Nicotine and Ethanol Activate Protein Kinase A Synergistically via G\textsubscript{i} \(\beta\gamma\) Subunits in Nucleus Accumbens/Ventral Tegmental Cocultures: The Role of Dopamine D\textsubscript{1}/D\textsubscript{2} and Adenosine A\textsubscript{2A} Receptors

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ABSTRACT

Tobacco and alcohol are the most commonly used drugs of abuse and show the most serious comorbidity. The mesolimbic dopamine system contributes significantly to nicotine and ethanol reinforcement, but the underlying cellular signaling mechanisms are poorly understood. Nicotinic acetylcholine (nACh) receptors are highly expressed on ventral tegmental area (VTA) dopamine neurons, with relatively low expression in nucleus accumbens (NAcb) neurons. Because dopamine receptors D\textsubscript{1} and D\textsubscript{2} are highly expressed on NAcb neurons, nicotine could influence NAcb neurons indirectly by activating VTA neurons to release dopamine in the NAcb. To investigate this possibility in vitro, we established primary cultures containing neurons from VTA or NAcb separately or in cocultures. Nicotine increased cAMP response element-mediated gene expression only in cocultures; this increase was blocked by nACh or dopamine D\textsubscript{1} or D\textsubscript{2} receptor antagonists. Furthermore, subthreshold concentrations of nicotine with ethanol increased gene expression in cocultures, and this increase was blocked by nACh, D\textsubscript{2} or adenosine A\textsubscript{2A} receptor antagonists, G\beta\gamma or protein kinase A (PKA) inhibitors, and adenosine deaminase. These results suggest that nicotine activated VTA neurons, causing the release of dopamine, which in turn stimulated both D\textsubscript{1} and D\textsubscript{2} receptors on NAcb neurons. In addition, subthreshold concentrations of nicotine and ethanol in combination also activated NAcb neurons through synergy between D\textsubscript{2} and A\textsubscript{2A} receptors. These data provide a novel cellular mechanism, involving G\beta\gamma subunits, A\textsubscript{2A} receptors, and PKA, whereby combined use of tobacco and alcohol could enhance the reinforcing effect in humans as well as facilitate long-term neuroadaptations, increasing the risk for developing coaddiction.

Alcoholism and nicotine addiction are significant public health problems (see Dani and Harris, 2005). The vast ma-

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; A\textsubscript{2A}R, adenosine 2A receptor; MSN, medium spiny neuron; D\textsubscript{1}R, dopamine D\textsubscript{1} receptor; D\textsubscript{2}R, dopamine D\textsubscript{2} receptor; GAD, glutamic acid decarboxylase; NAcb, nucleus accumbens; CRE, cAMP-response element; PBS, phosphate-buffered saline; PKA, protein kinase A; SCH23390, R-(++)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; eticlopride, S-(++)-3-chloro-5-ethyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxy- benzamide hydrochloride; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide dihydrochloride; MSX-3, 3,7-dihydro-3-[1(1E)-2-[3-methoxyphenyl]ethenyl]-7-methyl-3-[3-(phosphonoxy)]-5-propyl-1-[2-propynyl]}-1H-purine-2,6-dione disodium salt hydrate; FITC, fluorescein isothiocyanate; TH, tyrosine hydroxylase; VTA, ventral tegmental area; Luc, luciferase; βARK, β-adrenergic receptor kinase.
and smoking (Miller and Gold, 1998), the cellular molecular mechanisms that underlie simultaneous addiction to ethanol and nicotine remain unclear.

