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Cocaine perturbs neurodevelopment and increases neuroinflammation in a prenatal cerebral organoid model

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Prenatal exposure to cocaine causes abnormalities in foetal brain development, which are linked to later development of anxiety, depression and cognitive dysfunction. Previous studies in rodent models have indicated that prenatal cocaine exposure affects proliferation, differentiation and connectivity of neural cell types. Here, using cerebral organoids derived from the human iPSC cell line HPSI1213i-babk_2, we investigated cocaine-induced changes of the gene expression regulatory landscape at an early developmental time point, leveraging recent advances in single cell RNA-seq and single cell ATAC-seq. iPSC-cerebral organoids replicated well-established cocaine responses observed in vivo and provided additional information about the cell-type specific regulation of gene expression following cocaine exposure. Cocaine altered gene expression patterns, in part through epigenetic landscape remodelling, and revealed disordered neural plasticity mechanisms in the cerebral organoids. Perturbed neurodevelopmental cellular signalling and an inflammatory-like activation of astrocyte populations were also evident following cocaine exposure. The combination of altered neuroplasticity, neurodevelopment and neuroinflammatory signalling suggests cocaine exposure can mediate substantial disruption of normal development and maturation of the brain. These findings offer new insights into the cellular mechanism underlying the adverse effects of cocaine exposure on neurodevelopment and point to the possible pathomechanisms of later neuropsychiatric disturbances.

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INTRODUCTION

Cocaine use during pregnancy is increasing in prevalence and is a major public health issue worldwide [1]. As a psychoactive stimulant, cocaine mediates its neuroplastic effects through modulation of a number of neurotransmitters such as dopamine, serotonin and glutamate. Prenatal Cocaine Exposure (PCE) in the first trimester can substantially impact the development of the foetus' brain leaving individuals at risk of neurologic deficits in language development in early adolescence [2, 3], attentional and behavioural deficits [4], as well as issues with cognition and working memory [5].

Early pregnancy cocaine exposure *in utero* occurs at a critically important time during neurodevelopment where the timing of neurogenesis, synaptogenesis, and neuronal migration will ultimately govern the structure and function of the subsequent adult brain. In addition, PCE induces morphological changes in the prefrontal cortex, the hippocampus, and the nucleus accumbens. Furthermore, *in utero* cocaine exposure can alter the development of hippocampal and cortical neurons. Studying the effects of cocaine on human neurodevelopment is challenging due to limited access to foetal tissues and ethical concerns. Consequently, animal models have been used to illustrate a number of cellular and molecular changes that occur as a consequence of cocaine-induced neurotoxicity in the early foetal brain. In animals, PCE results in increased apoptosis [6], increased oxidative stress and neuroinflammation [7], disruption of neuronal migration and, hence, cortical architecture and epigenetic changes in the embryonic neocortex [8].

Although animal models have been a fundamental tool in unravelling the biological complications caused by PCE, specific biological processes involved in human brain development are now known to differ significantly from other species [9, 10]. For example, timing of neurogenesis and maturation of neuronal receptors and transmitters differs between humans and rodents [11]. Furthermore, there is often high variability in cocaine response among different animal models. Human iPSC-derived cerebral organoids (COs) have emerged as an excellent model for investigating brain development and related disease pathomechanisms due to their capacity to self-organise and reproduce specific neural cytoarchitecture and lineage trajectory. In addition, COs have a proteomic profile reminiscent of early foetal to postnatal human cerebral tissue [12]. Lastly, COs have been used to successfully model effects of drugs of abuse such as cocaine [13–16], ethanol [17, 18], methamphetamine [19] and opiates [20]. With the advances of single cell sequencing and lack of accessibility to human *in utero* models, COs are proposed as an alternative human model system to study prenatal cocaine-induced neurotoxicity at a single cell resolution.

Cocaine-induced molecular and cellular changes in the early developing human brain *in utero* are still not well understood. This

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study describes a model of PCE at an early developmental timepoint using cerebral organoids derived from human induced pluripotent stem cells. The model recapitulates a binge intoxication event in the mid-first trimester of pregnancy and demonstrates that such cocaine exposure alters neurogenesis and drives neuroinflammation in the developing human brain tissue.

METHODS

Ethics statement

All methods were performed in accordance with the relevant guidelines and regulations.

Human iPSC maintenance and organoid generation

All experiments were performed in the human female iPSC line, HPSI1213i-babk_2 purchased from the European Collection of Authenticated Cell Cultures. iPSCs were cultured as previously described [21] and were routinely checked for pluripotency and genomic integrity. Cells were mycoplasma tested using the Lonza Mycoalert detection kit and all cells tested negative.

Cerebral organoids were generated using a previously published protocol [22]. On Day 36, organoids were treated with 25 µM cocaine hydrochloride. This treatment was repeated after 24 h for a further 24 h, followed by a 24 h period with no cocaine.

Immunofluorescence

Organoids were fixed in 2% paraformaldehyde, cryoprotected in 30% (w/v) sucrose, snap frozen at −70 °C and cryosectioned. Sections were hydrated, incubated in blocking buffer (5% (v/v) goat serum, 0.06% (v/v) Triton X-100, DPBS) for 1 h at RT, and stained with primary antibodies (Supplementary Data 1) overnight at 4 °C. After washing, sections were stained with Hoechst 33342 Trihydrochloride Trihydrate and the corresponding AlexaFluor secondary antibody (1:500) or rhodamine phalloidin (1:500, Invitrogen). Sections were mounted with Ibidi mountant (Ibidi, Germany). All images were acquired using a Leica SP8 Confocal Microscope.

Statistics and reproducibility

Results are representative of the similar observations and analyses made across multiple independent experiments and technical replicates. Independent experiments were classed as organoids derived from separate passages and/or freeze-backs. The number of replicates for each experiment is indicated in the legends of the corresponding figures. Organoid characterisation was performed on 3–4 organoids from a minimum of three independent experiments.

Western blotting

Protein extracts were isolated from three pooled organoids per condition and western blotting was performed using standard western blotting protocols and 10% polyacrylamide gels. Primary antibodies are outlined in Supplementary Data 1. Secondary antibodies used were Dylight700/800 fluorescent secondaries and membranes were imaged using a Licor Odyssey.

RT-qPCR

RNA was extracted using an E.Z.N.A Tissue RNA extraction kit (Omega Biotek, Ireland). 500 ng cDNA was synthesized using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. Applied Biosystems Taqman Universal PCR mastermix was used for RT-qPCR (QuantStudio Flex 7 real-time PCR instrument) with specific FAM-labelled Taqman primers (Supplementary Data 1). Results of real time RT-qPCR data were analysed using the delta Ct (ΔC_T) method. Samples with amplification Ct values above 37.00 were excluded.

Single cell RNA-seq (scRNAseq)

Organoids were differentiated from a single well of passage 34 hiPSCs. scRNAseq was performed on three untreated and three cocaine-treated pooled organoids. Organoids were dissociated into single-cell suspension as previously described [23]. Single cell suspensions were resuspended in 2% (v/v) BSA/DPBS and cell concentrations and viability were assessed using trypan blue staining. scRNAseq libraries were generated from 5000 cells using the 10X Genomics Chromium Single cell 3′ Library and Gel Bead Kit V3.1 (10X Genomic, PN-1000092) as per manufacturers instructions. scRNA-seq libraries were sequenced using an Ilumina NextSeq 550 platform.

Single cell ATAC-seq (scATACseq)

Organoids used in scATACseq experiments were differentiated from a single well of passage 35 hiPSCs. scATACseq was performed on nine untreated and nine cocaine-treated organoids. Organoids were dissociated into single-cell suspension as previously described [21]. scATACseq libraries were generated using 10X Genomics Chromium ATAC library and Gel Bead kit (v1.1) according to the manufacturer's protocol. Libraries were sequenced using an Illumina NextSeq 550 platform.

