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Differential Expression of Arc mRNA and Other Plasticity-Related Genes Induced by Nicotine in Adolescent Rat Forebrain

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Abstract

Relatively little attention has been focused on mechanisms related to neural plasticity and drug abuse in adolescence, compared with abundant research using adult animal models. As smoking is typically initiated in adolescence, an important question to address is whether the adolescent brain responds differently to nicotine compared with the adult. To investigate this question, we examined the expression of a number of early response genes (*arc*, *c-fos* and *NGFI-B*) that have been implicated in synaptic plasticity and addiction, following acute nicotine in adolescent and adult rats. Baseline expression of *arc* and *c-fos* was higher in adolescent brains compared with adults. Following acute

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Abbreviations:

DS dorsal striatum

fmi forceps minor of the corpus callosum

gcc genu of corpus callosum

LO lateral orbital

mPFC medial prefrontal cortex

nic nicotine

QDR quinoid dihydropteridine reductase

sal saline

SmCtx somatosensory cortex

VDB nucleus of the vertical limb of the diagonal band

VLO ventral and lateral orbital cortex

VS ventral striatum.

nicotine treatment (0.1, 0.4 mg/kg), we found a marked induction of *arc* mRNA in the prefrontal cortex of nicotine-treated adolescents compared with a less pronounced increase of *arc* in the adult. *c-fos* and *NGFI-B* were also upregulated by nicotine, but not in an age-related manner. In contrast, nicotine induced less *arc*, *c-fos*, and *NGFI-B* expression in the somatosensory cortex of adolescents compared with adults. A fourth gene, quinoid dihydropteridine reductase was expressed at lower levels in white matter of the adolescent forebrain compared with the adult, but was not affected by nicotine. These results suggest that in adolescence, the activity of specific early response genes is higher in brain regions critical for emotional regulation and decision-making. Further, nicotine affects key plasticity molecules in these areas in a manner different from the adult. Thus, adolescence may represent a neurobiologically vulnerable period with regard to nicotine exposure.

Keywords

plasticity; development; drug abuse; prefrontal cortex; immediate-early gene

Smoking is an addictive habit that typically develops in adolescence. Despite its clear clinical relevance, relatively little is known about the neurobiology of immediate and long-term consequences of smoking during adolescence, or age-specific contributions to nicotine addiction. Nicotine has a number of well-established neurochemical and molecular effects on adult neural systems (Dani and Heinemann, 1996), including upregulation of nicotinic acetylcholine receptors (nAChRs) following chronic nicotine treatment (Wonnacott, 1990;Perry et al., 1999). However, beyond central cholinergic effects, systems involved in cellular plasticity and learning are also markedly affected by nicotine. Nicotine has been linked to increased dopamine levels in mesocortical limbic regions (Di Chiara and Imperato, 1988), and its behavioral and rewarding effects are partially dependent on dopaminergic activation (Clarke et al., 1988;Di Chiara, 2000). Nicotine also interacts with glutamate systems in the brain, enhancing fast excitatory synaptic transmission at glutamatergic synapses (McGehee et al., 1995). Since current major theories of addiction and memory implicate interactions between dopaminergic and glutamatergic systems, this profile of nicotine effects suggests nicotine may induce long-term synaptic alterations at the level of gene expression. Indeed, in the adult rat model, studies have shown acute and chronic nicotine administration activates the immediate-early gene *c-fos* in multiple limbic and cortical regions (Nisell et al., 1997;Pich et al., 1997;Salminen et al., 1999). Other immediate early genes implicated in the mesocorticolimbic response to nicotine include cAMP-response element CREB and deltaFosB (Kelz et al., 1999;Pandey et al., 2001).

The extent to which neurochemical and molecular mechanisms identified as important in the adult are applicable to the adolescent brain is undetermined. Adolescent rats display altered behavioral responses to nicotine compared with adults in a number of paradigms (Vastola et al., 2002;Faraday et al., 2003;Levin et al., 2003;Belluzzi et al., 2004;O'Dell et al., 2004). We have recently reported that although the overall locomotor doseresponse sensitivity to nicotine is similar in adolescents and adults, adolescent rats fail to display long-term contextual cue conditioning (Schochet et al., 2004). Changes in nicotinic acetylcholine, serotonergic, dopaminergic and glutamatergic receptor systems in forebrain and midbrain regions following prolonged adolescent nicotine exposure have all been reported (Slotkin, 2002; Abreu-Villaca et al.,2003a,2003b;Ad-riani et al., 2003;Collins et al., 2004b).

Relatively little is known about the effect of nicotine on plasticity-related genes in the adolescent brain. As cortical substrates are actively developing in the adolescent (Lewis, 1997), we hypothesized that nicotine might differentially affect expression of these genes in adolescents compared with adults. We report here that *arc*, *NGFI-B*, and *c-fos*, three plasticity-related early response genes, are upregulated in cortical and striatal sites following acute

nicotine. Moreover, the expression of *arc*, a dendritically targeted mRNA whose protein product is involved in synaptic modification and learning, is more strongly induced by nicotine in adolescence than in adulthood.

EXPERIMENTAL PROCEDURES

Subjects and handling

For all experiments, male Sprague-Dawley rats (Harlan, Madison, WI, USA) were used. Animals were housed in pairs in clear plastic cages in an animal colony. Food and water was available at all times. Lighting in the animal colony was on a 12-h light/dark cycle, with lights on at 07:00-19:00 h. Rats arrived in the colony 4 days prior to testing, and were handled daily in order to minimize stress during testing. All animal care was in strict accordance with University of Wisconsin-Madison Institutional Animal Care and Use Committee guidelines as defined by the NIH. Care was taken to minimize the number of animals used and their suffering.

The effects of nicotine on gene expression were assessed in two separate conditions. Our first experiment examined the acute effects of nicotine and the second experiment examined the doseresponsivity of the gene expression. For the examination of the acute nicotine effects on gene expression, we used a total of 32 rats. Of these rats, 16 were tested at approximately 70 days of age (adult, average weight 306 g), and 16 were tested at 30 days (early adolescent, average weight 84 g). On the test day, rats were given a single s.c. injection of nicotine [*n*=8 adolescent, *n*=8 adult, 0.4 mg/ml/kg s.c. nicotine hydrogen tartrate salt (Sigma, St. Louis, MO, USA), dissolved in saline, pH adjusted to 7.2 with NaOH] or a single saline injection (*n*=8 adolescent, *n*=8 adult, 1 ml/kg, s.c.). We have previously found that adolescent and adult animals show similar locomotor responses to increasing doses of nicotine. The moderate dose of 0.4 mg/kg nicotine used in our previous sensitization and conditioning experiments was used in this study (Schochet et al., 2004).

