Cross-generational impact of a male murine pheromone 2-sec-butyl-4,5-dihydrothiazole in female mice

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The current understanding of the activity of mammalian pheromones is that endocrine and behavioural effects are limited to the exposed individuals. Here, we demonstrate that the nasal exposure of female mice to a male murine pheromone stimulates expansion of mammary glands, leading to prolonged nursing of pups. Subsequent behavioural testing of the pups from pheromone-exposed dams exhibited enhanced learning. Sialic acid components in the milk are known to be involved in brain development. We hypothesized that the offspring might have received more of this key nutrient that promotes brain development. The mRNA for polysialyltransferase, which produces polysialylated neural cell adhesion molecules related to brain development, was increased in the brain of offspring of pheromone-exposed dams at post-natal day 10, while it was not different at embryonic stages, indicating possible differential brain development during early post-natal life.

1. Introduction

In diverse animal species, chemical signals relay information between individuals, triggering specific behaviours (releaser effect) and stimulating changes in a physiological state (primer effect) [1–3]. This chemical communication is mediated by pheromones that are detected by the olfactory system [1–3]. The first examples of primer effects in the house mouse (Mus domesticus) were oestrous suppression among females (Lee–Boot effect) [4,5], which was followed by the finding of oestrus induction and synchronization by exposure to males (Whitten effect) [6,7] and disturbed pregnancy establishment (Bruce effect) [8,9], all mediated by olfactory messengers. Mouse urine was used as a source to identify the chemical structures of putative pheromones responsible for these primer effects [10–13], and synthetic analogues were able to induce similar physiological changes [14–16]. The pheromone compounds 3,4-dihydro-exo-brevicomin and 2-sec-butyl-4,5-dihydrothiazole (SBT) [14] and a mixture of farnesene (sesquiterpene) isomers [15] were found to effectively induce the Whitten effect. Seemingly, the initially perceived pheromone at the target olfactory tissue triggers a cascade of hormonal effects that induce highly specific responses even in distant tissues such as the ovaries or epidermis.

More recently, olfactory communication was also implicated in neurogenesis. When females were exposed to bedding soiled by males, neurogenesis was enhanced [17–19]. We have recently demonstrated that the synthetic analogues of SBT and the farnesenes stimulate proliferation in the neurogenic regions of murine female brains [20,21]. Studies using transneuronal markers have shown that exposure to male murine pheromones activates gonadotropin-releasing hormone (GnRH) neurons in females [22], thus stimulating...
the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which enhances the secretion of oestrogen and progesterone. Studies have also shown that oestrogen stimulates prolactin secretion by upregulating dopamine-activated K⁺ channels in pituitary lactotrophs, producing increased levels of the hormone typical of the proestrous stage [23,24]. The Whitten effect can be explained by similar pheromonal activation of GnRH neurons and the subsequent modulation of ovarian function by the enhanced production of FSH and LH. Neurogenesis in the brain then appears to be stimulated by the pheromone-induced increases in prolactin and oestrogen. These studies suggest that the exposure to pheromones from the opposite sex triggers changes in the sex hormone secretion altering physiological states and would probably impact some other organs regulated by oestrogen, progesterone and prolactin [25].

Here, we report the profound effect of synthetic male pheromone SBT on the development of mammary glands. The presence of a larger mammary tree in the SBT-treated females suggested a potential for enhanced lactation capacity and subsequent ability to more efficiently nurse offspring. To determine whether prior exposure to the male pheromone can alter mammary gland development during pregnancy, we mated adult virgin females after exposure to SBT and compared the development of mammary glands in the mid-post-delivery period during lactation. We also measured the growth and cognitive function of the offspring. Offspring nursed by SBT-treated females demonstrated significantly enhanced brain expression in polysialyltransferases, which are involved in brain development [26], coupled to higher cognitive function in spatial learning tests.

2. Material and methods
See the electronic supplementary material for details.

(a) Animals and husbandry
C57BL/6 mice were used (a total of 325 females and males including pups). Mice were socially housed at two to five mice per cage to avoid the influence of isolation [27–29].

