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Regulation of CART mRNA by Stress and Corticosteroids in the Hippocampus and Amygdala

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Abstract

CART (Cocaine- Amphetamine-Regulated Transcript) has been shown to be regulated by corticosteroids in the hypothalamus, but its regulation by corticosteroids and stress has not been well examined in the hippocampus or the amygdala. Further, CART has been implicated in the transition to puberty. In this study we examine the effects of acute (30 minute) on CART mRNA in prepubescent and adult rats. In addition, we examined chronic (21 day \times 6 hours) restraint stress upon the expression of CART mRNA in the hippocampus and the amygdala and the effects of 7 days of adrenalectomy and corticosteroid replacement upon CART expression in these regions of the adult rat brain. We found an up-regulation of CART mRNA in the central amygdala induced by acute but not chronic stress and an up-regulation in the dentate gyrus induced by chronic but not acute stress.

Adrenalectomy reduced CART expression in the dentate gyrus but not the amygdala and this effect was blocked by corticosterone but not RU28,362 or aldosterone replacement, suggesting a synergism of mineralocorticoid and glucocorticoid receptors. Our data establish that CART expression is regulated by stress in a regionally and time specific manner and that CART is regulated by corticosteroid actions in the hippocampus.

Keywords

HPA axis; anxiety; depression

Introduction

Stress and corticosteroids play a role in a number of neuropsychiatric disorders, particularly major depressive disorder. Both stress and corticosteroids have been linked to the structural changes seen in these disorders and in animal models such as chronic restraint stress (McEwen and Olie, 2005). CART (Cocaine- Amphetamine-Regulated Transcript) plays a role in a number of different behaviors, such as feeding and drug use (Hunter and Kuhar, 2003), and a number of studies have suggested that CART may have a role in the central response to stressful stimuli. Early anatomical work demonstrated its localization in a number of regions associated with stress and anxiety (Douglass et al., 1995; Couceyro et al., 1997; Koylu et al., 1997; Koylu et al., 1998) which led to several studies demonstrating the anxiogenic effects of centrally injected CART peptides in the elevated plus maze and social interaction (Kask et al., 2000;

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Asakawa et al., 2001; Chaki et al., 2003; Stanek, 2006). More recently, it has been shown that human adolescents carrying the Leu34Phe substitution in the proCART peptide, had a higher level of anxiety and depression than control subjects (Miraglia Del Giudice et al., 2006). It seems likely that CART may have a role in anxiety and depression that is only beginning to be understood.

In parallel to the studies of CART's influence on anxious behaviors, a more substantial literature has demonstrated a number of interactions between CART and the HPA axis, especially in regard to corticosteroids. Central CART injection produces increased plasma corticosterone and ACTH (Vrang et al., 2000; Stanley et al., 2001) and, reciprocally, CART expression in the brain and blood is subject to regulation by corticosteroids (Balkan et al., 2001; Vrang et al., 2003; Vicentic et al., 2004; Hunter et al., 2005; Koylu et al., 2006). More recently Balkan has shown that a forced swim stress increases CART peptide levels in the amygdala of male rats while lowering it in females (Balkan et al., 2006). Other evidence implicates CART in the HPG axis as well as the HPA, particularly in the regulation of GnRH secretion (Lebrethon et al., 2000a; Lebrethon et al., 2000b; Parent et al., 2000) and, controversially, the onset of puberty (Adam et al., 2000; Lebrethon et al., 2000b; Brann et al., 2002). On this basis we decided to examine changes in the levels of CART mRNA in the hippocampus and amygdala following acute stress in adolescent and adult animals and the effects of chronic stress and corticosteroid manipulations in adults.

Results

Acute stress

Based upon the aforementioned findings that CART may have a role in the onset of puberty in rodents we chose to analyze the effect of acute stress upon both adult and prepubertal animals. Analysis of CART mRNA expression in the dentate gyrus showed a significant effect of age ($n=6$, $F(1,18)=10.66$) but not stress with higher levels in unstressed prepubertal animals than adults in either condition (see Figure 1) and a trend towards higher levels in the stressed condition.

In the central amygdala, a similar pattern emerged with regard to age, though not to stress. There was a significant main effect of age ($F(1,16)=59.93$) such that both stressed and unstressed prepubertal animals showed substantially higher CART mRNA expression than unstressed adults. There was also a significant main effect of stress ($F(1,16)=9.20$) which was observed in adult animals but not the prepubertal rats. This increase did not produce CART mRNA levels comparable to those of prepubertal animals (see Figure 2), suggesting the possibility that the younger animals may be subject to a ceiling effect. Corticosterone levels for these animals was reported in (Romeo et al., 2004a) where it was shown that peak levels of corticosterone did not differ between adults and prepubescent rats, though the younger animals showed a longer elevation in corticosterone levels than the adults.

