



Published in final edited form as:

Metabolism. 2016 April ; 65(4): 522–532. doi:10.1016/j.metabol.2015.12.011.

Visualizing estrogen receptor- α -expressing neurons using a new ER α -ZsGreen reporter mouse line

Kenji Saito^{1,4}, Yanlin He^{1,4}, Xiaofeng Yan¹, Yongjie Yang¹, Chunmei Wang¹, Pingwen Xu¹, Antentor Othrell Hinton Jr.¹, Gang Shu¹, Likai Yu¹, Qingchun Tong³, and Yong Xu^{1,2,*}

¹Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

²Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

³Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston, Houston, TX 77030

Abstract

Background—A variety of biological functions of estrogens, including regulation of energy metabolism, are mediated by neurons expressing estrogen receptor- α (ER α) in the brain. However, complex intracellular processes in these ER α -expressing neurons are difficult to unravel, due to the lack of strategy to visualize ER α -expressing neurons, especially in unfixed brain tissues.

Results and Conclusions—Here we generated a novel ER α -ZsGreen reporter mouse line in which expression of a green fluorescent reporter protein, ZsGreen, is driven by a 241 kb ER α gene promoter. We validated that ZsGreen is highly colocalized with endogenous ER α in the brain. Native ZsGreen signals were visualized in unfixed brain tissue, and were used to assist single cell collection and electrophysiological recordings. Finally, we demonstrated that this ER α -ZsGreen mouse allele can be used in combination with other genetic reporter alleles to allow experiments in highly selective neural populations.

Keywords

transgenic mouse; brain; estrogen receptor- α ; electrophysiology

*Correspondence should be addressed to: Yong Xu, 1100 Bates Street, Rm 8070, Houston, Texas 77030. yongx@bcm.edu, Telephone: (713)-798-7199, Fax: (713)-798-7187.

⁴These authors contributed to this work equally.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest. No potential conflicts of interest relevant to this article were reported.

Introduction

Estrogen receptor- α (ER α) plays important roles in many functions throughout the central nervous system (CNS). For example, during early development, signals mediated by brain ER α , as well as other ER isoforms (e.g. ER β), are essential to establish sex differences, promote neurite growth, and regulate synaptic patterning [1]. In addition, ER α in adult brains plays important roles in a variety of physiological processes, including female fertility [2, 3], sexual behaviors [4], cardiovascular activities [5] and cognition [6].

In particular, accumulating evidence indicates that ER α -expressing neurons in the brain are critically involved in the regulation of energy homeostasis [7–9]. This notion was first supported by early observations that microinjections of ER α agonists into various brain regions change animal's feeding behavior and body weight [10, 11]. Confirming a critical role of brain ER α in body weight control, we showed that mutant mice lacking ER α selectively in the brain develop obesity despite higher circulating levels of 17 β -estradiol [12]. Recent efforts in the field have led to an appreciation that estrogenic actions on different aspects of energy balance are mediated by distinct ER α neural populations in the brain. For example, the inhibitory effects of 17 β -estradiol on food intake are mediated by ER α expressed in the arcuate hypothalamic nucleus (ARH) [12], the dorsal nucleus raphe (DR) [13] and the nucleus of the solitary tract (NTS) [14]; ER α in the ventrolateral subdivision of the ventromedial hypothalamic nucleus (vlVMH), however, mediates estrogenic actions to enhance energy expenditure but does not affect feeding [12, 15–17]; ER α in the medial amygdalar nucleus (MeA) is functionally required to maintain normal physical activity [18].

With these advances in understanding the critical brain ER α populations in energy homeostasis, increasing efforts have been spent on unravelling the complex cellular behaviors and the underlying mechanisms of these ER α neurons [15, 19, 20]. However, characterizing the phenotype and understanding the functions of ER α -expressing neurons in the brain has been challenging, mainly because of the lack of tools to allow *direct* visualization of ER α -expressing cells, which are often located in brain regions comprised of heterogeneous neurons with various neurochemical identities. For example, ER α expressed by pro-opiomelanocortin (POMC) neurons in the ARH has been implicated in the regulation of food intake [12]. However, the effects of ER α signals on POMC neuron functions are difficult to assess, because only a portion (about 30%) of POMC neurons express ER α while many other POMC neurons do not [12]. Adding to the complexity, within the ARH, there are abundant ER α -expressing neurons that do not express POMC [21]. Thus, in order to rigorously study a specific ER α population (e.g. ER α (+) POMC neurons), one would need a tool to reliably identify ER α -expressing cells of interest.

In this study, we used the bacterial artificial chromosome (BAC) engineering to generate a new transgenic mouse line, namely ER α -ZsGreen, in which expression of a green fluorescent reporter protein (ZsGreen) was driven by a long (241 kb) ER α gene promoter. We used several different approaches to validate that the ZsGreen reporter can be used, in either fixed or unfixed brain tissue, to identify ER α -expressing cells. We further tested effects of a selective ER α agonist on the firing activities of ZsGreen (+) cells in the ARH.

Finally, we demonstrated that this ER α -ZsGreen mouse allele can be used in combination with other genetic reporter alleles to allow experiments in double-selected neural populations.

Methods

Generation of ER α -ZsGreen transgenic mice

A BAC clone (#RP23-283O2) was obtained from BAC PAC Resource Center at Children's Hospital Oakland Research Institute (CHORI). This BAC clone was derived from the C57BL/6J mouse strain and covers exon 1 to 4 of ER α gene. ZsGreen1 cDNA was prepared from pIRES-ZsGreen1 plasmid (Clontech, Mountain View, CA). The engineering of BAC transgenic vector was conducted in *E. coli* DH10B cells, using the Counter-Selection BAC Modification Kit (Gene Bridges, Heidelberg, Germany) following the manufacturer's manual. As illustrated in Figure 1, the ZsGreen cDNA was introduced into translation start site in exon 2 of the ER α gene in the BAC clone.

Large scale of the recombinant ER α -ZsGreen BAC DNA was prepared by NucleoBond Xtra BAC Kit (Macherey-Nagel, Germany), which were linearized by PI-SceI digestion, purified with phenol-chloroform, precipitated with ethanol. Transgenic mice were obtained by injecting the linearized ER α -ZsGreen BAC DNA into pronuclei of C57BL/6 mice in the Genetically Engineered Mouse Core at Baylor College of Medicine. ER α -ZsGreen founders were maintained at a C57BL/6 background. Tail biopsies were used for the genotyping on PCR with primers Esr1ATG-393 F 5'-CCAGCAGCGAAGACCTGGAAAGT-3' and ZsGr +236 R 5' - ATGTCCTGGGGGTACTCGGTGAA-3' producing the amplicon of 629 bp long.

Mice

ER α -ZsGreen mice were bred with C57Bl6 mice to produce ER α -ZsGreen mice for studies. In parallel, ER α -ZsGreen mice were bred with mice that carried both POMC-CreER [22] and Rosa26-tdTOMATO alleles [23] to produce ER α -ZsGreen/POMC-CreER/ Rosa26-tdTOMATO triple transgenic offspring. These ER α -ZsGreen/POMC-CreER/ Rosa26-tdTOMATO mice received i.p. injection of tamoxifen (0.2 mg/g) at 6 weeks of age to induce Cre recombinase activity.

Care of all animals and procedures were approved by Baylor College of Medicine Institutional Animal Care and Use Committee. Mice were housed in a temperature-controlled environment at 22°C–24°C using a 12 hr light/12 hr dark cycle. The mice were fed on standard chow with minimal phytoestrogens (4% fat, #2916, Harlan-Teklad) and water was provided ad libitum. All mice were on a pure C57Bl/6 background.

Tissue processing for histology

Both male and female ER α -ZsGreen mice (8–10 weeks of age) were anesthetized with isofluorane inhalation and were perfused intracardially with saline followed by 10% formalin. To avoid the influences of the estrous cycle on ER α expression, all female mice were perfused at the diestrus which was determined by vaginal cytology. The brains were

removed, postfixed two hours in the same fixative, and then immersed in 20% sucrose in PBS at 4°C until sinking in the solution. 25- μ m-thick coronal sections throughout whole brains were cut by freezing microtome, collected in cryoprotective solution (20% glycerol, 30% ethylene glycol in PBS) and stored at -20°C until use.

