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Contextual cues associated with nicotine administration increase arc mRNA expression in corticolimbic areas of the rat brain

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Abstract

Conditioned responses to cues associated with the administration of drugs of misuse are an impediment to continued abstinence for drug-free addicted individuals. In order to study the neuroanatomical and cellular response of the brain to cues associated with nicotine administration, we conditioned Sprague–Dawley rats to receive an ascending dose regimen of nicotine over 14 days in two distinct non-home cage environments and assessed expression of the early response gene *arc* in corticolimbic areas in response to the nicotine-associated context. All of the rats received the same dose regimen of nicotine. Three days after the last training day, the rats were exposed to the test environment. The rats that had previously received nicotine exhibited increased motor activity compared with the rats that had received saline in the test environment. After 45 min in the test environment, brains were taken for Northern blotting and *in situ* hybridization analysis, which revealed an increase in levels of activity-regulated, dendritically localized mRNA for *arc* in a variety of brain regions (medial and lateral prefrontal cortices, cingulate cortex, primary sensory cortex, sensorimotor cortex, ventral striatum and amygdala). Plasma corticosterone levels were not different between the groups, suggesting that exposure to nicotine cues is insufficient to activate the hypothalamo-pituitary-adrenal axis. Given that Arc plays a direct role in neuronal plasticity and memory consolidation, its induction by nicotine-associated cues in brain regions critical for cognitive and emotional processing suggests that rats may be learning that these cues are no longer necessarily predictive of nicotine administration. Further work will be needed in order to assess the role of *arc* expression in the extinction of conditioned responses to drug-paired cues.

Keywords

addiction; immediate-early gene; prefrontal cortex; relapse; Sprague—Dawley

Abbreviations

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ISH, *in situ* hybridization

Introduction

Continued abstinence from drugs of misuse is a major challenge for addicted individuals. Although current therapeutic interventions for addiction are effective on a par with treatments for other chronic medical conditions (Leshner, 1999), advances in understanding the neurobiological factors leading to relapse are needed for significant gains in positive outcomes to occur. Three precipitants of relapse (and reinstatement of drug-seeking behavior in animal

models) include administration of the drug itself ('priming'), acute stress and the presentation of drug-associated cues (Shalev *et al.*, 2002; Shaham *et al.*, 2003).

Environmental cues associated with drug administration are capable of eliciting a variety of physiological and psychological conditioned responses when animals are re-exposed to them. Interactions between the environment and internal states generated by nicotine underlie associative learning in corticolimbic circuits conferring reinforcing and motivational properties to drug-associated cues (Robbins & Everitt, 2002). For example, administration of drugs of misuse in a novel environment potentiates the expression of immediate-early genes necessary for neuronal plasticity in cortical and limbic brain regions (Uslaner *et al.*, 2001, 2003; Klebaur *et al.*, 2002; Ostrander *et al.*, 2003; Ferguson *et al.*, 2004). Subsequent administration of drug within the same environment results in increases in motor responses to the same dose of the drug, a phenomenon known as behavioral sensitization (Badiani *et al.*, 1995; Anagnostaras & Robinson, 1996; Anagnostaras *et al.*, 2002). Thus, neuronal plasticity resulting from repeated pairings of drugs and cues underlies alterations in behavior such as sensitization, conditioned locomotor hyperactivity, conditioned place preference and reinstatement of drug-seeking behavior in response to drug-cues, including those with nicotine (Caggiula *et al.*, 2001, 2002a, Caggiula *et al.*, b; Robbins & Everitt, 2002).

The neuroanatomical substrates underlying conditioned responses to drug-cues in humans have been identified using functional neuroimaging. Regional brain metabolism is increased in the anterior cingulate and orbitofrontal cortices and anterior temporal lobe by presentation of visual and tactile cues associated with smoking (Brody *et al.*, 2002). These authors showed that metabolic activity in bilateral orbitofrontal and dorsolateral prefrontal cortices and anterior insula and the right sensorimotor cortex were positively correlated with self-reports of cue-induced cigarette craving. Fos immunohistochemistry reveals a similar neuroanatomical activation profile in the rat brain after rats are exposed to a context associated with passive nicotine administration (Schroeder *et al.*, 2001).

It is unclear whether neural activation within corticolimbic circuits in response to drug cues results in alterations in the expression of genes known to play a role in synaptic plasticity. Arc, unlike activity-regulated transcription factors such as Fos, has direct effects on neuronal processes governing postsynaptic signaling efficacy (Shepherd *et al.*, 2004) and is necessary for long-term memory formation (Guzowski *et al.*, 2000). The expression of *arc* mRNA can therefore be used not only as a marker for alterations in neuronal activity (Lyford *et al.*, 1995; Guzowski *et al.*, 1999) but also as a marker for neuronal plasticity. In order to characterize further the neuroanatomical and cellular response to nicotine cues, we used an animal model of conditioned responses to contextual cues associated with prior administration of nicotine combined with Northern blotting and *in situ* hybridization (ISH) analysis for *arc* in a variety of corticolimbic areas.

Materials and methods

Subjects

Twenty-four male Sprague–Dawley rats (Harlan, Madison, WI, USA) weighing between 250 and 300 g at the beginning of training were used in this study. Pairs of rats were housed in clear plastic cages in an animal colony. Food and water were available at all times except during training and testing. Lighting in the animal colony was on a 12-h light/dark cycle with lights on at 07:00–19:00 h. Rats were handled daily for 3 days prior to the beginning of training. All efforts were made to minimize the number of animals used. All animal care was in strict accordance with our Institutional Animal Care and Use Committee guidelines.

Nicotine conditioning procedure

All rats received a daily nicotine injection using an escalating dose regimen ($n = 24$, 0.40 mg/kg days 1–5, 0.50 mg/kg days 6 and 7, 0.63 mg/kg days 8 and 9, 0.80 mg/kg days 10–14, s.c., dissolved in saline, pH 7.2, Sigma, St Louis, MO, USA) in one of two distinct, non-home cage contexts for 14 days (Fig. 1A). An escalating dose regimen was used in order to minimize any effect of stress from higher-dose nicotine treatments on conditioning. Subjects were randomly assigned to receive injections of nicotine or normal saline (s.c., 1 mL/kg) in context 1 and the alternative treatment in context 2. Although training and housing were carried out in similar polycarbonate cages (19 × 10 × 8 in, San Diego Instruments, San Diego, CA, USA), context 1 and context 2 were distinct from the home cage environment and from each other in a variety of ways. Cages in context 1 had wire mesh floors over aspen chips providing distinct visual, somatosensory and olfactory cues. Cages in context 2 had plain plastic floors and were scented with vanilla extract. Home cages had ground corn cob bedding and food and water present at all times. Daily, rats were placed in context 1 for 90 min following an injection of nicotine or saline. A photobeam activity system (San Diego Instruments) kept track of total horizontal, ambulatory and rearing activity counts (as described in Schroeder *et al.*, 2001) during the daily training sessions. Data were collected on an attached PC in 10-min intervals for 90 min. After 90 min, all rats were returned to the home cages for 30–45 min. The rats were then placed in context 2 after receiving the alternative injections (i.e. rats that received nicotine in context 1 received saline in context 2 and vice versa). After 90 min in context 2, all rats were returned to their home cages until the next day of training. Training was always conducted between 12:00 and 17:00 h. All rats received the same number of injections and the same nicotine dose regimen but in two distinct environments (see Fig. 1A).

The ‘saline cues’ control group in these experiments addresses some caveats revealed in our previous studies. The use of this control group, which received the same nicotine dose regimen as the test group but received nicotine in a distinctly different environment from the test cages and the home cages, ensured that any behavioral conditioned responses observed on the test day, and associated changes in gene expression, were due to the environment alone and not treatment history or an interaction of prior treatment with general arousal.

Three days following the final training day, rats were reintroduced into context 1, where they were exposed to either nicotine or saline cues depending on which group they were assigned to during training. Following a mock injection, the rats remained in context 1 where motor activity was measured for 45 min; they were then anesthetized with halothane and decapitated for collection of trunk blood and for brain dissection. Trunk blood was used to assay levels of plasma corticosterone in order to assess whether a stress response is part of the conditioned response. The brain was used for Northern blot analysis and ISH analysis.

Tissue collection

After decapitation, approximately 10 mL of trunk blood was collected in 15-mL conical tubes containing 100 μ L of 0.1 M EDTA (Sigma). Tubes were kept on ice until centrifugation at 1700 g at 4 °C for 15 min. Plasma was then stored at –80 °C until assayed. A rat corticosterone EIA kit (ICN Diagnostics, Costa Mesa, CA, USA) was used to quantify plasma corticosterone levels. Intra-assay variability was < 6%.

Heads were cooled for 30 s in –70 °C isopentane. Brains were rapidly dissected from cooled heads and frozen in –40 °C isopentane. Frozen brains were kept at –80 °C until they were used. Two of the brains ($n = 1$ per group) were sectioned at 20 μ m and thaw mounted onto Superfrost slides (Fischer Scientific), warmed at 57 °C for 1 min, and stored at –80 °C for ISH. Twelve of the brains ($n = 6$ per group) were sectioned for ISH (20 μ m) and for lateral prefrontal cortex dissection (see below). Ten of the brains ($n = 5$ per group) were sectioned (500 μ m) and the

lateral prefrontal cortex (corresponding to the ventral orbital and lateral orbital cortices, Fig. 1B) was dissected away from the rest of the tissue and stored at -80°C for subsequent RNA isolation and Northern blot analysis. To analyse the expression of *arc*, ten (RNA from one sample in each group was not analysed owing to degradation of the samples) animals from the saline- and nicotine-paired groups were analysed via Northern blot analysis and seven animals per group were analysed via ISH analysis.

arc Northern blot

RNA from lateral prefrontal cortex was isolated using the Nucleo-Spin® RNA II Kit (Macherey-Nagel, Palo Alto, CA, USA) using the manufacturer's suggested protocol. Briefly, tissue punches from brain were homogenized in GITC solution using a tissue tearor™ (Biospec Products, Inc., Bartlesville, OK, USA). Nucleic acids were ethanol precipitated, resuspended in GITC, and then bound to a NucleoSpin™ column (Macherey-Nagel). GITC was removed by centrifugation and samples were DNase treated on the column. DNase treatment was halted with a GITC wash. RNA was then washed twice with 100% ethanol and eluted from the column with RNase-free water. The concentration of RNA was determined using the RiboGreen method (Molecular Probes, Eugene, OR, USA) and RNA quality was assessed using agarose electrophoresis.

One microgram of total RNA from each rat was electrophoresed in individual lanes on a 1.2% agarose/formaldehyde/1×MOPS gel. The gel was transferred to GeneScreen™ plus (Perkin Elmer Life Sciences, Inc., Boston, MA, USA) using Stratagene's PosiBlotter™ (Stratagene, Inc., La Jolla, CA, USA). ^{32}P -labeled cDNA (2×10^6 c.p.m./mL) was hybridized to the membrane in Hybrisol™ I (Intergen, Inc., Norcross, GA, USA). Northern blots were washed and exposed to a phosphorimager screen for 1 day. Screens were scanned on a Storm™ phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA). Quantification was performed using Image-Quant™ software (Molecular Dynamics).

Probes used for Northern blotting were generated from rat prefrontal cortex cDNA using PCR amplification. The following primers were used to generate PCR product for *arc*: forward primer 5'-CCAGGAAGCTGATGGCTACGAC-3' (693–714), reverse primer 5'-GTGTCAGCCCCAGCTCAATCAAG-3' (1471–1494). Numbers in parentheses after the primer sequence represent the base number as defined by the rat *arc* mRNA sequence (accession number NM_019361) in the NCBI nucleotide database. A 983-bp PCR product for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generated as described previously (Roseboom *et al.*, 1996) and was used as a loading control. mRNA from one rat in the nicotine- and saline-paired group was precluded from Northern blot quantification and statistical analysis owing to obvious degradation of the samples (data not shown).

In situ hybridization

Sections on slides were fixed in 4% paraformaldehyde in PBS for 2 h at 4°C . Slides were then washed for 5 min in $2 \times \text{SSC}$ three times. Slides were incubated in 0.2 $\mu\text{g/mL}$ Proteinase K (Qiagen, Hilden, GmbH, Germany) in 0.1 M Tris base and 50 mM EDTA, pH 8.1, for 10 min at 37°C . Slides were washed in $2 \times \text{SSC}$ at room temperature for 2 min. Slides were incubated in 0.1 M TEA at room temperature with rapid stirring, and acetic anhydride was added to a final concentration of 0.25% (v/v) with rapid stirring for 10 min. Slides were then washed in $2 \times \text{SSC}$ for 5 min. Finally, sections were dehydrated in an ascending ethanol series and air dried for 15 min.

Arc template for *in vitro* transcription was generated as described above except that either the forward (sense *arc* probe) or reverse (antisense *arc* probe) was tagged with a T7 polymerase recognition sequence. The specific primer sequences were: forward primer 5'-

CCAGGAAGCTGATGGCTACGAC-3', reverse primer 5'-GGA
TCCTAATACGACTCACTATAGGGAGGGTGTCAGCCCCAGCTC AATCAAG-3' for
antisense template; forward primer 5'-GGATCCTA
ATACGACTCACTATAGGGAGGCCAGGAAGCTGATGGCTACG AC-3', reverse primer
5'-GTGTCAGCCCCAGCTCAATCAAG-3' for sense template (the underlined bases are the
T7 polymerase tag and anchor regions). An *in vitro* transcription reaction using amplified
template was used to generate ³⁵S-labeled *arc* antisense or sense probe. The *in vitro*
transcription was carried out in 1 × Transcription Optimized Buffer, 10 mM DTT, 1 U/μL
RNasin, 0.375 mM ATP, CTP, GTP, 1 U/μL T7 RNA polymerase (all Promega, Madison, WI,
USA), 3.5 μCi/μL [α -³⁵S]UTP (PerkinElmer) and 100 ng template DNA and incubated for 2
h at 37 °C. RQ1 RNase free DNase (Promega) was added at a concentration of 0.15 U/mL and
incubated for an additional 15 min at 37 °C. The labeled probes were purified using ProbeQuant
G-50 Micro columns (Amersham Biosciences, Piscataway, NJ, USA). Radioactivity of labeled
probe was determined via scintillation counting and riboprobe was diluted in hybridization
solution (3 × SSC, 10% dextran sulphate, 1× Denhardt's solution, 0.2 mg/mL tRNA, 50 mM
NaPO₄ buffer, and freshly added DTT to a final concentration of 50 mM) to ~10⁶ c.p.m./100
μL. One hundred microlitres of the hybridization solution at 55 °C with labeled probe was
applied to each slide. Slides were then coverslipped and incubated at 55 °C in a hybridization
chamber saturated with 75% formamide for 16 h.

After hybridization, coverslips were removed and slides were washed in 2× SSC with 2 mM
DTT at room temperature for 10 min, three times. Slides were incubated in 1.5 U/mL RNase
A (Qiagen) in RNase buffer (10 mM Tris/HCl and 0.5 M NaCl, pH 8.0) at 37 °C for 1 h followed
by washes in 1 × SSC with 1 mM DTT at room temperature for 5 min, 0.5 × SSC with 1 mM
DTT at room temperature for 5 min and 0.1 × SSC with 2 mM DTT at 70 °C for 1 h. The sections
were then dehydrated in an ascending series of ethanol and then air dried. Sections were
exposed to a phosphorimager screen for 4 days. Screens were scanned on a Storm
phosphorimager and quantification of signal in particular brain regions (Fig. 1B) was
performed using ImageQuant software as with the Northern blot. Values for each hemisphere
were averaged together in order to arrive at a single value for each region and each rat. Slides
were then covered with NTB2 emulsion (Eastman Kodak Co., Rochester, NY, USA) and
exposed for 28 days for analysis of silver grain distribution. After development, slides were
counterstained with Nissl stain and dehydrated through a graded series of ethanol and xylene.
A coverslip was then applied. Images were taken with a Leica DC 300F digital camera linked
to Image Pro-Plus software on a PC through a Leica DMRX microscope.

Data analysis

Behavioral data from the activity cages were analysed using StatView software (SAS Institute,
Cary, NC, USA). For the 14-day nicotine treatment, a two-factor, between–within analysis of
variance (ANOVA) was performed with treatment as the between-subjects factor and days as the
within-subjects factor. For the conditioning (test) day, a two-factor, between–within ANOVA was
performed with treatment as the between-subjects factor and time (intervals) as the within-
subjects factor. For the *arc in situ* hybridization data, quantification of phosphorescence was
performed using ImageQuant software as described above. The areas analysed were chosen
based on the regions previously implicated in our drug-conditioning paradigm (Schroeder *et al.*,
2001). We focused the active search within these specified regions; however, the analysis
performed does not preclude the involvement of other brain regions (see Fig. 1B for a depiction
of analysed areas). The average pixel intensities for each region were entered into StatView
and were analysed first by performing a two-way between–within ANOVA with treatment as the
between-subjects factor and brain region as the within-subjects factor. After determining
whether a significant group effect or interaction was present, comparisons between individual
experimental and control brain regions were performed using a one-way between-subjects

ANOVA. Because different coronal levels of the rat brain were run through ISH at different times, all data from ISH were normalized to the control values for each region analysed. Presentation of the data in this manner is meant to avoid inappropriate comparisons of *arc* mRNA levels between regions.

For qualitative analysis of emulsion autoradiography, images were captured at a magnification of 100× from sensorimotor cortex or 630× from prelimbic and lateral orbital prefrontal cortices. All images were processed using Adobe Photoshop.

Results

During the 14-day treatment period, nicotine had a strong stimulatory effect on locomotor activity (Fig. 2A). Horizontal activity ($F_{1,22} = 16.919$, $P < 0.0005$) and ambulatory activity ($F_{1,22} = 17.008$, $P < 0.0004$) were significantly increased in rats receiving nicotine compared with rats receiving saline injections in context 1 (only horizontal activity is shown in Fig. 2A). The behavioral activation following increasing nicotine dose administration became progressively greater over time (Fig. 2A), reflected by day $\times$ time interactions that were significant for horizontal activity ($F_{13,286} = 20.418$, $P < 0.0001$), ambulatory activity ($F_{13,286} = 18.874$, $P < 0.0001$) and rearing ($F_{13,286} = 4.825$, $P < 0.0001$).

Nicotine treatment also resulted in the development of a conditioned motor response (Fig. 2B). When the rats were re-exposed to the nicotine- or saline-paired environment 3 days later without any drug treatment, rats previously treated with nicotine in context 1 showed significant enhancement of motor activity (horizontal activity $F_{1,22} = 15.223$, $P < 0.0008$; ambulatory activity $F_{1,22} = 10.938$, $P < 0.0032$; rearing $F_{1,22} = 2.849$, $P < 0.1056$). The conditioned motor activation was not accompanied by a significant difference in plasma corticosterone in the two groups of rats ($F_{1,14} = 0.967$, $P < 0.3421$; Fig. 2B, lower right panel).

To determine whether changes in gene expression accompanied the conditioned motor response to nicotine-paired cues, we examined expression of the mRNA for the activity-regulated, cytoskeletal-associated protein Arc. Northern blot analysis indicated that *arc* levels in the ventrolateral orbitofrontal cortex were increased in rats exposed to nicotine cues compared with rats exposed to saline cues (Fig. 3A and B; $F_{1,18} = 6.933$, $P < 0.0169$). There was also a strong positive correlation between conditioned total horizontal activity and the *arc* mRNA phosphorescence signal (Fig. 3C; $y = 0.581 + 0.0011x$; $R^2 = 0.582$, $t = 5.009$, d.f. = 18, $P < 0.0001$).

To expand our analysis of *arc* mRNA expression during the conditioned response, we used ISH histochemistry so that multiple brain regions could be examined for potential changes in *arc* mRNA levels. Arc expression was measured in selected brain regions (Fig. 1B) 45 min following exposure to the activity cage environment previously associated with nicotine or saline administration using ISH with a ^{35}S -labeled *arc* antisense riboprobe. Sections from representative animals in the saline- and nicotine-cue groups are shown in Fig. 4A–L. Sections on which ISH with *arc* sense probe were performed were devoid of signal (data not shown). The overall between-within analysis of the *arc* data revealed a significant environment effect ($F_{1,12} = 38.717$, $P < 0.0001$), as well as a significant environment $\times$ brain region interaction ($F_{10,120} = 2.283$, $P < 0.0174$). Further analysis revealed significant increases in *arc* mRNA in several cortical, striatal and limbic brain regions (Fig. 4M) including lateral prefrontal cortex (ventral orbital and lateral orbital cortices) ($F_{1,12} = 27.545$, $P < 0.0002$), medial prefrontal cortex (including Cg1, prelimbic and infralimbic cortices) ($F_{1,12} = 19.106$, $P < 0.0009$), anterior cingulate cortex ($F_{1,12} = 11.979$, $P < 0.0047$), primary sensory cortex ($F_{1,12} = 10.721$, $P < 0.0066$), posterior cingulate cortex ($F_{1,12} = 9.301$, $P < 0.0101$), ventral striatum ($F_{1,12} = 7.066$, $P < 0.0209$), sensorimotor cortex ($F_{1,12} = 10.677$, $P < 0.0067$) and amygdala ($F_{1,12} = 12.228$,

$P < 0.0044$). Trends for increased *arc* mRNA signal existed in agranular insular cortex ($F_{1,12} = 3.216$, $P < 0.0982$) and the septum ($F_{1,12} = 3.697$, $P < 0.0786$). There were no significant changes in *arc* mRNA expression in the posterior dorsal striatum ($F_{1,12} = 1.449$, $P < 0.2518$), indicating that these findings were not simply a result of a generalized increase in *arc* mRNA expression in the brain. Graphical representation of these data is shown in Fig. 4M.

Emulsion autoradiography of forebrain sections revealed that *arc* was expressed primarily in cells that contained large, pale stained nuclei (arrows in Fig. 5A–D). A dramatic increase in *arc*, as evidenced by enhanced silver grain accumulation, was apparent in the prelimbic region of medial prefrontal cortex (Fig. 5A and B) and the ventral orbital region of the lateral prefrontal cortex (Fig. 5C and D) following exposure to nicotine cues. These labeling patterns are consistent with the expression of *arc* in neurons. Autoradiographic analysis of sensorimotor cortex further revealed silver grain accumulation over cells in layers II–IV and enhanced expression in layers V–VI in animals exposed to nicotine cues (Fig. 5F). Control *arc* expression was confined primarily to layers V–VI in animals exposed to saline cues (Fig. 5E).

Discussion

This paper describes an elevation in *arc* mRNA expression in specific regions of rat forebrain following the reintroduction of rats into an environment previously paired with nicotine administration. It is the first report that illustrates a nicotine-paired, context-elicited increase in the expression of mRNA encoding a protein (Arc) that is directly involved in modifying postsynaptic signaling efficacy. Changes in the expression levels of *arc* occurred in neuronal populations within specific corticolimbic regions involved in memory, cognitive functions and reward processing, and imply that pronounced signaling and biochemical changes within these regions occur during exposure to nicotine cues.

The importance of Arc in synaptic plasticity is well documented. *Arc* mRNA levels are increased by neuronal activity in an NMDA receptor-activation-dependent manner in the hippocampus (Steward & Worley, 2001b). *Arc* message is trafficked to activated dendrites (Steward & Worley, 2001a) where it is presumably translated by temporally and spatially defined intracellular signals (Steward & Worley, 2002). Arc protein interacts with elements of the cytoskeleton including microtubules and microtubule-associated protein-2 (Fujimoto *et al.*, 2004) and F-actin (Lyford *et al.*, 1995), suggesting that it plays a role in the organization of the cytoskeleton–plasma membrane interface. More recently, Arc has been shown to interact with PSD-95 and CaMKII (Okuno *et al.*, 2004) and the endocytic machinery responsible for trafficking AMPA receptors (Shepherd *et al.*, 2004). These observations support a role for Arc in directly modifying postsynaptic properties responsible for membrane excitation. This role stands in contrast to other immediate-early genes, such as *c-Fos* (Morrow *et al.*, 1999), that exert their indirect effects on synaptic plasticity and memory by regulating the transcription of other genes.

Arc appears to play a role in classical and operant conditioning. Up-regulation of *arc* has been observed in a variety of brain regions and the anatomical distribution of its induction depends on the type of stimulus or the type of experience provided to rats. Rats in the early stages of learning how to press a lever for a food reward have higher levels of *arc* mRNA and protein in brain regions known to be involved in the consolidation of this instrumental behavior compared with rats lever pressing for food but that have already learned how to perform the task (Kelly & Deadwyler, 2002, 2003). Expression of *arc* in hippocampal neurons depends on the environment in which a rat is placed and it depends on how familiar the rat is with the environment (Guzowski *et al.*, 1999, 2001; Vazdarjanova *et al.*, 2002). Blockade of Arc expression by local infusion of antisense in the hippocampus blocks long-term potentiation

and long-term memory, but not acquisition, of a hippocampus-dependent, spatial memory task in rats (Guzowski *et al.*, 2000). *Arc* is also expressed in response to acute treatment with cocaine (Fosnaugh *et al.*, 1995), acute or chronic treatment with amphetamine (Kodama *et al.*, 1998) and chronic treatments with morphine (Marie-Claire *et al.*, 2004). The environmental context as well as the rate of psychostimulant administration (two factors influencing behavioral sensitization and drug conditioning) modulate the up-regulation of *arc* (Klebaur *et al.*, 2002; Samaha *et al.*, 2004). The changes in expression of *arc* in response to drugs of misuse and paired sensory stimuli are thought to underlie some of the changes in behavior associated with repeated drug treatment such as sensitization and tolerance, dependence and withdrawal, and the development of conditioned responses to drug-related stimuli (Ujike *et al.*, 2002). Therefore, the elevation of *arc* message we observe during the first non-reinforced exposure to the nicotine-paired context may reflect new learning that this context is no longer necessarily predictive of nicotine administration. *Arc* expression in the mesocorticolimbic system, then, may play a role in the extinction of the conditioned response to the nicotine-paired context.

Remarkable similarities between *arc* expression patterns and neural activation profiles were found between this study and human neuroimaging studies of cue-elicited drug craving. These similar neural activation profiles extend across cues associated with administration of drugs of misuse from various chemical classes. For example, subregions of the prefrontal cortex in human addicts are activated in response to cigarette (Brody *et al.*, 2002; Due *et al.*, 2002), cocaine (Grant *et al.*, 1996; Maas *et al.*, 1998; Wang *et al.*, 1999; Garavan *et al.*, 2000; Bonson *et al.*, 2002) and alcohol cues (George *et al.*, 2001; Wrase *et al.*, 2002; Tapert *et al.*, 2003; Tapert *et al.*, 2004). Subregions of the cingulate cortex are responsive to cues associated with cigarettes (Brody *et al.*, 2004), cocaine (Maas *et al.*, 1998; Childress *et al.*, 1999; Garavan *et al.*, 2000; Kilts *et al.*, 2001; Wexler *et al.*, 2001), alcohol (Tapert *et al.*, 2003) and opiates (Daglish *et al.*, 2001). The ventral striatum is activated in addicted individuals in response to cigarette (Due *et al.*, 2002) and cocaine cues (Kilts *et al.*, 2001, 2004). The amygdala also exhibits increased activity after presentation of cues associated with cigarettes (Due *et al.*, 2002), cocaine (Grant *et al.*, 1996; Childress *et al.*, 1999; Kilts *et al.*, 2001; Bonson *et al.*, 2002) or alcohol (Schneider *et al.*, 2001). The increases in activity within these structures are often correlated with increases in self-reported drug-craving. We observe a similar correlation between conditioned locomotor hyperactivity and *arc* expression in the lateral prefrontal cortex. These similarities suggest that the paradigm used here for drug-cue-conditioned responses is an appropriate model for studying cue-elicited drug craving at a cellular and molecular level.

One criticism of the positive correlation between conditioned motor activity and immediate-early gene expression in the prefrontal cortex is that the increased motor activity caused the increase in immediate-early gene expression within the prefrontal cortex. We find this interpretation unlikely because rats exposed to cues associated with highly palatable food have increased Fos expression in prefrontal cortical areas but have no increased motor activity (Schroeder *et al.*, 2001). In addition, rats exposed to cues associated with the anxiogenic drug yohimbine showed no alteration in Fos levels in prefrontal cortical areas yet exhibited a robust conditioned motor activation (Schroeder *et al.*, 2003). We favor the interpretation that the increased neuronal activity resulting in changes in immediate-early gene expression in prefrontal cortical areas (as well as other areas) to drug-associated cues is responsible for the behavioral conditioned response in rats and the psychological conditioned response in humans. This interpretation is further supported by studies showing disruption of appetitive conditioned responses after lesions in prefrontal cortical subregions (Isaac *et al.*, 1989; Pears *et al.*, 2003).

Activity within subregions of the medial and lateral prefrontal cortex (McLaughlin & See, 2003; Fuchs *et al.*, 2004b), nucleus accumbens (Fuchs *et al.*, 2004a) and amygdala (Fuchs & See, 2002; Kantak *et al.*, 2002; McLaughlin & See, 2003) is responsible for cue-induced

reinstatement of drug-seeking behavior in animal models of relapse. Cue-induced reinstatement also elicits immediate-early gene expression in these corticolimbic areas (Ciccocioppo *et al.*, 2001). The observations that activity-dependent changes in gene expression in these brain areas parallel activation in human addicts suggest that related processes within corticolimbic circuits are occurring during cue-induced reinstatement as well as during expression of classically conditioned responses to drug cues.

The role of stress in drug-cue-conditioned responses is unclear. Because stress itself can interact with drug-paired cues to elicit or enhance drug-seeking behavior (Liu & Weiss, 2002), it becomes important to understand if stress plays a role in the conditioned response to nicotine-paired cues. We found no appreciable increase in plasma corticosterone in rats exposed to nicotine cues compared with rats exposed to saline cues. Furthermore, plasma corticosterone levels were not correlated with motor activity on the test day or with *arc* expression in the lateral prefrontal cortex (correlations not shown). Plasma corticosterone levels have been shown to increase in rats during cue-elicited reinstatement of cocaine-seeking behavior (Goeders & Clampitt, 2002) and during exposure to a cocaine-paired context (DeVries *et al.*, 1998). Further work will be needed to determine if the differences in hypothalamic-pituitary-adrenal (HPA) activation in response to nicotine vs. cocaine cues are due to differences in the pharmacological and conditioning properties of nicotine and cocaine or to differences in methodology between studies. Nonetheless, exposure to nicotine cues in this paradigm seems not to activate the HPA axis and stress, *per se*, seems to not contribute to the behavioral or cellular response to nicotine cues.

The enhanced expression of *arc* in layers II–VI in cortical regions involved in the conditioned response to nicotine cues is of interest because principal cells from different layers have differing inputs and outputs. In the parietal cortex, hippocampus and dentate gyrus, *arc* is expressed in excitatory neurons whereas in the striatum *arc* is expressed in inhibitory neurons (Vazdarjanova *et al.*, 2004). The pattern of *arc* expression elicited by nicotine cues suggests a distributed cortical and subcortical neuronal activation possibly resulting in plasticity of both excitatory and inhibitory principal neurons within the circuit.

Conditioned responses to cues associated with drug administration play a role in relapse to drug use. Here we report an animal model that can be used to study classically conditioned responses to cues previously paired with nicotine administration. Expression of at least two immediate-early genes (*Fos* and *arc*) has now been shown to increase in a variety of corticolimbic areas in response to nicotine cues. As these genes are known to participate in long-term memory formation (by differing mechanisms), their induction during the first unpaired exposure to conditioned stimuli (i.e. cues associated with nicotine administration) likely signifies that regions involved in the conditioned response may be undergoing plastic changes in order to store information that cues previously paired with nicotine are no longer necessarily predictive of nicotine administration. This hypothesis is especially interesting in terms of addiction and prefrontal cortical function as it relates to decision making, perseverance, reversal learning (Birrell & Brown, 2000; McAlonan & Brown, 2003) and extinction of conditioned responses (Morgan & LeDoux, 1995; Milad & Quirk, 2002; Herry & Mons, 2004). Neuronal activity in the prefrontal cortex thus may not only have a role in the manifestation of conditioned responses to drug-paired cues but also, upon non-reinforced presentation of these cues, a role in the eventual extinction of aspects of the conditioned response to the drug-paired cues. Indeed, in a recent study, extinction training was capable of reversing biochemical neuroadaptations within areas of the mesocorticolimbic system that are a result of chronic drug self-administration, like increased tyrosine hydroxylase immunoreactivity within the nucleus accumbens shell (Self *et al.*, 2004). More work will be necessary to ascertain the role of neuronal activity and immediate-early gene expression in

corticolimbic circuits on drug-induced biochemical neuroadaptations and subsequent behavior in response to drug-related cues.

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Fig. 1.

Schematic diagrams of the experimental procedure and brain areas analysed. (A) Rats were trained to receive an ascending dose regimen of nicotine in one of two distinct, non-home cage environments. Rats were placed in context 1 after an injection of nicotine or saline while motor activity was measured. After 90 min in context 1, rats were returned to their home cages for 30–45 min. Rats were then taken to context 2 where they received the counterbalanced injection (rats that received an injection of saline in context 1 received an injection of nicotine and vice versa). After 90 min in context 2, rats were returned to their home cages where they remained until the next training day. The conditioning (training) phase of the experiment lasted 14 days. Three days after the last day of training rats were exposed to context 1 without any injections where they remained for 45 min while motor activity was measured. (B) Brain regions analysed for expression of *arc* mRNA: (a) lateral prefrontal cortex, (b) medial prefrontal cortex, (c) anterior cingulate cortex, (d) primary sensory cortex, (e) agranular insular cortex, (f) posterior cingulate cortex, (g) ventral striatum, (h) septum, (i) posterior dorsal striatum, (j) sensorimotor cortex, (k) amygdala. Numbers represent distance, in millimeters, from bregma (Paxinos & Watson, 1998).

Fig. 2.

Motor activity data during conditioning and motor activity and corticosterone data during the test day. (A) Mean total horizontal activity ($\pm$ SEM) during the 14 days of training in context 1 for rats receiving saline or nicotine ($n = 12$ per group). The bars at the bottom of the graph designate the dose of nicotine the rats received for the overlying days. Rats receiving nicotine in context 1 exhibited greater total horizontal activity than rats receiving saline injections in context 1 ($**P < 0.001$, see text for statistical details). There was a significant treatment $\times$ day interaction in the nicotine group ($††P < 0.0001$). (B) Conditioned mean total horizontal activity (upper left), mean ambulatory activity (upper right), mean rearing activity (lower left) and mean plasma corticosterone (lower right) on the test day ($\pm$ SEM). Rats exposed to nicotine cues ($n = 12$) exhibited significantly higher activity counts for total horizontal activity and ambulatory activity ($**P < 0.01$, see text for statistical details) compared with rats exposed to saline cues ($n = 12$). Levels of plasma corticosterone did not differ between rats exposed to saline cues and nicotine cues.

Fig. 3.

Northern blot analysis of *arc* mRNA in lateral prefrontal cortex. (A) Representative Northern blot of RNA from the lateral prefrontal cortex of two rats exposed to nicotine cues and two rats exposed to saline cues probed for *arc* mRNA (upper panel) and normalized to GAPDH mRNA levels (lower panel). Sal = saline cues, Nic = nicotine cues, *gapdh* = glyceraldehyde-3-phosphate dehydrogenase mRNA. (B) Average pixel intensity of *arc* signal normalized to *gapdh* ($\pm$ SEM) for the saline cues ($n = 10$) and the nicotine cues ($n = 10$) groups. mRNA from one rat in the saline cues and in the nicotine cues group each was not included for quantification and statistical analysis due to obvious degradation of the samples (data not shown). A one-way ANOVA indicated an increased level of *arc* mRNA in the lateral prefrontal cortex of the nicotine cues group compared with the saline cues group ($*P < 0.05$, see text for statistical details). (C) Total horizontal activity vs. normalized *arc* mRNA levels in lateral prefrontal cortex. *Arc* levels in lateral prefrontal cortex are positively correlated with total horizontal activity ($R^2 = 0.5822$, $t = 5.009$, d.f. = 18, $P < 0.0001$).

Fig. 4.

In situ hybridization analysis for the immediate-early gene *arc* in coronal sections from the brains of rats exposed to saline cues or nicotine cues. (A–L) Representative phosphorescence images of coronal sections hybridized with ^{35}S -labeled *arc* antisense riboprobe from which pixel intensity measurements were taken. (A, C, E, G, I and K) Brain sections from a rat exposed to saline cues. (B, D, F, H, J and L) Brain sections from a rat exposed to nicotine cues. (A and B) Lateral prefrontal cortex. (C and D) Medial prefrontal cortex. (E and F) Anterior cingulate cortex, primary sensory cortex and agranular insular cortex. (G and H) Posterior cingulate cortex and ventral striatum. (I and J) Septum, posterior dorsal striatum and sensorimotor cortex. (K and L) Amygdala. Note how *arc* mRNA expression is recruited in upper layers of cortex especially in E compared with F, I compared with J, and K compared with L. (M) Mean *arc* hybridization signal intensity ($\pm$ SEM) in the saline-paired ($n = 7$) and nicotine-paired ($n = 7$) groups for each area analysed (ANOVA: $P < 0.01$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *post-hoc* one-way ANOVA). Signal intensities from the brain regions of rats exposed to saline cues and nicotine cues were normalized to the average value of the saline cues group for each brain region. Presentation of the data in this manner is meant to prevent inappropriate comparisons of *arc* expression between regions as ISH was carried out on different areas at different times.

Fig. 5.

Emulsion autoradiography of forebrain cortical regions hybridized to *arc* antisense probe. (A and B) Silver grains in layer V/VI of the prelimbic regions of the prefrontal cortex accumulate preferentially over cells with lightly stained, large nuclei (arrows in A and B) in a rat exposed to nicotine cues (B) compared with saline cues (A). This silver grain pattern is consistent with neuronal localization of induced *arc* (scale bar, 50 μ m for A–D). (C and D) Silver grains in layers V/VI of the lateral orbital prefrontal cortex also accumulate preferentially over cells with lightly stained, large nuclei (arrows in C and D) in a rat exposed to nicotine cues (D) compared with saline cues (C). This silver grain pattern is again consistent with neuronal localization of induced *arc*. (E and F) Lower power magnification, dark-field photomicrographs of sensorimotor cortex (corresponding to Fig. 4I and J). Recruitment of cells expressing *arc* occurs preferentially in layers II–IV in the sensorimotor cortex of rats exposed to nicotine cues (F) compared with rats exposed to saline cues (E). This laminar pattern of expression was evident in most cortical areas analysed (scale bar, 200 μ m for E and F).