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Limbic-Cortical-Ventral Striatal Activation during Retrieval of a Discrete Cocaine-Associated Stimulus: A Cellular Imaging Study with γ Protein Kinase C Expression

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We investigated the neuronal activation associated with reexposure to a discrete cocaine-associated stimulus using in situ hybridization to quantify the expression of the plasticity-regulated gene, γ protein kinase C (γ PKC), in the limbic-cortical-ventral striatal system. Groups of rats were trained to self-administer cocaine paired with a light stimulus (Paired) or paired with an auditory stimulus but also receiving light presentations yoked to those in the Paired group (Unpaired). Additional groups received noncontingent cocaine–light pairings (Pavlovian) or saline–light pairings (Saline) that were yoked to the Paired group. After acquisition of self-administration by the Paired and Unpaired groups, all groups had a 3 d drug- and training-free period before being reexposed to noncontingent presentations of the light conditioning stimulus during a 5 min test session in the training context. There were four major patterns of results for regional γ PKC expression 2 hr later. (1) Changes occurred only in groups in which the light was predictive of cocaine. (2) Increases were seen in the amygdala, but decreases were seen in the medial prefrontal cortex. (3) No changes were seen in the hippocampus. (4) Although changes were observed in the basal and central nuclei of the amygdala and the prelimbic cortex in both the Paired and Pavlovian groups, additional changes were observed in the nucleus accumbens core, lateral amygdala, and anterior cingulate cortex in the Pavlovian group. These results suggest not only that regionally selective alterations in γ PKC expression are an index of the retrieval of Pavlovian associations formed between a drug and a discrete stimulus, but also that a distinct neural circuitry may underlie Pavlovian stimulus–reward associations in cocaine-experienced rats.

Key words: γ protein kinase C; cocaine; memory retrieval; nucleus accumbens; frontal cortex; limbic system

Exposure to cocaine-paired environmental stimuli produces craving in cocaine-dependent humans (Gawin, 1991; Childress et al., 1993) that may contribute to relapse after abstinence (O’Brien et al., 1992). Brain imaging studies have reported increases in metabolic activity in several limbic regions in response to presentation of discrete cocaine-associated stimuli that were correlated with self-reports of craving for cocaine (Grant et al., 1996; Maas et al., 1998; Childress et al., 1999). These data suggest that Pavlovian learning mechanisms are critical to drug addiction. However, the neural circuitry and molecular substrates associated with these mechanisms are poorly understood (Berke and Hyman, 2000).

Both the formation of long-term memory and stimulus-cued retrieval of memories are dependent on new protein synthesis (Davis and Squire, 1984; Nader et al., 2000) and, in the case of exposure to cocaine cues, expression of the plasticity-associated gene c-fos (Brown et al., 1992; Crawford et al., 1995; Franklin and Druhan, 2000; Neiseawander et al., 2000). However, c-fos expression may not be the optimal marker of neuronal activity. For example, c-fos induction is refractory to multiple psychostimulant challenges in some brain regions (Graybiel et al., 1990; Hope et al., 1994). Furthermore, in the hippocampus there is a dissociation between the induction of the activity-dependent form of plasticity, long-term potentiation (LTP), and c-fos expression (Abraham et al., 1993; Worley et al., 1993). The expression and activity of the brain-specific γ isoform of the intracellular serine–threonine protein kinase C (γ PKC) family, however, is highly correlated with learning (Van der Zee et al., 1992; 1997; Abelsonich et al., 1993a,b; Douma et al., 1998) and with hippocampal LTP (Thomas et al., 1994). γ PKC is a brain-specific isoform of PKC that, unlike c-fos, is constitutively expressed in the majority of CNS neurons (Nishizuka, 1988; our unpublished observations), thus also allowing decreases in its expression to be quantified. These features indicate that studying γ PKC expression provides a useful molecular tool with which to investigate the neural system that is activated during retrieval of cocaine-associated memories.

The dopaminergic mesolimbic pathway projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) is widely accepted to be the critical substrate for the reinforcing effects of cocaine (Roberts et al., 1977; Wise and Bozarth, 1987). Dopamine itself may play a crucial role in learning associated with reinforcement within both the NAcc and other brain regions (Schultz and Dickinson, 2000). Glutamate-dependent plasticity processes in the NAcc, as well as in cortical areas providing the major glutamatergic innervation of the NAcc, namely regions of the medial prefrontal cortex (mPFC), the amygdala, and the hippocampus, have also been shown to be critical for learning in both aversive and appetitive settings (Morris et al., 1986; Miserendino et al., 1990; Burns et al., 1994; Kelley et al., 1997; Baldwin et al., 2000). Glutamate transmission in the NAcc is important in mediating cocaine-induced reinstatement of self-administration

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(SA) in rats (Cornish and Kalivas, 2000), suggesting that plasticity processes may also underlie drug-seeking behavior and may do so within a distributed corticolimbic-striatal system.

