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Novel Biomarkers to Assess In-Utero Effects of Maternal Opioid Use: First Steps towards Understanding Short and Long Term Neurodevelopmental Sequelae

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Abstract

Maternal opioid use disorder is common, resulting in significant neonatal morbidity and cost. Currently, it is not possible to predict which opioid-exposed newborns will require pharmacotherapy for neonatal abstinence syndrome. Further, little is known regarding the effects of maternal opioid use disorder on the developing human brain. We hypothesized that novel methodologies utilizing fetal central nervous system-derived extracellular vesicles isolated from maternal blood can address these gaps in knowledge. Plasma from opioid users and controls between 9 and 21 weeks was precipitated and extracellular vesicles were isolated. Mu opioid and cannabinoid receptor levels were quantified. Label free proteomics studies and unbiased small RNA next generation sequencing was performed in paired fetal brain tissue. Maternal opioid use disorder increased mu opioid receptor protein levels in extracellular vesicles independent of opioid equivalent dose. Moreover, cannabinoid receptor levels in extracellular vesicles were upregulated

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Laura Goetzl has submitted a full patent for techniques to isolate FCEs. No other author has a conflict of interest.

This study was conducted following approval by the Temple University human subjects IRB.

with opioid exposure indicating cross talk with endocannabinoids. Maternal opioid use disorder was associated with significant changes in extracellular vesicle protein cargo and fetal brain micro RNA expression, especially in male fetuses. Many of the altered cargo molecules and micro RNAs identified are associated with adverse clinical neurodevelopmental outcomes. Our data suggest that assays relying on extracellular vesicles isolated from maternal blood extracellular vesicles may provide information regarding fetal response to opioids in the setting of maternal opioid use disorder. Prospective clinical studies are needed to evaluate the association between extracellular vesicle biomarkers, risk of neonatal abstinence syndrome, and neurodevelopmental outcomes.

Keywords

extracellular vesicles; maternal opioid use disorder; fetal; micro RNA; cannabinoid

Introduction

A widespread opioid epidemic has reached record levels in the United States (U.S.) over the last decade (1). Since 2000, the number of neonatal abstinence syndrome (NAS) cases has increased 5-fold; an infant with NAS is born every 25 minutes in the U.S. (2–4). Extended hospitalizations associated with NAS contribute to the financial burden of addiction on our society. Among the devastating health consequences of NAS are poor postnatal growth, seizures, and deleterious effects on neurodevelopment (5; 6). Identifying the best treatment options for mothers and newborns exposed to opioids remains highly debated (7). *In utero* opioid exposure has been associated with reduced brain volumes (8) and abnormal childhood neurodevelopment/cognitive function (9–12).

Currently, it is not possible to predict which opioid-exposed newborns will require pharmacotherapy for NAS. Between 47-81% of newborns with gestational methadone or buprenorphine exposure will require pharmacotherapy. Opioid dose is a poor predictor (13); some factors that have been associated with NAS severity include: co-occurring exposure to benzodiazepines (14; 15), anti-depressants (16; 17), marijuana use or heavy smoking (18; 19), as well as genetic factors (20; 21). None of these factors are sufficient to reliably predict NAS severity, and the mechanisms by which they impact NAS are not well understood. Further, decisions to initiate pharmacotherapy rely partly on subjective observer-rated scoring. Biomarkers indicative of individual fetal brain responses to opioid exposure would be invaluable to augment observer-rated assessments that guide pharmacotherapy decision-making and contribute to personalized risk assessments for mothers antenatally.

Efforts to delineate the mechanisms underlying human neural development *in utero* are fraught with challenges, especially with regard to opioid exposure. Experimental studies have significant ethical limitations. Direct fetal brain tissue examination is essentially impossible in ongoing pregnancies. Non-invasive examination of the fetal brain has been limited to expensive and technically challenging *in utero* functional and anatomic imaging. Consequently, much of what we know has been derived from *in vitro* human cell line studies and *in vivo* animal studies. However it is not clear which preclinical findings have translational value. Incompletely answered questions include: a) what are the effects of

opioid exposure on human brain development? b) does maternal exposure to opioids prior to conception and/or *in utero* exposure to opioids increase future risk of opioid addiction, and if so, what is the mechanism? and c) how does fetal sex influence neuro-programming and subsequent phenotype?

