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Cocaine- and amphetamine-regulated transcript promoter regulated by nicotine in nerve growth factor-treated PC12 cells

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Nicotine and cocaine- and amphetamine-regulated transcripts (CART) have several overlapping functions, such as the regulation of reward, feeding behavior, stress response, and anxiety. Previous studies showed that nicotine regulates CART expression in various brain regions. However, the molecular mechanisms underlying this regulation are not known. This study investigated the regulatory effect of nicotine on promoter activity of the CART gene in PC12 cells, which were differentiated into a neuronal phenotype by nerve growth factor (NGF) treatment. Two vectors containing reporter genes (Gaussia luciferase or mCherry) and the 1,140-bp upstream of the transcriptional start site of the mouse CART gene are used to analyze the CART promoter activity. Transient transfection of PC12 cells with either vector displayed strong promoter activity in both undifferentiated and differentiated PC12 cells. CART promoter activity in the PC12 cell line is increased by forskolin or NGF treatment. In differentiated PC12 cells, exposure to 50 nM nicotine for 6 h increased CART promoter activity. However, treatment with higher nicotine doses for 6 h and treatment with all nicotine doses for 24 h showed no effect. A nicotine concentration of 50 nM is comparable to brain nicotine levels experienced by chronic smokers over long periods of time. Taken together, these data indicate that nicotine may exert some of its actions through the regulation of CART transcription in the brain.

Keywords: nicotine, CART, forskolin, PC12, promoter

Introduction

Nicotine, the nicotinic acetylcholine receptor (nAChR) agonist, enters the body primarily by smoking tobacco. After nicotine is absorbed into the bloodstream, it readily crosses the blood–brain barrier (37) and reaches the brain. Nicotine regulates various neurobiological processes underlying reward, learning and memory, stress response, anxiety, food intake, and metabolism (13, 32, 42, 51). These actions of nicotine are mediated by neuronal nAChRs, which are ionotropic receptors permeable to cations including Na^+ and Ca^{2+} (11). Homo- or heteromeric combinations of α7, α9, and α10 nAChR subunits and heteromeric combinations of α2-6 and β2-4 nAChR subunits are present in the mammalian nervous system (54). α4β2 heteromeric and α7 homomeric subunit combinations are the most common nAChR subtypes found in the brain (44). They are expressed especially in the prefrontal cortex, the striatum, the hippocampus, the amygdala, the hypothalamus, and the ventral tegmental area (49, 54). In contrast, $\alpha3\beta4^*$ and $\alpha3\beta2^*$ subtypes are highly expressed in the peripheral nervous system and in some brain regions, such as the medial habenula and the interpeduncular nucleus (44).

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Cocaine- and amphetamine-regulated transcript (CART) mRNA, very similar to nicotinic receptors, is highly expressed in the striatum, the hippocampus, the amygdala, and the hypothalamus (17, 23). Furthermore, CART peptides also play important roles in reward, feeding behavior, stress response, and anxiety (29, 47), leading to the hypothesis that nicotine may exert some of its regulatory actions via CART signaling. Studies suggest that systemic nicotine administration regulates CART expression in the medial prefrontal cortex, the amygdala, the hypothalamus, and the ventral tegmental area (12, 28, 30, 31, 53). This effect can arise by direct action through nAChRs. Borkar et al. (6) showed α7-nicotinic receptor immunoreactivity on hippocampal CART neurons. In addition, CART is highly coexpressed with proopiomelanocortin in hypothalamic neurons (19), which are suggested to contain α4β2 and α7 nAChRs (22, 39).

Previous studies (5, 16, 17, 26, 27, 33) demonstrated that the proximal promoter region of the mouse and rat CART gene contains an overlapping STAT/CRE/AP1 site, which is responsive to the cAMP-protein kinase A (PKA)-CREB (cAMP response element-binding protein) pathway and the Ca^{2+}-calmodulin-dependent protein kinase (CaMK)-CREB pathway. Similarly, activation of nAChRs also triggers intracellular signaling pathways, which results in CREB activation (11, 32). This overlap in the signal transduction pathways has led us to hypothesize that nicotine may regulate CART promoter activity.

Although there is some evidence that nicotine may regulate CART expression in the brain, the mechanisms underlying this putative regulation have not been identified. This study aimed to examine the effect of nicotine on promoter activity of the CART gene in cultured adrenal pheochromocytoma PC12 cells differentiated into neuronal cells by nerve growth factor (NGF) treatment (18, 45). We hypothesized that the downstream effects of nicotine increase CART promoter activity. The results of this study will shed light on the involvement of CART in numerous neurobiological effects of nicotine.

