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Reproductive steroid receptors and actions in the locus coeruleus of male macaques: Part of an aggression circuit?

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Abstract

This study was initiated to determine whether the noradrenergic (NE) neurons of the locus coeruleus (LC) could mediate the stimulatory action of androgens on serotonin-related gene expression in male macaques. These experiments follow our observations that serotonin neurons lack androgen receptors (ARs), and yet respond to androgens. Male Japanese macaques (*Macaca fuscata*) were castrated for 5–7 months and then treated for 3 months with [1] placebo, [2] T (testosterone), [3] DHT (dihydrotestosterone; non-aromatizable androgen) *plus* ATD (steroidal aromatase inhibitor), or [4] FLUT (Flutamide; androgen antagonist) *plus* ATD ($n = 5/\text{group}$). The noradrenergic (NE) innervation of the raphe was determined with immunolabeling of axons with an antibody to dopamine- β -hydroxylase (DBH). Immunolabeling of tyrosine hydroxylase (TH) dendrites and corticotropin releasing hormone (CRH) axons innervating the LC was also determined. Due to the longer treatment period employed, the expression of the cognate nuclear receptors was sought. Androgen receptor (AR), estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) immunostaining was accomplished. Quantitative image analysis was applied and immunopositive neurons or axons with boutons were measured. Double-label of NE neurons for each receptor *plus* TH determined whether the receptors were localized in NE neurons. Androgens with or without aromatase activity significantly stimulated DBH axon density in the raphe (ANOVA, $p = 0.006$), and LC dendritic TH (ANOVA, $p < 0.0001$), similar to serotonin-related mRNA expression in the raphe. There were significantly more AR-positive neurons in T- and DHT + ATD-treated groups compared to placebo or FLUT + ATD-treated groups (ANOVA, $p = 0.0014$). There was no difference in the number of positive-neurons stained for ER α or ER β . The CRH axon density in the LC was significantly reduced with aromatase inhibition, suggesting that CRH depends on estrogen, not androgens (ANOVA, $p = 0.0023$). Double-

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Disclosure

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Grace Mammerella, undergraduate summer intern, has nothing to disclose.

immunohistochemistry revealed that NE neurons did not contain AR. Rather, AR-positive nuclei were found in neighboring cells that are likely neurons. However, >80% of LC NE neurons contained ER α or ER β . In conclusion, the LC NE neurons may transduce the stimulatory effect of androgens on serotonin-related gene expression. Since LC NE neurons lack AR, the androgenic stimulation of dendritic TH and axonal DBH may be indirectly mediated by other neurons. Estrogen, either from metabolism of T or from de novo synthesis, appears necessary for robust CRH innervation of the LC, which differs from female macaques.

Keywords

Male macaque; Androgen receptors; Estrogen receptors; Tyrosine hydroxylase; Dopamine- β -hydroxylase; Corticotropin releasing hormone

1. Introduction

The locus coeruleus (LC) is a dense association of noradrenergic (NE) neurons on both sides of the fourth ventricle in the caudal midbrain. It plays an important role in vigilance, stress reactivity, anxiety, and transmission of these signals to neuroendocrine neurons that regulate reproduction (Bethea et al., 2014b). Of current interest is the LC NE projection to the raphe nucleus and its regulation by androgens in male macaques. We showed that androgens increase tryptophan hydroxylase 2 (TPH2) and serotonin reuptake transporter (SERT) mRNA expression in the raphe serotonin neurons (Bethea et al., 2014a), but serotonin neurons do not contain nuclear androgen receptors (ARs). Rather, ARs were found in neighboring non-serotonin neurons (Bethea et al., 2015a). This observation evoked the possibilities that (a) androgens stimulated excitatory input to the serotonin neurons, or (b) neighboring AR-positive excitatory neurons stimulated serotonin neurons or (c) a membrane AR was involved. This study examined the important NE stimulatory input to the dorsal raphe from the LC.

Electrophysiology data have demonstrated a midbrain amine circuit that controls many forebrain functions (El Mansari et al., 2010; Guiard et al., 2008a; Guiard et al., 2008b, Hamon and Blier, 2013). Within this circuit, LC NE stimulates serotonin neurons and serotonin blocks excitatory amino acid or glutamate stimulation of NE (Aston-Jones et al., 1991; Charlety et al., 1993). The A10 ventral tegmental area (VTA) dopamine (DA) neurons (VTA DA) also stimulate raphe serotonin neurons. In turn, serotonin and NE neurons inhibit VTA DA neurons (Grenhoff et al., 1993). VTA DA has been reported to both stimulate and inhibit LC NE neurons via adrenergic α 1 versus α 2 receptors (Guiard et al., 2008a). The stimulation of the raphe serotonin by NE was mediated by α 1 adrenergic receptors (Pudovkina et al., 2003). Along this line of reasoning, we questioned whether the LC NE innervation of raphe serotonin neurons could transduce the stimulatory effect of androgens on TPH2 mRNA expression (Bethea et al., 2014a).

Nuclear steroid receptors are gene transcription factors. In neurons, the presence of the cognate receptor for a steroid defines the neuron as a steroid target. Steroids bind to their receptors that in turn bind to response elements and induce gene transcription within the neuron. This laboratory has interest in defining cognate reproductive steroid receptor target

neurons in the brain with a focus on the nuclear receptors and their actions as gene transcription factors. We use steroid replacement paradigms in macaques that extend a month or more, and which would engage nuclear receptors. In more specific terms, estrogens bind to two types of nuclear estrogen receptors, ER α and ER β . Testosterone (T), the major androgen produced by the testis, is metabolized to (1) estradiol (E) and (2) dihydrotestosterone (DHT), a non-aromatizable androgen. T and DHT bind to androgen receptors (ARs) while E binds to both ERs. Thus T, via metabolites, can act through ARs and/or ERs. In addition, each nuclear receptor has a membrane counterpart that mediates rapid actions of steroid hormones (Razandi et al., 1999). Moreover, these rapid actions may initiate and support longer term actions on gene expression (Levin, 2015). For example, the estrogen specific membrane protein, GPER1, is ubiquitously expressed and found in many areas of the brain (Almey et al., 2015).

