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Differential Gene Expression in Differentiated Human Neuroblastoma SH-SY5Y Cells in Response to a Cocktail of Monoamine Oxidase Inhibitors

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Abstract

Differentiated human neuroblastoma (SH-SY5Y) cells were exposed to either 0.2 μM nicotine, a tobacco smoke preparation (TPM) diluted to the same nicotine concentration, or a cocktail of seven tobacco smoke monoamine oxidase inhibitors (MAOIs) at the concentrations measured in the TPM. Treatment occurred for 3 days, such that the cellular monoamine oxidase (MAO) concentration was reduced by approximately 50% in both the TPM and MAOI cocktail exposure groups. Changes in *MAO* gene expression after exposure to the different treatments were determined using qPCR, and the effect of these exposure treatments on global gene expression was also examined using mRNA sequencing. No change in *MAOA* and *MAOB* gene expression levels was observed, after any treatment, either using qPCR or mRNA sequencing. The MAOI versus control treatment comparison revealed that four genes were >2-fold down-regulated (*ZNF727*, *RP11-310E22.4*, *CRYM*, *SEMA3F*), and 19 genes were up-regulated after 3 days' exposure to the MAOI cocktail. Many of these differentially expressed genes were linked with disease conditions related to smoking and addiction. Exposure to nicotine and TPM each resulted in up- and down-regulation of different sets of genes. The results indicate that changes in MAO gene expression are unlikely to be responsible for the changes in MAO activity. The association between genes whose expression changes with tobacco MAO treatment and smoking-related diseases and addiction suggests the central role that MAO inhibition may play in mediating the effects of smoking on smokers.

Keywords: monoamine oxidase inhibitors; tobacco dependence; nicotine; tobacco particulate matter; global gene expression; qPCR

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1. Introduction

Monoamine oxidase inhibitors (MAOIs) are a well-known drug class [1] useful for treatment of depression and Parkinson's Disease. Several MAOIs are approved by the United States of America (USA) Food and Drug Administration for treatment of depression in humans, but side effects have limited their use. Monoamine oxidase (MAO) enzymes have a very wide range of substrates. Drugs affecting their activity against neurotransmitters can also affect their ability to detoxify unwanted metabolites. In early uses of these drugs, this led to life-threatening complications, limiting their use [2]. However, searches for new MAOIs are ongoing [3,4], and interest in using and developing MAOIs for treatment of depression continues [5].

Tobacco smoke is known to contain MAOIs [6]. While smoking tobacco is very harmful, the components which cause the severe health consequences of smoking are not the same as the major psychoactive components, nicotine and MAOIs [7]. Whether MAOIs in tobacco smoke are present in sufficient quantity to affect nicotine dependence is strongly suggested, but not yet proven [8]. Even less certain is the relationship of tobacco MAOIs to smoking and depression.

1.1. MAOIs in Tobacco Smoke

MAO enzymes have a broad specificity. Their function is to oxidise amine moieties in the body—to detoxify unwanted metabolites and to remove neurotransmitters such as serotonin, dopamine and norepinephrine. There are two isozymes, MAOA and MAOB, each with different substrate specificities and present in differing amounts within the various tissues in the body [6]. They are mitochondrial enzymes, tethered into the outer mitochondrial membrane, each with two subunits, with an active site next to an FAD cofactor which acts as the electron acceptor [9] in oxidation of the substrate.

Over 5000 different compounds have been detected in tobacco smoke [10] and a subset of these are known to act as MAOIs [6,11]. The most well-studied of these are the ß-carbolines, harman and norharman [12,13]. However, these do not potentiate addictive responses to nicotine unless they are at about ten times the concentration (relative to nicotine) that is found in tobacco smoke [14–16].

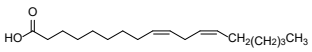
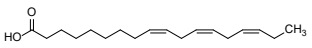
Recently we reported the identification of two new classes of MAOI—the polyunsaturated fatty acids (PUFAs) linoleic and linolenic acid, and some small phenolic molecules, catechol, hydroquinone, 4-ethyl catechol and 4-methyl catechol—present in tobacco smoke [17]. The PUFAs proved to be reversible competitive inhibitors, while the small phenolic molecules are likely to be irreversible inhibitors, long suspected to be an important part of the MAOIs in tobacco smoke [18,19]. These MAOIs are less potent than the ß-carbolines, but more abundant in tobacco smoke (Table 1), raising the possibility that they could be major contributors to tobacco smoke's overall MAO inhibitory activity [17]. Indeed, nicotine self-administration experiments in rats, using a mixture of tobacco smoke MAOIs made up to replicate their concentration in tobacco smoke, showed that this mixture, at only twice the concentration found in tobacco smoke relative to nicotine, could potentiate nicotine self-administration [20]. Thus, with the addition of these newly described tobacco MAOIs we are significantly closer to showing that tobacco MAOIs can affect addictive behaviour, at least in rats.

Overall, it is now clear that tobacco smoke delivers to the smoker a complex mixture of MAOIs, both competitive reversible inhibitors (the ß-carbolines and PUFAs [17]) and irreversible inhibitors (the small phenolic compounds), with potential to be present in sufficient amounts together to affect behaviour in smokers. Of the newly discovered tobacco MAOIs, the PUFAs have already been shown to function as antidepressants, as detailed below.

Table 1. Amounts of major phenolic, fatty acid and ß-carboline MAOIs identified in tobacco smoke.

