

TABLE 1. Sequences of oligonucleotide primers used for the real-time PCR analyses.

Gene	Primer ^a
<i>Gapdh</i>	F: 5'-AAATGGTGAAGGTCGGTGTG-3' R: 5'-TGAAGGGTCGTTGATGG-3'
<i>Cyp19a1</i>	F: 5'-GCCCAATGAATTTACCCTCGAA-3' R: 5'-AAGCCAAAAGGCTGAAAGTACCT-3'
<i>Cyp11a1</i>	F: 5'-TCGACTCCTCAGAACTAAGACCTG-3' R: 5'-GTACCCTGGTGTCTTTATAGCCT-3'
<i>Nr5a1</i>	F: 5'-CCTGGGCTGGCTACCTCTATC-3' R: 5'-CGAACTAGAGCCAGAGGAGGAC-3'
<i>Esr1</i>	F: 5'-GCACAGGATGCTAGCCTTGTCTC-3' R: 5'-AATTGTCACCAGCTTGCAGGTTTC-3'
<i>Ar</i>	F: 5'-GGCGGTCCTTCACTAATGTCAACT-3' R: 5'-CTGACTGTGCATGCGGTACTCAT-3'
<i>Esrrg</i>	F: 5'-CCGAGAGTTGGTGGTTATCATTTGG-3' R: 5'-GGAAGACCCTCGCCGTGC-3'

^a F, forward; R, reverse.

with 5% fetal bovine serum and plated out on four-well glass slides (BD Falcon). After several days, cells were fixed in methanol and processed for immunocytochemical analysis.

Immunocytochemical Staining

The sections were first incubated for 15 min in 0.01 M PBS. After inhibition of endogenous peroxidases (10 min in 0.6% H₂O₂ diluted in 0.01 M PBS plus 0.2% Triton X-100 [PBST]) and saturation (2 h in a 5% normal goat serum solution), sections were incubated overnight at 4°C in a polyclonal affinity-purified antiaromatase antibody or estrogen-related receptor gamma (ESRRG) antibody raised in rabbit against quail recombinant aromatase or ESRRG diluted 1:500 in 0.01 M PBST. The next day, the sections were immersed for 2 h at room temperature in a biotin-conjugated goat anti-rabbit immunoglobulin G (DakoCytomation, Inc.) diluted 1:400 in PBST and then for 2 h in a streptavidin-fluorescein complex (Rhodamine; DakoCytomation, Inc.) diluted 1:50 in PBST. Between each step, sections were extensively rinsed in PBST. The sections were mounted onto microscope slides, coverslipped with a gelatin-based mounting medium, and stored in the dark at 4°C. For double-labeling immunofluorescence, Alexa Fluor 488- or 594-conjugated secondary antibodies were used. Rabbit polyclonal anti-aromatase antibody was kindly provided by Prof. Nobuhiro Harada (Department of Biochemistry, Fujita Health University School of Medicine, Aichi, Japan) [22]. The rabbit polyclonal anti-ESRRG antibody used in the present study was established and characterized as

previously reported [23]. The mouse monoclonal anti-Ran antibody (Santa Cruz Biotechnology, Inc.) was used to detect nucleus in cells. Ran, also called TC4, is the small RAS-related protein that is localized in the nucleus.

Statistical Analysis

Results are expressed as the mean ± SD. Differences among the three groups were determined using Student *t*-test with Dunnett multiple comparison. A value of *P* < 0.05 was considered to be statistically significant.

RESULTS

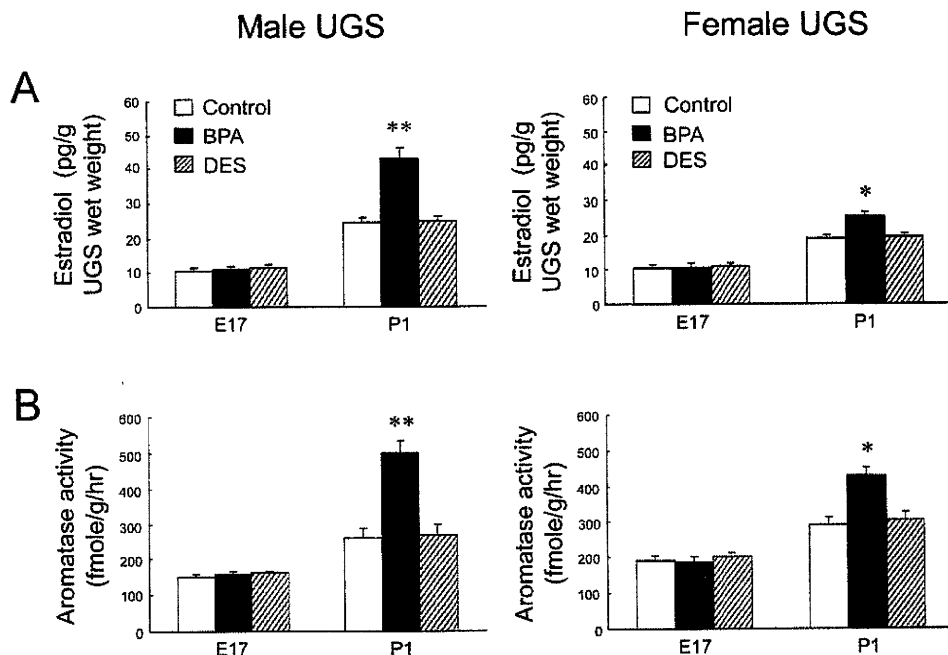
BPA-Specific Increases of E₂ Levels and CYP19A1 (Aromatase) Activity in Mouse UGS

The pregnant mice were exposed to low-dose BPA during the onset of prostatic budding (E13–E16), and the UGS of fetuses were collected during bud elongation (E17–P1). In analyses of in situ sex steroid hormonal environment, E₂ levels and CYP19A1 (aromatase) activity were significantly increased only at P1 in BPA-treated UGS, not at P1 in the DES-treated UGS (Fig. 1). At E17 and P1, both the E₂ levels and CYP19A1 (aromatase) activity in untreated male UGS were not significantly different compared with those in untreated female UGS.

BPA-Specific Up-Regulation of Steroidogenic Enzyme and Sex-Determining Gene mRNA in Mouse UGS

To investigate the BPA-specific gene alterations related to increases of the E₂ levels and aromatase activity, we performed preliminary GeneChip analysis with the Percellome method in the BPA- or DES-treated male UGS at E17 and P1. The results showed BPA-specific mRNA up-regulation of steroidogenic enzymes, such as *Cyp11a1*, *Cyp11b1*, and *Cyp17a1*, and sex-determining factors, such as *Nr5a1*, *Nr0b1*, *Gata4*, and *Amhr2* (data not shown). Furthermore, quantitative PCR analysis confirmed the mRNA up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* only in the BPA-treated neonatal (P0 and P1) UGS, not in the DES-treated neonatal UGS (Fig. 2). No difference in mRNA expression levels was found between E17 and P1 when comparing the untreated male UGS to that of the female. In

FIG. 1. BPA-specific increases of E₂ levels and CYP19A1 (aromatase) activity in mouse UGS. E₂ levels (A) and CYP19A1 (aromatase) activity (B) were measured in the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. **P* < 0.01, ***P* < 0.001 vs. control.



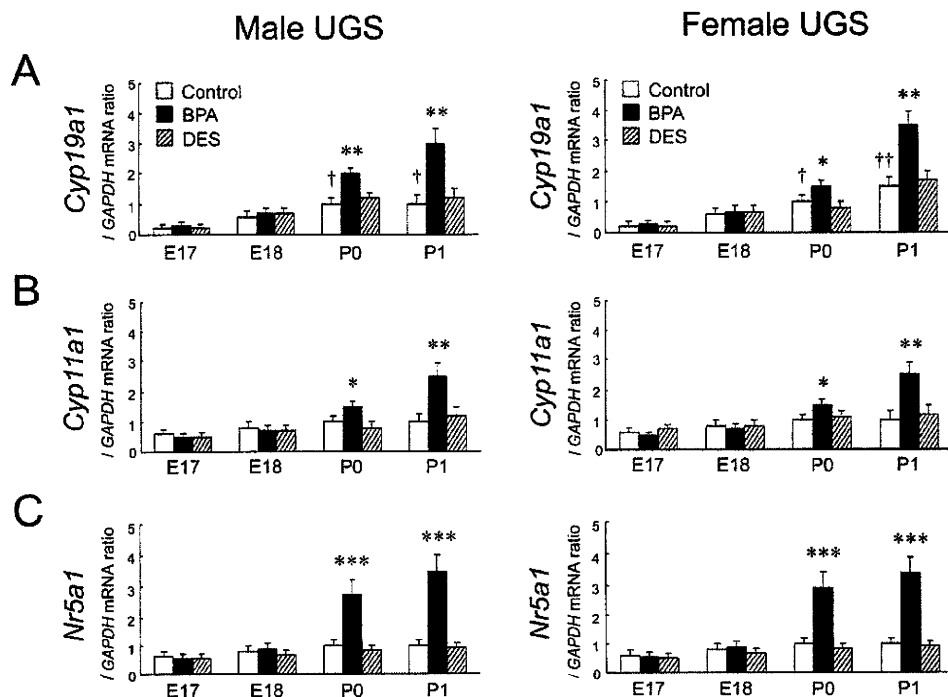


FIG. 2. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in mouse UGS. The relative mRNA expressions of *Cyp19a1* (A), *Cyp11a1* (B), and *Nr5a1* (C) were determined in the untreated control (open bar), BPA-treated UGA (closed bar), and DES-treated UGS (slashed bar) between E17 and P1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control at each time point; † $P < 0.01$, †† $P < 0.001$ vs. control at E17.

untreated male and female UGS, the mRNA of *Cyp19a1* was gradually increased between E17 and P1.

Restricted BPA-Specific Up-Regulation of Steroidogenic Enzyme and Sex-Determining Gene mRNA in UGE and UGM

In male fetuses at P1, it was not feasible to separate UGE and UGM components within the male UGS because of the formation of prostatic buds. In the female at P1, the up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA was observed only in

UGM, not in UGE, of the BPA-treated group (Fig. 3). In both male and female UGE, expressions of such mRNAs were quite low and not up-regulated, even in the BPA-treated group. At E17, no difference in mRNA expression levels was found when comparing the untreated male UGM with that of the female.