Due to the outcomes of the experiments performed, the regulation of stimulatory input to the LC was questioned. Corticotropin releasing hormone (CRH) plays a role in the regulation of the LC (Bangasser et al., 2010; Bangasser et al., 2012, Curtis et al., 2012; Reyes et al., 2005). CRH stimulates LC NE neurons in rodents (Curtis et al., 2012; Valentino et al., 1993; Valentino et al., 2001, Van Bockstaele et al., 1998); and the LC receives a prominent CRH innervation in female macaques that increases with stress and stress sensitivity (Bethea et al., 2013a). Neurons in the caudal PVN project to the midbrain rather than the median eminence (Reyes et al., 2005); and non-neuroendocrine CRH neurons are found widely in the brain (Bassett and Foote, 1992; Delville et al., 1992; Luiten et al., 1985; Palkovits et al., 1985; Portillo et al., 1998). CRH in the PVN, particularly in the caudal region, of ovariectomized female macaques was decreased by E replacement (Bethea and Centeno, 2008), and sex differences in the CRH innervation of the rodent LC have been reported (Bangasser et al., 2010). To further understand androgen actions mediated by the LC, it was important to examine its CRH innervation.

Therefore, to determine whether the LC mediates the effect of androgens on TPH2 gene expression, this study examined androgen regulation of the density of the NE innervation to the raphe with DBH as a marker. In addition, to further demonstrate that the LC NE neurons were targets of androgens, the regulation of tyrosine hydroxylase (TH) in the LC was determined; and the co-localization of ARs and ERs in LC NE neurons was performed. Since the results of these studies indicated that androgen action in NE neurons was not mediated through nuclear AR, we examined the well-known CRH stimulatory innervation of the LC.

2. Materials and methods

This experiment was approved by the IACUC of the Oregon National Primate Research Center and conducted in accordance with the 2011 Eight Edition of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male Japanese macaques (*Macaca fuscata*) were utilized for study.

2.1. Troop

The Japanese macaques were born and raised in a 2-acre outdoor corral at ONPRC with approximately 300 individuals. The troop has been the subject of extensive behavioral studies since it arrived at ONPRC in 1965 (Eaton, 1974; Eaton et al., 1990). The troop composition is relatively stable and the age structure is comparable to that of a natural troop (Maruhashi, 1982). Like other macaque species, the hierarchical organization of the troop is along matriarchal lineages. The matriarchal lines and dominance hierarchies within the troop are well documented, and have remained stable for the past 50 years. In the wild, males normally leave the natal troop and so their dominance is less a function of their mother's status and more a function of their age, size and social skills. Males cannot leave our troop on their own so in that respect, there are more males than a natural troop although they are removed for research and sales with attention to troop stability.

2.2. Study animals and treatments

The animals used in this study were the same animals used in previous studies as quoted below (Bethea et al., 2014a; Bethea et al., 2015a; Bethea et al., 2013b). Twenty adult male Japanese macaques were assigned to this project, castrated and housed as previously described (Bethea et al., 2013b). The experimental period was conducted for 3 months during the mating season when aggression is highest amongst males in the troop. In year 1, half of the animals were treated with placebo or testosterone (*T*) ($n = 5/\text{group}$) and then euthanized. In year 2, the remaining animals were treated with dihydrotestosterone (*DHT*) + aromatase inhibitor (*ATD*) or an androgen antagonist (*Flutamide*, *FLUT*) + *ATD* ($n = 5/\text{group}$) and then euthanized. The *T* treatment achieved serum *T* concentrations previously reported in intact Japanese macaques (Eaton and Resko, 1974; Rostal et al., 1986). Dosing and inhibition of brain aromatase with *ATD* administration were also previously reported in macaques (Ellinwood et al., 1984). The dose of *DHT* significantly elevated DHT ~20-fold over the normal concentration observed in intact or *T* treated macaques (Bethea et al., 2013b).

The rationale for the different treatment groups was explained in detail previously (Bethea et al., 2014a). Briefly, *T* treatment exposed the brain to DHT via 5 α -reductase metabolism and to E via aromatase metabolism, yielding high androgen receptor (AR) and high estrogen receptor (ER) activation. The *placebo* group would have little or no activity at AR, but independent de novo production of neural E from cholesterol could remain, yielding negligible AR and some ER activation (Mukai et al., 2006; Tsutsui, 2012). The *DHT* + *ATD* group would have significant androgen activity with ~90% inhibition of aromatase (Ellinwood et al., 1984), yielding high AR and no ER activation. It was previously reported that *ATD* nonspecifically activated androgen receptors (ARs) in castrated macaques (Resko et al., 1993). Therefore, the *FLUT* + *ADT* group was expected to have no androgen activity and inhibition of aromatase, yielding no AR and no ER activation. Please see Table 1 and Bethea et al. (2014a) for a diagrammatic summary of the treatments and their effects.

Blood samples were obtained for immunoassay of T, DHT, and E to verify treatment efficacy and the results were reported (Bethea et al., 2014a; Bethea et al., 2013b). The age,

weight, ranks and behavior of the animals have been published [(Bethea et al., 2013b) and supplement].

2.3. Euthanasia

Monkeys were euthanized at the end of the treatment periods for collection of the brain and other tissues according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association and performed by an expert veterinary pathologist. They were transported to the necropsy suite under sedation with ketamine anesthesia (ketamine HCl, 10 mg/kg, s.c. Fort Dodge Laboratories, Fort Dodge, IA). They were then administered pentobarbital (30 mg/kg, i.v. Hospira, Lake Forest, IL) and exsanguinated with severance of the descending aorta.

2.4. Tissue preparation

Perfusion, fixation, preparation of the brain and sectioning have been previously described in detail (Bethea et al., 2014a; Bethea et al., 2015a).

