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Maternal Exposure to Isobutyl-Paraben Impairs Social Recognition in Adult Female Rats

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Abstract: Isobutyl-paraben (IBP), a widely used preservative, exhibits estrogenic activity. We analyzed the effects of exposure to IBP during gestation and lactation *via* dam on social recognition behavior in ovariectomized offspring of Sprague-Dawley rats. Offspring were ovariectomized at 7 weeks of age, and were used in a social recognition test at 16 weeks of age. Each offspring was exposed to a novel ovariectomized rat four times and to a second novel rat in a fifth exposure. We counted the investigations by offspring of intruder rats. The IBP-exposed rats showed impaired social behavior compared with controls. These data imply that early exposure to IBP may have an effect on adult social behavior, which is reported to be an autism spectrum disorders in humans.

Key words: isobutyl-paraben, rat, social recognition

Autism spectrum disorders (ASDs) comprise a range of behavioral phenotypes including impaired social recognition, communication and imagination [1]. The Centers for Disease Control and Prevention indicates a dramatically increased prevalence of identified ASDs among children in the United States [29]. For example, the average prevalence of ASDs among children aged 8 years increased by 57% across 10 locations in the United States from 2002 to 2006. While genetic factors are clearly important, [23, 31] these increasing rates of ASDs suggest that environmental factors are involved in causing this developmental disorder [11, 14, 30, 39].

Exposure to synthetic chemicals through air, water and food is unavoidable in the modern way of life. Synthetic chemicals that mimic or inhibit the action of gonadal steroid hormones are referred to as endocrine-disrupting chemicals [8]. Gonadal steroid hormones are required for development during gestation and the early

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postnatal period, a time when numerous systems including the central nervous system are vulnerable to endocrine-disrupting chemicals. Early exposure to estrogenic chemicals may alter brain development and subsequent social behaviors, since early exposure to estrogenic chemicals alters monogamous behavior in the female pine vole [12], and alters mother-infant interactions in monkeys [26]. To date, however, the effect of early exposure to estrogenic chemicals on adult social recognition of animals of the same sex has not been reported.

Maternal glucocorticoid changes due to environmental factors may be involved in the manifestation of ASDs, because stressful life events during pregnancy correlate with the prevalence rates of ASDs in humans [2, 21, 36]. In addition, maternal glucocorticoids modulate the developing hypothalamic-pituitary-adrenal axis (HPA-axis) and social or maladaptive behavior associated with ASDs-like behaviors in rodents [10, 15, 22, 25, 37, 38] and primates [7, 32, 33].

Parabens are widely-used as preservatives in foods, cosmetics and pharmaceutical products [5, 9, 28], and have been studied to examine their estrogenic activity *in vitro* and *in vivo* [13]. Isobutyl-paraben (IBP) shows a comparatively high potency of estrogenic activity among parabens [13, 24]. In this study, to clarify whether early exposure to estrogenic chemicals predisposes to autistic behaviors, we analyzed the effect of maternal exposure to IBP on social recognition in adult offspring. In addition, we analyzed female offspring, because the major effect of maternal adrenalectomy on maladaptive behavior occurs in female offspring [38], and we previously reported that maternal IBP exposure decreased plasma corticosterone levels in dams [19]. Furthermore, IBP exposure may change estrogen susceptible systems, such as social recognition of female offspring, because we previously elucidated that maternal IBP exposure increased uterine sensitivity to estrogen in adult female offspring [19].

Sprague-Dawley rats were obtained from Charles River Laboratories Japan, Inc. (Ibaraki, Japan), and were maintained under conditions of controlled lighting (lights on, 07.00–19.00), temperature (22.5 ± 0.5°C) and humidity (55 ± 10%). The rats were given *ad libitum* access to food (CE2; CLEA Japan, Inc., Tokyo, Japan) and distilled water from glass bottles. Adult rats and nursing dams with pups were housed in stainless steel cages lined with paper bedding (Paper Clean; Japan SLC, Inc., Shizuoka, Japan). Animals were maintained and used according to the guidelines of the Musashino University Animal Care and Use Committee and the National Institute for Environmental Studies Animal Care and Use Committee.

