Single-Nucleus RNA Sequencing Exploration of Adolescent Rhesus Macaques in Relation to Anxious Temperament

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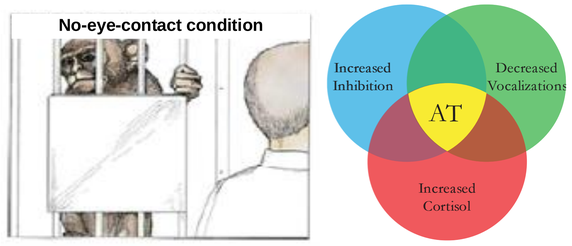
Background

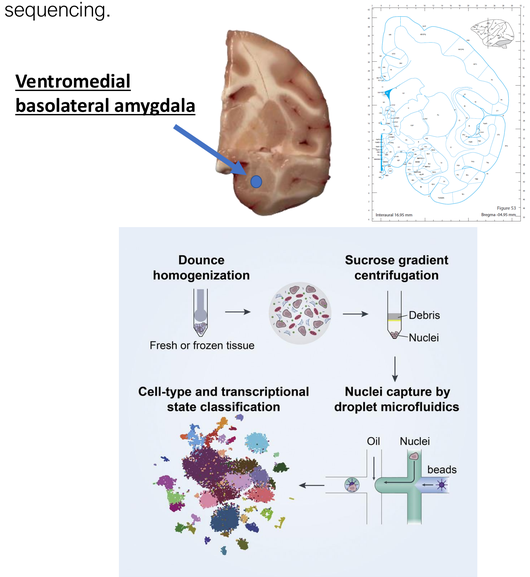
Anxiety disorders are among the most prevalent psychiatric disorders, with symptoms often beginning early in life, and the societal impact and severity of these disorders has been increasing[1]. Therefore, there is a compelling need to develop novel treatment strategies. To establish these strategies, it is critical to identify the molecular substrates underlying these disorders. Nonhuman primates (NHP) provide an important translational bridge for exploring human psychopathology due to similarities to humans in brain morphology, social structure and behavior,[2] and DNA and amino acid sequence. In collaboration with Dr. Elisabeth Binder's laboratory at the Max Planck Institute of Psychiatry in Germany, we have begun a study to identify cell type-specific gene expression patterns in the amygdala and other components of the anxiety circuit as a step towards understanding the cells and genes within those regions that are related to the phenotype that confers risk to develop stress-related psychopathology, termed anxious temperament (AT). From a cohort of 72 preadolescent rhesus macaques, phenotyped for AT and brain function and structure with multimodal imaging, we sampled tissue from the ventromedial basolateral amygdala region for single-nucleus RNA sequencing (snRNAseq) and bulk RNA sequencing (RNAseq[3,4]. We describe the preliminary snRNAseq results, including quality control metrics and a characterization of the various cell types that comprise the young rhesus amygdala. Annotation of cell types based on known marker genes revealed large proportions of excitatory and inhibitory neurons, and glia cells, including oligodendrocytes, microglia, astrocytes, and oligodendrocyte progenitor cells[5,6,7]. Preliminary results have identified genes with expression patterns related to age and cortisol levels. Future work will examine relations between gene expression and AT.

Methods

Rhesus macaques (N=72; age 1.8 – 4.3 years; N=24 female), were phenotyped for AT using the no-eye-contact (NEC) condition of the human intruder paradigm (HIP) that consisted of a human intruder presenting their profile to the monkey without making direct eye contact. The AT score was calculated as a composite of freezing behavior and coo vocalizations during the NEC and plasma cortisol values assessed immediately after the NEC.



Following euthanasia, ventromedial basolateral amygdala tissue punches (3 mm diameter) were collected from fresh frozen coronal tissue slabs (3.5 mm thick) and sent to the Binder Laboratory for sequencing.



Nuclei were extracted from ~20 mg of frozen tissue. For single-nucleus RNA sequencing (snRNA-seq), libraries were prepared using the 10x Genomics Chromium kit (v3.1), targeting 10,000 nuclei per sample. Libraries were sequenced on an Illumina NovaSeq X Plus System. Sequence reads were demultiplexed, aligned to the Macaca mulatta genome (NCBI 103), and processed using cell ranger count (Cell Ranger v8.0.1; 10x Genomics) including the argument --include-introns. As shown in Figure 1, SoupX was used for ambient RNA removal. Low-quality nuclei were filtered based on median absolute deviation (MAD) using scanpy v 1.10.2. Nuclei with MAD >= 4 were excluded. Any nuclei with a mitochondrial gene expression > 2% were excluded, then a MAD cutoff of >= 3 was set. Also shown, in Figure 1, doublets were identified using both doubletdetection v4.2 and scDblFinder v1.16.0 and removed. Genes expressed in fewer than 500 nuclei were removed. Quality control excluded four samples, resulting in a final dataset of 799,290 nuclei from 68 subjects, with 22,270 expressed genes. Data were normalized using scran, followed by feature selection, dimensionality reduction, clustering, and cell type annotation