DAB-immunohistochemistry for ZsGreen

Whole immunostaining procedures were carried out with free-floating sections at room temperature. After thorough washing in PBS, to block endogenous peroxidase activity, sections were treated with 3% H₂O₂ in methanol for 30 min and washed in PBS. Sections were blocked for 1 hr with blocking solution (PBS containing 3% normal goat serum and 0.25% Triton-X), and then incubated overnight in blocking solution containing rabbit anti-ZsGreen antibody (1:1000, Clontech, Mountain View, CA). After washing in PBS, sections were incubated in blocking solution containing biotinylated goat anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for 2 hours, then washed again and incubated in avidin-biotin complex (Vectastain ABC Elite kit, Vector laboratories, Inc. Burlingame, CA). Immunolabeled ZsGreen was visualized with 0.1% diaminobenzidine/0.01% cobalt chloride/0.01% nickel sulfate/0.01% H₂O₂ in PBS. Coloring was stopped by rinsing sections thoroughly in PBS. Sections were then mounted on Poly-L-Lysine glass slides and air dried. Finally, they were dehydrated with ascending series of ethanol, cleared in xylene, and coverslipped with Cytoseal (Richard Allen Scientific, Kalamazoo, MI). Photographs were taken with Leica Microscope using MM AF software. Anatomical identification of brain structures was conducted with the aid of Allen Reference Atlas (mouse.brain-map.org).

Colocalization of ER α and ZsGreen

Brain sections were prepared as same as in immunoperoxidase labelling. After thorough washing in PBS, sections were incubated for 1 hr with blocking solution (PBS containing 3% normal goat serum and 0.25% Triton-X), and then incubated overnight in blocking solution containing rabbit anti-ER α antibody (1:1000, Millipore, Billerica, MA). After washing in PBS, sections were incubated in blocking solution containing biotinylated goat anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch Laboratories, Inc) for 2 hours, then washed again and incubated in blocking solution containing Dylight 594 streptavidin (1:500, Jackson ImmunoResearch Laboratories, Inc.). After washing in PBS, the sections were mounted on Poly-L-Lysine glass slides and air dried. Finally, they were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories). Under the Leica 5500 fluorescence microscope equipped with optigrad illumination, native ZsGreen signals were directly visualized with the green fluorescence filter and ER α immunoreactivity was visualized with the red fluorescence filter.

Single-cell RT-PCR analysis

Six to twelve-week old ER α -ZsGreen mice were deeply anesthetized with isoflurane inhalation in early morning. Mice were then decapitated, and the entire brain was removed and immediately submerged in ice-cold sucrose-based cutting solution (adjusted to pH 7.3) containing (in mM) 10 NaCl, 25 NaHCO₃, 195 Sucrose, 5 Glucose, 2.5 KCl, 1.25 NaH₂PO₄, 2 Na pyruvate, 0.5 CaCl₂, 7 MgCl₂ bubbled continuously with 95% O₂ and 5%

CO₂, as we did before [13]. The slices containing the ARH region (250 μm) were cut with a Microm HM 650V vibratome (Thermo Scientific). Slices were incubated for 15 min at room temperature in PIPES buffer (in mM): 120 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 25 D-glucose, 20 PIPES, 100% O₂ [24], and then for 60 min at 33°C in PIPES containing trypsin (Sigma-Aldrich, type XI; 0.5 mg/ml). After enzymatic treatment, slices were rinsed and maintained in PIPES buffer at room temperature for 60 min. Slices were transferred to DMEM buffer (Invitrogen, Life Technologies), and the ARH region was identified and excised under a fluorescence-equipped dissecting microscope. The tissue was triturated gently in DMEM buffer using a series of fire-polished Pasteur pipettes (600, 300, and 150 μm, inner diameter) and the DMEM/neuron suspension was placed in a recording chamber on a fixed-stage fluorescence microscope (Eclipse FN-1, Nikon).

16 ZsGreen(+) and 16 ZsGreen(−) cells were visualized and collected via micropipette, and put in a PCR tube containing reverse transcription reaction mixture. cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, MD), according to the manufacturer's instruction. After the RT reaction, regular PCR was performed to detect ERα and β-actin (as an internal control) using two-step PCR protocol with REExtract-N-Amp PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The first round multiplex PCR contained 3 μl of cDNA, 2 μl of primer sets, 10 μl of REExtract-N-Amp PCR Reaction Mix, and 5 μl of water. The PCR condition was composed of an initial 4 min at 94°C, then 30 cycles of 15 sec at 94°C, 60 sec at 60°C, 90 sec at 72°C, and 10 min at 72°C. The first PCR was purified using NucleoSpin PCR Clean-up Kit (Macherey-Nagel, Germany) and eluted with 25 μl of the bundled Buffer NE. The second round PCR was performed individually for each gene using SsoAdvanced Universal SYBR Green Supermix with 5 μl SYBR Green, 1.2 μl primer set, 3.8 μl purified first round PCR. The amplicons of the second round PCR were separated on 12% polyacrylamide gel electrophoresis for ERα or on 1.5% agarose gel electrophoresis for β-actin. Primer sequences and the size of amplicon are shown below: ERα (5'-TGATGATTGGTCTCGTCTG-3' and 5'-TACCTTGATTCTGTCCAG-3', 87 bp), and β-actin (5'-ATGGAGGGGAATACAGCC-3' and 5'-TTCTTTGCAGCTCCTTCGTT-3', 148 bp). For the ERα primers, the forward and reverse primers hybridize to exon 6 and exon 6/7 junction, respectively, which are not covered by the BAC clone. As a positive control, total cDNA extracted from a mouse hypothalamus was used and subjected to regular PCR using the same ERα primers.

Electrophysiology

ERα-ZsGreen mice or ZsGreen/POMC-CreER/Rosa26-tdTOMATO mice (at 6–10 weeks of age) were used for electrophysiological recordings. Briefly, mice were deeply anesthetized with isoflurane and then decapitated, and the entire brain was removed and immediately submerged in ice-cold sucrose-based cutting solution. Using a Microm HM 650V vibratome (Thermo Scientific), the brains were cut into coronal slices (without trimming at other dimension) at the thickness of 250 μm each. Usually, three to four consecutive brain slices per mouse, ranging from Bregma −2.54 mm to −1.46 mm, contained the ARH and adjacent nuclei (the ventromedial hypothalamic nucleus, the dorsal medial hypothalamus and the

lateral hypothalamus) were used for recordings. The slices were recovered for 1 h at 34°C and then maintained at room temperature in artificial cerebrospinal fluid (aCSF, adjusted to pH7.3) containing (in mM) 126 NaCl, 2.5 KCl, 2.4 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, 11.1 glucose, and 21.4 NaHCO₃) saturated with 95% O₂ and 5% CO₂ before recording.

Slices were transferred to the recording chamber and allowed to equilibrate for at least 10 min before recording. The slices were superfused at 34°C in oxygenated aCSF at a flow rate of 1.8–2 ml/min. ZsGreen(+) neurons, ZsGreen(-) neurons, or ZsGreen(+)/TOMATO(+) neurons in the ARH were visualized using epifluorescence and IR-DIC imaging on an upright microscope (Eclipse FN-1, Nikon) equipped with a moveable stage (MP-285, Sutter Instrument). Patch pipettes with resistances of 3–5 MΩ were filled with intracellular solution (adjusted to pH 7.3) containing (in mM) 128 K gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl₂, 0.3 Na-GTP and 3 Mg-ATP. Recordings were made using a MultiClamp 700B amplifier (Axon Instrument), sampled using Digidata 1440A and analyzed offline with pClamp 10.3 software (Axon Instrument). Series resistance was monitored during the recording, and the values were generally < 10 MΩ and were not compensated. The liquid junction potential was +12.5 mV, and was corrected after the experiment. Data were excluded if the series resistance increased more than 20% during the experiment or without overshoot for action potential. Currents were amplified, filtered at 1 kHz, and digitized at 20 kHz. Frequency and peak amplitude were measured using the Mini Analysis program (Synsoft, Inc.). Current clamp was engaged to test neural firing frequency at the baseline and after bath perfusion of 100 nM propyl pyrazole triol (PPT, a selective ERα agonist) [25] for 6 min. The concentration of PPT was chosen based on previous studies showing that effects of PPT at this dose were blocked by genetic deletion of ERα [13]. The values for resting membrane potential and firing frequency were averaged within 2-min bin at the baseline or after PPT perfusion. In some experiments, the aCSF solution also contained 1 μM tetrodotoxin (TTX) [26] and a cocktail of fast synaptic inhibitors, namely bicuculline (50 μM; a GABA receptor antagonist) [27], AP-5 (30 μM; an NMDA receptor antagonist) [28] and CNQX (30 μM; an AMPA receptor antagonist) [28] to block the majority of presynaptic inputs.

