

Article

Developmental Nicotine Exposure Induces Intergenerational Transmission of an Ensemble of Neurodevelopmental Disorder-Related Translational Perturbations in DRD1-Expressing Striatal Cells of Adolescent Male Mice

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Abstract

Background/Objectives: Coupled with the already-problematic background rates of traditional cigarette consumption during pregnancy, the surging epidemic of electronic cigarette usage among pregnant women redoubles the importance of understanding the impacts of nicotine exposure during critical periods of development. To date, a burgeoning body of human epidemiological and animal model research indicates that not only the children but also the grandchildren of maternal smokers are at higher risk for neurodevelopmental disorders such as ADHD, autism, and schizophrenia and are predisposed to neurodevelopmental abnormalities which transcend these diagnoses. However, the roles of discrete cellular sub-populations in these and other intergenerational consequences of smoking during pregnancy remain indeterminate. **Methods:** Toward the resolution of this void in the literature, the present study characterized alterations in the gene expression profiles of dopamine receptor D1-expressing striatal cells from the first- and second-generation male progeny of female mice that were continuously exposed to nicotine beginning prior to conception, continuing throughout pregnancy, and concluding upon weaning of offspring. **Results:** Dopamine receptor D1-expressing striatal cells from our mouse models of the children and grandchildren of maternal smokers exhibit differential expression patterns for a multitude of genes that are (1) individually associated with neurodevelopmental disorders, (2) collectively overrepresented in gene set annotations related to brain, behavioral, neurobiological, and epigenomic phenotypes shared among neurodevelopmental disorders, and (3) orthologous to human genes that exhibit differential DNA methylation signatures in the newborns of maternal smokers. **Conclusions:** Together with our and others' previous findings, the results of this study support the emerging theory that, by inducing extensive alterations in gene expression that in turn elicit cascading neurobiological changes which ultimately confer widespread neurobehavioral abnormalities, nicotine-induced epigenomic dysregulation may be a primary driver of neurodevelopmental deficits and disorders in the children and grandchildren of maternal smokers.

Keywords: nicotine; pregnancy; neurodevelopment; intergenerational; translational; striatum; epigenetics



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1. Introduction

The rates of combustible and electronic cigarette use among pregnant women in the United States are approximately ten and fourteen percent, respectively, and are greater still in other nations [1,2]. Concerningly, the majority of individuals surveyed perceive electronic cigarettes as a safer and healthier alternative to conventional cigarettes, and this misconception is most widespread among women of reproductive age [3,4]. In truth, maternal smoking (MS) of traditional or electronic cigarettes, or consumption of any tobacco- or nicotine-containing products during the gestational and/or postpartum lactational periods, invariably exposes three generations to nicotine: the mother herself, her unborn or newborn child, and (via germline exposure of the child) her grandchildren. MS carries dire consequences for children and grandchildren regardless of the timing or method of nicotine consumption. For example, MS is linked to increased fetal and neonatal mortality in the forms of stillbirth, miscarriage, and sudden infant death syndrome and is associated with a variety of neonatal morbidities including prematurity, low birth weight, and birth defects. Many of these outcomes associated with MS become more likely as the quantity consumed increases [5–7]. In addition to costing the lives and livelihoods of numerous newborns each year, MS results in massive annual healthcare costs which exceeded \$1.16 billion in 2020 [8].

Beyond the infant morbidities and mortality associated with MS, there are also enhanced risks for neurodevelopmental deficits and neurodevelopmental disorders (NDDs) including ADHD, autism, and schizophrenia in the children of maternal smokers [9–14]. Of even greater concern, contemporary research links not only MS but also grandmaternal smoking (GMS) to enhanced ADHD risk and autism symptom severity, implying that the predisposition to NDDs conferred by MS may be heritable, a highly unsettling prospect in light of the human and monetary costs of NDDs like ADHD, autism, and schizophrenia, which totaled over \$400 billion in 2014 and have steadily risen ever since [15–18].

Independent of NDD diagnosis, the children of maternal smokers disproportionately exhibit biobehavioral markers consistent with several transdiagnostic features of NDDs, which are defined as core symptom domains shared across NDDs including but not limited to ADHD, autism, and schizophrenia [19]. MS-related child phenotypes align with transdiagnostic features of NDDs including *neurobehavioral deficits* (e.g., hyperactivity, inattention, impulsivity, impaired learning, memory, and cognition, and predispositions to smoking and substance abuse), *developmental encephalopathy* (e.g., reduced brain and gray matter volume and myelination, cortical thinning, and aberrant neural and brain-regional development and differentiation), *neuroteratogenicity* (e.g., long-term neurological morbidity, decreased neuronal density, impaired neuronal activity, and increased oxidative stress and apoptosis), *neurotransmitter system dysfunction* (e.g., anomalies within cholinergic, monoaminergic, and amino-acidergic neurotransmitter systems), *neurotrophic dysfunction* (e.g., atypification of BDNF levels, BDNF signaling, neuronal differentiation and development, learning and memory, and synaptic plasticity), *HPA axis alterations* (e.g., hypocortisolemia, aberrant glucocorticoid receptor expression, and altered stress responses), *neuroimmune and neuroinflammatory aberrations* (e.g., dysfunction of innate and adaptive immune systems, aberrant crosstalk between neurons and glia, and deregulation of inflammatory factors such as cytokines, chemokines, and eicosanoids), and *epigenomic perturbations* (e.g., anomalous global and gene-specific DNA methylation profiles, histone modifier expression and modification patterns, and nucleosome/chromatin architectures) [9,14,20–47]. For many of the abovementioned phenotypes, the relative risks associated with MS exhibit positive dose–response relationships with quantities smoked during pregnancy [48,49]. Despite robust correlational evidence from epidemiological studies linking MS to NDDs and trans-

diagnostic features thereof, the molecular mechanisms underpinning these phenomena are poorly understood.

However, the etiology of the heritable neurodevelopmental impacts of MS is difficult to elucidate in human subjects due to the scarcity of multigenerational datasets and tissue banks along with inherent experimental limitations, and developmental nicotine exposure (DNE) animal models of MS are ideally suited for this task. Cumulatively, DNE animal model studies reveal extensive brain and behavioral anomalies congruent with each of the eight aforementioned transdiagnostic features of NDDs, and many of these phenotypes appear to undergo intergenerational transmission in DNE animal models of GMS. For instance, we and others have demonstrated that not only DNE progeny but also DNE grand-progeny exhibit NDD-like hyperactivity and risk-taking behaviors, aberrant rhythmicity of activity, increased nicotine consumption and preference, therapeutic-like behavioral responsivity to methylphenidate and nicotine, impaired brain and neuronal development (e.g., premature differentiation, atypical morphology, apoptosis, disrupted maturation, and aberrant migration of neurons), reduced cortical volume and thickness, and multiple corticostriatal perturbations including nicotinic acetylcholine receptor (nAChR) and dopamine transporter (DAT) dysfunction, impaired proBDNF proteolysis and glucocorticoid receptor (GR) activation, hypocortisolemia, alterations in the global DNA methylome, and deficits in DNMT3A, MeCP2, and HDAC2 expression and function [50–80]. These findings in first- and second-generation DNE animal models recapitulate the pathosymptomatology and transdiagnostic features of several NDDs, including ADHD, autism, and schizophrenia [23,24,34–36,81–89]. Our previous research developing DNE mouse models and examining the intergenerational transmission of NDD-like phenotypes is recollected in the supplement (Figure S1), as the design and results of our prior work set the stage for the current study.

Collectively, the human and animal model studies indicate that MS and GMS confer a constellation of multigenerational neurodevelopmental anomalies that may be mediated by changes in cell type-specific gene expression programs which could be underpinned by epigenomic aberrations. However, the specific cell types and gene expression programs potentially impacted by MS-induced epigenomic alterations remain unknown.

Among the many neuronal subtypes that could be substrates for both the intergenerational impacts of MS/DNE and the etiology of NDDs, a primary candidate is D1-type striatal medium spiny neurons (MSNs), which selectively express the stimulatory G-protein (G α S)-coupled DA receptor D1 (DRD1) and are key components of corticostriatothalamic circuits, where they receive inputs from the frontal cortices (and thalami) and relay outputs to the thalami via the direct pathway [90–94]. Underscoring their relevance to MS and NDDs, D1-type striatal MSNs are subject to activity regulation by cholinergic interneurons, undergo plasticity changes in response to nicotine, are substrates for nicotine dependence, and are mediators of locomotor activity, behavioral impulsivity, and rhythmic behaviors [95–100]. Moreover, both DRD1 polymorphisms and dysfunction of D1-type striatal MSNs are linked to increased risks for ADHD, autism, and schizophrenia and to transdiagnostic features of thereof including hyperactivity, impulsivity, inattention, proclivity to smoke, psychostimulant treatment responsivity, and neurocircuitual, DAergic, and epigenetic anomalies [97,99–107]. Despite these findings implicating DRD1 and D1-type striatal MSNs in neurodevelopmental deficits and NDDs, D1-type striatal MSNs have been understudied compared to other neuronal subtypes. Toward a mechanistic understanding of the intergenerational NDD-like phenotypes conferred by DNE and the NDD liabilities and neurodevelopmental abnormalities linked to MS and GMS, this study endeavored to elucidate the hitherto unknown impacts of DNE on the gene expression profiles of DRD1-expressing striatal cells in first- and second-generation progeny.

2. Materials and Methods

2.1. Reagents

Reagents and suppliers are listed throughout the Materials and Methods.

2.2. Animals

All experimental and housing conditions for the animals in this study were preapproved by the Institutional Animal Care and Utilization Committee at the University of Colorado Boulder and conform to the guidelines for animal care and use established by the NIH and the Guide for the Care and Use of Laboratory Animals (8th Ed.). All mice were bred and maintained in the same on-site animal facility using a standard 12-hour light/dark cycle (lights on at 07:00) and with food (Envigo Teklad 2914 irradiated rodent diet, Harlan, Madison, WI, USA) and fluids provided ad libitum. Male DRD1-EGFP/Rpl10a mice (RRID: IMSR_JAX:030254), which express enhanced green fluorescent protein (EGFP)-tagged ribosomes in DRD1-positive cells, were used for all experiments. DRD1-EGFP/Rpl10a DNE offspring and grandoffspring were bred via the intergenerational DNE paradigm utilized for our prior studies [108,109]. As schematized in Figure 1 and further exemplified in the supplement (Figure S1), at PND 60 randomly selected DRD1-WT (non-transgenic) female mice (zeroth generation, F0 dams) began passive oral exposure to 0.2% sodium saccharin (ThermoFisher, Waltham, MA, USA) (developmental vehicle exposure) or 0.2% sodium saccharin and 200 µg/mL freebase nicotine (MilliporeSigma, Burlington, MA, USA) (DNE) in place of drinking water. All solutions were replaced twice weekly. Following 30 days of vehicle or nicotine treatment, at PND 90, randomly selected DRD1-WT (non-transgenic) F0 dams were mated with drug-naïve DRD1-EGFP/Rpl10a (hemizygous for the DRD1-EGFP/Rpl10a transgene) sires. Vehicle or nicotine treatment of DRD1-WT F0 dams continued until weaning of F1 offspring at PND 21, whereafter water was provided to all F1 progeny as the sole fluid source. To foster second-generation (F2) DNE (F2 NIC) offspring, at PND 90 randomly selected DRD1-WT female F1 NIC mice (F1 NIC dams) were crossed with drug-naïve DRD1-EGFP/Rpl10a sires. Water was provided to all F1 NIC dams and all F2 NIC progeny as the sole fluid source. As such, all female F1 NIC offspring used for the breeding of F2 NIC progeny were not directly exposed to nicotine following weaning, and thus the transmission of any DNE-induced phenotypes from F1 NIC to F2 NIC offspring occurred solely via the F1 maternal germline (oocytes). To control for between-litter and between-breeder variability and to minimize other potential confounders, mice (or tissues therefrom) originating from a minimum of fifteen litters spawned by a minimum of ten total breeder pairs were assayed for each group in each experiment.

