Addiction Biology

BRIEF REPORT

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# Decreased methylation of the NK3 receptor coding gene (*TACR3*) after cocaine-induced place preference in marmoset monkeys

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## ABSTRACT

Epigenetic processes have been implicated in neuronal plasticity following repeated cocaine application. Here we measured DNA methylation at promoter CpG sites of the dopamine transporter (*DAT1*) and serotonin transporter (*SERT*) and neurokinin3-receptor (NK3-R)-receptor (*TACR3*) coding genes in marmoset monkeys after repeated cocaine injections in a conditioned place preference paradigm. We found a decrease in DNA methylation at a specific CpG site in *TACR3*, but not *DAT1* or *SERT*. Thus, *TACR3* is a locus for DNA methylation changes in response to repeated cocaine administration and its establishment as a reinforcer, in support of other evidence implicating the NK3-R in reinforcement- and addiction-related processes.

**Keywords** Cocaine, conditioned place preference, DNA methylation, monkeys, NK3-receptor, *TACR3*.

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Epigenetic mechanisms play an important role in addiction-related behaviors. Repeated cocaine administration is known to be associated with alterations to histone modifications and DNA methylation although the precise genomic loci affected have not yet been delineated (LaPlant *et al.* 2010; Wong, Mill & Fernandes 2011).

The neurokinins, in particular the neurokinin3-receptor (NK3-R), are involved in reinforcement-related processes (Hasenöhrl *et al.* 2000) and interact with the behavioral and neurochemical actions of cocaine in rats (Jocham *et al.* 2007) and monkeys (De Souza Silva *et al.* 2006, 2008). In humans, the *TACR3* gene, coding for the NK3-R, is associated with alcohol and cocaine addiction (Foroud *et al.* 2008). Dopamine transporter (*DAT1*) and the serotonin transporter (*SERT*) are primary targets of cocaine in the brain, which essentially modulate its behavioral effects (Rothman, Blough & Baumann 2006). Based on the evidence for addiction-related involvement of the NK3-R, we hypothesized that methylation of its encoding gene *TACR3*, together with the genes that code cocaine's influence on reuptake of dopamine and serotonin, would respond to repeated cocaine administration. We investigated DNA methylation in the promoter regions of the *TACR3*, *DAT1* and *SERT* after repeated cocaine injections that led to the acquisition of a conditioned place preference (CPP) in marmoset monkeys.

All procedures were approved by the Animal Ethics Committee of the University of Brasilia and complied with NIH guidelines for care and use of laboratory animals. Ten adult male black tufted-ear marmosets (*Callithrix penicillata*), weighing 290–420 g, were used (for housing conditions, see: De Souza Silva *et al.* 2006). Testing for CPP was conducted in a rectangular chamber (150 × 30 × 35 cm) placed 1 m from the floor, consisting of two conditioning compartments (60 × 30 × 35 cm) containing different visual and tactile cues, connected by a central zone (30 × 30 × 35 cm); the walls and floor were of metal, and the top of glass (see Supporting Information Fig. S1).

*These authors contributed equally to the study.

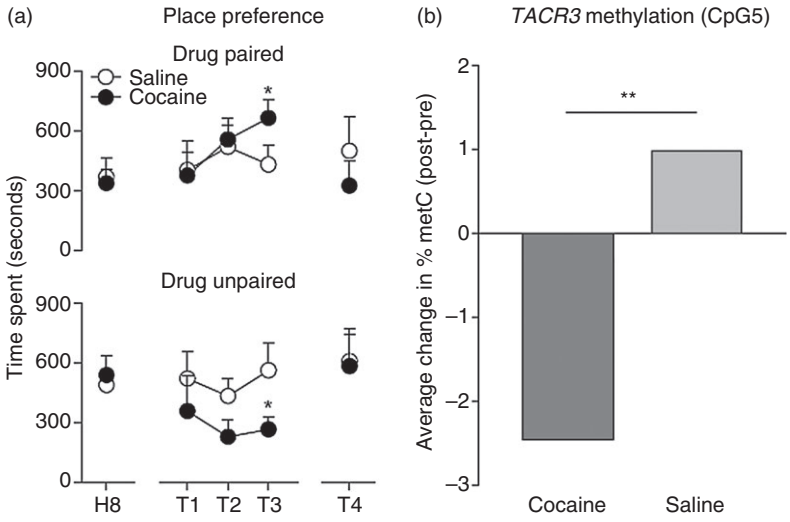


**Figure 1** Acquisition and extinction of cocaine (5 mg/kg; i.p.)-induced conditioned place preference and DNA methylation of the *TACR3* promoter. (a) Time (mean + SEM) spent in drug-paired (top) and drug-unpaired (bottom) compartments during last habituation (H8), three acquisition tests (T1–T3) and after extinction (T4; $*P < 0.05$ versus H8). (b) Effect of repeated cocaine or saline exposure on DNA methylation at CpG5 of the promoter region of *TACR3* (duplicate means; $**P < 0.01$)