2.5. Steroid receptor immunohistochemistry (IHC)

Midbrain sections containing the LC were removed from -20°C storage in cryoprotectant buffer and washed in 0.2 M potassium phosphate buffered saline (KPBS) buffer 4 times for 15 min each (rinsed), immersed in 1% hydrogen peroxide in KPBS for 30 min, rinsed, and incubated for 60 min with normal serum diluted in KPBS based upon the secondary antibody used in each assay as follows: Vector normal horse serum (NHS; Vector Laboratories, Burlingame, CA) for AR, or Vector normal goat serum (NGS) for ER α , or Vector NHS for ER β . Normal serum was followed by Vector avidin for 20 min and then Vector biotin for 20 min. Sections were incubated for 24 h at 4°C in rabbit anti-human AR (SC-816, Santa Cruz, Dallas, Texas), or rabbit anti-human ER α (C1355, Cat #06-935, Millipore, Billerica, Massachusetts), or mouse anti-human ER β (GTX70174, GeneTex, Irvine, California) diluted in 2% normal serum corresponding to the secondary antibody species, 0.02 M KPBS, and 0.4% Triton. The antibody to AR was used at a concentration of 1/400; the antibody to ER α was used at a concentration of 1/1000; and the antibody to ER β was used at a concentration of 1/400. Sections were then rinsed, and incubated for 60 min in Vector biotinylated horse anti-rabbit IgG (for AR) or Vector goat anti-rabbit IgG (for ER α), or Vector horse anti-mouse IgG (for ER β) diluted in 0.02 M KPBS and 0.4% Triton at 1/500. The sections were rinsed, incubated with Vector ABC reagent, rinsed, incubated with 0.05% diaminobenzidine (Vector DAB kit) containing 3% hydrogen peroxide for 1–10 min and lastly rinsed. Sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA) and dried overnight under vacuum. The sections were further dehydrated through a graded series of ethanols and xylene. The sections were finally mounted under glass with DPX. The antibodies were characterized across a range of titers with positive and negative controls.

2.6. TH, DBH and CRH immunohistochemistry (IHC)

Midbrain sections were removed from -20°C storage in cryoprotectant and washed in KPBS buffer 4 times for 15 min each (rinsed), immersed in 1% hydrogen peroxide for 30

min, rinsed and then incubated with the following blocking solutions: Vector normal goat serum for 60 min; 3% bovine serum albumin (BSA; Sigma, St. Louis, MO) for 60 min; Vector avidin for 20 min; and Vector biotin for 20 min. For CRH, sections were additionally blocked with 0.1% human α -globulin (Sigma, St. Louis, MO). Sections were then incubated for 24–48 h in primary antibody to tyrosine hydroxylase (rabbit anti-human TH, from Chemicon acquired by Millipore, Temecula, CA); dopamine- β -hydroxylase (rabbit anti-human DBH, from Immunostar, Hudson, WI; formerly Incstar), or corticotropin releasing factor (rabbit anti-human CRH; gift of Dr. Wylie Vale, Salk Institute, La Jolla, Calif., USA). They were extensively characterized across a range of titers with positive and negative controls; and were previously applied to primate brain (Bethea et al., 2013a; Bethea et al., 2014b; Kohama et al., 1992; Sanchez et al., 2010). All of the primary antibodies were diluted in 2% NGS, 0.02 M KPBS, and 0.4% Triton. The TH antibody was diluted 1/1000; the DBH antibody was diluted 1/4000; and the CRH antibody was diluted 1/6000. Sections were then rinsed, incubated in Vector biotinylated goat anti-rabbit serum at 1/500 for 60 min, rinsed, incubated with Vector ABC reagent for 60 min, rinsed, incubated with 0.05% diaminobenzidine containing 3% hydrogen peroxide for 1–10 min, and finally rinsed. Sections were mounted on Superfrost Plus slides and dried overnight under vacuum. The sections were further dehydrated through a graded series of ethanols and xylene and finally mounted under glass with DPX. Each IHC assay contained one level from all animals.

2.7. Double immunostaining for TH and steroid receptors

To determine the localization of the steroid receptors, double immunostaining was conducted. TH was used as the marker for NE neurons. Each steroid receptor antibody was processed as described above, with 1-hour 4% formaldehyde fixation between the steroid receptor reactions and the TH reactions to inactivate any residual peroxidase activity from the first reaction.

To double label AR + TH, rabbit anti-human AR antibody was applied at a concentration of 1/400 followed by Vector biotinylated horse anti-rabbit IgG (1/500). The conjugate was developed with Vector ABC kit and Vector Nickel-DAB kit, yielding black nuclear staining of AR. Afterwards the sections were fixed in 4% formaldehyde for 1 h. Then, rabbit anti-TH antibody was applied at a concentration of 1/1000 followed by Vector biotinylated goat anti-rabbit serum. The conjugate was developed with Vector ABC reagents and Vector DAB kit, yielding brown cytoplasmic staining.

To double label ER α + TH, rabbit anti-human ER α was applied at a concentration of 1/1000 followed by Vector biotinylated goat anti-rabbit serum (1/500). The conjugate was developed with Vector ABC reagents and Vector Nickel-DAB kit, yielding black nuclear staining of ER α followed by fixation. TH immunostaining proceeded as described above.

To double label ER β + TH, mouse anti-human ER β was applied at a concentration of 1/200 followed by Vector biotinylated horse anti-mouse serum (1/500). The conjugate was developed with Vector ABC kit and Vector Nickel-DAB kit, yielding black nuclei with significant cytoplasmic staining of ER β . After fixation and rinse, TH immunostaining proceeded as described above.

2.8. Positive controls

The antibody to ER α has been well characterized in mouse brain (McClellan et al., 2010), and the antibody to ER β produced nuclear staining in the human lung (Taniuchi et al., 2014). Positive controls for ER α and ER β in this laboratory were macaque endometrium and macaque prostate gland (Brenner and Slayden, 1994; Kuiper et al., 1996). Both antibodies worked well in peripheral tissue and in the brain of macaques (Bethea et al., 2015a). The AR antibody has been well characterized by Santa Cruz (<http://www.scbt.com/datasheet-816-ar-n-20-antibody.html>) in the literature (Satoh et al., 2001; Saunders et al., 2000; Vija et al., 2014), and it works well in macaque brain (Bethea et al., 2015a). Immunostaining for TH, DBH and CRH in monkey brain has been published (Bethea et al., 2013a; Bethea et al., 2014b).