Data Cleaning

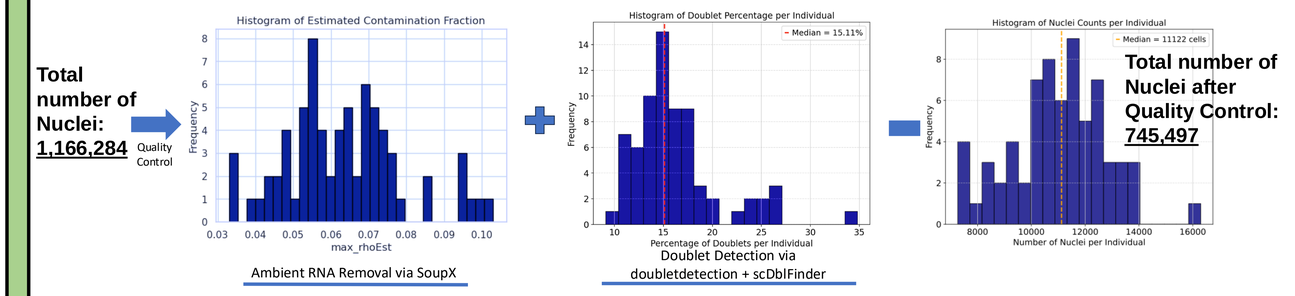


Figure 1. Data processing to remove ambient, non-nuclear RNA (left panel) and doublets (where two nuclei are contained in the same droplet; middle panel) followed by normalization yielding the frequency distribution plot of the number of nuclei represented across the 68 subjects (x-axis).

Neuronal Subtype Analysis

Clustering and uMAP - Using the Leiden algorithm at a resolution of 1.0 yielded 29 clusters of nuclei annotated using known marker genes for major cell types (Figure 1): For example, astrocytes (*AQP4*, *GFAP* and *GJA1*), excitatory (glutamatergic) neurons (*SLC17A7*, *SLC17A6* and *SATB2*), inhibitory (GABAergic) neurons (*GAD1*, *GAD2*), microglia (*CX3CR1*, *C3* and *P2RY12*), oligodendrocytes (MPB, MOBP and PLP1) and oligodendrocyte precursor cells (*OLIG1*, *PDGFRA*). Uniform manifold approximation and projection (uMAP) was used to visualize significant clusters. See below panel for more.

Results: Clustering of Nuclei into Specific Cell Types

Figure 2. Left panel, through Leiden clustering of snRNA-seq data, we found numerous clusters of nuclei that were subtyped based on known markers and data from previous publications[5,6,7]. Notable clusters are those of excitatory neurons, inhibitory neurons, and oligodendrocytes showcasing new insight into the cellular composition of the NHP ventromedial basolateral amygdala region. Right panel, number of nuclei representing each cell type. Future studies will examine the relation between the ventromedial basolateral amygdala cell type composition and phenotypic factors such as AT, age, and levels of the stress hormone cortisol.

Results: Differential Gene Expression Analysis

Figure 3. Differential gene expression (DGE) analyses were performed to explore the relationship between gene expression and physiological measures. For example, a number of genes were identified whose expression levels are significantly correlated with individual differences in cortisol or age across the 68 subjects included in the study. Significant correlations were identified using an FDR adjusted p-value of 0.1. Examples of significant correlations are shown in the accompanying graphs. Left panel –Cortisol levels and *FKBP5* (FKBP Prolyl Isomerase 5) in astrocytes, and *PER1* (Period Circadian Regulator 1) in microglia. Right panel – Age and *EFNA5* (Ephrin A5) in astrocytes, and *ETV1* (ETS Variant Transcription Factor 1) in excitatory neurons.

The Importance and Value of snRNAseq

To characterize the transcriptional populations present within the ventromedial basolateral amygdala, we are leveraging single-nuclei RNA-Seq methods, which allow for the characterization of the RNA contents of individual nuclei, which can be derived from frozen tissue[8,9]. These methods have the potential to characterize the transcriptional heterogeneity present within amygdala neurons, both with respect to excitatory and inhibitory populations as well as non-neuronal cell types. Interestingly, in other brain regions, studies have shown that there are primate-specific interneuronal cell types that are not present in rodent species supporting the critical importance of performing these studies in NHPs[10]. Furthermore, analyses can be performed to identify transcripts that are differentially expressed within transcriptomic sub-populations, providing highly specific insight into the molecules associated with either extreme anxiety or the anti-anxiety effects of pharmacological, chemogenetic or behavioral manipulations.

Conclusions

The analyses presented here represent a preliminary exploration of the cellular composition of the NHP ventromedial basolateral amygdala region based on results from 68 subjects. The differential gene expression results are exciting to see genes that are being expressed across neuronal and non-neuronal cell subtypes in relation to cortisol and age at necropsy. Bulk RNAseq is currently being performed on the same tissue punch samples used here. This within-animal approach allows us to take advantage of the in-depth sequencing possible with "bulk" RNA-Seq along with the cell type-specific gene expression data afforded by snRNA-Seq.

Acknowledgments

This project was made possible through the support of the Neuroscience Training Program, the Max Planck Institute of Psychiatry, the UW HeatlhEmotions Research Institute, and the following NIH grants to NHK: R01 MH081884 and R01 MH046729. The authors thank the staff of the Harlow Center for Biological Psychology and the Wisconsin National Primate Research Center.

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