

Effects of Repeated Nicotine Treatment on the Changes in Glutamate Receptor Subunits Levels in Mesocorticolimbic Dopamine Areas

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Recent studies suggest that alterations in glutamate receptor subunit levels in mesocorticolimbic dopamine areas could account for neural adaptations in response to psychostimulant drugs. Although many drugs of abuse induce changes in ionotropic glutamate receptor subunits in mesocorticolimbic dopamine areas, the changes of ionotropic glutamate receptor subunits by repeated nicotine treatment in these areas are not known. To answer this question, we injected male Sprague-Dawley rats twice daily with nicotine (0.4 mg/kg) or saline (1 ml/kg) for 10 days. The immunoreactivity of NR1, GluR1, and GluR2 glutamate receptor subunits was examined 16~18 h after the last injection of saline or nicotine. Repeated nicotine treatment significantly increased NR1 levels in the ventral tegmental area (VTA). In addition, repeated nicotine treatment showed a tendency towards an increase in GluR1 levels in the VTA as well as in striatum. However, there was no significant change in glutamate receptor subunits in other areas including nucleus accumbens (NAc). These results demonstrate that repeated nicotine treatment increases NR1 levels in VTA similarly to other drugs of abuse, suggesting that elevated glutamate receptor subunits in the VTA, but not NAc may be involved in the excitation of mesocorticolimbic dopamine neurons by nicotine.

Key Words: Addiction, Dopamine, Glutamate receptor, Nicotine, Synaptic plasticity, Ventral tegmental area

INTRODUCTION

Tobacco smoking is addictive and its use is the single most important preventable health risk in the developed world (Fagerstrom, 2002). Nicotine is known as a component of tobacco that induces habitual smoking (Nisell et al, 1995). Considerable evidence suggests that mesocorticolimbic dopamine (DA) system, which originates in the ventral tegmental area (VTA) and innervates the nucleus accumbens (NAc) and prefrontal cortex (Fallon & Moore, 1978), has been attributed to the positive reinforcement of many drugs of abuse, including nicotine. Nicotine increases the firing rate and burst firing of DA neurons in VTA (Grenhoff et al, 1986; Johnson et al, 1992; Nisell et al, 1996; Fisher et al, 1998), which, in turn, leads to increased release of DA in NAc (Johnson et al, 1992; Chergui et al, 1993). This increase in extracellular DA in the NAc is thought to mediate the positive reinforcement (Corrigall et al, 1992; Corrigall et al, 1994) and rewarding action of nicotine (Schilstrom et al, 1998b). Moreover, nicotine also acts at presynaptic $\alpha 7$ nicotinic acetylcholine receptors (nAChRs)

located on glutamatergic afferents to VTA to increase glutamate release (Schilstrom et al, 2000), thereby increasing DA release in NAc through NMDA receptors on dopamine neurons in VTA (Schilstrom et al, 1998a; Svensson et al, 1998; Fu et al, 2000).

Recently, in addition to changes in glutamate release in VTA, it has been suggested that many drugs of abuse induce changes in ionotropic glutamate receptor subunits in mesocorticolimbic dopamine areas. For example, repeated treatment of cocaine (Churchill et al, 1999), morphine (Fitzgerald et al, 1996) or ethanol (Ortiz et al, 1996) increases GluR1 levels, one of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits, in the VTA, which could contribute to increased sensitivity to the locomotor stimulating and rewarding actions of drugs of abuse (Carlezon et al, 1997). Although a single systemic injection does not change GluR1 expression in VTA (Ferrari et al, 2002), changes of ionotropic glutamate receptor subunits by repeated nicotine treatment in mesocorticolimbic dopamine areas have not been studied.

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ABBREVIATIONS: VTA, ventral tegmental area; DA, dopamine; NAc, nucleus accumbens; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; mPFC, medial prefrontal cortex; SN, substantia nigra.

To answer this question, we injected rats with nicotine for 10 days, and observed the changes of ionotropic glutamate receptor subunits in mesocorticolimbic dopamine areas.

