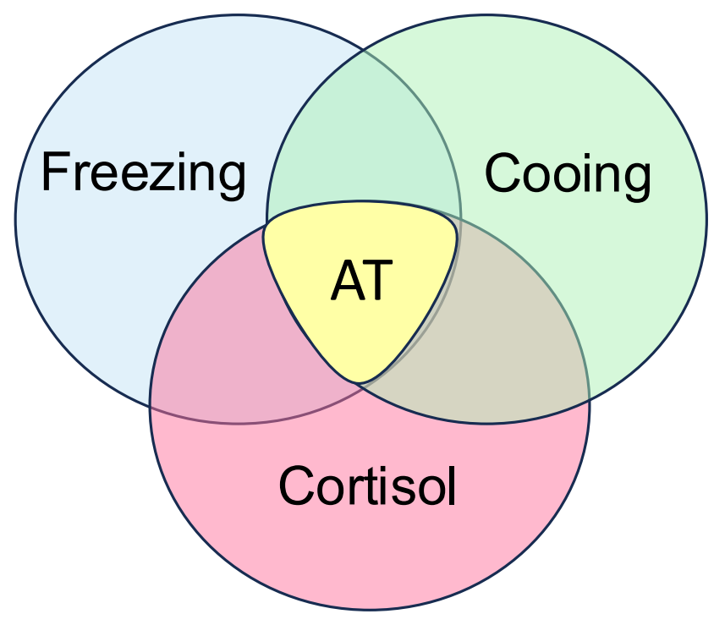
Exploration of the molecular basis of early life pathological anxiety through cell-type-specific gene expression in the basal amygdala region of nonhuman primates

LG Parkins[1,4], AS Fröhlich[2], PH Roseboom[1,4], JA Oler[1,4], NA Stowe[1,3,4], R Puralewski[1], EB Binder[2], NH Kalin[1,4]
[1]Department of Psychiatry, [2]Max Planck Institute of Psychiatry, [3]Neuroscience Training Program, [4]University of Wisconsin-Madison

