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Unique Responses of Midbrain CART Neurons in Macaques to Ovarian Steroids

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Abstract

CART (cocaine and amphetamine regulated transcript) is a neuropeptide involved in the control of several physiological processes, such as response to psychostimulants, food intake, depressive diseases and neuroprotection. It is robustly expressed in the brain, mainly in regions that control emotional and stress responses and it is regulated by estrogen in the hypothalamus. There is a distinct population of CART neurons located in the vicinity of the Edinger-Westphal nucleus of the midbrain that also colocalize urocortin-1. The aims of this study were 1) to determine the distribution of CART immunoreactive neurons in the monkey midbrain, 2) to examine the effects of estrogen (E) and progesterone (P) on midbrain CART mRNA and peptide expression and 3) to determine whether midbrain CART neurons contain steroid receptors. Adult female rhesus monkeys (*Macaca mulatta*) were spayed and either treated with placebo (OVX), estrogen alone (E), progesterone alone (P) or E + P. Animals were prepared (a) for RNA extraction followed by microarray analysis and quantitative (q) RT-PCR (n=3/group); (b) for immunohistochemical analysis of CART and CART +tryptophan hydroxylase (TPH), CART+estrogen receptors (ER) or CART+progesterone receptors (n=5/group) and (c) for western blots (n=3/group). Both E– and E+P-administration decreased CART gene expression on the microarray and with qRT-PCR. Stereological analysis of CART immunostaining at five levels of the Edinger-Westphal nucleus indicated little effect of E or E + P administration on the area of CART immunostaining. However, P administration increased CART-immunopositive area in comparison to the OVX control group with Student's t-test, but not with ANOVA. CART 55–102 detection on western blot was unchanged by hormone administration. ER β and PR were detected in CART neurons and CART fibers appeared to innervate TPH-positive serotonin neurons in the dorsal raphe. In summary, E decreased CART mRNA, but this effect did not translate to the protein level. Moreover, P administration alone had a variable effect on CART mRNA, but it caused an increase in CART immunostaining. Together, the data suggest that CART neurons in the midbrain have a unique steroid response, which may be mediated by nuclear receptors, neuroactive steroids or interneurons.

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Keywords

Cocaine and amphetamine regulated transcript (CART); estrogen; progesterone; serotonin; Edinger-Westphal nucleus

INTRODUCTION

Cocaine and amphetamine regulated transcript (CART) is a novel peptide which was originally described by Douglass et al. (1995) in the rat striatum as a transcript regulated by acute cocaine and amphetamine administration. CART mRNA and protein are expressed in various areas of the brain of humans, monkeys and rats, including hypothalamus, pituitary and adrenal (Douglass et al. 1995; Vrang 2006; Koylu et al. 1997; Valera et al. 2006); limbic system (Hurd & Fagergren 2000), and sensory cortex (Hurd & Fagergren 2000). CART is modified by post-translational processing and several CART peptide fragments exist in the brain (Dylag et al., 2006; Thim et al. 1999; Kuhar & Yoho 1999). However, CART- (55–102) is the best described fragment and it shows the most diverse profile of biological actions (Kristensen et al. 1998; Bannon et al. 2001).

CART is robustly expressed in the hypothalamic paraventricular nucleus (PVN) and in the locus coeruleus, both of which control stress responses (Koylu et al. 2006). Moreover in PVN, the CRH-immunoreactive neurons are activated by i.c.v. injection of CART-(55–102) (Vrang et al. 2000). Also, CART- (55–102) markedly induces plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels in male rats (Stanley et al. 2001). In vitro, CART- (55–102) increases release of CRH from hypothalamic explants (Stanley et al. 2001). Together, these data suggest that CART-(55–102) may activate the hypothalamus–pituitary–adrenal axis, possibly by interacting with the CRH system, thereby controlling emotional and stress responses (Chaki et al. 2003).

CART mRNA is robustly expressed in the Edinger-Westphal nucleus in humans (Hurd & Fagergren 2000) and rats (Kozicz 2003). Moreover in rats, CART colocalizes with urocortin-1 in the Edinger-Westphal nucleus (Kozicz 2003) and urocortin-1 expressed the Edinger-Westphal nucleus plays a role in the regulation of the hypothalamus-pituitary-axis in response to stress (Weninger et al. 1999; Skelton et al. 2000; Gaszner et al. 2004; Kozicz 2007). CART and urocortins-1 are present in fiber terminals that project to the lateral septal nucleus (LS) as well. However, the anatomy of the Edinger-Westphal nucleus in monkeys differs from that of humans. In monkey, the Edinger-Westphal nucleus contains choline acetyltransferase-positive, presumed pre-ganglionic neurons of the ciliary ganglion whereas the human Edinger-Westphal consists of noncholinergic urocortin-positive neurons (Horn et al., 2008). We found that the anatomical distribution of the midbrain CART neurons in the monkey is identical to that of the midbrain urocortins-1 neurons as recently described (May et al., 2008). Hence in monkey, midbrain CART neurons are located between the Edinger-Westphal nucleus and the oculomotor (III) nucleus.

