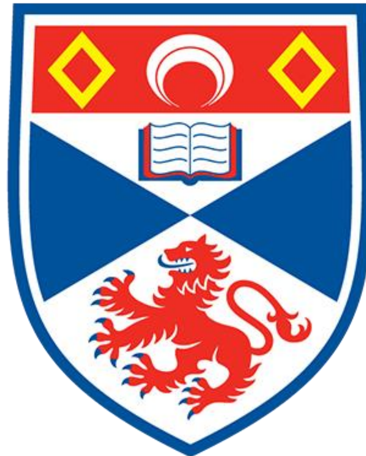
Understanding the Transgenerational Neurogenetic Effects of Maternal Opioid Use



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Introduction

Opioid use disorder has increased at a phenomenal rate since the turn of the century, fed by over-prescription and socioeconomic challenges, such as the lack of education.[1,2] As of 2021, 3.3 percent of the US population struggled with opioid misuse.[3] This trend has been consistent among reproductive age women, resulting in more infants being born with neonatal opioid withdrawal syndrome (NOWS).[2] Illustrating this, births associated with opioid use rose from 1.5 cases to 6.5 per 1000 births in the United States between 1999 and 2014, with some states seeing values as high as 48.6 affected births per 1000, equating to nearly 5 percent.[4]

The presentation of NOWS varies greatly due to polypharmacy, opioid-identity, and the frequency, duration, and dosage of intake. Generally, though, fetal exposure results in a combination of neurologic and gastrointestinal symptoms due to the high concentration of opioid receptors in these systems.[5] Currently, the criteria for a NOWS diagnosis are "in utero exposure to opioids [...and...] the presence of 2 of 5 of the most common clinical signs [...] i.e., high-pitched/excessive cry, poor sleep, hypertonia, tremors, and gastrointestinal issues."[6] While these are the most common, other signs include a reduction in birth weight and altered brain development, specifically in cortical thickness and ventricle size.[2] Even before birth there can be consequences, with higher rates of pre-term labor and miscarriage seen amongst women with opioid use disorder (OUD).[2,7]

There are also the behavioral effects: ramifications of in-utero opioid exposure may persist into adulthood, with animal models being more likely to exhibit a hyperactive phenotype, triangulating human studies that show children born with NOWS experience a higher incidence of attention-deficit/hyperactivity disorder (ADHD) and learning disorders.[2] Additionally, there is the question of whether maternal opioid use increases offspring's addiction liability.[8] With these facts in mind, the need for further research into the issue is critically important.

Despite this demand, many existing studies on the subject fail to capture the nuances of the issue. For one, most research has been conducted using morphine as the prototypical opioid, despite it not being a common drug of misuse compared to oxycodone (Oxy), which is more frequently prescribed for short and long-term pain management.[9] Additionally, models tend to fall short in their iniation of opioid delivery – starting exposure at the beginning of or right before pregnancy, when in humans, OUD is more likely to be well-established before conception. Finally, the delivery of opioids across research varies – some animals are given the appropriate drug through continuous release pellets or at experimenter-selected dosages and intervals. Again, this does not fully capture the human experience wherein individuals have some degree of agency over their intake. In summary, while no animal model can fully recapitulate human drug use, there is evident need for further pre-clinical study into how self-administration of prevalent opioids in well-established users affects offspring throughout life.[8]

As part of a larger program guided by these considerations, this research aims to better understand the epigenetic effects of maternal opioid use and how changes may correlate to offspring behaviour – particularly addiction. Specifically, this investigation will look at how the opioid use patterns of mothers (F0 generation; referred to as dams) affects long-term gene expression of 5 implicated genes – Arc, C-Fos, MeCP2, Oprm1, and Drd2 – in the nucleus accumbens (NAC) of their adult offspring (F1 generation). These target genes are a combination of epigenetic regulator genes important for neurodevelopment (MeCP2),[10] immediate early genes with important roles in synaptic activity (Arc and C-Fos),[11-13] as well as genes that code for opioid and dopamine receptors (Oprm1 and Drd2 respectively).[14-15] The NAC was chosen for its key role in the regulation of addictive behavior which is due to its dopamine activity associated with pleasure, motivation, and reinforcement responses – essentially, driving one to continue to perform pleasurable behaviors.[16]

Through comparing offspring gene expression to maternal opioid self-administration patterns, the goal is to determine whether correlations between the two can be discerned – whether these key genes' expression is modulated due to fetal opioid exposure. Moreover, comparing gene expression to offspring intake, when offspring were given the chance to

self-administer opioids themselves, will determine whether these genes can be linked to increased opioid addiction risk in fetally exposed males and/or females. In all, gaining insight into potential biological mechanisms associated with intergenerational transmission of abuse liability could help to better understand the consequences of opioid use on individuals, communities, and families.

