Chemistry of clitoral gland secretions of the laboratory rat: Assessment of behavioural response to identified compounds

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The present investigations were carried out to find out the chemical nature of clitoral gland extracts and their involvement in reproductive and social behaviour. Homogenates of clitoral glands of mature estrous female rats were extracted with *n*-hexane and dichloromethane (1 : 1 ratio v/v) and analysed by gas chromatography linked mass spectrometry (GC-MS). Three peaks were found to be in higher concentration, which were identified as 6,11-dihydro-dibenz-b,e-oxepin-11-one (I); 2,6,10-dodecatrien-1-ol-3,7,11-trimethyl(Z) (II); and 1,2-benzene-dicarboxylic acid butyl(2-ethylpropyl) ester (III). Odour preference tests demonstrated that the first compound attracted conspecifics of the opposite sex. By contrast, the second and third compounds were found to attract both sexes. The results conclude that the clitoral gland of laboratory rat contains three major chemical compounds which have a unique function in maintaining social and reproductive status.

1. Introduction

Among mammals chemical signals can send powerful messages with behaviour modulating effects that may be of considerable social importance. The study of pheromone cueing systems in relation to complex behaviours has been hampered by the lack of identification of specific compounds functioning as behaviour modifiers. Pheromones, like chemical signals, are detected by special receptor neurons in the olfactory system. The major difference between pheromones (species-specific) and other chemical signals (inter-specific) is in the output: when processed by the brain, chemical signals result in the sensation of smell, whereas pheromone signals trigger a unique characteristic behavioural or physiological response (Ben-Ari 1998). Mammalian pheromones are found to be involved in many reproductive behaviours, such as sexual attraction (Kannan *et al* 1998), interference with puberty, oestrous cycle and pregnancy (Dominic 1991), as well as social behaviours namely territorial marking (Doty 1980; Prakash and Idris 1992), individual identification (Poddar-Sarkar and Brahmachary 1999), mother-young interaction (Leon 1983) and initiation of aggression (Mugford and Nowell 1971). The major sources of physiologically and behaviourally important chemical cues are the secretions of specialized scent glands (Mykytowycz 1970; Adams 1980; Balakrishnan and Alexander 1985; Johnston 1990; Kannan and Archunan 1997a, b; Kennaugh *et al* 1997), urine (Jemiolo *et al* 1987; Hurst 1990) and faeces (Mykytowycz 1970; Asa *et al* 1985).

The secretions of scent glands have a distinct function in rodent behaviour. Among the scent glands present in rodents, the preputial glands of the rat play an important role in the production of olfactory substances which attract the opposite sex (Noble and Collip 1941; Merkx *et al* 1988; Kannan *et al* 1998). The preputial glands appear as paired structures on either side of the penis in the male rat; in females, the homologous gland has been observed to be associated with the clitoris (Balakrishnan and Alexander 1985). In rats, the preputial gland has a well developed capsule with a distinct excretory duct system (Montagna and Noback 1946).

It has been demonstrated that female rats prefer the odour of normal male preputial gland extracts to that of castrated rats and that the sex attractant compound(s) is present in the preputial gland extract (Orsulak and

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Gawienowski 1972; Gawienowski *et al* 1975). Likewise, experiments have shown that male rats prefer the odour of intact over that of preputialectomized estrous female rats (Thody and Dijkstra 1978). Further, Orsulak and Gawienowski (1972) observed that sexually experienced male rats are attracted by the odour of a homogenate of clitoral glands. An earlier report indicated that the preputial gland of the oestrous female has volatile and other lipid-like substances which attract the intact male (Gawienowski *et al* 1975). We have chemically characterized the preputial gland secretion of the male rat and have found that the identified volatile compounds have distinct social functions (Kannan *et al* 1998). However, the chemical identification of such odorant molecules has not yet been carried out in the clitoral gland.

Hence, the present investigation was carried out to find out the chemical nature of clitoral gland homogenates and to assay the biological activity of identified compounds.

2. Materials and methods

Male and female rats were housed separately in polypropylene cages (40 × 25 × 15 cm) with 2 cm of rice husk lining the bottom as bedding material. The bedding material was changed before every odour preference test. Nine females [160 ± 15·6 g (± SD)] were 12–14 week-old regularly cycling virgins and nine intact males [185 ± 14·7 g (± SD)] were 14–18 weeks old, having scrotal testes.

