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The Effect of Long-term Ovariectomy on Midbrain Stress Systems in Free Ranging Macaques

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Abstract

Communication between the serotonin system and the CRF system plays a pivotal role in the mediation of stress and stress reactivity. CRF appears to be inhibitory of serotonin neurotransmission through the CRF receptor type 1 (CRF-R1). Serotonin neurons also detect the urocortins, which are thought to be anxiolytic. Components of the CRF system in the serotonergic dorsal raphe region were examined in macaques that were ovary-intact or ovariectomized for 3 years living in a relatively natural environment. Female Japanese macaques (*Macaca fuscata)* were ovariectomized or tubal-ligated (n=5/group) and returned to their natal troop for 3 years. Quantitation of (1) CRF innervation of the serotonergic dorsal raphe, (2) CRF-Receptor type 1 (CRF-R1) in the dorsal raphe, (3) Urocortin 1 (UCN1) cells near the Edinger-Westfal nucleus and (4) UCN1 axons, was obtained with immunocytochemical staining and image analysis. There was no statistical difference in CRF axonal staining in the dorsal raphe, or in UCN1 axonal staining near the dorsal raphe. However, the average number of detectable UCN1 postive cells was significantly lower in the Ovx group than in the Intact group ($p = 0.003$). Average CRF-R1 positive pixel number and positive cell number were significantly higher in the Ovx group than in the Intact group ($p=0.005$ and 0.02, respectivly). The higher expression of CRF-R1 and lower expression of UCN1 in the Ovx group indicates they may be more vulnerable to stress. The greater expression of CRF-R1 could cause a greater inhibition of serotonin upon a stress-induced increase in CRF as well.

1. Introduction

Women experience ovarian failure and loss of ovarian steroid production around 50 years of age. Thus, with extended life spans, a woman may live 35–40 years without ovarian steroid secretion. After menopause, a significant number of women become more anxious, and less able to cope with stress, leading to new onset of depression (Conde et al., 2006; Heikkinen

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et al., 2006; Maki et al.; Tangen and Mykletun, 2008). The role of ovarian hormones in depression and stress sensitivity is of great interest for women transitioning through menopause. In addition, a significant number of young women lose ovarian function due to chemotherapy and require hormone therapy (Blumenfeld, 2012; Goswami and Conway, 2005).

The serotonin system plays a pivotal role in stress and affective disorders, including depression and anxiety. The administration of selective serotonin reuptake inhibitors (SSRIs) is to date, the most effective pharmacological intervention for depression and anxiety disorders (Cipriani et al., 2012; Gartlehner et al., 2011). The hypothalamic-pituitary adrenal axis has also been implicated in the etiology of depression (Morimoto et al., 1993; Nemeroff, 2004; Weiss et al., 1994), and an elevation in corticotropin releasing factor (CRF) in the hypothalamic paraventricular nucleus (PVN) is thought to underlie the hyperactivity of the HPA axis in depression (de Kloet et al., 2005; Holsboer, 1999; Keck and Holsboer, 2001). However, the CRF ‘system’ has multiple compnents including the CRF receptors 1 and 2, and the related peptide urocortins 1, 2 and 3 (UCN). (Clark and Kaiyala, 2003). The urocortins appear to have higher affinity for CRF-R2 than CRF; and they have been implicated in reducing the response to acute stress (Reul and Holsboer, 2002). On the other hand, CRF activation of CRF-R1 increases anxiety in mice and humans (Bailey et al., 2011; Smith et al., 1998).

This laboratory has shown that the ovarian steroids, estrogen (E) and progesterone (P) increase serotonin neural function (Bethea et al., 2002), protect serotonin neuronal health (Bethea et al., 2009) and increase gene and protein expression underlying dendritic spine proliferation on serotonin neurons (Bethea and Reddy, 2010; Bethea and Reddy, 2011; Rivera and Bethea, 2012). All of these actions could translate to improved mood and stress resilience in women. In addition, we found that administration of E or E+P to ovariectomized macaques decreased CRF gene and protein expression in the PVN of macaques (Bethea and Centeno, 2008), decreased CRF innervation of the dorsal and median raphe, decreased CRF-R1 receptors and increased CRF-R2 receptors in the dorsal raphe, increased detectable midbrain urocortin (UCN1) cell number and increased UCN1 fiber densitiy (Sanchez et al., 2010). Together these data suggest that ovarian hormone administration may improve stress resilience through the serotonin, CRF and UCN systems.

However, all of the former studies utilized a macaque model of hormone replacement therapy in which healthy adult monkeys were acquired that had been ovariectomized (Ovx) in other programs. The animals were rested for up to 8 months and then treated for 1 month with placebo, E or E+P via silastic capsules. This model was extremely cost effective and it demonstrated robust differences in serotonin-related, CRF and UCN1 gene and protein expression between treatment groups. However, it has certain limitations in comparison to natural menopause, although it resembles young women with chemotherapy-induced infertility. Of significance, the animals were Ovx for a relatively short period of time. In addition, the hormone therapy was continuous for one month rather than cycling for many months in the manner of intact females.

Therefore, we questioned the regulation of serotonin and CRF/UCN systems in intact monkeys compared to ovariectomized individuals, and whether the length of ovariectomy was an issue. To this end, we ovariectomized or ligated the fallopian tubes of young female Japanese macaques and maintained them for three years in an outdoor troop at the Oregon National Primate Research Center (ONPRC). These animals were not intended to fully model menopause, but rather to examine the effects of long-term ovariectomy, in a semi-natural, social environment. Other variables under current examination include diet and age.

We recently reported that expression of pivotal serotoin-related genes was reduced by long-term ovariectomy, which may partially reflect a decrease in serotonin cell number (Bethea et al., 2012). There were subtle differences between the regulation of serotonin-related genes by hormone replacement in the short-term Ovx monkeys compared to the differences in expression observed in the Intact versus long-term Ovx monkeys, again possibly related to the loss of serotonin neurons in the latter. We also reported that the same Ovx monkeys exhibited a decrease in positive social behavior in the troop and exhibited an increase in certain anxiety-related behaviors in temperament tests (Coleman et al., 2011).

In this study we extend our examination to the midbrain CRF and UCN1 systems in the long-term Ovx monkeys in comparison to their Intact troop mates.

2. Results

It was not possible to monitor the menstrual cycle in the individual tubal-ligated animals while they were in the outdoor corral or group housing; and they were in single cages for too brief a time to determine menstrual cycle stage from the presence of blood in the waste pans. Therefore, we measured estradiol (E), progesterone (P) and cortisol concentrations in serum obtained prior to euthanasia. Although previously published, this data is re-presented for convenience in Table 1. We found that serum E and P were very low in the ovariectomized animals. Low levels of E are produced by fat tissue and these concentrations are consistent with previous analyses of serum from ovariectomized monkeys (Bethea et al., 2009). Different concentrations of serum E and P were present among the Intact females, indicating that the Intact animals were at various stages of the menstrual cycle, as designated on Table 1. The cortisol concentrations were obtained prior to fenfluramine challenge, which has been published. There was no difference in basal cortisol between the groups. In addition, the cortisol response to fenfluramine did not differ between the groups (Bethea et al., 2012). We could not euthanize the animals at a particular stage of the cycle since this information was not available a priori. We hypothesize that the neurobiology results reflect the presence of cycling ovarian hormones in the Intact group; and reflect the near absence of these steroids in the Ovx group for a 3-year period. However, we cannot completely rule out that the possibility that the stage of the menstrual cycle at euthanasia increased the variance in the tubal-ligated (ovary) Intact group.

The location of the area analyzed for CRF fiber staining relative to the raphe subnuclei is illustrated in Figure 1 (dashed red box). Since the CRF fibers do not correspond to a particular subnucleus, the subnuclei were located based upon TPH2 gene expression as previously published (Sanchez et al., 2005). CRF staining in representative sections from the rostral, medial and caudal areas are shown. Routinely, representative sets of sections at 250 μm intervals contain 2 sections in the rostral area, 2 sections in the middle area and 3 sections in the caudal area. In our sections, the subnuclei at the rostral levels appear slightly different from reports with human brain (Bonkale et al., 2006). The Dorsal Raphe Ventrolateral (DRvl) subnucleus emerges in the middle levels rather than the rostral levels. This could be a species difference or it could be due to our plane of sectioning.

Representative CRF fiber staining in the dorsal raphe is shown in Figure 2. The CRF staining is largely in the boutons. An Ovx and an Intact animal are shown in panels A and B as observed with a Leica brightfield microscope. An Ovx and an Intact animal are shown in panels C and D following segmentation (red) as observed in Slidebook 4.2 and ImageJ. Panel E illustrates the rostral dorsal raphe as it appears in the montage generated by the Marianas and the area that was magnified and analyzed. The box contains the densest area of CRF fiber staining. This area corresponds roughly to the DRv and the middle of the DRd

subnuclei as illustrated in Figure 1. Seven anatomical levels of the dorsal raphe from a rostral to a caudal level were subjected to image analysis.