Bioinformatic analysis

scRNAseq. Cell Ranger (v3.1.0) was used to demultiplex, align, and generate single cell feature counts. scDblFinder (v1.6.0) was used to remove suspected doublets. Seurat (v3.0) [24] was used to filter low-quality cells (containing unique genes or UMIs >2 standard deviations above the median for all samples or containing >25% mitochondrial reads). scRNAseq datasets were processed using SCTransform pipeline. Differential gene expression was calculated in Seurat by Wilcoxon Rank Sum test.

scATACseq. Cell ranger ATAC (v.1.2.0) was used to demultiplex BCL2Fastq files. The resulting data was analysed using the Signac package (v1.2.1) using a standard protocol. For QC, cells were retained where: fragments in peaks were >30%, nucleosome score <4, TSS enrichment score >2, ENCODE-determined blacklist regions were <0.05%. Cell type assignments were performed by transferring labels from scRNAseq data in Signac using canonical correlation analysis to identify anchor points between datasets, with manual clean up performed by manual reference back to the scRNAseq dataset. Differential peak accessibility was calculated in Seurat using the Logistical Regression method and nearest genes were calculated. Motif analysis was performed in Signac using chromVAR package.

Ingenuity pathway analysis (IPA)

Differential gene expression analysis was conducted using Seurat (v3.0), where DEG were filtered by *P*-value (0.05) and avgLog2FC (≤−0.4, ≥0.4) to form filtered shortlists. Genes were uploaded onto the IPA platform (v.23; QIAGEN Inc., Hilden Germany). GSEA (Gene set enrichment analysis) was conducted on each gene list in comparison with the Entrez Gene library to calculate significant pathway enrichment (predicted activation Z-score >1, *p*-value < 0.05).

SCENIC

Regulatory network inference and regulon enrichment were performed using Scenic [25] as part of the VSN pipeline (v0.27.0) [26]. The Scenic workflow was iterated 100 times, retaining only regulons present in over 80% of runs.

CellChat interaction analysis

Inference of the cell-cell interaction network was performed using CellChat v1.6.0 R package according to the CellChat default workflow [27] (https://github.com/sqjin/CellChat).

RESULTS

The effect of cocaine on 36-day old cerebral organoids

We generated and characterised COs grown for 36 days (D36) using an adapted protocol of Lancaster and colleagues [22] (Supplementary Fig. 1a). COs contained typical cell types including $SOX2^{+ve}$ and $OTX2^{+ve}$ progenitors/radial glia, $TUJ1^{+ve}$, DCX^{+ve} and $MAP2^{+ve}$ neurons, $CDH2^{+ve}$ adherens junctions and $PROX1^{+ve}$ hippocampal neurons (Supplementary Fig. 1b).

To model the effect of PCE on human neurodevelopmental processes, D36 COs were treated with 25 µM cocaine for a total of 48 h, followed by abstinence for a further 24 h to recapitulate a binge-like episode (Supplementary Fig. 1a). This concentration was selected as it is representative of concentrations found in the brain tissue of animals self-administering cocaine [28] and, therefore, is representative of exposure levels that would be

encountered in those abusing the drug. Moreover, this concentration has been used previously to successfully model cocaine-mediated neurotoxicity [29]. Importantly, the D36 COs expressed the machinery to respond to cocaine including dopamine receptors, D1 and D2, dopamine transporter DAT1, serotonin receptors, 5HT1A and 5HT2A, serotonin transporter SERT, tryptophan hydroxylase (TPH2) (Supplementary Fig. 1c–e), the glutamate metabotropic receptor 5 (*GRM5*) and the glutamate transporter (*VGLUT1*) (Supplementary Fig. 1f). With the exception of 5HT1A, all these genes were found to have substantially greater expression levels in the organoids compared to undifferentiated hiPSCs. Moreover, the expression levels of these genes did not alter in response to cocaine.

Differentially expressed genes (DEGs) that characterised cell clusters in the control organoids were used to annotate the scRNAseq data. Three distinct superclusters were identified representing astrocyte-like (AS1 and AS2), cortical neurogenic niche (radial glia (RG), proliferating RG (PRG), intermediate progenitors (IP1 and IP2), and immature neurons (IN)), and choroid plexus-like/roof plate (CP1, CP2, RP) lineages (Fig. 1a–d). Although not tested statistically, the proportion of RG and PRG cells seemed to be greater following cocaine treatment while IP1 and IN cells made up a smaller proportion of cells in the cocaine-treated organoids (Fig. 1d, e). DEG analysis revealed that in response to cocaine, 1180 genes were upregulated amongst individual clusters with an additional 230 genes that were upregulated in two or more clusters [AS1(92), AS2(317), RG (37), PRG(117), IP1(95), IP2(45), IN(131), CP1(191), CP2(126), RP(260)]. Moreover, 900 unique genes were downregulated amongst individual clusters with 142 additional genes that were downregulated in two or more clusters [AS1(97), AS2(230), RG (83), PRG(69), IP1(44), IP2(113), IN(15), CP1(28), CP2(155), RP(108)] (Fig. 1f; Supplementary Data 2). We next performed gene ontology (GO) and IPA analysis on the DEGs. Most upregulated genes across clusters were enriched in metabolic pathways while downregulated genes were associated with the GO:biological processes related to central nervous system development and regulation of cell proliferation (Supplementary Data 3). IPA analysis identified protein translation, particularly EIF signalling, to be disrupted by the cocaine exposure (Fig. 1g; Supplementary Data 4). We compared upregulated DEGs with data from a PCE rodent model [6] (Supplementary Fig. 2) and cocaine-associated genes from adult humans and rodents [30–33] (Fig. 1h) and identified a clear overlap of gene expression and activated biological pathways offering further reassurance that the COs are accurately capturing aspects of cocaine-mediated action in neural tissues.

Cocaine alters neural plasticity, developmental signalling & neuroinflammatory tone

Neuronal plasticity, facilitated by immediate early genes (IEGs), plays a central role in long term synaptic changes observed following administration of drugs of abuse like cocaine [31, 34, 35]. Components of this core machinery are clearly modulated in the cocaine-treated COs, in particular, in the presumptive neurogenic niche. For example, increased FOSB protein expression (Fig. 2a) and *FOS*, *JUNB*, and *JUND* gene expression was evident compared to control (Fig. 2b). Key elements of the signalling cascade associated with neuronal plasticity and activation of the IEGs are also altered by cocaine in the neurogenic niche cell types, for example, mitogen-activated protein kinase 1 (*MAPK1*) was increased in PRG (Fig. 2c). Furthermore, there was increased expression of calcium/calmodulin-dependent protein kinase II inhibitor 1 (*CAMK2N1*), an inhibitor of CaMKII previously implicated in cocaine-induced plasticity [36] (Fig. 2d). *TOX3*, a calcium-dependent transactivator [37], and the axon neurofilament cytoskeletal regulator, synuclein gamma (*SNCG)* were increased in the neurogenic niche (Fig. 2d). Finally, the synaptic plasticity gene, neuritin (*NRN1*) was increased in the IN and the choroid plexus clusters (Fig. 2e).

Of potential interest, decreased expression of the Kv7.2 voltage and lipid-gated potassium channel (*KCNQ2*) was observed in RG and IP clusters (Supplementary Fig. 3a). Disruption of KCNQ2 channels is thought to influence cocaine's rewarding effects and increase cocaine place preference in adult rodents [38].

Within the neurogenic niche, several developmental genes were downregulated (*ID1, ID2, ID3, ID4, MSX1, HES1, HES5*) (Supplementary Fig. 3b) supporting the idea that PCE causes neurodevelopmental deficits. Furthermore, *NFIB*, a key transcription factor (TF) which is involved in the developing nervous system [39] was decreased in the CP2 and AS2 clusters (Supplementary Fig. 3c). Finally, cocaine reduced the expression of *BEX1* in RG and CP1 clusters. The BEX gene family are thought to mediate neuronal differentiation [40] and are decreased in humans with cocaine addiction [41] (Supplementary Fig. 3d).

