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Perinatal nicotine treatment induces transient increases in NACHO protein levels in the rat frontal cortex

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Abstract

The nicotinic acetylcholine receptor (nAChR) regulator chaperone (NACHO) was recently identified as an important regulator of nAChR maturation and surface expression. Here we show that NACHO levels decrease during early postnatal development in rats. This decrease occurs earlier and to a greater degree in the frontal cortex compared with the hippocampus. We further show that rats exposed to nicotine during pre- and postnatal development exhibit significantly higher NACHO levels in the frontal cortex at postnatal day (PND) 21, but not at PND60. Repeated exposure to nicotine selectively during early (PND8-14) or late (PND54-60) postnatal stages did not affect NACHO protein levels in the frontal cortex or hippocampus. Neither did exposure to high doses of the selective $\alpha 7$ nAChR agonists SSR180711, A-582941, or PNU-282987. However, we found significantly increased NACHO protein levels in the frontal cortex of PND36 rats after a single exposure to a combination of nicotine and the type II $\alpha 7$ nAChR positive allosteric modulator (PAM) PNU-120596, but not the type I PAM AVL-3288. These findings suggest that exposure to nAChR agonism affects NACHO protein levels, and that this effect is more pronounced during pre- or early postnatal development. The effect of PNU-120596 further suggests that the increase in NACHO expression is caused by activation rather than desensitization of nAChRs.

Keywords

TMEM35; nicotinic acetylcholine receptor; nicotinic acetylcholine receptor regulator; chaperone; development; allosteric modulator

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Conflict of interest

The authors declare no competing financial interests.

Introduction

Nicotinic acetylcholine receptors are ligand-gated ion channels that are important for a wide range of cellular functions, and altered nAChR levels and function have consequently been implicated in disorders ranging from cognitive dysfunction, over inflammation to pain states (Bencherif et al., 2011; Dineley et al., 2015; Thomsen et al., 2016).

It is well known that exposure to nicotine increases nAChR levels in vitro and in vivo, and that this effect is to some extent subtype and region-selective (Govind et al., 2009). Several mechanisms may underlie this upregulation. The cellular mechanisms by which nicotine produces upregulation of nAChRs are unknown, but it has been suggested to be due to a chaperoning effect of nicotine (Lester et al., 2009) or other posttranscriptional events (Govind et al., 2009). However, this effect is not exclusive to nicotine; we have previously shown that exposure to selective $\alpha 7$ nAChR agonists produce upregulation of $\alpha 7$ nAChR levels in the rat brain (Christensen et al., 2010), and that this effect is abolished by the type II $\alpha 7$ nAChR positive allosteric modulator PNU-120596, but not the $\alpha 7$ nAChR type I PAM AVL-3288 (Thomsen and Mikkelsen, 2012). Since the difference between type I and II PAMs is that the latter, but not the former, reduces receptor desensitization (Grønlien et al., 2007) it is possible that receptor desensitization causes nAChR upregulation.

The nicotinic acetylcholine receptor regulator chaperone (NACHO) was recently demonstrated to be an endoplasmic reticulum-resident protein that interacts with $\alpha 7$ and $\alpha 4\beta 2$ nAChRs and enhance their cell surface expression (Gu et al., 2016). This effect was particularly pronounced for $\alpha 7$ nAChRs, and the importance of NACHO for $\alpha 7$ expression is emphasized by the finding that NACHO knockout mice exhibit complete absence of $\alpha 7$ nAChRs (Gu et al., 2016).

Given the importance of NACHO for the maturation and functionality of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, this protein could be key to understanding how nicotine leads to increased surface expression of nAChRs. One possibility is that change in NACHO gene expression is the underlying mechanism. In order to test this possibility, we here investigate the expression of NACHO during development and in response to administration of nAChR agonists.

Materials and Methods

Compounds and Animals

2-methyl-5-(6-phenyl-pyridazin-3-yl)-octahydro-pyrrolo[3,4-c]pyrrole (A-582941) (Bitner et al., 2007), (4-bromophenyl) 1,4-diazabicyclo[3.2.2]nonane-4-carboxylate (SSR180711) (Biton et al. 2007), N-[(3'R)-1'-azabicyclo[2.2.2]oct-3'-yl]-4-chlorobenzamide (PNU-282987) (Bodnar et al. 2005), and 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) (Hurst et al., 2005) were synthesized at the Department of Medicinal Chemistry at NeuroSearch A/S (Ballerup, Denmark). N-(4-chlorophenyl)- α -[[[(4-chloro-phenyl)amino]methylene]-3-methyl-5-isoxazoleacet-amide (AVL-3288, also known as XY4083 and CCMI) (Ng et al., 2007) was a kind gift from Kelvin W. Gee,

University of California, Irvine. (–)-Nicotine hydrogen tartrate salt was purchased from Sigma Aldrich (St. Louis, MO).