Chronic Stress

We followed up our acute stress studies with an examination of the effects of chronic restraint stress on adult male rats. Prepubertal animals were not included due to the absence of an effect in the acute stress study and due to the fact that the chronic restraint stress paradigm would include most if not all of the period of puberty.

In contrast to the acute stress study, we found that chronic restraint stress increased the expression of CART mRNA in the dentate gyrus by more than 85% ($\pm 20\%$, $p < 0.05$, see Figure 3.) and had no impact on CART mRNA levels in the central amygdala (Figure 4).

Adrenalectomy and Corticosteroid Treatment

There was a significant main effect of treatment ($F(4,30)=5.62$) in the dentate (Figure 5.) but no effect in the central amygdala (Figure 6). Adrenalectomy reduced CART message by more than 50% ($\pm 12\%$, $p < 0.05$). Neither the selective MR agonist aldosterone nor the selective GR agonist RU28,362, given alone, reversed this effect, but treatment with corticosterone, which occupies both MR and GR, did restore CART mRNA expression in the dentate.

Discussion

Our results demonstrate that stress alters CART mRNA expression in a regionally specific manner in response to stress duration. In addition we show that acute stress has no impact on CART expression in adolescent animals relative to adults, though this may be a ceiling effect. We also show that CART is regulated by chronic corticosterone and adrenalectomy in the hippocampus but not in the amygdala by mechanisms potentially involving both MR and GR.

We first examined the effect of an acute restraint stress on CART mRNA expression in the prepubertal and adult rat brain. We found that acute stress elevates CART mRNA expression in the central amygdala of adults but not prepubertal animals. The change observed in the adults is in agreement with Balkan's observations of CART peptide levels after an acute swim stress (Balkan et al., 2006). The elevated levels of CART mRNA in the prepubertal animals, which did not respond to stress, suggest that CART may contribute to the differences in stress response between this age group and adults (Romeo et al., 2004a; Romeo et al., 2004b; Romeo et al., 2006), though only further study will establish the actual relationship between CART and the juvenile stress response.

Our confirmation of Balkan's result led us to examine whether chronic stress had the same impact on CART expression. The effect of chronic stress was in fact quite different from acute stress. CRS increased CART expression in the dentate gyrus and had no impact on CART mRNA levels in the central amygdala. Further, adrenalectomy reduced CART levels in the dentate gyrus, an effect which was reversed by corticosterone replacement. Adrenalectomy had no effect on CART expression in the amygdala. These data suggest that the regulation of CART in the amygdala is largely independent of corticosteroids and may depend on other elements of the stress response, such as catecholamines or endogenous neural activity.

In the hippocampus it is clear that corticosterone plays a role in the chronic regulation of CART expression. Adrenalectomy has been shown to down regulate CART expression in the brain and blood (Balkan et al., 2001; Vicentic et al., 2004) and glucocorticoid administration has been shown to elevate it in the hypothalamus and nucleus accumbens (Vrang et al., 2003; Hunter et al., 2005). It would appear that corticosterone acting in a non-classical fashion with regard to CART as neither activation of GR with the pure agonist RU28362 nor activation of the MR with the specific MR agonist, aldosterone, appears to have an effect upon CART expression after adrenalectomy. Given that the drugs in this study were systemically administered it is of course impossible to be certain, based on the present data, of the mechanism by which corticosteroids or stress are altering CART expression. It is possible, however, that it may be due to interaction with CREB, as CREB has been shown to regulate CART expression in other systems (Dominguez et al., 2002; Lakatos et al., 2002; Dominguez and Kuhar, 2004; Jones and Kuhar, 2006) and the CART promoter is not known to possess a GRE site (see (Dominguez, 2006) for a review on CART promoter structure). Alternatively, CART may be regulated by the action of MR/GR heterodimers (Trapp et al., 1994; Trapp and Holsboer, 1996). Corticosterone binds to both receptors with high affinity and both are present within the dentate gyrus (Reul and de Kloet, 1985; Reul and de Kloet, 1986). In fact, the both adrenalectomy (Sloviter et al., 1989) and the GR selective agonist dexamethasone (Hassan et al., 1996) can produce cell death in the dentate, whereas corticosterone prevents these effects

and there is evidence CART is neuroprotective (Louis, 1996; Wu et al., 2006; Xu et al., 2006). At present the mechanism by which CART is regulated by corticosterone is unclear.