(b) Exposure to synthetic pheromones
We exposed virgin female mice to an aqueous solution of male murine pheromone SBT [20,21], dissolved in OmniSolv® water (EM Science, Gibbstown, NJ, USA) (250 ppm, 50 μl), twice daily for 7 days. Synthesis of SBT is described elsewhere [30]. The control group was either exposed to water or kept without treatment. Mammary glands were harvested on the eighth day while some females were mated at that time. For breeding, females were mated for 10 days after one week of exposure to SBT or water, and singly housed thereafter. On the day of delivery, the litter size was adjusted to seven pups. For the cross-fostering experiment, the whole litter was exchanged on the day of birth when there were litters born on the same day.

(c) Recording and analysis of the time dams are out of the nest
On post-delivery day 10, the behaviours of dams were recorded for 5 h using Logitech QuickCam Vision Pro (Logitech, Newark, CA, USA). Total time out of the nest per hour was measured and average per hour for the 5 h of recording was calculated.

(d) Collection of milk and measurement of weights of mammary gland
To make the boundary of no. 4 and no. 5 mammary glands visible (figure 1a), and also to control the condition of the mammary gland in measuring the weight, milk was collected from no. 4 mammary gland. Post-delivery day 10 dams were separated from pups 2 h before the milk collection was started, then the skin above the mammary glands was massaged and milk was collected. Following the milk collection, no. 4 mammary glands were collected and the weights were measured.

(e) Whole mount staining of mammary glands
The no. 4 mammary fat pads including the mammary glands (figure 1a) of virgin females were collected and whole mount stained [31].

(f) qRT-PCR
Brains of post-delivery day 10 pups and embryonic stage 17 embryos of dams exposed to SBT and control group dams were dissected out, submerged in RNALater (Ambion, Austin, TX, USA), and kept at −20°C until use. Embryonic stage was confirmed by Theiler’s atlas of embryonic development of the house mouse [32]. RNA was extracted, converted to cDNA, and PCR was then performed on cDNA.

(g) Morris water maze tests
Studies supplementing sialic acid in rats during pregnancy and lactation have shown that the offspring of these dams demonstrated enhanced learning abilities [33]. Offspring of females exposed to SBT and control groups went through spatial memory tests for 5 days using a Morris water maze after postnatal day 70. Whether they could reach the hidden platform and the latency to reach there was measured each day.

(h) Statistics
A Student t-test was used to compare the weight of mammary glands, time spent in the nest by dams, number of terminal end buds (TEB, the proliferative structures of bulbous shape on the tips of mammary ducts) and branches of the mammary glands depending on the oestrous status. For results of RT-PCR and qRT-PCR on offspring, a Mann–Whitney U-test was used. ANOVA was used for statistical analyses of the TEB and branches of the mammary glands, growth of offspring and results of the Morris water tests between pheromone conditions. SYSTAT software was used for these statistical analyses.

3. Results
(a) Expansion of mammary glands by exposure to male pheromone
Adult virgin females exposed to SBT showed significantly more TEB (Student t-test: t₁₁₄ = 7.113, p < 0.001) and branches in their no. 4 mammary glands (Student t-test: t₁₄ = 6.374, p < 0.001) (figure 1a–c). The difference was significantly higher at all stages of the oestrous cycles (the electronic supplementary material, figure S1, shows results at oestrous stage).

In our previous studies, we observed the influence of exposure to SBT on cell proliferation in the brain only in adult females and not in pre-, post-puberty and young adult females [20,21]. We tested if there are age-dependent
effects on the influence of exposure to SBT on mammary gland expansion as well. We found that post-pubescent (exposed to SBT during P30 and P37) and young adult (exposed to SBT during P40 to P47) female mice did not display significant mammary gland changes but adult females did following SBT exposure (electronic supplementary material, figure S2), a result analogous to that found in the earlier studies on central nervous system cell proliferation [20,21].