For the experiment with perinatal nicotine exposure, timed-pregnant Sprague Dawley (SD) rats were obtained from Harlan Laboratories (Frederick, MD, USA). For the remaining experiments SD rats were obtained from Charles River Laboratories (Germany) and housed individually (pregnant dams) or two per cage. In total we used tissue from 145 male rats.

For all experiments, pups were weaned at postnatal day (PND) 21, only male animals were analyzed, and animals were kept on a 12/12 h light/dark cycle provided with standard rodent diet and water *ad libitum*. Animals were acclimatized for a minimum of 7 days after arrival before experiments began. Animal procedures were conducted with approval of the Howard University Animal Care and Use Committee (HU-IACUC) adhering to the guidelines set forth by the National Research Council, or in accordance with the Danish National Guide for Care and Use of Laboratory animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Developmental study

A total of 33 male rats from 5 litters were assigned to each experimental group in a pseudo-random fashion and decapitated at PND1, 7, 14, 26, 42, or 60. The hippocampus (HIP) and frontal cortex (FC) were dissected from each hemisphere and frozen in microfuge tubes in dry ice. Samples were kept at –80°C until use.

Nicotine administration

To study the effect of perinatal nicotine administration, nicotine was administered to pregnant rats through a mini-osmotic infusion pump (Model #2006, Alzet, Cupertino, CA) surgically implanted between the scapulae at gestational day 7, as previously described (Wang et al., 2011; Wang and Gondré-Lewis, 2013). Nicotine hydrogen tartrate (Sigma-Aldrich, St Louis, MO) was prepared fresh on the day of pump implantation and delivered to dams at a rate of 4 mg/kg/day (free base weight) from pregnancy until pups reached PND21. At PND21, the pups were weaned and allowed to reach adulthood (PND60). HIP and FC were dissected from a total of 46 male rats and stored at –80°C.

To study the effect of nAChR agonists when administered directly to young and adult rats, vehicle (0.9% saline, n=8 and 10 for comparing with nicotine and selective agonists, respectively), 0.4 mg/kg nicotine (free base weight, n=8), 10 mg/kg A-582941 (n=6), 10 mg/kg SSR180711 (n=6), or 20 mg/kg PNU-282987 (n=6) was administered subcutaneously (s.c.) twice daily for 7 days (PND8–14 or 54–60) and the FC and HIP were dissected 4 hours after the last administration. To study the effect of allosteric modulators in combination with nicotine a total of 24 male 36 days old rats were injected with 3 mg/kg PNU-120596, 1 mg/kg AVL-3288 or vehicle (5% DMSO and 8% Solutol in 0.9% saline). Fifteen minutes later, they were injected with 1 mg/kg nicotine (free base weight) or vehicle (0.9% saline). For both of the latter experiments rats were decapitated 4 hours after the last administration, and HIP and FC were dissected and stored at –80°C.

