Single nuclei transcriptomics of human and monkey striatum implicates DNA damage, neuroinflammation, and neurodegeneration signaling in opioid use disorder

BaDoi N. Phan[1,2,3], Madelyn H. Ray[4,5], Xiangning Xue[6], Robert J. Fenster[7,8], Stephen J. Kohut[7,9], Jack Bergman[7,9], Suzanne N. Haber[7,10], Kenneth M. McCullough[11], Madeline K. Fish[12], Jill R. Glausier[13], Qiao Su[1], Allison E. Tipton[4], David A. Lewis[13], Zachary Freyberg[13,14], George C. Tseng[6], Shelley J. Russek[4,12], Yuriy Alekseyev[15], Kerry J. Ressler[7,8], Marianne L. Seney[13], Andreas R. Pfenning[1,2†], Ryan W. Logan[4,16,17†]

[1]Computational Biology Department, Carnegie Mellon University, Pittsburgh, PA, United States, 15213.

[2]Neuroscience Institute, Carnegie Mellon University, Pittsburgh, PA, United States, 15213.

[3]Medical Scientist Training Program, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States, 15213.

[4]Department of Pharmacology, Physiology & Biophysics, Boston University School of Medicine, Boston, MA, United States, 02118.

[5]Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA, United States, 02118.

[6]Department of Biostatistics, University of Pittsburgh, Pittsburgh, PA, United States, 15213.

[7]Department of Psychiatry, Harvard Medical School, Boston, MA, United States, 02115.

[8]Division of Depression and Anxiety, McLean Hospital, Department of Psychiatry, Harvard Medical School, Belmont, MA, 02478.

[9]Behavioral Biology Program, McLean Hospital, Belmont, MA, 02478.

[10]Department of Pharmacology and Physiology, University of Rochester, School of Medicine, Rochester, NY, 14642.

[11]Basic Neuroscience Division, Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA, 02478.

[12]Center for Systems Neuroscience, Boston University, Boston, MA, United States, 02118.

[13]Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States, 15219.

[14]Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States, 15219.

[15]Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA, United States, 02118.

[16]Department of Psychiatry, University of Massachusetts Chan Medical School, Worcester, MA, United States, 01605.

[17]Department of Neurobiology, University of Massachusetts Chan Medical School, Worcester, MA, United States, 01605.

[†]Co-corresponding Authors:

Ryan W. Logan, PhD
364 Plantation Street, LRB 611
University of Massachusetts Chan Medical School
Worcester, MA 01605
ryan.logan@umassmed.edu

Andreas R. Pfenning, PhD
Gates Hillman Center Room 7711
Carnegie Mellon University
Pittsburgh, PA 15213
apfenning@cmu.edu

Abstract

The striatum in the brain is involved in various behavioral functions, including reward, and disease processes, such as opioid use disorder (OUD). Further understanding of the role of striatal subregions in reward behaviors and their potential associations with OUD requires molecular identification of specific striatal cell types in human brain. The human striatum contains subregions based on different anatomical, functional, and physiological properties, with the dorsal striatum further divided into caudate and putamen. Both caudate and putamen are associated with alterations in reward processing, formation of habits, and development of negative affect states in OUD. Using single nuclei RNA-sequencing of human postmortem caudate and putamen, we identified canonical neuronal cell types in striatum (*e.g.*, dopamine receptor 1 or 2 expressing neurons, D1 or D2) and less abundant subpopulations, including D1/D2 hybrid neurons and multiple classes of interneurons. By comparing unaffected subjects to subjects with OUD, we found neuronal-specific differences in pathways related to neurodegeneration, interferon response, and DNA damage. DNA damage markers were also elevated in striatal neurons of rhesus macaques following chronic opioid administration. We identified sex-dependent differences in the expression of stress-induced transcripts (*e.g.*, *FKBP5*) among astrocytes and oligodendrocytes from female subjects with OUD. Thus, we describe striatal cell types and leverage these data to gain insights into molecular alterations in human striatum associated with opioid addiction.

Main

Rates of deaths from opioid overdose and people diagnosed with opioid use disorder (OUD) are continuing to rise in the United States[1]. Efforts to generate new treatment strategies and to bolster avenues of therapeutic development to treat opioid addiction require a deeper understanding of the changes that occur in the human brain with chronic opioid use. To date, few studies have investigated the cellular and molecular changes in human brain associated with OUD[2–6]. Recently, we reported alterations in molecular pathways related to neuroinflammation, extracellular matrix[7], and synaptic signaling in postmortem brains from unaffected subjects and subjects with OUD[2,5]. Markers for microglia were also significantly enriched in OUD subjects, along with significant changes in pathways related to neurodegenerative disorders.

Prior studies highlight putative roles for several pathways related to neurodegeneration in OUD, including dysfunctional aggregation of the tau protein[8] and activation of proinflammatory cascades. Activation of inflammatory signaling is initiated by a myriad of processes in the brain. Across several neurodegenerative disorders, DNA damage is associated with neuroinflammation and strongly implicated in early pathogenesis[9–12] of Alzheimer's disease, Parkinson's Disease, and Amyotrophic Lateral Sclerosis[13–15]. Elevated DNA damage can activate microglia to release proinflammatory cytokines[16,17], leading to the activation of oxidative stress[18–20], metabolic, and immune signaling cascades in other glial cell types and neurons[14,17,21]. Thus, emerging evidence in human brain reveals changes in pathways involved in the pathogenesis of neurodegenerative disorders to be associated with opioid addiction, with strong implications for the involvement of pathways that regulate DNA damage and neuroinflammation in OUD. Further investigating these molecular alterations associated with OUD using single cell technologies may yield important insights into the potential roles for DNA damage and neuroinflammation in specific cell types in human brain.

Single cell RNA-based approaches provide high-throughput analyses to characterize the transcriptomes of specific cell types in the human brain. Studies using single nuclei RNA-sequencing (snRNA-seq) can resolve neural cell type specificity from disease-associated molecular alterations previously found using other approaches (*e.g.*, bulk tissue RNA-seq). By stratifying pathways across different cell types, the aim is to uncover new disease-associated molecular mechanisms related to specific neuronal and glial cell types. In human brain, the striatum is anatomically divided into ventral and dorsal regions, whereby the dorsal region comprises the caudate and the putamen. Altered functioning of the dorsal striatum is implicated in several psychiatric and neurological disorders. In OUD, the dorsal striatum is of particular

interest, given the brain region's critical roles in reward processing, habitual drug-seeking, craving, and relapse, as well as the development of negative affective states during opioid withdrawal[22,2324,25]. However, only a few studies have characterized the various cell types in the human dorsal striatum and essentially none have explored transcriptional alterations in striatal cell types in subjects with OUD.

To identify transcriptional alterations associated with OUD in specific striatal cell types, we conducted snRNA-seq on both the caudate and putamen of dorsal striatum from unaffected comparison subjects and subjects with OUD (98,848 total nuclei from both caudate and putamen of 6 unaffected subjects and 6 subjects with OUD, evenly divided between females and males). Using these data, we identified transcriptionally and anatomically heterogeneous cell types in human striatum, followed by differential expression analysis comparing unaffected and OUD subjects. We found significant transcriptional changes in neuroinflammatory and neurodegenerative pathways, implicating neuronal DNA damage responses and activated glial responses of microglia in OUD. Elevated DNA damage markers were also found in specific neuronal cell types in the striatum of rhesus macaques following chronic opioid administration. Additionally, we explored the interaction between sex and disease, finding sex- and cell type-specific transcriptional programs associated with OUD. As a resource, we deposited the annotated and filtered single nuclei transcriptomes for human caudate and putamen in unaffected and OUD subjects on the CZ CELLxGENE Discover Portal.

