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Long-term changes in connexin32 gap junction protein and mRNA expression following cocaine self-administration in rats

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Abstract

Considerable evidence indicates a critical role for dopamine in the reinforcing effects of cocaine. Because dopamine has been shown to be a critical modulator of gap junction communication in both eye and brain, we sought to examine whether extended intravenous cocaine self-administration would affect the expression of gap junction channel-forming proteins (connexins). Using ELISA, Western analysis, immunohistochemistry, semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), and non-radioactive *in situ* hybridization, we demonstrate that withdrawal from chronic cocaine self-administration causes lasting changes in connexin32 (Cx32) expression in the nucleus accumbens and hippocampus at 2, 7 and 21 days after the last cocaine injection. A sustained decrease in Cx32 protein and mRNA levels is noted in areas that have been implicated in cocaine craving (i.e. nucleus accumbens and subfields of the hippocampal formation). A progressive increase in gap junction protein and mRNA expression is noted in areas that become hyperexcitable after chronic cocaine exposure (i.e. CA1 hippocampal neurons). We speculate that gap junction communication may be critically involved in reinforcement processes and neuroadaptive changes produced by drugs of abuse.

Introduction

Animal models of drug self-administration have helped identify not only the neural mechanisms responsible for the acute reinforcing effects of cocaine but also many of the long-term neurochemical changes correlated with the development of the addictive process. These studies indicate that the site of action for acute cocaine reinforcement is at the dopamine (DA) transporter in the nucleus accumbens, striatum and other terminal fields of the mesolimbic/mesocortical DA system (Roberts *et al*., 1977; Koob, 1992; Roberts, 1992; Woolverton & Johnson, 1992). Self-administration paradigms also demonstrate that long-term exposure to cocaine results in lasting alterations in DA and other neurotransmitter levels as well as changes in the expression of DA receptors, transporters and catecholamine synthetic enzymes, thereby influencing the development of cocaine craving and cocaine addiction (Trulson *et al*., 1986; Kleven *et al*., 1990; Beitner-Johnson & Nestler, 1991; Laurier *et al*., 1994; Wilson *et al*., 1994; Pilotte *et al*., 1996).

While changes in chemical synaptic transmission following cocaine administration have been extensively documented, the effect of cocaine on gap junction communication has not been explored. Gap junctions consist of intercellular channels composed of connexin (Cx) proteins. At least 13 distinct Cx genes have been characterized with a high degree of conservation across vertebrate species (Bruzzone *et al*., 1996). Cx43, Cx32 and Cx26 are the most extensively studied in the central nervous system. Cx43 expression is found in ependymal cells, astrocytes, radial glia, and certain neurons of the occipital cortex, neocortex, hippocampus and cerebellum (Nadarajah *et al*., 1996, 1997; Simburger *et al*., 1997). Cx32 is abundant in oligodendrocytes (Scherer *et al*., 1995; Dermietzel *et al*., 1997; Li *et al*., 1997), while Cx32 and Cx26 are co-expressed in neurons (Micevych & Abelson, 1991; Nadarajah *et al*., 1996, 1997). Given that connexin-mediated gap junctions support both ionic flux and the transcellular movement of low molecular weight compounds (for review see Bennett, 1997), the presence of gap junctions can be inferred from either the demonstration of electrotonic coupling or by the transfer of a dye marker between neurons. Dye transfer has identified intercellular channels in the nucleus accumbens and striatum (Cepeda *et al*., 1989; O'Donnell & Grace, 1993; Onn & Grace, 1994b), and the rationale for evaluating the effect of cocaine on gap junctions is further strengthened by demonstrations that dye transfer in retina and brain can be modulated by DA (Hampson *et al*., 1992; Onn & Grace, 1994a, 1996; O'Donnell & Grace, 1995). Salient to this study, gap junction communication in the nucleus accumbens is decreased by D1 agonists and is increased by lesions of the nigrostriatal pathway or by administration of D1 or D2 antagonists (Cepeda *et al*., 1989; Onn & Grace, 1994a; 1996; O'Donnell & Grace, 1995).

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We sought to determine in the present study whether withdrawal from chronic cocaine self-administration elicits long-lasting changes in Cx32 expression. We report that sustained decreases in Cx32 expression are observed up to 21 days after the last cocaine injection in regions involved in cocaine reinforcement (nucleus accumbens and CA3 hippocampal fields), while sustained increases in Cx32 expression are observed in areas implicated in cocaine-precipitated epileptigenesis (CA1 hippocampal fields). These data suggest that alterations in gap junction communication may contribute to the behavioural alterations associated with psychostimulant addiction.

Materials and methods

Cocaine self-administration

Male Wistar rats ($n = 90$), weighing 275–300 g (Charles River Farms, Quebec, Canada), were group housed in shoe-box style cages and quarantined for 1 week. A reverse 12 h light/dark cycle (lights off from 15.00 h to 03.00 h) was instituted and maintained for the duration of training and testing. Twenty rats served as drug-naive control subjects. Following quarantine, rats ($n = 70$) were trained to press a lever under a fixed ratio (FR1) schedule of food reinforcement. Animals were food deprived for 23 h/day over the 2–3 days of food-reinforced training. Following this training period, Purina Rat Chow and water was available *ad libitum*. Each rat was anaesthetized with pentobarbital (65 mg/kg with supplements as required) and implanted with a chronically indwelling Silastic jugular cannula. Bard Marlex hernia mesh was secured under the skin in the region of the scapulae to provide an anchor for the cannula to exit on the dorsal surface. Subjects received post-surgical antibiotic (Neosporin, Wellcome Medical, Kirkland, Canada) and suppository analgesic (Abenol, SmithKline Beecham, Oakville, Canada). Complete details have been described elsewhere (Roberts & Goeders, 1989). Following cannulation, rats were singly housed in $25 \times 25 \times 25$ cm operant testing apparatus. The cannula exited the cage through a protective spring and was connected to a counter-balanced swivel apparatus which allowed the animal free movement within the operant chamber. The chamber was equipped with an operant lever and two stimulus lights. Rats were given access to a response lever for a 4-h period each day beginning at ~09.30 h. Each lever response activated an injection pump that delivered 1.5 mg/kg cocaine (in sterile saline over 5 s). Coincident with the start of the injection, a stimulus light was activated signalling a 20-s post infusion time-out period during which time responses produced no programmed consequence. The session was terminated after 40 injections or after 4 h, whichever occurred first. Typically, animals acquired a stable pattern of cocaine self-administration within 2–3 days. In order to have a homogeneous population with a consistent duration of exposure, animals that did not self-administer a minimum of 38 injections by the third test session were dropped from the study. The remaining subjects ($n = 60$) were given access to cocaine for 14 consecutive days. All procedures were carried out in accordance with the MRC and NIH guidelines for care and use of laboratory animals.