Statistics

The data for electrophysiology are presented individually for each recorded neuron. Statistical analyses were performed using GraphPad Prism. Comparisons between before and after PPT treatment were made by paired t-test. P<0.05 was considered to be statistically significant.

Results

Distribution of ZsGreen in ERα-ZsGreen mouse brains

Three transgenic ERα-ZsGreen founder lines were obtained. We used an anti-ZsGreen antibody to detect ZsGreen immunoreactivity in the brains from each line. Line 1 did not express any ZsGreen in the brain; ZsGreen immunoreactivity was detectable in both line 2 and line 3, with stronger signals in line 2 than in line 3. We then systemically characterized the distribution of ZsGreen in fixed brain sections from line 2, using DAB-

immunohistochemistry (Figure 2). As summarized in Table 1, the distribution pattern of ZsGreen in our ER α -ZsGreen mouse is largely consistent with distribution of endogenous ER α immunoreactivity in mouse brains as reported previously [29].

Colocalization of ZsGreen and endogenous ER α

We then directly visualized native ZsGreen signals, without any immunohistochemical staining procedures, in fixed brain sections obtained from both male and female mice. ZsGreen signals were distributed broadly in the cells, including the nucleus and the cytosol (Figure 3). Interestingly, in some cells, ZsGreen signals appeared to punctuate in intracellular organelles (asterisk-pointed neurons in Figure 3). We also used immunofluorescence to detect the endogenous ER α in the same brain sections, and examined the colocalization of native ZsGreen and ER α immunoreactivity in several brain regions with abundant ER α , including the bed nucleus of the stria terminalis (BNST), the arcuate hypothalamic nucleus (ARH) and the ventrolateral subdivision of the ventromedial hypothalamic nucleus (vlVMH). In all these three regions, the majority of ZsGreen (+) cells co-expressed ER α ; similarly, most of ER α (+) cells co-expressed ZsGreen (Figure 3). In particular, more than 90% of ZsGreen (+) neurons in female brains were confirmed to co-express ER α ; this overlapping pattern was slightly lower in male brains (80–95%, Table 2). In addition, we found that about 70–87% ER α (+) neurons in male/female brains were labelled by native ZsGreen (Table 2).

ZsGreen (+) single cells express ER α mRNA

To further confirm that ZsGreen (+) cells do express ER α mRNA, we collected ZsGreen (+) single cells in the ARH from unfixed brain slices, and performed single cell RT-PCR to detect ER α mRNA. Remarkably, ER α mRNA was detected in 14 out of 16 ZsGreen (+) cells (see Figure 4 for representative data). As a negative control, we also isolated ZsGreen (–) single cells from the same brain slices, and only detected ER α mRNA in 4 out of 16 cells (see Figure 4 for representative data). β -actin mRNA was detected in most ZsGreen (+) and ZsGreen (–) single cells, confirming good quality of mRNA samples (Figure 4).

An ER α agonist stimulates firing of ZsGreen (+) cells in the ARH

Using the whole-cell current clamp electrophysiology, we examined effects of a highly selective ER α agonist, propyl pyrazole triol (PPT) [25] on the firing activities of ZsGreen (+) cells in the ARH (Figure 5A). We showed that PPT (100 nM, bath for 6 min) depolarized 13/15 ZsGreen (+) cells tested (see Figure 5B for representative traces and Figure 5D for summary). We further analysed effects of PPT on firing frequency in 12 ZsGreen (+) cells with spontaneous firing activity and found that PPT significantly increased firing frequency in these cells (see Figure 5B for representative traces and Figure 5E for summary). We also tested effects of PPT on 8 ZsGreen (–) cells, and failed to detect any effects (see Figure 5C for representative traces and Figure 5D–5E for summary). In addition, we found that PPT depolarized 13/13 ZsGreen (+) cells in the presence of TTX and a cocktail of fast synaptic inhibitors (CNQX, AP-5 and bicuculline) (see Figure 5F for representative traces and Figure 5G for summary), which confirmed a direct action of PPT on these ZsGreen (+) cells.

Electrophysiological recordings from identified ER α (+) POMC neurons in the ARH

We then crossed both the POMC-CreER [22] and Rosa26-tdTOMATO alleles [23] onto our ER α -ZsGreen mice to produce ER α -ZsGreen/POMC-CreER/Rosa26-tdTOMATO mice. Tamoxifen was injected into these mice to induce Cre recombinase activity in POMC neurons, which led to expression of the red fluorescence protein, TOMATO, specifically in POMC neurons. Thus, cells labelled by both TOMATO and ZsGreen in the ARH were identified as ER α (+) POMC neurons (Figure 6A). Using the whole-cell current clamp electrophysiology, we examined effects of PPT on the firing activities of ER α (+) POMC neurons, and found that PPT (100 nM, bath for 6 min) significantly depolarized these neurons and increased their firing frequency (n = 10; see Figure 6B for representative traces and Figure 6C–6D for summary).

Discussions

In the present study, we generated a new reporter mouse line, in which expression of a green fluorescence protein, namely ZsGreen, is driven by a long ER α promoter (about 241 kb). Several different approaches were used to validate this model. First, we showed that the distribution of ZsGreen is largely consistent with reported ER α expression pattern in mouse brains [29]. We further confirmed that the majority of neurons labelled by native ZsGreen signals co-express endogenous ER α . In addition, using native ZsGreen signals as a guide, we collected single cells from unfixed brain slices, and showed that most of these ZsGreen (+) cells express ER α mRNA. At the functional level, we showed that a selective ER α agonist exclusively stimulated ZsGreen (+) cells in the ARH, but not ZsGreen (–) cells in the same region. Collectively, these results validated that ZsGreen is faithfully expressed in ER α -expressing cells in the brain.

Prior to our studies, several ER α promoter-driven reporter lines have been described. However, the fidelity of reporter expression in these lines was less than ideal. For example, Hamada et al. generated a transgenic rat expressing eGFP under the control of a 2.1 kb rat ER α gene promoter [30]. In these rats, eGFP was barely detected in the ARH and vVMH, regions where abundant ER α is expressed [29] and functional [12]. Further, in the BNST, only 70% of eGFP (+) neurons co-express ER α , and even a smaller portion of ER α (+) neurons are labelled by eGFP [12]. More recently, Matsuda et al. generated a transgenic mouse line in which expression of eGFP is driven by a 6 kb mouse ER α gene promoter [31]. Similar to the rat reporter, many ER α (+) neurons in the BNST, vVMH and ARH do not express eGFP in this ER α -eGFP mouse line [31]. On the other hand, we demonstrated a greatly improved fidelity in our ER α -ZsGreen reporter line. We speculate that this improvement resulted from the much longer promoter (approximately 241 kb) we used to drive ZsGreen expression, which may have been more faithful to the endogenous ER α promoter activity.