Methodology

Due to timing constraints, subjects were conceived, born, reared, and euthanized before the research period started, with sample brains originating from 2021-2023.

Pre-Natal

F0 Experimental Groups

The F0 generation consisted of 32 drug-naïve Sprague Dawley female rats divided into three groups: (1) the ‘Oxy Leader’ or OL group in which dams self-administered Oxy pre-pregnancy and during gestation, (2) the ‘Oxy Follower’ or OF group who received an equivalent dosage of Oxy as a paired OL dam, regardless of their own behaviour in the operant conditioning chamber, essentially helping to evaluate whether agency over dosage has a bearing over outcomes or whether outcomes are primarily explained by pre-natal exposure itself, or (3) the saline group which received an equivalent volume of saline as their paired OL’s dose of Oxy.

Drug Administration

Prior to conception, all dams underwent catheter implantation surgery to place in-dwelling intrajugular catheters for Oxy or saline intravenous infusions. They were then introduced to an operant conditioning chamber with a two-lever system. OL dams self-administered Oxy at a fixed ratio of a 0.1 mg/kg per infusion during 6-hour access windows, five days per week, for approximately three weeks. Dams in the OF and S groups received an equivalent dose of Oxy or saline, respectively, regardless of their own active lever presses. This same

pattern of self-administration carried through mating and pregnancy. The only difference: during pregnancy, drug administration occurred seven days per week.

Mating

As female rats only mate during the estrus phase of their cycle, all dams were given an intraperitoneal injection of luteinizing-hormone-releasing-hormone, which made them ready to mate 3-4 days after. They were mated by placing a male rat into the same cage overnight. Pregnancy was assessed in the morning via vaginal lavage. If sperm was present, pregnancy was confirmed, and the male rat was removed from the cage. If not, mating was repeated nightly until the dam became pregnant.

Neonatal

F1 Rearing

Most dams ($n = 29$) gave birth to their litter on gestational day 21, with a small cohort giving birth on day 22 ($n = 3$). From these 32 litters, 71 offspring were included in this study: 37 females and 34 males. On post-natal day 1, pups were separated from their biological mothers and fostered with drug-naïve dams to mitigate any confounding effects of opioid-induced alterations to maternal care behaviour.

Adulthood

F1 Drug Administration

Once the F1 generation reached adulthood, they also underwent intrajugular catheter implantation surgery and were trained in the same operant conditioning chamber as the F0 generation. All the offspring self-administered either Oxy or cocaine (Coc). However, due to more significant addiction liability observed in female rats exposed to Oxy and male rats to Coc, these two conditions made up the subject groups for this project of gene expression analysis (see table 1 for F1 generation distribution).

F0 Drug Administration Group	F1 Sex	F1 Drug Exposure	Number of F1 Samples
Saline	Female	Oxycodone	12
Saline	Male	Cocaine	11
Oxy-Leader	Female	Oxycodone	13
Oxy-Leader	Male	Cocaine	11
Oxy-Follower	Female	Oxycodone	12
Oxy-Follower	Male	Cocaine	12

Table 1: Distribution of F1 Subjects by Sex and F0 Drug Administration Group

The F1 generation self-administered the appropriate drug at a fixed ratio of 1 (FR1) during a 2-hour session 5 days/week, meaning that for every active lever press, they received one infusion. This was either a 0.1 mg/kg infusion of Oxy for females or a 0.5 mg/kg infusion of Coc for males (with a maximum of 100 infusions per session of Coc to avoid overdose). This was followed by five days at a fixed ratio of 5 (FR5), requiring 5 lever presses per infusion. Then, they completed 3 days in a progressive ratio condition wherein, each time the subject received an infusion, the number of lever presses needed to receive another increased exponentially. The sessions continued until they hit a break point where they did not meet the necessary number of lever presses for their next infusion within 30-minutes. Finally, all subjects had an extinction session where offspring all received saline at FR1: no drug was available. Three days after the extinction session, rats were given an acute injection of the drug they self-administered (1 mg/kg Oxy or 10 mg/kg Coc) 1-hour before euthanization. The subjects' brains were collected and flash-frozen in methylbutane and then stored at -80°C until the start of this research period.