In our second experiment, we examined whether the effect of nicotine on *arc* expression in adolescents and adults was dose-dependent using a total of 16 rats. Of these rats, eight were tested at approximately 70 days of age (adult, average weight 303 g) and eight were tested at 30 days (early adolescent, average weight 99 g). On the test day, rats were given a single s.c. injection of nicotine (*n*=4 adolescent, *n*=4 adult, 0.1 mg/ml/kg s.c. nicotine hydrogen tartrate salt (Sigma, St. Louis, MO, USA), dissolved in saline, pH adjusted to 7.2 with NaOH) or a single saline injection (*n*=4 adolescent, *n*=4 adult, 1 ml/kg, s.c.).

In all studies, injections were administered between 12:00 and 14:00 h. One hour after the injection, rats were anesthetized with halothane, and their brains rapidly removed, frozen, and stored at -80 °C. Cryostat sections (20 μm) were collected onto Superfrost plus microscope slides (Fisher, Pittsburgh, PA, USA) and stored dry at -80 °C until use.

In situhybridization

Sections were post-fixed in 4% paraformaldehyde for one 1/2 h at 4 °C. Following three washes of 2× SSC (1× SCC=150 mM NaCl, 15 mM citrate, pH 7.0), slides were briefly digested with Proteinase K (0.2 μg/μL; Qiagen, Valencia, CA, USA) for 10 min at 37 °C. Slides were then acetylated for 10 min at room temperature with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8, washed in 2× SSC and dehydrated in a graded ethanol series. Sections were hybridized overnight at 55 °C in a hybridization solution containing 10% dextran sulfate, 3× SSC, 0.5 M $NaPO_4$, 50% formamide, 1× Denhart's, and 200 μg/ml tRNA, pH 7.5, 0.05 M DTT and 0.1 ng/μL[^{35}S]-labeled antisense cRNA probe. Following hybridization, sections were washed in 500 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and digested for 1h at 37 °C in the same solution containing 20 μg/ml pancreatic RNAse A (Ambion, Austin, TX,

USA). Slides were then washed in 1× SSC, 0.2 M DTT (5 min), 0.5× SSC, 0.2 M DTT (5 min), and 0.1 M SSC, 0.2 M DTT (1 h, 70 °C), dehydrated in a graded ethanol series and dried. For autoradiography, sections were exposed to a phosphorimager screen for one week and scanned with a Molecular Dynamics Typhoon phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA). Slides were also subjected to autoradiography for 6 weeks using Kodak NTB-2 liquid emulsion (Eastman Kodak Co, Rochester, NY, USA) and stained as previously described (Landry et al., 1989). Photomicrographs were taken on a Leica DMRX microscope equipped with a Leica DC300F camera.

Probe preparation

Four genes were chosen for analysis, *arc*, *c-fos*, *NGFI-B* and quinoid dihydropteridine reductase (*QDR*). *Arc*, *c-fos*, and *NGFI-B* are immediate early genes selected for study based on their role in synaptic plasticity and sensitivity to drugs of abuse. Increases in *arc*, *c-fos*, and *NGFI-B* expression have been described following treatment with drugs including cocaine, morphine, amphetamine and nicotine (Fosnaugh et al., 1995;Konradi et al., 1996;Pich et al., 1997;Werme et al., 2000a;Steward and Worley, 2002). We previously found that the gene *QDR* (Turner et al., 1974) was expressed at higher levels in the adult compared with the adolescent brain based on preliminary gene expression microarray analysis (Schochet et al., 2002). Total RNA from rat brain was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and used to generate a cDNA library using reverse transcriptase as described by the manufacturer (Amersham Biosciences, UK). cDNA for *arc*, *NGFI-B* and *QDR* used for *in situ* hybridization was amplified from this library using standard PCR conditions. The following primers were used to generate PCR products for the gene indicated. Numbers in parentheses after the primer sequence represent the base number as defined by the gene sequence in the Unigene database. The sequence in italics corresponds to the T7 polymerase recognition sequence used for ^{35}S-labeled antisense cRNA probe generation as described by the manufacturer (Promega Corporation, Madison, WI, USA).

Arc, forward primer 5-CCCCAGGAAGCTGATGGCTAC-GAC-3′(693-714), reverse primer ′ 5′-CAGAGATGCATAATAC-
GACTCACTATAGGGAGAGAGTGTCAGCCCCAGCTCAATCA-AG-3′(1472-1494).

NGFI-B, forward primer 5-GGTGTATGGCTGCTACCC-TGG-3′(428-448), reverse primer ′ 5′-CAGAGATGCATAAT-
ACGACTCACTATAGGGAGAGAGTCCAAATGTGCTCGAATG-AGG-3′(1208-1229).

QDR, forward primer 5-TCAGTTCCGCGGGAGTCTT-3 (20-39), reverse primer 5-CAGAGATGCATAATACGACTCACTAT-′
AGGGAGAGATTACAGGCCCCCACTCATTC-3 ′ (970-950).

c-fos, forward primer 5′-AATAAGATGGCTGCAGCCAA-′ 3′(573-592), reverse primer 5′-CAGAGATGCATAATACGACT-
CACTATAGGGAGAGAGGATGGCTTGGGCTCAAGGT-3′ (890-871).

Plasmid pBluescript-II-SK containing *c-fos* cDNA was kindly provided by T. Curran. Plasmid was linearized with BamH1 and transcribed with T7 polymerase for the generation of ^{35}S-labeled antisense cRNA probe [573-890].

Data analysis

Optical density data values were generated using the ImageQuant software (Molecular Dynamics, Amersham Biosciences). For *arc*, *c-fos*, and *NGFI-B*, six brain regions were chosen for analysis, based on data in adult rats showing their involvement in the overall drug response. These regions were the medial prefrontal cortex (mPFC), ventral and lateral orbital cortex

(VLO), cingulate cortex, somatosensory cortex (SmCtx), ventral striatum (VS), and dorsal striatum (DS). As shown in Fig. 1, analysis was performed by delineating standard geometric shapes around the appropriate anatomical region. One section was analyzed per brain region, and counting was carried out on both sides of each chosen section. Following automatic calculation, density values were subsequently normalized to white matter density values calculated for each slice. This normalization was performed to adjust for differential binding of probe to each section; as these genes are not expressed in white matter, the levels of intensity in these regions would represent background nonspecific binding. For *QDR*, a gene found primarily in white matter, expression levels were measured in the forceps minor of the corpus callosum (fmi), the genu of the corpus callosum (gcc) and the nucleus of the vertical limb of the diagonal band (VDB). A region of gray matter not expressing *QDR* was selected for each section to provide background binding values.

Two kinds of statistical analysis were carried out on the *in situ* hybridization data. To determine cortical versus subcortical effects, a three-factor, between-within ANOVA was carried out with treatment and age as between-subjects factors, and brain region (cortical vs. subcortical) as the within-subjects factor. The cortical regions were analyzed by compacting optical density values for mPFC, VLO, cingulate and SmCtx as one variable. Subcortical or striatal regions were composed of the optical density values for VS and DS compacted as one variable. If overall interactions indicated significance, a two-factor ANOVA was performed with treatment and age as the between-subjects factors for each individual brain region.

Cell counts of *arc* mRNA-expressing cells in the VLO were performed on images collected on a Leica DMRX microscope equipped with a Leica DC300F camera. Silver grain accumulations corresponding to *arc*-expressing cells were visualized in dark field at 20× objective magnification and counted using Image Pro Plus 5.0.0.39. Two animals in each of four groups (adolescent saline and nicotine, adult saline and nicotine) were analyzed and data from the VLO from each hemisphere were pooled (*N* per group=4). An area of 0.55 mm by 0.68 mm corresponding to the boxed region shown in Fig. 4A was counted and a size cutoff of 100 um^2 was employed to eliminate individual and non-specific clusters of silver grains from contributing to the total count.

RESULTS

Arc mRNA is differentially expressed in specific regions of the prefrontal cortex

Gene expression profiling using *in situ* hybridization was used to determine whether acute nicotine treatment differentially affects the expression of early response, plasticityrelated genes in the adolescent compared with the adult forebrain. One of the genes that was selected for this analysis, the activity regulated, cytoskeletal-associated gene *arc*, undergoes dynamic changes in dendritic mRNA localization in response to specific stimuli in the hippocampus and is important in long-term potentiation (Steward and Worley, 2002). Following acute nicotine treatment at a dose of 0.4 mg/kg, an increase in *arc* expression was evident in specific forebrain regions of both the adolescent and adult compared with saline controls (Fig. 2B). In regions of the adolescent cortex, however, this nicotine-induced increase was much greater than that observed in the adult, particularly in prefrontal and sensorimotor cortical regions (Fig. 2B). However, baseline (saline treated) *arc* mRNA expression was found to be higher in the adolescent compared with the adult brain. This baseline difference may be linked to alterations in active synaptogenesis in the adolescent brain, as *arc* expression in dendritic spines has been correlated to synaptogenesis in opioidresponsive neurons in rat caudate-putamen (Wang and Pickel, 2004). To determine whether the induction of *arc* in specific cortical regions was due to a differential increase over baseline levels, we used densitometry to compare *arc* expression levels between regions and ages.

Statistical analysis confirmed the enhanced nicotine-induced increase in *arc* expression observed in adolescent cortical regions (Fig. 2A). A significant age×treatment interaction was present [$F(1,20)=4.853$, $P=0.0395$] for *arc* in cortical, but not striatal regions, indicating a differential effect of nicotine on *arc* expression in adolescent animals. ANOVA analysis of cortical regions further indicated significant main effects of age [$F(1,20)=31.210$, $P<0.001$], treatment [$F(1,20)=28.490$, $P<0.001$], and region [$F(3,30)= 8.881$, $P<0.001$]. Significant main effects were also present in the subcortical (striatal) regions for age [$F(1,20)=17.613$, $P<0.001$], treatment [$F(1,20)=6.525$, $P=0.05$], and region [$F(1,20)=11.887$, $P<0.01$]). This overall profile as well as significant age×region interactions in both the cortical [$F(3,60)=2.797$, $P>0.05$) and subcortical analyses ($F(3,60)=10.841$, $P>0.005$) indicates that the brains of adolescent animals expressed more *arc* mRNA overall.

A more focused ANOVA on select brain areas was performed to further determine region-specific effects of nicotine on *arc* expression. Most strikingly, a significant age×treatment interaction was present in the VLO cortex [$F(1,20)=4.932$, $P=0.0381$] indicating a differential induction of *arc* mRNA in this region of the adolescent prefrontal cortex compared with the adult. In the VLO, adolescent rats given nicotine displayed a 182% increase in *arc* expression relative to their saline counterparts compared with a 98% increase in adults given nicotine compared with adults given saline. Trends toward a significant age×treatment interaction were also present in the adolescent medial prefrontal [$F(1,20)=2.684$, $P=0.1170$] and sensorimotor cortex [$F(1,20)=3.939$, $P=0.0611$], suggesting that the differential induction of *arc* expression in the adolescent may be present in a number of cortical regions. In the mPFC, *arc* expression in nicotine-treated adolescents was increased 114% from saline treatment, whereas adult nicotine treatment values were 53% higher than saline counterparts. Interestingly, in sensorimotor cortex, adolescents expressed *arc* only 130% more when given nicotine compared with saline, whereas adults given nicotine expressed 229% more *arc* than saline-treated animals. Significant main age effects were present in all regions examined, indicating increased *arc* expression in adolescent brain occurred in a global manner. Significant main effects of treatment for both adolescents and adults were present for all regions except DS, indicating that *arc* expression increased after nicotine administration.

A lower dose of nicotine, 0.1 mg/kg, was tested to determine if the observed increases in *arc* expression were dose dependent. At this dose of nicotine, no age×treatment effects were present, although adolescents again expressed more *arc* overall [$F(1,88)=29.342$, $P<0.001$], and nicotine tended to increase *arc* mRNA expression [$F(1,88)=3.784$, $P=0.05$] (Fig. 3), confirming the previous experiment. This suggests that the differential expression of *arc* observed at 0.4 mg/kg was not due merely to a general increased sensitivity of adolescents to nicotine.

The elevation in *arc* expression in the adolescent cortex following nicotine administration occurred in a layer-specific pattern (Fig. 4). In saline-treated adolescents, *arc* expression was primarily evident within the deeper layers of the cortex. An example of this expression pattern in S1 region of the frontal cortex is shown in Fig. 4A. Following nicotine treatment, in addition to an accumulation of *arc* mRNA within the deeper cortical layers, a dramatic elevation in *arc* mRNA was evident within layer IV (Fig. 4B).

High magnification micrographs of sections subjected to *arc in situ* hybridization and emulsion autoradiography indicated that silver grain accumulation occurred primarily over cells containing large, pale-staining nuclei, consistent with labeling over cells of a neuronal morphology (arrows in Fig. 5). An example of a typical labeling pattern in adult and adolescent cortex is shown for the VLO (Fig. 5), a region where a differential induction of *arc* in adolescents relative to adults was evident. Although basal levels of *arc* in the VLO were higher

in the adolescent than the adult (compare Fig. 5C and D), a greater nicotine-induced increase in *arc* was present in the adolescent (Fig. 5E) compared with the adult (Fig. 5F).

Cell counts of the VLO were performed to determine whether the increase in *arc* expression following nicotine administration was due to an increase in the number of cells expressing *arc* or an increase in *arc* expression within cells that express the mRNA in the basal state. We found that the number of *arc* mRNA-expressing cells in VLO was similar in adults and adolescents and did not change after nicotine treatment (adolescent nicotine, 211±33; adolescent saline, 197±23; adult nicotine, 180±15; adult saline, 191±9) suggesting that *arc* expression increased in cells that expressed *arc* in the basal condition.

Fos mRNA is induced in adolescent forebrain following nicotine treatment

We also examined the influence of acute nicotine treatment on the expression of the early response transcription factor, *c-fos*. Interestingly, *c-fos* expression patterns following nicotine treatment in both the adolescent and adult forebrain were similar to the expression patterns observed for *arc* (compare Fig. 6B with 2B). As with *arc* expression, the highest levels of *c-fos* were observed in adolescent VLO and SmCtx (Fig. 6A). However, no significant age×treatment interactions were found for cortex, indicating that nicotine increases *c-fos* expression to the same degree in adolescents as in adults. Statistical analysis of cortical regions indicated significant main effects of age [$F(1,20)=14.999$, $P<0.001$], treatment [$F(1,20)=21.648$, $P<0.001$], and region [$F(3,60)=26.878$, $P<0.001$]. Significant main effects were also present in the subcortical (striatal) regions for age [$F(1,20)=17.613$, $P<0.001$], treatment [$F(1,20)=13.867$, $P=0.001$], and region [$F(1,20)= 5.919$, $P<0.05$). However, a significant age×region interaction was present only for cortex [$F(3,20)=5.067$, $P>0.05$], as the adolescents displayed greater cortical expression of *c-fos*, suggestive of altered plasticity mechanisms in these areas.

The more focused ANOVA indicated that significant main effects of age and treatment were present in all regions, with the exception of DS, in which there were no significant treatment effects. In contrast with the effects of nicotine on *arc* expression, no significant interactions were present for any regions, although a strong trend toward a significant age×treatment interaction was present in the VS [$F(1,20)=4.210$, $P=0.0535$)], where acute nicotine appeared to cause a larger increase in *c-fos* mRNA levels in the adolescent than in the adult. High magnification micrographs of sections subjected to emulsion autoradiography again indicated that silver grain accumulation occurred primarily over cells containing large, pale-staining nuclei, consistent with labeling over cells of a neuronal morphology (Fig. 5C). We found no difference in *c-fos* mRNA-expressing cells in VLO between adults and adolescents and no change after nicotine treatment.

A similar induction of NGFI-B is evident in adolescent and adult forebrain after acute nicotine

NGFI-B is an immediate early gene encoding an orphan nuclear receptor that is rapidly recruited under a variety of stimuli (Svenningsson et al., 1995; Werme et al.,2000a,2000b), and we therefore examined the expression of this gene following nicotine administration. Unlike the pattern of expression seen for *arc* and *c-fos*, acute nicotine did not produce any differential age effects for *NGFI-B*, although nicotine treatment generally increased *NGFI-B* expression (Fig. 7). The overall analysis of *NGFI-B* revealed significant main effects of treatment in both cortical [$F(1,20)= 5.087$, $P>0.05$] and subcortical regions [$F(1,20)=4.463$, $P>0.05$]. Main effects of region were also present for both cortex [$F(3,60)=11.598$, $P<0.001$] and striatum [$F(3,60)=33.055$, $P>0.001$]. However, no main effects of age were present. A significant treatment×region interaction was found for cortex [$F(3,60)=6.476$, $P>0.001$], but not striatum, suggesting that nicotine affected *NGFI-B* expression more strongly in cortical regions than subcortical. Conversely, a significant age×region interaction was present in the

striatum [$F(3.60)=9.942$, $P=0.005$] but not cortex. In Fig. 7, it is apparent that the overall levels of *NGFI-B* expressed in the DS may be higher in adolescents than adults. Silver grain accumulations in emulsion-treated slides were similar to *arc* and *c-fos* suggesting expression of *NGFI-B* in neurons (Fig. 7c).

QDR expression is not affected by acute nicotine treatment

We were interested in determining whether acute nicotine administration affected the expression of *QDR*, an enzyme implicated in monoamine and nitric oxide biosynthesis (Turner et al., 1974;Gorren and Mayer, 2002). In a preliminary microarray analysis directed at identifying genes whose expression patterns differed in the adolescent compared with the adult brain, *QDR* was found to be expressed at higher levels in adult brain (Schochet et al., 2002). Further, *QDR* was present primarily in white matter and was expressed in oligodendrocytes (not shown). An examination of major white matter regions following saline or nicotine treatment did not reveal any effect on *QDR* expression (Fig. 8A), although intense expression of *QDR* was evident in the adult forebrain (Fig. 8B). Therefore, *QDR* served as a non-affected "control" gene for our analysis.

DISCUSSION

These studies report three novel observations. First, the expression of *arc*, a dendritically targeted gene important for synaptic plasticity and involved in learning and memory, is upregulated in adolescence, and moreover, differentially increased in specific cortical regions of the adolescent compared with the adult following acute nicotine administration. Second, the plasticity-related genes *arc* and *c-fos*, but not *NGFI-B*, are higher in the baseline state in adolescent forebrain compared with adults. Third, the oligodendrocyte-enriched gene *QDR* is markedly more abundant in the adult compared with the adolescent brain, although its expression is not affected by nicotine. Taken together, these results suggest that a dynamic developmental profile of the expression of specific molecular markers is present in adolescent brain, and that acute nicotine influences the expression of plasticityassociated markers.

Maturation of the adolescent brain

The prefrontal cortex, an area of adolescent brain where we found differential induction of *arc* following acute nicotine, undergoes dramatic changes during adolescence including extensive synaptic pruning, alterations in dopaminergic input and changes in intrinsic circuitry (Rosenberg and Lewis, 1995;Cunningham et al., 2002;Erickson and Lewis, 2002;Cruz et al., 2003). Anatomical tracing studies indicate that projections from the amygdala to mPFC continue to increase in density throughout adolescence (Cunningham et al., 2002), suggesting that connectivity between emotional and cognitive areas undergoes refinement during this period. Further, cortical gray matter volume changes dynamically during adolescence in areas including mPFC and orbital prefrontal cortex (Kolb and Nonneman, 1976;Seeman et al., 1987;Giedd, 1999;Giedd et al., 1999;Seeman, 1999). Similarly, cerebral white matter volume increases throughout the adolescent period (Giedd et al.,1996a,1996b) in parallel with myelination of intrinsic fiber connections (Nauta, 1971;Benes, 1989;Paus et al., 1999). These findings suggest that plastic changes occur within the mammalian forebrain throughout the adolescent period.

Basal and nicotine-induced acute arc expression levels in the brain are age-dependent

Arc appears to be part of this developing substrate. *Arc* is an immediate-early gene that marks synapses undergoing modification (Lyford et al., 1995), is implicated in activitydependent plasticity and memory (Steward et al., 1998;Steward and Worley, 2002;Kelly and Deadwyler, 2003) and accumulates in dendrites at sites of recent synaptic activity (Guzowski et al., 2000;Guzowski, 2002). Additionally, *arc* is upregulated following amphetamine and cocaine

administration in a number of brain regions (Fosnaugh et al., 1995;Yamagata et al., 2000). For the first time, we show that *arc* is also strongly induced by nicotine in adult and adolescent rats in a dose-dependent manner, suggesting that the effects of nicotine involve alterations in genes and proteins regulating the post-synaptic density. Higher basal and drug-induced expression of *arc* in the adolescent suggests that altered underlying cortical plasticity reflective of an active state of synaptic modeling is present in the forebrain during this stage of development.

Within the cortex, nicotine altered the distribution of *arc* expression in a layer-specific manner. *Arc* expression was higher in the adolescent than the adult in cortical layer VI, a cortical output layer. Following acute nicotine, layer IV, which receives heavy input from the thalamus, was strongly recruited (Guillery and Sherman, 2002). Thus, this profile of increased *arc* expression suggests that nicotine has a profound effect on plasticity in integrative thalamocortical networks.

Differential induction of *arc* in the nicotine-treated adolescent was greatest in the ventrolateral orbital region of the prefrontal cortex, an area of brain important for maintaining representations of reward value and in guiding goal-directed responses (Pickens et al., 2003;Rolls, 2004). The occurrence of nicotine-induced gene induction primarily in cortical rather than striatal regions perhaps reflects a preferential effect of nicotine on cognitive and attentional functions, which may be immature in the adolescent (Benes et al., 2000). Immaturity of systems involved in reward and cognition is implied by recent electrophysiological data showing NMDA-D1 enhancement of depolarized “up states” in prefrontal cortex is not present in early adolescence and develops only in adulthood (Tseng and O’Donnell, 2005). In addition, a less pronounced effect of nicotine on *arc* expression in adolescent striatum may be due to differences in relative maturity of this region compared with cortical regions, as in fact, striatal neurons reach morphological and electrophysiological maturityprior to adolescence (Tepper and Trent, 1993). Given that *arc* is known to be regulated by NMDA and dopamine receptors, the upregulation of *arc* induced by nicotine may be mediated by these receptor systems. Additionally, given the well-established effects of nicotine on cholinergic function, a role for nicotinic receptors in the gene response to nicotine should not be excluded. Indeed, upregulation of nicotinic receptor subtypes and increases in cholinergic receptor binding have been reported in midbrain, cortex and hippocampus following adolescent nicotine exposure (Trauth et al., 1999;Adriani et al., 2004). Adolescent nicotine exposure also increases nicotine self-administration in adulthood (Adriani et al., 2004).

As our analysis only considered forebrain regions, other age-related effects may be present in additional brain areas. In fact, in contrast to more rostral brain regions, induction of *arc, c-fos* and *NGFI-B* in SmCtx was less pronounced in adolescents given nicotine than in adults.

These results are consistent with recent studies showing that the time-course of the development of nicotine-induced *c-fos* expression varies in different sensory cortical and limbic regions (Leslie et al., 2004). It will be interesting to determine whether other brain regions not examined in the current study, such as the hippocampus and amygdala, show similar differences in nicotine-induced early response gene expression between adolescents and adults.

The possibility that plasticity mechanisms with regard to drug exposure are different in adult and adolescent brains is intriguing, given there are differences in the behavioral response to nicotine and other drugs of abuse in adolescent and adult rats (Spear, 2000;Vastola et al., 2002;Belluzzi et al., 2004). For example, adolescent rats show blunted locomotor sensitization to repeated nicotine, compared with adults, and reduced cue-conditioning (Collins et al., 2004a;Schochet et al., 2004). In fact, the lack of nicotine cue conditioning in the adolescent compared with the adult may relate to ceiling levels of *arc* expression in the prefrontal cortex,

a region critical for expression of contextual conditioning (Schroeder et al., 2001; Schiltz et al., 2003;Schochet et al., 2004).

The immediate early genes c-fos and NGFI-B are upregulated by nicotine, but not in an age-dependent manner

c-fos, a well-characterized transcription factor, was induced in the adolescent brain following acute nicotine. Consistent with these data, age-dependent effects have been demonstrated for *c-fos*, which has been shown to progressively increase in the prefrontal cortex and striatum during the adolescent period (Kellogg et al., 1998;Andersen et al., 2001;Leslie et al., 2004). Since higher levels of *c-fos* mRNA were evident in the adolescent fore-brain following saline treatment, it is likely that the threshold for induction of this gene is lower in the adolescent brain than in the adult, suggesting that basal neuronal activity may be higher in the adolescent. Nicotine and other drugs of abuse increase *c-fos* expression under D1 and NMDA receptor control (Pagliusi et al., 1996;Nisell et al., 1997;Pich et al., 1997;Ostrander et al., 2003) and can be blocked by dopamine and glutamate antagonists (Kiba and Jayaraman, 1994;Konradi et al., 1996;Liu and Weiss, 2002).

An induction of *NGFI-B* (*Nur77*) following acute nicotine administration has not been previously reported. *NGFI-B* is an orphan nuclear receptor belonging to a larger class of steroid-thyroid hormone receptors and has been associated with dopaminergic target structures (Zetterstrom et al., 1996). Chronic morphine or cocaine administration, as well as other manipulations, alters the expression of *NGFI-B* in cortex, striatum, and accumbens (Svenningsson et al., 1995; Werme et al.,2000a,2000b). Of the three early-response genes studied, *NGFI-B* was the only gene whose expression pattern was the same in adolescents and adults regardless of treatment, indicating it is less influenced by a developmental context. Taken as a whole, the observation of changes in *arc* and *c-fos*, but not *NGFI-B* expression, suggests that the downstream targets of *arc* and *c-fos* may be more affected by adolescent development than targets of *NGFI-B*.

QDR is expressed in an age-dependent manner, but is not affected by nicotine administration

QDR was expressed at higher levels in the adult compared with the adolescent brain in a distribution suggesting principal expression in white matter (oligodendrocytes). *QDR* is required in the regeneration of tetrahydrobiopterin, a cofactor essential for the function of aromatic amino acid hydroxylases and nitric oxide synthases, implicating the enzyme in several important biosynthetic pathways (Turner et al., 1974;Kaufman et al., 1975;Gorren and Mayer, 2002). Our observation that *QDR* is expressed primarily in oligodendrocytes suggests age-related differences in biosynthetic mechanisms that may be related to differences in white matter volume and myelination observed in the adolescent.

CONCLUSIONS

These results have special relevance to the problem of nicotine addiction, a major contributor to morbidity and mortality in this country. Although the precise underlying causes and substrates of addiction are unknown, a major theory posits that addictive drugs interact with and influence primary motivational systems, and in particular the plasticity mechanisms within these systems (Jentsch and Taylor, 1999). Our results show that in adolescence, not only is certain gene activity higher in brain areas crucial for emotional regulation and decision-making, but that nicotine affects key plasticity molecules in these areas in a manner different from that the adult brain. Thus, adolescence may represent a neurobiologically vulnerable period for drug abuse.

Acknowledgements

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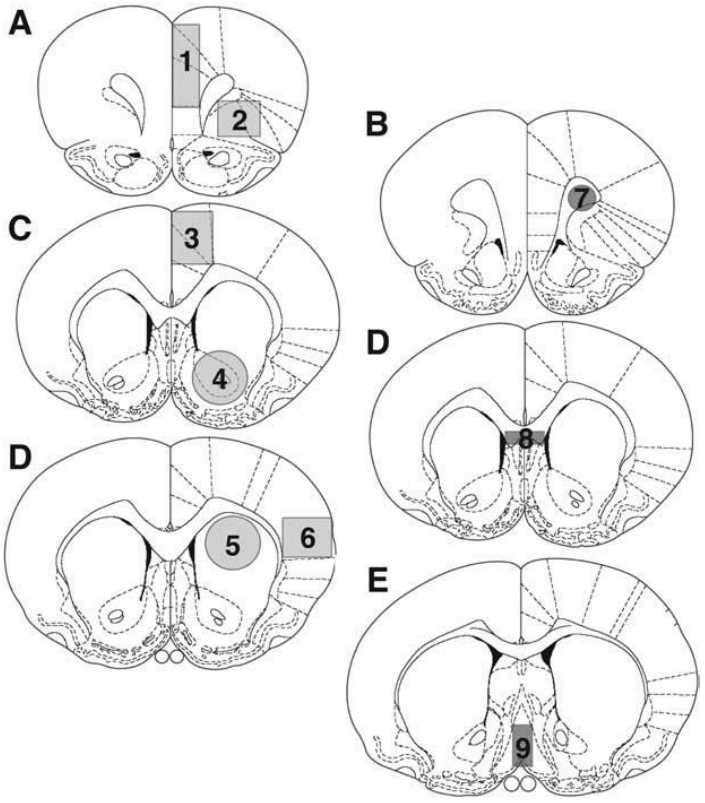

Fig. 1.

Schematic diagrams of brain regions selected for gene expression analysis. Coronal forebrain sections were subjected to *in situ* hybridization and the numbered regions further analyzed using densitometry as described in Experimental Procedures. The light gray numbered regions were analyzed for *arc*, *c-fos* and *NGFI-B* and the dark gray numbered regions for QDR. Distance in mm from bregma: A, 3.70; B, 2.70; C, 1.60; E, 1.60; D, 1.20; F, 0.70. 1, Medial prefrontal; 2, ventrolateral/lateral orbital; 3, cingulate; 4, VS; 5, DS; 6, sensory motor; 7, fmi; 8, the gcc; 9, the VDB.

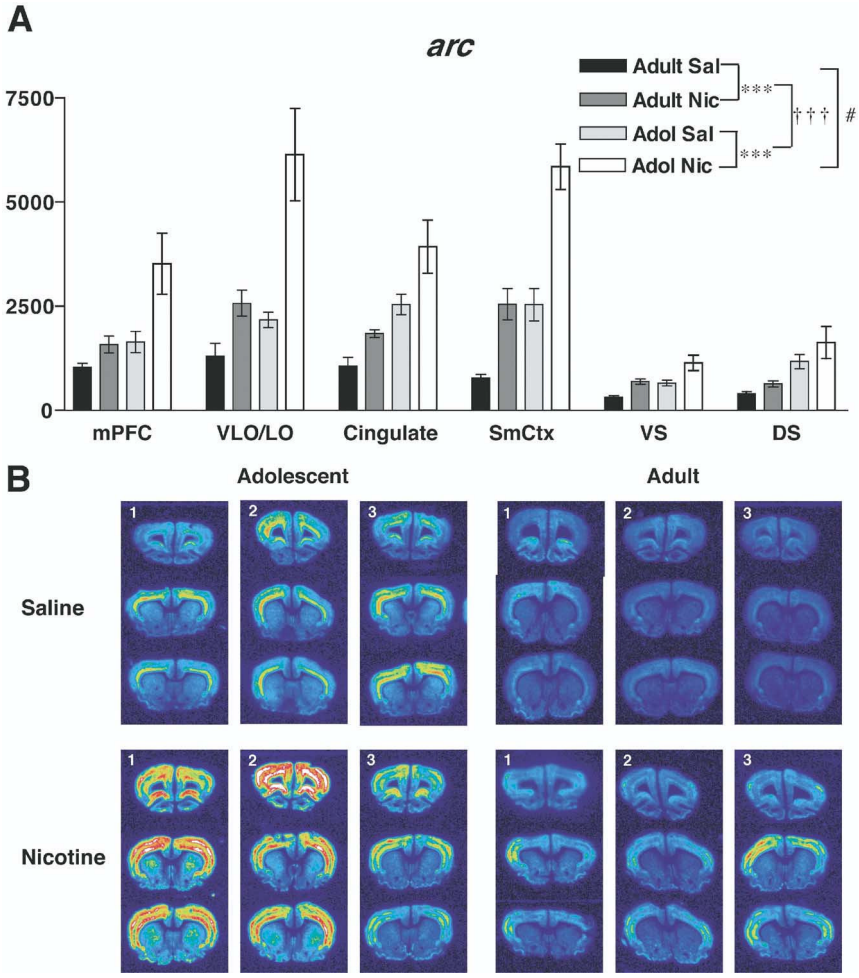

Fig. 2.

Arc expression is differentially induced in specific regions of the adolescent forebrain following acute nicotine (Nic) administration at 0.4 mg/kg. (A) Regions of the adolescent and adult forebrain were analyzed using densitometry after sections were subjected to *in situ* hybridization using ^{35}S-labeled probe to *arc* mRNA. Relative signal intensity is shown in optical density units. Note the dramatic elevation in *arc* mRNA expression in the adolescent brain following acute Nic (white bars). Although basal levels of *arc* were higher in adolescent (light gray bars) compared with adult (black bars), a differential induction of *arc* mRNA was evident in the VLO/lateral orbital area (LO) of the prefrontal cortex. (B) Coronal forebrain sections from three representative adolescent and three adult animals treated with either saline (Sal) or Nic were processed for *in situ* hybridization with *arc* probe. Images from each section were color rendered to enable visualization of *arc* induction (white-red, highest and blue, lowest levels). A pronounced induction of *arc* was evident in specific regions of adolescent forebrain following Nic treatment. *** Treatment effect, $P<0.001$; ††† age effect, $P<0.001$; $^{\#}$ age×treatment interaction, $P<0.05$.

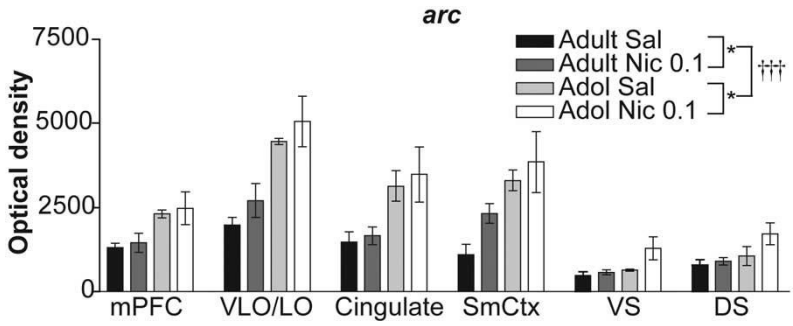

Fig. 3.

Arc expression is not differentially induced following acute administration at 0.1 mg/kg. Bar graph of densitometry data showing signal density in specific forebrain regions. Nic, nicotine, Sal, saline; LO, lateral orbital cortex; * treatment effect, $P=0.05$; ††† age effect, $P<0.001$.

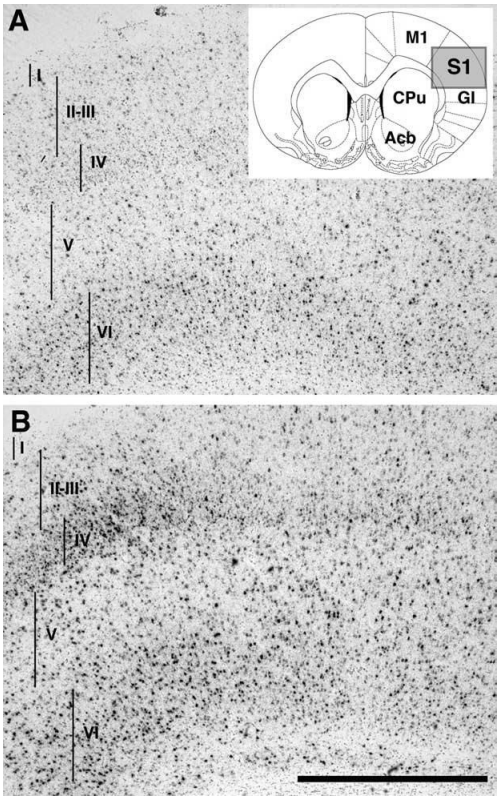

Fig. 4.

Arc mRNA accumulation in adolescent cortex following nicotine administration occurs primarily in layers IV to VI. (A) Representative cortical section from a saline-treated adolescent rat following *in situ* hybridization and emulsion autoradioagraphy showing silver grain accumulations primarily in layer VI. Gray square in inset represents the region of cortex depicted in A and B. (B) Coronal section from a nicotine-treated adolescent showing a dramatic induction of *arc* signal in layer IV and enhanced expression of arc in layers V and VI. Acb, nucleus accumbens; CPu, caudate-putamen; G1, M1 and S1, regional subdivisions of frontal cortex. Scale bar=1mminA,B.

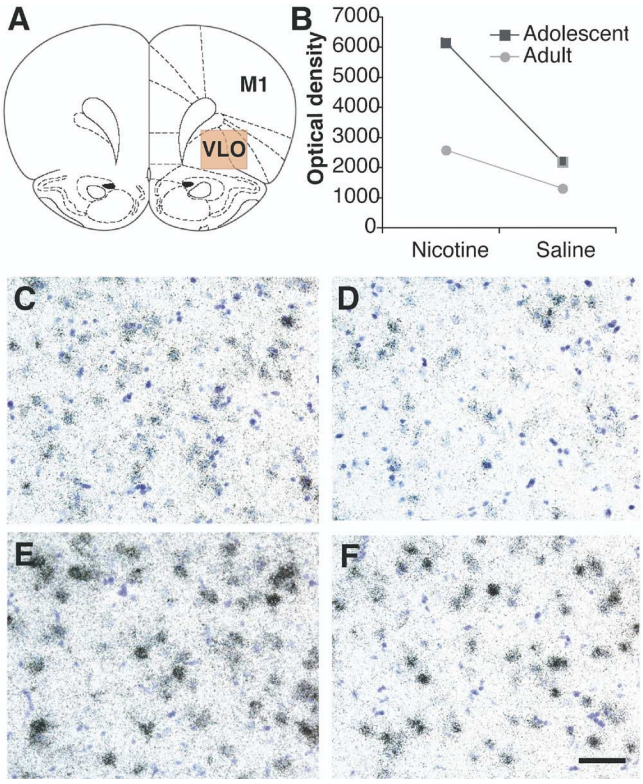

Fig. 5.

Nicotine induces *arc* expression in neurons of the ventrolateral orbital cortex. (A) Coronal forebrain sections were analyzed for optical density on a phosphorimager and autoradiography using liquid emulsion. Boxed area represents the regions of the VLO depicted as an interaction plot in B and photomicrographs in C-F. (B) Interaction plot showing the differential elevation of *arc* mRNA in adolescent VLO following nicotine administration. High magnification of counter stained emulsion sections of adolescent (C) and adult (D) saline and adolescent (E) and adult (F) nicotine. Silver grain accumulation corresponding to *arc* mRNA is evident over violet Nissl-stained tissue. Note the pronounced accumulation of signal in large, spherical patterns suggestive of *arc* mRNA in neurons. M1, region of frontal cortex. Scale bar=20 μm in C-E.

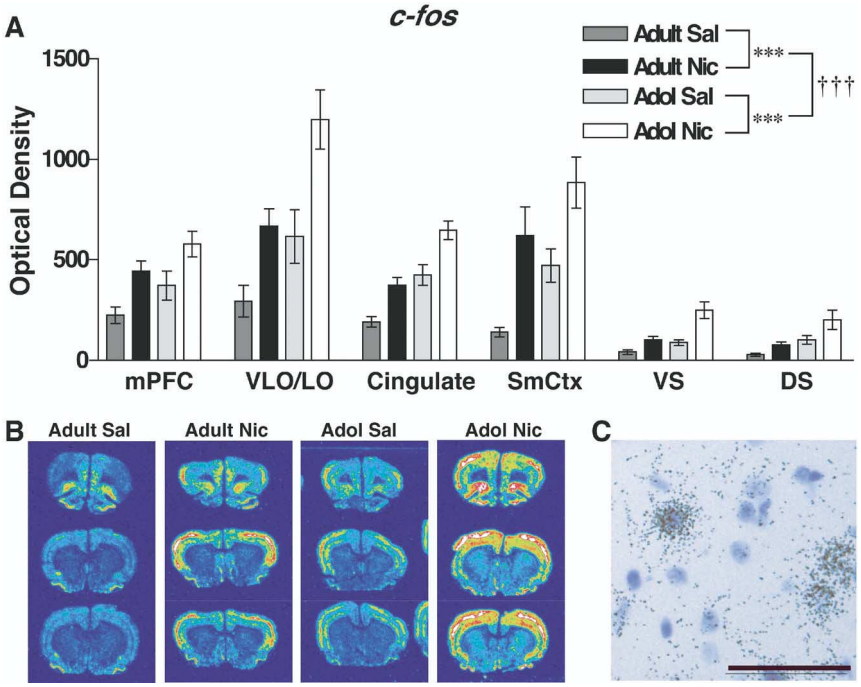

Fig. 6.

c-fos Is induced in the adolescent and adult forebrain following acute nicotine (Nic) administration. (A) Bar graph of densitometry data showing signal density in specific forebrain regions. (B) Representative sections from adolescent and adult animals treated with either saline (Sal) or Nic. Color rendering depicts heightened expression of *c-fos* in adolescent forebrain following acute Nic treatment. (C) Photomicrograph of an emulsion-dipped section from Nic-treated adolescent brain showing silver grain accumulation in a pattern suggestive of *c-fos* mRNA in neurons. *** treatment effect, $P<0.001$; ††† age effect, $P<0.001$. Scale bar=20 μminC.

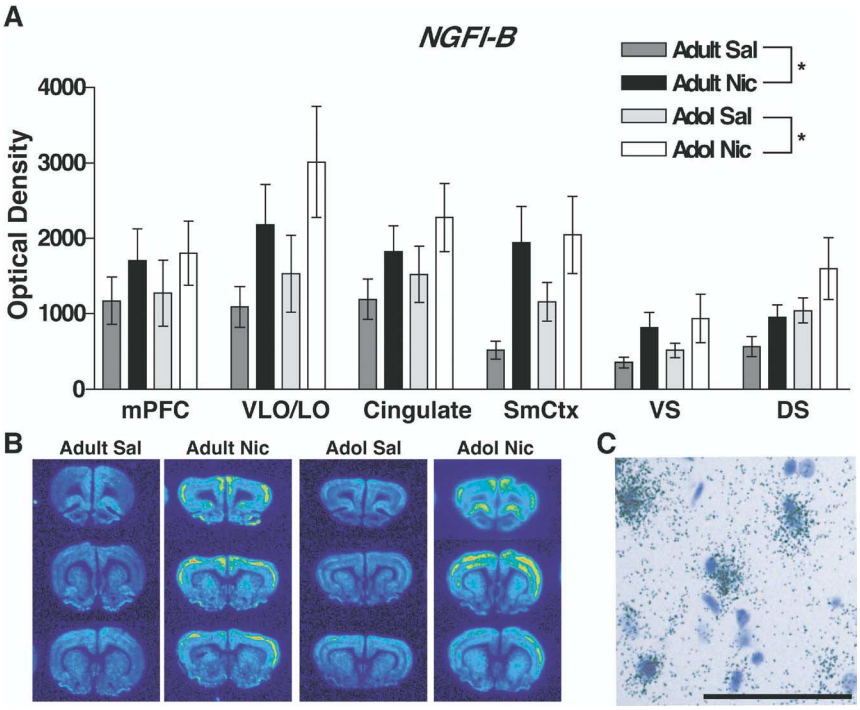

Fig. 7.

NGFI-B mRNA expression was elevated in the forebrain of adolescent and adult rats following nicotine (Nic) treatment. (A) Bar graph of optical density scans showing a moderate induction of *NGFI-B* in specific forebrain regions. (B) Coronal forebrain sections from adolescent and adult rats color rendered to show *NGFI-B* mRNA expression. (C) Photomicrographs of sections subjected to emulsion autoradiography showed silver grain accumulation over cells containing large, pale, Nissl-stained nuclei consistent with neurons. Sal, saline; * treatment effect, $P<0.05$. Scale bar=20 μm in C.

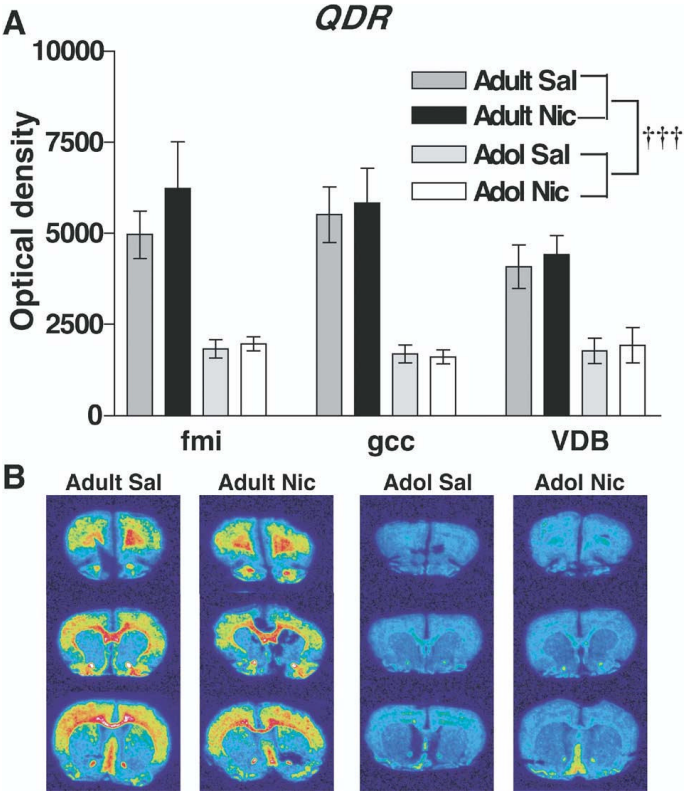

Fig. 8.

QDR is expressed at higher levels in adult compare with adolescent forebrain. (A) Optical density bar graphs of specific white matter regions from adolescent and adult rats treated with either saline (Sal) or nicotine (Nic). Although Nic had no affect on expression, *QDR* mRNA was more abundant in adult forebrain. (B) Representative coronal forebrain sections from Nicor Sal-treated adolescent or adult rats color rendered to illustrate expression of *QDR* mRNA. ††† Age effect, $P<0.001$.