Ingestive behavior in rat pups is modified by maternal sodium depletion

Carmen Perillán, Paula Núñez, Marina Costales, Manuel Vijande and Juan Argüelles
Universidad de Oviedo

Developmental programming by maternal stress during pregnancy is found to influence behavioral development in the offspring. The main objective of this study was to investigate the effect of maternal sodium depletion in rats during pregnancy on the development of thirst mechanisms in the offspring. Pregnant rats underwent 3 episodes of saline depletion, induced by injecting sc 10 mg of Furosemide in saline (0.5 ml). The treatment, given on the 14th, 17th and 20th days post-conception, is thought to induce acute sodium depletion on dams. The offspring were tested for their drinking responses to Isoproterenol (500 μg/kg sc). In accordance to the known sequence of ontogenic development of drinking mechanisms, all groups of pups drunk after being stimulated with Isoproterenol at 6 days of age. The offspring from Furosemide-treated dams drank significantly less than the control group after Isoproterenol ($p<0.001$). Nevertheless, basal intake (water drunk after vehicle-saline only) was also significantly lower in these pups ($p<0.001$). In conclusion, offspring exposed to saline depletion in utero, modify their thirst responses at 6 day of age. This confirms that in utero conditions determine thirst responses in the offspring and they could provide adaptive advantages.

La depleción sódica materna durante la preñez modifica el comportamiento ingestivo de la descendencia. Episodios de estrés materno acaecidos durante la fase de preñez pueden afectar al desarrollo normal del comportamiento ingestivo de la descendencia. El objetivo de este estudio es investigar el efecto de la depleción sódica durante la preñez sobre el comportamiento ingestivo de la descendencia. Ratas preñadas son sometidas a tres episodios de depleción sódica por inyección de furosemida (10 mg en salino sc) los días 14, 17 y 20 postconcepción. En la descendencia se estudió su respuesta ingestiva al Isoproterenol (500 μg/kg s.c.). Según la secuencia establecida de desarrollo de los comportamientos ingestivos, todos los grupos de animales beben en respuesta al isoproterenol a los 6 días de edad, pero descendientes de madres tratadas con furosemida bebían menos que los hijos de madres control ($p<0,001$). También la bebida basal (crías inyectadas con salino) se veía afectada, siendo menor en el grupo de descendientes de madres tratadas con furosemida ($p<0,001$). En conclusión, los descendientes expuestos a depleción sódica en el útero materno modifican su comportamiento ingestivo a los 6 días de edad. Esto confirma que las condiciones uterinas determinan las respuestas dípsicas en la descendencia y podrían proveer ventajas adaptativas.

The development of hunger or thirst in response to expanded periods of food or water deprivation is a basic feature of motivated ingestive behavior. If we want to have a complete understanding of thirst, we need to understanddifferent phenomena: how the brain of the animals or humans creates the urge to drink water, how it generates an anticipation of water, how it emits the behaviors that lead an animal to water and allow it to be selected from among other ingestible commodities, how it makes thirst and water drinking inherently reinforcing and memorable.

The particular development of dipsogenic and additional regulatory mechanisms for the control of hydrosaline homeostasis through ingestive behavior occurs during foetal and early stages of life and may be susceptible to changes in the pregnant environment (Ross & Nijland, 1998). Responsiveness to thirst challenges is precocious since it is present before the animal freely ingests water as a separate substance. The dipsic response to the β-adrenergic agonist Isoproterenol begins at six days of age in the rat (Wirth & Epstein, 1976).

Recently, programming influences of maternal distress during pregnancy on long-term behavioral and cognitive development of the offspring have received increased interest. During pregnancy adaptations in maternal haemodynamics, hormonal and biochemical variables occur that could have a potential impact and in some extent do "program" postnatal development (Barker, 1995; Godfrey & Barker, 2000).

Vomiting during gestation in humans has been reported to have some influence on systolic blood pressure in the offspring (Málaga et al., 2005). An increased salt appetite in the rat offspring when acute and repeated mineralo fluid losses had occurred during pregnancy after diuretic-natriuretic treatment has also been found (Galaverna, Nicolaïdis, Yao, Sakai, & Epstein, 1995). Moreover a high salt environment "in utero" seems to change pressor responsiveness to Angiotensin II in the adult offspring (Argüelles,

López-Sela, Brime, Costales, & Vijande, 1996). While pups from mother rats with diet-induced hypertension were insensitive to the hypertensive effects of sodium chloride (Langley-Evans & Jackson, 1996). Argüelles, Brime, López-Sela, Perillán and Vijande (2000) and Perillán, Costales, Díaz, Vijande and Argüelles (2004) have described modifications in the ingestive behavior of the offspring from hypereninemic, hypertensive and natriophilic mother rats.

Changes in the ontogeny of drinking behaviour after the manipulation of the renin-angiotensin-aldosterone system (RAAS) during pregnancy has already been reported (Perillán et al., 2004; Perillán, Costales, Vijande, & Argüelles, 2007, 2008). The purpose of the present study is to elucidate the influence of maternal sodium depletion during pregnancy on drinking behaviour in offspring after early beta-adrenergic stimulation.

Methods

Subjects

All experiments were performed on Wistar rats (from the *vivarium* of the Universidad de Oviedo) housed individually in a light and temperature controlled room (12 h light/dark; 21±1ºC). Animal care was in accordance with guidelines from 86/609/EEC Directive and the study had the approval of the Institutional Animal Ethical Committee.

Procedure

Pregnant rats (dams) were given a sodium depletion acute treatment in days 14, 17 and 20 of their pregnancies. Offspring from these dams was tested at six days of age for their dipsic response to a betadrenergic dipsogenic stimulus. Control animals were established according to the protocol described below.

A group of 10 female rats (250-300 g) were mated and pregnancy assessed by daily vaginal smears revealing spermatozoids presence the day 1 of pregnancy. They had free access to a standard laboratory diet, tap water and 2.7% NaCl (liquids available from graduated glass tubes fitted with glass spouts) where appropriate. In all groups daily water intake and 2.7% saline was recorded. Then they were divided in three groups as follows:Four "Furosemide dams" (D-FUR) received two 5 mg s.c. injections of Furosemide (Seguril® HOECHST, Barcelona, Spain) separated by 2 h on day 14, 17, and 20 postconception; three "Saline dams" (D-sFUR) were injected two times with saline vehicle on day 14, 17, and 20 postconception and used as control for the D-FUR group; three "Intact dams" (D- INTACT) received no treatment.

In order to avoid neophobia, D-FUR and D-sFUR groups who will be fed with a hyposodic diet during 24 h hours after furosemide treatment, were fed the day before with a 50% mixture of standard laboratory and sodium-deficient food. Once injected with furosemide, the dams were housed in cages rinsed with distilled water to remove all ambient sodium and drinking tubes containing saline solution (2.7%) were also removed. Then standard laboratory food was replaced by a sodium *deficient* diet (PANLAB, Barcelona, Spain) to prevent the rats from compensating for their treatment-induced sodium depletion. Only sodium-deficient food and water were available during the overnight period of sodium depletion by furosemide.

Twenty four hours after furosemide or saline treatment dams were given 2 hours access to 2.7% saline and water. Water and saline intakes were recorded at 5, 10, 15, 30, 60, 120 min.

Dams were sacrificed by decapitation immediately after the end of the intake test period of their pups. Haematocrit was determined and the remaining blood was centrifuged at 4 ºC. Plasma osmolality (vapour pressure osmometer -WESCOR) was measured.

Offspring

Daily inspection of litters was made at 17:00 hours. The day of birth was designated as "day 0". Litters, usually of 8-12 pups, were kept intact until the day of ingestive testing. The protocol used, as described by Wirth and Epstein (1976), starts adjusting the litter size to 8 pups (4 females and 4 males), four hours prior to testing. It consists of five steps: A) Deprivation: The pups are separated from the dam during 2 hours and placed under a 25-W lamp; skin temperature is monitored with a thermistor probe and maintained at 33 ºC. Pups are weighed to the nearest 0.01g, before and after deprivation. B) Nursing: pups are then returned to the dam for 1h and 45min and re-weighed. In order to minimizing the effect of hunger on subsequent thirst testing, one pup of each sex that had gained the least during the nursing period is excluded. C) Challenge: Three pups from each litter are subcutaneously injected with isoproterenol (SIGMA): 500 μg/kg, in 6 day-old pups. The other three pups from each litter are subcutaneously injected with 0.15 M NaCl (control). All subcutaneous injections are made over the scapulae at a volume of 1.25 mL/100 g body wt just before testing. D) Testing: The pups are weighed and replaced in the box after the challenge for a 2 hours test period. Skin temperature is still maintained under the heating lamp. The apparatus for pups thirst testing consisted of an infusion pump delivering room temperature distilled water through a PE-50 plastic spout extending 2 mm beyond the blunted end of a needle. The infusion rate was 0.7 mL/min. Each pup was offered a 15 seconds bout every 15 minutes, during two hours (9 bouts in total for each pup). It can either lick, struggle or remain active. E) End of Testing. At the end of the 2 h test period, each pup is weighed and percent weight gain during the test period calculated. Weight gain is used as a measure of water ingested, since there is no other source of weight gain (no mother available). Evaporative loss is assumed to be the same for both challenge and control pups, and there is no spontaneous excretion in the suckling pup according to Wirth and Epstein (1976).

At the end of the test each pup is decapitated with a razor blade, its blood collected in heparinized capillary tubes and hematocrit and osmolality determined.

Data analysis

The results are presented as means ± SE. Unpaired "t" Student test was used where appropriated. Values of p<0.05 were deemed as statistically significant.Statistical analysis was performed with SPSS software (version 18.0 SPSS Inc., Chicago, IL, USA).

Results

Mothers

The duration of pregnancy and the number of pups per litter were not significantly affected by sodium depleting treatment (Table 1). As no differences were found between D-sFUR and D-INTACT, these two groups of rats were joined for statistical analysis and labelled as D-CONTROL. This applies also to the offspring.

D-FUR salt intake significantly increased during the test period after depletion by furosemide treatment on days 14th, 17th and 20th of pregnancy in comparison to D-CONTROL (p<0.01). On the contrary, no increase was found in water intake (Figure 1 A and B).

On the rest of days of pregnancy, neither water intake nor salt intake was statistically different in D-FUR or D-CONTROL (data no shown).

Maternal blood parameters are shown in Table 2. No significant differences in hematocrit and plasma osmolality were found between D-CONTROL and. D-FUR groups on the day six after delivery.

Offspring

At six days of age O-FURpups (offspring from D-FUR) showed higher body weight than O-CONTROL pups (offspring from control dams) (Table 3).

Isoproterenol challenge at six days (Fig. 2) caused a significant water intake both in O-CONTROL (t= -4.45, df= 34, p<0.001) and O-FUR (t=-5.09, df= 20, p<0.001). Nevertheless O-FUR showed a smaller intake than O-CONTROL, after either vehicle (t= 3.74, df= 26, p<0.001) or isoproterenol (t= 2,81, df= 28 p<0.01).
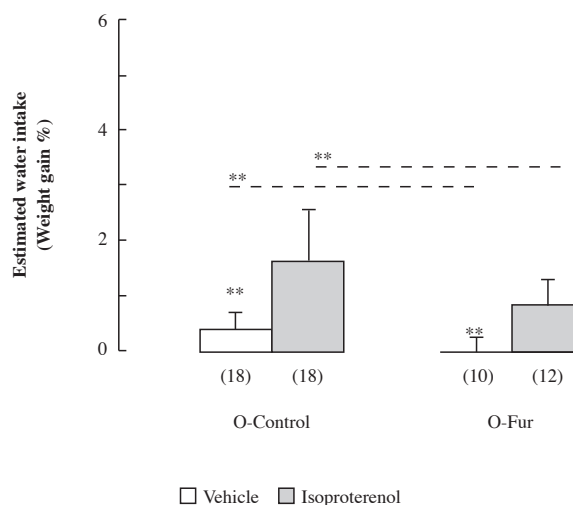
Blood parameters of 6 days old pups measured at the end of the ingestive test are shown in Table 3. Hematocrit was significantly lower in O-FUR than in O-CONTROL after vehicle (p<0.05) or

Table 1		
Influence of treatment on pregnancy		
Group	Duration of Pregnancy (days)	Pups per litter
D-CONTROL (6)	21	12.2±0.6
D-FUR (4)	21	9.8±1.7

Values are presented as mean ± SEM, (n)
D-CONTROL = saline +no treated dams; D-FUR = Furosemide treated dams

Table 2		
Blood parameters (dams)		
Group	Hematocrit (%)	Osmolality (mOsm)
D-CONTROL (6)	42.3±0.7	307.0±8.0
D-FUR (4)	43.0±0.4	304.2±1.2

Values are presented as mean ± SEM, (n)
D-CONTROL = saline +no treated dams; D-FUR = Furosemide treated dams