Unravelling the mechanisms underlying opioid-related neurodevelopmental disruptions in humans will require non-invasive methods. We have developed an innovative platform to isolate Fetal Central Nervous System-Derived Extracellular Vesicles (**FCEs**) from maternal blood (22). Our published findings demonstrate that purified FCEs can be used to detect fetal CNS damage caused by *in utero* alcohol exposure, hypoxia, and viral infection (22; 23). Thus FCEs could be a new tool to interrogate *in vivo* neurodevelopment involving elements of opioid signaling pathways, including proteins (mu-opioid receptor (**μOR**), cannabinoid receptors) and microRNAs (miRNAs). miRNAs are a class of small (17-24 nucleotides) non-coding RNAs that target the untranslated region of messenger RNAs to silence genes post-transcriptionally. There are over 1000 miRNAs in the human genome, which target over 60% of expressed genes (24; 25). MiRNAs are highly regulated by exposure to drugs of abuse (26–31) and emerging evidence also suggests that they play an important role in the etiology of addiction (27–29; 31–33). Specifically, opioid consumption drastically alters the miRNA landscape in the brain (28–31; 34–38). Additionally many miRNAs regulate the expression of molecules involved in opioid signaling (30; 34; 36; 38), which may have important implications for fetuses chronically exposed to opioids over the course of pregnancy. The aforementioned studies have focused on adult tissues, often using pre-clinical approaches. This study evaluates the potential of FCEs to address the gaps in knowledge regarding the fetal effects of Maternal opioid use disorder (MOUD) as well as the potential clinical utility of FCEs as a predictor of NAS severity.

Materials and Methods

Patient groups and sampling

A case control study was performed. Subjects were enrolled between 9 and 21 weeks gestation under a protocol approved by our Institutional Review Board. Maternal opioid exposure was determined via a face-to-face questionnaire that included questions regarding all types of drugs/medications used, dose and frequency. Cases (opioid users) were matched to controls (non-users) depending on biobank availability by gestational age (± 2 weeks) and smoking status. Where possible controls were also matched by other medication exposures, most commonly benzodiazepines and selective serotonin reuptake inhibitors. Women with recent or current heroin use were excluded. Clinical characteristics of participants are shown (Table 1). Following written informed consent and immediately prior to elective pregnancy termination, 20 ml of venous blood were drawn into 1 ml of saline with EDTA or heparin, incubated for 10 min at room temperature and centrifuged for 15 min at 2,500 xg. Plasma was stored in 0.5 ml aliquots at -80°C . Surgical tissue samples were collected fresh and snap frozen.

Preparation of FCEs and Synaptosomes

Two hundred-fifty μ L of plasma as used to isolate FCEs as previously described(22). Briefly, total ECVs were prepared from maternal plasma (EXOQ; System Biosciences, Inc., Mountainview, CA). To isolate ECVs from fetal neural sources, total exosome suspensions were incubated with mouse monoclonal IgG1 anti-human Contactin-2/TAG1 antibody (clone 372913, R&D Systems, Inc., Minneapolis, MN), that had been biotinylated (EZ-Link sulfo-NHS-biotin System, Thermo Scientific, Inc.) and antibody bound ECVs were precipitated with Streptavidin-Plus UltraLink resin (Pierce-Thermo Scientific, Inc.). Contactin-2/TAG1 is a glycosylphosphatidylinositol-anchored neuronal membrane adhesion protein of the immunoglobulin superfamily, that is transiently expressed in early human developmental stages to guide initial axonal connections and, in association with other proteins, promote molecular organization of myelinated nerves (39; 40). In our prior work, we found that approximately 3% of ECVs purified from non-pregnant controls cross reacted with Contactin-2/TAG1(22). This may indicate a low level of ongoing neuronal remodeling that extends into adult life or alternatively persistence of fetal neural ECVs from past pregnancies.

Fetal brain tissue in matched samples was used to prepare synaptosomes using standard commercial kits (ThermoFisher Scientific, Syn-PERTM Synaptic Protein Extraction Reagent (87793), Waltham, MA). Specific anatomic areas of the brain could not be definitively identified but the majority of tissue appeared grossly to be cortex.

ELISA quantification of exosomal and synaptosomal proteins.