It is also clear, given that adrenalectomy does not abolish CART expression in the hippocampus, that corticosteroids are not the only regulators of CART in that region. Interestingly, the hippocampus also expresses leptin receptors (Huang et al., 1996; Mercer et al., 1996; Shioda et al., 1998) and leptin is a regulator of CART expression in the hypothalamus (Kristensen et al., 1998). It is possible, therefore, that CART is also subject to regulation by leptin in the hippocampus as well.

Given that CART is known to be anxiogenic (Stanek, 2006), it is possible that the changes in CART expression observed here relate to the increases in anxiety levels produced by stress (Adamec and Shallow, 1993; Vyas et al., 2002; Gameiro et al., 2005). The up regulation of CART we observed in the hippocampus may also be neuroprotective, and there is evidence that the effects of chronic stress on the hippocampus may be reversible (Conrad et al., 1999; Sousa et al., 2000) and do not of themselves lead to neuron loss. In fact it has recently been shown that CART promotes the survival of cultured hippocampal neurons (Wu et al., 2006) and is also a mediator of the neuroprotective properties of estradiol (Xu et al., 2006).

In conclusion, evidence in this paper indicates that CART is robustly regulated by stressors in a regionally and temporally specific manner. Our findings extend and expand the emerging body of evidence that CART is a potentially important element of the stress response.

Experimental Procedures

Animals

Male Sprague Dawley rats were obtained from Charles River (Kingston, NY) at either 21 or 70 days of age. Animals were housed 2–3 per cage (same age cage mates) in clear polycarbonate cages with wood chip bedding. All animals were maintained on a 12 h light-dark schedule (lights on at 0800 h) and the temperature was kept at $21\pm 2^{\circ}\text{C}$. All animals had *ad libitum* access to food and water. All procedures were carried out in accordance with the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals.

Acute and chronic restraint stress

Animals were left undisturbed after arrival for one week (i.e., until either 28 or 77 days of age). For acute stress, animals were weighed and rapidly decapitated by a guillotine either immediately without being stressed (basal) or two hours after a 30 min session of restraint stress. CART was examined at the two hour time point based on previous studies of the time course of changes in CART expression (Hunter et al., 2006). Animals were restrained in wire mesh restrainers, secured at the head and tail ends with clips. Chronic stress experiments used the same restraint methodology as the acute stress, but the restraint was administered for 6 hours daily for 21 days. These animals were sacrificed 18 hours after the last stress. Further we chose not to examine prepubescent animals in the CRS and corticosteroid treatment experiments due to the absence of an effect in the acute stress experiment. Stressed animals were returned to their home cages immediately after termination of the stressor, until sacrifice. At the time of sacrifice, trunk blood samples were collected and hormone levels for these animals can be found in (Romeo et al., 2004a). Brains were removed and flash frozen on dry ice then stored at -80°C until processing. All animals were killed between 1300 and 1700 h.

Steroid Treatments

Doses were chosen based on those shown to have an effect upon a number of neurotransmitters and receptors in previous studies (Albeck et al., 1994; Kuroda et al., 1994; Watanabe et al.,

1995) and these treatments follow those administered by Watanabe (Watanabe et al., 1995) with some modification. Animals were anesthetized using ketamine and xylazine and the adrenal glands removed, save for one group which received a sham surgery. During the same surgery, osmotic mini-pumps (Alzet, Cupertino, CA) were implanted s.c. between the scapula of all groups, including sham adrenalectomized animals. These pumps delivered either vehicle (propylene glycol), the mineralocorticoid receptor agonist aldosterone at 10mg/hour or the glucocorticoid receptor agonist RU28,362 at 10µg/hour. Animals who underwent ADX received 0.9% saline in their drinking water and one group received 400µg/ml corticosterone in addition to the saline, sham animals received tap water. Seven days after the completion of the surgeries the animals were sacrificed and their brains frozen as described above.

In Situ Hybridization

Brain sections were cut at 14 µm on a cryostat and placed on Fisher Biotech ProbeOn Plus slides (Fisher, Pittsburgh, PA). In situ hybridization began with a tailing reaction to radioactively label the oligonucleotide probes with ³⁵S. The CART probe was 5'-ATC GGA ATG CGT TTA CTC TTG AGC TTC TTC AGG-3'. Processing of the slides followed methods as previously described (Couceyro et al., 1997) with some modification as described in (Hunter et al., 2006). Anatomical locations were determined with the assistance of the atlas of Paxinos and Watson (Paxinos and Watson, 1986), as well as previous studies of CART distribution in the hippocampus and amygdala (Douglass et al., 1995; Koylu et al., 1998; Hurd et al., 1999) and light microscopic examination of the relevant sections. Optical density was determined using MCID 5.0 (Imaging Research, St. Catharines, OT, Canada).