Lee et al. developed and validated an *Esr1*^{Cre} mouse line that expresses Cre recombinase in ER α -positive cells [32]. In combination with viral vectors expressing Cre-inducible optogenetic channels (e.g. channelrhodopsin and halorhodopsin), this *Esr1*^{Cre} mouse line was used to achieve photostimulation or photoinhibition of ER α (+) neurons in the vVMH [32]. Notably, one can use the similar strategy, injecting Cre-inducible reporter viral vectors,

to label ER α (+) neurons in any region of mouse brains, although precise stereotaxic viral injections could be technically challenging. Alternatively, one can cross this Esr1^{Cre} mouse line to a Cre-inducible reporter line (e.g. Rosa26-tdTOMATO) to generate offspring mice expressing the reporter selectively in ER α (+) cells. However, one caveat is that the reporter will be expressed not only in “current” ER α (+) cells, but also in cells with only transient ER α promoter activity. Given the dynamic changes in brain ER α expression during development [33] and depending on the estrogen milieu [34], these “transient” ER α (+) cells are expected to exist in large numbers and may substantially confound the experiments.

Our ER α -ZsGreen reporter mouse line offers a reliable and easy-to-use tool to visualize ER α (+) cells in mouse brains, which could benefit the field of estrogen research through various experimental designs. Obviously, the ZsGreen fluorescence reporter can replace the conventional staining procedures (e.g. immunohistochemistry or in situ hybridization) required to visualize ER α (+) cells; ZsGreen can also be easily combined with another staining protocol to determine if ER α (+) cells express other proteins or mRNAs. In addition to these histological applications in usually fixed tissues, one can also use ZsGreen as a marker to directly identify ER α (+) cells in unfixed tissues. For example, we took advantage of this feature to collect single ER α (+) cells. In addition, one can use flow cytometry to sort out highly purified ER α (+) cells; the similar approach has been applied to purify other neural populations which facilitated the discovery of novel factors that regulate functions of specific neural populations [35, 36].

In addition, as we did in this study, one can prepare fresh brain slices from ER α -ZsGreen mice, and perform electrophysiological recordings in identified ER α (+) cells. One step further is to cross the ER α -ZsGreen transgene onto mice that express another fluorescent reporter in selective neural population. For example, here we crossed the ER α -ZsGreen mice with POMC-CreER/Rosa26-tdTOMATO mice to generate mice carrying all these 3 transgenic alleles. In the brain slices obtained from the triple genetic offspring, we easily identified ER α (+) POMC neurons as double-labelled (yellow) neurons; electrophysiological recordings from these neurons demonstrated that the ER α agonist stimulated firing activities of these ER α (+) POMC neurons. The significance of these results is at least two folds. First, we provided direct evidence that activation of ER α can stimulate firing of hypothalamic POMC neurons, which could mediate the physiological functions of estrogenic actions in these neurons to prevent overeating and obesity [12]. In addition, the fact that all double-labelled neurons responded to the ER α agonist provided the proof of the principle that the ER α -ZsGreen mouse line could be used in combination with other genetically labelled reporter lines to allow experiments in highly selective neural populations. These will no doubt advance our understanding about molecular and cellular actions of ER α in various neural populations.

In summary, we generated and validated a new reporter mouse line which faithfully expresses a fluorescence protein in ER α (+) cells in the brain. Although the expression of this reporter in peripheral tissues warrants further characterizations in future, we propose that this tool can be used in a variety of experimental designs, with fixed or unfixed brains, to study estrogenic actions in specific neural populations.

Acknowledgments

This work was supported by grants from the NIH (R01DK093587 and R01DK101379 to YX; R01DK092605 to QT), American Heart Association postdoctoral fellowship (PX), American Heart Association National Scientist Development Grant (QT).

References

1. McCarthy MM. Estradiol and the developing brain. *Physiol Rev.* 2008; 88:91–124. [PubMed: 18195084]
2. Dubois SL, Acosta-Martinez M, DeJoseph MR, Wolfe A, Radovick S, Boehm U, et al. Positive, but not negative feedback actions of estradiol in adult female mice require estrogen receptor alpha in kisspeptin neurons. *Endocrinology.* 2015; 156:1111–1120. [PubMed: 25545386]
3. Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, et al. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron.* 2006; 52:271–280. [PubMed: 17046690]
4. Lee H, Kim DW, Remedios R, Anthony TE, Chang A, Madisen L, et al. Scalable control of mounting and attack by Esr1 neurons in the ventromedial hypothalamus. *Nature.* 2014
5. Spary EJ, Maqbool A, Batten TF. Oestrogen receptors in the central nervous system and evidence for their role in the control of cardiovascular function. *J Chem Neuroanat.* 2009; 38:185–196. [PubMed: 19505570]
6. Hara Y, Waters EM, McEwen BS, Morrison JH. Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiol Rev.* 2015; 95:785–807. [PubMed: 26109339]
7. Saito K, Cao X, He Y, Xu Y. Progress in the molecular understanding of central regulation of body weight by estrogens. *Obesity (Silver Spring).* 2015; 23:919–926. [PubMed: 25865677]
8. Frank A, Brown LM, Clegg DJ. The role of hypothalamic estrogen receptors in metabolic regulation. *Front Neuroendocrinol.* 2014; 35:550–557. [PubMed: 24882636]
9. Faulds MH, Zhao C, Dahlman-Wright K, Gustafsson JA. The diversity of sex steroid action: regulation of metabolism by estrogen signaling. *J Endocrinol.* 2012; 212:3–12. [PubMed: 21511884]
10. Butera PC, Beikirch RJ. Central implants of diluted estradiol: independent effects on ingestive and reproductive behaviors of ovariectomized rats. *Brain Res.* 1989; 491:266–273. [PubMed: 2765887]
11. Palmer K, Gray JM. Central vs. peripheral effects of estrogen on food intake and lipoprotein lipase activity in ovariectomized rats. *Physiol Behav.* 1986; 37:187–189. [PubMed: 3737718]
12. Xu Y, Nedungadi TP, Zhu L, Sobhani N, Irani BG, Davis KE, et al. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metab.* 2011; 14:453–465. [PubMed: 21982706]
13. Cao X, Xu P, Oyola MG, Xia Y, Yan X, Saito K, et al. Estrogens stimulate serotonin neurons to inhibit binge-like eating in mice. *J Clin Invest.* 2014; 124:4351–4362. [PubMed: 25157819]
14. Asarian L, Geary N. Estradiol enhances cholecystokinin-dependent lipid-induced satiation and activates estrogen receptor-alpha-expressing cells in the nucleus tractus solitarius of ovariectomized rats. *Endocrinology.* 2007; 148:5656–5666. [PubMed: 17823256]
15. Martinez de Morentin PB, Gonzalez-Garcia I, Martins L, Lage R, Fernandez-Mallo D, Martinez-Sanchez N, et al. Estradiol Regulates Brown Adipose Tissue Thermogenesis via Hypothalamic AMPK. *Cell Metab.* 2014; 20:41–53. [PubMed: 24856932]
16. Musatov S, Chen W, Pfaff DW, Mobbs CV, Yang XJ, Clegg DJ, et al. Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. *Proc Natl Acad Sci U S A.* 2007; 104:2501–2506. [PubMed: 17284595]
17. Correa SM, Newstrom DW, Warne JP, Flandin P, Cheung CC, Lin-Moore AT, et al. An estrogen-responsive module in the ventromedial hypothalamus selectively drives sex-specific activity in females. *Cell Rep.* 2015; 10:62–74. [PubMed: 25543145]
18. Xu P, Cao X, He Y, Zhu L, Yang Y, Saito K, et al. Estrogen receptor-alpha in medial amygdala neurons regulates body weight. *J Clin Invest.* 2015; 125:2861–2876. [PubMed: 26098212]