Here we focus on the mesolimbic system because of its central role in the regulation of reward, motivation, and addiction (see Wise, 2004). Cell bodies of dopaminergic neurons originate in the VTA and substantia nigra and project to forebrain structures such as the striatum, including the nucleus accumbens (NAcb). Acute exposure to addictive substances such as nicotine and ethanol increases extracellular dopamine in the NAcb, and dopamine seems to mediate some of the reinforcing actions of these drugs (see Wise, 2004), including nicotine (Balfour et al., 2000) and ethanol (Hodge et al., 1997; Weiss and Porrino, 2002). The reinforcing actions of nicotine are probably mediated in part by the VTA, because activation of nAChRs on dopaminergic neurons in the VTA enhances their firing rate and causes dopamine release from nerve terminals in the NAcb/striatum (see Wonnacott et al., 2005). Furthermore, behavioral studies suggest that nAChRs on VTA neurons are necessary for the reinforcing effects of nicotine (Corrigall et al., 1994) and ethanol (Ericson et al., 1998). In addition, nAChRs are strongly expressed in VTA neurons and their axon terminals, but NAcb/striatal medium spiny GABAergic neurons (MSNs) express relatively few postsynaptic nAChRs (Pakkanen et al., 2005).

Here, we used primary neuronal cultures prepared from the VTA/ventral midbrain (hereafter called VTA) and NAcb/striatum (hereafter called NAcb) to identify signaling events underlying synergistic interactions between nicotine and ethanol. Our studies suggest that nicotine binding to VTA neurons enhances the release of dopamine, which in turn activates dopamine receptors on NAcb neurons. Activation of NAcb dopamine receptors induces CRE-mediated gene expression hours later. It is noteworthy that subthreshold concentrations of nicotine and ethanol were ineffective when applied separately in NAcbVTA cocultures, but coinapplication of nicotine and ethanol to cocultures enhanced gene expression synergistically via dopamine and adenosine A2A receptors (A2AR) on NAcb neurons.

**Materials and Methods**

**Materials.** Reagents were purchased from Sigma-Aldrich (St. Louis, MO) except where indicated, including R-(+)-7-chloro-8-hydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH2390), S-(−)-3-chloro-5-ethyl-N-(1-ethyl-2-pyrrolidinyl)methyl-6-hydroxy-2-methoxy-benzamide hydrochloride (etioclone), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), and 3,5-dihydroxy-8-[1(E)-2-(3-methoxyphenyl)ethenyl]-7-methyl-3-[3-(phosphonyoxy)yl]-5-propyl-1-(2-propynyl)-1H-purine-2,6-dione disodium salt hydrate (MSX-3). Neurobasal medium, B-27, GlutaMAX-I supplement, and Hank’s balanced salt solution were from Invitrogen (Carlsbad, CA); papain was from Worthington Biochemicals (Freehold, NJ); Hibernate E was from Brain Bits LCC (Springfield, IL); polyclonal rabbit anti-glutamic acid decarboxylase (GAD) and tyrosine hydroxylase (TH) antibodies were from Chemicon International (Temecula, CA); and FITC- or Texas Red-conjugated anti-rabbit or anti-mouse secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); Luciferase assay system was from Promega (Madison, WI); β-galactosidase assay system was from Stratagene (La Jolla, CA).

**Primary Neuronal Cultures.** Neuronal cultures were prepared according to Yao et al. (2005), with some modifications. Pregnant Sprague-Dawley rats with 17-day-old embryos were anesthetized with CO2. Two coronal brain slices containing either the NAcb or the VTA were made. NAcb and VTA were dissected with tweezers according to Alvarez-Bolado and Swanson (1995) and transferred to dissection buffer. After mincing into small pieces, tissues were digested by papain (20 U/ml) for 30 min at 37°C. Proteolytic papain activity was then stopped by adding 0.5 mg/ml trypsin inhibitor solution (type I-S; Sigma-Aldrich). Single-cell suspensions were made by gentle trituration through edge-narrowed Pasteur pipettes in Hibernate E containing 1× B-27. After centrifugation at 1000 rpm for 5 min, cell pellets were suspended in neurobasal medium containing 1× B-27 and 0.5 mM GlutaMAX-I supplement. Cells (16 × 10^4 or 7 × 10^4) were plated on 24-well plates or 8-well slides precoated with poly-d-lysine and laminin and incubated at 37°C with 5% CO2/95% air. Half of the medium was changed every 4 days. Neuronal cultures were used for experiments 13 days after plating on day 0. All procedures were performed with protocols approved by the Gallo Center Institutional Animal Care and Use Committee and the Institute of Laboratory Animal Resources (1996).

