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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-

trations, cleared in xylene, and coverslipped with Eukitt (Kindler O. & Co., Freiburg, Germany). Control immunocytochemical procedures were performed in parallel to the normal Fos assay on ~5% of the brain sections in selected animals by pre-incubation of the primary antibody solution for 4 h with the c-Fos blocking peptide (sc-253 P, Santa Cruz), or omission of the primary antibody. The possibility that the Fos antibody used here also recognizes other Fos-related antigens has been discussed elsewhere (Ubink et al., 1997). The photomicrographs presented were scanned with a digital camera (AxioCam HR, Carl Zeiss, Inc., Jena, Germany).

Cell counting

Fos-like immunoreactive cell nuclei were counted visually under light microscopy in brain sections encompassing the whole brain, bilaterally. This method proved best for three reasons: 1) plethodontid salamanders possess few neurons for vertebrates and very large cell nuclei due to their large genome (Roth et al., 1994, 1997) making visual cell counts a relatively easy task; 2) labeling of low contrast was of a lesser clarity on photomicrographs; 3) light microscopy allowed the possibility of adjusting the focal plane to insure the authenticity of the labeled nuclei. Some brain sections were missing, damaged, or used for immunocytochemical controls thus, some counting rules were followed: 1) missing/damaged half sections were attributed the value observed on the opposite side; 2) the number of labeled cells in a region that had one or more missing whole sections was calculated according to the formula x=total number of labeled cells divided by the number of brain sections multiplied by the average number of brain sections for a given region across animals. Only full diameter nuclei were counted in order to avoid double cell counts on adjacent sections. Still, two error types could not be avoided: 1) double cell counts on adjacent sections occurred when the cell nuclei were cut in their middle; 2) cell nuclei exactly superposed within a brain section could not be distinguished. Note that only two or three neurons could fit in the 40 μm-thick sections since mean neuron soma diameter lies between 13.3 and 25 μm in *Plethodon* (Dicke et al., 1998). These errors would respectively overestimate or underestimate the number of labeled cells, but are likely minor.

Nomenclature of the telencephalon is according to Laberge and Roth (2005) with the exception that the rostral part of the caudal amygdala (cA) is here identified as the vomeronasal amygdala. Nomenclature of the more caudal parts of the brain is adapted from Dicke and collaborators (1998) and Wicht and Himstedt (1988). The nucleus accumbens and ventral cellular prominence form a continuum in the subpallial telencephalon. Here, labeled cells in the region found just below the ventral cellular prominence were counted on seven transverse brain sections from its caudal beginning just rostral to the anterior commissure and further rostrally (280 μm in the rostrocaudal direction). Labeled cells in the nucleus accumbens were counted on all sections from the caudal end of the AOB up to three sections rostral to the most rostral section belonging to the ventral cellular prominence (up to 800 μm). Labeled cells in the migrated part of the medial pallium as well as in its ventral periventricular part were counted only on 10 caudal sections beginning at the level of the rostral preoptic area. In the extended vomeronasal amygdala (see Laberge et al., 2006), labeled cells were counted in the whole extent of the striatopallial transition area (SPTA, up to 840 μm), which is replaced caudally by the vomeronasal amygdala defined here as comprising four sections. The structure identified here as the caudal amygdala consists of three more sections caudally adjacent to the vomeronasal amygdala. All labeled cells in the thalamic eminence/ventral thalamus (up to 360 μm) and preoptic area (up to 640 μm) were counted. The latter region begins rostrally slightly rostral to the level of the anterior commissure and ends at the optic chiasm, where the rostral hypothalamus begins. All labeled cells in the hypothalamus were counted. A complete cellular bridge across the dorsal hypothalamus, an arbitrary feature, was chosen as the dividing boundary between the rostral hypothalamus (up to 640 μm) and its more caudal regions (up to 480 μm). The caudal hypothalamus was further divided into dorsolateral, dorsomedial and ventromedial regions since labeling was regularly divided into these three regions. Note that the ventromedial hypothalamus extends much further caudally as the dorsal regions (typically for 200 μm). Labeled cells were counted on the 15 most caudal sections of the torus semicircularis. Finally, labeled cells were counted on all available sections comprising the raphe median (up to 960 μm).