Statistics

Optical density measurements were analyzed by a two way ANOVA (stress versus age for the acute stress study), one way ANOVA for the chronic steroid study and Student's t-test for the chronic stress study. Significant main effects and interactions in ANOVA were further analyzed using Fisher's protected least significant difference test and Tukey's test, respectively. Differences are considered significant at p<0.05. All data are presented as mean ±SEM.

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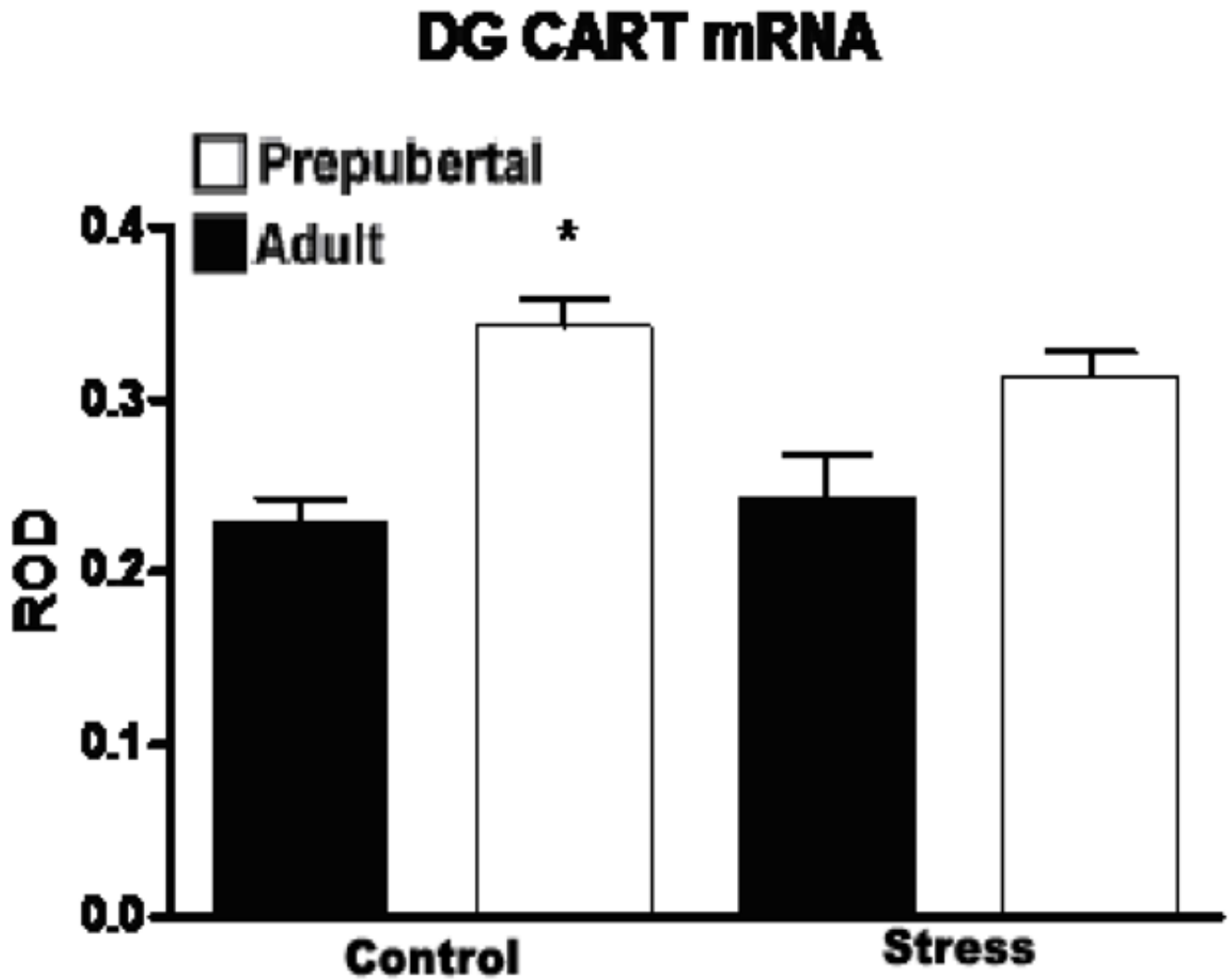
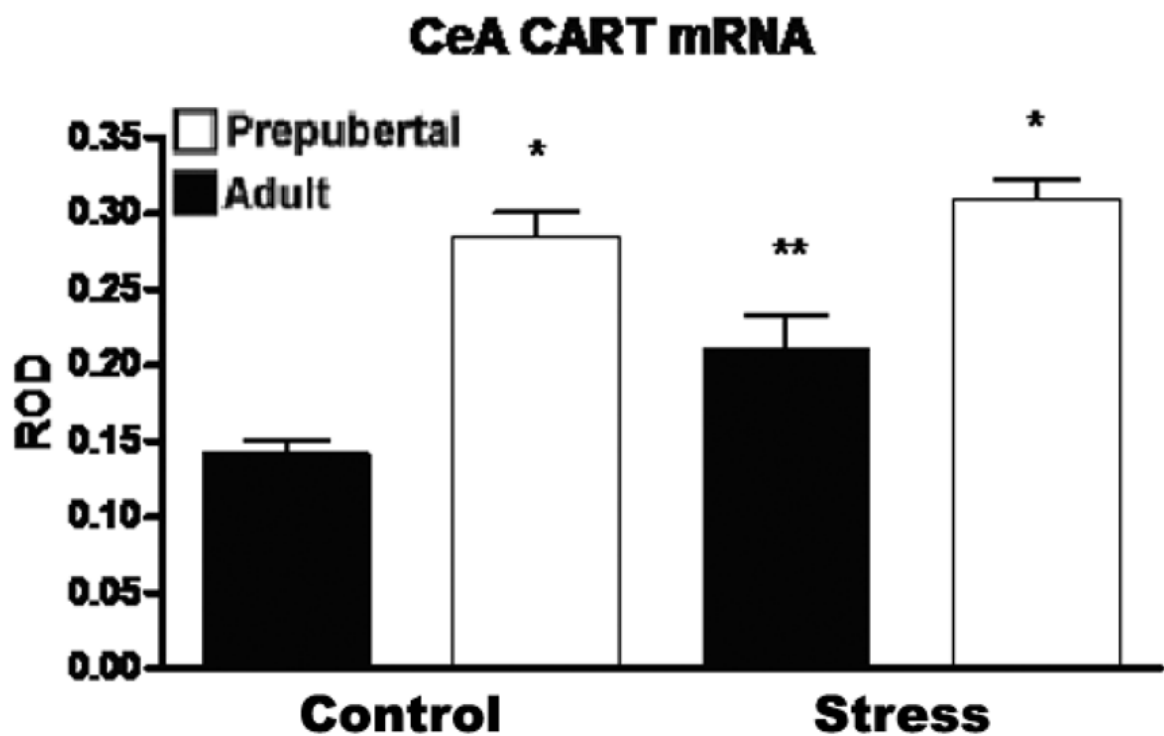
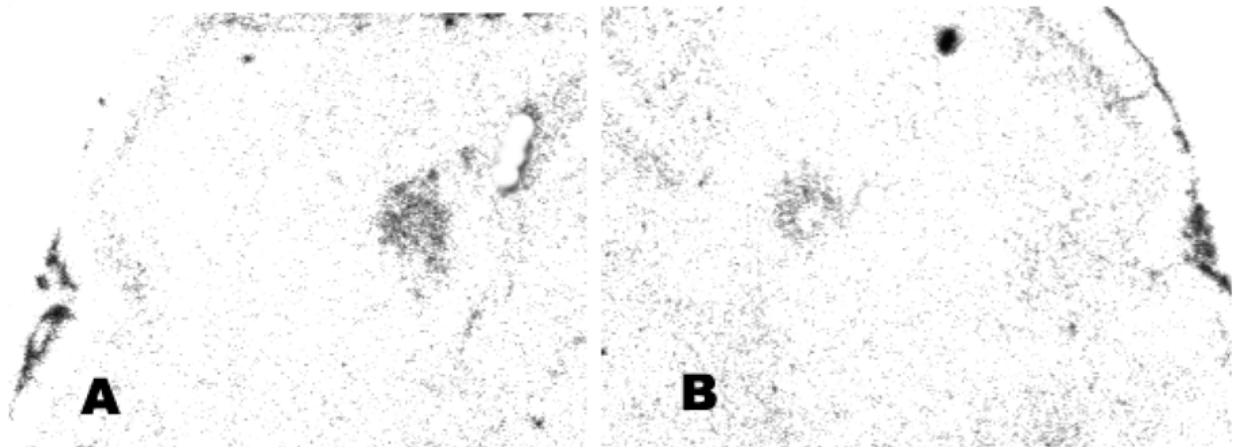


Figure 1.