Western blotting

Tissue samples were lysed in 2–300 μ l lysis buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% (v/v) Triton X-100, 1% (v/v) NP-40, 1 μ l/ml protease inhibitor cocktail (Sigma-Aldrich, Brøndby, Denmark) pH 7.4) by manual grinding with a pestle for 20 seconds followed by sonication on ice. Homogenates were centrifuged 10 minutes at $10,000 \times g$ at 4°C and the pellet discarded. Total protein content of the supernatant was measured using the Pierce™ 660nm Protein Assay (ThermoFisher Scientific, Copenhagen, Denmark). Equal amounts of lysates (10–30 μ g) were then diluted in loading buffer (120 mM Tris, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol, 4% (w/v) SDS, 0.05% (w/v) bromophenol blue, pH 6.8), incubated for 5 minutes at 95°C and submitted to gel electrophoresis using AnykD™ gels (Biorad, Hercules, CA), and blotted onto polyvinylidene fluoride membranes (BioRad). The membranes were washed in tris-buffered saline with 0.1% Tween 20 (TBS-T) and blocked in TBS containing 5% (w/v) dry milk powder (Biorad), which was also used for antibody incubations. The membranes were incubated with 0.4 μ g/ml primary rabbit antiserum directed against NACHO protein (HPA048583, Sigma) overnight at 4°C on parafilm in a humidified container, followed by 3×10 minute washes in TBS-T. This antibody has previously been shown to stain NACHO wild-type, but not knock-out brain samples using western blotting (Gu et al., 2016). This was followed by 1 hour incubation at 20–22°C with horseradish peroxidase-conjugated secondary antibody (1:2,000, Dako, Glostrup, Denmark). After thorough washing in TBS-T, enhanced chemiluminescence Western blotting detection reagents (Western Lightning® ECL Pro, Perkin Elmer, Waltham, MA) were used for signal detection and protein bands were visualized using a Chemidoc XRS+ digital image analyser (Biorad). Mean optical densities of bands were measured and their corresponding background measurement subtracted. The total protein content of each sample, as measured by Stain-Free™ technology (Biorad), was used to normalize band intensity. Molecular weight was estimated by comparing with dyed protein markers (PageRuler Prestained Protein Ladder, ThermoFisher Scientific).

Statistical analysis

Data sets that passed Bartlett's test for standard deviations and the D'Agostino & Pearson omnibus normality test (only tested where $n \geq 7$) were analyzed using two-way ANOVA followed by Sidak's multiple comparisons test, one-way followed by Tukey's, Dunnett's or Sidak's multiple comparisons tests, or by unpaired t-tests. The statistical calculations were performed using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA). All data are presented as mean $\pm$ standard error of the mean, and a *P*-value of less than 0.05 was considered statistically significant.

Results

NACHO protein levels decrease during early postnatal development

In the FC of male rats there was a statistically significant difference between groups as determined by one-way ANOVA ($F(5,26)=10.02$, $P<0.0001$) with NACHO protein levels decreasing to 31% of the level at PND1 during the first 26 postnatal days, after which it remained stable into adulthood. A Tukey's multiple comparison test revealed significant decreases at PND26, 42, and 60 compared with PND1 ($P<0.01$, Figure 1), as well as

decreases at PND14, 26, 42, and 60 compared with PND7 ($P<0.05$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively). In the hippocampus there was a statistically significant difference between groups as determined by one-way ANOVA ($F(5,25)=2.810$, $P=0.038$) with NACHO levels remaining stable until adolescence, but decreasing during or after adolescence to 48% of the level at PND1 ($P<0.05$ for PND14 compared with PND60).

NACHO protein levels are increased by perinatal nicotine exposure

Male mice were exposed to nicotine from embryonic day 7 to PND21 through osmotic minipumps implanted into the maternal parent. I.e. the exposure came through the placenta during pregnancy and during the mother's milk until weaning at PND21.

In the FC of the offspring a two-way ANOVA revealed a significant main effect of age ($F(2,39)=17.97$, $P<0.0001$) and nicotine exposure ($F(1,39)=6.908$, $P=0.0122$) on NACHO protein levels (Figure 2). Subsequent Sidak's multiple comparisons test revealed a significant increase (5.5 fold, $P<0.05$) in NACHO levels at PND21 in nicotine-exposed animals. In the HIP of the same animals, a two-way ANOVA revealed a main effect of age ($F(2,50)=66.88$, $P<0.0001$), but no effect of nicotine exposure ($F(1,50)=0.01727$, $P=0.8960$).

Staining with an antibody directed against NACHO produced a single band at ~18 kDa, which corresponds with the molecular weight of the protein.

It should be noted that we have previously published mRNA expression levels for the $\alpha 2$, $\alpha 4$, $\alpha 7$, and $\beta 2$ nAChR subunits as well as $\beta 2$ protein from the brains of the same rats as used here (Thomsen et al., 2014).

