

# Juvenile Play Behavior in Neonatally Underfed and Sensory Stimulated Wistar Rats

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## Abstract

Play development in juvenile rats depends on specific sensory signals integrated at cortical, limbic and brain stem levels to modulate motoric, metabolic, motivational and social responses. Neonatal undernourishment disrupts the morphological and functional organization of the brain for adaptive responses including play performance. These alterations may be restored by preweaning exposure to sensory-enriched environments. This study was designed to determine in four experimental groups, Control (LC), Underfed (LU), Control Ligated/Stimulated (LCS), and Underfed Ligated/Stimulated (LUS), whether changes in juvenile play of neonatally underfed male rats by the nipple-ligated procedure of F0 dams and/or the handling of F1 rats may restore the deficiencies in juvenile play performance. The pinning frequency values in LC, LCS and LUS groups consistently increased until reaching a significant peak between postnatal days (PDs) 25 and 50 and then gradually declining until PD 60, when the play in pairs was significantly higher compared with the play in groups that follows the same sequence but with lower values in the stimulated groups. The results may reflect poor maternal care and lower somatosensory stimulation; and the sensory massage of LU F1 pups compared with the LC, LUS, and LCS rats. Fewer dorsal body contacts occurred in LU and LUS rats when playing in pairs than in groups. Results suggest that although handling has salutary effects on neuronal play structures, the reduced levels of total pinning and dorsal contacts, mainly in the play of rat pairs in LCS vs. LUS groups, were not fully recovered.

## Keywords

Juvenile Play, Early Handling, Neonatal Undernutrition, Rats

## 1. Introduction

During the lifespan of rats, the neuronal mechanisms underlying juvenile play

development and maternal-like responses are partly activated and integrated by ancient neuronal networks concentrated in subcortical and cortical brain regions that elicit autonomic and motivational states, including the olfactory system, amygdala, and hypothalamus; and by the prefrontal and the anterior cingulate cortices, which modulate and refine postural and detailed adaptive movement displacements, respectively [1] [2] [3] [4] [5]. Play behavior in rats is a basic response of immature neonatal stages that enhances the acquisition of both competitive and noncompetitive social skills to facilitate learning and social bonding ranks later in life [6] [7]. Furthermore, juvenile play is a complex response in which the participants are exposed to multisensory environment signals that may influence the juvenile play. Unfortunately, the available information is sometimes contradictory or provides poor support to the neuronal mechanisms underlying this basic juvenile neuromotor activity both in healthy and in handicapped subjects [8]. Thus, it is known that in the study of play in rats, the punctured bilateral tympanic membrane with the pinna of the ear folded over the ear opening significantly reduced juvenile play, although the sealed eyelids with cyanoacrylate adhesive to block the sight had no effects on juvenile play [9]. Furthermore, induced anosmia by either zinc-sulfate or bilateral bullectomy had no effect on males but caused minimal frequency increments in the play outcome of juvenile females [10]. Somatosensory stimulation is a relevant source of influence to modulate the expression of specific motor responses of young rats, including maternal anogenital licking, huddling, grooming, suckling, and retrieving, elicited by sensitive skin receptors located in strategic areas of the body to provoke adaptive motoric responses including juvenile play [11] [12] [13] [14] [15].

Perinatal food restriction and the associated impoverishment of neonatal sensory stimulation are some of the non-genetic factors that may interfere with the developing brain assembly and are determinants of social behavior disruption in adults [16] [17] [18]. In the rat, early undernutrition or malnutrition significantly interferes with brain growth and functions by reducing neurogenesis, myelination, and the number and density of dendritic arbors, spines and synaptic contacts in different areas of the brain [19]-[25]. These anatomical alterations and the early sensory manipulations result in an immature brain with impaired sensory organization that may disturb the transmission, encoding and integration of ascending neuronal messages from the periphery to the cerebral cortex, which is related to the integration of complex locomotion and deficient early social motivation including juvenile play responses [26] [27].

During the rat's nursing period, the mother-litter bonds are relevant for newborn development and to maintain the maternal care activity for pup survival and breeding. Thus, the lactating mother provides important olfactory, tactile, thermal, and vestibular stimulation to the pups through body and anogenital licking, retrieving, whisking, and manipulation, which are significantly reduced in early underfed lactating dams, causing long-term behavioral, anatomical, and neurochemical deficits for the progeny [28] [29] [30].

During lactation, neonatally underfed F1 young interact poorly with their F0 mothers and littermates, and the somatosensory cues of the mother decrease significantly [31] [32]. Therefore, we hypothesized that these conditions may disrupt juvenile play performance because they possibly interfere with the neuronal circuit organization of F1 underfed rats. Additionally, because various preweaning handling routines of enriched environment and chronic tactile stimulation or body massage increase neurogenesis and the number of dendrites and spines in the ambiguous nucleus, hippocampus, amygdala, and cerebral cortex [33] [34] [35], we tested the role of the preweaning-increased sensory stimulation on juvenile pinning and body contact performance of early underfed rats.

## 2. Materials and Methods

### 2.1. Animals

Thirty-six male Wistar rats (*Rattus norvegicus*), descendants of a stock originally obtained from Harlan Sprague-Dawley (IN-USA) were used as subjects. Animal care and protocols were approved by Local Animal Committees that were in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research [36]; and the Norma Oficial Mexicana (SAGARPA NOM-062-ZOO-1999). Subjects were obtained from 8 pregnant, nulliparous dams, 100 - 120 days of age (200 - 250 g). The animals were maintained in a room with a temperature of  $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , 50% humidity, a light/dark cycle of 12 h/12h (lights on 07:00 h), and food (Purina chow) and water ad libitum. For mating, groups of four control adult virgin female rats were housed with two males of similar nutritional conditions in a plastic cage ( $60 \times 40 \times 20 \text{ cm}^3$ ). Sperm-positive females were individually housed in plastic cages ( $50 \times 40 \times 20 \text{ cm}^3$ ) with grill tops and wood shavings as nesting material one week before parturition. Births were checked daily at 08:00 and 18:00 h. The day after birth, pups were weighed and sexed, and the litters were adjusted to 8 pups per mother (four males and four females). The redistribution intended to minimize possible genetic and nutritional differences that may influence the experimental results. The distinctive bilateral thoracic and abdominal line of nipples and the shorter anogenital distance in the females were used as criteria for sex recognition [37].

### 2.2. Nutritional Paradigms

#### 2.2.1. Control (LC) and Undernourished (LU) Groups

Protocols of early malnutrition or undernutrition in rodents are associated with different levels of sensory deprivation that may interfere with brain growth and behavioral development, despite the consumption of a balanced diet from weaning onwards [17]. Moreover, neonatal handling or the exposure to a sensory-enriched environment does not totally compensate the behavioral alterations of early undernutrition [38]. To separate the effects of early food restriction from those of sensory deprivation in newborns, we used the underfeeding procedure with nipple-ligated mothers, which minimize the adverse effects of sensory deprivation

inherent to other underfeeding techniques [39]. Additionally, to evaluate the nipple-ligation paradigm associated or not with early neonatal sensory stimulation, we used the combination of these procedures and evaluated the behavioral development of juvenile play in male rats.

Eight of the 32 male Wistar rats between 20 to 60 days of age used for juvenile play derived from 8 litters (n = 4 males and 4 females each); they had been undernourished during the lactating period by means of the subcutaneous nipple-ligation procedure of one of the mothers in each pair of the sham non-ligated, or the ligated mothers. Thereafter, they were daily alternated between the paired litters every 12 h, from PDs 1-25. Undernourished ligated subjects (LU) were marked for recognition at postnatal day (PD) 40 by the right-ear clipping procedure. Thus, F1 LU male rats (n = 8) were weaned on PD 25 and thereafter were tested and recorded in pairs or in groups of four F1 animals for juvenile play development on PDs 20, 25, 30, 40, 50, and 60.

The eight F1 sham-ligated control (LC) subjects came from 4 paired litters where the non-ligated lactating dams were gently alternated every 12 h between litters. The male and female subjects from these litters were weaned at PD 25, and the males were reared with an ad lib balanced diet until PD 60 in the colony room. This LC group of males (n = 8) was tested and recorded for juvenile play on the same six PDs as the LU group.

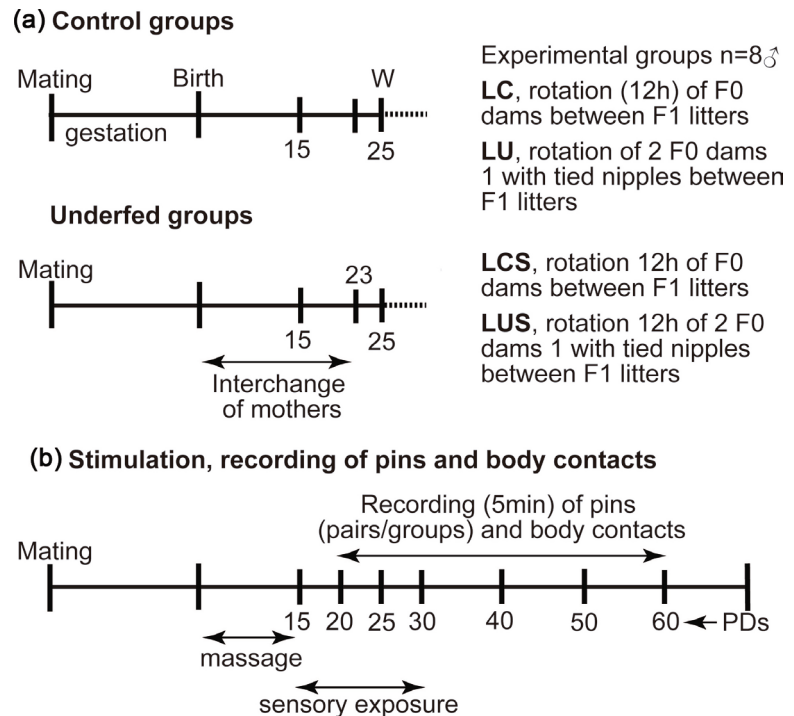
### **2.2.2. Control Stimulated (LCS) and Undernourished Stimulated (LUS) Groups**

The neonatally control-stimulated (LCS) and undernourished stimulated (LUS) rats were the stimulated subjects of this study. LCS (n = 8/group) and LUS rats (n = 8/group) were obtained with the same nutritional paradigms as the non-stimulated subjects. Moreover, these groups were maintained under the same environmental conditions and received gentle somatosensory handling (5 min) from PDs 1 to 7; thereafter, they were exposed (30 min) to a sensory enriched environment every day from PDs 8 to 30.

All non-stimulated and neonatally stimulated subjects were weaned at PD 25, after which they were given free access to water and food (Purina chow). The subjects were kept in groups of 4 - 6 males until reaching 60 days of age, when the juvenile play sessions ended (**Figure 1**).

### **2.3. Early Sensory Stimulation**

The LCS and LUS male subjects tested for play behavior performance were separated from their mothers to receive individual handling consisting of gentle touching, holding, shaking, and rubbing for 5 min from PDs 1 to 7. Then, from PDs 8 to 30, infant rats were exposed for 30 min a day to a sensory-enriched environmental ( $60 \times 50 \times 20 \text{ cm}^3$ ) containing six novel “toys”. The “toys” were randomly changed every day to avoid habituation. Additionally, during the sensory exposure, a radio was turned on for auditory stimulation. When the daily sensory stimulation ended, pups of the stimulated groups were returned to their



**Figure 1.** Experimental design for (a) nutritional paradigms and (b) sensory stimulation and recording procedures.

home cage where they received additional increased sensory stimulation during the encounter with the mother [40]. Early sensory stimulation sessions were carried out once a day between 09:00 and 12:00 h under controlled temperature ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) and 40% - 50% humidity in a sound-proof chamber to block out noise from the main laboratory. A fan was used to minimize odors remaining from the previous sensory exposure.

## 2.4. Play Behavior Measurements

In all cases, 5 min tests of play behavior were recorded between 10:00 and 12:00 h on PDs 20, 25, 30, 40, 50, and 60. The play behavior of subjects in all experimental groups was recorded by means of a closed-circuit television system located 100 cm away from a round translucent plastic arena (40 cm in diameter) with sawdust on the floor and continuous dim illumination provided by a red light bulb (25 W). Videotape records were assigned a random number to ensure that play behavior measurements obtained by a rater were blind with respect to the hypothesis, age and treatment. These measurements were compared with the counts obtained in four litters per group, randomly chosen by another experimenter. After a 12 h period of individual isolation during the dark phase of the cycle, the play of four pairs of rats or four groups of four rats of the same sex was measured in 5 min recording sessions as follows: frequency of pinning, where one rat is on its dorsal surface with the other rat on top; pinning is a reliable indicator to measure the levels of juvenile play [7]. Additionally, the frequency of contacts directed to the dorsum, nape and flanks of one rat to demand play of a

partner was also recorded because tactile stimulation has emerged in both humans and animals as a relevant sensory modality to facilitate growth and brain development [41] [42].

To evaluate the physical development of control and underfed subjects with or without sensory stimulation, we also recorded the body weight of animals after each play session on PDs 20, 25, 30, 40, 50, and 60.

## 2.5. Statistics

Body weight measurements of the experimental groups were compared using a two-way ANOVA 4 (experimental groups)  $\times$  6 (ages). In all cases, two-way ANOVA comparisons of the frequency of pinning and body contacts in paired or group play interactions, 4 (experimental groups)  $\times$  6 (days of testing), were used. Post hoc comparisons of scores between nutritional regimes and session days were made using the Fisher LSD post hoc test. Statistical significance was set at  $p = 0.05$ .

## 3. Results

### 3.1. Effects on Physical Growth

Neonatal undernutrition significantly reduced the body weight development of both LU and LUS subjects compared with their controls. However, this reduction was greater in LU rats (**Table 1**). The ANOVA comparisons indicated significant

**Table 1.** Mean values of body weight ( $\pm$ SEM) and percentage of reductions in the four juvenile experimental groups of rats.

Variable	df	F	p =
Groups (A)	3124	184.84	0.0001
Age (B)	5620	7882.09	0.0001
AXB	15,620	18.70	0.0001
Age	LC	LU	Different %
20	038.49 $\pm$ 0.97	022.62 $\pm$ 0.54*	41.21
25	057.66 $\pm$ 9.70	037.32 $\pm$ 5.30*	35.27
30	093.70 $\pm$ 2.56	059.62 $\pm$ 1.37*	36.36
40	172.73 $\pm$ 2.67	122.03 $\pm$ 2.07*	29.18
50	246.67 $\pm$ 3.77	189.00 $\pm$ 2.42*	23.37
60	317.28 $\pm$ 3.26	270.03 $\pm$ 2.66*	14.89
	LCS	LUS	
20	036.79 $\pm$ 0.73	022.13 $\pm$ 1.04*	39.23
25	062.86 $\pm$ 4.97	038.20 $\pm$ 8.53*	37.79
30	094.89 $\pm$ 1.06	059.02 $\pm$ 2.66*	27.03
40	168.44 $\pm$ 1.73	122.87 $\pm$ 4.37*	18.91
50	224.57 $\pm$ 2.10	182.12 $\pm$ 5.53*	12.82
60	295.07 $\pm$ 2.83	257.24 $\pm$ 4.09*	12.8

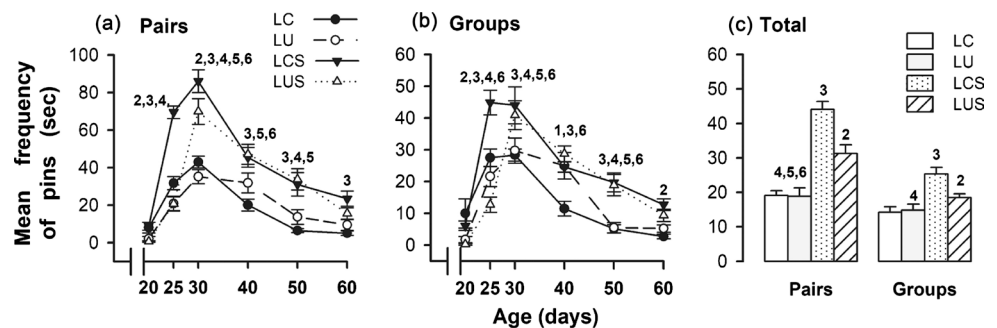
\* $p = 0.05$ .

body weight reductions associated with diet,  $F(3, 124) = 184.84, p = 0.0001$  and age,  $F(5, 620) = 7882, p = 0.0001$ , and a significant interaction between diet and age factors,  $F(15, 620) = 18.70, p = 0.0001$  (Table 1).

### 3.2. Effects on the Frequency of Pinning

The ANOVA comparisons showed that the play in pairs was reduced by the diet,  $F(3, 28) = 30.08, p = 0.0001$  and age,  $F(5, 140) = 90.72, p = 0.0001$ , and a significant interaction between diet and age,  $F(15, 140) = 7.91, p = 0.0001$ . Post hoc comparisons of pinning between groups showed consistent increments ( $p = 0.02$ ) associated with sensory stimulation in LC vs. LCS across sessions; LCS vs. LU ( $p = 0.02$ ) at PDs 25, 30, 40, and 50; and LU vs. LUS at PDs 30, 40, and 50 (Figure 2(a)). See Table for more comparisons.

The ANOVA also showed that group play was reduced by the diet,  $F(3, 28) = 11.49, p = 0.0004$ , modified by age,  $F(5, 140) = 79.68, p = 0.0001$ , and a significant interaction between diet and age,  $F(15, 140) = 6.77, p = 0.0001$ . Post hoc comparisons between groups throughout the sessions of play follow a similar inverted U shape sequence to that of the play in pairs, although group play values associated with sensory stimulation were attenuated. Thus, attenuated increased values ( $p = 0.009$ ) were observed between LC vs. LCS at PDs 25, 30, 40, and 50; LCS vs. LU at PDs 25, 30, and 50; and LU vs. LUS at PDs 30 and 50



(d) Statistical comparisons of grouped pins behavior during development

Pairs						
days	LC vs. LU <sup>1</sup>	LCS vs. LUS <sup>2</sup>	LC vs. LCS <sup>3</sup>	LCS vs. LU <sup>4</sup>	LU vs. LUS <sup>5</sup>	LUS vs. LC <sup>6</sup>
20	NS	NS	NS	NS	NS	NS
25	NS	***	***	***	NS	NS
30	NS	*	***	***	***	***
40	NS	NS	**	NS	*	***
50	NS	NS	**	*	*	***
60	NS	NS	*	NS	NS	NS
Groups						
20	NS	NS	NS	NS	NS	NS
25	NS	***	**	***	NS	**
30	NS	NS	**	*	*	*
40	*	NS	*	NS	NS	**
50	NS	NS	**	*	*	*
60	NS	*	NS	NS	NS	NS

\* $p = 0.05$ , \*\* $p = 0.001$ , \*\*\* $p = 0.0001$ . NS, non-significant differences.

Figure 2. Temporal course of the frequency of pins in pairs (a) and groups (b). Note the U shape curve of all groups with higher values in LCS and LUS. The total pin values in pairs or groups (c) showed that stimulation did not revert the effects of undernutrition. Numbers above graphs are comparisons as (d) table depicted.

(Figure 2(b)). See Table for more comparisons.

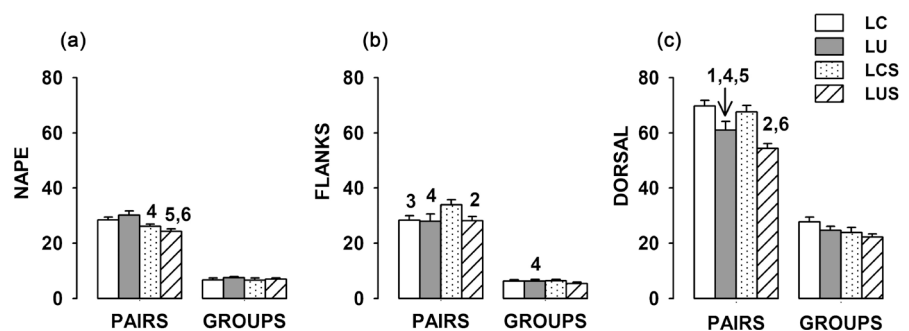
The ANOVA comparisons of the total frequency of pinning in pairs and groups of subjects showed significant effects of diet,  $F(3, 28) = 23.53$ ,  $p = 0.0001$  and the type of play,  $F(1, 28) = 237.56$ ,  $p = 0.0001$ , and a significant interaction between diet and type of play,  $F(3, 28) = 27.98$ ,  $p = 0.0001$  (Figure 2(c)).

### 3.3. Effects on the Number of Body Contacts

The ANOVA comparisons of the total number of dorsal region contacts during the play in pairs showed significant reductions by diet,  $F(3, 28) = 8.44$ ,  $p = 0.0003$  and age,  $F(5, 140) = 13.11$ ,  $p = 0.0001$ , and significant interaction between diet and age,  $F(15, 140) = 4.18$ ,  $p = 0.0002$ . Post hoc comparisons between the experimental groups throughout the days of play yielded reduced values in LU ( $p = 0.05$ ) compared with LC at PDs 20 and 25; in LUS vs. LCS at PDs 20, 25, and 30; in LU vs. LCS at PDs 40 and 60; and in LUS vs. LU at PDs 30 and 40 (Figure 3(a)). The findings showed that early undernutrition consistently reduced dorsal contacts, and early sensory stimulation did not ameliorate this effect. Post hoc comparisons of the number of dorsal contacts during group play showed reduced effects by diet in LU vs. LC at PDs 20, 25 and 40; with lower effects of sensory stimulation that did not compensate the effects of early undernutrition in LUS vs. LCS at PD 20 (Figure 3(a)). Post hoc comparisons of the number of contacts with the nape or flanks in the play in pairs and groups were poorly modified by underfed rats to demand play activity of the partners. Thus, LCS and LUS showed significant reductions in stimulation of the nape region during the play in pairs ( $p = 0.05$ ), with negligible effects on group play (Figure 3(b)). Moreover, a significant increment in the number of contacts with the flank region in the LCS group was observed compared with the other experimental groups ( $p = 0.05$ ). No relevant effects were detected in the rest of the comparisons, including the number of contacts during group play (Figure 3(c)).

