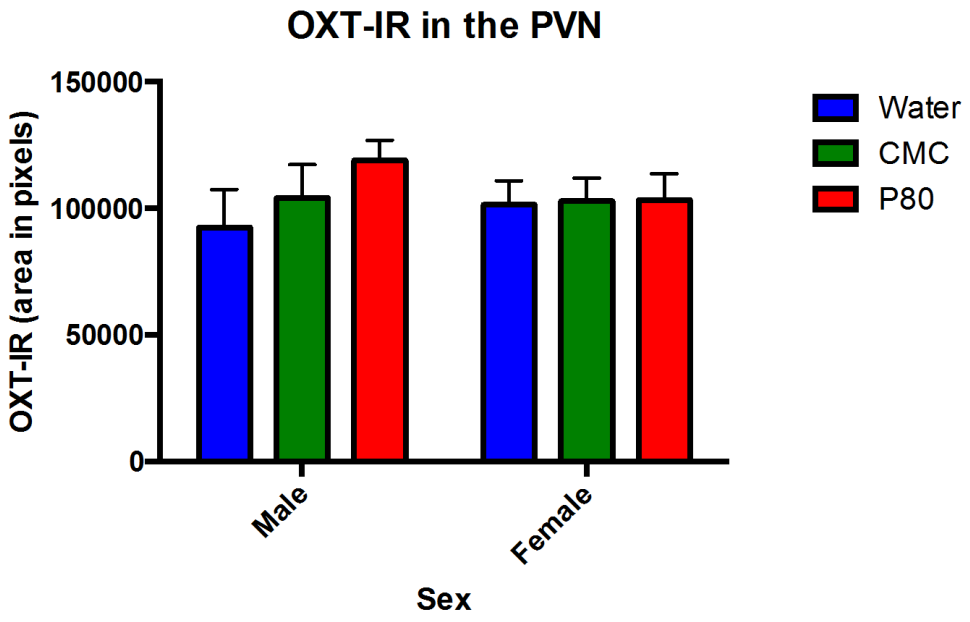


FIGURE 3. Effects of emulsifiers on oxytocin immunoreactivity in the PVN

DISCUSSION

Our results demonstrated that there were no significant effects of emulsifier treatment on the AVP-IR and OXT-IR in the PVN and PVT areas of the mouse brain. Thus, our hypothesis that administration of emulsifiers would increase AVP-IR in the PVN and PVT and decrease OXT-IR in the PVN was refuted. This was a surprising finding as previously discovered effects of emulsifier treatment on anxiety-like and social behavior indicated changes that may be linked to alterations in the vasopressin and oxytocin systems of the brain. There are several explanations and future directions for these results, including expanding on the various brain areas analyzed and analysis using the age and developmental stage of the animals.

The PVN and PVT were two brain areas chosen for analysis due to their prominent functions in the vasopressin and oxytocin systems and in stress-related and social behavior. The lack of significant effects of emulsifier treatment in the PVN and PVT of the mice may indicate the need to study other brain areas, such as the amygdala and bed nuclei of the stria terminalis (BNST). The central amygdala has been shown to be critical in the processing of anxiety and fear responses to stress. AVP is also synthesized in the medial amygdala (Smith et al., 2017) and there are well-documented AVP projections to the amygdala from AVP-containing magnocellular neurosecretory neurons (Hernández et al., 2016). Both AVP and OXT levels are increased in the amygdala in response to stress (Dabrowska et al., 2011). Due to profound behavior effects that AVP likely exerts in the amygdala, this area should be investigated as a region where emulsifiers may induce changes in AVP. The BNST is another brain area involved in the stress response, synthesizes AVP (Smith et al., 2017), and contains one of the highest levels of OXT receptor mRNA. There is also anatomical support for the existence of direct synaptic pathways linking hypothalamic OXT-IR neurons with the BNST, indicating that this

may be another region of action for stress-induced changes in OXT expression (Dabrowska et al., 2011). OXT-IR in the PVT was not able to be analyzed due to time constraints, but should also be performed to determine differences in this area. Although no significant differences were found in the PVN and PVT from this study, other brain areas should be analyzed to determine possible region-specific influences of microbiota on AVP and OXT immunoreactivity.

