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Regulation of Homer and group I metabotropic glutamate receptors by nicotine

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Abstract

The present study focuses on the nicotine-induced modulation of mRNA and protein expression of a number of genes involved in glutamatergic synaptic transmission in rat brain over different time periods of exposure. A subchronic (3 days) but not the chronic (7 or 14 days) administration of nicotine resulted in the up-regulation of Homer2a/b mRNA in the amygdala while in the ventral tegmental area (VTA) no change in expression of either Homer2a/b or Homer1b/c was observed. Although the increase in Homer2a/b mRNA was not translated into the protein level in the amygdala, a slight but significant up-regulation of Homer1b/c protein was observed in the same region at day 3. Both Homer forms were up-regulated at the protein level in the VTA at day 3. In the nucleus accumbens, 14 days of nicotine treatment up-regulated mRNA of Homer2b/c by 68.2% ($P < 0.05$), while the short form Homer1a gene was down-regulated by 65.0% at day 3 ($P < 0.05$). In regard to other components of the glutamatergic signalling, we identified an acute and intermittent increase in the mRNA and protein levels of mGluR1 and mGluR5 in the amygdala. In the VTA, however, the effects of nicotine on mGluR mRNA expression were long-lasting but rather specific to mGluR1. Nevertheless, mGluR1 protein levels in the VTA area were up-regulated only at day 3, as in the amygdala. These data provide further evidence for the involvement of nicotine in the glutamatergic neuronal synaptic activity *in vivo,* suggesting a role for the newly identified Homer proteins in this paradigm.

Introduction

Previous microarray experiments completed in this laboratory to study the effects of nicotine in different rat brain regions provided a set of candidate genes that may be responsible for the changed cellular environment in the presence of nicotine (Konu *et al*., 2001; Li *et al*., 2002). Among them, a candidate gene named Homer2b/VESL2 was found to be up-regulated by nicotine in the amygdala and ventral tegmental area (VTA) of the rat brain. Homer proteins can bind to and regulate the cellular distribution of the metabotropic glutamate receptors (mGluRs) (Brakeman *et al*., 1997; Kato *et al*., 1997; Kato *et al*., 1998). Homer proteins also function as scaffolding proteins connecting the metabotropic glutamate group I receptors (i.e. mGluR1 and mGluR5) to the intracellular phosphatidylinositol signalling pathway (Tu *et al*., 1998; Soloviev *et al*., 2000a).

The Homer family is made up of three genes named Homer1, 2 and 3 that have several alternatively spliced transcripts (Xiao *et al*., 2000). The long isoforms of Homer (Homer1b/c and Homer2a/b), but not the short isoform Homer1a, are constitutively expressed (Soloviev *et al*., 2000b) and contain a carboxyl terminal coiled-coil domain used in forming multimeric Homer complexes. The Homer long isoforms are enriched at postsynaptic sites and coimmunoprecipitate with the group I mGluRs (Xiao *et al*., 1998). Homer also associates with the Shank–PSD-95–NMDA complex, providing a role for mGluR and Homer to modulate *N*-methyl-D-aspartate receptors (NMDARs) (Naisbitt *et al*., 1999; Tu *et al*., 1999). Homer1b prevents mGluR5 expression on the cell surface by keeping it on the endoplasmic reticulum of the secretory pathway (Roche *et al*., 1999). Homer1a, on the other hand, does not impede the expression of mGluR5 and has been shown to increase cell surface expression of mGluR1α (Ciruela *et al*., 1999) while preventing clustering of group I mGluRs (Tu *et al*., 1998). Further study into Homer1 proteins reveals a complex interplay of Homer1 isoforms and the cell surface targeting of mGluR5 upon neuronal excitation (Ango *et al*., 2000). Homer is thus becoming an important player in the postsynaptic response to glutamatergic activation.

mGluRs are coupled to G protein second messenger pathways and modulate glutamate neurotransmission in the brain. Both the fast excitatory transmission ionotropic glutamate receptors (iGluRs) and the mGluRs are involved in neuroplasticity (Ottersen & Landsend, 1997; Anwyl, 1999). NMDA and alpha-amino-3-hydroxy-5-methyl-4-isopropinonate (AMPA) ionotropic glutamate receptors are known to participate in long-term potentiation (LTP) and long-term depression (LTD) of neurons (Asztely & Gustafsson, 1996; Xie *et al*., 1992). The number, location and gating characteristics of these ionic receptors are important in determining synaptic strength. It has been suggested that drugs of abuse, such as nicotine, that produce long-term neuronal changes by altering the steady state of intracellular signalling molecules, share mechanisms similar to those used in learning and memory (Nestler, 2002).

mGluRs and Homer proteins have been implicated in playing an important role in neural stimulation and drug addiction (Brakeman

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et al., 1997; Kato *et al*., 1997; Ango *et al*., 2000; Chiamulera *et al*., 2001; Swanson *et al*., 2001; Bottai *et al*., 2002; Paterson *et al*., 2003). It has been demonstrated that mGluR5-knockout mice lack the psychostimulant effect of cocaine injections, and a decrease in locomotor activity was observed (Chiamulera *et al*., 2001). These mice also had no interest in self-administering cocaine. In a separate study (Swanson *et al*., 2001), repeated cocaine administration blunted glutamate release into the nucleus accumbens and resulted in increased locomotor activity that accompanies intra-accumbens administration of the group I mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG). An investigation into this response revealed a decrease in Homer1b/c protein levels in the nucleus accumbens, suggesting that mGluR1 and Homer1b/c are involved in the neurochemical and behavioural response to the effects of a drug of abuse like cocaine (Swanson *et al*., 2001). Moreover, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a specific mGluR5 antagonist, decreased nicotine self-administration by rodents, providing further support for the involvement of mGluR5 in mediating the reinforcing effects of the drugs of abuse (Paterson *et al*., 2003). Finally, Homer-knockout mice displayed behavioural and biochemical characteristics similar to those observed after withdrawal from repeated cocaine administration, pointing to the role of Homers in sensitivity to drugs of abuse (Szumlinski *et al*., 2004).