Thirteen-week-old virgin female rats were paired with 11-week-old male rats from the evening of proestrus to the morning of estrus. The stage of the estrous cycle was determined based on vaginal cytology. Mating was verified by the presence of sperm in vaginal smears (gestational day 0; GD0), and when sperm was found the females were separated from the males. The pups were counted on postnatal day (PD) 3, were culled to four males and four females per litter, and were kept with their respective dams until weaning on PD21 (the day of birth was designated PD0). Three weeks before the mating, under ether anesthesia, female rats were implanted (under the skin of the flank region) with a 20-mm-long Silastic capsule, 2 mm *i.d.*, 3 mm *o.d.* (Kaneka Medix Co., Osaka, Japan) that was either filled with IBP (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) or empty (control). We prevopisly showed that Silastic capsules filled with IBP release about 4.36 mg/l/day IBP into 37°C saline [19]. At 7 weeks of age, female offspring were gonadectomized to remove the effects of inter-animal variability in circulating hormone levels.

Female offspring of treated and untreated dams were subjected to a social recognition test at the age of 16 weeks. Daily, for 3 days before the social recognition test, the rats were individually placed for 10 min in a square open-field apparatus constructed of vinyl chloride walls (50 × 50 × 50 cm) with paved polyethylene paper on the floor, and allowed to acclimate to the environment. On the fourth day, an unfamiliar ovariectomized 16-week-old female intruder was placed in the field for 60 s, and we counted the amount of time and the frequency with which the test animals spent investigating the intruder. Interaction was defined as sniffing of the intruders by the rat being tested. The tested rats were removed and then returned to the field after a 10-min interval. The rats were repeatedly tested for four additional trials (inter-trial interval, 10 min). On the fifth trial, a novel,

ovariectomized, 16-week-old intruder was placed into the field. During the 10-min interval between the trials, the apparatus was cleaned with 70% aqueous ethanol and the paving papers were replaced unless otherwise stated.

All results are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) for repeated measures was applied to examine the effects of IBP exposure on the social recognition behavior (n=6 control litters and n=5 IBP-exposed litters, with one female offspring per litter randomly). If ANOVA showed significant interactions, Fisher's *post-hoc* tests were applied to search for significant differences among the groups. If ANOVA did not show significant interactions, the Tukey-Kramer test, which can be used in the absence of significant ANOVA results was performed. Differences were considered significant when the *P* value was below 0.05. All analysis were performed using StatView 5.0J software (SAS, Inc., Cary, NC, USA) for Microsoft Windows.

Rats treated with IBP showed impaired social recognition. Two-way ANOVA revealed significant interactions between treatment and test. In the control group, the social response decreased through trials 1–4 (with repeated presentation of the same intruder rat) but increased again during the fifth trial when presented with a novel rat (Fig. 1a). On the other hand, IBP-exposed rats did not show a change in frequency of social interaction toward the repeatedly presented rat and the subsequent novel rat (Fig. 1a). In the fourth trial, the social interest of control rats was significantly lower than that of IBP-exposed rats. The same pattern was observed for the time spent in social interaction: control rats spent progressively less time investigating the repeatedly introduced intruder, then more time interacting with the novel intruder. However, there were no significant differences between IBP-exposed and control rats (Fig. 1b).

The present study demonstrated that female rats given early exposure to IBP had impaired social recognition in adulthood. This provides the first evidence that developmental exposure to estrogenic chemicals *via* the placenta or milk can exert permanent or long-lasting influences on social recognition in offspring.

These results cannot be explained by a generalized difference in total activity or anxiety, because there are