Figure 3 illustrates the quantitative analysis of CRF fiber density in the dorsal raphe. There was no significant difference in the CRF axonal bouton staining in the dorsal raphe. In the panel labeled ALL (7 sections), the overall average CRF positive pixel number and positive bouton number were not significantly different between the Intact group and the Ovx group (t (df 7) = 1.22; p= 0.26 and t (df 7)=1.088; p=0.31,respectively). Identical results were obtained with comparison of the total positive pixel number and total positive bouton number (t (df 7) = 1.22, p=0.26, and t (df 7)=1.088, p=0.31, respectively). Moreover, similar results were observed when the rostral (4 sections) and caudal (3 sections) areas were parsed. There did not appear to be a greater contribution from either region. The average of the areas analyzed for each animal equaled 817,394 pixels^2 or 294,262 μm^2.

CRF-R1 immunostaining in the dorsal raphe is illustrated in Figure 4. CRF-R1 staining in the dorsal raphe of an Ovx animal is shown in panel A as observed with a Leica brightfield microscope. The staining is largely cellular and at higher power, it can be seen as patches on the cell surface depending on the plane of sectioning. Panels B and C illustrate representative CRF-R1 staining in an Ovx animal and in an Intact animal as captured with Slidebook 4.2 from the Marianas stereology workstation. Panels D and E illustrate the same sections following segmentation of positive signal with ImageJ. CRF-R1 immunostaining was not confined to any particular subnucleus. Three levels of the dorsal raphe, one each at a rostral, mid- and caudal level (250 – 500 μm apart) were immunostained from each animal and subjected to image analysis.

There was a higher expression of CRF-R1 in the Ovx group compared to the Intact group as illustrated in Figure 5. The overall average CRF-R1 positive pixel number (t (df 7)=3.983, p=0.005) and positive cell number (Mann-Whitney test U=0, p=0.016) were significantly higher in the Ovx group than in the Intact group. The standard t-test analysis of the positive cell number exhibited a significant difference in variance (F=11.44, p=0.04), which required the nonparametric Mann-Whitney test. Identical results were obtained with comparison of the total positive pixel number and total positive bouton number (t (df 7)=3.983, p=0.005 and U=0, p=0.016, respectively). The average of the areas analyzed for each animal equaled 556,711 pixels^2 or 200,416 μm^2.

Representative UCN1 cell staining in an Ovx animal and in an Intact animal as observed with a Leica brightfield microscope is illustrated in Figure 6. The staining was clearly cytoplasmic. These cells are located in the supraoptic area (SOA) adjacent to the Edinger-Westfal nucleus (May et al., 2008; Sanchez et al., 2010). Six levels of the rostral midbrain containing the SOA were subjected to image analysis.

Figure 7 illustrates the quantitative analysis of UCN1 cell staining. The variance was significantly different between the groups (F=12.93, p=0.03), which necessitated the Mann-Whitney nonparametric test. The overall average number of detectable UCN1 postive cells was significantly lower in the Ovx group than in the Intact group (Mann-Whitney U=0, p=0.016). The average of the areas analyzed equaled 264,988 pixels^2 or 95,396 μm^2.

Figure 8 illustrates representative UCN1 fiber staining located rostral to the raphe of an Ovx and an Intact animal as observed with a Leica brightfield microscope. The staining was largely in the boutons. This little cluster of UCN1 fibers is located caudal to the Edinger-Westfall nucleus, but rostral to the dorsal raphe nucleus and it provides a reliable area for analysis. It is found in 3 levels of the midbrain at 250 μm intervals, which were subjected to image analysis.

Figure 9 illustrates the quantitative analysis. There was no significant difference in the UCN1 axonal bouton staining in the midbrain although there was a trend toward higher values in the Intact versus the Ovx animals. The overall average UCN1 positive pixel number and positive bouton number were not significantly different between the Intact group and the Ovx group (t (df 7)=1.38, p= 0.21 and t (df 7)=1.33, p=0.22, respectively). Identical results were obtained with comparison of the total positive pixel number and total positive bouton number (t (df 7)=1.38, p= 0.21 and t (df 7)=1.33, p=0.22, respectively). The average of the areas analyzed equaled 180,037 pixels^2 or 64,813 μm^2.

3. Discussion

During and after menopause a significant number of women report an increase in anxiety and vulnerability to stress (Conde et al., 2006; Heikkinen et al., 2006; Maki et al.; Tangen and Mykletun, 2008). Both the CRF and serotonin systems respond to stress and play roles in depression and anxiety disorders (Arango et al., 2002; Holsboer, 1999; Keck and Holsboer, 2001; Mann et al., 1996). Moreover, increasing evidence suggests that their function is inextricably linked. In humans, CRF terminals appose serotonin neurons in the raphe region (Ruggiero et al., 1999), and a postmortem study reported that patients with major depressive disorder had significantly more CRH-positive neurons in the PVN than normal controls (Raadsheer et al., 1994). Antidepressant SSRIs that increase available serotonin reduce the sensitivity of CRF neurons in the PVN (Stout et al., 2002), and cortisol levels return to normal in depressed patients treated with a variety of antidepressants (Himmerich et al., 2006; Schule et al., 2006). Moreover, repeated treatment with citalopram, an SSRI, decreased CRF and HPA axis activity in rodents (Moncek et al., 2003).

CRF producing neurons and projections are widespread and play a role in stress-related psychiatric disorders (Clark and Kaiyala, 2003). CRF innervation of the serotonergic dorsal raphe nucleus has been described and CRF receptors are found in the dorsal raphe of rodents (Linthorst, 2005; Lowry et al., 2000; Valentino et al., 2010) and primates (Sanchez et al., 2010). Within the CRF family, UCN1 plays a role in attenuation of the stress response (Pan and Kastin, 2008). Subsequently, the opposing effects of CRF and UCN1 on serotonin were established in rodents (Valentino et al., 2010). Administration of CRF directly into the dorsal raphe nucleus inhibits serotonergic activity, and CRF-R1 antagonists block this effect (Denihan et al., 2000). Conversely, the stimulatory effect of UCN and CRF-R2 is supported by increased 5-HT efflux in the basolateral amygdala (a projection region of the dorsal raphe) with intra-raphe administration of the CRF-R2 agonist, UCN2. This effect was completely blocked by antisauvagine-30 (ASV-30), a relatively selective CRF-R2 antagonist (Amat et al., 2004).

In the absence of hormone therapy, postmenopausal women exhibit higher release of ACTH and cortisol when administered a challenge of dexamethasone plus CRH (Kudielka et al., 1999) suggesting that their CRF system may be hyper-responsive. In animal models, the effects of ovarian steroids on the HPA axis are complex. Several studies initially indicated that treatment of ovariectomized female or male rats with estrogen increased CRF mRNA (Li et al., 2003; Lund et al., 2004). Subsequently, it was recognized that acute E treatment increases CRF expression, but low dose chronic E treatment decreased stress-induced ACTH in a rodent model (Dayas et al., 2000). In addition, 5 days of E treatment to ovariectomized macaques prevented the elevation in cortisol induced by icv administration of interleukin 1-alpha (Xia-Zhang et al., 1995).

We previously found that 1 month of hormone replacement in short-term (5–8 months) ovariectomized monkeys decreased CRF mRNA and protein in the PVN (Bethea and Centeno, 2008). In the same paradigm, we showed that E ± P supplementation decreased

CRF innervation of the dorsal raphe and median raphe; decreased CRF-R1 in the dorsal raphe; increased CRF-R2 in the dorsal raphe; increased UNC1 immunostained neurons in the SOA near the Edinger-Westfal nucleus; and increased UNC1 axon density in a cluster of fibers rostral to the dorsal raphe (Sanchez et al., 2010). We also found that CRF axon density in the caudal portion of the dorsal raphe was increased in ovarian intact individuals who were stress-sensitive, whereas UCN1 axon density was decreased (Weissheimer, 2010). Hence, ovarian steroids, stress and stress sensitivity can all regulate CRF and UCN1. It is important to note that all of these studies were done indoors with animals in either single cages (Weissheimer, 2010) or paired housing (Bethea and Centeno, 2008; Sanchez et al., 2010).

In this study with Japanese macaques in an outdoor troop setting, long-term Ovx had no effect on CRF innervation of the dorsal raphe although the possibility of a type 2 error is present due to the small number of animals. Hormone replacement is not equivalent to having intact ovaries so comparisons to the earlier study are limited, and our current observation has several interpretations or caveats. It may suggest that in an outdoor social environment (that can only be described as very ‘laid-back’), the presence or absence of ovarian hormones did not significantly impact CRF production. The short-term Ovx in indoor caged housing had an elevation in CRF gene and protein expression that was ameliorated by hormone therapy (Sanchez et al., 2010). This may imply that indoor caged animals are under low, but chronic stress that is not present in the outdoor troop; a notion that is supported by other studies (Olsson, 2007; Refinement, 2009; Schapiro et al., 2000; Schapiro, 2002; Schapiro, 1996). Alternatively, we may not have the power to detect a difference. However, the trend toward Intact animals having *higher* CRF fibers in the dorsal raphe than the Ovx group is the opposite of earlier data. Although the ranks of the individuals did not change, the Intact individuals participated in sexual behavior to a significantly greater extent than the Ovx individuals (Coleman et al., 2011). There is an increase in fighting in the troop during mating season that would have involved the Intact animals to a much larger extent than the Ovx animals. Since the animals were euthanised in November (mating season), the Intact animals may have had more monkey-type stress at that time, which would contribute to higher CRF immunodetection in the Intact animals. It is possible that a different result would have been found in a different season. In addition, the animals in this study were ovariectomized at a younger age than in previous studies with indoor rhesus macaques.

