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COURTSHIP PHEROMONE–INDUCED c-Fos-LIKE IMMUNOLABELING IN THE FEMALE SALAMANDER BRAIN

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Abstract—Plethodontid salamanders display intricate courtship behaviors. Proteinaceous courtship pheromones were recently discovered in the submandibular (mental) gland of the male *Plethodon shermani*, the red-legged salamander. Behavioral studies showed that these male pheromones are delivered by direct contact to the female snout and modulate her receptivity during courtship. Previous reports demonstrated that experimental application of courtship pheromones activates vomeronasal sensory neurons in *P. shermani*. The present study investigated the CNS response to courtship pheromones in that species using immunocytochemical detection of the immediate-early gene product c-Fos. The results show that application of a male gland extract to females activated Fos-like immunolabeling in the extended vomeronasal amygdala of the accessory olfactory system, as well as in the preoptic area and ventromedial hypothalamus; regions of the brain known to mediate reproductive responses in vertebrates. The gland extract additionally activated Fos-like labeling in the raphe median, possibly indicating a serotonergic activation. Application of individual purified courtship pheromone proteins resulted in increases in Fos-like labeling in some of the regions activated by the complete submandibular gland extract, but the pattern of labeling was not as clear as that of the complete extract. Unlike other known vertebrate reproductive pheromones, courtship pheromones in *P. shermani* were effective only at a high concentration. This could result from the particular mode of pheromone transfer in that species, which involves sustained direct contact between male and female. It is concluded that salamander courtship pheromones exert their influence on behavior through the vomeronasal pathway and its direct projections to the preoptic and hypothalamic regions.

Key words: amphibians, pheromone, vomeronasal, immediate-early gene, preoptic area, hypothalamus.

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Abbreviations: AOB, accessory olfactory bulb; BSA, bovine serum albumin; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; PMF, plethodontid modulating factor; PRF, plethodontid receptivity factor; SPTA, striato-pallial transition area.

Mating behavior in plethodontid terrestrial salamanders involves the transfer of a spermatophore from the male to the female during courtship. Females can choose to walk out of courtship at any time. Females eventually use sperm from the spermatophores they accept for fertilization at a later time, weeks to months following the courtship season. Thus, courtship and fertilization are separate events. In *Plethodon shermani* (previously referred to as *P. jordani*; Highton and Peabody, 2000), the male uses a mixture of persistent approaches, nudging and 'persuasive' behaviors directed at a female (Arnold, 1976). A later stage of courtship behavior involves the male and female together in a tail-straddling walk of variable duration (up to ~90 min) until the pair stops while the male deposits a spermatophore. Additionally, the male slaps its mental gland repeatedly onto the female snout in the early stages of the tail-straddling walk. Spermatophore deposition lasts approximately 7 min at the end of which the male guides the female forward so that her cloaca lies above the spermatophore. Courtship terminates after the female inserts the sperm mass into her cloaca.

Secretions of the mental gland can decrease courtship duration when applied experimentally to the snouts of females paired with males that had their mental glands removed by surgical ablation (Houck et al., 1998), which was interpreted as an increase in female receptivity. Subsequently, different proteins with opposing effects on female receptivity were characterized: plethodontid receptivity factor (PRF) decreases courtship duration, whereas plethodontid modulating factor (PMF) increases courtship duration (Rollmann et al., 1999; Houck et al., 2007). Experimental application of courtship pheromone solutions mixed with the cation channel permeant molecule agmatine revealed that salamander vomeronasal sensory neurons are labeled by agmatine in greater numbers when pheromones are present compared with saline alone (Wirsig-Wiechmann et al., 2002, 2006; Schubert et al., 2006). Axons of vomeronasal sensory neurons reach the accessory olfactory bulb (AOB) directly, their only target (Schmidt et al., 1988). Projection neurons of the AOB then project to the extended vomeronasal amygdala (Schmidt and Roth, 1990; Laberge and Roth, 2005; Laberge et al., 2006), the latter of which displays more diverse connections, but among others it targets strongly the preoptic area and hypothalamus; brain centers known to mediate reproductive responses in vertebrates (Butler and Hodos, 1996; Swanson, 2000).

The immediate-early gene *c-fos* and its product c-Fos have been used extensively as indirect markers of neuronal activation (Morgan and Curran, 1991; Herdegen and

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Leah, 1998). Notably, this method has been used in rodents to map brain pathways activated by reproductive pheromones (Fernandez-Fewell and Meredith, 1994; Bressler and Baum, 1996; Swann et al., 2001). The c-Fos method has also been used to study brain responses in anuran amphibians on a few occasions (Ubink et al., 1997; Cobellis et al., 1999; Tonosaki et al., 2004; Yao et al., 2004; Calle et al., 2006; Noronha-de-Souza et al., 2006). We used immunocytochemistry against c-Fos to study the brain pathways activated by experimental application of courtship pheromones in the female salamander *Plethodon shermani*. It is to our knowledge the first time that this method has been used in a urodele amphibian.

EXPERIMENTAL PROCEDURES

Animals

A total of 72 adult female *Plethodon shermani* were collected during the courtship season in late August 2005 and 2006 from a single locality in Macon Co., NC, USA (35°10′48″ north, 83°33′38″ west). The females were pre-screened for their tendency to court with males before they were shipped to Bremen. Only females that readily courted with males were used in this study. Upon arrival in Bremen, the animals were held by groups of 10 in 80 l terrariums equipped with soil bedding, several hiding covers and water. They were fed once a week with crickets. The experimental procedures conformed to the guidelines of the veterinary office of the Ministry of Health of the state of Bremen, Germany. Care was taken to minimize the number of animals used and their suffering.