(b) Long-term influence in post-delivery stages

As the exposure to a male pheromone affected mammary gland expansion, mating of the control group females may allow their mammary glands to expand to the level of females exposed to SBT because they will be exposed to males’ odours. Or, if the influence of SBT is long-term and also allows the altered mammary gland to differentiate better when they are mated, the differences between the groups may still exist when both of these groups of females are mated. To answer this question, we exposed a group of females to SBT and another group to water for one week, and mated them on the eighth day. We chose post-delivery day 10, the middle of nursing period, to determine if there are still differences between the groups. TEBs and branches of the highly differentiated mammary glands at this stage are impossible to measure, so we measured the weights of mammary glands. In measuring the weights, it is also impossible to see the boundaries of the adjacent no. 4 and no. 5 mammary glands. However, when milk was collected from one of the adjacent mammary glands, the boundary became clearly visible (figure 2a), which enabled specific removal of individual glands. Collecting milk before measurement also controls differential suckling of the glands by offspring. We found that the no. 4 mammary glands of the females exposed to SBT before mating (SBT dams) were about 30% heavier than those of control group dams (figure 2b–d) (Student t-test: t8 = 3.725, p = 0.006), indicating that the larger mammary gland obtained through the SBT exposure had a long-term impact that was maintained throughout pregnancy and lactation.

Such long-lasting differences in the mammary glands suggest that the SBT dams may nurse their offspring more frequently. As pups suckle nipples even when the dams are not couching over the pups, we decided to compare the time the dams stayed in their nests to represent potential nursing time. We recorded this on post-delivery day 10. The SBT dams stayed in their nests about 25% longer than controls (Student t-test: t10 = 4.409, p = 0.001) (figure 2e). The longer time that SBT dams spent in the nest suggests the possibility that their offspring may be heavier because of the larger volume of milk they could have received, however the bodyweight at P10 did not differ between SBT dams’ and control dams’ offspring (electronic supplementary material, figure S3).

(c) Enhanced expression of polysialyltransferase in the brain of offspring

The heavier mammary glands of SBT dams and the longer time they spent in the nest suggested that their pups may grow larger, however there were no differences in the bodyweight at P10.
weight of the offspring. We hypothesized that the more developed mammary glands might have affected milk synthesis to change the quality of milk rather than the quantity of it. There are various milk components that could be affected. We focused on sialic acid-containing glycoconjugates. They are known as components of maternal milk, which could become an exogenous source of polysialic acids in the offspring’s brain [26]. The amount of the expression of polysialyltransferases, which polysialylate neural cell adhesion molecules, is known to positively correlate with the amount of polysialic acids in the brain. We tested if there are any differences in the mRNA expression of polysialyltransferase in the brain of offspring of SBT dams and controls, which could indirectly implicate changes in the quality of milk. Expression of one of the polysialyltransferases, ST8SiaIV, was increased in the brain of P10 pups from SBT dams (ST8SiaIV, Mann–Whitney U-test: \(U = 176, p < 0.05\)) (figure 3a). If this difference were regulated by the specific quality of the milk the pups were exposed to, ST8SiaIV expression should not be different in embryos prior to birth. We tested this hypothesis by comparing the mRNA of polysialyltransferase between groups at an embryonic stage. At late embryonic stage (E17), polysialyltransferase transcripts’ levels were similar in the offspring of SBT dams and those from controls (figure 3b).

(d) Higher cognitive function in the offspring as adults

Higher expression of polysialyltransferase suggests a possibility that the offspring of SBT dams could have improved brain development and cognitive function. We used Morris water maze to test this hypothesis. We found that the offspring of SBT dams exhibited a significantly enhanced spatial memory (ANOVA: day 3, \(F_{1,69} = 7.615, p = 0.007\); day 4 and 5, \(F_{1,69} = 5.315, p = 0.024\)) (figure 4a). Learning was apparently faster in the offspring of pheromone-treated mice. The results of mRNA for polysialyltransferase showed that the differences of the expression of polysialyltransferase are post-natal. This suggests that if control group offspring were raised by SBT dams they would show higher cognitive function. To test this hypothesis, we cross-fostered the offspring on post-natal day 0, between the pheromone group and control group. The pups of control group dams raised by SBT dams performed better in the Morris water maze (ANOVA: day 3, \(F_{1,18} = 11.52, p = 0.003\); day 4, \(F_{1,18} = 16.2, p = 0.001\); day 5, \(F_{1,18} = 3.299, p = 0.086\)) (figure 4b), indicating the differences between the groups are produced post-natally.
Figure 3. Expression of polysialyltransferase, ST8SiaII and ST8SiaIV in the brains of offspring. (a) mRNA transcripts of polysialyltransferase, ST8SiaIV, were increased in the post-natal day 10 offspring of SBT dams. Error bars indicate standard error. *p < 0.05. (b) There was no difference in the expression of polysialyltransferase mRNA at embryonic stage 17.