CRF-R1 is considered the ‘anxiogenic’ receptor. We found that dorsal raphe CRF-R1 protein was significantly elevated in the long-term Ovx females. This could make the animals more sensitive to stress, and cause a greater inhibition of serotonin when stress is present. Although there was little effect of long-term Ovx on anxiety behaviors in the troop environment, certain parameters of anxiety behavior were increased in temperament tests that were conducted indoors with brief single caging.

In our previous study of hormone therapy in short-term Ovx macaques, we found that CRF-R1 immunoreactivity was elevated in Ovx and suppressed by ovarian steroid treatment (Sanchez et al., 2010), which is consistent with this study in which CRF-R1 immunoreactivity is suppressed in the Intact group compared to the Ovx group. Given that the protein expression and immunoreactivity are derived from a change in gene expression, then the mechanism by which ovarian steroids suppress CRF-R1 gene expression is of interest. In general, the ability of E to decrease gene expression appears to rely on indirect mechanisms. In one pathway, ligand activated steroid receptors sequester NFkB and thereby decrease gene transcription that is dependent upon binding of NFkB to its response element, NRE (Cerillo et al., 1998; McKay and Cidlowski, 1999). Hence, genes that are driven by NFkB can be deactivated with E treatment. We have shown that E treatment decreases

NFkB translocation to the nucleus in serotonin neurons of the dorsal raphe (Bethea et al., 2006). An unexplored mechanism involves repressor element silencing transcription factor/ neuron-restrictive silencing factor (REST/NRSF). REST is a repressor of transcription of TPH2 (Patel et al., 2007) and CRF (Seth and Majzoub, 2001). Hence, it is present in serotonin neurons. It is attractive to speculate that E affects REST in some fashion, which may influence CRF-R1 expression. It is also important to note that CRF terminals are on serotonin neurons as well as GABA neurons (Waselus et al., 2005). Therefore, CRF via CRF-R1 may stimulate GABA neurons that in turn inhibit serotonin neurons.

We also observed that there were fewer UCN1 positive cells in the midbrain SOA. There was a trend toward lower axon density in the Ovx group, but due to the small number of monkeys there remains the possibility of a type 2 statistical error in which a real effect is not detected. Nonetheless the data suggest that there may be a reduction in UCN1 production and transport resulting in fewer detectable cells or axons. Or, the reduction in UCN1 neurons in the long-term Ovx may be a decrease in cell number rather than a decrease in UCN1 production. Nonetheless, the UCN1 reserve appears to be reduced in the long-term Ovx and upon stress, further changes might be observed. In the short-term Ovx animals housed indoors, there was a significant decrease in the number of detectable UNC1 positive neurons and also significantly decreased UNC1 axon density (Sanchez et al., 2010), which mirrors this study. Whether these observations are due to a decrease in UCN1 cell number or a decrease in UCN1 expression is unresolved.

UCN1 decreases feeding behavior, and also participates in the stress response (Oki and Sasano, 2004; Pan and Kastin, 2008; Spina et al., 1996). We previously showed that relative UCN1 gene expression was 1000x higher than UCN2, and 10,000x higher than UCN3 in the raphe region (Sanchez et al., 2010). UCN1 and 2 bind to CRF-R2 with greater affinity than CRF, suggesting that in the raphe, low extracellular concentrations of UCN1 would preferentially bind CRF-R2. Both UCN1 knockout mice and CRF-R2 knockout mice exhibit increased anxiety-like behavior (Pan and Kastin, 2008). Thus, it has been generalized that activation of CRF-R1 by CRF increases anxiety-like behavior whereas activation of CRF-R2 by UCN1 decreases anxiety-like behavior. The elevated anxiety behavior exhibited by the long-term Ovx animals when brought indoors for temperament tests is consistent with stress causing an increase in CRF release which binds to the increased CRF-R1 receptors. A decrease in UCN1 would not act to ameliorate the stress response, which may last longer in the Ovx group.

Altogether the increase in CRF-R1 and the decrease in UCN1 in the Ovx group may lead to lower serotonin availability in the Ovx group compared to the Intact group. This suggestion is supported by our previous observation that fenfluramine-induced serotonin release as indicated by subsequent prolactin release, was significantly lower in the Ovx group than in the Intact group (Bethea et al., 2012). In addition, Ovx animals have fewer serotonin neurons and decreased gene expression of Fev, TPH2, SERT and 5HT1A. However, as previously discussed, the age at which the animals were ovariectomized contributes developmental variables in addition to the presence or absence of ovarian steroids (Bethea et al., 2012). Altogether, the increase in anxiety behavior in the temperament tests exhibited by the Ovx group together with their lower response to fenfluramine, lower serotonin-related gene expression, higher CRF-R1 and lower UCN1 compared to intact animals encourages the hypothesis that removal of ovarian steroids in women may lead to decreased serotonin and consequent mood deterioration.

The usual computation of human years to monkey years is 3 to 1, so the Ovx group is somewhat comparable to women after 9 years of steroid deprivation, though significantly younger than menopausal women. Nonetheless, these studies promote further understanding

of the effects of long-term ovarian steroid loss in females. For example, women with a relatively stress-free life or who are very stress-resilient may not notice changes in anxiety after menopause. Others may find that they are easily stressed or experience increases in anxiety and decreased coping mechanisms. In addition, monkey chow is significantly lower in fat and sugar than the typical western diet. In light of the inflammatory conditions induced by a western diet (Pistell et al., 2010; Posey et al., 2009), the endpoints examined in this study may be worsened with ovariectomy of older animals on a western diet.

In conclusion, we found (a) increased protein expression of CRF-R1 in the dorsal raphe, (b) decreased protein expression of UCN1 in the SOA cell body region and (c) a trend toward decreased UNC1 in midbrain axons, in long-term Ovx macaques living in an outdoor, stable troop. This was similar to results obtained with short-term Ovx rhesus macaques housed indoors. The pivotal difference between indoor short-term and outdoor long-term Ovx animals was in the CRF innervation of the dorsal raphe. CRF axon density was similar in outdoor long-term Ovx and Intact macaques, whereas it was significantly higher in caged short-term Ovx macaques compared to animals administered E±P therapy. We speculate that Ovx alone increases vulnerability to stress or anxiety by increasing CRF-R1 and decreasing UCN1, but many factors may impact CRF delivery to the dorsal raphe including age at ovariectomy, stress, stress sensitivity and season.

4. Materials and Methods

The Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC) approved this study, which followed the guidelines of the Animal Welfare Act.

Animals

Ten young females ~3.5 years of age, born into a troop of Japanese macaques housed in a naturalistic setting (corral, see below) were chosen. At the time, there were 12 matriarchal lineages in the troop; and there were enough animals born in Spring 2003 to use 10 individuals with 2 alternates. It was not feasible to remove 10–12 adult females from our troop because of their contribution to social stability. The Japanese macaques go through puberty between 3 and 4 years of age. A recent paper examining the timing of puberty in rhesus macaques found that animals born in the spring ovulate in the fall at the age of approximately 30 months (Wilson et al., 2012). This suggests that the animals assigned to this study likely had their first ovulation in Fall, 2005. Other than that, we were unable to determine the stages of puberty, such as Tanner stage in girls (Lloyd et al., 1996; Oldehinkel et al., 2011; Travers et al., 1995).

Five pairs of young females were selected that represented different ranks in the dominance hierarchy. In late November 2006 (about 1 year after first ovulation), one individual of each pair was ovariectomized (Ovx; reproductive tract remains) and the other individual was sterilized by tubal ligation to prevent pregancy, but the ovaries remained intact. The surgeries were conducted during the annual round up of all of the animals in the corral. The animals were returned to the troop and allowed to mature for 3 years. Behavioral observations were conducted during their 3 years in the corral. One animal from the Ovx group died in the troop during year 1 and was replaced with an animal of similar age and lineage. Thus, 1 animal in the Ovx group was treated for slightly less than 3 years. Unfortunately, another Ovx animal died at the end of the 3 year period resulting in a final n=4 in the Ovx group. After 3 years, the animals were collected for temperament tests, pharmacological challenges and euthanasia. The results of the behavioral observations and pharmacological challenge are published (Bethea et al., 2012; Coleman et al., 2011).

Description of the troop

The monkeys used in this study were part of a troop of approximately 300 individuals housed in a two-acre outdoor corral at the Oregon National Primate Research Center (ONPRC). The corral is surrounded by steel walls, and contains several platforms and climbing structures for play and exploration. Monkeys are fed commercially available monkey chow twice daily, supplemented by daily fresh produce or grains. Water is available *ad libitum*. As part of general animal husbandry of ONPRC, all animals were given unique markings on their backs, allowing for individual identification. Animal care-staff walk through the corrals daily to look for sick or injured monkeys. Japanese macaques are seasonal breeders, and at the ONPRC, the birth season is typically between May and August, with the majority of births occurring in June and July. The mating season is typically November through February.