Both sexes of albino rats used in the present investigation were housed under laboratory conditions and reared on pelleted food (Hindustan Lever Ltd., Bangalore) and water *ad libitum.* Twenty-five estrous female rats were sacrificed by cervical dislocation under anesthesia. Following autopsy, clitoral glands were removed and placed in double distilled solvent mixture (*n*-hexane and dichloromethane 1 : 1 ratio v/v) and ground well for about 10 min with a glass homogenizer under ice cold condition. Immediately after homogenization, the supernatant was filtered through silica gel (50–60 μm mesh size). The pure supernatant was collected in a glass vial sealed with airtight screw cap. The sample vials were stored at – 20°C until they were used for gas chromatography linked mass spectrometry (GC-MS) study.

The GC-MS analyses were done in a Shimatzu QP5000 instrument under computer control at 70 eV. Chemical ionization was performed using ammonia as reagent gas at 95 eV (Kannan *et al* 1998). The identified compounds were then compared with standards run under the same conditions. These data were already stored in a compact library of chemical substances (NIST62.LIB). As mentioned earlier, fresh samples were fractionated and collected in separate storage glass vials to carry out the behavioural study.

The homogenate of clitoral glands (20 ml) was distilled for 30 min at room temperature under a vaccum of 0·2 torr. The distillate was condensed by cooling with liquid nitrogen and concentrated to 2 ml. The volatiles from the distilled fractions were subjected to GC for cross checking and confirmation of compounds in each fraction (Pause *et al* 1997). Assuming the importance of the compounds in pheromone activity, Y-maze odour preference tests were conducted using the modified procedure of Ferkin and Seamon (1987).

The experimental animals were segregated into three different sets of the same and opposite sexes. Three individuals randomly taken from a pool of 20 rats (male and female colonies maintained separately) were used in each set for behaviour analyses, and the experiment for each set was repeated thrice. Fresh samples were used for each trial. The behaviour on exposure to the identified compound was assessed with the help of a Y-maze apparatus. The Y-maze apparatus contains three arms namely, a middle common arm (where the responder was released in the apparatus), and of the remaining two arms, the scented sample was placed in one (experimental arm), and the pure solvent mixture was placed in the other (control arm). The time spent in investigating both the scented sample and control was recorded for each animal. The odour preference test was assessed for 15 min with the identified compounds (experimental) and the solvent mixture was used as control. The responders were members of the same and opposite sex. The data were analysed using the *t*-test (Zar 1984) to compare the behaviour of males and females in response to exposure to the different compounds.

3. Results

The extracts prepared from female clitoral glands of laboratory rats contained more than 21 different fractions. Of these, the following three chemical substances constituted the major portion of the glandular homogenate. These were 6,11-dihydrodibenz-b,e-oxepin-11-one (I); 2,6,10-dodecatrien-1-ol-3,7,11-trimethyl(Z) (II); and 1,2-benzenedicarboxylic acid butyl(2-ethyl-propy1) ester (III). The gas chromatography clearly showed that the major compounds mainly fall between the retention time of 30 and 50 min (figure 1). Figures 2 and 3 show the mass spectra and chemical structure of the identified compounds respectively. Further, the computer matched data of all these compounds showed above 95% similarity with the compounds identified from the clitoral gland.

Among the three different compounds identified in the present study, the bioassay data revealed that compound I evoked the maximum response in the attraction of the opposite sex (males) than the same sex, while compounds

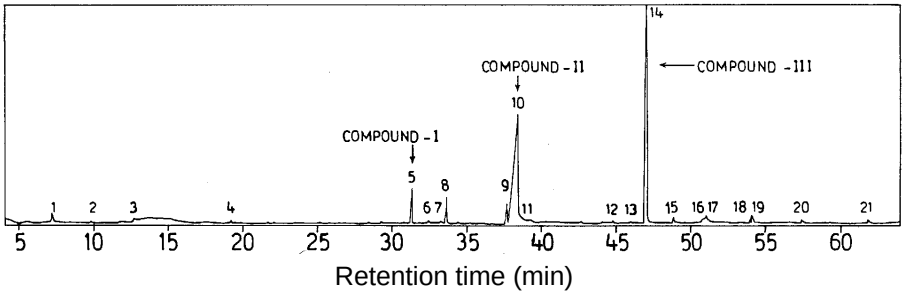

Figure 1. Gas chromatographic profiles of the clitoral gland secretions of laboratory rat.

