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Impact of aflatoxin B1 on hypothalamic neuropeptides regulating feeding behavior

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ABSTRACT

The presence of mycotoxins in food is a major problem of public health as they produce immunosuppressive, hepatotoxic and neurotoxic effects. Mycotoxins also induce mutagenic and carcinogenic effects after long exposure. Among mycotoxins that contaminate food are aflatoxins (AF) such as AFB1, which is the most powerful natural carcinogen. The AF poisoning results in symptoms of depression, anorexia, diarrhea, jaundice or anemia that can lead to death, but very few studies have explored the impact of AF on neuroendocrine regulations. To better understand the neurotoxic effects of AF related to anorexia, we explored in rat the impact of AFB1 on the major hypothalamic neuropeptides regulating feeding behavior, either orexigenic (NPY, Orexin, AgRP, MCH) or anorexigenic (α-MSH, CART, TRH). We also studied the effect of AFB1 on a novel neuropeptide, the secretogranin II (SgII)-derived peptide EM66, which has recently been linked to the control of food intake. For this, adult male rats were orally treated twice a week for 5 weeks with a low dose (150 μg/kg) or a high dose (300 μg/kg) of AFB1 dissolved in corn oil. Repeated exposure to AFB1 resulted in reduced body weight gain, which was highly significant for the high dose of AF. Immunocytochemical and quantitative PCR experiments revealed a dose-related decrease in the expression of all the hypothalamic neuropeptides studied in response to AFB1. Such orexigenic and anorexigenic alterations may underlie appetite disorders as they are correlated to a dose-dependent decrease in body weight gain of treated rats as compared to controls. We also found a decrease in the number of EM66-containing neurons in the arcuate nucleus of AFB1-treated animals, which was associated with a lower expression of its precursor SgII. These findings show for the first time that repeated consumption of AFB1 disrupts the hypothalamic regulation of neuropeptides involved in feeding behavior, which may contribute to the lower body weight gain associated to AF exposure.

1. Introduction

Mycotoxins are secondary metabolites produced by various groups of fungi that contaminate agricultural crops prior to harvest or during post-harvest storage, mostly observed in the hot and humid climate regions (Ciegler et al., 1981; Williams et al., 2004). The presence of mycotoxins in food has serious implications for human and animal health. To date, there are many reports of disorders caused by the effects of mycotoxins on the digestive, urinary, immune and reproductive systems (Elis and DiPaolo, 1967; Goerttler et al., 1980; Grice et al., 1973).

Among the well-known mycotoxins, aflatoxins (AF) are a group of hepatocarcinogenic agents primarily produced by *Aspergillus flavus* parasiticus (Alpert et al., 1971; Newberne and Butler, 1969). According to the International Agency for Research on Cancer, aflatoxins are classified as class I human carcinogens (Aquino et al., 2010; Herrold, 1969; Newberne and Butler, 1969). Among the 18 different types of aflatoxins identified so far, aflatoxin B1 (AFB1) is the most prevalent form and also the most potent genotoxic and hepatocarcinogenic agent of all mycotoxins (Barros et al., 1976). AFB1 is well known for its carcinogenic, mutagenic, teratogenic (Wu et al., 2009) and immunosuppressive effects (Giambrone et al.,

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1985; Hatori et al., 1991; Reddy et al., 1987), exerted in various organs such as liver (Lai et al., 2014), lungs (Liu et al., 2015), gastrointestinal tract (Gursoy et al., 2008), kidney (Gupta and Sharma, 2011), heart (Wangikar et al., 2005), nervous system (Ahmed and Singh, 1984; Coulombe and Sharma, 1985; Ikegwuonu, 1983; Jayasekara et al., 1989), and different endocrine glands (Goerttler et al., 1980). AFB1 exerts its biological effects after metabolic activation by cytochrome P-450-dependent monooxygenase to its reactive form, *i.e.* AFB1-8, 9-epoxide (AFBO), which then interacts with cellular macromolecules, particularly DNA (Essigmann et al., 1982; Swenson et al., 1977), to exert its gonotoxic and carcinogenic effects. Chronic forms of aflatoxicosis result in decreased performance and body weight gain, and growth stunting in farm animals (Robens et al., 1992). Acute or chronic aflatoxicosis in chicken results in decreased meat/egg production and growth rates, negative fee conversions, immunosuppression and increased sensitivity to different diseases (Hussain et al., 2010; Tedesco et al., 2004), which can cause death (Ahmed and Singh, 1984; Ikegwuonu, 1983).

Very few studies have explored the impact of AFB1 exposure on endocrine, neuroendocrine and neuronal regulations. Exposure of rats to AFB1 causes significant alterations in biochemical parameters, which are indicators of neurotoxicity, suggesting a possible degeneration in the central and peripheral nervous systems (Ikegwuonu, 1983). At the behavioral level, it has been proposed that AFB1 intoxication induces appetite disorders that can lead to anorexia and weight loss (Bruchim et al., 2012; Dereszynski et al., 2008). However, the hypothalamic processes at the origin of impaired appetite are not known. Consequently, the effects of AFB1 on the hypothalamic neuropeptides that regulate food intake in the hypothalamus deserve investigation.

