Regulation of insect steroid hormone biosynthesis by innervating peptidergic neurons

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In insects, steroid hormones named ecdysteroids elicit molting and metamorphosis. The prothoracic gland (PG) is a predominant source of ecdysteroids, where their biosynthesis (ecdysteroidogenesis) is regulated by several neuropeptides. Here, we report that FMRFamide-related peptides (FaRPs) regulate ecdysteroidogenesis through direct innervation of the PG in the silkworm Bombyx mori. We purified a previously uncharacterized Bombyx FaRP, DPFSLRFamide, and identified the corresponding Bombyx FMRFamide gene (Bomomo-FMRFamide, BRFa), which encodes three additional FaRPs. All BRFa peptides suppressed ecdysteroidogenesis in the PG by reducing cAMP production by means of the receptor for Bomomo-myosuppressin, another FaRP we have previously shown to act as a prothoracostatic factor. BRFa is predominantly expressed in neurosecretory cells of thoracic ganglia, and the neurons in the prothoracic ganglion innervate the PG to supply all four peptides to the gland surface. Electrophysiological recordings during development confirmed the increased firing activity of BRFa neurons in stages with low PG activity and decreased ecdysteroid levels in the hemolymph. To our knowledge, this study provides the first report of peptides controlling ecdysteroidogenesis by direct innervation.

steroid hormones constitute a very important class of compounds that regulate development and homeostasis in most animals. In insects, steroid hormones named ecdysteroids direct development and elicit molting and metamorphosis (1). They act by means of the nuclear ecdysone receptor/ultraspiracle complexes, which mediate ecdysteroid-induced gene expression (2, 3).

The activity of the prothoracic gland (PG), a principal endocrine organ producing ecdysteroids, is regulated by a cerebral peptide that acts as a prothoracicostatic hormone (PTTH). It is widely accepted that PTTH stimulates the PGs to synthesize and release ecdysteroids before each molting and during early metamorphosis (1, 4, 5). However, various reports on proprioceptive and environmental inputs that affect molting and metamorphosis suggest the existence of additional regulators of PG activity (6, 7). In fact, there is growing evidence that the CNS exerts a prothoracostatic effect in addition to the tropic effect, thus regulating the complex pattern of the ecdysteroid titer in the hemolymph during development (8–10). It is important to elucidate these prothoracostatic factors and their modes of action to understand the complexities of insect morphogenesis as well as the peptide regulatory mechanisms of steroid hormone biosynthesis, which exist in both invertebrates and vertebrates (11).

We previously identified Bommo-myosuppressin (BMS), which is an FMRFamide-related peptide (BMS), as a previously unidentified prothoracostatic factor in the silkworm Bombyx mori (12). BMS was shown to suppress ecdysteroidogenesis in the PG, where the BMS receptor (BMSR) is highly expressed. Interestingly, BMSRs respond to two other FaRPs from another lepidopteran species (Manduca sexta) that also exert inhibitory effects on Bombyx PGs. These results suggest that some other endogenous FaRPs in Bombyx may also act as prothoracostatic factors.

Here, we report the purification and identification of four Bombyx extended FMRFamides (Bomomo-FMRFamides, BRFAs) as previously uncharacterized prothoracostatic factors. These neuropeptides are encoded by the same gene. We show that BRFa peptides are produced in the CNS neurons that suppress PG activity by direct innervation. Although the importance of the PG-innervating neurons in the control of ecdysteroidogenesis has been well documented (13–18), studies revealing molecular basis of the PG regulation have been restricted to hormonal substances throughout the last century (1). To our knowledge, this study is the first report of peptides controlling ecdysteroidogenesis by direct innervation, which may shed light on the regulatory mechanisms of insect development.