Furthermore, the lack of significant effects of emulsifier treatment on AVP-IR and OXT-IR may be due to the developmental stage of the animals tested. In this experiment, there was only one time schedule of treatment and all animals were administered a constant level of emulsifiers throughout their entire development. In addition, the effects of dietary emulsifiers were only determined at adulthood. It is possible that emulsifiers demonstrate their effects at earlier time periods of development, such as neonatally or at juvenile age and that these effects are abolished after the onset of puberty or during adulthood. Developmental differences in AVP and OXT regulation of social behavior has been shown in that juvenile rats aged P35 demonstrate increased social behavior compared to their adult counterparts, and AVP and OXT receptor binding density was significantly higher in juveniles than in adults in several brain areas (Smith et al., 2017). Future studies can test the effects of these emulsifiers across various ages through different scheduling of administration from birth through adulthood to pinpoint developmental effects of emulsifier-induced intestinal inflammation.

Although there were no significant main effects of treatment on AVP-IR and OXT-IR in the brain areas studied, there was a significant main effect of sex in the AVP-IR in the PVT, in that males had increased AVP-IR than females. This result indicates a basal difference in AVP expression in this area, unaffected by emulsifier treatment. Although this sex difference in this particular area is a novel finding, AVP has been consistently shown to be sexually dimorphic in

the same manner in the BNST, and AVP-IR projections from the BNST to the lateral septum, lateral habenular nucleus, and periaqueductal gray was significantly higher in males than in females (De Vries and al-Shamma, 1990). In addition, conventionally colonized Swiss Webster mice demonstrate a sex difference in AVP-IR in the PVN with males showing increased AVP-IR compared to females (Mehta et al., 2015). It would be interesting to see whether OXT-IR demonstrates a similar basal sex difference in the PVT.

In conclusion, this research did not reveal the vasopressin and oxytocin systems as possible molecular mechanism for the effects of emulsifier treatment on physiological and behavior changes in mice. Further findings in this field may uncover an underlying neural mechanism for neurological and gastrointestinal diseases, leading to possible targets for treatment.

REFERENCES

Bienenstock, J., Kunze, W., & Forsythe, P. (2015). Microbiota and the gut–brain axis. *Nutrition reviews*, *73*(suppl 1), 28-31.

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends in molecular medicine*, *20*(9), 509-518.

Chassaing, B., Aitken, J. D., Malleshappa, M., & Vijay-Kumar, M. (2014). Dextran Sulfate Sodium (DSS)-Induced Colitis in Mice. *Current Protocols in Immunology / Edited by John E. Coligan ... [et Al.]*, *104*, Unit–15.25. http://doi.org/10.1002/0471142735.im1525s104

Chassaing, B., Koren, O., Goodrich, J., Poole, A., Srinivasan, S., Ley, R. E., & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature*, *519*(7541), 92–96. http://doi.org.ezproxy.gsu.edu/10.1038/nature14232

Dabrowska, J., Hazra, R., Ahern, T. H., Guo, J.-D., McDonald, A. J., Mascagni, F., … Rainnie, D. G. (2011). Neuroanatomical evidence for reciprocal regulation of the corticotrophin releasing factor and oxytocin systems in the hypothalamus and the bed nucleus of the stria terminalis of the rat: Implications for balancing stress and affect. *Psychoneuroendocrinology*, *36*(9), 1312–1326. http://doi.org.ezproxy.gsu.edu/10.1016/j.psyneuen.2011.03.003

De Palma, G., Collins, S. M., & Bercik, P. (2014). The microbiota-gut-brain axis in functional gastrointestinal disorders. *Gut Microbes*, *5*(3), 419–429. http://doi.org.ezproxy.gsu.edu/10.4161/gmic.29417

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G., & Cryan, J. F. (2014). Microbiota is essential for social development in the mouse. *Molecular Psychiatry*, *19*(2), 146–148. http://doi.org.ezproxy.gsu.edu/10.1038/mp.2013.65

De Vries GJ, al-Shamma HA. Sex differences in hormonal responses of vasopressin pathways in the rat brain. J Neurobiol. 1990;21:686–693. doi: 10.1002/neu.480210503.

