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Chronic Nicotine Alters Nicotinic Receptor-induced Presynaptic Ca^{2+} Responses in Isolated Nerve Terminals

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Abstract

Brain nicotinic receptors display pronounced permeability for Ca^{2+} and localize to presynaptic nerve terminals, in addition to postsynaptic sites. Chronic exposure to nicotine has been shown to alter brain nicotinic receptor expression, but the functional consequences for presynaptic Ca^{2+} have not been directly examined. Here, we used confocal imaging to assess Ca^{2+} responses in individual nerve terminals from cortices of mice treated up to 14 days with nicotine as compared to vehicle-treated controls. Chronic nicotine treatment led to substantially enhanced amplitudes of presynaptic Ca^{2+} responses to acute application of nicotine at concentrations of 50 nM (2-fold) and 500 nM (1.7-fold), but not 50 μM . In addition, increased expression of high-affinity nicotinic receptors on isolated terminals was observed following chronic treatment, as determined immunocytochemically and pharmacologically. These findings suggest that chronic exposure to nicotine may lead to enhanced sensitivity to nicotine at select presynaptic sites in brain via upregulation of high-affinity nicotinic receptors.

Keywords

Chronic nicotine; Presynaptic nerve terminal; Synaptosomes; Calcium regulation; Nicotinic receptors; Calcium imaging; Receptor up-regulation; Receptor immunocytochemistry

Introduction

Habitual use of tobacco products results from the addictive properties of nicotine. The time-dependency for development of addiction appears to be based on adaptation of the nicotinic acetylcholine receptor (nAChR) signaling systems in the brain, as well as those systems regulated by nicotine (e.g. mesolimbic dopamine and GABA), to chronic but intermittent stimulation by nicotine [1,2]. In particular, up-regulation of nAChRs has been noted for a wide variety of preparations, from cell lines to intact animals, following chronic nicotine treatment. It has been found that the high-affinity $\alpha 4\beta 2$ containing nAChR was significantly and preferentially up-regulated in animals chronically treated with nicotine on a daily-dose basis [3-5], though the $\alpha 6$ subunit, likely part of $\alpha 4\alpha 6\beta 2$ nAChRs, was also found to be dramatically up-regulated in animals self-administering nicotine [6]. In addition, exposure to nicotine levels well above those encountered by smokers results in up-regulation of other nAChR subtypes [7]. Up-regulation occurs post-transcriptionally and post-translationally, as nicotine appears to induce up-regulation via an action in the ER on receptor maturation [see 8]. Overall, previous findings suggest that high-affinity nAChRs are particularly sensitive to chronic nicotine,

consistently resulting in up-regulation, but with mixed results with regard to functional changes.

Nicotinic receptors are localized to both pre- and post-synaptic sites in brain [2]. Presynaptic regulation by nAChRs of dopamine release in striatum and prefrontal cortex and glutamate release in several brain regions, including prefrontal cortex, has been noted in response to acutely applied nicotine and involves several nAChR subtypes [reviewed in 1,9]. The regulation of neurotransmitter release by presynaptic nAChRs is also Ca^{2+} -dependent, involving Ca^{2+} influx via the nAChR ion pore and/or voltage-gated ion channels (VGCCs) activated by nAChR-mediated depolarization [10]. Direct measurement of presynaptic $[\text{Ca}^{2+}]_i$ in individual brain nerve terminals, coupled with pharmacological assessment, has demonstrated substantial and relatively sustained Ca^{2+} increases to acute application of nicotine, the nature of which depends upon the specific brain region [11-13]. The sustained nicotine-induced presynaptic Ca^{2+} responses are consistent with the sustained nicotine-induced increases in spontaneous excitatory release observed in a variety of preparations [14-18]. Together, these findings indicated that Ca^{2+} influx on activation of presynaptic nAChRs may function in the direct neuromodulation of synaptic transmission.

Here, we addressed whether the nicotine-evoked changes in presynaptic $[\text{Ca}^{2+}]_i$ are affected following chronic systemic exposure of mice to nicotine. In particular, the degree to which high-affinity presynaptic nAChRs appear to be up-regulated and the functional consequences of such an up-regulation were assessed via confocal imaging of individual isolated nerve terminals.