In this study, rats were trained to self-administer cocaine by responding on one of two levers; each drug infusion was accompanied by presentation of a light stimulus [conditioning stimulus (CS)] (Paired group). Using quantitative in situ hybridization, we measured the expression of γ PKC in response to presentation of this drug CS alone in the NAcc, VTA, and regions providing glutamatergic afferents to the NAcc. We were thus able to image the cellular neuroanatomical correlates of the motivational properties of a drug CS, including its ability to support drug-seeking behavior and reinstatement after extinction (Stewart et al., 1984; Arroyo et al., 1998). In addition to a Saline control group, two other important groups of rats were included in this study: (1) rats trained to self-administer cocaine, but for which light presentations were explicitly unpaired with drug (Unpaired group); and (2) rats receiving light–cocaine pairings, but in which the cocaine administration was not contingent on an instrumental response (Pavlovian group). These groups provided controls for the neuroadaptations that can accompany chronic cocaine exposure and the incentive properties of reward-associated stimuli. Guided by imaging studies of human cocaine abusers and our own studies of the neural basis of cue-controlled drug seeking in rats (Whiteal et al., 1996; Weissenborn et al., 1997), we predicted changes in γ PKC expression in limbic-cortical-ventral striatopallidal networks in response to presentation of the cocaine-associated CS.

**MATERIALS AND METHODS**

**Animals.** Male Lister hooded rats (300–400 gm; Charles River) were housed under a 12 hr reverse light/dark cycle (lights off 9:00 A.M.). Experiments were performed between 10:00 A.M. and 4:00 P.M. Animals were maintained at 90 % body weight by restricting food (20 gm lab chow daily), starting after surgery and continuing for the duration of the experiment. During the cocaine self-administration training period, the food was given to the rats once they had returned to their home cages after training. Water was available *ad libitum* in the home cages. The experiments were undertaken in accordance with the UK 1986 Animals (Scientific Procedures) Act (project license PPL 80/00684).

**Intravenous catheterization.** Rats were intraperitoneally anesthetized with 1 ml/100 gm body weight Avertin (10 gm of 2,2,2-tribromoethanol; Sigma-Aldrich, Poole, UK) and 4.5 ml of Dulbecco’s “A” solution (5 mg of tertiary amyl alcohol in 4.5 ml of PBS; Unipath Ltd.) in 40 ml of absolute ethanol. Indwelling jugular catheters (manufactured in-house) were attached via a metal spring-protected plastic lead (Plastic One) to a wall housing the levers was available to provide an auditory CS (see below). The external guide cannula of the implanted catheter on each rat was attached via a metal spring-protected plastic lead (Plastic One) to a ceiling-mounted, counter-balanced single-channel liquid swivel (Stoelting) that allowed the animal free movement within the operant chamber. Tygon plastic connected the swivel to an operant box-designated Razel infusion pump (Semat Technical Ltd.) situated outside the light–sound attenuating chamber from which (drug) solution was to be delivered. All cannulas were plugged with monofilament, and protected with a stainless steel cap.

**Apparatus.** Operant chambers (24 × 22 × 20 cm, Campden Instruments, Loughborough, UK) were housed in individual, ventilated sound- and light-attenuated boxes. Each chamber was lit by a red 2.5 W, 24 V light positioned centrally in the ceiling of the chamber. Two retractable levers (3.8 cm wide, 5.5 cm from the grid floor, and 10 cm apart) were positioned on one (22 × 20 cm) wall of the chamber. White 2.5 W, 24 V lights (2 cm) were positioned 2.8 cm above the levers, which could be illuminated to serve as a visual CS. In addition, a tone generator (RS Components, Northants, UK) located in the ceiling to the left of the wall housing the levers was available to provide an auditory CS (see below). The external guide cannula of the implanted catheter on each rat was attached via a metal spring-protected plastic lead (Plastic One) to a ceiling-mounted, counter-balanced single-channel liquid swivel (Stoelting) that allowed the animal free movement within the operant chamber. Tygon plastic connected the swivel to an operant box-designated Razel infusion pump (Semat Technical Ltd.) situated outside the light–sound attenuating chamber from which (drug) solution was to be delivered. All catheters were plugged with monofilament, and protected with a stainless steel cap.

**Procedure and acquisition criteria.** At least 7 days after recovery from catheter implantation, rats were trained to self-administer 0.1 ml of cocaine hydrochloride (0.25 mg of base per infusion, i.v., dissolved in 0.9% sterile saline; McFarlan-Smith). Rats were placed into the operant chambers and attached to the infusion pumps. The 2 hr training day began with presentation of a light stimulus (Saline group). The design of the experimental groups was as follows: (1) rats trained a group of rats to self-administer cocaine in which the conditioned cue was an auditory tone. However, these rats received light presentations in the Pavlovian group. We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group). We predicted that those areas involved in learning Pavlovian stimulus–drug presentations (Pavlovian group).

