Extracellular vesicles from morphine-exposed prefrontal cortex carry transcriptomic and proteomic signatures of synaptic dysfunction

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Abstract

Extracellular vesicles (EVs) released by neurons and glial cells mediate intercellular communication in the brain and regulate synaptic function, neuronal survival, and neuropathological processes. Although chronic opioid exposure induces widespread neuroadaptations, the role of brain-derived EVs (BDEVs) in these processes remains poorly understood. We isolated BDEVs from the prefrontal cortex of rats subjected to chronic morphine exposure and performed integrative analyses of their RNA and protein cargo. Total RNA sequencing was combined with unbiased proteomics to define morphine-induced alterations. Functional validation was conducted by exposing primary cortical neurons to BDEVs from morphine- or vehicle-treated animals, followed by transcriptional profiling. Morphine significantly reprogrammed the transcriptome and proteome of BDEVs, with enrichment of pathways related to synaptic plasticity, endoplasmic reticulum stress, mitochondrial dysfunction, and neurodegeneration. Among the most robust changes, the synaptic regulator **ARC** and the ER stress marker **Hspa5** were consistently modulated at both mRNA and protein levels. In functional assays, morphine-derived BDEVs altered neuronal expression of genes associated with synaptic remodeling and excitability, including **Ncam1** and **Kcnn1**. These findings provide the first evidence that chronic opioid exposure reprograms BDEV cargo in a brain region critical for addiction, and that these vesicles are sufficient to induce transcriptional changes in recipient neurons. BDEVs emerge as active mediators of morphine-induced neuroadaptations and hold promise as biomarkers and therapeutic targets in opioid-related neuropathologies.

extracellular vesicles; morphine; prefrontal cortex; transcriptomics; proteomics; synaptic plasticity

Introduction

Extracellular vesicles (EVs) are nanosized particles secreted by virtually all cell types, serving as mediators of intercellular communication by transferring proteins, nucleic acids (DNA, mRNA, microRNAs, and long noncoding RNAs), and lipids to recipient cells (1, 2). Within the central nervous system, neurons, astrocytes, and microglia secrete EVs that play essential roles in brain function, including synaptic transmission, trophic support, and dendritic spine remodeling (3-7). Disruptions in these processes have been increasingly implicated in psychiatric conditions such as substance use disorder (SUD) (8, 9).

Emerging evidence suggests that brain-derived extracellular vesicles (BDEVs) may act as conduits through which addictive substances reshape neural circuitry. Exposure to drugs of abuse alters the molecular cargo of BDEVs, thereby influencing neurobiological responses to substances (8-12). To date, most studies have centered on small RNA species, particularly microRNAs, based on the long-standing assumption that EVs are enriched for this RNA class (13, 14). Yet recent findings challenge this view, showing that BDEVs can selectively package and transfer intact mRNAs that are translated in recipient neurons (15). One striking example is the activity-regulated cytoskeleton-associated protein (ARC) mRNA, which has been shown to be selectively incorporated into EVs and delivered functionally to neurons in vivo (16).

Despite this progress, a major knowledge gap remains: it is unknown whether chronic opioid exposure reprograms the full transcriptomic and proteomic composition of BDEVs, and whether these vesicles are sufficient to drive transcriptional reorganization in recipient neurons. Addressing this gap is critical, given the escalating opioid crisis and the urgent need to identify novel molecular mechanisms underpinning opioid-induced neuroadaptations. Here, we directly test the hypothesis that chronic morphine exposure remodels BDEV cargo and that these vesicles, once secreted, are sufficient to reshape neuronal gene expression in ways relevant to opioid-evoked behavioral phenotypes.

We isolate BDEVs from the prefrontal cortex (PFC)—a region crucial for addiction-related plasticity—and comprehensively profile their proteome and transcriptome. To assess functional impact, we expose primary cortical neurons to BDEVs from vehicle- and

morphine-treated donors and quantify transcriptional responses. This approach establishes a mechanistic framework for understanding EV-mediated contributions to opioid-induced neuroplasticity, with implications for both biomarker discovery and therapeutic innovation.

Methods and Materials

Methods are summarized below. A complete description of all experimental procedures is available in Supplementary Materials.

Animals and drug treatment

Male Sprague–Dawley rats (8 weeks old, Janvier Labs) were pair-housed under standard conditions with ad libitum access to food and water. After two weeks of habituation, animals received intraperitoneal injections of saline or escalating doses of morphine (10–50 mg/kg, twice daily for 5 days). Four hours after the final injection, rats were sacrificed, and bilateral prefrontal cortex (PFC) was dissected and frozen at −80 °C. All procedures were approved by the Danish Animal Experiments Inspectorate and complied with EU Directive 2010/63/EU.

Isolation and characterization of brain-derived extracellular vesicles (BDEVs)

BDEVs were isolated from PFC tissue by enzymatic dissociation, differential centrifugation, filtration, and ultracentrifugation, as previously described (18). Vesicle purity and morphology were assessed by BCA protein assay, Western blotting, transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA). Full protocols are provided in Supplementary Methods.

RNA extraction, sequencing, and analysis

RNA was extracted from BDEVs, DNA was removed, and total RNA libraries were prepared using a stranded RNA-seq kit. Libraries were sequenced on an Illumina NovaSeq X Plus platform (150 bp, paired-end). Quality control, differential gene expression analysis (DESeq2), and pathway enrichment (Metascape, GO, and Reactome) followed standard procedures, with significance defined at FDR-adjusted $p < 0.1$ and log2FC ±0.5.

Proteomic analysis

BDEV proteins were extracted, digested with trypsin, and analyzed by LC–MS/MS on an Orbitrap Q Exactive HF system. Data were searched against the Rattus norvegicus UniProt database and filtered at 1% FDR. Proteins were quantified based on unique peptides, and enrichment analyses were conducted using Cytoscape, IPA, and R-based workflows.

Primary neuronal cultures and BDEV treatment

Cortical neurons were prepared from embryonic day 17 (E17) C57BL/6 embryos and plated in Neurobasal medium with B27 supplement. On DIV7, neurons were treated with BDEVs isolated from saline- or morphine-exposed rats, and RNA was extracted 24 h later.

NanoString gene expression profiling

Gene expression in primary neurons was measured using the NanoString nCounter® Mouse Neuropathology Panel (770 genes). Data were normalized to housekeeping genes using nSolver™ 4.0, and pathway enrichment was conducted in Reactome.

Results

Characterization of EVs from the prefrontal cortex of chronically morphine-treated rats

BDEVs were isolated from the prefrontal cortex of rats via ultracentrifugation as previously described (17). We used NTA to determine the size distribution and particle number, with the latter normalized to the individual prefrontal cortex weight. Results revealed no significant differences between animals treated with morphine and those treated with vehicle **(Fig. 1B).** Using TEM, we characterized the BDEVs, revealing their characteristic morphology (**Fig. 1C**). We also analyzed BDEVs via specific positive and negative markers via Western blotting. As expected, the positive markers flotillin-1 and Hsp70 were detected in the BDEV fractions, whereas the negative marker GM130 was absent from the BDEV fractions but present in the whole cortex lysates (**Fig. 1D**). Together, these findings confirm the reliability of our isolation technique.

Effect of morphine treatment on the RNA profile of BDEVs

The RNA-seq analysis identified a total of 12,224 RNA molecules that surpassed the quality control threshold for downstream analysis, comprising 49 long noncoding RNAs (0.40%), 94 small noncoding RNAs (0.77%), 11,948 protein-coding mRNA molecules

(97.74%), and other RNAs (e.g., ribosomal RNA, transfer RNA, mitochondrial RNA, and pseudogenes—1.09%). Prior to conducting the differential gene expression (DGE) analysis, the RNA-seq data were deconvoluted from publicly available single-cell RNA sequencing data from rodent brains. We analyzed the proportions of microglia, glutamatergic corticothalamic (CT), intratelencephalic (IT), and pyramidal tract (PT) neurons, as well as GABAergic interneurons. The proportions of oligodendrocytes, astrocytes, and glutamatergic NP neurons were negligible. No significant group differences in cell-type proportions were observed (all p values > 0.05; see **Fig.** 2A). However, we observed that almost 40% of the RNA-seq data were from BDEVs originating from GABAergic interneurons.