Relative optical density (ROD) of CART mRNA expression (\pm SEM) in the dentate gyrus of adult and prepubertal rats at baseline and 2 hours after a 30 minute restraint stress. Prepubertal rats showed significantly ($F(1,18)=10.66$) higher levels of CART mRNA expression than adults, though no effect of stress was observed at either age. Asterix indicates a significant difference between unstressed adult and prepubertal rats ($n=6$, $p<0.05$).



C

Figure 2.

A) shows an autoradiogram of the region of the central amygdala in an adult male rat acutely restrained for 30 minutes prior to sacrifice two hours later. B) shows the central amygdala of an unrestrained control. C) ROD of CART mRNA expression (\pm SEM) in the central amygdala of adult and prepubertal rats at baseline and 2 hours after a 30 minute restraint stress. Prepubertal rats showed significantly higher CART mRNA levels than adults ($F(1,16)=59.93$, $n=6$, $p<0.001$ vs. unstressed adults, $p<0.05$ vs. stressed adults), though no effect of stress. Adult animals showed a significant ($F(1,16)=9.20$, $n=6$, $p<0.05$ vs. unstressed adults and prepubertal rats) post-stress elevation of CART expression. Asterix indicates a significant difference

between adult and prepubertal rats and a double asterix indicates a significant difference from both unstressed adults and prepubertal animals.

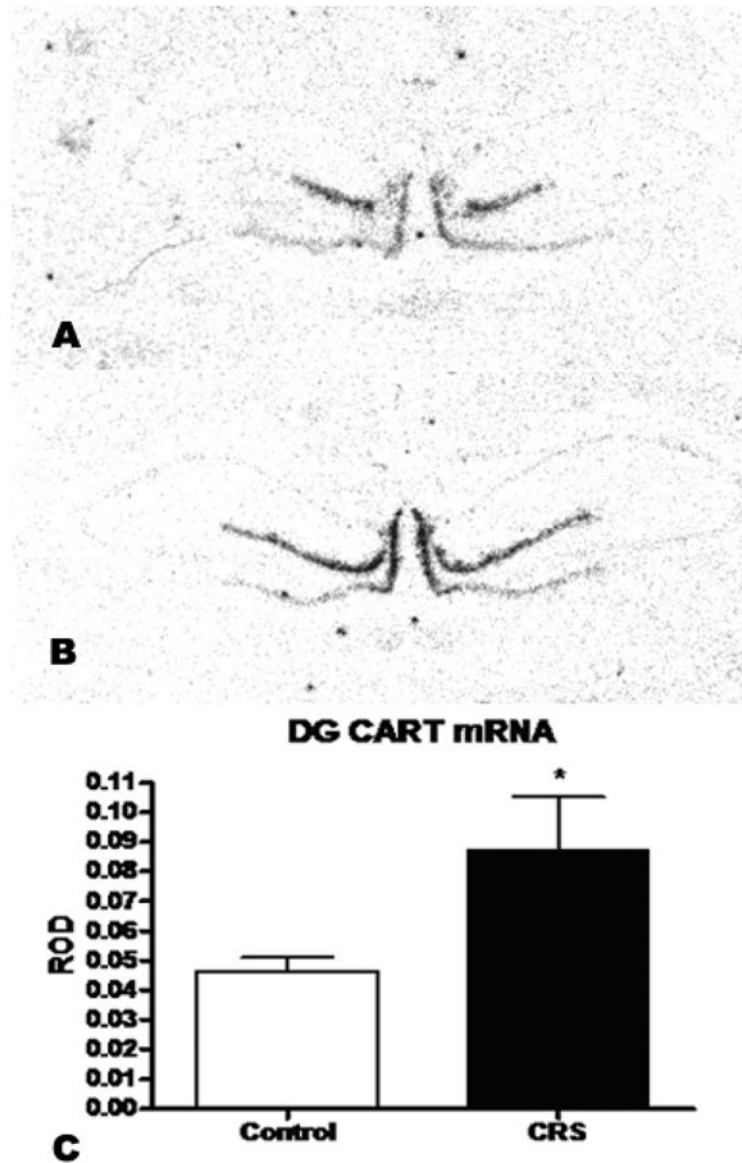


Figure 3.