Preparation of pheromone stimuli

Male *P. shermani* were collected in parallel to the females mentioned above in order to obtain pheromone stimuli. These males were anesthetized in a mixture of 7% ether in water and each male's mental gland was excised and placed in a solution of 0.8 mM acetylcholine chloride in amphibian Ringer's solution for approximately 60 min. The gland solution was processed by centrifuging for 10 min (at 14,000×*g*), removing the supernatant and centrifuging the supernatant again for 10 min, then removing the supernatant and freezing at −80 °C. The frozen pheromone solution represented gland secretions pooled from approximately 120 males. The frozen gland extract was shipped to R.C.F. and P.W.F. at the University of Louisville, Louisville, KY, USA for processing to obtain the different test solutions. The purification and characterization of PRF and PMF pheromones by anion-exchange and gel filtration chromatography, at pH 8.0 and 7.4, respectively, have been previously described (Rollmann et al., 1999; Feldhoff et al., 1999; Houck et al., 2007). The use of a high resolution anion-exchange column permits full separation of the PMFs (which have an extremely high net negative charge) from the PRFs (e.g. Fig. 1 of Rollmann et al., 1999; Fig. 2 and Fig. 3 of Feldhoff et al., 1999). After gel filtration chromatography, the purity of each pheromone preparation was estimated to be ~99% by reverse phase-high pressure liquid chromatography and sodium dodecyl sulfate polyacrilamide gel electrophoresis. Briefly, pooled gland extracts were filtered (0.2-μm non-protein-binding filter), and then applied to a Mono-Q column (FPLC HR 5/5; Pharmacia, Piscataway, NJ, USA) equilibrated at 50 mM Tris–HCl, pH 8.0. The column was then eluted (same buffer) at 1 ml/min using a NaCl gradient (5.0 mM of NaCl/min). Enriched pheromone fractions were further purified by rechromatography on the Mono-Q column followed by gel filtration chromatography on a G75 Superfine column (1.6×15.5 cm; Pharmacia) previously equilibrated with 0.5× phosphate-buffered saline (PBS). Courtship pheromones were previously estimated to represent 85% of the whole mental gland extract protein content at a ratio of 2:1 PMF:PRF (Feldhoff et al., 1999). Note that PMF and PRF are 7 and 22 kDa, respectively. The protein content of the solutions was standardized to 2.0 μg/μl (whole mental gland extract), 0.7 μg/μl (or 3.2×10^{-6} M for PRF) and 0.5 μg/μl (or 7.1×10^{-6} M for PMF) in 0.5× PBS so that protein concentration was consistent for all trials and reflected the relative concentrations of PRF and PMF in the whole pheromone extract. These concentrations were selected because they elicited female behavioral or physiological responses in earlier studies (Houck et al., 1998; 2007; Rollmann et al., 1999; Wirsig-Wiechmann et al., 2002, 2006). The pheromone solutions were shipped to Bremen, aliquoted, frozen, and then thawed just before use.

Delivery of courtship pheromones

Each female was transferred to a 2-l plastic box for acclimatization at least 7 days before stimulus delivery took place. They were not fed during that period, but treated every day with a saline drop to the snout to accustom them to the process of stimulus delivery. The experiments took place in Sept.-Nov. 2005 and Nov. 2006 with all treatments represented equally in both years, except the bovine serum albumin (BSA) treatment, which took place only in 2006. On experimental day, a salamander received 10 drops of 5 μl of pheromone or control solutions (0.5× PBS vehicle or BSA) to the nasolabial grooves at 5 min interval. Different pheromone dilutions were used. After this 45 min stimulus delivery period there was a survival period of 120 min followed by quick anesthesia in 2% tricaine methanesulfonate and decapitation. Five animals received no treatment at all before kill. The brain was dissected out and put into 4% phosphate-buffered paraformaldehyde within 15 min. Brain tissue was then processed for Fos immunocytochemistry within the next 7–40 days. Preliminary experiments used survival periods between 90 and 360 min to study the time-course of Fos-like immunolabeling, and showed that the 120 min survival period was adequate.

Fos immunocytochemistry

The brains were embedded in 4.4% agar–agar and 40 μm-thick transverse sections were cut on a vibratome (VT 1000S, Leica Microsystems, Wetzlar, Germany) and transferred in phosphate buffer (PB, 0.08 M Na_2HPO_4, 0.02 M KH_2PO_4, pH 7.4). Free-floating sections were incubated in 0.5% hydrogen peroxide PB for 15 min, then washed 3×10 min in PB, incubated 60 min in 3% BSA PB followed by 60 min in 1.5% normal goat serum (NGS), 0.1% Triton X-100 PB. The sections were then incubated overnight at room temperature in a solution containing a 1:4000 dilution of the primary antibody rabbit anti-c-Fos (sc-253, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 3% BSA, 0.5% NGS, and 0.5% Triton X-100. The next day, sections were washed 3×10 min in PB before a 90 min incubation in a solution containing a 1:200 dilution of the biotinylated secondary antibody goat anti-rabbit IgG (Vectastain Rabbit PK-4001 kit, Vector Laboratories, Burlingame, CA, USA), 0.5% NGS, and 0.1% Triton X-100. The sections were washed again 3×10 min in PB before a 90 min incubation in the avidin–biotin–peroxidase complex (prepared according to instructions in PK-4001 kit, Vector Laboratories) diluted in 0.1% Triton X-100 PB, then washed again 3×10 min in PB, and mounted on gelatinized microscope slides. The antibody–peroxidase complex was visualized using diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as chromogen with heavy-metal intensification (Adams, 1981) in the presence of 0.0009% hydrogen peroxide. Sections were dehydrated in ascending ethanol concen-