MOLECULAR SEX DIFFERENCES IN CGRP-EXPRESSING NORADRENERGIC NEURONS IN THE MOUSE A7

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BACKGROUND

Norepinephrine (NE) is a neurotransmitter that modulates many behaviors and physiological processes. NE plays a key role in both the central and peripheral nervous systems (1), making it a unique target of pathological treatments. NE function is affected by various genes which innervate its axons, including calcitonin gene-related peptide (CGRP).

CGRP PLAYS A KEY ROLE IN:

- THE ONSET OF MIGRAINE (2)
- CANCER-INDUCED ANOREXIA (3)
- CARDIOVASCULAR DISEASE (4)

Prior research has confirmed the presence of six anatomically distinct NE subpopulations in the mature mouse brain (5): A1, A2, A5, A7, locus coeruleus (LC) and subcoeruleus (SubC). The NE system has also been found to have projections to some forebrain regions, including the thalamus, hypothalamus, and BNST.

Figure 1. Anatomically defined NE subpopulations in the adult mouse brain (5).

In existing neuroscience literature, sex bias is a prevalent issue. A large majority of published data fails to examine sex as an experimental variable. In order to contribute to the growing body of research in neuroscience which does examine and analyze sex differences, we aim to investigate sex differential expression of CGRP in the norepinephrine system, specifically in the A7 region.

HYPOTHESIS

Based on prior research regarding sex differential expression of CGRP and its receptor components, we hypothesize that **female animals will show higher co-localization of CGRP and NE in A7.**

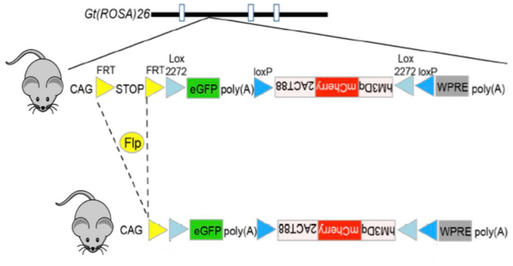

Figure 2. Gene targeting scheme for generation of transgenic mice.

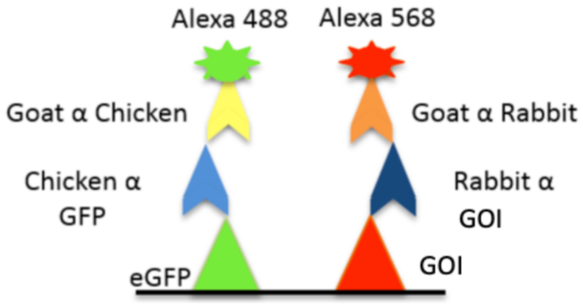

Figure 3. Visual representation name and order of antibodies used.

METHODS

In this experiment, we used transgenic mouse brain slices (Figure 1) and double immunofluorescence labelling in order to determine the level of CGRP expression in A7 NE neurons in male vs. female animals. In the generation of these mice, Dbh driven Flp was used to recognize FRT sites in mice containing Dbh::Flpo; R/C::FL-hM3Dq, excising the stop cassette in the NE neurons, resulting in GFP expression in all NE neurons, allowing for the visualization of all anatomical NE subpopulations under the microscope with green fluorescence.

PRIMARY ANTIBODY INCUBATION

A dual primary antibody solution was used to label eGFP expressing NE neurons.

SECONDARY ANTIBODY INCUBATION

A secondary antibody solution was used to label NE neurons with green fluorescence, and CGRP-expressing neurons with red fluorescence.

IMAGE ANALYSIS

Images were analyzed using Fiji ImageJ software. Neurons were visualized under 385 nm light with DAPI stain in order to locate NE subpopulations. eGFP was visualized under 470 nm light, and CGRP expressing-neurons were visualized under 590 nm light.

RESULTS: DOUBLE IMMUNOFLUORESCENCE OF GFP AND CGRP IN A7 REGION

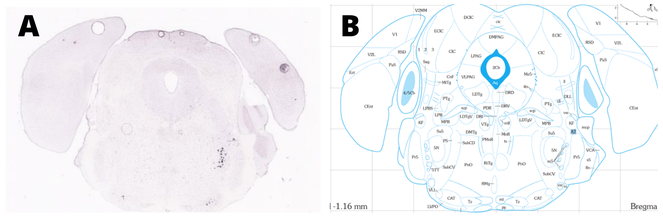

Figure 4. a) ISH imaging from Allen Brain Institute confirming CGRP expression in area A7 (coronal view). b) Digital anatomical map from Franklin and Maxinos locating A7 (coronal view).

IMMUNOREACTIVITY. Based on the size and visibility of eGFP-expressing norepinephrine neurons under 470 nm light (Figure 5), as well as the presence of some visible CGRP expression visualized using 590 nm light (Figure 5), we determined that the labelling we performed using antibodies was successful. Significant co-expression of norepinephrine and CGRP was found in both male and female mice, with no significant sex-differential expression (Figure 6).