The neurobiology studies of drug addiction and neuropsychiatric and many neurodegenerative diseases has focused primarily on deciphering the framework that comprises the mesocorticolimbic dopamine system. This system is generally defined by the dopaminergic neurons that originate within the ventromedial mesencephalon, such as the VTA and the substantia nigra, and terminate in limbic regions such as the striatum, amygdaloid and accumbal regions, as well as cortical areas such as the prefrontal cortex. In addition to the dopaminergic projections, there is a complex arrangement of glutamatergic, cholinergic and noradrenergic neurons that profoundly modulate dopamine's action and physiological effects (Fallon & Moore, 1978). The primary reinforcement pathway has been characterized by the excitation of dopaminergic neurons that project from the VTA of the midbrain to the nucleus accumbens. Nicotine administration activates mesolimbic dopaminergic neurons by acting directly at postsynaptic nicotinic acetylcholine receptors (nAChRs) within the VTA. Further study has indicated that enhanced glutamatergic excitation coupled with a depressed GABAergic inhibition contribute to mesolimbic dopamine plasticity (Pidoplichko *et al*., 2004).

The amygdala plays a predominant role in relaying the communication network between the primary reinforcement motivation toward an abused drug and the memories that are associated with that action. The basolateral amygdala has traditionally been associated with conditioned reinforcement behaviours (Everitt *et al*., 1989). Lesions of the amygdala have been shown to disrupt the cue conditioning of reward-related stimuli such as drug reinforcement (Hayes & Gardner, 2004). Stimulation of basolateral amygdala produced an increase in dopamine release in the nucleus accumbens, suggesting amygdaloid influence on the primary motor drive underlying drug reinforcement (Howland *et al*., 2002; Phillips *et al*., 2003).

In the present study, we report the region- and time-specific differences in the expression of the group I mGluR and Homer mRNA and protein levels in the nucleus accumbens, amygdala and VTA of nicotine-treated rats over a period of 3, 7 or 14 days. Based on prior evidence implicating these proteins in neuronal strength and synaptic plasticity, we hypothesize that these expressional changes are part of the cellular mechanisms responsible for the initial physiological response to nicotine administration.

Materials and methods

Animals, nicotine administration and brain punches

Male Holtzman rats (250–350 g; HSD, Madison, WI, USA) received nicotine dihydrochloride at a dose of 4.0 mg/kg/day in saline (pH 7.4), or saline alone, by i.p. injection. The dose was determined in previous studies demonstrating consistent expression levels with the least amount of behaviour changes (Li *et al*., 2000). An initiation phase of 48 h was implemented to habituate the animals to the stress of the injection; this has been shown to reduce c-fos levels in response to the stress of i.p. injection (Ryabinin *et al*., 1999). Moreover, saline-control rats were handled and treated exactly as the nicotine-treated group, thereby exposing controls to the same stress-induced effects of i.p. injection. All experiments had six animals in each of the control and treatment groups except when noted. Rats were housed in wire-bottomed cages at 22 °C and maintained on a 12 : 12-h light : dark cycle. Standard laboratory rat chow and water were freely available. Nicotine was administrated five times a day from 09.00 to 17.00 h at 2-h intervals over a period of 3, 7 or 14 days. After the indicated time of nicotine administration, rats were injected with a lethal overdose of sodium pentobarbital (100 mg/mL i.p.) and decapitated, and the brains were removed immediately for sectioning. Coronal 2-mm sections were prepared from fresh brains, using a Stoelting tissue slicer (Chicago, IL, USA). Brain punches from selected brain regions were excised using a brain punch tissue set from myNeuroLab.com (St Louis, MO, USA) based on coordinates from Paxinos & Watson (1986). For the ventral tegmental area, each sample contained a single 1.5-mm-diameter punch that was centred in the dense field of dopamine-containing cells. For the amygdala region, each sample contained bilateral 2.0-mm-diameter dissections that were centred in the basolateral nuclear complex. Each sample for the nucleus accumbens contained the core and shell of the accumbens dissected using bilateral 2.0-mm-diameter punches. All procedures were conducted in accordance with animal use guidelines, with the approval of our Institutional Animal Care and Use Committee.

RNA isolation, reverse transcription and real-time polymerase chain reaction (real-time quantitative RT-PCR)

Total RNA was isolated from corresponding regions using TRIzol reagent according to the manufacturer's protocol (Qiagen Inc., CA, USA). Three brain regions were examined in this study: the amygdala, VTA and nucleus accumbens which are all implicated to play a significant role in drug addiction. Before use, RNA samples were treated with RNAse-free DNAse I at 37 °C for 30 min. Assessments were made with respect to the integrity of the samples through viewing of the eithidium bromide-stained 28S and 18S ribosomal RNA bands.

Real-time RT-PCR conditions reported here were determined by the strategy described previously (Li *et al*., 1997) but some modifications were made to optimize conditions (Bustin, 2000; Konu *et al*., 2001). Briefly, total RNA concentration of each sample was measured in duplicate using a RIBOGreen^{TM} kit (Molecular Probes, Inc., Eugene, OR, USA). The 0.5 μg of total RNA was reverse-transcribed in a final volume of 20 μL containing 4 μL of 5× reverse transcriptase buffer (Tris-HCl, pH 8.8, 0.1 M; KCl, 0.5 M; and Triton X-100, 1%), $MgCl_2$, 5 mM; DTT, 10 mM; each dNTP, 0.625 mM; RNasin, 20 units; 50 μM random hexamers, 1 μL; and SuperScript II RNase H^- reverse transcriptase, 200 U (Gibco BRL Life Technologies, Grand Island, NY, USA). The RT mixtures were incubated at 42 °C for 1 h and then heated at 95 °C for 5 min to inactivate the reverse transcriptase. Amplification of 4 μL RT mixture (equivalent to 0.1 μg total RNA)

