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Regulation of Brain DNA Methylation Factors and of the Orexinergic System by Cocaine and Food Self-Administration

Lamis Saad[1,2] · Maxime Sartori[1,3] · Sarah Pol Bodetto[1] · Pascal Romieu[1] · Andries Kalsbeek[2,4] · Jean Zwiller[1] · Patrick Anglard[1,5]

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Abstract

Inhibitors of DNA methylation and orexin type-1 receptor antagonists modulate the neurobiological effects driving drugs of abuse and natural reinforcers by activating common brain structures of the mesolimbic reward system. In this study, we applied a self-administration paradigm to assess the involvement of factors regulating DNA methylation processes and satiety or appetite signals. These factors include Dnmts and Tets, miR-212/132, orexins, and orx-R1 genes. The study focused on dopamine projection areas such as the prefrontal cortex (PFCx) and caudate putamen (CPu) and in the hypothalamus (HP) that is interconnected with the reward system. Striking changes were observed in response to both reinforcers, but differed depending on contingent and non-contingent delivery. Expression also differed in the PFCx and the CPu. Cocaine and food induced opposite effects on *Dnmt3a* expression in both brain structures, whereas they repressed both miRs to a different extent, without affecting their primary transcript in the CPu. Unexpectedly, orexin mRNAs were found in the CPu, suggesting a transport from their transcription site in the HP. The o*rexin receptor1* gene was found to be induced by cocaine in the PFCx, consistent with a regulation by DNA methylation. Global levels of 5-methylcytosines in the PFCx were not significantly altered by cocaine, suggesting that it is rather their distribution that contributes to long-lasting behaviors. Together, our data demonstrate that DNA methylation regulating factors are differentially altered by cocaine and food. At the molecular level, they support the idea that neural circuits activated by both reinforcers do not completely overlap.

Keywords Cocaine and food self-administration · Drugs of abuse · DNA methylation · Epigenetics · Orexins/hypocretins · Addiction

Introduction

While drugs of abuse and natural reinforcers, such as food or sugar, activate common brain structures of the mesolimbic reward system, it is still not clear whether their activated neural circuits completely overlap or not. As illustrated by electrophysiological studies, neurons exhibit non-overlapping firing patterns in response to cocaine vs. sucrose [1–4]. Both reinforcers also induce significant differences in the reactivity of reward brain structures after long-term self-administration, as illustrated by functional magnetic resonance imaging, suggesting the development of neuroadaptative mechanisms related to the emergence of addiction-like behavior occurring only in cocaine self-administering rats [5]. Other studies have reported that neuropeptides modulating feeding and drug reward increase ventral tegmental area (VTA) dopamine neuron firing and responses to both drugs of abuse and food. Nevertheless, there are clearly more complex interactions over-ruling this relationship, since some of them like opioids,

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✉ Patrick Anglard
anglard@neuro-cnrs.unistra.fr; patrick.anglard@inserm.fr

[1] Laboratoire de Neurosciences Cognitives et Adaptatives (LNCA), UMR 7364 CNRS, Neuropôle de Strasbourg, Université de Strasbourg, Strasbourg, France

[2] The Netherlands Institute for Neuroscience (NIN), Royal Netherlands Academy of Arts and Sciences (KNAW), Amsterdam, The Netherlands

[3] Present address: IGBMC, Inserm U 964, CNRS UMR 7104, University of Strasbourg, Illkirch, France

[4] Department of Endocrinology and Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam, Netherlands

[5] INSERM, Institut National de la Santé et de la Recherche Médicale, Paris, France

orexin (hypocretin), ghrelin, and NPY (AgRP) show similar behavioral effects on food intake and cocaine reward, whereas others like CART or galanin do not [6].

In addition, agents modulating histone acetylation or DNA methylation affect cocaine-induced behavior, but not the intake induced by natural reinforcers in experimental rodent models. Indeed, HDAC inhibitors reduce cocaine self-administration without affecting sucrose self-administration or preference [7]. Treatment with methionine, a methyl donor increasing DNA methylation, inhibits the establishment of cocaine, but not food conditioned place preference [8]. It also reduces cocaine-induced locomotor sensitization and seeking without affecting sucrose self-administration [9]. These studies support the concept that epigenetic regulation of gene expression is part of the responses of brain cells to repeated exposure to drugs of abuse [10–12]. It is also consistent with data showing that repeated cocaine administration modulates the expression of methyl-DNA binding proteins [13, 14] and DNA methyltransferases (DNMTs) [15–17]. Drugs of abuse also induce alterations in brain DNA methylation, as observed for specific genes, some of them being involved in learning and memory processes [15, 17, 18], but also in global [8, 19] or genome-wide DNA methylation studies [20, 21], showing that gene hypermethylation and hypomethylation simultaneously occur in response to drugs of abuse. Conversely, that DNA methylation contributes to the rewarding effects of cocaine has been well illustrated by manipulating the expression or the activity of MeCP2, mir-212 [14], and Dnmt3a [16] and by using methyl donors such as methionine, S-adenosylmethionine (SAM), or DNMT inhibitors, [11, 22, 23] for review.

Following excessive intake, cocaine or food reward may ultimately lead to persistent habits or phenotype effects, but the aforementioned studies did not address how they differ in regulating epigenetic mechanisms involving DNA methylation. We hypothesize that DNA methylation and demethylation processes triggered by each of the two reinforcers could lead to stable long-lasting changes contributing to different addictive-like behaviors. Thus, one aim of the present study was to compare the effect of cocaine with that of food delivery by using a self-administration paradigm in rats exposed to the same schedule of reinforcement. In addition, passive administration was compared with voluntary administration representing a valid model for aspects of human drug addiction [24]. Expression of genes playing a major role in DNA methylation like Dnmt and Tet genes was investigated in the prefrontal cortex (PFCx) and the caudate putamen (CPu). Based on reported homeostatic interactions between Mecp2 and mir-212 [14], a microRNA together with mir-132 belonging to the same intronic cluster activated by CREB [25] were also analyzed.

Since orexinergic neurons project throughout the mesolimbic reward system and play a major role in arousal, feeding behavior/satiety, anxiety, and addictive behaviors [26–28], the orexinergic system was also investigated in response to both reinforcers. Expression of the orexin-receptor-1 was evaluated in the PFCx and the CPu, since its antagonists, similar to methyl donors, inhibit cocaine self-administration in preclinical trials [29–36]. Orexin expression itself was also examined in both brain structures, as well as in the hypothalamus where it is synthesized. Since in operant conditioning for food self-delivery food restriction is required, we also analyzed the effect of food restriction itself, with the general aim of better understanding the molecular mechanisms triggered by each reward in specific neuroadaptations.

Materials and Methods

Animals

Male Wistar rats (Janvier, France), weighting 160–180 g, were housed individually in standard home cages, in a temperature- and humidity-controlled room, under an inverted 12 h/12 h light/dark cycle (lights on at 7:00 PM). Animals were allowed to acclimate to laboratory conditions and were handled during 1 week before experimental procedures. Each behavioral experiment started 3 weeks after their arrival in the laboratory and was conducted during the dark period. Animals used for the cocaine operant self-administration experiment had ad libitum access to food and water, whereas animals used for the food pellet operant self-administration experiment had ad libitum access to water, but were food restricted. Their body weight was progressively reduced to 85% of its free-feeding value. It was maintained at this level throughout the experiment by providing an individually adjusted amount of food after each daily test session. All procedures involving animal care were conducted in compliance with national laws and policies (Council directive 87848, 1987, Service Vétérinaire de la Santé et de la Protection animale, permission 67-165 to J.Z. and 67-370 to P.R.), with the Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche (project permission number *APAFIS#2133-20151 00221 087072* to P.A.) and international guidelines (NIH publication 5586-23, 1985). A total of 72 rats have been used in the present study not including the genomic DNA methylation analysis [20].

