

Development of the Gusto Facial Reflex in Perinatally Undernourished Rats*

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ABSTRACT

In the rat, the taste system plays a critical role in motivating the animal to consume nutrients and avoid toxic substances. In neonatal rat the orofacial movements can be modified by the application of sucrose and quinine in the mouth, producing ingestion or rejection responses, respectively, but there is no information available on the gusto facial reflexes (GFR) under perinatal fasting. The aim of the present study was to determine how undernutrition can affect the development of orofacial responses to sucrose, quinine, and NaCl during early development. Pregnant dams were undernourished by being given 50% of a balanced diet from G6 to G12, 60% from G13 - G18, and 100% from G 19- G21. On postpartum days 0 - 9, perinatally underfed (UG) pups continued the undernourishment by remaining for 12 h with a foster dam and 12 h with a nipple-ligated mother. Stimuli were presented as a single droplet of sucrose (S), sodium chloride (NaCl), quinine hydrochloride (Q), and water (W) onto the lips at 1, 3, 5, 7, and 9 days of age, and mouth-opening frequency (MO) and lip-licking frequency (LL) were analyzed. The results show consistent effects associated with age, but not with diet, except in the cases of 0.01 M Q, which provoked in the UG subjects increased MO and the reduction of LL, and 0.1 M Q, which consistently reduced both responses. Moreover, S provoked increases in both MO and LL at 0.1 M while NaCl and water elicited minimal effects on GFR. These data suggest that perinatal undernutrition affects the basic components of the gustatory system necessary to produce GFR in the first postnatal days by possibly interfering with the integration of taste input for food learning and the hedonic aspects of gustatory cues.

Keywords: Gusto Facial Reflex; Newborn Rats; Undernutrition

1. Introduction

In the past, a number of studies have shown that perinatal undernutrition in the rat delays the maturation of sensory systems and causes the expression of altered motor patterns; these effects lead to a significant deficiency of somatosensory, auditory, visual, and chemosensory stimulation that may be essential for long-term behavioral performance [1-8]. Perinatal undernutrition mainly interferes with the anatomical organization of central nervous system (CNS) areas which undergo a phase of postnatal cell proliferation; these areas include the cerebral and cerebellar cortices, the hippocampus, central amygdaloid nucleus, olfactory bulb, and solitary tract nucleus, and they participate in the head movements, mouth opening and closing, chewing, food ingestion, and chemosensory CNS integration [8,9-13]. Early undernutrition affects neuronal development by reducing the cell number and their dendritic branching, and by the formation of spines with small perikarya alterations that interfere with their

connectivity and neuronal interactions [7,8,14-16].

The GFR evoked by the activation of gustatory receptors in the oral cavity generates two physiological processes, one related to the quality characteristics of the taste (sensorial) for food learning, and the other to the hedonic aspect (reward-aversion) of taste [17,18]. Because acceptance or rejection of taste stimuli depends on the hedonic rather than the sensory aspect, the hedonic component of taste stimulus may be indirectly evaluated by using relative fluid consumption tests or by analyzing taste mimetic responses [19-21]. Furthermore, the initial activation of specific taste receptors in the oral cavity triggers electrical impulses which are transmitted along two separate sensory and hedonic afferent pathways that are constituted by different relays and that ultimately reach the insular and the orbitofrontal cortices, respectively [22-24].

Although no facial motor reaction is specific to a particular taste sensation, there are clear variations of GFR with age and with the concentration of the different tastes. It is also known that an observer can make a good eva-

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luation of a newborn's facial expression because it shows reliable components of taste palatability or hedonic impact [17,25].

To our knowledge there is a lack of information about how perinatally underfed pups discriminate among different taste cues, and if the GFR evoked is different from that of normally fed pups. The current study investigates if the gustatory system of early underfed rats is functionally modified shortly after birth, as defined by MO and LL in response to different gustatory cues.