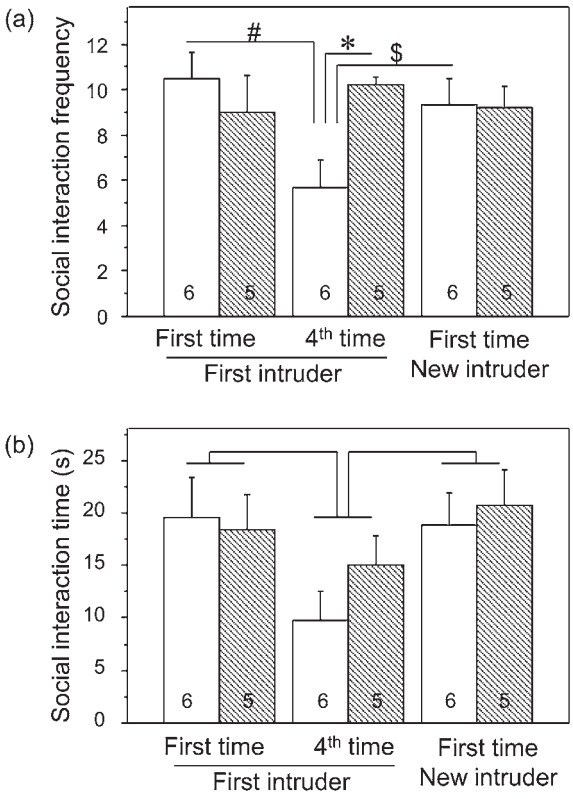

Fig. 1. Effects of developmental exposure to isobutyl-paraben (IBP) on social recognition performance. The following behaviors were quantitated: social interaction frequency (a) and social interaction time (b). The open and hatched columns show the control and IBP-exposed groups, respectively. Values shown are mean ± SEM. Numbers within the bars indicate the number of rats in each group. *: $P<0.05$ for control vs IBP-exposed rats on the fourth trial with the same intruder. #: $P<0.05$ for control rats on the first trial vs the fourth trial with the same intruder. $: $P<0.05$ for control rats on the fourth trial with the same intruder vs the first trial with a new intruder.

no effects of IBP exposure on general, anxiety and learning behaviors in female offspring [18]. In contrast, early exposure to IBP increases anxiety, and disturbs passive avoidance performance in male offspring [18]. Further elucidation is required for an understanding of the effects of IBP on the male offspring.

IBP treatment has been shown to decrease plasma corticosterone levels in dams, suggesting that IBP may inhibit endogenous estrogen activities [19] that stimulate the HPA-axis [4, 17]. Many studies have suggested that changes in maternal glucocorticoids levels may be in-

volved in the manifestation of ASDs, because maternal stress associates with the prevalence rates of ASDs in humans [2, 21, 36]. Further more, maternal glucocorticoids modulate the developing HPA-axis and ASDs-like behaviors in rodents [10, 15, 22, 25, 37, 38] and primates [32, 33]. For example, maternal adrenalectomy increases maladaptive behavior in the forced swim test [38], and maternal stress alters social behavior and the oxytocinergic system in the adult offspring of rodents [22]. The removal of, or an increase in, maternal glucocorticoids leads to similar consequences: immobility in the forced swim test and hypothalamic glucocorticoid receptor expression in adult offspring [38]. Impairment in social recognition behavior may reflect a decrease in plasma corticosterone concentration in IBP-exposed dams [19]; however, further studies are, necessary to confirm this.

Previously, we showed that maternal exposure to IBP decreased uterine sensitivity to estrogen, which may be reflected in estrogen receptor (ER) expression in adult female offspring [19]. Similarly, previous works have shown that ER expression in animals treated with other estrogenic compounds is significantly lower than that in control animals [20, 35]. Interestingly, ER plays a role in mediating social recognition in female mice, as *ERα* and *ERβ* knockout mice have specifically impaired social recognition [6]. In addition, ERα and ER*β* play a crucial role in oxytocin-dependent social recognition [6]. Therefore, impairment of social recognition may reflect low sensitivity to estrogen from fat, adrenal and brain in IBP-exposed female rats, though puberty, estrous cycle, plasma estradiol and gonadotropin appear normal [19].

In conclusion, this study represents the first investigation of the effects of endocrine-disrupting chemicals on social recognition. Social recognition in rodents has been implicated in oxytocin, vasopressin and cell adhesion molecule 1 [3, 27, 34], and impairment of these chemicals' function is associated with human ASDs [16, 40]. One of the prominent social dysfunctions in autism is abnormal social recognition. Environmental factors including exposure to chemicals are likely to account for a major proportion of the increased prevalence of autism. Early exposure to chemicals may have played a role in the increasing prevalence of ASDs, through changes in the HPA-axis or estrogen sensitivity.

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