The Japanese macaque troop arrived at ONPRC in 1965. The troop composition is relatively stable and the age structure is comparable to that of a natural troop (Eaton, 1984; Maruhashi, 1982; Rostal et al., 1986). 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 45 years.

Annual round ups

At least once a year, the animals were "rounded up", or brought from the corral into a capture run ("catch area") adjacent to the corral. This round up allowed collection of the monkeys for procedures that could not be done in an open field, such as annual veterinary exams, weighing and dye marking. Typically, the monkeys were brought into this area one or two times per year, and so most were acclimated to this procedure. During the round-up, monkeys ran through tunnels into the catch area, where they jumped into transport boxes for transfer to single cages. Annual exams were done on all members of the corral in the fall, after the birthing season, and before the mating season. In addition to the physical examination, animals were also weighed and tested for tuberculosis, and new infants were identified. The animals were typically kept in cages in the catch area for approximately one week.

Surgeries

Subjects were either ovariectomized (Ovx; n=5) or tubal ligated (tubal-ligated or ovary intact; n=5) after puberty (at age 3.5 yrs), and returned to their semi-free ranging troop, for 3 years until approximately age 6.5 yrs. For ovariectomy or tubal ligation, each animal was sedated with ketamine (10 mg/kg) in its cage in the catch area and transported to a surgical suite. Ovariectomy and tubal ligation were performed by the surgical personnel of ONPRC using a laparoscopic approach.

Hormone assays

Cortisol, estradiol and progesterone concentrations in serum were performed utilizing a Roche Diagnostics Cobas e411 automated clinical platform instrument, which has been validated in the Endocrine Technology and Support Laboratory, ONPRC, for macaque serum by comparison with traditional radioimmunoassays. The sensitivity and range of the cortisol assay was 2 and 500 ng/ml, respectively. The sensitivity and range of the estradiol assay was 5 and 4250 pg/ml, respectively. The sensitivity and range of the progesterone assay was 0.035 and 59 ng/ml, respectively. Within-assay coefficients of variation were less than 5% and between-assay coefficients of variation were less than 10% for all assays.

Tissue Preparation

The monkeys were euthanized according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, transported to the necropsy suite, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

Following euthanasia, the left ventricle of the heart was cannulated and the head of each animal was perfused with 1 liter of saline followed by 7 liters of 4% paraformaldehyde in 3.8% borate, pH 9.5 (both solutions made with DEPC-treated water [0.1% diethyl pyrocarbonate] to minimize RNase contamination). The brains were removed and dissected. Tissue blocks were post-fixed in 4% paraformaldehyde for 3 h, then transferred to 0.02M potassium phosphate-buffered saline (KPBS) containing 10%, followed by 20% glycerol and 2% dimethyl sulfoxide at 4°C for 3 days to cryoprotect the tissue. After infiltration, the block was frozen in isopentene cooled to −55°C, and stored at −80°C until sectioning. Sections (25 μm) were cut on a sliding microtome, and half were stored in cryoprotectant at −20°C until processing for immunocytochemistry (the other half were mounted on slides, dehydrated under vacuum and then frozen at −80°C until processing for ISH). Immunocytochemistry was employed in this study because all of the sections for ISH were used in a previous study and were depleted (Bethea et al., 2012).

Immunocytochemistry

Brain sections were removed from frozen storage and washed in KPBS for an hour. They were blocked in the appropriate normal serum for an hour, and then blocked in avidin and biotin for 20 minutes (Vector labs, Burlingame, CA). The primary antibody was diluted in 0.02 M KPBS/2% normal rabbit or goat serum/0.4% Triton X-100 and exposed to the experimental tissue for 48 hours at 4°C. After 48h, sections were washed in KPBS for an hour. The appropriate secondary antibody (Vector) was diluted 1:200 in KPBS/0.4% triton and incubated with the tissue for 60 minutes. After another KPBS wash, the tissue sections were incubated in VECTASTAIN Elite ABC solution (Vector) for one hour and washed in buffer again. Sections were then incubated in 0.02M KPBS with 0.05% DAB and 0.003% H_2O_2. Finally, the sections were washed, mounted on slides, dehydrated, dipped in xylene, and then coverslipped in DPX mountant for stereological analysis.

The CRF antibody was a gift from Dr. Wylie Vale (Salk Institute, La Jolla, CA) and was raised in rabbit against human CRF conjugated to human alpha globulin. Thus, in the CRF ICC, we used an additional blocking step with 1% human alpha globulin (Sigma G-2011, St. Louis, MO) for 20 minutes. Rabbit anti-human CRF (1/3,000) was diluted in 0.02 M KPBS/ 2% normal goat serum/ 0.4% triton/0.1% human alpha globulin. The secondary antibody was biotinylated goat anti-rabbit IgG (Vector). The antisera to CRF has been extensively characterized and previously applied to primate brain (Bassett and Foote, 1992).

The UCN1 antibody (Sigma-Aldrich, St. Louis, MO; Catalog No. U4757) was raised in rabbit against amino acids 25–40 of the human peptide. Prior to incubation with the primary antibody, an additional blocking step with 3% bovine serum albumin for an hour was included. Rabbit anti-UCN1 (1/15,000) was diluted in 0.02 M KPBS/2% normal goat serum/ 0.4% triton. The secondary antibody was biotinylated goat anti-rabbit IgG (Vector). The antisera to UCN1 has been extensively characterized and previously applied to rat and human brain (May et al.).

The CRF-R1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; Catalog No. 12381) was an affinity purified goat polyclonal antibody raised against an internal region of CRF-R1 of human origin. Increasing dilutions of anti-CRF-R1 produced decreasing signal up to a titer of 1/4000, beyond which the signal disappeared. Anti-CRF-R1 was diluted 1/1000 in 0.02 M

KPBS/2% normal rabbit serum/0.4% triton and incubated with the experimental tissue for 48 hours at 4°C. The secondary antibody was biotinylated rabbit anti-goat IgG (Vector). Absorption controls for this antibody were published for rhesus macaque (Sanchez et al., 2010). The antibody previously used for CRF-R2 was no longer available. Currently available CRF-R2 antibodies were not satifactory.

Densitometric Analysis

Each section was video-captured with the Marianas Stereology Workstation and Slidebook 4.2. A montage of the dorsal raphe was created by Slidebook, which was subjected to further analysis with Image J. The dorsal raphe changes shape from rostral to caudal levels. Unless otherwise specified, the representative sections were 250 μm apart and exhibited different anatomical levels of the dorsal raphe. The same size square was then used for all of the animals at that anatomical level. On each section, the operator outlined (boxed) the area of interest and the image was segmented into positive (stained) and negative (unstained) pixels. Three measurements were generated from the segmented image. The area covered by the positive pixels was obtained, called positive pixel area and the total area was obtained. The CRF and UCN fiber staining was confined to boutons. The number of CRF-R1 and UCN positive cells, or CRF and UCN boutons, was obtained using size cut-off values. The mean signal intensity is also obtained but it is not considered reliable with nonlinear assays such as ICC. The same procedure was applied at each anatomical level from the rostral to the caudal extent of the dorsal raphe nucleus. There is ~0.6 pixel/μm linear and 0.36 pixels^2/μm^2.

Statistical Analysis

The positive pixel area and the positive cell or bouton number were (a) averaged across levels and (b) totalled across levels, generating one overall average or total for each animal. The individual animal means and totals were subjected to statistical analysis with 2-tailed Student's t–test. The variance in the groups reflects the difference between animals. F values were computed to assure the variances were equal and when not, the Mann-Whitney nonparametric test for comparison of 2 groups was applied.

All statistical analyses were conducted using the Prism Statistic Program 5.0 (GraphPad, San Diego, CA). A confidence level of $p<0.05$ was considered significant.

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Highlights

- CRF innervation of the dorsal raphe was similar in Ovx and Intact macaques in an outdoor troop.
- CRF-R1 immunostaining was higher in long-term Ovx than Intact macaques in an outdoor troop.
- UCN1 cell number was lower in long-term Ovx than Intact macaques in an outdoor troop.