Statistics

Potential differences in the number of Fos-like immunoreactive cells across treatments were analyzed using Prism 4 (GraphPad Software, San Diego, CA, USA). The statistical procedure was as follows: 1) for each brain site selected, equality of variances was first tested on the raw data using the Bartlett test; if this test was not significant (α=0.05), an ANOVA was also performed on the raw data; 2) when the Bartlett test value proved significant, the data were square-root transformed (this procedure eliminated significant Bartlett test results in the five times it was needed out of 30 analyses) before the ANOVA was performed. In all cases, significant ANOVA results ($P<0.05$) were followed by the Tukey-Kramer post hoc test in order to establish which treatment(s) was responsible for the observed difference.

RESULTS

The immunocytochemical procedure used here labeled cell nuclei in many brain regions (Figs. 1 and 2). There was great inter-individual variability in the overall abundance of labeling, irrespective of treatment. Labeled cell nuclei displayed a variety of staining intensities and sizes, but could generally be easily distinguished from a homogeneous gray background staining of the cellular layer found throughout the brain. The immunocytochemical control procedures confirmed the specificity of the c-Fos-like immunolabeling: 1) pre-incubation of the Fos primary antibody solution with the c-Fos peptide abolished cellular labeling, while leaving the gray background staining of the cellular layer; 2) omission of the primary Fos antibody eliminated all labeling, background staining included. A number of brain regions of interest were chosen for Fos-like immunoreactive cell counting on the basis of their consistent labeling across animals (see Tables 1 and 2). A few notes about the extent of some brain regions must be mentioned here. Labeling in the ventral thalamus extended rostrally into the thalamic eminence, where cell nuclei were slightly larger. These two structures appear to form a continuum. The caudal torus semicircularis and dorsal tegmentum are hard to tell apart, and therefore are identified as TOR/TEG in Fig. 1J. Fos-like immunoreactive cells were also observed in small numbers in the AOB, medial septum and ventral cellular prominence, but there was clearly no difference between control and pheromone treatments in these regions (not shown). Fos-like immuno-

