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Research Report

Serotonin and 5-hydroxy-indole-acetic acid contents in dorsal raphe and suprachiasmatic nuclei in normal, malnourished and rehabilitated rats under 24 h of sleep deprivation

A. Alfaro-Rodríguez[a,*], R. González-Piña[b], A. González-Maciel[c], E. Arch-Tirado[d]

[a]Laboratorio de Neuroquímica, Instituto Nacional de Rehabilitación, SSA, México
[b]Laboratorio de Neuroplasticidad, Instituto Nacional de Rehabilitación, SSA, México
[c]Laboratorio de Microscopía Electrónica, Instituto Nacional de Pediatría, SSA, México
[d]Laboratorio de Bioacústica, Instituto Nacional de Rehabilitación, SSA, México

ARTICLE INFO

Article history:
Accepted 19 June 2006
Available online 31 July 2006

Keywords:
Serotonin
5-Hydroxy-indole-acetic acid
Dorsal raphe nuclei
Suprachiasmatic nuclei
Malnutrition
Sleep deprivation

ABSTRACT

It has been discussed that serotonin (5-HT) could be involved in the effects of sleep deprivation (SD) and/or malnutrition (M) on the sleep–wake cycle. The aim of this work was to study the effects of the M, SD and its interaction on 5-HT and 5-hydroxy-indole-acetic acid (5-HIAA) contents in the dorsal raphe (DR) and the suprachiasmatic nuclei (SCN), two sleep–wake cycle regulators. Forty-eight puppets rats were obtained from mothers fed with low or normal casein diet. They were allocated in 3 groups ($n=16$ each): prenatal/postnatal casein malnutrition (6/6%), prenatal casein malnutrition/nutritional casein rehabilitation (6/25%) and prenatal/postnatal casein well-nourished state (25/25%). When rats were 60 days old, 24 animals were exposed to sleep deprivation by means of forced locomotion during 24 h. The remaining 24 were kept under normal conditions of sleep–wake cycle. Then, all animals were sacrificed by decapitation. DR and SCN were dissected and processed to determine the 5-HT and 5-HIAA contents by means of HPLC. It was observed that 6/6% rats showed a 5-HT increase (DR $p<0.011$; SCN $p<0.019$) as well as in SD (DR $p<0.0008$; SCN $p<0.0009$) with respect to 25/25% rats. No differences were found in 6/25% rats. Therefore, 5-HIAA decreased significantly in both nuclei in all the groups, notably in M+SD animals (DR $p<0.001$; SCN $p<0.001$). We conclude that the sleep–wake cycle disruptions produced by chronic M and SD are mediated in part by a synergistic effect on 5-HT in the DR-SCN pathway, perhaps due to a delay in the development of such brain structures.

1. Introduction

The growth of the central nervous system (CNS) and its developmental processes, such as neuronal differentiation and synaptogenesis, occurs during the prenatal and suckling periods in the rat, when the brain is more vulnerable to several types of aggressions (Morgane et al., 1993). Thus, nutritional insults can cause irreversible alterations (Morgane et al., 1992).

* *Corresponding author.* Laboratorio de Neuroquímica y Farmacología, Torre de Investigación 1er piso. Instituto Nacional de Rehabilitación, Av. México-Xochimilco No. 289, Col. Arenal de Guadalupe, Tlalpan, México, D.F. C.P. 14389.
E-mail address: alfa1360@yahoo.com.mx (A. Alfaro-Rodríguez).

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doi:10.1016/j.brainres.2006.06.069

In the man, it begins in the last quarter of gestation, continuing until the first years of life (Morgane et al., 1993). The effects of nutritional insults on the neurotransmitter systems, in particular the serotonergic one, deserve special attention since this system participates in a wide variety of CNS functions, such as sleep control (Borbély et al., 1980; Cespuglio et al., 1990; Steriade and McCarley, 1990; Gonzalez-Pina and Alfaro-Rodriguez, 2003; Penalva et al., 2003) and it is also an important regulator of the mammalian circadian clock in the suprachiasmatic nucleus (SCN; Albers et al., 1991). There are several experimental evidences about the effects of malnutrition on the serotonergic system (Stern et al., 1975; Wiggins et al., 1984; Chen et al., 1992). However, there are few works on the effects of manipulations of this system in undernourished subjects, such as sleep deprivation.