**Table 1. The design of experimental groups used**

<table>
<thead>
<tr>
<th>Group</th>
<th>Training (context A)</th>
<th>Testing (context A)</th>
<th>Association examined in testing</th>
</tr>
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<tbody>
<tr>
<td>Paired</td>
<td>L→CS&lt;sub&gt;1&lt;/sub&gt;→cocaine</td>
<td>CS&lt;sub&gt;1&lt;/sub&gt;→cocaine</td>
<td>Pavlovian CS→cocaine Instrumental CS→response</td>
</tr>
<tr>
<td>Pavlovian</td>
<td>L→CS&lt;sub&gt;2&lt;/sub&gt;→cocaine</td>
<td>CS&lt;sub&gt;2&lt;/sub&gt;→cocaine</td>
<td>Pavlovian CS→cocaine Instrumental CS→response</td>
</tr>
<tr>
<td>Unpaired</td>
<td>L→CS&lt;sub&gt;1&lt;/sub&gt;→cocaine</td>
<td>CS&lt;sub&gt;1&lt;/sub&gt;→cocaine</td>
<td>Pavlovian CS→cocaine Instrumental CS→response</td>
</tr>
<tr>
<td>Saline</td>
<td>L→CS&lt;sub&gt;2&lt;/sub&gt;→cocaine</td>
<td>CS&lt;sub&gt;2&lt;/sub&gt;→cocaine</td>
<td>Pavlovian CS→cocaine Instrumental CS→response</td>
</tr>
</tbody>
</table>

1. Discriminated level press; CS<sub>1</sub>, light stimulus; CS<sub>2</sub>, tone stimulus; →, associated with; ↔, not associated with; CS<sub>1</sub> is presented to all four experimental groups during training, whether contingent on an instrumental response or not.
session was initiated by the experimenter pressing rapidly three times on one of the two levers and thereby designating this lever as the active drug-delivering lever for the duration of the experiment for that rat. These initial depresions of the active lever did not result in a drug infusion. The levers were randomly assigned as active and inactive drug levers but were left–right counter-balanced across the experimental group. Illumination of the red house light signaled the beginning of the training session, and rats were allowed to acquire intravenous cocaine self-administration under a continuous reinforcement schedule. Depression of the active lever by the rat resulted in the immediate extinction of the red house light and either a 20 sec illumination of the white light directly above the active lever (Paired group; n = 18) or a 20 sec intermittent tone (Unpaired group; n = 6). One second after active lever depression, both levers were retracted for 19 sec, and the pump concomitantly delivered a 3.6 sec duration cocaine infusion. The rats effectively received a 20 sec time-out period in which neither lever and, consequently, no further cocaine infusions were available before the extinction of the CS, red house light reillumination, and extension of both levers back into the operant chamber. Depressive of the inactive lever had no programmed consequence. The Pavlovian group (n = 12) was exposed to the same number of light–cocaine infusion pairings yoked to the Paired group and therefore pairings were given at the same time, and resulted in identical illumination periods as the Paired group. Although the levers were present in the operant chambers of the Pavlovian group, their depression had no consequence for the animal. The Saline rats were yoked to the Paired group in terms of white light stimulus presentation and time-out conditions, but rats of this group received an intravenous infusion of 0.1 ml of sterile saline (n = 6). The two levers were present in the operant chambers of the Saline group, but their depression had no consequence for the animal. The Saline rats were randomly assigned as active and inactive drug levers to rats of the Paired group in terms of CS presentations until their master Paired animals reached the discriminated lever-pressing criteria.

Lever-pressing data were analyzed by repeated measures. Violations of the sphericity assumption (Mauchly’s test) within the repeated measures ANOVAs were corrected using the Huynh-Feldt (Mauchly’s test) within the repeated measures ANOVAs was corrected using the Huynh-Feldt. Test phase. After the training criterion had been met, all rats then received a 3 d drug washout period in which they remained in their home cages and received no drug infusions. On the fourth day after conditioning, all rats were placed back in the training chambers. The levers in the chambers were not available to the animals. After 2 min, rats were presented with five 1 sec white light CS with an interstimulus interval of 90 sec. Thirty minutes after the last lever press, the rats were transferred back to their home cages; 2 hr later, they were killed by CO2 inhalation, and their brains were removed rapidly and frozen on dry ice. The 2 hr test time point after the CS presentations was chosen on the basis of a previous study showing that maximal levels of mRNA for γ PKC were measured 2 hr after the induction of the activity-dependent form of plasticity, LTP, in the hippocampus (Thomas et al., 1994). The brains were stored at −80°C until they were processed for in situ hybridization.