We previously conducted a microarray analysis on a small block of tissue that contained the dorsal raphe nucleus from ovariectomized (OVX) monkeys treated with placebo or ovarian steroids (Reddy & Bethea 2005). Data from the human Affymetrix chip indicated that CART mRNA was present in the block and that it was significantly decreased by estrogen (E) and E + progesterone (E+P). These data suggested that the Edinger-Westphal region might be part of the block and that midbrain CART may be regulated by ovarian steroids. We sought to confirm the data from the human array and re-probed new Rhesus Affymetrix gene chips with the same RNA from the midbrain blocks. In addition, we characterized CART mRNA expression with quantitative (q) RT-PCR.

Based upon the expression of CART mRNA, we then examined the distribution of CART immunoreactive neurons in the monkey midbrain and determined the effects of steroid administration on CART protein expression. In addition, we determined whether CART neurons express estrogen and/or progesterone receptors (ER α , ER β and PR, respectively) and whether CART neurons project to serotonin neurons of the dorsal raphe nucleus.

RESULTS

1. CART microarray in blocks from dorsal raphe nucleus in macaques

RNA extracted from midbrain tissue blocks was hybridized to Affymetrix Rhesus Gene Chips and analyzed with GeneSifter software (n=3 animals/group). The results of the analysis of CART gene expression are shown in Figure 1. The P group was elevated compared to the OvX group, but it was highly variable. There was a significant difference between the treated groups when the P group was omitted (ANOVA, $p<0.05$). Post-hoc comparison indicated that both E and E+P-administration decreased CART gene expression in the midbrain significantly compared with OVX animals (Student Newman-Keuls post hoc, $p<0.05$).

2. CART mRNA expression – (q) RT-PCR

RNA extracted from the midbrain tissue blocks (n=3 animals/group) was subjected to qRT-PCR for CART mRNA and GAPDH, a housekeeping gene. The pg of cDNA produced for GAPDH and for CART is shown in Table 1. There was no difference between the groups in GAPDH cDNA, but CART cDNA was significantly decreased by E and E+P. CART gene expression was normalized by GAPDH and the results are shown in Figure 2. There was a significant difference between all of the treatment groups (Kruskal-Wallis, $p=0.04$), but Dunn's post hoc comparisons yielded no differences. When the P group was omitted, there was a significant difference between the treated groups (ANOVA, $p<0.05$). Post-hoc comparison indicated that both E and E+P-administration decreased CART gene expression in the midbrain significantly compared with OVX animals (Student Newman-Keuls post hoc, $p<0.05$).

3. CART immunostaining near the Edinger-Westphal nucleus

3.1. CART single staining quantification—Figure 3 contains the montages of representative sections from a P-treated animal that were immunohistochemically stained for CART at 5 levels of the Edinger-Westphal nucleus, which were 250 μ apart in a rostral to caudal direction. CART immunoreactive neurons were prominent at each level. CART immunoreactivity was abolished by omission of the primary antibody and by immunoneutralization of primary antibody with the corresponding immunizing peptide (results not shown).

Figure 4 illustrates the immunostaining for CART at level 3 of one animal from each treatment group. Visually, there was an obvious and robust increase in CART immunostaining in the P-treated animals. Yet, there was no difference in the positive pixel area, in the percentage of the total area that was positive, or in the number of CART neurons by ANOVA. However, comparison of individual groups with the OVX group by Student's t-test indicated that P alone caused a significant increase both in the positive pixel area for CART (Figure 5A) and in the percentage of the total area that was positive for CART (Figure 5B). The total examined area did not differ between groups (Figure 5C). Also, in the direct comparison with Student's t-test, there was a significant increase in the total number of CART neurons in the P treated animals compared with the OVX group, both in the absolute number of cells (Figure 6A) and in the number of cells/mm³ (Figure 6B).