A comprehensive analysis revealed 625 RNA transcripts with unadjusted p values less than 0.05 (Supplemental Table 1), indicating an effect of morphine treatment on the EV transcriptomic cargo. Four hits were identified by DGE analysis as having significant group differences that surpassed the FDR correction thresholds: Arc, Hspa5, Hivep3, and RT1-CE16 **(Fig. 2B)**. These four molecules are mRNAs derived from protein-coding genes. Arc, Hspa5, and Hivep3 were upregulated in EVs from morphine-treated animals, whereas RT1-CE16 was downregulated. Additionally, we cross-referenced our RNA-seq data with a large-scale human genome-wide association study (GWAS) meta-analysis of opioid use disorder (18) to identify differentially regulated transcripts originating from previously identified genes associated with the disorder, such as Oprm1, Cdkal1, Btnl2, Rabepk, Fbxw4, Ncam1, Furin, Kcnn1, and Rnf114. Among these, two transcripts, Ncam1 (log2FC = 0.45; p value = 0.02) and Kcnn1 (log2FC = 0.49; p value = 0.04), had unadjusted p values below 0.05, indicating a greater abundance of EVs in morphine-treated animals than in control animals **(Fig. 2C).**

Pathway enrichment analysis was conducted using all RNAs with an unadjusted p value < 0.05 on the basis of the KEGG 2019 mouse database (*Rattus norvegicus* gene codes were converted to mice before enrichment). Among the enriched pathways, only Lysosome, Protein Processing in the Endoplasmic Reticulum, and Axon Guidance were significantly represented, predominantly comprising upregulated transcripts (Figure y1 a-b). Notably, the enriched RNAs within each pathway were mutually exclusive, with no individual RNA shared across multiple pathways. This suggests a limited overlap and low interpathway correlation among the transcriptional alterations observed (Figure y2a-b).

Effect of morphine treatment on the protein profile of BDEVs

A total of 1007 proteins were reliably quantified, 379 of which passed the quality control criteria for downstream analysis. Among these, 82 proteins were found to be dysregulated on the basis of unadjusted p values < 0.05 (Supplemental Table 2), including 44 downregulated and 38 upregulated proteins. However, none of these changes remained statistically significant after false discovery rate (FDR) correction. Consequently, we adopted a more permissive threshold, selecting proteins with unadjusted p values < 0.01 and an absolute log2-fold change (log2FC) ≥ 0.5 to highlight potentially relevant alterations, which is consistent with prior proteomic studies applying exploratory thresholds in complex biological models (19, 20). Using these criteria, ten proteins were identified as differentially regulated in brain-derived extracellular vesicles (BDEVs) following morphine exposure (Fig. 3). Among these proteins, Krt33a, Fabp5, Krt72, Sprr1a, and Pkp1 were downregulated, whereas Stx1b, Ywhag, Grm8, Hspa5, and Pdia3 were upregulated.

A volcano plot was used to visualize the overall distribution of protein abundance changes, clearly highlighting the most significantly dysregulated proteins (p value < 0.05). Furthermore, dynamic range analysis revealed a broad spectrum of protein abundances, indicating that both low- and high-abundance proteins were successfully identified and quantified, an important aspect of exosome proteomics that enables the detection of diverse cargo molecules (Figure 3a-b) (21, 22).

To elucidate the biological implications of these morphine-associated protein alterations, pathway enrichment analysis was performed via the KEGG Mouse 2019 database (23). The dysregulated proteins were significantly associated with pathways involved in neurodegenerative diseases, oxidative phosphorylation, nonalcoholic fatty liver disease, the citrate (TCA) cycle, retrograde endocannabinoid signaling, thermogenesis, and protein processing in the endoplasmic reticulum (Figure 3 C).

Inspection of the dysregulation patterns revealed notable enrichment of mitochondrial proteins, particularly members of the NDUF family, as well as COX1 and COX7A2L (Figure 3 D-E), which play essential roles in oxidative phosphorylation and energy metabolism, mechanisms implicated in neuropsychiatric disorders (24-26). Additionally, the upregulation of the GRM5-encoded metabotropic glutamate receptor in the exosomal cargo of morphine-exposed subjects suggests potential activation of the phosphatidylinositol–calcium second messenger signaling pathway, which is known to influence synaptic plasticity and drug-related neuroadaptation (27, 28).

Integrative analysis of differentially regulated RNAs and proteins

Proteomic analysis identified 1,084 proteins, of which 1,007 were quantifiable. Notably, more than 85% overlapped with transcripts detected at the mRNA level, demonstrating substantial concordance between the transcriptomic and proteomic datasets. However, this overlap was not preserved within statistically dysregulated molecules were considered (p value < 0.05, unadjusted). Only five genes—Hspa5, S100a6, Plp1, Slc24a2, and Uggt1—were commonly dysregulated at both the mRNA and protein levels (Figure 4 A-B). In addition to the complexity of EVs, this limited concordance likely reflects the influence of posttranscriptional and posttranslational regulatory mechanisms, including mRNA stability, translational efficiency, and protein turnover, which are well documented contributors to the discordance between transcriptomic and proteomic data.

Among the enriched pathways identified independently from the transcriptomic and proteomic datasets, protein processing in the endoplasmic reticulum was the only pathway common to both analyses. Interestingly, within this pathway, Hspa5 is upregulated at both the mRNA and protein levels in response to morphine exposure in BDEVs, suggesting conserved stress response activation (29, 30). In contrast, other transcripts contributing to this pathway were upregulated at the mRNA level, while their corresponding proteins were downregulated, further supporting the role of translational regulation or differential protein degradation in shaping the exosomal proteome under the influence of morphine (Figure 4 C-D) (31, 32).

Morphine-BDEVs modulate neurodegenerative gene expression and activate opioid signaling in cross-species recipient neurons

To investigate the functional role of BDEVs and their potential to modulate neurodegenerative processes, we treated primary neurons with morphine-BDEVs and control-BDEVs, followed by a comprehensive multiplex gene expression analysis targeting 770 neuropathology-associated genes **(Fig. 5A)**. After controlling for multiple comparisons, we identified significant differential expression of 15 genes in primary neuronal cells following treatment with morphine-derived BDEVs (FDR q value < 0.1). Among these genes, *Mnat1*, a gene implicated in blood brain barrier (BBB) damage in Alzheimer's disease (AD), was downregulated, whereas *PSEN1*, *NSF*, and *NRG1*, which are associated with neurodegenerative processes, particularly in AD, were upregulated **(Fig. 5B)**. This evidence supports previous findings suggesting that extracellular vesicles (EVs) may contribute to the neurodegeneration and dementia observed with long-term opioid use(9).

Pathway analysis revealed a selective enrichment of non-canonical WNT signaling components in neurons exposed to BDEVs derived from morphine-treated rats, despite the absence of direct morphine exposure. The top-ranked pathway was “Ca2+ pathway” (FDR = 2.75□×□10□□; ratio = 0.005), followed by “Beta-catenin independent WNT signaling” (FDR = 5.71□×□10□□; ratio = 0.01), and “Signaling by WNT” (FDR = 1.64□×□10□□; ratio = 0.02). These non-canonical pathways are critical for cytoskeleton remodeling, intracellular calcium flux, and neuronal polarity. Remarkably, neurons also showed enrichment for “DARPP-32 events” and “Opioid signalling” (both FDR = 0.004), indicating activation of dopaminergic integration and GPCR-related neuromodulatory cascades. These results suggest that morphine-induced BDEVs can transfer biologically active signals capable of reprogramming recipient neurons toward a phenotype consistent with drug exposure, engaging both polarity-regulating pathways and classical neuroadaptive circuits **(Fig. 5C)**.

Discussion

This study demonstrates that chronic morphine induces significant transcriptomic and proteomic reprograming in the cargo of BDEVs isolated from the rat prefrontal cortex. wich are capable of dysregulating neuronal function in in vitro. Through transcriptomic, proteomic, and integrative analyses, we identified BDEV-enriched gene and protein signatures involved in synaptic plasticity, neurodegenerative pathways, and ER stress, including upregulation of key modulators such as ARC and Hspa5. Functional assays further revealed that morphine-altered BDEVs actively reshape neuropathology-associated gene expression in recipient neurons, implicating these vesicles as mediators of opioid-induced neuroadaptation and contributors to long-term neuropathological processes.

EVs play critical roles in intercellular communication, and alterations in their cargo have been increasingly linked to opioid responses (3, 9). Previous studies have shown that the EV cargo changes, mainly in microRNAs in blood-derived EVs in adult individuals and animals exposed to opioids (33, 34). Similarly, BDEVs from animals exposed to opioids in utero and postnatally showed an upregulation of miR-504 and a downregulation of miR-30c-1 (10). However, these studies profiled small RNAs, limiting insights into the long transcript diversity of EVs (e.g. mRNAs and lncRNAs) (35). In this study, we employed total RNA sequencing with rRNA removal, allowing the identification of mRNAs in BDEVs, which is consistent with recent advances showing functional mRNAs in EVs (15).

Our RNA sequencing data revealed an increase in the expression of the ARC gene in BDEVs from animals treated with morphine. ARCs play a central role in synaptic plasticity and neuronal adaptation to chronic drug exposure, with previous studies reporting elevated ARC expression in the bulk prefrontal cortex of animals with a history of opioid exposure (36-38). Interestingly, ARC has also been implicated in the biogenesis and release of EVs. It can self-assemble into mRNA-containing, virus-like capsids that are packaged into EVs and released by neurons, where they can be endocytosed by recipient cells at postsynaptic sites (16, 39, 40). These ARC-containing EVs are proposed to modulate synaptic plasticity by delivering ARC mRNA to neighboring neurons, where local translation facilitates AMPA-type glutamate receptor (AMPAR) endocytosis and synaptic remodeling (40). Therefore, the observed upregulation of the ARC gene in BDEVs following morphine treatment may reflect an EV-mediated mechanism contributing to opioid-induced synaptic plasticity, a pivotal process underlying addiction-related neuroadaptation.