A) Shows an autoradiogram of CART mRNA hybridization signal in the DG of an unrestrained control rat. B) shows the CART hybridization signal in a chronically restrained animal. C) ROD of CART mRNA expression (\pm SEM) in the dentate gyrus of adult male rats after 21 days of chronic restraint stress. CRS significantly elevated CART expression in the dentate ($p < 0.05$, $n=8$).

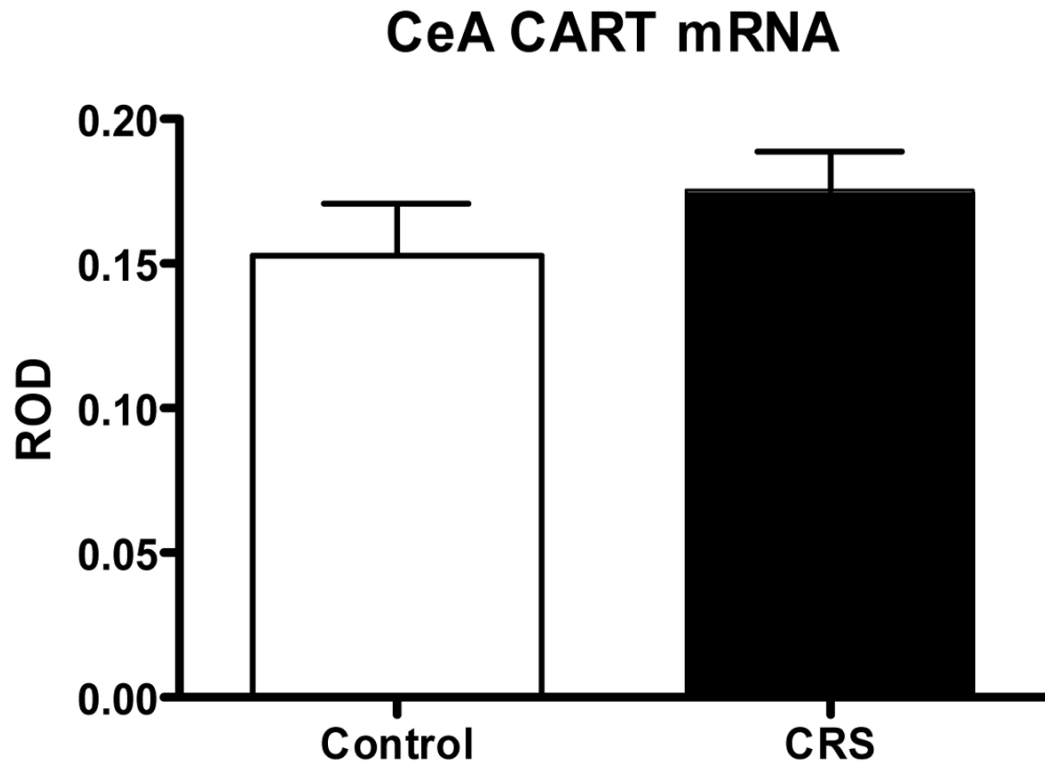


Figure 4. ROD of CART mRNA expression (\pm SEM) in the central amygdala of adult rats after 21 day CRS. No effect of chronic stress was observed in this region (n=8).