BPA-Specific Increases of Aromatase-Expressing Cells in Primary Cultured UGM

In both the male and female, P1 UGM was primary cultured in vitro. Representative pictures of aromatase-positive cells are shown in Figure 4, A–C. The aromatase-positive staining was

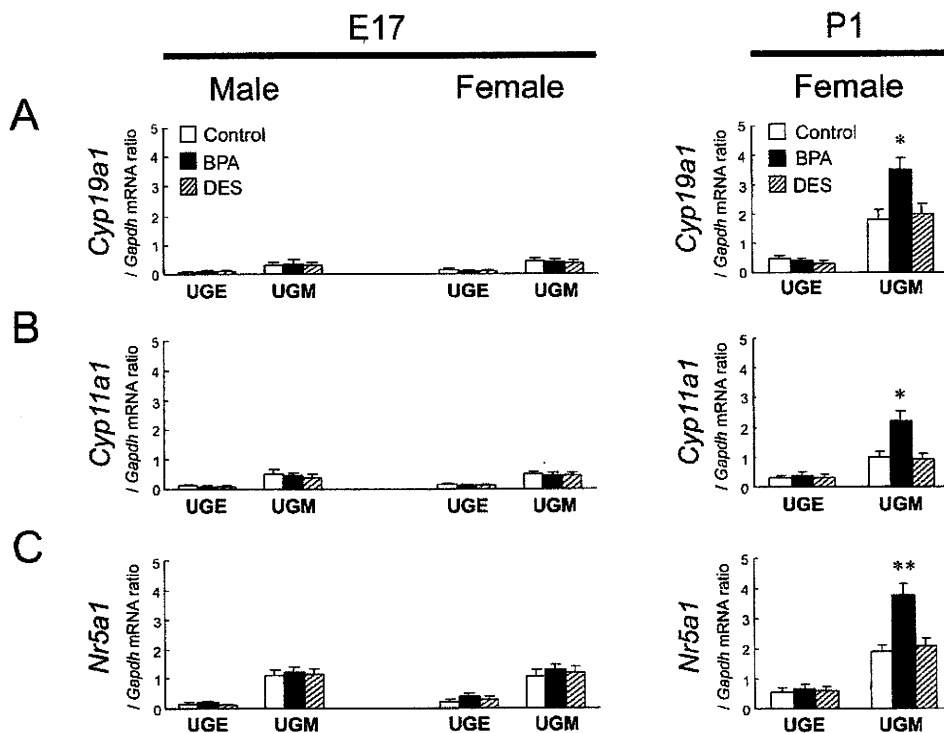
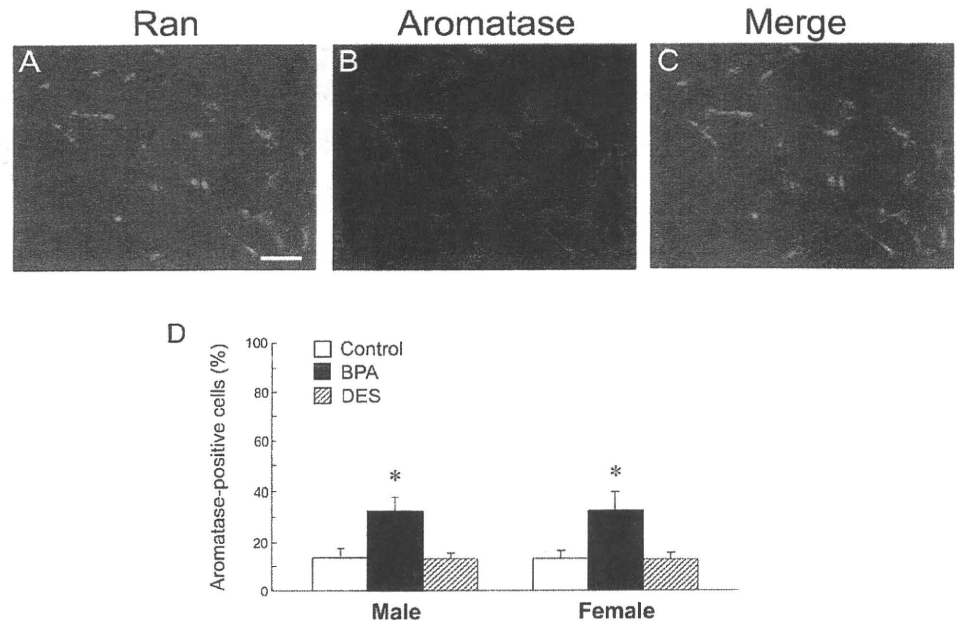


FIG. 3. Restricted BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in UGE and UGM. The relative mRNA expressions of *Cyp19a1* (A), *Cyp11a1* (B), and *Nr5a1* (C) were determined for UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. * $P < 0.01$, ** $P < 0.001$ vs. control.

FIG. 4. BPA-specific increases of aromatase-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the CYP19A1 (aromatase) protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μ m, magnification \times 400. **D**) The number of aromatase-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar), and the percentage of aromatase-positive cells was calculated from at least 10 areas. * $P < 0.01$ vs. control.



observed in the cytoplasm of cultured UGM. The rate of positivity (i.e., the percentage of cells that expressed CYP19A1 [aromatase] protein), was approximately 10% in the untreated and the DES-treated groups, whereas it was as high as approximately 30% in the BPA-treated group (Fig. 4D). No difference in the rate of positivity of CYP19A1 (aromatase) was found when comparing the untreated male UGM to that of the female.

Restricted BPA-Specific Up-Regulation of *Esrrg* mRNA in UGE and UGM

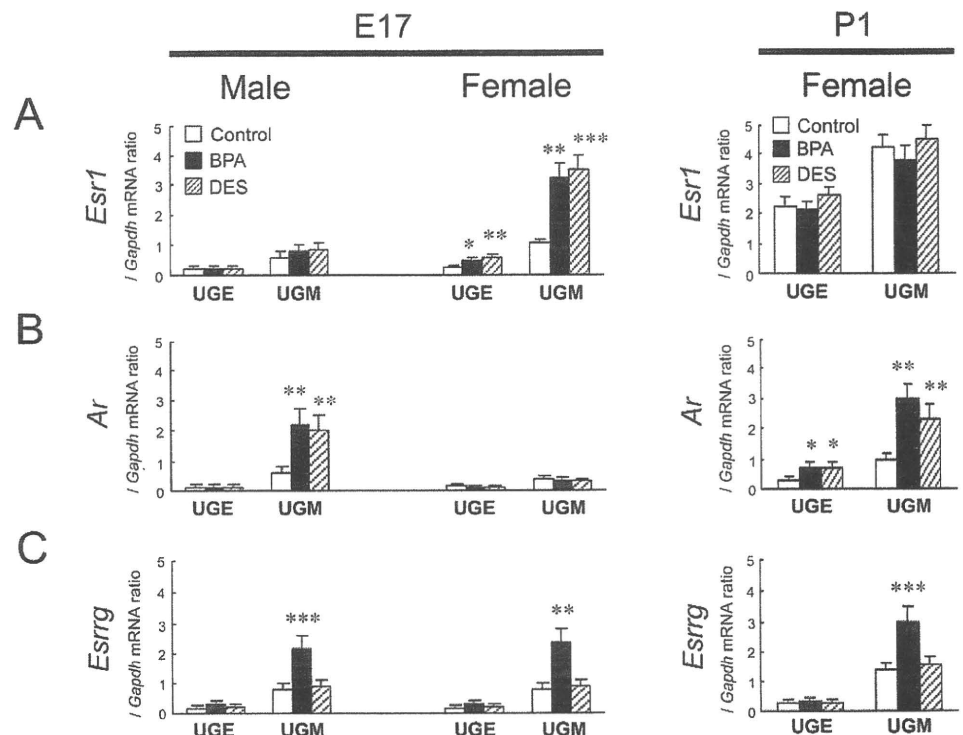
In E17 female UGM, the mRNA expression of *Esr1* was up-regulated by both BPA and DES treatment (Fig. 5A). At E17, however, the mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the male UGS (Fig. 5B). At

P1, mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the female UGS (Fig. 5B). In both the male and female, the up-regulation of *Esrrg* mRNA was observed at E17 and restricted in UGM, but not in UGE, of the BPA-treated group (Fig. 5C). In both the male and female UGE, the expression of *Esrrg* mRNA was quite low and not up-regulated, even in the BPA-treated group. At E17, no difference in mRNA expression levels was found when comparing the untreated male UGS with that of the female.

BPA-Specific Increases of *ESRRG*-Expressing Cells in Primary Cultured UGM

In both the male and female, E17 UGM was primary cultured in vitro. Representative pictures of *ESRRG*-positive

FIG. 5. Restricted BPA-specific up-regulation of *Esrrg* mRNA in UGE and UGM. The relative mRNA expressions of *Esr1* (A), *Ar* (B), and *Esrrg* (C) were determined in UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.