Materials and Methods

Cell culture

PC12 cells (CRL-1721 and ATCC) were maintained in RPMI 1640 (Gibco) supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS). Culture vessels were coated with 5 ng/cm^2 collagen to facilitate cell adherence. Cells were maintained in a humidified incubator at 37 °C under 5% CO_2. Culture medium was replaced with fresh medium every 3 days.

Plasmid constructs

Luciferase- or mCherry-expressing constructs (-1140-CART-LUC and -1140-CART-mCherry, respectively) containing 1,140-bp upstream and 47-bp downstream of the transcription start site (TSS) of the Cartpt (NM _001081493) promoter sequence were obtained commercially (GeneCopoeia Inc., Rockville, MD, USA). Plasmid constructs were sequenced to assure the accuracy of the cloned sequences (sequencing primers, forward: 5′-AGTTACTTAAGCTCGGGCCC-3′; reverse: 5′-TTGTTCTCGGTGGGCTTGGC-3′). The -1140-CART-LUC construct expressed Gaussia luciferase (GLuc), which is secreted into the culture medium. GLuc activity was assayed in the growth media of transfected cells.

Transfection and luciferase assay

To determine the optimal transient transfection conditions, PC12 cells were transfected with various X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Indianapolis, IN, USA)

and plasmid DNA (-1140-CART-mCherry) ratios ranging from 4 to 1 µl/1 µg. Cells were visualized after 72 h and images were quantified with ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/, 1997–2019). PC12 cells were transfected using 2 µl/µg ratio in all subsequent experiments, which yielded the highest transfection efficiency. The DNAs were mixed with X-tremeGENE HP DNA reagent in serum-free medium and incubated at room temperature for 15 min as described by the manufacturer. For 100 mm Petri dishes, 1 ml of the complexed reagent/DNA mixture was added to 6×10^6 cells. Cells were maintained in RPMI 1640 supplemented with 1% HS and 0.5% FBS for 48 h until they were seeded at a density of 3×10^4/well onto 96-well plates. All cells used in an experiment were sourced from the same transfected Petri dish to maintain cell transfection homogeneity.

Twenty-four hours after plating, the culture medium was replaced with RPMI 1640 supplemented with 2% HS, 1% FBS, and 100 ng/ml 7S-NGF to facilitate differentiation into neuronal phenotype and maintained for 7 days. The state of differentiation was monitored over time using traditional morphologic methods. Morphological analysis was performed on digitized images of live cells taken under phase contrast illumination with a Visiscope IT404 inverted microscope. Undifferentiated and differentiated cells were treated with forskolin (20 µM, 24 h) to induce cAMP-dependent promoter activation. Fluorescence levels reflecting CART promoter activity were analyzed in five random sections from each condition using an Olympus BX53 microscope equipped with Olympus DP72 camera (Olympus Corp., Tokyo, Japan) and ImageJ v1.49 (Rasband, W.S.). ImageJ software was used to subtract the background of images by selecting an area without any cells and to select the cells. Area integrated density values were used to analyze fluorescence.

In the second part of the experiment, NGF-differentiated cells were treated with nicotine (50 nM–100 nM–300 nM–1 µM–3 µM–10 µM–100 µM), forskolin (20 µM, positive control) or saline (control) in low serum conditions. Culture medium samples were collected at 6 and 24 h after the treatment. Luciferase activity was quantified using a luminometer equipped with injectors (Varioskan Flash Reader, Thermo Scientific, Waltham, MA, USA) and GLuc Assay Kit (New England Biolabs, Ipswich, MA, USA) as recommended by the manufacturer. Briefly, 20 µl of culture medium samples were loaded in 96-well plates. Luciferase substrate (50 µl) was dispensed into the wells by the injector, and luminescence was measured for 3 s in each well.

Statistical analyses

The differences in mCherry fluorescence between forskolin- or NGF-treated groups and their controls were analyzed by using two-way analysis of variance (ANOVA). Differences in GLuc activity between forskolin- or nicotine-treated groups and their controls were analyzed by one-way ANOVAs followed by *post-hoc* Tukey's tests. The SPSS Statistics for Windows (version 17.0, SPSS Inc., Chicago, USA) software was used for all statistical analyses.