Introduction

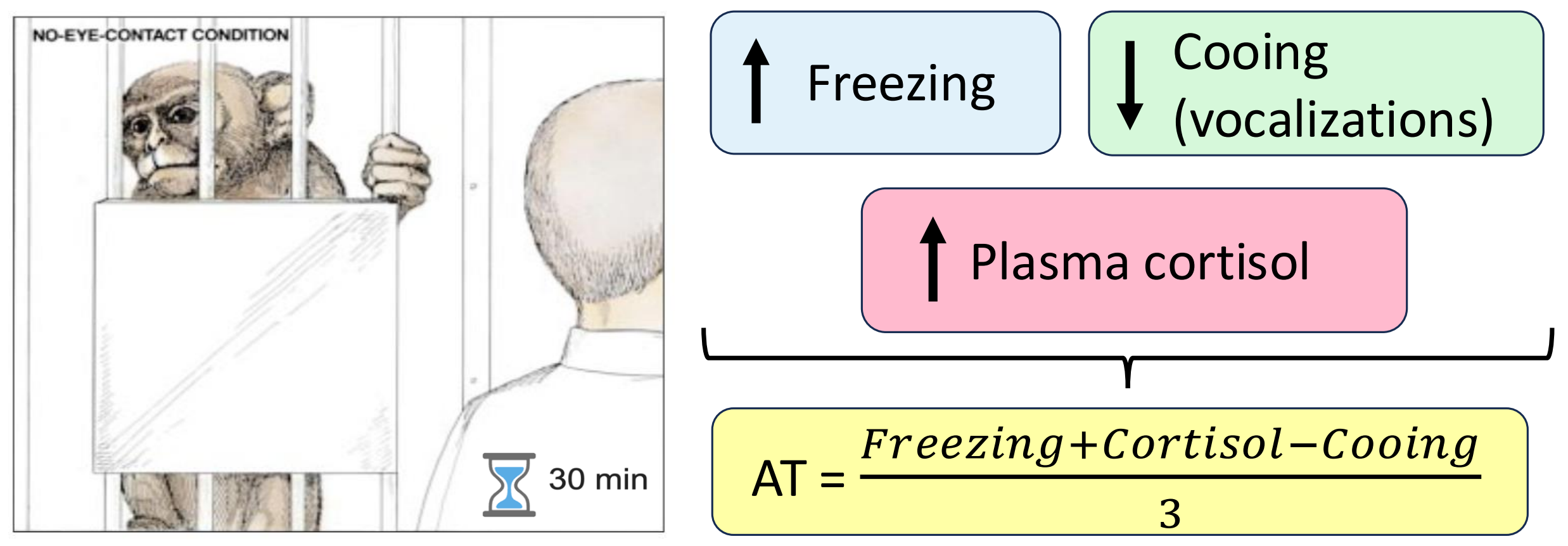


- Anxiety disorders (ADs) are highly prevalent and often emerge in early life.[1]
- Anxious Temperament (AT) is a nonhuman primate (NHP) model of early life trait anxiety and the risk for the development of anxiety disorders (ADs).[2]
- AT is a combination of behavioral and physiological responses to an indirect and potential novel threat.[2]
- The basolateral amygdala (BLA) is a critical component of AT-related neural circuitry and overall threat processing[1,3,4]
- The molecular mechanisms within the BLA that contribute to anxiety responses in early life remain unclear.
- Investigating the cell-type-specific molecular signatures that associate with AT and its components is critical for understanding the biological basis of ADs and may reveal therapeutic targets for early life interventions.

Aim: Characterize cell-type-specific gene expression in the basal amygdala region in relation to individual differences in AT and its components.

Methods

- 72 peri-pubescent *rhesus macaques* (age = 2.61 +/- 0.72, 24 female) were phenotyped for AT using the No-Eye-Contact Condition (NEC) of the Human Intruder Paradigm (HIP).
- During NEC, an unfamiliar human intruder stands presenting their profile to the monkey for 30 minutes, eliciting increases in freezing behavior and reductions in cooing vocalizations. Blood is collected from monkeys directly after NEC to assess threat-related increases in cortisol.



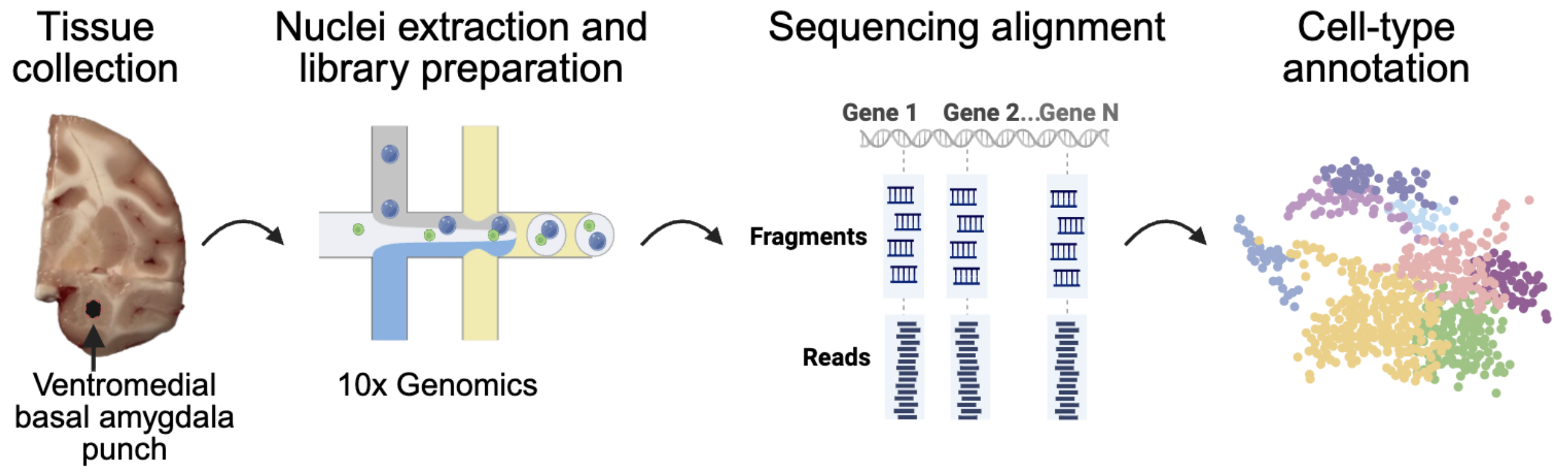
↑ Freezing

↓ Cooing (vocalizations)

