MOLECULAR SEX DIFFERENCES IN CGRP-EXPRESSING NORADRENERGIC NEURONS IN THE MOUSE SUBCV AND A5

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BACKGROUND

Norepinephrine (NE) is a neurotransmitter that modulates multiple diverse behaviors and psychological processes throughout the central and peripheral nervous systems (1). NE's function is affected by many genes that innervate its axons including calcitonin-gene related peptide (CGRP).

CGRP PLAYS A UNIQUE ROLE IN:

- THE ONSET OF MIGRAINES[2]
- VASODILATION[3]
- FEEDING BEHAVIOR[4]

While recent research has revealed unrecognized NE neuronal diversity outside of the LC subpopulation (5), other genetically distinct norepinephrine subpopulations are understudied. **Are there other genetically defined subpopulations of NE neurons?** Here, we seek to identify subpopulations of NE neurons, defined by their expression of CGRP, our gene of interest (GOI).

Systematic analysis of several neuroscientific publications using rats and mice has revealed that sex bias towards male subjects persists in neuroscience research (6), increasing the current demand for research which classifies sex as an experimental variable. To address this knowledge gap, we also seek to investigate whether CGRP-expressing NE neurons exhibit sex-differential expression within the SubCV and A5 regions of both male and female mice.

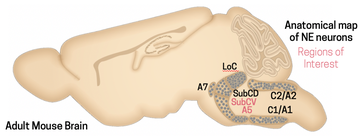

Figure 1. Adult mouse brain and NE subpopulations including the SubCV & A5 regions of interest

Previous investigations have found significant sex differences in CGRP and CGRP receptor component expression suggesting that females have a higher expression level of CGRP-encoding gene in the medulla (7). Sex-linked physiological differences in inflammatory response may be related to estrogen regulation of trigeminal ganglia sensitivity. Additionally, due to CGRP's association with migraine pathology and reports that migraines are more prevalent in human females than males, CGRP actvivity in the trigeminal ganglia likely differs by sex (8). Based on this prior literature, **we hypothesize that CGRP will predominate in female mice over males in both the A5 and SubCV.**

METHODS

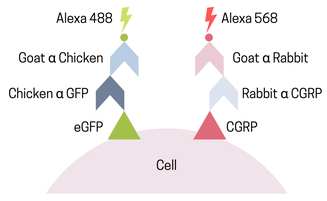

Figure 2. Dual primary antibody and secondary antibody protocol distinguishes eGFP- and CGRP-expressing neurons.

EXPERIMENTAL TIMELINE

PRIMARY ANTIBODY
We used a dual primary antibody solution to label eGFP-expressing norepinephrine neurons and CGRP.

SECONDARY ANTIBODY
We used a secondary antibody solution to label NE neurons with green fluorescence and CGRP neurons with red fluorescence.

IMAGE ANALYSIS
We visualized all hindbrain neurons under 385 nm light, eGFP-expressing NE neurons under 470 nm light, and mCherry-expressing CGRP neurons under 590 nm light

ANTIBODY INFORMATION

Table 1. Description of primary and secondary antibodies.

	Name	Host	Dilution
Primary antibodies	Chicken Anti-GFP (AB13970)	Chicken	1:10,000
	Rabbit Anti-CGRP (AB15360)	Rabbit	1:2,000
	Name	**Against**	**Dilution**
Secondary antibodies	AlexaFluor 488 (A-11039)	Goat	1:1000
	Goat Anti-Rabbit AlexaFluor 568 (A-11036)	Rabbit	1:1000

RESULTS

IMMUNOREACTIVITY

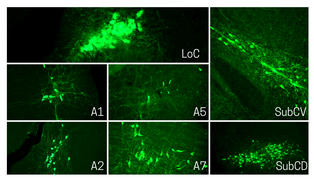

Figure 3. Immunofluorescent imaging of all NE subpopulations

The density and size of immunoreactive eGFP-expressing NE neurons in all subpopulations (A1, A2, A5, A7, SubCD, SubCV, LoC) indicates a successful immunohistochemistry antibody protocol for the anti-GFP and AlexaFluor 488 antibodies (Figure 3). Immunoreactivity of CGRP fibers in subjects including animal A206 female showed evidence of CGRP expression within the test subjects (Figure 4). These immunoreactive CGRP fibers indicate a successful antibody protocol for the anti-CGRP and ALexaFluor 569 antibodies. Although both eGFP-expressing NE neurons and CGRP neurons were immunoreactive, overlayed images of the A5 (Figure 5) and SubCV (Figure 6) did not exhibit coexpression of NE and CGRP within the same anatomical location of the two subpopulations.

