

# Regulation by Nicotine of *Gpr51* and *Ntrk2* Expression in Various Rat Brain Regions

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Our previous genetic studies demonstrated that variants of the  $\gamma$ -Aminobutyric acid B receptor subunit 2 (*GPR51*) and neurotrophic tyrosine kinase receptor type 2 (*NTRK2*) genes are significantly associated with nicotine dependence (ND) in smokers. However, whether such genetic associations lead to changes in the expression of the two genes in response to nicotine remains undetermined. In this study, we investigated the regulatory effect of nicotine on the expression of *Gpr51* and *Ntrk2* in seven rat brain regions during the administration of nicotine in a daily dose of 3.15 mg/kg for 7 days. With quantitative real-time RT-PCR, we found that nicotine increased the mRNA of *Gpr51* by 70, 78, and 32% in the amygdala, striatum, and prefrontal cortex (PFC), respectively, but decreased by 54% in the nucleus accumbens (NA). The *Gpr51* protein was upregulated by nicotine in the amygdala (26%), striatum (73%), PFC (28%), and medial basal hypothalamus (MBH; 19%) but downregulated in the NA (–72%). Similarly, the mRNA level of *Ntrk2* was enhanced by nicotine in the striatum (86%) and PFC (38%), but decreased in the NA (–46%) and ventral tegmental area (VTA; –49%). A significant change in protein expression was also obtained for *Ntrk2* in the PFC (24%), MBH (33%), NA (–33%), and VTA (–70%). Interestingly, these two genes showed a closely coordinated expression pattern in response to nicotine in most of the brain regions examined. In summary, our results demonstrate that the expression of *Gpr51* and *Ntrk2* is significantly regulated by nicotine at both the mRNA and protein levels in various brain regions, which provides further evidence that these two genes are involved in the etiology of ND, as reported in our previous genetic association studies in humans.

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## INTRODUCTION

Nicotine dependence (ND) is a complex quantitative trait that is influenced by both genetic and environmental factors (for recent reviews, see Sullivan and Kendler, 1999; Li *et al*, 2003a). Meta-analysis of 17 twin studies determined that the weighted mean heritability of liability for ND is 0.56 for all smokers (Li *et al*, 2003a). The habit-forming actions of nicotine appear to be triggered through the stimulation of dopamine secretion in the ventral tegmental area (VTA), which projects to the nucleus accumbens (NA), in a manner similar to other drugs of abuse such as cocaine, amphetamine, or morphine (Pontieri *et al*, 1996; Rose and Corrigall, 1997).

Previously, we reported that several chromosomal regions are likely to harbor susceptibility gene(s) for ND in the 313 extended Framingham Heart Study (FHS) families (Li *et al*, 2003b; Wang *et al*, 2005). Of these regions, that on chromosome 9q22–23 appears to be interesting because three additional studies reported a possible linkage, at a nominally significant level, with smoking behaviors (Bergen *et al*, 1999; Bierut *et al*, 2004; Gelernter *et al*, 2004). The genes for both  $\gamma$ -Aminobutyric acid (GABA)<sub>B</sub> receptor subunit 2 (*GPR51*) and neurotrophic tyrosine kinase receptor type 2 (*NTRK2*) have been mapped within this linkage region (Nakagawara *et al*, 1995; Valent *et al*, 1997; Martin *et al*, 1999) and suggested to play a significant role in drug addiction. To determine if these two genes are involved in the etiology of ND, we conducted family-based association studies using an independent cohort recruited by us during 1999–2004 and found that these two genes indeed are significantly associated with ND (Beuten *et al*, 2005a; Beuten *et al*, 2006). With the same cohort, we also found a significant association of brain-derived neurotrophic factor (*BDNF*), which exerts its regulatory effects through the activation of *NTRK2*, with ND in European-American male smokers (Beuten *et al*, 2005b).