Sleep deprivation has been used as a tool in the study of function and disorders of sleep itself (Borbély et al., 1984; López-Rodriguez et al., 2003; Walker and Stickgold, 2004). It causes increase in the cerebral 5-HT concentration, while an increase in slow wave sleep (SWS) and a decrease in paradoxical sleep (PS) have been observed (Cintra et al., 1988; Datta et al., 2000; López-Rodriguez et al., 2003, Hipolide et al., 2005). It is known that the DR, the main serotonergic reservoir, plays a role in sleep control since a depression of DR neuronal firing during sleep has been described (McGinty and Harper, 1976). Previous reports showed that electrical stimulation of the dorsal raphe nucleus (DRN) or median raphe nucleus (MRN) evoked 5-HT release in the SCN, which has been identified as an important oscillator with pacemaker functions which takes part in different circadian rhythms such as the sleep–wake cycle (Moore, 1983; Vinogradova et al., 1998; Lister et al., 2005; Ertugrul and Rezaki, 2004).

Diet is determinant in the production of tryptophan, the precursor of 5-HT. Prenatal protein malnutrition significantly elevates brain levels of serotonin in rats, and these levels remain throughout their lives. This increase seems to be directly related to an increase in the free fraction of plasma L-tryptophan (Hernandez et al., 1989; Manjarrez et al., 2003; Lister et al., 2005). Carbohydrates consumption produces an increase in insulin which results in an increase of brain tryptophan (Harel and Tannenbaum, 1995; Bronzino et al., 1999). Then, it is possible that the serotonergic DR plays an important role in the SCN 5-HT modulation in the effects reported by malnutrition and sleep deprivation, which constitute an important health issue in human beings. However, the specific role of the DR-SCN pathway serotonergic mechanisms involved in the sleep–wake cycle disruptions produced by pre- and postnatal malnutrition and sleep deprivation is not clarified. Therefore, it is also unclear the 5-HT status in malnutrition rehabilitated rats.

2. Results

2.1. *Removal of discrete fresh regions*

Removal of individual plugs of tissue was accomplished by placing the cannula at the periphery of the region to be removed at a slight angle pointing away from the individual. Freshly cut sections have the advantage of having easily recognizable ventricle and fiber tract landmarks, as compared to frozen sections (Fig. 1, DR and Fig. 2, SCN).

2.2. *Effects of undernourishment on the concentrations of 5-HT (ng/mg of wet tissue) of DR and SCN*

A significant increase of 5-HT levels in DR (Fig. 3A, left) and SCN (Fig. 3B, left) in 6/6% animals was found. Such an increase was of 130% in DR ($p<0.0111$) and 123% in SCN ($p<0.0119$), when they were compared to the amount observed in 25/25% rats.

Animals of the 6/25% group did not show any significant differences, when compared to 25/25% animals (DR $p<0.189$; SCN $p<0.204$).

2.3. *Effects of undernourishment and sleep deprivation on the concentrations of 5-HT of DR and SCN*

A remarkable increase of 5-HT in DR (Fig. 3A, left) and in SCN (Fig. 3B, right) was observed in the 6/6%+SD, when they were compared with the 25/25% animals (DR 588% $p<0.0008$; SCN 426% $p<0.0009$) and the 25/25%+SD rats (DR 241% $p<0.0010$, SCN 173% $p<0.0010$). The 6/25% and 6/25%+SD animals did not show differences in the concentration of 5-HT with respect to 25/25% and 25/25%+SD groups, respectively. However, 6/25%+SD animals showed a light increase of 5-HT in SCN with respect to 25/25% group ($p<0.0154$). There were also statistical differences between 25/25% and 25/25%+SD rats, which consisted of an increase in 5-HT in DR ($p<0.0135$) and in SCN ($p<0.0143$), as an effect of sleep deprivation.

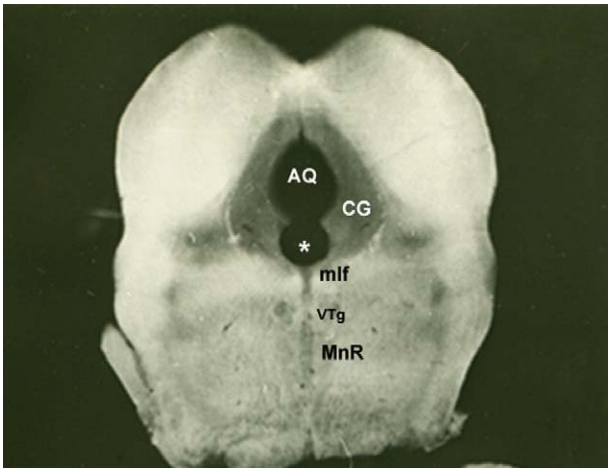

Fig. 1 – Vibratome section of the rat brain; 300-μm-thick slice; punch holes were made with a 500-μm-thick cannula, observed in the dorsal raphe nucleus region and marked with one asterisk (*). Reference regions, the cerebral aqueduct (AQ), the central periaqueductal gray (CG), the medial longitudinal fasciculus (mlf), the ventral tegmental nucleus (VTg) and the median raphe nucleus (MnR) are shown according to Paxinos and Watson (1998), interaural=0.7 mm, bregma=−8.3 mm.

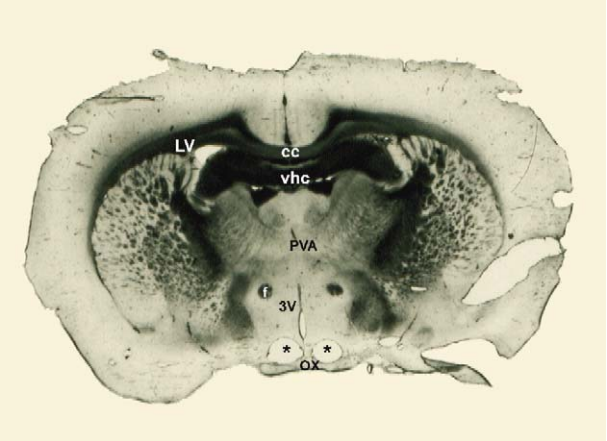

Fig. 2 – Punch holes were made with the 500-μm cannula, observed in the suprachiasmatic nucleus region and marked with one asterisks each (*). Reference regions, the lateral ventricle (LV), the corpus callosum (cc), the ventral hippocampal commissure (vhc), the third ventricle (3V), the optic chiasm (OX), the fornix (f) and PVA are shown according to Paxinos and Watson (1998), interaural = 7.7 mm, bregma = −1.3 mm.

2.4. *Effects of undernourishment on the concentrations of 5-HIAA (ng/mg of wet tissue) of DR and SCN*

The values of the concentration of 5-HIAA in ng/mg of wet tissue in DR (Fig. 4A, left) and SCN (Fig. 4B, left) in non-sleep deprivation and sleep deprivation animals, for each of the subgroups according to nutritional condition, are shown in Fig. 4. It can be observed that the 6/6% animals show an increase of 5-HIAA in DR of 66% with respect to 25/25% animals ($p<0.0149$) and of 100% in SCN ($p<0.0109$).

A significant 5-HIAA decrease in DR ($p<0.0165$) and an increase in SCN ($p<0.0168$) were observed in 6/25% animals.

2.5. *Effects of undernourishment and sleep deprivation on the concentrations of 5-HIAA of DR and SCN*

An important increase of 5-HIAA concentration in DR ($p<0.001$; Fig. 4A, right) and SCN ($p<0.001$; Fig. 4B, right) was observed in 6/6% + SD rats.

