SUPPLEMENTAL ONLINE SECTION

METHODS AND MATERIALS

In situ hybridization

[^{35}S]-labeled UTP was used to synthesize cRNA riboprobes in the sense and antisense orientation from a pGEM-3Z plasmid containing a 680bp fragment of c-fos cDNA between T7 and SP6 promoter sites. All tissue sections were pretreated with Proteinase K (1 µg/ml) for 10 min at 22°C, acetylated, dehydrated through graded ethanols and then air dried. Sections were incubated for 18 hr at 60°C, with hybridization solution (50% formamide, 10% dextran sulfate, 0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin, 500 µg/ml tRNA, 10 mM DTT, 0.3 M NAcCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) containing the [^{35} S]-labeled riboprobe (107 cpm/ml). After hybridization, sections were incubated with RNase A (20 µg/ml) for 30 min at 37°C, and washed twice at 22°C for 5 min each with 2x SSC buffer/10 mM DTT and 1x SSC buffer/10 mM DTT buffer, followed by a 30 min wash in 1x SSC at 60ºC. Tissue sections were dehydrated and apposed in light-tight cassettes to βmax film with ^{14}C standards of known radioactivity for 1 day. Film was then developed and rapidly fixed. Sections were postfixed with 4% paraformaldehyde and then processed for Nissl staining using cresyl violet for anatomical analysis.

Ligand Binding Autoradiography

Sections for DAT binding were incubated for 2 hr with 10 pM [^{125}I]RTI-55 (Perkin Elmer NEN, Waltham, MA, USA) in buffer containing 10 mM Na phosphate, 0.1 M sucrose, with 50 nM citalopram HBr and 5 nM desipramine to block binding to SERT and NET, respectively. Non-specific binding was determined in adjacent tissue sections by the addition of GBR 12909 (10 µM). SERT binding conditions were identical to DAT except 50 nM citalopram was replaced

with 1 μM GBR 12935, to block binding to DAT, and non-specific binding was defined by 50 nM citalopram. Sections were rinsed in ice-cold phosphate buffer. After a brief dip in ice-cold water, slides were dried and apposed to Kodak Biomax MR film with autoradiographic standards for 48 (SERT) or 72 hr (DAT) in light-proof X-ray cassettes. Film was developed and rapidly fixed. Sections were postfixed with 4% paraformaldehyde and then processed for Nissl staining using cresyl violet for anatomical analysis.

Tissue Catecholamine Content

Tissues samples from PFC, DS, NAc, and BLA were dissected using a 1 mm diameter tissue punch (Stoelting, Wood Dale, IL, USA), expelled into 300 ml of ice-cold 0.1 M perchloric acid, and homogenized. Samples were centrifuged at 10 000*g* for 10 min. The supernatants were used for the measurement of DA, 5-HT and metabolites using HPLC-ED. The pellets were resuspended in 300 ml of 0.1 M NaOH to measure protein content using a Bio-Rad protein assay kit (Hercules, CA). Remaining tissue sections were examined anatomically after tissue puncture to verify correct localization of tissue samples. Samples were filtered and then injected onto a 150x3.2 mm ODS C^{18} column (ESA Inc.; Chelmsford, MA) connected to an ESA 580 HPLC pump. The column was kept at 35°C and perfused by MD-TM mobile phase (ESA, Chelmsford, MA) at a rate of 0.6 ml/min. 5HT, DA and metabolite levels were determined using an electrochemical ESA 5600 detector with an ESA 5020 guard cell with the dominant potential of 300 mV. The sensitivity of the detector is 500 fg. Measurements were analyzed using CoulArray for Windows[32] Software 2.0 (ESA Inc.; Chelmsford, MA). Standard curves were generated with catecholamine (ESA Inc.; Chelmsford, MA), DOPAC, 5-HT and 5-HIAA (Sigma-Aldrich; St Louis, MO) standards, and levels in experimental samples were interpolated from the curve and adjusted for tissue sample protein levels.

Erectile Response:

Immediately after locomotor testing, animals were scored for erectile response, using a scoring scale (0-3), based on degree of penile erection: 0 = none; 1 = mild; 2 = moderate; 3 = high.

RESULTS

Nicotine pretreatment on acquisition of methamphetamine and alcohol self-administration

Age differences were also observed in nicotine-pretreatment effects on methamphetamine (0.02 mg/kg/inf) reinforced responding (Fig. S1), as shown by an effect of age [$F(1,31) = 4.55$, $p = 0.04$] and age × pretreatment interaction [$F(1,31) = 4.05$, $p = 0.05$]. Nicotine pretreatment at P28-31 animals induced a significant enhancement in reinforced responding at P32 as compared to saline-treated controls [$F(1,16) = 7.44$, $p = 0.015$], and significantly higher reinforced to non-reinforced responding ($p = 0.012$). In contrast, there was no nicotine pretreatment effect on methamphetamine self-administration [$F(1,15) = 0.25$, $p = 0.62$] in adult animals, and reinforced responding was not significantly greater than non-reinforced (Saline, $p = 0.07$; Nicotine, $p = 0.22$).

Adolescent nicotine pretreatment also enhanced acquisition of i.v. self-administration of a low dose of ethanol (1 mg/kg/inf,; Fig. S1). An overall ANOVA showed a significant age effect on ethanol-reinforced and non-reinforced responding [$F(1,37) = 9.79$, $p = 0.003$]. Adolescent nicotine-treated rats self-administered significantly more ethanol than saline-treated controls [$F(1,16) = 4.48$, $p = 0.05$]. Both groups demonstrated acquisition, determined by a significant preference for the reinforced hole compared to non-reinforced (Saline $p = 0.04$, Nicotine $p = 0.003$). Adult animals showed no significant effect of pretreatment [$F(1,21) = 0.04$, $p = 0.85$].

However, post-hoc comparisons found only saline-treated adults to show a significant acquisition of the task (Saline, p = 0.02; Nicotine, p = 0.27).

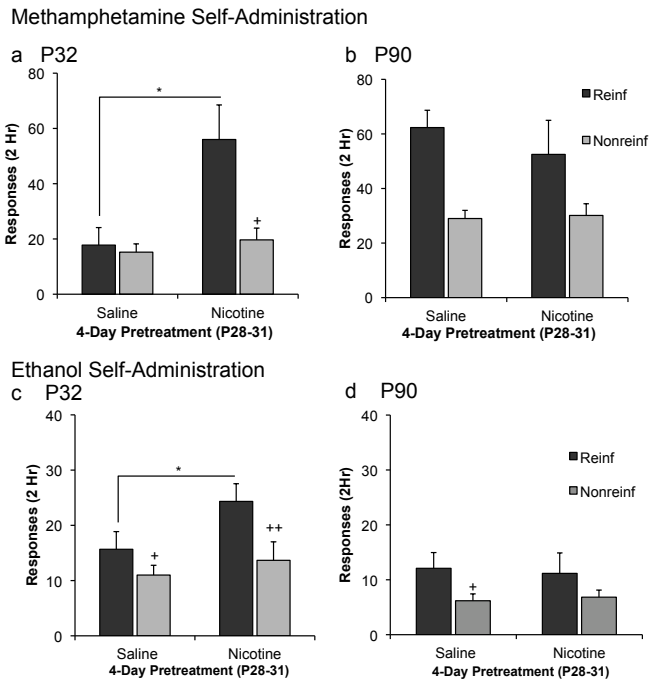


Figure S1: Effects of nicotine pretreatment on methamphetamine and ethanol self-administration. Adolescent (P32, left) and adult (P90, right) animals were given i.v. nicotine or saline daily for four days prior to methamphetamine (0.02 mg/kg/inf) or ethanol (1mg/kg/inf) self-administration tests. Figure S1 shows the mean (± SEM) reinforced (dark) and nonreinforced (light) responses in a 2 hr session on day 1. (a,b) Saline-treated adolescent rats self-injected significantly less methamphetamine than the nicotine-treated adolescents or adult animals. Nicotine-treated P32 rats had significantly greater responses at the reinforced hole for methamphetamine. *p < 0.03 vs. P32 Saline, +p < 0.02 Reinf vs. Nonreinf. (c,d) Nicotine-treated adolescent rats administered significantly more ethanol compared to saline controls. P32 rats had significantly greater responding than P90 animals. All groups but nicotine-treated adults had significantly greater responses at the reinforced hole. *p = 0.05 vs. P32 Saline;

+p<0.04, ++p<0.004 Reinf vs. Nonreinf. Methamphetamine: n = 8-9/group; Ethanol: n = 9-12/group

5-HT1A-R Blockade of Methamphetamine Self-Administration

In order to determine whether 5-HT_{1A}-Rs mediate nicotine-induced enhancement of psychostimulant self-administration in adolescents, the selective antagonist, WAY 100,635 (0.4 mg/kg), was co-administered with nicotine during pretreatment (Fig. S4). Animals were subsequently tested for acquisition of methamphetamine (0.02 mg/kg/inf) self-administration.

Drug pretreatment produced a significant effect on reinforced responses [F(3,17) = 4.66, p = 0.015]. Whereas nicotine pretreatment enhanced methamphetamine responding (p = 0.045), WAY 100,635 pretreatment (0.4 mg/kg) significantly decreased this effect (p =0.02), without influencing saline controls (p = 1.0).

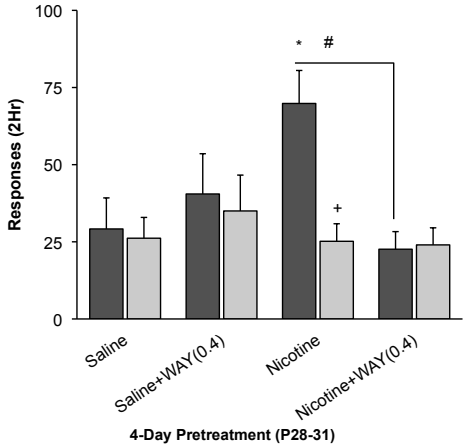
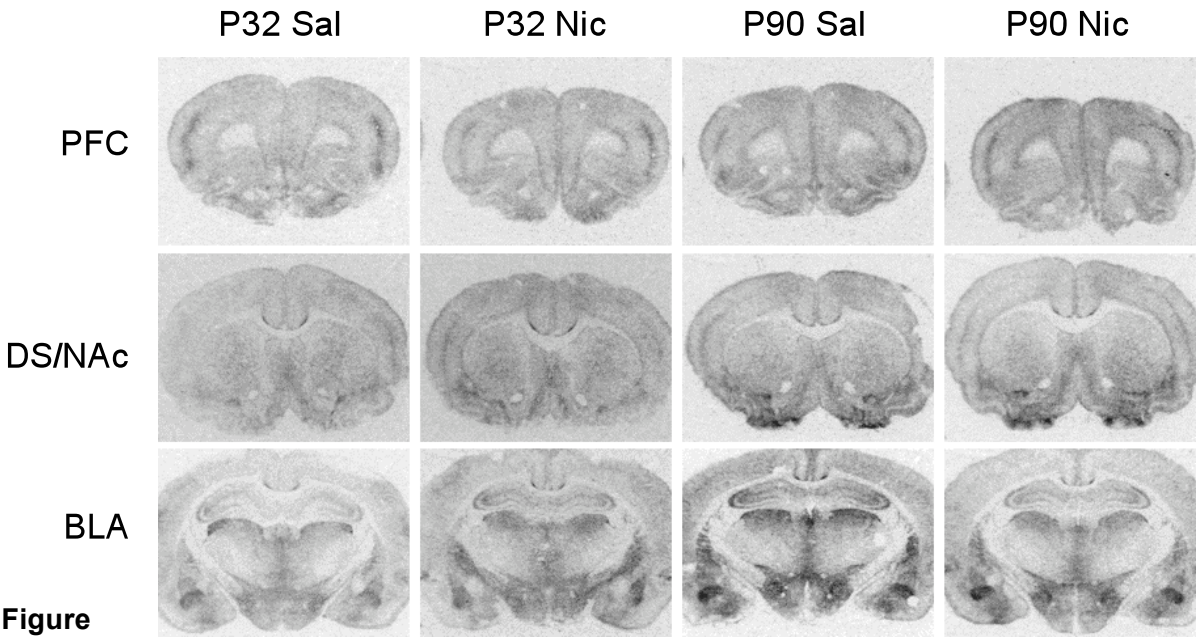


Figure S2. 5-HT1a receptor antagonism blocks nicotine enhancement of methamphetamine self-administration. Adolescent animals received daily infusions of WAY 100,635 (0 or 0.4 mg/kg/0.1 ml,i.v.) prior to each nicotine pretreatment. WAY blocked the

nicotine-pretreatment effect on methamphetamine (0.02 mg/kg/inf, right) intake and acquisition on Day 1. * p = 0.02 vs. Saline; + p = 0.01 Reinf vs. Nonreinf; # p = 0.01 vs. Nicotine. Methamphetamine: n = 4-6/group.

Representative autoradiograms for SERT radioligand binding



Figure

S3. Autoradiographic analysis of regional SERT density. Representative sections from adolescent (P32) and adult (P90) animals treated for four days prior with i.v. saline (Sal) or nicotine (Nic). BLA, basolateral amygdala; DS, dorsal striatum; NAc, nucleus accumbens; PFC, prefrontal cortex.

Age and nicotine pretreatment effects on dopaminergic system

There were significant age differences in DAT density in the BLA [$F(1,22) = 5.13$, $p = 0.03$] and an interaction of age × pretreatment [$F(1,22) = 4.39$, $p = 0.048$]. Adolescent saline-treated animals had significantly lower binding than adult controls ($p = 0.015$), but not nicotine-treated adolescents ($p = 0.11$ vs. P90Sal). No age or nicotine pretreatment effects were found in DAT

density in the DS or NAc. There was a significant effect of age on DA levels in the PFC [F(1,21) = 7.28, p = 0.013] and DS [F(1,21) = 15.09. p = 0.001], with adults showing higher tissue content than adolescents; however, there was no effect of nicotine pretreatment at either age. Nicotine pretreatment did enhance DA levels in NAc [F(1,21) = 7.34, p = 0.013]; however this effect was not age-specific. No significant effect of age or pretreatment was found for DA content in the BLA, nor for DOPAC content in any of the brain regions examined.

Representative autoradiograms for DAT radioligand binding

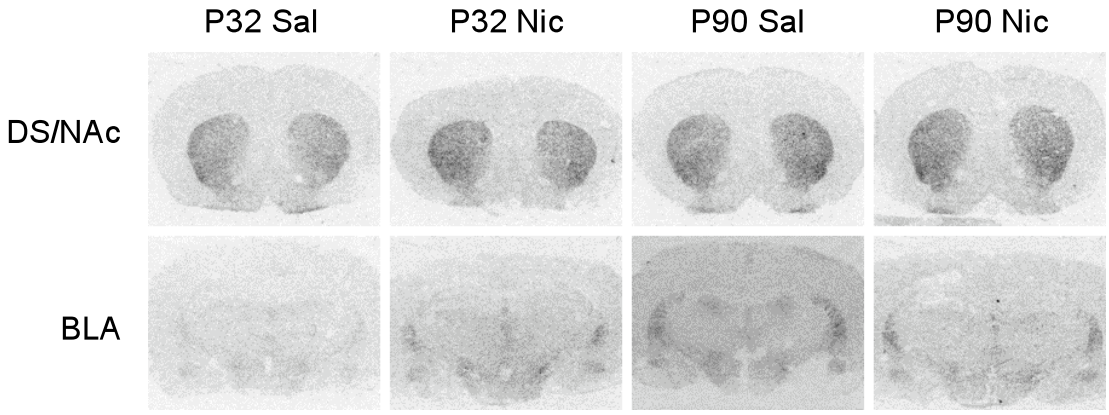


Figure S4. Autoradiographic analysis of regional DAT density. Representative sections from adolescent (P32) and adult (P90) animals treated for four days prior with i.v. saline (Sal) or nicotine (Nic). BLA, basolateral amygdala; DS, dorsal striatum; NAc, nucleus accumbens.

Effect of Adolescent Nicotine Pretreatment on Penile Erection

The D2-like agonist, quinpirole is known to induce penile erection when administered centrally or peripherally. In order to evaluate if nicotine exposure alters male rat erectile response, penile erections were measure immediately after locomotor testing. There were significant age differences in penile erection score [F(1,57) = 45.24, p < 0.0001] and an interaction of age × pretreatment [F(1,57) = 31.7, p < 0.0001]. Thus, groups were split and

analyzed by age group. Administration of quinpirole (0.4 mg/kg, i.p.) in adolescents (P32) elicited an age-specific erectile response [$F(1,31) = 49.716$, $p<0.0001$]. Adolescents pretreated with nicotine displayed significantly enhanced penile erections [$F(1,31) = 8.04$, $p=0.008$]. Adult animals did not elicit an erectile score in response to quinpirole [$F(1,26) = 2.01$, $p = 0.925$.

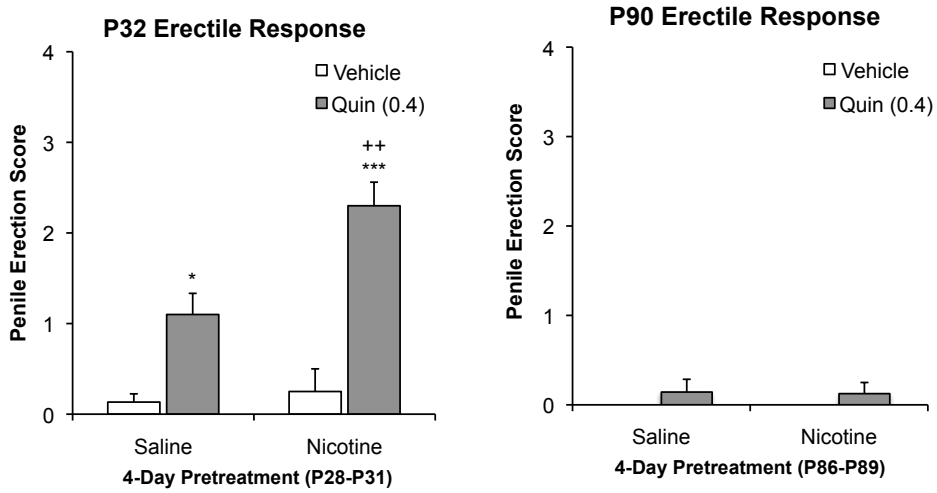


Figure S5. Effect of aage and nicotine pretreatment on erectile response. Adolescent and adult rats were injected i.p. with quinpirole (0.4 mg/kg). Penile erection was assessed 30 minutes post-drug administration. Penile erection score is defined as degree of penile erection (0-3). Data are expressed as Mean ± SEM. Quinpirole elicited an erectile response in adolescent animals. * p =.01; *** p < .0001 vs. vehicle control. Nicotine exposure during adolescence enhanced penile erection score. ++ p = .001 versus saline-pretreated controls.

DISCUSSION

In the present study, quinpirole also induced penile erection in adolescent rats, with nicotine pretreatment enhancing this proerectile drug effect. Preliminary studies showed that erectile response is blocked by L741,626, suggesting the control of penile erection in adolescent rats is D_2R-mediated. A significant body of literature supports the role of D_2R in the induction of erectile response. Proerectile activity of apomorphine, quinlelorane, 7-OH-DPAT, and

pramiprexole are thought to be mediated by activation of D_2R, based on studies showing that response could only be antagonized by L741,626 ((Millan et al. 2000; Collins et al. 2009; Collins et al. 2007). However, a specific role for the D_3R and D_4R in the induction of PE by D_2R-like agonists has also been reported. SB-277011 and PG01037, compounds with preferential selectivity for D_3R over D_2R (Grundt et al. 2005), dose dependently antagonized apomorphine-induced penile erection (Collins et al. 2009). Dose-dependent increases in the incidence of penile erection were also found after systemic and i.c.v. administration of selective D_4R agonists, PD168077 and CP226269 (Hsieh et al. 2004). Nonetheless, the positive data with L741,626 obtained in this study suggests that D_2R are the most likely candidates for mediating penile erection in adolescent rats.

Interestingly, rat strains differ in their sensitivity to the effects of D_2R like agonists on erectile response, with adult Sprague-Dawley rats being nonresponsive (Depoortère et al. 2009), with the present study replicated this finding. However, the effect of DA agonist administration on erectle response has yet to be explored. This is the first study to identify adolescent sensitivity to quinpirole-induced erectile response.

REFERENCES

Collins, G.T. et al., 2007. Yawning and hypothermia in rats: effects of dopamine D3 and D2 agonists and antagonists. *Psychopharmacology*, 193(2), 159-170.

Collins, G.T. et al., 2009. Proerectile Effects of Dopamine D2-Like Agonists Are Mediated by the D3 Receptor in Rats and Mice. *Journal of Pharmacology and Experimental Therapeutics*, 329(1), 210-217.

Depoortère, R. et al., 2009. Penile erection and yawning induced by dopamine D2-like receptor agonists in rats: influence of strain and contribution of dopamine D2, but not D3 and D4

receptors. *Behavioural Pharmacology*, 20(4), 303-311.

Grundt, P. et al., 2005. Novel heterocyclic trans olefin analogues of N-{4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl}arylcarboxamides as selective probes with high affinity for the dopamine D3 receptor. *Journal of Medicinal Chemistry*, 48(3), 839-848.

Hsieh, G.C. et al., 2004. Central mechanisms regulating penile erection in conscious rats: the dopaminergic systems related to the proerectile effect of apomorphine. *Journal of Pharmacology and Experimental Therapeutics*, 308(1), 330.

Millan, M.J., Lejeune, F. & Gobert, A., 2000. Reciprocal autoreceptor and heteroreceptor control of serotonergic, dopaminergic and noradrenergic transmission in the frontal cortex: relevance to the actions of antidepressant agents. *Journal of Psychopharmacology*, 14(2), 114 -138.