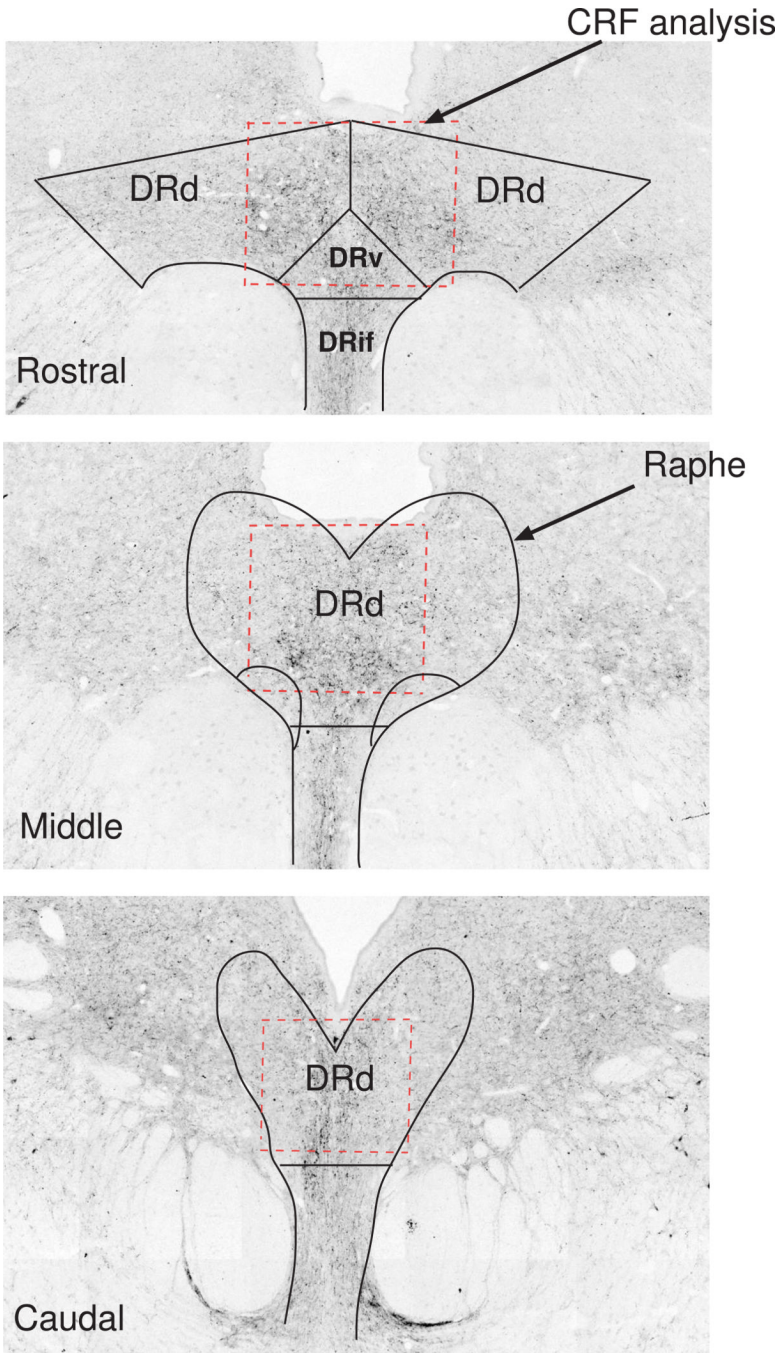

Figure 1.
Illustration of the CRF fiber area that was analyzed (red dashed box) relative to the subnuclei of the dorsal raphe nucleus at a rostral, middle and caudal level (solid black lines). The rostral levels contain the subnuclei called dorsal raphe dorsal (**DRd**), dorsal raphe ventral (**DRv**), and dorsal raphe infundibularis (**DRif**). The area identified as dorsal raphe ventral lateral (**DRvl**) makes an appearance in the middle region of our raphe sections in monkeys.

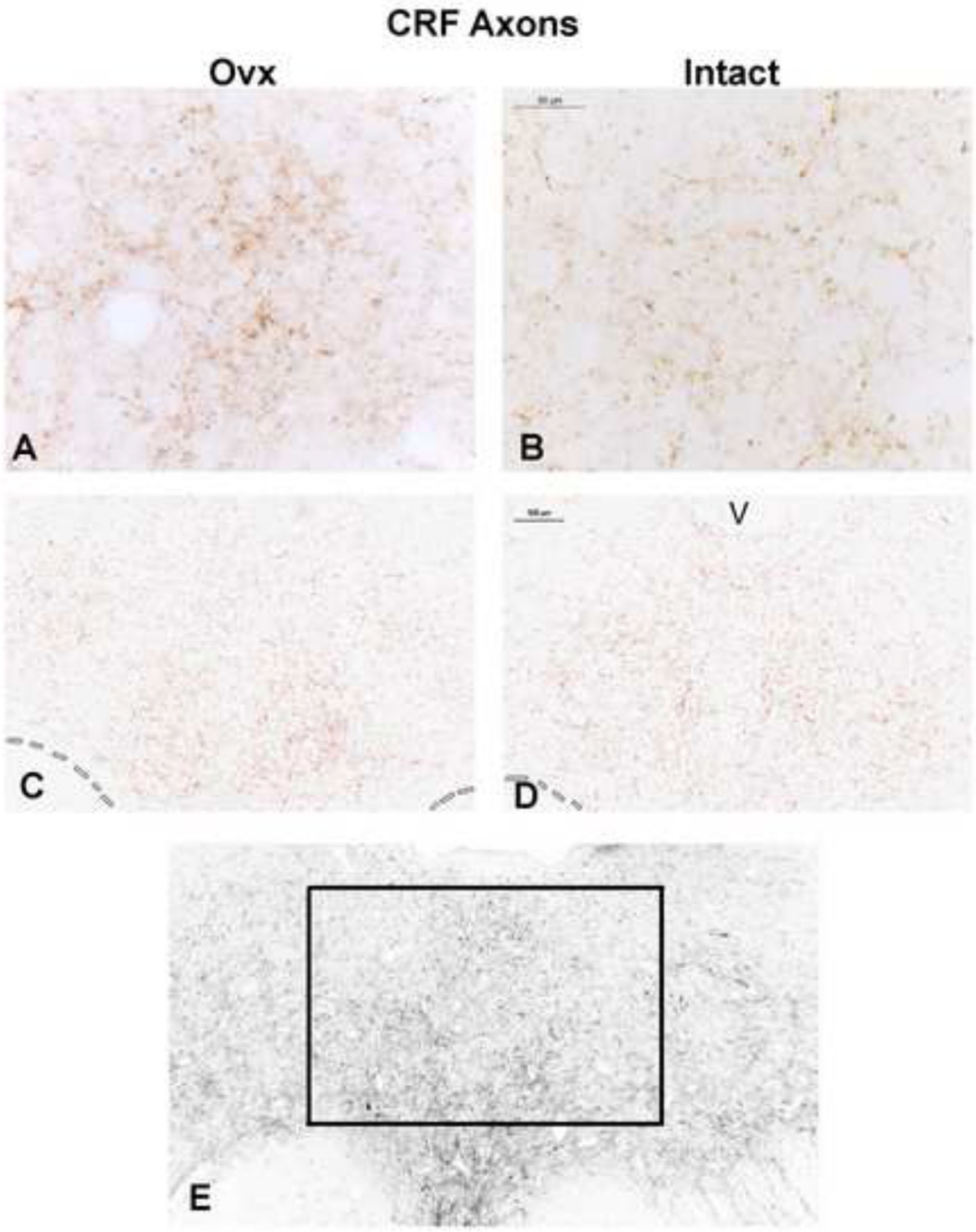

Figure 2.

Representative photomicrographs of CRF axon staining in the dorsal raphe of an Ovx and Intact Japanese macaque. Panels A and B illustrate the appearance of the CRF axons with boutons under brightfield optics at a high magnification. Panels C and D illustrate images captured with Slidebook 4.2 on the Marianas stereology workstation that were segmented into positive (red) and negative pixels in ImageJ for quantitation. Panel E illustrates the entire montage of a rostral section and the area that was cropped and analyzed. The montage was generated with Slidebook 4.2 on the Marianas stereology workstation. Slidebook 4.2 builds a montgage from multiple pictures taken at low power over the entire field (the optics are not like a brightfield microscope).

