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Diego Cabral Lacerda, Raul Manhães-De-Castro, Henrique José Cavalcanti Bezerra Gouveia, Yves Tourneur, Barbara Juacy Costa de Santana, Renata Emmanuele Assunção Santos, Jacques-Olivier Coq, Kelli Nogueira Ferraz-Pereira, Ana Elisa Toscano

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Treatment with the essential amino acid L-tryptophan reduces masticatory impairments in experimental cerebral palsy --Manuscript Draft--

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ABSTRACT

Purpose Children with cerebral palsy (CP) often exhibit difficulties in feeding resulting from deficits in chewing. This study investigates the therapeutic potential of L-tryptophan (TRI) to reduce deficits in chewing in rats subjected to an experimental model of CP.

Methods A total of 80 Wistar albino rats were used. Pups were randomly assigned to 4 experimental groups: Control Saline, Control TRI, CP Saline, and CP TRI groups. The experimental model of CP was based on the combination of perinatal anoxia associated with postnatal sensorimotor restriction of the hind limbs. TRI was administered subcutaneously during the lactation period. Anatomical and behavioral parameters were evaluated during maturation, including body weight gain, food intake, chewing movements, relative weight and the distribution of the types of masseter muscle fibers.

Results The induction of CP limited body weight gain, decreased food intake and led to impairment in the morphological and functional parameters of chewing. Moreover, for a comparable amount of food ingested, CP TRI animals grew the most. In addition, supplementation with TRI improved the number of chewing movements, and increased the weight and proportion of type IIB fibers of the masseter in rats subjected to CP.

Conclusion These results demonstrate that experimental CP impaired the development of mastication and that TRI supplementation increased masticatory maturation in animals subjected to CP.

Keywords: Cerebral Palsy; Tryptophan; Feeding and Eating Disorders; Chewing; Masseter Muscle.

INTRODUCTION

Cerebral palsy (CP), the most common physical disability in children, is a group of disorders of movement and posture and of motor function, including spasticity (85-91% of cases), and is caused by a lesion, interference or abnormality that originates in the immature brain⁽¹⁾⁽²⁾. On average, 40% of these children present feeding difficulties due to the appearance of orofacial impairments⁽³⁾. Orofacial impairments include deficits in sucking, mastication, drooling, swallowing, and pharyngeal and esophageal movements⁽³⁾⁽⁴⁾. In addition, a delay in the maturation of feeding behaviors, characterized by the transition from sucking to chewing, can aggravate the feeding disorder⁽⁴⁾⁽⁵⁾⁽⁶⁾. Among the chewing difficulties observed in children with CP, oral motor performance is compromised by decreased activity in muscle contractions due to the weakness of masticatory muscles⁽⁷⁾. In addition, atypical coactivations of agonist and antagonist masticatory muscles decrease food grinding in the mouth and lead to poor bolus formation and swallowing difficulties⁽⁸⁾⁽⁹⁾. Thus, meals become prolonged, painful and stressful, generating frustrations for parents and especially for children with CP⁽¹⁰⁾.

Rhythmical oral motor activity such as sucking and chewing is under the control of a network of brainstem neurons⁽¹¹⁾. The regulation of these activities occurs in the brainstem, particularly in the central pattern generators (CPGs)⁽¹²⁾⁽¹³⁾. Sucking and chewing CPGs extend from the rostral poles of the trigeminal motor nucleus (TMN) to the rostral pole of the facial nucleus⁽¹²⁾. In addition, the maturation of sucking and chewing involves neurotransmitter systems, including serotonin (5-HT)⁽¹⁴⁾. Several studies demonstrate that the serotonergic system is crucial to control TMN activities⁽¹⁴⁾⁽¹⁵⁾⁽¹⁶⁾. The TMN receives a dense serotonergic input and contains serotonergic receptors⁽¹⁴⁾, which facilitate the discharges of trigeminal motoneurons. Moreover, 5-HT is involved in the morphogenesis of the craniofacial, dental, bone and

muscle structures of the stomatognathic system⁽¹⁷⁾⁽¹⁸⁾, and all 5-HT receptor subtypes are expressed in the developing craniofacial structures as well⁽¹⁸⁾. Thus, an intact serotonergic system is necessary for the maturation of the neural structures involved in sucking and chewing.

The development of the serotonergic system appears impaired and disrupted after hypoxia-ischemia in a rat model of CP²⁰⁾⁽²¹⁾. According to these studies, experimental CP leads to a reduction in the 5-HT levels in the brain, and decrease in the quantity and length of serotonergic neurons²⁰⁾The serotonergic system was also affected in many segments of brain, including: somatosensory cortex, primary motor cortex, thalamus, and hypothalamus and around the sucking and chewing CPG areas in the brainstem²⁰⁾⁽²¹⁾. Thus, the serotonergic system appears crucial in the emergence of chewing deficits, as described in experimental models of atypical development of the central nervous system (CNS), such as CP

In a rodent model of CP, we recently showed a reduction in body weight and feeding intake, as well as deficits in the functional and morphological parameters of chewing, compared to control animals⁽²³⁾. Thus, this rodent model appears to replicate some orofacial clinical findings commonly found in children with CP and can also be used to test treatment strategies in reducing chewing damage, in particular 5-HT manipulations⁽²⁴⁾. In this context, the administration of the precursor essential amino acid L-tryptophan (TRI) is widely used in experimental studies to increase brain levels of 5-HT⁽²⁵⁾. Experimental CP promoted changes in TRI metabolism, triggering the activation of microglia and excitotoxic lesions that resulted in the production of markers of oxidative damage⁽²⁶⁾⁽²⁷⁾.

Conversely, TRI administration reduced the production of pro-inflammatory cytokines in the cerebral cortex in animals with CP, demonstrating its neuroprotective

effect⁽²⁸⁾. However, the effects of administration of TRI and the increased levels of 5-HT on the development of masticatory movements and the phenotype of chewing muscles remain unclear, especially in the case of CP. Thus, we hypothesized that supplementation with TRI increases CNS levels of 5-HT, which restores serotonergic activity in sucking and chewing CPGs, leading to an increase in the quantity and quality of sucking and chewing movements and thus enhancing body weight in an experimental CP model. To further explore our hypothesis, we evaluated the effects of TRI treatment on the chewing parameters in animals subjected to CP. Encouraging findings have shown that the manipulation of the serotonergic system has been used for the treatment of depression and stroke disease, suggesting the possibility of similar improvements in CP⁽²⁹⁾⁽³⁰⁾. Therefore, a possible increase in serotonergic activity induced by postnatal therapy with its precursor amino acid may improve masticatory maturation in animals subjected to CP and contribute to the inclusion of therapeutic nutritional strategies in attenuating the chewing impairments observed in children affected by CP.

MATERIALS AND METHODS

Animals

A total of 80 Wistar albino rats were obtained from 15 litters. In each litter, animals were randomly assigned to 4 experimental groups according to CP induction and TRI administration: Control Saline (n = 25); Control TRI (n = 16); CP Saline (n = 23); and CP TRI (n= 16) group. The litters used to compose the experimental groups were standardized, containing 8 pups in each litter, and only male rats were used for experimental analysis. The pups received TRI (dose of 50 mg/kg, Control TRI and CP

TRI) or saline solution (NaCl 0.9%, Control Saline and CP Saline) subcutaneously during lactation, daily starting at postnatal day 1 (P1) until P21. According to a recent systematic review, this dose of TRI supplementation seems to be an efficient method to increase brain levels of 5-HT²⁵. Moreover, we administered TRI at the beginning of the dark cycle, since it corresponds to a peak of expression of 5-HT in many segments of the CNS⁽³¹⁾. All rats were kept in an inverted cycle of 12/12h at a temperature of 22 ± 1°C and fed standard chow *ad libitum* (53.59g/100g carbohydrate, 25.51g/100g protein and 4.51g/100g lipids and 354.65 Kcal/100g) Presence®. The diet used contains on average 0.96mg/kg of TRI, based on previous studies which describes the composition and the content of TRI in animal diet⁽³²⁾⁽³³⁾.

All experiments and animal use were carried out in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines⁽³⁴⁾ and with the guidelines laid down by NIH (NIH Publication #80-23) and EC Council Directive (2007/526/EEC). This study was approved by the ethics committee of our university.

Experimental Model of CP

The experimental model of CP was based on the combination of neonatal anoxia with movement deprivation of the hind limbs, which corresponds to a sensorimotor restriction (SMR), as described earlier⁽³⁵⁾⁽³⁶⁾⁽³⁷⁾⁽³⁸⁾. First, the pups were subjected to two episodes of anoxia at P0 and P1. The rats were placed inside a glass chamber partially immersed in water at 37°C and exposed to nitrogen (100%) at 9 L/min for 12 minutes each time. From P2 to P28, the sensorimotor restriction of the hind paws was performed for 16 hours per day. During SMR, only limited movements of the hip joint were allowed, leaving the hind limbs extended in the epoxy mold (Poly Epox, Pulvitec). The elimination of urine and stools and maternal care was not impaired during SMR.

Measurement of Body Weight

To evaluate the effects of CP induction and neonatal treatment with TRI on the physical development of animals, their body weight was measured at several postnatal days (P1, P8, P14, P21 and P29) using a digital electronic scale (Marte, BL3200H model, capacity of 3.2 kg and sensitivity of 0.1 g).

Analysis of Food Intake

Feeding intake by sucking milk was evaluated during lactation following the methods described by Perillan et al. 2007⁽³⁹⁾. First, pups were separated from their mothers for 2 hours and kept in a heated box containing wood shavings. Then, pups were returned to their mothers for 1 hour to evaluate milk intake. Body weight was precisely measured 2 h after mother separation and 1 h after return to the mother. The difference in body weight obtained between the 2 measurements was used to determine the feeding intake in the first (W1, at P7/P8), second (W2, at P14/P15) and third (W3, at P19/P20) weeks of postnatal life. In addition, we measured the food intake by chewing in the third (W3, at P21) week of postnatal life⁽²³⁾ to quantify the amount of food ingested by chewing after the regression of sucking behavior. **In addition, we verified the relative food intake by sucking and chewing (quotient between food intake by body weight x 100).** The analysis of food intake by sucking and chewing was performed using a precision balance (*Shimadzu* model, capacity of 220 g, and sensitivity of 0.01 g).

Chewing Movements

The motor parameters of chewing were evaluated at P21, as previously described by Ferraz-Pereira et al. (2015)⁽⁴⁰⁾ and Lacerda et al. (2017)⁽²³⁾. First, the animals were food-deprived for 3 hours. Then, the chewing movements of each animal were videotaped and analyzed offline. The following parameters were evaluated: the

duration of the rhythmic chewing phase (s), the number of chewing cycles (n) and the frequency of chewing cycles (Hz). The rhythmic chewing phase is the period in which the animal performed vertical and regular movements of the mandible (chewing cycles) to grind and pulverize the food pellets (circular pellets, *Presence Rats and Mice*) between the upper and lower jaws. The frequency of chewing cycles corresponds to the number of chewing cycles performed during the rhythmic chewing phase.

