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Evidence of a Relationship between Aversive Learning and Hippocampal Interleukin 1 Beta Levels in Neonatal Handled Rats

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Indíce

Introdução

No presente trabalho, buscamos uma relação entre as alterações neuroendócrinas e comportamentais demonstradas no modelo de Manipulação Neonatal (MN) sob a perspectiva de descobertas recentes da neuroimunologia. Dentre essas se destaca a existência fisiológica de uma interação reguladora entre o sistema imune (SI) e o sistema nervoso central (SNC). Na visão atual da ciência as citocinas, que pareciam existir no SNC apenas durante respostas inflamatórias intensas, são ativamente secretadas por neurônios e células gliais em níveis regulatórios. No hipocampo, destacamos a IL-1 beta, que tem sido relacionada ao aprendizado contextual e LTP, e que pode ter sua expressão reduzida em resposta a glicocorticoides, como os produzidos na resposta ao estresse.

Ratos que recebem a Manipulação Neonatal têm demonstrado diversas alterações comportamentais, e também apresentam uma resposta reduzida ao estresse, com menor secreção de corticosterona, e retorno mais rápido desta aos níveis basais. Baseando-se no último fato, decidimos investigar se a redução na secreção de glicocorticoides em resposta a um evento estressor, e a redução da imunossupressão, possibilitariam aumentos nos níveis de IL-1 beta hipocampal, e dessa forma no aprendizado. Para isso, expusemos ratos Wistar juvenis e adultos manipulados a um protocolo de esquiva inibitória, e em seguida coletamos amostras de hipocampo desses animais; com isso pudemos correlacionar à ocorrência de aprendizado aversivo com possíveis alterações nos níveis de interleucina 1 beta hipocampal. Mais detalhes serão discutidos no artigo a seguir, escrito aos moldes do periódico Brain, Behavior and Immunity.

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ABSTRACT

The Neonatal Handling (NH) model presents a “blunted” corticosterone response to stress as well as an enhancement in contextual fear tasks. So the aim of this work was to examine a possible relationship between diminished imunosuppression over Interleukin 1 beta (IL-1) hippocampal levels and enhancement in aversive learning. To achieve that, we subjected male handled Wistar rats 30-35 and 90 days old to an inhibitory avoidance task, comparing the avoidance scores with respective hippocampal IL-1 levels at different time points. We found that handled adult animals learned the aversive task concomitantly with higher levels of hippocampal interleukin 1 beta 2h after footshock, while control animals showed diminished aversive learning and lower IL-1 beta levels at the same time. In the other hand, both handled and non-handled juvenile rats learned the aversive task, with no signal of immunosuppressant efect over interleukin 1 beta. However, basal levels of IL-1 beta were different between handled and non-handled animals in both ages, pointing a novel neuroimmunomodulation involvement to the NH model.

Keywords: Hippocampus, Inhibitory Avoidance, IL-1 b, Interleukin 1 beta, Neonatal Handling

1. Introduction

The neonatal handling (NH), is an animal model of moderate early intervention that has been used to investigate the impact of early developmental experiences on neurobehavioral plasticity in both infants and adults animals. In previous studies, the NH has been reported to reduce emotional reactivity (Levine and Mulin, 1996; Valee et al., 1997), protect against age related declines on hippocampal-dependent learning (Meaney et al., 1988), enhance two-way active avoidance learning (Escorihuela et al., 1994) and to generate a “blunted” hypothalamic–pituitary–adrenal (HPA) response to stress. The “blunted” response to stress can be resumed as a lower plasmatic CORT elevation after a stressor stimuli and a quickly return to CORT basal levels (Ader and Grota, 1969; Levine, 1957, 1962, 2001; Meaney et al. 1989). Handled animals achieve better scores in contextual fear tasks, when compared to non-handled controls (Beane et al., 2002; Kosten et al., 2007), and such differences are related with corticosterone levels as the increased density of glucocorticoids receptors in the hippocampus of handled animals (Champagne et al., 2003, 2008). Still, data on the mechanisms for corticosterone effects on memory are incomplete.