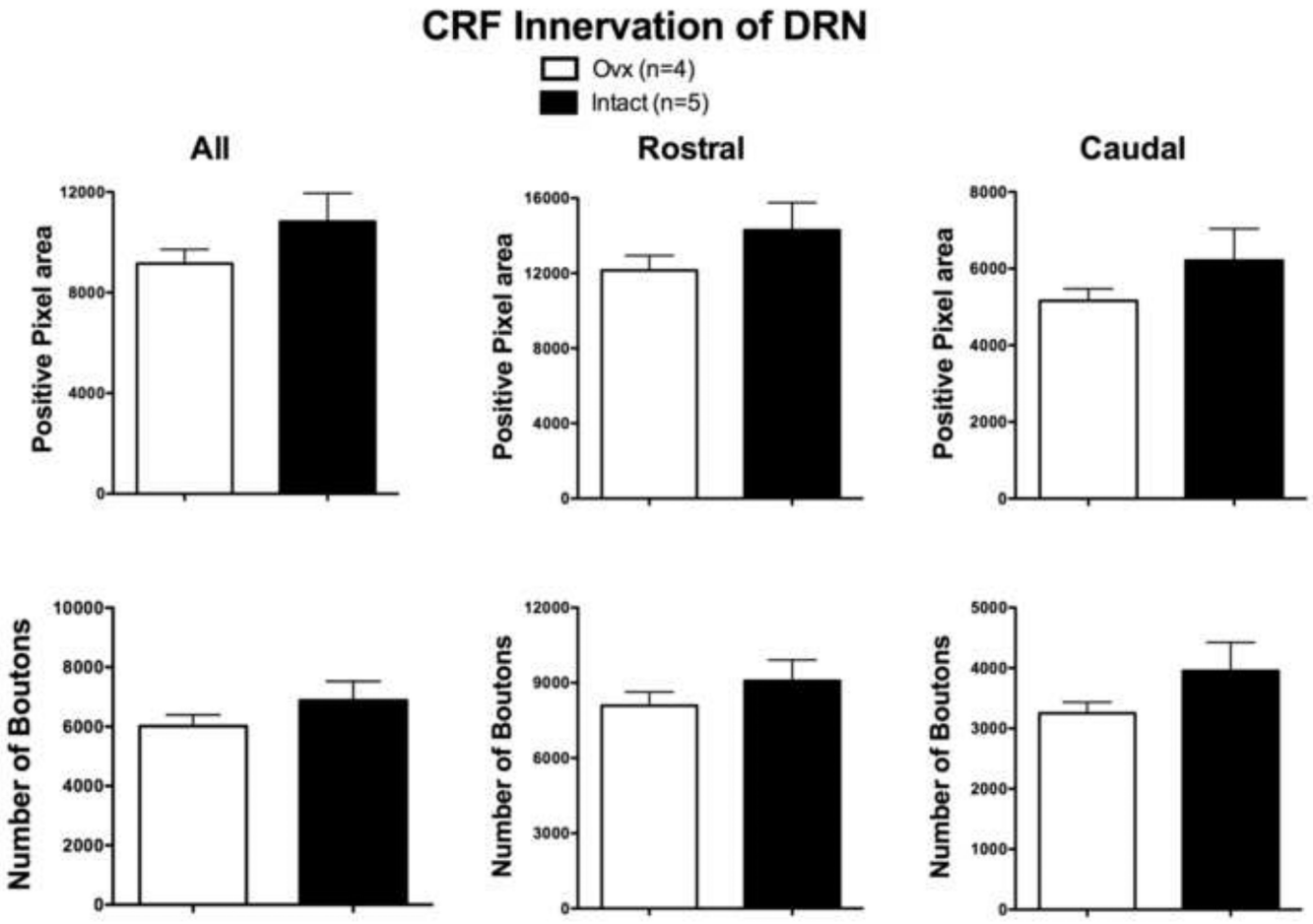

Figure 3.

Histograms illustrating the quantitative analysis of CRF boutons in the dorsal raphe of long-term Ovx and Intact macaques. There was no difference in the overall positive pixel area or the number of boutons in the areas analyzed (labeled All). There was no difference in the rostral + middle positive pixel area or the number of boutons (labeled Rostral). There was no difference in the caudal positive pixel area or the number of boutons (labeled Caudal). The average of the areas analyzed for each animal equaled 817,394 pixels^2 or 294,262 μm^2.

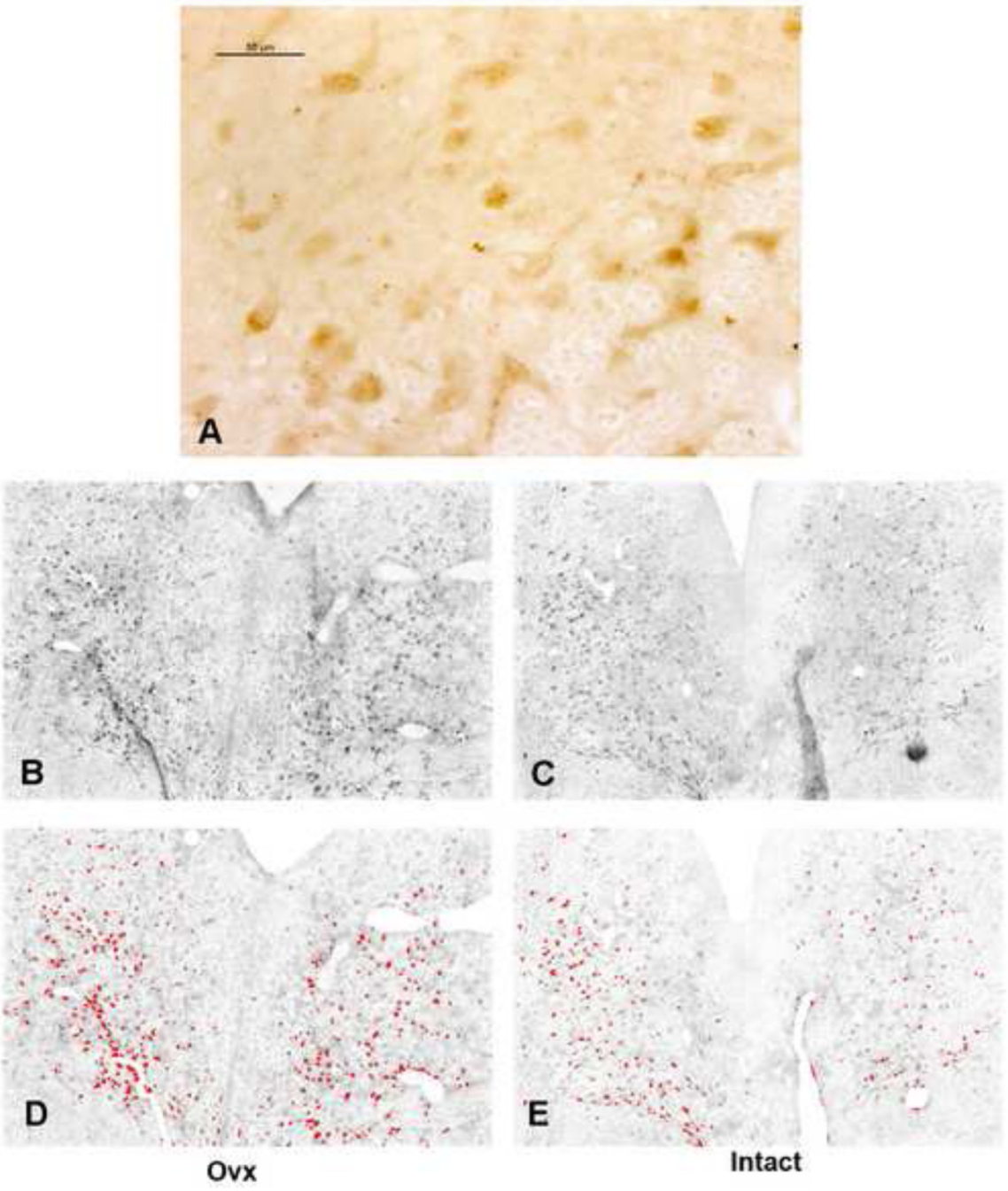

Figure 4.

Representative photomicrographs of CRF-R1 staining in the dorsal raphe of an Ovx and Intact Japanese macaque. Panel A illustrates the appearance of the CRF-R1 immunostaining in the dorsal raphe under brightfield optics at a high magnification. Panels B and C illustrate images of CRF-R1 immunostaining captured with Slidebook 4.2 on the Marianas stereology workstation. Slidebook 4.2 builds a montgage from multiple pictures taken at low power over the entire field. Panels D and E illustrate the same sections after background subtraction, erasure of folds or artifacts, and segmentation into positive (red) and negative pixels in ImageJ for quantitation.

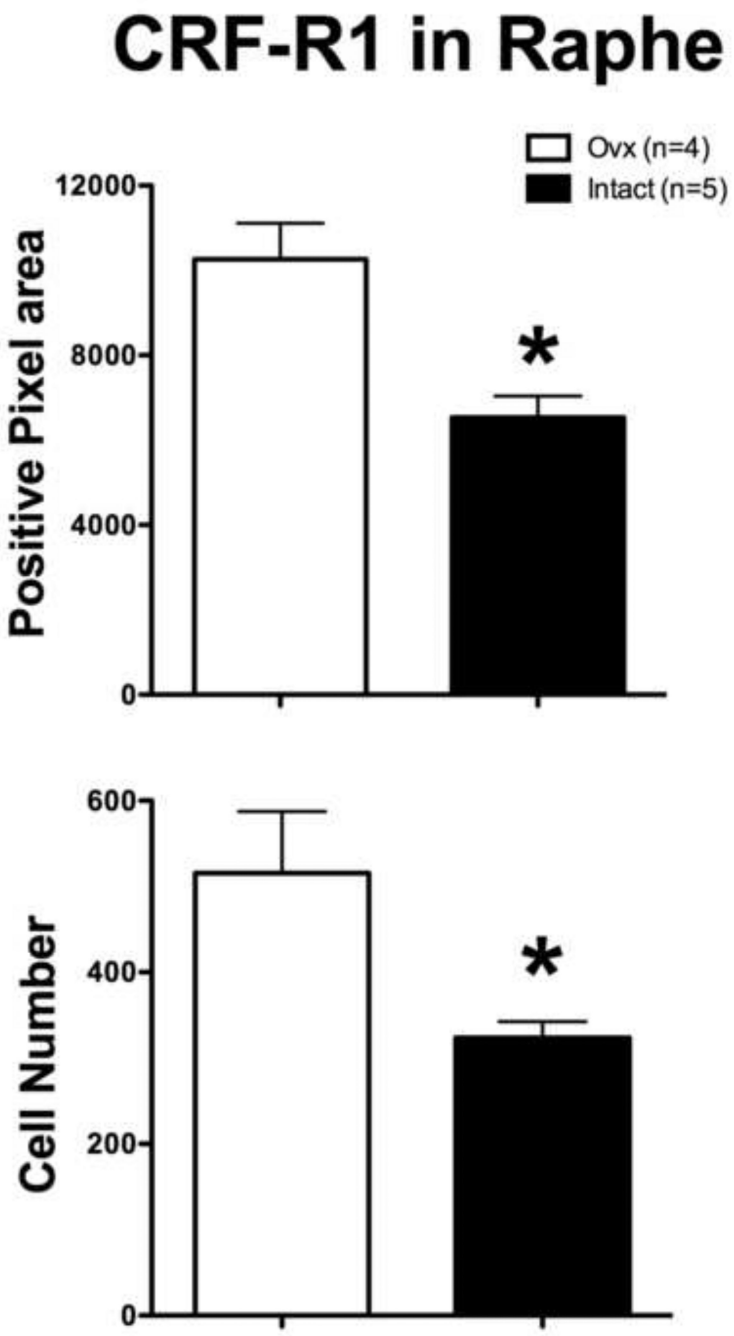

Figure 5.
Histograms illustrating the quantitative analysis of CRF-R1 immunostaining in the dorsal raphe of long-term Ovx and Intact macaques. The Ovx group had significantly greater positive pixel area ($p=0.005$) and positive cell number ($p = 0.02$). The average of the areas analyzed for each animal equaled 556,711 pixels^2 or 200,416 μm^2.

