Investigating the genetic profile of dopaminergic neurons in the VTA in response to perinatal nicotine exposure using mRNA-miRNA analyses

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Maternal smoking during pregnancy is associated with an increased risk of developmental, behavioral, and cognitive deficits. Nicotine, the primary addictive component in tobacco, has been shown to modulate changes in gene expression when exposure occurs during neurodevelopment. The ventral tegmental area (VTA) is believed to be central to the mechanism of addiction because of its involvement in the reward pathway. The purpose of this study was to build a genetic profile for dopamine (DA) neurons in the VTA and investigate the disruptions to the molecular pathways after perinatal nicotine exposure. Initially, we isolated the VTA from rat pups treated perinatally with either nicotine or saline (control) and collected DA neurons using fluorescent-activated cell sorting. Using microarray analysis, we profiled the differential expression of mRNAs and microRNAs from DA neurons in the VTA in order to explore potential points of regulation and enriched pathways following perinatal nicotine exposure. Furthermore, mechanisms of miRNA-mediated post-transcriptional regulation were investigated using predicted and validated miRNA-gene targets in order to demonstrate the role of miRNAs in the mesocorticolimbic DA pathway. This study provides insight into the genetic profile as well as biological pathways of DA neurons in the VTA of rats following perinatal nicotine exposure.

Maternal smoking during pregnancy is associated with adverse birth outcomes including stillbirth and low birth weight as well as sudden infant death syndrome (SIDS). In addition, many developmental abnormalities are associated with gestational nicotine exposure including behavioral disorders like attention deficit hyperactivity disorder (ADHD), learning disabilities, cognitive dysfunction, and a predisposition towards smoking later in life. Studies have shown that exposure to nicotine, the biologically active substance in tobacco, changes the intensity and timing of brain cell development, and also the programming of neurodevelopmental events on a cellular level. During pregnancy, nicotine readily diffuses across the placental barrier entering the fetal blood and amniotic fluid. After birth, the offspring continue to receive nicotine through breast milk, which contains two to three times more nicotine than the mother’s plasma.

Nicotine activates dopaminergic (DA) neurons of the mesocorticolimbic dopamine pathway, also known as the reward pathway, which consists of the ventral tegmental area (VTA), the prefrontal cortex (PFC), the nucleus accumbens (NAc), and other limbic areas. The VTA is believed to be central to the neural adaptations that underlie addiction. Several studies have been conducted on gestational nicotine exposure using a subcutaneously implanted osmotic minipump for nicotine treatment in order to investigate biochemical, behavioral, and genetic changes that occur due to nicotine exposure during pregnancy. In the brain, a diminished response to nicotine demonstrated by lowered levels of DA in the NAc and striatum has been found in offspring exposed to gestational nicotine or comorbid gestational nicotine and ethanol. Additionally, several receptor subunits have been found to be modulated by gestational nicotine exposure, including nicotinic acetylcholine receptors and glutamate receptors, within the VTA DA system.

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Because of the importance of the VTA in addiction, several studies have been conducted to determine genetic profiles of the VTA in response to exposure from different addictive substances\(^1\)\(^-\)\(^3\). These studies focused on the VTA in its entirety, providing a profile of the overall area, but do not separate the contributions of different cell types to the underlying mechanism of addiction. Other studies have investigated different regions that directly or indirectly receive input from the VTA in order to identify genetic changes\(^4\)\(^-\)\(^8\). Additionally, studies have investigated gender differences present after perinatal nicotine exposure. Despite extensive research on addiction, the mechanisms remain unclear. In contrast to previous studies, we sought to focus our experiment by investigating changes that occur specifically in DA neurons in the VTA in response to perinatal nicotine exposure.

Recently, several studies have focused on the influence of microRNAs (miRNAs) in addiction\(^9\)\(^-\)\(^12\). miRNAs are short, non-coding RNA sequences that post-transcriptionally regulate genes by directly targeting the 3′-untranslated region (3′-UTR) of mRNA. In addition, several studies have also been conducted integrating the analysis of mRNA and miRNA in order to elucidate the regulatory interactions that occur following exposure to substances of abuse\(^13\)\(^-\)\(^15\). Perinatal nicotine exposure is the most common approach to study the association between maternal smoking during pregnancy and associated disorders\(^6\). Therefore, given the importance of VTA DA neurons and the potential regulatory role of miRNAs, in this study we investigated the genetic profiles and biological pathways of DA neurons in the VTA in response to perinatal nicotine exposure using high-throughput microarray analyses of mRNA and miRNA expressions.