2.9. Densitometric analysis of immunostaining signal

Four sections at different morphological levels of the LC were immunostained for TH and CRH from each animal. Three sections per animal of the LC were immunostained for steroid receptors; and three sections per animal of the rostral dorsal raphe were immunostained for DBH. LC data was obtained from both sides of the midbrain on each slide. The sections were 250 μ m apart and morphologically matched between animals using anatomical reference points. The morphology of the LC was defined according to the appearance of the nucleus of the mesencephalic tract of the trigeminal nerve (5th facial nerve; mv). The Marianas Stereological workstation with Slidebook 5.0 was used for image capture. A montage of a consistent area was built by the workstation. The image was exported as a .tif file and ImageJ was used to perform the analysis. The LC was defined, cropped and kept constant across the levels and animals for TH, CRH and each receptor. The same procedure was applied to DBH axon staining in the dorsal raphe nucleus.

After contrast adjustment, the images were segmented into positive (highlighted with red) and negative pixels (not highlighted) with the same saturation parameters. For TH, the dendritic halo was targeted. The size of the area analyzed was held constant, and the positively stained dendrites were segmented from background and highlighted in red. The program provided the positive pixel area of the TH-stained dendrites, which reflects dendrite density. The data were then averaged across 4 LC-sections from each individual animal. DBH and CRF images were obtained and cell bodies that appeared stained were removed for consistency. The positive pixel area and bouton number of the axons was obtained from each section and averaged to obtain the individual animal's axon density and bouton number. With receptors, the program provided the positive pixel area of the red highlighted nuclei. A filter was applied that counted objects (groups of pixels that are touching) within a range of sizes corresponding to nuclei. This provided the number of positive nuclei in the defined area. Therefore, for each receptor-stained section the following data were obtained: positive pixel area, the number of positive nuclei, and total area examined (constant). There were 10-pixels/linear micron, and 100 pixels per square micron. The pixels on the y-axis should be divided by 100 to determine the area in square microns.

2.10. Double-label images

Photomicrographs of the double-labeled sections were captured with a Zeiss Axioplan bright field microscope and a Zeiss AxioCam digital camera with Zen software at magnifications of 200× and 400×.

2.11. Statistical analysis

TH, DBH, CRH and steroid receptor measurements were averaged across 3–4 sections from each animal, generating one overall value for an individual animal. These values were used to obtain the group average and for statistical analysis. Therefore, the variance around the mean of each group reflects the difference between animals. All data were compared with analysis of variance (ANOVA) followed by Newman–Keuls post hoc pairwise comparison. The data were tested for unequal variance and if present, a non-parametric ANOVA was applied (Kruskal–Wallace).

3. Results

3.1. TH and DBH immunohistochemistry and image analysis

Fig. 1 illustrates the immunostaining for TH and DBH as well as the steps of the image analysis executed by ImageJ. Immunostaining for TH in the LC dendritic halo was robust (panels A, B, C and D). Immunostaining for DBH, the enzyme that converts dopamine to NE, was used to detect the NE innervation in the dorsal raphe. The DBH immunostaining was also robust in the dorsal raphe (panels E, F, G, H, I and J).

The analysis of the TH dendrites was straightforward, but analysis of DBH required an extra step in ImageJ because of apparent cell body staining. The initial DBH staining in a consistent area of the dorsal raphe is illustrated in panel E. High magnification examination of the cell body staining, revealed a range of DBH boutons on individual neurons. Panels I and J illustrate patterns of DBH staining associated with neuronal cell bodies. In panel I, a neuron is present with dense bouton DBH staining (black arrow) and another neuron that appears to have axons circling the neuron with several synaptic boutons present (white arrow). Panel J illustrates adjacent neurons with different degrees of bouton staining on the neuron cell body. Technically, removal of the densely stained neuronal cell bodies was necessary to prevent oversaturation of pixels on the cell body as the axons were brought up to threshold by segmentation. In addition, the presence of the densely stained neuronal cell bodies varied from section to section, which compounded the variance. We elected to omit them and measure only axons with the caveat that a small amount of DBH staining was omitted from each section.

3.2. Quantification of dendrite TH and axon DBH

Quantification of TH staining in the dendritic halo and DBH staining in the dorsal raphe is illustrated in Fig. 2. The regulation of TH in the dendritic halo of the LC and regulation of DBH in axons innervating the dorsal raphe were similar. TH immunolabeling was increased by androgens, both T and DHT ($F [3,15] = 67.1$; $p < 0.0001$). DBH immunolabeling was also increased by androgens ($F [3,16] = 9.94$; $p = 0.006$). The presence of E via T metabolism (T treatment) or the complete absence of E (DHT + ATD) did not affect the

stimulation of TH and DBH by androgens. As illustrated in Fig. 3, *top*, there was a significant positive correlation between the DBH innervation of the raphe and the density of TH dendrites in the halo of the LC ($p = 0.0022$; $r^2 = 0.43$). R-squared indicates that 43% of the variance in DBH is explained by variance in LC TH, which suggests that not all of the DBH innervation of the raphe originates in the LC. As illustrated in Fig. 3, *bottom*, there was also a positive correlation between the DBH innervation of the raphe and TPH2 gene expression in the raphe ($p = 0.0072$; $r^2 = 0.37$). TPH2 mRNA expression was previously described (Bethea et al., 2014a). R-squared indicates that 37% of the variance in TPH2 is explained by variance in DBH, which suggests that not all of the regulation of TPH2 in the raphe depends on DBH/NE innervation.

3.3. Steroid receptor single and double immunohistochemistry

Single immunostaining for AR, ER α and ER β and double immunostaining for TH plus each receptor are shown in Fig. 4 at different magnifications. ARs were plentiful in the area of the LC (left column). However, AR did not co-localize in the TH positive neurons of the LC (right column). ER α and ER β immunostaining was robust in the LC and surrounding area (left column). In addition, a majority of TH positive neurons of the LC contained both ER α and ER β (right column). As previously observed in the raphe of male macaques (Bethea et al., 2015a), ER β immunostaining exhibited different staining patterns within the LC NE neurons, ranging from diffuse throughout the cell body to concentrated and dense in the nucleus. Nonetheless, ER β immunopositive neurons were abundant in the LC.

3.4. Quantification of receptor immunostaining in LC

Quantification of AR, ER α and ER β single immunostaining is illustrated in Fig. 5. AR positive pixel area and the number of AR-positive nuclei were both increased by androgens ($F [3,14] = 4.9$; $p = 0.015$ and $F [3,14] = 3.7$; $p = 0.036$, respectively). That is, ARs were upregulated by androgens in the presence of E (from T) or absence of E (with DHT + ATD). Neither ER α nor ER β exhibited significant changes in the presence or absence of AR or ER activity ($p = 0.46$ to 0.08) Thus, the expression of ER α and ER β could be considered constitutive.

3.5. CRH immunohistochemistry and image analysis

LC CRH immunostaining and analysis are illustrated in Fig. 6. However, there was nonspecific staining of some neuronal cell bodies. In addition to the axon and bouton staining, CRH-positive neurons were present at varying numbers. The unwanted cell staining was removed from the image. The remaining axons were segmented from background and measured, yielding positive pixel area and bouton number. Black arrows follow the same axons through the analysis and turquoise arrowheads follow the same neurons through the analysis.

3.6. Quantification of CRH axonal immunohistochemical staining

The quantification of CRH axonal immunostaining is illustrated in Fig. 7. The positive pixel area and the number of positive boutons were obtained. The regulation of CRH differs markedly from TH, DBH or AR. CRH was significantly suppressed in the absence of ER

activation (+ADT treatment), in the presence or absence of AR activity (DHT + ADT and FLUT + ATD). Indeed, T and placebo treatment were similar, and both should have E activity in the brain but of different origins ($F [3,14] = 8.04$; $p = 0.0023$ for pixel area, and $F [3,14] = 10.78$; $p = 0.0006$ for bouton number).

4. Discussion

4.1. Associations between NE, androgens and aggression

The NE neurons in the male LC have been linked to vigilance and attendance to the environment (Aston-Jones et al., 1996). Elevated vigilance or irritability appears to be a precursor to provoked aggression; and NE has also been implicated in aggression (Haller et al., 1998; Haller et al., 2006; Levine et al., 1990). Pathological anxiety in humans may be likened to unwarranted vigilance and patients with anxiety disorders are largely treated with noradrenergic reuptake inhibitors, or SNRIs that may also act on the serotonin system (Kapczinski et al., 2003). Pharmacological challenge studies with the adrenergic α_2 agonist, clonidine, indicate hyperactive NE in major anxiety disorder (Cameron et al., 2004; Coccaro et al., 1991); and clonidine per se can induce aggression (Coccaro et al., 1991; Nikulina and Klimek, 1993). Behavioral observations of the animals in this study found an increase in yawning with T and DHT + ATD treatments (Bethea et al., 2013b); and yawning has been questionably characterized as a mild aggressive behavior in macaques (Troisi et al., 1990). The LC NE neurons project to the serotonergic dorsal raphe (Bortolozzi and Artigas, 2003), the hypothalamus (Jones et al., 1977) and A10 ventral tegmental area or VTA (El Mansari et al., 2010; Guiard et al., 2008a), highlighting their importance in the regulation of a wide number of autonomic systems. Although androgens and LC NE had been independently linked to aggression, little was known about the specific actions of androgens on the function of the LC NE neurons or their mechanism of action in NHPs (Breuer et al., 2001; Gerra et al., 1996; Haller et al., 1998; Marino et al., 2005; Patki et al., 2015).

4.2. Androgen stimulation of serotonin via NE

Because serotonin neurons lacked AR, this study was initiated to determine whether LC NE could mediate the androgenic stimulation of raphe serotonin-related gene expression. Indeed, the androgenic action of T or DHT + ATD increased TH positive-dendrite density and increased the density of the DBH/NE axonal projection to the dorsal raphe. Moreover, androgen stimulation occurred in the presence or absence of aromatase or ER activity as observed for serotonin-related mRNA expression. Thus, the DBH/NE innervation of the raphe could stimulate TPH2 mRNA expression. Although DBH was highly correlated to TPH2 mRNA expression, R-squared indicated that about 37% of the DBH innervation contributed to TPH2 mRNA expression. This makes sense, since a number of different factors impinge upon serotonin-related gene expression. Likewise, only 43% of the change in LC TH contributed to the DBH innervation of the raphe. This suggests that a portion of the DBH/NE innervation of the raphe is derived from elsewhere. As in our monkeys, gonadectomy of male mice reduced TH in the LC and T-treatment of gonadectomized mice restored TH expression (Pendergast et al., 2008). Another study reported castration of male mice increased LC TH (Thanky et al., 2002), which differs from Pendergast et al. (2008) and our data in monkeys. Timing and stress of surgery may play a role in the different reports.

4.3. Indirect androgen stimulation of NE

The male macaque LC contained AR-positive neurons, as observed with ISH in male rats (Simerly et al., 1990). However, AR *did not* co-localize in the NE neurons. Thus, nuclear AR could not directly mediate the androgen-induced increase in LC dendrite TH and raphe DBH axon density. Rather, this observation suggests that androgens act elsewhere, perhaps in neighboring LC excitatory neurons, or in AR-positive VTA DA neurons that project to LC (Kritzer, 1997). Although the AR-positive nuclei appear small, this is only in comparison to the very large NE neurons. We think that the AR-positive nuclei in the LC were located in smaller neurons that could be GABAergic or glutamatergic. Furthermore, the AR-positive nuclei do not resemble nuclei in astrocytes, oligodendrites or microglia. The phenotypic identity of the AR-positive neurons in the LC awaits investigation, and the postulated VTA DA innervation of the LC needs histological verification (Guiard et al., 2008a).

4.4. Estrogen receptors in LC NE neurons

Male macaque LC NE neurons robustly expressed ER α and ER β . In male rats and mice; ER α and ER β were detected in the LC (Pendergast et al., 2008; Simerly et al., 1990; Yamaguchi and Yuri, 2012). Decreasing the activity of the ERs by blocking aromatase did not affect the ability of DHT to increase dendrite TH or DBH axon density. That is, the presence or absence of ER activation had no effect on the density of DBH-positive axons projecting to the raphe, unlike the reciprocal raphe serotonin projection to the LC, which is affected by E (Bethea et al., 2014a). Therefore, the question of what ERs do in LC NE neurons of male macaques is unanswered at this time. Of interest, ER α knock-out mice (ERKO) exhibited increased aggressive behavior compared to wild type mice (Ogawa et al., 1996). Since the steroid receptors are gene transcription factors, it would be enlightening to probe a large microarray with mRNA extracted from LC NE neurons and find genes that are regulated by E.

