

The Role of Harmane and Norharmane in In Vitro Dopaminergic Function


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Abstract Background. Nicotine is one of the most frequently used drugs of abuse. It has long been speculated that in addition to nicotine, tobacco contains other compounds that reinforce addiction. Two promising candidates are the tobacco smoke constituents, harmane and norharmane. Purpose. Our study evaluated the effects of exposure to harmane and norharmane alone and in combination with nicotine on midbrain neuronal cultures. Experimental design. After 24 h exposure, cytotoxicity, dopamine (DA) levels, pTH, TH, MAO-A, and MAO-B expression levels were investigated. Results. At low-to-moderate doses (1 μM–100 μM), harmane or norharmane exposure caused significant dose-dependent decreases in intracellular DA without causing high rate of cell death, mitochondrial dysfunction or generation of ROS. Harmane exposure also resulted in subsequent increase in extracellular DA levels. Conclusion. Along with downregulation of protein expression, notably MAO-B, our data indicate the potential of harmane and norharmane to act in synergy with nicotine in midbrain neurons. Keywords dopamine; harmane; monoamine oxidase; nicotine; norharmane; tyrosine hydroxylase

1. Introduction

The presence of the β-carbolines harmane (HAR) and norharmane (NOR) in the human brain has been interpreted as possible mediators in addiction [1]. Nicotine (NIC) is one of the most frequently used drugs of abuse. Worldwide, tobacco smoking leads to 5 million deaths annually, mainly as a consequence of lung cancer and cardiovascular diseases [2,3]. It has long been speculated that tobacco, in addition to NIC, contains other compounds that are addictive or act in concert with NIC [3,4]. A promising candidate among the 5,000 tobacco smoke constituents is acetaldehyde, one of the major smoke constituents [3]. HAR (1-methyl-9H-pyrido[3,4-b]indole), a condensation product of acetaldehyde and biogenic amines, is a sufficient monoamine oxidase A (MAO-A) inhibitor [3]. NOR (9H-pyrido[3,4-b]indole), the demethylated equivalent of HAR, is a MAO-A and MAO-B inhibitor [4].

Drug reward, dependence, and addiction are integrally connected to catecholamine synthesis, storage, release, and reuptake, particularly that of dopamine (DA). NIC exposure causes the release of many different neurotransmitters, notably DA [5,6]. It has also been demonstrated that NIC exposure causes significant increases in tyrosine hydroxylase (TH) expression, the rate-limiting step in catecholamine synthesis [7,8,9]. However, to date, few studies have investigated the regulation of TH expression in midbrain DA neurons. Those that have investigated expression in response to NIC exposure report conflicting findings, highlighting the need for clarification [8].

Inhibition of MAO, which catalyses the oxidative deamination of DA, increases DA levels, thereby facilitating the reinforcing effects of addictive drugs [1,4]. Even though NIC has a high affinity for MAO-A and a low affinity for MAO-B, smokers have been found to have a 28% reduction in brain MAO-A and a 40% reduction in brain MAO-B when compared to former or nonsmokers [4,10,11,12,13]. This raises a question about what compounds are responsible for the reported MAO-B inhibition and what roles they play in addictive behaviors and epidemiology. It has been suggested that HAR and NOR generation after smoking may contribute to the MAO inhibition seen in smokers [3,14,15,16].

Smoking a pack a day yields blood plasma levels of NIC in the micromolar range [17]. Large variations in HAR and NOR levels have been reported in tobacco smoke with HAR and NOR levels ranging from 2.05 nM to 20.55 nM and from 5.35 nM to 66 nM per cigarette, respectively [18]. Therefore, the potential exposure for a pack a day smoker would be 411 nM HAR and 1.32 μM NOR. Given that both HAR and NOR are also produced endogenously, found in alcoholic beverages and in various foods, doses in the range of 1 μM–100 μM were the focus of this study. This study aims to determine how HAR or NOR exposure alone or in combination with NIC affects DA metabolizing in differentiated murine mesencephalic cells (MN9D), a model of midbrain DA neurons [8].
2. Materials and methods