Several previous studies have shown that PCE is associated with increases in neuroinflammation and oxidative stress [13, 42–44]. Consistent with such a neurotoxic effect, cocaine exposure clearly led to activation of astrocyte-like cells (AS2) within the COs, with upregulation of several markers associated with reactive astrocytes and their inflammatory response (*AQP1, RGS4, PDPN, SLC3A2*) and oxidative stress (*PRDX6, FAM107B, TXNIP*) (Fig. 2f) [45–49]. The neurotoxic effects of cocaine have been partially attributed to its ability to increase intracellular calcium [50, 51] and, here, increased expression of the calcium binding protein, *S100A10* was observed in the CO astrocyte-like populations (Fig. 2f).

In several cell types, increased expression of glutamine synthetase (*GLUL*), the glutamate ionotropic delta 2 receptor GluRδ2 (*GRID2*), the vesicle glutamate transporter VGLUT3 (*SLC17A8*), and Na^+-dependent excitatory amino acid transporters, EAAT1 and SNAT1 (*SLC1A3, SLC38A1*), were evident (Fig. 2). These findings point to a cocaine-mediated disruption of the key excitatory neurotransmitter glutamate and further suggest the possibility of a drug-induced excitotoxic state.

To summarise, COs replicate well-established cocaine responses observed in vivo and provide additional information about the cell-type specific regulation of gene expression following cocaine exposure.

Cocaine-mediated remodelling of the epigenetic landscape

In the adult brain, cocaine mediates its long-lasting effects in key neural circuitries through molecular reprogramming and chromatin remodelling [52, 53]. In the current scRNA-seq study, cocaine increased the expression of several epigenetic mediators such as chromatin remodellers (*ARID1A, SMARCA4, CHD1*), histone methyltransferase (*SET*), the polycomb complex (*SUZ12*), histone deacetylases (*HDAC1, HDAC2*), histone demethylase (*KDM6B*), histone acetyltransferase (*KAT6B*) and bromodomain-containing proteins (*TRIM28, BRD4*) (Fig. 3a).

To further probe cocaine effects within the chromatin landscape, we performed scATACseq to map genome accessibility throughout the cell clusters in control (3647 cells) and cocaine-treated (3328 cells) organoids (Fig. 3b). Cell types and proportions were comparable between the scRNAseq and scATACseq datasets (Supplementary Fig. 4a–c). Pseudotemporal analysis of chromatin state signatures identified the same three supercluster cell lineages found in the scRNAseq dataset (Fig. 3c). In total, we identified 1726 differentially accessible regions (DARs) (AS1(274), AS2(267), RG (10), PRG (80), IP1(33), IP2(525), IN(0), CP1(89), CP2(108), RP(340)) with increased accessibility across cell types between control and cocaine-treated organoids (Supplementary Data 5; Fig. 3d). In addition, 9004 DARs (AS1(355), AS2(757), RG (809), PRG(2011), IP1(1407), IP2(1450), IN(147), CP1(500), CP2(1918), RP(1460)) were identified to be less accessible following cocaine treatment (Supplementary Data 5). Cocaine decreased global accessibility at transcription start sites (TSS), promoters and DNase I hypersensitivity sites (Fig. 3e; Supplementary Data 6). There was no significant difference in global

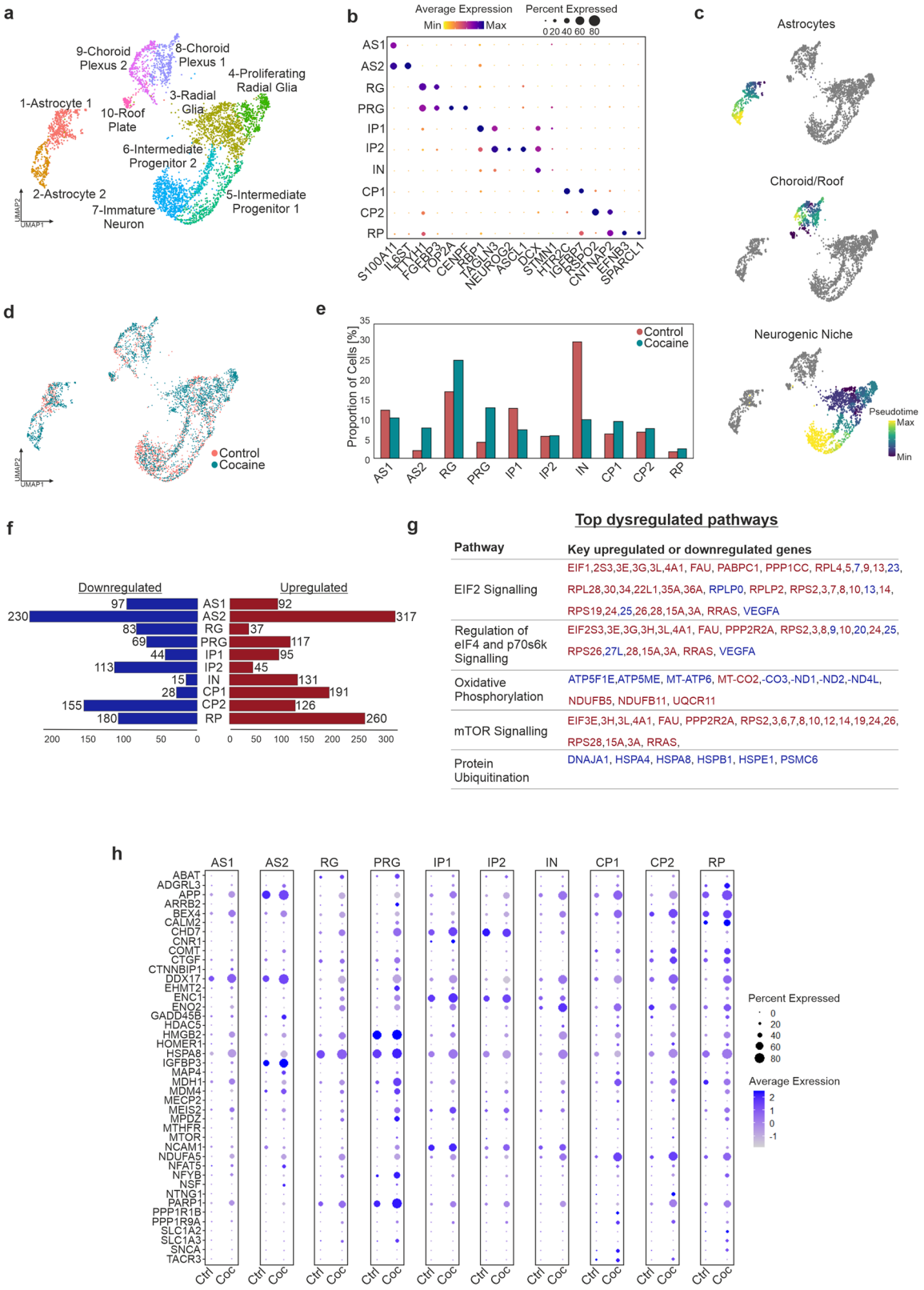


Fig. 1 scRNAseq characterisation of the effect of cocaine on the 36-day old cerebral organoid. a UMAP projection of 4437 single cells revealing 10 distinct clusters within control and cocaine-treated organoids. **b** Differentially expressed genes used for annotation within each cluster. **c** UMAP projection of astrocyte, neurogenic niche and choroid/roof plate lineage cell types in cocaine-treated organoids coloured by pseudotime. **d** Overlayed UMAP of 1681 cells for control and 2756 cells for cocaine. **e** Proportion of cells within each cluster in control and cocaine-treated organoids. **f** Differentially expressed genes within each cluster in response to cocaine. Genes were selected based on a $|\log_2 FC| > 0.4$, pct > 0.25, and $p < 0.05$. **g** Top dysregulated pathways in cocaine-treated organoids identified by IPA analysis. **h** A selection of previously identified cocaine-associated genes are increased in cocaine-treated organoids.