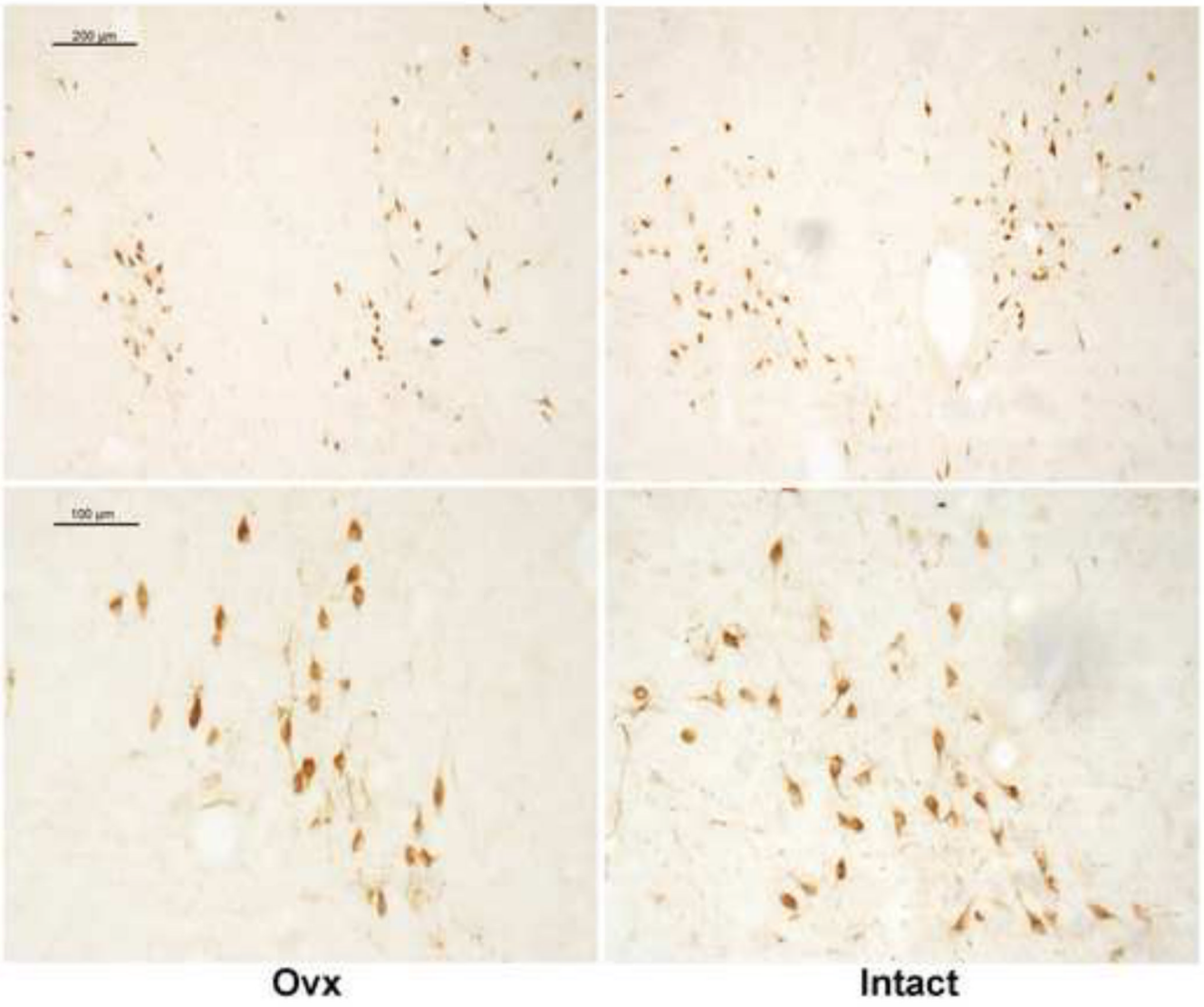

Figure 6.

Representative photomicrographs of UCN1 immunostaining in the SOA at matching levels of an Ovx macaque and an Intact macaque under brightfield optics at a low (top) and higher (bottom) magnification. For analysis, the entire area shown at low magnification was captured on the Marianas stereology microscope with Slidebook 4.2. The images were segmented into positive and negative pixels with Image J for quantitative analysis. This process is not illustrated, but it is identical to that shown in Figure 4.

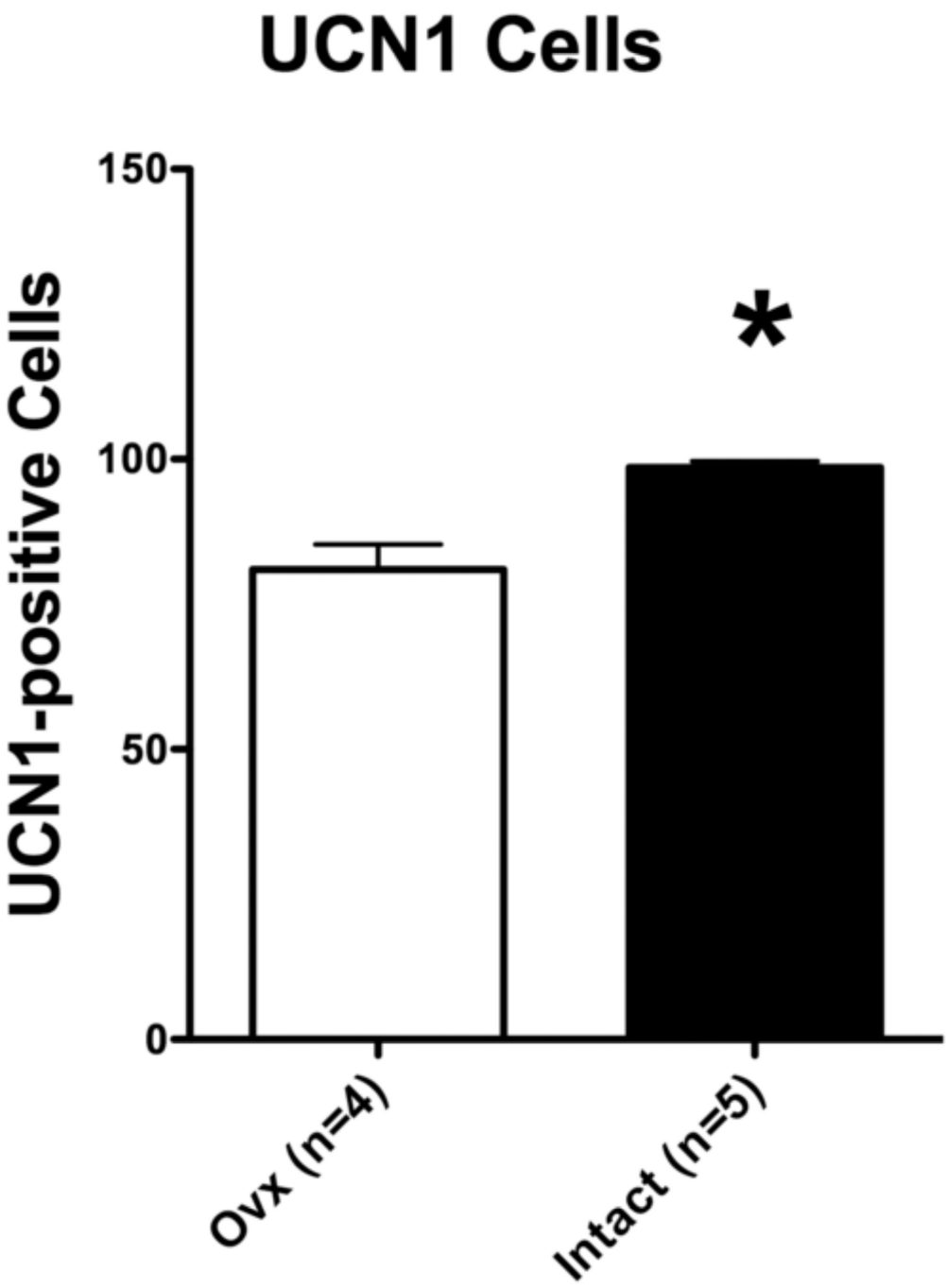

Figure 7.
Histograms illustrating the quantitative analysis of UCN1 immunostaining in the cell body area of long-term Ovx and Intact macaques. There were significantly fewer detectable UCN1 positive cells in the Ovx group than in the Intact group ($p = 0.003$). The average of the areas analyzed equaled 264,988 pixels^2 or 95,396 μm^2.