19. Park CJ, Zhao Z, Glidewell-Kenney C, Lazic M, Chambon P, Krust A, et al. Genetic rescue of nonclassical ERalpha signaling normalizes energy balance in obese Eralpha-null mutant mice. *J Clin Invest.* 2011; 121:604–612. [PubMed: 21245576]
20. Zhu L, Zou F, Yang Y, Xu P, Saito K, Othrell Hinton A Jr, et al. Estrogens prevent metabolic dysfunctions induced by circadian disruptions in female mice. *Endocrinology.* 2015; 156:2114–2123. [PubMed: 25807042]
21. Frazao R, Cravo RM, Donato J Jr, Ratra DV, Clegg DJ, Elmquist JK, et al. Shift in Kiss1 cell activity requires estrogen receptor alpha. *J Neurosci.* 2013; 33:2807–2820. [PubMed: 23407940]
22. Berglund ED, Liu C, Sohn JW, Liu T, Kim MH, Lee CE, et al. Serotonin 2C receptors in pro-opiomelanocortin neurons regulate energy and glucose homeostasis. *J Clin Invest.* 2013; 123:5061–5070. [PubMed: 24177424]
23. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci.* 2010; 13:133–140. [PubMed: 20023653]
24. Kay AR, Wong RK. Isolation of neurons suitable for patch-clamping from adult mammalian central nervous systems. *J Neurosci Methods.* 1986; 16:227–238. [PubMed: 3523050]
25. Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, et al. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem.* 2000; 43:4934–4947. [PubMed: 11150164]
26. Sohn JW, Harris LE, Berglund ED, Liu T, Vong L, Lowell BB, et al. Melanocortin 4 receptors reciprocally regulate sympathetic and parasympathetic preganglionic neurons. *Cell.* 2013; 152:612–619. [PubMed: 23374353]
27. Liu Y, Liu H, Sauvey C, Yao L, Zarnowska ED, Zhang SC. Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. *Nat Protoc.* 2013; 8:1670–1679. [PubMed: 23928500]
28. Liu T, Kong D, Shah BP, Ye C, Koda S, Saunders A, et al. Fasting activation of AgRP neurons requires NMDA receptors and involves spinogenesis and increased excitatory tone. *Neuron.* 2012; 73:511–522. [PubMed: 22325203]
29. Merchenthaler I, Lane MV, Numan S, Dellovade TL. Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses. *J Comp Neurol.* 2004; 473:270–291. [PubMed: 15101093]
30. Hamada T, Wada-Kiyama Y, Sakuma Y. Visualizing forebrain-specific usage of an estrogen receptor alpha promoter for receptor downregulation in the rat. *Brain Res Mol Brain Res.* 2005; 139:42–51. [PubMed: 15953656]
31. Matsuda KI, Yanagisawa M, Sano K, Ochiai I, Musatov S, Okoshi K, et al. Visualisation and characterisation of oestrogen receptor alpha-positive neurons expressing green fluorescent protein under the control of the oestrogen receptor alpha promoter. *Eur J Neurosci.* 2013; 38:2242–2249. [PubMed: 23601009]
32. Lee H, Kim DW, Remedios R, Anthony TE, Chang A, Madisen L, et al. Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. *Nature.* 2014; 509:627–632. [PubMed: 24739975]
33. Yokosuka M, Okamura H, Hayashi S. Postnatal development and sex difference in neurons containing estrogen receptor-alpha immunoreactivity in the preoptic brain, the diencephalon, and the amygdala in the rat. *J Comp Neurol.* 1997; 389:81–93. [PubMed: 9390761]
34. Shughrue PJ, Bushnell CD, Dorsa DM. Estrogen receptor messenger ribonucleic acid in female rat brain during the estrous cycle: a comparison with ovariectomized females and intact males. *Endocrinology.* 1992; 131:381–388. [PubMed: 1612018]
35. Ren H, Orozco IJ, Su Y, Suyama S, Gutierrez-Juarez R, Horvath TL, et al. FoxO1 Target Gpr17 Activates AgRP Neurons to Regulate Food Intake. *Cell.* 2012; 149:1314–1326. [PubMed: 22682251]
36. Henry FE, Sugino K, Tozer A, Branco T, Sternson SM. Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. *Elife.* 2015; 4

Schematic construct of the ER α -ZsGreen mouse allele

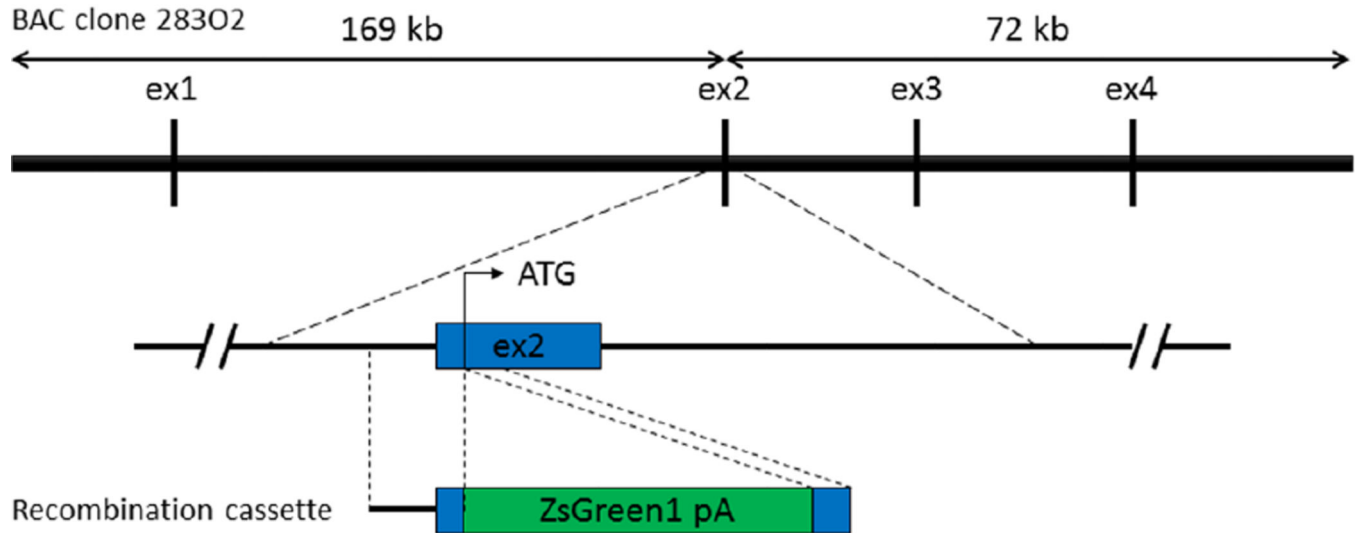


Figure 1. Schematic construction of the ER α -ZsGreen transgenic mouse allele

The BAC clone 283O2 covers 241 kb around *Esr1* genomic sequence (5', 169 kb; 3', 72 kb), which contains exon1–4 out of the *Esr1* gene. For BAC recombination cassette, 393-bp of 5' flanking region and 148-bp of 3' flanking region were used as homology arms. By homologous recombination in bacteria, the ZsGreen cDNA was introduced into translation start site of the *Esr1* gene in the BAC clone.