## 4. Discussion

The current findings indicate that neonatal food restriction resulted in consistent



**Figure 3.** Mean values  $\pm$  SEM of total body contacts directed at partners during the play in pairs or groups. Note the higher values in the dorsal area in the play in pairs and groups, with reductions in the LU and LUS groups and negligible values directed at other areas. Numbers above graphs are comparisons like those of Figure 2.

body weight reductions. This effect was more severe in the LU group than in the LUS group when compared with their respective controls. Furthermore, neonatal sensory stimulation was unable to ameliorate the body weight reductions associated with early undernutrition. The results were also in agreement with studies showing that neonatal undernutrition in the rat delays physical and sensory maturation such as ear- and eye-opening, huddling response, digging, sniffing, and sensory-evoked electrocortical activity [43] [44].

The total number of pins directed at the partner during the play in pairs or in groups of LCS and LUS subjects significantly increased on the play testing days. Early stimulation in F1 males of these groups differed in both their nutritional histories and in the reduced somatosensory input provided by the dams; hence, differences in their juvenile play may result from the effects of one or both factors. Our findings suggest that differences in the reduced sensory regulation of nursing behaviors in the LC and LU groups versus the increased effects in the LCS and LUS groups may partly be related to the disrupted motoric juvenile play outcome. Supporting this assumption is the fact that in pairs of rats anesthetized with dorsal xylocaine, the frequency of pinning and the number of dorsal contacts were significantly reduced because of deficiencies in their ability to be pinned [9]. Furthermore, early malnutrition or undernutrition in rats at later ages disrupted coordination and the ability to perform basic precise movements required for similar motoric activities including locomotion, grooming, nursing, retrieving, and visuospatial performance, among others [45] [46] [47]. In this regard, our findings suggest that tactile stimulation deficiencies elicited by pinning in the underfed subjects may affect the insula and integration in the somatosensory cortex of the ascending somatosensory impulses, disrupting the elaboration of play responses through the corticospinal tract with possible myelinating deficits and reduced axon diameter-myelin sheath ratio and cortical neuronal hypoplasia [48] [49].

Another point of interest was to analyze if during the juvenile play in pairs or in groups of subjects the somatosensory contacts given by underfed rats to invite partners to play, could have different effects on the frequency of hairy skin body contacts throughout the testing sessions. Our findings on the total number of body contacts given by an early underfed rat to a partner showed that the stimulation of the nape and flanks was unaffected or poorly activated; however, in the hairy dorsal area, the contacts increased during the juvenile play in pairs compared with the play in groups. Additionally, we detected significant increments in the number of contacts directed at the dorsal area in the stimulated LCS and LUS groups playing in pairs, with negligible effects in the play in groups directed at the three areas. This peculiar profile of hairy skin contacts may be related to modified pleasantness effects of tactile stimulations that interfere with the juvenile play associated with early undernutrition [50]. The reductions in LCS and LUS groups may be related to fewer dorsal somatosensory receptors area or to a low tactile threshold to elicit deficient tactile pleasant sensitivity to stimulate the

juvenile play performance of partners that were not compensated by sensory stimulation [48]. To support this assumption, we suggest that sensory deprivation may lead to an incomplete or distorted sensory image of neuronal representation during ontogenetic development, thus affecting juvenile play performance.

## 5. Conclusion

Our results suggest that early undernutrition resulted in significant reductions of juvenile pinning and increased hairy tactile dorsal stimulation in LCS and ULS groups, reflecting diminished excitability levels of the neuronal circuit underlying play performance. Moreover, sensory stimulation did not compensate for the deleterious effects of neonatal undernutrition.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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