METHODS

Animals and nicotine administration

Male Sprague-Dawley rats (initial weight 240~260 g, Samtaco, Osan, Korea) were received 4 days before experiment and were group housed 3 per cage under a 12 h light-dark cycle (light on at 6:00 A.M.). Food and water were available *ad libitum*. All experimental procedures performed on the animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). All rats received injections twice daily (at 10:00 A.M. and 16:00 P.M.) for 10 days with either nicotine [0.4 mg/kg, s.c., (-)-nicotine hydrogen tartarate salt, Sigma, St. Louis, MO] or 0.9 % saline (1 ml/kg, s.c.). (-)-Nicotine hydrogen tartrate salt was dissolved in 0.9% saline solution, and adjusted to pH 7.2~7.4 with 1 N NaOH. This dose of nicotine has been shown to enhance levels of extracellular dopamine in the nucleus accumbens and increase locomotor activity in response to subsequent injection of nicotine (Benwell & Balfour, 1992; Benwell et al, 1995).

Western blot analyses of glutamate receptor subunits

Rats were killed by decapitation 16~18 h after the last injection of nicotine or saline, as reported previously for cocaine (Fitzgerald et al, 1996; Boundy et al, 1998) or morphine (Lane-Ladd et al, 1997). Brains were placed in ice-cold artificial cerebrospinal fluid and 1.0 mm-thick coronal slices of brain were obtained with McIlwain tissue chopper (Brinkmann Instruments, Westbury, USA). The appropriate brain regions were dissected on an ice-cooled plate using a 12 or 15-gauge tissue punch according to the method of Wolf et al (1999). Bilateral tissue samples were homogenized in 1% sodium dodecyl sulfate (SDS) (Berhow et al, 1996; Fitzgerald et al, 1996) and frozen in aliquots at -80°C . Protein content was determined using Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, USA). Samples (15~30 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis with resolving gels containing 8% acrylamide and transferred electrophoretically to PVDF membranes (Bio-Rad Laboratories Inc., Hercules, USA). Proteins were probed for glutamate receptor subunit GluR1 (1:2000; Chemicon, Temecula, USA), GluR2 (1:2000; Chemicon, Temecula, USA) or NR1 (1:2000; Chemicon, Temecula, USA) separately for 48 h at 4°C . The specificity of these antibodies has been established (Manabe et al, 2000; Terashima et al, 2004; Priel et al, 2005) and these antibodies recognized specific bands at their predicted molecular weights. Proteins were detected using horseradish peroxidase-conjugated IgG (1:2000; Vector, Burlingame, USA) followed by chemiluminescence (Amersham Biosciences, Arlington Heights, USA) and exposed to Hyperfilm (Amersham Biosciences, Arlington Heights, USA). Equal loading and transfer of proteins were confirmed in every experiment by analyzing blots with amido black staining (Lane-Ladd et al, 1997).

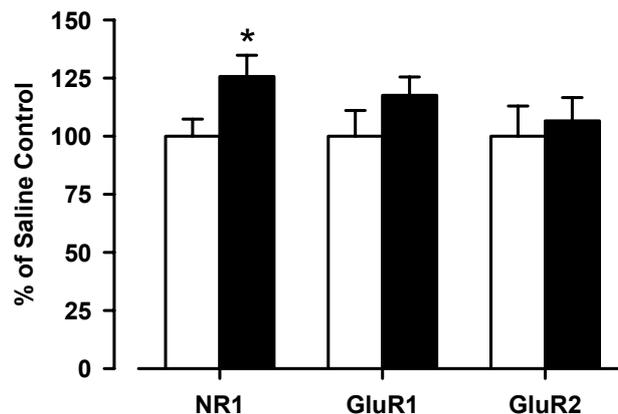


Fig. 1. Densitometric analysis of glutamate receptor subunit levels in ventral tegmental area (VTA) 10 days after repeated injection with saline (white bar) or nicotine (black bar). Data are mean \pm SEM percent change from values for repeated saline-treated control (abbreviated as saline control) for each immunoblot. Repeated nicotine-treated rats demonstrated a significant increase of NR1 ($p < 0.05$) compared to saline control ($n = 9$ per group). * $p < 0.05$ compared to saline control.