Collection of brain tissue

At various intervals following the last cocaine self-administration session (2, 7 or 21 days), rats ($n = 20$/time point) were anaesthetized with sodium pentobarbitol, and killed by perfusion or decapitation. Controls were age-matched (between 3.5 and 4.5 months old) drug-naive animals ($n = 20$). Following decapitation, brains were rapidly removed, and hippocampus, striatum, neocortex, posterior cortex, thalamus, medulla and cerebellum were dissected, flash-frozen in dry-ice-chilled isopentane, and stored at –100 °C until processing. In some animals ($n = 6$/time point), dorsal striatum and nucleus accumbens were separately dissected from total striatal tissue and flash-frozen. Protein and total RNA were simultaneously extracted from dissected samples using Triazol (Life Sciences Technologies, Gaithersburg, USA) according to the protocol provided by the manufacturer. Rats destined for *in situ* analyses were perfused transcardially with 60 mL of 100 mM phosphate-buffered saline (PBS: 100 mM sodium phosphate buffer pH 7.2, 154 mM NaCl), followed by freshly prepared 4% paraformaldehyde in 100 mM PBS. Brains were postfixed for 4 h in the same fixative at 4 °C and paraffin embedded using routine histological procedure. Sections (4 or 8 µm) were cut on a rotary microtome and mounted on sterile, gelatin-coated slides (Fisher, Ottawa, Canada).

Specificity of Cx32 antibodies

The Cx32 mouse monoclonal antibody M12.13 used in this study has been previously characterized (Goodenough *et al*., 1988). Briefly, M12.13 detects a 27-kDa band in liver tissue and brain corresponding to Cx32 (Goodenough *et al*., 1988; Scherer *et al*., 1995). Punctate immunoreactivity in regions of contact between adjacent hepatocytes, consistent with the known distribution of gap junctions is observed (Goodenough *et al*., 1988). Sections of liver or brain and immunoblots of liver or brain tissue incubated with preadsorbed antibody or preimmune serum show no reactivity (Goodenough *et al*., 1988; Scherer *et al*., 1995). The rat monoclonal antibody R5 detects a single 27-kDa band in liver immunoblots (Dr D.L. Paul, unpublished data).

Cx32 ELISA analysis

Cx32 protein was immobilized on 96-well polystyrene plates coated with rat monoclonal R5 (1 : 2) and detected with mouse monoclonal M12.13 (1 : 2). Plates were blocked with 10 mM PBS containing 1% bovine serum albumin. R5 was diluted in coating buffer (10 mM PBS containing 2% bovine serum albumin and 0.2% Triton-X100, pH 7.4) and incubated overnight at 4 °C. Total protein (20 µg) was incubated in coating buffer for 3 h at 37 °C. Cx32 was detected by M12.13 diluted in 10 mM PBS containing 1% bovine serum albumin for 3 h at 37 °C followed by peroxidase-linked anti-mouse IgG (1 : 2000, Jackson Immunolabs, Mississauga, ON, Canada) for 2 h at 37 °C. Plates were washed repeatedly between incubations with PBS, pH 7.4. Reactions were visualized by incubation for 20 min at room temperature with peroxidase blue substrate according to the protocol provided by the manufacturer (Boehringer Mannheim, Montreal, Canada) and absorbance (456 nm/690 nm) determined.

Cx32 Western analysis

Protein (30 µg) was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions on 12.5% polyacrylamide gels containing 0.1% (w/v) SDS and electroblotted to nitrocellulose membrane. Western analysis was performed using monoclonal M12.13 (1 : 10) (Goodenough *et al*., 1988), biotinylated goat anti-mouse IgG (1 : 20 000; Sigma, St Louis, USA), and extravidin peroxidase (1 : 1000; Sigma). Membranes were blocked in 10 mM PBS (10 mM sodium phosphate buffer, pH 7.5, 154 mM NaCl) containing 1% heat-denatured casein. Antibodies were diluted in the same blocking solution. Immunoreactive bands were visualized by chemiluminescence according to the protocol provided by the manufacturer (Boehringer Mannheim).

Cx32 and NeuN immunohistochemistry

Sections were deparaffinized, rehydrated and equilibrated in 10 mM PBS. Sections were reacted overnight with primary M12.13 (1 : 2) or primary NeuN (1 : 100; Chemicon, Mississauga, Canada) antibody at

4 °C, labelled for 1 h at room temperature with biotinylated goat anti-mouse IgG (1 : 300; Sigma), incubated for 1 h in extravidin-peroxidase (1 : 100, Sigma), and reacted with 1 mg/mL diaminobenzidine in 50 mM Tris–HCl, pH 8.0 containing 0.003% H_2O_2. Primary and secondary antibodies and tertiary reagents were diluted in Ab buffer (10 mM PBS, 0.3% Triton-X100, 3% BSA, 2% lamda carrageenan, pH 7.5). With respect to adjacent (4 µm) sections labelled with anti-Cx32 and anti-NeuN, respectively, identical fields were established visually on a Nikon immunofluorescent microscope and captured in 16-channel greyscale using a Nikon digital camera. These adjacent-field digital micrographs were converted to eight-channel greyscale and then overlaid using AdobePhotoshop 5.0. NeuN immunoreactivity was pseudo-coloured red in the overlay to permit identification of NeuN-positive neurons expressing Cx32.

Semi-quantitative RT-PCR analysis of Cx32 transcript

RNA (4 µg) was treated with 3 U RQ1 RNAse-free DNAse (Promega, Whitby, Canada) at 37 °C for 30 min in a final volume of 20 µL according to the protocol provided by the manufacturer. Total template RNA and 60 pmol pdN_6 (Life Science Technologies) were heated for 10 min at 70 °C. dNTPs (0.125 mM each, Promega), dithiothreitol (DTT: 10 mM) and 1 × RT buffer (Life Sciences Technologies) were added and incubated at 37 °C for 2 min. Superscript RT (200 U; Life Sciences Technologies) was added and samples were incubated at 25 °C for 10 min, 42 °C for 1 h, and 50 °C for 30 min. Two microlitres of this RT reaction was PCR amplified with dNTPs (0.3 mM each), 1 × PCR buffer (Life Sciences Technologies), 2.5 U DNA Taq polymerase (Life Sciences Technologies), 20 pmol Cx32 primers, and 10 pmol glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Samples were incubated at 95 °C for 5 min then 72 °C for 1 min. Samples were denatured at 95 °C for 30 s, annealed at 55 °C for 90 s, and extended at 72 °C for 120 s. Cx32 priming occurred over 30 cycles. GAPDH primers were added at the onset of cycle 8 and amplified over the remaining 23 cycles. RT-PCR-amplified samples were separated by electrophoresis on a 2% agarose gel. Positive and negative film photomicrographs of ethidium bromide-stained gels were obtained. Densitometry was performed on negative film photomicrographs using MCID Imaging software (St. Catherines, Canada). Cx32 primer sequences were 5-CTGCTCTACCCGGGCTATGC-3 (forward) and 5-CAGGCTGAGCATCGGTCGCTCTT-3 (reverse), defining a 386-bp fragment of the rat liver cDNA (Paul, 1986). GAPDH primer sequences were 5-TGGTGCTGAGTATGTCGTGGAGT-3 (forward) and 5-AGTCTTCTGAGTGGCAGTGATGG-3 (reverse) defining a 292-bp fragment of the rat GAPDH cDNA (Piechaczyk *et al*., 1984). Primers were synthesized at the Adirondack Biomedical Research Institute, Lake Placid, NY, USA. Single-primed Cx32 PCR products were digested with *Alu*I to demonstrate appropriate restriction enzyme digestion characteristics.