This interpretation aligns with our pathway enrichment analysis, which revealed significant enrichment of axon guidance, lysosome, and protein processing in the endoplasmic reticulum (ER) pathways, which were driven primarily by upregulated transcripts. These pathways are relevant to neuronal adaptations triggered by opioids (41). For example, previous studies have demonstrated altered expression of specific axon guidance genes in the adult mouse brain following chronic self-administration of oxycodone (42), which may reflect alterations in axon–target connectivity and synaptogenesis, potentially contributing to disrupted neural circuits and the behavioral changes associated with opioid addiction. Together, these findings suggest that morphine exposure provokes transcriptional changes in BDEVs that may underline core molecular mechanisms associated with opioid-related neuroplasticity.

We applied deconvolutional analysis to estimate the cell type origin of BDEVs by aligning the EV transcriptome to establish expression profiles of specific brain cell types (43). We found that approximately 40% of BDEV RNA originated from GABAergic interneurons, which are known to modulate reward functions affected by opioids (44). These findings, together with recent evidence showing the behavioral effects of GABAergic EVs (45), point to a role for interneuron-derived EVs in opioid-related neuroplasticity.

To increase our understanding of the functional implications of morphine-induced changes in BDEV cargo, we cross-referenced our RNA-seq data with a large-scale

human GWAS meta-analysis for opioid use disorder (OUD) (18). This integrative approach allowed us to identify differentially expressed transcripts originating from genes previously associated with OUD susceptibility, such as Oprm1, Furin, Ncam1 Kcnn1, and Rnf114 (18). We observed that Ncam1 and Kcnn1 transcripts were significantly more abundant in BDEVs from morphine-treated animals than in those from control animals. These genes are functionally relevant to opioid effects, with Ncam1 playing a role in synaptic plasticity, whereas Kcnn1 is involved in regulating neuronal excitability, highlighting a potential role of BDEVs in morphine-induced synaptic remodeling (46-48).

Our proteomic analyses r further demonstrated significant changes in the cargo of BDEVs following chronic morphine treatment by identifying dysregulated proteins implicated in morphine-induced neuroadaptation. Specifically, we observed increased levels of proteins associated with synaptic plasticity and neurodegenerative processes, including PDIA3, STX1B, YWHAG, and HSPA5, n previouslylinked to synaptic dysfunction and pathways relevant to neurodegenerative diseases (49-52). These findings align with previous evidence suggesting that chronic opioid exposure may increase the risk of neurodegeneration through mechanisms involving EVs (53). For example, increased levels of protein biomarkers of neurodegenerative processes have been detected in EVs isolated from the plasma of monkeys exposed to oxycodone (34). Similarly, morphine-treated astrocytes have been shown to secrete EVs enriched with amyloid-related proteins, contributing to neurodegenerative processes in vitro (54).

Consistent with these findings, our pathway enrichment analysis revealed significant associations between the dysregulated proteins and pathways implicated in neurodegenerative diseases. Notably, inspection of the dysregulation patterns revealed an enrichment of mitochondrial proteins, including COX1, COX7A2L, and members of the NDUF family, such as NDUFV1 and NDUFC2. These proteins play critical roles in mitochondrial function and are important for ATP production and cellular energy metabolism (55-57). Chronic opioid use has been linked to mitochondrial dysfunction, potentially driven by increased production of reactive oxygen species, which can damage mitochondrial DNA and disrupt cellular energy metabolism, processes closely associated with neurodegenerative pathologies (25, 58). Therefore, the observed alterations in mitochondrial proteins in BDEVs may contribute to increased neuronal vulnerability and the progression of opioid-induced neurodegenerative processes. Similarly, abnormal levels of mitochondrial proteins in EVs

from plasma have also been reported in neurodegenerative conditions, including Parkinson's disease and Alzheimer's disease (59, 60)

Moreover, our proteomic analysis also demonstrated the upregulation of the GRM5-encoded metabotropic glutamate receptor in BDEVs from morphine-exposed animals. This observation suggests potential alterations in glutamatergic signaling pathways, possibly affecting synaptic plasticity mechanisms relevant to neurodegenerative processes. Aberrant neuronal signaling mediated by mGluR5, which leads to synaptic dysfunction, has been recognized as an emerging mechanism in the biology of both drug addiction and neurodegeneration(61, 62). In support of this concept, a recent study reported elevated mGluR5 expression in the hippocampus of individuals with Alzheimer's disease, with expression levels positively correlated with disease progression in both human patients and mouse models (63). Although the presence of mGluR5 has not previously been described specifically in extracellular vesicles isolated from brain tissue after morphine treatment, its occurrence in EVs has been reported in animal models of Alzheimer's disease (64). Our findings align with these observations and further suggest that mGluR5 in BDEVs could represent a novel mechanism contributing to opioid-induced neurodegenerative changes.

We also performed an integrative analysis, which revealed substantial overlap (>85%) between proteins identified via proteomics and their corresponding transcripts detected via RNA sequencing, supporting the overall consistency and quality of our datasets. However, when statistically dysregulated molecules are specifically evaluated, this concordance notably diminishes. Five genes, *Hspa5*, *S100a6*, *Plp1*, *Slc24a2*, and *Uggt1*, exhibited common dysregulation at both the mRNA and protein levels. This limited overlap highlights the inherent complexity involved in selectively packaging cargo into EVs. Cargo loading into EVs is governed by an interplay of mechanisms, including several posttranslational modifications, such as ubiquitination, and interactions with RNA-binding proteins (65). Although these mechanisms have not been fully elucidated in BDEVs, it has been suggested that chronic opioid exposure may alter some of these selective cargo-loading processes, potentially influencing the BDEV cargo of both proteins and mRNAs (66, 67). Moreover, beyond selective cargo packaging, discrepancies between transcriptomic and proteomic profiles may be further influenced by factors such as mRNA stability, translational efficiency, and protein turnover, as previously described, including in the neural context (68-70).

Among the biological pathways independently enriched in both datasets, protein processing in the endoplasmic reticulum (ER) emerged as the common pathway, suggesting a convergent stress response signature in BDEVs following morphine exposure. Notably, Hspa5 was upregulated at both the transcript and protein levels, highlighting its potential as a biomarker for ER stress induced by chronic opioid treatment. Previous studies have demonstrated that the activation of opioid receptors triggers cellular events leading to ER stress, which significantly contributes to common opioid-related phenomena such as tolerance (71). Indeed, substantial upregulation of Hspa5, a well-established ER stress marker, has been reported in the spinal cord and several mesolimbic brain regions, including the prefrontal cortex, following opioid exposure (71-74). Taken together, these findings indicate that elevated Hspa5 expression in BDEVs is associated with morphine-induced cellular responses, suggesting that chronic opioid exposure modulates EV cargo composition through ER stress pathways. Thus, BDEVs represent promising platforms for investigating and potentially monitoring the neurobiological changes associated with opioid use.

Finally, to explore whether the molecular reprogramming observed in the BDEVs from morphine-treated rats would have functional consequences for recipient neurons, we evaluated the transcriptional response of primary neuronal cells to BDEVs isolated from morphine- and vehicle-exposed animals. A targeted multiplex gene expression analysis of 770 neuropathology-associated genes revealed significant differential expression of 15 genes following morphine-BDEV treatment. Among these genes, Mnat1, a gene involved in blood brain barrier integrity and linked to Alzheimer's disease, was downregulated (75). Conversely, Psen1, Nsf, and Negr1, all of which are associated with neurodegenerative processes, were upregulated (76-78). These findings indicate that morphine-associated BDEVs are sufficient to induce transcriptional programs in naïve neurons that mimic canonical features of opioid exposure. Notably, the selective enrichment of pathways such as DARPP-32 events and Opioid Signalling emerged in the absence of any direct pharmacological stimulus, suggesting that BDEVs carry bioactive cargo capable of reprogramming neuronal signaling cascades. In addition, the co-enrichment of non-canonical WNT and calcium-related pathways points to intracellular remodeling processes linked to neuronal polarization, cytoskeletal plasticity, and synaptic modulation, processes associated with neuroadaptive responses typically observed during the initial stages of drug exposure or sensitization (79-81).

Crucially, transcriptomic profiling of the BDEV cargo revealed robust overexpression of ARC mRNA, a key regulator of synaptic scaling and long-term depression (LTD) (82, 83) . ARC mRNA is known to be selectively packaged into neuronal EVs and has been shown to transfer plasticity-related signals between cells(84). Its presence in morphine-altered BDEVs likely contributes to the observed activation of non-canonical WNT pathways and calcium flux components, both of which underlie actin cytoskeleton rearrangement, spine destabilization, and polarity shifts. Because the recipient cultures were never exposed to morphine, these results suggest that morphine BDEVs act as vectors of neuroplasticity, reprogramming target neurons toward a transcriptional and structural state resembling that of drug-exposed circuits. This supports a model in which BDEVs mediate a trans-synaptic form of memory encoding and adaptation, potentially contributing to the systems-level persistence of drug-induced neuroadaptations.

In summary, our findings demonstrate that chronic morphine exposure significantly alters the molecular cargo of BDEVs, especially those associated with pathways involved in synaptic function, cellular stress responses, and neurodegeneration. Functional assays confirmed that these vesicles can modulate gene expression in recipient neurons, including genes implicated in neurodegeneration. Together, these results highlight BDEVs as crucial mediators of the persistent neural adaptations induced by chronic opioid use and suggest their potential as biomarkers or therapeutic targets in opioid-related neuropathologies.

In summary, our findings are the first demonstrating that BDEVs derived from the brain of rats chronically exposed to morphine present significant changes in their molecular content reflecting pathways associated with synaptic (dys)function, cellular stress responses, and neurodegeneration. Furthermore, our functional assays originally demonstrate that these vesicles trigger transcriptional reprogramming in recipient neurons, including the regulation of genes implicated in neurodegeneration. Together, these results highlight BDEVs content as crucial mediators of the persistent neural adaptations induced by chronic opioid use and suggest their potential as biomarkers and/or therapeutic targets in opioid-related neuropathologies.

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Disclosures

All authors report no biomedical financial interests or potential conflicts of interest.

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Figure 1. Characterization of BDEVs from rat prefrontal cortex.

(A) Experimental design. (B) Nanoparticle tracking analysis (NTA) showing no significant changes in concentration or size distribution of isolated BDEVs between groups.(C) Representative electron micrographs of EVs. (D) Western blot analysis confirming the presence of EV markers Flotillin and HSP70, and absence of the exclusion marker GM130 in the EV samples.

Figure 2. Effect of morphine treatment on the RNA profile of BDEVs

(A) Volcano plot of differentially expressed mRNAs in BDEVs from morphine-treated versus control animals. (B) KEGG pathway enrichment analysis of morphine-induced RNA alterations. (C) Dot plot showing upregulated and downregulated transcripts within enriched pathways. (D) Heatmap of significantly dysregulated mRNAs across enriched pathways (log□ fold-change). (E) Network visualization of enriched pathways and associated transcripts. (F) Integration with a human GWAS for opioid use disorder highlights risk-associated genes differentially expressed in BDEVs. (G) RNA deconvolution estimates brain cell-type composition, with nearly 40% of transcripts inferred from GABAergic interneuron-derived BDEVs.

Figure 3. Effect of morphine treatment on the protein profile of BDEVs

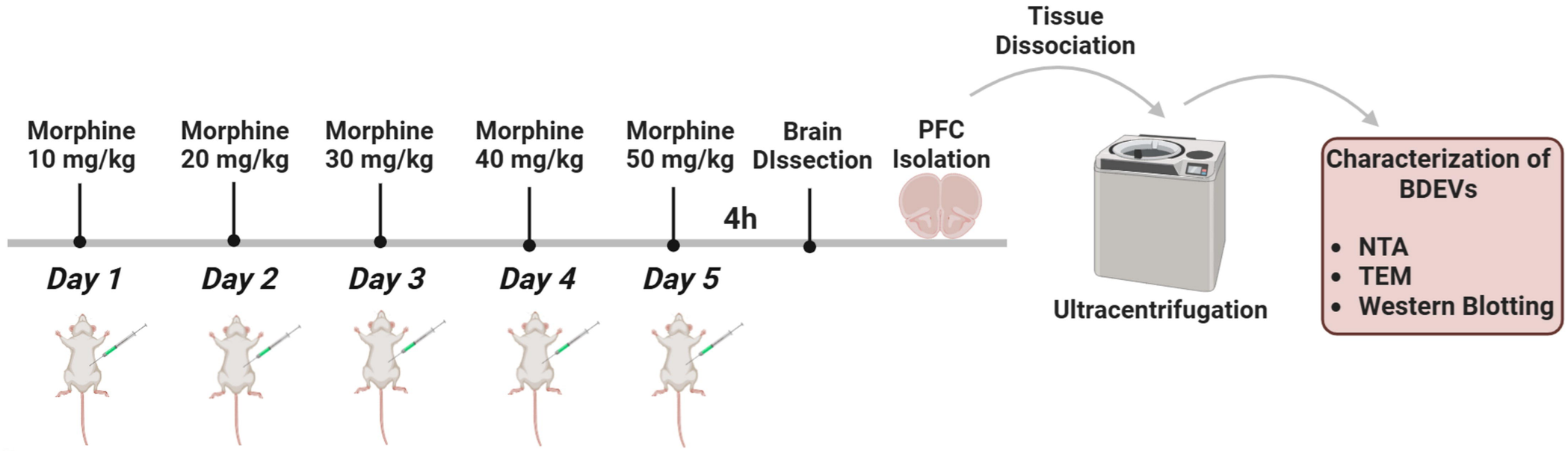
(A) Volcano plot showing differentially expressed proteins in BDEVs from morphine-treated versus control animals. (B) Dynamic range of protein abundance (left) and MA plot (right), highlighting significantly dysregulated proteins. (C) KEGG pathway enrichment analysis identifying pathways associated with morphine-induced protein alterations. (D) Network visualization of enriched pathways and associated proteins. Gray nodes represent KEGG pathways; colored nodes represent proteins, with node color indicating log□ fold-change (red = upregulated, blue = downregulated). (E) Heatmap of log□ fold-change values for dysregulated proteins across enriched KEGG pathways.

Figure 4. Integrated transcriptomic and proteomic analysis of BDEVs from morphine-treated rats.

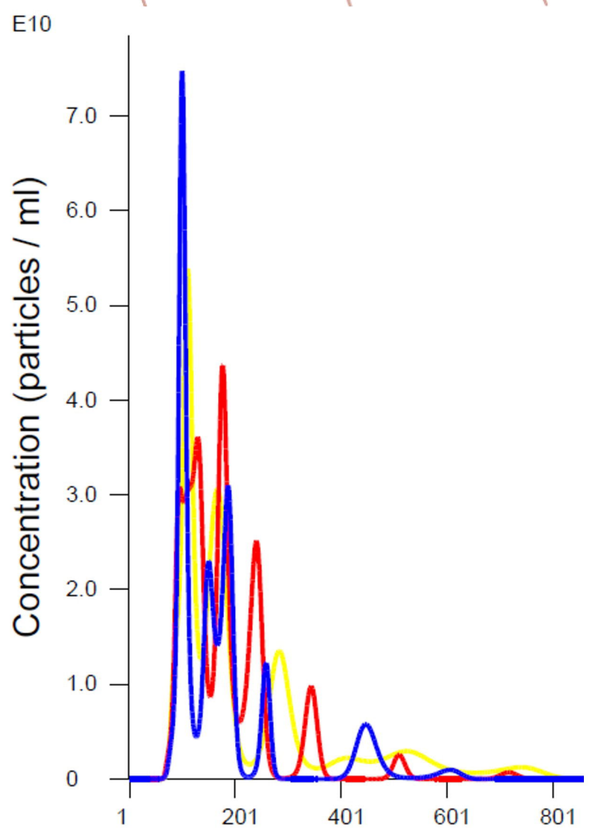
(A) Circos plot linking significantly dysregulated mRNAs (left) and proteins (right). Node colors represent log□ fold-change (red/orange = upregulated, blue = downregulated). (B) UpSet plot showing the overlap between significantly altered RNAs and proteins; bar height represents the number of molecules in each set. (C) Comparative KEGG pathway enrichment analysis of dysregulated mRNAs and proteins. Bubble size reflects the number of molecules per pathway, and color indicates –log□ □(p). (D) Chord diagram of the protein processing in endoplasmic reticulum pathway, depicting concordance and divergence between RNA and protein levels. Ribbons connect matched RNA–protein pairs, with ribbon color indicating log□ fold-change.

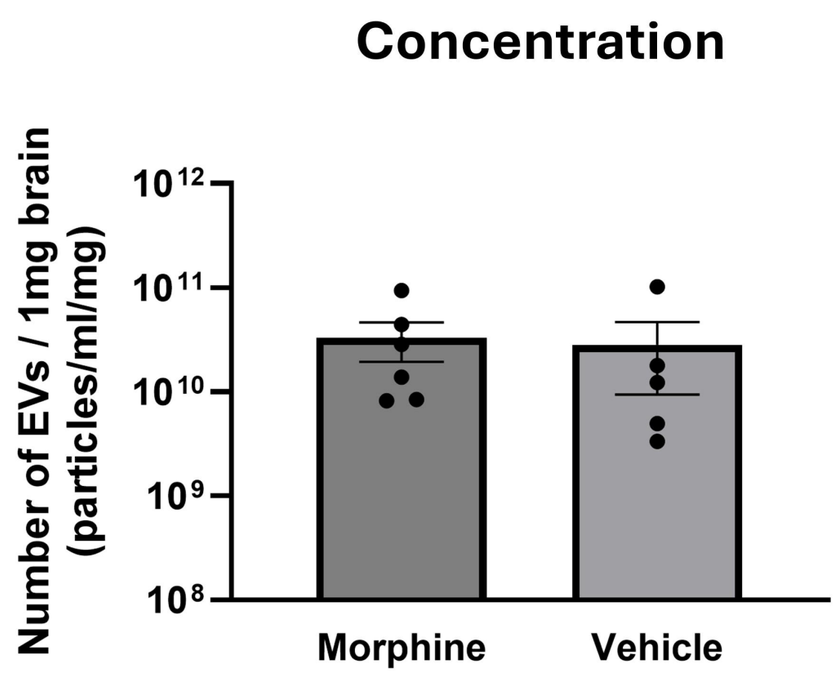
Figure 5. Effect of morphine-derived BDEVs on primary neuronal cultures. (A) Experimental design: BDEVs from morphine- and vehicle-treated rats were applied for 24 h to primary cortical neurons, followed by NanoString analysis. (B) Volcano plot showing differentially expressed transcripts in neurons exposed to morphine-derived BDEVs; selected significant genes are labeled. (C) Reactome pathway enrichment of dysregulated genes, highlighting Ca^{2}□, WNT, opioid, and DARP 32-related signaling pathways.

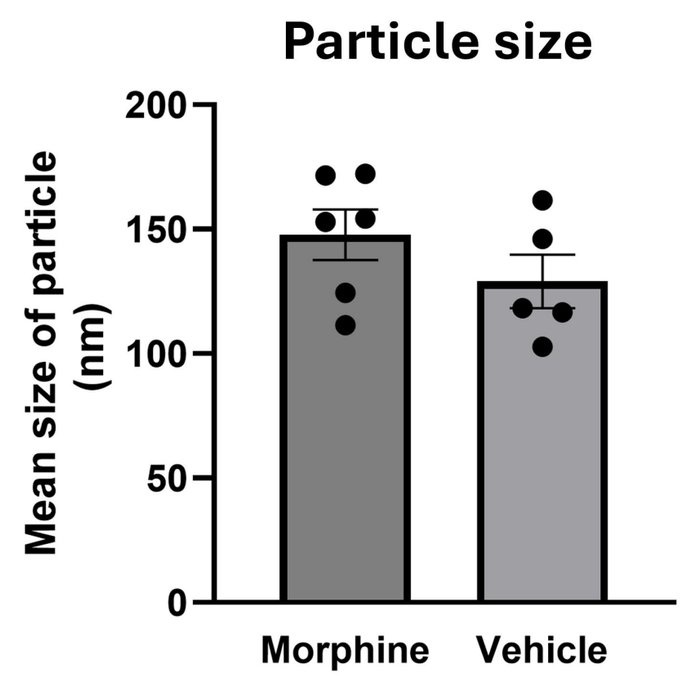
A


Morphine 10 mg/kg
Morphine 20 mg/kg
Morphine 30 mg/kg
Morphine 40 mg/kg
Morphine 50 mg/kg
Brain DIssection
PFC Isolation
4h
Day 1
Day 2
Day 3
Day 4
Day 5
Tissue Dissociation
Ultracentrifugation
Characterization of BDEVs
NTA
TEM
Western Blotting

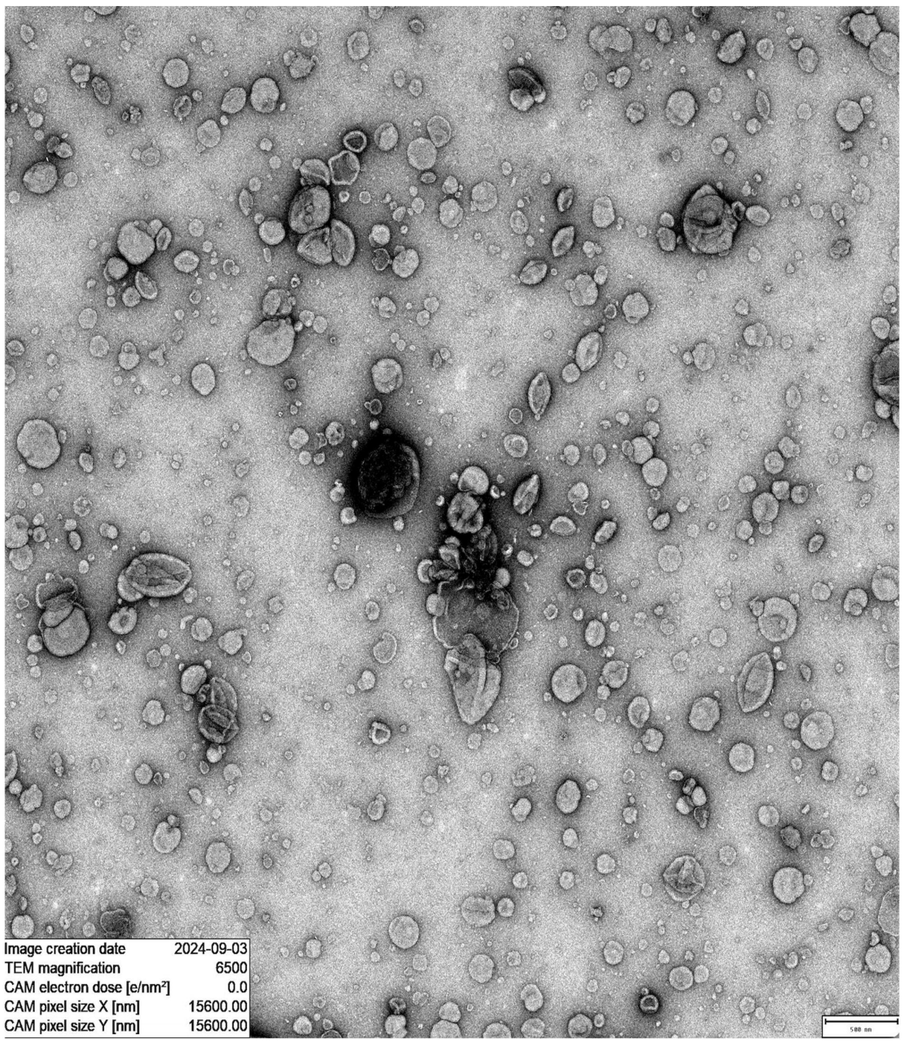
B


E10
Concentration (particles / ml)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
201
401
601
801


Concentration
Number of EVs / 1mg brain (particles/ml/mg)
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
Morphine
Vehicle


Particle size
Mean size of particle (nm)
200
150
100
50
0
Morphine
Vehicle

C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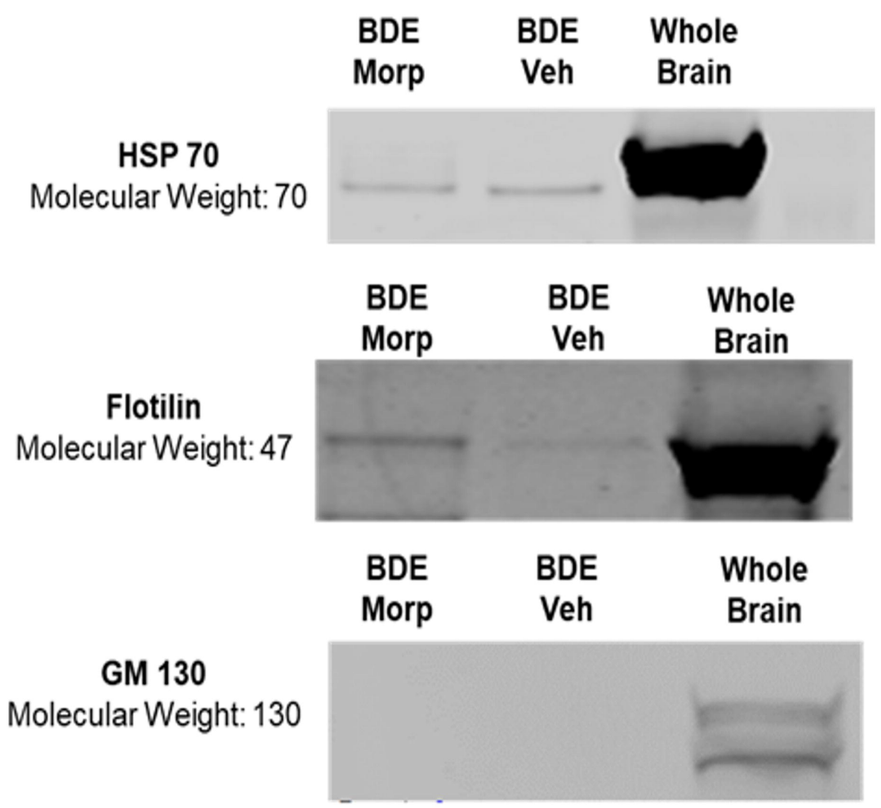
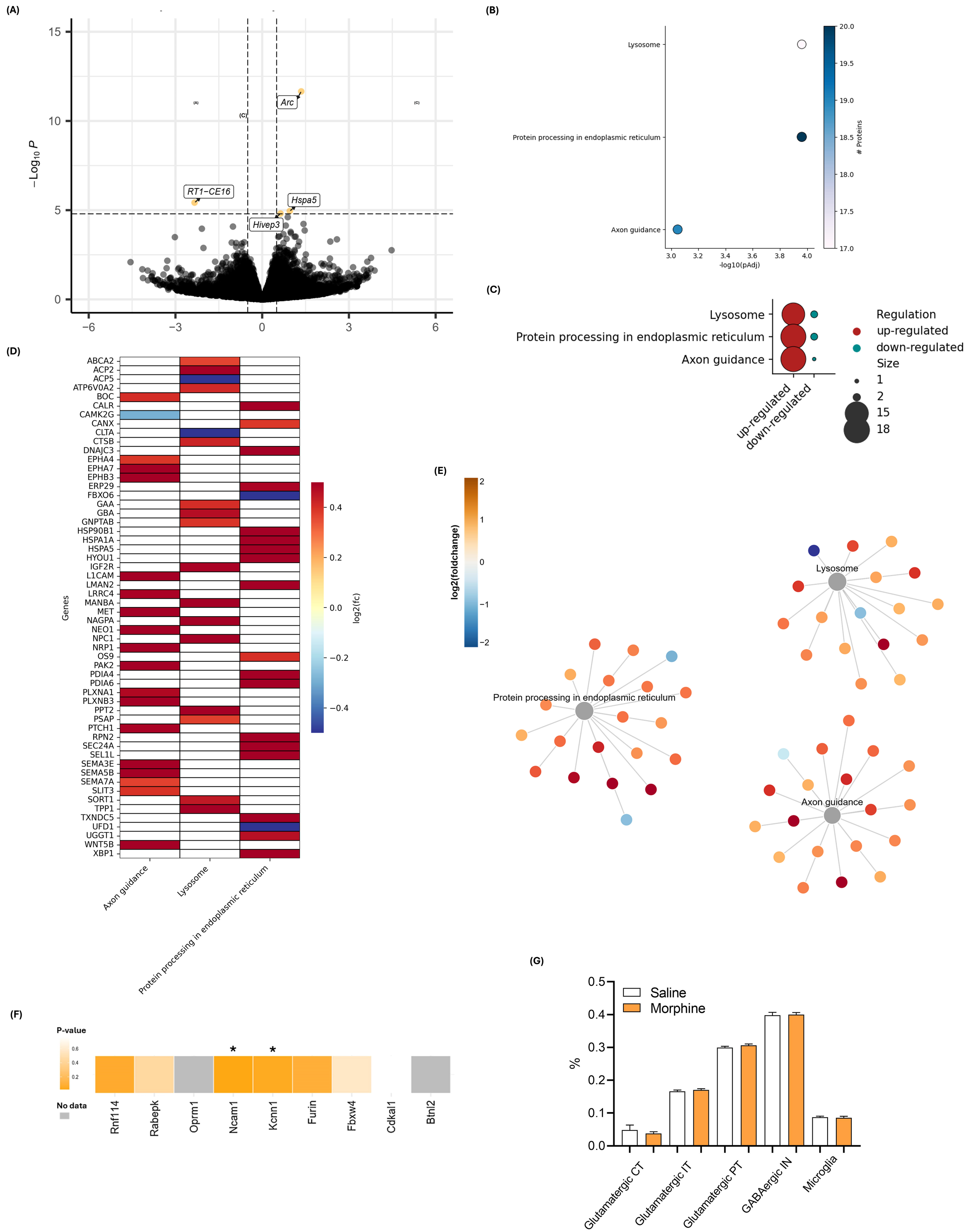
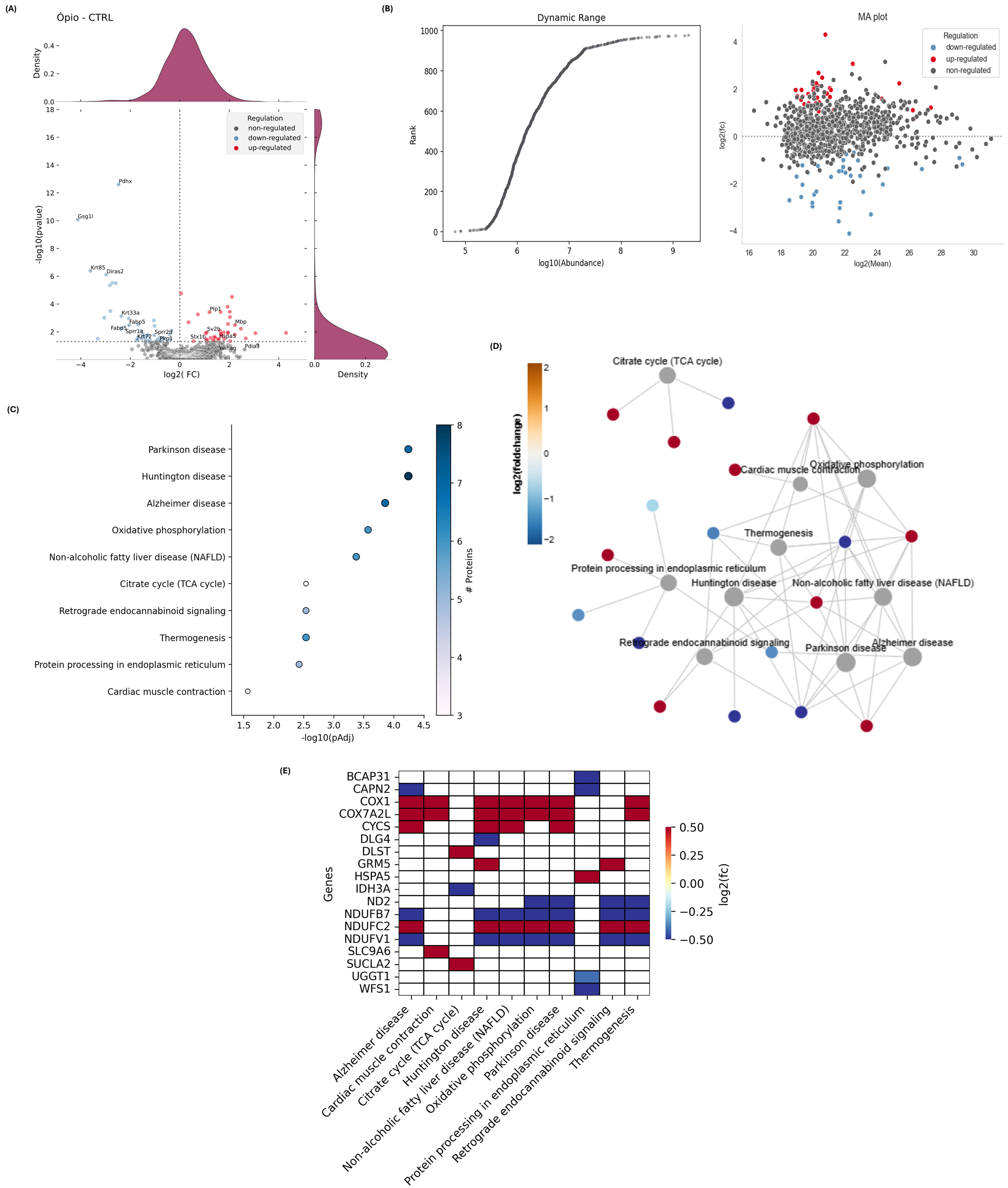
CAM pixel size Y [nm] 15600.00