2.3. Tissue Collection and Processing

Brains from 32 DRD1-EGFP/Rpl10a-positive male mice per group (96 mice total) were harvested at PND 45 (Figure 1) using RNase-decontaminated tools [108]. Bilateral striata were immediately dissected using an ice-chilled RNase-decontaminated brain matrix, immersed in chilled RNA stabilization reagent (RNAlater, ThermoFisher, Waltham, MA, USA), and stored at −80 °C. Striata were exclusively collected from male F1 Veh, F1 NIC, and F2 NIC mice, as our prior work revealed no sex differences in the multigenerational impacts of DNE, and sample size constraints disallowed controlling for estrus cycle.

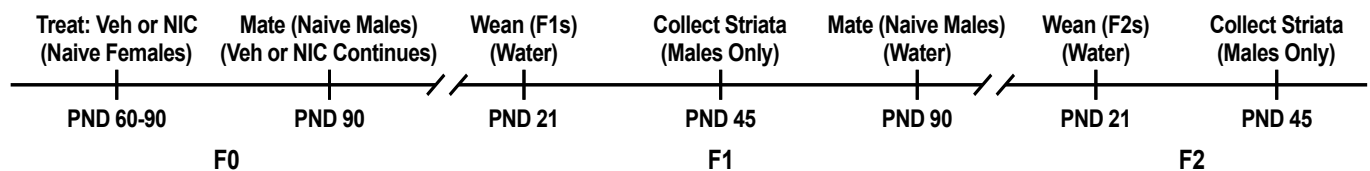


Figure 1. Workflows for DNE mouse model husbandry and striatal tissue collection. At PND 60, DRD1-WT (non-transgenic) dams (zeroth generation, F0) began passive oral exposure to either 0.2% saccharin alone (vehicle control) or 0.2% saccharin with 200 µg/mL nicotine (DNE). At PND 90, treated dams were mated with drug-naïve sires hemizygous for the DRD1-EGFP/Rpl10a transgene. Vehicle or nicotine exposure of F0 dams persisted until weaning of first-generation (F1) developmental vehicle (F1 Veh)- or nicotine (F1 NIC)-exposed progeny at PND 21. Thereafter, water was provided as the sole fluid source for all offspring. To breed second-generation (F2) DNE (F2 NIC) offspring, at PND 90 randomly selected DRD1-WT female F1 NIC mice were crossed with drug-naïve DRD1-EGFP/Rpl10a (hemizygous) sires. Following humane euthanasia on PND 45, whole brains were harvested from male F1 Veh, F1 NIC, and F2 NIC offspring hemizygous for DRD1-EGFP/Rpl10a, and bilateral striata were collected therefrom via manual dissection. PND, post-natal day; Veh, 0.2% aqueous sodium saccharin; NIC, 200 µg/mL nicotine in Veh.

2.4. Behavioral Characterization

To ascertain whether DRD1-EGFP/Rpl10a DNE offspring and grandoffspring exhibit intergenerational NDD-like behavioral anomalies consistent with those evinced by our prior studies in C57BL/6J mice as well as mice possessing a human SNP (rs16969968) on a C57BL/6J background (D379N), discrete cohorts of first- and second-generation DNE and first-generation vehicle/control DRD1-EGFP/Rpl10a mice (20 mice per group for all groups; 60 mice total) were administered the same battery of baseline behavioral assays utilized for our previous studies, which is detailed in Figure S2 [109,110]. This standardized behavioral testing protocol yielded measures of homecage locomotor activity patterns, homecage locomotor activity rhythms (see Figure S3 for more information), activity and risk-taking behaviors in the open field, and nicotine consumption and preference in DRD1-EGFP/Rpl10a DNE offspring and grandoffspring which were suitable for cross-comparison with baseline behavioral data from our previous investigations of first- and second-generation C57BL/6J and D379N DNE mice.

2.5. Translating Ribosome Affinity Purification and RNA Isolation

EGFP-tagged ribosome-mRNA complexes of DRD1-expressing striatal cells were isolated via translating ribosome affinity purification (TRAP) as schematized in the supplement (Figure S4A) [108,109]. Therein, thawed striatal tissues were transferred to chilled polysome extraction buffer (10 mM HEPES, pH 7.4; 150 mM KCl; 5 mM MgCl₂; 0.5 mM dithiothreitol; 10 µg/mL cycloheximide, protease inhibitors, and RNase inhibitors) and homogenized using a rotary homogenizer. Tissue homogenates were centrifuged at 2000× g for 10 min at 4 °C to remove cellular debris via fractionation. The resultant supernatants were adjusted to 1% NP-40 (EMD Biosciences, San Diego, CA, USA) and 30 mM 1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine (DHPC; Avanti Polar Lipids, Alabaster, AL, USA), incubated on ice for five minutes, and centrifuged for 10 min at 13,000× g to fractionate insoluble materials for discard. The supernatants obtained were then mixed with goat anti-EGFP (HtzGFP-19F7, RRID: AB_2716736; HtzGFP-19C8, RRID: AB_2716737 (Antibody and Bioresource Core Facility, Memorial Sloan Kettering Cancer Center, New York, NY, USA))-conjugated streptavidin Dynabeads (ThermoFisher, Waltham, MA, USA) and incubated with rotation for 30 min at 4 °C. Dynabead-polysome complexes were eluted using a magnetic rack and washed thrice with high-salt polysome wash buffer (10 mM HEPES, pH 7.4; 350 mM KCl; 5 mM MgCl₂; 1% NP-40, 0.5 mM dithiothreitol, and 100 µg/mL

cycloheximide). Lastly, RNA was purified from eluted polysomes using an RNeasy Kit (Qiagen, Redwood, CA, USA) in accordance with the manufacturer's protocol.

The cell type-selectivity of the TRAP-isolated RNA utilized for this study is an important factor that warrants consideration when assessing both the methods employed and the results obtained. Therein, while D1-type MSNs are the predominant DRD1-expressing cell type in the striata, single-cell RNA sequencing datasets such as that from the Q175 mouse model of Huntington's disease (available in Geo, GSE103345) indicate that DRD1 is expressed in both microglia and oligodendrocytes [111]. More broadly, DRD1 is expressed by multiple immune cell types sampled from both within and without the central nervous system [46,47]. Likewise, immune-related genes are expressed in DRD1-expressing neurons such as D1-type MSNs [112]. Accordingly, the translatomic profiles and differential translatomes reported herein likely represent composites of the individual translatomes of multiple DRD1-expressing striatal cell types, including but not limited to D1-type MSNs, oligodendrocytes, microglia, and various other classes of immune cells.

2.6. Quantification, Qualification, and Pooling of RNA Isolates

RNA isolates underwent initial quantification and qualification using a NanoDrop2000 spectrophotometer (ThermoFisher, Waltham, MA, USA). Results indicated that quadruplicate pooling of samples was necessary to achieve the minimum per-sample RNA input quantity for downstream cDNA library preparation and sequencing. Accordingly, RNA isolates from randomly selected subgroups of four mice were pooled in quadruplicate, yielding eight pooled samples per group which were subsequently assayed for RNA degradation and RNA purity by agarose gel electrophoresis and for precise RNA quantity and RNA integrity number (RIN) via an Agilent 2100 Bioanalyzer (Figure S4B). A single pooled sample (belonging to the F1 Veh control group) received an unsatisfactory RIN (RIN < 7.0) and was therefore replaced with a reserve pooled sample.

2.7. Library Preparation, Quality Control Screening, and High-Throughput Sequencing

After sample pooling, all RNA sequencing procedures were performed by Novogene (Sacramento, CA, USA) as described hereafter. The workflows for mRNA enrichment, cDNA library synthesis and quality control screening, and high-throughput sequencing are diagrammed in Figure S4B. Pooled RNA isolates that passed quality control screening were first subjected to polyA-selection using oligo(dt) beads. cDNA libraries were then synthesized from polyA-selected mRNA using a NEBNext Ultra II RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) in accordance with a modified protocol [113]. The cDNA libraries obtained were subjected to size selection (150 bp) followed by PCR amplification, after which the resulting cDNA libraries were submitted to quantification and quality control screening. Therein, a Qubit 2.0 was utilized to estimate cDNA library concentrations, an Agilent 2100 Bioanalyzer used to assess insert sizes, and quantitative PCR was performed to precisely quantify the effective cDNA library concentrations. Qualifying cDNA libraries were then subjected to paired-end sequencing via an Illumina NovaSEQ6000, which yielded approximately 20 million paired-end 150 bp reads (40 million actual 150 bp reads) for each pooled sample [113]. The raw data obtained were transformed to sequenced reads by base calling and stored in FASTQ files.

2.8. Bioinformatic Analyses

The bioinformatic analysis pipeline (Figure S4C) utilized for this study was designed in accordance with best practices [114]. Sequenced read datasets were quality-controlled ($Q > 30$) by FastQC, trimmed of adapter sequences via BBDuk, and aligned to the current Ensembl mouse genome using STAR [115–118]. Rsubread was then used to count aligned reads, yielding read count datasets which in turn underwent normalization and regularized log-transformation via DESeq2 [119,120].

2.9. Consensus Translatome Construction

A consensus translatome for DRD1-expressing striatal cells of DRD1-EGFP/Rpl10a mice was constructed via union (non-redundant aggregation) of the lists of all translating mRNA detected across F1 Veh, F1 NIC, and F2 NIC samples. Union of said lists was performed using the List Operations tool available at <https://molbiotools.com/listoperations.php> (accessed on 25 May 2024).

2.10. Comparative Translatomic Analysis

Comparative translatomic analysis was performed to indirectly evaluate the cell type-specificity of the translatomic data and the validity of the consensus translatome for DRD1-expressing striatal cells of DRD1-EGFP/Rpl10a mice which was utilized as the reference translatome for all annotation overrepresentation analyses. To this end, the number of elements shared between the consensus translatome (all translating mRNA detected) for DRD1-expressing striatal cells of DRD1-EGFP/Rpl10a mice and a previously reported consensus protein-coding transcriptome (all protein-coding mRNA detected) for DRD1-expressing striatal cells of male Drd1-tdTomato mice was determined by counting the number of intersecting elements. The degree of intersection (overlap) between the consensus translatome of DRD1-EGFP/Rpl10a mice and the consensus protein-coding transcriptome of Drd1-tdTomato mice was quantified by determination of the Jaccard similarity index [121]. Both shared element identification and Jaccard similarity indexing were conducted using the Multiple List Comparator tool accessible at <https://www.molbiotools.com/listcompare.php> (accessed on 25 May 2024).

2.11. Principal Component Analysis

To decrease the dimensionality of the translatomic data collected for DRD1-expressing striatal cells of F1 Veh, F1 NIC, and F2 NIC DRD1-EGFP/Rpl10a mice and to explore overarching themes therein, principal component analysis was conducted via DESeq2 [120–122].

