Rats Subjected to Extended L-Tryptophan Restriction During Early Postnatal Stage Exhibit Anxious–Depressive Features and Structural Changes

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Abstract
Serotonin transmission dysfunction has been suggested to play an important role in depression and anxiety. This study reports the results of a series of experiments in which rats were subjected to extended maize-based tortilla diets during early postnatal stages. This diet contains only approximately 20% of the L-tryptophan in normal diets of laboratory rodents. Compared with controls, experimental rats displayed a significant increase of immobility counts in the forced swimming test and exhibited anxiety-like behavior in the elevated plus maze test after 1 month of diet treatment. Low levels of serotonin contents were found in prefrontal cortex, striatum, hippocampus, and brainstem using high-performance liquid chromatography. Immunocytochemical reactions against 5-Bromo-2-deoxyuridine revealed a significant decrease in the proliferation rate for the subgranular zone of dentate gyrus. c-Fos expression after the forced swimming test was found reduced in prefrontal cortex, dentate gyrus, CA1, and hilus of hippocampus and amygdala. Moreover, dendrite arbor atrophy and decreased spine density were evident in Golgi-Cox-impregnated CA1 pyramidal neurons. Abnormal dendrite swelling in dentate gyrus granule cells was also observed. These findings indicate an involvement of hyposerotonnergia in emotional disturbance produced by L-tryptophan restriction during critical developmental stages and suggest that neuroplasticity changes might underlie these changes.

Key Words: Animal model of depression, BrdU, c-Fos, Dendrite spines, Dendrite varicosities, Golgi-Cox, Serotonin.

INTRODUCTION
It is generally believed that serotonin (5-HT) neurotransmission dysfunction plays an important role in the etiology of depression, in which despair, anxiety, and anhedonia are the central symptoms. This hypothesis is mostly supported by the findings of reduced 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in postmortem studies of brain areas and cerebrospinal fluid of suicide victims (1). The postmortem studies also suggested an upregulation of 5-HT2 and 5-HT3 receptors and abnormal reductions of brain 5-HT transporter sites (2). That serotonin plays a role in depression was suggested earlier from the observed depressed mood after drug administration depleting this biogenic amine (3, 4). This observation led to development of pharmacologic drugs that increase 5-HT levels such as selective serotonin reuptake inhibitors (5, 6).

Serotonin also acts as a regulator of brain development and neuroplasticity. There are several experimental evidences suggesting this regulation: 1) serotonin autoregulates development of neurons producing serotonin (7); 2) serotonin exerts effects on the development of target tissue (8); 3) changes in serotonergic transmission alter gene expression of brain derived neurotrophic factor (9, 10); 4) depletion of brain 5-HT by the tryptophan hydroxylase inhibitor parachlorophenylalanine decreases expression of MAP2, a dendritic protein (11) as well as synaptic density (12); and 5) the immediate early gene c-Fos, a transcriptional factor, has been shown to be induced by increase in brain 5-HT (13–15) and the expression of immediate early gene Arc (activity-related cytoskeleton-associated protein), a so-called “effector” immediate early gene, can be induced in the cortical neuronal dendrites by increasing extracellular 5-HT (16).

L-tryptophan (TRP) depletion challenge has provided a physiological tool to examine the 5-HT system in depression (17). TRP depletion induces depressive symptoms in healthy controls at high familial risk for depression (18–21). Moreover, relapses were observed in depressed patients who had previous improvements by serotonin reuptake inhibitors and were later administered a cocktail of amino acids without L-tryptophan (22).

Maize corn has been a main dietary component for American indigenous populations for many centuries. The native maize possesses low contents of several essential amino acids, among them TRP (23), the precursor of 5-HT. The Nixtamal process to prepare tortilla, which is a traditional method used in Mexico and Central America, depletes further tryptophan contents (24). It has also been documented that American indigenous populations have high...
The early development of the serotonin system and its extensive brain connections suggest that the 5-HT could play an important role in the development of the brain (28). Therefore, we hypothesized that a deficit of serotonin’s substrate TRP in crucial developmental stages might produce structural changes that could be reflected in the behavioral patterns. The aim of the present study was to evaluate whether a long-term, low-tryptophan tortilla diet (TD) during early postnatal stages induces depression-like and anxiety-like behaviors and, if so, ascertain if neurochemical and neuroanatomic changes after this diet restriction are involved.

### Materials and Methods

#### Diet Preparation

All experimental groups were fed ad libitum with pure TD made from maize landrace race Bolita from the Central Valley of Oaxaca, Mexico (donated by M. Bellon, International Maize and Wheat Improvement Center, CIMMYT). The Nixtamal procedure is described elsewhere (29). Vitamin compound (Berocca t; Roche, 8.4 mg tab/100 g body weight) was added to the TD to avoid malnutrition symptoms produced by deficit of vitamin B complex, which is lacking in maize, although this aspect is beyond the scope of the present study. Rats from control groups were fed with the same TD but supplemented with 0.2% L-tryptophan (Sigma, St. Louis, MO) to match the tryptophan level of the commercial diets (e.g. LabDiet).

#### Subjects and General Procedures

Wistar male rats of 2 postnatal ages (S1: P1–P28; S2: P28–P56) from the animal house at Faculty of Medicine, UNAM, were separated into 2 experimental groups: control and experimental. For the S1 group, 2 litters of lactating P1 (5 each) were housed with the corresponding females. For the S2 groups, rats were housed singly in standard Plexiglas cages. In both stages, animals were fed with corresponding diets and water ad libitum. Experimental subjects were kept in artificial light–dark cycles (light on at 3:00 PM and off at 3:00 AM), with controlled temperature and adequate ventilation. Distribution of animals and experimental design is shown in the Table.

#### Forced Swimming Test

After 4 weeks of TD treatment, 10 rats from each group were subjected to the forced swimming test during the late part of dark period of the artificial light–dark cycle. The original description and validation of the forced swimming test was published by Porsolt et al in 1978 (30) and modified by Detke et al in 1995 (31). For the present experiment, on day 1, as the pretest, the rats were immersed for 15 minutes in a vertical Plexiglas cylinder (45 cm height × 30 cm diameter) containing 25 cm of water kept at 24°C (32). Twenty-four hours later, the rats were immersed again and the swimming behavior was recorded by a video camera for 5 minutes. Analysis was done offline using the counting criteria described elsewhere (31). Briefly, the observer gives a score either “swimming” or “immobile” every 5 seconds. A rat was judged to be immobile when it remained floating, making only minimum movements necessary to keep its head over the surface. There were 60 counts per each record. Immobility in the forced swimming test provides a measure of behavioral despair.

#### Elevated Plus Maze Test

After 4 weeks of the TD treatment, 10 rats of each group underwent the elevated plus maze test. The plus maze was made of wood and consisted of 2 open arms (50 cm × 10 cm) and 2 opposite arms of equal dimensions enclosed by 40-cm high surrounding walls. The arms were connected by a central
square of 10 cm × 10 cm. The apparatus was elevated 50 cm from the floor and lit uniformly by white light (33, 34). Rats were placed at the center of the maze heading into an enclosed arm and then left for free exploratory activity for 5 minutes. A video camera was mounted vertically over the maze, and the behavior was recorded and scored offline. The percentage of time spent in the open arms of the maze provides the measure of anxiety (33).

The Sucrose Preference Test for Anhedonia Assessment

This test has been used for anhedonia assessment and was first described by Willner et al in 1987 (35). Briefly, the test cages were equipped with 2 liquid drop dispensers and placed next to each other. One dispenser contained plain water and another contained 1% sucrose solution. The animals were allowed to consume the fluids for a period of 24 hours. Daily consumption was determined by weighing the bottles before and after the test. A reduction of the sucrose liquid intake provides an indirect measure of desensitization of the brain reward mechanism.

Determination of Serotonin Contents

Serotonin contents in specific brain areas were measured with high-performance liquid chromatography (HPLC) with fluorescence detector. Immediately after the elevated plus maze test, 10 rats of each group were killed by decapitation; the prefrontal cortex, striatum, hippocampus, and brainstem were dissected quickly on an ice-cooled Petri dish. Tissues were kept in labeled Eppendorf microtubes, weighed, and quickly frozen with liquid nitrogen and kept in −70°C freezer until processed. Serotonin contents (ng of 5-HT/mg of protein) were measured by HPLC coupled to a fluorescence detector (Waters 474; 290-nm λ excitation and 330-nm λ emission) according to Peat and Gibb (36). Briefly, 100 μL of ice-cooled 0.1 M perchloric acid containing 4 mM of sodium metabisulfite/30 mg of wet tissue were added to all samples. After homogenization, the extract was centrifuged at 15,000 × g for 10 minutes at 4°C. The supernatants were passed through a Millipore HV filter of 0.45-μm of pore size and 5 mm and maintained at −3.9-mm internal diameter) with a particle size of 5 μm and maintained at 30°C. The mobile phase consisted of a mixture of methanol, double-distilled deionized water (3:2), and 0.02 mM of monobasic potassium phosphate with 3:2, m of the following brain regions: prefrontal cortex (4.2–3.2 mm, rostral to bregma), striatum (2.2–1.6 mm, rostral to bregma), dorsal hippocampus and amygdala (2.56–3.80 mm caudal to bregma), and brainstem (7.30–8.30 mm caudal to bregma), according to Paxinos and Watson (38), were obtained using a vibratome (Leica VT 1000, Heidelberg, Germany). Free-floating sections were processed for 10 minutes. For BrdU-IR, sections from dorsal hippocampus were incubated in 2N HCl for 30 minutes at 37°C for DNA denaturation. After this step, sections were rinsed twice with 0.1 M borate buffer (pH 8.5) followed by a rinse with phosphate buffer. All the immunoreactive sections were incubated with 0.3% Triton X-100 (Sigma, T-7878) and 0.3% bovine serum albumin (Sigma, A2153) in phosphate buffer for 1 hour at room temperature. This solution was used in the following steps unless specified. The following primary antibodies were used: rat anti-BrdU (Accurate Scientific, OBT003, 1:1000); mouse anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA; SC52, 1:1000); and sheep antitryptophan hydroxylase (Chemicon International, Temecula, CA; AB1541, 1:1000). After overnight incubation with primary antibodies, sections were rinsed 3 times for 10 minutes and then incubated for 4 hours at room temperature with the following biotinylated secondary antibodies: rabbit antirat (Vector Labs, Burlingame, CA; 1:200) for BrdU, horse antimouse (Vector Labs;1:200) for c-Fos, and rabbit antitrypsin (Vector Labs;1:200) for tryptophan hydroxylase. Finally, sections were incubated in avidin–biotin–peroxidase complex (Elite ABC kit; Vector Labs) for 1 hour at room temperature. Peroxidase was detected using diaminobenzidine (DAB) as chromogen. Sections were developed using Liquid DAB-Plus substrate kit (Zymed Laboratories, San Francisco, CA; 00–2020).

Golgi-Cox Impregnation

For Golgi-Cox impregnation procedures, rats at P28 (S1) and P56 (S2) received an overdose of anesthesia and were decapitated. Brains were removed from the skull and their central one-third parts (along anteroposterior axes) were sliced with a sharp blade into blocks of approximately 10-mm thickness. Tissues were briefly rinsed with phosphate buffer 0.1 M (pH 7.4) and then immersed in sequenced impregnation solutions (FD Rapid GolgiStain kit; FD Neuro Technologies, Ellicott City, MD) for 2 weeks in the dark. Sections of 200 μm were sliced using a CO2-cooled glider microtome and mounted on gelatin-coated glass slides.

Histologic Procedures

Ninety minutes after the forced swimming test, rats were anesthetized with an overdose of sodium pentobarbital and perfused transcardially with 0.9% saline followed by cold fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) plus 15% v/v of saturated picric acid for 15 minutes. Brains were removed, blocked, and then thoroughly rinsed with phosphate buffer. Coronal sections of 50 μm of the following brain regions: prefrontal cortex (4.2–3.2 mm, rostral to bregma), striatum (2.2–1.6 mm, rostral to bregma), dorsal hippocampus and amygdala (2.56–3.80 mm caudal to bregma), and brainstem (7.30–8.30 mm caudal to bregma), according to Paxinos and Watson (38), were obtained using a vibratome (Leica VT 1000, Heidelberg, Germany). Free-floating sections were collected for use in subsequent immunohistochemical reactions with 3% sodium peroxide in phosphate buffer for 10 minutes. For BrdU-IR, sections from dorsal hippocampus were incubated in 2N HCl for 30 minutes at 37°C for DNA denaturation. After this step, sections were rinsed twice with 0.1 M borate buffer (pH 8.5) followed by a rinse with phosphate buffer. All the immunoreactive sections were incubated with 0.3% Triton X-100 (Sigma, T-7878) and 0.3% bovine serum albumin (Sigma, A2153) in phosphate buffer for 1 hour at room temperature. This solution was used in the following steps unless specified. The following primary antibodies were used: rat anti-BrdU (Accurate Scientific, OBT003, 1:1000); mouse anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA; SC52, 1:1000); and sheep antitryptophan hydroxylase (Chemicon International, Temecula, CA; AB1541, 1:1000). After overnight incubation with primary antibodies, sections were rinsed 3 times for 10 minutes and then incubated for 4 hours at room temperature with the following biotinylated secondary antibodies: rabbit antirat (Vector Labs, Burlingame, CA; 1:200) for BrdU, horse antimouse (Vector Labs;1:200) for c-Fos, and rabbit antitrypsin (Vector Labs;1:200) for tryptophan hydroxylase. Finally, sections were incubated in avidin–biotin–peroxidase complex (Elite ABC kit; Vector Labs) for 1 hour at room temperature. Peroxidase was detected using diaminobenzidine (DAB) as chromogen. Sections were developed using Liquid DAB-Plus substrate kit (Zymed Laboratories, San Francisco, CA; 00–2020).

Immunocytochemical Reactions for 5-Bromo-2-deoxyuridine (BrdU), c-Fos, and Tryptophan Hydroxylase

BrdU Injections

Rats from the subgroup of the forced swimming test received intraperitoneal injections of BrdU (Sigma, B9285), 10 mg/mL of 0.9% NaCl solution during 3 consecutive days 24 hours before the forced swimming test (daily dose: 50 mg/kg body weight fractionated in 3 injections per day).
These sections were dried naturally at room temperature in the dark and then stained with staining solution provided by the kit mentioned previously. Dendritic patterns of 2 representative CA1 pyramidal neurons were reconstructed using a drawing tube at C2 magnification. Spine density was calculated by tracing a length of dendrite (20 μm long) at C2 from 5 randomly chosen segments of hippocampal CA1 pyramidal neurons: left primary basilar branch, right primary basilar branch, primary apical branch, secondary apical branch, and tertiary apical branch. The number of spines was counted with the help of a light microscope (Nikon T550i) equipped with a drawing tube. No attempt was made to correct for the fact that some spines were obscured from view, so the measure of spine density necessarily underestimates total spine number.

Statistical Analyses
Quantitative results were expressed as mean ± standard error of mean. Groups were tested for differences by performing analysis of variance followed by Student-Newman-Keuls test using Instat (GraphPad Software, San Diego, CA). Differences were considered statistically significant at a value p < 0.05 (*, p < 0.05; **, p < 0.01; ***, p < 0.001: vs control group).

RESULTS
The TD prepared for this study contained 0.056% of TRP, which is approximately one fifth of the TRP for normal rodent diet (e.g. LabDiet contains 0.27% of TRP [www.LabDiet.com]). The fat and protein contents of TD are 1.0% and 5.4%, which represent 25% and 20%, respectively, of the commercial rat diet mentioned previously. The average amount of dried TD taken by rat per day is approximately 10 g/100 g body weight. The average body weight of the control and experimental groups was approximately 20% lower than in rats from the animal house of the same age and fed a commercial rodent diet. This weight loss is mainly attributable to low caloric consumption. Pilot studies to assess the related behavioral and anatomic aspects of rats both fed a commercial rat diet (having same weight or having same age) and fed a TD supplemented with L-tryptophan revealed no significant differences.

Animals with TRP depletion showed a significant augmentation of immobility counts during the forced swimming test: 38.375 ± 3.246 versus 23.44 ± 2.102 of the control (n = 10, p < 0.01, Fig. 1A). Also, in the test that was used to evaluate anxiety-like behavior (i.e. elevated plus maze), a significant difference was observed between both groups (Fig. 1B). Experimental animals spent less time...
A loss of pleasure (anhedonia) is an important clinical feature of major depressive episodes. Anhedonia is commonly tested in rats as a decreased consumption of a sweet palatable solution. In the present experiment, rats had the opportunity to choose between water and a solution with 1% of sucrose and did not show any difference between experimental and control groups (17.790 g ± 3.214 g vs 17.001 g ± 1.629 of control, Fig. 1C).

Serotonin levels in the prefrontal cortex, striatum, hippocampus, and brainstem were reduced in all regions in the experimental group with a greater difference in the prefrontal cortex (Fig. 2).

Ninety minutes after completing the forced swimming test, rats were perfused and expression of one of the immediate early genes, c-Fos, was measured by immunocytochemistry against c-Fos in prefrontal cortex, hippocampus, and amygdala nuclei, the main brain regions that receive dorsal raphe nucleus serotoninergic input. An overall reduction of the c-Fos-positive nuclei density was observed (Fig. 3A). Figures 3B and 3C show the dramatic differences between a control animal and experimental animal in the dentate gyrus (DG) of dorsal hippocampus.

In terms of the enzymatic machinery of 5-HT synthesis, there was no significant effect in tryptophan hydroxylase-immunoreactive expression as can be seen in Figure 4, in which a pontine medium raphe area from an experimental animal is depicted in detail. This means that serotonin cells were not reduced in number as a result of the restriction of 5-HT precursor.

To elucidate the mechanisms underlying the behavioral changes, we studied the cytoarchitecture of hippocampal pyramidal neurons and DG granule cells that are related to some symptoms of major depression (e.g. memory...
impairment and anxiety). First, there was a significant decrease of BrdU-positive cells in the subgranular zone of DG (Fig. 5). Second, dendrite arborization and dendritic spine density were found reduced (Fig. 6). We also found structural changes such as abnormal dendrite swelling in DG granule cells of L-tryptophan-restricted animals (Fig. 6).

DISCUSSION

This study concerns the effects of feeding rats with a pure native maize-based TD, which contains low L-tryptophan (≤20% of normal laboratory diets for rodents) in the early postnatal stages for an extended period. This tryptophan restriction produced diminished levels of brain serotonin as determined by neurochemical procedures. The behavioral consequences of this restriction were a significant increase of immobility during the forced swimming test and a decreased exploratory activity measured using elevated plus maze. These behaviors have been reported in some animal models of both major depression and anxiety disorders (39, 40).

In addition to the depression/anxiety behavior-like constructs, this study also reports a change of the immediate early gene c-Fos expression through its induced protein c-Fos immunoreactivity in the main brain regions that receive dorsal raphe nucleus serotonergic input such as prefrontal cortex, dorsal and ventral striatum, and amygdala (41). Fadda et al reported a decrease in frontocortical 5-HT release after a TRP-free diet for 3 days (42). Therefore, one potential consequence of low TRP intake produced hypo-serotoninergia, a dysregulation of 5-HT mediated neuronal physiological responses.

It is generally believed that changes in neuronal morphology and cell number represent fundamental mechanisms in neuronal plasticity that allow an animal to adapt to environmental and pharmacologic stimuli and thereby make appropriate long-term responses (43). In this study, it was observed that an extended restriction of TRP during early postnatal stages produces dendritic structural changes in pyramidal neurons of the CA1 sector and in DG granule cells of hippocampus. The normal production of new DG granule cells also seemed to be affected in this animal model. The pontine dorsal raphe nucleus and median raphe nucleus project extensively to the hippocampus (44–46) with CA1 and CA2 regions receiving the most innervation (47). Virtually all subtypes of 5-HT receptors are present in hippocampal neurons (48). This innervation should be modified in a time- and activity-dependent manner because it has been observed that there is a decrease (approximately 37%) of both dorsal raphe nucleus and median raphe nucleus serotonergic neurons during the first 8 postnatal weeks (49). Our experimental observation of the morphologic changes and the decreased BrdU labeling, which most likely indicates a decreased neurogenesis in DG when compared with controls, strengthens the hypothesis that the serotonin system plays an important role during the critical period of neural development. Furthermore, the weakening of the 5-HT transmission caused by substrate restriction in early postnatal stages could modify the hippocampus neuronal circuitry and neuronal plasticity, which in turn could influence the neuronal turnover in the granular cell layer of the DG. There is evidence indicating that serotonin itself could work as a neurotrophic factor (50) and its deficit in the hippocampus most likely affect neurogenesis in the DG.

The chronic mild stress paradigm developed by Willner et al in 1987 exhibits considerable face, construct, and predictive validity and has been widely accepted for experimental use (51). In that model, rats are exposed to daily sessions of uncontrollable, inescapable stressors. The primary behavioral expression in that model is the subsensitivity to a reward, which may reflect anhedonia, a major symptom comprising the major depression disorder. Several studies have documented that the chronic mild stress model alters mainly the mesocorticolimbic dopaminergic pathway to produce a functional hypodopaminergia (51–53). This is mainly related to reward and motivational processes that are
FIGURE 6. Golgi-Cox impregnation. Dendrite structural and spine density changes in hippocampus CA1 pyramidal neurons and granule cells impregnated with Golgi-Cox procedure. (A) (tracing) and (B) (tissue immunoreacted with MAP-2) show the region where 2 representative CA1 pyramidal neurons were traced using a drawing tube ([C], control and [D], experimental) (scale bar = 40 μm). DG, dentate gyrus; A, alveus; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. 

(E–J) Photomicrographs of control (E–G) and experimental (H–J) animals showing dendrite spine density on primary (E, F), secondary (G, H), and tertiary (I, J) branches at ×1000 magnification (scale bar = 10 μm). Histograms show the analysis of dendritic spines in 20-μm lengths along the dendrite from animals treated with tryptophan-restricted diet or controls in stage 1 (K) and stage 2 (L). “Left b” and “right b” refer to the counting from basilar primary branches. “Primary,” “secondary,” and “tertiary” refer to the counting from apical branches. *** p < 0.001 (M, N) Representative microphotographs illustrating dendrite structural changes on granule cells of DG; a noticeable augment of dendrite swellings (arrows) in the experimental group which is an abnormal condition in these structures. Scale bar = 40 μm.
largely based on dopaminergic neurotransmission (54, 55). In our experiment, we found no difference between control and experimental groups with the sucrose intake test. This finding suggested that dopamine transmission-related anhedonia is not involved in the present model of serotonin precursor reduction.

Monoamine neurotransmission deficits had been widely accepted as the main factors in the pathogenesis of major depression disorder. These theories were mostly based on the principle of “reverse engineering” of antidepressants. However, this monoamine hypothesis has been challenged by the adaptive neuroplasticity hypothesis that emerged from the observation of the significant time delay before antidepressants begin to work in depressed patients (56). Duman et al proposed that depression could result from an inability to make the appropriate response to stress as a consequence of a dysfunction of the normal mechanism underlying neural plasticity (57). Chronic treatment with different classes of antidepressants, including selective 5-HT and noradrenergine reuptake inhibitors, upregulates the cyclic adenosine monophosphate transduction cascade, leading to the activation of cAMP-dependent protein kinase A, which phosphorylates proteins having a key role in cell signaling (57, 58). The diet tryptophan restriction paradigm and the behavioral and neurochemical changes observed from this study apparently were in concordance with the early hypotheses on the pathophysiology of major depression that were based on aberrant intrasynaptic concentration of mainly the neurotransmitters serotonin and norepinephrine. However, the pyramidal cells’ dendrite structural changes and the decreased cell turnover in the subgranular zone of DG observed in this study might implicate a temporal or longlasting structural neuroplasticity impairment and contribute to some, but not all, of the behavioral disturbances. In this context, the cellular and molecular mechanisms underlying the present findings still need further investigations.

Although it is naïve to expect that dysfunction of a single transmitter could be responsible for the constellation of symptoms that comprise depression and anxiety, and that an animal model for a disease involving higher human emotions cannot be fully validated, this experimental attempt represents a novel approach to the longlasting effects of a specific dietary constraint and could serve as an animal model to study the effects of hyposerotonergia during development on mechanics of brain activity related to measurable behaviors. In this sense, these results might give some insights about the influences of dietary culture on affective stereotype and social behaviors. However, it is worth stressing that we do not pretend to draw any conclusions about the human brain, and in particular no connection should be made with the cultural and social context of the American indigenous people.

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