2. Materials and Methods

2.1. Animals

Experiments were approved by local Animal Committees and were in accordance with the NIH Guide for Care and Use of Laboratory Animals. Subjects were male and female Wistar rats (*Rattus norvegicus*), born and reared in the animal colony at the Institute of Neurobiology, University of Mexico. All animals were maintained in an automatically controlled room at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 50% humidity, and a 12 h light/dark cycle (lights on at 7:00). For mating, a male was placed in a plastic cage ($60 \times 32 \times 20$ cm) containing three females (200 - 250 g). Sperm-positive females were placed in individual plastic maternity cages ($35 \times 27 \times 17$ cm) with grill tops and wood shavings as nesting material one week before parturition. The day of birth was referred to as PN 0, and 24 h later pups were randomly mixed, redistributed and adjusted to 8 pups per mother (four males and four females). The redistribution was intended to balance possible genetic and prenatal biological differences between litters and give them equal probability of development.

2.2. Nutritional Procedures

2.2.1. Undernourished Group (UG)

The UG males and females ($n = 56$) came from at least nine different litters. Pregnant dams were fed from G6 to G12 with 50% (7.8 g) of a balanced diet (Purina chow) received by a normal dam, from G13 to G18 with 70% (10.9 g), and with the 100% (15.6 g) of the same diet until parturition, to avoid cannibalism of pups (Figure 1). After birth, prenatally underfed newborns were nursed by a pair of gestationally underfed dams, one of whose main galactophorous ducts had been subcutaneously tied [26]. To continue the neonatal underfeeding method, these two lactating dams were interchanged between litters every 12 h from postnatal days 1 to 9. This cross-fostering procedure reduces the effects on the newborns of maternal sensory deprivation that may interfere with the expression of GFR [2,26,27]. This paradigm was chosen because the oromotor response is regulated at the brainstem level, and most brain stem neurogenesis occurs

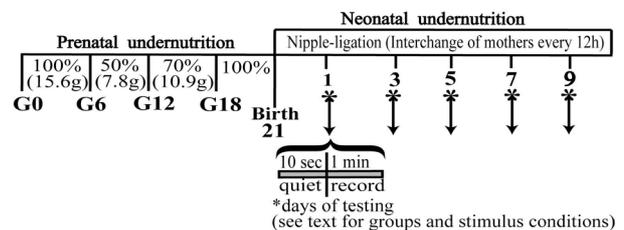


Figure 1. Schematic representation of the underfeeding procedure.

prenatally following first a rostrocaudal and then a mediolateral cytogenetic gradient; facial nerve efferent projections to the facial muscles and taste buds also occur during this period and continue during the early postnatal period [28,29]. Approximately 80% of the total underfed subjects included here were undernourished during the light phase and the remaining 20% during the dark phase of the cycle.

2.2.2. Control Group (CG)

The CG subjects consisted of 56 male and female pups obtained from nine, well-nourished litters, nursed by well-fed dams that had free access to water and balanced food (Purina chow) during gestation. After birth, CG pups were fed by two, normally lactating mothers, who were interchanged every 12 h between litters. To evaluate the effects of the nutritional treatment on body weights of subjects from the different dietary conditions a different group of six rats in each treatment was sampled.

2.3. Behavioral Testing

All GFR tests were performed between 10:00 and 12:00 in a sound-proof chamber that was illuminated with red light (60 W), maintained at $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and separated from the ambient noise of the main laboratory. The GFR test was carried out with a minor modification of the procedure previously described for neonatal rats [20]. A total of fourteen groups were used, including control (CG) and undernourished groups (UG), each with 4 males and 4 females. The animals were labeled with different colors; for each stimulus, subjects came from different litters, and the sapid stimuli tested were as follows: water (CGW) and (UGW) and three different sapid solutions diluted with distilled water to two different concentrations: 0.1 M and 0.3 M sucrose (CGS) and (UGS), 0.1 M and 0.3 M NaCl (CG NaCL) and (UG NaCl); and 0.01 M and 0.1 M quinine (CGQ) and (UGQ); each group was tested at different postnatal ages: 1, 3, 5, 7, and 9 days. As a preliminary study in addition to the water stimulus we used only gustatory cues with an opposite palatability, and a neutral stimulus to characterize the GFR. Pups under the two dietary treatments were maintained with the mother in their habitat for at least 30 min before the gustatory

stimulation test began. The taste stimuli were presented centrally on the pup's lips as a single droplet (about 8 μ l) via a 2-cm length of polyethylene tubing (P. E. 160) attached to a blunted tip of a 2.0-mm plastic micropipette. A single micropipette was used for each tastant during the recording tests. The hand-held and relaxed, swaddled pups were observed and videotaped for 10 sec before recording the sapid solution base line, and then for a 1-min interval under the effect of a tastant until the droplet disappeared into the pup's mouth under red-lamp illumination (**Figure 1**). Because the newborn rats possess reliable mimetic rhythmic facial, head, and body movements in response to oral gustatory stimulation, in each behavioral test both the MO and LL of the pups were analyzed. These early mimetic responses have been shown to be a reliable index of taste sensitivity associated with the GFR [19,20,30]. To ensure blind observations with respect to taste stimulus presentation, the different dietary treatments and gustatory exposure sequences were randomly modified from one test to the next. Because of the small body size of neonatal rats, and because a certain level of experience is required for accurate recording of GFR events, some experiments included data from naive, partially experienced, and very experienced observers to assess the reliability of the measurements.

2.4. Statistical Analyses

The statistical package Statistica, version 6 was used to perform all data comparisons body weight differences between the CG and UG subjects were compared by using a two-way ANOVA, (diet) \times (age). To analyze the MO and LL score differences in the case of the W stimulus, a two-way ANOVA (diet) \times (age) was used. MO and LL differences in each gustatory cue were analyzed with a two-way ANOVA, 2 dietary conditions (control and undernutrition) \times 5 (ages), followed by a post hoc, Fisher LSD test where appropriate. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Body Weight

Preliminary comparisons between males and females showed no significant differences, and they were then combined in the same group. Body weight comparisons among experimental groups showed that UG subjects weighed significantly less than CG. Body weight was also modified by age in both dietary conditions. Additionally, significant interactions between the diet and age factors were detected. Post hoc comparisons at each developmental age indicated consistently low body weight in the UG throughout the study as compared to their con-

trols. In summary, the data showed that the perinatal undernutrition paradigm interfered consistently with the physical growth of undernourished subjects during the study (**Table 1**).

3.2. Effects of W on GFR

Water was utilized to examine the GFR to a neutral stimulus and also because the other sapid stimuli were diluted in water. The ANOVA comparisons of MO and LL following W application were not modified by diet or by age (data not shown). The mean values of the scores obtained over the study are indicated in **Figure 2**.

3.3. Effects of NaCl on GFR

The ANOVA comparisons of the MO after exposure to 0.1 M NaCl showed no modification by the diet, but a significant difference with age, $F(4,56) = 51.20$, ($p < 0.05$) and a significant interaction between diet and age, $F(4,56) = 2.52$, ($p < 0.05$). The LL following 0.1 M NaCl was affected by the diet, $F(1,14) = 24.36$, ($p < 0.05$) but was not modified by age; furthermore, a significant interaction between the diet and age, $F(4,56) = 8.21$, ($p < 0.05$) was observed. Post hoc comparison of LL between UG NaCl 0.1 and CG NaCl 0.1 shows a reduction in UG NaCl 0.1 on days 1, 5, and 9 of age, but a significant increase on day 3 of age (**Figure 2(b)**, low left panels).

The MO after exposure to 0.3 M NaCl was only modified by age, $F(4,56) = 15.03$, ($p < 0.05$) (**Figure 2(b)**, upper right panels). The ANOVA comparison of the LL shows significant differences associated only with age, $F(4,56) = 2.95$, ($p < 0.05$) (**Figure 2(b)**, low right panels).

Table 1. Body weight (\pm SEM g) of pups reared by control and perinatally underfed dams ($n = 6$) during development.