4.5. Regulation of steroid receptor expression

The regulation of the different reproductive steroid receptors in male macaque LC was similar to that observed in the male macaque dorsal raphe (Bethea et al., 2015a). That is, androgens increased expression of AR in the presence or absence of aromatase activity. The stimulatory effect of androgens on AR expression has also been documented in the hypothalamus of macaques (Roselli and Resko, 1989) and rodents (Roselli and Resko, 1984). The up-regulation of nuclear AR by androgens could increase responsiveness to androgens and further increase dendrite TH or axonal DBH expression in a feed forward manner. Neither of the ERs was affected by the treatments, suggesting that their expression may be constitutive in the LC NE neurons. We proposed that ER α and ER β are constitutively expressed in serotonin neurons of male macaques (Bethea et al., 2015a), and that ER β in serotonin neurons of female macaques is also constitutively expressed (Gundlah et al., 2000).

All three steroid receptors were widely expressed outside of the LC at this level of the midbrain. Robust expression of AR, ER α and ER β was observed in the median raphe, in all divisions of the parabrachial nucleus, in the medullary tegmentum and throughout the pontine neurophil. The expression of ER α in the median raphe was consistent with our

report of ER α in the dorsal raphe of the same male macaques using the anti-human ER α antibody C1355 from Millipore (Bethea et al., 2015a). We previously showed AR in the dorsal raphe, PAG, ventral to the pons and lateral to the decussation of the cerebellar peduncles in male macaques. Kritzer et al. reported that ARs were also expressed in male rats in VTA, lateral terminal and peripeduncular nuclei, subregions of the substantia nigra, lateral margins of the retrorubral fields and others (Kritzer, 1997). We have not examined AR in more rostral sections containing VTA or substantia nigra in male macaques, but we hope future funding will enable these experiments.

GPER1, the membrane E-receptor, has been found throughout the rodent brain with high levels in the olfactory bulbs, hypothalamus, LC, the hippocampus, the habenular nucleus of the epithalamus, nucleus of the solitary tract, cortical regions including the motor, somatosensory piriform cortices, as well as the Purkinje and granule cells of the cerebellum (Almey et al., 2015). While many functions of GPER1 have been described, information on the hormonal regulation of GPER1 is limited. One article reported no effect of age or E treatment on GPER1 in the dorsolateral prefrontal cortex of ovariectomized female macaques (Crimins et al., 2016). We have not pursued GPER1 at this point. However, it is reasonable to speculate that GPER1 will be present in the macaque LC, and like nuclear ER receptors, not subject to regulation by steroid hormones.

4.6. Discrepancies in ER α expression between male and female macaques

The detection of ER α in male macaque LC differs from results in female macaques in which ER α was undetectable in the LC using the 1D5 anti-human ER α antibody from Neomarkers, or the anti-human ER α H222 antibody from Abbott Laboratories (Vanderhorst et al., 2009), or a combination of H222 and D75 gifted by Dr. Geoffrey Greene (Schutzer and Bethea, 1997). Both 1D5 and H222 detected ER α in the neighboring periaqueductal gray area (PAG) and hypothalamus, so the relative absence of ER α in the female LC was not due to any obvious antibody or technical issue (Schutzer and Bethea, 1997; Vanderhorst et al., 2009). However, both ER α and ER β were detected in female macaque LC by in situ hybridization (Pau et al., 2000). With our detection of ER α and ER β in the male macaque LC with Millipore C1355, it may behoove us to re-examine the female LC with the same antibody.

A parallel situation exists in the female dorsal raphe with respect to ER α . ER α is undetectable in the dorsal raphe of female macaques with H222 or 1D5 (Vanderhorst et al., 2009). It is also undetectable in male and female rats with polyclonal anti-ER α from Santa Cruz (Sheng et al., 2004). However, Alves et al. (Alves et al., 1998) detected ER α in the female rat raphe with a unique anti-rat ER α -beta galactosidase fusion protein made by Okamura et al. (Okamura et al., 1992), which agrees with ISH results (Simerly et al., 1990). How or why these antibodies would differ in their ability to detect ER α in amine neurons versus neighboring neurons is a mystery.

4.7. Midbrain amine circuit

Electrophysiological studies in rats indicate a midbrain circuit whereby the LC NE, raphe serotonin and VTA DA neurons communicate as illustrated in Fig. 8. Most studies support a

stimulatory effect of DA on LC NE and serotonin, as well as a stimulatory effect of LC NE on serotonin. Serotonin is thought to inhibit LC NE and VTA DA. At this point, the regulations of the rate limiting enzymes (TH and TPH2), as well as, the steroid receptor compliment of the LC and serotonergic raphe in male macaques have been described. Lacking is a histological examination of the VTA DA neurons in male macaques with attention to DA regulation and localization of the reproductive steroid receptors. In male rats, acute castration depleted cortical TH axon densities, and T replacement restored cortical-TH-axon densities (Kritzer et al., 1999), which apparently involves a mesocortical projection from VTA (Kritzer, 2000). If these observations translate to monkeys, then castrated-only male macaques should exhibit relatively low serotonin, NE and DA in the midbrain circuit. Androgen treatment may elevate all three amines leading to (1) increased awareness through increased NE (Aston-Jones et al., 1999); (2) increased yawning associated with increased serotonin, DA and oxytocin (Bethea et al., 2014a; Bethea et al., 2015b, Sanna et al., 2012), and (3) increased contentment with increased DA (Volkow et al., 2011).

4.8. Possible role of the midbrain amine circuit in aggression

Both androgens and LC NE have been linked to aggression in males, and now it is clear that androgens increase TH in LC. Hence, some discussion of aggression is warranted. The neurobiological basis of provoked aggression in male macaques is poorly understood. We speculate that androgens increase all of the amines to a physiologically consistent homeostasis as described above. Indeed, the androgen-treated males in this study demonstrated increased yawning over placebo and FLUT + ATD groups (Bethea et al., 2013b). However, they had no access to females, little provocation and little overt aggression. The question remains as to how a provocation leads to aggression, and whether the midbrain circuit, as diagramed in Fig. 8, plays a role. It is reasonable to suggest that a negative provocation (stress) further increases LC NE activity. In turn, this could decrease VTA DA and increase serotonin. A decrease in VTA DA to the nucleus accumbens could cause hostility. This idea is based upon concepts of VTA DA reduction to the nucleus accumbens during drug withdrawal and the accompanying hostility (McCormick and Smith, 1995). To further this hypothesis, closer examination of androgen regulated DA in the VTA, hypothalamus, nucleus accumbens and frontal cortex is needed in primates. Moreover, pharmacologically *reduced* VTA DA or *increased* LC NE should increase hostility or aggressive acts in androgen-treated male macaques.

4.9. Other links between aggression and the midbrain amine circuit

From another avenue, aggression in humans and animals was associated with increased serotonin due to low activity or deletion of MAO-A. Therefore, if provoked-increases in LC NE also increase serotonin, then both LC NE and serotonin would inhibit VTA DA and further decrease DA projecting to nucleus accumbens, thereby precipitating hostility. This could explain the correlation of elevated serotonin and aggression in humans and animals with deficient MAO-A.

4.10. Estrogen regulation of CRH innervation in male macaques

CRH directly activates LC NE neurons (Curtis et al., 2012; Jedema and Grace, 2004). Since LC NE neurons responded to androgens without nuclear AR, the stimulatory CRH innervation to the LC was examined. The regulation of CRH axon density differed markedly from the regulation of TH or DBH and it is unlikely that CRH mediated the stimulatory effect of androgens on the LC NE. The CRH innervation of the LC was suppressed by aromatase inhibition, which means that it is normally maintained or stimulated by E. The data further suggest that the E may be derived from metabolism of T or from de novo synthesis in the brain in the placebo group. The innervation is thought to partly derive from the hypothalamic PVN, which has a descending component in the caudal part of the nucleus (Reyes et al., 2005). If true, then E *stimulated* PVN CRH in castrated male macaques. Conversely, E *decreased* PVN CRH in ovariectomized female macaques (Bethea and Centeno, 2008). Thus, the effect of E on PVN CRH is potentially opposite in male and female macaques. In mice, sex differences in CRH receptor internalization were also noted (Bangasser et al., 2010; Bangasser et al., 2012). Another possibility is that E is needed to maintain transport of CRH to the LC. We found the same pattern of regulation in the serotonergic innervation of the male LC. That is, the serotonin axon density was significantly reduced in animals treated with aromatase inhibitors, and this pattern differed from TPH2 gene expression (Bethea et al., 2014a). To resolve this question, CRH expression needs to be examined in the PVN of the same males used in this study. In addition, the presence of GPER1 in rodent LC offers another mechanism deserving of study (Almey et al., 2015).

4.11. Questions remaining

Altogether, these observations raise important questions that need resolution such as (1) how do androgens increase TH or TPH2 expression if not through nuclear AR receptors? (2) What is the neuronal phenotype of AR-positive neurons in the LC and raphe? (3) Do VTA DA neurons contain AR and does AR stimulate TH expression in VTA DA neurons in macaques? (4) Do VTA DA neurons stimulate LC NE or raphe serotonin neurons upon androgen administration? (5) What genes are regulated by ER α and ER β in LC NE neurons? (6) Could an acute pharmacological block of VTA DA lead to hostility in androgen-treated macaques? (7) Does the expression of CRH in the hypothalamic PVN reflect the CRH innervation of the LC, or does aromatase inhibition prevent trafficking of CRH to the LC? It is hoped that these questions will be approached in the future by the author's laboratory or by the next generation of neuroscientists.

5. Conclusions

Androgens increased TH in the LC dendrites and increased DBH in axons innervating the dorsal raphe. LC NE neurons lacked nuclear AR, so the effect of androgens was not mediated directly, although a membrane action cannot be ruled out. LC NE neurons contained nuclear ER α and ER β , but their specific effects on gene transcription are unknown. Androgens increased AR expression in the LC, but there was no regulation of ER α or ER β in this paradigm. In addition, the CRH innervation of the LC was suppressed by aromatase inhibition, meaning CRH was maintained or normally stimulated by E in male

macaques, which is the opposite in female macaques. Also, the CRH innervation of the LC NE did not correlate with androgen regulation of TH or DBH. The regulation of the rate limiting enzymes and co-localization of steroid receptors was the same in the LC as in the dorsal raphe.

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Abbreviations

LC	locus coeruleus
ERα and ERβ	nuclear estrogen receptors
ARs	androgen receptors
T	testosterone
DHT	dihydrotestosterone, non-aromatizable androgen
E	estradiol
ATD	aromatase inhibitor
TH	tyrosine hydroxylase
TPH2	tryptophan hydroxylase, type 2
SERT	serotonin reuptake transporter
mRNA	messenger RNA
VTA	A10 dopamine cell group
CRH	corticotrophin releasing hormone
IACUC	Institutional Animal Care and Use Committee
ERKO	ER α knock-out
BSA	bovine serum albumin
ISH	in situ hybridization
IHC	immunohistochemistry
DPX	mounting media

ABC	avidin biotin complex
NGS	normal goat serum
NHS	normal horse serum
KPBS	potassium phosphate buffered saline
FLUT	Flutamide
DA	dopamine
NE	norepinephrine, noradrenergic
PVN	hypothalamic paraventricular

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Fig. 1.

Photomicrographs of TH in the dendritic halo of the LC and DBH in the dorsal raphe of male monkeys. Panel A contains the original image, which illustrates the LC with respect to other anatomical structures. The image is then cropped to a consistent dimension containing the dendrites as shown in panel B. The TH-positive dendrites are highlighted in panel C; the image is converted to a binary image, and positive pixel area is measured as shown in panel D. Panels E, F, G and H illustrate DBH axons innervating the dorsal raphe. The original image is cropped to a consistent dimension (E). The positive pixels are highlighted, and only groups of pixels corresponding to the size of a neuronal cell body are obtained by a filter (F). The pixels corresponding to the cell bodies are subtracted from the cropped image (E minus F). The remaining DBH-positive axons are highlighted (G) and converted to a binary image, which is measured. The measurements yield the positive pixel area and the total area. The boutons of the DBH axons could also be measured by applying a filter that finds groups of pixels in the bouton size range. Different types of DBH staining in the dorsal raphe are illustrated in Panels I and J. Both panels illustrate neuronal cell bodies that exhibit a very dense or less dense concentration of DBH boutons, or a peri-cellular location of DBH boutons. *Arrowheads*—neuronal cell bodies in the raphe with different densities of DBH boutons. *mv*—nucleus of the mesencephalic tract of the trigeminal (5th facial) nerve.