Recent advances in the field of neuroimmunology, allowed us to think about some additional mediators that could explain the alterations in learning and memory found in handled animals. One of them is the Interleukin 1 beta, this cytokine was recognized in the past only as a pro-inflammatory agent, and as an inducer of sickness behavior, but in the last years that has been demonstrated as an enhancer of contextual learning, memory and long term potentiation (LTP) (Kunz-Ebrecht et al., 2003; Yirmiya and Goshen, 2011). Yet, the IL-1 beta works in an inverse U-shaped way, in which moderate levels

are required to healthy hippocampus functioning, but too low or too high concentrations are deleterious (Goshen et al., 2007).

Work of Nguyen, obtained in adrenalectomized rats (ADX), demonstrated that, these animals answered a stressor stimuli with huge augments in IL-1 beta hippocampal levels. The ADX rats, lack not only the corticosterone response to stress, but also lack the immunosuppressive effect exerted by CORT (Nguyen et al., 2000). So we asked ourselves if, in NH rats, the corticosterone response to stress could be "blunted" enough to diminish immunosuppression over the hippocampus, and if this could allow IL-1 beta elevations at levels required to enhance aversive learning.

To answer our question, we subjected neonatal handled male adult Wistar rats, to the inhibitory avoidance task, so we could evaluate aversive hippocampal-dependent learning and compare these results with the current interleukin 1 beta levels. Also, we replied the experiment in 30-35 days-old handled male rats with the aim of observe IL-1 beta levels in response to stress at young age.

2. Materials and Methods

2.1. Subjects

Pregnant female Wistar rats from the institution's central colony were kept on our setorial animal room, and were observed daily to determine the day of delivery, which was designated as day 0. After day 21, male pups were housed in groups of four animals in plastic cages, under controlled temperature (21°C ± 2°C), and artificially lit in a 12-h cycle period. Subjects were allowed to growth until 30 to 35 days (juvenile group) or until 90 days (adult group). Standard rat chow and water were freely available. All animal procedures were previously evaluated and carried out according to the rules and standards for the use of laboratory animals pointed out by the Ethical Committee of Animal Use of Universidade Federal do Rio Grande do Sul (CEUA-UFRGS).

2.2. Neonatal Handling

Litters were randomly assigned to either the handled or the non-handled condition. Handling was performed on days 1 through 10, once a day, between 1200 and 1400 h. For the handling procedure, the dam of each handled litter was removed temporarily from the home cage and placed singly into a clean maternity cage. Then, each litter of pups was gentle handled by the researcher. After 1 min of handling, the pups were returned to their home cage. The dam was then returned to the home cage. To avoid olfactory learning biases, a clean pair of latex gloves was used in each handling procedure. For days 1 through 10, cage maintenance was not performed. During this time, other than handling, all litters were left undisturbed.

2.3.Inhibitory avoidance

Inhibitory avoidance was made as follows (Bernabeu et al., 1997; Izquierdo et al., 1998): rats were placed on a 2.5-cm-high, 8.0-cm wide platform at the right side of a 24.0 x 20.0 x 18.0-cm acrylic training apparatus, whose floor was a series of parallel 0.2-cm caliber bronze bars spaced 1.0 cm apart. Latency to step down onto the grid with all four paws was measured. In the training session, immediately after this, the animals received a 0.6-mA, 3.0-s scrambled footshock. In the test session, performed 24 h (long-term memory) after training, the procedures were similar, except that footshock was omitted. All sessions were recorded for 5 minutes and analyzed by a blinded observer.

2.4. IL-1b protein quantitation

Briefly after capitulation, hippocampus were dissected and frozen at -80°C for later quantitation. Levels of IL-1b protein were determined using a commercially available rat IL-1b ELISA kit (eBioscience©). Samples were homogenized in a specific protein extraction buffer and the assay was performed according to the manufacturer instructions.

2.5. Statistical analysis

All data are expressed as means ±SEM. A two-way analysis of variance (ANOVA) was performed to compare IL-1 beta levels, followed by Bonferroni post test when appropriate; t test was also used for comparison between two groups. Inhibitory avoidance data was compared by means of Mann-Whitney non-parametric analysis. The level of statistical significance was set at $p<0.05$.