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GABA is a major inhibitory neurotransmitter in the mammalian central nervous system and plays a key role in modulating neuronal activity via the ionotropic GABA<sub>A</sub> and metabotropic GABA<sub>B</sub> receptors (for a review, see Bettler *et al*, 2004). GABA neurons are part of the mesolimbic DA system, and GABAergic transmission regulates reinforcement by several drugs of abuse through an inhibitory action on mesolimbic dopaminergic neurons via GABA<sub>B</sub> receptors (Bardo, 1998; Gong *et al*, 1998; Cousins *et al*, 2002). Preclinical and clinical studies indicate that GABA<sub>B</sub> receptor agonists, that is, baclofen, reduce addictive behaviors associated with nicotine, cocaine, and morphine use in rats (Cousins *et al*, 2002; Fadda *et al*, 2003). On the other hand, expression of BDNF, a member of the neurotrophin family, has been found to be coregulated with that of *NTRK2* by ethanol at both the mRNA and protein levels, and by brain injury at the mRNA level (Hicks *et al*, 1999; Zhang *et al*, 2000; Ge *et al*, 2004; Zaidi *et al*, 2005). Several studies have demonstrated that BDNF is involved in the survival and differentiation of DA neurons and regulates the reward pathways associated with the actions of drug abuse (Hyman *et al*, 1991; Spina *et al*, 1992; Horger *et al*, 1999; Kenny *et al*, 2000; Meredith *et al*, 2002). Cocaine, methamphetamine, morphine, alcohol, and nicotine can increase Bdnf expression in the rat cortical area, hippocampus, and hypothalamus (Tapia-Arancibia *et al*, 2001; Le Foll *et al*, 2005). Microinjection of either Bdnf or Ntrk2 antibodies decreases the elevation of DA stimulated by methamphetamine and cocaine and induces DA-related behaviors in rat NA (Horger *et al*, 1999; Narita *et al*, 2003). Further, BDNF modulates GABAergic transmission in the visual cortex of rats and mice (Huang *et al*, 1999; Mizoguchi *et al*, 2003b) and accelerates the responsiveness to GABA in cultured granule cells (Kubo *et al*, 1995; Lin *et al*, 1998; Rieff *et al*, 1999; Borghesani *et al*, 2002; Mizoguchi *et al*, 2003b).

As for regulation of *Gpr51* and *Ntrk2* by nicotine, however, only a few studies have been reported (French *et al*, 1999; Kenny *et al*, 2000; Amantea *et al*, 2004; Li *et al*, 2004). Therefore, the primary purpose of this study was to determine whether nicotine has regulatory effects on the expression of *Gpr51* and *Ntrk2* in the rat brain.

## MATERIALS AND METHODS

### Animals, Nicotine Administration, and Brain Punches

Adult male Holtzman rats (250–350 g; HSD, Madison, WI) were randomly divided into nicotine-treated and control groups. For the former, nicotine bitartrate was administered through osmotic minipumps (Model 2ML1; Azlet Corp., Palo Alto, CA) in a daily dose of 3.15 mg/kg (calculated as the base) in saline (pH 7.4) for 7 days, as originally reported by Malin *et al*, 1992 and replicated by several other laboratories (Hildebrand *et al*, 1997; Epping-Jordan *et al*, 1998; Watkins *et al*, 2000). This model produces a stable plasma nicotine concentration of ca. 44 ng/ml, which is very similar to that in smokers consuming 30 cigarettes daily (Murrin *et al*, 1987; Benowitz, 1988). Rats in the control group were handled and treated exactly the same, except that only saline was delivered by the minipumps. All rats were housed at 22°C on a 12 h light/dark cycle. Standard laboratory rat chow and water were freely available. To

ensure we had enough sample size for real-time RT-PCR or Western blotting assays, seven animals were included for each experimental group. Brain punches used for real-time RT-PCR and Western blotting assays were from two independent animal experiments under an identical treatment regimen.

After 7 days of nicotine treatment, rats were anesthetized with isoflurane, and the brains were removed immediately for sectioning. Coronal 2-mm sections were prepared using a Stoelting tissue slicer (Chicago, IL). Punches from the selected brain regions were excised using a tissue set from myNeuroLab.com (St Louis, MO) based on coordinates from Paxinos and Watson (1986). For the NA, each sample contained the core and shell of the accumbens dissected using bilateral 2.0-mm-diameter punches. For the amygdala region, each sample contained bilateral 2.0-mm-diameter dissections centered in the basolateral nuclear complex. For the VTA, each sample contained a single 1.5-mm-diameter punch that was centered in the dense field of dopamine-containing cells. All animal-related experimental procedures were approved by the Institutional Animal Use and Care Committee.

### RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction (Quantitative RT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was reverse transcribed in a final volume of 20 µl containing 4 µl of 5 × first-strand buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM MgCl<sub>2</sub>), 10 mM DTT, 0.5 mM each dNTP, 40 U RNaseOUT™, 1 µl of 50 nM random hexamers, and 200 U of Superscript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen).

The TaqMan<sup>®</sup> probes and primers were designed according to the cDNA sequences of rat *Gpr51* (GenBank Accession No. NM031802) and *Ntrk2* (GenBank Accession No. NM012731) and synthesized by Applied Biosystems (Foster, CA). The forward and reverse primer sequences were 5'-TTCAACATCAAGAACCGG-3' and 5'-CAGAAAA GGTTAGAAATC-3', respectively, for *Gpr51*, and 5'-AGATC TCGCTTCCACTGTATAGCAT-3' and 5'-GGCTTGACATCT TAATCAGCTTTTG-3' for *Ntrk2*, with an expected PCR product size of 123 bp for *Gpr51* and 81 bp for *Ntrk2*. The TaqMan probes used for *Gpr51* and *Ntrk2* were 5'-TTCAACATCAAGAACCGG-3' and 5'-CAGAAAAAGGTTA GAAATC-3', respectively. Amplification of 2 µl of cDNA was carried out in a total volume of 20 µl according to the manual of TaqMan<sup>®</sup> Gene Expression Assays (Applied Biosystems). The mRNA level of each gene was determined using a calibration method (Winer *et al*, 1999) and normalized to that of 18S rRNA in each sample.

### Western Blotting Analysis

Total protein was extracted from individual frozen brain punches of an independent animal experiment by homogenization with a sonicator in RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), and the protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of total protein was separated by 8% SDS-PAGE followed by

transfer to nitrocellulose membranes (0.45  $\mu$ m) at 80 V for 1.5 h. The membrane was first incubated in a blocking buffer (6% nonfat milk and 0.2% Tween 20) for 0.5 h at room temperature and then overnight at 4°C in the blocking buffer containing rabbit anti-Gpr51 antibody (dilution 1:200; Chemicon, Temecula, CA) or mouse anti-Ntrk2 antibody (dilution 1:1000; BD Biosciences, San Jose, CA). After three washes in TBST (10 mM Tris-HCl, pH 8.0; 0.15 M NaCl; 0.2% Tween 20) for 10 min each, the membranes were exposed to horseradish peroxidase-conjugated secondary antibodies at 4°C for 3 h, and then exposed to X-ray film. After hybridization with the antibody of interest, membranes were stripped and re-probed with antibody to  $\beta$ -actin (dilution 1:10000; Santa Cruz Biotechnology, Santa Cruz, CA), which was used for normalization of the protein content of each sample. Then, the films were scanned for quantitative analysis with ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA).

### Statistical Analysis

The significance of differences between the nicotine-treated and control groups was analyzed by Student's *t*-test, and a *P*-value of  $\leq 0.05$  was considered statistically significant.

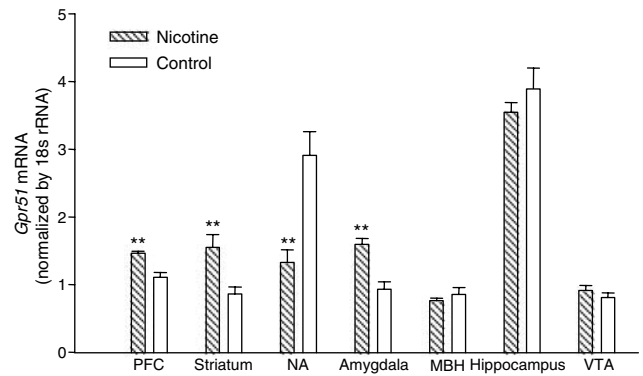
## RESULTS

### Changes in Expression of Gpr51 mRNA

To identify the regulatory effects of chronic nicotine treatment on *Gpr51* mRNA in rat brain, quantitative RT-PCR analysis was employed. After normalization with the corresponding 18S rRNA mRNA level of each sample followed by Student's *t*-test, we found that the mRNA level of *Gpr51* was significantly upregulated, by 70% ( $P < 0.01$ ), 78% ( $P < 0.01$ ), and 32% ( $P < 0.01$ ), in the amygdala, striatum, and prefrontal cortex (PFC), respectively, of the nicotine-treated rats compared with saline-treated controls. On the other hand, we found significant downregulation of *Gpr51* mRNA, by 54% ( $P < 0.01$ ), in the NA of nicotine-treated rats relative to controls (Figure 1).

### Protein Expression Changes of Gpr51

To determine if the protein level is also changed by nicotine, we performed Western blotting analysis of the seven brain regions. To check the loading efficiency and protein concentration, we conducted Western blotting analysis for  $\beta$ -actin on the same samples. The Western blotting images for Gpr51 and  $\beta$ -actin in the seven brain regions are shown in Figure 2. The protein level was significantly upregulated, by 26% ( $P < 0.05$ ), 73% ( $P < 0.01$ ), and 28% ( $P < 0.01$ ), in the amygdala, striatum, and PFC, respectively, and downregulated by 72% ( $P < 0.05$ ) in the NA. By comparing the expression trends of Gpr51 in response to nicotine, we found a closely parallel pattern between the mRNA and protein levels in six of the seven brain regions. The only difference was in the MBH, in which no significant difference was obtained in mRNA, but a significant difference was achieved in the protein (18%,  $P < 0.05$ ).



**Figure 1** Comparison of mRNA levels of *Gpr51* between nicotine-treated and control groups in seven rat brain regions after 7 days of nicotine administration, which showed that the mRNA level of *Gpr51* was increased by nicotine by 70, 78, and 32% in the amygdala, striatum, and PFC, respectively, and decreased by 54.1% in the NA. Values are the mean  $\pm$  SEM of six animals per group. \* $P < 0.05$ , \*\* $P < 0.01$ .

### Expression Changes of Ntrk2 mRNA

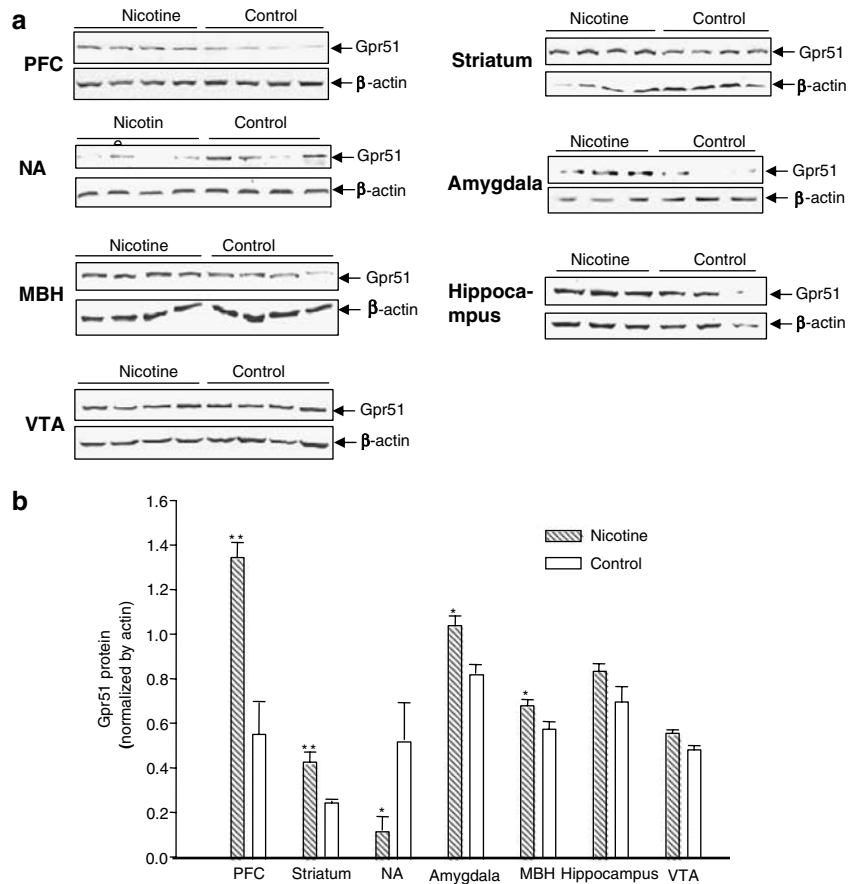
Figure 3 shows a summary of mRNA level of *Ntrk2* in the rat brain regions, which indicated that the gene was significantly altered at the mRNA level in four of the seven regions. Together, we found that mRNA level of *Ntrk2* was significantly increased, by 86% ( $P < 0.05$ ) and 38% ( $P < 0.05$ ), in the striatum and PFC, respectively, but decreased by 46% ( $P < 0.05$ ) and 49% ( $P < 0.05$ ) in the NA and VTA, respectively, in the nicotine-treated group compared with the control animals.

### Protein Expression Changes of Ntrk2

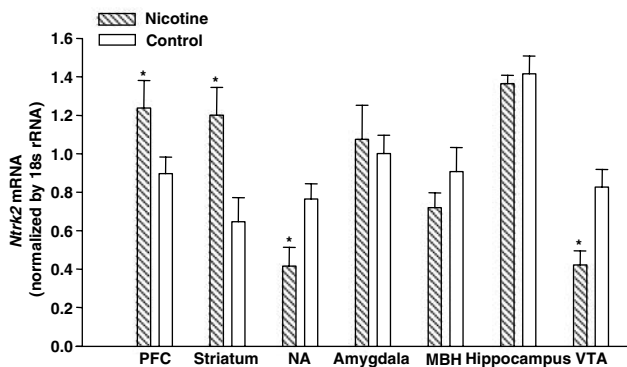
With Western blotting analysis, we found that nicotine significantly increased the level of Ntrk2 protein, by 24% ( $P < 0.01$ ), in the PFC and decreased it by 33% ( $P < 0.05$ ) and 70% ( $P < 0.05$ ) in the NA and VTA, respectively (Figure 4), which is consistent with the change in the amount of mRNA. In addition, we observed a significant increase, 30% ( $P < 0.05$ ), in Ntrk2 protein in the MBH and no significant increase in the striatum, which is different from what we observed for *Ntrk2* mRNA in the two regions.

## DISCUSSION

Previously, we reported identification of an approximately 13-cM genomic region on chromosome 9q22–q23 that showed a suggestive linkage to smoking quantity in the FHS samples (Li et al, 2003b). This linkage to ND at a nominally significant level was supported by three independent linkage studies for smoking behavior (Bergen et al, 1999; Bierut et al, 2004; Gelernter et al, 2004). Furthermore, this genomic region has been linked to alcohol consumption in the FHS cohort (Ma et al, 2003). Within this region, *GPR51* and *NTRK2* represent two plausible candidate genes for ND based on their biological functions and locations (Nakagawa et al, 1995; Valent et al, 1997; Martin et al, 2001). Recently, we conducted a family-based association analysis of *GPR51* and *NTRK2* with ND in an independent cohort, namely the mid-South Tobacco families, that we recruited



**Figure 2** Western blotting analysis of Gpr51 expression in seven rat brain regions. (a) shows the results for Gpr51 and  $\beta$ -actin in seven brain regions. (b) summarizes the statistical analysis of the Gpr51 protein level normalized by the corresponding  $\beta$ -actin level, which indicated that nicotine increased Gpr51 protein level by 26, 28, 73, and 19% in the amygdala, striatum, PFC, and MBH, respectively, and reduced its expression by 72% in the NA. Values are the mean  $\pm$  SEM of 3–5 animals per group. \* $P < 0.05$ , \*\* $P < 0.01$ .

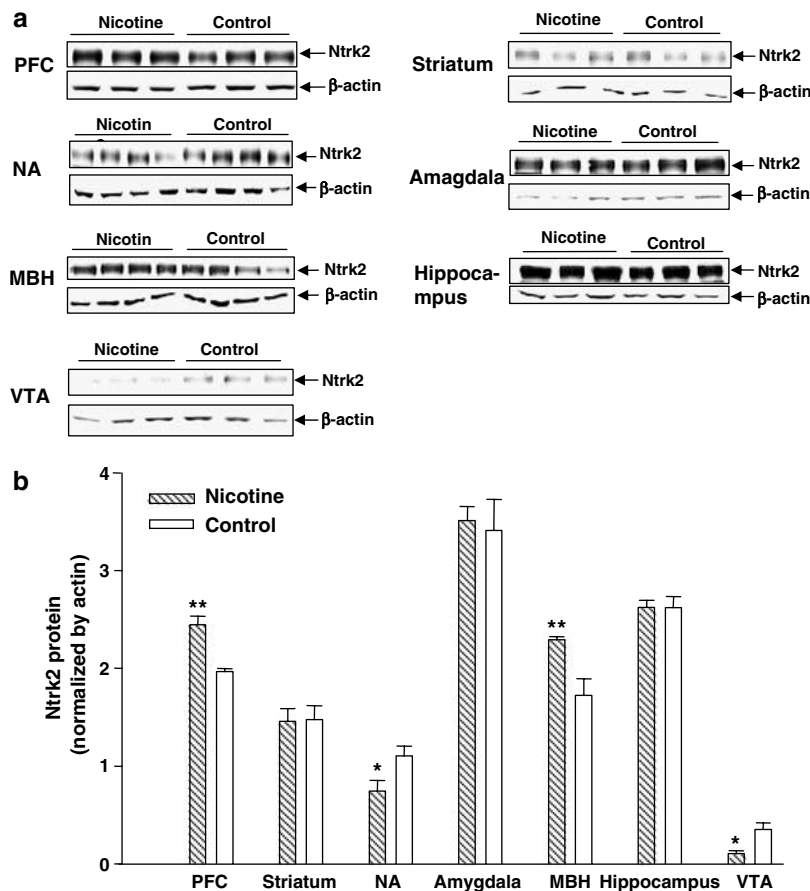


**Figure 3** Comparison of mRNA levels of *Ntrk2* between nicotine-treated and control groups in seven rat brain regions after seven days of nicotine administration, which showed that the mRNA level of *Ntrk2* was enhanced by nicotine in the striatum (86%) and PFC (38%) but decreased in the NA (–46%) and VTA (–49%). Values are the mean  $\pm$  SEM of 6 animals per group. \* $P < 0.05$ .

during 1999–2004, which revealed that these two genes are significantly associated with ND (Beuten *et al*, 2005a; Beuten *et al*, 2006). In the present study, we demonstrated that chronic nicotine treatment significantly modulated *Gpr51* expression in the amygdala, PFC, striatum, NA, and

MBH, and *Ntrk2* expression in the PFC, NA, MBH, and VTA. Furthermore, the mRNA patterns of the two genes in response to nicotine were consistent with the protein levels in most of these brain regions. The only difference noticed between mRNA and protein for *Gpr51* was in the MBH (no change in mRNA vs a 19% increase in protein) and that for *Ntrk2* in the MBH (no change in mRNA vs 33% increase in protein) and striatum (86% increase in mRNA vs no change in protein). This may be attributable to the regulation by nicotine at the translation level in these regions or to protein degradation after translation.

In recent years, growing experimental evidence has supported the hypothesis that activation of GABAergic transmission and *BDNF/NTRK2* is closely connected with the mesolimbic dopaminergic pathway during rewarding processes for drug abuse (Bowerly *et al*, 1987; Horger *et al*, 1999; Tapia-Arancibia *et al*, 2001; Cousins *et al*, 2002; Fadda *et al*, 2003; Narita *et al*, 2003; Tapia-Arancibia *et al*, 2004; Le Foll *et al*, 2005; Paterson *et al*, 2005). However, to date, there have been only a few studies reported on the regulation of *Gpr51* and *Ntrk2* expression by nicotine. For example, Amantea *et al* (2004) reported that G-protein coupling to GABA<sub>B</sub> receptors is significantly reduced in the PFC and NA of nicotine-treated rats, whereas GABA<sub>B</sub> receptor density and affinity are not altered. Another study found that



**Figure 4** Western blotting analysis for Ntrk2 expression in seven rat brain regions. (a) shows the results for Ntrk2 and  $\beta$ -actin in seven brain regions. (b) summarizes the statistical analysis of the Ntrk2 protein level normalized by the corresponding  $\beta$ -actin level, which indicated that nicotine enhanced Ntrk2 protein by 24 and 33% in the PFC and MBH, respectively, and reduced its expression by 33 and 70% in the NA and VTA, respectively. Values are the mean  $\pm$  SEM of 3–5 animals per group. \* $P < 0.05$ , \*\* $P < 0.01$ .

nicotine slightly decreased *Gpr51* mRNA in the rat PFC (Li et al, 2004), whereas we found herein that nicotine increased *Gpr51* expression in the PFC but decreased it in the NA. Such discrepancies in the PFC may result from different nicotine doses or treatment times or heterogeneity of the PFC region. In the present study, for example, nicotine was administered by osmotic minipump infusion in a daily dose of 3.15 mg/kg in saline for 7 days, whereas in the other two studies, nicotine was administered by subcutaneous injection (0.4 mg/kg for 14 days) (Amantea et al, 2004) or orally (3 mg/day for 4 weeks) (Li et al, 2004). Different doses of nicotine might lead to different degrees of activation of nAChRs on the GABA and DA neurons, which may impact the expression of GABA receptor and GABA release in these brain areas. Further, various treatment times could cause different extents of desensitization of nAChRs, which may subsequently produce different effects on the expression of *Gpr51*.

On the other hand, our finding that *Ntrk2* mRNA was enhanced by nicotine treatment in the striatum is consistent with the results reported by Maggio et al (1998), in which chronic nicotine treatment increased the expression of *Bdnf* in rat striatum. Conversely, another report showed that chronic nicotine administration, in a dose of 0.4 mg/kg through injection twice daily for 7 days, inhibited the

expression of *Bdnf* mRNA and protein in rat striatum (Yeom et al, 2005). Also, we found no alteration of *Ntrk2* expression in the hippocampus, which is contradictory to the report that chronic nicotine treatment by injection (0.5 mg/kg) twice daily for 7 days increased, and acute nicotine treatment decreased, the *Bdnf* mRNA level in rat hippocampus (French et al, 1999; Kenny et al, 2000). Such differences among studies are likely secondary to the different doses and times of nicotine treatment. Coincident with *Gpr51* in the present study, the expression of *Ntrk2* showed a region-specific pattern. On the basis of the function of *BDNF/NTRK2* pathway in the mesolimbic dopaminergic process, both interaction between BDNF and DA and network among various brain regions could lead to the different expression levels of *Ntrk2* in different regions.

In addition, we found that the expression pattern of *Gpr51* almost coincided with that of *Ntrk2*. Similarly, previous studies indicated the interactions between BDNF and GABAergic processes in cultured granule cells and rat or mouse visual cortex (Kubo et al, 1995; Lin et al, 1998; Huang et al, 1999; Rieff et al, 1999; Borghesani et al, 2002; Mizoguchi et al, 2003a). Further, both *Bdnf/Ntrk2* and *Gpr51* are involved in protein kinase C (PKC)-related intracellular signal transduction (Kaplan and Stephens,

1994; Zirrgiebel *et al*, 1995; Huang and Reichardt, 2001; Kubota *et al*, 2003). These findings imply that a common pathway coregulates the expression of *Gpr51* and *Bdnf*/*Ntrk2* and leads to some interactions between them during the dopaminergic process. The variation between the expression pattern of *Gpr51* and *Ntrk2* in the amygdala, striatum, and VTA may mean that the expression of *Gpr51* in the VTA and *Ntrk2* in the amygdala and striatum is not sufficient to be detectable by the techniques used in the present study.

In summary, our results demonstrated that chronic nicotine administration modulated the expression of *Gpr51* and *Ntrk2* in various rat brain regions, providing further support for our earlier observations that both *GPR51* and *NTRK2* are important players in the etiology of ND in human smokers. Therefore, these two genes represent critical candidates for future investigation on ND.

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