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ABSTRACT: In addition to be a primary risk factor for type 2 diabetes and cardiovascular disease, obesity is associated with learning disabilities. Here we examined whether a dysregulation of the kynurenine pathway (KP) of tryptophan (Trp) metabolism might underlie the learning deficits exhibited by obese individuals. The KP is initiated by the enzymatic conversion of Trp into kynurenine (KYN) by indoleamine 2,3-dioxygenase (IDO). KYN is further converted to several signaling molecules including quinolinic acid (QA) which has a negative impact on learning. Wistar rats were fed either standard chow or made obese by exposure to a free choice high-fat high-sugar (fcHFHS) diet. Their learning capacities were evaluated using a combination of the novel object recognition and the novel object location tasks, and the concentrations of Trp and KYN-derived metabolites in several brain regions determined by ultra-performance liquid chromatography-tandem mass spectrometry. Male, but not female, obese rats exhibited reduced learning capacity characterized by impaired encoding along with increased hippocampal concentrations of QA, Xanthurenic acid (XA), Nicotinamide (Nam), and oxidized Nicotinamide Adenine Dinucleotide (NAD+). In contrast, no differences were detected in the serum levels of Trp or KP metabolites. Moreover, obesity enhanced the expression in the hippocampus and frontal cortex of kynurenine monooxygenase (KMO), an enzyme involved in the production of QA from kynurenine. QA stimulates the glutamatergic system and its increased production leads to cognitive impairment. These results suggest that the deleterious effects of obesity on cognition are sex dependent and that altered KP metabolism might contribute to obesity-associated learning disabilities.

KEYWORDS: Tryptophan, kynurenine, brain, obesity, learning, memory

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Introduction

Obesity is a current worldwide public health issue that affects the quality of life and increases the risk of developing metabolic, cardiovascular and musculoskeletal diseases as well as some types of cancer.[1] Mounting evidence indicates that obesity has also a detrimental impact on cognitive function.[2] In particular, several clinical studies have shown that individuals with a high body mass index display poor cognitive performances including reduced memory abilities and impaired decision making.[3-5] Consumption of high-calorie diets, either rich in fat, sugar or both, is also positively associated with deficiencies in prospective memory and memory recall.[6,7] Experimental studies in rodents have confirmed that obesity is accompanied by cognitive impairment and have further shown that hippocampal-dependent memory tasks are particularly sensitive to diet-induced obesity[8,9,10] (see Abbott et al[11] for review).

Obesity is characterized by a low-grade inflammatory state which is assumed to be at the heart of the metabolic and cardiovascular comorbidities experienced by obese individuals. Multiple lines of evidence show that obesity-related inflammation also underlies the cognitive impairments associated with this pathological condition. Actually, high-fat feeding results in the disruption of the blood-brain barrier (BBB) leading to neuroinflammation via the entry of pro-inflammatory cytokines and peripheral immune cells into the brain and the activation of microglia, the resident immune cells of the brain.[12-16] Moreover, a positive association has been found between neuroinflammation and learning impairment in genetic or diet-induced experimental models of obesity.[17-20] In addition, the activation of the

immune response by the administration of lipopolysaccharide (LPS), results in reduced learning.[21,22] The existence of a causal link between neuro-inflammation and cognitive dysfunction, is also supported by data showing that the intracerebral administration of high concentrations of the pro-inflammatory cytokines IL-1β or IL-6 impairs memory formation in several learning paradigms.[23-25] Similarly, transgenic mice overexpressing tumor necrosis factor alpha (TNF-α) in brain, exhibit impaired memory acquisition in the Morris water maze and passive avoidance tests,[26,27] and IL-1β, IL-6 and TNF-α inhibit long-term potentiation (LTP), a cellular model of memory.[28-33]

However, although the correlation between neuroinflammation and cognitive impairment is well established, the mechanisms by which an increase in the cerebral concentration of pro-inflammatory interleukins leads to learning and memory deficits are far from being fully established. In this respect, it is worth mentioning that IL-1β and TNF-α enhance the expression of indoleamine 2,3-dioxygenase (IDO).[34-37] This enzyme catalyses the conversion of tryptophan (Trp) to kynurenine (KYN) which is the first and rate-limiting step of the kynurenine pathway (KP) of Trp metabolism. The subsequent transformation of KYN through a series of enzymatic reactions leads to the production of several neuroactive compounds and eventually to the synthesis of nicotinamide adenine dinucleotide (NAD+). Among the different products generated by the kynurenine pathway, quinolinic acid (QA), and kynurenic acid (KA) stand out for their impact on learning and memory as a result of their respective agonist and antagonist actions at the ionotropic glutamate and α7 nicotinic acetylcholine receptors.[38-42]

Cerebral biosynthesis of KYN, takes place from Trp transported to the brain from the bloodstream where it is bound to albumin or in free form. Only in its free form Trp is able to cross the blood-brain barrier. Non-esterified fatty acids (NEFAs) bind to albumin at the same site as Trp so that an increase in plasma concentrations of NEFAs results in the displacement of Trp from its site of interaction with albumin, leading to an increase in the proportion of free Trp in the circulation and increased transport of Trp into the brain.[43-45] The concentration of free tryptophan also increases as a result of its dissociation from albumin within the blood-brain barrier.[46] In addition to diet, this process is modulated by physiological and metabolic factors.[47]

On the basis of all above observations, it can be hypothesized that obesity-related cognitive alterations involve a signaling cascade triggered by the exacerbated production of pro-inflammatory interleukins in the brain that would lead to the activation of tryptophan metabolism through the KP and eventually to the impairment by KA and QA of the memory process mediated by cholinergic and glutamatergic receptors. This signaling pathway would be favored by an enhanced entry of Trp into the brain resulting from an elevation of free Trp in blood as consequence of the displacement of Trp from albumin by NEFAs. Here we have tested this hypothesis by examining the learning capacities and the concentrations of tryptophan and kynurenine-derived metabolites in several brain regions of obese rats.

Materials and Methods

Animals and diet-induced obesity model

We used the free-choice high-fat high-sugar diet (fc-HFHS) obesity model as described by La Fleur et al[48] with minor modifications. This model has the advantage of simulating the modern-day dietary environment of humans and of leading to a rapid increase in adiposity.[48,49] Male and female Wistar rats (Janvier Labs, Le Genest Saint Isle, France), were fed either standard chow (controls), or exposed from weaning to high-fat food and to a 30% sugar solution in addition to standard chow and tap water (obese). High-fat food consisted in powdered standard chow mixed with pig fat in the proportion of 30 g of fat per 70 g of food. Sweetened condensed milk was used as a base to prepare the 30% sugar solution. Animals were housed 4 per cage until they reached 300 g of body weight and 2 per cage thereafter and maintained under an inversed 12-/12-hour dark-light cycle (lights off at 7:00 AM), and at 21 ± 1°C with food and water ad libitum through all the experiment.