Surgery, Apparatus, and Cocaine Operant Self-Administration

Surgical implantation of a chronic indwelling catheter in the jugular vein was performed 10 days after the arrival of the rats in the laboratory. The intravenous (i.v.) catheterization procedure was performed as described previously [7]. After surgery, animals were allowed to recover for 7–10 days before the

beginning of the cocaine operant self-administration procedure. Nose pokes (NPs) into both holes were recorded. One hole was selected as the active hole for delivering either the cocaine or the saline solution depending on the experimental group considered and the other as the inactive hole. They were counterbalanced between right and left positions in each experimental group and in the various groups of rats. Rats were divided into three experimental groups. A first group of rats self-administering cocaine, called "SA cocaine" was compared to two yoked groups. Each rat from the two yoked groups was paired with a rat from the "SA cocaine" group and received passively i.v. injections of either cocaine ("Yoked cocaine" group) or saline ("Yoked NaCl" group). These passive injections together with their associated cues were computer-delivered, independently of the rat's behavior and strictly following the number and timing of those determined by the NPs responses of the "SA cocaine" paired rat. For the "SA cocaine" group, NPs into the inactive hole (NPi) had no programmed consequence. When the required number of NPs into the active hole was reached, a 40-μl cocaine (Cooper, Melun, France) solution (0.33 mg/kg diluted in 0.9% NaCl) was delivered by i.v. injection for 2 s under the control of the computer. A stimulus light, located 20 cm above the active hole, was paired contingently with the delivery of cocaine (light on during 5 s), and then turned off, replaced by the house-light of the operant chamber, which materialized a 40-s time-out period. "SA cocaine" rats were first submitted to a fixed-ratio 1 (FR1) schedule of reinforcement during daily 2-h sessions for 4 days. Rats were then submitted to a FR5 schedule of reinforcement during daily 2-h sessions for 6 days (Fig. 1). No cutoff was applied concerning the number of self-infusions the rat was able to perform during each session.

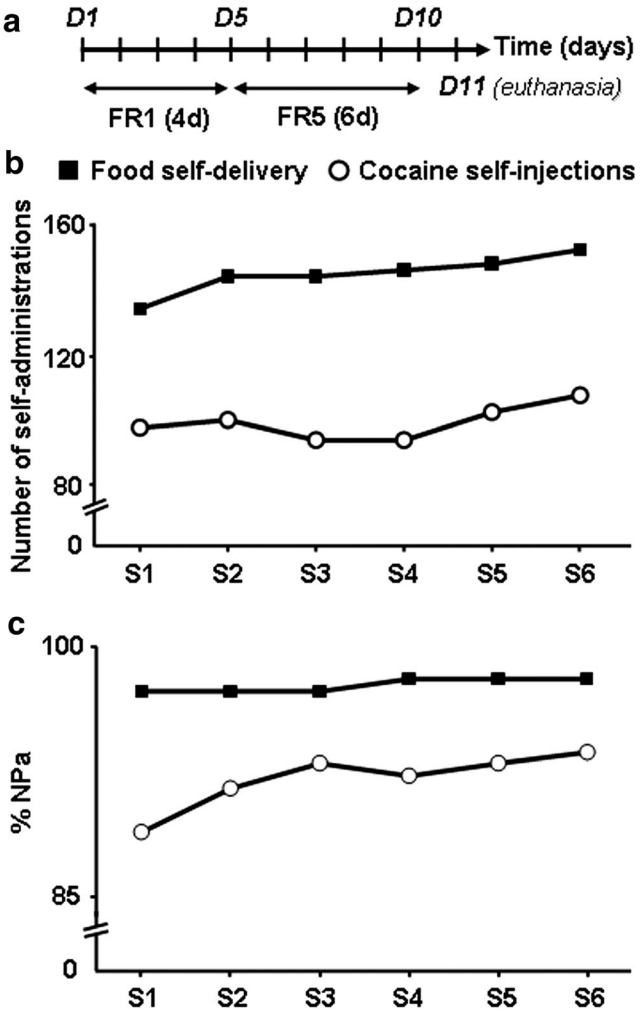

Fig. 1 Cocaine and food pellets operant self-administration paradigm in FR5 schedule of reinforcement. The effect of cocaine administration and food delivery was measured following a FR1 schedule of reinforcement for 4 days and then a FR5 schedule for 6 days (**a**). The number of cocaine injections in self-administering rats and food pellets self-delivery during the FR5 schedule is depicted as black squares for food pellets delivery and white circles for cocaine administration (**b**). Similar circles and squares are used for the percentage of nose pokes in the active hole (NPa) for food and cocaine (**c**). Data represent the mean ($n = 8$–12 per group) with interexperimental variation not exceeding 10%

Apparatus and Food Pellets Operant Self-Administration Procedure

Dark five-choice operant chambers (25.2 × 28 × 24 cm, Bioseb, BP 89 92,370 Chaville, France) placed in sound-attenuated and ventilated enclosures were used to test food pellets operant self-delivery. The curved rear wall comprised nine contiguous 2.3-cm square holes, located 2.2 cm above the grid floor. Each hole was equipped with an infrared photocell beam to detect NPs. The two farthest holes were used as the active and the inactive holes, the other holes being obstructed by a metal cover. NPs into both open holes were recorded. The active and inactive holes were counterbalanced between right and left positions in each experimental group and in the various groups of rats. The active hole was associated with the delivery of a food pellet (45 mg, Bioserv) into a magazine located at the opposite side of the chamber and equidistant from each hole. The rat collected the delivered food pellet by pushing a Perspex panel covering the magazine. Each chamber was automatically controlled by the Packwin software (Panlab S.P., Cornella, Barcelona, Spain).

As for the cocaine experiments, rats were divided into three different groups: "SA pellets," "Yoked pellets," and "Yoked control." "SA pellets" and "Yoked pellets" rats were initially given access to food pellets in their home cage (10 pellets per day during 5 consecutive days) to get them used to the reinforcer. Each rat was placed in the same chamber throughout the experiment. In a first training phase (one session), rats from each group were placed in the chamber for 15 min with the house-light off and the panel of the magazine in open position. During the training phase, for the "SA pellets" and the "Yoked pellets" groups, the magazine was filled with 15

food pellets to familiarize rats to eat the reinforcer from the magazine, but it remained empty for the "Yoked control" rats. In a second training phase, rats received two food magazine training sessions (20 min/session, 1 session/day), in which 20 food pellets were delivered according to a variable time schedule (mean = 60 s) for the "SA pellets" and the "Yoked pellets" groups. No pellet was delivered to animals of the "Yoked control" group. The house-light of the operant chamber was turned off during this phase. On the first session, the panel of the magazine was blocked in an upward position in order to maintain the food magazine open. For the second session, as for the sessions of the food pellets operant self-administration procedure, rats had to push away the panel in front of the food magazine to retrieve the food pellet.

Briefly, as for the cocaine operant self-administration experiment, "SA pellets" rats were first submitted to a FR1 schedule of reinforcement during daily 2-h sessions for 4 days and to a FR5 schedule during daily 2-h sessions for 6 days. When the required number of NPs into the active hole was reached, a food pellet was delivered into the magazine. NPs into the inactive hole had no programmed consequence. No cutoff was applied concerning the number of food pellets delivered the rat was able to induce during each session. Two yoked groups were included and exposed to the same associated cues based on the "SA pellets" group. Each rat from these groups was paired with a rat from the "SA pellets" group. Rats from the "Yoked pellets" group received passively the same amount of food pellets as that of "SA pellets" paired rats, whereas no food pellet was delivered to rats from the "Yoked control" group.

Reverse Transcription-Quantitative PCR Analysis

Animals were sacrificed 24 h after the beginning of the last session of cocaine self-administration or food pellet self-delivery and brain structures of interest were dissected, as described in supplementary Fig. S1. RNA was extracted from rat PFCx and CPu and first strand cDNA was generated from 0.5 μg of total RNA using random primers and reverse transcriptase (MLV), and the reaction product was used for real time PCR performed with Hot Pol EvaGreen (Euromedex, Souffelweyersheim, France) with a Light Cycler instrument and technology (Roche Applied Science, Indianapolis, IN) and CFX Connect Real Time PCR detection System (Bio-Rad), as previously described [18]. Primers were from Sigma-Aldrich (Saint Louis, MO) and are listed in Table 1. Results were normalized to *36B4* (*RPLP0*) used as an internal control for gene expression and to *U6 snRNA* for miR-212/132. Cycling conditions were 95 °C for 14 min, then 55 cycles of 95 °C for 14 s, 60 °C for 18 s, and 72 °C for 18 s. PCR products were verified by melting curve analysis and their sizes were confirmed by 2% agarose gel electrophoresis. Real-time PCR was conducted three times for each gene/miR of interest, using duplicate samples. Primers were designed with the Primer 3 software (http://frodo.wi.mit.edu/primer3/input.htm).

Immunohistochemistry

Twenty-four hours after the beginning of the last session of self-administration or self-delivery, rats were sacrificed by an overdose of pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 100 ml saline followed by 1% paraformaldehyde in phosphate-buffered saline. The brain were post-fixed in 1% paraformaldehyde in PBS for 1 h and kept overnight at 4 °C in 15% sucrose, then frozen in isopentane at − 40 °C, and finally stored at − 80 °C. Coronal tissue sections (16 μm thick) were prepared using a Microm HM560 S_18 Cryostat. Immunohistochemistry was carried out essentially as described previously [13, 37]. Antibody incubation was performed overnight with a polyclonal goat primary Orexin A-specific antibody (C-19, sc-8070, 1:5000). Images of each region of interest were obtained using a microscope (Olympus: Vanox AHBT3; × 20 objective) equipped with a digital camera. For each experimental condition, the number of orexin immunoreactive cells in the hypothalamus was determined using the Image J software (N.I.H.). The number of immunoreactive cells was counted bilaterally on four consecutive sections per rat, before being averaged to generate the final mean value. Each counting was performed twice by an investigator blinded to the identity of the samples.