Phenotype of the Masseter Muscle

The animals were sacrificed by decapitation to dissect the superficial bundle of the masseter muscle at P29. After dissection, the muscle was immediately weighed and immersed in frozen n-hexane. The muscles were stored at -80°C and subsequently evaluated for the proportion of different types of muscle fibers. Cross-sections (10 µm) were obtained using a cryostat maintained at a temperature of -20°C. The transverse sections were fixed on transparent microscope slides at room temperature. Afterward, histochemical analysis was performed according to the method proposed by Brooke and Kaiser (1970)⁽⁴¹⁾. This technique allows the identification of different types of muscle fibers according to the intensity of the ATPase enzyme reaction after preincubation in acidic medium (pH 4.7). The following types of muscle fibers were analyzed: type I fibers (oxidative, tonic and resistant to fatigue, reacts with maximum intensity with the ATPase enzyme, stained black); type IIA fibers (glycolytic, phasic, intermediate characteristic, reacts minimally with the enzyme ATPase, remains clear) and type IIB fibers (glycolytic, phasic, explosive muscular contraction, reacts in an intermediate way with the enzyme ATPase, stained gray). Images were captured with the 40x objective of an optical microscope. To determine the proportions of muscle fibers, the program Mensurin-PRO version 3.2 software (Jean-François Mother, Amiens, France) was used for counting the cells. This analysis

was performed to assess how muscle properties could be affected by the induction of the experimental CP, as well as to evaluate the therapeutic potential of L-TRI. In addition, the masseter muscle was chosen because it plays an important role in the execution of the chewing strokes and movements performed for grinding the food.

Statistical Analysis

Data analysis and statistics were performed using R (The R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Wien, Austria). The results were expressed as the mean \pm standard error of the mean and analyzed by two-way ANOVA repeated measures and Tukey *post hoc* tests for analysis of body weight and food intake and two-way ANOVA and Tukey *post hoc* tests for the other parameters. To assess possible relationships between sets of variables within the same animals, we used principal components analysis (PCA) to reduce high dimensionality. PCA is defined as an orthogonal linear transformation of the dataset into a reduced subspace with new coordinates so that the variance is maximized on each new coordinate axis or principal component, thus minimizing the loss of information. A normalization method, based on data mean adjustment to 0 and standard deviation (SD) adjustment to 1, was applied to allow comparisons of data with disparate values and variances. PCA was performed on 3 sets of variables, including 1) body weight during 5 time periods of body weight measurement (P1, P8, P14, P21 and P29), 2) feeding intake with 4 variables (sucking in week 1, sucking in week 2, sucking in week 3, chewing in week 3), and 3) chewing movements composed of 3 variables (rhythmic chewing phase, number of chewing cycles, frequency of chewing cycles). We thus obtained a score for each rat and for each set of variables along the PC1 or x-axis that optimally differentiated the different groups of animals. The aim was to correlate the scores of each rat between the 3 variables, i.e., the scores that

optimally characterized each animal for a given set of variables. We used these optimal scores to perform subsequent ANOVA between all 4 groups and linear correlations (Pearson or Spearman correlations according to data normality) within the same animals by using R.

RESULTS

Body Weight

Body weight in both control groups gradually increased from P1 to P29 [$F(4,375) = 1201.9$; $p < 0.0001$] without significant differences between the 2 control groups treated with either saline or TRI (Tukey *post hoc* test: P : n.s.; Fig. 1). Compared to the 2 groups of control rats, the body weight of CP rats with either saline or TRI was significantly smaller at P14, P21 and P29 [$F(3,375) = 75.1$; $P < 0.001$], but both CP groups did not differ (Tukey *post hoc* test: P : n.s.), suggesting an impact of CP treatment and no effect of TRI on body weight gain (Fig. 1). In addition, we found an interaction between the time period and groups, suggesting that the body weight of Control and CP rats increased differentially over time [$F(12,375) = 16.5$; $P < 0.0001$].

Analysis of Food Intake

Using PCA to compute individual scores along PC1 from the 4 variables dealing with the amount of food intake [$F(3,60) = 4.83$; $P < 0.005$], the scores of Control Saline rats (0.8 ± 0.4) did not differ from Control TRI rats (0.5 ± 0.3), and CP Saline rats (-0.9 ± 0.3) did not differ from CP TRI rats (-0.5 ± 0.3 ; Tukey *post hoc* tests: P : n.s.). In addition, the scores of Control rats with either treatment differed from those of CP rats with either treatment, suggesting an impact of CP on food intake by sucking and chewing (Tukey *post hoc* test $P < 0.01$; Fig. 2A) but no specific impact of TRI.

More precisely, the food intake by sucking in both control groups increased from week 1 to week 3 [$F(3,188) = 49.9$; $P < 0.0001$] without significant differences from

week 1 to week 2 (Tukey *post hoc* test: P : n.s.; Fig. 2B). In week 3 [$F(3,188) = 2.76$; $P < 0.05$]. Milk intake was statistically reduced only in CP Saline rats compared to Control Saline rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B). We found no interaction between periods and groups [$F(9,188) = 1.0$; P : n.s.]. Thus, the induction of CP promoted a specific negative impact on milk intake in week 3; however, the milk intake did not differ between Control TRI and CP TRI rats. Regarding food intake by chewing in week 3 [$F(3,60) = 4.83$, $P < 0.05$], we found a reduction in the number of food pellets eaten by CP TRI rats compared to that eaten by Control TRI rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B), while no significant changes were observed between other groups. **Based on relative food intake by sucking and chewing, CP Saline animals showed a reduction on food intake by sucking in week 3 (P19-P20), compared with Control Saline rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B-Table). An increase on food intake by sucking in week 3 was verified in CP TRI rats compared with CP Saline animals (Tukey *post hoc* test: $P < 0.05$; Fig. 2B-Table).**

To gain details about the impact of CP and TRI treatment, we evaluated the Pearson correlations between body weight and food intake by sucking and chewing in each experimental group. We found a moderate correlation between these variables in Control Saline ($r = 0.57$, $P < 0.05$), Control TRI ($r = 0.69$, $P < 0.05$), and CP Saline rats ($r = 0.67$, $P < 0.05$). Interestingly, the combination of CP and TRI showed the highest correlation (0.89 , $P < 0.0001$) in the CP TRI group, showing that for a comparable amount of food ingested, CP TRI animals grew the most. To complete this analysis on the impact of treatments with CP and TRI, we evaluated the execution of chewing movements.

Chewing Movements

Based on PCA computed with 3 variables characterizing chewing movements, ANOVA [$F(3,36) = 36.94$; $P < 0.0001$] showed that the scores of CP Saline rats (-2.1 ± 0.3) differed significantly from those of both CP TRI rats (-0.3 ± 0.2) and Control Saline rats (0.4 ± 0.3) along PC1 (Tukey *post hoc* tests: $P < 0.0001$) (Fig. 3A). However, no difference was found between CP TRI and Control Saline rats (Tukey *post hoc* test: $P = 0.24$, n.s.), while Control TRI animals (2.0 ± 0.2) differed from Control Saline animals (Tukey *post hoc* test: $P < 0.0001$; Fig. 3A). These results suggest that treatment with TRI attenuated the deleterious impact of CP on chewing movements.

To better understand the effect of CP and TRI, we analyzed specific parameters of chewing at P21. First, both CP groups had a reduced duration of rhythmic chewing phase compared to their Control groups (Tukey *post hoc* test: $P < 0.05$; Fig. 3B) [$F(3,36) = 10.31$; $P < 0.0001$]. Second, CP TRI rats showed a tendency for fewer chewing cycles compared to Control TRI rats (Tukey *post hoc* test: $P = 0.07$; Fig. 3C) [$F(3,36) = 3.733$; $P < 0.05$]. Finally, ANOVA [$F(3,36) = 10.73$; $P < 0.0001$] showed that CP Saline rats exhibited a lower frequency of chewing cycles than both Control Saline rats and CP TRI rats (Tukey *post hoc* test: $P < 0.05$), yet Control and CP rats treated with TRI did not differ (Tukey *post hoc* test: P : n.s.; Fig. 3D). These results suggest that CP impaired chewing efficiency, while treatment with TRI attenuated the deleterious impact of CP.

Phenotype of the Masseter Muscle

The relative weight of the masseter in CP rats with either treatment was significantly smaller (Tukey *post hoc* tests: $p < 0.001$; Fig. 4A) than that of control rats treated either with saline or TRI [$F(3,16) = 133$; $p < 0.0001$]. Conversely, the relative weight of the masseter was higher in both groups treated with TRI compared with their respective Saline groups, and this relative weight was higher in CP TRI rats than in CP

Saline rats (Tukey *post hoc* tests: $P < 0.001$), suggestive of a partial recovery of the weight of the masseter after TRI treatment (Fig. 4A).

We also evaluated the proportion of different fiber types in the masseter at P29 (Fig. 4 C). Both CP groups showed an increase in the percentage of type IIA fibers [$F(3,16) = 15.5$; $P < 0.0001$] associated with a decrease in type IIB fibers [$F(3,16) = 13.8$; $P < 0.0001$] compared to their respective Control groups (Tukey *post hoc* tests: $P < 0.05$) (Fig. 4B). Interestingly, treatment with TRI in both Control and CP rats promoted the opposite effect (Tukey *post hoc* test: $P < 0.05$), suggesting that TRI therapy partially reversed the adverse effect on the phenotype of the masseter after CP treatment (Figure 4 B, C).

Functional Relationships Between Specific Variables Using PCA

To evaluate the relationships between specific variables, individual scores for each rat in 3 sets of variables were computed using PCA. We found moderate but significant correlations between functional (the number of chewing cycles and the frequency of chewing cycles) and morphological (relative weight, proportion of type IIA and IIB fibers of the masseter muscle) parameters (Table 1). According to Table 1, the greater the relative weight and proportions of type IIB fibers, the greater the numbers and the higher the frequency of chewing cycles. Conversely, the greater the proportion of masseter type IIA fibers, the lower the number and frequency of chewing cycles, which means that the increase in the number of chewing movements led to an increase in the proportion of type IIB masseter fibers.

DISCUSSION

We analyzed the effects of experimental CP on body weight, food intake, masticatory movements and the phenotype of the masseter muscle. In addition, this study was the first to evaluate the therapeutic potential of the amino acid TRI in

minimizing chewing deficits in our rat model of CP. Our main findings show that experimental CP leads to the following: 1) a reduction in food intake and body weight during the first weeks of postnatal life; 2) a decrease in the number of chewing movements; and 3) a lower weight and proportion of type IIB fibers of the masseter. Regarding the data obtained from animals subjected to CP and treated with TRI, we found 1) no effect on body weight, but for a comparable amount of food ingested, the CP rats treated with TRI grew the most; 2) an improvement in the number of chewing movements; and 3) increases in weight and the proportion of type IIB fibers of the masseter. These findings demonstrated that nutritional manipulation by TRI administration may contribute to reducing chewing deficits in rats with experimental CP.

Body Weight

Experimental CP limited body weight gain when compared to Control animals. These data corroborate the findings obtained in studies that evaluated the same experimental model of CP and observed a similar reduction in body weight⁽²³⁾⁽³⁷⁾. The impact on body weight gain may be related to the motor damage produced by SMR during development. First, SMR rats developed locomotion deficits and a spasticity of the leg muscles⁽³⁵⁾⁽³⁷⁾⁽³⁸⁾. Second, SMR replicates deficits in sucking and chewing movements and decreases food intake⁽²³⁾. Thus, our results may be explained by the capacity of SMR to induce locomotion problems⁽³⁷⁾⁽³⁸⁾, sucking and chewing deficits and a reduction in food intake⁽²³⁾.

The absence of effects on body weight after TRI supplementation is corroborated by a previous study in which no difference in body weight evaluated at similar ages was found in Wistar rats treated with a TRI-enriched diet during the first month of life⁽⁴²⁾. We did not observe changes in body weight after the administration of

TRI. However, we found a high correlation between body weight and food intake in CP animals treated with TRI. Our results showed that for a comparable amount of food ingested, CP TRI animals grew the most. Therefore, we suggested that TRI supplementation increases somatic development through feeding intake stimulation⁽⁴³⁾.