Control of food intake is an integrated function that contributes to energy homeostasis and involves nervous, endocrine and neuroendocrine mechanisms. The hypothalamus is an integrative center that controls several functions, including autonomic, neuroendocrine and behavioral regulations, allowing the maintenance of homeostasis. It fulfills this role *via* neuronal and neuroendocrine circuitries involving several bioactive neuropeptides. More specifically, the arcuate nucleus (Arc) of the hypothalamus plays a key role in regulating food intake and energy homeostasis (Schwartz et al., 2000). This structure contains notably an anorexigenic neuronal population synthesizing the proopiomelanocortin (POMC)-derived peptide, α-melanocyte-stimulating hormone (α-MSH) (Cyr et al., 2013; Khachaturian, 1985; Mezey et al., 1985) and the cocaine and amphetamine-regulated transcript (CART) (Stanley et al., 2001), and an orexigenic neuronal system synthesizing neuropeptide Y (NPY) (Cyr et al., 2013; El Ouezzani et al., 2001) and Agouti gene-related peptide (AgRP) (Small et al., 2001). In this regard, our previous data among others demonstrate that various neuropeptides (orexigenic/anorexigenic) contribute to regulate feeding behavior and energy balance (Chartrel et al., 2011; El Ouezzani et al., 2005; Lectez et al., 2009) and any imbalance affecting the expression of these neuropeptides alters energy and weight homeostasis. The lateral hypothalamic area (LH) is another hypothalamic structure well known to be involved in the control of feeding behavior as it expresses major orexigenic neuropeptides such as orexin (de Lecea et al., 1998; Sakurai et al., 1998a,b), MCH (Skofitsch et al., 1985) and 26RFa (Chartrel et al., 2003). The possible alteration of appetite regulation by AFB1 may involve classical appetite-regulating neuropeptides, as well as novel hypothalamic neuropeptides such as the secretogranin II-derived (SgII) peptide EM66. The EM66 peptide is strongly expressed in the arcuate nucleus (Boutahricht et al., 2007, 2005; El Yamani et al., 2010, 2013), and it was proposed that this peptide could be involved in the control of food intake.

The aim of the present study was to explore a possible alteration of hypothalamic neuropeptides involved in the control of feeding behavior following AFB1 ingestion in rat, in order to elucidate the central mechanisms of appetite disorders that can be induced by AFB1. In this context, the hypothalamic expression of NPY, α-MSH, EM66 (within the arcuate nucleus) and orexin (within the lateral hypothalamus) was investigated by immunohistochemistry and quantitative PCR (qPCR) in control and AFB1-treated rats. The study was extended to other hypothalamic neuropeptides regulating food intake such as AgRP, MCH, CART and TRH whose expression was explored by qPCR following AFB1 exposure.

2. Materials and methods

2.1. Animals and treatment

Adult Male Wistar rats initially weighing 240–300 g were randomized and housed singly in a room maintained at 22 ± 1 °C with a 12-h light/12-h dark schedule, with free access to food and water throughout the duration of the study. All animals were acclimated to the animal care facility 10 days prior to the beginning of the study. Rats were treated twice a week for 5 weeks with AFB1 (from *Aspergillus flavus*, Sigma–Aldrich, Lyon, France) at a low dose (150 μg/kg, cumulative dose 1.5 mg/kg) or a high dose (300 μg/kg, cumulative dose 3 mg/kg). AFB1 was dissolved in 5% ethanol and 95% corn oil and administered by oral gavages. The gavages volume did not exceed 0.5 ml to avoid any activation of the stress response. The mode of aflatoxin administration usually used is oral, with corn oil as a vehicle, which is one of the most used vehicles in toxicology studies. Control animals were treated similarly with 0.5 ml corn oil alone. All the animals were weighed every week during the five-week AFB1 exposure, and the body weight gain was calculated. One week after the last gavage, the animals devoted to immunohistochemistry ($n = 3$ for each group) were anesthetized and sacrificed by intracardiac perfusion 24 h after intracerebroventricular (i.c.v) injection of 100 μg/10 μl volume of colchicine (necessary to visualize NPY and EM66-immunoreactive cell bodies). The rats destined for quantitative polymerase chain reaction (q-PCR) ($n = 5$ for each group) were decapitated, and the hypothalamic brain region was quickly dissected, and kept at −80 °C until use for q-PCR. All animal manipulations were performed according to the recommendations of the Local Ethical Committee. Three experimental animal groups were constituted, *i.e.* one control group and two AFB1-treated groups (with either a low or a high dose of AFB1).

2.2. Tissue processing

The expression of NPY, α-MSH, orexin, and EM66 within the hypothalamic cell bodies and fibers was studied by immunohistochemistry using rabbit polyclonal antibodies raised against NPY (Bakkali-Kassemi et al., 2011), orexin A (Alexandre et al., 2014), α-MSH (Tramu and Dubois, 1977) and EM66 (Montero-Hadjadje et al., 2003). The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (35 mg/kg) and perfused through the aorta with 50 ml of a saline solution followed by 300 ml of a fixative solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were dissected, cut into 5-mm thick slabs and post-fixed for 48 h at 4 °C with the same fixative. Brain sections (50 μm) at the hypothalamic level were cut on a vibratome (Electron Microscopy Sciences 5000) and rinsed with phosphate-buffer saline (PBS) solution.

2.3. Immunohistochemistry

The tissue sections were rinsed several times in PBS and processed for indirect immunohistochemistry. Tissue slices were

Table 1
Primers for q-PCR analysis.