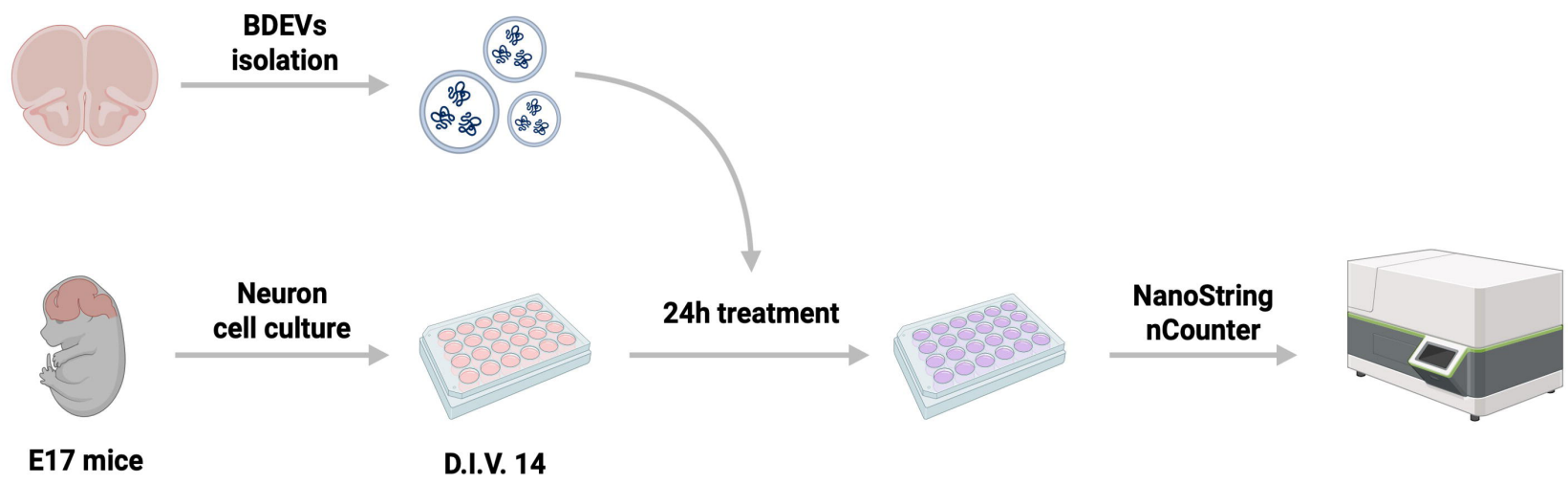
D


BDE Morp
BDE Veh
Whole Brain
HSP 70
Molecular Weight: 70
BDE Morp
BDE Veh
Whole Brain
Flotilin
Molecular Weight: 47
BDE Morp
BDE Veh
Whole Brain
GM 130
Molecular Weight: 130

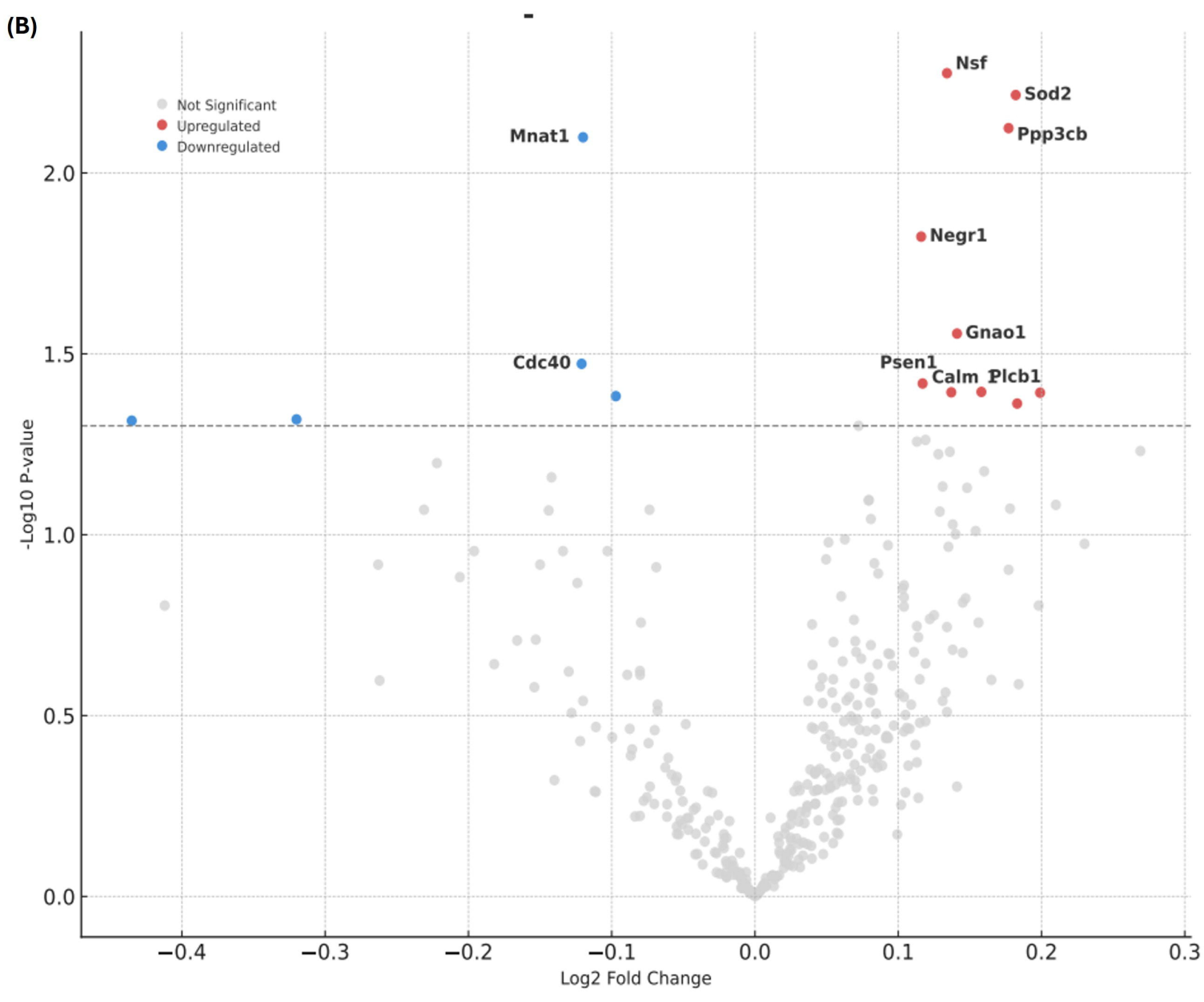

(A)
−Log10 P
Arc
RT1−CE16
Hspa5
Hivep3
(B)
Lysosome
Protein processing in endoplasmic reticulum
Axon guidance
-log10(pAdj)
Proteins
(C)
Lysosome
Protein processing in endoplasmic reticulum
Axon guidance
up-regulated
down-regulated
Regulation
up-regulated
down-regulated
Size
(D)
Genes
log2(fc)
Axon guidance
Lysosome
Protein processing in endoplasmic reticulum
(E)
log2(foldchange)
Lysosome
Protein processing in endoplasmic reticulum
Axon guidance
(F)
P-value
No data
Rnf114
Rabepk
Oprm1
Ncam1
Kcnn1
Furin
Fbxw4
Cdkal1
Btnl2
(G)
Saline
Morphine
%
Glutamatergic CT
Glutamatergic IT
Glutamatergic PT
GABAergic IN
Microglia


(A)
Ópio - CTRL
Density
Regulation
non-regulated
down-regulated
up-regulated
Pdhx
Gsg1l
Krt85
Diras2
Krt33a
Fabp5
Sprr1a
Sprr2d
Krt72
Pkp1
Plp1
Mbp
Sv2b
Stx1b
Hspa5
Ywhag
Pdia3
-log10(pvalue)
log2(FC)
(B)
Dynamic Range
Rank
log10(Abundance)
MA plot
Regulation
down-regulated
up-regulated
non-regulated
log2(fc)
log2(Mean)
(C)
Parkinson disease
Huntington disease
Alzheimer disease
Oxidative phosphorylation
Non-alcoholic fatty liver disease (NAFLD)
Citrate cycle (TCA cycle)
Retrograde endocannabinoid signaling
Thermogenesis
Protein processing in endoplasmic reticulum
Cardiac muscle contraction
-log10(pAdj)
Proteins
(D)
Citrate cycle (TCA cycle)
log2(foldchange)
Cardiac muscle contraction
Oxidative phosphorylation
Thermogenesis
Protein processing in endoplasmic reticulum
Huntington disease
Non-alcoholic fatty liver disease (NAFLD)
Retrograde endocannabinoid signaling
Parkinson disease
Alzheimer disease
(E)
Genes
BCAP31
CAPN2
COX1
COX7A2L
CYCS
DLG4
DLST
GRM5
HSPA5
IDH3A
ND2
NDUFB7
NDUFC2
NDUFV1
SLC9A6
SUCLA2
UGGT1
WFS1
Alzheimer disease
Cardiac muscle contraction
Citrate cycle (TCA cycle)
Huntington disease
Non-alcoholic fatty liver disease (NAFLD)
Oxidative phosphorylation
Parkinson disease
Protein processing in endoplasmic reticulum
Retrograde endocannabinoid signaling
Thermogenesis
log2(fc)

