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Dopamine deficits and regulation of the cAMP second messenger system in brains of simian immunodeficiency virus–infected rhesus monkeys

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The basal ganglia, structures rich in the neurotransmitter dopamine, are primarily affected during human immunodeficiency virus (HIV) infection. The authors measured levels of dopamine and its metabolites, homovanillic acid and 3,4-dihydroxyphenylacetic acid, in brains of uninfected and simian immunodeficiency virus (SIV)-infected rhesus monkeys during the asymptomatic stage of the infection. Moreover, the authors investigated changes in cyclic adenosine monophosphate (cAMP) and cAMP response element–binding protein (CREB), two factors involved in the signaling pathway of dopamine. The brain regions examined were the nucleus accumbens and the corpus amygdaloideum, which are limbic structures of the basal ganglia that are involved in the pathophysiology of psychiatric disorders and substance abuse. Dopamine content was reduced in both regions of SIV-infected monkeys compared to uninfected animals. Moreover, dopamine deficits were associated with a decrease in expression of total CREB. Intracellular concentrations of cAMP were decreased in nucleus accumbens and remained unchanged in corpus amygdaloideum of SIV-infected macaques. Changes in dopamine signaling were not related to pathology or viral load of the investigated animals.The results suggest that dopamine defects precede neurologic deficits and implicate dysfunction of the dopaminergic system in the etiopathogenesis of HIV dementia. Therefore, affective complications in HIV subjects should not be interpreted only as reactive psychological changes. The alterations in the mesolimbic dopaminergic system during asymptomatic stage of SIV infection implicate a biological background for psychiatric disorders in HIV infection. *Journal of NeuroVirology* (2004) **10,** 163–170.

Keywords: brain; cAMP; CREB; dementia; dopamine; HIV; monkeys; SIV

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Introduction

Several lines of evidence report vulnerability of the basal ganglia in subjects with immunodeficiency infection (Berger and Nath, 1997). Basal ganglia atrophy and global hypometabolism were indicated in late stages of human immunodeficiency virus (HIV) disease by positron emmission tomography (PET) scans and radiologic imaging studies, respectively (Aylward *et al*, 1993; Rottenberg *et al*, 1987). Pathologic studies demonstrated reactive gliosis, great viral load, and multinucleated giant cell infiltrates in the basal ganglia, particularly in motor regions such as

the putamen and the caudate nucleus (Cornford *et al*, 1992; Kure *et al*, 1990; Navia *et al*, 1986; Reyes *et al*, 1991). In the caudate nucleus, dopamine concentrations were decreased in patients with HIV dementia (Sardar *et al*, 1996) and parkinsonian symptoms were reported (Koutsilieri *et al*, 2002c). In the putamen of simian immunodeficiency virus (SIV)-infected monkeys, dopamine deficits were evident already within the first 2 months of infection (Czub *et al*, 2001). In accordance, early subclinical motor dysfunction related to basal ganglia involvement has been reported in asymptomatic HIV patients (Arendt *et al*, 1990).

Although regions of the basal ganglia involved in motor regulation have been studied in immunodeficiency infection, structures among the basal ganglia involved in behavioral and affective regulation have not received the same attention. Such structures are part of the mesolimbic dopaminergic pathway that is functionally involved in the pathophysiology of a number of neuropsychiatric disorders (e.g., schizophrenia, depression, mania, etc.), also observed in HIV-infected individuals (Koutsilieri *et al*, 2002a). The lack of focus on the mesolimbic dopaminergic system may have resulted from the fact that psychiatric illnesses in HIV infection are often overlooked or wrongly interpreted as reactive psychological changes. In the current study, we measured the concentrations of dopamine and its metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in limbic structures, such as nucleus accumbens and corpus amygdaloideum, of SIV-infected rhesus monkeys. Moreover, we investigated whether the signaling pathway of dopamine is altered and determined the intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP) and the expression of the transcription factor cAMP response element–binding protein (CREB).

Results

All SIV-infected animals remained asymptomatic during the course of infection. Asymptomatic status was asserted by lack of clinical symptoms, low to moderate cell-associated viral load (median of 24 infectious cells/million peripheral blood mononuclear cells [PBMCs], log transformation = 1.39 ± 0.36), and no evidence for a rise in urinary neopterin (data not shown), usually found in preterminal stages. $CD4^+$ T-cell numbers in blood were significantly decreased from 970 ± 94 cells/μl in uninfected to 646 ± 114 cells/μl in infected monkeys. According to histopathological examination, there was no evidence of SIV-specific pathology. Microglial nodules, multinucleated giant cells, perivascular cuffing, or meningitis were not observed. The basal glanglia were completely inconspicuous (Figure 1). Astrocytic gliosis was absent in the regions of basal ganglia and rest subcortical gray matter.