Table 3	
Pups body weight at six days of age	
Group	B.W. (g)
O-CONTROL (36)	12.94±0.27
O-FUR (23)	14.28 ±0.53*

Values are presented as mean ± SEM, (n). t= -2.48, df= 57; *p= 0.016)
D-CONTROL = saline +no treated dams; D-FUR = Furosemide treated dams
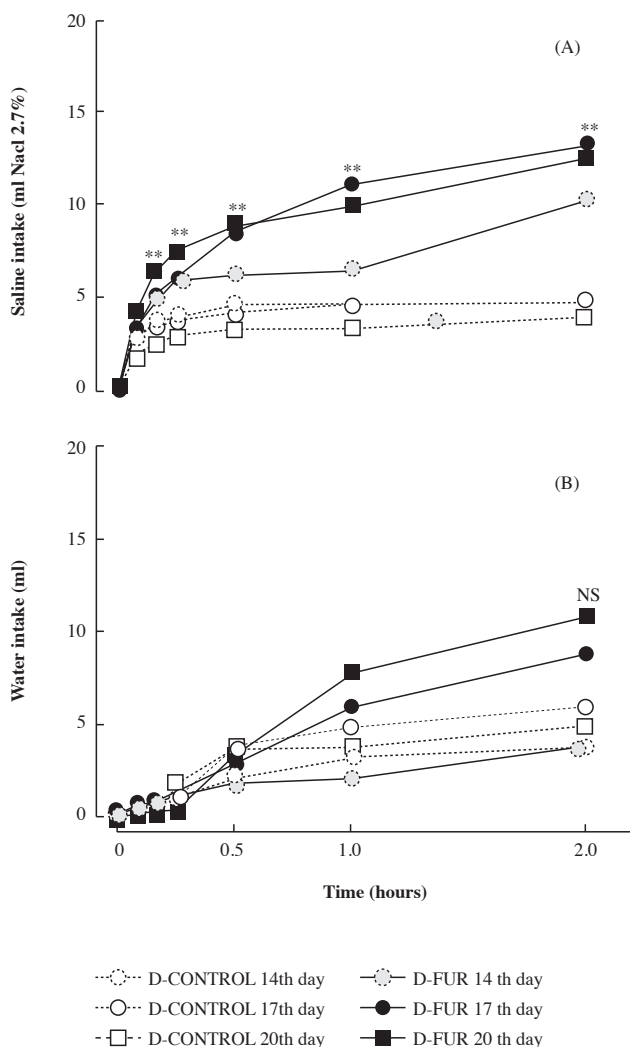
Figure 1. *Water (A) and Saline (NaCl 2.7%) intake (B), by Control dams (D-CONTROL; n= 6) and by Furosemide-treated dams (D-FUR; n= 4) during a 2-h test period (** p<0.01)*

Figure 2. *Water Intake by offspring from Control dams (O-CONTROL) and by offspring from furosemide dams (O-FUR) during a 2-h test period. Thirst challenges: Isoproterenol at 6 days of age (n in brackets at the bottom of bars; ** p<0.01)*

Isoproterenol (p<0.001) treatments. However no changes have been found in osmolality regardless of the treatment.

Table 4
Offspring blood parameters

Treatment	Hematocrit (%)		Osmolality (mOsm)	
	O-CONTROL	**O-FUR**	**O-CONTROL**	**O-FUR**
Vehicle	29.9 ± 0.6**ᵃ (16)	25.7 ± 0.7 (10)	296.0 ± 4.18 (9)	293.9 ± 10.1 (11)
Isoproterenol	29.2 ± 10.8*ᵇ (18)	26.4 ± 0.6 (11)	295.3 ± 3.03 (9)	291.6 ± 1.6 (12)

Values are presented as mean ± SEM. (n); * p<0.05; t= 2.45; df= 17, ** p<0.005; t= 4.06; df= 24
O-CONTROL= offspring from saline + non treated dams; O-FUR= Offspring from furosemide treated dams. a= O-CONTROL vs O-FUR for Vehicle; b= O-CONTROL vs O-FUR for Isoproterenol

Discussion

The behavior of suckling is most frequently studied in terms of its role in nutrition and food-getting, but from our point of view, in this study it is clearly an ingestive behavior in the sense of being fluid consumption, a response that has evolved to serve fluid needs.

