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ORIGINAL INVESTIGATION

Desensitization of δ-opioid receptors in nucleus accumbens during nicotine withdrawal

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Abstract
Rationale The synthesis and release of met-enkephalin and β-endorphin, endogenous ligands for δ-opioid peptide receptors (DOPrs), are altered following nicotine administration and may play a role in nicotine addiction.
Objectives To investigate the consequences of altered opioidergic activity on DOPr expression, coupling, and function in striatum during early nicotine withdrawal.
Methods Mice received nicotine-free base, 2 mg/kg, or saline, subcutaneously (s.c.), four times daily for 14 days and experiments performed at 24, 48, and 72 h after drug discontinuation. DOPr binding and mRNA were evaluated by [^{3}H]naltrindole autoradiography and in situ hybridization. DOPr coupling and function were investigated by agonist pCl-DPDPE-stimulated [^{35}S]GTPγS binding autoradiography and inhibition of adenylyl cyclase activity.
Results During nicotine withdrawal DOPr binding was unaltered in caudate/putamen (CPu) and nucleus accumbens (NAc) shell and core. Receptor mRNA was slightly increased in the shell at 72 h, but significant elevations were observed in prefrontal cortex and hippocampus. pCl-DPDPE-stimulated [^{35}S]GTPγS binding was attenuated in NAc, but not CPu. In the shell, binding was decreased by 48 h and remained decreased over 72 h; while in the core, significant reduction was seen at 72 h. Basal adenylyl cyclase activity was suppressed in striatum at 24 h, but recovered by 48 h. DOPr stimulation with pCl-DPDPE failed to inhibit adenylyl cyclase activity at 24 h and produced attenuated responses at 48 and 72 h.
Conclusions These observations suggest that DOPr coupling and function are impaired in the NAc during nicotine withdrawal. DOPr desensitization might be involved in the affective component of nicotine withdrawal.

Keywords Nicotine withdrawal · δ-Opioid receptors · Binding · mRNA · [^{35}S]GTPγS binding · Adenylyl cyclase · Desenstitization · Caudate/putamen · Nucleus accumbens

Introduction

Nicotine withdrawal in rodents induces somatic symptoms (Malin et al. 1992; Isola et al. 1999), disruption of reward systems (Epping-Jordan et al. 1998; Johnson et al. 2008), aversion (Suzuki et al. 1996a; Shram et al. 2008), depression (Zaniewska et al. 2010), and anxiety (Costall et al. 1989; Stoker et al. 2008). These behaviors model symptoms that have been proposed as contributing factors to tobacco smoking relapse in dependent humans. A growing body of experimental evidence supports the idea that the endogenous opioid system might play a role in the nicotine withdrawal syndrome. The nicotine withdrawal somatic symptoms resemble those of opiates (Malin et al. 1992; Isola et al. 1999), and opioid antagonists precipitate some of the somatic, motivational, and affective features of

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nicotine abstinence (Malin et al. 1993; Ise et al. 2000; Watkins et al. 2000). Studies with transgenic mice have provided clues for significant and distinct roles for the endogenous opioid system in nicotine addiction. Nicotine-rewarding effects and physical dependence are attenuated in mice lacking the preproenkephalin or μ-opioid receptor (MOPr) gene (Berrendero et al. 2002, 2005), while proopiomelanocortin gene disruption decreases the anxiogenic and rewarding effects of nicotine without affecting physical dependence (Trigo et al. 2009). In addition, lack of dynorphin increases the sensitivity to nicotine self-administration without affecting the somatic signs of nicotine withdrawal (Galeote et al. 2009). Consistent with the behavioral observations, nicotine alters the synthesis and release of opioid peptides, met-enkephalin, β-endorphin, and dynorphin in brain areas associated with addiction (Rosecrans et al. 1985; Marty et al. 1985; Pierzchala et al. 1987; Houdi et al. 1991, 1998; Dhatt et al. 1995; Boyadjieva and Sarkar 1997; Rasmussen 1998; Isola et al. 2000, 2002, 2008, 2009). In line with the animal data, human studies have provided corroborating evidence for the involvement of opioids in the behavioral pharmacology of nicotine (Pomerleau 1998 and references therein).

δ-Opioid peptide receptors (DOPrs) are highly expressed in neuronal circuits that control pain perception, motivation, affective behaviors, cognitive function, and locomotion (Bausch et al. 1995; Svingos et al. 1998, 1999; Cahill et al. 2001) and are thought to participate in the rewarding effects of drugs of abuse (Le Merrer et al. 2009). Met-enkephalin and β-endorphin are the endogenous ligands for DOPrs, with met-enkephalin having a tenfold higher affinity for DOPrs than MORrs and β-endorphin being equally active at μ- and δ-receptors (Corbett et al. 1993), and changes in their synthesis and/or release, as after nicotine, could influence DOPr signaling and associated behaviors. DOPr stimulation increases reward and decreases anxiety and depression-like behaviors (Shippenberg et al. 1987; Suzuki et al. 1996b; Broom et al. 2002; Saitoh et al. 2004; Vergura et al. 2008). Congruently, DOPr knockout mice exhibit an increase in anxiety and depression-like behaviors (Filliol et al. 2000), and the DOPr antagonist naltrindole is anxiogenic in rodents (Saitoh et al. 2005; Perrine et al. 2006). In humans, nicotine displays both rewarding and aversive properties (Foulds et al. 1997; Heishman and Hennigfield 2000), and its withdrawal syndrome is characterized by a constellation of behavioral symptoms, including anxiety, anhedonia, depression, anger, difficulty in concentrating, impatience, insomnia, and restlessness (Hughes 2007), some of which are regulated by DOPrs.