Gene Expression Measurement

Brains were sliced using a cryostat (Leica CM1510S) until the NAC's depth, where 2mm bilateral precision brain punches were collected. Gene expression in the NAC was then analyzed through utilizing the quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Following the protocol of the Qiagen mRNA Extraction Kit, mRNA was collected from each sample and concentrations were measured using a BioTek Synergy H1 Multimode reader. The average mRNA concentration across samples was 280.778 ng/µL. Sample quality was

assessed through 260/280 ratios, of which values between 2.100 and 2.200 were considered ideal. mRNA Samples were stored at -80℃.

The collected mRNA was then used to synthesize complementary DNA (cDNA) using components of a Retroscript kit, plus a Veriti thermocycler. For each NAC sample, 20μL of cDNA with a concentration of 60 ng/μL were synthesized, each using 120 ng/μL concentrations of mRNA. The cDNA samples were stored at –20°C until use in PCR.

For PCR, in addition to the five target genes, two housekeeping genes were run as controls: Actb and Gusb. By comparing housekeeping gene expression across all samples – which should theoretically be unaffected by group identity – one can evaluate sample quality and correct for large inconsistencies between groups. In advance of PCR, a master mix for each of the seven genes being measured was prepared with RNAse free water, the appropriate primer, and TaqMan master mix (sourced from Life Technologies). In each well of the 96-well PCR plates, 1μL of cDNA was added to 24μL of the appropriate master mix. All samples were run in duplicate to account for random error. Each plate was centrifuged then run in the Applied Biosystems QuantStudio3 PCR System, wherein 40 heating and cooling cycles were performed. Each cycle doubled the amount of DNA containing the gene of interest, and measured the fluorescence of the sample's primer, which only fluoresces in the presence of the gene of interest.

This process provided cycle threshold (Ct) values: the number of cycles before the fluorescent signal began increasing at an exponential rate. Lower Ct values indicated higher expression of a gene as less replication was needed to reach the critical threshold. The two Ct values from duplicate wells were compared: averaged to generate a mean-Ct and their standard deviation (SD) calculated. If a sample had a gene wherein the SD between the two wells was above 1.000, the sample was re-run, and if it was a housekeeping gene with the high SD, all genes for that sample were re-prepared and re-run, and the new Ct values used for analysis.

Gene Expression Analysis

To determine relative gene expression (RE) across groups, the delta-delta-Ct (**ΔΔ**Ct) method was used. Initially, the delta-CT (**Δ**Ct) was determined by finding the difference between a sample's mean-Ct of a target gene and the average of the mean-Ct of both housekeeping genes. This normalized the data for each sample. Next, the **ΔΔ**Ct was found by averaging all the **Δ**Ct values of the saline group for each gene to produce a control **Δ**Ct average, and then finding the difference between each sample's **Δ**CT and the control **Δ**Ct average of that same gene. This allowed for comparisons between experimental groups (OL and OF) and the control (S). Finally, to determine relative gene expression, one uses the formula: $2^{-\Delta\Delta Ct}$ = RE. This process standardizes gene expression.[17]

RE Should ideally be close to 1 for the control group. A value significantly less than 1 in an experimental group would indicate reduced expression of that gene and a value above 1 indicates increased expression.

Statistical Analysis

To evaluate the data seen in figures 1A and 1B, a two-way ANOVA was performed. For figures 1C, 1D, and 3, a one-way ANOVA was used. Finally, linear regression evaluated the strength of the correlations in figures 2, 4, and 5. Each statistical test had a significance threshold of $P < 0.05$.

Results

Self-Administration Data

The fixed-ratio self-administration phase focused on establishing consistent drug use. There was a significant main effect of day ($P < 0.001$) across both sexes in FR1 and FR5, with the number of active lever presses increasing, demonstrating learning of the operant task.

The PR phase measured subject motivation to acquire opioids, which was used as a metric of addiction liability. One can see in both figures 1A and 1C that females self-administering Oxy saw a significant increase in intake in the OF group compared to the control (Fig. 1A: $P = 0.0013$ and 1C: $P = 0.0227$). Additionally, there was a noticeable but non-significant increase in intake between the female OF and OL groups. There were no significant differences in the male self-administration data, but there was a clear trend of offspring with fetal opioid exposure having higher Coc intake.