Fig. 2. Effect of repeated nicotine treatment on the levels of glutamate receptor subunit immunoreactivity in rat ventral tegmental area (VTA) and nucleus accumbens (NAc) as determined by Western blotting. -: repeated saline-treated control, +: repeated nicotine-treated group.

Data analysis

Levels of immunoreactivity were quantified by measuring the optical density of each band using computer-assisted densitometry (NIH Image analysis program, version 1.61). Values of each bands obtained in repeated saline-treated control rats (abbreviated as saline control) were averaged, and the remaining data were normalized as percentage of saline control. Data were combined across blots for statistical analysis. The percent values in the Western blots between saline control and repeated nicotine-treated rats were compared using a two-tailed unpaired Student's *t*-test with a significance set at $p < 0.05$.

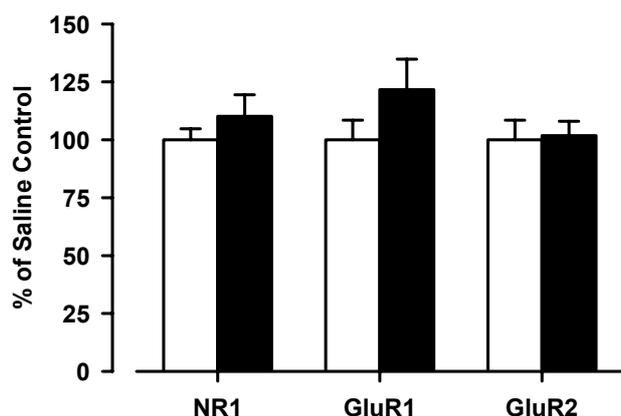
RESULTS

Effects of repeated nicotine treatment on glutamate receptor subunit changes in the VTA

Repeated nicotine treatment caused a significant increase in NR1 levels ($125.8 \pm 9.1\%$ of saline control) in the VTA, whereas the same treatment did not alter GluR2

Table 1. Effects of repeated nicotine treatment on percent change of glutamate receptor subunits in nucleus accumbens (NAc), medial prefrontal cortex (mPFC), and substantia nigra (SN). Data are mean \pm SEM percent change in optical density from saline control

	NR1		GluR1		GluR2	
	Saline	Nicotine	Saline	Nicotine	Saline	Nicotine
NAc	100.0 \pm 8.3	94.7 \pm 8.8	100.0 \pm 10.1	100.0 \pm 6.7	100.0 \pm 8.3	94.7 \pm 8.8
mPFC	100.0 \pm 6.3	96.4 \pm 6.6	100.0 \pm 10.6	106.1 \pm 9.8	100.0 \pm 9.5	106.4 \pm 5.7
SN	100.0 \pm 15.4	99.7 \pm 17.0	100.0 \pm 9.1	103.4 \pm 9.0	100.0 \pm 6.3	108.9 \pm 8.3

**Fig. 3.** Densitometric analysis of glutamate receptor subunit levels in striatum 10 days after repeated injection with saline (white bar) or nicotine (black bar). Data are mean \pm SEM percent change from values for repeated saline-treated control. There was a trend for an increase in GluR1 levels of striatum in repeated nicotine-treated rats (n=9 per group).

levels in the VTA. In addition, repeated nicotine treatment increased GluR1 levels in the VTA (117.6 \pm 7.9% of saline control), but this difference did not reach a statistical significance (Fig. 1, 2).

Effects of repeated nicotine treatment on glutamate receptor subunit changes in the NAc

Repeated nicotine treatment slightly decreased NR1 and GluR2 levels compared to saline control, but this effect was not statistically significant ($p > 0.05$). There was no significant change in GluR1 levels following repeated nicotine treatment compared with saline control (Fig. 2, Table 1).