Genomic DNA Methylation Analysis of the PFCx

High molecular weight DNA was extracted, as previously described [38]. Whole genome methylation analysis was performed on PFCx samples from rats killed 24 h after the last session of FR5 schedule of reinforcement. The comparison was carried out between cocaine self-administering rats with control rats (Yoked NaCl). Briefly, genome-wide 5mC were quantified by Active Motif (Carlsbad, CA, USA) and purification was achieved through the formation of binding complexes between methylated DNA and the methyl-binding proteins MBD2b and MBD3L1, as previously reported [20].

Statistical Analysis

In behavioral experiments, the preference for the active vs. inactive hole was evaluated by comparing the percentage of NPs performed into the active hole (%NPa) to 50% considered as random. Daily performance and the mean performance realized during the FR1 period and the FR5 period were compared to 50%. The acquisition of the task was evaluated by comparing the %NPa to 80%, which was considered as the acquisition criterion. Daily performance and the mean performance realized during both FR1 (mean (FR1)) and FR5

Table 1 Primer sequences

Gene	Primer sequence (5′–3′)	GeneBank Acc. #
MiR-212/132	FP: AAGGTCCCCGTGGGTTACA RP: TCCGGTTCCCACAGTAACAA	AC_000078.1 from 59053356 to 59053749
MiR-212	FP: GGCACCTTGGCTCTAGACTG RP: GCCGTGACTGGAGACTGTTA	NR_031925.1
MiR-132	FP: ACCGTGGCTTTCGATTGTTA RP: GGCGACCATGGCTGTAGACT	NR_031878.1
MiR-U6	FP: CTTCGGCAGCACATATACTAAAA RP: GAATTTGCGTGTCATCCTTG	K00784.1
Dnmt3 A	FP: GCTGAAGGAGAGGGAACTGA RP: TGCCTGGAAGGTGAGTCTTG	NM_001003958
Dnmt3 B	FP: TGCGGTAAGAAGAACCCTGT RP: CTGATAGCCGTCCTCATCGT	NM_001003959
Tet1	CTGTGGGGAATGCACCTACT TGGCTTCTTTTTGAGCACCT	XM_008774952.1
Tet 2	AGAAGCGTAAGAAGCGCAGT TCTTTTTCATTTGACCGTCTCTTCC	XM_006224264.2
Tet 3	TGTGTGCAAGAGGACTTTCG TACTGACGGGTGGTTTCTCC	XM_006224966
Orx	TCCTTGGGTATTTGGACCAC CCCAGGGAACCTTTGTAGAAG	NM_013179
Orx R1	CCATCAGTGTCCTCAATGTCC AGAAGGTGAAGCAGGCGTAG	NM_013064.1
36B4	CTGCCCGAGCCGGTGCCATC TTCAATGGTACCTCTGGAGAT	NM_022402

periods (mean (FR5)) were compared to 80%, as previously described [37]. The number of NPs into the active (NPa) and the inactive hole (NPi) was evaluated with two-way ANOVA ("active/inactive" and "FR1/FR5"). The number of rewards self-delivered was assessed by one-way ANOVA with repeated measures in order to evaluate "Day" effect, as previously described [37]. In RT-qPCR and immunohistochemistry analyses, one-way ANOVA was performed to evaluate "Group" effect. Neuwman-Keuls post hoc was performed, if required. Significance was set at $p \leq 0.05$. Data are expressed as means ± S.E.M.

Results

Operant Conditioning in Cocaine and Food Pellets Self-Administering Rats

Cocaine and food self-administering rats were submitted to a FR1 schedule of reinforcement for four consecutive days, and then to a FR5 schedule for 6 days during 2-h daily sessions (Fig. 1a). During both schedules, rats self-administered about 100 cocaine injections and self-delivered about 150 food pellets per day, as described during the FR5 schedule (Fig. 1b). This performance reveals a rapid preference for the active hole, since the percentage of nose pokes in the active hole (NPa) was above 80% (Fig. 1c) for each reinforcer. In contrast, all yoked control groups displayed no preference for the active or the inactive hole in none of the sessions with a percentage of NPa of about 50% (data not shown). Only self-administering rats differentiate the hole associated with cocaine or food pellets delivery from the inactive hole and therefore fulfill behavioral acquisition rules, as previously described [20, 37]. However, despite food restriction during the operant conditioning for food self-delivery, rats self-administering cocaine made 2.5 more NPa during the time-out period associated with a stimulus light than those self-delivering food pellets (data not shown), consistent with our previous report [37]. These excessive NPa in drug seeking may result from cocaine-induced impulsive behavior, locomotor hyperactivity, and/or stereotypies, but could also represent deficits in behavioral adaptations, since cocaine decreases behavioral flexibility [39, 40].

Cocaine and Food Induce Opposite Effect on *Dnmt3a* Expression in the PFCx and the CPu

Cocaine-induced DNA methylation changes are mainly mediated by de novo *Dnmt3a* and *3b*, as previously described by their biphasic time-course regulation following chronic passive treatment or by using a self-administration paradigm [15–17]. Their expression was compared in response to cocaine and food in self-administering rats exposed to the same schedule of reinforcement. In the PFCx reward brain structure,

voluntary cocaine intake induced a 1.6-fold increase in *Dnmt3a* upon voluntary intake, whereas a 2-fold decrease was found upon passive and voluntary food intake, the latter compensating the food restriction effect (Fig. 2a). This opposite effect between cocaine and food was also observed in the CPu, but the other way around (Fig. 2b). Indeed, while *Dnmt3a* was repressed by cocaine, it was induced by food in the CPu. In contrast to the PFCx, this latter activation was