Dopamine turnover

Dopamine levels were reduced in nucleus accumbens of SIV-infected animals by 66% compared to uninfected monkeys. The loss of dopamine was accompanied by a statistically significant decrease in HVA levels as indicated (Figure 2). DOPAC concentrations did not statistically change, although a trend for reduction was evident. Dopamine deficits were observed also in corpus amygdaloideum of SIV-infected monkeys compared with uninfected animals. The decrease was 57.3% of the uninfected animals. However, in this region, the concentrations of both dopamine metabolites remained unchanged (Figure 2).

No difference in dopamine metabolism was observed in animals infected with different strains of SIV.

Dopamine signaling pathway

To elucidate whether changes in dopamine content were associated with alterations in the signaling pathway of dopamine, we measured the intracellular concentrations of the second messenger of dopamine, cAMP, in the brains of uninfected and SIV-infected rhesus monkeys. cAMP levels were reduced in nucleus accumbens of SIV-infected macaques by 39.2% (Figure 3). No changes in cAMP concentrations were apparent in the region of corpus amygdaloideum.

Brain samples of SIV-infected and uninfected monkeys were subjected to Western blot to analyze the expression levels of the transcription factor CREB, a downstream target in the signaling pathway of dopamine/cAMP. Concentrations of total CREB were significantly lower in nucleus accumbens of SIV-infected compared to uninfected animals. A decline in the expression of total CREB was also observed in corpus amygdaloideum (Figure 4), although not statistically significant. A positive correlation was evident between dopamine content and CREB immunoreactivity in both regions investigated (Figure 5). To further elucidate to what degree the decrease in CREB expression in brains of SIV-infected monkeys was associated with dopamine deficits, we treated SIV-infected monkeys with selegiline, a substance that increases dopamine availability (Gerlach *et al*, 1994). Selegiline treatment resulted in a rise in CREB levels of 52.8% (to 152.8% ± 57.6% from 100% ± 26.08%) and 45.6% (to 145.62% ± 37.22% from 100% ± 33.3%) in nucleus accumbens and corpus amygdaloideum, respectively, compared to SIV infection alone.

Discussion

A new body of evidence has arisen during the last years that suggests that many clinical features of HIV dementia are attributed to abnormalities in dopaminergic systems (Berger and Arendt, 2000; Koutsilieri *et al*, 2002b; Lopez *et al*, 1999; Nath *et al*, 2000). In