The role of DOPrs in the behavioral responses of nicotine is relatively unexplored (Ise et al. 2000; Balerio et al. 2005; Ismayilova and Shoaib 2010). We have shown that the synthesis of the DOPr endogenous ligand met-enkephalin is increased for a protracted time in the nucleus accumbens (NAc) of nicotine-dependent and nicotine-withdrawn mice (Isola et al. 2002), suggesting that the δ-opioidergic system might be involved in some aspects of nicotine addiction. These studies expand our previous work and explore the consequences of nicotine withdrawal on DOPr expression, coupling, and function in the caudate/putamen (CPu) and NAc. Agonist availability is central for DOPr regulation, and whether changes in endogenous ligand activity during nicotine withdrawal could alter DOPr regulation and the significance of this dysregulation, with regard to the neurochemical adaptive responses and the natural course of the behavioral syndrome, are important questions. DOPrs mediate their actions through coupling to G_i/G_o proteins and subsequent recruitment of downstream effectors, such as adenylyl cyclase and potassium and calcium channels (Waldhoer et al. 2004), and undergo desensitization, internalization, and downregulation or recycling following agonist stimulation (Varga et al. 2004). Accordingly, receptor binding autoradiography and in situ hybridization were employed to estimate possible changes in receptor density and mRNA, and receptor G-protein functional coupling was assessed by δ-agonist (pCl-DPDPE)-stimulated [^{35}S]GTPγS binding auroradiography and adenylyl cyclase responsiveness.

Materials and methods

Animals and treatments

Male Swiss–Webster mice (Harlan) 3 months old, 30–35 g, were used for the studies, which were approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. They were housed in our animal facility on a 12 h light:dark schedule, with free access to food and water, and were treated with nicotine according to a protocol that induces a somatic withdrawal syndrome over a 72 h abstinence period (Isola et al. 1999). Mice were injected four times daily (8 a.m., 12 p.m., 4 p.m., 8 p.m.) with nicotine-free base, 2 mg/kg, or saline, subcutaneously (s.c.), for 14 consecutive days. At various times during nicotine withdrawal (24, 48, and 72 h), they were decapitated, brains were removed, were either frozen whole or striatum–dissected, and were used immediately or stored at −70°C.

Procedures

DOPr binding autoradiography

Frozen whole brains were cut into coronal sections, 20 μm, and thaw-mounted onto gelatin-coated slides in an alternative

manner such as one slide to be used for total binding and the other, containing the adjacent sections, for nonspecific binding. After drying, slides were stored at −70°C until use. Binding autoradiography was performed as described by Kitchen et al. (1997). Slides were incubated for 30 min at 25°C in assay buffer (50 mM Tris–HCl, pH 7. 4, 5 mM $MgCl_2$, 1 mg/ml BSA) containing 100 mM NaCl to remove bound endogenous opioids and rinsed. DOPr binding was performed with [^3H]naltrindole, 0.4 nM, (33 Ci/mmol; Perkin-Elmer) at 25°C for 60 min, and nonspecific binding was determined in the presence of naloxone, 10 μM. Slides were exposed to X-ray film for 3–6 weeks at room temperature in parallel with tritium standards (American Radiochemicals). Brain structures of interest, CPu and NAc shell and core, were hand-traced using anatomical coordinates, bregma 1.70 mm–bregma 0.86 mm (Paxinos and Franklin 2001), and average gray values estimated and converted to units of radioactivity tissue equivalents by quantitative image analysis (MetaMorph).

Opioid receptor binding

DOPr and μ-opioid peptide receptor (MOPr) binding in striatal membranes was performed following established methods (Chan et al. 1995; Kelly et al. 1998). For DOPr labeling, 0.4 nM [^3H]naltrindole (Perkin-Elmer, 33 Ci/mmol) was used at 25°C for 90 min. While for MOPr labeling, 0.7 nM [^3H]DAMGO (Amersham Biosciences, 65 Ci/mmol) was used at 37°C for 60 min. Nonspecific binding was determined with naloxone, 10 μM. Reactions were terminated by rapid filtration through a cell harvester and thorough washing, and radioactivity on filters estimated by liquid scintillation.