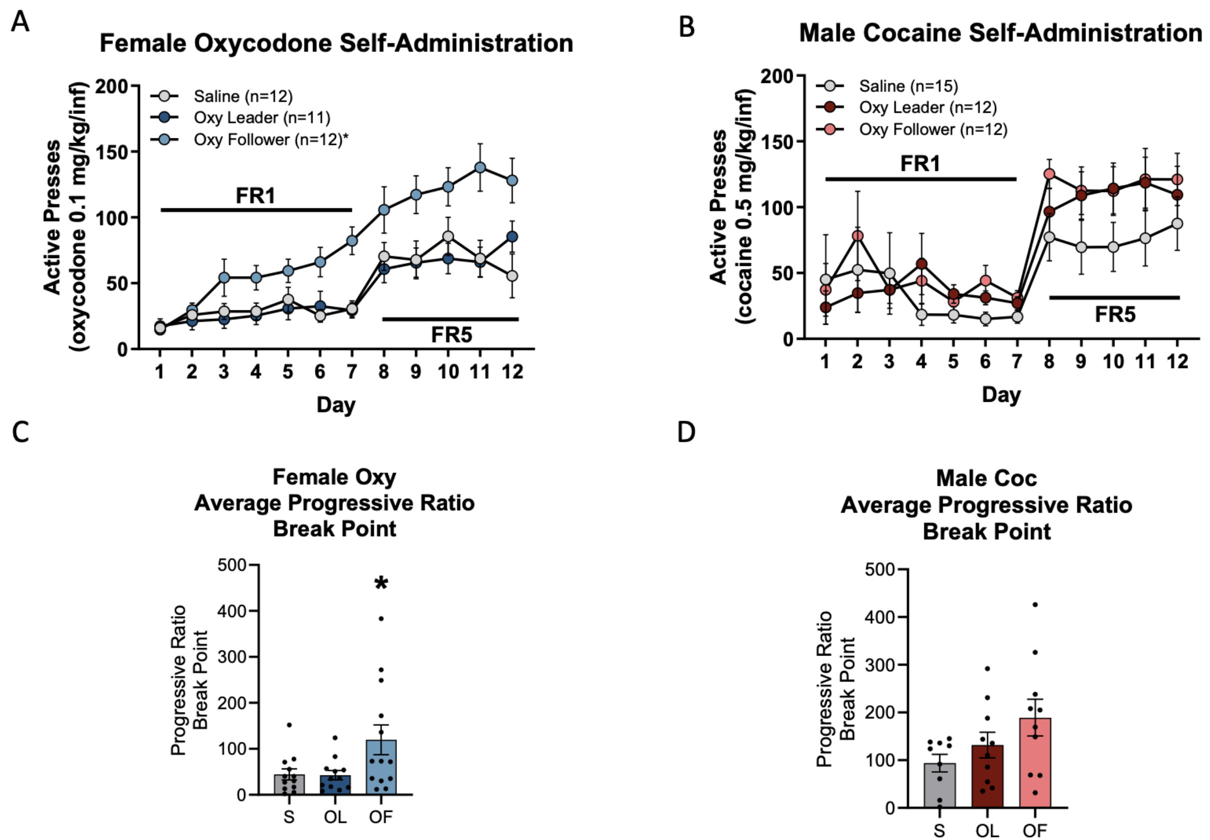

Figure 1: F1 Opioid Self-Administration. Significance of P < 0.05 denoted by '∗'. Discrepancies in the subject counts in figures 1A and 1B compared to table 1 are the result of the self-administration data that was available. Despite the differential counts, all rats whose data was included in these graphs underwent the same pre-natal treatment, rearing, and opioid self-administration procedure outlined in the methodology. **A**: F1 Female Self-Administration of Oxy throughout FR1 and FR5. **B**: F1 Male Self-Administration of Coc throughout FR1 and FR 5. **C**: F1 Female Average Breakpoint in Self-Administration of Oxy at a Progressive Ratio across experimental groups. One OL subject was excluded due to being an outlier with a breakpoint average of 630. **D**: F1 Male Average Breakpoint in Self-Administration of Coc at a Progressive Ratio across experimental groups. Three S subjects, one OL subject, and two OF subjects were excluded for having average breakpoints below 6, demonstrating lack of understanding of the progressive-ratio task.