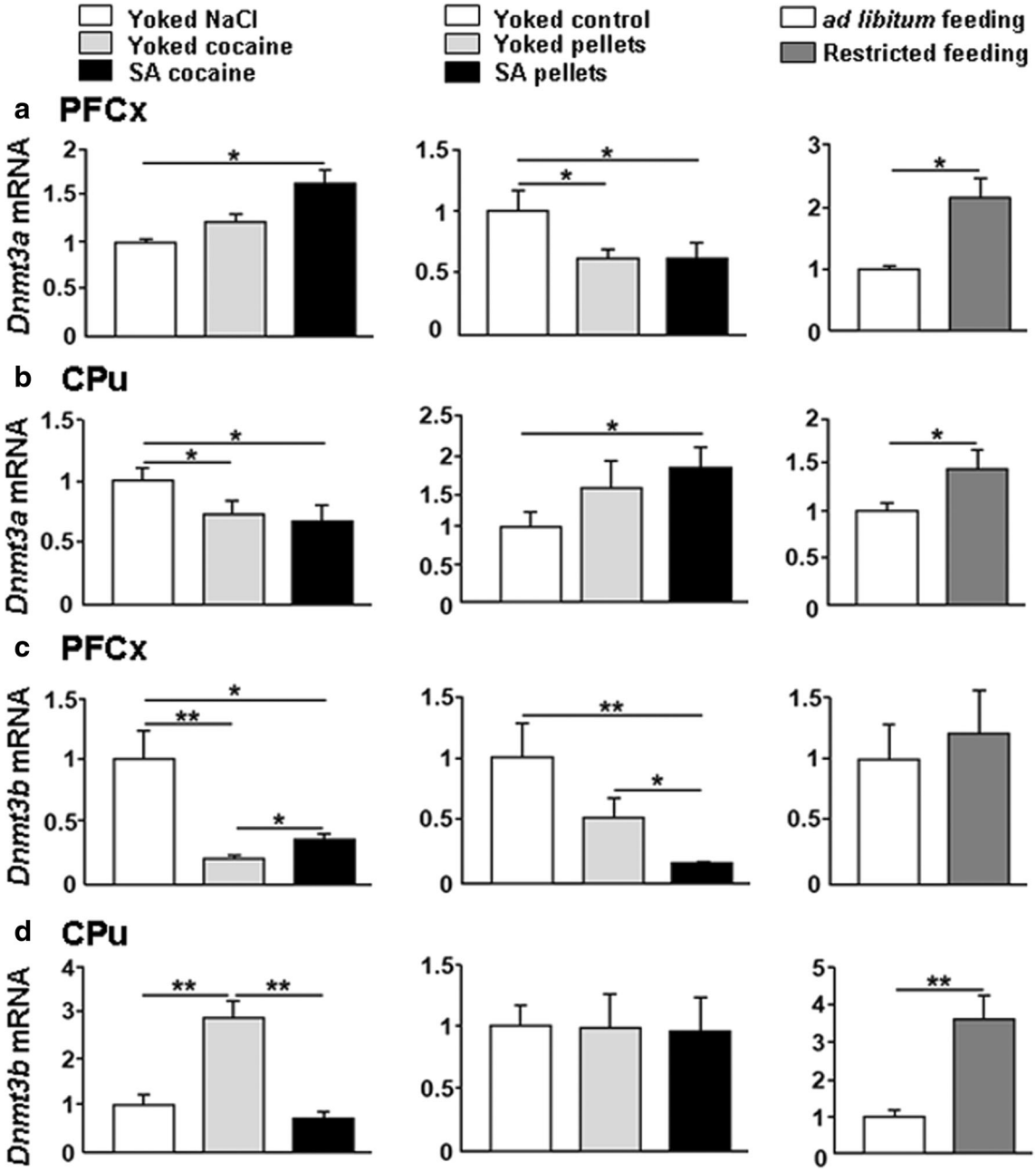

Fig. 2 *Dnmt3a* and *Dnmt3b* mRNA expression in the prefrontal cortex (PFCx) and the caudate putamen (CPu) in response to passive vs. voluntary cocaine, food pellets delivery, and food restriction. The effect of cocaine and food reward and that of operant conditioning on *Dnmt3a* (**a**, **b**) and *Dnmt3b* (**c**, **d**) levels were evaluated by quantitative RT-PCR 24 h after the beginning of the last session of cocaine and food pellets administration in the PFCx (**a**, **c**) and in the CPu (**b**, **d**). Yoked control rats from the cocaine experiments that were not food restricted were compared with those from the food pellet experiments that were food restricted to check the food restriction effect. The effect of passive cocaine or food pellets intake was investigated by comparing either "Yoked cocaine" to "Yoked control" rats or "Yoked pellets" to "Yoked control" rats, whereas the effect of voluntary intake was evaluated by comparing either "SA cocaine" to "Yoked cocaine" rats or "SA pellets" to "Yoked pellets" rats. "Yoked control" rats were compared to either "SA cocaine" or "SA pellets." The amount of *Dnmt3a* and *Dnmt3b* transcripts were normalized to that of *36B4*. Data represent the mean ± S.E.M., n = 4–6 per group (**a**, **b**, **c**, **d**). Statistical analysis performed was one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc, when required. *$p < 0.05$, **$p < 0.01$

actually exacerbating the positive effect of food restriction observed in both brain structures.

In the PFCx, a repression of *Dnmt3b* was found simultaneously in response to passive and voluntary cocaine and food intake, suggesting less de novo methylation triggered by *Dnmt3b* whose expression was not affected by food restriction (Fig. 2c). In the CPu, *Dnmt3b* was surprisingly only activated by passive cocaine intake. No change was observed upon passive or voluntary food delivery, despite a significant induction resulting from food restriction (Fig. 2d). These data highlight opposite effects of the two rewarding stimuli on *Dnmt3a* in the PFCx and the CPu, effects that do not always require learning and memory processes associated with operant conditioning. They also indicate that de novo DNA methylation patterns mediated by *Dnmt3a* are different between cocaine and food and from one brain structure to another. On the other hand, the selective repression of *Dnmt3b* in the PFCx suggests less DNA methylation of its target sequences in response to both reinforcers independently of the mode of administration.

Tet Genes Are Widely Repressed by Cocaine and Food

The ten eleven translocation (Tet) family of enzymes oxidizes 5-methylcytosine (5mC) and promotes locus-specific reversal of DNA methylation. Three Tet proteins catalyze the successive oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). The oxidation products are intermediates in the conversion of 5mC to unmodified cytosine, providing the first steps for active DNA demethylation and implying that DNA methylation patterns are not as static as previously assumed [41, 42].

In the PFCx, independently of the mode of administration, all *Tet* genes were found to be repressed by cocaine relative to saline treatment (Fig. 3a–c). The effect of food was more heterogeneous, since only *Tet3* was repressed by both passive and voluntary delivery (Fig. 3c). In contrast to cocaine, food did not affect *Tet1* mRNA levels (Fig. 3a) and *Tet2* was intriguingly selectively induced by passive food delivery without any change in self-administering rats relative to yoked control rats (Fig. 3b). No significant change in *Tet* gene expression was detected upon food restriction. Thus, *Tet1* and *Tet2* are not similarly regulated by cocaine and food (Fig. 3a, b), whereas *Tet3* is repressed by both reinforcers in the PFCx in which the reward and its associated cues are sufficient without requiring learning and memory processes (Fig. 3c). In the CPu, the three *Tet* genes were repressed by both rewarding stimuli to a similar extent by passive and voluntary intake (Fig. 3d–f). However, *Tet2* repression by food should be taken with caution, as it compensates the stimulatory effect of food restriction that was of the same range (Fig. 3e).

Taken together, our data underline a repression of *Tet* genes in most experimental conditions, suggesting that less 5mC oxidation products are generated by both a drug of abuse and a natural reinforcer. The repression would ultimately prevent DNA demethylation following the involvement of the thymine DNA glycosylase-mediated base excision DNA repair pathway involved in the final step of demethylation [43].

Selective Repression of miR-212 and miR-132 by (Passive and Voluntary) Cocaine and Food Intake in the Dorsal Striatum

miR-212 and miR-132 belong to an intronic polycistronic cluster activated by CREB and modulate dendritic plasticity by controlling MeCP2 expression, as a validated target playing a critical role in cocaine intake [44, 45]. Indeed, increased MeCP2 expression by cocaine in the dorsal striatum inhibits miR-212 expression that, in turn, represses MeCP2 expression and their homeostatic interactions have been suggested to be important in regulating vulnerability to cocaine addiction [14, 46]. In addition, an upregulation of microRNA-212 in the dorsal striatum of rats exposed to cocaine decreased operant responding during the post-infusion time-out period, thereby identifying its role in regulating compulsive-like cocaine intake [47]. We compared the expression of the primary miR-212/132 transcript (pri-miR-212/132) with that of each miRNA in brain structures of rats exposed to passive or voluntary cocaine and food intake. The gene cluster structure and sequence with the relative position of the primers used are depicted in supplementary Fig. S2. When examining the PFCx, overall, cocaine did not affect their levels relative to control saline-treated rats, except a slight increase observed for miR-212 in voluntary relative to passive administration (Fig. 4a–c). Similarly, miR-212 and miR-132 levels were not altered by food delivery, despite a significant increase of their primary transcript in response to passive food pellet delivery. This increase was of the same range as that of its decrease resulting from food restriction (Fig. 4a), showing that intriguingly only passive food delivery compensates the food restriction effect. Indeed, since food restriction was used to motivate rats in operant conditioning experiments, its effect was addressed by comparing yoked control rats from food pellet experiments under food restriction with yoked control rats from cocaine experiments that were not. Food restriction decreased pri-miR-212/132 expression, as that of miR-212 and miR-132 (Fig. 4a–c), suggesting that a transcriptional repression mediates the effect of food deprivation by altering the regulation of their target genes in the PFCx. However, their role in triggering the effect of cocaine and food intake is not likely in this brain structure.

We next examined the CPu (Fig. 4d–f) that serves as a key brain area for habits, reward association learning and compulsive drug-seeking behaviors [48, 49]. The pri-miR-212/132 levels were not altered neither by passive and voluntary cocaine and food intake, nor by food restriction (Fig. 4d), unlike in the PFCx, suggesting a lack of transcriptional effect.