Results

Neuronal differentiation of PC12 cells

Neuronal differentiation of PC12 cells was achieved by NGF treatment. Studies report that 50–100 ng/ml NGF for 3–7 days promotes neurite outgrowth, which is a useful indicator

of neuronal differentiation (18, 45). In this study, PC12 cells were incubated with NGF (100 ng/ml) for 7 days. NGF-induced neurite formation was initiated on the third day and increased until the last day of treatment.

CART expression in undifferentiated PC12 cell line

CART mRNA is expressed endogenously in PC12 cells (35). Forty-eight hours after the transfection with -1140-CART-mCherry vector, a notable red fluorescence was observed in transfected PC12 cells. Strong mCherry fluorescence visualized under fluorescence microscope showed that intracellular signaling pathways in PC12 cells are capable of inducing CART promoter activity without any drug exposure. Strong mCherry protein expression in PC12 cells also confirmed the efficiency of transfection.

Regulation of CART promoter by forskolin and NGF

Seventy-two hours after the transfection with -1140-CART-mCherry vector, PC12 cells were treated with NGF (100 ng/ml) for 7 days to achieve neuronal differentiation. NGF-differentiated and -undifferentiated PC12 cells were exposed to forskolin (20 μM), a potent activator of adenylate cyclase, for 24 h. Figure 1 demonstrates increased mCherry fluorescence in undifferentiated and differentiated PC12 cells 24 h after forskolin treatment. Fluorescence levels in the PC12 cells transfected with -1140-CART-mCherry vector were used for the statistical analysis of CART promoter activity. A two-way ANOVA was conducted to examine the effect of NGF and forskolin treatments on CART promoter activity (Fig. 2). The main effect of NGF yielded an F ratio of $F(1, 12) = 8.79$, $p = 0.012$, indicating a significant difference between undifferentiated (mean = 286.71, SD = 85.18) and differentiated (mean = 368.33, SD = 109.20) PC12 cells. The main effect of forskolin yielded an F ratio of $F(1, 12) = 69.96$, $p = 0.000002$, indicating a significant difference between forskolin-treated (mean = 422.88, SD = 61.50) and untreated (mean = 242.38, SD = 34.56) PC12 cells. CART promoter activity increased significantly in PC12 cells after forskolin exposure. There was no statistically significant interaction between the effects of NGF and

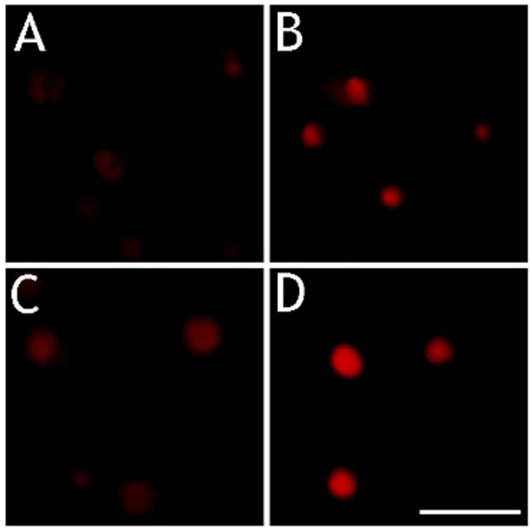

Fig. 1. Regulation of CART promoter by forskolin and NGF in PC12 cells transfected with -1140-CART-mCherry vector. (A, B) mCherry expression in undifferentiated PC12 cells; (C, D) mCherry expression in NGF-differentiated PC12 cells; (A, C) mCherry expression in PC12 cells without forskolin treatment; (B, D) mCherry expression in forskolin-treated PC12 cells. Scale bar: 50 μm

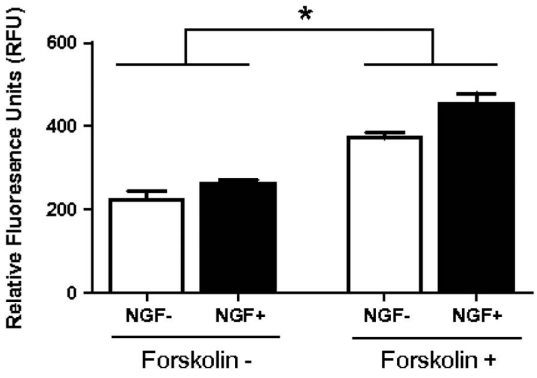

Fig. 2. Effect of forskolin and NGF treatment on mCherry fluorescence in PC12 cells transfected with -1140-CART-mCherry vector. Two-way ANOVA showed main effects for forskolin and NGF treatments. mCherry fluorescence representing the CART promoter also increased significantly in forskolin-treated groups when compared to untreated groups (*$p = 0.000002$). CART promoter activity increased also in NGF-treated groups when compared to untreated groups ($p = 0.012$, not annotated). There was no statistically significant interaction between the effects of NGF and forskolin on CART promoter activity

forskolin on CART promoter activity [$F(1, 12) = 1.125$, $p = 0.31$],indicating that the effect of forskolin was equivalent for undifferentiated and differentiated PC12 cells.