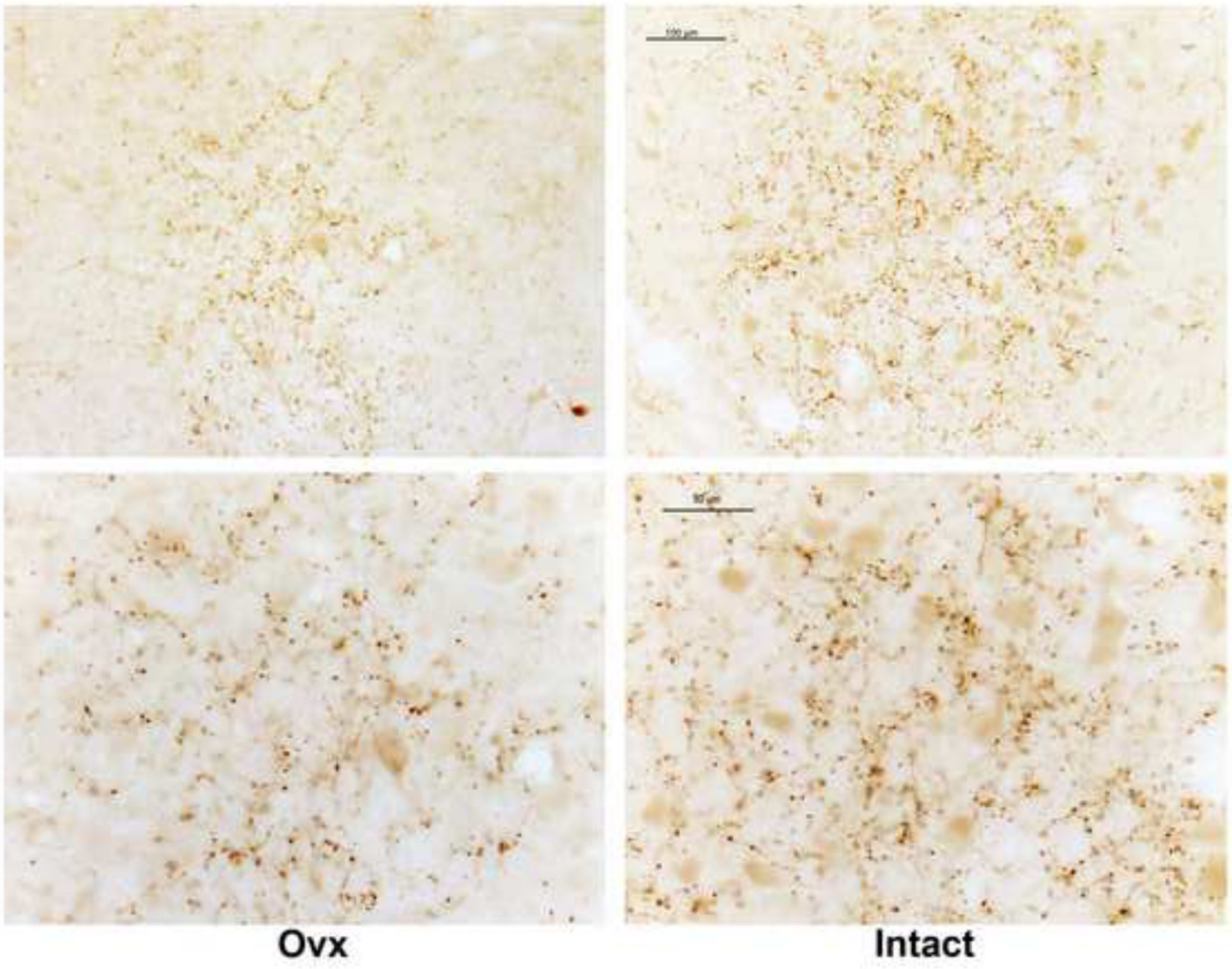

Figure 8.

Representative photomicrographs of UCN1 axon immunostaining anatomically located between the SOA and dorsal raphe of a long-term Ovx- and an Intact-macaque under brightfield optics at a low and higher magnification. The cluster of axons appears larger in the Intact animal with more detectable butons. The images captured on the Marianas workstation contained the entire area. The images were segmented into positive and negative pixels with Image J for quantitative analysis. This process is not illustrated, but it is identical to that shown in Figure 2.

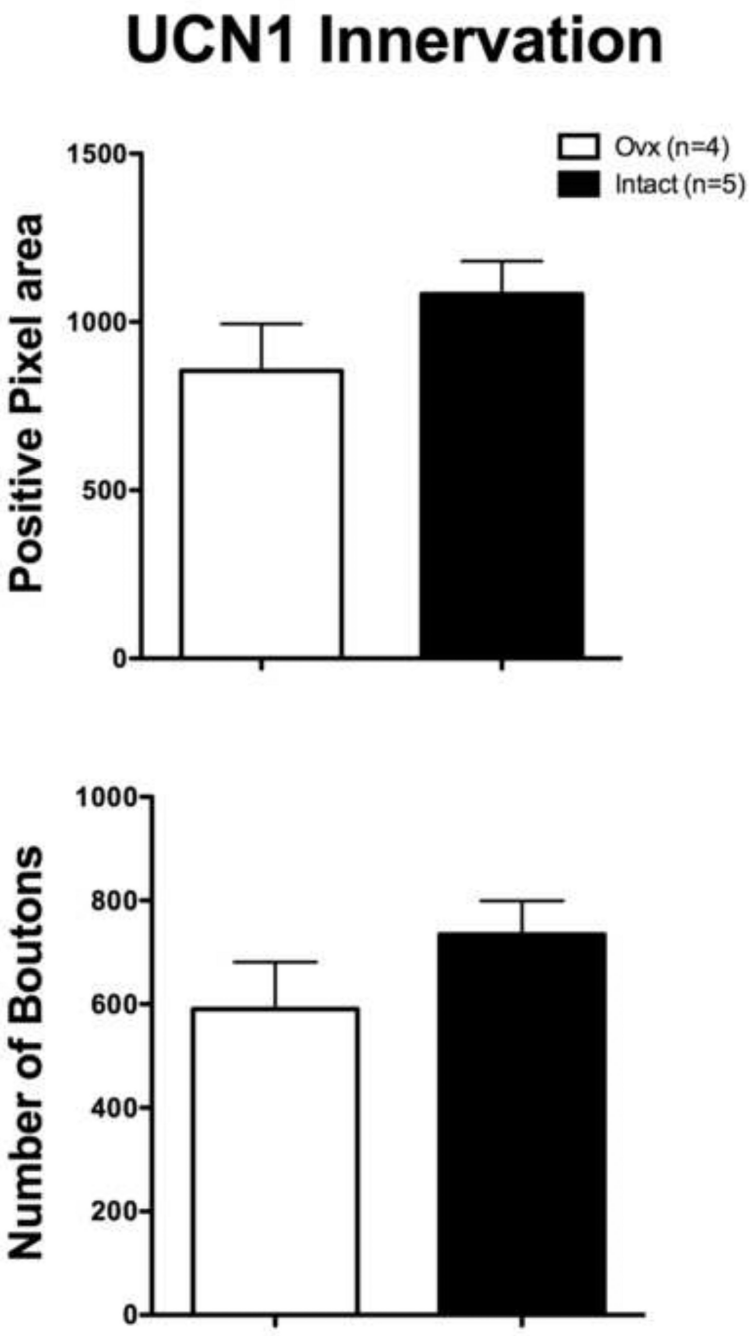

Figure 9.
Histograms illustrating the quantitative analysis of UCN1 boutons in the small fiber plexus between the SOA and the dorsal raphe of long-term Ovx and Intact-macaques. There was no statistical difference in the positive pixel area or the number of boutons in the area analyzed. The average of the areas analyzed equaled 180,037 pixels^2 or 64,813 μm^2.

Table 1

Steroid hormone concentrations in serum of female Japanese macaques prior to euthanasia and under propofol anesthesia.

Treatment Group	Cortisol	Estradiol-17β	Progesterone	Stage
Animal ID	**ng/ml**	**pg/ml**	**ng/ml**	
Ovx				
24018	319	14	0.30	n/a
24020	317	28	0.85	n/a
24028	194	20	0.69	n/a
24029	188	10	0.60	n/a
Avg	254±37	18±4	0.61±0.12	
Intact				
24032	250	19	0.23	menstrual
24037	175	64	0.12	early-follicular
24040	202	431	1.17	early luteal
24042	275	18	1.20	late luteal
24049	217	107	0.86	mid-follicular
Avg	**223±18**	127±77	0.72±0.23	