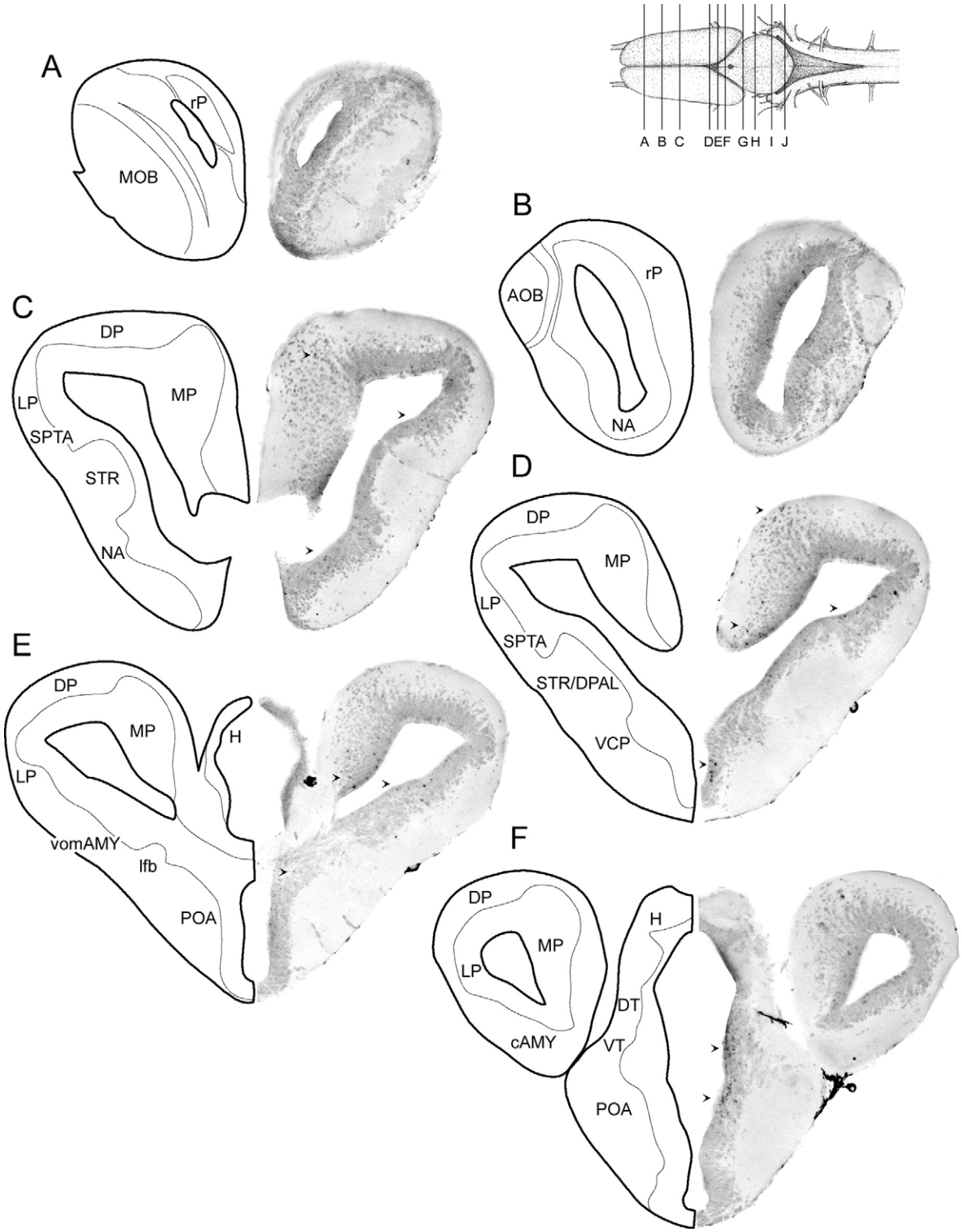

Fig. 1. Pattern of c-Fos-like immunolabeling observed after experimental application of courtship pheromones in the salamander. An example of Fos-like labeling is shown on the right side micrographs of transverse brain sections obtained from an animal treated with a high concentration of PRF. Arrowheads point to sites of labeling found in Tables 1 and 2. Brain structures are identified schematically on the left side. Scale bar=1 mm. Letters (A–J, from rostral to caudal) correspond to the level of transverse section illustrated on the schema of the dorsal view of the salamander brain.

reactive cells were sometimes seen in the following brain regions: main olfactory bulb, region below the AOB, striatum, rostral pallium, dorsal pallium, lateral pallium, dorsal thalamus, nucleus fasciculi longitudinalis medialis, tuber-