Figure 2 explores if F0 gestational intake can directly predict offspring intake. No significant relationship was found, although it is interesting to consider the dissimilar ways OF offspring were affected in the Oxy-using females versus Coc-using males. The divergent effects highlight the value of researching the unique implications of different opioids.

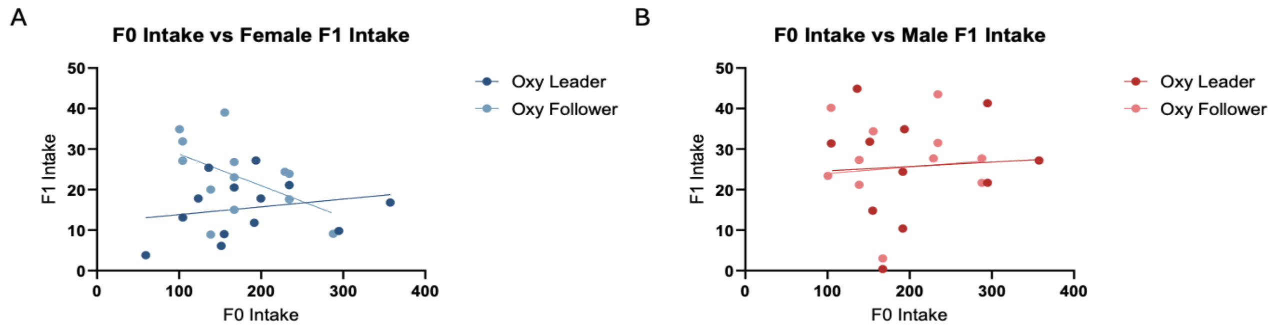

Figure 2: Direct Effect of F0 Gestational Intake (mg) on F1 Oxy (♀) or Coc (♂) Intake Across FR1 and FR5 (mg). The saline group was excluded as F0 dams in this group did not have any exposure to opioids.

Gene Expression Analysis

F1 Gene Expression

Figure 3 displays RE of the target genes in the adult NAC. No significant differences in expression are apparent. This may be the result of utilizing bulk tissue samples and varying opioid intake across subjects. Despite the lack of significance, there are some observations of note.

Firstly, Arc and C-Fos appear reduced in females as a general effect of pre-natal opioid exposure. These are both immediate early genes, which are expressed rapidly upon neuronal stimulation.[13] Arc, for example, is expressed upon synaptic activation and is therefore a marker of brain activity in response to stimuli.[11] C-Fos is also a marker of cell activation and is implicated in memory formation.[12,18] Thereby, the observed decrease in expression of these genes in fetally-exposed females could indicate lower NAC stimulation in response to Oxy. Conversely, in Coc-males, C-Fos expression appears increased in the OF and OL groups, particularly the former. This could be interpreted as higher levels of synaptic activity in the NAC. Although contrasting, the fact that both opioids induce possible alterations in expression in the OL and OF groups signifies that pre-natal

exposure, to either opioid, likely modulates normal motivation and pleasure responses, but perhaps via different mechanisms. Or, potentially, the decrease in expression seen in the Oxy-females could point to tolerance, while the increased C-Fos expression seen in the males may be more of an acute effect of Coc.

Another observation was that MeCP2 expression appears increased in OF males, which may be correlated to F1 intake. As MeCP2 is a key epigenetic regulator, with important functions in neuronal maturation and brain development, changes to its expression could have far-reaching consequences in motivation and opioid-related behaviour.[10,19] This trend triangulates existing findings that Coc exposure increases MeCP2 production in the NAC in male rats and plays a role in reinforcing Coc use.[20]

Finally, Drd2 expression seems reduced in females with pre-natal exposure, but most notably in the OF group. The Drd2 gene codes for dopamine receptor D_2 which is important in reward learning.[15] This reduction could indicate alterations in dopamine processing, which could have implications on reward learning and drug-seeking behaviour.