Fig. 2.

Histograms of the quantitative analysis of LC dendrite TH immunostaining and raphe DBH axon immunostaining. TH and DBH were elevated by T and DHT + ATD treatments ($F [3,15] = 67.1$; $p < 0.0001$, and $F [3,16] = 9.94$; $p = 0.006$, respectively). Columns with different letters were significantly different by Newman–Keuls post hoc pairwise comparison at $p < 0.05$.

Fig. 3.

Illustration of the linear regression analyses performed to determine the relationship of DBH in the raphe to TH in LC dendrites and the relationship of DBH in the raphe to TPH2 gene expression. Individual animal means of each outcome were used in the correlation ($n = 20$). The top panel indicates that there is a significant correlation between raphe DBH innervation and TH in LC dendrites ($p = 0.0022$; $r^2 = 0.43$). The bottom panel indicates there was a significant correlation between raphe DBH innervation and TPH2 mRNA expression ($p = 0.0072$; $r^2 = 0.37$).

Fig. 4. Photomicrographs of the LC illustrating single labeled AR, ERα and ERβ (left column) and double labeling for each receptor plus TH in male monkeys (right column). *Left columns.* Illustration of single-labeled AR, ERα and ERβ in the LC and surrounding neuropil. There is robust and widespread expression of all three nuclear receptors. However, ERβ staining is most dense within the LC, and it presents with different patterns of cellular staining; that is, (i) strongly nuclear, (ii) moderate nuclear concentration plus cytoplasmic staining, or (iii) largely diffuse cytoplasmic staining. *Top right.* A double labeled area of the LC showing TH

positive neurons with clear nuclei and AR-positive nuclei in other neurons that were not stained for TH. *Middle right.* A double labeled area of the LC showing TH positive neurons that contain nuclear ER α . There were also neurons that were positive for ER α and do not exhibit TH. *Bottom right.* A double labeled area of the LC showing TH positive neurons that contain nuclear ER β . There are double-labeled neurons with darkly stained nuclei and others with gray and brown staining that may be TH neurons with diffuse ER β . *Black arrowheads* point to positively stained nuclei. *White arrowheads* point to neurons with only cytoplasmic staining for TH. *Black arrows* point to neurons that are double labeled for TH and a nuclear receptor. *White arrows* point to neurons that appear to be labeled for TH and contain diffuse staining for ER β .

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Fig. 5.

Quantitation of steroid receptor immunostaining. *Left column.* AR in the LC of male macaques. AR-positive pixel area and the number of AR-positive neurons are illustrated in the top and bottom panels, respectively. AR-positive pixel area and the number of AR-positive neurons were significantly increased in the T and DHT + ATD groups compared to placebo or FLUT + ATD ($F [3,14] = 4.9$; $p = 0.015$ and $F [3,14] = 3.7$; $p = 0.036$, respectively). In the top panel, columns with different letters are significantly different with Newman–Keuls post hoc pairwise comparison ($p < 0.05$). In the bottom panel, there was a significant difference with ANOVA, but no pairwise differences were detected with the post hoc test. *Middle column.* ERα in the LC of male macaques. ERα-positive pixel area and the number of ERα-positive neurons are illustrated in the top and bottom panels, respectively. There was no difference in ERα-positive pixel area or -positive neurons between the groups ($F [3,13] = 2.8$; $p = 0.08$ and $F [3,13] = 2.4$; $p = 0.114$, respectively). *Right column.* ERβ in the LC of male macaques. ERβ-positive pixel area and the number of ERβ-positive neurons are illustrated in the top and bottom panels, respectively. There was no difference in ERβ-positive pixel area or -positive neurons between the groups ($F [3,14] = 1.8$; $p = 0.19$ and $F [3,14] = 0.92$; $p = 0.45$, respectively).

CRH in LC

Fig. 6.

Photomicrographs of CRH image analysis in the LC of male macaques. (A) The original picture was cropped to a consistent area. The same axons are marked with black arrows and the same neuronal cell bodies are marked with turquoise arrowheads in all panels. (B) The immunostained cells and axons were highlighted and pixel groups within a range corresponding to cell body size were selected and the results are shown. (C) The cell pixels were subtracted from the original image ($A - B = C$) and the remaining axons were highlighted. (D) The highlighted axons were converted to a binary image and the pixel area was measured. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

Histograms illustrating the quantitative analysis of CRH innervation of the LC in male macaques. There was a significant difference across the groups in the CRH-positive pixel area and in the number of CRH-positive boutons ($F [3,14] = 8.04$; $p = 0.0023$ for pixel area, and $F [3,14] = 10.78$; $p = 0.0006$ for bouton number). CRH axon density was significantly suppressed by ADT in the presence or absence of an androgen. a — significantly different from the T-treated group ($p < 0.05$, Newman–Keuls post hoc test). b — significantly different from the placebo-treated group ($p < 0.05$, Newman–Keuls post hoc test).

Fig. 8.

Hypothetical midbrain circuit of amine nuclei. This diagram was based upon an amalgam of electrophysiological and immunological reports in rodents and monkeys. Reports on the LC NE innervation of VTA DA indicate both stimulatory and inhibitory effects (El Mansari et al., 2010; Grenhoff et al., 1993; Guiard et al., 2008a; Guiard et al., 2008b; Velasquez-Martinez et al., 2012). In male monkeys, we have confirmed that LC NE and raphe serotonin neurons lack nuclear AR and that steroid hormones regulated communication between LC and raphe. The steroid receptor compliment and regulation of VTA DA neurons in male macaques are lacking, but reliance on published rodent studies and female macaque studies enables viable speculation (Aubele and Kritzer, 2012; Creutz and Kritzer, 2004; Kritzer, 1997; Kritzer et al., 2003).

Table 1

Treatments and their activation of androgen receptors (ARs) or estrogen receptors (ERs).

Treatments and activation of steroid receptors				
Treatment	T	Placebo	DHT + ATD	FLUT + ATD
<i>n</i>	5	5	5	5
Receptor activation	+AR+ER	−AR + ER ^{low}	+AR−ER	−AR−ER

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