Experimental procedures

Chronic nicotine treatment

Adult male C57Bl/6J mice (Jackson Labs, Bar Harbor, ME) were housed in an AAALAC-accredited, environmentally controlled animal facility for 1–2 weeks prior to any treatment. The weights of the mice were checked prior to commencing injections and also served to help the animals adjust to handling. Nicotine bitartrate (2 mg/kg containing 0.7 mg/kg free nicotine) in sterile saline or sterile saline alone (control) was injected subcutaneously twice a day [19]. Injections were done quickly into the scruff, with gentle handling in order to minimize any risk of stress to the mice, following a protocol approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee. The rationale for using intermittent nicotine exposure is that the timing and amount delivered can be tightly controlled.

Purification of isolated presynaptic nerve terminals

Intact presynaptic nerve endings (synaptosomes) were purified from rodent brain as previously described [12]. In brief, cerebral cortices from adult C57Bl/6J mice, treated with nicotine or sterile saline vehicle (controls) were dissected under ice-cold 0.32 M sucrose. The cortices were rapidly homogenized in ice-cold 0.32 M sucrose with a glass-Teflon tissue grinder. Synaptosomes were isolated using the Percoll step gradient method [20]. The purified synaptosomes were washed with oxygenated HEPES-buffered saline (HBS, pH 7.4) containing 142 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4 , 1 mM MgCl_2 , 5 mM D-glucose, and 10 mM HEPES, containing 1 mM Ca^{2+} and stored as pellets on ice until used. The animal procedure used for this study followed a protocol approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee.

Measurement of relative Ca^{2+} levels

Fluo-4 was loaded at 5 μM final concentration into the purified cortical synaptosomes, which had been resuspended in HBS containing 1 mM Ca^{2+} , for 60 min at 37°C, using the

acetoxymethyl ester derivative of the dye as previously described [12]. The dye-loaded synaptosomes were subsequently washed by centrifugation and resuspended in HBS. The preparations were plated onto coverslips coated with Cell-Tak and inserted into a rapid-exchange Warner perfusion system mounted on a Nikon Diaphot microscope on a Nikon PCM 2000 laser-scanning confocal imaging system. Fluorescent images were recorded during excitation of the preparation with 488 nm light, while the preparations were under constant perfusion at ~3–5 ml/min with HBS. Images were typically collected at 4-s intervals, using the first five consecutive images as baseline. Fluorescence intensities associated with individual synaptosomes in digitized images were calculated using OPTIMAS image analysis software (Optimas Co., Seattle, WA) and corrected for photobleaching based on the baseline images (typically <3%). Each experiment corresponds to image sequences recorded from single preparations. Response to depolarization evoked by elevated extracellular K^+ was used as a criterion for synaptosomal viability for each preparation [21]. Results are presented as normalized responses (F/F_0 , where F_0 is the fluorescence intensity associated with a given structure at t_0). Maximal amplitudes ($F_{\max}/F_0$) were determined from peak or plateau values, as appropriate.

Immunocytochemistry

Immunocytochemistry staining was performed as described [12,22]. In brief, the synaptosomes on Cell-Tak-coated coverslips were fixed with freshly prepared 4% paraformaldehyde in HBS for 30 min. The preparations were subsequently washed with Tris-buffered saline (TBS: 50 mM Tris pH 7.2, 0.9% NaCl). The synaptosomes were then incubated for 45–60 min at room temperature with TBS alone (control) or with rat monoclonal antibodies raised against the $\alpha 4$ nAChR subunit (mAb 299) or the $\beta 2$ nAChR subunit (mAb 270) at 1:500. After extensive washing with TBS, the synaptosomes were incubated for 1 h at room temperature with fluorescein-conjugated goat anti-rat IgG secondary antibodies at 1:100 in TBS containing 10% goat serum. After extensive washing again with TBS, imaging of the synaptosomes was performed using the confocal microscope and the fluorescence emitted in response to excitation at 488 nm (fluorescein) was recorded as described [23]. A threshold was set for each experiment by imaging the control synaptosomes processed in parallel without primary antibodies but with secondary antibody and adjusting the 'black-level' to eliminate the background fluorescence due to the secondary antibody alone. Synaptosomes stained with primary antibody plus the same secondary antibody were then immediately imaged using this threshold. The use of strict thresholding selects for immunostained structures having high signal-to-noise ratios and ensured that the fluorescence observed was specifically due to the primary antibody. Synaptosomes were identified by size and shape [see 22].