Figure 4. Results of Morris water maze tests on offspring when adult. (a) Average percentage of the number of mice that reached the hidden platform in the water maze during 5 days of testing. Equivalent performance of the two groups on day 1 suggests that subsequent differences in maze success are due to learning rather than to motor or motivational differences. (b) Results of a Morris water maze tests on cross-fostered offspring at post-natal day 70. Note the high incidence of floating behaviours by SBT offspring cross-fostered and raised by control group dams. Error bars indicate standard error. *p < 0.05, **p < 0.01, ***p < 0.001. (Online version in colour.)

4. Discussion

In this study, the mammary glands were harvested on the eighth day after females were exposed to SBT for 7 days. We did not investigate whether the enlarged mammary gland was maintained after exposure in virgin mice. However, the females, which were mated after pheromone exposure, continued to have larger glands into mid-lactation. We also found higher expression of ST8SiaIV in the brains of post-natal day 10 offspring, but not in E17 foetuses of SBT dams. The offspring from SBT dams also showed increased learning ability as assayed by the Morris water maze. Cross-fostered offspring born to control dams and raised by SBT dams also showed enhanced learning.

Recent studies in mice have shown that fear conditioning with the odour of acetophenone affects the sensitivity to that compound in the following generations (F1 and F2). These subsequent generations also showed significantly larger numbers of olfactory neurons with the receptors for acetophenone (M71, Olfr151 gene) [34]. Acetophenone is not known to be a pheromone in mice and there have been few studies so far showing trans-generational pheromone effects in mammals. However, in insects, there have been some studies showing that exposure to alarm pheromone increases wing polyphenism in the next generation [35,36].

Our results suggest that some post-natal factor(s) had affected the expression of polysialyltransferase in the brain of offspring and their cognitive functions as adults. Polysialyltransferase is involved in oligomerization of sialic acids. Free oligosaccharides are major constituents of mammalian milk [37], which contains N-acetylgalactosamine, galactose, fucose and sialic acid as its main monosaccharide components. Sialic acid, as a component in milk oligosaccharides, is known to be important for brain development [26,37–39]. It is polymerized by polysialyltransferase, ST8SiaII and ST8SiaIV [40], and this polymerized form, polysialic acid, is found at a high level in the early brain developmental stages, i.e. embryonic and early post-natal stages. In adults, it is maintained at a high level in the hippocampus and olfactory system, where adult neurogenesis occurs [41,42]. The degree of expression of polysialyltransferase always correlates with the occurrence of polysialic acid within proteins and lipids [40]. The offspring of SBT dams could have received more sialic acid-containing oligosaccharides. This could function as an exogenous source of polysialic acids, and subsequently the offspring of SBT dams expressed more polysialyltransferase in their brains.

With heavier mammary glands and longer nursing time, it is possible that the offspring of SBT dams received more sialic acid-containing oligosaccharides through milk. One would expect that the situation could be similar to dams with a small litter size, in which each pup can receive a larger volume of milk compared with the pups in larger litter sizes. However, the body weights of offspring from each group were not different (electronic supplementary material, figure S3). It is more likely that
the altered qualitative composition of milk of the SBT dams provided the observed benefits as opposed to the enhanced volume. This possibility needs to be addressed in future studies.

There is an increasing interest in the early nutrition and epigenetic processes in humans [43]. The processes in which the exposure to specific nutrients during the early postnatal stage affecting the expression of genes later in life has been termed nutritional epigenetics [43,44]. Our research suggests that neuroendocrine changes in adult life can in turn impact the neonatal nutritional environment resulting in modulation of behaviours and cognitive function in the preceding generation. The precise mechanisms by which nutrition may influence neurological function still remain to be elucidated.

A factor other than milk that may have affected the offspring post-natally is the maternal behaviour. The SBT dams spent more time in their nests (figure 2c). Higher maternal stimulation (licking and grooming) has been linked to higher ERα and mGluR1 gene expression in the brain of the offspring [45,46]. In this study, the exposure of females to SBT before mating may have induced some changes in the gene expression of their brains in a way that affected a spectrum of maternal behaviours, resulting in subsequent neurological function changes in the offspring. This possibility also needs to be addressed in detail in the future.