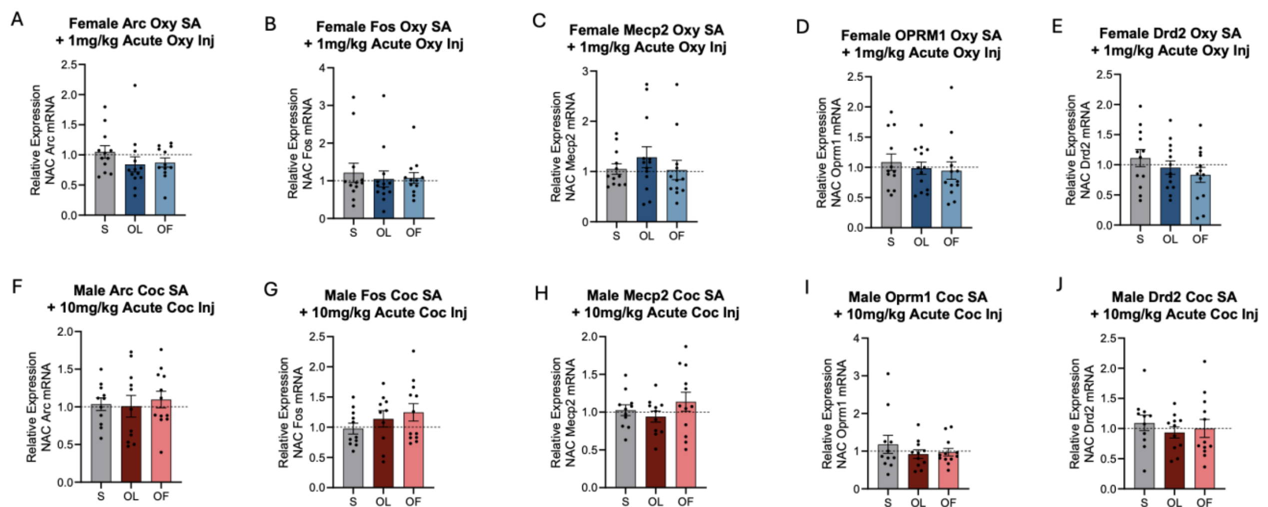

Figure 3: Effects of Maternal Opioid Exposure Pattern on F1 Relative Gene Expression in the Nucleus Accumbens Following Either Oxy (♀) or Coc (♂) Self-Administration.

Correlations Between Gene Expression and Intake

Due to the opioid self-administration technique used here, total drug intake was inconsistent, even between subjects in the same group. This variability helps to explain the lack of significance in target gene RE, with the effects of direct dosage potentially playing a role in shaping outcomes. Therefore, the relationship between total intake and the RE of target genes was explored (figures 4 and 5).

In figure 4C, one can see a significant negative correlation ($P = 0.0466$) between F1 Female Oxy intake and C-Fos expression in the OL condition, indicating a dose-dependent reduction of C-Fos expression in this group. This data is consistent with the observations of figure 3 and could suggest that tolerance to Oxy – when higher doses are needed to achieve the same effect – is correlated to decreased C-Fos expression in the NAC.

Moreover, in figure 4G, there is a significant negative correlation between F1 Female Oxy intake in the control group and Oprm1 expression ($P = 0.0138$). The Oprm1 gene codes for the Mu-opioid receptor and thus its expression can be a predictor of opioid sensitivity, with decreased expression likely indicating a developed drug tolerance.[14]

Finally, in figure 5, maternal opioid intake quantity is compared to gene expression. In figure 5J, there is a significant positive correlation between F0 intake in the OF group and male Drd2 expression ($P = 0.0229$). Changes to Drd2 expression, with its role in modulating reward learning, are likely to affect pleasure-seeking behaviour and thereby motivation and dependency on opioids.