Materials

Fluo-4/AM was purchased from Molecular Probes (Eugene, OR, USA). The adhesive matrix Cell-Tak was from BD Sciences (Bedford, MA, USA). Percoll was originally from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Ultrapure sucrose was from ICN Biomedicals (Aurora, OH, USA). HEPES (ULTROL grade) was from Calbiochem (San Diego, CA, USA). Nicotine was from Sigma (St. Louis, MO, USA). The rat monoclonal antibodies mAb 299 and mAb 270 [24] were originally from RBI (Natick, MA, USA). All other chemicals were of the highest reagent grade.

Data analysis and statistics

All experiments were independently replicated at least 3 times. As the responses were heterogeneous in nature, maximal amplitudes were compared (peak or plateau). Where indicated, the significance of the difference between mean values of maximal amplitudes was

determined by one-way ANOVA followed by Scheffé's *F*-test or by *t*-test. Differences were considered significant when *P* was minimally <0.05.

Results

Presynaptic Ca^{2+} responses following chronic treatment with nicotine

Nicotine at 50 nM evoked robust but transient increases in presynaptic $[\text{Ca}^{2+}]_i$ in individual nerve endings from cerebral cortices of control mice (Fig. 1), consistent with previous observations of responses to stimulation of cortical presynaptic nAChRs [21]. Increased presynaptic $[\text{Ca}^{2+}]_i$ likely underlies the nicotine-evoked release of neurotransmitter from cortical synaptosomes described previously to involve predominantly $\alpha 7$ nAChRs whose activation, in turn, triggers the opening of N- and P/Q-type VGCCs [25,26], though $\beta 2$ nAChRs are likely involved as well [27,28]. In addition, increased presynaptic $[\text{Ca}^{2+}]_i$ also likely underlies nicotine enhancement of neurotransmitter release measured in cortical slices [29-33] and the intact cortex using microdialysis [19,34,35].

In contrast, following chronic exposure to nicotine for 14 days, acute presynaptic Ca^{2+} responses to 50 nM nicotine were sustained and larger in amplitude (plateau) as compared to that observed for control (peak) responses (Fig. 1). Enhancement of evoked Ca^{2+} responses after chronic nicotine treatment was only evident for acute application of 50 nM (1.95-fold) and 500 nM (1.75-fold) nicotine but not 50 μM (0.85-fold) nicotine (Fig. 2a). The lack of a significant difference in presynaptic responses to 50 μM nicotine in preparations treated chronically with nicotine may reflect dose-dependent desensitization [2]. The enhanced responses to nM nicotine were sensitive to dihydro- β -erythroidine (DHBE), an antagonist with some selectivity for $\alpha 4$ containing nAChRs, such as the high-affinity $\alpha 4\beta 2$ nAChRs (Fig. 2b). There was no significant effect of DHBE on control preparations (Fig. 2b, legend), which likely indicates that non- $\alpha 7$ nAChRs are a more minor component of presynaptic nAChRs in cortex [cf. 26-28]. The partial sensitivity of the presynaptic Ca^{2+} responses to 50 nM nicotine to DHBE following chronic nicotine, essentially reducing the amplitudes to those observed for control preparations, may indicate that presynaptic non- $\alpha 7$ nAChRs, such as $\alpha 4\beta 2$ nAChRs, were selectively up-regulated.