Behavior was monitored via cameras, one mounted 1 m above and 1.5 m away from the chamber. The behavior was tracked using AnyMaze Software (Stoelting Co., Wood Dale, IL, USA). Marmosets were randomly assigned to two treatment groups: saline ($n = 5$) or cocaine ($n = 5$; 5 mg/kg; i.p., Sigma-Aldrich, St Louis, MO, USA). On days 1–8, they had daily 20-minute free access to the entire apparatus (habituation). On days 9–11, 13–15 and 17–19, they underwent CPP conditioning trials in one chamber with an unbiased counterbalanced design. Five minutes after cocaine or saline injection (1 ml/kg), the subject was released into the CPP compartment for 20 minutes. On days 12, 16 and 20, test trials (T1–T3) were performed, during which subjects accessed the entire apparatus for 20 minutes (no treatment). On days 21 and 22, all subjects were submitted to 20-minute extinction trials to the entire apparatus, 5 minutes after a saline injection. Another test trial (T4) was performed on day 23. Blood was taken prior to the initial habituation trials and after T3 via femoral venipuncture.

DNA was extracted from blood using a commercial kit (Qiagen, Crawley, UK). All samples were assessed for purity and quantity, and global DNA methylation was estimated using luminometric methylation assay combined with pyrosequencing (Karimi *et al.* 2006) for each treatment group and sample time. All experiments were performed in duplicate and fully methylated and fully unmethylated control samples were processed in parallel to ensure assay efficiency. As the *C. penicillata* genome is not publicly available, we designed genomic DNA polymerase chain reaction (PCR) primers based on the March 2009 *Callithrix jacchus* assembly so that the regions could be sequenced to enable us to design locus-specific assays for DNA methylation analysis. Standard PCR protocol was employed and sequenced with ABI BigDye chemistry (Applied Biosystems, Foster City, CA, USA) using a standard protocol. For *TACR3* and *SERT*, no sequence differences were found between the two strains. In the *DAT1* promoter, however, three single nucleotide changes were found (see Supporting Information Table S1 and Supporting Information Fig. S2). DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA) and pyrosequencing assays were designed specific to the *C. penicillata* sequence (see Supporting Information Table S2). Following bisulfite-PCR amplification, DNA methylation was quantitatively assessed using the Pyromark Q24 pyrosequencer according to the manufacturer's protocol (Qiagen, Hilden, Germany). Data were analyzed by two-way ANOVAs and Dunnett's *post hoc* test (behavior) or paired *t*-tests (two-tailed; methylation) with $P < 0.05$ for significance.

Cocaine, but not saline treatment, increased the time spent in the conditioning compartment during the third test trial ($F_{4,8} = 3.38$, $P < 0.05$), compared with the last habituation trial (H8); time in the unpaired compartment decreased ($F_{4,8} = 3.25$, $P < 0.05$). The cocaine-induced CPP was extinguished when tested at T4 ($P > 0.05$; Fig. 1a).

Repeated cocaine administrations had no effect on global DNA methylation levels or on average DNA methylation across all three target amplicons ($P > 0.05$). Ten CpGs were analyzed in the promoter region of *DAT1*, and there was no cocaine effect on *DAT1* methylation at any specific site ($P > 0.05$). However, at CpG11, a trend towards a decrease was observed after cocaine exposure ($P = 0.055$; saline: $P = 0.77$). Between-group differences were not observed for any of the 11 *SERT* CpG sites assessed ($P > 0.05$). Ten CpGs were assayed in the promoter region of *TACR3*. At CpG5, a small but significant decrease in DNA methylation was seen after cocaine ($P = 0.002$), but not after saline exposure ($P > 0.05$; Fig. 1b).

 

In this study, repeated exposure to cocaine which led to the acquisition of a CPP in monkeys, decreased methylation of *TACR3*, but not of *DAT1* or *SERT* at a CpG site of the promoter. Previous studies showed that changes in the methylation status of a single intronic CpG site are sufficient to change gene expression (Nile *et al.* 2008; Zhang *et al.* 2010). A decrease in methylation may reduce binding of MeCP2, a methyl CpG-binding transcriptional regulator, which was shown to control psychostimulant-induced CPP and cocaine intake in rodents (Deng *et al.* 2010; Im *et al.* 2010). Because promoter DNA methylation has been associated with gene silencing, a decrease in methylation may lead to enhanced expression of NK3-R. In rats, NK3-R control nucleus accumbens dopamine responses to cocaine and cocaine's locomotor-activating effects (Jocham *et al.* 2007). NK3-R ligands did not affect cocaine-induced CPP in rats. However, significant species differences between rats and primates in NK3-R function limit the interpretation of previous data (De Souza Silva *et al.* 2008).

The present results in monkeys support the hypothesis of a significant role for NK3-receptors in the control of addiction-related processes in primates, as they suggest an involvement of *TACR3* methylation in the neuronal adaptations that follow repeated cocaine treatment.

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**Authors Contribution**

JPH, MB, MDS, JM, CT and CPM conceived and designed the study. SC, RSM, ED and NI performed the experiments. MB, MDS, RSM and JM performed data analysis. CPM, JPH and MB wrote the manuscript. All authors critically reviewed content and have approved the final manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** CPP chamber

 

**Figure S2** Differences in DNA of the dopamine transporter gene (*DAT1*) between *Callithrix penicillata* and *Callithrix jacchus*

**Table S1** Genomic PCR primers for the candidate genes

**Table S2** Primer and PCR assay details (*Biotinylated primer)

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