3.2. Double immunofluorescence for CART plus TPH and CART plus ER α —

Sections from a P-treated animal were examined for CART plus TPH and CART plus ER α . Double-label immunofluorescence revealed that CART neurons (red-Figure 7A) did not express TPH (green-Figure 7B; overlay-Figure 7C) although TPH positive neurons were observed in the dorsal raphe nucleus, which is caudal to the Edinger-Westphal nucleus (Figure 7D). Moreover, CART fibers (red) were present in the vicinity of TPH positive neurons (green) in the dorsal raphe nucleus. Although we cannot conclude that these fibers are in synaptic contact with the serotonin neurons, they appear to be juxtaposed (Figure 7E and 7F). ER α was undetectable (not shown).

3.3. Double peroxidase staining for CART and ER β —Sections from a P-treated monkey were examined for CART plus ER β . In double-labeled neurons, CART stained brown in the cytoplasm and ER β stained black in the nucleus (Figure 8, panels A–E, double head arrows). ER positive neurons that are not CART expressing were present as well (single head arrows). Omission of only the ER β antibody resulted in CART labeled neurons with no nuclear staining (Figure 8, panels F–H). There was no staining when the primary antibodies for CART and ER β were omitted (results not shown).

3.4. Double peroxidase staining for CART and PR—Sections from an E+P treated animal were examined for CART plus PR. In double-labeled neurons, CART stained brown in the cytoplasm and PR stained black in the nucleus. PR was undetectable in the absence of the primary antibody for PR (Figure 9, panels A, B). Numerous CART neurons contained PR (double head arrows, Figure 9, panels C–H). Figures 9F and 9G also show PR positive neurons that do not contain CART (single head arrows).

4. Western Blots

There was no difference between the groups (n=3/group) in cytosolic CART protein levels (Figure 10).

5. Hormone Assays

Serum estradiol concentrations (pg/ml $\pm$ SEM) equaled 18.42 $\pm$ 3.35, 108.05 $\pm$ 14.05, 21.02 $\pm$ 3.97 and 112.83 $\pm$ 10.82 in the placebo, E, P and E+P treated groups, respectively (n=11/treatment). Serum progesterone concentrations (ng/ml $\pm$ SEM) equaled 0.11 $\pm$ 0.02, 0.39 $\pm$ 0.11, 6.99 $\pm$ 0.63 and 6.16 $\pm$ 0.63 in the placebo, E, P and E+P treated groups (n=11/treatment), respectively.

DISCUSSION

The present work showed that in monkeys, CART protein is expressed in midbrain neurons adjacent to the Edinger-Westphal nucleus and that CART fibers project to serotonin neurons in the dorsal raphe nucleus. CART neurons contain ER β , thus corroborating previous work showing that urocortin-1 cells, which colocalize CART, contain ER β but not ER α in the mouse brain (Derks et al. 2007). We also demonstrated that CART neurons colocalize PR and together, these findings implicate a direct regulation of CART gene expression by steroids. Although E decreased CART mRNA expression in the midbrain, this was not reflected in the peptide expression, perhaps due to post-transcriptional regulatory mechanisms. However, P alone increased CART immunostaining in the cell bodies in the midbrain, but there was no change in total protein. The mechanism by which P exerts this effect requires further investigation.

Gene Expression versus Protein Expression

CART mRNA expression in the midbrain population was significantly suppressed by E and E+P administration as determined with microarray and qRT-PCR. Since the E+P treated group was similar to the E group, we attribute the decrease in both groups to E action. CART mRNA

was slightly but not significantly elevated by P only administration. Thus, it appeared that E regulated CART gene expression in the midbrain. However, in the immunohistochemistry assay, neither E nor E+P had any effect, but there was an indication that P increased immunostaining. Thus, changes in CART mRNA did not translate to changes in immunostaining. Finally, there was no regulation of CART on the western blots. CART is heavily edited by post-transcriptional events yielding numerous splice variants (Dylag et al., 2006; Thim et al. 1999; Kuhar & Yoho 1999). These data suggest that even though RNA copy number decreases, there may be adjustments in translational efficiency, or peptide turnover and degradation, which maintain CART peptide expression within a narrow range. Likewise, if there is decreased release of CART along with decreased transcription of CART in the E and E+P treatment groups, then protein levels may not change significantly.

Effects of E and P administration

In rats, a sexually dimorphic modulation of CART expression was observed in the PVN and ARC nuclei with the forced swim test (Gozen et al. 2007). Another study in rats showed that E administration reduced the elevated CART expression due to reoccurring insulin hypoglycemia. However, E did not regulate basal CART expression in that area (Nedungadi & Briski 2007). During pregnancy, when serum P is elevated, rats show increased CART expression in the anteroventral periventricular nucleus (AVPV), a nucleus governing maternal behavior and GnRH release (Valera et al. 2006). The latter data are consistent with our observation that P increased CART immunostained area, as well as the number of detectable neurons in the midbrain.