**Immunocytochemistry.** Primary neurons were fixed for 15 min in 4% formaldehyde containing 120 mM sucrose. Fixed cells were rinsed with phosphate-buffered saline (PBS) and preincubated with blocking buffer (5% normal donkey serum in PBS) followed by incubation with primary antibodies specific for GAD (1:100) or TH (1:300). Cells were rinsed with PBS, incubated with FITC- or Texas Red-conjugated anti-rabbit or anti-mouse secondary antibody (diluted at 1:200), rinsed, and coverslipped. No staining was evident when primary antibodies were preincubated with excess peptide antigen or in the absence of FITC- or Texas Red-conjugated anti-rabbit or anti-mouse secondary antibody. Antibody specificity was confirmed by Western blots; no bands were detected after the primary antibodies were preabsorbed with antigen (data not shown).

**Confocal Microscopy.** Images were obtained as a single plane near the center of the cell with Zeiss 510 laser scanning confocal microscope (Yao et al., 2005) and processed using Adobe Photoshop software.

**Viral Vectors.** HSVLacZ/CRE-Luc was prepared and transfected into neurons as described by Yao et al. (2003). Construction and production of recombinant Ad5βARK1 and Ad5LacZ vectors were as described in Yao et al. (2002).

**CRE-Luciferase Reporter Assay.** CRE-mediated luciferase was assayed as a functional marker of cAMP/PKA signaling. Primary VTA and/or NAcb neurons were plated at a total of 16 × 10^4 cells per well of 24-well plates and grown for 12 days in neurobasal medium supplemented with 1× B-27 and GlutaMAX. Cells were then infected overnight with HSVLacZ/CRE-Luc at 1 multiplicity of infection in neurobasal-only medium. Cells were preincubated with dopamine transporter inhibitor nomifensine (10 μM) for 20 min and then treated with drugs for 10 min in the presence of nomifensine, washed, and cultured for an additional 4 h before the luciferase assay. Nomifensine inhibition of transporter activity was used to enhance the otherwise low endogenous dopamine signaling (Murray and Gillies, 1993) and the ability of nicotine to elevate free dopamine levels. Inhibition of dopamine uptake was required to demonstrate a receptor response to extracellular dopamine in cell cultures, because 10 μM nicotine alone had no effect on CRE-luciferase activation in the absence of nomifensine (data not shown). In addition, nomifensine alone decreased luciferase activity by ~30% relative to controls (data not shown). Nevertheless, CRE-mediated gene activation by high concentrations of nicotine or subthreshold nicotine and ethanol in combination was blocked by dopamine and/or adenosine receptor antagonists. Thus, our studies reliably determined the receptor and signaling mechanisms through which nicotine and ethanol interact to increase gene expression in cultured neurons. Luciferase activity was normalized to nomifensine control levels and expressed as relative luciferase units.

**Statistical Analysis.** All values were expressed as the mean ± S.E.M. Data were analyzed by one-way analyses of variance, fol-
owed by the Dunnett’s test. A $t$ test was used when a single comparison between two means was required. The minimal level of significance accepted was set at $p < 0.05$. All data represent the mean ± S.E.M. ($n = 3$) and are representative of at least three experiments.

**Results**

**Histochemical Characterization of VTA and NAcb Cultures and Cocultures.** Primary neuronal cultures containing VTA alone, NAcb alone, and NAcb/VTA in coculture were prepared as described under Materials and Methods. Figure 1 shows that more than 90% of the neurons in NAcb cultures were GABAergic MSNs, indicated by GAD immunoreactivity (Fig. 1) (also see Yao et al., 2005). VTA cultures consisted primarily of dopaminergic neurons, indicated by TH immunoreactivity, and GABAergic neurons, indicated by GAD immunoreactivity (Fig. 1).