Behavioral analyses

Memory tests were performed under red light 2 hours after the beginning of the dark phase of the light-dark cycle in a laboratory adjacent to the housing room of the animals and recorded using the Viewpoint Videotrack system (Point Grey Research Inc., Richmond B.C. Canada), for offline analysis by 2 investigators blind to the nutritional status of the animals. In a first set of experiments, the learning capabilities of 180-day-old male and female rats of both experimental groups (control and obese), were assessed using the novel object recognition test (NOR) as described by Ennanceur and Delacour.[50] Subsequently, the impact of obesity on the different components of the memory process was evaluated in a different set of 105-day-old male rats using the combination of the NOR and the novel object location (NOL) tasks.[51] The NOR test was divided into 3 sessions: habituation, training and testing. During the habituation session, rats were allowed to freely explore an open-field arena (50 × 50 × 40 cm) for 20 minutes during 3 consecutive days. The fourth day, 2 identical objects were placed into the arena before introducing the rat and each animal was allowed to explore the objects for 7 minutes. 2 hours after this training phase, animals were returned to the arena in which one of the familiar objects has been exchanged for a new one. The time exploring each object was recorded and the learning capacity determined from the difference in time spent exploring the novel versus the familiar object. Preferential exploration of the novel object versus the familiar one indicates memory acquisition whereas an identical or similar time spent exploring the 2 objects means no learning. Object exploration was defined as sniffing or touching the object with the vibrissae or when the animal's head was oriented toward the object with the nose placed at a distance of less than 2 cm from the object. The NOR-NOL test was carried out in the same way excepting that 2 additional testing sessions were carried out 24 hours

and 7 days after the first test session to evaluate, respectively, long term (LTM) and consolidated (CM) memories. In addition, in each of the test sessions, including the 2-hour session to assess short term memory (STM), both the new and the familiar objects were placed in a different location from the one they occupied in the previous session (see Pérez-García et al[51] for a complete description of the test).

Biological samples collection and processing

The day following the last NOR or NOR-NOL testing session, animals were deeply anesthetized with isoflurane 2 hours after the beginning of the dark phase of their light-dark cycle to obtain a blood sample by cardiac puncture and sacrificed immediately after by cervical dislocation. The frontal cerebral cortex, brainstem and hippocampus were dissected and the serum separated from blood was split into 2 fractions. One of them was filtrated using an ultrafiltration device endowed with an Ultracel YM-T membrane with a pore size of 30 kDa (Millipore, France), to obtain serum free tryptophan. All brain samples were immediately frozen after dissection in liquid nitrogen and stored at −70° C together with serum samples until analysis. Mediastinal and abdominal (omental, perirenal, retroperitoneal, epididymal, periovarian, perivesical, and parametrial), fat depots were dissected, weighed and summed to provide a measure of body fat.

Metabolite determinations

Serum was assayed for insulin using an assay kit from Linco Research Inc. Triglycerides, cholesterol and fatty acids were analyzed by enzymatic methods (Triglycérides enzymatiques PAP 150, BioMérieux; Cholesterol RTU, Biomérieux; NEFA FS, DiaSys). Serum glucose concentrations were determined with a blood glucose monitor (Accu-Check®, Roche Diagnostics).

Analyses of tryptophan metabolism

Tryptophan and the metabolites generated by its catabolism through the serotonin (5-HT) and KYN pathways were quantified in the brain-stem, hippocampus, frontal cortex and serum by ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the methodology described in detail by Zheng et al and Martimiano et al.[52,53] In brief 50 mg of tissue or 50 µL of serum were homogenized in 200 µL of an ice-cold solution of 2.7 mM EDTA containing 1% formic acid and mixed with 10 µL of a pool of exogenous internal standards (500 µM D_5-TRP, 2000 µM cafeic acid and 50 µM 2-CAD). Subsequently, 200 µL of the homogenate were mixed with 500 µL of ice-cold acetonitrile and, after centrifugation at 15 000*g* for 15 minutes at 4°C, the supernatant was recovered and evaporated to dryness under a stream of nitrogen at room temperature. Calibration curves were generated using serial dilutions of Trp, QA,5-HT, KYN, 5-HIAA, KA, and Xanthurenic acid (XA), in 2.7 mM EDTA containing 0.1% formic acid which were processed in the same way that the tissue and serum samples. At the end, all dried samples were dissolved in 100 µL of EDTA 2.7 mM containing 0.1% formic acid and 5 µL of each sample were injected into the LC-MS/MS system for analysis. Compounds were separated on a reverse Acquity HSS T3 column (2.1 × 100 mm, 1.7 µm, Waters Corporation, Milford, MA, USA) at 30°C with a linear gradient of the mobile phase B (acetonitrile containing 0.5% formic acid and 2.5 mM ammonium acetate) in the mobile phase A (water containing 0.5% formic acid and 2.5 mM ammonium acetate) and at a flow rate of 400 µL/min. The electrospray interface of the mass spectrometer was operated in positive ion mode and the multiple reaction monitoring mode was chosen for metabolite detection. Data acquisition and analysis were performed using MassLynx® and TargetLynx® software, respectively (version 4.1; Waters). Compound concentrations were calculated using calibration curves plotted from standard solutions.

Real time quantitative RT-PCR

A conventional Trizol extraction method was used to obtain total RNA from a fraction of the same tissue homogenate that was used for the quantification of Trp metabolites by mass spectrometry. Thereafter, 1 µg of purified RNA was treated with a DNase and reverse transcribed using the MultiScribe™ Reverse Transcriptase kit from Thermo Fisher according to the manufacturer's instructions. The resulting cDNA was diluted 40-fold in DNAse and RNAse free water and 5 µL of each cDNA diluted sample were used as template for amplification using an iCycler iQ Real-Time PCR detection system and SYBR Green as fluorogenic intercalating dye. Relative gene expression differences between control and obese animals were determined by the comparative $2^{-\Delta\Delta Ct}$ method,[54] using 18 S, β2-microglobulin or GAPDH transcripts as housekeeping genes. The applicability of the $2^{-\Delta\Delta Ct}$ method was first validated by determining how the amplification efficiencies of the different transcripts, including those of the reference genes, varied with template dilution. These experiments showed that the efficiency of the PCR amplification was the same for all the genes and that the expression of 18 S, β2-microglobulin or GAPDH was not influenced by obesity. The sequences of the primers used for the amplification are as follows : *Tryptophan hydroxylase 2 (TPH2)*, forward: ATC CCA AGT TCG CTC AGT TTT CTC, reverse: ACG CCC GCA GTT GAC CTT C; *Indoleamine 2,3-Dioxygenase 1(IDO1)*, forward: CGT TGG TGA AGG ATC TGC TG, reverse : AGG GCT CTT CTG ACT TGT GG; *Indoleamine 2,3-Dioxygenase 2 (IDO2)*, forward: *GGT GAC AGT CTT GGT GGA GAA G*, reverse*: GGT GTC CTG GCT GTG TTG C; Tryptophan 2,3-Dioxygenase (TDO2)*, forward: CAA GGT GAT GAC TCG GAT GC,

Table 1. Anthropometric characteristics and serum metabolic profile of male and female 180 days-old rats exposed to the FcHFHS diet.