TABLE 1. A list of genes and their primers used in real-time quantitative RT-PCR

Gene name	Accession number	Slope of 10× dilutions	Primer sequence (5′–3′)	Product size (bp)
18S	X01117	−3.3	AGA AAC GGC TAC CAC ATC CAA G TGT TAT TTT TCG TCA CTA CCT CCC	84
Homer1a			AAC TCA GAG CCA AGG GCT GA CAT GAT TGC TGA ATT GAA TGT G	78
Homer1b/c			GCT ATA TTC TCC GCG CAA CCT T GCA ACT CAA CGA GGC AGA CAA T	116
Homer2a/b	NM053309	−3.5	GAG TGG AAA GCG TGT GTG AG CGC ATT ACA GAA GCA AAC GGA G	65
mGluR1	NM017011	−3.4	ATC ATT GCC AAA CCT GAG AGG AAC GCC GTT AGA ATT GGC ATT	141
mGluR5	NM017012	−3.0	TTC TCT GTC CAC CAC CAA CC TAT TGC TCA CGA ACT GCA CC	51

was carried out using core reagents from the SYBR® Green kit (PE Biosystems, Foster City, CA, USA), 5 μL of 10× SYBR® Green PCR buffer, 4.0 μL of 25 mM $MgCl_2$, 1.0 μL of 12.5 mM dNTP mix with dUTP, 1 μL of sense or antisense primers (0.1 μg/μL) and 2.5 U of AmpliTaq DNA polymerase in a total volume of 50 μL. The real-time RT-PCRs were initially denatured at 94 °C for 3 min and then were subjected to 40 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s) and extension (72 °C, 45 s). The iCycler (Bio-Rad, Hercules, CA, USA) was used to perform the real-time quantitative RT-PCR. After the last cycle, a dissociation curve was run to check for product purity. Intensity values were based on the incorporation of fluorescent SYBR® Green dye (excitation 497, emission 520 nm; Molecular Probes) into the double-stranded PCR products and were measured by the Bio-Rad iCycler. All genes were normalized to 18S RNA expression level.

The primer sequences used for real-time RT-PCR and related information on each gene are shown in Table 1. To confirm the efficacy of the real-time PCR technique and demonstrate that each set of gene-specific primers accurately estimates a two-fold induction per PCR cycle, a 10× serial dilution of cDNA was used as template for amplification of 18S, Homer2a/b, mGluR1a and mGluR5 genes using gene-specific primers. The slope of the linear regression demonstrates a difference ranging from 3.0 to 3.5 cycles between each 10× dilution, which is very close to the theoretically expected value of 3.3 cycles (see Table 1). The expression level of each gene was determined using the calibration method (Winer *et al*., 1999). Threshold cycles (C_t) were determined by the iCycler for both the target gene and the housekeeping gene. In this method, one of the unknown samples is termed the 'calibrator sample'; the expression of this sample is compared against all other samples analysed. The formula used is: fold induction $= 2^{-[\Delta\Delta Ct]}$ where $\Delta\Delta C_t = [C_t$ GI (gene of interest, unknown sample) $- C_t$ 18S (unknown sample)] $- [C_t$ GI (threshold cycles, calibrator sample) $- C_t$ 18S (calibrator sample)].

Western blotting analysis

Total protein was extracted from rat brain punches obtained from an independent time-course experiment conducted specifically for protein analysis under the same paradigm as described above except that three rats in each of the control and treatment groups were used. Individual frozen brain punches were suspended in 100 μL of 240 mM Tris–HCl, pH 6.8, containing 20% β-mercaptoethanol, 8% SDS and 40% (v/v) glycerol, and homogenized with the Sonic Dismembrator 550 sonicator (Fisher Scientific, Houston, TX, USA). Tissue debris were removed by centrifugation at 12 000 ***g*** for 1 h. The supernatant was electrophoresed using 8% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) followed by blotting to nitrocellulose membranes (Immobilon-P; Sigma). Blots were then blocked with 2% nonfat dry milk for 1 h at room temperature and probed with antibodies against group I mGluRs (BD Sciences, San Jose, CA, USA) and Homers (kindly provided by Dr Paul Worley, The Johns Hopkins University). The respective dilution of each antibody was as follows: Homer1b/c, 1 : 5000 and Homer2a/b, 1 : 5000; mGluR1, 1 : 2500; mGluR5, 1 : 2500; and α-tubulin, 1 : 2500. Immune complexes were detected with appropriate secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce Inc., Rockford, IL, USA). Blots were stripped with Restore Western Blot Stripping Buffer (Pierce Inc.). After it was determined that the membranes were free of the immunodetection reagents, they were re-probed with antibodies to α-tubulin (for protein normalization). After the films were developed, they were scanned on a Microtek ScanMaker 8700 scanner with ScanWizard 5.5 at a resolution of 600 dpi for quantitative analysis. Scanned images were analysed with ImageQuant 5.1 (Molecular Dynamics, Sunnyvale, CA, USA). Normalized data to either 18s rRNA or tubulin were analysed using ANOVA (Systat 6.0. SPSS Inc, Chicago, IL, USA). Significant *F*-tests were followed by comparison using the Bonferroni procedure. Comparisons yielding more than 95% statistical confidence ($P < 0.05$) were considered significantly different.

Results

Amygdala

At day 3 of nicotine administration, an 82% increase in the expression of the longer form of Homer2a/b was observed by real-time RT-PCR analysis ($P < 0.05$; Fig. 1A) within the amygdala region. Because long forms of Homers are known to cluster and aid in the intracellular signalling of group I metabotropic receptors, quantitative real-time RT-PCR was used to measure the mRNA levels of mGluR1 and mGluR5 in the amygdala region. As with Homer2a/b, mRNA expression levels of mGluR1α (37%, $P < 0.05$; Fig. 1B) and mGluR5 (45%, $P < 0.05$; Fig. 1C) were induced on day 3 of nicotine administration but returned to control levels thereafter (see below and Fig. 4A for details).

To determine whether the protein levels also reflect an acute up-regulation by nicotine as shown with the RNA expression studies, Western blot analysis was employed under the same experimental model as used for quantitative real-time RT-PCR experiments. While

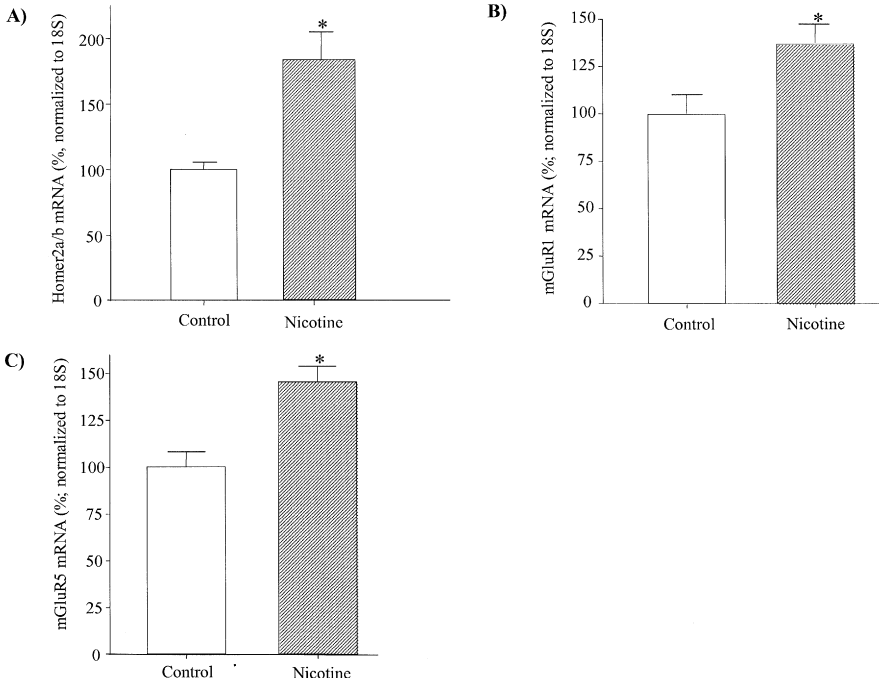

FIG. 1. Comparison of mRNA expression levels for Homer2a/b and group I mGluRs between nicotine and saline groups in the amygdala region after 3 days of nicotine administration. Using gene-specific primers, real-time quantitative RT-PCR showed (A) an 83% increase in Homer2a/b, (B) a 37% increase in mGluR1α mRNA level and (C) a 45% increase in mGluR5 mRNA level in the amygdala at day 3. $*P < 0.05$.

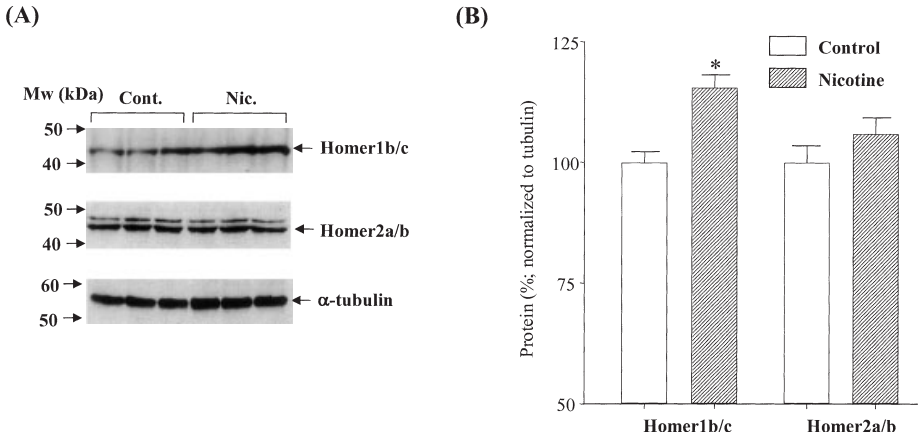

FIG. 2. (A) Western blotting analysis for Homer1b/c and Homer2a/b at 3 days of nicotine treatment in the amygdala region. (B) Analysis indicated a 16% increase in Homer1b/c protein levels while no change in Homer2a/b protein levels was observed. $*P < 0.05$.

we did in fact observe an increase in Homer1b/c protein levels at day 3 (16%, $P < 0.05$), no significant increase in Homer2a/b protein levels was present (Fig. 2). Using commercially available monoclonal antibodies against mGluR1α and mGluR5, we also measured their protein levels at 3, 7 and 14 days of nicotine administration and found that mGluR1α was increased by 20% ($P < 0.05$) by nicotine at day 3 (Fig. 3A). mGluR5 was also shown to be increased by 20% ($P < 0.05$) in the amygdala at day 3 (Fig. 3B). Homer1b/c, mGluR1α and mGluR5 were all observed to return to control levels by days 7 and 14 of nicotine administration (Fig. 4B).

Ventral tegmental area

Glutamatergic neurotransmission within the VTA is important in modulating the mesolimbic dopaminergic system, thought to be critical in the primary reward response to drugs of abuse such as nicotine. We investigated the levels of the group I mGluRs and various Homer isoforms because nicotine administration is known to increase glutamatergic neurotransmission in this region. Western blot analysis showed an 18% increase in Homer1b/c ($P < 0.05$) and a 31% increase in Homer2a/b ($P < 0.05$; Fig. 5) proteins without a significant change at the transcript level at day 3 of nicotine treatment. Western blotting analysis also showed a significant increase in mGluR1α protein at day 3 only (15%, $P < 0.05$; Fig. 6). No significant changes were observed for mGluR5 at both the mRNA and protein levels in the VTA at any time point (Fig. 7B). On the other hand, quantitative real-time RT-PCR demonstrated a consistent increase in the expression level of mGluR1α in the VTA (60, 67 and 46% at 3, 7 and 14 days, respectively, $P < 0.05$; see Fig. 7A). While the mRNA expression levels of mGluR1α and Homer2a/b seemed to be somewhat elevated over the 14 days, the protein levels of Homer1b/c, Homer2a/b and

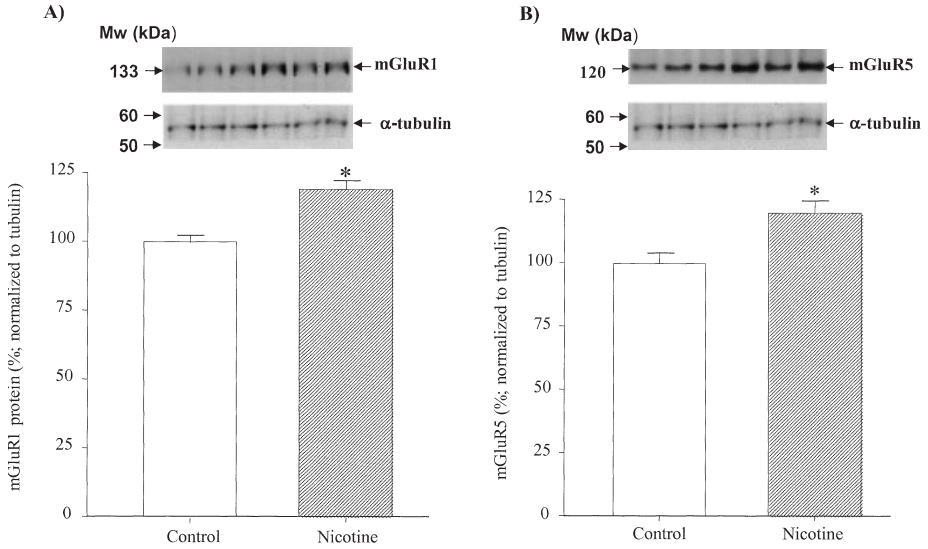

FIG. 3. Western blotting analysis for the group I mGluRs in the amygdala after 3 days of nicotine treatment. Monoclonal antibodies against the group I mGluRs revealed a complementary 20% increase in protein levels at day 3 in the amygdala for both (A) mGluR1α and (B) mGluR5. *$P < 0.05$.

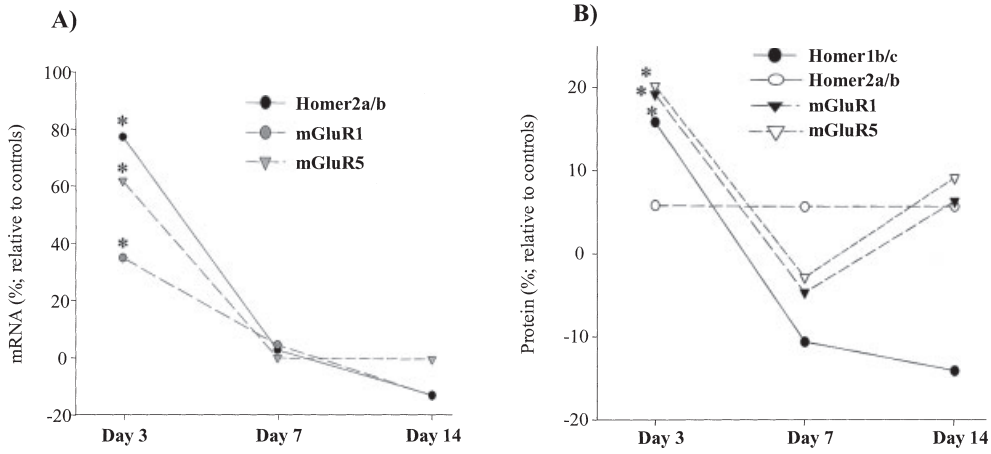

FIG. 4. Summary of expression changes in (A) mRNA and (B) protein levels for Homers and group I mGluRs over 14 days of nicotine treatment in the amygdala. There appears to be an early up-regulation of both mRNA and protein levels of group I mGluRs and Homer genes in the amygdala that return to control levels thereafter. *$P < 0.05$.

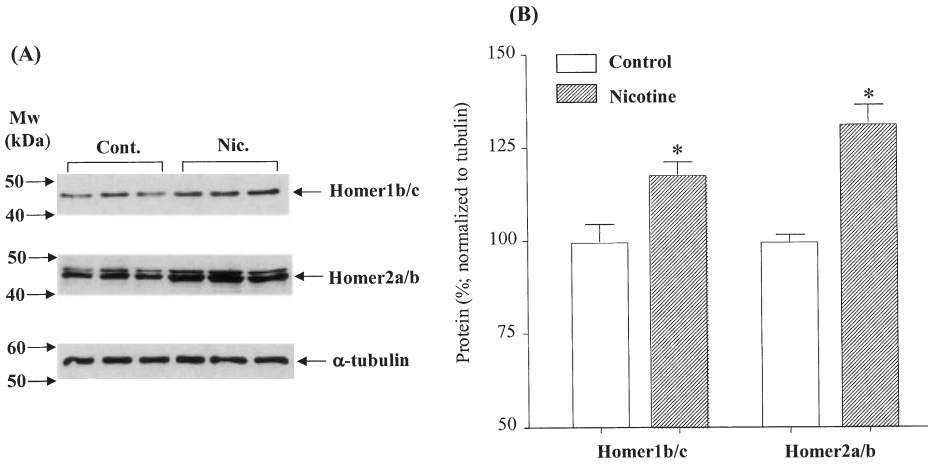

FIG. 5. Western blotting analysis for Homer1b/c and Homer2a/b in the VTA region. While microarray (data not shown) and quantitative RT-PCR (see Fig. 8) show no difference in mRNA levels of Homer2a/b, Western blot demonstrated a 31% increase in protein levels. (A) A representative autoradiograph of Western analysis. (B) Consistent with an increase in Homer2a/b levels, Western blot analysis shows an 18% increase in Homer1b/c protein levels at day 3 in the VTA. *$P < 0.05$.

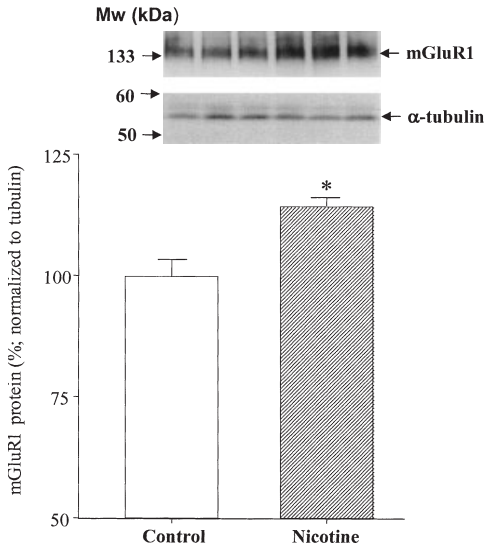

FIG. 6. Western blot analysis of the group 1 mGluR1 in the VTA. A 15% increase was observed in mGluR1α protein levels. *$P < 0.05$.

mGluR1α dropped to control levels after day 3 in a fashion analogous to the amygdala region.

Nucleus accumbens

Real-time RT-PCR assays indicated that the short form Homer1a and mGluR1α mRNA levels in the nucleus accumbens were down-regulated by 65.3% (Fig. 8A; $P < 0.05$) and 37.1% (Fig. 8B; $P < 0.05$), respectively, in response to 3 days of nicotine administration. However, no significant differences were detected in these mRNA levels at 7 or 14 days of nicotine treatment (see Fig. 8D). In contrast, Homer2b/c was up-regulated by 68.2% after 14 days of administration (Fig. 8C; $P < 0.05$). Similarly, we also assayed for the expression levels of other genes mentioned in the present study at both the RNA and protein levels and found that they displayed no significant differences between the nicotine-treated and saline control groups in the nucleus accumbens (data not shown).

Discussion

Previous microarray analyses demonstrated that nicotine increased the constitutive expression of the Homer2a/b gene (Konu *et al.*, 2001). The present study confirms the modulation of Homer2a/b mRNA expression in nicotine-treated rats. Interestingly, although protein levels of Homer1b/c increased by 16% ($P < 0.05$), Homer2a/b protein did not show significant change in the amygdala at day 3 (Fig. 2). It is possible that the protein levels for Homer2a/b had already begun to return to baseline at day 3, at which time the first samples were assayed. As noted at day 7 in the amygdala, the mRNA and protein levels of the genes under study were all back to control levels (Fig. 4).

Homer is a scaffolding protein involved in a complex interplay between membrane receptors and intracellular signalling. It represents an important link in the modulation of a complicated system driven by the excitatory amino acid glutamate upon target neurons. Nicotine affects glutamatergic neurotransmission by acting at nAChRs. nAChRs are located presynaptically where they modulate the release of neurotransmitters such as glutamate and postsynaptically where they affect signalling pathways (McGehee *et al.*, 1995; Wonnacott, 1997; Aramakis & Metherate, 1998; Guo *et al.*, 1998). Coupled with the widespread CNS distribution of nAChRs, nicotine administration affects physiological conditions such as addiction, learning, memory and more (Levin, 1992; Levin & Simon, 1998). The cellular adaptations upon nicotine exposure are of particular interest in attempting to explain these effects of nicotine. In this report, mRNA and protein expression analyses suggest that the different forms of Homer and mGluRs are being regulated by nicotine in these brain regions.

It has been demonstrated that nicotine regulates glutamatergic neurotransmission within the amygdala region (Girod *et al.*, 2000; Barazangi & Role, 2001). Our data support this with expression levels of the two group I metabotropic receptors being acutely up-regulated in the amygdala after nicotine administration. The use of monoclonal antibodies against mGluR1 and mGluR5 demonstrated that the increase in mRNA expression levels was translated into an increase in protein levels in the amygdala at day 3. The amygdala region is known to play a central role in conditioned reinforcement (Everitt *et al.*, 1999; Kalivas & Nakamura, 1999; Koob, 1999; Parkinson *et al.*, 2001) and in establishing different types of learning and memory (Maren, 1999; Walker & Davis, 2002). Group I mGluRs are involved in the formation of synaptic plasticity (O'Connor *et al.*, 1994; Huber

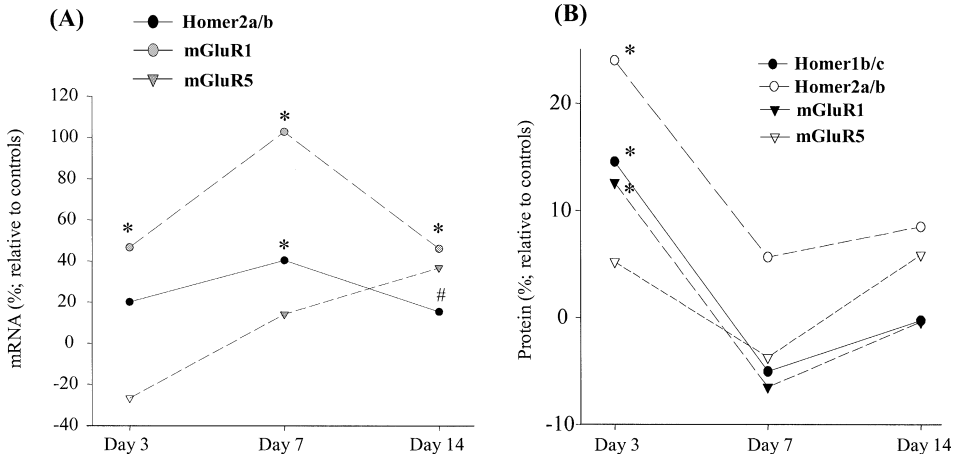

FIG. 7. Summary of the target gene (A) mRNA expression and (B) protein levels presented as the percent change relative to control in the VTA. While the protein levels of Homer1b/c and mGluR1α dropped in a similar fashion to that observed in the amygdala, the expression level of mGluR1α remained elevated throughout the full 14 days of nicotine administration. *$P < 0.05$.

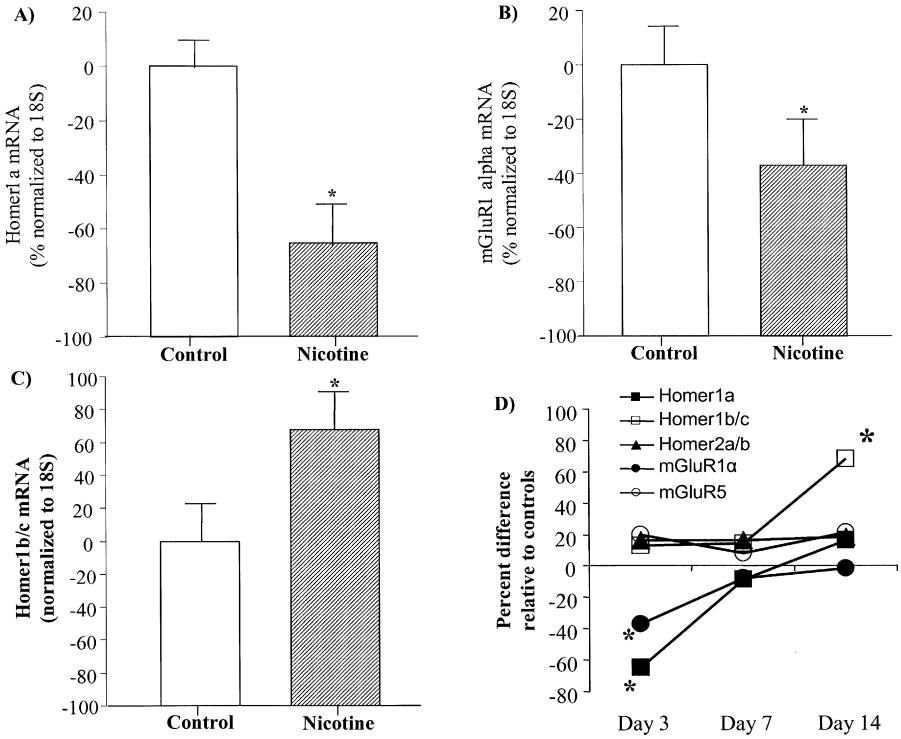

FIG. 8. A comparison of the mRNA levels of (A) Homer1a and (B) mGluR1α at day 3 and (C) Homer1b/c at day 14 in the nucleus accumbens of control and nicotine-treated rats. (D) mRNA expression profiles of Homer1a, Homer1b/c, Homer2a/b, mGluR1a and mGluR5 in the nucleus accumbens at different time points of nicotine administration. *$P < 0.05$.

et al., 1998; Balschun *et al*., 1999). Group I mGluRs and Homers can modulate LTP and affect the firing pattern of the target neuron (Bortolotto *et al*., 1999; Zheng & Gallagher, 1992; Brakeman *et al*., 1997; Kato *et al*., 1997; Rodrigues *et al*., 2002). Through changing the synaptic strength of the target neuron, these processes are believed to be involved in the formation of learning and memory (Barnes, 1995; Kato *et al*., 1997; Turrigiano & Nelson, 2000). The increase in glutamatergic activity in response to nicotine administration may be driving the up-regulation of group I mGluRs, as observed in the amygdala at day 3, thereby providing a mechanism for a new level of responsiveness to the presence of glutamate. An up-regulation in the mGluR and Homer protein levels may help explain a change in the neuroplasticity within the amygdala region that is responsible for the formation of learning and memory events associated with nicotine administration.

mGluRs are also known to function postsynaptically through interaction with other glutamate receptors such as NMDARs (Naisbitt *et al*., 1999; Rodrigues *et al*., 2002). An up-regulation of both mGluR1α and Homer proteins may indicate an increased association with the NMDA receptor (Fig. 9). Tu *et al*. (1999) demonstrated the existence of the coupling complex, mGluRs–Homer–Shank–F-actin–GKAP–PSD-95–NMDAR. This complex places all receptors in close proximity to one another and also is believed to provide a bridge allowing for the efficient modulation of postsynaptic LTP via modulation of the actin cytoskeleton (Tu *et al*., 1999). NMDA receptors play an important role in LTP formation and the group I mGluRs are known to modulate LTP through the NMDA receptors (Anikszteijn *et al*., 1991; Fitzjohn *et al*., 1996; Pisani *et al*., 1997; Yu *et al*., 1997; Rodrigues *et al*., 2002). Recently, Manahan-Vaughan *et al*. (2003) reported an increase in mGluR1 and mGluR5 protein

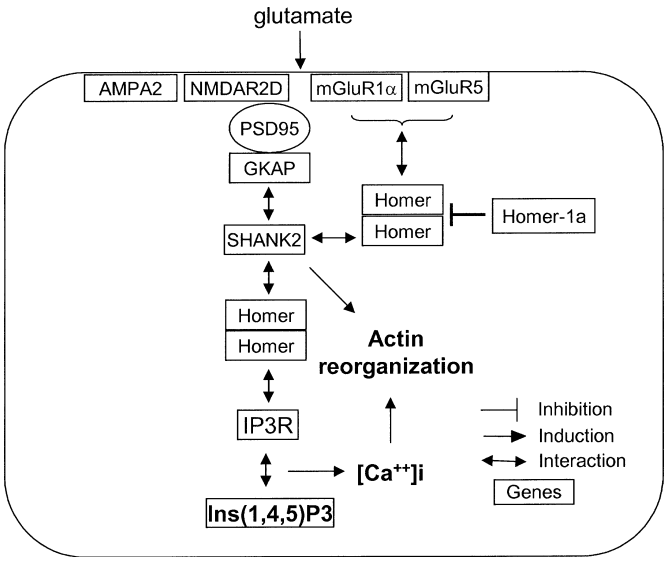

FIG. 9. Graphical representation of various interacting components of glutamatergic signalling that lead to restructuring of the actin cytoskeleton in the context of synaptic plasticity. Homer dimers allow for receptor clustering at the cell surface while increasing the intracellular Ca^{++} levels via their interaction with IP3Rs. Homer-1a, on the other hand, may compete with Homer long forms and thus is able to modulate the strength of the synaptic complexes and cytoskeletal organization. Abbreviations of genes: AMPA2, ionotropic glutamate receptor AMPA 2; NMDAR2D, NMDA ionotropic glutamate receptor; mGluR1a, glutamate receptor metabotropic alpha 1; mGluR5, glutamate receptor metabotropic 5; PSD-95, postsynaptic density protein 95 kDa; GKAP, guanylate kinase-associated protein; SHANK2, proline-rich synapse associated protein 2; IP3R, inositol 1,4,5-trisphosphate receptor.

levels and mGluR5 mRNA levels in the hippocampus after LTP induction, demonstrating regulation of these genes during LTP. Thus, it is reasonable to assume that the presence of nicotine increases the release of glutamate presynaptically from nerve terminals where, upon activation, postsynaptic metabotropic and ionotropic glutamate receptors modulate LTP in target neurons (see Fig. 9).

The mGluR–Homer–Shank–GKAP–PSD-95–NMDAR complex is also believed to play a role in the effective control of intracellular calcium levels through the modulation of the phosphatidylinositol signalling pathway (Nakanishi, 1994; Kawabata *et al*., 1998; Emptage *et al*., 1999; Tu *et al*., 1999). An increase in glutamatergic activity at this complex would indicate an increase in phosphoinositide and intracellular calcium levels (Fig. 9). In addition, Homer dimers are connected to F-actin whose reorganization is essential for spine growth and synapse formation (Sala *et al*., 2001). In addition to the up-regulation of mGluRs and Homer proteins, the phosphatidylinositol pathway might be modulated by nicotine via changes in the pool of available phosphatidylinositol-4,5-bisphosphate (PIP2) levels in the membrane, which can buffer actin-binding proteins. Nicotine has been shown to increase calcium levels in different types of neuronal cells (Barrantes *et al*., 1995; Sabban & Gueorguiev, 2002). Recently, Conroy *et al*. (2003) have identified the PDZ-containing proteins that are receptor-specifically associated with neuronal nAChRs in the chick ciliary ganglion. They have suggested that PDZ-associated proteins may play a role in the maturation of nicotinic synapses as well as the transduction of the calcium signal to the downstream components. Our findings therefore directly implicate Homer proteins as intrinsic components of nAChR signalling at the postsynaptic densities.

The mesolimbic dopaminergic projection is modulated by glutamatergic action within the VTA (Kalivas *et al*., 1989; Taber *et al*., 1995). Our data revealed an increase in mGluR and Homer proteins in the VTA, suggesting a role for mGluR pathways in the glutamatergic modulation of mesolimbic dopamine neurons. The difference in responses between VTA and amygdala of Homer2a/b and mGluR5 at day 3 may be due to the different glutamatergic input and/or response mechanisms utilized by the two separate regions. However, despite the differences there is a similar up-regulation of these related gene families at the early time points of nicotine administration. Interestingly, the expression and protein levels of Homers and mGluRs generally returned to baseline at the later time points, suggesting a new level of tolerance. Behaviour observation mirrors this chronology with an increased response to nicotine administration at the earlier time points than to later administrations. Given the fundamental role the amygdala and VTA play in the physiological response of an organism to drug treatment, the increase in Homer and mGluR levels suggest a role and possible target for the type I mGluR signalling pathway in the primary reinforcement actions of nicotine.

While nicotine produces a transient activation of mesolimbic dopaminergic neurons acting at somatic nAChRs (Pidoplichko *et al*., 1997), Mansvelder & McGehee (2000) demonstrated that glutamate release from glutamatergic nerve terminals was facilitated by the activation of presynaptic α7-containing nAChRs, and is required for prolonged dopamine release in the nucleus accumbens. Furthermore, NMDA receptor antagonism in the VTA has been shown to decrease nicotine-elicited accumbal dopamine release (Schilstrom *et al*., 1998). Regardless, further pharmacological experiments will be required to determine whether the group I metabotropic glutamate signalling complex acting either at distal sites, such as the amygdala or VTA, or directly within the nucleus accumbens is involved in nucleus accumbens dopamine release in response to nicotine.

Our findings also implicated the activity-induced short form of Homer (i.e. Homer1a) as a potential modulator of the nicotine-induced synaptic activity within the nucleus accumbens region. The expression analysis demonstrated a significant decrease in Homer1a and mGluR1α mRNAs in the nucleus accumbens at the early time point (Fig. 8A and B), although these transcriptional changes were not immediately reflected at the level of protein expression. Interestingly, a previous study (Kato *et al*., 1998) indicated that LTP induced expression of Homer1a mRNA but not that of the protein, possibly due to the rapid Homer1a protein turnover. Homer1a, which lacks a coiled-coil (CC) domain, was shown to exert negative feedback on Homer-mediated IP3 signalling possibly by competing with Homer1b/c and other long isoforms (CC–Homer), and disrupting the complex formation between CC–Homer and Shank, a component of postsynaptic density (PSD; Sala *et al*., 2003). Furthermore, Sala *et al*. (2003) have demonstrated that Homer1a is important for synaptic plasticity because its presence results in the restructuring of dendritic spine morphology via changes in the dendritic dimensions and number and in the molecular composition of PSD components. Our findings suggest that the nicotine-induced synaptic activity and calcium signalling may be altered by changes in Homer1a and mGluR1 expression in the nucleus accumbens. However, the cellular localization of mGluRs as well as long and activity-induced isoforms of Homer proteins should be determined upon nicotine treatment because Homer proteins are known to regulate cell-surface targeting of mGluRs upon ligand activation (Ango *et al*., 2002; Minami *et al*., 2003). Furthermore, studies that incorporate shorter intervals between experimental time points may be needed to address in detail the degree to which Homer1a expression is modulated during the course of nicotine administration.

In summary, our data provide evidence for the regulation of the group I mGluRs and Homer proteins in the three brain regions (VTA, amygdala and nucleus accumbens) by systemic nicotine administration. This represents the first report demonstrating Homer regulation by nicotine. Nicotine, acting through presynaptic nAChRs on glutamatergic terminals, drives glutamate release, thereby regulating excitatory input on target neurons. Activation of the group I mGluRs, Homer proteins and the phosphatidylinositol signalling pathway may reflect a new synaptic state in the target neurons. The change in synaptic strength of these neurons may be involved in several physiological responses to nicotine administration. Future experiments are needed to investigate these connections.

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Abbreviations

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isopropinonate; CC, coiled-coil; iGluR, ionotropic glutamate receptor; LTD, long-term depression; LTP, long-term potentiation; mGluR, metabotropic glutamate receptor; nAChR, nicotinic acetylcholine receptor; NMDAR, *N*-methyl-D-aspartate receptor; PDZ, the postsynaptic protein PSD-95/SAP90; the *Drosophila* septate junction protein Discs-large, and the tight junction protein ZO-1; PSD, postsynaptic density; real-time quantitative RT-PCR, reverse transcription and real-time polymerase chain reaction; VTA, ventral tegmental area.

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