In situ *hybridization to Cx32 transcript*

Sections were deparaffinized, rehydrated in 10 mM PBS, incubated successively in 20 mM HCl for 10 min at room temperature, 10 mM PBS containing 0.1% Triton-X100 for 90 s at 4 °C, 20 µg/mL proteinase K dissolved in 10 mM Tris–HCl, pH 7.5 for 20 min at 37 °C, 2 mg/mL glycine for 5 s, and cold 20% glacial acetic acid for 15 s at 4 °C. Sections were rinsed in PBS between incubations. Cx32 in pGEM3 (Paul, 1986) was digested with *Avr*II and transcribed with T7 RNA polymerase to generate sense probes, or digested with *Nco*I and transcribed with SP6 RNA polymerase to generate antisense probes. Probes were biotinylated during synthesis using a Boehringer Mannheim biotinylation transcription kit according to the protocol provided by the manufacturer. Labelled Cx32 sense or antisense riboprobe (final concentration of 20 ng/mL) was added to hybridization buffer (Amersham, Arlington Heights, IL, USA) diluted 1 : 1 with deionized formamide and applied to the sections. Sections were heated at 80 °C for 1 min and hybridization was carried out at 42 °C for 16 h. Sections were washed twice in 1 × SSC (150 mM NaCl, 15 mM sodium citrate) containing 0.1% SDS and twice in 0.2 × SSC containing 0.1% SDS at 42 °C for 5 min with shaking. Sections were rinsed in PBS and incubated with extravidin peroxidase (1 : 20, Sigma) in 10 mM PBS containing 3% BSA for 1 h at room temperature. Hybridization was detected by reaction with 1 mg/mL diaminobenzidine in 50 mM Tris–HCl containing 0.01% H_2O_2.

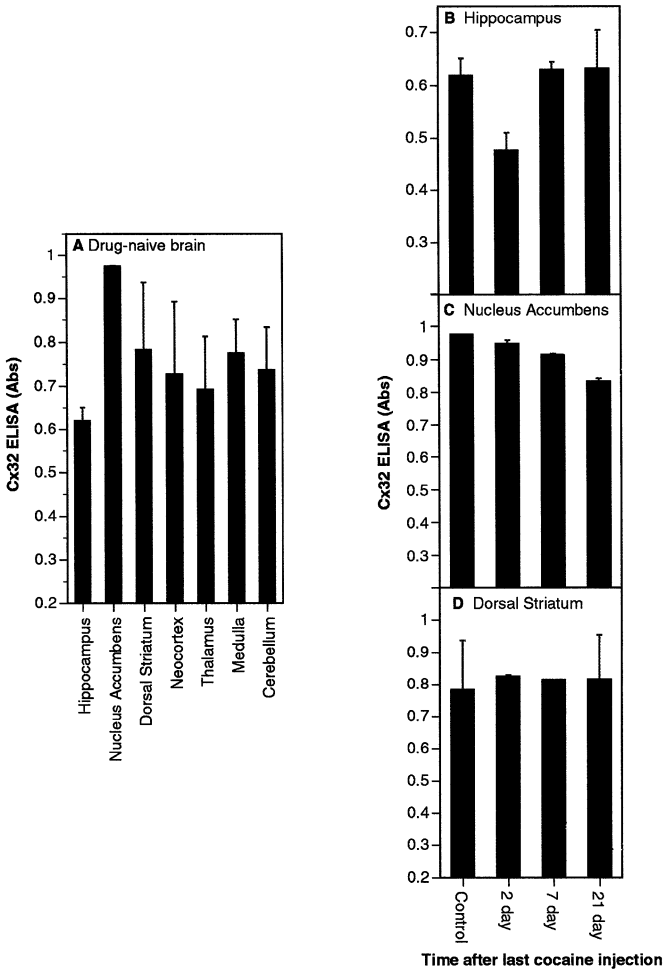

FIG. 1. Regional distribution of Cx32 protein in Wistar rat brain. Sandwich ELISA analysis of 20 µg of protein dissected from hippocampus, nucleus accumbens, dorsal striatum, neocortex, thalamus, medulla and cerebellum using rat monoclonal R5 as the capture antibody and mouse monoclonal M12.13 (Goodenough *et al*., 1988) as the detection antibody. Details are as described in Materials and methods. Data represent the mean absorbance ± standard error of measurement (SEM) from $n = 2$ animals/ region.

Statistics

Densitometric data were analysed using analysis of variance (ANOVA). Following detection of a statistically significant result ($P < 0.05$), *post hoc* Dunnett's *t*-tests were performed to determine which condition differed significantly from control levels.

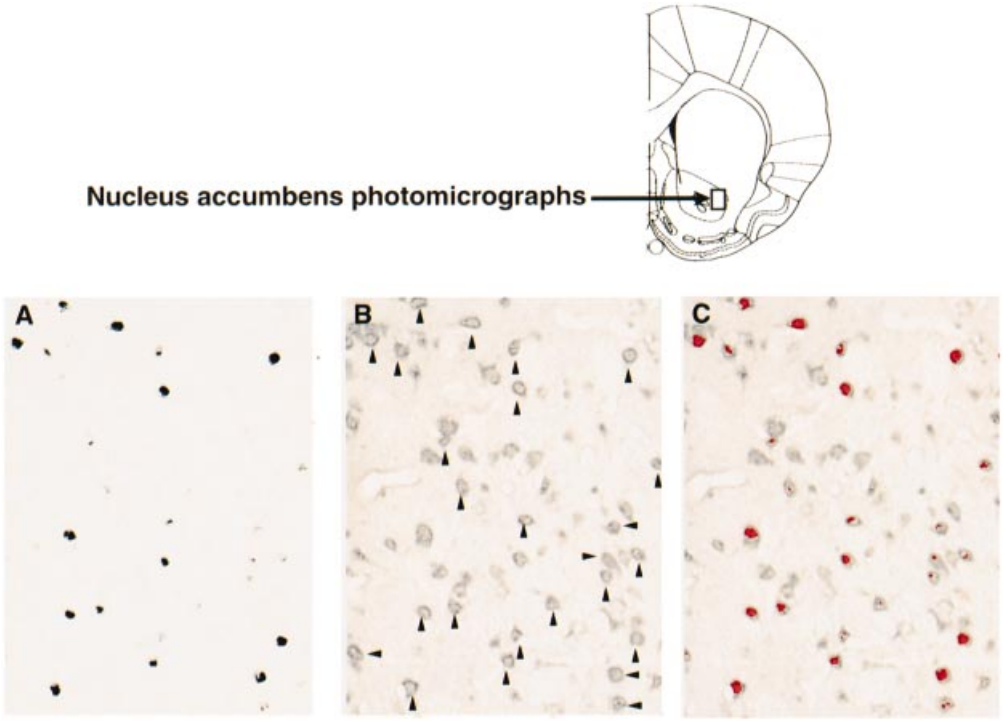

FIG. 2. Cx32 is expressed by neurons in nucleus accumbens. Localization of Cx32 immunoreactivity to neurons in the nucleus accumbens was determined by reacting adjacent paraffin-embedded sections (4 µm in thickness) with monoclonal antibodies raised against the neuron-specific nuclear antigen NeuN (A) or Cx32 (M12.13) (B). Digital photomicrographs were then overlaid using AdobePhotoshop and NeuN immunoreactivity pseudo-coloured in red to visualize cells expressing both Cx32 and NeuN (C). Details are as described in Materials and methods. Note that all of the neurons detected in A are Cx32 positive (arrowheads in B and red nuclear profiles in C). These data provide clear evidence that Cx32 is expressed by neurons in the nucleus accumbens. Additional cells, not detected in A, are also Cx32 positive. These cells may be glia or may be neurons whose nuclear profiles were not present in the 4 µm section reacted in A.