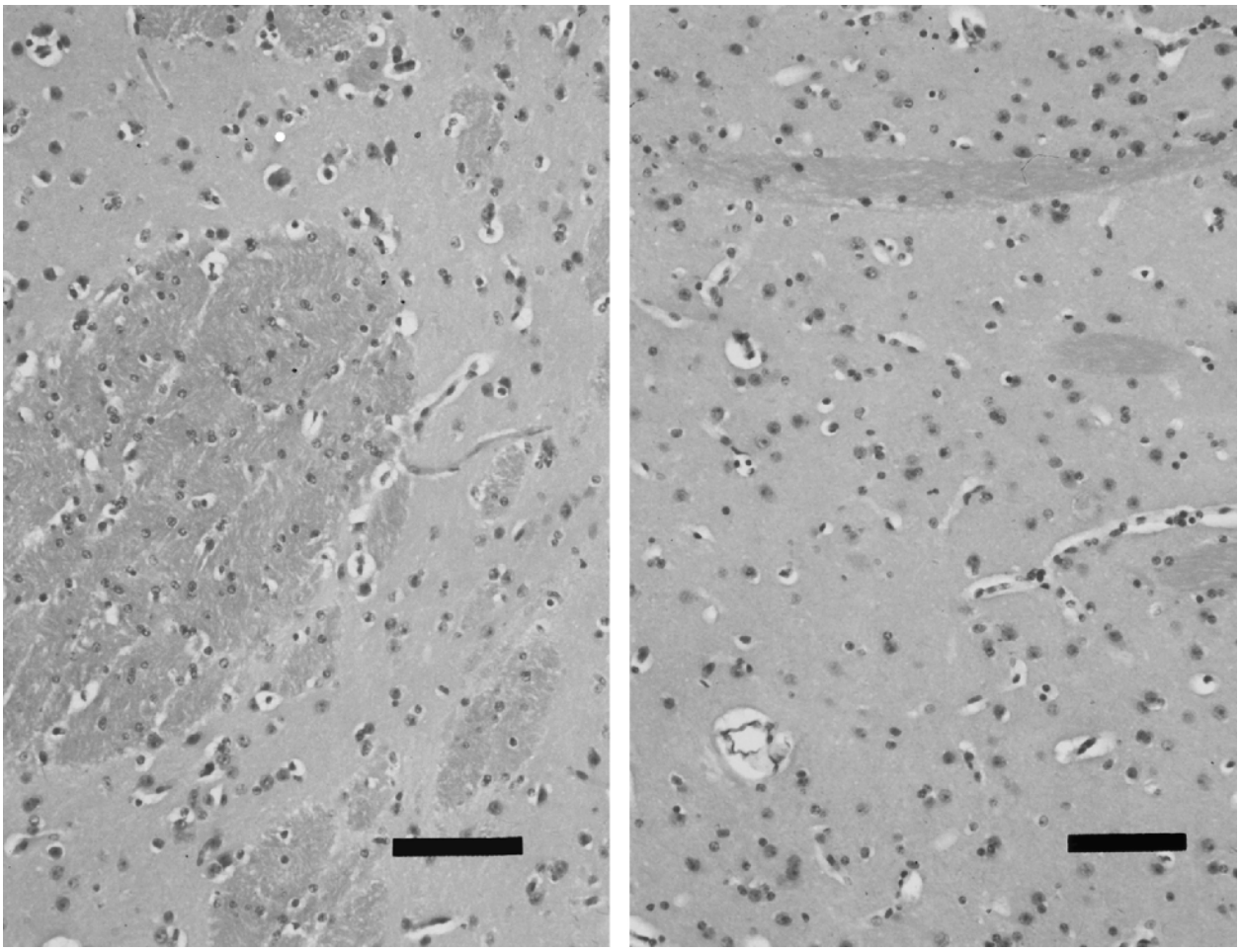

Figure 1 Routine histology of basal ganglia of SIV-infected animals. *Left*, monkey with the lowest dopamine concentration in nucleus accumbens; *right*, monkey with the lowest dopamine concentration in corpus amygdaloideum (H&E, Luxol-fast blue, and Masson's trichrome; bar = 100 μm).

the current study, we investigated the impact of SIV infection on dopamine and its signaling pathway in limbic structures of rhesus monkeys, the most relevant animal model to explore the influence of HIV infection on brain functions (Sopper *et al*, 2002). We found striking dopamine deficits in both nucleus accumbens and corpus amygdaloideum during asymptomatic SIV infection. Such a reduction may have been either a direct result of dopaminergic cell loss in these regions or a compensative cellular adaptation following an initial increased dopaminergic neurotransmission. The dopaminergic deficiency in the limbic regions is known to result in dysfunction in the dopamine-mediated reward system, anhedonia, and loss of motivation, which are primary symptoms of depression (Fibiger, 1984; Klimek *et al*, 2002; Nestler *et al*, 2002). Affective disorders emerge in the course of HIV infection and they dramatically increase around 6 to 18 months preceding clinical acquired immunodeficiency syndrome (AIDS) (Treisman *et al*, 1998). Potential dopamine deficiency in HIV-infected patients, as we found in SIV-infected monkeys, may be the etiologic factor associated with the pathological brain process producing affective dysfunction.

cAMP concentrations were reduced in nucleus accumbens of SIV-infected monkeys but remained unchanged in the corpus amygdaloideum of the same animals. Similar results were observed with expression of CREB. These findings may be explained by a differential distribution of D1/D2-like receptors between these regions, as it is well-known that dopamine receptors are differently coupled to the cAMP second messenger cascade. D1 receptors stimulate the effector enzyme adenylate cyclase whereas D2-like receptors inhibit it (Senogles *et al*, 1988). Further, it is important to keep in mind that although the data concerning dopamine turnover are directly associated to dopaminergic cells, our data on targets in the signaling pathway of dopamine such as cAMP and CREB may represent net results originated by various cell types in the brain. However, the positive correlation between dopamine content and relative CREB immunoreactivity suggest a specific involvement of dopamine in regulation of CREB in dopaminergic regions. Additionally, treatment of