	MALES		FEMALES	
	CONTROL	OBESE	CONTROL	OBESE
Body weight (g)	620 ± 11	770 ± 19****	341 ± 6	454 ± 11****
Adiposity Index	6.35 ± 0.43	9.80 ± 0.38****	6.45 ± 0.38	10.25 ± 0.39****
Leptin (ng/mL)	13.56 ± 2.02	24.28 ± 2.34**	7.27 ± 0.96	22.69 ± 3.03***
Insulin (ng/mL)	1.58 ± 0.21	2.99 ± 0.67	1.36 ± 0.45	3.41 ± 0.76*
Glucose (mg/dL)	163 ± 10.76	193 ± 19.97	175 ± 4.61	183 ± 10.06
Cholesterol (ng/dL)	73.62 ± 5.55	74.83 ± 2.90	63.98 ± 4.28	85.11 ± 5.23*
Triglycerides (ng/dL)	101 ± 10.96	151 ± 20.08*	137 ± 15.72	251 ± 84.09**

*$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$ compared to their respective male or female control group as determined by Student's *t*-test.

reverse: GGA ACT GAA GAC TCT GGA AGC; *Kynurenine aminotransferase (KAT)*, forward GAA TTA CTC AAG GTT CCT CAC TG, reverse *GAT GGT GCT TCC GTT CTC C; .Kynurenine mono-oxygenase (KMO)*, forward: GCT TCC AAC GCA TAC TGA TG, reverse: GGC AGG CAA CAG AAA GAA ATC; *3-hydroxyanthranilate 3,4-dioxygenase (HAAO)*, *IL-6*, forward: GCC CTT CAG GAA CAG CTA TGA, reverse: TGT CAA CAA CAT CAG TCC CAA GA; *IL-1*β, forward: AAT GCC TCG TGC TGT CTG ACC, reverse: GGG TGG GTG TGC CGT CTT TC; *TNF*-α, forward: CCA CCA CGC TCT TCT GTC TAC TG, reverse: GGC TAC GGG CTT GTC ACT CG; *TLR2*, forward: GTC TCC AGG TCA AAT CTC, reverse: CAG CAT CAC ATG ACA GAG, *TLR4*, forward: GAT TGC TCA GAC ATG GCA GTT TC, reverse: CAC TCG AGG TAG GTG TTT CTG CTA A; *18 S*, forward: GAT GCG GCG GCG TTA TTC C, reverse: CTC CTG GTG GTG CCC TTC C; β*2-microglobulin*, forward: GAT GGC TCG CTC GGT GAC, reverse: CGT AGC AGT TGA GGA AGT TGG; *GAPDH*, forward: CAG TAT GAC TCT ACC CAC GGC A, reverse: ATC TCG CTC CTG GAA GAT GGT G.

Statistical analysis

Experimental results were analyzed using software GraphPad Prism version 9. Data were first checked for normality by the Shapiro–Wilk normality test and the statistical differences assessed by unpaired Student's *t*-test or 2-way ANOVA. Statistical significance was set at $P < .05$. Data are expressed as means ± SEM.

Results

Obesity impairs learning in male but not female rats

One hundred eighty day-old male and female rats exposed to the fc-HFHS diet developed a clearly obese phenotype characterized by increased body weight and body fat as well as by enhanced serum levels of leptin and triglycerides (Table 1). In addition, obese females displayed higher serum concentrations of insulin and cholesterol in comparison to their respective control group (Table 1).

To address whether fc-HFHS feeding-induced obesity was affecting learning, animals were exposed to the NOR test using the standard protocol described by Ennaceur and Delacour.[50] This test assesses the animal's capacity to distinguish a novel from a familiar object therefore providing a measure of recognition memory. We chose this learning paradigm because it does not require the use of positive or negative reinforcements and it does not rely on the achievement of a physical activity that could be compromised by obesity. As illustrated in Figure 1, obese male rats showed a reduced ability to distinguish the novel object from the familiar one, indicating a decreased learning capacity, during a single test session performed 2 hours after the training phase of the NOR test. In contrast, no difference in exploration time for the novel versus the familiar object was observed in obese female rats compared to their control counterparts.

Fc-HFHS feeding enhances the expression of proinflammatory cytokines in brain

With the aim of identifying the components of the memory construction affected by the fc-HFHS diet, we performed a second experiment using only male rats. In addition, we decided to assess learning skills in younger animals (105-day-old) in order to distinguish the effects of obesity from those induced by aging. Indeed, aging entrains the decline of certain cognitive abilities, such as memory, visuospatial, and executive function abilities,[55] and obesity accelerates the rate of aging.[56]

Similar to the 180-day-old animals, the 105-day-old rats that consumed the fc-HFHS diet, showed an obese phenotype characterized by enhanced body weight and increased adiposity index in comparison with control rats (Figure 2A and B). In addition, they presented elevated levels of leptin (Figure 2C),

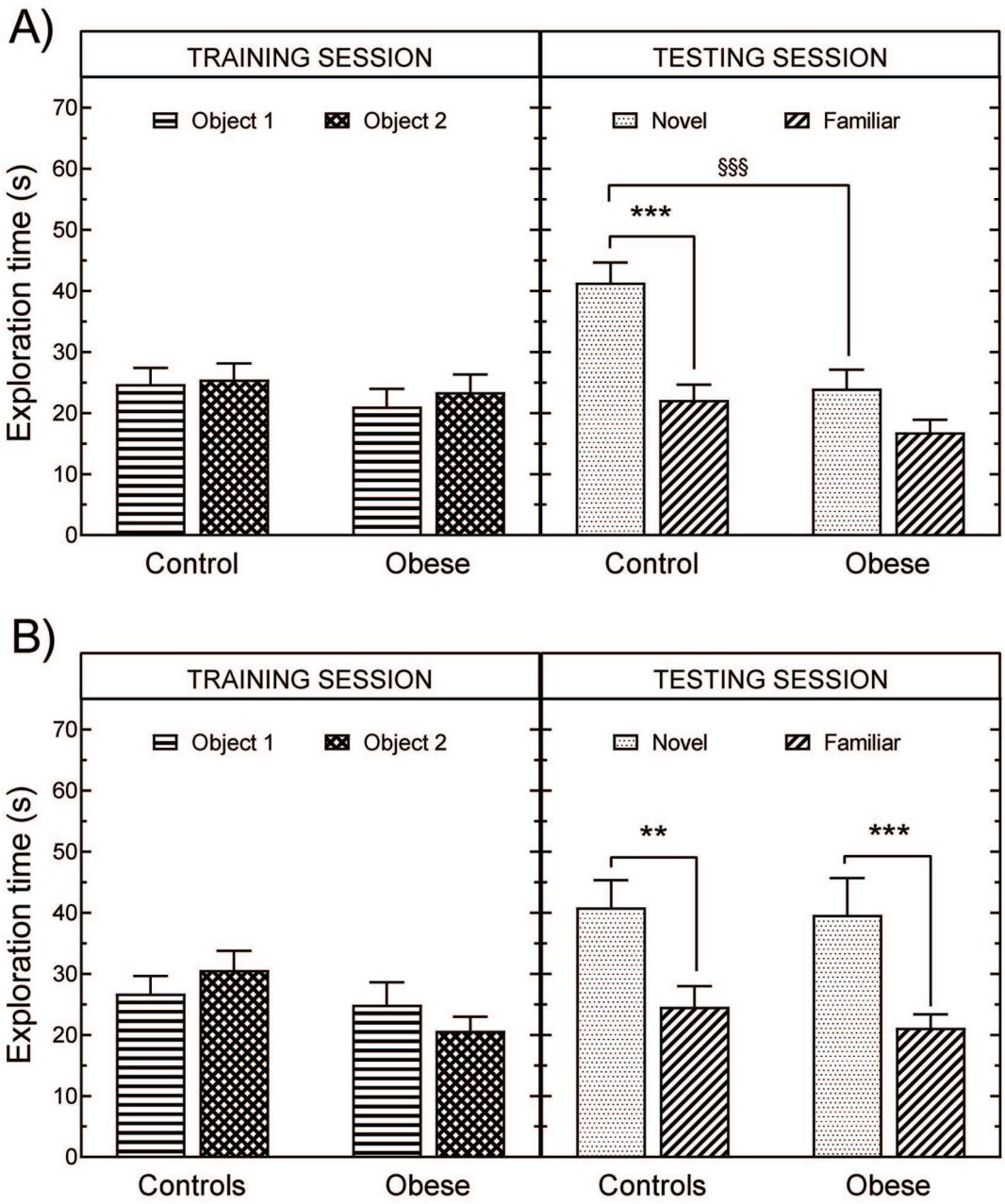

Figure 1. Impact of obesity on the cognitive skills of 6-month-old male (A) and female (B) rats. The learning capacity of the animals was tested using the standard NOR test. Bars correspond to the exploration time of each of the objects during a 7-minute period. No difference in object exploration time was observed between the different groups during the training session. In contrast, obese male animals were unable to distinguish the novel object from the familiar one as shown by the almost identical exploration time of the 2 objects. Learning tests were performed at the beginning of the dark phase of the animal's dark-light cycle with a total number of 8 to 12 animals per experimental group.
$^{**}P < .01$; $^{***}P < .001$ (Student's *t-test).* $^{§§§}P < .001$ (2-way ANOVA).