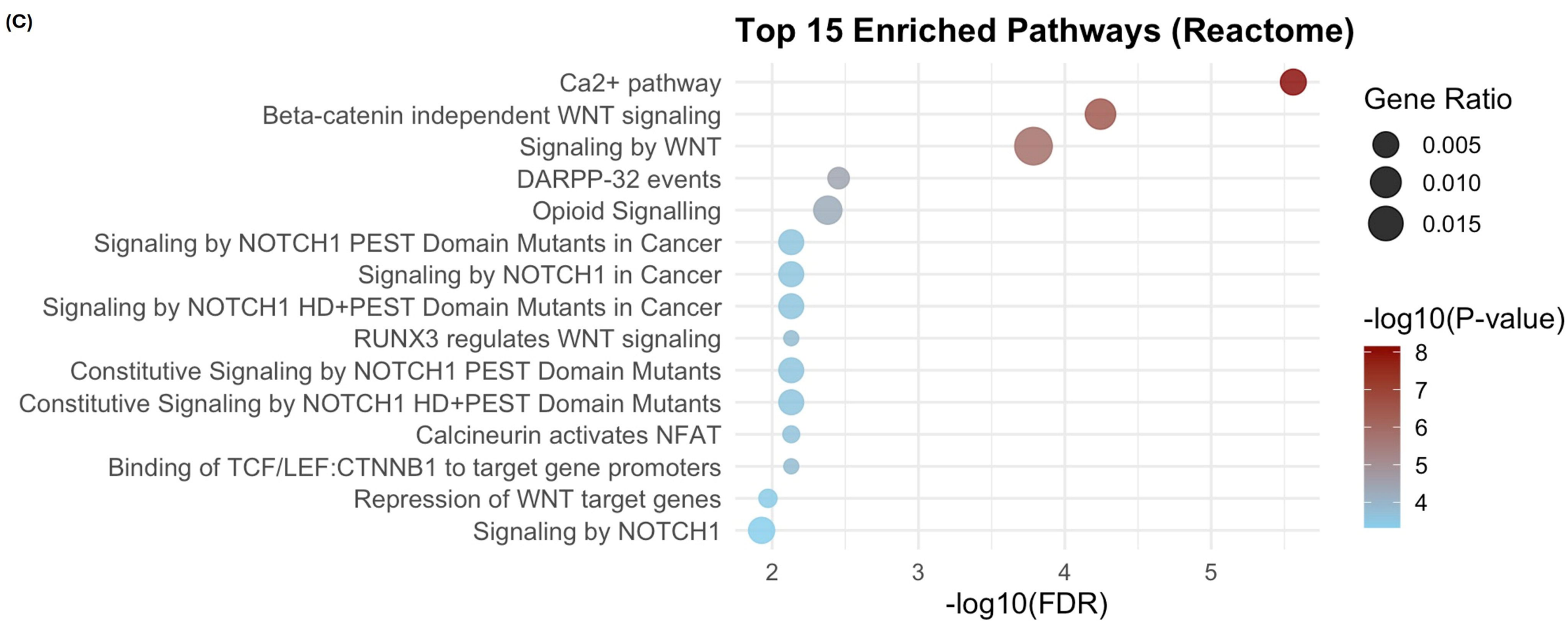
(A)

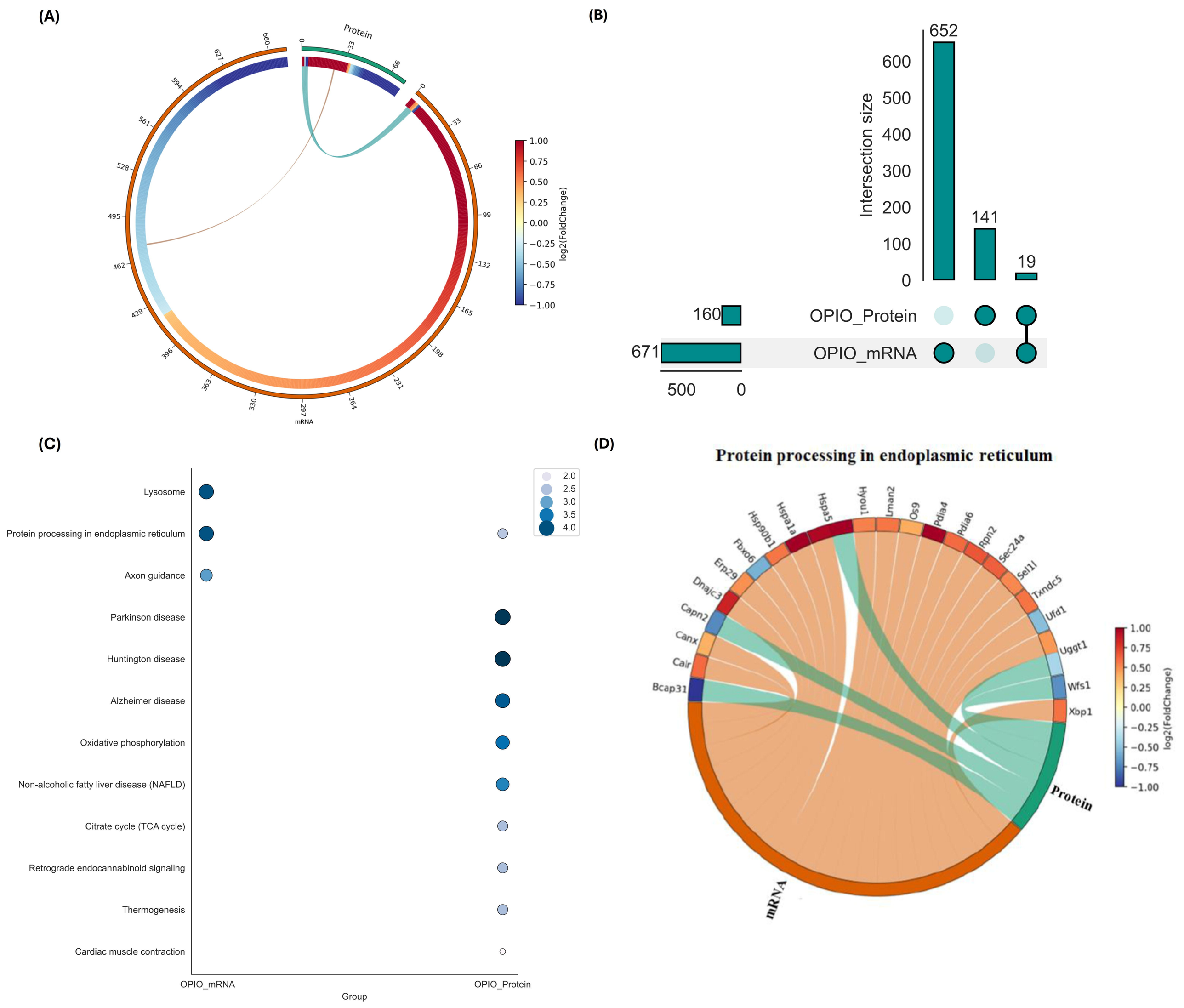

BDEVs
isolation
Neuron
cell culture
24h treatment
NanoString
nCounter
E17 mice
D.I.V. 14

(B)


Not Significant
Upregulated
Downregulated
Nsf
Sod2
Ppp3cb
Mnat1
Negr1
Gnao1
Cdc40
Psen1
Calm
Plcb1
-Log10 P-value
Log2 Fold Change
2.0
1.5
1.0
0.5
0.0
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3

(C)


Top 15 Enriched Pathways (Reactome)
Ca2+ pathway
Beta-catenin independent WNT signaling
Signaling by WNT
DARPP-32 events
Opioid Signalling
Signaling by NOTCH1 PEST Domain Mutants in Cancer
Signaling by NOTCH1 in Cancer
Signaling by NOTCH1 HD+PEST Domain Mutants in Cancer
RUNX3 regulates WNT signaling
Constitutive Signaling by NOTCH1 PEST Domain Mutants
Constitutive Signaling by NOTCH1 HD+PEST Domain Mutants
Calcineurin activates NFAT
Binding of TCF/LEF:CTNNB1 to target gene promoters
Repression of WNT target genes
Signaling by NOTCH1
2
3
4
5
-log10(FDR)
Gene Ratio
0.005
0.010
0.015
-log10(P-value)
8
7
6
5
4


(A)
Protein
mRNA
log2(FoldChange)
(B)
Intersection size
652
141
19
160
671
OPIO_Protein
OPIO_mRNA
500
0
(C)
Lysosome
Protein processing in endoplasmic reticulum
Axon guidance
Parkinson disease
Huntington disease
Alzheimer disease
Oxidative phosphorylation
Non-alcoholic fatty liver disease (NAFLD)
Citrate cycle (TCA cycle)
Retrograde endocannabinoid signaling
Thermogenesis
Cardiac muscle contraction
OPIO_mRNA
OPIO_Protein
Group
(D)
Protein processing in endoplasmic reticulum
Hspa1a
Hspa5
Hyou1
Lman2
Os9
Pdia4
Pdia6
Rpn2
Sec24a
Sel1l
Txndc5
Ufd1
Uggt1
Wfs1
Xbp1
Bcap31
Calr
Canx
Capn2
Dnajc3
Erp29
Fbxo6
Hsp90b1
Protein
mRNA
log2(FoldChange)