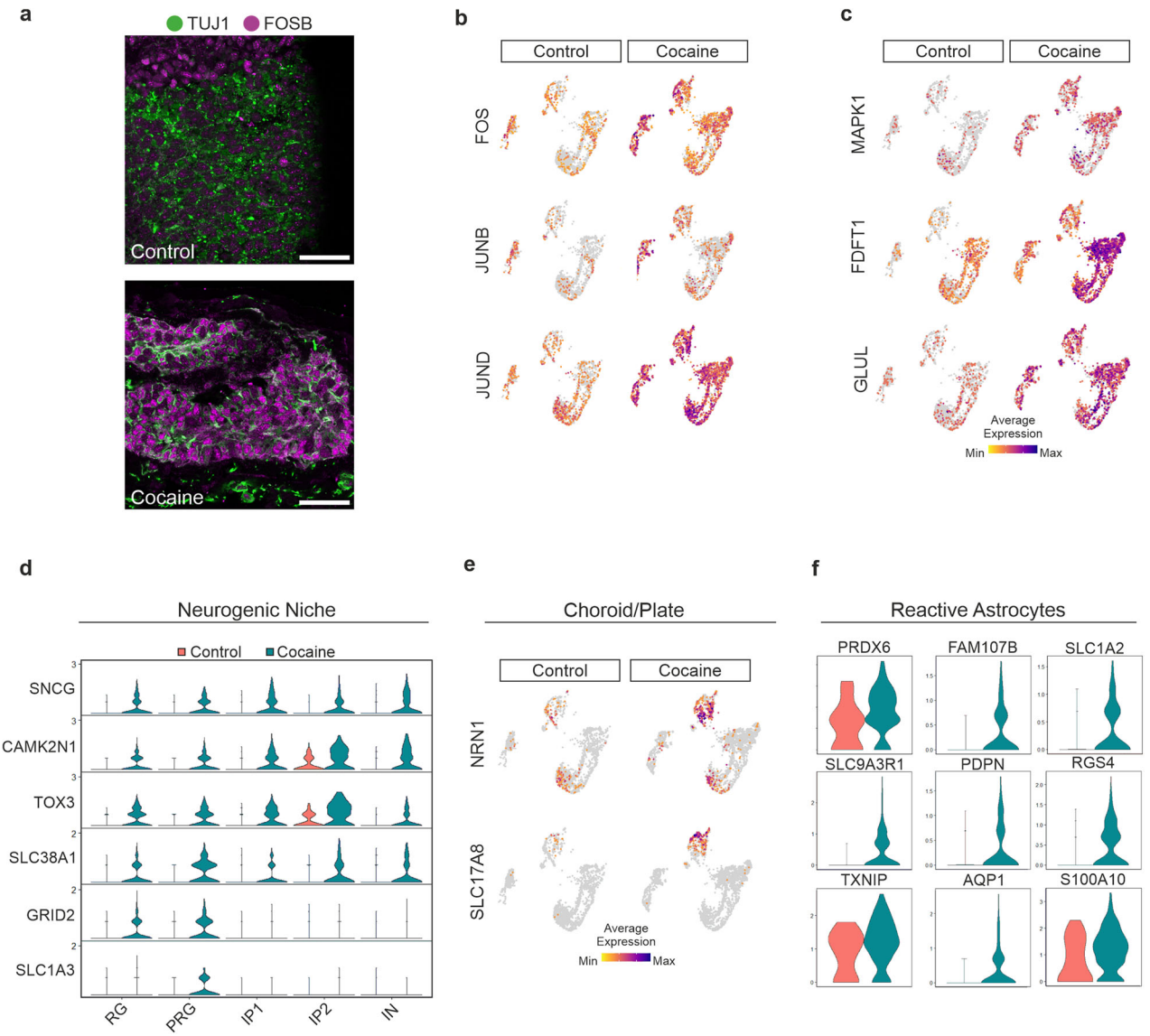
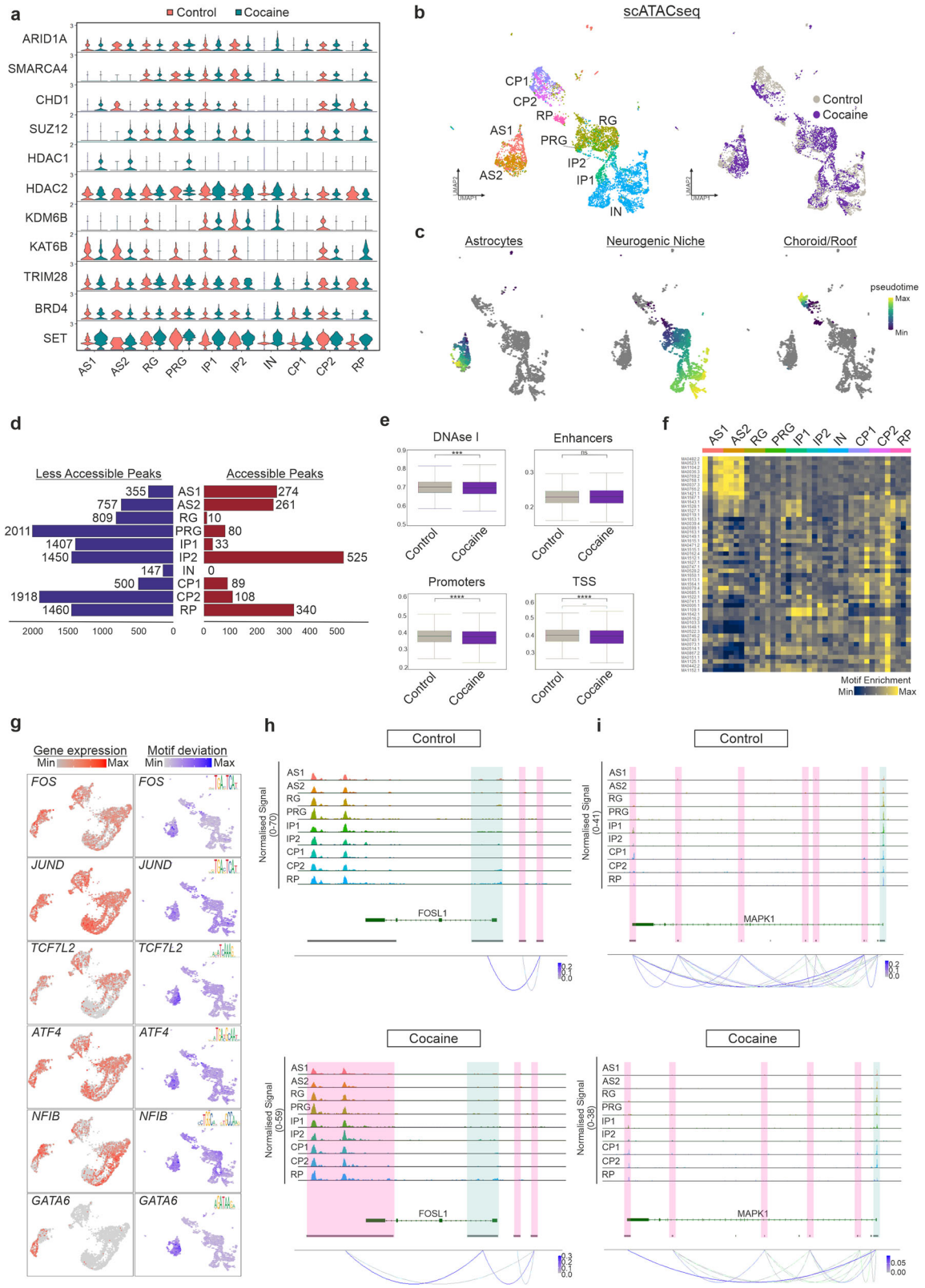


Fig. 2 Cocaine alters neural plasticity, developmental signalling and neuroinflammatory tone. a Immunostaining revealed increased FOSB expression in organoids treated with cocaine. Magnification 40X. Scale: 50μm. **b** Increased expression of *FOS*, *JUNB*, and *JUND* and **c** Immediate early genes (IEGs) were evident across the organoids. **d** Increased expression of synaptic plasticity-associated genes, the glutamate ionotropic receptor GluD2 (*GRID2*), and Na^{+}-dependent excitatory amino acid transporters, EAAT1 (*SLC1A3*), and SNAT1 (*SLC38A1*) were observed in the neurogenic niche clusters. **e** Increased expression of the vesicle glutamate transporter VGLUT3 (*SLC17A8*) and *NRN1* was observed in the choroid/plate niche. **f** Increased expression of oxidative stress and neuroinflammation associated genes were observed in the astrocyte-like clusters.

accessibility at enhancers (Fig. 3e). We did however note, cluster-to-cluster differences (Supplementary Fig. 5; Supplementary Data 7).