↑ Plasma cortisol

$$AT = \frac{Freezing + Cortisol - Cooing}{3}$$

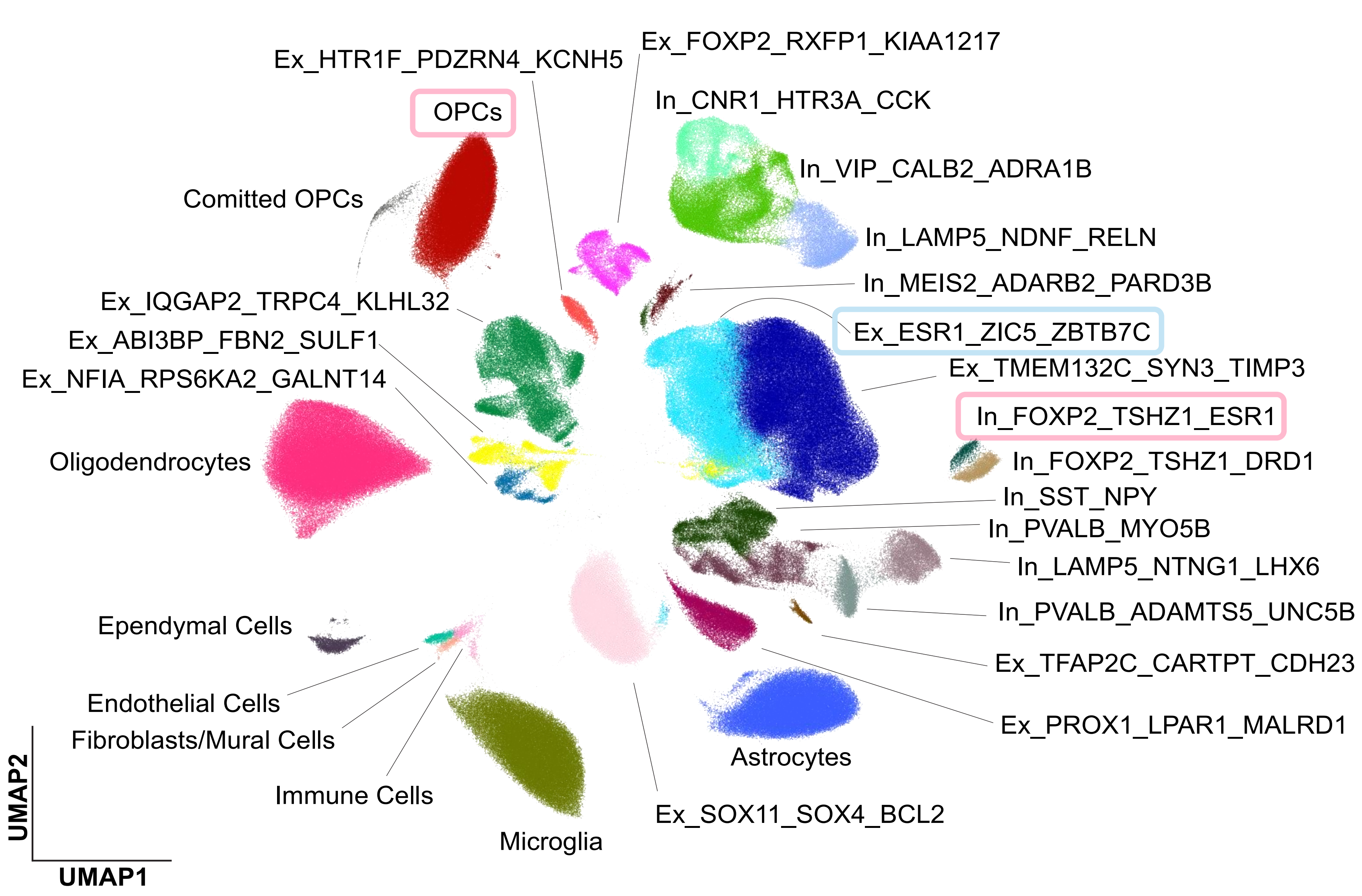
- At the time of euthanasia, blood was collected for basal cortisol assessment, and brain slabs were flash frozen.
- 3-mm punches from the basal amygdala region were collected for nuclei isolation and 10x single-nucleus RNA sequencing.
- Libraries were sequenced on a NovaSeq platform, aligned to the *Macaca mulatta* genome (NCBI 103), and processed with Cell Ranger. After quality control, 68 subjects remained.
- Cell types were defined using Leiden clustering and canonical marker genes as previously described.



- Differentially expressed (DE) genes were assessed by aggregating gene counts across cells within each cell type to generate cell-type-level profiles. Models accounted for effects due to batch, age, study/sex, RIN, cortisol at euthanasia, and either AT or NEC-related cortisol and freezing. FDR correction was applied both across and within cell types.