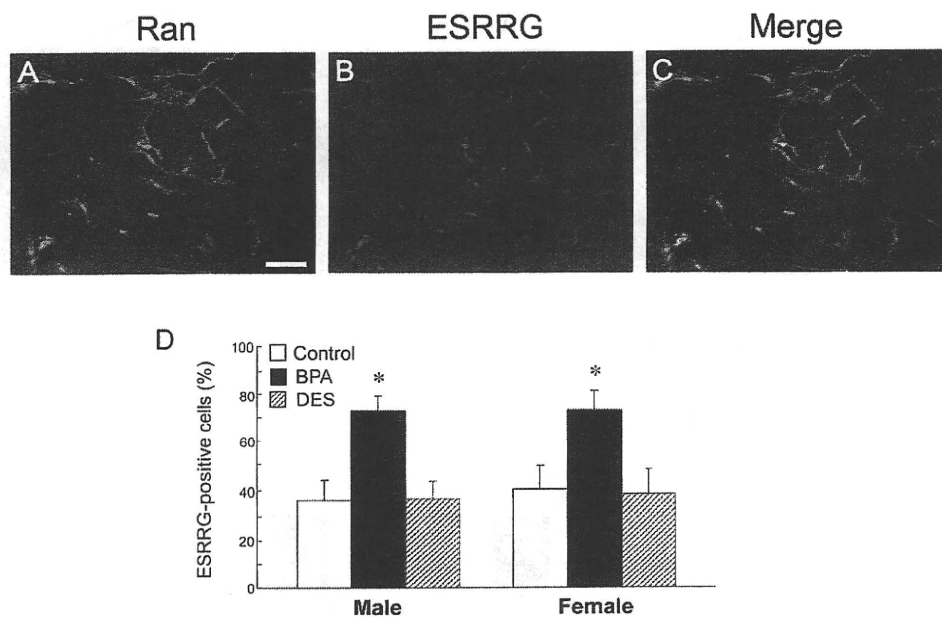


FIG. 6. BPA-specific increases of ESRRG-expressing cells in primary cultured UGM. A–C) Fluorescence signals were detected for the ESRRG protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μ m, magnification \times 400. D) The number of ESRRG-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar), and the percentage of ESRRG-positive cells was calculated from at least 10 areas. * $P < 0.01$ vs. control.

cells are shown in Figure 6, A–C. The ESRRG-positive staining was observed in both the nucleus and the cytoplasm of cultured UGM. The number of ESRRG-positive UGM was significantly increased only in the BPA-treated group and showed a 2.2-fold increase in males and a 1.6-fold increase in females (Fig. 6D). No difference was found in the rate of positivity of ESRRG when comparing the untreated male UGM with that of the female.

BPA-Specific Up-Regulation of *Esrrg* and Steroidogenic Enzyme mRNA in Sex Hormone-Related Organs

To investigate the BPA-specific up-regulation of in situ steroidogenesis in other organs, we first examined the changes in *Esrrg* mRNA expression in sex hormone-related organs, such as the cerebellum, heart, kidney, ovary, and testis. At P1, the mRNA expression of *Esr1* in the cerebellum, heart, kidney, and ovary, but not in the testis, was up-regulated by both BPA and DES treatment (Fig. 7A). However, no significant difference in *Ar* mRNA expression was observed in all organs examined (Fig. 7B). In the untreated group, the mRNA expression of *Esrrg* was not detected in the testis at E17 and P1 (Fig. 7C). The up-regulation of *Esrrg* mRNA was observed at E17 and restricted to the cerebellum, heart, kidney, and ovary (Fig. 7C). The BPA-specific up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA was observed only at P1 in the cerebellum, heart, kidney, and ovary, but not in the testis (Fig. 8).

DISCUSSION

Concern about the effects of EDCs such as BPA on human health has been increasing [24]. Although the majority of EDCs have the potential to alter functioning of the reproductive and endocrine system, the actual mechanism responsible for such alterations has not been identified thoroughly. BPA is of concern because its chemical structure resembles that of DES. Several studies have reported that BPA can mimic estrogen action, such as induction of vaginal cornification, uterine vascular permeability, growth and differentiation of the mammary gland, and synaptic plasticity in the hippocampus [25–28]. In the prostate, alterations in normal development can

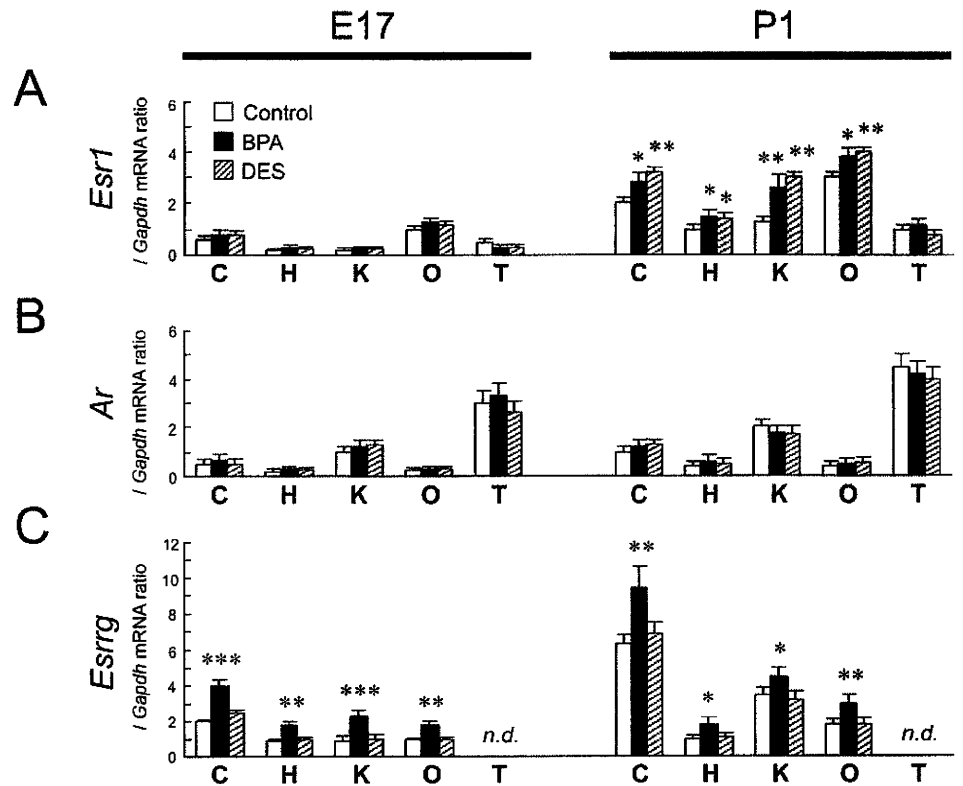
produce permanent changes that persist throughout adulthood and may increase the risk of disease in later life [9]. Thus, our objective was to investigate the biological effects of low-dose BPA on the initial development of primary ducts in the fetal prostate.

During prostatic development, alteration of sex steroid hormone synthesis may be responsible for prostatic anomalies associated with fetal exposure to EDCs. In the present study, fetal exposure to low-dose BPA increased E_2 levels in P1 UGS of both the male and female, whereas DES-induced changes were not detected. This alteration was also correlated with increased activity of CYP19A1 (aromatase) in UGS at P1, suggesting the unique action of BPA for in situ steroidogenesis in UGS. The BPA-specific increase of E_2 levels in UGS at P1 was correlated with the following: mRNA up-regulation of steroidogenic enzymes, such as *Cyp19a1* and *Cyp11a1*, and an increased number of aromatase-expressing UGM. The enzyme CYP19A1 (aromatase) is responsible for in situ E_2 production and the crucial testosterone/ E_2 balance necessary for normal embryonic and fetal development, even in males. The data presented here shows that the up-regulation of *Cyp19a1* mRNA in BPA-treated UGM was comparable to changes in both in situ E_2 production and CYP19A1 (aromatase) activity.

In the present study, we demonstrated that the BPA-specific increase in steroidogenic enzyme mRNA and aromatase-expressing cell number were observed in both the male and female UGM. During embryonic development, the mesenchymal component is involved in the induction and organogenesis of various organs, including the prostate, mammary gland, lung, kidney, and pancreas. It has been well established that subpopulations of the mesenchymal component are a source of potent molecules that regulate epithelial growth and differentiation [29]. In the prostate, androgen-responsive signals derived from UGM permissively and instructively induce UGE to form primary ducts of the prostate [30].

Comparison between the neonatal male and female UGS shows a similarity in the condensed mesenchyme of the ventral areas—that is, the ventral prostate mesenchyme (VPM) in the male and the ventral mesenchymal pad (VMP) in the female [31]. In the male, a defined VPM is specifically associated with ductal branching morphogenesis and cytodifferentiation of the ventral prostate. Females do not usually form a prostate. In a

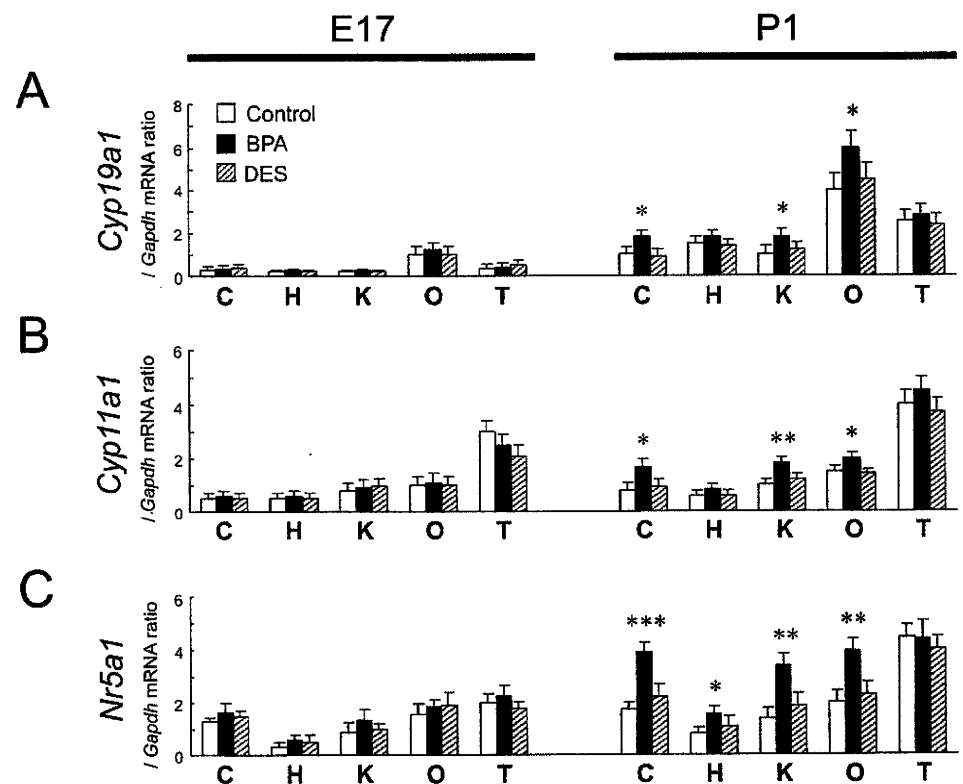
FIG. 7. BPA-specific up-regulation of *Esrrg* mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Esr1* (A), *Ar* (B), and *Esrrg* (C) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis; *n.d.*, not detected. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.



tissue recombination model, the female VMP induces prostate development in response to androgens [32], suggesting that cells within the female VMP have prostatic-inductive activity. Moreover, an earlier tissue recombination study showed that the ability of the female UGS to respond to androgens in forming prostate was gradually lost between P1 and P5 [33]. These results suggest strongly that androgen-responsive regulatory

molecules are expressed constitutively even in the female VMP. Although the female VMP forms in the absence of androgens, androgen receptor (AR) expression was observed in the neonatal female VMP in a pattern similar to that observed in the male VPM [34]. Therefore, the BPA-specific increase in E_2 levels might interact with the intracellular AR signaling in both the male VPM and the female VMP. However, to our knowledge,

FIG. 8. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Cyp19a1* (A), *Cyp11a1* (B), and *Nr5a1* (C) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.



the morphological changes in neonatal female UGS have not yet been investigated.