2.1. Materials

2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), sodium butyrate, NIC, obtained as (−)-nicotine hydrogen tartrate salt, and NOR, obtained as NOR hydrochloride, were obtained from Sigma-Aldrich (St. Louis, MO, USA). HAR was obtained as HAR dihydrochloride from American Custom Chemicals Corp. (San Diego, CA, USA). DCFH-DA (2′,7′-di-chlorofluorescin di-acetate) was obtained from Invitrogen (Grand Island, NY, USA). Lactate dehydrogenase (LDH) kits were obtained from Roche Applied Science (Indianapolis, IN, USA). DA ELISA kits were obtained from Eagle Biosciences (Nashua, NH, USA). Antibodies were obtained from Millipore (Billerica, MA, USA), Proteintech (Manchester, UK), Invitrogen, and Jackson ImmunoResearch (West Grove, PA, USA). Western blot supplies were obtained from Bio-Rad (Hercules, CA, USA), Cell Signaling (Beverly, MA, USA), Roche, and Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). All media constituents, protein assay kits, and culture dishes were obtained from Thermo Fisher Scientific (Hanover Park, IL, USA). MN9D cells (mixed sex) were a gift from Drs. Alfred Heller and Lisa Won, University of Chicago. All assays requiring use of a plate reader utilized the Synergy Mx with Gen5 software from Biotek Instruments (Winooski, VT, USA). Immunofluorescent images were captured with a Nikon epifluorescence microscope (Melville, NY, USA). ChemiDoc XRS system obtained from Bio-Rad was used to image western blots.

2.2. Cell culture

MN9D cells were grown on collagen coated plates at 37 °C and 5% CO₂ in complete media consisting of DMEM (high glucose: 4,500 mg/mL) supplemented with 10% FBS. Complete media was supplemented with 1 mM sodium butyrate for 5–7 days to induce differentiation. Upon ~ 70% confluence, cells were exposed to various concentrations of HAR, NOR, and/or NIC for 24 h. Molecular weight adjustments for each compound were performed such that doses were calculated and reported as the free base concentration, not the salt.

2.3. Membrane integrity

LDH release, an indicator of membrane integrity, from the cytoplasm of damaged cells was measured according to manufacturer specifications. Briefly, after cells were treated with varying doses of HAR, NOR, and/or NIC (1 μM–100 μM), 100 μL of media was incubated with 100 μL of LDH substrate mixture in a 96 well plate. After 15 min in an incubated shaker, 50 μL of stop solution was added to each well and the absorbance measured at 490 nm with a reference wavelength of 650 nm. Data were analyzed and presented as percent of control.

2.4. Mitochondrial function

The mitochondrial function of MN9D cells was determined using previously described methods [19]. Briefly, cells were treated with varying doses of HAR or NOR (1 μM–1 mM) at 120 μL well for 24 h. Mitochondrial dehydrogenase-induced cleavage of XTT to generate a water-soluble orange colored formazan derivative was then measured [20]. Fresh XTT reagent (30 μL at 0.2 mg/mL) in the presence of 25 μM phenazine methosulfate (PMS) was added to each well and the plates were incubated for 2 h at 37 °C. Formazan production was measured at an absorbance wavelength of 450 nm with a reference wavelength of 650 nm. Data were analyzed and presented as percent of control.

2.5. Reactive oxygen species (ROS)

The ROS assay uses DCFH-DA, a nonionic, nonpolar compound that crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. DCFH is oxidized by ROS to highly fluorescent dichlorofluorescein (DCF). As a result, intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells [21]. Briefly, after 24 h exposure to HAR or NOR (10 μM–10 mM) with 200 μM DCFH-DA (final concentration), fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm. Data are expressed as percent of fluorescent intensity relative to the control.

2.6. DA ELISA

Ultrasensitive DA ELISAs were performed on both intracellular and extracellular samples in accordance to the manufacturer’s specifications. Briefly, for sample preparation, cell culture supernatants were transferred to a sterile tube and centrifuged at 1000 rpm for 5 min at 4 °C to remove cellular debris. Then 900 μL was transferred to a 2 mL Costar Spin-X centrifuge tube with a 0.45 μm filter and centrifuged at 3000 rpm for 3 min at 4 °C. Subsequently, 90 μL of stabilizer was added to each tube and the samples were stored at −20 °C until the following day. For cell lystate sample preparation, cells were gently washed with PBS, followed by addition of 2 mL PBS/well and vigorous pipetting to dislodge the cells, which were then transferred to a tube and sonicated for 10 s. Next, 100 μL was transferred to an Eppendorf tube for subsequent protein assay for normalization. The sonicated sample was then centrifuged at 13,000 rpm for 10 min at 4 °C. Afterwards, 650 μL was transferred to a 2 mL Costar Spin-X centrifuge tube and centrifuged at 3,000 rpm for 3 min at 4 °C. Then, 65 μL of stabilizer was added to each tube and the samples were stored at −20 °C until the following
day. Concentrations of samples were extrapolated from the standard curve and are expressed as percent of control.