Regulation of CART promoter activity by nicotine and forskolin in differentiated PC12 cell line

In the first part of the experiment, PC12 cells transfected with -1140-CART-LUC vector and differentiated with NGF are exposed to forskolin (20 μM) or nicotine (50, 100, 300 nM or 1, 3, 10 μM) for 6 h. Differences in luciferase activity measured in control, forskolin- and nicotine-treated PC12 cells were determined by one-way ANOVA (Fig. 3). This test showed that there is a statistically significant difference between the groups [$F(7, 87) = 8.481$, $p < 0.000001$]. A Tukey's *post-hoc* test revealed that CART promoter activity was significantly higher in PC12 cells treated with 50 nM nicotine (mean ± SEM = 141.33 ± 14.15, $p = 0.024$) or forskolin (mean ± SEM = 140.97 ± 8.84, $p = 0.032$) when compared to control (mean ± SEM = 100.0 ± 5.92). There was no statistically significant difference between groups treated with higher nicotine doses and the control group or between 50 nM nicotine- and forskolin-treated groups.

In the second part of the experiment, when the duration of drug exposure was increased to 24 h, one-way ANOVA also detected a significant difference between groups [$F(7, 88) = 24.38$, $p < 0.000001$]. The *post-hoc* Tukey's test showed that forskolin

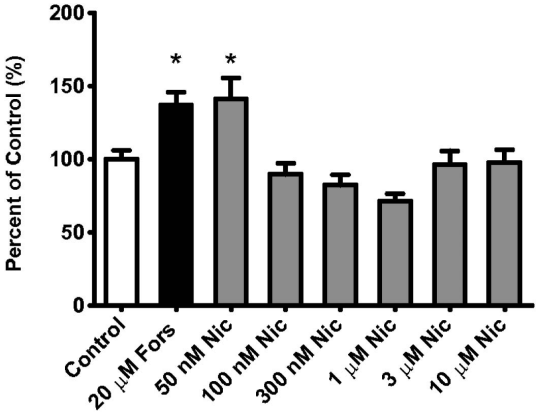

Fig. 3. Effect of 6-h nicotine and forskolin treatment on GLuc activity in differentiated PC12 cells transfected with -1140-CART-mCherry vector. Difference from saline-treated control was demonstrated by one-way ANOVA followed by *post-hoc* Tukey's tests. The bars represent arbitrary units (mean ± SEM) expressed as % of the mean values of the controls. Luminescence representing CART promoter activity increased significantly in differentiated PC12 cells after forskolin (20 μM) or 50 nM nicotine treatment (*$p < 0.05$, compared to control, $n = 9$–10 for each group). Nicotine treatment with doses higher than 50 nM for 6 h did not change CART promoter activity

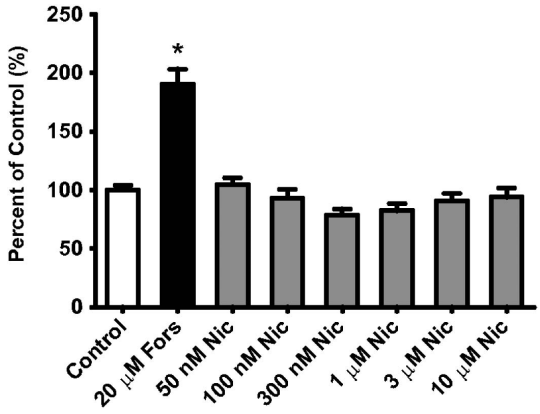

Fig. 4. Effect of 24-h nicotine and forskolin treatment on GLuc activity in differentiated PC12 cells transfected with -1140-CART-mCherry vector. Difference from saline-treated control was demonstrated by one-way ANOVA followed by *post-hoc* Tukey's tests. The bars represent arbitrary units (mean ± SEM) expressed as % of the mean values of the controls. Luminescence representing the CART promoter activity increased significantly in differentiated PC12 cells after forskolin (20 μM) treatment (*$p < 0.000001$, compared to control, $n = 10$ for each group). None of the nicotine doses used for 24 h-exposure changed CART promoter activity

(mean ± SEM = 190.61 ± 12.77, $p < 0.000001$) treatment increased CART promoter activity significantly when compared to control (mean ± SEM = 100.0 ± 3.87; Fig. 4). However, none of the nicotine doses regulated CART promoter activity after 24 h of exposure.