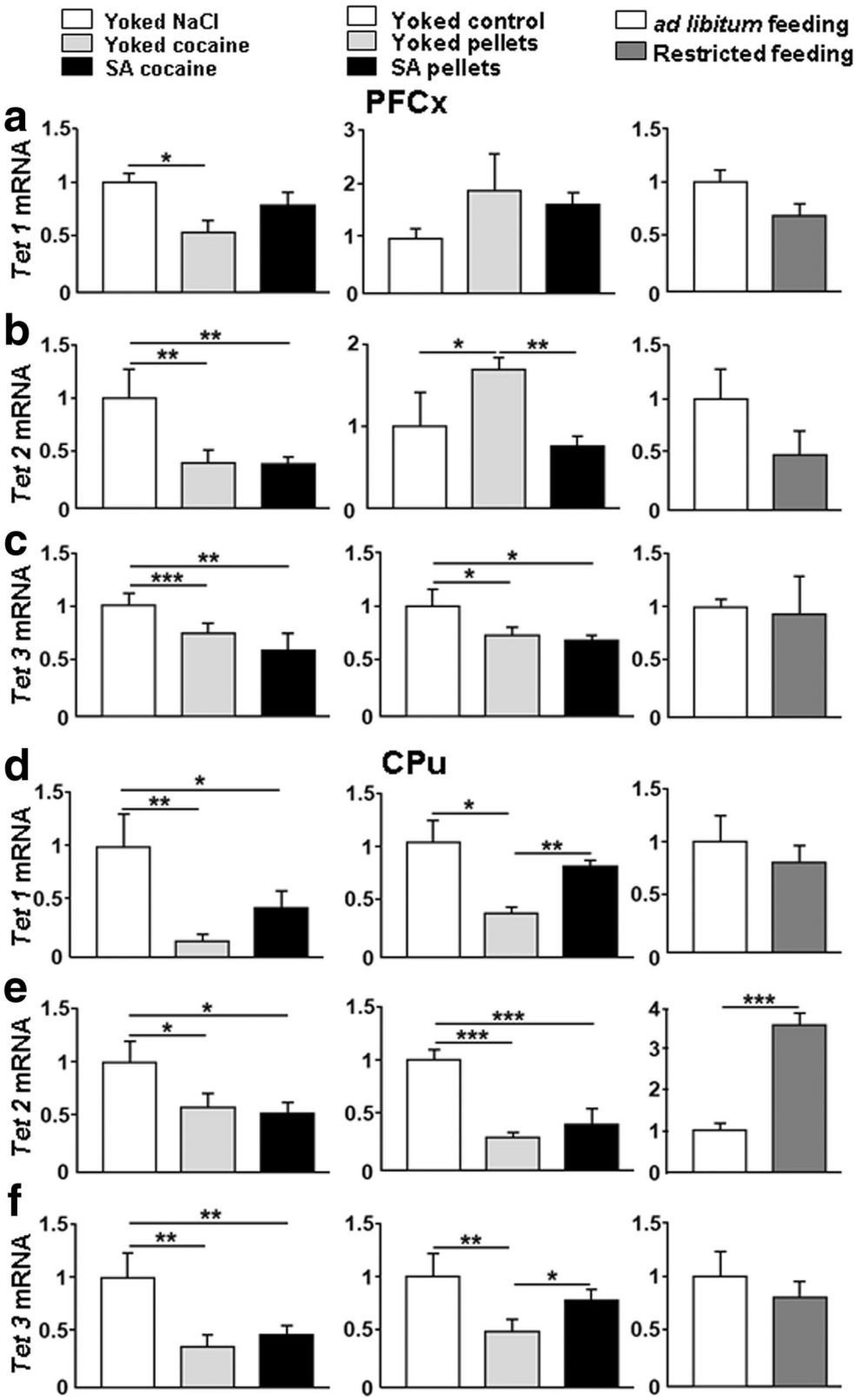

Fig. 3 *Tet1*, *Tet2*, and *Tet3* mRNA expressions in the PFCx and the CPu in response to passive vs. voluntary cocaine or food pellets delivery. The effects of cocaine and food reward and that of related operant conditioning on *Tets*' mRNA levels were evaluated by quantitative RT-PCR 24 h after the beginning of the last session of cocaine and food pellets administration in the PFCx (**a**, **b**, **c**) and in the CPu (**d**, **e**, **f**). Food restriction was measured by comparing yoked control rats from the cocaine experiments that were not food restricted with those from the food pellet experiments that were food restricted. Expression of the three genes was normalized to that of *36B4* mRNA. Data represent the mean ± S.E.M., $n = 4–6$ per group. Statistical analysis performed was one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc, when required. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

However, both miRs were strongly repressed following contingent and not contingent cocaine administration (Fig. 4e, f), consistent with increased Mecp2 expression previously reported in various experimental models [23, 50] and with its inhibitory effect on mature miR-212 expression [45]. A repression was also observed in response to food pellet

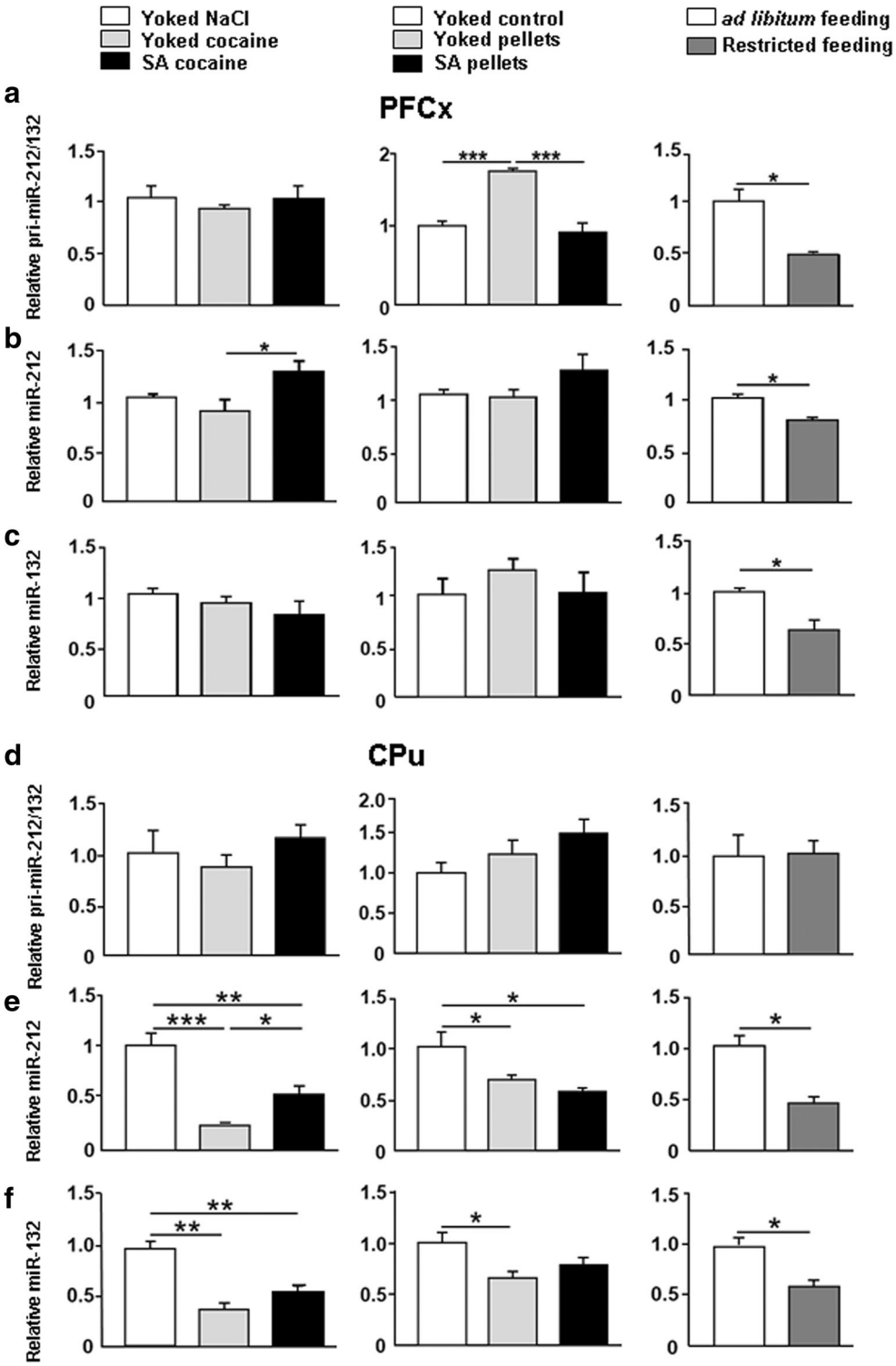

Fig. 4 Expression of primary miR-212/132 transcript (*pri-miR-212/132*) and its precursors in the prefrontal cortex (PFCx) and the caudate putamen (CPu) in response to passive vs. voluntary cocaine or food pellets delivery and to food restriction. Quantitative RT-PCR was applied to evaluate the effects of cocaine and food reward and that of operant conditioning on *pri-miR-212/132* (**a**, **d**), *miR-212* (**b**, **e**), and *miR-132* (**c**, **f**) levels, 24 h after the beginning of the last session of cocaine and food pellets administration in the PFCx (**a**, **b**, **c**) and in the CPu (**d**, **e**, **f**). Yoked NaCl rats from cocaine experiments that are not food restricted were compared to yoked controls from the food pellet experiments that were food restricted to measure the food restriction effect. The amount of *pri-miR-212/132* transcript and its precursors were normalized to that of *miR-U6* RNA. Data represent the mean ± S.E.M., n = 4–6 per group. Statistical analysis performed was one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc, when required. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

delivery 24 h after the beginning of the last session and intriguingly, providing limited amount of food, independently of the administration mode further emphasizing the food restriction effect. Nevertheless, the repression of both miRs observed in cocaine groups was of a higher magnitude than that in food pellets groups, as for the NPas during the time-out period, supporting the role of miR-212 in compulsive behavior for drug-seeking [47]. The data underline a specific repression of both miRs by cocaine and food reward with their associated cues in the CPu, a repression that does not require learning and memory processes involved in operant conditioning.