Effects of repeated nicotine treatment on glutamate receptor subunit changes in the striatum

Repeated nicotine treatment caused an increase in GluR1 levels (121.6 \pm 13.2% of saline control) and NR1 levels (110.2 \pm 9.3% of saline control), respectively, but these effects were not statistically significant ($p > 0.05$). There was no change in GluR2 levels following repeated nicotine treatment compared with saline control (Fig. 3).

Effects of repeated nicotine treatment on glutamate receptor subunit changes in the medial prefrontal cortex (mPFC) and substantia nigra (SN)

Although there was a trend toward an increase in GluR1 levels (106.1 \pm 9.8% of saline control) and GluR2 levels (106.4 \pm 5.7% of saline control) in mPFC as well as GluR2 levels (108.9 \pm 8.3% of saline control) in the substantia nigra, this effect was not significant. Moreover, there was no significant effect of nicotine on NR1 levels in mPFC or SN (Table 1).

DISCUSSION

The present study demonstrates that repeated nicotine treatment significantly increased NR1 levels in VTA, which is similar to that reported after long-term treatments with ethanol (Ortiz et al, 1995) or cocaine (Fitzgerald et al, 1996; Churchill et al, 1999). Extensive pharmacological and physiological evidence shows the presence of NMDA and AMPA receptors in midbrain dopaminergic neurons (Overton & Clark, 1992; Chergui et al, 1993; Wang & French, 1993). The electrical activity of VTA DA neurons and the release of DA in the NAc are strongly regulated by the activation of these glutamate receptors on VTA DA neurons (Suaud-Chagny et al, 1992). The activation of NMDA or AMPA receptors increases firing of VTA DA neurons, but there is a difference in firing nature: NMDA receptor activation increases burst firing while AMPA receptor activation increases sustained firing. Apart from the sustained firing, the burst firing of VTA DA neurons are known to enhance the DA release in the NAc (Suaud-Chagny et al, 1992; Chergui et al, 1993). Furthermore, repetitive nicotine treatment increases burst firing of VTA neurons in a manner that is antagonized by NMDA receptor antagonists (Nisell et al, 1996). Interestingly, $\alpha 7$ nAChRs are located on presynaptic glutamatergic terminal and, when these receptors are activated by nicotine, glutamate release from presynaptic glutamatergic terminal in VTA is increased (Schilstrom et al, 1998a). Thus, activation of presynaptic $\alpha 7$ nAChR by nicotine enhances glutamate release, which in turn leads to an increased activation of NMDA and AMPA receptors on VTA DA neurons. This hypothesis is supported by the finding that decreased function of NMDA receptor by NR1 antisense oligonucleotide reduces the number of bursts and percentage of DA neurons exhibiting a bursting pattern (Tajiri et al, 2001). Considering these together, increased NR1 levels by repeated nicotine treatment in the present experiment may be one of the

mechanisms by which nicotine increases the excitatory synaptic transmission in the VTA. It would be likely that repeated nicotine treatments increase the sensitivity of the NMDA receptors to subsequent stimulation by nicotine, which may be accompanied by burst firing of DA neuron in VTA (Nisell et al, 1996) as well as an increase in DA release in NAc (Benwell & Balfour, 1992).

Although GluR1 increase in VTA following repeated nicotine treatment was not significant, a tendency toward an increase of GluR1 in VTA may further contribute to the excitation of mesocorticolimbic dopamine neurons (Grillner & Svensson, 2000). Similar to our findings, repeated treatment with ethanol, morphine or cocaine increases GluR1 levels in the VTA (Ortiz et al, 1995; Fitzgerald et al, 1996; Churchill et al, 1999). Elevated GluR1 levels in the VTA may increase the formation of GluR1-homomeric AMPA receptors which can enhance Ca^{2+} influx to glutamate stimulation (Carlezon et al, 1997; Neve et al, 1997). Thus, VTA DA neurons may be more sensitive to AMPA-receptor initiated Ca^{2+} influx by nicotine after repeated nicotine treatment. As with increased NR1 levels in VTA, AMPA receptor activation may contribute further to the increase in the excitability and function of VTA dopaminergic neurons. However, it was reported that chronic administration of nicotine, using osmotic minipump, lead to a decreased firing rate of dopamine neuron in VTA (Rasmussen & Czachura, 1995). The reasons for this different result are not clear, but may be related to the treatment dose, regimen, duration and the time after the last drug dose (Carlezon & Nestler, 2002). For example, sensitization of dopamine release to subsequent challenge dose of nicotine is only observed after daily nicotine injection, but not after constant subcutaneous infusion of nicotine via Alzet minipumps (Benwell et al, 1995). Interestingly, repeated intermittent, but not constant, administration of morphine induces an increase of GluR1 subunit in VTA (Fitzgerald et al, 1996), suggesting that intermittent injection of nicotine causes a different result compared with osmotic minipump injection. In addition to increase in the excitability of VTA DA neurons, elevated GluR1 in the VTA may contribute, in part, to the increased sensitivity to the locomotor stimulating and rewarding actions of nicotine, as suggested by previous studies (Carlezon et al, 1997; Carlezon & Nestler, 2002).

In contrast with the VTA, repeated nicotine treatment did not affect the glutamate receptor subunit levels in the NAc. Similarly, repeated treatment with cocaine (Churchill et al, 1999) or amphetamine (Lu et al, 1999) does not change glutamate receptor subunits levels in the NAc. Given the importance of NAc in expression of drug sensitization, it is surprising that repeated nicotine treatment did not change the subunits of glutamate receptor. However, accumulating evidence shows that nicotinic receptors within VTA, rather than the NAc, are of major importance for nicotine's stimulatory actions on behavior and dopamine release in NAc (Corrigall et al, 1994; Nisell et al, 1994a, b). Although previous studies have focused on the role of the mesocorticolimbic system as a mediator for nicotine addiction, relatively less is known about the role of the nigrostriatal DA systems. Recent study shows that stimulation of nigrostriatal DA neurons may be involved in the expression of behavioral sensitization by repeated nicotine treatment (Shim et al, 2001). For example, the magnitude of increase in dopamine release in striatum by local infusion of nicotine is very similar to that observed in NAc

(Shim et al, 2001). In the present study, repeated nicotine treatment showed only a tendency towards an increase in GluR1 levels in striatum, but not other glutamate receptor subunits. In line with this result, no change of MK-801 binding in striatum is observed in chronically nicotine-treated animals (Shoaib et al, 1997). It has been suggested that nicotine acts at $\alpha 7$ nAChRs on striatal glutamate terminals, and then it stimulates dopamine release in vitro (Kaiser & Wonnacott, 2000) and in vivo (Marchi et al, 2002). Moreover, perfusion of AMPA into the striatum causes a dose-dependent increase of dopamine release, which is prevented by AMPA antagonist, indicating that AMPA receptor may exert an excitatory influence on the release of dopamine in the striatum (Hernandez et al, 2003). These results raise the possibility that striatum may be involved in long-term changes following repeated nicotine treatment (Shim et al, 2001). However, there was no significant change in glutamate receptor subunits in medial prefrontal cortex and substantia nigra by repeated nicotine treatments. This result suggests that glutamate receptor subunits in PFC or SN may not be involved with long-term changes following nicotine treatment. However, we cannot exclude possible changes in glutamate receptor subunits after a different paradigm of nicotine injection.

Taken together, the present results demonstrate that repeated nicotine treatment increases NR1 levels in VTA similarly to other drugs of abuse, suggesting that elevated glutamate receptor subunits in the VTA, but not NAc may be involved the excitation of mesocorticolimbic dopamine neurons by nicotine. In addition, these results raise the possibility, although speculative, that elevated glutamatergic transmission in the VTA may be implicated in the increased sensitivity to the locomotor stimulating and rewarding actions of nicotine.

ACKNOWLEDGEMENT

This work was supported by grants from the MRC Research Center for Environmental Toxicogenomics and Proteomics (Korea Science and Engineering Foundations and Ministry of Science & Technology; R13-2003-016-02004-0) and the Korea Health 21 R&D Project (Ministry of Health & Welfare; 03-PJ10-PG13-GD01-0002).

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