Discussion

Previous studies showed that the expression of CART mRNA or peptides in various brain regions may be regulated by the psychostimulant drug nicotine (12, 28, 30, 31, 53). However, the mechanisms underlying this regulation have not been studied. This study aimed to explore the nicotinic regulation of CART promoter in PC12 cells, which are differentiated into neuronal phenotype when induced by NGF. Our results showed that nicotine regulates the promoter activity of CART gene in a dose-dependent manner.

PC12 cells display endogenous CART mRNA expression (35). Consistent with this, we were able to detect baseline promoter activity without any induction in PC12 cells (Fig. 1A). This study showed that strong promoter activity was induced in both undifferentiated and differentiated PC12 cells when transfected with vectors containing 1,140-bp upstream and 47-bp downstream of the TSS of the mouse CART gene. This finding indicates that intracellular signaling pathways in PC12 cells are capable of inducing CART promoter activity. Similar results were obtained by Dominguez et al. (16) and Barrett et al. (4) in GH3 pituitary cells, undifferentiated PC12 cells, and SH-SY5Y neuroblastoma cells. Dominguez et al. (16) demonstrated that transfection with two vectors containing 3,451- or 641-bp upstream of TSS of the mouse CART gene induced intense luciferase activity in GH3 cells and undifferentiated PC12 cells. Barrett et al. (4) showed CART promoter activity also in SH-SY5Y neuroblastoma cells transfected with vectors containing 1,167- and 265-bp upstream of the start codon of the rat CART gene. In addition, this study showed that NGF treatment increased CART promoter activity significantly in differentiated PC12 cells. NGF-induced neuronal differentiation is a result of TrkA receptor signaling, which leads to the activation of many transcription factors such as CREB (41). CREB is also known as an important regulator of CART promoter (33).

Dominguez et al. (16) suggested that the 320-bp region located upstream from the TSS of mouse CART gene contained an overlapping STAT/CRE/AP1 site. In this study, CART promoter inserted into the vectors contained 1,140 bp of upstream sequence, therefore

including the STAT/CRE/AP1 site. Forskolin, a compound that binds and activates adenylate cyclase directly, increased CART promoter activity significantly both in undifferentiated and differentiated PC12 cells. Accordingly, forskolin was used as a positive control in this study. CART promoter activity is induced 6 h after the forskolin exposure and persisted for 24 h. In parallel, previous studies showed that forskolin exposure for 6, 12, and 24 h increases CART transcription in GH3 cells (5, 16, 33). Forskolin also increases luciferase activity in GH3 (12) and SH-SY5Y (3) neuroblastoma cells transfected with CART promoter-containing vectors. Furthermore, Lakatos et al. (33) showed that PKA-phosphorylated CREB protein binds to the CRE site located on the CART promoter. Although there may be other pathways leading to CART promoter activation such as Ca^{+2}/CaMK signaling, the results of these *in vitro* studies suggest that the CART promoter may be regulated by the activation of the cAMP-PKA-CREB signaling pathway. *In vivo* studies also support this suggestion. Jones and Kuhar (26) demonstrated that injections of forskolin into the nucleus accumbens (NAcs) induced CREB phosphorylation and increased CART mRNA levels, which is attenuated by the inhibition of PKA. Moreover, when herpes simplex virus-1 vectors that overexpressed CREB were injected into rat NAc, CART mRNA expression increased (27).

Main nAChR subunits expressed on undifferentiated and differentiated PC12 cells are α3, α5, α7, β2, and β4 (21, 46). Nicotine applied to the differentiated PC12 cells may act via $\alpha3\beta4^*$, $\alpha3\beta2^*$, and homomeric α7 nAChRs, which are reported as functional nicotinic receptor subtypes in PC12 cells (15, 40). When NGF-differentiated PC12 cells were treated with 50 nM nicotine for 6 h, CART promoter activity increased to levels comparable to those induced by forskolin (Fig. 3). Cigarette smoking increases brain nicotine levels to the range of ∼50–500 nM within seconds to minutes (43). Studies suggest that nicotine concentrations at the lower end (20–80 nM) of this concentration range persist in smokers for longer times (52). Therefore, we can postulate that nicotine, especially when found at low concentrations, may increase CART transcription in neurons. Supporting this suggestion, Kaya et al. (28) and Kramer et al. (30) reported increased CART mRNA expression in amygdala and paraventricular nucleus of hypothalamus following daily nicotine injections. In another study by Balkan et al. (3), forced swim stress increased CART peptide expression in rat adrenal gland. This effect may be mediated via the activation of nicotinic receptors on chromaffin cells (10) during sympathetic stimulation of the adrenal medulla.