2.12. Differential Translatome Analyses

Differential translation in DRD1-expressing striatal cells of F1 NIC and F2 NIC mice was assessed by comparison to F1 Veh translatomes using DESeq2 [120–122]. Enriched and depleted differentially expressed genes (DEGs) assigned FDR-adjusted p -values satisfying the significance threshold of $p_{\text{adj}} < 0.05$ were designated as differentially translated and were included in the differential translatome compilations (lists of enriched or depleted DEGs) for the corresponding group. Enriched and depleted DEGs shared between F1 NIC and F2 NIC mice were identified by comparative analysis of the intersections (overlap) between enriched and depleted DEG lists for DRD1-expressing striatal cells of F1 NIC and F2 NIC mice. The degrees of similarity between enriched and depleted differential translatomes in DRD1-expressing striatal cells of F1 NIC and F2 NIC mice were quantified by determination of Jaccard similarity indices. Both shared DEG identification and Jaccard similarity indexing were conducted using the aforementioned Multiple List Comparator tool.

To illustrate key biological themes characteristic of the enriched and depleted differential translomes in DRD1-expressing striatal cells of F1 NIC and F2 NIC mice as well as the subsets of DEGs shared therebetween, STRING protein annotations of DEGs in each dataset were submitted to word frequency analysis via the Genes2WordCloud tool available at <http://www.maayanlab.net/G2W/> (accessed on 25 May 2024) [123]. The results from this analysis were visualized as word clouds via the WordItOut tool accessible at <https://worditout.com/> (accessed on 25 May 2024).

2.13. Annotation Mapping and Overrepresentation Analyses of Shared Enriched and Depleted DEGs

Functional (UniProt Keyword), ontological (G.O. Biological Process), and pathway (Reactome Pathway) annotation mapping and overrepresentation analyses were conducted via STRING (<http://string-db.org/>, accessed on 27 September 2024), PANTHER (<http://www.pantherdb.org/>, accessed on 27 September 2024), and STRING, respectively [124,125]. Due to the lack of an official reference translome (or transcriptome) for mouse DRD1-expressing striatal cells, all annotation mapping and overrepresentation analyses utilized the consensus translome (described in the preceding *consensus translome construction* subsection) as the reference translome in lieu of the entire mouse genome or transcriptome. All mapped annotations were filtered by overrepresentation analysis with the following inclusion criteria: p -value (FDR-adjusted) < 0.10 and fold change (FC) > 2.0 , where p -values represent statistical significance (defined as the probability that a given result is due to chance) and where FC values convey biological significance (calculated as a ratio of the quantity of DEGs mapped to a given annotation vs. the quantity that would be expected by chance).

A proxy measure (π -value, where $\pi = \text{Log}_2\text{FC} \times \text{Log}_{10}P$) of the ‘biostatistical significance’ (FC-adjusted statistical significance) of DEGs and DEG-mapped annotations was utilized for ranking and visualization of both individual DEGs and the results of annotation overrepresentation analyses [126]. Importantly, π -values consolidate p -value (statistical significance) and FC (biological significance) into a singular composite metric that increases with increasing ‘biostatistical significance’ of DEGs and annotations thereof [126]. For a given π -value, $\text{FC} = -2$ or $+2$ is neutral toward p -value, $-2 < \text{FC} < +2$ penalizes p -value, and $\text{FC} < -2$ or $\text{FC} > +2$ strengthens p -value.

2.14. Trans-Omic Ortholog Analysis

Trans-omic ortholog analysis was conducted to compare the differential translomes in DRD1-expressing striatal cells of F1 NIC and F2 NIC mice to the differential cord blood DNA methylomes in newborns of maternal smokers reported by the Pregnancy and Childhood Epigenetics (PACE) Consortium meta-analysis thereof [28]. To this end, human orthologs were mapped to all DEGs comprising the differential translomes in DRD1-expressing striatal cells of F1 NIC and F2 NIC mice, and the subsets of DEG orthologs that intersected (overlapped with) differentially methylated genes (DMGs) comprising the differential cord blood DNA methylomes in newborns of maternal smokers were identified using the aforementioned Multiple List Comparator tool. Hypergeometric distributions were then calculated for F1 NIC vs. PACE, F2 NIC vs. PACE, and F1 NIC vs. PACE vs. F2 NIC intersections using the Hypergeometric Distribution Calculator at https://molbiotools.com/math_calculators/hypergeometric.html (accessed on 30 September 2024). G.O. Biological Process annotation mapping and overrepresentation analyses of DEG orthologs comprising F1 NIC vs. PACE, F2 NIC vs. PACE, and F1 NIC vs. PACE vs. F2 NIC intersections were conducted using PANTHER.

2.15. Perturbagen Analysis

The online CLUE analysis environment (CMap Linked User Environment, <https://clue.io>, accessed on 13 January 2025) was used to investigate whether the differential gene expression profiles shared between F1 NIC and F2 NIC mice were comparable to those resulting from other experimental treatments. The CLUE query tool searches over 1.3M L1000 gene expression profiles found in CMap (Connectivity Map) to identify compounds or genetic manipulations ('perturbagens') that evoke differential gene expression profiles with similarities to a user-provided input [127]. To conduct perturbagen analysis, human orthologs mapped to subsets of enriched or depleted DEGs that were shared among or unique to F1 NIC and F2 NIC mice were submitted to CLUE. Each perturbagen was assigned a standardized value ranging from -100 to 100 (connectivity score) determined based on the degree of similarity between the input gene expression profile (DEG subset) and the gene expression profiles cataloged for each perturbagen in the database (perturbagens are characterized at multiple doses and time points across up to nine discrete cell lines). Perturbagen classes—subsets of individual perturbagens with similar mechanisms of action and impacts on gene expression profiles—were also scored for connectivity to subsets of enriched and depleted DEGs that were shared or unique among F1 NIC and F2 NIC mice.

For visualization of perturbagen analysis results, heatmaps were created which compare across groups the connectivity scores for all significant perturbagen classes and for an exemplary subset of significant individual perturbagens selected from the same perturbagen classes. For example, if Group X (out of the possible Groups X, Y, and Z) had a significant score (e.g., 92.1) for one of five individual perturbagens (e.g., P3 out of P1, P2, P3, P4, or P5) with the same descriptor (e.g., 'glutamate inhibitor'), the heatmap would include a row labeled 'glutamate inhibitor' displaying a score of 92.1 for compound P3 in Group X alongside the highest/lowest (nearest-to-significant) score obtained in Group Y and Group Z for any compound with a matching descriptor (P1, P2, P3, P4, or P5). Perturbagens with positive connectivity scores to DNE elicit similar gene expression profiles, while those with negative connectivity scores elicit opposing gene expression patterns.

2.16. Experimental Design and Statistical Analyses

All experimental and statistical designs and methodologies utilized for this study are detailed in the preceding and ensuing subsections as well as the Supplementary Materials. By necessity, JMB was aware of group allocation throughout all experiments. Conversely, the technicians responsible for all RNA sequencing procedures were blind to all sample identities. Sample sizes for behavioral experiments ($n_{F1Veh} = 20$ mice, $n_{F1NIC} = 20$ mice, $n_{F2NIC} = 20$ mice,) were determined based on our prior behavioral studies in DNE mouse models. Sample sizes for translomic profiling (32 mice yielding eight pooled samples per group such that $n_{F1Veh} = 8$ samples, $n_{F1NIC} = 8$ samples, $n_{F2NIC} = 8$ samples) were the maximum allowed by the study budget. All behavioral data analyses compared F1 Veh vs. F1 NIC mice, F1 Veh vs. F2 NIC mice, and F1 NIC vs. F2 NIC mice. Differential expression data for F1 Veh vs. F1 NIC mice and F1 Veh vs. F2 NIC mice served as the input for all translomic analyses, which were further subdivided by whether the DEGs analyzed were shared or unique among F1 NIC and F2 NIC mice. Aside from the single F1 Veh sample with RIN < 7.0 that was replaced, no animals or data were excluded from any dataset or analysis. A protocol was prepared but not registered before this study was conducted.

3. Results

Since F1 NIC mice were directly exposed to nicotine from conception until weaning, phenotypes observed in first-generation DNE mice could constitute direct pharmacologic consequences of nicotine exposure which persist following cessation of nicotine treatment. On the other hand, since F2 NIC mice were the progeny of female F1 NIC mice that were (1) exposed to nicotine from conception until weaning, (2) provided only water post-weaning, and (3) mated with drug-naïve sires, second-generation DNE mice were devoid of direct exposure to nicotine aside from the oocytes from which they were conceived. As such, behavioral or translational changes that F2 NIC mice share with F1 NIC mice constitute intergenerational transmissions (via the F1 NIC maternal germline) rather than direct pharmacologic effects of nicotine. Co-detection of behavioral or translational phenotypes in first- and second-generation DNE mice suggests that the given phenotypes are heritable, likely via epigenetic mechanisms involving the maternal germline.

3.1. Behavioral Characterization of *DRD1-EGFP/Rpl10a* DNE Mice

For baseline (BL) behavioral characterization, all mice were first subjected to open-field testing (single 15-minute trial) on PND 35 and then immediately placed in modified homecages to undergo homecage (HC) activity monitoring for three consecutive days while singly housed. Four-bottle choice testing (FBCT) was then conducted to quantify total nicotine consumption and compare relative preferences for bottles containing 0 (water), 25, 50, and 100 µg/mL nicotine.

Supportive of the utility and validity of our *DRD1-EGFP/Rpl10a* DNE mouse model, the baseline behavioral experiments summarized in Figure 2A,B revealed that first- and second-generation *DRD1-EGFP/Rpl10a* DNE mice exhibit NDD-like phenotypes including hyperactivity in a novel environment and exacerbated risk-taking behaviors (Figure S5A,B), total, active phase, and inactive phase homecage hyperactivity (Figure S6A–D), aberrant rhythmicity of homecage activity (Figure S6A,E–G), and enhanced voluntary nicotine consumption and preference (Figure S7A,B). As evinced in Figure S8, the breadth and magnitude of these multigenerational NDD-related behavioral perturbations are congruent with our previous findings in the offspring and grandoffspring of C57BL/6J and rs16969968 knock-in (D397N) DNE mice [64,110]. For all outcome measures evaluated in baseline behavioral testing of *DRD1-EGFP/Rpl10a* DNE mice, comprehensive raw datasets (formatted as inputs for statistical analyses) and the percent vs. control values calculated therefrom are provided in the supplement (Table S1 and Table S2, respectively). Hourly binned homecage activity datasets (formatted as inputs for rhythmometry analyses) are also provided in the supplement (Table S3).

3.2. Consensus Translatome Construction

Union (non-redundant aggregation) of the comprehensive lists of translating mRNA detected across all F1 Veh, F1 NIC, and F2 NIC mice yielded the consensus translatome for *DRD1*-expressing striatal cells from *DRD1-EGFP/Rpl10a* mice provided in Table S4. Consistent with the portrayal of this study as an assessment of the multigenerational impacts of DNE on the translational signatures of not just D1-type striatal MSNs but also *DRD1*-expressing striatal oligodendrocytes, microglia, and other classes of immune cells, the consensus translatome constructed for *DRD1-EGFP/Rpl10a* mice and the whole translatomes for each group included several genes with expression patterns thought to be restricted to either oligodendrocytes, microglia, or other immune cell types (e.g., *GFAP*, *ALDH1A1*, and *SLC1A2*) [121,128].