Results

Purification and Identification of Bombyx FMRFamides. We developed an ELISA system to purify endogenous FaRPs in Bombyx, in addition to BMS. Competitive ELISA revealed that some HPLC fractions, originally prepared from Bombyx pupal brains for the purification of BMS (12), show FMRFamide-like immunoreactivity (Fig. 1A). Three more steps were required to purify one of the immunoreactive fractions (no. 19) to homogeneity, sufficient for MALDI-TOF MS analysis (Fig. 1B). Two other FaRPs were purified from fraction no. 16 (unpublished data). An aliquot of the isolated fraction was subjected to MALDI-TOF MS, resulting in the detection of a monoisotopic mass of 880.5 ([M+H]+). Further analysis of this putative peptide with postsource decay (PSD) yielded the sequence DPSFLRFamide (Fig. 6A), which is published as supporting information on the PNAS web site. Because a glycine residue is required for the amidation at the C terminus, the sequence DPSFLRFamide was used as a query for the TBLASTN search against Bombyx whole-genome shotgun sequence contigs. This search resulted in identification of contig Io5432 with a putative exon sequence coding for DPSFLRFamide flanked by basic arginine residues. Using this putative coding sequence and its flanking regions, RACE was performed to obtain the nucleotide sequence of the corre-

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Abbreviations: FaRP, FMRFamide-related peptide; PG, prothoracic gland; PTTH, prothoracotrophic hormone; BMS, Bomomo-myosuppressin; BMSR, BMS receptor; BRFa, Bomomo-FMRFamide; BRFa receptor; NS-VII, ventrolateral neurosecretory cells 1 and 2; PBST, PBS containing 0.05% (vol/vol) Tween 20.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB234100 and AB253536).

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three additional purification steps, which resulted in a homogeneous peak immunoreactive fractions (fraction no. 19, arrowhead in UV absorbance; the dashed line denotes the acetonitrile gradient. One of the fourth (C-terminal glycine residue is necessary for amidation. mature peptides are underlined with gray bars. For each mature peptide, the most likely site of the signal peptide cleavage is indicated with an arrowhead. The purified peptide (BRFa-3) is underlined with a black bar, and putative purified BMS. (A) BLASTP search of BMS, and FaRP content in this fraction was presented as the amount of BMS, and FaRP content in this fraction was presented as the amount of purified BMS. (C) The deduced amino acid sequence of the BRFa precursor. The most likely site of the signal peptide cleavage is indicated with an arrowhead. The purified peptide (BRFa-3) is underlined with a black bar, and putative mature peptides are underlined with gray bars. For each mature peptide, the C-terminal glycine residue is necessary for amidation.

BRFa Peptides Are Prothoracicostatic Factors. To elucidate the prothoracicostatic activity of BRFa, cAMP and ecdysteroid assays were performed by using PGs of the fifth larval instar on day 4, as described for BMS in ref. 12. As shown in Fig. 2 A and B, BRFa peptides inhibited both basal and PTTH-stimulated CAMP accumulation in the PGs in a dose-dependent manner. In the ecdysteroid assay, BRFa peptides inhibited PTTH-stimulated ecdysteroidogenesis, although the effects were less pronounced (Fig. 2C). These results suggest that BRFa peptides, like BMS, inhibit ecdysteroidogenesis through activation of BMSR to lower the cAMP level in the PGs.

To clarify that the prothoracicostatic effects of BRFa are actually mediated by BMSR, Ca2+ imaging analysis was performed by using a heterologous expression system. Human embryonic kidney (HEK) 293 cells expressing both BMSR and Ga15 responded to BRFa peptides in a dose-dependent manner (Fig. 2D). Moreover, the relative responsiveness of BMSR to four BRFa peptides corresponded well to that in the cAMP assay and the ecdysone assay, demonstrating that BMSR actually mediates the prothoracicostatic effects of BRFa.

As for FMRFamides in Drosophila, a highly specific receptor that is different from the myosuppressin receptor has been reported (20). Using this receptor sequence, we identified a homologous gene coding a putative BRFa receptor (BRFaR) in the Bombyx genome and elucidated its high responsiveness to BRFa peptides (Fig. 7 A–C, which is published as supporting information on the PNAS web site). However, no expression of BRFaR was detected in the PG (Fig. 7D), further demonstrating that BMSR is the functional receptor for BRFa in the PG.

Although these results indicate that BRFa peptides actually act as prothoracicostatic factors by means of BMSR, their effective concentrations were always much higher than those of BMS. Whereas BMS has been suggested to work as a humoral inhibitory factor, BRFa peptides appear to suppress the PG
activity via a different pathway, possibly by direct innervation, because the local concentrations of neuropeptides can be much higher if they are released in close proximity of the target organ. This possibility was further verified by experiments described below.