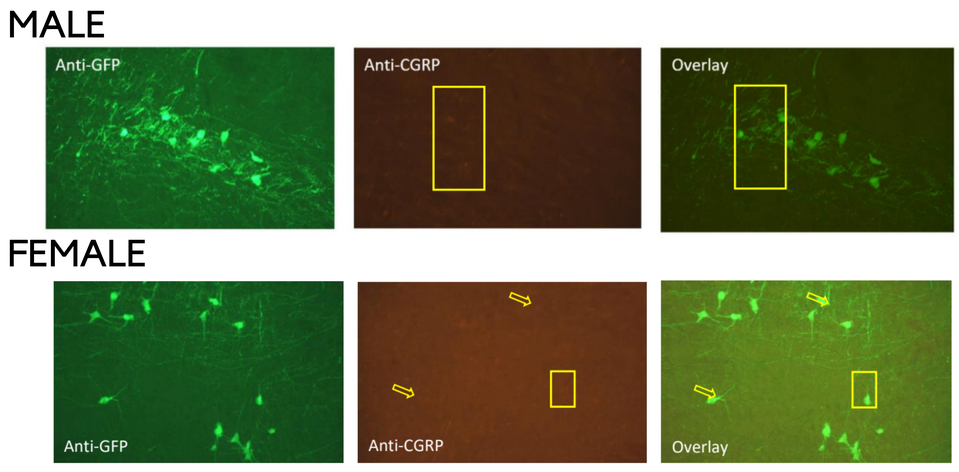

Figure 5. IHC results showing norepinephrine neurons (green) and CGRP expression in the exact same regions (red) in the male vs. female mouse A7. Merged images are also shown to display any co-localization.

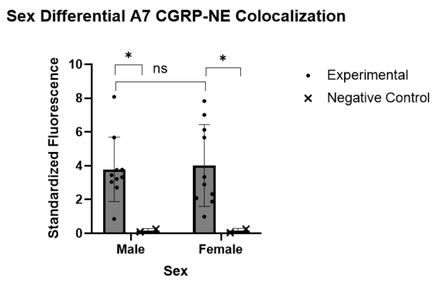

Figure 6. Sex differential A7 CGRP-norepinephrine co-localization.

STATISTICAL ANALYSIS. In order to determine if the sex differential CGRP expression we found was significant, we performed an unpaired two-tailed T-test (Figure 6) using the standardized fluorescence values for the male and female A7 region. The p-value we found was 0.68 (*n* males = 10, *n* females = 10), which is much higher than the p-value required in order to declare the results of the T-test statistically significant (p=0.05). This indicates that there was so significant difference between male and female CGRP expression in A7. In order to determine if co-localization of NE and CGRP was significant, we performed a single factor ANOVA analysis (figure 6). Both p-values were > 0.05, meaning co-localization was significant in both sexes (p-values: female = 0.01 , male = 0.02).

CONCLUSION

In this experiment, we aimed to determine if there is sex differential expression of CGRP in brain area A7 and if so, to what degree. Based on qualitative analysis, as well as statistical tests, we determined that **there is significant co-expression of norepinephrine and CGRP in A7.** We also found that **there is no significant sex differential expression of CGRP in A7.**

Our experiment did have a few limitations which could be improved upon in further research. Our sample size was only 10 male and 10 female mice. More animals could have provided us with more representative data. Additionally, it may be useful to use a positive primary antibody control in future experiments, as we only used a negative control in this experiment.

Previous research has found a link between CGRP expression and the norepinephrine system. Our experimentation supported existing data, as **we did find significant co-expression in A7.** This suggests that **future research may focus on the expression and location of CGRP and its various receptor components in A7 as targets for therapeutic migraine-treatments, as well as for other CGRP-mediated diseases.**

RESOURCES

1 Norepinephrine - Rice University. https://www.caam.rice.edu/~cox/wrap/norepinephrine.pdf. **2.** Durham, Paul L. "Calcitonin gene-related peptide (CGRP) and migraine." Headache vol. 46 Suppl 1,Suppl 1 (2006): S3-8. doi:10.1111/j.1526-4610.2006.00483.x **3.** Campos, Carlos A et al. "Cancer-induced anorexia and malaise are mediated by CGRP neurons in the parabrachial nucleus." Nature neuroscience vol. 20,7 (2017): 934-942. doi:10.1038/nn.4574 **4.** Kee, Zizheng, et al. "The Role of Calcitonin Gene Related Peptide (CGRP) in Neurogenic Vasodilation and Its Cardioprotective Effects." Frontiers, Frontiers, 1 Jan. 1AD, https://www.frontiersin.org/articles/10.3389/fphys.2018.01249/full#h5. **5.** Robertson, S., Plummer, N., de Marchena, J. et al. Developmental origins of central norepinephrine neuron diversity. Nat Neurosci 16, 1016–1023 (2013). https://doi.org/10.1038/nn.3458

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