Orexin Receptor-1 Gene Methylation and Expression in PFCx

Among the orexin receptors, Orx R1 plays an important role in regulating the reinforcing and reward-enhancing properties of cocaine, as well as in the neurobiological effects driving various drugs of abuse [29–31, 33, 51, 52]. Having previously performed a genome-wide DNA methylation study in the PFCx of rats after cocaine intake [20], we know the methylation status of the genes examined here. The most prominent difference in gene methylation was found for *Orx-R1*. Indeed, hypomethylated regions were identified in the gene promoter, within the gene body and downstream of the gene with a ratio of 5mC (cocaine]/[saline] ranging from 0.5 to 0.9 (Fig. 5a). Since hypomethylation of 5mC results from enzymatic oxidation, the levels of 5-hydroxymethylcytosine (5hmC) were investigated in *Orx-R1* differentially methylated regions (DMRs) by hydroxymethylated DNA immunoprecipitation (hMeDIP). A 40% increase was found in 5hmC density within the gene, whereas no significant change was found for the Dnmt3a gene (supplementary Fig. S4c, d).

When next analyzing its expression in the PFCx (Fig. 5b), passive and voluntary cocaine intake was found to induce *Orx R1* gene expression. In contrast, no differences were observed in *Orx R1* mRNA levels neither upon food pellets intake, nor upon food restriction. In the CPu, only food restriction was affecting its levels with a 3.8-fold increase (Fig. 5c), whereas surprisingly passive and voluntary food intake remained without significant effect. Since orexins control feeding behavior/satiety, as indicated by previous studies [26], the limited amount of food provided during the daily sessions may not be sufficient to compensate the increase to palliate a body weight maintained at 85% throughout the whole experiment. Thus, decreased DNA methylation in the PFCx accompanied by an increased hydroxymethylation is in agreement with cocaine-induced mRNA expression (Fig. 5b) and the *Orx-R1* therefore appears to be regulated by cocaine at least in part through DNA methylation.

Cocaine and Food Induced Different Regulation of Prepro-Orexin mRNA and Protein-Polypeptide in the Hypothalamus

Orexin A and orexin B belong to the same prepro-orexin gene which is transcribed and translated before secretion and

a ***Orexin R1*** **gene Differentially Methylated Regions**

Position	Distance to start (bp)	Length (bp)	5mC [Coc]/[NaCl]
upstream	-9 935	1105	0.70
	-8 967	617	0.82
	-7 926	575	0.90
within gene (9.4 Kb)	6 167	580	0.58
	1 776	478	0.51
downstream	13 706	582	0.70

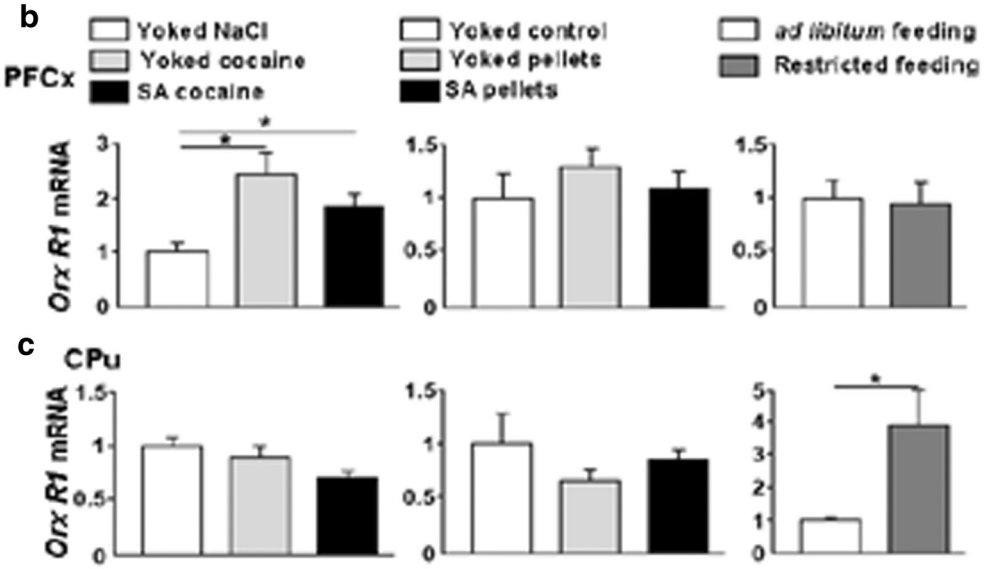

Fig. 5 *Orexin receptor 1* DNA methylation and gene expression *OrxR1* gene methylation including location and number of differentially methylated regions and their patterns in cocaine self-administering and control rats are depicted in **a**, consistent with genome-wide DNA methylation analysis. Cocaine, food, and food restriction effects on *orxR1* mRNA levels were evaluated in the PFCx (**b**) and the CPu (**c**), as indicated in previous figures. Data represent the mean ± S.E.M., $n = 4–6$ per group. Statistical analysis performed was one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc, when required. $^*p < 0.05$

cleavage producing each single peptide. Its transcription exclusively occurs in the lateral hypothalamus (LH) in orexinergic neurons that widely project throughout the brain including key structures of the limbic system which are "multi-tasking" neurons regulating functions like arousal, sleep/wake states, feeding behavior, energy homeostasis, anxiety, and addictive behaviors [26–28].

We examined expression of the prepro-orexin peptide by immunohistochemistry (Fig. 6a–c) and gene expression by RT-qPCR (Fig. 6d) in the lateral hypothalamus 24 h after the last session of cocaine and food pellet administration. Passive administration of cocaine and food did not alter the number or the intensity of immunoreactive neurons, nor orexin gene expression, since no differences were detected between "yoked control" rats and rats receiving cocaine or food passively. However, voluntary administration of cocaine and food triggered striking different responses. Indeed, cocaine self-administration resulted in a significant increase in the number of orexin immunoreactive cells (Fig. 6 a, c) and was found to slightly increase prepro-orexin gene expression without