Our results suggest that BPA has a stimulatory effect on in situ steroidogenesis in P1 UGS of both the male and female at low-dose exposure levels. Recently, ESRRG has been reported to bind strongly with BPA [35]. Susens et al. [36] have reported that expression of ESRRG in the mouse is organ-specific: ESRRG is expressed in the brain, heart, kidney, and skeletal muscle but not in the lung, spleen, and testis. In the present study, the up-regulation of *Cyp19a1* and *Cyp11a1* mRNA by BPA treatment was detected only in organs expressing *Esrrg* mRNA. These data suggest that the possibility of a stimulatory effect on in situ steroidogenesis by fetal exposure to low-dose BPA may be a concern not only in UGS but also in organs expressing ESRRG, such as the brain, heart, kidney, and ovary. It is important to note that Takeda et al. [23] have recently reported that ESRRG was detected in the human testis, suggesting that the distribution of ESRRG differs slightly between mice and humans.

In the present study, the BPA-specific up-regulation of steroidogenic enzyme mRNA in UGS, cerebellum, heart, kidney, and ovary was observed only during the neonatal period (i.e., P0 and P1) and not during the prenatal period (i.e., E17 and E18). During pregnancy in rodents, large amounts of estrogens produced in the maternal ovaries are continuously delivered to the fetus through the placenta. After birth, however, the fetus may be released from the maternal, high-estrogen environment. Thus, one possibility is that the maternal, high-estrogen environment in pregnancy may protect the fetus from the effect of BPA on in situ steroidogenesis during the prenatal period. However, we did not investigate the effects of neonatal BPA treatment on in situ steroidogenesis.

The EDC-induced alterations of the in situ estrogen environment depend on each compound. In addition to atrazine and dioxin, the organotin compound tributyltin also increases E_2 production in human placental choriocarcinoma cells [37]. Tributyltin has been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, which is a phenomenon known as imposex [38]. These studies suggest strongly that EDCs might affect fetal development not only by mimicking the actions of sex steroid hormones but also by alteration of in situ steroidogenesis.

In the prostate, AR expressed in mesenchyme is required for directing growth and branching morphogenesis of epithelia, presumably by induction of growth factors [39]. In the present study, fetal exposure to BPA or DES increased *Ar* mRNA expression in E17 UGM of the male, whereas *Esrl* mRNA expression was up-regulated in E17 UGM of the female. Recently, Richter et al. [40] have reported that in vitro BPA treatment stimulates *Ar* and *Esrl* mRNA expression in mesenchymal cells isolated from fetal mouse prostate. Thus, our results support the idea that BPA-induced cell proliferation of the primary prostatic ducts may be caused by inducing *Ar* mRNA expression in the male UGM. In contrast, the induction of *Esrl* mRNA expression by BPA or DES may create a positive-feedback loop in the female UGM. Further investigation and morphological analysis will be necessary to confirm the effects of up-regulated ESRRG in the female UGS.

In conclusion, we have shown the unique action of BPA in the mouse UGS. Specifically, we have demonstrated that the increases in E_2 levels and CYP19A1 (aromatase) activity were observed in the BPA-treated UGS but not in the DES-treated UGS. Ricke et al. [41] have recently reported that stromal hormone imbalance, a potential source of local E_2 production, may be responsible for prostatic disease, such as benign

prostatic hyperplasia and prostate cancer. The data in the present study give rise to the concept that the development and differentiation of UGS in mouse fetuses is very sensitive to fetal exposure to low-dose BPA via the mother. Further investigation of various aspects of BPA-specific action is necessary to fully understand the role of BPA as an EDC.

ACKNOWLEDGMENTS

We thank Prof. Nobuhiro Harada at Department of Biochemistry, Fujita Health University School of Medicine, Aichi, Japan, for kindly providing rabbit polyclonal antiaromatase antibody. We also thank Mrs. Hiroko Nishii for technical support.

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Pigment-Dispersing Factor Affects Nocturnal Activity Rhythms, Photic Entrainment, and the Free-Running Period of the Circadian Clock in the Cricket *Gryllus bimaculatus*

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Abstract Pigment-dispersing factor (PDF) is a neuropeptide widely distributed in insect brains and plays important roles in the circadian system. In this study, we used RNA interference to study the role of the *pigment-dispersing factor (pdf)* gene in regulating circadian locomotor rhythms in the cricket, *Gryllus bimaculatus*. Injections of *pdf* double-stranded RNA (*dspdf*) effectively knocked down the *pdf* mRNA and PDF peptide levels. The treated crickets maintained the rhythm both under light-dark cycles (LD) and constant darkness (DD). However, they showed rhythms with reduced nocturnal activity with prominent peaks at lights-on and lights-off. Entrainability of *dspdf*-injected crickets was higher than control crickets as they required fewer cycles to resynchronize to the LD cycles shifted by 6 h. The free-running periods of the *dspdf*-injected crickets were shorter than those of control crickets in DD. These results suggest that PDF is not essential for the rhythm generation but involved in control of the nocturnality, photic entrainment, and fine tuning of the free-running period of the circadian clock.

Key words circadian rhythm, entrainment, locomotor activity, nocturnality, pigment-dispersing factor, RNAi

Circadian rhythms are about 24-h periodicities widely observed in a variety of insect behaviors, such as locomotion (Abe et al., 1997; Tomioka et al., 1997), hatching (Tomioka et al., 1991; Sauman et al., 1996; Sakamoto and Tomioka, 2007), and eclosion

(Chang, 2006). The neural mechanism controlling the overt rhythms still largely remains to be elucidated. The pigment-dispersing factor (PDF), an octadecanoneuropeptide, has been reported as a key circadian neuromodulator/neurotransmitter functioning in the

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output pathway of the insect circadian networks regulating locomotor rhythms (Renn et al., 1999; Helfrich-Förster et al., 2000; Park et al., 2000; Isaac et al., 2007). In *Drosophila*, PDF is expressed in 16 cerebral clock neurons and required for normal circadian locomotor rhythms (Taghert et al., 2001; Taghert and Shafer, 2006). PDF is thus considered as the principal neurotransmitter of the clock neurons responsible for organizing daily locomotor rhythms. It is required to adjust cycling amplitude, period, and phase in a variety of clock neurons in the brain (Miyasako et al., 2007; Tomioka et al., 2008; Yoshii et al., 2009). The discovery of the PDF receptor gene (*pdfR*) and the behavioral analysis of *pdfR* mutants confirmed the critical role of PDF signaling in maintaining robust locomotor rhythms (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005).

PDF plays important roles also in circadian rhythms of hemimetabolous insects. In cockroaches, it seems to be a primary neurotransmitter for regulation of the locomotor rhythm since transplantation of the accessory medulla including the PDF-immunoreactive neurons restores the locomotor rhythm in arrhythmic animals whose optic lobes had been removed (Reischig and Stengl, 2003). The importance of PDF is confirmed by the recent finding that *pdf* RNAi disrupted the circadian rhythmicity in German cockroaches (Lee et al., 2009). However, in the cricket, the regulatory role of PDF in overt locomotor rhythms remains to be examined since locomotor rhythm could be disrupted by removal of the outer half of the optic lobe without eliminating the PDF neurons in the proximal optic lobe (Okamoto et al., 2001).

Besides the regulatory role in locomotor activity, PDF regulates circadian responsiveness rhythms of the visual system. In the cricket, PDF levels undergo daily cycling in the optic lobe (Abdelsalam et al., 2008) and enhance the photoresponsiveness of the visually responding interneurons during night (Saifullah and Tomioka, 2003). Thus, PDF seems to be involved in fine tuning of behavioral rhythms under light-dark cycle as well as in photic entrainment. In fact, injection of PDF causes phase shifts of locomotor rhythms in a phase-dependent manner (Petri and Stengl, 1997; Singaravel et al., 2003). However, no detailed studies are available on the role of PDF in these light-associated behavioral regulations.

In this study, to elucidate the role of PDF in the cricket's circadian locomotor rhythm, we examined the profiles of circadian locomotor rhythms of *pdf* RNAi crickets, including the locomotor activity patterns in

light-dark cycles and constant darkness, free-running period in constant darkness, and resynchronization to light-dark cycles shifted by 6 h. The results show that knocking down of the *pdf* mRNA and PDF peptide levels drastically reduced the nocturnal activity, accelerated resynchronization to shifted LDs, and shortened free-running periods in DD. Based on these results, we hypothesize the multiple roles of PDF in the cricket circadian system.

MATERIALS AND METHODS

Experimental Animals

Newly emerged adult male crickets, *Gryllus bimaculatus*, were used in all experiments. They were obtained from a laboratory colony maintained under standard environmental conditions: a lighting regimen of 12-h light and 12-h dark (LD12:12; light, 0600-1800 h; Japanese standard time) and a constant temperature of 25 °C. They were fed laboratory chow (CA-1, Nihon Clea, Tokyo, Japan) and water ad libitum.