3.3. Comparative Translatomic Analysis

Comparison of the consensus translome for DRD1-expressing striatal cells of male DRD1-EGFP/Rpl10a mice to the consensus protein-coding transcriptome reported for DRD1-expressing striatal cells of male *Drd1*-tdTomato mice revealed a high degree of similarity (overlap) [121]. As depicted in Figure S9, 11,542 transcripts intersected (overlapped) between the two datasets, while 1179 and 2147 elements were distinct to the consensus translome for DRD1-EGFP/Rpl10a mice and the consensus protein-coding transcriptome for *Drd1*-tdTomato mice, respectively, yielding a Jaccard similarity index of 0.7763 for pairwise intersections between said datasets. These findings corroborate the cell type-selectivity of the translomic data reported herein and the support the validity of the consensus translome constructed for DRD1-expressing striatal cells of DRD1-EGFP/Rpl10a mice, which was used as the reference translome for all annotation overrepresentation analyses.

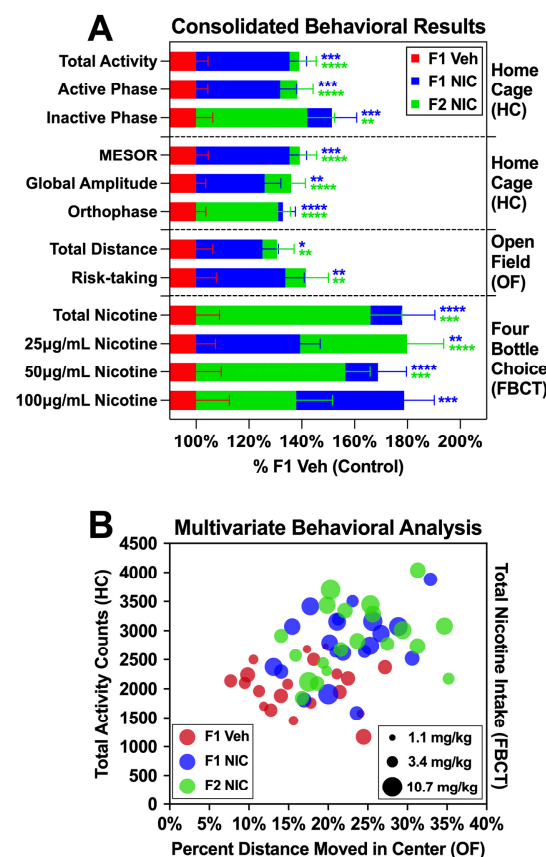


Figure 2. Behavioral validation of the DRD1-EGFP/Rpl10a DNE mouse model. **(A)** Stacked bar plot displaying percent vs. control values for all behavioral metrics used to assess NDD-related phenotypes in first-generation (F1 NIC) and second-generation (F2 NIC) DRD1-EGFP/Rpl10a DNE mice. A percent vs. control value of 100% indicates no difference for F1 NIC or F2 NIC mice relative to F1 Veh control, whereas values of 150% and 200% indicate 1.5-fold and two-fold increases in an outcome measure relative to F1 Veh control, respectively. Raw data from each of the four clusters of outcome measures separated by dashed lines were analyzed by one-way or mixed ANOVA. Adjusted *p*-values from pairwise comparisons are represented by the colored asterisks affixed to each dataset (stacked bar). Blue asterisks denote F1 NIC vs. F1 Veh comparisons, and green asterisks denote F2 NIC vs. F1 Veh comparisons. No significant differences were identified for F1 NIC vs. F2 NIC mice. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001; **** *p* < 0.0001. **(B)** Bubble graph of within-subject datapoints for key NDD-related phenotypes. Vertical axis indicates total daily HC activity. Horizontal axis indicates percent distance moved in the OF center, a proxy for risk-taking. Circular area of each bubble is proportional to nicotine intake (mg/kg).

3.4. Principal Component Analysis

Exploratory principal component analysis of read count data for each pooled sample revealed divergent translomic clustering patterns between F1 Veh versus F1 NIC and F2 NIC mice (Figure 3), indicative of substantial translomic distance between DRD1-expressing striatal cells of control mice compared to first- and second-generation DNE mice.

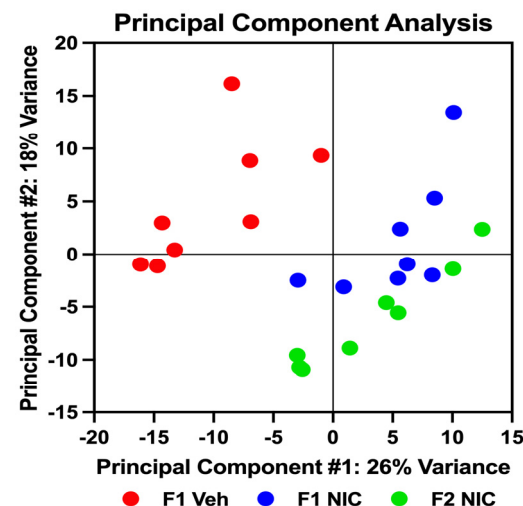


Figure 3. DNE precipitates anomalous translomic clustering in both offspring and grandoffspring. XY plot visualizing the intercept of two principal variance components for each sample partitioned by principal component analysis. Principal Component #1 (horizontal axis) accounted for 26% of the variance, and Principal Component #2 (vertical axis) accounted for 18% of the variance.

3.5. Differential Translatome Analyses

Differential translatome analyses revealed that, compared to F1 Veh mice, DRD1-expressing striatal cell translatomes of F1 NIC (Figure 4A) and F2 NIC (Figure 4B) mice exhibited enrichment of 239 and 278 DEGs and depletion of 193 and 218 DEGs, respectively (Table S5). Analysis of the intersections between enriched and depleted differential translatomes in F1 NIC and F2 NIC mice (Figure 4C) revealed that 102 enriched and 80 depleted DEGs were shared between F1 NIC and F2 NIC mice, respectively, yielding Jaccard similarity indices of 0.2458 for enriched and 0.2417 for depleted differential translatomes in F1 NIC versus F2 NIC mice.

Table S6 provides comprehensive differential gene expression analysis results for enriched and depleted DEGs shared between F1 NIC and F2 NIC mice. Table S7 and Table S8 provide the same for enriched and depleted DEGs uniquely detected in F1 NIC or F2 NIC mice, respectively. Table S9 contains a reference index of gene attributes for all DEGs comprising the differential translatomes of F1 NIC and F2 NIC mice. Table S10 provides a glossary of gene symbols, gene names, Entrez Gene summaries, and UniProt summaries for all enriched and depleted DEGs shared among DRD1-expressing striatal cells of F1 NIC and F2 NIC mice.

As portrayed in Figure 5, top enriched DEGs shared between F1 NIC and F2 NIC mice included *Npsr1*, *Parp1*, *Phf24*, *Scg5*, and *Ccl21b*, and top depleted DEGs shared between F1 NIC and F2 NIC mice included *Pnpt1*, *Nme7*, *Elof1*, *Ints7*, and *Rbis*. Other noteworthy DEGs shared between F1 NIC and F2 NIC mice included the enriched DEGs *Fkbp5*, *Dnajb1*, and *Slc2a13* along with the depleted DEGs *H2bc21*, *Erbp2*, and *Dmac2*. Many DEGs unique to either F1 NIC or F2 NIC mice (Figure S10) were also noteworthy. Top enriched DEGs unique to F1 NIC mice included *Cpne8*, *Dnah6*, *Gjc2*, *Tcf23*, and *Cort*, while top enriched DEGs unique to F2 NIC mice included *Zkscan16*, *Hspb11*, *Usp18*, *Adgre1*, and *Arnt2*. Other enriched DEGs unique to F1 NIC mice included *Polr2f*, *H2ax*, and *Cops6*, while those unique

to F2 NIC mice included *Snrpf*, *Hsp90aa1*, and *Mybbp1a*. On the other hand, top depleted DEGs unique to F1 NIC mice included *Pde4a*, *Crhr1*, *Plch2*, *Golt1a*, and *Cabin1*, while top depleted DEGs unique to F2 NIC mice included *Zfp982*, *Tmem44*, *Gdf10*, *Zfhx2*, and *Ube2d1*. Other depleted DEGs unique to F1 NIC mice included *Gpr75*, *Rmdn3*, and *Adh1*, while those unique to F2 NIC mice included *Gpr55*, *Dctn3*, and *Polr3b*, respectively. Table S11 provides DEG-encoded protein annotations for all DEGs detected in F1 NIC and F2 NIC mice. Word frequency analyses elucidated several NDD-related high-frequency words within DEG-encoded protein annotations for DEGs that were shared (Figure 5) or unique (Figure S11) among F1 NIC and F2 NIC mice.

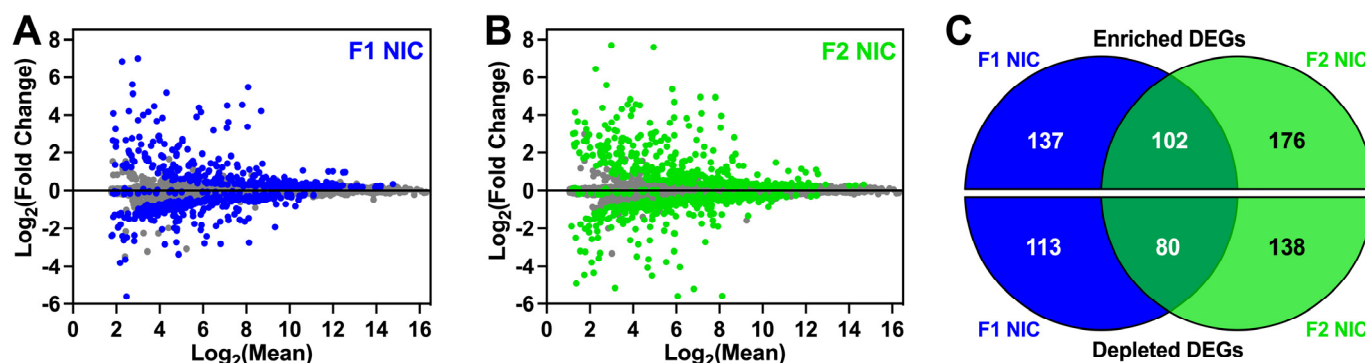


Figure 4. DNE confers broad spectra of shared and unique translomic perturbations in DRD1-expressing striatal cells of first- and second-generation progeny. (Top) MA plots portraying differential expression profiles relative to F1 Veh control mice for DRD1-expressing striatal cells of (A) F1 NIC and (B) F2 NIC mice, which exhibited significant (colored points) upregulation (enrichment) of 239 and 278 and downregulation (depletion) of 193 and 218 DEGs, respectively. (C) Venn diagram displaying the quantities of enriched (upper semicircles) and depleted (lower semicircles) DEGs that are shared among (overlapping semicircular areas) and unique to (non-overlapping semicircular areas) DRD1-expressing striatal cells of F1 NIC (blue) and F2 NIC (green) mice. 102 enriched (Jaccard similarity index = 0.2458) and 80 depleted (Jaccard similarity index = 0.2417) DEGs were shared (blue-green) between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice. Colored points in MA plots denote individual transcripts exhibiting significant ($p_{adj} < 0.05$) fold-enrichment or fold-depletion.

3.6. Annotation Mapping and Overrepresentation Analyses of Shared Enriched and Depleted DEGs

Functional, ontological, and pathway annotation analyses mapped the enriched and depleted DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice to a variety of overrepresented UniProt Keyword (Table S12 and Table S13, respectively), G.O. Biological Process (Table S14 and Table S15, respectively), and Reactome Pathway (Table S16 and Table S17, respectively) annotations. Noteworthy overrepresented UniProt Keyword annotations mapped to the depleted (Figure 6A) differential translomes shared between F1 NIC and F2 NIC mice included *mRNA splicing*, *mRNA processing*, *neurotransmitter transport*, *gastrulation*, *annexin*, and *plasminogen activation*, while those mapped to the enriched (Figure 6B) differential translomes shared between F1 NIC and F2 NIC mice included *cell adhesion*, *stress response*, *inflammatory response*, *MHC I*, *neuropeptide*, and *spermidine biosynthesis*. Moreover, noteworthy overrepresented G.O. Biological Process annotations mapped to the enriched (Figure 6C, upper) differential translomes shared between F1 NIC and F2 NIC mice included *brain development*, *regulation of behavior*, *necrotic process*, *S-adenosylmethionine metabolic process*, *detoxification*, and *regulation of I-kappaB kinase/NF-kappaB signaling*, while those mapped to the depleted (Figure 6C, lower) differential translomes shared between F1 NIC and F2 NIC mice included *trans-synaptic signaling by BDNF*, *chromatin-mediated maintenance of transcription*, *intracellular steroid hor-*

monine receptor signaling pathway, neuron fate specification, neuron development, and response to interleukin-4, respectively. In addition, noteworthy overrepresented Reactome Pathway annotations mapped to the enriched (Figure 6D, upper) differential translomes shared between F1 NIC and F2 NIC mice included *hedgehog ligand biogenesis, GPCR ligand binding, acetylcholine neurotransmitter release cycle, HSP90 chaperone cycle for steroid hormone receptors, peptide ligand-binding receptors, and neutrophil degranulation*, while those mapped to the depleted (Figure 6D, lower) differential translomes shared between F1 NIC and F2 NIC mice included *tight junction interactions, metabolism of RNA, cell–cell communication, intrinsic pathway for apoptosis, PRC2 methylates histones and DNA, and glutathione synthesis and recycling*.

3.7. Trans-Omic Ortholog Analysis

The PACE consortium identified genes that were differentially methylated in cord blood from the newborns of maternal smokers [28]. To assess whether there is overlap between the DEGs induced by DNE in this study and the differentially methylated genes (DMGs) attributed to maternal smoking by the PACE consortium, we performed a trans-species, trans-omic ortholog analysis of these datasets. Comparisons of the human orthologs of all DNE-evoked DEGs (Table S18) and those shared among F1 NIC and F2 NIC mice (Table S19) with all DMGs detected in newborns of maternal smokers revealed intersections (Figure 7) encompassing 69 (F1 NIC vs. PACE), 84 (F2 NIC vs. PACE), and 30 (F1 NIC vs. PACE vs. F2 NIC) trans-omic ortholog pairs (Table S20), respectively. Hypergeometric distributions calculated for F1 NIC vs. PACE (fold change = +1.87; $p = 7.55 \times 10^{-8}$), F2 NIC vs. PACE (fold change = +1.88; $p = 1.29 \times 10^{-9}$), and F1 NIC vs. PACE vs. F2 NIC (fold change = +4.94; $p = 7.07 \times 10^{-14}$) intersections revealed significant over-enrichment of trans-omic orthologs.

Noteworthy trans-omic orthologs comprising the F1 NIC vs. PACE intersection included *PBX2, KCNH3, LEFTY1, DDIT4, and DTNBP1*, while those comprising the F2 NIC vs. PACE intersection included *CRELD2, HSPB1, CREB3L2, H1FO, and LRRK2*, and those comprising the F1 NIC vs. PACE vs. F2 NIC intersection included *ASB3, ITPK1, DNAJB1, HMGN3, and CBLN3*. Annotation mapping and overrepresentation analyses of all trans-omic orthologs comprising each intersection yielded a wide array of overrepresented G.O. Biological Process annotations (Figure 7). Exemplary G.O. Biological Process annotations for trans-omic orthologs comprising the F1 NIC vs. PACE (Table S21) intersection included *general adaptation syndrome, regulation of dopamine receptor signaling pathway, regulation of corticosterone secretion, neurotrophin signaling pathway, negative regulation of nervous system development, positive regulation of acute inflammatory response, regulation of interleukin-13 production, positive regulation of immune system process, and astrocyte cell migration*, while those for trans-omic orthologs comprising the F2 NIC vs. PACE (Table S22) intersection included *positive regulation of DNA demethylation, regulation of histone deacetylase activity, regulation of dopamine receptor signaling pathway, striatum development, positive regulation of neuroinflammatory response, regulation of cytokine production, innate immune response, and regulation of microglial cell activation*, and those for trans-omic orthologs comprising the F1 NIC vs. PACE vs. F2 NIC (Table S23) intersection included *cellular response to nicotine, maintenance of blood–brain barrier, protein processing, regulation of multicellular organismal development, locomotion, inflammatory response, response to prostaglandin, positive regulation of immune response, and glial cell migration*.

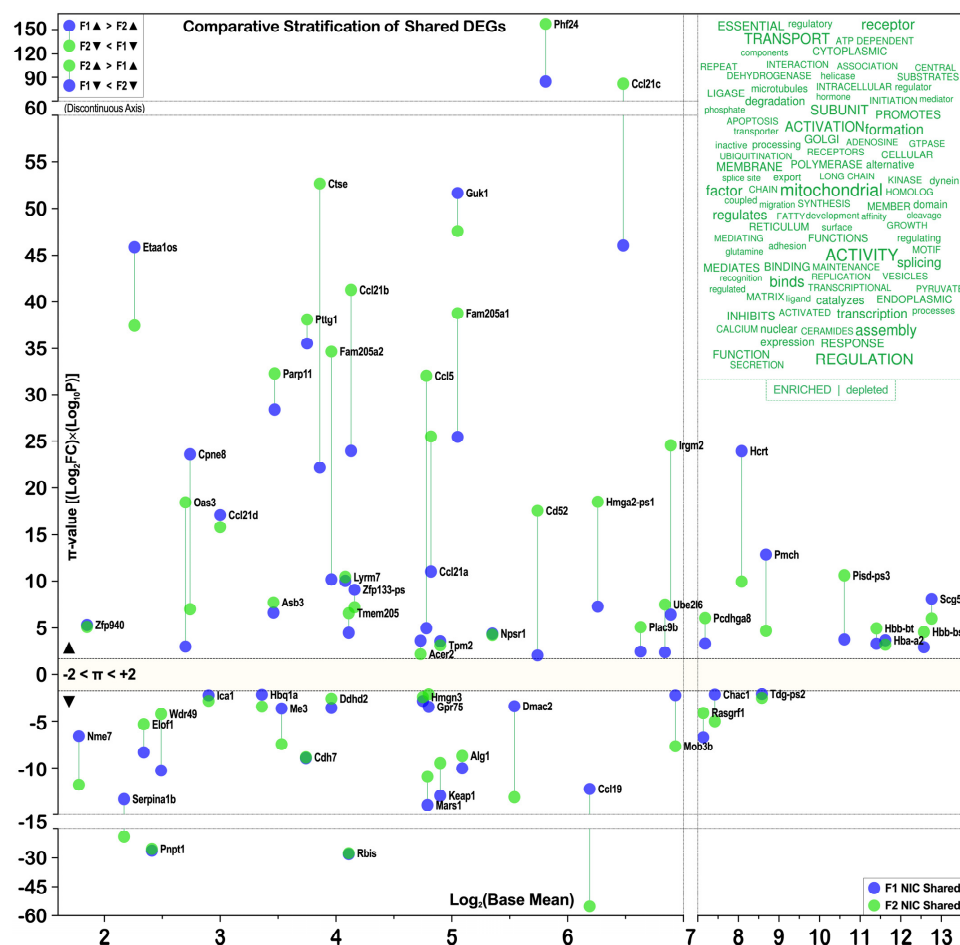


Figure 5. Comparative stratification of shared DEGs. π -values ($\pi = \text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$) were calculated for all shared DEGs to provide a composite ranking metric that integrates both fold change (magnitude or biological significance) and p -value (statistical significance). π -value cutoffs of -2π and $+2\pi$ were applied to stratify depleted ($\blacktriangledown$) and enriched ($\blacktriangle$) DEGs, respectively, into subsets with higher ($\pi < -2$ or $\pi > +2$) or lower ($-2 < \pi < +2$) degrees of differential expression, the former being more likely to robustly mediate the phenotypic impacts of DNE and the intergenerational transmission thereof. π -values for shared DEGs with $\pi < -2$ or $\pi > +2$ and corresponding Log_2 (base mean) values (mean of normalized counts across all samples) were plotted on the vertical and horizontal axes, respectively, with the latter functioning to sort DEGs by average translational abundance. The shaded region flanked by dashed lines and spanning the vertical axis from -2π to $+2\pi$ denotes the π -value thresholds used to stratify shared DEGs. The lengths (in units of π) of the blue-green vertical lines connecting corresponding datapoints (circles labeled with gene symbols) are proportional to differences in the degree of depletion or enrichment for DEGs shared between F1 NIC (blue) and F2 NIC (green) mice (longer lines indicate larger differences). The legend (upper left) conveys how the direction ($\blacktriangledown$ or $\blacktriangle$) and relative degree ($\text{F1} < \text{or} > \text{F2}$) of differential expression define the appearance of the interconnected points representing each shared DEG. Discontinuously scaled axes were utilized to facilitate visualization of more shared DEGs than would otherwise be feasible. Interfaces between discontinuously scaled axis segments (segments along the same axis that were assigned different numerical scales) are marked by a gap in the composite axis and are flanked by dotted lines. The number plotted at each axis segment interface is the value of π or Log_2 (base mean) at which the scale of the composite axis changes, but no range of values is omitted from any composite axis. Word frequency analysis attributed broad biological themes to shared DEGs by quantifying absolute word frequencies in DEG-encoded STRING protein annotations and in turn rendering a word cloud (upper right) wherein higher frequency words are assigned larger font sizes, words mapped to shared enriched DEGs are ‘UPPERCASE’, and words mapped to shared depleted DEGs are ‘lowercase’.