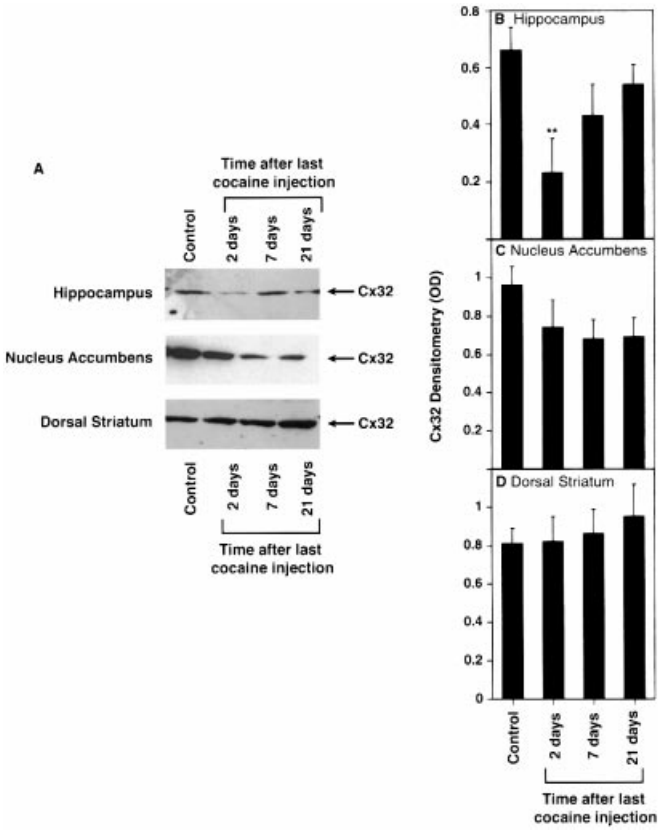

FIG. 3. Cx32 protein expression is altered in multiple brain regions following withdrawal from chronic cocaine self-administration. Western analysis using monoclonal M12.13 of protein (30 µg) dissected from hippocampus, nucleus accumbens and dorsal striatum from control (drug-naive) rats and animals at 2, 7 and 21 days after withdrawal from 14 days of chronic cocaine self-administration (A). Details are as described in Materials and methods. Blots were stripped following Cx32 analysis and reprobed for tubulin to ensure equivalent protein concentrations across wells (data not shown). Densitometric analysis of five independent immunoblots was performed from protein extracted from five different animals per time point from hippocampus (B), nucleus accumbens (C) and dorsal striatum (D). Data represent the mean optical density (OD in arbitrary units) ± SEM from $n = 5$ animals/time point. A statistically significant decrease in protein levels was noted in the hippocampus 2 days after the last cocaine injection (asterisk in B, ANOVA, *post hoc* Dunnett's *t*-test, $P < 0.01$). A sustained decrease in protein expression was noted in nucleus accumbens following cocaine self-administration, but this reduction failed to reach statistical significance. No change in Cx32 levels was noted at any time point in dorsal striatal samples.

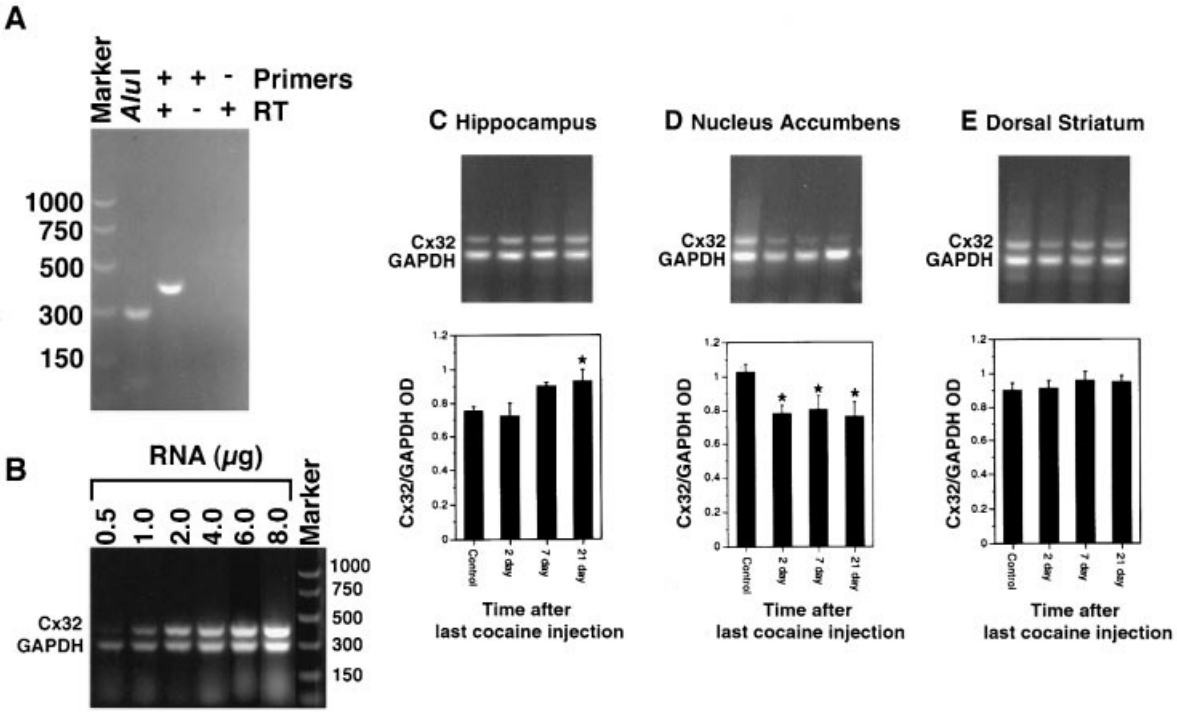

FIG. 4. Cx32 mRNA expression changes in rat hippocampus and nucleus accumbens but not dorsal striatum during cocaine withdrawal. Semi-quantitative RT-PCR analysis of Cx32 expression was performed at 2, 7 and 21 days after withdrawal from 14 days of chronic cocaine self-administration. Total RNA was extracted from whole brain tissue of control animals. Four micrograms of RNA was treated with 3 U of RQ1 RNAse-free DNAse and subjected to RT-PCR as described in Materials and methods. Amplification produced a single Cx32 amplicon with the correct *Alu*I digestion characteristics (287 bp and 99 bp). RT-PCR reactions processed in the absence of RNA or primers did not amplify Cx32 cDNA demonstrating specificity for mRNA template and lack of genomic contamination (A). For semi-quantitative analyses, Cx32 expression was standardized against GAPDH expression (internal standard). Optimal cycling times were established for co-amplification and GAPDH primers were added at the onset of cycle 8 and amplified for 23 cycles as described in Materials and methods. The linearity of co-amplification was established by adding 2 μL of RT product transcribed from 0.5 to 8 μg of whole brain template RNA. One to six micrograms of template RNA fell within a linear range of amplification (B). The kinetics of Cx32 expression in rat hippocampus (C), nucleus accumbens (D) and dorsal striatum (E) in drug-naive control animals, and at 2, 7 and 21 days after chronic cocaine self-administration were determined. Total RNA was extracted from dissected brain regions from five animals per time point, and 4 μg total RNA was DNAse-treated and RT-PCR amplified as described in Materials and methods. A representative photograph from one series of samples is depicted. For semi-quantitative analyses, densitometry was performed on negative film exposures of ethidium bromide-stained gels. Cx32 OD (in arbitrary units) was standardized against co-amplified GAPDH OD. Data represent the mean standardized Cx32 OD ± SEM of five animals per time point in nucleus accumbens (D), and six animals per time point in hippocampus (C) and dorsal striatum (E). A statistically significant increase in mRNA levels compared with basal expression was detected in the hippocampus 21 days after the last cocaine injection (ANOVA, *post hoc* Dunnett's *t*-test, $P < 0.05$, asterisks in C). A statistically significant decrease in mRNA levels compared with basal expression was detected in nucleus accumbens 2, 7 and 21 days after the last cocaine injection (ANOVA, *post hoc* Dunnett's *t*-test, $P < 0.05$, asterisks in D). No change in protein levels was detected at any time point in dorsal striatal samples (E) (ANOVA, $P > 0.05$).