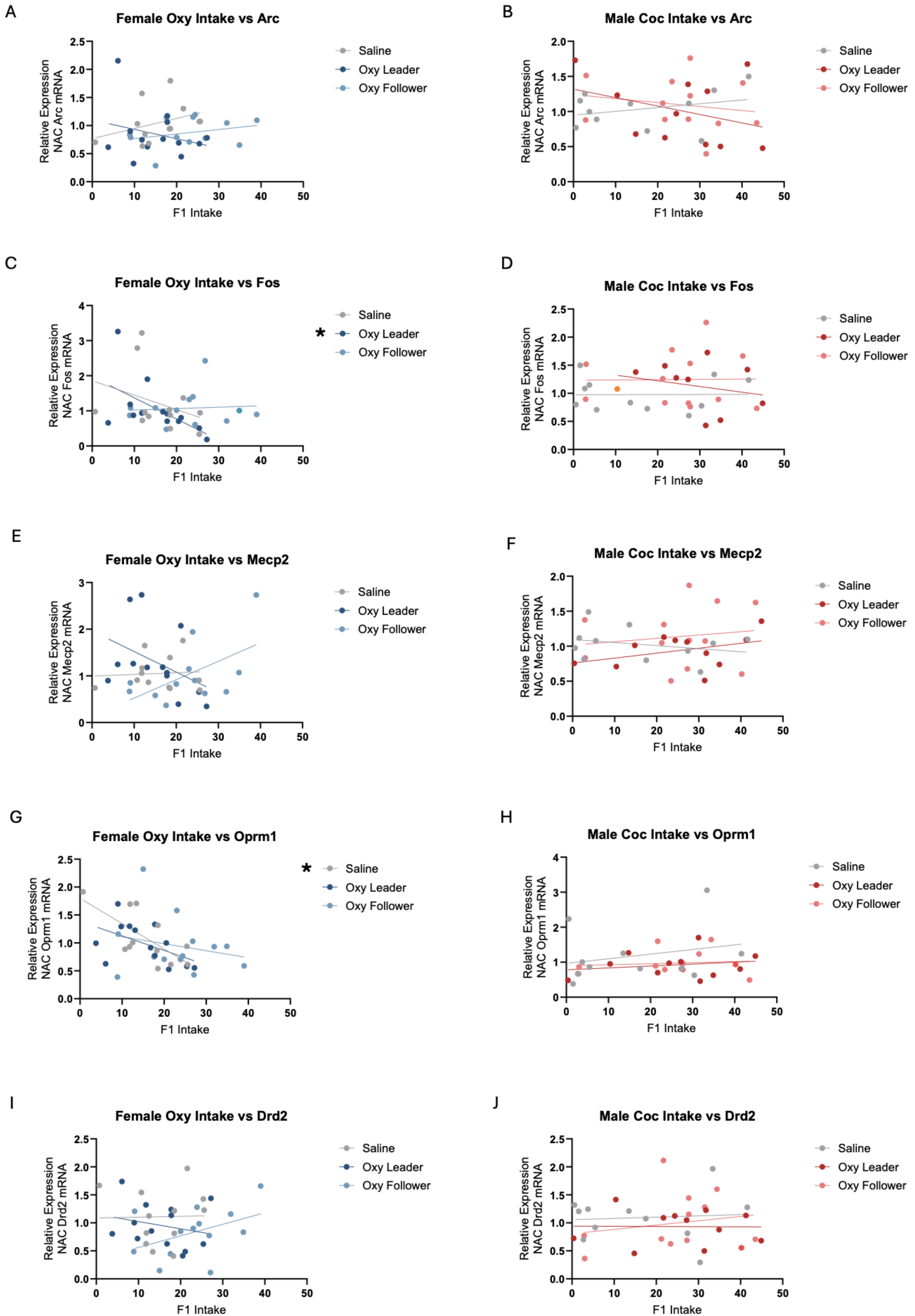

Figure 4: Effects of F1 Total Opioid Intake (mg) Across FR1 and FR5 on Relative Gene Expression. Significance of $P < 0.05$ denoted by '∗'.