RNA Interference

Double-stranded RNAs (dsRNA) for *Gryllus bimaculatus pdf* (*Gb'pdf*) and *DsRed2* were synthesized as previously described for the *Gb'per* gene using MEGAscript High Yield Transcription Kit (Ambion, Austin, TX) (Moriyama et al., 2008). A fragment (35-285 bp) of the *Gb'pdf* cDNA (GenBank accession no. AB047800), cloned into pBluescript II SK+ (Toyobo, Osaka, Japan), was amplified with M13 forward and reverse primers. *DsRed2* in pDsRed2-N1 (Clontech, Mountain View, CA), which is derived from a coral species (*Discosoma sp.*), was amplified with the forward and reverse primers containing T7 promoter. With these DNA fragments, RNAs were synthesized with T7 and T3 RNA polymerases. The same amounts of synthesized sense and antisense RNAs were mixed, denatured for 5 min at 98 °C, and annealed by a gradual cool down to room temperature. After ethanol precipitation, the obtained dsRNA was suspended in Ultra Pure Water (Invitrogen, Carlsbad, CA) at a concentration of 20 µM. The dsRNA solutions were stored at -80 °C until use. 759 nL of dsRNA solution was injected with a nanoliter injector (WPI, Sarasota, FL) into the thorax of newly emerged adult male crickets anesthetized with CO₂.

Quantification of mRNA Levels

Gb'pdf mRNA levels were measured using a quantitative real-time RT-PCR (qPCR). Total RNA was extracted from 20 adult male optic lobes with TRIzol Reagent (Invitrogen) and was treated with DNase I (Ambion) to remove contaminating genomic DNA. 450 ng of total RNA of each sample was reverse transcribed with random hexamers using PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio, Otsu, Japan). qPCR was performed with Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) using Fast Start Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland) including SYBR Green with primers 5'-GCTGCTCGACAAGGAGGTAG-3' and 5'-TTTCCGAGTTCTTTTGTGG-3'. The *Gryllus bimaculatus* ribosomal protein L18a (*Gb'rpl18a*, GenBank accession no. DC448653) was used as an internal reference gene. The primers used for *Gb'rpl18a* were 5'-GCTCCGATTACATCGTTGC-3' and 5'-GCCAAATGCCGAAGTTCTTG-3'. The results were analyzed using the software associated with the instrument: quantification of mRNA levels was performed by the standard curve method (Rutledge and Cote, 2003). The values for *Gb'pdf* were normalized with the values for *Gb'rpl18a* at each time point. Results of 3 or 4 independent experiments were pooled to calculate the mean \pm SEM.

Immunohistochemistry

For immunohistochemistry, dissected brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for about 12 h. After washing with phosphate-buffered saline (PBS), they were immersed overnight in a blocking buffer containing normal donkey serum. They were then incubated with rabbit polyclonal anti-*Gryllus* PDF (1/6000) for 72 h at 4 °C as previously described by Abdelsalam et al. (2008). Specimens were incubated in Cy3-conjugated goat anti-rabbit secondary antibodies (1/2000) (Jackson ImmunoResearch, West Grove, PA). Preparations were washed with PBS containing 1% Triton X-100 between all steps. Brains were mounted in 50% glycerine (in PBS). Preparations were scanned and photographed with the Olympus Fluoview300 laser confocal imaging system (Tokyo, Japan). Photographs were taken at 2- and 4- μ m intervals for the optic lobes and midbrain areas, respectively. PDF immunoreactivity in the cerebral lobes and optic lobes were calculated with ImageJ computer software

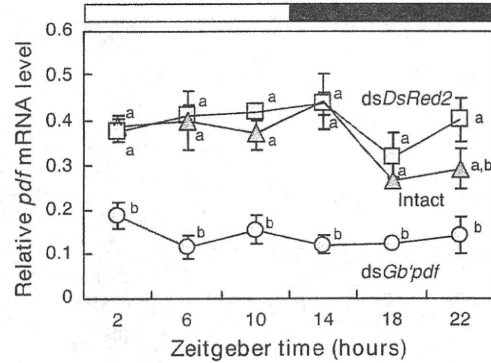


Figure 1. *Gb'pdf* mRNA levels relative to *Gb'rpl18a* mRNA levels in the optic lobes of intact, *dsDsRed2*-injected control, and *dsGb'pdf*-injected crickets under LD12:12 at 25 °C. Measurement was performed 5 days after the injection in case of injected crickets. mRNA levels were measured by quantitative real-time RT-PCR. The white and black bars indicate light and dark fractions of the LD cycle, respectively. Plotted are the mean \pm SEM of 3 or 4 replicate samples; the mRNA in each sample was extracted from 20 optic lobes of 10 adult male crickets. Values with different letters significantly differ from each other ($p < 0.05$; ANOVA with Tukey test).

(W. Rasband, National Institutes of Health, Bethesda, MD) by measuring the gray brightness value of the photographs. The background values were also measured and subtracted from the staining intensity. The values were obtained from 9 to 11 specimens for each time point and shown as mean \pm SEM.

Behavioral Assay

Locomotor activity was monitored and recorded using a computerized system described previously (Moriyama et al., 2008). Newly emerged adults were individually housed in a transparent plastic box (18 \times 9 \times 4.5 cm) with a rocking substratum, of which movement caused by a moving cricket was detected by a magnetic reed switch placed on the bottom of the box. The number of rocking was recorded every 6 min by a computer. The actographs were placed in an incubator (MIR-153, Sanyo, Moriguchi, Japan), in which temperature was kept at 25 °C and lighting conditions were given by a cool white fluorescent lamp connected to an electric timer. The light intensity was 500 to 900 lux at the animal's level, varying with the proximity to the lamp. The raw data were displayed as conventional double-plotted actograms to evaluate activity patterns, and free-running periods were calculated by the χ^2 periodogram (Sokolove

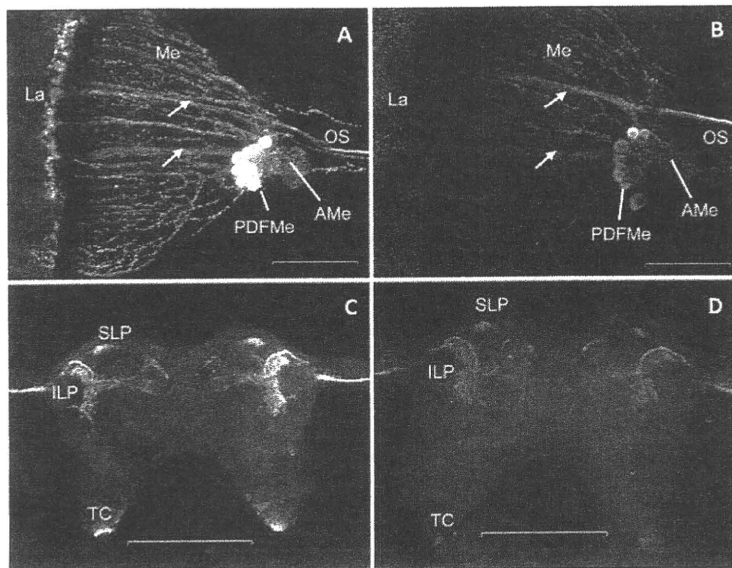


Figure 2. Confocal laser microscope images of the PDF-immunoreactive neurons in the optic lobes (A, B) and protocerebral lobe (C, D) of male crickets injected with *dsDsRed2* (A, C) or *dsGb'pdf* (B, D). The neurons were labeled with anti-*Gryllus* PDF 10 days or 15 days after injection in optic and protocerebral lobes, respectively. Note the weaker immunoreactivity in *dsGb'pdf*-treated specimens. Bars indicate 100 μm (A, B) and 500 μm (C, D). La = lamina; Me = medulla; OS = optic stalk; PDFMe = medulla PDF-immunoreactive cells; AMe = accessory medulla; SLP = superior lateral protocerebrum; ILP = inferior lateral protocerebrum; TC = tritocerebrum. Arrows (A, B) point to axon bundles of PDFMe cells in the medulla.

and Bushell, 1978). In the periodograms, the peaks appearing above the 0.05 confidence level were designated as statistically significant.

The daytime and nighttime activities and the ratio of nighttime/daytime activity were calculated where necessary. In free-running conditions, the daily average activity profiles with 6-min resolution were first calculated with a free-running period. The onset of subjective night was determined when the activity exceeded the average activity level and the duration of the subjective night was set at 12 circadian hours. The subjective night/subjective day activity ratio was then calculated.

RESULTS

pdf RNAi Knocked Down *Gb'pdf* mRNA Levels in the Cricket's Optic Lobe

Levels of *Gb'pdf* mRNA transcripts were measured under LD12:12 in the optic lobes of intact, *DsRed2* dsRNA (*dsDsRed2*)-injected control and *Gb'pdf*

dsRNA (*dsGb'pdf*)-injected adult male crickets using qPCR (Fig. 1). In intact and *dsDsRed2*-injected crickets, *pdf* mRNA levels were rather stable except a decrease at ZT18; no significant changes were detected by ANOVA ($p > 0.05$). There was no significant difference between the intact and the *dsDsRed2*-injected crickets at all the examined zeitgeber times. For *dsGb'pdf*-injected crickets, measurement was performed 5 days after injection. The results showed that the *Gb'pdf* mRNA levels were significantly suppressed to about 30% of the control crickets (ANOVA followed by Tukey test, $p < 0.05$), with an exception at ZT22, where the value was not significantly different from intact crickets; no daily changes were observed (ANOVA, $p > 0.05$).

pdf dsRNA Reduced PDF Immunoreactivity

Effects of *dsGb'pdf* on PDF levels were examined by measuring intensity of PDF immunolabeling using anti-*Gryllus* PDF antibody. Levels of PDF immunoreactivity were measured, after 5, 10, 15, and 20 days of injection, at PDFMe cell bodies, accessory medulla, medulla, and lamina areas in the optic lobe and at superior lateral protocerebrum and inferior lateral protocerebrum in the protocerebral lobe with 7 to 10 samples. In *Gb'pdf* RNAi crickets, PDF levels were significantly reduced in both the optic lobe and the cerebral lobe (Figs. 2 and 3). In the optic lobe, a significant reduction was observed 5 days after injection in all measured areas, while in the protocerebral lobe, 10 days were required until a significant reduction was detected (Fig. 3). Further gradual reduction of immunoreactivity proceeded with days both in the optic lobe and protocerebral lobe. The same pattern of reduction was evident in the axonal bundles in the medulla area and the optic stalk and in the tritocerebrum area (data not shown). It is noteworthy that PDF immunoreactivity gradually decreased even in intact and *dsDsRed2*-injected crickets except cell bodies, where a high level of immunoreactivity was maintained throughout the examined period. The reduction probably related to aging. No significant differences were found in staining levels between intact and *dsDsRed2*-injected crickets in all examined tissues.