2.6. Experimental Design

Neonatal handled animals and their age paired controls (non-handled) were divided into three main groups: 0h (basal) –that were not exposed to the inhibitory avoidance task–, 2h –that were capitulated 2 hours after the training session (to which foot shock was used as an stressor stimuli) –, and 24h –that were killed immediately after the test session. All experimental groups had at least 8 animals.

3. Results

3.1. Aversive learning

We measured aversive learning in the 24h groups, by comparing the latency to step down in the training phase to the latency to step down in the test phase. In the juvenile rats both non-handled (N=13) and handled (N=11) showed no remarkable differences in the mean time to step on the grid at the training phase. The two groups appeared to remember the stressor event in the test phase, as they freeze and stayed over the platform (data not shown).

Adult rats showed small differences in the median time to descend the platform during the training phase ($md_{control}$ = 5.5s (3.25/8.75) vs $md_{handled}$ = 11.5s (10.25/15.75) p = 0.027), however bigger differences were found in the test phase, in which handled 24h rats (N=8) took longer time to descend the platform in the test phase when compared with 24h non-handled (N=8) ($md_{control}$ = 23s (3.75/146.5) vs $md_{handled}$ = 180s (66/180) p = 0.049) (Fig. 1).

3.2. Hipoccampal interleukin 1 beta levels

To access differences in juvenile hippocampal IL-1 beta levels, Two-way ANOVA was applied. Differences were found between the 0h control group and the 24h control rats (Fig. 2). Further t test of data also revealed a significant difference between the 0h control and the 0h handled animals (Fig. 3).

Two-way ANOVA was also used to analyze adult data (Fig. 4). Interleukin 1 beta levels increased along the time ($F_{time(1,16)} = 28.51$, $p < 0.001$), although handled animals showed a higher increase in hippocampal IL-1 beta 2h after shock. Twenty-four hours after shock non-handled rats presented the same levels of IL-1 beta as handled ones ($F_{treatment(1,16)} = 86.24$, $p < 0.001$).

4. Discussion

We found no avoidance learning differences between the 24h juvenile (30-35 days-old) individuals at the inhibitory avoidance task. However, Beane and colleagues (Beane et al., 2002) found that 30 days-old handled rats subjected to a fear conditioning protocol very similar the avoidance task applied by us, presented greater levels of freezing behavior. Although, in their work the freezing times were accounted, while we only regarded to the step-down response. In our analysis, both non-handled and handled animals remembered the stressor stimuli 24h after the shock, so it is possible that the behavioral task applied and the parameters analyzed were not appropriate to juvenile rats, as it could not show minor variations (if they exist) in the aversive learning performance. Due to experimental issues, plasma CORT levels were not accessed, so we look upon corticosterone data in literature in order to make assumptions over the results.

Young rats IL-1 beta levels behave quite interestingly between handled and non-handled individuals, as basal levels (0h groups) of interleukin 1 beta were higher in controls. However, interleukin basal concentrations for both juvenile groups are huge in comparison to the adults basal levels, a plausible hypothesis is that, at young age, hippocampus still in development and levels of IL-1 beta must be signalizing neuronal proliferation and network adjustments, functions already described for adults in literature (Depino et al. 2004; Goshen and Yirmiya, 2009; Labrousse et al., 2009). At 2h both groups elevated their interleukin 1 beta levels; again, according to the effects of this cytokine over LTP and learning already described in research literature, it is possible to link the aversive learning of both groups at test phase with the 2h elevations. Tough no cytokine inhibition was observable over non-handled rats, our hypothesis was

based upon experimental work made in adult subjects, thus it is possible that 30-35 days-old rats have not yet developed all feedback mechanisms to form a corticosterone driven immunosuppressive response as significant as the observed in adults (Levine, 1962), also, the current finding suggest an intriguing involvement of IL-1 beta in hippocampal maturation processes for neonatal handled rats. Both juvenile groups equaled their cytokine 1 beta levels at 24h, finding that will be further discussed bellow.