Age (days)	Groups		
	CG	UG	% of reduction
1	7.38 \pm 1.95	5.70 \pm 0.15**	22.7
3	9.55 \pm 0.18	7.16 \pm 0.29**	25.0
5	12.26 \pm 0.20	7.71 \pm 0.31**	37.1
7	15.13 \pm 0.32	12.35 \pm 0.58**	18.3
9	18.15 \pm 0.31	14.51 \pm 0.44**	20.0
Factor	df	F	p <
(A) Nutrition	1, 10	13921.42	0.05
(B) Age	4, 40	284.32	0.05
A \times B	4, 40	5.53	NS

** $p < 0.0001$, Fisher LSD test; NS = Nonsignificant values.

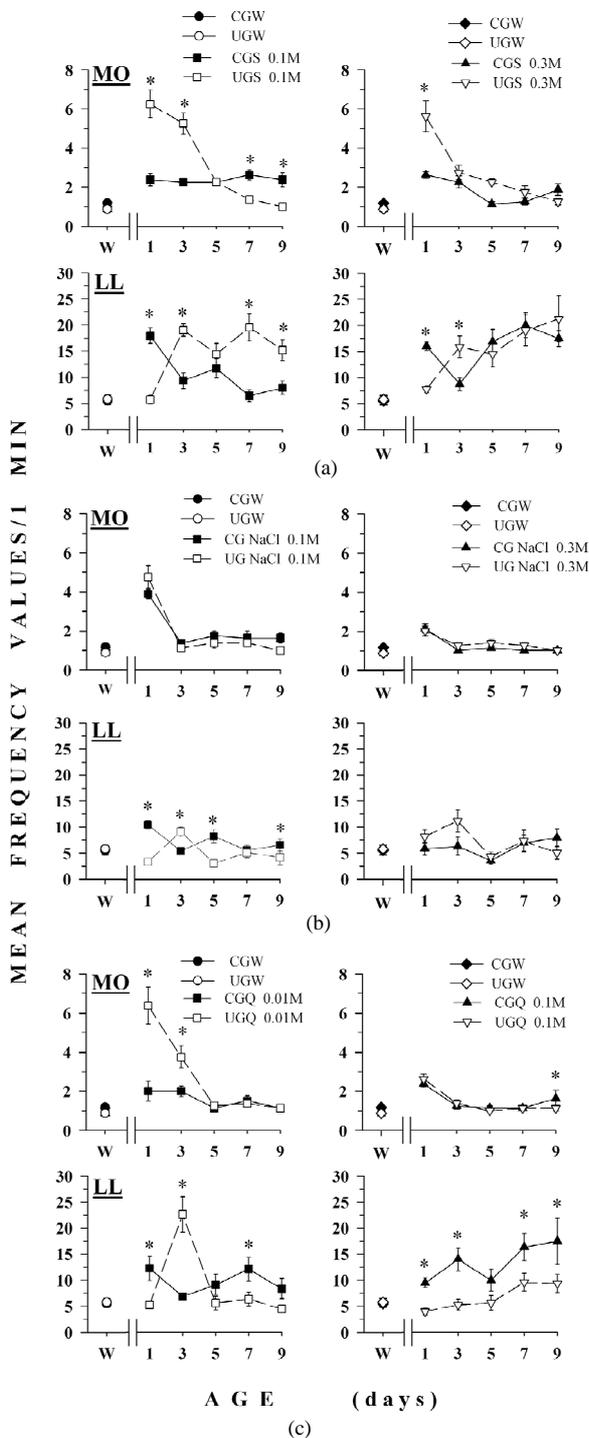


Figure 2. Mean frequency of MO and LL at different ages in response(s) to different tastants: (a) sucrose; (b) NaCl; and (c) quinine in both CG and UG groups. Squares (left) represent the low and triangles (right) the high concentration. Solid symbols represent the (CG) and empty symbols the underfed (UG). Note that the MO in UGS 0.1 M and UGQ 0.01 M are increased compared to the corresponding CGS on days 1 and 3 of age. Comparing the same groups at day 3 LL is increased in UGS 0.1 M, and in UGQ 0.01 M. Water (W); *Significant differences associated with diet ($p < 0.05$).