To identify potential regulators of the cocaine transcriptional response, we generated motif activity scores using ChromVAR to find transcription factors (TFs) with specifically enriched motif accessibility across the cell clusters (Fig. 3f). Motif enrichment analysis for each set of DARs identified over representation of TF binding motifs included the IEG AP1 family (*JUN, JUNB, FOS, FOSL2, JDP2*), the T-cell factor/lymphoid enhancer-binding factor family (*TCF7, TCF7L2, TCF12, LEF1*), and the bZIP transcription factor, *ATF4* (Fig. 3f, g). We also identified cell-type specific motif enrichment in response to cocaine. For example, enrichment of *GATA6* was observed in astrocyte-like clusters and increased *NFIB* in the neurogenic niche (Fig. 3g). GATA6 has previously been shown to promote inflammatory phenotypes in astrocytes [54] while NFIB is associated with synaptic plasticity [55]. Changes in cis co-accessibility were, for example, observed at IEG-associated genes such as *FOSL1* and *MAPK* (Fig. 3h, i).

We next explored global gene regulatory networks associated with the cocaine response using SCENIC which integrates scRNAseq data with known protein-DNA interactions to infer TF regulons/networks. SCENIC analysis identified high regulon activity associated with the IEG AP1 family, chromatin mediators (*HDAC2, YY1*) and other plasticity associated TFs (*EGR2, ATF4, MXI1, POU3F2*, and *FOXO6*) [56–59] across clusters (Fig. 4a, b). Increased putative footprint depth, associated with putative TF binding, was also observed globally for IEG-associated regulons such as JUND, FOS, and ATF4 (Fig. 4b–d). This establishes a transcriptional regulatory mechanism centred on IEG-associated genes that seems to mediate substantial proportions of the cocaine response across multiple cell types in the CO.



Cocaine-mediated alteration in cellular communication in the CO

To better understand changes in global cell-cell communication networks contributing to the actions of cocaine, we interrogated cell surface protein interactions using the R package, CellChat. CellChat infers the probability for cell-cell communications to occur via specific ligand-receptor systems. CellChat revealed increased inferred interactions and new signalling pathways with

Fig. 3 Cocaine-mediated remodelling of the epigenetic landscape. a Increased expression of chromatin modifiers was observed across clusters in response to cocaine. **b** UMAP plot of annotated scATACseq dataset with overlayed UMAP of 3647 cells for control and 3328 cells for cocaine. **c** UMAP projection of chromatin accessibility in astrocytes, neurogenic niche, and choroid/roof plate cell types in cocaine-treated organoids coloured by pseudotime. **d** Differentially accessible regions (DARs) within each cluster in response to cocaine. DARs were selected based on a $\log_2$FC ≤ −0.5 or ≥ 0.5, pct ≥ 0.25, and $p < 0.05$. **e** Global changes in chromatin accessibility at DNase I hypersensitivity sites, transcription start sites, promoters and enhancers. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns ≥ 0.05; pairwise *t*-test with Bonferroni correction. **f** Heatmap of chromVAR computed deviations z-score for the topmost variable TF motifs enriched in cocaine-treated organoids. **g** UMAPs of scRNAseq gene expression (left panel) and scATACseq based ChromVAR motif deviation scores (right panel) for *FOS, JUND, TCF7L2, ATF4, GATA6*, and *NFIB*. Prediction of cis co-accessibility networks between enhancer (blue) and promoter (red) elements at sample Immediate Early Gene loci, **h** *FOSL1* and **i** *MAPK* in response to cocaine. Higher co-accessibility score indicates higher co-accessibility between promoter and enhancer elements.

significant differences in overall information flow between cell types in the cocaine organoid compared to the control (Fig. 5a, b; Supplementary Fig. 6a). Specifically, we found increased signalling in midkine (MDK), pleiotrophin (PTN), neural cell adhesion molecule (NCAM), CD99, cadherin (CDH) and junctional adhesion molecule (JAM) pathways and 'de novo' cocaine-mediated signalling pathways like laminin, non-canonical Wnt (ncWnt), notch and collagen (Fig. 5c). We explored which cell types were involved in the cell-cell communication patterns in response to cocaine. Increased incoming interactions were identified in most clusters (AS2, RG, PRG, CP1, CP2, and RP) while increased outgoing interactions were identified in five clusters: AS1, AS2, RG, PRG, and RP (Supplementary Fig. 6b-c).

Increased putative notch and ncWnt signalling between AS2 and PRG clusters was suggested in cocaine-treated organoids (Fig. 5d–f, g–i). This is of potential relevance as notch and ncWnt are key regulators of neuronal proliferation and differentiation in the neurogenic niche during development. The notch pathway is also important for astrocyte differentiation into a pro-inflammatory phenotype [60, 61] and glutamate-mediated astrocyte-neuron communication and synaptic plasticity following injury [62, 63]. Of various ligand-receptor pairs, DLK1-NOTCH1 displayed the strongest putative signal from AS2 to PRG clusters while DLK1-NOTCH2 displayed the strongest putative signal from AS2 to CP1, CP2, and RP clusters (Fig. 5j). Increased WNT5B-FZD3 ligand-receptor signalling between AS2 and other clusters was suggested by the CellChat analysis (Fig. 5j). ncWnt signalling, in particular, through WNT5B, has been implicated in neuroinflammation [64] and in inflammation in other contexts [65].

Midkine was one of the top dysregulated cellular signals in the scRNAseq analysis across most cell types. We examined how MDK signalling patterns differed between control and cocaine-treated organoids using network centrality analysis, which uses the outgoing and incoming reaction strengths of each cell cluster to determine their likelihood as signalling sources or targets. Increased MDK signalling was observed between several groups within the cocaine COs (Fig. 6a) and we identified the dominant senders of MDK signalling to be both astrocyte-like populations and roof plate cells, while the PRG cluster was the dominant receiver (Fig. 6a, b). Increased signalling in the astrocyte-like populations is consistent with current literature which states that astrocytes upregulate midkine during neuroinflammatory events [66, 67]. Furthermore, increased expression of not only MDK and PTN but also their related receptors (*SDC2*, *PTPRZ1, LRP1, NCL, ITGA6*, and *ITGB1*) was evident in cocaine-treated organoids (Fig. 6c). The ligand-receptor pair, MDK-NCL displayed the strongest putative signal (Fig. 6d, e). In cocaine-treated organoids, increased PTN pathway signalling was seen amongst the two RG clusters (RG and PRG) and the roof plate cells (Fig. 6f, g). Finally, signalling through NCL was predicted to mediate the PTN signal in the RG and RP clusters (Fig. 6h, i).