The development of enhanced nicotine-evoked increases in presynaptic $[\text{Ca}^{2+}]_i$ required over 1 week of chronic intermittent treatment of mice with nicotine (Fig. 3), consistent with previous reports demonstrating time-dependent changes in nicotine-evoked neurotransmitter release [19] or Rb^+ efflux [36]. The enhancement in acute presynaptic Ca^{2+} responses remained after several days of withdrawal of nicotine (not shown). The increase in nicotine-evoked presynaptic Ca^{2+} following chronic nicotine treatment was paralleled by apparent increases in nAChR expression in the nerve terminals, specifically $\alpha 4$ and $\beta 2$ subunits (Fig. 4). Though not quantitative, the overall increased intensities of immunostaining for the nAChR subunits after chronic nicotine treatment may reflect a combination of increased expression on individual terminals and increased numbers of terminals expressing the nAChR subunits.

Discussion

Following chronic treatment with nicotine under an intermittent schedule (twice a day injection s.c. of 0.7 mg/kg nicotine base), acute activation of mouse cortical presynaptic nAChRs with nM nicotine resulted in a relatively enhanced, sustained increase in $[\text{Ca}^{2+}]_i$ in comparison to control preparations from vehicle-injected mice, whereas no significant difference was detected on acute application of μM nicotine. In addition, the enhancement was strongly attenuated in the presence of DHBE, a nAChR antagonist with selectivity for non- $\alpha 7$ containing nAChRs. Finally, chronic treatment with nicotine resulted in an apparent increase in the expression of $\alpha 4$ and $\beta 2$ nAChR subunits on presynaptic terminals from cortex. Taken together,

these findings suggest that chronic systemic exposure to nicotine results in a selective up-regulation of high-affinity $\alpha 4\beta 2$ nAChRs on presynaptic nerve terminals in mouse cortex.

The direction of the change in functional presynaptic nAChR response after chronic nicotine appears to depend upon dose and brain region [37]. More importantly, the means by which nicotine is chronically administered appears to be important [19], but the results are mixed. Continuous infusion, using, for example, osmotic mini-pumps, has been found either to be without effect [19] or to cause up-regulation [38] or up-regulation with concomitant down-regulation of functional presynaptic responses [39] or increased functional presynaptic nAChR responses with receptor up-regulation [36]. Typically, high-affinity $\alpha 4\beta 2$ nAChR is the species most susceptible to up-regulation [40], but with continuous nicotine administration up-regulation of several nAChR subtypes has been observed. By contrast, chronic but intermittent exposure leads, in most cases, to selective up-regulation of high-affinity nAChRs [3-5; however see 40]. Interestingly, very long-term treatment (11 months) of aged mice (to 24–28 months) with nicotine led to a preservation of the high-affinity $\alpha 4\beta 2$ nAChRs as compared to age-matched control mice which displayed a significant decline [41]. Thus, though exposure to nicotine for 1–2 weeks results in up-regulation of high-affinity nAChRs, the consequence of chronic activation of nAChRs over long periods (months to years) may be to maintain nAChR signaling, which otherwise declines with age.

The mechanism by which nicotine induces up-regulation of nAChRs in cells appears to involve its direct interaction intracellularly with nAChRs undergoing assembly [42] in the ER-Golgi pathway [43,44], resulting in stabilization of nAChR pentameric complexes. For upregulation of presynaptic nAChRs, this would require subsequent transport of an augmented pool of receptors down the axon. Unlike the cell body, such a requirement might limit the extent of upregulation and/or greatly extend the time frame over which upregulation occurs. Indeed, in oocytes upregulation of $\alpha 4\beta 2$ nAChRs can be measured in hours [40].

Though up-regulation of nAChRs by nicotine likely occurs post-translationally, chronic nicotine has been nonetheless shown to alter transcriptional activity of a number of genes [45], including several components of intracellular signaling pathways. Of particular interest are components of the PI3/Akt pathway, which have been linked to neurotrophins, especially BDNF [46]. BDNF has also been found to regulate neurotransmitter release [47], but the understanding of the impact of up-regulation of presynaptic nAChRs by chronic nicotine on presynaptic stability and function, possibly via intersection with neurotrophic regulation, remains to be elucidated.