Mu-opioid receptor (μ OR, American Research Products-Cusabio, Waltham, MA) and cannabinoid receptor (CB1, Elabscience, Houston, TX) protein levels were quantified using commercial ELISA kits and normalized to the tetraspanning exosome marker CD81 (American Research Products-Cusabio, Waltham, MA). The mean value for all determinations of CD81 in each assay group was set at 1.00 and the relative values for each sample used to normalize their recovery. FCE protein levels between exposure groups were compared using ANOVA testing (IBM SPSS Statistics for Windows, Version 25.0.; 2017; Armonk, NY). The level of significance was set at 0.05.

Proteomic Analysis

For label free proteomics studies, extracted proteins were digested with trypsin (41; 42). Peptides were then acidified, loaded onto an activated in-house-made cation stage tip, purified and eluted into six fractions (43). Mass spec analysis was performed on these fractions using QE mass spectrometer as previously described (41).

Small RNA Next Generation Sequencing and qPCR

Small RNAs were extracted from fetal brain tissue using the Qiagen miRNeasy Kit according to the manufacturer's instructions. RNA concentration, size and purity was assessed by Bioanalyzer electrophoresis (Agilent Technologies) and fluorometric quantitation (Invitrogen). Generation of small RNA libraries was carried out using the TruSeq Small RNA Library Preparation Kits (Illumina) with 10-50 ng of RNA as template.

Libraries were size-selected using electrophoresis to enrich for mature miRNAs. Constructed libraries were sequenced on an Illumina NextSeq 500 using NextSeq 500 High Output Cartridge 75 cycles (Illumina), producing 10-20 million reads per sample. The sequencing results were trimmed for their adapters, quality filtered, and then aligned to an up-to-date human miRNA database (mirbase.org) using the miRDeep2 pipeline to identify and quantify miRNA content of brain tissue. Hierarchical clustering and principal component analyses was conducted to rule out potential biological outliers and to assess intra- and inter-group variability. Given the scarcity of available tissue and the inherent costs of an exploratory RNAseq analysis, we chose a sample size of 5 for each group. Power analyses are complex to conduct for RNA sequencing experiments, as average number of reads and dispersion can vary from gene to gene, but estimations using different sets of permissive average parameters for number of reads per gene, dispersion, and effect size provided us with estimated ideal sample size of 4-9 individuals. After a dispersion-based normalization of miRNA counts in each sample, miRNA expression in all groups was then compared using the DESeq package to isolate candidate miRNAs with significantly altered expression. Two statistical approaches were used to that end: first, differences in sex response to buprenorphine exposure were assessed by fitting two generalized linear models (GLMs) to the miRNA count data. A full model regressed miRNA expressions on both sex and drug exposure condition, whereas a reduced model regressed them only on drug exposure condition. The fits of both models were subsequently compared for each miRNA in order to identify miRNAs for which additional specification of the sex had a significant effect. We also adopted a second and separate sex-specific analytical approach, in which we compared miRNA expression separately in both sexes: miRNA gene counts between control tissues and buprenorphine-exposed tissues in each sex were compared using a simple binomial test. A p-value cutoff of 0.05 was set for each individual comparison; to correct for multiple comparisons, we used a False Discovery Rate (FDR), calculated according to the Benjamini-Hochberg procedure. An FDR cutoff was set at 0.1 for all of our analyses. For qPCR validation, generation of cDNA was carried out using the miScript II RT kit (Qiagen) with 250ng of RNA as template. Reactions were prepared in 96-well optical reaction plates (ABI) with optical adhesive covers (ABI) using miScript SYBR green PCR kit (Qiagen). Three technical replicates were used for each sample. Reactions were carried out in the qTower Real Time PCR system (Analytik Jena) with an initial incubation at 95°C for 15 minutes, and 40 subsequent cycles of 95°C for 15 seconds, 60°C for 30 seconds. The miScript primer assays (Qiagen) used were for Hs-miR-196a-5p, Hs-miR-196b-5p, Hs-miR-183-5p, Hs-miR-10a-5p, Hs-miR-10b-5p, Hs-miR-28-3p, RNU6 (control) and SNORD42 (control). C_t values were corrected using expression levels of control probes for each sample and fold change was calculated as $2^{\exp(-C_t)}$. The data presented is the calculated mean for the biological replicates with n being equal to the number of biological replicates (i.e. the number of cases examined).

