Brain nitric oxide metabolites in rats preselected for nicotine preference and intake

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**HIGHLIGHTS**

- Rats were exposed to oral nicotine choice starting at adolescence or adulthood.
- Brain nitrate + nitrite determined in rats with maximum/minimum nicotine preference.
- Control rats received only water from both bottles, naive rats were group housed.
- Isolation stress increased NO metabolites in the hippocampus (naive-control).
- Preference had no effect; adolescent-onset rats had higher NO activity in frontal cortex.

**ABSTRACT**

Nicotine addiction is a serious health problem resulting in millions of preventable deaths worldwide. The gas messenger molecule nitric oxide (NO) plays a critical role in addiction, and nicotine increases nitric oxide metabolites (NOx) in the brain. Understanding the factors which underlie individual differences in nicotine preference and intake is important for developing effective therapeutic strategies for smoking cessation. The present study aimed to assess NO activity, by measuring its stable metabolites, in three brain regions that express high levels of nicotinic acetylcholine receptors in rats preselected for nicotine preference. Rats (n = 88) were exposed to two-bottle, free choice of oral nicotine/water starting either as adolescents or adults; control animals received only water under identical conditions. Following 12 or six weeks of exposure, levels of NOx (nitrate + nitrite), were determined in the hippocampus, frontal cortex, and amygdala. Since the rats were singly housed during oral nicotine treatment, naive rats were also included in the study to evaluate the effect of isolation stress. Isolation stress increased NOx in the hippocampus. Nicotine preference did not have a significant effect on NO activity, but rats with adolescent exposure had higher NOx levels in the frontal cortex compared to adult-onset rats. Our findings suggest that nicotine exposure during adolescence, regardless of the amount of nicotine consumed, results in higher NO activity in the frontal cortex of rats, which persists through adulthood.

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**1. Introduction**

Nitric oxide (NO) is a gaseous biological messenger molecule in the central nervous system (CNS), which is synthesized by various isoforms of nitric oxide synthase (NOS) that catalyze the conversion of arginine to citrulline and NO [4,5]. Calcium/calmodulin is needed for the activation of NOS, and Ca\textsuperscript{2+} influx is at least partly caused by the stimulation of glutamate receptors. However, the activation of other ion channels, such as the nicotinic acetylcholine receptor, has also been shown to result in increased NO levels in the brain [26]. NO regulates neurotransmitter uptake and mediates in the neurotoxic actions of glutamate, as well as in learning and memory processes [27].

The nitergic system is also suggested to play a critical role in addiction (see for examples [1,20,22,29,32]). In rats, NOS inhibition attenuates nicotine abstinence syndrome, suggesting the involvement of NO in nicotine dependence [19]. We have shown that acute and repeated nicotine administration increases levels of the stable NO metabolites nitrite and nitrate (collectively, NOx) in rat brain and that this effect is region-specific [26]. A follow-up study confirmed our results regarding increased NOx following repeated nicotine administration; however, nicotine treatment did not affect the number of NOS/nicotinamide dinucleotide

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phosphate (NADPH)-diaphorase-positive NO-synthesizing neurons [36]. Inhaled NO from cigarette smoke is also suggested to contribute to nicotine addiction in smokers [35].

Oral nicotine self-administration to rats, using a two-bottle free choice design, mimics the human smoking condition by providing rats with a choice of nicotine or water for 24 h/day for extended periods. In this paradigm the animals have the opportunity to dose their nicotine intake, based on their preferences [6]. In the present study, rats were preselected based on their oral nicotine intake as maximum and minimum nicotine preferring rats. Since the starting age of smoking varies in smokers, we used two groups of rats with different ages at onset of nicotine treatment. Sex was included as a factor because sex differences in the central effects of nicotine and nicotine/tobacco dependence are well documented [reviewed in [25,28]]. The aim of the study was to assess NO activity, by measuring its stable metabolites, in selected brain regions that express high levels of nicotinic acetylcholine (ACh) receptors [34] in rats with different preferences and different durations of nicotine exposure.

2. Materials and methods

2.1. Animals

Male and female adult Sprague Dawley rats (n = 88), obtained from Ege University Laboratory Animal Breeding Facility, were used in the experiments. All animals were kept under standard laboratory conditions (20–22°C; humidity 45–65%), were maintained on a 12:12 h light:dark cycle (lights on:07:00–19:00), and fed ad libitum (standard rat food pellets).

The animals were treated under the prescriptions for animal care and experimentation of the pertinent European Communities Council Directive of 24 November 1986 (86/609/EEC). The Institutional Animal Ethics Committee of Ege University approved all the procedures.