Results

Long-term cocaine self-administration

Rats ($n = 60$) received between 35 and 40 injections of 1.5 mg/kg cocaine each day for 14 days prior to Cx32 evaluation. Controls were drug-naive rats ($n = 20$).

Changes in Cx32 protein expression following cocaine self-administration are regionally specific in rat brain

Basal expression levels of Cx32 protein in control rats were established by ELISA in hippocampus, nucleus accumbens, dorsal striatum, neocortex, thalamus, medulla and cerebellum (Fig. 1A). Maximal Cx32 protein levels were detected in the nucleus accumbens and minimal levels in the hippocampus (Fig. 1A).

Localization of connexin protein in neurons of hippocampus, nucleus accumbens and dorsal striatum was determined by immunohistochemistry for NeuN, a neuronal-specific nuclear marker (Fig. 2A) and Cx32 (Fig. 2B). Immunohistochemistry was performed on adjacent paraffin sections (4 μm apart) and photomicrographs were then overlaid using AdobePhotoshop 5.0 to identify NeuN/Cx32-positive cells (arrows in Fig. 2B, red cells in Fig. 2C). All of the NeuN-positive neurons in the nucleus accumbens (Fig. 2C) and dorsal striatum (data not shown) were Cx32 positive. The majority (~ 60–70%) of all NeuN-positive neurons in the pyramidal cell fields of the hippocampus were Cx32 positive (data not shown), although the exact percentage could not be precisely quantitated as stereological analyses were not performed.

Three brain regions (hippocampus, Fig. 1B, nucleus accumbens, Fig. 1C, and dorsal striatum, Fig. 1D) were screened for changes in Cx32 protein levels by ELISA at various time points following the last injection of long-term cocaine self-administration. In hippocampus, a transient 23% decrease in Cx32 protein levels was observed 2 days after the last drug injection in cocaine-exposed animals compared with age-matched drug-naive rats (Fig. 1B). In nucleus accumbens, a progressive decrease in Cx32 protein expression was observed 2–21 days after chronic cocaine self-administration with levels dropping by 14% at 21 days compared with control rats (Fig. 1C). In dorsal striatum, no change in Cx32 protein levels was observed at any time point (Fig. 1D).

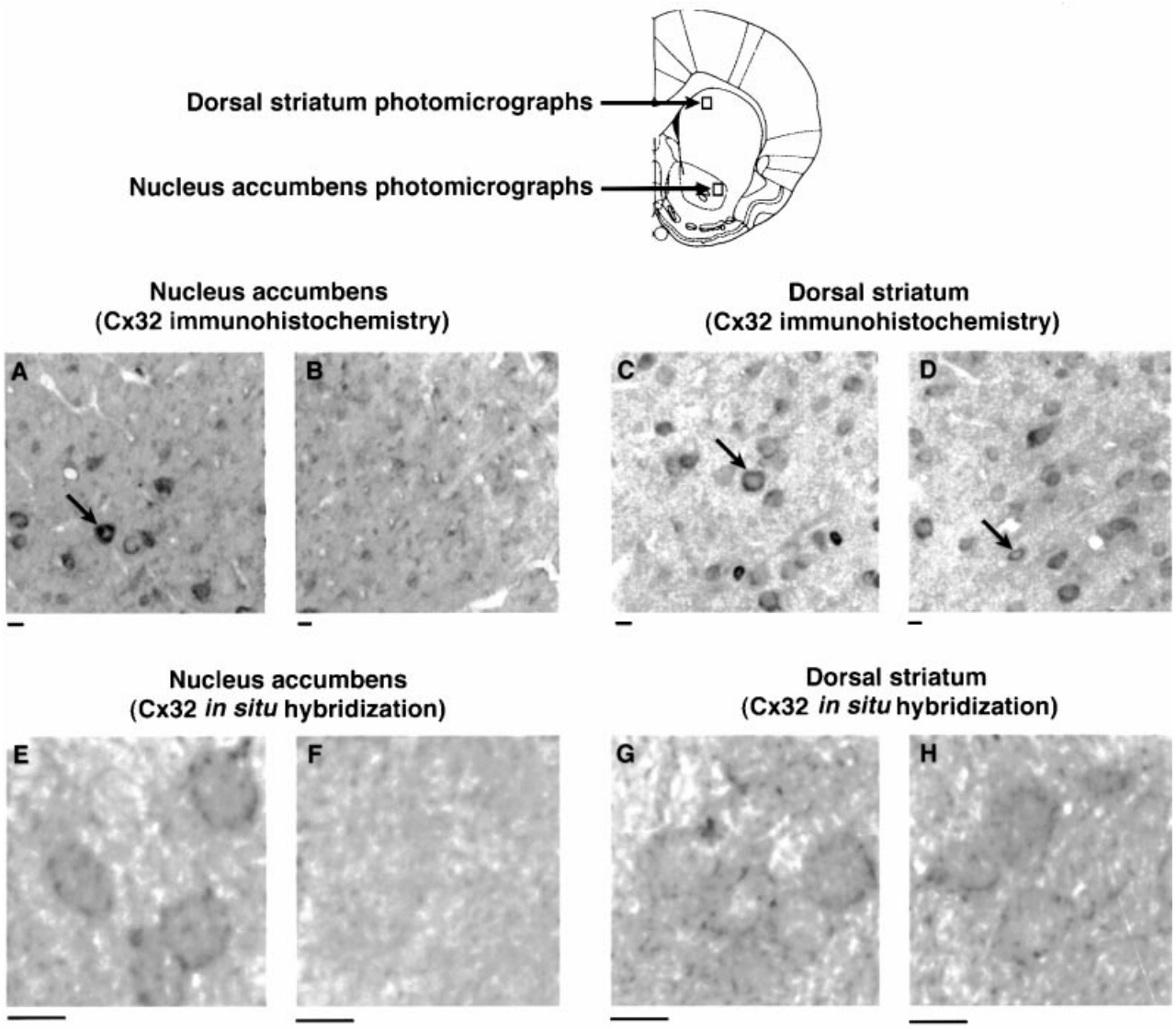

FIG. 5. Cellular localization of Cx32 mRNA and protein in striatal neurons following chronic cocaine self-administration. (A, C, E and G) Drug-naive rats. (B, D, F and H) Seven days after last cocaine injection. (A–D) Cx32 immunohistochemistry using monoclonal M12.13. (E–H) Cx32 *in situ* hybridization using Cx32 antisense riboprobe. Details as described in Materials and methods. No cellular signal was observed with sense probe (data not shown). A decrease in Cx32 protein and mRNA expression is observed 7 days after cocaine self-administration in nucleus accumbens but not dorsal striatal neurons. Scale bar, 20 μm.