Tissue preparation, in situ hybridization, and grain density analysis. Cellular imaging studies have successfully investigated the genomic response to environments associated with cocaine administration using the expression of the immediate early gene c-fos as a marker for neuronal activation (Brown et al., 1992; Crawford et al., 1995; Franklin and Drahman, 2006; Neisewander et al., 2000). The use of c-Fos as a marker of cellular activation after exposure to cocaine-associated cues has some interpretational difficulties. First, c-fos expression in the NAcc exhibits tolerance with chronic cocaine administration (Graybiel et al., 1990; Hope et al., 1994). Second, c-Fos is not always a marker for cellular activation (Dragunow and Faull, 1989), particularly in the hippocampus (Abraham et al., 1993; Worley et al., 1993). We have seen more than fivefold increases in γ PKC expression in the hippocampus in response to a context that was associated with mild footshock (K. L. Thomas, J. Hall, and B. J. Everett, unpublished observations), suggesting that changes in γ PKC expression can be measured in the hippocampus on retrieval of contextual memories. Third, the basal expression of c-fos in many brain regions is very low. Therefore, quantitative measures of alterations in expression not only are quite variable, but also do not permit the detection of potential decreases in regional activity.

Tissue sections (14 μm) were cut at −18°C on a freezing Microtome (Leica Instruments) and thaw-mounted onto poly-L-lysine HBr (0.02 mg/ml diethyl pyrocarbonate-treated water; molecular mass >300,000; Sigma)-coated glass slides. Sections at equivalent anteroposterior levels from different experimental groups were mounted onto the same slide. Sections from the different groups were mounted onto two or three individual rats from the Paired group. The sections were air-dried for not<30 min, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, for 5 min, rinsed in PBS for 1 min, delipidated in 70% ethanol for 4 min, and stored in 95% ethanol at 4°C. A DNA antisense probe complementary to nucleotides 1157–1191 of the rat γ PKC gene (Ono et al., 1988) was synthesized on an Applied Biosystems DNA synthesizer. The resulting oligonucleotide was end-labeled with [α-35S]dATP (1200 Ci/mm; DuPont NEN, Hounslow, UK) in a 30:1 molar ratio of radiolabeled ATP/oligonucleotide using terminal deoxynucleotidyl transferase (Promega, Southampton, UK) as described previously (Wisden and Morris, 1994). Specific activity of the 35S-labeled probe was between 2.0 × 108 and 3.0 × 109 dpm/μl probe.

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Silver grain density was assessed in discrete neuronal populations using OpenLab imaging software (ImproVision). Coordinates were as follows: anterior cingulate (Cg1) and prelimbic (PL) cortical pyramidal neurons, bregma +3.5 mm; nucleus accumbens core and shell, bregma +1.7 mm; central nucleus of the amygdala (CeN) neurons, bregma −2.0 mm; CA1 pyramidal neurons and dentate gyrus (dg) granule cells, bregma −3.3 mm; lateral (L) and basal (B) amygdala pyramidal neurons, bregma −3.0 mm; VTA neurons, bregma −6.1 mm (Paxinos and Watson, 1997) (Fig. 1). Briefly, silver grains (total and nonspecific) were counted over sufficiently random selected neurons from each region for each animal such that the SE of the counts for any region was <10% of the population mean (typically 24 cells). In each case, cells were selected from at least three separate sections that were right–left side counterbalanced. Then a specific grain count was calculated for each region by subtracting total and nonspecific counts. All neurons in the 10 regions that we studied showed constitutive levels of γ PKC expression. The mean silver grain count in each region for each animal was then divided by the mean grain count in that region for the Paired group × 100 to give a standardized grain count for each group. Results were standardized to the common Paired group to control for differences in absolute grain densities across several in situ hybridization repetitions. The analysis of silver grain density was made by an investigator who was blind to the treatment regimen of the individual sections. Standardized results were analyzed by ANOVA, and individual post hoc comparisons were made using Tukey's test for pairwise comparisons.

RESULTS

Acquisition of intravenous cocaine self-administration

The number of days for the Paired and Unpaired groups to reach the criterion for drug lever discrimination ranged from 9 to 12 d. Active (drug) and inactive lever responses during the first (Fig. 2a) and last (Fig. 2b) 9 d of intravenous self-administration for each experimental group were analyzed. The use of backward analysis in which the data were time-locked to each rat that was self-administering drug and had reached training criterion permits the comparison of lever-pressing behavior between the four experimental groups immediately before the test phase, regardless of the length of training. There was a significant Group × Lever × Day interaction over the first F(24,304) = 3.007; $\epsilon = 0.501; p < 0.01$ and last F(24,304) = 2.076; $p = 0.522; p < 0.05$ d of training. In addition, there was a significant Group × Lever interaction over the first $F(3,38) = 15.974; p < 0.001$ and last
although there were differences in the discriminated lever
there were no differences in the total number of cocaine infusions
either on the degree of lever discrimination or on the vigor of
pairing a tone rather than a light with cocaine SA during training
no significant difference between the Paired and Unpaired groups

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Regional γPKC expression after exposure to a
cocaine-associated cue

Analysis of the regional density of silver grains as a measure of γ
PKC expression 2 hr after exposure of the experimental animals to
a light stimulus on the test day resulted in a significant Group x
Region interaction \(F_{(3,372)} = 2.976; p < 0.001\), reflecting different
levels of γPKC expression between both the experimental groups
\(F_{(3,372)} = 2.787; p < 0.05\) and the distinct regions \(F_{(9,372)} =
4.098; p < 0.001\).