Results

High quality single nuclei transcriptomics identifies canonical and low abundant cell types in human dorsal striatum

To investigate the molecular features of human dorsal striatum, we collected both caudate and putamen from unaffected subjects (n=6, 3 females and 3 males, for each subregion). Unaffected subjects were then compared to subjects with OUD to identify cell type-specific molecular alterations associated with opioid addiction (n=6, 3 females and 3 males, for each subregion). Both striatal regions were dissected from frozen postmortem tissue samples and processed to generate nuclei suspensions for snRNA-seq (Figure 1b). Based on quality control assessments, we used a total of 98,848 sequenced nuclei for subsequent analyses (~70% of captured nuclei with an average of 5,757 nuclei per caudate and 3,440 nuclei per putamen; Fig 1d). The number of nuclei or percent nuclei captured were similar across all subjects (p=0.40, Table S1). Each nucleus was deeply sequenced at 70% saturation rates, detecting an average of 3,513 ± 77 genes and 13,635 ± 560 unique transcripts per nucleus per subject (Figure 1e). The average number of genes detected and the number of unique mapped identifiers for transcripts were similar between unaffected and OUD subjects (p=0.55, Table S1).

We used multiple rounds of graph-based clustering, cell label transfers and refinement from a high-quality, high-resolution snRNA-seq dataset from non-human primate striatum[26,27] (Figure 1c). The first round identified major cell classes of glia and neurons: astrocytes (*AQP4*+), endothelial cells (*CLDN*+), microglia (*CX3CR1*+), mural cells (*CD44*+), oligodendrocytes (*ASPA*+), oligodendrocyte precursors (OPC, *PDGFRA*+), and neurons (*RBFOX3*+, *GAD2*+) (Fig S1). The second round identified major striatal neuronal subtypes, including medium spiny neurons (MSN, *PPP1R1B*+) and interneurons (*LHX6*+) (Fig S1). Recapitulating the heterogeneous striatal neuronal subtypes previously described in our non-human primate datasets[26], our snRNA-seq analysis identified canonical medium spiny neuron (MSNs), including *DRD1*+ and *DRD2*+ MSNs within distinct neurochemical compartments, the striosome (*KCNIP1*+) and matrix (*EPHA4*+).

In addition, we identified less abundant neuronal subtypes including MSNs expressing both *DRD1* and *DRD2* (D1/D2 hybrid cells, known as eccentric MSNs or D1H in mice)[26–29], mural cells, and several types of interneurons (*e.g.*, *CCK*+, *PVALB*+, *SST*+, and *TH*+; Figure 1f). Previously, interneuronal subtypes have only been described in mouse brain single cell RNA-seq datasets[30]. For subsequent analyses, we pooled interneuronal subtypes because of their comparatively lower abundance to other neurons, and largely shared transcriptional signatures. Overall, relative proportions of low or

high abundance striatal cell types were consistent across brain regions, between sexes, and between unaffected and OUD subjects (FDR=0.25, mixed effects linear regression, Fig S2, Table S2).

Neuronal and glial cell type-specific expression of opioid receptors in human striatum

Opioids, including endogenous opioids, enkephalin, dynorphin, and β-endorphin, differentially activate several classes of opioid receptors, including mu, delta, and kappa, encoded by *OPRM1*, *OPRD1*, and *OPRK1*, respectively. Previous efforts have mapped striatal expression of opioid receptors in non-human primates and rodents, characterizing species-specific and cell type-specific patterns of expression[31]. We investigated the expression of opioid receptors and cognate ligands within human dorsal striatum, revealing several notable results related to specific cell types and potential species differences in expression. First, *OPRM1* was detected in each MSN subtype with the highest level of expression in D1/D2 hybrid and D2-striosome MSNs (Fig S3), indicating that, in contrast to rodents, *OPRM1* expression is also in the striosome in human brain. *OPRM1* in other human striatal regions is consistent with prior findings in non-human primate striatum[26]. Additionally, we reproducibly detected *OPRM1* in microglia across subjects and biological samples, consistent with findings in rodents[32,33], establishing a potential pathway for opioids to directly modulate neuroimmune processes[5].

D1-striosome MSNs expressed the gene encoding the preproprotein, prodynorphin (*PDYN*), while D2-matrix and D2-striosome MSNs coexpressed *OPRD1* and proenkephalin (*PENK*) at markedly higher levels than other striatal cell types. Preferential expression of *OPRD1* and *PENK* in D2 MSNs in human striatum is consistent with rodent striatum[34]. Across MSNs, we detected *OPRK1* expression at many folds lower than *OPRM1*, *OPRD1*, *PENK*, or *PDYN*, suggesting that targets of the dynorphin-kappa opioid receptor signaling by D1-striosome MSNs may primarily be outside of dorsal striatum[35,36]. Between unaffected and OUD subjects, we found no significant differences in opioid receptor and/or endogenous ligand expression within specific cell types. Other mechanisms involved in the post-transcriptional and/or post-translational regulation of opioid receptor and ligand expression, along with receptor binding activity, may be altered in OUD.

Identification of cell type-specific transcription factor and gene regulatory networks in human dorsal striatum

Following cell annotations, we investigated novel transcriptional networks that represent putative regulatory networks in specific striatal cell types. First, we identified marker genes for each of the annotated cell types that were reproducible across biological replicates (mean ± standard error, 722 ± 172 specific marker genes per cell type, Figure 1g,

Table S3). Glial cell types yielded more marker genes (1,134 ± 204) compared to neuronal cell types (310 ± 131). Next, we inferred gene regulatory networks directly from each of the cell type-specific marker datasets using machine-learning (SCENIC)[37]. Briefly, SCENIC is based on the premise that transcriptional regulation of gene expression is, in part, based on transcription factor binding to proximal gene promoters. Based on single cell gene expression and the enrichment of promoter binding sites, we used SCENIC to build transcription factor gene regulatory modules that were specific to each of the neural cell types identified in human striatum. Collectively, we found many transcription factors and gene regulatory modules per cell type (47 ± 15 specific modules; glial subtypes, 90±19; neuronal subtypes, 12 ± 6; Figure 1h, Table S4) Together, the annotated cell type clusters in human dorsal striatum, their associated marker genes, and related transcription factor gene regulatory modules offer new insights into the molecular signaling pathways that contribute to the heterogeneity of cellular identity in human striatum.

Differentially expressed genes in specific striatal cell types associated with OUD

We assembled a cohort of subjects with OUD based on time since clinical diagnosis of at least four years and deceased from opioid overdose, as described previously[4,5]. Subjects with OUD were matched with unaffected subjects on sex, age, postmortem interval (PMI), and RNA integrity number (RIN). (Table S5, n=6 unaffected subjects and n=6 OUD subjects with 3 females and 3 males for both caudate and putamen; Figure 1a). We first aggregated gene expression profiles across cell types to generate pseudobulk profiles for each major cell type. We then directly compared gene expression profiles across neuronal and glial subtypes between unaffected and OUD subjects to identify differentially expressed genes (DEGs), weighted for cell type proportions between groups, while also considering covariates (age, PMI, RIN, cell type abundance, gene detection rate, and surrogate variables). We identified 1,765 DEGs across any cell types (197 ± 43 genes per cell type; FDR < 0.05, Table S6*)*, with more DEGs found in glial cells relative to neurons (Figure 2a, 3a). Further, DEGs in OUD subjects were largely unique to each glial cell type, while DEGs overlapped more between neuronal cell types (Fig S4).

Using gene set enrichment analyses (GSEA), we identified pathways significantly enriched in aggregate cell types and across specific cell types. Across cell types, we found 286 pathways (34 ± 7 pathways per cell type; FDR < 0.05, Table S7) and 51 differentially regulated transcription factor regulatory modules (5.1 ± 0.7 per cell type, FDR < 0.05, Table S8). The cell type-specific transcriptional differences in OUD subjects were represented largely by distinct

biological processes between neurons and glia. Among MSNs, DEGs had a higher tendency to be shared between spiny neuron subtypes, while DEGs tended to be more distinct between glial subtypes and between interneuron subtypes. We identified a higher number of DEGs in interneuron subtypes and D1/D2 hybrid MSNs compared to canonical D1 or D2 MSNs. Further, DEGs in D1/D2 hybrid MSNs did not overlap with canonical MSNs, suggesting these sparser populations of striatal neurons displayed a unique transcriptional response to OUD (Fig S4).