Acknowledgments

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Abbreviations

DHBE, Dihydro- β -erythroidine; HBS, HEPES-buffered saline; TBS, TRIS-buffered saline; VGCC, Voltage-gated calcium channel; nAChR, Nicotinic acetylcholine receptors.

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Fig. 1.

Nicotine-evoked increases in $[Ca^{2+}]_i$ in cortical nerve terminals from control mice and from mice chronically treated with nicotine. Top, representative Ca^{2+} responses to acute application of 50 nM nicotine in individual isolated nerve terminals from saline-treated control mice (left) and from mice treated twice a day with nicotine for 14 days (right). (Note different ordinate scales.) Similar results were obtained with 500 nM or 50 μ M nicotine (not shown). Increases in presynaptic $[Ca^{2+}]_i$ measured in control mouse cortical terminals in response to nAChR activation are α -bungarotoxin-sensitive and absent in preparations from $\alpha 7$ nAChR subunit null mutant mice [21]. Graph shows averaged responses

Fig. 2.

Comparison of presynaptic Ca^{2+} responses evoked by various concentrations of acutely applied nicotine in the absence or presence of nAChR antagonist in cortical nerve terminal preparations from control mice and mice chronically treated with nicotine. **(a)** Normalized average maximal responses (F_{max}/F_0) to 50, 500 and 50 μM nicotine. (Data for 50 nM nicotine was derived from the results shown in Fig. 1.) Fold change in nicotine-evoked Ca^{2+} responses in preparations from chronically treated mice (14 days) as compared to controls were: 1.95-fold for 50 nM; 1.74-fold for 500 nM; 0.85-fold for 50 μM . An increased proportion of terminals responding to nM nicotine was observed for preparations from chronically treated mice (numbers of individual terminals given in parentheses). * $P < 0.05$ compared to control **(b)** Normalized

average responses to 50 nM and 50 μ M nicotine in the absence or presence of the $\alpha 4^*$ nAChR-selective antagonist dihydro- β -erythroidine (DHBE; 100 nM) in preparations from mice chronically treated with nicotine. The numbers of individual terminals are given in parentheses. * $P < 0.05$ compared to the absence of antagonist. Amplitudes for responses in cortical terminal preparations from control mice were: 50 nM nicotine, 1.05 ± 0.04 ($n = 8$); 50 nM nicotine + DHBE, 1.13 ± 0.02 ($n = 8$); 50 μ M nicotine, 1.16 ± 0.02 ($n = 17$); 50 μ M nicotine + DHBE, 1.21 ± 0.02 ($n = 16$)

Fig. 3.

Comparison of presynaptic Ca^{2+} responses evoked by acute application across various days of treatment of mice with nicotine. Graph shows normalized average responses to 50 nM and 50 μM nicotine in preparations from mice treated twice a day for 1st day, 3rd day or 10th day with nicotine. Maximal responses were determined from peak or plateau values. Data obtained with preparations from mice treated for 10 days were similar to those obtained from mice treated for 7 days or 14 days (not shown). The numbers of individual nerve terminals examined under each condition are shown in parentheses. * $P < 0.05$ compared to vehicle-treated (control) preparations

Fig. 4.

Immunostaining for the $\alpha 4$ and $\beta 2$ nAChR subunits in individual nerve terminals from control mice compared to mice chronically treated with nicotine. **(a)** Immunostaining for $\alpha 4$. **(b)** Immunostaining for $\beta 2$. Probing for the $\alpha 7$ nAChR subunit using either antibody or biotin-linked α -bungarotoxin displayed either weak staining over background or high background, respectively (not shown). Synaptosomal preparations were plated onto coverslips at the same densities under all conditions and immunostained as described in Experimental Procedures. To assess background staining, a set of preparations were incubated with secondary antibody only (no primary Ab). The background fluorescence was then eliminated by using these preparations to set the threshold (black) levels for confocal imaging [see 22]. Synaptosomes

are identifiable by their characteristic size (0.5–2 μm in diameter) and round shape, and such structures fully colocalize with synaptic vesicle markers (22, 26, 28). Bar = 5 μm