Results

μ Opioid Receptor and Cannabinoid Receptors in FCEs and Synaptosomes

First, we examined the effects of MOUD on protein levels of the μ opioid receptor (μ OR) in fetal CNS-derived extracellular vesicles isolated from maternal blood. Samples were

analyzed from 4 groups: control (n=19), methadone (n=10), buprenorphine (n=5) and buprenorphine/naloxone (n=6). Mean μ OR was not different based on naloxone exposure (data not shown); therefore the two buprenorphine groups were combined for analysis (n=11). FCE μ OR increased with buprenorphine and methadone (Figure 1a, $p=0.002$). There was no correlation between morphine equivalent dose (MED) and FCE μ OR levels ($r=0.21$, $p=0.36$). Next, we examined the effects of MOUD on μ OR protein levels in matched fetal synaptosomes. In contrast to our observations in FCEs, and consistent with the known effects of opioid exposure, both methadone and buprenorphine were associated with down regulation of μ OR at the synapse (Figure 1b, $p=0.01$). There was significant negative correlation between FCE and synaptosome μ OR ($r=-0.38$; $p=0.02$) in matched samples. No correlation was seen between gestational age and FCE μ OR ($r=0.013$; $p=0.947$). In addition, we explored cross talk between opioid and endocannabinoid pathways. We found that MOUD was associated with up regulation of protein levels of the cannabinoid receptor CB1 in FCEs (Figure 2). Of interest, we found a positive correlation between FCE CB1 levels and gestational age that was not seen in FCE μ OR ($r=0.508$, $p=0.004$). In addition, we saw a significant positive correlation between FCE μ OR and CB1 levels ($r=0.51$, $p=0.004$) further supporting developmental crosstalk between the two systems. When patients using cannabinoids were excluded, the correlation between FCE μ OR and CB1 levels strengthened ($r=0.64$, $p<0.001$) and the association between FCE CB1 and gestational age was unchanged (data not shown).

miRNA Sequencing Reveals MOUD Alters Expression Patterns in a Sex Dependent Fashion

The amount of miRNA present in FCEs was not of sufficiently high quantity to generate sequencing libraries. Therefore we performed pilot sequencing on male and female fetal brain tissue from buprenorphine-exposed and control cases (n=4-7). To explore the main sources of variance in miRNA expression, we first performed a principal component analysis (Figure 3). The first component (PC1) appears to be attributable to drug treatment based on sex. Consistent with this possibility, a general linear model-based analysis (DESeq R package) revealed an interaction between sex and treatment for the expression levels of certain miRNAs. Most notably, the expression of hsa-miR-196a-5p (q value = 3.39×10^{-5}) and hsa-miR-196b-5p (q value = 6.51×10^{-4}) was altered in drug-exposed brain tissues in a sex-dependent fashion. These results clearly indicated that opioid exposure alters miRNA expression differently in males and females. A list of some of miRNAs most altered with MOUD are shown (Supplemental Table 1). To uncover sex-specific miRNA expression changes, we analyzed our results in male and female cases separately and found that miR-128 is selectively affected in male, but not female cases (Supplemental Table 2).

Validation of miRNA Targets in Fetal Brain

We measured the expression of 6 miRNAs that were substantially changed by *in utero* opioid exposure in fetal brain using qPCR; candidate miRNAs were chosen on the basis of their significance and their fold-change ranking in the list, since both considerations are critical elements of a reliable biomarker. Drastic reduction in the expression of miR-196b-5p was confirmed in female cases (Figure 4A). Interestingly, the expression patterns in male subjects trended in the opposite direction, which was consistent with our sequencing

findings. Expression of miR-196a-5p was not changed in fetal brain tissue by maternal opioid exposure using the qPCR approach, suggesting that we may have had one false positive result from our sequencing results. The other 5 miRNAs screened using in this experiment showed changes in gene expression that matched our sequencing results (see Figure 4B and Supplemental Table 2). None of the changes for male cases reached statistical significance, largely due to high variability across samples and small sample size. Future studies examining expression of these miRNAs in separate cases would to confirm these changes in a separate cohort of subjects.

MOUD Associated with Altered FCE Protein Cargo: Pilot analysis

A pilot proteomic analysis was performed on FCEs isolated from maternal plasma in women with current daily buprenorphine use (n=3) and gestational age matched controls (n=3). Multiple altered proteins were identified. An abbreviated list of some of the most intriguing proteins is shown as well as the associated miRNAs either known or predicted to target each transcripts encoding each protein (Table 2). As expected, based on the demonstrated fetal brain origin of FCEs, the majority of proteins identified are expressed primarily in the CNS and especially in cortical tissue. Further, based on studies in rats, the majority of proteins we identified have their highest expression during development (44). Statistical analysis was not performed due to the small samples size and the results presented are descriptive.

Discussion

EV miRNAs have already shown utility as a diagnostic tool for neuropsychiatric diseases (45). FCE protein- and miRNA-based assays have the perfect marriage of protection from degradation, peripheral accessibility, and highly specific opioid-responsivity, making them promising NAS biomarkers. Our data provide the first human in-vivo information regarding the molecular effects of MOUD on the developing fetal brain and the use of a novel methodology, fetal CNS derived extracellular vesicles (FCEs), to interrogate these alterations. Our primary findings are that MOUD is associated with down regulation of μ OR at the fetal synapse and upregulation of μ OR in FCEs. The intermediate effect of buprenorphine in both analyses is concordant with reduced severity of NAS with buprenorphine compared to methadone (46). The lack of a direct relationship between μ OR levels and opioid dose mirrors the difficulty in accurately predicting clinical NAS based on maternal opioid dose (13). The lack of correlation seen between gestational age and FCE μ OR suggests that MOUD may have effects on the fetus across the entirety of pregnancy. Taken together, our data suggest that neurons may down-regulate μ OR through the disposal of μ OR as FCE cargo and that FCE μ OR may be a biomarker for individual fetal response to MOUD. The biological implication is that receptor down regulation during times of exposure (*in utero*) may lead to NAS when the exposure is withdrawn (at birth). The clinical potential of our data are that in the future, molecular-based tools may aid clinicians in monitoring the individual fetal response to MOUD, independent of maternal opioid dose. If successful, this would address a significant clinical gap in knowledge as currently there are no available tests to monitor in utero effects of opioids.

As a secondary finding, we found evidence of cross talk between MOUD and fetal brain cannabinoid pathways. The positive correlation between FCE CB1 levels and gestational age suggests that CB1 FCE expression increases with increasing gestation. Therefore, fetal opioid exposure may have the greatest effect on the endocannabinoid pathway in the third trimester of pregnancy. In rodent models, prenatal cannabinoid exposure is associated with conditioned place preference to morphine that disproportionately affects males(47; 48), suggesting sex-specific interactions between the opioid and cannabinoid pathways.

Next, we present exploratory findings regarding the effects of MOUD on fetal brain miRNA expression and FCE protein content. These results have several important implications 1) they provide important hypothesis generating information that can link human in-vivo mechanisms with data from current epidemiologic studies and preclinical animal models 2) findings of sex-specific changes in miRNA expression supports growing epidemiologic evidence that male infants are at a substantially higher risk of NAS (49–51). In animals, maternal pre-conceptual opioid exposure is associated with altered sex-specific behavioral outcomes in both the first and second generations(52–55). Recently, an association was reported between NAS and poorer high school performance which was worse in males(56).

Although we do not wish to over interpret our results, we will briefly review the potential biologic and clinical implications of some of the specific miRNA and protein changes we observed associated with MOUD. The overall picture that emerges is one of potential alterations in cortical migration, synaptogenesis and synaptic transmission. The expression of key regulators of neuronal differentiation (class III beta-tubulin (TUBB3); (57; 58)), synapse formation and neuronal migration (cell division control protein 42 homolog (Cdc42); (59; 60) was upregulated in FCEs following opioid exposure. Interestingly, μ OR and OR regulate JNK through Cdc42 dependent mechanisms(61; 62) and Cdc42 may play a role in the crosstalk between opioid and endocannabinoid pathways through GPR55 (63). The FCE proteins down-regulated by *in utero* opioid exposure have been associated with opioid signaling and neuronal plasticity elicited by morphine in adults. For example the opioid binding cell adhesion molecule (OBCAM) and neural cell adhesion molecule 1 (NCAM-1), which are both critical for synaptic function (64; 65) have been shown to both respond to (66) and regulate opioid signaling and tolerance in response to chronic opioid exposure (67–71). Taken together, alterations in fetal proteins by *in utero* opioid exposure highlighted in our studies could provide a potential mechanism for changes in opioid tolerance or addiction potential in the offspring of women with MOUD.

Buprenorphine exposure was associated with significant down regulation of miR-196a and miR-196b. Multiple predicted and/or validated target genes of miR-196a and miR-196b have been associated with developmental disorders (ZMYND11 (72) for autism (73; 74) and intellectual disability (75); HOXB7 (76) for Huntington's disease (77) and neural tube defects (78; 79)). Other targets of down regulated miRs in opioid cases also have a role in opioid addiction (AQP4 (80; 81), NR2C2 (82)) and co-dependence of alcohol and nicotine (NR2C2 (83)). Other targets that have not yet been validated in the literature also show involvement in the pathogenesis of neurodevelopmental disorders (SLC9A6 (84; 85), PPP1R15B (86) are associated with intellectual disability (87)). Upregulated miRs of interest in the buprenorphine-exposed group include miR 10b-5p, miR 10a-5p, miR 183-5p

and miR 182-5p and alter the expression of molecules known to modulate synaptic plasticity that also contribute to the etiology of psychiatric diseases. BDNF is a validated target of miR 10b-5p (88) and miR10a-5p (89; 90) that is involved in the pathogenesis of alcohol abuse (89; 91), opioid use (92; 93) and cocaine use (94), Huntington's disease (88) and schizophrenia (95). Overall, our findings indicate that changes in miRNA expression driven by *in utero* opioid exposure could play an important role in regulating the expression of genes associated with developmental disorders and psychiatric diseases during critical stages of embryonic development.

There are several limitations to our study. First, it is important to remain aware that up-regulation in FCEs may be associated with down-regulation in the brain and vice versa as seen with μ OR. Therefore proteomic analysis of FCEs may not directly reflect brain protein changes. The small number of samples analyzed requires that future studies are performed on larger samples to confirm these preliminary findings. Next, Exoquick methodology (even using individually optimized protocols such as we have employed rather than "out of the box" manufacturers protocols) can result in some contamination with other elements. However, in other disease states, our FCE findings have been validated against clinical outcomes(42) suggesting that any minor contamination does not invalidate the clinical utility of FCE based tests. In addition, there is an approximately 3% contamination with maternal neural ECVs that cannot be avoided. However, given that the contamination is relatively minor we do not expect our overall results to be significantly affected. All of our analyses used total brain/cortical samples or ECVs limiting conclusions regarding alternations in other specific anatomic or functional areas of the fetal brain. We have been developing technologies to isolate ECVs from specific brain areas using secondary antibodies however that work is not yet validated. We acknowledge that ELISA tests for the Mu-opioid receptor are notoriously fickle. However an inaccurate/imprecise test would inevitably bias results towards the mean, therefore while our results may underestimate the differences in μ OR between groups, the observed results should still be valid. Finally; our miRNA findings need to be validated in FCEs to determine if non-invasive testing can act as a marker of neurodevelopmental perturbations.

Overall, the targets we have identified to date: 1) support the origin of FCEs from developing CNS cells, 2) are consistent with the existing literature regarding the effects of opioid exposure in animal models and humans, and 3) highlight potential interconnections between the opioid and endocannabinoid pathways. In summary, our data suggest that maternal blood FCEs based assays have the potential to provide additionally clinically useful information to clinicians caring for women with MOUD regarding individual fetal response to opioids and NAS risk independent of opioid use. In addition, we have shown the neural exosomes can also be purified from neonatal samples(42). Prospective clinical studies are needed to evaluate the association between FCE biomarkers, risk of NAS, and neurodevelopmental outcomes. MOUD or FAS cohorts should collect and bank plasma or serum samples to allow for novel biomarker discovery and validation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fetal exposure to opiates is associated with up-regulation of the μ opioid receptor (μ OR) in FCEs (Panel A, $p=0.002$) and down-regulation in matched fetal brain synaptosomes (Panel B, $p=0.01$). Changes are most extreme with pure opioid agonists with an intermediate response with mixed agonist/antagonists.

Figure 2.

Fetal exposure to opiates is associated with up regulation of the cannabinoid CB1 receptor in fetal CNS-derived FCEs ($p=0.02$).

Figure 3.

In utero buprenorphine exposure elicits distinct brain miRNA profiles in male and female fetuses. The first two principal components (PC1 and PC2), which account for the majority of the variance are depicted on the x- and y-axes, respectively. Here we show that miRNA expression data cluster based on sex and treatment. In males and females, PC1 seem to be attributable to buprenorphine exposure.

Figure 4.

Validation of change in miRNA expression in fetal brain following *in utero* opioid exposure using qPCR. A. Expression of miR-196b-5p was reduced in female cases exposed to buprenorphine *in utero*, which matches our sequencing results. B. Expression of the miRs examined trended in the same direction as we observed using the sequencing approach. (p values for male data; miR-196a-5p = 0.1937; miR-196b-5p = 0.2084; miR-183-5p = 0.0789; miR-10a-5p = 0.1429; miR-10b-5p = 0.1269; miR-128-3p = 0.0574). * p < 0.05

Table 1.

Clinical Characteristics of Subjects by Exposure

	Controls (n=19)	Buprenorphine (n=11)	Methadone (n=10)
Maternal Age (years $\pm$ SD)	24.8 $\pm$ 5.4	26.4 $\pm$ 3.3	27.63 $\pm$ 3.42
Parity ($\pm$ SD)	1.7 $\pm$ 1.0	1.4 $\pm$ 1.1	1.5 $\pm$ 0.9
Gestational Age (weeks $\pm$ SD)	14.8 $\pm$ 2.8	13.8 $\pm$ 3.1	16.8 $\pm$ 3.4
Body Mass Index ($\pm$ SD)	24.9 $\pm$ 5.6	26.21 $\pm$ 6.6	27.6 $\pm$ 3.5
Ethnicity (%)			
Hispanic	10.5%	0%	0%
Race (%)			
White	52.6	100%	90%
Black	47.4	0%	10%
Opioid Dose (mg $\pm$ SD)		9.3 $\pm$ 6.6	90.6 $\pm$ 40.7
Naloxone Exposure (%)	0%	54.5%	0%
Cannabinoid Use (%)	26.3%	18.2%	20.0%
Tobacco Use (%)	78.9%	90.9%	80%
Cig/day ($\pm$ SD)	7.3 $\pm$ 5.3	13.5 $\pm$ 5.8	14.8 $\pm$ 7.5
Male Fetal Sex (%)	42.1%	60.0%	54.5%

Table 2.Novel protein targets associated with *in utero* opioid exposure

FCE Protein	Fold : Opioid	Tissue/Cell Location	Potential Relevance	Associated miRNAs	Rat/ Mouse Brain Expression
TUBB3 Tubulin beta 3	2.15	Primarily CNS microtubules Cell projections	Primarily expressed in neurons and may be involved in neurogenesis	None reported	Almost Exclusively in Brain; Highest expression at 2 weeks of life
CDC42	1.84	Many tissues Microtubules Cell projections Vesicles	BDNF signaling pathway; Dendritic cell migration, Autism like social behavior in mice.	miR-124 miR-132 miR-137 miR-608	Expressed from early conceptus Highest express at 2 weeks of life
RAB3A	0.66	Primarily CNS, Synapse, Vesicles Cell projections	Involved in neurotransmitter release; regulates late step in synaptic vesicle fusion.	miR124a	Very high in brain; Highest expression at 2 weeks of life
SYT1	0.46	Largely cortex Synapse membrane Vesicles Cell Projections	Synaptic Vesicle Pathways. Trigger for neurotransmitter release at the synapse. Dendrite formation; Binds neurexins, syntaxin and AP2	miR 137 miR-34a	Almost exclusively in brain; Highest expression at 2 weeks of life
OBCAM	0.44	Primarily CNS, Largely Cortex Membrane	Accessory role in opioid receptor function ; selective for mu ligands in rats	None reported	Brain>>other organs; highest at 2 weeks
NCAM1	0.44	Primarily CNS Membrane; Cytosol Cell projections	CNS development; neuron-neuron adhesion, outgrowth of neurites. Polymorphisms: Heroin Dependence. miR-375 also diminishes BDNF dependent neurite outgrowth.	miR-572 miR-290-5p miR-377 miR-375 miR-128	Brain>>other organs; Highest expression at 2 weeks of life Embryo ectoderm