Pathways in neuronal subtypes converge on neurodegenerative processes in OUD

We conducted separate pathway analyses on upregulated and downregulated transcripts between unaffected and OUD subjects. Among neuronal subtypes, several upregulated pathways in OUD were associated with neurodegenerative processes. In MSNs, enriched pathways from upregulated DEGs in OUD included DNA replication and cell cycle re-entry (Figure 2b, Table S9), a common feature in neurodegeneration[38]. For example, both *MCM8* ($\log_2$ fold-change, log2FC > 1.06, FDR < 0.041) and myosin light-chain 6, *MYL6*, (log2FC >1.13, FDR < 0.045) were significantly upregulated in striatal MSNs. MCM8 is involved in DNA replication, forming a complex with MCM9 to facilitate repair of double-stranded DNA breaks[39,40]. MYL6 is a key factor in rho-GTPase signaling, known to facilitate glutamate receptor endocytosis[41] and neuroplasticity[42,43], and may be involved in DNA damage via the activation of RAC1[44]. Other genes related to neurodegeneration included *APOE*[45–47], which was upregulated in striosome MSNs and D2-matrix MSNs (log2FC >2.50, FDR < 0.025, Figure 2d, Figure 3d), and *GPX4*, which was upregulated in D1-striosome MSNs (log2FC>1.17, FDR < 0.044) in OUD subjects. Both *APOE* and *GPX4* are involved in oxidative stress within neurons, where GPX4 buffers the accumulation of reactive oxygen species to prevent apoptosis[45–47]. Accumulation of reactive oxygen species and oxidative stress in neurons involves alterations in redox signaling and mitochondrial respiration[48]. Indeed, across multiple MSN subtypes, genes involved in mitochondrial respiration were significantly upregulated in OUD, including a complex III subunit of the mitochondrial respiratory chain, *UQCR11* (log2FC >1.36, FDR < 0.042), and a subunit of complex I, *NDUFA4* (log2FC>1.29, FDR < 0.030).

Consistent with cell type-specific alterations in pathways related to neurodegeneration, we identified several transcription factor gene regulatory modules that were significantly upregulated in different neuronal subtypes (FDR < 0.05) linked to cell senescence, DNA damage, and inflammation, and stress (Figure 2). For example, gene regulatory module linked to BCLAF1 was upregulated in OUD, a transcription factor involved in the transduction of NFkB-

dependent signaling and the activation of DNA-damage-induced senescence[4249,50] (Figure 2e). Another module was associated with the upregulation of the neuron-specific glucocorticoid receptor transcription factor, NR3C1, previously related to the impact of psychosocial stress on human brain[51–53] (Figure 2e). Other stress-related transcription factor gene regulatory modules were upregulated in OUD, including ATF2[54] and ZNF518A[55]. Both transcription factor modules were upregulated primarily in D1-matrix and D2-matrix MSNs (FDR < 0.04). ATF2 is induced by stress in mouse dorsal striatum[54] and phosphorylated downstream of delta opioid receptor activation[56], suggesting delta opioid receptor activation of ATF2-dependent transcription in striatal matrix MSNs[57,58] may be due to an interplay between stress and opioids in OUD.

Compared to upregulated modules, we identified almost twice as many significantly downregulated transcription factor gene regulatory modules in OUD subjects (Fig 2c). We identified several downregulated pathways that are related to neuroprotection, further implicating processes involved in neurodegeneration in OUD. For example, the humanin-like 8 gene, *MTRNR2L8*, is significantly downregulated in OUD across every neuronal subtype (log2FC <-3.79, FDR < 0.0054, Figure 2d). *MTRHR2L8* and the mitochondrial paralog, *MTRNR2*, may act as neuroprotective factors in response to neurodegeneration and the induction of DNA damage[13,59,60]. The top DEGs within clusters of pathways involved in UV irradiation, another potential link to DNA damage (FDR < 0.029, Figure 2c), included *FOXO1*, *TLE4*, *SHOC2*, *MEIS2*, and *THBS1* (log2FC <-0.64, FDR < 0.0422) in interneurons and CLASP1, PIK3C3, and MTUS1 (log2FC <-0.64, FDR < 0.042) in D1/D2 hybrid MSNs. Additionally, downregulation of MEF2B and BACH1 transcription factor gene regulatory modules were found in D1-matrix MSNs in OUD, accompanied by downregulation of MAF, EST1, ETV1, and PRRX1 modules in D1/D2 hybrid MSNs (FDR < 0.043), each of which are implicated in neurodegeneration[61–64]. Notably, BACH1, BCL11A, and PRRX1 have downstream targets with concordant differential expression in OUD (*e.g.*, *FOXP2*, *POUF3*, *PTPRD*, *RAB31*, *RCBTB2*, Fig 2e), indicating coordinated changes in striatal gene networks implicated in neurodegeneration associated with opioid addiction. Additional links to neurodegeneration included modules for NFATC2 and RXRG (FDR < 0.042). Both nuclear factor of activated T-cell C2 (NFATC2) and the retinoid X receptor gamma (RXRG) of the RXR family of receptors link changes in neuronal activity associated with addictive drugs to processes involved in neuroprotection, neurodegeneration,[65–68] and reward-related behaviors[69–73].

Enrichment of DNA damage markers in specific neuronal subtypes in OUD

To investigate more broadly whether specific processes related to neurodegeneration were altered in OUD, we tested the possible enrichment of DNA damage markers in specific cell types[17] between unaffected and OUD subjects (Figure 3a). Overall, we confirmed an upregulation of DNA damage gene markers within striatal neurons of OUD subjects (p=0.046; linear regression t=2.18; Figure 3b). Within neuronal subtypes, we found a significant increase in DNA damage gene signature specifically within interneurons of OUD subjects (p=0.035, linear regression t=2.14, Figure 3c, Table S10). Together with the significant changes in the expression of *APOE* and *MTRNR2L8* in OUD (Figure 3d), our findings suggest striatal neurons may incur DNA damage in response to opioids and other factors, such as stress, neuroinflammation, and hypoxia, associated with opioid addiction. Interestingly, we found a significant downregulation of pathways involved in hypoxia (Figure 2c, Table S9), including decreased expression of genes that are activated in response to hypoxic events (*e.g.*, *NMRK1, BHLHE40*)[74–76], or overexpression of hypoxia-inducible transcription factor, HIF1A. Therefore, while respiratory depression is a hallmark of opioid overdose, downregulation of hypoxia-responsive genes points toward possible compensation in neurons secondary to periodic hypoxia and altered redox states, consistent with rodent models of substance use[77].

Given the potential confounding factors in our postmortem human brain cohort between chronic opioid use disorder and overdose, we explored how chronic opioids could alter transcriptional markers of DNA damage in striatal neurons in nonhuman primates (Figure 3e). Rhesus macaques were dosed with morphine twice daily for a year, matched for age and sex to control treatment monkeys (Figure S5a). After a year of opioid administration, brain tissue was collected and processed for snRNA-seq (Methods, Figure S5b). Similar to our human dataset, cell types were clustered across monkeys, consistent with our previously described cell populations in rhesus striatum (Figure S5c,d). Our gene signature analysis identified a marginal increase of DNA damage marker enrichment among neurons of monkeys chronically treated with morphine ($P = 0.054$, linear regression, Figure 3f), with significant increases in specific striatal neuronal cell types ($P < 0.00066$, linear regression, Figure 3f). Thus, elevated neuronal DNA damage in non-human primates corroborates those findings in postmortem brain tissue from subjects with OUD, providing additional, cross-species validation of the impact of chronic opioids on striatum.

Enriched pathways among glial cells support molecular signatures related to neuroinflammation and neurodegeneration in OUD

Compared to neurons, we identified more than twice as many DEGs in glial cells between unaffected and OUD subjects (Figure 4a). The number of DEGs were independent of cell type proportions, as microglia and endothelial cells had the most DEGs among glial cells, and expression patterns were largely distinct between glial cell types (Figure S4). An exception was the robust enrichment of genes involved in interferon response across multiple glial cell types, including astrocytes and oligodendrocytes. Interferon response genes were significantly upregulated in glial cell types in OUD subjects (FDR < 0.047, Table S11). Upregulated hub genes within the interferon response pathway included: *CMP2*, *HLA-F*, *IFI44*, *PPM1B*, and *RSAD2*. Furthermore, we observed an upregulation of the NFKB1 transcription factor gene regulatory module in astrocytes and the upregulation of the STAT3 module in oligodendrocyte precursor cells (OPC, Fig 4f, FDR< 0.036). Several of the predicted NFKB1 gene targets are also differentially expressed including the inhibitor of this pathway, *NFKBIA* (log2FC = 1.86, FDR = 0.039). Other glial cell types showed non-significant activation of this module, with the weakest being microglia. In response to cell stress, including neurodegeneration, microglia release pro-inflammatory cytokines that may trigger an interferon response in other cell types, potentially responsible for the more broad patterns of interferon activation we observe across multiple cell types in human striatum associated with OUD[17,78].