Gene	Forward primer	Reverse primer
GAPDH	ACTCCACTCACGGCAAATTCA	TCTCGCTTCTGGAAGATGGT
NPY	TCCGCTCTGCGACACTACAT	TGCTTTCCTTCATTAAGAGGTCTG
POMC	ACCGCAGAAAGATCGGTTGT	GGCAGACCGTGAGTTACGAG
SgII	AAGACGAGTGGATGCGGATAA	GCAGATGGCGGCTCATTT
Orexin	TTCTACAAAGGTTCCCTGGGC	AGTTCGTAGAGACGGCAGGA
CART	GGCGCTGTGTTGCAGATTG	CAGCGTCACACATGGGGACTT
AgRP	GCTTTGGCAGAGGTGCTAGA	TTGAAGAAGCGGCAGTAGCA
MCH	ACATTCAGGATGGGGAAAGCC	TGGAGCCTGTGTTCTTTGTGG

incubated overnight at 4 °C with the primary NPY, α-MSH, orexin A or EM66 antisera, diluted 1/2000 in PBS containing 0.3% Triton X-100. The sections were rinsed in PBS for 30 min and incubated for 2 h at room temperature with a biotinylated goat anti-rabbit antiserum (Vector Laboratories, Paris, France) diluted 1:400. Finally, brain sections were incubated for 2 h at room temperature with standard avidin–biotin peroxidase (Vector Laboratories) diluted 1:400 in PBS. The peroxidase activity was revealed according to the method of Shu et al. (1988) using diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich) as chromogen, and intensified with nickel ammonium sulfate (Sigma–Aldrich). Rinsing the sections in PB stopped the reaction. Tissue sections were placed on gelatin-coated slides, air-dried, dehydrated in graded alcohol and mounted in Eukit (O. Kindler GmbH & Co, Freiburg, Germany). The sections were examined under a Nikon microscope (Tokyo, Japan).

2.4. RNA extraction, reverse transcription and qPCR

Total RNA from hypothalamic tissue was extracted with the Tri-reagent (Sigma–Aldrich), purified by using a Nucleospin kit (Macherey–Nagel, Hoerdt, France), and quantified with a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, DE). Contaminated genomic DNA was removed by treatment with deoxyribonuclease I, and cDNAs were synthesized from approximately 1 μg RNA using the ImProm II Reverse Transcriptase (Promega Corp., Madison, WI). Quantitative PCR was performed by using the 7900 HT Fast Real-time PCR System and Gene Expression Master Mix 2X assay (Applied Biosystems, Courtaboeuf, France). Primers for genes examined using real time PCR are described in Table 1. The fold change in the expression of each target mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was calculated by the comparative threshold (Ct) method and expressed by means of the $2^{-\Delta\Delta Ct}$ method according to Applied Biosystems instructions using GAPDH as an internal control.

2.5. Statistical analysis

Quantification of NPY, α-MSH or EM66-immunoreactive cell bodies within the Arc and orexin-immunopositive neurons in the LH of control and AFB1-treated rats was performed on four sections per animal ($n = 3$ animals/group) and an average value was calculated for each animal. The results are expressed as mean ± SEM. Analysis of statistical significance was performed using one-way ANOVA followed by a Bonferroni *post hoc* test for the immunohistochemical data and the Mann–Whitney *U* test for the qPCR data.

3. Results

3.1. Effect of a five-week AFB1 exposure on body weight gain

Oral administration of AFB1 induced a dose-dependent decrease in body weight gain (Fig. 1). The more pronounced effect of AFB1 was observed for a dose of 3 mg/kg. At this dose, a significant decrease in body weight gain was observed as soon as the first week of treatment (Fig. 1). Then, decreased body weight gain became more highly significant from the second to the fifth week of AFB1 ingestion (Fig. 1). Rats treated with the low dose of AFB1 (1.5 mg/kg) displayed a slight, but significant, decrease of body weight gain that occurred only at the fourth and fifth weeks of AFB1 treatment as compared to the control group (Fig. 1). Of note, only the animals treated with the highest dose of AFB1 displayed a noticeable behavioral effect of weakness during the experimental process.

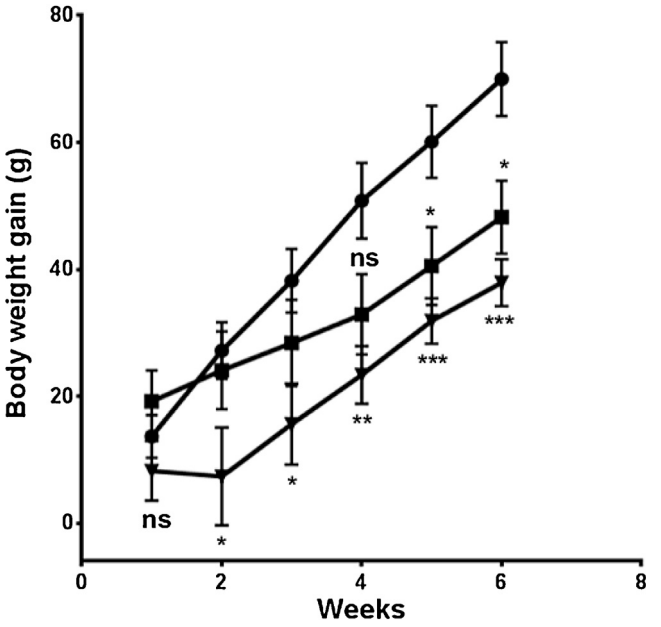

Fig. 1. Effect of a five-week aflatoxin B1 (AFB1) exposure on body weight gain in rat. The closed circles, closed squares and closed triangles represent control animals, rats treated with a low dose of AFB1 (1.5 mg/kg) and rats treated with a high dose of AFB1 (3 mg/kg), respectively. AFB1 at the two doses used provoked a dose-dependent decrease of the body weight gain in comparison to controls. The more pronounced effect is observed for a high dose of AFB1 (3 mg/kg) as this group displayed a significant decrease in body weight gain after the first week of AFB1 administration. The animals treated with the low dose of AFB1 (1.5 mg/kg) displayed a moderate but significant decrease of body weight gain after 4 weeks of AFB1 treatment as compared to the control group. Values represent mean ± SEM of 10 animals. ns, not significant, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3.2. Effect of a five-week AFB1 exposure on the expression of hypothalamic neuropeptides involved in the control of feeding behavior