Food Intake

We found a reduction in food intake by sucking in animals subjected to CP compared to control animals injected with saline. This effect may be related to an incoordination of sucking and swallowing⁽⁴⁴⁾⁽⁴⁵⁾, a reduction in tactile perception through rat vibrissae⁽⁴⁶⁾ and disorganized cortical somatosensory maps⁽³⁶⁾ in previous studies with experimental models of CP⁽⁴⁴⁾. Thus, it is suggested that the reduction in maternal milk intake found in the present study may result from difficulties in the animal's perception of the maternal nipple.

The milk intake did not differ between Control TRI and CP TRI rats at any age evaluated. CP TRI animals also presented a reduction in food intake by chewing compared to Control TRI rats. However, it is important to highlight that the administration of TRI improved feeding efficiency (more weight gain for the same food intake). Under normal conditions, the maternal milk of rats is composed on average of 11 g/dl of protein content and 0.29-0.39g/dl of TRI content⁽⁴⁷⁾. Regarding TRI therapy, we estimate how much the supplementation of this amino acid contributed to the total intake of protein and TRI during lactation period, on first week (Control TRI: 2.72%, 48.38%; CP TRI: 2.07%, 42.16%), second week (Control TRI: 2.07%, 42.16%; CP TRI: 2.43%, 46.21%) and third week (Control TRI: 1.22%, 29.92%; CP TRI: 1.12%, 28.08%) of postnatal life. In addition, we estimate how much the supplementation with TRI contributed to the total intake of protein and TRI at P21, through chewing movements

(Control TRI: 1.13%, 99.96%; CP TRI: 1.16%, 99.96%). This result revealed that almost all TRI was offered by the subcutaneous administration of TRI. Thus, the increase in feeding efficiency may result from the TRI intake offered by maternal milk together with the TRI supplementation provided by the subcutaneous route during first weeks of postnatal life.

According to previous studies, subcutaneous administration of 50 mg/kg of TRI was able to increase the brain levels of 5-HT⁽²⁵⁾. Humans and experimental evidences demonstrated that 5-HT signaling promoted suppression of food intake⁽⁴⁸⁾⁽⁴⁹⁾. Moreover, it has been reported that administration of TRI suppresses total food intake in rats⁽⁵⁰⁾. However, the reduction of food intake promoted by TRI supplementation showed minimum effects on protein intake⁽⁵⁰⁾. In addition, its well known that protein intake is critical for the integrity of muscle tissue⁽⁵¹⁾. Thus, we suggested that muscle tissues may be preserved after treatment with TRI.

Chewing Movements

The induction of CP promoted a reduction in mastication efficiency when compared to control animals. Thus, these results suggest that CP treatment reduces the capacity to grind food into small particles and to form a feeding bolus. These data corroborate the study of Lacerda et al. (2017)⁽²³⁾. Our experimental CP model leads to multiple deficits in orofacial functions, including difficulty in manipulating food, a reduction in head movements during feeding, impaired olfactory response and an incoordination of sucking and swallowing⁽⁵²⁾. As the integrity in chewing movements appears to depend on the maturation of orofacial functions, deficits in some of these functions can lead to overall damage to food behaviors⁽⁵³⁾. Thus, all of these alterations in orofacial functions may affect chewing performance, as evidenced by the reduction

in the time for oral food processing and the decrease in the number of chewing cycles required to pulverize food.

To gain details on mastication efficiency, we evaluated the frequency of chewing cycles and the number of closing and opening jaw movements per second. Animals subjected to experimental CP exhibited a lower frequency of chewing cycles than control groups. In contrast, TRI treatment increased the frequency of chewing cycles in CP animals. Thus, supplementation with TRI increased the frequency of chewing cycles, suggesting greater chewing efficiency in preparing the food for digestion and the subsequent absorption of nutrients. The putative increase in serotonergic activity related to the supplementation with TRI, its precursor amino acid, seems to improve chewing maturation in CP animals⁽²⁴⁾. The maturation of chewing is gradual and depends on the typical development of the CNS and neurotransmitter systems, including 5-HT⁽¹⁴⁾. Our results may be related to increased levels of 5-HT in the CNS after TRI administration⁽²⁴⁾. The dose used in our study promotes an increase in 5-HT levels, especially in the brainstem, where sucking and chewing CPGs are located⁽²⁵⁾. Experimental evidence shows that 5-HT activity promotes an increase in the firing properties of trigeminal neurons⁽¹⁴⁾⁽¹⁶⁾. According to these studies, elevated levels of 5-HT promote a fast activating and rapid inward current in trigeminal motor neurons. Thus, increased serotonergic activity appears to facilitate the depolarization of trigeminal motor neurons and subsequently increase neural excitability⁽¹⁴⁾. Thus, we suggest that the administration of TRI promotes changes in the electrical properties of the trigeminal nuclei, which may lead to increased jaw movements during chewing.

Phenotype of the Masseter Muscle

The induction of CP promoted a reduction on masseter weight compared to Control animals. This result corroborated our previous study⁽²³⁾. This result may related

to reduction of functional demand of masseter muscle, since animals subjected to CP performed less masticatory movements. Moreover, treatment with TRI increased masseter weight suggestive of partial recovery of this muscle after nutritional therapy. To complete the evaluation of morphological properties, we investigated the proportions of different masseter fiber types. Animals subjected to CP and treated with saline showed an increase in the proportion of type IIA fibers and a concomitant decrease in the proportion of type IIB fibers compared to the corresponding control group. This result corroborated our previous findings⁽²³⁾. In the first two weeks of typical development, the rat masseter muscle is composed predominantly of type IIA fibers⁽⁵⁴⁾⁽⁵⁵⁾. The transition from sucking behavior to chewing movements begins after P12⁽⁵⁴⁾. This change in food behavior results in an increase in the proportion of type IIB fibers, since chewing is an activity that requires greater strength, velocity and an explosion of muscle contraction, characteristics of type IIB fibers⁽⁵⁶⁾⁽⁵⁷⁾. Thus, the lower proportion of type IIB fibers found after experimental CP suggests a delay in the postnatal maturation of the masseter muscle.

Supplementation with TRI in both groups preserved a higher proportion of type IIB fibers than in the Saline groups. Thus, our results suggest that TRI treatment can decrease the deleterious effect of CP on the masseter muscle and chewing. This effect appears to be related to the critical role of 5-HT in the morphogenesis of craniofacial structures, including the maturation of teeth, the jaw bone and chewing muscles⁽¹⁷⁾⁽¹⁸⁾⁽⁵⁸⁾. During the typical maturation of food behaviors, 5-HT receptors are expressed early in the developing craniofacial structures⁽¹⁸⁾⁽⁵⁹⁾. Thus, our results highlight that the modulation of 5-HT by TRI therapy may improve the maturation of craniofacial structures after experimental CP, which could reflect the maintenance of a higher proportion of type IIB fibers of the masseter muscle. The correlations between

functional and morphological parameters found in the present study revealed that the quality and quantity of chewing movements appear to determine the development of the morphological aspects of mastication. In addition, we suggest that mechanical stimulation through chewing movements also contributes to the maturation of other structures of the stomatognathic system, including the teeth, jaw bones and temporomandibular joints⁽⁵³⁾⁽⁶⁰⁾. We also emphasize that mastication movements seem to have high potential for neuroplasticity, which may be reflected by the electrical properties of the trigeminal nuclei⁽⁶⁰⁾. Considering that supplementation with TRI led to an increase in the number of chewing movements, we suppose that this treatment is able to improve the development of the components of the craniofacial system during atypical development.

Proper nutrition is essential for craniofacial maturation during critical periods of development (prenatal, birth and first days of postnatal life)⁽⁴⁰⁾⁽⁵¹⁾. In addition, experimental studies demonstrated that protein and amino acid intake during early postnatal period, contribute for development of muscle skeletal tissue⁽³⁷⁾⁽⁵¹⁾. Our study did not evaluate the effect of others essential amino acids, which limit the elucidation of the specific role of TRI on chewing maturation. However, since TRI content and 5-HT activity are involved on morphogenesis of masticatory system⁽¹⁸⁾⁽⁵⁸⁾⁽⁶⁰⁾, we suggested that ours results may be related to TRI treatment offered for animals subjected to CP.

Thus, the data obtained in our study give new and promising insights into the protective role of TRI in the development of chewing in animals subjected to experimental CP. These data suggest the important role of treatment with TRI as a nutritional intervention in the neonatal intensive care unit (NICU)⁽⁶¹⁾. The milk of mothers who deliver prematurely contains inadequate amounts of nutrients⁽⁶²⁾.

Adequate nutrition during the first postnatal week is associated with the optimal growth and neurodevelopment of children with CP⁽⁶¹⁾. In addition, evidence from human studies has demonstrated the safety of providing higher levels of amino acids, including TRI⁽⁶¹⁾⁽⁶³⁾. Therefore, from these experimental data, we demonstrated the efficacy of therapy with TRI to guarantee the appropriate development of chewing during the breastfeeding period.

CONCLUSIONS

In summary, we investigated the therapeutic potential of TRI in reducing deficits in mastication in experimental CP. From our findings, we confirm our hypothesis that TRI minimizes the appearance of the masticatory impairments observed in CP animals. Thus, our study was the first to evaluate the therapeutic potential of nutrition, particularly treatment with TRI, in attenuating the functional and morphological damage of CP. Therefore, the data obtained in our study may contribute to the inclusion of therapeutic nutritional strategies to attenuate the chewing impairments observed in children affected by CP.

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LEGENDS

Figure 1 Effect of CP and TRI on Body Weight at Postnatal days: Body weight of rats of the Control Saline (n = 25), Control TRI (n = 16), CP Saline (n = 23), CP TRI (n = 16) groups at 1, 8, 14, 21 and 29 days postnatal life. The body weight of both control groups treated either with saline or TRI increased similarly over time, but was larger than that of CP groups treated either with saline or TRI (*P <0.001).

Figure 2 Effect of CP and TRI on Feeding Intake: (A) PCA Analysis of feeding intake variables (sucking on week 1, sucking on week 2, sucking on week 3, chewing on week 3). CS: Control Saline; CT: Control L-Tryptophan; CPS: Cerebral Palsy Saline; CPTRI: Cerebral Palsy L=Tryptophan. (B) and feeding intake by sucking and chewing and relative food intake of pups from the Control Saline (n = 13), Control TRI (n = 14), CP Saline (n = 13) and CP TRI (n = 11). (A) The scores of PCA analysis of Control Saline rats did not differ from Control TRI rats, as well as CP Saline rats did not differ from CP Tri rats. The scores of Control rats with either treatment differed from those of CP rats with either treatment (P <0.01). (B) In week 3, milk intake was reduced in CP Saline rats, compared to Control Saline rats (*P <0.05). A reduction in the amount of food

pellets eaten by CP TRI rats was verified when compared to Control TRI rats (* $P < 0.05$), while no significant changes between other groups. Based on relative food intake by sucking and chewing, CP Saline animals showed a reduction on food intake by sucking in week 3 (P19-P20), compared with Control Saline rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B-Table). An increase on food intake by sucking in week 3 was verified in CP TRI rats compared with CP Saline animals (Tukey *post hoc* test: $P < 0.05$; Fig. 2B-Table).