High-dose (>50 nM) nicotine treatment for 6 h did not regulate CART promoter activity in differentiated PC12 cells. In addition, none of the nicotine doses changed CART promoter activity 24 h after exposure. This result may be explained by desensitization of nicotinic receptors. It is well-known that nAChRs undergo desensitization upon prolonged agonist exposure. Desensitization of nicotinic receptors prevents channel opening and receptor signaling (7, 16). nAChRs have distinct desensitization properties depending on the extracellular nicotine concentration and the duration of nicotine exposure(20). Chronic (20–60 min) incubation of Xenopus oocytes expressing $\alpha3\beta4^*$ or $\alpha3\beta2^*$ nAChRs with varying concentrations of nicotine showed that half-maximal concentration for inhibition (IC_{50}) by nicotine is 1.15 μM for $\alpha3\beta4^*$ and 330 nM for $\alpha3\beta2^*$ subtypes (20). Similarly, the IC50 value reported for the α7 subtype ranges from ∼640 nM to 1.3 μM (1, 20). There is no desensitization of these nAChR subtypes at low nicotine concentrations (<100 nM), whereas desensitization increases with nicotine concentrations higher than 100 nM (20, 38). Furthermore, Wooltorton et al. (52) reported that low nicotine concentrations (20–80 nM), which model nicotine levels experienced by chronic smokers over long time periods, do not induce any significant α7 desensitization in rodent midbrain slices. In the light of these data, we can

suggest that $\alpha3\beta4^*$, $\alpha3\beta2^*$, and homomeric α7 receptor signaling may lead to the increased CART promoter activity observed 6 h after 50 nM nicotine exposure in this study. Disappearance of the regulatory effect at nicotine concentrations above 50 nM or with 24-h exposure may be explained by the desensitization of these nAChR subtypes.

In vitro studies showed that nicotine at concentrations in nano- and micromolar ranges trigger measurable electric currents in neurons (1, 14, 36, 43). nAChRs are ligand-gated channels and allow the passage of cations including Ca^{2+} (11). In addition, nAChR activity increases cytosolic Ca^{2+} by triggering voltage-gated Ca^{2+} channels on the plasma membrane or intracellular Ca^{2+} release channels on the endoplasmic reticulum membrane. Ca^{2+} induces several downstream signaling enzymes including PKA, CaMK, and mitogen-activated protein kinase, which leads to the activation of transcription factors such as CREB (11, 32). On the other hand, previous studies also showed that the CART gene is regulated by the cAMP/PKA/CREB and Ca^{+2}/CaMK/CREB pathways (26, 27). Nicotine-induced CART promoter activity observed in this study may be a result of CRE site activation.

To our knowledge, this is the first study showing that the promoter region of the CART gene is regulated by nicotine. Only low nicotine concentrations (50 nM) increased CART promoter activity in PC12 cells, which are differentiated into neuronal phenotype. Such low nicotine concentrations mimic brain nicotine levels experienced by chronic smokers over long periods of time. Therefore, it is possible that nicotine in smokers' brains may increase CART transcription in neurons, which subsides with long exposure times due to nAChR desensitization. Rogge et al. (47) suggested that CART blunts the effects of dopamine in NAc and assigned a homeostatic function to CART in reward. Job et al. (25) reported that the homeostatic function of CART is lost during chronic cocaine exposure and this may play a role in the development of addiction. Based on our results, we can suggest that desensitization of nicotinic receptors leading to decreased CART synthesis and function may have a role in nicotine addiction. Nicotine may exert its actions on the stress response, anxiety and appetite also through the regulation of CART transcription in the brain. Both acute nicotine exposure and intracerebroventricular CART injections activate the hypothalamic–pituitary–adrenal axis (2, 50), increase anxiety-like behavior (8, 24), and reduce feeding (7, 34). However, these responses disappear with chronic nicotine administration in rodents (9, 24, 48), which indicate desensitization of nAChRs.

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Conflict of interest

The authors declare no conflict of interest.

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