Significant increases of 5-HIAA in DR of 25/25% + SD rats with respect to 25/25% group ($p<0.0135$) as well that in SCN ($p<0.0143$) were also observed. Rats of 6/25% group also showed significant increases of 5-HIAA content in DR ($p<0.0165$) and SCN ($p<0.0168$) while the 6/25% + SD group did not show 5-HIAA changes with respect to 25/25% group, although a significant decrease was observed in DR ($p<0.0126$) and SCN ($p<0.0113$) when it was compared with 25/25% + SD group.

3. Discussion

Sleep deprivation and malnutrition diet increase 5-HT and 5-HIAA concentrations in both DR and SCN. Results of control group are in concordance with previous reports in which an increase in 5-HT in both nuclei has been speculated as stated in Chen et al. (1997) and Datta et al. (2000). The increase in 5-HT shown in our malnutrition model could be due to an enhanced brain tryptophan uptake, which has been reported after a lesser severe sleep deprivation (Datta et al., 2000; Blaise and Bronzino, 2000; Smith and Kennedy, 2003).

Malnutrition during early stages of CNS development accelerates brain 5-HT functioning biosynthetic chain. Increases in this neurotransmitter depend on the elevation of its plasmatic precursor (Manjarrez et al., 1996; Morgane et al., 1978). Thus, the higher tryptophan 5-hydroxylase affinity by L-tryptophan could explain the increase in enzymatic activity in malnourished rats. However, the fact that this enzyme is more active in phosphorylation conditions suggests that enzymatic mechanisms are implied in the activation of an excessive neurotransmitter synthesis. This way, the greater enzyme affinity and its major phosphorylation activity occur during the critical periods of development in

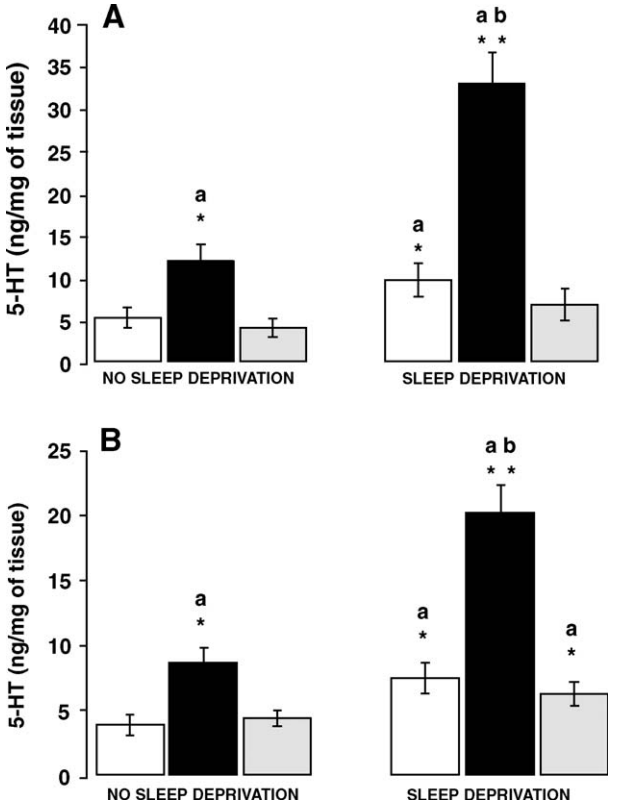

Fig. 3 – This figure shows (A) 5-HT concentration in DR and (B) 5-HT concentration in SCN. In the left plots, 25/25% control group (white bar), 6/6% malnourished group (black bar) and 6/25% rehabilitated (gray bar) rats, without sleep deprivation (SD). In the right plots: 25/25% + SD (white bar), 6/6% + SD (black bar) and 6/25% + SD (gray bar) rats, in which a significant increase was generated in 5-HT levels in both DR and SCN. The nutritionally rehabilitated group showed a reversible effect. Mean values (mean ± SD) were statistically compared using two-way ANOVA and Tukey test (*$p\leq0.01$, **$p\leq0.0001$) and found (a) a statistical difference as compared to control (no sleep deprivation) and (b) a statistical difference as compared to malnourished rats under sleep deprivation.

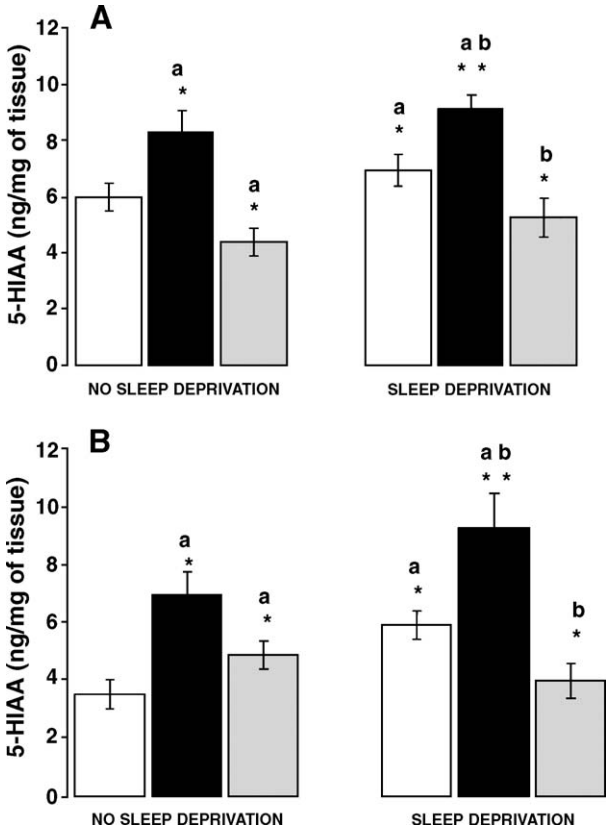

Fig. 4 – This figure shows (A) 5-HIAA concentration in DR and (B) 5-HIAA in SCN. Right plots: 25/25% control group (white bar), 6/6% malnourished rats (black bar) and 6/25% rehabilitated (gray bar) rats. Left plots show animals in different nutritional state plus SD, where the pattern of changes seems to the 5-HT in DR and SCN. Mean values (mean ± SD) were statistically compared using two-way ANOVA and Tukey test (*$p \leq 0.01$, **$p \leq 0.001$) and found (a) a statistical difference as compared to control (no sleep deprivation) rats and (b) a statistical difference as compared to malnourished rats under sleep deprivation.

malnourished animals, producing structural changes in the tryptophan 5-hydroxylase enzymatic complex, which plays a fundamental role in the serotonin synthesis mechanisms (Growdon and Wurtman, 1976; Bronzino et al., 1999; Manjarrez et al., 2003; Sing and Sanyal, 2004). This could explain the increase in 5-HT levels found in DRN and SCN of malnourished rats.

Then, the increase in 5-HT concentrations produced by malnutrition is a consequence of a higher plasmatic tryptophan availability, eliciting a high incorporation of this amino acid in CNS (Hernandez et al., 1989; Bronzino et al., 1999; Mokler et al., 2003). These facts correlate with the results of our study since the highest concentration of 5-HT was found in DR, which is the main source of 5-HT in the CNS and whose afferences are interconnected with the forebrain. It is important to note that we also found a significant increase in 5-HT concentrations at SCN.

We have to keep in mind the effect of sleep deprivation, which has also been reported to increase the levels of indolamine (Borbély et al., 1980). Under this condition, 5-HT levels are low, but they increase during wakefulness, which highly suggests that these effects are produced by a forced wakefulness during 24 h (Borbély et al., 1980; Bergmann et al., 1989, 1994).

Another line of evidence has demonstrated that chronic malnutrition produces a delay in the development of vigilance states and more time spent in SWS and PS in rats. The conclusion has been reached that malnutrition affects the nervous structures that control the homeostatic functions of vigilance states and those that regulate their circadian rhythmicity, which is another fact in agreement with our results. We may propose that malnutrition and sleep deprivation produce a synergic effect, shown by the important 5-HT elevations observed in both DRN and SCN. However, this increase is not reflected in 5-HIAA concentrations. This way, the enzymatic system would be acting normally in terms of degradation. Monoamine oxidase could become saturated, in such a way that it would reach its limit without producing a higher activity. This results in a 5-HT storage so that production index does not match with its degradation (Morgane et al., 2002; Barreto-Medeiros et al., 2004).

On the other hand, sleep deprivation affects 5-HT and 5-HIAA levels, in both control and chronically malnourished animals. Malnutrition and sleep deprivation present a synergic effect, producing a significant increase in 5-HT levels in both nuclei. These levels in rehabilitated animals with and without sleep deprivation are similar to controls, both in DRN and SCN.

These findings suggest that the serotonergic system was affected by malnutrition during the critical period of brain development and persisted even after a long period. This effect seems to be differentially modulated by sleep deprivation and malnutrition (Barreto-Medeiros et al., 2004; De Vasconcelos et al., 2004). Maturation of the nervous system and the consequent behavior depends in part on prenatal nutritional factors and postnatal environmental stimulation.

In particular, DR and SCN are two important CNS areas that are vulnerable to such pre- and postnatal manipulations. Maturation of the nervous system and the consequent behavior depend in part on both prenatal nutritional factors and postnatal environmental stimulation (Penalva et al., 2003; Kehoe et al., 2001). These pre- and postnatal challenges may cause a specific pattern of modifications in the CNS and, in combination, may be additive, particularly in the serotonergic functioning in both hypothalamus (SCN) and midbrain (RD), which is a fact with important clinical implications. There have been many studies describing abnormalities in experimental animals who have been undernourished. In humans, early protein–caloric malnutrition can be associated with a poor school performance, a memory deficiency and a diminished intellectual quotient (Galler et al., 1984; Valadares and De Sousa Almeida, 2005). Then, it is important to be able to separate the contributions of an impoverished environment (as in the case of sleep disruptions) and diet to a diminished brain function (Sutker et al., 1992; Lieberman et al., 2005). In examining the many effects of malnutrition insult on the CNS, the developmental approach is essential in furthering our understanding of the relationships between brain alterations and behavioral outcome, particularly regarding learning and

memory functions. The brain processes underlying intelligence, learning and memory receive, integrate and respond to both endogenous and exogenous stimulation. These processes, including the various neurotransmitter interactions that facilitate their operation, derive from numerous biochemical and metabolic systems in the various interacting parts of the brain. All of these interrelated processes are affected by both nutrition and sleep deprivation (Miklos et al., 2004; Mu et al., 2005).

This means that protein malnutrition has important effects on the circadian and homeostatic mechanisms driving sleep. Therefore, the temporal structure of the undernourished rats may not allow proper synchronization of some sleep parameters, particularly REM sleep (Cintra et al., 2002; Smith and Kennedy, 2003; Ertugrul and Rezaki, 2004), to environmental time cues.

We conclude that the restriction of food intake is an important factor that may generate a delay in the maturation of some areas of CNS. Malnutrition and sleep deprivation play a synergic role on 5-HT levels, which could interfere with the normal development and synchronization of many parts of the CNS, in relationship with structures that control the homeostatic and circadian functions of vigilance states.

4. Experimental procedures

Protocol and the use of animals were sanctioned by the Research Committee of Instituto Nacional de Rehabilitación, SSA, México, the Institute where the study was performed (Protocol N0.10/04). Animals were maintained in a 12:12 h light–dark condition (light period was from 8:00 to 20:00 h) and had free access to water and food.

The composition of the diets, in form of pellets, was as follows (in %): protein (21.8), fat (15.4), carbohydrates (50.9), salts (4.7), L-methionine (0.4), vitamins (1.0), water (2.2), and non-nutritive components (4.2) for 25% casein diet, and protein (5.3), fat (15.0), carbohydrates (68.9), salts (4.0) complemented with monobasic calcium phosphate (0.6) and zinc carbonate (0.001), L-methionine (0.4), vitamins (1.0), water (1.2) and non-nutritive components (4.2) for 6% casein diet. In both cases, the caloric supplementation was 4.3 kcal/g.

Forty-eight Sprague–Dawley male rats were studied under three nutritional conditions: 16 pre- and postnatal chronically casein malnourished rats (6/6%), 16 prenatal casein malnourished rats rehabilitated after birth (6/25%) and 16 control well-nourished casein rats (25/25%) were used. Ten female rats, assigned for breeding, were started in either casein malnutrition (6%) or well-nutrition (25%) diets 5 weeks prior to mating and were maintained on the respective diet during pregnancy through weaning of the litters. After birth and during suckling, pups of group 6/6% were maintained fed by mothers with casein malnutrition, the 25/25% group was maintained fed by well-nourished mothers and the rehabilitated group (6/25%) consisted of cross-fostering pups at birth from mothers fed with the 6% casein diet to foster mothers fed with the well-nutrition (25%) diet.

When rats were 60 days old, 24 of them were sleep deprived for 24 h (from 8:00 am to 8:00 am next morning), while the remaining 24 were kept in normal sleep–waking conditions. Sleep deprivation (SD) was performed by the forced locomotion method, according to Cintra et al. (1988). Briefly, rats were placed on a revolving disk coupled to a horizontal acrylic cylinder that had a partition. The disc was continuously rotating, for 24 h, at 2.5 mm/s.

Brain concentration of 5-HT and 5-HIAA in DR and SCN was estimated according to Digory and Bucket's technique (Diggory and Buckett, 1984). All the rats were decapitated between 8:30 and 9:30 am in order to avoid 5-HT and 5-HIAA changes due to the circadian cycle of this neurotransmitter and its metabolite. Brain was quickly dissected out and placed in a cooled rat's brain coronal matrix (BAS), where it was sliced. Slices closely corresponding to bregma –8.3 mm and –1.3 mm, according to the Paxinos and Watson (1998) stereotaxic atlas, were placed in a glass plate containing 6–8 ml ice-cooled perchloric acid in order to prevent 5-HT for oxidation until the DR and SCN extraction. Removing of such structures was performed according to Palkovits (1973) and Jacobowitz (1974) techniques. Briefly, brains were frozen on dry ice and cut in a cryostat. The sections were quickly thawed and frozen in order to enable adherence of the tissue slice to a glass slide. The observation of standard neuroanatomic landmarks through a stereomicroscope enabled us to remove both DR and SCN using special stainless steel hollow cannulas (500 μm diameter). Tissues were then homogenized by an ultrasonic homogenizer to 60 MHz for 5 s in 250 ml perchloric acid 0.1 N with 0.1% sodium metabisulfite followed by 5 min of centrifugation to 9000 rpm. Supernatants were filtered using a swinnex filter with a 0.22-μm pore and kept to –70 °C during 15 days, until their analysis.

5-HT and 5-HIAA contents were analyzed by high performance liquid chromatography (HPLC). A Perkin Elmer chromatography series 3-B with a loop of 20 μl, with electrochemical detector LC-4B (BAS) and an integrator, was used. Chromatograms of experimental samples were interpolated with chromatograms of three known standards. A reverse phase column μBondapak C-18, with particle size of 10 μm, 3.9×300 mm Waters Millipore and a mobile phase, was made of an acetate buffer solution (0.1 M, pH 4.1) containing 0.1 M citric acid (40%), 0.1 M sodium acetate (60%), 0.2 mM octylsulfate and 20% v/v methanol. The flow rate was 1 ml/min, while the potential was set at 600 mV against a reference electrode of Ag/AgCl. Obtained values are reported as ng/mg of tissue. Results were analyzed using a two-way ANOVA and a post hoc Tukey test.

Acknowledgments

This work was supported by Centro de Neurobiología, UNAM and Instituto Nacional de Padiatria, SSA.

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