DOPr in situ hybridization

Adjacent whole brain coronal sections, 12 μm, were thaw-mounted on SuperfrostR/Plus slides, dried, and stored at −70°C until use. Prior to hybridization, they were fixed in 4% paraformaldehyde followed by 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. [^{35}S]-labeled (Amersham Biosciences) sense and antisense riboprobes were prepared from an 800 bp cDNA fragment from the mouse DOPr (a gift from Dr. H. Akil, University of Michigan) and were incubated with brain sections for 22 h at 55°C in 50% formamide, 10% dextran sulfate, 1×Denhardt's solution, 0.3 M NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 M dithiothreitol, and 0.3 μg of tRNA. Sections were washed and treated with RNase A and exposed to X-ray film along with [^{14}C] standards (Amersham Biosciences). Brain structures of interest were hand-traced using anatomical coordinates (Paxinos and Franklin 2001): CPu and NAc shell and core (bregma 1.70 mm–bregma 0.86 mm); prefrontal cortex (Preuss 1995; bregma 2.96 mm–bregma 1.94 mm); hippocampus, CA1, CA2, CA3, and dentate gyrus (DG; bregma −1.46 mm–bregma −3.40 mm); and midbrain, substantia nigra pars compacta (SNC) and ventral tegmental area (VTA, bregma −3.40 mm–bregma −3.88 mm). Average gray values were estimated and converted to units of radioactivity tissue equivalents by quantitative imaging analysis (MetaMorph).

[^{35}S]GTPγS binding autoradiography

[^{35}S]GTPγS binding autoradiography was performed as described by Sim et al. (1995), with modifications based on studies by Happe et al. (2001). Frozen whole brains were cut into coronal sections, 20 μm, and thaw-mounted onto gelatin-coated slides in an alternative manner such as one slide to be used for basal [^{35}S]GTPγS binding and the other, containing the adjacent sections, for agonist-stimulated binding. After drying, slides were stored at −70°C until use. Slides were rinsed in assay buffer containing 50 mM glycylglycine, pH 7.4, 100 mM $NaCl_2$, and 1.0 mM EGTA and incubated in assay buffer containing 2 mM GDP for 30 min at 25°C. Then they were incubated at 25°C for 3 h in assay buffer containing [^{35}S]GTPγS, 0.1 nM (1,065 Ci/mmol; Amersham Biosciences) and 2 mM GDP in the presence or absence of the selective DOPr agonist pCl-DPDPE, 10 μM. In some studies, MOPr-stimulated [^{35}S]GTPγS binding was evaluated with DAMGO, 10 μM. Specific binding was demonstrated by the addition of 10 mM unlabeled GTPγS to the assay buffer. Sections were rinsed, dried, and exposed to X-ray film along with [^{14}C] standards (Amersham Biosciences) for 12–24 h. Brain structures of interest were hand-traced using anatomical coordinates: bregma 1.70 mm–bregma 0.86 mm for CPu, NAc shell, and NAc core and bregma 2.96 mm–bregma 1.94 mm for prefrontal cortex (Paxinos and Franklin 2001). Average gray values were estimated and converted to units of radioactivity tissue equivalents by quantitative image analysis (MetaMorph). Agonist-induced [^{35}S]GTPγS binding stimulation was determined by subtracting basal [^{35}S]GTPγS binding, estimated in the absence of agonist, from the stimulated [^{35}S]GTPγS binding, estimated in the presence of agonist.

Adenylyl cyclase activity

Adenylyl cyclase activity was measured as described by Izenwasser et al. (1993) with minor modifications. Striata (CPu and NAc) from a single mouse were homogenized in ice-cold buffer containing 20 mM Tris–HCl, 2 mM EGTA, 1 mM $MgCl_2$, 25 mM sucrose, pH 7.4, centrifuged, and the pellet resuspended in 2 mM Tris, 2 mM EGTA,

Table 1 δ-Opioid receptor density in the striatum during nicotine withdrawal

	[^3H]Naltrindole binding (nCi/g ± SEM)			
	Saline	Nicotine		
		24 h	48 h	72 h
Caudate/putamen	15±0.3	15±0.6	16±0.6	16±1.0
Nucleus accumbens				
Shell	17±0.5	17±0.5	18±0.4	17±1.0
Core	14±1.0	14±1.0	14±1.0	15±2.0

Animals were treated with nicotine 2 mg/kg, or saline, sc, four times daily as described in "Materials and methods" and euthanized at the indicated times. DOPr density in caudate/putamen and nucleus accumbens shell and core was evaluated by [^3H]naltrindole, 0.4 nM, autoradiography and analyzed by image analysis, as described in "Materials and methods". Estimated [^3H]naltrindole binding was not different in the saline-treated controls for the various nicotine groups, and values have been pooled for presentation purposes N=7–11 animals/group

pH 7.4. Membrane aliquots, 20–40 μg protein, were added to the assay buffer (80 mM Tris, 10 mM theophylline, 1 mM $MgSO_4$, 0.4 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 10 μM GTP, pH 7.4), incubated at 30°C for 5 min in