Nicotine Induces CRE-Mediated Gene Expression in NAcb Neurons Only If Cocultured with VTA Neurons. Cultured neurons were infected with an herpes simplex virus-packaged LacZ/CRE-Luc construct to examine nicotine- and/or ethanol-induced activation of cAMP-dependent gene expression, which was taken to be an indirect indicator of neuronal activity. To determine whether CRE-mediated gene transcription was cell context-dependent, we assayed nicotine- and/or ethanol-induced CRE-luciferase activity 4 h after drug exposure (Asher et al., 2002; Yao et al., 2002) in cultures containing VTA neurons alone, NAcb neurons alone, or both VTA and NAcb neurons in coculture. Treatment with 1 or 10 μM nicotine for 10 min was without effect in cultures containing either VTA or NAcb neurons alone (Fig. 2A and B). By contrast, in combined NAcb/VTA cocultures, 10 μM nicotine for 10 min significantly enhanced luciferase activity (Fig. 2C), suggesting that both neuronal cell types were required. Shorter exposure (2 min) to 10 μM nicotine was not sufficient to induce luciferase activity in NAcb/VTA cocultures, and nicotine treatment for 30 min produced a similar increase in CRE-mediated gene expression as a 10-min exposure (Fig. 2D). Therefore, a 10-min time point was used in all subsequent experiments with nicotine. In addition, dopamine transporter inhibition with nomifensine (10 μM) was used to enhance the otherwise low endogenous dopamine signaling (Murray and Gillies, 1993) and the ability of nicotine to elevate free dopamine levels, because 10 μM nicotine alone had no effect on CRE-luciferase activation in the absence of nomifensine (data not shown). Furthermore, continuous nicotine exposure can desensitize nAChRs (Dani and Harris, 2005), perhaps explaining why higher concentrations of nicotine were necessary for enhancement of gene expression.

Our results demonstrate that nicotine promoted CRE-mediated gene expression only in cultures containing both VTA dopaminergic and NAcb GABAergic neurons. Nicotine-dependent stimulation of luciferase activity in these cocultures was prevented by the nAChR antagonist d-tubocurarine (Fig. 3A, dTc), confirming a requirement for nAChR. Nicotine activates nAChRs on dopaminergic neurons to promote the release of dopamine (see Dani and Harris, 2005; Wonnacott et al., 2005). In addition, nAChRs are expressed only at relatively low levels postsynaptically on GABAergic neurons in the NAcb (Pakkanen et al., 2005). Therefore, we hypothesized that nicotine-dependent gene expression in cocultures was due to dopamine released from VTA neurons, which then activated NAcb GABAergic neurons postsynaptically. Previous studies found that most NAcb/striatal neurons in culture express both D₁R and D₂R on the same neurons (Aizman et al., 2000) and that combined D₁R and D₂R activation is required for dopamine-induced increases in NAcb-firing rates in brain slices (Hopf et al., 2003). In agreement, inhibition of either the D₁R alone with SCH23390 (2.5 μM) or the
the PKA inhibitor H-89 (10 nM) induced luciferase activity in NAcb/VTA cocultures was prevented by inhibition of D1R or D2R alone or together prevented nicotine-induced enhancement in gene expression (Fig. 3B), indicating no role for A2AR (see below).

Because activation of CRE-luciferase under these conditions required cAMP/PKA signaling (Asher et al., 2002; Yao et al., 2002, 2003), we next examined the role of PKA in nicotine-induced luciferase activity in NAcb/VTA cocultures. As predicted, the PKA inhibitor H-89 (10 nM) prevented nicotine-dependent CRE-mediated luciferase activation in cocultures (Fig. 3B). These results indicate that the presence of both VTA and NAcb neurons in coculture. In addition, the A2aR antagonist MSX-3 (100 nM) did not prevent the nicotine-induced enhancement in gene expression (Fig. 3B), indicating no role for A2aR (see below).