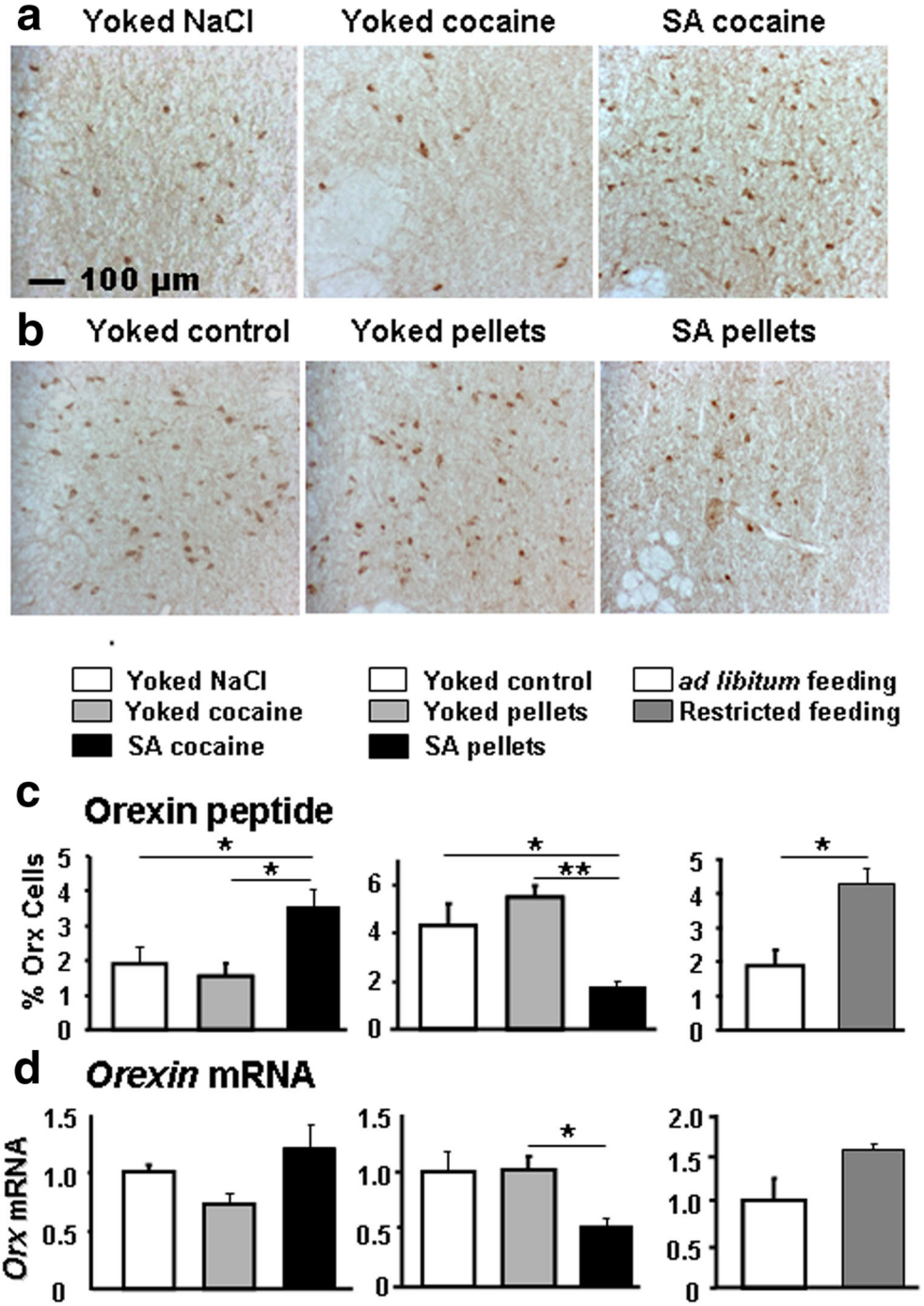

Fig. 6 Passive and voluntary cocaine or food pellets delivery effect on orexin mRNA and peptide expression in the lateral hypothalamus. The effect of cocaine and food pellets on orexin peptide expression was evaluated by immunohistochemistry (a, b, c) and that of its mRNA was evaluated by RT-qPCR (**d**) in the LH. Representative immunostainings are shown for cocaine (**a**) and food pellets (**b**) experiments. The level of positive cells has been evaluated relative to nuclear Hoechst staining and the negative controls without primary antibody did not exhibit any significant signal. Food restriction effect (**c**, **d**) and normalization of mRNA levels (**d**) were measured, as in previous figures. Data represent the mean ± S.E.M., $n = 4–6$ per group. Statistical analysis performed was one-way ANOVA followed by Newman-Keuls post hoc, when required. $*p < 0.05$, $**p < 0.01$. Immunohistochemistry of ORX in the LH (**c**, **d**), scale bar applicable to all micrographs, 100 µM

reaching statistical significance relative to the two other controls (Fig. 6d). This increase appears consistent with the role of orexin in the control of arousal [28, 53]. In contrast, food self-delivery resulted in a significant decrease of the number of orexin positive cells (Fig. 6 b, c) and in gene expression (Fig. 6d), compared with the two other control groups. The repression, solely observed following contingent food pellet delivery, appeared to compensate for the induction of peptide and gene expression that result from the food restriction effect (Fig. 6c, d). Thus, orexin induction by cocaine self-administration and repression by food restriction underline a marked divergence between the two reinforcers.

Detection of Orexin mRNA Controlled by Cocaine and Food in the CPu

Although orexins are known to be exclusively transcribed in neurons within the lateral hypothalamus [54], we nevertheless checked their mRNA levels in the CPu and PFCx. Surprisingly, *orx* mRNA was detected in the CPu, but not in the PFCx (Fig. 7a). The detection of orexin mRNA in the CPu was confirmed with a similar extent relative to HP in additional experiments performed with home cage rats of similar age (data not shown). Considering that orexin-expressing neurons project over virtually the entire brain and spinal cord, this result suggests an unexpected mRNA transport from the hypothalamus to the CPu. However, the peptide was detected neither in the CPu, nor in other areas including the nucleus accumbens (data not shown), while a strong staining was observed in cell bodies in the HP (Fig. 6a, b). The absence of staining is probably due to the limited amount of peptide present in axons or axon terminals of these structures, which may not be sufficient to be detected by conventional immunohistochemistry. No significant changes were detected in the CPu upon cocaine administration (Fig. 7b). Nevertheless, orx mRNA levels were significantly downregulated by passive and voluntary food intake (Fig. 7c), as well as by food restriction (Fig. 7d). This modulation appears consistent with that observed in food SA-rats in the LH in food restricted rats (Fig. 6), underlining a correlation between orx mRNA levels in the CPu and those observed in the hypothalamus where they are synthesized.

Discussion

In the present study, we demonstrated that at the molecular level neural circuits activated by drugs of abuse and natural reinforcers do only partially overlap, thus supporting previous suggestions based on electrophysiological and functional magnetic resonance imaging (fMRI) studies previously suggested. This is illustrated by striking changes in the expression of genes controlling DNA methylation/demethylation processes as well as in orexins and orx-R1 involved in the control of satiety or appetite signals. These behavior-dependent changes were observed in response to both reinforcers, but differed depending on passive or voluntary intake, feeding conditions, and from one brain structure to another.

When comparing *Dnmt3* expression in response to cocaine and food under the same schedule of reinforcement, the most prominent difference was observed for *Dnmt3a*. Opposite responses to cocaine and food passive and voluntary intakes were observed in both brain structures, despite a stimulatory effect of food restriction (Fig. 3a, b). In addition, our data highlight its tissue-specific regulation, since *Dnmt3a* was repressed and activated in response to each rewarding agent. In contrast, *Dnmt3b* levels were similarly repressed by both reinforcers in the PFCx independently of the administration mode (Fig. 3c), while voluntary intake of both did not lead to significant changes in the CPu, a key brain structure altered in cocaine-addicted humans involved in reward association learning [48, 49]. Taken together with other available data, our results demonstrate that Dnmt3a represents the major Dnmt differentially regulated by cocaine and food. Overall, DNA methylation patterns triggered by a drug of abuse and a natural reinforcer differ within brain structures. Identification of its targets sequences may certainly shed light into the mechanism by which DNA methylation contributes to addictive

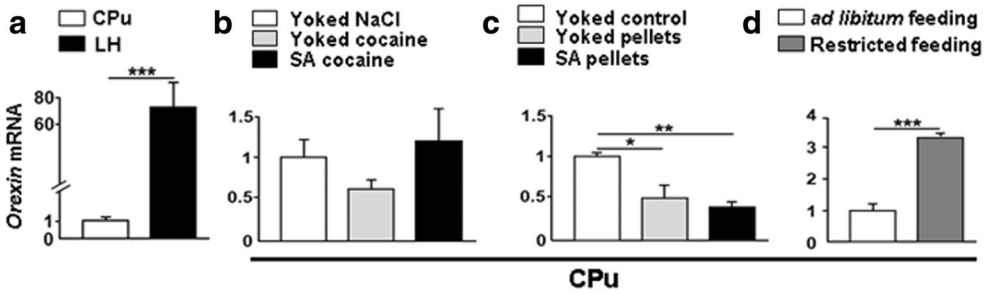

Fig. 7 *Orexin* mRNA expression in the CPu and the LH. *Orx* mRNAs in the CPu and the LH were analyzed by RT-qPCR by comparing their expression in yoked NaCl/control rats between both brain structures (**a**). In the CPu, their expression was evaluated in cocaine (**b**), food pellets (**c**), and food restriction experiments (**d**). Data represent the mean ± S.E.M., $n = 4–6$ per group. Statistical analysis performed was one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc, when required. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

behaviors. Among Dnmts, Dnmt1 is essential for maintaining DNA methylation patterns and Dnmt3a and Dnmt3b are required for de novo methylation [55]; it is noteworthy that these enzymes have overlapping and different target genes and functions [56, 57]. Moreover, a pharmacological agent like cocaine induces DNA methylation and gene repression by a Mecp2-mediated mechanism that was initially reported for the *Cdkl5* [18] and the *PP1c* [15] genes. In the mean time, the effect of inhibition of DNA methylation in drug-induced learning and memory was also described [58]. Consistent with these studies, de novo Dnmt3a and Dnmt3b enzymes were found to be modulated by repeated cocaine exposure with a daily biphasic expression [15–17]. Cocaine-induced brain DNA methylation changes were thereafter documented for several genes and by genome-wide studies, suggesting that these dynamic changes are important in cocaine-induced behavior [11, 23, 50]. Interestingly, a relationship between feeding behavior or obesity and DNA methylation has been documented as well: (i) Mecp2 deletion in the hypothalamus alters social and feeding behavior [59]; (ii) Mecp2 mutation disrupts body weight balance in Rett patients [60], while in mutant mice, it alters leptin-signaling components regulating Pomc and Agrp expression which are essential signals for satiety and appetite [61]; (iii) high fat or methyl donor supplementation in early life alters DNA methylation in the brain [62]; (iv) maternal methyl donor-deficient diet causes a reduction of the body weight and length of embryos during the gestational period [63] and reduces global brain DNA methylation [64]. These studies support the concept that DNA methylation provides a strong mechanistic link between environment, nutrition, and diseases, including addictive behaviors [65]. In addition, the link between DNA methylation and food reward was illustrated by studies having demonstrated that (i) human males with hypomorphic mutations overexpressing the MeCP2 or females with milder variants of Rett syndrome often become obese, as in some Mecp2 mutant mice [59] and (ii) Mecp2 expression was increased in the CPu upon food restriction [37].