Compound	Structure	Amounts in Tobacco Particulate Matter (TPM)	
		(µg/g Tobacco)	Reference
Catechol		100–360	[21]
4-Methylcatechol		32–46	[21]
4-Ethylcatechol		10–46	[21]

Table 1. *Cont.*

Compound	Structure	Amounts in Tobacco Particulate Matter (TPM)	
		(µg/g Tobacco)	Reference
Hydroquinone		110–300	[21]
Linoleic acid		50–146	[22]
α-Linolenic acid		52–329	[22]
Harman		0.5–2.2	[12]
Norharman		1.1–5.8	[12]

1.2. PUFAs and Depression

PUFAs taken orally have long been suggested as effective therapies for depression [23]. High α-linolenic acid (an *n*-3 PUFA) intakes are associated with low scores in standard tests for depression, with high linoleic acid (an *n*-6 PUFA) intakes having a similar effect on scores for anxiety [24]. The ratio of linolenic and linoleic acids consumed appears to also be important for depression risk [25].

The early observation that eating oily fish appeared protective against depression has prompted a significant amount of work to test the efficacy of eicosopentanoic acid (EPA) and docosahexanoic acid (DHA) supplementation for treatment of depression [26,27] since these are major components of fish oils. Such supplementation shows some promise, particularly for those whose baseline diet is deficient in PUFAs.

EPA and DHA are long-chain *n*-3 PUFAs produced from linolenic acid in the body, although they can also be obtained by eating oily fish. Amounts used in supplementation regimes are typically at least one gram per day [26]. The *n*-3 PUFAs are thought to work by increasing membrane fluidity, particularly in the brain, and via their effects on reducing inflammation [27].

Thus, there is significant evidence that the *n*-3 PUFAs play some role in preventing depression; however, the discovery that linolenic and linoleic acids are MAOIs adds another dimension to consideration of mechanisms by which they might act, particularly when they are obtained by smoking tobacco, where inhalation and consequent rapid delivery to the brain are the mode of delivery, rather than ingestion of these compounds.

1.3. Does Smoking Relieve Depression, or Cause It?

Smoking is well known to reduce MAO activity in the human body [19] and it seems increasingly likely that the sum of the known MAOIs in tobacco smoke is sufficient to cause the MAO-modulating effects of smoking, as opposed to indirect mechanisms such as modulation of gene expression, or epigenetic mechanisms. Thus, an anti-depressant effect from smoking could be anticipated. However, smoking's relationship to depression is complex and not well understood.

Anecdotal evidence suggests that smokers smoke not only for the nicotine, but also to help regulate mood and cope with stress, anxiety and depression. Some studies support this, showing that, independent of nicotine's effects, the ß-carbolines are able to alleviate depression in mice [28] and rats [29] and anxiety responses in zebrafish [30], and that smoking is able to decrease pain and anxiety responses in humans [31,32]. In contrast, other studies on humans suggest that smoking cessation relieves depressive symptoms [33], suggesting that smoking may cause some degree of depression. In summary, the association

between depression and smoking is strong [34,35], although the direction of causation is not clear [36].

It seems likely, since smokers feel better after smoking, that people suffering from depression might be at high risk for smoking initiation and subsequent nicotine dependence. If smoking also includes effective MAO inhibition, sufficient to noticeably relieve depression, then attempts to quit smoking will be less likely to succeed, because the smoker trying to quit smoking will be withdrawing not only from nicotine, but from the effects of MAO inhibition. The depression and irritability experienced during smoking cessation will not only be caused by nicotine withdrawal, but also by gradually rising MAO enzyme activity in the brain [19]. The long-known association between smoking and depression and the limited success of nicotine replacement therapy in supporting smoking cessation [37] both support the notion that tobacco smoke may contain MAOIs capable of helping relieve depression.

Since these tobacco smoke components are not, in themselves, contributors to the toxic and carcinogenic effects of smoking, they have the potential to be useful contributors to our arsenal of pharmacotherapies against depression.

In this paper we present results of work examining the effects of combined tobacco MAOIs on both MAO gene and global gene expression in a human neuroblastoma cell line, differentiated SH-SY5Y cells [38].

The differentiated cells were exposed to various reagents (TPM, nicotine, cocktail of seven MAOIs) for 3 days before harvesting. Our aims were two-fold: firstly, to determine whether physiologically relevant concentrations of TPM, nicotine, or the MAOI cocktail caused changes in MAO gene expression, and secondly, to examine changes in global gene expression after exposure to these different treatments, which could be linked to behavioural changes.

2. Results

2.1. Relative Gene Expression of MAOA and MAOB at Day 3

The relative expression of *MAOA* and *MAOB* genes normalised to the housekeeping gene *POLR2F* is shown in Figure 1. There was no significant change in the expression of *MAOA* following nicotine, TPM or MAOI treatment with respect to the control ($F(3, 11) = 0.3462$, $p = 0.7927$). There was a slight, but non-significant decrease in *MAOB* gene expression following nicotine, TPM and MAOI treatments ($F(3, 11) = 2.382$, $p = 0.1251$). *MAOB* expression in nicotine-treated ($p = 0.1481$), TPM-treated ($p = 0.1736$) and MAOI-treated ($p = 0.2322$) cells was not significantly altered from the control. Similar results were obtained when the results were normalised to *GAPDH* (Figure 1).