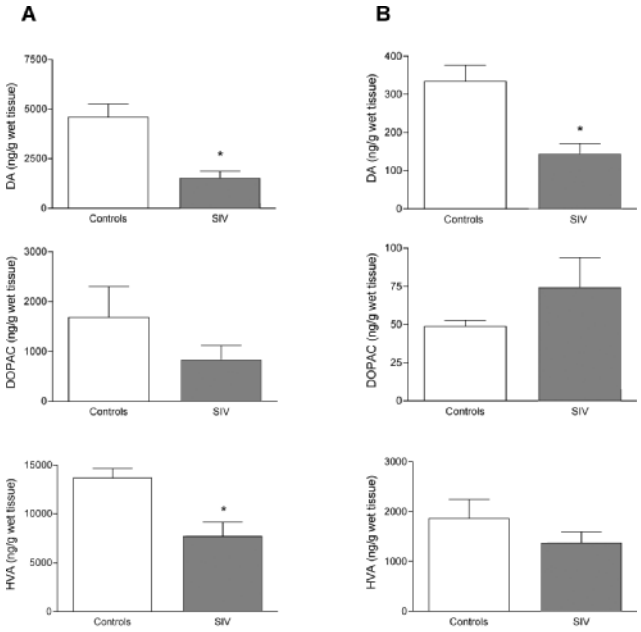

Figure 2 Dopamine, DOPAC, and HVA concentrations in nucleus accumbens (**A**) and corpus amygdaloideum (**B**) of uninfected (Controls) and SIV-infected (SIV) rhesus monkeys. Data represent means ± SEM, $n = 4$–6. $^{*}P < .05$, significantly different from Controls (unpaired *t* test with Welch's correction).

SIV-infected animals with the dopaminergic drug selegiline increased CREB immunoreactivity in both nucleus accumbens and corpus amygdaloideum, indicating that reductions in CREB immunoreactivity are at least partly "associated" with dopamine deficits. To our knowledge, there are no data available on intracellular concentrations of cAMP in brains of either HIV- or SIV-infected subjects. Regarding the direct effects of HIV on cAMP signaling pathway in cells of the nervous system, some information is available from *in vitro* studies: HIV Tat protein induced a decline of intracellular cAMP after 2 h in

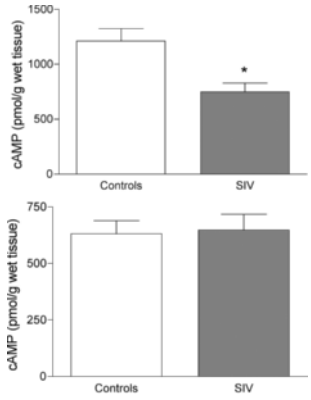

Figure 3 Intracellular cAMP concentrations in nucleus accumbens (*upper panel*) and corpus amygdaloideum (*lower panel*) of uninfected (Controls) and SIV-infected (SIV) rhesus monkeys. Data represent means ± SEM, $n = 5$–6. $^{*}P < .05$, significantly different from Controls (unpaired *t* test with Welch's correction).

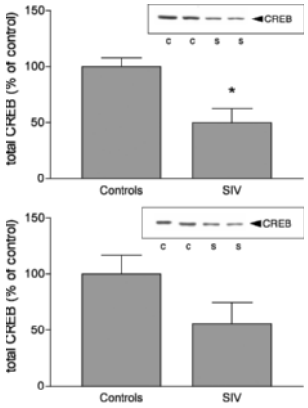

Figure 4 Densitometric analysis of total CREB protein levels in nucleus accumbens (*upper left panel*) and corpus amygdaloideum (*lower left panel*) of uninfected (Controls) and SIV-infected (SIV) rhesus monkeys. Data are means ± SEM and are shown as % of control, $n = 4$–7. $^{*}P < .05$, significantly different from Controls (unpaired *t* test with Welch's correction). Right panels show representative immunoblots of total CREB in nucleus accumbens (*upper panel*) and corpus amygdaloideum (*lower panel*) from Controls (c) and SIV-infected animals (s). The immunoreactivities are observed at the expected molecular mass of 43 kDa.