2.7. TH and MAO localization

Cells seeded on eight-chamber slides were treated for 24 h with HAR (10 μM), NOR (10 μM), NIC (50 μM) or a combination of HAR and/or NOR with NIC. After media removal, slides were fixed with 4% formaldehyde for 15 min and permeabilized with 0.25% Triton X-100 for 10 min. After blocking for 30 min with 2% bovine serum albumin (BSA), cells were incubated with either rabbit anti-TH (1:200, 62 kDa, Millipore) or rabbit polyclonal anti-pTH (1:200, Santa Cruz) in combination with mouse monoclonal MAO-A IgG (1:200) or goat polyclonal MAO-B IgG (1:200, Santa Cruz) overnight at 4 °C. Slides were then incubated with a combination of the secondary antibodies Alexa Fluor 488 goat antirabbit IgG, Alexa Fluor 568 donkey antigoat IgG or Alexa Fluor 594 goat antimouse IgG (1:400, Invitrogen) for 2 h at room temperature. Slides were allowed to dry overnight. The next day, slides were mounted with Vectashield plus DAPI (Vector Labs Inc.). Photomicrographs were taken at 20× using an epifluorescence microscope with DAPI (64 ms exposure), FITC (105 ms exposure), and RodX (333 ms exposure) filters.

2.8. Western blot

Cells seeded on 100 mm dishes were treated for 24 h with HAR (10 μM), NOR (10 μM), NIC (50 μM) or a combination of HAR or NOR with NIC, with treatments in triplicate. Plates were then placed on ice and 500 μL RIPA buffer (Cell Signaling) with 1% complete Ultra Mini EDTA-free protease inhibitor cocktail (Roche), 1% protease inhibitor cocktails 2 and 3 (Sigma), and 1 mM PMSF (Cell Signaling) with 1% complete Ultra Mini EDTA-free protease inhibitor cocktail (Roche) were added per dish. After 5 min, dishes were scraped and the suspensions were transferred to 1.5 mL Eppendorf tubes. Tubes were placed on a shaker (300 rpm) at 4 °C for 30 min and then sonicated briefly and centrifuged at 14,000 g for 10 min at 4 °C. The protein concentration for each sample was then determined (Pierce BCA protein assay kit). Samples were diluted in Laemmlı sample buffer (Bio-Rad) to a protein concentration of 30 μg. Samples were incubated for 5 min at 95 °C and transferred to corresponding lanes in 4%–15% Tris-HCl Criterion gels (Bio-Rad) for electrophoresis at 200 V for 50 min–55 min. Precision Plus Protein Standards (Bio-Rad) were used for molecular weight markers. After separation, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) using a Criterion Blotter (Bio-Rad) run at 100 V for 30 min. After transfer was completed, nonspecific binding was blocked by incubation with 5% BSA in TBS. Membranes were incubated with anti-TH (1:250, 62 kDa, Millipore), anti-pTH (1:250, 60 kDa, phosphoSer 40, Millipore), anti-MAO-A (1:150, 60 kDa Proteintechn) or anti-MAO-B (1:150, 53 kDa, Proteintechn) antibody for

Figure 1: Change in membrane integrity after 24 h exposure to HAR or NOR (1 μM–100 μM, N = 18) indicated by lactate dehydrogenase (LDH) assay. Analysis by ANOVA with Tukey’s post hoc. *P < .05, **P < .001 with respect to control.

48 h on a shaker (300 rpm) at 4 °C. Afterwards, membranes were washed and exposed to HRP-conjugated secondary antibody (1:800; Jackson ImmunoResearch) for 2 h at room temperature. Bands were imaged using Pierce ECL Western Blotting Substrate (Thermo Scientific) and ChemiDoc XRS system. As a control for equal protein loading, membranes were stripped and reprobed with goat antimouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:100, 36 kDa, Calbiochem) and HRP-conjugated goat antimouse (1:5,000; Jackson ImmunoResearch). Images were quantified using Image Studio Lite V3.1 (Licor).

2.9. Statistical analysis

Results are expressed as mean values ±SEM and analyzed by one-way ANOVA followed by Tukey post-hoc analysis using GraphPad Prism 6. In all instances, α = 0.05.