**Results**

The VTA was isolated from rat pups treated perinatally with nicotine or saline (control). After dissociating and sorting our samples, an average of 27.9% ± 3.3% of the NeuN-positive neurons were positively stained by TH. These results confirm the proportion of double-stained neurons reported by Guez-Barber et al., who found that 30.2% of all NeuN-positive events were double stained for TH and NeuN from midbrain samples. Another study by Chung et al. followed the same protocol and reported approximately 25% of NeuN-positive cells were also TH-positive\(^30\). The exact percentage of TH-positive neurons in the VTA is not known, but is speculated to be about 50–60%\(^31\); therefore, we considered the estimated 25–30% of TH-positive neurons after fluorescent-activated cell sorting (FACS) to be adequate for the suggested experiment, consistent with other studies\(^30\)\(^-\)\(^32\). There was no significant difference in the number of collected DA neurons per VTA between the treatment groups since the saline-treated and nicotine-treated groups had 27.1% ± 3.7% and 28.5% ± 3.1% double-positive neurons from the total NeuN-positive neurons, respectively. Furthermore, statistical analysis using Student’s t-test showed no significant difference (p > 0.5).

After collecting DA neurons using FACS, samples were processed using mRNA and miRNA microarrays in order to find the differential expression profiles of DA neurons after perinatal exposure to nicotine. The transcriptome and miRNome were then used to identify potential points of regulation and enriched pathways following perinatal nicotine exposure in VTA DA neurons.

**mRNA expression changes following perinatal nicotine exposure.** For VTA DA neurons, 2,636 genes were found to be differentially expressed at a q-value < 0.05 using Benjamini & Hochberg (BH) method and an absolute log2 fold change > 1. More specifically, 862 differentially expressed genes (DEGs) were upregulated and 1,774 DEGs were downregulated showing that the majority of genes in VTA DA neurons are downregulated due to nicotine exposure. Of these genes, 2,095 were annotated genes (639 upregulated and 1,456 downregulated). Figure 1 shows a heatmap of the top overall results for up and downregulated genes in DA neurons. Details about the top up and downregulated genes are shown in Table 1.

**miRNA expression changes following perinatal nicotine exposure.** For DA neurons, there were 74 differentially expressed miRNAs (DEmiRs) of which 58 miRNAs were found to be upregulated, while 16 were downregulated showing that the majority of miRNAs were upregulated due to perinatal nicotine exposure (see Fig. 1 for heatmap). Applying parameters q-value < 0.05 (BH) and an absolute log2 fold change > 0.5\(^29\), we found 11 DEmiRs that were upregulated and 9 DEmiRs that were downregulated. DEmiR results are shown in Table 2.

**miRNA-mRNA target predictions.** MultimiR\(^33\)\(^-\)\(^34\) was used to find validated and predicted miRNA-gene targets using inversely regulated DEmiRs and DEGs. Considering the top 20% of predicted scores and conserved targets sites, 618 unique miRNA-gene target pairs were found with 290 nodes (58 miRNAs and 232 genes) based on conserved prediction sites. The majority of the miRNA-gene pairs were from upregulated DEmiRs and downregulated DEGs. Pair-wise Pearson correlation analysis was performed on expression values of DEGs and DEmiR. Multiple testing was corrected using BH method. Figure 2a shows the predicted network for miRNA-mRNA in VTA DA neuron after perinatal nicotine exposure. Notably, rno-miR-125a-5p was predicted to target the most number of unique genes (Fig. 2b).

**Enriched pathway analysis and integrated miRNA-mRNA network.** According to DAVID, pathway analysis of the downregulated DA DEGs revealed significant enrichment of many Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with addiction, including neuroactive ligand-receptor interaction (p < 0.001), calcium signaling (p < 0.001), cAMP signaling (p < 0.01), and long-term potentiation (p < 0.05). Additionally, multiple signaling pathways and synapse pathways were enriched as well as drug addiction pathways (see Table 3 for more details). Notably, the DAergic synapse pathway was significantly enriched (p < 0.001). Significant enrichment was determined using a modified hypergeometric test. Upregulated DA
DEGs also showed non-significant enrichment of glutamatergic and serotonergic synapse pathways and neuroactive ligand-receptor interaction pathway.

Figure 1. Heat maps of DEGs (left) and DEmiRs (right) in DA neurons in the VTA following perinatal nicotine exposure compared to saline control. Displayed are expression profiles of the most altered genes and miRNAs based on greatest absolute log fold change selected from the microarrays. Red denotes decreased expression; while green denotes increased expression.

A gene network was created using KEGGgraph\(^\text{35}\) incorporating enriched pathways found using DAVID\(^\text{36,37}\). Additional relevant KEGG pathways were included from the literature\(^\text{18,21}\). A subset graph was created removing genes that were not significant in the microarray results in order to visualize gene-gene interactions within our results. Results from miRNA-gene target predictions were included in order to show the relationship between...
genes and pathways and their corresponding miRNA predictions. Figures 3 and 4 show the mRNA network created from the dopaminergic synapse pathway and neurotrophin signaling pathway, respectively, with miRNA targeting interaction. According to the hypergeometric test, we found two pathways with significant miRNA-gene target prediction with significant miRNA-pathway interaction results: DAergic synapse pathway and rno-miR-30b-5p was (p < 0.01) and the neurotrophin signaling pathway and rno-miR-195-5p and rno-miR-204-5p (p < 0.01).