NACHO protein levels are not altered by postnatal exposure to nAChR agonists

Exposure to 0.4 mg/kg nicotine twice daily for 7 days in adult male rats did not alter NACHO protein levels in the FC or HIP, as determined by unpaired t-tests (Figure 3A and B, $T(13)=0.3284$, $P=0.7478$ and $T(14)=0.2009$, $P=0.8436$, respectively). Neither did 10 mg/kg A-582941, 10 mg/kg SSR180711, or 20 mg/kg PNU-282987 twice daily for 7 days, as determined by a one-way ANOVA ($F(3,24)=0.2183$, $P=0.8827$). Exposure to 0.4 mg/kg nicotine twice daily for 7 days in 8–14 days old rats also had no effect on NACHO protein levels in the FC and HIP, as determined by a one-way ANOVA (Figure 3C, $F(2,19)=0.5522$, $P=0.5847$ and $F(2,21)=2.283$, $P=0.1267$, respectively). In a separate experiment we saw no effect of A-582941, administered in the same way as above, on NACHO levels in animals euthanized 1, 10, 24, 72, or 168 hours after the final administration (data not shown).

Since the dosage regimen influences the long-term effects of nicotine (Matta et al., 2007), we cannot preclude that the lack of effect of nicotine is due to a difference in the dosage regimen compared with the experiment with perinatal exposure via minipumps.

NACHO protein levels are increased by a combination of nAChR agonism and positive allosteric modulation

When co-administering nicotine with the type II $\alpha 7$ nAChR positive allosteric modulator (PAM) PNU-120596 or the type I $\alpha 7$ nAChR PAM AVL-3288 a one-way ANOVA revealed no significant main effect on NACHO levels in the FC or HIP ($F(3,19)=2.149$, $P=0.1277$

and $F(3,20)=0.1975$, $P=0.8969$, respectively). However, subsequent Sidak's multiple comparisons revealed that the combination of nicotine and PNU-120596 resulted in significantly increased NACHO levels in the FC of adult rats (2.9 fold, $P<0.05$, Figure 4). No such increase occurred with nicotine alone or with co-administration of nicotine with AVL-3288. Neither of the compound combinations altered NACHO levels in the HIP in the same animals.

Discussion

Our main findings are that NACHO expression decreases during early postnatal development in rats, and that perinatal exposure to nicotine transiently increases NACHO levels. This effect of nicotine during early development cannot be recapitulated by postnatal exposure to nicotine alone, but it occurs with concomitant exposure to nicotine and a type II PAM.

We find that NACHO levels are downregulated earlier and to a larger degree in the FC compared with the HIP, indicating that NACHO plays different roles during development in different brain regions. Interestingly, the developmental expression pattern of NACHO is similar to that of [125 I]- α -bungarotoxin and [3 H]-epibatidine in the cortex and HIP (Zhang et al., 1998; Tribollet et al., 2004), reflecting $\alpha 7$ and non- $\alpha 7$ nAChR binding, respectively. However, there are notable differences as binding studies as well as mRNA studies often show a peak expression around PND7 (Zhang et al., 1998; Thomsen et al., 2014), and we have previously shown higher levels of $\beta 2$ nAChR subunit protein at PND 60 compared with PND14 (Thomsen et al., 2014). Notably, the latter study used tissue from the very same animals as presented here. Therefore, although the developmental pattern of NACHO levels supports the notion that NACHO may be an important regulator of the levels of mature $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in the rat brain, it is certainly not the only one.

In this light, it is interesting that exposure to nicotine during pre- and postnatal development transiently increases NACHO levels in the FC approximately 5-fold, since this opens the possibility that an increase in NACHO levels may underlie part of the nicotine-induced upregulation of NACHRs during development (Heath and Picciotto, 2009; Arvaniti et al., 2016). However, since nicotine does not affect NACHO levels in the HIP, other factors besides NACHO must also be involved.

In contrast to the finding above, repeated nicotine exposure selectively during early or late postnatal stages did not affect NACHO levels in the FC or HIP. This suggests that NACHO expression is more sensitive to manipulation during development. However, it should be noted that we have used different dosing paradigms for the perinatal versus postnatal experiments. The exact dosage regimen has been shown to have an influence on the long-term effects of nicotine (Matta et al., 2007), and this may therefore explain the different findings from these experiments. Although specifically in terms of nAChR upregulation, this effect of nicotine is present across various dosage regimens (Matta et al., 2007).

Repeated exposure to selective $\alpha 7$ nAChR agonists in young or adult rats did not affect NACHO levels. These agonists have previously been shown to robustly increase [125 I]- α -

bungarotoxin binding in the brain of the very same animals (Christensen et al., 2010). Our data therefore suggest that $\alpha 7$ nAChR agonists increase $\alpha 7$ nAChR levels in a NACHO-independent manner.