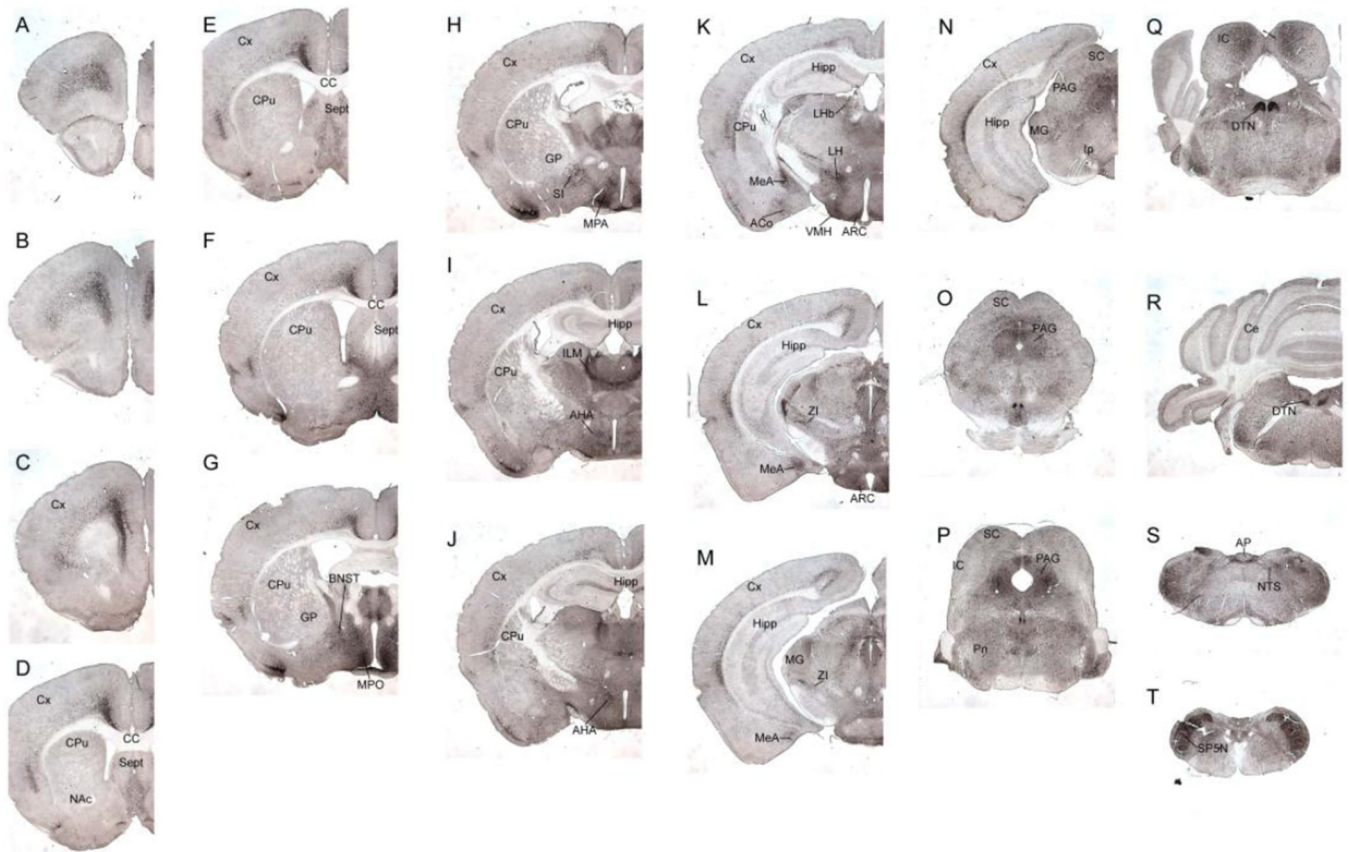


Figure 2. Distribution of ZsGreen (+) cells throughout the brain

Images of coronal sections of the ER α -ZsGreen transgenic mouse brain are arranged from rostral to caudal separated by a distance of 400–600 μ m. Images shown here were taken in same magnification. Scale bars=1 mm. AHA, anterior hypothalamic area; AON, anterior olfactory nucleus; AP, area postrema; ARH, arcuate hypothalamic nucleus; BNST, bed nucleus of the stria terminalis; CA, CA1–3; Cbx, cerebellum; CoA, cortical amygdalar nucleus; CPU, striatum; CT, central nucleus of the thalamus; Ctx, cerebral cortex; DG, dentate gyrus; DTN, dorsal tegmental nucleus; ENT, entorhinal cortex; IC, inferior colliculus; LG, lateral geniculate complex, thalamus; LHA, lateral hypothalamic area; LHb, lateral habenula; LPO, lateral preoptic area; LS, lateral septum; MeA, medial amygdalar nucleus; MPO, medial preoptic area; MS, medial septum; NAc, nucleus accumbens; NDB, diagonal band of Broca; NTS, nucleus of the solitary tract; PAG, periaqueductal grey; PIR, piriform cortex; PM, premammillary nucleus; PV, periventricular hypothalamic nucleus; PVT, paraventricular nucleus of the thalamus; SC, superior colliculus; SP5N, spinal trigeminal nucleus; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta.

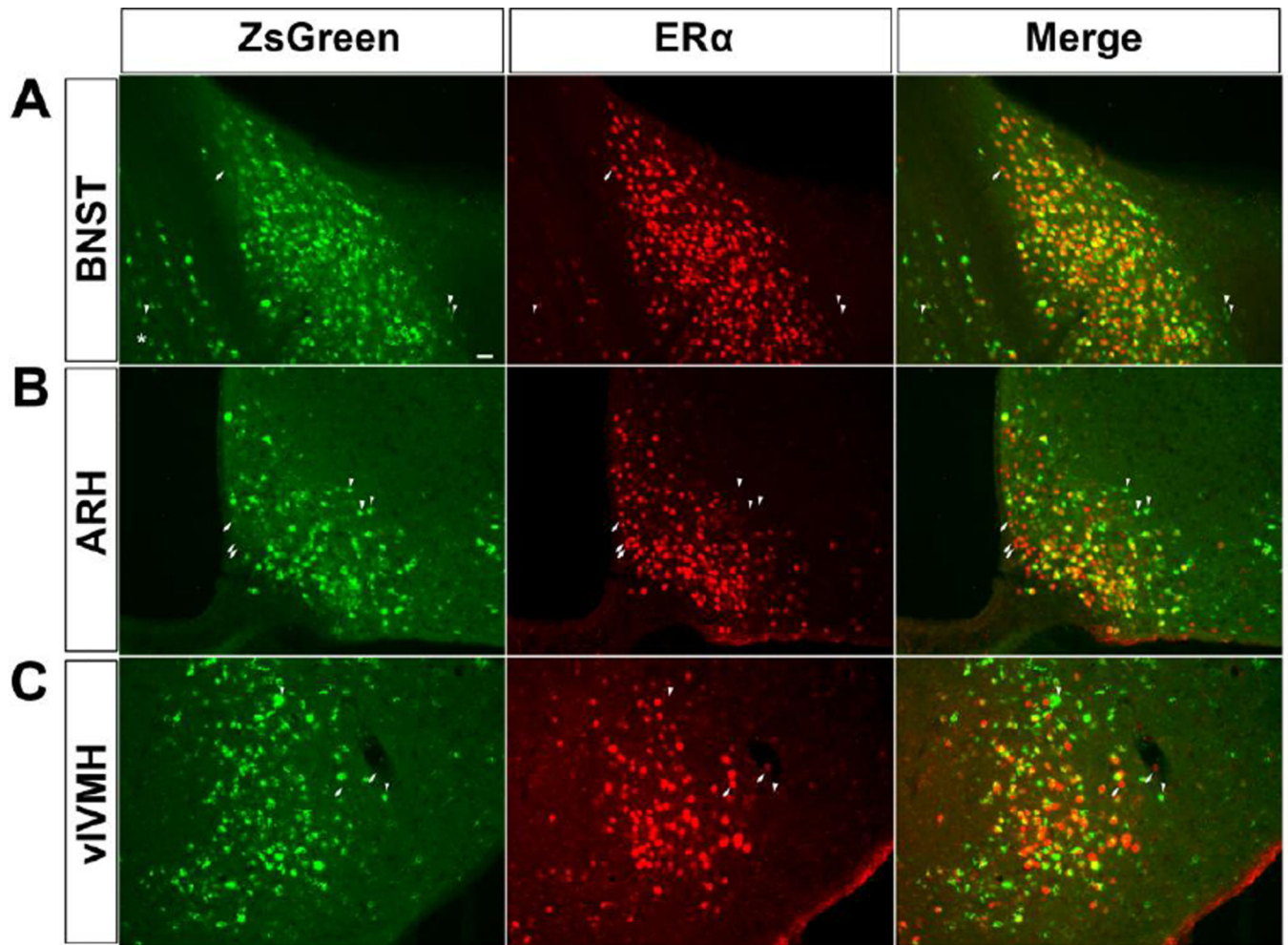


Figure 3. Colocalization of ZsGreen and ER α

Colocalization of native ZsGreen (left), ER α immunoreactivity (middle), and the merge (right) in the BNST (A), ARH (B) and vVMH (C). Scale bar = 50 μ m. Note that most cells co-express both ZsGreen and ER α ; a few cells that only express ZsGreen are indicated by arrowheads; a few cells that only express ER α are indicated by arrows; the asterisk indicates a cell with punctuated ZsGreen signals.