insulin (Figure 2D), triglycerides (Figure 2E) and glucose (Figure 2F). In contrast, there was no difference in the circulating levels of free fatty acids between obese (29.43 ± 8.42 mg/dL) and control animals (33.48 ± 8.25 mg/dL). fcHFHS fed rats displayed also increased levels of mRNAs encoding for IL-6, IL-1β, TNF-α, TLR2, and TLR4 receptors in the brain stem (Figure 3). In contrast, only the expression of TLR2 mRNAs was enhanced in the hippocampus and frontal cortex of obese rats (Figure 3).

Obesity delays the acquisition of the memory trace but does not impair memory retrieval

To assess the learning and memory skills of young male rats, we used a behavioral paradigm based on the combination of the NOR and the Novel Object Location (NOL) tests (NOR-NOL). This test allows the analysis of the 3 mains stages of the memory process,. that is, encoding, consolidation and retrieval (see Pérez Garcia et al,[51] for a detailed description of the test and of its theoretical ground).

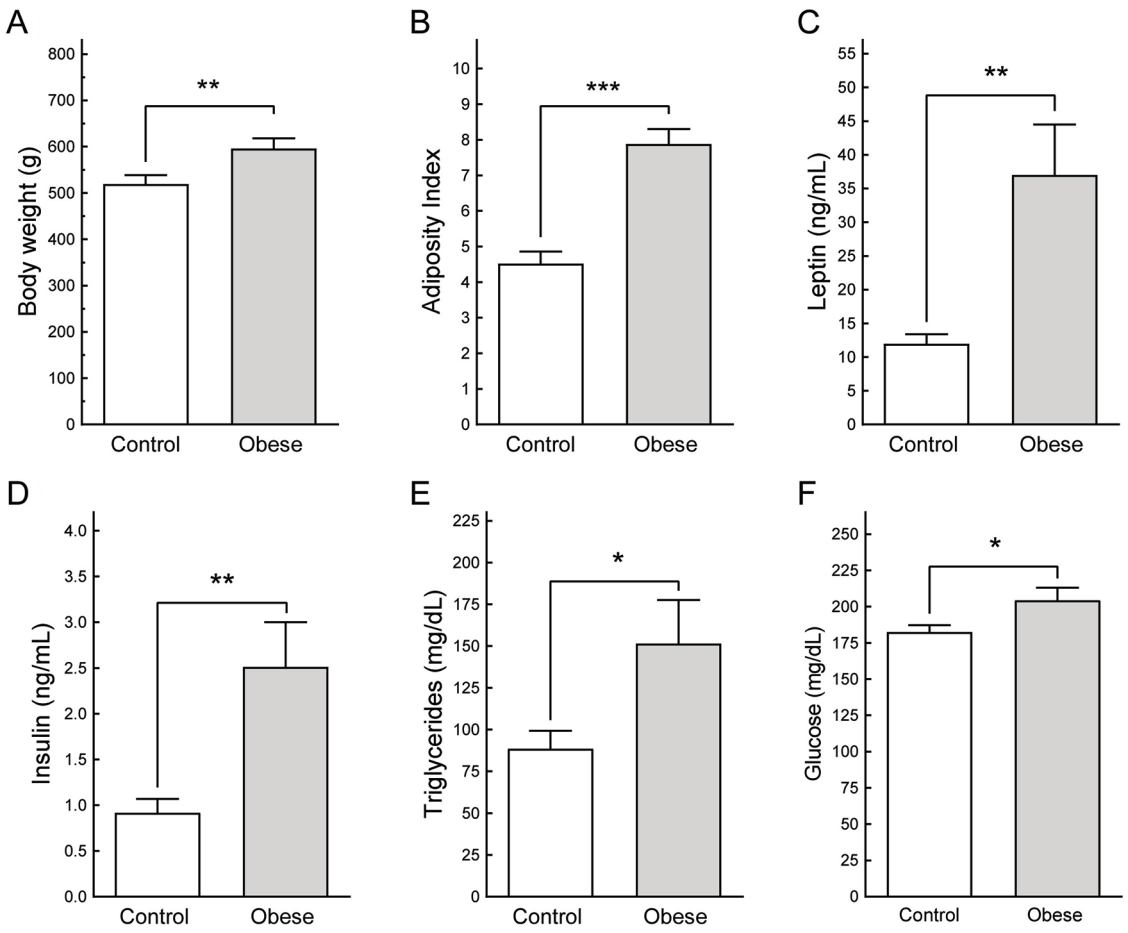

Figure 2. Body weight (A), adiposity index (B), and serum levels of leptin (C), insulin (D), triglycerides (E) and glucose (F) exhibited by 105-day-old control and obese male rats. Animals were made obese by exposure to a FcHFHS diet from weaning. Data represent the mean ± SEM from a total number of 8 animals per experimental group.
*$P < .05$; **$P < .01$; ***$P < .001$ as determined by Student's *t*-test.

As illustrated in Figure 4, obese rats displayed reduced ability to distinguish the novel object from the familiar one 2 hour after the encoding session. In contrast, clear differences in exploration time for the novel versus the familiar object were observed in both control and obese animals during the test sessions performed 24 hours and 7 days after training. This result indicates that, at least under our experimental conditions, obesity impairs the capacity to encode new information but not the capacity to remember a previously encoded and stored memory trace.

Obesity impairs brain kynurenine pathway metabolism

With the aim of determining the potential contribution of Trp metabolites to the learning deficits observed in obese animals, Trp and several products spanning its metabolism through the 5-HT and KYN pathways were quantified by LC-MS/MS in the brain stem, hippocampus and frontal cortex of control and obese rats. Quantified metabolites included 5-HT and 5-HIAA (5-HT pathway); KYN, KA, XA, QA, nicotinic acid (NA) nicotinamide (NAm), and NAD+ (KYN pathway). The results of these analyses showed that the concentrations of XA, QA, Nam, and NAD+ in the hippocampus are enhanced by fc-HFHS feeding-induced obesity (Figure 5A). We also found increased levels of KYN in the brain stem (Figure 5B) and of NAD+ in the frontal cortex (Figure 5C) of obese rats in comparison to the control group. Otherwise, there were no differences between control and obese rats in the levels of all other quantified tryptophan metabolites (Supplemental Table 1).

To get insight into the mechanisms underlying the enhanced concentration of KYN breakdown products in the brain of obese animals, the expression of mRNA transcripts encoding key enzymes of Trp and KYN metabolism was evaluated by quantitative real-time PCR. We observed an enhanced expression of TPH2 and of kynurenine 3 monooxygenase (KMO), which is involved in the metabolic pathway leading the conversion of KYN to QA, in the brain stem of obese rats as compared to controls (Figure 6). Likewise, mRNA levels encoding for KAT—the enzyme that catalyses the catabolism of KYN into KA- and KMO were enhanced in the hippocampus and frontal cortex of obese rats (Figure 6).

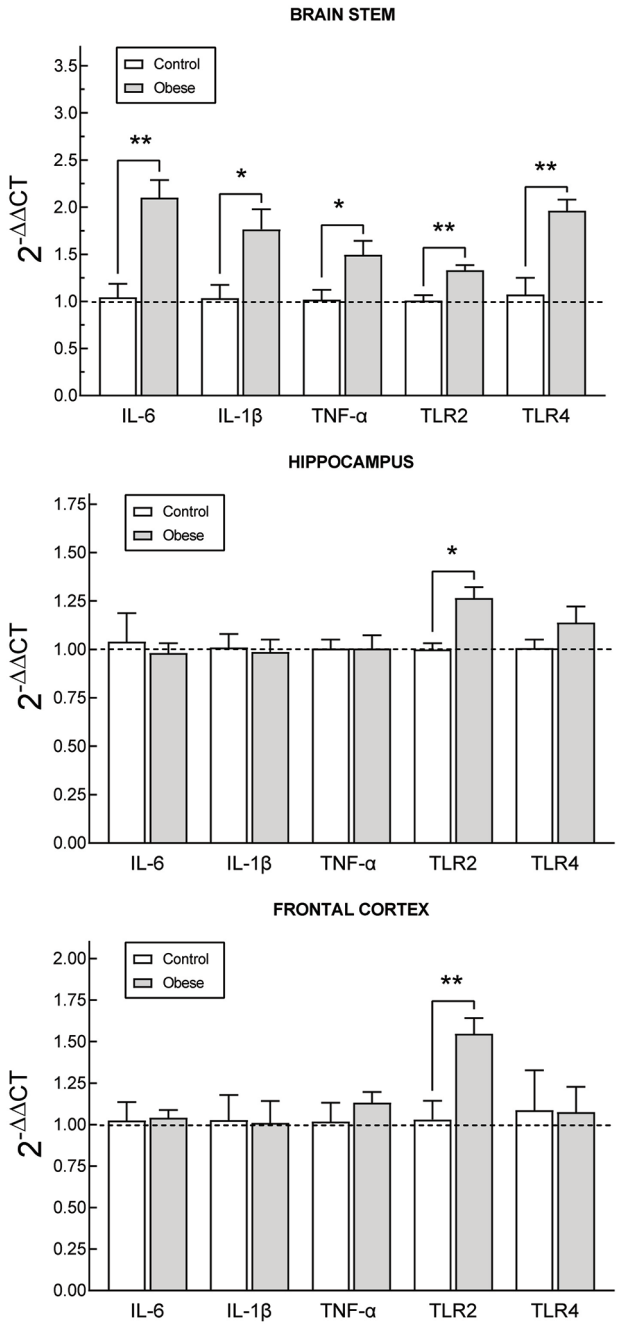

Figure 3. Brain expression levels of mRNAs encoding for genes involved in inflammatory responses. Male rats were fed standard chow or rendered obese by exposure to a fcHFHS diet. Variations in gene expression were calculated by the 2-ΔΔCT method using the expression of control animals as a calibrator. Data represent the mean ± SEM from a total number of 8 animals per experimental group.
* $P < .05$; ** $P < .01$ (Student's *t*-test).

Impact of obesity on peripheral kynurenine pathway metabolism

The metabolism of KYN within the brain, is in part dependent on the availability of free Trp in blood as well as on the peripheral concentration of KYN which can readily enter into the brain. We therefore evaluated the concentrations of total and free Trp as well as the levels of KYN and its metabolites in serum. Consumption of the fcHFHS diet did not alter neither the total nor the free circulating levels of Trp (Supplemental Table 2). Likewise, no differences were detected in the serum levels of KYN pathway metabolites with the exception of a decrease in the concentration of QA (Figure 7A), which was paralleled by a decrease in the expression levels of IDO2 and of HAAO mRNAs in liver (Figure 7B and C).

Discussion

A large number of epidemiological studies and animal investigations show that obesity is associated with learning and memory impairment. However, the mechanisms underlying this cognitive alteration remain largely to be determined. The results presented herein provide compelling evidence for the involvement of the kynurenine pathway of tryptophan metabolism in obesity-induced cognitive deficits. Namely, adult rats that became obese by exposure to a fc-HFHS diet, showed learning difficulties characterized by a decreased ability to acquire new information but not to remember a previously encoded memory trace. These alterations were associated enhanced production of QA, XA, Nam, and NAD+ in the hippocampus along with upregulated expression of mRNAs coding for KMO, an enzyme involved in the transformation of KYN into XA and QA.

QA is known for its neurotoxic action and its deleterious effects on learning,[41,57] resulting from its action as an agonist of glutamate NMDA receptors. Indeed, overstimulation of these receptors by QA, leads to massive calcium entry into the cell which results into free radical production, cytoskeleton disorganization, apoptosis, and mitochondrial dysfunction.[58] XA has also been shown to modulate the glutamatergic system via its interaction with the metabotropic receptors mGlu2 and mGlu3 and the inhibition of vesicular glutamate transporters (see Fazio et al[59] for review). Although the effects of XA on memory are yet to be determined, it has been shown that administration of this compound reduces the amplitude of field excitatory postsynaptic potentials (fEPSP) in the hippocampus,[60] suggesting that an increase in the brain concentration of XA could inhibit the neuronal plasticity which underlies the learning process.

Both QA and XA are by products of the neurotoxic branch of KYN metabolism. This pathway is initiated by the conversion of KYN to 3-hydroxykynurenine (3-HK), by KMO. 3-HK is then converted to XA by kynurenine amino transferase and, subsequently, to 3-hydroxyanthranilic acid, QA and NAD+ by, respectively, kynureninase, 3HAAO and quinolinate phosphoribosyl transferase. Consistent with the increase in the concentration of QA, the hippocampal content of NAD+ and of Nam, a by-product of the KP which serves as a precursor for NAD + synthesis via the salvage pathway, was also enhanced by obesity. Supplementation with Nam[61,62] or NAD+[63,64] in animal models of Alzheimer's disease, aging or diabetes, have been shown to restore the cognitive defects that are associated

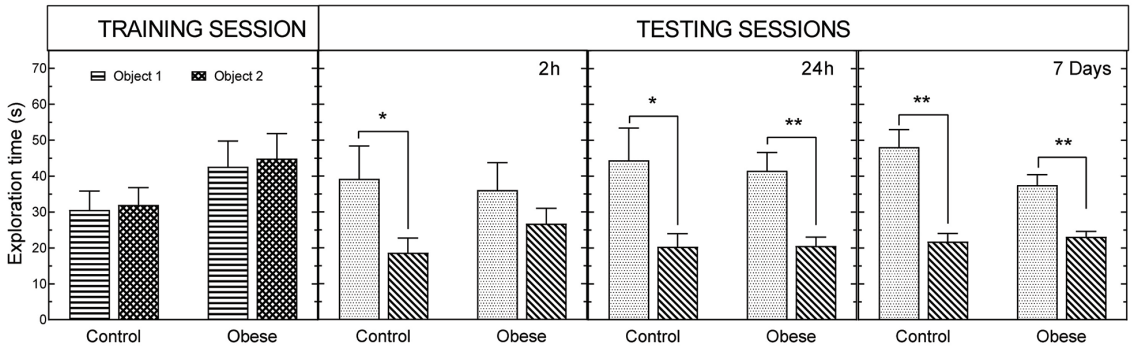

Figure 4. Evaluation of the impact of obesity on the different components of the memory process. The learning capacity of 105-day-old male rats, was tested using a combination of the novel object recognition and novel object location tests. Bars represent the capacity of the animals to encode (2 hours), consolidate (24 hours), and retrieve (7 days), new information. Learning tests were performed at the beginning of the dark phase of the animal's dark-light cycle. Data represent the mean ±SEM of the data from a total number of 9 to 12 animals per experimental group.
*$P < .05$ as determined by Student's *t*-test.

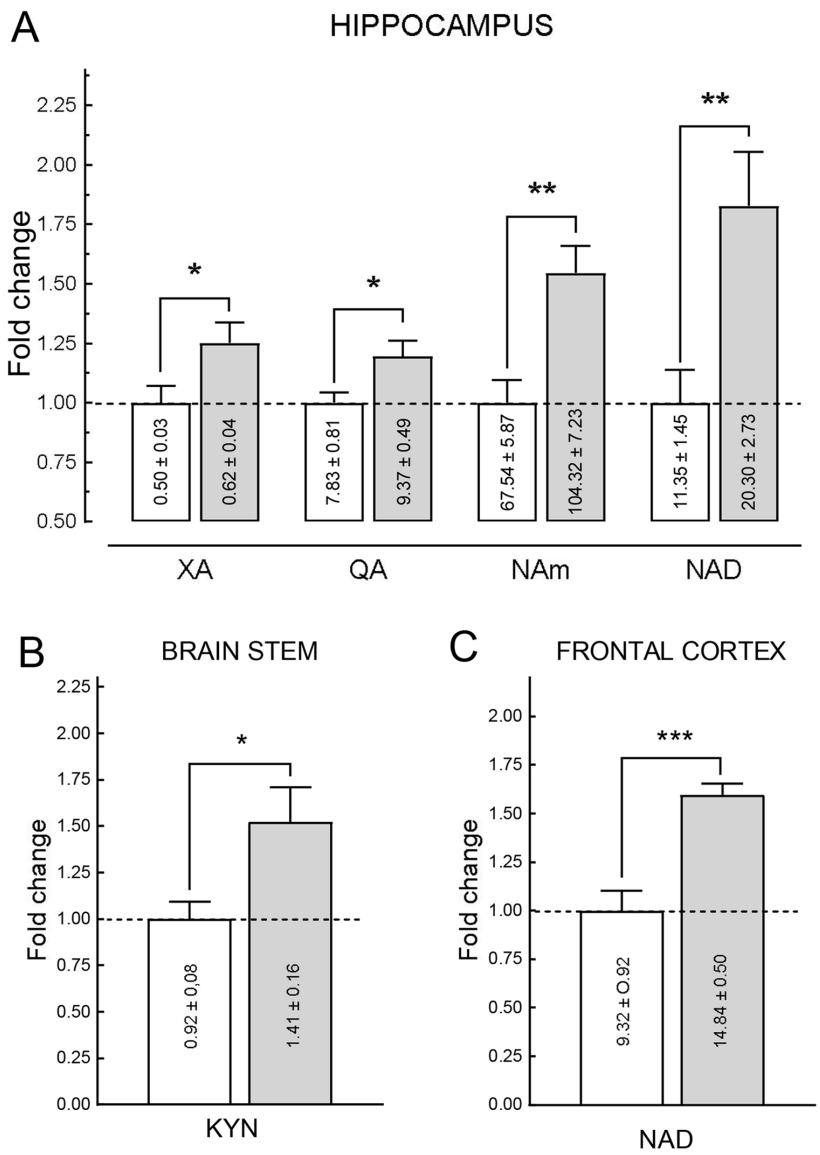

Figure 5. Concentration of several metabolites derived from the metabolism of tryptophan through the kynurenine pathway in the hippocampus (A), brain stem (B) and frontal cortex (C) of 105-day-old control (white bars), and obese (grey bars), male rats. The concentration of the different metabolites was determined by liquid chromatography–tandem mass spectrometry using brain samples from 7 to 8 animals per group. Data are expressed in percentage of values in adult animals fed a standard diet. Absolute values in pmol/g of tissue of xanthurenic (XA) and quinolinic (QA) acids as well as of kynurenine (KYN) and NAD+, for control animals in the different brain regions are indicated in the corresponding white bars.
*$P < .05$; **$P < .01$, ***$P < .001$ as determined by Student's *t*-test.

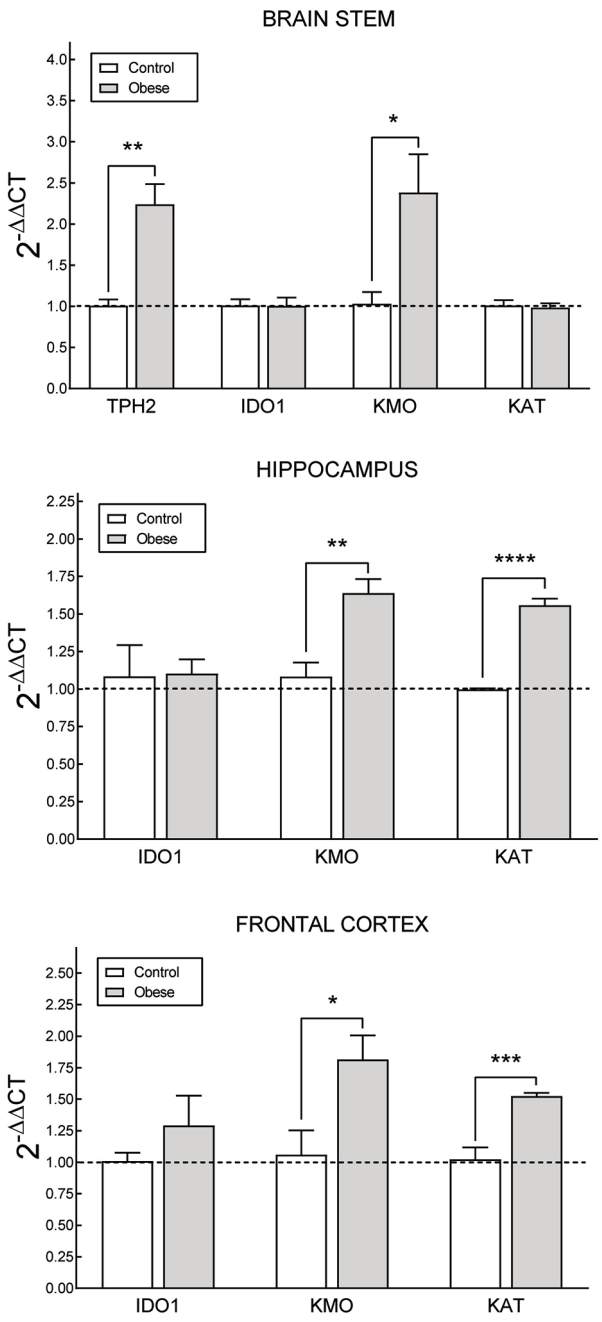

Figure 6. Expression levels of mRNAs encoding for key enzymes of tryptophan metabolism through the kynurenine pathway in the brain of control and obese 105-day-old male rats. The expression of TPH2, IDO1, KMO, and KAT, was determined by real-time quantitative PCR. Variations in gene expression were calculated by the 2-ΔΔCT method using the expression of control animals as a calibrator. Data represent the mean ±SEM from a total of 8 animals per experimental group. White bars, control group; Grey bars, obese group.
*$P < .05$. **$P < .01$. ***$P < .001$ (Student's *t*-test).

with these pathological conditions. It may therefore seem paradoxical that the concentration of Nam and NAD+ is increased in obese rats while they show learning deficits. Nevertheless, negative effects on brain function have also been reported following the administration of these compounds.[65,66] In particular, the degeneration of dopaminergic neurons and the motor deficit characteristic of Parkinson's and Huntington's disease are exacerbated by Nam injection.[67,68] Similarly, in vitro studies in cell cultures or brain slices have shown that exposure to NAD + results in astrocyte cell death.[69] Further research is needed to establish the relationship between increased brain NAD+ and Nam concentration and the learning deficits identified in the obese animals in this study.

The results of the present study also show that the changes in brain KYN metabolism in response to obesity differ by brain region. Thus, in contrast to the increase in QA, XA, Nam, and NAD + concentrations in the hippocampus, only the content of NAD+ was increased in the brain stem of obese animals. On the other hand, consumption of the high-calorie diet increased the concentration of KYN only in the frontal cortex. This differential response pattern, can be explained by the phenotypic and functional heterogeneity of microglial cells. Indeed, in the brain, the transformation of KYN into KA takes place predominantly in astrocytes, whereas the synthesis of 3-HK and other metabolites resulting from its transformation, including QA, is carried out mainly in the microglia.[70,71] The gene expression profile and physiological characteristics of microglial cells vary from one brain region to another, including in response to an immunological challenge.[72,73] In this respect, an observation of particular relevance to the present study, is that stimulation of the immune response by peripheral administration of LPS increases the proliferation, and thus density, of microglial cells in the dentate gyrus and subventricular zone of the hippocampus but not in the cortex.[74]

In order to determine the mechanisms underlying the alterations in the brain concentration of Trp metabolites in obese animals, the expression level of the genes coding for the main enzymes regulating its metabolism was assessed by RT-PCR. The results of these analyses showed that obesity increases the expression of KMO in the 3 brain regions examined and upregulates the levels of mRNAs coding for TPH2 in the brain stem along with those of KAT in the hippocampus and frontal cortex. The increased levels of XA, QA and NAD+ in the hippocampus, could therefore be consecutive to the enhanced KMO expression detected in this brain region. In contrast, obesity enhanced the expression of TPH2 in the brain stem without affecting the concentration of 5-HT. Similarly, there is no correlation between the increased expression of KAT in the hippocampus and frontal cortex and the absence of change in KA levels in these structures or between the increase in KYN concentration and the lack of change in KAT expression in the brain stem. It must be considered, however, that there are several levels of regulation between the synthesis of a given mRNA and its translation into a functional protein. These include the control of mRNA stability and translation efficiency as well as the post-translational modifications and the level of degradation of the protein it encodes. Consequently, there is not always a direct correlation between the expression levels of an mRNA and the concentration and/or functional activity of the protein which results from its translation. Another factor to consider is the lack of cellular resolution of our analyses. Indeed, TPH2 expression takes

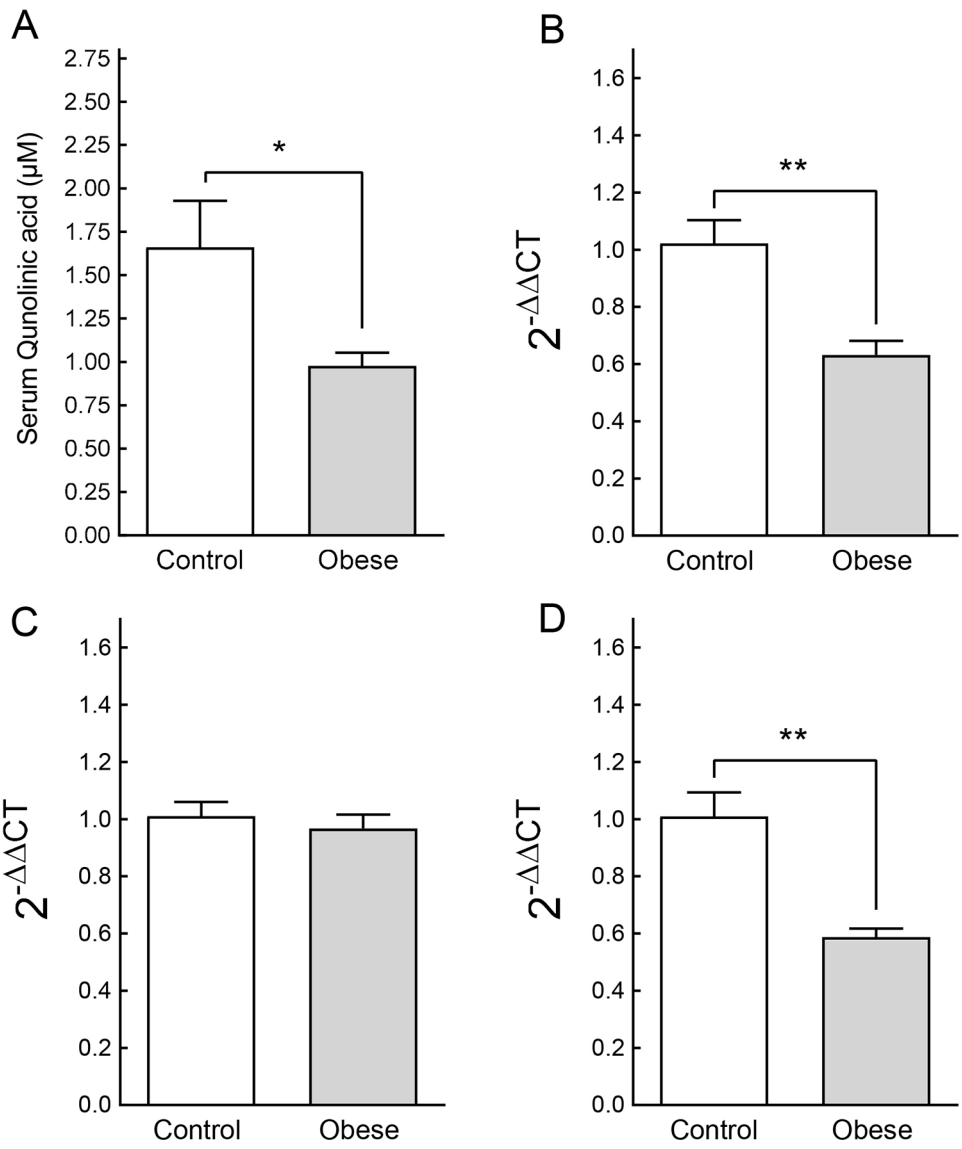

Figure 7. Concentration of quinolinic acid in serum (A) and gene expression levels of IDO2 (B), TDO2 (C) and HAAO (D) mRNAs in liver of control and obese 105-day-old rats. The concentration of QA was determined by liquid chromatography–tandem mass spectrometry whereas the expression of IDO2, TDO2, and HAAO mRNAs was determined by real-time quantitative PCR. Variations in gene expression were calculated by the 2-ΔΔCT method using the expression of control animals as a calibrator.
Data correspond to the mean ± SEM from a total number of 7 to 8 animals per experimental group *$P < .05$. **$P < .01$ (Student's *t*-test).

place in serotonergic neurons located in the raphe nuclei which are part of, but that do not constitute, the entire brainstem. On the other hand, Trp metabolism via the KP in the brain takes place in glial cells, which are present in a slightly lower proportion than neurons.[75] Hence, the mixture of cell types on which the analyses were performed is a limitation of the present study. To overcome this limitation, it would be necessary to perform experiments on enriched preparations or primary cultures of glial cells. However, the separation of glial cells from neurons and their subsequent segregation into astrocytes and microglia in the adult brain, remains a highly challenging task.

We conducted analyses in the brain stem because both positive and negative actions on learning have been described for 5-HT[76,77] and because the raphe nuclei located within this brain region are the seat of serotoninergic neurons which innervate the whole brain. Concerning the hippocampus and the prefrontal cortex, these regions are considered to be the main areas underlying the memory process. It is indeed accepted that the encoding and retrieval of information relies on a bi-directional communication network between the hippocampus and the prefrontal cortex. Within this network, the acquisition and consolidation of new information is carried out in the hippocampus while the retrieval of a memory trace is done in the frontal cortex (reviewed by Preston[78]). The increased concentration of QA in the hippocampus but not in the frontal cortex in obese animals, is therefore consistent with their cognitive deficit characterized by a difficulty in encoding new information. On the other hand, the fact that there were no differences between obese and control animals in the cerebral content of 5-HT or 5-HIAA, indicates that the cognitive disturbances linked to obesity result mainly from the alteration of Trp metabolism through the kynurenine pathway.

It should be noted, however, that in contrast to our observations, Haleem and Mahmood,[79] recently reported an increase in the concentration of Trp and 5-HIAA in the hippocampus of obese rats. Several factors may explain these discrepancies. First, the housing conditions of the animals and the obesity model differed significantly between the 2 studies. Thus, while in our case the animals were made obese by exposure to a free-choice high-fat, high-sugar diet and were always housed at least 2 per cage and at a controlled temperature of 21°C, in Halem and Mahmood's study the rats were fed exclusively with a high-fat diet and were housed individually at a temperature of 24 ± 2°C. Another important difference concerns the phase of the circadian cycle during which the animals were sacrificed, that is, during the dark phase in the present study versus the light phase in the study by Haleem and Mahmood. Interestingly, despite these experimental differences and the use of 2 distinct learning tests, the NOR-NOL test in the present study and the water maze test in Haleem and Mahmood, in both studies obese animals showed reduced memory encoding. The analysis of other functional parameters of the serotonergic system, such as the quantification of the expression and the evaluation of the sensitivity of the 5-HT receptors involved in the regulation of the memory process, should allow to draw a definitive conclusion as to the involvement of 5-HT in the cognitive alterations induced by obesity.

The brain concentration of KYN is determined by its synthesis in microglial cells from Trp and by the amount of kynurenine transported into the brain from the bloodstream since, unlike 5-HT, KYN can cross the blood-brain barrier.[80] At the peripheral level, 85% to 95% of Trp is bound to albumin and the remaining 15% to 5% flows in free form.[81-83] Only free Trp can cross the blood-brain barrier via the large neutral amino acids (LNAA) transporter. The binding site of Trp on albumin is common to free fatty acids[84] and, in fact, NEFAs inhibit, in vitro, the binding of Trp to albumin, thereby increasing the concentration of free Trp.[43,44] Furthermore, it has been shown that the administration of insulin increases the cerebral transport of Trp.[85,86] This effect would be the result of a decrease in the blood concentration of LNAA, whose uptake by the muscle is stimulated by insulin, and thus of a greater availability of the LNAA carrier for the transport of free Trp into the brain. On the basis of these data from the literature, we hypothesized that the neuroinflammation triggered by the consumption of a high-calorie diet would activate the KP and that this process would be favored by an increased passage of Trp to the brain due to the stimulatory action of insulin on this process and the increase in free Trp in the bloodstream resulting from its displacement from albumin by free fatty acids.

However, contrary to our expectations, there was no difference in serum free Trp levels between control and obese animals despite the hyperinsulinemia of the latter. Obese animals also showed the same circulating levels of KYN and NEFAs than control animals. Several arguments can be advanced to explain these results. First, we deliberately euthanized the animals at the same time of the day and in the same nutritional status at which the memory tests were performed. That is, 2 hours after the beginning of the dark phase of their light-dark cycle and under fed conditions. However, the blood concentration of NEFAs decreases after each meal so that to observe any variation in the circulating level of these lipids, a period of food deprivation of at least 12 hours is necessary even in obese individuals. On the other hand, the deregulation of lipid metabolism induced by obesity is directly related to its severity. Actually, more than 8 weeks of exposure to a hyperlipidic diet were required to observe an increase in serum NEFAs levels after an overnight fast in rats.[87] Finally, the correlation between increased NEFAs concentration at the peripheral level and enhanced brain Trp[88,89] as well as the stimulatory effect of insulin on brain Trp transport,[86] have been documented only in fasted animals. Therefore, the results obtained in this study lead us to conclude that Trp metabolism at the peripheral level is not involved in the cognitive alterations induced by obesity.

The first experimental series that was performed showed that obesity reduces learning capabilities in male animals but not in female rats. This observation is in agreement with several reports in the literature indicating that there are sex differences in how new information is processed within the hippocampus,[90] and with the results of Hwang et al[91] and Lord et al[92] who showed that male mice or male rats made obese by exposure to a high-fat diet show learning deficits while their female counterparts exposed to the same nutritional insult do not display any decrease in their memory capacity as compared to control animals. However, learning deficits in obese female rats have also been reported.[93] These discordant results could be explained by the impact of sex hormones on memory. Indeed, estrogens positively impact learning such that female rats generally exhibit better cognitive performances during the estrous phase of the estrous cycle when estrogen levels are at their highest.[94-97] Unfortunately, the phase of the estrous cycle during the memory tests was not documented either in the present investigation nor in the aforementioned studies. In consequence, it remains to be determined whether the negative effects of obesity on learning in female rats are attenuated or exacerbated as a function of the phase of their estrous cycle.

In conclusion, the results of the present study show that, in rats, the learning deficit induced by obesity is characterized by a reduction of the capacity to acquire new information. This cognitive deficit is associated with a dysfunction of the cerebral metabolism of tryptophan through the kynurenine pathway which results in increased concentrations of QA, XA, Nam, and NAD+ in the hippocampus. Given the major role of the hippocampus in the encoding process of memory and the negative effects of QA on learning, it can be reasonably proposed that altered kynurenine pathway metabolism contributes to obesity-induced learning disabilities. The reduction of QA synthesis in obese animals through the blocking of IDO1 or KMO enzymatic activity with specific inhibitors, would add further support to this proposal and could pave the way for pharmacological treatment of obesity-related cognitive alterations. The deleterious effects of obesity on cognition

were observed only in male animals indicating that they are sex dependent. Additional studies are needed to determine the extent to which the effect of obesity on cognition in female rats is modulated by sex hormones.

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Author Contributions

FBJ conceived the study and designed the experiments. CEMG, ADA, LARC, SOH and CA performed the experiments and analysed the data. MC and AA performed the mass spectrometry analysis. ECG and EZ contributed with conceptual ideas. CEMG and FBJ wrote the original draft. All authors revised the manuscript and agreed to publish it.

Supplemental Material

Supplemental material for this article is available online.

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