Table 2 δ-Opioid receptor mRNA during nicotine withdrawal

	DOPr mRNA (percent of control ± SEM)			
	Saline	Nicotine		
		24 h	48 h	72 h
Caudate/putamen	100±1	97±9	100±6	106±4
Nucleus accumbens				
Shell	100±5	115±11	108±5	123±5*
Core	100±1	100±10	98±6	103±4
Prefrontal cortex				
Superficial layers	100±2	122±5*	123±3*	123±3*
Deep layers	100±1	107±8	122±3*	120±3*
Hippocampus				
Dentate gyrus	100±1	124±5*	134±4*	143±7*
CA1	100±2	135±5*	141±7*	156±11*
CA2	100±1	150±9*	141±7*	162±10*
CA3	100±1	123±3*	127±6*	141±9*

Animals were treated with nicotine or saline, as described in Table 1, and euthanized at various times after drug discontinuation as indicated. DOPr mRNA in the brain regions of interest were evaluated by in situ hybridization as described in the "Materials and methods" section. Average gray values were estimated by image analysis and data presented as percent of the corresponding saline treatment (control). Estimated hybridization densities were not different in the saline-treated animals for the various nicotine groups, and values have been pooled for presentation purposes

*P<0.05 compared with respective saline-treated animals. N=4–5 animals/group

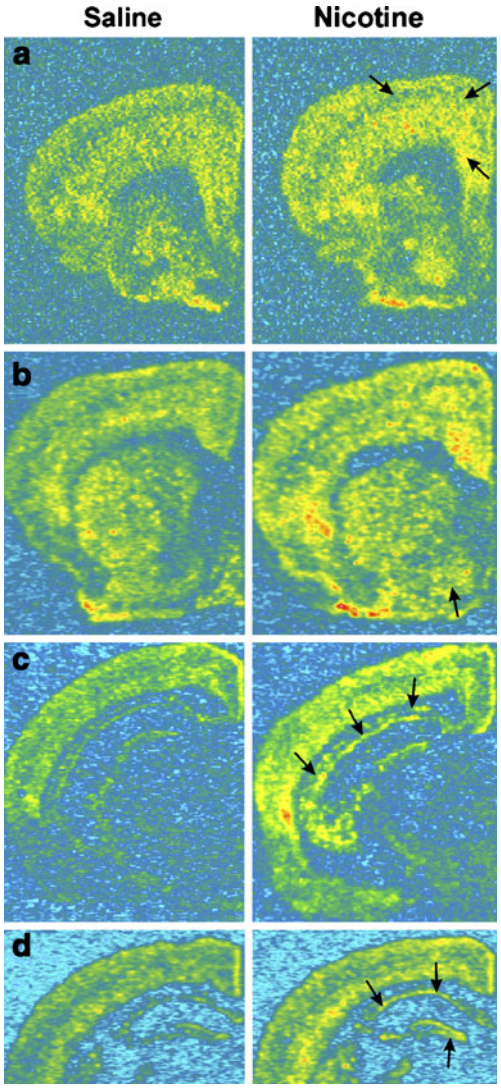

Fig. 1 DOPr mRNA in the brain of nicotine-withdrawn mice. Representative in situ hybridization images showing the levels of DOPr mRNA in saline-treated and nicotine-treated mice 72 h after treatment discontinuation. DOPr mRNA is elevated in prefrontal cortex (**a**); nucleus accumbens shell (**b**); and hippocampus CA1, CA2, and CA3 region and dentate gyrus (**c** and **d**), indicated by the *arrows*

the presence of pCl-DPDPE, 10 μM, or vehicle, and the reaction terminated by boiling for 2 min. The cAMP content was assayed by competitive [^3H]cAMP (36.3 Ci/mmol; Perkin-Elmer) binding to bovine adrenal cAMP binding protein.

Statistical analysis

For the statistical analysis of the data, one- or two-way analysis of variance (ANOVA) was used followed by a Tukey–Kramer's post hoc test for group comparisons. When appropriate, a Student's t test was employed for