Figure 3 Effect of CP and TRI on Chewing Movements: (A) PCA analysis of chewing movements composed by 3 variables (rhythmic chewing phase, number of chewing cycles, frequency of chewing cycles). CS: Control Saline; CT: Control L-Tryptophan; CPS: Cerebral Palsy Saline; CP TRI: Cerebral Palsy L=Tryptophan, (B) duration in seconds of rhythmic chewing phase, and (C) number of chewing cycles and (D) frequency of chewing cycles of the Control Saline ($n = 10$), Control TRI ($n = 10$), CP Saline ($n = 10$), CP TRI ($n = 10$) in the twenty-first (P21) day postnatal life. (A) The scores of PCA analysis of CP Saline differed from those of both CP TRI and Control Saline ($P < 0.0001$). No difference was found between CP TRI and Control Saline rats, while Control TRI animals differed from Control Saline animals ($P < 0.0001$). (B) Both CP groups had a reduced duration of rhythmic chewing phase compared to their Control groups (* $P < 0.05$). CP TRI showed a tendency for lesser chewing cycles, compared to Control TRI rats ($P = 0.07$). CP Saline rats exhibited a lower frequency of chewing cycles, compared to both Control Saline rats and CP TRI rats (* $P < 0.05$), yet Control and CP rats treated with TRI did not differ.

Figure 4 Effect of CP and TRI on Phenotype of Masseter: (A) Relative weight, (B: CS = I, CT = II, CPS = III, CPT = IV) histological sections and (C) percentage of the fibers type I (dark), IIA (light) and IIB (gray) of the masseter muscle, of the Control Saline (n = 5), Control TRI (n = 5), CP Saline (n = 5), CP TRI (n = 5) at 29 days postnatal life. (A) The relative weight of masseter in CP rats with either treatment was smaller than that of control rats treated either with saline or TRI (*P < 0.001). The relative weight of masseter was higher in both groups treated with TRI compared with their respective Saline groups, and this relative weight was higher in CP TRI than in CP Saline (*P < 0.001). (C) Both CP groups showed an increase in the percentage of type IIA fibers associated to a decrease in type IIB fibers compared to their respective Control groups (*P < 0.05). Treatment with TRI promoted the opposite effect when compared Control Saline with Control TRI (P = 0.06), and CP saline with CP TRI (*P < 0.05).

Table 1: Correlations between specific variables, based on the individual scores along PC1 (x-axis) of animals obtained by using PCA. Pairwise two-sided p-values: *p < 0.05.

Treatment with the essential amino acid L-tryptophan reduces masticatory impairments in experimental cerebral palsy

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ABSTRACT

Purpose Children with cerebral palsy (CP) often exhibit difficulties in feeding resulting from deficits in chewing. This study investigates the therapeutic potential of L-tryptophan (TRI) to reduce deficits in chewing in rats subjected to an experimental model of CP.

Methods A total of 80 Wistar albino rats were used. Pups were randomly assigned to 4 experimental groups: Control Saline, Control TRI, CP Saline, and CP TRI groups. The experimental model of CP was based on the combination of perinatal anoxia associated with postnatal sensorimotor restriction of the hind limbs. TRI was administered subcutaneously during the lactation period. Anatomical and behavioral parameters were evaluated during maturation, including body weight gain, food intake, chewing movements, relative weight and the distribution of the types of masseter muscle fibers.

Results The induction of CP limited body weight gain, decreased food intake and led to impairment in the morphological and functional parameters of chewing. Moreover, for a comparable amount of food ingested, CP TRI animals grew the most. In addition, supplementation with TRI improved the number of chewing movements, and increased the weight and proportion of type IIB fibers of the masseter in rats subjected to CP.

Conclusion These results demonstrate that experimental CP impaired the development of mastication and that TRI supplementation increased masticatory maturation in animals subjected to CP.

Keywords: Cerebral Palsy; Tryptophan; Feeding and Eating Disorders; Chewing; Masseter Muscle.

INTRODUCTION

Cerebral palsy (CP), the most common physical disability in children, is a group of disorders of movement and posture and of motor function, including spasticity (85-91% of cases), and is caused by a lesion, interference or abnormality that originates in the immature brain⁽¹⁾⁽²⁾. On average, 40% of these children present feeding difficulties due to the appearance of orofacial impairments⁽³⁾. Orofacial impairments include deficits in sucking, mastication, drooling, swallowing, and pharyngeal and esophageal movements⁽³⁾⁽⁴⁾. In addition, a delay in the maturation of feeding behaviors, characterized by the transition from sucking to chewing, can aggravate the feeding disorder⁽⁴⁾⁽⁵⁾⁽⁶⁾. Among the chewing difficulties observed in children with CP, oral motor performance is compromised by decreased activity in muscle contractions due to the weakness of masticatory muscles⁽⁷⁾. In addition, atypical coactivations of agonist and antagonist masticatory muscles decrease food grinding in the mouth and lead to poor bolus formation and swallowing difficulties⁽⁸⁾⁽⁹⁾. Thus, meals become prolonged, painful and stressful, generating frustrations for parents and especially for children with CP⁽¹⁰⁾.

Rhythmical oral motor activity such as sucking and chewing is under the control of a network of brainstem neurons⁽¹¹⁾. The regulation of these activities occurs in the brainstem, particularly in the central pattern generators (CPGs)⁽¹²⁾⁽¹³⁾. Sucking and chewing CPGs extend from the rostral poles of the trigeminal motor nucleus (TMN) to the rostral pole of the facial nucleus⁽¹²⁾. In addition, the maturation of sucking and chewing involves neurotransmitter systems, including serotonin (5-HT)⁽¹⁴⁾. Several studies demonstrate that the serotonergic system is crucial to control TMN activities⁽¹⁴⁾⁽¹⁵⁾⁽¹⁶⁾. The TMN receives a dense serotonergic input and contains serotonergic receptors⁽¹⁴⁾, which facilitate the discharges of trigeminal motoneurons. Moreover, 5-HT is involved in the morphogenesis of the craniofacial, dental, bone and muscle structures of the stomatognathic system⁽¹⁷⁾⁽¹⁸⁾, and all 5-HT receptor subtypes are expressed in the developing craniofacial structures as well⁽¹⁸⁾.

Thus, an intact serotonergic system is necessary for the maturation of the neural structures involved in sucking and chewing.

The development of the serotonergic system appears impaired and disrupted after hypoxia-ischemia in a rat model of CP²⁰⁾⁽²¹⁾. According to these studies, experimental CP leads to a reduction in the 5-HT levels in the brain, and decrease in the quantity and length of serotonergic neurons²⁰⁾The serotonergic system was also affected in many segments of brain, including: somatosensory cortex, primary motor cortex, thalamus, and hypothalamus and around the sucking and chewing CPG areas in the brainstem ²⁰⁾⁽²¹⁾. Thus, the serotonergic system appears crucial in the emergence of chewing deficits, as described in experimental models of atypical development of the central nervous system (CNS), such as CP

In a rodent model of CP, we recently showed a reduction in body weight and feeding intake, as well as deficits in the functional and morphological parameters of chewing, compared to control animals⁽²³⁾. Thus, this rodent model appears to replicate some orofacial clinical findings commonly found in children with CP and can also be used to test treatment strategies in reducing chewing damage, in particular 5-HT manipulations⁽²⁴⁾. In this context, the administration of the precursor essential amino acid L-tryptophan (TRP) is widely used in experimental studies to increase brain levels of 5-HT⁽²⁵⁾. Experimental CP promoted changes in TRP metabolism, triggering the activation of microglia and excitotoxic lesions that resulted in the production of markers of oxidative damage⁽²⁶⁾⁽²⁷⁾.

Conversely, TRP administration reduced the production of pro-inflammatory cytokines in the cerebral cortex in animals with CP, demonstrating its neuroprotective effect⁽²⁸⁾. However, the effects of administration of TRP and the increased levels of 5-HT on the development of masticatory movements and the phenotype of chewing muscles remain unclear, especially in the case of CP. Thus, we hypothesized that supplementation with TRP increases CNS levels of 5-HT, which restores serotonergic activity in sucking and chewing

CPGs, leading to an increase in the quantity and quality of sucking and chewing movements and thus enhancing body weight in an experimental CP model. To further explore our hypothesis, we evaluated the effects of TRI treatment on the chewing parameters in animals subjected to CP. Encouraging findings have shown that the manipulation of the serotonergic system has been used for the treatment of depression and stroke disease, suggesting the possibility of similar improvements in CP⁽²⁹⁾⁽³⁰⁾. Therefore, a possible increase in serotonergic activity induced by postnatal therapy with its precursor amino acid may improve masticatory maturation in animals subjected to CP and contribute to the inclusion of therapeutic nutritional strategies in attenuating the chewing impairments observed in children affected by CP.

MATERIALS AND METHODS

Animals

A total of 80 Wistar albino rats were obtained from 15 litters in the Department of Nutrition, Federal University of Pernambuco, Brazil. In each litter, animals were randomly assigned to 4 experimental groups according to CP induction and TRI administration: Control Saline (n = 25); Control TRI (n = 16); CP Saline (n = 23); and CP TRI (n= 16) group. The litters used to compose the experimental groups were standardized, containing 8 pups in each litter, and only male rats were used for experimental analysis. The pups received TRI (dose of 50 mg/kg, Control TRI and CP TRI) or saline solution (NaCl 0.9%, Control Saline and CP Saline) subcutaneously during lactation, daily starting at postnatal day 1 (P1) until P21. According to a recent systematic review, this dose of TRI supplementation seems to be an efficient method to increase brain levels of 5-HT²⁵. Moreover, we administered TRI at the beginning of the dark cycle, since it corresponds to a peak of expression of 5-HT in many segments of the CNS⁽³¹⁾. **All rats were kept in an inverted cycle of 12/12h at a**

temperature of $22 \pm 1^\circ\text{C}$ and fed standard chow *ad libitum* (53.59g/100g carbohydrate, 25.51g/100g protein and 4.51g/100g lipids and 354.65 Kcal/100g) Presence®. The diet used contains on average 0.96mg/kg of TRI, based on previous studies which describes the composition and the content of TRI in animal diet⁽³²⁾⁽³³⁾.

All experiments and animal use were carried out in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines⁽³⁴⁾ and with the guidelines laid down by NIH (NIH Publication #80-23) and EC Council Directive (2007/526/EEC). This study was approved by the ethics committee for animal use of the Federal University of Pernambuco (#0050/2016; approved in July 2016).

Experimental Model of CP

The experimental model of CP was based on the combination of neonatal anoxia with movement deprivation of the hind limbs, which corresponds to a sensorimotor restriction (SMR), as described earlier⁽³⁵⁾⁽³⁶⁾⁽³⁷⁾⁽³⁸⁾. First, the pups were subjected to two episodes of anoxia at P0 and P1. The rats were placed inside a glass chamber partially immersed in water at 37°C and exposed to nitrogen (100%) at 9 L/min for 12 minutes each time. From P2 to P28, the sensorimotor restriction of the hind paws was performed for 16 hours per day. During SMR, only limited movements of the hip joint were allowed, leaving the hind limbs extended in the epoxy mold (Poly Epox, Pulvitec). The elimination of urine and stools and maternal care was not impaired during SMR.

Measurement of Body Weight

To evaluate the effects of CP induction and neonatal treatment with TRI on the physical development of animals, their body weight was measured at several postnatal days (P1, P8, P14, P21 and P29) using a digital electronic scale (Marte, BL3200H model, capacity of 3.2 kg and sensitivity of 0.1 g).

Analysis of Food Intake

Feeding intake by sucking milk was evaluated during lactation following the methods described by Perillan et al. 2007⁽³⁹⁾. First, pups were separated from their mothers for 2 hours and kept in a heated box containing wood shavings. Then, pups were returned to their mothers for 1 hour to evaluate milk intake. Body weight was precisely measured 2 h after mother separation and 1 h after return to the mother. The difference in body weight obtained between the 2 measurements was used to determine the feeding intake in the first (W1, at P7/P8), second (W2, at P14/P15) and third (W3, at P19/P20) weeks of postnatal life. In addition, we measured the food intake by chewing in the third (W3, at P21) week of postnatal life⁽²³⁾ to quantify the amount of food ingested by chewing after the regression of sucking behavior. **In addition, we verified the relative food intake by sucking and chewing (quotient between food intake by body weight x 100).** The analysis of food intake by sucking and chewing was performed using a precision balance (*Shimadzu* model, capacity of 220 g, and sensitivity of 0.01 g).

Chewing Movements

The motor parameters of chewing were evaluated at P21, as previously described by Ferraz-Pereira et al. (2015)⁽⁴⁰⁾ and Lacerda et al. (2017)⁽²³⁾. First, the animals were food-deprived for 3 hours. Then, the chewing movements of each animal were videotaped and analyzed offline. The following parameters were evaluated: the duration of the rhythmic chewing phase (s), the number of chewing cycles (n) and the frequency of chewing cycles (Hz). The rhythmic chewing phase is the period in which the animal performed vertical and regular movements of the mandible (chewing cycles) to grind and pulverize the food pellets (circular pellets, *Presence Rats and Mice*) between the upper and lower jaws. The frequency of chewing cycles corresponds to the number of chewing cycles performed during the rhythmic chewing phase.

Phenotype of the Masseter Muscle

The animals were sacrificed by decapitation to dissect the superficial bundle of the masseter muscle at P29. After dissection, the muscle was immediately weighed and immersed in frozen n-hexane. The muscles were stored at -80°C and subsequently evaluated for the proportion of different types of muscle fibers. Cross-sections (10 µm) were obtained using a cryostat maintained at a temperature of -20°C. The transverse sections were fixed on transparent microscope slides at room temperature. Afterward, histochemical analysis was performed according to the method proposed by Brooke and Kaiser (1970)⁽⁴¹⁾. This technique allows the identification of different types of muscle fibers according to the intensity of the ATPase enzyme reaction after preincubation in acidic medium (pH 4.7). The following types of muscle fibers were analyzed: type I fibers (oxidative, tonic and resistant to fatigue, reacts with maximum intensity with the ATPase enzyme, stained black); type IIA fibers (glycolytic, phasic, intermediate characteristic, reacts minimally with the enzyme ATPase, remains clear) and type IIB fibers (glycolytic, phasic, explosive muscular contraction, reacts in an intermediate way with the enzyme ATPase, stained gray). Images were captured with the 40x objective of an optical microscope. To determine the proportions of muscle fibers, the program Mensurin-PRO version 3.2 software (Jean-François Mother, Amiens, France) was used for counting the cells. This analysis was performed to assess how muscle properties could be affected by the induction of the experimental CP, as well as to evaluate the therapeutic potential of L-TRI. In addition, the masseter muscle was chosen because it plays an important role in the execution of the chewing strokes and movements performed for grinding the food.

Statistical Analysis

Data analysis and statistics were performed using R (The R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Wien, Austria). The results were expressed as the mean ± standard error of the mean and analyzed by two-way ANOVA repeated measures and Tukey *post hoc* tests for analysis of body weight and food intake

and two-way ANOVA and Tukey *post hoc* tests for the other parameters. To assess possible relationships between sets of variables within the same animals, we used principal components analysis (PCA) to reduce high dimensionality. PCA is defined as an orthogonal linear transformation of the dataset into a reduced subspace with new coordinates so that the variance is maximized on each new coordinate axis or principal component, thus minimizing the loss of information. A normalization method, based on data mean adjustment to 0 and standard deviation (SD) adjustment to 1, was applied to allow comparisons of data with disparate values and variances. PCA was performed on 3 sets of variables, including 1) body weight during 5 time periods of body weight measurement (P1, P8, P14, P21 and P29), 2) feeding intake with 4 variables (sucking in week 1, sucking in week 2, sucking in week 3, chewing in week 3), and 3) chewing movements composed of 3 variables (rhythmic chewing phase, number of chewing cycles, frequency of chewing cycles). We thus obtained a score for each rat and for each set of variables along the PC1 or x-axis that optimally differentiated the different groups of animals. The aim was to correlate the scores of each rat between the 3 variables, i.e., the scores that optimally characterized each animal for a given set of variables. We used these optimal scores to perform subsequent ANOVA between all 4 groups and linear correlations (Pearson or Spearman correlations according to data normality) within the same animals by using R.

RESULTS

Body Weight

Body weight in both control groups gradually increased from P1 to P29 [$F(4,375) = 1201.9$; $p < 0.0001$] without significant differences between the 2 control groups treated with either saline or TRI (Tukey *post hoc* test: P : n.s.; Fig. 1). Compared to the 2 groups of control rats, the body weight of CP rats with either saline or TRI was significantly smaller at P14, P21 and P29 [$F(3,375) = 75.1$; $P < 0.001$], but both CP groups did not differ (Tukey *post hoc* test: P : n.s.), suggesting an impact of CP treatment and no effect of TRI on body weight gain

(Fig. 1). In addition, we found an interaction between the time period and groups, suggesting that the body weight of Control and CP rats increased differentially over time [$F(12,375) = 16.5$; $P < 0.0001$].

Analysis of Food Intake

Using PCA to compute individual scores along PC1 from the 4 variables dealing with the amount of food intake [$F(3,60) = 4.83$; $P < 0.005$], the scores of Control Saline rats (0.8 ± 0.4) did not differ from Control TRI rats (0.5 ± 0.3), and CP Saline rats (-0.9 ± 0.3) did not differ from CP TRI rats (-0.5 ± 0.3 ; Tukey *post hoc* tests: P : n.s.). In addition, the scores of Control rats with either treatment differed from those of CP rats with either treatment, suggesting an impact of CP on food intake by sucking and chewing (Tukey *post hoc* test $P < 0.01$; Fig. 2A) but no specific impact of TRI.

More precisely, the food intake by sucking in both control groups increased from week 1 to week 3 [$F(3,188) = 49.9$; $P < 0.0001$] without significant differences from week 1 to week 2 (Tukey *post hoc* test: P : n.s.; Fig. 2B). In week 3 [$F(3,188) = 2.76$; $P < 0.05$]. Milk intake was statistically reduced only in CP Saline rats compared to Control Saline rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B). We found no interaction between periods and groups [$F(9,188) = 1.0$; P : n.s.]. Thus, the induction of CP promoted a specific negative impact on milk intake in week 3; however, the milk intake did not differ between Control TRI and CP TRI rats. Regarding food intake by chewing in week 3 [$F(3,60) = 4.83$, $P < 0.05$], we found a reduction in the number of food pellets eaten by CP TRI rats compared to that eaten by Control TRI rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B), while no significant changes were observed between other groups. **Based on relative food intake by sucking and chewing, CP Saline animals showed a reduction on food intake by sucking in week 3 (P19-P20), compared with Control Saline rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B-Table). An increase on food intake by sucking in week 3 was verified in CP TRI rats compared with CP Saline animals (Tukey *post hoc* test: $P < 0.05$; Fig. 2B-Table).**

To gain details about the impact of CP and TRI treatment, we evaluated the Pearson correlations between body weight and food intake by sucking and chewing in each experimental group. We found a moderate correlation between these variables in Control Saline ($r = 0.57$, $P < 0.05$), Control TRI ($r = 0.69$, $P < 0.05$), and CP Saline rats ($r = 0.67$, $P < 0.05$). Interestingly, the combination of CP and TRI showed the highest correlation (0.89, $P < 0.0001$) in the CP TRI group, showing that for a comparable amount of food ingested, CP TRI animals grew the most. To complete this analysis on the impact of treatments with CP and TRI, we evaluated the execution of chewing movements.

Chewing Movements

Based on PCA computed with 3 variables characterizing chewing movements, ANOVA [$F(3,36) = 36.94$; $P < 0.0001$] showed that the scores of CP Saline rats (-2.1 ± 0.3) differed significantly from those of both CP TRI rats (-0.3 ± 0.2) and Control Saline rats (0.4 ± 0.3) along PC1 (Tukey *post hoc* tests: $P < 0.0001$) (Fig. 3A). However, no difference was found between CP TRI and Control Saline rats (Tukey *post hoc* test: $P = 0.24$, n.s.), while Control TRI animals (2.0 ± 0.2) differed from Control Saline animals (Tukey *post hoc* test: $P < 0.0001$; Fig. 3A). These results suggest that treatment with TRI attenuated the deleterious impact of CP on chewing movements.

To better understand the effect of CP and TRI, we analyzed specific parameters of chewing at P21. First, both CP groups had a reduced duration of rhythmic chewing phase compared to their Control groups (Tukey *post hoc* test: $P < 0.05$; Fig. 3B) [$F(3,36) = 10.31$; $P < 0.0001$]. Second, CP TRI rats showed a tendency for fewer chewing cycles compared to Control TRI rats (Tukey *post hoc* test: $P = 0.07$; Fig. 3C) [$F(3,36) = 3.733$; $P < 0.05$]. Finally, ANOVA [$F(3,36) = 10.73$; $P < 0.0001$] showed that CP Saline rats exhibited a lower frequency of chewing cycles than both Control Saline rats and CP TRI rats (Tukey *post hoc* test: $P < 0.05$), yet Control and CP rats treated with TRI did not differ (Tukey *post hoc* test:

P: n.s.; Fig. 3D). These results suggest that CP impaired chewing efficiency, while treatment with TRI attenuated the deleterious impact of CP.

Phenotype of the Masseter Muscle

The relative weight of the masseter in CP rats with either treatment was significantly smaller (Tukey *post hoc* tests: $p < 0.001$; Fig. 4A) than that of control rats treated either with saline or TRI [$F(3,16) = 133$; $p < 0.0001$]. Conversely, the relative weight of the masseter was higher in both groups treated with TRI compared with their respective Saline groups, and this relative weight was higher in CP TRI rats than in CP Saline rats (Tukey *post hoc* tests: $P < 0.001$), suggestive of a partial recovery of the weight of the masseter after TRI treatment (Fig. 4A).

We also evaluated the proportion of different fiber types in the masseter at P29 (Fig. 4 C). Both CP groups showed an increase in the percentage of type IIA fibers [$F(3,16) = 15.5$; $P < 0.0001$] associated with a decrease in type IIB fibers [$F(3,16) = 13.8$; $P < 0.0001$] compared to their respective Control groups (Tukey *post hoc* tests: $P < 0.05$) (Fig. 4B). Interestingly, treatment with TRI in both Control and CP rats promoted the opposite effect (Tukey *post hoc* test: $P < 0.05$), suggesting that TRI therapy partially reversed the adverse effect on the phenotype of the masseter after CP treatment (Figure 4 B, C).

Functional Relationships Between Specific Variables Using PCA

To evaluate the relationships between specific variables, individual scores for each rat in 3 sets of variables were computed using PCA. We found moderate but significant correlations between functional (the number of chewing cycles and the frequency of chewing cycles) and morphological (relative weight, proportion of type IIA and IIB fibers of the masseter muscle) parameters (Table 1). According to Table 1, the greater the relative weight and proportions of type IIB fibers, the greater the numbers and the higher the frequency of chewing cycles. Conversely, the greater the proportion of masseter type IIA fibers, the lower

the number and frequency of chewing cycles, which means that the increase in the number of chewing movements led to an increase in the proportion of type IIB masseter fibers.

DISCUSSION

We analyzed the effects of experimental CP on body weight, food intake, masticatory movements and the phenotype of the masseter muscle. In addition, this study was the first to evaluate the therapeutic potential of the amino acid TRI in minimizing chewing deficits in our rat model of CP. Our main findings show that experimental CP leads to the following: 1) a reduction in food intake and body weight during the first weeks of postnatal life; 2) a decrease in the number of chewing movements; and 3) a lower weight and proportion of type IIB fibers of the masseter. Regarding the data obtained from animals subjected to CP and treated with TRI, we found 1) no effect on body weight, but for a comparable amount of food ingested, the CP rats treated with TRI grew the most; 2) an improvement in the number of chewing movements; and 3) increases in weight and the proportion of type IIB fibers of the masseter. These findings demonstrated that nutritional manipulation by TRI administration may contribute to reducing chewing deficits in rats with experimental CP.

Body Weight

Experimental CP limited body weight gain when compared to Control animals. These data corroborate the findings obtained in studies that evaluated the same experimental model of CP and observed a similar reduction in body weight⁽²³⁾⁽³⁷⁾. The impact on body weight gain may be related to the motor damage produced by SMR during development. First, SMR rats developed locomotion deficits and a spasticity of the leg muscles⁽³⁵⁾⁽³⁷⁾⁽³⁸⁾. Second, SMR replicates deficits in sucking and chewing movements and decreases food intake⁽²³⁾. Thus, our results may be explained by the capacity of SMR to induce locomotion problems⁽³⁷⁾⁽³⁸⁾, sucking and chewing deficits and a reduction in food intake⁽²³⁾.

The absence of effects on body weight after TRI supplementation is corroborated by a previous study in which no difference in body weight evaluated at similar ages was found

in Wistar rats treated with a TRI-enriched diet during the first month of life⁽⁴²⁾. We did not observe changes in body weight after the administration of TRI. However, we found a high correlation between body weight and food intake in CP animals treated with TRI. Our results showed that for a comparable amount of food ingested, CP TRI animals grew the most. Therefore, we suggested that TRI supplementation increases somatic development through feeding intake stimulation⁽⁴³⁾.

Food Intake

We found a reduction in food intake by sucking in animals subjected to CP compared to control animals injected with saline. This effect may be related to an incoordination of sucking and swallowing⁽⁴⁴⁾⁽⁴⁵⁾, a reduction in tactile perception through rat vibrissae⁽⁴⁶⁾ and disorganized cortical somatosensory maps⁽³⁶⁾ in previous studies with experimental models of CP⁽⁴⁴⁾. Thus, it is suggested that the reduction in maternal milk intake found in the present study may result from difficulties in the animal's perception of the maternal nipple.

The milk intake did not differ between Control TRI and CP TRI rats at any age evaluated. CP TRI animals also presented a reduction in food intake by chewing compared to Control TRI rats. However, it is important to highlight that the administration of TRI improved feeding efficiency (more weight gain for the same food intake). **Under normal conditions, the maternal milk of rats is composed on average of 11 g/dl of protein content and 0.29-0.39g/dl of TRI content⁽⁴⁷⁾. Regarding TRI therapy, we estimate how much the supplementation of this amino acid contributed to the total intake of protein and TRI during lactation period, on first week (Control TRI: 2.72%, 48.38%; CP TRI: 2.07%, 42.16%), second week (Control TRI: 2.07%, 42.16%; CP TRI: 2.43%, 46.21%) and third week (Control TRI: 1.22%, 29.92%; CP TRI: 1.12%, 28.08%) of postnatal life. In addition, we estimate how much the supplementation with TRI contributed to the total intake of protein and TRI at P21, through chewing movements (Control TRI: 1.13%, 99.96%; CP TRI: 1.16%, 99.96%). This result revealed that almost all TRI was offered by the subcutaneous administration of TRI.**

Thus, the increase in feeding efficiency may result from the TRI intake offered by maternal milk together with the TRI supplementation provided by the subcutaneous route during first weeks of postnatal life.

According to previous studies, subcutaneous administration of 50 mg/kg of TRI was able to increase the brain levels of 5-HT⁽²⁵⁾. Humans and experimental evidences demonstrated that 5-HT signaling promoted suppression of food intake⁽⁴⁸⁾⁽⁴⁹⁾. Moreover, it has been reported that administration of TRI suppresses total food intake in rats⁽⁵⁰⁾. However, the reduction of food intake promoted by TRI supplementation showed minimum effects on protein intake⁽⁵⁰⁾. In addition, it's well known that protein intake is critical for the integrity of muscle tissue⁽⁵¹⁾. Thus, we suggested that muscle tissues may be preserved after treatment with TRI.

Chewing Movements

The induction of CP promoted a reduction in mastication efficiency when compared to control animals. Thus, these results suggest that CP treatment reduces the capacity to grind food into small particles and to form a feeding bolus. These data corroborate the study of Lacerda et al. (2017)⁽²³⁾. Our experimental CP model leads to multiple deficits in orofacial functions, including difficulty in manipulating food, a reduction in head movements during feeding, impaired olfactory response and an incoordination of sucking and swallowing⁽⁵²⁾. As the integrity in chewing movements appears to depend on the maturation of orofacial functions, deficits in some of these functions can lead to overall damage to food behaviors⁽⁵³⁾. Thus, all of these alterations in orofacial functions may affect chewing performance, as evidenced by the reduction in the time for oral food processing and the decrease in the number of chewing cycles required to pulverize food.

To gain details on mastication efficiency, we evaluated the frequency of chewing cycles and the number of closing and opening jaw movements per second. Animals subjected to experimental CP exhibited a lower frequency of chewing cycles than control

groups. In contrast, TRI treatment increased the frequency of chewing cycles in CP animals. Thus, supplementation with TRI increased the frequency of chewing cycles, suggesting greater chewing efficiency in preparing the food for digestion and the subsequent absorption of nutrients. The putative increase in serotonergic activity related to the supplementation with TRI, its precursor amino acid, seems to improve chewing maturation in CP animals⁽²⁴⁾. The maturation of chewing is gradual and depends on the typical development of the CNS and neurotransmitter systems, including 5-HT⁽¹⁴⁾. Our results may be related to increased levels of 5-HT in the CNS after TRI administration⁽²⁴⁾. The dose used in our study promotes an increase in 5-HT levels, especially in the brainstem, where sucking and chewing CPGs are located⁽²⁵⁾. Experimental evidence shows that 5-HT activity promotes an increase in the firing properties of trigeminal neurons⁽¹⁴⁾⁽¹⁶⁾. According to these studies, elevated levels of 5-HT promote a fast activating and rapid inward current in trigeminal motor neurons. Thus, increased serotonergic activity appears to facilitate the depolarization of trigeminal motor neurons and subsequently increase neural excitability⁽¹⁴⁾. Thus, we suggest that the administration of TRI promotes changes in the electrical properties of the trigeminal nuclei, which may lead to increased jaw movements during chewing.

Phenotype of the Masseter Muscle

The induction of CP promoted a reduction on masseter weight compared to Control animals. This result corroborated our previous study⁽²³⁾. This result may be related to reduction of functional demand of masseter muscle, since animals subjected to CP performed less masticatory movements. Moreover, treatment with TRI increased masseter weight suggestive of partial recovery of this muscle after nutritional therapy. To complete the evaluation of morphological properties, we investigated the proportions of different masseter fiber types. Animals subjected to CP and treated with saline showed an increase in the proportion of type IIA fibers and a concomitant decrease in the proportion of type IIB fibers compared to the corresponding control group. This result corroborated our previous

findings⁽²³⁾. In the first two weeks of typical development, the rat masseter muscle is composed predominantly of type IIA fibers⁽⁵⁴⁾⁽⁵⁵⁾. The transition from sucking behavior to chewing movements begins after P12⁽⁵⁴⁾. This change in food behavior results in an increase in the proportion of type IIB fibers, since chewing is an activity that requires greater strength, velocity and an explosion of muscle contraction, characteristics of type IIB fibers⁽⁵⁶⁾⁽⁵⁷⁾. Thus, the lower proportion of type IIB fibers found after experimental CP suggests a delay in the postnatal maturation of the masseter muscle.

Supplementation with TRI in both groups preserved a higher proportion of type IIB fibers than in the Saline groups. Thus, our results suggest that TRI treatment can decrease the deleterious effect of CP on the masseter muscle and chewing. This effect appears to be related to the critical role of 5-HT in the morphogenesis of craniofacial structures, including the maturation of teeth, the jaw bone and chewing muscles⁽¹⁷⁾⁽¹⁸⁾⁽⁵⁸⁾. During the typical maturation of food behaviors, 5-HT receptors are expressed early in the developing craniofacial structures⁽¹⁸⁾⁽⁵⁹⁾. Thus, our results highlight that the modulation of 5-HT by TRI therapy may improve the maturation of craniofacial structures after experimental CP, which could reflect the maintenance of a higher proportion of type IIB fibers of the masseter muscle. The correlations between functional and morphological parameters found in the present study revealed that the quality and quantity of chewing movements appear to determine the development of the morphological aspects of mastication. In addition, we suggest that mechanical stimulation through chewing movements also contributes to the maturation of other structures of the stomatognathic system, including the teeth, jaw bones and temporo-mandibular joints⁽⁵³⁾⁽⁶⁰⁾. We also emphasize that mastication movements seem to have high potential for neuroplasticity, which may be reflected by the electrical properties of the trigeminal nuclei⁽⁶⁰⁾. Considering that supplementation with TRI led to an increase in the number of chewing movements, we suppose that this treatment is able to improve the development of the components of the craniofacial system during atypical development.

Proper nutrition is essential for craniofacial maturation during critical periods of development (prenatal, birth and first days of postnatal life)⁽⁴⁰⁾⁽⁵¹⁾. In addition, experimental studies demonstrated that protein and amino acid intake during early postnatal period, contribute for development of muscle skeletal tissue⁽³⁷⁾⁽⁵¹⁾. Our study did not evaluate the effect of others essential amino acids, which limit the elucidation of the specific role of TRI on chewing maturation. However, since TRI content and 5-HT activity are involved on morphogenesis of masticatory system⁽¹⁸⁾⁽⁵⁸⁾⁽⁶⁰⁾, we suggested that ours results may be related to TRI treatment offered for animals subjected to CP.

Thus, the data obtained in our study give new and promising insights into the protective role of TRI in the development of chewing in animals subjected to experimental CP. These data suggest the important role of treatment with TRI as a nutritional intervention in the neonatal intensive care unit (NICU)⁽⁶¹⁾. The milk of mothers who deliver prematurely contains inadequate amounts of nutrients⁽⁶²⁾. Adequate nutrition during the first postnatal week is associated with the optimal growth and neurodevelopment of children with CP⁽⁶¹⁾. In addition, evidence from human studies has demonstrated the safety of providing higher levels of amino acids, including TRI⁽⁶¹⁾⁽⁶³⁾. Therefore, from these experimental data, we demonstrated the efficacy of therapy with TRI to guarantee the appropriate development of chewing during the breastfeeding period.

CONCLUSIONS

In summary, we investigated the therapeutic potential of TRI in reducing deficits in mastication in experimental CP. From our findings, we confirm our hypothesis that TRI minimizes the appearance of the masticatory impairments observed in CP animals. Thus, our study was the first to evaluate the therapeutic potential of nutrition, particularly treatment with TRI, in attenuating the functional and morphological damage of CP. Therefore, the data obtained in our study may contribute to the inclusion of therapeutic nutritional strategies to attenuate the chewing impairments observed in children affected by CP.