If we focus on prenatal life, previous research has established that factors that disrupt fetal development in utero may contribute to the risk of diseases in the adulthood and influence behavioral development. Such factors include exposure of the mother during critical gestational periods to hypoxia, malnutrition or glucocorticoids (Mao, Shi, Xu, Zhang, & Xu, 2009). Alterations of the renin angiotensin system (Fitzsimons, 1998), blood pressure homeostasis (Argüelles et al., 2000) and ingestive behavior could have been developed too in utero (Nicolaïdis, Galaverna, & Metzler, 1990; Galaverna et al., 1995). There is also a strong evidence of links between perinatal natriophilic behaviour and adult pathological blood pressure (Katovich, Aerni, Céspedes, & Rowland, 2001). Conversely, other studies demonstrated that salt deprivation in pregnant rats determined an increased water intake in the adult offspring (Mouw, Vander, & Wagner, 1978; Contreras, 1993). McBride, Culver & Flynn (2008) shown that early life experience with low- and high-sodium diets, during the prenatal or early postnatal period, is a stress that produces long-term changes in responsiveness to amphetamines and to subsequent stressors.

The hypothesis in the present study is that maternal exposure to sodium depletion and dehydration, treatments that severely affects the fetal ambiance in utero, could affect the early onset of thirst responses to well known dipsogenic agents. We studied the effect of maternal saline depletion by repeated Furosemide injections on early water intake response in the descendents. Furosemide treatment in days 14, 17 and 20th of pregnancy produced a progressive enhancement of natriophilic induced response in dams. This is in accordance with Na (Na, Morris, Johnson, Beltz, & Johnson, 2007) who reported that sodium depletion enhanced salt intake and may have a direct impact on central structures implicated in sodium appetite and reward signalling.

In our experiments, Isoproterenol increased water intake of 6-days old pups, in full accordance to the ontogenic thirst sequence described by Wirth & Epstein (1976), independently of their maternal condition (O-FUR or O-CONTROL). Moreover, O-FUR drunk always less water than O-CONTROL when injected with isotonic saline or with Isoproterenol. Therefore pups O-FUR whose early development occurred in a modified uterine environment (maternal sodium depletion and dehydration) have their spontaneous or induced thirst mechanisms altered. These results confirm the hypothesis of this work.

There could be some plausible non excluding explanations to the mechanisms of such an early alterations in drinking behavior: 1) Endocrine alteration (including RAAS) in the mothers and/or foetuses may modify the development of the neural substrate necessary for appropriate thirst responses; 2) maternal behaviour is altered by the experimental procedure and this in turn affects the foetuses and pups development pattern.

The implication of RAAS in the modification of the thirst mechanisms has already been demonstrated in different paradigms. Enhanced thirst responses were found in 6 days old pups from hyperreninemic dams (Perillán et al., 2004). On the contrary, offspring from DOCA treated dams respond normally to beta-adrenergic activation at 6 days of age (Perillán et al., 2007). Therefore there is strong evidence supporting that RAAS alterations during pregnancy are able to modify the ingestive behaviour in the infant rats. Similar effects in adult offspring have been also reported elsewhere (Galaverna et al., 1995; Argüelles et al., 2000).

It has been demonstrated that maternal salt loading during pregnancy induces changes in angiotensinogen mRNA in the foetal liver and brain (Mao et al., 2007). This provides unequivocal proof that hydromineral challenges in mothers during pregnancy affect the expression of foetal genes implicated in hydromineral homeostasis.

According with the above information, the influence on the offspring by acute changes in maternal salt and water balance starts now to be enlightened.

With regard to the second explanation – effects due to maternal behaviour alterations, several studies implicating high salt diet during gestation and lactation, produced nursing changes accompanied by modifications in the consummatory behaviour of the offspring (Contreras et al., 1993, 2000). In any case this means that the developing organism as Gluckman & Hanson (2004) already stated, may make predictive adaptive responses of no immediate advantage but with long-term consequences.

This kind of developmental analysis provides an important means for learning about how the system is put together and for understanding the factors that create, modulate and control components of ingestive behavior. Always taking into account that at these early stages of development some ingestive systems may be immature simplified and thus more easily understood.

In conclusion, these findings, all together, suggest that a hydrosaline and hormonal altered environment during pregnancy modify some thirst responses in the first days of extrauterine life. This developmental approach permits control, manipulation, and evaluation of the significance or importance of the history of an animal's ingestion-related experience. We provide more evidence supporting the "developmental origins of health and disease" concept that has important biological, psychological, medical, and socio-economic implications.

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