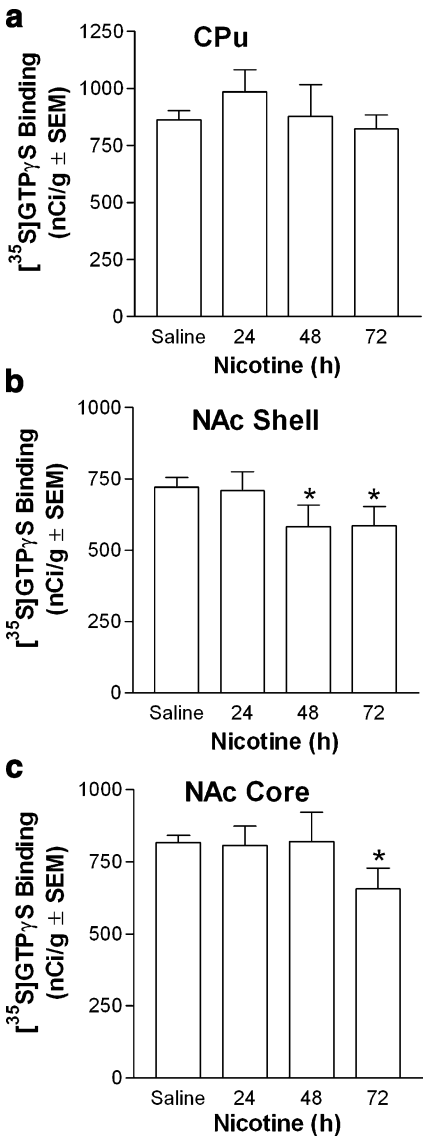

Fig. 2 DOPr agonist-stimulated [^{35}S]GTPγS binding in the striatum during nicotine withdrawal. Animals were treated as described in Table 1 and euthanized at the indicated times after treatment discontinuation. [^{35}S]GTPγS, 0.1 nM, binding was performed under basal (vehicle) and stimulated (pCl-DPDPE, 10 μM) conditions in the presence of 2 mM GDP, as described in the "Materials and methods" section. Average gray values in the caudate/putamen (CPu; **a**) and nucleus accumbens (NAc) shell (**b**) and core (**c**) were estimated by image analysis. Data are presented as the difference between pCl-DPDPE-stimulated, 10 μM, and corresponding basal [^{35}S]GTPγS binding for each treatment group. The calculated basal [^{35}S]GTPγS binding (nCi/g±SEM) is, CPu: Control 975±97, Nicotine 24 h 1,025±133, Nicotine 48 h 900±123, Nicotine 72 h 910±72; NAc shell: Control 922±101, Nicotine 24 h 922±127, Nicotine 48 h 769±83, Nicotine 72 h 825±72; NAc core: Control 936±96, Nicotine 24 h 955±134, Nicotine 48 h 860±79, Nicotine 72 h 848±79. N=7–9 animals/group. *P<0.05 compared with respective saline-treated animals

two group comparisons. Data expressed as percent of control were analyzed prior to their percent conversion. The Graph-Pad InStat software was used, and results were considered significant when $P \leq 0.05$.

Results

DOPr binding and mRNA

In initial studies, the number of DOPrs was evaluated with [^{3}H]naltrindole, 0.4 nM, binding in striatal membranes prepared from nicotine-withdrawn animals from 4 to 96 h. These studies revealed no appreciable changes in DOPr binding (data not shown). Likewise, Northern blots did not show any mRNA change in the striatum during the same time span (data not shown). To evaluate the possibility of anatomically discrete regulation of striatal DOPrs during nicotine withdrawal, receptor binding autoradiography and mRNA in situ hybridization were utilized. For these studies, three time points, 24, 48 and 72 h, during nicotine withdrawal were chosen. [^{3}H]Naltrindole binding density was similar to control values in CPu and NAc shell and core at all time points studied (Table 1). No changes in the receptor mRNA expression were observed in the CPu and NAc core during the duration of the studies (Table 2; Fig. 1b); however, there was a trend for a small rise in the NAc shell that reached significance at 72 h, about 24% over control, t test, P=0.0170 (Table 2; Fig. 1b). Notably, significantly increased DOPr mRNA expression was observed in the cortex, particularly, in the deep [F(3,10)=5.467, P=0.0174] and superficial layers [F(3,12)=7.219, P=0.0050] of prefrontal cortex and in the hippocampus, CA1 [F(3,160=4.846, P=0.0138], CA2 [F(3,16)=4.976, P=0.0126], CA3 [F(3,16)=4.990, P=0.0124], and DG [F(3,16)=7.655, P=0.0021], which was evident at 24 h and lasted over 72 h (Table 2; Fig. 1a, c, d). DOPr hybridization signal was low in VTA and SNC, and no measurable changes were observed during nicotine withdrawal (data not shown).

DOPr/G-protein coupling

DOPr/G-protein coupling was investigated by autoradiographic measurement of agonist-stimulated [^{35}S]GTPγS binding from control and nicotine-withdrawn mice at 24, 48, and 72 h. Basal [^{35}S]GTPγS binding in CPu and NAc shell and core was similar across the various treatment groups. ANOVA analysis showed no differences in basal [^{35}S]GTPγS binding in CPu [F(3,26)=0.3602, P=0.7822], NAc shell [F(3,27)=0.6853, P=0.5688], and NAc core [F(3,29)=0.8651, P=0.4703]. In control animals, activation of DOPrs with pCl-DPDPE resulted in enhanced [^{35}S]GTPγS binding in CPu and NAc shell and core, whose magnitude was about 116% over basal for CPu, 74% over basal for NAc shell, and 80% over basal for NAc core. In nicotine-withdrawn mice, the pCl-DPDPE-stimulated [^{35}S]GTPγS binding, expressed as stimulated [^{35}S]GTPγS binding over the corresponding basal, was not different in

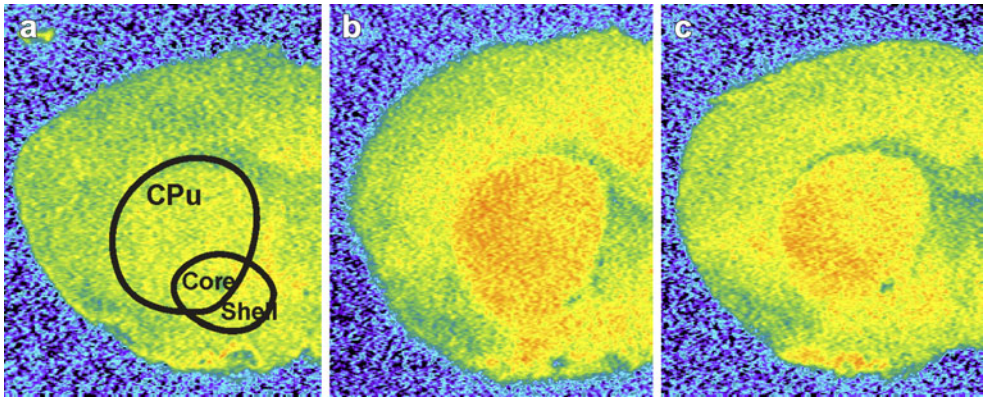

Fig. 3 Attenuated DOPr agonist-stimulated [^{35}S]GTPγS binding in the nucleus accumbens shell and core of nicotine-withdrawn mice. Animals were treated as described in Table 1, and [^{35}S]GTPγS binding estimated in caudate/putamen (CPu) and nucleus accumbens shell and core (**a**), as described in Fig. 2. Representative images shown basal (**a**) and pCl-DPDPE-stimulated, 10 μM, [^{35}S]GTPγS binding in saline-treated mice (**b**), as well as in nicotine-treated mice at 72 h (**c**) during nicotine withdrawal

the CPu from control at any time tested (Figs. 2a and 3c). In contrast, the nicotine-withdrawn animals displayed a reduced response to DOPr stimulation in NAc. In the shell, significant decreases in the ability of pCl-DPDPE to stimulate [^{35}S]GTPγS binding were observed at 48 and 72 h (Figs. 2b and 3c) and the observed binding stimulation accounted about 80% of the control response [$F(3,27)=2.990$, $P=0.0485$]. In the core, stimulated [^{35}S]GTPγS binding was reduced only at 72 h (Figs. 2c and 3c). Despite that DOPr mRNA was increased in the prefrontal cortex during nicotine withdrawal, the estimated pCl-DPDPE-stimulated [^{35}S]GTPγS binding was not statistically different [$F(2,9)=2.889$, $P=0.1074$] from that seen in saline-treated mice (Saline 998±66; Nicotine 24 h 1,041±101; Nicotine 72 h 858±40 nCi/g±SEM).

Table 3 δ-Opioid receptor-stimulated adenylyl cyclase activity in the striatum during nicotine withdrawal

	Adenylyl cyclase activity (cAMP pmol/mg prot/min ± SEM)	
Treatment	Basal	pCl-DPDPE
Saline	176±14	118±8*
Nicotine		
24 h	125±10*	127±9
48 h	169±7	139±6**,***
72 h	175±9	141±8**,***

Animals were treated with nicotine or saline as described in Table 1 and euthanized at the indicated times. Adenylyl cyclase activity was estimated in membranes prepared from striatum under basal, vehicle, and stimulated, pCl-DPDPE, 10 μM, conditions for 5 min, as described in the "Materials and methods" section. Basal adenylyl cyclase activity in the saline-treated controls for the various nicotine groups was not different, and values have been pooled for presentation purposes. $N=10$–12 animals/group

*$P<0.05$ compared with basal in saline-treated animals; **$P < 0.05$ compared with respective basal in nicotine-treated animals; ***$P < 0.05$ compared with pCl-DPDPE-stimulated in saline-treated animals

DOPr-mediated inhibition of adenylyl cyclase

Across all experiments, incubating membrane preparations of striatum from control animals with pCl-DPDPE decreased adenylyl cyclase activity by 25% below basal levels, as was expected (Table 3). Basal adenylyl cyclase activity was significantly reduced in the striatum of mice after 24 h of nicotine withdrawal, and the δ-agonist did not inhibit further the suppressed enzyme activity. By 48 and 72 h, basal enzyme activity had recovered to control levels, but stimulation with pCl-DPDPE only partially decreased enzyme activity (Table 3).

μ-Opioid receptors

Since enkephalins and endorphins are endogenous ligands for MOPrs as well, MOPr/G-protein coupling was evaluated in some studies. Basal [^{35}S]GTPγS binding was not different between saline- and nicotine-withdrawn mice in CPu [$F(3,11)=0.2.619$, $P=0.1033$], NAc shell [$F(3,11)=0.2019$, $P=0.8929$], and NAc core [$F(3,11)=0.6526$, $P=0.5978$]. Likewise, no difference was observed in the DAMGO-stimulated [^{35}S]GTPγS binding in CPu [$F(3,10)=0.3374$, $P=0.7988$], NAc shell [$F(3,10)=0.4888$, $P=0.6978$], and NAc core [$F(3,10)=0.4654$, $P=0.7128$] between saline- and nicotine-withdrawn animals at all times studied, 24–72 h (Fig. 4a). MOPr binding density, estimated in striatal membrane preparations by [^{3}H]DAMGO, 0.7 nM, was not altered at any time studied during nicotine withdrawal (Fig. 4b; [$F(3–26)=1.165$, $P=0.3419$]), and similar observations were made for the receptor mRNA, estimated by Northern blot in striatum (data not shown).

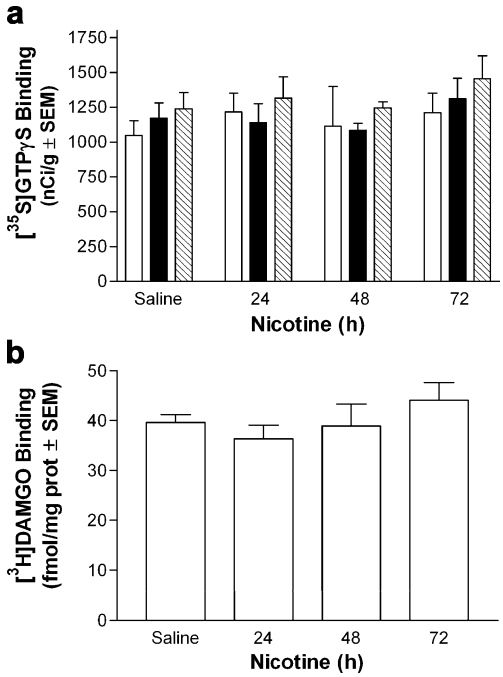

Fig. 4 MOPr agonist-stimulated [^{35}S]GTPγS binding and MOPr binding density in striatum during nicotine withdrawal. Animals were treated as described in Table 1 and euthanized at the indicated times after treatment discontinuation. **a** [^{35}S]GTPγS, 0.1 nM, binding was performed under basal (vehicle) and stimulated (DAMGO, 10 μM) conditions in the presence of 2 mM GDP, as described in the "Materials and methods" section. Average gray values in the caudate/putamen (*open bars*), nucleus accumbens shell (*solid bars*), and core (*hatched bars*) were estimated by quantitative image analysis. Data are presented as the difference between DAMGO-stimulated, 10 μM, and corresponding basal [^{35}S]GTPγS binding for each treatment group. The calculated basal [^{35}S]GTPγS binding (nCi/g±SEM) is, CPu: Control 1,267±101, Nicotine 24 h 1,125±111, Nicotine 48 h 1,469±145, Nicotine 72 h 1,174±80; NAc shell: Control 1,187±72, Nicotine 24 h 1,132±139, Nicotine 48 h 1,244±47, Nicotine 72 h 1,185±81; NAc core: Control 1,237±100, Nicotine 24 h 1,149±139, Nicotine 48 h 1,354±31, Nicotine 72 h 1,191±84. N=3–4 animals/group. **b** [^{3}H]DAMGO, 0.7 nM, binding in striatal membranes was performed as in the "Materials and methods" section. N=5–15 animals/group

Discussion

The data presented provide evidence that following chronic treatment, discontinuation of nicotine differentially impairs the coupling of DOPrs to G-proteins in striatum without changing receptor binding density. The DOPr agonist pCl-DPDPE-stimulated binding of [^{35}S]GTPγS was attenuated in the NAc shell and core, but not CPu, 48–72 h into nicotine withdrawal, suggesting decreased coupling to $G_{i/o}$ proteins and reduced number of active receptors. The concomitant diminution of the ability of pCl-DPDPE to inhibit adenylyl cyclase in striatal membranes provides further support for DOPr uncoupling during nicotine withdrawal. The attenuated pCl-DPDPE-stimulated [^{35}S]GTPγS binding is unlikely to be caused by a decrease in $G_{i/o}$ proteins, as basal binding was not different among saline- and nicotine-treated mice at the times studied, and we have not found appreciable changes in the levels of $G_{i1,2,3}$ and G_o proteins in the striatum 24–72 h during nicotine withdrawal (McCarthy et al. 2010). Loss of agonist activity, e.g., reduced [^{35}S]GTPγS binding, has been interpreted to indicate receptor desensitization at the G-protein level (Harrison and Traynor 2003). Desensitization of DOPrs occurs upon prolonged or repeated agonist exposure and has been attributed to receptor phosphorylation by G-protein receptor kinases, followed by interaction with β-arrestins, and internalization (Marie et al. 2006 for review). In our animal model, the content of met-enkephalin and its precursor peptide mRNA are augmented in NAc shell and core early and for over 72 h after nicotine discontinuation (Isola et al. 2002), implying a link between peptide change and DOPr desensitization. Notably, DOPr coupling was not altered in the prefrontal cortex, where met-enkephalin synthesis appears intact in our nicotine-withdrawn animals (Isola et al. 2002). Reports of altered synthesis and release of β-endorphin in the hypothalamus and striatum (Rosecrans et al. 1985; Boyadjieva and Sarkar 1997; Rasmussen 1998; personal observations) after chronic nicotine treatment and withdrawal, as well as in smokers (Wewers et al. 1994; del Arbol et al. 2000), raise the possibility of involvement in DOPr dysregulation.

Both membrane and autoradiography binding studies showed that the density of DOPrs was not altered in the striatum and its subregions following chronic nicotine administration and withdrawal. Thus, despite the DOPr desensitization in NAc shell and core, no receptor down-regulation was evident. Little is known about the trafficking of DOPrs in vivo (Wang et al. 2008 and references therein). It has been suggested that DOPrs are slowly recycled/degraded after agonist-induced internalization in vivo (Pradhan et al. 2009), and there is evidence in vitro that the post-endocytotic fate of the receptors is influenced by the type of agonist with the natural enkephalins promoting recycling and resensitization of receptor (Lecoq et al. 2004). Perhaps, receptor externalization is the preferable route following long-term pharmacological manipulation of the endogenous ligand in vivo. In striatal neurons, the majority of DOPrs is located in the cytosol (Wang and Pickel 2001; Wang et al. 2008 and discussion therein), and a compensatorily maintained intracellular receptor pool during prolonged agonist exposure might influence DOPr trafficking by facilitating the insertion of new receptors into the cell surface membrane. The observation that the receptor mRNA in NAc shell tended to rise over the time indicates that compensatory receptor protein synthesis may occur in order to maintain receptor number. This might be also true for brain regions with inputs to NAc, such as prefrontal cortex and hippocampus (Shirayama and Chaki 2006), where DOPr mRNA was upregulated. In NAc,

DOPrs are present on cholinergic and GABAergic intrinsic neurons, medium spiny neurons. as well as excitatory afferent neurons (Bausch et al. 1995; Svingos et al. 1998, 1999; Cahill et al. 2001), but our studies cannot distinguish the cell type of the desensitized receptors.

There is ample evidence that DOPrs contribute to the expression of the emotional responses of enkephalins (Filliol et al. 2000; Broom et al. 2002; Saitoh et al. 2004, 2005; Mas Nieto et al. 2005; Perrine et al. 2006; Vergura et al. 2008), whereas MOPrs participate in the enkephalins' regulation of basal hedonic tone (Hayward et al. 2002; Skoubis et al. 2005) and hedonic responses to drugs of abuse (Shippenberg et al. 2008; Berridge et al. 2009; Le Merrer et al. 2009). Investigations concerning the involvement of DOPrs in the behavioral pharmacology of nicotine are scarce but, overall, indicative of a DOPr contribution in various aspects of nicotine addiction. For example, DOPrs are involved in the anxiety-like behaviors of acute nicotine (Balerio et al. 2005), δ-agonists attenuate mecamylamine-precipitated nicotine-withdrawal aversion (Ise et al. 2000), and δ-antagonists have some effect on nicotine self-administration (Ismayilova and Shoaib 2010). Despite differences in nicotine treatment schedules, our studies and these in literature show a temporal overlap of DOPr desensitization with anhedonia (Epping-Jordan et al. 1998; Bevins and Besheer 2005; Johnson et al. 2008), anxiety (Costall et al. 1989; Stoker et al. 2008), and depression-like behavior (Zaniewska et al. 2010) in nicotine withdrawal, which could imply a link between negative affective symptoms and δ-signaling dysregulation in NAc. In contrast to DOPrs, the coupling of MOPrs to G-proteins appears to be normal in NAc, especially shell, in early nicotine withdrawal, suggesting that MOPrs might not be involved in the reward deficits associated with this stage of nicotine addiction. However, prevalent MOPr activity over DOPr may facilitate anxiogenic and depressive behaviors (Filliol et al. 2000). Taken together, a case can be made that the accumbal δ-enkephalinergic system might be involved in the emergence of the negative affect experienced in nicotine withdrawal (Markou et al. 1998). We propose that enhanced enkephalin synthesis in NAc (Isola et al. 2002) in response to diminished dopaminergic tone (Duchemin et al. 2009; Hadjiconstantinou et al. 2010; Angulo and McEwen 1994) leads to prolonged DOPr activation and desensitization with subsequent alteration of molecular mediators of affective modalities. That DOPr coupling was not changed in the prefrontal cortex of our animals, where met-enkephalin is unaltered in nicotine withdrawal (Isola et al. 2002), provides added support to the proposed met-enkephalin/DOPr nexus in NAc. Given that affect regulation is complex and arbitrated by multiple neuronal systems, it is likely that the accumbal δ-opioidergic system is only a part of the story (Barrot et al. 2002; Shirayama and Chaki 2006; Wallace et al. 2009).

In conclusion, during nicotine withdrawal, enhanced synthesis and release of enkephalins in the NAc might lead to desensitization of DOPrs. Diminished endogenous δ-opioidergic signaling in NAc can be viewed as disadvantageous by promoting the negative affective component of the nicotine addiction cycle and tobacco smoke continuation/relapse. Although, δ-agonists display antidepressant and anxiolytic effects and appear to be good candidates for nicotine withdrawal pharmacotherapy, they may have limited utility early into withdrawal because of desensitized receptors and possible exacerbation of receptor hypoactivity. Understanding the plasticity of the DOPr system during the various stages of nicotine addiction and identifying the brain regions and the neuronal populations involved will provide useful clues for the development of DOPr-based therapeutics for tobacco smoking cessation. In this regard, low-internalizing δ-agonists are an interesting class of drugs with translational potential.

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