Fodor, A., Pintér, O., Domokos, Á., Langnaese, K., Barna, I., Engelmann, M., & Zelena, D. (2013). Blunted HPA axis response in lactating, vasopressin-deficient Brattleboro rats. *Journal of Endocrinology*, *219*(2), 89-100.

Foster, J. A., & Neufeld, K. A. M. (2013). Gut–brain axis: how the microbiome influences anxiety and depression. *Trends in neurosciences*,*36*(5), 305-312.

Hernández, V. S., Hernández, O. R., Perez de la Mora, M., Gómora, M. J., Fuxe, K., Eiden, L. E., & Zhang, L. (2016). Hypothalamic Vasopressinergic Projections Innervate Central Amygdala GABAergic Neurons: Implications for Anxiety and Stress Coping. *Frontiers in Neural Circuits*, *10*, 92. http://doi.org.ezproxy.gsu.edu/10.3389/fncir.2016.00092

K.D. Mehta, M.K. Holder, N.V. Peters, G.J. de Vries. Effects of microbiota on vasopressin and microglia in swiss webster mice. Poster presented in Atlanta, GA: Brain and Behavior Summer Symposium, 2015.

Li, Q., & Zhou, J. M. (2016). The microbiota–gut–brain axis and its potential therapeutic role in autism spectrum disorder. *Neuroscience*, *324*, 131-139.

Luna, R. A., Oezguen, N., Balderas, M., Venkatachalam, A., Runge, J. K., Versalovic, J., … Williams, K. C. (2017). Distinct Microbiome-Neuroimmune Signatures Correlate With Functional Abdominal Pain in Children With Autism Spectrum Disorder. *Cellular*

and Molecular Gastroenterology and Hepatology, *3*(2), 218–230. http://doi.org.ezproxy.gsu.edu/10.1016/j.jcmgh.2016.11.008

M.K. Holder, B. Chassaing, N.V. Peters, J. Whylings, A. Gewirtz, G.J. deVries. Sex differences in the effects of dietary emulsifiers on physiology and behavior in mice.Program No. 446.04 2016 Neuroscience Meeting Planner San Diego: Society for Neuroscience, 2016

Montiel-Castro, A. J., González-Cervantes, R. M., Bravo-Ruiseco, G., & Pacheco-López, G. (2013). The microbiota–gut–brain axis: neurobehavioral correlates, health and sociality. *Beyond the borders: The gates and fences of Neuroimmune interaction*, *7*, 63-78.

Murray, E. K., Varnum, M. M., Fernandez, J. L., de Vries, G. J., & Forger, N. G. (2011). Effects of neonatal treatment with valproic acid on vasopressin immunoreactivity and olfactory behaviour in mice. *Journal of neuroendocrinology*, *23*(10), 906-914.

Neufeld, K. M., Kang, N., Bienenstock, J., & Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, *23*(3), 255-e119.

Rood, B. D., Murray, E. K., Laroche, J., Yang, M. K., Blaustein, J. D., & De Vries, G. J. (2008). Absence of progestin receptors alters distribution of vasopressin fibers but not sexual differentiation of vasopressin system in mice. *Neuroscience*, *154*(3), 911-921.

Smith, C. J. W., Poehlmann, M. L., Li, S., Ratnaseelan, A. M., Bredewold, R., & Veenema, A. H. (2017). Age and sex differences in oxytocin and vasopressin V1a receptor binding densities in the rat brain: focus on the social decision-making network. *Brain Structure & Function*, *222*(2), 981–1006. http://doi.org.ezproxy.gsu.edu/10.1007/s00429-016-1260-7