Because activation of CRE-luciferase under these conditions requires cAMP/PKA signaling (Asher et al., 2002; Yao et al., 2002, 2003), we next examined the role of PKA in nicotine-induced luciferase activity in NAcb/VTA cocultures. As predicted, the PKA inhibitor H-89 (10 nM) prevented nicotine-dependent CRE-mediated luciferase activation in cocultures (Fig. 3B). These results are consistent with our earlier observation that PKA is required for the D1R/D2R-dependent activation of NAcb-firing rates in brain slices (Hopf et al., 2003). Taken together, our data suggest that nicotine seems to activate nAChRs on VTA neurons in coculture, causing the release of dopamine. In turn, dopamine activates D1 and D2 receptors simultaneously on NAcb neurons in coculture, leading to a PKA-dependent increase in CRE-mediated luciferase activity. This working hypothesis is supported by control studies in primary cultures containing either VTA alone or NAcb alone. Unlike in coculture, nicotine did not increase luciferase activity in separate cultures of VTA or NAcb (Fig. 2B).

**Subthreshold Concentrations of Nicotine and Ethanol in Combination Activate CRE-Mediated Gene Expression in NAcb/VTA Cocultures.** We are interested in identifying molecular mechanisms that contribute to reinforcing comorbidity of alcoholism and smoking in humans. Our earlier studies suggest that synergy between addicting agents, such as opiates and ethanol, activates PKA signaling and CRE-mediated gene expression (Yao et al., 2002, 2003; 2005, 2006). Therefore, we next asked whether subthreshold concentrations of nicotine and ethanol in combination would induce cAMP-dependent CRE-mediated gene expression synergistically in cocultures. A low concentration of ethanol (25 mM), previously shown to be subthreshold for PKA activation in cultured neurons (Yao et al., 2002), did not alter CRE-mediated gene expression in cultures containing VTA neurons alone (Fig. 4B) or NAcb neurons alone (Fig. 4A) or in NAcb/VTA cocultures (Fig. 4C). Likewise, a low concentration of nicotine (3 μM) alone did not affect CRE-mediated gene expression in separate VTA (Fig. 4B) or NAcb (Fig. 4A) cultures or in cocultures (Fig. 4C). However, combined application of subthreshold concentrations of ethanol (25 mM) and nicotine (3 μM) for 10 min synergistically induced CRE-mediated luciferase activity 5 h later in cocultures containing VTA and NAcb neurons together (Fig. 4C). It is noteworthy that combined application of subthreshold concentrations of ethanol and nicotine had no effect in separate VTA (Fig. 4B) or NAcb (Fig. 4A) cultures. Thus, synergy between subthreshold concentrations of nicotine and ethanol required the presence of both VTA and NAcb neurons in coculture.

We next identified specific receptor requirements for activation of CRE-mediated gene expression by nicotine and ethanol. VTA neurons express β2α- and α7-containing nAChRs (see Wonnacott et al., 2005). Synergistic enhancement of CRE-mediated gene expression by nicotine and ethanol required β2-containing nAChRs but not α7-containing nAChRs; luciferase activation was blocked by dihydro-β-erythroidine (50 μM), a selective inhibitor of β2-containing nAChRs, but not by α-bungarotoxin (10 nM), a selective inhibitor of α7-containing nAChRs (Fig. 5A). Dopamine receptor requirements were more complex. Activation of CRE-
mediated gene expression in cocultures by high concentrations of nicotine required both D_{1}Rs and D_{2}Rs (Fig. 3B). However, synergistic activation between subthreshold concentrations of nicotine with ethanol required only D_{2} receptors. Thus, the D_{2}R antagonist eticlopride inhibited luciferase activity by nicotine/ethanol, but the D_{1}R antagonist SCH 23390 did not (Fig. 5B). In an earlier study, we reported that synergy for PKA signaling involving ethanol required D_{2}Rs and adenosine A_{2A}Rs (Yao et al., 2002). Here we find that the specific A_{2A}R antagonist MSX-3 (100 nM) prevented synergistic induction of CRE-dependent luciferase activity by nicotine and ethanol (Fig. 5B). In contrast, the A_{1}R receptor 1,3-dipropyl-8-cyclopentylxanthine (100 nM) was without effect (Fig. 5B). Consistent with a requirement for A_{2A}Rs and with the observation that adenosine is continually released by neuronal cells (Nagy et al., 1990; Brundege and Williams, 2002), degradation of extracellular adenosine by adenosine deaminase (ADA, 1 U/ml) also prevented nicotine/ethanol activation of D_{2}Rs and A_{2A}Rs (Fig. 5B). However, synergistic activation between subthreshold concentrations of nicotine and ethanol for CRE-mediated gene expression required D_{2}Rs and A_{2A}Rs (Yao et al., 2002). Our results suggest that synergy between subthreshold concentrations of nicotine and ethanol required coactivation of D_{2}R and A_{2A}R. These findings are similar to a reported synergy between a D_{2}R agonist and ethanol (Yao et al., 2002).