Altered neuroinflammatory signaling in microglia has previously been reported in postmortem brains from subjects with OUD[5]. Several DEGs in microglia of OUD subjects lend further support for associations between augmented inflammatory signaling, synaptic plasticity, and neurodegenerative processes and OUD. For example, the PAX-FOXO1, CDH1, and neuron projection signaling pathways were enriched among microglia DEGs (FDR < 0.049). PAX-FOXO1 signaling regulates cellular senescence in the brain and is involved in the pathogenesis of several neurodegenerative disorders[19,79], while CDH1 signaling is involved in glial cell migration and axonal projections[80]. In the neuronal projection pathway, *ADGRB3* was among the top downregulated genes in microglia of OUD subjects (FDR = 3.5e-7). *ADGRB3* (known as BAI3, brain-specific angiogenesis inhibitor 3) encodes for a protein that has high-affinity for complement, C1q, an innate immune component released by neurons to eliminate damaged or inactive synapses[81–83]. Downregulation of *ADGRB3* in microglia may lead to aberrant synaptic formation. In support of synaptic changes associated with opioid addiction, several genes involved in glutamatergic and GABAergic synaptic functions and plasticity were altered in subjects with OUD (astrocytes: metabotropic glutamate receptor 5, *GRM5*, log2FC = -3.13, FDR

= 1.06 E -14 and glutamate ionotropic receptor AMPA type subunit 1, *GRIA1*, log2FC = -1.98, FDR = 2.8 E -8; oligodendrocytes: GABA type A receptor subunit beta1, *GABRB1*, log2FC = -0.717, FDR = 0.023; microglia: GABA type A receptor subunit gamma 2, *GABRG2*, log2FC = -1.45, FDR = 0.038). Together, our cell type-specific findings suggest an interplay between microglial-dependent signaling and synaptic plasticity in OUD, consistent with our previous bulk transcriptomics findings in other striatal subregions from subjects with OUD[5]. With single nuclei resolution, we report that the elevation of neuroinflammatory pathways related to microglial activation in OUD is likely due to transcriptional alterations within microglia, rather than pronounced changes in the number of microglia within striatum in subjects with OUD (FDR = 0.48, linear mixed effect regression for differential abundance, Figure S2, Table S2).

Endothelial cells displayed the second most DEGs behind microglia between unaffected and OUD subjects. Upregulated pathways in endothelial cells included several growth factors, including VEGFR, EGFR, and PDGFR signaling[84–86] (Fig 4b). These growth factors regulate nociception and opioid tolerance via actions in peripheral circulation and spinal cord[85,87], suggesting similar pathways in endothelial cells may be involved in alterations associated with OUD in human brain[6]. Other pathways included leukocyte chemotaxis and dendrite morphogenesis. Among the densely connected endothelial pathways, the top significantly upregulated hub gene was the brain-specific angiogenesis inhibitor 1-associated protein 2, *BAIAP2* (log2FC=2.31, FDR = 0.0017), and, interestingly, the Alzheimer's related microtubule-associated protein tau, *MAPT* (log2FC = 1.58, FDR=0.036). The upregulation of *MAPT* may contribute to neurovascular brain insults in OUD[6,88].

The identification of differentially expressed transcription factor gene regulatory modules across glial cell types further implicated neurodegenerative processes in OUD. In oligodendrocyte precursor cells, the CEBPB module was upregulated in OUD (Fig 4e-f, FDR = 0.031), with prior studies showing CEBPB directly regulates the expression of *APOE* in Alzheimer's disease-related pathologies[89,90]. Notably, *APOE* is concordantly upregulated in oligodendrocyte precursor cells (Figure 3d, log2FC = 2.56, FDR = 5.9e-4), along with other predicted CEBPB targets (Figure 4e). The RXRG TF-gene module, which was downregulated in neuronal subtypes, was also downregulated in microglia (Figure 4e, FDR = 0.027). An inferred target of RXRG is *FOXP2* (Figure 4e), which was recently significantly associated with OUD and other substance use disorders at the genome-wide level[91]. FOXP2 expression in microglia may be unique to humans compared to other primates[92]. Thus, downregulation of the FOXP2 transcription factor gene regulatory module in human microglia may link the genetic risk of OUD to neurodegenerative processes.

Sex-specific transcriptional alterations in specific striatal cell types associated with OUD

Rates of OUD and responses to opioids are dependent on sex[93–96]. Between unaffected and OUD subjects, we found a significant effect of sex on DEGs in both neuronal and glial cell types. Overall, the impact of sex on DEGs in OUD was magnified in neurons (Figure 2d) relative to glia (Figure 4d). We therefore conducted secondary analyses to identify female-biased and male-biased transcriptional alterations in neurons and glia associated with OUD (Table S12). Pathways enriched primarily in females with OUD were associated with the upregulation of DNA repair processes in MSNs, upregulation of interferon response in glia, and upregulation of synaptic-related function in astrocytes (Figure 5a, Table S13, Table S15). We also identified the FK506 binding protein 5, *FKBP5*, as a potential factor in the role of sex in opioid use. FKBP5[97,98] in the brain has been proposed as a key factor mediating the relationship between sex and OUD[99]. *FKBP5* was significantly upregulated in astrocytes, oligodendrocytes, and oligodendrocyte precursor cells of female OUD subjects (Figure 5b; $\log2FC_{F\ glia}>1.30$, $FDR_{F\ glia} < 0.0165$, $\log2FC_{M}= -1.62$, $FDR_{M\ interneurons} < 0.040$). Prior studies in rodents identified sex differences in *Fkbp5* expression in dorsal striatum in response to opioids[100,101]. We investigated whether transcriptional alterations by opioids in rodents were enriched in human dorsal striatum by sex, cell type, and brain region. Indeed, rodent gene sets were significantly enriched in DEGs from glia and oligodendrocytes of female OUD subjects ($FDR_{F\ glia} < 0.027$, Table S16). Cross-species enriched DEGs were primarily associated with the downregulation of synaptic regulatory proteins, particularly in astrocytes (Fig 5a, cluster 6). The genes encoding synaptic regulatory proteins spanned both pre- and postsynaptic compartments, consistent with the role of astrocytes in synaptic modulation and the tripartite synapse (Fig 5c, Table S14, Table S15). In contrast, male-biased transcriptional alterations in OUD were mostly found in microglia, MSNs, and interneurons (Figure 5d, clusters 12, 15, 16). Male-biased pathways consisted of respiratory electron transport and related mitochondrial complexes (Figure 5e). Collectively, our evidence of sex differences in cell type-specific molecular signaling suggests an augmented, magnified glial cell response in females compared to males in OUD.

Discussion

Using single nuclei transcriptomics technologies, we identified both canonical neuronal and glial cell types in human striatum (*e.g.*, D1 and D2 MSNs), along with several of the less abundant cell types, including subclasses of

striatal interneurons and D1/D2 hybrid neurons. We also characterized the expression of specific opioid receptor and endogenous ligand subclasses across cell types, highlighting both expression patterns unique to human striatum and those patterns consistent with non-human primate and rodent findings. In addition, we investigated the impact of opioid addiction on transcriptional patterns among specific striatal cell types between unaffected and OUD subjects. Our single nuclei findings provide further insights into the specific cell types that may be impacted in OUD, while highlighting several putative mechanisms in human striatal subtypes of neurons and glia involved in opioid addiction. A common theme that emerged from our analyses was the involvement of processes related to neuroinflammation and neurodegeneration in OUD, including a broad interferon response among glial cells and elevated DNA damage in neurons (Figure 6). DNA damage markers were identified in both the striatum of humans and monkeys, suggesting chronic opioid use associated with OUD leads to augmented DNA modifications (*e.g.*, double-stranded breaks, chromatin accessibility). Elevated markers for neuroinflammation, the involvement of microglia-dependent signaling in striatum, and alterations in synaptic signaling in OUD are consistent with previous bulk transcriptomics findings from postmortem brains of subjects with OUD.