Immunohistochemical analysis performed after a five-week AFB1 treatment revealed an apparent decrease in the immunoreactivity of several hypothalamic neuropeptides involved in the regulation of food intake (Figs. 2–5). Thus, the density of NPY immunoreactivity in the Arc was reduced in a dose-dependent manner in AFB1-treated rats (Fig. 2B and C) as compared to controls (Fig. 2A). Indeed, quantitative analysis of Arc slices revealed a significant and dose-dependent decrease in the number of NPY-immunoreactive (IR) cell bodies following the two distinct AFB1 treatments as compared to the control group (Fig. 2D). The present immunocytochemical data revealed also a significant decrease in α-MSH immuoreactivity in the Arc following the administration of AFB1 at low and high doses (Fig. 3B and C) as compared to untreated animals (Fig. 3A). Consistent with this finding, quantitative analysis revealed a dose-dependent decrease in the number of α-MSH-IR cell bodies in Arc slices (Fig. 3D). Similar observations were done for hypothalamic expression of

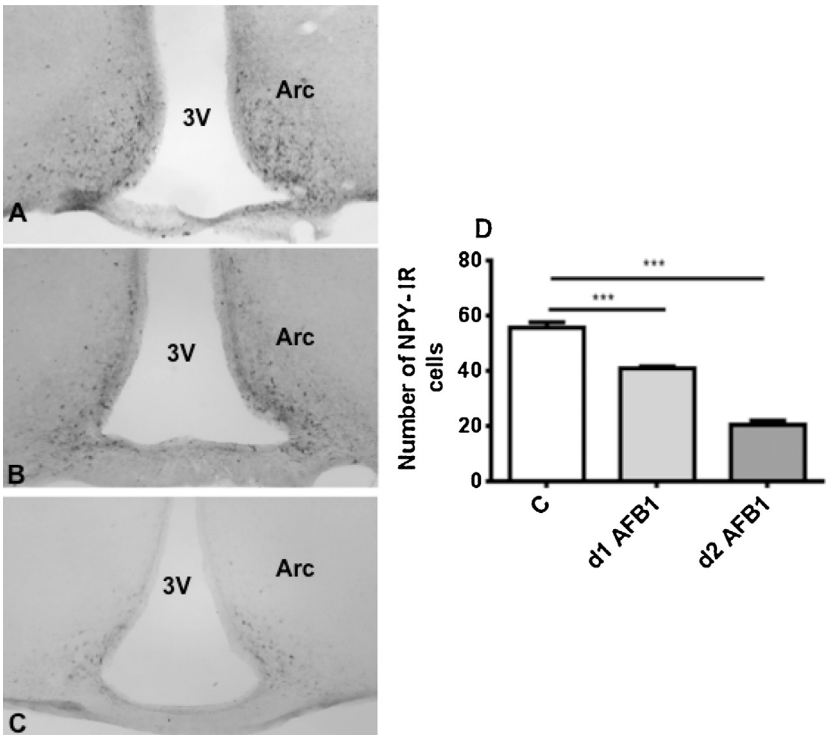

Fig. 2. Frontal sections of rat brains showing NPY immunoreactivity at the mediobasal hypothalamus level following a five-week AFB1 treatment. (A) NPY-immunoreactive (IR) cell bodies are concentrated in the medial part of the arcuate nucleus (Arc) of control rats. (B) A decrease in the density of the NPY-IR cells appears in the Arc of rats treated with AFB1 at a low dose (1.5 mg/kg) as compared to untreated animals (A). (C) The decrease is more pronounced in the Arc of rats treated with the high dose of AFB1 (3 mg/kg). Third ventricle (3V). Magnification 100×. (D) Quantitative analysis of the NYP-IR cell bodies in the medial part of the Arc of controls *vs* animals treated with a low (1.5 mg/kg) or high (3 mg/kg) dose of AFB1. A significant decrease of NPY-IR cells is observed in the two groups of rats treated with AFB1. Immunopositive cells were counted on four sections per animal. The data are expressed as mean ± SEM ($n = 3$ animals in each group). $^{***}p < 0.001$.