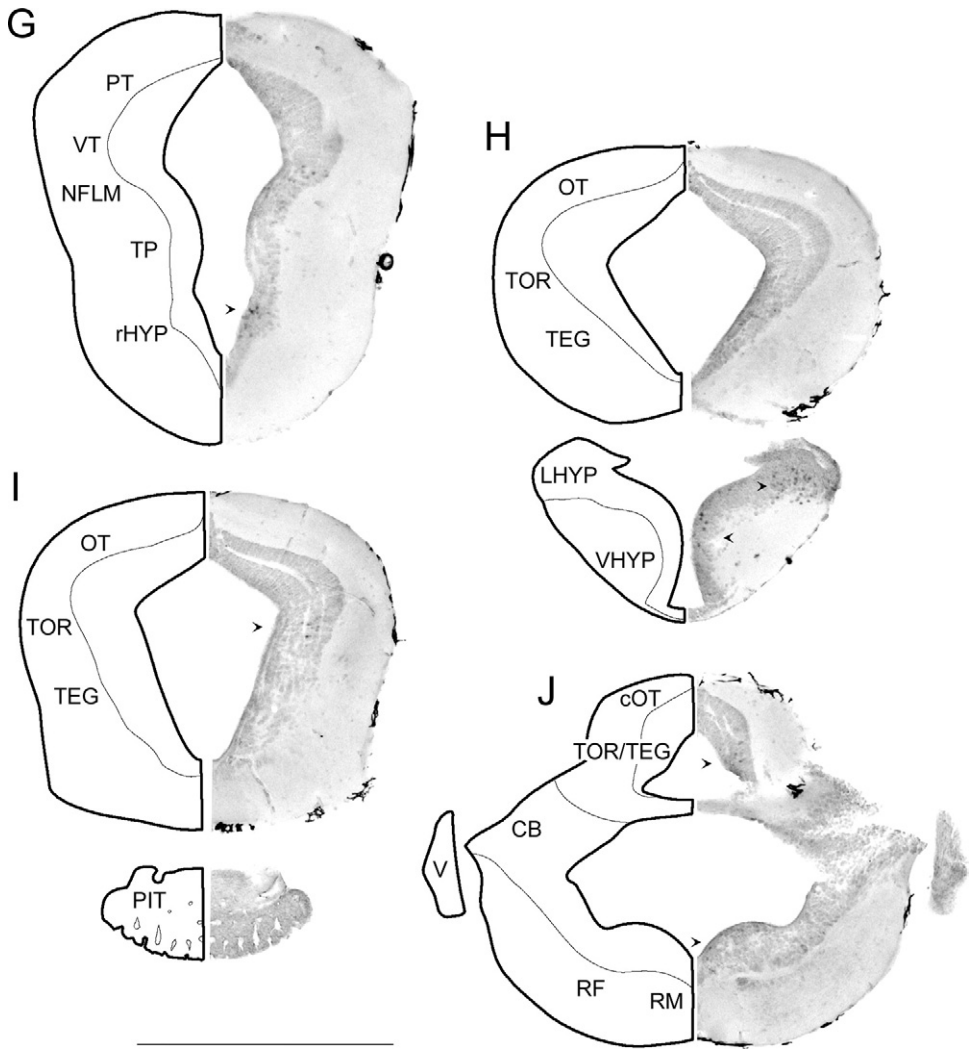

Fig. 1. *(Continued).*

Abbreviations used in the figures

ac	anterior commissure
AMY	amygdala
AOB	accessory olfactory bulb
c	caudal
CB	cerebellum
DP	dorsal pallium
DPAL	dorsal pallidum
DT	dorsal thalamus
H	habenula
HYP	hypothalamus
lfb	lateral forebrain bundle
LHYP	dorsolateral hypothalamus
LP	lateral pallium
MOB	main olfactory bulb
MP	medial pallium
NA	nucleus accumbens
NFLM	nucleus fasciculi longitudinalis medialis
OT	optic tectum
P	pallium
PIT	pituitary gland
POA	preoptic area
PT	ptetectum
RF	reticular formation
RM	raphe median
r	rostral
SPTA	striato-pallial transition area
STR	striatum
TEG	tegmentum
TOR	torus semicircularis
TP	tuberculum posterius
V	trigeminal nerve
VCP	ventral cellular prominence
VHYP	ventromedial hypothalamus
vom	vomeronasal
vomAMY	vomeronasal amygdala
VT	ventral thalamus

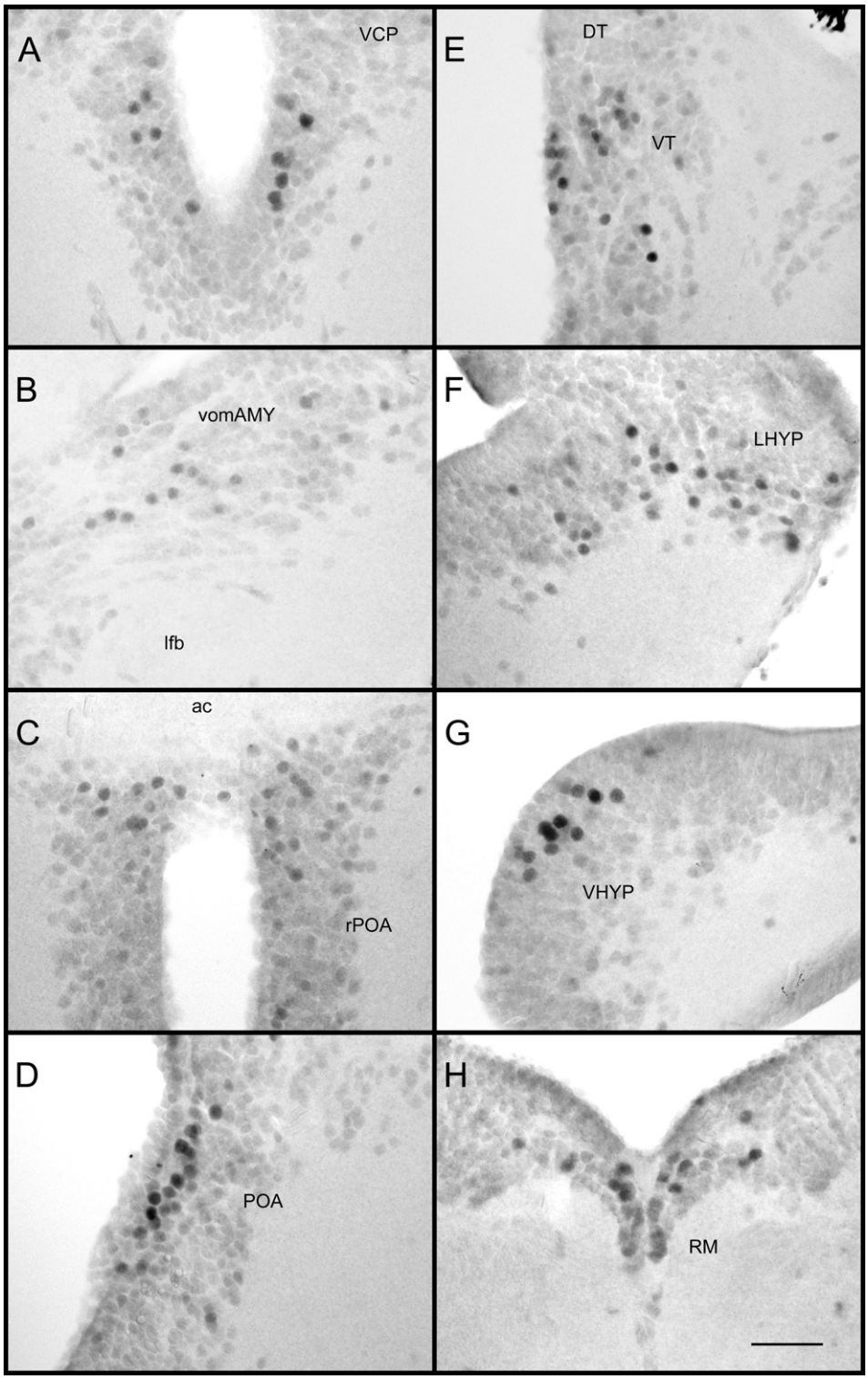

Fig. 2. Examples of Fos-like nuclear labeling at a high magnification. The micrographs are transverse brain sections taken from animals treated with a high concentration of PRF. The labeling shown is found below the ventral cellular prominence (A), in the vomeronasal amygdala (B), rostral preoptic area (C), dorsal magnocellular preoptic area (D), ventral thalamus (E), lateral hypothalamus (F), ventromedial hypothalamus (G), and median raphe (H). Scale bar=100 μm.

culum posterius, ventral tegmentum, nucleus cerebelli, and lateral part of the medulla oblongata. Cells were not counted in the latter brain regions because labeling was inconsistent across animals.

Table 1. Number of Fos-like immunoreactive cells in salamanders treated with the whole mental gland extract compared to controls

Brain region	No treatment (n=5)	Vehicle (n=7)	WE 1 μg/μl (n=7)	WE 2 μg/μl (n=3)	BSA 0.35 μg/μl (n=2)
NA	45.6±38.6	44.7±25.3	40.9±18.1	71.7±38.5	44.3±6.7
MPmig	14.4±2.4	18.9±9.6	22.1±9.5	17.7±2.3	18±4.2
MPv	56.4±16.9	62.3±18	76.4±25	85.3±27.1	81±24
SPTA*	19.8±12.8^{A}	16.9±8.2^{A}	35.6±8.6^{B}	26.7±4.5	15.5±2.1
vomAMY*	3.2±1.8^{A}	6.7±4.2^{A}	19±8.9^{B}	16.3±3.1	1±1.4
cAMY*	4.8±3.4^{A}	5.29±4.15^{A}	13.9±4.8^{B}	9.7±2.1	1±0
Below VCP	19.4±19.1	23.1±12.4	29.7±6.3	36±7	35.5±2.1
TE/VT	80.6±51	89.1±47.7	130.6±33.8	130±44	97±28.3
POA*	10.6±10.8^{A}	17.4±12.8^{A}	48.6±25.3^{B}	86.3±81.7	13.5±7.8
rHYP*	6.6±7.8^{A}	15.4±5.7^{AB}	21.4±7.4^{B}	35±8.7	17.5±6.4
LHYP	62.6±51.1	63.9±41.3	90.6±30.8	116±30.4	79.5±5
MHYP*	4.6±4.2^{A}	6±1.9^{AB}	10.9±5.2^{B}	14.3±6.1	9.6±10.5
VHYP*	5.2±5.8^{A}	13.3±8.9^{A}	37.2±15.1^{B}	35±4.6	25±4.2
TOR	118.6±18.3	114.9±33.5	111.7±32.9	75.3±20.4	182±45.3
RM*	9.8±15.6	12.9±14.7	37.6±26.3	37.7±15	12±8.5

The uppercase letters display the groups resulting from the Tukey-Kramer post hoc test, except in the case of RM, where the post hoc test showed no significant difference between groups. The statistical analysis did not include the WE 2 μg/μl and BSA animals. Values are mean±standard deviation. AMY: amygdala; Below VCP: region below the ventral cellular prominence; c: caudal; LHYP: laterodorsal hypothalamus; MHYP: mediodorsal hypothalamus; mig: migrated part; MP: medial pallium; NA: nucleus accumbens; POA: preoptic area; rHYP: rostral hypothalamus; RM: raphe median; TE: thalamic eminence; TOR: torus semicircularis; v: ventral; VHYP: ventromedial hypothalamus; vom: vomeronasal; VT: ventral thalamus; WE: whole mental gland extract.