Within the amygdala, individual analysis for each region of γ
PKC expression revealed that there was a group effect on grain
density in the lateral nucleus (Fig. 3a) \(F_{(3,38)} = 7.834; p < 0.001\)
that reflected an increase in γPKC expression in the Pavlovian
group compared with the Saline controls (Fig. 3b,c) and also with
the Unpaired and Paired groups. The effect of group on γPKC
expression in the basal amygdala (Fig. 3d) \(F_{(3,38)} = 2.995; p <
0.05\) and the CeN (Fig. 3e) \(F_{(3,38)} = 8.099; p < 0.001\) resulted
from an increase in expression in the Paired and Pavlovian groups
compared with the Saline group. In addition, γPKC expression in
the CeN in the Paired and Pavlovian groups was elevated with
respect to that measured in the Unpaired group.

There were differences in the density of silver grains between
the experimental groups in the Cg1 (Fig. 4a) \(F_{(3,372)} = 7.246; p <
0.001\) and also in the PrL (Fig. 4b) \(F_{(3,38)} = 2.833; p < 0.05\)
region of the rat frontal cortex. In the Cg1 area, this was attribu-
table to a decrease in γPKC expression in the Pavlovian group
in comparison with the Saline, Unpaired, and Paired groups. In
the PrL, both the Pavlovian (Fig. 4d) and Paired groups also
showed decreased γPKC expression with respect to the Saline
control group (Fig. 4c).

No changes in γPKC expression were seen in the CA1 region
of the hippocampus (Fig. 5a) \(F_{(3,38)} = 0.174; p = 0.913\) or dg
(Fig. 5b) \(F_{(3,38)} = 0.262; p = 0.852\). There was an effect of group in the core region of the NAcc
(Fig. 6a) \(F_{(3,38)} = 7.867; p < 0.001\) but not in the NAcc shell
(Fig. 6b) \(F_{(3,38)} = 1.178; p = 0.331\) or in the VTA (Fig. 6c)
\(F_{(3,38)} = 1.316; p = 0.283\). The significant effect of the group in the
NAcc core resulted from an increase in grain density in the
Unpaired and Paired groups, compared with the Saline control
group.

DISCUSSION
Presentation of a light CS that had been associated previously
with self-administered cocaine produced discrete, regional alter-
tations in the expression of the γ isoform of PKC. Three general
patterns of altered gene response were evident (Table 2). (1) The
first pattern was dependent on whether rats had received the
cocaine infusion initially and the CS contingent on a behavioral
response (Paired group) or whether they were administered drug–CS
pairings noncontingently (Pavlovian group). (2) Al-
though increased γPKC expression was measured within the
amygdala in both the Paired and Pavlovian groups, decreased
expression was seen within areas of the mPFC. Thus, both groups
showed increased γPKC expression in the B and CeN amygdala
and decreased γPKC expression in the PrL cortex. However, the
Pavlovian group showed additional changes in γPKC mRNA
levels in the NAcc core, LA of the amygdala, and anterior
cingulate cortex. (3) In marked contrast to the increased γPKC
expression within the amygdala, no changes in its expression
within the hippocampus were seen in any group on exposure to
cocaine cues. This result is in accord with data supporting a role
for the amygdala, but not the hippocampus, in conditioning to
discrete cues (Selden et al., 1991; Holland and Gallagher, 1998;
LeDoux, 2000). By contrast, the hippocampus has been suggested
to underlie conditioning to contextual and spatial cues (Eichen-
baum et al., 1999; Holland and Bouton, 1999; Fanselow, 2000;
Maren and Holt, 2000). Moreover, the acquisition of an associa-
tion between a neutral discrete cue and food reward in both
Pavlovian and instrumental learning tasks has been shown not to
codifies a representation of the effects of cocaine. The anterior cingulate cortex may form part of the neural circuitry that encodes a representation of the effects of cocaine. (Neisewander et al., 2000), again suggesting that the anterior cingulate cortex but only in rats that had undergone conditioning injection of cocaine also induced c-fos in the anterior cingulate cortex (Unpaired group). The genetic responses were not attributable to nonspecific neuronal adaptations to the chronic administration of cocaine because no differences were measured between the Saline control group and the cocaine-experienced Unpaired group. Moreover, the experimental design determined that all cocaine-experienced groups received a similar number of drug infusions. All rats had been presented with the light stimulus during training, regardless of whether it was a CS, so that the effects of the novelty of presentation of the light on the test day were equated across groups. Therefore, the changes in γPKC expression in the instrumentally and Pavlovian-conditioned rats were specific to retrieval of CS-associated memories.