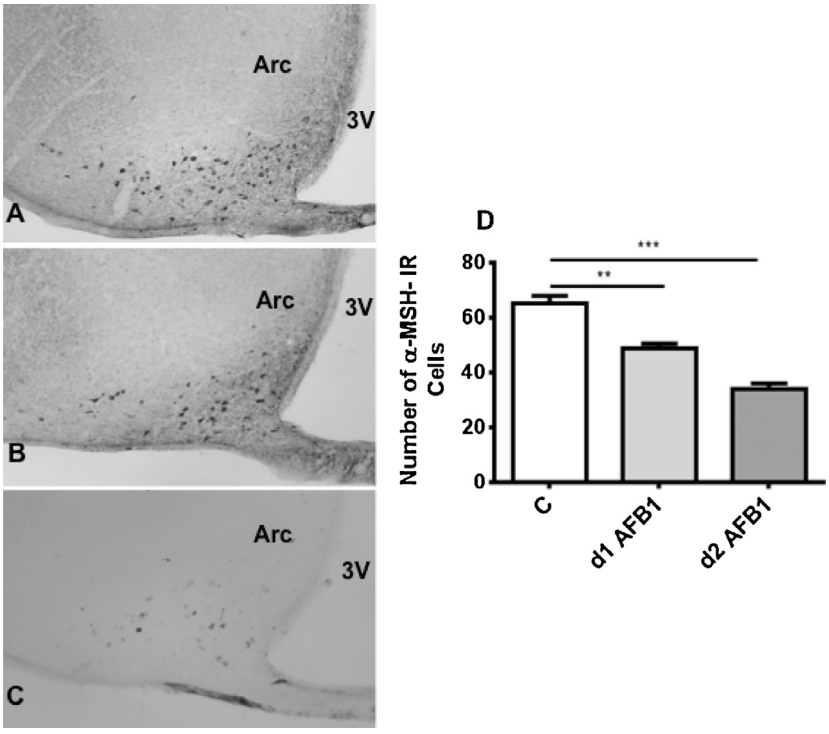

Fig. 3. Frontal sections of rat brains showing α-MSH immunoreactivity at the mediobasal hypothalamus level following a five-week AFB1 treatment. (A) α-MSH-immunoreactive (IR) cell bodies are primarily localized in the lateral part of the arcuate nucleus (Arc) of control rats. (B) A decrease in the density of the α-MSH-IR cells is observed in the Arc of rats treated with AFB1 at a low dose (1.5 mg/kg) as compared to untreated animals (A). (C) The decrease is more pronounced in the Arc of rats treated with the high dose of AFB1 (3 mg/kg). Third ventricle (3V). Magnification 100×. (D) Quantitative analysis of the α-MSH-IR cell bodies in the lateral part of the Arc of controls *vs* animals treated with a low (1.5 mg/kg) or high (3 mg/kg) dose of AFB1. A significant decrease of NPY-IR cells is observed in the two groups of rats treated with AFB1. Immunopositive cells were counted on four sections per animal. The data are expressed as mean ± SEM ($n = 3$ animals in each group). $^{**}p < 0.01$; $^{***}p < 0.001$.

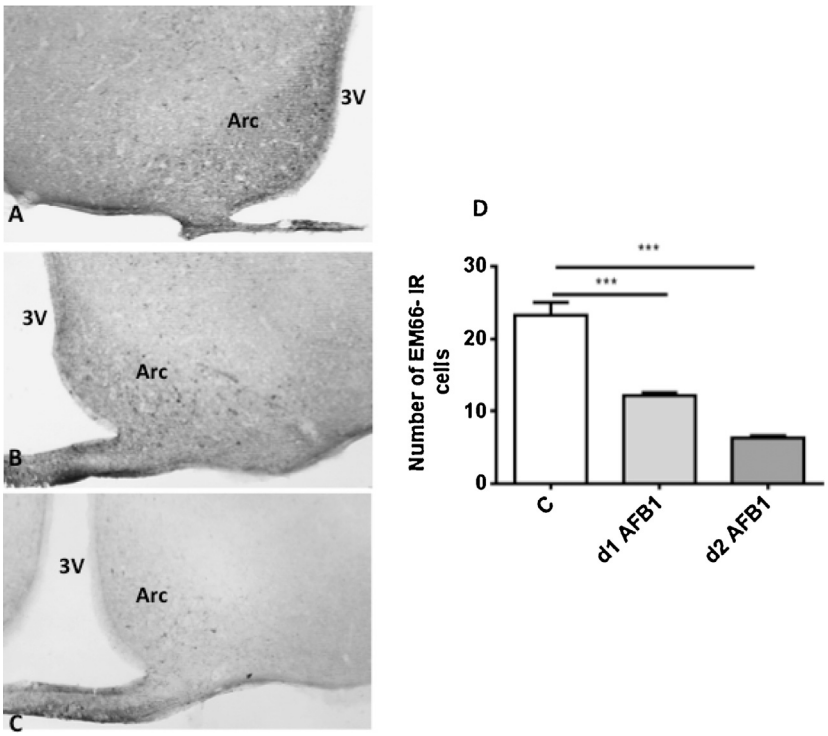

Fig. 4. Frontal sections of rat brains showing EM66 immunoreactivity at the mediobasal hypothalamus level following a five-week AFB1 treatment. (A) EM66-immunoreactive (IR) cell bodies are concentrated in the medial part of the arcuate nucleus (Arc) of control rats. (B) A decrease in the density of the EM66-IR cells appears in the Arc of rats treated with AFB1 at a low dose (1.5 mg/kg) as compared to untreated animals (A). (C) The decrease is more pronounced in the Arc of rats treated with the high dose of AFB1 (3 mg/kg). Third ventricle (3V). Magnification 100×. (D) Quantitative analysis of the EM66-IR cell bodies in the medial part of the Arc of controls *vs* animals treated with a low (1.5 mg/kg) or high (3 mg/kg) dose of AFB1. A significant decrease of EM66-IR cells is observed in the two groups of rats treated with AFB1. Immunopositive cells were counted on four sections per animal. The data are expressed as mean ± SEM (n = 3 animals in each group). $^{***}p < 0.001$.