Results

Clustering of single-nucleus RNA sequencing data identifies 29 cell types within the basal amygdala region



Uniform manifold approximation and projection (UMAP) of 745,497 nuclei from 68 subjects with clusters colored by cell type. Leiden clustering identified 10 inhibitory neuron subtypes, 10 excitatory neuron subtypes, and 6 glial cell types (OPCs, committed OPCs, oligodendrocytes, microglia, astrocytes, and ependymal cells). This highlights the extensive cell type diversity within the basal amygdala region. Highlighted cell types are those with threat-related freezing-associated DE genes (blue) or threat-related cortisol-associated DE genes (pink) featured in later analyses. In, inhibitory neurons; Ex, excitatory neurons.

Number of DE genes within cell types that show significant associations with behavioral or physiological components of AT

Threat-Related Cortisol Genes

Cell Type	Number of Genes
*Astrocytes	2
Committed OPCs	3
In_FOXP2_TSHZ1_DRD1	142
In_FOXP2_TSHZ1_ESR1	8
*In_MEIS2_ADARB2_PARD3B	2
*OPCs	1
Oligodendrocytes	2

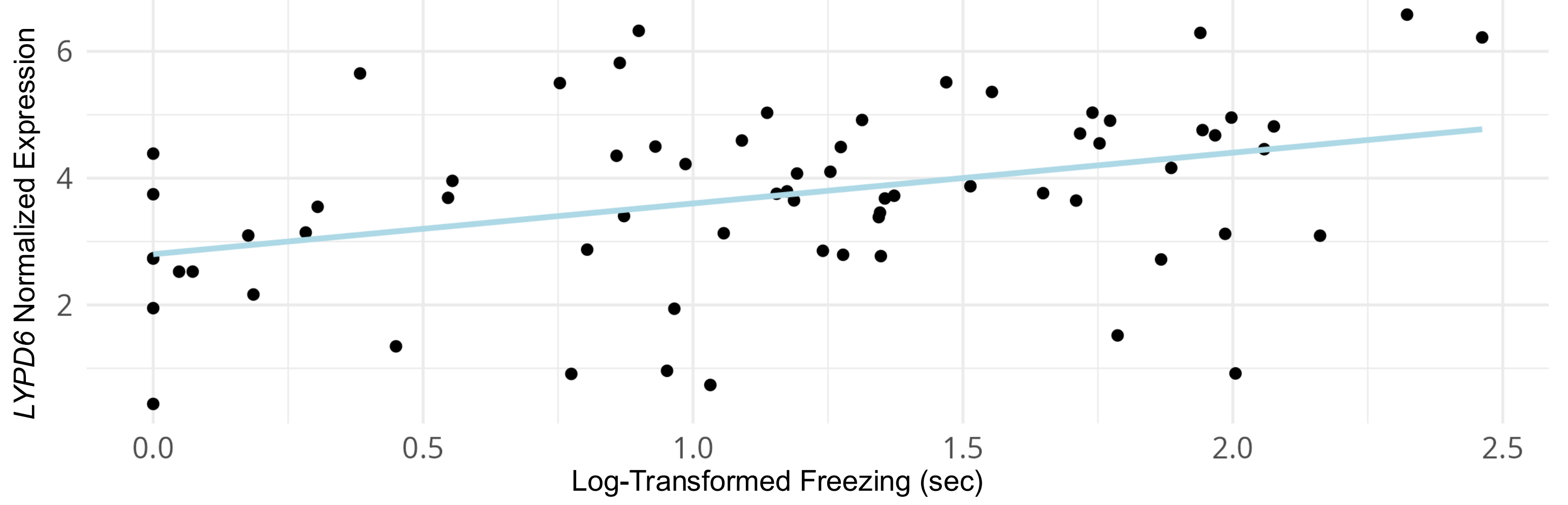
Threat-Related Freezing Genes

Cell Type	Number of Genes
Ex_ESR1_ZIC5_ZBTB7C	3
Ex_IQGAP2_TRPC4_KLHL32	1
In_LAMP5_NTNG1_LHX6	1
OPCs	1

DE genes associated with threat-related cortisol or freezing at within cell-type FDR-adjusted $P < 0.05$. For three cell types, a single gene remained significant after FDR correction across cell types ($P < 0.1$; denoted by *). No DE genes were found for AT.