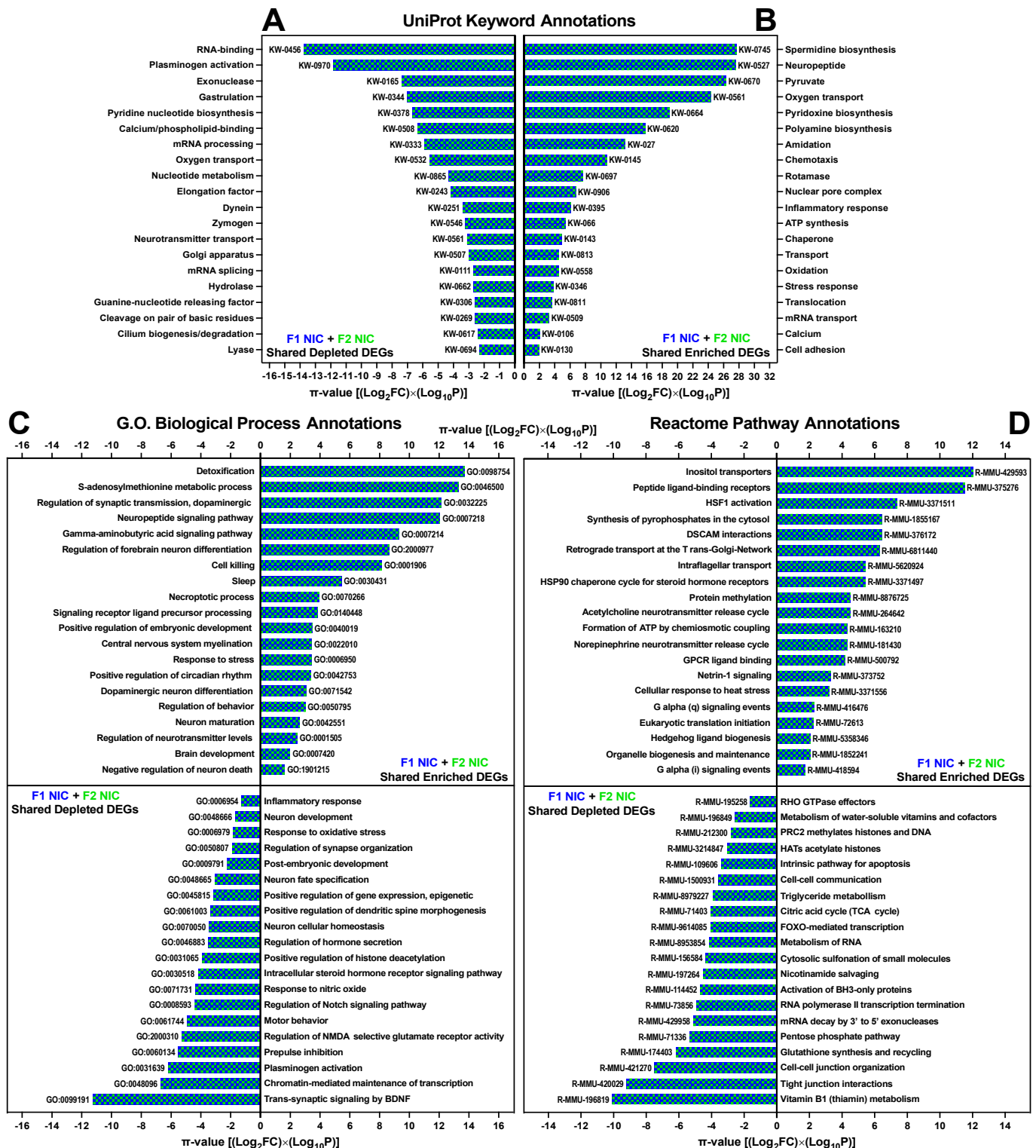


Figure 6. Overrepresented functional, ontological, and pathway annotations mapped to enriched and depleted DEGs shared among DRD1-expressing striatal cells of DNE offspring and grandoffspring recapitulate key transdiagnostic features of NDDs. Bar plots depicting π -values ($\pi = \text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$) for (A,B) functional (UniProt Keyword), (C) ontological (G.O. Biological Process), and (D) pathway (Reactome Pathway) annotations of enriched (rightward bars) and depleted (leftward bars) DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice. For all annotations, p -value (FDR-adjusted) < 0.10 and fold change (FC) > 2.0 . Alphanumeric identifiers are labeled adjacent to each plotted annotation. The data visualized in this figure constitute a subset of findings selected from a larger pool of significant results.



Figure 7. Human orthologs of DEGs identified in DRD1-expressing striatal cells of DNE progeny and grandprogeny are over-enriched for DMGs reported in cord blood from newborns of maternal smokers. Results from trans-omic ortholog analysis comparing the differential transcriptomes of F1 NIC and F2 NIC mice to the differential cord blood DNA methylomes detected in newborns of maternal smokers by the PACE Consortium meta-analysis thereof. (Top) Venn diagram quantifying intersections among human orthologs of DEGs in DRD1-expressing striatal cells from F1 NIC (blue circle) and F2 NIC (green circle) mice and DMGs in cord blood from newborns of maternal smokers (gray circle). F1 NIC vs. PACE (blue-gray), F2 NIC vs. PACE (green-gray), and F1 NIC vs. PACE vs. F2 NIC (blue-green-gray) intersections totaled 69, 84, and 30 trans-omic ortholog pairs, respectively. Hypergeometric distributions revealed over-enrichment of F1 NIC vs. PACE (fold change = +1.87; $p = 7.55 \times 10^{-8}$), F2 NIC vs. PACE (fold change = +1.88; $p = 1.29 \times 10^{-9}$), and F1 NIC vs. PACE vs. F2 NIC (fold change = +4.94; $p = 7.07 \times 10^{-14}$) intersections. (Bottom) Lists of select overrepresented NDD-related G.O. Biological Process annotations mapped to F1 NIC vs. PACE (blue-gray), F2 NIC vs. PACE (green-gray), and F1 NIC vs. PACE vs. F2 NIC (blue-green-gray) intersections. Alphanumeric identifiers and π -values are provided (in parentheses) for each annotation. π , π -value ($\pi = \text{Log}_2\text{FC} \times \text{Log}_{10}P$); FC, fold change. The data shown are a subset of the statistically significant findings.

3.8. Perturbagen Analysis

Lastly, we assessed whether DNE-evoked gene expression patterns resemble those conferred by other pharmacologic ‘perturbagens’. To this end, lists (Table S24) of human orthologs of enriched and depleted DEGs shared or unique among F1 NIC and F2 NIC mice were queried using the CLUE analysis environment, yielding connectivity scores for both pharmacologic perturbagen classes (Table S25) and individual pharmacologic perturbagens (Table S26). Analysis of pharmacologic perturbagen classes (Figure 8A) revealed that *HDAC inhibitor* (+) was the sole pharmacologic perturbagen class connected to shared DEGs, while *PI3K inhibitor* (−) and *MTOR inhibitor* (−) were connected to DEGs unique to F1 NIC mice,

and *HSP inhibitor* (+) and *proteasome inhibitor* (+) were connected to DEGs unique to F2 NIC mice. Individual pharmacologic perturbagens contributing to pharmacologic perturbagen classes with connectivity scores >90 or <-90 are detailed in the supplement (Table S27). Noteworthy individual pharmacologic perturbagens (Figure 8B) connected to shared DEGs included *ICAM1 inhibitor* (+), *protein synthesis inhibitor* (−), and *protein phosphatase inhibitor* (−), while those connected to DEGs unique to F1 NIC mice included *glutamate inhibitor* (−), *acetylcholinesterase inhibitor* (+), and *glucocorticoid receptor agonist* (+), and those connected to DEGs unique to F2 NIC mice included *dopamine receptor agonist* (+), *calcium channel blocker* (−), and *cytochrome P450 inhibitor* (+). Data for genetic perturbagen classes (Table S28) and individual genetic perturbagens (Table S29) are provided in the supplement but are otherwise outside the scope of this report.

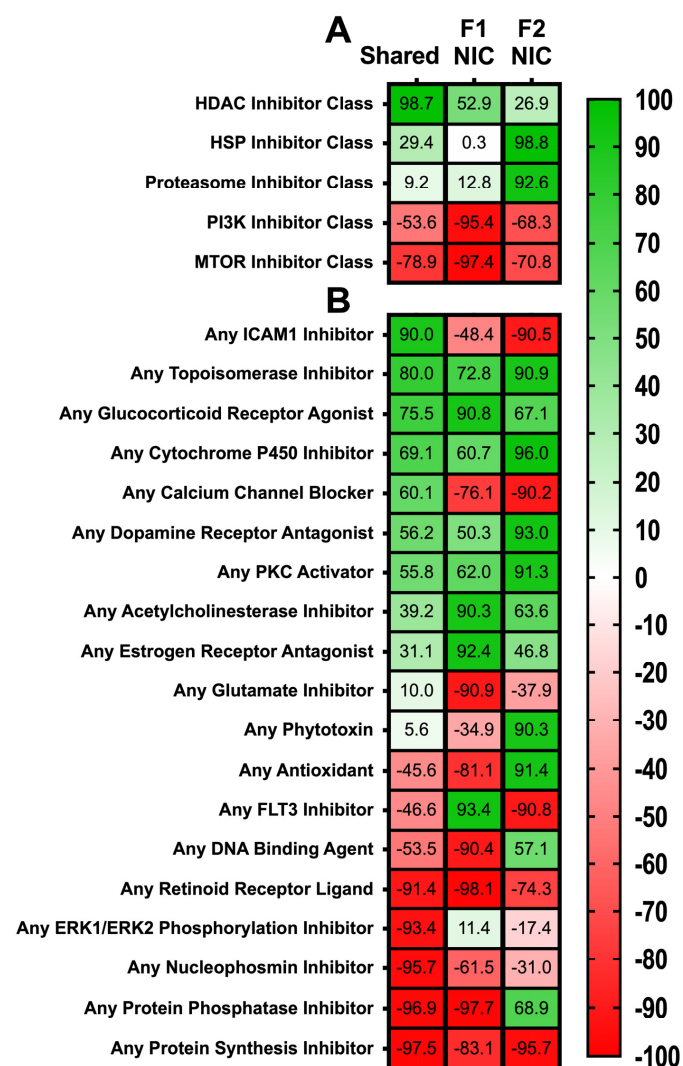


Figure 8. Translational signatures shared among and unique to DNE offspring and grandoffspring mirror gene expression changes elicited by both classes and specific subtypes of pharmacologic perturbagens. (A) Connectivity scores for all perturbagen classes found to be significant (>90 / <-90) in any group. (B) Highest or lowest connectivity scores in each group for any individual perturbagen with a description matching one with a significant (>90 or <-90) score selected from any group. Shared, shared among F1 NIC and F2 NIC mice, F1 NIC, unique to F1 NIC mice, F2 NIC, unique to F2 NIC mice. Perturbagens with positive and negative connectivity scores produce analogous and opposing transcriptional profiles, respectively, compared to those detected in DNE offspring and/or grandoffspring. The data visualized in this figure constitute a subset of findings selected from a larger pool of significant results.

4. Discussion

The current study endeavored to characterize the intergenerational impacts of DNE on translating mRNA levels in DRD1-expressing striatal cells of adolescent male mice. To this end, we first performed a series of baseline behavioral tests which confirmed the validity of the DNE mouse model developed for this study. A battery of bioinformatic assessments including principal component analyses, differential translome analyses, word frequency analyses of DEG summaries, and annotation overrepresentation analyses were then performed. We also conducted a novel trans-omic ortholog analysis which compared the differential translomes in DRD1-expressing striatal cells of first- and second-generation DNE progeny with the differential DNA methylomes documented in cord blood from the newborns of maternal smokers. Finally, we performed a perturbagen analysis to ascertain whether and to what extent any tested pharmacologic agents elicit differential gene expression signatures that resemble those conferred by DNE. Importantly, given that the translomic anomalies shared among DNE offspring and grandoffspring are most likely to be directly involved in the intergenerational transmission of DNE-evoked NDD-like phenotypes and to be mediated by epigenetic transmission, the ensuing discussion exclusively addresses shared DNE-induced translomic changes. A thorough description and discussion of the DNE-induced translomic changes unique to either first- or second-generation DNE mice is provided in the Supplemental Discussion.

Differential translome analyses identified 239 and 278 enriched DEGs along with 193 and 218 depleted DEGs in DRD1-expressing striatal cells of DNE progeny and grandprogeny, respectively, of which 102 enriched and 80 depleted DEGs were shared therebetween. Briefly, the findings of exploratory word frequency analyses revealed frequent occurrences of words within DEG-encoded protein annotations that are compatible with both the core transdiagnostic features of NDDs and various ancillary phenotypes such as metabolic abnormalities [129,130]. We next considered the relevance to NDDs of specific enriched and depleted DEGs shared among DNE offspring and grandoffspring.

Npsr1, *Phf24*, *Scg5*, and *Fkbp5* exemplify NDD-related DEGs enriched in both first- and second-generation DNE progeny. *Npsr1* encodes neuropeptide S receptor 1, a stimulatory G-protein-coupled receptor that is rhythmically expressed throughout the corticostriothalamic circuitry, wherein it enhances intracellular cAMP and Ca²⁺ levels and modulates GABA, glutamate, acetylcholine, corticotropin-releasing factor, and corticosterone signaling [131–135]. Notably, both upregulation (as detected in DNE progeny and grandprogeny) and overstimulation of NPSRs are known to elicit hyperactivity, sleep disruptions, hyperarousal, impaired stress-responsivity, reduced anxiety-like and increased risk-taking behaviors, and impaired prepulse inhibition, each of which phenotypes are characteristic of ADHD, autism, and/or schizophrenia and have been documented in DNE offspring and in some cases DNE grandoffspring [23,24,54,131,133–136]. The multigenerational upregulation of *Phf24* is also interesting considering that PHF24 regulates locomotor activity patterns and rhythms, anxiety-like behaviors, and risk-taking behaviors [137]. Moreover, since the *Scg5*-encoded protein 7B2 inhibits the proBDNF-processing protease proconvertase 2 (PC2), the upregulation of *Scg5* in first- and second-generation DNE offspring could contribute to the DNE-evoked multigenerational disruption of corticostriatal proBDNF proteolysis that we previously reported [138,139]. However, as our prior research revealed no changes in the abundance of the GR chaperone/inactivator FKBP5 in the striata of first- or second-generation DNE mice, the upregulation of *Fkbp5* in DNE progeny and grandprogeny might constitute a cell type-specific neurotranslomic anomaly capable of mediating the DNE-evoked intergenerational striatal GR hypoactivity that we previously reported, perhaps via FKBP5-driven overinhibition of GC-induced activation and/or activation-induced nuclear translocation of cytosolic GRs [72,140].

Exemplary NDD-related DEGs found to be depleted in DRD1-expressing striatal cells of DNE offspring and grandoffspring include *Pnpt1*, *ErbB2*, *Elof1*, and *Ints7*. Interestingly, *Pnpt1* encodes the polynucleotide phosphorylase and exosome complex subunit PNPT1, and both *Pnpt1* mutations and PNPT1 malfunctions delay myelination and are implicated in NDDs [141,142]. *ErbB2*, which mediates neurocircuit assembly, neurotransmission, axon ensheathment, and synaptic plasticity, is linked to the etiology of schizophrenia and may be causal in ADHD [143–146]. The transcription factors *Elof1* and *Ints7* directly interact with RNA polymerase II, are crucial for both normal development and transcriptional regulation across the lifespan, and are implicated in ADHD, autism, and schizophrenia [147].

The annotation overrepresentation analyses reported in this study exclusively assessed enriched and depleted DEGs shared among DRD1-expressing striatal cells of first- and second-generation DNE progeny. Consistent with the generalized, modular deregulation of gene expression characteristic of NDDs, a multitude of functional (UniProt Keyword), gene ontological (G.O. Biological Process), and pathway (Reactome Pathway) annotations were overrepresented in the differential translomes shared among DNE offspring and grandoffspring [146,148–150]. As categorized in Table 1 and detailed in the Supplemental Discussion, distinct subsets of annotations mapped to the enriched and depleted differential translomes shared among DNE offspring and grandoffspring recapitulate eight discrete transdiagnostic features of NDDs, namely neurobehavioral deficits, developmental encephalopathy, neuroteratogenicity, neurotransmitter system dysfunction, neurotrophic dysfunction, HPA axis dysregulation, neuroimmune and neuroinflammatory aberrations, and epigenomic perturbations [9,14,20–42,50–73,75–77,81–89,151–166].

Beyond the eight transdiagnostic features of NDDs which are the focus of this report, many overrepresented annotations of shared DEGs are congruent with other aspects of NDDs including ADHD, autism, and schizophrenia. Namely, the *pyruvate*, *oxygen transport*, *citric acid cycle* (TCA cycle), *pentose phosphate pathway*, and *formation of ATP by chemiosmotic coupling* annotations are consistent with metabolic and mitochondrial dysfunction in NDDs, and the *metabolism of RNA*, *mRNA processing*, *mRNA splicing*, and *mRNA transport* annotations recapitulate the broad transcriptional dysregulation common among NDDs [146,148–150].

In aggregate, the overrepresented annotations of enriched and depleted DEGs shared among DRD1-expressing striatal cells of DNE offspring and grandoffspring are congruent with (1) the assortment of DNE-induced intergenerational phenotypes that we and others previously delineated, (2) the aberrant neurodevelopmental profiles documented in the children and increasingly the grandchildren of maternal smokers, and (3) the core etiological and transdiagnostic features of NDDs such as ADHD, autism, and schizophrenia. More generally, the extensive DNE-evoked translomic perturbations detected in DNE progeny and grandprogeny corroborate the purported involvement of DRD1 and DRD1-expressing striatal cells in features of NDDs such as hyperactivity, impulsivity, inattentiveness, anomalous circadian rhythmicity, aberrant DA signaling, neuroimmune activation, and neuroinflammation [46,47,54,72,76,99–101].

To relate our findings in DNE mice to the offspring of human smokers, we next performed a novel trans-species, trans-omic ortholog analysis which compared changes in the cord blood DNA methylomes of MS-exposed human neonates to the DNE-induced translomic changes detected in DNE offspring and grandoffspring. This analysis identified 69, 84, and 30 genes that overlap between the DNA methylomes of prenatal tobacco exposed neonates and the translomes of first-generation, second-generation, and both first- and second-generation DNE mice, respectively. MS-associated DMGs found to be orthologous to DNE-induced DEGs shared among DNE offspring and grandoffspring included *ASB3*, *ITPK1*, *DNAJB1*, *HMGN3*, and *CBLN3*. *ASB3* is noteworthy in light of its

modulation of inflammatory responses, forebrain development, and neuronal migration coupled with its implication in autism and Down syndrome; *ITPK1* is distinguished by its role in neural tube development, neural function, cognition, and brain microstructure along with its linkage to autism; *DNAJB1* is notable given its contributions to neurite outgrowth, neurodegeneration, and autism risk; *HMGN3* stands out for its regulation of chromatin architecture and transcriptional accessibility, development and differentiation, and cellular identity maintenance alongside its association with autism; *CBLN3* warrants consideration due to its involvement in brain function, motor behavior, and synaptic organization and maintenance along with its linkage to autism [89,167–174].

G.O. Biological Process annotations overrepresented among trans-omic orthologs shared between MS-associated DMGs, DEGs detected in first-generation DNE mice, and DEGs detected in second-generation DNE mice (three-way intersection) are emblematic of several transdiagnostic features of NDDs. These annotations include *cellular response to nicotine*, *maintenance of blood–brain barrier*, *protein processing*, *regulation of multicellular organismal development*, *locomotion*, *inflammatory response*, *positive regulation of immune response*, and *glial cell migration*. In addition to aligning with transdiagnostic features of NDDs, these annotations are consistent with the aberrant nAChR, DA, and (pro)BDNF signaling, hyperactivity, impulsivity/risk-taking, and altered rhythmicity of activity linked to MS and shown to undergo multigenerational transmission in DNE animal models.

Perturbagen analysis revealed that the *HDAC inhibitor* perturbagen class contains compounds which produce an altered transcriptional profile most connected to the translatomic changes shared among DNE progeny and grandprogeny. The shared translatomic perturbations evoked by DNE exhibited a connectivity score of 98.7 with the *HDAC inhibitor* perturbagen class, indicating that only 1.3% of other tested perturbagens had a connectivity score with the *HDAC inhibitor* class greater than that for DEGs shared among DRD1-expressing striatal cells of first- and second-generation DNE mice. No other perturbagen class exceeded a connectivity score of +90 or −90 for DEGs shared between DNE offspring and grandoffspring. This finding is congruent with epigenomic perturbations as a transdiagnostic feature of NDDs and relates to the aberrant histone modifier expression and histone modification patterns, atypical nucleosome and chromatin architectures, and other epigenetic changes associated with MS and found to undergo intergenerational transmission in DNE animal models.

Among individual perturbagens with descriptions other than *HDAC inhibitor*, DEGs shared among DNE offspring and grandoffspring had positive connectivity to individual perturbagens with the description *ICAM1 inhibitor* and negative connectivity to individual perturbagens with the description *protein synthesis inhibitor* and *protein phosphatase inhibitor*. These findings highlight the impacts of DNE on fundamental cellular processes, which in turn may trigger cascades of multidimensional changes that contribute to the overall complexity and diversity of the intergenerational impacts of DNE.

Altogether, the findings of this study indicate that DNE elicits sweeping changes in gene expression programs within DRD1-expressing striatal cells of DNE progeny and grandprogeny which relate to transdiagnostic features of ADHD, autism, and schizophrenia and, by extension, provide newfound insights into the cell type-selective translatomic substrates for the multigenerational transmission of DNE-induced and MS-associated neurodevelopmental abnormalities. These results have clinical relevance, public health implications, and potential therapeutic applications.

Table 1. Select annotations mapped to DEGs shared among F1 and F2 NIC mice are clustered by relevance to specific transdiagnostic features of NDDs. Δ , enriched (+) or depleted (–).

Annotation ID	Annotation Term: UniProt Keyword, G.O. Biological Process, or Reactome Pathway	Δ	Transdiagnostic Feature of NDDs
GO:0061744	Motor behavior	–	Neurobehavioral Deficits
GO:0042753	Positive regulation of circadian rhythm	+	
GO:0060134	Prepulse inhibition	–	
GO:0050795	Regulation of behavior	+	
GO:0030431	Sleep	+	
GO:0007420	Brain development	+	Developmental Encephalopathy
GO:0022010	Central nervous system myelination	+	
R-MMU-5358346	Hedgehog ligand biogenesis	+	
R-MMU-373752	Netrin-1 signaling	+	
GO:0048666	Neuron development	–	
GO:2000977	Regulation of forebrain neuron differentiation	+	Neuroteratogenicity
GO:0098754	Detoxification	+	
R-MMU-9614085	FOXO-mediated transcription	–	
GO:0070266	Necroptotic process	+	
GO:1901215	Negative regulation of neuron death	+	
GO:0070050	Neuron cellular homeostasis	–	Neurotransmitter System Dysfunction
KW-0970	Plasminogen activation	–	
R-MMU-264642	Acetylcholine neurotransmitter release cycle	+	
GO:0071542	Dopaminergic neuron differentiation	+	
GO:0007214	Gamma-aminobutyric acid signaling pathway	+	
R-MMU-181430	Norepinephrine neurotransmitter release cycle	+	Neurotransmitter System Dysfunction
GO:2000310	Regulation of NMDA selective glutamate receptor activity	–	
GO:0032225	Regulation of synaptic transmission, dopaminergic	+	

Table 1. Cont.

Annotation ID	Annotation Term: UniProt Keyword, G.O. Biological Process, or Reactome Pathway	Δ	Transdiagnostic Feature of NDDs
GO:0048665	Neuron fate specification	—	Neurotrophic Dysfunction
KW-0527	Neuropeptide	+	
R-MMU-375276	Peptide ligand-binding receptors	+	
GO:0050807	Regulation of synapse organization	—	
GO:0140448	Signaling receptor ligand precursor processing	+	
GO:0099191	Trans-synaptic signaling by BDNF	—	
R-MMU-3371497	HSP90 chaperone cycle for steroid hormone receptors	+	HPA Axis Dysregulation
GO:0009725	Response to hormone	—	
GO:0030518	Intracellular steroid hormone receptor signaling pathway	—	
KW-0906	Nuclear pore complex	+	
GO:0046883	Regulation of hormone secretion	—	
KW-0346	Stress response	+	
KW-0041	Annexin	—	Neuroimmune and Neuroinflammatory Aberrations
KW-0395	Inflammatory response	+	
R-MMU-174403	Glutathione synthesis and recycling	—	
R-MMU-6798695	Neutrophil degranulation	+	
GO:0043122	Regulation of I-kappaB kinase/NF-kappaB signaling	+	
GO:0070670	Response to interleukin-4	—	
GO:0048096	Chromatin-mediated maintenance of transcription	—	Epigenomic Perturbations
R-MMU-3214847	HATs acetylate histones	—	
GO:0045815	Positive regulation of gene expression, epigenetic	—	
GO:0031065	Positive regulation of histone deacetylation	—	
R-MMU-212300	PRC2 methylates histones and DNA	—	
KW-0745	Spermidine biosynthesis	+	

From a clinical perspective, the overlap between DEGs in DNE mice and DMGs in newborns of maternal smokers implies that biomarkers may exist which could enable earlier identification of individuals at risk for NDDs and other DNE-associated neurodevelopmental anomalies. To this end, genes such as *ASB3*, *ITPK1*, *DNAJB1*, *HMGN3*, and *CBLN3*, which exhibited both differential translation in DNE mice and differential methylation in newborns of maternal smokers, could be explored as candidates for screening the children of maternal smokers and other populations at heightened risk for NDDs.

Through the lens of public health, the findings of this study provide supporting evidence for initiatives promoting abstinence from nicotine and tobacco products and underscore the importance of mitigating nicotine exposure during pregnancy at the population level. Public dissemination of this research could bolster awareness campaigns by emphasizing that not only maternal but also grandmaternal smoking or vaping may inflict neurodevelopmental harms that span multiple generations, thereby providing additional motivation for nicotine cessation among pregnant women and those planning to conceive.

The results of this study also inform the development of novel therapies for NDDs and the neurodevelopmental consequences of DNE. For instance, the strong connectivity between DNE-induced translational changes and HDAC inhibitors implicates epigenetic dysregulation in the DNE-NDD axis and supports the exploration of novel and repurposed therapies targeting histone modifiers as putative mediators of DNE-associated neurodevelopmental abnormalities. Similarly, specific genes and pathways identified herein, including those involved in HPA axis function (*Fkbp5*), neuropeptide signaling (*Npsr1*), and neurotrophic support (*Scg5*), represent candidate therapeutic targets that could be pursued in future preclinical and clinical studies. However, further research is required to validate these inferences and to develop safe and effective interventions to combat the neurodevelopmental consequences of maternal smoking.

Several limitations of this study warrant consideration. Firstly, translational analyses were performed exclusively in male offspring, the rationale for which was twofold: (1) our earlier studies revealed no sex differences in the multigenerational behavioral or biological impacts of DNE, and (2) budgetary constraints prevented testing of a sufficient quantity of female mice to properly control for estrous cycle. As such, it is feasible that sex differences in the intergenerational impacts of DNE on gene expression profiles in DRD1-expressing striatal cells exist but remain undetected, a possibility that subsequent studies should address. A second limitation is that the current study focused on DRD1-expressing striatal cells, which are but one of many cell types implicated in both the etiology of NDDs and the neurotoxicology of DNE. The multigenerational translational impacts of DNE in other NDD-related cell types should be characterized by future research. Thirdly, while the TRAP-seq method utilized for the present study enables cell type-selective isolation of translating mRNAs, DRD1 is expressed by multiple striatal cell types including D1-type MSNs, oligodendrocytes, microglia, and other immune cells. Thus, the differential translational profiles delineated by this study are likely composites of multiple DRD1-expressing striatal cell types rather than a single homogeneous population. Fourthly, the pooling of translating mRNA samples required to achieve sufficient input quantities for sequencing may have masked or distorted interindividual variability. Fifthly, the intergenerational DNE mouse model evaluated for this study does not recapitulate exposure to the thousands of other constituents present in tobacco smoke, which may limit the generalizability of the study's findings to the human condition. Finally, while the trans-omic ortholog analysis conducted for this study yielded meaningful insights, inherent cross-species and cross-omic differences in neurodevelopment, gene regulatory mechanisms, and epigenomic landscapes warrant caution when interpreting these results.

5. Conclusions

This study reveals that DNE elicits intergenerational transmission of a multitude of NDD-related translational alterations in DRD1-expressing striatal cells. These abnormalities span individual genes, protein functions, and biological pathways associated with NDDs and appear to disproportionately impact loci that are known to be epigenetically dysregulated in newborns of maternal smokers. The alignment of DNE-induced changes in gene expression with transdiagnostic features of NDDs and the cooccurrence of multi-layered epigenomic perturbations strongly suggest that epigenomic disturbances may be the conduit through which DNE evokes neurodevelopmental deficits and disorders in the children and grandchildren of maternal smokers and animal models thereof. Future human and animal model studies should pursue all manner of preventatives, prophylactics, and treatments to counteract the heritable neurodevelopmental consequences of MS. However, maternal abstinence remains the sole guarantor of complete protection.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/genes17020128/s1>, Figure S1: Procedural Schematic for breeding of DNE progeny and compilation of key findings from our prior studies utilizing the same DNE mouse model paradigm; Figure S2: Procedural timeline for baseline behavioral characterization; Figure S3: Schematic depicting the global (composite) parameters used to characterize HC activity rhythms; Figure S4: Workflow visualizations for translating ribosome affinity purification, RNA sequencing, and bioinformatic and differential translational analyses; Figure S5: DNE elicits multigenerational hyperactivity and risk-taking behaviors in adolescent DRD1-EGFP/Rpl10a mice; Figure S6: DNE elicits multigenerational homecage hyperactivity and aberrant rhythmicity of homecage activity at baseline in adolescent DRD1-EGFP/Rpl10a mice; Figure S7: DNE elicits multigenerational enhancement of voluntary nicotine intake and preference in adolescent DRD1-EGFP/Rpl10a mice; Figure S8: Comparison of baseline behavioral phenotypes among C57BL/6J, D397, and DRD1-EGFP/Rpl10a DNE offspring and grandoffspring; Figure S9: Comparison of the consensus translateome for DRD1-expressing striatal cells of DRD1-EGFP/Rpl10a mice from the present study to a consensus protein-coding transcriptome for DRD1-expressing striatal cells of male *Drd1*-tdTomato mice; Figure S10: Comparative stratification of DEGs uniquely detected in F1 NIC or F2 NIC mice; Figure S11: Word clouds generated by word frequency analyses of STRING protein annotations retrieved for enriched and depleted DEGs uniquely detected in F1 NIC or F2 NIC mice; Table S1: Outcome measures from experiments utilized for baseline behavioral analyses of DRD1-EGFP/Rpl10a DNE offspring and grandoffspring; Table S2: Percent vs. control data derived from baseline behavioral analyses of DRD1-EGFP/Rpl10a DNE offspring and grandoffspring; Table S3: Raw hourly binned home cage activity data for all DRD1-EGFP/Rpl10a DNE offspring and grandoffspring; Table S4: Consensus D1-type striatal MSN translateome for DRD1-EGFP/Rpl10a mice; Table S5: Comprehensive lists of enriched and depleted DEGs unique to and shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S6: Excerpted differential expression analysis results for enriched and depleted DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S7: Excerpted differential expression analysis results for enriched and depleted DEGs uniquely detected in DRD1-expressing striatal cells of F1 NIC but not F2 NIC mice; Table S8: Excerpted differential expression analysis results for enriched and depleted DEGs uniquely detected in DRD1-expressing striatal cells of F2 NIC but not F1 NIC mice; Table S9: Reference index of gene attributes for all DEGs comprising the differential translateomes of F1 NIC and F2 NIC mice; Table S10: Glossary of gene symbols, gene names, Entrez Gene summaries, and UniProt summaries for enriched and depleted DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S11: Index of DEG-encoded protein annotations used for word frequency analysis; Table S12: UniProt Keyword annotations mapped to depleted DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S13: UniProt Keyword annotations mapped to enriched DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S14: G.O. Biological Process annotations mapped to enriched DEGs shared

between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S15: G.O. Biological Process annotations mapped to depleted DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S16: Reactome Pathway annotations mapped to enriched DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S17: Reactome Pathway annotations mapped to depleted DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S18: Human orthologs mapped to all DEGs identified among DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S19: Grouped human-mouse ortholog pairs for all DEGs detected in DRD1-expressing striatal cells of F1 NIC and F2 NIC mice and for all DEGs shared between F1 NIC and F2 NIC mice; Table S20: Grouped trans-omic ortholog pairs for all DEGs detected in F1 NIC and F2 NIC mice and for all DEGs shared between F1 NIC and F2 NIC mice that overlap with human gene orthologs exhibiting differential methylation signatures in the PACE meta-analysis of cord blood DNA methylation profiles among maternal smokers; Table S21: G.O. Biological Process annotations mapped to DEGs from DRD1-expressing striatal cells of F1 NIC mice that are orthologs of DMGs in cord blood from newborns of maternal smokers reported by the PACE meta-analysis thereof; Table S22: G.O. Biological Process annotations mapped to DEGs from DRD1-expressing striatal cells of F2 NIC mice that are orthologs of DMGs in cord blood from newborns of maternal smokers reported by the PACE meta-analysis thereof; Table S23: G.O. Biological Process annotations mapped to DEGs shared between DRD1-expressing striatal cells of F1 NIC and F2 NIC mice that are orthologs of DMGs in cord blood from newborns of maternal smokers reported by the PACE meta-analysis thereof; Table S24: Perturbagen analysis input lists of human orthologs mapped to DEGs shared between and unique to DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S25: Pharmacologic perturbagen classes mapped to DEGs shared between and unique to DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S26: Individual pharmacologic perturbagens mapped to DEGs shared between and unique to DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S27: Individual pharmacologic perturbagens contributing to pharmacologic perturbagen classes with connectivity scores >90 or <-90 mapped to DEGs shared between and unique to F1 NIC and F2 NIC mice; Table S28: Genetic perturbagen classes mapped to DEGs shared between and unique to DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Table S29: Individual genetic perturbagens mapped to DEGs shared between and unique to DRD1-expressing striatal cells of F1 NIC and F2 NIC mice; Supplemental Discussion [175–197].

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