**Validation of miRNA and mRNA microarray results using RT-qPCR.** The microarray results were validated for both miRNA and mRNA using the same approach as detailed in Bosch et al.29. Candidate genes were selected based on previously reported gene expression profiling of DA neurons in the VTA and biological interest

| Gene Symbol | Entrez ID | Log FC | adj p val | Description | miRNA target |
|-------------|-----------|--------|-----------|-------------|--------------|
| Nepal       | 309775    | 1.635  | 0.00093  | nephrocan    |              |
| Spert       | 498572    | 3.824  | 0.00118  | spermatid associated |              |
| Map2        | 25595     | 1.365  | 0.00124  | microtubule-associated protein 2 |              |
| Pced1b      | 315283    | 3.652  | 0.00138  | PC-esterase domain containing 1B |              |
| LOC103691883| 103691883| 2.475  | 0.00153  | uncharacterized LOC103691883 |              |
| Sept11      | 305227    | 1.017  | 0.00166  | septin 11   | rno-miR-30b-5p, rno-miR-30d-5p, rno-miR-290 |
| Ptp4a2      | 85237     | 1.114  | 0.00175  | protein tyrosine phosphatase type IVA, member 2 |              |
| S100a1      | 291254    | 1.243  | 0.00175  | S100 calcium binding protein A1 |              |
| LOC102547613| 102547613| 2.412  | 0.00175  | uncharacterized LOC102547613 |              |
| LOC103691014| 103691014| 1.545  | 0.00175  | uncharacterized LOC103691014 |              |
| Rnf126      | 314613    | 2.96   | 0.00186  | ring finger protein 126 |              |
| LOC103693092| 103693092| 2.542  | 0.00191  | uncharacterized LOC103693092 |              |
| Sept11      | 29360     | 1.164  | 0.00194  | selenoprotein P, plasma, 1 |              |
| Lrcc25      | 498605    | 1.536  | 0.00208  | leucine rich repeat containing 25 |              |
| Nkain3      | 689576    | 1.466  | 0.00219  | Na+/K+ transporting ATPase interacting 3 |              |

**Table 1.** Top 20 DEGs based on p-value from microarray expression analysis of DA neurons following perinatal nicotine exposure compared to saline control. Predicted miRNA targets are also included.
Significantly differentially expressed miRNAs (DEmiRs) in DA neurons of the VTA following perinatal nicotine exposure.

| miRNA Accession | miRNA name | logFC | adj p val |
|-----------------|------------|-------|-----------|
| MIMAT000836     | rno-miR-130a-3p | 0.862 | 0.00001   |
| MIMAT000779     | rno-let-7i-5p   | 0.912 | 0.00001   |
| MIMAT000830     | rno-miR-125b-5p | 1.106 | 0.00007   |
| MIMAT000781     | rno-miR-9a-5p   | 1.544 | 0.00029   |
| MIMAT000560     | rno-miR-326-3p  | 0.541 | 0.00662   |
| MIMAT000470     | rno-miR-9a-3p   | 0.996 | 0.00777   |
| MIMAT000552     | rno-miR-301a-3p | 0.669 | 0.02011   |
| MIMAT000821     | rno-miR-99b-5p  | 0.185 | 0.03329   |
| MIMAT0024854    | rno-miR-6215    | 0.350 | 0.03372   |
| MIMAT000796     | rno-miR-26a-5p  | 0.380 | 0.03383   |
| MIMAT000575     | rno-miR-335     | 0.368 | 0.04411   |
| MIMAT000826     | rno-miR-107-3p  | 0.354 | 0.04429   |
| MIMAT000791     | rno-miR-22-3p   | 0.460 | 0.04945   |
| MIMAT000855     | rno-miR-153-3p  | 0.574 | 0.05339   |
| MIMAT000842     | rno-miR-136-5p  | 0.738 | 0.00586   |
| MIMAT000812     | rno-miR-33-5p   | 0.190 | 0.00610   |
| MIMAT003383     | rno-miR-497-5p  | 0.509 | 0.00635   |
| MIMAT000801     | rno-miR-29b-3p  | 0.459 | 0.00758   |
| MIMAT000806     | rno-miR-30b-5p  | 0.247 | 0.00759   |
| MIMAT000596     | rno-miR-551b-3p | 0.372 | 0.00788   |
| MIMAT0000553    | rno-miR-324-5p  | 0.461 | 0.00814   |
| MIMAT000615     | rno-miR-101b-3p | 0.410 | 0.01005   |
| MIMAT000877     | rno-miR-204-5p  | 0.477 | 0.01066   |
| MIMAT000820     | rno-miR-99a-5p  | 0.293 | 0.01115   |
| MIMAT0012833    | rno-miR-582-5p  | 0.220 | 0.01237   |
| MIMAT0000573    | rno-miR-140-5p  | 0.407 | 0.01283   |
| MIMAT0003178    | rno-miR-542-5p  | 0.191 | 0.01377   |
| MIMAT000823     | rno-miR-101a-3p | 0.409 | 0.01401   |
| MIMAT000870     | rno-miR-195-5p  | 0.389 | 0.01430   |

| miRNA Accession | miRNA name | logFC | adj p val |
|-----------------|------------|-------|-----------|
| MIMAT0024853    | rno-miR-6216 | −1.375| 0.00003   |
| MIMAT0012827    | rno-miR-1224 | −0.718| 0.00026   |
| MIMAT0024853    | rno-miR-3473 | −0.736| 0.00568   |
| MIMAT0005278    | rno-miR-466b-5p| −0.863| 0.00802   |
| MIMAT0005301    | rno-miR-188-5p| −0.249| 0.00864   |
| MIMAT0033572    | rno-miR-1896 | −0.818| 0.01267   |
| MIMAT0025048    | rno-miR-3099 | −0.861| 0.01347   |
| MIMAT0003382    | rno-miR-664-3p| −0.593| 0.01739   |