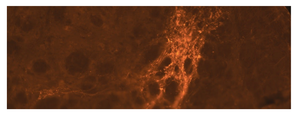

Figure 4. Immunofluorescent CGRP fibers from animal A206, female

In addition to coexpression of CGRP and NE, we also explored sex differential expression of CGRP through overlayed image analysis. Figure 5 shows A5 immunofluorescence in both male (A-C) and female (D-F) mice. Figure 6 follows the same layout. There is an absence of CGRP immunoreactivity in both subpopulations (5B,E & 6B,E), and across sexes. **We did not find evidence of sex differential CGRP expression in the A5 or the SubCV through this qualitative analysis.**

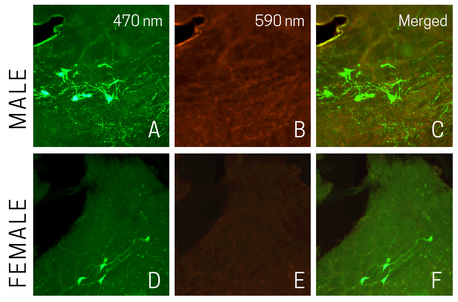

Figure 5. Immunofluorescent imaging of A5 NE subpopulation in male A946 and female B177 3HB mice.

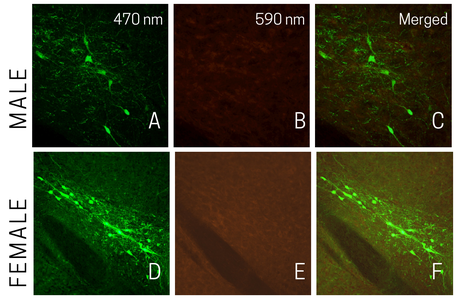

Figure 6. Immunofluorescent imaging of SubCV NE subpopulation in male B342 and female A020 3HB mice.

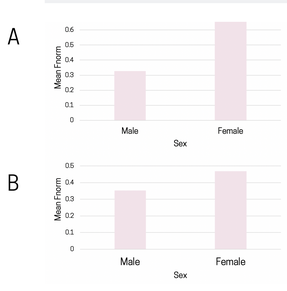

Figure 7. Graphical representation normalized fluorescence values. (A) Mean fnorm values across sexes in A5 (B) Mean fnorm values across sexes in SubCV

FLUORESCENCE

To test whether mean levels of normalized fluorescence differed between the sexes specifically in the A5 or SubCV subpopulations, we conducted independent-samples t-tests. Results of the two-tailed t-test for the A5 subpopulation indicated that females had the same level of normalized fluorescence (M = 0.67, SD = 0.38) relative to males (M = 0.33, SD = 0.35), t (6) = -1.34, p =.229.

Table 2. Average normalized fluorescence values for each animal, both A5 and SubCV subpopulations.

A5				SubCV			
Male Animal	Mean Fnorm	Female Animal	Mean Fnorm	Male Animal	Mean Fnorm	Female Animal	Mean Fnorm
A018	0.132	A020	1.000	A018	0.230	A020	0.076
A946	0.217	A206	0.925	A946	0.400	A206	0.710
B342	0.108	B177	0.621	B342	0.519	B177	0.427
C108	0.850	B349	0.154	C108	0.261	B349	0.662

Results of the two-tailed t-test for the SubCV subpopulation indicated that females had the same level of normalized fluorescence (M = 0.47, SD = 0.29) relative to males (M = 0.35, SD = 0.13), t (6) = -.730, p = .493.

Figure 7A compares the mean bias scores for males and females in the A5 subpopulation, and Figure 7B compares the mean normalized fluorescence levels for males and females in the SubCV subpopulation. **There was not a significant difference between males and females in either subpopulation. Therefore, sex differences in fluorescence for each subpopulation were likely due to chance.**

MORE RESULTS

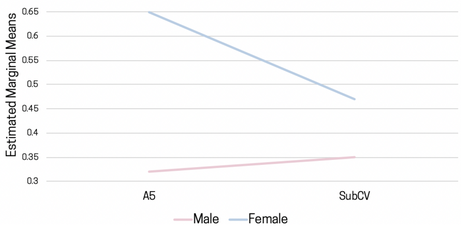

Figure 8. Interaction between sex and subpopulation on normalized fluorescence

To determine whether the effect of sex on level of fluorescence differed as a function subpopulation, we conducted a 2 (Sex: male, female) X 2 (Subpopulation: A5, SubCV) analysis of variance (ANOVA). We did not find a significant main effect of sex on normalized fluorescence. We also did not find a significant main effect of subpopulation on normalized fluorescence. Finally, there no significant interaction between sex and subpopulation on normalized fluorescence level, F (1, 15) = .578, p = .462. Figure 8 displays a plot of the analysis of variance. **There were no significant sex differences in normalized fluorescence levels between subpopulations.**

CONCLUSION

This study aimed to identify sex-differential expression of CGRP neurons in the A5 and SubCV. Based on our qualitative and quantitive analysis, significant expression of CGRP was not found in the A5 and the SubCV. Additionally, no significant differences were found in the expression of CGRP between the sexes. Some limitations of this study were its small sample size (n=4 per sex) and the absence of a negative primary antibody control.

Although prior literature suggested a link between NE and CGRP, future investigations should focus on the expression of receptor components in addition to the CGRP neuropeptide itself **The information to be gained from future research could be used to improve sex-specific differential diagnosis and treatment of migraines and psychiatric disorders.**

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