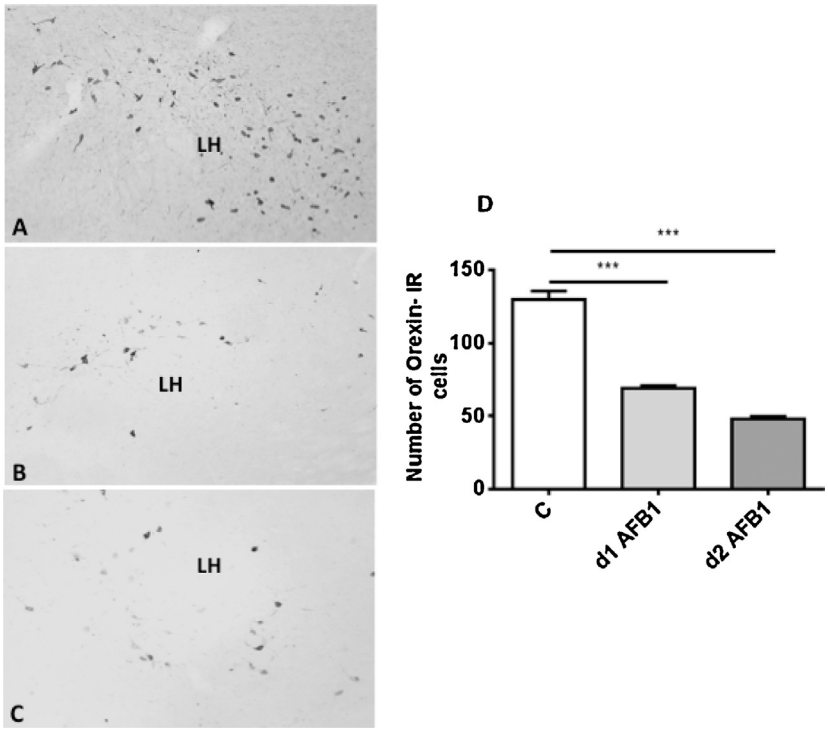

Fig. 5. Frontal sections of rat brains showing orexin A immunoreactivity in the lateral hypothalamic area (LH) following a five-week AFB1-treatment. The density of Orexin A-IR cell bodies is significantly decreased in AFB1-treated rats with the dose 1.5 mg/kg (B) as compared to controls (A). (C) The decrease in body weight gain is more pronounced for the high dose of AFB1 (3 mg/kg). Magnification 100×. (D) Quantitative analysis of the Orexin A-IR cell bodies in LH of controls *vs* animals treated with a low (1.5 mg/kg) or high (3 mg/kg) dose of AFB1. A significant decrease of orexin A-IR cells is observed in the two groups of rats treated with AFB1. Immunopositive cells were counted on four sections per animal. The data are expressed as mean ± SEM (n = 3 animals in each group). $^{***}p < 0.001$.

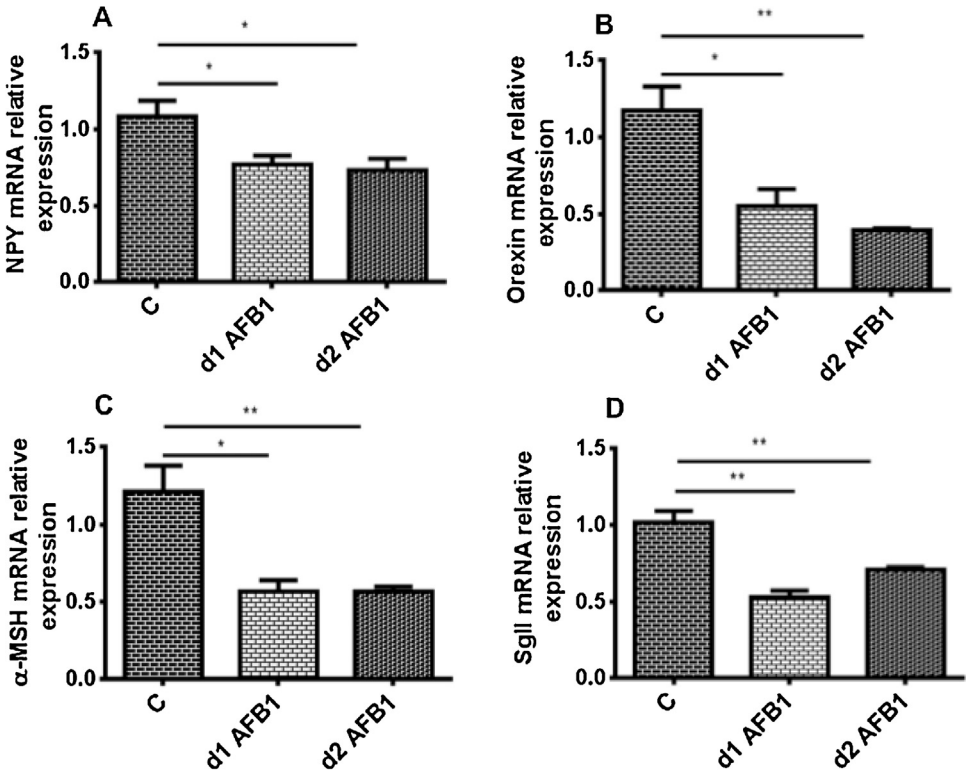

Fig. 6. Effect of a five-week AFB1 exposure, at low (1.5 mg/kg) or high (3 mg/kg) doses, on the mRNA levels of hypothalamic neuropeptides evaluated by q-PCR. (A) NPY mRNAs levels show a moderate but significant decrease following AFB1 exposure whatever the dose of aflatoxin administered. (B) Orexin mRNAs levels in the hypothalamus display a decrease following AFB1 treatment at the two doses used. This decrease in mRNA expression is slightly significant for the low dose of AFB1 and more significant for the high dose of AFB1. (C) POMC mRNA levels in the hypothalamus show a decrease following AFB1 treatment at the two doses used. This decrease in mRNA expression is slightly significant for the low dose of AFB1 and more significant for the high dose of AFB1. (D) SgII mRNAs levels show also a significant decrease following AFB1 treatment at the two doses used. Values represent mean ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$.