PC12 cells (Zauli *et al*, 2001) and inhibited the expression of tyrosine hydroxylase, the rate-limiting enzyme for the dopamine biosynthetic pathway. This was accompanied by inhibition of the production and release of dopamine into the culture medium through involvement of a member of the cAMP-responsive element modulator transcription factor family (Zauli *et al*, 2000). Further, reduced cAMP synthesis in microglia was reported following Tat treatment (Patrizio *et al*, 2001).

Intracellular levels of cAMP are relevant in immunodeficiency infection. *In vitro* observations indicate that the production of several substances from activated glial cells is regulated by the intracellular concentrations of the second messenger cAMP. cAMP enhances the microglial production of potentially neuroprotective molecules such as interleukin (IL)-10 (Aloisi *et al*, 1999) and prostaglandin (PG) E2 (Minghetti *et al*, 1997). Furthermore, cAMP

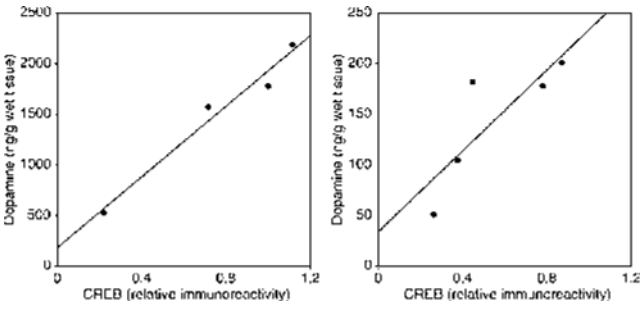

Figure 5 Correlation between dopamine content and relative immunoreactivity of total CREB in SIV-infected monkeys. Nucleus accumbens (*left*), corpus amygdaloideum (*right*). Linear regression, $r^2 = .97$ ($P < .05$) and $r^2 = 0.69$ for nucleus accumbens and corpus amygdaloideum, respectively.

down-regulates potentially neurotoxic substances, such as tumor necrosis factor (TNF)-α (Aloisi *et al*, 1999; Suzumura *et al*, 1999) and nitric oxide (Minghetti *et al*, 1997), found to be increased in the brain of HIV-infected individuals (Adamson *et al*, 1996; Nuovo and Alfieri, 1996; Tyor *et al*, 1992). Consequently, agents that raise intracellular cAMP levels, such as pentoxyphilline or rolipram, have been tested for beneficial actions (Biswas *et al*, 1994). The reduction of intracellular concentrations of cAMP in nucleus accumbens in our SIV-infected animals may thus implicate a deficit in the protective cellular mechanisms and indicate an enhanced possibility for neuronal dysfunction in this region during asymptomatic SIV infection. An impairment in this limbic region has neurobehavioral consequences such as dysfunction of the reward systems, psychosis, and affective disorders.

The cascade of events triggered by cAMP influences gene transcription patterns via CREB (Montminy, 1997), a downstream target in the cAMP signaling pathway. CREB is involved in regulation of genes essential for neuronal function and production of proteins, such as brain-derived neurotrophic factor (Zafra *et al*, 1992), which is important for cell survival, particularly of dopaminergic cells (Hyman *et al*, 1991). In addition, CREB has been reported to link immediate postsynaptic events with neuronal changes in affective disorders (Duman *et al*, 1997). Decreased levels of CREB were reported in brains of depressive patients who had not received antidepressants compared to controls and treated subjects (Dowlatshahi *et al*, 1998; Yamada *et al*, 2003). The decrease in the expression of CREB found in SIV-infected animals may, therefore, indicate an affective dysfunction in immunodeficiency infection. However, our results on CREB levels must be interpreted with caution. The levels of phosphorylated CREB could not be reliably measured in postmortem brain tissue, making it difficult to determine the functional relevance of decreased CREB levels with respect to neuronal gene expression.

Our present findings indicate that altered signal transduction regarding the impaired dopamine/cAMP signaling in limbic regions of SIV-infected monkeys precedes neurologic deficits and they implicate defects in the dopaminergic system in the etiopathogenesis of immunodeficiency-associated neuropsychiatric disease. Our results suggest that affective complications in HIV subjects should not be interpreted only as reactive psychological changes and implicate a biological background for psychiatric disorders in HIV infection.

Materials and methods

Animals

Juvenile (3.7 $\pm$ 0.6 years) rhesus monkeys (Macaca mulatta) were housed individually in indoor facilities on a 12:12 light:dark schedule at the German Primate Center (Göttingen, Germany). Dry food with fresh fruits as a dietary supplement was provided twice a day and water was available *ad libitum*.