In both cases, ethanol acts through the A_{2A}R, consistent with observations that ethanol inhibits an adenosine transporter to increase extracellular adenosine levels (Nagy et al., 1990). Furthermore, activation of G_{a_{3}}-linked receptors, such as the D_{2}R, releases G_{βγ} subunits from G_{i} (Sunahara et al., 1996; Yao et al., 2002, 2003), and G_{βγ} subunits can act synergistically with G_{α_{olf}} subunits (e.g., from A_{2A}Rs) to stimulate adenyl cyclase isoforms II and IV to increase cAMP and activate PKA. Both adenyl cyclases II and IV are expressed in cultured NAc neurons (Yao et al., 2002). Therefore, we asked whether synergy between subthreshold concentrations of nicotine and ethanol for CRE-mediated gene expression required G_{βγ} subunits and subsequent activation of PKA. In support of this hypothesis, we found that synergistic activation of CRE-mediated luciferase expression by subthreshold levels of nicotine and ethanol was prevented by viral expression of the dominant-negative G_{βγ} inhibitor βARK1 (Fig. 5C), which scavenges G_{βγ} subunits (Yao et al., 2002), and also by the PKA inhibitor H-89 (Fig. 5B). Control viral construct (β-galactosidase) was without effect (Fig. 5C). Because the βARK construct is derived from the site where G protein-coupled receptor kinase GRK2 interacts with G_{βγ}, we cannot completely rule out the possibility that this construct might inhibit GRK2 in addition to scavenging G_{βγ} subunits. However, the βARK construct used here is widely used to examine the contribution of G_{βγ} to intracellular signaling (e.g., Blackmer et al., 2001), and results from previous studies have shown similar inhibition of receptor synergy with βARK and the QEA peptide, which interferes with G_{βγ} function via a different mechanism from βARK (Yao et al., 2002, 2003). Thus, synergistic enhancement of gene expression by nicotine and ethanol required activation of G_{α_{olf}}- and G_{α_{olf}}-linked receptors, as well as G_{βγ} and PKA, suggesting that this molecular mechanism of synergy might represent a final common pathway during action of several addictive drugs.

**Discussion**

Our major findings in cocultures containing VTA and NAc neurons support the hypothesis that nicotine, acting on VTA neurons, promotes the release of dopamine, which activates postsynaptic dopamine receptors on NAc neurons to stimulate CRE-mediated gene transcription (Fig. 6). In particular, we propose that, with higher concentrations of nicotine, there is greater activation of VTA neurons and dopamine release and thus activation of both the higher affinity D_{2}Rs and lower affinity D_{1}Rs on MSNs (Missale et al., 1998). With subthreshold concentrations of nicotine, activation of dopamine neurons and dopamine release may be more modest, leading to activation of D_{2}Rs but not D_{1}Rs on MSNs. When a lower concentration of ethanol, which is insufficient by itself to increase gene activation (Yao et al., 2002), is combined with the subthreshold levels of nicotine, we propose that this dose of ethanol moderately inhibits the adenosine transporter and increases extracellular adenosine (Nagy et al., 1990), resulting in activation of A_{2A}Rs on MSNs. A_{2A}Rs can then interact with D_{2}Rs to activate PKA and enhance CRE-mediated gene expression. Nicotine and ethanol had no effect in cultures containing either VTA neurons alone or NAc neurons alone. Thus, our results represent a novel transynaptic cellular mechanism by which nicotine and ethanol
concentrations of nicotine release less dopamine, activating only the high adenylyl cyclase (AC)-cAMP-PKA-CREB pathway, inducing CRE-mediated gene expression, perhaps via Gi or Gs coupled from the Gi/o-coupled D2Rs synergistically stimulates the release of Gi or Gs/olf subunits from D2Rs (Hopf et al., 2003). B, low concentrations of nicotine release less dopamine, activating only the high affinity D2R. Ga<sub>q/11</sub> were released from activated A2ARs due to ethanol-induced increase of extracellular adenosine. A<sub>2a</sub>R activation coupled with release of Gβγ from the G<sub>i</sub>-coupled D2Rs synergistically stimulates the adenylyl cyclase (AC)-cAMP-PKA-CREB pathway, inducing CRE-mediated gene expression. CREB, cAMP-response element-binding protein.