The effect of P alone on CART immunostaining is unusual. Visually, there is an obvious and robust increase in CART immunostaining density with P administration, which was not adequately portrayed by the stereological analysis. P could increase CART peptide by an action through nuclear PR; but without E, nuclear PR may be scarce, although P tended to increase CART gene expression. Alternatively, the action of P on CART expression may depend upon its metabolite, allopregnanolone, interacting with potential GABA-A receptors on CART neurons (Barbaccia et al. 2001). However, this is thought to be an inhibitory “GABA-like” action, so it is difficult to understand how this mechanism could increase CART translation or stability. Yet, if activation of GABA receptors inhibited cell firing and CART release from the terminals and/or trafficking of CART to the terminal regions, then CART may accumulate in the cell bodies thereby producing more immunostaining. Allopregnanolone could also act via stimulatory interneurons. Immunohistochemistry is more sensitive than the western blots, which did not detect any difference in total CART protein in the midbrain region.

Gender and Sensitivity to Cocaine

It is attractive to speculate that gender differences in the sensitivity to cocaine may involve CART. Women are more sensitive to cocaine effects than men (Festa & Quinones-Jenab 2004) and studies during the menstrual cycle have reported an increased effect of cocaine during the follicular phase. Since the luteal phase of the cycle is characterized by the highest levels of P, it was suggested that P attenuated the subjective effects of cocaine (Evans et al. 2002; Evans & Foltin 2006). In this study, CART accumulated in cell bodies with P administration. Although we are not sure if this represents increased CART synthesis or decreased CART trafficking, perhaps this response plays a role in the attenuated effects of cocaine during the luteal phase. Further studies to determine the effect of E and P on the density of CART fibers in the dorsal raphe nucleus could be informative regarding CART trafficking.

CART, Stress and the HPA axis

CART stimulates CRF and ACTH, and responds to glucocorticoids, indicating that CART impacts activity of the HPA axis. Hypothalamic nuclei involved with the control of stress, like

PVN, ARC and supraoptic nuclei (SON) display high CART expression and there is a body of evidence indicating that CART plays a role in the stress response (Koylu et al. 1997; Vrang et al. 1999; Smith et al. 2004). Of note, the Edinger-Westphal region is one of the areas where CART regulates CRH release [for review see (Kuhar et al. 2000)]. Although CART is widely expressed, in the rat brain the Edinger-Westphal region demonstrates robust CART mRNA expression (Douglass et al. 1995). In macaques, the neurons expressing CART are outside of the Edinger-Westphal nucleus. Initially, neurons from the Edinger-Westphal nucleus were described in rats as a parasympathetic component of the oculomotor complex, projecting to the ciliary ganglion and leading to pupilloconstriction (Klooster et al. 1993). The Edinger-Westphal nucleus receives input from the spinal cord and the hypothalamus and sends projections to different areas expressing the CRH type I (Potter et al. 1994) and type II receptors (Bittencourt et al. 1999; Van Pett et al. 2000; Korosi et al. 2007), suggesting a role in autonomic regulation. Recently the Edinger-Westphal region has been postulated to exert a regulatory control over stress responses. Neurons colocalizing CART and urocortins located adjacent to Edinger-Westphal, respond to acute stress and can modulate several physiological functions (Kozicz 2003). Since E and P regulate CART expression in the midbrain near the Edinger-Westphal nucleus, it is possible that steroids are modulating the stress response by an action in this area.

CART and Serotonin

It has been reported in rats that CART may have an antidepressant effect through increases in extracellular serotonin (Ma et al. 2007). Specifically, a CART infusion in the dorsal raphe nucleus leads to a significant increase of serotonin efflux in the nucleus accumbens, a major target of dorsal raphe serotonergic projections. This work is consistent with our demonstration that CART terminals are in close apposition with serotonergic cell bodies in the dorsal raphe nucleus. Clearly, evidence of CART receptors on serotonin neurons is needed.

CART, Estrogen and Neuroprotection

CART is also involved in neuroprotection mechanisms. A recent study showed that CART is highly induced by E in the cerebral cortex during ischemia, suggesting that the up-regulation of this neuropeptide by E may play a role in neuroprotection (Xu et al. 2006). In our study without insult, CART mRNA was decreased by E administration but there was no effect on the peptide. Thus, the CART gene may react to E differently under different condition (ischemia versus normal) or in different areas of the brain.