2.2. Data collection and selection of animals

Three different groups of male and female rats were used in the study.

(a) Naive (four months of age); housed in same-sex groups, 3–4 animals/cage and received tap water from one bottle.
(b) Adolescent onset: housed singly after weaning (four weeks of age). One week after acclimatization to single housing, rats were given a free choice of oral nicotine (10 mg/L for two weeks, 20 mg/L for the remaining period)/water (n = 87; 41 male) or water/water (controls, n = 13; 7 male) for twelve weeks, both containing saccharine (10 g/L).
(c) Adult onset: housed singly after 13 weeks of age. One week after acclimatization to single housing, rats were given a free choice of oral nicotine (10 mg/L for two weeks, 20 mg/L for the remaining period)/water (n = 67; 27 male) or water/water (controls, n = 23; 12 male) for six weeks, both containing saccharine (10 g/L).

The protocol used in the present study was reported recently [6,23]. Following oral nicotine exposure, rats were divided into three different groups (Ward method, p < 0.01: maximum, median and minimum nicotine preferring rats) according to their average nicotine consumption (mg/kg/week) over 6 weeks (weeks 1–6 in adult onset, and weeks 7–12 in adolescent onset rats). Maximum and minimum nicotine preferring rats were used in the experiments. Nicotine intake values are given in Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Nicotine consumption of maximum and minimum nicotine preferring rats.</th>
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<tr>
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<td><strong>MAXIMUM</strong></td>
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<tr>
<td>ADOLESCENT ONSET</td>
<td>MALE 6.86 ± 0.60 (n = 8) ***</td>
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<tr>
<td></td>
<td>FEMALE 8.49 ± 0.84 (n = 9) **</td>
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<tr>
<td>ADULT ONSET</td>
<td>MALE 4.34 ± 0.84 (n = 6)</td>
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<td></td>
<td>FEMALE 6.13 ± 1.05 (n = 6)</td>
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Rats were exposed to oral nicotine starting at the age of five (adolescent onset) or twelve (adult onset) weeks of age. Average nicotine intake during six weeks as adults (mg/kg body weight/week) ± SEM are given; the number of animals/group are in parentheses. One-way ANOVA with eight groups revealed a significant difference between the groups [F(7,53) = 20.09, p < 0.0001]. The results of post hoc LSD are: different from MAXIMUM preferring, same sex “p < 0.001, “p < 0.01; different from ADULT ONSET, same preference ***p < 0.001, **p < 0.01.

2.3. Chemicals

Nicotine [(–) nicotine hydrogon tartrate]; nicotinamide dinucleotide phosphate (NADPH) (N9125), flavin adenine dinucleotide (F2253), sulphanilamide (S9251) and N-1 naphthylethylenediamine (N9125) were purchased from Sigma. Nitrate reductase from Aspergillus sp. was obtained from Roche (10981249001).

2.4. Tissue preparation

Rats were taken from their home cages where the nicotine/water choice was available and were decapitated without delay. Brains were rapidly removed and dissected (frontal cortex, hippocampus, and amygdala) on ice.

2.5. Nitrite and nitrate determination

Brain nitrate was determined after its reduction to nitrite, followed by the Griess reaction. Tissue samples were homogenized in phosphate buffer (pH 7.5) and centrifuged at 2000 × g for 5 min. From the supernatant, 0.25 ml was diluted 4 times and added to 0.25 ml of 0.3 M NaOH. After incubation for 5 min at room temperature, 0.25 ml of 5% (w/v) ZnSO4 was added for deproteinization. This mixture was then centrifuged at 3000 × g for 20 min and the supernatant was used for the assays. Total NOx levels in tissue homogenates were determined spectrophotometrically, based on the reduction of nitrate to nitrite by nitrate reductase (EC 1.6.6.2) from Aspergillus sp. in the presence of NADPH.

Sodium nitrate solutions were used for standard measurements. NOx levels in the tissues were expressed as mmol/g wet weight. Protein concentrations were determined by Bradford assay.

2.6. Statistical analyses

SPSS (v17.0) software was used for all statistical analyses. Data was analyzed by ANOVAs; factors and dependent variables are given in the Section 3. Post hoc tests were used to compare groups.

3. Results

The differences in the nicotine consumption of the rats were analyzed by ANOVA with sex (male, female), preference (maximum, minimum), and age at onset (adolescent, adult) as factors and average nicotine consumption as the dependent variable. Preference, as expected, emerged as a significant main effect [F(1,72) = 428.334, p < 0.0001] and interacted with age [F(1,72) = 20.181, p < 0.0001]. Maximum nicotine preferring rats drank significantly more nicotine solution than minimum preferring rats; and although the adolescent-onset maximum preferring
rats consumed more nicotine than adult-onset maximum preferring rats, age at onset did not affect nicotine consumption in minimum preferring groups (Table 1).