**γ PKC as a cellular activity marker**

Several cellular imaging studies have investigated the expression of the immediate early gene c-fos in response to environments associated with cocaine administration (Brown et al., 1992; Crawford et al., 1995; Franklin and Druhan, 2000; Neisewander et al., 2000). Although each study reported conditioned changes in c-Fos protein, there were some differences in the neuroanatomical responses that may be at least partly attributed to methodological differences. However, regions of the mPFC were engaged consistently by the cocaine-associated environments and also activated by a discrete, cocaine-associated cue in this study, as measured by changes in γPKC expression. Furthermore, a priming injection of cocaine also induced c-fos in the anterior cingulate cortex but only in rats that had undergone conditioning (Neisewander et al., 2000), again suggesting that the anterior cingulate cortex may form part of the neural circuitry that encodes a representation of the effects of cocaine.

Imaging studies in human cocaine users have revealed that exposure to discrete, cocaine-associated stimuli results in increased metabolic activity in frontal cortical regions, including the anterior cingulate and dorsolateral prefrontal cortex and the amygdala, activations that were correlated with self-reports of craving for cocaine (Grant et al., 1996; Maas et al., 1998; Childress et al., 1999). Although the NAcc in these human brain imaging studies may not have been resolved anatomically, the concordance of the present, cellular imaging data with such studies of human addicts indicates the face validity of using in situ hybridization of γPKC mRNA as a tool to investigate at the cellular and systems levels the neural basis of associative mechanisms critical to addictive behavior.

**Processing the CS in the amygdala**

The cocaine-associated light stimulus consistently increased γPKC expression in the CeN and B. However, the increase in the LA was seen only in rats having undergone purely Pavlovian pairings of cocaine and light CS.

There is significant support for a role of the CeN in processing Pavlovian stimulus–reward associations (Kapp et al., 1979; Gallagher et al., 1990; Davis, 1992; Parkinson et al., 2000b) and for the B and LA (the BLA complex) in mediating the affective motivational properties of reward-associated cues (Hatfield et al., 1996; Everitt et al., 2000), including cocaine-associated cues (Whitelaw et al., 1996; Meil and See, 1997). The selective induction of γPKC mRNA in the CeN and B of the Paired and Pavlovian groups may index the reactivation of these two associations but additionally may reflect the role of the CeN to influence conditioned somatic, autonomic, and endocrine responses on exposure to the drug cue (Everitt et al., 1999).

Few studies have investigated a functional role for the LA independent of the B, and as a consequence there is a paucity of data that allow a dissociation of LA and B function. However, specific excitotoxic lesions of the LA that left the B and CeN intact were reported to prevent the acquisition of a conditioned place preference for amphetamine, leading to the suggestion that

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**Figure 2.** Acquisition of intravenous cocaine SA. a, b. The mean number of responses on the active (cocaine or saline SA,) or inactive (no programmed consequence) levers in the 2 hr training sessions in the first 9 d of training (a) or the last 9 d of training (b). c. The total number of cocaine (0.25 mg base/0.1 ml saline) infusions in rats self-administering (Unpaired and Paired groups) or receiving yoked administrations (Pavlovian) of the drug. *p < 0.05, **p < 0.01, ***p < 0.001, in comparison with the responses at the inactive lever; Sidak’s test. Error bars indicate SEM.
the LA is a possible neural site of association of cues with the primary rewarding effect of amphetamine (Hiroi and White, 1991). Similarly, the LA has been shown to be the site of storage of associations formed between discrete auditory stimuli and an aversive unconditioned stimulus during Pavlovian fear conditioning (Fanselow and LeDoux, 1999).

If the increase in γ PKC expression in the LA does indeed reflect the stimulus–reward association, then an increase would have been expected in the Paired as well as in the Pavlovian group, but this was not the case. There may be two kinds of explanation for this dissociation in responses in amygdala nuclei. The first concerns the fact that stimulants, such as cocaine, have both appetitive and aversive effects (Spealman, 1979; Ettenberg and Geist, 1991), especially when administered noncontingently (Everitt et al., 1999). Given the strong association between the LA and aversive (fear) conditioning (Fanselow and LeDoux, 1999), this may explain in part the selective activation of the LA by cues associated with cocaine given noncontingently, because it is under such conditions that aversive effects may predominate (Everitt et al., 1999). The second explanation is that for the Paired group, the CS may selectively engage neural circuitry involving the B and NAcc core to mediate drug-seeking because it retrieves information about the CS associated with the instrumental contingency, and this process might be independent of the LA.

**Processing the CS in the medial prefrontal frontal cortex**

In rats, there is evidence that the anterior cingulate and prelimbic cortices subserve dissociable functions. Anterior cingulate cortex lesions, but not mPFC lesions that encompass PrL cortex, disrupt Pavlovian stimulus–reward associations, whereas more rostral mPFC lesions result selectively in attentional deficits (Bussey et
Paired group (100%). Error bars indicate SEM. The results are presented as silver grain density relative to the self-administer cocaine paired with a light C S (Paired group) or a tone CS (Unpaired group) in rats trained with noncontingent administration of cocaine and light CS (Pavlovian group) or saline and light CS (Saline group). The results are presented as silver grain density relative to the Paired group (100%). Error bars indicate SEM.