Opposed to the juvenile group, basal levels of IL-1 beta were lower in handled adults when compared to non-handled controls. At 2h the interleukin response behave as predicted by our hypothesis, so that control rats showed a minor increase in interleukin 1 beta levels while a huge increase was observed in the handled group. The IL-1 beta increase in 2h-handled animals can be perfectly explained by a diminished immunosuppressive response, which could be caused by diminished and/or brief CORT response, as described in the neonatal handling literature (Levine, 1957, 1962, 2001; Meaney et al. 1989). By the same way, the modest interleukin 1 beta elevation in 2h non-handled group could be explained as an effect of immunosuppressive corticosterone activity. Interestingly, at 24h both non-handled and handled individuals reached the same levels of cytokine 1 beta, tough handled maintained interleukin amounts while controls demonstrated a growth from 2h to 24h. When linking behavior with molecular data in adults, we see a link between avoidance learning and the 2h results. The higher concentrations found at 2h could certainly exert an effect over hippocampal response by reaching ideal levels, that could signalize long term potentiation, hippocampal proliferation and memory consolidation (Goshen et al. 2007; Kunz-Ebrecht, 2003). Differences in behavior response at training phase correlates with increased exploratory behavior found in handled animals, as these spent more time sniffing the platform (Padoin et al. 2001).

Tough we were unable to prove our hypothesis for juvenile rats, two points in IL-1 beta patterns must be observed with care. The first was that basal levels of interleukin 1 beta were different between non-handled and handled animals at both ages, while we cannot correlate the basal results between young and older animals, this finding alone provide us with valuable insight about interleukin 1 beta effects over hippocampus both in development as in adult life of handled animals. Also, variation in patterns of neural and glial development must be involved with the differences found in NH, as these cells are shown to produce IL-1 beta in the SNC, (Depino et al. 2004; Goshen and Yirmiya, 2009; Labrousse et al., 2009). It is also possible that changes in the basal levels of interleukin 1 beta in handled animals might be over spread across other brain areas. The second point was that 24h individuals achieve the same interleukin levels; tough juvenile reduced their cytokine levels while adults elevated it. This could be related with latter memory consolidation mechanisms, and is plausible to think that IL-1 is unrelated to post-24h memory consolidation mechanism for both handled and non-handled individuals.

In the present work we prove our hypothesis for the adult group but not for the juvenile ones, however, the original experiment was designed to adult animals, with juvenile being included with a screening purpose. Clearly, maturation processes are fully running in 30-35 days-old rats, and for them CORT related immunosuppression do not seem to exert effect on aversive learning. The relationship between hippocampal interleukin 1 beta and aversive learning in adults can alone explain many behavior changes described in NH model, also, CORT levels must be accessed in future studies in order to endorse these results. The unexpected findings about IL-1 beta basal levels of handled animals gave us a dozen of possibilities to investigate involvement of this cytokine in hippocampal development of NH model, as it turns possible the

involvement of another brain structures. In the end, we could not validate our hypothesis for both ages, but instead, we gain insight of a clear involvement of a perinatal intervention over neuroimmunomodulation processes.

ABREVIATIONS:

CORT: Corticosterone

IL-1 beta: Interleukin 1 beta

NH: Neonatal Handling

SNC: Central Nervous System

Graphs:

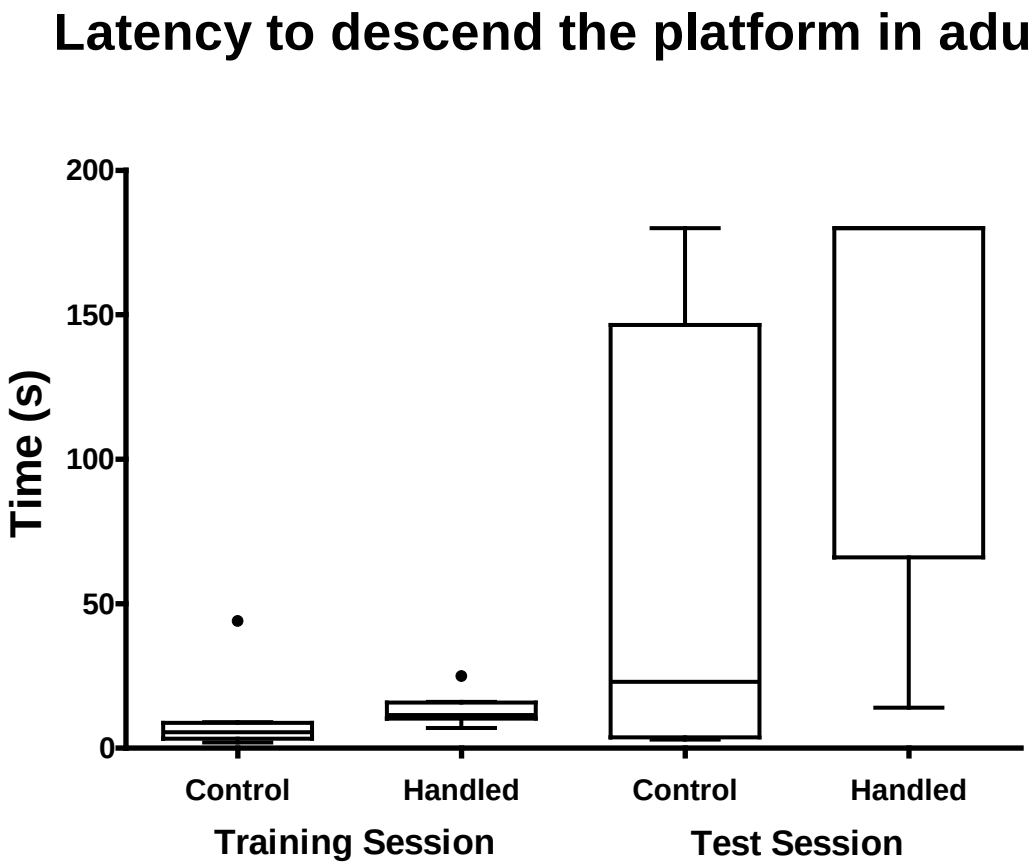

Figure 1. Time to descend the platform in seconds: There is a small difference on the median time to descend the platform during the training ($md_{control}$ = 5.5s (3.25/8.75) vs $md_{handled}$ = 11.5s (10.25/15.75) p = 0.027). In the test session the median time to descend the platform was higher in handled animals ($md_{control}$ = 23s (3.75/146.5) vs $md_{handled}$ = 180s (66/180) p = 0.049). Points floating over training session bars indicate outlayers animals.

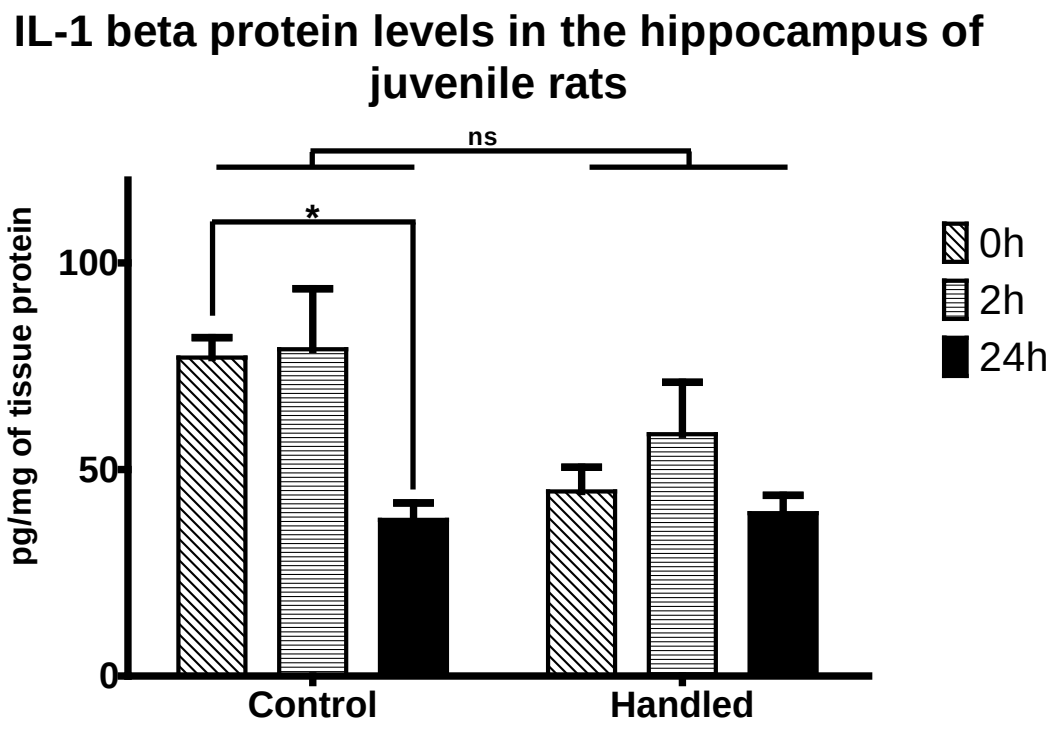

Figure 2. Interleukin 1 beta protein levels in the hippocampus of handled and control male juvenile rats with no shock (0h) or 2 and 24 hours after foot shock: Time accounts for approximately 21.44% of the total variance (F= 6.24 p= 0.048) and treatment accounted for 9.96% of the total variance (F= 5.80 p= 0.0214), but treatment and time do not interact ($F_{(1,16)}$ = 1.99, *p* =0.1519). Il1 beta levels in 0h controls subjects were statistically different from 24h controls. Both groups presented similar levels of the cytokine at 24h post shock.

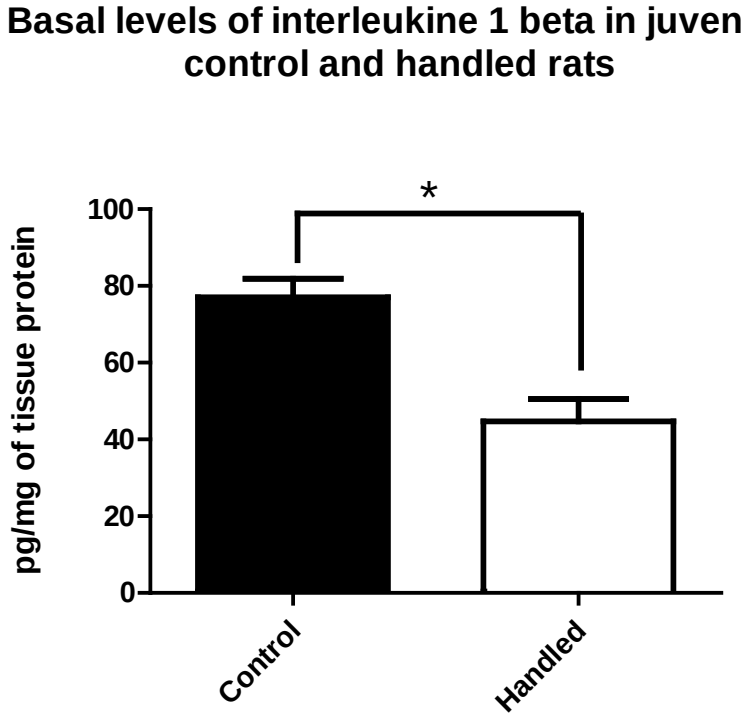

Figure 3. Basal levels of interleukine 1 beta in the hippocampus of juvenile rats, handled and controls: There is a significant difference in the levels of IL-1 beta between control and neonatal handled individuals that do not experienced any stressor stimuli ($Mean_{control}$= 77.08 ± 4.826 N=7 vs $mean_{handled}$= 44.69 ± 5.866 N=8 p= 0,0011).