DISCUSSION

This study provides a deep insight into cocaine-mediated perturbations of neurodevelopmental processes in a model of the early developing human brain, roughly corresponding to the mid-first trimester of pregnancy [12, 22]. Cocaine alters gene expression patterns, in part, through epigenetic landscape remodelling, to dysregulate neurodevelopmental cellular signalling and drive an inflammatory-like activation of astrocyte-like populations. Any one of these actions can clearly disrupt the normal development and maturation of the brain. In keeping with a considerable body of literature on the effect of cocaine in the adult brain [68–71], clear evidence emerges of regulation of IEG TFs, remodelling of the epigenetic landscape and synaptic plasticity in our D36 organoids. These functions and signalling appear to be altered across multiple cell types following cocaine exposure. Importantly, the D36 cerebral organoids used here do express D1 and D2 receptors and DAT1, serotonin receptors and SERT and, hence, possess all the cellular machinery to enable the usual mechanism of cocaine action seen in the adult brain. It is important to note, however, that cocaine-mediated impact on the cerebral organoids may involve/include other mechanisms. Indeed, the same is likely true of the detrimental effects of cocaine on neurodevelopment *in utero*, especially early in development, prior to the emergence of functional dopamine neurons. In this regard, the D36 organoids represent an ideal model to address questions related to early stages of neural development.

Our data assigns a central and substantial role to transcriptional regulators of the IEG family in cocaine-mediated changes in the developing neural tissues of the COs. IEGs have long been established as key mediators of gene expression regulation downstream of glutamatergic neurotransmission and drivers of synaptic plasticity in neuronal circuits in the setting of information encoding and memory formation [72, 73]. Our D36 CO data is in line with others who have also shown robust IEG responses in rodent [31] and organoid models of cocaine exposure [16]. Similarly, IEGs such as FOS, JUN and others are implicated in the aberrant plasticity created by initial exposure to cocaine; plasticity that underpins significant dysregulation of brain circuitries responsible for reward registration, memory formation and stress regulation [36, 68, 74]. Moreover, in the present study, we see a substantial remodelling of the epigenetic landscape in the CO. It is clear that this is related to the modulation of IEG TF activity as, for example, we observed epigenetic changes associated with the AP1 family, JUND and FOS. This establishes a transcriptional regulatory mechanism centred on IEG-associated genes that contribute substantially to the cocaine response across multiple cell types in the D36 CO. Again, there are distinct parallels with findings from the adult brain where elegant studies have mapped regulation of components of the epigenetic machinery, including HDACs, histone methyltransferases and DNMTs, and demonstrated loci-specific alterations in chromatin structure that subserve long-lasting change in gene expression tone and gene responsivity [53, 75, 76].

mRNA translation is a fundamental cellular process which is regulated at multiple levels including initiation and elongation, to promote synaptic plasticity in the central nervous system [77, 78]. Cocaine-mediated alteration of mRNA translational machinery

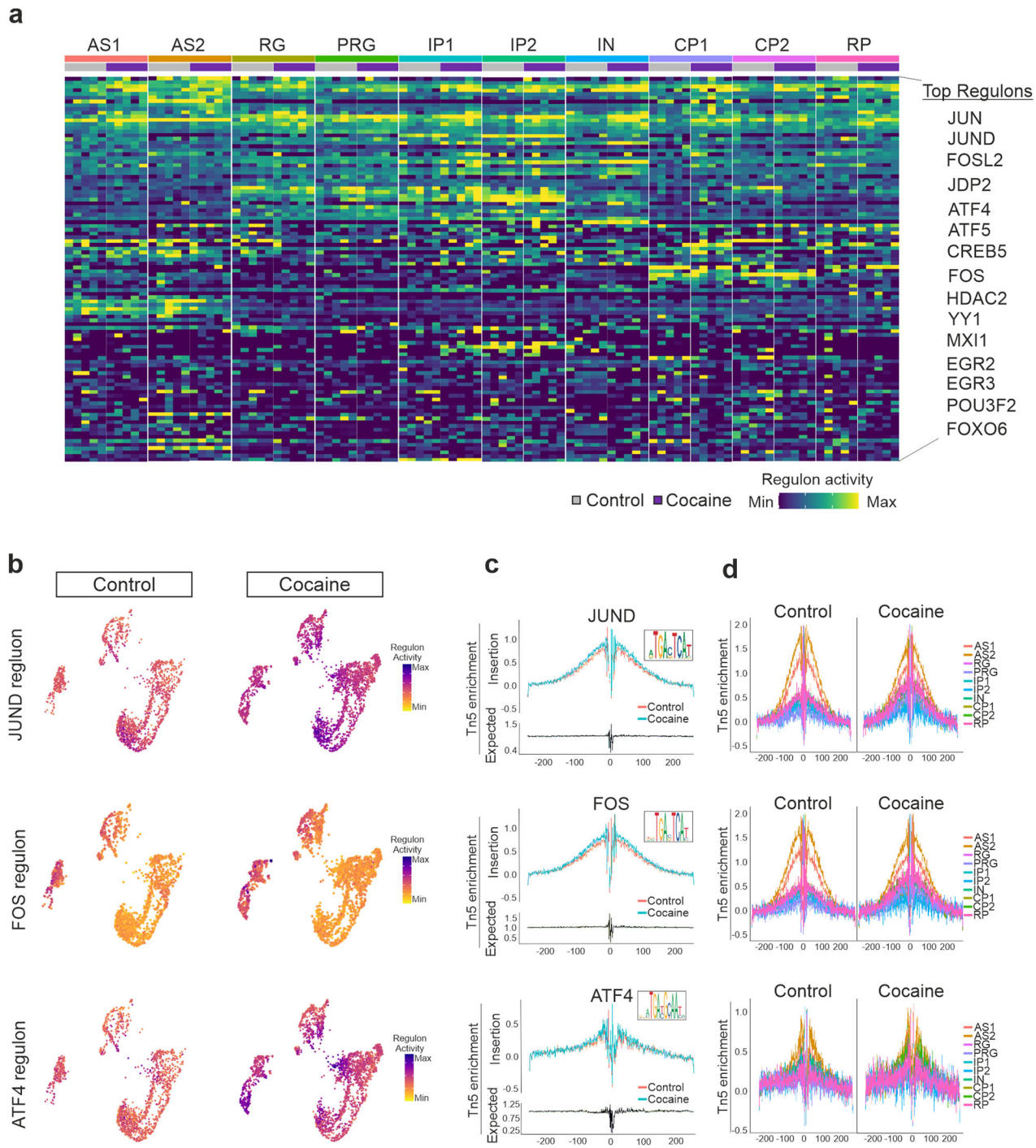


Fig. 4 Identifying the transcription factor networks underpinning cocaine-mediated actions in the cerebral organoid. a Heatmap of top differentially regulated transcription factor networks ("regulons") across all clusters in response to cocaine. **b** UMAP visualisation of TF regulons for JUND, FOS, and ATF4. **c** Motif-centric footprinting estimating transcription factor binding for JUND, FOS, ATF4 in response to cocaine and **d** across clusters.

across all cell types of the developing brain emerged as the top dysregulated cellular function in our study of COs, with several of the top pathways up and down regulated relating to this vital function such as EIF signalling, regulation of eIF4 and mTOR signalling (specific genes included *EIF2A*, *EIF4A1*, and *EIF4B*). Recent studies have identified a central role for disrupted translation initiation and elongation processes in the actions of cocaine and other drugs of abuse [79, 80]. For example, both acute and repeated cocaine administration-mediated conditioned place preference has been linked to decreased phosphorylation of eIF2 in the ventral tegmental area (VTA) in adult and adolescent mice [79] and increased eIF2 signalling is observed in other cocaine-treated cell [81] and organoid models [14].

Repeated cocaine administration in vivo results in the formation of long-lasting drug association memories which are maintained after cocaine withdrawal. Chronic cocaine use has been linked to changes in glutamate signalling that contribute to cocaine self-administration and reinstatement of cocaine seeking [82, 83]. Glutamate receptors

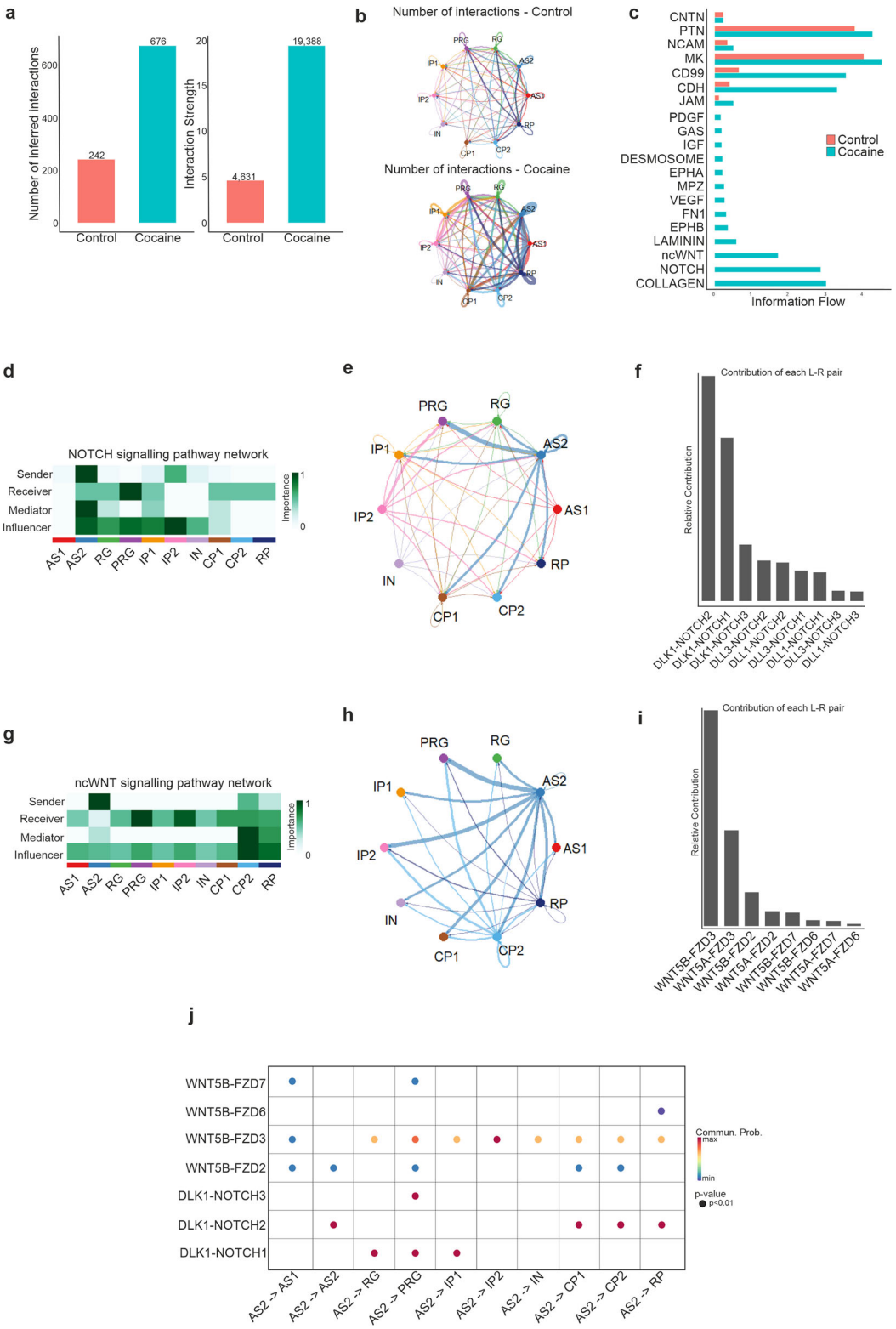


Fig. 5 Cocaine-mediated alteration in cellular communication in the cerebral organoid. a Increased number of inferred interactions between most clusters were apparent in cocaine-treated organoids relative to control. **b** Circle plots depicting interaction numbers and strength between all cell clusters in control and cocaine-treated organoids. Line thickness represents increased interaction between clusters. **c** Signalling in PTN, NCAM, MK, CD99, JAM, ncWNT, Laminin, Notch, and Collagen pathways were increased in cocaine-treated organoids**. d** Heatmap of notch signalling pathway network contributing to mostly outgoing or incoming signalling between cell groups. **e** Circle plot depicting the notch signalling pathway network. **f** Overall contribution of each ligand-receptor pair to the notch signalling pathway amongst cell types. **g** Heatmap of non-canonical Wnt signalling pathway network contributing to mostly outgoing or incoming signalling between cell groups. **h** Chord diagram showing the non-canonical Wnt (ncWnt) signalling pathway network. **i** Overall contribution of each ligand-receptor pair to the ncWnt signalling pathway amongst cell types. **j** Notch and ncWnt ligand receptor interactions between AS2 and all other cell subtypes.