Since the rats were singly housed during the two-bottle free choice oral nicotine treatment, naïve rats and control animals were compared to assess the possible effect of single vs. group housing. NOx levels did not differ between brain regions of control rats in the adolescent- and adult-onset groups. Therefore, all the control groups were pooled and compared with the NOx levels of naïve rats. ANOVAs with sex and single housing (naïve, control) as the factors revealed a main effect of housing in the hippocampus \(F(1,36) = 12.646, p = 0.001\); NOx levels were higher in control rats than naïve animals (Fig. 1).

NOx levels in the control and experimental groups in each brain region are shown in Fig. 2, panels (a), (b) and (c). To assess the effect of nicotine treatment, we performed a series of ANOVAs. First we did a repeated measures ANOVA with brain region (amygdala, frontal cortex, hippocampus) as the within-subjects factor; age at onset (adult, adolescent), sex (male, female), and preference (control, minimum, maximum) as the between-subjects factors; and NOx levels as the dependent variable. A significant effect of brain regions emerged and interacted with age at onset \(F(2,130) = 15.965, p < 0.0001\). Overall, the highest levels were in the hippocampus, followed by frontal cortex and amygdala. While this pattern was seen in the rats exposed to nicotine as adolescents, the rats exposed to nicotine as adults still had the highest levels of NOx in the hippocampus, but levels were higher in the amygdala than in the frontal cortex. When we analyzed our results for adult-onset and adolescent-onset rats separately, the only significant effect was region: \(F(2,32) = 14.576, p < 0.0001\) and \(F(2,98) = 31.787, p < 0.0001\) for adult and adolescent exposure, respectively. Since region emerged as a significant main effect, we continued our analyses for each region separately. A significant effect of age at onset was found in the amygdala \(F(1.52) = 8.105, p = 0.007\) and frontal cortex \(F(1.52) = 50.847, p < 0.0001\); in the amygdala, levels in adult-onset rats were higher than adolescent-onset, while in the frontal cortex levels were lower in the adult-onset rats than adolescent-onset.

To compare the age and sex effects for each group separately (control, minimum and maximum preferring rats), we performed two-way ANOVAs. There was no effect observed in the control groups. Both in the minimum \(F(1,29) = 23.207, p < 0.0001\) and maximum \(F(1,2) = 32.351, p < 0.0001\) nicotine preferring rats, a main effect of age at onset was observed in the frontal cortex; adolescent-onset rats had higher NOx levels than adult-onset animals. In the amygdala, the reverse trend was seen in age effect in both minimum and maximum nicotine preferring rats, but it was not significant: \(F(1,29) = 4.006, p = 0.056\) and \(F(1,2) = 4.088, p = 0.057\), respectively.
4. Discussion

When designing this study, we hypothesized that in rats with maximum preference for nicotine, brain levels of NO metabolites will be higher than in those with minimum preference and that there will be sex and regional differences. While our data clearly show that there are regional differences in NOX levels, we did not observe a significant effect of nicotine preference or sex following long-term nicotine intake. On the other hand, age at onset of nicotine exposure did have an effect on NO activity in the frontal cortex.

Single housing can be a stress factor, namely isolation stress, in oral nicotine intake experiments. In the present study we observed a main effect of housing on nitric oxide metabolites in the hippocampus; levels were significantly higher in the singly housed control rats compared to naive animals housed 3–4 same sex animals per cage. Similar to our findings, chronic isolation stress is reported to increase NO metabolites in the hippocampus of male Wistar rats, while no significant effect was observed in the prefrontal cortex [38]. We have previously shown that chronic saline injections increased NO metabolites in the hippocampus in Sprague Dawley rats [26]. The regulation of neuronal nitric oxide synthase (nNOS) activity and expression in the rodent hippocampus by psychological stress has been shown in many studies [9,11,17,18,37]. We have recently shown that forced swim stress and chronic nicotine administration increased nNOS expression in the hippocampus of female, but not male rats [16]. Exposure to stressors during adolescence increases vulnerability to psychostimulant abuse, and female rats stressed during puberty show enhanced locomotor sensitization to nicotine treatment [21]. Although the emphasis of the current study is not to study the effect of single housing stress on the nitrergic system in rat brain, we have to consider this effect when discussing the nicotine-induced changes in the levels of NO metabolites. Subsequently, the higher levels of NO metabolites in rats exposed to nicotine as adolescents compared to those exposed as adults may be reflecting the effect of pubertal stress exposure.