A more detailed examination of changes in Cx32 protein expression during cocaine withdrawal was performed by Western analysis. Protein was extracted from five different animals per time point ($n = 20$). Immunoblots were quantified by densitometry. Figure 3A shows a representative Western blot analysis for each region extracted from a drug-naive control and cocaine self-administrators at 2, 7 and 21 days after the last cocaine injection. Immunoblot exposure time was optimized for each region to allow for detectable increases and decreases in signal intensity. In hippocampus, following densitometric analysis of five animals per time point, a statistically significant decrease in protein levels was detected 2 days after the last cocaine injection compared with basal (control) expression (ANOVA, $F = 3.4$, d.f. = 3,16, $P \leq 0.05$; *post hoc* Dunnett's *t*-test, control versus 2 day time point, $P \leq 0.01$). Cx32 protein levels dropped to 65% of that of control levels 2 days after cocaine withdrawal (Fig. 3B). In nucleus accumbens, a sustained decrease in Cx32 levels was observed over time after cocaine withdrawal with expression dropping to 70% that of basal (control) expression 7 days after the last cocaine injection (Fig. 3C). While this decrease failed to reach statistical significance (ANOVA, $F = 1.6$, d.f. = 3,16, $P > 0.05$), the trend was consistent with the reduction in protein levels detected by ELISA (Fig. 1C). No change in dorsal striatal Cx32 levels was detected at any time point (ANOVA, $F = 0.2$, d.f. = 3,16, $P > 0.05$, Fig. 3D).

Alterations in Cx32 mRNA are consistent with protein changes in nucleus accumbens but not hippocampus

RT-PCR semi-quantitative analyses of Cx32 expression were performed on total RNA extracted from drug-naive animals and cocaine self-administrators at 2, 7 and 21 days after drug withdrawal. A 386-bp fragment of Cx32 was amplified from whole brain total RNA (Fig. 4A). *Alu*I digestion of the amplicon (Fig. 4A, lane '*Alu*I') produced fragments of the predicted size demonstrating specificity of amplification for Cx32. Control reactions without RNA or RT demonstrated the lack of template or genomic contamination (Fig. 4A). Cx32 mRNA levels were normalized to GAPDH mRNA levels

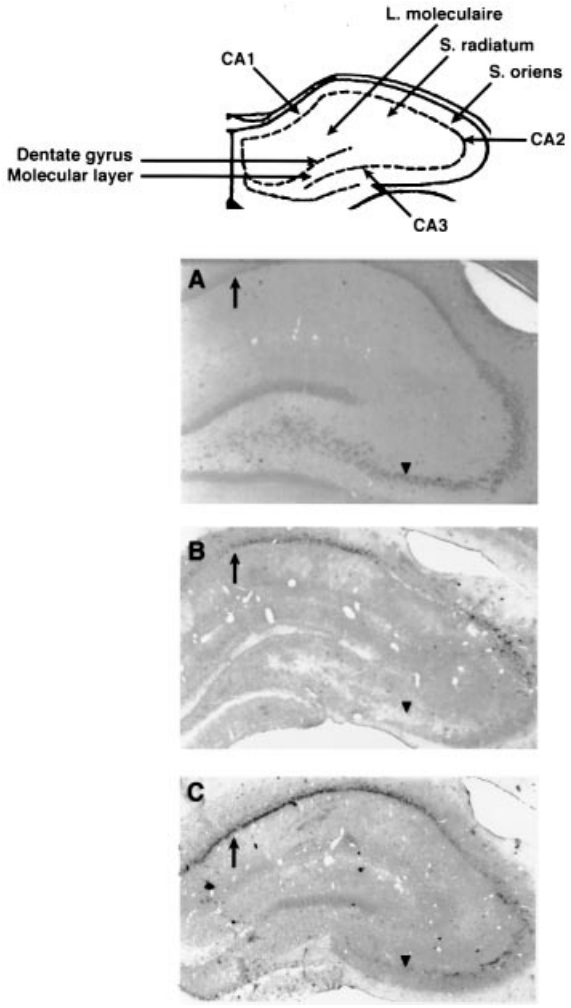

FIG. 6. Cellular localization of Cx32 protein in rat hippocampus during cocaine withdrawal. Immunohistochemistry was performed using monoclonal M12.13 on 10 µm sections from control (drug-naive) rats and animals at 2 and 7 days after withdrawal from chronic cocaine self-administration as described in Materials and methods. A decrease in Cx32 immunoreactivity and *in situ* hybridization is observed in lateral CA1 and CA3 cell fields and dentate gyrus 2 days after cocaine self-administration. Partial restoration of Cx32 levels can be seen at 7 days (CA3, arrowheads). An increase in immunoreactivity in medial CA1 cells (arrows) is observed 2–7 days after cocaine withdrawal.

to control for variations in RNA template concentration. GAPDH was chosen as an internal standard based on previously published results (Szaflarski *et al*., 1995). Because GAPDH mRNA is more abundant than Cx32 in the brain, optimal cycling times for Cx32 (30 cycles) and GAPDH (23 cycles) were established, and GAPDH primers were added to ongoing PCR reactions at the onset of cycle 8. In this manner, the reaction rates were adjusted so that a linear increase in the amount of template resulted in linear increases in the amount of both GAPDH and Cx32 amplicons (Fig. 4B).

In the hippocampus, a statistically significant increase in Cx32 mRNA expression was observed following chronic cocaine exposure (ANOVA, $F = 3.5$, d.f. = 3,20, $P \leq 0.05$; *post hoc* Dunnett's *t*-test, control versus 21 day time point, $P \leq 0.05$) with mean levels over the 2–21-day observation period reaching 124% of control (Fig. 4C). These data contrast with the statistically significant decrease in protein levels observed 2 days after cocaine withdrawal (Figs 1B and 3A and B).

In striatal samples, changes in mRNA levels were consistent with the amounts of protein observed at the various time points following cocaine withdrawal. A statistically significant decrease in Cx32 mRNA was detected in nucleus accumbens 2–21 days after cocaine injection (ANOVA, $F = 3.2$, d.f. = 3,16, $P \leq 0.05$; *post hoc* Dunnett's *t*-test, control versus 2 day time point, $P \leq 0.05$; control versus 7 day time point, $P \leq 0.05$; control versus 21 day time point, $P \leq 0.05$, Fig. 4D). No change in dorsal striatal Cx32 mRNA expression was noted (ANOVA, $F = 0.4$, d.f. = 3,20, $P > 0.05$, Fig. 4E).

Localization of Cx32 mRNA and protein in neurons of the nucleus accumbens, dorsal striatum and hippocampus

Immunohistochemistry and non-radioactive *in situ* hybridization were performed to more precisely localize changes in Cx32 expression.