Table 2. Significantly differently expressed miRNAs (DEmiRs) in DA neurons of the VTA following perinatal nicotine exposure.

from pathway enrichment and miRNA-gene target prediction. Significant miRNAs and mRNAs were selected for further analysis using RT-qPCR in order to validate the results of the microarrays. For mRNA microarray validation, two previously reported DA neuron markers were chosen, Cck and Gabrg2, as well as the significant DEGs found in the integrated miRNA-gene network, Scn1a, Ntrk2, and Ablim3. The direction of regulation resulting from RT-qPCR was consistent with the microarray results from our experiments, indicating that the data produced by the microarray experiments were valid. Regulation was determined using Student’s t-test comparing nicotine and saline treatment groups and corrected for multiple testing using the BH method with false discovery rate of 0.05 (Fig. 5).

Discussion

The current study is the first to conduct a large-scale profile of both miRNA and mRNA expression in DA neurons located in the VTA of rats following perinatal nicotine exposure. In our current work, the offspring was exposed to nicotine over a period equivalent to the three trimesters of human pregnancy. Our study sought to identify significant genes and miRNAs in DA neurons modulated by perinatal nicotine exposure. After differential expression analysis, our results showed 1,774 DEGs were downregulated and 862 DEGs were upregulated. Additionally, 16 DEmiRs were downregulated and 58 DEmiRs were upregulated. The gene expression profile of DA neurons was analyzed using DAVID for functional enrichment analysis. Enriched KEGG pathways were analyzed in order
to summarize putative processes modulated by perinatal nicotine exposure compared to saline. In addition, target predictions for DEmiRs were compared with DEG results in order to identify miRNA-gene pairs that may be changed after perinatal nicotine exposure. Further analysis of these results revealed potential points of pathway regulation by DEmiRs that may be involved in altered mechanisms following perinatal nicotine exposure.

Many gestational or perinatal nicotine exposure studies have focused on nicotinic acetylcholine receptors (nAChRs), which were reduced following gestational nicotine exposure in adolescent rats. One study exposed rats to nicotine during fetal and neonatal brain development (similar to the time of exposure used in our study)\(^1\). They found that during P7-14, the number of high-affinity nAChRs reach their highest levels in most brain regions\(^1\), which is the time period of our experiment. They also found decreased expression of multiple nAChR subunits including \(\beta_4\) mRNA subunit (Chrnb4), which we found significantly reduced as well. Additionally, Chrnb4 is involved in the neuroactive ligand-receptor interaction and cholinergic synapse pathways, which were significantly enriched by downregulated DEGs. The \(\beta_4\) subunit is only expressed in about 10% of DA neurons with a much higher expression in GABA neurons, but the time point of our study and equivalent amount of DA neuron total RNA may account for the expression pattern of nAChRs found in this experiment. Further studies focusing specifically on the nAChR subunits are necessary in order to determine if nAChR mRNA expression is decreased due to nicotine exposure or if reduced expression is caused by decreased DA neuron population.

Notably, the nicotine addiction KEGG pathway was enriched from our downregulated DEGs and four genes were found to be significant among our results. The enrichment of the nicotine addiction pathway indicates that there may be common genetic alterations in the brain that occur between gestational nicotine-treated offspring and adults with nicotine dependence. The first two genes, Gabrd and Gabrg2, are subunits of the \(\gamma\)-Aminobutyric

Figure 2. Predicted miRNA-mRNA target network. (a) Network of predicted and experimentally validated miRNA-mRNA target interactions using inversely correlated DEG targets and their DEmiR with highest degree of connectivity. (b) rno-miR-125a-5p is predicted to target the most number of genes within our DEG results using negative correlation.
results. A recent study by Stojakovic et al. investigated the selective deletion of Gabrg2 on DA neurons. Mice with the deletion showed a high risk of developing alcohol addiction based on behavior and an increased concentration of alcohol in blood32. These results suggest that despite the expression of other subunits of the GABA-A receptor, Gabrg2 expression is important in DA neurons and DA neurotransmission in response to drugs. Another study investigating DA neurons in the VTA following cocaine exposure found GABA-A receptor subpopulations to be significantly downregulated15. While this study did not use a model of gestational drug exposure, it focused on alterations within VTA DA neurons following chronic drug exposure.