**BRFa Is Predominantly Expressed in Thoracic Ganglia.** The expression pattern of **BRFa** was determined by RT-PCR analysis. As shown in Fig. 3A, **BRFa** was expressed only in the CNS and predominantly in the thoracic ganglia of wandering fifth-instar larvae on day 6. A similar pattern was observed in fifth-instar larvae on day 2 (data not shown).

The relative expression level of **BRFa** in the prothoracic ganglion was determined throughout the last instar to the first day of the pupal stage by using quantitative RT-PCR (Fig. 3B). **BRFa** is expressed throughout this developmental period, with highest expression levels observed in the early half of the fifth instar, similar to those of **BMS**.

**BRFa Neurons Innervate the PG.** *In situ* hybridization with the DNA probe revealed strong **BRFa** expression in two pairs of large neurons in each thoracic ganglion (Fig. 4A). Only a few other small neurons were stained in the CNS. Subsequent double immunohistochemical staining of the same CNS showed that large thoracic neurons are ventrolateral neurosecretory cells 1 and 2 (NS-VTL1,2) (Fig. 4B), which project their axons through transverse nerves into the periphery. Axons originating in **BRFa** neurons of the prothoracic ganglion innervate the PG (Fig. 4C).

Moreover, these axons and axon terminals contain numerous varicosities, suggesting that **BRFa** is released in close proximity of the PG (Fig. 4D).

To further confirm that all four **BRFa** peptides are actually delivered to the gland surface, direct MS analysis of the axons running on the gland surface was performed. This analysis resulted in the detection of four major signals, which corresponded to the theoretical masses of the four **BRFa** peptides (Fig. 4E).

**Developmental Profile of the BRFa Neuron Activity.** To investigate the physiological function of **BRFa** neurons, their temporal patterns of activity were investigated electrophysiologically. Using a suction electrode, the firing frequency of **BRFa** neurons was measured throughout the last instar (Fig. 5). In all of the larval stages tested, the recordings showed a combination of regular firing patterns, reflecting the signals from multiple neurosecretory cells (Fig. 5A). However, the spikes rarely overlapped with each other (arrowhead in Fig. 5A), which made it possible to monitor the firing frequency. The recorded spikes exhibited a slow time course and a waveform typical of neurosecretory cells (Fig. 5B) (21, 22). Moreover, the time course of recorded action potentials was highly uniform in all of the experiments, suggesting that the activity emanated only from NS-VTL1,2 (Fig. 5B). Ultrastructure of the nerve at the location from which recordings were made indicates that only four neurosecretory axons exist, further
confirming that all of the recorded spikes are from four BRFa neurons (data not shown).

The activity of BRFa neurons is high during the feeding period of the last instar, when the hemolymph ecdysteroid titer is low. Activity decreases remarkably on the day before larvae start wandering, in line with the gradual increase of the ecdysteroid titer. The firing frequency recovers after the onset of wandering, followed again by a marked decrease to almost complete inactivation. The overall fluctuation pattern of BRFa neuron activity is shown in Fig. 5C, with the schematic illustration of the hemolymph ecdysteroid titer (based on refs. 5 and 23).

Discussion

In this study, we identified four BRFa peptides as previously unidentified prothoracicostatic factors and elucidated their mode of action on the PG. Although the importance of PG innervation has been well documented (13–18), to our knowledge, this study is the first report on the molecular basis for neural regulation of ecdysteroidogenesis.

BRFa peptides were shown to regulate ecdysteroidogenesis by means of BMSR, the receptor previously reported to mediate the prothoracicostatic effect of BMS (12). Although effective concentrations of BRFa were much higher than BMS, this difference reflects their different modes of action on the PGs. It is well known that hormonal factors are usually effective at nanomolar concentrations, whereas peptides delivered by direct innervation act at micromolar concentrations (24–26). The even higher effective concentrations of BRFa in the heterologous expression system compared with those in PG incubated in vitro (cAMP and ecdysteroid assays) can be explained by different sensitivities of these two systems. As for BMSR, the EC50 for BMS in the cAMP assay (0.089 nM) was ~400 times lower than that in the heterologous expression system (32 nM) (12). However, this relatively high value is comparable with the EC50 of Drosophila myosuppressin (DMS) (40 nM) that is necessary for the activation of two DMS receptors expressed in a similar heterologous system (27). Taking these relative differences into consideration, it appears that the responsiveness of BMSR to BRFa corresponds well to the effective concentrations in the cAMP and ecdysteroid assays (see Fig. 2). The absence of highly responsive BRFaR in the PG further suggests that BMSR is the functional receptor for BRFa in the PG (Fig. 7).

Why does Bombyx use two different factors for the BMSR-mediated signaling? Although much needs to be clarified to answer this question, we propose that insects use BMSR-mediated signaling for both general and specific inactivation. Our previous results indicate that BMS is secreted into the hemolymph from the brain neurosecretory cells and works as a multifunctional neuropeptide. We showed expression of BMSR in various tissues, indicating that homologs of BMS simultaneously acts on a number of organs (12). On the other hand, BRFa is delivered directly to the PG surface by the innervating neurons to act as direct and tissue-specific inhibitors of ecdysteroidogenesis. In Manduca, the BMS homolog (F10) was the dominant FaRP in the hemolymph of feeding larvae, whereas BRFa homologs (F7D and F7G) were not detected as circulating hormones (28), in line with our hypothesis.

The overall developmental fluctuation of BRFa neuron firing frequency showed an inverse relationship with the hemolymph ecdysteroid titer (Fig. 5C), in accordance with the prothoracicostatic activity of BRFa. BRFa neuron activity is high during the feeding period of last-instar larvae (days 0–4), corresponding to the low ecdysteroid titer in the hemolymph. The activity decreases on the day before larvae start wandering (day 5), when the ecdysteroid titer increases gradually. BRFa neurons are completely inactive when the ecdysteroid titer reaches its highest level on the day before pupation (day 9). It is interesting that BRFa neurons transiently recover their activity after the hemolymph ecdysteroid titer begins to increase (days 6–8). This possible brief inactivation of the PG may prevent the sudden increase of the ecdysteroid titer, giving the larvae enough time to prepare for metamorphosis. This neural regulatory mechanism of ecdysteroidogenesis is summarized in Fig. 5D and E.

It is important to note that the general fluctuation of the hemolymph ecdysteroid titer can basically be explained by the developmental profile of the basal and PTTH-stimulated PG activity (5, 29, 30), although slight discrepancies still remain. For
example, a surge of basal activity of isolated PG during the feeding period has been reported in *Bombyx* and *Manduca*, which is not reflected by the increased hemolymph ecdysteroid titer in intact animals (29, 30). This discrepancy is presumably due to the removal of active neural inhibition from isolated PGs. As we stated above, BRFas may thus function as fine regulators of ecdysteroidogenesis, especially during feeding and wandering periods, when slight fluctuations of the ecdysteroid titer play important roles in insect development (23).

In addition to PTTH and FaRPs, there are a few more factors that are known to regulate the PG (1, 10, 31). Insects may use these factors in response to different signals that are important for regulation of ecdysteroidogenesis. These signals are then integrated as PG activity, thus coordinating the proper timing for regulation of ecdysteroidogenesis. These signals are then used. Wells were blocked with 0.5% (vol/vol) Tween 20 (PBST) just before use. Wells were blocked with 0.5% (vol/vol) Tween 20 (PBST) just before use. Wells were blocked with 0.5% (wt/vol) skim milk in PBST for 1 h, followed by rinsing with PBST. Sample or standard in 50 μl of PBST was then added to each well, followed by 50 μl of antiserum (anti-FMRFamide rabbit polyclonal antiserum, Bachem) diluted 1:2,000 with 0.5% skim milk in PBST. The plate was incubated for 3 h with shaking. After rinsing with PBST, 100 μl of enzyme-labeled secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate, Promega) diluted 1:5,000 in PBST was added to each well and incubated further for 2 h. Substrate (100 μg of p-nitrophenyl phosphate, Sigma-Aldrich) was added to each well in 100 μl of substrate buffer [50 mM sodium carbonate buffer containing 1 mM MgCl₂ (pH 9.6)] after rinsing with PBST, and the color was allowed to develop for 100 min. The reaction was stopped by adding 10 μl of 1 M NaOH to each well, and the absorbance was measured at 405 nm with a Model 550 Microplate Reader (Bio-Rad). Because BMS was used as a standard, the FMRamide content of each sample was expressed in BMS equivalents.

**Materials and Methods**

**Experimental Animals.** *B. mori* racial hybrids were fed on the artificial diet called Silkmate (Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 16-h light/8-h dark photoperiod and staged after the final (fourth) larval ecdysis. Most larvae started wandering behavior on day 6 of the fifth instar and pupated on day 10.

**FMRFamide ELISA.** Equal amounts of neuropeptides DVHVF-LRamide, DVVHSFLRamide, DVGHVFLRamide, and GOERNFLRamide were coupled to human serum albumin (HSA) as reported in ref. 35. ELISA plate wells were coated with the HSA-peptide conjugates overnight at 4°C and washed with PBS containing 0.05% (vol/vol) Tween 20 (PBST) just before use. Wells were blocked with 0.5% (wt/vol) skim milk in PBST for 1 h, followed by rinsing with PBST. Sample or standard in 50 μl of PBST was then added to each well, followed by 50 μl of antiserum (anti-FMRFamide rabbit polyclonal antiserum, Bachem) diluted 1:2,000 with 0.5% skim milk in PBST. The plate was incubated for 3 h with shaking. After rinsing with PBST, 100 μl of enzyme-labeled secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate, Promega) diluted 1:5,000 in PBST was added to each well and incubated further for 2 h. Substrate (100 μg of p-nitrophenyl phosphate, Sigma-Aldrich) was added to each well in 100 μl of substrate buffer [50 mM sodium carbonate buffer containing 1 mM MgCl₂ (pH 9.6)] after rinsing with PBST, and the color was allowed to develop for 100 min. The reaction was stopped by adding 10 μl of 1 M NaOH to each well, and the absorbance was measured at 405 nm with a Model 550 Microplate Reader (Bio-Rad). Because BMS was used as a standard, the FMRamide content of each sample was expressed in BMS equivalents.

**Purification of BRFa-3.** Residual fractions of the first purification step of *Bombyx* pupal brain extracts, which were previously used for the purification of BMS (12), were applied to the FMRFamide ELISA. One of the immunoreactive fractions (no. 19 in Fig. 1A) was loaded onto a PEGASIL-300 C8P column (4.6 × 250 mm, Senshu Kagaku, Tokyo) using a Waters 2695 Separations Module. Elution was performed with a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 60 min at a flow rate of 1 ml/min. The FMRFamide-positive fraction was then applied onto a PEGASIL-300 ODS-II column (4.6 × 250 mm, Senshu Kagaku). The column was eluted with a linear gradient of 10–40% acetonitrile in 0.1% TFA over 60 min at a flow rate of 0.5 ml/min. The immunoreactive fraction was further purified by passing over a PEGASIL-300 ODS-II column (4.6 × 250 mm), eluting it with a linear gradient of 10–40% acetonitrile in 0.1% heptfluorobutyric acid over 60 min at a flow rate of 0.5 ml/min.

**MALDI-TOF MS.** Mass spectra and postsource decay mass spectra of peptides were obtained with an AXIMA-CFR (Shimadzu) mass spectrometer. Recrystallized α-cyano-4-hydroxycinnamic acid was used as the matrix with a concentration of 5 mg/ml of acetonitrile/water (1:1 by volume) containing 0.1% trifluoroacetic acid. All spectra were measured in reflector mode.

**Synthetic and Recombinant Peptides.** BMS and BRFa peptides were all custom-synthesized and further purified by HPLC. The recombinant PTTH was prepared as described in ref. 36.

**Cloning of BRFa.** Using the amino acid sequence of the purified peptide as a query, BLAST analysis (BLASTN) was performed against *Bombyx* whole-genome shotgun sequence contigs by using KAIKOBLAST. Based on the putative coding sequence of the obtained contig (contig 105432, GenBank accession no. BAA0102501), RACE primers were designed as follows: sense primer, 5′-CGATTGGGAGGCCACCCTAGGC-3'; antisense primer, 5′-CTGAAGTGTGACCGACCTGCTGCTGCTG-3'. To obtain the whole cDNA sequence of *BRFa* (GenBank accession no. AB234100), RACE was performed by using the above primers and the SMART RACE cDNA Amplification Kit (BD Biosciences), according to the manufacturer's instructions. First-strand cDNA prepared from larval brain total RNA was used as a template.

**cAMP Assay and Ecdysteroid Assay.** The cAMP assay and the ecdysteroid assay were performed as described in ref. 12. For the cAMP assay, prothoracocostatic activity of a sample was expressed as an inhibition/activation index calculated with the following formula: inhibition/activation index = ([cAMP content of PG incubated with sample/cAMP content incubated without sample]).

**HEK293 Cell Expression and Ca2+ Imaging Analysis of BMSR.** BMSR cloned into the pME18S mammalian expression vector was transfected into HEK293 cells with the promiscuous G protein Gα15 as described in ref. 37. Ca²⁺ imaging analysis was performed as reported in ref. 37.

**Cloning and Characterization of BRFaR.** BRFaR (GenBank accession no. AB255356) was annotated from the genome sequence and cloned into the pME18S vector. The expression analysis was performed by using RT-PCR. See Supporting Materials and Methods, which is published as supporting information on the PNAS web site, for detailed information.
Cycler System (Cepheid, Sunnyvale, CA) essentially as described in ref. 39. After 1 min at 95°C, 40 cycles (95°C for 10 s and 68°C for 20 s) were carried out for the amplification. BRFa specific primers used were as follows: sense primer, 5'-CGATTTG-

In Situ Hybridization and Immunohistochemistry. For detection of BRFa expression in the CNS, we used a digoxigenin-labeled single-stranded DNA probe. Dig-11-dUTP (Roche Applied Science, Basel) was incorporated into single-stranded antisense DNA by asymmetric PCR according to a modified procedure (40). See Supporting Materials and Methods for the modified in situ hybridization procedure used in this study. After staining of neurons expressing the BRFa mRNA, the CNS was rinsed and incubated with a mixture of rabbit polyclonal antiserum to IF and rabbit polyclonal antiserum to GFAP. The tissue was then washed with PBST and incubated with a mixture of Alexa Fluor 488-labeled goat anti rabbit IgG and Alexa Fluor 555-labeled goat antimouse IgG (Invitrogen). BRFa expression was observed under a confocal laser microscope (510, Zeiss) using Nomarski DIC optics and FITC and rhodamine filters.

Direct MS Analysis. Direct MS analysis was performed based on previous reports (19, 41), which were slightly modified. Larvae were anesthetized in water for 10–20 min, and the PG-innervating nerve was dissected rapidly from the PG surface in sterile saline [0.85% NaCl (wt/vol)]. The dissected piece of the nerve was transferred onto a MALDI sample plate, and the droplet of saline transferred along with the nerve was blotted with cellulose paper. The tissue was then washed on the sample plate with 1 ml of pure water, which was again completely removed with cellulose paper. After drying the sample completely, it was covered with ∼100 nl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in methanol/water) by using a fine pipette. The whole preparation was again dried completely, and spectra were obtained with a Voyager-DE STR mass spectrometer (Applied Biosystems).

Electrophysiology. Efferent electrical activity of the BRFa neurons was recorded in situ throughout the last instar. Larvae were anesthetized briefly under CO2 and immobilized in silicon tubes. After the prothoracic region was opened ventrally, the BRFa nerve was cut just posterior to the prothoracic ganglion. The nerve was then drawn rapidly into a suction electrode filled with insect Ringer’s solution (154.8 mM NaCl/12.3 mM KCl/4.0 mM MgCl2/4.5 mM CaCl2/2.1 mM NaHCO3/0.1 mM NaHPO4/67.1 mM glucose/10.0 mM HEPES, pH 6.8). Spike activities of the efferent neurons were recorded with respect to an indifferent electrode placed in the hemolymph and amplified with a DAM50 biological amplifier (WPI Instruments, Waltham, MA) for extracellular recordings. Signals were digitized with an analog/digital converter (IDAC, Syntech, Hilversum, The Netherlands), and the number of spikes during the first 3 min of each recording was counted by using computer software (AUTO-SPIKE32, Syntech).

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