Many of the genes and pathways enriched in specific striatal cell types of OUD subjects have been implicated in neurodegenerative disorders (Figure 6a). DNA damage, in particular, has been broadly studied in Alzheimer's disease[9,10], Parkinson's disease[102], and ALS[103]. Integrity of DNA is critical for neural cells to prevent insertions, deletions, or mutations that can lead to aberrant synaptic plasticity and/or neurodegeneration. Double-stranded breaks and subsequent DNA repair may allow neurons to rapidly respond at the transcriptional level to changes in neuronal activity[104–107]. Opioids likely lead to persistent changes in neuronal activity and synaptic plasticity within striatum. Prolonged or repeated alterations in neuronal activity combined with dysfunction within DNA repair pathways hampered by persistent cell stress (*e.g.*, oxidative stress) may lead to the accumulation of DNA damage. Neurons may be particularly vulnerable to DNA damage[108]. Our results suggest elevated DNA damage in neurons of OUD subjects, particularly within striatal interneurons (Figure 6b). Several subclasses of striatal interneurons are found in striatum[30], possessing an array of transcriptional and physiological properties. We separately clustered TH+, CCK+, PTHLH+, and SST+ interneurons in human striatum. The functionality of interneurons in the striatum is diverse, with several subclasses modulating the excitability and inhibition of MSNs in response to various substances[109], including opioids[110]. Although we were unable to reference the overall elevation of DNA damage signature to a specific subset of interneurons, our findings suggest striatal

interneurons may be particularly vulnerable to a loss of DNA integrity and repair mechanisms in response to changes in neuronal activity by opioids and related perturbations.

The DNA damage response in neurons can lead to a proinflammatory response in microglia and other glial cells[16,17]. In OUD, there were a relatively large number of genes that were upregulated specifically in microglia, enriched for genes involved in neuroinflammation. Microglial activation may propagate to other neural cell types, evident by an increase in interferon responses[17,78]. Indeed, we found significantly upregulated interferon response in both neuronal and glial cells of OUD subjects. Interferon responses may modulate opioid actions in the brain and could be involved in the emergence of symptoms of opioid withdrawal[111].

Other pathways related to neurodegenerative disorders include oxidative stress, mitochondrial respiration, and neuroprotection. The coordinated alterations in the expression of genes involved in these pathways may reflect compensation to changes in neural activity impacted by opioid use. For example, glutamatergic hyperactivity at striatal MSNs[112] may cascade towards excitotoxicity and elevated DNA damage[12,18,113], both of which have been recently associated with OUD[114,115]. GRM5 was specifically downregulated in astrocytes in OUD. GRM5 is typically transiently expressed in astrocytes to detect and respond to extracellular glutamate, suggesting downregulation in OUD may be due to changes in glutamatergic activity in striatal neurons[116]. Alterations in other glutamatergic and GABAergic transcripts were also found in specific cell types in OUD (*e.g.*, downregulation of *GRIA1* in astrocytes and upregulation of *GABRG2* in microglia). In parallel, enrichment of neurodegenerative and neuronal activity markers in certain striatal cells in OUD may be a result of high energetic demand on MSNs to maintain a hyperpolarized state, a possible mechanism of vulnerability of striatal MSNs proposed in Huntington's disease[117]. We also found OUD-associated changes in neuronal energetics with the downregulation of nicotinamide riboside kinase 1, *NMRK1*, and the basic helix-loop-helix family member e40, *BHLHE40* (DEC1), both of which are involved in regulating cellular metabolism, oxidative stress, and inflammation[74–76]. Together, the broad set of changes in genes and pathways associated with OUD were associated with various processes involved in neurodegeneration.

Sex differences in the vulnerability to substance use, development of substance use disorders, and treatment outcomes, along with the biological response to drugs are evident from both preclinical and clinical studies[118–120]. Our findings suggest sex-specific alterations in the transcriptional response to opioids in specific striatal cell types. Based on the transcriptional patterns, we found a more pronounced inflammatory response associated with striatal microglia in

females compared to males with OUD, suggesting microglia in the human brain also exhibit sex-specific responses to stress and substances[120–122]. In addition, we identified the upregulation of *FKBP5* as a potential female-specific factor in several glial subtypes in OUD. *FKBP5* acts as a co-chaperone of the glucocorticoid receptor activated in response to stress, with major implications in the pathology of several psychiatric disorders and the impact of stress on substance withdrawal, craving, and relapse[123–125]. Further, the transcriptional alterations within microglia, oligodendrocytes, and astrocytes in female OUD subjects were significantly enriched for *FKBP5* target genes identified in rodents administered opioids. Genes related to synaptic functions were also enriched in astrocytes of females, while enrichment was mostly in MSNs and interneurons in male OUD subjects, suggesting sex-specific alterations of striatal neuronal and glial cell signaling in opioid addiction.

Several limitations of our resource stem from challenges of profiling single cell transcriptomes in postmortem human brains. While the nuclear transcriptome correlates on whole with the transcriptomes of other subcellular compartments, there are notable differences in mRNA trafficking within certain neural cell types and synapses. Furthermore, the nuclear transcriptome does not capture post-transcriptional regulation. Further, the sex-biased differential expression in OUD is likely influenced by cultural and behavioral factors that are confounded with biological sex. Lastly, while this resource will be useful to identify the consequences of OUD in the postmortem brain transcriptome, integration of our findings with other types of approaches from genetics to functional genomics in clinical cohorts to animal models of opioid addiction could contribute to a greater understanding of opioid addiction and aid in the realization of new treatment strategies.

