INTRODUCTION

Gonadotropin-releasing hormone (GnRH) neurons govern ovulation by responding to rising levels of estradiol with a phasic burst of neuronal firing, which leads to robust luteinizing hormone (LH) secretion (the LH surge). However, the neuronal and molecular basis for the generation of the GnRH/LH surge is still unclear. Glutamate, the major excitatory transmitter in the central nervous system, has been implicated in the generation of the LH surge (Iremonger et al., 2010). For example, glutamate release in the preoptic area increases during the LH surge (Jarry et al., 1995), and blockade of ionotropic glutamate receptors abolishes the surge (Brann and Mahesh, 1991; Ping et al., 1997). Furthermore, the expression patterns of each glutamate AMPAR subunit in GnRH neurons change dynamically during the estrous cycle (Bailey et al., 2006). However, it is largely unknown how glutamate regulates the LH surge.

Excitatory glutamatergic synaptic transmission is mainly mediated by the cation-permeable AMPARs. Synaptic trafficking of AMPARs, including the calcium permeable (CP)-AMPARs, is a major mechanism of circuit reorganization in vivo (Takahashi et al., 2003; Rumpel et al., 2005; Clem and Barth, 2006; Plant et al., 2006; Kessels and Malinow, 2009; Jitsuki et al., 2011; Mitsushima et al., 2011; Miyazaki et al., 2012a,b, 2013). CP-AMPARs lack the GluR2 subunit and exhibit prominent inward rectification, due to voltage-dependent blockade of the channel pore by polyamines at positive membrane potentials (Bowie and Mayer, 1995). The stoichiometric change in AMPARs that produces the GluR2-lacking CP-AMPARs plays a crucial role in the dynamics of synaptic functions (Clem and Barth, 2006; Plant et al., 2006). Here, we investigated the postsynaptic change in the stoichiometry of AMPAR subunits in GnRH neurons during the estrous cycle.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats (7–8 weeks of age, Charles River, Yokohama, Japan) were maintained at a constant temperature of 24–26 °C under controlled lighting conditions (lights on 5:00–19:00) with food and water available ad libitum. In electrophysiological studies, Wistar rats (7–12 week-old males; sexually mature females, 7–8 weeks old; juvenile
females, 4–5 weeks old) carrying an eGFP-tagged transgene driven by the GnRH promoter (Kato et al., 2003) were used. Daily vaginal smears were taken from female rats to determine the estrous cycle stage. Ovariectomized rats were prepared as described previously (Nishihara et al., 1994). Estradiol treatment was performed under anesthesia by isoflurane gas at around noon, 3 days before the experiment by implanting a silastic tube (inner diameter, 1.5 mm; outer diameter, 2.5 mm; length, 25 mm) containing 20% 17β-estradiol (Sigma Chemical Co., USA) subcutaneously. We showed previously that this procedure results in a surge of LH secretion 3 days later (Nishihara et al., 1994). All animal housing and surgical procedures were in accord with the guidelines laid down by the institutional animal care and use committee of the Yokohama City University School of Medicine.

(A) (B) (C) (D) (E) (F) (G) (H) (I)
LH assay
An intra-atrial cannula was implanted through the jugular vein on the morning of proestrus, between 8:00 and 9:00, under ether anesthesia. Serum concentrations of LH were measured by an ELISA kit (AKRLH-010, Shibayagi, Co., Ltd., Shibukawa, Japan). Blood samples (approximately 120 μl each) were collected at 1-h intervals from 13:00 to 19:00 from freely moving rats. The minimally detectable amount (95% confidence limit of buffer controls) of LH was 0.12 ng/ml. The inter-assay and intra-assay coefficients of variation estimated in five replicated assays of pooled serum samples with a mean LH concentration of 9.2 ng/ml were 4.2% and 3.8%, respectively.

Brain surgery
A bilateral cannula guide system, with stainless steel cannuli (outer diameter, 0.65 mm; length, 13 mm) was stereotaxically placed into the preoptic area (stereotaxic coordinates: A = 7.0, V = 3.2 and L = ±0.5, interaural 0) of animals under sodium pentobarbital anesthesia (31.5 mg/kg i.p.). The animals were allowed to recover for at least 10 days. After the intraventricular cannulation, 3 mmol of NASPM (Tsubokawa et al., 1995), a specific antagonist for GluR2-lacking AMPARs, dissolved in 1.0 μl saline was injected through the implanted cannula into the preoptic area at 9:00. The same volume of saline was injected into control animals. The injection sites were verified after the experiment.