In striatal sections of control animals, Cx32 immunolabelling was identified in neurons of nucleus accumbens (Fig. 5A, arrow) and dorsal striatum (Fig. 5C, arrow). Neuronal immunoreactivity was confirmed by double-labelling for Cx32 and a neuronal nuclear antigen (Fig. 2). Immunoreactivity was localized primarily to cell body with little or no labelling of cell processes (Fig. 5A and C, arrows). Seven days after chronic cocaine exposure, Cx32 immunoreactivity could no longer be detected in cells of the nucleus accumbens (compare Fig. 5A and B), although some neurons in the lateral reaches of the ventral striatum remained Cx32 positive (data not shown). No change in the distribution or intensity of Cx32 immunoreactivity was detected in dorsal striatum during cocaine withdrawal (compare Fig. 5C and D). These data are in complete accord with Western and ELISA analyses.

Similar changes in mRNA expression were detected by colorimetric *in situ* hybridization. Cx32 mRNA was observed in neurons of both the nucleus accumbens (Fig. 5E) and dorsal striatum (Fig. 5G) in control sections. Seven days after chronic cocaine exposure, little or no mRNA was detected in cells of the nucleus accumbens (compare Fig. 5E and F) although, as with protein labelling, some neurons in the lateral reaches of the ventral striatum continued to express Cx32 transcript (data not shown). No change in mRNA levels or distribution was detected at any time point in dorsal striatum (compare Fig. 5G and H). These data are consistent with RT-PCR analyses.

In midbrain sections, Cx32 immunoreactivity in control hippocampus was detected at low levels in the majority of neurons of the CA fields and cells of the dentate gyrus (Fig. 6A). In CA neurons, Cx32 protein was primarily localized to the cell body with some punctate staining of processes (data not shown). Astrocytes, abundant in the lacunosum moleculaire, did not label for Cx32. Two days after cocaine self-administration, a marked increase in the intensity of the Cx32 signal was detected in the medial CA1 cell field (compare arrows in Fig. 6A and B). However, a consistent decrease in Cx32 label was observed in the pyramidal cell region of CA3 (compare arrowheads in Fig. 6A and B) and in the dentate gyrus (Fig. 6B). Seven days after the last cocaine self-administration session, a return of Cx32 signal was evident in some dentate gyrus and CA3 cells (Fig. 6C), while immunoreactivity within the CA1 cell field remained more intense compared with drug-naive subjects (Fig. 6C).

Similar changes in mRNA expression were noted by *in situ* hybridization. Two days after cocaine self-administration, a marked increase in the number of Cx32 mRNA-positive cells was observed in the medial region of CA1 (compare Fig. 7A and B), while a decrease in the number of cells exhibiting Cx32 mRNA was observed in the lateral reaches of CA1 (compare Fig. 7D and E), CA2 and CA3 cell fields and dentate gyrus (data not shown). Seven days after cocaine administration, transcript levels in neurons located in the medial

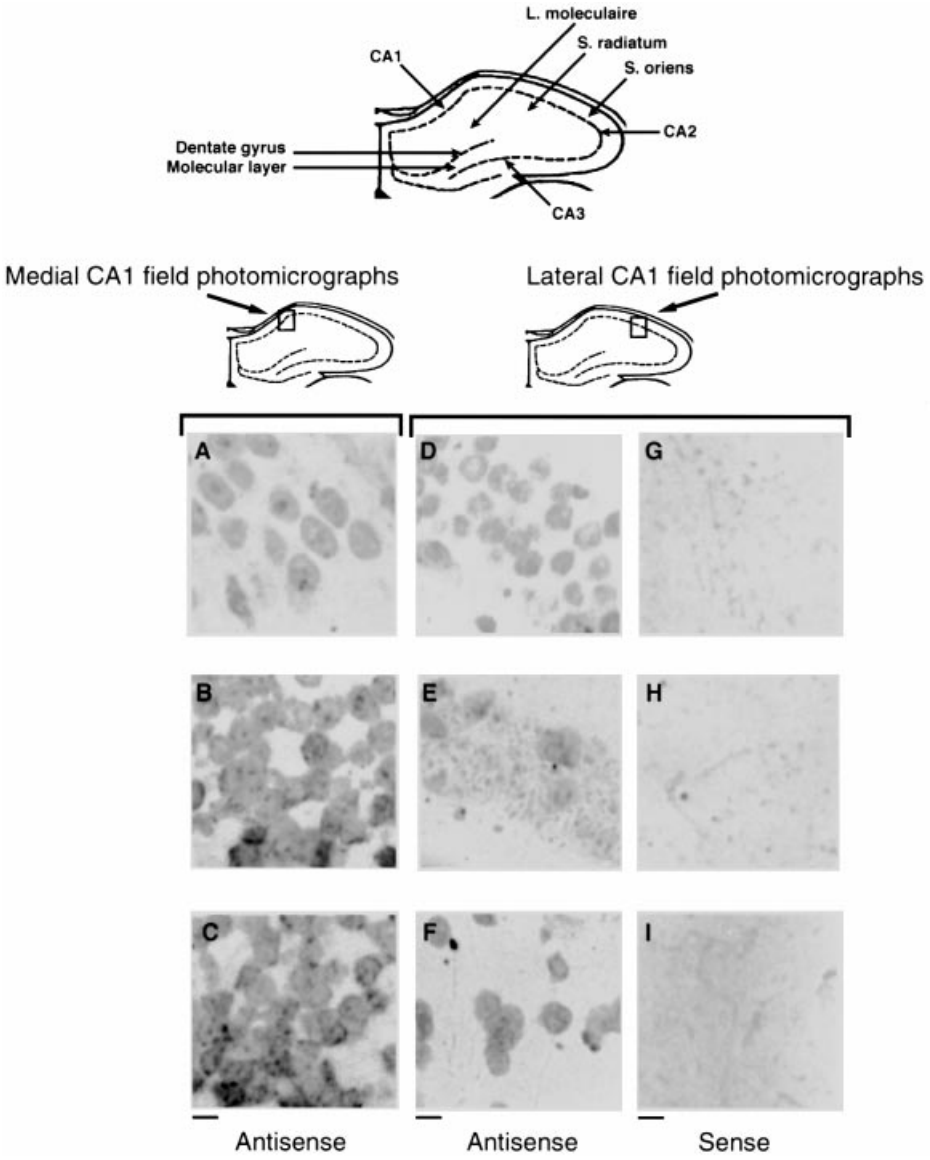

FIG. 7. Cellular localization of Cx32 mRNA in CA1 pyramidal neurons of rat hippocampus following chronic cocaine self-administration. Cx32 mRNA was localized in RNAse-free sections (10 μm) from control (drug-naive) rats (A, D and G), and animals at 2 days (B, E and H) and 7 days (C, F and I) after withdrawal from chronic cocaine self-administration. (A–C) Medial CA1 cells. (D–I) Lateral CA1 cells. (A–F) *In situ* hybridization to Cx32 mRNA antisense riboprobe. (G–I) Sections adjacent to (D–F) probed with Cx32 sense riboprobe. Details as described in Materials and methods. An increase in Cx32 *in situ* hybridization is observed in medial CA1 neurons at 2 and 7 days following the last cocaine injection (compare A with B and D). A decrease in the number of cells exhibiting Cx32 transcript is noted in lateral CA1 cells 2 days after cocaine self-administration (compare D and E) with partial restoration at 7 days (F). Scale bar, 20 μm.

reaches of CA1 remained elevated (Fig. 7C), and a partial restoration of Cx32 mRNA in some lateral CA1 (Fig. 7F), CA2, CA3 and dentate gyrus cells (data not shown) was apparent. No signal was obtained when sections were hybridized with sense riboprobe at any of the time points analysed (Fig. 7G–I), demonstrating the specificity of antisense hybridization for Cx32 mRNA.