Figure 5. CS-induced γ PKC expression in the hippocampus. a, b. The expression of γ PKC in CA1 (a) and dg (b) neurons in rats trained to self-administer cocaine paired with a light CS (Paired group) or a tone CS (Unpaired group) in rats trained with noncontingent administration of cocaine and light CS (Pavlovian group) or saline and light CS (Saline group). The results are presented as silver grain density relative to the Paired group (100%). Error bars indicate SEM.

Figure 6. CS-induced γ PKC expression in mesoaccumbal DA system. The density of silver grains, standardized to the number of grains per neuron, measured in animals self-administering cocaine and receiving paired light CS (Paired group) in the core of the NAcc (a), the shell of the NAcc (b), and VTA 2 hr after presentation of a light stimulus (c) in groups of rats that, during training, received either yoked noncontingent presentations of the light CS and cocaine (Pavlovian group) or saline (Saline group), or rats that had self-administered cocaine paired with an auditory tone (Unpaired group). *p < 0.05, **p < 0.01; Tukey’s test. Error bars indicate SEM.

Table 2. Summary of regional changes in γ PKC expression

<table>
<thead>
<tr>
<th>Region</th>
<th>Saline</th>
<th>Unpaired</th>
<th>Paired</th>
<th>Pavlovian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>0</td>
<td>↑</td>
<td>↑</td>
<td>0</td>
</tr>
<tr>
<td>Shell</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VTA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CA1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cg1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>↓</td>
</tr>
<tr>
<td>PrL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>↓</td>
</tr>
<tr>
<td>LA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>↑</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CeN</td>
<td>0</td>
<td>0</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

↑, Increased expression; ↓, decreased expression; 0, no change in expression with respect to the Saline group.

Conditioned stimuli generally produce decreases in the activity of neurons in the anterior cingulate cortex and the PrL regions, whether predictive of a natural food reward or an aversive footshock (Garcia et al., 1999; Takenouchi et al., 1999). Although there is no clear evidence that the direction of a change in gene expression is directly correlated with neuronal activity, the decrease in expression of γ PKC in medial prefrontal cortical regions in response to the cocaine-associated cue shown here may reflect a similar conditioned decrease in neuronal activity.

Processing the CS in the nucleus accumbens and ventral tegmental area

Expression of γ PKC was increased in the NAcc core in both the Paired and Unpaired groups. This stands in marked contrast to those changes found in the amygdala and mPFC in which alterations were seen only in the Paired and Pavlovian groups and therefore related specifically to the cue that was predictive of cocaine. There are two possible explanations for this result. First, the genomic response in the NAcc core may not be specific to the associative recall of the drug cue and instead reflects some other sensory-dependent process. Second, molecular neuroadaptations in the NAcc in response to repeated cocaine administration are well established (Nestler and Aghajanian, 1997), and the large number of cocaine infusions received during training for the Paired, Unpaired, and Pavlovian groups may have induced an increased basal expression of γ PKC in the NAcc core. Indeed, acute intravenous cocaine does increase γ PKC expression within the NAcc core (our unpublished observations). Therefore, presentation of the cocaine CS may have led to decreased γ PKC expression in the NAcc core selectively in the Pavlovian group, just as decreased γ PKC mRNA levels were seen in the Cg1 for this group. Presumably, genomic responses in the NAcc core are determined by patterns of input from limbic cortical areas, and because the Cg1 projects richly to the core (Parkinson et al., 2000), the decreased response seen here in the Pavlovian group may reflect coordinated responses by these areas.

Lesions of the NAcc apparently do not disrupt measures of instrumental learning, such as the knowledge of the contingency between a rewarded outcome and the action to gain the reward (Balleine and Killcross, 1994). However, selective lesions of the NAcc core, but not the shell, do abolish both the acquisition and the performance of a purely Pavlovian learning task, autoshaping (Parkinson et al., 1999, 2000a). Moreover, lesions of the core made after learning a Pavlovian association between a CS and

al., 1997a,b). Moreover, rostral anterior frontal cortex neurons (encompassing the Cg1 region studied here) respond selectively to stimuli predictive of food reward (Takenouchi et al, 1999). In the context of addictive behavior, not only is the anterior cingulate cortex activated on exposure to cocaine cues, but selective lesions of the anterior cingulate or prelimbic cortices prevent the control over cocaine-seeking behavior by drug-associated cues (Weissenborn et al., 1997).
food reward do not prevent the subsequent acquisition of a new response with conditioned reinforcement (Parkinson et al., 2000a). These data suggest a dissociation in the role for the NAcc, especially the core region, in the neural mechanism underlying Pavlovian and instrumental learning. In addition, they suggest that the CS-induced decrease in \( \gamma \) PKC expression in the NAcc core in the Pavlovian, but not in the Paired, group that initially had been responding instrumentally for cocaine reflects the selective engagement or activation of the core during the processing of Pavlovian-conditioned stimuli.