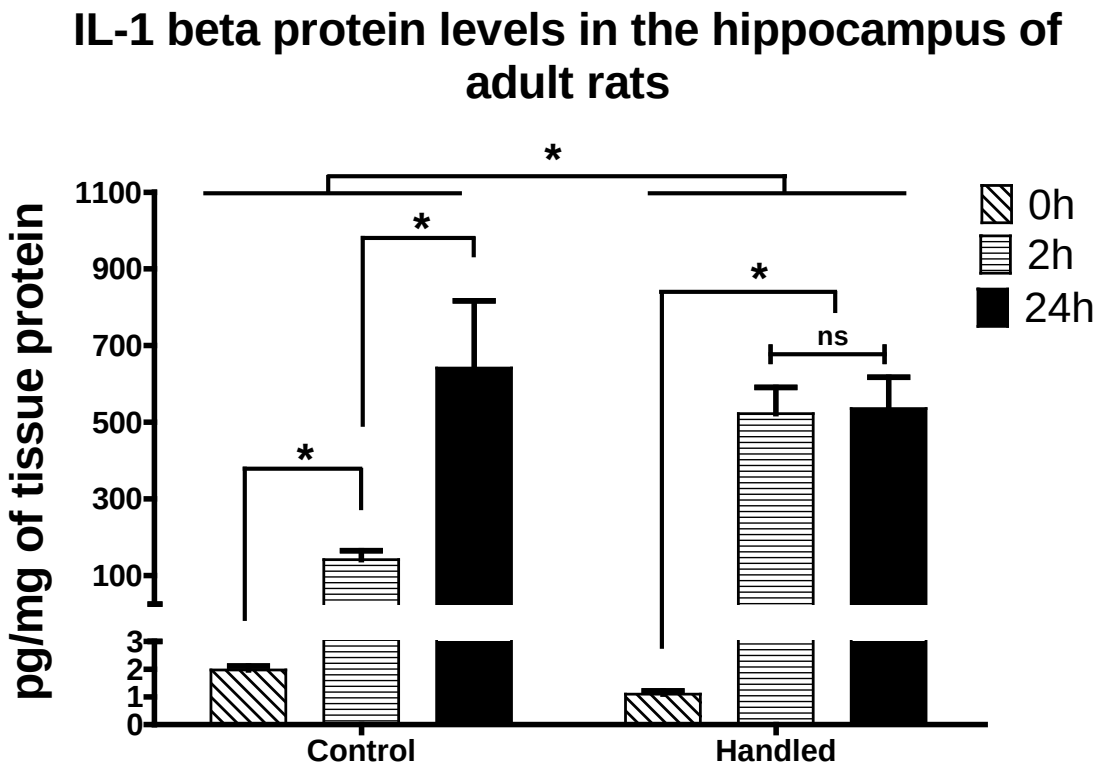

Figure 4. Interleukin 1 beta protein levels in the hippocampus of handled and control male adults with no shock (0h) or 2 and 24 hours after foot shock: treatment and time interacts to form the phenotype ($F_{(1,16)} = 28.77$, $p < 0.001$) in witch time acts different in the two groups: Interleukin 1 beta levels increases along time ($F_{time(1,16)} = 28.51$, $p < 0.001$) although handled animals have a higher increase in hippocampal IL-1 beta 2h after shock. 24h after shock controls have the same levels of IL-1 beta as handled animals ($F_{treatment(1,16)} = 86.24$, $p < 0.001$).

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Conclusão:

No presente trabalho, conseguimos provar nossa hipótese para o grupo adulto, mas não para o juvenil, entretanto, a ideia original do experimento original foi proposta para animais adultos, sendo os juvenis incluídos numa fase posterior. É claro para nós que a negação da hipótese para animais entre 30 e 35 dias se deve ao fato de que processos de maturação cerebral ainda estão em pleno desenvolvimento, assim como os grandes níveis basais de IL-1 beta para ambos os grupos indicam para uma atividade hipocampal elevada. Já a relação entre os níveis de interleucina e o aprendizado aversivo em ratos adultos pode sozinha, explicar muitas alterações comportamentais até então descritas para a manipulação neonatal, entretanto, é mandatório que os níveis de CORT sejam aferidos em experimentos futuros para dar respaldo a nossos resultados. Os resultados observados nos níveis de IL-1 beta de manipulados nos abrem um vasto leque de possibilidades para investigar o envolvimento desta citocina no desenvolvimento hipocampal de animais submetidos à manipulação neonatal. Ao fim, não pudemos validar nossa hipótese para ambas a idades, mas conseguimos detectar uma relação clara, de uma intervenção perinatal sobre um processo de neuroimunomodulação.