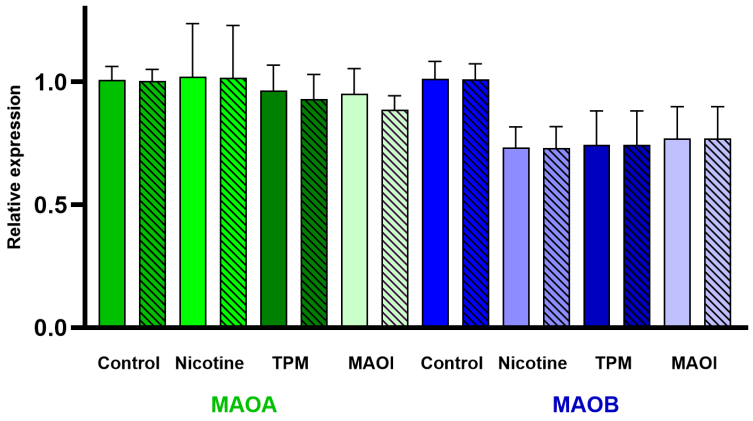


Figure 1. Expression of *MAOA* and *MAOB* genes after treatment with nicotine, TPM or mixed MAOIs, relative to the untreated control. The results are normalised to the expression of housekeeping genes

GAPDH (unhatched columns) and *POLR2F* (hatched columns) and are shown as mean ± standard error of the mean. Green columns relate to *MAOA* gene expression, and blue ones relate to *MAOB* gene expression.

2.2. Differential Global Gene Expression with Different Treatments

The effect of different treatment exposures for 3 days on gene expression was determined using RNA sequencing technology (RNAseq). Table 2 shows the total number of transcripts with differentially expressed genes in nicotine versus control treatment after the application of the two filters (p-adj < 0.05 and $\log_2$FoldChange > 2 or <−2). Ten genes showed differential expression, with six genes having increased expression and four genes having decreased expression compared to the control. Similarly, Tables 3 and 4 show the total number of transcripts with differentially expressed genes for TPM treatment versus control and MAOI treatment versus control, respectively. TPM versus control had 17 genes with differential expression, where four genes were down-regulated, and 13 genes were up-regulated. Table 3 shows the list of 23 genes with differential expression associated with MAOI treatment. The MAOI versus control treatment comparison showed four genes were down-regulated, and 19 genes were up-regulated. Tables 2–4 also detail the putative functions of the differentially expressed genes from the three comparison groups obtained from the NCBI gene database. It was found that many genes with significant differential expression were yet to be annotated and had no putative functions assigned to them. No significant change in the expression of *MAOA* or *MAOB* from any of the treatments compared to control was observed.

Table 2. List of genes with significant expression changes after treatment with nicotine compared to the control.

Gene ID	Gene Name	log2FoldChange	p-Value
NOS1	nitric oxide synthase 1	−2.008	5.91×10^{-8}
GATA4	GATA binding protein 4	−2.140	1.07×10^{-8}
HOOK3	hook microtubule tethering protein 3	−2.699	4.87×10^{-6}
RP1-178F10.1	Not annotated	−2.484	5.11×10^{-5}
LINC00342	Long intergenic non-protein coding RNA 342	2.350	8.80×10^{-6}
DNAH12	dynein axonemal heavy chain 12	2.743	3.40×10^{-5}
ZNF658B	Zinc Finger Protein 658B	2.568	6.56×10^{-5}
LINGO1	leucine rich repeat and Ig domain containing 1	2.060	9.53×10^{-5}
RP11-848G14.2	Not annotated	2.183	0.000450
RP11-10A14.3	Not annotated	2.281	0.000614

The genes with differential expression in the three comparison groups are presented in a three-way Venn diagram in Figure 2. *DNAH12* (Dynein Axonemal Heavy chain 12) and *ZNF658B* (Zinc Finger Protein 658B) are common for nicotine and MAOI treatments, while *CTD-2012J19.1* and *RP11-80514.5* are common for TPM and MAOI treatments. The common genes identified in the comparisons of TPM versus control and MAOI versus control are yet to be annotated and do not have any functions assigned to them. No common genes with differential expression for nicotine versus control and TPM versus control or for all three comparison groups were found.

Table 3. List of genes with significant expression changes after treatment with TPM compared to the control.

Gene ID	Gene Name	log2FoldChange	*p*-Value
ZSCAN20	zinc finger and SCAN domain containing 20	−2.759	1.38×10^{-6}
PRH2	proline rich protein HaeIII subfamily 2	−6.431	2.52×10^{-6}
RP11-566K11.5	Not annotated	−5.315	6.60×10^{-5}
SYNGR4	synaptogyrin 4	−2.900	0.000279
RP11-220I1.5	Not annotated	6.472	8.04×10^{-6}
RP11-104F15.9	Not annotated	4.457	1.30×10^{-5}
RP11-328C8.4	Not annotated	2.220	6.23×10^{-5}
USP7	ubiquitin specific peptidase 7	2.006	3.60×10^{-9}
RP11-805J14.5	Not annotated	2.117	0.000103
CTD-2012J19.1	Not annotated	3.964	0.000183
TENM4	teneurin transmembrane protein 4	2.142	0.000254
TNFRSF10A	TNF receptor superfamily member 10a	2.448	0.000272
ZNF34	zinc finger protein 34	2.019	1.17×10^{-7}
ALPK1	Alpha Kinase 1	2.378	0.000293
AC106869.2	Not annotated	2.001	0.000445
AC007036.5	Not annotated	2.276	0.000448
FOXR2	forkhead box R2	2.314	0.000522

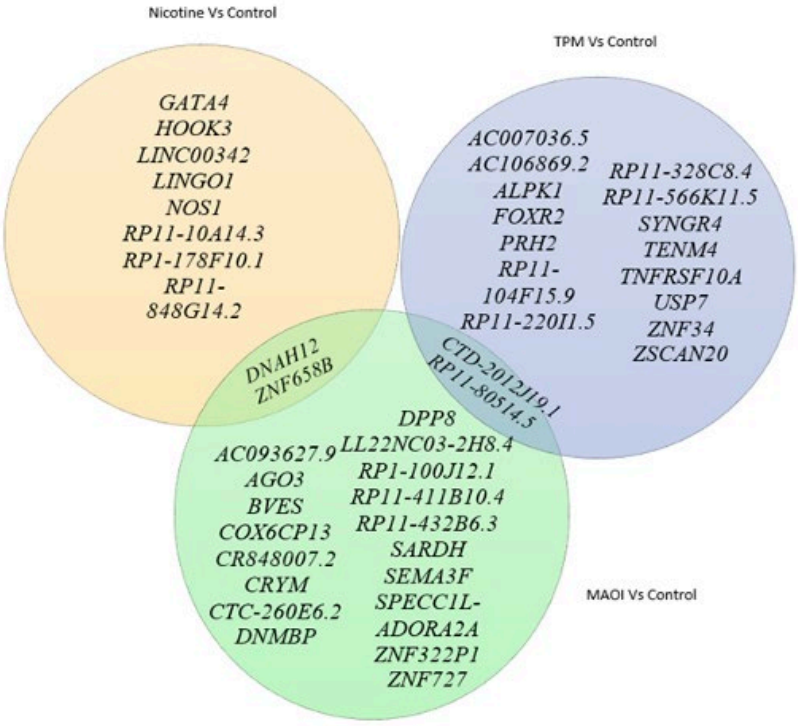


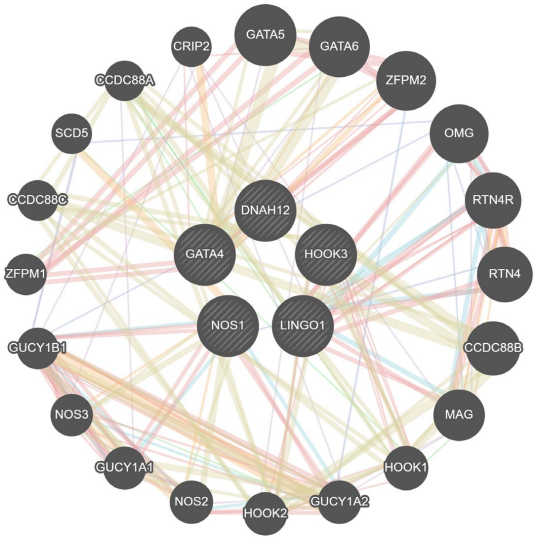
Figure 2. Three-way Venn diagram showing differentially expressed genes in different comparison groups.

Table 4. List of genes with significant expression changes after treatment with the MAOI cocktail compared to the control.

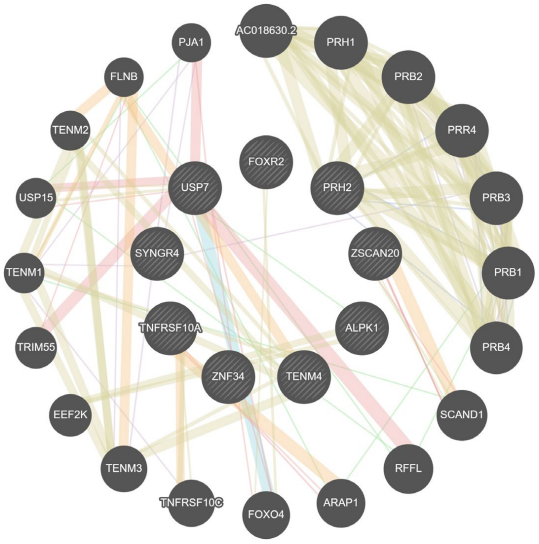
Gene ID	Gene Name	log2FoldChange	*p*-Value
ZNF727	zinc finger protein 727	−3.881	0.0000548
RP11-310E22.4	Not annotated	−2.439	0.000448
CRYM	crystallin mu	−2.903	0.0000572
SEMA3F	semaphorin 3F	−2.200	0.0000068
RP11-805J14.5	Not annotated	2.517	0.0000098
BVES	blood vessel epicardial substance	2.328	0.0000184
LL22NC03-2H8.4	Not annotated	2.212	0.0000224
ZNF658B	Zinc Finger Protein 658B	3.828	0.0000002
CTD-2012J19.1	Not annotated	4.241	0.0000853
ZNF322P1	Zinc Finger Protein 322 Pseudogene 1	4.041	0.0000977
AC093627.9	Not annotated	2.251	0.000106
SPECC1L-ADORA2A	Not annotated	3.257	0.000120
SARDH	sarcosine dehydrogenase	2.159	0.000144
DNMBP	dynamin binding protein	2.198	0.000147
DNAH12	dynein axonemal heavy chain 12	2.236	0.000233
DPP8	dipeptidyl peptidase 8	2.048	<0.0000001
AGO3	argonaute RISC catalytic component 3	2.269	0.000461
RP11-411B10.4	Not annotated	2.927	0.000489
RP1-100J12.1	Not annotated	2.754	0.000512
CR848007.2	Not annotated	3.674	0.000624
RP11-432B6.3	Not annotated	2.656	0.000846
CTC-260E6.2	Not annotated	2.045	0.00101
COX6CP13	Not annotated	3.255	0.00106

Functional annotation was performed on the list of differentially expressed genes in each of the three comparison groups using DAVID version 2021q4 software. Pathway analysis revealed that the differentially expressed genes from the nicotine versus control comparison were enriched in pathways for amyotrophic lateral sclerosis ($p = 0.045$), neurodegeneration (multiple diseases) ($p = 0.058$) and microtubules ($p = 0.062$) as an enriched cellular component.

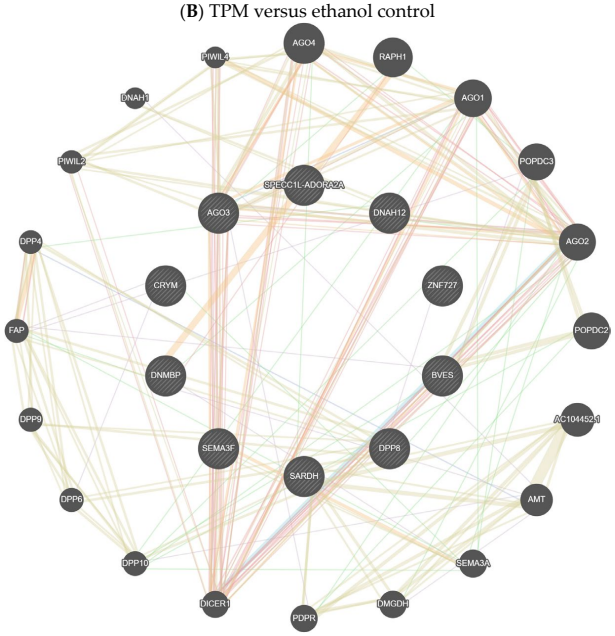
Figure 3A consists of 5 of 10 differentially expressed genes after nicotine treatment compared to control and 20 further genes in the gene network derived by GeneMANIA (https://genemania.org/). Similarly, Figure 3B consists of 9 of 17 differentially expressed genes after TPM treatment compared to control and 20 further genes in the derived gene network from GeneMANIA. Figure 3C consists of 10 of 23 differentially expressed genes after MAOI treatment compared to control and 20 further genes in the derived gene network from GeneMANIA.



(**A**) Nicotine versus ethanol control

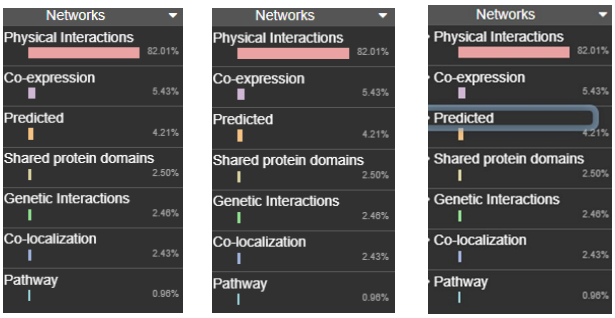


(**B**) TPM versus ethanol control



(**C**) MAOI cocktail versus ethanol control.

Figure 3. *Cont*.



(**D**) Key to the connections between genes mapped by GeneMANIA.

Figure 3. GeneMANIA networks built for differentially expressed genes in SH-SY5Y cells, using Gene Ontology weighting (molecular function-based). (**A**) shows the genes changing when cell are treated with nicotine only. (**B**) shows the genes changing when cells are treated with TPM (contains both nicotine and tobacco MAOIs). (**C**) shows the genes changing when cells are treated with mixed tobacco MAOIs. The larger, striped circles in the centre are the genes submitted to the system having known links to other proteins and pathways. The smaller circles are genes that interact with them. The links are coloured according to the type of connection, as shown in (**D**).

3. Discussion

Both RNA sequencing data and qPCR analysis showed that there was no notable change in gene expression of *MAOA* and *MAOB* genes in SH-SY5Y cells post-exposure to nicotine, TPM or MAOI cocktail treatments when compared with control. Thus, the observed reduction in enzyme activity was unlikely to be due to changes in MAO gene expression.

RNA sequencing analysis provided important information about the effects of treatments on differential gene expression. Nicotine treatment caused down-regulation of genes such as *NOS1* and *GATA4* and up-regulation of genes such as *LINC00342*. These genes have been found in previous studies to be related to nicotine's effects on cells. A previous study reported that smokers have reduced levels of exhaled nitric oxide. However, the mechanism of this reduction is not clear [39]. Nitric oxide synthase 1 synthesises nitric oxide from L-arginine [40]. The reduction in *NOS1* gene expression levels induced by nicotine exposure as seen in our study may be one of the possible mechanisms for the reduction in nitric oxide synthase enzyme, which in turn causes a reduced level of exhaled nitric oxide in smokers.

We showed that nicotine treatment also caused significant down-regulation of *GATA4*, which encodes a member of the GATA family of zinc-finger transcription factors involved in regulation of genes having roles in myocardial differentiation and embryogenesis [41]. In another study, a suppressive effect of nicotine exposure was observed on myocardial differentiation, regulated by decreased expression of the gene *GATA4* [42]. They further found nicotine-induced inhibition of *GATA4* was blocked when hexamethonium, a global nicotinic acetylcholine receptor (nAChR) antagonist, was added. This suggests a role of nAChRs in nicotine-mediated gene suppression.

The increased expression of LINC00342 seen in our results may be one of the mechanisms by which nicotine induces cell proliferation and invasion. Nicotine has been shown to induce cell proliferation and invasion in different human cancer cells [43]. *LINC00342* is a non-protein-coding RNA which is also associated with cell proliferation and tumour progression [44].

Two separate studies, performed to observe the change in gene expression induced by 1 mM nicotine exposure of SH-SY5Y cells for 24 h [45] or 1 h [46], showed 163 and 295 differentially expressed genes respectively. None of the genes with altered expression in our study matched with those of either of the earlier studies. A reason for the discrepancy

and much lower number of differential gene expressions observed in our study could be the 5000-fold lower concentration of nicotine used here.

The pathway enrichment analysis using DAVID revealed that differentially expressed genes in the nicotine treatment group compared to control were enriched for amyotrophic lateral sclerosis (ALS) pathway genes. ALS is a neurodegenerative disorder marked by the degeneration of motor neurons [47]. Cigarette smoking has been found to be associated with ~70% increased risk of ALS in a case study [48]. *DNAH12* and *NOS1* were found to be related genes, and these genes could be potential targets when studying ALS.