The null effect of the CS presentation on gene expression in the largely dopaminergic neurons of the VTA does not necessarily indicate that activity in this region remained unaltered under these conditions. Indeed, increased electrophysiological activity in the dopaminergic neurons of the VTA has been shown to occur in response to the presentation of stimuli associated with reward (Ljungberg et al., 1992). Our results simply indicate that this possibly enhanced neuronal activity is not associated with increases in \( \gamma \) PKC expression. Indeed, it is only patterns of neuronal activity that induce long-term changes in synaptic strength which correlate with changes in \( \gamma \) PKC expression (Thomas et al., 1994). It remains a possibility that although the VTA itself may not be a site of associative synaptic plasticity involving \( \gamma \) PKC, activity there may mediate plasticity in other brain regions such as the striatum and prefrontal cortex (Schultz and Dickinson, 2000).

**Synthesis**

The oppositional nature of the changes in \( \gamma \) PKC expression in the amygdala and Cg1 and PrL regions of the mPFC observed in this study on presentation of a drug-associated CS closely corresponds with the changes in BLA-induced prefrontal cortical activity seen in electrophysiological studies. Although presentation of reward-related stimuli increases firing responses in neurons of the BLA complex (Muramoto et al., 1993; Ono et al., 1995; Pratt and Mizumori, 1998), stimulation of glutamatergic BLA afferents elicits excitatory and inhibitory responses in the mPFC, with the latter responses predominating (Perez-Jaranay and Vives, 1991).

Individual neurons of the medial NAcc core and shell receive convergent glutamatergic afferents from the amygdala, hippocampus, and mPFC (O'Donnell and Grace, 1995; Finch, 1996). The outcome of simultaneous stimulation of two sources of glutamatergic afferents in terms of neuronal firing of these NAcc neurons is complex (Pennartz et al., 1994; O'Donnell and Grace, 1995; Mulder et al., 1998). The stimulation of BLA neurons elicits firing of NAcc neurons, whereas activation of hippocampal afferents does not reliably initiate NAcc neuronal firing but does cause NAcc neurons to switch to an “active” state that permits activation by mPFC inputs (O'Donnell and Grace, 1995). The processing of discrete conditioned cues apparently does not involve hippocampal activation (Burns et al., 1993; LeDoux, 2000), and \( \gamma \) PKC expression was not increased in the hippocampus by the CS in the present study. Thus, the pattern of \( \gamma \) PKC expression induced by the cocaine-associated CS in this study indicates that motivationally salient, discrete stimuli are processed by specific elements of limbic-cortical-ventral striatopallidal circuitry. However, the precise ways in which interactions between the amygdala and the medial prefrontal cortical areas determine the activity of, and expression of \( \gamma \) PKC within, accumbens neurons remains to be determined. It should also be considered that the changes we observed in \( \gamma \) PKC expression may reflect plasticity-related processes initiated on stimulus-cued retrieval. Thus, it has been demonstrated that Pavlovian associations require a protein synthesis-dependent “reconsolidation” (Nader et al., 2000). This reconsolidation may involve plasticity-related processes (Przybysz-lawski and Sara, 1997), and the selective changes in the regional expression of the plasticity-related gene \( \gamma \) PKC in those groups in which the light stimulus was associated with cocaine seen in the present study may form part of the molecular mechanism underlying the lasting storage of this Pavlovian association. Furthermore, the associative storage of input patterns to the NAcc may be mediated by the strengthening sets of prefrontal and amygdala inputs. Indeed, LTP-like associative plasticity processes have been described in the BLA, anterior cingulate cortex, and NAcc (Pennartz et al., 1993; Jay et al., 1995; Maren and Fanselow, 1995).

**Conclusion**

Exposure of rats to a discrete cocaine-associated stimulus in the absence of both the drug and the ability to self-administer it altered \( \gamma \) PKC expression in the NAcc and its cortical afferent structures. The expression of \( \gamma \) PKC was selective to those rats in which there was a predictive stimulus–cocaine relationship. The pattern of expression was dependent on whether, during training, the rats self-administered cocaine or received it noncontingently. Although changes in \( \gamma \) PKC expression were common to these groups, additional changes were observed in the NAcc core, LA, and Cg1 in rats having received explicitly Pavlovian pairings of noncontingent drug and CS. Further investigations of gene expression patterns in response to cocaine-associated stimuli presented as contingent on lever pressing, i.e., as conditioned reinforcers, may clarify the mechanisms within cortical-ventral striatopallidal circuits engaged by the dissociable consequences of Pavlovian conditioning (Everitt et al., 1999; Parkinson et al., 2000a,b).

**REFERENCES**