In summary, we found that in monkeys CART is expressed in midbrain neurons adjacent to the Edinger-Westphal nucleus and that CART fibers project to serotonin neurons in the dorsal raphe nucleus. CART neurons contain ER β and PR, which may act to regulate gene expression. However, peptide expression did not reflect gene expression. P alone increased CART immunostaining in the cell bodies in the midbrain, but there was no change in total protein. The mechanism by which P exerts this effect requires further investigation. Nonetheless, CART neurons exhibit a unique steroid response profile, which may play a role in stress sensitivity and subjective affects of cocaine.

EXPERIMENTAL PROCEDURES

The Oregon National Primate Research Center Institutional Animal Care and Use Committee approved this study.

1- Animals and treatments

Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized by the surgical personnel of ONPRC according to accepted veterinary surgical protocol. All animals were born in China

and were aged between 7 and 14 years by dental exam. They weighed between 4 and 8 kg, and were in good health. Steroid hormone treatments were initiated 3–8 months after ovariectomy. Animals were either treated with placebo (Ovx control group; n=11), or treated with E for 28 days (E group; n=11), or with E for 28 days and then supplemented with P for the final 14 of the 28 days (E+P group; n=11), or with placebo for 14 days and then treated with P for 14 days (P group; n=11). The spayed control monkeys received empty Silastic capsules (s.c.). The E-treated monkeys were implanted with a 4.5-cm E-filled Silastic capsules (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI) filled with crystalline estradiol [1,3,5(10)-estratrien-3,17-b-diol; Steraloids, Wilton, NH]. The E+P-treated monkeys received an E-filled capsule and days later, received one 6-cm capsule filled with crystalline P (4-pregnen-3,20 dione; Steraloids). The P treated monkeys received a placebo capsule and 12 then one 6-cm capsule filled with crystalline P on day 14. All capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA). The monkeys were euthanized at the end of the treatment periods according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta. Twenty animals (n=5/group) were processed for immunohistochemistry. Twelve animals were perfused with RNA later and processed for mRNA extraction (n=3/group). Twelve animals were processed for subcellular fractionation of the dorsal raphe region and western blotting (n=3/group). The animals were processed in consecutive sets containing one OvX control animal and one animal from each hormone treatment group.

2- Tissue preparation for microarray analysis and (q) RT-PCR

The left ventricle of the heart was cannulated, and the head of each animal was perfused with 3 l of 1X cold RNA-later buffer (Ambion Inc., Austin, TX). The brain was removed from the cranium and blocked. The midbrain block was dissected as previously described (Reddy & Bethea 2005), and extends from the Edinger-Westphal nucleus through the dorsal raphe nucleus (5 mm wide, 6 mm high and 3 mm thick). The tissue was immediately frozen in liquid nitrogen. For RNA extraction, the frozen block was dropped directly into TriReagent (Sigma, St. Louis, MO).

2.1- RNA extraction, analysis and Affymetrix Hybridization—RNA was extracted from the microdissected blocks of midbrain, cleaned, quality tested, labeled and hybridized to Affymetrix Rhesus Gene Chips by the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource as previously described (Reddy & Bethea 2005; Bethea & Reddy 2008). The data was processed with Affymetrix GCOS interface software, compressed into CHP files and uploaded to GeneSifter (VisX Labs, Seattle, WA)

2.2- Quantitative (q) RT-PCR—The RNA from the microdissected midbrain blocks was subjected to qRT-PCR for CART. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as the internal reference gene, as the array hybridization data indicated that this gene was not regulated (data not shown). Complementary DNA (cDNA) synthesis and quantitative polymerase chain reaction (qRT-PCR) was conducted as previously described with Sybr green (Bethea and Reddy, 2008). Fluorescence was detected with the ABI 7900 thermal cycler during forty cycles. The reaction (final volume 20 µl) contained 1–10,000 ng of pool cDNA (standard curve) or 250 ng of sample cDNA, 100 nM of forward and reverse primers and 1X Platinum Sybr green PCR mix. The amount of cDNA added to the reaction mix was approximated from the amount of RNA used for reverse transcriptase and Nanodrop quantitation of single strand cDNA. The slope of the standard curve was used to calculate the relative pg of each transcript in the RNA extracted from the raphe blocks. The primers were obtained from Invitrogen:

CART—Accession # L26318

Forward primer (284–302 bp)— CTG ATC GAA GCG CTG CAA G

Reverse primer (494–475 bp)— GAG AAT GGA CGC CCC TTC AT

GAPDH

Forward primer (348–369bp)—CTG ACA CTG AGT ACG TCG TGG A

Reverse primer (615–595 bp)—TGG ACT GTG GTC ATG AGT CCT T

3- Tissue Preparation for immunohistochemistry

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 serial sections were collected in a cryoprotection buffer (30% ethylene glycol, 20% glycerol in 0.05 M PBS) and then frozen at – 20°C until processing for ICC.