* One-way ANOVA significant (P<0.05).

Effect of the whole mental gland extract on Fos-like labeling in the female brain

Statistical analysis showed that the number of Fos-like immunoreactive cells was higher in select brain regions after application of a mental gland extract (1 μg/μl for a total of 50 μg extract) to the female's snout, namely, the SPTA, vomeronasal amygdala, caudal amygdala, preoptic area and ventromedial hypothalamus (Table 1). On the other hand, despite a significant increase in labeled cells between untreated animals and animals that received the mental gland extract in the rostral and dorsomedial hypothalamus, the difference was not significant when the pheromone-treated animals were compared with vehicle-treated animals as revealed by the post hoc test. In the case of the raphe median, a significant effect was detected by the ANOVA test, but surprisingly not by the post hoc test. Since the number of Fos-like immunoreactive cells in the raphe median is clearly higher in animals treated with the mental gland extract (Table 1), it is concluded that this treatment increased Fos-like labeling. In support of the latter conclusion, the number of Fos-like immunoreactive cells

Table 2. Number of Fos-like immunoreactive cells in salamanders treated with purified mental gland pheromones compared to controls

Brain region	Vehicle (n=10)	PRF 0.07 μg/μl (n=5)	PRF 0.35 μg/μl (n=5)	PMF 0.05 μg/μl (n=5)	PMF 0.25 μg/μl (n=5)
NA	54.4±26.2	44.2±15.5	50±19.7	50.8±14.9	63±37.7
MPmig	22.4±5.1	17.8±5.4	19.8±4.9	16.6±6.4	22.2±7
MPv	98.4±37.4	72.2±17.1	70.2±23.3	88.8±21.4	84±29.8
SPTA*	25.4±12.2^{A}	25±7^{AB}	42.4±18.9^{AB}	23.2±10.8^{A}	53.8±26.8^{B}
vomAMY*	9.7±6.4^{A}	12.8±7.1^{AB}	31±13.5^{B}	9.8±14.3^{A}	26.2±12.3^{AB}
cAMY	7.1±6.8	6.4±3.7	13.6±7.2	7.8±6.1	14.6±8.4
Below VCP	34.8±9.6	19.7±7.3	31.4±8.2	28.2±12.7	31.8±3.4
TE/VT	111±45.2	80.8±24	131.6±38.2	74.4±31.6	115±25.3
POA*	36.8±34.6^{AB}	16.8±8.6^{A}	88.2±52.9^{B}	31±29.5^{AB}	75.6±34.2^{B}
rHYP	30.9±17.6	15.4±7.5	36.4±17	22±8.1	23.6±11.1
LHYP	67.9±33	53.4±17.4	87.4±23.5	54.2±49.3	88.6±30
MHYP	14.1±8.7	10±12	41.4±46.3	18±15.3	35.6±25.8
VHYP*	19.92±13.5^{A}	23.2±9.2^{A}	36.6±15.4^{AB}	33.2±24.9^{AB}	56.4±24.4^{B}
TOR	110.5±18.5	129±32.7	108.8±29	130.2±38.9	103.4±7.8
RM*	23.2±17.1^{AB}	8.8±12.7^{AB}	25.5±15.1^{AB}	3.2±4.1^{A}	37.7±29.2^{B}

The uppercase letters display the groups resulting from the Tukey-Kramer post hoc test. Values are mean±standard deviation. AMY: amygdala; Below VCP: region below the ventral cellular prominence; c: caudal; LHYP: laterodorsal hypothalamus; MHYP: mediodorsal hypothalamus; mig: migrated part; MP: medial pallium; NA: nucleus accumbens; POA: preoptic area; rHYP: rostral hypothalamus; RM: raphe median; TE: thalamic eminence; TOR: torus semicircularis; v: ventral; VHYP: ventromedial hypothalamus; vom: vomeronasal; VT: ventral thalamus.