TPM exposure was shown to cause changes in the expression of genes including *USP7*, *ZNF34*, *ALPK1*, *TNEM4*, and *FOXR2*, which have been found to have roles in the pathophysiology of different diseases and conditions. Research performed by Zeng and co-workers agrees with our result, where cigarette smoke was observed to cause increased gene expression of *USP7* [49]. They used a mouse model to show the USP7/p300-dependent pathway was responsible for inhibited cell activity, resulting in cell arrest and depletion of endothelial progenitor cells that might lead to COPD. Son and coworkers observed that *ZNF34* was among the top 10 genes out of the 140 genes associated with asthma exacerbation in smokers, but not in non-smokers [50]. This suggests that smoking can influence *ZNF34* expression, which might have a role in asthma exacerbation. TPM has also been shown to increase gene expression of *TENM4*, a candidate gene for schizophrenia and a novel cancer stem cell molecule [51,52]. *FOXR2* is another notable gene having increased expression with TPM treatment. This gene has been demonstrated to have high expression in several types of cancer, such as breast, liver and lung cancer, and tumour samples of patients [53,54]. It is well-known that smoking causes several types of cancer, and increased expression of *FOXR2* by TPM may have an important role in the promotion of cancer cell proliferation.

We observed that MAOI treatment caused a significant increase in the expression of the *BVES* gene. *BVES* is included in the list of genes that are likely to encode for cell adhesion molecules (CAM). The variation in CAM gene expression is believed to cause effects on different circuits that are specific to addiction and drug reward [55]. MAOIs present in cigarette smoke may impart some of the addictiveness of smoking by increasing the expression of *BVES*.

Some research suggests that smoking poses a significantly increased risk of development of Alzheimer's disease [56]; however, some research has linked cigarette smoking with decreased risk of Alzheimer's disease [57,58]. Expression of mRNA of the *DNMBP* gene has been found to be lower in Alzheimer's brains, and increasing the expression of *DNMBP* may help in preserving long-term memory formation [59]. The up-regulation of *DNMBP* post-exposure to MAOIs in our study may be involved in this partly protective feature of cigarette smoking against Alzheimer's disease. However, more research is required to better understand the reason behind the decreased risk observed in some studies.

SARDH was found to be up-regulated after MAOI exposure in our study. This gene encodes sarcosine dehydrogenase, which is responsible for catalysing the oxidative demethylation of sarcosine (NCBI gene database). Sarcosine dehydrogenase, which is found in the soluble portion or the matrix space of mitochondria, is covalently bound to flavin adenine dinucleotide (FAD) along with succinic dehydrogenase, which is a mitochondrial inner membrane enzyme and MAO, which is located in the outer membrane [60]. The increased gene expression of SARDH may influence the regulation of MAO inhibition due to TPM or MAOIs, as both SARDH and MAO have covalent bonding to FAD in common. Further study is required to establish such a relationship and understand the effect it might have on the regulation of MAO inhibition.

Increased gene expression of DPP8 was observed with MAOI treatment. This gene has been found to have increased expression in the nucleus accumbens of rhesus macaques following long-term cocaine self-administration [55] and is a potential target of substances of abuse.

Another gene, *Sema3F*, had decreased expression following MAOI treatment. *Sema3F*, along with other genes in the semaphorin pathway, has been found to have altered expression in mice exposed to morphine or heroin or in post-mortem brains of patients with chronic alcohol or cocaine use, suggesting a role in addiction [61].

Our results did not clearly link nicotine, TPM or tobacco MAOIs to depression, an expected result, since the MAOIs were most likely affecting MAO activity directly, and not through a change in MAO gene expression.

Changes seen in one cell type in vitro may not replicate what happens in the brain, where many different cell types interact to form the functioning brain, and many different transport and metabolic processes will affect just what substances these different cells "see" when someone smokes. In particular, it should be noted that our study was performed using one neural cell type at one time point (3 days of exposure) with concentrations of exposure substances relevant to smokers, which are quite low compared to other in vitro studies. However, even at these low concentrations, the TPM treatment results appeared to be dominated by toxicity and cancer-related responses. Statistically significant nicotine and MAOI treatment results were distinct from the TPM results. Interestingly some MAOI treatment gene expression changes were related, via other research findings, to addiction [55,61].

If these tobacco MAOIs can be effective in relieving depressive symptoms, it is of interest that the reduction in MAO activity, even in heavy smokers, is less than 50% [19], unlike the 90% inhibition required for efficacy with irreversible MAO inhibitor drugs [2]. Further, smoking is not associated with difficulties in removing tyramine from the body so that, if tobacco MAOIs can be developed to help treat depression, side effects from their use are likely to be minimal.

When tobacco is smoked, a bolus of nicotine (and other accompanying smoke molecules) is taken up to the bloodstream in the lung and travels rapidly, and with minimal opportunities for dilution and metabolism, to the brain. In contrast, when injected or administered intravenously as in most animal experiments, or when ingested as in human trials, these MAOIs will be metabolised and diluted significantly before ever reaching the brain. Inhalation, as a means of delivery of these particular MAOIs, may prove to be critical for efficacy. The PUFAs are expected to be metabolised to more complex lipids quite rapidly, or incorporated into membranes, or both [62]. The MAO inhibitory activity of many metabolites is unknown, as is the length of time for which PUFA MAOIs will remain in the cell cytoplasm and be physically able to interact with MAO enzymes. When delivered by inhalation, the brain will "see" more of the MAOI activity than by other delivery routes. PUFAs will, however, have a very low toxicity profile. By analogy with smoker intakes of these PUFAs, an effective dose of linolenic acid, inhaled, should be in the low milligram range. Patients in trials of oral supplementation with PUFAs use doses of at least 1 g, for efficacy. Thus, the effective dose of inhaled linolenic or linoleic acid could be 100-to 1000-fold lower than the effective oral dose of EPA or DHA.

The small phenolic inhibitors in the MAOI cocktail are expected to be oxidised rapidly in the body (potentially by MAO enzymes). They appear to be irreversible inhibitors of MAOA and MAOB, taking several hours of contact with the enzyme to have their full effect [17]. Their effects may take time to build up, but should be effective in a similar dose range, if these MAOIs are inhaled. For both the PUFAs and the small phenolic inhibitors,

administration by inhalation would have a further advantage of minimising side effects arising from MAO inhibition occurring elsewhere than in the brain.

3.1. Limitations

None of the gene expression changes observed have been formally validated. Further, they are in vitro experiments, pertaining to only one neuronal cell line. Changes seen in one cell type in vitro may not replicate what happens in the brain, where many different cell types interact to form the functioning brain. SH-SY5Y cells differentiate towards a neuronal cell type with neurite outgrowths [63], but will not have functional connections with other brain cell types. Taking the in vitro situation even further from the in vivo reality, many different transport and metabolic processes will affect just what substances these different cells "see" when someone smokes.

Also our study was performed at one time point (3 days of exposure). This time point represents something akin to a new "steady state" rather than capturing the dynamic changes occurring after initial exposure. The concentrations of exposure substances used were, however, relevant to smokers. They are low compared to other in vitro studies.

Even at these low concentrations the TPM treatment results appeared to be dominated by toxicity and cancer-related responses. Statistically significant nicotine and MAOI treatment results were distinct from the TPM results. Interestingly some MAOI treatment gene expression changes were related, via other research findings, to addiction [55,61].

3.2. Future Work

Future work could include validation and further exploration of the links between MAO inhibition and addictive responses in these cells, looking at both mRNA transcription responses and proteomic changes. The studies could also be expanded towards studies of rodent brains following inhalation exposure to these MAOIs. It will also be important to link these MAOIs with behavioural changes in rodents, related to depression, such as the forced swim test and the sucrose preference test [64] or analysis of ultrasonic vocalisations [65].

It is possible that we still have more tobacco MAOIs to find. However, the PUFAs and the small phenolic tobacco MAOIs each have interesting characteristics that may make them important in modulating the behavioural effects of smoking, and both types of MAOI deserve further exploration.

4. Materials and Methods

4.1. Cell Line Culture

The SH-SY5Y (human bone marrow neuroblast) cell line (catalogue number CRL-2266, American Type Culture Collection), was cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (GIBCO, Invitrogen, Carlsbad, CA, USA) with 10% v/v heat-inactivated Foetal Bovine Serum (FBS) (Sigma, St. Louis, MO, USA) and 1% v/v Penicillin–Streptomycin (P/S) (10,000 Units Penicillin and 10 mg streptomycin per mL) (Sigma). Genomic and transcriptomic characterisation of SH-SY5Y cells has been reported elsewhere [66]. The SH-SY5Y cells were maintained in a 75 cm^2 (T-75) Nunc TM cell culture flask (ThermoFisher, Waltham, MA, USA) in a cell culture CO_2 incubator (Esco, Shinjuku, Japan) at 37 °C in a humidified atmosphere of 5% CO_2. Cell manipulations were carried out in an aseptic environment in a horizontal biological safety cabinet (Esco, Shinjuku, Japan), sterilised by UV irradiation and decontamination with 70% ethanol before and after each use. Cells were routinely subcultured once per week at an approximately 1:10 ratio. Trypsin was not required for subculturing, since these cells will detach when medium is replaced by calcium- and magnesium-free phosphate-buffered saline (pH7.2, PBS).

4.2. Cell Differentiation

An aliquot of 4×10^6 cells was seeded in a T-25 cell culture flask in RPMI medium with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The medium was replaced with a differentiation medium comprising RPMI medium with 3% FBS, 1% P/S and retinoic acid two days later. Retinoic acid (10 μM) was used as a differentiation agent. The differentiation medium was refreshed every other day after the first addition. Differentiation was completed 7 days after the addition of retinoic acid. Differentiation of cells was confirmed by cell images showing dendrites and Western blots comparing the changes in the level of neuronal marker synaptophysin in differentiated and undifferentiated cells.

4.3. Cell Treatment

Aliquots of differentiated SH-SY5Y cells (n = 3 per treatment) received one of four different treatments—0.05% ethanol as a vehicle control; nicotine dissolved in distilled water to achieve a final concentration of 0.2 μM nicotine (reported to be the mean concentration of nicotine found in the blood of smokers [67]; tobacco particulate matter (TPM); or mixed tobacco MAOIs. All treatments included ethanol at 0.05%. The replicates of SH-SY5Y cells were cultured in 75 cm^2 culture flasks and exposed to the treatments for a period of 3 days, with medium replaced every second day to maintain the concentration of retinoic acid and catechols in the medium. Cell lysates were collected at the end of the exposure period and utilised for MAO gene expression and RNA seq analysis.

TPM was prepared using a smoking machine as previously described [68]. Briefly, tobacco (1 kg roll-your-own tobacco, "Drum" brand manufactured by Imperial Tobacco), was hand-rolled into cigarettes that each contain approximately 1 g of tobacco. Cigarettes were individually smoked, and TPM was collected onto filters using 5 cigarettes per filter. The TPM was stored on the filters at −80 °C until being extracted with ethanol and dried by vacuum centrifugation (Eppendorf South Pacific, Macquarie Park, Australia). Prior to use, the contents of one vial containing TPM from 0.03 gm of tobacco were dissolved in ethanol. The TPM treatment contained ethanol in the final concentration of 0.05% in the cell differentiation medium, and TPM sufficient to give a final concentration of 0.2 μM of nicotine. The required dilution was based on the amount of nicotine present in each TPM sample obtained from the assay of nicotine.

For the MAOIs two different stock solutions were prepared, one for polyunsaturated fatty acids (PUFAs) and the other for catechols. For the PUFAs, a concentration of 34.12 μM linoleic acid and 32.59 μM linolenic acid was prepared, dissolved in ethanol in a microfuge tube, and stored at −80 °C. A stock solution of the small phenolic inhibitors had a concentration of 115.85 μM quinol, 166.24 μM catechol, 25.75 μM 4-methylcatechol and 22.29 μM 4-ethylcatechol, which was dried using vacuum centrifugation and stored at −80 °C. Prior to use, the contents of one vial of each stock solution were dissolved and diluted in ethanol and pooled before adding to the differentiated SH-SY5Y cells. The MAOI treatment contained ethanol in the final concentration of 0.05% in the differentiation medium, and the MAOIs (1.32 nM linoleic acid, 1.26 nM linolenic acid, 4.48 nM quinol, 6.43 μM catechol, 0.99 μM 4-methylcatechol, 0.86 μM 4-ethylcatechol). The required concentration of MAOIs was calculated from the assays of nicotine and MAOIs in TPM, based on the molar amount of individual MAOIs relative to that of nicotine in the TPM sample. The stock solutions of MAOIs were diluted to the required MAOI concentration using absolute ethanol and added to the differentiation medium immediately before use.

Treatment with ethanol concentrations up to 0.5% has been found to be non-toxic for SH- SYY cells [69,70]. Both TPM and the mixed MAOIs inhibited cellular MAO activity by approximately 50% under the conditions used [71], whereas nicotine itself did not inhibit MAO activity, as expected [72].

4.4. mRNA Extraction

After 3 days of treatment, total RNA was extracted from the cells using an RNeasy extraction kit (Qiagen, Hilden, Germany) and the RNA concentration and quality were determined. RNA samples were stored at −80 °C until required for further analysis.

4.5. MAOA and MAOB Gene Expression Assays

Gene expression assays were performed to compare the expression level of specific genes in the sample and were conducted using the LightCycler96 (Roche, Copenhagen, Denmark) PCR system. TaqMan Gene Expression assays that comprised predesigned primer and probe sets and TaqMan fast advanced master mix (ThermoFisher, Waltham, MA, USA) were used to perform quantitative gene expression studies on mRNA samples as described by the manufacturer.

Equivalent amounts of total RNA in the stored samples were converted to cDNA using an iScript® cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA).

cDNA samples were amplified for qPCR in a Lightcycler96 using a pre-set protocol with the following cycling conditions: an initial step of 50 °C for 2 min, followed by pre-incubation at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

There were three replicates for each cDNA sample, and gene expression of *MAOA* and *MAOB* relative to the housekeeping genes GAPDH and POLR2F [73] was determined using the $2^{-\Delta\Delta CT}$ method.

4.6. Global Gene Expression Analysis

Read quality was assessed for each RNA sample using FastQC [74] and the results were collected by MultiQC [75]. The reads that passed the quality filtering were used for downstream analysis. All reads were mapped using splice-aware mapper HISAT2 against the HG38 version of the reference human genome obtained from the NCBI website [76].

StringTie 2.2.3 was used to assemble the reads into transcripts for each sample. The tool GFFcompare v0.11.2 was used to assess the overall quality of the assembled transcripts by comparison to the HG38 reference [77]. An analysis of normalised counts' profiles was performed and plotted as a heatmap for all the treatments and principal component analysis (PCA) plots; PCA plot analysis was performed using the top 50 most varying genes.

DESeq2 was used to generate differential expression calls and statistics for the treatment comparisons based on the observed read counts for each gene. Filters (p-adj < 0.05; log2FoldChange > 2 or <−2) were applied to the results for the entire set of genes.

The functional roles of the differentially expressed genes were then explored using the Database for Annotation, Visualisation, and Integrated Discovery (DAVID) to better understand the biological functions of the genes with differential expression [78,79]. Gene enrichment was determined using the Gene Ontology function and pathway analysis of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) from the list of differentially expressed genes for each treatment compared to the control.

The GeneMANIA online tool was used to get more information about the functional relatedness of the genes identified including co-expression, co-localization, genetic interactions, pathways, physical interactions, predicted networks, and shared protein domains in humans [80].

4.7. Statistical Analysis

Statistical tests on PCR data were performed using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA). These include the Shapiro–Wilk test for normality and one-way Analysis of Variance, with Dunnett's multiple-comparison correction.

5. Conclusions

The gene expression changes seen, together with behavioural data [20], strongly indicate the potential of these tobacco MAOIs to affect addictive behaviour. Importantly, we used concentrations of nicotine and MAOI that are physiologically realistic for smokers' brains to experience. Given the known efficacy of MAOIs in depression, the observed effects of ingested PUFAs on depression, and the links between smoking and depression we suggest that further work on tobacco MAOIs as potential antidepressants is indicated.

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Abbreviations

The following abbreviations are used in this manuscript:

CAM	cell adhesion molecule
DHA	docosahexanoic acid
EPA	eicosopentanoic acid
FAD	flavine adenine dinucleotide
FBS	foetal bovine serum
PUFA	polyunsaturated fatty acid
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
P/S	penicillin–streptomycin
RPMI 1640	Roswell Park Memorial Institute 1640
TPM	tobacco particulate matter

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