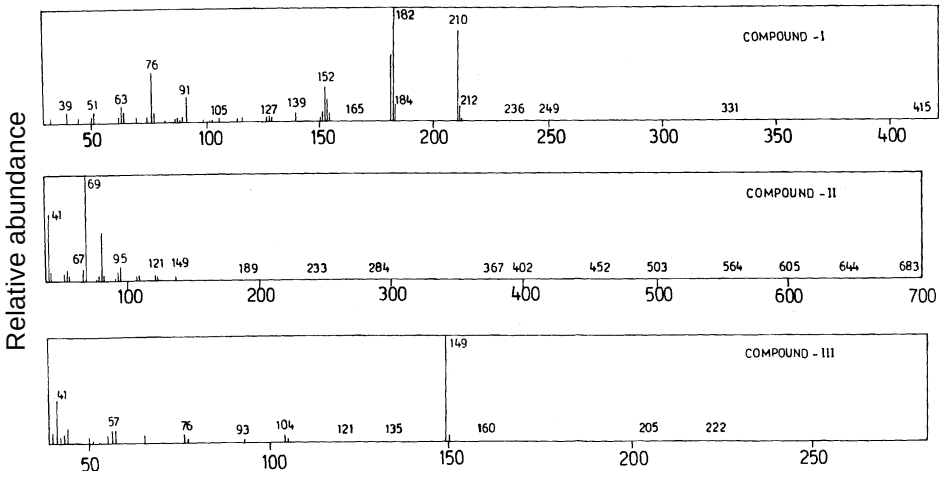

Figure 2. Mass spectra of the identified compunds of female laboratory rat clitoral gland.

II and III were involved in the attraction of both male and female rats (table 1). In addition, it was observed that of the three identified compounds, the second one (II) was found to be most attractive for all responders used in this investigation.

4. Discussion

Odours are extremely important for rodents and other mammals for many types of behavioural communication (Gosling 1985). Our earlier study (Kannan *et al* 1998) indicated that the male preputial glands of laboratory rats contain eleven different compounds of which three compounds constitute major fractions. Similarly, in the present study three major compounds have been identified. Further, it is striking to note that the compounds 2,6,10-dodecatrien-1-ol-3,7,11-trimethyl(Z) (II) and esters of 1,2-benzene-dicarboxylic acid (III) identified in the clitoral glands have already been recorded in the male rat and were found to attract both sexes. Therefore, the present study further confirms that the compounds II and III serve as both sex attracting chemical moities and also convey signals to individuals irrespective of sex.

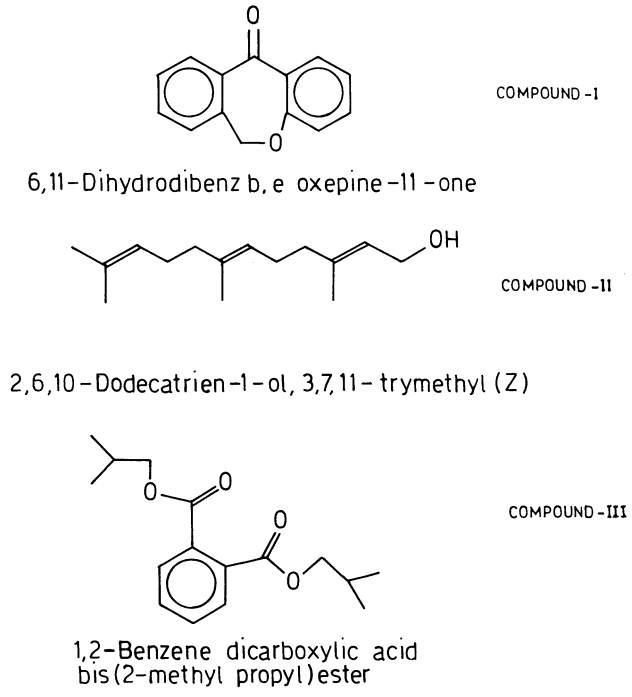

Figure 3. Chemical structure of the identified compounds of female clitoral gland of laboratory rat.

Table 1. Investigation time in seconds (mean ± SE) of male and female rats exposed to the identified compounds (I, II, III) of clitoral gland extracts under laboratory conditions.