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LEGENDS

Figure 1 Effect of CP and TRI on Body Weight at Postnatal days: Body weight of rats of the Control Saline (n = 25), Control TRI (n = 16), CP Saline (n = 23), CP TRI (n = 16) groups at 1, 8, 14, 21 and 29 days postnatal life. The body weight of both control groups treated either with saline or TRI increased similarly over time, but was larger than that of CP groups treated either with saline or TRI (*P <0.001).

Figure 2 Effect of CP and TRI on Feeding Intake: (A) PCA Analysis of feeding intake variables (sucking on week 1, sucking on week 2, sucking on week 3, chewing on week 3). CS: Control Saline; CT: Control L-Tryptophan; CPS: Cerebral Palsy Saline; CPTRI: Cerebral Palsy L=Tryptophan. (B) and feeding intake by sucking and chewing and relative food intake of pups from the Control Saline (n = 13), Control TRI (n = 14), CP Saline (n = 13) and CP TRI (n = 11). (A) The scores of PCA analysis of Control Saline rats did not differ from Control TRI rats, as well as CP Saline rats did not differ from CP Tri rats. The scores of Control rats with either treatment differed from those of CP rats with either treatment ($P < 0.01$). (B) In week 3, milk intake was reduced in CP Saline rats, compared to Control Saline rats ($*P < 0.05$). A reduction in the amount of food pellets eaten by CP TRI rats was verified when compared to Control TRI rats ($*P < 0.05$), while no significant changes between other groups. **Based on relative food intake by sucking and chewing, CP Saline animals showed a reduction on food intake by sucking in week 3 (P19-P20), compared with Control Saline rats (Tukey *post hoc* test: $P < 0.05$; Fig. 2B- Table). An increase on food intake by sucking in week 3 was verified in CPTRI rats compared with CP Saline animals (Tukey *post hoc* test: $P < 0.05$; Fig. 2B- Table).**

Figure 3 Effect of CP and TRI on Chewing Movements: (A) PCA analysis of chewing movements composed by 3 variables (rhythmic chewing phase, number of chewing cycles, frequency of chewing cycles). CS: Control Saline; CT: Control L-Tryptophan; CPS: Cerebral Palsy Saline; CPTRI: Cerebral Palsy L=Tryptophan, (B) duration in seconds of rhythmic chewing phase, and (C) number of chewing cycles and (D) frequency of chewing cycles of the Control Saline (n = 10), Control TRI (n = 10), CP Saline (n = 10), CP TRI (n = 10) in the twenty-first (P21) day postnatal life. (A)The

scores of PCA analysis of CP Saline differed from those of both CP TRI and Control Saline ($P < 0.0001$). No difference was found between CP TRI and Control Saline rats, while Control TRI animals differed from Control Saline animals ($P < 0.0001$). (B) Both CP groups had a reduced duration of rhythmic chewing phase compared to their Control groups ($*P < 0.05$). CP TRI showed a tendency for lesser chewing cycles, compared to Control TRI rats ($P = 0.07$). CP Saline rats exhibited a lower frequency of chewing cycles, compared to both Control Saline rats and CP TRI rats ($*P < 0.05$), yet Control and CP rats treated with TRI did not differ.

Figure 4 Effect of CP and TRI on Phenotype of Masseter: (A) Relative weight, (B: CS = I, CT = II, CPS = III, CPT = IV) histological sections and (C) percentage of the fibers type I (dark), IIA (light) and IIB (gray) of the masseter muscle, of the Control Saline ($n = 5$), Control TRI ($n = 5$), CP Saline ($n = 5$), CP TRI ($n = 5$) at 29 days postnatal life. (A) The relative weight of masseter in CP rats with either treatment was smaller than that of control rats treated either with saline or TRI ($*P < 0.001$). The relative weight of masseter was higher in both groups treated with TRI compared with their respective Saline groups, and this relative weight was higher in CP TRI than in CP Saline ($*P < 0.001$). (C) Both CP groups showed an increase in the percentage of type IIA fibers associated to a decrease in type IIB fibers compared to their respective Control groups ($*P < 0.05$). Treatment with TRI promoted the opposite effect when compared Control Saline with Control TRI ($P = 0.06$), and CP saline with CP TRI ($*P < 0.05$).

Table 1: Correlations between specific variables, based on the individual scores along PC1 (x-axis) of animals obtained by using PCA. Pairwise two-sided p-values: * $p < 0.05$.

Figure 1

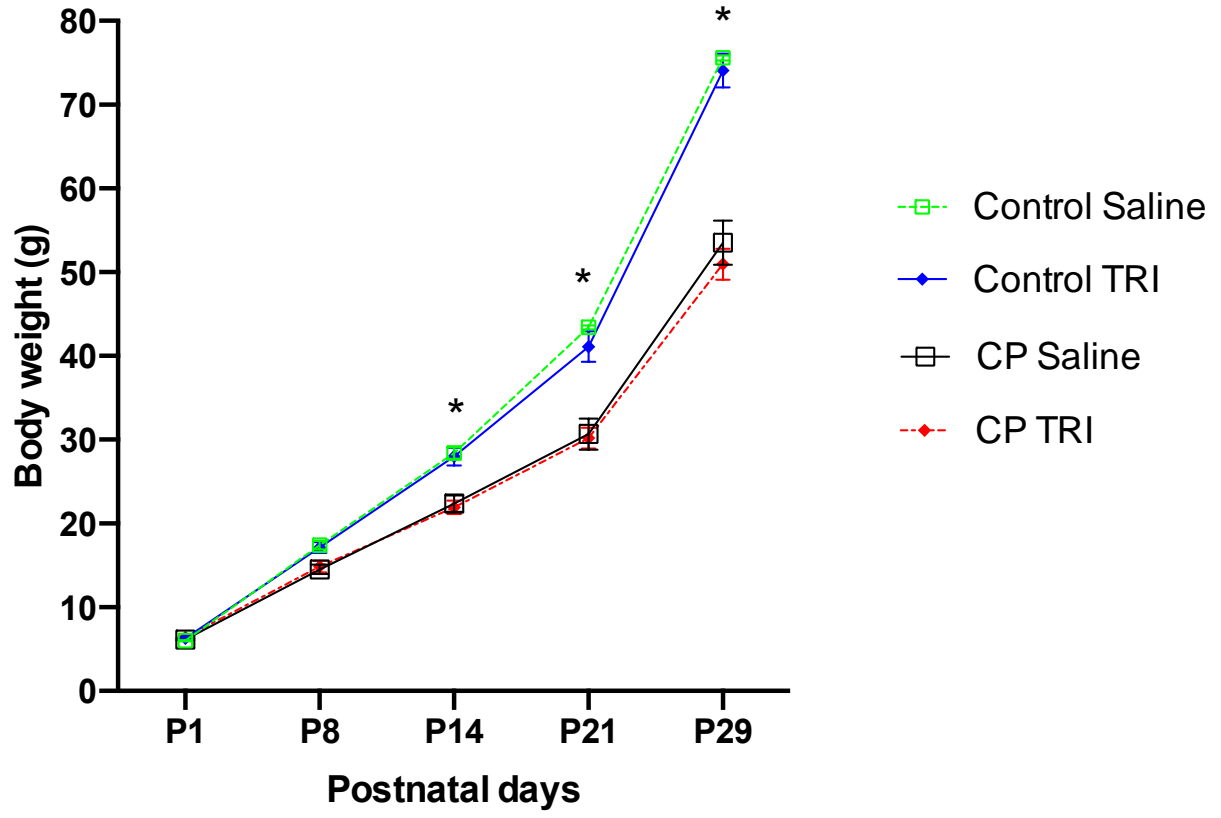
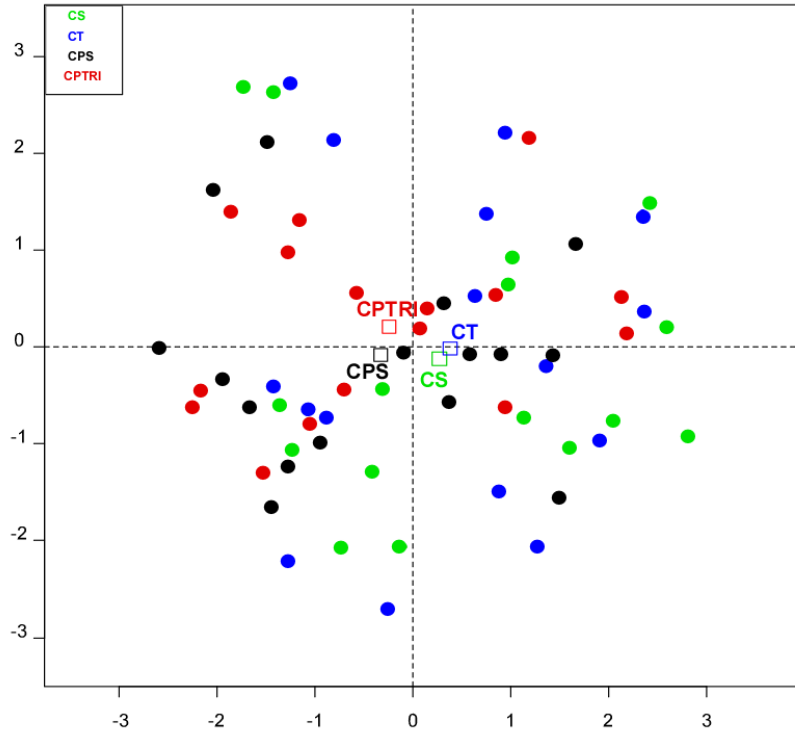
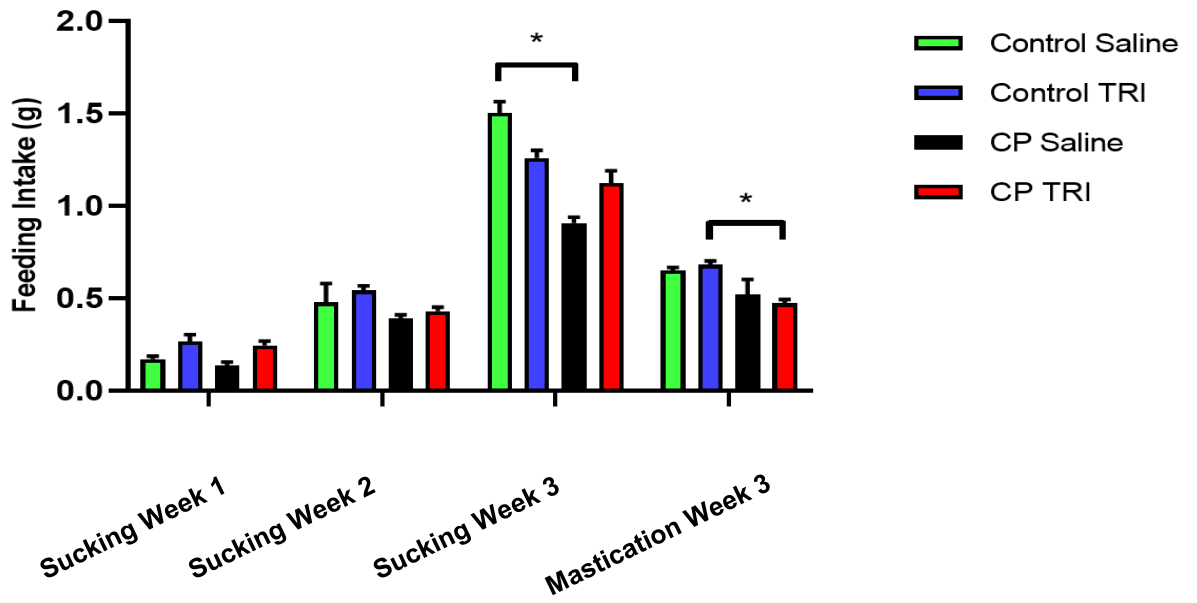


Figure 2

A

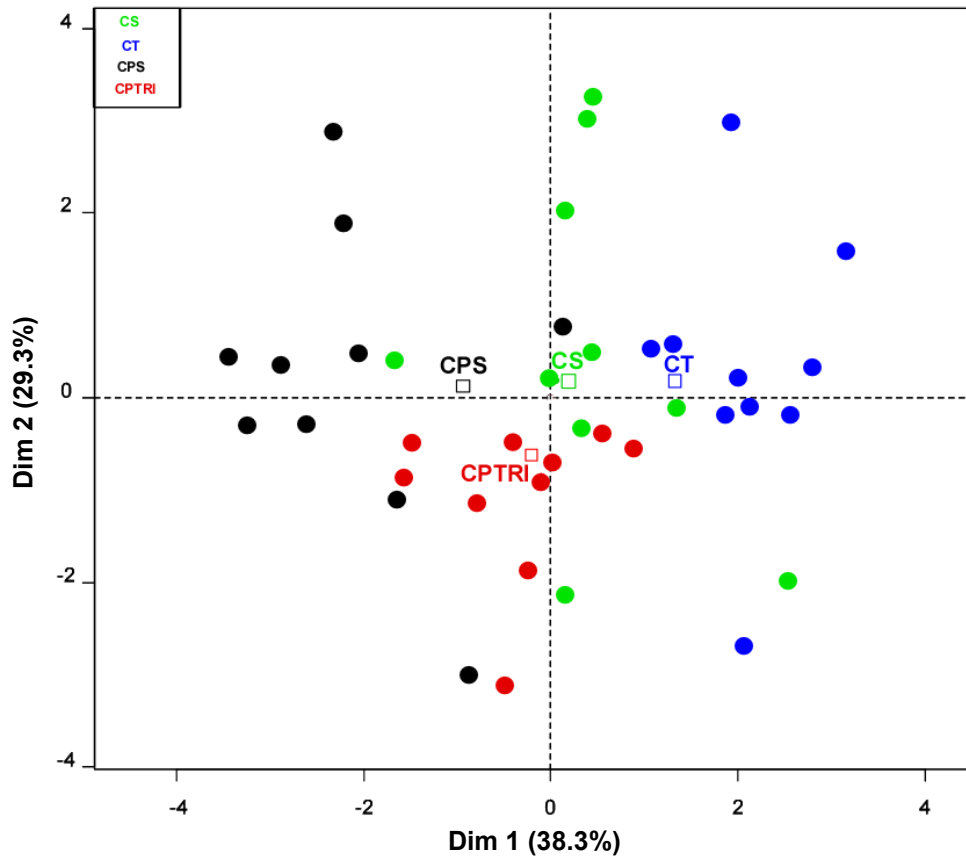


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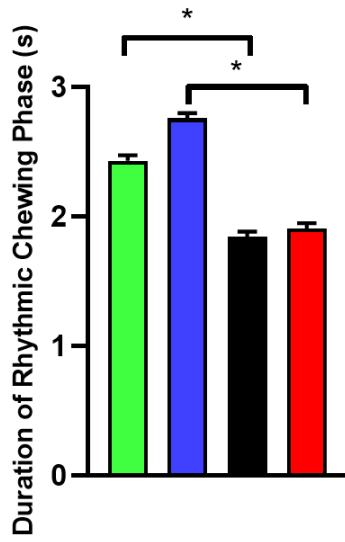


| Experimental Groups | Sucking | Sucking | Sucking | Mastication Week 3 (P21) |
|----------------------------|----------------------------|------------------------------|------------------------------|-------------------------------------|
| | Week 1 (P7- P8) | Week 2 (P14- P15) | Week 3 (P19- P20) | |
| Control Saline | 11.42 | 17.07 | 35.12 | 15.45 |
| Control TRI | 15.45 | 18.59 | 30.75 | 17,00 |
| CP Saline | 13.18 | 20.17 | 23.29* | 13.41 |
| CP TRI | 18.61 | 21.31 | 29.48* | 12.82 |

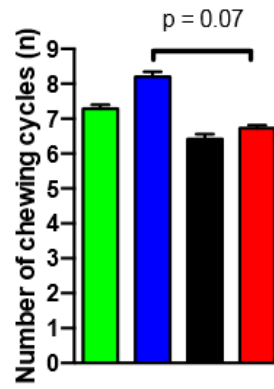
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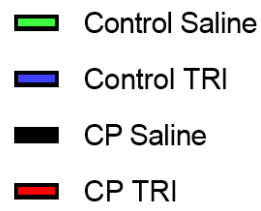
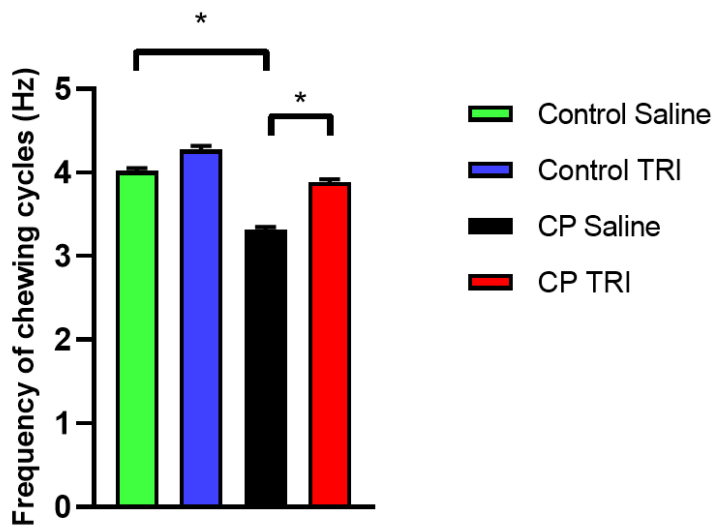
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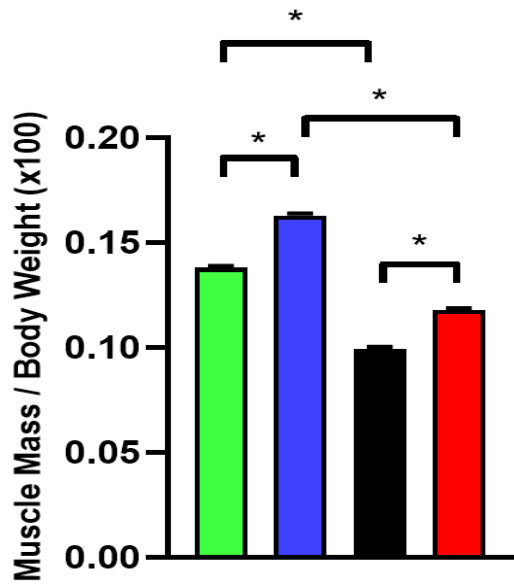
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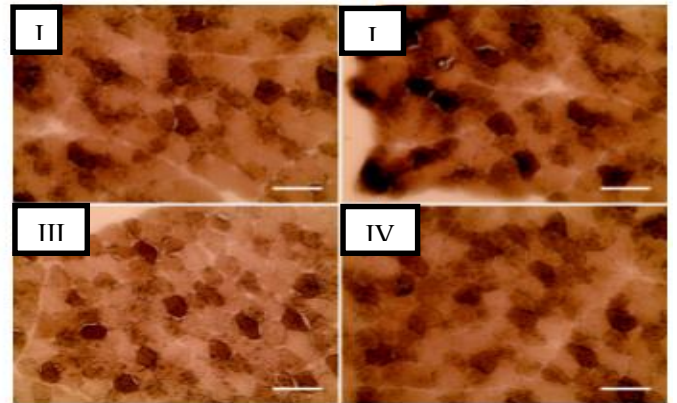
D



A



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C

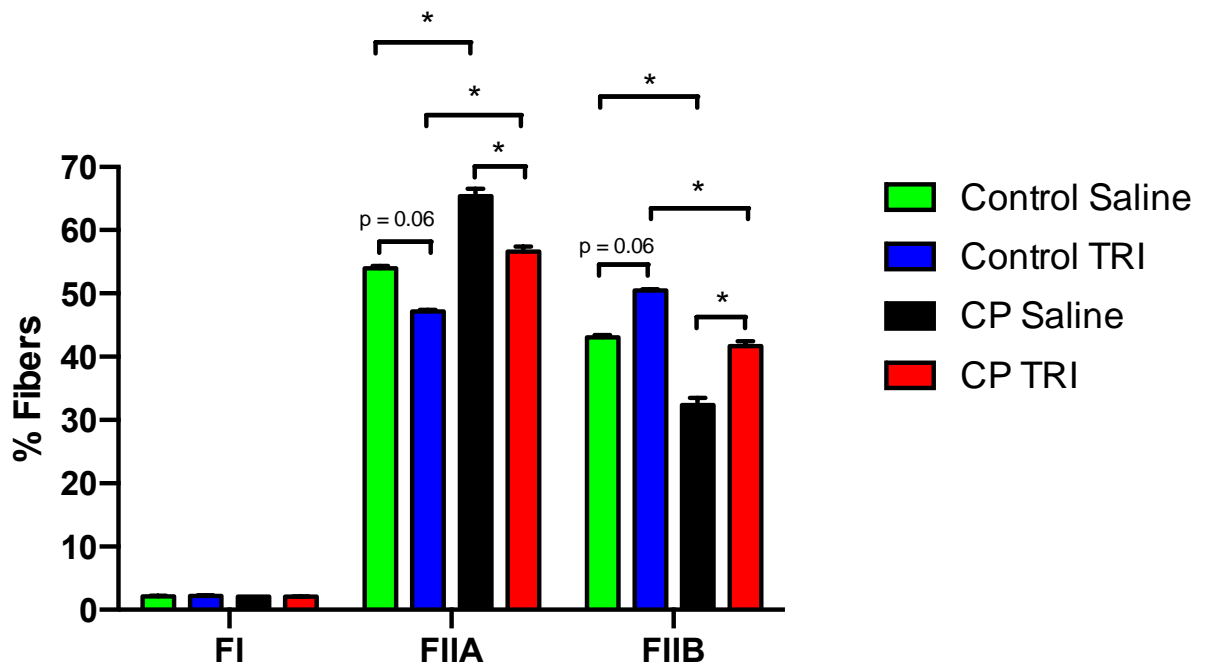


Table 1

| Variables | Number of Chewing Cycles | Frequency of Chewing Cycles |
|-------------------------|---------------------------------|------------------------------------|
| Masseter (g) | 0.449*; P = 0.05 | 0.686*; P = 0.01 |
| Masseter – FIIA | - 0.428*; P = 0.05 | - 0.735*; P < 0.01 |
| Masseter – F IIB | 0.428*; P = 0.05 | 0.749*; P < 0.01 |

Dear George E. Barreto,

We were very grateful with the evaluation of our revised manuscript. The authors have made few minor formatting points raised by the referee. We hope the revised version will fit the requirements for publication in Nutritional Neuroscience.

Comments from the Editors and Reviewers:

Reviewer #5: The headings in the newly provided table should be corrected since "Relative Food intake (Food Intake/Body Weight x 100)" is not suitable for the group column.

Reply: The authors thank Reviewer for the recommendation to adjust the identification of group column. We removed "Relative Food intake (Food Intake/Body Weight x 100)" and corrected as follow "Experimental groups" . Please check the changes in the Figure 2B.

We hope that after the modifications made, the manuscript is suitable for publication.

Best regards,

Professor Ana Elisa Toscano