3. Results

Membrane integrity, as indicated by LDH, after 24 h exposure to HAR or NOR (Figure 1) indicated that 5 μM HAR increased LDH leakage by 23% with respect to control. Both HAR and NOR at 100 μM yielded LDH levels 40% and 25% less than control, respectively, in addition to being significantly less than all other doses. NIC exposure (5 μM–100 μM) did not affect membrane integrity (data not shown; ANOVA, N = 18). Mitochondrial function was only affected after 24 h exposure to 1 mM HAR or NOR (data not shown; ANOVA, N = 6, P < .001). Increase in reactive oxygen species (ROS) was only seen after 24 h exposure to 1 mM HAR; this dose resulted in ROS levels that were significantly greater than control and all other treatments (data not shown; ANOVA, N = 18, P < .001).

Highly sensitive DA ELISAs indicated a dose-dependent decrease in intracellular DA (Figures 2(a) and 2(b)) after
24 h exposure to HAR or NOR, with concentrations at 1 μM being 55% and 54% lower than control, respectively. Additionally, exposure to 10 μM NOR resulted in intracellular DA levels that were significantly higher than that of HAR (P < .01).

NIC (Figure 2(c)) also displayed a dose-dependent decrease in intracellular DA, with 25 μM, 50 μM, and 100 μM being 24.5%, 42%, and 49% lower than control, respectively. Levels after 50 μM and 100 μM exposure were significantly less than after 5 μM exposure (P < .001). Levels after 100 μM exposure were also significantly less than after 25 μM exposure (P < .05). Extracellular concentrations of DA correspondingly increased after 24 h exposure to HAR (Figure 2(d)). Notably, 100 μM HAR yielded extracellular DA levels significantly higher than all other doses of HAR, NOR, and NIC (P < .001). No significant change in extracellular concentration of DA was seen after NOR or NIC exposure, although NOR displayed a dose-dependent trend in which extracellular DA increased with respect to dose (data not shown).

Immunofluorescent assessment of TH and MAO-B localization (Figure 3) indicated that MAO-B signal decreased after exposure to HAR, NOR, and/or NIC. Most notable visual decrease in TH and MAO-B signal occurred after 24 h exposure to HAR + NOR + NIC. Immunofluorescent assessment of phosphorylated tyrosine hydroxylase
(pTH) and MAO-A localization (Figure 4) indicated that MAO-A likely plays a minute role in the effects of HAR, NOR, or NIC exposure. pTH signal decreased notably with exposure to NIC, HAR or NOR coexposure with NIC, and exposure to HAR + NOR + NIC. Quantification of protein expression was accomplished with western blot.

Once quantified, western blot data, representative image in Figure 5(a), indicated that HAR + NIC resulted in 0.56 fold decrease in pTH expression relative to control (Figure 5(b)). HAR + NOR + NIC resulted in 0.52 fold decrease in TH expression relative to control. TH expression after exposure to HAR + NOR + NIC was also significantly

**Figure 3:** Immunofluorescent assessment of TH (green) and MAO-B (red) localization after 24 h exposure to HAR (10 μM), NOR (10 μM), NIC (50 μM) or a combination thereof. Nuclei were stained with DAPI (blue). Images were taken at 20×.

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**Figure 4:** Immunofluorescent assessment of pTH (green) and MAO-A (red) localization after 24 h exposure to HAR (10 μM), NOR (10 μM), NIC (50 μM) or a combination thereof. Nuclei were stained with DAPI (blue). Images were taken at 20×.
Figure 5: Western blot: (a) representative western blot of pTH, TH, MAO-A, MAO-B, and GAPDH after 24 h exposure to HAR (10 μM), NOR (10 μM), NIC (50 μM) or a combination thereof. (b) Quantification of pTH and TH expression. (c) Quantification of MAO-A and MAO-B expression. In all cases, control = 1, *P < .05, **P < .01, ***P < .001 significantly less than control; analysis by ANOVA with Tukey’s post hoc, N = 3.

Changes in MAO-A and MAO-B expression relative to control can be seen in Figure 5(c). A notable decrease, 0.83 fold relative to control, in MAO-A expression was seen after exposure to NOR + NIC. Exposure to NOR + NIC also resulted in a significantly decreased expression of MAO-A when compared to exposure to NIC alone (P < .05). MAO-B expression after 24 h was significantly less than control for all treatments. Additionally, MAO-B expression after 24 h exposure to NOR + NIC was significantly less than expression after exposure to NOR alone (P < .05). MAO-B expression levels after HAR or NOR exposure were also significantly higher than levels after NIC exposure (P < .05).