3.4. Effects of Sucrose (S) on GFR

Statistical comparisons of the MO provoked by S 0.1 was modified by diet, $F(1,14) = 23.12$, ($p < 0.05$) and by age, $F(4,56) = 22.93$, ($p < 0.05$). Moreover, a significant interaction between diet and age, $F(4,56) = 22.93$, ($p < 0.05$) was observed. Post hoc comparisons between UGS 0.1 and CGS 0.1 indicate a significant increase on days 1 and 3 of age without differences on day 5 and a significant reduction on days 7 and 9 (**Figure 2(a)**, upper left panels). ANOVA comparison of the LL elicited by S 0.1 shows that it was modified only by diet, $F(1,14) = 14.23$, ($p < 0.05$) with a significant interaction between diet and age, $F(4,56) = 18.21$, ($p < 0.05$). Post hoc comparison shows a significant reduction of LL in UGS 0.1 on day 1 compared with CGS 0.1; in contrast, on days 3, 7, and 9 it shows a significant increase in UGS 0.1 compared with CGS 0.1 (**Figure 2(a)**, low left panels).

MO in S 0.3 was modified by diet, $F(1,14) = 9.09$, ($p < 0.05$) and by age, $F(4,56) = 26.55$, ($p < 0.05$), and a significant interaction between these factors $F(4,56) = 9.57$, ($p < 0.05$). Post hoc comparison shows a significant increase in UGS 0.3 compared with CGS 0.3 on day 1 of age, but the other ages analyzed did not reveal significant effects (**Figure 2(a)**, upper right panels). ANOVA comparison of the LL elicited by S 0.3 shows significant differences only due to age, $F(4,56) = 5.07$, ($p < 0.05$) and a significant interaction diet by age, $F(4,56) = 3.27$, ($p < 0.05$). Post hoc comparisons show a significant decrease in UGS 0.3 relative to CGS 0.3 only on day 1 of age, with significant increase on day 3 ($p < 0.05$) (**Figure 2(a)**, low right panels).

3.5. Effects of Quinine (Q) on GFR

ANOVA comparison of the MO in response to Q 0.01 shows significant differences due to diet, $F(1,14) = 34.79$, ($p < 0.05$) and age, $F(4,56) = 19.25$, ($p < 0.05$) and a significant interaction between the diet and age, $F(4,56) = 9.90$, ($p < 0.05$). Post hoc comparisons between UGQ 0.01 and CGQ 0.01 indicated a significant increase ($p < 0.05$) on days 1 and 3 of age (**Figure 2(c)**, upper left panels). Statistical comparisons of the LL in responses to Q 0.01 did not show significant differences due to diet, but the LL changed with age, $F(4,56) = 6.03$, ($p < 0.05$), and there was a significant interaction between diet and age, $F(4,56) = 12.73$, ($p < 0.05$). Post hoc comparisons show that the LL in UGQ 0.01 is reduced on days 1 and 7 compared CGQ and there is a significant increase at 3 days of age (**Figure 2(c)**, low left panels).

Comparisons of the MO in response to Q 0.1 do not show significant differences due to diet, but MO was modified by age, $F(4,56) = 15.60$, ($p < 0.05$) without a significant interaction effect. Post hoc comparisons show a significant reduction ($p < 0.05$) in UGQ 0.1 compared

to CGQ 0.1 on day 9 of age (**Figure 2(c)**, upper right panels). ANOVA comparison of LL shows significant differences due to diet, $F(1,14) = 24.03$, ($p < 0.05$) and to age, $F(4,56) = 3.90$, ($p < 0.05$) without interaction. Post hoc comparisons showed a significant reduction of LL in UGQ 0.1 relative to CGQ on days 1, 3, 7, and 9 of age (**Figure 2(c)**, low right panels). In summary the ANOVA comparisons indicated consistent effects associated with age, with minimal effects of the diet, except in the UG subjects that exhibited an increase in MO and a decrease of LL with 0.01 M Q, and consistently reduced responses with 0.1 M Q.

4. Discussion

The present results indicated that perinatal undernutrition may induce functional alterations that may, in turn, modify the oromotor expressions in the UG subjects in response to aversive and pleasant gustatory cues. These behavioral alterations may be relevant because they could be reflecting the disruption of UG pup's hedonic component during the early associative gustatory learning that occurred in the maternal environment and is fundamental for the newborn survival [31-33]. The present data also indicate that the versatile oromotor responses to different types and concentrations of tastants change depending on several factors, including age, mother-litter bonds, and nutritional conditions of the pups [33-35]. Although newborns exhibit a complex of mimetic facial responses, head, body, and flexor-extensor movements in responses to different tastants [20]; according to our previous observations the MO and LL responses were the more reliable measurements of taste responsivity. The statistical comparisons of the different low and high tastants concentrations indicated consistent significant interactions between diet and age in the case of S, and only to a low NaCl and Q concentrations. These findings suggest that the gustatory circuits organization of the C and U subjects at each developmental age, and the two tastants concentrations could be reciprocally influenced in the case of the S, and partially by the NaCl and Q stimuli concentrations. By contrast, the interactions between diet and age are lost when the NaCl and Q at high concentrations are presented, because they probably saturate the circuits functional capabilities; although, this possibility requires further investigation.

The GFR alterations in response to tastants found in the present study are in line with neuronal hypoplasia and the disorganization of the dendritic tree and spines observed along the relay nuclei of the gustatory and motor facial nucleus [7,13,15,36,37]. In this regard, it is known that descending input from the insular cortex, lateral hypothalamus, and central amygdale modulate the brainstem sensory and motor mechanisms of the newborn to

produce fluid and food intake, nipple attachment, and the GFR integration that seems to be disrupted in the UG animals studied here [19,38-41]. Thus, it is possible that early in life brainstem structures that modulate GFR and are widely connected to forebrain areas may be vulnerable to food restriction and altered mother-litter bonds that disrupt various adaptive behavioral processes related to the early physiological needs of the newborn [35,42]. The fact that W and NaCl stimulation provoked negligible MO and LL responses may be related to the reduction of sensitivity to these two stimuli that maintain a relatively constant concentration as normal components of the amniotic fluid in the uterine environment during gestation [43-45]. Additionally, in the present study, the somatosensory system could participate in orosensory perception of different tastes, because the taste stimuli were diluted in water and oral movements would facilitate their spread to all parts of the oral cavity. Thus, we can not discard the possibility that tactile signals could contribute to these orofacial responses because palatability depends on their sensory characteristics of the food, which are detected by the taste trigeminal, and olfactory systems; more specifically, behavioral studies have shown that palatability can be modulated by trigeminal input [41,46].

Another point of interest concerns the fact that in the UG subjects there were reduced MO and LL, in response to Q administration, suggesting then that they preserve the aversive responsivity to unpleasant and possibly toxic tastants. This is a relevant finding because the UG animals maintain their capacity to gain experience through associative learning, and to revert the cognitive deficiencies commonly associated with perinatal undernutrition, by the combination of food and early sensory rehabilitation [5,6,33,47-49]. However, further studies are required to test that in the wild life these mechanisms are over expressed as in the case of UG subjects, surrounded by an environmental condition that frequently elicits stressful responses.

5. Conclusion

The basic brain stem mechanisms underlying the GFR arise during gestation and they are affected by prenatal and neonatal food restriction. This conclusion is supported by the increase in MO and LL in response to low sucrose concentration, and the increased MO and reduced LL in response to low Q concentration during the first days of life. By contrast W and NaCl stimulation provoked negligible affects on MO and LL. The current findings could be helpful in the design of protocols to study of early associated learning guided by gustatory stimulation.

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