Similarly to Dnmts, Tet proteins may also display tissue-specific biological functions and recognize common and specific 5mC target sequences [42, 66]. Since all of them are repressed by cocaine and food in the CPu, less 5mC oxidation products should be generated in this brain structure. Hence, less cytosine demethylation in response to both reinforcers is expected. Changes in the relative levels of intermediate oxidation products (5hmC, 5fC, 5caC) may lead to altered gene expression. In the PFCx, all *Tet* genes were repressed by cocaine, as in the CPu (Fig. 4a–c). Interestingly, three DMRs were identified within the *Tet1* gene in the PFCx and all were hypermethylated with a ratio of 5mC (cocaine/saline) ranging from 1.3 to 2.7 (supplementary table), indicating that as in promoter sequences, 5mC located within the gene correlates with repression by cocaine. In contrast, food only repressed *Tet3* without altering *Tet1* and *Tet2* mRNA levels under contingent delivery, suggesting no change in the oxidation of 5mC triggered by the two family members (Fig. 4a–c). The data are again consistent with non-overlapping neural circuits activated by a drug of abuse and a natural reinforcer and underline brain structure specific regulations of *Tet* genes.

The regulation of de novo *Dnmta* and *Tet* genes by cocaine and food appears quite complex, the food data being sometimes biased by the food restriction that is required to train rats in the food self-delivery experiments [37]. Considering this limitation, data interpretation for food delivery must be taken with caution. Nevertheless, genome-wide methylation patterns in response to cocaine in the whole brain, the PFCx, or the NAc have been reported. No or little changes have been detected in global DNA methylation [8, 9, 19, 20, 67, 68], the pattern of which being not random, but tightly regulated in a tissue-specific manner [69]. Our data further support these previous studies, since no significant changes in 5mC were found in response to cocaine, neither by analyzing 188,926 DMRs identified in genome-wide DNA methylation analysis (supplementary Fig. S3), nor by analyzing global 5mC by methylated DNA immunoprecipitation (supplementary Fig. S4a, b). Hence, the striking changes in the expression of major factors controlling this process, including Dnmts with specific target sequences [56, 57], indicate that cocaine and food-induced changes in DNA methylation modifications differ, but mainly affect the distribution of modified cytosines, rather than their whole amount throughout the brain genome, as previously suggested for cocaine [23].

Previous findings have shown that in the dorsal striatum, Mecp2 acts as a pro-addiction transcriptional repressor that, by attenuating miR-212 expression in response to cocaine increases vulnerability to addiction [45]. miR-212 was also proposed to play an important role in regulating compulsive-like cocaine intake [47], a proposal that is further supported by its stronger repression by cocaine than by food administration associated with more NPas during the time-out period. We show here that miR-132 is also repressed by cocaine and food in the CPu, independently of the administration mode. This is consistent with the hypothesis that neural circuits activated by drugs of abuse and natural reinforcers overlap in the CPu. Moreover, both miRs belong to the same polycistronic cluster located in a CpG enriched region with conserved regulatory elements activated by CREB (Fig. S2), the activity of which affects drug rewards as well as preference for natural reinforcers [70]. Hence, their common transcriptional regulation may appear likely. Indeed, their conserved upstream region could be methylated and recognized by cocaine-induced Mecp2 [23, 50]. In addition, Mecp2 could act as a transcriptional repressor in agreement with its negative homeostatic relationship with both miRs [71–73]. Finally, knock down of Mecp2 increases mature miR-212 and miR-132 expressions in cultured cells [74] underlining its role as a transcriptional

repressor. However, despite a striking inhibition of both pre-miRs expression in response to cocaine and food, no change was observed in the primary miR-212/132 transcript levels (Fig. 2d). Although this repression is consistent with a positive correlation between mature and pre-miR expression [75, 76], a transcriptional regulation of the pri-miR is not likely, unlike a previous suggestion [45]. MiRs are also regulated at the level of processing in which pri-miRs are cleaved by the Microprocessor complex including DGCR8 and Drosha into pre-miRNA and exported from the nucleus to the cytoplasm. Interestingly, inconsistencies between levels of pri-miR and of their derived precursor and mature forms have been reported to be more obvious for so-called polycistronic or miR clusters, indicating a miR-specific regulation [77]. Hence, we propose that Mecp2 mediates cocaine and food effects by modulating the processing of the polycistronic pri-miR-212/132 cluster into pre- and mature forms, in agreement with studies having reported that Mecp2 regulates RNA splicing [78] and suppresses nuclear pri-miR processing by regulating the DGCR8/Drosha complex [79].

Interestingly, the *Orx R1* gene was found to be hypomethylated within six DMRs identified in the gene promoter, within the gene or downstream in the PFCx of cocaine self-administering rats, indicating DNA methylation changes, as previously reported for *Orx* and *orx R2* genes [80–82]. Gene hypomethylation was associated with its overexpression that is likely to contribute to addictive-like behavior, since orexin receptor antagonists attenuate motivational and hedonic properties of cocaine [34]. By looking at the role of orexins involved in feeding and addictive behaviors, their expression was found to be induced in cocaine self-administering rats and repressed in food pellet self-delivering rats (Fig. 6). The repression observed following voluntary food intake was actually only compensating the known food restriction effect to reach levels that are similar as those observed in control rats having ad libitum access to food. Nevertheless, despite this compensation, the activation in response to cocaine highlights a marked divergence between the two reinforcer effects. That orexin levels were not affected by passive cocaine and food intake indicates the requirement of learning and memory processes involved in operant conditioning.

The presence of orx mRNA in the CPu is surprising and suggests an axonal transport from LH neurons cell bodies to distal sites. Delivery and mRNAs translation in axons or dendrites have been documented [83–85]. Its level is much lower than in the hypothalamus (Fig. 7a) which appears consistent with a low density of orexin-containing fibers reported in the anterior CPu [86]. The mechanism initiating orx mRNA transport and local translation requires further investigation. Alternatively, a contamination from an adjacent tissue such as the nucleus accumbens cannot be ruled out completely, but it would still mean a distal site from the HP, which is still recognized to be the exclusive site for orexins to be transcribed and synthesized. Since both orexin A and orexin B peptides are present in the striatum, as in many brain structures of the mesolimbic dopamine system, they may result from peptide transport and/or local translation of mRNA initially transcribed in the hypothalamus. On one hand, the transport of mRNA encoding orx A and orx B prior to a local translation may require less energy, compared with transport of both peptides. It may represent an additional control of orx peptide levels at sites distal from their transcription site. Whether orx mRNA that was not detected in the PFCx is present in other brain structures expressing both receptors remains to be determined. On the other hand, thorough in situ hybridization analyses of orexin receptors in the whole brain have shown that the non-selective Orx R2 was not detected in the PFCx [87], although present in various layers of the neocortex, in the piriform cortex, or in the bed nucleus of the stria terminalis [27]. Hence, axonal transport of both the polypeptide and its mRNA to the PFCx may not be required.

In summary, the present data demonstrate that DNA methylation factors and the orexinergic system are not similarly regulated by a drug of abuse and a natural reinforcer, providing new insight into the mechanism by which each factor triggers reward. They well support at the molecular level the concept that neural circuits activated by either of them only partially overlap, a conclusion previously reported based on electrophysiological and functional magnetic resonance imaging studies. De novo changes in gene DNA methylation including various 5mC oxidative products resulting from each stimulus are therefore likely to be different in the brain. Whether and how each of these products contributes to long-lasting behaviors requires further investigation. Elucidation of the molecular mechanisms dissociating these reinforcers is required prior to conceive novel diagnostic and therapeutic tools for addictive disorders.

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Compliance with Ethical Standards

All procedures involving animal care were conducted in compliance with national laws and policies (Council directive 87848, 1987, Service Vétérinaire de la Santé et de la Protection animale, permission 67-165 to J.Z. and 67-370 to P.R.), with the Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche (project permission number *APAFIS#2133-20151 00221 087072* to P.A.) and international guidelines (NIH publication 5586-23, 1985).

Conflict of Interest The authors declare that they have no conflict of interest.

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