The last two genes were downregulated in our results and encode subunits of glutamate receptors. Grin2d is the 2D subunit of N-methyl-D-aspartate (NMDA) receptors, which are a class of glutamate ionotropic receptors. Gria3 is subunit 3 of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) type glutamate ionotropic receptors. A study by Roguski et al. investigated intra-VTA NMDA receptors and concluded that a subpopulation of NMDA receptors were responsible for increased vulnerability to DA release16. These results suggest that despite the expression of other subunits of the GABA-A receptor, Gabrg2 expression is important in DA neurons and DA neurotransmission in response to drugs.

To date, a vast body of research has been conducted investigating the implication of miRNAs in addiction using different drugs of abuse. However, our present study investigates, for the first time, the large-scale effects of miRNAs in VTA DA neurons after perinatal nicotine exposure. We recently reported the upregulation of miR-140-3p, which we confirmed with our current results26. Further studies should be conducted on all receptor subunits in order to determine if GABA and glutamate receptors on DA neurons are significantly modulated by perinatal nicotine exposure.

Table 3. KEGG pathways enriched by down or upregulated DEGs and the corresponding genes identified in pathway analysis.

| KEGG Term                          | p value | Genes                                                                 |
|------------------------------------|---------|-----------------------------------------------------------------------|
| Downregulated                      |         |                                                                       |
| Neuroactive ligand-receptor interaction | 8.56E-06 | Htrr1, Grnk2, Slpr2, Gabrg2, Htr4, Tac3, Sotr3, Adra1b, Gabrd, Grnk3, Grnk2d, Galr2, Gria3, Chrnb4, Chrnm3, Gria4 |
| Insulin secretion                  | 1.59E-04 | Camkg2, Stx1a, Atplb1, Kcnmb4, Kcn1, Kcn2, Chrnm3                     |
| Dopaminergic synapse                | 3.55E-04 | Mapk8, Caly, Camkg2, Scn1a, Camkg2, Ppp1ca, Gsk3b, Itpr1, Gria3       |
| Calcium signaling pathway          | 9.03E-04 | Camkg2, Camkg2, Grin2d, Itpr1, Htr4, Cacna1g, Cacna1h, Tac3, Adra1b, Chrnm3 |
| Aldosterone synthesis and secretion | 9.54E-04 | Camkg1, Camkg2, Camkg2, Itpr1, Camk1, Cacna1g, Cacna1b                |
| African trypanosomiasis            | 6.01E-03 | LOC689064, Hbb, Hbb-b1, LOC10134871, Hba2                            |
| Amphetamine addiction             | 6.01E-03 | Camkg2, Camkg2, Ppp1ca, Grin2d, Stx1a, Gria3                         |
| Circadian entrainment              | 6.01E-03 | Camkg2, Camkg2, Grin2d, Itpr1, Gria3, Cacna1g, Cacna1h                |
| Cholinergic synapse                 | 6.01E-03 | Camkg2, Camkg2, Kcn12, Kcnq1, Itpr1, Chrnb4, Chrnm3                  |
| Glutamatergic synapse              | 6.01E-03 | Camkg2, Camkg2, Grin2d, Itpr1, Gria3, Gls, Grnm3, Gria4               |
| AMPK signaling pathway             | 6.01E-03 | Mapk8, Camkg2, Camkg2, Ppp1ca, Gnm2d, Gria3, Htr4, Atplb1, Pak1       |
| Oxytocin signaling pathway         | 6.01E-03 | Camkg1, Camkg2, Camkg2, Ppp1ca, Kcn12, Map2k5, Itpr1, Camk1           |
| Malaria                            | 6.01E-03 | LOC689064, Hbb, Hbb-b1, LOC10134871, Hba2                            |
| Long-term potentiation             | 6.01E-03 | Camkg2, Camkg2, Ppp1ca, Grin2d, Itpr1                                 |
| Nicotine addiction                 | 6.01E-03 | Grin2d, Gabrg2, Gria3, Gabrd                                         |
| Gastric acid secretion             | 6.01E-03 | Camkg2, Camkg2, Itpr1, Atplb1, Chrnm3                                |
| ErbB signaling pathway             | 6.01E-03 | Mapk8, Camkg2, Camkg2, Gsk3b, Pak1                                   |
| Alzheimer’s disease                | 6.01E-03 | Atplb1, Gsk3b, Grin2d, Atp5c1, Itpr1, App, Apvh1                      |
| Uregulated                         |         |                                                                       |
| Inflammatory mediator regulation of TRP channels | 6.01E-03 | Il1r1, Adcy2, Cyp2c7, Htr2a, Trpv3                                    |
| HTLV-I infection                   | 6.01E-03 | Il1r1, Pdgfa, Aacy2, Pold4, RT1-N2, RT1-A3, Katri                        |
| Pancreatic secretion               | 6.01E-03 | Slc4a2, Pla2g2, Aacy2, Clca4                                         |
| Choline metabolism in cancer       | 6.01E-03 | Cbpt1, Pdgfa, Slc22a4, Pcyt1b                                        |
| Glutamatergic synapse              | 6.01E-03 | Adcy2, Grin2d, Gnsq4, Slc1a6                                         |
| Serotonergic synapse               | 6.01E-03 | Htr6, Cyp2c7, Gnsq4, Htr2a                                           |
| Neuroactive ligand-receptor interaction | 6.01E-03 | Gira1, Htr6, Grin2d, Htr2a, Chrma1, Htr4                             |

acid (GABA) A receptor which were both significantly downregulated in our results. Our microarray tested the α-, β-, and γ-subunits of the GABA-A receptor, but these were not significantly differentially expressed in our results. A recent study by Stojakovic et al. investigated the selective deletion of Gabrg2 on DA neurons. Mice with the deletion showed a high risk of developing alcohol addiction based on behavior and an increased conditioned place preference to alcohol. These results suggest that despite the expression of other subunits of the GABA-A receptor, Gabrg2 expression is important in DA neurons and DA neurotransmission in response to drugs. Another study investigating DA neurons in the VTA following cocaine exposure found GABA-A receptor subunits, including Gabrg2, to be significantly downregulated. While this study did not use a model of gestational drug exposure, it focused on alterations within VTA DA neurons following chronic drug exposure.

The last two genes were downregulated in our results and encode subunits of glutamate receptors. Grin2d is the 2D subunit of N-methyl-D-aspartate (NMDA) receptors, which are a class of glutamate ionotropic receptors. Gria3 is subunit 3 of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) type glutamate ionotropic receptors. A study by Roguski et al. investigated intra-VTA NMDA receptors and concluded that a subpopulation of NMDA receptors were responsible for increased vulnerability to DA release in NAc. In addition, previous studies indicated that the expression of NMDA subunits was unchanged due to chronic ethanol exposure followed by withdrawal, but the function of the receptor was changed. Further investigation needs to be conducted on all receptor subunits in order to determine if GABA and glutamate receptors on DA neurons are significantly modulated by perinatal nicotine exposure.

To date, a vast body of research has been conducted investigating the implication of miRNAs in addiction using different drugs of abuse. However, our present study investigates, for the first time, the large-scale effects of miRNAs in VTA DA neurons after perinatal nicotine exposure. We recently reported the upregulation of miR-140-3p after perinatal nicotine exposure. From our miRNA expression results, miR-140-3p and miR-140-5p were both significantly differentially expressed and have both been implicated in nicotine addiction. Huang et al. found that miR-140-3p expression was increased by nicotine, which then repressed the expression of dynamin 1 (Dnm1) by direct base-pairing to the 3′-untranslated region of the gene. In accordance with their results, miR-140-3p was significantly upregulated and Dnm1 was downregulated in our results, although not significantly. In our previous study, we investigated the modulation of addiction-related miRNAs by nicotine and found upregulation of miR-140-5p, which we confirmed with our current results. Further studies should be conducted on...
miR-140-3p and miR-140-5p and their potential gene targets in order to elucidate their relationship to nicotine exposure.

The miRNA-gene network indicated that miR-125a-5p was the most highly predicted miRNA based on DEG targets (Fig. 2b). A study by Bosch and colleagues identified miR-125a-5p as an addiction-related miRNA in a study investigating the changes in RNA expression in the VTA due to the self-administration of methamphetamine in rats29. Their study found an overall upregulation of miR-125a-5p in the VTA 29, which confirms the upregulation of miR-125a-5p in VTA DA neurons in our results. The significance of miR-125a-5p in the VTA in both of these studies implicates its role in regulating pathways related to addictive drugs, including methamphetamine and nicotine. Further study into the genes targeted by miR-125a-5p could provide a clearer picture of the precise nature of this miRNA in pathways related to substance abuse.

Our integrated network of DEGs and DEmiRs was created from the enriched KEGG pathways with the addition of significantly upregulated predicted miRNA in order to visualize putative points of pathway regulation by miRNAs. These pathways were enriched by the downregulated DEGs indicating important putative pathways for miRNA regulation as tested by hypergeometric testing with correction for multiple testing. Significantly upregulated predicted miRNAs were added to this network in order to visualize points of pathway regulation by miRNA. In the DAergic synapse pathway, miR-30b-5p was predicted to target Scn1a, a protein-coding gene involved in voltage-gated sodium channels (Fig. 3). As a whole interaction network, the probability of the involvement of miR-30b-5p in the DAergic synapse pathway was significant ($p < 0.01$). The enrichment of this pathway in our results demonstrates that perinatal nicotine exposure leads to genetic alterations in VTA DA neurons that are in accordance with addiction mechanisms. In addition, we have highlighted a potential gene-miRNA target interaction, which may contribute to the observed alterations via post-transcriptional gene regulation.
The neurotrophin signaling was enriched in our results at a significance $p < 0.1$. Our analysis revealed that Ntrk2 (also known as TrkB) was predicted to be targeted by miR-204-5p (dark blue). Additionally, rno-miR-195-5p (dark blue) was predicted to target Ikbkb. The predicted interaction of both miRNAs on the neurotrophin signaling pathway was significant ($p < 0.01$). Light blue nodes are significantly downregulated DEGs in the results and yellow nodes are genes that were not detected or not significantly in the results. In addition to significance, location of the mRNA has been indicated by color (pink for membrane, orange for nucleus, otherwise cytoplasm).

**Figure 4.** Enriched KEGG neurotrophin signaling network following perinatal nicotine exposure in DA neurons. The neurotrophin signaling pathway describes the activation of multiple intracellular signal transduction pathways through the binding of Ntrk2 (TrkB) to Bdnf on the cell membrane. Our results predicted that Ntrk2 was targeted by miR-204-5p (dark blue). Additionally, rno-miR-195-5p (dark blue) was predicted to target Ikbkb. The predicted interaction of both miRNAs on the neurotrophin signaling pathway was significant ($p < 0.01$). Light blue nodes are significantly downregulated DEGs in the results and yellow nodes are genes that were not detected or not significantly in the results. In addition to significance, location of the mRNA has been indicated by color (pink for membrane, orange for nucleus, otherwise cytoplasm).

In summary, we focused on the response of VTA DA neurons to perinatal nicotine exposure and investigated transcriptional and post-transcriptional regulation disruptions. We also concentrated on the identification of biological pathways modulated by nicotine within the DA neurons of the VTA. Our study suggested that the dopaminergic synapse pathway was significantly altered by perinatal nicotine exposure as well as significant interactions with miRNAs. Although our results indicate that perinatal nicotine exposure alters the expression of miRNAs and genes, demonstrating the involvement of several biological pathways in the mechanism of addiction in DA neurons of the VTA, we did not investigate the effect of gender on DA neurons in the VTA following perinatal nicotine exposure in the current study. Such an investigation merits further exploration as there is evidence indicating nicotine exposure differentially affects gene expression changes in many different regions of the brain depending on gender[41]. Additionally, further investigation needs to be performed to develop an interactive model to evaluate the effect of miRNAs on biological pathways.
Materials and Methods

Animal treatment. All experiments were performed in accordance with the protocols and surgical procedures that were approved by the Institutional Animal Care and Use Committee (IACUC) and the University of Houston Animal Care Operations (ACO). Pregnant female Sprague–Dawley (SD) rats (Charles River, Wilmington, MA, USA) were maintained on a 12-h light/12-h dark schedule at a temperature of 22 ± 2 °C and 65% humidity. Access to standard food and water was ad libitum. Rats were acclimated to the animal facility for 72 hours before they received treatment via an osmotic minipump (Alzet, Cupertino, CA) that was implanted subcutaneously containing either nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, MO, USA) released at a rate of 6 mg/kg/day (moderate to heavy smokers5,12), or an equal volume of saline vehicle for the control. Nicotine continues to be released from the osmotic minipump for 4 weeks from gestational day 6 to postnatal day 14. Seven-to-fourteen day-old pups (male and female) were anesthetized with isoflurane before decapitation. On a VT1200 semiautomatic vibrating blade microtome (Leica, Nussloch, Eisfeld, Germany), horizontal slices containing VTA were cut at a thickness of 1000 μm. Brain punches containing the VTA were collected bilaterally using a 1 mm biopsy punch (Integra Miltex, VWR, Radnor, PA, USA) and placed in 1 mL of Hibernate A (Gibco, Thermo Fisher Scientific, USA) on ice to maintain cell viability. Brain punches were pooled so that one litter resulted in one litter. A total of four samples for both saline and nicotine treated groups were processed for RNA extraction and microarray processing.

Cell Dissociation, FACS, and RNA extraction. Tissue was dissociated into a single cell solution and sorted by FACS as reported by Guez-Barber et al.32. Briefly, tissue punches were placed in 1 mL of Accutase (Gibco, Thermo Fisher Scientific, USA) and shaken for 30 minutes at 4 °C in order to dissociate the tissue punches. Accutase is a mixture of proteolytic and collagenolytic enzymes, which showed to produce the most single cells with the least cell damage32. Cells were pelleted at 425 x g and resuspended in Hibernate A (Gibco, Thermo Fisher Scientific, USA) on ice to maintain cell viability. Brain punches were pooled so that one litter resulted in one litter. A total of four samples for both saline and nicotine treated groups were processed for RNA extraction and microarray processing.

Figure 5. RT-qPCR validation results of microarray data comparing gene (top) and miRNA (bottom) expression in DA neurons in the VTA comparing perinatal exposure to nicotine and saline control. Six DEGs (top) were tested and five DEmiRs to assess validity of microarray experiments using the same total RNA sample used in microarray experiments. The results from RT-qPCR validation experiments are shown as ΔCt values relative to control: GAPDH for mRNA (top) and U6 snRNA for miRNA (bottom) experiments. Significance was evaluated using Student’s t-test (n = 3) and corrected for multiple comparisons using Benjamini–Hochberg procedure with false discovery rate of 0.05 (*p < 0.05, **p < 0.01).
resuspending them in equal parts Hibernate A and 100% cold ethanol, gently vortexed, and kept on ice for 15 minutes. Cells were co-labeled with conjugated primary antibodies neuronal marker, NeuN/Alexa Fluor 488 (NeuN/AF488, ab190195, Abcam, Cambridge, MA, USA), and tyrosine hydroxylase/phycocerythrin (TH/PE, ab209921, Abcam, Cambridge, MA, USA). Using conjugated antibodies simplifies the staining procedure by eliminating the need for a secondary antibody, which can bind unspecifically and reduces the time it takes to stain cells.