EM66 (Fig. 4). Indeed, the number of EM66-IR neurons in the Arc was significantly reduced in AFB1-treated rats at both the low and high doses (Fig. 4B–D) as compared to the control group (Fig. 4A). A significant decrease in orexin A immunoreactivity was also found in LH of the two AFB1-treated groups as compared to the control group (Fig. 5).

3.3. *Effect of a five-week AFB1 exposure on hypothalamic neuropeptide mRNA levels*

Concurrently, the expression of a several neuropeptides involved in the regulation of food intake was investigated in the hypothalamus by q-PCR. After a five-week treatment, the expression of NPY mRNA was significantly reduced whatever the dose of AFB1 applied, as compared to control animals (Fig. 6A). AFB1 treatment also induced a significant and dose-dependent decrease in orexin A mRNA levels in the rat hypothalamus as compared to the control group (Fig. 6B). Our data also reveled a significant decrease in POMC mRNA levels following AFB1 treatment in the two treated groups as compared to controls (Fig. 6C). Repeated exposure to AFB1 also provoked a significant decrease in SgII mRNA levels in the hypothalamus (Fig. 6D). In addition, the gene expression of other hypothalamic neuropeptides was also explored. We found that the expression of AgRP (Fig. 7A), MCH (Fig. 7B), CART (Fig. 7C) and TRH (Fig. 7D) mRNAs was significantly reduced in AFB1-treated rats at the two doses used as compared to the control rats.

4. Discussion

Our results show that AFB1 at two distinct doses (1.5 and 3 mg/kg) induces a decrease in body weight gain, which is moderate at the low dose and highly significant at the higher dose. These data are in agreement with a previous study reporting that AFB1 at a dose of 2 mg/kg provokes a decrease in body weight gain, although this study showed that lower doses had no effect (Coulombe and Sharma, 1985). However, Raisuddin et al. (1990), using the same mode of AFB1 administration in weaning Wistar rats, showed that a higher dose of AFB1 (14 doses of 700 μg/kg, cumulative dose 9.8 mg/kg, oral gavage) was required to induce a significant reduction in body weight gain. The doses used in the present study (respectively 10% and 30% of oral LD50) are considered to be below the toxicity threshold previously reported in rat, as the oral LD50 of AFB1 is approximately 10 mg/kg (Wong and Hsieh, 1980). Of note, body weight alteration in our study represents the only toxicity symptom of AFB1 observed; no other apparent symptoms or mortality occurred. Several observations reveal variations in the efficiency of AFB1 to affect different biological parameters probably due to the different animal species genders used and/or experimental conditions *i.e.* the toxin purity, the time of day of toxin treatment, 1*etc.*

In order to assess the physiological process underlying the body weight alteration following AFB1 administration, we investigated the hypothalamic expression of several orexigenic or anorexigenic neuropeptides that control feeding behavior. We explored by immunohistochemistry and by qPCR the expression of the orexigenic neuropeptides NPY, AgRP, MCH and orexin (de Lecea et al., 1998; El Ouezzani et al., 2001; Sakurai et al., 1998a,b; Small et al., 2001), and the anorexigenic neuropeptides α-MSH, CART and TRH (Cyr et al., 2013; Mezey et al., 1985; Stanley et al., 2001). Our immunohistochemical data show a significant and dose-related decrease in the immunoreactivity of NPY and α-MSH in the Arc which is associated with a reduced density of their respective