Main Figure Legends

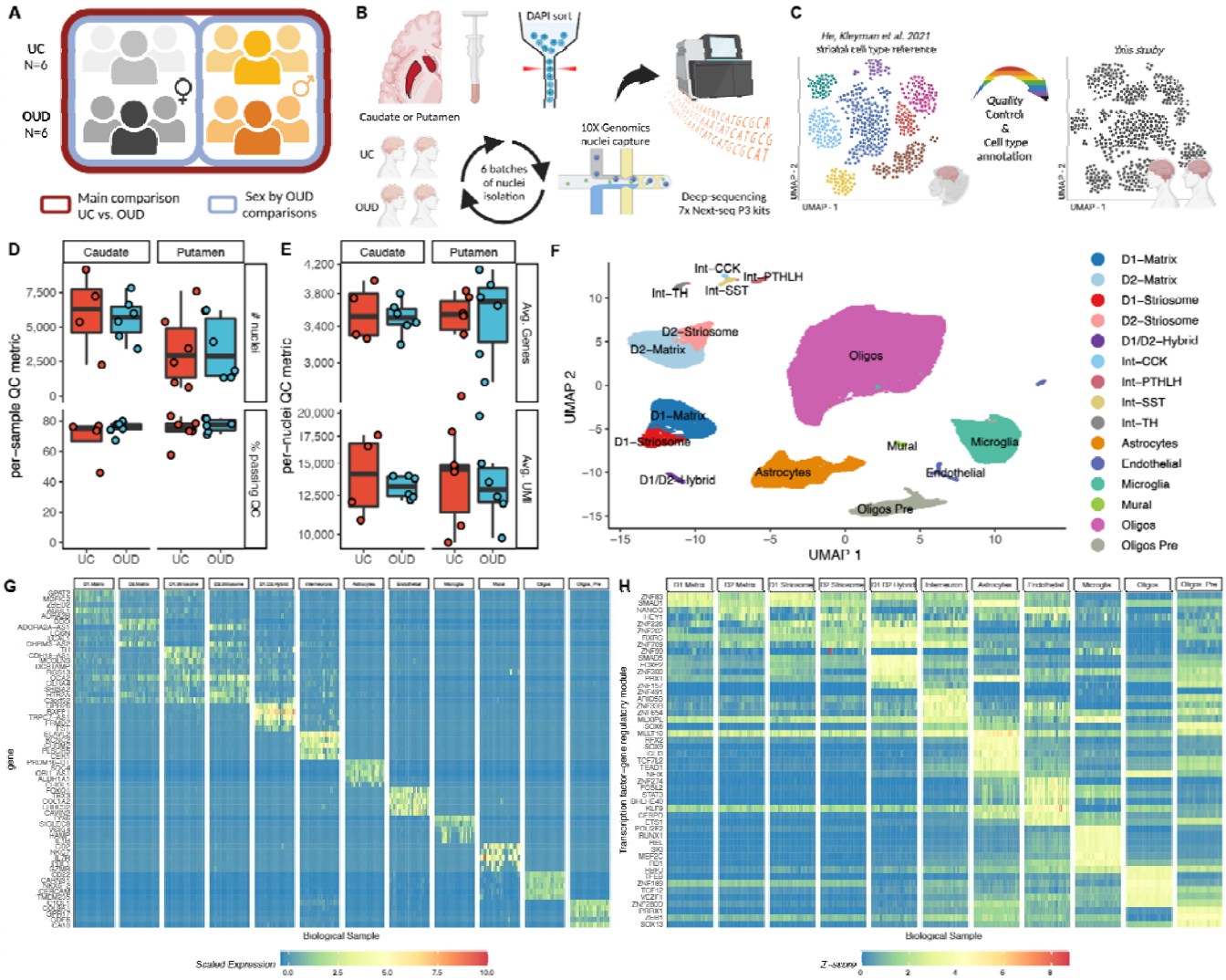

Figure 1. Single nuclei RNA-sequencing of postmortem brain to identify specific cell types in human striatum

(A) Post-mortem human brain cohort design and analysis to compare unaffected comparison (UC) subjects with subjects with opioid use disorder (OUD). (B) Schematic of the sample collection process to isolate single nuclei and generate single nuclei RNA-seq libraries in balanced batches from the caudate nucleus and putamen. (C) Single nuclei clustering and label annotation of dorsal striatal cell types using a high-quality reference[26]. (D) Per-sample quality control (QC) metrics and (E) average per-nuclei QC metrics across tissue sources and diagnoses. (F) A low dimensionality projection of striatal cell types after QC filtering and annotation. (G) Cell type-specific marker genes and (H) marker transcription factor-gene regulatory networks discovered from single nuclei RNA-seq striatal tissues.

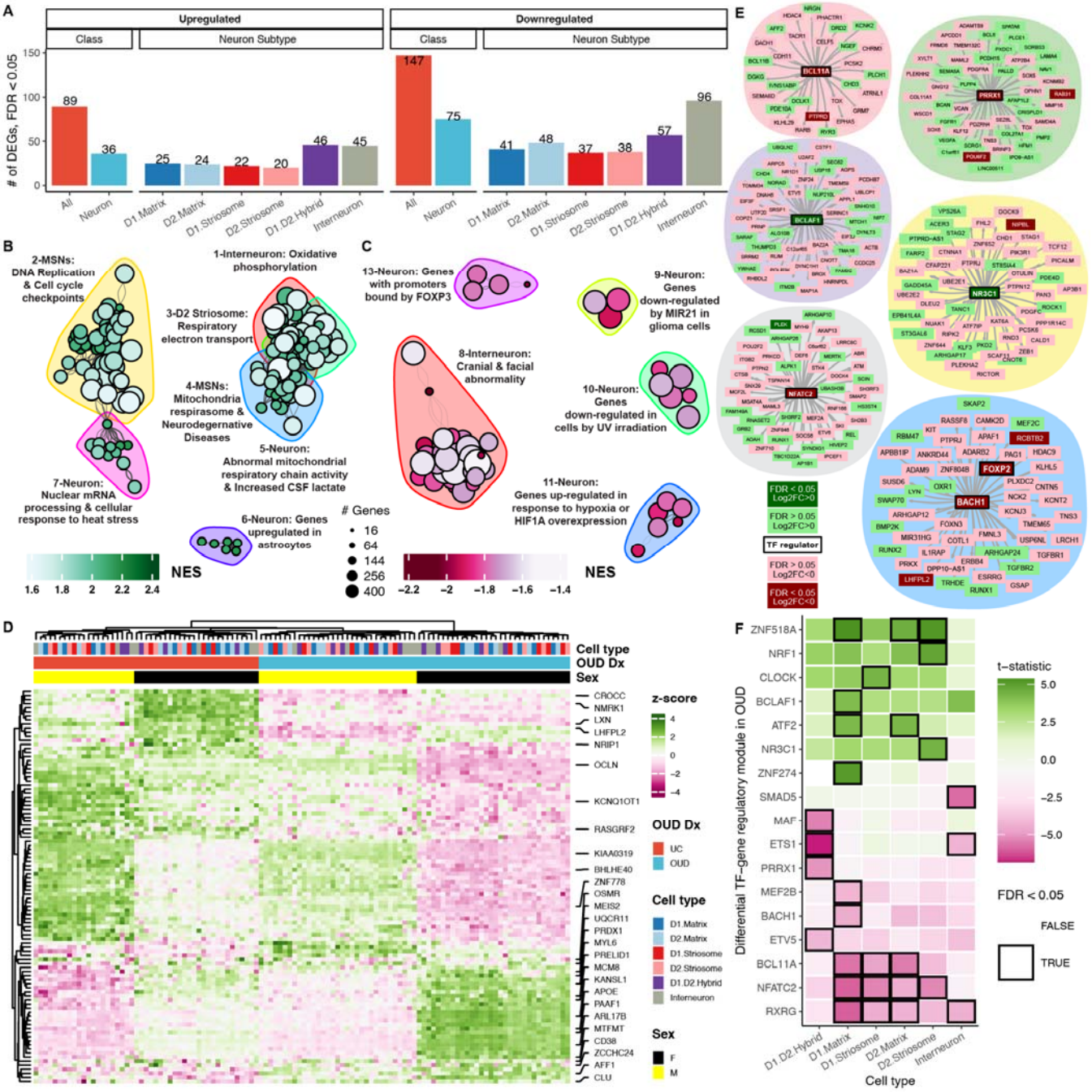

Figure 2. Transcriptional alterations in specific neuronal cell types in human striatum associated with OUD

(A) Barplot of differentially expressed genes (DEGs) either up- or down-regulated in striatal neurons at a false discovery rate less than 0.05 (FDR < 0.05). (B-C) Network plot of significant clusters and enriched pathways, where each point is a significantly enriched pathway in a neuronal cell type and lines represent the proportion of shared genes between two pathways. Each point is colored by the normalized enrichment score (NES), indicating whether a pathway is enriched in up- or down-regulated genes. The color outlines represent the unique clusters of interconnected pathways by shared genes

and the nearby text labels the unique cluster number and briefly summarizes which cell types and pathways are represented. (D) Expression heatmap of z-normalized pseudobulk gene expression, where the rows are DEGs and the columns are the biological replicates labeled by cell type, diagnosis, and sex of each subject. Genes that are represented by the enriched pathways in (B-C) are labeled along the right margins. (E) Cluster maps of select transcription factor gene regulatory networks. Each transcription factor or gene is colored to denote the direction and significance of DE in neuronal subtypes in OUD. (F) A heatmap plotting the t-statistic of differentially activated transcription factor gene regulatory networks across neuronal subtypes in OUD.