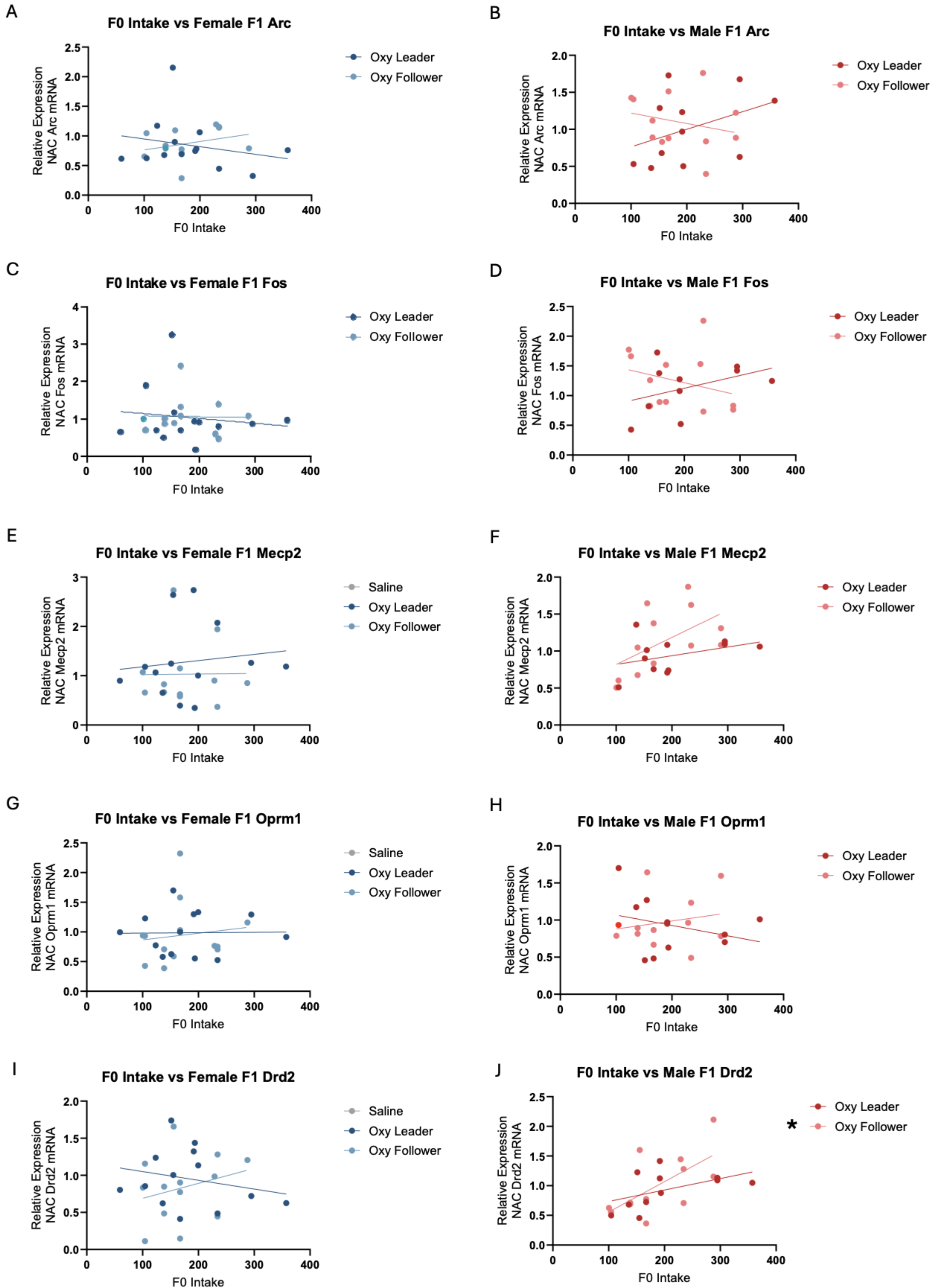

Figure 5: Effects of F0 Total Gestational Opioid Intake (mg) on Relative Gene Expression. Significance of $P < 0.05$ denoted by '∗'. The saline group was excluded as the F0 Saline dams did not have any exposure to opioids.

Conclusion

The results show that agency plays a crucial role in shaping offspring outcomes and drug-related behaviour, underscoring the need for more research utilizing self-administration models for greater transferability. Moreover, there were significant sex-specific effects of maternal opioid use on Drd2 and offspring intake on Oprm1 and C-Fos, implicating these genes in the pathways by which opioids affect behaviour and development, especially at greater doses. Thereby, they are important to keep in mind either as therapeutic targets or as metrics by which to evaluate therapies aimed at reducing negative effects of prenatal opioid exposure.

Another takeaway was the divergent sex-specific and drug-specific results. This emphasized the need for research to expand and include the range of opioids used in society since opioids are all unique in their receptor-binding, mechanisms of action, and degrees of trophoblast transferability. Oxy, for example, has a high trophoblast transfer permeability compared to many other opioids of abuse. Better yet, it would be valuable to look at the effects of opioid polypharmacy for improved realism as fetal drug exposure more commonly occurs as mixtures.[9]

One direction to explore in future research would be looking at regional and cell-type-specific effects on gene expression. The bulk samples collected in this experiment contained a large variety of cell types, which respond to opioids via different mechanisms and thereby presumably see different alterations in expression. Greater specificity and knowledge in this domain could help predict offspring outcomes and identify protective factors. Furthermore, it may be valuable to ascertain how opioid use prior to pregnancy, but not during, affects expression. Even without in-utero exposure, opioid use may induce epigenetic changes that are passed down to the next generation, having ramifications on behaviour. [21]

In sum, the epigenetic link between OUD and offspring behaviour is an area of continued interest and demands further research to mitigate any negative consequences of in-utero opioid exposure for individuals and families with a history of drug misuse.

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