An Influx (BD Biosciences, San Jose, CA, USA) instrument was used to sort the cells at the Flow Cytometry and Cellular Imaging Core Facility (MD Anderson – South Campus, Houston, TX, USA). Samples were sorted based on double-positive NeuN⁺/TH⁺ staining. Once sorted, cells were centrifuged into a pellet at 2650 × g for 8 minutes at 18°C. Total RNA was isolated using miRNeasy Micro Kit (Qiagen, Hilden, Germany) following manufacturer’s instructions, including DNase treatment and used in subsequent microarray and RT-qPCR validation experiments. RNA quality and quantity was established according to the optical density (OD) of each sample at 260 nm and 280 nm determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only total RNA samples with A260/280 ratio of 1.9 or greater were used in subsequent experiments.

Data Analysis. All pre-processing, normalization, and statistical analyses were performed using several Bioconductor packages in R version 3.4.2. The quality of microarray data was assessed using arrayQualityMetrics package. Raw mRNA expression data was imported using limma package. Before normalization, probes expressing Agilent flags for feature and background outliers were removed from the analysis. Raw median intensity values were background corrected using “normexp” method and quantile normalized. Control probes were filtered out as well as low expressed probes, which were defined by expression intensity less than 10% brighter than 95% intensity of negative controls. Replicated probes were averaged and a total of 27,791 genes were selected. Genes that remained for analysis. Quality assessment was performed to check for outliers among microarrays. A linear fit model was used to calculate fold change and standard errors for each gene of interest. To increase the power of the data, standard errors were moderated using the eBayes function, which applies a simple empirical Bayes model and corrects for multiple testing. Hypergeometric testing was performed on target DEGs negatively correlated with DEmiRs using eBayes, similar to mRNA data analysis. Differential expression was identified using q-value threshold <0.05 (calculated using BH method)28,29.

Integrated analysis of miRNA-mRNA. Functional enrichment analysis was performed on differentially expressed genes using DAVID v6.8.20,21. Enriched KEGG pathways were identified and analyzed. Further analysis of KEGG pathways was done using DEgraph and KEGGgraph. MultiMiR33,34 was used to identify predicted and validated miRNA-gene target pairs based on inversely correlated regulation of DEmiRs and DEGs. multiMiR is a comprehensive compilation of predicted and validated miRNA-gene target interactions from 14 external databases. Hypergeometric testing was performed on target DEGs negatively correlated with DEmiRs using pairwise Pearson correlation analysis and then BH corrected for multiple comparisons. Remaining significant miRNA-gene target pairs were integrated into gene network models based on enriched KEGG pathways. Following the procedure used in the miRPathDB26, we performed a hypergeometric test with multiple testing correction using BH method and an FDR < 0.05 for each miRNA-gene predicted interaction. miRPathDB is a tool to measure the association between miRNAs and putative target pathways.
Quantitative RT-PCR validation of microarray data. All reagents and kits for quantitative reverse transcription polymerase chain reaction (RT-qPCR) were purchased from Applied Biosystems (Thermo Fisher Scientific, Carlsbad, CA, USA) unless otherwise stated. Seven genes and five miRNAs were chosen for validating microarray data using RT-qPCR. Total RNA was isolated from FACS samples for each experimental group as described above. For gene expression validation, cDNA was prepared using High Capacity cDNA Reverse Transcription Kit according to manufacturer’s instructions. For miRNA expression validation, cDNA was prepared and preamplified using TaqMan Advanced miRNA cDNA Synthesis Kit according to manufacturer’s instructions. For gene validation and miRNA validation, quantitative PCR (qPCR) was carried out using TaqMan Fast Advanced Master Mix and corresponding TaqMan Assay (TaqMan Gene Expression Assay or TaqMan Advanced miRNA Assay) on a StepOnePlus Real-Time PCR System according to manufacturer’s instructions using the following parameters: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 1 sec at 95 °C and 20 sec at 60 °C. Each reaction was prepared in triplicate for both validation sets. Comparative Ct method was used to find the relative quantity of the target genes or miRNAs. Student’s t-test was performed comparing nicotine and saline treatment groups (n = 3) and corrected for multiple testing using the BH method with false discovery rate of 0.05.

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Acknowledgements

We would like to thank Tina Kazemi for her technical assistance with the experiment and John Hite for editing this manuscript. This study was funded by the University of Houston. Cells were sorted at MD Anderson FACS Core facility, which is partially funded by NCI Cancer Center Support Grant P30CA16672.

Author Contributions

R.F., A.D., Y.F., Y.M.A. and M.A. designed the experiment. R.F. conducted the experiments. All authors interpreted the data and wrote and reviewed the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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