* One-way ANOVA significant (P<0.05).

observed in the three animals treated with the highest concentration of mental gland extract (2 μg/μl) was also high (Table 1). The sample size with the latter treatment was not large enough to perform statistics, but it proved useful to support the increases in Fos-like labeled cells observed when the 1 μg/μl solution was used, and further suggest that the mental gland extract might also be effective in stimulating Fos-like labeling in the rostral and dorsomedial hypothalamus.

The number of Fos-like immunoreactive cells was not different between the group that received no treatment and the group that received a vehicle saline solution. Application of BSA (0.35 μg/μl), a protein likely not detected by the olfactory system, in two animals produced a pattern of Fos-like labeling highly similar to the pattern seen in untreated or vehicle-treated animals. The same was true of animals treated with 0.2 μg/μl ($n=2$) and 0.02 μg/μl ($n=2$) of the mental gland extract (not shown).

Effect of purified courtship pheromones on Fos-like labeling in the female brain

Statistical analysis showed that after treatment with 0.35 μg/μl PRF (17.5 μg total) the number of Fos-like immunoreactive cells was increased in the vomeronasal amygdala compared with vehicle controls (Table 2). After treatment with 0.25 μg/μl PMF (12.5 μg total), the number of Fos-like immunoreactive cells was greater in the SPTA and ventromedial hypothalamus compared with vehicle controls. Significant effects detected by the ANOVA test in the preoptic area and raphe median resulted from low numbers of Fos-like immunoreactive cells with the 0.07 μg/μl PRF (3.5 μg total) and 0.05 μg/μl PMF (2.5 μg total) treatments, respectively, and not from differences between vehicle controls and a pheromone treatment. Despite the lack of statistical significance, large differences in the number of labeled cells could sometimes be observed between controls and treatments with high concentrations of purified pheromones (e.g. preoptic area). It is important to note here that the sample size was only five for this experiment, compared with seven for the experiment using the mental gland extract.

At the concentrations used in the current study, it was not possible to distinguish between the response patterns to the whole mental gland extract and individual pheromone components. A clear distinction between responses to PRF and PMF was also not obvious.

DISCUSSION

The present study found increased Fos-like labeling in the extended vomeronasal amygdala, preoptic area, ventromedial hypothalamus, and median raphe of the female salamander brain after experimental application of male courtship pheromones. Vehicle application showed no effect on Fos-like labeling when compared with a group that received no treatment. It was additionally found that dilution of the pheromone solutions abolished all responses.

Methodological considerations

Methods for the preparation of highly purified *P. shermani* pheromones were developed several years ago in order to be able to test individual components for biological activity and to determine amino acid sequences (Rollmann et al., 1999; Feldhoff et al., 1999; Houck et al., 2007). In the whole mental gland extract, the stoichiometric ratio of PMF:PRF is about 2:1 and this is reflected in the concentrations employed in the current study and earlier work used to determine biological activity (Rollmann et al., 1999; Houck et al., 2007).

The Fos-like immunoreactive cell counts presented here include the whole brain (corrected for missing sections), and thus, the difference between pheromone and control treatments provides an approximation of the total number of neurons activated by courtship pheromones in *P. shermani*, on the assumption that all pheromone-responsive neurons express the Fos-like protein. This latter assumption may not be appropriate at least for the AOB since neurons in that structure, an obligatory relay station between the vomeronasal sensory neurons and the extended vomeronasal amygdala, displayed a very low Fos-like labeling that was not affected by pheromone treatment. This observation came as a surprise since AOB neurons in rodents show increased Fos expression after treatment with reproductive pheromones (Fernandez-Fewell and Meredith, 1994; Bressler and Baum, 1996). Activated AOB neurons might express different transcription factors than the homologue of c-Fos in salamander. Also, the present study could not have highlighted brain regions that showed decreased activity, which could play a role in the effects of pheromones, if they did not display basal Fos-like labeling. In the future, we will use alternative functional neuroanatomical methods and electrophysiology to investigate the response of AOB neurons to courtship pheromones in *P. shermani*.

Functional organization of the female reproductive brain

In the present study, Fos-like labeling induced by courtship pheromones was observed in the extended vomeronasal amygdala, secondary prosencephalon, i.e. preoptic area and hypothalamus, and median raphe. These results combined with previous neuroanatomical work (Laberge and Roth, 2005) suggest strongly that a direct pathway between the vomeronasal system and the secondary prosencephalon mediates the effects of pheromones on courtship in *P. shermani*. The results cannot however rule against the involvement of local projections between the preoptic area and ventromedial hypothalamus as being involved to some extent. The existence of a direct pathway between the amygdala and the reproductive centers of the secondary prosencephalon was recently demonstrated in the mouse (Choi et al., 2005).