The impact of external stimuli on the ovariatic activities of females has been shown from the 1950s in mice, in which the stimulus was the odour of male-soiled bedding, and studies have shown that male-soiled bedding induces oestrous in females [3,6,7]. More recently, it was also found that male-soiled bedding stimulates adult neurogenesis in females [17–19]. Our study represents one of the few cases in which the exposure to a male pheromone has a positive cross-generational impact on brain function in mammals. It seems apparent that the exposure to male murine pheromones produces a much broader effect on the female neuroendocrine system than previously anticipated.

The impact is not limited to their oestrous cycles [3,6,7] and adult neurogenesis [17–21], but extends to the development of mammary glands, and possibly includes the entire spectrum of physiology and behaviours affected by oestrogen and prolactin.

The particular pheromone (SBT) that has been linked to the enhanced development of the mammary gland and to the learning ability of their offspring is secreted at higher levels in dominant male mice [47]. While the experimental conditions for the control group females that completely lacked exposure to SBT prior to breeding is a laboratory artefact and, in nature, these mice would most likely be exposed to the pheromone at varying levels, this effect of SBT we found on females suggests that mating with dominant males would enhance the reproductive success. This can also be explained from the perspective of investment on reproduction depending on conditions, i.e. activating the reproductive system when environmental conditions are best for reproductive success and suppressing it when the situation is not suitable for breeding. It has been known that female pheromones suppress the oestrous cycles when there are no male odours in the environment and females are in high density (the Lee–Boot effect) [3–5]. Our finding represents the opposite situation, i.e. when there are cues indicating males with dominant status in the environment, suggesting better possibility of reproductive success, the female reproductive system becomes activated. The primer effects found so far in females and males [3] suggest that the house mice have evolutionarily developed a sophisticated system to adjust their reproductive capabilities, using chemical signalling in a way to respond to a changing environment. In the case of a mammalian species such as the house mouse, which breeds typically in a communal breeding system [48] with one sire male and multiple breeding females [49], it will be beneficial to breed with a dominant male that secretes a high concentration of male murine pheromones. Consequently, the offspring in the communal breeding nest, rather than the individual mother–litter, can receive benefits that may enhance their survival. This suggests that female mate choice not only affects the inheritance of specific traits and access to resources, but can also increase maternal fitness.

Ethics. The study was approved by the Indiana University Institutional Animal Care and Use Committee (IACUC).

Authors’ contributions. S.K. designed and conducted the experiments other than qRT-PCR. J.W.-M. conducted qRT-PCR of the brains of the offspring in the Kenneth Mackie laboratory at Indiana University. J.F., M.V.N. and C.L. provided resources for the experiments conducted by S.K., and shared the mice housing cost. The Morris water maze test was conducted in the laboratory of J.D.C. by S.K. with the help of set-up by M.J.P. C.R. helped to develop the method of collecting milk with S.K. J.A. provided Pitocin, which was used to stimulate milk letdown. The synthetic analogue of pheromone used in the study was originally generated in the laboratory of M.V.N. H.A.S. prepared pheromone solutions and monitored their stability. S.K. wrote the paper and J.W.-M., H.A.S., M.V.N., J.F., C.R., W.A.Jr., M.J.P., J.D.C., C.L., J.A. edited the paper.

Competing interests. The authors declare no competing financial interests.

Funding. This work was supported by NIH/NIH 5R01MH094130-01 (S.K. and J.A.), the Gill Center for Biomolecular Science and the Lilly Endowment Excellence in Indiana grant (C.L.). Pheromone analyses were supported through the funds from the Lilly Chemistry Alumni Chair (M.V.N.).

Acknowledgements. We sincerely thank Kenneth Mackie of Indiana University, The Linda and Jack Gill Center for Biomolecular Science and Department of Psychological and Brain Sciences, Bloomington, for his support and advice on the project. We thank Joseph K. Leffel II of Indiana University, Department of Psychological and Brain Sciences, Bloomington, for his advice on the Morris water maze and Tadasu Urashima of Obihiro University of Agriculture and Veterinary Medicine, Department of Animal and Food Hygiene, Japan, for his advice on milk biochemistry.

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