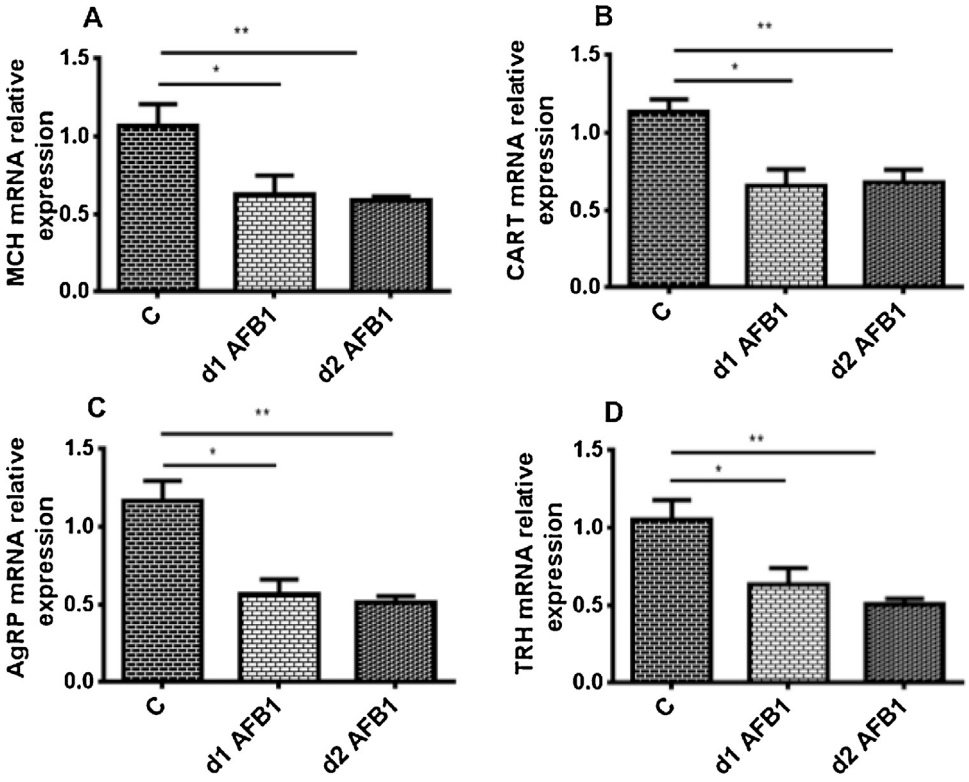

Fig. 7. Effect of a five-week AFB1 exposure, at low (1.5 mg/kg) or high (3 mg/kg) doses, on the mRNA levels of hypothalamic neuropeptides evaluated by q-PCR. (A) MCH mRNA levels in the hypothalamus show a significant decrease following AFB1 treatment at the two doses used. This decrease in mRNA expression is slightly significant for the low dose of AFB1 and more significant for the high dose of AFB1. (B) CART mRNAs levels in the hypothalamus display a decrease following AFB1 treatment at the two doses used. This decrease in mRNA expression is slightly significant for the low dose of AFB1 and more significant for the high dose of AFB1. (C) AgRP mRNAs levels in the hypothalamus display also a decrease following AFB1 treatment at the two doses used. This decrease in mRNA expression is slightly significant for the low dose of AFB1 and more significant for the high dose of AFB1. (D) Similarly, TRH mRNAs levels in the hypothalamus show a decrease following AFB1 treatment at the two doses used. This decrease in mRNA expression is slightly significant for the low dose of AFB1 and more significant for the high dose of AFB1. Values represent mean ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$.

neuronal populations. The orexin immunoreactivity also underwent a significant decrease in the LH after exposure to AFB1. Consistent with these immunohistochemical data, the hypothalamic expression of NPY, α-MSH and orexin mRNAs was significantly attenuated in AFB1-treated rats as compared to the controls. Given the similarity of AFB1 action on these orexigenic and anorexigenic peptides, we extended our investigations to the expression of the peptides AgRP, MCH, CART and TRH in order to confirm this effect. We investigated therefore their gene expression by qPCR. Our data show that AgRP, MCH, CART and TRH mRNA levels are also significantly reduced in AFB1-treated rats at the two doses used, as compared to the controls. Furthermore, we explored the effect of AFB1 on the novel neuropeptide EM66 as previous data from our group revealed that this SgII-derived peptide is strongly expressed in hypothalamic neuroendocrine areas such as the Arc and the median eminence (Boutahricht et al., 2007), and is therefore potentially involved in the control of food intake (Boutahricht et al., 2005). Following repeated exposure to AFB1, EM66-IR as well as the number of EM66-containing neurons evaluated by immunohistochemistry was significantly reduced in the Arc, as compared to the control group. Consistently, the gene expression of the precursor, *i.e.* SgII, determined by q-PCR was also reduced in the Arc of AFB1-treated compared to control animals. This finding supports the notion that EM66 is a neuropeptide involved in the control of appetite in physiological and pathological conditions.

Overall, the present data show that repeated consumption of AFB1 decreases the expression of both orexigenic and anorexigenic hypothalamic neuropeptides, suggesting that the associated body weight gain impairment could be a consequence of the alteration of the hypothalamic neuronal systems expressing these peptides, leading to disruption of the orexigenic/anorexigenic peptide balance. These alterations of the neuropeptidergic systems in the hypothalamus as evidenced notably by the reduction in the number of neurons expressing the neuropeptides in the Arc or the LH, could be due to a neurodegenerative process induced by AFB1. Indeed, previous studies reporting on the effects of AFB1 in the central and peripheral nervous systems have shown an increase in the β-glucuronidase and β-galactosidase activities which represent early signs of neuronal degeneration (Dewar and Moffett, 1979; Ikegwuonu, 1983). It has also been shown that repeated AFB1 treatment significantly decreases the concentrations of RNA and phospholipid of several nervous tissues in rat, and alters specific peripheral and central neuronal ATPases (Ikegwuonu, 1983). AFB1 is able to induce reactive oxygen species (ROS) generation that causes oxidative stress, leading to oxidation of proteins, lipids and DNA (Mary et al., 2012), thus acting as a direct and indirect initiator as well as promoter of genotoxicity and apoptotic processes (Golli-Bennour et al., 2010).

In addition to these neuropeptidergic alterations, other neurochemical impairments in relation with AFB1 intoxication were also reported. The greatest effect of AFB1 was observed on the major amines, dopamine and serotonin. Indeed, following repeated oral administration of AFB1 (2 mg/kg), a dramatic decrease in dopamine, catecholamine metabolites and serotonin was found in the corpus striatum, in addition to minor alterations of the amines detected in other brain structures such as the hypothalamus (Coulombe and Sharma, 1985). In agreement, the brain tryptophan hydroxylase activity has been shown to be altered by aflatoxin as revealed by its Km increase (Weekley and Llewellyn, 1984).

Besides, cholinergic neurotransmission is also affected by AFB1 as revealed by a decrease in the level of acetylcholinesterase in the cerebellum and hippocampus in response to AFB1 acute treatment, and a drastic increase in the mesencephalon and amygdala following chronic treatment, associated to behavioral deficits (Egbunike and Ikegwuonu, 1984).

It has been reported that higher doses of AFB1 (cumulative doses of 4.9–21 mg/kg) induce immunosuppressive effects in rat (Reddy and Sharma, 1989), mouse (Hatori et al., 1991; Reddy et al., 1987) and chicken (Giambrone et al., 1985), but the hypothalamic–pituitary–adrenal axis does not appear to be significantly affected by AFBl-induced immunotoxicity (Hatori et al., 1991).

In conclusion, the present immunocytochemical and q-PCR data show for the first time a significant decrease in the expression of numerous hypothalamic neuropeptides regulating food intake following repeated exposure to AFB1. These alterations affecting orexigenic as well as anorexigenic peptides may underlie food intake control dysregulation and thus the body weight gain alteration.

Conflict of interest

The authors declare that there are no conflicts of interest.

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