Swanson (2000) proposed that a medial longitudinal zone of the mammalian secondary prosencephalon forms a behavioral control column critical for social behaviors,

with distinct interconnected networks within this zone dedicated to reproductive and defensive behaviors. Brain regions displaying increased Fos expression following mating in female mammals (rodents, sheep, monkey) show some similarities with the regions found in salamander, i.e. vomeronasal amygdala, preoptic area and ventromedial hypothalamus (Flanagan-Cato and McEwen, 1995; Polston and Erskine, 1995; Michael et al., 2005; Gelez and Fabre-Nys, 2006). Male pheromones are sufficient to induce Fos responses in the vomeronasal pathway of rodents, with prior sexual experience increasing responses in central regions (Halem et al., 1999; Hosokawa and Chiba, 2007). In the ferret, male body odors also increase Fos expression in the medial amygdala, preoptic area and ventromedial hypothalamus of females, but this effect is mediated by the main olfactory bulb (Kelliher et al., 1998). The present results in salamander suggest that the amygdala and a medial zone of the secondary prosencephalon have conserved functions in reproductive behavior across vertebrates, at least in the female brain. Future studies should investigate responses in the male brain, since courtship pheromones have been shown to activate vomeronasal sensory neurons in male *P. shermani* (Schubert et al., 2006).

Relevant functional studies in amphibians are few. Interestingly, mating calls modulate the expression of the immediate-early gene egr-1 in the túngara frog suprachiasmatic (rostral) hypothalamus and posterior tuberculum, but not in the preoptic area or medial hypothalamus (Hoke et al., 2005). A previous study that implicated the preoptic area in acoustic aspects of anuran mating behavior suggests that immediate-early gene labeling cannot alone reveal the complete functional organization of mating pathways (Schmidt, 1989). More studies are needed in order to establish a framework of the functional subdivisions of the amphibian reproductive brain.

Possible role of raphe activation

The position of Fos-like immunoreactive cells in the raphe median of the medulla oblongata corresponds closely to the position of raphe serotonergic neurons in *Plethodon*, but Fos-like labeling and 5-HT localizations are distinct in the hypothalamus (see Dicke et al., 1997). The salamander raphe median displays a large number of serotonergic neurons known to make up the 5-HT ascending neurotransmitter system (Halliday et al., 1995; Dicke et al., 1997). In *P. shermani*, pheromone-activated brain regions that could project to the raphe median are restricted to the preoptic area and ventromedial hypothalamus (see Dicke et al., 1998). Therefore, it is proposed that salamander courtship pheromones activate the raphe median through a connection with the secondary prosencephalon. The raphe, in turn, could modulate pheromone perception through its widespread ascending projections, probably serotonergic in nature. Interestingly, 5-HT has been suggested to improve pheromone detection both in lepidopteran insects and rat (Kloppenburg et al., 1999; Inamura and Kashiwayanagi, 2000; Gatellier et al., 2004). It would be of interest to investigate the effects of drugs that act on the serotonergic system on courtship in the salamander.

Courtship pheromones are not effective at low concentration

Vertebrate reproductive pheromones can normally be detected by sensory neurons at extremely low concentrations with response thresholds in the picomolar to nanomolar range (Holy et al., 2000; Leinders-Zufall et al., 2000; Toyoda and Kikuyama, 2000; Stacey and Sorensen, 2005). The present study showed that dilution of courtship pheromone solutions by a factor of 5 (still at micromolar concentrations) induced no Fos-like labeling. It is thus reasonable to believe that courtship pheromones do not stimulate the vomeronasal system at low concentrations in *P. shermani*. Since our purified PRF and PMF solutions contained a lesser amount of protein compared with the gland extract, they could have been comparatively less effective due to a low concentration. These two proteins could be acting synergistically on Fos-like activity in some brain regions. Alternatively, a smaller sample size in the experiment testing PRF and PMF could explain the more restricted pattern of statistically significant effects that was observed in comparison with the test of the gland extract. Future functional experiments of courtship pheromones will need to test undiluted secretions or their equivalent content in purified proteins at the amount they occur in male mental glands naturally. Courtship pheromone transfer from male to female involves sustained physical contacts in plethodontid salamanders. Therefore, it appears that these animals had no need to evolve extremely sensitive sensory neurons for detection of diluted pheromone signals as is found in other vertebrates.

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