Electrophysiology
Rats were anesthetized with isoflurane gas, and the brain was removed. Each brain was quickly transferred into ice-cold dissection buffer (25.0 mM, NaHCO₃, 1.25 mM NaH₂PO₄; 2.5 mM KCl, 0.5 mM CaCl₂, 7.0 mM MgCl₂; 25.0 mM glucose, 110.0 mM chloride chloride, 11.6 mM ascorbic acid, 3.1 mM pyruvic acid), bubbled with 5% CO₂/95% O₂. Coronal brain slices were cut (295 μm, Leica vibratome, Germany) in dissection buffer and transferred to physiological solution (22–25 °C, 118 mM NaCl, 2.5 mM KCl, 26.2 mM NaHCO₃, 1 mM NaH₂PO₄, 11 mM glucose, 1.3 mM MgCl₂, 2.5 mM CaCl₂, pH 7.4, gassed with 5% CO₂/95% O₂). The recording chamber was perfused with physiological solution containing 0.1 mM picrotoxin, 4 mM 2-chloroadenosine at 22–25 °C to reduce the basal activity.

Patch recording pipettes (4–6 MΩ) were filled with intracellular solution (115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM Na₃ATP, 0.4 mM Na₃GTP, 10 mM sodium phosphocreatine, 0.6 mM EGTA at pH 7.25). Whole-cell recordings were obtained from GnRH neurons with an Axopatch-1D amplifier (Axon Instruments, USA). Concentric tungsten-stimulating electrodes were placed in the organum vasculosum laminae terminalis. Synaptic AMPAR-R-mediated responses at −60 mV and +40 mV were averaged over 30 trials and their ratio was used as an index of rectification. The data were expressed as the percentage of the control values, and statistical comparisons were made by non-parametric analysis. Measurements of the frequency and amplitude of mEPSCs were recorded in bath solution (118 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 10 mM glucose, 1 mM MgCl₂, and 4 mM CaCl₂) containing 0.5 μM TTX and 0.1 mM picrotoxin. The miniature excitatory postsynaptic currents (mEPSCs) were detected by setting the amplitude threshold to the background noise level × 3. PPR (paired-pulse ratio) was measured by using two-paired stimuli with an interval of 50, 100, 200, 300, or 400 ms. The PPR (2nd/1st EPSP slope) was evaluated for each interpulse interval.

Drug application
All drugs were applied by bath perfusion. In our hands, 2 min was required for the entire chamber solution to be replaced with another solution. Thus, drug effects were tested 5 min after their administration.

Statistics
For comparison between means, we used the Kruskal–Wallis test, unpaired t-test, paired t-test, one-way factorial
analysis of variance (ANOVA) with post hoc test, (Fisher PLSD test), or repeated measures ANOVA. Data are reported as means and SEM, unless otherwise noted.

RESULTS

To identify GnRH neurons in brain slices, we utilized transgenic rats expressing green fluorescent protein (GFP) driven by the GnRH-promoter (Kato et al., 2003). We prepared acute brain slices that included the preoptic area of male and female transgenic rats, and performed whole-cell recordings from the GnRH neurons in response to stimulation of the organum vasculosum laminae terminalis (Fig. 1A). We measured the rectification index (RI: response at −60 mV/response at +40 mV) of AMPA transmission. Because bath applications of 2 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocked the evoked responses (data not shown), these responses were necessarily mediated by AMPA receptors.

We found an increased RI on the day of proestrus in female rats compared to male rats or female rats on other days of the estrus cycle (Fig. 1B, C), indicating that postsynaptic CP-AMPARs are inserted into GnRH neuron synapses upon proestrus. In the presence of NMDA receptor antagonist (APV) or kainate receptor antagonist (UBP301), we still observed a prominent increase of RI on the day of proestrus (Fig. 1D), indicating that this effect is due to the change in the stoichiometry of AMPARs. This increase was similar when mature proestrus females were compared with juvenile females (Fig. 1E). Therefore, we hypothesized that the rise of blood estrogen levels during proestrus is required for the synaptic incorporation of CP-AMPARs. To test this, ovariectomized (OVX) rats were primed with estradiol at a dose that induces the LH surge (see Experimental procedures). The RI was significantly higher in OVX rats primed with estradiol than in vehicle-injected animals (Fig. 1F). These results indicate that CP-AMPARs are inserted into synapses onto GnRH neurons on the day of proestrus in female rats by the transient rise of estradiol.