Our results underscore the importance of the interaction of the nicotinic and dopaminergic systems in the etiology of nicotine and ethanol addiction. Blockade of nAChRs prevented gene activation in NAcB neurons by both a higher concentration of nicotine and by subthreshold levels of nicotine and ethanol in combination. In this regard, a number of studies have shown that nicotine can enhance VTA neuron activity and NAcB dopamine release (see Dani and Harris, 2005; Wonnacott et al., 2005). Furthermore, nicotine facilitates ethanol self-administration and reinstatement (Le et al., 2003), and inhibition of nAChRs within the ventral midbrain reduces ethanol self-administration and prevents ethanol-mediated enhancement of DA release in the NAcB (Ericson et al., 1998; Tizabi et al., 2002). A similar pattern was observed for nicotine self-administration (Corrigall et al., 1994), suggesting that the VTA and NAcB contribute to the primary reinforcing effects of nicotine, although some components of nicotine reinforcement do not require dopamine (reviewed in Wonnacott et al., 2005). In addition, animals will self-administer ethanol directly into the VTA (Gatto et al., 1994), and ethanol alone or nicotine and ethanol in synergy could enhance VTA neuron activity (Brodie et al., 1990; Clark and Little, 2004; but see Ericson et al., 1998). Here, nicotine and ethanol did not enhance gene expression in cultures containing only VTA, and thus any effects on CRE-mediated gene expression will probably occur indirectly through dopamine released from VTA neurons. Electron microscopy studies have also shown a low density of nAChRs postsynaptically within the NAcB (Pakkonen et al., 2005); however, these nAChRs do not seem to be activated significantly in our cell culture studies, because nicotine did not enhance gene expression in NAcB-only cultures. Thus, our results support the hypothesis that nicotine increased gene expression in NAcB neurons indirectly by enhancing release of dopamine from VTA neurons.

We propose that higher concentrations of nicotine result in more robust VTA activation and dopamine release and that both D<sub>2</sub>R and D<sub>1</sub>R are activated and necessary for CRE-mediated gene activation. These results agree with a previous study in NAcB brain slice showing a D<sub>1</sub>R/D<sub>R</sub>-mediated and Gβγ- and PKA-dependent synergistic enhancement in firing (Hopf et al., 2003). In addition, ethanol self-administration is significantly reduced by antagonism of Gβγ (Yao et al., 2002) or D<sub>2</sub>R or D<sub>1</sub>R (Hodge et al., 1997) in the NAcB. However, D<sub>1</sub>R/D<sub>2</sub>R colocalization might not be as prevalent in adult NAcB (Lee et al., 2006) relative to the more immature cultured neurons studied here (Aizman et al., 2000). Although not directly tested here, the requirement for both D<sub>2</sub>R and D<sub>1</sub>R in the nicotine-induced gene activation in cultured NAcB neurons perhaps suggests a role for Gβγ in the effects of higher concentrations of nicotine as well.

In contrast, under conditions of synergy, it seems that a requirement for the G<sub>i</sub>-linked D<sub>2</sub>R was replaced by another G<sub>olf</sub>-linked receptor, the adenosine A<sub>2a</sub>R, which is colocalized with the D<sub>2</sub>R in adult NAcB neurons (Svenningsson et al., 1999). Interestingly, ethanol can activate A<sub>2a</sub>Rs in neuronal cultures (Yao et al., 2002) by increasing extracellular concentrations of adenosine (Nagy et al., 1990), and an A<sub>2a</sub>R antagonist or enzymatic degradation of extracellular adenosine by adenosine deaminase prevented synergy between nicotine and ethanol. Moreover, A<sub>2a</sub>R activation probably occurred only on NAcB neurons, because VTA neurons express few A<sub>2a</sub>Rs relative to NAcB (Svenningsson et al., 1999). Our results also predict that ethanol-mediated A<sub>2a</sub>R activation should synergize with D<sub>2</sub>R agonists in MSNs, which we have observed previously (Yao et al., 2002).

Thus, under all experimental conditions examined here, activation of CRE-mediated gene expression in cocultures required both G<sub>i</sub>-linked and G<sub>olf</sub>-linked receptors. We have previously shown that synergy between G<sub>i</sub>- and G<sub>olf</sub>-linked receptors in the same NAcB neuron was observed during the interaction of several addicting drugs, including ethanol, cannabinoids, and opioids (Yao et al., 2003, 2005). In all cases, these examples of postsynaptic synergy require Gβγ subunits, stimulation of cAMP production by adenylyl cyclase II and IV, and activation of PKA. Here, synergistic stimulation of CRE-mediated gene expression by nicotine and ethanol in cocultures of VTA and NAcB neurons also required both Gβγ subunits and PKA. Synergy between receptors involved in addiction also seems to share a common requirement for A<sub>2a</sub>R activation in cultured neurons (Yao et al., 2002, 2003) and in vivo (Yao et al., 2006). Thus, Gβγ- , PKA- , and A<sub>2a</sub>R-mediated synergy in the NAcB might represent a final common pathway through which addictive drugs exert their reinforcing effects. Our results are also in agreement with a number of studies suggesting the importance of cAMP/PKA signaling in cellular and animal models of addiction and withdrawal (Wise, 2004; Carlezon et al., 2005), including ethanol self-administration (Wand et al., 2001).

The requirement for nomifensine in order to observe nicotine-mediated and nicotine/ethanol-mediated enhancement in gene expression could suggest that the results here might be more relevant to humans with addiction to psychostimu-
lants in combination with nicotine or nicotine/ethanol. Although we cannot rule out this possibility, we considered it more likely that the requirement for nomifensine reflects otherwise low endogenous dopamine signaling (Murray and Gilles, 1993), and we speculate that, in the intact brain of the addicted animal or human, there are likely to be a number of other factors released, in particular glutamate (Adell and Artigas, 2004), which may increase the activity of and facilitate dopamine release from dopamine neurons.

In summary, our results are the first to show that a Gβγ- and A2AR-dependent PKA pathway mediates a trans-synaptic interaction between nicotine and ethanol, resulting in CRE-mediated gene expression in NAcb neurons in coculture with VTA. Our results support a working model in which higher concentrations of nicotine activate dopamine neurons of the VTA, resulting in sufficient dopamine release to activate D1Rs and D2Rs on NAcb neurons, and enhance gene activation. With subthreshold levels of nicotine and ethanol in combination, nicotine activation of VTA neurons may produce dopamine release sufficient for activation of D2Rs but not D1Rs, and thus simultaneous ethanol activation of A2ARs on NAcb neurons by increased extracellular adenosine levels (Nagy et al., 1990) is required for CRE-mediated gene activation. Thus, nicotine/ethanol interaction through coactivation of D1R and A2AR might contribute both to the initial reinforcing effect of these drugs and to the longer-term neurotransaptations through CRE-mediated gene expression that contribute to the development of addiction (Carlezon et al., 2005). It is tempting to speculate that synergy between receptor signaling pathways may account for the central role of the NAcb in regulating nicotine and ethanol intake. Thus, drugs that inhibit Gβγ function and/or synergy between A2ARs and D2Rs might prevent, attenuate, or reverse excessive smoking and drinking and their serious health risks.

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References


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