Four monkeys were infected under ketamine anesthesia (10 mg/kg) with SIVmac 251 MPBMC and two with SIVmac 239. Three additional SIV-infected monkeys inoculated with SIVmac 251 MPBMC were treated with 2 mg/kg selegiline, intramuscularly, until sacrifice. Five uninfected animals served as controls. Animals were monitored clinically, and physical examinations and blood samplings were performed at regular intervals under ketamin anesthesia from experienced veterinarians.

Animal experiments were approved by and performed according to the guidelines set out by the ethics committee for animal experimentation of the Bezirksregierung Braunschweig (604.42502/08-02.95).

Peripheral markers of the disease

Cell-associated viral load was determined by limiting dilution assay. Briefly, PBMCs from the infected monkeys, isolated by a Ficoll-Paque (Pharmacia) densitiy centrifugation, were cocultured with the permanent T-cell line C81-66 as indicator cells (Sauermann *et al*, 1997). For three-color flow cytometry, 50-μl citrated blood samples were incubated with the following antibodies as described recently (Sopper *et al*, 2003): anti-monkey CD3 FN18 (M. Jonker, TNO, Rijswijk, The Netherlands), biotinylated using standard techniques; CD20 $\sim$ FITC (B1, Coulter, Krefeld, Germany); CD4 $\sim$ PE (L200, Pharmingen, Heidelberg, Germany); and CD8 $\sim$ FITC (RPAT8, Pharmingen, Heidelberg, Germany). After lysis of erythrocytes and fixation of cells using fluorescence-activated cell sorting (FACS) lysing solution (Becton-Dickinson, Heidelberg, Germany), bound biotinylated antibodies were detected with streptavidin-coupled Cy-Chrome (Pharmingen, Heidelberg, Germany). Cells were analyzed with a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany) using Lysis II and cellquest software. Quadrants were set according to the staining pattern obtained by isotype matched control antibodies.

For determination of absolute numbers of lymphocyte subsets, the proportion of the respective lymphocyte subset within a forward and side light scatter gate, including $CD3^+$ T cells, $CD20^+$ B cells, and $CD3^-CD8^+$ natural killer (NK) cells, was multiplied by the absolute number of lymphocytes obtained with a coulter counter.

Collection and dissection of monkey brain tissue

Animals were sacrificed the same time of the day by exsanguination during the asymptomatic phase of SIV infection (4 to 19 weeks post infection, wpi; mean time $\pm$ SD: 9.6 $\pm$ 5.3). Additionaly, for cAMP

measurements, tissue from an asymptomatic animal, which was sacrificed 32.4 wpi, was used.

The anesthetized animals were perfused with RPMI 1640. The brains were quickly removed, partly immersion fixed (5% neutral-buffered formaldehyde for light microscopy), and partly frozen at −70°C for biochemical analysis.

Prior to neurochemical investigations nucleus accumbens and corpus amygdaloideum were dissected on a Teflon plate at −20°C according to a stereotactic atlas using standardized procedures (Gsell *et al*, 1993).

Histopathology and immunohistochemistry

Routine neuropathological assessment was performed on 3-μm paraffin-embedded sections stained by hematoxylin and eosin (H&E), Luxol-fast blue, and Masson's trichrome. In order to detect astrocytic gliosis, additional immunohistochemical staining was performed using a two-step staining system in which incubation with the primary antibody is followed by incubation with a polymeric substrate (DAKO Envision detection kit). Briefly, paraffin-embedded tissue sections were deparaffinized, hydrated in buffer, and subjected to antigen retrieval in a microwave oven. After blocking of the endogenous peroxidase by hydrogen peroxide, the slides were incubated with a commercially available polyclonal antibody against the glial fibrillary acid protein (GFAP) and consequently incubated with a peroxidase-labeled dextran coupled with goat antibodies to rabbit and mouse immunoglobulins. Diaminobenzidine was utilized as chromogen, and the slides were counterstained with hematoxylin. The reagents were purchased from DAKO Diagnostica (Hamburg, Germany).