These *in situ* analyses of Cx32 expression in hippocampus demonstrate an increase in protein and mRNA in medial CA1 cells, and a decrease in protein and mRNA in lateral CA1, CA2, CA3 and dentate gyrus neurons during cocaine withdrawal. These regional differences are sufficient to account for the apparent discrepancy between Western/ELISA analyses and RT-PCR. RT-PCR is exquisitely sensitive to minute increases in RNA template concentration, and results reflect the regional increase in transcript in medial CA1 cells. Protein analyses are consistent with the overall decrease in Cx32 expression observed throughout the rest of the hippocampus.

Discussion

Dopaminergic transmission has been shown to be critically involved in the acute reinforcing effects of cocaine, and withdrawal from cocaine has been shown to cause marked alterations in striatal DA efflux, receptor levels, transporter levels and synthetic enzyme levels (Trulson *et al*., 1986; Kleven *et al*., 1990; Beitner-Johnson & Nestler, 1991; Laurier *et al*., 1994; Wilson *et al*., 1994; Pilotte *et al*., 1996). Abstinence from cocaine produces affective disorders and recurring episodes of drug craving. Given evidence that gap junction coupling between neurons is sensitive to dopaminergic modulation (Cepeda *et al*., 1989; Llinas, 1984; Onn & Grace, 1994a,b; O'Donnell & Grace, 1995; Perez-Velazquez *et al*., 1994) we hypothesized that some of these drug-induced disorders might be accounted for by alterations in gap junction communication. To address this possibility, we evaluated whether withdrawal from chronic cocaine injection elicits long-lasting changes in connexin levels. Expression of Cx32 in drug-naive animals and in rats undergoing withdrawal from 2 weeks of chronic cocaine self-administration was examined. Our data reveal protracted alterations in Cx32 protein levels in rat hippocampus and nucleus accumbens during cocaine withdrawal by ELISA, Western analysis, semi-quantitative RT-PCR, immunohistochemistry and *in situ* hybridization.

Although the distribution of Cx32 in the striatum has not been extensively studied, Naus *et al*. (1990) have documented the presence

of Cx32 message in striatal tissue by Northern analysis. The present results confirm the presence of Cx32 transcript in striatum and extend this observation by localizing protein expression to neurons. Following chronic cocaine self-adminstration, the levels of Cx32 protein and mRNA are decreased in nucleus accumbens within 2 days of the last cocaine injection and fail to recover after 21 days of cocaine withdrawal. No change in dorsal striatal Cx32 expression is observed. This distinction is important given that both the nucleus accumbens and dorsal striatum are responsive to dopaminergic stimulation and hence modulated by chronic cocaine abuse. However, only the nucleus accumbens has been directly linked to the reinforcing effects of cocaine (Roberts, 1992), while dorsal striatal activity is associated with cocaine-induced stereotypic and motoric behaviour (Amalric & Koob, 1993). The fact that the alterations in Cx32 expression are restricted to the ventral rather than dorsal striatum leads us to speculate that these changes may be functionally related to cocaine reinforcement and/or motivational changes associated with cocaine withdrawal.

Cx32 expression in the hippocampus was also found to be sensitive to the effects of cocaine withdrawal. Recent MRI studies have indicated that elevated metabolic activity in the hippocampus, parahippocampal gyrus and nucleus accumbens correlate with craving ratings in cocaine-dependent human subjects (Breiter *et al*., 1997). The ability of cocaine to enhance acetylcholine release in the hippocampus has led to the hypothesis that cocaine-induced hippocampal activity may play a role in cocaine addiction by regulating the memory of a DA-dependent reinforcing event (Imperato *et al*., 1993). In the present study, the baseline distribution of Cx32 protein and mRNA observed in the hippocampus is in general accord with previously published reports (Shiosaka *et al*., 1989). During cocaine withdrawal, overall protein levels in the hippocampus decline 2 days after the last cocaine session and exhibit partial recovery 7–21 days after drug administration. Consistent with this recovery, a moderate increase in Cx32 mRNA is observed at later time points during cocaine withdrawal compared with basal expression in control animals. Close examination of neuronal subpopulations by *in situ* hybridization and immunohistochemistry reveal differential effects within distinct hippocampal cell fields. In CA3, CA2, lateral CA1 and dentate gyrus cells, the number of neurons expressing Cx32 is markedly decreased immediately after cocaine withdrawal followed by a partial restoration of neuronal expression 7–21 days after the last cocaine session. These kinetics are substantiated on the protein level by ELISA and Western analysis. Conversely, a profound increase in Cx32 protein and mRNA is detected in medial CA1 neurons 2–21 days after cocaine injection. This regionally restricted increase was confirmed on the mRNA level by RT-PCR. Thus, cocaine withdrawal increases gap junction protein expression in CA1 and transiently decreases connexin expression in CA3. This regional specificity is particularly interesting given evidence that spontaneous bursting of individual CA1 but not CA3 pyramidal neurons is enhanced by administration of dopamine or cocaine to hippocampal slice preparations (Stein & Belluzzi, 1989), and that subconvulsive doses of cocaine increase CA1 electrical activation in response to afferent stimulation (Lesse & Collins, 1979). These data are consistent with the proposed role of cocaine in precipitating epileptic seizure. Chronic exposure to subconvulsant doses of cocaine increases the likelihood of seizure activity in response to low doses of a convulsant drug (Zhai *et al*., 1997). This reduction in seizure threshold is apparently caused by enhanced membrane channel permeability in CA1 pyramidal neurons resulting in spontaneous synchronous bursting of CA1 cells (Zhai *et al*., 1997). Increases in spontaneous electrical coupling, specifically through gap junction channels in CA1, has already been implicated in the generation and spread of seizure activity in mammalian brain (Perez-Velazquez *et al*., 1994). Our data provide the first direct evidence that cocaine alters gap junction protein expression in CA1 neurons *in vivo*. While we have not determined whether cocaine withdrawal affects gap junction function, the changes in Cx32 levels support speculation that chronic cocaine exposure may affect the properties of neuronal gap junction assemblies within the hippocampus.

Thus, the regional variations in Cx32 expression in both hippocampus and nucleus accumbens observed during cocaine withdrawal are consistent with a putative role for gap junctions in both cocaine craving (via decreased gap junction protein expression in nucleus accumbens and subfields of the hippocampus) and psychomotor stimulant-precipitated epileptigenesis (via enhanced gap junction protein expression in CA1 hippocampal neurons). Functional correlates documenting the effect of cocaine-induced changes in Cx32 expression are needed to confirm this hypothesis.

Conclusions

This is the first study to show that pharmacological manipulation *in vivo* can affect the expression of gap junction proteins, specifically Cx32. A decrease in gap junction protein and mRNA expression is noted in areas predicted to mediate acute cocaine reinforcement and chronic cocaine craving (i.e. nucleus accumbens). An increase in gap junction protein and mRNA expression is noted in areas that become hyperexcitable after chronic cocaine exposure (i.e. CA1 hippocampal neurons). These data lead us to hypothesize that changes in neuronal gap junction communication are responsible, in part, for the long-term alterations in behaviour produced by drugs of abuse.

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Abbreviations

Cx, connexin; DA, dopamine; DTT, dithiothreitol; FR, fixed ratio; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OD, optical density; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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