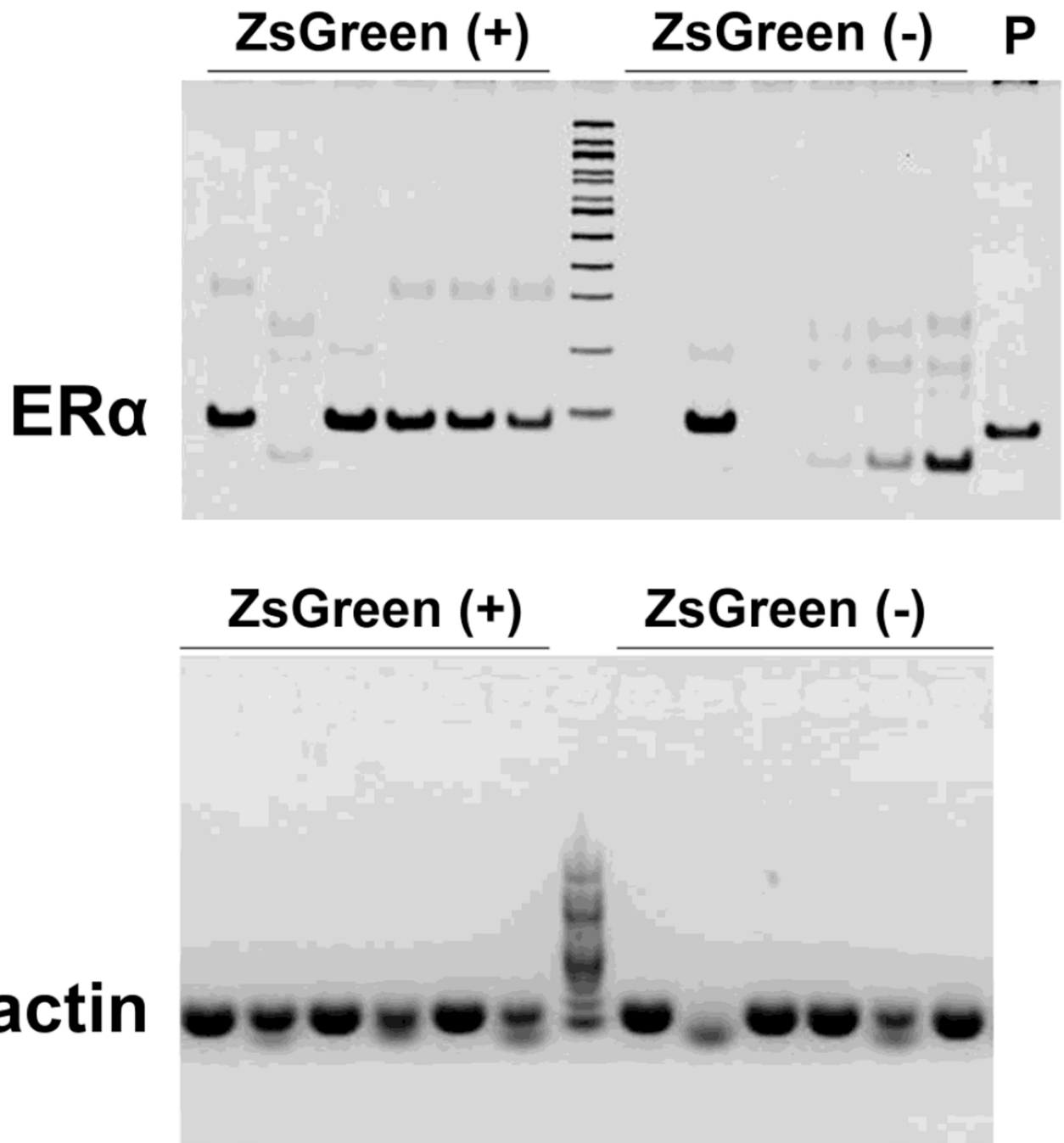


Figure 4. ZsGreen(+) cells express ER α mRNA

RT-PCR detecting ER α (upper panel) and β -actin (lower panel) mRNAs in single ZsGreen (+) or ZsGreen (-) cells isolated from the ARH of ER α -ZsGreen mice. P indicates a positive control, showing that the same ER α primers produced an amplicon from total cDNA extracted from a mouse hypothalamus.

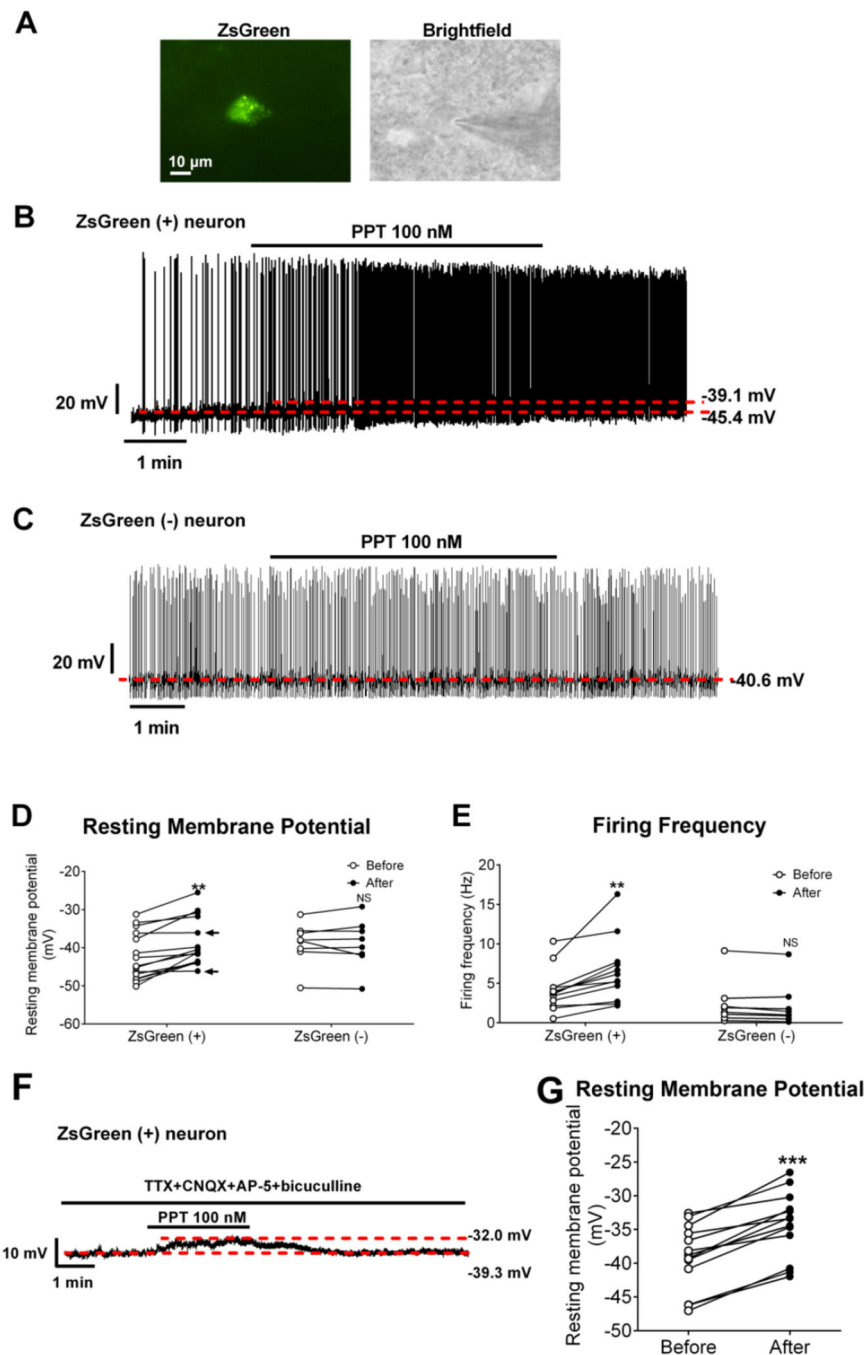


Figure 5. Effects of a selective ER α agonist on neural activities of ZsGreen (+) and ZsGreen (-) cells in the ARH