Increased expression of *LYPD6* associates with higher levels of threat-related freezing

Ex_ESR1_ZIC5_ZBRB7C: Expression of *LYPD6* vs Threat-Related Freezing

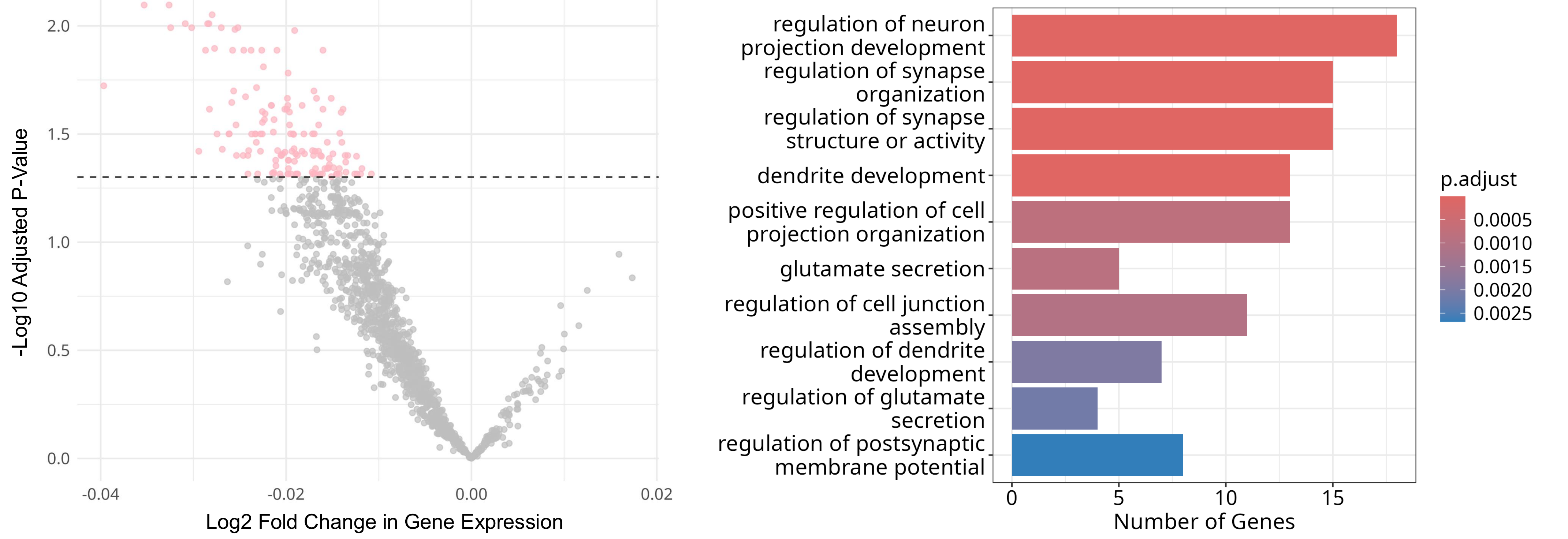


Relationship between *LYPD6* in Ex_ESR1_ZIC5_ZBTB7C neurons and threat-related freezing (within cell-type FDR-adjusted P = 0.0234). **LYPD6 modulates nicotinic acetylcholine receptors and is associated with intellectual disability and autism.[5,6]**

Downregulation of genes involved in neuron development and synaptic organization/function in relation to threat-related cortisol