Measurement of dopamine, HVA, and DOPAC

Brain homogenates: Brain tissues were disrupted in 150 mM phosphoric acid (Merck, Darmstadt, Germany) supplemented with 500 μM diethylenetriamine pentaacetic acid (DTPA) by means of a sonifier (Branson W-250, efficacy 50%, 40 pulses) in an argon atmosphere on ice. The homogenates were centrifuged at 44593 × *g* (Sorvall, Germany), the supernatants were filtered through molecular weight filters (molecular weight cut off 5 kDa; Millipore, Bedford, USA) at 4°C, to remove high-molecular-weight compounds, by spining down with a MicroRapid centrifuge (Hettich, Tuettlingen, Germany) and stored at −70°C until analysis.

HPLC: Filtrates of 20 μl were injected into a Rheodyne injector (type 7725, Eppelheim, Germany) and analyzed for dopamine, HVA, and DOPAC by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC consisted of a solvent delivery pump (P580, Dionex, Germany), and an electrochemical detector (EC3000, Recipe, Germany) with a glassy carbon electrode. The detector potential was set at +750 mV *versus* Ag/AgCl, with the sensitivity setting at 5 nA. A 5-μm analytical column (250 × 4.0 mm nucleosil 120-5C18) was used, operating at a flow rate of 1.0 ml/min. The mobile phase consisted of 0.1 M sodium phosphate buffer (Merck), pH 3.35, 84% (*v*/*v*), and 16% (*v*/*v*) methanol containing 0.1 mM EDTA (Sigma, St. Louis, USA), 0.65 mM 1-octanesulfonic acid, and 0.5 mM triethylamine. Qualitative and quantitative analyses were performed by comparing retention times and peak heights with those of commercially available standards (Sigma).

cAMP assay

Nucleus accumbens and corpus amydaloideum were homogenized in 20 volumes of 0.1 M HCl or 150 mM phosphoric acid on ice using a glass on glass homogenizer. Homogenates were centrifuged at 600 × *g* (Hettich, Tuettlingen, Germany), at 0°C, for 5 min. The supernatants were then diluted 1:2 to 1:8 and assayed immediately. cAMP measurement was based on a competitive binding technique in which cAMP present in the sample competed with a fixed amount of alkaline phosphatase–labeled cAMP for sites on a rabbit polyclonal antibody. The assay was performed with a commercially available kit according to the instructions of the manufacturer (cAMP low pH; R&D Systems, Germany). To monitor the performance of the cAMP immunoassay, a quality control program was established in our laboratory.

Total CREB determination

Homogenate preparation: Whole-cell homogenates for immunobloting were prepared from nucleus accumbens and corpus amygdaloideum of rhesus monkeys, as previously described (Yamamoto-Sasaki *et al*, 1999), with minor modification. Briefly, brain tissue was homogenized with a glass on glass homogenizer in 10 volumes of a buffer (pH 7.4) containing 20 mM HEPES, 1 mM $MgCl_2$, 0.3 M phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 1 mM EGTA, 5 nM calycalin A, and 1 μM okadaic acid and centrifuged at 9500 × *g* (Hettich), at 0°C, for 5 min. The supernatants were stored at −70°C until required. Protein concentrations were determined by the Coomassie blue binding method using bovine serum albumin as a standard (Bradford, 1976). Protein content in brain homogenates did not differ between uninfected and SIV-infected animals (data not shown).

Gel electrophoresis and immunoblotting: The proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes for subsequent immunoblotting. Homogenates (10 to 20 μg) were subjected to SDS-PAGE with 10% polyacrylamide gels at 125 to 130 V for 90 to 120 min. Proteins were transferred to nitrocellulose membranes at 30 V for 75 min at room

temperature. The membranes were blocked with 3% low fat milk and 1.5% Tween 20 in Tris-buffered saline (TBS) for 30 min at room temperature and then incubated with total CREB antiserum (1:1000 Biomol) for 60 min at room temperature. Membranes were washed and incubated with secondary antibody (anti-rabbit immunoglobulin G [Ig], horseradish peroxidase [HRP] linked, 1:3000; Cell Signaling) in 3% milk/1.5% Tween 20 in TBS for 60 min at room temperature. Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) system (Amersham) and analyzed by laser densitometry (NIH 1.55 Image Analysis System). Identical control human cortical homogenate was routinely loaded onto each gel as an internal standard to ensure linearity of each analysis and to enable comparisons across blots on different gels. In the immunoblots using the standard homogenate, the immunoreactivities showed linearity within the ranges of 5 to 75 μg.

Statistical analysis

The unpaired *t* test with Welch's correction was used for statistical analysis. Samples from uninfected and SIV-infected animals were compared. The significance level was set at $P < .05$.

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