(A) Micrographic images showing a recorded ZsGreen (+) neuron in the ARH. Left panel: the neuron visualized under the green fluorescence microscope; right panel: the same neurons patch clamped by a micropipette visualized under the brightfield microscope. Scale bar=10 μ m. (B–C) Representative responses to PPT (100 nM, bath) in ZsGreen (+) cells (B) and in ZsGreen (-) cells (C). (D–E) Summary data of resting membrane potential (D) and firing frequency (E) before and after PPT treatment. Data are presented for each cell. **, $P < 0.01$ in paired t-tests. Note that the arrows in (D) point to the two ZsGreen (+) cells that

were not depolarized by PPT. (F) Representative responses to PPT (100 nM, bath) in ZsGreen (+) cells in the presence of 1 μ M TTX, 30 μ M CNQX, 30 μ M AP-5 and 50 μ M bicuculline. (G) Summary data of resting membrane potential before and after PPT treatment in the presence of 1 μ M TTX, 30 μ M CNQX, 30 μ M AP-5 and 50 μ M bicuculline. Data are presented for each cell. ***, $P < 0.001$ in paired t-tests.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

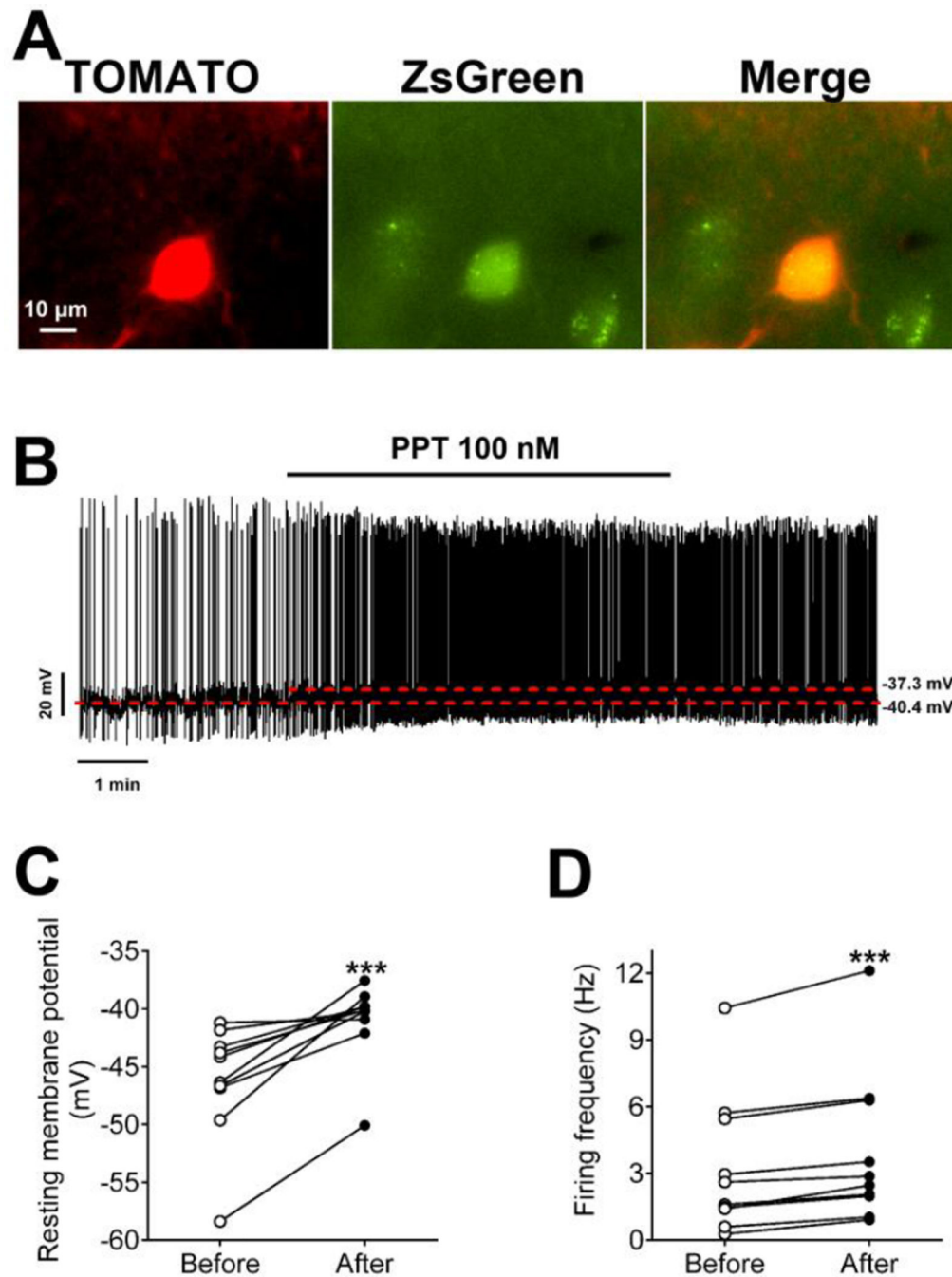


Figure 6. Effects of a selective ER α agonist on neural activities of ER (+) POMC neurons in the ARH

(A) Micrographic images showing a recorded ER α (+) POMC neuron in the ARH. Left panel: TOMATO signal visualized under the red fluorescence microscope; middle panel: ZsGreen signal visualized under the green fluorescence microscope; right panel: merge. Scale bar=10 μ m. (B) Representative responses to PPT (100 nM, bath). (C–D) Summary data of resting membrane potential (C) and firing frequency (D) before and after PPT treatment. Data are presented for each cell. ***, $P < 0.001$ in paired t-tests.

Table 1

Distribution of ZsGreen expressing cells throughout the brain

Anatomical structure	ZsGreen expression
<i>Telencephalon</i>	
Olfactory bulb	
Anterior olfactory nucleus	+/-
Isocortex	
Layers 2-3	-
Layer 6	++
Piriform cortex	-
Entorhinal cortex	+
Hippocampal formation	
CA1 – CA3	+
Dentate gyrus	+
Septum	
Medial	++
Lateral	++
Diagonal band of Broca	+
Bed nucleus of the stria terminalis	++
Amygdala	
Basolateral nucleus	+/-
Basomedial nucleus	+
Central nucleus	+/-
Cortical nucleus	++
Medial nucleus	++
Nucleus accumbens	+
Striatum	+
<i>Diencephalon</i>	
Thalamus	
Anterior nuclei (paraventricular)	++
Central nuclei	++
Lateral geniculate nucleus	-
Zona incerta	++
Habenula	
Medial	-
Lateral	++
Hypothalamus	
Preoptic area	+/-
Periventricular	+/-
Anterior hypothalamic area	++
Periventricular nucleus	+/-
Paraventricular nucleus	+/-

Anatomical structure	ZsGreen expression
Retrochiasmatic area	-
Suprachiasmatic nucleus	-
Supraoptic nucleus	-
Arcuate nucleus	++
Dorsomedial nucleus	+/-
Lateral hypothalamus	++
Ventromedial nucleus	
Dorsal	-
Ventral	++
Premammillary nuclei	+
<i>Mesencephalon</i>	
Interpeduncular nucleus	-
Periaqueductal gray	++
Raphe nuclei, dorsalis	+/-
<i>Metencephalon</i>	
Cerebellum (Purkinje cells)	+/-
<i>Myelencephalon</i>	
Area postrema	++
Nucleus of the solitary tract	+
Spinal nucleus of the trigeminal	++

ZsGreen expression was evaluated based on number of intense ly-stained cells in each brain sites. Anatomical structures were defined based on Allen Reference Atlas. -, not detected; +/-, low/faint; +, moderate; ++, high.

Table 2Colocalization of native ZsGreen and ER α immunoreactivity

Brain Regions	Male		Female	
	Double/ZsGreen	Double/ER α	Double/ZsGreen	Double/ER α
BNST	81.05 \pm 2.11%	71.68 \pm 5.70%	97.88 \pm 0.34%	83.12 \pm 2.29%
ARH	94.12 \pm 0.88%	55.35 \pm 1.55%	95.22 \pm 0.49%	70.06 \pm 1.7%
vVMH	83.78 \pm 1.94%	86.81 \pm 3.61%	90.62 \pm 1.02%	73.20 \pm 5.33%

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript