Effects of Nicotine Pretreatment on Dopaminergic and Behavioral Responses to Conditioned Fear Stress in Rats: Dissociation of Biochemical and Behavioral Effects

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Background: We have examined the effects of nicotine pretreatment on dopaminergic and behavioral responses to conditioned fear stress in the rat.

Methods: Rats were pretreated daily with saline or nicotine for 20 days then challenged with nicotine or saline on day 21. Animals were trained in a classical conditioned fear paradigm. Dopamine utilization in the medial prefrontal cortex and nucleus accumbens shell and conditioned fear stress–induced immobility responses were assessed.

Results: Saline pretreated animals rapidly acquired the conditioned fear stress response as assessed by preferential activation of mesoprefrontal dopamine metabolism and tone-elicited immobility responses. Repeated, but not acute, nicotine pretreatment significantly reduced conditioned fear stress–induced dopamine metabolism in the medial prefrontal cortex and nucleus accumbens shell. Repeated nicotine pretreatment did not modify the acquisition or expression of conditioned fear stress responses, however.

Conclusions: The dissimilar effects of repeated nicotine exposure on the cortical dopamine and behavioral responses to conditioned fear stress suggest that nicotine differs from other agents with anxiolytic activity that produce coordinated changes in conditioned fear stress–induced cortical dopaminergic and behavioral responses. Furthermore, compared with results of acute footshock stress, repeated nicotine pretreatment appears to have differential effects on physical versus psychological stressors. Results are discussed within the clinical context of stress-related psychopathology syndromes and comorbid nicotine dependence.

Key Words: Nicotine, dopamine, prefrontal cortex, nucleus accumbens, conditioned fear stress, immobility response

Introduction

Nicotine dependence through tobacco smoking has a prevalence in the general population of ~25%. In psychiatric populations, smoking rates are much higher; patients with major depression and anxiety disorders have rates of nicotine dependence of 40 to 50%, whereas rates in alcohol and cocaine dependence (60–80%) and schizophrenic disorders (58–88%) are much higher (Dalack et al 1998; Hughes et al 1986; Ziedonis and George 1997). The high rates of comorbid nicotine dependence in psychiatric disorders may relate to 1) self-medication of dysfunctional affective and cognitive states, 2) enhanced rewarding properties of nicotine in patients with these disorders, 3) attenuation of side effects of psychotropic medications (e.g., neuroleptic-induced parkinsonism; Ziedonis and George 1997; George et al 1998), 4) a shared susceptibility for both disorders. As such, nicotine dependence through habitual tobacco use may attenuate symptoms in individuals with neuropsychiatric disorders, as has been documented in major depression, anxiety disorders, posttraumatic stress disorder (PTSD), Alzheimer’s disease, Parkinson’s disease, and Tourette’s syndrome (Jarvik 1991). Furthermore, nicotine appears to have stress-reducing effects in both animal models and in human smokers (Breslau 1995; Brioni et al 1993; George et al 1998).

Dopaminergic (DA) projections from the ventral tegmental area in the midbrain to the prefrontal cortex appear to have an important role in cognition (Goldman-Rakic and Selemon 1997; Knable and Weinberger 1997) and affective responses (Horger and Roth 1996). Dysfunction of mesoprefrontal DA pathways may be involved in neuropsychiatric diseases such as schizophrenia and affective disorders (Roth and Elsworth 1995). Mild stressors including acute footshock stress (George et al 1998, 2000) and conditioned fear stress (CFS; Goldstein et al 1998;
Morrow et al. (1997) produce preferential activation of mesoprefrontal DA neurons. This may relate to unique properties of this subset of DA neurons compared with nigrostriatal and mesoaccumbal DA neurons including a lack of (or reduced numbers of) terminal field impulse-modulating autoreceptors and complex afferent regulation by other neurotransmitter systems. Nicotinic acetylcholine receptors (nAChRs) may play a role in regulation of mesoprefrontal DA neurons because nicotine stimulates these neurons acutely and, when given repeatedly, also alters DA cell activity and neurotransmitter release and metabolism (George et al. 1998, 2000; Nisell et al. 1996; Vezina et al. 1992).

We have shown that repeated but not acute nicotine preexposure reduces the stress responsivity of mesoprefrontal DA neurons and associated stress-induced behavioral responses in rats subjected to acute inescapable footshock stress, a physical stressor (George et al. 2000). The effect of nicotine was found to be dose dependent, with maximal responses at low to moderate nicotine doses, and these effects were dependent on stimulation of mecamylamine-sensitive nAChRs (George et al. 1998) and activity of the endogenous opioid peptide system (George et al. 2000).

In our study, we tested the effects of nicotine pretreatments on dopaminergic and behavioral responses to CFS, a psychologic stressor resulting from the pairing of neutral (conditioned) and aversive (unconditioned) stimuli. Our results suggest that nicotine pretreatment has differential effects on mesocorticolumbic dopaminergic and behavioral responses to CFS, suggesting that nicotine dependence may produce clinical effects on biochemical and behavioral responses to stress through different mechanisms. These findings are discussed in the context of the observed high rates of nicotine dependence in patients with stress-exacerbated psychiatric disorders, such as major depression, schizophrenia, and PTSD.

Methods and Materials

Materials

Male Sprague–Dawley rats initially weighing 150 to 175 g were obtained from CAMM (Rutgers, NJ). The weights of rats at the conclusion of the experiments was 300 to 350 g. S-(-)-nicotine bitartarate was obtained from Sigma Chemical (St. Louis).

Treatment Paradigm

Rats were given daily, subcutaneous (SC) injections of saline (1 cc/kg) or nicotine bitartarate (0.15 mg/kg, expressed as the freebase) for 20 days, and then given a challenge injection with saline or nicotine on day 21. All injection solutions were freshly prepared daily, and the pH of the saline and nicotine solutions was adjusted to pH 7.4 with sodium hydroxide. Biochemical measures were obtained 0.5 hours after challenge injections.

Conditioned Fear Stress Procedure

The procedures for the CFS procedure have been described in detail elsewhere (Goldstein et al. 1994; Morrow et al. 1997). On day 19, rats were placed in sound-attenuated paired chambers (24 × 30 × 27 cm) with a stainless steel rod floor wired for footshock for ~0.5 hours (“habituation”) for 30 min. On day 20, after being given a saline or nicotine (0.15 mg/kg, SC) injection, rats receiving the conditioning procedure were placed into the chambers and given 10 tones (2.8 kHz, 5 sec duration) in combination with an incandescent red signal light (placed behind the chambers) paired with footshocks (0.8 mA 160 msec duration; Davis and Astrachan 1978) over a 30-min period using a pulse-shock generator (BR5/LVE Division of Tech Serv, Beltsville, MD) and a pulse stimulator (Grass Medical Instruments, Quincy, MA) connected to an IBM personal computer (the “acquisition” period). The intervals between the tones were randomly selected by the computer to be between 1 and 4 min. Unstressed control subjects were given tones paired with the signal light only. On day 21, after receiving challenge injections, both stressed and unstressed rats were reintroduced to the chamber and given 10 tones only paired with the signal light (the “expression” period).

Biochemical Analysis

Immediately after the conclusion of the “expression” period (0.5 hours after challenge injection), rats were sacrificed by decapitation. Samples of mPFC (+2.7–1.2 mm from bregma) were harvested by block dissection (1.5 mm thick), and samples of the shell portion of NAS (+1.2–0.2 mm from bregma) and dorso-lateral striatum (~0.2–1.2 mm from bregma) were obtained by tissue punch (Figure 1; Paxinos and Watson 1986) and were 2 mm in diameter and 1 mm in thickness. All tissue samples were stored frozen at −70°C until prepared for extraction.

Dopamine (DA) and its major metabolite, dihydroxyphenylacetic acid (DOPAC), were quantitated using high-performance liquid chromatography with electrochemical detection (HPLC-ED) with a glassy carbon electrode set at +0.7 V and an Ag/AgCl reference electrode. Tissue was sonicated before the extraction procedure, which involved alumina extraction before HPLC analysis as described previously (Elsworth et al. 1996). A reverse phase 3-µm C18 HPLC column (Ranin Instruments, Woburn, MA) was utilized. The mobile phase, delivered at 0.65 mL/min, was comprised of sodium citrate (30 mmol/L), sodium dihydrogen phosphate (14 mmol/L), sodium octanesulphonate (2.3 mmol/L), EDTA (0.025 mmol/L), acetonitrile (6.5%), tetrahydrofuran (0.6%), and diethylamine (0.1%), adjusted to pH 3.10 with concentrated phosphoric acid. Dihydroxybenzamine was used as an internal standard and used to calculate percentage recovery of DOPAC and DA. Results are expressed as the ratio of DOPAC to DA, with tissue levels calculated in ng/mg protein. Protein determination was performed using the method of Lowry et al. (1957) with bovine serum albumin as standard.
Behavioral Ratings

All footshock sessions were recorded by videotaping. The percentage of freezing (% immobility) behavior each minute after presentation of the 10 sequential tones (acquisition and expression) was scored by blinded examiners (TPG, CDV) who manually rated the taped sessions post hoc. There was excellent inter-rater reliability ($k = .80$) in the scoring of immobility (George et al 1998, 2000).

Statistics

SuperANOVA (Abacus Concepts, Berkeley, CA) was used for statistical analysis. For biochemical analyses, comparisons were performed using one- and three-factor analysis of variance (ANOVA) with pretreatment, challenge, and conditioned fear stress as the independent variables. For analysis of tone presentation effects on immobility responses, two-factor ANOVA with repeated measures (one within and one between-groups comparison) was used. Post hoc comparisons were performed using Fisher’s least significant difference procedure; differences were considered significant when $p < .05$.

Results

Effects of Nicotine Pretreatment on Conditioned Fear Stress–Induced Mesoprefrontal and NAS DA Utilization

mPFC. In mPFC (Figure 2, upper panel), there were significant effects of nicotine pretreatment [$F(1,43) = 17.88, p < .01$] and stress [$F(1,43) = 10.23, p < .01$], but not nicotine challenge [$F(1,43) = 1.04, p = .31$] on DA utilization and a nearly significant pretreatment $\times$ challenge $\times$ stress interaction [$F(1,43) = 3.38, p = .07$].

Acute nicotine augmented mPFC DA utilization ($p < .01$), whereas repeated nicotine pretreatment (0.15 mg/kg, SC) produced tolerance to saline or nicotine challenge (Figure 2, upper panel). Conditioned fear stress produced a significant increase in mPFC DA utilization ($p < .01$), and this was significantly reduced by repeated ($p < .05$), but not acute ($p = .56$) nicotine pretreatment. Furthermore, when the challenge injection after repeated nicotine administration was saline, the mesoprefrontal DA responses to footshock stress were also reduced ($p < .01$).

There were no significant differences in tissue DA levels between the various pretreatment groups (data not shown), suggesting that changes in the DOPAC/DA ratio were metabolite driven (i.e., enhanced formation of DOPAC). The 21-day saline or nicotine pretreatments...
used in our present study produced similar effects on mesoprefrontal DA utilization compared with our previously established pretreatment paradigm using 5-day saline or nicotine pretreatments (George et al 1998, 2000).

**SHELL SUBDIVISION OF NAS (NASsh).** In the NASsh (Figure 2, lower panel), there were significant effects of nicotine pretreatment \[F(1,43) = 33.83, p < .01\] and nicotine challenge \[F(1,43) = 10.49, p < .01\], but not stress \[F(1,43) = 1.33, p = .26\] on DA utilization, and a significant pretreatment \(\times\) challenge \(\times\) stress interaction \[F(1,43) = 5.33, p = .03\].

Acute nicotine challenge augmented NASsh DA utilization \((p < .01)\), whereas repeated nicotine pretreatment \((0.15 mg/kg, SC)\) produced tolerance to saline or nicotine challenge. Nicotine withdrawal for 24 hours \((NIC/SAL)\) under nonstress conditions led to a reduction in NASsh DA utilization compared with SAL/SAL control subjects \((p < .05)\). Stress produced a significant increase in NASsh DA utilization \((p < .01)\), and, similar to the mPFC, this was significantly reduced by repeated nicotine whether the challenge was with nicotine \((p < .01)\) or saline \((p < .01)\). Similar to the mPFC, acute nicotine pretreatment did not alter CSF-induced NASsh responses \((p = .41)\). There were no differences in tissue DA levels in NASsh among the various pretreatment groups.

There were no significant effects of nicotine pretreatments or CSF on DA utilization in the core subdivision of the NAS or the dorsolateral striatum (data not shown), consistent with our previous results with acute footshock stress (George et al 1998, 2000).

**Effects of Nicotine Pretreatments on Conditioned Fear Responses**

**ACQUISITION OF CFS (FIGURE 3, UPPER PANEL).** In unstressed rats, there were no differences in immobility responses between the treatment groups during tone presentation. Tones paired with footshock produced a rapid increase in immobility with a significant effect of tone–shock presentation \[F(9,225) = 23.10, p < .01\] and a tones \(\times\) pretreatment interaction \[F(27,225) = 1.58, p = .04\], but not nicotine pretreatment \[F(3,225) = 2.71, p = .07\]. Post hoc analysis revealed differences between saline pretreated control subjects \((SAL/SAL)\) and repeated nicotine pretreated animals at Tone 6 \((NIC/NIC; p < .05)\) and Tone 8 \((NIC/NIC and NIC/SAL; p < .05)\).

**EXPRESSION OF CFS (FIGURE 3, LOWER PANEL).** In unstressed rats, there were no differences in immobility between the treatment groups during tone presentation. Before the presentation of the first tone during the expression session, animals pretreated with nicotine versus saline and trained in the CFS procedure displayed a modest increase in freezing behavior (data not shown) and subsequently displayed robust immobility responses when the first tone was presented. This is consistent with data in mice suggesting that repeated nicotine administration enhances contextual, but not cued, fear conditioning when given during both CFS training and testing phases (Gould and Wehner 1999).

The presentation of tones alone produced a significant but modest effect on reduction of the expression of CFS-induced immobility responses \[F(9,216) = 2.74, p < .01\], and there was a reduction of tone-induced immobility with successive tone presentation, consistent with partial extinction of the conditioned fear response. There were no significant effects of nicotine pretreatment \[F(3,216) = 0.93, p = .44\] and no pretreatment \(\times\) tones interaction \[F(27,216) = 0.74, p = .82\]. There were few significant post hoc differences among the various pretreatment group across the series of tones presented. The NIC/NIC and NIC/SAL groups demonstrated a decrease in immobility.
responses compared with SAL/SAL control subjects at the Tone 6 presentation only (Figure 3, lower panel).

Discussion

The results of our study suggest that repeated nicotine pretreatment differentially affects mesocorticolimbic dopaminergic (DA) and behavioral responses to conditioned fear stress (CFS). Similar to our previous studies, repeated, but not acute, nicotine administration attenuated CSF-induced mesoprefrontal DA metabolism (George et al 1998). In contrast to our previous findings, the uncoupling of the effects of repeated nicotine administration on conditioned fear stress-induced cortical DA and immobility responses suggests that the situation with a conditioned fear stressor is more complex. Different neuroanatomic pathways appear to subserve immobility responses to acute footshock and conditioned fear stressors. Acute footshock (physical) stress appears to recruit nociceptive pathways and the involvement of endogenous opioid peptide systems (George et al 2000), whereas conditioned fear (psychologic) stress utilizes circuits involving the amygdala (i.e., basolateral and central nuclei), hippocampus, and medial prefrontal cortex (Armony and LeDoux 1997; Morgan and LeDoux 1995). Several studies have suggested that augmentation of mesocortical DA function is linked to conditioned fear immobility responses and that agents that reduce mPFC dopaminergic responses to CFS (i.e., diazepam and the NMDA/glycine site antagonist (+)-HA-966) also reduce CFS immobility responses (i.e., block the acquisition of or enhance the extinction of immobility responses (Goldstein et al 1994; Ida et al 1989; Morrow et al 1997, 1999b; Yoshioka et al 1996). This coordinated effect of putative anxiolytic agents on dopaminergic and behavioral responses to CFS is not seen in the present experiments involving nicotine pretreatment. Our findings are consistent with those of Gould and Wehner (1999), who observed that repeated nicotine pretreatment (during both training and testing phases of the fear conditioning procedure) did not alter the expression of conditioned fear responses in C57BL/6 mice at the nicotine doses used in our study. This group also found that nicotine pretreatment (0.1–0.3 mg/kg) during training and testing phases of the CFS procedure enhanced contextual fear conditioning, which we observed to be elevated during the testing (expression phase) before tone presentation. Contextual fear conditioning is known to be dependent on hippocampal function, whereas cued conditioned fear responses are thought to be mediated through the amygdala (Phillips and LeDoux 1992). This is consistent with the differential effects of repeated nicotine administration on contextual versus cued conditioned fear responses observed in the study of Gould and Wehner (1999) and in our study. In addition, we used a higher footshock intensity (0.8 mA) compared with previous conditioned fear studies in this laboratory (0.3–0.4 mA; Goldstein et al 1994; Morrow et al 1999a) to produce more consistent activation of the mesoprefrontal DA system (George et al 1998, 2000). Thus, the degree of extinction of immobility responses achieved with presentation of the 10 tones during the 30 minute expression phase (20–30%) in our present study was less than in our previous studies (40–60%).

The finding that repeated nicotine modulates stress-induced DA metabolism in both the medial prefrontal cortex and the shell subdivision of the nucleus accumbens, both of which are A10 DA neuron-innervated terminal fields with preferential responsiveness to mild stressors, suggests that these previously observed findings in the mesoprefrontal DA projection extend to the shell portion of the NAS (Morrow et al 1999). In contrast, there were no effects of repeated nicotine or CFS on DA utilization in the core subdivision of NAS or dorsolateral striatum, suggesting that A9 DA neurons are not involved in this process. There is both physical (Clarke and Pert 1985) and functional (George et al 1998, 2000; Nisell et al 1996; Vezina et al 1992) evidence for the presence of nicotinic acetylcholine receptors (nAChRs) on both mesolimbic and mesoprefrontal DA neurons, and the similar effects of nicotine of both mesocortical and shell subdivision mesoaccumbal DA pathways probably relates to common afferent regulation of these DA systems by nicotinic cholinergic systems, either directly or indirectly.

CFS may be phenomenologically similar to some of the psychopathologic sequelae of PTSD (Yehuda and Antelmann 1993). There are few studies that have characterized the prevalence rates of nicotine dependence in PTSD or the modulation of stress-exacerbated symptomatology in this disorder. One study found that Vietnam combat veterans with PTSD have rates of cigarette smoking similar to those of veterans without PTSD (53 vs. 45%), but that PTSD veterans were heavier smokers and that heavy smoking was correlated with avoidance and numbing and with hyperarousal symptoms (Beckham et al 1997). These symptoms may resemble features of behavioral responses seen with CFS. It is notable that in the fear-potentiated startle paradigm, which resembles CFS (Davis 1992), nicotine reduced fear-potentiated startle responses without affecting baseline startle. Studies by several groups (Jarvik et al 1989; Pomerleau and Pomerleau 1987; Rose et al 1983; Schachter 1978; Warburton 1992) have suggested that cigarette smoking increases during periods of stress, especially with respect to psychologic stressors, and smoking severity is known to be robustly linked to the number of life stressors in childhood (Anda et al 1999). Nonetheless, a controlled demonstra-
tion that psychologic stress increases nicotine use has not been reported (Pomerleau and Pomerleau 1991). The issue of whether nicotine exerts anxiolytic effects or produces withdrawal attenuation is still unresolved (Parrott 1995; Silverstein 1982) and requires further study in nicotine-naive human subjects.

Taken together, our findings with respect to a lack of an effect of repeated nicotine administration (presumably similar to habitual smoking) in modulating behavioral responses to CFS, but reducing cortical DA responses, suggests a complex relationship between psychologic stress and the effects of repeated nicotine administration. It is possible that 1) the CFS-reducing effects of repeated nicotine pretreatment correlate with blunted cortical DA responses, but not behavior responses; 2) the stress-reducing effects of repeated nicotine are not sensitively measured by conditioned fear immobility; and 3) conditioned fear immobility responses are not closely linked to stress-induced evoked changes cortical DA function. Our previous work has demonstrated that the repeated effects of nicotine on acute footshock (physical) stress are mediated by endogenous opioid peptide systems (George et al 2000), and thus behavioral responses to conditioned fear stress (a psychological stressor) may not be mediated by these systems. Furthermore, the relationship between nicotine dependence and stress-responsive psychiatric disorders such as PTSD, depression, and schizophrenia, as well as the effects of nicotine dependence on the course of these disorders, requires further evaluation.

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