We next analyzed the AMPAR-mediated synaptic responses of GnRH neurons before and after the application of NASPM (1-naphthyl acetyl spermine), an antagonist of CP-AMPARs, during different stages of the estrus cycle. We detected decreased AMPA responses after the application of NASPM compared to baseline, but only on the proestrus day (Fig. 1G), indicating that the synaptic insertion of CP-AMPARs contributes to AMPAR-mediated synaptic transmission to GnRH neurons only in proestrus rats. We also detected a significant increase in the frequency of AMPAR-mediated mEPSCs during proestrus compared to other estrus cycle stages. There was no significant difference in the mEPSC amplitude on any day of the estrus cycle, except the day of estrus, when it decreased (Fig. 1H). The PPR was not modified by the estrus cycle stage (Fig. 1I), suggesting that the increased frequency of mEPSCs on the day of proestrus can be due to the synaptic incorporation of CP-AMPARs at newly synthesized spines as well as existing synapses, rather than to presynaptic changes in glutamate release. The decreased amplitude of mEPSC on the day of estrus could be due to a homeostatic plasticity mechanism. Thus, the glutamatergic synapses are strengthened only transiently, on the day of proestrus.

To investigate the effect of the synaptic insertion of CP-AMPARs in proestrus rats on the LH surge generation, we examined whether the increase of synaptic CP-AMPARs in GnRH neurons on the day of proestrus was required for the LH surge. We injected NASPM (3 nM, 1 μl) bilaterally into the preoptic area (POA) of proestrus rats at 9:00AM, and measured serum LH levels by serial blood sampling. NASPM infusion significantly attenuated the LH surge (Fig. 2), supporting the idea that synaptic delivery of CP-AMPARs is involved in its generation.

DISCUSSION

Taken together, our data support the idea that the transient rise of estradiol on the day of proestrus strengthens glutamatergic synapses on GnRH neurons by the synaptic trafficking of CP-AMPARs, and that the newly incorporated receptors are involved in the generation of the LH surge.

Since GnRH neurons receive only a few synapses (Witkin et al., 1995), GnRH neurons involved in the LH surge are largely regulated extra-synaptically by a variety of neuropeptides (Kalra, 1993; Blaustein, 2010; Campbell and Suter, 2010). Indeed, it was difficult to obtain synaptic responses from the GnRH neurons, indicative of the limited number of excitatory synapses on them. Nevertheless, GnRH neurons possess a glutamatergic synaptic architecture (Witkin et al., 1995; Kiss et al., 2003; Khan et al., 2010), and the expression patterns of each glutamate AMPAR subunit in GnRH neurons change dynamically during the estrus cycle (Bailey et al., 2006). These previous findings are consistent with our present results, which suggest a
physiological role for the phasic synaptic incorporation of postsynaptic CP-AMPARs by GnRH neurons, at the time of the LH surge.

CP-AMPARs (which lack GluR2) exhibit a larger single-channel conductance than GluR2-containing AMPARs, and they have been implicated in synaptic plasticity (Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007; Conrad et al., 2008). In the present study, we investigated phasic changes in the stoichiometry of AMPA subunits in GnRH neurons, and found that CP-AMPARs were incorporated into GnRH synapses only on the day of proestrus. Thus, the synaptic incorporation of CP-AMPARs could regulate the generation of the LH surge by enhancing the synaptic responses of GnRH neurons. Our results represent the first evidence of a phasic change in the stoichiometry of synaptic AMPARs, and they delineate its physiological role.

The remaining question is what induces phasic changes in the stoichiometry of AMPA subunits? It appeared that an increase in the AMPARs lacking the GluR2 subunit could be in response to an increase in serum estrogen, because the increase in the RI coincided with the day of proestrus, when elevated serum estrogen is observed (Butcher et al., 1974). In support of this, we showed that estrogen-priming in ovariectomized rats induced a daily LH surge (Nishihara et al., 1994) and increased the RI, as a result of increasing the synaptic GluR2-lacking CP-AMPARs at GnRH neurons. However, how estrogen regulates the stoichiometry of AMPARs at GnRH synapses remains to be elucidated.

Acknowledgments—This project was supported by the Special Coordination Funds for Promoting Science and Technology (T.T.), the Sumitomo Foundation (T.T.), and “Development of biomarker candidates for social behavior,” carried out under the Strategic Research Program for Brain Sciences by the Ministry of Education, Culture, Sports, Science and Technology of Japan (T.T.). We also thank Yoshiko Kanno for excellent technical assistance.

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(Accepted 19 June 2013)
(Available online 27 June 2013)