	Compound I		Compound II		Compound III	
Responders	SS	BS	SS	BS	SS	BS
Female (n = 3)*	$127{\cdot}14^{S} \pm 2{\cdot}30$	$27{\cdot}14 \pm 1{\cdot}38$	$375{\cdot}20^{NS} \pm 1{\cdot}94$	$29{\cdot}16 \pm 2{\cdot}48$	$233{\cdot}70^{NS} \pm 2{\cdot}66$	$25{\cdot}20 \pm 1{\cdot}48$
Male (n = 3)*	$415{\cdot}70^{S} \pm 3{\cdot}08$	$29{\cdot}50 \pm 1{\cdot}84$	$357{\cdot}71^{NS} \pm 3{\cdot}09$	$31{\cdot}80 \pm 0{\cdot}98$	$231{\cdot}40^{NS} \pm 2{\cdot}21$	$28{\cdot}43 \pm 1{\cdot}82$

Unit: s/15 min odour preference test.
SS, Scented slide; BS, blank slide (contains only the solvent).
*3 individuals per set and 3 trials for each set using the same individuals; hence values are means of means for 3 individuals; values for males and females compared using t-test.
^{S}, Statistically significant pairs ($P < 0{\cdot}05$); ^{NS}, Non significant pairs ($P > 0{\cdot}05$).
Compound I, 6,11-Dihydrodibenz-b,e-oxepin-11-one; compound II, 2,6,10-Dodecatrien-1-ol-3,7,11-trymethyl(Z); compound III, 1,2-Benzene-dicarboxylic acid butyl(2-ethylpropyl) ester.

Compound I found in the clitoral gland has not been reported in the male preputial glands of rats (Kannan *et al* 1998). The odour preference tests in the present study demonstrate that this compound acts as an attractant for the opposite sex. However, in the earlier study, two compounds identified in the male preputial gland, i.e. 2,6,10-dodecatrien-1-ol-3,7,11-trimethyl and di-*n*-octyl phthalate are reported to be involved as attractants for female rats (Kannan *et al* 1998). This results in the conclusion that a single volatile compound secreted by the female clitoral gland is involved in attracting the opposite sex, whereas two volatile compounds identified in the homogenate of the male preputial gland are necessary to bring about attraction of the opposite sex. Besides, each fraction has a unique extent of attraction of other individuals of the same species. This present observation gains support from reports related to pheromone identification studies in the mouse (Hurst 1990; Drickamer 1999), rat (Kannan *et al* 1998; Kannan and Archunan 1999), bobcat (Mattina *et al* 1991), horse (Ma and Klemn 1997), bovids (Ramesh Kumar *et al* 2000), white-tailed deer (Jemiolo *et al* 1995), tiger (Brahmachary 1996), elephant (Rasmussen *et al* 1997) and humans (Stern and McClintock 1998). These studies indicate that mammalian pheromone(s) may be a single compound or a mixture of compounds and that each of the major fractions is faithfully involved in conveying specific signals related to reproductive and social behaviours. For instance, Jemiolo *et al* (1985) have reported that mouse urine contains many volatile fractions. Of these, 2-sec-butyl-4,5-dihydrothiazole and dehydro-exo-brevicomin occur in greater proportion and are involved in the maintenance of the estrous cycle. Meanwhile, isobutyl amine and isoamyl amine in the urine of male mice accelerate puberty in female mice (Nishimura *et al* 1989). Thus the results of the present work lend support to the concept that females advertise their readiness for mating by liberating some kind of odourous substance.

The present findings are in agreement with the previous report of Gawienowski *et al* (1976) that the odour of the clitoral glands extract is attractive to male rats. Based on the compounds identified in the present study, we provide further evidence that the clitoral gland extract may also have other odorous substances, which may be involved in the attraction of both sexes. Gawienowski *et al* (1975) reported that female rats did not respond to the odour of female preputial extract and preferred the odour of normal male preputial extracts. It is to be noted however that they used the total extract of the female preputial gland for odour preference tests with females. Moreover, these authors identified the active compounds by gas liquid chromatography. But we have identified the major chemical constitutents of clitoral gland homogenates through GC-MS and have carried out the odour preference tests of the identified compounds individually. When the compounds are individually tested, the nature of the behaviour relevance of the compounds can be observed. Our results demonstrate that the clitoral gland is an important site for pheromone production to attract conspecifics.

In rat scent glands, pheromones exist as a mixture of alcohols, aldehydes, acids of saturated or unsaturated aliphatic or aromatic compounds (Dominic 1991; Gassett *et al* 1996; Kannan *et al* 1998; Kannan and Archunan 1999). Gawienoski and Orsulak (1973) reported that the attractive compounds of female preputial extracts include aliphatic alcohols having methyl-substituted 7 and 8 carbon atoms. The present study shows the identified compounds are aliphatic unsaturated alcohols (compound II) and aromatic acids (compound III) and demonstrates that the clitoral gland has three major components which appear to be involved in reproductive and social behaviours.

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