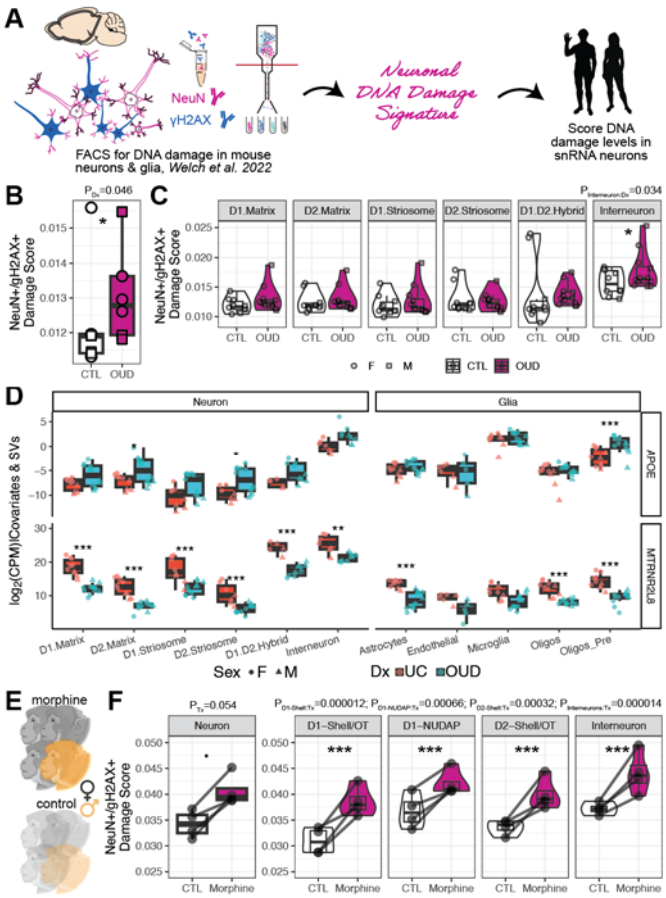

Figure 3. Elevated markers of DNA damage in striatal neurons associated with OUD and chronic morphine administration in non-human primates

(A) Schematic of the application of DNA damage gene signatures from a mouse model of Alzheimer's Disease[17] to score DNA damage in human striatal neurons. (B) Boxplot of subject-level pseudobulk average DNA damage scores across striatal neurons between unaffected and OUD subjects (P_{Dx} = 0.046, linear regression). (C) Violin-boxplot of cell type-level pseudobulk average DNA damage scores between unaffected and OUD subjects. The significant effect of diagnosis P-values from linear regressions are reported above each plot. (D) Boxplots showing the log2(counts per million) normalized gene expression values regressing covariates and surrogate variables for the *APOE* and *MTRNR2L8* genes stratified by neuronal and glial cell types. Each point is a biological replicate colored by diagnosis and shaped by biological sex. (E) Schematic of chronic morphine exposure or unexposed rhesus macaques (N=4 monkeys per treatment). (F) Boxplots of subject level pseudobulk average DNA damage scores across striatal neurons or neuronal subtypes between control monkeys or those exposed to chronic morphine. The significant effect of chronic morphine treatment P-values from linear regressions are reported above each plot. (* P < 0.05; ** P < 0.01; *** P < 0.001).

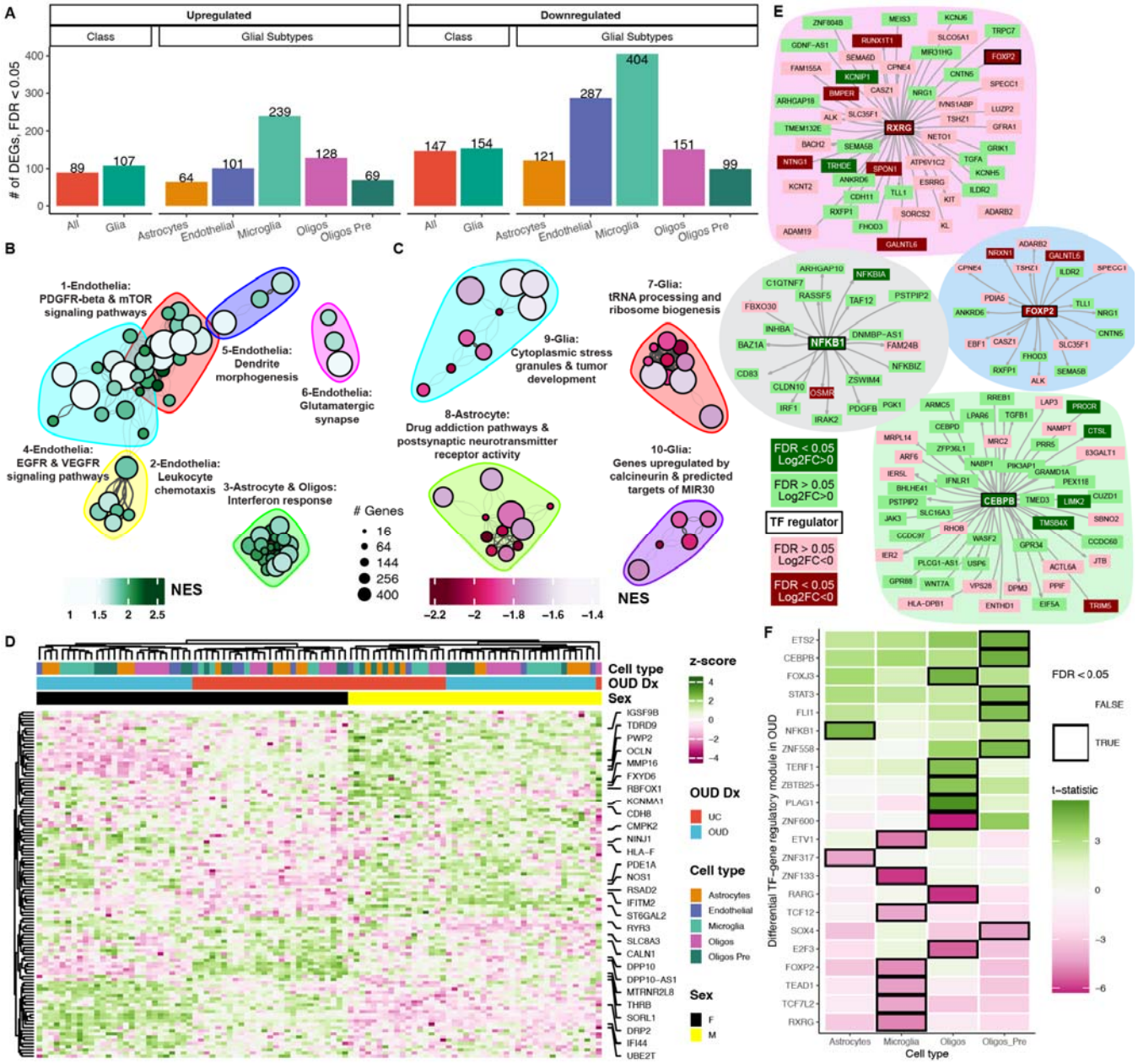

Figure 4. Transcriptional alterations in specific glial cell types in human striatum associated with OUD

(A) Barplot of differentially expressed genes (DEGS) either up- or down-regulated in striatal glia at a false discovery rate less than 0.05 (FDR < 0.05). (B-C) Network plot of significant clusters and enriched pathways, where each point is a significantly enriched pathway in a glial cell type and lines represent the proportion of shared genes between two pathways. Each point is colored by the normalized enrichment score (NES), indicating whether enriched in up- or down-regulated genes. The color outlines represent the unique clusters of interconnected pathways by shared genes and the nearby text labels the unique cluster number and briefly summarizes which cell types and pathways are represented. (D)

Expression heatmap of z-normalized pseudobulk gene expression where the rows are DEGs and the columns are the biological replicates labeled by cell type, diagnosis, and sex of each individual. Genes that are represented by the enriched pathways in (B-C) are labeled along the right margins. (E) Cluster maps of select transcription factor gene regulatory networks each transcription factor or gene is colored by the direction and significance of being differentially expressed in glial cell types in OUD. (F) A heatmap plotting the t-statistic of differentially activated transcription factor gene networks across glial cell types in OUD.