In_FOXP2_TSHZ1_DRD1 Threat-Related Cortisol DE Genes

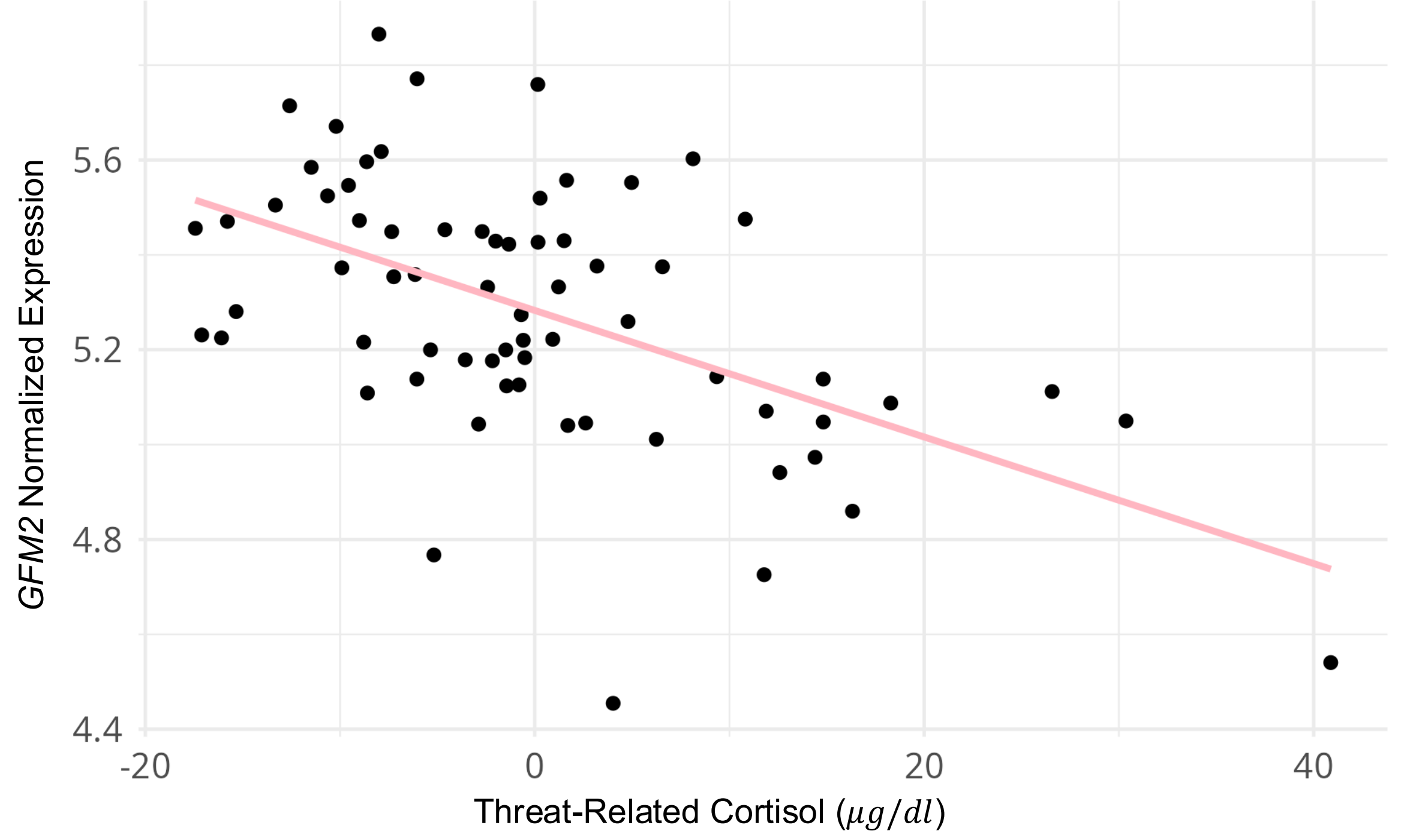
Gene Ontology of In_FOXP2_TSHZ1_DRD1 Threat-Related Cortisol DE Genes



In_FOXP2_TSHZ1_DRD1 neurons showed downregulation of 142 genes with threat-related cortisol (within cell-type FDR-adjusted P < 0.05). Gene ontology associated these genes with biological processes such as neuron development and synaptic organization/function. High *FOXP2* expression is characteristic of intercalated cells, groups of inhibitory neurons between amygdala subnuclei that mediate information flow between amygdala regions.[7]

Decreased expression of *GFM2* associates with higher levels of threat-related cortisol

OPCs: Expression of *GFM2* vs Threat-Related Cortisol



Relationship between *GFM2* in OPCs and threat-related cortisol (across cell-type FDR-adjusted P = 0.0812). Cortisol values were residualized for time of day. **GFM2 is involved in mitochondrial translation and is associated with mitochondrial diseases.[8]**

Discussion

- Cell-type-specific gene expression in the basal amygdala region predicts individual differences in threat-related freezing and cortisol of peri-pubescent monkeys.
- *LYPD6* upregulation with increased threat-related freezing suggests the possibility that altered cholinergic signaling may contribute to maladaptive anxiety responses.
- Intercalated cells show gene expression patterns robustly associated with threat-related cortisol, suggesting altered development/function of this cell type may contribute to dysregulated stress responses.
- These findings in NHPs advance mechanistic insight into early life AD risk in humans and highlight candidate molecular targets for therapeutic interventions.

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Acknowledgements

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