Interestingly, we find that a single exposure to nicotine in combination with a type II, but not a type I, $\alpha 7$ nAChR PAM in young animals increases NACHO levels in the FC. Both PAM types increase the amplitude of response of $\alpha 7$ nAChRs, but only type II PAMs inhibit desensitization (Grønlien et al., 2007). This suggests that the effect of nicotine on NACHO is dependent on the degree of receptor activation, and it further indicates that it is possible to manipulate NACHO levels in adulthood. Notably, we have previously found that nicotine-induced upregulation of $\alpha 7$ nAChRs was unaffected by the presence of PAMs in vivo, whereas upregulation induced by several experimental selective $\alpha 7$ nAChR agonists was abolished by a type II PAM (Thomsen and Mikkelsen, 2012). These findings suggest that nicotine has a unique mechanism of action in relation to nAChR upregulation, and further suggest that type II PAMs may differentially affect nAChR levels and NACHO levels dependent on properties of the agonist.

In summary, our data are in line with a physiological role for NACHO in the maturation and function of nAChRs, and indicate that nAChR activation can increase NACHO levels in the FC both during early and late developmental stages, dependent on the degree of activation.

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- NACHO protein levels decrease during early postnatal development in rats.
- This decrease is earlier and greater in the frontal cortex (FC) compared with the hippocampus.
- Nicotine exposure during early development, but not during later developmental stages, increases NACHO levels in the FC
- Combining a positive allosteric modulator with nicotine at later developmental stages reinstates nicotine's effect on NACHO
- Our data suggest that nicotine can have long-term effects on cholinergic neurotransmission via NACHO.

Figure 1. NACHO protein levels during development and perinatal nicotine exposure

(A) NACHO protein levels were determined using Western blotting on homogenates from the frontal cortex and hippocampus of male rats at different postnatal days (P, n=4–6). ** $P < 0.01$ and # $P < 0.05$, ### $P < 0.001$, and \$ $P < 0.05$ indicate significant difference from P1, P7 and P14, respectively, in a one-way ANOVA with Tukey's multiple comparison test. NACHO protein levels are normalized to total protein content of the samples and set to 1 for P1 separately for each region.

Figure 2. Perinatal nicotine treatment induces transient increases in NACHO protein levels
NACHO protein levels were determined using Western blotting on homogenates from the frontal cortex and hippocampus of male exposed to nicotine (4 mg/kg/day) from embryonic day 7 to postnatal day (P) 21 through osmotic minipumps implanted into pregnant dams. Animals were euthanized at P7 (n=5), 21 (n=10) or 60 (n=13). Shown are representative Western blot images with the bands placed over the respective group indicating that the anti-NACHO antibody detected a single band at 18 kDa as well as graphs showing the quantification of the detected band. Main effect of nicotine treatment in a two-way ANOVA with age and treatment as the fixed factors is indicated in the graphs. * $P < 0.05$ indicates statistical difference from P21 vehicle in a Sidak multiple comparisons test. NACHO protein levels are normalized to total protein content of the samples and set to 1 for the vehicle group separately for each region.

Figure 3. NACHO protein levels are not altered by postnatal exposure to nAChR agonists
 NACHO protein levels were determined using Western blotting on homogenates from the frontal cortex and hippocampus of (A) adult (54–60 days old) male rats injected with vehicle (Veh) or 0.4 mg/kg nicotine (Nico) twice daily for 7 days (n=7–8), (B) adult male rats injected with vehicle (Veh), 10 mg/kg A-582941 (A-58), 10 mg/kg SSR180711 (SSR), or 20 mg/kg PNU-282987 (PNU) twice daily for 7 days (n=6–10), or (C) Young (8–14 days old) male rats exposed to vehicle, 0.4 mg/kg nicotine, or 10 mg/kg SSR180711 (n=7–8).

NACHO protein levels are normalized to total protein content of the samples and set to 1 for the vehicle group separately for each region.

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Figure 4. Co-administration of nicotine and PNU-120596 increase NACHO protein levels in the frontal cortex

NACHO protein levels were determined using Western blotting on homogenates from the frontal cortex and hippocampus of adolescent (36 days old) male rats injected with vehicle, 3 mg/kg PNU-120596, or 1 mg/kg AVL-3288 followed by injection with 1 mg/kg nicotine or vehicle (n=6). * $P < 0.05$ indicates statistical difference from vehicle in a one-way ANOVA followed by a Sidak multiple comparisons test. NACHO protein levels are normalized to total protein content of the samples and set to 1 for the vehicle group separately for each region.