In the mammalian brain, nine of the total 12 nAChR subunit genes are expressed: α2–α7, β2–β4. Of these, α4, β2 and α7 subunits are the most abundant and widespread in the rodent brain (reviewed in [7,12,13]). Genetic analyses and manipulations point to the involvement of receptors containing α4, β2 and α7 subunits in nicotine addiction [2,8]. The frontal cortex, hippocampus and amygdala are rich in these three types of subunits. Therefore, the subunit composition of the nAChR receptors is unlikely to underlie the differences in NO activity in these three brain regions.

The present study can be considered an observational study; we did not employ a mechanistic approach. We used an outbred rat strain (Sprague Dawley) and preselected rats (minimum and maximum nicotine preferring) based on their nicotine consumption in a two-bottle free choice oral nicotine intake paradigm. To study the effect of age at onset of nicotine exposure, we used two groups of rats: rats first exposed to nicotine as adolescents or as adults. Rats exposed to the nicotine choice as adolescents were singly housed for 13 weeks and received nicotine for 12 weeks, while rats exposed as adults were housed singly for seven weeks and received nicotine for six weeks. Our findings show that the amount of nicotine consumed does not affect the levels of nitric oxide metabolites in the three brain regions studied. If the different nicotine preferences and intake of the rats have a genetic basis, then our results suggest that genes regulating NO activity and nicotine preference are not related. On the other hand, there was a significant effect of age at onset in the frontal cortex; adult-onset animals had lower levels of NOX compared to adolescent-onset animals. This difference may be attributed to the effect of longer isolation stress or to the effect of extended nicotine intake. However, considering that isolation stress affected NOX levels in the hippocampus but not in the frontal cortex when naïve rats were compared to singly housed control animals, the effect of nicotine is more likely to underlie this difference. Adolescence is a critical phase in development and adolescent nicotine exposure produces long-lasting neurochemical effects that persist into adulthood in rodents (reviewed in [15,31]). Our findings suggest that the neuroplastic changes induced by nicotine exposure starting in adolescence resulted in increased NO activity in adulthood.

There are a few reports on the comparison of NOS expression in adult and adolescent brain regions. Terada et al. [33] studied the ontogeny of nNOS in the developing rat brain (between embryonic day 13 and postnatal day 14). It is reported that nNOS-positive cells at postnatal day 14 resemble those in the adult rat brain. Neuronal NOS mRNA is widely distributed in the rat brain, including the olfactory bulbs, the islands of Calleja, the amygdala, thalamus, hypothalamus, cerebellum, cerebral cortex, basal ganglia, hippocampus and the brain stem [14]. It would be illuminating to study the effect of nicotine intake in rats preselected for nicotine preference in brain regions rich in dopaminergic innervation.

Neurotoxic insults are reported to increase nitric oxide metabolites in the brain. For example, METH-induced dopaminergic neurotoxicity is accompanied by increases in the number of nNOS expressing cells [10] and in NO metabolites in brain regions rich in dopaminergic cells [3]. Glutamate was the first reported trigger that increased NO levels in the brain (reviewed in [24]). Nicotine stimulates dopaminergic neurons through alpha-7-containing nicotinic ACh receptors on glutamatergic afferents in the ventral tegmental area; activated NMDA receptors increase NO levels. Schilstrom et al. [30] suggest that increased NO production may be involved in plastic changes which underlie nicotine addiction. Along these lines, the time-course of nicotine exposure may be related to the extent of the plastic changes. In the current study, while the amount of nicotine consumed by rats did not have an effect on NOx, adolescent-onset rats had higher levels in the frontal cortex than adult-onset animals. These findings suggest that nicotine, possibly through dopaminergic and glutamatergic mechanisms, increases NOx in the frontal cortex of rat brain, and that the increase is related to the duration and developmental stage of exposure rather than the amount of nicotine consumed.

In conclusion, we have confirmed that isolation stress increases brain NOx in male and female Sprague Dawley rats in the hippocampus. Nicotine preference did not affect brain NO activity, implying differences in the genetic regulation of the nicotinic cholinergic and nitrergic systems in rat brain. On the other hand, age at onset of nicotine exposure had a significant impact on NOx in the frontal cortex, suggesting long-lasting effects of nicotine on NO synthesis in the frontal cortex. These findings underscore the importance of nicotine exposure during adolescence.

Acknowledgements

This study was supported by Ege University Research Fund Grant 2006/BAM/001 to LK. The authors would like to thank Jacqueline Renee Gutenkunst, (Izmir University of Economics, School of Foreign Languages) for her effort in refining the language of this paper.

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