3.1- CART single immunostaining—The polyclonal antiserum was generated against CART peptide fragment 55–102 (a gift of Dr Michael J. Kuhar, Emory University, Atlanta, Georgia). The immunohistochemical characteristics of the antibody to CART have been published (Koylu et al., 1997). Sections of midbrain were removed from cryoprotectant and immediately washed 4 times with 0.02 M KPBS (15 min each). Then, they were immersed in 0.6% H₂O₂ for 30 min, washed 4 times in KPBS buffer (15 min each) and then incubated with the following blocking solutions: normal goat serum (0.6% NGS, Vector Laboratories, Burlingame, CA) for 1 hour; bovine serum albumin (3% BSA, Sigma, St. Louis, MO) for 1 hour; avidin for 20 min and biotin for 20 min (Vector Laboratories, Burlingame, CA). Sections were then incubated at 4°C for 48 h in antibody to CART diluted 1/15,000 in 0.6% NGS, 0.4% triton and KPBS. Sections were rinsed 4 times in KPBS buffer (15 min each), incubated in biotinylated goat anti-rabbit serum for 1 hour (Vector Laboratories, Burlingame, CA), washed 4 times in KPBS buffer (15 min each), incubated with ABC reagent (Vector Laboratories, Burlingame, CA) for 1 hour, washed 4 times in KPBS buffer (15 min each), incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan)-KPBS plus 3% hydrogen peroxide for approximately 2 min, washed 4 times in KPBS buffer (15 min each). Sections were mounted on Superfrost Plus slides (Fischer, Santa Clara, CA) and dehydrated through a graded series of ethanols, xylene, and HistoClear. Sections were mounted under glass with DPX.

The sections were processed in consecutive assays containing one OvX control animal and one animal from each hormone treatment group. The animals were previously treated with hormones and processed together. Thus, 5 assays were conducted on 5 matched sets of animals. A covariance analysis for differences between the assays was not necessary.

3.2- Double immunostaining

CART + tryptophan hydroxylase (TPH) and CART + ERα: Sections of midbrain were incubated with the rabbit anti-CART (1:5000) and sheep anti-TPH antibody (Chemicon, Temecula, CA) or mouse anti-ERα 1D5 (1:25, Neomarkers, Fremont, CA). The TPH antibody intensely labels serotonin neurons in the monkey midbrain (Bethea et al., 2000) and intensely labels embryonic stem cell derived serotonin neurons (Salli et al., 2004). The ERα antibody provides intense nuclear staining in the ERα-positive human breast cancer cell line T-47D

(Bethea et al., 2003). The staining was visualized by incubating with fluorescence-conjugated secondary antibodies. To visualize CART neurons a donkey anti-rabbit IgG conjugated to Alexa Fluor 555 (Molecular Probes, Eugene, OR, USA) was used. For visualization of TPH positive neurons a donkey anti-sheep IgG conjugated to Alexa Fluor 488 was used (Molecular Probes, Eugene, OR, USA). For visualization of ER α positive neurons, a donkey anti-mouse IgG conjugated to Alexa Fluor 488 was used.

First, sections were mounted on slides, incubated at room temperature for 20 minutes in an autofluorescence eliminator reagent (Chemicon, Temecula, CA) and rinsed for 1 minute in 70% ethanol. Then sections were incubated at 4°C for 48 h in a cocktail containing both primary antibodies in 2% normal donkey serum (NDS-Jackson Immunoresearch Laboratories, West Grove, PA) and KPBS. They were then rinsed in KPBS buffer four times for 15 min each, incubated for 1 hour in a cocktail of fluorescent secondary antibodies and washed again four times for 15 min each. Then, a drop of a solution containing 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, SlowFade Gold, Molecular Probes) was applied in each section, followed by the coverslip. To determine the relationship between serotonin neurons (TPH positive) and CART neurons, the sections were examined with a Marianas stereology workstation (Intelligent Imaging Innovations, Denver, CO, USA) and images were acquired with Slidebook 4.2 (Olympus Imaging Systems, Center Valley, PA).