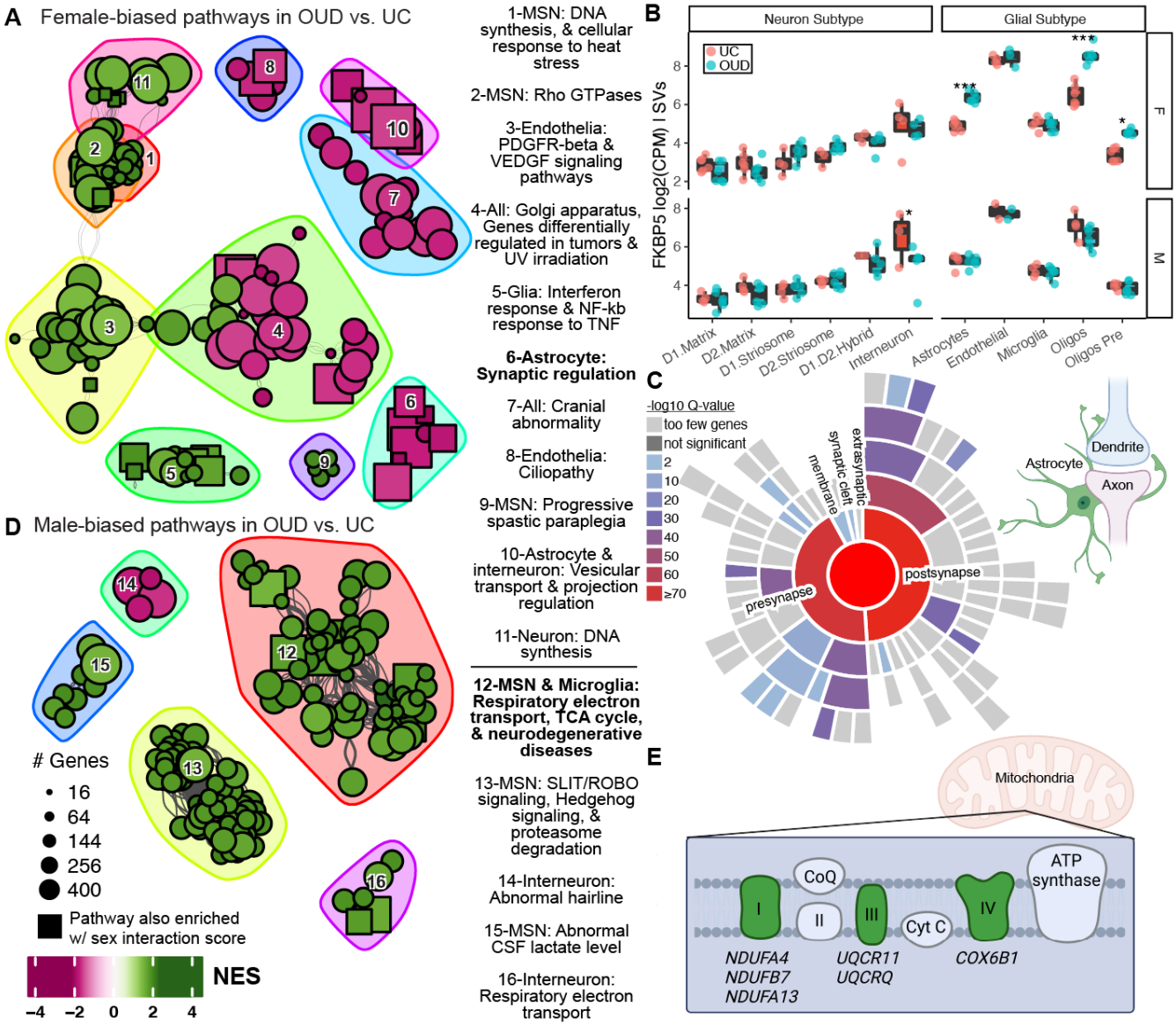
A Female-biased pathways in OUD vs. UC
D Male-biased pathways in OUD vs. UC
Genes
16
64
144
256
400
Pathway also enriched w/ sex interaction score
NES
−4 −2 0 2 4
1-MSN: DNA synthesis, & cellular response to heat stress
2-MSN: Rho GTPases
3-Endothelia: PDGFR-beta & VEDGF signaling pathways
4-All: Golgi apparatus, Genes differentially regulated in tumors & UV irradiation
5-Glia: Interferon response & NF-kb response to TNF
6-Astrocyte: Synaptic regulation
7-All: Cranial abnormality
8-Endothelia: Ciliopathy
9-MSN: Progressive spastic paraplegia
10-Astrocyte & interneuron: Vesicular transport & projection regulation
11-Neuron: DNA synthesis
12-MSN & Microglia: Respiratory electron transport, TCA cycle, & neurodegenerative diseases
13-MSN: SLIT/ROBO signaling, Hedgehog signaling, & proteasome degradation
14-Interneuron: Abnormal hairline
15-MSN: Abnormal CSF lactate level
16-Interneuron: Respiratory electron transport
B
Neuron Subtype
Glial Subtype
UC
OUD
FKBP5 log2(CPM) I SVs
F
M
D1.Matrix
D2.Matrix
D1.Striosome
D2.Striosome
D1.D2.Hybrid
Interneuron
Astrocytes
Endothelial
Microglia
Oligos
Oligos Pre
C
-log10 Q-value
too few genes
not significant
2
10
20
30
40
50
60
≥70
extrasynaptic
synaptic cleft
membrane
presynapse
postsynapse
Dendrite
Astrocyte
Axon
E
Mitochondria
CoQ
ATP synthase
I
II
III
IV
Cyt C
NDUFA4
NDUFB7
NDUFA13
UQCR11
UQCRQ
COX6B1

Figure 5. Sex-biased transcriptional alterations in striatal cell types associated with OUD

(A) Network plot of significant clusters and enriched pathways as in Figure 2B-C and 4B-C. Here, enriched pathways include only DEGs calculated within female subjects if less significant than those calculated within male subjects. Pathways with a square point are also enriched using an alternate calculation of a sex-interaction score. Clusters are labeled and the corresponding summaries listed along the right side of the cluster plot. (B) Boxplots showing sex- and cell type-specificity of differential expression of *FKBP5*. The boxplot labels the log2(counts per million) normalized gene expression values regressing out covariates and surrogate variables. Each point is a biological replicate colored by diagnosis (* $P_{Dx\ within\ Sex} < 0.05$; ** $P_{Dx\ within\ Sex} < 0.01$; *** $P_{Dx\ within\ Sex} < 0.001$). (C) A sunburst plot representing curated synaptic gene sets from SynGO from genes enriched in cluster 6, a female-biased set of pathways enriched in astrocytes. (D) A network plot as in (A); however, displaying the male-biased pathways. (E) Diagram showing the male-biased DEGs that are components of the electron transport gene.

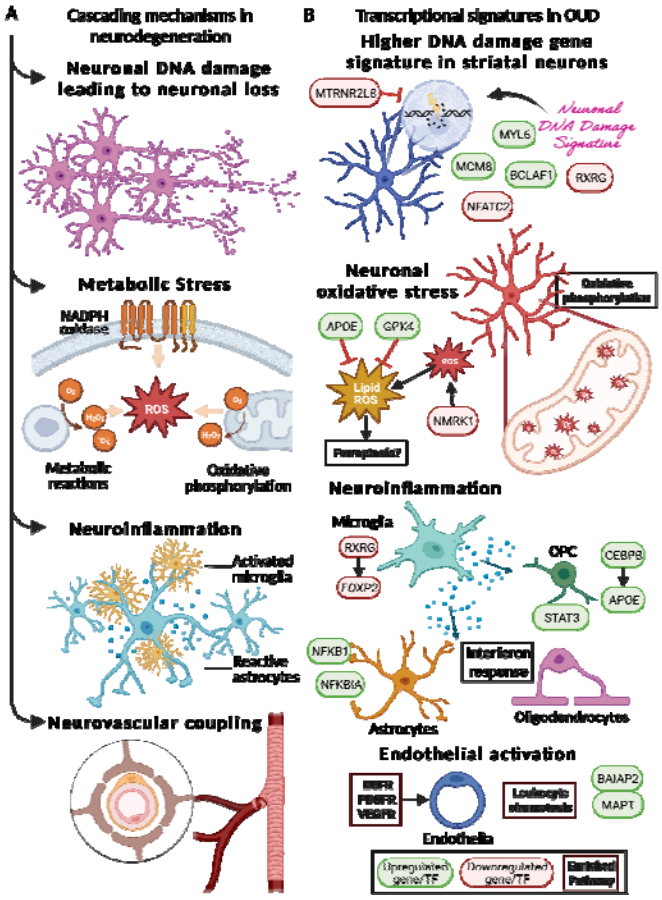

Figure 6. Parallel cellular and molecular pathways in neurodegeneration and opioid use disorder

(A) Molecular cascade of mechanisms that are shared across neurodegenerative processes. Figure created in Bio Render, an adaptation from ref[126]. (B) A summary of cell type-specific transcriptional alterations in the human postmortem dorsal striatum that parallel the cascading mechanisms in neurodegeneration.

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