4. Discussion

As relatively large variations in the levels of HAR and NOR have been reported in tobacco smoke [18], the focus of this study was to determine if HAR or NOR alter DA metabolism in midbrain neurons, possibly implicating them as potential contributors to NIC addiction. As loss or dysregulation of midbrain DA neurons is also associated with drug addiction and exposure to dopaminergic toxins [8, 22], intracellular and extracellular DA levels after exposure to the HAR, NOR or NIC were investigated. At low-to-moderate doses (1 μM–100 μM), HAR or NOR exposure caused significant dose-dependent decreases in intracellular DA (Figures 2(a) and 2(b)) without causing a high rate of cell death (Figure 1), mitochondrial dysfunction or generation of ROS. NIC displayed similar effects, with intracellular DA being affected beginning at 25 μM (Figure 2(c)). Lower intracellular DA levels could be the result of MAO-A and/or MAO-B inhibition. Only HAR exposure resulted in a subsequent increase in extracellular DA levels (Figure 2(d)), possibly due to prolonged depolarization and/or MAO-B inhibition (Figure 6). HAR in combination with NIC did significantly decrease the expression of pTH, and NOR + HAR + NIC significantly decreased the expression of TH. This could be the result of inhibition or downregulation of 14-3-3 protein or α-synuclein. The lack of evidence for increased extracellular DA after NOR or NIC exposure is likely the result of inhibition or downregulation of DA production. However, because neither NOR nor NIC resulted in a significant decrease
Figure 6: Proposed cellular mechanism of HAR and NOR effects in the presence of nicotine: nicotine elicits a response to neurons by binding to acetylcholine receptors (nAChR) that are ligand-gated ion channels. This causes prolonged depolarization, which in turn causes the neuron to release more dopamine (DA). Reuptake of dopamine by nAChRs is also inhibited by nicotine. HAR and NOR are transported into neurons possibly via β-carboline receptors, at which point they inhibit MAO-A and MAO-B, which slows the degradation of DA. It is also possible that 14-3-3 proteins simultaneously bind to α-synuclein and, in the presence of nicotine, causes tyrosine hydroxylase (TH) to be converted into its active, phosphorylated form (pTH), which in turn generates ROS as a byproduct of DA synthesis.

in pTH or TH expression (Figure 5(b)), the decrease in intracellular DA without an increase in extracellular DA may be the result of inhibition or downregulation of 14-3-3 protein or α-synuclein (Figure 6), both of which are required to act in concert with TH to synthesize DA. Additionally, all treatments decreased MAO-B expression, which would result in a slower breakdown of DA. As HAR + NIC significantly decreases pTH expression, HAR + NOR + NIC significantly decreases TH expression (Figure 5(b)), and NOR + NIC significantly decreases MAO-A and MAO-B expression (Figure 5(c)), it is possible that in midbrain neurons HAR or NOR in combination with NIC may play a role in reinforcing NIC addiction. A proposed mechanism of action is given in Figure 6. It is important to note that how HAR and NOR enter neurons is still uncertain although the GTPase proteins RhoA and/or Cdc-42 in conjunction with PIP 5-kinase have been suggested [23]. The DA reuptake transporter portrayed at the synapse may be a nAChR.

It is possible that inappropriate regulation of TH and MAO gene expression may play a role in drug addiction. The present study indicated that HAR and NOR may play a role in NIC addiction. Further studies are warranted to determine whether the changes in TH and MAO expression seen in this study occur as a result of glucocorticoid interactions, are transcriptional and/or posttranscriptional, and/or are the result of a direct effect on nAChRs. Additional studies are also needed to investigate the role α-synuclein and 14-3-3 protein play in NIC addiction and DA synthesis after HAR or NOR exposure. The ability of HAR or NOR to interact and/or inhibit P450 enzymes, specifically 1A1, 2D6, and 2E1 [24], and whether this plays a role in NIC addiction also warrants further investigation.

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Meeting presentations Portions of the data were previously presented at the following:

Authors' contributions S. M. Lantz, E. Cuevas, and S. Z. Imam participated in research design. S. M. Lantz, E. Cuevas, and B. L. Robinson conducted experiments. M. G. Paule and S. F. Ali contributed new reagents or analytical tools. S. M. Lantz performed data analysis. S. M. Lantz and S. Z. Imam wrote or contributed to the writing of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References