CART + ER β and CART +PR: Double peroxidase immunohistochemistry was performed to determine if CART neurons contained ER β or PR. Sections were first immunostained for the nuclear steroid receptor and developed with DAB + nickel (0.05% DAB-KPBS plus 8% nickel and 3% hydrogen peroxide) yielding black nuclei, and then subsequently immunostained for CART yielding a brown cytoplasm, as described in section 3.1. The antibody used against PR was diluted to 2.5 μ g/ml in 0.6% NGS, 0.4% triton and KPBS. B39 (a gift of Dr Geoffrey Green, University of Chicago) was raised against PR isolated from the human breast carcinoma cell line T-47D (Press and Greene, 1988) and cross-reacts with monkey PR (Bethea et al., 1992). ER β was detected with a mouse anti-ER β (1:200; Neomarkers). The antibody to ER β intensely stains the nucleus of the ER β -positive human breast cancer cell line T47D (Salli et al., 2004). After the dehydration process, sections were mounted under glass with DPX. Sections were examined and photographed with direct optical microscopy (Zeiss microscope with Olympus digital camera).

3.3- Quantitative Tissue Analysis—Sections were anatomically matched between animals using anatomical reference points. The Marianas stereological workstation with Slidebook 4.2 was used for analysis. Each section was examined through the rostro-caudal extent of the Edinger-Westphal nucleus (five levels, 250 μ m interval between sections). A montage of the entire area containing CART positive neurons was built by the workstation. The area containing the CART positive neurons was defined and measured (μ^2 total area). Then, the image was segmented into positive and negative pixels with Slidebook 4.2. The positive pixel area was reported in square microns (μ^2 positive area). The percent of the total area that was positively stained for CART was also computed.

For cell counting, the area of interest was outlined on the montage and detectable CART-immunoreactive cells were identified and counted by hand. All cell counts refer to detectable neurons only. The number of CART-immunoreactive neurons in the designated area of the five sections was summed, generating one value for each animal. The individual sums were averaged to obtain the group mean. In addition, Slidebook 4.2 provides the square microns of the outlined area. The thickness of each section (25 μ m $\times$ 5 sections = 125 μ m) plus the intervals between the sections (250 μ m $\times$ 4 intervals = 1000 μ m) in microns was used as the length of the region (125 + 1000 = 1125 μ m). The average area of the region for each animal (μ^2 outlined by Slidebook 4.2), was multiplied by the length to obtain the volume of the region ($\mu^3/10^9$ =

mm³). The total number of CART-immunoreactive neurones in each animal was divided by the volume of the area examined to obtain the number of CART neurons per cubic millimeter for each animal. The individual numbers of CART neurons /mm³ were then averaged to obtain the group means. Differences in cell numbers between the groups were determined by ANOVA followed by Newman-Keuls post-test or by Student's t-test using Prism Statistical software (Graph-Pad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

4- Western Blots

The midbrain raphe region was obtained and subjected to subcellular fractionation as previously described (Tokuyama et al. 2008). Cytosolic samples containing 50 µg of protein were run on a 16% nongradient Tris-Tricine gel (Jule, Inc., Milford, CT) as previously described (Kuhar & Yoho 1999). The antibody to CART was generated against the 55–102 fragment and was previously characterized (Kuhar & Yoho 1999). In this study, CART 55–102 was subjected to densitometric analysis with NIH Image. The area under the curve in arbitrary units was analyzed with ANOVA.

5- Hormone Assays

Assays for E and P were performed utilizing a Roche Diagnostics 2010 Elecsys assay instrument. Before these analyses, measurements of E and P on this platform were compared to traditional RIAs as reported previously (Bethea et al. 2005).

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Figure 1.

Microarray analysis of CART gene expression in the midbrain area of monkeys from each treatment group (n=3 animals/group) using new Rhesus Affymetrix gene chips. The P group was highly variable and not different from the OVX group. There was a significant difference between the treated groups when the P group was omitted (ANOVA, $p < 0.05$). Post-hoc comparison indicated that both E and E+P administration decreased CART gene expression in the midbrain significantly compared with OVX animals (Student Newman-Keuls post hoc, $p < 0.05$).

Figure 2. qRT-PCR analysis of CART transcript expression in the midbrain area of monkeys from each treatment group (n=3 animals/group). CART gene expression was normalized by GAPDH. There was a significant difference between all of the treatment groups (Kruskal-Wallis, $p=0.04$), but Dunn's post hoc comparisons yielded no differences. When the P group was omitted, there was a significant difference between the treated groups (ANOVA, $p<0.05$). Post-hoc comparison indicated that both E and E+P-treatments decreased CART gene expression in the midbrain significantly compared with OVX animals (Student Newman-Keuls post hoc, $p<0.05$).

Figure 3.

Montages of all levels (1–5) of the midbrain from a P treated monkey, showing positive immunostaining for CART below the Edinger-Westphal nucleus (EW) and above the oculomotor (III) nucleus. A diagram showing the relative positions of the periaqueductal gray (PAG), Edinger-Westphal nuclei (EW), the oculomotor nuclei (III) and the CART neurons (dots) is shown below the montages. The area where the CART positive neurons are located has been referred to as the supraoculomotor area, or SOA (May et al., 2008). The montages were built automatically by Slidebook 4.2 and the stereology microscope. Scale bar: 500 μ m

Figure 4. Comparison of immunohistochemical staining for CART at level 3 of the Edinger-Westphal nucleus from representative animals in each treatment group. Scale bar: 500 μ m

CART Immunocytochemical Signal Analysis

Figure 5.

Stereological analysis of CART immunostaining in the midbrain. (A) Histograms illustrating the positive pixel area stained for CART, (B) the percentage of the total area that was positive, and (C) the total area examined (all in μ^2) in the midbrain area across five levels of the Edinger-Westphal nucleus in each treatment group (n=5 animals/group). Comparison with ANOVA showed no significant differences between the groups. However, comparison of individual groups with the OVX group by Students t-test indicated a significant increase in the positive area immunostained for CART (A) and a significant increase in the percentage of the total area stained for CART (B) in the P treatment group. The total area examined did not differ between groups (C)

Immunostained CART Neuron Number

Figure 6.

Stereological examination of the number of CART positive neurons in the midbrain. (A) Analysis of total number of CART cells or (B) CART neurons per volume in the midbrain area across five levels of the Edinger-Westphal nucleus in each treatment group (n=5 animals/group). CART neurons were counted on the montage within a defined area (μ^2). There was a significant increase in the total number of CART neurons in the P treated group compared with the OVX group, either in the absolute number of cells (A) or in the number of cells/mm³ (B) with direct comparison using Student *t*-test ($p<0.05$). ANOVA comparison did not show significant differences among groups.

Figure 7.

CART and tryptophan hydroxylase (TPH)-positive neurons. Confocal photomicrographs from midbrain of a P-treated monkey following double-immunofluorescence for CART (red; A) and TPH (green; B). CART neurons do not express TPH as shown in the overlaid image (C). However, TPH neurons (arrows) were detected in the dorsal raphe nucleus, which is caudal to the CART neurons. CART fibers (red) were observed adjacent to TPH positive neurons (green) in the dorsal raphe nucleus (D,E). Scale bar: 50 μ m

Figure 8.

Colocalization of CART and ERβ. CART stained brown in the cytoplasm and ERβ stained black in the nucleus (panels A–E, double head arrows). ER positive neurons that are not CART expressing were present as well (single head arrows). Omission of only the ERβ antibody resulted in CART labeled neurons with no nuclear staining (panels F–H). There was no staining when the primary antibodies for CART and ERβ were omitted (results not shown).

Figure 9.

Colocalization of CART and PR. CART stained brown in the cytoplasm and PR stained black in the nucleus. PR was undetectable in the absence of the primary antibody for PR (panels A, B). Numerous CART neurons contained PR (double head arrows, panels C–H). Figures 9F and 9G also show PR positive neurons that do not contain CART (single head arrows).

Figure 10.

Top. Representative western blot showing CART signal at 9kD and the same blot reprobed for actin. Three sets of the 4 treatment groups were run on one gel and the band density was obtained with NIH Image as area under the curve in arbitrary units. The density of the CART band was normalized by the actin band and the results expressed as the mean±SEM are shown in the bottom panel. There was no difference between the treatment groups with ANOVA.

GAPDH and CART cDNA (pg) generated during qRT-PCR. There was no difference between the groups in GAPDH cDNA, but CART cDNA was significantly decreased by E and E+P. Normalization of CART by GAPDH is shown in Figure 2.

Table 1

	Ovx	E	P	E+P	
GAPDH cDNA (pg±sem)	9261 ± 5138	12259 ± 3798	18478 ± 2271	14502 ± 5001	ns
CART cDNA (pg±sem)	13348 ± 3917	524 ± 197	67409 ± 31795	1010 ± 213	p < 0.03