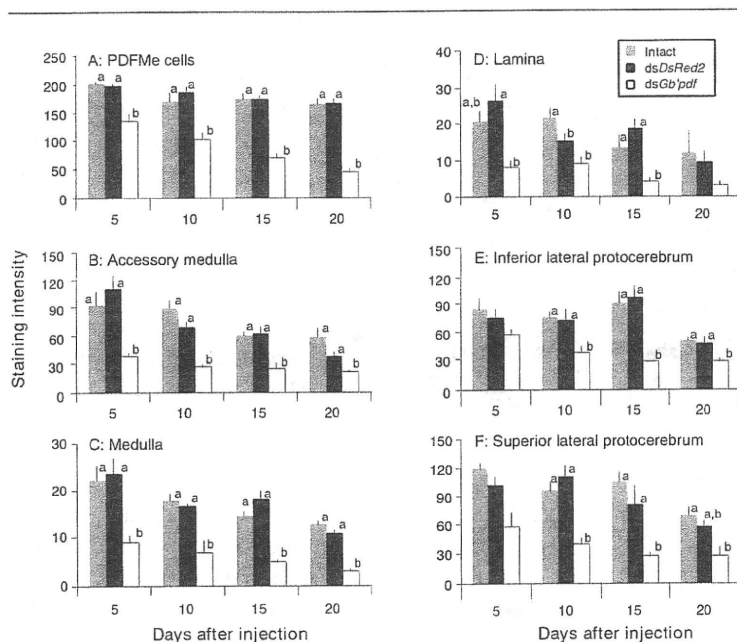


Figure 3. Effects of *dsGb'pdf* on the PDF immunoreactivity in cell bodies of the PDFMe neurons (A), accessory medulla (B), medulla (C), lamina (D), inferior lateral protocerebrum (E), and superior lateral protocerebrum (F). Plotted are the mean \pm SEM of 7 to 10 samples. Values with different letters significantly differ from each other ($p < 0.05$; ANOVA with Tukey test).

pdf RNAi Altered Circadian Locomotor Activity

To investigate the effects of *pdf* RNAi on the locomotor rhythm, we measured the locomotor activity in 76 male crickets injected with *dsGb'pdf*. We also used 109 intact and 80 *dsDsRed2*-injected crickets as controls. The representative records of locomotor activity are shown in Figure 4. Analysis of activity levels for the first 13 days after imaginal molt revealed that *Gb'pdf* dsRNA significantly reduced activity during night time (Fig. 5). In intact and *dsDsRed2*-injected control crickets, average nighttime activity gradually increased from 20 to approximately 70 during the first 10 days (Fig. 5B). The daytime activity showed an increase from 40 to approximately 50 to 60 during the first 5 days, followed by a slight decrease (Fig. 5A). The night/day activity ratio increased rapidly between day 5 to day 10 to reach a peak level around 2.5 and then maintained the level thereafter (Fig. 5C). These changes are associated with a reversal from nymphal diurnality to adult nocturnality during the first several days after the imaginal molt (Tomioka and Chiba, 1982). The *dsGb'pdf* RNAi crickets showed significantly less activity only

for night time (Figs. 4, 5A and 5B). The night/day activity ratio only slightly increased and stayed at nearly 1 (Fig. 5C), being mostly significantly smaller than both *dsGb'pdf* RNAi and intact crickets after 5 days of injection onwards (ANOVA followed by Tukey test, $p < 0.05$) (Fig. 5C).

pdf RNAi Resulted in a Faster Resynchronization to Shifted LD Cycles

Gb'pdf RNAi crickets showed shorter transient cycles required for resynchronization to LD cycles advanced or delayed by 6 h than intact and *dsDsRed2*-injected crickets (Figs. 4 and 6) (ANOVA followed by Tukey test, $p < 0.05$). *DsRed2* RNAi slightly increased the transient cycles only for delay shifts (Tukey test, $p < 0.05$). In intact and *dsDsRed2*-injected controls, a remarkable positive masking effect of light was observed at lights-on several days after phase advance of LD (Fig. 4A and 4C), while the positive masking associated with advance shifts was significantly reduced (79.0 ± 54.6 v. 137.5 ± 102.3 [*DsRed2* RNAi], t test, $p < 0.01$) and sometimes disappeared in the *dsGb'pdf*-injected crickets (Fig. 4E). Weak masking effects of light were often observed even in these *dsGb'pdf* RNAi crickets in steady-state entrainment (arrowheads in Fig. 4E and 4F).

pdf RNAi Shortened Free-Running Period in DD

To examine the effect of *Gb'pdf* RNAi on free-running rhythms under DD, we recorded the locomotor activity of 11 *dsGb'pdf*-injected crickets under DD. The *Gb'pdf* RNAi crickets showed significantly shorter free-running periods than those of intact and *dsDsRed2*-injected crickets (ANOVA followed by Tukey test, $p < 0.05$) (Fig. 7). The *dsDsRed2*-injected crickets showed a slightly longer free-running period than intact crickets (Tukey test, $p < 0.05$). When activity was analyzed for free-running conditions, there were no significant differences in total daily activity and subjective night/subjective day activity ratio among the 3 groups of animals (ANOVA, $p > 0.05$) (Fig. 7E and 7F).

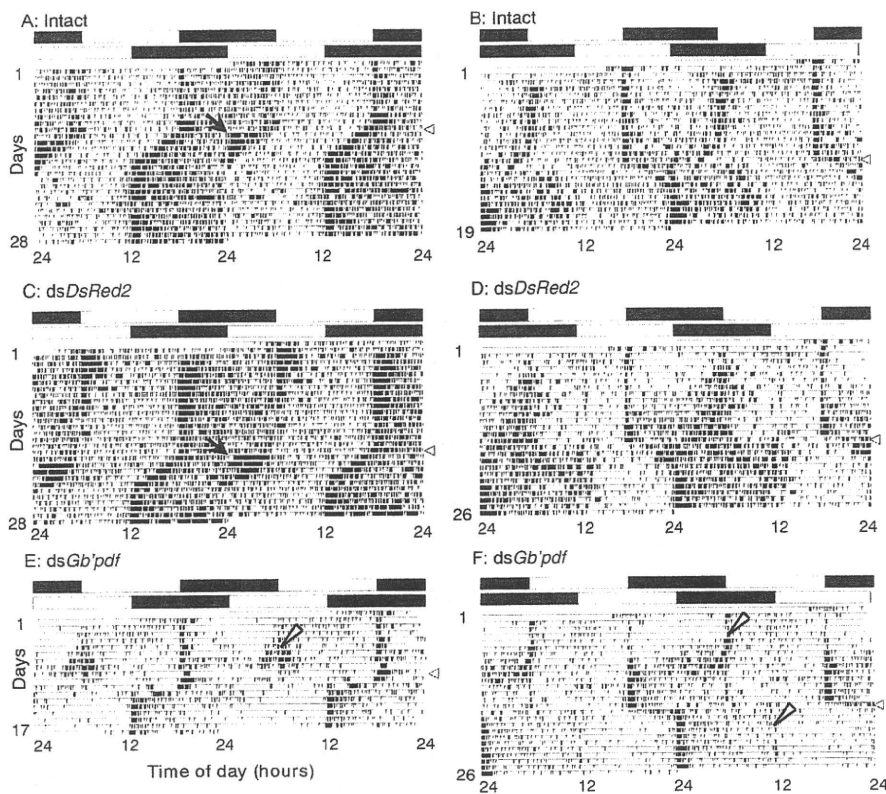


Figure 4. Double-plotted actograms of intact (A, B), ds*DsRed2*-injected (C, D), and ds*Gb'pdf*-injected crickets (E, F). Newly emerged intact or dsRNA-treated crickets were first kept in LD12:12 at 25 °C for about 10 days until full nocturnality is established, then subjected to either 6-h advance (A, C, E) or 6-h delay shifts (B, D, F) by shortening or lengthening the light phase, respectively. White and black bars indicate light and dark phases, respectively. Arrowheads indicate the day when LD was shifted. Arrows (A, C) indicate masking effect. White arrowheads indicate positive masking effects in ds*Gb'pdf*-injected crickets (E, F). Note the weak nocturnal activity levels and quicker resynchronization in *pdf* dsRNA-injected crickets.

DISCUSSION

Effectiveness of *pdf* RNAi

We showed here that a single injection of dsRNA of the *pdf* gene effectively knocked down its mRNA levels and subsequently its product PDF peptide levels, leading to altered circadian rhythms. The results of systemic *pdf* RNAi gene silencing are in accordance with a similar study on *pdf* in cockroaches (Lee et al., 2009) and on clock genes of crickets and other insects (Moriyama et al., 2008; Moriyama et al., 2009; Danbara et al., 2010; Kamae et al., 2010). Like clock genes *period* and *timeless* (Moriyama et al., 2008; Danbara et al., 2010), the effects of ds*Gb'pdf* continued for up to 4 weeks. The prolonged effect of *Gb'pdf* after a single injection

might involve RNA-dependent RNA polymerase (RdRp) to convert the single-stranded target mRNA to dsRNA using the antisense strands of primary siRNAs as primers (Zamore et al., 2000; Lipardi et al., 2001; Sijen et al., 2001). This possibility is supported by a recent finding of RdRp in *Drosophila* (Lipardi and Paterson, 2009). The systemic RNAi thus provides an efficient way to unravel molecular machineries underlying various physiological functions by switching the genes "off" at the posttranscriptional level through a minimally invasive way. This is especially helpful for insects in which forward genetics is less practical comparable to *Drosophila*.