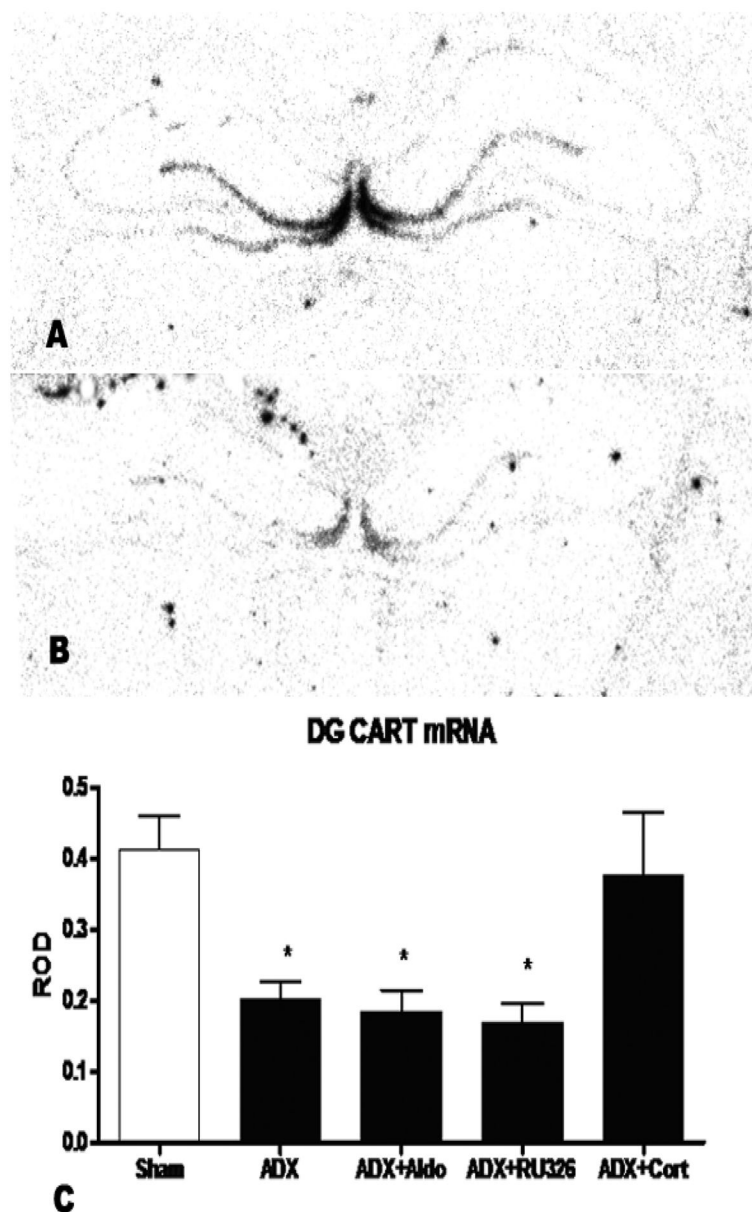


Figure 5. A) Shows an autoradiogram of CART mRNA hybridization signal in the DG of a control rat subjected to sham surgery. B) shows the CART hybridization signal in an adrenalectomized animal. C) ROD of CART mRNA expression (\pm SEM) in the dentate gyrus after either sham surgery, or adrenalectomy and 7 days of either vehicle, 10mg/hour aldosterone, 10 μ g/hour RU28,362 or vehicle plus corticosterone in the drinking water. Adrenalectomy produced a significant reduction in dentate CART mRNA ($F(4, 30)=5.62, p<0.05$ sham versus ADX, $n=7$). This effect was blocked by corticosterone in the drinking water.

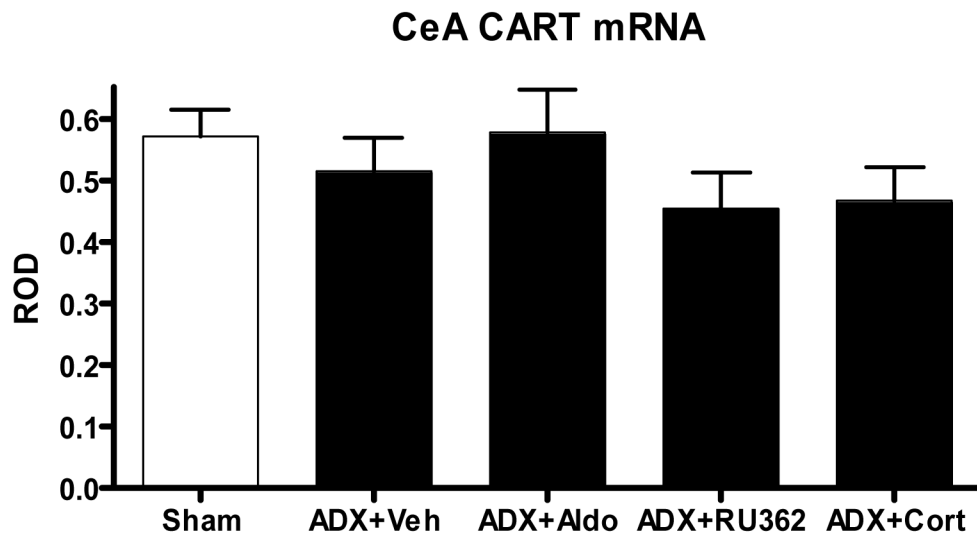


Figure 6. ROD of CART mRNA expression (\pm SEM) in the central amygdala after either sham surgery, or adrenalectomy and 7 days of either vehicle, 10mg/hour aldosterone, 10 μ g/hour RU28,362 or vehicle plus corticosterone in the drinking water. There was no effect of any of the treatments on CART mRNA levels in this region.