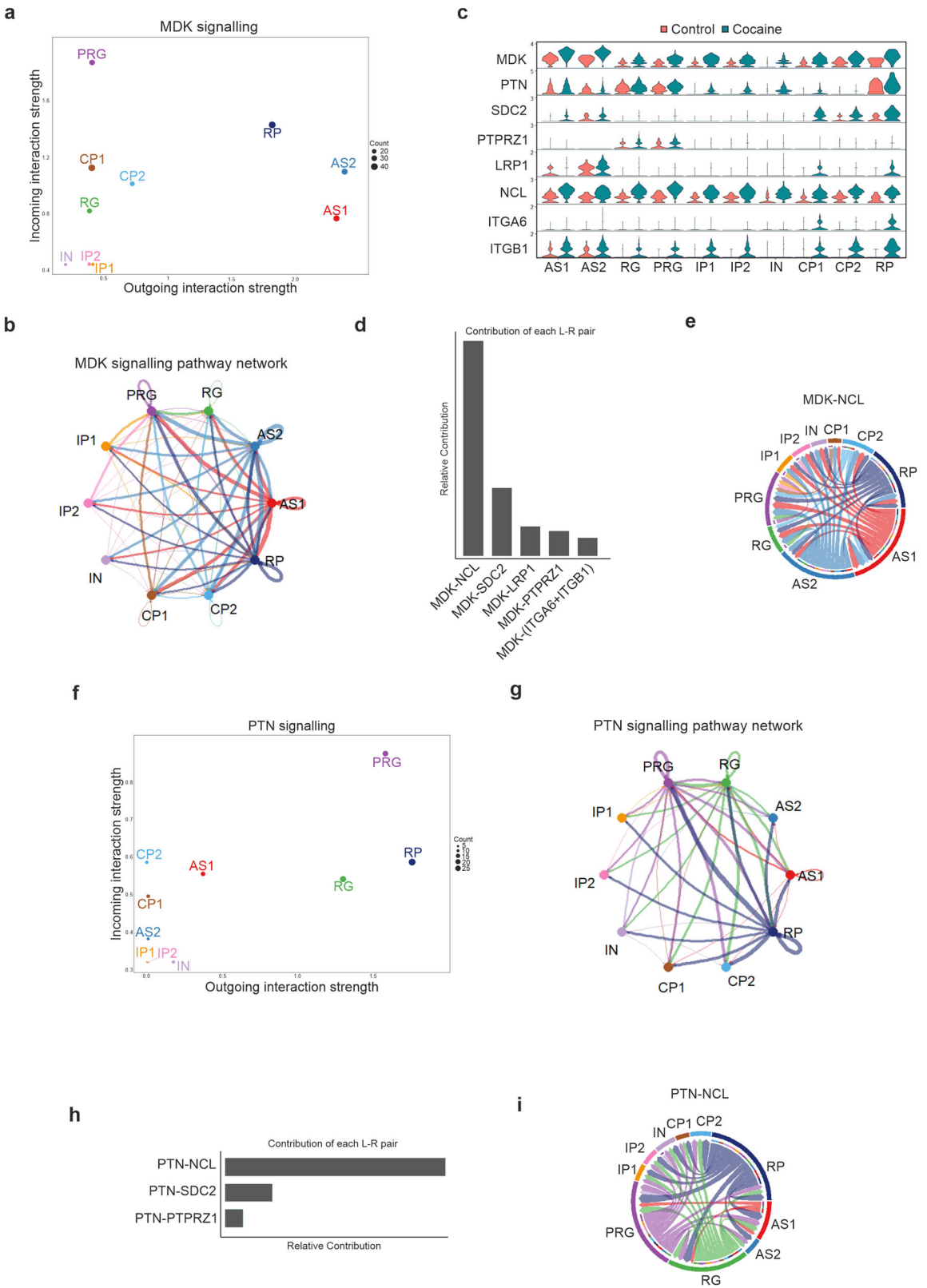


Fig. 6 Altered midkine and pleiotrophin signalling in cocaine-treated organoids. a Roof plate and astrocyte-like cells contributed to the majority of midkine (MDK) signalling in cocaine-treated organoids. **b** Circle plot depicting the MDK signalling pathway network. **c** Increased expression of midkine and pleiotrophin (PTN)-associated genes were evident in cocaine-treated organoids. **d** Overall contribution of each ligand-receptor pair to the MDK pathway amongst cell types. **e** Cell-cell communication network for the MDK-Nucleolin (NCL) ligand-receptor pair. **f** Proliferating radial glia, roof plate, and radial glial cells contribute to the majority of pleiotrophin signalling. **g** Circle plot depicting the PTN signalling pathway network. **h** Overall contribution of each ligand-receptor pair to the PTN pathway amongst cell types. **i** Cell-cell communication network for the PTN-NCL ligand-receptor pair.

are key mediators of synaptic plasticity and reduced glutamate levels are observed after both short and long periods of withdrawal [84]. Several alterations in glutamate neurotransmission known to occur in cocaine models in vivo were recapitulated here in the COs. For example, the expression of EAAT1 was increased in organoids following cocaine treatment and this is in line with rodent models in which elevated protein expression of EAAT1 is observed in the nucleus accumbens core and shell [85, 86].

Altered neurodevelopmental signalling following cocaine exposure is supported by the pathway analyses (GO and IPA) which identify key dysregulations in the general control of balance between proliferation and differentiation and neurodevelopmental signalling. Moreover, CellChat analysis allowed a deeper understanding of the nature of the altered developmental signalling tone created by exposure to cocaine. Specific neurodevelopmental signalling molecules are substantially altered by cocaine in the cerebral organoid including Wnt, notch, midkine and pleiotrophin. It is clear that the signalling tone of these secreted factors is substantially imbalanced with change in the cellular crosstalk suggested between multiple cell type pairs, revealing errant proliferation/differentiation control, particularly in the neurogenic niche. This is reminiscent of alterations in adult hippocampal neurogenesis seen with cocaine and other drugs of abuse [87, 88] and would be predicted to underlie a decrement in neuronal populations as the brain develops.

Substantial upregulation in MDK- and PTN-mediated signalling was revealed following cocaine exposure. The cytokines MDK and PTN are multifunctional growth factors implicated in a range of physiological and pathological functions including cellular proliferation, migration and differentiation, apoptosis, synaptic formation and gene expression regulation [89–91]. Both MDK and PTN are widely expressed during the mid-gestational period of embryogenesis, becoming highly downregulated after birth, but heavily induced in many tissues accompanying injury, inflammation and cancer [89, 92, 93]. The diverse range of functions of MDK and PTN seem to be underpinned by the assembly of hetero-receptor complexes of distinct composition in different cellular contexts [92–95]. Thus, given these roles of MDK and PTN, the increase in their intercellular signalling following cocaine exposure could clearly contribute not only to the altered gene expression, synaptic plasticity and neurodevelopmental deficits discussed above, but also the enhanced neuroinflammatory environment discussed below.

Likely an interdependent factor in all of the alterations discussed above is the neuroinflammatory environment that appears to be generated, in particular, through activation of astrocyte-like cells in the developing COs by cocaine exposure. There is a rich literature on the detrimental effects of *in utero* infection and inflammation on brain development and it is a key environmental risk factor contributing to later emergence of a range of mental illnesses including depression, psychosis and addiction [96–98]. There is a clear upregulation in cytokine neuroinflammatory signalling and oxidative stress in the CO following cocaine exposure. This is particularly reflected in the activated astrocyte-like population (AS2 cluster) which are almost unique to the cocaine-treated organoid. The appearance of this cell cluster strongly points to an injury/damage type response. Such a response may relate to attempted repair and/or neurodegenerative-associated neuroinflammation. The latter is known to occur in response to cocaine exposure in the adult brain and to contribute to the neurodegenerative component of severe cocaine use disorder [99, 100]. Furthermore, increased oxidative stress is also observed in response to cocaine in other organoid models [13, 15]. In our study, for example, genes such as GJA1 (connexin 43) and IL6ST were significantly upregulated in response to cocaine. GJA1 upregulation identifies activated astrocytes in setting such as amyloid plaque formation in Alzheimer's disease [101, 102]. IL6ST is a hub protein found in several receptor complexes where it serves to transduce a range of inflammatory signals including IL6, LIF, OSM, CNTF, IL11, CTF1 and BSF3 [103]. Recently, Ho and colleagues showed an upregulated interferon response to oxycodone in opioid use disorder patient-derived cerebral organoids [104]. Similarly, several other studies have shown upregulated interferon and immune responses to oxycodone [101], cocaine [14, 15] and dopamine [15] using cerebral organoids. Taken with our data, these studies suggest parallels between the neurodevelopmental signalling perturbations associated with opioid and cocaine exposure *in utero* and reveal that cocaine-mediated neuroinflammatory damage is a pathological mechanism common to the adult and developing brain.

In conclusion, the human iPSC-derived CO is confirmed as a robust model of *in utero* cocaine exposure and the resultant alteration of neurodevelopment. While it is important to note that the cocaine response in this study may be specific to the HPI1213i-babk_2 cell line used, other studies cited above, using different iPSC lines, have implicated similar molecular and cellular changes in iPSC-derived models of cocaine exposure [13–15]. In particular, the presence of cocaine in the developing brain substantially dysregulates gene expression patterning including IEG and neurotransmission-associated programmes and neurodevelopmental signalling systems to skew neurodevelopment. This action is in part underpinned by altered tone of cell-cell signalling critical for neurodevelopment such as that mediated by MDK and PTN. Finally, cocaine-mediated damage is associated with a reactive inflammatory phenotype in astrocyte-like and other cell types suggestive of induction of a neurodegenerative environment in the developing brain tissue. All these signalling perturbations offer opportunities for the future development of novel treatments for foetal cocaine exposure.

DATA AVAILABILITY

Correspondence and requests for data should be addressed to JLD, JC and KJM.

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AUTHOR CONTRIBUTIONS

J.C, K.J.M and J.L.D conceived the project. J.L.D performed differentiation experiments, sample preparation, western blotting, scRNAseq and scATACseq analysis, interpretation, and data visualisation. C.K performed scRNAseq and scATACseq bioinformatic analysis. C.L.McM assisted with differentiation experiments and scRNAseq analysis and interpretation. L.K assisted with differentiation experiments and performed qPCR and western blotting. A.E.H and S.C performed sample preparation, immunofluorescence and imaging. Y.K performed IPA analysis. N.T. assisted with differentiation experiments and sample preparation. D.F.B, J.C and K.J.M acquired funding. J.L.D, J.C, and K.J.M wrote the manuscript with feedback from all authors.

COMPETING INTERESTS

The authors declare no competing interests.

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