Injections of *pdf* dsRNA induced a gradual decrease of PDF immunoreactivity in both the optic lobe and protocerebrum (Figs. 2 and 3), indicating the reduction of PDF peptide levels. The gradual decrease is probably due to the lifetime of already present PDF because the mRNA level was strongly reduced on day 5 after the *dspdf* injection (Fig. 1). Reduction of PDF in the neurons projecting into the protocerebrum seems important since these neurons are responsible for relaying the circadian output signal to the locomotor center located in the brain (Stengl and Homberg, 1994). Both daytime and nighttime activity reductions preceded the statistically significant reduction of PDF levels in the cerebral lobe (Figs. 3 and 5), suggesting that a slight decrease of PDF affects the locomotor activity. Thus, PDF seems to be an output neurotransmitter/neuromodulator for regulation of the locomotor activity as has been reported for *Drosophila* (Renn et al., 1999) and German cockroaches (Lee et al., 2009). In cockroaches, *pdf* RNAi effectively decreases PDF in the optic lobe and the brain below

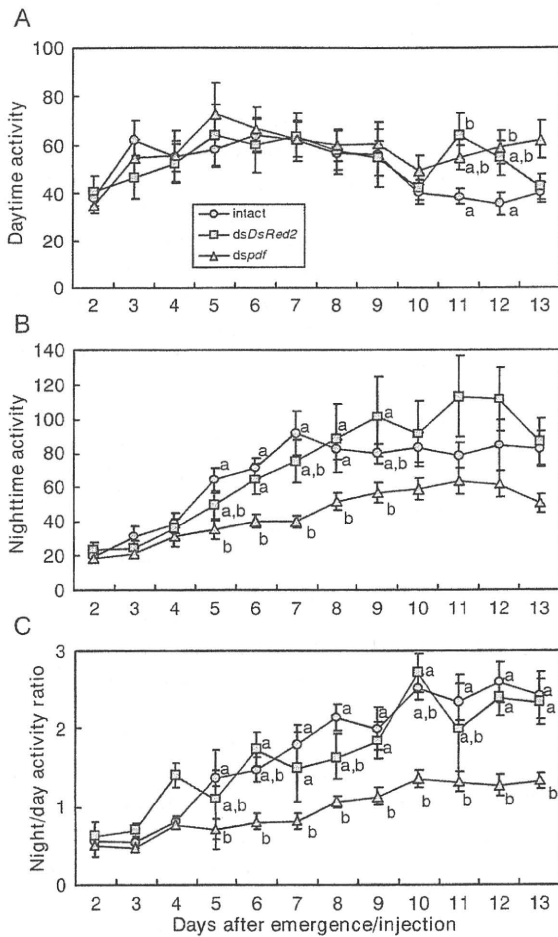


Figure 5. Effects of *dsGb'pdf* RNAi on daily diurnal (A) and nocturnal activity (B) and night/day activity ratio (C) in the cricket *G. bimaculatus* in LD12:12. The abscissa represents days after injection of *dsGb'pdf* or *dsDsRed2* or days after imaginal molt for intact crickets. Error bars indicate SEM. Values with different letters significantly differ from each other ($p < 0.05$; ANOVA with Tukey test).

the detectable level by immunohistochemistry within 10 days (Lee et al., 2009). However, in the present study, complete disappearance was never observed during 20 days after *dsPdf* injection. This might be attributable to species specificity in RNAi efficiency, the difference of expression levels of *pdf*, or the lifetime of PDF peptide. In the cricket, PDF peptide seems to have a very long lifetime because PDF immunoreactivity persisted in axons projecting to the optic stalk and protocerebral lobe for more than 30 days after the cell bodies were removed (Stengl, 1995; Okamoto et al., 2001).

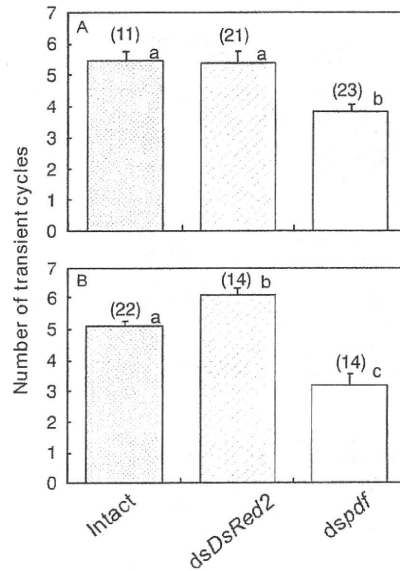


Figure 6. The average number of cycles required for resynchronization to LDs shifted by 6 h in the advance (A) or delay (B) direction in intact, *dsDsRed2*-injected, and *dsGb'pdf*-injected crickets. Data are represented as mean \pm SD. n = number of insects. Values with different letters significantly differ from each other ($p < 0.05$; ANOVA with Tukey test). Numbers in brackets indicate the number of animals used. Note that fewer numbers of cycles are required for resynchronization in *dsGb'pdf*-injected crickets.

Possible Roles of PDF in the Cricket Circadian System

In contrast to the results in cockroaches where arrhythmicity was induced by *pdf* RNAi (Lee et al., 2009), the crickets treated with *dsPdf* maintained a clear locomotor rhythm in both LD and DD (Figs. 4 and 7). It is premature to exclude the possibility that PDF is indispensable for the circadian locomotor rhythm because our immunohistochemistry revealed that a still certain amount of PDF remains in both the optic lobes and protocerebrum (Figs. 2 and 3), whereas the PDF became below detectable level by immunohistochemistry within 10 days of *dsPdf* treatment in cockroaches (Lee et al., 2009). The preserved locomotor rhythm in the cricket might be attributable to the still remaining PDF. Several alterations of the locomotor rhythm were yet observed in *pdf* RNAi crickets. First, suppression of nocturnal activity was the most prominent feature of *pdf* RNAi crickets (Figs. 4 and 5). Second, *pdf* RNAi resulted in shortening of the free-running period under DD (Fig. 7). Third, the resynchronization to the LDs

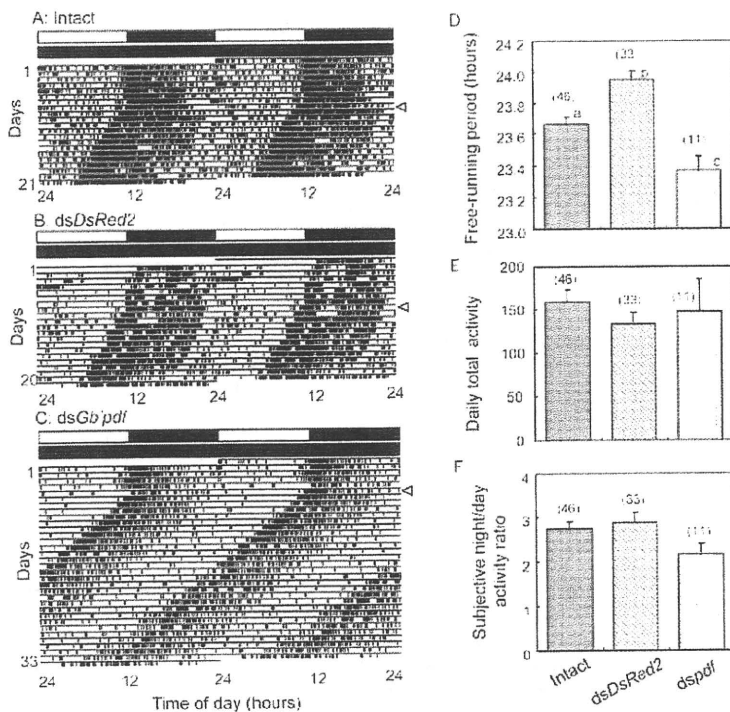


Figure 7. Representative free-running locomotor activity rhythms under DD in intact (A), dsDsRed2-injected (B), and dsGb'pdf-injected crickets (C) and their average free-running periods (D), average daily total activity (E), and subjective night/subjective day activity ratio (F). White and black bars indicate light and dark phases of the LD cycle, respectively. Arrowheads indicated the day transferred to DD. *n* indicates the number of insects. Values with different letters significantly differ from each other ($p < 0.05$; ANOVA with Tukey test). Note the free-running period was significantly shorter in dsGb'pdf-treated crickets.

shifted by 6 h occurs more quickly than control crickets (Fig. 4).

Gb'pdf RNAi suppressed nocturnal activity (Fig. 5), suggesting that PDF is released under the regulation of circadian clock as has been previously suggested (Saifullah and Tomioka, 2003; Abdelsalam et al., 2008). This fact is reminiscent of the finding that PDF regulates arousal in *Drosophila*: pdf⁰¹ flies have increased sleep (reduced locomotor activity) during the day (Parisky et al., 2008). The suppression of nocturnal activity in pdf RNAi crickets is consistent with what was observed in German cockroaches, where only nocturnal activity was reduced and eventually resulted in arrhythmicity (Lee et al., 2009). It could be thus hypothesized that PDF regulates the nocturnal activity in these nocturnal hemimetabolous insects. In *Drosophila* pdf⁰¹ mutant flies that genetically lack PDF, however, the evening peak is clearly maintained but with advanced phase, while the morning peak is

generally absent (Renn et al., 1999; Blanchardon et al., 2001). Thus, the role of PDF in activity regulation seems different between diurnal and nocturnal insects. In this regard, the nymphal crickets *G. bimaculatus* seem a good subject to examine this hypothesis because they are diurnally active (Tomioka and Chiba, 1982).

The shortening of the free-running period under DD is consistent with the results in pdf⁰¹ *Drosophila* mutant flies, showing the free-running period much shorter than wild-type flies (Renn et al., 1999; Yoshii et al., 2009). PDF is hypothesized in *Drosophila* that it lengthens free-running periods and simultaneously couples the multiple clock neurons (Peng et al., 2003; Lin et al., 2004; Sheeba et al., 2008; Yoshii et al., 2009). A similar role of PDF is proposed in the cockroach (Schneider and Stengl, 2005). It seems likely that PDF lengthens the period of the molecular oscillation via the cAMP-dependent pathway (Shafer et al., 2008).

The quick resynchronization of pdf RNAi crickets to the shifted LDs suggests involvement of PDF in the photic entrainment pathway. It has been suggested that PDF regulates the circadian rhythm of the cricket's visual interneurons, which directly connect the bilateral medulla area of the optic lobe, increasing their photoresponsiveness during the night (Saifullah and Tomioka, 2003). This hypothesis is supported by the finding that pdf RNAi crickets showed reduced positive masking effect at lights-on during advance shifts after phase advance of LD by 6 h (Fig. 4) but seems to be inconsistent with the finding that reduction of PDF levels enhances the photic entrainability (Fig. 6). However, there are at least 2 possible explanations for the role of PDF in photic entrainment. First, the PDF enhances the photoresponsiveness of the visual interneurons, which may negatively act in the photic entrainment pathway. The second explanation is based on the *Drosophila* clock system including light-sensitive and relatively light-insensitive oscillators for the control of locomotor rhythm (Miyasako et al., 2007; Yoshii et al., 2009): a reduced level of PDF may weaken the PDF-dependent decelerating influence of light-insensitive oscillator on the light-sensitive oscillators, enabling the light-sensitive clock neurons to synchronize more

quickly. The other possibility is that extraretinal photoreception might be involved in the photic entrainment as has been suggested in the New Zealand weta and the band-legged crickets (Waddell et al., 1990; Shiga and Numata, 1997): reduced PDF levels may reduce the photic input through the visual system; then, input from the extraretinal entrainment pathway may become prominent. This might be plausible considering that *Drosophila* has multiple photoreceptive systems for photic entrainment including the compound eye, ocelli, H-B eyelet, and cryptochrome, an extraretinal photoreceptor (Rieger et al., 2003; Hanai and Ishida, 2009).

In addition to the entrainment pathway, PDF likely mediates direct effect of light. A startle response, or positive masking effect of light, occurring at lights-on after an advance shift of LD (Fig. 4A and 4C) was significantly reduced in *pdf* RNAi crickets (Fig. 4E). The startle response may be caused through the PDF pathway. The fact that no significant difference in activity levels was observed between *pdf* RNAi crickets and controls (Fig. 7) also suggests the PDF pathway regulates the activity in response to light. The PDF pathway probably plays a similar role in *Drosophila* because *pdf⁰¹* mutant flies show only a weak morning peak (Yoshii et al., 2009) and the ILNvs that express PDF are responsible for the morning startle response (Sheeba et al., 2010). In summary, the PDF pathway plays multiple roles in the cricket circadian system, regulating the nocturnal rhythm, its photic entrainment, and the free-running period. With the aid of PDF, the cricket may maintain its robust nocturnal rhythm and adjust it to given LD cycles.

ACKNOWLEDGMENTS

This study was supported in part by grants to K.T. from the Japan Society for the Promotion of Science (JSPS). E.H. is supported by a grant from the Egyptian Ministry of Higher Education. Y.M. is a JSPS Research Fellow.

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Structure-Activity Studies on Nociceptin and Its Receptor ORL1

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A novel diagram of receptor assays was established to discriminate the characteristic receptor responses, exemplifying the antagonists of nociceptin ORL1 receptor and nuclear receptor ERR γ . In particular, the novel idea of inverse-type of agonist/antagonist was introduced for constitutively active receptors.

Keywords: agonist; antagonist; bisphenol A; inverse agonist; nociceptin

Introduction

Nociceptin or orphanin FQ, a heptadecapeptide FGGFTGARKSARKLANQ, is the endogenous ligand of opioid receptor-like 1 (ORL1) receptor. The nociceptin receptor is a G protein-coupled receptor and involved in the regulation of numerous brain activities, particularly instinctive and emotional behaviors. We have performed a series of structure-activity studies on these neuropeptide and its target receptor ORL1. Those include (1) development of superagonists, (2) development of potent pure antagonists, (3) exploration of ORL1 receptor binding sites of superagonists, (4) identification of receptor residues essential for activation, and (5) identification of receptor residues essential for interactions between transmembrane domains.

In the present study, we concentrated the research scheme on the principal diagram to evaluate or design receptor antagonists. As shown in Fig. 1, there are two different types of receptor antagonist: *i.e.*, a regular receptor antagonist for ordinary receptors and an inverse-type antagonist for constitutively active receptors. We attempted to obtain a pure antagonist of nociceptin ORL1 receptor, and we have discussed its activity profile in comparison with bisphenol A, inverse antagonist of nuclear receptor ERR γ .

Results and Discussion

Nociceptin ORL1 receptor possesses a considerably low constitutive activity, although some mutants exhibit higher levels of activity. Thus, ORL1 receptor antagonist is to be regular-type to inhibit nociceptin. In order to pursue a pure antagonist, a series of analogs were prepared for parental Ac-Arg-Arg-Tyr-Arg-Ile-Lys-NH₂ (Ac-RRYRIK-NH₂), a partial agonist of ORL1. Eventually we reached a conclusion that the most important structural element for antagonism is the N-terminal acetyl group, and then several highly potent antagonists were designed and synthesized [1]. As a pure antagonist, for example, isovaleryl-RRYRIK-NH₂ was found with no agonist activity [2].

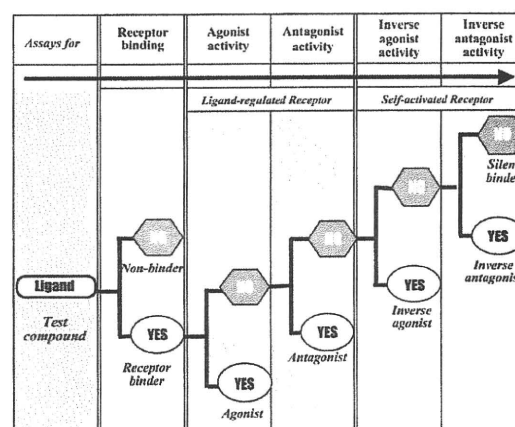


Fig. 1. Schematic flow diagram of receptor assays to discriminate the characteristic receptor responses.

It is not unusual to have a compound with no agonist activity and simultaneously with no antagonist activity. In this case, the target receptor must be constitutively active. Constitutively active receptors possess inverse-type agonists and their neutral antagonists. We have recently discovered bisphenol A as an inverse-type neutral antagonist of human nuclear receptor ERR γ [3]. Bisphenol A was found to reverse the deactivation activity of inverse agonist 4-hydroxytamoxifen in ERR γ [4].

For receptors, there are two different types, namely, ligand-activated receptors and self-activated constitutively active receptors (Fig. 1). The ordinary antagonist is a compound that inhibits the agonist activity in ligand-activated receptors, whereas the inverse antagonist is a compound that inhibits the inverse-agonist activity in self-activated receptors. Thus, the antagonist should be designed, depending upon the receptor type to which a ligand exhibits either activation or deactivation activity.

This study was supported in part by Health and Labour Sciences Research Grants for Research on the Risk of Chemical Substances from the Ministry of Health, Labor and Welfare of Japan.

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Drosophila Neuropeptide hugy Present in the Clock Cells Important for Circadian Rhythm Oscillation

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We prepared polyclonal antibodies against *Drosophila* neuropeptides hugy and PK2. Prepared anti-hugy-pAb stained dorsal lateral neuron in a time-dependent manner. Since this neuron works to generate an evening activity, hugy is expected as a neurotransmitter candidate to emit an evening locomotor activity.

Keywords: *Drosophila melanogaster*; neuropeptide; circadian rhythm; hugy; PK2

Introduction

The *Drosophila* gene *hugin* encodes two different neuropeptides named hugy (QLQSNQEPAYRVRTPL-NH₂) and PK2 (SVPFKPRL-NH₂). These peptides having C-terminal PRL-NH₂ were said to modulate feeding behaviors [1]. A mammalian homolog of this *hugin* is the gene encoding neuromedin U (NMU), having C-terminal PRN-NH₂ structure. It should be noted that NMU is involved in the neurotransmission of circadian rhythm [2]. Thus, hugy and/or PK2 are likely to play a similar role of NMU in *Drosophila*. In the present study, we performed immunostaining examination with specific antibodies for hugy and PK2, respectively, to confirm the relations of these peptides with the circadian clock system.

Results and Discussion

Anti-hugy polyclonal antibodies (anti-hugy-pAb) and anti-PK2 polyclonal antibodies (anti-PK2-pAb) were prepared by immunizing KLH-conjugated antigen peptides into New Zealand white rabbits. The serum was treated for purification by the immunoprecipitation followed by affinity chromatography. The specificity of antibodies was determined by ELISA, and found not to interact with other PRX-NH₂ peptides such as PK1 (*Drosophila* PBAN like peptide), ETH1, ETH2, CAPA1, and CAPA2.

When brains of the fruit fly *Drosophila melanogaster* was examined, the cells stained by both antibodies were found in the subesophageal ganglion (SOG), and also dorsal lateral neurons (LNds) were stained (Fig. 1). This immunostaining of LNds was confirmed by co-expression of TIMELESS for brains of transgenic fly *tim-gal4/UAS-GFP* genotype. It should be noted that LNds are clock neurons that drive evening activity of *Drosophila* behavior [3].

Given the production of hugy and PK2 peptides was

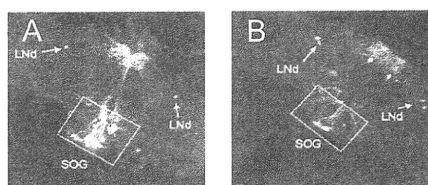


Fig. 1. Immunohistochemistry of anti-hugy-pAb (A) and anti-PK2-pAb (B), in *Drosophila* brain. Immunoreactive cells were found in the dorsal lateral neurons (LNd) (white arrows) and the subesophageal ganglion (SOG) (white box).

involved in a circadian rhythmicity, these peptides were undoubtedly circadian neurotransmitter. Peptide expression profiles were evaluated by measuring the strength of immunostaining with anti-hugy-pAb and anti-PK2-pAb at 4-hr intervals. It was found that the strength of hugy-immunofluorescence exhibits clearly a circadian rhythmicity. In the LD/DD condition, the profiling of hugy-immunofluorescence intensities in LNd cells peaked at 0-4 hr and reached to the lowest levels at 16-20 hr. On the other hand, the levels of PK2-immunofluorescence intensities have retained in almost a constant level.

In order to examine whether or not hugy expression is regulated in the clock gene feedback system, we tested four clock mutant flies. Those include *period*-null mutant fly (*per⁰¹*), *timeless*-null mutant fly (*tim⁰¹*), *clock*-null mutant fly (*clk^{rk}*), and *pdf*-null mutant fly (*pdf⁰¹*). The *per⁰¹* mutant fly exhibited decrease in immunofluorescence at Zeitgeber time (ZT) 4 as compared with the wild type fly. However, similar reduction was not observed for any other null mutant flies. These results implied that hugy production is regulated in the clock gene feedback system, again suggesting that hugy is a neurotransmitter peptide to emit an evening locomotor activity in the *Drosophila* circadian clock.

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